# Targeting Premalignant Lesions: Implications for Early Breast Cancer Detection and Intervention

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Breast cancer progression constitutes a multistep process through a series of intermediate hyperplastic and neoplastic stages to invasive carcinoma. In this study, we aimed to identify peptides that specifically recognize premalignant lesions in the mammary tissue. To achieve this goal, we utilized the power of phage display to probe hyperplastic lesions associated with premalignant disease in a transgenic MMTV-PyMT animal model. We have identified a peptide CISQ that targets to the stroma in premalignant lesions and binds to cancer-associated fibroblasts (CAFs) in MMTV-PyMT mice. Considerable numbers of CAFs are frequently observed within the tumor-associated stroma of various human cancers, including those of the breast, prostate, lung, colon and pancreas and have been also reported in the premalignant lesions. This peptide could provide us with an opportunity to therapeutically intervene to successfully inhibit or even reverse tumor progression.

**15. SUBJECT TERMS**
- Early breast cancer, early detection, homing peptides, premalignant lesions, targeted nanomedicine

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1. INTRODUCTION:

Screening for breast cancer using mammography and MRI has improved the mortality, however these methods still lack the sensitivity and specificity needed to reliably detect early-stage disease. Over 60% of breast cancer patients are diagnosed after the cancer cells have already invaded surrounding tissues and metastasized throughout the body, limiting the success of subsequent therapies. Therefore, methods to detect early premalignant lesions in the mammary gland are needed to expand our understanding of breast cancer progression and for early intervention in patients at high-risk of breast cancer based on their genetic predisposition.

Our laboratory has successfully employed in vivo screening of phage libraries to develop new probes for breast tumors. Progression of breast cancer constitutes a multistep process wherein each stage is characterized by distinct phenotypic changes that occur in the mammary gland. We proposed to utilize this animal model to probe early stage (premalignant) lesions with phage libraries to identify novel peptides that specifically recognize the premalignant stage of breast cancer. These peptides and the identification of their putative receptors will help our understanding of the underlying biology of breast cancer progression. Furthermore, these probes will be used to develop targeted therapeutic nanoparticles for early intervention in breast cancer.

2. KEYWORDS:

Early breast cancer, extra cellular matrix, homing peptides, premalignant lesions, targeted nanomedicine

3. ACCOMPLISHMENTS:

Specific Aim 1: Identify peptides that specifically home to premalignant breast lesions (Months 1-12)

Task 1. To screen phage libraries for new peptides that specifically recognize premalignant lesions (Months 1-9):

Task 2. To validate the homing specificities of individual phage and synthetic peptides

Specific Aim 2: Identify and characterize putative receptors in premalignant lesions (Months 12-24).

Task 1: To identify putative receptors of these peptides in these early lesions (Months 12-15)

Specific Aim 3: Target premalignant lesions utilizing peptide-conjugated nanoparticles to prevent/delay progression of premalignant lesions to invasive breast cancer (Months 18-36)
Task 1: To engineer and characterize peptide conjugated therapeutic nanoparticles (Months 18-24)

Task 2: Study the effect of targeted delivery of therapeutic nanoparticles on the onset of the disease (Months 24-36)

Major goals and objectives completed for this project are as follows:
1. Tested a panel of previously identified tumor homing peptides for homing to premalignant lesions in PyMT-MMTV and found two peptides (IGD and CSG) that homed to these early tumor lesions.
2. Initiated phage library screening in PyMT-MMTV model and identified a novel peptide CAGK that shows strong homing to the extracellular matrix at premalignant stage.
3. Conducted protein expression analysis and observed a significant change in ECM composition of premalignant lesions in PyMT mice as compared to normal mice.
4. Utilized the homing peptides to enhance targeting of a nanoparticle payload to these lesions.

Results

Aim 1: Identify peptides that specifically home to premalignant breast lesions

We characterized and studied the early stages of the MMTV-PyMT transgenic model of de novo breast cancer. The hyperplasia appears around 8 week of age in the PyMT mice as observed by carmine red staining (method described at end of results section) (Fig. 1). Using this model, we have performed phage library screens to identify a panel of peptides that can target to the premalignant lesions from an intravenous injection. The peptides that are described below show distinct localization pattern in these early tumor lesions. For instance, IGD peptide and CISQ peptide when injected intravenously localized into cellular structures as shown in Fig. 2 and 3.

Fig. 1: Whole mounts of mammary fat pad of MMTV-PyMT mice. Mammary fat pad (#4) was isolated at 8 weeks of age from wild-type (left) and PyMT (right) and fixed in Carnoy’s fixative and stained with Carmine alum. Microscopic evaluation revealed the hyperplastic lesion consisting of the clusters of densely packed lobules formed at the ends of growing immature ducts shown in higher magnification in the right inset.
Fig. 2: IGD homes to early (premalignant) lesions in mammary fat pad isolated from PyMT-MMTV mice. Immunofluorescence staining on whole mount sections of mammary fat pad isolated following 1 hour in-vivo circulation of 0.15 μmol FAM-IGD injected intravenously in normal Blk6 mouse (A) or day 48 PyMT-MMTV mouse (B). Green – anti-FAM-IGD; Blue - Nuclear Stain. FAM- fluorescein.

Fig. 3: CISQ accumulates in premalignant lesions in mammary fat pad isolated from PyMT-MMTV animals. Immunofluorescence staining overlapped with H&E staining on whole mount sections of mammary fat pad isolated following FAM-CISQ injection in PyMT-MMTV mouse. Green – anti-FAM-CISQ; Purple - Nuclear Stain.
On the other hand, two other peptides CSG and CAG showed binding to components in the extracellular matrix. A control peptide did not show any accumulation in these early lesions.

**Fig. 4:** CAG homes to early (premalignant) hyperplastic lesions in mammary fat pad isolated from PyMT-MMTV animals. Immunofluorescence staining on whole mount sections of mammary fat pad isolated following 1 hour in-vivo circulation of FAM-CAG in PyMT-MMTV mouse (B) or normal Blk6 mouse (C). A shows the H&E staining on the corresponding section in B. Green – anti-FAM-CAG; Blue - Nuclear Stain.

**Fig. 5:** CSG homes to premalignant lesions in mammary fat pad isolated from PyMT-MMTV animals. Immunohistochemical staining on whole mount sections of mammary fat pad isolated following 1 hour in-vivo circulation of FAM-CSG in normal Blk6 mouse (A) or PyMT-MMTV mouse (B). Brown – Peptide signal, blue – nuclear staining.

CSG peptide also recognizes human breast tumors. Tumor-specific binding of CSG on tissue cross sections was observed in all patient samples (work done in Dr. Hamzah’s laboratory).
Aim 2: Identify and characterize putative receptors in premalignant lesions

In this aim, we confirmed the target for the peptides described above in Aim 1. IGD and CISQ targeted cellular structures that stained positive for Vimentin and ER-TR7 staining, suggesting that they were fibroblasts (Fig. 6 and 7). We noticed an overall increase in the fibroblast activation as visualized by immunostaining for FAP positive cells in these sections (Fig. 8). Fibroblast activation in early tumor lesions and their role in tumor progression was recently reported (Erez N, et. al Cancer Cell 2010).

Fig. 6: IGD colocalizes with fibroblasts in early (premalignant) hyperplastic lesions in mammary fat pad isolated from PyMT-MMTV animals. Immunofluorescence staining on whole mount sections of mammary fat pad isolated following FAM-IGD injection in PyMT-MMTV mouse. Green – anti-FAM-IGD; Red – anti-Vimentin-1, Blue - Nuclear Stain.

Fig. 7: CISQ colocalizes with fibroblasts in early (premalignant) lesions in mammary fat pad isolated from PyMT-MMTV animals. Immunofluorescence staining on whole mounts of mammary fat pad isolated following 1 hour in-vivo circulation of FAM-CISQ stained with
fibroblast marker ER-TR7 (A) and vimentin (B). Note the colocalization of FAM-CISQ and fibroblasts.

We have also characterized the homing of the other two peptides, CSG and CAGK that localized into the ECM of the premalignant lesions in the PyMT model. It is known that tumor initiation and progression are accompanied by complex structural changes in the extracellular matrix (ECM) and cellular architecture (Butcher, D. T., Alliston, T. & Weaver, V. M. A tense situation: forcing tumor progression. Nature Rev. Cancer 9, 108–122 (2009). Our studies on peptide homing to ECM in early lesions, suggested that the ECM changes start early to facilitate the tumor initiation and progression in this model. As a next step, we decided to study the global expression of ECM proteins to characterize their composition at the premalignant stage. For this, we enriched the ECM proteins from PyMT and WT mice at 10 weeks of age by following a series of steps that included a decellularization procedure adapted from (Naba et al. in J. Vis. Exp. (101), e53057, doi:10.3791/53057 (2015) that consisted of sequential incubations in buffers of different pH and salt and detergent concentrations. This procedure resulted in the extraction (or depletion) of cytosolic, nuclear, membrane and cytoskeletal proteins and the enrichment of ECM proteins. We then digested the ECM-enriched protein preparations into peptides for subsequent analysis by mass spectrometry. The results from the proteomics analysis on these samples are summarized in Fig. 9. Many ECM proteins such as Elastin, Collagens, Nidogen showed a dramatic increase in PyMT lesions compared to the WT mice. Next, we analyzed the expression of three ECM components, Nidogen-1, laminin and collagen IV by immunofluorescence staining in these early lesions, (Fig. 10). All of these members show re-organization of the matrix in the mammary glands containing the early cancer lesions. On further analysis, we confirmed that CSG peptide co-localized with the ECM protein Nidogen-1 (Fig. 11). Recently, a report showed that Nidogen-1 possessed prometastatic functions as it aided in tumor progression (Aleckovic et al, Genes & Dev, 2017).
Fig. 9: Changes in extra cellular matrix proteins in premalignant lesions. Mammary glands were isolated from the mice and the ECM fraction was separated and analyzed on Mass spectrometry. Data was compared for normal mice and PyMT mice at 9 weeks of age. Bar graph represents fold change of PyMT vs WT, + fold increase and – fold decrease.

Fig. 10: Expression of ECM components in premalignant lesions of PyMT mice. Immunofluorescence staining on whole mount sections of mammary fat pad in PyMT-MMTV mouse or normal Blk6 mouse.
**Fig. 11:** CSG colocalized with Nidogen in early (premalignant) hyperplastic lesions in mammary fat pad isolated from PyMT-MMTV animals. Immunofluorescence staining on whole mount sections of mammary fat pad isolated following 1 hour in-vivo circulation of FAM-CSG in normal Blk6 mouse (A) or PyMT-MMTV mouse (B). Green – anti-FAM-CSG; Red – anti-Nidogen-1, Blue - Nuclear Stain. Scale Bar is 2 micron.

**Aim 3:** Target premalignant lesions utilizing peptide-conjugated nanoparticles to prevent/delay progression of premalignant lesions to invasive breast cancer

In this aim, we developed a nanoparticle drug delivery system to target to the premalignant lesions. The silver nanoparticle (AgNP) system used for this aim has been previously used for targeted drug delivery (Braun et al. Nature Materials, 2014). First, the peptides were chemically synthesized with an extra cysteine for coupling to AgNP. AgNPs of around 35 nm diameter were synthesized as described previously in (Braun et al. Nature Materials, 2014) and coupled with two homing peptides IGD and CAGK (Fig. 12).

**Fig. 12. Schematic of targeted AgNPs.** AgNPs were coated with polyethylene glycol linker (black lines) onto which fluorescently-labeled cysteine peptides were attached. The size of the AgNP was measured around 35nm using dynamic light scattering (DLS, Malvern instruments, IL, USA).
Both IGD-AgNP and CAGK-AgNP targeted the premalignant lesions in MMTV-PyMT mice (Fig. 14). The control peptide conjugated AgNP did not show any specific accumulation and no AgNP homing was observed in the WT mice. This data demonstrates that the homing peptides IGD and CAGK can mediate delivery of nanoparticles to the premalignant lesions in the PyMT mice.

**Fig. 13. Peptide mediated nanoparticle delivery to premalignant lesions.** Peptide conjugated AgNP were injected intravenously in mice and allowed to circulate for 2 hours. The mice were then perfused with saline and their mammary glands were isolated and analyzed for nanoparticle accumulation by silver staining (brown). A. IGD-AgNP in PyMT lesions. B. CAGK-AgNP in PyMT lesions. C. Control AgNP in PyMT lesions.

The original approved proposal described an experiment to treat the PyMT mice at early stages of the disease with these targeted therapeutics to delay or prevent the tumor progression. However, there were difficulties in breeding of the transgenic PyMT mice and to generate enough mice for a large therapy experiment. However, our collaborator, Dr. Juliana Hamzah at The University of Western Australia, has utilized the CSG peptide to systemically deliver a therapeutic agent (Tumor necrosis factor, TNFα) for treatment of breast tumor (manuscript submitted).

**Opportunities for training and professional development:**

During this project the following training and professional development has been undertaken

1. “Pathobiology of the Mouse Tier 1A” online course offered through UC Davis Extension
2. “The Art of Science Communication” course offered by Sanford-Burnham and American Society for Biochemistry and Molecular Biology (ASBMB).
3. Appointed as a founding member of the Sanford Burnham Prebys Postdoc Training Advisory Group to strategize in developing training strategies for postdocs at SBP.
4. Recently promoted to Research Assistant Professor and appointed as an associate member of the Tumor Microenvironment and cancer immunology program of the NCI-Designated Cancer Center at Sanford Burnham Prebys Medical Discovery Institute.
Dissemination of results:

1. Presented highlights of this work on December 3, 2014, at the Annual Holiday Symposium of Group of 12 - a community service group in La Jolla stressing health, education, and friendship.
2. Presented at the inaugural Postdoc Open-Mic Night conducted in La Jolla by Scripps Research Institute in collaboration with The Salk Institute and UCSD, 2015.
3. Presentation at the Annual Postdoctoral symposium held at Sanford Burnham Medical Research Institute, 2015.
4. Participated in collaborative meetings with Hamzah group in University of Western Australia and Teesalu group in University of Tartu, Estonia (see collaborations).

4. IMPACT:

**Impact on the development of the principal discipline(s) of the project:** We have identified four homing peptide that can target premalignant lesions of breast cancer. We also demonstrated targeted delivery of nanoparticles to these lesions. This approach can be applied to delivery of therapeutics to these lesions as a method of early intervention of breast cancer.

**Impact on other disciplines:** The homing ability of the peptide to premalignant lesions can also be utilized for diagnostic applications. This can be done by combining the peptides with imaging agents such as iron oxide nanoparticles for non-invasive detection of these premalignant lesions.

**Impact on technology transfer:** Nothing to report

**Impact on society beyond science and technology:** Nothing to report

5. CHANGES/PROBLEMS - None

6. PRODUCTS:

**Journal publications.**

1. **Authors:** Aman P Mann, Sazid Hussain, Gary B. Braun, Venkata Ramana Kotamraju Tambet Teesalu, Erkki Ruoslahti
   **Title:** Peptides for detecting early changes in tumor progression
   **Status of publication:** In preparation
   **Acknowledgement of federal support:** Yes

Title: Immune-mechanical effects of matrix-bound TNFα effectively degrade multiple components of extracellular matrix and enhance perfusion of nanoparticles in solid tumors

Status of publication: Submitted; in Process

Acknowledgement of federal support: No

Books or other non-periodical, one-time publications: Conference proceedings for American Association for Cancer Research Annual Meeting (see appendix).

Conference: American Association for Cancer Research Annual Meeting 2014

Title of Presentation: Targeting Premalignant lesions for early breast cancer detection and intervention

Authors: Aman P Mann, Venkata Ramana Kotamraju, Tambet Teesalu, and Erkki Ruoslahti

Status of publication: Published

Bibliographic information: Cancer Res October 1, 2014 74; 3258

Acknowledgement of federal support: Yes

Website(s) or other Internet site(s): None

Technologies or techniques – Peptides that can target premalignant lesions

Inventions, patent applications, and/or licenses - None

Other Products - None

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS:

Name: Aman Mann
Project Role: PI
Research Identifier: N/A
Nearest Person Month Worked: 12
Contribution to Project: Principal Investigator and oversee all scientific, experimental and administrative aspects
Funding Support: This Grant

Name: Erkki Ruoslahti
Project Role: Mentor
Research Identifier: N/A
Nearest Person Month Worked: 0
Contribution to Project: Serves as a mentor to Dr. Aman Mann
Funding Support: This Grant
• Active collaboration with Tambet Teesalu, head of the Laboratory of Cancer Biology at University of Tartu, Estonia.
• Initiated collaboration with Dr. Juliana Hamzah, Laboratory Head, Targeted Drug Delivery, Imaging and Therapy at the Harry Perkins Institute of Medical Research in Australia. Joint manuscript submitted for publication.

8. SPECIAL REPORTING REQUIREMENTS: None

9. APPENDICES:
   1. Conference proceedings for American Association for Cancer Research
Abstract 3258: Targeting premalignant lesions for early breast cancer detection and intervention

Aman P. Mann, Ramana Kotamraju, Tambet Teesalu, and Erkki Ruoslahti

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Abstract

Breast cancer patients' outcome and survival depends on the timely diagnosis of early malignant lesions. The lack of molecular understanding of the early changes in breast tissue that facilitate tumor progression has limited the development of tools to non-invasively distinguish early stage disease from normal breast tissue. Therefore, probes to target and better understand early breast tumors are much needed. Our laboratory has pioneered in vivo screening of phage libraries to identify peptides that specifically recognize tumor vessels, including breast cancer vasculature. These peptides have been employed to specifically deliver drugs, diagnostic agents, and nanoparticles to breast tumors.

Breast cancer progression constitutes a multistep process through a series of intermediate hyperplastic and neoplastic stages to invasive carcinoma. In this study, we aimed to identify peptides that specifically recognize premalignant lesions in the mammary tissue. To achieve this goal, we utilized the power of phage display to probe hyperplastic lesions associated with premalignant disease in a transgenic MMTV-PyMT animal model. After multiple ex-vivo and in-vivo rounds of selection, we identified a peptide, Prem-1, that on intravenous administration, specifically homed to premalignant mammary lesions. Prem-1 also homed to fully developed breast tumors in the same animal model, suggesting that the putative receptor for Prem-1 is expressed throughout the progression of the disease. Interestingly, Prem-1 did not show any affinity to normal breast tissue. Furthermore, we also identified 2 other candidate peptides that showed significant homing to premalignant lesions with a very different binding pattern as compared to Prem-1. We hypothesized that all three peptides recognize early changes in the breast tissue microenvironment but each bind a different target receptor in the tissue. We are currently investigating these receptors and analyzing their expression in breast cancer progression. Secondly, we are testing the peptides identified herein for the delivery of therapeutic nanoparticles as a mode of early intervention in breast cancer progression. This project utilized the natural environment in the early breast tumor to probe for new markers for detection of early disease. Methods to detect and study early premalignant mammary lesions will expand our understanding of the natural history of the disease, especially the changes in...
breast tissue that likely lead to tumor development. Hence, the knowledge gained from this study would provide a basis for therapies aimed at suppressing or eradicating premalignant breast lesions. This would be particularly beneficial for women at high-risk of breast cancer based on their genetic predisposition (mutations in BRCA genes).


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