The Role of microRNAs in Pain

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Motivation for molecular analysis of Pain

• Chronic pain one of the most common causes for seeking medical treatment
• Approximate cost to the US economy 635 billion dollars
• Current treatments effective only in 1/2 of patients.
• Complex etiology
  – damage or disease affecting any part of the nervous system
  – spinal cord injury, diabetes, alcoholism, chemotherapy, chronic viral infection, multiple sclerosis, and strokes
• Subjective assessment
• Need Biomarkers

![Image of Wong-Baker Faces Pain Rating Scale]
Pain Biology

microRNA

• Short RNA sequences
  – 17-22 nucleotides
  – Lin-4 and let-7 identified in C. Elegans
  – Classified in 2000

• miRNA causes downregulation of target genes
  • Degradation of mRNA transcript (more common in plants)
  • Translational repression is more common in mammals

• Involved in many diseases including cancer
Biogenesis of miRNAs

- miRNA transcribed by RNA Pol II into pri-miRNA
- Pri-miRNA cleaved into pre-miRNA by Drosha and DGCR8
- Transported into cytoplasm by exportin-5
- Cleaved into miRNA by Dicer
Mechanisms of Post-Transcriptional Regulation

Filipowicz et al. 2008 Nature Reviews
Computational Target Prediction

• Step 1: Identify miRNA binding sites in mRNA sequence
• Step 2: Assess prediction confidence by examining cross-species conservation of binding site
Circulating miRNAs

• miRNAs stable in extracellular fluid
  – Blood, saliva, CSF, urine
• In blood miRNAs may be packaged in exosomes or bound to Ago proteins
• miRNAs released into circulation
  – Macrophage derived exosomes
  – Dead or dying cells
  – In response to tissue injuries
• Circulating miRNAs are a source of biomarkers.
Goals of this study

• Identify circulating miRNAs as potential pain biomarkers
  – miRNA profiles to predict patient responsiveness to treatment
  – Enrich pre-clinical drug discovery

• Explore mechanisms of regulation of pain by circulating miRNAs
RT-PCR

1. Prepare Sample
   - PAXgene Blood miRNA kit

2. Reverse Transcribe
   - Megaplex™ RT Primers & TaqMan® MicroRNA Reverse Transcription Kit

3. Amplify cDNA (optional)
   - Megaplex™ PreAmp Primers & TaqMan® PreAmp Master Mix

4. Dilute cDNA & Load TaqMan® Array
   - Diluted RT product & TaqMan® Universal PCR Master Mix

5. Run TaqMan® Array
   - Applied Biosystems 7900HT Fast Real-Time PCR System

6. Analyze Data

- MATLAB

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[Diagram of RT-PCR process with time durations: 30 min, 150 min, 90 min, 10 min, 120 min]

- Cycle 1: 1 copy
- Cycle 2: dNTPs, Taq pol
- Cycle 3: 

http://www.scq.ubc.ca/polymerase-chain-reaction/
Cycle Threshold

Data Analysis Pipeline

1. Raw CT values
2. Quality control, outlier detection
3. Normalization
4. T-Test, FDR
5. Calculate ΔCT
6. Calculate CT₀
7. Statistically significant miRNAs
8. Known/predicted target genes
9. Gene set enrichment
Normalization and Calculation of Fold Change

\[ \Delta CT = CT - CT_0 \]  
\[ \Delta \Delta CT = \Delta CT - \Delta CT_{ctrl} \]  
\[ FC = 2^{-\Delta \Delta CT} \]

• Select endogenous control \((CT_0)\) and use it to adjust CT values of all samples
  – Endogenous controls have high expression and low variance across multiple samples
  – Small nuclear RNAs (snRNAs) often used
Normalization Methods

• Endogenous Controls such as RNU44 and RNU48
  – Caution: Detected to be significantly different
  – Many endogenous controls are differentially expressed in cancer

• Mean normalization
• Z-normalization
• Quantile normalization
Gene Set Enrichment

• Optimize pathway enrichment methodologies in order to characterize the gene regulatory networks and molecular interaction pathways affected by differential miRNA regulation in pain
An Example Pathway
Probability that $k$ significant genes are also in the Pathway

Array (N)

Significant Genes (m)

(k)

Pathway genes (n)
Exercise

• From a deck of cards (N=52), m=3 cards are randomly selected.
• What is the probability of exactly k=2 cards being Aces? (Number of Aces in deck: n=4)
Probability at least $k$ significant genes are present in the pathway

• Hypergeometric Probability (exact):

$$P(X > k) = 1 - \sum_{r=0}^{k} \frac{\binom{m}{r} \times \binom{N-m}{n-r}}{\binom{N}{n}}$$

• $X^2$ (approximation):

$$X^2 = \frac{N(n_{11}n_{22} - n_{12}n_{21})^2}{N_1rN_2rN_{1c}N_{2c}}$$

$$df = (r - 1)(c - 1)$$

<table>
<thead>
<tr>
<th>Genes on Array</th>
<th>Significant Genes</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>In Pathway</td>
<td>$n_{11}$</td>
<td>$n_{12}$</td>
</tr>
<tr>
<td>Not in Pathway</td>
<td>$n_{21}$</td>
<td>$n_{22}$</td>
</tr>
<tr>
<td>N$<em>{1c}$=n$</em>{11}$+n$_{21}$</td>
<td>N$<em>{2c}$=n$</em>{12}$+n$_{22}$</td>
<td>N=n$<em>{11}$+n$</em>{12}$+n$<em>{21}$+n$</em>{22}$</td>
</tr>
</tbody>
</table>
Enrichment

- Over-representation Analysis
  - Hypergeometric
  - Chi-squared
  - Binomial

- Functional Class Scoring
  - GSEA
  - PAGE
  - SAM-GS

- Pathway Topology
  - Pathway-Express
  - SPIA
  - ScorePage
CRPS

• Characterize the miRNA expression in the blood of Complex Regional Pain Syndrome (CRPS) patients
Complex Regional Pain Syndrome (CRPS)

• CRPS is a chronic, painful and progressive neurological disease that affects skin, muscles, joints and bones
• CRPS is characterized by various degrees of burning pain, excessive sweating, swelling and sensitivity to touch
• Compare expression of miRNAs in blood from patients with CRPS and healthy age and sex matched controls
Complex Regional Pain Syndrome (CRPS)

Scores recorded for different types of pain

McGill Pain Index

- CRPS (RSD/Causalgia) 50
- childbirth (1st labour) 40
- phantom limb pain 30
- toothache 20
- amputation of digit 10
- chronic back pain 0
- cancer pain non-terminal
- fracture

Figure adapted from Katz and Melzack (1999)
CRPS Study Design

- Profiled serum microRNAs CRPS patients and controls
  - Identify potential miRNA biomarkers
- 20 Controls
- 41 CRPS Patients
- Analyzed blood miRNA levels by qPCR
- Correlated miRNA expression with other data
  - Cytokine levels
  - Co-morbidities
  - Pain level
## CRPS Circulation

<table>
<thead>
<tr>
<th>miRNA</th>
<th>Fold change</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>hsa-miR-939</td>
<td>-4.59358</td>
<td>5.55E-06</td>
</tr>
<tr>
<td>hsa-miR-25#</td>
<td>-3.92328</td>
<td>1.09E-06</td>
</tr>
<tr>
<td>hsa-let-7c</td>
<td>-2.53502</td>
<td>2.07E-05</td>
</tr>
<tr>
<td>hsa-let-7a</td>
<td>-2.45923</td>
<td>0.002308</td>
</tr>
<tr>
<td>hsa-let-7b</td>
<td>-2.40344</td>
<td>5.49E-05</td>
</tr>
<tr>
<td>hsa-miR-320B</td>
<td>-2.0527</td>
<td>6.91E-06</td>
</tr>
<tr>
<td>hsa-miR-126</td>
<td>-2.02362</td>
<td>0.002469</td>
</tr>
<tr>
<td>hsa-miR-629.A</td>
<td>-1.71231</td>
<td>0.000653</td>
</tr>
<tr>
<td>hsa-miR-664</td>
<td>-1.54881</td>
<td>0.001487</td>
</tr>
<tr>
<td>hsa-miR-320</td>
<td>-1.44273</td>
<td>7.28E-05</td>
</tr>
<tr>
<td>hsa-miR-1285</td>
<td>-1.41594</td>
<td>0.003077</td>
</tr>
<tr>
<td>hsa-miR-625#</td>
<td>-1.33174</td>
<td>0.003542</td>
</tr>
<tr>
<td>hsa-miR-532-3p</td>
<td>-1.27226</td>
<td>0.001226</td>
</tr>
<tr>
<td>hsa-miR-181a-2#</td>
<td>-1.25927</td>
<td>0.000229</td>
</tr>
<tr>
<td>RNU48</td>
<td>1.348125</td>
<td>0.000391</td>
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<tr>
<td>hsa-miR-720</td>
<td>1.476853</td>
<td>0.003243</td>
</tr>
<tr>
<td>RNU44</td>
<td>1.854213</td>
<td>0.000904</td>
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<tr>
<td>hsa-miR-1201</td>
<td>2.14584</td>
<td>3.17E-05</td>
</tr>
</tbody>
</table>

Fold changes and p values of significantly altered miRNAs (minus sign indicates down-regulation; data sorted based on fold change). The statistical significance was calculated using 2-tailed t-tests on the miRNA expressions in CRPS patients versus control samples.

CRPS Clustering

Correlation Analysis
miRNA correlations

- mir-532-3p → CRPS type
- mir-151-5p → CRPS duration
- mir-296-5p, 361-3p, 532-3p, 30d → Pain Level
- mir-92a, 484 → hypertension
- 219-1-3p, 204 → high cholesterol
- 150 → headache
- 339, 30c, 192, 140-3p, 152, 145 → narcotics
- 296-5p, 1290, 423-5p, 25, 1270 → BMI
miRNA meta-analysis

• Evaluate changes in miRNA expression in the blood of several rodent models of pain and characterize miRNA response to therapeutic intervention
Animal Models of Pain

• Inflammatory Pain
  – Mice injected with Complete Freund's Adjuvant (CFA).
  – Relieved by COX-inhibitor celecoxib
• Chemotherapy Induced Pain
  – Mice treated with JNJ-26481585, an HDAC inhibitor currently in Phase II clinical trials for cancer
• Neuropathic Pain
  – Spinal Nerve Ligation (SNL) in rats
  – Spared Nerve Injury (SNI) in rats and mice
• Pain behavior confirmed by:
  – Mechanical sensitivity (von Frey test)
  – Thermal nociception (Hargreaves’ test)
• Blood samples collected, RNA isolated. miRNA measured by rodent TLDA cards on ABI rt-PCR.

Circulating microRNA Signatures in Rodent Models of Pain
Rodent Neuropathic Pain Models

• Spinal Nerve Ligation
  – Peripheral neuropathic pain model
  – L5 spinal nerve or L5 and L6 spinal nerves bound with silk
  – 5 sham operated rats, and 4 SNL rats

• Spared Nerve Injury
  – Peripheral nerve injury model
  – Targets common peroneal nerve and tibial nerve
  – Nerves bound with silk and cut distal to ligation site
  – 5 Sham and 5 SNI rats
  – 5 Sham and 5 SNI mice

Adapted from Decosterd and Woolf, Pain, 2000
Commonly Enriched KEGG Pathways
Enriched KEGG Pathways

Enriched KEGG pathways (continued)

- **Axon guidance pathway**
- **Neurotrophin Signaling Pathway**
- **ErbB signaling Pathway**
  - Calvo et al. Neuregulin-ErbB signaling promotes microglial proliferation and chemotaxis contributing to microgliosis and pain after peripheral nerve injury. J. Neurosci. 2010
- **TGF-Beta Signaling Pathway**
  - Utreras et al. TGF-β1 sensitizes TRPV1 through Cdk5 signaling in odontoblast-like cells. Molecular Pain 2013
- **mTor Signaling Pathway**
  - Lyu et al. The mTOR signaling pathway regulates pain-related synaptic plasticity in rat entorhinal-hippocampal pathways. Molecular Pain
- **GnRH Signaling**