VIA MEDICA

ORIGINAL ARTICLE

Folia Morphol.
Vol. 76, No. 3, pp. 372–378
DOI: 10.5603/FM.a2017.0016

ISSN 0015–5659
www.fm.viamedica.pl

The expression of inhibitor of nuclear factor kappa-B kinase epsilon (IKKε) in human aortic aneurysm

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[Received: 5 September 2016; Accepted: 17 November 2016]

Background: *Aortic aneurysm (AA) is one of the most common causes of sudden death among elderly people. Although AA can be detected by non-invasive imaging techniques, there are no pharmacological treatments currently available to prevent progression at any stage of the disease. In this study we will explore the expression of inhibitor of nuclear factor kappa-B kinase epsilon (IKKε) in AA and its potential underlying molecular mechanism in AA.*

Materials and methods: *Human aortic tissue was taken from 14 patients who underwent surgical repair of AA for the AA group and another 11 patients with normal aorta who underwent aortic valve replacement surgery for the control group. After excision, we used haematoxylin-eosin staining, Masson staining, immunohistochemistry analysis and Western blot analysis to observe the expression, location and morphological changes of the IKKε, P50 and the extracellular matrix within the AA.*

Results: *In the AA group, haematoxylin-eosin staining revealed a loss of medial integrity and inflammatory cell infiltration. Masson staining confirmed the degradation of the extracellular matrix in the AA group. Immunohistochemistry analysis showed increased infiltration of inflammatory cells and up-regulation of proinflammatory cytokines in the AA group when compared to the control group. Based on immunohistochemistry and Western blot analysis, there was clearly over-expression of IKKε, P50 and MMP2 in AA group, mainly in the intrinsic aortic cells of the media.*

Conclusions: *The over-expression of IKKε may play an important role in the origination and progression of AA and might be a vital target for their treatment.*
(Folia Morphol 2017; 76, 3: 372–378)

Key words: IKKε, P50, inflammation, aortic aneurysm, metalloproteinase

INTRODUCTION

Aortic aneurysm (AA) is a degenerative disease of the aorta with a natural history of progressive dilation and rupture. In the past decade, AA have been detected using colour Doppler, multislice spiral computed tomography angiography (MCSTA) and digital subtraction angiography, but no pharmacological therapy can limit their progression or prevent their rupture [16]. AA are char-

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acterised by the medial degeneration, and investigations of the pathogenesis of AA focus mainly on the effects of hypertension, age, and apoptotic pathway activation, which finally results in the apoptosis of smooth muscle cells and degradation of the extracellular matrix [11, 15]. Hinterseher et al. [4] showed that gene such as *GATM, FOSB, ADCY7* and proteins such as CXCR4, PLEK, and GATM participated in the development of AA using a custom-designed "AA-chip", Western blot and immunohistochemical staining. However, the precise underlying aetiology of AA remains unclear.

A recent study showed that inhibitor of nuclear factor kappa-B kinase epsilon (IKKε) is related to vascular inflammation, which accounts for its important role in the atherosclerosis, and it also has a potential relationship with the hypertension [13]. The role of IKKε in the development of atherosclerosis was further verified in our previous investigation, and recent studies have confirmed that atherosclerosis is a risk factor for AA [1, 6, 13]. Matrix metalloproteinase-2 (MMP-2), a downstream of IKKε, is widespread and responsible for extracellular collagen degradation and remodelling [8]. Therefore, we assumed that IKKε might play a role in the development and progression of AA.

In this study, we examined the activation of IKKε and its related downstream factors in aortic tissues from the patients in the aortic aneurysm group and the control group, to observe whether IKKε has an underlying role in AA.

MATERIALS AND METHODS

Patient enrolment and aortic specimen collection

The study conforms to the principles outlined in the Declaration of Helsinki. The trial was approved by the ethics committee of Nanjing Medical University affiliated Nanjing First Hospital and each patient provided written informed consent. Between 2011 and 2013, 102 patients were admitted to in Nanjing First Hospital for the treatment of the AA. Patients meeting the criteria for the study were then divided into one of the groups (AA group and control group). We randomly selected 14 patients who underwent open surgical repair of AA to avoid bias for the AA group and another 11 patients who underwent aortic valve replacement surgery with normal aortas for the control group. Patients were excluded if they had a history of heritable connective tissue disease, infected pseudoaneurysms, post-traumatic AA and peripheral artery diseases. The patient age, gender and characteristics were also obtained. The study population characteristics are summarised in additional information Table 1. Samples of human aortic tissue were excised from the area of the aneurysm during surgery. After excision, we used cold saline to store the samples and removed thrombi. The aortic specimens were either snap-frozen in liquid nitrogen or fixed in 4% formalin until analysis.

Table 1. Clinical characteristics of the aortic aneurysms patients undergoing emergent surgical repair and the control group of patients

Item	Control group (n = 11)	Patients (n = 14)
Age [years]	70.0±8.8	69.7 ± 7.7
Sex (male/female)	9/2	11/3
Hypertension	2 (18.2%)	2 (14.2%)
Hyperlipidaemia	5 (45.4%)	4 (28.6%)
Diabetes	2 (18.2%)	3 (21.4%)
Coronary artery disease	1 (9.09%)	0 (0.00%)
Chronic kidney disease	0 (0.00%)	0 (0.00%)

Haematoxylin and eosin staining

After 48 h in 4% formalin, the tissue samples were embedded in paraffin and then sliced into serial aortic sections (3 μm thickness) with a slicing machine before being stained with haematoxylin and eosin (H&E) and Masson trichrome.

Masson trichrome stain

The fixed aorta samples were dehydrated, and paraffin-embedded, and then sectioned at a thickness of 3 μm. The sections were used for Masson trichrome staining and were observed with an Olympus BX-URA2 camera.

Western blotting analysis

The frozen aortic tissue samples were homogenised, and the proteins were collected and then separated by SDS-PAGE. Then, the proteins were transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA), which were washed twice for 10 min each in Tris-buffered saline (TBS) with Tween® diluted 1:1000 (TBST; Promega, Madison, WI, USA) and then blocked with TBS containing 5% non-fat milk powder for 1 h. Next, the following primary antibodies were incubated with the membranes in TBS with Tween plus 5% milk overnight at 4°C: anti-IKKε (1:500; Novus Biologi-

Table 2. The details of antibodies

Antibody name	Catalogue code	Lot No.	Primary/secondary antibody	Company	The experimental subjects	Use for
Anti- IKKε	AF3199		Primary antibody	Novus Biological		Western blot
Anti-P50	sc-114	#H2178	Primary antibody	Santa Cruz	Human breast tumour	Western blot, immunohistochemical staining
Anti-GAPDH	sc-25778		Primary antibody	Santa Cruz	HeLa cells, human lung tissue	Western blot
Anti-MMP2	ab110186	GR52940-7	Primary antibody	Abcam	Human mammary cancer tissue	Western blot, immunohistochemical staining
Goat-anti-rabbit IgG	ZB-2301	127760	Secondary antibody	ZSGB-BIO		Western blot, immunohistochemical staining
Anit-CD68	ab125212	GR77386-17	Primary antibody	Abcam	Murine spleen tissue	Immunohistochemical staining
Anti-IL-6	ab6672	GR106735-3	Primary antibody	Abcam	THP1 cell, human lung, mouse mesenchymal SC cells	Immunohistochemical staining

cals, Littleton, USA), anti-p50 (1:500; Santa Cruz Biotechnology, Santa Cruz, CA), anti-GAPDH (1:5000; Santa Cruz Biotechnology) and anti-MMP-2 (1:500; Abcam). The next day, after washing with TBST four times for 10 min each time at room temperature, the PVDF membranes were incubated with proportionally diluted peroxidase-conjugated goat anti-rabbit secondary antibodies (1:1000; Beijing ZhongShan Biotechnology Co.) for 1 h. Furthermore, for the specific proteins, we used an ECL reagent (GE Healthcare, Piscataway, NJ, USA) to detect and capture images on Hyperfilm (Amersham, GE Healthcare). Ultimately, we evaluated the results using Image J software [1] for semi-quantitation of the mean grey value of each blot. All the details of antibodies are in additional information Table 2.

Immunohistochemical staining

The perfusion-fixed aortas were embedded, cut into cross sections (3 μm) and rehydrated in graded alcohol for morphological analysis. To prevent the non-specific binding of antibodies and to eliminate endogenous peroxidase activity, the sections were incubated in buffered normal horse serum and treated with 3% hydrogen peroxide for 15 min. Then, we incubated the sections with anti-CD68 (1:200; Abcam), anti-P50 (1:100; Santa Cruz), anti-MMP (1:500; Abcam), and anti-IL-6 (1:500; Abcam) primary antibodies for 14 h. The sections were incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (Beijing Zhongshan Biotechnology Co., Beijing, China) for 1 h at 37°C in a humidified box. Subsequently, upon application of the substrate diaminobenzidine (DAB, Beijing Zhongshan Biotechnology Co.), the signal for each antibody appeared. The sections were then counterstained, and the differences in these signals were determined. As a negative control, the primary antibodies were omitted from the reaction sequence. The data were recorded using an Olympus BX-URA2 camera via photomicrographs. All the details of antibodies are in additional information Table 2.

Statistical analysis

All continuous variables are presented as the means ± standard errors. Statistical significance was defined as a p-value of 0.05. The analyses were performed using SPSS17.0 (SPSS Inc, Chicago, IL, USA) software. Categorical variables were compared using an independent-sample t test.

RESULTS

Morphological differences of the aortic tissue samples

The results of the H&E staining showed that the patients in the AA group had a damaged and thinner medial layer with disordered smooth muscle cells and a fibrin clot in the edge between the inner media and intima. Furthermore, the entire aortic vessel wall showed neutrophil infiltration, particularly in the medial layer. By contrast, there was no neutrophil infiltration in the control group, and the aortic morphology was normal (Fig. 1A).

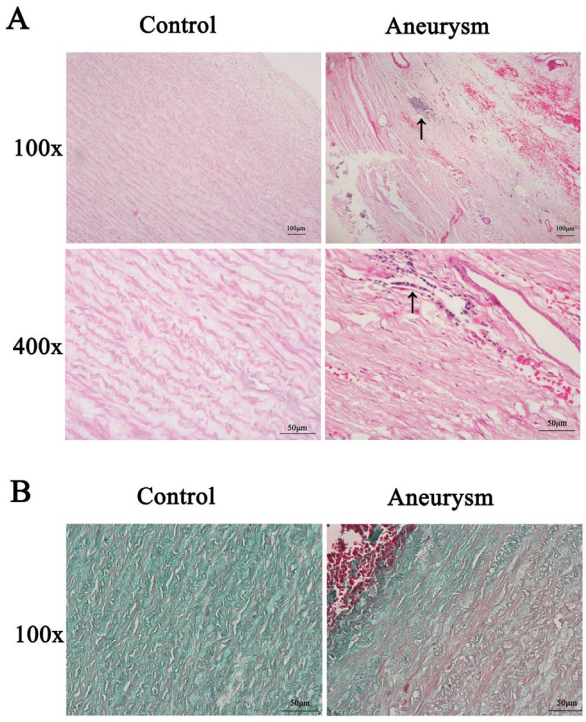

Figure 1. Morphological changes in the aortic aneurysm (AA) group and control group; **A.** Haematoxylin-eosin staining of the aortic vessel wall of AA and the normal aortic media of the control group. The integrity of the media was maintained and smooth muscle cells were arranged in an orderly fashion in the control group. In the aortic vessel wall of the aneurysm group, there were disarranged smooth muscle cells in the media, representing the infiltration of neutrophils throughout the entire aortic vessel wall, particularly in the media (the above are magnification ×100, the down are magnification ×400, arrow to indicate the inflammatory infiltrations); **B.** Changes in the extracellular proteins between the AA and control groups. Green represents collagen fibres, whereas red represents muscle fibres. The collagen of the aorta was significantly degraded in the AA group compared to the control group (magnification ×400).

The Masson trichrome stain is mainly used for the identification of collagen fibres (green) and the muscle fibres (red). As it is shown, the collagen of the aorta was degraded in the AA group compared to the control group (Fig. 1B).

Inflammatory infiltration in AA

To assess inflammatory infiltration, we used immunostaining for macrophages (CD68) and the pro-inflammatory cytokine interleukin 6 (IL-6). Our results showed that CD68 and IL-6 were observed throughout the tunica media at a uniformly distributed density in the AA group while the control group showed no of such expression (Fig. 2).

Expression of IKKε in AA

Western blot analysis showed a significant difference in the expression of IKKε (independent t test, $t = 5.878$, $df = 18$, $p < 0.01$) between two groups (Fig. 3A, B). Similar results were also obtained using immunohistochemical staining for IKKε. In addition, the area of increased expression of IKKε was primarily in the medial layer of the aortic vessel wall in AA patients (Fig. 3C).

Expression of P50 in AA

Western blot analysis showed the expression of P-P50 (independent t test, $t = 10.561$, $df = 18$, $p < 0.01$) was significantly increased in the AA group compared to the control group (Fig. 4A). Immunohistochemical staining analysis was used to assess the activity and location of P-P50, and the results showed that P-P50 was located in the nuclei of stromal cells.

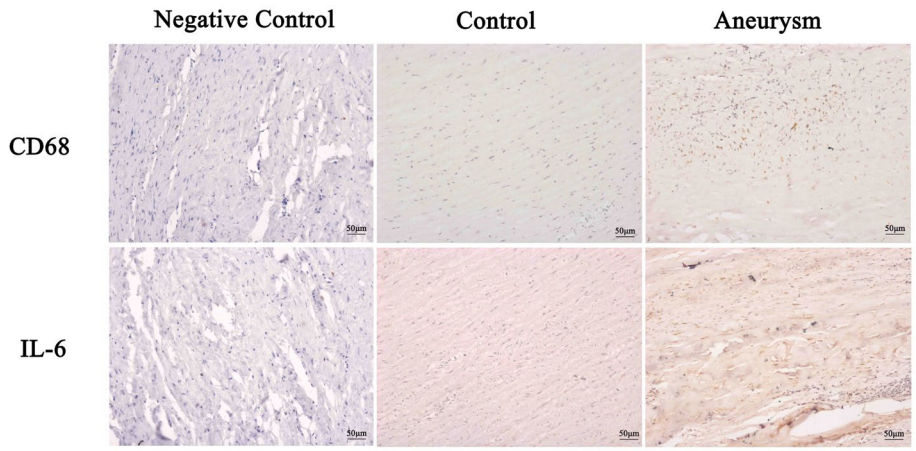

Figure 2. Immunohistochemistry staining for interleukin 6 (IL-6) and CD68 showed their increased expression in the aortic aneurysm group compared to the control group (magnification ×400).

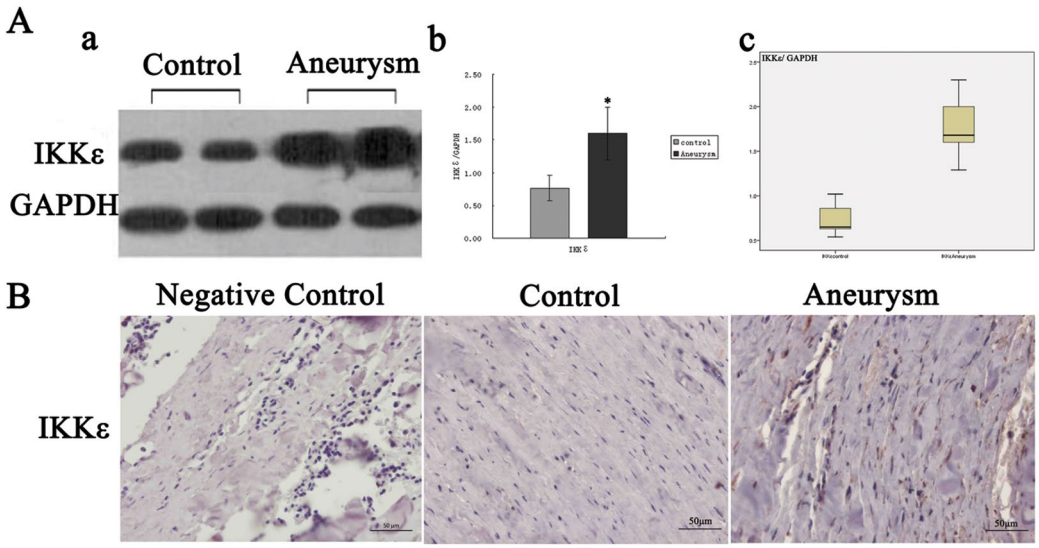

Figure 3: Protein expression and localisation of IKKε in the aortic aneurysm (AA) group and control group; **A.** The protein level of IKKε was measured by Western blotting; **a.** Western blotting of IKKε and GAPDH; **b.** The expression of IKKε in the aortic vessel wall after normalisation to GAPDH was significantly increased in the AA group compared to the control group. The values are the means ± standard error; *p < 0.01; **c.** Box-and--whisker plots of IKKε; **B.** Immunohistochemistry staining also showed that IKKε were up-regulated in the AA group when compared with the control group, and the expression of IKKε was mainly distributed in the medial area of the aortic vessel wall (magnification ×400).

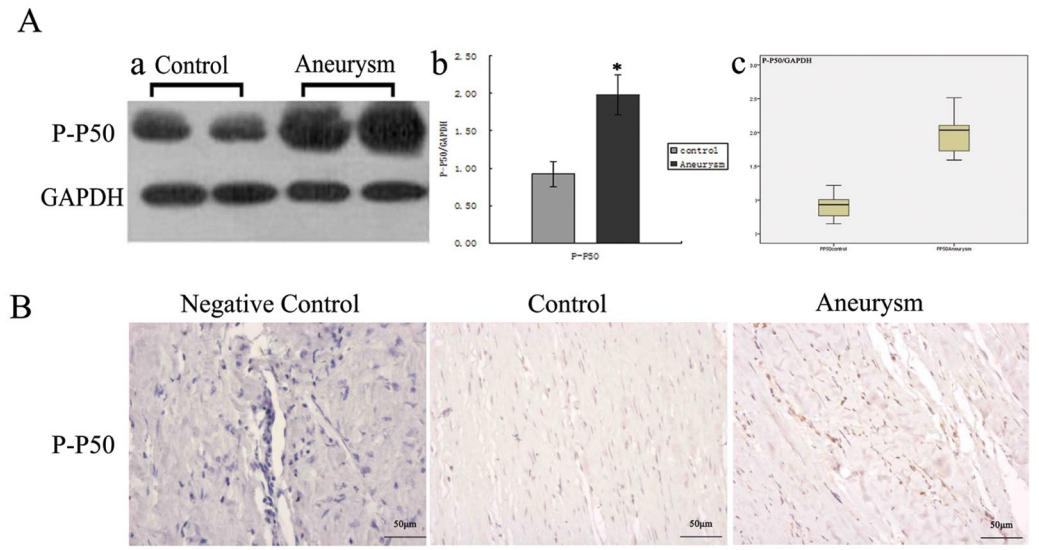

Figure 4. Protein expression and localization of P50 determined in the aortic aneurysm (AA) group and control group; **A.** The protein level of P50 was measured by Western blotting; **a.** Western blotting of P50 and GAPDH; **b.** The expression of P50 in the aortic vessel wall after normalisation to GAPDH was significantly increased in the AA group compared to the control group. The values are the means ± standard error; *p < 0.01; **c.** Box-and-whisker plots of P50; **B.** Representative images showed the up-regulated expression of P50 in the aorta of the AA group compared to the control group, which was spread throughout the entire media and adventitia, particularly in the inner media at the border between the intima and media (magnification ×400).

These results indicate that the protein is biologically active in the AA group (Fig. 4B).

Expression of MMP-2 in AA

To verify the expression and location of MMP-2, Western blot analysis and immunostaining were used to detect MMP-2. Western blot analysis showed that the expression of MMP-2 was significantly increased in the AA group compared to the control group (independent t test, $t = 5.221$, $df = 18$, $p < 0.01$) (Fig. 5A). Immunostaining confirmed that MMP-2 was primarily distributed in the media in the AA group but not in the control group (Fig. 5B).

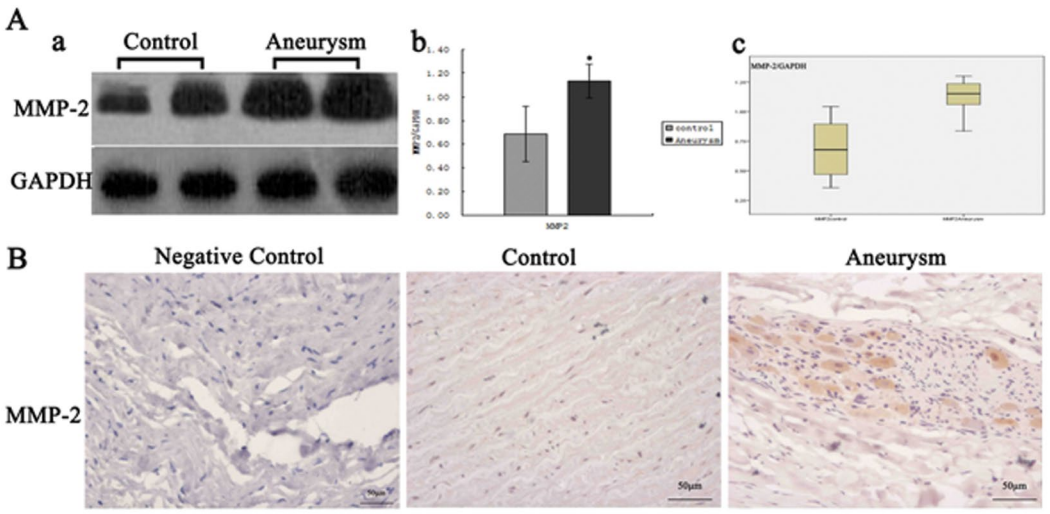

Figure 5. Protein expression, localisation and activity of MMP-2 in the aortic aneurysm (AA) and control groups; **A.** The protein level of MMP-2 was measured by Western blotting; **a.** Western blotting of MMP-2; **b.** The expression level of MMP-2 in the aortic vessel wall after normalisation to GAPDH was significantly increased in the AA group compared to the control group. The values are the means ± standard error; *$p < 0.01$; **c.** Box-and-whisker plots of MMP-2; **B.** The representative images show the upregulated expression of MMP-2 in the aorta of the AA group compared to the control group, which was spread throughout the entire media and adventitia, particularly in the inner media at the border between the intima and media (magnification ×400).

DISSCUSION

Aortic aneurysm is a progressive disease of the aorta and is mainly associated with smoking, advanced age, male sex and atherosclerosis [3]. Existing evidence shows that the occurrence of AA is associated with the degradation of the tunica medial and an inflammatory reaction [7, 12]. Histological analysis has shown that the extracellular matrix degeneration is characterised by disruption and degradation of medial elastin and gross medial collagen deposition, whereas inflammation is exemplified by inflammatory cell infiltration of the aortic wall. We observed the same results in our experiment. The integrity of the tunica media was disrupted and there was a thinner medial thickness in the AA group, which accompanied with main pathological findings of this disease.

The main pathological characteristics of the experimental group included neutrophil infiltration and disordered of the smooth muscle cells which could be observed. In addition, we also detected increased infiltration of macrophages by immunohistochemical staining for CD68. IL-6 is a downstream factor of the nuclear factor-κB cascade, which is secreted at high levels in human AA disease [5, 9]. It has also been identified as an independent biomarker for severe coronary artery disease [2], and is associated with the risk of aneurysm rupture [10]. There was a significance difference between the AA group and the control group in the expression of IL-6, which was mainly located in the medial area in the AA group.

IKKε influences nuclear factor-κB signalling and induces inflammation, cell proliferation, tumour cell survival and cell transformation [14]. A significant overexpression of IKKε was observed in the AA group compared to the control group. Together with the Western blotting results, immunohistochemical staining showed upregulation of IKKε in the AA group, which was distributed particularly in the medial layer. These results indicate that the pathological and morphological changes of the AA might be due to the upregulation of IKKε.

We assessed changes in P50 in the AA group to determine whether the activation of IKKε promotes AA through the activation of P50. Our study demonstrated that the expression of total and phosphorylated P50 in the aortic vessel wall was increased in the AA group compared to the control group, suggesting a role of P50 in the progression and formation of AA. MMP-2 plays an important role in the degradation of extracellular proteins and the remodelling of the extracellular matrix. The expression of MMP-2 was significantly increased in the AA group compared to the control group.

In this study, immunohistochemistry was used to visualise the localisation of IKKε, P50 and MMP-2. Our results revealed that IKKε, P50 and MMP-2 were upregulated in the aortic tissue of the AA group compared to the control group.

Based on these results, our study suggests that the expression of IKKε and P50 signalling may regu-

late the transcription of diverse genes encoding cytokines (IL-6), which could lead to the infiltration of macrophages and the secretion of MMPs. Moreover, the extracellular matrix of the medial layer was degenerated, which resulted in the formation of the AA. This study also provides us a deeper insight of the AAs at the molecular level, which may lead to a better understanding and future treatment and also prevention of patients with AA through the inhibition of IKKε.

CONCLUSIONS

The over-expression of IKKε may play an important role in the origination and progression of AA and might be a vital target for their treatment.

Sources of funding

This study was supported by grants from the National Natural Science Foundation of China (No. 81370259).

Acknowledgements

Dr. Xin Chen is a fellow at the Collaborative Innovation Centre for Cardiovascular Disease Translational Medicine.

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