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·Original Article·

Gene expression profiles in intracranial aneurysms

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ABSTRACT

In this study, we extracted total RNA from 15 intracranial aneurysms and 17 superficial temporal artery samples, then performed genome-wide expression profiling using the Affymetrix U133 Plus 2.0 GeneChip. Genes that were differentially expressed between intracranial aneurysms and arterial samples were identified using significance analysis for microarrays, and the expression patterns of three randomly-selected genes were verified by real-time polymerase chain reaction analysis. We identified 3 736 differentially-expressed genes out of the 47 000 assayed transcripts. A total of 179 genes showed a >10-fold change in expression between the aneurysms and the arterial samples. Genes involved in the proliferation, migration, and apoptosis of vascular muscle cells, atherosclerosis, extracellular matrix disruption, and inflammatory reactions were associated with the formation of intracranial aneurysms. There were no significant differences in gene expression profile between unruptured and ruptured aneurysms.

Keywords: intracranial aneurysms; microarray; gene expression profiles

INTRODUCTION

Aneurysmal subarachnoid hemorrhage is an event with high mortality and morbidity. It accounts for 3–11% of all stroke cases[1, 2], and causes death in 5% and disability in 25% of stroke patients. The prevalence of intracranial aneurysms is ~1% in adults[3-6]. Studies have shown that both environmental and genetic factors are involved in the pathogenesis of cerebral aneurysms, and inflammatory factors such as interleukin (IL)-1β, IL-6, IL-8, IL-18, interferon (IFN)-γ, and tumor necrosis factor (TNF)-α also play essential roles in the development, progression, and rupture of aneurysms. However, studies focused on single-nucleotide polymorphisms of these genes, and their association with aneurysms, have yielded inconsistent results[7-11]. Defects or aberrant changes in gene transcription and translation underlie the development of many diseases, since they regulate the structure, concentration, and activity of specific proteins in disease-related tissues. Thus, gene expression changes in intracranial aneurysms have received widespread attention in recent years. However, there has been limited success in identifying the significance of gene expression in aneurysms, due to ethnic differences underlying differential gene-expression profiles and difficulties in tissue-sampling and control selection. For example, aneurysm tissues were obtained from autopsies in some studies[12], and controls were obtained from diverse tissues, such as the superficial temporal artery[13], the middle meningeal artery[14], normal arteries adjacent to an arteriovenous malformation[15], and other intracranial arteries[16]. In this study, we obtained aneurysm tissue samples from 15 patients and superficial temporal artery samples from 17 patients as a control during surgery, and examined the gene expression profiles using cDNA microarray-based methods.

PARTICIPANTS AND METHODS

Informed Consent and Ethics Committee Approval

All experimental protocols in this study were approved by the Ethics Committee of Beijing Tiantan Hospital, Capital Medical

University. All participants and their legal representatives were informed of the purposes and procedures of the study, and provided written informed consent.

Selection and Treatment of Participants

A total of 32 patients who underwent aneurysm resection with craniotomy at Beijing Tiantan Hospital, Capital Medical University from March to June 2012 were enrolled. They were all of Han Chinese origin and aged 18–66 years. Patients were diagnosed with intracranial aneurysms using cerebrovascular digital subtraction angiography. Clinical data of the 32 participants are listed in Table 1. Fifteen patients were in the experimental group and 17 in the control group. In the experimental group, aneurysm tissue samples were collected from patients who underwent aneurysm resection by craniotomy; in the control group, superficial temporal artery (STA) samples were collected from intracranial aneurysm patients by the frontotemporal craniotomy. The number of patients with hypertension, coronary heart disease, diabetes, and other diseases were matched in the two groups. The samples were washed, soaked in the RNALater (Qiagen, Hilden, Germany) RNA stabilization reagent immediately after collection, and preserved at −80°C until use.

RNA Extraction, Reverse Transcription, Amplification, and Probe Labeling

Tissues were ground in liquid nitrogen and homogenized in 600 μL β-mercaptoethanol containing RLT buffer (Qiagen). RNA was extracted and purified using an RNEasy kit according to the manufacturer's instructions (Catalog No. 74104, Qiagen) and quantified using a UV spectrophotometer (NanoDrop ND-1000; Thermo Fisher Scientific, Waltham, MA). Quality inspection of extracted RNA was performed using agar gel electrophoresis or a 2100 Bioanalyzer (Agilent Technologies, Inc., Waldbronn, Germany).

Reverse transcription was performed with 100 ng of purified and quality-inspected RNA as template, which was labeled using the labeling reagent from the GeneChip Poly-A RNA Control kit (Catalog No. 900433; Affymetrix, Santa Clara, CA). First-strand cDNA was synthesized using a T7 promoter-containing oligonucleotide primer, and second-strand cDNA synthesis was performed in accordance with the instructions with the MessageAmp™ Premier RNA Amplification kit (Catalog No. AM1792; Life Technologies Corp., Carlsbad, CA). *In vitro* transcription was performed with the synthesized double-stranded DNA as template, and cRNA was labeled with biotin according to the instructions with the MessageAmp™ kit.

Probe Preparation and Microarray Hybridization

A total of 15 μg purified biotin-labeled cRNA was fragmented as probe using a MessageAmp™ II-Biotin Enhanced kit (Catalog No. AM1791; Life Technologies Corp.). The fragmented probe was then mixed with the pre-made hybridization solution according to the instructions with the Eukaryotic Hybridization Control kit (Catalog

Table 1. Clinical data from 32 patients with aneurysms

	Total	Aneurysm group	Control group	*P* value	Statistic
Age (years)		51.67 ± 8.457	54.88 ± 9.313	0.317	t = -1.017 df = 17
Gender (female)	21	11	10	0.472	
Hypertension	17	7	10	0.723	
Diabetes	2	0	2	0.486	
Coronary disease	3	0	3	0.229	
Hypertension medication	10	3	7	0.265	
Diabetes medication	1	0	1	1.000	
Coronary disease medication	3	0	3	0.229	

Statistics on age was conducted using *t*-test; Other data were analyzed with χ^2 test.

No. 900433; Affymetrix). The mixture was hybridized onto U133 Plus 2.0 GeneChips (Affymetrix) for 16 h at 45°C. The chips were washed and stained with the Affymetrix GeneChip Hybridization Wash and Stain kit (P/N 900720) using a GeneChip Fluidics Station 450 (Affymetrix), and finally scanned with a GeneChip Scanner 3000 (Affymetrix).

Microarray Analysis

Affymetrix GeneChip Human Genome U133 Plus 2.0 software was used to analyze gene transcription in human vascular tissues. This array covers ~38 500 human genes and assays ~47 000 transcripts.

Data generated by the GeneChip Scanner 3000 were then transformed with Affymetrix GeneChip Command Console software (Affymetrix), and saved as .CEL files. Transformed data pre-processing (background correction, normalization, and expression level calculation) was performed using Robust Multiarray Analysis. Significance analysis of microarrays (SAM) was used to select genes that exhibited differential expression between the aneurysm-derived and arterial samples, and the R language pack was used to calculate the significance of such differences (*q* value). A significant difference was defined as a fold-change >2 and *q* <0.01.

Real-time Quantitative PCR (qPCR)

We further randomly selected three differentially-expressed genes (*TLR4*, *MYD88*, and *IRAK-1*), and obtained their full-length cDNA sequences from Genbank. PCR primers were designed by Capital Biochip Corp., Beijing. The expression of these genes in the aneurysms and arterial samples was analyzed by real-time qPCR using the ABI 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA) to verify the results of the GeneChip analysis.

RESULTS

Clinical Features

All 32 patients were of Han-Chinese origin. Clinical data are summarized in Table 1. The locations of the intracranial aneurysms in the experimental group were: anterior cerebral artery (13.3%, *n* = 2), anterior communicating artery (13.3%, *n* = 2), middle cerebral artery (26.7%, *n* = 4), internal carotid artery (13.3%, *n* = 2), and multiple sites (33.3%, *n* = 5).

Based on the history of aneurysm rupture, patients in the experimental group comprised: unruptured aneurysm (40%, *n* = 6), primary aneurysm rupture (46.7%, *n* = 7), and secondary aneurysm rupture (13.3%, *n* = 2).

The aneurysm diameters in the experimental group were: small (≤5 mm) (33.3%, *n* = 5), medium (6–15 mm) (26.7%, *n* = 4), large (16–25 mm) (6.7%, *n* = 1), and huge (>25 mm) (33.3%, *n* = 5).

There were no significant differences in age, gender, comorbidities, and medication history between the experimental and control groups.

Nucleotide Extraction

After purification, RNA extraction yielded 0.21–10.30 μg RNA, with RNA integrity numbers >6.60 and A_{260}:A_{280} ratios close to 1.

Microarray Quality Control

For the 32 arrays, the average background was 41.85 (range 40.58–44.00); the average noise signal was 1.47 (range 1.33–1.68); and the average 3'/5' ratio was 1.46 (range 1.08–2.24).

Cluster Analysis

Cluster analysis was performed using Cluster 3.0 (http://bonsai.hgc.jp/~mdehoon/software/cluster/software.htm#ctv). Microarray gene expression data are provided in matrix form, with rows referring to genes and columns referring to samples. Data in each column were calculated with zero mean, and the intra-sample and inter-group differences were normalized. The results of cluster analysis are shown in Figure 1.

Gene Expression Profiles

A total of 3 736 differentially-expressed transcripts [fold-change ≥2, false discovery rate (FDR) <0.01] were identified using SAM, and 179 showed a ≥10-fold change (FDR <0.01). Among all differentially-expressed transcripts in aneurysms, the greatest up-regulation reached 88.1-fold (*COL11A1*), and the greatest down-regulation reached 59.9-fold (*RERGL*).

Real-time qPCR

The real-time qPCR analysis revealed fold-changes in the expression of *TLR4* (encoding Toll-like receptor 4), *MYD88* (encoding myeloid differentiation primary response 88),

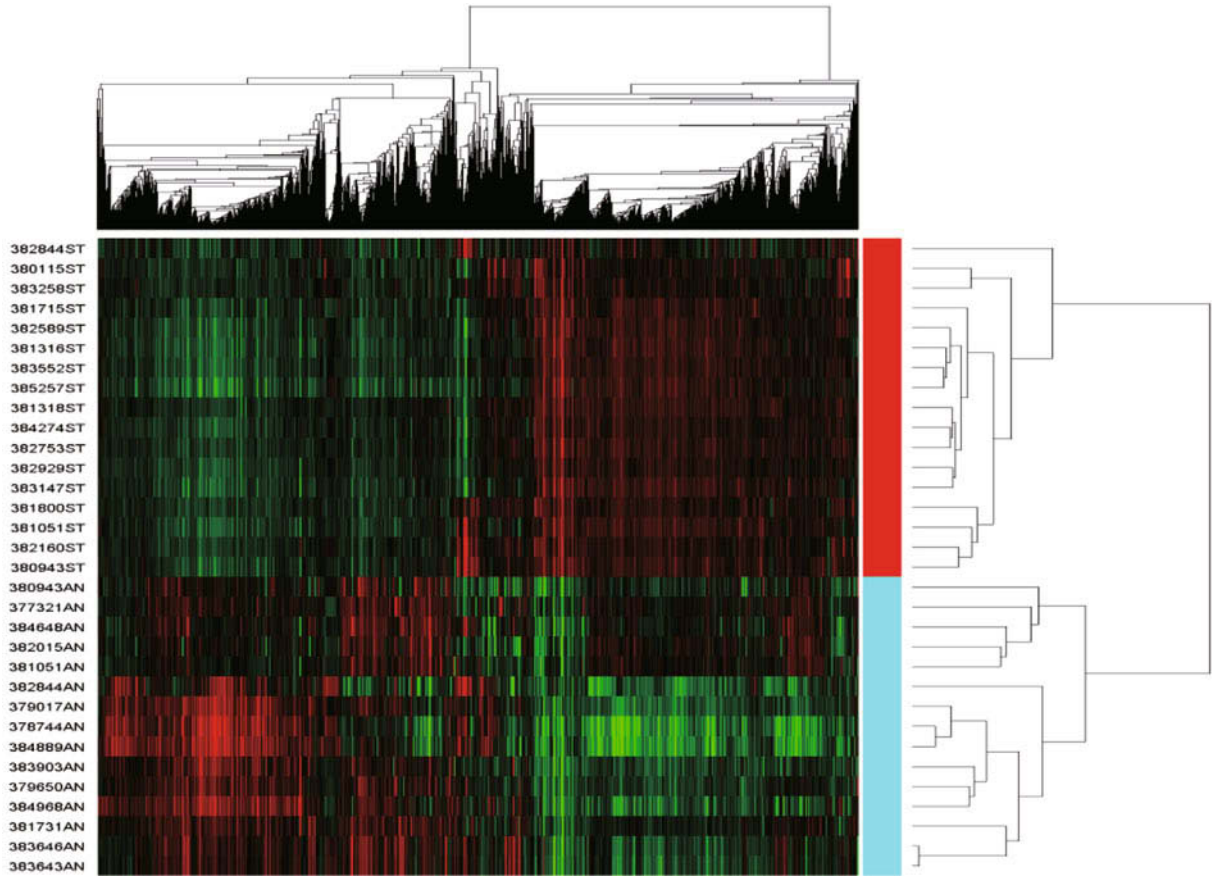

Fig. 1. Results of cluster analysis. Red, up-regulated genes; green, down-regulated genes. The abscissa represents the genes the chip included and the ordinate represents the tissues we used. The numbers on the left are the IDs of patients. AN means the aneurysm tissue while ST means the superficial temporal artery tissue. The trees on the top is the result of cluster analysis in accordance with the gene expression respectively in all samples and the tree on the right is the cluster analysis in accordance with all genes expression in every sample.

and *IRAK-1* (encoding interleukin-1 receptor-associated kinase-1) between the experimental and control groups, differences consistent with the microarray-based analyses, indicating the accuracy and reliability of the microarray-based method in the detection of differentially-expressed genes in aneurysms.

DISCUSSION

Stroke is the third leading cause of death world-wide. The annual incidence rate of intracranial aneurysms is 2%, and these cause 500 000 cases of stroke, resulting in severe neurological dysfunction and even death[17]. Family and twin studies have found that the incidence of intracranial hemorrhage in twins with intracranial aneurysms is four times higher than that in the general population[18], indicating the importance of genetic factors in their development. However, no major genotypes or mutations have been confirmed to be associated with intracranial aneurysms based on genome-wide linkage and candidate-gene studies[3]. Studies have also examined the gene expression profiles in intracranial aneurysms to identify differentially-expressed genes, albeit with limited success, due to difficulties in tissue sampling and control selection[12, 14-16, 19]. Also, inconsistent results were obtained due to racial differences in gene expression profiles, which hampered the identification of aneurysm-specific changes in gene expression.

Here, we used a microarray-based method to reveal the gene expression profiles in intracranial aneurysms

(experimental) and superficial temporal artery (control) samples from Han-Chinese patients. SAM identified 3 736 differentially-expressed genes (fold-change ≥2, FDR <0.01), of which 179 showed >10-fold changes and had FDRs <0.01 (Table 2). Among all the differentially-expressed transcripts, the most marked up-regulation reached 88.092-fold (*COL11A1*), and the greatest down-regulation reached 59.880-fold (*RERGL*).

Increasing numbers of studies have demonstrated that the proliferation, migration, and apoptosis of vascular muscle cells, atherosclerosis, extracellular matrix (ECM) destruction, and inflammatory reactions are associated with the formation of intracranial aneurysms[20-23]. Among the 179 genes that exhibited significant differences in expression levels between aneurysms and arterial samples (>10-fold change), those involved in vascular muscle cell proliferation, migration, apoptosis, and atherosclerosis were *ALOX5*, *APOC1*, *APOE*, *HMOX1*, *MSR1*, *OLR1*, *PLA2G7*, *SPP1*, *AGTR1*, *PDE4C*, and *RASL12*, of which *ALOX5*, *APOC1*, *APOE*, *HMOX1, MSR1*, *OLR1*, *PLA2G7*, and *SPP1* were up-regulated, while *AGTR1*, *PDE4C* and *RASL12* were down-regulated in aneurysm-derived tissues. Genes involved in ECM destruction were *APOE*, *IBSP*, *COL1A1*, *POSTN*, *SPP1*, and *COL4A6*, of which *APOE*, *IBSP*, *POSTN*, and *SPP1* were up-regulated, while *COL4A6* was down-regulated in aneurysm-derived tissues. Genes involved in inflammatory reactions were *ALOX5*, *APOE*, *CCL18*, *CCL3*, *CD86*, *CXCR4*, *FCGR1A*, *FCGR3A*, *HMOX1*, *IL8*, *LYZ*, *PLA2G7*, *RGS1*, *SERPINA1*, *SPP1*, *TYROBP*, *AGTR1*, *AOC3*, *COL4A6*, *CXCL14*, *PDE4C*, *TNC*, and *TRPV1*, of which *ALOX5*, *APOE*, *CCL18*, *CCL3*, *CD86*, *CXCR4*, *FCGR1A*, *FCGR3A*, *HMOX1*, *IL8*, *LYZ*, *PLA2G7*, *RGS1*, *SERPINA1*, *SPP1*, and *TYROBP* were up-regulated, while *AGTR1, AOC3*, *COL4A6*, *CXCL14*, *PDE4C*, *TNC*, and *TRPV1* were down-regulated in aneurysm-derived tissues.

The formation of intracranial aneurysms has been associated with vessel-wall damage caused by lymphocytes, antibodies, and complement-induced inflammatory reactions[20, 21]. Immune and inflammatory reactions can cause leukocyte adhesion, which then stimulates immune responses and the subsequent secretion of various inflammatory factors and proteolytic enzymes, which results in vascular wall damage. Chemokines are a class of small chemotactic molecules that interact with their

Table 2. Significance Analysis of Microarray showing the differential expression of genes between intracranial aneurysms and superficial temporal artery samples

RefSeq Transcript ID	Gene Symbol	Fold Change
NM_000582	SPP1	39.597
NM_004967	IBSP	33.7671
NM_001645	APOC1	24.0686
NM_002543	OLR1	20.33
NM_002922	RGS1	18.6297
NM_000569	FCGR3A /// FCGR3B	17.5669
NM_002922	RGS1	16.7418
NM_001004340	FCGR1B	15.6071
NM_001135934	POSTN	15.5631
NM_006332	IFI30	15.2804
NM_002133	HMOX1	15.0894
NM_005084	PLA2G7	14.4612
NM_000566	FCGR1A /// FCGR1C	14.4536
NM_002445	MSR1	14.291
NM_000295	SERPINA1	14.2094
NM_002988	CCL18	14.0653
NM_000584	IL8	13.197
NM_000088	COL1A1	12.5983
NM_000239	LYZ	12.1781
NM_006889	CD86	12.1323
NM_001008540	CXCR4	11.6774
NM_001001437	CCL3 /// CCL3L1 /// CCL3L3	10.884
NM_000698	ALOX5	10.163
NM_003332	TYROBP	10.0903
NM_000041	APOE	10.0606
NM_000923	PDE4C	0.0993
NM_031426	AIF1L	0.0973
NM_018727	TRPV1	0.0934
NM_000779	CYP4B1	0.0875
NM_004887	CXCL14	0.0822
NM_000685	AGTR1	0.0697
NM_003734	AOC3	0.0695
NM_016563	RASL12	0.0659
NM_001847	COL4A6	0.0467
NM_002160	TNC	0.1687

All *q*-values were 0%. These genes were reported to participate in atherosclerosis, extracellular matrix destruction, and inflammatory reactions, which are associated with the formation of aneurysm.

receptors to recruit and activate inflammatory cells such as neutrophils, lymphocytes, monocytes, and dendritic cells[24]. In this study, we not only identified up-regulated chemokine genes, such as *CCL18*, *CCL3*, *CCL3L3,* and *CCL3L1*, but also elevated expression of a chemokine receptor gene (*CXCR4*) in aneurysm tissues. A variety of inflammatory mediator genes including *IL8* and *IFI30* were up-regulated in the aneurysms, and the expression of various complement genes, such as *FCGR1A*, *FCGR1B*, *FCGR1C*, *FCGR3A*, and *FCGR3B*, was also significantly higher in the aneurysms than in the control arterial samples. In addition, many immune reaction-associated genes, such as *HMOX1* (encoding hemoxygenasge-1) showed significantly elevated expression in the aneurysms. High expression of immune and inflammation-associated genes in the aneurysms indicated that widespread immune and inflammatory reactions might be one of the most important causes of inflammatory injury and the formation of aneurysms. Nine immune and inflammatory reaction-associated genes, *AGTR1*, *AOC3*, *COL4A6*, *CXCL14, PDE4C*, *TNC*, *TRPV1*, *AIF1L*, and *CYP4B1*, were down-regulated in the aneurysms in our study. Generally, the magnitude of gene expression does not necessarily correspond to the pathophysiological process. And the functions of these up- or down-regulated genes in the pathogenesis and pathogenic pathways of intracranial aneurysms are still unknown.

Since human intracranial arteries lack an external elastic lamina, disruption of the ECM, which provides strength and elasticity, is suggested to play a role in the pathogenesis of intracranial aneurysms[22]. The ECM of the arterial wall contains collagen, elastic and reticular fibers, as well as a variety of non-collagenous sugar proteins. The ECM helps maintain the elasticity and resistance-to-expansion of the vessel wall, and participates in the regulation of endothelial and muscle cell functions by interacting with vascular wall cells. Collagen is assembled through the twisting of 3 polypeptide strands (α1, α2, and α3), and based on the structure of these strands, collagen can be classified into 27 types. There are 5 types of collagen in the arterial wall: types I, III, IV, V, and VI. Types I and III account for 80–90% of the total collagen in the arterial wall, and type III is the major factor contributing to the tensile properties of the arterial wall[25]. Here, we found that *COL1A1* (encoding collagen type I, α1) was significantly up-regulated, while *COL4A6* (encoding collagen type IV, α6) was significantly down-regulated in aneurysms. Type III is often co-expressed with type I in many tissues, and can form heterologous fibrils with type I. The diameter of the collagen fibril is negatively correlated with the ratio of type III to type I, and a higher III/I ratio leads to a finer fibril and a more pliant artery. The decrease in expression of type III and the concomitant increase in expression of type I would result in a decreased III/I ratio, thereby causing vessel dilation and the formation of aneurysms[22].

Apolipoprotein abnormalities can cause abnormal blood lipid levels and induce atherosclerosis, which then leads to intracranial arterial wall damage and decreases arterial resistance to expansion, and finally, intracranial aneurysms. The apolipoprotein E gene (*ApoE*) is located on chromosome 19, and the 3 most-frequent genotypes are ε2/ε2, ε3/ε3, and ε4/ε4, which encode the 3 main isoforms ApoE2, ApoE3, and ApoE4. We found that the concentration of *ApoE* in aneurysms was 10-fold higher than in the control samples. As an important apolipoprotein, ApoE participates in the pathogenesis of various cerebrovascular diseases through the regulation of lipid metabolism, and is significantly associated with the development of atherosclerosis. Different ApoE isoforms may differentially regulate the plasma concentration of low-density lipoprotein (LDL), thus affecting the atherosclerotic process. Previous studies have suggested that ApoE2 protects against cerebrovascular diseases by reducing the concentration of plasma LDL; on the other hand, ApoE4 increases it, thereby promoting the development of atherosclerosis, and is therefore a risk factor for atherosclerosis[23].

In accord with the data reported in previous studies[12, 14-16, 19], our results showed that genes with differential expression in aneurysms are mainly involved in immune and inflammatory processes, in cell growth, proliferation, differentiation and migration, as well as in intercellular signal transduction. We also found that these genes were most highly correlated with immune cell trafficking, tissue morphology, humoral immune responses, and blood and cardiovascular system development. However, we found no significant differences in the gene expression profiles of unruptured and ruptured aneurysms, which contradicts previous findings[16]. The

inconsistent results might be explained by the development of microarray technology, differences in the number of samples, different statistical methods, and by differences in control selection. For instance, Weinsheimer *et al.*[12] used intracranial vascular samples from autopsies; Krischek *et al.*[15] used arteriovenous malformations in the feeding artery; Shi *et al*.[19] used the superficial temporal artery; and Pera *et al*.[16] used meningeal artery samples as controls. All these arteries differ from intracranial vascular samples in both structure and hemodynamics. Also, racial differences in the participants in various studies are another important factor that may yield inconsistent results. Our study had a few limitations. First, the sample size was small, especially when aneurysms were divided into ruptured and unruptured groups. Second, the microarray results need to be verified by other experimental methods, such as real-time qPCR and immunohistochemistry. We verified the differential expression in three randomly-selected genes using real-time qPCR. Further validation of the microarray data and analysis of a larger number of samples is required in future studies to address the role of gene expression changes in the pathogenesis and progression of aneurysms.

In sum, the results of our study suggest that the genes differentially expressed in aneurysms are mainly those involved in immune and inflammatory responses, in cell growth, proliferation, differentiation, and migration, and in intercellular signal transduction. We also found that these genes were most-highly correlated with immune cell-trafficking, tissue morphology, humoral immune responses, and blood and cardiovascular system development. In contrast to the results of previous studies, our findings showed no significant differences in gene expression profiles between unruptured and ruptured aneurysms.

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