PLENARY LECTURE

CELINA KLEER, MD
BREAST PATHOLOGY

“Metaplastic Carcinomas of the Breast: Pathology, Proteomics and Mouse Models”
Metaplastic carcinomas of the breast: Pathology, proteomics, and mouse models

Celina G. Kleer, M.D.
Harold A. Oberman Collegiate Professor
University of Michigan
Clinical History

• 32 year old woman
• Nodule in right breast
• Abnormal mammogram- 0.7 cm lesion
• No personal or family history of breast cancer
Imaging Findings

- Mammography: 0.7 cm density
Imaging Findings

- Ultrasound: circumscribed lesion with posterior shadowing
First impression

• Sclerosing lesion, perhaps papilloma or adenosis
• Area of infarction - difficult to interpret
• Scar/fibrous area- secondary to previous biopsy?
• Does not really fit well in a diagnostic category- carcinoma?
Summary of Features

- Small, expansile lesion with abundant sclerosing adenosis
- Too many spindle cells separating the acini of sclerosing adenosis
- Thick rim of scar-like tissue with myxoid change and immersed epithelioid cells
- Squamous differentiation
Diagnosis?

Pathologist 1: Complex sclerosing lesion with reactive stromal changes and infarction.

Pathologist 2: Metaplastic carcinoma arising in a complex sclerosing lesion.

Other: Fibromatosis surrounding a complex sclerosing lesion
High molecular weight cytokeratin
Diagnosis
Metaplastic carcinoma arising in a complex sclerosing lesion
Metaplastic carcinomas

- **Definition**
  Heterogeneous group of malignant tumors in which part or all of the carcinomatous epithelium is transformed into a non-glandular (metaplastic) growth process
Epidemiology

- Less than 1% of invasive carcinomas of the breast, but up to 14% in African Americans
- Average age at presentation is 55 years
- Subtype of triple negative breast cancer (TNBC)

Bae SY, Breast Cancer Res Treat 2011, 126:471-78
Abouharb S Curr Oncol Rep 2015, 17:431
Histological classification

- Metaplastic carcinoma of no special type
  low grade adenosquamous fibromatosis-like
  Squamous
  Spindle
- Metaplastic ca with mesenchymal differentiation
  Chondroid
  Osseous
  other types of mesenchymal differentiation
- Mixed metaplastic carcinoma
Immunohistochemical work-up

• **Cytokeratin** is positive in the epithelial elements and also in the spindle cells (sometimes focally)
  
  $34\beta E12$ (high molecular weight cytokeratin),
  CK5, CK14, CAM5.2, AE1/AE3

• **P63**- positive in epithelial and spindle cells.

**ALWAYS DO CYTOKERATIN STAIN(S)**
HMW cytokeratin
Metaplastic carcinoma vs. TNBC outcomes

Metaplastic carcinomas:
• More distant metastasis
• More chemo-resistant
• Worse overall survival

There are currently no targeted therapy

Molecular alterations in metaplastic breast carcinomas

- Whole exome sequence studies
- Complex pattern of copy number alterations
- TP53 mutations, PI3K/AKT/mTOR and Wnt pathway activation
- Our lab has reported Wnt pathway gene mutations, including CCN6/WISP3
- Limited models that recapitulate the histologic subtypes of metaplastic carcinomas
- The proteomic landscape is unknown

Our research efforts...

- Development of a mammary gland specific CCN6 knockout mouse model for metaplastic carcinomas
- Discovery of the proteomic landscape of human metaplastic carcinomas
Our research efforts...

• Development of a CCN6 knockout mouse model for metaplastic carcinomas
• Discovery of the proteomic landscape of human metaplastic carcinomas
CCN6 belongs to the CCN protein family

• CCN family contains 6 members in humans (CCN1-CCN6)

• Matricellular proteins

• Implicated in angiogenesis, chondrogenesis, and wound healing. They also participate in cell proliferation, migration, and differentiation.

• Secreted proteins and also may translocate to the nucleus – e.g. CCN3

Multimodular structure

- SP: Signal peptide
- IGFBP: IGFBP motif
- VWC: von Willebrand factor C motif
- TSP1: Thrombospondin type 1 motif
- CT: cysteine knot

Perbal B, The Lancet, 2004
Planque and Perbal J Cell Biochem, 2006
CCN6/WISP3

- Wnt-1 induced secreted protein 3
- Located at 6q21-22
- CCN6 mutations cause progressive pseudo-rheumatoid dysplasia
- Secreted, matrix associated protein with regulatory roles

**CCN6 is reduced in metaplastic ca**

<table>
<thead>
<tr>
<th></th>
<th>Invasive ductal ca</th>
<th>Metaplastic ca</th>
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<tbody>
<tr>
<td>CCN6 low</td>
<td>27 (33%)</td>
<td>19 (67.9%)</td>
</tr>
<tr>
<td>CCN6 high</td>
<td>55 (67%)</td>
<td>9 (32.1%)</td>
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</table>

P = 0.0018

Martin EE. Oncogene 2017
CCN6 expression is associated with the aggressiveness of breast cells

MCF10A benign spontaneously immortalized cells derived from a patient with fibrocystic changes
HME benign cells immortalized with HPV E6/E7
MDA-MB-231 ER negative breast cancer cells; form tumors and metastasis in nude mice
MCF-7 ER positive breast cancer cell; non-invasive
SUM149 highly aggressive ER neg breast cancer cell
CCN6 knockdown (KD) disrupts acinar development and promotes invasion

HME cells

Control

CCN6 KD

Day 15 in matrigel
Recombinant human CCN6 protein rescues invasion due to CCN6 knockdown

CCN6 downregulation promotes survival and decreases apoptosis

**WB.**

<table>
<thead>
<tr>
<th></th>
<th>HME</th>
<th>MCF10A</th>
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<tbody>
<tr>
<td>CCN6 shRNA:</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>CCN6</td>
<td>1 0.2</td>
<td>1 0.3</td>
</tr>
<tr>
<td>β-Actin</td>
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</table>

**Serum-free medium**

![Graph showing the number of viable cells over time](image)

**Annexin V apoptosis Assay**

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<tr>
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<th>control</th>
<th>CCN6 KD</th>
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<td>apoptotic cells (%)</td>
<td><img src="image" alt="control" /></td>
<td><img src="image" alt="CCN6 KD" /></td>
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CCN6 overexpression reduces tumorigenesis


MDA-MB-231

<table>
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<th>Flag-vector</th>
<th>CCN6-Flag</th>
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<tr>
<td>CCN6-Flag</td>
<td>- 37 kD</td>
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<tr>
<td>β-Actin</td>
<td>- 42 kD</td>
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MDA-MB-231:

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<tr>
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<th>CCN6-Flag</th>
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<tr>
<td>Distant metastasis rate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>14/14</td>
<td>9/15*</td>
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CCN6 has extracellular functions

Binds to IGF-1

Binds to BMP4

TSP1 domain is required
Conditional deletion of *Ccn6* in mammary epithelial cells may alter mammary gland development and may induce mammary tumors with features of epithelial to mesenchymal transition (EMT).
Conditional knockout (KO) of the mouse *Ccn6* gene in mammary epithelial cells using the Cre-Lox system
Genotyping

400 bp → 200 bp

wt/wt, wt/fl, fl/fl

Ccn6

5 wks 8 wks 4 mo 12 mo

wt/wt wt/fl wt/fl fl/fl

Immunoblots of mammary glands

Ccn6

Ponceau

qRT-PCR of mammary glands

Ccn6 mRNA/GAPDH

80

60

40

20

0

**

wt/wt fl/fl wt/wt fl/fl

8 weeks 4 months
Whole mount of a normal mammary gland of 8 week-old female virgin mouse (Puberty)

- Mammary fat pad
- Ductal branches
- Lymph node
- Terminal end buds (TEBs)
Ccn6 KO leads to reduced mammary gland complexity at 4 months

NORMAL (Ccn6wt/wt)

Ccn6fl/fl

Martin EE et al. Oncogene, 36(16):2275-85, 2017
Ccn6 KO mammary glands have fewer ducts at 4 months of age
Ccn6 epithelial specific KO leads to reduced ductal thickness
72% (13/18) Ccn6 KO mice developed mammary tumors and 46% metastasized.

\[ \text{Ccn6}^{fl/fl} \] mammary tumors

Age range at tumor development 10-21 months, mean 15 months

Martin EE et al. Oncogene, 36(16):2275-85, 2017
Ccn6^{fl/fl} tumors share 87 genes with human metaplastic carcinomas
Ccn6^{fl/fl} tumors share 87 genes with human metaplastic carcinomas

- Oncofetal proteins
- Reciprocal regulation
- Regulated by WNT signaling
Ccn6^{fl/fl} tumors share 87 genes with human metaplastic carcinomas.
CCN6 low/ IMP2 high/ HMGA2 high phenotype in metaplastic carcinomas with spindle and squamous differentiation

<table>
<thead>
<tr>
<th>Metaplastic Component</th>
<th>H&amp;E</th>
<th>CCN6</th>
<th>IMP2</th>
<th>HMGA2</th>
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<table>
<thead>
<tr>
<th>Metaplastic Component</th>
<th>CCN6 Low</th>
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<tr>
<td></td>
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<td></td>
<td>HMGA2</td>
<td>HMGA2</td>
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<tr>
<td></td>
<td>High</td>
<td>High</td>
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<tr>
<td>Spindle</td>
<td>4</td>
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<tr>
<td>Squamous</td>
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<td>9</td>
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<tr>
<td>Chondroid</td>
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<tr>
<td>Total</td>
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<td>14</td>
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p-value

McMullen et al. Breast Cancer Res Treat, 2018
Ongoing experiments

1. We are currently investigating how CCN6 regulates HMGA2 and IMP2 and their contribution to tumor phenotypes

2. The role of CCN6 as an antagonist of WNT/β-catenin signaling in metaplastic carcinomas
Our research efforts...

• Development of a CCN6 knockout mouse model for metaplastic carcinomas

• Discovery of the proteomic landscape of human metaplastic carcinomas

NEW DATA
<table>
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<tr>
<th>Case #</th>
<th>Age (yrs)</th>
<th>Tumor size (cm)</th>
<th>Diagnosis</th>
<th>Histologic Subtype</th>
<th>Tumor grade</th>
<th>Stage at diagnosis</th>
<th>Distant Metastasis</th>
<th>Time to metastasize (months)</th>
<th>Status at follow up</th>
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<td>43</td>
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<td>12</td>
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<td>21-26</td>
<td>33-73</td>
<td>–</td>
<td>Normal</td>
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</table>
Hypothesis: Each metaplastic carcinoma (MBC) subtype has a unique proteomic signature
Workflow used in metaplastic carcinoma samples

TMT vs iTRAQ labeling:
- Robust hyperplexing
- Constant MW mass tags
- Time, cost effectiveness
- Simpler quantitation

Collab. with Alexey Nesvizhskii
Hierarchical Clustering

- R scripts
- Cluster 3.0
- Tree View 1.1
The Human Metaplastic Carcinoma Proteome

- 5670 unique proteins

- Chondrocyte differentiation
- Cytokine activity / Ras-related
- Metabolism and stemness reprogramming
- Downregulation of tumor suppressors
- Chromatin remodeling, epigenetic responses
- Keratinization, immune activities
- Immune system, wound healing, EMT
- SARCOMATOID
- NORMAL
- SPINDLE
- MIXED
- TNBC
- SQUAMOUS
A majority of differentially expressed proteins are downregulated.

1,298 differentially expressed proteins, ~ 500 shared.
Differentially expressed proteins in MBC and TNBC relative to normal tissues

- 1,298 differentially expressed proteins
Downregulated Proteins

• Common or shared downregulated protein profiles of histologic MBC subtypes

• Spindle most in common with TNBC

• Sarcomatoid is most distinct

• MBCs and TNBC may arise through common process
We found distinct upregulated protein profiles in each subtype of metaplastic carcinoma. May relate to a unique active differentiation program.
Summary

• A first-time proteomic subtyping analysis of the histological diversity of metaplastic breast carcinomas

• Identified a set of upregulated proteins that may mark the different MBC subtypes:
  o Spindle: metabolism rich
  o Squamous: inflammation rich
  o Sarcomatoid: highly along mesenchymal lineage, inorganic ion (angiogenesis)

• Found a set of proteins commonly deregulated in MBC and TNBC

• May be useful to understand the biology of MBC, discover new biomarkers, and potential treatment strategies
Ongoing studies

• Currently investigating mutational profile of human metaplastic carcinomas to perform a proteogenomic analysis

• Proteomic profile of our CCN6 knockout mouse model and compare to human tumors for further model validation

• We have developed cell lines from the metaplastic tumors and will use them to study different biological processes identified by proteomics (e.g. inflammation in squamous subtype)

• GOAL: understand how these tumors develop and find targets for treatment based on their protein profiles.
Conclusions

- CCn6 knockout results in defective development leading to hypoplastic mammary glands.
- CCn6 knockout in the mammary gland leads to development of late but aggressive carcinomas with EMT features recapitulating human metaplastic carcinomas of spindle cell type.
- CCN6 loss leads to upregulated expression of oncofetal protein pathways in metaplastic carcinomas.
- Currently investigating how CCN6 loss induces WNT signaling.

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