A microarray study of gene and protein regulation in human and rat brain following middle cerebral artery occlusion

Nick Mitsios†1, Mohamad Saka†1, Jerzy Krupinski2, Roberta Pennucci1, Coral Sanfeliu3, Qiuyu Wang1, Francisco Rubio2, John Gaffney1, Pat Kumar1, Shant Kumar4, Matthew Sullivan1 and Mark Slevin*1

Address: 1School of Biology, Chemistry and Health Science, John Dalton Building, Manchester Metropolitan University, Chester Street, Manchester, UK, 2Department of Neurology, Stroke Unit, Hospital Universitari de Bellvitge (HUB), Fundacio IDIBELL, L’ Hospitalet de Llobregat, Barcelona, Spain, 3Departamento de Farmacologia i Toxicologia, Institut d’ Investigacions Biomediques de Barcelona (IIBB), CSIC-IDIBAPS, Barcelona, Spain and 4Department of Pathology, Medical School, University of Manchester and Christie Hospital, Manchester, UK

Email: Nick Mitsios - n.mitsios@mmu.ac.uk; Mohamad Saka - myassin3@hotmail.com; Jerzy Krupinski - krupinski@csub.scs.es; Roberta Pennucci - camilbea@hotmail.com; Coral Sanfeliu - csfpat@iibb.csic.es; Qiuyu Wang - q.wang@mmu.ac.uk; Francisco Rubio - frubio@cebas.csic.es; John Gaffney - j.gaffney@mmu.ac.uk; Pat Kumar - p.kumar@mmu.ac.uk; Shant Kumar - shant.kumar@man.ac.uk; Matthew Sullivan - m.sullivan@mmu.ac.uk; Mark Slevin* - m.a.slevin@mmu.ac.uk

* Corresponding author    †Equal contributors

Abstract

Background: Altered gene expression is an important feature of ischemic cerebral injury and affects proteins of many functional classes. We have used microarrays to investigate the changes in gene expression at various times after middle cerebral artery occlusion in human and rat brain.

Results: Our results demonstrated a significant difference in the number of genes affected and the time-course of expression between the two cases. The total number of deregulated genes in the rat was 335 versus 126 in the human, while, of 393 overlapping genes between the two array sets, 184 were changed only in the rat and 36 in the human with a total of 41 genes deregulated in both cases. Interestingly, the mean fold changes were much higher in the human. The expression of novel genes, including p21-activated kinase 1 (PAK1), matrix metalloproteinase 11 (MMP11) and integrase interactor 1, was further analyzed by RT-PCR, Western blotting and immunohistochemistry. Strong neuronal staining was seen for PAK1 and MMP11.

Conclusion: Our findings confirmed previous studies reporting that gene expression screening can detect known and unknown transcriptional features of stroke and highlight the importance of research using human brain tissue in the search for novel therapeutic agents.

Background

Ischaemic stroke results from obstruction of blood flow in a major cerebral vessel and leads to deregulation of genes whose expression promotes ischemic neuronal death and subsequent neurological dysfunction [1,2]. Under ischemic conditions, energy metabolism fails, and severe reduction in mRNA and protein synthesis occurs in the ischemic core region. The tissue surrounding this area (peri-infarcted region) is able to maintain some functions, such as ionic homeostasis and can be partially salvaged by blood recirculation [3,4].
The precise molecular mechanisms involved in ischemia-induced brain injury remain poorly understood. Limited knowledge of the molecular mechanisms involved in tissue regeneration has been gained from animal experiments using the middle cerebral artery occlusion (MCAO) model which replicates, in many aspects, the neuropathological changes following stroke in humans [5]. Although the contralateral side of the brain is not totally unaffected by ischemic damage, the collection of experimental and reference control tissue from the same animal is a better comparison than using sham-operated control in rats, while in human samples the only control tissue available is contralateral hemisphere. In addition, using contralateral tissue as a control and the direct comparison with stroke hemisphere provides the best model for validation as it removes inter-patient genetic variation and also minimises the differences in potential degradation between the target and reference mRNAs. This has been applied previously in both human [6,7] and animal [8-10] studies. Rao et al. in particular observed very few differences in gene expression between sham and contralateral cortex at 24 h of reperfusion following MCAO in the rat [9].

Analysis of ischemic brain tissue with techniques capable of studying multiple transcripts simultaneously can identify gene expression changes previously not known to be implicated in ischemic pathophysiology and may lead to development of new targets for stroke therapy [11]. DNA microarray technology has been used to investigate the expression of thousands of genes in a single hybridization experiment. Several experimental studies have examined alteration of gene expression in the posts ischemic rat brain using microarray technology [8-10,12-18], while blood genomic profiling in human stroke have been investigated in recent pilot studies [19,20] (Table 1). Critical comparison of gene expression profiles after stroke in humans with those in animal models may lead to a better understanding of the pathophysiology of brain ischaemia and allow an evaluation of the usefulness of animal models in stroke research.

Until recently, gene expression profiling had not been applied to patients dying of ischemic stroke, in part because human brain autopsies are not regularly obtained. Although tissue obtained from brain autopsies is generally of lower quality than that of brain biopsies obtained from living patients, the majority of RNA transcripts and proteins in the human brain are reasonably stable (compared to other tissues such as blood and kidney) and degrade to only a minor degree following death, thus making autopsy tissue a useful source for the isolation of nucleic acids and proteins [21]. Previous studies evaluating the mRNA quality in human post-mortem brain tissue have demonstrated a minimal effect upon their overall relative stability and indicated that frozen brains up to 72 hours post-mortem can be efficiently analyzed [22]. In line with that, in previous human brain studies, tissue was obtained up to a maximum of 6 hours [7,40] hours [23], 45 hours [24] and 69 [6] hours following death. Moreover, after comparing mRNA levels in autopsies and biopsies, Castensson et al. [25] found a general similarity in the levels between the two groups, and suggested that mRNA levels in brain autopsy samples can provide clues about the brain in vivo. Interestingly, Almeida et al. [26] found that, even if performed on degraded RNA, RT-PCR can be used to provide a reliable estimate of in vivo mRNA levels, maybe due to the similarities in the rates of degradation between the target and reference mRNAs. Recently, Vikman and Edvinsson [27] investigated the gene expression in human brain after ischaemia using samples 7–10 days post-stroke; however, they obtained their samples after a considerable delay of 2–3 days post-mortem and they focused mainly on mRNA expression of receptors.

To identify the genes whose expression was changed in the human brain following ischaemia, we investigated the dynamic changes in gene expression in brain samples (collected within 6 h of death) from patients with various times of survival (2–37 days; Table 2) following stroke and compared them with those at various time-points (1 hour – 21 days) following middle cerebral artery occlusion (MCAO) in rats. The Atlas 1.2 cDNA microarray was used to screen for differential expression of 1176 genes and significantly de-regulated genes were selected through image analysis. We further investigated whether the altered mRNA and protein levels of a subset of deregulated molecules in the postischemic brain could be reproduced in an in vitro model of neuronal and endothelial cell culture under conditions of oxygen-glucose deprivation (OGD). The findings confirmed previous studies reporting that parallel screening of gene expression can detect both previously documented and novel transcriptional features of the cerebral response to ischemia, and demonstrated significant differences in gene expression between human stroke and the animal model.

**Results**

**cDNA microarray analysis**

The expression of ischemia-related genes was determined by comparing the infarct-induced expression (combined samples from infarcted and peri-infarcted areas) to that in the contralateral hemisphere: 77, 92 and 15 genes were de-regulated in stroke-affected regions in the 3 patient survival groups respectively, while 9, 51, 48, 166, 253, 117 and 261 genes were altered at the 7 different time-points in the animal model compared to the controls (Figure 1). The combined number of differentially expressed transcripts in stroke patients represented 6.5%, 7.8% and 1.3% respectively in each survival group of the total...
Table 1: Previous studies employing microarray approaches to study stroke

| Material used         | Model of ischemia                     | No of genes | Time after ischemia | Cut-off values | Confirmation of results |
|-----------------------|---------------------------------------|-------------|---------------------|----------------|-------------------------|
| Soriano et al. 2000   | Permanent focal MCAO                  | 750         | 3 hours             | 2.0-fold       | In situ hybridization, western blotting, immuno-histochemistry |
| Jin et al. 2001       | Transient focal MCAO                  | 374         | 4 hours             | 1.7-fold       | Real-time PCR, antisense knockdown, western blotting, immuno-histochemistry |
| Kim et al. 2002       | Permanent focal MCAO                  | 1176        | 6 hours             | 2.0-fold       | Microarray analysis only |
| Rao et al. 2002       | Transient focal MCAO                  | 1263        | 6 hours             | 2.5-fold       | Real-time RT-PCR        |
| Schmidt-Kastner et al. 2002 | Permanent focal MCAO               | 9044        | 5 hours             | 1.7-fold       | Cell culture, in situ hybridization, western blotting, immuno-fluorescence |
| Tang et al. 2002      | Transient focal MCAO                  | −8,000      | 24 hours            | 2.0-fold       | Real-time RT-PCR        |
| Roth et al. 2003      | Permanent focal MCAO                  | −13,000     | 24 hours            | 0.5-fold       | Microarray analysis only |
| Kim et al. 2004       | Transient focal MCAO                  | 5,000       | 5 hours             | 3.0-fold       | Microarray analysis only |
| Lu et al. 2004        | Blood from ischemic stroke patients   | 1,322       | 1 hours             | 2.0-fold       | Real-time PCR, immuno-histochemistry |
| Moore et al. 2005     | Permanent and transient focal MCAO    | −19,000     | As soon as possible after hospitalization | -             | Cell culture, RT-PCR, western blotting, immuno-histochemistry, immuno-fluorescence |
| Ford et al. 2006      | Blood from ischemic stroke patients   | 8784        | 24 hours            | 2.0-fold       | -                       |
| Tang et al. 2006      | Post-mortem brain tissue from 11 stroke patients | 39,000      | 3 hours             | 1.5-fold       | -                       |
| Vikman and Edvinsson 2006 | Post-mortem brain tissue from 12 stroke patients and permanent focal rat MCAO | 7458        | 5 hours             | 2.0-fold       | -                       |
| Our data              |                                       | 1176        | 24 hours            | 2.0-fold       | -                       |

Selected molecules: NGFI-C, ARC, GRB2, SMN1, IFN-IP, NDPAP-1, NPR, SOCS-3, NARP, SPR, SPIN2C, ARG1, LBP, PC4, FAK, Synaptic proteins, CD14, CD36, FcGR1A, IFNGR1, caspase-1, a-catenin, LY64, ELK3, POLIIJ3F4, RHOA, PAK1, MPP1, INII
Table 2: Clinical Details of Patients

| Patient no. | Age/sex | Survival after stroke | NIHSS on admission | Hypertension | Coronary artery disease | Atrial fibrillation | History of TIA/previous stroke | Hypercholesterolemia | Smoking | Obesity | Cause of death | Antiplatelets | Statins | RSA-be |
|-------------|---------|-----------------------|--------------------|--------------|-----------------------|--------------------|---------------------------|---------------------|---------|---------|----------------|---------------|---------|--------|
| 1           | 63/F    | 2 days                | 26                 | Yes          | No                    | No                 | No                        | No                  | No      | Yes     | Large ischemic stroke | No            | No      | No     |
| 2           | 84/M    | 3 days                | 21                 | Yes          | Yes                   | No                 | No                        | No                  | Yes     | No      | Malignant stroke | No            | No      | No     |
| 3           | 68/M    | 3 days                | 24                 | Yes          | Yes                   | No                 | No                        | Yes                 | No      | No      | Brain oedema | No            | No      | Yes    |
| 4           | 84/M    | 6 days                | 22                 | Yes          | Yes                   | No                 | No                        | No                  | No      | No      | Cardiac failure | Yes           | Yes     | No     |
| 5           | 51/M    | 9 days                | 25                 | Yes          | No                    | No                 | Yes                       | No                  | No      | No      | Respiratory infection | Yes           | No      | No     |
| 6           | 74/M    | 15 days               | 22                 | Yes          | Yes                   | No                 | No                        | No                  | No      | Yes     | Heart attack | Yes           | No      | Yes    |
| 7           | 86/M    | 15 days               | 14                 | Yes          | Yes                   | No                 | No                        | Yes                 | No      | No      | Urinary infection | Yes           | No      | No     |
| 8           | 58/M    | 17 days               | 16                 | Yes          | Yes                   | No                 | No                        | Yes                 | No      | No      | Cardiac infarction | Yes           | No      | No     |
| 9           | 74/M    | 20 days               | 12                 | Yes          | Yes                   | No                 | No                        | No                  | No      | No      | Bronchial aspiration  | Yes           | No      | No     |
| 10          | 73/M    | 26 days               | 14                 | Yes          | Yes                   | No                 | No                        | Yes                 | No      | Yes     | Respiratory infection | Yes           | No      | Yes    |
| 11          | 75/M    | 29 days               | 20                 | No           | Yes                   | Yes                | No                        | No                  | Yes     | No      | Septic shock | Yes           | No      | Yes    |
| 12          | 60/F    | 37 days               | 18                 | Yes          | Yes                   | No                 | No                        | Yes                 | Yes     | Yes     | Pulmonary embolism | Yes           | No      | No     |

a Blood pressure greater than 135/85 mmHg.
b Serum total cholesterol levels greater than 5.2 mmol.
c Body mass index greater than 30.
d Patients who were on statins before the ischemic stroke.
e Patients taking either angiotensin converting enzyme inhibitors or angiotensin type I receptor antagonists.
M = male; F = female; NIHSS = NIH Stroke Scale; TIA = Transient Ischaemic Attack.
Figure 1
**Statistical analysis of microarray data.** Total number of genes and number of overlapping genes (between the two array sets) deregulated following stroke in human and rat (A). Scatter plots representing the data dispersion over two logarithmic scales for all time-points in human (B) and rat (C).
number of the genes on the microarray. These findings compare with 0.8%, 4.3%, 4%, 14.1%, 21.5%, 10% and 22.2% of genes respectively at each time-point in rats.

In total, 126 genes were deregulated after stroke in humans and 335 in the rat MCAO model. However, these data are not directly comparable since many transcripts in the human array were not present in the rat array and vice versa. Out of a total of 393 genes present in both arrays, 31, 49 and 5 showed deregulated expression in the 3 patient groups respectively, whilst 7, 27, 26, 62, 107, 34 and 107 genes were deregulated at each of the 7 time-points respectively following rat MCAO (Table 3, Figure 1). Of the 393 overlapping transcripts, the expression of 36 was changed only in the human study, compared with 184 that were altered only in the animal model, while only 41 deregulated genes were shared between the two studies. Interestingly, the mean fold changes in the human data were much higher than in the rat.

Amongst these genes we examined in more detail a small subset with no prior report of a role in stroke (PAK1, MMP11 and INI1). PAK1 was only induced in the human study although present in both microarray sets, MMP11 was induced in both cases, while INI1 was induced in the human but was not present in the rat microarray set. To confirm the microarray data, RT-PCR was carried out on selected deregulated genes. The temporal expression patterns of these genes following RT-PCR showed good agreement with the corresponding expression profiles obtained from the microarray analysis, supporting the validity of the data obtained from the microarrays. Using Western blotting and immunohistochemistry, PAK1, INI1 and MMP11 protein expression and localization was determined in the contralateral and ipsilateral brain areas of individual stroke patients and rats subjected to MCAO, and in HBMEC and HFN exposed to OGD and reperfusion.

Integrase Interactor 1 (INI1)

In agreement with the microarray data, RT-PCR demonstrated an increase in ini1 mRNA levels in peri-infarcted and infarcted areas of patients who survived between 2 and 6 days following stroke (Figure 2A). Analysis of INI1 protein expression in samples from individual stroke patients showed that protein levels were increased in peri-infarcted and infarcted regions in 8 of 12 samples (Table 4; Figure 2Bi and 2Bii). Only one patient who survived for 3 days after stroke showed decreased protein expression. Cells from contralateral white matter were not stained for INI1 but some weak neuronal cytoplasmic staining was seen in grey matter (Figure 2Ci). An increase in its expression was observed in the cytoplasm of cells with the morphological appearance of glia and microvessels from peri-infarcted and infarcted areas of patients surviving for 3 to 29 days after stroke (Figure 2Cii and 2Ciii). In the rat, RT-PCR and Western blotting demonstrated no notable changes in INI1 mRNA and protein expression respectively following MCAO. Weak cytoplasmic staining was observed in contralateral neurons but no differences in the level of INI1 neuronal expression occurred following MCAO (data not included). Finally, HFN and HBMEC exposed to OGD and/or reperfusion showed no difference in mRNA and protein levels for INI1 when compared with untreated cells.

Matrix Metalloproteinase II (MMP11)

For MMP11, RT-PCR data agreed with the findings from the microarray study, showing increased mRNA levels in infarcted and peri-infarcted tissue from patients surviving 2–20 days following stroke (Figure 3Ai). Western blotting in individual patient samples demonstrated that 6 of 12 patients had elevated MMP11 protein levels (Table 4; Figure 3Bi and 3Bii). The majority of cells from contralateral grey and white matter were not stained for MMP11 (Figure 3Ci). In patients surviving from 3 days to 4 weeks, endothelial cells and neurons from both infarcted and peri-infarcted tissue were stained positive for MMP11 (Figure 3Cii and 3Ciii). In the rat model, RT-PCR confirmed the microarray data for some of the time-points, showing no notable change in mRNA levels at 1 and 12 h but a prolonged upregulation at 3 days following MCAO (Figure 3Aii). Protein levels were elevated at 1 h, 24 h and 3 days, after which they returned to control levels. No staining for MMP11 was seen in contralateral areas (Figure 3Di), but an increase in its expression occurred in neurons following MCAO, in particular at 12 and 24 h (Figure 3Dii). MMP11 mRNA and protein levels remained unchanged in HFN and HBMEC exposed to conditions of oxygen-glucose deprivation.

P21-activated kinase I (PAK1)

RT-PCR confirmed the upregulation of pak1 determined by the microarrays in pooled samples from stroke patients who survived between 2 and 6 days following stroke (Figure 4A). Western blotting showed an upregulation in the protein levels of PAK1 in 6 of 12 patients (Table 4; Figure 4Bi and 4Bii). No staining was seen in contralateral white matter, while, in grey matter, PAK1 stained weakly the cytoplasm of some neurons (Figure 4Di). In patients surviving for 3 days to 4 weeks after stroke, increased PAK1 nuclear staining was seen in neurons in both peri-infarcted and infarcted regions (Figure 4Dii). In the rat, RT-PCR showed no significant changes in the mRNA levels for pak1 at most of the time-points examined. However, Western blotting showed an upregulation in protein levels 1, 12 and 24 h after MCAO, returning to control levels at 3 days, and becoming downregulated at 7 days following MCAO (Figure 4Ci and 4Cii). Weak staining was observed in neurons from the contralateral hemisphere (Figure
Table 3: Genes deregulated in both human and animal stroke microarrays

| Gene name                                      | Human GenBank | Human SwissProt | Rat GenBank | Rat SwissProt | Human Max/min | Human Days | Rat Max/min | Rat Time |
|-----------------------------------------------|---------------|-----------------|------------|---------------|---------------|------------|-------------|----------|
| c-jun proto-oncogene                           | J04111        | P05412          | X17163     | P17325        | 4.4-fold      | 9 – 20     | 3.5-fold    | 1 h – 24 h|
| Matrix metalloproteinase II                   | X57766        | P24347          | U46034     | P97568        | 3.2-fold      | 2 – 20     | 2.6-fold    | 3 days   |
| Calcium/calmodulin-dependent kinase (CAMKI)   | L41816        | Q14012          | L24907     | Q63450        | 17.2-fold     | 2 – 20     | 0.05-fold   | 21 days  |
| LIM domain kinase I                            | D26309        | P53667          | D21873     | P53669        | 3.6-fold      | 2 – 20     | 2.4-fold    | 3 days   |
| T-Lymphocyte maturation-associated protein     | M15800        | P21145          | U11367     | Q64349        | 1.7-fold      | 2 – 6      | 0.2-fold    | 21 days  |
| Retinoic Acid Receptor beta                    | M84820        | P28702          | M81766     | P49743        | 2.0-fold      | 2 – 6      | 0.1-fold    | 21 days  |
| Tyrosine Phosphatase 1B                        | M31724        | P18031          | M33943     | P20417        | 3.6-fold      | 2 – 20     | 2.4-fold    | 3 days   |
| Glutamate Decarboxylase 67                     | M81883        | Q92959          | M34445     | P18088        | 5.6-fold      | 2 – 6      | 2.5-fold    | 3 days   |
| Growth arrest & DNA damage-inducible 153      | S40706        | P33638          | U30186     | Q62804        | 2.4-fold      | 2 – 6      | 2.1-fold    | 3 days   |
| Interleukin 10                                 | M62138        |                 |            |               |               |            |             |          |
| Heat Shock Protein 27                          | M84827        | P23201          | L02926     | P29456        | 2.4-fold      | 2 – 20     | 6.4-fold    | 21 days  |
| Heat Shock Protein 70                          | M11717        | P80107          | Z27118     | Q63718        | 0.6-fold      | 2 – 6      | 9.4-fold    | 21 days  |
| Thioredoxin Peroxidase I                       | M62829        |                 |            |               |               |            |             |          |
| Platelet-Derived Growth Factor A               | X06374        | P31945          | U06099     | P35704        | 4.9-fold      | 2 – 20     | 3.9-fold    | 21 days  |
| Matrix Metalloproteinase 14                    | X83335        | Q23678          | X83337     | Q10739        | 6.9-fold      | 2 – 6      | 3.3-fold    | 24 – 3 d|
| Kinase receptor TYRO3Sky proto-oncogene         | D17517        | Q66148          | D37880     | P5146        | 3.1-fold      | 9 – 20     | 4.0-fold    | 24 – 3 d|
| CSF-1-Receptor                                | X03663        | P07333          | X61479     | Q00495        | 89.2-fold     | 9 – 20     | 2.8-fold    | 3 days   |
| Integrin-like Growth Factor Binding Protein 2  | M35410        | P18065          | J04486     | P12843        | 2.7-fold      | 9 – 20     | 2.1-fold    | 3 days   |
| Mitogen activated kinase 1/2                   | M84489        | P28492          | M64300     | P27703        | 4.8-fold      | 9 – 20     | 0.3-fold    | 21 days  |
| Aquaporin 4                                    | U34846        | P55087          | U14007     | P47863        | 18-fold       | 9 – 20     | 3.2-fold    | 3 days   |
| erbB2 proto-oncogene Neu proto-oncogene        | M56667        | P04626          | X03362     | P06494        | 11.2-fold     | 9 – 20     | 2.7-fold    | 12 hrs   |
| L-type calcium channel (I3)                    | M11770        | Q14236          |           |               |               |            |             |          |
| LIM domain kinase 1                            | D26309        | P53667          | D21873     | P53669        | 3.6-fold      | 2 – 20     | 2.4-fold    | 3 days   |
| Signal Transducer & Activator of Transcription 3| L29277        | P40763          | X91810     | P52631        | 0.4-fold      | 9 – 20     | 6.6-fold    | 4 – 3 d  |
| Glutathione S-Transferase P                    | X08058        | P09311          | X02904     | P04906        | 3.1-fold      | 9 – 20     | 0.1-fold    | 1 hr     |
| Glucose transporter 1                          | K03195        | P11166          | M12979     | P11167        | 0.6-fold      | 26 – 37    | 11.6-fold   | 4 – 21 d |
4Ei), but an increase in cytoplasmic and nuclear staining in neurons occurred following MCAO, in particular at 1 h and 24 h (Figure 4Eii). Finally, an increase in PAK1 expression was also seen in human foetal neurons following oxygen-glucose deprivation (Figure 4Fi and 4Fii).

Discussion and Conclusion
In the human brain, many differentially expressed genes were observed from 2 to 6 days and from 9 to 20 days after stroke, with the majority being upregulated. The number of deregulated genes declined during 26 to 37 days after stroke, indicating that dynamic changes in gene expression occur during the first days to few weeks in the human postischaemic brain. In the rat brain, few differences were observed at 1 hour, while the number of differentially expressed genes steadily increased with time after MCAO, with a peak after 3 days, supporting the concept of active mechanisms initiated during the acute phase after experimental stroke and lasting for several days. The number of upregulated genes gradually increased, peaking at 3 days, while downregulated genes were detected 24 h after MCAO and increased dramatically until the final measured time-point at 21 days (Figure 1).

The limitations of post-mortem brain samples in cDNA microarray analysis concern the small sample size and potential low quality and the genetic heterogeneity and diversity in terms of age, sex and previous medical history within a group of patients [28,29]. We found that analysis of postischaemic gene expression using a cDNA microarray can allow identification of known and novel transcriptional events, molecular participants and signalling mechanisms in cerebral ischaemia as previously suggested, but can also detect differences in gene expression between distinct organisms.
The present gene expression profile study is the first large-scale microarray report showing altered expression of several genes following human stroke. These included genes participating in transcription, apoptosis, inflammation and neuroprotection. Many genes/proteins previously shown to be deregulated following stroke were reported in our study too e.g. IL-10 [30,31], PDGF [32], STAT3 [33,34], MAPK1/2 [35]. To test whether our microarray analysis could predict novel candidate genes involved in the cerebral response to ischaemia with possible functional importance and significance in stroke-induced neuronal damage, we measured protein expression and
cellular localisation for three induced genes, INI1, PAK1 and MMP11. They were chosen because they showed at least 2-fold mRNA induction and there was no prior published evidence implicating them in human cerebral ischaemia.

PAK1 is a downstream Rac effector and a major cyclin-dependent kinase 5 (Cdk5) substrate and target that colocalizes with p35/Cdk5 at neuronal peripheries. P35/Cdk5 causes PAK1 hyperphosphorylation, which results in PAK1 down-regulation and is likely to have an impact on the dynamics of the reorganization of the actin cytoskeleton in neurons during dendrite development [36]. Based on this evidence, these authors proposed the existence of a neuron-specific signalling complex involving Cdk5/p35-PAK1 that inhibits PAK1 activity in neurons. We have recently provided evidence for a potential role of Cdk5/p35 in the response to ischaemic injury as we showed association of Cdk5 with nuclear damage, by demonstrating co-expression of Cdk5 in TUNEL-positive neurons following human stroke and in propidium iodide-positive human foetal neurons following OGD [37]. Here, we have reported for the first time an upregulation in PAK1 protein levels in human and rat brain samples following MCAO and in HFN following oxygen-glucose deprivation. Although in the animal model PAK1 protein levels returned to normal 3 days following stroke, some patients showed elevated levels for PAK1 at later time-points too. In both human and the animal model, neurons were the predominant type of cells stained positive for PAK1.

MMP11 or stromelysin-3 (ST3), first isolated as a breast cancer-associated protease, is not expressed in the majority of normal adult organs but is expressed during a number of pathological processes, including wound healing and atherosclerotic lesions [38,39]. Although other metalloproteinases have been studied extensively following stroke [40,41], there is no report of the expression of MMP11 following stroke in vivo or in vitro. Here we report an increase in protein levels of MMP11 following stroke in both human and rat brain, although the increase seen in man remained elevated much longer. Although MMP11 shares many similarities with other MMPs, it also differs in that it exhibits anti-apoptotic properties, a first-known activity for a MMP [42]. Moreover, although it is expressed in many processes involving tissue remodelling, cell migration and cell death, the pathways through which it participates in pathogenesis remain unclear, largely due to the lack of information on its substrates in vivo [43].

INI1 is a tumour suppressor gene, thought to exert its tumour suppressor function by mediating cell cycle arrest [44]. It was initially identified as a human homolog of yeast transcriptional activator SNF5 that binds to the HIV-1 integrase and stimulates its DNA-joining activity [45]. Brains of AIDS patients had been shown to manifest neuronal injury and apoptotic-like cell death raising the question about the way HIV-1 resulted in neuronal damage, since neurons themselves are very rarely infected by the virus [46]. Adler et al. [47] also reported an association of the human SNF5/INI1 protein with growth arrest and DNA damage-inducible protein 34 (GADD34) that mediates growth arrest and apoptosis in response to stress signals [48,49]. Our study is the first to suggest a potential role for INI1 in pathways activated after stroke with a possible role in brain injury. However, in the animal model study, INI1 levels remained unchanged following stroke. The reason for this discrepancy warrants further studies.

Many experimental trials of stroke therapies have failed to translate to human clinical trials and one possible way to improve the success rate can be through comparative genomics. As it has been recently commented, it is very surprising that the exciting developments observed in basic and clinical stroke research over the past two decades have occurred in parallel, with too little direct translation between bench and bedside [50]. Here, we have provided substantial evidence that, although the available animal models of MCAO may well be suitable to study the pathophysiological changes following the occlusion of a cerebral vessel, they may not entirely reflect the pathophysiological process through which stroke evolves in humans. The species difference is one of the main reasons accounting for the lack of success of bench to bedside translation in the stroke area. Limitations of our study include the fact that early acute phase changes in gene expression may have been missed since genes induced and returning to normal during the first 48 hours post-ischaemia in man could not have been detected. Moreover, since we analyzed pooled RNA samples, small changes in gene expression occurring in a minority of the samples may have been missed. However, there was only a small overlap of our results with prior studies in experimental stroke involving brain tissue, and the successful identification of novel ischaemia-related genes reported here suggests that performing a further study using whole genome microarrays would be valuable.

Methods

Human brain autopsy specimens

Human brain tissue samples were obtained from 12 patients who died from acute ischaemic stroke, with the approval of the local Ethics Committee and Brain Bank at the Department of Neuropathology, Collegium Medicum, Jagiellonian University, Krakow, Poland. All patients were admitted with large middle cerebral artery strokes confirmed by CT-scan or MRI. The patients, 10 male and 2 female, were aged between 51 and 86 years and had survived between 2–37 days following ischaemic stroke...
(Table 2). Routine blood parameters were determined on admission. Full clinical examinations, including NIH Stroke Scale, were also carried out on admission. Excluded from the study were patients with recent history of head trauma, major cardiac, renal, hepatic or cancerous disease and obvious signs of infection after admission. Immediately after death the body was put in a cold chamber and tissue was collected within 6 h of death. Tissue samples were taken from infarct and peri-infarcted zones while controls were obtained from the contralateral hemisphere at the same time. The peri-infarcted areas were defined in tissue sections as the tissue immediately surrounding the infarcted core which contained some necrotic cells and showed evidence of tissue disorganisation confirmed by histology. Sections were stained with 2,3,5-triphenyltetrazolium chloride which stains active mitochondria pink; therefore, non stained areas represented stroke affected cortical regions (data not included). Tissue specimens were immediately frozen in liquid nitrogen, kept at -70°C and a portion of each sample was processed for histology and stained with haematoxylin and eosin to determine tissue morphology [51].

Rat middle cerebral artery occlusion

Stroke experiments were performed on female Sprague-Dawley rats (weight: 230–270 g) as they suffer less than male during ischaemia. Cerebral ischaemia was produced using a modified method of Baron [52] by distal, permanent occlusion of the MCA by electrocautery as described elsewhere [53,54]. The mortality in this model is very low. Sets of six animals (3 for morphological studies and 3 pooled together) for each time-point were sacrificed at 1 h, 4 h, 12 h, 24 h and 3, 7 and 21 days.

In vitro oxygen-glucose deprivation (OGD)

Human brain microvascular endothelial cells (HBMEC) were obtained from TCS CellWorks (Buckingham, UK) and cultured according to the supplier's instructions. Human foetal (cerebral cortical) neurons (HFN) were extracted and cultured with permission from the Local Ethics Committee. Brain tissue from foetus specimens of 14–19 weeks gestational age, legally aborted and with the appropriate written consent, were collected in cold preservation medium and cells were isolated and cultured as described elsewhere [55]. For OGD experiments, the culture medium was replaced by glucose-free medium containing 2% foetal bovine serum (TCS CellWorks, Buckingham, UK) and cells were cultured at 37°C in a humidified chamber with 94% N₂, 1% O₂, and 5% CO₂ for 6 h (HBMEC) or 95% N₂ and 5% CO₂ for 14 h (HFN) followed by 24 h reperfusion in fresh medium containing 4.5 g/l glucose. This resulted in approximately 30% of cells undergoing apoptosis after OGD and 60% following reoxygenation, as determined from our pilot studies. Cells cultured in normoxic conditions without glucose depriva-
25, 30, 35 and 40 cycles to ensure the semi-quantitative nature of the results. The results were normalized using housekeeping gene GAPDH and semi-quantitatively analyzed using Scion Imaging Software version 4.02 (Scion Corporation, Maryland, USA). Sense and antisense oligonucleotide primers containing 18–27 nucleotides based on previously reported mRNA sequences in the GenBank depository were designed with the aid of the Primer3 Output Program (Version 0.2). InVitrogen plc. (Paisley, UK) synthesized the primer sets (Table 5).

### Protein extraction and Western blotting

Proteins were extracted from tissues and the protein concentration of each sample was determined using the Bio-Rad assay. For Western blotting, 10 μg of protein were separated by SDS-PAGE (13% w/v) and the proteins were electro-botted onto nitrocellulose filters as described previously [57]. Filters were blocked in 1% w/v bovine serum albumin (BSA) in Tris-buffered saline Tween (TBS Tween) and stained overnight at 4°C with antibodies to the following proteins (obtained from Autogen Bioclear, Wiltshire, UK, unless stated otherwise) diluted in normal serum: MMP11, INI1, and α-actin (Sigma, 1:1000) used as a loading control. Membranes were washed in TBS-Tween before staining with the appropriate peroxidase-conjugated secondary antibody, diluted 1:1000 in 5% w/v milk in TBS-Tween for 1 h. Blots were developed with the ECL detection system (Amersham, UK). The relative intensities of the bands were measured in an LKB densitometer. Results are semi-quantitative and are given as a numerical (fold) change compared to the control (contralateral tissue) which was given an arbitrary value of 1.0. All experiments were performed twice and a representative example of patient(s) showing an increase in protein expression is given.

### Immunohistochemistry/Immunofluorescence

Paraffin-embedded tissue samples were processed and serial 5 μm sections were cut. The Avidin-Biotin Peroxidase (ABC Vectastain kit, Vector Laboratories, Peterborough, UK) method was used and antibodies to MMP11, PAK1 and INI1 were used at a dilution of 1:50. Paraffin-embedded sections were deparaffinized, rehydrated and boiled for 10 min in an antigen unmasking solution of concentrated citric acid pH 6.0 as described elsewhere [57]. Slides were incubated in 0.5% v/v H₂O₂ in methanol for 30 min, with normal serum for 20 min and then with a primary antibody (diluted in normal serum) for 30 min, followed by 30-min incubation with biotinylated secondary antibody (diluted 1:50) and finally with ABC complex (diluted 1:50) for 30 min at RT. Staining was completed after incubation with DAB substrate chromogen solution for 3–10 min. Slides were counterstained with haematoxylin, dehydrated, cleared and mounted in DPX. For immunofluorescence, cultured cells were fixed in 4% paraformaldehyde for 20 min, permeabilized with 0.2% Triton ×100 for 10 min, blocked with normal serum and stained with the primary antibody as above, followed by 1 h incubation with Alexa-fluor conjugated dye at RT. Negative control slides were performed in parallel, where primary antibody was replaced with washing buffer and processed as above (data not included).

### Abbreviations

GAPDH: glyceraldehyde 3-phosphate dehydrogenase; HBMEC: human brain microvascular endothelial cells; HFN: human foetal neurons; INI1: integrase interactor 1; MCAO: middle cerebral artery occlusion; MMP11: matrix metalloproteinase; OGD: oxygen-glucose deprivation; PAK1: p21-activated kinase 1.
Authors’ contributions
NM carried out the human studies and drafted the manuscript. MOS carried out the experimental work in rats. JK and FR provided the material for the study. RP and CS carried out the in vitro work. QW participated in the analysis of the microarray results. MS performed the statistical analysis. JG, PK and SK participated in the design and coordination of the study and helped to draft the manuscript. MAS conceived of the study, and directed the research project. All authors read and approved the final manuscript.

Acknowledgements
This work was supported by the Higher Education Funding Council for England (HEFCE) and the Research Institute for Health and Social Change (RIHSC). We also thank Mick Hoult for his assistance in the preparation of this manuscript.

References
1. Mitsios N, Gaffney J, Kumar P, Krupinski J, Kumar S, Slevin M: Pathophysiology of acute ischaemic stroke: An analysis of common signalling mechanisms and identification of new molecular targets. Pathobiology 2006, 73(4):159-175.
2. Slevin M, Krupinski J, Kumar P, Gaffney J, Kumar S: Gene activation and protein expression following ischaemic stroke: strategies towards neuroprotection. Journal Of Cellular And Molecular Medicine 2005, 9(1):85-102.
3. Wechsler B, Graf R: Cerebral ischemia and reperfusion: The pathophysiological concept as a basis for clinical therapy. Journal Of Cerebral Blood Flow And Metabolism 2004, 24(4):351-371.
4. Slevin M, Kumar P, Gaffney J, Kumar S, Krupinski J: Can angiogenesis be exploited to improve stroke outcome? Mechanisms and therapeutic potential. Clinical Science 2006, 111(3):171-183.
5. Longa EZ, Weinstein PR, Carlson S, Cummings R: Reversible Middle Cerebral-Artery Occlusion Without Cranioectomy In Rats. Stroke 1989, 20(1):84-91.
6. Hasselblatt M, Jelkmann A, Riesmeier B, Maintz D, Schabitz WR: Granulocyte-colony stimulating factor (G-CSF) and G-CSF receptor expression in human ischemic stroke. Acta Neurochirurgica 2007, 113(1):45-51.
7. Rosell A, Ortega-Aznar A, Alvarez-Sabin J, Fernandez-Cadenas I, Ribo M, Molina CA, Lo EH, Montaner J: 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 2006, 26(8):1089-1102.
8. Byrd MR, Lewohl JM, Scott HL, Dodd PR: Biochemical and molecular studies using human autopsy brain tissue. Journal Of Neurochemistry 2003, 85(3):543-562.
9. Schramm M, Falkai P, Tepest R, Schneider-Axmann T, Przoroka R, Waha A, Pietsch T, Bonne W, Bayer TA: Stability of RNA transcripts in post-mortem psychiatric brains. Journal Of Neural Transmission 1999, 106(3-4):329-335.
10. Saarinen T, Karijaalainen-Lindborg ML, Paetau A, Ijas P, Lindersborg PJ: Apoptosis dominant in the perifarct area of human ischaemic stroke - a possible target of antiapoptotic treatments. Brain 2006, 129:189-199.
11. Saarinen T, Ristimaki A, Karijaalainen-Lindborg ML, Paetau A, Kaste M, Lindersborg PJ: Cyclooxygenase-2 is induced globally in infarcted human brain. Annals Of Neurology 1998, 43(6):738-747.
12. Castensson A, Emilsson L, Preece P, Jazin E: Molecular studies using human autopsy brain tissue. Journal Of Neurochemistry 2000, 73(4):751-371.
13. Almeida A, Thiery JP, Magdelena H, Radvanyi F: Gene expression analysis by real-time reverse transcription polymerase chain reaction: influence of tissue handling. Analytical Biochemistry 2004, 328(2):101-108.
14. Vikman P, Edvinsson L: Gene expression profiling in the human middle cerebral artery after cerebral ischemia. European Journal Of Neurology 2006, 13(12):1324-1332.
15. Iwamoto K, Kato T: Gene expression profiling in schizophrenia and related mental disorders. Neuroscientist 2006, 12(4):349-361.
16. Mirnics K, Pevsner J: Progress in the use of microarray technology to study the neurobiology of disease. Nature Neuroscience 2004, 7(5):434-439.
17. Perini P, Piarra M, Alecci M, Galloni E, Marchi M, Toso V: Temporal profile of serum anti-inflammatory and pro-inflammatory interleukins in acute ischaemic stroke patients. Neurological Sciences 2001, 22(4):289-296.
18. Tarkowski E, Rosenegger M, Blomstrand C, Wikkelso C, Jensen C, Eholm S, Tarkowski A: Intrathelial release of pro- and anti-inflammatory cytokines during stroke. Clinical And Experimental Immunology 1997, 110(3):492-499.
19. Krupinski J, Iisa R, Bujny T, Slevin M, Kumar P, Kumar S, Kaluza J: A putative role for platelet-derived growth factor in angiogenesis and neuroprotection after ischemic stroke in humans. Stroke 1997, 28(3):564-573.
20. Choi JS, Kim SY, Cha JH, Choi YS, Sung KW, Oh ST, Kim ON, Chung JW, Chun MH, Lee SB, Lee MY: Uregulation of gp130 and...
STAT3 activation in the rat hippocampus following transient forebrain ischemia. Glia 2003, 41(3):237-246.

34. Justicia C, Lluserna JL, Planas AM: Activation of the JAK/STAT pathway following transient focal cerebral ischemia: Signal- ing through Jak1 and Stat3 in astrocytes. Glia 2000, 30(3):253-270.

35. Slevin M, Krupinski J, Slowik A, Rubio F, Szczudlik A, Gaffney J: Activation of MAP kinase (ERK-1/ERK-2), tyrosine kinase and VEGF in the human brain following acute ischemic stroke. Neuroreport 2000, 11(12):2759-2764.

36. Nikolic M, Chou MM, Lu WG, Mayer BJ, Tsai LH: The p35/Cdk5 kinase is a neuron-specific Rac effector that inhibits Pak1 activity. Nature 1998, 395(6698):194-198.

37. Matsios N, Pennucci R, Krupinski J, Sanfelici C, Gaffney J, Kumar P, Kumar S, Juan-Babot O, Slevin M: Expression of cyclin-dependent kinase 5 mRNA and protein in the human brain following acute ischemic stroke. Brain Pathology 2007, 17(1):11-23.

38. Wang C, Cheseard MP, Degrossouvre PD, Bellocc JP, Chambon P, Basset P: Breast-Cancer Associated Stromelysin-3 Gene Is Expressed In Basal-Cell Carcinoma And During Cutaneous Wound-Healing. Journal Of Investigative Dermatology 1992, 99(6):870-872.

39. Montaner J, Alvarez-Sabin J, Molina C, Angles A, Abilleira S, Arenillas J, Gonzalez MA, Monasterio J: Matrix metalloproteinase expression after human cardiogenic stroke - Temporal profile and relation to neurological impairment. Stroke 2001, 32(8):1759-1766.

40. Montaner J, Alvarez-Sabin J, Molina CA, Angles A, Abilleira S, Arenillas J, Monasterio J: Matrix metalloproteinase expression is related to hemorrhagic transformation after cardiogenic stroke. Stroke 2001, 32(12):2762-2767.

41. Matsios N, Dive V, Yotsakis A: Matrix metalloproteinase I I (MMP-11; stromelysin-3) and synthetic inhibitors. Medicinal Research Reviews 2007, 10.1002/med.20066.

42. Wei L, Shi YB: Matrix metalloproteinase stromelysin-3 in development and pathogenesis. Histology And Histopathology 2005, 20(1):177-185.

43. Zhang ZK, Davies KP, Allen J, Zhu L, Pestell RG, Zagzag D, Kalpana GV: Cell cycle arrest and repression of cyclin D1 transcription by INI1/SMN5. Molecular And Cellular Biology 2002, 22(16):5975-5988.

44. Kalpana GV, Marmon S, Wang WD, Crabtree GR, Goff SP: Binding And Stimulation Of Hvi-1 Integrase By A Human Homolog Of Yeast Transcription Factor Snf5. Science 1994, 266(5193):2002-2006.

45. Lipton SA: Neuronal injury associated with HIV-1: Approaches to treatment. Annual Review Of Pharmacology And Toxicology 1998, 38:159-177.

46. Adler HT, Chinery R, Wu DY, Kussjik SJ, Payne JM, Fornace AJ, Tchkchuk DC: Leukemic HRX fusion proteins inhibit GADD34-induced apoptosis and associate with the GADD34 and hNSF/INI1 proteins. Molecular And Cellular Biology 1999, 19(10):7070-7060.

47. Hollander MC, Sheikh MS, Yu K, Zhan QM, Iglesias M, Woodworth C, Fornace AJ: Activation of Gadd34 by diverse apoptotic signals and suppression of its growth inhibitory effects by apopto- sis inhibitors. International Journal Of Cancer 2001, 96(1):22-31.

48. Hollander MC, Zhan QM, Bae I, Fornace AJ: Mammalian GADD34, an apoptosis- and DNA damage-inducible gene. Journal Of Biological Chemistry 1997, 272(21):13731-13737.

49. Dinrgat U, Bench to bedside: the quest for quality in experimental stroke research. Journal Of Cerebral Blood Flow And Metabolism 2006, 26(12):1464-1478.

50. Eke A, Conger KA, Anderson M, Garcia JH: Histologic Assessment Of Neurons In Rat Models Of Cerebral-Ischemia. Stroke 1990, 21(2):299-304.

51. Baron JC: Perfusion thresholds in human cerebral ischemia: Historical perspective and therapeutic implications. Cerebrovascular Diseases 2001, 11(2):8.