Inhibition of S-adenosylhomocysteine hydrolase (SAHH) Induces Fas Ligand Gene Expression and Apoptotic Death in Leukemic T Lymphocytes.

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The SAHH is a methyltransferase that catalyzes the transfer of a methyl group from S-adenosylmethionine (SAM) to acceptors. The enzyme is critical for methylation reactions in the cell. Inhibition of SAHH has been shown to induce apoptosis in various cell types.

**ABSTRACT**

The inhibition of SAHH decreases the cellular methylation potential of human T leukemic cells. The data obtained showed that SAHH inhibition led to the induction of apoptosis in leukemic T cells. The TM pathway involves the methyltransferase (TM) pathway, entails a methyl ligand gene. The SAHH inhibition led to the induction of Fas, gene expression. We are currently examining the molecular changes and apoptosis in the promoter region of the Fas gene that are induced by SAHH inhibition in T leukemic cells. Overall, the data indicate that SAHH could be a potential therapeutic target in the treatment of T leukemic cells.

**RESULTS**

Inhibition of S-adenosylhomocysteine hydrolase (SAHH) Decreases the Cellular Methylation Potential of Human T Leukemic Cells

**CONCLUSIONS**

Inhibition of SAHH Decreases the Cellular Methylation Potential of Human T Leukemic Cells.

**MATERIALS AND METHODS**

Human Leukemic Cells: Cultured human leukemic target lymphocytes (Jurkat) were propagated in RPMI medium supplemented in 10% fetal bovine serum, 1% penicillin/streptomycin, and maintained in a 37°C and 5% CO2 environment. Jurkat cells at a density of 5.2x10⁵/mL were maintained as untreated controls, or treated with DZNep for 4 days (for RNA),12h (for protein), or 24h (for RNA and cell viability).

Cell viability: (A) Trypan Blue Dye Exclusion Assay: Cells were stained with Trypan blue dye, and live and dead cells were counted. (B) DNA Fragmentation Apoptosis Assay: The cell death ELISA kit from Roche (Indianapolis IN) was used to detect DNA Fragmentation.

Western Blot analysis: After treatments, cells were lysed with RIPA lysis buffer. The lysates were separated by electrophoresis and immunoblotting.

**CONCLUSIONS**

Inhibition of SAHH leads to upregulation of FasL and formation of Death Initiating Signaling Complex (DISC). Inhibition of SAHH induces Fas/FasL mediated apoptosis and decreases cell viability in T leukemic cells.

Overall our data demonstrate that SAHH inhibitors maybe a potential therapeutic strategy for treatment of T cell leukemia.
Biomarker Significance of Exosomes During Breast Cancer Initiation and Progression

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**Introduction**

The American Cancer Society estimates approximately 232,670 new cases of invasive breast cancer in women in the United States for 2014, with an estimated 40,000 deaths\textsuperscript{1}. Women who have prolonged elevated estrogen levels have a higher susceptibility to be diagnosed with breast cancer. Current methods of breast cancer diagnosis require invasive methods such as a biopsy.

Exosomes are endosomal vesicles (~40-200 nm) that are released from all cell types and can be found in most or all bodily fluids\textsuperscript{2}. Recent studies show that exosomes are released more abundantly from tumor cells than normal cells\textsuperscript{3}.

Exosomes facilitate intercellular communication via the unique cargo it carries including lipids, proteins, RNA and mRNAs. We hypothesize that exosomes could be used as a potential biomarker for the initiation and progression of breast cancer.

**Results**

**Exosome Size Determination**

Table 1. Exosome size (nm) and polydispersity index (PDI) of serum exosomes.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Size (nm)</th>
<th>PDI</th>
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<tbody>
<tr>
<td>Control A1</td>
<td>151.2</td>
<td>0.41</td>
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<tr>
<td>Control A2</td>
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<td>E2 C6</td>
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<td>E2 C7</td>
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<td>Control A8</td>
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<td>Control A14</td>
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<tr>
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<tr>
<td>E2 C30</td>
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</table>

**Protein Yield of Serum-Exosomes**

Mean protein yield (mg/ml) of serum-derived exosomes at different time points (n=4). Statistical analysis was done using paired t-test.

**Protein Yield of CD63-Enriched Exosomes**

Total protein yield (µg) of CD63-enriched exosomes. Two hundred µg of exosomes isolated by precipitation method were used for enrichment with CD63 magnetic beads (n=3). Statistical analysis was done using paired t-test.

**Key Findings**

- Exosomes isolated from serum samples were in the size range of 40-200 nm.
- The mean exosomal protein yields of E2-treated samples were higher than their respective control for each time period. However, the differences did not attain statistical significance.
- Enrichment of serum exosomes using CD63 magnetic beads resulted in higher protein yield in a) E2-treated than control and b) 7 m E2-treated compared to 3 m E2-treated samples; however, these differences did not attain statistical significance.
- Serum derived exosomes were positive for hallmark exosomal proteins, including Alix, CD61, CD63 and CD44.
- Expression levels of Alix and CD44 were significantly higher in E2-treated serum exosomes compared to control. While this trend was also observed with other exosomal markers, this difference did not attain statistical significance.
- Previous reports indicate CD44 positive exosomes to have the ability to increase extracellular matrix degradation and augment tumor invasion\textsuperscript{4}.
- Estrogen growth factor receptor (EGFR) expression levels were higher in E2-treated serum exosomes compared to control; this difference was even greater after enrichment for CD63-positive exosomes.
- Our preliminary observations suggest the potential of serum exosomes to reflect the increase in proliferation of rat mammary tissue. However, significance was not attained for either condition presumably due to small sample size.

**Conclusions**

Higher levels of serum exosomes positive for exosomal surface proteins and proliferation markers with E2-treatment indicate their potential as biomarker during initiation and progression of breast cancer.

**Future Directions**

- Increase sample size to reduce deviations
- Use enrichment techniques to improve quality of data
- Correlate data to tumor size and multiplicity for each animal

**Methods**

- **ACI Rat Model**: Female ACI rats (5-8 week old) were acclimated for 1 week on rat chow diet and later fed either purified AIN-93M diet. Two weeks later, animals were imprinted with 1.2 cm long silicon implant containing 3 mg of 17β-estradiol. Body weight, diet consumption and tumor incidence were monitored weekly until euthanasia. Animals were euthanized after 3 weeks (3 w), 3 months (3 m) and 7 month (7 m). Serum was separated from blood for further analysis.
- **Exosomes Isolation**: Total Exosome Isolation (from serum) reagent (Invitrogen, Carlsbad, CA) was added to each serum sample (100 µl). After incubation at 4°C for 30 min, the sample was centrifuged at 10,000 g for 10 min at room temperature. Exosomes were suspended in 200-300 µl of PBS.
- **Exosome Enrichment**: Isolated exosomes were enriched using CD9-capturing magnetic beads. Beads were washed 3 times with 0.1% BSA in PBS and exosome samples were incubated overnight at 4°C with continuous mixing. At the end of incubation the beads were washed 3 times and CD9-specific exosomes were eluted using RIPA lysis buffer.
- **Exosome Size Determination**: Zetasizer Nano (Malvern, Westborough, MA) was used to determine the average size and polydispersity index (PDI) of exosomes isolated from serum. Samples of 50 µl were appropriately diluted and analyzed by measuring the dynamic light scattering.
- **BCA Protein Assay**: The protein concentration was determined by using a BCA Protein Assay Kit (Pierce, Rockford, IL), where the serum exosomes were run against known standards. After 10 minute incubation in 37°C, optical density was measured at 562 nm using UV-visible spectrophotometer (Molecular Devices,Sunnyvale, CA).
- **Western Blot Analysis**: 15 µg protein was loaded and separated on SDS-PAGE gel and then transferred to PVDF membrane. The membrane was blocked using either 4% non-fat dry milk or 4% BSA, and the appropriate primary antibody was added (Alix, EGFR, CD61, CD63, and CD44) overnight at 4°C. After washing, membranes were probed with HRP-conjugated secondary antibody. Proteins were analyzed using chemiluminescent kit. Blot intensities were quantified using ImageJ software. Statistical analysis was determined using Sigma Plot software.

**References**

The Role of Carbon Chain and Carbonyl Group in AHL-induced Caspase-9-dependent Apoptosis

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Abstract

N-Acyl-Homoserine lactones (AHLs) are essential quorum-sensing molecules that play vital roles, such as regulating virulence factors in pathogens. Our interest has been sparked by C12, an AHL produced by the opportunistic bacterium, Pseudomonas aeruginosa. We have recently discovered that C12 induces human tumor cell apoptosis, following a pathway that is caspase-9-dependent. Recent studies have also found that it preferentially kills transformed cells over normal cells. For this study, our goal was to identify other AHLs that induce caspase-9-dependent apoptosis. WT and Caspase-9 KO Mouse Embryonic Fibroblast (MEF) cells were treated with increasing concentrations of various derivatives of C12 for 24 hours. We found that reduction of the carbon chain length by treating with C6 and C8, eliminated the ability to induce cell death based upon the propidium iodide (PI) assay. However, C14, with increased carbon chain length, like C12 was able to induce the apoptosis cascade. Simply removing the carbonyl group from C14, forming C14 HSL, resulted in a completely different cell death pathway independent of caspase-9. We suspect that pathway to be autophagy, as many of the cells have an accumulation of LC3-B positive autophagosomes and are not Annexin V or PI positive. Our results indicated that both the carbon chain length and carbonyl group are important for AHL-induced caspase-9-dependent apoptosis.

Results

1. C12 and C14 induce apoptotic cell death in MEF cells

   - N-3-oxodecanoyl-L-Homoserine Lactone (C12)
   - N-3-oxodecanoyl-L-Homoserine Lactone (C14)

2. The Cytotoxicity of AHLs is dependent on the length of the carbon tail

   - N-3-oxodecanoyl-L-Homoserine Lactone (C6)
   - N-3-oxodecanoyl-L-Homoserine Lactone (C8)

3. The carbonyl group is essential for both C12 and C14 induced apoptosis

   - N-dodecanoyl-L-Homoserine Lactone (C12 HSL)
   - N-dodecanoyl-L-Homoserine Lactone (C14 HSL)


   - Caspase-9 KO MEF cells

5. C14 HSL induces caspase-9-independent cell death through autophagy

   - WT and Caspase-9 KO MEF cells

Figure 1: (A-C) Upon 24 hours-treatment with various concentrations of C12 in MEF WT cells, the percentage of PI positive cells (A), relative caspase-3/7 activity (B), and annexin V positive cells (C) were measured. (D-F) MEF WT cells were treated with C14 for 24 hours and the percentage of PI positive cells (D), relative caspase-3/7 activity (E), and annexin V positive cells (F) were measured.

Figure 2: (A-B) C6 and C8, which have shorter carbon chain length, have no cytotoxic effects on the WT MEF cells after 24 hours-treatment.

Figure 3: (A-B) No apoptosis was found in MEF cells following 24 hour treatment with C12 HSL and C14 HSL, which have no carbonyl group.

Figure 4: (A-B) Caspase-9 KO MEF cells were treated with increasing concentrations of C12 (A) and C14 (B) for 24 hours. There was no caspase-3/7 activity relative to control. This suggests that the induction of apoptosis by C12 and C14 is dependent upon caspase-9. (C) Western blot showing caspase-9 expression in MEF cells.

Figure 5: (A) WT and Caspase-9 KO MEF cells were treated for 24 hours with increasing concentrations of C14 HSL. Both WT and Caspase-9 KO cells were killed by C14 HSL. (B) Percent annexin V positive cells was measured in WT MEF cells following 24 hours-treatment with C14 HSL. (C) 60X Maximum Intensity Projection confocal images are shown of MEF WT cells treated for 24 hours with either vehicle control, 200µM C14, or 200µM C14 HSL. Arrows point to LC3-B markings.

Conclusions

- C12 and C14 induce caspase-9-dependent apoptotic cell death in MEF cells.
  - Reducing the length of carbon chain decreases the cytotoxicity of the AHLs, C12 and C14.
  - The carbonyl group is vital for both C12 and C14 induced apoptosis.
  - C14 HSL, which lacks the carbonyl group, causes cell death through autophagy but not through apoptosis or necrosis.

Acknowledgements

- Research was supported by a grant from NCI R25 grant University of Louisville Cancer Education Program NIH/NCI (R25-CA134283) and the School of Medicine Summer Research Scholar Program.
Cannabinol Modulates the Efficacy of Cannabinoids On CB2 Receptor

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University of Louisville School of Medicine

Abstract
Cannabinol (CBG) is a non-psychoactive phytocannabinoid. It is currently unknown what the interaction of CBG is with the cannabinoid receptor 2 (CB2). This project measured the modulation of CBG on the effects of known cannabinoid agonists including endo- and synthetic cannabinoids. A homogeneous time resolved fluorescence method was used to quantify CB2 mediated decrease in cyclic adenosine monophosphate (cAMP) levels. CBG by itself had no effect on cAMP levels. However, CBG was found to increase the efficacy of AEA and WIN55,212-2, but no effect was observed on the other cannabinoids.

Methods
1. Drug
2. cells Stimulate 7 min
3. D2 Cryptate
Incubate for 2 hours
4. 337 nm light to excite cryptate transfer from cryptate to D2-labeled cAMP
5. 2 wavelengths, 665 and 620 nm to measure emission

Results
Figure 2A. Effect of CBG on Forskolin-stimulated cAMP accumulation. HEK293 cells stably expressing CB2 were treated with different concentrations of CBG.
Figure 2B. Modulation of endocannabinoid-induced inhibition of forskolin-stimulated cAMP accumulation by CBG. HEK 293 cells stably expressing CB2 were pre-incubated for 10 minutes with vehicle or 1 μM CBG before subject to stimulation with (B) AEA or (C) CP-2AG for 7 minutes. Results are expressed as percent forskolin-stimulated cAMP accumulation.
Figure 2D,E,F. Effect of CBG on forskolin-stimulated cAMP accumulation by synthetic cannabinoids. Cells stably expressing CB2 were pre-incubated with vehicle or 1 μM CBG for 10 minutes before subject to stimulation with synthetic cannabinoid agonists (D) WIN55,212-2, (E) CP-55,940, and (F) HU-210 for 7 minutes. Results are expressed as percent forskolin-stimulated cAMP accumulation.

Conclusions
1. CBG alone did not affect forskolin-stimulated cAMP accumulation at concentrations up to 10 μM.
2. CBG did not modify cAMP inhibition induced by synthetic cannabinoids CP-55,940 or HU-210, or endocannabinoid 2AG.
3. CBG increased the efficacy of cAMP inhibition induced by endocannabinoid AEA and synthetic WIN55,212-2.

Acknowledgements
This research was partially supported by NCI grant R25 CA134283 to the University of Louisville

Introduction
CBG was first identified by Gaoni an Mechoulam in 1964. This phytocannabinoid has multiple potential targets including Cox1/2 enzymes, TRP channels, cannabinoid, 5-HT, α1 adrenergic, and PPAR receptors. CBG has been shown to exhibit antiproliferative and pro-apoptotic on a number of human cancer cell lines. CB2 is a G protein coupled receptor found primarily in the peripheral tissues of the immune system, but not extensively in the CNS. CB2 ligands have immunomodulary, anti-inflammatory, and pain modulatory effects.

Figure 1: Structures of Cannabinoids used

- Cannabigerol (CBG)
- N-arachidonylethanolamine (AEA)
- 2-Arachidonoylglycerol (2AG)
- WIN55,212-2
- CP-55,940
- HU-210
The Novel Interaction of NORE1A and RIT in Lung Cancer

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School of Medicine, University of Louisville

INTRODUCTION

Lung cancer remains the largest cause of cancer-related deaths and diagnosis worldwide [1]. Lung cancer cases are classified based on size and biochemical alterations in the cells of origin, with non-small cell lung carcinomas being the predominant form witnessed clinically [2]. Mutations in the Ras gene remain a signature genetic contributor to development of non-small cell lung carcinomas and can be found in roughly 30% of cases [3, 4]. Members of the RAS Subfamily, class of small GTPase proteins, have long been implicated in the etiology of lung cancers and play an important role in cell signal transduction [5]. Activated RAS contributes to a pro-growth and survival phenotype mediating the hallmarksof cancer by activating and suppressing an array of effectors with many downstream targets associated with diverse changes in cell behaviors [5]. NORE1A (RASSF5) is a novel RAS death effector and potent tumor suppressor [6]. Its functions by interacting with other apoptotic effectors thereby promoting the pro-apoptotic effects of RAS [6]. Death effectors of RAS are believed to serve as a protection mechanism against over-stimulation of RAS signaling, and loss of these negative effectors of RAS shifts the cell toward transformation [6]. NORE1A is suppressed by an epigenetic mechanism in at least 30% of NSCLC. Recently, a novel branch of the RAS Superfamily has also recently been identified as having a role in the establishment and progression of lung cancer [7]. Activating mutations have now been detected in the RIT protein in lung cancer [7]. RIT (Ras-like protein in tissues) is remarkably similar to RAS in both domain and sequence homology and it has been shown to powerfully induce activation of p38, ERK, and AKT signaling depending on cellular context [7].

The mechanisms with which RIT drives cellular transformation remain only partially characterized. A yeast-two hybrid screen identified wild type RIT as a binding partner of NORE1A. We sought to determine if this interaction could be detected in mammalian cell lines and if there are any functional consequences of the interaction for lung cancer cells. Here we show that RIT does in fact complex with NORE1A in cells and acts to suppress the pro-apoptotic and tumor suppressor phenotype of NORE1A.

METHODOLOGIES

Cell Lines: HEK293, HEK293T, and NCI-H1299 cells were maintained in DMEM supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin and RPMI supplemented with 10% FBS and 1% penicillin-streptomycin, respectively.

Luciferase Assays: PUMA signaling was measured by luciferase assays using the PUMA-Luc promoter construct with the Promega Dual Luciferase System.

Protein Analysis: Protein expression was monitored by transfection of HEK293, HEK293T or NCI-H1299 cells and Western blotting analysis. Immunoprecipitations were performed with GFP-conjugated sepharose beads.

ACKNOWLEDGEMENTS

This work was supported by NCI R25-CA134283 Cancer Education Grant awarded to the University of Louisville and R01 CA133171-01A2 funded to JGC. Additionally, would like to thank Dr. Clark for his mentorship, Lee Schmidt for his training and mentorship, and the entire Clark Lab Staff for their entertainment and patience.

REFERENCES

7. Sanger, A.H., et al., Oncogenic RIT (Ha-Ras) and lung cancer.
Breast Cancer Diagnosed through the Mobile Mammography Van in Jefferson County, KY
S Mudra¹, J Pan², SN Rai², EC Riley¹
Department of Medicine, Division of Oncology/Hematology¹ and Department of Bioinformatics and Biostatistics²
James Graham Brown Cancer Center, University of Louisville School of Medicine

BACKGROUND

• The purpose of this study is to investigate and understand demographic, clinical and biologic trends among breast cancer patients diagnosed through the Mobile Mammography Unit (MMU) in Jefferson County, KY from 2000-2010.
• This study is a retrospective institutional review designed to examine demographic, clinical and biologic trends among women diagnosed with invasive breast cancer or DCIS via the MMU.

MATERIALS AND METHODS

• 21,857 individuals visited the MMU during the study period.
• 247 unique subjects were identified through the database as requiring biopsies.
• 165 individuals were ineligible for analysis due to benign pathology or high risk status.
• Data were unavailable for 4 patients because surgical consult was recommended; however, pathology was unavailable for review (e.g. went to another institution).
• 78 invasive cancers (stage I, II, III) or DCIS treated at our institution remained for analysis.
• Demographic data (age, race and insurance status) and clinical and biologic factors (histologic diagnosis, biologic subtype, stage, BMI and family history) were collected on those with a cancer diagnosis.
• For categorical variables, the descriptive statistics frequency, percentage and cumulative percentage, related to different predictors (such as age, race and insurance) were produced using SAS procedure FREQ. All calculations were performed with SAS statistical software (SAS, 2003).

RESULTS

• Most women diagnosed with cancer were uninsured (63%), despite a majority of insured women (57%) visiting the MMU (Table 1).
• Consistent with the known incidence of biologic subtypes of breast cancer, DCIS was 23% of diagnoses (Table 3).
• Consistent with the goals of mammography, early stage breast cancer (stage 0, I, or II) represented nearly half of the diagnoses. Locally advanced disease (stage III) only represented 5%, although 35% of staging data are unavailable for review.
• Cancers diagnosed were more likely to be ER positive. Triple negatives represented only 8% of diagnoses. 13% of tumors were Her2Neu positive.
• Over ¼ of the cancer dataset was individuals aged 40-49.

CONCLUSIONS

• A higher density of breast cancer was observed among black women, with blacks representing 41% of all cancer diagnoses.
• Additionally, over ¼ of women diagnosed were aged 40-49, a higher incidence than would be expected given historical controls.
• Although there was a higher cancer incidence among black and uninsured women, these data may be biased given this is the targeted population of the MMU.
• Known risk factors for breast cancer, namely obesity and family history, were consistent with the dataset.
• Due to the retrospective nature of this study, large amounts of data were unavailable for review. Therefore, results should be confirmed with additional studies.
• Despite these limitations, this dataset suggests that cancer incidence among historically disadvantaged populations (blacks, uninsured) as well as younger aged women may be disproportionately high in Jefferson County and should be analyzed in a larger study to confirm this finding.

Table 1: Insurance

<table>
<thead>
<tr>
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<th>Total (N=21,857)</th>
<th>Biopsy (N=247)</th>
<th>Cancer (N=78)</th>
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<tr>
<td>Insured</td>
<td>12,435 (57%)</td>
<td>56 (23%)</td>
<td>29 (37%)</td>
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<tr>
<td>Uninsured</td>
<td>9,422 (43%)</td>
<td>191 (77%)</td>
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Table 2: Clinical and Biologic Factors

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<td>IDC</td>
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<td>DCIS</td>
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<td>ILC</td>
<td>3 (4%)</td>
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<td>Stage</td>
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<td>0</td>
<td>17 (22%)</td>
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<td>I</td>
<td>19 (24%)</td>
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<td>II</td>
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<td>III</td>
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Table 3: Age

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<td>20 (26%)</td>
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<td>60-69</td>
<td>23 (29%)</td>
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Table 4: BMI

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<td>10 (10%)</td>
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<td>Overweight</td>
<td>12 (16%)</td>
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<td>Obese</td>
<td>26 (33%)</td>
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Table 5: Family History

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<td>19 (24%)</td>
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<td>24 (31%)</td>
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Identification of an Internal Reference microRNA from the Plasma of Multiple Cancer Types

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Methods and Materials

Our study investigated endogenous reference microRNA expression in plasma from a variety of different neoplasias.

- Colorectal Cancer (n=20)
- Colorectal Adenoma (n=10)
- Breast Cancer (n=10)
- Lung Cancer (n=10)
- Pancreatic Cancer (n=10)
- Controls (n=10)

Peripheral blood was collected from all patients and plasma was isolated.

Total RNA was extracted using Ambion TRIzol LS Reagent Protocol and measured with a Nanodrop 2000 Spectrophotometer.

cDNA was produced for 381 microRNAs by reverse transcription, with Megaplex Reverse Transcription Pool A v2.1 (Life Technologies, Foster City, CA).

After preamplification, quantitative Real-Time Polymerase Reaction (PCR) was performed using a 381 microRNA TaqMan low-density array card (TLDA) with a ViiA™7 Real-Time PCR system (Life Technologies, Foster City, CA).

A fixed cycle threshold bar of 0.03 was for all screening arrays.

The mean and standard deviation were calculated for each microRNA in each group.

microRNAs were assessed for minimal standard deviation in each group and then analyzed for consistent means across the groups.

Review Results

We conducted a literature review of 203 publications on the subject of “plasma microRNAs” from the first half of 2014. We queried three main questions:

- Did the publication use absolute or relative quantification?
- If it used relative quantification, than which reference microRNA(s) was used?
- If it used absolute quantification, which spike in control was used?

Relative quantification was used 69% of the time, whereas 31% used absolute quantification.

- For absolute quantification, 88% of the absolute papers used cel-miR-39.
- For relative quantification, miR-16 (30%) and U6 (25%) were the two most commonly used housekeeping microRNAs, between the relative papers.

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