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TITLE: Identifying Metastasis Susceptibility Genes for Estrogen Receptor-Negative Breast Cancer

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The goal of this project is to identify metastasis susceptibility genes for estrogen receptor-negative breast cancer. Genetic analysis in mouse models has localized candidate genes to the distal end of mouse chromosome 6. Combined genomic and in vivo analysis has identified at least three candidate genes in this that are associated with metastatic progression in the mouse. Preliminary human association studies provide suggestive support for at least one of the candidate genes. Furthermore, evidence accumulated to date suggest that the effects on metastasis may be tumor-autonomous for at least some of the genes in question. To gain further information regarding additional candidate genes full genome sequencing has been performed on the MOLF/Ei strain of mice. In depth analysis of the polymorphic content of this strain may provide additional candidate genes for analysis as well as greater insight into the potential causative polymorphisms association with metastatic susceptibility.

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Introduction

Estrogen receptor-negative (ER-) tumors have the poor prognosis compared to ER+ breast cancer subtypes [1]. Mortality from ER- breast cancers, as with all solid tumors, is primarily due to sequela associated with metastatic lesions, rather than the primary tumor [2]. Despite of this, relatively little is understood regarding the mechanisms and origins of metastatic breast cancer. Better understanding of the etiology and mechanisms underlying metastatic disease would likely provide additional avenues for clinical intervention, particularly for ER- breast cancer for which no targeted therapies currently exist. The purpose of this project is to therefore investigate the factors contributing to metastatic disease in ER- breast cancer, using mouse genetically engineered cancer models and a meiotic genetic approach. Candidate genes identified by this screen are currently being validated in representative ER- mammary cancer mouse models, prior to evaluation in human data sets and cohorts. Successful completion of this project should provide additional etiologic information regarding the mechanisms of metastatic disease that may provide additional clinical intervention for the prevention or treatment of ER- breast cancer patients.

Keywords

Breast cancer, metastasis, ER-negative, mouse models, genetic susceptibility, prognosis

Overall Project Summary

Task 1: Identification of candidate metastasis susceptibility genes for ER- breast cancer (Hunter laboratory, NCI; months 1-12)

Tasks 1a-1c have been completed as anticipated. High quality RNA was isolated from the polyoma middle T (PyMT)-induced mammary tumors. Gene expression analysis was performed by submitting the PyMT mammary tumor RNA to the NCI Laboratory of Molecular Technology core facility, where it was arrayed on the Affymetrix Mouse ST 1.0 array. Expression data was provided to the Hunter laboratory who subsequently analyzed the tumor expression data for genes associated with metastatic disease. A total of 19 candidate genes were identified for further analysis in Task 2.

Task 1d was also completed during the proposed time period. Sequencing analysis of the Welcome Trust Sanger Centre Mouse Sequence Database was performed to identify those genes that contained amino acid substitutions. A total of 67 genes were identified, based on comparisons between the CAST/Ei strain, the closest related sequenced strain to MOLF/Ei, and FVB/NJ, the companion strain in the mouse cross used for this research proposal. These genes were subsequently analyzed for possible associations with human breast cancer progression under Task 3 (see below). To provide direct identification and analysis of putative functional polymorphisms in the MOLF/Ei strain, whole genome sequencing has been performed at 40x.
coverage. Identification of polymorphisms in the MOLF/Ei genome is currently underway in collaboration with Dr. Thomas Keane at the Welcome Trust Sanger Centre.

Task 2: Screening mouse candidate genes for correlations with metastasis free survival using retrospective gene expression meta-analysis (Hunter laboratory, NCI; months 6-14)

The 19 genes whose expression was associated with metastatic disease in the mouse gene expression analysis were screened for the association of gene expression and distant metastatic disease in human gene expression data set using the Gene expression-based Outcome for Breast cancer Online (GOBO) [3] tool. This tool permits the meta-analysis of more than 1800 human breast cancer samples that were arrayed on the Affymetrix U133A chip. Each of the 19 candidate genes was screened to determine whether expression levels were correlated with distant metastasis free survival (DMFS) in patients stratified for ER status. No data was available for 6 of the 19 candidate genes (C12orf72, KLHDC5, FGFROP2, LRRN1, IL17RE, CLEC6A). Of the remaining genes, 3 (C12orf35, ARNTL2, EMP1) had significant gene expression correlations with DMFS in the ER- GOBO data sets and were considered for further analysis (Figure 1).

Task 3: Candidate genes identified in task 1 will be screened in a retrospective breast cancer cohort for associations with distant metastatic disease (Shu laboratory, Vanderbilt University; months 6-18).

The 67 genes with potential amino acid substitutions and the three genes displaying correlations with DMFS in the expression data were analyzed on the Vanderbilt breast cancer cohort. Genetic polymorphisms for each of the genes were examined for their association with disease free survival (DFS) and overall survival (OS) with adjustment for age at diagnosis, TNM stage and cancer treatments. A number of SNPs were significantly associated with DFS or OS but none of them retained a statistical significance after correction for multiple testing. Based on these results, in vivo modeling of candidate genes is being performed in the mouse prior to further investigation of this suggestive human association data. SNPs with suggestive associations in

![Figure 1: Kaplan-Meier analysis of candidate genes in the ER- samples of the GOBO meta-data analysis tool.](image)
the discovery cohort whose associated genes are significant in the in vivo metastasis assays will be considered for genotyping in the human validation cohort in future studies.

Task 4: Validation of DMFS-associated SNPs in the validation cohort (months 18-30)

As indicated above in Task 3, work on this task has not yet been initiated.

Task 5: In vivo modeling of metastasis susceptibility genes (Hunter laboratory, NCI; months 6-36)

Task 5a&b: Knockdown cell lines have been generated for four candidate genes, Arntl2 (Figure 2A), 2810474O19Rik (C12orf35) (Figure 3A+B), 4833442J19Rik (C12orf72), and Emp1. 2810474O19Rik, 4833442J19Rik and Arntl2 were selected because they reside under the maximal peak of association with DMFS in the mouse cross. In addition, these genes demonstrate the strongest association with DMFS in the mouse gene expression data. Furthermore the human orthologs of 2810474O19Rik and Arntl2 correlate with DMFS in retrospective human gene expression data. Expression data for the human ortholog for 4833442J19Rik was not available and therefore could not be evaluated. Emp1 was also selected due to a significant association with DMFS in the genetic data, the human gene expression data and a suggestive association with an enhancer SNP in the first intron in the human discovery cohort data (Task 2, data not shown).

Orthotopic implantation of the knockout cell lines was performed and the effect on pulmonary metastasis evaluated. No significant difference was observed between the control
and knockdown cell lines for 4833442J19Rik in the preliminary experiment. Further work on this gene was therefore discontinued (data not shown). Significant associations between metastatic disease were observed for knockdowns for 2810474O19Rik, Arntl2 and Emp1 (Figure 2 and 3). Over-expression of 2810474O19Rik, Arntl2 and Emp1 cell lines is currently in progress.

Task 5c: Importation and colony establishment for genetically engineered models of Arntl2 and 2910474O19Rik have been established in the Hunter laboratory animal colonies. Both models were obtained from the KOMP Project resources, as planned. Importation of Emp1 models will be considered after the over-expression studies have been completed.

Task 5d: Experimental crosses between the Arntl2 and 2810474O19Rik genetically engineered models and the MMTV-PyMT model is currently in progress. Preliminary evidence suggests that constitutional reduction of 2810474O19Rik levels significantly influences metastatic progression of the MMTV-PyMT induced mammary tumors (Figure 4). At this time, the Arntl2 cross does not have sufficient data to make any preliminary evaluation. Additional animals are required for both crosses for conclusive analysis.

Task 5e: Orthotopic implantation of unmodified tumor cell lines into mice carrying a knockout of 2810474O19Rik heterozygously does not demonstrate any difference when compared to wildtype control animals (Figure 5). This suggests that the effect of 2810474O19Rik on metastasis is a tumor-autonomous effect, rather than a stromal effect or a combination of a stromal and tumor effect. Similar
Key Research Accomplishments

- Identification of 2810474O19Rik, Arntl2 and Emp1 as potential metastasis susceptibility genes in a model of ER- breast cancer

- In vivo validation of 2810474O19Rik, Arntl2 and Emp1 as metastasis susceptibility genes, as demonstrated by gene knockdown experiments in models of ER- human breast cancer.

- Demonstration that 2810474O19Rik and Arntl2 exert their metastasis-associated effects through tumor-autonomous mechanism.

- Complete genome sequence of the MOLF/Ei inbred mouse strain at 40x coverage

Conclusions

The data gathered to date suggest that multiple genes contribute to susceptibility to metastasis potential for ER- breast cancer. Identification and characterization of these genes and their mode of action in breast cancer progression will lead to novel insights and potentially point toward new therapeutic strategies for this class of breast cancer. In the future the Hunter experiments for Arntl2 also demonstrated no difference indicating that the effect of Arntl2 is also a tumor-autonomous effect (Figure 6).

Task 5f: Genome editing experiments have not yet been initiated at this time.
laboratory will continue to explore the role of these genes in breast cancer by a combination of \textit{in vitro} and \textit{in vivo} methods to refine our understanding of the mechanisms involved and the pathways that play important roles in tumor progression. These efforts will consist of experimental crosses between knockout strains and the MMTV-PyMT model, as described in task 5, as well as investigations into whether alterations of these genes affect any of the intermediate processes in the metastatic cascade such as invasion, migration, apoptosis, anoikis etc.

\textbf{Publications, Abstracts and Presentations}

Presentation at the 27\textsuperscript{th} International Mammalian Genome Conference, Salamanca, Spain “A metastasis susceptibility gene for estrogen receptor negative breast cancer maps to the distal end of chromosome 6” Ngoc-Han Ha, Ying Hu, Mia Williams, Rosan Nieves and Kent Hunter

\textbf{Inventions, Patents and Licenses}

Nothing to report

\textbf{Reportable Outcomes}

Nothing to report

\textbf{Other Achievements}

Nothing to report

\textbf{References}


\textbf{Appendices}

None