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Original Article

The roles of phenotypic transformation of vascular smooth muscle cells regulated by AGEs in the thoracic aortic dissection in neonatal rats

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Abstract: Objective: This study aims to observe the expression of advanced glycation end products (AGEs) and its receptor (RAGE) and explore the roles of phenotypic transformation of vascular smooth muscle cells (VSMCs) regulated by AGEs in the thoracic aortic dissection (TAD) in neonatal rats. Methods: The thoracic aortic dissection in neonatal rat model was established. The expression levels of AGEs and RAGE in thoracic aortic dissection tissues were detected. VSMCs phenotype transformation model was established by recombinant platelet derived growth factor BB (PDGF-BB) stimulation and starvation culture methods. The expression of RAGE was silenced by siRNA to explore the roles of phenotypic transformation of VSMCs regulated by AGEs in the thoracic aortic dissection in neonatal rats. The expression changes of SM α-actin and calponin were detected by western blotting method. The expression changes of AGEs and RAGE were detected by ELISA and RT-PCR methods. Results: The expression levels of AGEs and its receptor RAGE in thoracic aortic dissection were significantly higher than that in normal thoracic aorta ($P<0.01$). The expression of AGEs and RAGE were negatively correlated with that of SM α-actin and calponin. The regulation of AGEs on phenotypic transformation of VSMCs was blocked after silencing RAGE. Conclusions: AGEs can promote the transformation of VSMCs from the contractile type to the synthetic type in the thoracic aortic dissection and aortic aneurysm tissue.

Keywords: Thoracic aortic dissection, advanced glycation end products, receptor for advanced glycation end products, vascular smooth muscle cells

Introduction

Thoracic aortic dissection (TAD) refers to the lesions that longitudinal separation between the aortic intima and the middle occurred and true and false lumen were formed in the arteries, which was caused by a sudden pressure increase within the vascular lumen after the lesion in the tunicae media occurred, and then a direct, retrograde, or bidirectional dissecting aneurysm formed or ruptured. Its mortality is very high [1, 2]. At present, the main treatment methods include endovascular graft replacement surgery and traditional graft replacement surgery, the mortality and postoperative complication rates were high [3].

The aortic wall has contractibility and elasticity. Vascular smooth muscle cells (VSMCs) play an important role in the maintenance of vascular homeostasis and repair of vascular injury. The phenotype of VSMCs was divided into two types: synthetic VSMCs and contractile VSMCs. Synthetic VSMCs have strong ability of migration, proliferation and secretion. Contractile VSMCs highly expressed smooth muscle specific genes and had strong contractibility [4, 5]. The mature VSMCs were able to convert between the two types. VSMCs could transform from the contractile type to the synthetic type in the thoracic aortic dissection and the middle membrane tissue of aneurysm [6]. Smooth muscle alpha-actin (SMα-actin) and calponin can be used to evaluate the phenotypic transformation of VSMCs.

Advanced glycation end products (AGEs) were first discovered by Maillard in 1912 [7, 8]. AGEs can be combined with a variety of human tis-

Table 1. The primer used in Real-time PCR

Gene	GenBank Accession NO.	Primer (5'-3')	Length of product (bp)
RAGE	NM_001271424.1	F: CAGGGTCACAGAAACCGG	214
		R: ATTCAGCTCTGCACGTTCCT	
β-actin	NM_007393.4	F: GACAGGATGCAGAAGGAGAT	273
		R: GCCATGCCAATGTTGTCTCTTA	

F: forward; R: reverse.

Table 2. The synthetic system of inverse transcription

Components	Volume per Reaction
dNTP Mix	4 μl
Primer Mix	2 μl
RNA Template	4 μl
5×RT Buffer	4 μl
DTT	2 μl
HiFiScript	1 μl
RNase-Free Water	Up to 20 μl

sues or cells to destroy them. The biological effect of AGEs is related to the expression of RAGE (Receptor for AGE) [9]. Interactions between AGEs and RAGE can activate a variety of signaling pathways related to cell proliferation and apoptosis, which is associated with the occurrence and development of diabetes, Alzheimer's disease, atherosclerosis and other diseases [10-13]. Vascular endothelial cells, smooth muscle cells, fibroblasts, macrophages and even neurons express RAGE or have AGEs binding site. At present, the roles of AGEs in the transformation of the arterial VSMCs phenotype in patients with TAD remain unclear. In this study we observe the expression of AGE and RAGE and explore the roles of phenotypic transformation of VSMCs regulated by AGEs in the thoracic aortic dissection in neonatal rats, which could provide theoretical basis for the prevention, diagnosis and treatment of the disease.

Materials and methods

Cell culture

USMC cell lines were purchased from American type culture collection (ATCC). They were cultured with DMEM medium containing 10% fetal calf serum at 37°C with 5% CO_2.

Experimental animals

A total of 45 SD rats (weight 200±20 g, 15 males and 30 females) were obtained from the experimental animal center. They were pre-feeding for one week with free access to food and water to adapt to the environment and 5 per cage. Room temperature maintained at 18~25°C. The neonatal rat thoracic aortic dissection model was established in pregnant rats (n=25). Semicarbazlde (25 mg/kg.d) was casted to the implantable capsule osmotic pump and implanted inside the abdomen of pregnant 14 d rats. Neonatal mice were sacrificed after anesthesia and thoracic aortic tissues were taken out [14]. All experimental procedures were approved by the Care of Experimental Animals Committee of our hospital.

HE staining

The thoracic aortic tissue was cut into small pieces and fixed in 10% formalin and embedded in paraffin routinely. The paraffin blocks of specimen were cut into continuous sections with 4 μm respectively. The sections were dewaxed with xylene and washed with ethanol and water. They were stained with hematoxylin after that and then differentiated, washed and stained with eosin, then dehydrated, hyalinized and finally mounted on slides and observed under microscope, pictures were taken.

Preparation and detection of VSMCs phenotype transformation model

VSMCs phenotype transformation model was established by platelet derived growth factor BB (PDGF-BB) stimulation and starvation culture methods respectively. Different concentration of PDGF-BB (0 ng/ml, 2 ng/ml, 5 ng/ml and 7 ng/ml) was added into DMEM medium and culture for 48 h and then detection. VSMCs were detected after starvation treatment for 0 h, 24 h, 48 h and 72 h respectively. Successfully established model cells were performed AGEs stimulation experiment, they were divided into control group, 40 μg/mL AGEs group, 20 μg/mL AGEs group, 10 μg/mL AGEs group and 5 μg/mL AGEs group respectively.

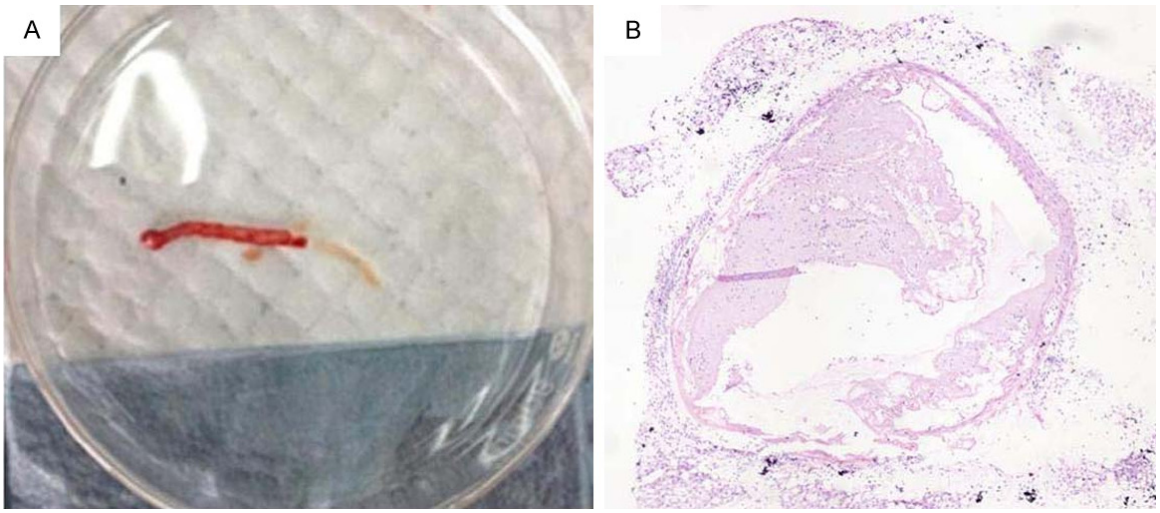

Figure 1. Establishment of thoracic aortic dissection model. A: Thoracic aortic tissue; B: HE staining of thoracic aortic dissection model.

RAGE siRNA

SiRNA oligonucleotides corresponding to RAGE (5'-CGAGAATCACGCTGC ATGACCATGT-3') were used for transfection of VSMCs cells using lipofectamine 2000 (Life Technologies) according to the manual. The cells were divided into control group, AGEs 40 μg/mL group and RAGE siRNA+AGEs 40 μg/mL group.

Enzyme linked immunosorbent assay (ELISA)

AGEs was detected after grinding the tissue samples with ELISA kit according to the manual. OD values at 450 nm were determined by ELISA detector. Standard curve was drawn to calculate the concentration.

Protein extraction and western blotting

The tissues were lysed with RIPA lysis buffer and total proteins were extracted and analyzed with SDS-PAGE electrophoresis. Then it was electrotransferred to the PVDF membrane. After the transmembrane, PVDF membrane was rinsed with TBS for 10 to 15 min, placed in TBS/T blocking buffer containing 5% (w/v) skimmed milk powder and shook at room temperature for one hour. It was incubated at room temperature for two hours after added with appropriate dilution degree of primary antibody (diluted with TBST containing 1% (w/v) skimmed milk powder). Then the membrane was rinsed with TBST for three times (5 to 10 minutes one time). The membrane was incubated at room temperature for one hour with HRP labeled secondary antibody (1:10000) diluted with TBST containing 0.05% (w/v) skimmed milk powder and rinsed for three times with TBST (5 to 10 minutes at a time). The protein bands were scanned and quantified as a ratio to β-actin.

RNA extraction and real-time PCR

The harvested cells were washed with RNase free PBS. Total RNA was extracted using RNeasy Mini Kit (Qiagen) according to the manufacturer's protocol. Their concentration and purity were detected with Qubit Fluorometer. 1 μg RNA was subjected to reverse transcription using reverse transcription kit (Promega). Real-time PCR were performed using SYNBR Green PCR Master Mix (Qigen). The primers used in this study were shown in **Table 1**, the synthetic system of PCR were shown in **Table 2**. β-actin gene was used as an internal control for normalization of RNA quantity and quality differences in all samples.

Statistical analysis

The results are expressed as mean ± S.D. and analyzed with SPSS 16.0 software, student t-test was used to evaluate the differences between groups. A value of $P<0.05$ and $P<0.01$ were taken to denote statistical significance.

Results

Establishment of thoracic aortic dissection model

As shown in **Figure 1**, the histopathological features of thoracic aortic dissection were middle

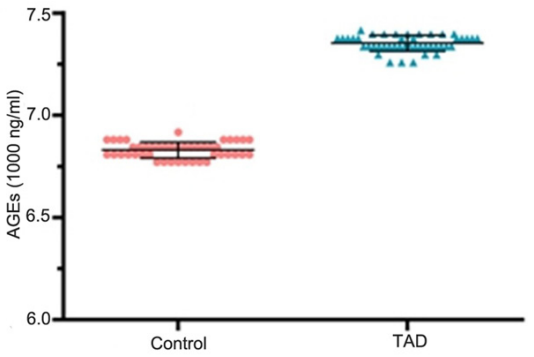

Figure 2. Expression of AGEs in thoracic aortic tissues.

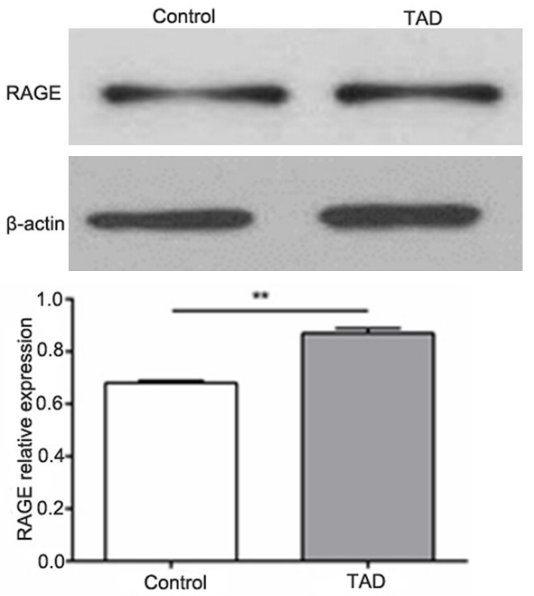

Figure 3. Expression of RAGE in thoracic aortic tissues.

arterial degeneration, lamellar necrosis with smooth muscle cell loss. We established thoracic aortic dissection model successfully.

Expression of AGEs in thoracic aortic tissues

The expression levels of AGEs were shown in **Figure 2**. It showed that the expression levels of AGEs in thoracic aortic dissection tissues were significantly higher than that of normal thoracic aortic dissection tissues (7350.97± 31.01 ng/ml vs 6830.88±35.46 ng/ml, P< 0.01).

Expression of RAGE in thoracic aortic tissues

Western blotting results were shown in **Figure 3**. It showed that the expression levels of RAGE

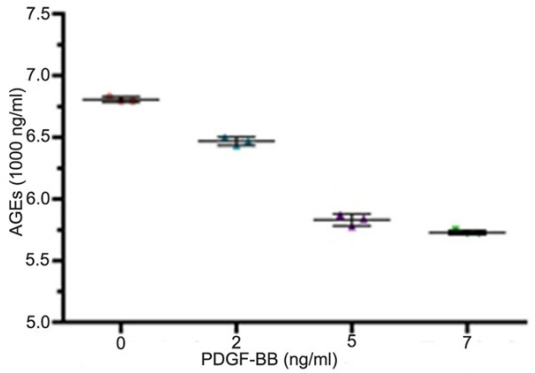

Figure 4. AGEs expression changes with different concentrations of PDGF-BB.

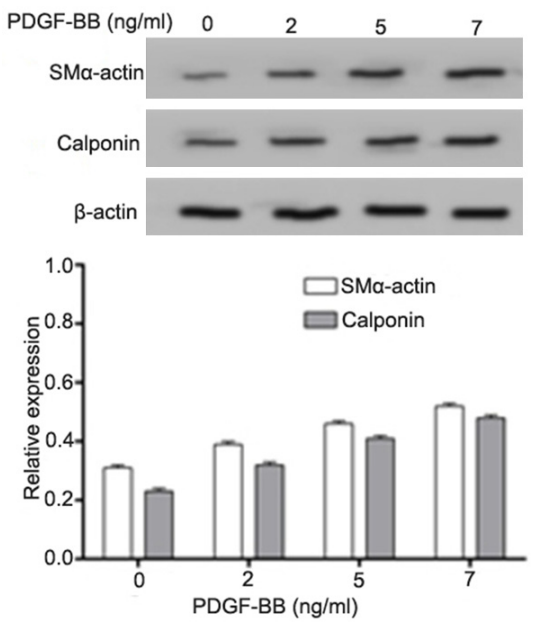

Figure 5. Western blotting results of SMα-actin and Calponin expression under different concentrations of PDGF-BB.

in thoracic aortic dissection tissues were significantly higher than that of normal thoracic aortic dissection tissues (P<0.01).

Establishment of VSMCs phenotype transformation model by PDGF-BB stimulation

As shown in **Figure 4**, the concentration of AGEs gradually decreased with the increase of PDGF-BB concentration. Western blotting results showed that the expression levels of SMα-actin and Calponin increased with the increase of PDGF-BB concentration (**Figure 5**). RT-PCR results showed that the expression levels of RAGE decreased with the increase of PDGF-BB concentration (**Figure 6**).

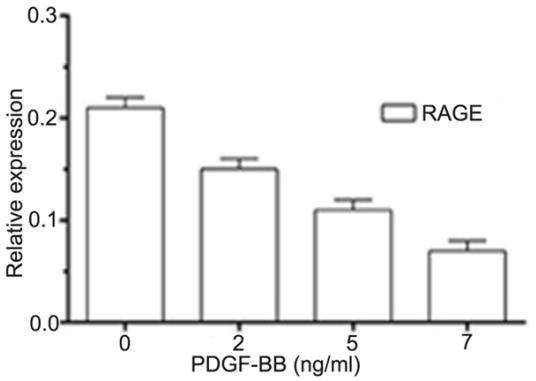

Figure 6. RT-PCR results of RAGE expression under different concentrations of PDGF-BB.

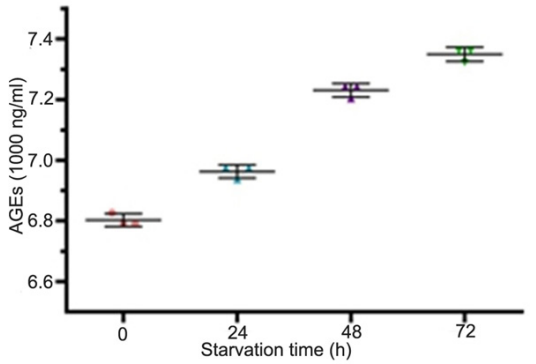

Figure 7. AGEs gradually increased with the time of starvation treatment.

Establishment of VSMCs phenotype transformation model by starvation treatment

ELISA results of AGEs were shown in **Figure 7**. It showed that the concentration of AGEs gradually increased with the time of starvation treatment. Western blotting results showed that the expression levels of SMα-actin and Calponin decreased with the time of starvation treatment (**Figure 8**). RT-PCR results showed that the expression levels of RAGE increased with the time of starvation treatment.

Phenotype transformation of VSMCs in different concentration of AGEs groups

Western blotting results were shown in **Figure 9**. It showed that the expression levels of SMα-actin and Calponin decreased with the increase of AGEs concentration. RT-PCR results showed that the expression levels of RAGE increased with the increase of AGEs concentration.

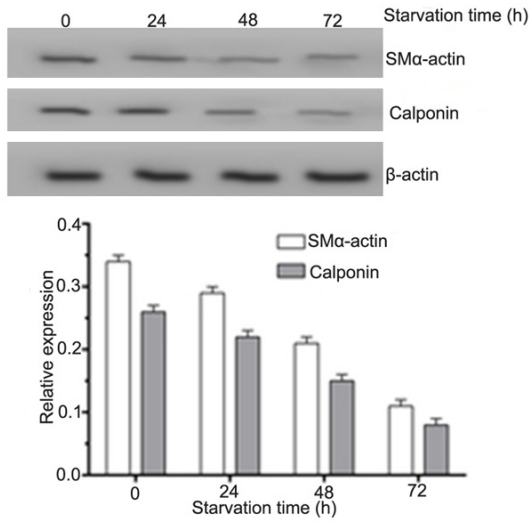

Figure 8. Western blotting results of SMα-actin and Calponin expression on different starvation treatment time.

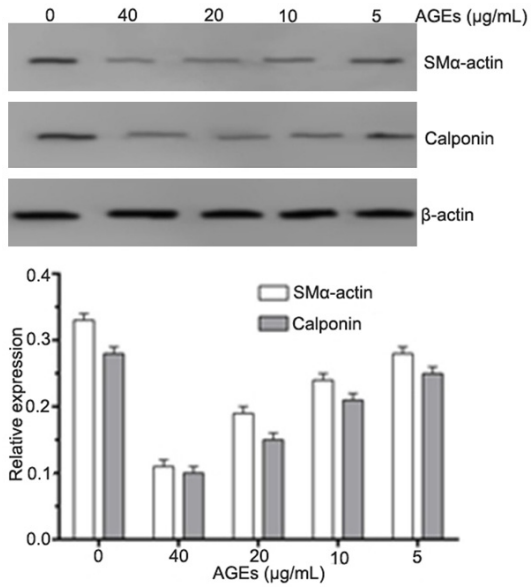

Figure 9. Western blotting results of SMα-actin and Calponin expression under different concentrations of AGEs treatment.

RAGE siRNA results

Western blotting results were shown in **Figure 10**. It showed that AGEs could down-regulate the expression levels of SMα-actin and Calponin, while the expression levels of SMα-actin and Calponin increased after silencing RAGE. **Figure 11** showed that RAGE expression decreased in RAGE siRNA group.

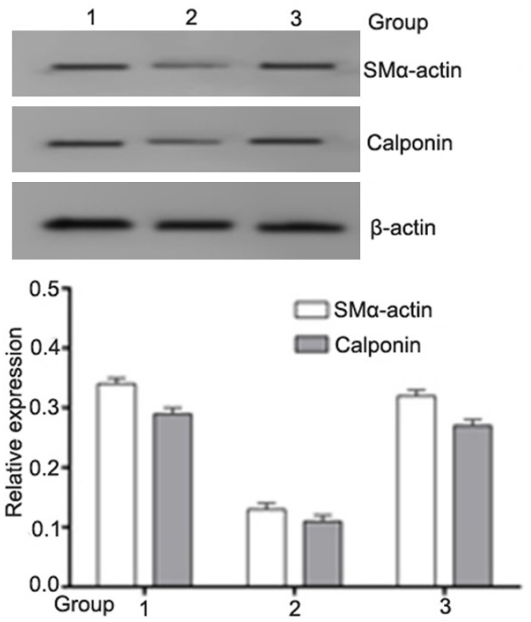

Figure 10. Western blotting results of SMα-actin and Calponin expression under AGEs and RAGE siRNA treatment. 1: Control group; 2: AGEs 40 μg/mL group; 3: RAGE siRNA+AGEs 40 μg/mL group.

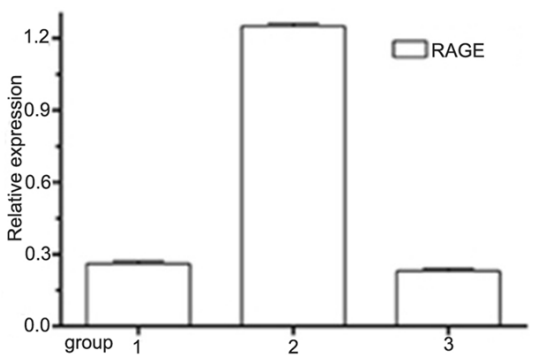

Figure 11. RT-PCR results of RAGE expression under AGEs and RAGE siRNA treatment. 1: Control group; 2: AGEs 40 μg/mL group; 3: RAGE siRNA+AGEs 40 μg/mL group.

Discussion

TAD is a common serious cardiovascular disease and its prognosis is poor, the incidence rate was about 3 cases/100,000 [14, 15]. It was found that the interaction of RAGE and AGEs is related to the development of a variety of diseases [11-13]. In this study we found that AGEs levels in TAD tissues were higher than that of normal tissue, which suggested that AGEs may play an important role in the development of TAD. We also found that the expression changes of RAGE was consistent with AGEs, the regulation of AGEs on phenotypic transformation of VSMCs was blocked after silencing RAGE. These results suggested that AGEs activated signal pathway, regulated the expression of related molecules and phenotypic transformation depending on its receptor RAGE.

SMα-actin and Calponin were specific genes of VSMCs. Their expression levels could change with different VSMCs phenotypes [16, 17]. Increased SMα-actin and Calponin predicated that VSMCs transformed to synthetic type, otherwise they transformed to contractile type. PDGF-BB is an important promoting mitogenic factor; it could stimulate VSMCs transform to contractile type. Starvation treatment could stimulate VSMCs transform to synthetic type [18]. We found that the expression levels of SMα-actin and Calponin increased with the increase of PDGF-BB concentration, while the expression levels of RAGE decreased with the increase of PDGF-BB concentration. The expression levels of SMα-actin and Calponin decreased with the time of starvation treatment, while the expression levels of RAGE increased with the time of starvation treatment. These results suggested that the expression level of RAGE was negatively correlated with the expression of SMα-actin and Calponin. AGEs expression was also negatively correlated with the expression of SMα-actin and Calponin. Therefore, we thought that AGEs and RAGE could promote the transformation of VSMCs from the contractile type to the synthetic type in the thoracic aortic dissection.

We analyzed the interaction of AGEs and RAGE and related pathway through STRING database (http://string-db.org) (**Figure 12**). We found that Saa1/2, Hmgb1, S100b and other proteins got higher credibility. These proteins abnormally expressed in diabetes, rheumatoid arthritis and other diseases, they played important roles in the development of the diseases. These suggested that AGEs was involved in the occurrence and progression of the disease through its receptor and signaling pathway, AGEs had complex biological effects.

In a word, AGEs and RAGE could promote the transformation of VSMCs from the contractile type to the synthetic type in TAD, its specific molecular mechanism need further study.

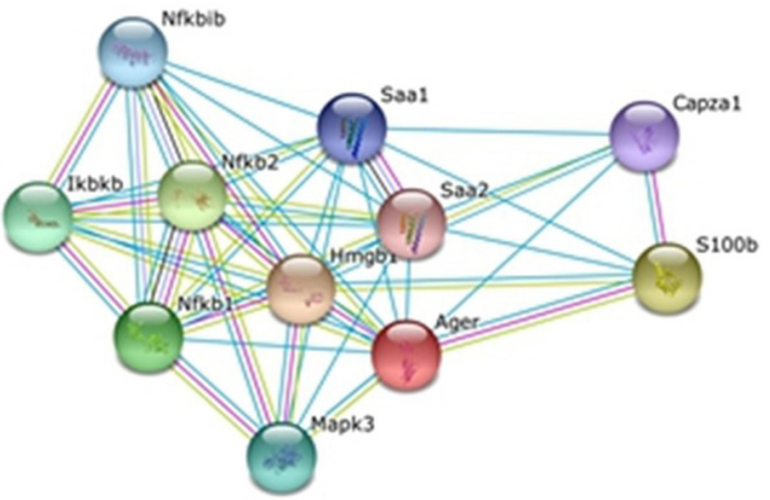

Figure 12. Bioinformatics analysis of RAGE. Hmgb1: High mobility group box 1; S100b: S100 protein beta polypeptide; Nfkbib: Nuclear factor of kappa light polypeptide gene enhancer in B cells inhibitor beta; Capza1: Capping protein (actin filament) muscle Z-line alpha 1; Saa2: Serum amyloid A 2; Mapk3: Mitogen-activated protein kinase 3.

Disclosure of conflict of interest

None.

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References

[1] Shalhub S, Dua A and Brooks J. Biomarkers in descending thoracic aortic dissection. Semin Vasc Surg 2014; 27: 196-199.

[2] Roselli EE. Thoracic endovascular aortic repair versus open surgery for type-B chronic dissection. J Thorac Cardiovasc Surg 2015; 149: S163-167.

[3] Olsson C, Thelin S, Stahle E, Ekbom A and Granath F. Thoracic aortic aneurysm and dissection: increasing prevalence and improved outcomes reported in a nationwide population-based study of more than 14,000 cases from 1987 to 2002. Circulation 2006; 114: 2611-2618.

[4] Yu X and Li Z. MicroRNAs regulate vascular smooth muscle cell functions in atherosclerosis (review). Int J Mol Med 2014; 34: 923-933.

[5] Mao N, Gu T, Shi E, Zhang G, Yu L and Wang C. Phenotypic switching of vascular smooth muscle cells in animal model of rat thoracic aortic aneurysm. Interact Cardiovasc Thorac Surg 2015; 21: 62-70.

[6] El-Hamamsy I and Yacoub MH. Cellular and molecul-ar mechanisms of thoracic aortic aneurysms. Nat Rev Cardiol 2009; 6: 771-786.

[7] Stirban A and Tschope D. Vascular Effects of Dietary Advanced Glycation End Products. Int J Endocrinol 2015; 2015: 836498.

[8] Tessier FJ. The Maillard reaction in the human body. The main discoveries and factors that affect glycation. Pathol Biol (Paris) 2010; 58: 214-219.

[9] Fritz G. RAGE: a single receptor fits multiple ligands. Trends Biochem Sci 2011; 36: 625-632.

[10] Lin L, Zhong K, Sun Z, Wu G and Ding G. Receptor for advanced glycation end products (RAGE) partially mediates HMGB1-ERKs activation in clear cell renal cell carcinoma. J Cancer Res Clin Oncol 2012; 138: 11-22.

[11] Abe R, Shimizu T, Sugawara H, Watanabe H, Nakamura H, Choei H, Sasaki N, Yamagishi S, Takeuchi M and Shimizu H. Regulation of human melanoma growth and metastasis by AGE-AGE receptor interactions. J Invest Dermatol 2004; 122: 461-467.

[12] Abe R and Yamagishi S. AGE-RAGE system and carcinogenesis. Curr Pharm Des 2008; 14: 940-945.

[13] Mittal SR. Presystolic flow in ascending aorta in a case of left ventricular diastolic dysfunction. Indian Heart J 2015; 67: 152-155.

[14] Elefteriades JA. Thoracic aortic aneurysm: reading the enemy's playbook. Yale J Biol Med 2008; 81: 175-186.

[15] Yu SY, Lee CH, Hsieh HC, Chou AH and Ko PJ. Treatment of primary infected aortic aneurysm without aortic resection. J Vasc Surg 2012; 56: 943-950.

[16] Chen S, Liu B, Kong D, Li S, Li C, Wang H and Sun Y. Atorvastatin calcium inhibits phenotypic modulation of PDGF-BB-induced VSMCs via down-regulation the Akt signaling pathway. PLoS One 2015; 10: e0122577.

[17] Zhang J, Wang L, Fu W, Wang C, Guo D, Jiang J and Wang Y. Smooth muscle cell phenotypic diversity between dissected and unaffected thoracic aortic media. J Cardiovasc Surg (Torino) 2013; 54: 511-521.

[18] Li PC, Sheu MJ, Ma WF, Pan CH, Sheu JH and Wu CH. Anti-Restenotic Roles of Dihydroaustrasulfone Alcohol Involved in Inhibiting PDGF-BB-Stimulated Proliferation and Migration of Vascular Smooth Muscle Cells. Mar Drugs 2015; 13: 3046-3060.