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Award Number: W81XWH-08-1-0306

TITLE: Characterizing an EMT Signature in Breast Cancer

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REPORT DATE:
April 2009

TYPE OF REPORT:
Annual Summary

PREPARED FOR:
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT:
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REPORT DOCUMENTATION PAGE				Form Approved OMB No. 0704-0188	
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1. REPORT DATE 01-04-2009		2. REPORT TYPE Annual Summary Report		3. DATES COVERED (From - To) 01 Apr 2008-31 Mar 2009	
4. TITLE AND SUBTITLE Characterizing an EMT Signature in Breast Cancer				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER W81XWH-08-1-0306	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Melanie Bocanegra EMAIL: mbocaneg@stanford.edu				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Leland Stanford Junior University Stanford, CA 94305-5176				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited					
13. SUPPLEMENTARY NOTES:					
14. ABSTRACT: Epithelial-to-mesenchymal transition (EMT), a switch of polarized epithelial cells to a migratory, fibroblastoid phenotype, is increasingly considered a determining step for cancer cell invasiveness and metastasis. To identify new molecular markers and potential key regulators of EMT, we performed expression profiling comparing 10 epithelial-like and 5 fibroblast-like breast cancer cell lines, subtracting the gene signatures from 5 early-passage normal breast fibroblast cell cultures. Differentially expressed genes were used to generate a 200-gene EMT signature, which was evaluated for its association with overall survival in patients with breast cancer. In published microarray cohorts there was significant association between the EMT signature and clinical outcome in patients with breast cancer, which was independent of established clinical and pathological variables (p<0.05). Among highly-ranked EMT signature genes, we also investigated the role of LYN, a member of the Src-family kinases. LYN expression, assayed by immunohistochemistry on tissue microarrays, was observed in 14.2% of breast cancer cases, and was associated with shorter overall survival (p=0.02), and correlated with the basal-like/triple-negative phenotype. Knockdown of LYN by RNA interference in fibroblast-like breast cancer cell lines inhibited cell migration and invasion, but not cell proliferation. Our findings define a prognostically-relevant EMT signature in breast cancer, and implicate LYN as a mediator of invasion and possible new therapeutic target, with particular relevance to clinically-aggressive basal-like/triple-negative breast cancer.					
15. SUBJECT TERMS: None					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	8	19b. TELEPHONE NUMBER (include area code)

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Introduction

Breast cancer is the second leading cause of cancer death in women in the United States, effecting women of all races and ethnicities. Breast cancer mortality results from the tumor acquiring the ability to invade and metastasize to different sites in the body. In order to invade and metastasize, cancer cells must first dissociate from one another and become motile. This process mirrors the epithelial-mesenchymal transition (EMT), a highly conserved process in embryonic development that occurs during tissue patterning. During EMT, epithelial cells lose polarity and the ability to make cell-cell contacts by altering the expression of several genes, ultimately leading to a more migratory, fibroblast-like “mesenchymal” cell phenotype. There is strong evidence EMT occurs, at least transiently, during breast cancer progression [1] and we propose to exploit the phenotypic differences between “epithelial-like” and “fibroblast-like” breast cancer cell lines in culture to explore the mechanisms of EMT.

Body

Our first goal was to identify gene-expression signatures of EMT from breast cancer cells in culture. A discriminatory gene-expression signature was identified from the microarray data, using supervised analysis methods. The EMT signature comprised the top ranked 100 genes overexpressed in fibroblast-like breast cancer cells compared to epithelial-like breast cancer cells and normal breast fibroblasts, and in epithelial-like breast cancer cells compared to fibroblast-like breast cancer cells and normal breast fibroblasts (Figure 1A).

Our second goal was to determine the prognostic value of the EMT signature in breast cancer. The prognostic value of the EMT gene signature was evaluated by analyzing its coordinated expression and correlation with clinical outcomes in primary breast tumors, using publicly-available clinically-annotated breast cancer microarray expression datasets. Prognostic value was determined by clustering samples in the space of the set of 200 EMT signature genes, and comparing clinical outcomes (both recurrence-free survival and disease-specific survival) among the top sample clusters by Kaplan-Meier analysis. In published microarray cohorts there was significant association between the EMT signature and clinical outcome in patients with breast cancer, which was independent of established clinical and pathological variables $p < 0.05$ (Figure 1B).

Our third goal was to assess the functional role of EMT signature genes in the EMT process. We attempted to identify candidate EMT “driver” genes by intersecting the EMT signature with genes found to be locally amplified by array-based comparative genomic hybridization (array CGH); however, no linear relationships were uncovered. Additionally, we evaluated the top 20 genes (Figure 1A) involved with key EMT processes and prioritized the LYN gene, a member of the Src-family kinases, for subsequent analysis. LYN expression, assayed by immunohistochemistry on tissue microarrays, was observed in 133 of 970 breast cancer cases (14.2%) (Figure 2A), and was significantly associated with shorter overall survival ($p = 0.02$), and correlated with the basal-like/triple-negative phenotype (CK5/6 and/or EGFR positive, HER2 and ER negative) (Figure 2B). LYN overexpression in fibroblast-like breast cancer cell lines relative to epithelial-like cell lines was verified using western blot (Figure 3A).

In order to assess LYN's role as a regulator of EMT, we performed RNA interference (RNAi) on fibroblast like breast cancer cell lines to knockdown LYN levels. Western blot confirmed a 70% reduction of both active and total forms of LYN, as well as altered EMT molecular profiles (e.g. loss of vimentin) in response to LYN knockdown (Figure 3B). Although phase-contrast microscopy did not yield altered morphologies (i.e. loss of cell-cell contact), migration and invasion were significantly downregulated in response to LYN knockdown (Figure 3C). We also examined the expression profiles the Src-family of kinases in a cohort of 50 breast cancer cell lines and discovered that LYN expression is most significantly correlated with the basal-like breast cancer cell lines (Figure 4).

Key Research Accomplishments

- An EMT signature, derived by comparing morphologically distinct breast cancer cells, exhibits prognostic utility.
- LYN tyrosine kinase, a highly ranked EMT signature gene, is overexpressed in a subset of primary breast tumors, and is associated with basal-like phenotype and unfavorable outcome.
- RNA-interference (RNAi) knockdown studies demonstrate a role of LYN in promoting cell motility and invasion.

Reportable Outcomes

"Prognostic signature of epithelial-mesenchymal transition in breast cancer highlights role of LYN in invasion and likely target of dasatinib." 2009 American Association for Cancer Research Conference, Denver, CO

Conclusions

Our findings define a prognostically-relevant EMT signature in breast cancer, and implicate LYN as a mediator of invasion and possible new therapeutic target with particular relevance to clinically-aggressive basal-like/triple-negative breast cancer. Furthermore, LYN belongs to a class of tyrosine kinases (Src-family of protein kinases) which several small molecule inhibitors exist. We are currently focusing on understanding LYN's role as a theragnostic target for the drug dasatinib, presently in clinical trials for the treatment of basal-like breast tumors [2]. Additionally, future studies will center on supporting the RNAi studies with overexpression studies of LYN in "epithelial-like" breast cancer cell lines as outlined in the original statement of work.

Taken together, this body of work furthers our understanding of the molecular mechanisms tumors utilize to invade and metastasize. Due to LYN's significant role in cell motility and correlation to the invasive basal-like breast tumors, it's a promising molecular marker for predicting drug sensitivities which will ultimately improve clinical response.

References

1. Thiery JP (2002). Epithelial-mesenchymal transitions in tumour progression. *Nat Rev Cancer* 2:442-454
2. Huang F, et. al. (2007). Identification of Candidate Molecular Markers Predicting Sensitivity in Solid Tumors of Dasatinib: Rationale for Patient Selection. *Cancer Res* 2007;67;2226-238

Appendices: None

Supporting Data: *Figure Legends*

Figure 1. Cell line derived EMT signature shows prognostic relevance. (A) Expression profiling of morphologically distinct breast cancer cell lines defines signature of EMT. The EMT signature (heatmap shown) comprises the top ranked 100 genes overexpressed in fibroblast-like breast cancer cells compared to epithelial-like breast cancer cells and normal breast fibroblasts, and in epithelial-like breast cancer cells compared to fibroblast-like breast cancer cells and normal breast fibroblasts. The top 20 genes, including the LYN tyrosine kinase (arrow), are shown. **(B)** EMT signature is prognostically relevant. Primary breast tumors from a publically-available microarray dataset (Sotiriou et al., JNCI, 2006) are clustered in the space of the EMT signature genes, then the two major sample clusters are compared by Kaplan-Meier analysis (*P*-values shown).

Figure 2. LYN immunostaining is associated with unfavorable outcome. (A) LYN immunostaining. Shown are representative breast cancer cases negative (arrow identifies region with tumor cells) or positive for expression of the LYN tyrosine kinase. (B) Kaplan-Meier analysis of LYN immunostaining in relation to relapse-free and overall survival.

Figure 3 LYN overexpression contributes to invasiveness. (A) LYN exhibits relative overexpression (by Western blot) in fibroblast-like breast cancer cell lines. (B) Validation of LYN knockdown by siRNA. (C) LYN knockdown leads to significantly decreased cell migration (left) and invasion (right) (*, $P<0.05$, **, $P<.01$).

Figure 4. LYN is overexpressed in basal-like breast cancer cell lines. Heatmap of microarray expression profiles of SRC family kinase genes in a collection of 50 breast cancer cell lines (clustering based on ~5000 variably expressed genes). LYN is significantly overexpressed in basal-like lines ($P<0.01$), previously reported to be selectively sensitive to dasatinib.

Figure 1

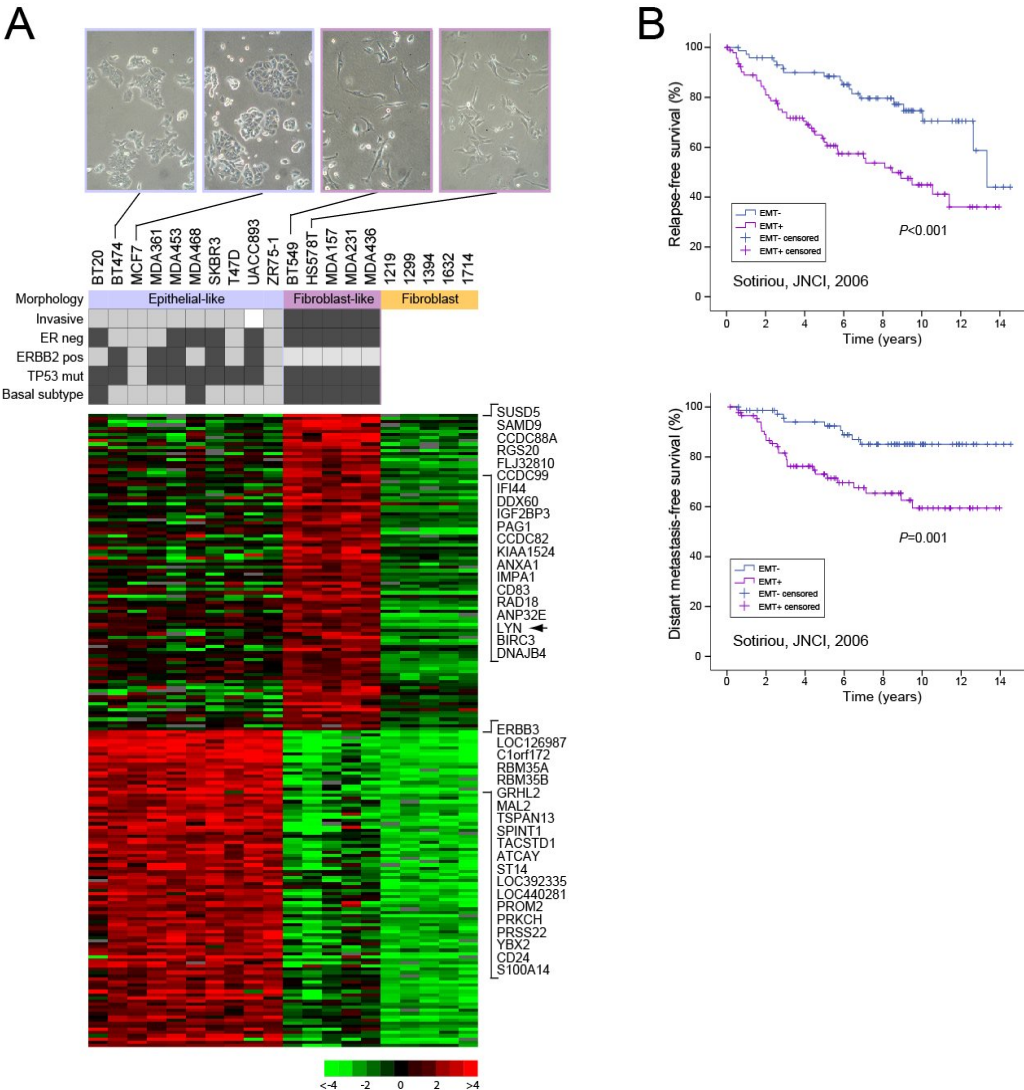


Figure 2

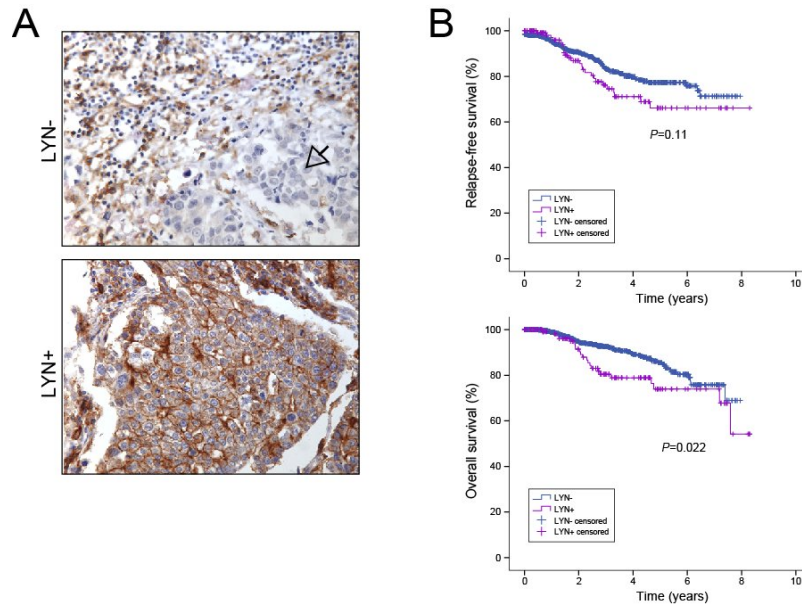


Figure 3

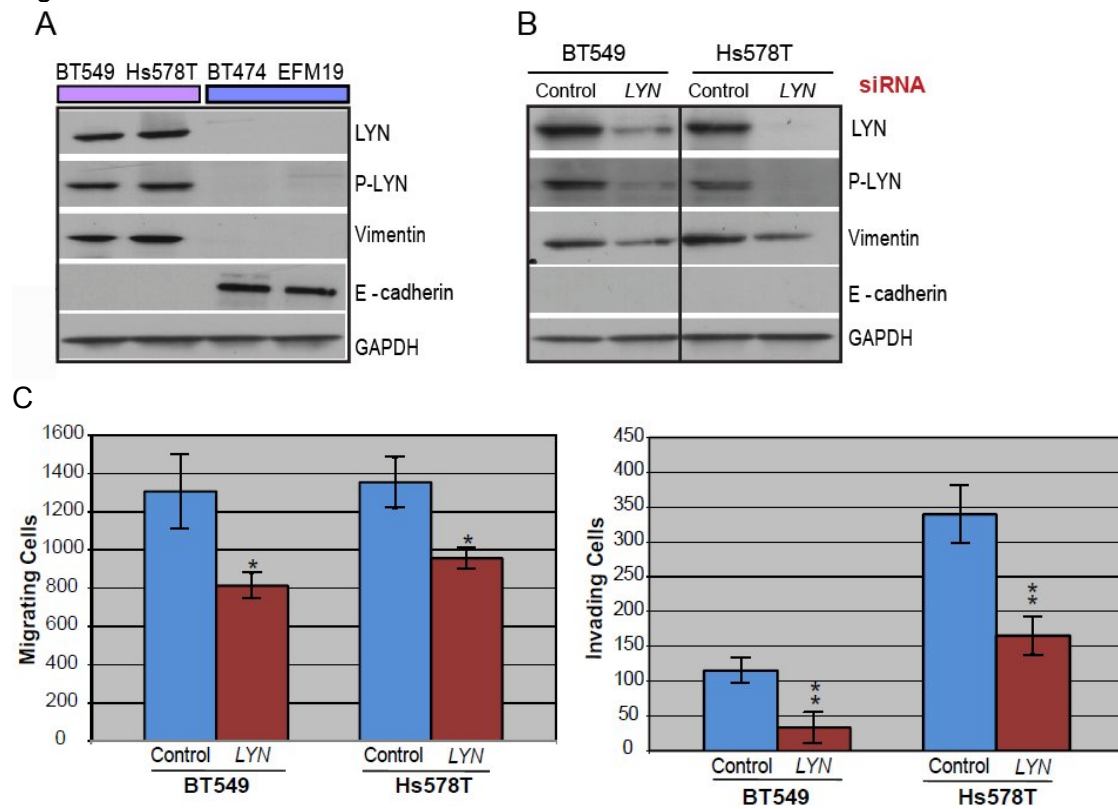


Figure 4

