Effects of Pre-analytic Variables on Circulating MicroRNAs Using a CCSG Biorepository

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What are MicroRNAs?

- Small RNA molecules (~21 nt)
- Found in almost every species
- Highly evolutionarily conserved
- Regulate gene expression
Currently there are 1,527 human microRNAs annotated in miRBase (Release 18.0, Nov. 2011)

On average, each microRNA has 200 targets

Over 60% of all human protein encoding genes are regulated by microRNAs

Important to all biological functions and pathways
MicroRNAs and Cancer

- microRNA may play a role as tumor suppressor genes (down regulated in cancer) or oncogenes (up regulated in cancer)

- microRNA disregulation is involved in initiation, progression, and resistance to therapy of human cancers

- microRNA as promising biomarkers of cancers
MicroRNAs Detected in:

URINE

BLOOD (SERUM/PLASMA)

TISSUES

CELLS

SAMPLES

RNA 5S

t-RNA

miRNA

FFPE
formalin-fixed
paraffin-embedded

FROZEN
Over 100 studies have been done in a variety of cancers, including colorectal, prostate, ovarian, breast, lymphoma, etc.

Biospecimens include: serum, plasma, and whole blood.

A few microRNA based biomarkers have been reported:
- miR-141 in prostate cancer
- miR-195 and let-7a in breast cancer
- miR-155, miR-210 and miR-21 in lymphoma, etc
Detection of Human Prostate Cancer by Serum miR-141

miR-141 (tumor-associated miRNA)

Wilcoxon (W) = 63
p-value = 1.47 x 10^{-7}

100% specificity,
60% sensitivity

Mitchell P S et al. PNAS 2008
Blood $miR-195$ and $let-7a$ in Breast Cancer

Heneghan, et al, Annual of Surgery, 2010
The inconsistency might be due to:

- Unclear biological mechanisms
- Variations in study design and execution
- Variations among the cancer patient population
- Tumor heterogeneity
- Variations in biospecimens, from sample procurement to final analyses
Challenges from Biospecimen Perspective

- Types of biospecimens: serum, plasma or whole blood, etc
- Reliable internal controls to normalize the data
- The effects of pre-analytic variables on circulating microRNAs
Levels of Circulating MicroRNAs Are Affected by Types of Biospecimens

Study design

- Number of freeze/thaw: 1 vs 2
- Compare number of microRNAs that can be detected in plasma
- Compare expression of selected microRNAs used as internal controls in literatures (miR-15 and miR-16)

Results

- 1 cycle: 137 microRNAs were detected
- 2 cycles: 117 microRNAs were detected
- The expression of miR-15 was reduced 15% (1 cycle vs 2 cycles)
- The expression of miR-16 was reduced 20% (1 cycle vs 2 cycles)

Conclusions

- The number of freeze/thaw affects circulating microRNA expression
- Biospecimen research in circulating microRNA is needed
I. Discover a panel of “housekeeping” circulating microRNAs which can be used as internal controls.

II. Development of circulating microRNA QC tools by studying the effects of pre-analytic variables on the internal control microRNAs identified in Milestone 1.
Perform microRNA profiling in 40 plasma samples to identify a panel of circulating microRNAs based on the following criteria:

- occur in all tested samples
- expression levels are not significantly different between cancer cases and controls
- show little inter-individual variations among cases and controls

Further evaluated individually in an additional 200 plasma samples from 100 cancer cases and 100 healthy controls using quantitative real-time PCR based analysis

In parallel, we repeated microRNA profiling and validation analysis in the same study subjects as above, but using whole blood samples collected using PAXgene Blood RNA System
DataBank and BioRepository (DBBR) of Roswell Park Cancer Institute (RPCI) has been used to:

- recruit study subjects
- obtain high quality biospecimens
- collect epidemiological/clinical data

Standard Operation Procedure (SOP) has been developed and strictly followed.

Blood collection, sample processing, and storage are completed in one hour.
20 Cases

- 8 prostate cancer patients (men) and 12 breast cancer patients (women)
- Average age of cancer diagnosis: 56 years old
- All Caucasians

20 Controls

- Matched with cases on age, gender and ethnicity
Exiqon MicroRNA Ready-to-use PCR array was used

742 human microRNAs and 6 reference RNAs

Spike RNAs were used to normalize plate-plate variations
Data Analysis Procedure

Step 1: identify miRNAs expressed in all biospecimens

Step 2: identify miRNAs without significant expression changes

Step 3: rank miRNAs based on a composite score of FC (fold change) and CV (correlation of variation)

748 miRNAs profiled in 40 whole bloods using Exiqon PCR array

209 miRNAs with Ct value <38 in all 40 whole blood samples

52 miRNAs with FC<1.5 & FDR>0.1 in all three case vs. control comparisons.

3 miRNAs whose CV rank and FC rank are consistently among the top 10.

1) All group: 20 vs.20
2) Breast group: 12 vs. 12
3) Prostate group: 8 vs. 8

miR-134, miR-346 and miR-934
Whole Blood miR-346 Levels Across The Study Subjects

- FC=-1.03, CV=0.04
- FC=-1.05, CV=0.03
- FC=1.01, CV=0.04
Whole Blood miR-134 Levels Across The Study Subjects

FC = 1.02, CV = 0.03

FC = -1.05, CV = 0.03

FC = 1.02, CV = 0.03

FC = -1.16, CV = 0.03

![Graph showing miR-134 levels across different groups: BCase, BControl, PCase, PControl.](chart)
Whole Blood miR-16 Levels Across The Study Subjects

- hsa.mir.16
  - FC=1.04, CV=0.07
- FC=1.33, CV=0.07
- FC=-1.38, CV=0.07
Comparison Between miR-346 and miR-16 Across The Study Subjects
Plasma miR-346 Levels Across The Study Subjects

- FC = 1.07, CV = 0.04
- FC = 1.01, CV = 0.03
- FC = -1.08, CV = 0.03

Bar chart showing hsa.mir.346 (plasma) levels across different categories:
- BCase
- BControl
- PCase
- PControl
Cases (100)

- 56 breast cancer, 36 prostate cancer, 3 colon cancer, and 5 lung cancer cases
- 91 Caucasians, 6 Blacks, and 3 Hispanics
- 62 women and 38 men
- Mean age of cancer diagnosis: 57 years old

Controls (100)

- 93 Caucasians, 6 Blacks, and 1 Hispanics
- 80 women and 20 men
- Mean age of enrollment: 56 years old
Molecular and data analysis

- MiScript PCR System (Qiagen) is used to quantify each selected microRNAs
- Selected microRNAs include: miR-346, miR-134, miR-934, miR-16, miR-421, miR-222, miR-1207, miR-339, miR-505, miR-183, miR-374b, miR-1260, miR-345, and miR-323.
- Linear regression analysis was applied.
## microRNA Internal Controls in Whole Bloods

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We have identified several internal control candidates for whole blood based circulating microRNA analysis, including miR-346, miR-134, miR-934 and miR-16.

miR-16 is the only microRNA which shows relatively consistent expression in plasma samples.
Development of the circulating microRNA QC tools by studying the effects of the pre-analytic variables on the “housekeeping” microRNAs identified in Milestone 1.
Objective 1 in Milestone 2

- **Processing delay time**
  - Plasma: No delay vs 24 hours delay
  - Whole blood: 2 hours vs 24 hours delay

- **Recruitment**
  - 7 cases (completed)
  - 7 controls (completed)

- **Selection of microRNAs**
  - Whole bloods: miR-346, miR-134 and miR-934
  - Plasma: miR-16
Whole bloods: 2 vs 24 hours

- No significant changes in miR-346 levels between 2 hrs and 24 hrs delay
  - P=0.892
- Similar trends for miR-134 and miR-934
• Systematical decrease miR-16 levels between no delay and 24 hrs delay
  
  • P=0.004

• Processing time delay affects quantity of circulating microRNAs in plasma
Objective 2 in Milestone 2

- **Storage conditions**
  - Plasma: cryovials at -80°C vs straws in liquid nitrogen
  - Whole blood: -20°C vs -80°C

- **Recruitment**
  - 7 cases (completed)
  - 7 controls (completed)

- **Selection of microRNAs**
  - Whole bloods: miR-346, miR-134 and miR-934
  - Plasma: miR-16
Whole blood: -20°C vs -80°C

- No significant changes in miR-346 levels between -20°C and -80°C
  - P=0.852
- Similar trends for miR-134 and miR-934
Plasma: cryovials at -80°C vs straws in liquid nitrogen

- No significant changes in miR-16 levels between cryovials at -80°C and straw in liquid nitrogen
- \( P=0.792 \)
Objective 3 in Milestone 2

- **Storage duration**
  - Plasma: 0 vs 6 months
  - Whole blood: 0 vs 6 months

- **Recruitment**
  - 7 cases (Completed)
  - 7 controls (completed)
Objective 4 in Milestone 2

- **Number of freeze-thaw**
  - Plasma: 0 vs 1, 2, and 4
  - Whole blood: 0 vs 2

- **Recruitment**
  - 7 cases (Completed)
  - 7 controls (completed)
Acknowledgement

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