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Original Research

The expression and significance of NF-κB, MMP1, and MMP2 in rats with abdominal aortic aneurysm

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Abstract: This study aimed to investigate the expression and significance of NF-κB, matrix metal protein1 (MMP1), and matrix metal protein2 (MMP2) in rats with an abdominal aortic aneurysm (AAA). 48 Wistar rats were collected, then they were divided into an experimental group and a control group (n=24). The rats in the experiment group were used to carry out an abdominal aortic aneurysm modeling, while the rats in the control group were infused with the same dose of saline through catheters. qRT-PCR and ELISA were used to detect the expressions of EGF and VEGF-C in skin tissues and mRNA of NF-κB, MMP1, and MMP2 in their abdominal aorta. A vernier caliper was used to measure the diameter of their abdominal aorta. Western blot was used to detect the expressions of NF-κB, MMP1, and MMP2 in their abdominal aorta. In the experiment group, the abdominal aorta of the rats expanded to some extent after they were infused compared to that before they were infused ($P< 0.05$), and the abdominal aorta of them was significantly larger than that of the rats in the control group. On the 3rd week after modeling, the abdominal aorta of the rats expanded more obviously compared to that after they were infused for a while in the experiment group, and the expansion rate of the diameter was (57.19 ± 4.67) % ($P< 0.05$). Protein expression levels of NF-κB, MMP1, and MMP2 in the abdominal aorta of the rats increased significantly in the experiment group compared to those of the rats in the control group ($P<0.05$). The increase of NF-κB, MMP1, and MMP2 may be related to the pathogenesis of abdominal aortic aneurysm in abdominal aortic aneurysm models of rats.

Key words: Abdominal aortic aneurysm; NF-κB; MMP1; MMP2; Aortic diameter.

Introduction

An abdominal aortic aneurysm (AAA) is an aortic dilated disease (1). In recent years, its morbidity has increased year by year and ranks the first in all aneurysms. When patients with AAA are hospitalized, their survival rate is slightly more than 50%, and their prognosis is very poor (2,3). Clinically AAA with a large diameter can be treated effectively by an aortic reconstruction surgery or aortic aneurysm endovascular repair, but the early pathogenesis of AAA remains unclear. Thus, it is important to research the biological indicators existing in the formation of AAA (4-12).

Matrix metal proteins (MMPs) is a general term of some enzymes. They can degrade some extracellular matrix, such as collagen, elastin, fibrin, and proteoglycan (13, 14). MMPs have some effects, for example, it can degrade proteins and injure the barrier, which can stop cancer cells from invading surrounding tissues, moreover, it can also facilitate the angiogenesis of tumor tissues. These effects facilitate the invasive growth of tumors. Therefore, it plays an important role in cancers and is involved in many developmental stages of cancers such as vascular remodeling and cell migration (15, 16). Many studies have shown that MMPs play an important role in the occurrence and development of AAA (17). Matrix metal protein 1 (MMP1) is a member of MMPs. Some studies have shown that the expression of MMP1 increases in tumor cells and the overexpression of MMP1 is closely related to the migration speed of tumor cells (18, 19). MMP2 is a gelatinase, and it is involved in many physiological and pathological processes such as wound healing, pregnancy, bone resorption, tumor invasion, and tumor metastasis. Some studies demonstrate that MMP2 is related to the formation and development of aneurysm (20). Nuclear factor-κB (NF-κB) is an intranuclear transcription factor. It plays an important role in regulating inflammation, immune responses and cell apoptosis and is involved in the occurrence, development, metastasis and anti-apoptosis of many tumors. It provides new ideas for researches on tumors (21-25).

It is generally believed that the abnormality of the extracellular matrix is one of the main reasons for the formation of an aneurysm (26), and some studies have shown that the abnormal activation of NF-κB in the aorta can facilitate the expression of inflammatory factors and MMP hydrolyzed proteins. NF-κB becomes one of the factors inducing the occurrence of an aneurysm (27). However, the expression of NF-κB, MMP1, and MMP2 in the abdominal aortic aneurysm of rats is still unclear. Therefore, in this study, the expression of NF-κB, MMP1, and MMP2 in the abdominal aortic aneurysm of experimental rats was investigated by constructing abdominal aortic aneurysm models of rats. The purpose was to provide references for clinical practice.

Materials and Methods

Experimental animals

48 Wistar rats in a clean grade were collected, they were purchased from Nanjing Junke Biological Co., Ltd. Their mass was between 280g and 320g and their age was between 3 weeks old and 7 weeks old. They were fed in an environment with good ventilation, in which the indoor humidity was (50.00 ± 5.00)% and the temperature was (24.00 ± 2.00) ℃. They were fed with SPF experimental rat foods provided by Jiangsu Xietong Organism Co., Ltd. They could drink water and eat food freely. This study was approved by the ethics committee of the hospital. The experiment operations complied with "*The Guide for Care and Use of Laboratory Animals*" (18).

The preparation of animal models and the measurement of the diameter of the abdominal aorta

According to the principle of similar weight, the rats were randomly divided into two groups. There were 24 rats in each group. The rats in the experiment group were anesthetized by injecting chloral hydrate (300 mg/kg) into their enterocoelia. The renal artery and the hypogastrium aorta, as well as the two-iliac total artery, were dissociated in a surgical microscope. After the blood flow was blocked, the left iliac total artery was cut, then a PE-10 catheter was inserted into the cut and it extended to the upside of the abdominal aorta furcation. The abdominal aorta was ligated at the furcation (it was connected to the catheter), then I porcine pancreatic elastase (ICN Biomedicals, 25U/ml) was infused through the catheter. After l hour, the raffinate was extracted, then the catheter was pulled out, lastly, the cut of the left iliac total artery was anastomosed. The rats in the control group were infused with the same dose of saline through the catheter in the same period. Before and after the rats in two groups were infused with I porcine pancreatic elastase and saline, the diameter of their abdominal aorta was measured by a vernier caliper, and the expansion rate of their abdominal aorta was calculated. They were anesthetized on the 3rd week after they were infused with I porcine pancreatic elastase and saline, then their abdominal aorta was dissociated after they were laparotomized, next the diameter of their abdominal aorta was measured, lastly their abdominal aorta was excised and was frozen in liquid nitrogen.

Quantitative RT-PCR (qRT-PCR)

The expression of NF-κB mRNA, MMP1 mRNA, and MMP2 mRNA in abdominal aorta tissues was detected by RT-qPCR. Some abdominal aorta tissues were collected, then they were rinsed twice by PBS, next they were ground into powder in a bowl with liquid nitrogen. The total RNA was extracted according to the instructions of TRIzol extraction kits (Shanghai Yeasen Biotech Co., Ltd., China), and the operations were carried out in strict accordance with the instructions. Lastly, an ultraviolet-visible spectrophotometer (Eppendorf, Germany, 6135000041) was used to measure the optical density values of the total RNA, and concentrations of the total RNA were calculated in order to carry out other experiments. The total RNA was reversely transcribed into cDNA according to the instructions of M-MLV reverse transcription kits (Solarbio, USA, RP1100). 10μl reaction system, PCR Premix, double distilled water, ROX Dye, and 200nM primers were prepared according to the instructions of TAKARA real-time PCR (Beijing TransGen Biotech Co., Ltd. Article number: ER101-01, AT351-01, AQ301-01, China). B-actin was used as an internal parameter. The primers are shown in Table I. A Light Cycler qRT-PCR instrument (Roche, Switzerland, 05815916001) was used to carry out amplification reactions. Reaction conditions: Denaturation at 95 ℃ for 10min; 95 ℃ for 30s; 60 ℃ for 30s; a total of 40 cycles. The experiment was carried out three times repeatedly. The software of the manufacturers was used to analyze the data of the amplification reactions. Relative expression levels of NF-κB mRNA, MMP1 mRNA, and MMP2 mRNA were expressed as $2^{-\Delta Ct}$. All tests were carried out for three times repeatedly. The results were averaged.

Western-Blot

Western Blot was used to detect the expression of NF-κB protein, MMPl protein, and MMP2 protein in tissues: the tissue samples stored in liquid nitrogen were collected and were homogenized conventionally. RIPA was used to extract the total protein in the tissues, then the protein was denatured. Coomassie brilliant blue method was used to measure the concentration of the protein, then SDS-PAGE electrophoresis was carried out. After the electrophoresis was finished, the protein was rinsed by deionized water and was transferred to a film with nitrocellulose, then it was sealed in 5% skim milk powder overnight at 4 ℃. After blocking solution was removed, the protein was rinsed by TBST twice or three times, then a primary antibody was added into the protein (the dilution rate of the primary antibody was 1:500), next it was incubated in a shaker at room temperature for 2 hours, then it was rinsed by TBST again, next to a goat anti-mouse secondary antibody labeled by horse radish peroxidase (HRP) was added into it, then it was incubated at 37 ℃ for 1 hour after it was rinsed by TBST again, the color of it was developed by diaminobenzidine (DAB), lastly, its gray value was analyzed by Quantity One (Molecular Devices Corp, The Bay Area, CA, USA). The relative expression level of the protein = gray value of the target protein band / gray value of the β-Actin protein band.

Table 1. Primer sequences of NF-κB mRNA, MMP1 mRNA, MMP2 mRNA, and β-actin.

Gene	Forward primer	Reverse primer
NF-κB	5'-CGCATCCAGACCAACAACA-3'	5'- TGC CCA GAA GGA AAC ACC A -3'
MMP1	5'- AACATCACTTCTCCCCGAAT -3	5'- GTTCCCAAAATCCTGTCCA -3'
MMP2	5'-CATCGCTGCACCATCGCCCATCATC-3',	5'-CCCAGGGTCCACAGCTCATCATCATCA-3’
β-actin	5'-AAATCGTGCGTGACATTAA-3'	5'-CTCGTCATACTCCTGCTTG-3'

Statistical methods

SPSS 22.0 (Shanghai Yuchuang Network Technology Co., Ltd.) was used to carry out a statistical analysis. The measurement data were expressed as (mean value ± standard deviation) and were compared by an independent sample t-test. The count data between groups were expressed as the number of case/percentage [n (%)] and were compared by a chi-square test. The data were compared by repeated-measures ANOVA at several time points. The pairwise comparison in groups was carried out by the Bonferroni test at different time points. When P< 0.05, differences were statistically significant.

Results

The general data of rats

There were no differences between gender, age, length, blood glucose (Glu), body weight before and after modeling, indoor temperature and indoor humidity in the experiment group and those in the control group. The differences were not statistically significant (P> 0.05). As shown in Table 2.

General changes of the abdominal aorta of the rats in the experiment group and the control group

There was no significant difference between the diameter of the abdominal aorta of the rats in the control group and that of the rats in the experimental group before they were infused (P> 0.05). In the control group, there was no significant difference between the diameter of the abdominal aorta of the rats after they were infused for a while and that before they were infused (P> 0.05). In the experiment group, the abdominal aorta of the rats expanded to some extent after they were infused compared to that before they were infused (P< 0.05), and the abdominal aorta of them was significantly larger than that of the rats in the control group after they were infused. On the 3rd week after modeling, when the samples were collected, it was found that there was no significant difference between the abdominal aorta of the rats after they were infused for a while and that before they were infused in the control group (P> 0.05), while the abdominal aorta of the rats expanded more obviously compared to that after they were infused for a while in the experiment group, and the expansion rate of the diameter was (57.19 ± 4.67) % (P< 0.05). As shown in Table 3 and Figure 1.

The comparison of the diameter of the abdominal aorta of the rats in two groups before they were infused (A). There were no significant differences in the diameter of the abdominal aorta of the rats in two groups before they were infused (P> 0.05). The comparison of the diameter of the abdominal aorta of the rats in two groups after they were infused for a while (B). The abdominal aorta of the rats in the experiment group expanded more obviously compared to that of the rats in the control group (P<0.05). The comparison of the diameter of the abdominal aorta of the rats in two groups when the samples were collected (C). The abdominal aorta of the rats in the experiment group expanded more obviously compared to that of the rats in the control group (P< 0.05). The comparison of the expansion rate of the abdominal aorta of the rats in two groups when the samples were collected (D). The expansion rate of the abdominal aorta of the rats in the experiment group was significantly higher than that of the rats in the control group (P< 0.05). Note: compared to the control group, * P< 0.05.

Protein expression levels of NF-κB, MMP1, and MMP2 in the abdominal aorta of the rats in the experiment group and the control group

Protein expression levels of NF-κB, MMP1, and MMP2 in the abdominal aorta of the rats in the experimental group increased significantly compared to those

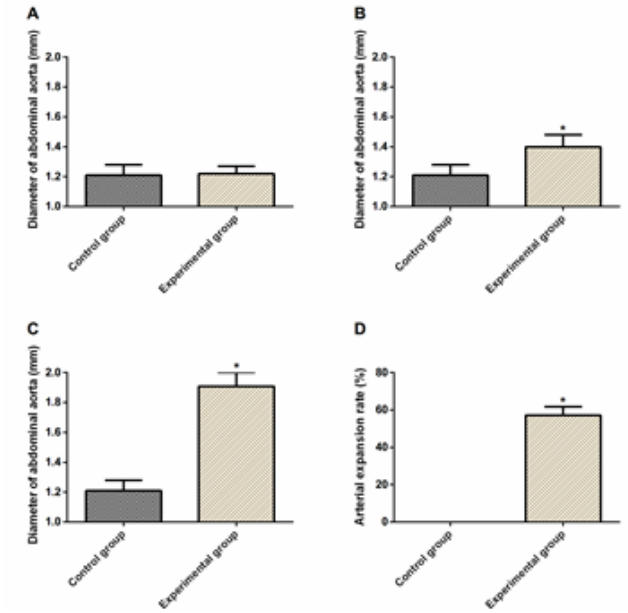

Figure 1. Changes in the diameter of the abdominal aorta of the rats in the experiment group and the control group.

Table 2. The general data of rats [n (%)]/(x±sd).

Category	The control group (n=24)	The experiment group (n=24)	t / χ2 value	P
Gender			0.343	0.558
Male	15(62.50)	13(54.17)		
Female	9(37.50)	11(45.83)		
Age (week)	8.26±0.41	8.34±0.28	0.789	0.434
Length (cm)	18.84±1.12	19.08±0.85	0.836	0.407
Glu (mmol/L)	71.29±4.18	72.42±4.43	0.909	0.368
Body weight before modeling (g)	226.29±14.34	222.43±14.47	0.928	0.358
Body weight after modeling (g)	210.18±7.83	208.12±8.16	0.892	0.377
Indoor temperature (°C)	24.11±1.15	23.89±1.02	0.701	0.487
Indoor humidity (%)	50.16±2.81	51.22±1.95	1.518	0.136

Table 4. The comparison of protein expression levels of NF-κB, MMP1, and MMP2 in the abdominal aorta of the rats in two groups ($\bar{x}$±sd).

Group	n	NF-κB	MMP1	MMP2
The control group	24	1.54±0.71	5.08±0.65	1.96±0.55
The experiment group	24	2.76±0.86	7.19±0.63	3.41±1.08
t	-	5.359	11.420	5.861
P	-	< 0.001	< 0.001	< 0.001

Table 5. The comparison of expression levels of mRNA of NF-κB, MMP1, and MMP2 in the abdominal aorta of the rats in two groups ($\bar{x}$±sd).

Group	n	NF-κB	MMP1	MMP2
The control group	24	4.19±0.71	9.19±0.95	8.45±1.25
The experiment group	24	4.76±0.82	16.13±0.53	10.34±1.18
t	-	2.574	31.250	5.386
P	-	0.013	< 0.001	< 0.001

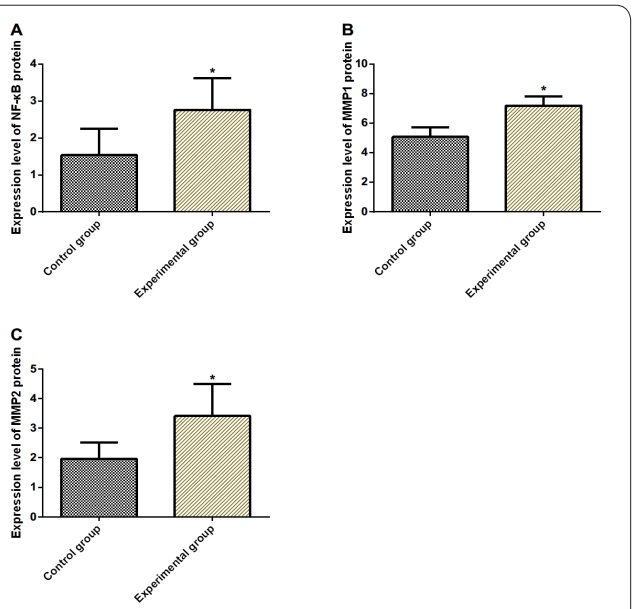

Figure 2. The comparison of protein expression levels of NF-κB, MMP1, and MMP2 in the abdominal aorta of the rats in the experiment group and the control group.

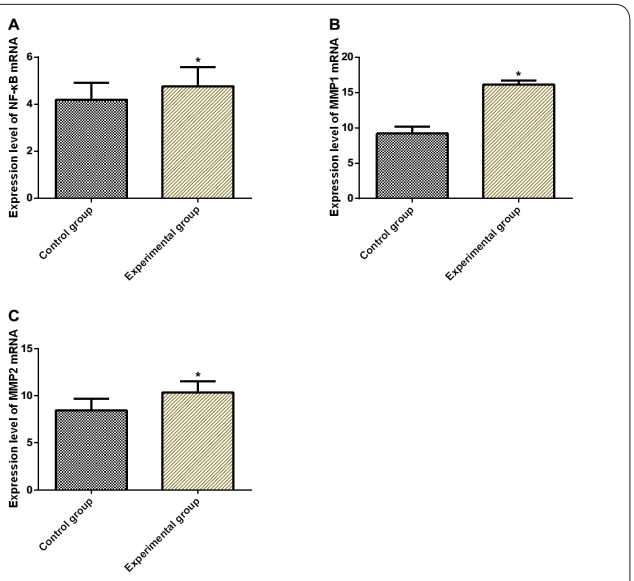

Figure 3. The comparison of expression levels of mRNA of NF-κB, MMP1, and MMP2 in the abdominal aorta of the rats in the experiment group and the control group.

in the control group (P< 0.05). As shown in Table 4 and Figure 2.

The comparison of protein expression levels of NF-κB in the abdominal aorta of the rats in the experiment group and the control group (A). The results of Western Blot showed that protein expression levels of NF-κB in the abdominal aorta of the rats in the experimental group increased significantly compared to those in the control group (P< 0.05). The comparison of protein expression levels of MMP1 in the abdominal aorta of the rats in the experiment group and the control group (B). The results of Western Blot showed that protein expression levels of MMP1 in the abdominal aorta of the rats in the experimental group increased significantly compared to those in the control group (P<0.05). The comparison of protein expression levels of MMP2 in the abdominal aorta of the rats in the experiment group and the control group (C). The results of Western Blot showed that protein expression levels of MMP2 in the abdominal aorta of the rats in the experimental group increased significantly compared to those in the control group (P< 0.05). Note: compared to the control group, * P< 0.05.

Expression levels of mRNA of NF-κB, MMP1, and MMP2 in the abdominal aorta of the rats in the experiment group and the control group

Expression levels of mRNA of NF-κB, MMP1, and MMP2 in the abdominal aorta of the rats in the experimental group increased significantly compared to those in the control group (P< 0.05). As shown in Table 5 and Figure 3.

The comparison of expression levels of NF-κB mRNA in the abdominal aorta of the rats in the experiment group and the control group (A). The results of qRT-PCR showed that expression levels of NF-κB mRNA in the abdominal aorta of the rats in the experimental group increased significantly compared to those in the control group (P< 0.05). The comparison of expression levels of MMP1 mRNA in the abdominal aorta of the rats in the experiment group and the control group (B). The results of qRT-PCR showed that expression levels of MMP1 mRNA in the abdominal aorta of the rats in the experimental group increased significantly compared to those in the control group (P< 0.05). The comparison of expression levels of MMP2 mRNA in the abdominal aorta of the rats in the experiment group and the control group (C). The results of qRT-PCR showed

that expression levels of MMP2 mRNA in the abdominal aorta of the rats in the experimental group increased significantly compared to those in the control group ($P< 0.05$). Note: compared to the control group, $^{*}P< 0.05$.

Discussion

An abdominal aortic aneurysm is a partial or integral abnormal expansion. It will appear when the structure of the aortic wall is injured by various congenital or acquired factors and then the aortic wall gradually swells in the blood flow. As this expansion easily worsens and ruptures, patients easily die due to massive hemorrhage (28, 29). An abdominal aortic aneurysm is common among men aged more than 55 years old, and its morbidity is rising in recent years (30). Currently, it is believed that the surgery is the main treatment method of AAA, but AAA of many patients is insidious. When patients are in the initial stage, they may don't have symptoms or symptoms that are easily concealed by other comorbidities. Thus AAA attracts little attention from patients and is extremely dangerous. Therefore, it is important to seek the biological indicators affecting the occurrence and development of AAA to improve the survival rate of patients (31).

An aortic aneurysm is a partial or integral abnormal expansion. It will appear when the structure of the aortic wall is injured by various congenital or acquired factors and then the aortic wall gradually swells in the blood flow. As this expansion easily worsens and ruptures, patients easily die due to massive hemorrhage (32). In this study, AAA models of rats were constructed by the Elastase perfusion method. There was no significant difference between the diameter of the abdominal aorta of the rats in the control group and that of the rats in the experimental group before they were infused ($P> 0.05$). In the control group, there was no significant difference between the diameter of the abdominal aorta of the rats after they were infused for a while and that before they were infused ($P> 0.05$). In the experiment group, the abdominal aorta of the rats expanded to some extent after they were infused compared to that before they were infused ($P< 0.05$), and the abdominal aorta of them was significantly larger than that of the rats in the control group after they were infused. On the 3rd week after modeling, when the samples were collected, it was found that there was no significant difference between the abdominal aorta of the rats after they were infused for a while and that before they were infused in the control group ($P>0.05$), while the abdominal aorta of the rats expanded more obviously compared to that after they were infused for a while in the experiment group, and the expansion rate of the diameter was $(57.19 \pm 4.67)\%$. This result indicates that the aorta wall of the rats in the experiment group expands abnormally during the experiment. Nuclear factor NF-κB is a transcriptional regulatory factor and is ubiquitous in eukaryotic cells. Currently, it is proved that NF-κB that is in a dissociated and activated condition can transfer to cell nuclei quickly and specifically combine with the specific sites in many gene promoters or enhancer sequences to facilitate transcription and expression. The abnormal expression of NF-κB is closely related to many biological processes, such as the regulation of cardiovascular diseases. In some studies, activated NF-κB was detected in endothelial cells, smooth muscle cells, and macrophages in atherosclerosis hard zones, and the expression of corresponding target genes was found in it, while the expression of NF-κB was rare or there was no expression of NF-κB in blood vessels without atherosclerosis. This result indicates that NF-κB plays an important role in the occurrence and development of atherosclerosis. BRAND et al. initially confirmed that NF-κB was activated in human atherosclerotic tissues by using the new mouse antibody α-p65 mAb, and they demonstrated that the chronic inflammatory reaction mediated by NF-κB/Rel led to atherosclerosis (33-45) .

The relationship between MMPs and the occurrence and development of tumors and the internal mechanism of MMPs is currently one of the areas researched frequently and deeply. In many studies about tumors, it was found that the degradation of the extracellular matrix was the precondition of the metastasis of tumor cells. Moreover, some studies have shown that osteopontin may up-regulate the expression of MMP and uPA in tumor cells through the NF-κB pathway, thus the degradation of the extracellular matrix is facilitated and the invasive ability and metastasis ability of tumor cells are improved greatly. MMP1 is an interstitial collagenase and can degrade collagen fibers and gelatin in the extracellular matrix. However, some studies indicate that the gene mutation or abnormal expression of MMP1 is closely related to the occurrence, development, and efficacy of tumors. MMP2, also known as gelatinase A, is an important member of MMPs. The substrate degraded by MMP2 is mainly IV collagen. It is reported that MMP2 is expressed abnormally in some tumors, such as laryngeal cancer (46-48).

This study showed that protein expression levels of NF-κB, MMP1, and MMP2 in the abdominal aorta of the rats in the experiment group were significantly higher than those in the control group, and expression levels of mRNA of NF-κB, MMP1, and MMP2 in the abdominal aorta of the rats in the experiment group were significantly higher than those in the control group. In the past, many studies demonstrated that NF-κB, MMP1, and MMP2 were expressed abnormally in AAA. In the study of Wilson et al. (49), it was found that the increase of MMP1 in plasma was associated with the rupture of abdominal aortic aneurysm. The level of MMP1 in the plasma of patients with ruptured AAA was significantly higher than that of patients with unruptured AAA. By doing some animal experiments, Sinha et al. (50) found that the activity of MMP2 increased in AAA tissues on the 7th day after the tissues were infused with elastase. Freestone et al. (51) pointed out that MMP2 might trigger the degradation of elastin and expansion of arteries, and MMP2 was positively correlated with the diameter of AAA. The chronic inflammatory reaction mediated by MMP2 leads to atherosclerosis. In the study of Parodi et al. (52), it was found that pyrrolidine dithiocarbamate could inhibit the expression of NF-κB in the rat abdominal aortic aneurysm induced by elastase and reduce the inflammatory reaction. This result suggests that NF-κB, MMP1, and MMP2 are likely involved in the occurrence and development of AAA and play important roles in the development of AAA, which is important for them to become the new biomarkers or

therapeutic targets of AAA.

In this study, the differences in expression levels of NF-κB, MMP1, and MMP2 of the rats in two groups were analyzed by constructing rat AAA models. However, there are still some shortcomings in this study due to the limited experimental conditions. The specific mechanism of NF-κB, MMP1, and MMP2 in tumors needs to be researched deeply. There are differences between animal models and the human body, thus experiments on human beings will be carried out as soon as possible to validate some views in this study and obtain the best study results.

In summary, expression levels of NF-κB, MMP1, and MMP2 in aortic tissues of AAA rat models are significantly higher than those of healthy rats. NF-κB, MMP1, and MMP2 may play important roles in the occurrence and development of AAA.

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Availability of data and materials
The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions
ZX and TF conceived and designed the study, collected, analyzed and interpreted the experiment data, drafted this paper, and revised the manuscript critically for important intellectual content. Both authors read and approved the final manuscript.

Ethics approval and consent to participate
The study was approved by the Ethics Committee of Yantaishan Hospital.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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