Correlation of Adrenomedullin gene expression in peripheral blood leukocytes with severity of ischemic stroke

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Human adrenomedullin (ADM), a 52-amino acid peptide, belongs to the calcitonin/calcitonin gene-related peptide (CGRP)/amylin peptide family. ADM acts as a multifunctional regulatory peptide and is upregulated in response to hypoxia. Previous microarray studies have found increased ADM gene (ADM) expression in peripheral blood cells of patients with stroke, however, it is unknown if an increased ADM level is correlated with severity of human ischemic stroke. This study investigated ADM expression in peripheral blood leukocytes (PBL) of healthy controls and subjects at day 1, week 1 and week 3 postacute ischemic stroke using rtPCR methodology. We found that ADM expression was significantly upregulated on the first day of stroke compared to the healthy subjects and the disease controls; the levels remained elevated for up to week 3. Further, ADM expression at day 1 was correlated with stroke severity measured by the National Institute of Health Stroke Scale (NIHSS), the modified Barthel Index (mBI) and the modified Rankin Scale (mRS). This could indicate that ADM expression level is related to the severity of tissue damage. We suggest that increased ADM expression in PBL after acute ischemic stroke is most likely to indicate that these cells have been subjected to hypoxia and that the magnitude of expression is likely to be related to the volume of hypoxic tissue. Hypoxia can affect lymphocytes function and could affect the immune response to stroke. The correlation of ADM expression level with the measures of stroke severity implicates ADM – a potential blood bio-marker in studies of ischemic stroke.

KEYWORDS: gene expression, rtPCR, microarray, NIHSS, mBI, mRS

Introduction

Acute ischemic stroke is resultant from a sudden loss of blood supply to focal areas of the brain. This leads to cell death and local inflammation. Systemic responses induced by ischemic stroke include modulation of the immune system and alteration of gene expression in peripheral blood cells [1–3].

Adrenomedullin (ADM) is a 52 amino acid peptide, belongs to the calcitonin/CGRP (calcitonin gene-related peptide)/amylin peptide family. The ADM gene (ADM) is located on chromosome 11 with a single locus [4]. ADM was originally isolated from human pheochromocytomas [5,6]. The ADM product exists in plasma and a variety of tissues including blood vessels, heart and lungs [7], however, measurement of the ADM protein presents difficult due to a short half-life [8,9]. The biological activities of ADM has been investigated intensively and include vasodilation, diuresis and natriuresis, positive inotropic effect, inhibition of endothelial cell apoptosis, induction of angiogenesis, inhibition of cardiomyocyte apoptosis, suppression of aldosterone production, anti-inflammatory activity and antioxidant activity [7].

ADM has been shown to be protective in ischemic stroke. In a rat model, postischemic infusion of ADM has been shown to protect against ischemic stroke by inhibiting apoptosis and promoting angiogenesis [10]. ADM also plays important autocrine and paracrine roles by regulating circulation and endothelial blood-brain barrier functions [11]. Evidence from cell culture and animal model studies have shown that ADM has...
protective effects against vascular injury, including the effects of oxidative stress [7]. Increased expression of both ADM and a component of the ADM receptor, Calcitonis-receptor-like receptor (CRLR), have been found in cultured endothelial cells under hypoxic conditions [7,11]. Treatment with ADM has been thought to limit infarct size through mitochondrial KCa in PKA signaling pathways [12].

A study using the microarray technique examined gene expression profile in peripheral blood mononuclear cells of human acute ischemic stroke and showed that ADM level was increased [13]. Further studies have shown that ADM level is regulated by hypoxia-inducible factor-1 in other diseases or tissues [14–16].

It is unknown if endogenous ADM levels are correlated with stroke severity posts ischemia. Our study aimed to investigate ADM expression in peripheral blood leukocytes (PBL) in patients at day 1, week 1 and week 3 after acute ischemic stroke using rtPCR, and correlate this with stroke severity, as measured by the most common stroke severity scales the National Institute of Healthy Stroke Scale (NIHSS) (http://www.ninds.nih.gov/doctors/NICH_Stroke_Scale.pdf) [17] and with disability using the modified Barthel Index (mBI) and the modified Rankin Scale (mRS) [18–21]. The NIHSS measures the impairment of a patient’s ability caused by stroke using 42 scores, with a low score correlating to lesser symptoms and a high score correlating with more severe stroke symptoms. The mBI is a modification of the original Barthel Index initially developed in 1965 [18,19]. The mBI measures the daily performance of patients in a range from 0–100, with high score showing better performance. The mRS measures patient’s independence, not performance, in 6 grades, from 0 to 5, with low score corresponding to better symptoms.

Our results indicated that the ADM RNA level at the initial stroke period is correlated with severity compared to healthy controls and the subjects with motor neurone disease (MND), and suggests that ADM expression level at day 1 poststroke may be a potential blood bio-marker in studies of ischemic stroke.

**Material and methods**

**Participants**

The study design and protocol was approved by the Human Research Ethics Committees of the Royal Brisbane and Women Hospital, the Wesley Hospital and the Medical Research Ethics Committees of the University of Queensland. All participants provided informed consent. Detail of patients and controls are summarized in Table 1.

Stroke patients were recruited from the Royal Brisbane and Women Hospital and Wesley Hospital, Brisbane, Australia, between August 2008 and December 2011. Ischemic stroke was diagnosed on clinical history, presence of focal neurological signs and symptoms, and by MRI or CT brain scans. The strokes were classified according to the Oxfordshire Stroke Classification [22] into total anterior circulation infarcts (TACI), partial anterior circulation infarcts (PACI), posterior circulation infarcts (POCI) and lacunar infarcts (LACI). Patient stroke severity was evaluated on day 1, week 1 and week 3 by NIHSS, mBI and mRS. Demographic data was collected from the patient or their family members.

Blood was collected from stroke subjects at three time points: day 1, 7–10 d (referred as Week 1 in the text) and 3–6 weeks (referred as Week 3 in the text) after stroke. We also recruited age and sex matched healthy subjects and the patients with MND, recruited from the MND clinic, as a disease control. Blood from these subjects was collected only once. In total, samples from 44 patients with acute ischemic stroke, 19 healthy subjects and 27 patients with MND were studied (Table 1).

<table>
<thead>
<tr>
<th>Table 1. General condition of participants.</th>
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<tbody>
<tr>
<td><strong>Acute ischemic stroke</strong></td>
</tr>
<tr>
<td><strong>Number</strong></td>
</tr>
<tr>
<td><strong>Total</strong></td>
</tr>
<tr>
<td><strong>Female</strong></td>
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<tr>
<td><strong>Male</strong></td>
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<tr>
<td><strong>Median age in years (range)</strong></td>
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<tr>
<td><strong>Hypertension N (%)</strong></td>
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<td><strong>Hyperlipidemia N (%)</strong></td>
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<td><strong>Smoking N (%)</strong></td>
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<tr>
<td><strong>NIHSS median (range)</strong></td>
</tr>
<tr>
<td><strong>mBI median (range)</strong></td>
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<tr>
<td><strong>mRS median (range)</strong></td>
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</tbody>
</table>

N.A. = not applicable
Not all stroke patients provided blood samples at all 3
time points: 24 patients provided blood samples on day 1
after onset, 37 patients at week 1 and 34 patients at
week 3.

RNA extraction and first strand cDNA
synthesis
Peripheral blood (2.5 ml) was collected into PAX-
geneTM Blood RNA tubes (Qiagen, Australia). The
blood RNA tubes were left at room temperature for 8 h
and then kept in −20 °C until RNA extraction (up to
36 months). RNA was extracted using a PAXgeneTM
Blood RNA Extraction Kit (PreAnalytiX, Qiagen, Aus-
tralia). RNA concentration was determined by the Nano-
drop and RNA quality was determined using the Expe-
rionTM Automated Electrophoresis System (Bio-Rad,
Australia). All RNA samples used in this study had a
RNA quality index >7.

First strand cDNA was synthesized using an RT2
Easy first strand synthesis kit (PreAnalytiX, Qia-
gen, Australia). The final concentration of extracted
RNA samples was adjusted to 125 ng/μl. First strand
cDNA synthesis reactions were conducted with 8 μl of
125 ng/μl RNA samples, according to the manufacture’s
protocol (SABiosciences, Qiagen, Australia). Briefly the
samples were incubated at 42°C for 5 min, followed by
incubation at 42°C for 15 min and 95°C for 5 min for
the reverse transcription.

qPCR array
Customized qPCT plate and the primers for ADM gene
were prepared by SABiosciences (Qiagen, Australia).
SYBER green master mix including Taq polymerase was
activated at 95°C for 10 min. Thermal cycling was done
using an iQ5 qPCR machine (Bio-Rad. Australia) with
40 cycles at 95°C for 15 s and 60°C for 1 min. β-actin
was used to normalize the sample loading. The thresh-
old value of each reaction and the quality of controls
were analyzed using Web-based software, supplied by
SABiosciences.

Quantification of ADM serum level
A commercial enzyme linked immune-sorbent assay
kit (ADM EIA kit, Phoenix pharmaceuticals, Inc, Califor-
ia, USA) was used to detect the serum level of ADM.
The sequence of the kit detected was Tyr – Arg – Gln –
Ser – Met – Asn – Asn – Phe – Gln – Gly – Leu – Arg
– Ser – Phe – Gly – Cys – Arg – Phe – Gly – Thr – Cys
– Thr – Val – Gln – Lys – Leu – Ala – His – Gln – Ile –
Tyr – Gln – Phe – Thr – Asp – Lys – Asp – Lys – Asp
Pro – Gln – Gly – Tyr – NH2. The serum samples were
tested in a serial dilution in preliminary experiments. As
the level of ADM was low, we decided to use undiluted
serum in the experiment. A standard curve and negative
controls were included in each plate. A 50 μl of undi-
luted serum from each patient and healthy control was
added in triplicate. The assay was carried out according
to manufacturer’s instruction.

Flow cytometry of PBL
Cell populations (T cells, B cells and memory and naive
cells) of PBL were analyzed by flow cytometry according
to our previously published methods [3].

Statistical analysis
Statistical analysis was performed using InStat version
4 (GraphPad Software, San Diego, California, USA).
Normality tests were performed to determine whether
data normally distributed. If data were normally dis-
btributed, then mean values ± SE are shown, pairs of
data were compared using student’s t-test, and ANOVA
was used to compare more than three groups. If analy-
ysis of the group as a whole showed a significant dif-
ference (p < 0.05), then the Bonferroni was used as a
posttest to compare pairs of group. If the data were not
normally distributed, data is presented as median ± in-
terquartile range, pairs of data were compared used the
Mann–Whitney u-test and three or more groups were
compared by Kruskal–Wallis test. Spearman’s rank cor-
relation coefficient test, which gives a Spearman r value,
was performed to assess the degree of correlation be-
tween ADM expression fold-changes with age and gen-
der, and severity of stroke patients at each time point
following stroke. Correlation test and nonlinear regres-
sion (curve fit) were used for analysis of the association
between ADM expression level with NIHSS, mBI and
mRS scores. The slope of the curves of male and female
was compared to determine if there was significant dif-
ference in different gender, or between total stroke and
gender.

Results
Increased ADM gene expression in PBL
of stroke patients compared to controls
ADM expression was examined in the samples col-
clected from patients after stroke, patients with MND
and healthy controls. Since the data were not normally
distributed across all groups, the median fold-changes
were compared between the groups. There was a highly
statistically significant elevation in ADM mRNA levels
at day 1 after stroke compared to healthy controls and

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patients with MND and this was maintained at week 1 and week 3 (Figure 1). The elevated ADM expression level in stroke was not affected by gender or age (data not shown).

**ADM expression levels in different stroke types**

Stroke patients were classified according to the Oxfordshire Stroke Classification (OSSC) as TACI, PACI, LACI and POCI [22]. The patients with LACI were excluded due to small sample size. ADM expression levels in each type of stroke was all significantly different from healthy controls at three time points; patients with TACI, the most severe clinical stroke group showed the greatest increase in ADM expression levels at week 1 and week 3, and these were significantly different to healthy controls ($p < 0.01$) (Figure 2).

**Correlation of ADM expression with measures of stroke severity and disability**

The severity of stroke was assessed using the NIHSS, the mBI and the mRS for the stroke subjects. Figure 3 shows that patients with more severe disease and/or greater disability had significantly high ADM fold-changes at day 1 after stroke ($p < 0.006$ for NIHSS, $r = 0.5553; p = 0.004$ for mBI, $r = 0.5719$ and $p < 0.001$ for mRS, $r = 0.7023$), and remained high at week 1 ($p = 0.006$ for NIHSS, $r = 0.4665; p < 0.027$ for mBI, $r = 0.3861$ and $p = 0.035$ for mRS, $r = 0.6657$). The correlations of stroke severity

![Figure 1. Fold-change in expression of the ADM gene. Box and Whisker plots showing fold-change in gene expression of ADM. Box extends from 25% to 75% percentile, with horizontal line at median (50% percentile). Whiskers extend down to smallest value and up to largest value. Stroke samples at all time points showed significant fold-change compared to healthy controls (HEL) or patients with MND. $***p < 0.001$.](image1.png)

![Figure 2. ADM gene expression in subjects classed with OSSC. ADM expression levels in stroke patients at day 1, week 1 and week 3 (grouped according to OSSC) compared to healthy controls (HEL). Bars represent median ± interquartile range. Significant differences were found between stroke groups and healthy controls for each time point. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. There were no significant difference when compared within the stroke groups, for example, between TACI and POCI.](image2.png)
with NIHSS, mBI and mRS were also analyzed against gender (Figures 3 and 4; Tables 2 and 3). The correlations between ADM fold-change and NIHSS, mBI or mRS in week 3 patients had reduced significance (Table 2).

**Possibility of ADM as a prognosis marker of stroke**

In order to assess if ADM can be a prognostic biomarker, we correlated ADM expression on day 1 with the clinical severity score measured on week 1 and 3. Figure 4 and Table 3 show that ADM expression on day 1 stroke had significant positive correlation with NIHSS and mRS week 1 and 3 scores, and negative correlation with mBI. These results indicated severe stroke insult induces high ADM expression at the earliest stage of stroke. Alternatively, early ADM expression levels are correlated to stroke severity and appear prognostic of longer term disability.

**ADM peptide levels**

We measured the levels of ADM peptide in the blood of 15 healthy subjects and 21 subjects sampled at day 1, day 7 and week 3 after stroke. There was no significant difference between the levels of healthy subjects and patients with stroke (data not shown).

**Flow cytometry analysis**

To exclude the possibility that the changes in gene expression are due to changes in the circulating cell populations, we measured the total CD3+ T cells, CD20+ B cells, CD45RO+, CD45RA+ memory and naïve cell populations (Figure 5). There were no significant differences between stroke subjects at day 1, week 1 and week...
Figure 4. Correlation of ADM gene expression on day 1 with stroke severity scores on week 1 and week 3. The scores of NIHSS, mBI and mRS measured at week 1 and week 3 versus ADM expression levels on day 1. Statistically significant of the correlations (the $p$ values) were calculated by Spearman’s rank correlation in GraphPad Prism. The lines, which indicating the trend of the correlation, were done by nonlinear regression in GraphPad Prism.

3 stroke compared to healthy controls. There were no significant differences in DC and NK cell populations between stroke and healthy controls (data not shown).

**Discussion**

In this study, we have investigated expression of ADM in PBLs of subjects with acute ischemic stroke. We studied the levels of ADM expression over time and also correlated the expression of ADM with severity of stroke. In accordance with previously published microarray data [13], we observed an increased ADM gene expression in PBL of stroke patients using an rtPCR technique. Examining ADM expression over a 3-week period allowed the observation of the temporal profile of ADM expression. Notably, levels remained significantly increased from day 1 for 3 weeks after stroke, suggesting that ADM expression occurs as an early event postinsult, and participate in the responses induced by stroke over at least the next three weeks.

It is known that gender and age can influence gene expression within blood [23]. In our study, these confounding factors have been excluded through the

| Table 2. ADM level versus stroke severity measured on each time point. |
|-----------------------------|-----------------|-----------------|-----------------|
|                            | Day 1           | Week 1          | Week 3          |
|                            | Total Female    | Male            | Total Female    | Male            | Total Female    | Male            |
| NIHSS                      |                 |                 |                 |
| Spearman R                | 0.555           | 0.343           | 0.511           | 0.467           | 0.806           | 0.280           |
| ($p$ value)               | (0.006)         | (ns)            | (ns)            | (0.006)         | (0.003)         | (ns)            |
| mBI                        |                 |                 |                 |
| Spearman R                | −0.572          | −0.708          | −0.477          | −0.386          | −0.676          | −0.299          |
| ($p$ value)               | (0.004)         | (0.033)         | (ns)            | (0.027)         | (0.022)         | (ns)            |
| mRS                        |                 |                 |                 |
| Spearman R                | 0.702           | 0.756           | 0.662           | 0.371           | 0.504           | 0.344           |
| ($p$ value)               | (0.001)         | (0.03)          | (0.019)         | (0.037)         | (ns)            | (ns)            |

ns = not significant
Table 3. ADM expression level on day 1 versus stroke severity measured on week 1 and week 3.

<table>
<thead>
<tr>
<th></th>
<th>Day 1/week 1</th>
<th>Day 1/week 3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total</td>
<td>Female</td>
</tr>
<tr>
<td>NIHSS</td>
<td></td>
<td>0.555</td>
</tr>
<tr>
<td>Spearman R</td>
<td>(p value)</td>
<td>(0.006)</td>
</tr>
<tr>
<td>mBI</td>
<td></td>
<td>−0.606</td>
</tr>
<tr>
<td>Spearman R</td>
<td>(p value)</td>
<td>(0.004)</td>
</tr>
<tr>
<td>mRS</td>
<td></td>
<td>0.666</td>
</tr>
<tr>
<td>Spearman R</td>
<td>(p value)</td>
<td>(0.004)</td>
</tr>
</tbody>
</table>

ns = not significant

correlation of ADM expression levels with the gender and age of stroke subjects. There was no significant difference between females and males or any differences among different age groups of patients.

We have also correlated ADM levels with stroke severity, measured as NIHSS and patient disability measured as the mBI and mRS scores. One limitation of the study is that we chose to analyze the correlation in consideration of stroke severity scores as continuous variables, although these are ordinal variables. There was a significant positive correlation on day 1 and week 1 with NIHSS and mRS, and negative correlation at the same time points with mBI (Figure 3 and Table 2) potentially indicating that ADM expression occurs as an adaptive response to hypoxic tissue injury. Our results found that the ADM fold-change was influenced by the type of stroke.
of ischemic stroke (Figure 2). It was observed that the most severe stroke subtype TACI has the highest expression level at week 1 and week 3 points following stroke (Figure 2). These results suggested that ADM expression could be related to stroke severity. Furthermore, the r values for the ADM levels and stroke severity scores measured at week 1 and week 3 indicated a strong correlation of ADM level to the stroke severity and disability.

It is possible that the changes in gene expression in PBL are due to exposure of these cells to hypoxic environments in the region of the stroke. It has been reported that ADM expression is regulated by hypoxia in rat ventricular cardiac myocytes [15], implying tissue ischemia and inflammation response related activity. Although we have reported that there are increased levels of activated T cells in subjects with stroke [3,24], there was no difference in the overall percentages of cell populations in stroke compared to healthy controls. This suggests that increased ADM gene expression was not affected by variation of cell populations. We do not think that death of neurons alone is the cause of increased ADM expression because there was no significant difference between the subjects with MND, another severely disabling neurological disease and the healthy controls.

This increased ADM expression could possibly be a neuro-protective mechanism in line with current literature [25,26]. A key function of the ADM product is to produce substantial vasodilation of arterioles [5, 27]. In an animal model, ADM-induced vasodilation has been suggested to occur through the binding of the CGRP receptor and ADM-R [28,29]. ADM has also been shown to inhibit reactive oxygen species (ROS) in ADM knock-out heterozygous mice [26]. Additionally, in a rabbit model, the neuroprotective mechanisms of ADM has been suggested to function through the cAMP/PKA-mediated priming of mitochondrial Calcium activated Potassium (mitoKca) channels [12,26].

There is a need for a biomarker of stroke. The optimal blood bio-marker should be rapidly detected, be sensitive and specific. Thus, far there are over 58 proteins and 7 panels of proteins which have been described to possess the features to be potential biomarkers for the diagnosis and prognosis of ischemic stroke [30–33]. However, several studies have described the possibility of using peripheral blood gene expression in the diagnosis in stroke [34–36], and the advantages of using a RNA biomarker in stroke have also been considered. Gene transcription is affected by stroke rapidly; it can occur within minutes, whereas protein translation occurs later than gene transcription. Another reason for considering marker of gene expression is that, circulating proteins present detection and accuracy issues [36]. RNA expression in possible ischemic stroke patients has the potential to yield a sensitive and specific marker for measurement.

The clinical observation of an early response ADM expression in our study, has suggested that it could be a potential bio-marker of ischemic stroke. The correlation of the ADM expression levels on day 1 with the stroke severity scores measured on week 1 and week 3 was to examine if early stroke-induced ADM expression levels can predict disease severity. The results have shown that high ADM level on day 1 was correlated with worse stroke (Figure 4 and Table 3).

ADM protein expression has previously been recommended as a biomarker for the other disease [37,38]. However, despite expression levels, we found no significant difference in levels of ADM peptide in the serum of subjects with stroke and healthy controls. This could potentially be due to the rapid degradation or utilization of ADM in the circulation. Another possibility is that there are many sources of ADM and increased ADM produced by hypoxic leukocytes might not be sufficient to change the overall systemic levels. ADM is released from pro-ADM, and methods for ADM or pro-ADM serum levels using radioimmunoassay or an immunoluminometric assay have been described [8,9]. It is possible that pro-ADM measurements may differ between stroke patients and controls. It would be worth investigation in the future.

**Conclusion**

The novel contribution of our results to the study of ADM in ischemic stroke includes the time-course of ADM expression and correlation with clinical features. The potential of ADM as a clinical blood prognostic marker for ischemic stroke was also explored. In summary, elevated expression of ADM postischemic stroke was observed, and this was correlated with stroke severity and disability. Further, there is potential for consideration of ADM expression as a useful clinical biomarker. Future investigations aim to examine whether hypoxia is the causative factor in increased ADM expression in leukocytes postischemic stroke.

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**Declaration of Interest**

No conflict of interest declared. The authors alone are responsible for the content and writing of this paper.
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