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14. ABSTRACT: Apoptosis, or programmed cell death, is frequently triggered through the release of cytochrome c from mitochondria. Cytosolic cytochrome c then binds to a cytosolic protein known as Apaf-1, which binds to and activates the cell death protease, caspase 9. Many cancers are resistant to apoptosis induced by chemotherapies. During the course of this work we demonstrated that breast cancer cells, while resistant to release of mitochondrial cytochrome c are actually hypersensitive to cytochrome c, once it is released. Our work provides a unique point of sensitivity that might be exploited for the successful treatment of breast cancers.					
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Table of Contents

	<u>Page</u>
Introduction.....	4
Body.....	4-7
Key Research Accomplishments.....	7
Reportable Outcomes.....	7
Conclusion.....	7
References.....	
Appendices.....	

Chemotherapeutic agents act, at least in part, by triggering mitochondrial cytochrome c release, which promotes activation of a cell death machine known as the apoptosome. Comprised of a protease (caspase 9) and a caspase 9 activator (Apaf-1) which can bind the released cytochrome c, this multiprotein complex acts to activate other caspase protease which can promote cell death and destruction. The basis for our original proposal was a yeast two hybrid screen using Apaf-1 as a bait. In this screen, we isolated a clone encoding a protein known as AFG3L2, which appeared to be a AAA ATPase. Our goal was to both characterize AFG3L2 and undertake a study of apoptotic regulation at the level of the apoptosome. Although our AFG3L2 studies met with many technical hurdles, the apoptosome work proved very fruitful, uncovering a novel point of cell death regulation in breast cancer cells.

Task I. Determine the role of AFG3L2 in the apoptotic response

Although we produced AFG3L2 recombinantly, we were unable to determine that AFG3L2 had any direct effects on apoptosome formation or function, despite its having been isolated in screen for Apaf-1 interactors. However, in the course of performing this work, we became very interested in the notion that cancers might regulate apoptosis not only upstream of mitochondria, which had been reported by many others, but also downstream of mitochondrial cytochrome c release, at the level of the apoptosome. Towards this end, we tested a panel of oncoproteins and cancer types to determine if the apoptosome was regulated. In this work, we made several surprising and interesting discoveries. First, we found that the apoptosome was very inactive in number of tumor types, including multiple types of leukemia. We found that the oncoprotein Bcr-Abl, found in chronic myelogenous leukemias and the Tel-PDGF fusion could both prevent apoptosis even after mitochondrial cytochrome c release. We also found that activation of the MAPK pathway, as seen in many tumor types, can also prevent activation of caspase 9 in the presence of cytochrome c.

Unlike almost all of the other tumors we tested, breast cancer cells appeared to be hypersensitive to cytochrome c, rather than resistant (Fig. 1).

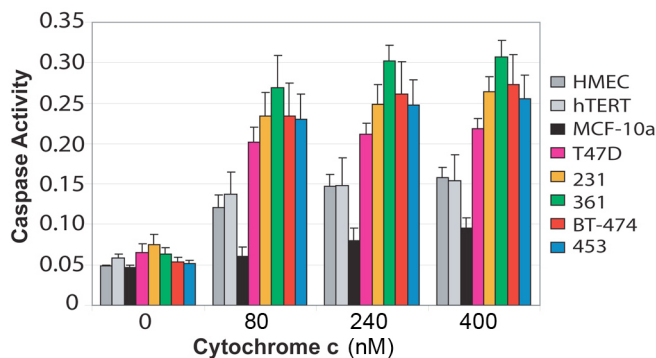


Fig. 1 Cytosolic extracts from various breast cancer cell lines and control cells (HMEC, hTERT) were assayed for caspase activation using the caspase-3 substrate DEVD-pNA.

Conversely, all of the breast cancer cell lines tested appeared to be relatively insensitive to chemotherapy-induced cytochrome c release. Therefore, despite their hypersensitivity, the cells never “saw” cytosolic cytochrome c, making the chemotherapeutic agents ineffective. This raised the intriguing possibility that cytochrome c or a mimetic might serve as a therapeutic in breast cancers. As a proof of principle, we microinjected cytochrome c into normal and breast cancer cells and found that breast cancer cells were notably sensitive to the injection (Fig 2). Recently, we have tested a broader battery of cancer types and have found that brain tumors, like breast cancers, are hypersensitive to cytochrome c, so cytochrome c-mimetic compounds might prove of great utility in the treatment of both breast and brain tumors.

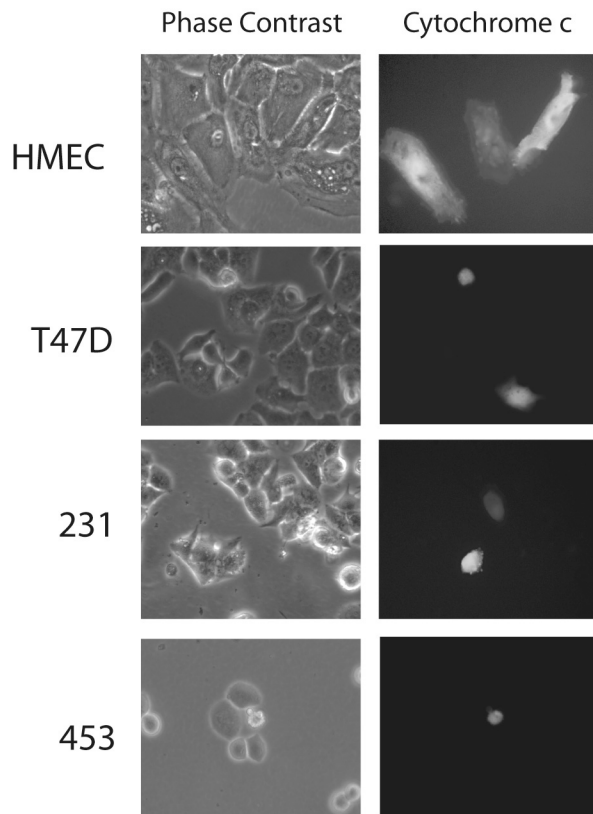


Fig. 2 10mg/mL cyt c microinjection (with rhodamine co-injection) of breast cells preferentially kills malignant mammary epithelial cells while leaving HMEC controls unharmed.

Task II. To determine the mechanism of AFG3L2-mediated control of the apoptosome

Because we were successful in identifying several mechanisms that could control the apoptosome, we wished to determine whether they all shared a common mechanism. Moreover, we wished to elucidate the molecular details of this regulation, particularly because this regulation proved to be altered in breast cancers. In the case of MAPK signaling, it was reported soon after our initial publication that MAPK could phosphorylate and inhibit caspase 9. We found that Bcr-Abl could prevent the assembly of caspase 9 into the apoptosome and are currently continuing investigation of the basis for that phenomenon. However, in breast cancers, we made the interesting discovery that apoptosome hypersensitivity was due to up-regulation of a protein known as PHAPI, previously reported to be an apoptosome activator. PHAPI was upregulated in both breast cancer cell lines and primary breast tumors (Fig 3).

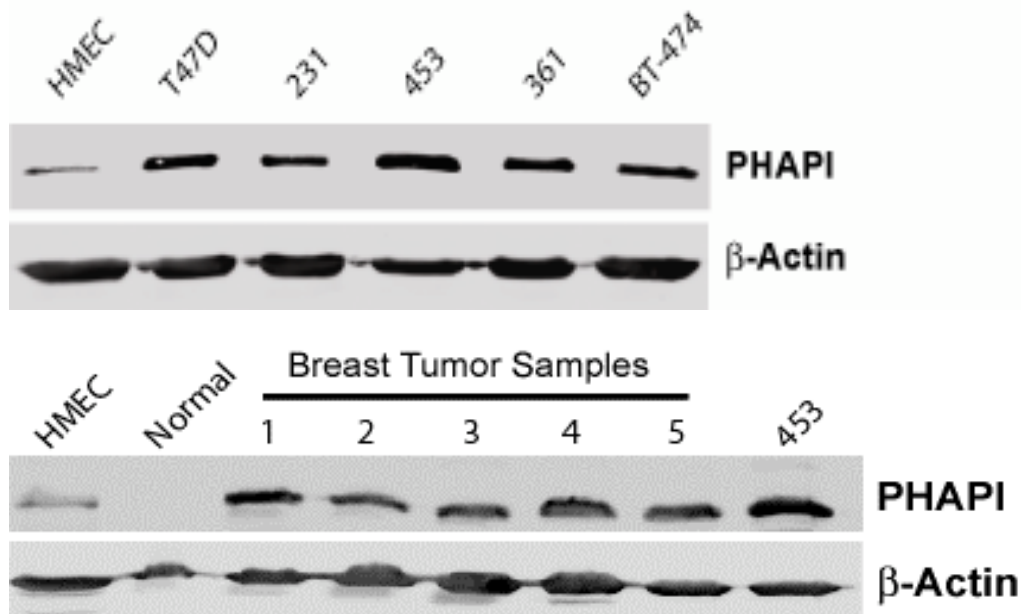


Fig. 3. (Upper panel) Lysates prepared from normal (HMEC) mammary epithelial cells and a variety of breast cancer cell lines were immunoblotted for PHAPI. (Lower panel) Lysates prepared from normal breast tissue and 5 distinct primary breast tumors were immunoblotted for PHAPI. The HMECs and transformed 453 cells are shown for comparison.

Moreover, we found that ablation of PHAPI by RNA interference could dampen the sensitivity of breast cancer cells to cytochrome c and that PHAPI overexpression in normal mammary epithelial cells could confer the hypersensitivity characteristic of breast cancer cells. Hence, we are confident that we have identified a fundamental mechanism for controlling apoptosis in breast cancer cells. Moreover, the hypersensitivity of breast

tumors to cytochrome c might be a point of vulnerability amenable to therapeutic approaches.

Key research accomplishments:

- Demonstration that MAPK can prevent apoptosis in response to cytochrome c
- Demonstration that Bcr-Abl prevents apoptosis downstream of mitochondrial cytochrome c release
- Determination that the Apaf-1/caspase 9 apoptosome is hyperactive in breast cancer cells
- Identification of PHAPI as a protein overexpressed in breast cancers, which underlies the hypersensitivity of the apoptosome

Reportable outcomes: (note that this project was undertaken by two different students as the first PI graduated and the award was reassigned. Hence, publications by both students, Jessica Tashker and Zachary Schafer are listed below.

Tashker, J.S., M. Olson, and S. Kornbluth (2002). Post-cytochrome c protection from apoptosis conferred by a MAPK pathway in *Xenopus* egg extracts. *Mol Biol Cell* **13**: 393-401.

Deming, P.B., Z.T. Schafer, J.S. Tashker, M. Potts, M.Deshmukh, and S. Kornbluth. (2004). Bcr-Abl-mediated protection from apoptosis downstream of mitochondrial cytochrome c release. *Mol. Cell Biol.*, **24** ; 10289-10299.

Schafer, Z.T., A.B. Parrish, K.M. Wright, J.R. Marks, M. Deshmukh, and S. Kornbluth. (2006). Increased apoptosome activation and enhanced sensitivity to cytochrome c-induced apoptosis in breast cancer cells. *Cancer Research*, **66**: 2210-2218

Schafer, Z.T. and S. Kornbluth (2006). The Apoptosome: Physiological, Developmental, and Pathological Modes of Regulation. *Devel. Cell*, **10**: 549-561.

Conclusion: This study was designed to understand regulation of the apoptosome through characterization of Apaf-1 interactors. Investigation of the original interactor, AFG3L2, did not prove as fruitful as the parallel studies on apoptosome regulation. Perhaps most importantly, we found that breast cancer cells have hypersensitive apoptosomes. This finding raises the possibility that this sensitivity might be exploited for breast cancer chemotherapy through the isolation of apoptosome activators.