

**A STUDY OF MICRO RNA PROFILES IN
ISCHAEMIC STROKE AND ITS RISK FACTOR IN
DEVELOPMENT OF THE NOVEL BIOMARKER OF
YOUNG STROKE PATIENTS**

GAN CHYE SHENG

SUBMISSION OF DISSERTATION IN FULFILMENT
OF THE REQUIREMENT FOR THE DEGREE OF
MASTER OF MEDICAL SCIENCES

**FACULTY OF MEDICINE
UNIVERSITY OF MALAYA**

2012

Abstract

Recent global stroke surveillance conducted by World Health Organization (WHO) has reported 15 million people suffer from stroke per annum. Approximately one-third of the stroke causes mortality placing it as the second leading cause of death after cardiac arrest. Knowledge pertaining stroke has been valuable and utmost desired in order to deliver effective education, prevention, diagnosis/classification and management. Emergence of microRNA expression level delivering promising biomarker has driven diligent researches in developing novel biomarkers for stroke. MicroRNA is a class of ~22 nucleotide-long non-coding RNAs, which is shown to orchestrate protein expression at posttranscriptional levels, hence earlier detection prior manifestation. Acquiring the-state-of-the-art Real-time PCR technology in RNA quantification, this study was aimed to study the microRNA-145, -214, -222, -223, -23b and -339 present in peripheral blood and its temporal expression profiles of chronic young ischaemic stroke patients; having an insight of the molecular regulation of cerebrovascular maladies origin. Peripheral blood of chronic ischaemic stroke patients (n = 32) aged between 18-49 years, characterized based on WHO clinical criteria which subsequently classified in accordance to TOAST guideline were tested in this study. Subsequent peripheral blood sampling of these patients (n = 11) were performed between 3 to 16 months after the initial sampling in order to study the long-term stroke temporal expression profiles of each microRNAs studied. Peripheral blood from normal volunteers (n = 14) were used as controls. Ribosomal RNA, 18S rRNA expression was used as housekeeping gene. Students' t-Test was employed for statistical calculation. P value of lesser than 0.05 ($p < 0.05$) is considered significant. Results revealed the circulating microRNA-145, -222, -223 and -23b that are implicated in the vascular biology and immune response in young ischaemic stroke patients demonstrated ectopic

expression profile relative to the normal control. The temporal expression profile of circulating microRNA-214, -222, -223 and -23b demonstrated plummet in expression; this suggests recovery and downregulation of microRNAs expression is associated with good outcome following stroke. Comparative analysis of the 6 circulating microRNAs and the subtypes of ischaemic stroke suggested that the microRNA-145, 223, 23b and 339 expression profile in cardioembolic subtype of stroke might differ from the atherosclerotic stroke that comprises of large and small vessel subtypes. In addition, the expression profile of circulating microRNA-23b was also shown to be significantly different between the cardioembolic and undetermined subtypes of ischaemic stroke. Therefore, this study demonstrates the potential of peripheral blood microRNAs to be developed as diagnostic and prognostic biomarkers of cerebral ischaemic stroke.

Abstrak

Hasil kaji selidik global terkini yang dilangsungkan oleh World Health Organization (WHO) telah melaporkan 15 juta individu mengalami stroke setiap tahun. Daripada itu, lebih kurang satu pertiga daripada kes strok memudaratkan lantas menempatkannya penyebab kedua kematian sejurus serangan jantung. Namun, pengetahuan mengenai strok penting dan diperlukan demi penyampaian pendidikan efektif, pencegahan, diagnostik/klasifikasi dan penyelenggaraan. Kemunculan taraf ekspresi mikroRNA sebagai biomarker telah mencetus kegigihan para penyelidik dalam menempuh penemuan biomarker unggul bagi strok. MikroRNA merupakan kumpulan RNA tidak mengkod yang terdiri daripada ~22 rantaian nukleotida yang mampu mengolah ekspresi protein pada tahap pasca-transkripsi, yakni membolehkan pengesana awal sebelum menifestasi. Dengan penggunaan teknologi pengkuantitan Real-time PCR, kajian ini dijalankan dengan tujuan mengkaji mikroRNA-145, -214, -222, -223, -23b dan -339 yang hadir dalam darah periferi dan profil ekspresi temporalnya dalam pesakit kronik muda strok iskemia; demi penghayatan regulasi molekul yang berpunca daripada masalah cerebrovaskular. Darah periferi pesakit kronik strok iskemia ($n = 32$) yang berumur diantara 18-49 tahun, diklasifikasi dengan merujuk kepada kriteria klinikal WHO yang selanjutnya diklasifikasi mengikut tatacara TOAST telah digunakan bagi menjalankan kajian ini. Pensampelan darah periferi lanjutan ($n = 11$) dijalankan diantara 3 hingga 16 bulan selapas pensampelan pertama dilakukan bagi mengkaji profil ekspresi temporal setiam mikroRNA yang dikaji. Darah periferi yang diperolehi daripada sukarelawan normal ($n = 11$) digunakan sebagai piawai. Ekspresi RNA ribosomal, 18S rRNA dirujuk sebagai gen housekeeping. Students' t-Test digunakan dalam penghitungan statistik. Nilai p yang kurang daripada 0.05 ($p < 0.05$) dikira signifikan. Keputusan menunjukkan mikroRNA-145, -222, -223 dan -23b yang terdapat

dalam pengaliran darah yang memunyai implikasi terhadap biologi vaskular dan tindak balas immune dalam pesakit muda stroke iskemia menunjukkan profail ekspresi ectopic relatif kepada piawai normal. Profail ekspresi temporal mikroRNA-214, -222, -223, dan -23b yang terdapat dalam pengaliran darah menunjukkan penjunaman ekspresi. Hasil analisis 6 mikroRNA yang di kaji melalui perbandingan yang dibuat terhadap sub-jenis strok iskemia telah mecadangkan bahawa profile ekspresi mikroRNA-145, -222, -23b dan -339 pada sub-jenis cardioembolic mungkin berbeza daripada atherosclerotic strok yang terdiri daripada sub-jenis large vessel dan small vessel. Tambahan pula, profile ekspresi mikroRNA-23b dari darah periferi juga menunjukkan perbezaan yang signifikan diantara sub-jenis cardioembolic dan sub-jenis strok iskemia yang tidak dikenalpasti. Ini mecadangkan pemulihan dan pengurangan regulasi ekspresi microRNA dikaitkan dengan hasil baik selanjut strok. Dengan ini, hasil kajian ini telah menyerlahkan potensi microRNA darah periferi dalam penghasilan biomarker prognostik strok iskemia cerebral.

Acknowledgements

The process of getting this thesis written up has been more than just spending time in the laboratory, but inspirational and metamorphosing. The experiences gained have driven my motivation high into research and reflected my goals in life. Hereby, I would like to acknowledge those key people and institutes that have had contributed into this dynamic development.

I would like to send my heartiest thank and warmest gratitude to my supervisors, Professor Dr. Wang Chee Woon and Professor Dr. Tan Kay Sin for their guidance and teaching throughout the completion of this thesis. Thank you all very much.

I would also like to acknowledge the University of Malaya for the offer of the Master programme and for granting me the UM Fellowship scholarship in support of my Master programme. My appreciation also goes to the Faculty and Professor Dr. Onn Hashim, the Head of Department of the Department of Molecular Medicine and staffs that gave considerable help during the time in need. My special thanks goes to Professor Dr. Jeyaseelan and co-workers from National University of Singapore for the introductory training. Thank you all very much.

For their presence and support that encourages my perseverance in completion of this thesis. I would like to send my most sincere appreciation to all of my friends within the department and beyond, especially to Ryan Heh. Thank you!

Above all, I shall reserve my greatest acknowledgement to my family. Their unconditional love, trust and support strengthen my confidence to face all my endeavors with forbearance and pride. To my mum, brother and late dad- who shall live eternally within me, I love you and thank you from the bottom of my heart. *Thank you!*

Contents

Abstract	ii
Acknowledgements	vi
Contents	vii
List of Figures	xii
List of Tables	xiv
List of Appendixes	xv
Abbreviations	xvi
1. Introduction	1
1.1. Stroke	1
1.1.1. Definitions, stroke types and subtypes	1
1.1.2. TOAST classification of ischaemic stroke	2
1.1.3. Biomarkers in ischaemic stroke	3
1.2. MicroRNA, maturation and mechanism of action	4
1.3. Expression of microRNA in animal model of stroke mimic	7
1.4. Expression of RNA in stroke accessed from blood	7
1.5. Expression of microRNA in chronic stroke accessed from blood	8
1.6. Study objectives	10
2. Literature Review	12
2.1. Stroke	12
2.2. Biomarkers for stroke	14
2.3. MicroRNA and microRNA based biomarkers	16
2.3.1. MicroRNA-145 and vascular biology	18
2.3.2. MicroRNA-214 and vascular biology	22
2.3.3. MicroRNA-222 and vascular biology	24
2.3.4. MicroRNA-223 and vascular biology	26
2.3.5. MicroRNA-23b and vascular biology	27
2.3.6. MicroRNA-339 and vascular biology	29

3. Materials and Methods	31
3.1. Study outline	31
3.2. Peripheral blood collection	31
3.2.1. Ethics statement	31
3.2.2. Study subjects	32
3.2.3. Control subjects	32
3.2.4. Blood collection and stabilization of RNA in <i>RNAlater</i> Solution	33
3.2.5. Study subjects blood sample recollection	34
3.3. Solutions and buffers preparation	34
3.3.1. 2.5 M Natrium Chloride (NaCl) stock solution preparation	34
3.3.2. 10X 3-(N-morpholino)propanesulfonic acid (MOPS) buffer stock solution preparation	34
3.3.3. 1X MOPS buffer solution preparation from stock solution	34
3.3.4. 10X Tris/Borate/Ethylenediaminetetraacetic acid (TBE) buffer stock solution preparation	35
3.3.5. 1X TBE buffer solution preparation from stock solution	35
3.3.6. Wash Solution 1 (70% Ethanol/30% Denaturing solution) preparation	35
3.3.7. Wash Solution 2 (80% Ehanol/50 mM NaCl) preparation	36
3.3.8. 40% Acrylamide/Bis-acrylamide (29:1) monomer preparation	36
3.3.9. 10% Ammonium Persulfate (APS) preparation	36
3.4. Total RNA extraction	37
3.4.1. Background	37
3.4.2. Methods and procedures	38
3.4.2.1. Cell lysis	38
3.4.2.2. Acid-Phenol/Chloroform extraction	38
3.4.2.3. RNA purification	39

3.5. DNase 1 treatment	40
3.5.1. Background	40
3.5.2. Methods and procedures	41
3.6. Quantification of RNA	42
3.7. Assessment of the integrity of RNA: Denaturing Agarose Gel Electrophoresis	42
3.7.1. Background	42
3.7.2. Methods and procedures	42
3.7.2.1. Gel preparation	43
3.7.2.2. RNA samples preparation	43
3.7.2.3. Electrophoresis and gel visualization	44
3.8. Assessment of the total RNA extracted: Polyacrylamide Gel Electrophoresis	45
3.8.1. Background	45
3.8.2. Methods and procedures	45
3.8.2.1. Gel preparation	45
3.8.2.2. RNA samples preparation	46
3.8.2.3. Electrophoresis and gel visualization	46
3.9. Reverse Transcription PCR	47
3.9.1. Background	47
3.9.2. Total RNA → cDNA (microRNA)	47
3.9.2.1. RT master mix preparation	47
3.9.2.2. RT reaction preparation	48
3.9.2.3. Reverse transcription	48
3.9.3. Total RNA → cDNA	48
3.9.3.1. RT reaction preparation	48
3.9.3.2. Reverse transcription	49
3.10. Real-time quantitative PCR	49
3.10.1. Background	49
3.10.2. TaqMan microRNA assay	50
3.10.2.1. Master mix preparation	51
3.10.2.2. Performing Real-time quantitative PCR	52

3.11. Data and statistical analyses	53
3.11.1. Background	53
3.11.2. Derivation of the $2^{-\Delta\Delta C_T}$ method	53
3.11.3. Data analysis using the $2^{-\Delta\Delta C_T}$ method	56
3.11.4. Statistical analysis of Real-time quantitative PCR data	57
3.12. Bioinformatic analysis: incorporating microRNA in pathways	57
4. Results	58
4.1. Study subjects and demographic data	58
4.2. Control subjects	58
4.3. Study subjects blood sample collection	59
4.4. Total RNA extraction	59
4.5. Quantification of RNA	59
4.6. Assessment of the integrity of RNA: Denaturing Agarose Gel Electrophoresis	61
4.7. Assessment of the total RNA extracted: Polyacrylamide Gel Electrophoresis	61
4.8. Expression profile of studied circulating microRNAs of ischaemic stroke patients	62
4.9. Paired expression profile of studied circulating microRNAs in ischaemic stroke patients	63
4.10. Correlation study of microRNAs expression	64
4.11. Pathway analysis	65
4.12. Expression of microRNA in subtypes of ischaemic stroke	66
5. Discussion	68
5.1. Real-time quantitative PCR technique in RNA analysis	68
5.2. Expression of circulating microRNAs in chronic ischaemic stroke patients	69
5.2.1. Circulating microRNA-145	70
5.2.2. Circulating microRNA-214	73
5.2.3. Circulating microRNA-222	74
5.2.4. Circulating microRNA-223	76

5.2.5. Circulating microRNA-23b	78
5.2.6. Circulating microRNA-339	81
5.3. Correlation of microRNAs expression profiles	82
5.4. Expression of microRNA in subtypes of ischaemic stroke	85
5.5. Overview of stroke mechanotransduction	87
5.6. Study limitations	90
6. Conclusion	91
References	94

List of Figures

- Figure 1.1 MicroRNA biosynthesis
- Figure 1.2 MicroRNA mechanism of action
- Figure 3.1 TaqMan chemistry during PCR
- Figure 3.2 Real-time quantitative PCR amplification plot
- Figure 4.1 Plot produced by NanoDrop ND-1000 spectrophotometry
- Figure 4.2 Box plot of A_{260}/A_{280} ratio of RNA extracted from blood samples
- Figure 4.3 Box plot of concentration of RNA extracted from blood samples
- Figure 4.4 Gel picture of denaturing agarose gel electrophoresis
- Figure 4.5 Gel picture of polyacrylamide gel electrophoresis (PAGE)
- Figure 4.6 Circulating microRNA-145 relative expression profiles of ischaemic stroke patients (n = 32)
- Figure 4.7 Circulating microRNA-214 relative expression profiles of ischaemic stroke patients (n = 32)
- Figure 4.8 Circulating microRNA-222 relative expression profiles of ischaemic stroke patients (n = 32)
- Figure 4.9 Circulating microRNA-223 relative expression profiles of ischaemic stroke patients (n = 32)
- Figure 4.10 Circulating microRNA-23b relative expression profiles of ischaemic stroke patients (n = 32)
- Figure 4.11 Circulating microRNA-339 relative expression profiles of ischaemic stroke patients (n = 32)
- Figure 4.12 Paired relative expression profiles of circulating *Homo sapiens* microRNA-145
- Figure 4.13 Paired relative expression profiles of circulating *Homo sapiens* microRNA-214
- Figure 4.14 Paired relative expression profiles of circulating *Homo sapiens* microRNA-222
- Figure 4.15 Paired relative expression profiles of circulating *Homo sapiens* microRNA-223
- Figure 4.16 Paired relative expression profiles of circulating *Homo sapiens* microRNA-23b
- Figure 4.17 Paired relative expression profiles of circulating *Homo sapiens* microRNA-339
- Figure 4.18 Circulating microRNAs relative expression profiles showing congruent expression profile

- Figure 4.19 Positive correlation between circulating microRNA-145 relative expression profile and the other circulating microRNAs studied
- Figure 4.20 Positive correlation between circulating microRNA-222 relative expression profile and the other circulating microRNAs studied
- Figure 4.21 Positive correlation between circulating microRNA-223 relative expression profile and the other circulating microRNAs studied
- Figure 4.22 Positive correlation between circulating microRNA-23b relative expression profile and the other circulating microRNAs studied
- Figure 4.23 Positive correlation between circulating microRNA-339 relative expression profile and the other circulating microRNAs studied
- Figure 4.24 Poor correlation between circulating microRNA-214 relative expression profile and the other circulating microRNAs studied
- Figure 5.1 Physiological response of microRNA-mediated postnatal neovascularization at ischaemic site

List of Tables

Table 2.1	Stroke candidate genes
Table 2.2	Ischaemic stroke candidate protein biomarkers
Table 2.3	MicroRNAs in vascular biology
Table 3.1	Risk factors and definition considered in this study
Table 3.2	Master mix components and measurement for reverse transcription of total RNA → cDNA (microRNA)
Table 3.3	Reverse transcription parameters for total RNA → cDNA (microRNA)
Table 3.4	Reverse transcription parameters for total RNA → cDNA
Table 3.5	Master mix components and measurement for Real-time quantitative PCR
Table 3.6	Pre-optimized thermal cycling parameters for Real-time quantitative PCR
Table 4.1.1	Patients' demographic data
Table 4.1.2	Data of individual control subjects
Table 4.1.3	Data of individual patient subjects
Table 4.2	List of patients who participated the second part of the study and the time interval after the first sample collection
Table 4.3	Circulating microRNAs relative expression values of ischaemic stroke patients (n = 32)
Table 4.4.1	Correlation of studied circulating microRNAs relative expression profiles: strong correlation
Table 4.4.2	Correlation of studied circulating microRNAs relative expression profiles: week correlation
Table 4.5.1	Pathways and target genes that circulating microRNA-145 possibly regulate
Table 4.5.2	Pathways and target genes that circulating microRNA-214 possibly regulate
Table 4.5.3	Pathways and target genes that circulating microRNA-222 possibly regulate
Table 4.5.4	Pathways and target genes that circulating microRNA-223 possibly regulate
Table 4.5.5	Pathways and target genes that circulating microRNA-23b possibly regulate
Table 4.5.6	Pathways and target genes that circulating microRNA-339 possibly regulate

List of Appendixes

Appendix 1. Blood collection tube used in blood collection of study subjects

Appendix 2. RNA quantification using NanoDrop

Appendix 3. Agarose Gel Electrophoresis pictures of total RNA extracted

Appendix 4. Polyacrylamide Gel Electrophoresis pictures of total RNA extracted

Appendix 5. Circulating microRNAs ΔC_T value of 14 control subjects

Appendix 6. Data presentations/publications

Abbreviations

-	negative/minus
%	percent
+	positive/plus
∞	infinity
®	registered
°C	degree Celsius
Δ	delta
μl	micro liter
A ₂₆₀	absorbance at 260 nano meter wave lengths
A ₂₈₀	absorbance at 280 nano meter wave lengths
Ago	argonaute
AHA	the American heart association
BP	blood pressure
bp	base pair
cDNA	complimentary ribonucliec acid
C _T	cycle threshold
CT	computed topography
DGCR8	DiGeorge syndrome critical region 8 gene
dNTP	deoxyriboneucleotide triphosphate
ECG	electrocardiography
Elk-1	E twenty-six-like transcription factor 1
eNOS	endothelial nitric oxide synthase
EPC	endothelial progenitor cells
FRET	foster resonance energy transfer through space
g	gram
G1 phase	gap 1 phase
G ₀	gene ontology
has	<i>Homo sapien</i>
HbA1c	hemoglobin A1c
HbF	fetal hemoglobin
HDL	high-density lipoprotein

HUVEC	human umbilical vein endothelial cells
ICAM-1	intracellular cell adhesion molecule-1
KEGG	Kyoto encyclopedia of genes and genomes
KLF	kruppel-like factor
LMO2	LIM-only protein 2
M phase	Mitotic phase
MADS	MCM1-agamous-deficient serum response factor
Mef2c	myocyte enhancer factor 2c
MGB	minor groove binder
MHC	myosin heavy chain
miR	micro ribonucleic acid
ml	mili liter
mM/l	mili molar per liter
MMAC1	mutated in multiple advance cancers
mmHg	mili meter mercury
MMP9	matrix metalloproteinase 9
MRI	magnetic resonance imaging
mRNA	messenger ribonucleic acid
NFQ	nonflorescent quencher
ng	nano gram
nm	nano meter
NSE	neuron specific enclose
OD	optical density
p	p value
p53	protein 53
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PDE4D	phosphodiesterase 4D
PI3K	phosphoinositide 3 kinase
pre	precursor
pri	primary
PTEN	phosphate and tensin homolog protein

RISC	ribonucleic acid induced silencing complex
RNA	ribonucleic acid
RNAi	ribonucleic acid interference
rRNA	ribosomal ribonucleic acid
RT-PCR	reverse transcription polymerase chain reaction
S	svedberg
S phase	Synthesis phase
S.E.M.	standard error mean
S100B	S100 calcium binding protein B
SCF	stem cell factor
SM	smooth muscle
SMC	smooth muscle cell
SRF	serum response factor
TIA	transient ischaemic attack
™	trade mark
TOAST	trial of ORG 10172 in acute stroke treatment
tRNA	transfer ribonucleic acid
UMMC	University Malaya Medical Centre
UTR	untranslated region
UV	ultra-violet
V	volt
VCAM-1	vascular cell adhesion protein-1
VSMC	vascular smooth muscle cell
WHO	world health organization