Serum MicroRNA Profile and Serum Neuron Specific Enolase, S-100, and Interleukin-6 Level as Biomarkers for Differentiating Acute Vertigo Between Cerebellar or Brainstem Infarction and Peripheral Vertigo

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การแสดงออกของไมโครอาร์เอ็นเอและระดับโปรตีน นิวรอนสเปซิฟิกอิโนเลส, เอส-100, และ อินเตอร์ลิวคิน-6 จากซีรั่มผู้ป่วยที่มีอาการเวียนศีรษะบ้านหมุนจากโรคหลอดเลือดสมองตีบส่วนเซเรเบลลัมหรือก้านสมองเทียบกับผู้ป่วยเวียนศีรษะบ้านหมุนจากโรคในหูชั้นใน

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นฤชร กิจไพศาลรัตนา : การแสดงออกของไมโครอาร์เอ็นเอและระดับโปรตีน นิวรอนสเปซิฟิกอิโนเลส, เอส-100, และ อินเตอร์ลิวคิน-6 จากซีรั่มผู้ป่วยที่มีอาการเวียนศีรษะบ้านหมุนจากโรคหลอดเลือดสมองตีบส่วนเซเรเบลลัม หรือก้านสมองเทียบกับผู้ป่วยเวียนศีรษะบ้านหมุนจากโรคในหูชั้นใน. (Serum MicroRNA Profile and Serum Neuron Specific Enolase, S-100, and Interleukin-6 Level as Biomarkers for Differentiating Acute Vertigo Between Cerebellar or Brainstem Infarction and Peripheral Vertigo) อ.ที่ปรึกษาหลัก : ศ. พญ.นิจศรี ชาญณรงค์, อ.ที่ปรึกษาร่วม : ศ. ดร.สัญชัย พยุงภร,อ. นพ.ไตรรักษ์ พิสิษฐ์กุล

*บทนำ*อาการเวียนศีรษะบ้านหมุนเป็นอาการที่พบได้บ่อยในโรคของหูชั้นใน อย่างไรก็ตามอาการดังกล่าวอาจมีสาเหตุมาจากโรคสมองขาดเลือดของระบบไหลเวียนส่วนหลัง การแยกอาการเวียนศีรษะบ้านหมุนในระยะเฉียบพลันจากสาเหตุทั้ง 2 ประการอาจทำได้ยากทั้งจากอาการแสดงของผู้ป่วยหรือการใช้ภาพถ่ายทางรังสี ในการศึกษานี้จึงมีวัตถุประสงค์เพื่อศึกษาการแสดงออกของไมโครอาร์เอ็นเอและระดับโปรตีนนิวรอนสเปซิฟิกอิโนเลส เอส-100 และอินเตอร์ลิวคิน-6 ในซีรั่มเพื่อเป็นตัวชี้วัดทางชีวภาพในการแยกผู้ป่วยที่มีอาการเวียนศีรษะบ้านหมุนจากโรคหลอดเลือดสมองตีบส่วนเซเรเบ ลลัม หรือก้านสมองเทียบกับผู้ป่วยเวียนศีรษะบ้านหมุนจากโรคในหูชั้นใน*วิธีการ*สำหรับขั้นตอนการค้นหา การศึกษานี้ใช้เทคโนโลยีนาโนสตริงเอ็นเคาน์เตอร์ ในการศึกษาแสดงออกของไมโครอาร์เอ็นเอในผู้ป่วยที่มีอาการเวียนศีรษะบ้านหมุนจากโรคหลอดเลือดสมองตีบส่วนเซเรเบลลัม หรือก้านสมอง (3 ราย) และผู้ป่วยที่มีอาการเวียนศีรษะบ้านหมุนจากโรคในหูชั้นใน (3 ราย) ที่ระยะเวลาเฉียบพลันภายใน 72 ชั่วโมงหลังมีอาการเวียนศีรษะบ้านหมุน (วันที่ 0) และวันที่ 90 ไมโครอาร์เอ็นเอ ที่มีการแสดงออกเฉพาะในระยะเฉียบพลันของผู้ป่วยที่มีอาการเวียนศีรษะบ้านหมุนจากโรคหลอดเลือดสมองจะถูกคัดเลือกเพื่อศึกษาในขั้นตอนการยืนยันด้วยวิธีปฏิกิริยาลูกโซ่พอลิเมอเรสแบบย้อนกลับในซีรั่มของผู้ป่วยที่มีอาการเวียนศีรษะบ้านหมุนจากโรคหลอดเลือดสมองตีบส่วนเซเรเบลลัมหรือก้านสมอง (23 ราย) และผู้ป่วยที่มีอาการเวียนศีรษะบ้านหมุนจากโรคในหูชั้นใน (35 ราย) การศึกษานี้ใช้วิธีการวัดปริมาณของโปรตีนนิวรอนสเปซิฟิกอิโนเลส เอส-100 และอินเตอร์ลิวคิน-6 โดยหลักการ electrochemiluminescence immunoassay*ผลการศึกษา*ในขั้นตอนการค้นหาจากเทคโนโลยีนาโนสตริง เอ็นเคาน์เตอร์ พบ miR-342-3p, miR-376-3p และ miR-433-5p เป็นไมโครอาร์เอ็นเอที่มีความเป็นไปได้ในการเป็นตัวชี้วัดทางชีวภาพ ซึ่งในขั้นตอนการยืนยัน พบว่าเฉพาะ miR-433-5p เท่านั้นที่มีปริมาณสูงอย่างมีนัยสำคัญทางสถิติในผู้ป่วยเวียนศีรษะบ้านหมุนจากโรคหลอดเลือดสมอง [92.13 (49.06-183.2) ก๊อปปี้ต่อไมโครลิตร] เมื่อเทียบกับผู้ป่วยที่มีอาการเวียนศีรษะบ้านหมุนจากโรคในหูชั้นใน [53.45 (35.37-102.3) ก๊อปปี้ต่อไมโครลิตร], *P*=0.0056 นอกจากนี้ miR-433-5p ยังมีอำนาจในการจำแนกผู้ป่วยที่มีอาการเวียนศีรษะบ้านหมุนจากโรคสมองขาดเลือดออกจากโรคในหูชั้นใน โดยมีค่าพื้นที่ใต้กราฟ receiver operating characteristic เป็น 0.71 และเมื่อใช้ระดับของซีรั่ม miR-433-5p สูงกว่า 46 ก๊อปปี้ต่อไมโครลิตร ในการวินิจฉัยอาการเวียนศีรษะบ้านหมุนจากโรคหลอดเลือดสมองพบว่ามีความไวร้อยละ 87 และความจำเพาะร้อยละ 49 นอกจากนี้ระดับ เอส-100 [โรคหลอดเลือดสมอง: 0.111 (0.049-0.335) ไมโครกรัมต่อลิตร เทียบกับ โรคในหูชั้นใน: 0.054 (0.039-0.082) ไมโครกรัมต่อลิตร, *P*=0.005] และอินเตอร์ลิวคิน-6 [โรคหลอดเลือดสมอง: 7.42 (4.23-14.47) พิโคกรัมต่อมิลลิลิตร เทียบกับ โรคในหูชั้นใน: 2.44 (0.70-4.68) พิโคกรัมต่อมิลลิลิตร, *P*<0.001] ในผู้ป่วยเวียนศีรษะบ้านหมุนจากโรคหลอดเลือดสมองสูงกว่าผู้ป่วยจากโรคในหูชั้นในอย่างมีนัยสำคัญทางสถิติ*สรุป* การศึกษานี้แสดงให้เห็นว่าไมโครอาร์เอ็นเอ miR-433-5p ในซีรั่มมีความสามารถในการเป็นตัวชี้วัดทางชีวภาพเพื่อแยกระหว่างโรคหลอดเลือดสมองตีบส่วนเซเรเบลลัมหรือก้านสมองและโรคของหูชั้นใน ในผู้ป่วย

สาขาวิชา	อายุรศาสตร์	ลายมือชื่อนิสิต .....
ปีการศึกษา	2562	ลายมือชื่อ อ.ที่ปรึกษาหลัก .....
		ลายมือชื่อ อ.ที่ปรึกษาร่วม .....
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KEYWORD: acute vertigo, stroke biomarker, microRNA, posterior circulation stroke, central vertigo, peripheral vertigo

Naruchorn Kijpaisalratana : Serum MicroRNA Profile and Serum Neuron Specific Enolase, S-100, and Interleukin-6 Level as Biomarkers for Differentiating Acute Vertigo Between Cerebellar or Brainstem Infarction and Peripheral Vertigo. Advisor: Prof. Dr. NIJASRI CHARNNARONG, M.D. Co-advisor: Prof. Dr. SUNCHAI PAYUNGPORN, Ph.D.,Dr. TRAIRAK PISITKUN, M.D.

*Background and Purpose*Acute vertigo is a common presentation of inner ear disease. However, it can also be caused by more serious conditions, especially posterior circulation stroke. Differentiation between these 2 conditions by clinical presentations and imaging studies during acute phase can be challenging. This study aims to evaluate the serum microRNA profile and serum neuron specific enolase (NSE), S-100, and interleukin-6 (IL-6) level as potential biomarkers to differentiate between patients with central vertigo due to cerebellar or brainstem infarction and peripheral vertigo.*Methods*In the discovery phase, miRNA expression profiling was performed by Nanostring nCounter Technology in serum of patients with central vertigo due to cerebellar or brainstem infarction (n=3) and peripheral vertigo (n=3) during acute phase within 72 hours after vertigo onset (day 0) and on day 90. MiRNAs that expressed only in acute phase of patients with stroke were selected as potential candidates. Subsequent validation was performed by quantitative reverse-transcription polymerase chain reaction (RT-qPCR) in the serum of patients with posterior circulation stroke (n=23) and peripheral vertigo (n=35). The serum NSE, S100 and interleukin-6 (IL-6) measurements were performed by electrochemiluminescence immunoassay (ECLIA).*Results*In the discovery phase, miR-342-3p, miR-376-3p, and miR-433-5p were identified by Nanostring nCounter Technology as potential biomarker candidates. In subsequent validation phase, serum miR-433-5p was the only miRNA expressed at significantly high levels in patients with central vertigo during acute phase (median (IQR) central: 92.13 (49.06-183.2) copies/μL vs. peripheral: 53.45 (35.37-102.3) copies/μL, *P*=0.0056). Only miR-433-5p had discriminative ability to differentiate between central and peripheral vertigo with area under the receiver operating characteristic curve (AUROC) of 0.71. Using a serum miR-433-5p cut off at level >46 copies/μL, the sensitivity and specificity were 87% and 49% respectively. Both S100 (central: 0.111 (0.049-0.335) μg/L vs. peripheral: 0.054 (0.039-0.082) μg/L, *P*=0.005), and IL-6 central: 7.42 (4.23-14.47) pg/mL vs. peripheral: 2.44 (0.70-4.68) pg/mL, *P*< 0.001) had significantly higher level in patients with central vertigo.*Conclusion*This is the first study to demonstrate the potential of serum miR-433-5p as a biomarker to differentiate between cerebellar or brainstem infarction and peripheral vertigo among patients with acute vertigo.

Field of Study:	Medicine	Student's Signature .....
Academic Year:	2019	Advisor's Signature .....
		Co-advisor's Signature .....
		Co-advisor's Signature .....

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Naruchorn Kijpaisalratana

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Chapter 1 Introduction

1.1 Background and rationale

Ischemic stroke is one of the leading causes of death and disability in Thailand. The most effective way of managing acute ischemic stroke is based on an early detection of symptoms as well as an accurate diagnosis. The thrombolytic therapy for a patient with acute ischemic stroke requires time-dependent management to help reduce morbidity and mortality. Patients with acute ischemic stroke have variety of clinical presentations such as hemiparesis, facial weakness, dysarthria, and aphasia.

Vertigo is one of the common presentations of ischemic stroke in cerebellum or brainstem. Vertigo related stroke should be promptly evaluated and treated since it is a life-threatening condition. The diagnosis of stroke in the cerebellum or brainstem is problematic since it is, most of the time, difficult to differentiate between central (brain lesion) and peripheral (inner ear lesion). History taking and physical examination are usually limited due to patient's vertiginous symptoms. Computed tomography (CT) is not sensitive for diagnosis of cerebellar or brainstem disease. Although magnetic resonance imaging (MRI) has higher sensitivity, initial diffusion-weighted imaging done within 48 hours after onset can miss the infarction in 12% of the patients (1).There are patients who presented with acute vertigo which require extensive physical examination and investigation to differentiate between acute vertigo due to acute ischemic stroke in brainstem or cerebellum and diseases of inner ear such as benign paroxysmal positional vertigo (BPPV), Meniere's disease, and vestibular neuritis. Despite the patient's history, extensive and specific physical examination which should be performed and interpreted by an experienced physician, as well as an investigation by neuroimaging either CT scan or MRI, the differentiation between central and peripheral vertigo remain indistinguishable in many cases.

Nowadays, there has been extensive research on biomarkers to help diagnosing acute ischemic stroke. The objectives of using biomarkers for stroke diagnosis include differentiating between ischemic stroke and hemorrhagic stroke, differentiating between ischemic stroke and stroke mimics, and predicting clinical outcome and prognosis. Most of previous studies focused on using protein known to participate in stroke pathophysiology as stroke biomarkers. However, to date, there is no known single protein biomarker that has good sensitivity and specificity enough to help diagnose acute ischemic stroke. Following studies tend to use the combination of proteins or protein panel to help increase the sensitivity and specificity. In addition to protein biomarkers, there is an effort among researchers to identify a new source of the biomarker.

MicroRNA (miRNA) is one of the small non-coding ribonucleic acid, which regulates protein synthesis. Currently, biomarker studies have been focused on miRNA and its potential to be a biomarker of various diseases such as cancers and neurodegenerative diseases. Due to its stability in various body fluids and its property of protein synthesis regulation, miRNA is one of the promising candidates in the field of the biomarker. The study on miRNA profile from patients with central and peripheral vertigo may result in the discovery of a new biomarker that can help differentiating between two conditions. Identification of the new biomarker will help physicians who are inexperienced with meticulous physical examination in vertigo patients make diagnosis more confidently and accurately.

1.2 Research Questions

Primary research question

Among patients who presented with acute vertigo at King Chulalongkorn Memorial Hospital within 72 hours after onset, are the serum microRNA expression profiles analyzed by NanoString nCounter Technology followed by quantitative reverse transcription polymerase chain reaction from patients presented with acute vertigo due to cerebellar/brainstem infarction different from patients with peripheral vertigo by at least one miRNA?

Secondary research questions

1. Is the level of serum Neuron-Specific Enolase (NSE) from patients with acute vertigo presented at King Chulalongkorn Memorial Hospital (KCMH) within 72 hours after onset due to cerebellar/brainstem infarction different from patients with peripheral vertigo?
2. Is the level of S-100 from patients with acute vertigo presented at King Chulalongkorn Memorial Hospital (KCMH) within 72 hours after onset due to cerebellar/brainstem infarction different from patients with peripheral vertigo?
3. Is the level of serum Interleukin-6 (IL-6) from patients with acute vertigo presented at King Chulalongkorn Memorial Hospital (KCMH) within 72 hours after onset due to cerebellar/brainstem infarction different from patients with peripheral vertigo?
4. Are the serum microRNA expression profiles analyzed by NanoString nCounter Technology followed by quantitative reverse transcription polymerase chain reaction in patients with acute vertigo due to cerebellar/brainstem infarction at acute phase within 72 hours after onset different from serum microRNA expression profiles at 3 months follow up by at least one miRNA?

5. Are the serum microRNA expression profiles analyzed by NanoString nCounter Technology followed by quantitative reverse transcription polymerase chain reaction in patients with acute vertigo due to peripheral vertigo at acute phase within 72 hours after onset different from serum microRNA expression profiles at 3 months follow up by at least one miRNA?

1.3 Objectives

1. To compare the serum microRNA expression profiles from NanoString nCounter Technology followed by quantitative reverse transcription polymerase chain reaction among acute vertigo patients due to cerebellar/brainstem infarction and peripheral vertigo who presented at King Chulalongkorn Memorial Hospital (KCMH) within 72 hours after onset.
2. To compare the level of serum Neuron-Specific Enolase (NSE) between patients with acute vertigo due to cerebellar/brainstem infarction and peripheral vertigo who presented at King Chulalongkorn Memorial Hospital (KCMH) within 72 hours after onset.
3. To compare the level of serum S-100 between patients with acute vertigo due to cerebellar/brainstem infarction and peripheral vertigo who presented at King Chulalongkorn Memorial Hospital (KCMH) within 72 hours after onset.
4. To compare the level of serum Interleukin-6 (IL-6) between patients with acute vertigo due to cerebellar/brainstem infarction and peripheral vertigo who presented at King Chulalongkorn Memorial Hospital (KCMH) within 72 hours after onset.
5. To compare the serum microRNA expression profiles from NanoString nCounter Technology followed by quantitative reverse transcription polymerase chain

reaction–among acute vertigo patients due to cerebellar/brainstem infarction between acute phase within 72 hours after onset and at 3 months follow up.

6. To compare the serum microRNA expression profiles from NanoString nCounter Technology followed by quantitative reverse transcription polymerase chain reaction–among acute vertigo patients due to peripheral vertigo between acute phase within 72 hours after onset and at 3 months follow up.

1.4 Hypothesis

Null Hypothesis

1. Serum microRNA expression profiles analyzed by NanoString nCounter Technology and followed by quantitative reverse transcription polymerase chain reaction from patients with acute vertigo presented at King Chulalongkorn Memorial Hospital within 72 hours after onset due to cerebellar/brainstem infarction are similar to serum microRNA expression profiles from patients with peripheral vertigo.
2. The level of serum Neuron-Specific Enolase (NSE) from patients with acute vertigo presented at King Chulalongkorn Memorial Hospital (KCMH) within 72 hours after onset due to cerebellar/brainstem infarction is similar to patients with peripheral vertigo.
3. The level of serum S-100 from patients with acute vertigo presented at King Chulalongkorn

   Memorial Hospital (KCMH) within 72 hours after onset due to cerebellar/brainstem infarction is similar to patients with peripheral vertigo.
4. The level of serum Interleukin-6 (IL-6) from patients with acute vertigo presented at King

Chulalongkorn Memorial Hospital (KCMH) within 72 hours after onset due to cerebellar/brainstem infarction is similar to patients with peripheral vertigo.

5. Serum microRNA expression profiles analyzed by NanoString nCounter Technology and followed by quantitative reverse transcription polymerase chain reaction in patients with acute vertigo due to cerebellar/brainstem infarction at acute phase within 72 hours are similar to serum microRNA expression profiles at 3 months follow up.
6. Serum microRNA expression profiles analyzed by NanoString nCounter Technology and followed by quantitative reverse transcription polymerase chain reaction in patients with acute vertigo due to peripheral vertigo at acute phase within 72 hours are similar to serum microRNA expression profiles at 3 months follow up.

Alternative Hypothesis

1. Serum microRNA expression profiles analyzed by NanoString nCounter Technology and followed by quantitative reverse transcription polymerase chain reaction in patients with acute vertigo presented at King Chulalongkorn Memorial Hospital within 72 hours after onset due to cerebellar/brainstem infarction are different from serum microRNA expression profiles in patients with peripheral vertigo by at least one miRNA.
2. The level of serum Neuron-Specific Enolase (NSE) from patients with acute vertigo presented at King Chulalongkorn Memorial Hospital (KCMH) within 72 hours after onset due to cerebellar/brainstem infarction is different from patients with peripheral vertigo.
3. The level of serum S-100 from patients with acute vertigo presented at King Chulalongkorn

Memorial Hospital (KCMH) within 72 hours after onset due to cerebellar/brainstem infarction is different from patients with peripheral vertigo.

4. The level of serum Interleukin-6 (IL-6) from patients with acute vertigo presented at King

   Chulalongkorn Memorial Hospital (KCMH) within 72 hours after onset due to cerebellar/brainstem infarction is different from patients with peripheral vertigo.

5. Serum microRNA expression profiles analyzed by NanoString nCounter Technology and followed by quantitative reverse transcription polymerase chain reaction in patients with acute vertigo due to cerebellar/brainstem infarction at acute phase within 72 hours are different from serum microRNA expression profiles at 3 months follow up by at least one miRNA.
6. Serum microRNA expression profiles analyzed by NanoString nCounter Technology and followed by quantitative reverse transcription polymerase chain reaction in patients with acute vertigo due to peripheral vertigo at acute phase within 72 hours are different from serum microRNA expression profiles at 3 months follow up by at least one miRNA.

1.5 Keywords

acute vertigo, cerebellar infarction, brainstem infarction, biomarker, microRNA

1.6 Operational definition

The operational definition of the etiology of acute vertigo is based on studies by Kattah et al.(1) and Newman-Toker et al (2).

a. Acute ischemic stroke due to cerebellar/brainstem infarction: The reference standard diagnosis of acute ischemic stroke is the evidence of acute ischemic

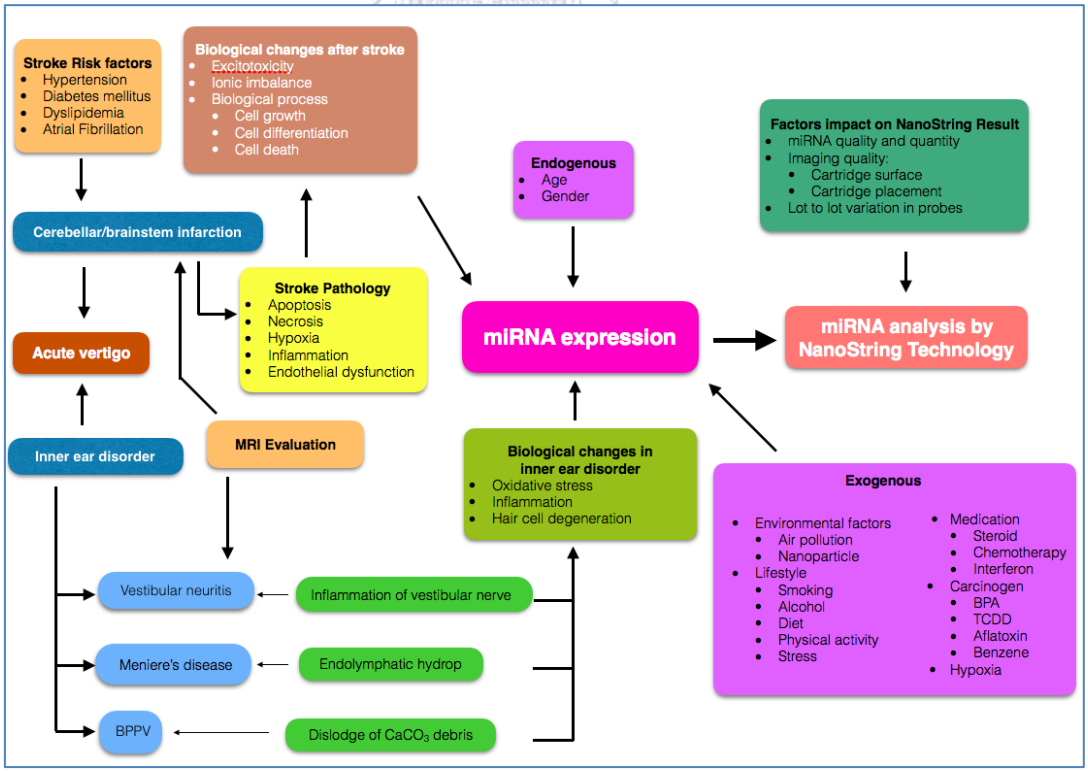
stroke by neuroimaging either CT scan or MRI with an evidence of acute ischemic stroke involving cerebellum or brainstem relevant to the clinical findings (1, 2).

b. Peripheral vertigo: The reference standard diagnosis of peripheral vertigo is the absence of evidence of acute ischemic stroke in cerebellum or brainstem by neurologic signs on serial examination by a neurologist, lack of evidence of acute ischemic stroke in neuroimaging study either CT scan or MRI, and characteristic clinical course during the follow-up period (1, 2).

1.7 Conceptual Framework

The conceptual framework of this project is based on the different pathophysiology between acute vertigo due to cerebellar or brainstem infarction and inner ear disorder. Several factors both exogenous and endogenous also contributed to miRNA expression(3).

Figure 1: Conceptual Framework



BPA=bisphenol A, TCDD = tetrachlorodibenzo-p-dioxin

1.8 Expected benefits and application

Acute vertigo is one of the common problems. Data from patient's history, physical examination, and neuroimaging sometimes cannot provide sufficient information to help identify the cause of acute vertigo. New microRNA biomarker might be discovered from this study which can be incorporate into the routine physical examination.

Chapter 2 Review of Literature

2.1 Incidence and disease burden of acute ischemic stroke

Acute stroke is one of the major public health problems. It is one of the leading causes of death and disability in Thailand and worldwide. Seventy-five to eighty percent of stroke is due to acute ischemic stroke (4). In Thailand, each year, 250,000 patients suffered from acute stroke and 50,000 patients died of stroke(5). According to the data from Thai Ministry of Public Health, stroke is the first leading cause of death. Regarding the disease burden, it was rank the first in female and the third in male by disability-adjusted life years (DALYs) (5). The prevalence of acute stroke in Thailand is 1.88% among participants aged more than 45 years according to Thai Epidemiology Stroke Study, TES (6).

2.2 Acute vertigo

Acute vertigo is one of the presenting symptoms of acute vestibular syndrome which is described as rapid onset of acute vertigo, nausea/vomiting, gait unsteadiness, head-motion intolerance and nystagmus (1, 7). Acute vertigo and dizziness are among the most common symptoms that bring patients to the emergency department (8). Approximately 10-20% of dizziness patients in emergency department have acute vestibular syndrome (9). In 2011, there were 3.9 million emergency department visits for the evaluation of vertigo and dizziness in the United States (10). It is estimated that 400,000-800,000 patients with acute vestibular syndrome visit emergency department annually (9, 10). The most common causes of acute vertigo are peripheral causes due to the inner ear diseases such as benign paroxysmal positional vertigo (BPPV), Meniere's disease, and vestibular neuritis (VN) (8, 11). Uncommon yet life-threatening is the central cause of acute vertigo which is most commonly due to posterior circulation ischemic stroke involving cerebellum or brainstem. Twenty to 25% of ischemic stroke involves in

posterior circulation (12, 13). According to the New England Medical Center Posterior Circulation Registry, 47% of patient with posterior circulation stroke experience dizziness which is the most common symptom (14). Prevalence of ischemic stroke in acute vertigo patient varies among studies which depends on definition used in the study. Stroke/TIA was diagnosed in 3.2% of patients age > 44 years presented with vertigo, dizziness, and imbalance according to a population-based study in Texas, United States (11). While a study from Sweden revealed that 25% of patients aged between 50-75 years old who presented with acute onset of isolated vertigo had acute ischemic stroke (15). There is no definitive prevalence of acute ischemic stroke among acute vertigo patients, however, it is estimated that 10-40% of acute vestibular syndrome is due to stroke (9). In addition to dizziness or vertigo other neurological symptoms and signs usually present in patient with posterior circulation stroke. However, isolated vestibular syndrome occurs in approximately 25% of posterior circulation stroke (16). Cerebellum, inferior or superior cerebellar peduncles, and caudal lateral or rostral dorsolateral medulla are among common areas present with isolated vestibular syndrome (16). A single-center retrospective study reported that 11% of patients with cerebellar infarction presented with isolated vertigo, most commonly involving in the area supplied by medial posterior inferior cerebellar artery (17). Although central vertigo is uncommon, the diagnosis of acute vertigo result from ischemic stroke should not be missed. The undetected cerebellar infarction as a cause of acute vertigo leads to an increase in mortality up to 8 folds (9).

2.3 Challenges in acute vertigo diagnosis

2.3.1 Clinical perspective

Patient's history and physical examination are required to help identify the cause of acute vertigo. Other neurological deficits such as hemiparesis, dysarthria, and limb ataxia, in addition to acute vertigo, are important clinical clues which help differentiate between central vertigo and peripheral vertigo. However, less than half of patients with acute vestibular syndrome have other obvious neurological deficits (2). Certain physical signs can distinguish vertigo from central and peripheral causes. In peripheral vertigo, the nystagmus is unidirectional and horizontal pattern, whereas in central vertigo, the direction of nystagmus is variable, gaze-evoked, spontaneous vertical or pure torsional pattern (8). However, unilateral horizontal nystagmus found in peripheral vertigo has been reported in cerebellar infarction (8, 15, 17). In addition to nystagmus, head impulse test for vestibulo-ocular reflex evaluation, is one of the necessary bedside evaluations to identify the cause of acute vertigo. In patients with acute vestibular neuritis, corrective saccade will be presented when performing a rapid head turning toward the side of the lesion, whereas in central vertigo, corrective saccade is absent (8). However, corrective saccade on head impulse test could be demonstrated in central vertigo result from anterior inferior cerebellar artery (AICA) infarction involving vestibular nucleus and flocculus (17, 18). Skew deviation, which is vertical ocular misalignment detected by alternate cover test, is consider to be a specific sign of central cause in patient with acute vestibular syndrome (1, 19). Despite being specific for central vertigo, skew deviation was found in a case report of patient with peripheral vestibulopathy (20).

Clinical manifestation of acute vertigo due to cerebellar or brainstem infarction can mimic acute vestibular neuritis, hence the name "pseudo vestibular neuritis" (2, 15, 17, 19). Acute stroke registry at Keimyung University Dongsan Medical Center showed that 10.4% of patients with cerebellar infarction presented with clinical features

suggesting vestibular neuritis (17). Therefore, diagnosis of acute vertigo has long been challenging especially in emergency setting. It is estimated that 35% of posterior circulation stroke presented with dizziness was misdiagnosed (1). Clinical signs of central vertigo such as vertical nystagmus, direction-changing gaze-evoked nystagmus, and severe postural instability have high specificity but low sensitivity for posterior circulation stroke diagnosis (18). Normal head impulse test is the best predictor for stroke; however, it is estimated that one out of ten strokes will be missed if other ocular findings are not considered (1, 2). A combination of bedside physical examinations includes head impulse test, nystagmus, and test of skew (HINTS) has been proposed to improve the diagnosis of stroke in patients with acute vestibular syndrome (1). The presence of normal head impulse test, direction-changing nystagmus, or skew deviation had 100% sensitivity and 96% specificity for diagnosis of stroke (1). Using HINTS for stroke diagnosis has higher sensitivity than magnetic resonance imaging (MRI) which has 77%-88% sensitivity when performed in first 24-48 hours after onset (1, 21). Further study adding acute hearing loss to the diagnostic paradigm, "HINTS Plus", help diagnose posterior circulation stroke with false positive head impulse test due to ischemic stroke in AICA territory (22). Despite promisingly high sensitivity and specificity for stroke diagnosis, HINTS has some limitations. In the original study, HINTS was performed by an experienced neuro-ophthalmologist who also used prism cross-cover test for ocular alignment. Therefore, high sensitivity and specificity of the test may not be translated into practical clinical setting where most of the acute vertigo patients were examined by general practitioner and emergency physician. HINTS Plus is valuable in case of acute vestibular syndrome patients presented with nystagmus. Head impulse test performed in patient without nystagmus can be misleading due to normal examination (23, 24). Therefore, HINTS Plus is not applicable to most of the patients with resolution of the vestibular symptoms (25). In

addition to the limitation of the clinical signs, uncooperative patients due to severe vertiginous symptoms might limit physical examination.

2.3.2 Neuroimaging perspective

Neuroimaging using either computed tomography (CT) scan or magnetic resonance imaging (MRI) is an important investigation for acute ischemic stroke diagnosis in order to differentiate between ischemic and hemorrhagic stroke. CT scan has lower sensitivity for acute ischemic stroke diagnosis compare to MRI (16% vs. 83%) (26). In addition, CT scan has low sensitivity for detecting acute ischemic stroke in the posterior fossa due to bony artifacts from the skull base (27). Therefore, acute ischemic stroke cannot be excluded when CT scan is normal in the patient presented with acute vertigo (8). MRI has higher sensitivity compare to CT scan with sensitivity of 80-95% when diffusion weighted image (DWI) is used within the first 24 hours (26, 28). Therefore, MRI is preferred for diagnosis of acute ischemic stroke especially in the posterior circulation (13). However, comparing with other brain regions, posterior fossa is the area where MRI has the lowest sensitivity (21). False negative MRI in ischemic stroke is not uncommon. Posterior circulation had false negative MRI more often than in anterior circulation ischemic stroke (19% vs. 2%) (21). When performing DWI within the first 24 hours, 31% of patients with posterior circulation ischemic stroke had false negative MRI (21). Among patients with acute vestibular syndrome, 12% had false negative MRI when performed within the first 48 hours (1). Furthermore, in patients with isolated vestibular syndrome, initial MRI with DWI was falsely negative in 17.6% of the patients during early phase (16). False negative initial MRI performed within 48 hours after onset were more common in patients with acute vestibular syndrome due to small stroke (≤ 10 mm. in axial diameter) than large stroke (29). Low signal-to-noise ratio due to short time interval from onset to scan, location and size of infarction, and magnetic susceptibility artifacts causing

brainstem distortion are causes of false negative MRI (16). Thus, MRI can be normal in posterior circulation stroke especially when the area of infarction is small (13). The availability of MRI is another limitation when considering acute ischemic stroke diagnosis. In several regional hospitals, MRI cannot be performed in the emergency department(8).

2.4 Biomarker and acute stroke

Due to several limitations from physical examination and neuroimaging, biomarkers for acute ischemic stroke including proteins, nucleic acids such as RNA, and metabolites have been studied extensively (30-32). There are several sources of biomarker in neurological diseases such as serum or plasma, cerebrospinal fluid, neuroimaging, and electroencephalogram. However, biomarkers acquired from peripheral blood are convenient to collect in the emergency clinical setting. The objectives of biomarker studies in acute ischemic stroke include confirmation of the diagnosis, differentiation between acute stroke and other stroke mimics, differentiation between ischemic and hemorrhagic stroke, assessment the severity of acute ischemic stroke, determination the cause of acute ischemic stroke, and prediction of neurological outcome with regards to early neurological deterioration and hemorrhagic transformation (32). In early studies, most of the biomarkers are proteins known to be participated in stroke pathophysiology. Protein biomarkers can be categorized into CNS tissue injury biomarkers, inflammatory biomarkers, and coagulation/thrombosis biomarkers (31, 32).

NSE

Neuron-specific enolase (NSE), located mainly within neurons and cells of neuroendocrine origin, is a dimeric isoenzyme of the glycolytic enzyme enolase (33, 34). NSE was released after neuronal injury in various conditions including acute ischemic stroke. Various studies have been done extensively on serum NSE level regarding its correlation with time of stroke onset, stroke severity, and functional outcome(34). NSE level

was found to be significantly increased as early as 4 hours after stroke onset (35) but the level peaks 24 hours after onset (34). The level of NSE is significantly higher in stroke patients and correlates with the volume of infarction (34). A study using serum sample from patients in the National Institute of Neurological Disorders and Stroke (NINDS) tissue plasminogen activator stroke study revealed that higher serum NSE level is associated with higher National Institute of Health Stroke Scale (NIHSS) at baseline (33). NSE level was also found to be correlated with NIHSS at 24 hours after thrombolytic treatment (36). In a serial examination of serum NSE level, the second elevation of NSE peak was associated with hemorrhagic transformation in patients with acute ischemic stroke (37).

S100

S100 is a cytosolic calcium-binding protein abundantly found in glial and Schwann cells. The protein consists of homo- or hetero-dimers of two subunits, alpha and beta. The combination of alpha-beta and beta-beta are known as S100B protein, which is highly specific for nervous tissue (33, 38). S100 was known to be a marker of blood-brain barrier dysfunction since it was released when there is structural damage to glial and Schwann cells (38, 39). S100 level was significantly higher in stroke patients as early as 6 hours after stroke onset (40) and reached its peak level after 24 hours after onset (41). The level of S100 was correlated with the volume of infarction (33, 41), stroke severity measured by baseline NIHSS (33, 38, 42), and functional outcome (33). A prospective study of 23 patients with MCA/M1 occlusion revealed that S100B value below 0.4 ug/ml measured 48-96 hours after stroke onset was associated with early recanalization (<6 hours after onset) (43). Pre-thrombolytic S100B level from serum of patients with acute ischemic stroke was found to be significantly higher in patients with hemorrhagic transformation (39).

IL-6

Interleukin-6 (IL-6) is a cytokine originally known to be a B cell differentiation factor, which induced B cell to be antibody-producing cells (BSF-2). Besides its role in B cell maturation, IL-6 also plays an important role in neurogenesis. Expression of IL-6 was altered in several of neurological conditions including acute ischemic stroke (44). Significant correlation was found between IL-6 level and infarction volume (45, 46). Clinical outcome determined by NIHSS and modified Rankin scale (mRS) was positively correlated with IL-6 level(45). Plasma IL-6 > 21.5 pg/ml was an independent factor for early clinical worsening (47).

Currently, there is no single protein biomarker that can be used in clinical setting for acute stroke diagnosis. This might be due to the heterogeneity of ischemic stroke enrolled in the studies and low sensitivity and specificity of the proteins. The combination of proteins or protein panel has been used in subsequent studies to help increase the sensitivity and specificities of the protein biomarkers (30, 32). The "high throughput screening" technology also facilitates the field of biomarker discovery to screen for multiple biomarkers simultaneously (32). This new technology has been adopted for not only protein biomarkers but also other types of biomarker including nucleic acids and metabolites.

2.5 MicroRNA

MicroRNA (miRNA) is a small non-coding RNA containing 20-24 nucleotides. It plays a critical role in the regulation of protein synthesis via the epigenetic process. MicroRNA inhibits protein synthesis by enhancing messenger RNA (mRNA) degradation and inhibiting of mRNA translation. These processes result in a reduction in protein synthesis (48-50). MicroRNA was synthesized in the nucleus. "Primary miRNA" (pri-miRNA), containing thousands of nucleotides with stem-loop, was the first product from

deoxyribonucleic acid (DNA) transcription via RNA polymerase II. Drosha (ribonuclease III) then cleave the stem loop into “precursor miRNA” (pre-miRNA). The pre-miRNA was export from the nucleus to the cytoplasm by exportin-5 protein and cleaved by Dicer (ribonuclease II) and TAR RNA-binding protein to form “mature miRNA” (49, 50). In protein synthesis regulation, miRNA and RNA-induce silencing complex (RISC) together bind the target mRNA which leads to miRNA destabilization and inhibition of mRNA translation(49). Regulation of protein synthesis by miRNA plays a critical role in various fundamental biological processes including cell growth, cell differentiation, and cell death (51).

2.5.1 miRNA as biomarker

MicroRNA has several properties essential for the biomarker. MicroRNA can be collected noninvasively due to its stability in various body fluids including plasma, serum, saliva, and urine. The biological stage and organ-specific properties of miRNA also contribute to its potential as a biomarker (51, 52). Currently, miRNA has been studied as a biomarker for several diseases. In the cardiovascular system, miRNA-499 has been studied for the diagnosis of myocardial infarction (53). Mir-122 and mir-192, liver-specific miRNAs, were studied as biomarkers of drug-induced liver injury (54). MiRNA has also been studied in hematologic malignancy as a diagnostic biomarker of diffuse large B-cell lymphoma (55). In neurodegenerative diseases, miRNA was known to be involved in the pathophysiology of Alzheimer’s disease including amyloid deposition, inflammation, and neuronal injury. Alteration in miRNA expression in the midbrain of an animal model of Parkinson’s disease was also demonstrated in a preclinical study (56). MicroRNA-4639 was shown to be up-regulated in Parkinson’s disease patients and has the potential to be early diagnostic biomarker for Parkinson’s disease (57).

2.5.2 miRNA as stroke biomarker

Due to the advancement in biological technology, biomarker has become a potential alternative diagnostic tool for stroke diagnosis. Due to miRNA properties as previously mentioned, many studies have been focus on miRNA as stroke biomarker. These studies acquired miRNA from different sources and had a different method of miRNA analysis.

1. miRNA analysis by RNA sequencing and selective quantitative PCR

In 2017, Tiedt et al. identified circulating microRNAs in plasma associated with acute ischemic stroke. RNA sequencing was used in the discovery phase including 20 patients with ischemic stroke and 20 matched healthy control subjects. Quantitative PCR was applied in the validation (40 patients with ischemic stroke and 40 matched controls) and replication phases (200 patients with ischemic stroke and 100 healthy controls). MiR-125a-5p, miR-125b-5p, and miR-143-3p were found to be upregulated in patients with ischemic stroke compare with both healthy controls and patients with transient ischemic attack. A temporal profile study up to 90 days after stroke revealed normalization of miR-125b-5p and miR-143-3p to control level at day 2 after stroke (58).

2. miRNA analysis by miRNA microarray and selective quantitative PCR

2.1) Whole blood as a source of miRNA

The first study on miRNA as stroke biomarker was done in 2009 by Tan et al. This prospective case-control study enrolled 19 ischemic stroke patients aged between 18-49 years old. Blood samples were collected from stroke patients within 6-18 months after onset. MiRNA expression profile from ischemic stroke patients were compared to 5 healthy control subjects. In this study, ischemic stroke etiology classified by TOAST classification including large vessel atherosclerosis, small vessel disease, cardioembolic, and undetermined were correlated with miRNA expression profile. MiRNA was extracted

from whole blood and miRNA expression was done by miRNA microarray followed by quantitative polymerase chain reaction (qPCR). In miRNA microarray, 157 miRNAs from 836 miRNAs presented on the array chip were found to be regulated differently between stroke and control samples. Among 157 dysregulated miRNAs, 138 miRNAs were upregulated (fold change>1.0), and 19 miRNAs were down-regulated (fold change <1.0) in stroke patients. Among these, 17 miRNAs were up regulated and 8 miRNAs were down regulated across 3 stroke subtypes including large vessel atherosclerosis, small vessel disease, and cardioembolic. These observed miRNAs were known to be involved in the hypoxic response, endothelial function, neural function, and angiogenesis. Besides, miRNA expression among stroke due to large vessel atherosclerosis was found to be different from that in small vessel disease (59).

In 2014, Sepramaniam et al. conducted a prospective case-control study. In this study, the miRNA expression profiles from 169 patients with acute ischemic stroke were compared to 24 controls with risk factors for acute ischemic stroke. Venous blood samples were obtained at day 1,2, and 7 after stroke onset. For the recovery period, blood samples were obtained from patients at outpatient clinic 2 months to 2 years after stroke onset. MiRNA was isolated from peripheral blood sample. Using TagMan Low-Density Array (TLDA) and selective qPCR, 5 miRNAs including miR-125b-2*, -27a*, -422a, -488, and -627 were found to be changed consistently in acute stroke patients regardless of age, severity, and risk factors. MiRNA expression patterns also found to be different between disease time course at acute phase (within 7 days after onset), 6 and 24 months recovery phases (60).

In 2014, a prospective case-control study by Jickling et al. evaluated the miRNA expression profiles in peripheral blood cells from 24 patients with acute ischemic stroke and compared to 24 vascular risk factor controls. Venous blood samples were obtained within 72 hours after stroke onset. Using Affymetrix Gene Chip miRNA 3.0 array

followed by selective qPCR, 6 miRNAs were down-regulated including miR-122, miR-148a, let-7i, miR-19a, miR-320d, and miR-4429. Two miRNAs were up-regulated including miR-363 and miR-487b. These miRNAs were known to be involved in stroke pathophysiology via different pathways such as NF- κβ signaling, Toll-like receptor signaling, interleukin signaling, and prothrombin activation pathway (61).

2.2) Plasma as a source for miRNA

A prospective case-control study by Wang et al. in 2014, enrolled total 136 ischemic stroke patients both MRI negative (n=60) and MRI positive (n=76) and 116 healthy controls. Blood samples were collected within 24 hours after stroke onset. MiRNAs were isolated from plasma. Using Agilent miRNA microarray system, 17 miRNAs were up regulated and 103 miRNAs were down regulated in MRI negative compare to MRI positive ischemic stroke patients. Thirty-three miRNAs were up regulated and 36 miRNAs were down regulated among MRI positive ischemic stroke patients compare to healthy control subjects. 105 miRNAs were up regulated and 37 miRNAs were down regulated among healthy control subjects compare to MRI negative ischemic stroke patients. Among these dysregulated miRNAs, 17 miRNAs candidates were obtained and confirmed by qPCR assay. Among 17 miRNAs candidates, hsa-miR-106b-5P and hsa-miR-4306 were significantly higher in acute stroke patients in a stepwise pattern among MRI negative and MRI positive ischemic stroke patients respectively, whereas hsa-miR320e and hsa-miR-320d were significantly and gradually lower than healthy controls among MRI negative and MRI positive ischemic stroke patients respectively (62).

2.3) Serum as a source for miRNA

In 2014, Li et al. evaluated miRNAs expression in serum of ischemic stroke patients. Pooled serum (10 serums per group) of 5 groups of patients including 1.) thrombotic stroke and hypertension, 2.) thrombotic stroke, hypertension, and hyperlidipemia, 3.) embolic stroke and heart disease, 4.) embolic stroke, hypertension,

and heart diseae, 5.) healthy control were studied in initial screening stage using miRNA microarray. Six miRNAs candidates were validated in 53 patients with ischemic stroke and 50 controls. MiR-32-3p, miR-106-5p, and miR-1246 were significantly increased in serum of ischemic stroke patients compared with control group (63).

3. miRNA analysis by quantitative PCR

3.1) Whole blood as a source of miRNA

In 2011, Zeng et al. examined the miR-210 expression profile, which is a master and pleiotropic hypoxia miRNA. This prospective case-control study enrolled 112 ischemic stroke patients and 60 healthy controls. The miRNA-210 expressions were measure at 3, 7, and 14 days after stroke using qPCR. MiRNA-210 was found to be significantly decreased in acute stroke patients compare to healthy controls especially at day 7 and day 14 after onset. The miR-210 level was also found to be significantly higher in acute stroke patients with good outcome measured by modified Rankin score $\leq$ 2 (64).

In 2012, a prospective case-control study by Gan et al., enrolled 32 ischemic stroke patients aged between 18-49 years old and 14 healthy controls. The miRNA-145 expression, which is a modulator of smooth muscle cell phenotype regarding the oscillating state, was evaluated. A vascular smooth muscle cell has plasticity and can adapt its phenotype in response to extrinsic stimuli. Using quantitative PCR, miRNA-145 expression in acute ischemic stroke patients is significantly higher than healthy control subjects (65).

3.2) Plasma as a source for miRNA

In 2013, Long et al. enrolled 197 ischemic stroke patients and 50 healthy controls in a prospective case-control study. The plasma miRNA-30a, miR-126 and let-7b expression in patients with acute ischemic stroke at 24 hours, 1 weeks, 4 weeks, 24 weeks, and 48 weeks after ischemic stroke onset were evaluated using qPCR. Regardless of

stroke etiology, miR-30a and miR-126 were markedly down regulated after acute ischemic stroke until 24 weeks. In patients with large vessel atherosclerosis, the let-7b level was lower than healthy volunteers whereas in among patient with stroke due to other etiology, the let-7b level was higher than healthy controls (66).

In 2017, Jin et al. evaluated 28 pro-angiogenic and anti-angiogenic miRNAs in patients with acute ischemic stroke compared with controls. Ten ischemic stroke patients and 10 controls were included in the exploring phase followed by validation in 106 stroke patients and 110 controls. Quantitative PCR was used for plasma level miRNAs evaluation. MiR-222, miR-218, and miR-185 plasma levels were elevated in patient with acute ischemic stroke. In addition, miR-126, miR-130a, miR-222, miR-218, and miR-185 were independent predicting factors for acute ischemic stroke in univariate and multivariate logistic regression analysis. A combination of these 5 miRNAs revealed good diagnostic value with an area under curve (AUC) value of 0.767 (95% CI 0.705-0.829), sensitivity of 87.7% and specificity of 54.5% (67).

3.3) Serum as a source for miRNA

Serum expression levels of miR-15a, miR-16, and miR-17-5p in 106 patients with acute ischemic stroke and 120 healthy controls were evaluated by Wu et al. in 2015. The serum expression levels of miR-15a, miR-16, miR-17-5p increased by 8.3, 42, and 9.9 fold respectively in patients with acute ischemic stroke compared to healthy controls. In addition, serum miR-17-5p level was an independent predictor for the presence of acute ischemic stroke in the multivariate logistic regression (68).

In 2015, Jia et al. evaluated 9 previously reported stroke-associated miRNAs in serum of 146 acute ischemic stroke patients and 96 healthy controls by using qPCR. In addition, plasma level of high-sensitivity C-reactive protein (hs-CRP) and serum interleukin-6 (IL-6) were measured by enzyme-linked immunosorbent assay. Serum miR-

145 was significantly upregulated within 24 hours after ischemic stroke. There was a strong positive correlation between serum miR-145 and hs-CRP and a moderate correlation between serum miR-145 and IL-6 (69).

The expressions of 17 previously reported stroke-associated miRNAs in serum of acute ischemic stroke patients and controls were evaluated by Chen et al in 2018. Serum miR-146b was significantly increased in stroke patients compared with controls. In addition, serum miR-146b had strong positive correlation with plasma hs-CRP, infarct volume, and NIHSS and moderate positive correlation with serum IL-6 (70).

Table 1: Summary of miRNA and ischemic stroke study

MiRNA analysis Methodology	Study design	Number of Case	Number of Control	Comparison	Sample Source	Results
RNA sequencing and selective qPCR						
Tiedt et al., 2017	Prospective case-control study	screen:20 validation:40 replication:200	screen:20 validation:40 replication:100	Ischemic stroke patient vs. healthy control	Plasma	miR-125b-5p, miR-143-3p significantly up regulated in acute stroke patients and normalized after day 2 miR-125a-5p up regulated in acute stroke patients up to 90 days
miRNA microarray and selective qPCR						
Tan et al., 2009	Prospective case-control study	19	5	Stroke in the young patient vs. healthy control	Whole blood	17 miRNAs => up regulated 8 miRNAs => down regulated across 3 stroke subtypes
Sepramaniam et al., 2014	Prospective case-control study	169	24	Ischemic stroke patient vs. Risk factors for ischemic stroke control	Whole blood	5 miRNAs consistently changed in stroke patients regardless of age, severity, risk factors
Jickling et al., 2014	Prospective case-control study	24	24	Ischemic stroke patient vs. risk factors for ischemic stroke control	Whole blood	6 miRNAs => up regulated 2 miRNAs => down regulated in stroke patients
Wang et al., 2014	Prospective case-control study	136	116	Ischemic stroke patient vs. healthy control	Plasma	17 miRNAs candidates hsa-miR-106b-5P,-4306 => up regulated in stroke patients hsa-moR-320e,-320d => down regulated in stroke patients
Li et al., 2015	Prospective case-control study	53	50	Ischemic stroke patient vs. healthy control	Serum	serum miR-32-3p, miR-106-5p, miR-1246 were significantly increased in ischemic stroke patients.
Quantitative PCR						
Zeng et al., 2011	Prospective case-control study	112	60	Ischemic stroke patient vs. healthy control	Whole blood	miRNA-210 significantly decreased in stroke patients
Gan et al., 2012	Prospective case-control study	32	14	Ischemic stroke patient vs. healthy control	Whole blood	miRNA-145 significantly increased in stroke patients
Long et al., 2014	Prospective case-control study	197	50	Ischemic stroke patient vs. healthy control	Plasma	miR-30a, miR-126 => down regulated in stroke patients let-7b => down regulated in large vessel atherosclerosis let-7b => up regulated in other aetiology
Jin et al., 2017	Prospective case-control study	screen:10 validate:106	screen:10 validate:110	Ischemic stroke patient vs. healthy control	Plasma	plasma miR-222, miR-218, and miR-185 levels were significantly increased in ischemic stroke patients
Wu et al., 2015	Prospective case-control study	105	121	Ischemic stroke patient vs. healthy control	Serum	serum miR-15a, miR-16, and miR-17-5p levels were significantly increased in ischemic stroke patients
Jia et al., 2015	Prospective case-control study	screen:30 validate:146	screen:30 validate:96	Ischemic stroke patient vs. healthy control	Serum	serum miR-145 was significantly increased in ischemic stroke patients
Chen et al.,2018	Prospective case-control study	screen:30 validate:128	screen:30 validate:102	Ischemic stroke patient vs. healthy control	Serum	serum miR-146b was significantly increased in ischemic stroke patients

As reviewed above, different body fluids were used as miRNA sources. Using different miRNA source may result in different miRNA expression profile. Consensus on optimal sample source is still lacking. A study by Shah et al. from 2,391 individuals revealed that miRNA from plasma has a discordant expression from that of whole blood (71). Wang et al. compare miRNA concentration between that from serum and plasma. Higher miRNA concentration was observed from serum samples compare to plasma samples. This may be in part due to the coagulation process (72).

2.5.3 Potential miRNA candidates as stroke biomarker

Several miRNAs had been studied as potential biomarker for diagnosis of acute stroke using different sources of miRNAs and different miRNA detection techniques. Among several studies, there are some potential miRNA candidates that had been replicated with subsequent study or had been studied through several cohorts in the discovery phase and validation phase. In 2011, Weng et al. identified tissue-specific miRNA by using miRNA microarray to evaluate the miRNA expression among various tissues. Among miRNAs significantly detected in the cerebrum and cerebellum, hierarchical clustering analysis revealed that there were 13 miRNAs specific to brain tissue. Among these, miR-124 showed the highest signal on the subsequent validation by qPCR. In addition, miR-124 was detected exclusively in brain tissue with the strongest signal from the cerebellum. In rat model of middle cerebral artery occlusion, plasma concentration of miR-124 was significantly increased in animal model compared to sham-operated group (73). In subsequent study, Ji et al. evaluated the serum exosomal miR-124 among patients with acute ischemic stroke. Serum exosomal miR-124 were significantly increased among acute ischemic stroke patients. In addition, there were significant correlations between serum exosomal miR-124 level and NIHSS score and the infarct volume (74).

Using RNA sequencing in the discovery phase of 20 ischemic stroke patients and 20 healthy controls, Tiedt et al. identified 4 miRNAs upregulated among stroke patients. Subsequent validation by qPCR performed in 40 ischemic stroke patients and 40 healthy controls, 3 out of 4 miRNAs including miR-143-3p, miR-125b-5p, and miR-125a-5p were significantly upregulated among ischemic stroke patients. The replication study consisted of 200 ischemic stroke patients 100 healthy controls, and 72 TIA patients revealed consistent result of significantly high expression levels of all 3 miRNAs among stroke patients. In longitudinal analysis using generalized linear mixed model, miR-125b-5p and miR-143-3p were associated with stroke severity assessed by NIHSS. However, there was no significant correlation between the miRNA expression levels and the infarct volume (58).

2.6 NanoString nCounter Technology

NanoString nCounter Technology is the novel gene expression analysis platform. The technology is based on high-throughput multiplex, fluorescence-based digital hybridization which assess gene expression up to 800 genes simultaneously. The system using molecular barcode chemistry consists of gene-specific molecular barcode reporter probes and biotin-labeled capture probes. The CodeSet probes including reporter and capture probes can be designed to hybridize RNA or DNA. After an overnight hybridization, the molecular barcodes are quantify by direct counting using automated digital analyzer. The counts represent the gene expression within the sample. This technology has major advantage including no requirement of library preparation and amplification, by which bias can be introduced. In addition, the technique can be applied in variety of specimens such as formalin-fixed tissues, cell lysates, and biological fluid samples (75, 76). This technique demonstrated higher sensitivity compared to microarray and similar sensitivity when compared to real-time PCR (76).

To date, miRNA expression analysis in acute stroke by NanoString nCounter Technology has not been studied. This technique is a promising tool for miRNA biomarker discovery. Since there are several problems regarding both physical examination and neuroimaging in patients with acute vertigo as previously mentioned, the discovery of biomarker to help diagnose this condition would be valuable to both physicians and the patients.

Chapter 3 Research Methodology

3.1 Research design

This is a prospective longitudinal study. The study was approved by the Institutional Review Board of the Faculty of Medicine, Chulalongkorn University (IRB No. 461/2560).

3.2 Research methodology

Population

Patients presented at King Chulalongkorn Memorial Hospital with acute vertigo

Target population

Patients presented at King Chulalongkorn Memorial Hospital with acute vertigo within 72 hours after onset.

Inclusion criteria

1. Age ≥ 45 years old
2. Clinical of acute vertigo including vertiginous symptoms with sense of spinning and imbalance.
3. Presented at King Chulalongkorn Memorial Hospital within 72 hours after onset of vertigo

Exclusion criteria

1. Alteration of consciousness
2. Body temperature > 38 Celsius
3. White blood cell count > 15,000 cell/μL
4. Abnormal renal function defined by serum creatinine > 2 mg/dl
5. Abnormal liver function defined by elevated liver enzyme > 3 fold of upper normal limit or cirrhosis

6. History of known active malignancy
7. History of autoimmune disease such as systemic lupus erythematosus, rheumatoid arthritis
8. Concurrent intracranial pathology other than acute ischemic stroke such as brain tumor, intracerebral hemorrhage

Observation and measurement

1. Neurological and otolaryngological examination were performed by a neurological resident or certified board neurologist and otolaryngological resident or certified board otolaryngologist respectively at King Chulalongkorn Memorial Hospital.
2. Ischemic stroke severity was measured by National Institute of Health Stroke Scale (NIHSS) and assessed by a trained physician.
3. Neuroimaging (computed tomography or magnetic resonance imaging) was done within 1 week after onset of vertigo
4. Blood sample was taken within 72 hours after onset of vertigo. Serum was isolated for microRNA and protein analysis.
5. Screening for miRNAs candidates was performed by nCounter® miRNA expression assay (NanoString® technologies, Seattle, WA). The miRNAs candidates were validated by quantitative polymerase chain reaction (qPCR).
6. Serum NSE, S-100, IL-6 were measured by electrochemiluminescence technique using Cobas® 6000 analyzer series (Roche Diagnostics, Mannheim, Germany).

3.3 Research maneuver

3.3.1 Clinical procedures

- *Patient screening*

1. History taking and physical examination will be done on patient presented with acute vertigo at King Chulalongkorn Memorial Hospital.
2. Patient will be screened for inclusion and exclusion criteria prior to enrollment in the project.
3. Research project information will be explained to the patient and his/her relatives. The patient and his/her relatives are able to ask the investigator regarding the project detail. The decision on participating in the research project is voluntary and withdrawal of the consent can be done at any time.

- *Diagnosis of acute vertigo*

a. **Central vertigo**

- Acute ischemic stroke due to cerebellar/brainstem infarction will be diagnosed by a presence of an episode of neurological dysfunction caused by focal cerebral infarction based on clinical evidence of persisting symptoms more than 24 hours or imaging evidence of cerebral infarction in a defined vascular distribution (77).

b. **Peripheral vertigo**

- Benign Paroxysmal Positional Vertigo (BPPV) will be diagnosed according to the Consensus document of the Committee for the Classification of Vestibular Disorders of the Barany Society (78). The definite diagnosis of BPPV requires the observation of a canal-specific positional nystagmus following the diagnostic positional maneuvers.

a) *Canalolithiasis of the posterior canal*: a combination of torsional nystagmus with the upper pole of the eyes beating toward the lower ear combined with vertical upbeating nystagmus typically lasting < 1 minute elicited by the Dix-Hallpike or Semont diagnostic maneuver.

b) *Canalolithiasis of the horizontal canal*: Geotropic direction changing horizontal nystagmus lasting < 1 minute elicited by the supine role test. Apogeotropic direction changing horizontal nystagmus may be observed when otoconia are located close to the cupula.

c) *Cupulolithiasis of the horizontal canal*: Apogeotropic direction- changing nystagmus lasting > 1 minute elicited by the supine roll test.

d) *Canalolithiasis of the anterior canal*: Positional nystagmus predominantly vertically downbeat lasting < 1 minute elicited by the Dix-Hallpike maneuver or the supine straight head-hanging position.

The diagnostic criteria of BPPV also require the recurrent attacks of positional vertigo or dizziness provoked by lying down or turning over in the supine position. In addition, the exclusion other disorder is necessary especially in a patient with direction-changing apogeotropic positional nystagmus which can be attributed to CNS disorder (78).

- Meniere's disease will be diagnosed according to the diagnostic criteria for Meniere's disease published in 2015 by the joint

committee from the Classification Committee of the Barany Society, the Japan Society for Equilibrium Research, the European Academy of Otology and Neurotology (EAONO), the Equilibrium Committee of the American Academy of Otolaryngology-Head and Neck Surgery (AAO-HNS), and the Korean Balance Society (79). The definite diagnosis of Meniere's disease requires

a) Two or more spontaneous episodes of vertigo, each lasting 20 minutes to 12 hours.
b) Audiometrically documented low- to medium- frequency sensorineural hearing loss in one ear before, during, or after one of the episodes of vertigo.
c) Fluctuating aural symptoms including hearing, tinnitus, or fullness in the affected ear.
d) Not better accounted for by another vestibular diagnosis.

- Vestibular neuritis is a diagnosis of exclusion and no confirmatory diagnostic tests are available (80). The diagnosis was primarily clinical based with the diagnostic hallmarks including (80)

a) Spontaneous horizontal-torsional nystagmus beating away from the lesion side.
b) Abnormal head impulse test for the involved semicircular canal
c) Ipsilesional caloric paresis
d) Decreased responses of vestibular-evoked myogenic potentials during stimulation of the affected ear
e) Ipsiversive ocular tilt reaction and ipsiversive tilt of the subjective visual vertical/horizontal

f) Unsteadiness with a falling tendency toward the lesion side

- *Data and specimen collection*

1. Patient's data regarding history, physical examination, and investigation will be a recorded in the case record form.
2. Venous blood 8 ml will be obtained and collected in two blood collection tubes containing clot activator (Red cap, vacuette®). Each blood collection tube contains 4 ml of venous blood. One tube will be used in microRNA analysis; the other will be used for serum NSE, S-100, and IL-6 assay. The blood collection in acute phase will be done in acute phase within 72 hours after onset of vertigo at day 0, and day 90 after enrollment.
3. Serum isolation
    a. Leave blood collection tube at room temperature for 30 min-1hour for complete coagulation
    b. Centrifuge tube for 15 minutes at 1000g, 4 ºC
    c. Transfer upper serum phase to new tube
    d. Centrifuge serum collected previously for 15 minutes at 2500g, 4 ºC to remove residual debris
    e. Transfer cleared supernatant to a new tube without disturbing the pellet.
    f. Store serum in 1.5 ml tube at -80 ºC until used in miRNA and protein analysis

- *Follow-up visit at 3 month*

The patients enrolled in the studies will have the follow-up visit scheduled at 3 months after acute vertigo onset both cerebellar infarction and peripheral vertigo group. Data will be collected in the case record form. The serum collection

for miRNA analysis and protein biomarker, miRNA isolation protocol, and miRNA sequencing will be similar to that described previously in acute onset vertigo.

3.3.2 Laboratory procedures

- *MiRNA isolation*

1. Thaw 200 µl of frozen serum.
2. microRNA isolation protocol from serum using Geneaid® miRNA isolation kit is as follows:
    a. Add 200 µl of Lysis Buffer to 200 µl of serum, then vortex vigorously and incubate at room temperature for 10 minutes
    b. Add 20 µl of Mi Buffer and 180 µl of double-distilled water (ddH_2O) saturated phenol and 40 µl of chloroform.
    c. Vortex vigorously for 2 minutes then centrifuge at 14,000 g for 3 minutes.
    d. Transfer the upper phase to 1.5 ml microcentrifuge tube.
    e. Add 35% volume of absolute ethanol to upper phase and mix well.
    f. Place a RNA Column in a 2 ml Collection Tube and transfer the ethanol-added mixture to the RNA Column.
    g. Incubate for 1 minute at room temperature then centrifuge at 14,000 g for 30 seconds.
    h. Transfer the filtrate to a new 1.5 ml microcentrifuge tube.
    i. Add a 70% volume of absolute ethanol to the filtrate and mix well.
    j. Place a new RNA Column in a 2 ml Collection Tube and transfer the mixture to the RNA Column.
    k. Incubate for 1 minute at room temperature then centrifuge at 14,000 g for 30 seconds to allow the miRNA to bind to the RNA Column membrane.
    l. Add 200 µl of Wash Buffer to the RNA Column and incubate for 1 minute at room temperature.

m. Centrifuge at 14,000 g for 1 minute to completely remove the liquid residue.

n. Add 30 µl of pre-heated at 65ºC into the center of RNA Column and incubate at room temperature for 3 minutes.

o. Centrifuge at 14,000 g for 3 minutes to recover the miRNA.

- *MiRNA expression assay*

The serum miRNA expression profile was performed using nCounter® miRNA expression assay as per the following protocol.

1. Day 1: miRNA sample preparation

a. Normalize RNA samples to 33 ng/µL using RNAse-free water.

b. Prepare a 1:500 dilution of the miRNA Assay Controls by adding 499 µL of DEPC water to 1 µL of the miRNA Assay Controls in a sterile microcentrifuge tube. Mix by vortexing and briefly spin down. Store on ice.

c. Prepare an annealing master mix by combining 13 µL of Annealing buffer, 26 µL of nCounter® miRNA Tag Reagent and 6.5 µL of the 1:500 miRNA Assay Controls dilution prepared in step b. Mix well by pipetting up and down.

d. Aliquot 3.5 µL of the annealing master mix into each tube of a 12*0.2 mL strip tube.

e. Add 3 µL (100 ng) of RNA sample to each tube. Cap tubes and flick tubes gently to mix. Spin down.

f. Place strip in the thermocycler and initiate Annealing Protocol (Table 2)

Table 2: Annealing Protocol for nCounter® miRNA expression assay

Temperature	Time
94 ºC	1 min
65 ºC	2 min
45 ºC	10 min
48 ºC	Hold
Total time	13 min

g. Combine 19.5 µL PEG and 13 µL Ligation Buffer to prepare a ligation master mix.

h. Following the completion of the Annealing Protocol, when the thermocycler has reached 48 ºC, add 2.5 µL of the ligation master mix to each tube. Flick tubes gently and spin down.

i. Return tubes to 48 ºC thermocycler, close lid, and incubate for 5 minutes

j. Open thermocycler, remove caps from tubes, leaving strip tube in place in the heat block, and add 1.0 µL of Ligase directly to each tube while incubating at 48 ºC. Check the pipette tip to make certain all of the ligase was added to the reaction.

k. Immediately after addition of Ligase to the final tube, recap tubes, close the thermocycler, and initiate Ligation protocol (Table 3).

Table 3: Ligation Protocol for nCounter® miRNA expression assay

Temperature	Time
48 ºC	3 min
47 ºC	3 min
46 ºC	3 min
45 ºC	5 min
65 ºC	10 min
4 ºC	hold
Total time	24 min

l. After completion of Ligation Protocol, add 1 µL Ligation Clean-Up Enzyme to each reaction. The tubes can be removed from the heat block. Flick tubes gently to mix. Spin down.

m. Return tubes to thermocycler and initiate Purification Protocol (Table4).

n. After completion of Purification Protocol, add 40 µL RNAse-free water to each sample. Mix well and spin down.

Table 4: Purification Protocol for nCounter® miRNA expression assay

Temperature	Time
37 ºC	1 hour
70 ºC	10 min
4 ºC	hold
Total time	1 hour 10 min

2. Day 1: Setting up for hybridization

    a. Remove aliquots of both the Reporter CodeSet and Capture ProbeSet reagent from the freezer and thaw on ice. Invert several times to mix well and briefly spin down reagent at <100 rpm.

    b. Create a master mix containing 130 µL of the Reporter CodeSet and 130 µL of hybridization buffer by adding the hybridization buffer to the tube containing the Reporter CodeSet. Invert to mix and spin down master mix.

    c. Label a provided 12 tube strip and cut it in half to fit in a picofuge.

    d. Add 20 µL of master mix to each of the 12 tubes.

    e. Denature samples from the miRNA sample prep protocol at 85 ºC for 5 minutes and quick-cool on ice. Add a 5 µL aliquot from the miRNA Sample Preparation Prococol to each tube.

    f. Pre-heat thermocycler to 65 ºC. Program the thermocycler using 30 µL volume, calculated temperature, heated lid and “forever” time setting.

    g. Add 5 µL of Capture ProbeSet to each tube immediately before placing at 65 ºC. Cap tubes and mix the reagents by inverting the strip tubes several times and flicking to ensure complete mixing. Briefly spin down at < 1000 rpm and immediately place the strip tube in the 65 ºC thermocycler. Minimizing the time between the addition of the Capture ProbeSet and the placement of the reaction at 65 ºC to increase the sensitivity of the assay.

    h. Incubate hybridization assays for at least 12 hours. Hybridizations should be left at 65 ºC until ready for processing.

    i. Removed strip tube from the thermocycler, proceed to post-hybridization processing with the nCounter® Prep Station.

3. Day 2: Setting up prep station run and data collection (automated processing)
    a. Data analysis was performed by nSolver version 4.0 software.
    b. MiRNAs of interest were selected using Venn diagram (81).

- *Quantitative reverse transcription polymerase chain reaction (RT-qPCR)*

MiRNAs of interest were quantified by RT-qPCR consisted of 3 steps including polyuridylation, reverse transcription (RT) reaction, and real-time PCR. MiRNA was polyuridylated by poly(U) polymerase. Then, cDNA molecules were reverse transcribed by the universal poly(A) stem-loop RT primers (SL-poly(A) or SL-Poly(A)-M13). Finally, the quantification was performed by real-time PCR analysis using the SYBR® Green fluorescence. During PCR, the 3'-end of the forward primer was miRNA-specific and was hybridized to the cDNA molecule of the specific miRNA; whereas the universal reverse primer was hybridized to the stem-loop portion (82). The detail protocol is as follow:

a. Prepare master mix for polyuridylation (Table 5)

Table 5: Polyuridylation master mix preparation

Master mix	1 Reaction (µL)
10X NEBuffer™ (New England BioLabs®)	2.5
50 mM UTP (Thermo Scientific™)	0.25
40U/µL RiboLock (Thermo Scientific™)	1.00
2U/µL PolyU polymerase (New England BioLabs®)	1.00
miRNA from serum extraction (fix volume)	10
Distilled Water to a final volume of	25
Total volume	25

b. Incubate at 37 ºC for 10 minutes

c. Add 0.4 µL of 10 µM SL-Poly(A) (or SL-Poly(A)-M13)

d. Incubate at 65 ºC for 5 minutes then place on ice for 2 minutes

e. Prepare master mix for reverse transcription (Table 6)

Table 6: Reverse transcription master mix preparation

Master mix (Thermo Scientific™)	**1 Reaction (µL)**
5X RT Buffer	8
10 mM dNTPs	4
200U RevertAid	2
40U/µL RiboLock	1
Total volume	15

f. Incubate at 42 ºC for 60 minutes

g. Inactivate at 70 ºC for 10 minutes

h. Prepare master mix for real-time PCR (Table 7)

Table 7: Real-time PCR master mix preparation

Master mix	**1 Reaction (µL)**
Luna qPCR Master Mix (New England BioLabs®)	5
10 µM Forward primer	0.25
10 µM Reverse primer	0.25
Distilled Water	3.5
cDNA template	1
Total volume	10

i. The designed primers of miRNAs of interest are showed in Table 8.

j. Set the thermocycler condition for PCR according to each miRNA (Table 9).

Table 8: Designed forward primers for miRNA candidate

No.	miRNA candidate	Sequence 5'-3'	Length (bp)	A/T	C/G	Tm
1	hsa-miR-124-3p	GCACGCGGTGAATGCCA	17	6	11	56
2	hsa-miR-124-5p	TGTTCACAGCGGACCTTG	18	8	10	56
3	hsa-miR-125a-5p	TCCCTGAGACCCTTTAACC	19	9	10	58
4	hsa-miR-125b-5p	TCCCTGAGACCCTAACTTG	19	9	10	58
5	hsa-miR-143-3p	AGATGAAGCACTGTAGCTC	19	10	9	56
6	hsa-miR-342-3p	ACACAGAAATCGCACCCGT	19	9	10	58
7	hsa-miR-376a-3p	ATCATAGAGGAAAATCCACG	20	12	8	56
8	hsa-miR-433-5p	GGTGAGCCTGTCATTATTC	19	10	9	56
9	hsa-miR-584-3p	TTCCAGGCCAACCAGGCT	18	7	11	58
10	hsa-miR-584-5p	GGTTTGCCTGGGACTGAG	18	7	11	58

Table 9: Thermocycler condition for real-time PCR

No.	miRNA candidate	Pre-denature	Denature	Annealing	Extension
1	hsa-miR-124-3p	95 ° C 3 minutes	95 ° C 15 seconds	51° C, 30 sec	72 ° C 20 seconds
2	hsa-miR-124-5p			51° C, 30 sec	
3	hsa-miR-125a-5p			51° C, 30 sec	
4	hsa-miR-125b-5p			53° C, 30 sec	
5	hsa-miR-143-3p			53° C, 30 sec	
6	hsa-miR-342-3p			51° C, 30 sec	
7	hsa-miR-376a-3p			51° C, 30 sec	
8	hsa-miR-433-5p			51° C, 30 sec	
9	hsa-miR-584-3p			51° C, 30 sec	
10	hsa-miR-584-5p			53° C, 30 sec	

- *Generating standard curve for absolute quantification of target miRNAs*

Absolute quantification to detect the amount of the target miRNAs in serum sample was performed by the standard curve method. Standard curve was generated by serial dilution of the known concentration of the target miRNAs. The absolute amount of the target miRNAs from qPCR was calculated based on the standard curve. The protocol for generating the standard curve is as follows:

1. Amplification of miRNA target

    a. Extract miRNAs from U87 and 5Y cell line (courtesy of Dr. Naphat Chantaravisoot) using miRNA extraction protocol as described previously.

    b. Reverse transcribe miRNAs into cDNA by using the universal poly(A) stem-loop RT primers (SL-poly(A) or SL-Poly(A)-M13) as per protocol described previously (Table 5,6).

    c. Amplify cDNA molecules of target miRNAs using *Taq* DNA polymerase (biotechrabbit™). Prepare the master mix as shown in Table 10.

    d. Pipet the master mix into thin-walled 0.2 ml PCR tubes.

    e. Mix and centrifuge briefly to collect the liquid in the bottom of the tube. Place PCR tubes in the thermocycler.

    f. Set the thermocycler condition as shown in Table 9.

Table 10: PCR master mix preparation

Master mix	1 Reaction (µL)
10X Reaction buffer	5
50 mM $MgCl_2$	1.5
10 mM dNTP Mix	1
10 µM Forward primer	1
10 µM Reverse primer	1
Template DNA	1
Taq DNA polymerase (biotechrabbit™)	0.5
Nuclease free water to a final volume of	50
Total volume	50

2. Run 2% agarose gel electrophoresis 100V for 45 minutes to analyze the PCR products.

3. Cut target band from gel electrophoresis and purify gel as per the following protocol.
    a. Excise the DNA fragment from the agarose gel with a clean and sharp scalpel.
    b. Weigh the gel slice in a colorless tube. Add 3 volumes Buffer QG to 1 volume gel (100 mg gel ~100 µL)
    c. Incubate at 50 ºC for 10 minutes or until the gel slice has completely dissolved. Vortex the tube every 2-3 minutes to help dissolve gel. After the gel slice has dissolved completely, check that the color of the mixture is yellow. If the color is orange or violet, add 10 µL 3M sodium acetate, pH 5.0, and mix.
    d. Add 1 gel volume isopropanol to the sample and mix.
    e. Place a QIAquick® spin column in a provided 2 ml collection tube. Apply the sample to the QIAquick® column and centrifuge for 1 minute. Discard flow-through and place the QIAquick® column back into the same tube.
    f. To wash, add 750 µL Buffer PE to QIAquick® column column and centrifuge for 1 minute. Discard flow-through and place the QIAquick® column back into the same tube. Centrifuge the QIAquick® column in the provided 2 ml collection tube for 1 minute to remove residual wash buffer.
    g. Place QIAquick® column into a clean 1.5 ml microcentrifuge tube.
    h. To elute DNA, add 30 µL Buffer EB (10 mM Tris.Cl, pH 8.5) or water to the center of the QIAquick® membrane and centrifuge the column for 1 minute.
4. Cloning PCR products

cDNA molecules of the miRNAs of interest are cloned for absolute quantification of the miRNAs as per the following protocol.

1. Ligation using 2X Rapid Ligation Buffer

a. Briefly centrifuge the pGEM®-T Easy Vector and Control Insert DNA tubes to collect contents at the bottom of the tube.

b. Set up ligation reaction as shown in Table 11. Vortex the 2X Rapid Ligation Buffer vigorously before each use. Use 0.5 ml tubes known to have low DNA binding capacity.

c. Mix the reactions by pipetting. Incubate the reactions 1 hour at 22 ºC.

Table 11: Ligation reaction master mix preparation

Master mix	1 Reaction (µL)
2X Rapid Ligation Buffer, T4 DNA Ligase (Thermo Scientific™)	5
pGEM®-T Easy Vector (Promega)	1
PCR product	X*
T4 DNA Ligase (Thermo Scientific™)	1
Distilled water to a final volume of	10

*X is 5:1 molar ratio of insert:vector. The amount of PCR product in master mix was calculated according to www.insilico.uni-dusseldorf.de/Lig_input.html

2. Transformation of DH5α High Efficiency Competent Cells

a. Prepare Luria Broth (LB)/ampicillin agar plates as shown in Table 12 and add 15 ul of XGal and 5ul of IPTG prior to use.

Table 12: LB/ampicillin plates preparation

Reagents	Amount
Yeast extract (HiMedia®)	0.5 g
Tryptone (HiMedia®)	1 g
NaCl (Merck)	0.5
Agar (HiMedia®)	1.5 g
Distilled water	100 µL

b. Centrifuge the ligation reactions briefly. Add 2 µL of each ligation reaction to a sterile 1.5 ml tube on ice. Prepare a control tube with 0.1ng of uncut plasmid.

c. Place the DH5α High Efficiency Competent Cells in an ice bath until just thawed (5 minutes). Mix cells by gently flicking the tube.

d. Mix 50 µL DH5α High Efficiency Competent Cells and 10 of µL of ligation reaction. Incubate on ice for 30 minutes. (Proceed similar method for uncut DNA control tube).

e. Heat-shock the cells for 45-50 seconds in a water bath at 42 ºC. Immediately return the tubes to ice for 2 minutes.

f. Add 950 µL room temperature Super Optimal broth with Catabolite repression (SOC) medium to the ligation reaction transformations and 900 µL to the uncut DNA control tube. Incubate for 1.5 hours at 37 ºC with shaking (150 rpm).

g. Plate 100 µL of each transformation culture onto duplicate LB/ampicillin/IPTG/X-Gal plates (prepared in step a.).

h. Incubate plates overnight at 37 ºC. Select white colonies.

5. Perform colony PCR to check the presence of the insert DNA in plasmid constructs.

   Using M13 Forward and Reverse primer with the following sequence.

   M13_F 5'-GTTTTCCCAGTCACGAC

   M13_R 5'-TCACACAGGAAACAGCTATGAC

Table 13: Thermocycler condition for colony PCR

Step	Temperature	Time
Pre-denature	95 ºC	2 min
Denature	95 ºC	30 sec
Annealing	55 ºC	30 sec
Extension	72 ºC	30 sec
Final extension	72 ºC	2 min

6. Propagate the selected colony in 1.5-2.0 ml. LB broth with ampicillin as prepared in Table 12 without adding agar.
7. Extract plasmid DNA from the cultured DH5α High Efficiency Competent Cells using Plasmid miniprep kit (BioHelix) as per the following protocol.
    a. Transfer 1.5 ml bacterial culture into a microcentrifuge tube and centrifuge at 14,000 g for 1 minute. Discard the supernatant.
    b. Resuspend pelleted bacterial cells in 200 µL of the Buffer S1
    c. Add 200 µL of Buffer S2 and mix thoroughly by inverting the tube 10 times and incubate at room temperature for 2 minutes or until the lysate is homogeneous.
    d. Add 300 µL of Buffer S3 and mix immediately and thoroughly by inverting the tube 10 times. Centrifuge at 14,000 g for 3 minutes.
    e. Place a PM column in a Collection Tube. Apply supernatant from step d. to the column. Centrifuge at 14,000 g for 30 seconds, then discard the flow-through, and place the PM column back into the same collection tube.

f. Add 400 µL of Buffer W1 into the PM column. Centrifuge at 14,000 g for 30 seconds. Discard the flow-through, and place the PM column back into the same collection tube.

g. Add 600 µL of Buffer W2 into the PM column. Centrifuge at 14,000 g for 30 seconds. Discard the flow-through, and place the PM column back into the same collection tube.

h. Centrifuge at 14,000 g for 2 minutes to remove excess Buffer W2.

i. Elute DNA by placing the PM column in a clean 1.5 ml microcentrifuge tube. Add 50-200 µL of the Buffer E to the center of each PM column. Incubate for 2 minutes then centrifuge at 14,000 g for 2 minutes.

8. Sending the plasmid construct for Sanger's sequencing (1st Base company, Singapore) to confirm the sequence of amplified cDNA molecule of target miRNA.

9. Generation of standard curve by serial dilution.

a. Measure the PCR product concentration by Qubit™ DNA-HS Assay Kit (Thermo Fisher Scientific)

b. Change the concentration from ng/µL to copies/µL by using the following formula.

$$\mathrm{N(copies/\mu l)} = \frac{\mathrm{conc.\,(ng/\mu l)} \times 10^{-9} \times 6.02 \times 10^{23}}{660 \times (\mathrm{vector + insert\ length\ (bp)})}$$

c. Calculate the volume of the PCR product required for serial dilution (V_1) by using the following formula.

$$C_1V_1=C_2V_2$$

C_1=stock concentration (as calculated in step b.)

C_2=final concentration (starting concentration for serial dilution: 10^9 copies/µL)

V_1=stock volume required for serial dilution

V_2=volume required for starting serial dilution

d. Generate 10 fold serial dilution starting at 10^9 copies/µL by adding 9 ml of distilled water to 1 ml of PCR product from previous concentration.

10. Perform RT-qPCR with absolute quantification using standard curve method to quantify the amount of miRNA of interest in serum.

- *Protein biomarker detection via electrochemiluminescence assay*

The serum protein biomarkers detection including NSE, S100, and IL-6 was performed by electrochemiluminescence immunoassay (ECLIA) using Cobas® 6000 analyzer series (Roche Diagnostics, Mannheim, Germany) as per the following protocol.

1. Thaw 20 µL of frozen serum until reaching 20-25 ºC.
2. Place sample into 5-position racks, then onto the sample loader.
3. Bring the cooled reagents to approximately 20 ºC and place on the reagent disc.
4. The sample is loaded into the automated analyzer.
5. The 1st incubation is the formation of a sandwich complex of sample, biotinylated monoclonal antigen (NSE, S100, or IL-6) specific antibody and a monoclonal antigen (NSE, S100, or IL-6) specific antibody labeled with ruthenium complex.
6. The 2nd incubation is the addition of streptavidin-coated microparticles to bound the sandwich complex to the solid phase via the interaction of biotin and streptavidin.
7. The reaction mixture is aspirated into the measuring cell.

8. The microparticles are magnetically captured onto the surface of the electrode. Unbound particles are removed.
9. A voltage is applied to the electrode to induce the chemiluminescent emission which is measured by a photomultiplier.
10. The level of the biomarkers of interest are determined via a calibration curve.

- *Magnetic Resonance Imaging data acquisition*

MRI studies were performed using a 3-Tesla MR scanner (Skyra, Siemens, Erlangen, Germany). The imaging sequences were: T1-weighted image(TE 9 milliseconds; TR 2000 milliseconds; NEX 1; DFOV 59.6*22 cm; matrix size 272*320; slice thickness/slice spacing 5/6 mm), T2-weighted image (TE 88 milliseconds; TR 5400 milliseconds; NEX 1; DFOV 37.9*22 cm; matrix size 272*320; slice thickness/slice spacing 5/6 mm), FLAIR (TE 81 milliseconds; TR 8000 milliseconds; NEX 1; DFOV 37.9*22 cm; matrix size 256*320; slice thickness/slice spacing 5/6 mm), DWI (TE 64 milliseconds; TR 4900 milliseconds; NEX 1; DFOV 63*23 cm; matrix side160*160; slice thickness/slice spacing 5/6 mm, b=0, 1000 s/mm^2). The ADC was generated automatically.

- *Volumetric measurement of cerebral infarction*

The cerebral infarction volume was quantified by using diffusion weighted images on MRI. The measurement of infarction volume was processed by Olea Sphere™ Version 3.0 software (Olea Medical®, La Ciotat, France).

3.4 Statistical analysis

3.4.1 Sample size calculation

The sample size calculation was performed after conducting a pilot study of total 28 patients. After collecting specimens consisted of 14 patients in each group, the miRNA extraction from serum was performed. However, due to the nature of low

concentration of miRNA in serum which was not suitable for next generation sequencing, the technique of identifying potential miRNA candidates was changed from next generation sequencing to molecular barcoding by NanoString nCounter Technology which requires lower concentration of miRNA in the specimen. We re-calculated the appropriate sample size using results from total 28 patients as a pilot study. Due to the non-normally distributed data in the pilot study, the following equation was used for sample size calculation to compare difference median (83).

$$n = \frac{(\sigma_1^2 + \sigma_2^2)\left(z_{\frac{\alpha}{2}} + z_\beta\right)^2}{(\log(m_1) - \log(m_2))^2}$$
$$= \frac{\left[\log\left(\frac{1}{2} + \sqrt{\frac{1}{4} + \frac{\phi_1^2}{m_1^2}}\right) + \log\left(\frac{1}{2} + \sqrt{\frac{1}{4} + \frac{\phi_2^2}{m_2^2}}\right)\right]\left(z_{\frac{\alpha}{2}} + z_\beta\right)^2}{(\log(m_1) - \log(m_2))^2}.$$

From the pilot study, the median and standard deviation for central and peripheral vertigo are m1 = 87.97, ϕ_1= 76 and m2 = 43.03, ϕ_2= 36.73 respectively. An appropriate sample size for detecting significance difference with alpha at 0.05 ($z_{\alpha/2}$ = 1.96) and power of 80% ($z_{\mathcal{B}}$ = 0.84) consisted of 28 patients per group. We estimate 25% of missing patients at 3 months follow up period. Therefore, a sample size of 35 patients in each group is required in the study.

3.4.2 Data collection

Variables collected in the study and method of data collection are shown in Table 14.

Table 14: Data collection

Variables		Method
Demographic Variables	Date and time of visit/symptom onset	Interview
	Date and time of blood drawn	Interview
	Age, Gender	Interview
Risk factors	HT, DM, DLP, IHD	Interview
	Smoking, Alc	Interview
Current medication	Name, dosage	Interview
Vital signs	BT, BP, PR, RR	Measure from calibrated device
Physical Exam	Neurovascular exam	Observation from examination
	Head impulse test	Observation from examination
	Nystagmus	Observation from examination
	Skew deviation	Observation from examination
	NIHSS	Observation from examination
	mRS	Observation from examination
Investigation	EKG	Observation
	CT	Observation
	MRI	Observation
	Vascular imaging	Observation
	CBC, blood chemistry	Measure from calibrated device
	miRNA expression	Measure from calibrated device
	NSE, S100, IL-6 level	Measure from calibrated device

3.4.3 Data summary

Summary of variable details are shown in Table 15.

Table 15: Data summary

Variables		Type of data	Central Tendency	Deviation
Demographic variables	Age	Ratio	Mean	SD (mean)
	Gender	Nominal	Proportion	SD (proportion)
Risk factors	HT, DM, DLP, IHD	Nominal (binary data)	Proportion	SD (proportion)
	Smoking, Alc	Nominal (binary data)	Proportion	SD (proportion)
Physical Exam	Neurovascular exam	Nominal (binary data)	Proportion	SD (proportion)
	Head impulse test	Nominal (binary data)	Proportion	SD (proportion)
	Nystagmus	Nominal (binary data)	Proportion	SD (proportion)
	Skew deviation	Nominal (binary data)	Proportion	SD (proportion)
	NIHSS	Ordinal	Proportion	SD (proportion)
	mRS	Ordinal	Proportion	SD (proportion)
Investigation	CBC, blood chemistry	Ratio	Mean	SD (mean)
	miRNA expression	Ratio	Mean	SD (mean)
	NSE, S100, IL-6 level	Ratio	Mean	SD (mean)

3.4.4 Data analysis

- *Statistical analysis* (Please refer to Data summary table for further detail)

  1. The absolute quantification of the miRNA expression level is calculated from the standard curves generated by serial dilution of the known concentration of the target miRNAs.

2. Kolmogorov-Smirnov test was used to determine the distribution of the data.
3. Continuous variables were presented in mean ± standard deviation (SD) or median with interquartile range (IQR) when appropriate.
4. Categorical variables were presented in percentages and frequencies.
5. Unpaired T test or Mann-Whitney U test was used for comparison between baseline characteristics of the continuous data between central and peripheral vertigo groups with normal distribution and non-normal distribution respectively.
6. Pearson's chi-squared or Fisher's exact test was used for comparison between categorical variables.
7. Mann-Whitney U test was used to compare miRNA or protein level between central and peripheral vertigo groups.
8. Wilcoxon signed rank test was used to compare miRNA or protein level within group at different time points.
9. Kruskal Wallis test with Dunn's post hoc test was used to compare miRNA level between different stroke etiologies and areas of infarction.
10. Spearman's correlation was used to demonstrate the correlation between miRNA level and infarct volume or severity of ischemic stroke defined by NIHSS.
11. Receiver operating characteristic curve was generated to demonstrate the diagnostic ability of the biomarkers.
12. Binary logistic regression was performed to identify factors that associated with central vertigo.
13. Youden's index was determined to identify the optimal cut off value both miRNA candidates and protein biomarkers

14. In case of undetectable level of serum protein biomarkers, imputation with half of the lower detection limit was performed.

- *Software for data analysis*

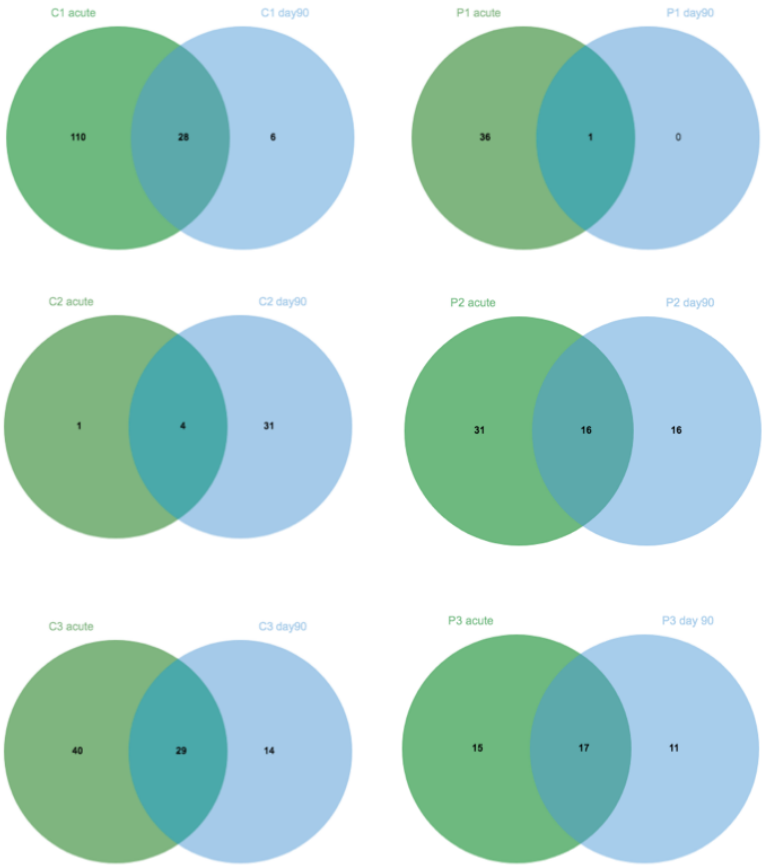
    1. Statistical analysis both qualitative and quantitative data was performed by SPSS 22.0 for Mac software package (SPSS, Inc.,Chicago, IL).
    2. Graphs demonstrated the comparison of the biomarker level between groups were generated by Prism 8 for OS X (GraphPad Software, Inc., San Diego, CA).
    3. Receiver operating characteristic curves were generated by STATA 15 for windows (StataCorp ,LLC., College Station, TX).

Chapter 4 Results

4.1 miRNA expression profiling from NanoString analysis

In the discovery phase, miRNA expression profiling was performed by using NanoString nCounter Technology in the serum of patients with posterior circulation stroke (n=3) and peripheral vertigo (n=3) in acute phase within 72 hours and at day 90 after onset. Normalized counts were obtained from raw counts normalized to exogenous synthetic spike-in miRNA, cel-miR-254. The average count of the synthetic spike-in miRNA was 10. MiRNAs with count above the average count of cel-miR-254 were selected for subsequent analysis. MiRNA candidates was selected by using Venn diagram (81) based on result from NanoString nCounter Technology. Firstly, miRNAs that expressed only within 72 hours from each sample were selected (Figure 2).

Figure 2: Venn diagram of miRNA expression profiles by NanoString analysis from serum of posterior circulation stroke patients (C1-C3) and peripheral vertigo patients (P1-P3) in acute phase within 72 hours and at day 90



Secondly, miRNAs that expressed only in acute phase from each group were evaluated. Only miRNAs that expressed in more than one serum sample from each group were selected for final analysis (Figure 3). Finally, common miRNAs that expressed in each group were analyzed. Only miRNAs that expressed in acute phase within 72 hours in patients with posterior circulation stroke were selected as potential candidates for subsequent validation phase (Figure 4).

Figure 3: Venn diagram of miRNA that expressed only in acute phase from each sample

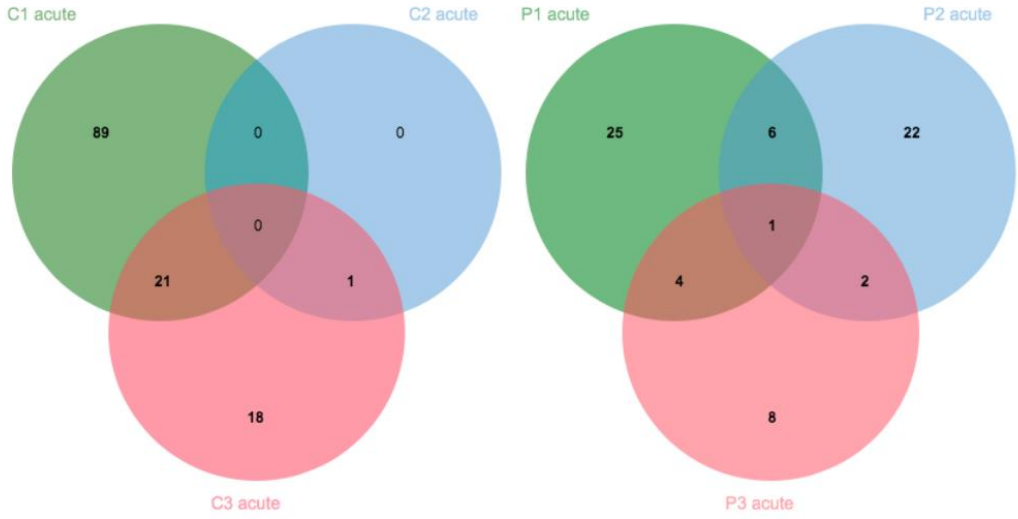
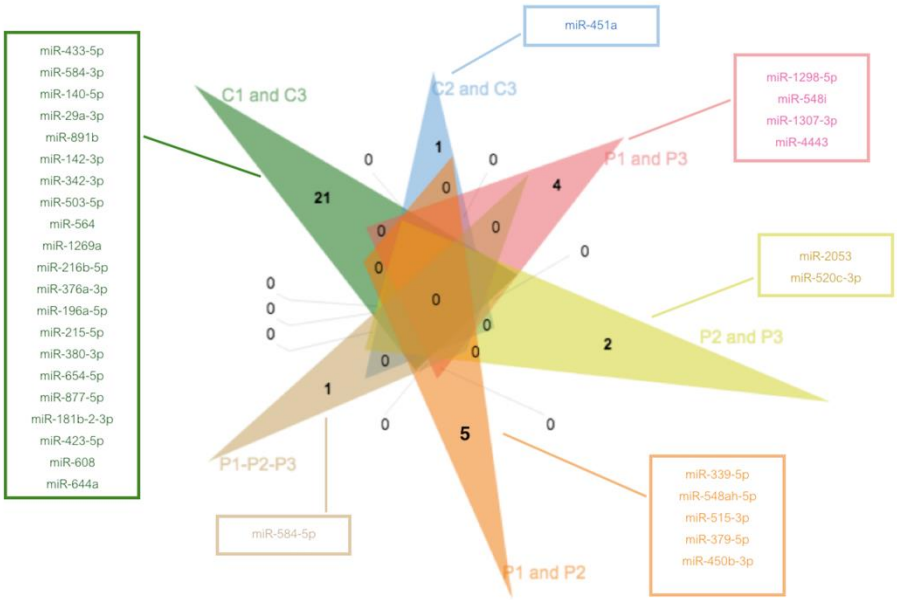


Figure 4: Venn diagram of potential miRNA candidates



4.2 miRNA candidates selection for validation phase

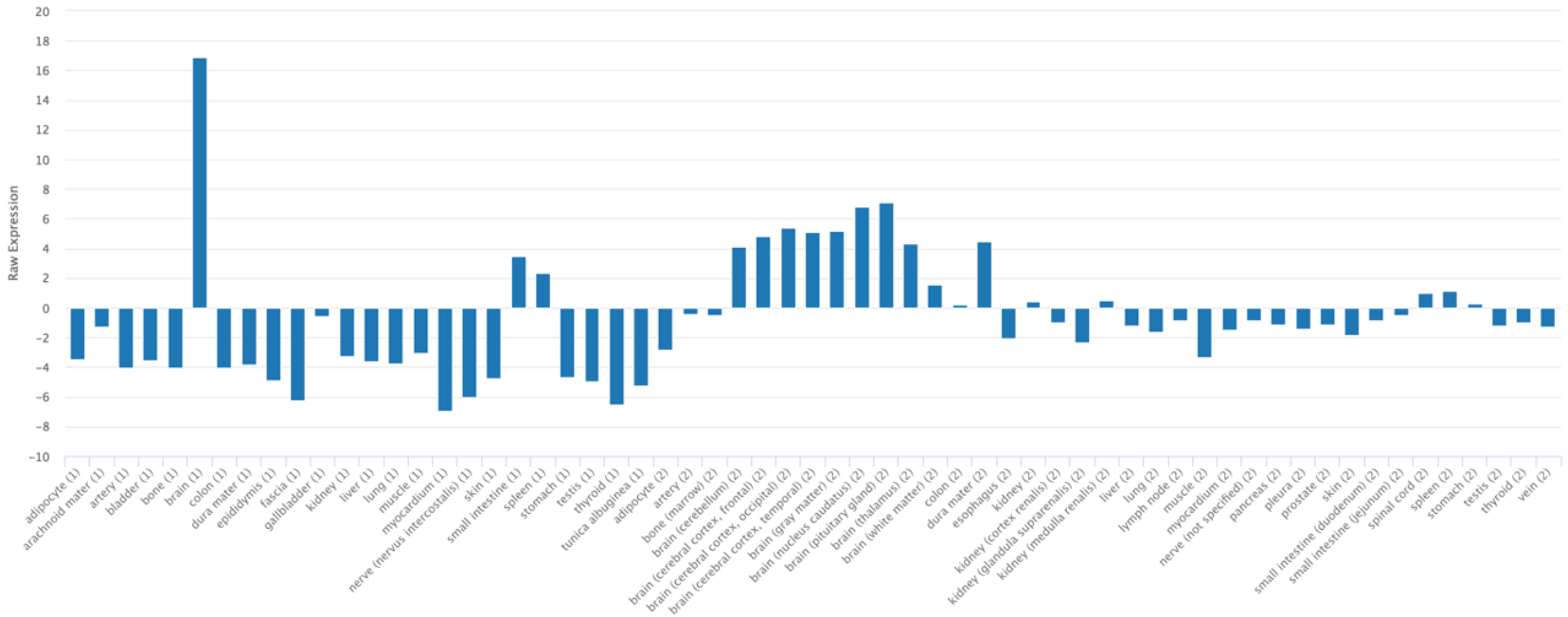
In this study, potential miRNA candidates for validation phase were selected by 2 pathways including from NanoString analysis and from reviewing potential candidates proposed by previous studies.

4.2.1 miRNA candidates from NanoString analysis

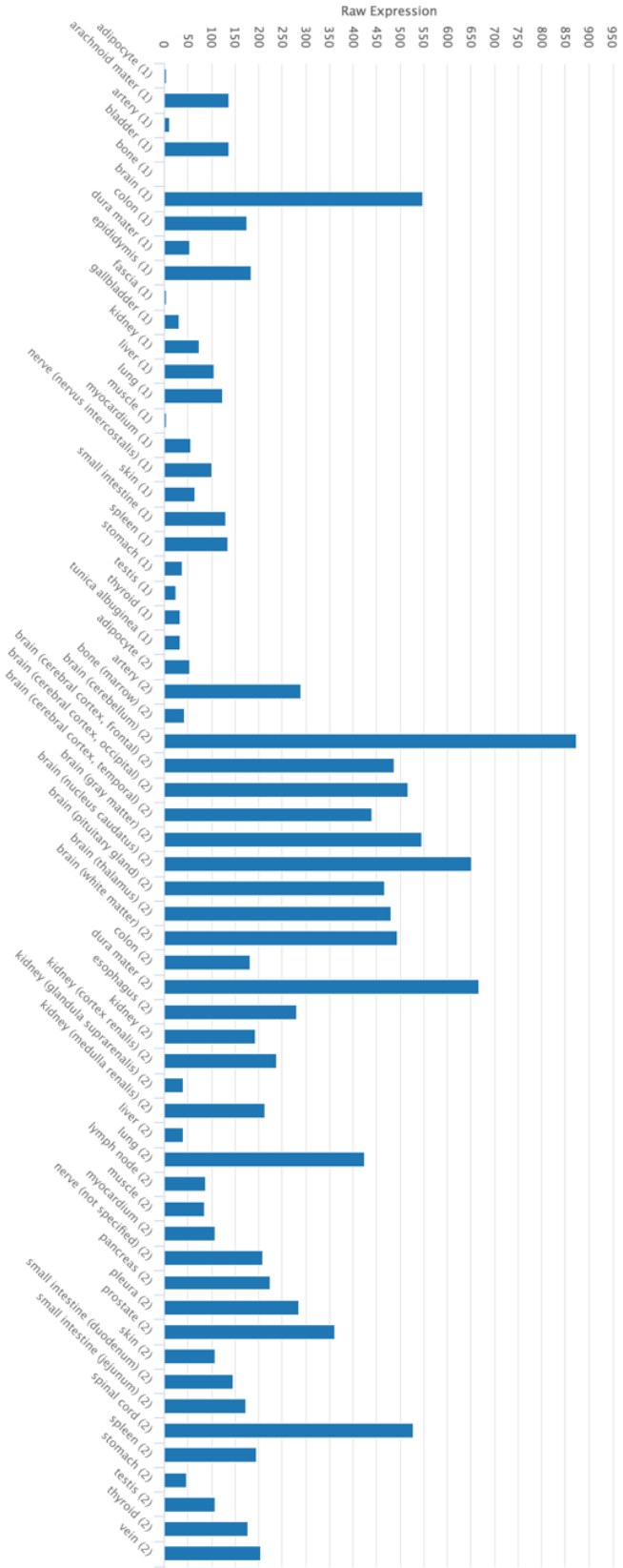
From the discovery phase by NanoString analysis, miRNAs that expressed in more than one patients with acute posterior circulation stroke in within 72 hours after onset were carefully reviewed in the human miRNA tissue atlas (https://ccb-web.cs.uni-saarland.de/tissueatlas/) which is an miRNA expression database (84). MiRNAs with high level of expression in the brain were selected for the validation phase. Among 22 miRNAs, there were 3 miRNAs with high expression in the brain including miR-433-5p, miR-342-3p, and miR-376-3p.

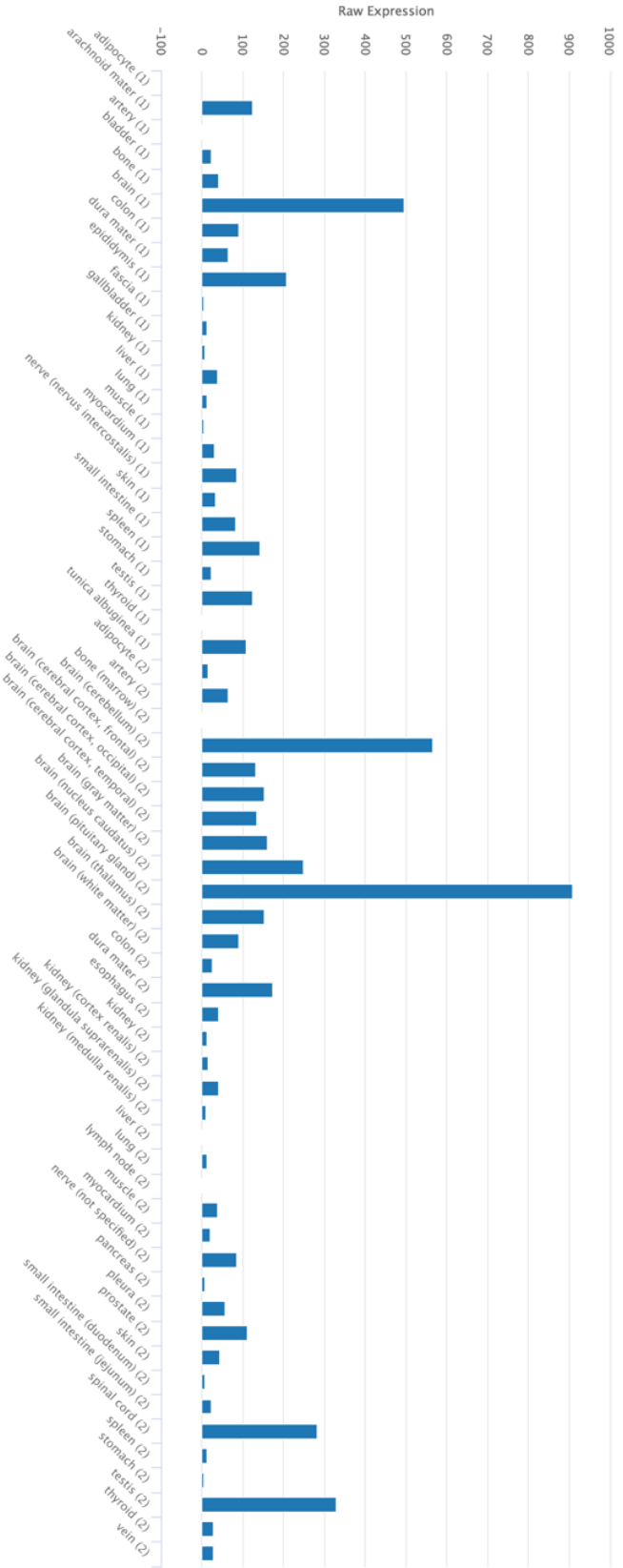
Figure 5: Raw expression level of miRNAs of interest from human miRNA tissue atlas

5A) miR-433



5B) miR-342-3p


Raw Expression
0
50
100
150
200
250
300
350
400
450
500
550
600
650
700
750
800
850
900
950
adipocyte (1)
arachnoid mater (1)
artery (1)
bladder (1)
bone (1)
brain (1)
colon (1)
dura mater (1)
epididymis (1)
fascia (1)
gallbladder (1)
kidney (1)
liver (1)
lung (1)
muscle (1)
myocardium (1)
nerve (nervus intercostalis) (1)
skin (1)
small intestine (1)
spleen (1)
stomach (1)
testis (1)
thyroid (1)
tunica albuginea (1)
adipocyte (2)
artery (2)
bone (marrow) (2)
brain (cerebellum) (2)
brain (cerebral cortex, frontal) (2)
brain (cerebral cortex, occipital) (2)
brain (cerebral cortex, temporal) (2)
brain (gray matter) (2)
brain (nucleus caudatus) (2)
brain (pituitary gland) (2)
brain (thalamus) (2)
brain (white matter) (2)
colon (2)
dura mater (2)
esophagus (2)
kidney (2)
kidney (cortex renalis) (2)
kidney (glandula suprarenalis) (2)
kidney (medulla renalis) (2)
liver (2)
lung (2)
lymph node (2)
muscle (2)
myocardium (2)
nerve (not specified) (2)
pancreas (2)
pleura (2)
prostate (2)
skin (2)
small intestine (duodenum) (2)
small intestine (jejunum) (2)
spinal cord (2)
spleen (2)
stomach (2)
testis (2)
thyroid (2)
vein (2)



5C) miR-376a-3p

Interestingly, from NanoString analysis, miR-584-5p is the only miRNA that commonly expressed in acute phase among 3 patients with peripheral vertigo, whereas miR-584-3p was commonly expressed in acute phase among 2 patients with posterior circulation stroke. Both miR-584-3p and miR-584-5p were included in the validation phase due to the complementary base sequences with different expression in peripheral vertigo and posterior circulation stroke respectively. Therefore, 5 miRNAs from NanoString analysis including miR-342-3p, miR-376a-3p, miR-433-5p, miR-584-3p, and miR-584-5p were selected for the validation phase.

4.2.2 miRNA candidates from previous studies

In addition to the miRNAs selected from the NanoString analysis, potential miRNA candidates from previous studies (as reviewed in chapter 2) were selected for the validation phase. MiR-124 has been shown to be brain-specific miRNA (73). Subsequent study revealed significantly high level of serum exosomal miR-124 in acute ischemic stroke patients (74). Therefore, in this study, both miR-124-3p and miR-124-5p were included for the validation study. Additional miRNA candidates including miR-125a-5p, miR-125b-5p, and miR-143-3p which had been evaluated in a well-designed study consisted of screening phase by miRNA sequencing with subsequent validation and replication by qPCR (58) were also included in the validation phase.

Table 16: List of miRNAs candidates for validation study

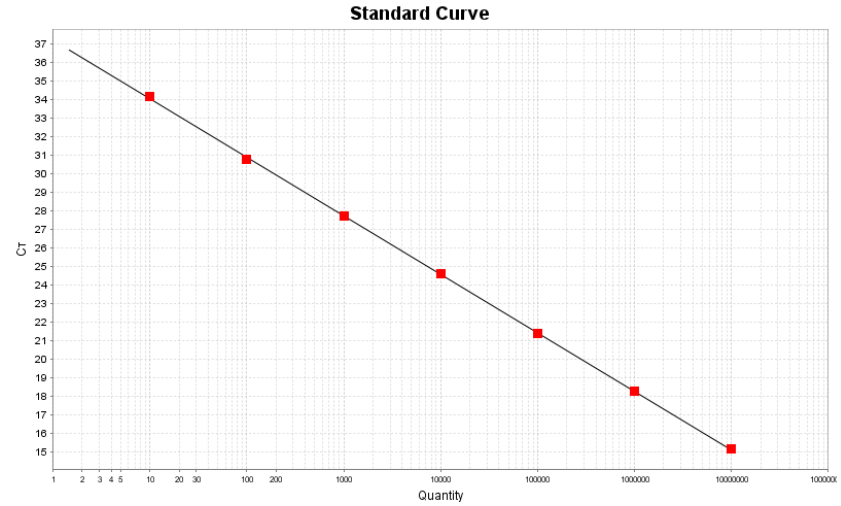
NanoString Analysis	Literature Review
miR-342-3p	miR-124-3p
miR-376a-3p	miR-124-5p
miR-433-5p	miR-125a-5p
miR-584-3p	miR-125b-5p
miR-584-5p	miR-143-3p

4.3 Standard curve generation of miRNAs of interest

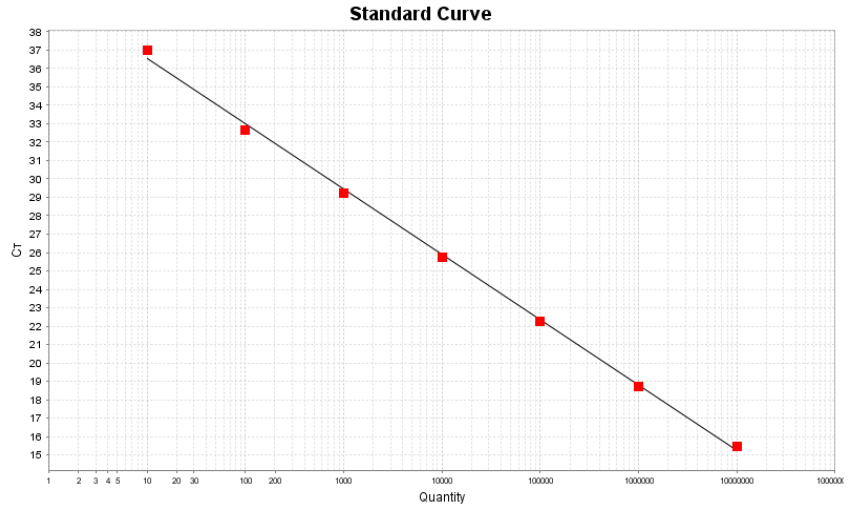
In order to perform the absolute quantification of the target miRNAs in serum sample, 10 standard curves of miRNA candidates were generated by serial dilution of the known concentration of the target miRNAs.

Figure 6: Standard curves from RT-qPCR of the miRNA candidates

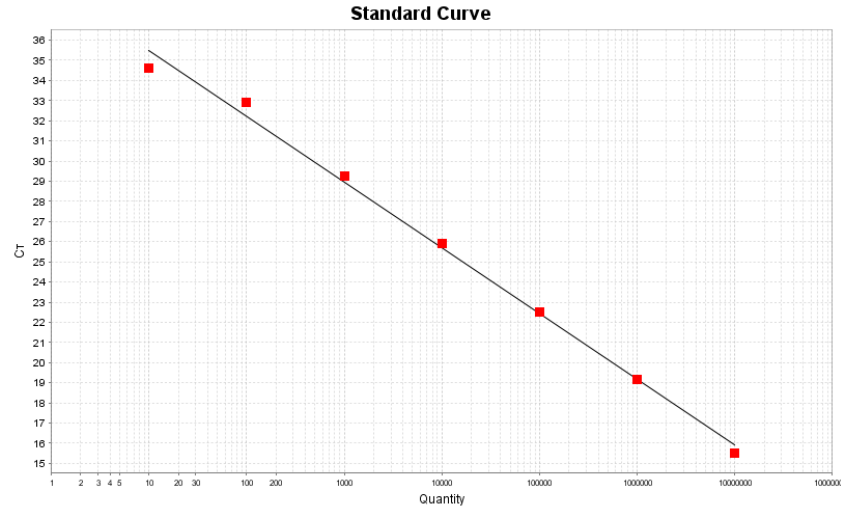
6A) miR-124-3p: Y= -3.155X + 37.213



6B) miR-124-5p: Y= -3.548X + 40.059

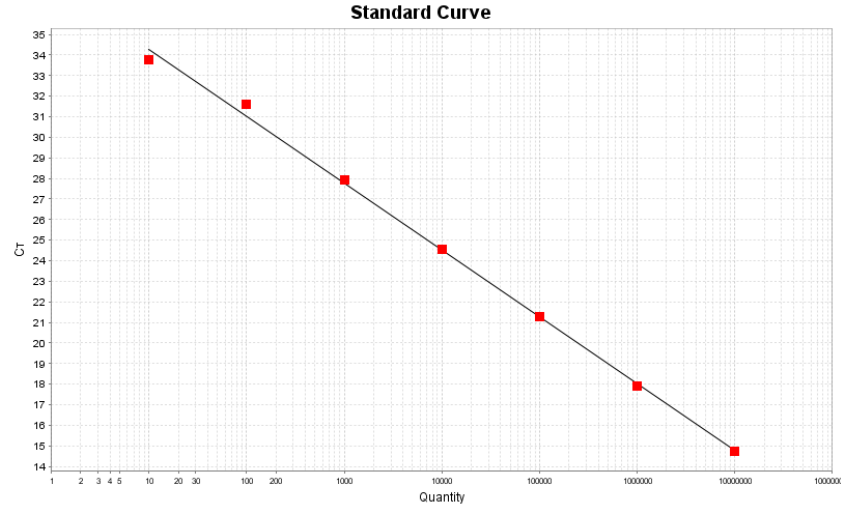


6C) miR-125a-5p: Y= -3.268X + 38.777



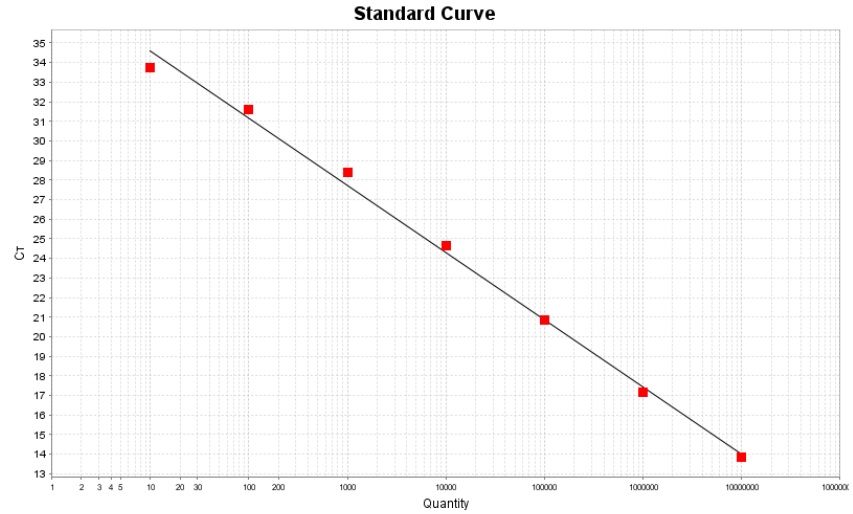
Target: miR-125a-5p Slope: -3.268 Y-Inter: 38.777 R^2: 0.995 Eff%: 102.283

6D) miR-125b-5p: Y= -3.252X + 37.549

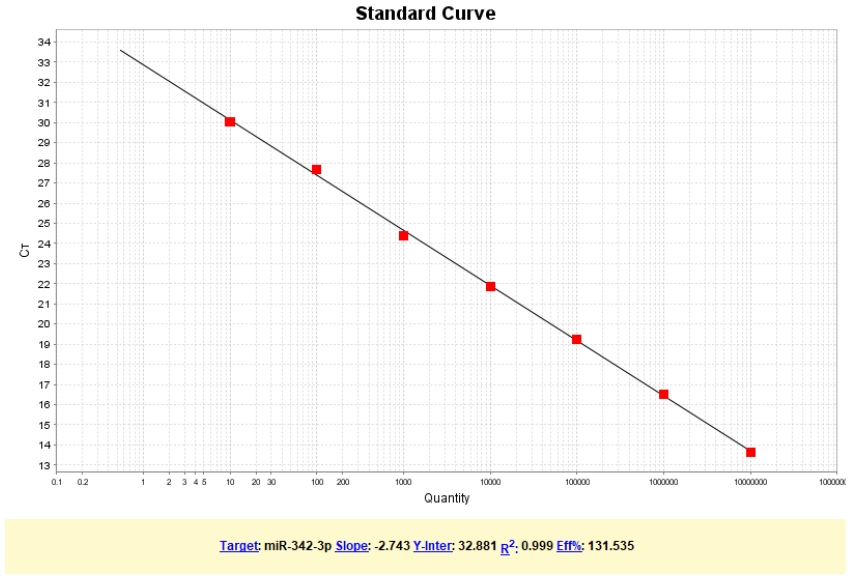


Target: miR-125b-5p Slope: -3.252 Y-Inter: 37.549 R^2: 0.998 Eff%: 103.017

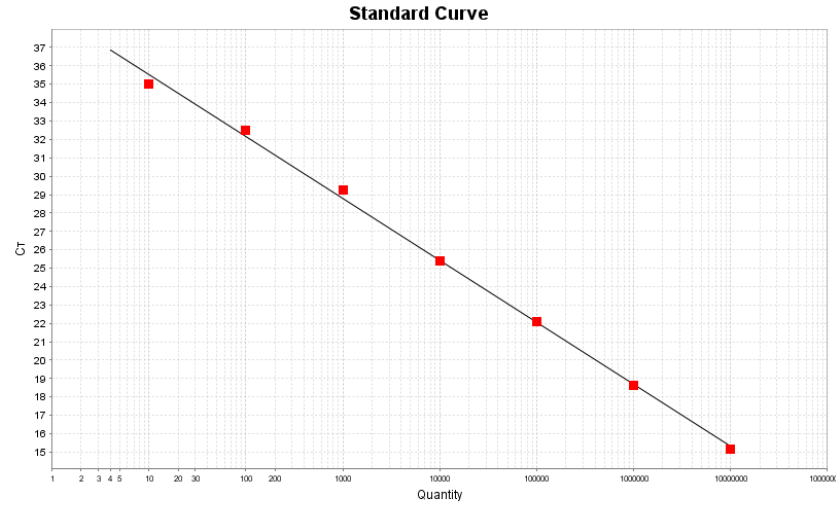
6E) miR-143-3p: Y= -3.431X + 38.05



6F) miR-342-3p: Y= -2.743X + 32.881

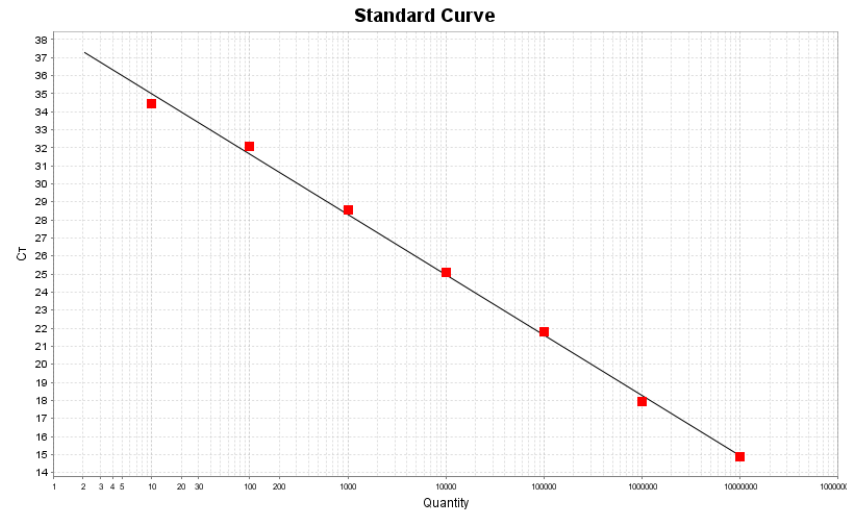


6G) miR-376a-3p: Y= -3.371X + 38.907



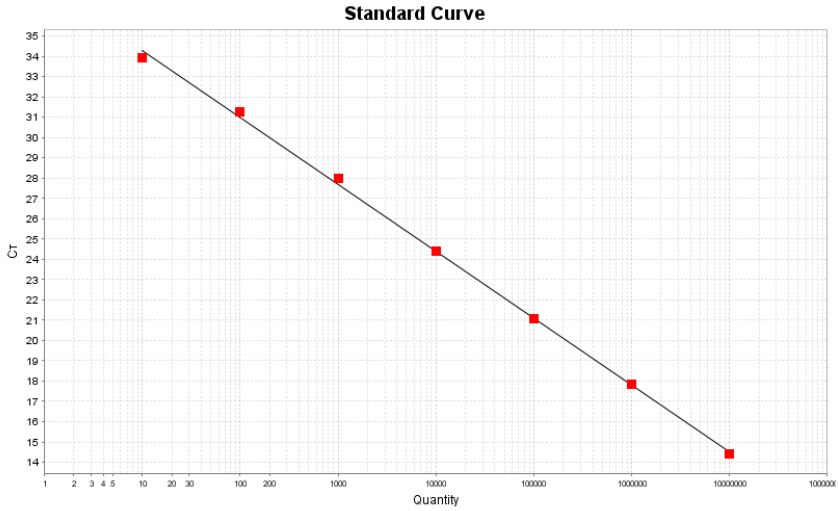
Target: miR-376a-3p Slope: -3.371 Y-Inter: 38.907 R^2: 0.998 Eff%: 98.008

6H) miR-433-5p: Y = -3.324X + 38.355



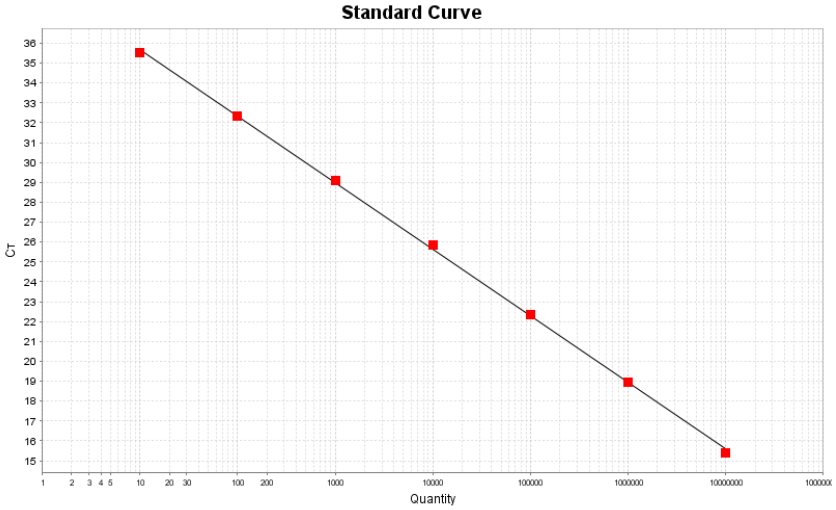
Target: miR-433-5p Slope: -3.345 Y-Inter: 38.355 R^2: 0.998 Eff%: 99.046

6I) miR-584-3p: Y = -3.297X + 37.605



Target: miR-584-3p Slope: -3.297 Y-Inter: 37.605 R^2: 0.999 Eff%: 101.047

6J) miR-584-5p: Y = -3.353X + 39.056



Target: miR-584-5p Slope: -3.353 Y-Inter: 39.056 R^2: 1 Eff%: 98.719

The standard curves of 9 out of 10 miRNA candidates (excepting miR-342-3p) had good PCR reaction as reflected by high efficiency (range between 90% and -110%) and the standard curve slope between -3.58 and -3.10. The presence of PCR inhibitors in the reagents might account for the lower efficiency of the PCR reaction seen in the miR-342-3p. The correlation coefficient of the standard curve of all 10 miRNA candidates were very high (0.99-1). The absolute amount of the miRNAs of interest from the serum sample was calculated based on the standard curve equation using the C_T result obtained from qPCR.

4.4 Study outcomes

4.4.1 Baseline Characteristics, laboratory parameters, and medication of patients with central and peripheral vertigo

During June 2017-January 2020, there were 76 patients screened for the study participation. Thirteen patients were ineligible due to the clinical presentation compatible with diagnosis other than true vertigo according to the inclusion criteria. Five patients were excluded based on exclusion criteria. There were a total of 58 patients including 23 patients with central vertigo and 35 patients with peripheral vertigo enrolled in the study. Among central vertigo patients, there were 12 patients with cerebellar infarction, 6 patients with brainstem infarction, and 5 patients with both cerebellar and brainstem infarctions. In peripheral vertigo group, there were 17 patients with benign paroxysmal positional vertigo, 4 patients with Meniere's disease, 4 patients with vestibular neuritis, and 10 patients with peripheral vertigo who had negative MRI study that could not be classified into any other peripheral vertigo diseases (Figure 7). The number of female patients was significantly higher among peripheral vertigo group (82.9% vs 21.7%, *P*=0.001). The onset to blood collection time was significantly higher in central vertigo group (37.59±16.94 vs 21.35±17.9, *P*=0.001). There were more patients who had diabetes mellitus, history of myocardial infarction, and smoking among central vertigo group. Other

baseline characteristics were comparable between study groups. Regarding the laboratory parameters, the level of hemoglobin, WBC count, absolute monocyte count, creatinine, fasting blood sugar, HbA_1C, and total bilirubin were significantly higher in central vertigo group. There was no significant difference regarding medication taken by patients in both groups (Table 17).

Figure 7: Flow diagram of participant recruitment

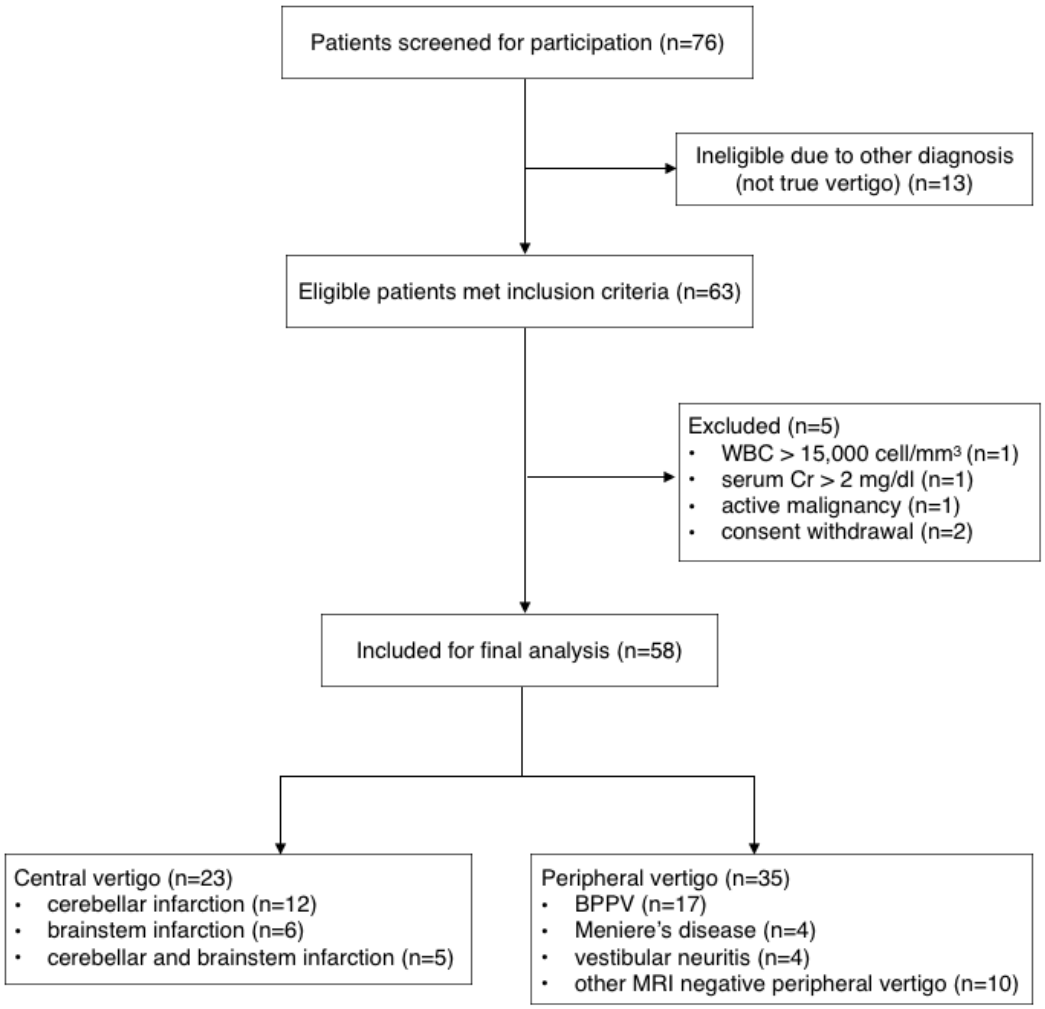


Table 17: Baseline characteristics, laboratory parameters, and medication in acute vertigo patients

	Central Vertigo (n=23)	Peripheral Vertigo (n=35)	*P* value
Age, years, mean (SD)	64.52(11.80)	63.69(9.42)	0.766
Female, % (n)	21.7% (5)	82.9% (29)	0.001*
Body weight, kg, mean (SD)	67.03 (8.12)	62.26 (11.23)	0.08
Body mass index, kg/m^2, mean (SD)	24.89 (3.96)	24.56 (4.62)	0.59
Onset to blood collection (hrs), mean (SD)	37.59 (16.94)	21.35 (17.91)	0.001*
Stroke risk factors, % (n)			
- Hypertension	65.2% (15)	51.4% (18)	0.30
- Diabetes mellitus	52.2% (12)	17.1% (6)	0.005*
- Dyslipidemia	56.5% (13)	48.6% (17)	0.55
- History of stroke	13.0% (3)	28.6% (10)	0.17
- History of myocardial infarction	21.7% (5)	2.9% (1)	0.02*
- Smoking	26.1% (6)	0	0.001*
Previous history of vertigo, % (n)	13% (3)	31.4% (11)	0.11
Systolic BP, mmHg, med (IQR)	161 (146-170)	149 (139-168)	0.10
Diastolic BP, mmHg, med (IQR)	90 (75-100)	80 (68-90)	0.14
Pulse rate, bpm, med (IQR)	80 (60-85)	76 (70-82)	0.87
Laboratory parameters			
- Hemoglobin, g/dL, med (IQR)	13.7 (13.1-15.4)	13.0 (12.5-13.4)	0.009*
- WBC count, *10^3/µL, mean (SD)	9.91(2.95)	8.29 (2.21)	0.03*
- Absolute neutrophil, *10^3/µL, mean (SD)	7.23(2.66)	6.02 (2.14)	0.06
- Absolute lymphocyte, *10^3/µL, med (IQR)	1.98 (1.43-2.77)	1.60 (1.25-2.02)	0.24
- Absolute monocyte, *10^3/µL, med (IQR)	0.50 (0.35-0.53)	0.36 (0.29-0.46)	0.02*
- Platelet, *10^3/µ, mean (SD)	227.61 (66.43)	266.24 (81.14)	0.07
- Creatinine, mg/dL, med (IQR)	0.84 (0.71-0.98)	0.67 (0.61-0.82)	0.04*
- Na, mEq/L, med (IQR)	138 .0 (137-140)	138 (137-140)	0.67

- K^+, mEq/L, med (IQR)	3.7 (3.6-4)	3.7 (3.5-4)	0.71
- Cl^-, mEq/L, mean (SD)	103.70 (2.79)	105.06 (3.02)	0.10
- $HCO3^-$, mEq/L, med (IQR)	22.0 (20-24)	23 (21-25)	0.18
- Fasting blood sugar, mg/dL, med (IQR)	130.0 (103-154)	100.0 (80-113.5)	0.008*
- HbA_1C, %, med (IQR)	6.3 (5.6-7.2)	5.6 (5.28-6.2)	0.02*
- Total cholesterol, mg/dL, mean (SD)	199.26 (49.85)	181.18 (54.81)	0.28
- Triglyceride, mg/dL, med (IQR)	109.0 (87-154)	103.0 (94-151)	0.68
- LDL, mg/dL, mean (SD)	136.0 (46.78)	110.35 (51.27)	0.11
- HDL, mg/dL, mean (SD)	41.09 (8.17)	45.18 (14.74)	0.27
- Total bilirubin, mg/dL, mean (SD)	0.77 (0.30)	0.6 (0.27)	0.04*
- Direct bilirubin, mg/dL, med (IQR)	0.30 (0.22-0.34)	0.23 (0.21-0.31)	0.10
- SGOT, mg/dL, med (IQR)	21.5 (19.75-25.25)	20.0 (18-26)	0.29
- SGPT, mg/, med (IQR)	22.5 (16.75-30.5)	19.5 (16.5-24.25)	0.23
- ALP, mg/dL, mean (SD)	69.56 (16.61)	71.72 (19.57)	0.70
- Albumin, mg/dL, med (IQR)	4.10 (3.9-4.4)	4.15 (4.0-4.5)	0.27
Medication, n(%)			
- Antiplatelets	17.4% (4)	37.1% (13)	0.11
- Anticoagulant	0% (0)	2.9% (1)	0.31
- ACEI/ARBs	26.1% (6)	31.4% (11)	0.66
- Calcium channel blockers	21.7% (5)	40%(14)	0.15
- ฺB-Blockers	13% (3)	17.1% (6)	0.67
- Diuretics	4.3% (1)	5.7% (2)	0.82
- Statins	21.7% (5)	45.7% (16)	0.06
- Glipizide (Sulfonylurea)	8.7% (2)	0.8% (3)	0.99
- Metformin (Biguanide)	26.1% (6)	14.3% (5)	0.26
- Proton Pump Inhibitor	4.3% (1)	14.3% (5)	0.22

WBC: white blood cell; LDL: low density lipoprotein; HDL: high density lipoprotein; SGOT: serum glutamic-oxaloacetic transaminase; SGPT: serum glutamic pyruvic transaminase; ALP: alkaline phosphatase; ACEI: angiotensin-converting enzyme inhibitor; ARBs: angiotensin II receptor blockers

*$P<0.05$

4.4.2 Clinical Presentations

The signs and symptoms of spinning, sense of imbalance, nausea and vomiting, oscillopsia, and nystagmus among acute vertigo patients with central and peripheral vertigo were indistinguishable. Physical signs including skew deviation, diplopia, and limb ataxia were found exclusively in patients with central vertigo. Positive HINTS test which is a combination of bedside physical examinations including head impulse test (with presence of corrective saccade), nystagmus (with characteristic of peripheral vertigo nystagmus), and test of skew (absence of skew deviation) were found only in peripheral vertigo patients. However, this was not statistically significant (Table 18).

Table 18: Clinical presentations of acute vertigo

Clinical presentations %(n)	Central Vertigo (n=23)	Peripheral Vertigo (n=35)	*P* value
Sense of spinning	65.2% (15)	82.9% (29)	0.13
Sense of imbalance	95.7% (22)	88.6% (31)	0.35
Nausea/vomiting	60.9% (14)	77.1% (27)	0.18
Positive HINTS	0% (0)	11.4% (4)	0.09
Nystagmus	47.8% (11)	54.4% (19)	0.63
Bilateral direction changing	17.4% (4)	0% (0)	
Vertical	4.3% (1)	0% (0)	
Torsional	0% (0)	0% (0)	
Unidirectional horizontal	13.0% (3)	17.1% (6)	
Multidirectional	13.0% (3)	0% (0)	
Diagnostic maneuver	0% (0)	37.0% (13)	
Skew deviation	13.0% (3)	0% (0)	0.03*
Diplopia	30.4% (7)	0% (0)	0.001*
Oscillopsia	8.7% (2)	8.6% (3)	0.99
Limb Ataxia	18 (78.3%)	0% (0)	<0.001*

HINTS: head impulse test, nystagmus, test of skew deviation

*$P<0.05$

4.4.3 miRNA candidates and protein biomarker level in acute phase of patients with central and peripheral vertigo

Among 58 patients, the level of 5 out of 10 miRNA candidates were significantly higher in patients with central vertigo during acute phase within 72 hours after onset. These miRNAs were miR-125a-5p (central: 568.7(338.2-924.5) copies/μL vs. peripheral: 244.5 (160.8-495.1) copies/μL, *P*=0.001), miR-125b-5p (central: 125.0 (98.01-199.8) copies/μL vs. peripheral: 48.6 (22.66-103.7) copies/μL, *P*<0.001), miR-143-3p (central: 321.6 (188.7-407.2) copies/μL vs. peripheral: 172.5 (102.2-242.2) copies/μL, *P*=0.014), miR-433-5p (central: 92.13 (49.06-183.2) copies/μL vs. peripheral: 53.45 (35.37-102.3) copies/μL, *P*=0.0056), and miR-584-5p (central: 879.6 (532.2-1889.0) copies/μL vs. peripheral: 557.3 (290.1-979.8) copies/μL, *P*=0.03) (Table 19, Figure 8).

Both S100 (central: 0.111 (0.049-0.335) μg/L vs. peripheral: 0.054 (0.039-0.082) μg/L, *P*=0.005), and IL-6 central: 7.42 (4.23-14.47) pg/mL vs. peripheral: 2.44 (0.70-4.68) pg/mL, *P*< 0.001) had significantly higher level in patients with central vertigo (Table 19, Figure 9).

Table 19: Biomarker level during acute phase in patients with central and peripheral vertigo

Biomarker level median (IQR)	Central Vertigo (n=23)	Peripheral Vertigo (n=35)	*P* value
miRNAs			
miR-124-3p	906.0 (437.7-1697.0)	753.7 (453.5-1502)	0.42
miR-124-5p	243.4 (172.6-476.3)	227.6 (123.5-385.3)	0.729
miR-125a-5p	568.7(338.2-924.5)	244.5 (160.8-495.1)	0.001*
miR-125b-5p	125.0 (98.01-199.8)	48.6 (22.66-103.7)	< 0.001*
miR-143-3p	321.6 (188.7-407.2)	172.5 (102.2-242.2)	0.014*
miR-342-3p	44.8 (16.39-118.7)	37.76 (14.57-136.4)	0.81
miR-376a-3p	134.3 (78.07-209.5)	87.93 (56.63-167.6)	0.056
miR-433-5p	92.13 (49.06-183.2)	53.45 (35.37-102.3)	0.006*
miR-584-3p	618.1 (406.0-1214.0)	439.3 (252.4-700.3)	0.056
miR-584-5p	879.6 (532.2-1889.0)	557.3 (290.1-979.8)	0.032*
Protein biomarker			

NSE (ng/mL)	13.84 (11.83-18.83)	13 (10.29-18.33)	0.248
S100 (µg/L)	0.111 (0.049-0.335)	0.054 (0.039-0.082)	0.005*
IL-6 (pg/mL)	7.42 (4.23-14.47)	2.44 (0.70-4.68)	< 0.001*

*P<0.05

Figure 8: miRNA level of central and peripheral vertigo patients in acute phase

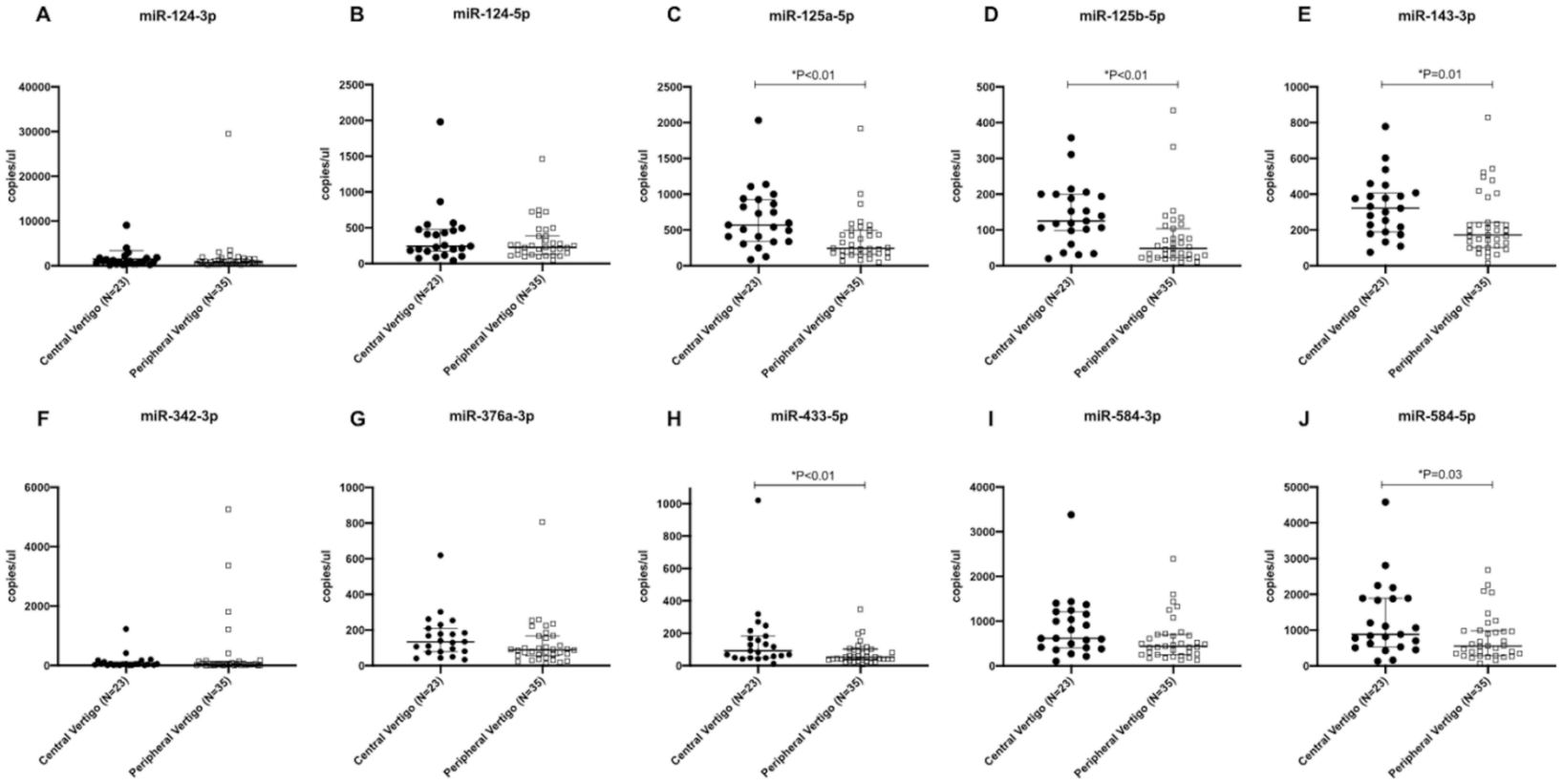
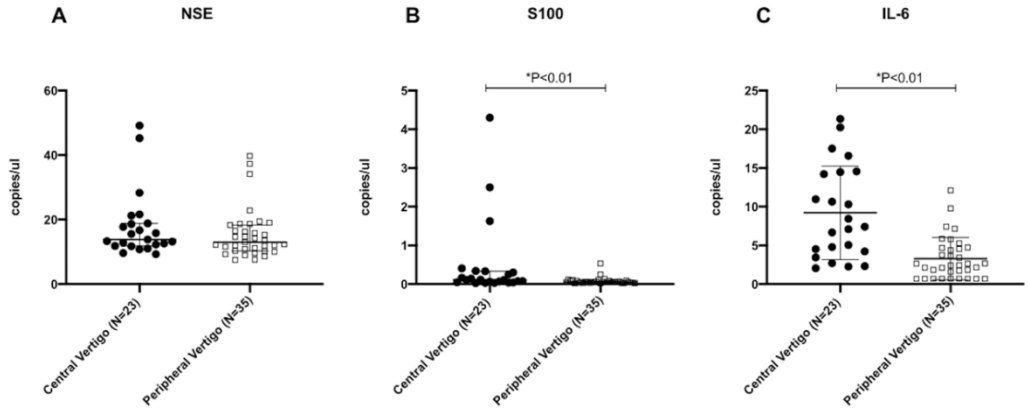


Figure 9: NSE, S100, and IL-6 level of central and peripheral vertigo patients in acute phase



4.4.4 Temporal expression profile of miRNA candidates and protein biomarker level in patients with central and peripheral vertigo

The expression level of serum biomarker from both miRNAs and protein including NSE, S100, and IL-6 was measured at day 0 and day 90 of the study. Among total 58 patients including 23 patients with central vertigo and 35 patients with peripheral vertigo, there were 1 patient from central vertigo group and 6 patients from peripheral vertigo group loss to follow up at day 90 of the study. Therefore, the result presented in Table 19 were median and interquartile range of biomarker level of 22 patients with central vertigo and 29 patients with peripheral vertigo. The level of serum IL-6 were below detectable level (<1.5 pg/mL) in 1 patients with central vertigo and 10 patients with peripheral. Imputation these values at half of the lower detection limit was done.

Table 20: miRNA candidates and protein biomarker level in central and peripheral vertigo patients

Biomarker level median (IQR)	Central Vertigo (n=22)		Peripheral Vertigo (n=29)	
	Day 0	Day 90	Day 0	Day 90
miRNAs(copies/μL)				
miR-124-3p	946.0 (435.9-1731)	860.3 (435.6-1297)	683.1 (400.5-1578)	1056 (308.5-1961)
miR-124-5p	241.9 (158.5-481.0)	238.1 (142.7-389.4)	222.7 (118.2-302.5)	279.3 (102.8-633.3)
miR-125a-5p	569.9 (337.2-927.8)	242.4 (120.0-326.9)	244.5 (156.0-496.6)	219.5 (117.0-481.6)
miR-125b-5p	122.2 (88.56-195.3)	38.2 (14.02-106.4)	56.0 (22.6-113.9)	51.95 (19.26-159.3)
miR-143-3p	326.4 (210.1-418.1)	141.9 (81.03-268.3)	165.4 (100.3-238.0)	158.1 (77.47-365.1)
miR-342-3p	46.59 (17.44-132.4)	16.98 (7.767-115.1)	49.23 (13.94-148.4)	45.54 (13.77-157.2)
miR-376a-3p	136.8 (80.12-214.5)	71.70 (30.65-123.3)	77.79 (50.01-160.8)	118.1 (36.41-173.7)
miR-433-5p	87.97 (48.78-172.3)	61.63 (33.58-118.7)	43.89 (32.87-101.1)	61.49 (31.67-89.37)
miR-584-3p	616.5 (399.3-1221.0)	367.8 (202.4-649.0)	415.1 (250.9-644.8)	401.0 (196.3-751)
miR-584-5p	971.6 (525.7-1889.0)	482.6 (256.9-870.8)	496.7 (283.2-971.8)	513.2 (274.1-1079.0)
Protein biomarker				
NSE (ng/mL)	13.58 (11.81-19.44)	11.50 (10.10-12.95)	12.33 (10.27-16.41)	12.80 (11.06-15.34)
S100 (μg/L)	0.1095 (0.048-0.3365)	0.0515 (0.0388-0.059)	0.053 (0.037-0.082)	0.06 (0.042-0.073)
IL-6 (pg/mL)	7.27 (4.035-14.28)	3.73 (2.223-4.928)	2.44 (1.67-4.71)	2.58 (1.115-5.24)

- *miRNA expression level*

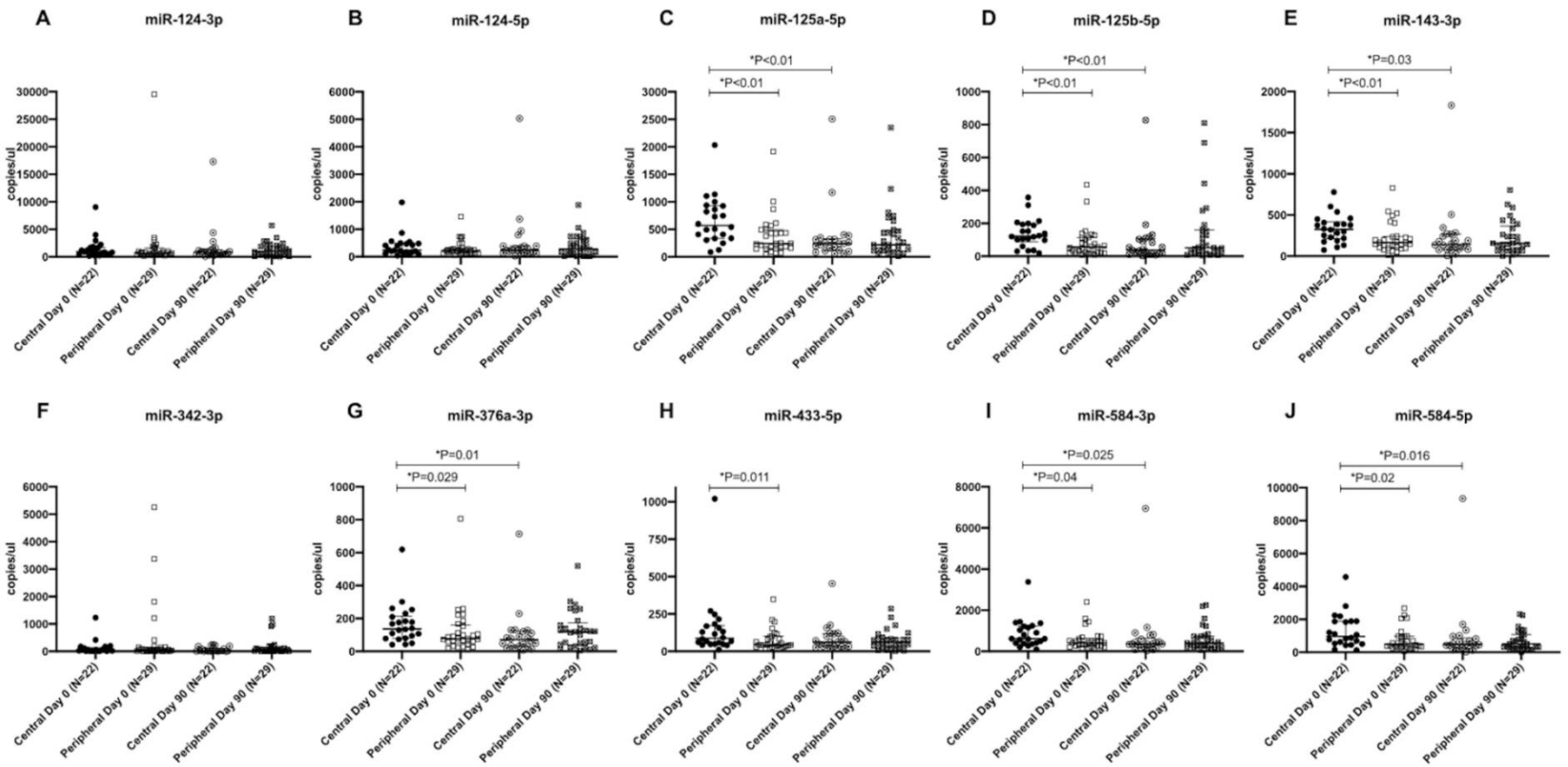
Ten miRNA candidates were selected for validation phase including 5 miRNAs from nanostring analysis and 5 miRNAs from literature review. Among miRNAs selected from nanostring analysis, miR-376a-3p (central: 136.8 (80.12-214.5) copies/μL vs. peripheral: 77.79 (50.01-160.8) copies/μL, *P*= 0.029), miR-433-5p (central: 87.97 (48.78-172.3) copies/μL vs. peripheral: 43.89 (32.87-101.1) copies/μL, *P*=0.011), miR-584-3p (central: 616.5 (399.3-1221.0) copies/μL vs. peripheral: 415.1 (250.9-644.8) copies/μL, *P*=0.04), and miR-584-5p (central: 971.6 (525.7-1889.0) copies/μL vs. peripheral: 496.7 (283.2-971.8) copies/μL, *P*=0.02) had significantly higher level of miRNA

expression in acute phase at day 0 among patients with central vertigo compared to those in peripheral vertigo. Among these miRNAs, the level of miR-376a-3p (day 0: 136.8 (80.12-214.5) copies/μL vs. day 90: 71.70 (30.65-123.3) copies/μL, *P*=0.01), miR-584-3p (day 0: 616.5 (399.3-1221.0) copies/μL vs. day 90: 367.8 (202.4-649.0) copies/μL, *P*=0.025) and miR-584-5p (day 0: 971.6 (525.7-1889.0) copies/μL vs. day 90: 482.6 (256.9-870.8) copies/μL, *P*=0.016) significantly decreased by day 90 in patients with central vertigo (Table 20 and Figure 10F-10J).

Among 5 miRNAs from literature review, miR-125a-5p (central: 569.9 (337.2-927.8) copies/μL vs. peripheral 244.5 (156.0-496.6) copies/μL, *P*=0.004), miR-125b-5p (central: 122.2 (88.56-195.3) copies/μL vs. peripheral: 56.0 (22.6-113.9) copies/μL, *P*=0.003) and miR-143-3p (central: 326.4 (210.1-418.1) copies/μL vs. peripheral: 165.4 (100.3-238.0) copies/μL, *P*=0.007) had significantly higher level of miRNA expression in acute phase among patients with central vertigo. The expressions level of these miRNAs among patients with central vertigo significantly decreased by day 90 (miR-125a-5p: day 0: 569.9 (337.2-927.8) copies/μL vs. day 90: 242.4 (120.0-326.9) copies/μL, *P*=0.008); miR-125b-5p: day 0: 122.2 (88.56-195.3) copies/μL vs. day 90: 38.2 (14.02-106.4) copies/μL, *P*=0.003); miR-143-3p: day 0: 326.4 (210.1-418.1) copies/μL vs. day 90: 141.9 (81.03-268.3) copies/μL, *P*=0.03) (Table 20 and Figure 10A-10E).

In patients with peripheral vertigo, there was no significant difference regarding the level of expression between acute phase at day 0 and follow up period at day 90 of 10 miRNA candidates.

Figure 10 : miRNA level at day 0 and day 90 in central and peripheral vertigo patients

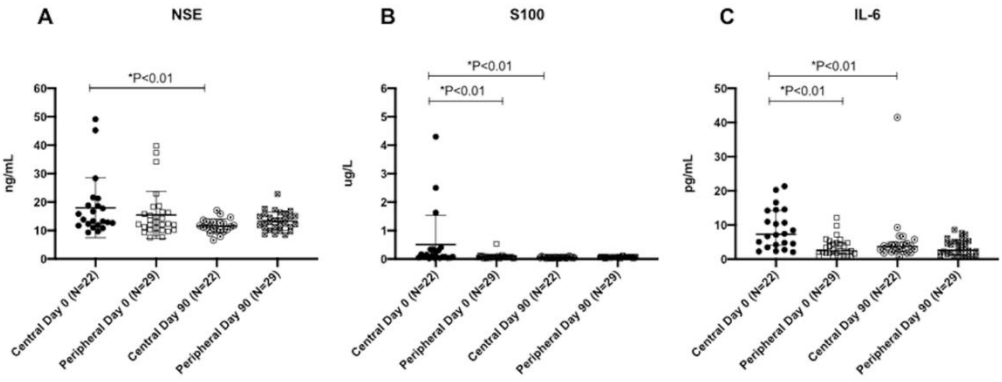


- *Protein biomarker level*

During acute phase at day 0, both serum S100 and IL-6 level in patients with central vertigo were significantly higher than in patients with peripheral vertigo (S100: central 0.1095 (0.2885) µg/L vs. peripheral 0.053 (0.045) µg/L, *P*=0.006; IL-6: central 7.27 (4.035-14.28) pg/mL vs. peripheral 2.44 (1.67-4.71 pg/mL, *P*<0.001).

The level of NSE, S100, and IL-6 significantly decreased at day 90 follow up period in patients with central vertigo (NSE: day 0: 13.58 (7.63) ng/mL vs. day 90: 11.50 (2.85) ng/mL, *P*<0.01; S100: day 0: 0.1095 (0.2885) µg/L vs. day 90: 0.0515 (0.02025) µg/L, *P*<0.001; IL-6: day 0: 7.27 (4.035-14.28) vs. day 90: 3.73 (2.223-4.928) pg/mL, *P*=0.001). There was no significant difference regarding serum level of NSE, S100, and IL-6 among patients with peripheral vertigo at day 0 and day 90 (Figure 11).

Figure 11: NSE, S100, and IL-6 level at day 0 and day 90 in central and peripheral vertigo patients



4.4.5 Biomarker level and ischemic stroke severity

The correlation between serum miRNA candidates, serum NSE, S100, and IL-6 and ischemic stroke severity were evaluated in 23 patients with central vertigo during acute phase at day 0. The ischemic stroke severity was determined by neuroimaging using cerebral infarction volume measurement and clinical evaluation by NIHSS.

- *Biomarker level and infarction volume*

Due to not normally distributed data of biomarker level and infarction volume, Spearman's Correlation was used in this analysis. The median (IQR) volume of infarction was 4.51 (15.6) ml. Most of the serum miRNA level did not correlate with the cerebral infarction volume. However, miR-125a-5p (r_s 0.53, *P*=0.009), miR-125b-5p (r_s 0.546, *P*=0.007), and miR-376a-3p (r_s 0.423, *P*=0.044), demonstrated low to moderate positive correlation with the cerebral infarction volume. In contrast, serum S100 level was highly correlated to the cerebral infarction volume (r_s 0.764, *P*<0.001) (Table 21).

Table 21: Spearman's Correlation between biomarker level and infarction volume

	r_s	*P* value
miRNAs		
miR-124-3p	0.04	0.858
miR-124-5p	-0.004	0.986
miR-125a-5p	0.53	0.009*
miR-125b-5p	0.546	0.007*
miR-143-3p	0.189	0.387
miR-342-3p	0.305	0.157
miR-376a-3p	0.423	0.044*
miR-433-5p	0.239	0.273
miR-584-3p	-0.034	0.879
miR-584-5p	0.116	0.598
Protein biomarkers		
NSE	0.272	0.21
S100	0.764	<0.001*
IL-6	0.387	0.068

- *Biomarker level and NIHSS*

The distribution of NIHSS among patients with central vertigo due to ischemic stroke were not normally distributed. Therefore, Spearman's correlation was performed in this analysis. The median (IQR) of NIHSS among patients with central vertigo was 3 (4). There was no correlation between the stroke severity determined by NIHSS and serum level of both miRNA candidates and protein biomarkers (Table 22).

Table 22: Spearman's Correlation between biomarker level and NIHSS

	r_s	*P* value
miRNAs		
miR-124-3p	0.014	0.948
miR-124-5p	0.053	0.808
miR-125a-5p	0.239	0.272
miR-125b-5p	-0.006	0.977
miR-143-3p	0.086	0.696
miR-342-3p	0.275	0.203
miR-376a-3p	0.200	0.360
miR-433-5p	0.085	0.698
miR-584-3p	-0.026	0.906
miR-584-5p	0.035	0.874
Protein biomarkers		
NSE	-0.217	0.320
S100	-0.037	0.867
IL-6	-0.018	0.935

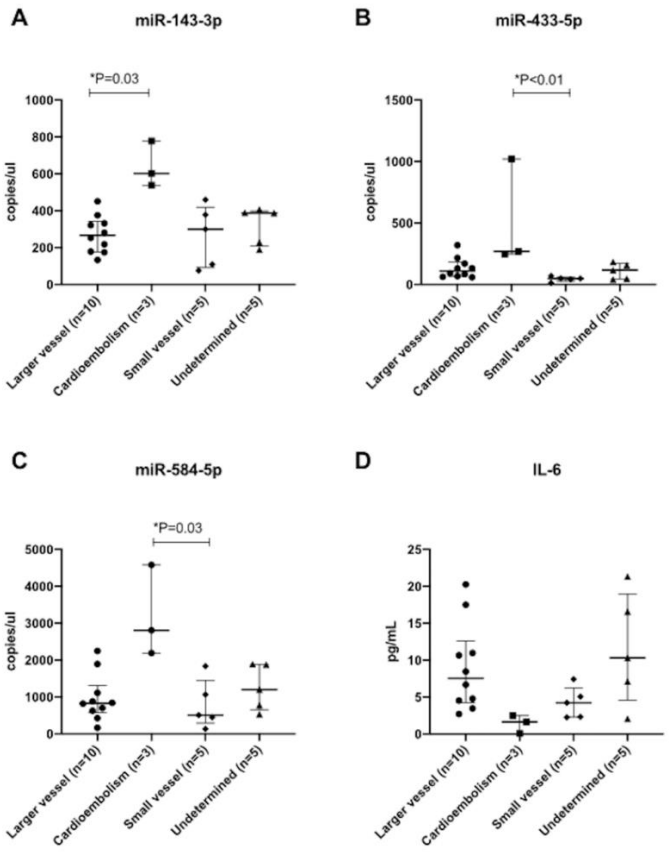
4.4.6 Biomarker level in different stroke etiologies and sites of infarction

The serum level of miRNA candidates and protein biomarkers including NSE, S100, and IL-6 were compared among different stroke etiologies determined by the Trial of Org 10172 in Acute Stroke Treatment (TOAST) classification (85) and regions of infarction.

- *Biomarker level and stroke etiology*

Among total 23 patients with central vertigo, there were 10 patients with ischemic stroke due to large vessel atherosclerosis, 3 patients due to cardioembolism, 5 patients due to small vessel disease, and 5 patients due to undetermined etiology. The level of miR-433-5p and miR-584-5p in patients with cardioembolic stroke were significantly higher than that in patients with small vessel disease. The level of miR-143-3p in patient with cardioembolic stroke were significantly higher than in patients with large vessel atherosclerosis. There was significant different in level of IL-6 among different stroke etiologies analyzed by Kruskal Wallis test (*P*=0.03). However, the post hoc Dunn's test did not reveal significant difference in any pairwise comparison of different stroke etiologies (Figure 12).

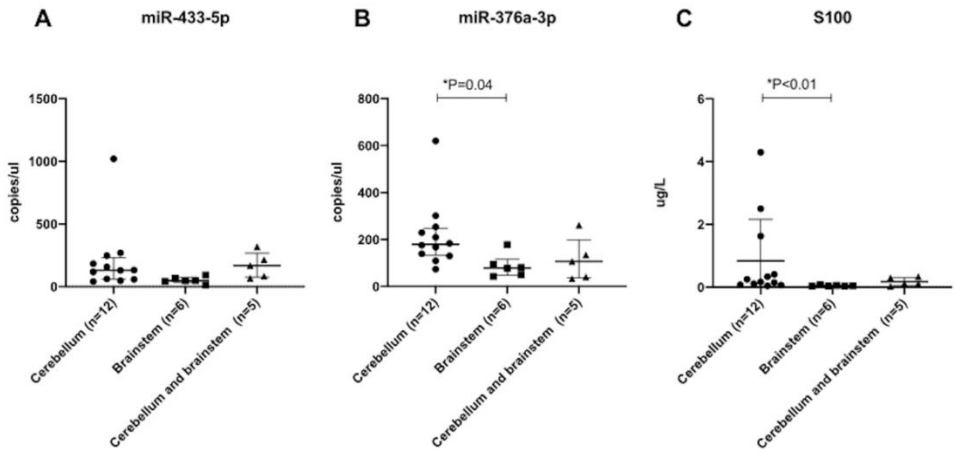
Figure 12: Serum miR-143-3p, miR-433-5p, miR-584-5p, and IL-6 by stroke etiology



- *Biomarker level and site of infarction*

Kruskal Wallis with post hoc Dunn's test was used to compare the different regarding the level of serum biomarker in both miRNA candidates and protein biomarkers at different sites of infarction including cerebellum, brainstem, cerebellum and brainstem. There was significant different regarding level of miR-433-5p expression among three infarction areas analyzed by Kruskal Wallis test (*P*=0.04). However, the post hoc Dunn's test did not reveal significant difference in any pairwise comparison of different areas of infarction. Both serum level of miR-376a-3p and S100 revealed significantly higher level in cerebellar infarction when compared to brainstem infarction (Figure 13). There was no significant difference regarding the level of serum biomarkers at different sites of infarction in other biomarkers.

Figure 13: Serum miR-433-5p, miR-376a-3p, and S100 level in different site of infarction



4.4.7 Biomarker as potential diagnostic test for central vertigo

The discriminative ability between central and peripheral vertigo of serum 10 miRNA candidates and protein biomarkers including NSE, S100, and IL-6 were demonstrated by ROC curves and area under the ROC curve.

- *Serum miRNAs*

The areas under the ROC curve of serum miR-125a-5p, miR-125b-5p, miR-143-3p, miR-433-5p, and miR-584-5p were 0.7516 (0.6196-0.8835), 0.7689 (0.6418-0.8961), 0.6919 (0.5523-0.8315), 0.7143 (0.5780-0.8506), and 0.6671 (0.5218-0.8315) respectively. These 5 miRNA candidates had acceptable discriminative ability to differentiate between central and peripheral vertigo (Figure 10). Among 5 miRNAs, serum miR-125b-5p, miR-125a-5p, and miR-433-5p had highest area under the ROC curve. By using cut off level above 89 copies/μL, the sensitivity and specificity of using serum miR-125b-5p were 78.26% and 74.29% respectively. Using serum miR-125a-5p level above 299 copies/μL yielded the sensitivity of 86.96% and specificity of 57.14%. Serum miR-433-5p level above 46 copies/μL had sensitivity and specificity of 86.96% and 48.57% respectively. The remaining 5 miRNA candidates had poor discriminative ability (Figure 14).

- *Serum NSE, S100, and IL-6*

Regarding serum protein biomarkers, S100 and IL-6 had area under the ROC curve of 0.718 (0.5725-0.8635) and 0.829 (0.7241-0.9343) respectively. Using cut off level above 0.1070 μg/L, serum S100 had sensitivity 56.52% and specificity of 88.57% to differentiate between central and peripheral vertigo. Serum IL-6 with the cut off level above 6.27 pg/mL had sensitivity of 60.87% and specificity of 84.62% (Figure 15).

- *Combination of serum biomarkers*

In order to increase the discriminative ability of the serum biomarker, miR-125b-5p and IL-6, which had highest area under ROC curve from miRNA candidate and protein biomarker respectively, were selected to combine with other biomarkers (Figure 15-16). The combination of serum miR-125b-5p with other biomarkers including miR-125a-5p, miR-143-3p, miR-433-5p, miR-584-5p and S100 revealed an increase in area under the ROC curve up to 0.76-0.82 when compared to using single biomarker (Figure 16, Table 23). However, this was not statistically significant (Table 23). Combining IL-6 and miR-584-5p significantly increase the area under ROC curve from 0.6671 (0.5218-0.8124) to 0.8248 (0.7139-0.9358) (P=0.029) (Figure 17, Table 24).

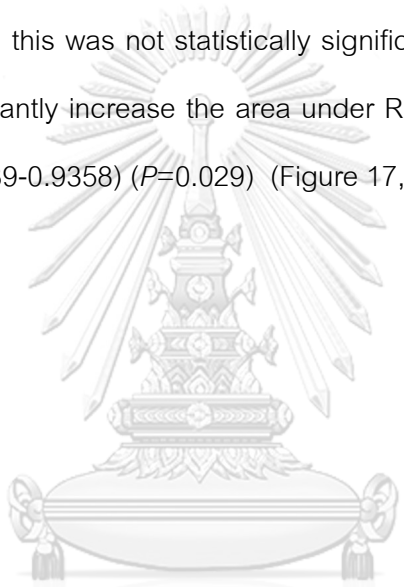


Figure 14: ROC curves of miRNA biomarkers

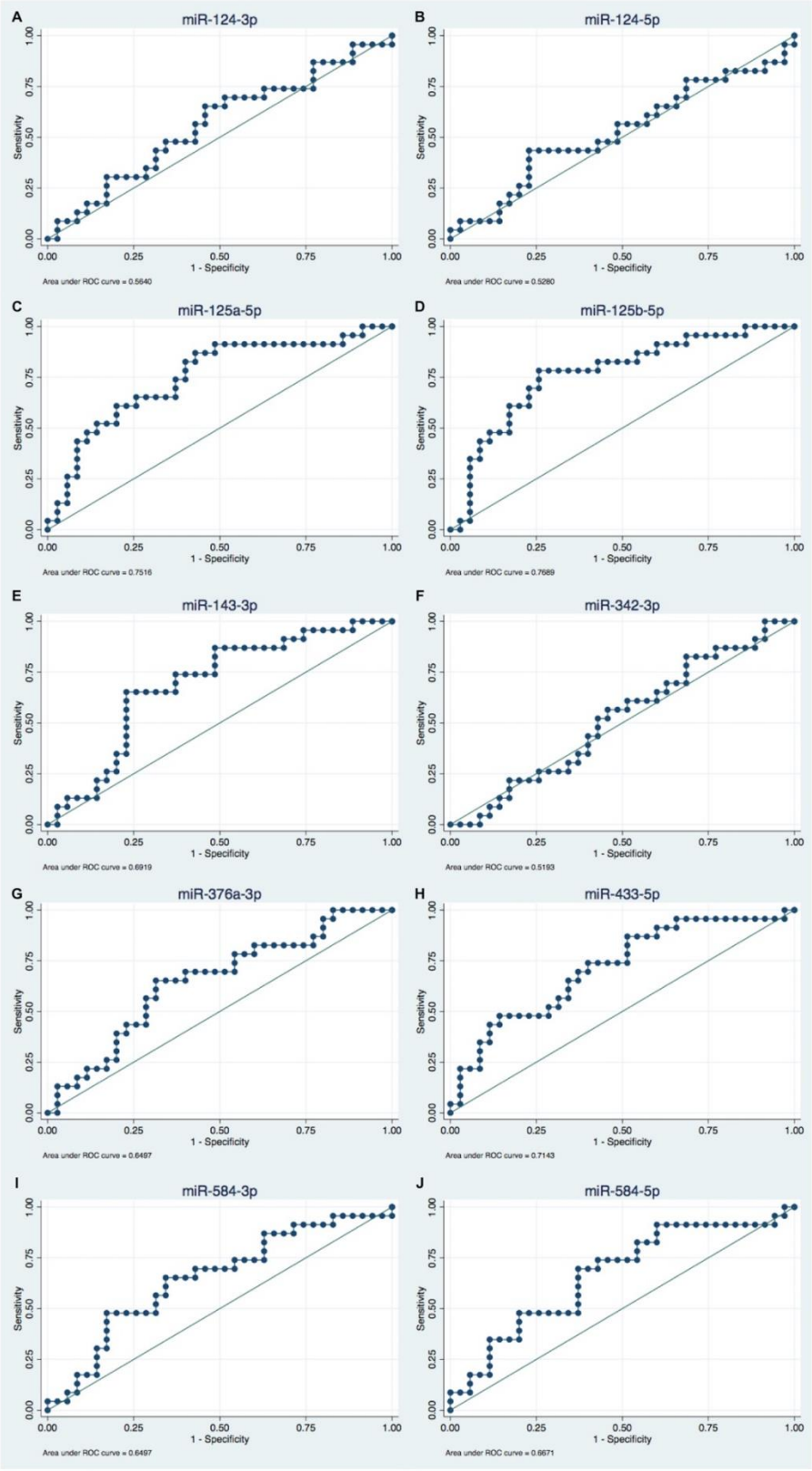


Figure 15: ROC curves of NSE, S100, and IL-6

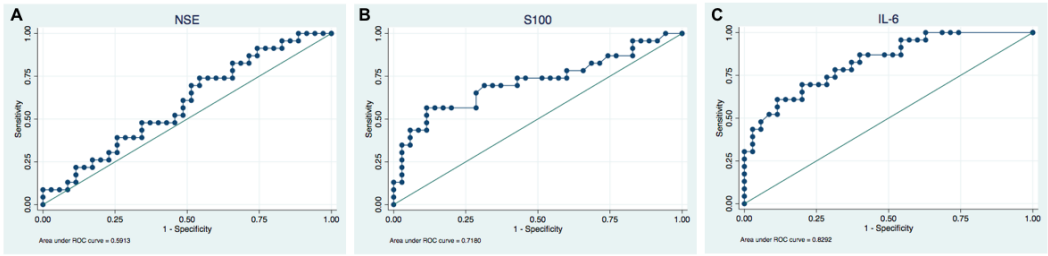


Figure 16: ROC curves of combination of biomarkers with miR-125b-5p

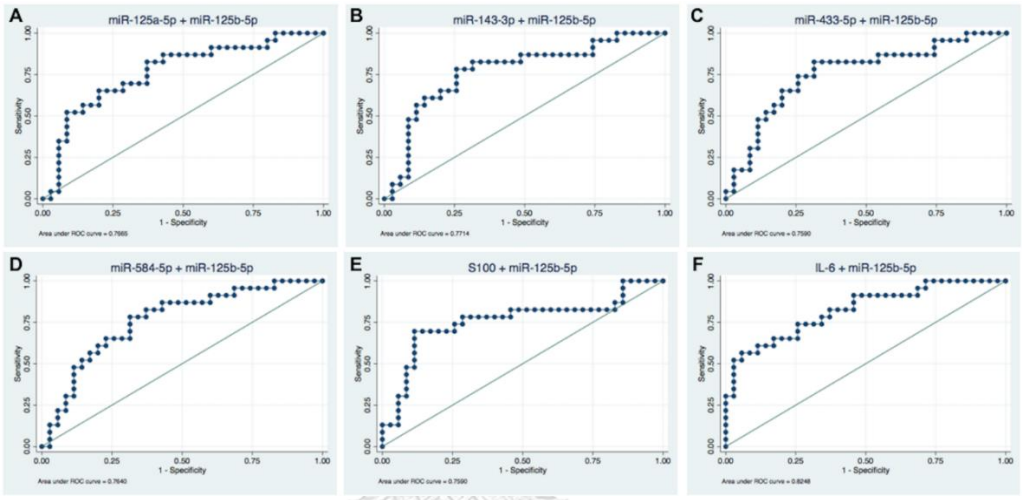


Table 23: AUROC of individual biomarker and combination with miR-125b-5p

	AUROC (individual) (95%CI)	AUROC (combine miR-125b-5p) (95%CI)	*P* Value†
miR-125a-5p	0.7516 (0.6196-0.8835)	0.7665 (0.6382-0.8948)	0.4682
miR-143-3p	0.6919 (0.5523-0.8315)	0.7714 (0.6419-0.9010)	0.1447
miR-433-5p	0.7143 (0.5780-0.8506)	0.7590 (0.6282-0.8899)	0.3703
miR-584-5p	0.6671 (0.5218-0.8124)	0.7640 (0.6380-0.8900)	0.0691
S100	0.7180 (0.5725-0.8635)	0.7590 (0.6174-0.9006)	0.4405
IL-6	0.8292 (0.7241-0.9343)	0.8248 (0.7148-0.9349)	0.8385

Figure 17: ROC curves of combination of biomarkers with IL-6

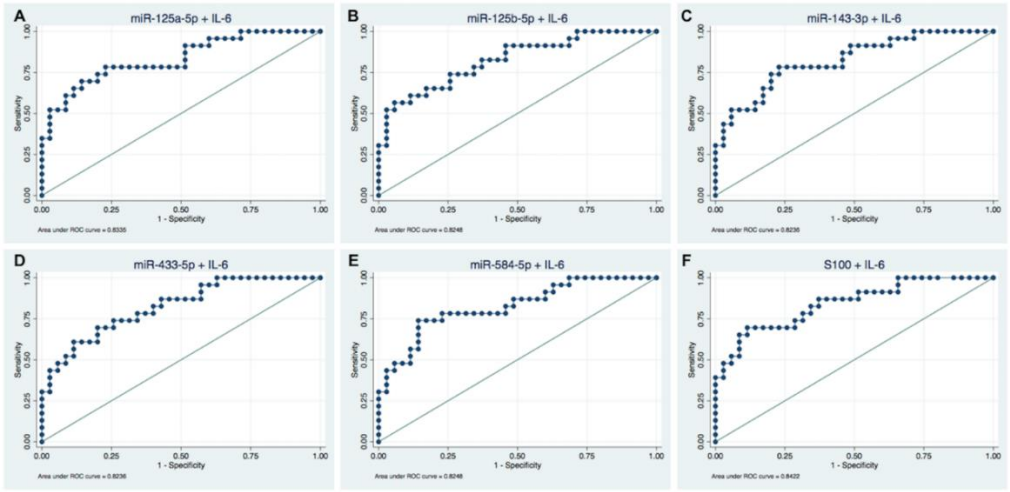


Table 24: AUROC of individual biomarker and combination with IL-6

	AUROC (individual) (95%CI)	AUROC (combine IL-6) (95%CI)	*P* Value†
miR-125a-5p	0.7516 (0.6196-0.8835)	0.8336 (0.7243-0.9248)	0.1591
miR-125b-5p	0.7689 (0.6418-0.8961)	0.8248 (0.7148-0.9349)	0.3421
miR-143-3p	0.6919 (0.5523-0.8315)	0.8236 (0.7140-0.9332)	0.0716
miR-433-5p	0.7143 (0.5780-0.8506)	0.8236 (0.7161-0.9311)	0.1077
miR-584-5p	0.6671 (0.5218-0.8124)	0.8248 (0.7139-0.9358)	0.0290*
S100	0.7180 (0.5725-0.8635)	0.8422 (0.7379-0.9466)	0.0651

*P<0.05

†P value for comparison with the combination of biomarker

4.4.8 Regression analysis

Binary logistic regression analysis was performed to evaluate the association between serum miRNA candidates and the diagnosis of central vertigo due to posterior circulation stroke. In the univariate analysis, miR-125a-5p, miR-125b-5p, miR-143-3p, miR-433-5p, and miR-584-5p were associated with diagnosis of central vertigo due to cerebellar or brainstem infarction. After adjusted for sex, onset to blood collection time, diabetes mellitus, history of myocardial infarction, and smoking, the multivariate analysis revealed that miR-125a-5p, miR-125b-5p, miR-143-3p, miR-433-5p, miR-584-3p, and

miR-584-5p were associated with central vertigo. The odds ratios (OR) and 95% confidence intervals of each biomarker were presented in Table 25.

Table 25: Univariate and multivariate logistic regression analyses for diagnosis of central vertigo

	Univariate analysis		Multivariate analysis†	
	Unadjusted OR (95% CI)	*P* Value	Adjusted OR (95% CI)	*P* Value
miRNAs				
miR-124-3p	1.000 (1.000-1.000)	0.765	1.000 (1.000-1.000)	0.307
miR-124-5p	1.001 (0.999-1.002)	0.493	1.002 (0.999-1.005)	0.159
miR-125a-5p	1.002 (1.000-1.004)	0.016*	1.004 (1.001-1.007)	0.013*
miR-125b-5p	1.009 (1.002-1.016)	0.016*	1.021 (1.007-1.035)	0.004*
miR-143-3p	1.003 (1.000-1.007)	0.046*	1.009 (1.002-1.015)	0.013*
miR-342-3p	0.999 (0.998-1.001)	0.323	1.000 (0.998-1.001)	0.81
miR-376a-3p	1.002 (0.998-1.006)	0.31	1.003 (0.998-1.008)	0.252
miR-433-5p	1.008 (1.000-1.016)	0.044*	1.017 (1.003-1.032)	0.016*
miR-584-3p	1.001 (1.000-1.002)	0.1	1.002 (1.000-1.004)	0.022*
miR-584-5p	1.001 (1.000-1.002)	0.039*	1.003 (1.001-1.005)	0.016*

*P<0.05

†Adjusted for sex, onset to blood collection time, diabetes mellitus, history of myocardial infarction, smoking

Chapter 5 Discussion and Conclusion

This study is the first to demonstrate the potential of serum miRNAs as biomarkers to differentiate between central vertigo due to cerebellar or brainstem infarction and peripheral vertigo among patients presented with acute vertigo. Total 10 miRNA candidates were selected for validation by RT-qPCR in this study. By using NanoString nCounter Technology in the discovery phase, 5 miRNA candidates were selected including miR-342-3p, miR-376a-3p, miR-433-5p, miR-584-3p, and miR-584-5p. Five miRNA candidates were selected from literature review including miR-124-3p, miR-124-5p, miR-125a-5p, miR-125b-5p, and miR-143-3p. In addition to miRNA candidates, 3 protein biomarkers including NSE, S100, and IL-6 representing neuronal specific, glial specific, and inflammatory biomarkers respectively were also included in the study.

During acute phase of vertigo, we found that the serum miR125a-5p, miR125b-5p, miR143-3p, miR433-5p, and miR-584-5p had significantly higher level in patients with central vertigo due to cerebellar or brainstem infarction than in patients with peripheral vertigo. Serial measurement of the serum miRNA candidates and protein biomarkers was performed at day 0 and day 90 in order to evaluate the temporal expression profile of the potential biomarkers. In acute phase, 7 out of 10 selected miRNAs demonstrated significantly higher level of expression in central vertigo compared to peripheral vertigo. These miRNAs included miR-125a-5p, 125b-5p, miR-143-3p, miR-376a-3p, miR-433-5p, miR-584-3p, and miR-584-5p. Among patients with central vertigo, all of these 7 miRNAs except miR-433-5p demonstrated significantly decreased in level of expression at day 90 when compared to the expression level in acute phase at day 0. Interestingly, there was no significant different regarding the expression level of miR-433-5p among patients with central vertigo in acute phase and at day 90. This might be due to the persistently elevated level of miR-433-5p expression which remained in high level until day 90. Regarding protein biomarkers, similar temporal expression profile were

demonstrated in serum S100 and IL-6 where there was significantly higher level of expression in central vertigo compared to peripheral vertigo in acute phase and significantly decreased in level of expression by day 90. None of the selected miRNA candidates and protein biomarkers demonstrated significant difference regarding the level of expression between patients with central and peripheral vertigo at day 90. In addition, there was no significant difference regarding the level of miRNA candidates and protein biomarker between day 0 and day 90 in patients with peripheral vertigo. These demonstrated that changes in miRNAs and protein biomarkers level occurred mainly in the acute phase of patients with central vertigo and emphasized the potential of miRNAs as biomarker for central vertigo diagnosis. MiR-125a-5p, miR-125b-5p, and miR-143-3p had been previously shown to have significant changes in level of expression over time. Similar temporal expression profile to our study, miR-125b-5p and miR-143-3p level of expression were significantly higher in acute phase on admission (at day 1) in patients with acute ischemic stroke when compared to healthy controls and significantly decreased after day 2. In contrast to our study, miR-125a-5p had significantly high level of expression in acute phase of patients with ischemic stroke compared to healthy control and remained elevated until day 90 (58). Despite the strong profiles of miR-124 as potential biomarker for acute ischemic stroke from previous studies (73, 74), there was no significant difference regarding the expression level of both strands of miR-124 including miR-124-3p and miR-124-5p between patients with central and peripheral vertigo in our study. The temporal expression profile of the miRNA candidates demonstrated in our study revealed the essential biomarker properties regarding the significant change in level of expression in the acute phase among patients with the central vertigo. This temporal expression pattern might indicate the possible mechanism of these miRNAs with regards to pathophysiology and pathogenesis of acute ischemic stroke. An elevation of these serum miRNAs in acute phase of ischemic stroke is possibly due to the passive

release from ischemia-vulnerable neurons (86). In addition, an increase in the level of the circulating miRNAs could result from active secretion via extracellular vesicles (58, 87). In our study, some of the selected miRNAs are novel candidates in the field of stroke biomarker. Therefore, it is possible that these novel miRNA candidates are released from injured neuron that specifically expressed in cerebellum or brainstem. However, further studies are required in order to demonstrate the organ specific properties of these miRNAs.

Regarding the correlation between level of potential biomarkers selected in our study and ischemic stroke severity, there was no correlation between expression level of 10 selected miRNA candidates and protein biomarkers and ischemic stroke severity determined by NIHSS. It has been widely demonstrated that symptoms of posterior circulation stroke are not well represented by NIHSS (88, 89). This could account for lack of correlation between selected biomarker candidates and NIHSS demonstrated in this study. Only miR-125a-5p, miR-125b-5p, and miR-376a-3p demonstrated low to moderate positive correlation with infarction volume. This finding is in contrast with previous study that demonstrated no correlation between expression level of miR-125a-5p, miR-125b-5p, miR-143p-3p and infarction volume (58). The poor correlation among serum level of miRNA candidates and infarction volume seems contradictory to the concepts of potential involvement in ischemic stroke pathogenesis and pathophysiology of these miRNAs. However, the independent expression level of both serum miRNA candidates and protein biomarkers and ischemic stroke severity determined by both infarction volume and NIHSS emphasize the potential of these biomarker candidates to be applicable in the diagnosis of acute ischemic stroke regardless of stroke severity.

The serum level of 10 selected potential miRNA candidates and 3 protein biomarkers were stratified by ischemic stroke etiology according to TOAST classification (85). Most of miRNAs had similar level of expression among different etiologic subgroups.

Interestingly, patients with ischemic stroke due to cardioembolic etiology had relatively higher level of serum miR-143-3p, miR-433-5p, and miR-584-5p. There were significantly high level of serum miR-433-5p and miR-584-5p in ischemic stroke due to cardioembolic stroke when compared to small vessel disease. This study also demonstrated significantly high level of serum miR-143-3p in cardioembolic stroke compared to ischemic stroke due to large vessel atherosclerosis. However, miR125a-5p, miR-125b-5p, and miR-143-3p had not been shown to have difference level of expression with regards to ischemic stroke subtypes in previous study (58). The level of expression of most of miRNAs selected for analysis in our study are independent of ischemic stroke etiology which also highlight the potential of these miRNAs as ischemic stroke biomarker candidates. Nonetheless, our findings should be interpret with cautions since the sample size in each ischemic stroke subtype was small.

To our knowledge, our study is the first to evaluate the level of miRNAs and protein biomarkers in different regions of cerebral infarction. Serum miR-376a-3p and S100 had significantly higher level in cerebellar infarction compared to brainstem infarction. Interestingly, the level of miR-376a-3p significantly elevated in acute phase of ischemic stroke and decreased by day 90 in the temporal expression profile study. Since the miR-376a-3p has not been previously reported as potential biomarker for acute ischemic stroke diagnosis, this finding from our study demonstrated potential of miR-376a-3p as possible brain region-specific biomarker. Further study focusing on serum miR-376a-3p expression and cerebellar infarction is recommended. Due to small number of patients in each subgroup in this analysis, the result should be interpret with caution.

The discriminative ability to diagnose central vertigo was demonstrated by analysis of the receiver operatic characteristic curves. Five miRNAs including miR-125a-5p, miR-125b-5p, miR-143-3p, miR-433-5p, and miR-584-5p and 2 protein biomarkers including S100 and IL-6 were shown to have satisfactory discriminative ability (AUROC

0.67-0.77). The sensitivity of CT scan for diagnosis of posterior circulation stroke is low (27). Although MRI has higher sensitivity, it is not widely available especially in the rural area where stroke specialist is lacking. Therefore, the basis of this study is to identify potential serum biomarker with high sensitivity to help diagnose patients with posterior circulation stroke who presented with acute vertigo. Using proposed cut off level above 299 copies/ μL in serum miR-125a-5p and above 46 copies/μL in serum miR-433-5p, we were able to achieve sensitivity of 86.96% with acceptable specificity. In order to improve the discriminative ability, we combine potential miRNA and protein candidates with miR-125b-5p and IL-6 which had highest discriminative ability demonstrated by AUROC. However, only combination of IL-6 and miR-584-5p shown significantly increase in discriminative ability. Further replication study with higher number of participants is required to evaluate the diagnostic accuracy of these potential biomarker candidates.

The predictive value of potential biomarker candidates were assessed by univariate and multivariate logistic regression analyses. All of 5 miRNAs including miR-125a-5p, miR-125b-5p, miR-143-3p, miR-433-5p, and miR-584-5p demonstrated to have satisfactory discriminative ability for central vertigo diagnosis in ROC analysis were associated with the diagnosis of ischemic stroke due to cerebellar or brainstem infarction in the univariate analysis. In addition to the 5 miRNAs, miR-584-3p was shown to be associated with the diagnosis of cerebellar or brainstem infarction after adjustment for baseline characteristics.

Out of 10 selected miRNA candidates from the discovery phase by NanoString analysis, we were able to demonstrate 5 miRNAs as potential posterior circulation stroke biomarkers. These miRNAs are miR-125a-5p, miR-125b-5p, miR-143-3p, miR-433-5p, and miR-584-5p. All of these 5 miRNAs demonstrated significantly high level in serum of patients with central vertigo due to acute cerebellar or brainstem infarction compared with those with peripheral vertigo. In addition, these 5 miRNAs were

shown to be associated with central vertigo and had satisfactory discriminative ability to differentiate between central and peripheral vertigo. Among these, miR-125a-5p, miR-125b-5p, and miR-143-3p had been previously identified as potential biomarkers for acute ischemic stroke by RNA sequencing analysis. Platelets were demonstrated to be potential source of these circulating miRNAs. The level of these 3 miRNAs increased by an increasing concentration of platelets that were spiked back into the platelet-poor plasma (58).

MiR-125a-5p may have multiple roles in ischemic stroke pathophysiology. MiR-125a-5p was shown to impair endothelial cell angiogenesis in elderly through the repression of related transcriptional enhancer factor-1 (RTEF-1) and modulation of endothelial nitric oxide synthase (eNOS) and vascular endothelial growth factor (VEGF) (90). An in vivo experiments demonstrated that miR-125a-5p regulate blood brain barrier function. There was significant increase in brain endothelial barrier function in overexpressed miR-125a-5p cell lines (91). In addition, miR-125a-5p plays an important role in regulating differentiation of macrophages by promoting M2 macrophages (alternative activation) which are anti-inflammatory and suppressing M1 macrophages which are proinflammatory (92).

MiR-125b expression in the endothelial cells was induced by tissue hypoxia in an animal model of murine hindlimb ischemia by femoral artery occlusion. This leads to inhibition of vascular endothelial (VE)-cadherin mRNA and *in vitro* tube formation by endothelial cells (93). MiR-125b was also shown to regulate synaptic structure and function by inducing long narrow dendritic spines and opposing normal dendritic protrusions (94). Furthermore, miR-125b-5p had previously been shown to be a potential predictive biomarker for ischemic stroke outcome. In a prospective cohort study, plasma level of miR-125b-5p was higher in patients who received thrombolytic therapy with

unfavorable outcomes defined by mRS scores of 2-6 and was correlated with stroke severity by NIHSS and infarction volumes (95, 96).

MiR-143 along with its counterpart, miR-145, which were co-transcribed together, were shown to be highly expressed in vascular smooth muscle cells of various organs and essential for controlling contractile phenotype (97, 98). Both miR-143 and miR-145 were demonstrated to promote differentiation and suppress proliferation of vascular smooth muscle cells by regulating serum response factor (SRF) to repress factors that promote proliferative smooth muscle phenotype (99). Plasma miR-143 was significantly higher in patient with large vessel atherosclerotic stroke and atherosclerotic patients without stroke when compared to healthy controls. In addition, plasma miR-143 level was shown to be correlated with the severity of cerebral atherosclerosis (100). Interestingly, upregulation of circDLGAP4, which is endogenous miR-143 inhibitor, were demonstrated to decrease blood brain barrier damage, decrease area of infarction and reduce the neurological deficits in the mouse stroke model of transient middle cerebral artery occlusion (101).

We discovered 2 novel miRNAs which were miR-433-5p and miR-584-5p as potential biomarkers for posterior circulation stroke diagnosis. MiR-433 is highly expressed in brain tissue (102). MiR-433 has previously demonstrated to be associated with some neurological disorders. It was shown that miR-433 regulates α-synuclein expression by controlling the translation of fibroblast growth factor 20 (FGF20). Disruption of miRNA-433 binding site increases the translation of FGF 20 both *in vitro* and *in vivo* (103). Subsequent study showed that plasma level of miR-433 was significantly reduced in patients with Parkinson's disease; therefore, was potential biomarker for Parkinson's disease (104). However, previous studies in animal model of cerebral ischemia and traumatic brain injury demonstrated contrast findings of miR-433 to our study in ischemic stroke patients. MiR-433-3p was down regulated in hippocampus of animal model of

controlled cortical impact injury (105). In addition, miR-433 was down regulated in hypoxia-induced human umbilical vein vascular endothelial cells (HUVECs) and rat neurons. Upregulation of miR-433 inhibited cell migration and proliferation of HUVECs and neurons by targeting Hypoxia-Inducible Factor-1α (HIF-1α) (106). Further study is recommended to assess whether the significant increase of miR-433 in acute ischemic stroke patient demonstrated in our study is related to patient outcomes.

However, previous studies has demonstrated the function of miR-584 in other neurological diseases. In patients with relapsing-remitting multiple sclerosis, miR-584 expression in blood cells was shown to be significantly higher compared to healthy controls (107). In addition, miR-584 has been demonstrated to have tumor suppressive functions in breast cancer (108), renal cell carcinoma (109), glioma (110), and neuroblastoma (111). In neuroblastoma, matrix metalloproteinase 14 (MMP-14) is essential for tumorigenesis and aggressiveness. Binding site of miR-584-5p was identified in MMP-14 promotor. It was shown that miR-584-5p was down regulated and inversely correlated with MMP-14 expression in neuroblastoma tissue (111).

Although previous studies demonstrated the potential of miRNAs as stroke biomarkers, none of these studies focusing on the diagnosis of ischemic stroke in the posterior circulation especially among patients presented with acute vertigo(58, 70, 112). However, due to the diagnostic challenges of acute vertigo based on clinical findings, several studies had focusing on the serum proteins as potential diagnostic biomarkers to identify patients with acute vertigo due to cerebrovascular cause. Serum S100B had been demonstrated to be present at significantly higher level in patients who had acute vertigo with positive MRI findings (113, 114). In addition, serum S100b level were significantly higher in patients with acute vertigo due to stroke when compared to patients with nonvascular vertigo (115). These findings were in keeping with the results from our study which demonstrated the significantly higher level of serum S100 among patients with

acute central vertigo due to cerebellar or brainstem infarction compared to those with peripheral vertigo in both acute phase and temporal expression profiles study. In contrast to our study which demonstrated non-significant difference regarding the serum level of NSE among patients with acute central and peripheral vertigo, Zuo L, et al. found that serum NSE were significantly increased in patients with acute vertigo who presented at the hospital within 24 hours after onset due to cerebral infarction (116). Acute vertigo patients with positive MRI had significantly higher level of serum NSE compared to those with MRI negative (113). In addition, an increased NSE was one of the strongest indicators of acute vertigo due to stroke in the the multivariate logistic regression (116). The difference in time of enrollment after onset of acute vertigo might explain the non-significant difference in the serum NSE level in our study. Although several studies evaluated the serum IL-6 level in stroke patients (45, 117, 118), none of these focusing in patients with ischemic stroke who presented with acute vertigo.

Our study demonstrated to be the pioneer study in the field of stroke biomarker. This study is the first to demonstrate the potential miRNA biomarkers in patients with posterior circulation stroke who presented with acute vertigo. This study is also the first study to use molecular barcoding technique by NanoString nCounter Technology in the discovery phase. This technique has been shown to have high sensitivity and less bias introduction which is due to no requirement of library preparation and amplification. In addition, serial measurement of the biomarkers was performed to demonstrate the temporal expression profile of each biomarker. This study also included protein biomarkers of ischemic stroke that represent different cell types involved in stroke pathophysiology including neurons, glial cells, and inflammatory cytokine in the analysis.

This study has some limitations. First, the result is based on discovery phase and validation phase including relatively small number of patients in each group. Independent replication cohort is essential to validate the novel findings from our study.

Second, this study enrolled patients from a single center. Finally, although we enrolled patients with acute vertigo symptoms within 72 hours upon hospital arrival, most of the patients in central vertigo group had onset to blood collection time after 24 hours. This may represent the fact that most of patients with acute vertigo usually arrive to the hospital late.

In conclusion, this study demonstrates the different miRNA expression profiles among patients presented with acute vertigo from cerebellar or brainstem infarction and peripheral vertigo. Specifically, this study discovered novel miRNA candidates including miR-433-5p and miR-584-5p and supports previously studied miR125a-5p, miR-125b-5p, and miR-143-3p as potential biomarkers for posterior circulation stroke diagnosis among patients presented with acute vertigo.

REFERENCES

1. Kattah JC, Talkad AV, Wang DZ, Hsieh Y-H, Newman-Toker DE. HINTS to Diagnose Stroke in the Acute Vestibular Syndrome. Three-Step Bedside Oculomotor Examination More Sensitive Than Early MRI Diffusion-Weighted Imaging. 2009;40(11):3504-10.

2. Newman-Toker DE, Kattah JC, Alvernia JE, Wang DZ. Normal head impulse test differentiates acute cerebellar strokes from vestibular neuritis. Neurology. 2008;70(24 Pt 2):2378-85.

3. Gulyaeva LF, Kushlinskiy NE. Regulatory mechanisms of microRNA expression. J Transl Med. 2016;14(1):143.

4. Thrift AG, Cadilhac DA, Thayabaranathan T, Howard G, Howard VJ, Rothwell PM, et al. Global stroke statistics. Int J Stroke. 2014;9(1):6-18.

5. Suwanwela NC. Stroke epidemiology in Thailand. J Stroke. 2014;16(1):1-7.

6. Hanchaiphiboolkul S, Poungvarin N, Nidhinandana S, Suwanwela NC, Puthkhao P, Towanabut S, et al. Prevalence of stroke and stroke risk factors in Thailand: Thai Epidemiologic Stroke (TES) Study. J Med Assoc Thai. 2011;94(4):427-36.

7. Hotson JR, Baloh RW. Acute Vestibular Syndrome. N Engl J Med. 1998;339(10):680-5.

8. Kerber KA. Vertigo and dizziness in the emergency department. Emerg Med Clin North Am. 2009;27(1):39-50, viii.

9. Tarnutzer AA, Berkowitz AL, Robinson KA, Hsieh YH, Newman-Toker DE. Does my dizzy patient have a stroke? A systematic review of bedside diagnosis in acute vestibular syndrome. CMAJ. 2011;183(9):E571-92.

10. Saber Tehrani AS, Coughlan D, Hsieh YH, Mantokoudis G, Korley FK, Kerber KA, et al. Rising annual costs of dizziness presentations to U.S. emergency departments. Acad Emerg Med. 2013;20(7):689-96.

11. Kerber KA, Brown DL, Lisabeth LD, Smith MA, Morgenstern LB. Stroke among patients with dizziness, vertigo, and imbalance in the emergency department: a population-based study. Stroke. 2006;37(10):2484-7.

12. Savitz SI, Caplan LR. Vertebrobasilar disease. N Engl J Med. 2005;352(25):2618-26.

13. Markus HS, van der Worp HB, Rothwell PM. Posterior circulation ischaemic stroke and transient ischaemic attack: diagnosis, investigation, and secondary prevention. The Lancet Neurology. 2013;12(10):989-98.

14. Searls DE, Pazdera L, Korbel E, Vysata O, Caplan LR. Symptoms and signs of posterior circulation ischemia in the new England medical center posterior circulation registry. Arch Neurol. 2012;69(3):346-51.

15. Norrving B, Magnusson M, Holtas S. Isolated acute vertigo in the elderly; vestibular or vascular disease? Acta Neurol Scand. 1995;91(1):43-8.

16. Choi JH, Kim HW, Choi KD, Kim MJ, Choi YR, Cho HJ, et al. Isolated vestibular syndrome in posterior circulation stroke: Frequency and involved structures. Neurol Clin Pract. 2014;4(5):410-8.

17. Lee H, Sohn SI, Cho YW, Lee SR, Ahn BH, Park BR, et al. Cerebellar infarction presenting isolated vertigo: frequency and vascular topographical patterns. Neurology. 2006;67(7):1178-83.

18. Kim H-A, Yi H-A, Lee H. Recent Advances in Cerebellar Ischemic Stroke Syndromes Causing Vertigo and Hearing Loss. The Cerebellum. 2016;15(6):781-8.

19. Cnyrim CD, Newman-Toker D, Karch C, Brandt T, Strupp M. Bedside differentiation of vestibular neuritis from central "vestibular pseudoneuritis". J Neurol Neurosurg Psychiatry. 2008;79(4):458-60.

20. Halmagyi GM, Gresty MA, Gibson WP. Ocular tilt reaction with peripheral vestibular lesion. Ann Neurol. 1979;6(1):80-3.

21. Oppenheim C, Stanescu R, Dormont D, Crozier S, Marro B, Samson Y, et al. False-negative diffusion-weighted MR findings in acute ischemic stroke. AJNR Am J Neuroradiol. 2000;21(8):1434-40.

22. Newman-Toker DE, Kerber KA, Hsieh YH, Pula JH, Omron R, Saber Tehrani AS, et al. HINTS outperforms ABCD2 to screen for stroke in acute continuous vertigo and dizziness. Acad Emerg Med. 2013;20(10):986-96.

23. Kattah JC. Update on HINTS Plus, With Discussion of Pitfalls and Pearls. J Neurol Phys Ther. 2019;43 Suppl 2 Supplement, Special Supplement: International Conference on Vestibular Rehabilitation:S42-s5.

24. Gurley KL, Edlow JA. Acute Dizziness. Semin Neurol. 2019;39(1):27-40.

25. Choi JH, Park MG, Choi SY, Park KP, Baik SK, Kim JS, et al. Acute Transient Vestibular Syndrome: Prevalence of Stroke and Efficacy of Table 26Bedside Evaluation. Stroke. 2017;48(3):556-62.

26. Chalela JA, Kidwell CS, Nentwich LM, Luby M, Butman JA, Demchuk AM, et al. Magnetic resonance imaging and computed tomography in emergency assessment of patients with suspected acute stroke: a prospective comparison. Lancet. 2007;369(9558):293-8.

27. Simmons Z, Biller J, Adams HP, Jr., Dunn V, Jacoby CG. Cerebellar infarction: comparison of computed tomography and magnetic resonance imaging. Ann Neurol. 1986;19(3):291-3.

28. Edlow JA, Newman-Toker DE, Savitz SI. Diagnosis and initial management of cerebellar infarction. Lancet Neurol. 2008;7(10):951-64.

29. Saber Tehrani AS, Kattah JC, Mantokoudis G, Pula JH, Nair D, Blitz A, et al. Small strokes causing severe vertigo: Frequency of false-negative MRIs and nonlacunar mechanisms. Neurology. 2014;83(2):169-73.

30. Jickling GC, Sharp FR. Biomarker panels in ischemic stroke. Stroke. 2015;46(3):915-20.

31. Glushakova O, Glushakov A, Miller E, Valadka A, Hayes R. Biomarkers for acute diagnosis and management of stroke in neurointensive care units. Brain Circ. 2016;2(1):28-47.

32. Jickling GC, Sharp FR. Blood biomarkers of ischemic stroke. Neurotherapeutics. 2011;8(3):349-60.

33. Jauch EC, Lindsell C, Broderick J, Fagan SC, Tilley BC, Levine SR. Association of serial biochemical markers with acute ischemic stroke: the National Institute of Neurological Disorders and Stroke recombinant tissue plasminogen activator Stroke Study. Stroke. 2006;37(10):2508-13.

34. Anand N, Stead LG. Neuron-specific enolase as a marker for acute ischemic stroke: a systematic review. Cerebrovasc Dis. 2005;20(4):213-9.

35. Stevens H, Jakobs C, de Jager AE, Cunningham RT, Korf J. Neurone-specific enolase and N-acetyl-aspartate as potential peripheral markers of ischaemic stroke. Eur J Clin Invest. 1999;29(1):6-11.

36. Lu K, Xu X, Cui S, Wang F, Zhang B, Zhao Y. Serum neuron specific enolase level as a predictor of prognosis in acute ischemic stroke patients after intravenous thrombolysis. J Neurol Sci. 2015;359(1-2):202-6.

37. Kim BJ, Kim YJ, Ahn SH, Kim NY, Kang DW, Kim JS, et al. The second elevation of neuron-specific enolase peak after ischemic stroke is associated with hemorrhagic transformation. J Stroke Cerebrovasc Dis. 2014;23(9):2437-43.

38. Nash DL, Bellolio MF, Stead LG. S100 as a Marker of Acute Brain Ischemia: A Systematic Review. Neurocrit Care. 2008;8(2):301-7.

39. Foerch C, Wunderlich MT, Dvorak F, Humpich M, Kahles T, Goertler M, et al. Elevated serum S100B levels indicate a higher risk of hemorrhagic transformation after thrombolytic therapy in acute stroke. Stroke. 2007;38(9):2491-5.

40. Wunderlich MT, Wallesch CW, Goertler M. Release of neurobiochemical markers of brain damage is related to the neurovascular status on admission and the site of arterial occlusion in acute ischemic stroke. J Neurol Sci. 2004;227(1):49-53.

41. Foerch C, Singer OC, Neumann-Haefelin T, du Mesnil de Rochemont R, Steinmetz H, Sitzer M. Evaluation of serum S100B as a surrogate marker for long-term outcome and infarct volume in acute middle cerebral artery infarction. Arch Neurol. 2005;62(7):1130-4.

42. Hill MD, Jackowski G, Bayer N, Lawrence M, Jaeschke R. Biochemical markers in acute ischemic stroke. CMAJ. 2000;162(8):1139-40.

43. Foerch C, du Mesnil de Rochemont R, Singer O, Neumann-Haefelin T, Buchkremer M, Zanella FE, et al. S100B as a surrogate marker for successful clot lysis in hyperacute middle cerebral artery occlusion. J Neurol Neurosurg Psychiatry. 2003;74(3):322-5.

44. Erta M, Quintana A, Hidalgo J. Interleukin-6, a major cytokine in the central nervous system. Int J Biol Sci. 2012;8(9):1254-66.

45. Shaafi S, Sharifipour E, Rahmanifar R, Hejazi S, Andalib S, Nikanfar M, et al. Interleukin-6, a reliable prognostic factor for ischemic stroke. Iranian Journal of Neurology. 2014;13(2):70-6.

46. Waje-Andreassen U, Krakenes J, Ulvestad E, Thomassen L, Myhr KM, Aarseth J, et al. IL-6: an early marker for outcome in acute ischemic stroke. Acta Neurol Scand. 2005;111(6):360-5.

47. Vila N, Castillo J, Davalos A, Chamorro A. Proinflammatory cytokines and early neurological worsening in ischemic stroke. Stroke. 2000;31(10):2325-9.

48. Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. Cell. 2004;116(2):281-97.

49. Bhalala OG, Srikanth M, Kessler JA. The emerging roles of microRNAs in CNS injuries. Nat Rev Neurol. 2013;9(6):328-39.

50. Ha M, Kim VN. Regulation of microRNA biogenesis. Nat Rev Mol Cell Biol. 2014;15(8):509-24.

51. Vijayan M, Reddy PH. Peripheral biomarkers of stroke: Focus on circulatory microRNAs. Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease. 2016;1862(10):1984-93.

52. Etheridge A, Lee I, Hood L, Galas D, Wang K. Extracellular microRNA: a new source of biomarkers. Mutat Res. 2011;717(1-2):85-90.

53. Adachi T, Nakanishi M, Otsuka Y, Nishimura K, Hirokawa G, Goto Y, et al. Plasma microRNA 499 as a biomarker of acute myocardial infarction. Clin Chem. 2010;56(7):1183-5.

54. Wang K, Zhang S, Marzolf B, Troisch P, Brightman A, Hu Z, et al. Circulating microRNAs, potential biomarkers for drug-induced liver injury. Proc Natl Acad Sci U S A. 2009;106(11):4402-7.

55. Lawrie CH, Gal S, Dunlop HM, Pushkaran B, Liggins AP, Pulford K, et al. Detection of elevated levels of tumour-associated microRNAs in serum of patients with diffuse large B-cell lymphoma. Br J Haematol. 2008;141(5):672-5.

56. Sheinerman K, Umansky S. Circulating cell-free microRNA as biomarkers for screening, diagnosis and monitoring of neurodegenerative diseases and other neurologic pathologies. Front Cell Neurosci. 2013;7(150).

57. Chen Y, Gao C, Sun Q, Pan H, Huang P, Ding J, et al. MicroRNA-4639 Is a Regulator of DJ-1 Expression and a Potential Early Diagnostic Marker for Parkinson's Disease. Front Aging Neurosci. 2017;9:232.

58. Tiedt S, Prestel M, Malik R, Schieferdecker N, Duering M, Kautzky V, et al. RNA-Seq Identifies Circulating miR-125a-5p, miR-125b-5p, and miR-143-3p as Potential Biomarkers for Acute Ischemic Stroke. Circ Res. 2017;121(8):970-80.

59. Tan KS, Armugam A, Sepramaniam S, Lim KY, Setyowati KD, Wang CW, et al. Expression Profile of MicroRNAs in Young Stroke Patients. PLoS One. 2009;4(11):e7689.

60. Sepramaniam S, Tan JR, Tan KS, DeSilva DA, Tavintharan S, Woon FP, et al. Circulating microRNAs as biomarkers of acute stroke. Int J Mol Sci. 2014;15(1):1418-32.

61. Jickling GC, Ander BP, Zhan X, Noblett D, Stamova B, Liu D. microRNA expression in peripheral blood cells following acute ischemic stroke and their predicted gene targets. PLoS One. 2014;9(6):e99283.

62. Wang W, Sun G, Zhang L, Shi L, Zeng Y. Circulating microRNAs as novel potential biomarkers for early diagnosis of acute stroke in humans. J Stroke Cerebrovasc Dis. 2014;23(10):2607-13.

63. Li P, Teng F, Gao F, Zhang M, Wu J, Zhang C. Identification of circulating microRNAs as potential biomarkers for detecting acute ischemic stroke. Cell Mol Neurobiol. 2015;35(3):433-47.

64. Zeng L, Liu J, Wang Y, Wang L, Weng S, Tang Y, et al. MicroRNA-210 as a novel blood biomarker in acute cerebral ischemia. Front Biosci (Elite Ed). 2011;3:1265-72.

65. Gan CS, Wang CW, Tan KS. Circulatory microRNA-145 expression is increased in cerebral ischemia. Genet Mol Res. 2012;11(1):147-52.

66. Long G, Wang F, Li H, Yin Z, Sandip C, Lou Y, et al. Circulating miR-30a, miR-126 and let-7b as biomarker for ischemic stroke in humans. BMC Neurol. 2013;13:178.

67. Jin F, Xing J. Circulating pro-angiogenic and anti-angiogenic microRNA expressions in patients with acute ischemic stroke and their association with disease severity. Neurol Sci. 2017;38(11):2015-23.

68. Wu J, Du K, Lu X. Elevated expressions of serum miR-15a, miR-16, and miR-17-5p are associated with acute ischemic stroke. Int J Clin Exp Med. 2015;8(11):21071-9.

69. Jia L, Hao F, Wang W, Qu Y. Circulating miR-145 is associated with plasma high-sensitivity C-reactive protein in acute ischemic stroke patients. Cell Biochem Funct. 2015;33(5):314-9.

70. Chen Z, Wang K, Huang J, Zheng G, Lv Y, Luo N, et al. Upregulated Serum MiR-146b Serves as a Biomarker for Acute Ischemic Stroke. Cell Physiol Biochem. 2018;45(1):397-405.

71. Shah R, Tanriverdi K, Levy D, Larson M, Gerstein M, Mick E, et al. Discordant Expression of Circulating microRNA from Cellular and Extracellular Sources. PLoS One. 2016;11(4):e0153691.

72. Wang K, Yuan Y, Cho J-H, McClarty S, Baxter D, Galas DJ. Comparing the MicroRNA Spectrum between Serum and Plasma. PLoS One. 2012;7(7):e41561.

73. Weng H, Shen C, Hirokawa G, Ji X, Takahashi R, Shimada K, et al. Plasma miR-124 as a biomarker for cerebral infarction. Biomed Res. 2011;32(2):135-41.

74. Ji Q, Ji Y, Peng J, Zhou X, Chen X, Zhao H, et al. Increased Brain-Specific MiR-9 and MiR-124 in the Serum Exosomes of Acute Ischemic Stroke Patients. PLoS One. 2016;11(9):e0163645.

75. Goytain A, Ng T. NanoString nCounter Technology: High-Throughput RNA Validation. Methods Mol Biol. 2020;2079:125-39.

76. Geiss GK, Bumgarner RE, Birditt B, Dahl T, Dowidar N, Dunaway DL, et al. Direct multiplexed measurement of gene expression with color-coded probe pairs. Nat Biotechnol. 2008;26(3):317-25.

77. Sacco RL, Kasner SE, Broderick JP, Caplan LR, Connors JJ, Culebras A, et al. An Updated Definition of Stroke for the 21st Century. A Statement for Healthcare Professionals From the American Heart Association/American Stroke Association. 2013;44(7):2064-89.

78. von Brevern M, Bertholon P, Brandt T, Fife T, Imai T, Nuti D, et al. Benign paroxysmal positional vertigo: Diagnostic criteria Consensus document of the Committee for the Classification of Vestibular Disorders of the Barany Society. 2015. 2015;25:105-17.

79. Lopez-Escamez JA, Carey J, Chung W, Goebel JA, Magnusson M, Mandala M, et al. Diagnostic criteria for Meniere's Disease Consensus document of the Barany Society, The Japan Society for Equilibrium Research, the European Academy of Otology and Neurotology (EAONO), the American Academy of Otolaryngology-Head and Neck Surgery (AAO-HNS) and the Korean Balance Society. J Vestib Res. 2015;25:1-7.

80. Jeong SH, Kim HJ, Kim JS. Vestibular neuritis. Semin Neurol. 2013;33(3):185-94.

81. Bardou P, Mariette J, Escudié F, Djemiel C, Klopp C. jvenn: an interactive Venn diagram viewer. BMC Bioinformatics. 2014;15(1):293.

82. Mei Q, Li X, Meng Y, Wu Z, Guo M, Zhao Y, et al. A Facile and Specific Assay for Quantifying MicroRNA by an Optimized RT-qPCR Approach. PLoS One. 2012;7(10):e46890.

83. O'Keeffe AG, Ambler G, Barber JA. Sample size calculations based on a difference in medians for positively skewed outcomes in health care studies. BMC Med Res Methodol. 2017;17(1):157.

84. Ludwig N, Leidinger P, Becker K, Backes C, Fehlmann T, Pallasch C, et al. Distribution of miRNA expression across human tissues. Nucleic Acids Res. 2016;44(8):3865-77.

85. Adams HP, Bendixen BH, Kappelle LJ, Biller J, Love BB, Gordon DL, et al. Classification of subtype of acute ischemic stroke. Definitions for use in a multicenter

clinical trial. TOAST. Trial of Org 10172 in Acute Stroke Treatment. Stroke. 1993;24(1):35-41.

86. Wu J, Fan CL, Ma LJ, Liu T, Wang C, Song JX, et al. Distinctive expression signatures of serum microRNAs in ischaemic stroke and transient ischaemic attack patients. Thromb Haemost. 2017;117(5):992-1001.

87. Creemers Esther E, Tijsen Anke J, Pinto Yigal M, van Rooij E. Circulating MicroRNAs. Circ Res. 2012;110(3):483-95.

88. Martin-Schild S, Albright KC, Tanksley J, Pandav V, Jones EB, Grotta JC, et al. Zero on the NIHSS does not equal the absence of stroke. Ann Emerg Med. 2011;57(1):42-5.

89. Schneck Michael J. Current Stroke Scales May Be Partly Responsible for Worse Outcomes in Posterior Circulation Stroke. Stroke. 2018;49(11):2565-6.

90. Che P, Liu J, Shan Z, Wu R, Yao C, Cui J, et al. miR-125a-5p impairs endothelial cell angiogenesis in aging mice via RTEF-1 downregulation. Aging Cell. 2014;13(5):926-34.

91. Reijerkerk A, Lopez-Ramirez MA, van Het Hof B, Drexhage JAR, Kamphuis WW, Kooij G, et al. MicroRNAs regulate human brain endothelial cell-barrier function in inflammation: implications for multiple sclerosis. The Journal of neuroscience : the official journal of the Society for Neuroscience. 2013;33(16):6857-63.

92. Banerjee S, Cui H, Xie N, Tan Z, Yang S, Icyuz M, et al. miR-125a-5p regulates differential activation of macrophages and inflammation. The Journal of biological chemistry. 2013;288(49):35428-36.

93. Muramatsu F, Kidoya H, Naito H, Sakimoto S, Takakura N. microRNA-125b inhibits tube formation of blood vessels through translational suppression of VE-cadherin. Oncogene. 2013;32(4):414-21.

94. Edbauer D, Neilson JR, Foster KA, Wang CF, Seeburg DP, Batterton MN, et al. Regulation of synaptic structure and function by FMRP-associated microRNAs miR-125b and miR-132. Neuron. 2010;65(3):373-84.

95. He X-W, Shi Y-H, Liu Y-S, Li G-F, Zhao R, Hu Y, et al. Increased plasma levels of miR-124-3p, miR-125b-5p and miR-192-5p are associated with outcomes in acute ischaemic stroke patients receiving thrombolysis. Atherosclerosis. 2019;289:36-43.

96. He XW, Shi YH, Zhao R, Liu YS, Li GF, Hu Y, et al. Plasma Levels of miR-125b-5p and miR-206 in Acute Ischemic Stroke Patients After Recanalization Treatment: A Prospective Observational Study. J Stroke Cerebrovasc Dis. 2019;28(6):1654-61.

97. Zampetaki A, Mayr M, van Rooij E. MicroRNAs in Vascular and Metabolic Disease. Circ Res. 2012;110(3):508-22.

98. Ramanujam D, Engelhardt S. Intercellular miRNA Traffic. Circ Res. 2015;116(11):1726-8.

99. Cordes KR, Sheehy NT, White MP, Berry EC, Morton SU, Muth AN, et al. miR-145 and miR-143 regulate smooth muscle cell fate and plasticity. Nature. 2009;460(7256):705-10.

100. Gao J, Yang S, Wang K, Zhong Q, Ma A, Pan X. Plasma miR-126 and miR-143 as Potential Novel Biomarkers for Cerebral Atherosclerosis. J Stroke Cerebrovasc Dis. 2019;28(1):38-43.

101. Bai Y, Zhang Y, Han B, Yang L, Chen X, Huang R, et al. Circular RNA DLGAP4 Ameliorates Ischemic Stroke Outcomes by Targeting miR-143 to Regulate Endothelial-Mesenchymal Transition Associated with Blood–Brain Barrier Integrity. The Journal of Neuroscience. 2018;38(1):32.

102. Davis E, Caiment F, Tordoir X, Cavaille J, Ferguson-Smith A, Cockett N, et al. RNAi-mediated allelic trans-interaction at the imprinted Rtl1/Peg11 locus. Curr Biol. 2005;15(8):743-9.

103. Wang G, van der Walt JM, Mayhew G, Li Y-J, Züchner S, Scott WK, et al. Variation in the miRNA-433 binding site of FGF20 confers risk for Parkinson disease by overexpression of alpha-synuclein. Am J Hum Genet. 2008;82(2):283-9.

104. Zhang X, Yang R, Hu B-L, Lu P, Zhou L-L, He Z-Y, et al. Reduced Circulating Levels of miR-433 and miR-133b Are Potential Biomarkers for Parkinson's Disease. Front Cell Neurosci. 2017;11:170-.

105. Redell JB, Liu Y, Dash PK. Traumatic brain injury alters expression of hippocampal microRNAs: potential regulators of multiple pathophysiological processes. J Neurosci Res. 2009;87(6):1435-48.

106. Zhang L, Zhang Y, Zhang X, Zhang Y, Jiang Y, Xiao X, et al. MicroRNA-433 Inhibits the Proliferation and Migration of HUVECs and Neurons by Targeting Hypoxia-Inducible Factor 1 Alpha. J Mol Neurosci. 2017;61(2):135-43.

107. Keller A, Leidinger P, Lange J, Borries A, Schroers H, Scheffler M, et al. Multiple Sclerosis: MicroRNA Expression Profiles Accurately Differentiate Patients with Relapsing-Remitting Disease from Healthy Controls. PLoS One. 2009;4(10):e7440.

108. Fils-Aime N, Dai M, Guo J, El-Mousawi M, Kahramangil B, Neel JC, et al. MicroRNA-584 and the protein phosphatase and actin regulator 1 (PHACTR1), a new signaling route through which transforming growth factor-beta Mediates the migration and actin dynamics of breast cancer cells. J Biol Chem. 2013;288(17):11807-23.

109. Ueno K, Hirata H, Shahryari V, Chen Y, Zaman MS, Singh K, et al. Tumour suppressor microRNA-584 directly targets oncogene Rock-1 and decreases invasion ability in human clear cell renal cell carcinoma. Br J Cancer. 2011;104(2):308-15.

110. Wang X-P, Deng X-L, Li L-Y. MicroRNA-584 functions as a tumor suppressor and targets PTTG1IP in glioma. Int J Clin Exp Pathol. 2014;7(12):8573-82.

111. Xiang X, Mei H, Qu H, Zhao X, Li D, Song H, et al. miRNA-584-5p exerts tumor suppressive functions in human neuroblastoma through repressing transcription of matrix metalloproteinase 14. Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease. 2015;1852(9):1743-54.

112. Chen Y, Song Y, Huang J, Qu M, Zhang Y, Geng J, et al. Increased Circulating Exosomal miRNA-223 Is Associated with Acute Ischemic Stroke. Front Neurol. 2017;8:57-.

113. Mozaffari Jea. Characteristics of S100B and Neuron Specific Enolase in Differentiating Acute Vertigo Cases with Central Causes; a Diagnostic Accuracy Study. . Archives of Academic Emergency Medicine. 2020.

114. Kartal AG, Yilmaz S, Yaka E, Pekdemir M, Sarisoy HT, Cekmen MB, et al. Diagnostic value of S100B protein in the differential diagnosis of acute vertigo in the emergency department. Acad Emerg Med. 2014;21(7):736-41.

115. Purrucker JC, Herrmann O, Lutsch JK, Zorn M, Schwaninger M, Bruckner T, et al. Serum Protein S100β is a Diagnostic Biomarker for Distinguishing Posterior Circulation Stroke from Vertigo of Nonvascular Causes. Eur Neurol. 2014;72(5-6):278-84.

116. Zuo L, Zhan Y, Liu F, Chen C, Xu L, Calic Z, et al. Clinical and laboratory factors related to acute isolated vertigo or dizziness and cerebral infarction. Brain and Behavior. 2018;8(9):e01092.

117. Smith CJ, Emsley HC, Gavin CM, Georgiou RF, Vail A, Barberan EM, et al. Peak plasma interleukin-6 and other peripheral markers of inflammation in the first week of ischaemic stroke correlate with brain infarct volume, stroke severity and long-term outcome. BMC Neurol. 2004;4:2.

118. Moreno VP, Subira D, Meseguer E, Llamas P. IL-6 as a biomarker of ischemic cerebrovascular disease. Biomark Med. 2008;2(2):125-36.

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PUBLICATION	1. Lumlertgul N, Kijpaisalratana N, Pityaratstian N, Wangsaturaka D. Cinemeducation: A pilot student project using movies to help students learn medical professionalism. Medical Teacher 2009; 31(7): e327-e332. 2. Sullivan GM, Mierzwa AJ, Kijpaisalratana N, Tang H, Wong Y, Song SK, Selwyn R, Armstrong RC. Oligodendrocyte lineage and subventricular zone response to traumatic axonal injury in the corpus callosum. J Neuropathol Exp Neurol 2013; 72(12): 1106-1125. 3. Davidson NL, Fengshan Y, Kijpaisalratana N, Le TQ, Beer LA, Radomski KL, Armstrong RC. Leukemia/lymphoma-related factor (LRF) exhibits stage- and context-dependent transcriptional controls in the oligodendrocyte lineage and modulates remyelination. J Neuro Res 2017;1-18. 4. Kijpaisalratana N, Chutinet A, Suwanwela NC. Hyperacute simultaneous cardiocerebral infarction: rescuing the brain or the heart first? Front. Neurol 2017;8:664.doi:10.3389/fneur.2017.00664

5. Travanichakul S, Kijpaisalratana N, Chutinet A, Suwanwela NC. Patent foramen ovale related stroke. J Thai Stroke Soc. 2017; 16(3): 14-26.

6. Travanichakul S, Kijpaisalratana N, Akarathanawat W, Chutinet A, Suwanwela NC. A man presented with acute polyopia: a case report. J Thai Stroke Soc. 2018 17(2): 41-46.

7. Suwanwela NC, Chutinet A, Kijpaisalratana N. (2018). Thrombolytic Treatment in Thailand. Journal of Stroke Medicine. 2018 1(1): 41–44.

8. Chutinet A, Keosodsay S, Vorasayan P, Samajarn J, Akarathanawat W, Kijpaisalratana N, Nosakpeseuth N, Phetsiriseng K, Thongsna S, Vongvasinkul P, Suwanwela NC. The first 10 thrombolysis for acute ischemic stroke in Lao People's Democratic Republic under teleconsultation from Thailand. J Stroke Cerebrovasc Dis. 2019 Sep 14: 104327. doi: 10.1016/j.jstrokecerebrovasdis.2019.104327. [Epub ahead of print]

AWARD RECEIVED

1. 2 AMEE Awards with support of the Patil Family i.e.

a) Teaching and Learning

b) Student Issues

From the short communication presentation of the research on "CINEMEDUCATION: Learning professionalism through films at Chulalongkorn Medical School" in the conference: The Association for Medical Education in Europe 2007 (AMEE 2007) during August 23rd -29th 2007 at Norwegian University of Science and Technology, Trondheim, Norway.

2.The award from the Faculty of Medicine, Chulalongkorn University in 2008 for bringing the fame to the University

(by receiving AMEE awards in 2007).

3. Awarded Prince Mahidol Youth Program Scholarship 2010 for one year research project in Regenerative Medicine in Traumatic Brain Injury at Center for Neuroscience and Regenerative Medicine, Bethesda, Maryland, United States of America

4. Outstanding extern achievement award of the Department of Pediatrics for year 2010 from Chulalongkorn University, Faculty of Medicine, Chulalongkorn University

5. Shield of academic award from Chulalongkorn University Savings Cooperative Limited for graduation Doctor of Medicine, with First Class Honors.

6. Shield of Honor award for bringing distinguished reputation and pride to the Faculty of Medicine, Chulalongkorn University on the occasion of honoring individuals of the Faculty of Medicine Chulalongkorn University for year 2011.

7. Honorary student of the year 2011 from Chulalongkorn University Demonstration School.

8. Awarded Certificate of Good Research from Graduate research competition 2016 of Faculty of Medicine, Chulalongkorn University