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Exosomes as Liquid Biopsy: From Molecular Phenotype to Functional Mechanisms

Wei Cao, Ph.D.
Welcome to our 4-part webinar series on exosomes

Exosomes as liquid biopsy – a 4-part webinar series

- **Part 1: Exosomes as Liquid Biopsy: From Molecular Phenotype to Functional Mechanisms**  
  March 4, 2015, 9:30 – 10:30 a.m. EST, 2:30 pm GMT, 3:30 CET

- **Part 2: Exosome Research – Maximize Quantity and Purity of Exosomal RNA**  
  March 11, 2015, 9:30 – 10:30 a.m. EST, 2:30 pm GMT, 3:30 CET

- **Part 3: Biomarker Discovery in Biofluids: from Sample to Biomarker**  
  March 18, 2015, 9:30 – 10:30 a.m. EST, 2:30 pm GMT, 3:30 CET

- **Part 4: Meeting the Challenges of Biomarker Research: miRNA Function, Profiling and Data Analysis**  
  March 25, 2015, 9:30 – 10:30 a.m. EST, 2:30 pm GMT, 3:30 CET

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Agenda

- Molecular biology of extracellular vesicles (EVs)
  - Composition
  - Biological functions

- The diagnostic, prognostic and therapeutic potentials
  - Exosomal RNAs as disease specific biomarker
  - Exosomal miRNA
  - Exosomal IncRNA

- Exosomal RNA isolation and characterization

- Solutions provided by QIAGEN

- Questions
The molecular composition of extracellular vesicles (EVs)

Extracellular vesicles (EVs) are membraneous vesicles released by a variety of cells into the extracellular microenvironment. Two major classes based on biogenesis:

1. Exosomes are small vesicles (<100nm), formed and stored within the cell before their release, released by fusion of MVEs with the plasma membrane
2. Microvesicles (MVs) (100-1000nm) are shed directly from the plasma membrane via ectocytosis

- No clear discrimination between exosomes and MVs.
- EVs are released by almost all cell types, and found in the plasma and other bodily fluids, including breast milk, semen, saliva, urine and sputum.
- Biological molecules (protein, RNA, miRNA, lncRNA) contained in exosomes and MVs are well protected by a lipid bilayer membrane that confers a high degree of stability.

http://www.microvesicles.org/
# Functional biomolecules of extracellular vesicles (MVs)

- **Proteins/peptides:** adhesion molecules, membrane trafficking molecules, cytoskeleton molecules, heat shock proteins, cytoplasmic enzymes, signal transduction proteins, cytokines, chemokines, proteinases and cell-specific antigens
- **Lipids**
- **DNA fragments** (exosomal DNA, exoDNA)
- **mRNA** (exoRNA)
- **Non-coding RNAs,** such as miRNAs and long non-coding RNAs (IncRNAs)

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**Vesiclepedia’s collection**

Play central roles in cell-to-cell communication, which is conferred by mediators that are expressed on the surface of the EVs.

http://www.microvesicles.org/
http://www.exocarta.org/

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<table>
<thead>
<tr>
<th>Statistics</th>
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<td>Lipid molecules</td>
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<td>Countries</td>
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</table>
Features and biological functions of exosomes

General features
- Extracellular vesicles with < 100nm in size
- Released into the extracellular milieu by fusion of the peripheral membrane of MVBs with the plasma membrane
- The composition of protein and lipid is different from other types of EVs
- Present in a quantity of 3,000,000 /ml
- Abundant in body fluids including blood, saliva, urine, and breast milk
- Float at a density of 1.15–1.19g / ml of sucrose gradient

Biological functions
- Mediators of intercellular communication of the messages including growth, division, survival, differentiation, stress responses, apoptosis, etc.
- Facilitators of the immune response and inflammatory response, and play antigen presenting roles.
- Essentially analogous to viruses.

The diagnostic, prognostic and therapeutic potentials of exosomes

Exosomes are involved in the pathogenesis, diagnostics and therapeutics of various diseases

**In cancers**
- Promote tumorigenesis, regulate metastatic niche formation, and tumor immune responses
- Potential serve as novel circulating biomarkers (e.g., miRNAs)
- Serve as a vehicle for the administration of anti-tumor compounds

**In cardiovascular diseases**
- Have pathological effects on cardiac remodeling, and pro-angiogenic & cardio-protective properties
- Potential biomarkers to assess cardiovascular risk
- Serve as delivery system or vectors in cardiovascular system via non-immunogenic means

**In liver disease**
- Some liver related cells are exosome-releasing and/or exosome-targeting cells
- Liver exosomes as diagnostic and prognostic biomarkers
- Exosomes as novel therapeutics for liver diseases

**In kidney diseases**
- Urinary exosomes reflect the state of the urinary system, from podocytes to renal-tubular cells
- Potential diagnostic biomarkers for kidney disorders: non-invasive biomarkers

**In neurodegenerative diseases**
- Vehicles for the transfer of toxic proteins associated with neurodegenerative diseases
- Potential diagnostic biomarkers for neurological conditions

Masyuk, A.I., et al. (2013) “Exosomes in the pathogenesis, diagnostics and therapeutics of liver diseases.” J Hepatol. 59 1
Ailawadi, S. et al. (2015) “Pathologic function and therapeutic potential of exosomes in cardiovascular disease” Biochimica et Biophysica Acta 1852 1
Exosomal RNAs as disease specific biomarkers

Exosomal RNAs contain fingerprints for various diseases, so have potential as liquid biopsy.

- minimally invasive and non-invasive, to traditional needle or excision biopsies.

- **Blood samples:** Specific expression patterns of serum miRNAs identified for lung cancer, colorectal cancer, prostate cancer, and diabetes (ex. serum miR25 and miR223 in lung cancer, miR-141 in prostate cancer)

- **Urine samples:** exosomal mRNA and non-coding RNAs serve as markers related to kidney disorders

- **Saliva-based diagnostics:** assess the health state of the oral cavities, and monitor systemic health

- **Cerebrospinal fluid (CSF) as brain liquid biopsy:** exosomal miRNAs have potential for biomarkers related to neurodegenerative disorders

mRNAs and non-coding RNAs (ncRNAs) in exosomes

**mRNAs**
- mRNA released into the circulation is stable, implying that it is protected from degradation by its packaging into exosomes
- The level of exosomal RNA implies genetic information

**miRNAs**
- miRNAs are ~21 nt, the most widely studies ncRNAs
- Have potential to serve as diagnostic markers
- miRNA expression is frequently deregulated in cancer
- In blood, miRNAs are highly stable, because most of them are included in apoptotic bodies, microvesicles, exosomes and can withstand known mRNA degradation factors

**Long non-coding RNAs (IncRNAs)**
- IncRNAs are novel class of RNAs, with >200 nucleotides in sizes
- Regulate protein-coding genes transcription in more complex ways than the miRNAs
- Changes in IncRNA can be correlated with diseases

The molecular functions of IncRNAs

1. Negatively affect expression
2. Positively affect expression
3. Hybridize to the pre-mRNA resulting in an alternatively spliced transcript
4. Hybridization of the sense and antisense transcripts allow Dicer to generate endogenous siRNAs
5. Binding to miRNA results in miRNA function silencing
6. The complex of IncRNA and specific protein partners can modulate the activity of the protein
7. Involved in structural and organizational roles of the cell
8. Alters the protein localizes in the cell
9. Affects epigenetic processes
10. Be processed to the small RNAs

IncRNA – new game player in liquid biopsy

Source of novel potential biomarkers for diagnosis, prognosis and therapeutics purposes.

- The long non-coding PCA3 RNA-based urine test is the first FDA-approved test for the diagnosis of prostate cancer patients
- New field, their expression and functions remain unclear, and reports regarding the circulating IncRNAs in bodily fluids are scant in the literature

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<td>Plasma sample</td>
<td>TUG1, MALAT1, HOTAIR, lincRNA-p21, GAS5</td>
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<tr>
<td>Prostate cancer</td>
<td>Plasma sample, tissue samples</td>
<td>MALAT-1 and PCA3</td>
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IncRNAs as novel biomarkers for prostate cancer from urine

- Comparison study of IncRNAs in
  - Human prostate cell line vs. normal
  - Prostate adenocarcinoma tissue vs. normal tissue
  - Urine from prostate cancer vs. urine from normal or benign cancers

- Identified 6 IncRNAs in prostate adenocarcinoma: AK024556, XLOC_007697, LOC100287482, XLOC_005327, XLOC_008559, and XLOC_009911)

- Same 6 IncRNAs detected in patient urine samples

  New biomarker?
Agenda

- Molecular biology of extracellular vesicles (EVs)
  - Composition
  - Biological functions

- The diagnostic, prognostic and therapeutic potentials
  - Exosomal RNAs as disease specific biomarker
  - Exosomal miRNA
  - Exosomal lncRNA

- Exosomal RNA isolation and characterization

- Solutions provided by QIAGEN

- Questions
Exosomes isolation methods and challenges

**Challenges:** improve and standardize methods for exosomes isolation

- Many current protocols to isolate exosomes use ultracentrifugation based on size
- Long processing time, and the process is unreproducible and not selective for tumor exosomes
- Different RNA isolation methods give extensive variation in exosomal RNA yield and patterns
- Often fail to distinguish between differently sized exosomes and membrane-free macromolecular aggregates, so should be taken cautiously

**Current methods**

- Differential centrifugation
- Size exclusion
- Immunoaffinity isolation
- Microfluidic devices
- Polymeric precipitation (ExoQuick)

**Issues**

- Long processing time
- Cannot achieve absolute separation
- Low reproducibility
- High contamination
- Low yields
- Low purity

**Needs**

- Rapid, exosome-specific extraction
- Simple, effective release from exosomes
- Highly sensitive, specific detection of endogenous biomolecules

Exosomal RNAs characterization and profiling

- **RNA-seq (Whole transcriptome sequencing)**
  - Discover new RNAs and splicing variants

- **Microarrays**
  - Use data analysis approaches to identify RNAs
  - Retrospective studies of microarray data

- **Real-time PCR based approaches**
  - Sensitive and quantitative for low expressing RNAs and small gene changes
Agenda

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Exosomes research workflow: from sample to insight

Sample collection & Analyte enrichment

Sample Isolation

Amplification

qPCR

Data Analysis & Interpretation

mRNA

exoRNeasy Serum/Plasma Kit

RT² PreAMP cDNA Kit

RT² PCR System

Free data analysis tool
Ingenuity Pathway Analysis

miRNA

exoRNeasy Serum/Plasma Kit

miScript PreAMP PCR Kit

miScript PCR System

Free data analysis tool
Ingenuity Pathway Analysis

lncRNA

exoRNeasy Serum/Plasma Kit

RT² IncRNA PreAMP PCR Kit

RT² IncRNA PCR System

Free data analysis tool

Sample to Insight
Isolating mRNA/IncRNA and miRNA from exosomes and other extracellular vesicles
exoRNeasy Serum/Plasma Kits

For efficient purification of RNA from exosomes and other extracellular vesicles out of serum or plasma samples

**Novel & efficient workflow:**
Sample from exosomes and microvesicles to total RNA, in just one hour.

![Diagram of workflow]

Pre-filter plasma to remove particles larger than 0.8 μm

exoRNeasy Serum/Plasma Kits

Deliver intact vesicles of the expected size, and capture all mRNA and vesicle-specific miRNAs in plasma

- Scanning electron microscopy (SEM) of isolated vesicles from ultracentrifugation and eluate from exoEasy column
- exoRNeasy Serum/Plasma Kits yield a cleaner preparation

- Capture all mRNA and vesicle-specific miRNAs in plasma
- Confirmed by raw $C_T$ values from RT-qPCR experiments
Advance circulating miRNA biomarker discovery
miScript miRNA PCR system

miScript miRNA PCR Arrays

- **miRNome**
  - Human: miRBase v21, covers 2,402 primer assays
  - Mouse: miRBase v21, covers 1,765 primer assays
  - Rat: 653 primer assays
  - Dog: 277 primer assays
  - Rhesus macaque: 469 primer assays
  - Cow: 744 primer assays

- **Pathway-focused arrays (>20 arrays)**
  - **Serum and Plasma**
  - miFinder
  - Cancer PathwayFinder
  - Liver miFinder
  - Brain Cancer
  - Breast Cancer
  - Ovarian Cancer
  - Prostate Cancer
  - Cancer Stem Cells
  - Apoptosis

**miScript PreAMP Kit**

- **Optional step for small or precious samples**
- Full miRNome profiling from as little as 1 ng RNA

Pre-formatted, single-use PCR arrays with wet-lab verified assays
Workflow for exosomal miRNA profiling

1. Isolate total RNA with exoRNeasy Serum/Plasma Kits → 1 hour
2. Perform reverse-transcription → 2 minutes
3. Prepare PCR pre-mix → 2 hours
4. Load PCR arrays and perform real-time PCR
5. Analyze data → 15 minutes
miScript Serum & Plasma 384HC PCR Array profiled 381 miRNAs isolated using exoRNeasy kits

Exosomal miRNAs distribution

Abundance of miRNAs inside and outside extracellular vesicles
Uncover the secrets hidden in exosomes
Are there any lncRNAs in my exosomal sample?

Sample Isolation → Amplification → qPCR → Data Analysis & Interpretation

exORNeasy Serum/Plasma Kit → RT² lncRNA PreAMP PCR Kit → RT² lncRNA PCR System → Free data analysis tool

Test and verify your hypothesis with RT² lncRNA qPCR Assays or Custom RT² lncRNA PCR Arrays
Application: discover IncRNA in serum of NSCLC patients

Background

- Non-small cell lung cancer (NSCLC) is the most common type of lung cancer (about 85%).
- The roles of IncRNA in lung cancer have drawn attention. IncRNAs such as MALAT1, BCYRN1, LINC00970, and H19 in lung cancer have been studied.
- Limited work has been done on serum/plasma IncRNA.
- The current recommended screening test is low-dose computed tomography, which is time consuming and costly.
- Need early diagnostic marker for mortality rate control.

IncRNAs hold the promise for potential circulating biomarker - more convenient and low cost.

→ RT² IncRNA qPCR Array system
Experiment design: discover IncRNA in serum of NSCLC patients

Samples:
- Control: healthy donor serum samples (n=24)
- Patient sample: serum from NSCLC patients (n=3)

1. Total RNA isolation: total RNA from 200 µl serum was purified with the miRNeasy Serum/Plasma Kit.
2. cDNA synthesis: convert to total RNA to cDNA using RT2 PreAMP cDNA Synthesis Kit
3. Pre-amplification: Pre-amplify the target IncRNAs with pre-amplification RT² PreAMP cDNA kit
4. Run PCR: detect IncRNAs with RT-PCR
   - Human RT2 IncRNA Cancer PathwayFinder PCR Array
   - Master mix: RT2 SYBR® Green qPCR Mastermix
   - qPCR cyclers: ABI 7900HT
5. Data analysis: GeneGlobe Data Analysis Center
Discover lncRNA biomarkers in serum of NSCLC patients

Successfully detected lncRNAs in serum samples with pre-amplification

Volcano plot of lncRNA gene expression changes in NSCLC serum samples compared with healthy donor serum samples
RT² IncRNA qPCR system

- **IncRNA databases:** In-house database at QIAGEN GeneGlobe provides cover > 40,000 human, 27,000 mouse IncRNA targets.

- **RT² IncRNA assays:** Assays laboratory-verified for optimal qPCR performance — high specificity, amplification efficiency, and sensitivity.

- **RT² IncRNA qPCR Arrays:** Pathway or disease relevant IncRNA assays
  - RT² IncRNA Cancer PathwayFinder Array (Human and Mouse)
  - RT² IncFinder PCR Array (Human and Mouse)

- **Custom option:** Flexible custom design from the IncRNA database and qPCR database to profile mRNA and IncRNA simultaneously.

- **IncRNA isolation:** exoRNeasy kits or miRNeasy kits

- **Data analysis:** free on-line data analysis tool
  
Cancers:
- Bladder Cancer: BLACAT1, H19, MALAT1, MEG3, SNHG16, TERC, TUG1, UCA1.
- Breast Cancer: BCAR4, GAS5, H19, HIF1A-AS2, HOTAIR, JADRR, LSINCT5, MIR31HG, MRPL23-AS1, TRERNA1, UCA1, XIST, ZFAS1.
- Cervical Cancer: MEG3.
- Colorectal Cancer: CCAT1, CCAT2, CRNDE, H19, HOTAIR, MALAT1, PCAT1, PVT1, SNHG16, TUSC7.
- Endometrial Cancer: H19, HOTAIR.
- Esophageal Squamous Cell Carcinoma: CBR3-AS1, HOTAIR.
- Gallbladder Cancer: MALAT1.
- Gastric Cancer: CCAT1, CDKN2B-AS1, GACAT1, H19, HOTAIR, HULC, LINC00152, LINC00261, LINC00312, MEG3, PVT1, SUMO1P3, TERC.
- Glioma: H19, HOTAIR, MEG3.
- Kidney Cancer: AFAP1-AS1, DGCR5, HIF1A-AS2, WT1-AS.
- Laryngeal Squamous Cell Carcinoma: HOTAIR, MALAT1.
- Leukemia: DLEU2, GAS5, H19, HOTAIR, HOXA-AS2, MALAT1, MEG3, TERC, TUG1, XIST.
- Liver Cancer: H19, HEIH, HOTAIR, HOTTIP, HULC, KCNQ1OT1, MALAT1, MEG3, MIR7-3HG, PANDAR.
- Non-Small Cell Lung Cancer: BANC, CCAT2, GAS6-AS1, HOTAIR, MALAT1, MEG3.
- Other Lung Cancers: ACTA2-AS1, LUCAT1.
- Melanoma: BANC, HOTAIR, PTENP1.
- Multiple Myeloma: MEG3, PVT1.
- Nasopharyngeal Carcinoma: HOTAIR, LINC00312.
- Neuroblastoma: HAND2-AS1, SNHG16.
- Oesophageal Squamous Cell Carcinoma: LINC01234.
- Osteosarcoma: TUG1.
- Ovarian Cancer: H19, HOTAIR, LSINCT5, WT1-AS, XIST.
- Pancreatic Cancer: GAS5, HOTAIR, PVT1.
- Pituitary Cancer: MEG3.
- Prostate Cancer: CBR3-AS1, GAS5, HOTAIR, LINC00963, MALAT1, PCA3, PCAT1, PCGEM1, PRNCR1, PVT1, TERC, XIST.
- Rhabdomyosarcoma: RMST.
- Sporadic Pediatric Adrenocortical Tumors: KCNQ1OT1.
- Testicular Cancer: XIST.
- Thyroid Cancer: NAMA, PTCSC3.

Oncogenes: ACTA2-AS1, AFAP1-AS1, BANC, BCAR4, BLACAT1, CBR3-AS1, CCAT1, CCAT2, CRNDE, H19, HAND2-AS1, HEIH, HIF1A-AS2, HNF1A-AS1, HOTAIR, HOTTIP, HOXA-AS2, HULC, JADRR, KCNQ1OT1, LINC00152, LINC00263, MALAT1, PCA3, PTCSC1, PTCSC3, PTCSP1, PVT1, SWI6, SWI6-AS1, TSS2, UCA1, XIST.

Tumor Suppressor Genes: ADAMTS9-AS2, CAHM, DLEU2, DLX6-AS1, GACAT1, GAS5, GAS6-AS1, GNAS-AS1, LINC00261, LINC00312, MEG3, MIR31HG, MIR7-3HG, NAMA, NEAT1, PTCSC1, PTCSC3, PTCSP1, TERC, TUSC7, WT1-AS, ZFAS1.

Other Cancer-Related lncRNAs: AIRN, EMX2OS, FTX, HIF1A-AS1, HOTAIRM1, HOXA11-AS, IPW, KRASP1, LINC00538, LINC00887, LINC01233, NBR2, NRON, RMRP, RPS6KA2-AS1, TSIX.
Flexible layout and patented controls

Each 96-well plate

- 84 lncRNA-specific assays
- 5 reference genes
- 1 genomic DNA
- 3 reverse transcription controls
- 3 PCR controls

Arrays are also available in 384-well plates & 100-well ring discs for the Rotor-Gene Q. Free online analysis tool

<table>
<thead>
<tr>
<th>Species</th>
<th>Number of qPCR assays designed (custom designs not included)</th>
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<tbody>
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<td>Human</td>
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<tr>
<td>Mouse</td>
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We provide service — send samples to us & receive results

**Whole genome**
- Illumina gene expression profiling
- Illumina genotyping

**Pathway/focused panels**
- Mutation profiling
- Methylation
- PCR arrays
- miRNA PCR arrays
- NGS

**Individual gene/locus**
- Mutation detection
- Methylation
- qPCR
- NGS

**Sample preparation — DNA, RNA extraction and purification**
- Cells, tissues, or biofluids
- Fixed tissue
- Small samples

http://www.qiagen.com/products/catalog/services/
Solutions for exosomes research at QIAGEN

Exosomal RNA isolation

- miRNA arrays
  - Profile exosomal miRNAs
- IncRNA arrays
  - Detect IncRNAs in exosomes
- Serve core
  - Send samples & receive results

exoRNeasy serum/plasma kits
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Wei Cao, Ph.D.
Wei.Cao@QIAGEN.com
Welcome! March Webinar Calendar

March 2015 webinars
Global Marketing Management, Translational Sciences Frederick, MD

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9:30 am EST
1–2 pm EST
Sign up here: http://sabiosciences.com/seminarlist.php