Gene Expression in Intracranial Aneurysms—Comparison Analysis of Aneurysmal Walls and Extracranial Arteries with Real-Time Polymerase Chain Reaction and Immunohistochemistry

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BACKGROUND: **This study was aimed at evaluating the gene expression levels of 4 genes in the intracranial aneurysm wall and comparing them with extracranial arteries. The analysis was done using real-time polymerase chain reaction (RT-PCR) and immunohistochemistry (IHC). Also, a correlation of the differential genetic expression was done with various patient clinical and radiologic factors.**

METHODS: **The quantitative assessment of ribonucleic acid levels was done with RT-PCR and was validated with IHC. The genes studied were collagen 1A2 *(COL1A2),* tissue inhibitor of metalloproteinase 4 *(TIMP4),* cathepsin B *(CTSB),* and alpha-1 antitrypsin *(α-1 AT).* The analysis was done on 24 aneurysm sacs and superficial temporal/occipital artery samples from patients undergoing surgical clipping.**

RESULTS: **The mean fold change of *COL1A2* in the aneurysm sample was 8.89, that of *TIMP4* was 10.16, that of *CTSB* was 1.02, and that of *α-1 AT* was 1.46 when compared with normal control vessel on PCR. On semiquantitative IHC, *COL1A2* was 94.44%, *α-1 AT* was 77.8% overexpressed, *CTSB* was positive in 50%, and the expression of *TIMP4* was 94.4% underexpressed in aneurysmal walls. There was no statistically significant correlation between patient profile and gene expression.**

CONCLUSIONS: **On RT-PCR and IHC analysis, *COL1A2* and *α-1 AT* were overexpressed, *CTSB* was marginally overexpressed, and *TIMP4* had equivocal expression in the aneurysmal sac when compared with the normal extracranial vessel. This is the first study of its kind in the Indian population with the largest sample size on live human patients.**

INTRODUCTION

Stroke is one of the leading causes of morbidity and mortality worldwide. Only approximately 13% of the patients had a stroke caused by hemorrhage, even less so (3%) due to subarachnoid hemorrhage (SAH).[1] A ruptured intracranial aneurysm (IA) represents the most frequent etiology, contributing up to 85% of the cases.[2] Even though IA rupture resulting in SAH is relatively rare (≈1 in 10,000 people), the

Key words
- Alpha-1 antitrypsin
- Aneurysm
- Aneurysm genetics
- Cathepsin B *(CTSB)*
- Collagen 1A2 *(COL1A2)*
- Immunohistochemistry
- Intracranial aneurysms
- PCR
- Tissue inhibitor of metalloproteinase 4 *(TIMP4)*

Abbreviations and Acronyms
α-1 AT: Alpha-1 antitrypsin or *SERPIN-A*
AComm: Anterior communicating
COL1A2: Collagen 1A2
CT: Cycle threshold
CTSB: Cathepsin B
IA: Intracranial aneurysm
IHC: Immunohistochemistry
MCA: Middle carotid artery
OA: Occipital artery
RT-PCR: Real-time polymerase chain reaction
SAH: Subarachnoid hemorrhage
STA: Superficial temporal artery
TIMP: Tissue inhibitor of matrix metalloproteinases
UIA: Unruptured intracranial aneurysm

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prevalence of unruptured IA (UIA) is common in the population. IA prevalence increases with age and peaks in the fifth to sixth decades, affecting around 1%–2% of the population.[3] The incidence of aneurysmal SAH has specific geographic and demographic patterns as is evident by the increased occurrence in countries like Japan and Finland.

The main modifiable risk factors for SAH are smoking, hypertension, and heavy alcohol intake. Nonmodifiable risk factors include increasing age, female sex, autosomal dominant polycystic kidney disease, and family history of SAH.[4] The inherent risk of an individual to harbor an aneurysm and suffer from SAH can be attributed to the constitutional structural elements and genetic factors. There is, however, no reliable way to identify individuals who are at risk for the formation of IAs.

Thus in the current study, we aim to identify the role of specific gene expression and function in the development of intracranial aneurysms and their rupture. We evaluated the differential expression of collagen 1A2 (*COL1A2*), cathepsin B (*CTSB*), tissue inhibitor of metalloproteinases-4 (*TIMP4*), and alpha-1 antitrypsin (*α-1 AT* or *SERPIN-A*) genes in the walls of aneurysm tissue compared with control tissue. We did real-time quantitative polymerase chain reaction (RT-PCR) for gene expression and validated the results with immunohistochemistry (IHC).

MATERIALS AND METHODS

Patient Selection

The cases were prospectively recruited during 1 year between July 2016 and June 2017. A total of 35 patients undergoing surgery for an IA were recruited for the study. Only adult patients between 18 and 75 years of age were recruited in the study. All of them had a sporadic aneurysm with no family history of aneurysm or SAH. None of the patients had any syndrome predilecting the formation of IA, like Marfan, adult polycystic kidney disease, Ehlers-Danlos, and other known syndromic associations. These 35 patients had favorable anatomy for excision of the sac as deemed by the operating surgeon. A booster clip distal to the first clip was included for reinforcement to prevent bleeding in the event of a single clip slipping out of the neck. Informed consent for recruitment in the study was taken before the procedure. The study was approved by the Departmental Scientific Committee and the Institutional Ethics Committee.

Data Collection, Sampling, and Storage

The data regarding patient demography, clinical presentation, laboratory and imaging reports, intraoperative findings, and postoperative clinical outcome were collected from individual case records. During surgery, once the aneurysm was clipped and its anatomy was deemed favorable (adequate size, distinct neck, no vessels/perforators wrapped around the dome) by the operating surgeon, the sac of the aneurysm distal to the clips was excised. A branch of the superficial temporal artery or a muscular branch from the occipital artery (in a patient who underwent retromastoid craniotomy) was also harvested from the same patient at the time of scalp dissection. Each sample was divided in half, 1 for ribonucleic acid (RNA) expression studies and the other for IHC. The tissue for expression studies was transferred immediately to RNA solution kit at 4°C in the operating room and transferred to a −80°C freezer within 24 hours until RNA extraction. The other was kept in 10% formalin solution at room temperature.

Real-Time Polymerase Chain Reaction

Isolation of Ribonucleic Acid. The samples stored at −80°C were thawed at 4°C for at least half an hour before isolation. RNA was isolated from tissue samples with Qiagen RNeasy Mini Kit. All the preliminary processes were carried out in an ESCO biosafety cabinet (ESCO Corporation Inc, Portland, Oregon, USA) with laminar airflow. The quality of RNA was analyzed with NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies Inc, Wilmington, Delaware, USA). Samples having an A260/A280 ratio of >1.8–2.0 were considered.

cDNA Synthesis and Real-Time Quantitative Polymerase Chain Reaction. We converted 200–1000 ng of purified RNA to cDNA using the Applied Biosystems High Capacity cDNA Reverse Transcription Kit. TaqMan Gene Expression Assays were used for quantitative RT-PCR. The housekeeping gene, *HPRT1* (Hs02800695_m1, VIC), was used as an endogenous control and run along with the genes tested. Corresponding assays were used to detect the target genes of interest: *COL1A2* (Hs00164099_m1, FAM), *TIMP4* (Hs00162784_m1, FAM), *CTSB* (Hs00947439_m1, FAM), and *SERPIN-A* (Hs00165475_m1).in the Applied Biosystems-7500.

Analysis of Results. The following formula calculated the fold change of the particular gene in the aneurysm wall with the superficial temporal artery (STA)[5,6]:

ΔCt (aneurysm samples) = Ct (target gene) − Ct (housekeeping gene)

ΔCt (STA) = Ct (target gene) − Ct (housekeeping gene)

Δ ΔCt (samples) = ΔCt (aneurysm samples) − ΔCt (STA)

Fold change expression= $2^{(-\Delta\,\Delta Ct\ [\text{target gene}])}$

The fold changes of individual genes were computed by considering the mean cycle threshold (CT) value of all the STA samples. For the patients from whom only the aneurysmal sac was harvested, without STA, the mean CT value of the STA samples taken from the other patients was used to compute the fold change of the genes studied.

Immunohistochemistry

4μ fixed sections from formalin-fixed embedded blocks were cut, and the sections were collected on poly-L-lysine coated slides and subjected for IHC. Briefly following deparaffinization and initial antigen retrieval steps, the sections were stained for anti-*TIMP4*, anti-*COL1A2*, anti-cathepsin B, and anti-*α-1 AT*.

The staining was done using the automated immunohistochemistry staining machine (Ventana Benchmark XT, Basel, Switzerland). After initial processing, cut sections were incubated with primary antibodies for 1 hour followed by incubation with secondary antibodies for 8 minutes. The negative controls were treated identically except that the primary antibody was omitted.

Statistical Analysis

Analysis of the data was done using IBM SPSS 19.0 (Armonk, New York, USA). Variables were not normally distributed. Categorical data were analyzed using the chi-squared and Fisher tests, while the independent Student's *t*-test was used for continuous

variables. The level of significance was at 5% with 95% confidence interval. P value of ≤ 0.5 was considered significant.

RESULTS

From the 35 patients included in the study, 35 samples of aneurysmal sac, 30 samples of the frontal branch of the superficial temporal artery, and 1 sample from a branch of occipital artery could be obtained. After RT-PCR and IHC analysis, data from only 24 samples could be taken for analysis. Further, data from both IHC and RNA expression were available for only 12 samples, though altogether data for 18 samples were available. The remaining samples had to be discarded at various stages of the experiments due to small size of the tissue, incomplete visualization of the wall under a microscope, low RNA yield, etc. A schematic illustration of the number of cases included in the study has been shown in **Figure 1**.

Patient Demography and Clinical Features

The mean age of the patients was 52.67 years (range 20–77 years) (**Table 1**). The study encompassed 10 males and 14 females. SAH was present in 22 (88.89%) cases. Two patients presented with an incidentally detected UIA with complaints of intermittent headache.

The most common presenting symptom was sudden onset of severe headache, in 21/24 (87.5%) patients. Seizures occurred in 3 patients. One patient presented with right hemiparesis after ruptured left middle carotid artery (MCA) aneurysm, while another patient had a complete right third nerve weakness due to a ruptured communicating internal carotid artery aneurysm. One patient with posterior inferior cerebellar artery aneurysm presented with seventh nerve weakness with cerebellar signs. **Table 1** summarizes the patient demography and clinical symptoms.

A history of smoking was present in 6 patients, while 4 had a history of chronic alcohol consumption. History of diagnosed hypertension was present in 7 patients. None of the patients gave a positive family history for SAH or intracranial aneurysm. Patients were graded on clinical presentation considering the Glasgow Coma Scale after resuscitation and the presence of neurologic deficits, and a World Federation of Neurosurgical Societies grade was assigned. The median presenting Glasgow Coma Scale score was 15 (**Table 2**).

Radiologic Features

All patients underwent a plain CT scan of the brain (**Table 3**). Seven patients had intraventricular hemorrhage. Four patients had the presence of hydrocephalus. All patients underwent catheter angiography (digital subtraction angiography) to localize and define the character and anatomy of the IA except for 2 who were operated on based on CT angiogram. Two patients had multiple aneurysms; the total was 27 aneurysms in 24 patients under study. The average maximum diameter of the sac was 6.67 mm, and the average diameter of the neck of the aneurysm was 3.54 (see **Table 3**).

Management and Outcome

Patients presenting with SAH were put on standard antiedema measures. Twenty patients underwent pterional craniotomy and clipping. In 2 patients frontal parasagittal and bifrontal craniotomies, respectively, were done to clip distal anterior cerebral artery aneurysms. Two patients underwent retrosigmoid suboccipital and far lateral craniotomies for posterior inferior cerebellar artery aneurysms. Temporary clipping of the proximal vessel was done in 19 of the 24 cases with a mean duration of 2.5 minutes in order to facilitate dissection of the aneurysm. Intraoperative rupture occurred in 4 cases. Infrared indocyanine green video angiography was used to identify patency of the parent vessels and obliteration of the sac in 12 cases.

Significant complications occurred in 3 patients after surgery. One patient developed a malignant MCA territory infarct post

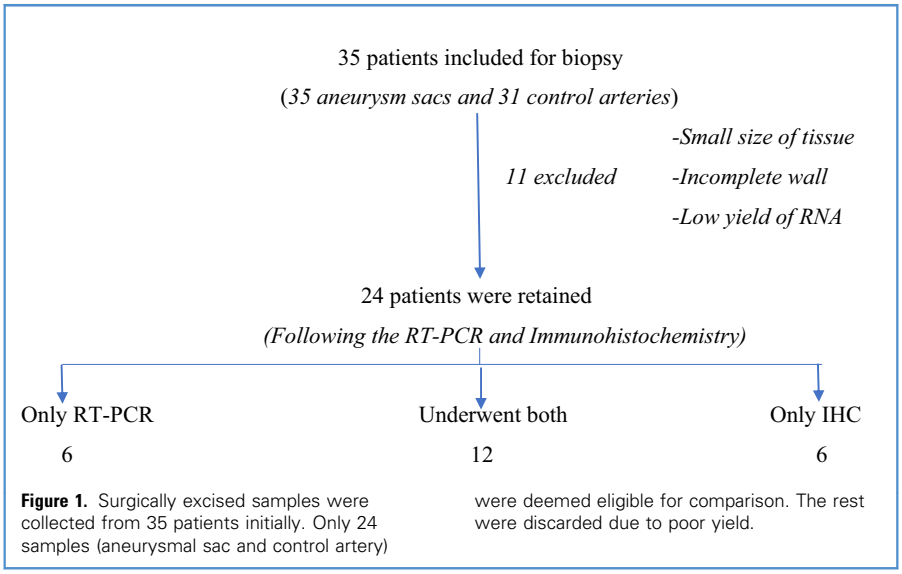

Figure 1. Surgically excised samples were collected from 35 patients initially. Only 24 samples (aneurysmal sac and control artery) were deemed eligible for comparison. The rest were discarded due to poor yield.

Table 1. Demographic Profile and Risk Factors of Patients Included in Study

Variables	Number of Patients (n = 24)	% Split
Sex		
Male	10	42%
Female	14	58%
Age		
<50 years	10	42%
>50 years	14	58%
Smoking		
Yes	8	33%
Alcohol		
Yes	6	25%
Hypertension		
Yes	10	41.67%
Rupture		
Yes	22	91.67%
No	2	08.33%

obliteration of the MCA lumen with clips and had to undergo decompressive hemicraniectomy on postoperative day 2. Another patient developed infarct in the anterior choroidal artery territory with postoperative hemiplegia. The last patient developed operative site hematoma and deterioration in sensorium and had to undergo reexploration and evacuation of hematoma. He later developed wound site infection and ventilator-associated pneumonia and expired due to septicemia.

Transient worsening of neurologic status developed in 8 patients due to vasospasm, which improved on conservative measures and intraarterial nimodipine infusion. Two patients developed postoperative hydrocephalus and underwent a ventriculoperitoneal shunt.

Three mortalities occurred during the postoperative hospital stay. The first patient died due to septicemia after multiple surgeries. One patient had severe diffuse vasospasm with multiple infarcts. The third mortality was due to myocardial dysfunction and severe pleural effusion following surgery (Table 4).

Table 2. World Federation of Neurosurgical Societies (WFNS) Grade of Patients in Immunohistochemistry Group Who Underwent Surgery

Grade	Number of Patients (n = 24)	Percentage
WFNS Grade		
I	15	66.67%
II	3	16.67%
III	5	16.67%
IV	1	04.16%

Table 3. Radiologic Findings of Patients Undergoing Surgery

Findings on Imaging	Number of Patients (n = 24)	Percentage
Computed tomography of brain		
Unruptured	2	08.33%
Cerebral edema	15	62.5%
IVH	7	29.17%
Hydrocephalus	8	33.33%
DSA		
Vasospasm	17	70.83%
Multiple aneurysms	2	11.11%
Maximum diameter of sac	6.67 mm	
Fundus height	4.44 mm	
Neck	3.54 mm	
Location of aneurysm (n = 27)		
Anterior communicating artery	9	33.33%
DACA	2	07.40%
Communicating ICA	5	18.52%
Ophthalmic ICA	1	03.70%
MCA bifurcation	8	29.62%
PICA	2	07.40%

IVH, intraventricular hemorrhage; DSA, digital subtraction angiography; DACA, distal anterior cerebral artery; ICA, internal carotid artery; MCA, middle cerebral artery; PICA, posterior inferior cerebellar artery.

Immunohistochemistry

On hematoxylin-eosin, monoclonal antibody (MAT)-1, and Verhoeff–Van Gieson staining, a definitive aneurysmal wall was noted in 19 out of the 28 cases and superficial temporal artery (control) in 25/28 cases (Figures 2 and 3). *TIMP4* was predominantly nuclear, staining noted in the fibroblastic cells of aneurysmal and smooth muscle cells of arterial controls. *CTSB*, *SERPIN-A*, and *COL1A2* stained the cytoplasm and background collagen. A few histiocytes stained positive for *SERPIN-A*. Visual assessment of the immunoreactivity in aneurysmal versus arterial (control) was carried out. The results are shown in Table 5.

Table 4. Summary of Glasgow Outcome Scale of Patients at Follow-up (n = 18)

Glasgow Outcome Scale	Number of Patients (n = 24)	Percent Split
Dead	3	14.28%
Vegetative state	-	-
Severe disability	2	14.28%
Moderate disability	2	07.14%
Good recovery	11	64.28%

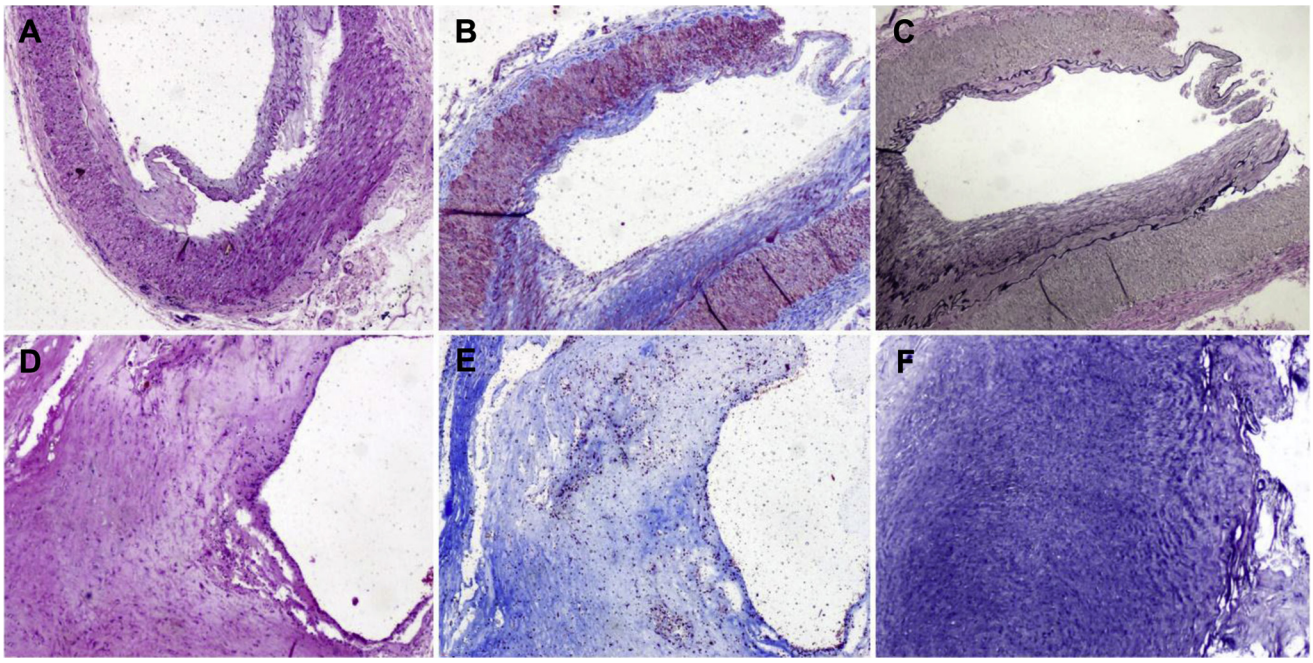

Figure 2. Histopathologic images of control temporal artery (**A**–**C**) and aneurysmal wall of 1 case (**D**–**F**). All images are original magnification ×160. (**A**) The control artery shows all layers; tunica intima, media, and adventitia in hematoxylin-eosin stain. (**B**) Masson trichrome stain highlights the smooth muscles in the media. (**C**) The elastic lamina is delineated by the Verhoeff–Van Gieson stain. (**D**–**F**) In contrast, the aneurysm has only the fibrous wall (**A**), devoid of smooth muscles (**B**) and elastic lamina (**C**).

Real-Time Polymerase Chain Reaction

Table 6 compares the various laboratory variables in RNA extraction.

Mean Fold Changes of Genes

The fold change of all the genes was calculated in 18 patients (Table 7). In 1 patient the fold change value of collagen 1A2 was 128.29, which was treated as an outlier and excluded from the study. While calculating the mean fold change of the aneurysmal sample, the average mean of the STA Ct value was used wherever the control vessel was not available for comparison. The results are summarized in Table 7.

The mean fold change values of RT-PCR and gene expression with IHC were statistically compared with the clinical and radiologic data of individual patients. However, we could not find any statistically significant correlation between them.

DISCUSSION

IA formation is a complex process with multifactorial pathogenesis. The role of mechanical factors like increased wall tension and shear stress has been described. However, the molecular changes that these mechanical factors bring about in the walls of blood vessels, leading to the formation and ultimately rupture of aneurysms, remain to be explored. Scores of studies involving latest technologies (microarray, genome sequencing, SNPs) have been used, but the quest to narrow down this complex multifactorial pathology to a defined etiology that can be targeted by therapy has been unrewarding.

On reviewing the present literature on genetics of intracranial aneurysm, we identified 4 genes—*SERPIN-A*, *COL1A2*, *CTSB*, and *TIMP4*—that had been studied for their association with IAs in multiple microarray and genome wide association study.

Various controls have been used in few studies including the STA, middle meningeal artery (MMA), arterial feeders of an arteriovenous malformation, and MCA segments during STA-MCA bypass procedures.[7] We used a frontal branch of the superficial temporal artery and muscular branch from the occipital artery (OA) as controls. We believe the scalp has an extensive blood supply with a network of anastomosis between the arteries. Thus taking a branch of either artery is less likely to cause a vascular compromise. Also, harvesting an extracranial vessel is far safer than harvesting an intracranial vessel.

Collagen 1A2

Collagen is one of the most important structural proteins in vessel walls. It not only provides structural and tensile strength but also plays a vital role in remodeling, wound healing, and inflammation. Upregulation of different types of collagens in aneurysm walls has been described.[8] *Collagen 1A2* encodes alpha 2 chains of type I collagen. Qi Gan et al,[9] in their systematic meta-analysis on polymorphism of the *COL1A2* gene, found that the rs42524 polymorphism is a significant risk factor for aneurysm development.

In microarray analysis, Shi et al[10] found an upregulation of the *COL1A2* gene between IA and STA samples. Li et al[11] and Marchese et al[12] showed similar results. Krischek et al[13] studied ruptured versus unruptured versus control arteries (arteriovenous malformation feeders) by microarray analysis and IHC and

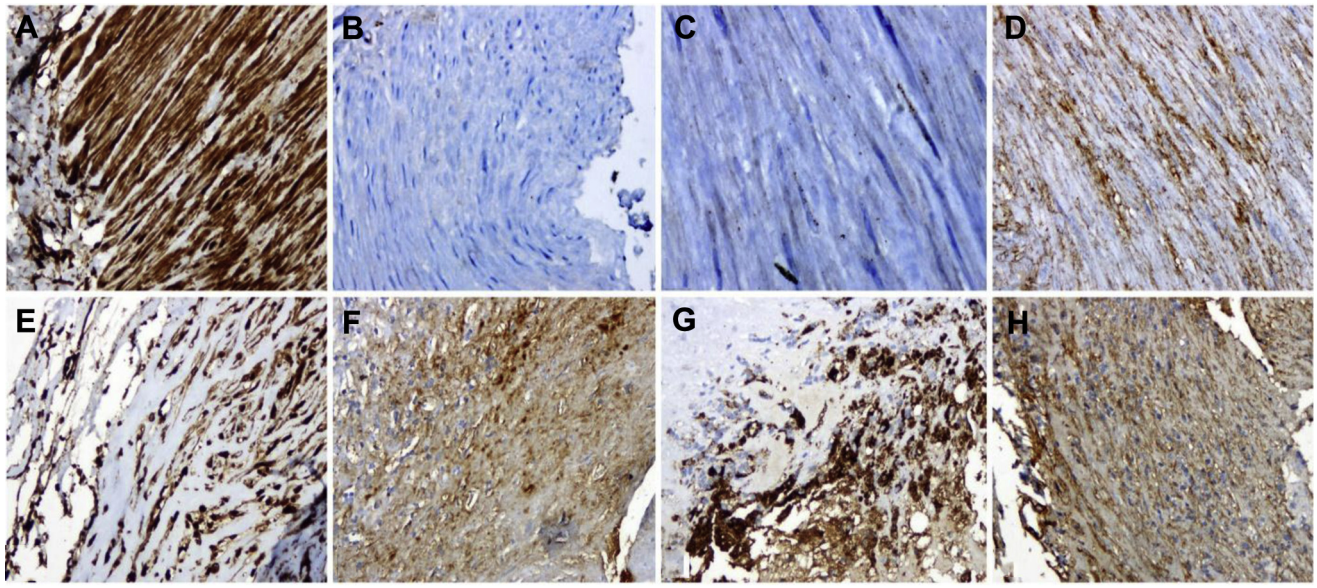

Figure 3. Immunohistochemical marker expression of control temporal artery (**A**–**D**) and aneurysmal wall of 1 case (**D**–**G**). All images are original magnification ×160. *TIMP4* expression is higher in the control (**A**) when compared with the aneurysmal wall (**E**). *α1-Antitrypsin* expression is higher in the aneurysmal wall (**F**) compared with the control (**B**). *Cathepsin-B* expression is higher in the aneurysmal wall (**G**) compared with the control (**C**). *COL1A2* expression is higher in the aneurysmal wall (**H** compared with the control, **D**).

found significantly increased expression of *COL1A2*. Lastly, Babu et al[7] reported an increased expression with a mean fold change of 2.46 by RNA PCR. Similar readings were found in our study (Figures 2 and 3).

The increased expression of collagen gene may seem paradoxical because there is increased degradation of collagen in the vessel walls due to increased activity of gelatinases, collagenases, and elastases. However, structural remodeling might occur with an accelerated production of collagen. This compensatory haphazard laying of collagen might cause a structural abnormality predisposing to vessel wall weakness and IA formation.

Cathepsin B

Cathepsins are part of a large family of lysosomal proteolytic enzymes. Cathepsins digest foreign or unwanted intracellular or endocytosed proteins.[14] Recently their collagenolytic, as well as elastolytic properties, have also been demonstrated extracellularly.[15] Their expression was found to be increased in multiple vascular diseases like arteriosclerosis, aortic abdominal aortic aneurysms, and IAs.[7,16,17] However, no immunoreactivity of cathepsins was found in healthy human vessels.

In their study on experimentally induced aneurysms in rats and human aneurysm walls, Aoki et al,[16] found increased expressions of cathepsin B, K, and S in smooth muscle cells. Similarly, endothelium and macrophage also expressed these enzymes in the later stages of IA progression. Increased degradation of ECM occurs, resulting in wall weakness, IAs formation, progression, and rupture. They also showed that cystatin C (NC-2300), a cysteine inhibitor of cathepsins prevented the progression of aneurysms.[16] Thus proteinase inhibitors such as NC-2300 may be considered as a promising candidate for targeting the development of IAs.

Table 5. Variable Expression of Genes Under Study in 18 Aneurysmal Walls as Seen by Immunohistochemical Staining, When Compared with STA Controls of Same Patient

	TIMP4	*Cat-B*	*α-1 AT*	*Coll-1A2*
Expression	Reduced	Increased	Increased	Increased
Sample	17/18	09/18	14/18	17/18
Percentage	94.44%	50%	77.78%	94.44%

Table 6. Laboratory Variables After Ribonucelic Acid Extraction from Tissue (n = 18)

Average weight of aneurysmal wall biopsied	25.2 mg
Average RNA quantity of aneurysm sample	41.9 ng/uL
RNA quality of aneurysm (260/280 nm value)	1.8
Average amount of RNA converted to cDNA per aneurysm sample	12 ng
Average weight of STA biopsied	30 mg
Average RNA quantity of STA sample	41.3 ng/ul
RNA quality of STA (260/280nm value)	1.8
Average amount of RNA converted to cDNA per STA/OA sample	12 ng

Table 7. Final Mean Fold Change Values of Gene Expression by PCR

Gene	Mean Fold Change	Expression
Collagen 1A2	8.89	Increased
Cathepsin B	00.98	Decreased
Tissue Inhibitor of Metalloproteinases	10.16	Increased
Alpha-1 Antitrypsin gene	01.46	Increased

CTSB gene expression was found to be 6.50 times more in the IA samples than in STA by Shi et al.[10] Similarly, Babu et al[7] found it to be more than 31 times that of control with PCR. Marchese et al[12] also found an overexpression of *CTSB* in the aneurysmal wall. However, Krischek et al[13] found no change in the expression of the cathepsin B gene, *CTSB*, in the aneurysm walls when compared with the control vessel. In the current study, *CTSB* had a mean fold change value of 0.98 with PCR. It was analyzed to be only marginally (1.02 times) underexpressed when compared with control values. This finding was also validated by the IHC where only 50% of the IA samples showed increased staining with CTSB antibody as compared with control vessels. These equivocal findings were consistent with Krishchek's observations.

We believe that decreased levels of cathepsin B, a lysosomal enzyme, could lead to an accelerated activity of intracellular and extracellular proinflammatory cytokines. An upregulated inflammatory process might result in increased remodeling of the vessel wall.

Matrix Metalloproteinases and Tissue Inhibitor of Metalloproteinases

MMPs, also known as *matrixins*, are endopeptidases belonging to the metzincin superfamily of proteases. MMPs are capable of degrading all kinds of extracellular matrix proteins. They also play a significant role in the cellular function like proliferation, differentiation, angiogenesis, migration, apoptosis, and host defenses.[18] MMPs degrade extracellular structural proteins (elastin and collagen) by their enzymatic action.[19] MMP-9 (gelatinase B) overexpression has been reported in aortic and cerebral aneurysms.[20]

Metalloproteinase inhibitors act as specific endogenous protease tissue inhibitors to MMPs. They are a family of 4 enzyme inhibitors: *TIMP1*, *TIMP2*, *TIMP3*, and *TIMP4*.[21] *TIMP4* has been shown to interact and inhibit the function of MMP2.

Jin et al[20] found the gene expression ratios of MMPs versus TIMPs were higher in ruptured than unruptured aneurysms. They considered this upregulation of TIMPs as an adaptive reaction to counter the degradation of ECM caused by MMPs.

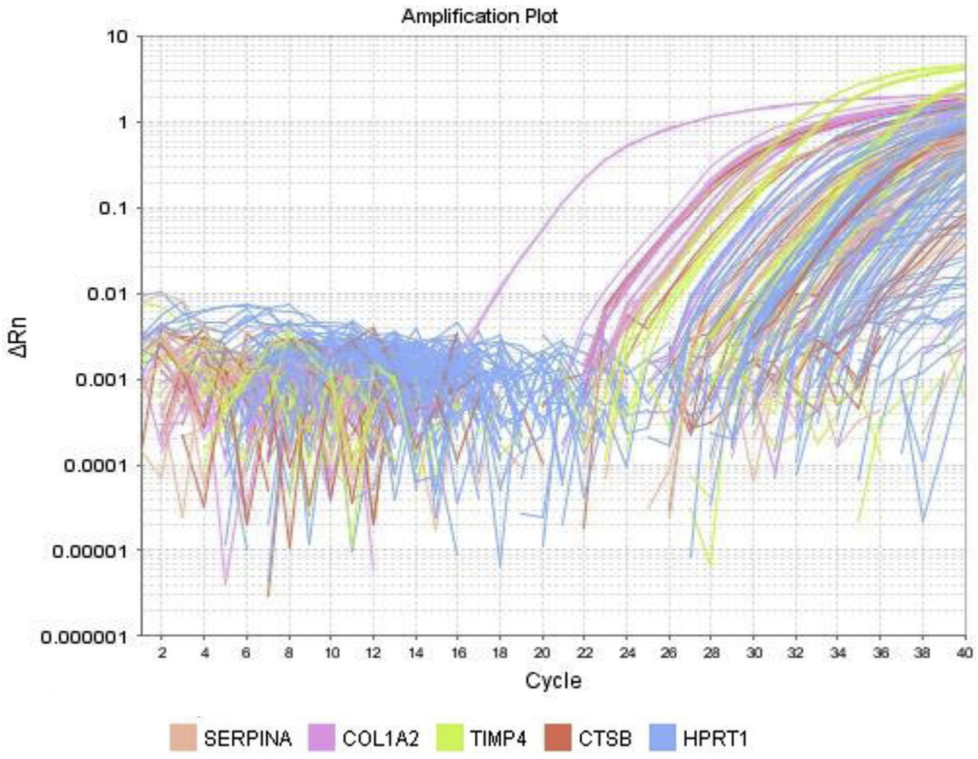

Figure 4. Amplification plot of ribonucleic acid expression for all 4 genes grouped together.

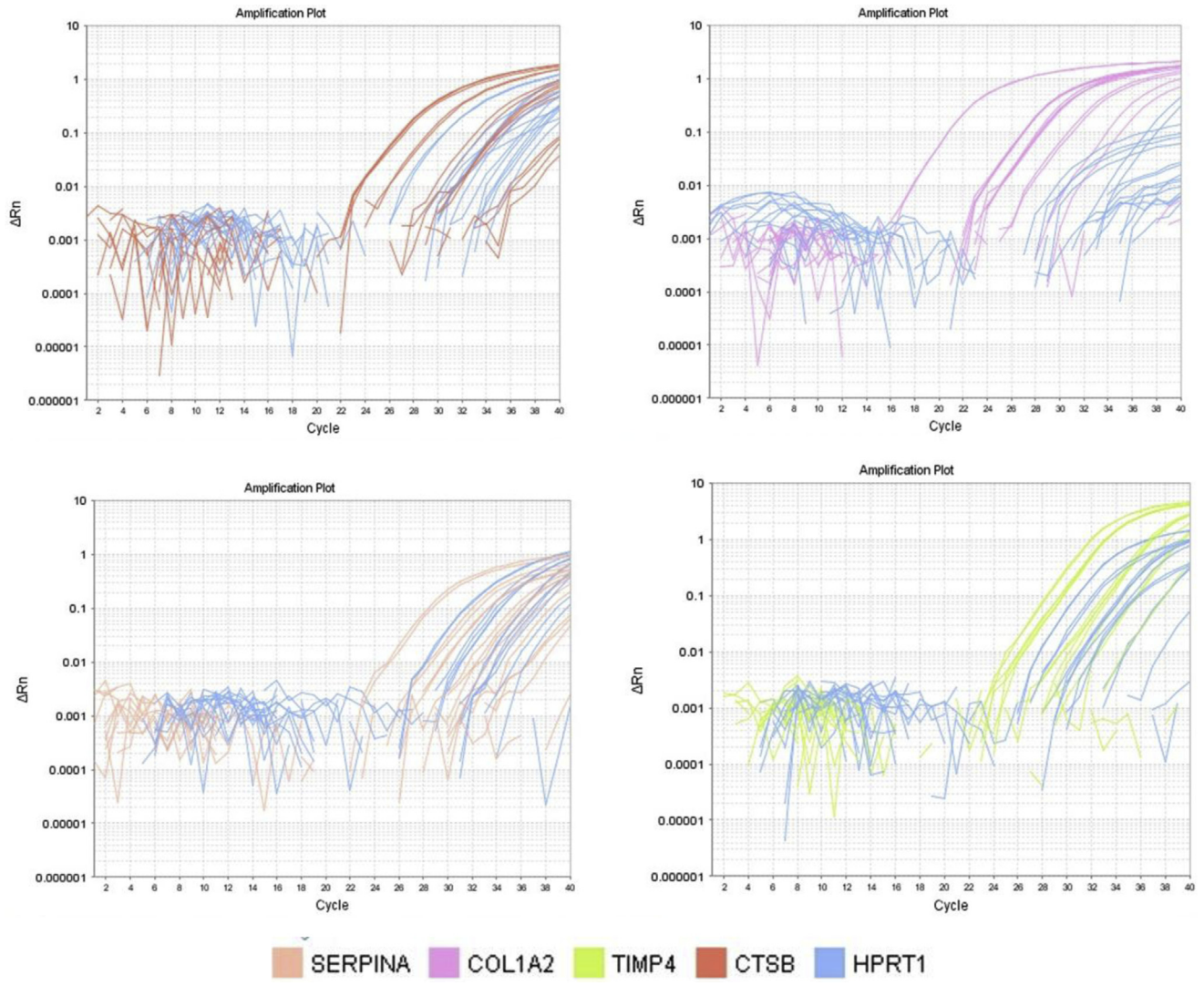

Figure 5. Amplification plots of individual genes, clockwise from top left: 1) *Cathepsin B,* 2) *Collagen 1 A2,* 3) *TIMP4,* and 4) *SERPIN-A.*

The authors concluded that this disproportional expression of MMP-2, MMP-9, and TIMP-1, 2, and 3 contributes to the evolution and rupture of IAs.[20] These findings suggest that this imbalance between MMPs and TIMPs in the late stages of IA formation may be 1 of the reasons for ECM degradation leading to the rupture of IAs.

Quantitatively, Pera et al[22] found an underexpression of *TIMP4* by 2.22 times in ruptured IAs and 2.32 times in UIA when compared with a normal MMA. Marchese and Krischek[12,23,24] found similar results in their respective studies. Babu et al[7] found the underexpression of *TIMP4* was 3.22 times as compared with the control STA by PCR. They also found a significant positive association of *TIMP4* expression and maximum diameter and fundus size of the aneurysm. However, no such statistically significant correlation was found in the current study. On analyzing the IHC staining, the *TIMP4* gene had an underexpression in 94.44% of the IA walls when compared with normal STA/OA samples. However, the RNA expression of *TIMP4* by RT-PCR was 10.16 times over normal.

Decreased activity of *TIMP4* might result in uninhibited activity of MMPs (especially MMP2). However, we believe it is not the absolute but a relative reduction in the levels of TIMP when compared with the levels of MMPs that result in the increased wall changes.

A similar observation was made by Jin et al,[20] who while working on other inhibitors of metalloproteinases found an increased expression on IHC and PCR. Koskivirta et al[25] investigated the role of *TIMP4* using IHC staining in various cardiovascular pathologies and found increased levels of *TIMP4*. These increased levels of TIMP4 may represent a compensatory

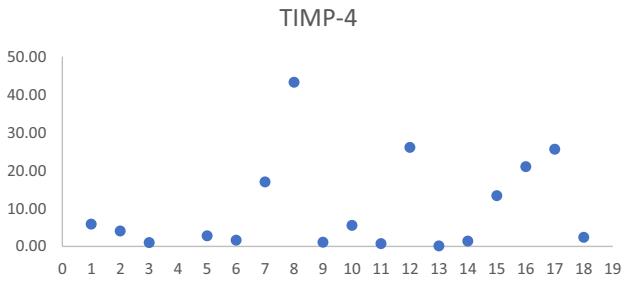

Figure 6. Scatter map of individual values for fold change of *TIMP4* RNA expression (x axis—fold change, y axis—patients).

mechanism to counterbalance the increased activity of MMPs since TIMP4 is capable of inhibiting the activity of all MMPs.[25]

The IHC findings in our study correlate with the literature we have reviewed (i.e., reduced expression of TIMP4) (Figures 4 and 5). The increased amount of RNA expression by RT-PCR may be due to increasing demands and stimulus for production of the enzyme in the tissue to counteract on the increased levels of MMPs. However, since the expression of the protein was reduced in the walls of the aneurysm on IHC, there is a possibility of a block at some stage in the process of the translation process of TIMP4 to the enzyme. Increased destruction of RNA or protein or accelerated utilization of the protein in the tissue can also be a cause for reduced expression in IHC.

Secondly, on reviewing the individual values of the patients and plotting them on a scatter map, the findings were found to be spread apart (Figure 6). The individual variation in the aneurysm wall constituents under examination of the aneurysm, along with modifications in the translation process or increased degradation of the RNA or protein, can be 1 possibility of reduced protein expression in the tissue on IHC. Thus there may be considerable variation of expression across patients at this locus and generalizations should be made with caution.

Alpha-1 Antitrypsin (*α-1 AT* or *SERPIN-A*)

The cardiovascular dysfunction associated with the deficiency of α-1 AT is the development of aneurysms and dissections in systemic and intracranial arteries.[26,27]

Multiple studies involving the serum values of α-1 AT in patients with aneurysms, as well as case reports or series documenting the prevalence of aneurysm in patients with α-1 AT deficiency, were published in the late 1990s and early 2000s.[27,28] The associated reduction in the levels of elastase in serum correlates with the reduction in α-1 AT levels, suggesting a common pathway and different function of the genes.[29] Also, the effects of smoking in the development of aneurysm and cardiovascular abnormalities are also thought to work down this pathway.[30] However, a multicenter study evaluating the α-1 AT deficiency alleles in 72 patients with IA has not revealed any significant results.[31]

The genetic analysis involving α-1 AT or SERPIN-A has mainly been restricted to serum analysis of the enzyme's levels. Few genomes and linkage studies have seen the expression of the SERPIN-A gene in the tissue samples. Li et al,[11] in a deoxyribonucleic acid microarray study on 3 aneurysm/STA samples, found an upregulation of SERPIN-A. In our study, SERPIN-A is 1.46 times overexpressed in the wall of the aneurysm as compared with the control vessels. On IHC 77.8% of the aneurysm samples showed increased expression when compared with the control. α-1 AT is an important marker of inflammation with increased levels at the site of the aneurysm. The enzyme acts against the neutrophilic proteases, especially elastases in various pathologic processes.

We believe that increased expression in the aneurysmal wall, when compared with a healthy control vessel, is suggestive of the enzymes role in countering the increased inflammatory response. The increased levels of proteases and elastases acting in degradation of the ECM and the structural proteins would cause increased formation of antiproteinases like α-1 AT. However, it is the disturbed balance or relative overactivity of the proteases that lead to vascular remodeling and subsequent aneurysm formation.

In our study, mRNA expression of the genes was studied with RT-PCR and validated with the IHC, which analyzes the final translated proteins. Although patients with IA are not uncommon, the minimal quantity of tissue that is available for analysis makes it challenging to carry out a dual assessment of RNA and protein levels as we have attempted.

However, we believe that there are a few limitations in our study (e.g., because the gene expression was studied between the aneurysmal wall and an extracranial artery from the same patient, an individual harboring an IA might have some amount of inflammatory, microscopic predisposing processes in his normal vessels as well). Also, there is a structural difference between the intracranial and extracranial vessels, so an ideal control for comparison with IA would have been a healthy intracranial vessel of a normal individual. However, such biopsy samples are ethically and technically challenging to harvest. Finally, to derive any significant association between the clinical profile and gene expression, a large population-based study is required as the numbers in our study seem inadequate to draw a meaningful conclusion. Whether these changes in the gene profiles are a cause or effect of aneurysm formation also needs validation.

Multicenter cohorts in different ethnic populations should ideally be included to negate locus and allelic heterogeneity between individuals. Further advancements in technology and statistical methods in genomics are developing quickly, and new approaches may also be used to identify new candidate genes. We hope that the genetic markers used in these studies may contribute to the development of a prognostic scoring system or a risk stratification strategy to identify individuals within families at high risk. Such individuals and their family members can be screened by imaging to identify aneurysms that can be treated prophylactically.

Such intervention can change the course of the natural history of this dreadful disease and bring down the morbidity and mortality associated with aneurysm rupture in the population.

We have reported our gene expression findings at 4 candidate loci in a sample of patients seeking treatment for IA. Although the sample set is modest, we hope that our findings contribute to the understanding of this debilitating illness to make way for prophylactic measures in the future.

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