CIRCULATING MICRORNAS AS BIOMARKERS FOR EARLY CANCER DETECTION

by

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BSc., St. Francis Xavier University, 2011

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

in

The Faculty of Graduate Studies

(Interdisciplinary Oncology)

THE UNIVERSITY OF BRITISH COLUMBIA

(Vancouver)

June 2013

Abstract

Cancer is a major cause of death worldwide. Patients diagnosed at an early stage have an improved prognosis and therefore efforts have been made into the development of methods to detect tumors at their earliest stages. MicroRNAs (miRNAs) are non-coding RNAs that negatively regulate gene expression by interfering with the translation of target mRNAs. Studies have found that miRNAs are present at stable levels in the circulation and that they are differentially expressed in patients with various diseases. In this thesis we used qRT-PCR to assess the utility of 742 serum miRNAs as biomarkers for early cancer detection. In aim 1 we examined the levels of serum miRNAs in patients with high-risk oral lesions. We identified five miRNAs that are significantly deregulated in the serum of these patients compared to demographically matched, non-cancer controls. Additionally, these miRNAs correspondingly decreased or increased after surgical resection of the lesion. In aim 2 we examined the effect of hemolysis, fasting, and smoking on the serum miRNA levels of healthy individuals. We also compared serum miRNA profiles of samples taken from healthy individuals over different time periods. We found that mechanical hemolysis of blood samples simulating blood drawing can significantly alter serum miRNA quantification and should be taken into consideration when identifying endogenous controls and candidate biomarkers for circulating miRNA studies. Fasting, smoking, and a time period up to 17 months between samples were demonstrated to not have a significant effect on the overall serum miRNA profiles of healthy individuals. In aim 3 we compared the miRNA profiles of paired samples collected during surgery from the same patient from a) pulmonary venous effluent draining the tumor vascular bed (tumor associated samples) and b) systemic arterial blood to identify lung adenocarcinoma biomarkers. We found 35

miRNAs that were significantly up-regulated in tumor-associated serum samples. However, when we tested the candidate miRNAs in cancer versus non-cancer peripheral venous blood samples they were not significantly differentially expressed. The results presented in this thesis demonstrate the need for standardized protocols for circulating miRNA studies and provide evidence for the utility of serum miRNAs as biomarkers of disease.

Preface

Portions of this thesis have been published as described below.

A version of chapter 3 has been published. MacLellan SA, Lawson J, Baik J, Guillaud M, Poh CF , and Garnis C. (2012) Differential expression of miRNAs in the serum of patients with high-risk oral lesions. *Cancer Medicine* 1(2): 268-274. I conducted the majority of the RNA extractions and qRT-PCR profiling with the assistance of J Lawson. I also wrote the manuscript and conducted the data analysis with the exception of the ROC curves which were made by J Baik and M Guillaud. CF Poh was the oral pathologist for the study and contributed to study design. C Garnis conceived and designed the study.

A version of chapter 4 has been submitted for publication. MacLellan SA, MacAulay C, Lam S, and Garnis C. (2013) Pre-profiling factors influencing serum microRNA levels. I conducted the majority of the blood processing and all of the RNA extractions and qRT-PCR profiling for the study. I also contributed to study design and wrote the manuscript. I conducted the data analysis with the help of C MacAulay. S Lam, C MacAulay, and C Garnis contributed to the study conception and design.

In the above chapters, edits have been made to the published version to better integrate the work into the thesis and clarify material where necessary. Most changes are minor with the exception of the introduction of each manuscript which has been incorporated into chapter 1 of this thesis.

Chapter 5 contains work from our collaborators. Dr. Stephen Lam and Dr. John Yee contributed to the study conception and design and provided all serum samples used in the study. Dr. Cathie Garnis performed RNA extractions for the surgical samples and contributed to study conception and design. I conducted all other RNA extractions and all qRT-PCR profiling with the exception of the surgical samples that were profiled by Exiqon qRT-PCR Services. I conducted all data analyses.

The work described in this thesis was approved by the Research Ethics Board of The University of British Columbia. Ethics Certificate Number: H10-02846.

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List of Abbreviations

miRNA	microRNA
OSCC	oral squamous cell carcinoma
NSCLC	Non-small cell lung cancer
CT	Computed tomography
Pre-miRNA	pre-miRNA
Exp5	Exportin-5
RISC	RNA-induced silencing complex
Argo	Argonaute
HDL	High density lipoprotein
CIS	Carcinoma in situ
AC	Adenocarcinoma
SCC	Squamous cell carcinoma
LCC	Large cell carcinoma
qRT-PCR	quantitative reverse transcription real-time polymerase chain reaction
HRL	High risk lesion
ROC	Receiver operating characteristic
AUC	Area under the curve
dPCR	Digital polymerase chain reaction
COPD	Chronic obstructive pulmonary disease

Acknowledgements

Firstly, I would like to thank my graduate supervisor Dr. Cathie Garnis for her guidance and patience throughout my degree. Working in her lab has been a wonderful experience and I've learned so many skills, both in the lab and outside of it, which I will keep with me as I continue my career.

I would also like to thank my supervisory committee, Dr. Stephen Lam and Dr. Calum MacAulay for all their help throughout this thesis. It has been a privilege to work with experts in this field.

I also want to thank all of the collaborators, fellow members of the Garnis lab, and volunteers who helped with the work described in this thesis. The studies presented here would not have been possible without their support and contributions.

I would like to acknowledge the financial support of the University of British Columbia Interdisciplinary Oncology Program, the Pacific Otolaryngology Foundation, and the Terry Fox Research Institute.

Dedication

To my parents, my brothers, and the rest of my amazing friends and family. Thank you so much for your support and encouragement throughout this whole process.

1. Introduction

1.1 Biomarkers for the early detection of cancer

Cancer is a devastating disease and a major cause of death worldwide accounting for ~ 13% of all deaths in 2008[1]. Patients diagnosed at an early stage have an improved prognosis compared to those diagnosed at a late stage and early detection can reduce cancer mortality[2]. Thus, a considerable amount of resources have been invested in the development of efficient screening methods to detect tumors at their earliest stages[3]. To develop screening programs many researchers are conducting studies dedicated to the identification of highly sensitive and specific tumor biomarkers[4]. The variety of proteins, nucleic acids, and other molecular elements in blood has made it a popular medium for such studies[5]. The major advantage of blood-based biomarkers is that they can be detected non-invasively[5]. In addition to early detection, blood-based biomarkers may also be useful for cancer diagnosis, monitoring progression and response to treatment, and for identifying disease recurrence.

Despite an increase in publications of potentially useful non-invasive cancer biomarkers, very few biomarkers are used in standard clinical practice[6]. This could be due to a variety of problems that can occur during the pre-analytical, analytical, and post-analytical stages of biomarker discovery and validation[7]. Blood-based cancer biomarkers currently used in the clinic are all proteins including: α-fetoprotein and human chorionic gonadotropin-β for testicular cancer, prostate-specific antigen for prostate cancer, and CA-125 for ovarian cancer[8]. However, even these biomarkers have limitations. For example, levels of prostate-specific antigen are elevated by a number of pathologies leading to a reduced specificity for cancer detection[8].

1.2 Early detection of oral cancer

Oral cancer is a cancer of the oral cavity or the oropharynx. Oral cancer is one of the most commonly diagnosed cancers worldwide and in high-risk countries (e.g. in South-East Asia) this disease represents ~25% of all new cancers[9]. Among the different subtypes of oral cancer, oral squamous cell carcinoma (OSCC) is the most common contributing to over 90% of oral malignancies[9]. OSCC carries a poor prognosis and, despite advances in treatment, overall survival rates for patients have seen little improvement. Early diagnosis is a major factor contributing to the survival rates of OSCC patients as those diagnosed at an early stage have a five-year survival rate of about 80% to 90% but for patients diagnosed at an advanced stage of disease, the five-year survival rate drops to about 30%[10]. A high incidence of recurrence is also a major factor contributing to the poor survival rates of patients diagnosed with OSCC with about 30% of males and 20% of females developing secondary primary tumors[11].

Although OSCC is preceded by visible lesions in the oral mucosa and the oral cavity is easily accessed for clinical inspection, ~ 64% of OSCCs are detected at a late stage with ~ 50% exhibiting regional cervical metastases[12, 13]. Studies examining the reasons for this delay in detection often cite a lack of public awareness as well as a need for more action on the part of health-care providers[14]. Therefore, in order to improve early detection rates and outcomes for OSCC patients the role of oral cancer screening programs have been examined[15]. A study in 2005 conducted over 9 years on over 130 000 participants provided evidence on the positive impact of screening high-risk individuals (those with high tobacco and/or alcohol intake). The results of this study suggested that oral cancer screening by visual inspection of the oral cavity has the potential to prevent ~37 000 oral cancer deaths worldwide[16].

In addition to visual inspection other minimally invasive techniques used for oral cancer screening include: brush biopsy, toluidine blue staining, and tissue fluorescence imaging[14]. Brush biopsies involve collecting a complete transepithelial specimen from mucosal lesions that appear clinically benign and would not otherwise receive a scalpel biopsy[17]. Studies examining the sensitivity and specificity of this technique show variable results and some suggest that brush biopsies show an increased rate of false-positive findings when used on benign-appearing oral lesions from low risk populations[14]. Toluidine blue is a dye that may stain nucleic acids and aid in the identification of potentially malignant tissue. This method is mainly used by experienced clinicians to aid in clinical examination and few studies have been carried out to evaluate toluidine blue as an oral cancer screening tool[18]. Fluorescence imaging involves exposing the tissue to blue light resulting in the autofluorescence of cellular fluorophores after excitation. Cellular alterations, such as those present in oral lesions, result in changes in the autofluorescence of the tissue[19]. Early studies of this technology are encouraging however more studies with larger sample sizes are required to determine if fluorescence imaging is a reliable screening tool for oral cancer[18, 19].

Although new technologies for oral cancer screening are currently under investigation there are currently no routine population-based screening programs in place for oral cancer[2]. Current recommendations for dentists and general practitioners involve visual inspection of the oral cavity and follow-up before a surgical biopsy for definitive diagnosis. These recommendations can reduce false positive rates but have not significantly improved early diagnosis rates in OSCC patients[20]. A blood-based biomarker used alone or in combination with other screening techniques for the early detection and monitoring of oral cancer will lead to improved outcomes for patients diagnosed with this disease.

1.3 Early detection of lung cancer

In 2008, lung cancer accounted for 18% of overall cancer deaths and was the leading cause of cancer death in men and the second leading cause of cancer death in women worldwide[21]. Lung cancer is classified into two main subgroups, small-cell lung cancer and non-small-cell lung cancer (NSCLC). NSCLC is the most common subtype accounting for ~87% of newly diagnosed lung cancers. NSCLC is classified into four subgroups: adenocarcinoma, squamous-cell carcinoma, large-cell carcinoma, and NSCLC-not otherwise specified[22]. Five-year survival rates for lung cancer remain low at ~16% and, as in oral cancer, this dismal prognosis is partly due to diagnosis at late stages[23].

The majority of current lung cancer diagnoses occur when patients present with symptoms and seek clinical examination. Unfortunately, these symptoms usually arise at an advanced, inoperable stage[24]. Therefore, efforts have been made to determine the advantages of lung cancer screening tests for high-risk individuals (such as heavy smokers)[22]. Studies examining the use of sputum cytology and chest x-ray as lung cancer screening tools have shown that although lung cancer was detected at an earlier stage in screened individuals, there was no significant decrease in mortality rates[25, 26]. In 2002 the National Lung Screening Trial compared lung cancer screening with chest radiography to screening with low-dose computed tomography (CT) to see if mortality rates would decline. This group found that screening using low-dose CT showed a 20% decrease in mortality from lung cancer compared to those screened using chest radiography[27]. However, this technique does have risks including: a high rate of false positive results with the downstream implication of unnecessary diagnostic procedures, radiation exposure, and overdiagnosis (discovery of a cancer that would never come to light, nor cause any

symptoms or problems for the remainder of the person's life either because of slow growth or because some other illness brought about death before the diagnosis of the cancer)[24].

Other techniques currently being examined for early lung cancer detection are autofluorescence imaging and biomarkers in sputum, exhaled breath, and blood[28]. Autofluorescence bronchoscopy has been used with white-light bronchoscopy to detect early stage lung cancer and preinvasive lesions in the central airways and has been shown to have an increased sensitivity compared to white-light bronchoscopy alone[29]. However this technique also identifies benign conditions including inflammation and is therefore associated with a low specificity[30]. Currently, because small lung cancers are unlikely to exfoliate detectable cancer cells, sputum cytology is not considered a useful screening tool for early lung cancer detection. However, enhanced molecular techniques have led to improvements in the diagnostic value of sputum cytology which may be used to identify individuals with an increased lung cancer risk[31]. Biomarkers have been identified in exhaled breath and blood however these biomarkers are not used in a clinical setting as they are still in the experimental phase and have not been validated in large studies[32-34]. A non-invasive test with the ability to detect early lung cancer and distinguish between benign and malignant nodules would greatly improve the prognosis of patients diagnosed with this disease.

1.4 microRNAs

microRNAs (miRNAs) are endogenous, non-coding RNAs that are ~22 nucleotides in length. miRNAs control gene expression by binding to target mRNAs leading to their degradation or repression of translation[35]. miRNAs are transcribed in the nucleus into primary

miRNA transcripts called pri-miRNA which are cleaved by the RNase III enzyme Drosha into a precursor miRNA (pre-miRNA)[36]. The pre-miRNA is a stem loop intermediate about 60-70 nucleotides long. The export receptor Exportin-5 (Exp5) binds to pre-miRNA and exports it from the nucleus into the cytoplasm[37]. Once in the cytoplasm, the terminal base pairs and loop of the pre-miRNA are cleaved off by another RNase III enzyme, Dicer. This cleavage leaves a small double stranded RNA referred to as the miRNA:miRNA* duplex[35]. One strand of this duplex forms the mature miRNA and is incorporated into the RNA-induced silencing complex (RISC). The RISC is a ribonucleoprotein that contains the mature miRNA and an Argonaute (Argo) protein which is believed to be responsible for translational repression[38]. The other strand from the miRNA:miRNA* duplex, referred to as the miRNA* strand, generally accumulates at a lower level than its partner strand an is assumed to be mostly degraded however, studies suggest some miRNA* strands may have an extensive regulatory role[39] (Figure 1).

Once the mature miRNA is incorporated into the RISC this complex inhibits the translation of target mRNA through 1) mRNA cleavage 2) mRNA destabilization or 3) translational repression[40]. Endonucleolytic cleavage of the target mRNA takes place mainly in plant miRNAs and occurs when the miRNA and mRNA show perfect complementarity[41]. In metazoans, miRNAs imperfectly base-pair with their target mRNAs with the exception of the seed regions of the miRNA (nucleotides 2-8) which must bind perfectly with its target binding site in the 3' UTR of the mRNA in order for optimal translational repression to take place[40]. Rather than mRNA cleavage, this imperfect binding leads to mRNA destabilization or translational repression. The exact mechanisms of translational repression by miRNAs are still under investigation[42]. Evidence exists that supports translational repression both at the initiation stage as well as post-initiation however, it is believed that the majority of translational repression

takes place at initiation[42]. Additionally, many studies have shown an inverse correlation in the levels of miRNAs and their target mRNAs suggesting that miRNAs can induce mRNA destabilization and subsequent degradation[43]. Further studies have shown that the mechanism of this decay is through the binding of the RISC complex followed by deadenylation of the target mRNA and degradation via an exonuclease[44]. Although our understanding of miRNA biology is increasing, further studies are required to fully understand the mechanisms by which miRNAs repress protein production.

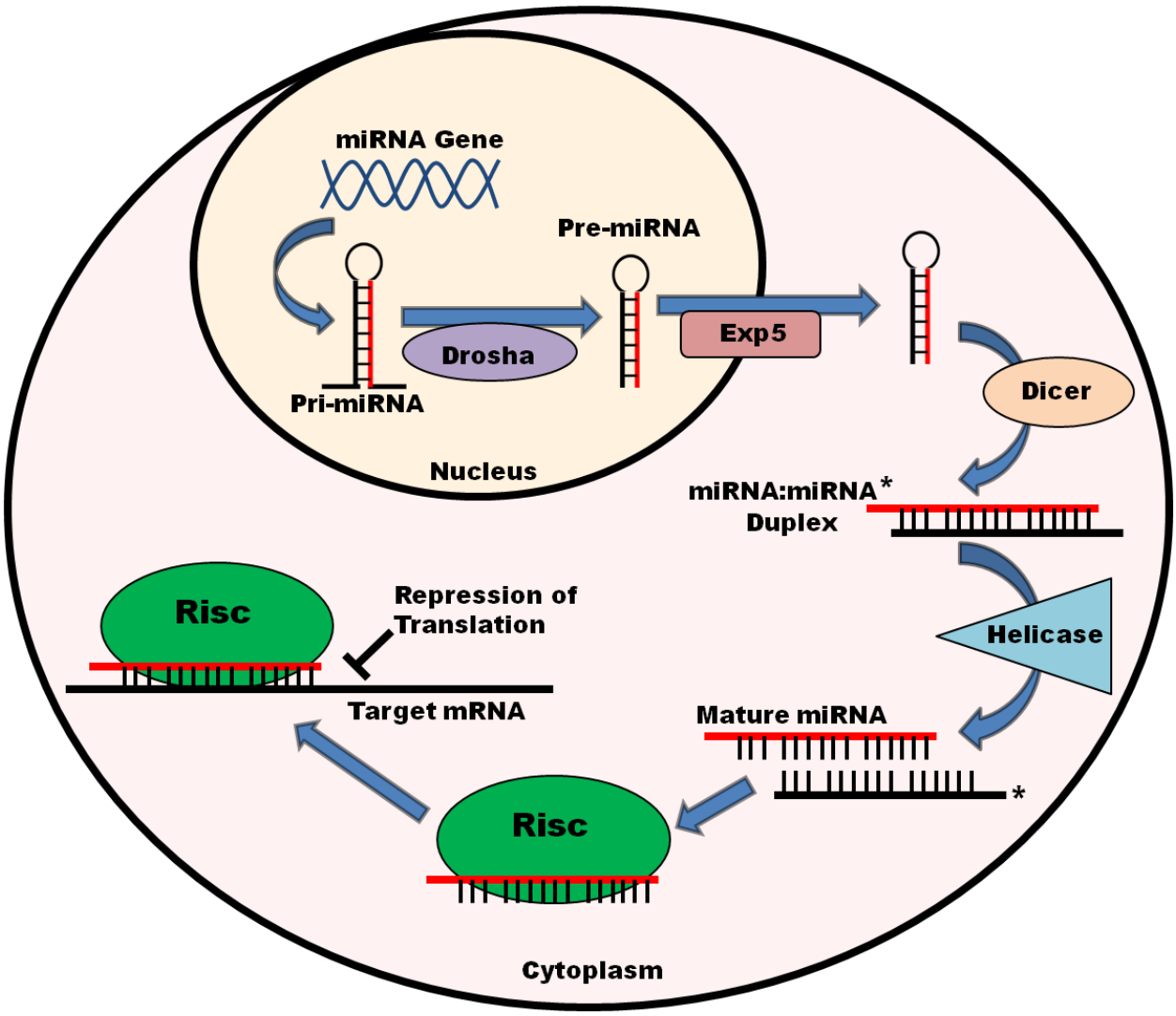


Figure 1. miRNA biogenesis and incorporation into the RISC complex.

1.5 miRNAs and cancer

Over 1500 mature miRNA sequences have been identified in humans (miRBase, Release 18.0, http://www.mirbase.org). This high number of miRNAs combined with the fact that each miRNA can target multiple mRNAs has led to the estimate that over 30% of the human genome is regulated by miRNAs[45]. Therefore, it is not surprising that studies have found miRNAs to be

involved in many cellular processes including development, cell proliferation, apoptosis, fat metabolism, and cell differentiation[46-51]. With such widespread regulatory functions in gene expression and key roles in cancer associated cellular processes, the roles of miRNAs in cancer are now being extensively explored[52].

In an early study examining the role of miRNAs in cancer, Calin et al mapped 186 miRNA genes and compared their chromosomal loci to areas commonly deleted or amplified in human tumors[53]. This work showed that 52% of the mapped miRNAs are located in cancer-associated genomic regions and demonstrated the potential involvement of miRNAs in many cancer types. This work has led to the classification of miRNAs as both tumor suppressors (miRNAs that target mRNAs of oncogenes and are frequently down-regulated in cancer) and oncogenes (miRNAs that target mRNAs of tumor suppressor genes and are frequently up-regulated in cancer)[52]. In addition to this new classification miRNAs have been shown to be involved in Hanahan and Weinberg's hallmarks of cancer[54] with deregulated miRNA expression allowing cancer cells to be self-sufficient in growth signals, evade growth suppressors, resist apoptosis, obtain limitless replicative potential, induce angiogenesis, and activate invasion and metastasis[55].

The connections between miRNAs and cancer have led to investigations into potential clinical uses for miRNAs as prognostic, predictive, and diagnostic molecules. In 2005, a group used a bead-based flow cytometric method to examine miRNA expression in 20 human cancers[56]. The authors found that miRNAs are generally down-regulated in tumor compared to normal tissues and that miRNA expression signatures may be useful for the classification of poorly differentiated cancers. Since 2005, many studies have been published examining the miRNA expression profiles of tumors. While the numerous studies on tumor miRNA expression

have yielded promising results, and some miRNA markers are currently being tested in clinical trials, further investigation in this area is needed before miRNAs can be used in the clinic as prognostic, predictive, and diagnostic biomarkers[57].

Another potential clinical application for miRNAs in cancer that is under increasing investigation is their use as therapeutic targets or tools[57]. One feature of miRNAs that makes them attractive molecules for therapeutic use is that, compared to single genes or proteins, miRNAs regulate the expression of multiple genes and can have implications for entire pathways[52]. For example, the reintroduction of tumor-suppressive miRNAs miRs 15a and 16-1 induces apoptosis in leukaemic cells both in vitro and in vivo[58]. Knock-down of oncogenic miR-21 inhibits breast tumor growth in a xenograft mouse model[59]. In addition to restoring tumor-suppressive miRNA expression or inhibiting oncogenic miRNA expression through anti-sense oligonucleotides, introduction of synthetic miRNAs to target oncogenic proteins has also been explored as a miRNA-based therapeutic[60]. Because miRNA function is so different from current targeted therapeutics, these molecules present new challenges regarding their integration into the clinic[61]. Nonetheless, the field of miRNAs as therapeutics for the treatment of cancer is a promising one.

1.6 Circulating miRNAs

In 2008, two independent groups published studies demonstrating that miRNAs are present at detectable levels in the serum and plasma of humans[62, 63]. These groups also illustrated that circulating miRNAs are very stable and could withstand multiple freeze-thaw cycles, long periods of storage, extreme pH conditions, and showed resistance to RNase A digestion. One

group hypothesized that the stability of these circulating miRNAs can be attributed to their packing in microvesicles which are then secreted by cells into circulation[63]. These initial studies sparked further research to explain the stability of circulating miRNAs and to elucidate their source and function. Since 2008, exosomes, microparticles, lipoproteins, and ribonucleoprotein complexes have all been found to be carriers of circulating miRNAs[64-67] (Figure 2).

Exosomes are 30-100 nm vesicles formed from the inward budding of endosomes, which are formed from inward budding of the plasma membrane. When endosomes bud inward they create multivesicular bodies (endosomes containing multiple exosomes). Exosomes are released into the extracellular environment when multivesicular bodies fuse with the plasma membrane[68]. Studies have shown that miRNAs contained in exosomes secreted from cancer cell lines and macrophages can be transferred to recipient cells in a paracrine fashion[69, 70].

Microparticles are vesicles that are 0.05-1 μm in size and are released from the plasma membrane of stimulated cells[71]. These particles play a role in vascular inflammation and coagulation and have been shown to contain miRNAs associated with cardiovascular diseases in a composition that is significantly different from their maternal cells[65].

Lipoproteins are complexes that consist of a lipid core surrounded by a shell of apolipoproteins that allow the lipids to travel in the bloodstream[72]. A recent study has shown that high-density lipoproteins (HDLs) are loaded with miRNAs both *in vitro* and *in vivo* and that HDLs transfer miRNAs to recipient cells[66].

Using size-exclusion chromatography, Arroyo et al (2011) demonstrated that the majority of certain miRNA species analyzed cofractionate with Argonaute2 proteins and not with vesicles[67]. Currently, it is unknown whether Argonaute2-bound circulating miRNAs can interact

with recipient cells and it has been suggested that these miRNAs are by-products of cellular activity[73].

Another important result to come out of the two seminal studies on circulating miRNAs was the finding that miRNAs are differentially expressed in the serum of individuals with certain diseases including prostate cancer, lung cancer, and diabetes[62, 63]. This finding opened the door for researchers to evaluate the utility of circulating miRNAs as biomarkers of disease. Circulating miRNAs possess a number of characteristics that make them ideal biomarkers including: 1) their stability under various conditions, 2) their ability to be detected through non-invasive means, and 3) their ability to be easily quantified with high sensitivity and specificity using methods such as qRT-PCR[74]. These characteristics have led to countless studies identifying potential circulating miRNA biomarkers for conditions such as sepsis[75], drug induced liver injury[76], cardiovascular disease[74], diabetes[77], and cancer[78].

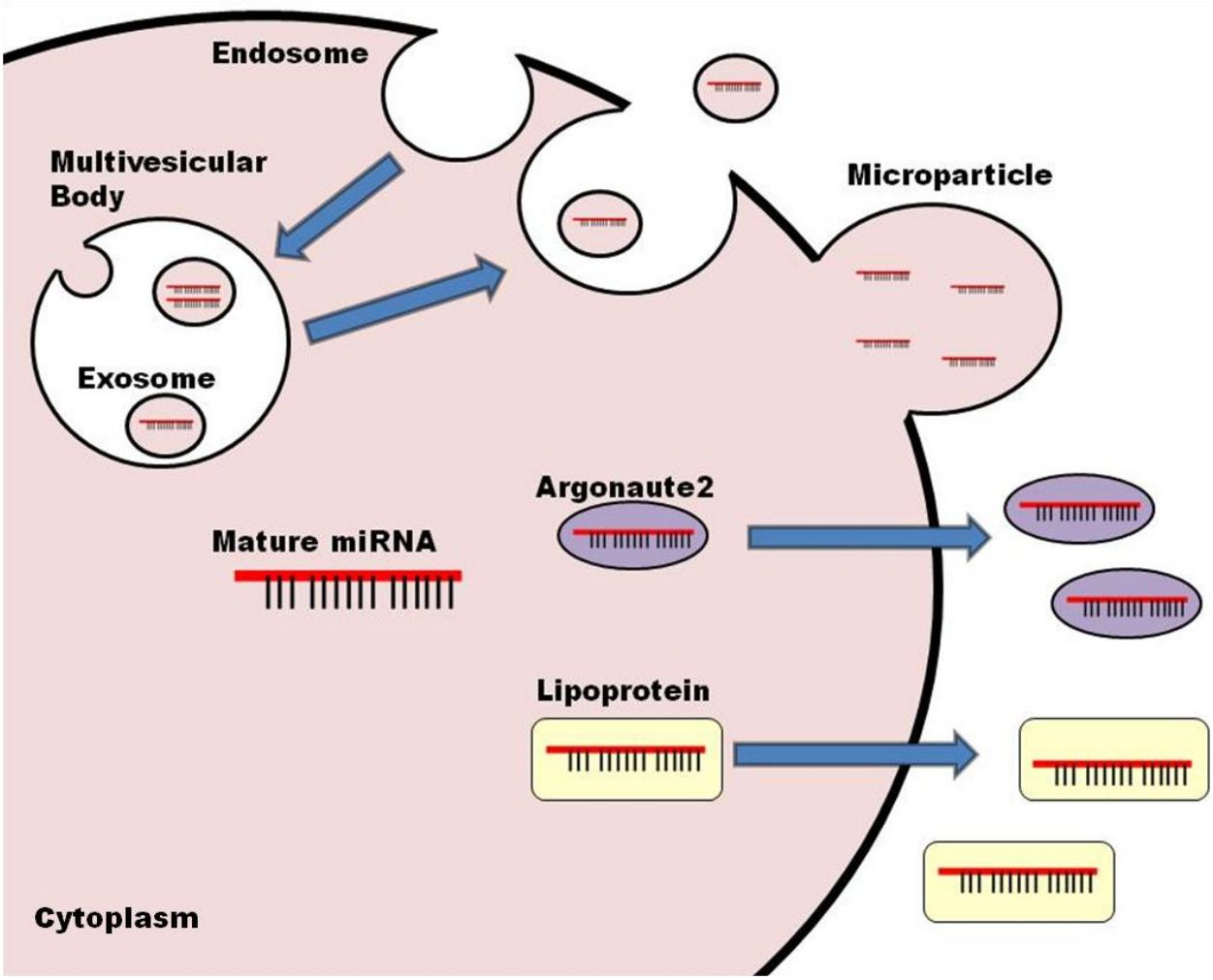


Figure 2. Current theories on how miRNAs enter circulation via exosomes, microparticles, proteins, and lipoproteins.

1.7 Circulating miRNAs and oral cancer

In 2008 Wong et al identified the up-regulation of miR-184 in tongue SCC tissue samples (N = 38 control 30 cancer) and found that plasma miR-184 levels were also increased in these patients compared to healthy controls. Furthermore, plasma miR-184 levels were significantly reduced after surgical resection of the tumor[79]. Similar studies in which plasma miRNA levels were shown to reflect oral cancer tissue miRNA profiles have identified miR-24 (N = 10 control

33 cancer)[80], miR-21 (N = 20 control 50 cancer)[81, 82], miR-181 (N = 12 control 39 cancer)[83], miR-31 (N = 21 control 43 cancer)[84], and miR-10b (N = 36 control 54 cancer)[85] as up-regulated in the plasma of oral cancer patients and miR-375 as down-regulated (N = 20 control 50 cancer)[81]. Together, these studies suggest the potential utility of circulating miRNAs as biomarkers for oral cancer detection and possibly as predictors of recurrence and progression of oral lesions. However, there have been few studies examining global miRNA expression in patients with OSCC and therefore many potential circulating miRNA biomarkers have yet to be investigated. Also, studies using samples collected from patients with pre-invasive oral cancer (carcinoma *in situ*, CIS) and oral dysplasia are required in order to identify candidate circulating miRNA biomarkers for the detection of oral cancer at its earliest stages.

1.8 Circulating miRNAs and lung cancer

The first study examining circulating miRNA levels in NSCLC used Solexa sequencing technology and found 91 miRNAs that were differentially expressed between the pooled sera from 11 patients with NSCLC and 21 healthy controls[62]. These 91 miRNAs were later tested in serum samples from 400 NSCLC cases and 220 controls leading to a panel of 10 miRNAs significantly up-regulated in NSCLC samples (miR-20a, miR-24, miR-25, miR-145, miR-152, miR-199a-5p, miR-221, miR-222, miR-223, and miR-320)[86]. The authors also demonstrated the utility of their panel for early lung cancer detection by accurately classifying 6 out of 7 patients with serum samples collected before (0.7-33.37 months) clinical diagnosis of NSCLC. Two studies with smaller sample sizes also identified signatures of multiple miRNAs for early lung cancer detection. Boeri et al (2011) identified a 16 ratio signature (using 15 miRNAs) that could

distinguish patients with NSCLC 1-2 years prior to clinical diagnosis with 80% sensitivity and 90% specificity[87]. While Bianchi et al (2011) identified a 34 miRNA signature that could accurately classify symptomatic and asymptomatic patients as well as benign and malignant lesions[88]. In addition to these studies, numerous groups have identified potential circulating miRNA biomarkers for the early detection of NSCLC (Table 1). Although the results of these studies are promising, there is little consistency in the miRNAs identified in each study. This variation could be due to multiple analytical and pre-analytical factors. These factors make comparisons between studies difficult and before the results of NSCLC circulating miRNA biomarker studies can be translated into the clinic, these factors must be identified and standardized.

Table 1. Current studies on the use of serum miRNAs as biomarkers of NSCLC.

Study	Samples[1]	Platform	Normalization	Candidate miRNAs	Hemolysis
62, 86	Discovery: 11 pooled NSCLC patients and 21 controls pooled. Training and Validation sets: 200 NSCLC (AC, LCC, and SCC patients stages I-IV) and 100 controls in each set.	Discovery: Solexa sequencing. Training and Validation: Taqman based qRT-PCR assay.	Directly normalized to serum volume	Discovery: 91 miRNA signature Validation: miR-20a, miR-24, miR-25, miR-145, miR-152, miR-199a-5p, miR-221, miR-222, miR-223, and miR-320 (all up-regulated).	Found differences in miRNA profiles of NSCLC serum versus blood cells
89	Discovery: 11 NSCLC (AC, SCC, and LCC stages I and II) and 11 controls. Validation: 22 NSCLC (AC, SCC, and LCC stages I and II) and 31 controls.	Discovery: microarray. Validation: SYBR Green based qRT-PCR.	Discovery: U6B, U6-337, 5S, and PC-HU5S. Validation: U6 and cel-miR-39.	miR-1254 and miR-574-5p (up-regulated)	Not mentioned

Study	Samples[1]	Platform	Normalization	Candidate miRNAs	Hemolysis
88	Training: 25 NSCLC (AC, Stages I-IV) and 39 controls. Testing: 34 NSCLC (AC and SCC, stage I-IV) and 30 controls. All samples were collected from asymtomatic patients.	Taqman based qRT-PCR assay	Geometric mean of miRs 197,19b, 24, 146, 15b, and 19a	34 miRNA signature	Not mentioned
87	Training: 19 NSCLC (AC, SCC, and other stages I-IV) and 5 control pools containing 5-7 samples. Validation: 22 NSCLC (AC, SCC, and other stages I-IV) and 10 control pools containing 5-7 samples	Taqman based qRT-PCR assay	Ratios between all miRNAs (N = 4,950 ratios)	16 ratio signature (using 15 miRNAs)	Not mentioned
90	70 NSCLC (AC and SCC, stages I-IV) and 44 controls.	Taqman based qRT-PCR assay	U6	miR-21 (up-regulated)	Not mentioned
91	Discovery: 5 NSCLC and 5 controls. Test: 88 NSCLC (AC, SCC, and other, stages I-III) and 17 controls.	Discovery: microarray. Test: Taqman based qRT-PCR assay.	U6	miR-21 (up-regulated)	Not mentioned
92	8 NSCLC samples collected at 3 time points (2 pre-diagnosis 1 post-diagnosis, histology and stage not available) and 6 controls	Microarray	Quantile normalization	17 miRNAs that change throughout tumor progression	miRNAs are also deregulated in NSCLC blood cells
93	Discovery: 193 NSCLC (AC and SCC, stages I-IV) and 110 controls	Taqman based qRT–PCR was performed directly in serum samples	Relative miRNA levels quantified using the formula 2^{50-ct}	miR-125b (up-regulated)	Not mentioned
94	Training: 30 NSCLC (AC and SCC, stages I-III) and 20 controls. Test: 55 NSCLC (AC and SCC, stages I-IV) and 75 controls.	Taqman based qRT-PCR assay	The ΔCt matrix approach	miR-15b and miR-27b (up-regulated)	Not mentioned
95	220 NSCLC (AC, SCC, and other, stages I-II) and 220 controls.	Taqman based qRT-PCR assay	Synthetic cel-miR-54 and cel-miR-238	miR-146b, miR-221, let-7a, miR-155, miR-17-5p, miR-27a and miR-106a (down-regulated) and miR-29c (up-regulated)	Not mentioned

Study	Samples[1]	Platform	Normalization	Candidate miRNAs	Hemolysis
[96]	82 NSCLC (pre- and post surgery, AC and SCC, stages I-IV) and 50 controls.	Taqman based qRT-PCR assay	miR-16	miR-21, miR-205, miR-24, miR-30d (up-regulated)	Not mentioned
[97]	Discovery: 21 NSCLC and 11 controls. Test: 97 NSCLC (AC, SCC, and LCC, stages I-IV) and 20 patients with benign lung disease and 30 controls.	Discovery: microarray Test: Taqman based qRT-PCR assay	miR-1233	miR-361-3p and miR-625* (down-regulated)	Not mentioned
[98]	260 NSCLC (AC, SCC, LCC, and other, stages III-IV) and 260 controls.	Taqman based qRT-PCR assay	miR-16	miR-125b (up-regulated)	Not mentioned
[99]	62 NSCLC (AC and SCC, stages I-IV) and 20 controls	SYBR Green based qRT-PCR	U6	miR-126 and miR-183 (down-regulated)	Not mentioned

1. AC = Adenocarcinoma, SCC= Squamous cell carcinoma, LCC= Large cell carcinoma

1.9 Challenges associated with circulating miRNA biomarker studies

The area of circulating miRNAs is a new field of research with many unknowns to be determined. The source of circulating miRNAs in both healthy individuals and those under various disease states have yet to be elucidated. Additionally, the exact mechanisms by which miRNAs are released into the bloodstream are not clear and further studies are required to determine the function (if any) of miRNAs in circulation. In addition to these functional studies, additional research on the pre-analytical and analytical features influencing serum miRNA quantification is required. One analytical area to be examined is the choice of platform for quantification.

Currently, there are three commonly used methods for the quantification of circulating miRNA: microarray chip technology, quantitative reverse transcription real-time PCR (qRT-

PCR), and sequencing technology[100]. Sequencing is advantageous for identifying novel miRNAs and modifications associated with different disease states but has a relatively low-input and high-cost compared to other methods[101]. However, with the recent improvements in this technology, sequencing may soon become a commonly used tool for miRNA screening[102]. Microarrays are high input and the most cost-efficient of the three methods but require a high concentration of input RNA and the low amount of RNA in serum and plasma samples can lead to less reliable results. qRT-PCR is more robust than microarray, has a moderate cost, and requires less starting material than both microarray and sequencing[101]. Considering these features, the most common study design for circulating miRNA biomarker studies is the use of microarray for initial screening to identify candidate miRNAs followed by validation by qRT-PCR[101]. However, more studies are required to determine the best platform to use and to standardize circulating miRNA quantification so results from various studies can be easily compared.

Another important analytical parameter that requires further investigation and standardization is the selection of a suitable endogenous control for circulating miRNA studies. Unfortunately, the well defined housekeeping genes used as endogenous controls in tissue and cell line miRNA studies are not expressed or show variable expression in serum and plasma[103, 104]. Potential endogenous controls have been suggested such as the small nuclear RNA U6[90]. However other studies have shown that U6 can be degraded in serum[62] and can have highly variable expression in some samples sets[105]. Another commonly used endogenous control is miR-16 which has been shown by some groups to have high and stable expression across samples[106, 107]. However this miRNA has also been shown to be significantly differentially expressed in patients with various disease states[108, 109]. To compensate for the lack of reliable endogenous controls some groups have used other methods of normalization such as normalizing

to the geometric mean of miRNAs detected in all samples[110], to total RNA[62], and to synthetic exogenous miRNAs such as cel-miR-39[63]. However, a reliable standard method of normalization that can be applied to various circulating miRNA studies has yet to be established.

The statistical methods used to determine differentially expressed miRNAs are also quite diverse among circulating miRNA studies. Many groups identify one or several differentially expressed miRNAs using Student's *t*-test[62, 111] or Mann-Whitney U test[112, 113] and assess clinical utility by determining a cut-off and calculating sensitivity and specificity. Other groups take advantage of the high number of miRNA species present in circulation and use computational networks to identify disease-specific miRNA profiles[77]. The advantages of this type of analysis over studying individual miRNAs alone is that clustering algorithms allows the identification of miRNAs that are likely to have similar biological functions and representing circulating miRNAs as networks may aid in revealing the role of specific miRNAs in properties of disease at the levels of organs and organ systems[114]. However, when dealing with large numbers of variables the study sample size needs to be quite large to avoid over-training the data. To ensure proper evaluation of circulating miRNA data and aid in the translation of biomarkers into the clinic, researchers need to seek the advice of experienced biostatisticians during all stages of their studies[115].

The identification of pre-analytical factors influencing circulating miRNA expression and quantification and appropriate experimental designs are required before circulating miRNAs can be used as reliable biomarkers. The majority of current candidate circulating miRNA biomarkers have been detected using case/control studies identifying differential expression between cancer and control subjects[116]. These studies can be useful in identifying biomarkers however important variables should be taken into consideration including differences in: sample

collection and handling, demographics, medical treatment (ie. medication), environment, and lifestyle that may influence circulating miRNA expression inducing bias in study results[117]. These factors are also important when considering the generalizability of the study[117]. Additionally, to confirm the utility of potential biomarkers for early detection, prospective studies in which blood samples are collected prior to onset of disease need to be conducted. Once these factors are addressed, the utility of circulating miRNA biomarkers in the clinic can begin to be assessed.

1.10 Rationale, hypotheses and aims

The development of new early detection techniques is essential to improve the outcome of patients with cancer. Blood-based biomarkers offer promising means of non-invasive detection however few molecules have been found that have a sensitivity and specificity high enough to be used in standard clinical practice. The finding that miRNAs are stably expressed in human plasma and serum and that they are differentially expressed in patients with cancer suggests the utility of these molecules as biomarkers not only for early detection, but also as means of monitoring disease progression and recurrence. However, further research into the factors that influence circulating miRNA expression and quantification is required before the full potential of these molecules as cancer biomarkers can be achieved.

The hypotheses examined in this thesis are 1) that miRNAs are differentially expressed in the serum of patients with oral and lung cancer compared to non-cancer control subjects and 2) that pre-analytical factors such as sample processing, patient life-style, and fasting status can have a significant effect on serum miRNA levels.

Aim 1: Identification of differentially expressed miRNAs in the serum of patients with high-risk oral lesions

Aim 1 of this thesis was to evaluate global miRNA expression in serum samples taken from patients with OSCC/CIS and from a group of demographically-matched control individuals with no cancer to identify miRNAs with disease-associated serum expression. Additionally, we compared global serum miRNA expression profiles in blood samples taken from individuals with disease that were collected before and after surgery, to hone the list of disease-associated serum miRNA candidates through a pairwise internally-matched comparison. This work represents the first-ever attempt to delineate serum miRNA biomarkers for head and neck malignancy and one of the first attempts to define such markers for any epithelial precancerous lesion.

Aim 2: Pre-profiling factors influencing serum microRNA levels

Before circulating miRNAs can reliably be used as biomarkers of disease, the pre-profiling factors that may contribute to inconsistent miRNA quantification must be identified and the variability of circulating miRNAs in healthy individuals must be determined. Aim 2 was to examine the effect of hemolysis, fasting, and smoking on serum miRNA profiles of healthy individuals and to compare serum miRNA profiles of samples taken from the same healthy individuals over different time periods. Using the results of these comparisons we also aimed to identify a miRNA that is stably expressed across all samples and unaffected by these pre-profiling variables to us as a suitable endogenous control for serum miRNA biomarker studies.

Aim 3: Identification of differentially expressed miRNAs in the serum of patients with early lung adenocarcinoma

A number of studies have been published examining the differential expression of miRNAs in the serum of patients with NSCLC[62, 88, 89]. However, the candidate miRNAs identified in these studies vary considerably; possibly due to the pre-analytical factors described above or to other sources of inter-patient variability. Aim 3 was to identify NSCLC-associated serum miRNAs using a novel approach that allows us to avoid inter-patient variability by using the patient as his/her own control. Specifically, we compared the miRNA profiles of serum collected during surgery from a) pulmonary venous effluent draining the tumor vascular bed and b) systemic arterial blood from the same patient[118]. Given the proximity to the tumor source, effluent blood samples are expected to have the highest concentration of tumor-associated miRNAs when compared to matched systemic arterial blood samples. Furthermore, we examined the possible utility of the differentially expressed miRNAs as biomarkers for lung cancer detection by comparing the serum miRNA profiles of patients with lung adenocarcinoma to a group of non-cancer controls.

2. Materials and methods

2.1 Sample collection

Aim 1 serum samples were collected from 30 patients with high-risk oral lesions (HRLs, i.e. CIS or OSCC cases) undergoing curative resection treatment between the dates of March 2010 and June 2011. Additional samples from ten of these patients were obtained six months after surgery for comparison to pre-surgery samples. For two of these patients, fresh frozen tissue

samples from biopsies of the lesion were also collected. Serum samples from a non-cancer control group (N=26) that were demographically matched to the above cohort for age, sex, smoking status, and ethnicity were also collected (Table 2). These samples were collected between the dates of December 2008 and March 2011.

All samples used in aim 2 were collected from healthy volunteers. To determine the effects of hemolysis on serum miRNA profiles, two vials of blood were drawn from 5 volunteers. To mimic mechanical hemolysis due to improper blood collection or specimen preparation[119] one vial was lysed immediately after collection by passing the blood through a 20 gauge needle several times. To assess the effects of fasting on serum miRNA profiles, blood samples were drawn from 7 healthy volunteers one hour after eating a fatty meal and again, three weeks later, after fasting overnight. Serum triglyceride levels were measured for each sample using a Triglyceride Assay Kit (Cayman Chemical Company, Ann Arbor, MI, USA). To determine the effects of smoking on serum miRNA profiles, blood samples were collected from 10 current smokers and from 10 age and sex-matched non-smokers without evidence of disease. Finally, to examine changes in serum miRNA levels over time two samples were collected from 12 volunteers over various time periods.

To identify an initial list of tumor associated miRNAs for aim 3, serum samples were collected during surgery from the pulmonary venous effluent draining the tumor vascular bed and from the systemic arterial blood of the same patient (N=20, 10 from each patient). To further examine the utility of differentially expressed miRNAs as NSCLC biomarkers, we compared the serum miRNA profiles of samples collected from patients with lung adenocarcinoma (N= 26) to samples from demographically matched, high risk smokers without cancer (N=43). All subject demographics are listed in Table 3.

Table 2. Aim 1 patient demographics.

	Oral carcinoma in situ patients	**Oral squamous cell carcinoma patients**	**Non-cancer control patients**	***P*-value Cancer (CIS and OSCC) vs Control**
Total patients	14	16	26	
Mean age	64	62	62	0.63[a]
Age range	50-84	51-93	50-75	
# of males	12	9	13	0.17[b]
Former Smokers	8	10	14	0.39[b]
Current Smokers	5	3	12	
Non-Smokers	1	3	0	
Mean smoker pack years	24	30	43	0.002[a]
Pack year range	0-60	0-156	30-80	
Ethnicity:				
Caucasian	13	14	26	0.24[b]
Other	1	2	0	

[a] Student's t-test
[b] Fisher's exact test

Table 3. Aim 3 patient demographics.

	Surgical Samples [a]	Lung Adenocarcinoma Patients	Non-cancer Controls	*P*-value (Adenocarcinoma vs Control)
Total patients	10	26	43	
Mean age	71.5	65	63	0.25[b]
Age range	62-79	49-80	50-75	
# of males	4	12	25	0.46[c]
Former Smokers	3	9	24	0.14[c]
Current Smokers	4	17	19	
Never Smokers	1	0	0	
Mean pack years	63.2	46	47	0.88[b]
Pack year range	35-88	16.5-87.5	30-80	
Stage (%):				
1A	4	8 (30.7)		
1B	1	5 (19.2)		
2A		3 (11.5)		
2B		2 (7.6)		
3A	1	6 (23)		
Unknown	4	2 (7.6)		
Ethnicity:				
Caucasian	10	26	43	

[a] Data missing data for some cases
[b] Student's t-test
[c] Fisher's exact test

2.2 Blood processing

All blood samples were collected in SST vacutainer tubes and kept at room temperature (30 minutes) to allow clotting. Clotted samples were centrifuged at room temperature for 15 minutes at 1500 rcf. Serum was aliquoted and frozen at -80°C. All samples were processed within 2 hours of initial collection.

2.3 Hemoglobin concentration

Serum absorbance levels were measured spectrophotometrically using a NanoDrop ND1000 spectrophotometer (NanoDrop Technologies). The hemoglobin concentration of each sample was calculated using an Allen Correction[120] with the following formula:

$$\text{Hemoglobin (g/L)} = 1.672\ A_{415} - 0.836\ A_{380} - 0.836\ A_{450}$$

2.4 RNA purification

Total RNA was extracted from 200 μL of serum using the miRNeasy Mini Kit (Qiagen) according to the manufacturer instructions, except that 1.25 μL of MS2 carrier RNA (Roche Applied Science) per 200 μL of serum was added to the QIAzol Lysis Reagent prior to RNA purification as the addition of a carrier has been shown to improve miRNA recovery[121]. Purified RNA was re-suspended in 50 μL of nuclease-free water and stored at -80°C prior to assaying miRNA expression. For some samples a synthetic spike-in consisting of 20 fmol of synthetic *C. elegans* miR-39-3p (cel-miR-39-3p; Qiagen, Toronto, ON, Canada) miRNA was added to each sample after a five minute incubation in QIAzol Lysis Reagent.

For the freshly frozen oral HRL tissues, RNA was purified via a standard TRIzol (Life Technologies Inc.) protocol after microdissection by an oral pathologist.

2.5 miRNA quantification by qRT-PCR

Due to low RNA yields in serum, the concentration of purified RNA could not be reliably measured, hence fixed volumes of eluted RNA (19.2 μL for 768 reactions) were used for miRNA expression assays as previously reported[81, 84, 121]. RNA was reverse transcribed using the miRCURY LNA Universal RT miRNA PCR, Polyadenylation and cDNA synthesis kit (Exiqon Inc.). cDNA was then quantified by quantitative real-time PCR (qRT-PCR) on the miRNA Ready-to-Use PCR, Human panel I and panel II with the miRCURY LNA Universal RT miRNA PCR, SYBR Green master mix according to the manufacturer's protocol (Exiqon Inc.). For samples profiled with a synthetic spike-in, 1μl of LNA™ control cel-mir-39-3p primer (Exiqon) was added to two empty wells on each panel. Ct (cycle threshold) values and ROX reference dye normalization were calculated using Viia7 Software (Applied Biosystems). All assays were inspected for distinct melting curves – those with >1 T_m and those detected within 5 Ct's of the negative control (Ct >35) were excluded from analysis.

The lung cancer surgical samples were quantified by Exiqon qPCR Services using the same reagents, panels, and quality controls as described above. Amplification for these samples was performed in a Roche Lightcycler480.

2.6 Data analysis

qRT-PCR results were exported to GenEx (MultiD Analyses AB) and normalized using inter-plate calibrators on the miRNA Ready-to-Use panels. The normalized (ΔCt) values for aim 1 samples were calculated using the mean expression value of all expressed miRNAs in a given sample (the global mean[110]) with the following equation: ΔCt = global mean - Ct (assay). Aim 2

and aim 3 samples were normalized as described in each corresponding results section. All matched samples (samples collected from the same person) were analyzed using a fold-change analysis ($2^{(\text{sample 1 } \Delta Ct - \text{sample 2 } \Delta Ct)}$).

Mann-Whitney, ANOVA, Tukey HSD *post hoc* testing, and box plots were calculated and generated using STATISTICA Software (Statsoft™, Tulsa, OK). For the aim 1 oral cancer samples, a permutation test was conducted using miRNAs that were present at detectable levels in ≥60% of samples and was used to identify miRNAs significantly deregulated between disease and control cases[122]. All *P*-values were corrected using the Benjamini and Hochberg method for multiple testing. Average-linkage hierarchical clustering was calculated and displayed using Genesis software (www.genome.tugraz.at). Receiver operating characteristic (ROC) curves and area under the curve (AUC) plots were generated using the ROCR package in R[123].

3. Differential expression of miRNAs in the serum of patients with high-risk oral lesions[1]

3.1 Introduction

There have been reports of differential miRNA expression in plasma from patients with oral squamous cell carcinoma (OSCC) or esophageal cancer[80, 81, 84]. Individual miRNAs examined in these studies were selected *a priori*, based on direct study of cancer tissues. Other data, however, suggest that miRNAs showing disease-associated expression changes in blood are

[1] A version of chapter 3 has been published. MacLellan SA, Lawson J, Baik J, Guillaud M, Poh CF , and Garnis C. (2012) Differential expression of miRNAs in the serum of patients with high-risk oral lesions. *Cancer Medicine* 1(2): 268-274.

not necessarily the same ones differentially expressed in cancer tissues; tumor cells may selectively release specific miRNAs into the extracellular environment. Hence miRNA expression patterns in tumor and serum samples from the same patient might not correlate[124, 125]. Further, plasma miRNA expression has been shown to be more variable (as compared to serum expression)[95]. This inconsistency may be due to contamination of platelet miRNAs in plasma samples[126], a reality that makes serum samples more attractive for blood biomarker analysis. Therefore, we evaluated global miRNA expression in serum samples taken from patients with OSCC/CIS and from a group of demographically-matched control individuals with no cancer to identify miRNAs with disease-associated serum expression.

3.2 Results

3.2.1 miRNA expression in patients with OSCC and CIS

To identify differentially expressed miRNA we use qRT-PCR to quantify miRNA expression in serum from 56 subjects (30 HRL patients and 26 non-cancer controls). Of the 742 miRNAs assayed, 565 were detected in ≥1 of our samples and 58 were detected in every sample. Additionally, 502 miRNAs were detected in ≥1 HRL sample and 69 miRNAs were detected in all HRL samples.

The HRL cohort consisted of 14 CIS and 16 OSCC cases. Since miRNA expression can vary based on histopathological stage[127], we first asked whether these two groups had distinct serum miRNA profiles compared to controls. A one way ANOVA of all 56 samples showed significant differential expression of six miRNAs that were specific to CIS versus control comparisons. Two miRNAs were found to have significantly different expression between CIS

versus control and CIS versus OSCC (though not different between control and OSCC). Also, ten miRNAs were found to have differential expression between control and both CIS and OSCC but not between CIS and OSCC. And four miRNAs showed a difference specific to the OSCC group when compared to control (Table 4).

Ultimately, since CIS has a high chance of progressing to OSCC, we treated CIS and OSCC as a single HRL cohort. A permutation test comparing HRL samples to the demographically-matched control samples was conducted on all 56 cases using the global-mean normalized data (ΔCt). A total of 45 miRNAs were significantly up-regulated ($p<0.001$) and ten miRNAs were significantly down-regulated in the HRL samples compared to controls (Table 5).

Table 4. Results of a one-way ANOVA followed by Tukey's HSD *post hoc* to determine significantly differentially expressed miRNAs between ctrl (non-cancer control), CIS (carcinoma *in situ*), and OSCC (oral squamous cell carcinoma) samples.

miRNA	**Corrected *P*-value**	**Groups that are significantly different**
miR-197	0.0150	ctrl/CIS
miR-125b	0.0219	ctrl/CIS
miR-342-3p	0.0282	ctrl/CIS
miR-19a	0.0331	ctrl/CIS
miR-486-5p	0.0417	ctrl/CIS
miR-23a	0.0469	ctrl/CIS
let-7d*	0.0046	ctrl/OSCC
miR-26b	0.0300	ctrl/OSCC
miR-26a	0.0329	ctrl/OSCC
miR-29c	0.0373	ctrl/OSCC
miR-320a	0.0177	ctrl/CIS , ctrl/OSCC
miR-142-5p	0.0040	ctrl/CIS, ctrl/OSCC
miR-16	0.0042	ctrl/CIS, ctrl/OSCC
miR-29a	0.0066	ctrl/CIS, ctrl/OSCC
miR-338-3p	0.0081	ctrl/CIS, ctrl/OSCC
let-7b	0.0093	ctrl/CIS, ctrl/OSCC
miR-17	0.0367	ctrl/CIS, ctrl/OSCC
miR-150	0.0373	ctrl/CIS, ctrl/OSCC
miR-18b	0.0373	ctrl/CIS, ctrl/OSCC
miR-181a	0.0480	ctrl/CIS, ctrl/OSCC
miR-223	0.0002	ctrl/CIS, CIS/OSCC
miR-423-3p	0.0026	ctrl/CIS, CIS/OSCC

Table 5. miRNAs significantly (P > 0.001) up or down-regulated in cancer serum samples as determined by a permutation test.

Up-regulated miRNAs	**Corrected *P*-value**	**Up-regulated miRNAs**	**Corrected *P*-value**	**Down-regulated miRNAs**	**Corrected *P*-value**
hsa-miR-16	1.00E-10	hsa-miR-451	4.77E-06	hsa-miR-29a	6.55E-07
hsa-let-7b	1.00E-10	hsa-miR-7	1.11E-05	hsa-miR-223	1.05E-06
hsa-miR-320a	1.00E-10	hsa-miR-93	1.22E-05	hsa-miR-338-3p	1.08E-06
hsa-miR-18b	1.00E-10	hsa-miR-25	1.52E-05	hsa-miR-142-5p	1.66E-06
hsa-miR-26a	1.23E-10	hsa-miR-151-3p	3.60E-05	hsa-let-7d*	7.54E-06
hsa-miR-17	1.35E-10	hsa-miR-660	3.98E-05	hsa-miR-150	1.07E-04
hsa-miR-19a	2.11E-10	hsa-miR-186	5.68E-05	hsa-miR-181a	1.18E-04
hsa-miR-26b	7.64E-10	hsa-let-7a	6.40E-05	hsa-miR-342-3p	1.93E-04
hsa-miR-486-5p	8.99E-10	hsa-miR-195	1.10E-04	hsa-miR-23a	4.96E-04
hsa-miR-92a	7.24E-09	hsa-miR-624*	1.30E-04	hsa-miR-423-3p	6.84E-04
hsa-miR-22*	8.24E-09	hsa-miR-146b-5p	1.33E-04	hsa-miR-140-5p	9.88E-04
hsa-miR-125b	2.19E-08	hsa-miR-106a	1.34E-04		
hsa-miR-205	5.09E-08	hsa-miR-20b	1.40E-04		
hsa-miR-301a	9.50E-08	hsa-let-7i	1.83E-04		
hsa-miR-185	1.33E-07	hsa-miR-21	2.02E-04		
hsa-miR-30e	1.77E-07	hsa-miR-16-2*	2.29E-04		
hsa-miR-20a	2.92E-07	hsa-miR-93*	2.34E-04		
hsa-miR-320b	9.79E-07	hsa-miR-324-3p	2.71E-04		
hsa-miR-15b*	1.06E-06	hsa-miR-27b	3.40E-04		
hsa-miR-126*	1.12E-06	hsa-miR-423-5p	6.91E-04		
hsa-miR-484	1.97E-06	hsa-miR-106b	7.13E-04		
hsa-let-7c	2.19E-06	hsa-miR-144	7.67E-04		
hsa-miR-128	2.22E-06				

3.2.2 Serum miRNA levels pre and post surgical resection of an oral HRL

To ensure that the observed differential miRNA expression between the HRL and control groups was due to the presence of an oral lesion – and to avoid inter-patient variability – we next compared miRNA profiles for serum samples taken from matched pre- and post-surgical serum samples (N=10). Serum samples contributing to this analysis were taken immediately prior to surgery and six months following surgical resection. An average-linkage hierarchical clustering analysis on the 50 miRNAs with the highest standard deviation showed that the serum miRNA profiles clustered according to the presence/absence of disease – and not according to the source patient (Figure 3).

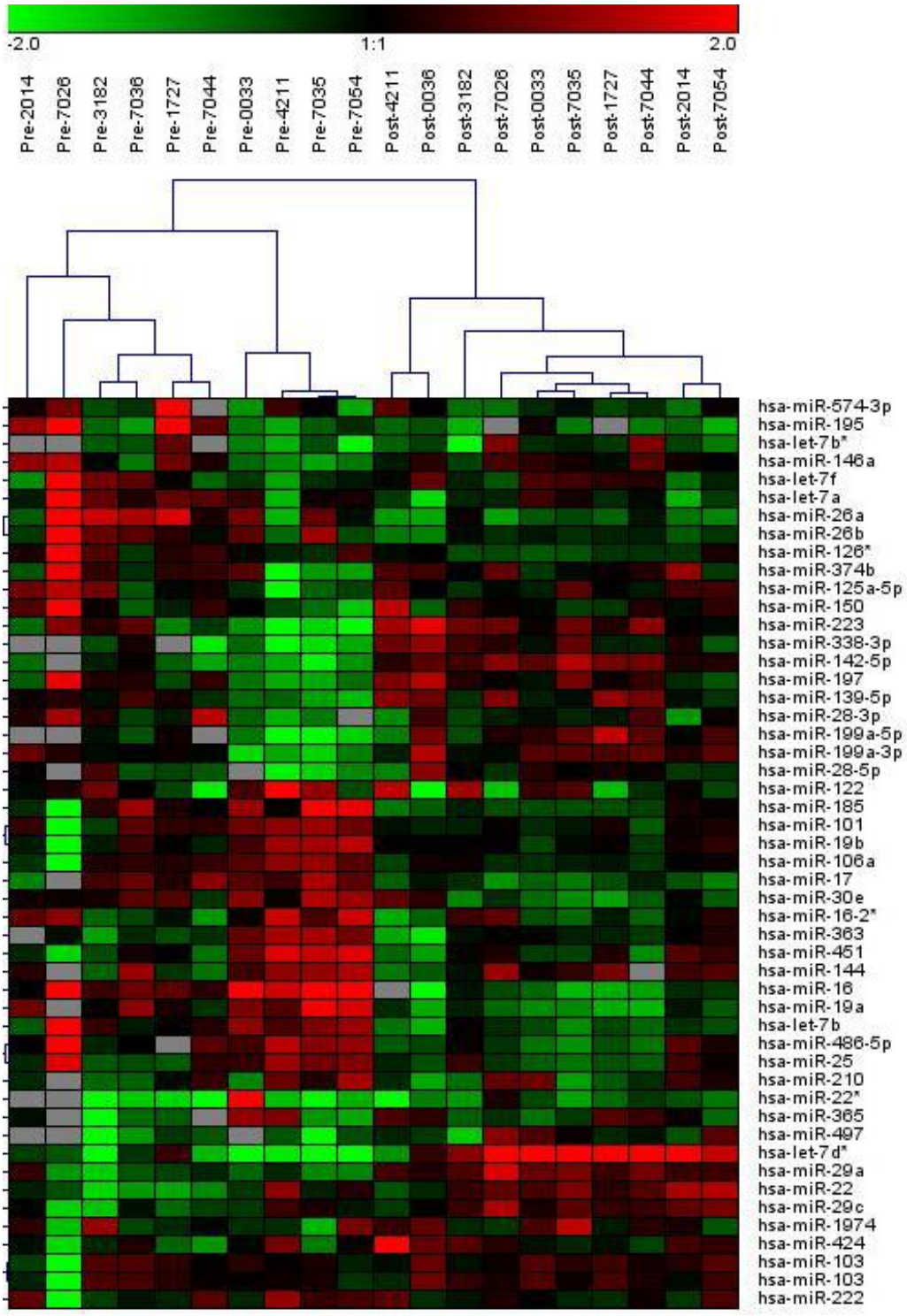
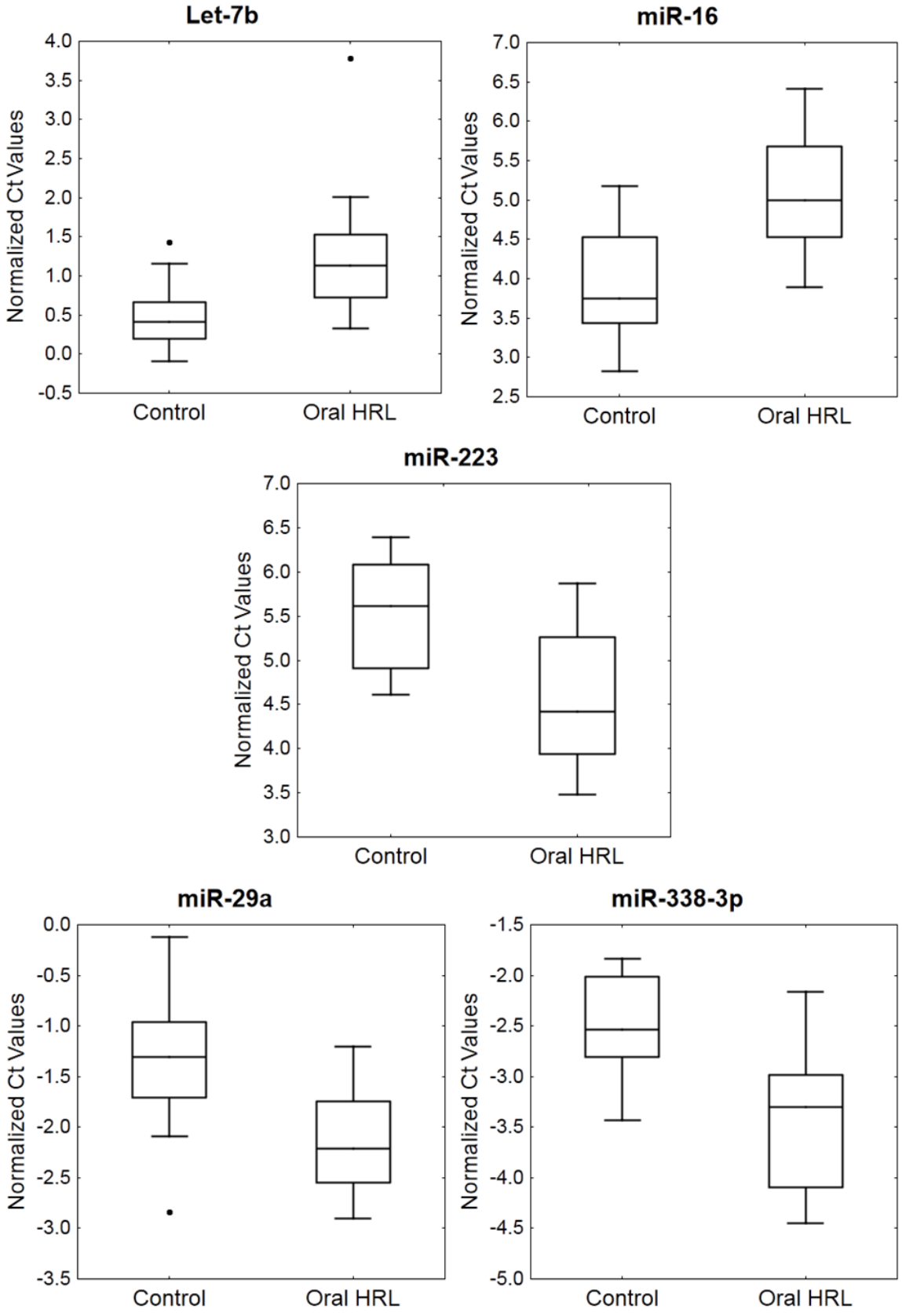


Figure 3. An average-linkage Pearson correlation hierarchical clustering analysis of the 50 miRNAs with the highest standard deviation across all pre- vs. post-surgery cases showing that disease state has more influence on serum miRNA profiles than inter-patient variability. The analysis was performed on mean centered, normalized data.

3.2.3. Serum miRNA biomarker candidates

A fold-change analysis of miRNA expression levels showed up-regulation of 32 miRNAs and down-regulation of 41 miRNAs by ≥2-fold in ≥50% of pre-surgical cases (versus post-surgical ones). Comparing the results from both above analyses – 1) analysis of HRL versus control samples and 2) analysis of pre- versus post-surgical samples – uncovered 15 miRNAs with expression that was significantly up-regulated with disease (i.e., cancer patients in comparison #1 and pre-surgical patients in comparison #2). Five miRNAs were also found to be significantly down-regulated with disease in this comparison (Table 5). Follow-up with ROC curve analysis of these 20 candidates identified five miRNAs with AUC values >0.8. Two of five were significantly up-regulated in HRL cases compared to controls (miR-16 and let-7b) and three were down-regulated in this same comparison (miR-338-3p, miR-223, and miR-29a) (Figure 4).

A


Let-7b
Normalized Ct Values
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
-0.5
Control
Oral HRL
miR-16
Normalized Ct Values
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
Control
Oral HRL
miR-223
Normalized Ct Values
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
Control
Oral HRL
miR-29a
Normalized Ct Values
0.0
-0.5
-1.0
-1.5
-2.0
-2.5
-3.0
-3.5
Control
Oral HRL
miR-338-3p
Normalized Ct Values
-1.5
-2.0
-2.5
-3.0
-3.5
-4.0
-4.5
-5.0
Control
Oral HRL

B

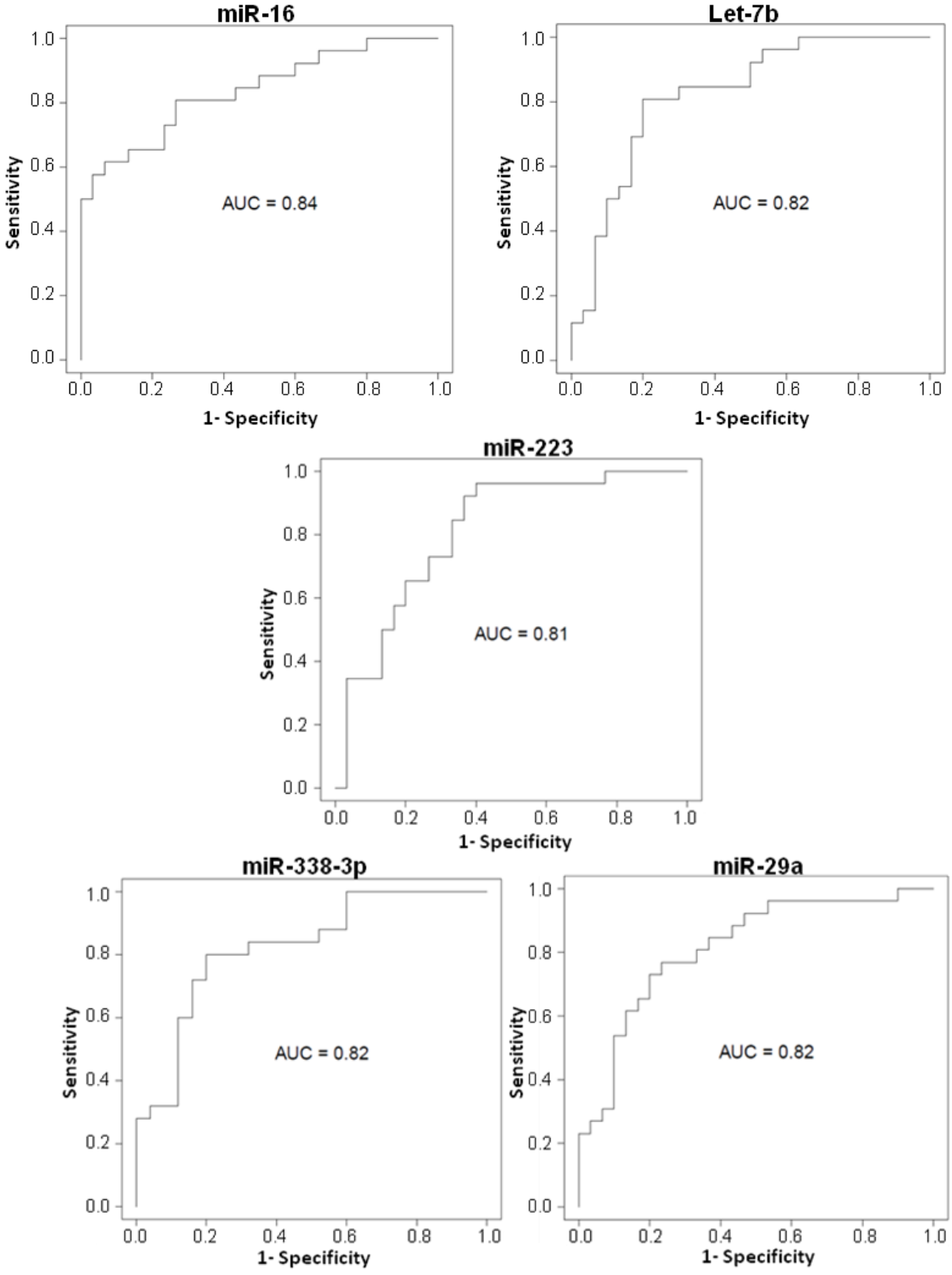

miR-16
AUC = 0.84
Let-7b
AUC = 0.82
miR-223
AUC = 0.81
miR-338-3p
AUC = 0.82
miR-29a
AUC = 0.82
Sensitivity
1- Specificity
0.0
0.2
0.4
0.6
0.8
1.0

Figure 4. (A) Expression levels of the five candidate miRNAs in the serum of oral HRL (N = 30) and non-cancer control (N = 26) patients. Scale at y-axis represents Ct values normalized to the global mean. Line inside the box: median, box: interval between the 25th and 75th percentiles, whiskers: interval between the 10th and 90th percentiles, circles: outliers. (B) ROC curve analysis conducted on the cancer versus control cases showing the five most significantly deregulated miRNAs. miR-333-3p yielded an AUC of 0.82 (95% CI: 0.71–0.94) with 80.0% specificity and 80.0% sensitivity in identifying oral HRLs, miR-29a yielded an AUC of 0.82 (95% CI: 0.70–0.93) with 76.7% specificity and 76.9% sensitivity, miR-223 yielded an AUC of 0.81 (95% CI: 0.69–0.92) with 60.0% specificity and 96.2% sensitivity, miR-16 yielded an AUC of 0.84 (95% CI: 0.73–0.94) with 93.3% specificity and 61.5% sensitivity, and let-7b yielded an AUC of 0.82 (95% CI: 0.71–0.93) with 80.0% specificity and 80.8% sensitivity.

3.2.4 Correlation between oral HRL tissue and serum miRNA profiles

We also sought to determine whether serum profiles were reflective of tissue miRNA expression in two cases where matched HRL tissue specimens were available. For the tissue samples, miRNA levels of tumor tissue were compared to that of matched adjacent normal tissue and a fold-change $\geq$ 2-fold was considered significant. For one patient, 30 miRNAs were up-regulated in both tissue and serum, no miRNAs were down-regulated in both serum and tissue, and 322 miRNAs were differentially expressed between tissue and serum. For the second patient, 39 miRNAs were up-regulated and 3 were down-regulated in both samples, while 346 were differentially expressed.

3.3 Discussion

Novel biomarkers detecting oral cancer at early stages could be used to more effectively stratify patients and ultimately improve disease outcomes. Serum-expressed miRNAs have great potential as biomarkers: miRNAs are understood to be key molecular drivers affecting cancer phenotypes (thus making them worthy of direct monitoring)[56] and serum samples for miRNA

expression are both cheaply and easily (i.e. non-invasively) obtained, making them feasible to apply in screening populations. By evaluating expression of circulating miRNAs in sera from patients with oral cancer, we identified 20 miRNAs that are significantly deregulated with oral HRLs. This work represents the first-ever global analysis of serum miRNA expression profiles in patients with any head and neck cancer or pre-cancer.

By comparing the serum miRNA profiles of patients with oral cancer to profiles from non-cancer controls, we identified 55 miRNAs that were significantly deregulated with disease, demonstrating that the presence of oral HRLs can significantly alter circulating miRNA expression. Six miRNAs were specifically deregulated in the serum of patients with precancerous lesions while four miRNAs were specifically deregulated in serum from OSCC patients (Table 4). These stage-specific miRNA profiles may represent alterations in exosomally-secreted miRNAs during cancer progression. The presence of specific serum miRNAs associated with precancerous lesions also suggests utility for such miRNAs in the early detection of oral cancer; these easily tested blood markers could be applied as an adjunct to direct investigation of the oral cavity to improve disease detection.

An analysis of serum miRNA levels in pre- and post-operative samples demonstrated that many circulating miRNAs are tumor-specific as the levels correspondently decrease or increase after tumor resection (Figure 3). Because miRNAs are involved in many cellular processes[35], the use of these patient-matched samples allowed us to remove inter-patient variability. These results suggest that serum miRNAs could also have utility for monitoring disease recurrence, a clinical reality that contributes significantly to continuing poor oral cancer outcomes[9]. Moreover, because treatment (surgery, radiation) can alter the appearance of normal tissue surrounding a

lesion, which can delay recurrence detection, serum miRNAs may be applicable as an alternative means of post-treatment monitoring.

Cross-comparison of results from the two above analyses identified 20 serum miRNAs consistently deregulated based on disease status. These miRNAs did not overlap with candidates defined by other oral cancer analyses[79, 80, 84]. One explanation for this could be the use of serum as the miRNA source instead of plasma, as recent reports have shown differential miRNA expression in patient-matched serum and plasma samples[95, 126]. Another significant result was the detection of serum miRNAs up-regulated in normal and post-surgery patients as compared to disease/matched pre-surgical samples (five of 20 miRNAs referenced above). The finding of serum miRNAs up-regulated in disease/pre-surgical samples relative to their comparators is intuitive as the given HRL still harbored by the patient could be the over-expressed serum miRNA source; absence or removal of this miRNA source would be associated with reduced expression. However, the finding of disease-associated serum miRNAs exhibiting reduced expression relative to non-disease/post-treatment states cannot be explained by this mechanism, suggesting that expression of disease-associated miRNAs in serum may be a product of factors beyond secretion of these miRNAs from diseased cells. Similar results have been reported in other cancer types[81, 95].

ROC analysis of the 20 miRNA candidates identified five miRNAs (miR-16, let-7b, miR-338-3p, miR-223, and miR-29a) that have an AUC >0.8, suggesting that these miRNAs could have utility as biomarkers for oral cancer detection. The two miRNAs that were highly up-regulated in sera from patients with oral HRLs (miR-16 and let-7b) have been reported to act as tumor suppressors (down-regulating oncogenes like RAS, BCL2) and are known to be down-regulated in many cancer types[128-130]. Our result may be a product of selective exosome-mediated

release of let-7 miRNA family members into the extracellular environment, with this exclusion serving to preserve cancer phenotypes[124]. Indeed, selective release of tumor suppressive miRNAs by cancer cells presents a possible explanation for the up-regulation of let-7b and miR-16 in the serum of patients with OSCC/CIS. It may also explain the relative lack of correlation between our matched tissue and serum miRNA profiles reported by other groups[125]. Interestingly, miR-16 has been used by a number of studies as an endogenous control to normalize qRT-PCR data from both OSCC tissue and plasma samples[79, 84], however our results show that it is the most significantly up-regulated miRNA in the serum of patients with oral cancer (suggesting it may not be a reliable endogenous control). The three miRNAs that were highly down-regulated in serum from patients with oral HRLs – miR-338-3p, miR-223, and miR-29a – have been shown to be down-regulated in esophageal squamous cell carcinoma tissue, OSCC cell lines, and hypopharyngeal squamous cell carcinoma tissue, respectively[131-133]. As mentioned above, the process by which disease-associated down-regulation of serum miRNAs is an outstanding issue requiring further investigation.

We report the first-ever analysis of global miRNA expression in serum samples from patients with oral cancer or pre-cancer. By incorporating matched, pre-/post-surgical serum samples into our combined analysis, we have reduced the impact of inter-patient variability on our findings. Due to the relatively small number of samples used in this study, further validation in a larger cohort is needed to fully assess the utility of candidate miRNAs as oral cancer biomarkers. We are currently drawing samples from a large clinical trial to facilitate this validation. Current clinical practice relies on histology to determine the malignant potential of dysplastic lesions however this method is a poor predictor for low grade lesions[134]. Therefore, we are also accruing serum samples from patients harboring this disease stage to determine whether

our serum miRNA biomarker candidates are applicable to this patient population. These markers could potentially be used alone or in combination with current promising molecular markers in the literature including chromosomal alterations[134] and quantitative cytology[135] to predict the progression of oral dysplasias. Our results provide a strong rationale for wider evaluation of serum miRNAs as biomarkers for management of oral malignancy. They also suggest that novel processes pertaining to the relationship between disease and serum miRNA expression remain to be uncovered.

4. Pre-profiling factors influencing serum microRNA levels

4.1 Introduction

Before circulating miRNAs can reliably be used as biomarkers of disease, the pre-profiling factors that may contribute to inconsistent miRNA quantification must be identified and the variability of circulating miRNAs in healthy individuals must be determined. Recent studies have shown that the majority of miRNAs found in serum and plasma are also present in blood cells and that hemolysis during sample collection or processing may affect the miRNA profile of the sample[62, 136, 137]. In particular, circulating miRs 451, 16, 24, 15b, 223, 486, 150 and 92a have all been shown to be inconsistently expressed in healthy individuals due to their presence in blood cells[126, 138]. However, the effects of mechanical hemolysis on whole micronome serum quantification have not been extensively studied. Additionally, it has been suggested that lipid intake before blood draw may interfere with miRNA extraction leading to a variable profile[139]. To date there have been no studies examining the effects of fasting status on miRNA expression profiles. Lifestyle may also affect serum miRNA expression. Because we (along with others[62, 88,

[93]) are interested in the utility of circulating miRNAs as lung cancer biomarkers and because ~85% of lung cancers in men and 45% in women are caused by smoking[140], there is a need to examine the effects of smoking on circulating miRNA profiles. One study has shown that serum levels of miR-625* are significantly lower in non-small cell lung cancer patients who smoke compared to those who do not smoke[97]. However, there have been no studies comparing the serum miRNA levels of healthy smokers to healthy non-smokers. Other unknown factors such as changes in hormone levels or lifestyle changes may influence the serum miRNA profiles of healthy individuals leading to a change in these profiles over time.

In this study, we examined the effect of hemolysis, fasting, and smoking on serum miRNA profiles of healthy individuals and compared serum miRNA profiles of samples taken from healthy individuals over different time periods. Our results contribute to a better understanding of the pre-analytical factors that may influence serum miRNA profiles.

4.2 Results

4.2.1 The effects of hemolysis on serum miRNA levels

To determine the effects of hemolysis on serum miRNA profiles, we measured the serum miRNA profiles of lysed and unlysed serum samples from ten healthy volunteers. Hemoglobin levels of all samples were measured spectrophotometrically, all unlysed samples had a hemoglobin concentration below 1 g/L and all lysed samples had a hemoglobin concentration above 1.5 g/L (Table 6).

Table 6. Hemoglobin concentrations of matched lysed and unlysed serum samples.

Sample ID	Hemoglobin Concentration (g/L) Unlysed	Hemoglobin Concentration (g/L) Lysed
11	0.51	5.52
12	0.48	12.17
14	0.55	6.20
15	0.56	2.20
16	0.42	1.54
17	0.74	10.91
18	0.69	10.48
19	0.75	24.28
20	0.37	12.35
21	0.70	22.49

miR-122 was used to normalize the data because it showed little variability between samples collected from the same person and has been shown by others to be unaffected by hemolysis[126, 138]. Additionally, a synthetic spike-in (cel-miR-39-3p) was added and measured in 14 of the 20 samples and the fold-change results of these samples normalized to miR-122 were not significantly different from the results of the spike-in normalized data. However, because the levels of miR-122 were highly variable from person to person, miR-122 normalized data was only used to make intra-individual comparisons.

Out of the 742 miRNAs profiled 109 miRNAs were detected in all 20 samples, 181 miRNAs were detected in all 10 hemolysed samples, and 116 miRNAs were detected in all 10 non-hemolysed samples. 36 miRNAs were detected in at least 5 of the 10 hemolysed samples at a Ct below 34 but were not detected in any of the matched non-hemolysed samples.

A cut-off of 2-fold is commonly used to identify significant changes in circulating miRNA levels[86, 141] however, to increase our stringency and identify only the most significantly deregulated miRNAs for exclusion of further analyses a 3-fold cut-off was applied to our fold-change analysis of matched samples. This analysis identified 4 miRNAs that were up-regulated by at least 3-fold in all hemolysed samples and 144 miRNAs that were up-regulated by at least 3-fold in 40% of hemolysed samples compared to unlysed samples (Table 7). No miRNAs were down-regulated by at least 3-fold in more than 1 hemolysed sample. The cel-miR-39-3p spike-in was not significantly differentially expressed between hemolysed and non-hemolysed samples suggesting that the difference in miRNA levels is not due to the presence of inhibitors in the samples. Because clinical samples often contain some hemolysis[142], we have identified a list of miRNAs significantly affected by mechanical hemolysis to exclude from serum miRNA biomarker studies. For our purposes, we decided to exclude miRNAs up-regulated by at least 3-fold in at least 50% of samples or detected in 80% of hemolysed samples and not detected in matched unlysed samples leaving a final list of 130 miRNAs to be excluded due to the influence of hemolysis (Table 7).

To measure the correlation between hemoglobin concentration and serum miRNA levels, the raw data was normalized to miR-99a (rationale described below). Of the miRNAs detected in at least 80% of the hemolysed samples or 80% of the non-hemolysed samples, 150 miRNAs were significantly (corrected P-value < 0.05) correlated with hemoglobin concentration (Table 7). A Pearson correlation cluster analysis of miRNAs show that the samples cluster according to hemoglobin concentration and not according to sample source (Figure 5).

Table 7. List of 144 miRNAs most affected by mechanical hemolysis.

miRNA	# of cases	Median Fold-change	miRNA	# of cases	Median Fold-change	miRNA	# of cases	Median Fold-change
miR-15b*	10	15.34	miR-335	8	5.62	miR-376c	6	4.43
miR-451	10	18.03	miR-140-5p	8	7.70	miR-423-3p	6	7.03
miR-20b	10	17.18	miR-21	8	6.89	miR-625*	6	5.36
miR-144	10	29.63	miR-151-3p	8	5.71	miR-942	6	6.13
miR-92a	9	13.98	miR-26b	8	9.09	miR-132	6	5.33
miR-19b	9	15.43	miR-378	8	8.65	miR-103-2*	6	11.36
miR-140-3p	9	14.95	miR-148a	8	9.49	miR-186	6	14.84
miR-324-3p	9	14.69	miR-320b	8	9.68	miR-30c	6	3.91
miR-590-5p	9	10.57	miR-30e	8	10.30	miR-152	6	5.33
miR-15a	9	15.28	miR-130b	8	7.48	miR-182	6	4.40
miR-19a	9	13.95	miR-502-3p	8	12.08	miR-338-3p	5	3.52
miR-660	9	16.63	miR-501-5p	8	9.91	miR-27a	5	2.75
miR-25	9	21.45	miR-454	7	7.50	miR-146a	5	2.97
miR-16	9	22.16	miR-196b	7	5.34	miR-221	5	3.31
miR-16-2*	9	15.68	miR-532-5p	7	10.45	miR-223*	5	3.11
miR-32	9	26.37	miR-215	7	13.16	miR-151-5p	5	4.79
miR-363	9	22.38	let-7d	7	4.02	miR-29a	5	3.06
miR-101	9	16.15	let-7d*	7	5.99	miR-24	5	3.51
miR-192	9	9.03	miR-191	7	5.69	miR-574-3p	5	3.20
miR-210	9	18.17	miR-107	7	7.53	miR-223	5	3.14
miR-500a	9	19.11	miR-301a	7	6.66	miR-1979	5	3.35
miR-194	8	8.86	miR-130a	7	13.46	miR-126	5	4.13
miR-423-5p	8	9.29	miR-584	7	6.61	miR-143	5	3.02
miR-106a	8	11.60	let-7g	7	8.56	miR-26a	5	3.43
miR-93	8	13.85	miR-345	7	5.37	miR-195	5	3.14
miR-20a	8	15.29	miR-7	7	12.53	miR-1974	5	3.06
miR-185	8	16.99	miR-181a	7	4.83	miR-501-3p	5	3.31
miR-425	8	12.87	miR-374a	7	5.41	miR-30e*	5	5.39
miR-29b	8	17.50	miR-421	7	8.25	let-7a	5	3.75
miR-106b	8	13.57	miR-331-3p	7	6.89	miR-146b-5p	5	3.63
miR-222	8	5.87	miR-324-5p	7	8.63	miR-27b	4	2.43
miR-598	8	6.22	miR-532-3p	7	5.90	miR-382	4	2.65
miR-320a	8	11.15	miR-30d	7	11.50	miR-33a	4	3.25
miR-652	8	11.32	miR-628-3p	7	6.63	miR-23a	4	2.47
miR-18a	8	9.94	miR-629	7	19.62	miR-505	4	2.52
miR-17	8	11.29	miR-133b	6	3.94	let-7b*	4	2.38

miRNA	# of cases	Median Fold-change	miRNA	# of cases	Median Fold-change	miRNA	# of cases	Median Fold-change
let-7b	8	11.99	miR-150	6	3.66	miR-28-3p	4	2.23
miR-424	8	9.65	miR-126*	6	3.49	miR-376a	4	3.69
miR-93*	8	8.64	let-7c	6	4.01	miR-142-3p	4	1.89
miR-18b	8	13.90	miR-103	6	5.63	miR-199a-5p	4	2.86
miR-29c	8	11.27	miR-142-5p	6	4.47	miR-582-5p	4	3.19
miR-148b	8	11.91	miR-144*	6	4.39	miR-339-3p	4	3.01
miR-22	8	14.27	miR-2110	6	5.79	let-7f	4	1.90
let-7i	8	13.58	miR-484	6	14.05	miR-18a*	4	11.20
miR-486-5p	8	17.85	miR-30b	6	3.44	miR-550a*	4	10.87
miR-328	8	4.52	miR-374b	6	5.65	miR-92b	4	10.62
miR-15b	8	6.64	miR-199a-3p	6	3.97	miR-486-3p	4	33.51
miR-425*	8	4.92	miR-190	6	3.97			

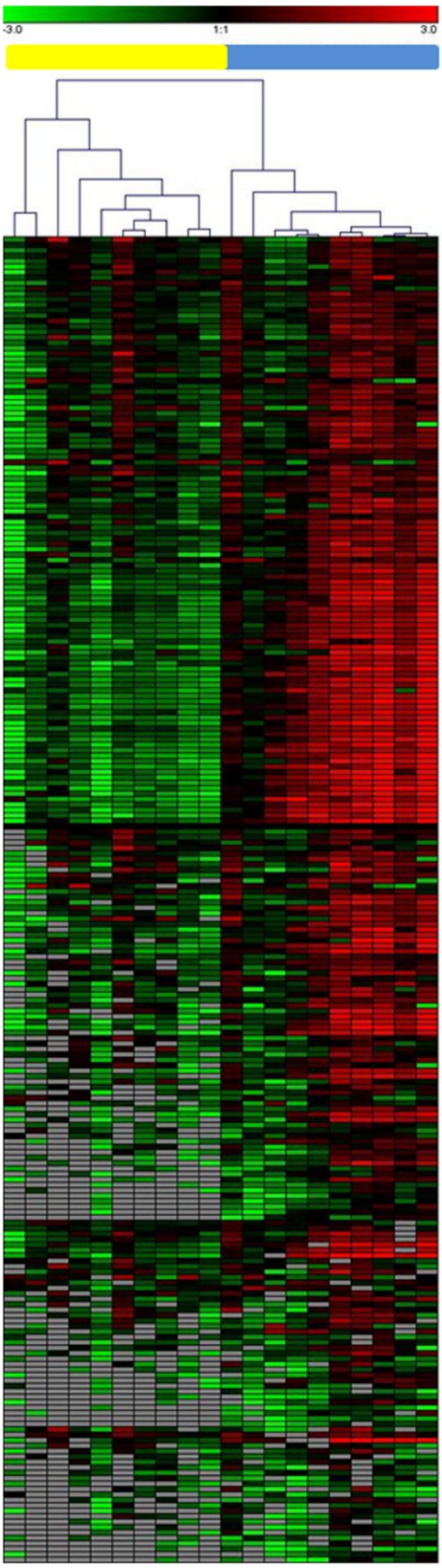


Figure 5. An average-linkage Pearson correlation cluster analysis of the serum miRNA profiles of matched unlysed (yellow) and lysed (blue) samples collected from 10 healthy individuals. Only miRNAs detected in at least 80% of lysed samples or 80% of unlysed samples are included. The analysis was performed on mean centered, normalized data.

4.2.2 Endogenous controls for serum miRNA studies

Currently, there are no standard endogenous controls for serum miRNA studies[143]. The varying levels of miRNAs observed in samples with high hemoglobin concentrations suggest that some current methods of normalization may be unreliable when samples exhibit hemolysis. Our data suggest that normalizing to liver specific miR-122 may be appropriate for matched samples as it is not affected by hemolysis and shows little variation in samples collected from the same person within a short period of time. However, because this miRNA is inconsistently expressed from person to person and has been shown to be deregulated under certain pathologies[144, 145] it is not a suitable endogenous control for inter-individual comparisons.

To identify a suitable endogenous control for serum miRNA studies, we examined the profiles of 29 serum samples from healthy individuals, 7 of which had a hemoglobin concentration above 1.5 g/L. Synthetic miR-cel-39-3p was added to all samples prior to extraction and the levels were measured along with all other RNAs contained on the Exiqon platform. The raw Ct values of all 742 miRNAs as well as U6 and the global mean[110] were converted to a linear scale and compared to the linear raw values of the synthetic miRNA. After including only miRNAs present at a detectable level in all samples along with U6 and the global mean, the levels of miR-99a showed the highest correlation (highest R-value) with miR-cel-39-3p levels. When all samples were normalized to cel-miR-39-3p, miR-99a also had the lowest standard deviation across all samples compared to the other potential controls. When hemolysed samples were removed, the correlation and standard deviation values for miR-99a remained similar, however when these samples are removed, 9 miRNAs (miR-29b, miR-144, miR-29c, miR-590-5p, miR-29a, miR-103-2*, miR-27a, miR-32, and miR-101) had a higher R-value than miR-99a. When compared to three commonly used endogenous controls (miR-16, U6, and the

global mean) miR-99a has a higher correlation to cel-miR-39 and a lower standard deviation when normalized to cel-miR-39-3p than all 3 controls, both with and without hemolysed samples (Table 8).

Table 8. The correlation of potential endogenous controls to spiked-in synthetic miR-cel-39-3p levels and the standard deviation of the controls when normalized to cel-miR-39-3p with and without samples exhibiting high hemoglobin concentrations.

Endogenous control	**Correlation to cel-miR-39-3p (R)**	**Standard Deviation when Normalized to cel-miR-39-3p**
miR-99a	0.66	0.74
U6[a]	-0.10	1.21
Global Mean	0.33	1.37
miR-16	0.30	2.10
Excluding samples with hemolysis:		
Endogenous control	**Correlation to cel-miR-39-3p (R)**	**Standard Deviation when Normalized to cel-miR-39-3p**
miR-99a	0.65	0.71
U6[b]	-0.10	1.26
Global Mean	0.47	0.78
miR-16	0.44	0.95

[a]U6 was detected in 17 of the 29 samples
[b]U6 was detected in 13 of the 22 samples

4.2.3 The effects of fasting on serum miRNA profiles of healthy individuals

It has been suggested that fasting status may alter serum miRNA profiles due to lipids interfering with the RNA extraction[139] or to miRNAs from food entering circulation[146]. However, to our knowledge, this effect has not yet been tested. To assess the effects of fasting on serum miRNA profiles we collected blood samples from 7 healthy volunteers one hour after eating a fatty meal and again three weeks later, after fasting overnight. Serum triglyceride levels were

measured enzymatically and were higher in the non-fasting samples (Table 9). A comparison of the total number of miRNAs detected in each sample showed that, after the exclusion of miRNAs significantly affected by hemolysis, the total miRNA count was slightly higher in the non-fasting samples compared to the fasting samples although not significant ($P = 0.27$, Figure 6). Pearson correlation coefficients between serum triglyceride concentration and miRNA levels showed no miRNAs were significantly correlated with serum triglyceride levels. A fold-change analysis between matched samples normalized to miR-122 showed that no miRNAs were significantly (by at least 3-fold in at least 50% of cases) differentially expressed between the fasting and non-fasting samples. No miRNAs were differentially detected in at least 4 of the 7 fasting or non-fasting samples and not detected in the corresponding matched sample. A Mann-Whitney U test of miRNAs normalized to miR-99a and expressed in at least 75% of fasting or 75% of non-fasting samples showed that no miRNAs were significantly ($P < 0.05$) differentially expressed between the two groups (data not shown).

Table 9. Triglyceride concentration of fasting and non-fasting serum samples.

Sample	Triglyceride concentration (mg/dl) Non-Fasting	Triglyceride concentration (mg/dl) Fasting
1	202.8	120.6
2	152.1	96.2
3	148.5	52.1
4	93.3	30.4
5	176.4	102.4
6	72.4	57.8
7	53.5	45.7

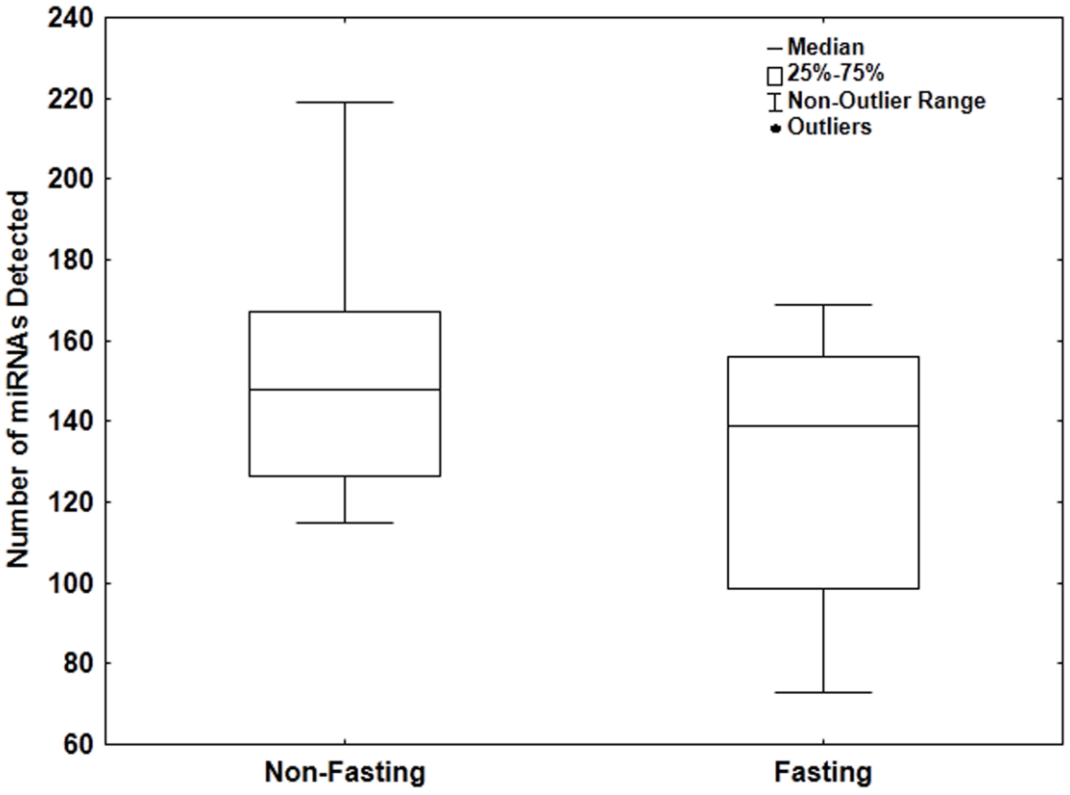


Figure 6. The total number of miRNAs (out of 742 profiled) present at detectable levels in fasting and non-fasting samples. *P*-value = 0.27, Mann-Whitney U test.

4.2.4 The effects of smoking on serum miRNA profiles

To examine the effects of tobacco smoke on serum miRNA expression we compared the profiles of 10 current smokers to 10 age and sex-matched controls (Table 10). A Mann-Whitney U test conducted on miRNAs normalized to miR-99a and detected in at least 80% of smokers or 80% of non-smokers showed that no miRNAs were significantly (corrected *P*-value < 0.05) differentially expressed. However, miR-128 showed the lowest uncorrected *P*-value (0.004) and is the only miRNA with an average fold-change greater than 3. This miRNA was detected in 7 non-smoker cases and 9 smoker cases and was slightly up-regulated in smokers (Figure 7). No miRNAs were present in only smoking samples or only non-smoking samples.

Table 10. Patient demographics for smoking/non-smoking samples.

	Current-Smokers	Non-Smokers
Males	3	3
Females	7	7
Median age	60.5	52
Age Range	52-67	40-64
Median Pack Year	46.3	0

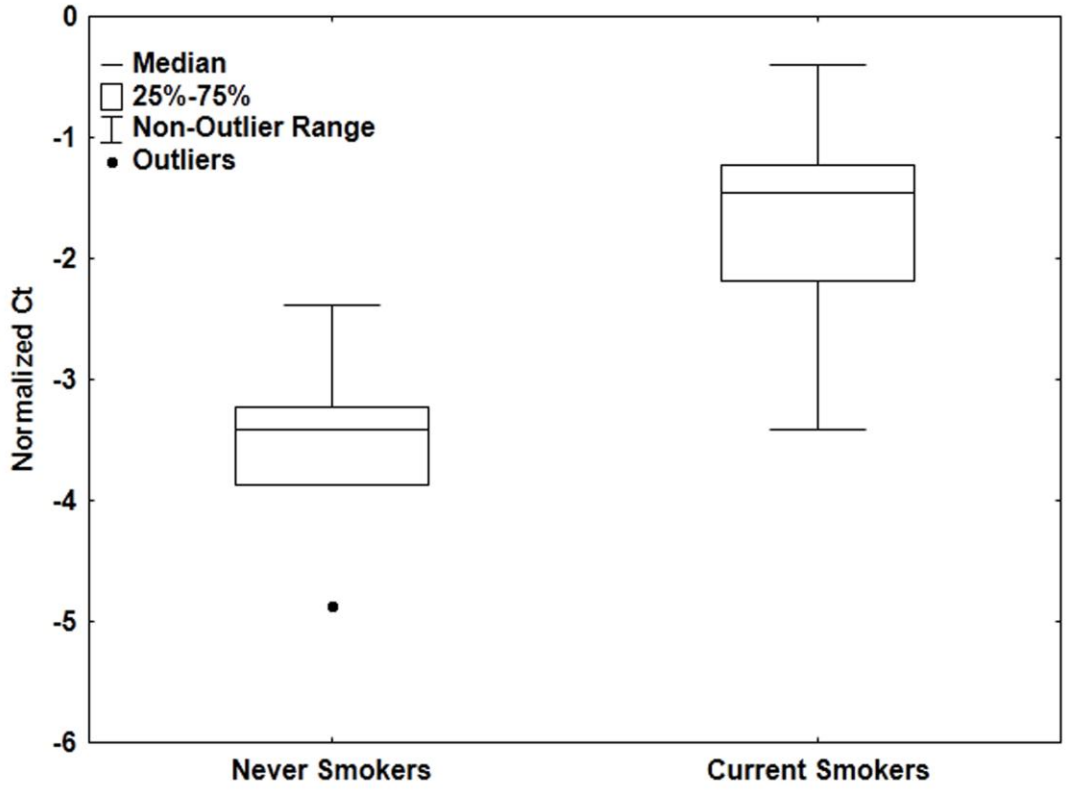


Figure 7. Levels of miR-128 in the serum of non-smokers and current smokers. Uncorrected *P*-value = 0.004, Corrected *P*-value = 0.78, Mann-Whitney U test.

4.2.5 Changes in serum miRNA levels over time

To assess changes in serum miRNA levels in healthy individuals over time we compared the profiles of two samples collected from the same individual over varying time periods. Pearson correlation coefficients of the levels of miRNAs detected in at least 19 of 24 samples showed a strong correlation between samples collected over different time periods from the same person (Table 11). A fold-change analysis showed no miRNAs were frequently significantly deregulated between matched samples (up or down-regulated by at least 3-fold in at least 50% of cases). Nine miRNAs (miR-888, miR-454, miR-10a, miR-181c, miR-1909, miR-20a*, miR-484, miR-501-5p, and miR-622) were detected in at least 50% of cases and not detected in the corresponding matched control. Of these nine miRNAs, three (miR-454, miR-484, and miR-501-5p) are also in the list of 130 miRNAs significantly affected by hemolysis and therefore the difference in miRNA levels could be due to differences in the amount of hemolysis in each sample.

Table 11. Pearson correlation coefficients for miRNA profiles of serum collected at two different time points from healthy individuals.

Sample	Age at first blood draw	Sex	Correlation Coefficient	Time Between Sample Collection
1	60	M	0.89	13 months 7 days
2	63	F	0.92	15 Months 7 days
3	54	F	0.92	17 months 3 days
4	75	M	0.93	11 months 9 days
5	67	M	0.94	7 months 10 days
6	55	M	0.94	5 Months 3 days
7	64	M	0.94	13 months
8	63	M	0.95	12 months 19 days
9	69	M	0.95	2 months 14 days
10	58	M	0.96	6 months 7 days
11	60	M	0.96	10 months
12	53	F	0.96	11 months 22 days

4.3 Discussion

An ideal biomarker can be measured non-invasively and is highly sensitive and specific to the disease state of interest. In order for a biomarker to have a high positive predictive value it should not be significantly influenced by technical variables or pathologic conditions unrelated to the disease being investigated. In this study we examined the effects of a range of technical and individual factors on serum miRNA levels. Our results have identified a list of miRNAs that are significantly affected by these factors and are therefore likely to have very limited utility as biomarkers of disease.

We have contributed to previous studies examining the role of blood cell contaminants in serum miRNA quantification[126, 137, 138]. While miRNA profiles of blood cells and blood microvesicles have been previously identified[137], to our knowledge, this is the first study

specifically examining the effect of mechanical hemolysis on the quantification of 742 miRNAs in the serum of healthy individuals. Other studies examining the effects of hemolysis on circulating miRNA profiles have done so by profiling blood cells directly or by adding hemolysate back into already separated serum or plasma samples[126]. However, Dimeski (2004) demonstrated that samples prepared in this way can have a different effect on analytes than samples prepared by mimicking mechanical hemolysis[119]. By mimicking hemolysis caused by improper blood collection or preparation we have identified a list of 63 miRNAs that are most likely to be affected by hemolysis in a clinical setting. Currently, researchers examining the role of hemolysis on plasma and serum miRNA profiles have suggested excluding hemolysed samples from studies[147]. This method may be useful in identifying biomarkers because it allows the measurement of all miRNAs while avoiding the effects of hemolysis. However, from a clinical standpoint, this method is impractical because the most common technique for identifying hemolysed samples in the clinic is by visual inspection[148] and our results as well as other studies[147] have shown that minor (not visually apparent) levels of hemolysis can significantly alter miRNA levels. Additionally, for samples with obvious hemolysis, asking patients to return for multiple blood draws requires further staff time and supplies and can delay diagnosis leading to increased stress on the patient[149]. Therefore, we propose that rather than excluding hemolysed samples from biomarker studies, researchers should eliminate miRNAs significantly affected by hemolysis when identifying potential biomarkers.

Several groups have addressed the importance of assessing analytical parameters for serum miRNA studies especially with regards to comparing results from multiple groups[101, 126]. In this study we've demonstrated a method for normalization that excludes miRNAs affected by hemolysis and is based on the levels of a synthetic spike-in. In cases with samples that may be

compromised by hemolysis, this method is more appropriate than some commonly used normalization methods. For example, normalizing to the geometric mean of miRNAs expressed in all samples[110] can produce skewed results if a sample is contaminated with miRNAs from blood cells. This occurs because more highly expressed serum miRNAs, which are used for global mean normalization, are also expressed in blood cells and will be present at higher levels in hemolysed samples[138]. Therefore, samples exhibiting hemolysis will appear to have high RNA input due to an overall lower normalization value and miRNAs that are not expressed in blood cells will seem to be down-regulated in that sample. Another commonly used method for normalization is the use of miR-16 as an endogenous control[79]. However, we have demonstrated that the levels of serum miR-16 are significantly increased in samples with high hemoglobin concentrations suggesting that this method is also inappropriate in cases where some samples may be contaminated with blood cell miRNAs. Finally, another commonly used method for miRNA normalization is the use of U6 as an endogenous control. U6 is a nuclear RNA and has been used to normalize tissue and cell miRNA expression[150]. However, because U6 is localized in the nucleus it should not be present at consistent, high levels in the non-cellular components of blood[143]. Indeed, of the 60 samples analyzed in this study U6 is only present at a detectable level in 31 samples suggesting U6 is not a suitable endogenous control for serum miRNA studies. We propose that miR-99a should be used for sample normalization instead of these commonly used endogenous controls due to its correlation with synthetic spike-in levels, its low standard deviation in spike-in normalized samples, and because it is unaffected by hemolysis (Table 7).

A recent report has shown that plant miRNAs are present in the sera of humans and that these miRNAs are acquired through food intake[146]. This finding suggests that miRNAs acquired through food intake would show variable expression over time and could be affected by fasting

status. Furthermore, fasting status alters the amount of lipids in the blood which could interfere with RNA extraction leading to variable miRNA levels[139]. Here we show that there is no significant difference in the total number of miRNAs detected in fasting versus non-fasting samples demonstrating that the presence of lipids in the blood does not lead to a loss of less abundant miRNAs during extraction. Furthermore, no miRNAs examined in our assay were significantly differentially expressed (P-value < 0.05) between fasting and non-fasting samples indicating that fasting status will not interfere with serum miRNA biomarker discovery in subjects with normal miRNA physiology. However, our study only examines short term dietary effects on circulating miRNAs and experiments with long-term, controlled diets need to be carried out in order to test the overall effects of diet on serum miRNA profiles[151]. Additionally, because lipoproteins have been shown to carry miRNAs in circulation[66] the correlation between high density and low density lipoproteins and serum miRNAs should also be examined in future studies.

As smoking plays an important role in the development of lung, oral, and other cancers and must be considered when identifying potential biomarkers for these diseases, we have also examined the influence of smoking on serum miRNA profiles. Because no miRNAs were significantly differentially expressed between smokers and non-smokers after correction for multiple testing, our results suggest that smoking status will not interfere with serum miRNA biomarker studies. However, a recent study using 6-month cigarette smoking in mice found that the levels of miR-128 were significantly changed in the lung tissue and plasma of exposed mice suggesting a role for this miRNA in the cellular response to cigarette smoke exposure[152]. We have found that although miR-128 was not significantly (P-value < 0.05) deregulated in the serum of smokers, this miRNA was detected in more current smoker samples than non-smoker

samples and was the only miRNA with an average fold-change over 3-fold higher in smoking samples compared to non-smoking samples. Therefore, further studies are required to determine if miR-128 is significantly affected by smoking status. Roth et al determined that miR-625* is down-regulated in the serum of NSCLC patients who smoke compared to those who do not smoke[97]. We did not see a significant difference in miR-625* between healthy smokers and non-smokers, however this miRNA was only detected in 7 of 10 non-smoking samples and only 5 of 10 smoking samples. Therefore, using more sensitive profiling techniques or a larger sample set, miR-625* could be found to be significantly down-regulated in the serum of healthy smokers. Taking the results of both studies into account, the role of smoke exposure should be considered when determining the possible utility of miR-128 and miR-625* as biomarkers of disease.

When identifying biomarkers for disease detection and/or monitoring, an important aspect to consider is the possibility of changes in biomarker levels over time in the healthy population. In order to evaluate serum miRNA variability in healthy individuals, we measured the levels of miRNAs in paired serum samples taken from 12 individuals over varying time periods. Pearson correlation coefficients of the matched samples show that the samples with the 3 lowest R-values are samples with the longest time between draws indicating that serum miRNA levels may show a slight change over longer time periods. However, the other 9 samples show no pattern between correlation coefficients and time between sample collections. This lack of a pattern and the fact that the matched samples with the lowest R-value (0.89) are still highly correlated suggest that, up to 17 months, overall serum miRNA levels show little variability in healthy individuals. Further, long-term studies are required to determine changes in serum miRNAs over several years. A fold-change analysis showed no individual miRNA was consistently deregulated in these matched samples implying that variability in serum miRNAs

over time should not interfere with biomarker studies examining abundant serum miRNAs. Among the less abundant miRNAs, 9 were differentially detected in the matched samples. This result demonstrates that these less abundant miRNAs are not suitable biomarkers due to their inconsistent expression.

Although this study has limitations such as small sample sizes, the results presented here identify factors that should be taken into consideration when selecting endogenous controls and biomarker candidates. In order for serum miRNA biomarkers to eventually be implemented in a clinical setting, pre-analytical and analytical variables affecting serum miRNA profiles should be examined and standardized. In addition to the variables described in this study there are other areas requiring further examination. Studies have shown that pregnancy can influence serum miRNA levels[153] suggesting that hormonal activity may also interfere with serum miRNA biomarker identification. Other factors requiring further investigation include the affect of benign conditions such as inflammation

and diurnal variation on serum miRNA profiles of healthy individuals as well as analytical factors such as the best platform and statistical tests to use for the detection and determination of differentially expressed miRNAs. Once these variables are well defined, the complete potential of circulating miRNAs as biomarkers can be fully explored.

5. Identification of differentially expressed miRNAs in the serum of patients with early lung adenocarcinoma

5.1 Introduction

A number of studies have been published examining the differential expression of miRNAs in the serum of patients with NSCLC[62, 88, 89]. However, the candidate miRNAs identified in these studies vary considerably. Some of this variation may be attributed to the analytical and pre-analytical factors described in chapter 4 while other possible sources of variation such as hormonal activity and long term differences in diets have yet to be examined. To avoid these inter-patient variables, we used a novel approach to identify tumour-associated serum miRNAs and examined their utility as non-invasive biomarkers by quantifying their levels in patients with lung adenocarcinoma and a group of non-cancer controls.

5.2 Results

5.2.1 Identification of differentially expressed miRNAs in patient-matched samples

To identify an initial list of tumor-associated miRNAs we compared the miRNA profiles of serum collected during surgery from a) pulmonary venous effluent draining the tumor vascular bed and b) systemic arterial blood from the same patient. A total of 63 miRNAs were detected in at least 50% of tumor associated samples and not detected in the matched systemic case and 3 miRNAs were detected in 50% of systemic samples and not detected in the matched tumor associated sample (Table 12). A fold-change analysis identified miR-375 as up-regulated

by at least 3-fold in all tumor associated (pulmonary venous) samples compared to systemic blood samples. In total, 64 miRNAs were up-regulated and no miRNAs were down-regulated by at least 3-fold in at least 50% of samples (Table 12). In chapter 4 we identified a list of miRNAs significantly affected by hemolysis. To identify a final list of candidate miRNA biomarkers, we removed miRNAs significantly (by 3-fold in at least 50% of samples) affected by hemolysis. After removal of miRNAs significantly affected by hemolysis, 35 miRNAs were up-regulated by at least 3-fold in at least 50% of samples (Table 12). In addition to fold-change values, the overall numbers of miRNAs detected were higher in tumor associated samples compared to systemic arterial samples both before and after the removal of hemolysis associated miRNAs with the exception of sample 95 (Figure 8).

Table 12. Results of a fold-change analysis of pulmonary venous and systemic arterial lung cancer samples.

microRNA	# of samples up-regulated	Median Fold-change	Detected in ≥ 50% of tumor associated samples	# of cases	Detected in ≥ 50% of systemic samples	# of cases
hsa-miR-375	10	13.23	hsa-miR-138	9	hsa-miR-604	5
hsa-miR-141	9	26.75	hsa-miR-216a	9	hsa-miR-20b*	5
hsa-miR-200c	9	22.46	hsa-miR-29c*	9	hsa-miR-509-3p	5
hsa-miR-125b	9	19.85	hsa-miR-100	8		
hsa-miR-34a	9	12.96	hsa-miR-187	8		
hsa-miR-497	9	8.43	hsa-miR-195*	8		
hsa-miR-145	9	7.65	hsa-miR-642	8		
hsa-miR-200b	8	14.29	hsa-miR-195	7		
hsa-miR-30a*	8	7.32	hsa-miR-203	7		
hsa-miR-10a	8	6.86	hsa-miR-30d*	7		
ǂhsa-miR-146b-5p	8	4.86	hsa-miR-424*	7		
ǂhsa-miR-133b	8	4.18	hsa-miR-663	7		
ǂhsa-miR-143	7	10.02	hsa-miR-99b*	7		
hsa-miR-204	7	6.47	hsa-miR-1201	6		
ǂhsa-miR-29b	7	4.75	hsa-miR-125a-3p	6		
hsa-miR-99a	7	4.6	hsa-miR-125b-2*	6		
ǂhsa-miR-532-3p	7	3.98	hsa-miR-1260	6		
ǂhsa-miR-29a	6	5.17	hsa-miR-135a	6		
hsa-miR-95	6	4.89	hsa-miR-148a*	6		
ǂhsa-miR-29c	6	4.88	hsa-miR-200a	6		
hsa-miR-133a	6	4.77	hsa-miR-200b*	6		
hsa-miR-199b-5p	6	4.34	hsa-miR-211	6		
hsa-miR-193b	6	4.18	hsa-miR-214	6		
ǂhsa-miR-30e*	6	3.56	hsa-miR-217	6		
hsa-let-7f-2*	6	3.45	hsa-miR-23a*	6		
hsa-miR-1	6	3.41	hsa-miR-23b*	6		
ǂhsa-miR-423-3p	6	3.39	hsa-miR-362-3p	6		
ǂhsa-miR-222	6	3.23	hsa-miR-381	6		
hsa-miR-99a*	5	6.79	hsa-miR-429	6		
hsa-miR-218	5	6.13	hsa-miR-449a	6		

microRNA	# of samples up-regulated	Median Fold-change	Detected in ≥ 50% of tumor associated samples	# of cases
hsa-miR-30a	5	5.33	hsa-miR-511	6
hsa-miR-361-3p	5	4.68	hsa-miR-548b-3p	6
ǂhsa-miR-338-3p	5	4.62	hsa-miR-708	6
hsa-miR-18a*	5	4.31	hsa-miR-92a-1*	6
hsa-miR-181b	5	4.1	hsa-miR-101*	5
hsa-miR-1972	5	3.85	hsa-miR-124	5
hsa-miR-551a	5	3.84	hsa-miR-1271	5
ǂhsa-miR-660	5	3.65	hsa-miR-135b	5
hsa-miR-99b	5	3.5	hsa-miR-1468	5
hsa-miR-337-3p	5	3.33	hsa-miR-146b-3p	5
hsa-miR-28-3p	5	3.32	hsa-miR-149*	5
ǂhsa-miR-629	5	3.31	hsa-miR-181a*	5
hsa-miR-769-5p	5	3.27	hsa-miR-183	5
ǂhsa-miR-574-3p	5	3.22	hsa-miR-185*	5
hsa-miR-365	5	3.21	hsa-miR-193a-3p	5
ǂhsa-miR-328	5	3.18	hsa-miR-196b*	5
hsa-miR-342-3p	5	3.17	hsa-miR-26a-1*	5
ǂhsa-miR-532-5p	5	3.16	hsa-miR-299-5p	5
ǂhsa-miR-7	5	3.15	hsa-miR-331-5p	5
ǂhsa-miR-152	5	3.13	hsa-miR-33a*	5
ǂhsa-miR-940	5	3.1	hsa-miR-342-5p	5
ǂhsa-miR-132	5	3.09	hsa-miR-34b*	5
ǂhsa-miR-502-3p	5	3.03	hsa-miR-362-5p	5
ǂhsa-miR-140-3p	5	2.98	hsa-miR-489	5
ǂhsa-miR-501-3p	5	2.88	hsa-miR-519a	5
ǂhsa-miR-324-3p	5	2.86	hsa-miR-520c-3p	5
ǂhsa-miR-148a	5	2.77	hsa-miR-579	5
ǂhsa-miR-21	5	2.72	hsa-miR-589*	5
hsa-miR-17*	5	2.71	hsa-miR-629*	5
hsa-miR-326	5	2.66	hsa-miR-663b	5
ǂhsa-miR-486-5p	5	2.63	hsa-miR-675b	5
ǂhsa-miR-320b	5	2.56	hsa-miR-760	5
hsa-miR-22*	5	2.53	hsa-miR-98	5
ǂhsa-miR-146a	5	2.19		

ǂ Significantly affected by hemolysis

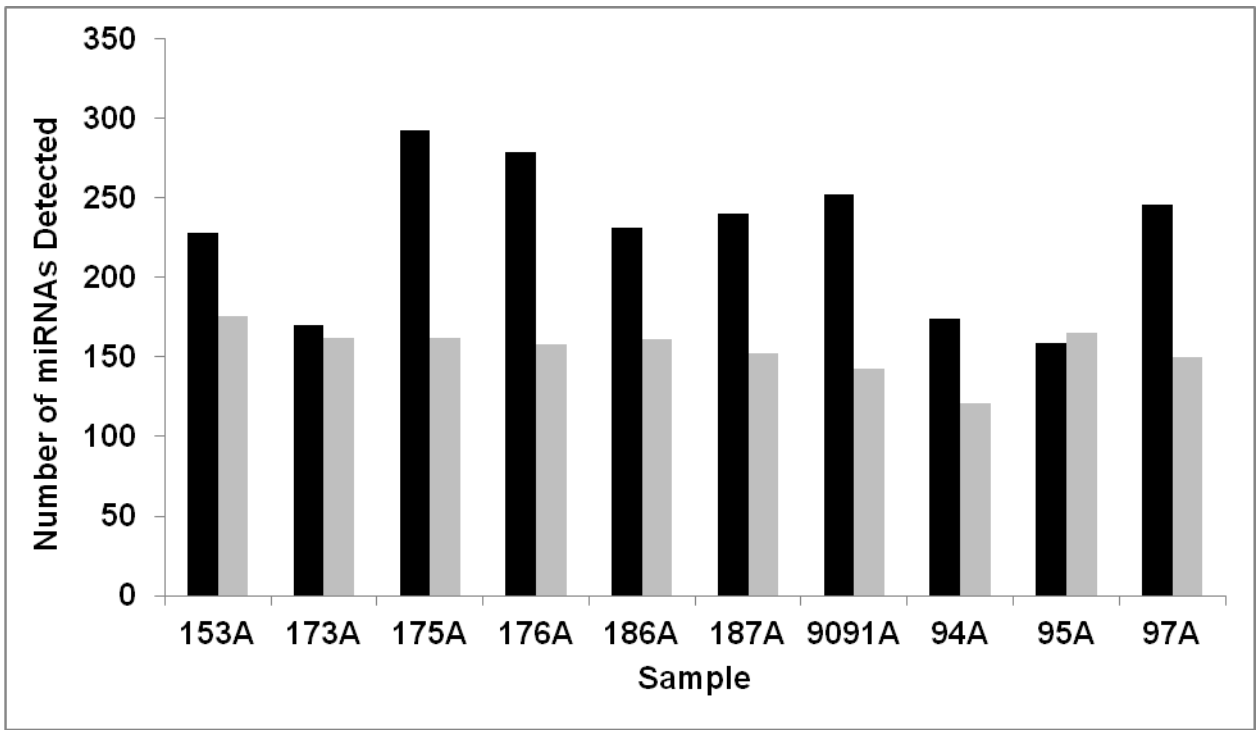


Figure 8. The number of miRNAs detected in pulmonary venous (black) and systemic arterial (light grey) blood samples after removal of miRNAs significantly affected by hemolysis. *P* = 0.001, Mann-Whitney U test.

5.2.2 Comparison of miRNA profiles from patients with lung adenocarcinoma and non-cancer controls

To examine the utility of differentially expressed miRNAs as non-invasive biomarkers we compared the serum miRNA profiles of 26 patients with lung adenocarcinoma a group of demographically matched non-cancer controls (Table 3). In total, 81 miRNAs were detected in all 26 cancer samples and 94 miRNAs were detected in all control samples. An uncentered Pearson correlation cluster showed no pattern in the clustering of cancer and control samples suggesting the presence of a tumour does not affect the overall serum miRNA profiles of patients (Figure 9). A Mann-Whitney U test conducted on miRNAs normalized to miR-99a and detected in at least 80% of adenocarcinoma samples or 80% of controls showed that 22 miRNAs were

significantly (*P*-value < 0.05) differentially expressed. However, after correction for multiple testing all *P*-values were above 0.1 (Table 13). Interestingly, of the miRNAs with significant uncorrected *P*-values, only 3 were up-regulated and 19 were down-regulated in cancer samples compared to controls (Table 13). Additionally, 12 of these 22 miRNAs are listed in chapter 4 as significantly affected by hemolysis.

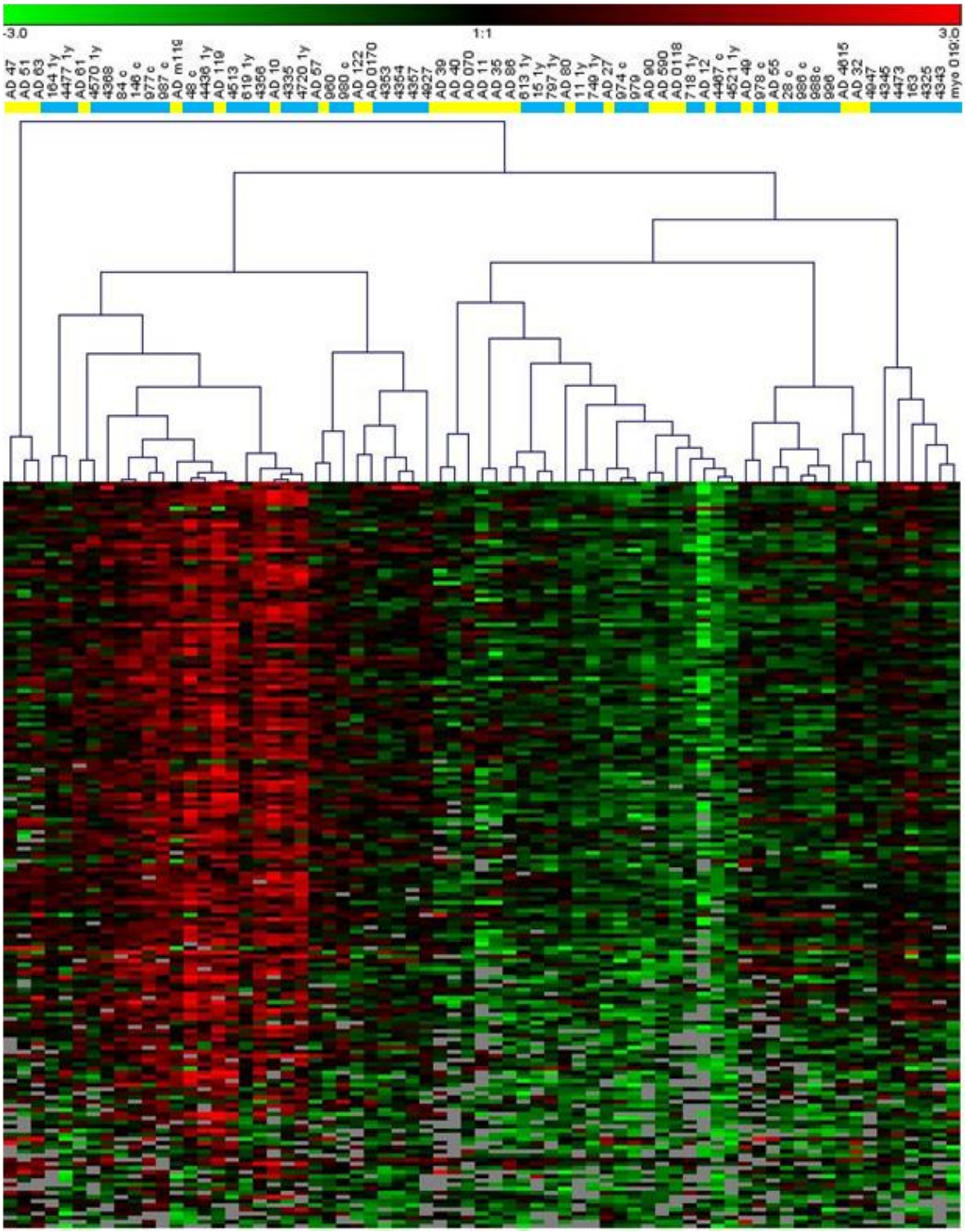


Figure 9. An average-linkage Pearson correlation cluster analysis of the serum miRNA profiles of lung adenocarcinoma patients (yellow) and non-cancer controls (blue). Only miRNAs detected in at least 80% of cancer or 80% of control samples are included. The analysis was performed on mean centered, normalized data.

Table 13. miRNAs significantly (uncorrected *P*-value < 0.05) up or down-regulated in lung adenocarcinoma serum samples compared to controls. Mann-Whitney U test, *P*-value corrected using Benjamini-Hochberg correction.

miRNA	*P*-value	Corrected *P*-value
Down-regulated		
hsa-miR-199a-5p	0.002	0.321
ǂhsa-miR-191	0.002	0.189
hsa-miR-28-5p	0.004	0.260
ǂhsa-miR-221	0.005	0.228
hsa-miR-139-5p	0.006	0.191
ǂhsa-miR-199a-3p	0.007	0.174
hsa-miR-99b	0.008	0.179
ǂhsa-miR-151-5p	0.011	0.216
hsa-miR-766	0.016	0.259
hsa-miR-142-3p	0.017	0.250
ǂhsa-miR-26a	0.021	0.266
hsa-let-7e	0.032	0.389
ǂhsa-miR-151-3p	0.033	0.369
ǂhsa-miR-30e*	0.033	0.357
ǂhsa-miR-146a	0.034	0.344
ǂhsa-miR-142-5p	0.035	0.331
hsa-miR-1	0.037	0.334
ǂhsa-miR-152	0.042	0.366
ǂhsa-miR-22*	0.048	0.393
Up-regulated		
hsa-miR-1266	0.005	0.184
hsa-miR-532-5p	0.015	0.271
hsa-miR-29b-1*	0.018	0.252

ǂ Significantly affected by hemolysis

5.2.3 Overlap between surgical and peripheral venous sample sets

Comparison of miRNAs significantly differentially expressed in surgical samples and significantly differentially expressed prior to correction in peripheral venous samples identified one miRNA (miR-532-5p) that was up-regulated in both sample sets (Figure 10). The most significantly up-regulated miRNAs (miRNAs with the highest median fold-change) in the

surgical samples were miR-375, miR-141, miR-200c, miR-200b, and miR-125b none of which are significantly affected by hemolysis (Table 12). In the set of peripheral venous samples the miR-200 family of miRNAs were present at very low levels or not detected in the majority of samples with miR-200c, miR-141, and miR-200b only detected in 50, 53, and 26 of the 69 samples respectively. While miR-125b and miR-375 were present at higher levels in these samples and were detected in 69 and 68 samples respectively. Of the samples in which these 5 miRNAs were detected, the average normalised Ct was slightly higher in cancer compared to control cases although not significant (Table 14).

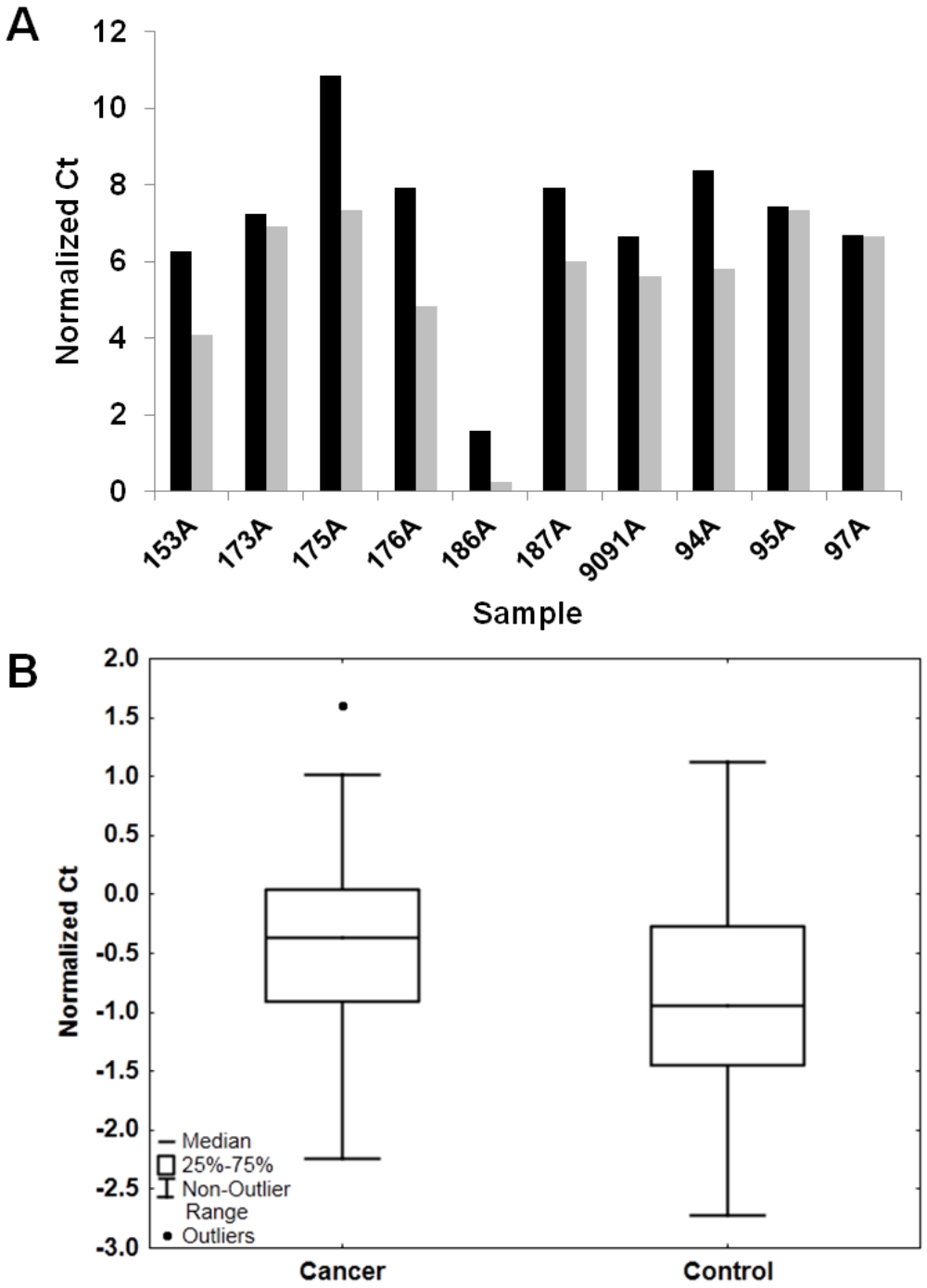


Figure 10. (A) miR-532 is up-regulated in pulmonary venous (black) samples compared to matched systemic arterial (light grey) samples. (B) miR-532-5p is also slightly up-regulated in lung adenocarcinoma peripheral venous samples compared to controls. Corrected P = 0.27, Mann-Whitney.

Table 14. Average normalized Cts from peripheral venous samples of the miRNAs most significantly up-regulated in surgical samples.

miRNA	Average Normalized Ct Cancer (Standard Deviation)	Average Normalized Ct Control (Standard Deviation)
hsa-miR-125b	0.73 (0.54)	0.54 (0.44)
hsa-miR-375	-1.29 (1.29)	-1.47 (1.17)
hsa-miR-141	-2.71 (1.24)	-2.91 (0.84)
hsa-miR-200c	-2.87 (1.17)	-3.22 (0.75)
hsa-miR-200b	-3.49 (0.75)	-3.61 (0.92)

5.3 Discussion

A number of studies have been published identifying serum miRNAs that can distinguish NSCLC patients from healthy controls with high sensitivity and specificity (Table 1). However, there is little overlap in the candidate miRNA biomarkers identified in each study. Here, we have used a novel approach to identify tumor-associated miRNAs while also eliminating differential miRNA expression caused by inter-patient variables by comparing two samples collected from the same patient during surgery. In these surgical samples, 35 miRNAs were significantly up-regulated and no miRNAs were significantly down-regulated in tumor associated samples. These results are consistent with studies examining miRNA profiles of NSCLC tissue and serum samples which have found a general up-regulation of miRNAs in tumor compared to matched normal samples[86, 154]. The most significantly up-regulated miRNAs were miR-375, miR-141, miR-200c, miR-200b, and miR-125b (Table 12). Our results support the findings of other studies demonstrating the up-regulation of miR-125b in serum of NSCLC patients[93, 98]. Studies have suggested both tumor suppressive and oncogenic roles for miR-125b[155, 156]. The exact role of this

miRNA in lung cancer is unclear. However, it has been demonstrated that miR-125b induces apoptosis and plays a tumor suppressive role in lung cancer cell lines[157]. miR-375 and miR-200b have been shown by other groups to be up-regulated in tissue and sputum but not plasma samples from patients with NSCLC[158, 159]. MicroRNAs miR-200b, miR-200c, and miR-141 are all members of the miR-200 family (along with miR-200a and mir-429). Interestingly, these three miRNAs were up-regulated in the tumor associated surgical samples compared to the systemic samples and miRs 200a and 429 were detected in 6 tumor associated samples but not detected in matched systemic samples. This family of miRNAs has been shown to inhibit lung adenocarcinoma metastasis[160] and have been shown to be over-expressed by ~11-fold in primary lung tumor cell lines compared to cell lines from metastatic sites[161].

Among the most significantly differentially expressed miRNAs (those with the lowest uncorrected *P*-values) in the cancer/control 19 miRNAs were up-regulated in the control samples compared to the cancer samples. This finding seems contradictory to the suggestion that lung tumors are releasing miRNAs into circulation. However, we have found that 11 of the 19 miRNAs are significantly up-regulated in samples exhibiting hemolysis (Table 13) suggesting that the control samples used in this study contained more blood cell contaminant miRNAs than the cancer samples. Additionally, 2 of the 3 miRNAs up-regulated in the cancer samples were found to be unaffected by hemolysis supporting this hypothesis. Of note, miR-532-5p was up-regulated in cancer samples and was also found to be significantly up-regulated in samples exhibiting hemolysis. If the higher expression of miRNAs in control samples is due to the presence of blood cell miRNAs, this result may be explained by suggesting that the amount of up-regulation in miR-532-5p due to the presence of a tumor is greater than that due to the presence of blood cell contaminants which is supported by the fact that miR-532-5p was also up-

regulated in the surgical samples. Because the most deregulated miRNAs in the surgical samples were shown to be unaffected by hemolysis, it is likely that the amount of hemolysis in these matched samples were relatively equal or that the influence of the tumor on serum miRNA levels is greater than the influence of blood cell miRNAs.

The miRNAs most deregulated in the surgical samples showed a slight but not significant increase in expression in cancer versus control samples. The presence of blood-cell associated miRNAs (and possibly miRNAs from other sources) could be interfering with the disease-specific miRNA profile in these samples. This issue could possibly be solved by examining the miRNA profiles of tumor-derived exosomes isolated from the serum of cancer patients and comparing the results to exosomes isolated from control patients[162]. Ideally, the miRNA profiles of tumor-derived exosomes should be unaffected by blood cell contaminants. This study uses a novel method to identify serum miRNAs that are up-regulated in the circulation of patients with lung adenocarcinoma however; further research is required to assess the utility of these miRNAs as non-invasive biomarkers.

6. Review of aims

Aim 1 of this thesis was to identify candidate circulating miRNA biomarkers for patients with high-risk oral lesions. The results of this study significantly contribute to this field of research as it was the first study to examine global miRNA expression for head and neck malignancy and one of the first attempts to identify miRNA expression in any epithelial precancer. The finding of stage-specific serum miRNAs associated with precancerous lesions provides evidence that circulating miRNA profiles can change with disease progression. These

results suggest utility for such miRNAs not only in the early detection of oral cancer but possibly as markers of progression. Additionally, these results imply that serum miRNA signatures derived from patients with invasive cancer in direct contact with the blood stream may not have the same utility in the detection of early stage cancers which are separated by the basement membrane from direct access to the blood vascular system. The identification of miRNAs that are both de-regulated in cancer versus control serum samples and that correspondingly decreases or increases with surgical resection of the tumor support the utility of serum miRNAs as biomarkers for oral cancer detection and disease monitoring.

In **aim 2** we sought to examine some of the pre-profiling factors that could influence serum miRNA variability as miRNAs significantly affected by these factors are likely to have little utility as biomarkers or endogenous controls for serum miRNA studies. We found that no miRNAs were significantly (P-value < 0.05) differentially expressed in healthy individuals with varying smoking and fasting statuses. However, further studies on are required to confirm these results. We have also found that mechanical hemolysis of blood samples prior to miRNA profiling significantly increases the levels of 130 miRNAs and should be taken into consideration in future circulating miRNA studies. Importantly, we also found that serum miRNA profiles of healthy individuals are stable and show little variation over a range of time periods with the exception of 9 less abundant miRNAs. The results of this study considerably contribute to the field of serum miRNA biomarkers as they highlight the potential and the limitations of serum miRNA biomarkers and call into question some miRNAs that are currently used as endogenous controls and candidate biomarkers in serum miRNA studies.

Finally, in **aim 3** of this thesis we used a novel approach that avoids inter-patient variability by using the patient as his/her own control to attempt to identify candidate serum

miRNAs with utility as lung adenocarcinoma biomarkers. We found 35 miRNAs that were significantly up-regulated in tumor-associated serum samples and 66 miRNAs that were differentially detected in matched tumor-associated and systemic samples. The most highly up-regulated miRNAs in the tumor-associated samples have been implicated in NSCLC suggesting the serum miRNA levels in these samples are reflective of tumor miRNA levels. Unfortunately, the most highly up-regulated miRNAs in the tumor-associated surgical samples were not significantly differentially expressed in the cancer/control peripheral venous samples. These results demonstrate the need for alternative methods to isolate miRNAs that are tumor-specific in order to avoid over-whelming the miRNA signatures of patients and controls with miRNAs unrelated to disease-state such as those from blood cell contaminants.

7. Conclusion

7.1 Future perspectives

Although we have identified potential biomarkers for the detection of oral and lung cancer, further studies need to be conducted before serum miRNA biomarkers can be implemented in the clinic or used for population screening. For oral cancer biomarkers, future experiments can be designed using previous work in the field of non-invasive cancer biomarker discovery as a guide to identify the analytical and pre-analytical hurdles that need to be overcome. For example, the use of prostate-specific antigen for prostate cancer screening has been problematic due to the fact that this molecule is also elevated in patients with benign prostate conditions[7]. Therefore, after potential serum miRNA biomarkers are examined in

healthy individuals they should also be tested and compared in screening subjects with a range of conditions rather than just those with the disease state of interest. In order to avoid this issue and the issue of data over-fitting, the candidate oral cancer miRNA biomarkers outlined in this thesis will have to be tested in larger cohorts. These cohorts should continue to include individuals who may benefit from an oral cancer screening test (ie. high-risk smokers) and include patients with benign conditions. A more recent study examining the levels of 1205 human miRNAs in serum samples from oesophageal SCC patients identified miR-1246 as the most significantly up-regulated miRNA in the serum of cancer patients. Interestingly, the group found that miR-1246 was not differentially expressed in oesophageal SCC tissue samples but was up-regulated in the proximal lymph-nodes of these patients. Additionally, the levels of miR-1266 were higher in isolated exosomes from an oesophageal SCC cell line than in the parent cells suggesting this miRNA is selectively secreted by oesophageal SCC cells and enters the circulatory and lymphatic systems[163]. Using this study as an example, future work on the candidate miRNAs identified here will include functional studies measuring the levels of these miRNAs in exosomes isolated from OSCC cell lines and possibly serum of patients with OSCC. Additionally, because we have shown that some candidate miRNAs are significantly affected by hemolysis, this issue will have to be addressed before moving on to the validation stage of our oral cancer study.

Of the pre-profiling variables examined in this thesis, lysis of blood cells prior to sample processing had the most significant impact on serum miRNA levels. Although an ideal biomarker would be unaffected by such conditions, it is reasonable to consider that one or more of the 130 miRNAs identified in chapter 4 as significantly affected by hemolysis could also exhibit deregulated serum expression due to the presence of a tumor. Therefore, an appealing

alternative to excluding these miRNAs from biomarker studies is to establish a method to adjust the quantification of circulating miRNAs according to the amount of hemolysis in the sample. Our results show a significant linear correlation between serum hemoglobin concentration and miRNA levels. Therefore, it is feasible that a correction factor for the quantification of miRNAs may be created using the hemoglobin concentration of the given sample. As part of our study we attempted to create such a correction factor using the 10 matched pairs of hemolysed/non-hemolysed samples from chapter 4. However the coefficients established were not applicable to other data sets. This lack of applicability may be due to the small sample size of this study but also due to the reliability of hemoglobin concentration measurements. Although hemoglobin can be measured spectrophotometrically in serum samples, interference from lipids and other proteins in the sample can lead to less accurate measurements[120]. Although the spectrophotometer data from the matched lysed and unlysed samples showed little interference from lipids and proteins, the absorbance graphs from the cancer and control samples were irregular demonstrating interference from other compounds. Therefore we were unable to determine a robust correction factor using the available samples. Future studies on blood cell miRNAs using larger sample sizes and more accurate measurements of blood cell lysis, such as a hematology analyzer, may offer a solution to this issue.

Unfortunately, the lung adenocarcinoma-associated miRNAs identified in the unique surgical samples used in our study were not significantly differentially expressed in the peripheral venous samples of patients with lung adenocarcinoma compared to samples from healthy controls. One possible reason for these results is that miRNAs released from the tumor are present at very high levels in pulmonary venous effluent directly draining the tumor vasculature bed but are so diluted in systemic circulation that their signals are overwhelmed by

other miRNAs such as those from the currently unknown source of circulating miRNAs in healthy individuals. Therefore, new methods of miRNA quantification need to be applied in future studies examining the utility of the miRNAs that are up-regulated in the pulmonary venous samples as biomarkers for lung adenocarcinoma. This analytic noise could be filtered out by isolating and comparing the miRNA profiles of exosomes from serum samples of patients with early stage lung adenocarcinoma to exosomes derived from the serum of healthy controls. Furthermore, limiting miRNA profiling to tumor-derived exosomes expressing certain surface proteins could further hone the list of miRNAs to tumor specific candidates. Alternatively direct miRNA profiling in these samples using more sensitive techniques could also result in more stratified miRNA profiles. One technique that we are currently examining is the use of digital PCR (dPCR) which is more sensitive than qRT-PCR and can directly calculate absolute miRNA concentrations. Our collaborator (Roza Bidshahri) was able to quantify serum miRNAs with a limit of detection of 0.2-0.1 copies/μl (data not shown). This method may allow us to measure differences in miRNA levels of candidate miRNAs that were up-regulated in pulmonary venous samples but were less abundant peripheral samples such as the miR-200 family of miRNAs. Other, more sensitive methods such as next generation sequencing could also be explored[102]. The results presented in chapter 5 show a modest deregulation of miRNAs in the serum of patients with lung adenocarcinoma. Therefore, future studies will also include the integration of serum miRNA signatures with other screening techniques including low dose spiral computed tomography, risk modeling (smoking pack-years, COPD status, etc.), and autofluorescence bronchoscopy rather than using serum miRNAs as stand-alone biomarkers.

7.2 Conclusion

The progression of cancer from a localized tumor to a metastatic disease is a devastating event that reduces effective treatment options for patients. Therefore, early detection of cancer is vital to improve the prognosis of patients diagnosed with this disease. Unfortunately, for many cancers, the disease progresses into a late, metastatic stage before symptoms arise and thus non-invasive screening tools are required to ensure the detection of early stage localized tumors. To this end, a number of studies have been conducted examining the utility of circulating miRNAs as biomarkers of disease. Here, we identify serum miRNAs with potential utility as biomarkers for the early detection of oral and lung cancer. Additionally, we've examined pre-profiling variables that should be taken into account when conducting serum miRNA biomarker studies. Much more work into standardizing the pre-profiling and analytical aspects of circulating miRNAs as well as functional studies determining their source and function are required before the full clinical potential of circulating miRNAs can be realized. However, the results presented in this thesis provide promising evidence for the utility of circulating miRNAs to improve cancer screening programs.

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