Appendix 6.

Data presentations/publications

(A) Poster Presentation

NEUROSCIENCE 2010

(B) Oral Presentation

International Union of Biochemistry and Molecular Biology (IUBMB) 2010

(C) Accepted Manuscript in ISI-cited Journal

Genetic and Molecular Research (GMR)

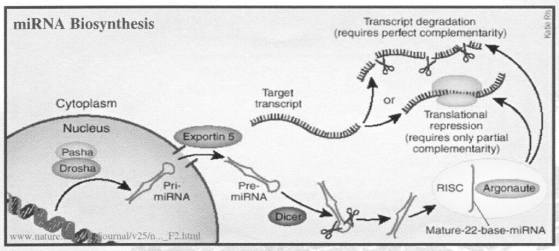
(D) Manuscript submitted for review in ISI-cited Journal

MicroRNAs Expression in Ischemic Young Stroke Patients: What are they trying to tell us?

CS Gan[1], CW Wang[1], KS Tan[2]
[1]Department of Molecular Medicine; [2]Department of Medicine, Faculty of Medicine, University of Malaya, 50603 Kuala Lumpur, Malaysia.

Background:

MicroRNAs (miRNAs) represent a class of small (about 22 nucleotides) single stranded non-coding RNA that regulate gene expression on the post transcriptional level.



Previously, microarray profiles of 19 pooled ischemic young stroke patients' blood sample revealed a group of miRNAs that were altered in expression level. miRNA-222, -339 and -214 are important candidates, correlate to angiogenesis and vascular function. The dysregulated miRNAs are also shown to be detectable even after months from the stroke onset in what is usually regarded as neurologically stable patients.[1]

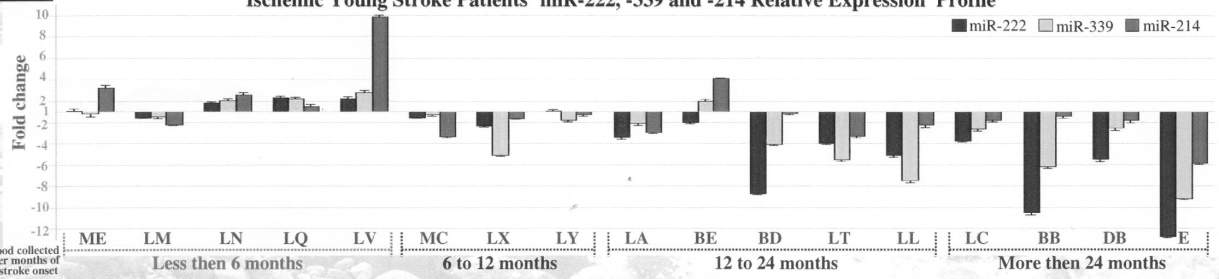
Objective:

To study the miR-222, -339 and -214 expression in individual ischemic young stroke patients.

Methodology:

- **Patient Selection and Blood Collection.**
  This study has been granted by the Medical Ethics Committee of University of Malaya Medical Centre (UMMC). Written consent was given by each patient and the information stored in the hospital database was used in this research.
- **Clinical Methodology.**
  Seventeen ischemic stroke patients between age 18 to 49 were selected among those admitted via the neurology service at UMMC. These subjects were further classified in accordance to TOAST classification. Blood samples were collected from patients who came back for clinical follow-up at the UMMC. Total of ten healthy non-stroke subjects between age 25 to 45 were randomly selected as control.
- **Laboratory Methodology**

Results:



Discussion:

Changes of studied miRNA profiles were presented in patients where samples were collected in the year of stroke onset concomitant with the clinical observation of active recovery in patients.

In this study, three miRNAs (miR-222, -339, and -214) were found to be up regulated during the first 6 months from the onset of stroke. However, after the first 6 months, these miRNAs were found to be down regulated where the graph shows that the expression magnitude increases over time.

Anti-angiogenic miR-222 expression was shown to negatively correlated with C-kit receptor expression[2]. C-kit is a cytokine stem cell factor protein that controls cell survival, proliferation and differentiation, thus promoting angiogenesis. miR-222 and miR-339 were shown to suppress intracellular adhesion molecule- 1 (ICAM-1) individually[3]. ICAM-1 is an endothelial- and leukocyte-associated transmembrane protein known for stabilizing cell-cell interactions and facilitating leukocyte endothelial transmigration. miR-214 was previously reported to be negatively correlated with endothelial nitric oxide synthase (eNOS) which, commonly associated with angiogenesis and maintenance of cerebral blood flow has thus established itself as an ischemic stroke candidate gene. [4, 6]

Conclusion:

We have revealed that the down regulation of miR-222, -339 and -214, which correlate with time in young ischemic stroke patients might up regulate C-kit receptor, ICAM-1 and eNOS which are required in cell survival, proliferation, differentiation, angigenesis and neovascularization. These observations are consistence with current literature. With the understanding of these microRNAs regulation, it is hoped that a reliable biomarker aiding the prognosis and treatment of ischemic stroke can be discovered. Further study using larger sample size is in progress to confirm our observations.

Alterations of microRNA

Time Point: Blood collected after months of stroke onset.	No. (n)	miR-222 Down regulated	miR-222 Up regulated	miR-339 Down regulated	miR-339 Up regulated	miR-214 Down regulated	miR-214 Up regulated
Less then 6 months	5	1	4	2	3	1	4
6 to 12 months	3	1	2	3	0	3	0
12 to 24 months	5	5	0	4	1	4	1
More then 24 months	4	4	0	4	0	4	0
Total	17	11	6	13	4	12	5

Patients' Demographic Data

Sample	Sex	Age	Time Point	TOAST Classification	mRS	Relative Fold Change (Patient vs. Control) miR-222	miR-339	miR-214
ME	M	47	1 month	Small Vessel	2	1.04811	-1.21235	3.21434
LM	M	41	2 months	Small Vessel	1	-1.59535	-1.50958	-2.25763
LN	M	45	4 months	Large Vessel	2	1.81835	2.02608	2.57610
LQ	M	49	1 months	Undetermined	3	2.31145	2.22251	1.48609
LV	F	40+	5 months	Small Vessel	1	2.20554	2.81771	9.85729
MC	F	45	9 months	Small Vessel	1	-1.57812	-1.31629	-3.35848
LX	M	39	9 months	Large Vessel	1	-2.34492	-5.10582	-1.67769
LY	M	37	9 months	Undetermined	-	1.08257	-1.82152	-1.28682
LA	M	41	2 years	Small Vessel	2	-3.42999	-2.11571	-3.00107
BE	F	36+	2 years	Cardioembolism	3	-2.01699	1.99934	4.08082
BD	F	40	2 years	Undetermined	2	-8.71522	-4.06750	-1.20176
LT	F	34	2 years	Undetermined	3	-3.98120	-5.54611	-3.34223
LL	M	38	2 years	Undetermined	0	-5.07663	-7.48400	-2.23738
LC	M	40	3 years	Large Vessel	1	-3.74562	-2.66933	-1.81942
BB	M	18	3 years	Large Vessel	1	-10.5004	-6.22602	-1.44191
DB	F	45+	3 years	Large Vessel	2	-5.50485	-2.55557	-1.82806
E	M	36	5 years	Large Vessel	2	-12.7451	-9.17352	-5.84881

Time Point: Blood collected after date of stroke onset; mRS: Modified Rankin Score (0-2 = Good Outcome, 3-6 = Poor Outcome)

Acknowledgements:

This study is funded by University of Malaya Research (UMR) grant and student research grant (PPP).

References:

1. Tan KS, et al. (2009) Expression Profile of MicroRNAs in Young Stroke Patients. PloS ONE 4(11): e7689. doi:10.1371/journal.pone.0007689
2. Felli N, et al. (2005) MicroRNAs 221 and 222 inhibit normal erythropoiesis and erytholeukemic cell growth via kit receptor down-modulation. PNAS. Vol.102 No.50.December 13,2005. doi:10.1073/pnas.0506216102
3. Ueda R, et al. (2009) Dicer-regulated microRNAS 222 and 339 promote resistance of cancer cells to cytotoxic T-lymphocytes by down-regulation of ICAM-1. PANAS. Vol.106 No.21 June 30, 2009. doi:10/1073/pnas.0811817106
4. Chan LS, et al. (2009) Role of MicroRNA-214 in ginsenoside-Rg1-induced angiogenesis. European Journal of Pharmaceutical Science. doi:10.1016/j.ejps.2009.08.008
5. Yang H, et al. (2008) MicroRNA Expression Profiling in Human Ovarian Cancer miR-214 Induces Cell Survival and Cisplastin Resistance by Targeting PTEN. doi:10.1158/0008-5472.CAN-07-2488
6. Endress M, et al. (2004) Targeting eNOS for stroke protection. TRENDS in Neurosciences. Vol.27 No.5, May 2004. doi:10.1016/j.tins.2004.03.009

Symposium 9 - CURRENT ADVANCES IN NEURAL REGENERATION

Sponsored by the School of Medicine, University of Tasmania, the Wicking Dementia Research and Education Centre and the Australia New Zealand Spinal Cord Injury Network

SYM-09-04

MICRO-RNA-145 EXPRESSION IN ISCHEMIC YOUNG STROKE PATIENTS: A POTENTIAL NOVEL BIOMARKER FOR CARDIOEMBOLIC & ATHEROTHROMBOTIC ISCHAEMIC STROKES

Gan C.S.[1], Wang C.W.[1] and Tan K.S.[2]
[1]Department of Molecular Medicine, Faculty of Medicine, University of Malaya, 50603 Kuala Lumpur, Malaysia. [2]Department of Medicine, Faculty of Medicine, 50603 Kuala Lumpur, Malaysia.

MicroRNAs represent a class of small single stranded non-coding RNA with genetic regulation through degradation and translational inhibition of target mRNA. Animal studies have identified microRNA-145 to be closely associated with vascular injury and vascular smooth muscle cell proliferation. The aim of this study was to determine if stroke aetiology evaluated clinically could be distinguished by samples of peripheral blood through microRNA-145 expression. In our study, we investigated the expression level of microRNA-145 in young ischemic stroke patients in 20 ischaemic stroke patients from the University of Malaya Medical Centre, an urban teaching hospital in Kuala Lumpur, Malaysia. The stroke aetiology has been previously subtyped according to the TOAST criteria. These patients were between the ages of 18-49 years and further subtyped according to the TOAST classification after extensive investigations. Total RNA was then extracted from whole blood and subjected to real-time quantitative PCR to generate the expression profile of microRNA-145 in young ischemic stroke patients, 18S rRNA was used as the endogenous control. MicroRNA-145 appeared to be down regulated in most ischaemic strokes of atherothrombotic aetiology (large or small vessel ischaemic strokes) and is up regulated in cardioembolic ischaemic stroke. MicroRNA-145 appeared to be differentially expressed in the peripheral blood and can distinguish between atherothrombotic and cardioembolic ischaemic strokes. This study is limited by a small sample size and further samples are to validate this finding. A reliable, stable and novel biomarker can augment clinical evaluation and future treatment of ischaemic stroke.

GMR Genetics and Molecular Research
Online Journal
ISSN:1676-5680

Short Communication

Circulatory microRNA-145 expression is increased in cerebral ischemia

C.S. Gan[1], C.W. Wang[1] and K.S. Tan[2]

[1]Department of Molecular Medicine, Faculty of Medicine, University of Malaya, Kuala Lumpur, Malaysia
[2]Department of Medicine, Faculty of Medicine, University of Malaya, Kuala Lumpur, Malaysia

Corresponding author: C.S. Gan
E-mail: mr.dennisgan@gmail.com

Genet. Mol. Res. 11 (1): 147-152 (2012)
Received July 21, 2011
Accepted October 10, 2011
Published January 27, 2012
DOI http://dx.doi.org/10.4238/2012.January.27.1

ABSTRACT. Cerebral ischemia or ischemic stroke is mainly attributed to vascular and circulation disorders. Among protein biomarkers, RNA profiles have also been identified as markers of ischemic stroke. MicroRNA-145 expression is ostensibly recognized as marker and modulator of vascular smooth muscle cell phenotype; however, expression levels in ischemic stroke had not been investigated. Employing real-time quantitative PCR, we examined the expression profile of circulatory microRNA-145 in healthy control subjects (N = 14) and ischemic stroke patients (N = 32). Circulatory microRNA-145 expression was significantly higher in ischemic stroke patients than in control subjects. This demonstrates that hemostatic mechanisms are affected by ischemic stroke. We conclude that circulating microRNA-145 has potential as a biomarker for ischemic stroke.

Key words: Biomarker; Circulation; Gene expression; Ischemic stroke; MicroRNA-145; Postnatal neovascularization

INTRODUCTION

Stroke is a leading cause of mortality and permanent disability, with two-thirds of onsets due to ischemia (Lloyd-Jones et al., 2009). A non-intrusive ancillary test to support conservative clinical diagnosis, classification and prognosis, aimed at improving stroke management is desirable. Protein biomarkers have been suggested and shown to be associated with the onset of cerebral ischemia, manifesting its potential use in clinical practice. Among many, C-reactive protein, interleukin-6, matrix metallopeptidase 9, vascular cell adhesion molecule 1, and intercellular adhesion molecule 1 have been shown to be dysregulated in stroke studies (Jickling and Sharp, 2011). RNA-based studies also unveil promising biomarker candidates for stroke. However, research on these biomarkers is ongoing and they are not yet used in clinical settings.

MicroRNAs are a class of small, non-coding RNAs that have the affinity to pair with sites in 3' untranslated regions in mRNAs, orchestrating their expression (Lagos-Quintana et al., 2001). This posttranscriptional regulation of nucleotide expression by microRNAs makes these small RNA pieces intriguing candidate biomarkers for potential detection before phenotypic projection. It is noteworthy that specific microRNA expressions have been shown in both brain tissue and blood following ischemic stroke mimic (Jeyaseelan et al., 2008). Moreover, circulatory microRNAs manifest the potential to be developed as ischemic stroke biomarkers in diagnosis and prognosis (Tan et al., 2009).

MicroRNA-145 has been extensively studied and its role in modulating the oscillating state of smooth muscle cells has been elucidated (Cheng et al., 2009; Cordes et al., 2009). Vascular smooth muscle cells (VSMC) are a critical cellular constituent of the vascular structure. They exhibit remarkable plasticity and readily change phenotype in response to a plethora of extrinsic stimuli, including mechanical injury, growth factors and oxidative stress (Owens, 1995). Compiling evidence strongly associates intriguing microRNA-145 expression as a modulator of smooth muscle cell (SMC) phenotype. These studies revealed the inverse correlation of microRNA-145 level to the Kruppel-like factor (KLF) family of transcription factor protein transcripts, thus identifying microRNA-145 as a novel SMC phenotype biomarker (Cheng et al., 2009; Cordes et al., 2009). However, the expression profile of circulatory microRNA-145 in vascular and circulatory disorders remains to be explored. Thus, this study aimed to examine the expression profile of circulatory microRNA-145 of ischemic stroke patients. This profile will serve as a reference and contribution to the knowledge pertaining to circulatory microRNA-145 in ischemic stroke.

MATERIAL AND METHODS

Sample collection and ethical statement

Ischemic stroke patients (N = 32) between the ages of 18 and 49 years were recruited among those admitted for neurology treatment at University of Malaya Medical Centre (UMMC). Ischemic stroke was confirmed by either MRI or CT imaging of the brain, and the risk factors, if any, were characterized based on the ancillary blood and routine tests (Tan, et al., 2009). Excluded from the study were subjects with hemorrhage stroke. The patients (N = 11) were recruited for second sampling, and healthy volunteers (N = 14) without risk factors or his-

tory of cardiovascular and cerebrovascular diseases were included as control subjects. The study protocol was approved by the Medical Ethics Committee of UMMC (reference number 607.20). Each volunteer prior to blood sampling gave written informed consent.

Total RNA isolation and preparation

Total RNA was isolated from peripheral whole blood using the Ribopure-Blood RNA isolation kit (Ambion, Austin, TX, USA), adhering strictly to the recommended protocol by the manufacturers. The eluted RNA was quantified by NanoDrop ND-1000 Spectrophotometer (Rockland, DE, USA) and assessed for integrity by 1% denaturing agarose and 15% denaturing polyacrylamide gel electrophoresis.

Quantification of microRNA-145

Quantification of microRNA was carried out with the TaqMan Real-Time PCR. TaqMan MicroRNA Reverse Transcription kit and High Capacity RNA-to-cDNA Master Mix were used for reverse transcription of the RNA. TaqMan MicroRNA Assay (P/N 4373133) and Universal PCR Master Mix were used for real-time quantitative PCR. Ribosomal 18s rRNA was used as an endogenous control. RNA template (10 ng) was subjected to reverse transcription. The cDNA product (1.33 mL) was used for PCR. Real-time PCR was carried out using the StepOnePlus Real-Time PCR system (Applied Biosystems, Foster City, CA, USA). All procedures adhered to the protocols were provided by the manufacturer.

Statistical analysis

The Student *t*-test was performed using the SPSS software for Windows version 11.0, and the level of significance was set at $P < 0.05$. The data are reported as means ± standard error of the mean.

RESULTS AND DISCUSSION

The circulatory microRNA-145 of patients, compared to that of controls, demonstrated significant upregulation ($P = 0.022$; Figure 1A). The altered expression profile of circulatory microRNA-145 in this study may mark the biological stages of vascular reendothelialization, a postnatal vasculogenesis response after cerebral ischemia (Dimmeler and Zeiher, 2004). The upregulation of circulatory microRNA-145 detected may derive from the pool of circulating progenitor cells that are in commitment to differentiate into VSMC lineage, since it has been shown that the introduction of microRNA-145 into neural crest stem cells is sufficient to propitiously guide the progenitor cells to differentiate into VSMC (Cordes et al., 2009). Thus, upregulation of circulatory microRNA-145 suggests positive reendothelialization, while downregulation or vice versa.

As has been delineated, microRNA-145 shows an inverse correlation with the expression of KLF4/5 proteins, regulating the mRNA transcripts, posttranscriptionally. High expression level of microRNA-145 will consequently down modulate its targets, KLF4/5; myocardin will not be suppressed and will form a complex with serum response factor, which eventually

promotes the transcription of the genes essential for VSMC differentiation and maintenance of the differentiated state (Figure 2) (Long et al., 2008; Cheng et al., 2009; Cordes et al., 2009).

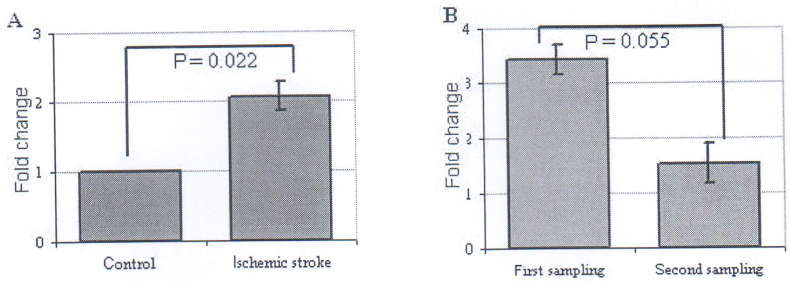


Figure 1. A. Circulatory microRNA-145 expression profile of ischemic stroke patients, N = 32. **B.** Paired expression profile of patients, N = 11. Peripheral blood was resampled and expression profile of circulatory microRNA-145 was generated and compared. Expression values are normalized to the mean of the expression values from healthy controls. All values are reported as mean fold changes ± standard error of the mean, and $P < 0.05$ is considered to be significant.

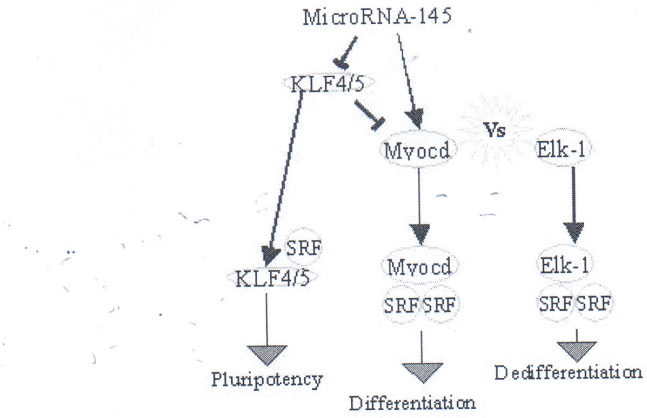


Figure 2. Effect of microRNA-145 congregate on serum response factor (SRF)-dependent transcription by regulation of co-activators and co-repressors directing cell fate. MicroRNA-145 potentiates myocardin (Myocd) and negatively regulates Kruppel-like factor (KLF4/5), which interacts with SRF and also inhibits Myocd. KLF is a transcription factor implicated in pluripotency. ETS-like transcription factor 1 (Elk-1) represses Myocd's activity by competing for common docking site (displacing Myocd from SRF), activating gene transcription for cell dedifferentiation. Upregulation of microRNA-145 renders Myocd functional in directing cell differentiation by repressing KLF4/5 and potentiates Myocd's effect over Elk-1 (Long et al., 2008; Cheng et al., 2009; Cordes et al., 2009).

On the other hand, postnatal neovascularization is shown to be no longer attributed to angiogenesis only. Postnatal vasculogenesis has been described as also being involved in vascular homeostasis. This process is involved in the recruitment and incorporation of precursor cells, which have also been shown to circulate postnatally in the peripheral blood to the injured and ischemic sites (Luttun et al., 2002). Besides neural crest stem cells, bone marrow-derived circulating endothelial precursor cells, common vascular progenitors, and SMC progenitors have been shown to possess the potential to participate in postnatal vasculogenesis, extending the pool of circulating multipotent precursor cells in vascular regeneration (Luttun et al., 2002; Dimmeler and Zeiher, 2004). These findings support the observation in this study.

Therefore, plummet of the upregulated circulatory microRNA-145 may suggest a good outcome in the completion of vascular regeneration, achieving homeostatic equilibrium (Tan et al., 2009). Ischemic stroke patients (N = 11) were called back months after the initial blood sampling. The pair expression profile of circulatory microRNA-145 (first sampling *vs* second sampling) demonstrated nonsignificant downregulation (P = 0.055; Figure 1B). This may be due to the limitations of the study in that only some of the patients agreed to subsequent samplings or the time of second collection was too parsimonious for effective postnatal vasculogenesis, which is affected by individual risk factors (Dimmeler and Zeiher, 2004).

CONCLUSION

The present study revealed that circulatory microRNA-145 expression is upregulated in ischemic stroke patients, as compared to the control. This finding may have implications for the development of a desirable biomarker and therapy for ischemic stroke. Since this study utilized peripheral whole blood, identification of the source of the upregulated circulatory microRNA-145 would be beneficial for the elucidation of mechanotransduction.

ACKNOWLEDGMENTS

Research supported by the University Malaya Research Grant (UMRG, #FS192/2008B) and the University Malaya Postgraduate Research Fund (PPP, #PS385/2009B) for K.S. Tan and C.S. Gan, respectively.

REFERENCES

Cheng Y, Liu X, Yang J, Lin Y, et al. (2009). MicroRNA-145, a novel smooth muscle cell phenotypic marker and modulator, controls vascular neointimal lesion formation. *Circ. Res.* 105: 158-166.

Cordes KR, Sheehy NT, White MP, Berry EC, et al. (2009). miR-145 and miR-143 regulate smooth muscle cell fate and plasticity. *Nature* 460: 705-710.

Dimmeler S and Zeiher AM (2004). Vascular repair by circulating endothelial progenitor cells: the missing link in atherosclerosis? *J. Mol. Med.* 82: 671-677.

Jeyaseelan K, Lim KY and Armugam A (2008). MicroRNA expression in the blood and brain of rats subjected to transient focal ischemia by middle cerebral artery occlusion. *Stroke* 39: 959-966.

Jickling GC and Sharp FR (2011). Blood biomarkers of ischemic stroke. *Neurotherapeutics* 8: 349-360.

Lagos-Quintana M, Rauhut R, Lendeckel W and Tuschl T (2001). Identification of novel genes coding for small expressed RNAs. *Science* 294: 853-858.

Lloyd-Jones D, Adams R, Carnethon M, De Simone G, et al. (2009). Heart disease and stroke statistics-2009 update: a report from the American Heart Association Statistics Committee and Stroke Statistics Subcommittee. *Circulation* 119: e21-181.

Long X, Bell RD, Gerthoffer WT, Zlokovic BV, et al. (2008). Myocardin is sufficient for a smooth muscle-like contractile phenotype. *Arterioscler. Thromb. Vasc. Biol.* 28: 1505-1510.

Luttun A, Tjwa M, Moons L, Wu Y, et al. (2002). Revascularization of ischemic tissues by PlGF treatment, and inhibition of tumor angiogenesis, arthritis and atherosclerosis by anti-Flt1. *Nat. Med.* 8: 831-840.

Owens GK (1995). Regulation of differentiation of vascular smooth muscle cells. *Physiol. Rev.* 75: 487-517.

Tan KS, Armugam A, Sepramaniam S, Lim KY, et al. (2009). Expression profile of MicroRNAs in young stroke patients. *PLoS One* 4: e7689.

Original article

Circulating MicroRNA Expression Profile: potential novel prognostic biomarker of ischemic stroke

Chye Sheng Gan [a]*; Chee Woon Wang [a]; Kay Sin Tan [b].

[a]Department of Molecular Medicine, Faculty of Medicine, University of Malaya, Lembah Pantai, 50603 Kuala Lumpur, Wilayah Persekutuan, Malaysia.

[b]Department of Medicine, Faculty of Medicine, University of Malaya, Lembah Pantai, 50603 Kuala Lumpur, Wilayah Persekutuan, Malaysia.

Circulating MicroRNA Expression Profile in Ischemic Stroke

*Corresponding author.

Name: Chye Sheng Gan

Address: Department of Molecular Medicine, Faculty of Medicine, University of Malaya, Lembah Pantai, 50603 Kuala Lumpur, Wilayah Persekutuan, Malaysia.

e-mail: mr.dennisgan@gmail.com

Abstract

MicroRNA expression orchestrating protein expression at posttranscriptional level is emerging as a new platform in transcriptome studies. We aim to explore the expression profiles of circulating microRNA-222 and -223 in young ischemic stroke patients as both of these microRNAs are reported to play a role in angiogenesis and ischemia. Thirty two ischemic stroke patients were recruited and their peripheral blood samples were subjected to quantitative PCR to determine their microRNAs expression profiles. Paired first and second peripheral blood samples from the same patient were investigated to determine the expression profiles after a period of time. Both circulating microRNA-222 and -223 showed significant dysregulation ($p<0.05$), respectively. Both expression profiles also revealed significant down regulation, after a period of time, which suggests recovery. The microRNAs studied show strong positive correlation ($r = 0.928$). These findings unveil the potential of circulating microRNAs profiles to be developed as biomarkers in the prognosis of ischemic stroke.

Keywords: Biomarkers; Expression profiles; Ischemic stroke; MicroRNA.

Abbreviations:

ERK (extracellular signal-regulated kinase), HbF (fetal hemoglobin), LMO2 (LIM-only protein 2), MADS (MCM1-agamous-deficient serum response factor), MAPK (mitogen-activated protein kinase), mTOR (mammalian targeted of rapamycin), RNAi (RNA interference), SCF (stem cell factor), TOAST (Trial of ORG 10172 in acute stroke treatment), UTR (untranslated region), VEGF (vascular endothelial growth factor).