1.Introduction

1.1. Stroke

1.1.1. Definitions, stroke types and subtypes

The phenomenon that causes interruption of pulsating blood circulation to the brain defines stroke. With persistence, the obstruction deprives brain tissues of oxygen and nutrients supply, thus causing infarction. World Health Organization (WHO) has characterized stroke as an event of a 'focal neurological deficit of sudden onset with symptoms lasting 24 hours or longer or leading to death'. Indeed, stroke is a medical emergency and can cause permanent neurological damage, complications, and even death. According to 2010 statistic by The American Heart Association (AHA), stroke is the second most common cause of mortality in Europe and in the European Union, and the third in Canada and the United States. Nevertheless, almost 2% of Asians aged 18 years and older have experienced a stroke (Lloyd-Jones *et al.*, 2010).

They are two types of stroke; namely ischaemic and haemorrhagic. One is diagnosed as ischaemic if the stroke is caused by the lack/blockade of blood flow consequently depriving brain tissue of nutrients and oxygen. Meanwhile, transient ischaemic attack (TIA), as the name suggests, is a temporary fault of the blood flow to part of the brain. Because the blood supply is restored quickly, cerebral infarct may be prevented, as it doesn't in a stroke, and therefore every event of TIA is given equal attention as ischaemic stroke. This is because there isn't any way, at this time point to differentiate TIAs from ischaemic stroke when the symptoms occur. For the matter of fact, TIA is also termed as mini stroke.

On the other hand, haemorrhage is defined as the release of blood into the extravascular space within the cranium. This event of bleed damages the brain tissue by cutting off connecting pathways and causes localized or generalized pressure injury. The biochemical substances released during this bleed also play a role in haemorrhagic stroke. The focus of this study is on ischaemic stroke.

Almost 80% of strokes are ischaemic and it can be caused by multiple genetic factors, environmental factors and/or interactions amongst them (Stanković *et al.*, 2008). Based on the etiology, ischaemic stroke is divided into several subtypes. Thrombosis is an event when the interruption is caused by an obstruction to blood flow by a thrombone. The lumen of the vessel is narrowed or occluded by an alteration in the vessel wall or by superimposed cloth formation of various origins. On the other hand, vascular system around the brain area that is blocked by materials originated from elsewhere (as in it is not due to localized process) it is then classified as embolism.

1.1.2. TOAST classification of ischaemic stroke

In order to meet standardization in classifying the different subtypes of ischaemic stroke, TOAST (Trial of ORG 10172 in Acute Stroke Treatment) criteria for ischaemic stroke classification was introduced (Adams *et al.*, 1993). The TOAST criteria denotes five diagnostic subtypes of ischaemic stroke namely, large-artery atherosclerosis, cardioembolism, small-vessel occlusion, stroke of other determined etiology, and stroke of undetermined etiology. It is noteworthy that there are other means of classification method being used (i.e. Stroke Data Bank classification), although TOAST criteria have been the most widely practiced standards for ischaemic stroke subtype

classification underlying pathophysiologic mechanisms based on comprehensive information and ancillary diagnostic tests outcome.

1.1.3. Biomarkers in ischaemic stroke

As there are limited biomarkers and therapy available, efforts in understanding stroke mechanotransduction, discovering new biomarkers, prevention and treatment for this metabolic disease has been diligently pursued. Many protein biomarkers have been identified and shown potential in discriminating ischaemic stroke. In a study utilizing those reported protein biomarkers coupled with the algorithm designed, a combination of 5 protein markers of the many was identified to enables the discrimination of ischaemic stroke from the healthy controls. These S-100b (marker for astrocytic activation), matrix metalloproteinase-9, B-type neurophic factor, von Willebrand factor and monocyte chemotactic protein-1 were reported to detect ischaemic stroke with 92% sensitivity and 93% specificity (Reynolds *et al.*, 2003).

With the advantage of early detection prior phenotypic manifestation, research in identification of RNA biomarkers has been diligent. This will be discussed further in Chapter 2: Literature Review.

1.2. MicroRNA, maturation and mechanism of action

MicroRNA is a form of small, single-stranded RNA consisting of 18- to 25- noncoding nucleotides that regulates the functions of other genes in protein synthesis mainly via the messenger RNA (mRNA) (Ambros, 2004; Lagos-Quintana *et al.*, 2001; Lee *et al.*, 2001). The role of microRNA was first observed in *C. elegans.* It was discovered during a study of the *lin*-4 gene, where the transcript demonstrates unusual means of information transfer (a variation to the law of central dogma). Instead of encoding for a protein, the transcript encodes a small (micro) RNA that forms imperfect base-pairing to its complimentary sequence on target mRNAs. This interaction subsequently blocked the translation of the protein, and therefore regulating the gene expression involved in the developmental timing of the worm (R. C. Lee *et al.*, 1993). It was found later that microRNAs are highly conserved endogenous RNA molecules and they occur in diverse eukaryotic organisms, ranging from nematods, to flies, to human (Lagos-Quintana *et al.*, 2003).

The processing and maturation of microRNAs have been delineated (Bartel, 2004). The primary (pri) transcript of the microRNA genes, often several hundred nucleotides long, are generated by RNA polymerase II (Lee *et al.*, 2004). The pri-microRNAs are then processed in the nucleus into shorter (around 70 nucleotides long) hairpin structure termed as precursor (pre)-microRNAs. This enzymatic process is mediated by the microprocessor that is made up of the ribonuclease (RNase) III protein family, Dorsha and the DiGeorge syndrome critical region 8 gene (DGCR8) (Gregory *et al.*, 2004; Lee *et al.*, 2003). Subsequently, the pre-microRNAs are transported to the cytoplasm via exportin-5 and further processed to mature microRNAs that is mediated by Dicer, another member of RNase III protein family (Cullen, 2004).

Figure 1.1 depicts the typical processing and maturation of microRNAs.

Most microRNAs orchestrate cellular pathways directly by regulating target mRNA translation having the affinity to the 3'-untranslated region (3'UTR) by a process known as RNA interference (RNAi) (Felli *et al.*, 2005; Felli *et al.*, 2009). mRNA decay or translation repression is with regard to the recruitment of the effector (Argonaute (Ago) 1- or Ago2-) that tolerates different degree of complimentarity of the microRNA against the mRNA (Forstemann *et al.*, 2007). Partition of double-stranded microRNA into Ago1-RNA-induced Silencing Complex (RISC) will result in mRNA translational repression that consists of central mismatches in its binding sites. However, if the double-stranded microRNA partitions into Ago2-RISC, mRNA decay occurs but only in the presence of precise match/total complement to its target sequence. **Figure 1.2** depicts the typical mechanism of action of microRNAs targeting its target mRNA 3'UTR.

As microRNAs are not restricted to any of the effectors, it is plausible for each microRNA to have multiple mRNA targets and more than one microRNA can either cooperatively or independently regulate the same target (Doench *et al.*, 2003; Thum *et al.*, 2008). It is estimated that one microRNA has approximately 200 target transcripts (Krek *et al.*, 2005). As an example, phosphate and tensin homolog protein (PTEN) mRNA is shown to be posttrascriptionally regulated by microRNA-21 (Meng *et al.*, 2007), microRNA-26a (Huse *et al.*, 2009), microRNA-214 (Jindra *et al.*, 2010), microRNA-216a and microRNA-217 (Kato *et al.*, 2009). To date, it is estimated that about one-third of the genes are regulated by microRNAs (Urbich *et al.*, 2008).

There were findings showing microRNA indirect regulation of protein synthesis involving transcription factor proteins, such as DNA methyltransferases and histone deacetylases. These enzymes/proteins control DNA transcriptions by editing the chromatin structure deciding gene expression/transcription in nucleus (Guil *et al.*, 2009). Another model describes the involvement of microRNAs in regulation of phenotypic switch of differentiated cells. For instance, in vascular smooth muscle cells (VSMC), microRNA-145 and -143 are shown to induce serum response factor (SRF, a transcription factor) activity and function to repress multiple factors that encourages proliferation over differentiation of the smooth muscles. The congregation of these two microRNAs, microRNA-145 and -143 dictates the proliferative or differentiated phenotype of VSCM influencing SRF-dependent transcription by regulation of the co-activators and co-repressors involved (Cordes *et al.*, 2009). These phenomena suggest that posttranscription regulation by microRNAs can involve an intricate pathways and our understanding of microRNAs are still at infancy.

Dysregulation of microRNA expression were observed in cerebral vascular diseases (Cordes *et al.*, 2009; Fasanaro *et al.*, 2008; Grundmann *et al.*, 2011; Harris *et al.*, 2008; Ouyang *et al.*, 2011). As an example, it was demonstrated the expression of brain-enriched microRNA-181 was altered following brain ischaemia (Yuan *et al.*, 2010). Force regulation of this microRNA in brain cells revealed the association to the expression of Bcl-2 and Mcl-1 proteins level that regulate the apoptotic cascade of the cell (Ouyang *et al.*, 2011). The elevation of microRNA-181 expression demonstrated reduced cell survival determined by the observation of higher number of condensed nuclear undergoing apoptosis, while the deprivation of microRNA-181 expression was demonstrated to increased cell survival subjected to stroke mimic. This, therefore

underscore the crucial role of microRNAs in biology, although the precise mechanisms remain to be explored (Ouyang *et al.*, 2011).

1.3. Expression of microRNA in animal model of stroke mimic

Study on microRNAs in stroke gained attention when highly expressed microRNAs in ischaemic rat brain was detected in peripheral blood sample (Jeyaseelan *et al.*, 2008). The microRNAs found to be present in both the blood and brain include *Rattus norvegicus* (rno*)*-microRNA-16, -23a, -103, -107, -150, -185, -191, -292-5p, -320, -451, -494, let-7 (a, c, d, f, and i), -26a, -26b, -140*, -150, -185, -195, -214, 320, 328, -352 and -494. The identification of microRNAs expression in ischaemic brain that can also be detected in peripheral blood brought us a step forward in developing microRNAs biomarkers (Jeyaseelan *et al.*, 2008; Lim *et al.*, 2010).

The hypothesis of differential expression of microRNA in injured brain and blood were proofed again by Liu and co-workers when pannel of common and unique expression of microRNA profile were observed in rats with sharm surgeries, ischaemic stroke, haemorrhage stroke and kainate-induces seizures (Liu *et al.*, 2010). These blood microRNAs were suggested to be associated to those expressed in the brain. These changes of microRNA expression were accounted for the different protein expression in brain and blood following brain injury (Liu *et al.*, 2010).

1.4. Expression of RNA in stroke accessed from blood

Referring to the proof-of-principals studies in animals, the quest in discovering blood based RNA biomarker for stroke formed the basis for subsequent human studies. The first report to access RNA expression in stroke patients unveiled a total of 190 genes to

be differently regulated in the 20 stroke patients as compared with 20 control subjects (Moore *et al.*, 2005). Of the differently regulated genes detected, a panel of 22 genes derived form the prediction analysis was able to detect stroke with 78% sensitivity and a specificity of 80%. Repeated with a more systematic study design, a different panel of 25-probe sets for 18 genes was reported to be able to identify ischaemic stroke with 93.5% sensitivity and 89.5% specificity (Stamova *et al.*, 2010; Tang *et al.*, 2006).

In addition, panel of 23 genes were reported to discriminate cardioembolic subtype from the large vessel atherosclerotic stroke with 95.2% specificity and 95.2% sensitivity (Xu *et al.*, 2008). These have therefore, demonstrated the feasibility of acquiring RNA as stroke biomarker, and to develop them to predict the etiology of ischemic stroke. However, it is noteworthy that these panels of mRNA genes were derived from the studies of acute ischaemic stroke patients. Expression profile of microRNA for both acute and chronic ischaemic stroke patients remains to be explored.

1.5. Expression of microRNA in chronic stroke accessed from blood

In order to access the microRNAs expression profile, whole blood from chronic ischaemic stroke patients and healthy control subjects was collected. The RNA was extracted and the microRNAs were hybridized on μParaflow microRNA microarray in generating the expression profiles. Among the 836 microRNAs, 138 microRNAs have shown upregulation and 19 microRNAs demonstrated downregulation as compared to the control group. Hierachical clustering of the expression profiles distinctively cluster the ischaemic stroke patients from the control subjects (Tan *et al.*, 2009). The microRNAs that were found dysregulated in these chronic stroke patients include *Homo sapien* (hsa)-microRNA-126, -23a, -23b, -1259, -142-3p, -186, -519c, -768-5p, -

1185, -1246, -1261, -1285, -1290, -181a, -550, -602, -222, -223, -320 (b, c and d) and -939. Hierachial clustering of the microarray heat map of the microRNAs seperates the control samples from the stroke samples. The peripheral blood microRNA expression profiles were also found to be of potential in development of biomarkers in diagnosis and prognosis of cerebral ischaemic stroke (Tan *et al.*, 2009).

The microRNAs that were shown dysregulated in stroke patients as compared to the healthy control were found to affect the key biological pathways such as MAPK singnaling pathways and Focal Adhesion pathways. The microRNA profiling from this study has also suggested the hypoxia related microRNAs (microRNA-23a, -23b, -24, -103, -93, -181a, -15a, -16, -101, -126, -320), angiogenesis related microRNAs (microRNA-19b, -130a, -145, -15a, -16, -222, -320), immune response related microRNAs (microRNA-15a, -16, -214, -23b, -24, -29a, -93, -223, -339) and haematopoiesis related microRNAs (microRNA-16, -24, -30c, -106b, -223) were regulated following stroke.

1.6. Study objectives

Hereby, this study aims to explore the expression profiles of selected 6 microRNAs, microRNA-145, -214, -222, -223, -23b and -339 in ischaemic stroke of young stroke patients using Real-time quantitative PCR method. The selection was made on the basis of dysregulated expression shown in microarray analysis (Tan *et al.*, 2009) and reports dictating their regulation as the key player in multi facets of vascular diseases (refer section 2.3 for review). Chronic ischaemic stroke patients and normal healthy control subjects were recruited to observe the different regulation of microRNAs expression in stable stroke condition as compared to the normal control. Ischaemic stroke patients were recalled back for blood sampling in order to study the expression of microRNAs in an event of long-term stroke recovery, as this information has not yet been reported. Therefore, this study was designed with the following objectives:

a) To generate and compare the microRNAs (-145, -214, -222, -223, -23b and -339) profiles of chronic young ischaemic stroke patients to the normal control.
b) To generate and compare the microRNAs (-145, -214, -222, -223, -23b and -339) temporal profiles of chronic young ischaemic stroke patients after a period of time against the initial profiles.

Null hypothesis (H_0) 1:

Circulating microRNA-145, -214, -222, -223, -23b and -339 expression profiles of the chronic ischaemic stroke patients do not differ from the control. The respective expression profiles of microRNAs generated form Real-time quantitative PCR are not consistent with the microarray results as reported previously.

Alternative hypothesis (H_A) 1:

Circulating microRNA-145, -214, -222, -223, -23b and -339 expression profiles of the chronic ischaemic stroke patients differ from the control. The respective expression profiles of microRNAs generated form Real-time quantitative PCR are consistent with the microarray results as reported previously.

Null hypothesis (H_0) 2:

The temporal expression of circulating microRNA-145, -214, -222, -223, -23b and -339 from the blood sample re-collection of the ischaemic stroke patients do not differ from the initial expression profile of the respective microRNAs. These microRNAs temporal expression profile may not have prognostic value for ischaemic stroke.

Alternative hypothesis (H_A) 2:

The temporal expression of circulating microRNA-145, -214, -222, -223, -23b and -339 from the blood sample re-collection of the ischaemic stroke patients differ from the initial expression profile of the respective microRNAs. These microRNAs temporal expression profile may have prognostic value for ischaemic stroke.

2. Literature Review

2.1. Stroke

Pathophysiology of stroke remains multifarious as a plethora of biochemical changes corresponding to the initiation of neurophysiological disturbances. Therefore, understanding stroke is far most important in improving the stroke management through development of new approach for both prevention and treatment of this neurological malady. For example, cardioembolic stroke due to atrial fibrillation typically requires oral anticoagulants such as warfarin to decrease the risk of recurrent stroke (McCabe *et al.*, 2007). On the other hand, large-vessel atherosclerotic stroke requires carotid endarterectomy for significant carotid stenosis and antiplatelet agent to be taken in decreasing the risk of recurrent stroke (Ocava *et al.*, 2006).

Over the years, laboratory based research on stroke has revolutionized from genomic study to proteins expression study and now microRNAs (a class of non-coding RNA) expression study. In genomic studies, it was reasoned that stroke could be caused by multiple genetic factors, environmental factors and/or interactions amongst them (Stanković *et al.*, 2008). The Mendelian approach has resulted in the discovery of a list of candidate monogenic stroke genes. The meta-analysis and linkage studies have gathered those candidate genes, which are found to be key players involved in homeostasis, inflammation, nitric oxide production, homocysteine and lipid metabolism, rennin-angiotensis-aldosteron system and etcetera. **Table 2.1** summarized the candidate genes and its association in the pathophysiology of stroke.

In terms of gene expression, global mRNA expression studies of patients with ischaemic stroke have demonstrated an intriguing expression profile as compared to healthy controls (Grond-Ginsbach *et al.*, 2008; Xu *et al.*, 2008). In a gene expression study of human peripheral blood mononuclear cells, transcript encoding phosphodiesterase 4D (PDE4D) protein revealed a significant altered expression in acute ischaemic stroke patients as compared to healthy recruits (Grond-Ginsbach *et al.*, 2008). Further analysis of the global expression profile associates the gene dysregulation with inflammatory response (Gene ontology (G_O): 0006954), which includes genes like interleukin 1 receptor antagonist and zinc finger family of proteins. Nevertheless, result of global gene expression in peripheral whole blood identified the difference between cardioembolic and large-vessel atherosclerotic stroke (Xu *et al.*, 2008). It was revealed that in large-vessel atherosclerotic stroke subtype, gene expression triggering homeostasis in platelets and monocytes was implicated. However, gene expression in neutrophiles implicated to immune response was described in cardioembolic subtype. These expression profiles that are unique, discriminating stroke subtypes shed light towards study of transcriptome.

To date, many proteins with unique expression have been continuously discovered and identified to be reliable biomarkers as their expression level was shown to be a hallmark of disease/stages of the disease (Chan *et al.*, 2009a; Sung *et al.*, 2011). In stroke, protein molecules such as C-reactive protein (CRP), matrix metallopeptidase 9 (MMP9), S100 calcium binding protein B (S100B), and neuron specific enclose (NSE) protein are amongst the many other proteins that were identified as biomarkers (Hergenroeder *et al.*, 2008; Jickling *et al.*, 2011; Laterza *et al.*, 2006). In **Table 2.2** are

the summarized reported protein biomarkers and their potential in clinical applications (Jickling *et al.*, 2011).

2.2. Biomarkers for stroke

Numerous attempts have been made to develop a blood test to diagnose stroke. And these attempts in developing the blood based biomarkers remains to be refined. By detecting the elevation of antibodies specific to NR2A/NR2B subunits of the glutamate *N*-methyl *D*-aspartate (NMDA)-receptor (NMDA-R), the data demonstrated as high as 97% sensitivity and 98% specificity in distinguishing ischaemic stroke form control subjects (Dambinova *et al.*, 2003; Dambinova *et al.*, 2002). However, the elevation of these antibodies level was also reported in patients with other vascular diseases such as hypertension and atheroscelosis (Jickling *et al.*, 2011). And because ischaemic stroke is caused by large-vessel atherosclerotic disease, cardioembolic disease and lacuna small vessel disease (Amarenco *et al.*, 2009), the elevation of these antibodies may only indicate the presence of vascular disease in subject and are not exclusive in characterizing the diseases.

Improvement in the approach in developing blood test to diagnose a stroke was attempted with the usage of a panel of proteins expression. Panels of four markers, which made up of S100B, von-Willebrand factor (vWF), MMP9 and VCAM were able to discriminate ischaemic stroke form controls with 90% sensitivity and 90% specifi. When VCAM was substituted with beta-nerve growth factor (BNGF) and monocyte chemoattractant protein-1 (MCP-1) proteins, the sensitivity and specificity of the proteins panel in detecting ischaemic stroke from controls were raised to 92% and 93%, respectively (Reynolds *et al.*, 2003). Despite to the achievements, the application of

proteins panel in clinical settings met with a drawback when the proteins panel fail to facilitate the diagnosis of acute stroke (Whiteley *et al.*, 2010).

With the advantage of detection prior protein synthesis, RNA based biomarker is much desired because it would deliver rapid detection well before events could be detected using protein biomarkers. Studies on the messenger RNAs expression profiles have revealed its association with ischaemic stroke. Recent RNA expression studies has identified eight genes that are at least 2 fold change difference in expression with one downregulated gene following stroke, as compared to the non-stroke controls (Barr *et al.*, 2010). And the eight-upregulated RNAs code for: arginase 1, carbonic anhydrase 4, chondroitin sulfate proteoglycan 2, IG motif-containing GTPase activation protein 1, lymphocyte antigen 96, matrix metalloproteinase 9, oroscomocoid 1, and S100 calcium binding protein A12. The downregulated RNA codes for chemokine receptor 7. Further studies using panel of gene probes demonstrated the possibilities for the RNA to be used to distinguish ischaemic stroke from healthy control and other vascular related diseases.

MicroRNAs, as described in section 1.2, is a class or regulatory RNAs that have been shown to be dysregulated following stroke (Jeyaseelan *et al.*, 2008; Yuan *et al.*, 2010). These observation prompt our interest to study the expression of microRNAs found in blood circulation of young ischaemic stroke patients versus the normal non-stroke control subjects.

2.3. MicroRNAs and their potential as biomarkers

The breakthrough in the discovery of microRNAs and their function in regulating protein translation during the early 20th century have opened a new dimension in transcriptome study. From the understanding of the importance of microRNAs in cellular pathway regulations in maintaining homeostasis and in disease etiology identification, numerous groups have contributed in delineting the mechenism of specific microRNAs in various clinical settings. Tabulated in **Table 2.3** is a list microRNAs that have been shown to impinge on vascular biology (Urbich *et al.*, 2008).

MicroRNA possesing distinctive function in gene regulation posttranscriptionally has gained more confidence when studies showing temporal and tissue-specific regulation of pri-microRNA transcription were demonstrated (Urbich *et al.*, 2008). For instance, microRNA-138 that has been shown to regulate human telomerase reverse transcriptase during cellular proliferation is spatially restricted to proliferating cells, while the pre-microRNA is ubiquitously expressed.

Study of microRNA-145 expression revealed its downregulation in both injured and atherosclerotic arteries (Cheng *et al.*, 2009). More to that, microRNA-145 is shown to be critical in modulating VSMC plastic phenotype; restroration of the microRNA-145 in dedifferentiated VSMC (that has low expression level of microRNA-145) resulted in the enhanced expression of VSMC differentiation marker genes and consequently impedeing cell proliferation (Cheng *et al.*, 2009; Cordes *et al.*, 2009). Interguiningly, *in vivo* restroration of microRNA-145 in insulted arteries inhibited neointimal lesion growth (Cheng *et al.*, 2009).

Another study delinated the microRNA-126 inhibitory reguation of vascular cell adhesion molecule-1 (VCAM-1) (Harris *et al.*, 2008). The expression of microRNA-126 was reported to be negatively correlated to the expression of VCAM-1 protein. Furthermore, inhibition of microRNA-126 in endothelial cells was shown to increase leukocyte adherece under pro-imflammatory condition via the regulation of VCAM-1. Conversely, overexpression of microRNA-126 in endothelial cells resulted in low adherence of leukocyte at site. This observation suggess that microRNA-126 can be aquired to mitigate the infiltration of leukocytes at injured vessels. Therefore, besides being a biomarker, the microRNA also served as a candidate target for therapeutic intervention. These findings have thus impinged on the approaches in developing biomarker and novel therapy at the molecular level.

As abovementioned, ischaemic stroke is a malady involving not only the brain tissue itself but it also involves the vascular biology, in which comprises of the vasculature and blood. Platelets, erythrocytes, clotting factors, and endothelium were those found involved (Sharp *et al.*, 2011). Compiling studies also dictate the involvement of inflammatory cytokines, chemokines, lymphocytes, monocytes, neutrophils and myriad of other adhesion molecules in determining stroke outcome (Becker, 2010; Downes *et al.*, 2010; Elkind, 2010; Hallenbeck, 2010; Sharp *et al.*, 2011). Therefore, the study of RNAs (microRNA-145, -214, -222, -223, -23b, -339) of peripheral blood in this study is relevant in understanding ischaemic stroke.

2.3.1. MicroRNA-145 and vascular biology

MicroRNA-145 is being extensively studied and its role in modulating the oscillating state of smooth muscle cells is elucidated (Cheng *et al.*, 2009; Cordes *et al.*, 2009). VSMC are critical cellular constituents of the blood vessel wall. Indeed, these cells exhibit remarkable plasticity and can readily change phenotype in response to a plethora of extrinsic stimuli including mechanical injury, growth factors and oxidative stress (Owens, 1995).

It has been described, at differentiated state of VSMC, where they are quiescent exhibiting contractile phenotype, microRNA-145 is found abundant in VSMC. In contrast, upon stimulation (injury), the level of microRNA-145 expression is substantially downregulated pararellel with the VSMC differentiation marker proteins, namely the smooth muscle (SM) α-actin, calponin and SM-myosin heavy chain (MHC) (Cheng *et al.*, 2009). The downregulation of differentiation marker proteins indicates VSMC dedifferentiation, in commitment towards proliferation, a typical VSMC response upon injury.

Lines of evidences strongly associate the intriguing expression of microRNA-145 as a fate and plasticity modulator of SMC (Cheng *et al.*, 2009; Cordes *et al.*, 2009). The studies revealed the inverse correlation of microRNA-145 level to the Kruppel-like factor (KLF) family of transcription factor proteins (KLF4 and KLF5) transcripts.

Kruppel-like factor 4 / KLF4

KLF4 is identified to impinge on a vast range of cellular biology that includes epithelial cell differentiation, endothelial pro-inflammatory activation, macrophage gene expression, tumor cell development and stem cell biology (Feinberg *et al.*, 2007; Haldar *et al.*, 2007; Hamik *et al.*, 2007). KLF4 expression is observed induced upon vascular injury when VSMC demonstrate active proliferation. Additionally, growth factors that regulate SMC proliferation, differentiation and extracellular matrix formation were shown to implicate the expression of KLF4 (Haldar *et al.*, 2007; Suzuki *et al.*, 2005). These findings confirm the role of KLF4 in determining the fate and plasticity of SMC. It has been elucidated that the mecanotransduction is associated to the KLF4 trans-repression of its downstream molecule, myocardin (an important transcriptional coactivator responsible for SMC differentiation) (Cordes *et al.*, 2009).

Nevertheless, accumulating studies underscored the importance of KLF4 in leukocyte biology that includes its role in lineage commitment, differentiation and function. In monocytes/macrophage, KLF4 is described to be the regulator of the stage specific monocyte-restricted maturation during myelopoiesis (Feinberg *et al.*, 2007). Introduction of KLF4 in promyelocytic progenitors, primary common myeloid progenitors or haematopoietic stem cells confers monocyte lineage differentiation, while KLF4 knockout model exhibits decreased monocyte counts in bone marrow, resident monocytes in spleen and trace amount of blood inflammatory monocytes (Alder *et al.*, 2008). In spite of that, KLF4 is also reported to play a role in macrophage activation. These findings have therefore highlighted KLF4 as a critical player in both monocyte differentiation and activation (Cao *et al.*, 2010).

Because monocyte and granulocyte derived from common precursors; i.e. primary common myeloid progenitor and granulocyte/macrophage progenitor; KLF4 expression is shown to be associated with the downstream lineage commitment. Introduction of KLF4 overexpression in either monocyte and granulocyte common precursors favors monocyte differentiation and at the same time repress granulocyte differentiation. On the other hand, the absences of KLF expression demonstrate the opposing observation. These findings suggest that KLF4 acts as monocyte differentiation promoter and active granulocyte differentiation inhibitor (Cao *et al.*, 2010).

The role of KLF4 in T-cell was tested using $CD8^+$ T-cells (leukemic cell line). Upon stimulation that promotes $CD8^+$ T-cells hyperproliferation, the level of KLF4 is found reduced. In line with this observation, overexpression of KLF4 in this cancerous cell activates the apoptosis pathway instead of cell proliferation suggesting that KLF4 is a tumor suppressor that maintains T-cell quiescence (Cao *et al.*, 2010).

In B-cells, KLF4 expression is reported to be low in progenitor-B cells and the expression increases with maturation into both precursor-B cells and resting mature B-cells. Similar to T-cells, overexpression of KLF4 retards B-cell proliferation (Cao *et al.*, 2010).

Kruppel-like factor 5 / KLF5

Similar to KLF4, KLF5 is also implicated as a prominent regulator of VSMC differentiation, proliferation and gene expression. Its expression is abundant in embryonic SMC and is downregulated with vascular formation. Nevertheless, KLF5 expression is induced in proliferating neointimal SMC and the development of restenosis upon vascular injury (Haldar *et al.*, 2007).

The role of KLF5 in vascular biology was derived from the lost-of-function experiments (Shindo *et al.*, 2002). The KLF5 knockout is embryonically lethal while heterozygous allele demonstrates normal vascular phenotype. However, in times of induced injury, heterozygous allele displays a substantial decrease of neointima formation and VSMC proliferation as compared to wild-type controls. Therefore, these results ostensibly suggest that KLF5 is the crucial transcription factor in VSMC remodeling and demonstrate its potential as the therapeutic target for cardiovascular/cerebrovascular diseases.

It is noteworthy that besides its roles in cardiovascular/cerebrovascular remodeling and angiogenesis, KLF5 is shown to implicate in lineage specific germline expression modulation of T-cells. It is shown that KLF5 possesses affinity to the promoter region of the gene locus, thus transactivating gene transcription (Miyamoto *et al.*, 2003). However, the pathway and the role of KLF5 regulating gene transcription in T-cell lineage development are yet to be elucidated.

Understanding the pivotal role of transcription factor KLF4/5 in VSMC differentiation, in addition to its mRNAs as the targets of microRNA-145, this study was aimed to study the expression of peripeheral blood microRNA-145 of chronic ischaemic stroke patients and healthy control subjects.

2.3.2. MicroRNA-214 and vascular biology

MicroRNA-214 is reported to associate with angiogenesis, cell survival, and cell differentiation (Chan *et al.*, 2009b). Silencing of microRNA-214 via RNAi is shown to enhance endothelial cell migration and neovascular formation. Moreover, increase in wound recovery activity is also observed upon attenuation of microRNA-214 expression (Chan *et al.*, 2009b). Conversely, enforcement of microRNA-214 transcripts in human umbilical vein endothelial cells (HUVEC) results in retardation of its angiogenic nature. This demonstrates that microRNA-214 is a repressor of endothelial cell migration and neovascular network formation, thus is classified as an anti-angiogenic factor. Noteworthy, concomitant to microRNA-214 expression, eNOS is shown to express in a negative correlated fashion (Chan *et al.*, 2009b).

It is shown that microRNA-214 anti-angiogenic ability acts through the negative regulation of PTEN, posttranscriptionally (Yang *et al.*, 2008). PTEN is also known as the 'mutated in multiple advanced cancers' (MMAC1) inhibitor as well as the transforming growth factor β- regulated and epithelial cell-enriched phosphatase, and was first identified as a tumor-repressor (Oudit *et al.*, 2004). Mutations in PTEN are often found in many cancers and, together with p53, PTEN serves as an essential governor of cell proliferation, differentiation, growth and apoptosis. The evidence of PTEN function in the regulation of cell cycle by preventing cells from growing and

dividing too rapidly became clear when PTEN null mice underwent hyperproliferation and embryonic lethality, while the heterozygote mice demonstrate elevated susceptibility to cancer (Oudit *et al.*, 2004).

PTEN is ubiquitously expressed in cells, which include cardiomyocytes, VSMC, and endothelial cells (Oudit *et al.*, 2004). The function mechanism of PTEN is involved preferentially in dephosphorylating phosphoinositide (PI) substrates and act as a growth regulator and tumor suppressor by negatively regulating AKT signaling pathway. Indeed, PI3K/AKT and PTEN signaling are involved in endothelial cells and VSMC, in regulation of vascular homeostasis and angiogenesis, as well as VSMC growth and proliferation. AKT/PKB, on the other hand, posphorylates eNOS and mediates nitric oxide (NO) release and vasodilatation upon sheer stress, estrogen and corticostroid stimulation.

Interestingly, PTEN is also shown to be an important regulator of T-cell fate (Buckler *et al.*, 2008). Activation of T-cells requires signaling through the T-cell receptor upon recognition of peptide-major histocompatibility complexes on the surface of antigen-presenting cells. The down stream networks include PI3K signaling pathway and AKT signaling pathway that promotes T-cell survival, cytokine production, and differentiation (Acuto *et al.*, 2003). PTEN negatively regulates the AKT signaling pathway by dephosphorylating phosphotidylinositol 3,4,5-triphosphate, a second messenger generated by PI3K that promotes the recruitment of 3-phosphonositide-dependant protein kinase 1 to the T-cell receptor signaling complex as co-stimulator (Buckler *et al.*, 2008).

Therefore, in this study we aimed to study the expression of peripheral blood microRNA-214 of chronic ischaemic stroke patients and healthy control subjects as specified in the objectives.

2.3.3. MicroRNA-222 and vascular biology

Labeled as an anti-angiogenic, microRNA-222 is reported to be abundantly expressed in endothelial cells and VSMC (Suarez *et al.*, 2007). The importance of microRNAs in vascular biology was demonstrated when the silencing of Dicer, a microRNA microprocessor, abrogates the angiogenic properties of HUVEC. As angiogenesis is a homeostatic process important for vascular maintenance in the adult organism. It involves the degradation of extracellular matrix, migration, proliferation and organization in neovascular network (Carmeliet, 2003). Dicer silencing in HUVEC also shows elevated eNOS protein expression besides the negative effect on cell proliferation. Introduction of microRNA-222 in HUVEC after Dicer silencing partially reduces the increased eNOS protein level (Suarez *et al.*, 2007).

eNOS is one of the constituent of nitric oxide synthases family of enzymes that catalyze the production of NO from L-arginine. eNOS is positively correlated with NO release. NO synthesized by eNOS is vital for endothelial cell survival, migration and angiogenesis (Yu *et al.*, 2005). Even so, the relation of microRNA-222 with eNOS regulation is yet to be elucidated.

Nonetheless, microRNA-222 is elucidated to be inversely correlated with c-Kit (a stem cell factor receptor) by having complimentary binding sequence between c-Kit 3'UTR mRNA and microRNA-222 seed sequence, which results in attenuation of protein translation, posttranscriptionally (Felli *et al.*, 2005; Gabbianelli *et al.*, 2010; Poliseno *et al.*, 2006). Transfection of microRNA-222 in HUVEC demonstrates a reduction of c-Kit protein, at the same time the cell was observed to lose the ability to form tubes or to heal wounds upon induction (Poliseno *et al.*, 2006).

c-Kit is a 145 kDa transmembrane tyrosine kinase protein that binds to the ligand, stem cell factor (SCF). Tyrosine phosphorylation by c-Kit mediates cell survival, differentiation, apoptosis, attachment and migration (Matsui *et al.*, 2004). High levels or altered c-Kit and its ligand, SCF, are associated with the 'angiogenic switch' that causes by the overwhelming angiogenic factors resulting in extravagant cell proliferation (Bergers *et al.*, 2003; Litz *et al.*, 2006). Thus, SCF/c-Kit interaction may promote cell proliferation/ differentiation.

MicroRNA-222 is also shown to regulate intracellular cell adhesion molecule-1 (ICAM-1); this will be later discussed together with microRNA-339 in section 2.3.6. Retrospective associations of microRNA-222 with cell adhesion proteins (i.e. c-Kit and ICAM-1) those crucial in mediating cell-cell interaction as in leukocyte trafficking at the site of injury were demonstrated. Therefore, it is to our interest in studying the expression of peripheral blood microRNA-222 in the chronic ischaemic stroke patients as compared to the healthy control subjects.

2.3.4. MicroRNA-223 and vascular biology

MicroRNA-223 is associated to immune response (Carissimi *et al.*, 2009). It has been described to exclusively express in the myeloid compartment as a 'fine-tuner' of granulocyte production (Chen *et al.*, 2004; Johnnidis *et al.*, 2008). The expression of microRNA-223 transcript is scarce in pluripotent haematopoietic stem cells but common in myeloid progenitors (Johnnidis *et al.*, 2008). Gradual increase in microRNA-223 expression is detected as granulocitic differentiation advances through granulocyte/macrophage progenitors till peripheral blood granulocytes. On the contrary, the repression of microRNA-223 expression is reported in granulocyte/macrophage progenitors that adopt the monocytic fate.

In the quest of delineating the role of microRNA-223 in the haematopoietic system, microRNA-223 demonstrates inverse correlation regulation with LIM-only protein 2 (LMO2), a member of LIM-only class of transcription cofactors (Felli *et al.*, 2009; Nam *et al.*, 2006). As a transcription cofactor, LMO2 forms multimeric transcriptional complexes in regulating the expression of target genes (Hansson *et al.*, 2007). Indeed, the importance of LMO2 was elucidated in haematopoietic stem cell development and erythropoiesis when mice with LMO2 gene deprivation show defects in blood maturation, as well as the maturation of fetal erythrocytes (Warren *et al.*, 1994).

Additionally, microRNA-223 is also unveiled to posses inverse correlation to myocyte enhancer factor 2c (Mef2c) transcription factor 3'UTR mRNA (Johnnidis *et al.*, 2008). The Mef2c transcription factor was first identified as a critical regulator of myogenic differentiation, and this protein belongs to the distinct class of MCM1-agamous-deficient serum response factor (MADS) family of transcription factors (Black *et al.*,

1998). Responding to developmental and extracellular environmental cues, Mef2c transcription factor (incorporate with a series of transcription cofactors) orchestrates transcription of specific sets of target genes, a typical criteria of MADS family domain proteins. Besides regulating myogenic differentiation and vessels development (Bi *et al.*, 1999), Mef2c is also important in modulating myeloid cell fate (Schuler *et al.*, 2008).

As genes expression in white blood cells of circulating blood were shown to be significantly altered following stoke, and for the elucidated role of microRNA-223 to exclusively express in the myeloid compartment as a 'fine-tuner' of granulocyte production; therefore, this study was aimed to study the expression of peripheral blood microRNA-223 of chronic ischaemic stroke patients and healthy control subjects.

2.3.5. MicroRNA-23b and vascular biology

MicroRNA-23b is notoriously found to be involved in cardiovascular functions (Divakaran *et al.*, 2008) and hypoxia regulation (Kulshreshtha *et al.*, 2008). Interestingly, microRNA-23b is recently revealed to be a key player in flow regulation and endothelial cell growth (Wang *et al.*, 2010). It was demonstrated that under pulsatile flow, level of microRNA-23b is upregulated in endothelial cell, resulting in growth arrest, as compared to the static condition that demonstrates consistence growth. Furthermore, by using RNAi approach, expression level of microRNA-23b is unveiled to be associated with the phosphorylation state of retinoblastoma protein.

Retinoblastoma protein so named because its inactivation or its absence causes retinoblastoma tumor (Korenjak *et al.*, 2005). It is understood later that its main role is to act as a signal transducer connecting the cell cycle clock with the transcriptional machinery. Loss of protein function causes failure of cell cycle regulation and thus leading to undesirable cell proliferation through deprivation of negative feedback mechanism of gene expression (Hatakeyama *et al.*, 1995; Weinberg, 1995).

Retinoblastoma protein is found to be in hypophosphorylated state during early gap 1 (G1) of the cell cycle. Conversely, during late G1 of the cell cycle, as the condition is propitious to advance into next phase of cell cycle, retinoblastoma protein undergoes phosphorylation and remains hyperphosphorylated throughout synthesis (S) phase until the end of mitotic (M) phase of cell cycle. Phosphorylated state of retinoblastoma protein is presumably functionally inactive (Weinberg, 1995).

Recently, other then its role as the cell cycle clock gatekeeper, retinoblastoma protein is also found to modulate cell differentiation and development (Korenjak *et al.*, 2005). Besides regulating the E2F family of transcription factors proteins, retinoblastoma protein is shown capable to modulate the activity of several tissue-specific transcription factors, subsequently orchestrating the transcription of tissue-specific genes, hence modulating cell differentiation and function.

The participation of pRb as a transcription factor in regulating gene expression was demonstrated by Decary and co-workers (2002) when pRb was shown to bind to the promoter of the anti-apoptosis Bcl-2 gene and directs its expression (Decary *et al.*, 2002). In a stroke model, exorbitant expression of Bcl-2 was detected in the microglia/microphage that infiltrates the site of infraction. The expression of this anti-apoptotic gene, Bcl-2 was not detected in those microglia/microphage of that control model (Benjelloun *et al.*, 1999).

In account to the studies and associations above, the basis of our objectives in studying the expression of peripheral blood microRNA-23b of chronic ischaemic stroke patients was defined.

2.3.6. MicroRNA-339 and vascular biology

There is scarce amount of literature about microRNA-339. However, microRNA-339 expression was reported to be downregulated in several cancer research studies (Roldo *et al.*, 2006; Visone *et al.*, 2008). This shows that microRNA-339 may have a role in cell cycle or/and cell survival againts immune response.

Together with microRNA-222, microRNA-339 is shown to downregulate ICAM-1 in glioma cell (Ueda *et al.*, 2009). The relation between both microRNA-222 and -339 to ICAM-1 mRNA 3'UTR were confirmed by luciferace reporter assays. The results demonstrated inhibition of luciferase activity that indicate ICAM-1 mRNA 3'UTR as the target of both microRNAs. Indeed, suppression of microRNA elevates of ICAM-1 expression and over expression of microRNA suppresses ICAM-1 expression.

ICAM-1 is one of the 5 receptors of the immunoglobulin gene superfamily that comprises namely, mucosal addressin (MAdCAM-1), platelet-endothelial cell adhesion molecule-1 (PECAM-1), vascular cell adhesion molecul-1 (VCAM-1), ICAM-2 and lastly ICAM-1 itself. The function of CAMs is to promote firm adhesion of leucocytes upon inflammation, hence the low amount on the cell membranes during pulsatile circulation (Carlos *et al.*, 1994).

The pivotal role of ICAM-1 is demonstrated when its upregulation increases the susceptibility of T-cell adhesion and activation (Ueda *et al.*, 2009). The down modulation of ICAM-1 expression resulted in reduced T-cell adhesion, thus promoting resistance of T-cell activation. This has enormous implication on stroke physiology as leukocytes activation is shown to induce the development of secondary injury after acute ischaemic infarction and inhibition of leukocytes accumulation mitigates ischaemic injury (Frijns *et al.*, 2002). In such, it is to our interest in studying the expression of peripheral blood microRNA-339 in the chronic ischaemic stroke patients as compared to the healthy control subjects.

3. Materials and Methods

3.1. Study outline

The peripheral blood of young ischaemic stroke patients and healthy individual as controls was collected. The ischaemic stroke patients were characterized based on WHO clinical criteria with magnetic resonance imaging (MRI) or computed tomography (CT) scans of the brain. Further classification of the etiology was according to the TOAST classification. In order to generate the microRNAs profiles, total RNAs were extracted and subjected to reverse transcription PCR and Real-time quantitative PCR. Relative expressions of the microRNAs profiles were then calculated as compared to the controls. Similar procedures were performed to the peripheral blood collected after some time from the first collection in generating the relative expressions of the microRNAs profiles. Comparison between these two profiles was subsequently carried out.

3.2. Peripheral blood collection

3.2.1. Ethics statement

The Medical Ethics Committee of University of Malaya Medical Center (UMMC) had granted this study with Ethics Committee/IRB reference number, **607.20**. Written consent was given by all recruited subjects (study and control) for blood collection and their information to be stored and used for this research. Consent was taken again for the subsequent blood collection, if applied.

3.2.2. Study subjects

Malaysian ischaemic stroke patients between age 18 to 50 not discriminating the gender and ethnicity were recruited for this study. These subjects were mainly from those admitted via the neurology service at the UMMC (a major 900 bed teaching hospital serving a population of about 800,000). The inclusion criteria for study subjects were in accordance to WHO criteria with or without risk factors (risk factors are described in **Table 3.1**). All patients had gone through standard neurological evaluation with subsequent review and follow up as out patients. Ischaemic stroke was confirmed either with CT or MRI scans of the brain. Further diagnostics work-up included were chest electrocardiography (ECG), routine blood test such as fasting blood glucose and hemoglobin A1c (HbA1c). When routine stroke investigations were normal or negative, thrombophilia screen and detailed immunologic studies (anti-nuclear, anti-DNA, and anti-RNA antibodies) were performed. Stroke event were classified when the patients was completely evaluated with the etiology identified. Overall, the basis of the above classification was based on clinical, imaging, routine and optional tests. Accordingly, the TOAST classification was applied. Demographic data, medical history and conventional vascular risk factors were recorded in a standardized computerize database and abstracted from the medical records.

3.2.3. Control subjects

Healthy Malaysian volunteers between age 18 to 50 not discriminating the gender and ethnicity were recruited for this study as control subjects. Control subjects were interviewed for demographic data after consent was given before blood collection being made. Peripheral blood samples were collected from all control subjects the same way as study subjects. Additionally, blood was tested for the risk factors investigation and

only subjects who are free from any risk factors (**Table 3.1**) were used as control subjects.

3.2.4. Blood collection and stabilization of RNA in RNA*later* Solution

Peripheral blood samples were collected from all subjects recruited. With the help from a nurse; around 10 ml of whole blood was collected from each subject according to standard procedure using a sterile 10 ml syringe with a 24G sterile clinical needle, a sterile 24G butterfly needle or using a sterile Flashback needle and BD Vacutainer® (BD, New Jersey, USA) blood collection tube containing sodium EDTA anticoagulant.

Complying with the manufacturer's recommendation, 1.3 ml of RNA*later* solution (Ambion, Texas, USA) was added to approximately 0.4 to 0.5 ml of whole blood in 2 ml labeled tubes. The mixture was allowed to mix thoroughly by inverting the tubes several times (about 30 inversions). All blood samples collection and RNA stabilization in RNA*later* solution were performed within 1 hour. RNA stabilized samples were stored at -20°C until being used.

NOTE:

As recommended by the manufacturer, blood samples were collected in tubes containing anticoagulant (potassium/sodium EDTA preferred) and RNA stabilized by adding RNA*later* solution, although, there were blood samples collected and RNA stabilized in RNA*later* solution without adding anticoagulant. In the later cases, the RNA*later* solution was however added before any coagulation of the blood formed. **Appendix 1** records the blood samples that were either collected in tubes containing anticoagulant or without adding anticoagulant prior RNA stabilization.

3.2.5. Study subjects blood sample recollection

Selected study subjects were contacted for blood sample recollection after a period of time for the second part of the study. Patients who agreed to participate in the study were treated as a new recruit starting from consent statement. Same procedures of peripheral blood collection, RNA stabilization and storage were practiced as mentioned in sections 3.2.1, 3.2.2 and 3.2.4.

3.3. Solutions and buffers preparation

3.3.1. 2.5 M Natrium Chloride (NaCl) stock solution preparation

The solution was prepared by adding MiliQ water up to 100 ml into 14.61 g of NaCl (MERCK, Darmstadt, Germany). The solution was stirred thoroughly to dissolve the NaCl crystals followed by autoclave and stored at room temperature until used.

3.3.2. 10X 3-(N-morpholino)propanesulfonic acid (MOPS) buffer stock solution preparation

The buffer was prepared by adding MiliQ water up to 1 l into MOPS powder (Sigma-Aldrich, St. Louis, USA) pre-packed for 10X 1 l preparation. The mixture was stirred thoroughly until all powder was completely dissolved. The buffer was autoclave and stored at room temperature until used.

3.3.3. 1X MOPS buffer solution preparation from stock solution

The 1X MOPS buffer solution was prepared by diluting the 10X MOPS buffer stock solution. One part of 10X MOPS buffer stock was diluted with 9 parts of autoclaved MiliQ water.

Example:

Autoclaved MiliQ water (9 ml) was added into 1 ml of 10X MOPS buffer to make 10 ml of 1X MOPS buffer. The mixture was allowed to mix prior use. The 1X MOPS buffer solution can be used fresh or stored at room temperature until used.

3.3.4. 10X Tris/Borate/Ethylenediaminetetraacetic acid (TBE) buffer stock solution preparation

The buffer was prepared by adding MiliQ water up to 1 l into TBE powder (GIBCO-BRL, New York, USA) pre-packed for 10X 1 l preparation. The mixture was stirred thoroughly until all powder was completely dissolved. The buffer was autoclave and stored at room temperature until used.

3.3.5. 1X TBE buffer solution preparation from stock solution

The 1X TBE buffer solution was prepared by diluting the 10X TBE buffer stock solution. One part of 10X TBE buffer stock was diluted with 9 parts of autoclaved MiliQ water.

Example:

Autoclaved MiliQ water (9 ml) was added into 1 ml of 10X TBE buffer to make 10 ml of 1X TBE buffer. The mixture was allowed to mix prior use. The 1X TBE buffer solution can be used fresh or stored at room temperature until used.

3.3.6. Wash solution 1 (70% Ethanol/30% Denaturation solution) preparation

Wash solution 1 was prepared according to 7:3 ratio of absolute ethanol (Fisher Scientific, Leicestershire, UK):Denaturation solution (Ambion, Texas, USA).

Example:

Absolute ethanol (7 ml) was mixed with 3 ml of Denaturation solution in preparation of 10 ml wash solution 1. The solution was stored at 4°C and allowed to thaw to room temperature prior use.

3.3.7. Wash solution 2 (80% Ethanol/50 mM NaCl) preparation

Wash solution 2 was prepared according to 4000:999:1 ratio of absolute ethanol:nuclease-free water:2.5 M NaCl.

Example:

Absolute ethanol (8 ml) was mixed with 0.2 ml of 2.5 M NaCl and 1.8 ml of nuclease-free water in preparation of 10 ml wash solution 2. The solution was stored at 4°C and allowed to thaw to room temperature prior use.

3.3.8. 40% Acrylamide/Bis-acrylamide (29:1) monomer preparation

The 40% monomer was prepared by dissolving 40 g of Acrylamide (MERCK, Darmstadt, Germany):Bis-acrylamide (Sigma-Aldrich, St. Louis, USA) at 29:1 ratio with autoclaved MiliQ water. The water was toped up to 100 ml. The monomer was protected from light and stored at 4°C until used.

3.3.9. 10% Ammonium Persulfate (APS) preparation

The 10% APS was prepared by dissolving 1 g of APS (MERCK, Darmstadt, Germany) with autoclaved MiliQ water. The solution was prepared fresh for every use.

3.4. Total RNA extraction

3.4.1. Background

Ribopure™ - Blood RNA isolation kit (Ambion, Austin, TX) was used to perform total RNA extraction. This isolation kit is based on phase separation by centrifugation of a mix of the aqueous sample and a solution containing water-saturated phenol, chloroform and guanidinium thiocyanate giving rise to an upper aqueous phase and a lower organic phase. Guanidinium thiocyanate was added to denature proteins, including RNases and separates ribosomal RNA (rRNA) from ribosome. Chloroform helps the poor solubility solvents used to separate completely into two phases.

After centrifugation, phase separation occurs, RNA is present in the aqueous phase, DNA in the interphase as a partition and proteins in the organic phase. In total RNA extraction, the aqueous phase is recovered and purified using glass fiber column approach.

Glass fiber column RNA purification is a solid phase purification method that allows positively charged ions to form a salt bridge between the negatively charged glass fibers and the negatively charged RNA backbone in the high salt concentration. The RNA can then be washed with high salt and ethanol (washing buffer) to remove impurities passing through the column and eluted out from the column using low salt solution (elution solution) giving a pure RNA preparation.

3.4.2. Methods and procedures

The whole blood mixed in RNA*later* Solution was allowed to thaw to room temperature prior extraction procedure. The total RNA extraction protocol is made up from three main parts: lysis, extraction, and purification of total RNA.

3.4.2.1. Cell lysis

The 2 ml tube containing whole blood and RNA*later* Solution was centrifuged at high speed for 2 minutes separating the pellet and the supernatant. The supernatant was decanted and any remaining fluid completely removed by taping the rim of the inverted tube against a clean paper towel and removing any fluid from inside the tube cap.

After, 800 µl of lysis solution from Ribopure-Blood Kit was added to the pellet and vigorously vortexed resuspending the pellet. Another 100 µl (often not exceeding total of 1 ml) of lysis solution was added if the mixture was turbid. Subsequently, 10 µl of glacial acetic acid was added and vortexed again to mix. The final mixture (cell lysate) was stored on ice for 1 hour.

3.4.2.2. Acid-Phenol/Chloroform extraction

Following incubation on ice, 500 µl of acid-phenol/chloroform withdrawn from beneath the overlaying layer of the aqueous layer was added into the cell lysate. As low as 300 µl of acid-phenol:chloroform was added when the tube was too full to permit adequate mixing. Then it was vortexed vigorously for about 30 seconds and centrifuged immediately at room temperature for 1 minute at maximum speed separating the aqueous (upper) and organic (lower) phases.

Using a pipette, the aqueous phase that contains the RNAs was transferred into a sterile 15 ml falcon screw cap tube that has been labeled accordingly to the sample code. This transferring step was performed carefully avoiding the organic phase being carried over. The aqueous phase volume transferred is usually around 1.2 ml.

Upon transfer, 1 ml of Denaturing solution was then added into each falcon tube, vortexed and 2.7 ml of absolute ethanol was added immediately. At this point the volume of the preparation should be about 5 ml. This preparation was then vortexed mixing content thoroughly. The preparation should be a clear solution. Otherwise, 300 µl increments of nuclease-free water, mixing after each, were added until solution turned clear.

3.4.2.3. RNA purification

The filter cartridge was placed onto the collection tube supplied by the manufacturer. The assembly was labeled according to the sample code. Then, the clear solution was filtered through the filter cartridge by applying 700 µl at a time, centrifuging at low speed for few seconds. The flow-through was discarded. This continued until all the clear solution passes through the filter cartridge.

Placed on the same collection tube, the filter cartridge was washed with washing solution 1 (70% Ethanol/30% Denaturation solution). Thus, 700 µl of washing solution 1 was aliquoted into the filter cartridge and centrifuged to allow the washing solution to pass trough the filter. The flow-through was discarded.

Using the same collection tube, the filter cartridge was then washed with washing solution 2 (80% Ethanol/ 50 mM NaCl) and this washing step was performed twice. Washing solution 2 (700 µl) was applied to the filter cartridge and centrifuged to assist flow through the filter. The flow-through was discarded. After discarding the flow-through from the last wash, the assembly was centrifuged for 1 minute at high speed to remove residual fluid from the filter.

To elute out the RNAs, the filter cartridge was transferred into a new-labeled collection tube and 150 µl of elution solution pre-heated to 80°C was applied to the filter cartridge. This assembly was allowed to incubate at room temperature for 1 minute before centrifuging at high speed for 2 minutes to yield the RNA in the collection tube.

Recovered RNA was stored in 1.5 ml labeled polypropylene tube at -20°C until used.

3.5. DNase I treatment

3.5.1. Background

DNase I treatment was performed to eliminate contaminating genomic DNA from the eluted RNA. Contamination of genomic DNA would lead to overestimation of RNA yield when estimated by spectrophotometer. This is because all nucleic acids absorb at 260 nm wavelengths.

3.5.2. Methods and procedures

To start the treatment, 7.5 µl of the 20X DNase buffer was added into 150 µl of sample. Three repetition of up down pipette motion was performed to mix the mixture gently. One (1) µl of DNase I (8 U/µl) was subsequently added.

Next, the mixture was incubated for 30 minutes at 37°C to allow optimal enzymatic activity degrading the genomic DNA.

In order to deactivate the enzyme activity, 30 µl of DNase inactivation reagent was added into the treated mixture. This was stored at room temperature for 2 minutes with occasional vortexing to resuspend the settled DNase inactivation reagent.

Finally, the sample was centrifuged at high speed for 1 minute to pellet the DNase inactivation reagent, and the RNA solution was transferred to a new RNase-free tube. Sample was stored at -20°C until used.

NOTE:

The DNase inactivation reagent was thawed on ice and vortex vigorously to resuspend the slurry reagent completely. When pipette, the pipette tip was inserted well below the surface and the aliquot was ensured to be mostly white, without significant amount of clear fluid. Nuclease-free water (100 µl) was added and vortexed thoroughly recreating a pipetteable slurry if reagent was challenging to aliquot.

3.6. Quantification of RNA

The concentration and purity of the RNA extracted was estimated using a Nanodrop ND-1000 spectrophotometry (Nanodrop Tech, Rockland, Del). The absorbance at 260 nm and 280 nm were determined against blank (elution solution used during RNA extraction). The concentration was automatically calculated from the absorbance reading, assuming 1 OD is produced from 40 μg/ml RNA solution. The ratio of A_{260}/A_{280} indicates the purity of the sample and values ranging from 1.8 to 2.2 are acceptable for the following protocols.

3.7. Assessment of the integrity of RNA: Denaturing Agarose Gel Electrophoresis

3.7.1. Background

Electrophoresis is termed as the movement of ions and charged macromolecules through a matrix when an electric current is applied. Agarose (a polysaccharides extracted from seaweed) was used as the primary stabilizing media/matrix in this electrophoresis of RNA. Nucleic acids like RNA migrate through the gel towards the anode based on size and structure, with little influence from base composition or sequence. Notably, the higher percentage of agarose used will increased the resistance of macromolecules migration, increasing the resolution but decelerates the migration rate. The denaturing gel system was applied to allow the RNA to migrate according to its size as most RNA forms extensive secondary structure via intramolecular base paring.

3.7.2. Methods and procedures

Denaturing agarose gel electrophoresis comprises 3 main steps, which include denaturing agarose gel preparation, sample treatment, and electrophoresis.

3.7.2.1. Gel preparation

Denaturing agarose gel (1% in 50 ml) was required. Agarose (0.5 g) was dissolved in 43.5 ml of MiliQ water in a microwave oven. The molten agar was cooled to about 60°C by swilling the glassware against running tap water to allow homogeneous cooling.

In the fume hood, 5 ml of 10X MOPS running buffer was added followed by 1.5 ml of 12.3 M (37%) formaldehyde. The mixture was swilled to mix and allowed to set in the gel-casting tray with the comb fixed in order to form wells for the samples. All air bubbles especially those formed around the comb teeth were made sure devoid.

After 30 minutes or until the gel was hardened, the gel was removed from the cast set and placed in the tank filled with 1X MOPS running buffer. The buffer was made sure to submerge the gel for at least 1 mm. The wells were made sure to be near the cathode because RNA will migrate to the anode according its size.

3.7.2.2. RNA samples preparation

For the band to be visible, a minimum of 500 ng of total RNA was used. To each sample including the RNA marker, 2 µl of 10X MOPS running buffer, 3.5 µl 12.3 M (37%) formaldehyde and 10 µl of deionized formamide was added followed by 2 µl of RNA loading dye supplied by the blood extraction kit.

Subsequently, the mixture was incubated at 65°C for 10 minutes to allow the denaturation of RNA. After, the sample was immediately stored on ice and centrifuged for 5 seconds prior to loading. The prepared samples were loaded using a micropipette and fine disposable micro-tips into each well created by the comb during casting. Care was taken by avoiding air bubbles during the drawing of samples by micropipette, as these would cause difficulties in loading the samples into the well. RNA marker (18S + 28S rRNA from calve liver) (Sigma-Aldrich, St. Louis, USA) was prepared same as the sample and loaded into one well as the positive control.

3.7.2.3. Electrophoresis and gel visualization

The electrophoresis process was performed using flow direct current at constant voltage (80 V) for 1 hour. After 1 hour, when the blue dye front has migrated to about ¾ of the way down the gel, away from the wells, the electric flow was switched off and the gel was ready to be analyzed. The gel was visualized under ultra-violet (UV) light where etedium bromide (EtBr) that intercalates with the nucleic acids would fluoresce. Sharp 28S and 18S rRNA bands with the band intensity ratio of 2:1 of 28S:18S rRNA determines the RNA integrity. Degraded RNA samples, appeared as low molecular weight smear were not used in the study.

3.8. Assessment of the total RNA extracted: Polyacrylamide Gel Electrophoresis

3.8.1. Background

Polyacrylamide gel electrophoresis (PAGE) is widely used for separating nucleic acids, such as RNA that differ in length by as little as one nucleotide. The polyacrylamide gels are prepared by the chemical reaction of free radical polymerization of acylamide and the cross-linking agent bis-acrylamide. The resistance created by the polyacrylamide can be manipulated by adjusting the percentage of the polyacrylamide, ratio of cross-linking or thickness of the gel depending to the need of the separation resolution desired. The conditions used in this protocol are intended to assess the presence of small RNA in the total RNA extracted, cross-checking the success of the RNA extraction procedure. Generally, the electrophoresis describes the process of acquiring electric flow to separate molecules inversely proportional to the resistance applied through a solution.

3.8.2. Methods and procedures

Similar to denaturing agarose gel electrophoresis, PAGE comprises the same 3 main steps, which are gel preparation, sample treatment, and electrophoresis.

3.8.2.1. Gel preparation

In order to cast a polyacrylamide gel, the gel apparatus was cleaned and set up. Leaving the apparatus aside, the gel was prepared by dissolving 3.6 g of urea (BDR, Poole, England) in 2.82 ml of the 40% acrylamide/bis-acrylamide solution and 750 µl of 10X TBE buffer at 37°C. After, 950 µl of autoclaved water, 37.5 µl of 10% APS and 7.5 µl of TEMED (Sigma-Aldrich, St. Louis, USA) were subsequently added and mixed prior

casting the mixture into the gel apparatus. The mixture was left to polymerize for approximately 1 hour at room temperature.

3.8.2.2. RNA samples preparation

In preparation of the samples for PAGE, 2 µl of 10X TBE, 10 µl of deionized formamide and 3 µl of RNA loading dye was mixed with the RNA (approximately 200 ng/µl). The mixture was later incubated at 95°C for 2 minutes and immediately chilled on ice after.

3.8.2.3. Electrophoresis and gel visualization

Prior to electrophoresis, the gel was fixed into the PAGE system and filled up with 1X TBE running buffer. The gel was then pre-run (without sample) using flow direct current at constant voltage (100 V) for 30 minutes. After, the wells of the gel were flushed with the running buffer and samples were then loaded into the wells and the electrophoresis process was performed under the same electric flow for 90 minutes. After 90 minutes, when the blue dye front has migrated to about ¾ of the way down the gel, away from the wells, the electric flow was switched off and the gel was stained with EtBr for approximately 1 hour. The gel was visualized under UV light where EtBr that intercalates with the nucleic acids would fluoresce. Three (3) distinct sharp bands indicating 18S and 28S rRNAs, 5.8S rRNA and 5S rRNA; while the transfer RNAs (tRNAs) appear as a thicker band furthest from the loading well. These bands demonstrate the presence of small RNAs indicating the success of the protocol in extracting total RNA.

3.9. Reverse Transcription PCR

3.9.1. Background

Reverse transcription (RT) is a method used in complimentary DNA (cDNA) synthesis. This method converts a single stranded RNA template into double stranded cDNA template by reverse transcriptase. Therefore, allowing downstream application i.e. PCR reactions to be carried out because the enzyme, DNA polymerase only recognizes double stranded DNA template and not RNA template.

Reverse transcriptase is a common name for an enzyme that functions as a RNA-dependent DNA polymerase. They are of retroviruses origin, where they copy the viral RNA genome into DNA prior to its integration into host cells and replicate.

3.9.2. Total RNA → cDNA (microRNA)

TaqMan® MicroRNA Reverse Transcription Kit was used to synthesize the single-stranded cDNA from total RNA samples. For each 15 µl of RT reaction, 10 ng per 5 µl input amount of total RNA was used. This process involved the following procedures:

3.9.2.1. RT master mix preparation

The deoxyriboneucleotide triphosphate (dNTP), reverse transcriptase, 10X Reverse Transcriptase Buffer and the RNase Inhibitor were thawed on ice prior to RT master mix preparation. The master mix was prepared in a polypropylene tube by scaling the volumes listed below to the number of RT reactions needed. Additional of 10% of total volume were prepared accounted for pipetting losses. **Table 3.2** lists the components and measurement for RT master mix preparation. Master mix was mixed, centrifuged and stored on ice while preparing the subsequent procedure.

3.9.2.2. RT reaction preparation

The microRNA RT primer provided by TaqMan MicroRNA Assay kit was allowed to thaw on ice, mixed, centrifuged and stored on ice prior use.

For each 15 µl RT reaction, 7 µl of RT master mix was mixed with 3 µl of microRNA RT primer aliquot and 5 µl of total RNA (10 ng) in 0.2 ml polypropylene PCR tube. Up down pipetting was done to mix the preparation and it was centrifuged to draw down the RT reaction preparation.

3.9.2.3. Reverse transcription

The reverse transcription was performed using Perkin Elmer Geneamp 2400 PCR System. The system was turn on and the conditions for the reverse transcription were set as in **Table 3.3**. The cDNA created was kept at -20°C until being used.

3.9.3. Total RNA → cDNA

High Capacity RNA-to-cDNA Master Mix was used to synthesize the single-stranded cDNA from total RNA samples. This kit utilizes random hexamers as primer. For each 15 µl of RT reaction, 10 ng per 5 µl input amount of total RNA was used. This process involved the following procedures:

3.9.3.1. RT reaction preparation

The High Capacity RNA-to-cDNA Master Mix was mixed and centrifuged drawing down the mix. For each reaction in 0.2 ml PCR polypropylene tube, 3 µl of High Capacity RNA-to-cDNA Master Mix was added into the RNA (10 ng per 5 µl) and 7 µl of nuclease-free water was toped up to make the final volume of 15 µl. Up

down pipetting was performed to mix the preparation gently, centrifuged and stored on ice until being loaded into the PCR system.

3.9.3.2. Reverse transcription

The reverse transcription was performed using Perkin Elmer Geneamp 2400 PCR System with the reaction volume set at 15 µl. The pre-optimized conditions were performed as listed in **Table 3.4**. The cDNA created was kept at -20°C until being used.

3.10. Real-time quantitative PCR

3.10.1. Background

Real-time PCR is an automated detection tool used to measure PCR product growth throughout the amplification processes. This was done by measuring the threshold cycle (C_T) value; the calculated fraction cycle number that the PCR product crosses a threshold of detection, where replication is at the exponential stage.

TaqMan MGB probe was used as mean of measurement. This probe is made of a reporter dye (FAM™ dye) linked to the 5' end of the probe, a minor groove binder (MGB) at the 3' end of the probe and a nonflorescent quencher (NFQ) at the end of the 3' end of the probe. NFQ provides a more reliable detection of reporter florescent for its unique quality, a quencher that does not fluoresce. Similar probe with TAMRA™ reporter dye for endogenous control (18S rRNA) was used in this study.

There will be no event of florescence from the reporter dye to be detected when the probe is intact. This is due to the contiguity of the reporter dye to the quencher dye that suppresses the florescence by FRET (Foster resonance energy transfer through space). Florescence will only occur when the reporter dye is separated from the quencher dye and only be detected if the florescence crosses the threshold value.

A passive reference dye (ROX dye) was included into the reaction as an internal reference that is necessary to correct for well-to-well signal fluctuations, due to the design of the instrument. ROX dye is not reactive toward the PCR component and proven not to inhibit PCR reaction at a final concentration of up to 1000 nM.

During PCR, the TaqMan MGB probe anneals specifically to the complementary target between the forward and reverse primers annealing sites. As the amplification proceeds toward the probe hybridization site, the DNA polymerase will cleave only the probes that hybridized to the target. This cleavage separates the reporter dye from the quencher dye resulting in the increase of florescence and because only the probes that hybridized to the complimentary target during PCR amplification are cleaved, non-specific amplification will not be detected **(Figure 3.1)**.

3.10.2. TaqMan microRNA assays

In this study, there were 6 microRNA assays (ABI, Foster City, USA) conducted and the assay names and product numbers are hsa-miR-145 (P/N 4373133), hsa-miR-214 (P/N 4373085), hsa-miR-222 (P/N 4373076), hsa-miR-223 (P/N 4373075), hsa-miR-23b (P/N 4373073) and hsa-miR-339 (P/N 4373042).

3.10.3.1. Master mix preparation

TaqMan MicroRNA Assay kit and Universal PCR Master Mix, No AmpErase UNG were used in this procedure. The microRNA assay mix was allowed to thaw on ice, mixed, centrifuged and stored on ice prior to use.

For each 20 µl PCR mixture, 10 µl of 2X Universal PCR Master Mix, No AmpErase UNG, 1 µl of TaqMan MicroRNA assay mix and 1.33 µl of RT-PCR product were mixed in 7.67 µl of nuclease free water.

One batch of the mix without the RT-PCR product was prepared enough for triplicates of each sample (RT-PCR product) with 10% excess accounted for pipetting losses per assay. **Table 3.5** lists the components and measurement in preparing the master mix for real-time quantitative PCR. The master mix was briefly mixed, centrifuged and stored on ice until use.

An aliquot of 18.68 µl of the mix was dispensed into the polypropylene tubes (MicroAmp™ Fast Reaction Tube) fixed on the Real-time PCR plate. Next, RT-PCR product were centrifuged and gently mixed with up down pipetting repetition and 1.33 µl of RT-PCR product was subsequently loaded into each of the triplicate tubes. The tubes were then sealed with MicroAmp® Optical 8-Cap Strip and the plate was centrifuged at 3500 rpm at 4°C for 5 minutes spinning down the content. This Real-time quantitative PCR reaction plate was stored on ice until placed in Real-time PCR machine.

3.10.3.2. Performing Real-time quantitative PCR

Real-time quantitative PCR was performed on ABI StepOne Plus (ABI, Faster City, USA). Plate documents were created using the following parameters:

Sample Volume: set at 20 µl

Auto Increment Setting: set as zero (0), default value.

Ramp Rate Settings: set as standard ramping rate, default value.

Data Collection: set at 60°C, default value

Thermal cycling parameters were set as in **Table 3.6.**

The reaction plate was then loaded into the instrument and run.

3.11. Data and statistical analyses

3.11.1. Background

The $2^{-\Delta\Delta C_T}$ method was used in quantifying the relative changes in test microRNA expression determined from real-time quantitative PCR assays. The derivation of the $2^{-\Delta\Delta C_T}$ equation, including assumptions, experimental design, and validation tests as described in Applied Biosystem Users Bulletin No. 2 (P/N 4303859) and Livak et al., 2001 was referred.

3.11.2. Derivation of the $2^{-\Delta\Delta C_T}$ method

The exponential amplification of PCR is described by the following equation:

$$X_n = X_0(1+E_X)^n,$$

[1]

Where X_n represent the number of target RNA molecule at cycle n of the reaction, X_0 is the initial number of target RNA molecule. E_X denotes the efficiency of PCR and n denotes the cycle number.

Additionally, the threshold cycle (C_T) indicating fraction cycle number at which the amount of amplified RNA/brightness of florescence reaches the threshold value.

Therefore,

$$X_T = X_0(1+E_X)^{C_T,X} = K_X$$

[2]

Where X_T represent the threshold number of target RNA molecules, $C_{T,X}$ is the threshold cycle for target amplification, and K_X denotes constant.

The endogenous control as reference can be described by a similar equation. That is,

$$R_T = R_0(1+E_R)^{C_{T,R}} = K_R, \quad [3]$$

Where R_T represent the threshold number of reference molecules, R_0 is the initial number of target reference molecules, E_R denotes the efficiency of reference amplification, $C_{T,R}$ is the threshold cycle for reference amplification and K_R is a constant.

Dividing X_T by R_T gives the expression,

$$\frac{X_T}{R_T} = \frac{X_0(1+E_X)^{C_{T,X}}}{R_0(1+E_R)^{C_{T,R}}} = \frac{K_X}{K_R} = K. \quad [4]$$

In real-time amplification using TaqMan probes, the exact values of the X_T and R_T depends on several factors that include the reporter dye used in the probes, the sequence context effects on the florescence properties of the probe, the efficiency of probe cleavage, purity of the probe, and setting of the florescence threshold. Therefore, the constant K does not have to be equal to one.

Assuming efficiencies of the target and the reference are the same,

$$E_X = E_R = E,$$

Equation [4] can be expressed as

$$\frac{X_0}{R_0} \quad (1+E)^{C_{T,X}-C_{T,R}} = K, \qquad [5]$$

Or

$$X_N \times (1+E)^{\Delta C_T} = K, \qquad [6]$$

Where X_N is equal to the normalized amount of target (X_0/R_0) and ΔC_T is equal to the difference in threshold cycle for target and reference ($C_{T,X} - C_{T,R}$).

Rearranging the equation [6] gives rise to the expression,

$$X_N = K_X(1+E)^{-\Delta C_T}. \qquad [7]$$

The final step is to divide the X_N for test RNA (q) by the X_N for the calibrator /control (cb),

$$\frac{X_{N,q}}{X_{N,cb}} = \frac{K(1+E)^{-\Delta C_{T,q}}}{K(1+E)^{-\Delta C_{T,cb}}} = (1+E)^{-\Delta\Delta C_T}. \qquad [8]$$

Here,

$$-\Delta\Delta C_T = -(\Delta C_{T,q} - \Delta C_{T,cb}).$$

As the amplicons length in this research is less then 150 bp and Applied Biosystem have had optimized the primer and magnesium concentration, the efficiency is close to one. Hereby, the amount of target, normalized to an endogenous reference and relative to a calibrator/control, is given by,

$$\text{Amount of target RNA} = 2^{-\Delta\Delta C_T}. \qquad [9]$$

3.11.3. Data analysis using the $2^{-\Delta\Delta C_T}$ method

The cycle threshold (C_T) value provided from real-time RCR instrumentation was used for analysis. The change in expression of the RNA of interest normalized to 18S rRNA was obtained in each sample patients and control subjects. Real-time PCR was performed in triplicates on the corresponding cDNA synthesized from each sample. The data was then analyzed using equation [9], where,

$$\Delta\Delta C_T = (C_{T,\,RNA} - C_{T,18S\ rRNA})_{patient} - (C_{T,RNA} - C_{T,18S\ rRNA})_{control\ mean}$$

Patient was referred to young ischaemic stroke patients and control was referred to healthy non-stroke subjects. The mean and standard error mean (S.E.M.) were then determined from the triplicate of each sample.

3.11.4. Statistical analysis of Real-time quantitative PCR data

The C_T value provided from Real-time quantitative PCR was determined from a log-linear plot of the PCR signal versus the cycle number (**Figure 3.2**). Analysis was performed using statistical tool, SPSS™ software for Windows version 11.0. Mean, S.E.M. and significance of the data were calculated using one-sample t-test, two-sample t-test and paired sample t-test. Significant level was set as $p < 0.05$. Pearson correlation analysis was performed to determine the correlation between the microRNA expression profiles studied.

3.12. Bioinformatic analysis: incorporating microRNAs in pathways

Pathway analysis of the circulating microRNAs was carried out using bioinformatic software tool, Diana Lab (http://diana.cslab.ece.ntua.gr/). This software provides computational predictive models based on the algorithms developed by Diana Lab and available databases. Therefore, this web-based computational tool allows the identification of molecular pathways potentially affected by each microRNA expression, in relation to all known Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways.

4. Results

4.1. Study subjects and demographic data

A total of 32 (21 males and 11 females) Malaysian ischaemic stroke patients with average age of 41.6 years were recruited for this study. These subjects were mainly from those admitted via the neurology service at the UMMC. Out of this 32 stroke patients that were recruited acknowledging the risk factors listed in **Table 3.1**, there were 11 subjects with hypertension, 10 with dyslipedemia, 8 with diabetes mellitus, 8 smokers and 5 alcoholic. Tabulated in **Table 4.1** summarizes the demographic data of all samples used in this study.

4.2. Control subjects

A total of 22 healthy Malaysian volunteers between the ages of 18 to 49 not discriminating the gender and ethnicity were recruited as candidates for the study control. As the inclusion factors for the control subjects are to be free of the risk factors as listed in **Table 3.1**; therefore, 14 (7 males and 7 females) healthy controls with no history of cardiovascular or cerebral vascular diseases, recent infection, or haematologic diseases were used in this study. The control group has an average age of 33.1 years. The demographic data is represented in **Table 4.1**. Others, which revealed risk factor/s after blood tests corresponding to list in **Table 3.1** were excluded from this study.

4.3. Study subjects blood sample recollection

A total of 11 patients out of the 32 agreed to participate the second part of this study. The time interval for the first and second collection was between 3 months up to 16 months. As tabulated in **Table 4.2,** describes the 11 patients and the time interval of second sample collection after the first sample collection.

4.4. Total RNA extraction

This study uses peripheral circulating RNAs for real-time quantitative PCR. All RNA (total RNA) from peripheral blood samples were successfully isolated for all patients and controls sample using acid phenol/chloroform extraction method and DNase I treated as described in sections 3.4 and 3.5, respectively.

4.5. Quantification of RNA

Quantification of RNA was performed as described in section 3.6. The ratios of A_{260}/A_{280} obtained ranged from 1.8 to 2.2, indicating that RNA isolated were of high quality and purity. The RNA concentration of each extraction calculated by the software was referred to for downstream application. **Figure 4.1** illustrates the plot produced by Nanodrop ND-1000 spectrophotometry. Listed in **Appendix 2** are the A_{260}/A_{280} ratio reading and RNA concentration of all extracts.

The A_{260}/A_{280} ratio reading of the blood collected in the presence of sodium EDTA anticoagulant additive showed no distinctive difference from the blood collected without sodium EDTA anticoagulant additive with $p = 0.472$. **Figure 4.2**, box plot, illustrates the mean A_{260}/A_{280} ratios of total RNA extracted from blood samples with and without anticoagulant. The mean A_{260}/A_{280} ratio of RNA extracted from blood with

sodium EDTA was 1.91 ± 0.015, while the mean A_{260}/A_{280} ratio of RNA extracted from blood sample without sodium EDTA was 1.93 ± 0.014. Therefore, high quality and purity of RNA was extracted from both blood collection methods.

The concentration of samples collected with sodium EDTA anticoagulant additive resulted in a higher RNA concentration as compared to those without sodium EDTA anticoagulant additive with p = 0.027. **Figure 4.3**, box plot, illustrates the mean concentration (ng/µl) of total RNA extracted from blood samples with and without anticoagulant. The mean concentration of RNA extracted from blood with sodium EDTA was 64 ± 6, while the mean concentration of RNA extracted from blood sample without sodium EDTA was 46 ± 5. Therefore, higher yield of RNA was recovered from the blood samples collected in the presence of sodium EDTA anticoagulant.

4.6. Assessment of the integrity of RNA: Denaturing Agarose Gel Electrophoresis

The integrity of total RNA was tested as described in section 3.7. The integrity was assured when 2 distinctive bands were visible, representing the 2 discrete ribosomal RNAs, the 28S rRNA and 18S rRNA. **Figure 4.4** illustrates the gel picture of good RNA integrity from denaturing agarose gel electrophoresis. The intensity of the 28S rRNA band was approximately twice then that of 18S rRNA band. There were no distinct differences of the agarose gel profile between the RNA extracted from blood collected in the presence of sodium EDTA anticoagulant additive and from the blood collected without sodium EDTA anticoagulant additive. **Appendix 3** illustrates the gel pictures of the other samples.

4.7. Assessment of the total RNA extracted: Polyacrylamide Gel Electrophoresis

The total RNA extracted was tested adhering protocol as described in section 3.8. The success of total RNA extraction, presence of small RNA was assured when distinctive bands of 18S + 28S rRNAs, 5.8S rRNA, 5S rRNA and tRNAs were visible. **Figure 4.5** illustrates the gel picture revealing the bands of small RNA from PAGE. There were no distinct differences of the polyacrylamide gel profile between the RNA extracted from blood collected in the presence of sodium EDTA anticoagulant additive and from the blood collected without sodium EDTA anticoagulant additive. **Appendix 4** illustrates the gel pictures of the other samples.

4.8. Expression profiles of studied circulating microRNAs of ischaemic stroke patients

In order to study the microRNAs expression profiles of ischaemic stroke patients, total RNA from peripheral blood was extracted and the relative expression profiles of circulating microRNAs were generated after converting the RNAs into cDNAs by methods in section 3.9 and the copy number of the transcripts of interest were successfully quantified using Real-time quantitative PCR as described in section 3.10.

Figure 4.6 – 4.11 depict the six microRNAs relative expression profiles and tabulated in **Table 4.3** is the fold change values (calculated upon equation [9] as described in section 3.11.2) to the corresponding circulating microRNAs of each patient. The blood from young ischaemic stroke patient revealed significant dysregulation in circulating microRNA-145, -222, -223, and -23b expression profiles at $p = 0.022$, $p = 0.011$, $p = 0.028$ and $p = 0.040$, respectively as compared to control.

Nevertheless, expression of circulating microRNA-214 and -339 were shown not to have ectopic profiles in young ischaemic stroke patients at $p = 0.780$ and $p = 0.436$, respectively. This is because both microRNAs demonstrated almost equal number of those up and down regulated expression of the microRNA, although the individual expression of respective microRNA was significantly different as compared to the control. This observation suggests that these microRNAs might be differently expressed in relative to the control with large interindividual variability.

Thus, results revealed four out of the selected six circulating microRNAs expression in patients, compared to that of control, demonstrated significant altered expression profiles.

4.9. Paired expression profiles of studied circulating microRNAs in ischaemic stroke patients

Peripheral blood was successfully recollected adhering to protocol described in section 3.2.5 and circulating microRNAs (microRNA-145, -214, -222, -223, -23b and -339) relative expression profiles were subsequently generated as described from section 3.4 to section 3.11 in order to compare the profiles after a period of time.

Depicted in **Figure 4.12 – 4.17** are the respective circulating microRNAs expression profiles and fold change values of first collection and the subsequent collection. The results unveil plummet of circulating microRNAs expressions in almost every patient after a period of time. However, only circulating microRNA-214, -222, -223 and -23b marked a significant downregulation of expressions at $p = 0.004$, $p = 0.038$, $p = 0.048$ and $p = 0.016$, respectively. The downregulation of circulating microRNA-145 and -339 was not significant at $p = 0.055$ and $p = 0.058$, respectively.

4.10. Correlation study of microRNAs expression

MicroRNAs expression profiles were studied and compared, and depicted in **Figure 4.18** are the expression profiles of circulating micrRNAs that show congruency. The figure shows the relative expression pattern of circulating microRNA-145, -222, -223, -23b and -339 shared a congruent expression profile, where the circulating microRNAs expression profile of individual patient agrees with the other microRNAs; leaving circulating microRNA-214 with its unique expression profile. Hereby, the expression profiles can be seprerated into two groups of expression pattern.

In order to determine the relationship between these studied circulating microRNAs relative expression profiles, correlation analysis was carried out using Pearson correlation analysis (SPSS for Windows, Version 11.5). Tabulated in **Table 4.4.1** and **Table 4.4.2** are the Pearson correlation values for the six circulating microRNAs studied. The relationships are also depicted in the form of scatter plots (**Figure 4.19 – 4.24**).

Strong positive correlations in the studied circulating microRNAs relative expressions except for circulating microRNA-214 were observed. Moreover, these positive correlations were significant at < 0.05 level (**Table 4.4.1**).

4.11. Pathway analysis

All six circulating microRNAs were analyzed by comparing their possible targets in all known KEGG pathways as described in section 3.12. **Table 4.5.1** to **Table 4.5.6** list the predicted pathways and target genes to be affected respective to the expression of the studied microRNAs (microRNA-145, -214, -222, -223, -23b, and -339).

The different altered expressions of circulating microRNAs appear to be involved in cell growth, immune responses, cell survival, maturation and proliferation. Amongst the predicted pathways, there were similar key biological pathways that are affected by microRNAs studied including MAPK signaling, focal adhesion, mTOR signaling, cytokine-cytokine receptor interaction, ECM-receptor interaction VEGF signaling pathway and etcetera. Taking together the findings from correlation (**Table 4.4.1 and Table 4.4.2**) and pathway analyses (**Table 4.5.1** to **Table 4.5.6**), these circulating microRNAs may function in synergy.

4.12. Expression of microRNA in subtypes of ischaemic stroke

The expression of microRNAs were expressed in mean fold change ± S.E.M according to the subtype of ischaemic stroke as depicted in **Figure 4.25**. Of the 32 ischaemic stroke patients, 11 of them were classified as large vessel, 9 were classified as small vessel, 2 were classified as cardioembolic, 3 were classified as other determined and 7 were classified as undetermined subtypes of ischaemic stroke.

The expression of circulating microRNA-145 of large and small vessel subtypes showed a significantly different expression from that of cardioembolic and other determined subtypes of ischaemic stroke (**Figure 4.25 B**). Expression of circulating microRNA-145 of large vessel subclass was significantly different from cardioembolic and other determined subtypes of ischaemic stroke with p value of 0.018 and 0.024, respectively. Meanwhile, expression of circulating microRNA-145 of small vessel subtype was significantly different from cardioembolic and other determined subtypes of ischaemic stroke with p value of 0.047 and 0.048, respectively.

The expression of circulating microRNA-223 of small vessel subtype showed a distinguish expression from that of cardioembolic and subtype of ischaemic stroke (**Figure 4.25 C**). The different expressions of circulating microRNA-223 between the small vessel and cardioembolic subtypes were significant with the p value of 0.011.

The expression of circulating microRNA-23b of cardioembolic subtype demonstrated a significantly different expression from that of small vessel and undetermined subtypes of ischaemic stroke (**Figure 4.25 E**). Expression of circulating microRNA-23b of cardioembolic subtype was significantly distinguishable from small vessel and undetermined subtypes of ischaemic stroke with p value of 0.003 and 0.042, respectively.

The expression of circulating microRNA-339 of small vessel subtype demonstrated a significantly different expression from that of cardioembolic and other determined subtypes of ischaemic stroke (**Figure 4.25 G**). Similar to the expression of microRNA-145, expression of circulating microRNA-339 of small vessel subtype was significantly different from cardioembolic and other determined subtypes of ischaemic stroke but with p value of 0.02 and 0.028, respectively.

On the other hand, the expression of circulating microRNA-214 and -222 demonstrated similar expression profile with insignificant differences across all subtypes of ischaemic stroke (**Figure 4.25**).

5. Discussion

5.1. Real-time quantitative PCR technique and RNA analysis

Real-time quantitative PCR is an established technique for RNA quantification and recognized as the gold standard for the validation of microarray data (Canales *et al.*, 2006). This state-of-the-art technology delivers higher sensitivity, larger dynamic range, has potential for higher throughput analysis and accuracy in the quantification of RNA compared to the conventional techniques (Thellin *et al.*, 2009; VanGuilder *et al.*, 2008). However, similar to every technology, there remains some limitations associated to its application. These include the inherent variability of RNA, variability of extraction protocols, and different reverse transcription and PCR efficiencies (Bustin, 2000). Therefore, recommended precautions and standardization have been practiced throughout the study in control of the aforesaid limitations.

Ensuring a uniform sample size, by sampling standardization, is the first recommendation to minimize experimental error. As human peripheral blood was used in this study, it would be rather difficult to ensure uniform cellular material obtained due to interindividual variations of the cell count per ml of blood (Huggett *et al.*, 2005). This may also be the underlying reason for the difference of RNA concentration yielded between whole blood sample collection with and without the addition of sodium EDTA prior RNA stabilization (section 4.5, **Figure 4.3**). It is noteworthy that the RNA concentration between these two cohorts of sample shared similar range of RNA concentration per volume. Nevertheless, absence of sodium EDTA in whole blood prior to RNA stabilization does not affect the RNA quality (section 4.5, **Figure 4.2**). Therefore, it would be misleading to directly perform comparison based on

sample volume alone. In order to make an accurate comparison, quantity of the RNA yielded was standardized instead of volume-based.

Gel electrophoresis (denaturing agarose gel electrophoresis and polyacrylmide gel electrophoresis) was employed as ancillary tests to confirm the RNA integrity and extraction purity ensuring only excellent quality of total RNA used in this study. RNA concentration of 10 ng/µl was standardized as starting concentration prior to reverse transcription and 1.33 µl of the product was standardized for the PCR reaction. Ribonucleic RNA, 18S rRNA was used as normalization reference. In order to minimize reagent efficiency variation, all enzymes and chemicals were ensured to be the same and respective recommended protocols were stringently adhered to.

5.2. Expression of circulating microRNAs in chronic ischaemic stroke patients

This experiments were conducted with the aims to study the expression of microRNA-145, -214, -222, -223, -23b and -339 in chronic ischaemic stroke patients and its expression in the event of long-term stroke by subsequent sampling of blood at a later time point. Findings from this study will serve as a reference and contribution to the knowledge pertaining these circulating microRNAs in chronic ischaemic stroke. In this study, we report that circulating microRNAs were differently expressed in chronic stroke patients as compared to control and the expression normalized/reduced after some time form the initial profile. The different altered expression profile in chronic stroke patients was supported with findings from the study conducted by McKenzie and co-workers (McKenzie *et al.*, 2008).

Study acquiring the quantification of the number of circulating endothelial progenitor cells were reported to be affected in chronic stroke patients when compared to the number of circulating endothelial progenitor cells found in healthy control (Chu *et al.*, 2008; Ghani *et al.*, 2005). In both studies, the authors reported that the number of circulating progenitor cells were significantly reduced in both acute and chronic stage of ischaemic stroke and the difference between acute and chronic stage was insignificant. This strongly suggests that changes in the pathophysiology of ischaemic stroke are still present in chronic stroke condition and this may account to the changes in microRNA expression.

5.2.1. Circulating microRNA-145 in chronic ischaemic stroke

The upregulation of circulating microRNA-145 expression shown in this study (**Figure 4.4.A**) is consistent with our previous report and it suggests positive reendothelialization, a vascular homeostasis response following cerebral ischaemia. Therefore, H_0 1 is rejected and H_A 1 is accepted. The peripheral blood microRNA-145 detected might be derived from the pool of circulating progenitor cells that are in commitment to differentiate into VSMC lineage as described in the literature. The fall in the upregulated circulating microRNA-145 expression may suggest completion of vascular regeneration achieving homeostatic equilibrium, however in this study the downregulation was shown to be insignificant (**Figure 4.5.1**). This might be due to the limitations of the study that only a portion of the patients agreed to be sampled for the second time or the time point of the second collection was too parsimonious for effective postnatal vasculogenesis that is affected by individual risk factors (Dimmeler *et al.*, 2004). Therefore, H_0 2 is accepted.

As one of the extensively studied microRNAs, compelling evidences provide the insight of microRNA-145 role in regulating smooth muscle cells' fate and plasticity, posttranscriptionally (Cheng *et al.*, 2009; Cordes *et al.*, 2009). Studies using loss-of-function and gain-of-function experimental designs unveiled the inverse correlation expression of microRNA-145 and its mRNA targets, KLF4 and KLF5 proteins. Understanding that KLF4/5 regulation in abrogating transcriptional cofactor myocardin, which has been shown to be responsible in maintaining SMC differentiation (Long *et al.*, 2008). High expression of KLF4/5 proteins will consequently promote its competitor E twenty-six-like transcription factor 1 (Elk-1) transcriptional cofactor in activating the transcription factor SRF. This will guide the VSMC to undergo dedifferentiation and subsequently proliferate.

Therefore, high expression level of microRNA-145 (as shown in VSMC) will render down modulation of its targets KLF4/5, thus myocardin will not be suppressed and will form complex with SRF that eventually potentiate the transcription of the genes essential to guide VSMC differentiation and maintain differentiated. Indeed, microRNA-145 is shown sufficient for directing the VSMC fate from multipotent stem cell. *In vitro* introduction of microRNA-145 into neural crest stem cells demonstrated approximately 75% of cells propitiously differentiate into VSMC (Cordes *et al.*, 2009).

In line with this, postnatal neovascularization has been shown to be no longer an attribute to angiogenesis only. Postnatal vasculogenesis has also been described to involve in vascular homeostasis. This process involved in the recruitment and incorporation of precursor cells that have been revealed to circulate postnatally in the peripheral blood to the injured and ischaemic sites (Luttun *et al.*, 2002a; Luttun *et al.*,

2002b). This compliments the findings that demonstrated reduction of progenitor cells in the peripheral blood as the cells are stimulated and recruited to incorporate to the ischaemic site (Chu *et al.*, 2008; Ghani *et al.*, 2005). Our result suggests the continuation of vascular resilience in the stable stage of stroke. As abovementioned, bone marrow-derived cells including circulating endothelial precursor cells were shown to aid the process of reendothelialization. In addition, common vascular progenitor and smooth muscle cell progenitor have been shown to potentially participate in postnatal vasculogenesis, hence extending the pool of circulating multipotent precursor cells in vascular regeneration (Dimmeler *et al.*, 2004; Luttun *et al.*, 2002b).

In this study, peripheral blood derived circulating microRNA-145 expression was significantly higher in chronic ischaemic stroke patients than in control subjects. This demonstrates that homeostatic mechanisms were affected by ischaemic stroke. This may leave implications for the development of the microRNA as biomarker for ischaemic stroke. However, since peripheral whole blood was acquired in this study, identification of the source of upregulated microRNA-145 would reveal cellular physiology following ischaemic stroke.

5.2.2. Circulating microRNA-214 in chronic ischaemic stroke

The dysregulation of circulating microRNA-214 in chronic ischaemic stroke patients was not significant (**Figure 4.4.B**); nevertheless the plummet in microRNA expression was significant in the second sampling compared to the first (**Figure 4.5.2**). Therefore, H_0 1 is accepted and H_A 2 is accepted in rejection of H_0 2. Upregulation of circulating microRNA-214 might indicate active proliferation of T-cells, while the plummet/normalization of circulating microRNA-214 expression might indicate the post-stroke mechanisms of immune system homeostasis via reinforcing PTEN negative regulation of AKT signaling pathway balancing the recruitment of costimulators during T-cell activation. This might subsequently regulate T-cells proliferation rate, which would restore the normalization of T-cells count. This is because upregulation of microRNA-214 expression was shown to increased proliferation of T-cell upon stimulation (Jindra *et al.*, 2010). The increase was demonstrated to be the consequence of PTEN function in negative regulation of AKT signaling pathway, which is normally activated in the presence of stimulator (Acuto *et al.*, 2003; Buckler *et al.*, 2008).

The change of T-cells count was documented after stroke onset (Vogelgesang et al., 2008). Significant deprivation of CD4+ and CD8+ T-cells were found in the ischaemic stroke patients in relative to control subjects. The drop of T-cells count was reported to normalize in a temporal fashion during the rehabilitation after stroke onset. Event of lymphocytopenia was supported by study acquiring murine ischaemic model (Prass *et al.*, 2006). Ischaemic stroke model demonstrated increased susceptibility to bacterial infections, while the sharm controls were not susceptible to infection even with the highest number of bacteria inoculation. This phenomenon therefore, underlines the attribution of ischaemic stroke to immune system and was validated when the increased

susceptibility to bacterial infection was abrogated when the suppression of immune responses halted (Prass *et al.*, 2006).

As such, the study results of peripheral blood derived circulating microRNA-214 expression that was revealed to reduce significantly in a temporal fashion (during the second sampling) might suggest the normalization of microRNA-214 expression as a feedback mechanism to regulate the proliferation rate of T-cells in achieving balanced homeostasis of the immune system. However, quantification of T-cells of ischaemic stroke patients as compared to the control subjects needs to be repeated. And the quantification of microRNA-214 expression of the T-cell isolates from ischaemic stroke patients and control subjects are needed to affirm their association.

5.2.3. Circulating microRNA-222 in chronic ischaemic stroke

The ectopic regulation of circulating microRNA-222 (**Figure 4.4.C**) might indicate the regulation of the erythropoesis compartments as microRNA-222 expression level has been shown to be abundant in haematopoietic progenitor cells and its level gradually and markedly declines during erythropoiesis differentiation/maturation (Felli *et al.*, 2005). Therefore, H_0 1 is rejected and H_A 1 is accepted. The decline of microRNA-222 expression level will unblock c-Kit protein production posttrascriptionally, thus leading to the expansion of early erythroblast link to prevalent fetal hemoglobin (HbF) synthesis during stress erythropoiesis (Gabbianelli *et al.*, 2010).

Downregulation of microRNA-222 in erythroid progenitor cells has shown to increase erythroid proliferation rate and reactivating HbF synthesis of approximately 50% via the c-Kit protein activation while vise versa was observed in erythroid progenitors over expressing microRNA-222 (Gabbianelli *et al.*, 2010). Therefore, those patients that demonstrated upregulation of circulating microRNA-222 expression (**Figure 4.4.C**) might be at unfavorable condition as upregulation of microRNA-222 expression retards progenitor cell recruitment that aids angiogenesis, as well as inhibiting normal erythropoiesis and/or halt HbF reactivation by blocking c-Kit protein translation.

However, it is also noteworthy that the microRNA-222 expression in erythropoesis compartments may be masked by the upregulation of this microRNA in myeloid compartments upon maturation, despite their abundance in haematopoietic progenitor cells (Brioschi *et al.*, 2010).

Despite the dysregulation, plummet in the circulating microRNA-222 expression at the second sampling as compared to the first sampling observed in this study (**Figure 4.5.3**) might derive from the net increase of microRNA-222 repression in the erythropoesis compartments. Therefore, H_0 2 is rejected and H_A 2 is accepted. The repression of microRNA-222 expression in the erythropoiesis compartments will unblock the c-Kit protein translation, thus increasing erythroid proliferation rate and reactivating HbF synthesis. This phenomenon may be due to homeostatic mechanotrusduction following chronic ischaemia.

Moreover, the recruitment of progenitor cells that would differentiate into vascular endothelial cells by the preexisting circulating endothelial cell precursor as abovementioned is mediated by SCF/c-Kit interaction. The activation of SCF/c-Kit signaling pathway has been shown to promote the survival, migration and capillary tube formation of endothelial cells (Matsui *et al.*, 2004). This suggest the regulation of c-Kit expression that is important for the recruitment of progenitor cells during the vascular resilience still occurs in chronic stage of ischaemic stroke.

Nevertheless, in order to have a better insight of peripheral blood derived microRNA-222 expression profile and to determine if its expression in erythroid lineage is being masked by the expression level in those myeloid lineage cells, the expression of microRNA-222 in peripheral blood TER119+ (erythroid lineage) cells and CD11+ (myeloid lineage) cells from both ischaemic stroke patients and control subjects should be compared. In addition, c-Kit receptor protein expression should be compared from these pure TER119+ and CD11+ cell isolates. Understanding the expression level of microRNA-222 and c-Kit protein can lead to in-depth study designs in developing microRNA-222 biomarker.

5.2.4. Circulating microRNA-223 in chronic ischaemic stroke

Notably, microRNA-223 expression is confined to myeloid lineages (Chen *et al.*, 2004). It is shown to act as a "fine-tuner" of granulocyte production and the inflammatory response via Mef2c posttranscriptional regulation (Johnnidis *et al.*, 2008). Augmentation in microRNA-223 expression was observed with granulocytes maturation, sustained during monocytes maturation, while extinguished during erythropoiesis (Chen *et al.*, 2004; Felli *et al.*, 2009; Johnnidis *et al.*, 2008). Therefore,

the upregulation of circulating microRNA-223 in this study might mark the biology of myeloid lineages and those demonstrating downregulation of circulating microRNA-223 expression might be the consequence of the overriding erythropoiesis compartments commitment (**Figure 4.4.D**). H_0 1 is rejected and H_A 1 is accepted. Moreover, this expression profile was in agreement with the expression profile of microRNA-222 (section 5.2.3) as the expression profiles were in congruency (**Figure 4.6** and **Figure 4.7.3.b**) and positive correlation, r = 0.928 (**Table 4.4.1**). Therefore, this strongly suggests that the myeloid compartment contributed to the upregulation of microRNA-222 in peripheral blood.

In line with this, expression of microRNA-223 showed inverse correlation with the expression of LMO2 protein-regulator of haematopoietic stem cell development and erythropoiesis. LMO2 activation in multipotent progenitor cells resulted in increased erythropoiesis, while LMO2 is not detected in mature myeloid lineages (Chen *et al.*, 2004). This has again suggested that the upregulated circulating microRNA-223 in this study may might myeloid compartments commitment. Indeed, microRNA-223 knock out mice models exhibited enhanced oxidative burst compared to wild type granulocytes. Hypersensitive neutrophiles are produced as a consequence of micoRNA-223 deletion. As low as 1 ng/ml of stimulus was able to elicit almost a 70% increase in superoxide production. Histological examination showed inflammatory lung pathology characteristics in microRNA-223 knock out mice, further demonstrating the hypersensitive neutrophile activity (Johnnidis *et al.*, 2008).

Therefore, the upregulated circulating microRNA-223 (**Figure 4.4.4**) observed in this study might postulate myeloid lineage commitment regulation and in prevention of the hypersensitive neutrophile production, which would lead to autoimmune and inflammatory responses. Thus, the significant plummet of circulating microRNA-223 observed in the second collection compared to the first (**Figure 4.5.4**) might reflect the homeostatic regulation of the myeloid lineage towards a better stroke outcome. Therefore, H_0 2 is rejected and H_A 2 is accepted. This suggests the homeostasis of immune response still occurs in chronic stage of ischaemic stroke.

However, future studies involving the isolation of peripheral blood mononuclear cells or even the CD18+ blood cells (neutrophile) of ischaemic stroke patients and control subjects need to be compared for their sensitivity towards commercial activating stimuli such as N-formyl-methionine-leucine-phenylamine and at the same time quantifying the microRNA-223 expression to validate current results.

5.2.5. Circulating microRNA-23b in chronic ischaemic stroke

Altered expression of circulating microRNA-23b observed in this study might mark the haematopoietic homeostasis during chronic stroke. As high level of microRNA-23b is correlated to high level of hypophosphorylated retinoblastoma protein (Wang *et al.*, 2010), was shown to be important in monocyte differentiation (Bergh *et al.*, 1999), the profile of circulating microRNA-23b shown (**Figure 4.4.E**) might indicate exclusive commitment of monocyte maturation. The result also rejects H_0 1 and accepts H_A 1. This might indicate recovery because monocyte was shown to be beneficial towards angiogenesis and arteriogenesis as it can differentiate into vascular endothelial cell, in addition to its ability of secreting angiogenic factors (Carmeliet, 2003).

MicroRNA-23b was revealed to play a role in response to flow regulation and endothelial cell growth (Wang *et al.*, 2010). The expression level of microRNA-23b was shown to be associated with the phosphorylation of retinoblastoma protein. It was demonstrated that under pulsatile flow, the level of microRNA-23b was upregulated in endothelial cell, resulting to growth arrest, as compared to static condition. The study suggested that the expression level of microRNA-23b causes cell growth arrest via the downregulation of E2F family of transcription factor proteins and retinoblastoma hypophosphorylation. Because the activation of E2F family proteins was shown to be governed by retinoblastoma protein (i.e. in hypophosphorylated or hyperphosphorylated condition) and their molecular pathway involving microRNA-23b remains elusive, it was postulated that the downregulation of E2F family proteins was an indirect cause of retinoblastoma hyperphosphorylation.

Despite its notorious role as the cell cycle clock gatekeeper, retinoblastoma protein was found to modulate cell differentiation and development (Korenjak *et al.*, 2005). Noteworthy, high level of retinoblastoma protein was found induced and sustained during erythroid maturation, while the level was suppressed during granulocytic differentiation (Condorelli *et al.*, 1995). Moreover, mechanism involving PU.1 transcription factor protein and retinoblastoma protein has been described during monocyte differentiation (Valledor *et al.*, 1998). This demonstrates that during monocyte maturation, hypophosporylated retinoblastoma protein (correlated to upregulation of microRNA-23b) sequestrated the inhibition of PU.1 transcription factor by binding to the inhibitor, therefore transactivating the PU.1 activity and allowing the transcription of genes required during maturation (Bergh *et al.*, 1999). These

mechanisms were in agreement with the microRNAs congruent expression profile (**Figure 4.6**).

On the other hand, the profile of circulating microRNA-23b shown in our study might also reflect the erythropoiesis compartments. This was reasoned after *in vivo* model incorporating phenylhydrazine inducing hemolytic anemia (mimicking stress erythropoiesis) had demonstrated vital activity of retinoblastoma protein during stressed erythropoiesis. The absence of retinoblastoma protein activity (correlated with the downregulation of microRNA-23b) was shown to confer advantage on erythroblast expansion under stress condition but the erythroblasts failed to enucleate due to defects in terminal differentiation, failing to promote normal maturation of erythroblast (Spike *et al.*, 2004). This had indeed demonstrated the requirement of retinoblastoma protein regulation of end stage erythrocytes differentiation under stress condition.

Taken these together, the significant plummet in the circulating microRNA-23b expression in the second collection (**Figure 4.5.5**) might indicate the balance regulation of postnatal vasculogenesis reaching homeostasis, corresponding to the biochemical changes in chronic cerebral ischaemia. Therefore, H_0 2 is rejected and H_A 2 is accepted. As for future studies, the correlation between microRNA-23b expression and the level of hypophosporylated retinoblastoma protein in erythroid (c-Kit- and TER119^{Hi}) and monocyte (CD34+) can be repeated. And comparison of the expression level of microRNA-23b and the phosphorylation level of retinoblastoma protein in erythroid and monocyte between ischaemic stroke patients and control subjects can reveal the homeostatic mechanism involving the expression of microRNA-23b following ischaemic stroke.

5.2.6. Circulating microRNA-339 in chronic ischaemic stroke

Current literature dictates the role of microRNA-339 and -222 in regulating the protein expression of ICAM-1, constituent of immunoglobulin gene superfamily. Functional test using luciferase reporter assays revealed the inverse correlation of ICAM-1 protein with the microRNAs, demonstrating congruent expression profile (Ueda *et al.*, 2009). Coherent with the findings, this study revealed correlation ($r = 0.962$) in microRNA-339 and -222 expressions (**Table 4.4.1**).

The study result of circulating microRNA-339 revealed a rather interesting expression profile. Of the 32 patients sampled, 50% demonstrated upregulation of microRNA-339 in the circulation, while 31% demonstrated downregulation of the same microRNA. However, the expression profile was insignificantly altered as compared to the control and the second sampling (**Figure 4.4.F** and **Figure 4.5.6**), in line with H_0 1 and H_0 2. With the elucidation of microRNA-339 in regulating ICAM-1; the results were reasoned to be in agreement with the literature dictating that expression of ICAM-1 was ectopic in ischaemic stroke patients with large interindividual variability (Frijns *et al.*, 2002), hence the insignificant observation.

ICAM-1 is a member of the immunoglobulin gene superfamily proteins that have been extensively studied and well characterized. Its role in promoting firm adhesion of leukocytes to the endothelial cell during an event of vascular insult was described. Noteworthy, of the 5 CAMs, only ICAM-1 and VCAM-1 can be stimulated in increasing the protein expression (Frijns *et al.*, 2002). Cells with downregulated ICAM-1 protein (upregulation of microRNA-339/-222) demonstrated low susceptibility towards leukocyte adhesion and activation (Ueda *et al.*, 2009).

Besides SCF/c-kit interaction, ICAM-1/CD18 interaction was also shown to be essential in mediating endothelial progenitor cells (EPC) recruitment during postnatal vasculogenesis (Wu *et al.*, 2006). Interception of ICAM-1/CD18 interaction with neutralizing antibody was shown to thwart EPC recruitment at ischaemic site by 95%. The blockade of ICAM-1 receptor will consequently lead to postnatal neovascularization without postnatal vasculogenesis and depending solely to the attribution of angiogenesis, which has been shown insufficient (Dimmeler *et al.*, 2004). These phenomenons were reflected in the clinical trial using enlimobab administration, the anti-ICAM-1 antibody, which resulted in adverse outcome ("Use of anti-ICAM-1 therapy in ischemic stroke: results of the Enlimomab Acute Stroke Trial," 2001). Taken all these together, peripheral blood derived microRNA-339 will not be a good candidate as biomarker.

5.3. Correlation of microRNA expression profiles

Results of the circulating microRNA expression profiles demonstrated congruent expression profiles except for circulating microRNA-214 (**Figure 4.6**). Within those microRNAs that showed congruency in their expression, their profile was indeed strongly correlated to each other with $p < 0.05$ when tested with Pearson Correlation analysis (**Table 4.4.1**), while microRNA-214 showed insignificant correlation with any of the microRNAs studied (**Table 4.4.2**). Furthermore, *in silico* analysis of the biological effects that are possibly regulated by each microRNA resulted in similar outcome of pathways involved (**Table 4.5.1** to **Table 4.5.6**). The pathways predicted include MAPK, focal adhesion, mTOR and VEGF signaling pathways. These signaling pathways are those highly conserved pathways that are involved in a plethora of

cellular functions, including cell proliferation, differentiation and migration (Nishida *et al.*, 1993; Zhang *et al.*, 2002).

The expression profile of strongly correlated microRNAs, namely microRNA-145, -222, -223, -23b and -339, and with the similar outcome of pathways that each microRNA would possibly regulate; these microRNAs were deduced to be working together in regulating similar responses. It is noteworthy, however, in this study the circulating microRNA-339 expression profile of ischaemic stroke patients was not significantly different from the control and the second collection, therefore its expression profile will not be discussed further. Nevertheless, these findings suggest supporting relationship to the 'cause-effect' logic employed pertaining to vascular physiology and ischaemic stroke. It is noteworthy that the deductions were in line with the current literature review with regard to the targets and pathways involved.

Figure 5.1 illustrates the relationship of the microRNAs in response to ischaemic stroke. Those microRNAs that were congruently expressed (microRNA-145, -222, -223 and -23b) were deduced to cooperatively mediate postnatal vasculogenesis; while the expression of microRNA-214 that showed a unique profile as compared to the other microRNAs studied, was inferred to regulate T-cells activation and infiltration, a different protective response following stroke.

In this study, circulating microRNA-145 dysregulation was inferred to correspond to its role in the regulation of postnatal vasculogenesis by directing the circulating vascular progenitor cells to differentiate into mature VSMC (section 5.2.1). This response of mechanotransduction that involved the regulation of KLF4/5, myocardin and Elk-1 was shown to signal via MAPK signaling pathway (Li *et al.*, 2010; Tamama *et al.*, 2008).

Circulating microRNA-23b that was also inferred to regulate postnatal vasculogenesis, not by directing VSMC differentiation but by mediating the differentiation of monocytes was shown to play a role in postnatal vasculogenesis by inducing reendothelialization process of the vascular wall (section 5.2.5). The mechanotransduction involved in the regulation of retinoblastoma protein phosphorylation via MAPK signaling pathway in determination of monocytic lineage from the progenitor cells were previously described (Bergh *et al.*, 1999; Chen *et al.*, 2003; Ji *et al.*, 2002).

Moreover, circulating microRNA-223 expression was inferred to regulate the myeloid lineage commitment. This commitment would include the differentiation of progenitor cells into monocytes (section 5.2.4). MicroRNA-223 was shown to regulate Mef2c transcription factor in confining myeloid lineage expansion; and the regulation of Mef2c activity was shown to be governed by MAPK signaling pathway (Khiem *et al.*, 2008; Olson, 2004).

Meanwhile, circulating microRNA-222 that was delineated to regulate the expression of c-Kit protein may mediate the recruitment of both endothelial and vascular progenitor cells to home in at the infarct site, where the agonist will be abundantly expressed (section 5.2.3). The c-Kit signaling pathway was shown to be mediated by MAPK signaling pathway (Matsui *et al.*, 2004; Wandzioch *et al.*, 2004).

On the other hand, the circulating microRNA-214 expression that showed insignificant correlation to other microRNAs studied was inferred to respond in a different mecahotransduction in mediating vascular recovery. It is inferred that the circulating microRNA-214 expression profile reflects the T-cell activity (section 5.2.2). Having the capability to negatively regulate PTEN, downregulation of circulating microRNA-214 might indicate the suppression of T-cell proliferation in mitigating the accentuation of T-cell infiltration at infarct site via regulating AKT signaling pathway (Acuto *et al.*, 2003; Buckler *et al.*, 2008; Jindra *et al.*, 2010).

5.4. Expression of microRNA in subtypes of ischaemic stroke

The comparison of microRNAs between the subtypes of ischaemic stroke revealed that the expression could be differed between the different subtypes of ischaemic stroke. Interestingly, the expression level of circulating microRNA-145, -223, -23b and -339 of cardioembolic subtype were significantly different from small vessel subtype of ischaemic stroke. This observation was similar to the current literature that dictates the unique expression was shown in cardioembolic subtype as compared to atherothrombotic aetiology (Jickling *et al.*, 2010; Montaner *et al.*, 2008; Xu *et al.*, 2008).

Genes regulated during an onset of cardioembolic subtype of ischeamic stroke was suggested to be involved in response to pathogens, including immune cell activation, defense response, proliferation and apoptosis (Xu *et al.*, 2008). And the detected ectopic genes expressions in cardioembolic subtype were mainly contributed by neutrophile, while the differently regulated genes expressions in atherothrombotic aetiology of ischaemic stroke were contributed by monocyte (Xu *et al.*, 2008). This was in support to our results, where the upregulation of circulating microRNA-145, -223, -23b and -339 were observed in large and small vessel subtypes of ischaemic stroke and their involvement were postulated to play a role in monocytic lineage. In contrast, these circulating microRNAs were expressed at a basal level in cardioembolic subtype of ischaemic stroke (**Figure 4.25 A**).

Results also demonstrated that the expression of circulating microRNA-23b of cardioembolic subtype was significantly differed from the undetermined subtype of ischaemic stroke (**Figure 4.25 E**). The expression profile also demonstrated that the undetermined subtype was similar as to large and small vessel subtypes of ischaemic stroke (**Figure 4.25 A**). Hence it is plausible to suggest that the samples with an undetermined aetiology might have resulted from atherothrombotic aetiology of ischaemic stroke. Thus, our results have demonstrated the possibility of microRNA expression to aid the prediction of the causes of ischaemic stroke. However, in order to address the issue of reproducibility, these observations needs to be repeated and validated in a larger cohort prior any definite conclusion being made.

5.5. Overview of mechanotransduction after stroke

At times of stroke, changes in blood flow that implicate an alteration of the pulsating sheer stress appear to be the key event in determining vascular homeostasis response. Specific molecules and chemoattractant molecules from the endothelium, in particular those responsible for the adherence, migration, and accumulation of leukocytes, will be stimulated, subsequently, leading to the accentuation of receptors and chemoattractants production on cell (endothelial) surfaces. Notably, 3 families of molecules have been identified to be responsible, namely the selectins, the immunoglobulin gene superfamily, and the intergrins (Carlos *et al.*, 1994). Expression of microRNA-222 and -339 were associated with the expression of ICAM-1 (section 5.2.6).

The infiltration starts with the rolling and adherence of circulating inflammatory cells, especially the monocytes and T-cells that take place on site as a result of ligand-receptor interaction. This interaction was shown to be mediated by the selectins. Mediated by the elevated receptors of the immunoglobulin gene superfamily (that promotes stronger attachment as compared to selectins), these inflammatory cells would firmly adhere and be activated (Carlos *et al.*, 1994). Activation of the inflammatory cells will be further accentuated by the upregulation of receptors, which include intergerins on their surfaces. As a result, inflammatory cell activation was exacerbated followed by proliferation to elicit local inflammatory response (Carmeliet, 2003; Ross, 1999). Expression of microRNA-214 and -23b were shown to be associated with circulating inflammatory cells proliferation (sections 5.2.2 and 5.2.5).

Ostensibly, infiltration of circulating inflammatory cells was reasoned to elicit secondary injury after acute ischaemic infarction. This theorem was formed based on the observations of circulating inflammatory cells infiltration after ischaemia causes injury such as microvessel occlusion (del Zoppo *et al.*, 1991), release of oxygen radicals, cytolytic proteases and proinflammatory cytokines (Hartl *et al.*, 1996). Coherent with the report, exacerbated circulating inflammatory cell count in acute stage was associated with relatively poor outcome and even deleterious (Pozzilli *et al.*, 1985; Yoon *et al.*, 2005). Moreover, line of studies also demonstrated neutropenia condition suppresses ischaemic injury (Frijns *et al.*, 2002).

Dispute to the theorem arises when compelling studies demonstrated evidences suggesting alternative physiology. Occurrence of the infiltration of circulating inflammatory cells was suggested not to be the contributing factor, but it was claimed as the result of the cerebral infarct (Emerich *et al.*, 2002). This alternative theorem was supported by the observation that the transactivation of the circulating inflammatory cells did not exacerbate the infarct volume, but occurred as a more delayed reaction to the infarct's formation (Hayward *et al.*, 1996).

Studies conducted to quantify the neutrophile infiltration and infarct formation between 6 and 168 hours of ischaemic stroke model revealed that small numbers of neutrophiles were recorded at affected tissue between 6 to 12 hour after occlusion. At the 12th hour after occlusion, mere maximal cell damage was exhibited, that was when neutrophile accumulation was still relatively low. Noteworthy, further observation unravels the maximum neutrophile accumulation to occur between 21st to 48th hour, much later after maximal damage was observed (Hayward *et al.*, 1996; Zhang *et al.*, 1994). These

observations have thus suggested that neutrophile accumulation did not contribute to the development of the infarct but was a homeostatic process of reendothelialization.

Reendothelialization is a regeneration process described after vascular injury (Dimmeler *et al.*, 2004). The first paradigm of this recovery process was attributed to the migration and proliferation of neighboring endothelial cells. However, it was found later that the rate and number of neighboring cells proliferating was low (Caplan *et al.*, 1973). Subsequent animal studies unveiled that additional repair mechanisms exist during reendothelialization. Bone-marrow-derived cells from CD34+ haematopoietic stem cells, immature CD133+ haematopoietic stem cells, and circulating endothelial precursor cells were shown to differentiate and home into the denuded parts of injured vessel aiding regeneration (Peichev *et al.*, 2000; Shi *et al.*, 1998; Walter *et al.*, 2002). The expression of microRNA-145 in neural crest stem cells propitiously caused differentiation of VSMC (section 5.2.1); while expression of microRNA-222 and -339 that are associated with the membrane protein receptors may play some roles in the process (sections 5.2.2 and 5.2.6).

Astonishingly, circulating inflammatory cells were also shown to be proangiogenesis such that they were able to release angiogenic factors or transdifferentiate into endothelial-like cells, expending the source of circulating endothelial progenitor cells that function as an additional repair mechanism. For instance, platelets derived from haemangioblast progenitor cells mediate the production of angiogenic factors and were shown capable of differentiating into endothelial-like cells in response to environmental cues (Carmeliet, 2003). Similarly, monocytes were also shown to participate in angiogenesis by having the capability to differentiate into endothelial-like cells, besides

their ability to secrete growth factors (Rehman *et al.*, 2003; Schmeisser *et al.*, 2001). Expression of microRNA-23b was associated with cell proliferation and myeloid lineage differentiation (section 5.2.5) and similarly, expression of microRNA-222 and -339 were associated with the membrane protein receptors that contribute to the process (sections 5.2.2 and 5.2.6).

Therefore, it is suggested that homeostasis involves an intricate mechanism of balancing of injury and repair in response to the myriad of biochemical changes that include the regulation of microRNA during an onset of the disturbance.

5.6. Study limitations

This study acquired subjects from those admitted via the neurology services at the UMMC, although the peripheral blood samples were obtained during their follow ups. A lot of time and patience was required as the enrollment of patient was on voluntary basis. Due to the same factors, low number of patients was enrolled in this study. More time will be needed in order to obtain a higher number of young ischaemic stroke patients. Failure of the patients returning for their subsequent check up reduced the number of patients studied at the long-term stroke recovery.

6. Conclusion

This study was performed with the aims to study the microRNAs present in peripheral blood and their temporal expression profiles in young chronic stroke patients, with the view of gaining an insight to the molecular regulation of cerebrovascular maladies origin. Real-time quantitative PCR was utilized in this study as a means of RNA analysis. The application in a 'real time' setting, being the most sensitive technique in detecting RNA has become the most popular means of quantifying transcriptome levels (Bustin, 2000).

Amongst the 6 selected microRNAs in this preliminary study, microRNA-145, -222, -223 and -23b demonstrated significant ectopic expression in chronic stroke patients as compared to healthy control subjects. Expression of circulating microRNA-145 was dysregulated with $p = 0.002$ (46% upregulation, 22% downregulation); microRNA-222 was dysregulated with $p = 0.011$ (50% upregulation, 19% downregulation); microRNA-223 was dysregulated with $p = 0.028$ (44% upregulation, 16% downregulation) and microRNA-23b was dysregulated with $p = 0.040$ (38% upregulation, 16% downregulation). This strongly suggests that changes in the pathophysiology of ischaemic stroke are still present in chronic stroke condition.

In silico prediction of the biological effects and existing literature relate the dysregulations to the pathology of vascular biology. MicroRNA-145 was shown to regulate KLF4/5, transcription factors implicated in VSMC plasticity fate and haematopoiesis. MicroRNA-214 was shown to regulate PTEN and has been associated with T-cells proliferation. MicroRNA-222 was shown to regulate synthesis of c-Kit and ICAM-1 protein receptors that facilitate inter-cell adherence. MicroRNA-223 was

shown to regulate production of Mef2c and LMO2, which are transcription co-factors associated with haematopoiesis development. MicroRNA-23b was shown to affect retinoblastoma protein phosphorylation that controls cell cycle progression thus regulating cell proliferation, while microRNA-339 was shown to regulate ICAM-1 implicated in inter-cell adherence.

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. This observation has demonstrated the potential feasibility of using microRNA expression to facilitate the determination of the cause of cryptogenic stroke.

Cumulatively, this study revealed the ongoing regulation of circulating microRNA-145, -222, -223, and -23b in young chronic ischaemic stroke, also known as stable stroke patients; this might mark the stages of vascular biology and immune response following ischaemic stroke. The reduction of the circulating microRNA-222, -223, -214, and -23b expressions after a period of time might indicate good progression of the vascular homeostasis; and consistent with the previous report dictating better stroke outcome in patients with downregulation of microRNAs expressions (Tan *et al.*, 2009). Therefore, this study demonstrated the potential of circulating microRNAs profile from peripheral blood as possible stroke indicators in encouraging the development of microRNA biomarkers in prognosis of cerebral ischaemic stroke, which can augment clinical

evaluation and future treatment of ischaemic stroke. However, given the complexity of postnatal neovascularization and vasculoprotection mechanotransductions, specific investigation and elucidation of microRNAs regulation is desired; and because of the many uncertainties it would be premature to draw any definitive conclusion from this study.

References

Acuto, O., Mise-Omata, S., Mangino, G., & Michel, F. (2003). Molecular modifiers of T cell antigen receptor triggering threshold: the mechanism of CD28 costimulatory receptor. *Immunol Rev, 192*, 21-31.

Adams, H.P., Bendixen, B.H., Kappelle, L.J., Biller, J., Love, B.B., Gordon, D.L., et al. (1993). Classification of Subtype of Acute Ischemic Stroke - Definitions for Use in a Multicenter Clinical-Trial. *Stroke, 24*(1), 35-41.

Alder, J.K., Georgantas, R.W., 3rd, Hildreth, R.L., Kaplan, I.M., Morisot, S., Yu, X., et al. (2008). Kruppel-like factor 4 is essential for inflammatory monocyte differentiation in vivo. *J Immunol, 180*(8), 5645-5652.

Alvarez-Garcia, I., & Miska, E.A. (2005). MicroRNA functions in animal development and human disease. *Development, 132*(21), 4653-4662.

Amarenco, P., Bogousslavsky, J., Caplan, L.R., Donnan, G.A., & Hennerici, M.G. (2009). Classification of stroke subtypes. *Cerebrovasc Dis, 27*(5), 493-501.

Ambros, V. (2004). The functions of animal microRNAs. *Nature, 431*(7006), 350-355.

Ariyaratnam, R., Casas, J.P., Whittaker, J., Smeeth, L., Hingorani, A.D., & Sharma, P. (2007). Genetics of ischaemic stroke among persons of non-European descent: A meta-analysis of eight genes involving; 32,500 individuals. *Plos Medicine, 4*(4), 728-736.

Banerjee, I., Gupta, V., & Ganesh, S. (2007). Association of gene polymorphism with genetic susceptibility to stroke in Asian populations: a meta-analysis. *J Hum Genet, 52*(3), 205-219.

Barr, T.L., Conley, Y., Ding, J., Dillman, A., Warach, S., Singleton, A., et al. (2010). Genomic biomarkers and cellular pathways of ischemic stroke by RNA gene expression profiling. *Neurology, 75*(11), 1009-1014.

Bartel, D.P. (2004). MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell, 116*(2), 281-297.

Becker, K.J. (2010). Modulation of the postischemic immune response to improve stroke outcome. *Stroke, 41*(10 Suppl), S75-78.

Benjelloun, N., Renolleau, S., Represa, A., Ben-Ari, Y., & Charriaut-Marlangue, C. (1999). Inflammatory responses in the cerebral cortex after ischemia in the P7 neonatal rat. *Stroke, 30*(9), 1916-1923.

Bergers, G., & Benjamin, L.E. (2003). Tumorigenesis and the angiogenic switch. *Nat Rev Cancer, 3*(6), 401-410.

Bergh, G., Ehinger, M., Olsson, I., Jacobsen, S.E., & Gullberg, U. (1999). Involvement of the retinoblastoma protein in monocytic and neutrophilic lineage commitment of human bone marrow progenitor cells. *Blood, 94*(6), 1971-1978.

Bi, W., Drake, C.J., & Schwarz, J.J. (1999). The transcription factor MEF2C-null mouse exhibits complex vascular malformations and reduced cardiac expression of angiopoietin 1 and VEGF. *Dev Biol, 211*(2), 255-267.

Black, B.L., & Olson, E.N. (1998). Transcriptional control of muscle development by myocyte enhancer factor-2 (MEF2) proteins. *Annu Rev Cell Dev Biol, 14*, 167-196.

Brioschi, M., Fischer, J., Cairoli, R., Rossetti, S., Pezzetti, L., Nichelatti, M., et al. (2010). Down-regulation of microRNAs 222/221 in acute myelogenous leukemia with deranged core-binding factor subunits. *Neoplasia, 12*(11), 866-876.

Buckler, J.L., Liu, X., & Turka, L.A. (2008). Regulation of T-cell responses by PTEN. *Immunol Rev, 224*, 239-248.

Bueno, M.J., Perez de Castro, I., Gomez de Cedron, M., Santos, J., Calin, G.A., Cigudosa, J.C., et al. (2008). Genetic and epigenetic silencing of microRNA-203 enhances ABL1 and BCR-ABL1 oncogene expression. *Cancer Cell, 13*(6), 496-506.

Bustin, S.A. (2000). Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. *J Mol Endocrinol, 25*(2), 169-193.

Canales, R.D., Luo, Y., Willey, J.C., Austermiller, B., Barbacioru, C.C., Boysen, C., et al. (2006). Evaluation of DNA microarray results with quantitative gene expression platforms. *Nat Biotechnol, 24*(9), 1115-1122.

Cao, Z., Sun, X., Icli, B., Wara, A.K., & Feinberg, M.W. (2010). Role of Kruppel-like factors in leukocyte development, function, and disease. *Blood, 116*(22), 4404-4414.

Carissimi, C., Fulci, V., & Macino, G. (2009). MicroRNAs: novel regulators of immunity. *Autoimmun Rev, 8*(6), 520-524.

Carlos, T.M., & Harlan, J.M. (1994). Leukocyte-endothelial adhesion molecules. *Blood, 84*(7), 2068-2101.

Carmeliet, P. (2003). Angiogenesis in health and disease. *Nat Med, 9*(6), 653-660.

Casas, J.P., Hingorani, A.D., Bautista, L.E., & Sharma, P. (2004). Meta-analysis of genetic studies in ischemic stroke - Thirty-two genes involving approximately 18 000 cases and 58 000 controls. *Archives of Neurology, 61*(11), 1652-1661.

Catto, A.J., Kohler, H.P., Bannan, S., Stickland, M., Carter, A., & Grant, P.J. (1998). Factor XIII Val 34 Leu - A novel association with primary intracerebral hemorrhage. *Stroke, 29*(4), 813-816.

Chan, H.P., Lewis, C., & Thomas, P.S. (2009a). Exhaled breath analysis: novel approach for early detection of lung cancer. *Lung Cancer, 63*(2), 164-168.

Chan, L.S., Yue, P.Y., Mak, N.K., & Wong, R.N. (2009b). Role of microRNA-214 in ginsenoside-Rg1-induced angiogenesis. *Eur J Pharm Sci, 38*(4), 370-377.

Chen, C.Z., Li, L., Lodish, H.F., & Bartel, D.P. (2004). MicroRNAs modulate hematopoietic lineage differentiation. *Science, 303*(5654), 83-86.

Chen, J.F., Mandel, E.M., Thomson, J.M., Wu, Q.L., Callis, T.E., Hammond, S.M., et al. (2006). The role of microRNA-1 and microRNA-133 in skeletal muscle proliferation and differentiation. *Nature Genetics, 38*(2), 228-233.

Chen, X.C., Xu, M.T., Zhou, W., Han, C.L., & Chen, W.Q. (2007). A meta-analysis of beta-fibrinogen gene-455G/A polymorphism and plasma fibrinogen level in Chinese cerebral infarction patients. *Biomed Environ Sci, 20*(5), 366-372.

Chen, Y., & Gorski, D.H. (2008). Regulation of angiogenesis through a microRNA (miR-130a) that down-regulates antiangiogenic homeobox genes GAX and HOXA5. *Blood, 111*(3), 1217-1226.

Chen, Y.F., Chiu, H.H., Wu, C.H., Wang, J.Y., Chen, F.M., Tzou, W.H., et al. (2003). Retinoblastoma protein (pRB) was significantly phosphorylated through a Ras-to-MAPK pathway in mutant K-ras stably transfected human adrenocortical cells. *DNA Cell Biol, 22*(10), 657-664.

Cheng, Y., Liu, X., Yang, J., Lin, Y., Xu, D.Z., Lu, Q., et al. (2009). MicroRNA-145, a novel smooth muscle cell phenotypic marker and modulator, controls vascular neointimal lesion formation. *Circ Res, 105*(2), 158-166.

Chu, K., Jung, K.H., Lee, S.T., Park, H.K., Sinn, D.I., Kim, J.M., et al. (2008). Circulating endothelial progenitor cells as a new marker of endothelial dysfunction or repair in acute stroke. *Stroke, 39*(5), 1441-1447.

Cimmino, A., Calin, G.A., Fabbri, M., Iorio, M.V., Ferracin, M., Shimizu, M., et al. (2005). miR-15 and miR-16 induce apoptosis by targeting BCL2. *Proc Natl Acad Sci U S A, 102*(39), 13944-13949.

Condorelli, G.L., Testa, U., Valtieri, M., Vitelli, L., De Luca, A., Barberi, T., et al. (1995). Modulation of retinoblastoma gene in normal adult hematopoiesis: peak expression and functional role in advanced erythroid differentiation. *Proc Natl Acad Sci U S A, 92*(11), 4808-4812.

Cordes, K.R., Sheehy, N.T., White, M.P., Berry, E.C., Morton, S.U., Muth, A.N., et al. (2009). miR-145 and miR-143 regulate smooth muscle cell fate and plasticity. *Nature, 460*(7256), 705-710.

Corral, J., Iniesta, J.A., Gonzalez-Conejero, R., Villalon, M., Rivera, J., & Vicente, V. (2000). Factor XIII Val34Leu polymorphism in primary intracerebral haemorrhage. *Hematol J, 1*(4), 269-273.

Cullen, B.R. (2004). Transcription and processing of human microRNA precursors. *Mol Cell, 16*(6), 861-865.

Dambinova, S.A., Khounteev, G.A., Izykenova, G.A., Zavolokov, I.G., Ilyukhina, A.Y., & Skoromets, A.A. (2003). Blood test detecting autoantibodies to N-methyl-D-aspartate neuroreceptors for evaluation of patients with transient ischemic attack and stroke. *Clin Chem, 49*(10), 1752-1762.

Dambinova, S.A., Khounteev, G.A., & Skoromets, A.A. (2002). Multiple panel of biomarkers for TIA/stroke evaluation. *Stroke, 33*(5), 1181-1182.

Decary, S., Decesse, J.T., Ogryzko, V., Reed, J.C., Naguibneva, I., Harel-Bellan, A., et al. (2002). The retinoblastoma protein binds the promoter of the survival gene bcl-2 and regulates its transcription in epithelial cells through transcription factor AP-2. *Molecular and Cellular Biology, 22*(22), 7877-7888.

del Zoppo, G.J., Schmid-Schonbein, G.W., Mori, E., Copeland, B.R., & Chang, C.M. (1991). Polymorphonuclear leukocytes occlude capillaries following middle cerebral artery occlusion and reperfusion in baboons. *Stroke, 22*(10), 1276-1283.

Dews, M., Homayouni, A., Yu, D., Murphy, D., Sevignani, C., Wentzel, E., et al. (2006). Augmentation of tumor angiogenesis by a Myc-activated microRNA cluster. *Nat Genet, 38*(9), 1060-1065.

Dimmeler, S., & Zeiher, A.M. (2004). Vascular repair by circulating endothelial progenitor cells: the missing link in atherosclerosis? *J Mol Med (Berl), 82*(10), 671-677.

Divakaran, V., & Mann, D.L. (2008). The Emerging Role of MicroRNAs in Cardiac Remodeling and Heart Failure. *Circulation Research, 103*(10), 1072-U1047.

Doench, J.G., Petersen, C.P., & Sharp, P.A. (2003). siRNAs can function as miRNAs. *Genes Dev, 17*(4), 438-442.

Downes, C.E., & Crack, P.J. (2010). Neural injury following stroke: are Toll-like receptors the link between the immune system and the CNS? *British Journal of Pharmacology, 160*(8), 1872-1888.

Elbaz, A., Poirier, O., Moulin, T., Chedru, F., Cambien, F., Amarenco, P., et al. (2000). Association between the Glu298Asp polymorphism in the endothelial constitutive nitric oxide synthase gene and brain infarction. *Stroke, 31*(7), 1634-1639.

Elkind, M.S.V. (2010). Inflammatory Mechanisms of Stroke. *Stroke, 41*(10), S3-S8.

Esquela-Kerscher, A., & Slack, F.J. (2006). Oncomirs - microRNAs with a role in cancer. *Nat Rev Cancer, 6*(4), 259-269.

Fasanaro, P., D'Alessandra, Y., Di Stefano, V., Melchionna, R., Romani, S., Pompilio, G., et al. (2008). MicroRNA-210 modulates endothelial cell response to hypoxia and inhibits the receptor tyrosine kinase ligand Ephrin-A3. *J Biol Chem, 283*(23), 15878-15883.

Feinberg, M.W., Wara, A.K., Cao, Z., Lebedeva, M.A., Rosenbauer, F., Iwasaki, H., et al. (2007). The Kruppel-like factor KLF4 is a critical regulator of monocyte differentiation. *EMBO J, 26*(18), 4138-4148.

Felli, N., Fontana, L., Pelosi, E., Botta, R., Bonci, D., Facchiano, F., et al. (2005). MicroRNAs 221 and 222 inhibit normal erythropoiesis and erythroleukemic cell growth via kit receptor down-modulation. *Proc Natl Acad Sci U S A, 102*(50), 18081-18086.

Felli, N., Pedini, F., Romania, P., Biffoni, M., Morsilli, O., Castelli, G., et al. (2009). MicroRNA 223-dependent expression of LMO2 regulates normal erythropoiesis. *Haematologica, 94*(4), 479-486.

Forstemann, K., Horwich, M.D., Wee, L., Tomari, Y., & Zamore, P.D. (2007). Drosophila microRNAs are sorted into functionally distinct argonaute complexes after production by dicer-1. *Cell, 130*(2), 287-297.

Frijns, C.J., & Kappelle, L.J. (2002). Inflammatory cell adhesion molecules in ischemic cerebrovascular disease. *Stroke, 33*(8), 2115-2122.

Funk, M., Endler, G., Lalouschek, W., Hsieh, K., Schillinger, M., Lang, W., et al. (2006). Factor VII gene haplotypes and risk of ischemic stroke. *Clinical Chemistry, 52*(6), 1190-1192.

Gabbianelli, M., Testa, U., Morsilli, O., Pelosi, E., Saulle, E., Petrucci, E., et al. (2010). Mechanism of human Hb switching: a possible role of the kit receptor/miR 221-222 complex. *Haematologica, 95*(8), 1253-1260.

Ghani, U., Shuaib, A., Salam, A., Nasir, A., Shuaib, U., Jeerakathil, T., et al. (2005). Endothelial progenitor cells during cerebrovascular disease. *Stroke, 36*(1), 151-153.

Giraldez, A.J., Cinalli, R.M., Glasner, M.E., Enright, A.J., Thomson, J.M., Baskerville, S., et al. (2005). MicroRNAs regulate brain morphogenesis in zebrafish. *Science, 308*(5723), 833-838.

Gregory, R.I., Yan, K.P., Amuthan, G., Chendrimada, T., Doratotaj, B., Cooch, N., et al. (2004). The Microprocessor complex mediates the genesis of microRNAs. *Nature, 432*(7014), 235-240.

Gretarsdottir, S., Thorleifsson, G., Reynisdottir, S.T., Manolescu, A., Jonsdottir, S., Jonsdottir, T., et al. (2003). The gene encoding phosphodiesterase 4D confers risk of ischemic stroke. *Nat Genet, 35*(2), 131-138.

Grond-Ginsbach, C., Hummel, M., Wiest, T., Horstmann, S., Pfleger, K., Hergenhahn, M., et al. (2008). Gene expression in human peripheral blood mononuclear cells upon acute ischemic stroke. *J Neurol, 255*(5), 723-731.

Grundmann, S., Hans, F.P., Kinniry, S., Heinke, J., Helbing, T., Bluhm, F., et al. (2011). MicroRNA-100 regulates neovascularization by suppression of mammalian target of rapamycin in endothelial and vascular smooth muscle cells. *Circulation, 123*(9), 999-1009.

Guil, S., & Esteller, M. (2009). DNA methylomes, histone codes and miRNAs: tying it all together. *Int J Biochem Cell Biol, 41*(1), 87-95.

Haldar, S.M., Ibrahim, O.A., & Jain, M.K. (2007). Kruppel-like Factors (KLFs) in muscle biology. *J Mol Cell Cardiol, 43*(1), 1-10.

Hallenbeck, J. (2010). How inflammation modulates central nervous system vessel activation and provides targets for intervention-a personal perspective. *Innate Inflammation and Stroke, 1207*, 1-7.

Hamik, A., Lin, Z., Kumar, A., Balcells, M., Sinha, S., Katz, J., et al. (2007). Kruppel-like factor 4 regulates endothelial inflammation. *J Biol Chem, 282*(18), 13769-13779.

Han, J., Jiang, Y., Li, Z., Kravchenko, V.V., & Ulevitch, R.J. (1997). Activation of the transcription factor MEF2C by the MAP kinase p38 in inflammation. *Nature, 386*(6622), 296-299.

Hansson, A., Zetterblad, J., van Duren, C., Axelson, H., & Jonsson, J.I. (2007). The Lim-only protein LMO2 acts as a positive regulator of erythroid differentiation. *Biochemical and Biophysical Research Communications, 364*(3), 675-681.

Harris, T.A., Yamakuchi, M., Ferlito, M., Mendell, J.T., & Lowenstein, C.J. (2008). MicroRNA-126 regulates endothelial expression of vascular cell adhesion molecule 1. *Proc Natl Acad Sci U S A, 105*(5), 1516-1521.

Hartl, R., Schurer, L., Schmid-Schonbein, G.W., & del Zoppo, G.J. (1996). Experimental antileukocyte interventions in cerebral ischemia. *J Cereb Blood Flow Metab, 16*(6), 1108-1119.

Hatakeyama, M., & Weinberg, R.A. (1995). The role of RB in cell cycle control. *Prog Cell Cycle Res, 1*, 9-19.

Hergenroeder, G., Redell, J.B., Moore, A.N., Dubinsky, W.P., Funk, R.T., Crommett, J., et al. (2008). Identification of serum biomarkers in brain-injured adults: potential for predicting elevated intracranial pressure. *J Neurotrauma, 25*(2), 79-93.

Hsiai, T.K., Cho, S.K., Wong, P.K., Ing, M., Salazar, A., Sevanian, A., et al. (2003). Monocyte recruitment to endothelial cells in response to oscillatory shear stress. *FASEB J, 17*(12), 1648-1657.

Huggett, J., Dheda, K., Bustin, S., & Zumla, A. (2005). Real-time RT-PCR normalisation; strategies and considerations. *Genes Immun, 6*(4), 279-284.

Hung, A.M., Qureishy, O., Wetmore, J.B., Lovett, D.H., & Johansen, K.L. (2002). Role of IL 1RA gene polymorphism in ESRD and cardiovascular disease. *Journal of the American Society of Nephrology, 13*, 435a-436a.

Huse, J.T., Brennan, C., Hambardzumyan, D., Wee, B., Pena, J., Rouhanifard, S.H., et al. (2009). The PTEN-regulating microRNA miR-26a is amplified in high-grade glioma and facilitates gliomagenesis in vivo. *Genes Dev, 23*(11), 1327-1337.

Hutvagner, G., McLachlan, J., Pasquinelli, A.E., Balint, E., Tuschl, T., & Zamore, P.D. (2001). A cellular function for the RNA-interference enzyme Dicer in the maturation of the let-7 small temporal RNA. *Science, 293*(5531), 834-838.

Jeyaseelan, K., Lim, K.Y., & Armugam, A. (2008). MicroRNA expression in the blood and brain of rats subjected to transient focal ischemia by middle cerebral artery occlusion. *Stroke, 39*(3), 959-966.

Ji, R., Cheng, Y., Yue, J., Yang, J., Liu, X., Chen, H., et al. (2007). MicroRNA expression signature and antisense-mediated depletion reveal an essential role of MicroRNA in vascular neointimal lesion formation. *Circ Res, 100*(11), 1579-1588.

Ji, Y., Kutner, A., Verstuyf, A., Verlinden, L., & Studzinski, G.P. (2002). Derivatives of vitamins D2 and D3 activate three MAPK pathways and upregulate pRb expression in differentiating HL60 cells. *Cell Cycle, 1*(6), 410-415.

Jickling, G.C., & Sharp, F.R. (2011). Blood Biomarkers of Ischemic Stroke. *Neurotherapeutics*.

Jickling, G.C., Xu, H.C., Stamova, B., Ander, B.P., Zhan, X.H., Tian, Y.F., et al. (2010). Signatures of Cardioembolic and Large-Vessel Ischemic Stroke. *Annals of Neurology, 68*(5), 681-692.

Jindra, P.T., Bagley, J., Godwin, J.G., & Iacomini, J. (2010). Costimulation-dependent expression of microRNA-214 increases the ability of T cells to proliferate by targeting Pten. *J Immunol, 185*(2), 990-997.

Johnnidis, J.B., Harris, M.H., Wheeler, R.T., Stehling-Sun, S., Lam, M.H., Kirak, O., et al. (2008). Regulation of progenitor cell proliferation and granulocyte function by microRNA-223. *Nature, 451*(7182), 1125-1129.

Kato, M., Putta, S., Wang, M., Yuan, H., Lanting, L., Nair, I., et al. (2009). TGF-beta activates Akt kinase through a microRNA-dependent amplifying circuit targeting PTEN. *Nat Cell Biol, 11*(7), 881-889.

Khiem, D., Cyster, J.G., Schwarz, J.J., & Black, B.L. (2008). A p38 MAPK-MEF2C pathway regulates B-cell proliferation. *Proc Natl Acad Sci U S A, 105*(44), 17067-17072.

Kim, R.J., & Becker, R.C. (2003). Association between factor V Leiden, prothrombin G20210A, and methylenetetrahydrofolate reductase C677T mutations and events of the arterial circulatory system: a meta-analysis of published studies. *Am Heart J, 146*(6), 948-957.

Korenjak, M., & Brehm, A. (2005). E2F-Rb complexes regulating transcription of genes important for differentiation and development. *Curr Opin Genet Dev, 15*(5), 520-527.

Krek, A., Grun, D., Poy, M.N., Wolf, R., Rosenberg, L., Epstein, E.J., et al. (2005). Combinatorial microRNA target predictions. *Nat Genet, 37*(5), 495-500.

Kuehbacher, A., Urbich, C., Zeiher, A.M., & Dimmeler, S. (2007). Role of Dicer and Drosha for endothelial microRNA expression and angiogenesis. *Circ Res, 101*(1), 59-68.

Kulshreshtha, R., Davuluri, R.V., Calin, G.A., & Ivan, M. (2008). A microRNA component of the hypoxic response. *Cell Death Differ, 15*(4), 667-671.

Ladeiro, Y., Couchy, G., Balabaud, C., Bioulac-Sage, P., Pelletier, L., Rebouissou, S., et al. (2008). MicroRNA profiling in hepatocellular tumors is associated with clinical features and oncogene/tumor suppressor gene mutations. *Hepatology, 47*(6), 1955-1963.

Lagos-Quintana, M., Rauhut, R., Lendeckel, W., & Tuschl, T. (2001). Identification of novel genes coding for small expressed RNAs. *Science, 294*(5543), 853-858.

Lagos-Quintana, M., Rauhut, R., Meyer, J., Borkhardt, A., & Tuschl, T. (2003). New microRNAs from mouse and human. *RNA, 9*(2), 175-179.

Laterza, O.F., Modur, V.R., Crimmins, D.L., Olander, J.V., Landt, Y., Lee, J.M., et al. (2006). Identification of novel brain biomarkers. *Clin Chem, 52*(9), 1713-1721.

Lee, D.Y., Deng, Z., Wang, C.H., & Yang, B.B. (2007). MicroRNA-378 promotes cell survival, tumor growth, and angiogenesis by targeting SuFu and Fus-1 expression. *Proc Natl Acad Sci U S A, 104*(51), 20350-20355.

Lee, J., Sayegh, J., Daniel, J., Clarke, S., & Bedford, M.T. (2005). PRMT8, a new membrane-bound tissue-specific member of the protein arginine methyltransferase family. *J Biol Chem, 280*(38), 32890-32896.

Lee, R.C., & Ambros, V. (2001). An extensive class of small RNAs in Caenorhabditis elegans. *Science, 294*(5543), 862-864.

Lee, R.C., Feinbaum, R.L., & Ambros, V. (1993). The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. *Cell, 75*(5), 843-854.

Lee, Y., Ahn, C., Han, J., Choi, H., Kim, J., Yim, J., et al. (2003). The nuclear RNase III Drosha initiates microRNA processing. *Nature, 425*(6956), 415-419.

Lee, Y., Kim, M., Han, J., Yeom, K.H., Lee, S., Baek, S.H., et al. (2004). MicroRNA genes are transcribed by RNA polymerase II. *EMBO J, 23*(20), 4051-4060.

Li, H.X., Han, M., Bernier, M., Zheng, B., Sun, S.G., Su, M., et al. (2010). Kruppel-like factor 4 promotes differentiation by transforming growth factor-beta receptor-mediated Smad and p38 MAPK signaling in vascular smooth muscle cells. *J Biol Chem, 285*(23), 17846-17856.

Li, M., Scott, D.E., Shandas, R., Stenmark, K.R., & Tan, W. (2009). High pulsatility flow induces adhesion molecule and cytokine mRNA expression in distal pulmonary artery endothelial cells. *Ann Biomed Eng, 37*(6), 1082-1092.

Lim, K.-Y., Chua, J.-H., Tan, J.-R., Swaminathan, P., Sepramaniam, S., Armugam, A., et al. (2010). MicroRNAs in Cerebral Ischemia. *Translational Stroke Research, 1*(4), 287-303.

Litz, J., & Krystal, G.W. (2006). Imatinib inhibits c-Kit-induced hypoxia-inducible factor-1alpha activity and vascular endothelial growth factor expression in small cell lung cancer cells. *Mol Cancer Ther, 5*(6), 1415-1422.

Liu, D.Z., Tian, Y.F., Ander, B.P., Xu, H.C., Stamova, B.S., Zhan, X.H., et al. (2010). Brain and blood microRNA expression profiling of ischemic stroke, intracerebral hemorrhage, and kainate seizures. *Journal of Cerebral Blood Flow and Metabolism, 30*(1), 92-101.

Lloyd-Jones, D., Adams, R.J., Brown, T.M., Carnethon, M., Dai, S., De Simone, G., et al. (2010). Heart disease and stroke statistics--2010 update: a report from the American Heart Association. *Circulation, 121*(7), e46-e215.

Long, X., Bell, R.D., Gerthoffer, W.T., Zlokovic, B.V., & Miano, J.M. (2008). Myocardin is sufficient for a smooth muscle-like contractile phenotype. *Arterioscler Thromb Vasc Biol, 28*(8), 1505-1510.

Luttun, A., Carmeliet, G., & Carmeliet, P. (2002a). Vascular progenitors: From biology to treatment. *Trends in Cardiovascular Medicine, 12*(2), 88-96.

Luttun, A., Tjwa, M., Moons, L., Wu, Y., Angelillo-Scherrer, A., Liao, F., et al. (2002b). Revascularization of ischemic tissues by PlGF treatment, and inhibition of tumor angiogenesis, arthritis and atherosclerosis by anti-Flt1. *Nature Medicine, 8*(8), 831-840.

Lynch, J.R., Blessing, R., White, W.D., Grocott, H.P., Newman, M.F., & Laskowitz, D.T. (2004). Novel diagnostic test for acute stroke. *Stroke, 35*(1), 57-63.

Maderazo, A.B., Belk, J.P., He, F., & Jacobson, A. (2003). Nonsense-containing mRNAs that accumulate in the absence of a functional nonsense-mediated mRNA decay pathway are destabilized rapidly upon its restitution. *Mol Cell Biol, 23*(3), 842-851.

Matsui, J., Wakabayashi, T., Asada, M., Yoshimatsu, K., & Okada, M. (2004). Stem cell factor/c-kit signaling promotes the survival, migration, and capillary tube formation of human umbilical vein endothelial cells. *J Biol Chem, 279*(18), 18600-18607.

McCabe, D.J., & Rakhit, R.D. (2007). Antithrombotic and interventional treatment options in cardioembolic transient ischaemic attack and ischaemic stroke. *J Neurol Neurosurg Psychiatry, 78*(1), 14-24.

McKenzie, M.J., Yu, S.Z., Macko, R.F., McLenithan, J.C., & Hafer-Macko, C.E. (2008). Human genome comparison of paretic and nonparetic vastus lateralis muscle in patients with hemiparetic stroke. *Journal of Rehabilitation Research and Development, 45*(2), 273-281.

Melton, D.A., Krieg, P.A., Rebagliati, M.R., Maniatis, T., Zinn, K., & Green, M.R. (1984). Efficient in vitro synthesis of biologically active RNA and RNA hybridization probes from plasmids containing a bacteriophage SP6 promoter. *Nucleic Acids Res, 12*(18), 7035-7056.

Meng, F., Henson, R., Wehbe-Janek, H., Ghoshal, K., Jacob, S.T., & Patel, T. (2007). MicroRNA-21 regulates expression of the PTEN tumor suppressor gene in human hepatocellular cancer. *Gastroenterology, 133*(2), 647-658.

Miyamoto, S., Suzuki, T., Muto, S., Aizawa, K., Kimura, A., Mizuno, Y., et al. (2003). Positive and negative regulation of the cardiovascular transcription factor KLF5 by p300 and the oncogenic regulator SET through interaction and acetylation on the DNA-binding domain. *Mol Cell Biol, 23*(23), 8528-8541.

Montaner, J., Perea-Gainza, M., Delgado, P., Ribo, M., Chacon, P., Rosell, A., et al. (2008). Etiologic diagnosis of ischemic stroke subtypes with plasma biomarkers. *Stroke, 39*(8), 2280-2287.

Moore, D.F., Li, H., Jeffries, N., Wright, V., Cooper, R.A., Elkahloun, A., et al. (2005). Using peripheral blood mononuclear cells to determine a gene expression profile of acute ischemic stroke - A pilot investigation. *Circulation, 111*(2), 212-221.

Nam, C.H., & Rabbitts, T.H. (2006). The role of LMO2 in development and in T cell leukemia after chromosomal translocation or retroviral insertion. *Mol Ther, 13*(1), 15-25.

Nelson, P.T., Wang, W.X., & Rajeev, B.W. (2008). MicroRNAs (miRNAs) in neurodegenerative diseases. *Brain Pathol, 18*(1), 130-138.

Nishida, E., & Gotoh, Y. (1993). The Map Kinase Cascade Is Essential for Diverse Signal Transduction Pathways. *Trends in Biochemical Sciences, 18*(4), 128-131.

Ocava, L.C., Singh, M., Malhotra, S., & Rosenbaum, D.M. (2006). Antithrombotic and thrombolytic therapy for ischemic stroke. *Clin Geriatr Med, 22*(1), 135-154, ix-x.

Olson, E.N. (2004). Undermining the endothelium by ablation of MAPK-MEF2 signaling. *J Clin Invest, 113*(8), 1110-1112.

Oudit, G.Y., Sun, H., Kerfant, B.G., Crackower, M.A., Penninger, J.M., & Backx, P.H. (2004). The role of phosphoinositide-3 kinase and PTEN in cardiovascular physiology and disease. *J Mol Cell Cardiol, 37*(2), 449-471.

Ouyang, Y.B., Lu, Y., Yue, S., & Giffard, R.G. (2011). miR-181 targets multiple Bcl-2 family members and influences apoptosis and mitochondrial function in astrocytes. *Mitochondrion*.

Owens, G.K. (1995). Regulation of differentiation of vascular smooth muscle cells. *Physiol Rev, 75*(3), 487-517.

Peichev, M., Naiyer, A.J., Pereira, D., Zhu, Z.P., Lane, W.J., Williams, M., et al. (2000). Expression of VEGFR-2 and AC133 by circulating human CD34(+) cells identifies a population of functional endothelial precursors. *Blood, 95*(3), 952-958.

Pezzini, A., Del Zotto, E., & Padovani, A. (2003). Search for coagulopathy does not obviate search for venous thrombosis in suspected paradoxical embolism - Response. *Stroke, 34*(9),

Poliseno, L., Tuccoli, A., Mariani, L., Evangelista, M., Citti, L., Woods, K., et al. (2006). MicroRNAs modulate the angiogenic properties of HUVECs. *Blood, 108*(9), 3068-3071.

Pouladi, M.A. (2004). A link between ALOX5AP and myocardial infarction and stroke. *Clinical Genetics, 66*(1), 18-20.

Pozzilli, C., Lenzi, G.L., Argentino, C., Bozzao, L., Rasura, M., Giubilei, F., et al. (1985). Peripheral white blood cell count in cerebral ischemic infarction. *Acta Neurol Scand, 71*(5), 396-400.

Prass, K., Braun, J.S., Dirnagl, U., Meisel, C., & Meisel, A. (2006). Stroke propagates bacterial aspiration to pneumonia in a model of cerebral ischemia. *Stroke, 37*(10), 2607-2612.

Rauch, B.H., Muschenborn, B., Braun, M., Weber, A.A., & Schror, K. (2007). ICAM-1 and p38 MAPK mediate fibrinogen-induced migration of human vascular smooth muscle cells. *Eur J Pharmacol, 577*(1-3), 54-57.

Reynolds, M.A., Kirchick, H.J., Dahlen, J.R., Anderberg, J.M., McPherson, P.H., Nakamura, K.K., et al. (2003). Early biomarkers of stroke. *Clin Chem, 49*(10), 1733-1739.

Roldo, C., Missiaglia, E., Hagan, J.P., Falconi, M., Capelli, P., Bersani, S., et al. (2006). MicroRNA expression abnormalities in pancreatic endocrine and acinar tumors are associated with distinctive pathologic features and clinical behavior. *Journal of Clinical Oncology, 24*(29), 4677-4684.

Ross, R. (1999). Atherosclerosis--an inflammatory disease. *N Engl J Med, 340*(2), 115-126.

Schetter, A.J., Leung, S.Y., Sohn, J.J., Zanetti, K.A., Bowman, E.D., Yanaihara, N., et al. (2008). MicroRNA expression profiles associated with prognosis and therapeutic outcome in colon adenocarcinoma. *JAMA, 299*(4), 425-436.

Schuler, A., Schwieger, M., Engelmann, A., Weber, K., Horn, S., Muller, U., et al. (2008). The MADS transcription factor Mef2c is a pivotal modulator of myeloid cell fate. *Blood, 111*(9), 4532-4541.

Sellner, L.N., & Turbett, G.R. (1998). Comparison of three RT-PCR methods. *Biotechniques, 25*(2), 230-234.

Sharp, F.R., Jickling, G.C., Stamova, B., Tian, Y.F., Zhan, X.H., Liu, D.Z., et al. (2011). Molecular markers and mechanisms of stroke: RNA studies of blood in animals and humans. *Journal of Cerebral Blood Flow and Metabolism, 31*(7), 1513-1531.

Shi, Q., Rafii, S., Wu, M.H.D., Wijelath, E.S., Yu, C., Ishida, A., et al. (1998). Evidence for circulating bone marrow-derived endothelial cells. *Blood, 92*(2), 362-367.

Shindo, T., Manabe, I., Fukushima, Y., Tobe, K., Aizawa, K., Miyamoto, S., et al. (2002). Kruppel-like zinc-finger transcription factor KLF5/BTEB2 is a target for angiotensin II signaling and an essential regulator of cardiovascular remodeling. *Nat Med, 8*(8), 856-863.

Smith, J.A. (1994). Neutrophils, host defense, and inflammation: a double-edged sword. *J Leukoc Biol, 56*(6), 672-686.

Spike, B.T., Dirlam, A., Dibling, B.C., Marvin, J., Williams, B.O., Jacks, T., et al. (2004). The Rb tumor suppressor is required for stress erythropoiesis. *Embo Journal, 23*(21), 4319-4329.

Stamova, B., Xu, H.C., Jickling, G., Bushnell, C., Tian, Y.F., Ander, B.P., et al. (2010). Gene Expression Profiling of Blood for the Prediction of Ischemic Stroke. *Stroke, 41*(10), 2171-2177.

Stanković, S., & Majkić-Singh, N. (2008). Advances in the genetic basis of ischemic stroke *JMB, 27*(2), 123-134.

Suarez, Y., Fernandez-Hernando, C., Pober, J.S., & Sessa, W.C. (2007). Dicer dependent microRNAs regulate gene expression and functions in human endothelial cells. *Circ Res, 100*(8), 1164-1173.

Sung, H.J., Ahn, J.M., Yoon, Y.H., Rhim, T.Y., Park, C.S., Park, J.Y., et al. (2011). Identification and validation of SAA as a potential lung cancer biomarker and its involvement in metastatic pathogenesis of lung cancer. *J Proteome Res, 10*(3), 1383-1395.

Suzuki, T., Aizawa, K., Matsumura, T., & Nagai, R. (2005). Vascular implications of the Kruppel-like family of transcription factors. *Arterioscler Thromb Vasc Biol, 25*(6), 1135-1141.

Szolnoki, Z., Havasi, V., Talian, G., Bene, J., Komlosi, K., Somogyvari, F., et al. (2006). Angiotensin II type-1 receptor A1166C polymorphism is associated with increased risk of ischemic stroke in hypertensive smokers. *J Mol Neurosci, 28*(3), 285-290.

Tamama, K., Sen, C.K., & Wells, A. (2008). Differentiation of Bone Marrow Mesenchymal Stem Cells into the Smooth Muscle Lineage by Blocking ERK/MAPK Signaling Pathway. *Stem Cells and Development, 17*(5), 897-908.

Tan, K.S., Armugam, A., Sepramaniam, S., Lim, K.Y., Setyowati, K.D., Wang, C.W., et al. (2009). Expression profile of MicroRNAs in young stroke patients. *PLoS One, 4*(11), e7689.

Tang, Y., Xu, H.C., Du, X.L., Lit, L., Walker, W., Lu, A.G., et al. (2006). Gene expression in blood changes rapidly in neutrophils and monocytes after ischemic stroke in humans: a microarray study. *Journal of Cerebral Blood Flow and Metabolism, 26*(8), 1089-1102.

Thellin, O., ElMoualij, B., Heinen, E., & Zorzi, W. (2009). A decade of improvements in quantification of gene expression and internal standard selection. *Biotechnol Adv, 27*(4), 323-333.

Thum, T., Catalucci, D., & Bauersachs, J. (2008). MicroRNAs: novel regulators in cardiac development and disease. *Cardiovasc Res, 79*(4), 562-570.

Ueda, R., Kohanbash, G., Sasaki, K., Fujita, M., Zhu, X., Kastenhuber, E.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. *Proc Natl Acad Sci U S A, 106*(26), 10746-10751.

Urbich, C., Kuehbacher, A., & Dimmeler, S. (2008). Role of microRNAs in vascular diseases, inflammation, and angiogenesis. *Cardiovasc Res, 79*(4), 581-588.

Use of anti-ICAM-1 therapy in ischemic stroke: results of the Enlimomab Acute Stroke Trial. (2001). *Neurology, 57*(8), 1428-1434.

Valledor, A.F., Borras, F.E., Cullell-Young, M., & Celada, A. (1998). Transcription factors that regulate monocyte/macrophage differentiation. *Journal of Leukocyte Biology, 63*(4), 405-417.

VanGuilder, H.D., Vrana, K.E., & Freeman, W.M. (2008). Twenty-five years of quantitative PCR for gene expression analysis. *Biotechniques, 44*(5), 619-626.

Varnholt, H. (2008). The role of microRNAs in primary liver cancer. *Ann Hepatol, 7*(2), 104-113.

Visone, R., Petrocca, F., & Croce, C.M. (2008). Micro-RNAs in gastrointestinal and liver disease. *Gastroenterology, 135*(6), 1866-1869.

Vogelgesang, A., Grunwald, U., Langner, S., Jack, R., Broker, B.M., Kessler, C., et al. (2008). Analysis of lymphocyte subsets in patients with stroke and their influence on infection after stroke. *Stroke, 39*(1), 237-241.

Walter, D.H., Rittig, K., Bahlmann, F.H., Kirchmair, R., Silver, M., Murayama, T., et al. (2002). Statin therapy accelerates reendothelialization - A novel effect involving mobilization and incorporation of bone marrow-derived endothelial progenitor cells. *Circulation, 105*(25), 3017-3024.

Wandzioch, E., Edling, C.E., Palmer, R.H., Carlsson, L., & Hallberg, B. (2004). Activation of the MAP kinase pathway by c-Kit is PI-3 kinase dependent in hematopoietic progenitor/stem cell lines. *Blood, 104*(1), 51-57.

Wang, A.M., Doyle, M.V., & Mark, D.F. (1989). Quantitation of mRNA by the polymerase chain reaction. *Proc Natl Acad Sci U S A, 86*(24), 9717-9721.

Wang, K.C., Garmire, L.X., Young, A., Nguyen, P., Trinh, A., Subramaniam, S., et al. (2010). Role of microRNA-23b in flow-regulation of Rb phosphorylation and endothelial cell growth. *Proc Natl Acad Sci U S A, 107*(7), 3234-3239.

Warren, A.J., Colledge, W.H., Carlton, M.B., Evans, M.J., Smith, A.J., & Rabbitts, T.H. (1994). The oncogenic cysteine-rich LIM domain protein rbtn2 is essential for erythroid development. *Cell, 78*(1), 45-57.

Weinberg, R.A. (1995). The retinoblastoma protein and cell cycle control. *Cell, 81*(3), 323-330.

Whiteley, W., Lowe, G.D., Rumley, N., Wardlaw, J.M., Green, V., Welsh, P., et al. (2010). Blood Markers and the Diagnosis of Stroke or Transient Ischaemic Attack in the Emergency Department: A Prospective Cohort Study. *Journal of Neurology Neurosurgery and Psychiatry, 81*(11), E28-E29.

Wu, Y., Ip, J.E., Huang, J., Zhang, L., Matsushita, K., Liew, C.C., et al. (2006). Essential role of ICAM-1/CD18 in mediating EPC recruitment, angiogenesis, and repair to the infarcted myocardium. *Circ Res, 99*(3), 315-322.

Xu, H., Tang, Y., Liu, D.Z., Ran, R., Ander, B.P., Apperson, M., et al. (2008). Gene expression in peripheral blood differs after cardioembolic compared with large-vessel atherosclerotic stroke: biomarkers for the etiology of ischemic stroke. *J Cereb Blood Flow Metab, 28*(7), 1320-1328.

Yang, H., Kong, W., He, L., Zhao, J.J., O'Donnell, J.D., Wang, J., et al. (2008). MicroRNA expression profiling in human ovarian cancer: miR-214 induces cell survival and cisplatin resistance by targeting PTEN. *Cancer Res, 68*(2), 425-433.

Yang, W.J., Yang, D.D., Na, S., Sandusky, G.E., Zhang, Q., & Zhao, G. (2005). Dicer is required for embryonic angiogenesis during mouse development. *J Biol Chem, 280*(10), 9330-9335.

Yoon, S.S., & Zheng, Z.J. (2005). Elevated total white blood cell count with high blood glucose is associated with poor outcome after ischemic stroke. *J Stroke Cerebrovasc Dis, 14*(2), 88-93.

Yu, J., deMuinck, E.D., Zhuang, Z., Drinane, M., Kauser, K., Rubanyi, G.M., et al. (2005). Endothelial nitric oxide synthase is critical for ischemic remodeling, mural cell recruitment, and blood flow reserve. *Proc Natl Acad Sci U S A, 102*(31), 10999-11004.

Yuan, Y., Wang, J.Y., Xu, L.Y., Cai, R., Chen, Z., & Luo, B.Y. (2010). MicroRNA expression changes in the hippocampi of rats subjected to global ischemia. *J Clin Neurosci, 17*(6), 774-778.

Zhang, W., & Liu, H.T. (2002). MAPK signal pathways in the regulation of cell proliferation in mammalian cells. *Cell Res, 12*(1), 9-18.

Zhao, S., Consoli, U., Arceci, R., Pfeifer, J., Dalton, W.S., & Andreeff, M. (1996). Semi-automated PCR method for quantitating MDR1 expression. *Biotechniques, 21*(4), 726-731.

Zhao, Y., Samal, E., & Srivastava, D. (2005). Serum response factor regulates a muscle-specific microRNA that targets Hand2 during cardiogenesis. *Nature, 436*(7048), 214-220.

Zinn, K., DiMaio, D., & Maniatis, T. (1983). Identification of two distinct regulatory regions adjacent to the human beta-interferon gene. *Cell, 34*(3), 865-879.