Identification of Targets for Delayed Mammary Tumor Onset and Aggressiveness Using Diversity Outbred Mice Expressing the Delta 16 Variant of HER2/neu

J.B. Jacob[1], W-Z. Wei[2], K. White[2], L. Polin[2], Q-S. Mi[3], J.D. Reyes[2]

[1]Department of Biochemistry & Molecular Biology, Michigan State University, E. Lansing, MI
[2]Department of Oncology, Wayne State University, Detroit, MI
[3]Immunology & Dermatology Division, Henry Ford Health System, Detroit, MI

ABSTRACT

Our research aims to identify genetic regulators of breast cancer oncogenes using diversity outbred (DO) mice. We previously established a filtering process to identify genes driving aggressive, early-onset tumors in HER2/neu-expressing NeuT mice (Jacob et al., 2023). This process includes identifying founding strain-specific gene variants, survival analysis in human breast cancer patients, and identifying actionable targets using single-cell RNA sequencing (scRNA-Seq) and cellular expression.

In our current study, DO mice were crossed with FVB d16HER2 mice, which carry the human HER2 Delta 16 mutation and develop spontaneous mammary tumors with 100% penetrance, beginning at ~20 weeks. NeuT mice on the DO background (BALBxDO) F1 NeuT developed earlier, more aggressive tumors, while (FVBxDO) F1 d16HER2 mice showed delayed onset. We monitored tumor onset, days to reach 500mm³, and growth rate. Through r/qtl2 analysis, we identified quantitative trait loci (QTL) in four regions associated with tumor onset, twelve with tumor growth, and four with growth rate.

We are now using the EMBL Strain Table and the Jackson Founder Variant Database to identify candidate genes contributing to the reduced aggressiveness of tumors in (FVBxDO) F1 d16HER2 mice. scRNA-Seq data from d16HER2 tumors and adjacent tissues are helping us correlate candidate gene expression with cells in the microenvironment and identify actionable targets for tumor inhibition. One promising target is NKG7, a gene essential for cytotoxic degranulation and expressed on NK cells and activated CD8+ cytotoxic T cells. NKG7-expressing cells are found in both mammary tumors and adjacent mammary tissue in FVB d16HER2 mice.

INTRODUCTION

We use genetic diversity to identify genes that regulate oncogenes, such as HER2/neu[1]. HER2 makes up 15-20% of all breast cancers and drives aggressive disease. Approximately 10% of HER2 is made up a variant that lacks exon 16. This variant - Delta 16 HER2 (d16HER2) – is found in metastatic sites such as the brain and correlates with enhanced signaling pathways and increased aggressivness[2].

GEMM mice expressing d16HER2 on the FVB background develop spontaneous mammary tumors with 100% penetrance[3]. To identify genes that regulate HER2-driven tumor onset and growth, we crossed FVB d16HER2 mice with Diversity Outbred (DO) and monitored the DO F1 females.

DO mice are derived from the random outbreeding of five common laboratory inbred mouse strains and three inbred wild strains. All 8 strains have been fully sequenced and tail tissue is collected and used to identify the haplotype of each individual DO F1 mouse (GigaMUGA array), then associated with phenotype(s) of interest.

Single cell RNA sequencing (scRNA-Seq) quantifies the RNA being generated within a dissociated tissue sample at a given point in time.

MATERIALS & METHODS

FVB d16HER2 mice were generously provided by Drs. Marchini and Amici[3]. Heterozygous males were crossed with non-sibling DO females to generate (FVBxDO) F1 d16HER2 pups. Female DO F1s were monitored 2x/wk for tumor onset and growth. The week of age when total tumor volume was ≥32 mm³ was used as tumor onset. The current data was generated using 115 females across multiple generations.

Tail tissue was collected and DO F1 haplotypes generated using the GigaMUGA array from Neogen.

Association between the mouse haplotypes and phenotypes were performed using the R package, r/qtl2, in RStudio (version 2024.04.0). Phenotypes used for association were:

1) Week of age of tumor onset (≥32 mm³)
2) Tumor latency as days to 500 mm³ or 1000 mm³ total tumor volume
3) Slope of the tumor growth for 7 or 15 weeks

Data from the Ensembl Strain Table (useast.ensemble.org) and the Founder Strain Variant Database (https://churchilllab.jax.org, Jackson Laboratories) are used to identify founder DNA variants within candidate genes.

scRNA-Seq was performed by enzymatic dissociation of whole mammary tissue from glands 4 & 9 of FVB or 129 females and libraries generated using 10X Genomics' Chromium system. Libraries were sequenced on an Illumina NextSeq500. Cell Ranger was used to analyze the sequenced data and Seurat to identify cell populations and compare gene expression of candidate gene(s).

REFERENCES

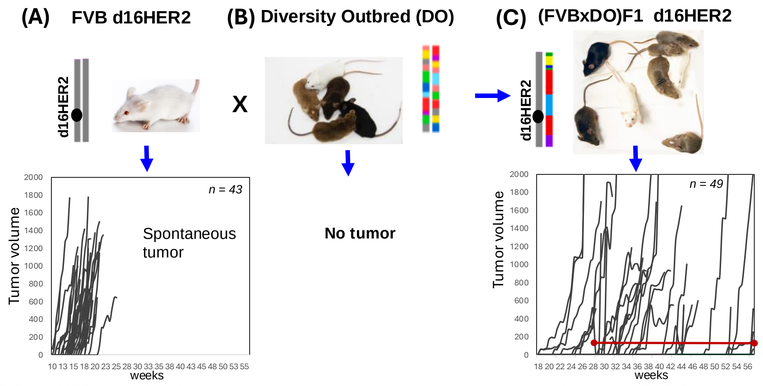
[1]Jacob, J.B. et al., 2023. iScience. PMID: 36968078.
[2]Volpi, et al., 2019. Scientific Reports. PMID: 30837627.
[3]Marchini et al., 2011. PLoS One. PMID: 21559085.

CONTACT INFORMATION

Jennifer Jacob – wellsje2@msu.edu
517-355-9724 (Office) 913-956-9795 (Mobile)

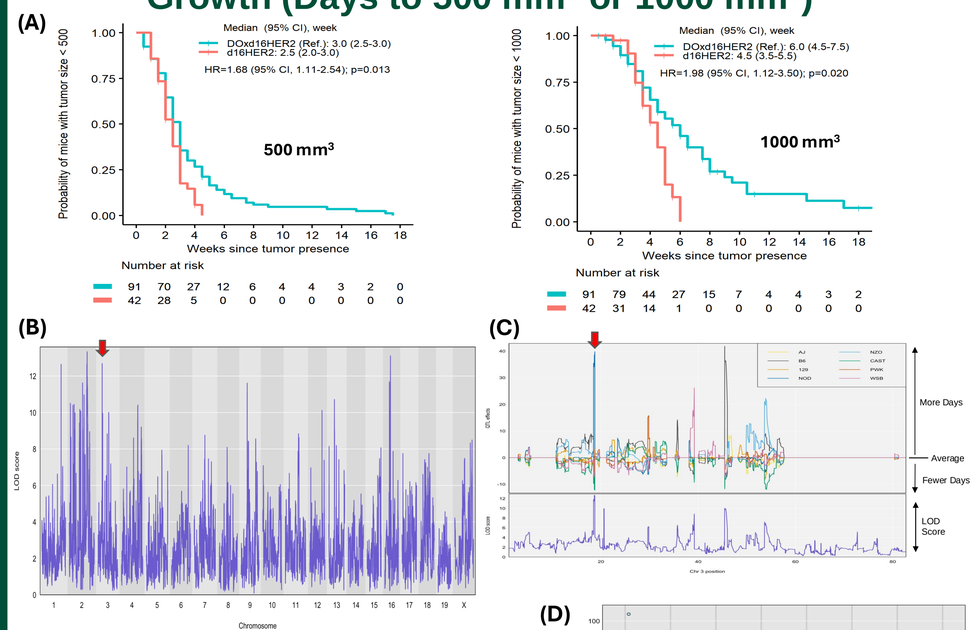
603 Wilson Road, 519 Biochemistry Building,
East Lansing, MI 48824

FVB d16HER2 Mice on the DO Background Have Delayed Tumor Onset and Growth



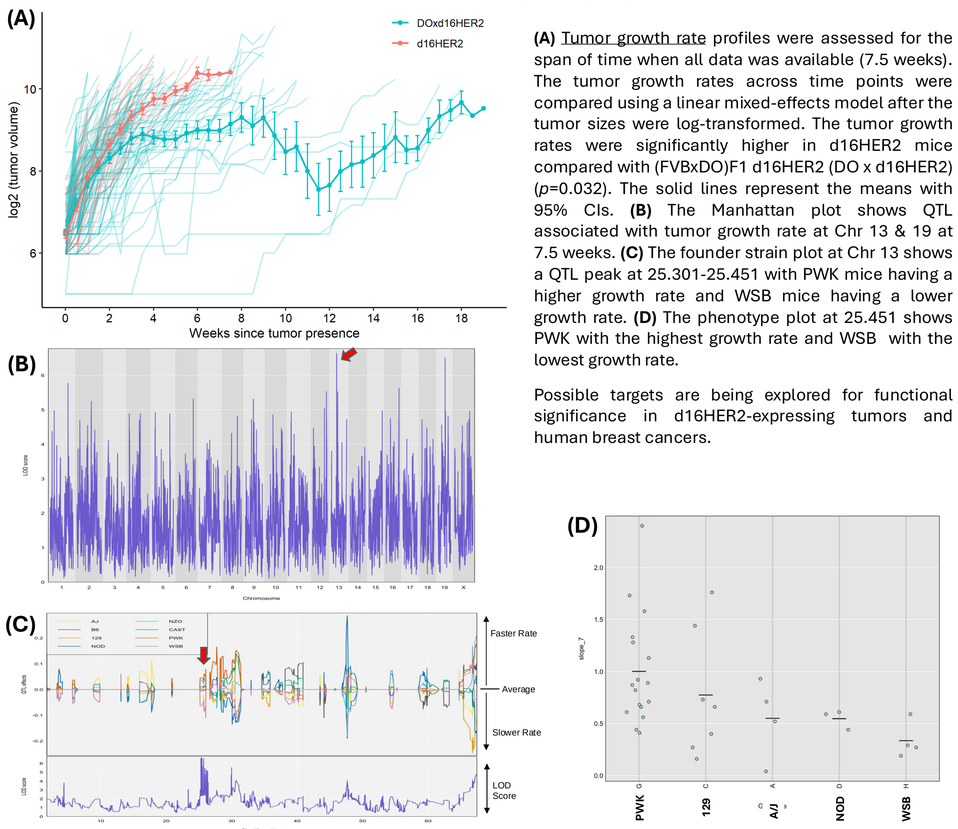
(A) FVB d16HER2 females develop mammary tumors between 12 and 19 weeks of age. **(B)** DO females do not develop tumors. **(C)** (FVBxDO)F1 d16HER2 females have variable tumor onset ages, better modeling onset in human breast cancer patients. *The red bar indicates late tumor onset.*

QTL in Chr 1,2,3,9 & 16 are Associated with Tumor Growth (Days to 500 mm³ or 1000 mm³)



(A) Tumor latency was determined by monitoring the number of days from tumor onset to either 500 mm3 (*left*) or 1000 mm3 *(right)*, and is summarized by Kaplan-Meier estimates, and comparisons were made using a Wald test in a Cox regression model. For latency to 500 mm³, mice in the d16HER2 group reached 500 mm³ earlier than those in the DO x d16HER2 group (HR [hazard ratio] = 1.68, 95% CI 1.11 to 2.54, p=0.013). In the case of 1000 mm³, mice in the d16HER2 group reached the target tumor volume earlier than those in the DOxd16HER2 group (HR = 1.98, 95% CI 1.12 to 3.50, p=0.020). **(B)** The Manhattan plot shows QTL in Chr 1, 2, 3, 9, 15 associated with tumor latency. **(C)** The founder strain plot for Chr 3 shows a QTL peak at 18.624 Mbp with a higher latency in NOD mice and a lower latency in CAST, and the resulting phenotype plot is shown in **(D)**. NOD have the longest tumor latency while CAST has the shortest. *Red arrows indicate the selected peaks.*

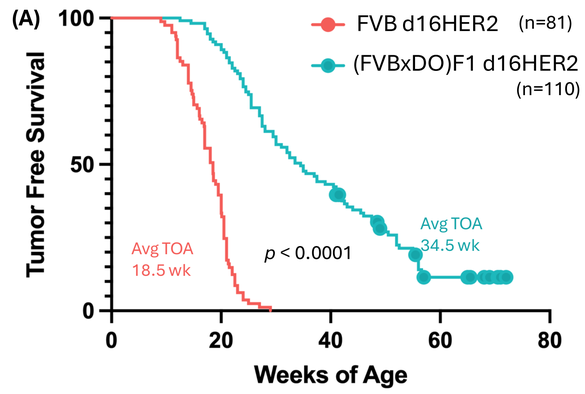
QTL in Chr 13 & 19 are Associated with Tumor Growth Rate (Slope)

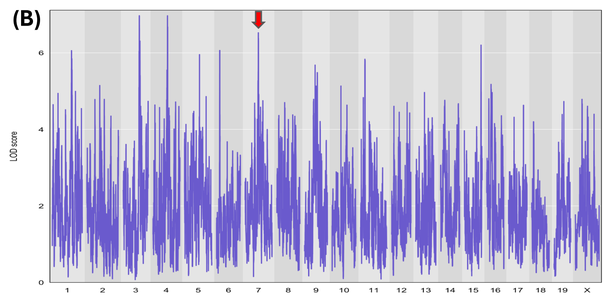


(A) Tumor growth rate profiles were assessed for the span of time when all data was available (7.5 weeks). The tumor growth rates across time points were compared using a linear mixed-effects model after the tumor sizes were log-transformed. The tumor growth rates were significantly higher in d16HER2 mice compared with (FVBxDO)F1 d16HER2 (DO x d16HER2) (p=0.032). The solid lines represent the means with 95% CIs. **(B)** The Manhattan plot shows QTL associated with tumor growth rate at Chr 13 & 19 at 7.5 weeks. **(C)** The founder strain plot at Chr 13 shows a QTL peak at 25.301-25.451 with PWK mice having a higher growth rate and WSB mice having a lower growth rate. **(D)** The phenotype plot at 25.451 shows PWK with the highest growth rate and WSB with the lowest growth rate.

Possible targets are being explored for functional significance in d16HER2-expressing tumors and human breast cancers.

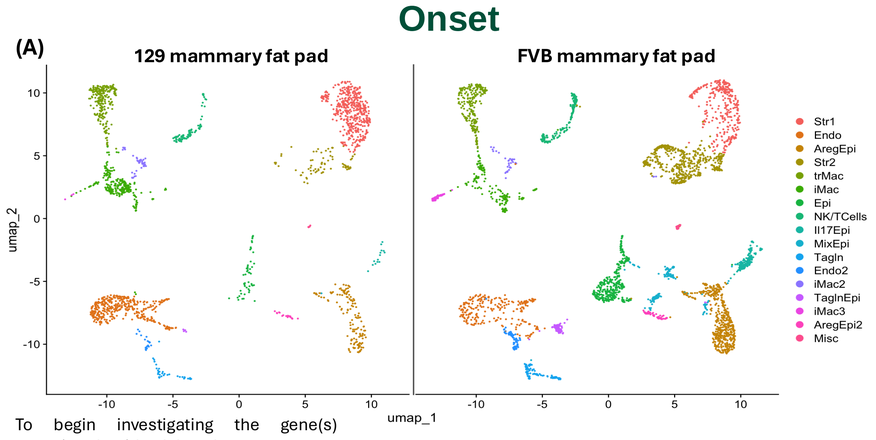
Tumor Onset-Associated QTL in Chr 3, 4, 7 & 15





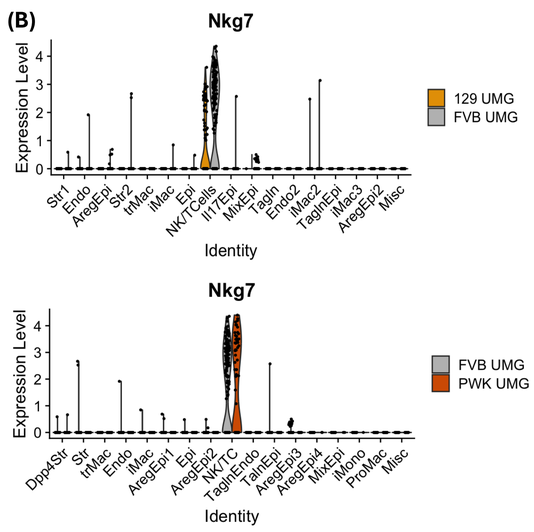
Previously, we reported QTL identified in mice that developed spontaneous HER2/neu tumors significantly earlier than the parental strain[1]. Here we are looking for QTL associated with delayed tumor onset, as ~10% of the mice remain tumor free at 76 weeks of age **(A).** Tumor-free survival was determined using using Kaplan-Meier analysis. The average tumor onset age (TOA) in d16HER2 mice was 18.5 while the TOA in (FVBxDO)F1 d16HER2 was significantly delayed at 34.5 weeks with 10% of the mice tumor-free at 76 weeks of age (p < 0.0001). These are the genotypes of particular interest. **(B)** The Manhattan plot shows QTL for tumor onset age at Chr 3, 4, 7 & 15. **(C)** The founder strain plot at Chr 7 shows a QTL peak at 44.484 with NZO mice developing tumors at a later timepoint. At the same peak, 129 mice are predicted to develop tumors earlier. **(D)** The phenotype plot shows that at the QTL region, the NZO genotype averages ~400 days to tumor onset, while B6 and 129 mice developed tumors at ~175 days.

Nkg7 as a Predicted Candidate Gene for Tumor Onset



To begin investigating the gene(s) associated with delayed tumor onset, expression of Nkg7 found the Chr 7 QTL peak was assessed.

(A) Single cell RNA sequencing was used to compare mice of the 129 genetic background with the parental FVB strain. Mammary fat pads of glands 4 & 9 from five mice were collected and dissociated using collagenases 2 and D. Libraries were generated following 10X Genomics' protocol and sequenced. Seurat was used to compare FVB and 129 clusters. Overall differences are most obvious in stromal and epithelial clusters, with changes in the density of macrophage and NK/T cell clusters. **(B)** Violin plots of Nkg7 expression in clusters of cells found in mammary fat pads. 129 mice had fewer NK and T cells with lower expression of Nkg7, while PWK mice showed no difference in expression. Nkg7 regulates degranulation of cytotoxic granules in cytotoxic T cells and natural killer cells, regulating inflammation and anti-cancer response.



CONCLUSIONS & FUTURE DIRECTIONS

- Growth of spontaneous mammary tumors are highly variable and delayed when FVB d16HER2 mice are crossed with DO to generate (FVBxDO)F1 d16HER2 mice. A subset of ~10% of the mice did not develop tumors by 76 weeks of age. All mice express HER2 as monitored by flow cytometry.
- In addition to significantly delayed tumor onset in (FVBxDO)F1 d16HER2 mice, both tumor latency and the growth rate (slope of the curve) were reduced.
- r/qtl2 analysis of tumor onset, tumor latency and growth rate in 115 DO F1 females identified QTL across multiple chromosomes.
- A Chr 7 QTL was associated with tumor onset, with Nkg7 as one of the genes within the region.
- scRNA-Seq analysis demonstrated that the 129 strain, predicted to have an earlier tumor onset, expressed less Nkg7, which is important for degranulation and cytotoxic effects of T cells and natural killer cells
- Additional mice are being accrued for analysis → ongoing work with a goal of 250-300 mice
- Identify eQTLs using scRNA-Seq libraries