Using Anti-Viral Biomarkers to Predict Breast Cancer Aggressiveness
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ABSTRACT
Metastasis of cancer cells from the breast to distal target organs is the single most important cause of mortality in women with breast cancer. One of the most challenging aspects of eradicating breast cancer and increasing the survival rates of women with breast cancer is the prevention and/or treatment of metastasis. Arguably, breast cancers that do not metastasize become a far more manageable form of the disease. Recent next generation sequencing studies have confirmed that TP53 and CDKN2A (ARF protein) mutations are the most frequent alterations in breast carcinomas, occurring in all subtypes of human breast cancer albeit at varying rates. Moreover, TP53 and CDKN2A mutations can enhance metastasis in basal-like tumors, providing evidence that the p53 and ARF tumor suppressor proteins act in part to prevent metastasis. There are two aspects to this disease that we must address to lengthen the lives of breast cancer patients. First, we must identify tumor markers that drive aggressive invasion and metastasis BEFORE the tumor is metastatic. Second, we must understand how these drivers work in order to prevent breast cancer metastasis in affected women.

We have discovered that a common anti-viral pathway, RIG-I/IFN-β, is elevated in aggressive metastatic breast TP53/CDKN2A mutant breast cancers. We hypothesize that loss of both TP53 and CDKN2A unleashes a pro-invasive/metastatic pathway in breast cancer cells. In order to address this hypothesis, we had proposed a single specific aim: (1) To determine whether activation of the anti-viral RIG-I pathway is required and/or sufficient for breast cancer invasion and metastasis. Through the work described in this summary statement, we have determined that activation of this anti-viral pathway is not only sufficient to drive aggressive breast cancer proliferation and metastasis, but that we can observe the induction of this pathway in the most aggressive forms of human breast cancer.

LAY SUMMARY
Can we identify, early on, why some breast cancers metastasize and become life-threatening? This question has remained at the top of the list for many clinicians, researchers, and patients. Metastasis of cancer cells from the breast to distal target organs is the single most important cause of mortality in women with breast cancer. One of the most challenging aspects of eradicating breast cancer and increasing the survival rates of women with breast cancer is the prevention and/or treatment of metastasis. Arguably, breast cancers that do not metastasize become a far more manageable form of the disease. Recent next-generation sequencing studies have confirmed that TP53 and CDKN2A gene mutations are the most frequent alterations in breast carcinomas, occurring in all subtypes of human breast cancer at varying rates. Our exciting new combined published and preliminary findings indicate that the activation of a common anti-viral signaling pathway is sufficient to drive enhance tumorigenesis and metastasis of breast cancer cells. This has led us to re-think the process of metastasis. Could it be that aggressive metastasis is the result of the activation of an anti-viral pathway? That is, can tumor cells use this anti-viral pathway to escape their local environment and move throughout the body to distal organs?

We hypothesize that a common anti-viral regulatory pathway provides p53/CDKN2A-mutant mammary tumor cells with a tremendous advantage in vivo and therefore, serves as a driver for growth and metastasis.
To test this hypothesis, we had proposed to deconstruct this pathway in highly metastatic cells that harbor TP53/CDKN2A mutations AND exhibit an activation of this pathway. We determined that we could trigger metastasis in TP53 mutant cells through the activation of this antiviral pathway. Our experiments uncovered both in vitro and in vivo aspects of growth, invasion, and metastasis, using established human breast cancer cells and primary mouse mammary epithelial cells. Our combined expertise in tumor suppressors and oncogenes along with our unlimited access to primary human tumor samples at Washington University gave us tremendous leverage in successfully obtaining large amounts of patient tumors to perform our analysis. Identification of required signaling components in aggressive TP53/CDKN2A mutant breast cancers will lead to the discovery of novel drugs targeting the key genes and genetic pathways responsible for breast cancer metastasis. How fast can we get there? The ant-viral pathway we have uncovered already has targeted drug therapies, suggesting that our timeline to the clinic could be within two years.

INTRODUCTION
Cancer is the accumulation of mutations in key regulatory genes that control cellular growth and proliferation. Two of the most frequently inactivated genes in human cancer are the TP53 and CDKN2A tumor suppressors. The p53 tumor suppressor is activated upon DNA damage or numerous forms of cellular stress, leading to the transcription or repression of p53 target genes that control DNA repair, cell cycle arrest, or apoptosis. p53 protein levels are tightly regulated by the MDM2 oncoprotein that binds to p53 and targets it for proteosomal degradation. The CDKN2A locus encodes both p14ARF (p19ARF mouse) and p16INK4A, which possess distinct tumor-suppressive roles. ARF’s canonical role as a tumor suppressor resides in its ability to activate p53 in response to oncogenic signals such as those emanating from the RAS, E2F1 and MYC oncogenes. Upon sensing oncogenic signals, ARF binds to MDM2 and sequesters it in the nucleolus, resulting in p53 accumulation. Thus, ARF and p53 work in conjunction with one another to sense oncogenic stress and halt unwarranted cell proliferation (Figure 1).

However, an additional layer of regulation exists between ARF and p53. Notably, p53 binds directly to the ARF promoter where it recruits histone deacetylases (HDAC) and polycomb group (PcG) proteins to effectively repress ARF transcription. Consequently, cells lacking p53 should express abnormally high levels of ARF through de-repression of the ARF locus. Indeed, in a p53-null setting, both ARF mRNA and protein expression levels are considerably elevated in vitro and in vivo.

The context behind the negative feedback between p53 and ARF has remained elusive. While one could argue that the primary role of p53 is to repress ARF in order to dampen the ARF response, an alternative view is that in this setting ARF becomes an effective sensor of p53 function, wherein loss of p53 releases ARF from this repression to act on new targets. The fact that TP53 and CDKN2A are co-inactivated in numerous cancers lends credence to the notion that p53 and ARF coordinate tumor suppression. Indeed, we recently showed that upon the acute loss of p53, de-repressed ARF attenuated tumorigenesis. Moreover, reduction of ARF in this p53-null context resulted in an induction of a type-I interferon (IFN) response, demonstrating that the type-I IFN network is a novel target for ARF in a p53-deficient setting. Although primarily studied as an antiviral pathway, increasing evidence has pointed to a tumor-promoting role for several members of the type-I IFN pathway, namely STAT1, JAK1, and ISG15.

In the antiviral setting, type-I IFN induction is predominantly triggered through the activation of cytosolic receptors that recognize double-stranded RNA (dsRNA). The dsRNA sensors retinoic acid-inducible gene-I (RIG-I) and melanoma differentiation-associated gene-5 (MDA5) each
contain a C-terminal DExD/H box RNA helicase domain that interacts with viral dsRNA. Their activation triggers downstream signaling cascades that lead to type-I interferon production (Figure 2). In this report, we show that in a p53-deficient setting, following ARF reduction, the canonical type-I IFN pathway is activated. Both JAK1 and phosphorylated-STAT1 are required for increased tumorigenicity. The JAK1 pathway is induced via the activation of both RIG-I and MDA5. However, activation is not through increases in dsRNA but instead due to aberrant RNA editing mediated by adenosine deaminase acting on RNA 1 (ADAR1). Significantly, restored ARF proteins readily prevented the increased expression of ADAR1 and ISG15 and reduced cell proliferation. Recently both ADAR1 amplification and augmented RNA editing via ADAR1 have been linked to enhanced tumorigenesis, underscoring the importance of ADAR1 as a new oncogenic target for ARF tumor suppression.

RESULTS

We have successfully utilized molecular biology, biochemical, and genetic techniques to uncover the dynamic role of the ADAR1/RIG-I/MDA-5 pathway in promoting the aggressive proliferation and metastasis of breast cancer cells in vitro and in vivo (Figure 3). The experimental procedures and resulting datasets are contained within the following manuscripts, in which we have acknowledged the support of the Longer Life Foundation:

ARF suppresses RIG-I/JAK1 signaling through nucleolar sequestration of the ADAR1 RNA editing enzyme, by Kuzmicki et al.; under review, Nature Communications.

ISGylation of novel substrates controls tumorigenesis, by Kuzmicki et al.; to be submitted January 2018.

ADAR1 expression drives triple negative breast cancer, by Maggi et al.; to be submitted March 2018.

Our findings clearly show:

• ADAR1 and RIG-I are required for the enhanced IFN-beta production of TP53/Arf-null cells.
• ADAR1 and RIG-I are required for the enhanced invasion phenotype of breast cancer cells.
• Elevated levels of RIG-I are required to drive the metastasis in mice.
• Elevated RIG-I expression alone is not sufficient to drive this phenotype. However, elevated ADAR1 is sufficient.
• Expression of ADAR1/RIG-I does predict aggressive triple-negative breast cancer in women.

We have successfully modeled this pathway in mice and have demonstrated its utility as a biomarker using three annotated databases:

• Human-in-mouse patient-derived breast tumor xenografts at the Xenograft Core Facility in the Siteman Cancer Center (Figure 4)
• Early-stage human breast cancer samples (210) through the St. Louis Breast Cancer Registry (Figure 5)
• Triple-negative human breast cancer samples (500) through Dr. Cynthia Ma at Washington University

We have also begun a preliminary analysis of this pathway in 100 primary ovarian cancer samples obtained by Dr. Katherine Fu at Washington University.
DISCUSSION

Recent studies have revealed a possible correlation between aberrant RNA editing and tumor progression, with both hyper and hypo RNA editing correlating with tumorigenicity. Coupled with increases in RNA editing, the overexpression of ADAR1 has been observed in numerous cancers, loss of which led to decreased tumorigenicity. Our data supports this notion and suggests that ADAR1 is critical in driving the transformation of cells lacking both functional p53 and ARF. Mechanistically, this is consistent with our data showing the nucleolar sequestration of ADAR1 by ARF. In this manner, ARF serves as a genetic sensor of p53 loss of function with ARFs heightened expression restraining ADAR1 and preventing A-to-I editing. Thus, when we overexpressed wild type ADAR1, we were in effect, overcoming ARF sequestration and driving free ADAR1 to edit RNAs. We suggest that the genetic context of the tumor is critical in determining ADAR1’s role, with co-inactivated p53 and/or ARF tumors exhibiting oncogenic ADAR1, perhaps explaining the inconsistencies of ADAR1’s role in tumorigenesis. We have also shown that this aberrant pathway is a functional driver of growth and metastasis in human breast cancers, underscoring its huge potential as a point of therapeutic intervention.

We have received the following NEW grant funding as a direct result of data obtained through the Longer Life Foundation sponsored work:

“Regulation of tumor suppression by ARF”
National Institutes of Health/National Cancer Institute
2016-2021
$228,000 Annual direct costs

“Targeting drivers of triple-negative breast cancer in African Americans”
Department of Defense
2018-2021
$200,000 Annual direct costs
Figure 1. The ARF and p53 pathway. ARF is negatively regulated by p53 while ARF is an indirect activator of p53 through its ability to sequester MDM2 in the nucleolus. ARF and p53 collaborate to negatively regulate the activation of JAK1 and the secretion of IFN-beta.

Figure 2. The ADAR1 pathway. ADAR1 edits RNAs in the cytosol where they are recognized by RIG-I and MDA-5 RNA helicases. TRIM25 activates both helicases leading to the downstream activation of JAK1 and production of IFN-beta.
**Figure 3.** In vivo breast cancer formation. When ADAR1 expression is elevated (left) in the absence of p53 and Arf, massive mammary tumors are observed along with aggressive metastasis to distal organs including the lung, liver and colon. When this pathway is off (right), only local disease is observed.

**Figure 4.** Human-in-mouse patient-derived breast tumor xenografts. Breast tumor samples were implanted into healthy immune-compromised recipient mice and allowed to grow over a period of several months. Resulting tumors were excised, fixed and stained with the indicated antibodies. Stained samples were scored using the Allred method with a 9 being the highest level of staining.
Figure 5. Primary human breast cancer samples. 200 primary human breast cancer samples were stained with the indicated antibodies and scored.