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14. ABSTRACT

We hypothesize that some leukocytes infiltrating breast cancers possess oncogenic mutations which affect breast cancer growth and metastasis. We are investigating this hypothesis in 3 different patient groups: 1) patients with newly diagnosed breast cancer and high amounts of tumor infiltrating leukocytes, 2) patients who developed secondary leukemias after treatment for breast cancer, and 3) breast cancer patients with clonal hematopoiesis (CH) incidentally found on genomic screens but with no apparent hematologic disorders. We have over 4,700 breast cancer patients who have undergone genomic testing of their tumors and peripheral lymphocytes. Of these patients, ~25% have CH. We have clinical information from these patients and are currently assessing whether key clinical features influence CH. For those CH patients who have primary tumor tissue available, we will also sequence the TILs in the primary breast cancer to evaluate for mutations associated with CH. 4) We are also prospectively collecting blood samples from patients pre/post neoadjuvant therapy and pre/post surgery to assess how chemotherapy and primary tumor presence may affect CH. From a functional standpoint, we have made progress in evaluating models to assess how mutant hematopoietic cells impact tumor growth. Specifically, we evaluated two genes, *Dnmt3A* and *Tet2,* in select models and have expanded our studies to assess the functional role of *Tet2* in a second transgenic model. Additionally, we have set up our assays to investigate first, whether select leukocytes produce inflammatory cytokines which impact breast cancer progression, second, whether Tet2 expression defines response to chemotherapy, and third, whether hypomethylating agents alone or in combination with chemotherapy lead to increased therapeutic efficacy.

15. SUBJECT TERMS

Characterization of the genomic landscape of tumor infiltrating hematopoietic cells in breast cancer. Evaluation of the functional interaction between mutant leukocytes and breast cancer cells.

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INTRODUCTION:

This proposal aims to identify a unique way in which the tumor microenvironment drives breast cancer growth and to determine novel ways to treat breast cancer based on these insights. Specifically, this proposal lays the foundation for understanding whether the acquisition of somatic mutations in cancer genes, in particular those related to leukemogenesis, within tumor-infiltrating leukocytes contributes to breast tumor initiation and/or progression. This study is based on our previous work which identified leukemia associated mutations within tumor infiltrating leukocytes in primary breast cancers. The mutations found in infiltrating leukocytes were not identified in the peripheral blood or epithelial cells of the same breast cancer patients. We hypothesize that breast cancers intimately interact with mutated leukocytes and that this interaction may contribute to breast cancer metastasis. To test this hypothesis clinically, we are investigating mutated leukocytes in 3 different patient groups: 1) patients with newly diagnosed breast cancer and high amounts of tumor infiltrating leukocytes, 2) patients who developed secondary leukemias after treatment for breast cancer, and 3) breast cancer patients with clonal hematopoiesis incidentally found on genomic screens but with no apparent hematologic disorders. From a functional standpoint, we are developing select models to determine whether mutations within hematopoietic cells can impact tumor growth and metastasis. We are utilizing these models to interrogate how breast cancer cells and mutant white blood cells interact and, importantly, how this affects therapeutic response. Despite efforts to develop targeted therapies against breast cancer cells and decrease metastatic growth, breast cancer metastasis continues to drive mortality. If the proposed aims are achieved, this work could dramatically transform breast cancer treatment for patients with newly diagnosed breast cancer, including those at risk for and with metastatic disease. Our studies are poised as the springboard from which we can develop novel therapeutic approaches, in which dual targeting of cancer cells and of tumor-associated leukocytes improves outcomes for breast cancer patients.

KEYWORDS: Provide a brief list of keywords (limit to 20 words).

Breast cancer, tumor infiltrating, leukocytes, mutations, *TET2*, *DNMT3A*, myeloid leukemia, secondary hematological malignancies, clonal hematopoiesis, inflammation, cytokines

ACCOMPLISHMENTS:

1. What were the major goals of the project?

Specific Aim 1: To evaluate the mutational spectrum of tumor infiltrating leukocytes in 150 sampes of primary triple-negative breast cancers. We will follow these patients and in those patients who develop metastasis, we will compare the mutational spectrum of tumor-infiltrating leukocytes in primary tumors to those found in metastatic lesions. We will also assess the mutation spectrum found in tumor-infiltrating leukocytes in 10 de novo metastatic breast cancer patients.

 Major Task 1: Recruitment of 150 triple-negative patients of 2.5 years with newly diagnosed, locally advanced (non-metastatic) breast cancer.

Subtask	**Month**	**Completion**
Request for Approval for Use of Human Subjects	1-3	100%
Accrual of 150 triple-negative newly diagnosed patients	4-24	~60%. We expanded our efforts to include not only triple-negative breast cancer patients but also hormone receptor positive and HER2+ breast cancer patients. We have over 85 samples collected from breast cancer patients. We also collected samples from 28 breast cancer patients who received neoadjuvant chemotherapy. We collected blood samples at each time point of chemotherapy as well as their pre-chemo biopsy and any remaining tissue at the time of surgery. We are in final stages of reviewing the sequencing data from the leukocytes in these samples.
Obtain samples from 10 patients with de novo metastatic disease with intact primary breast cancer	4-15	10%. To date we have re- focused our efforts on accruing biospecimens from patients with metastatic disease independently of their treatment status.
Obtain samples from patients who developed metastatic disease within 2.5 years	8-33	0% In our original cohort, only 1 patient developed presumed metastatic disease and she elected against a biopsy and further treatment. As part of a separate cohort, we are tracking patients with clonal hematopoiesis and metastatic disease.

 Major Task 2: Targeted sequencing analysis of tumor infiltrating leukocyte, germline, peripheral blood, and tumor DNA.

Subtask	**Month**	**Completion/Status**
Perform targeted sequencing on tumor- infiltrating leukocytes	4-32	At present, DNA samples from tumor infiltrating leukocytes, germline, peripheral blood, and tumor DNA

purified from primary and metastatic sites		have been submitted for sequencing analysis. We are finalizing the processing of 58 patient samples.
Validation of identified mutations in tumor- infiltrating leukocytes (including sequence analysis of peripheral blood and micro-dissected tumor cells)	18-36	In the original cohort of 27 patients, we did not identify somatic variants in sorted TILs from these patients. Whilst variants were present, the quality of the sequencing was insufficient to consider these mutations for verification by secondary analyses. We suspect this was due to limitations in our ability to sort and capture sufficient leukocytes in the samples processed. Moving forward, we have developed superior single cell sequencing analysis capacity such that identifying key mutations will be more rigorous. We have 58 samples that are being sequenced using superior methods, specifically Tapestri by Mission Bio.
Perform computational analysis of sequencing results and compare variants identified in tumor- infiltrating leukocytes to metastatic, peripheral blood, and tumor sequencing results	6-36	We are actively working with Mission Bio to optimize the dual DNA-sequencing and oligonucleotide-based antibody barcoding for immunophenotyping approach. We are final stages of optimization and anticipate the completion of this subtask within the next 9 months.

Milestone #1: *Identification of somatic mutations in tumor-infiltrating leukocytes in primary and metastatic disease (4-36).*

Specific Aim 2: To determine whether mutations within hematopoietic cells can impact tumor growth and metastasis.

 Major Task 3: Analysis of the impact of mutated hematopoietic cells on tumor growth and metastasis in mouse models of breast cancer.

Subtask	**Month**	**Completion/Status**
Request Approval for Use of Animals	1-3	100%
Assess the effect of *Tet2* mutated hematopoietic cells on tumor growth and metastasis in the E0771 breast cancer model	4-10	100%
Assess the effect of *Bcor* and *Tet2* mutated hematopoietic cells on tumor growth and metastasis in the transgenic	10-24	50%. We have completed an evaluation of *Tet2* loss of function in the MMTV/Neu model, however the experiments to evaluate the impact of *Bcor* mutations have not been performed. Although no differences in tumor

MMTV/*neu* model of breast cancer		latency between *Tet2* KO and WT mice have been observed, we are considering alternative models to determine if Tet2-deficiency alters tumor growth and metastatic potential as well as response to therapy, despite showing minimal difference in time to disease onset using MMTV/*neu* breast cancer models. Specifically, we are working with different types of breast cancer cell lines (MMTV-PyMY driven).

Milestone #2: *Determining the functional role of mutations in tumor-infiltrating leukocytes on tumor growth and metastasis using murine breast cancer models (4-24)*

Specific Aim 3: To interrogate functional interactions between breast cancer cells and white blood cells with somatic mutations and its relevance to therapeutic response

 Major Task 4: Elucidate inflammatory cytokine production from tumor-infiltrating leukocytes in murine models of breast cancer and human breast cancer.

Subtask	**Month**	**Completion/Status**
Assess the inflammatory cytokine production profile from specific hematopoietic CD45-positive subpopulations in the E0771 and MMTV/*neu* breast cancer models	10-24	10%. Since the submission of the last report, the first experiments have been ongoing. We are in the process of assessing the cytokine secretion potential of macrophages isolated from tumor-bearing wt or *Tet2* KO mice and macrophages from mammary fat pads of healthy mice (in collaboration with Dan Landau, Cornell). Instead of using E0771 and MMTV/*neu* breast cancer models we will use PY230 and PY8819 orthoropic breast cancer models because we have already robust and established CH models in the C57BL/6 background.
Interrogation of the therapeutic potential of hypomethylating agents and JAK inhibitors in murine models of breast cancer, alone and in combination with breast cancer therapy	16-30	10% (see pitfalls).
Determine secretomic profiles of tumor- infiltrating leukocytes in human breast cancers		

	12-30	0%

Milestone #3: *Mapping the deep functional phenotypes in major hematopoietic cell lineages in breast tumors using single cell cytokine profiling in mouse models and primary tumors of breast cancer (10-30)*

 Major Task 5: Present findings in national meetings and publish in peer-reviewed journals.

Subtask	**Month**	**Completion/Status**
Present at national meetings including the American Association for Cancer Research, American Society of Clinical Oncology, and the San Antonia Breast Cancer Symposium.	12-24	Not applicable
Subtask 2: Prepare work for publication in peer- reviewed journals	12-36	Not applicable
Subtask 3: Publish our work in peer-reviewed journals	12-36	As a result of the support from this grant, we published our work on secondary hematologic malignancies in breast cancer patients. Comen EA, Bowman RL, Selenica P, Kleppe M, Farnoud NR, Pareja F, Weigelt B, Hill CE, Alon A, Geyer FC, Akturk G, Reis-Filho JS, Norton L, Levine RL. Evaluating clonal hematopoiesis in tumor infiltrating leukocytes in breast cancer and secondary hematologic malignancies. J Natl Cancer Inst. 2019 Aug 27

Milestone #4: *To present out work in national meetings and to publish our findings in scientific journals in order to present out work to the breast cancer community, to build future collaborations, and to work towards the development of novel therapeutic approaches for breast cancer patients. (12-36).*

2. What was accomplished under these goals?

Specific Aim 1: To evaluate the mutational spectrum of tumor infiltrating leukocytes in 150 primary triple-negative breast cancer cases.

Major Activities:

1. Patient accrual and tumor tissue collection in newly diagnosed breast cancer patients
Specific Objective: To better characterize the genomic landscape of tumor-infiltrating hematopoietic cells in primary breast cancers.

Results:
A. Identification and tumor tissue collection: To test our hypothesis that select subsets of white blood cells harbor unique mutations, we enriched our primary breast cancer samples for tumors with a high leukocytic infiltrate. Towards this end, we expanded our screen search to include patients who were HER2+ and ER+. Because many patients with tumors >1cm are getting neoadjuvant therapy, we also started collecting blood samples from patients undergoing neoadjuvant chemotherapy. We collected blood samples from patients pre-, during and post-neoadjuvant therapy. These samples will allow us to evaluate for mutations associated with clonal hematopoiesis (CH) within peripheral blood samples. We are comparing these results with sequencing of TILs within biopsy and residual tumors (at time of surgery). We submitted 28 neoadjuvant breast cancer samples for sequencing and are finalizing the analysis currently.

Standard screening workflow (breast): Once we identify a potentially eligible patient, H&E slides are then reviewed by our breast pathologist (Dr. Hannah Wen, MSKCC) and scored for the level of tumor-infiltrating leukocytes by a breast pathologist within the Reis-Filho Lab (Dr. Fresia Pareja, MSKCC). Patients eligible for the study are then approached by Dr. Comen to obtain informed consent. In an effort to obtain more samples, Dr. Wen has involved the entire breast pathology department such that if any pathologist reviews a case with increased tumor- infiltrating leukocytes, we are notified and then try in reverse to consent the patient prior to planned surgery. Additionally, many tumors over 1>cm are funneled to neoadjuvant chemotherapy. To be able to include sample analysis on tumors <1cm, we are now employing a more streamlined tumor dissociation protocol as described below and single cell sequencing on a Mission Bio Tapestri device. This more sensitive technique has allowed us to collect not only samples from triple-negative breast cancer patients, but also smaller samples from ER+ and HER2+ patients. This approach has resulted in the collection of a total of 87 samples over the course of the project representative of all clinical subtypes defined by ER and HER2 status, and irrespective of tumor size. Furthermore, TIL yield has been improved using our new platform.

Standard sample collection workflow: For each patient, Dr. Pablo Sanchez Vela obtains fresh tumor specimens, peripheral blood and saliva samples at the time of their primary surgery. Tumor tissue, including stromal cells and tumor-infiltrating leukocytes, are dissociated from the primary tumor using the Miltneyi GentleMACS dissociator utilizing a papain based enzymatic dissociation. After generating a single cell suspension, CD45+ cells are viably frozen alongside an aliquot of CD45- tumor cells. We have optimized the freezing and thawing processing such that viably frozen cells can be batch processed for flow and sequencing by either traditional bulk NGS or single cell DNA seq with antibody-oligonucleotide barcoding for subpopulation identification.

Lung/colon: As of last year, we are also collecting samples from lung cancer patients as well, including primary tumors, saliva and blood samples. We will perform the same analysis as described above to investigate the presence of mutations in TILs in lung cancer patients. So far, we have successfully collected 19 lung cancer cases and 4 colon cancer cases. Our aim is to get 20 lung cancer cases and 20 colon cancer cancers. The rationale for this expansion was the recognition that CH and mutated TILs could be present in a variety of solid tumors, and our aim is to define the generalizability of the findings in breast cancer samples. Our pilot collection with lung cancer has been successful, and tumor size, a barrier experienced in the context of the accrual of breast cancer samples, has proven not to be an impediment in the case of lung cancers. We plan to sequence these samples using the Mission Bio Tapestri platform as described below.

B. Targeted capture sequencing: To date, we have accrued a total of 87 patients. The complete set of samples from 27 of these patients have been processed, and we were successful in the purification and extraction of sufficient amounts of DNA for downstream sequencing analysis in 24 out of these 27 patients. All samples including those obtained from tumor, different hematopoietic subsets, granulocytes from peripheral blood and saliva (germline control) have been submitted for sequencing. In our first batch analysis of these 27 samples (see below), the quality of the sequencing was insufficient to consider these mutations for verification by secondary analyses. Since Dr. Kleppe left the lab, Dr. Bowman has now employed new methods of analysis. For the remaining TIL cases and those collected prospectively, we will use Tapestri by Mission Bio improved mutation detection, as described below.

C. Analysis of sequencing data from primary breast cancer samples: To date, we submitted DNA from blood, tumor, saliva and different sorted leukocyte populations from 27 breast cancer patients for sequencing analysis. To generate mutational profiles, sequencing was performed using a targeted panel covering 156 myeloid genes at an average depth of 600x on Illumina HiSeq 4000 (~100 bp paired-end reads). The raw sequence data was aligned to GRCh37 reference genome using BWA-MEM algorithm (v. 0.7.12-r1039). The data quality was assessed using FastQC (v. 0.11.5). Candidate substitutions and insertions/deletions were called using cgpCaVEMan (v. 1.7.4) and cgpPindel (v. 1.5.4) algorithms. These methods provide post-hoc filters that remove systematic sequencing artifacts as well as artifacts that arise from mapping errors. All candidate mutations were compared to COSMIC (v. 81), ExAC (v. 03.12) and 1000 Genomes (phase 3 release) databases to provide further annotation that would help to exclude common mutations in normal populations and identify somatic mutations. Each identified variant was manually visualized using Integrated Genomics Viewer (v. 2.3.92) to ensure the high quality of the variant at the sequence level. We compared variants found in TILs, peripheral blood and tumor cells. Whilst variants were present, the quality of the sequencing was insufficient to consider these mutations for verification by secondary analyses.

Pitfalls and Alternative Approaches:

1. Sample Size: Since last year, we have significantly circumvented our issues with sample collection. By broadening the breast cancer spectrum, including breast cancers of any estrogen receptor and HER2 status, and smaller tumors, we have been able to collect over 50 more samples. Our sequencing methods as per above were not sufficiently sensitive in our original group of 27 patients to detect with confidence select mutations. As we have improved our sample collection, we will run our newly acquired samples through the below platform. Moreover, with the

appointment of Jorge Reis-Filho as the PI, we have established a better integration with the Precision Pathology Biobanking Center and the Department of Pathology. In addition, based on the infrastructure available at MSKCC, we have now appointed a dedicated research study assistant to identify cases for pathology review on a daily basis.

2. Sequencing Analysis: We have strong circumstantial evidence to suggest that the inadequate sequencing quality of the first batch of 27 patients was largely due to limitations in our ability to sort and capture sufficiently sized populations of leukocytes. Moving forward, we have developed superior single cell sequencing analysis capacity such that identifying key mutations will be more rigorous. We have recently optimized a platform that will allow for single cell DNA sequencing across a panel of genomic loci often mutated in CH. This platform, Tapestri by Mission Bio, has a greater detection capacity than that of traditional sequencing technologies, and provides a better approach for identifying rare CH clones within a tumor. This single cell sequencing system is now available in both the Reis-Filho and the Levine laboratories; the methods, protocols and standard operating procedures have been fully implemented. We have altered our cell isolation strategy to use a magnetic bead based isolation strategy for CD45+ cells. These cells, in addition to the CD45- flow through, were subsequently cryopreserved, and are being sequenced using the Mission Bio Tapestri device. We are currently optimizing oligonucleotide barcoding of antibodies for concurrent sequencing-based immunophenotyping paired to single cell DNA mutation detection. By utilizing this single cell technology, we will circumvent what has been the greatest limiting factor in our success to date, isolating quality DNA from limited cell quantities for bulk sequencing. Furthermore, since we are not limited by sorting only 4 immune populations on a FACS Aria, we can now resolve the cells that harbor the CH mutations with substantially greater precision and resolution. We anticipate processing our samples within the next few months.

2. Sequencing analysis of tumor infiltrating leukocytes from breast cancer patients with incidental clonal hematopoiesis

Specific Objectives: To assess whether clonal hematopoietic cells are enriched in primary breast tumors.

A. Approach and Results: The Levine laboratory and other groups have recently demonstrated that a subset of older individuals have clinically inapparent, CH characterized by recurrent somatic mutations in genes previously associated with myeloid cancer. Our original plan at the beginning of this proposal lead to the identification of 7 breast cancer patients with incidentally diagnosed CH. The specific mutations associated with CH had already been identified through sequencing analysis of the peripheral blood samples from these patients (this effort was previously described in last year's progress report). Breast cancer tumor blocks for these patients were also retrieved, reviewed and processed. Originally, laser capture microdissection was performed on representative tissue sections to isolate tumor infiltrating leukocytes. The original plan was to then use AmpliSeq sequencing to test for the absence/presence of the mutations identified in the peripheral blood in the tumor infiltrating leukocytes. In the past year, however, we have modified our approach to this project given the tremendous advance in assessing CH at MSKCC. MSKCC has now sequenced over 5,000 breast cancer patients tumors and peripheral blood. Moreover, over ~25% of these patients were found to have CH. We have access to all the clinical information for the aforementioned patients and the clonal hematopoietic clone is already known for each patient.

From the patients with incidental CH, the Jorge Reis-Filho and Levine laboratories are now collaborating together to reviewed TILs in select breast cancer patient breast tumor blocks. We are also working to optimize CH assays so that we can evaluate larger numbers of breast cancer patients.

Pitfalls and alternative approaches:
Given that we will retrospectively work with breast cancer tumor blocks, we cannot microdissect specific hematopoietic subsets. Hence, the implementation of the Mission Bio Tapestri device has been proven instrumental to circumvent the pitfalls we experienced earlier in the course of this project. We aim, however, to address the question of whether mutated leukoctyes are found within the tumors and, more importantly, if these cells are enriched in the tumor as compared to peripheral blood. Efforts to understand the role of mutated TILs in breast cancer patients align with our murine modelling efforts as described in this report.

B. Sequencing analysis of tumor infiltrating leukocytes from breast cancer patients with secondary hematological malignancies

Specific objectives: To investigate whether mutations found in the hematopoietic cells of patients with secondary hematological malignancies following breast cancer are already present in white blood cells infiltrating primary breast cancers.

Approach and Results: Our previous work raises the possibility that some patients may be at increased risk for secondary leukemias based on the presence of oncogenic mutations in infiltrating white cells, which pre-exist before systemic therapy, and would subsequently be selected for by cytotoxic chemotherapy. Breast cancer patients exposed to chemotherapy have an increased risk of secondary hematologic malignancies. To date, there is no reliable biomarker to determine who is at risk for a future leukemia. We performed a retrospective study to determine whether breast cancer patients harbor leukemia associated TILs years before their leukemia diagnosis. We screened for patients with secondary hematological malignancies following breast cancer using the following criteria: >20% blast count (bone marrow or PBMC), access to viably frozen MNC cells or bone marrow aspirate slides (diagnosis) and breast tumor blocks, and suitable for laser-capture microdissection. Using this approach, 12 cases were identified, of which seven samples yielded sufficient tumor infiltrating leukocytes and tumor cells for sequence analysis. First, we sequenced the matching secondary leukemia sample using a targeted sequencing panel of 585 genes commonly mutated in leukemia, lymphoma, and solid tumors and. In parallel, we subjected the DNA samples extracted from laser-capture microdissected and isolated TILs and tumor cells using a targeted amplicon sequencing to define whether the somatic genetic alterations found the leukemia samples would be present in the TILs and tumor cell samples retrieved from the breast cancer specimens from the respective patients. Our analysis led to a recent publication: **Comen EA, Bowman RL**, Selenica P, **Kleppe M**, Farnoud NR, Pareja F, Weigelt B, Hill CE, Alon A, Geyer FC, Akturk G, **Reis-Filho JS**, Norton L, Levine RL. Evaluating clonal hematopoiesis in tumor infiltrating leukocytes in breast cancer and secondary hematologic malignancies. J Natl Cancer Inst. 2019 Aug 27. Specifically, we demonstrated that in four out of the seven patients (Patients #2, 4, 6 and 7), mutations which were present in the leukemia were also present in the TILs of the breast cancer samples (**Figures A and B**, below). In patient #2, we observed mutations

in *TGFBR2* (p.E150K) and *DNMT3A* (p.S638P) in the pre-treatment breast cancer TILs (VAF of 3.10%, 10 mutant /32 total reads and 1.00%, 9 mutant /902 total reads respectively), which were enriched relative to tumor cell samples (VAF of 2.87%, 6 mutant /209 total reads and 0.21%, 1 mutant /477 total reads respectively). In patient #4, the breast cancer TILs were found to harbor *TET2* (p.C1263Y) and *DNMT3A* (p.L639H) mutations, which were clonal in the post-treatment leukemia, at VAFs of 5.87% 1297 mutant /22082 totals reads and 2.88%,12 mutant /417 total reads respectively, whereas these mutations were detected at a much lower proportion (VAFs of 0.73%, 156 mutant /21450 total reads and 0.56%, 5 mutant /894 total reads respectively) in the tumor cell samples. In Patient #6, we identified the known oncogenic *IDH2* R140Q mutation in the TILs, which was clonally present in the therapy-related leukemia. This alteration was detected at a VAF of 1.27% (100 mutant /7862 totals reads) in the TILs versus 0.49% (49 mutant /9962 totals reads) in tumor cell samples. In patient #7, we found that TILs had an increased VAF for the *NRAS* p.G12A mutation, which was present in the post-treatment leukemia. The *NRAS* mutation was present at a MAF of 0.72% in the TILs (2 mutant /277 totals reads), by contrast none of the sequencing reads from microdissected tumor cells harbored the mutation (229 total reads). See Figure 1 A/B below.

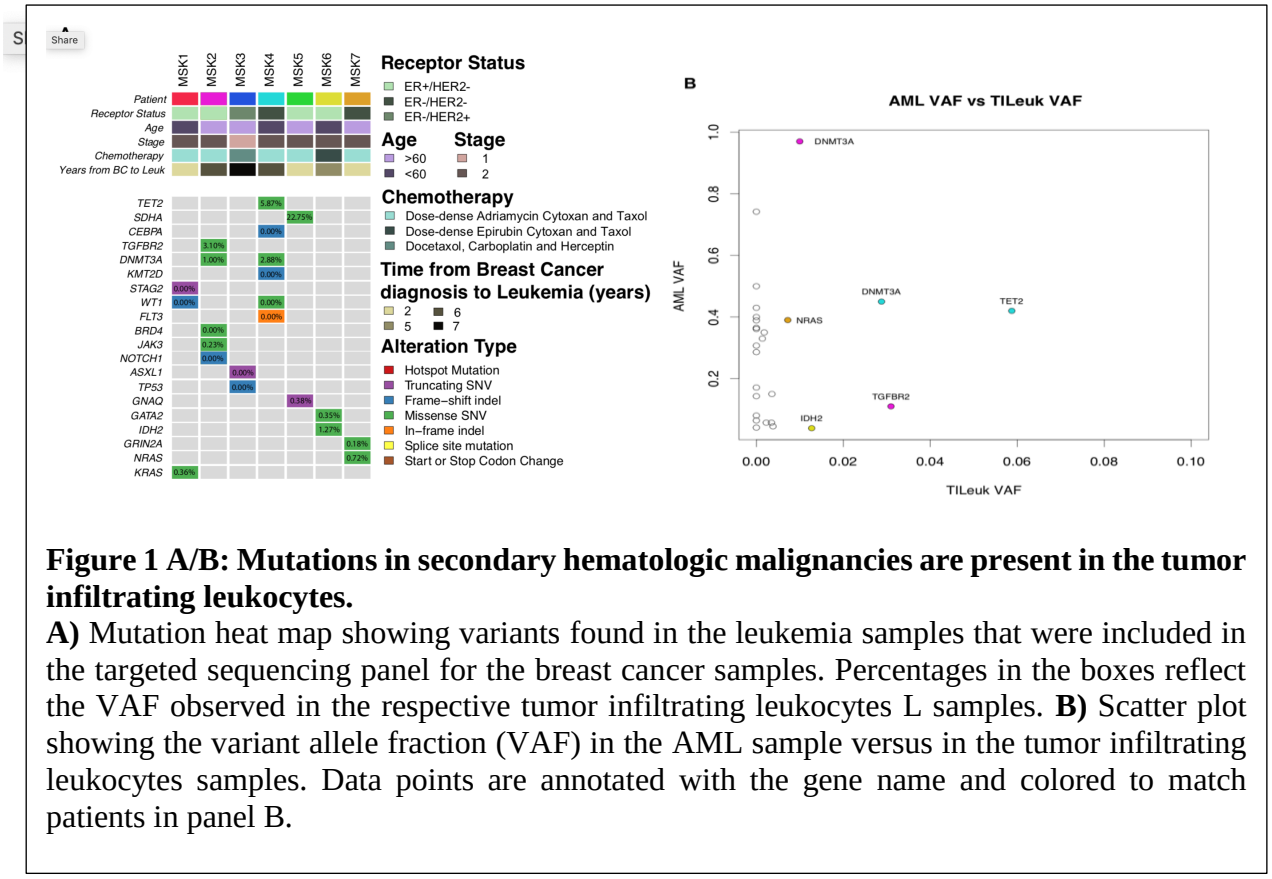


Figure 1 A/B: Mutations in secondary hematologic malignancies are present in the tumor infiltrating leukocytes.
A) Mutation heat map showing variants found in the leukemia samples that were included in the targeted sequencing panel for the breast cancer samples. Percentages in the boxes reflect the VAF observed in the respective tumor infiltrating leukocytes L samples. **B)** Scatter plot showing the variant allele fraction (VAF) in the AML sample versus in the tumor infiltrating leukocytes samples. Data points are annotated with the gene name and colored to match patients in panel B.

These analyses provide direct evidence that CH is not limited to peripheral blood leukocytes but rather can be detected in tumor-infiltrating hematopoietic cells. In patients who subsequently develop leukemia, TILs in the primary tumor harbored mutations that were present in the leukemic

clone years later. This is consistent with the notion that pre-leukemic clones are present at the time of a diagnosis of a solid tumor, pre-therapy, and precede the subsequent development of leukemia.

Pitfalls and alternative approaches:

1. **Processing of patient tumor specimen and approach to mutation identification:**

One limitation of the study was that blood samples at the time of each patient's breast cancer diagnosis were not available. Therefore, we were not able to sequence blood samples to assess for peripheral CH associated mutations in the peripheral blood at the time of breast cancer diagnosis. We have, however, previously shown that CH mutations were preferentially enriched in breast cancer TILs compared to peripheral blood samples. Furthermore, despite the limited number of patients analyzed in this study, we were able to detect the presence of the mutations present in the leukemic clone in the breast cancer TILs in four of the seven patients analyzed. Despite the use of laser capture microdissection, the presence of a small proportion of TILs in the tumor cell samples cannot be ruled out in breast cancers where leukocytes are admixed with tumor cell clusters.

2. Patient accrual

A. To date, it is not known when during the exposure to chemotherapy, CH evolves. This is particularly important to understand in light of those women who may be at risk for secondary hematologic malignancies as a result of receiving chemotherapy. To help elucidate this process, we are collecting blood samples among women undergoing neoadjuvant chemotherapy. Blood samples are taken pre-chemotherapy, during each cycle of chemotherapy and at the completion of chemotherapy. We will analyze these samples for CH and track the potential evolution of CH clones therein.

B. As a result of our pilot work evaluating the TILs in seven breast cancer patients who developed secondary hematologic malignancies, we sought to increase our sample size analyze of patients who developed secondary hematologic malignancies. We have since identified 36 epithelial cancer patients who developed t-MN, from whom clinical and genomic profiling data at the time of the leukemia diagnosis are available. In addition to the original untreated solid tumor samples from which we can isolate tumor cells and tumor-admixed hematopoietic cells, we have blood samples from 6 of the 36 patients within seven weeks or less of their solid tumor diagnosis. This will allow us to define whether the clone harboring the genetic alterations present in the t-MN is present in peripheral leukocytes compared to infiltrating solid tumor leukocytes at solid tumor diagnosis and to ascertain whether there is enrichment for mutant clones within the tumor compared to the peripheral hematopoietic compartment. By defining the presence and characteristics of mutated infiltrating leukocytes among solid tumor patients who developed secondary leukemias, we will address whether the identification of CH mutations in solid tumors can help risk stratify those at risk for leukemia.

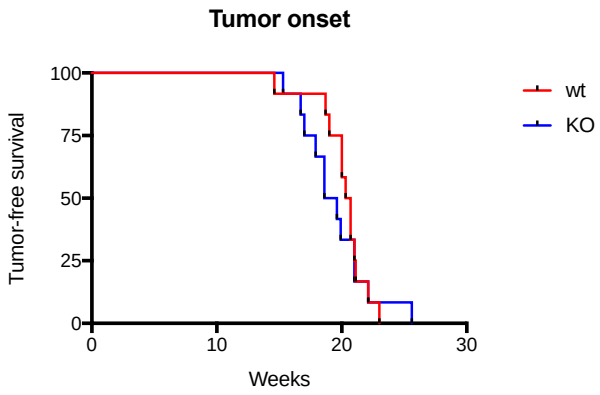
Specific Aim 2: To determine whether mutations within hematopoietic cells can impact tumor growth and metastasis.

Major Activities:

1. Effect of *Tet2* mutated hematopoietic cells on tumor growth and metastasis in different breast cancer models

Specific objective: Determine if hematopoietic deletion of TET2 alters time to disease onset in a Erbb2 driven model.

Approach and Results: Here, we bred the *Tet2* Vav:Cre mice to the MMTV:Neu/Erbb2 model of mammary tumorigenesis. To accelerate tumor formation, we synchronized female mice by inducing superovulation and pregnancy. This has been shown previously to accelerate the Erbb2 models and, critically, reduce the variability in disease onset and penetrance. We next monitored mice for the onset of disease and found no difference between *Tet2* KO and WT mice with regard to the time until tumors emerged (data shown to the right). We will continue to evaluate whether these tumors possess altered immune composition and patterns of metastatic outgrowth.



Pitfalls and alternative approaches: Although no differences in tumor latency between *Tet2* KO and WT mice have been observed, we considered alternative models to determine if Tet2-deficiency alters tumor growth and metastatic potential as well as response to therapy, despite showing minimal difference in time to disease onset. Specifically, we have tried to orthotopically implant various breast cancer cell lines (MMTV-PyMY driven) in c57bl/6 with the results as follows:

A. BRC-P cell line: Inconsistent engraftment and slow growth with only one of the nineteen mice showing tumor formation after four months.

B. Py8119 undifferentiated mesenchymal cell line: Full engraftment even at low cell number. Tumors develop after only one month.

C . Py230 epithelial-like cell line: High engraftment. Tumors develop more slowly than Py8119 after two months.

We will continue to work with the above breast cancer cell lines to see how Scl-CreERT^{+} (Tet2 fl/fl or Dnmt3a fl/-), and Scl-CreER^{T}- models impact immune composition, patterns of metastatic growth and response to therapy.

Specific Aim 3: To interrogate the functional interactions between breast cancer cells and white blood cells with somatic mutations and its relevance to therapeutic response

Major Activities:

1.Interrogation of the therapeutic potential of hypomethylating agents and JAK inhibitors in murine models of breast cancer, alone and in combination with breast cancer therapy.

Approach and Results: Given our difficulty in generating orthotopic breast tumors, we opted to evaluate JAK1 inhibition *in vitro* while we optimized tumor take *in vivo*. Here we directly

evaluated JAK1 inhibition in its capacity to affect *Tet2* mutant hematopoietic cell self-renewal. We found that prolonged treatment with the JAK1 inhibitor, INCB052793, led to decreased colony formation compared to no treatment alone.

Pitfalls and alternative approaches: The most significant pitfall thus far is an inability to reliably generate orthotopic breast cancer models. One alternative approach is to use genetic mouse models including both the MMTV/Neu and MMTV/PyMT model. Whilst we have employed these models, limited differences have been identified when crossed to a *Tet2* hematopoietic specific knockout mouse. The reasons for this may stem from 1) the kinetics of these models are either too fast or too asynchronous to identify a specific role for *Tet2* mutant cells or 2) the sheer number of mutant cells in the genetic mouse may overwhelm any subtle effects of *Tet2* mutations, 3) a purely genetic system in the hematopoietic compartment may mask any potentially subclonal interactions between *Tet2* mutant and WT immune cells, and lastly 4) *Tet2*-mutant cells may simply not play a role in tumor progression. To evaluate the first three possibilities fully we feel it is critical to implement orthotopic models so that we can control tumor burden, time of tumor initiation and implant tumors in mixed chimeras where the *Tet2* allele burden can also be controlled. In prior years, Dr Kleppe used transgenic mouse models in a BALBc strain background (MMTV:Neu/Erbb2 driven). As an alternative, this year we are expressly working out the best orthotopic breast cancer model in a c57bl/6 mouse strain background (the same that our lab has implemented for CH and leukemia models). We have also tried different injections techniques (open breast surgery under anesthesia and percutaneous intramammary injections). Percutaneous injections have proven to be faster and have shown similar (if not better) results compared to those of an open surgery. Now that we have a working model we will start the in vivo trials with the JAK1 inhibitor.

What opportunities for training and professional development has the project provided?

For Dr. Comen, this project has elicited tremendous interest and collaboration across not only the breast medicine service but associated hematologic and other solid tumor services. Specifically, Dr. Comen is now collaborating with not only her leukemia colleagues but the colon and lung cancer teams at MSKCC. This work is part of larger collaborative efforts at MSKCC to evaluate CH in solid tumor patients. Dr. Comen is helping to lead these efforts on the breast medicine service. As such, recognition for this work has also contributed to her being recently promoted. For Dr. Jorge Reis-Filho this approach has helped develop better bioinformatics pipelines for the detection of subclonal mutations in cancer and the detection of CH in tumor specimens; in turn, this resulted in the development of new collaborative endeavors with members of the Breast Medicine Service to study the impact of TILs harboring leukemogenic mutations in the biology and clinical behavior of human cancers.

How were the results disseminated to communities of interest?

Nothing to report.

What do you plan to do during the next reporting period to accomplish the goals?

Describe briefly what you plan to do during the next reporting period to accomplish the goals and objectives.

Specific Aim 1: To evaluate the mutational spectrum of tumor infiltrating leukocytes in 150 primary triple-negative breast cancer cases.

A. To accrue additional patients with primary and metastatic breast cancer: As previously described, we have changed our approach to accrual and are confident we can substantially increase our accrual to our study. In 2016, 165 women had breast surgeries for triple-negative breast cancer. Initially, we aimed to accrue only patients with locally advanced triple-negative breast cancer. In the last several years, almost all patients with triple-negative breast cancer or HER2 positive breast cancer are funneled into neoadjuvant therapy and/or clinical trials. This rendered patient's ineligible for our study. We have previously described our viable efforts to expand sample selection and employ new platforms which allow us to evaluate smaller tumors of any receptor status. As described previously, we have expanded our analysis to include evaluating CH in a variety of settings: First, we are evaluating patients undergoing neoadjuvant therapy (28 patients to date). Second, we are also analyzing their blood samples both pre and post surgery to evaluate whether the presence of a tumor changes CH (20 patients).Third, we are collecting surgical tissue specimens from primary untreated tissue specimens and metastatic samples independent of their treatment status. Fourth, we are continuing to track patients to evaluate for metastatic disease but are not focusing our efforts on *de novo* metastatic patients.

B. To analyze and validate sequencing data from primary breast cancer samples: We have over 50 primary samples that we will employ single sequencing techniques as described above.

C. To identify and analyze additional breast cancer cases with CH: Originally, we sought to evaluate CH in breast cancer patients from patients recently and prospectively sequenced (both blood and tumor for somatic mutations). The intent was to then select cases with CH and use laser-capture microdissection to purify tumor- infiltrating leukocytes from breast tumor tissue to assess whether mutations observed in the circulation can also be found in tumor-infiltrating leukocytes. More recently however we have gained access to a breast cancer cohort that will allow us to assess effects of CH over time in breast cancer patients. The samples for this aim come from banked samples of newly diagnosed women with early stage breast cancer between 1999 and 2010. There are 2,791 unique breast cancer patients (341 with DCIS, 2450 with invasive breast cancer) with banked blood samples at diagnosis and prior to any surgery or therapy. The invasive breast cancer and DCIS samples were obtained when patients underwent primary breast cancer surgery at MSKCC. In addition to blood samples from newly diagnosed breast cancer patients, we have an additional 1,730 blood samples from 1,730 unique nonaffected women (controls) that were collected under the same biospecimen protocol. To ensure the cohorts are similar, we will assemble the 800-sample cohort as follows:

A. Randomly choose 500 patients from 2,450 patients with invasive breast cancer;

B. Frequency match the 500 invasive patients with 150 DCIS patients by 5-year increments of age at the time of the banked blood draw and 2-year increments of the year that the blood was drawn;

C. Frequency match the 500 invasive patients with 150 controls on 5-year increments of age at the time of the banked blood draw and 2-year increments of the year that the blood was drawn.

The rates of CH in early-stage BC patients diagnosed and treated at Memorial Sloan Kettering Cancer Center between 1999 and 2010 will be compared to those of patients who had ductal carcinoma *in situ* (DCIS) and women who had no evidence of cancer (controls). We will frequency match invasive breast cancer patients with DCIS patients on 5-year increments of age at the time of the banked blood draw and 2-year increments of the year that the blood was drawn. Standardized and prospectively collected long-term follow-up and detailed clinical information since diagnosis has been collected for this patient population. For patients with invasive BC, we will sequence matched peripheral blood samples to enumerate for CH as well as primary breast tumor samples for somatic alterations within cancer cells. For breast cancer patients, this is an exploratory analysis of how CH may impact the spectrum of somatic alterations within breast cancer cells. The advantage of this cohort of patients is that we have long term follow up on these patients. For the aforementioned project, we have applied for additional funding from a DoD Expansion award.

D. To sequence breast tumor, and TIL sample from patients with secondary hematological malignancies by sequencing of microdissected samples and single cell sequencing: As an extension of the former manuscript, we will evaluate over 36 patients with a history of solid tumors who developed secondary hematologic malignancies. We will evaluate the TILs in the original primary solid tumor to see if solid tumor TILs possessed evidence of the future leukemia clone for each patient. Methods will be performed as described earlier.

Specific Aim 2: To determine whether mutations within hematopoietic cells can impact tumor growth and metastasis.

Effect of *Tet2* mutated hematopoietic cells on tumor growth and metastasis in a an orthotopic MMTVPyMT breast tumor model: We used MMTV:PyMT-derived cell line (BRC3) developed by our collaborator Nir Ben-Chetrit in the Landau lab at Cornell. This cell line has been shown to possess robust metastatic capacity to the lung when injected into either the mammary fat pad as a "spontaneous metastasis model" or through tail vein injection as a "colonization model". Despite the efforts expended with this cell line inconsistent engraftment in addition to slow growth was shown. After 5 months, only one of the nineteen mice showed signs of tumor formation. In contrast Py8119 and Py230 show consistent engraftment even at lower cell numbers, which makes them ideal candidates for these studies.

To expedite the execution of the experiments, we will transplant Scl-CreER^T + (Tet2 fl/fl or Dnmt3a fl/-) or Scl-CreER^T – bone marrow into irradiated recipients and allow for engraftment over the course of 8 weeks. At this time point, we will inject 1x10^6 BRC3, 0.5x10^6 Py8119 or 0.5x10^6 Py230 cells into the mammary fat pad. We will closely monitor for tumor growth. Eight weeks post injection, we will sacrifice mice and harvest the primary tumor, lungs and liver for histological and flow cytometric evaluation. Flow cytometric analyses will permit an investigation of the immune infiltrate into lung metastases to determine if Tet2 loss of function skews the quantity of T, B, NK or myeloid cells, while histological analyses will be used to evaluate any changes in spatial localization of these immune cells. We will also carry out immunohistochemical assays to assess tumor cell proliferation (Ki67) and apoptosis (cleaved caspase 3). We will subsequently repeat these studies in a tail vein model of lung colonization, evaluating tumor cell number in the lung at 48 hours post injection to assess metastatic seeding, and at 6 weeks to assess metastatic outgrowth.

As next steps, we recently transplanted several mice with either Scl-CreER^T-, Scl-CreER^T+Tet2 fl/fl or Scl-CreER^T +Dnmt3a fl/- bone marrow. We will repeat the orthotopic injections in this context and we will monitor for tumor growth and metastatic spread. We will also generate a stable cell line expressing a fluorescent reporter and luciferase to help tract the tumors when performing experiments in vitro and metastasis formation in vivo.

Specific Aim 3: To interrogate functional interactions between breast cancer cells and white blood cells with somatic mutations and its relevance to therapeutic response.

To evaluate the molecular interactions between breast cancer cells and mutant hematopoietic cells, we will utilize a tumor-stroma organoid 3D culture system developed by our collaborator Dr. Nir Ben-Chetrit from the Landau lab at Cornell. This multicellular co culture system allows for the interrogation of macrophage function and their capacity to support tumor cell proliferation as well as suppression of T and NK cell-mediated killing. Here we will co-culture *Tet2* mutant or wild-type bone marrow with BRC3, Py230 or Py8119 cells and their associated stroma. In these co-cultures, we will evaluate the proliferative and apoptotic indices of both the cancer cell and immune cells. This experimental setting will allow us to test whether mutations in genes such as *Tet2* or *Dnmt3a* lead to altered phenotypes in the cancer cell compartment. The unique feature of this platform is its capacity to measure T-cell activation *ex vivo*, and as such we will be able to assess whether the mutations described above will have alter the known immune suppressive effects of tumor-associated macrophages.

IMPACT:

What was the impact on the development of the principal discipline(s) of the project?

Our work has generated substantial interest in our leukemia and solid tumor colleagues for several reasons. First, tumor infiltrating leukocytes are not unique to breast cancers but are known to influence prognosis in a variety of solid tumors. Recent research and the successful development of immunotherapy approaches across many solid tumors underscore the essential role the immune system plays in surveillance, tumor initiation and progression. We have previously demonstrated that many of these white blood cells, while appearing morphologically normal, actually have acquired mutations in known cancer genes. These mutations were either not found or present at a lower frequency in peripheral white blood cells, tumor cells themselves or other normal cells in the body. Moreover, many of these mutations were associated with leukemia. This suggests that mutant infiltrating white blood cells may interact with cancer cells, which has significant clinical implications for tumor development and response to treatment. Our colleagues are interested in investigating this phenomenon not only in breast cancer patients but in a variety of other solid tumors, including cancers associated with *BRCA1, BRCA2* and *CHEK2* mutations. Second, it has long been believed that secondary leukemias after treatment for breast cancer are uniquely related to chemotherapy. Moreover, patients with a variety of solid tumors are at risk for future secondary leukemias. Third, roughly 25% of breast cancer patients harbor CH mutations. As a result of our preliminary data, we are now investigating additional groups of breast patients: 1) we are sequencing peripheral blood cells from newly diagnosed breast cancer patients before receiving neoadjuvant therapy and after treatment completion. This will provide insight as to whether CH mutations are present in newly diagnosed breast cancer patients and whether chemotherapy modifies the mutations in peripheral blood cells. This is particularly important since CH mutations in solid cancer patients are associated with adverse clinical outcomes; 2) we are sequencing peripheral blood cells from breast cancer patients (with no chemotherapy exposure) before and after surgical resurrection. We will compare the mutational landscape of peripheral cells in patients both pre and post-surgical resection. We believe that the presence of the tumor cells may effect the "fitness" of mutant white blood cells and in turn that mutated white blood cells may affect the behavior of tumors. 3) Our work has generated significant interest in how we genotype tumors. At present, when a mutation is identified in a given cancer the assumption is that this mutation is in the cancer cells themselves; we posit that in some instances the identifying mutation may actually be in an infiltrating white blood cell. This has important implications for targeted therapies which are matched to select mutations. 4) In a group of breast cancer patients with a history of secondary lethal leukemias, we have provided direct evidence of the presence of the leukemic clone in the TILs present in the breast cancer sample years before the leukemia diagnosis. To date, there is no ability to predict who is at risk for therapy-related/ therapy-induced neoplasms. Absent a predictive biomarker for secondary hematologic malignancies, clinicians blindly counsel patients on chemotherapy risks without an ability to refine treatment based on risk. Determining which early stage breast cancer patients are at highest risk for secondary hematologic malignancies is a crucial unmet medical need. Moreover, CH can increase the risk for other epithelial cancer as well as increase the risk of other co-morbidities including heart disease. Identifying patients early on in treatment who may be at risk for t-MN, other solid tumor malignancies as well as heart disease may help refine treatment rendered and screening for additional malignancies and disease. Alongside the clinical efforts, our models of disease provide a powerful platform to identify therapeutic avenues tailored to tumors with mutated immune cells. This fits a critical unmet need

in the field, especially given the large percentage of patients anticipated to possess mutant immune cells in their tumors, and the growing interest in immune-targeted therapy.

Fundamentally, we are confident that our findings will reveal whether mutated leukocytes contribute to tumor growth and metastasis. We also believe that mutated leukocytes may be clinically relevant in patients with CH and those patients who may develop secondary leukemias after treatment for breast cancer.

What was the impact on other disciplines?
Nothing to report.

What was the impact on technology transfer?
Nothing to report.

What was the impact on society beyond science and technology?
Nothing to report.

CHANGES/PROBLEMS:

I. Changes in approach and reasons for change.

Describe any changes in approach during the reporting period and reasons for these changes. Remember that significant changes in objectives and scope require prior approval of the agency.

A. Sequencing studies of peripheral blood cells from patients with breast cancer.

A1. Clonal hematopoiesis:

As explained in Specific Aim 1C above, we have identified a more robust and powerful cohort to examine CH in breast cancer patients. This aim addresses how CH may be a biomarker for breast cancer initiation as well as risk of poor outcome, including overall survival, disease free survival, and secondary cancers including leukemia. The rates of CH in early-stage BC patients diagnosed and treated at Memorial Sloan Kettering Cancer Center between 1999 and 2010 will be compared to those of patients who had DCIS and women who had no evidence of cancer (controls). We will frequency match invasive breast cancer patients with DCIS patients on 5-year increments of age at the time of the banked blood draw and 2-year increments of the year that the blood was drawn. Standardized and prospectively collected long-term follow-up and detailed clinical information since diagnosis has been collected for this patient population. For patients with invasive BC, we will sequence matched peripheral blood samples to enumerate for CH as well as primary breast tumor samples for somatic alterations within cancer cells. For breast cancer patients, this is an exploratory analysis of how CH may impact the spectrum of somatic alterations within breast cancer cells. The samples for this aim come from banked samples of newly diagnosed women with early stage breast cancer between 1999 and 2010. There are 2,791 unique breast cancer patients (341 with DCIS, 2450 with invasive breast cancer) with banked blood samples at diagnosis and prior to any surgery or therapy. The invasive breast cancer and DCIS samples were obtained when patients underwent primary breast cancer surgery at MSKCC. In addition to blood samples from newly diagnosed breast cancer patients, we have an additional 1,730 blood samples from 1,730 unique nonaffected women (controls) that were collected under the same biospecimen protocol. We will randomly select 500 patients from the invasive breast cancer cohort, 150 from the DCIS cohort and 150 from the control cohort. To ensure the cohorts are similar, we will assemble the 800-sample cohort as follows:

A. Randomly choose 500 patients from 2,450 patients with invasive breast cancer;

B. Frequency match the 500 invasive patients with 150 DCIS patients by 5-year increments of age at the time of the banked blood draw and 2-year increments of the year that the blood was drawn;

C. Frequency match the 500 invasive patients with 150 controls on 5-year increments of age at the time of the banked blood draw and 2-year increments of the year that the blood was drawn.

A2. Neoadjuvant therapy and clonal hematopoiesis:

Large patient cohort studies have demonstrated that the incidence of CH mutations is increased in cancer patients who have previously received chemotherapy and particularly radiotherapy. It remains unresolved, however, whether these therapies cause an increase in the variant allele frequency or severity of clonal hematopoiesis within a given patient. As of last year, we aimed to analyze the presence or absence of mutations in cancer- related genes in the peripheral blood cells using blood samples obtained before exposure and those obtained after completion of chemotherapy to determine whether any differences exist, and to correlate these differences with

clinical and pathological features. We have over 20 breast cancer patients identified. We are finalizing our analysis of blood samples from these patients. With the inclusion of the Reis-Filho lab, we now have access to state-of-the-art facilities for the laser-capture microdissection experiments, and to five bioinformaticians and a dedicated high-performance computer set up, which will undoubtedly expedite the analyses.

A3. Surgery and clonal hematopoiesis:
It is unknown how removal of the primary tumor influences clonal hematopoiesis (CH). To determine the impact of therapy and surgery on CH, we have collected blood from patients before and after removal of their primary tumor as well as before and after neoadjuvant therapy. Blood and isolated DNA from patients in the surgery cohort are being submitted through the MSKCC Heme-PACT platform for targeted sequencing.

B. Functional *in vivo and in vitro* studies:
B1. Change in tumor models: So far our studies have been hampered by inconsistent tumor growth in implantable models. In addition, with Dr. Kleppe, Corinne Hill and now Swetha Ravi no longer working at MSKCC, the technical expertise to achieve of consistent injectable tumors has become a limiting issue for in vivo studies. Paired with the negative results in the expeditious genetic models, we have refocused our studies on utilizing three new breast cancer models, two based on the Py230 and Py8119 cell lines and another based on the BRC3 cell line developed by Dr. Nir Ben-Chetrit. These lines are injected directly into the mammary fat pad requiring less surgical expertise and allow for grafting into C57BL6 mice gaining us increased access to our *Dnmt3a* and *Tet2* genetic mouse models in these mouse lines.
B2: Change in *ex vivo* co-cultures: Given that we are altering our *in vivo* models, we are also changing the cell lines we are using to evaluate inflammatory cytokine secretion and impact on tumor growth *in vitro*. We will focus on the BRC3 which is composed of both epithelial tumor cells and fibroblasts as well as the Py230 and Py8119 cell line which has a epithelia and mesenchymal characteristics respectively. These lines should allow for greater range in our capacity to evaluate how mutant myeloid cells interface with discrete component on the tumor including both tumor cells and cancer associated fibroblasts.

II. Actual or anticipated problems or delays and actions or plans to resolve them.
Describe problems or delays encountered during the reporting period and actions or plans to resolve them.

A. Acquisition of samples from patients with metastatic disease:
Given the bandwidth of the additional sample collection as above, we no longer are screening for de novo metastatic cases. We are continuing to screen for our early breast cancer patients who may have developed metastatic disease and those patients with stablished independently of their treatment status.

B. Number of patient samples:
We have entirely changed our screening as previously described to include all breast cancer samples and other primary and metastatic sites.

C. Isolation of sorted leukocyte DNA:

One of the major limitations of our study is the efficient isolation of DNA from sorted leukocytes, often from a very small number of cells. Whilst this has been fruitful in the past, there remain issues on reproducibility that has negatively impacted our progress. To remedy this, we have taken two distinct approaches. 1) We have revamped tumor processing by utilizing semi-automatic tissue processing using the GentleMACS Octo-dissociator by Miltenyi. Switching this processing step has dramatically increased our cell yield and viability, with cell viable cells now making up over 80% of the sample as opposed to previous ranges of 20-50%. 2) We have changed our strategy to focus on single cell DNA sequencing strategies as opposed to sorted cells. This allows us to freeze CD45+ leukocytes from patient samples and process them in bulk reducing batch errors and increasing reproducibility.

D. High variability in tumor growth in mouse models:
We have tested several different orthotopic models of breast cancer in mice and have observed high variability in tumor take as well as growth. To remedy this variability we are testing new cell lines derived from a MMTV:PyMT model as described above. We also are focusing on timed takedowns following the initiation of treatment and evaluation of metastatic outgrowth in the lungs, a site which is particularly relevant to breast cancer patients and a common site of metastasis for these cell lines.

Changes that had a significant impact on expenditures
Nothing to report.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents
Nothing to report.

PRODUCTS: List any products resulting from the project during the reporting period. Examples of products include:
Nothing to report.

PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS I. Individuals who have worked on the project (>1 calendar month):

Name:	Jorge Reis-Filho
Project Role:	Initiating PI
Researcher Identifier (e.g. ORCID ID):	C-6341-2014
Nearest person month worked:	3
Contribution to Project:	Dr. Reis-Filho is responsible for the project coordination, the direct supervision of the pathology analyses, tissue microdissection and bioinformatics analyses.
Funding Support:	Dr. Reis-Filho is funded by the Breast Cancer Research Foundation, DOD level III GC229671, Geoffrey Beene Cancer Research Center GC231509, Starr Cancer Consortium I11-0051, and internal funds.

Name:	Elizabeth Comen
Project Role:	Partnering PI
Researcher Identifier (e.g. ORCID ID):	Not applicable
Nearest person month worked:	2.28
Contribution to Project:	Dr. Comen oversees all aspects of the protocol related to human subjects, and in particular human sample collection and screening daily. Dr. Comen tracks eligible patients, reviews their study eligibility and follows patient's clinical course accordingly. Dr. Comen ensures that tissue/blood samples are obtained and processed appropriately. Dr. Comen participates with data analysis and interpretation. Dr. Comen also helps identify patients with histories of breast cancer and secondary hematologic malignancies.
Funding Support:	Funding support for Dr. Comen is provided by the present grant as well as philanthropic funds. She also has a grant from the Breast Cancer Research Foundation for work unrelated to this project.

	Damian Kim

Name:	
Project Role:	Research Study Assistant
Researcher Identifier (e.g. ORCID ID):	Not applicable
Nearest person month worked:	6
Contribution to Project:	Damian Kim functions as the clinical research study assistant for this project. He assists with patient screening, accrual and sample transport.
Funding Support:	Damian Kim is supported by funds through the Breast Medicine Service, MSKCC.

Name	Robert Bowman
Project Role:	Postdoctoral researcher
Researcher Identifier (e.g. ORCID ID):	Not applicable
Nearest person month worked:	2
Contribution to project:	Dr. Bowman is in charge of the bioinformatic analysis of the samples. He also supports Dr. Sanchez Vela work and is one of the points of contact between the Levine Lab and clinical collaborators including Dr. Comen and Dr. Reis-Filho.
Funding Support:	Dr. Bowman's salary is covered by the Damon-Runyon cancer research foundation.

Name	Dr. Pablo Sanchez Vela
Project Role:	Postdoctoral researcher
Researcher Identifier (e.g. ORCID ID):	Not applicable
Nearest person month worked:	4
Contribution to project:	Dr. Sanchez Vela is responsible for the supervision, processing of patient samples, DNA extraction and sample preparation for downstream analysis including flow cytometry and sequencing. He is also responsible for the murine studies and perform techniques such as tail vein injections, bone marrow harvests and mammary fat pad injections to support. Dr. Sanchez Vela is also responsible for cell culture techniques and mouse husbandry to maintain experimental cell lines and mouse models.

Funding Support:	Dr. Sanchez Vela salary is covered by the Levine laboratory at MSKCC.

II. Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?
No.

III. What other organizations were involved as partners?
Nothing to report.

SPECIAL REPORTING REQUIREMENTS: COLLABORATIVE AWARDS: For collaborative awards, independent reports are required from BOTH the Initiating PI and the Collaborating/Partnering PI. A duplicative report is acceptable; however, tasks shall be clearly marked with the responsible PI and research site. A report shall be submitted to **https://ers.amedd.army.mil** for each unique award.

APPENDICES: Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.