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Nothing to report

14. ABSTRACT
NF1 function is classically altered by gene deletion in breast cancer. An alternative-but-equally-important mechanism of NF1 deficiency is defective mRNA processing whereby mutant mRNA transcripts directly or indirectly affect the stability of wild type transcripts. This delicate balance of mutant versus wild type transcript abundance can dramatically affect protein synthesis and, ultimately, RAS signaling fates. In cancer, dysregulation of alternative splicing promotes malignant progression and therapeutic resistance by altering the expression and function of tumor suppressors and oncogenes. Little is known about NF1-related mRNA processing or how alternative transcripts affect NF-related phenotypes such as breast cancer. Our goal is to define the genetic and isoform changes in NF1 that occur in breast cancer with the ultimate goal of identifying prognostic biomarkers and targeted therapeutic strategies for both female and male NF patients with breast cancer. Our hypothesis is that alternative RNA splicing of NF1 abrogates NF1 gene function and RAS regulation to promote breast cancer progression and therapeutic resistance. Our experimental approaches leverage innovative sequencing methods, our established rat model of Nf1-deficient breast cancer, and comprehensive analysis of breast cancer datasets. The results of these studies will provide insight into the

15. SUBJECT TERMS
Neurofibromatosis Type I, breast cancer, alternative isoforms

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1. Introduction

NF1 function is classically altered by gene deletion in breast cancer. An alternative-but-equally-important mechanism of *NF1* deficiency is defective mRNA processing whereby mutant mRNA transcripts directly or indirectly affect the stability of wild type transcripts. This delicate balance of mutant versus wild type transcript abundance can dramatically affect protein synthesis and, ultimately, RAS signaling fates. In cancer, dysregulation of alternative splicing promotes malignant progression and therapeutic resistance by altering the expression and function of tumor suppressors and oncogenes. Little is known about *NF1*-related mRNA processing or how alternative transcripts affect NF-related phenotypes such as breast cancer. Our *goal* is to define the genetic and isoform changes in *NF1* that occur in breast cancer with the ultimate goal of identifying prognostic biomarkers and targeted therapeutic strategies for *both female and male* NF patients with breast cancer. Our *hypothesis* is that alternative RNA splicing of *NF1* abrogates *NF1* gene function and RAS regulation to promote breast cancer progression and therapeutic resistance. Our experimental approaches also leverage innovative gene and RNA sequencing methods, as well as our established rat model of *Nf1*-deficient breast cancer. The results of these studies will provide insight into the mechanisms whereby alternative *NF1* RNA isoforms and mRNA processing promote breast cancer. Moreover, the results of this study will reveal RNA isoform signatures of *NF1* deficiency that could serve as diagnostic or prognostic markers for both NF1-related and sporadic breast cancer.

2. Keywords

Neurofibromatosis Type 1, NF1, alternative isoforms, breast cancer, mRNA splicing

3. Accomplishments

See following pages 5-10

What were the major goals of the project?

Tasks and Milestones Achieved	**Timeline (Months)**
Submit for human subjects review and approval for work proposed in Aim 3.	Completed
Specific Aim 1: Determine *Nf1* isoform expression during *Nf1*-deficient breast cancer progression	
Subtask 1: Perform long-read RNA sequencing on Nanopore MinION	Completed
Subtask 2: Perform short-read RNAseq with Illumina platform	Completed
Subtask 3: Align to reference transcriptome, reassemble the transcriptome, identify RNA structural variants.	In progress
Subtask 4: Realign the direct RNA to the reassembled transcriptome. Reads will be grouped into isoforms based on exons/intron composition.	In progress
Specific Aim 2: Determine *Nf1* isoform expression in endocrine-resistant *Nf1*-deficient breast cancers.	
Subtask 1: Perform long-read RNA sequencing on Nanopore MinION	In progress
Subtask 2: Perform short-read RNAseq with Illumina platform	Completed
Subtask 3: Align to reference transcriptome, reassemble the transcriptome, identify RNA structural variants.	In progress
Subtask 4: Realign the direct RNA to the reassembled transcriptome. Reads will be grouped into isoforms based on exons/intron composition.	In progress
Specific Aim 3: Identify NF1 isoform expression in human breast cancers	
Subtask 1: Bioinformatic analysis of *NF1* isoform expression in METABRIC human breast cancer database.	Completed
Subtask 2: Bioinformatic analysis of *NF1* isoform expression in TCGA human breast cancer database.	Completed
Subtask 3: Determine correlation of alternative *NF1* isoform expression with survival, recurrence, and subtype.	Completed
Subtask 4: Perform WGCNA and unsupervised clustering to uncover connections between *NF1* status, RAS signaling, and ER signaling.	In progress
Subtask 5: Perform RNAseq on 20 ER+/PR+/HER2- and 20 ER-/PR-/HER2- breast cancer. These tissues were previously obtained under an approved IRB.	In progress
Subtask 6: Validate NF1 isoform expression in tissues assessed in Subtask 5.	In progress

What was accomplished under these goals?

Specific Aim 1: Determine *Nf1* isoform expression during *Nf1*-deficient breast cancer progression[i]

To interrogate Nf1 isoform expression it is essential to utilize several sequencing methods because of the extreme length of the *NF1* genes (60 exons and > 300 kb of genomic DNA). Standard short-read sequencing approaches have provided important but incomplete details regarding the *NF1* genome and RNA sequence variation. Even though short-read sequencing is commonly used for transcript and isoform analysis, reads that span exon–exon junctions (and are used to identify distinct isoforms) can map indistinctly when a junction is shared between isoforms [1]. This issue can complicate isoform analysis. Long-read cDNA methods can generate full-length isoform reads that substantially reduce these issues and improve differential isoform expression analysis. Although long-read sequencing has several advantages for isoform analysis, there are distinct limitations, as well, including lower throughput of long transcripts and higher error rates. To interrogate the range of *NF1* isoforms present in normal and malignant cells, we proposed to combine both long-read nanopore RNA sequencing technology and short-read RNA sequencing methods. For these experiments, we isolated RNA from WT control mammary epithelial cells, whole mammary pads, and mammary adenocarcinomas (from female and male animals) from $Nf1^{IF}$, $Nf1^{PS}$, and $Nf1^{IF\text{-}PS}$ rats.

Long-read sequencing of *Nf1 transcripts in* mammary glands and tumors

We developed RNA-probes that were used to pull down target sequences in *Nf1* in order to enrich for larger (>500 bp) transcript fragments. This hybridization method required large amounts of RNA template (>500 ng). For the RNA isolation, we isolated high molecular weight (HMW) RNA from $Nf1^{WT}$ and $Nf1^{mut}$ mammary epithelial cells and tumor cells. Using *Nf1* targeted probes, we were able to pulldown *Nf1* RNA transcript fragments. In two separate runs on the Nanopore MinION sequencer, our analysis of the read length revealed that the majority of sequencing reads were shorter than desired and ranged between 1-3Kb (Figure 1). Read length in Nanopore sequencing can be limited by the ability to deliver very HMW DNA to the pore as efficiently as shorter reads. This is a known challenge in the long-read sequencing field and an issue that several labs are working to optimize (Gordon Research Conference: Post-Transcriptional Gene Regulation, July 2022). In the next year, we will continue to optimize our long-read sequencing using the following methods. 1) Use ReadFish to select target regions – using this software algorithm we can analyze the signal after a DNA molecule enters a pore to determine whether that molecule lies within a specified region of interest. If it does, the pore continues to sequence the molecule; if not, the DNA molecule is ejected from the pore [2]. In this manner, we can focus on *Nf1* transcripts 2) We will utilize both polyA probes and *Nf1* probes during the targeted pulldown step. Since longer polyA tail length are more readily translated than shorter read tails, we will preferentially pulldown and sequence *Nf1* transcripts that are likely to be transcribed.

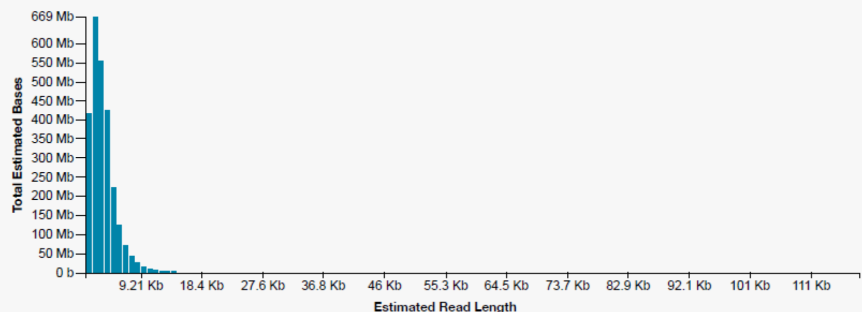

Figure 1: Sequencing read length on Nanopore MinION platform. The estimated read length of all sequences in a single run of combined samples.

Short-read sequencing of *Nf1* mammary glands and tumors

Standard short-read sequencing using the Illumina NovaSeq 6000 platform was performed on RNA isolated from the following cells and tissues: whole mammary pads, isolated mammary epithelial cells, and mammary adenocarcinomas from $Nf1^{WT}$, $Nf1^{IF}$, $Nf1^{PS}$, and $Nf1^{IF\text{-}PS}$ rats. Because the normal mammary gland is primarily stroma (adipocytes, fibroblasts, immune cells) whereas the tumor is primary epithelial with less stroma, we

included both whole mammary glands and mammary epithelial cells for our "normal" tissue controls. This will allow us to analyze the impact of *Nf1*-deficiency in non- and pre-tumorigenic cells. High quality RNA sequencing data was obtained from all samples. The initial transcriptome analysis identified differences between $Nf1^{WT}$ and $Nf1^{mut}$ tissues has been performed. To evaluate differences among Nf1-deficient lines, transcriptomic profiles for each tissue type were compared by two dimensionality reduction approaches including, Principal Components Analysis (PCA) and Uniform Manifold Approximation and Projection (UMAP) (Figure 2A). In addition, we utilized several approaches to identify significant gene expression differences between samples (Figure 2B-C). A comprehensive analysis of the gene expression differences between the genotypes in each tissue type is in progress.

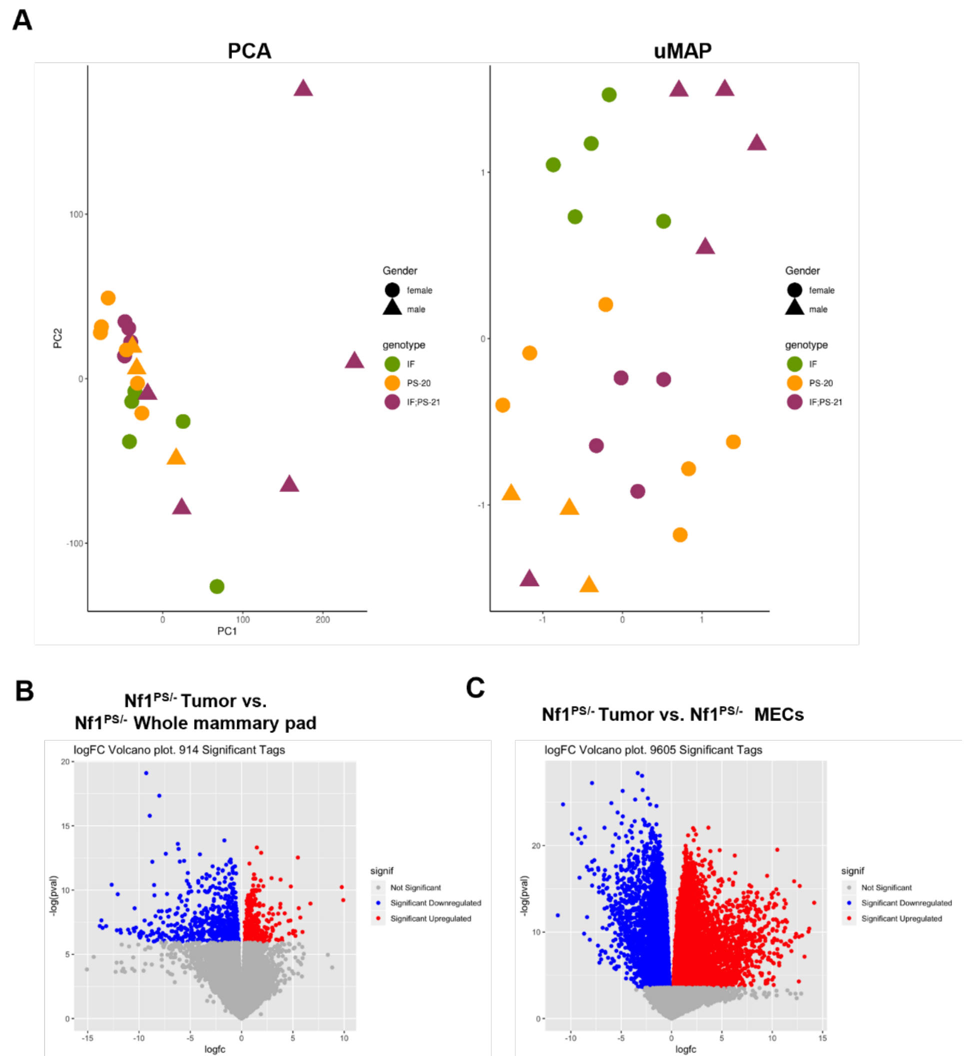

Figure 2: Clustering and differential gene expression analysis of RNAseq transcriptome A) PCA and uMAP analysis of mammary tumors from all Nf1 genotypes (female and male rats). B) Volcano plots of differential gene expression contrasts between $Nf1^{PS/+}$ tumors compared to B) $Nf1^{PS/+}$ whole mammary pads and C) $Nf1^{PS/+}$ MECs.

RNA analysis: *Short-read sequencing:* raw data were quality controlled with FastQC. Adapters were removed with Trimgalore and mapped with STAR [3] to the rn6 genome. STAR outputs counts for all genomic features/tags/genes (option: –quantMode GeneCounts). RNAseq and DGE analysis used the edgeR [4] framework for RNAseq analysis. The data was first filtered for low counts and then TMM normalization factors are calculated. These normalization factors adjust for library size and composition biases in the glm models. Significance was tested using the quasi-likelihood F test. P-values were then adjusted using the BH method. Genes were termed significant if they passed the FDR and logFC thresholds (indicated in the specific contrast tabs). These significant genes were used in the gene set enrichment analysis using the R package goseq which adjusts for gene length bias. *Long-read sequencing*: Nanopore Minion RNA-Seq data was processed following the procedure implemented in nfcore-nanoseq pipeline.

Specific Aim 2: Determine *Nf1* isoform expression in endocrine-resistant *Nf1*-deficient breast cancers.

The success of endocrine therapies has established the efficacy of estrogen modulation in estrogen-positive breast cancer; however endocrine-resistance in breast cancer is a significant clinical challenge. *NF1* loss-of-function mutations have been identified as a frequent event in endocrine resistant breast cancers. We have an existing collaboration with Genentech to evaluate the efficacy of several next-generation selective ER degraders

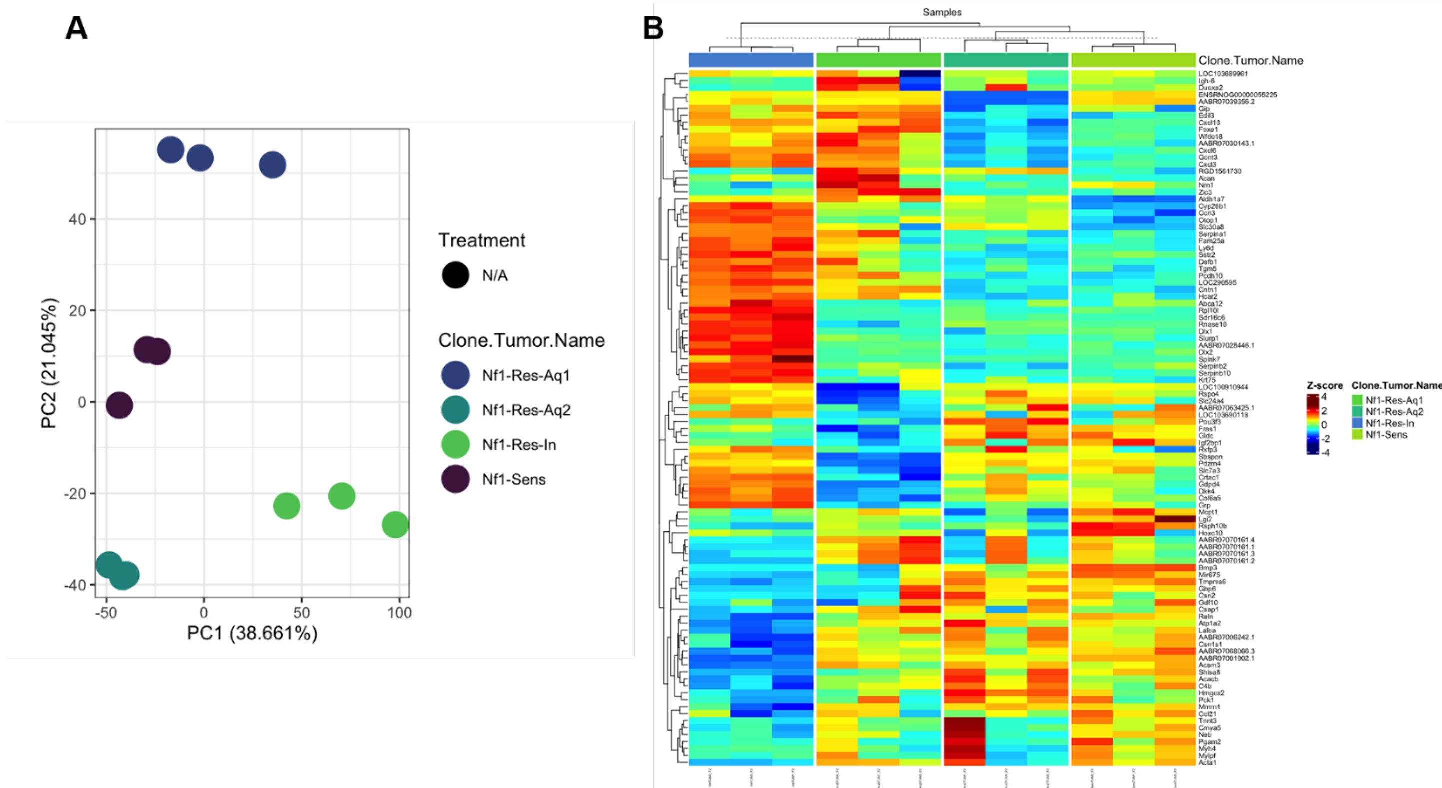

Figure 3: Clustering and differential gene expression analysis of RNAseq transcriptome A) PCA analysis of SERD-sensitive and -resistant tumors. B) Unsupervised clustering was performed and a heatmap of the top 100 differentially expressed genes is shown here.

(SERDs) in our ER-dependent breast cancer model. We previously demonstrated significant tumor inhibition with SERD treatment in our *Nf1*-deficient tumors, yet we have also been able to create three *Nf1* tumorgraft lines that are resistant to endocrine therapy. To investigate whether there are unique RNA isoforms or structural variations are present in these endocrine-resistant breast cancers, we performed direct RNA sequencing on both SERD-sensitive and SERD-resistant tumors from *Nf1*$^{PS/+}$rats. This included RNA isolated from a SERD-sensitive tumor (Nf1-Sens), an innately SERD-resistant tumor (Nf1-Res-In), and two tumors with acquired SERD resistance (Nf1-Res-Aq1 and Nf1-Res-Aq2). The transcriptome analysis was performed as described above in Aim 1 (Figure 3). We will perform long-read sequencing on these tumors once we have optimized the HMW transcript read length as discussed previously in Aim 1. We are currently in the process of analyzing the *Nf1* isoforms and genome-wide transcript changes using the short-read sequences present in SERD-sensitive vs. SERD-resistant tumors. The methods used for this analysis are described below in Aim 3.

Specific Aim 3: Identify NF1 isoform expression in human breast cancers

<u>Identify *NF1* transcript variants in breast cancer datasets</u>

To identify alternative RNA isoforms present in human breast cancers we assessed *NF1* transcript variants and alternative splicing events in the TCGA dataset containing 2,509 sporadic breast cancer samples. First, we utilized the computational tool SpliceSeq (Ryan et al., 2015) to identify alternative RNA splicing in the TCGA RNAseq breast cancer dataset. Using this tool, we identified several novel splicing events that occur in *NF1* transcripts (Figure 4). One of the alternative NF1 transcripts (Figure 4B) included alternative exon 23a has been reported previously identified in NF1 tissues [5-7]. Type I, which lacks exon 23a, has ten times higher Ras-GAP activity. The type II variant includes exon 23a, producing a 63 amino acid, in-frame, sequence in the GRD. Type I and type II variants are ubiquitously expressed across all tissue while type II is preferentially expressed in differentiated cells. Tight regulation and expression of type I and II has been shown to be crucial for neuronal

cell differentiation. Recent studies have investigated and correlated more severe NF phenotypes to be associated with lower expression of type I variant compared to type II in peripheral blood samples. The identification of the Type I and Type II transcripts in our study validated our sequencing and bioinformatic analysis approaches. In addition to these results, we also identified NF1 transcript variants with exon skipping of exon 52 and a transcript with the exclusion of exon 44 (Figure 4B-C). The impact on these transcript variants on the functionality of the NF1 gene product, neurofibromin, is unknown. However, we are excitedly following up on the exon 52 transcript, since skipping of exon 52 would result in the loss of the nuclear localization signal in the 3' end of Nf1. Through RT-PCR we validated the MCF7 and MCF10A cell lines express the *NF1*ΔNLS transcript variant. Interestingly, we see a shift in the ratio of type I and ΔNLS transcripts in the *NF1*-deficient cell lines. Because genomic alterations of *NF1* are an emerging driver of sporadic breast cancers and associated with endocrine resistance, we hypothesize that altered or imbalanced expression of *NF1* transcript variants are as damaging as genomic alterations in driving breast cancer.

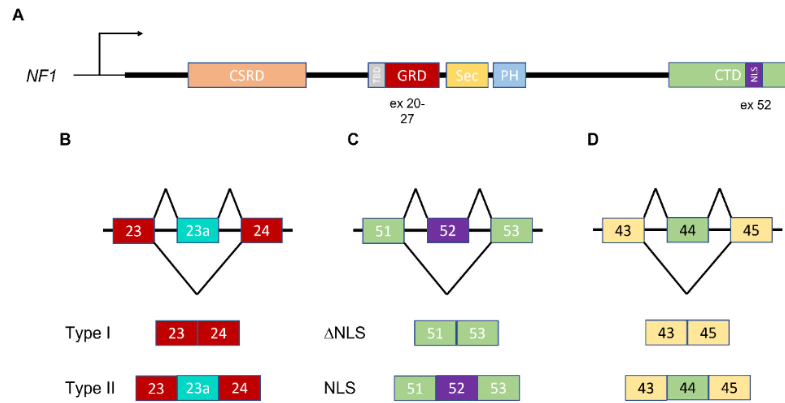

Figure 4: SpliceSeq analysis of TCGA breast cancers identified 3 alternative NF1 transcripts. A) NF1 gene with the main structural domains represented including the RAS-binding domain (GRD) and NLS, B) Alternative transcripts including B) inclusion of exon 23, C) exclusion of exon 52 and the NLS, and D) exclusion of exon 44.

<u>Correlate breast survival and signaling with *NF1* transcript variant expression</u>

Analysis of *NF1* transcripts in the TCGA breast cancer dataset, confirmed the expression of the *NF1*ΔNLS and the type I (ex23a) transcripts. Recent studies have demonstrated that alternative RNA isoform expression can be correlated with disease survival. We tested whether the *NF1*ΔNLS correlated with breast cancer survival and recurrence using the statistical method SURVIV [8]. This statistical method is used to associate patient survival time with mRNA transcript variants and simulations of SURVIV suggest a more robust model than Cox regression survival analysis. Previously, we have demonstrated that *NF1* genomic loss correlates with poor outcomes in breast cancer patients [9]. In this grant period, we performed SURVIV analysis of *NF1*ΔNLS transcripts +/- NF1 heterozygous deletions (NF1_HETLOSS) in ER+ breast tumors. This analysis revealed that ER+ cases with both *NF1* heterozygous deletions and *NF1*ΔNLS expression had significantly diminished survival compared to other ER+ breast tumors with and without *NF1* deletions (Figure 5). Currently, we are utilizing the computational tool rMATS turbo which has been previously used to identify oncogenic splicing alterations in prostate cancer [10]. The rMATS turbo analysis will compare breast cancer subtypes to detect any *NF1* transcript variants. With this experiment we will be able to identify *NF1* transcript variants in

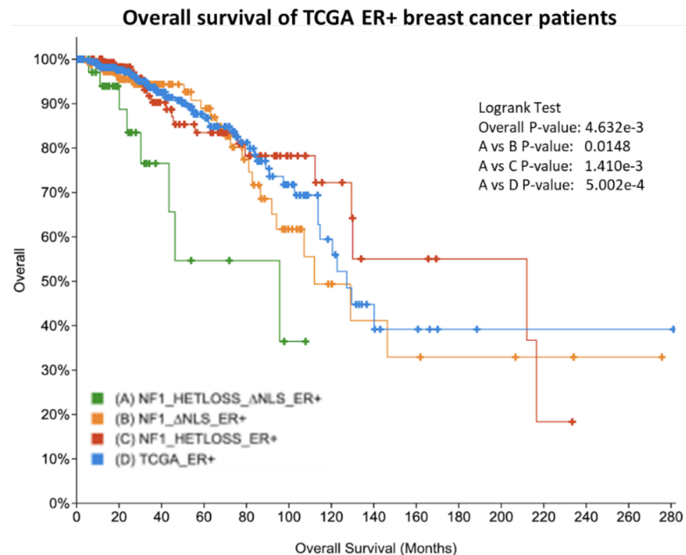

Figure 5: SURVIV analysis of NF1 NLS in ER breast cancers. Overall survival is decreased in breast cancer patients (TCGA) harboring *NF1* genomic loss (NF1_HETLOSS) and *NF1*ΔNLS transcripts.

breast cancer samples and if they are enriched in a particular breast cancer subtype. We have already implemented MATS to analyze transcript variants in MCF7 breast cancer cells with *NF1* mutations that are treated with various ER inhibitors. Our preliminary results suggest that certain alternative *NF1* transcripts are expressed during selective pressure with ER inhibitors. This data is being validated through additional experiments.

What opportunities for training and professional development has the project provided?
Nothing to report

How were the results disseminated to communities of interest?

We have an annual meeting with the NF-Michigan to update them on our research progress on NF1-related research. This will be held this year in September 2022. In addition, we expect to have at least one manuscript in submission for publication at the end of the next grant period.

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

Our plans for each Aim are discussed above with each aim.

4. Impact

What was the impact on the development of the principal discipline(s) of the project?
The results of these studies will define the impact of alternative RNA splicing on *NF1* function, breast cancer progression, and therapeutic resistance. This will fill a huge gap in our understanding of *NF1* expression in normal and malignant tissues. Moreover, the results of this study may reveal RNA isoform signatures of *NF1* deficiency that could serve as diagnostic or prognostic markers for both NF1-related breast cancer and sporadic breast cancer patients.

What was the impact on other disciplines?
We expect these results to advance our understanding of the role of NF1 alternative splicing in other NF-related cancers.

What was the impact on technology transfer?
Nothing to report

What was the impact on society beyond science and technology?
We have an annual meeting with the NF-Michigan to update them on our research progress on NF1-related research. This will be held this year in September 2022. These interactions are mutually beneficial. These meetings communicate research progress in NF research and helps us understand the challenges that individuals with NF face.

5. Changes/Problems

Changes in approach and reasons for change
One of the subtasks in Aim 3 was to analyze *NF1* isoform expression in several breast cancer datasets. We were able to complete an extensive analysis of the TCGA breast cancer dataset; however, the proposed METABRIC dataset was constructed using Illumina HT 12 microarrays and not RNA-seq platforms which are required for isoform analysis. When we originally wrote this grant, the cBioportal METABRIC Datasets page inaccurately stated that the METABRIC study was composed of 1904 samples with RNA-seq and 1904 samples with mRNA microarray. Due to this fact, we have focused our bioinformatic analysis on the TCGA dataset and included *NF1*-deficient MCF7 cell lines that we have developed in the lab using CRISPR-CAS genome editing. We are also planning to analyze smaller breast cancer datasets that include metastatic samples.

Actual or anticipated problems or delays and actions or plans to resolve them *Nothing to report*

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*

Significant changes in use or care of human subjects *Nothing to report*

Significant changes in use or care of vertebrate animals. *Nothing to report*

Significant changes in use of biohazards and/or select agents *Nothing to report*

6. Products

Journal publications. *Nothing to report*

Books or other non-periodical, one-time publications. *Nothing to report*

Other publications, conference papers, and presentations. Poster presentation at Gordon Research Conference: Post-Transcriptional Gene Regulation, July 10 - 15, 2022

7. Participants & Other Collaborating Organizations

Name:	Matthew Steensma
Project Role:	Principal Investigator
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	0.48 calendar months or 10% effort (of 40% appointment)
Contribution to Project:	Guiding the experimental design for the entire project and current overseeing the completion of experiments

Name:	Carrie Graveel
Project Role:	Senior Research Scientist
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	1.2 calendar months or 10% effort
Contribution to Project:	Designing, performing, and analyzing the experiments; contributing to the development of research strategies, and preparing the results for presentation and publication.

Name:	Elizabeth Tovar
Project Role:	Research Scientist
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	2.4 calendar months or 20% effort
Contribution to Project:	Designing, performing, and analyzing the experiments; contributing to the development of research strategies, and preparing the results for presentation and publication.

Name:	Patrick Dischinger
Project Role:	Graduate Student
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	6.0 calendar months or 50% effort
Contribution to Project:	Designing, performing, and analyzing the experiments, and bioinformatic analyses.

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period? Nothing to report

What other organizations were involved as partners? *Nothing to report*

8. Special Reporting Requirements *Nothing to report*

9. Appendices *Nothing to report*

10. References

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