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NETWORK-BASED GENE EXPRESSION ANALYSIS OF INTRACRANIAL ANEURYSM TISSUE REVEALS ROLE OF ANTIGEN PRESENTING CELLS

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Abstract—Little is known about the pathology and pathogenesis of the rupture of intracranial aneurysms. For a better understanding of the molecular processes involved in intracranial aneurysm (IA) formation we performed a gene expression analysis comparing ruptured and unruptured aneurysm tissue to a control artery.

Tissue samples of six ruptured and four unruptured aneurysms, and four cerebral arteries serving as controls, were profiled using oligonucleotide microarrays. Gene ontology classification of the differentially expressed genes was analyzed and regulatory functional networks and canonical pathways were identified with a network-based computational pathway analysis tool.

Real time reverse transcription polymerase chain reaction (RT-PCR) and immunohistochemical staining were performed as confirmation.

Analysis of aneurysmal and control tissue revealed 521 differentially expressed genes. The most significantly associated gene ontology term was antigen processing (P=1.64E-16).

Further network-based analysis showed the top scoring regulatory functional network to be built around overexpressed major histocompatibility class (MHC) I and II complex related genes and confirmed the canonical pathway "Antigen Presentation" to have the highest upregulation in IA tissue (P=7.3E-10).

Real time RT-PCR showed significant overexpression of MHC class II genes. Immunohistochemical staining showed strong positivity for MHC II molecule specific antibody (HLA II), for CD68 (macrophages, monocytes), for CD45RO (T-cells) and HLA I antibody.

Our results offer strong evidence for MHC class II gene overexpression in human IA tissue and that antigen presenting cells (macrophages, monocytes) play a key role in IA formation.

Key words: microarray, intracranial aneurysms, gene expression, macrophages.

While the incidence of stroke has declined in recent decades, primarily because of improved detection and management of hypertension, the incidence of subarachnoid hemorrhage has remained relatively constant. Its incidence is between 8/100,000 in the USA and 96/100,000 in certain regions in Japan (Riina and Spetzler, 2002; Morita et al., 2005). The overall mortality is still 40–50% (Juvela, 2002) while around 23% of the survivors suffer from a significant restriction of lifestyle requiring daily help up to full dependency (Molyneux et al., 2002). The definite mechanism and cause for formation and rupture still remain to be elucidated.

Due to the difficulty of obtaining suitable tissue samples of intracranial aneurysms there has been an abundance of defining novel points of therapeutic intervention through genetic analysis of tissue, e.g. microarray technique. Microarray analyses have been applied to several types of diseases, with cancer on the forefront (DeRisi et al., 1996), leading to recognition patterns and pointing to new forms of therapies. Furthermore, with the advent of "computational network based pathway analyses" new insights into disease mechanisms can be gained (Bredel et al., 2006).

In this study, we performed oligo microarray gene expression analyses in combination with a new data-analysis and mining approach to identify pathways of importance. Microarray simultaneously monitors the expression of a large number of genes covering various biological machineries. With progress in systems biology, numerous knowledge databases have been developed to host the constellation of genes, their functions, and relationships. The global influence of molecular events can be illustrated by combining the gene expression profiles detected by microarray and the gene functional profiles annotated by such knowledge databases. A systematic transcriptional expression examination of intracranial aneurysm tissues in comparison to other intracranial vessel tissue has not yet been available.

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Abbreviations: AAA, abdominal aortic aneurysm; AVM, arteriovenous malformation; Cy3, cyanine 3-dCTP; Cy5, cyanine 5-dCTP; FDR, false discovery rate; GO, Gene Ontology; HLA, human leukocyte antigen; RT-PCR, reverse transcription polymerase chain reaction; SMC, smooth muscle cells.

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We coupled these novel approaches to identify functions and pathways regulated differently in intracranial aneurysm tissue. The computational tool, Ingenuity Pathways Analysis (Ingenuity Systems, Mountain View, CA, USA), allowed us to identify regulatory networks of differentially expressed genes and the corresponding canonical pathways, that govern the changes in intracranial aneurysm tissue. Canonical pathways are core pathways established for a given molecule in the cell in which molecular interactions occur in a linear and stepwise manner. A significance *P* value was calculated for each function and pathway.

The functional profiles detected demonstrate that the previously described (Chyatte et al., 1999) and suspected functions (immune response) and pathways (antigen presentation) are significantly upregulated in intracranial aneurysm tissue. Already back in 1847, Virchow hypothesized the cause for intracranial aneurysm formation to be inflammation (Virchow, 1847).

These profiles served as benchmarks to assess the relative significance of other functions and pathways. The network-based computational pathway analysis also uncovered unique novel functional classes, gene networks, and pathways that have not yet been related to intracranial aneurysm formation, indicating that these approaches can complement one another in the exploration of the functional profile of microarray data. Additional experiments can be proposed based on the new findings to investigate possible new approaches to intracranial aneurysm treatment.

EXPERIMENTAL PROCEDURES

Tissue specimens

A total of 25 unruptured and ruptured intracranial aneurysm specimen were collected over a time period of 42 months at the Department of Neurosurgery of the Tokyo Women's Medical University after gaining the patients' consent and approval by the ethics committee. Part of the intracranial aneurysm dome was harvested after a standard procedure of microsurgical clipping which prevents rupturing. In the case of subarachnoid hemorrhage all resections were done within the first 24 h after initial hemorrhage. To serve as intracranial control tissue, arteriovenous malformation (AVM) feeders, which were located extranidally, were obtained during microsurgical resection. Prior to their excision and use the patients had given their informed consent. None of the patients who had undergone AVM surgery had suffered from postoperative stroke. Following their excision, all tissue samples were immediately snap-frozen in liquid nitrogen and stored at −80 °C until further use. Of the 25 intracranial aneurysms six ruptured and four unruptured specimens were used as well as four controls consisting of the AVM feeders. The mean age of the intracranial aneurysm patients was 56.4 years, that of the controls was 60.25 years (see Table 1). All were of Japanese ethnicity. Data from the other 15 aneurysms were not further analyzed due to insufficient RNA quality.

Microarray preparation

Total RNA from the respective vessel tissue was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The quantity and quality of the extracted RNA were examined with a 2100 Bioanalyzer (Agilent Technologies Inc., Palo Alto, CA, USA) using an RNA 6000 Nano LabChip (Agilent Technologies). For the deployment of the dye swap technique we used pooled total RNA as a common reference in two-color microarray experiments containing one of each groups of tissue: ruptured aneurysm, unruptured aneurysm and AVM feeder.

For fluorescent cRNA synthesis, high-quality total RNA (150 ng) was labeled with Low RNA Input Fluorescent Linear Amplification Kit (Agilent Technologies) according to the manufacturer's instructions. In this procedure, cyanine 5-dCTP (Cy5) and cyanine 3-dCTP (Cy3) (PerkinElmer, Boston, MA, USA) were used to generate labeled cRNA from the extracted patient RNA and the pooled reference RNA, respectively, or the dye assignments were reversed in the dye swap experiment. Labeled cRNAs (0.75 μg each) from one patient and the common reference were combined and fragmented in a hybridization mixture with the In Situ Hybridization Kit Plus (Agilent Technologies) according to the manufacturer's instructions. The mixture was hybridized for 17 h at 60 °C to an Agilent Human 1A(v2) Oligo Microarray (Agilent Technologies), which carries 60-mer probes to 18,716 transcripts of human. After hybridization, the microarray was washed with SSC buffer, and

Table 1. Clinical characteristics of the patients

ID	Age/sex	Location	Maximum diameter (mm)	SAH	Current or ex-smoker	Hypertension
UA#1	62/F	PcomA	20	−	−	+
UA#2	55/F	MCA	12	−	+	+
UA#3	54/F	MCA	15	−	+	−
UA#4	70/F	MCA	12	−	−	−
RA#1	64/M	PcomA	8	+	+	−
RA#2	77/F	ICA	6	+	−	−
RA#3	58/F	MCA	9	+	−	+
RA#4	70/M	AComA	7	+	+	−
RA#5	83/F	ICA	15	+	+	−
RA#6	54/F	AComA	25	+	+	+
C#1	53/M	AVM feeder		−	−	+
C#2	35/M	AVM feeder		−	+	−
C#3	37/F	AVM feeder		−	−	−
C#4	33/M	AVM feeder		−	+	−

C, control artery patient; RA, patient with a ruptured aneurysm; UA, patient with an unruptured aneurysm; SAH, subarachnoid hemorrhage; PcomA, posterior communicating artery; MCA, middle cerebral artery; ICA, internal carotid artery; AcomA, anterior communicating artery; –, no SAH, non-smoker and no hypertension.

Table 2. Immunohistochemistry results

Patient information				Immunohistochemistry			
ID	Age (yr)/Sex	SAH	Location	HLA I	HLA II	CD68	CD45RO
UA#5	55/F	−	MCA	R	D	R	NR
UA#6	73/F	−	MCA	D	D	D	D
UA#7	58/F	−	MCA	NR	D	NA	NR
RA#7	53/M	+ (OP day 1)	ACA	D	D	D	D
UA#8	70/F	−	MCA	D	D	D	R
RA#8	70/F	+ (OP day 3)	AcomA	R	R	R	R
RA#9	57/F	+ (OP day 1)	AcomA	R	R	R	NR
UA#9	49/F	−	MCA	R	R	R	NR

UA, unruptured aneurysm; RA, ruptured aneurysm; SAH, subarachnoid hemorrhage; MCA, middle cerebral aneurysm; ACA, anterior cerebral aneurysm; AcomA, anterior communicating aneurysm; R, regional immunoreactivity; D, diffuse immunoreactivity; NR, negative reaction; NA, not available; –, no SAH.

then scanned in Cy3 and Cy5 channels with the Agilent DNA Microarray Scanner (model G2565BA).

Data normalization and filtering

Signal intensity per spot was generated from the scanned image with Feature Extraction Software version 7.5 (Agilent Technologies) in the default settings. Spots that did not pass quality control procedures in this software were flagged and removed from further analysis (abiding by the software's default settings).

GeneSpring GX 7.3 (Agilent Technologies) was used to apply the Lowess (locally weighted linear regression curve fit) method to normalize the ratio (Cy5/Cy3) of the signal intensities generated in each microarray. On the basis of the Lowess-normalized ratios, analytical tools in GeneSpring GX were systematically employed to extract differentially expressed genes between all aneurysmal cases (ruptured and unruptured) and the controls, which contained 780 transcripts that were differentially expressed between IA and AVM feeder tissue at an average fold change difference >2. Later, a statistical significance of the difference was examined by Student's *t*-test controlling false discovery rate (FDR) at the level of 0.05 according to Benjamini and Hochberg (1995) which yielded a list of 521 genes. Gene Tree clustering was performed using the Pearson correlation as a similarity measure between genes.

Gene ontology and functional network analysis

As a supervised approach for analyzing the function of genes whose levels were modified by >twofold, Gene Ontology (GO) analysis was performed as an integrated function in the GeneSpring GX software version 7.3. The program automatically obtains the gene ontology annotations that are overrepresented in the inputted list of genes.

Analyses of gene canonical pathways, and functional networks were executed using tools from Ingenuity Pathways Analysis, a Web-delivered application that enables the discovery, visualization and exploration of molecular interaction networks in gene expression data. Detailed information about this analysis software can be found at www.ingenuity.com. The gene list identified by twofold selection and subsequent (FDR<0.05) statistical analysis containing the up- and downregulated genes with their corresponding log ratio differences were uploaded into the Ingenuity Pathways Analysis as a tab-delimited text file. Each clone identifier was mapped to its corresponding gene object in the Ingenuity Pathways knowledge base, which represents a proprietary ontology of 300,000 classes of biologic objects spanning genes, proteins, cells and cell components, anatomy, molecular and cellular processes, and small molecules. Semantically consistent pathway relationships are modeled based on a continual, formal extraction from the public domain literature and cover more than 10,300 human genes (www.ingenuity.com/products/pathways_knowledge.html). These mapped focus genes were then used as a starting point for generating biologic networks. A score was computed for each network according to the fit of the original set of significant genes. This score reflects the negative logarithm of the *P* value that indicates the likelihood of the focus genes in a network being found together as a result of random chance. Using a 99% confidence level, scores of 2 were considered significant. Significances for the enrichment of the genes in a network with particular biologic functions or canonical pathways were determined via right-tailed Fisher's exact test with 0.05 and

Table 3. Top 10 "biological process" ontology categories significantly overrepresented in the 521 differentially expressed genes

Category	Genes in list in category[a]	% Of genes in list in category[b]	*P*-value[c]
Antigen processing (GO:30333)	17	4.3	1.64E-16
Immune response (GO:6955)	69	17.6	3.75E-15
Organismal physiological process (GO:50874)	114	29.0	3.23E-14
Antigen presentation (GO:19882)	17	4.3	4.03E-14
Response to biotic stimulus (GO:9607)	73	18.6	8.57E-14
Defense response (GO:6952)	71	18.1	1.07E-13
Antigen presentation, exogenous antigen (GO:19884)	11	2.8	4.34E-13
Antigen processing, exogenous antigen via MHC class II (GO:19886)	11	2.8	2.17E-12
Response to stimulus (GO:50896)	105	26.7	3.58E-10
Response to external stimulus (GO:9605)	49	12.5	3.84E-07

[a] Number of differentially expressed genes of the data set in the respective category.

[b] Out of the 521 differentially expressed genes, 392 had a GO annotation in the ontology "biological process" and were used to calculate the percentage of genes in the respective category.

[c] The *P*-value was calculated by Fisher's exact test comparing the observed percentage of genes of the data set to that of all annotated genes found on the Agilent Human 1A(V2) microarray gene chip (13,011 genes).

the whole database as a reference set. The same computation was used for gene ontology analyses of the initial gene list.

Real time reverse transcription polymerase chain reaction (RT-PCR)

For cDNA synthesis, the total RNA (100 ng) subjected to microarray analysis was also used as a template in first-strand cDNA synthesis with SuperScript III First-Strand Synthesis System (Invitrogen). Real-time PCR was performed using TaqMan Gene Expression Assays (Applied Biosystems) (primer sequences available on request) with TaqMan Universal PCR Master Mix, No AmpErase UNG (Applied Biosystems) on an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems) according to the manufacturer's instructions. Relative expression levels for the respective genes were obtained by normalizing to GAPDH in all specimens. Statistical comparison in the quantitative analysis of the gene expression was performed by Student's *t*-test; $P<0.05$ was considered significant. Out of the 10 IA samples used for the microarray study there was only sufficient mRNA of seven to perform the RT-PCR analysis.

Immunohistochemistry

As the amount of original samples was not sufficient to perform an immunohistochemical study, intracranial aneurysm tissue from eight additional patients (see Table 2) was taken during surgical clipping and then fixed in 10% buffered formalin and embedded in paraffin. Five 4 μm-thick blocks were taken from all tissue paraffin blocks. One of the five sections was stained with H&E and the rest were immunostained with the following anti-human monoclonal antibodies: CD 68 (macrophage/monocyte marker) 1:100 (DakoCytomation, Glostrup, Denmark); CD 45RO (T-cell marker) 1:100 (Nichirei Co., Tokyo, Japan); human leukocyte antigen (HLA) class I 1:100 (heavy chain HLA-A, HLA-B and HLA-C marker) (Hokudo, Tokyo, Japan), and HLA class II (HLA-DP, -DQ and DR marker) 1:100 (DakoCytomation). All slides were prepared according to the Universal Immuno-Peroxidase Polymer method as described in the guidelines of the Histofine Simple Stain MAX-PO kit (Nichirei Co.). Hematoxylin was used for counterstaining. Specificity of the primary antibodies was established by omission in the immunocytochemical staining protocol. Positive controls were lymphocytes within the germinal center of autopsied human lymph nodes for anti-HLA class I, anti-HLA class II, CD68 and CD45RO.

RESULTS

Oligo microarray results of aneurysms vs. controls

A twofold filtration yielded 780 genes of which 59 genes had insufficient data for the following statistical comparison. After setting the *P* value cutoff to 0.05 based on the Student's *t*-test controlling FDR at 5% level, 521 genes were extracted. Of these 263 were overexpressed and 258 underexpressed.

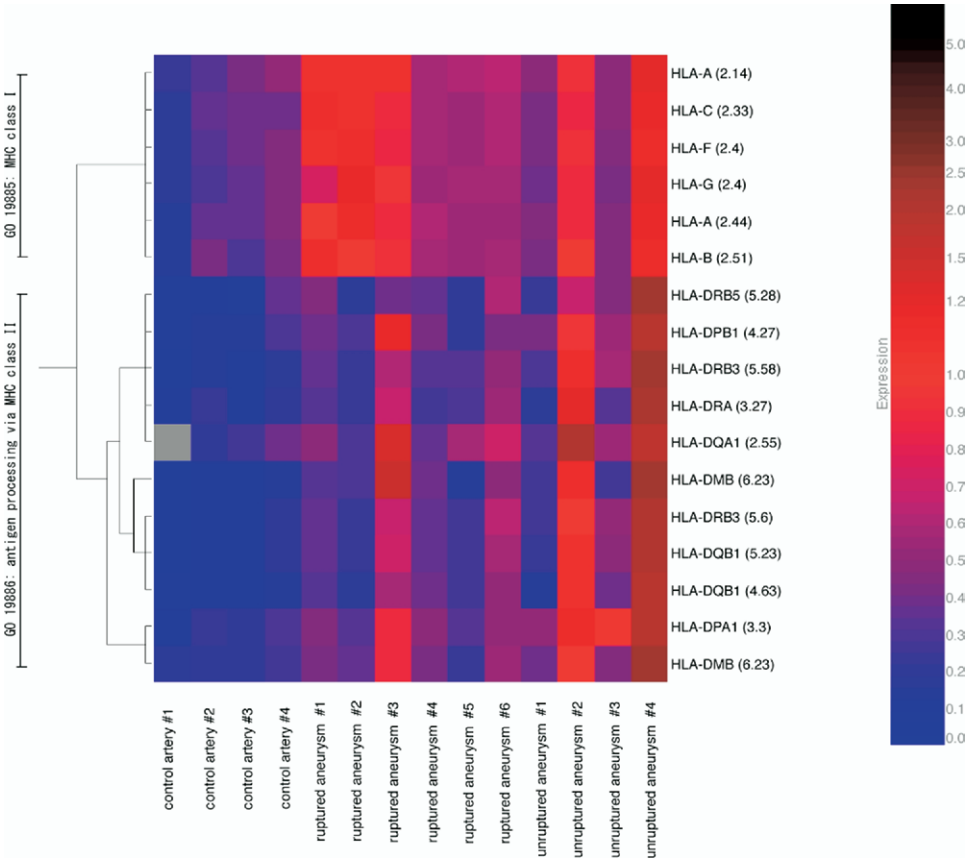

Fig. 1. Gene tree of the GO classification: antigen processing. Hierarchical clustering of antigen presentation genes. The 17 genes that were differentially expressed in the Agilent Human 1A(v2) Oligo Microarray G4110B Chip of the top GO category Antigen Processing (GO:30333) were clustered based on their expression patterns using GeneSpring GX software. The category can be subclassified into antigen processing via MHC class II (GO:19886) and antigen processing via MHC class I (G:19885) (left bar). The value in parentheses signifies the fold overexpression in the aneurysmal tissue as compared with the control. The scale on the right side shows arbitrary values.

GO classification of the 521 genes calculated with the GeneSpring software showed the top significant GO class to be antigen processing (GO:30333) (P=1.64E-16) with 17 associated genes overexpressed in our dataset (out of a total of 38 in that category) (see Table 3 and Fig. 1). Eleven of those genes were further subclassified into "Antigen processing, exogenous antigen via MHC class II (GO:19886)" (P=2.17E-12) and six to the group "Antigen processing, endogenous antigen via MHC class I (GO: 19885)" (P=4.51E-06) (see Fig. 1). The subsequent gene ontology terms for biological processes are listed in the top 10 table (Table 3). All showed a form of subordination and a direct affiliation with each other (see Fig. 2).

Network analysis based on predetermined knowledge about individually modeled relationships between genes identified 20 highly significant overlapping networks in the data set; the first five are shown in Table 4. Of note, in the first three networks, the genes affected in aneurysmal tissue comprised the maximum number of genes (n=35) allowed by the program. This analysis suggests that there is significant interaction between the protein products of these genes whose translational activity is modified in aneurysmal tissue, which in turn suggests that a variety of biochemical pathways at multiple points are altered in aneurysmal tissue. The top-scoring networks displayed high level functions in immune response, cell-to-cell signaling and interaction and immune and lymphatic system development and function. Moreover, the top scoring IPA-generated network is associated with immune response, a functional process consistent with that identified by the GO analysis.

The most significantly influenced canonical pathway seen in the aneurysmal tissue as analyzed by Ingenuity Pathways Analysis was the antigen presentation pathway (P=7.3E-10) followed by integrin signaling, chemokine signaling, complement and coagulation cascades, nitric oxide signaling and IL-10 signaling (Table 5).

Comparison of ruptured vs. unruptured aneurysm tissue

The genes from twofold differentially expressed genes between ruptured and unruptured intracranial aneurysms yielded only a slight difference. After multiple testing correction with FDR only two genes, AHNAK (AHNAK nucleoprotein) and RPL22L1 (ribosomal protein L22-like 1), were significantly differentially expressed.

Results of the real time RT-PCR

Of the tested MHC I genes HLA-A and HLA-B were overexpressed in the intracranial aneurysm tissue, but not significantly (P=0.058 and 0.07, respectively). Of the MHC II genes HLA-DQB and HLA-DMB were significantly overexpressed (P=0.003 and 0.0048, respectively) (see Fig. 3). After completion of this RT-PCR study further testing was not performed due to limited mRNA.

Immunohistochemistry results (Table 2)

MHC class I and MHC class II. Immunohistochemical studies were performed on tissue of eight unrelated aneurysms. All showed MHC II immunoreactivity throughout the vessel wall (HLA II, Fig. 4B). Diffuse staining was observed in five aneurysms, and three demonstrated discrete regions of immunoreactivity. Immunoreactive MHC I was present throughout the wall of most aneurysm samples (seven of eight). MHC I was diffusely present in four aneurysms and regional staining occurred in three aneurysms (HLA I, Fig. 4A).

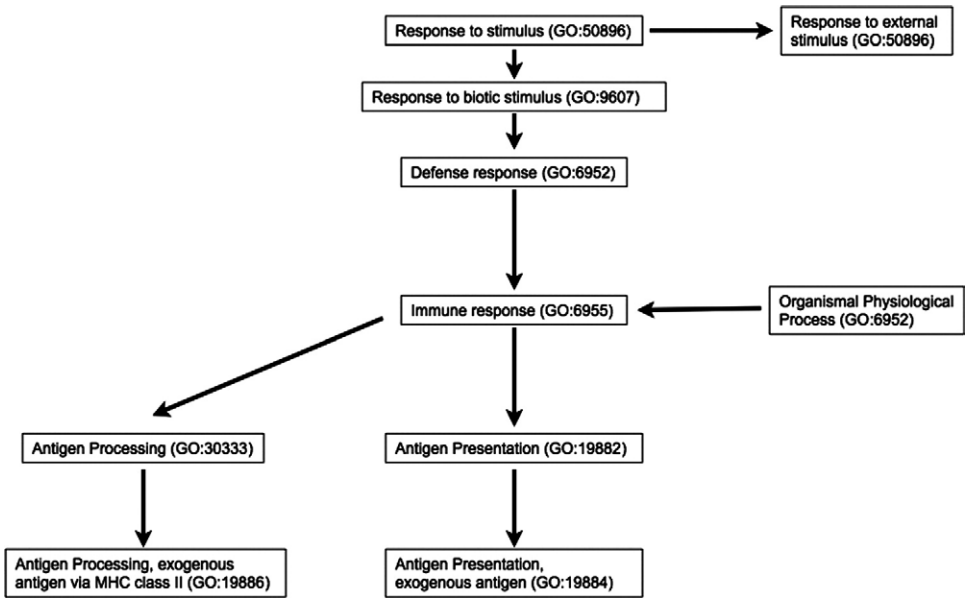

Fig. 2. GO subordination classification scheme. Subordination of the top 10 biological process ontology categories of Table 3.

Table 4. Functional network analysis

Network No.	Genes in the network	No. of genes in network	Score[a]	No. of focus genes[b]	High level functions	No. of associated genes	Significance[c]
1 Aneurysm vs. Normal (out of 521 genes FDR 0.05)	ABL2[d] **ALOX5, CCR7, CD33, CSF1R, CXCL9, FCGR1A, FCGR2B, G1P2, GPX1, HLA-A, HLA-B, HLA-C, HLA-DMB, HLA-DQA1, HLA-DQB1, HLA-DRA, HLA-DRB1, HLA-DRB5, HSPE1, IFIT1, IGJ,** IL6,[d] **INPP5D, IRF8, LYZ, MSR1, PPP1R15A, PSMB8, SCARB1, SIRPA, SLC7A7, SNX10,** SORBS2,[d] **TXNIP**	35	52	35	Immune response	25	4.98E-15-7.13E-3
					Cell-to-cell signaling and interaction	22	9.50E-14-4.34E-3
					Immune and lymphatic system development and function	24	1.12E-10-7.13E-3
2	ACTA1,[d] ACTC,[d] ADAMTS1,[d] ANGPTL1,[d] BBC3, C1ORF24,[d] CALD1,[d] CAPG, CASQ2,[d] **COL5A1, COL5A2,** CRYAB,[d] CSPG2, CTSB, CYFIP2,[d] DDX3X,[d] DSCR1,[d] **FBN1, FN1,** HEY2,[d] **HLA-G,** INHBA,[d] **LGALS3BP, LGMN,** MCL1,[d] **MFAP5,** MYC,[d] NOP5/NOP58,[d] PTMA,[d] **SPARC,** TAGLN,[d] TEAD3,[d] TNC,[d] TP53BP2,[d] **TP53I3**	35	52	35	Cell-to-cell signaling and interaction	10	9.51E-7-7.13E-3
					Cellular assembly and organization	12	9.51E-7-7.13E-3
					Cardiovascular system development and function	13	1.04E-5-7.13E-3
3	**ABCC3, AKR1B1,** BMP2,[d] CCL2,[d] **CCR5, CD68,** CEBPZ,[d] **COL1A1, COL1A2, COL3A1, CTSK, CXCL12, CXCR4,** DGKD,[d] F2,[d] FGF2,[d] FOSL2,[d] **GPNMB, HCLS1,** HMGB1,[d] **IFNGR2, IQGAP2,** NOV,[d] PLCB3,[d] **POSTN, PTGS1,** SMTN,[d] SYK,[d] TGFB3,[d] **TGFBR2,** THBD,[d] **TIMP2,** TIMP4,[d] TNFRSF12A,[d] **VAV1**	35	52	35	Cellular growth and proliferation	22	9.45E-10-5.47E-4
					Cancer	18	3.33E-9-1.64E-3
					Cellular movement	16	3.33E-9-1.26E-3
4	AKR1B10, **ARHGAP22,** BACH1,[d] CALCA,[d] CALCRL, **CD2,** CD74, CTSS, CXCL9, CYP27A1, DNAJB5,[d] ETS2,[d] **FTL,** GSTA1, H2-PB, **HLA-DPA1, HLA-DPB1,** INSIG1,[d] **KRT18,** LIFR, MAFF,[d] **MSR1,** NFE2L2, NTN1, ODC1,[d] RAB7, RAC1, RAMP1,[d] RAP1A,[d] RAPGEF5, RCP9, **RGL1,** TFPI2,[d] TIE1, TNF	35	19	19	Cancer	13	4.15E-7-6.47E-3
					Cell-to-cell signaling and interaction	13	4.15E-7-5.40E-3
					Skeletal and muscular disorders	4	4.15E-7-3.57E-3
5	BCL6,[d] CCT2,[d] **CD69, CTSZ,** (includes EG: 1522), HDAC4, IFRD1,[d] IL16, ITGA4, KIR2DS2, LAT, M-RIP, MARK1,[d] MECP2, NCOR1, NCOR2, PLCG2, PPP1R12A, PPP1R12B, PRKG1,[d] ROCK2, RUNX1T1, **S100A4,** SAP30,[d] **SRGAP2,** SU RB7,[d] SVIL,[d] SYK, THBS1,[d] **THBS2,** TNFAIP6, **TREM2, TYROBP,** USF2, YWHAG, ZBTB16	35	19	19	Cellular development	18	1.25E-6-1.07E-2
					Immune response	18	2.83E-6-7.13E-3
					Cellular movement	10	6.41E-6-3.57E-3

Note: Boldfacing indicates genes of the test set that were overexpressed in the tissue of ruptured aneurysm. Genes in plain type without footnotes are nonfocus genes that were not altered in expression in the test set.

[a] Reflects the negative logarithm of the *P* value that indicates the likelihood of the focus genes in a network being found together as a result of random chance (99% confidence level; scores ≥2 were considered significant.

[b] No. of genes identified as part of the test set mapping to the network.

[c] Range of significances of the associated genes for the high-level function (alpha=0.05).

[d] Genes in the test set that were underexpressed in the ruptured aneurysm tissue.

Table 5. Significant canonical pathways ($P<0.05$) involved in aneurysm tissue

Canonical pathway	*P* value	Differentially expressed genes in aneurysm tissue
Antigen presentation	0.00000000073	HLA-A, HLA-B, HLA-C, HLA-DMB, HLA-DPA1, HLA-DPB1, HLA-DQA1, HLA-DQB1, HLA-DRA, HLA-DRB1, HLA-DRB5, HLA-G, PSMB8
Integrin signaling	0.000445	ACTA1, ACTC, ACTN2, ARPC1B, COL1A1, COL1A2, FN1, ITGA7, ITGA11, ITGB2, LAMA4, LAMB1, PPP1R12A, PPP1R12B, RAP1A, RND3, TSPAN5
Chemokine signaling	0.00215	CCL2, CCR5, CXCL12, CXCR4, GNAI1, PLCB3, PPP1R12A, PPP1R12B
Complement and coagulation cascades	0.00797	C2, C3, C1QA, C1QB, C1QC, CFD, F2, THBD
Nitric oxide signaling in the cardiovascular system	0.0279	HSP90AA1, PLN, PRKAR1A, PRKG1, RYR2
IL-10 signaling	0.037	CCR5, CD14, FCGR2B, IL6, IL10RA, RELB

Inflammatory cells: Macrophages, T lymphocytes

Macrophages and monocytes identified by CD68 staining, were scattered throughout the wall in almost all aneurysms (seven/eight) (Fig. 4D). Diffuse infiltration was observed in three and regional infiltration occurred in four. T lymphocytes, identified by CD45RO staining, were found in almost half of the aneurysms (four/eight). Two aneurysms were diffusely infiltrated by T lymphocytes, and two showed regional infiltration (Fig. 4C). Macrophages and T lymphocytes were not always present in the same area of the aneurysms although overlap did occur.

DISCUSSION

In this article we present the first microarray-based gene expression analysis of several ruptured and unruptured aneurysms and compare their gene expression profile with that of an intracerebral control artery. Using three different classification schemes, one gene ontology– and two network-based, we are led to the same result: the key role of immune response in intracranial aneurysms, particularly MHC class II–related mechanisms. Focusing on the latter, the gene expression microarray-based results are confirmed by RT-PCR. As MHC II molecules are predominantly expressed by antigen presenting cells we sought to visualize our findings through immunohistochemistry of intracranial aneurysm tissue. Macrophages and T-cells showed immunoreactivity to MHC II antibodies also confirming previously published findings (Kosierkiewicz et al., 1994). Our immunohistochemical results show strong overlap of MHC II, MHC I and CD68 (macrophage) staining.

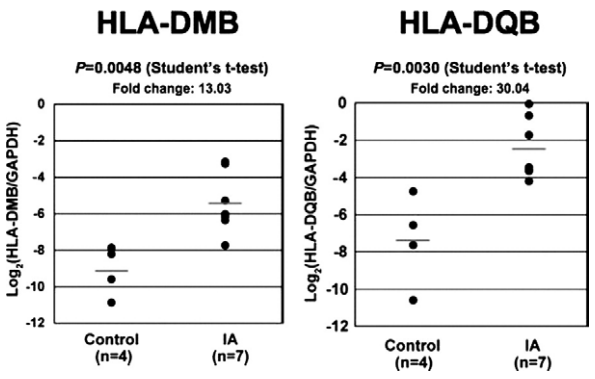

Fig. 3. Real Time RT-PCR of HLA-DMB and HLA-DQB. Graphs depicting the $\log_2$ relative expression levels of the respective genes obtained by normalizing to GAPDH. The horizontal bar indicates the average of the respective group's $\log_2$ expression values. Of ruptured intracranial aneurysms #3, 4, 5 the amount of mRNA was not sufficient to perform an RT-PCR test.

Antigen presenting cells found in the aneurysm wall

Macrophages are professional antigen presenting cells which display a fragment of the antigen, bound to a class II MHC molecule, on their membrane. Macrophages are the principal inflammatory cells in atherosclerosis and may stimulate SMC to change phenotype and proliferate, thus promoting fibrosis (Frosen et al., 2004). Leukocytes initially participate in the acute inflammatory process. They both secrete many kinds of proteases that destroy the extracellular matrix that lead to aneurysm wall restructuring and wall fragility (Sukhova et al., 1998; Kataoka et al., 1999).

As we did not use double staining techniques the exact type of cell in case of MHC class II expression could not be determined. Previous publications have shown that most macrophages and some T-lymphocytes as well as scattered smooth muscle cells (SMC) in advanced atheromatous lesions stained positively for MHC class II antigen (Kosierkiewicz et al., 1994). A possible SMC proliferation, T-cell and macrophage infiltration, indicated by MHC class II gene overexpression must be present before rupture because in healthy arterial wall, they occur in response to injury during the first 24 h or later (Jonasson et al., 1988; Okamoto et al., 2001). Our ruptured samples were all snap frozen within the first 24 h of SAH. The use of *extranidal* AVM feeders as the control vessel, although not ideal, is warranted by the fact that samples were obtained intraoperatively and not obtained postmortem. This approach ensures that RNA samples are freshly harvested and thereby avoids potential problems resulting from excessive RNA degradation that occurs when samples are harvested after decease. Inflammatory cell infiltration and SMC proliferation increase in the IA wall before rupture, which substantiates the hypothesis that they are part of the adaptation and repair mechanism of the IA wall. It is not known whether inflammation triggers the rupture of the IA wall causing subarachnoid hemorrhage. However, infiltrating

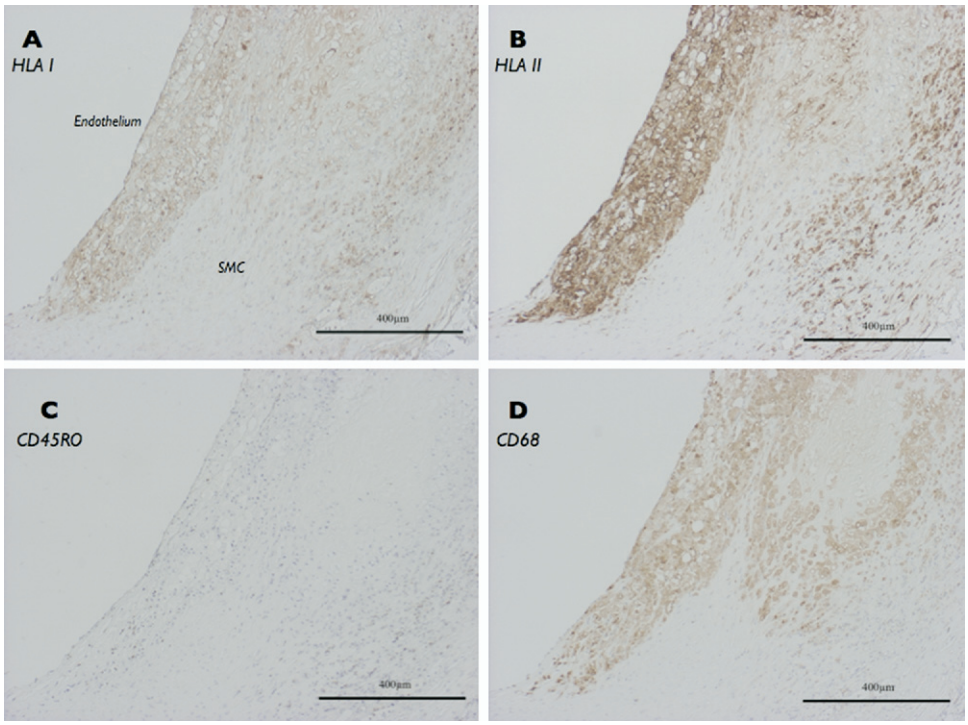

Fig. 4. Immunohistochemistry. Photomicrograph of immunohistochemical staining of intracranial aneurysm tissue (A–D are the same sections of a patient with an unruptured intracranial aneurysm, UA#8 in Table 2). Brown areas indicate positive immunostaining, cell nuclei appear blue. Diffuse immunoreactivity after HLA I, HLA II and CD68 antibody staining; CD45RO (T-cells) shows only little immunoreactivity. ECM: extracellular matrix. Magnification: 100×.

leukocytes, mainly T-cells and macrophages, stimulate SMC proliferation in areas of vascular wall thickening (Hansson et al., 2002).

Antigen expression

The difference in MHC class II gene expression between the IA and control tissue is highly significant and therefore seems to be a more specific immune response mechanism involved in intracranial aneurysm formation. Immunohistochemistry showed the prominent infiltration of macrophages into the IA tissue, which are the main antigen presenting cell to express MHC II molecules. Further activation may be due to previous antigen exposure, which has thus far not been elucidated. It may be possible that a component of the extracellular matrix poses as an antigen. In a recent study, we detected elastin to be a susceptibility gene (Akagawa et al., 2006). In combination with high shear stress through hypertension this could lead to the initial breakdown of the intima, followed by an initiation of an invasion of macrophages (Peters et al., 2001). Macrophage infiltration is associated with SMC proliferation, and both are increased in ruptured aneurysms resected <12 h from rupture, suggesting that these are not reactive changes (Frosen et al., 2004). In a study by Kataoka et al (1999) there were almost no changes in either the degree of inflammatory responses that could be attributed to the time elapsed between intracranial aneurysm rupture onset and fixation of the specimens.

In many inflammatory and autoimmune conditions, as well as vasculitides, vascular smooth muscle MHC class II antigen expression has been observed. This may result in an attack of SMC by specific T lymphocytes recognizing a smooth muscle autoantigen (Klareskog et al., 1982; Hanafusa et al., 1983; Moyer and Reinisch, 1984; Arnaud-Battandier et al., 1986). For aortic abdominal aneurysms there is mounting evidence that points to an immunological or autoimmunological process as formation cause. The presence of extensive inflammatory infiltrates in the tunica media and tunica adventitia leading to increased expression of proinflammatory cytokines and C-reactive protein has been reported (Rasmussen et al., 2001; Vainas et al., 2003). Recently, it had been shown that emphysema is an autoimmune disease characterized by the presence of antielastin antibody and T-helper type 1 responses suggesting the potential for autoimmune pathology for other elastin-rich tissues such as arteries (Lee et al., 2007).

In a previous serial analysis of gene expression comparing tissue of a single intracranial aneurysm compared with a matched superficial temporal artery, strong immune and inflammatory response was detected (Peters et al., 1999).

Signaling pathways

As shown in the top category of significant canonical pathways (Table 5), several signaling pathways and their respective genes may be associated with intracranial aneurysms. There have been several reports showing the involvement of extracellular matrix genes that are part of the integrin and chemokine signaling pathways (Krischek and

Inoue, 2006), as well as complement cascades (Tulamo et al., 2006) and nitric oxide signaling (Khurana et al., 2005; Krischek et al., 2006). Ongoing research will prove and elucidate to which proportion these pathways play a part and if there is a synergistic effect toward IA formation and rupture.

Approaches for treatment

Even though intracranial aneurysms may be considered an irreversible process there may be a way for pharmaceutical therapy to lead to a regression. Recently an abdominal aortic aneurysm (AAA) mouse model was described in which application of a jun N-terminal kinase inhibitor inhibitor led to a regression of the aneurysm's diameter. In the case of AAA the diseased aorta seems to have the potential to regress if exacerbating factors are eliminated and/or the tissue repair is reinforced (Yoshimura et al., 2005). Matrix metalloproteinase inhibitors that reduce proteolysis in mechanical arterial wall injury models (Zempo et al., 1996; Frosen et al., 2001) might inhibit harmful matrix degradation in the IA wall and prevent rupture. Endovascular seeding of vascular SMC or pharmaceutically covered stents with antiinflammatory agents may be an option of future therapy, that leads to regression of the aneurysmal outpouching (Ribourtout and Raymond, 2004; Mazighi et al., 2006).

Identification of new targets to suppress the inflammation and immune response may significantly contribute to the development of anti-aneurysm and anti-rupture treatment.

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