Receptor tyrosine kinases as prognostic biomarkers for cancers

Ron and EGFR as prognostic markers for epithelial cancers
Background

- Cell surface tyrosine kinase receptors
- Extracellular, transmembrane and intracellular domains
- Ligand binding activates receptor
- Receptor dimerization
- Intrinsic tyrosine kinase activity
- Activation of receptor activity leads to diverse biological responses
Background

Courtesy of Sean Boykevisch
Background

- Examples of cell surface tyrosine kinase receptors
- Her family of receptors
  - Her1 (EGFR), Her2, Her3 and Her4
- Met/Ron/Sea
- PDGFR\(\alpha\) and \(\beta\)
- Kit/Ret
- IGFR
- ..........
Background

- The Ron receptor: $\alpha$ and $\beta$ chains
- Cloned from a human keratinocyte cDNA library
- Ligand: MSP/HGFL
- Activation elicit diverse biological responses:
  - Cell-cell dissociation
  - Cellular proliferation
  - Motility and invasiveness
Background

α: 30kD
β: 150kD
Ron

Courtesy of Sean Boykevisch
Background

- The role of Ron in human epithelial cancers

  - Expressed/over-expressed in hepatocellular carcinomas (2/7), NSCLC (3/8), breast carcinomas (35/74, 23/52), transitional-cell bladder cancer (60/183), colorectal cancer (29/49, 42/53), HNSCC (6/8)

  - Short form Ron expressed in primary breast (6/21) and ovarian (9/9) cancer samples
Background

- The role of Ron in experimental animal models
- Ron transformed 3T3 cells exhibit oncogenic potential in vivo
- Transgenic mice overexpressing Ron in distal lung epithelial cells develop adenomas/adenocarcinomas
- Ron defective, pMT driven breast tumor transgenic mice have decrease mammary tumor growth
Background

Mouse Ron Receptor Gene

Extracellular  TM  Cytoplasmic

Germline Mutation, TK\(^{-/-}\)

Waltz SE et al. JCI (2001)
Background

$TK^{+/+}$  $TK^{-/-}$  

Week 8

$TK^{+/+}$  $TK^{-/-}$  

Week 13

Chan et al. Oncogene (2005)
Background

Week 8

Week 13

Tumor Volume (mm³)

Time (Weeks)

Chan et al. Oncogene (2005)
Background

**SCC/Sarcoma-like**

**Odontogenic Tumor**

**Salivary Gland Tumor**

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Chan et al. Oncogene (2005)
## Background

<table>
<thead>
<tr>
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<th>SCC/Sarcoma-like</th>
<th>Other Malignant Tumors</th>
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<td>TK&lt;sup&gt;++&lt;/sup&gt;</td>
<td>0.67% (10/1487)</td>
<td>27% (10/36) Ameloblastoma 5</td>
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<tr>
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<td>Salivary gland tumor 1</td>
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<tr>
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<td>Lymphoma 2</td>
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<td>Cartilage tumor 2</td>
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<td>TK&lt;sup&gt;--&lt;/sup&gt;</td>
<td>0.33% (8/2433)</td>
<td>2.7% (1/35) * Ameloblastoma 1</td>
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<tr>
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<td>*p &lt; 0.05</td>
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</table>

*Chan et al. Oncogene (2005)*
Background

- High level of Ron expression in the papillomas
- Our results are consistent with reports of other experimental animal models of epithelial cancer
- Ron overexpression is an important molecular event driving tumor growth

Chan et al. Oncogene (2005)
Ron and EGFR

pClneo expression vector

CMV I.E. Enhancer/Promoter

Sal I
Not I
T3

T7

Amp

Sal I
Not I

Ron

pClneo-Ron
Ron and EGFR

Ron:

Tubulin:

3T3Ron  COS1-pCl-neo-Ron  COS1-pCl-neo-Ron  COS1-pCl-neo  COS1 + EGF  3T3Ron
## Ron and EGFR

### IP Rabbit IgG
- COS1-pClneo-Ron
- COS1-pClneo + MSP
- COS1 + EGF
- A431 + EGF
- 3T3 Ron
- COS1-pClneo-Ron
- COS1-pClneo + MSP
- COS1 + EGF
- 3T3 Ron

### IP EGFR (rabbit Ab)
- COS1-pClneo-Ron
- COS1-pClneo + MSP
- COS1 + EGF
- COS1-pClneo-Ron
- COS1-pClneo + MSP
- COS1 + EGF
- COS1 + EGF

### p-Tyr:
- 170
- 130
- 100
- 72

### EGFR:
- 170
- 130
- 100
Ron and EGFR

IP Rabbit IgG
3T3 Ron
COS1-pClneo-Ron
COS1-pClneo
COS1-pClneo + MSP
A431 + EGF
COS1 + EGF

IP EGFR (rabbit Ab)
3T3 Ron
COS1-pClneo-Ron
COS1-pClneo
COS1-pClneo + MSP
3T3 Ron
Central Hypothesis

Ron interacts with EGFR to confer a more aggressive phenotype in epithelial cancers
Translational Research Model

Clinic

Laboratory
Tumor Model

- Human Epithelial Cancer Model to test our hypothesis
- Squamous cell carcinomas of the head and neck (HNSCC)

**Rationale**
- > 90% HNSCC overexpress EGFR
- EGFR is an independent prognostic biomarker for HNSCC
- EGFR inhibitors are currently in clinical trials for HNSCC
- Results of these trials are promising but not dramatic
Tumor Model

- Seek additional target(s) that work in conjunction with EGFR
- A small study also discovered that 75% HNSCC tumors (6/8) express Ron
- Recent reports also found crosstalk between EGFR and Ron
Specific Aim I

To determine if cancer cells that overexpress both Ron and EGFR are more aggressive
Ron in HNSCC cell lines

- IP Ron (mouse Ab)
  - 3T3 Ron
  - 3T3 Ron + MSP
  - SCC-25

- IP mouse Ab
  - 3T3 Ron
  - 3T3 Ron + MSP
  - SCC-25
  - SCC-25
  - 3T3 Ron

Ron:
- 170
- 130
- 100
- 72
- 55
- 40
Ron in HNSCC cell lines
EGFR in HNSCC cell lines

- IP rabbit Ab
- IP EGFR (rabbit Ab)
- COS1 + EGF

EGFR:
- SCC15
- CAL 27
- SCC 9
- MDA1386
- SCC15
- CAL 27
- SCC 9
- MDA1386
- MDA1386 + EGF

EGFR:
- SCC 25
- 3T3 Ron

Tubulin:
- 170
- 130
- 100
- 72
- 55
- 40
Response to EGFR inhibitors

IP EGFR

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<tr>
<th>+Tarveca +EGF</th>
<th>+Erbitux +EGF</th>
<th>+ EGF</th>
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<tbody>
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<td>COS1-pCneo-Ron</td>
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</tr>
<tr>
<td></td>
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<td>COS1-pC neo</td>
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</table>

p-Tyr:

EGFR:
Future Research Plan

Cell and Animal Model of Cancer

- Determine if endogenous Ron interacts with EGFR in SCC cells
- Evaluate and compare the in vitro response of Ron− and Ron+ SCC cells to EGFR inhibitors
- Evaluate and compare the in vivo response and behavior of Ron− and Ron+ SCC cells using a skin grafting SCID mouse model
Specific Aim II

To determine if co-expression of EGFR and Ron is associated with more aggressive disease
Background

- Two studies of fairly large sample size correlating Ron and Met expression with prognosis
- Node negative breast cancer ($T_{1-2}N_0M_0$)
- Transitional-cell carcinomas of the bladder (183 cohort)
- Expression of RON and/or MET confer a poor prognosis
- Predictors of metastasis, disease-free survival
Background

- High expression level of tyrosine kinase receptors correlates with poor prognosis in cancers (e.g. EGFR, Her2)

- Immunohistochemistry is a widely used method to assess expression level of receptor tyrosine kinase in primary tumor specimen
Background

Immunohistochemistry

- **Pros**: cancer cell specific, materials readily available, prospective collection not necessary, easy and fast procedure, internal staining control

- **Cons**: qualitative not quantitative, low sensitivity and specificity, examiner dependent, wide definition of positive versus negative staining, unpredictable staining pattern (esp. with Ron antibody)
Develop an array of complementary methods to increase the sensitivity and specificity of detecting and quantifying Ron and EGFR expression/phosphorylation

1. Immunohistochemistry: protein
2. Immunoprecipitation and Western blots technique: protein
3. Real-time PCR: mRNA
Immunoprecipitation and Western blots technique

**Background**

- **Tumors in Lysis Buffer**
  - 0.1 – 0.2g tumor → 10 – 40 mg protein

**Homogenizer**

**IP 1 mg protein**

1º antibody (mouse): recognizes extracellular ligand binding site of Ron

**Spin down, discard supernatant and wash**

**Heat**

1º antibody binds to Ron

**beads**
Background

Immunoprecipitation and Western blots technique

- Incubate with 2° antibody (rabbit): recognizes intracellular C-terminal end of Ron
- Incubate with fluorescent Ab.
- Red/Green Fluorescence
- Quantify band
Background

Immunoprecipitation and Western blots technique

- **Pros:** ↑ specificity, better method to detect phosphorylated receptors (i.e. activated receptors), quantification more reliable

- **Cons:** sensitivity may be lower, heterogenous population of cells (i.e. not cell type specific), no internal control, need prospective collection of tumors with systematic processing to yield reliable results
Tumor lysates for IP Western

- IP mouse Ab
  - Endo. Ca w/SCD
  - normal bladder
  - SCC bladder

- IP Ron (mouse Ab)
  - Endo. Ca w/SCD
  - normal bladder
  - SCC bladder
  - MDA-1386
  - 3T3 Ron + MSP
  - 3T3 Ron

Ron:

- Red arrows indicate the protein bands of interest.
Tumor lysates for IP Western

- **p-Tyr:**
  - IP EGFR
  - Endo. Ca w/SCD
  - EGF
  - COS1 +
- **EGFR:**
  - IP EGFR
  - Endo. Ca w/SCD
  - EGF
  - COS1 +
- **Tubulin:**
  - IP EGFR
  - Endo. Ca w/SCD
  - EGF
  - COS1 +
Background

Real Time PCR

Isolate RNA

RT Rxn.

mRNA
cDNA

Tumor tissue

Real Time PCR

Plateau

Log phase

Threshold

ΔRn

Cycle number

CT

Fluorescence

SYBR Green

Real Time PCR

Log phase

Threshold

ΔRn

Cycle number

CT

Fluorescence

SYBR Green
Real Time PCR

- **Pros:** Very sensitive (more sensitive than IP Western), allow the detection of low level of gene expression, quantification reliable
  
- **Cons:** Specificity heavily depends on primers, heterogenous population of cells (i.e. not cell type specific), no internal control, need prospective collection of tumors with systematic processing to yield reliable results, no information on receptor phosphorylation
Background

**FL Ron**

Extracellular  TM  Cytoplasmic

11 12 13  18  19

**SF Ron**

Primers Selection

Primers Selection

**FL Ron**

**SF Ron**

ATG

ATG

ATG

ATG

**Primers Selection**

**Primer A**

**Primer B**

**FL Ron**

**SF Ron**
Testing Primer B for QRT-PCR

Standard Curve Method of Quantification

**Primer B**

<table>
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<th>Serial Dilutions</th>
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<tr>
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<tr>
<td>100 copies/µl</td>
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<td>20</td>
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<tr>
<td>10,000 copies/µl</td>
<td>17</td>
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Future Research Plan

**Human Model of cancer**
- Collect HNSCC tumors from CHTN and Stony Brook Tissue Bank
- Determine the prevalence of Ron expression in HNSCC
- Characterize the level of Ron and EGFR expression/phosphorylation in HNSCC
- Determine if co-expression of Ron and EGFR is associated with more aggressive disease in HNSCC
Long Term Objective

- Ron and EGFR as prognostic biomarkers for other epithelial cancers
- Ron and EGFR as prognostic biomarkers for pediatric solid tumors
- Interaction among receptor tyrosine kinases of other family
- Receptor tyrosine kinases as prognostic biomarkers in cancers
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