European Review for Medical and Pharmacological Sciences 2017; 21: 3262-3269

Investigation into the correlations of expressions of Cav-3 and Smad3 with pathogenesis and prognosis of viral myocarditis

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Abstract. – OBJECTIVE: To investigate the correlations of expressions of Caveolae-3 (Cav-3) and sma and mad homologue (Smad3) with the pathogenesis and prognosis of viral myocarditis (VMC).

MATERIALS AND METHODS: VMC animal models were prepared and divided into the control group, the virus group and the Shenmai group. We detected the levels of creatine kinase isoenzyme (CK-MB) in the serum that was associated with the myocardial injuries, investigated the pathological features of VMC in BALB/C mice via hematoxylin-eosin (HE) staining, measured the mRNA expressions of Cav-3 and Smad3 via Real-time polymerase chain reaction (RT-PCR) and determined the protein expressions of Cav-3 and Smad3 through Western blotting method.

RESULTS: The expressions of CK-MB in the virus group and Shenmai group were significantly higher than those in the control group; in comparison with the virus group, obvious improvement was identified in the pathologic condition of the Shenmai group; also, there was a statistically significant difference in comparison of the pathologic scores of BALB/C mice between the Shenmai group and the virus group. The mRNA expressions of Cav-3 and Smad3 in the virus group and Shenmai group were significantly higher than those in the control group, and the differences had statistical significance; however, higher mRNA expressions were identified in the virus group. Besides, protein expressions of Cav-3 and Smad3 in the virus group and Shenmai group were remarkably higher than those in the control group with statistically significant differences, but those in the virus group were much higher.

CONCLUSIONS: Cav-3 and Smad3 may be involved in the occurrence and development of VMC, which provides some theoretical evidence for further research into the pathogenesis of VMC and the development of clinical drugs for treatment of VMC.

Key Words:
Viral myocarditis, Cav-3, Smad3.

Introduction

Viral myocarditis (VMC) is a cardiovascular disease frequently seen in clinical practice in Pediatric Department[1]. In recent years, a gradual increase on a year-by-year basis has been found in the incidence rate of VMC, severely affecting the mental and physical health of children[2]. At present, the pathogenesis of VMC remains unclear yet, and mainly involves the direct damages of toxins generated by virus, immunologic mechanism and myocardial fibrosis, etc.[3-5]. Caveolin, as the cytoskeleton protein of signal molecule on cell membrane and negative regulating protein, can directly bind with the downstream signal molecules that will be mounted in the caveolae on cell membrane[6]. Literatures have reported that over 40% signal transduction molecules aggregate in caveolae[7], and that caveolae-3 is mainly expressed in the myocardial cells[8]. The family of Smads, short for the Sma and Mad that were found in the Drosophila and the nematode, respectively, is a kind of important signal transduction factors; in myocardial tissues, activated Smads can contribute to the myocardial fibrosis and cell apoptosis in myocardium, in which the expression of Smad[3] plays a critical role[9,10]. Based on the facts above, we postulated that Cav-3 and Smad3 might be correlated with the occurrence and development of VMC. Thus, in this study, we investigated the correlations of Cav-3 and Smad3 expressions with the pathogenesis and prognosis of VMC, which will be served as the theoretical evidence for di-

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scovering the pathogenesis of VMC and the development of drugs for clinical treatment of VMC.

Materials and Methods

Experiment Materials

Eighty BALB/C mice aged between 3 and 5 weeks within 18 to 20 g were fed in a clean environment. Coxsackie virus B3 (CVB3). Shenmai injections purchased from Chia Tai Group (Qingchunbao Pharmaceutical Co., Ltd., Hangzhou, China), were used in this study. This study was approved by the Animal Ethics Committee of Shandong University Animal Center.

Major Reagents

Rabbit anti-mouse Cav-3 monoclonal antibody (Beijing Dingguo Changsheng Biotechnology Co., Ltd., Beijing, China); rabbit anti-mouse Smad3 antibody (BD Biosciences, Franklin Lakes, NJ, USA); 3,3'-diaminobenzidine (DAB) color-development agent (Shanghai Xin Yu Biotech Co., Ltd., Shanghai, China); powder preparation of citrate buffer (Shanghai Xin Yu Biotech Co., Ltd., Shanghai, China); hematoxylin and eosin (HE) (Nanjing DASF Biological Technology Co., Ltd., Nanjing, China); Real-time quantitative PCR kit (Vipotion Biotechnology Co., Ltd., Guangzhou, China); TRIGene reagent (Kangrun Chengye Biotechnology Co., Ltd., Beijing, China); reverse-transcription kit (Yuduo Biological Technology Co., Ltd. Zibo, China); extraction kit for total protein of cell (Keygen Biotech Co., Ltd. Nanjing, China).

Detection for Indexes of Myocardial Enzymology in Serum

For BALB/C mice in the experiment groups, they received the intraperitoneal injection of 0.1 mL 102 tissue culture infective dose (TCID) 50 virus bulk, while in the control group, mice received the intraperitoneal injection of Eagel's virus-free viral culture solution in the same volume. After BALB/C mice were infected using CVB3, CK-MB in serum that was associated with the myocardial injuries was detected using the serum enzymology-related kit produced by Beckman-Coulter (Miami, FL, USA). Experiment was carried out in strict accordance with the instructions.

HE Staining

Sections that were embedded using paraffin were dewaxed and rinsed using alcohol. Sections, after being thoroughly hydrated, were infected using hematoxylin for 10 min followed by washing using water. Next, the sections were differentiated using 1% hydrochloric acid alcohol for 3 s followed by washing using water. The sections were blued using saturated lithium carbonate for 3 s followed by rinsing in running water for 20 min. 1% alcohol-soluble eosin was used for 10 s of staining followed by regular dehydration, cleaning and sealing.

Detecting the mRNA Expressions of Cav-3 and Smad3 via RT-PCR

According to the instructions of TRIGene kit (Kangrun Chengye Biotechnology Co., Ltd., Beijing, China), the total RNA was extracted from the myocardial tissues; the concentration and purification of total RNA were detected using spectrometer; A260/A280 ratio was within 1.8 and 2.0. Following the instructions of reverted fist strand cDNA synthesis kit (K1622 Thermo Scientific, Waltham, MA, USA,), primer synthesis was performed by Jiran Biotech Co., Ltd. (Shanghai, China) (Table I). Reaction system in 20 μL was placed onto the RT-PCR apparatus for reverse transcription and synthesis of cDNA. According to the instructions of Real-time quantitative PCR kit (2×RealStar Green Power Mixture, GenStar, A311), RT-PCR was performed in 25 μL reaction system in following conditions: a total of 40 cycles of 95°C for 5 min, 94°C for 30 s and 54°C for 35 s; 95°C for 15 s followed by cooling down to 65°C. Fluorescence value was read, and with GAPDH as internal reference, the relative mRNA expression levels of Cav-3 and Smad3 were calculated automatically using RT-PCR apparatus.

Detecting the Protein Expressions of Cav-3 and Smad3 via Western Blotting Method

Total protein was extracted from the myocardial tissues according to the instructions of extraction kit of cell protein, and the concentration of protein was assayed followed by preservation at -70°C for later use. Gel was prepared for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The positions of two kinds of proteins on the gel were identified using the stripes of marker. Then, the proteins were transferred onto the membrane for 35 min of blotting, and the membrane was then blocked for 90 min at 37°C using 5% skimmed milk. Primary antibody was added onto the membrane for incubation at 4°C overnight; afterwards, tris buffer saline + Tween 20 (TBST) was added onto the membrane

Table I. Primer sequences of Cav-3 and Smad3

Cav-3	Forward primer	5'-CCCAAGAFCACACAGATCTGC-3'
	Reverse primer	5'-GAGCAGGGCCAGTGGAACACC-3'
Smad3	Forward primer	5'-TCTACTGCCGCTTGTGG-3'
	Reverse primer	5'-TGTGGTTCATCTGGTGGTC-3'
GAPDH	Forward primer	5'-ACCACAGTCCATGCCATCAC-3'
	Reverse primer	5'-TCCACCACCCTGTTGCTGTA-3'

Table II. Content of CK-MB in serum of BALB/C mice in each group at different time points ($\bar{x}$ ± S, U/L).

	n	7 d	14 d
Control group	10	312.84±29.04	302.88±30.42
Virus group	10	483.02±34.83[a]	538.96±39.27[a]
Shenmai group	10	390.55±33.51[ab]	448.03±35.58[ab]

Note: Compared with the control group: [a]$p<0.01$; Compared with the virus group: [b]$p<0.01$.

followed by shaking and rinsing on a shaker for 3 times (15 min/time); secondary antibody was added onto the membrane for 1 hour of incubation at 37°C followed by shaking on a shaker and rinsing using TBST for 3 times (15 min/time). Membrane was then transferred into the dark room, on which ECL (electro-chemi-luminescence) reagent was added for coloration, exposure, development and fixation; next, the membrane was scanned using ChemiDocTMMP imaging system (Silver Springs, MD, USA), the images were analyzed using the ImageJ software and the optical density was also recorded.

Evaluation of Results

Semi-quantitative assessment for inflammatory responses and pathological changes of necrosis in myocardial tissues. Methods in the literature were used for reference of semi-quantitative assessment for inflammatory responses and pathological changes of necrosis in myocardial tissues. After HE staining, the sections were placed under the microscope for observation, and accordingly, we calculated the ratios of areas of infiltration of inflammatory cells (I) and myocardial necrosis (N). 0 points were for no infiltration of inflammatory cells or myocardial necrosis, 1 point for ratios of areas of I and N less than 25%, 2 points for ratios of areas of I and N between 25% and 50%, 3 points for ratios of areas of I and N between 50% and 75%, and 4 points for ratios of areas of I and N higher than 75%.

Statistical Analysis

In this study, all data were analyzed using SPSS 17.0 software (Version X; IBM, Armonk, NY, USA), and presented as mean ± standard deviation. *t*-test was performed for statistical analysis of comparison between two groups, while Least Significant Difference was performed as the Post Hoc Test of One-way ANOVA test for comparisons between groups. $p<0.05$ suggested that the difference had statistical significance.

Results

Content of CK-MB in Serum of BALB/C Mice in Each Group

As result from Table II, we found that the content of CK-MB in serum of BALB/C mice in the control group remained basically at a same level in different time points without any remarkable changes, but after 7 days since model establishment, a significant increase in content of CK-MB was found in BALB/C mice in the virus group, and the persistent increase appeared at 14 d. Comparison between the control group and the virus group showed a statistically significant difference ($p<0.01$); in the Shenmai positive control group, corresponding changes were also observed in the content of CK-MB in serum of BALB/C mice at the same time period, but the content of CK-MB in this group was lower than that of the BALB/C mice in the virus group with a statistically significant difference ($p<0.01$).

The Results of HE Staining for Myocardial Tissues of BALB/C Mice in Each Group

In the control group (Figure 1), no pathological changes were observed in the myocardial

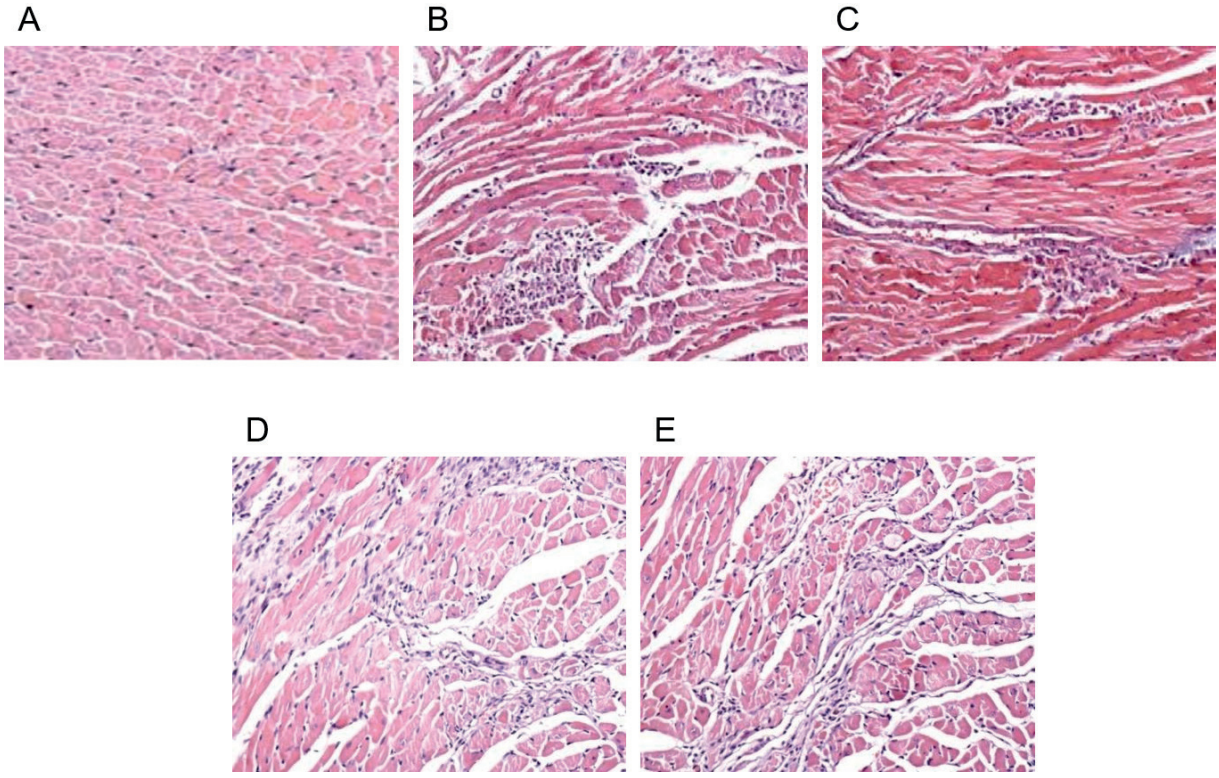

Figure 1. Pictures of HE staining for myocardial tissues of BALB/C mice in each group (×400). ***A***, control group; ***B-C.*** virus group at 7 d and 14 d; ***D-E.*** Shenmai positive control group at 7 d and 14 d protein expressions of Cav-3 and Smad3 in myocardial tissues of BALB/C mice via Western blotting method.

tissues of BALB/C mice, while ever since the 7th day, pathological changes were found in BALB/C mice in the virus group, including the visible inflammatory responses with infiltration of inflammatory cells, swelling in myocardial cells, vague transverse striation and necrosis in myocardial cells with minor fibroplasia in local parts. Since the 14th day, infiltration of inflammatory cells was gradually absorbed, and myocardial fibers were in malalignment with fibroplasia. Compared with the control group, fluctuations in pathological scores in the virus group at different time points were more marked with a statistically significant difference (p<0.01). After drug administration for intervention, there was an increasing trend in the pathological score of inflammatory responses of myocardial cells with the extension of time. However, in the virus group, mice enjoyed the recovery in pathological condition and significantly decreased pathological score, while there was a statistically significant difference in comparison of the pathological score of BALB/C mice between the Shenmai positive control group and the virus group, in which p was less than 0.05 at 7 d, and 0.01 at 14 d (Table III).

Table III. Pathological scores of myocardial tissues in BALB/C mice in each group ($\bar{x}$ ± S).

		Pathological score	
Group	**Quantity**	**7 d**	**14 d**
Control group	10	312.84±29.04	302.88±30.42
Virus group	10	483.02±34.83[a]	538.96±39.27[a]
Shenmai group	10	390.55±33.51[ab]	448.03±35.58[ab]

Note: [a]p<0.01; [b], c: Compared with the levels of virus group at 7 d and 14 [d], b p<0.05 and [c] p<0.01.

Table IV. Relative mRNA expressions of Cav-3 and Smad3 in myocardial tissues of BALB/C mice ($\bar{x} \pm S$).

		Cav-3		Smad3	
Quantity		**7 d**	**14 d**	**7 d**	**14 d**
Control group	10	0.19±0.04		0.21±0.02	
Virus group	10	0.28±0.04[a]	0.33±0.03[a]	0.28±0.05[a]	0.37±0.03[a]
Shenmai group	10	0.24±0.01[ab]	0.23±0.02[ab]	0.26±0.08[ab]	0.25±0.05[ab]

Note: [a] $p<0.05$; Compared with the Shenmai positive control group, [b]$p<0.05$.

Detecting the mRNA Expressions of Cav-3 and Smad3 in Myocardial Tissues of BALB/C Mice via RT-PCR

According to Table IV, mRNA expression of Cav-3 of BALB/C mice in the control group at different time points sustained a low level, while in the virus group, an increase in mRNA expression of Cav-3 of BALB/C mice was identified at the 1st and 2nd weeks, and the comparison with the control group showed a statistically significant difference ($p<0.05$). Compared with the virus group, mRNA expression of Cav-3 in BALB/C mice in the Shenmai positive control group was dramatically decreased with a statistically significant difference ($p<0.05$). In the control group, mRNA expression of Smad3 in BALB/C mice at different time points remained at a relatively low level, while in the virus group, mRNA expression of Smad3 in BALB/C mice at different time points was gradually increased with the extension of time, and the difference in comparison with the control group had statistical significance. In the Shenmai group, mRNA expression of Smad3 in mice was remarkably decreased in comparison with the virus group with a statistically significant difference ($p<0.05$).

Detecting the Protein Expressions of Cav-3 and Smad3 in Myocardial Tissues of BALB/C Mice via Western Blotting Method

As showed in Figure 2, we could figure out that with GAPDH as the internal reference, the stripes of Cav-3 and Smad3 were more obvious, and the protein expressions of Cav-3 and Smad3 in the virus group at different time points were significantly higher than those in the control group with statistically significant differences ($p<0.05$). In the Shenmai positive control group, protein expressions of Cav-3 and Smad3 in BALB/C mice were lower than those in the control group with statistically significant differences ($p<0.05$).

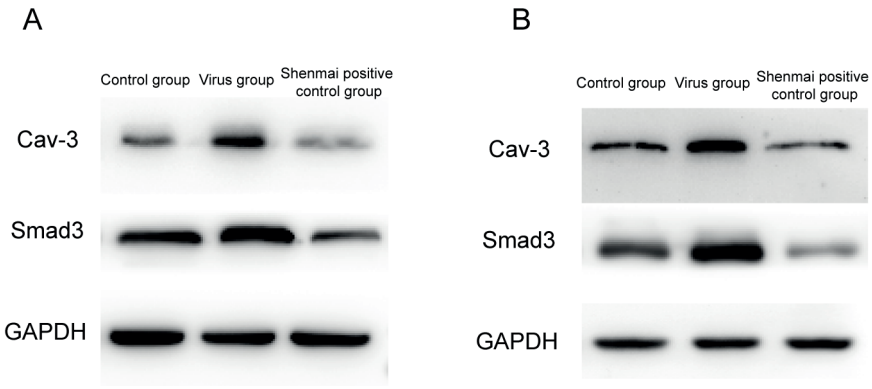

Figure 2. Protein expressions of Cav-3 and Smad3 in myocardial tissues of BALB/C mice via Western blotting method. The protein expressions of Cav-3 and Smad3 in the virus group were significantly higher than those in the control group; while, protein expressions of Cav-3 and Smad3 in the Shenmai positive control group were lower than those in the control group at 7 d (Figure 2A) and 14 d (Figure 2B).

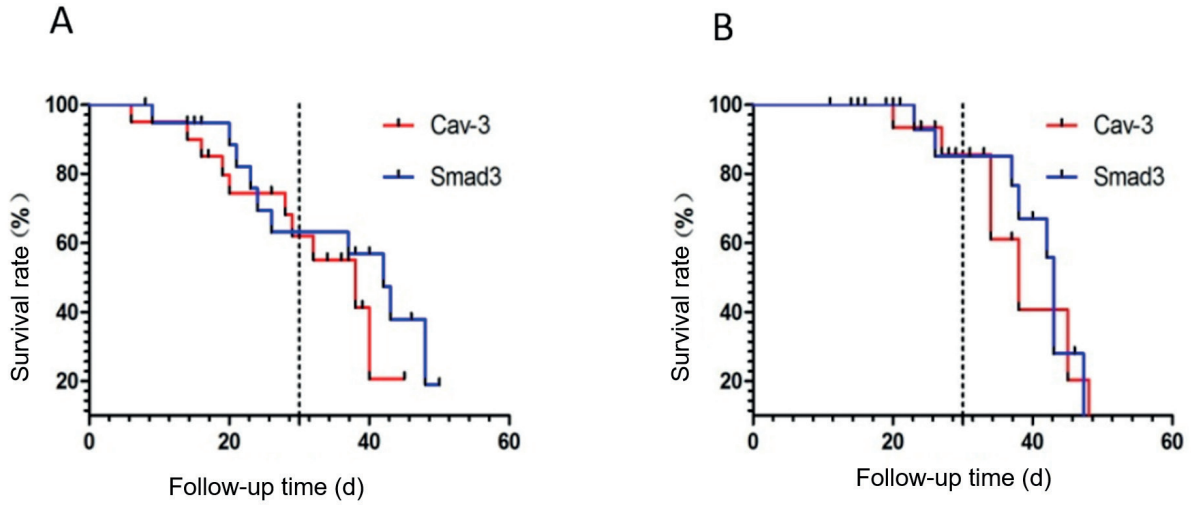

Figure 3. Protein expressions of Cav-3 and Smad3 and survival curves of BALB/C mice in the virus group and Shenmai group; ***A,*** In the virus group, the survival rate in 30 days is about 60%; ***B,*** in the Shenmai group, the survival rate in 30 days reaches 85%.

Correlation Between Protein Expressions of Cav-3 and Smad3 and Prognosis

Survival analysis was carried out using statistics software, and the results revealed that in the virus group, the survival rate in 30 days was about 60% (Figure 3A), while in the Shenmai group, the survival rate in 30 days reached 85% (Figure 3B), further suggesting that the survival rate of BALB/C mice was much higher when the protein expressions of Cav-3 and Smad3 sustain at a low level.

Discussion

VMC is a kind of non-specific interstitial inflammation in myocardium caused by infection of myocardiotropic virus11, and in recent years, the incidence rate of VMC has been increasing year by year in children and teenagers. However, the pathogenesis remains unclear so far. Direct damages caused by virus are considered as the most immediate factors, in which the coxsackie virus, as the most frequent risk factor, is associated with about 50% of VMC cases (mainly the CVB3)[12-14]. Research shows that in the myocardial cells infected by CVB3 virus, their cytoskeleton can be damaged through degradation of the dystrophin glycoprotein complex by protein kinase 2A, thereby causing myocardial dysfunction[15]. The family of Smads, short for the Sma and Mad that were found respectively in the drosophila and the nematode, is a kind of important signal transduction factors, which are involved in cell proliferation, apoptosis, differentiation, migration and inflammatory responses. In myocardial tissues, activated Smads can contribute to the myocardial fibrosis and cell apoptosis in myocardium[16,17]. According to a study, it was reported that during the occurrence of VMC, TGF-β1 and Smad3, downstream protein, play critical roles[18], but whether this process is regulated by other signal molecules, it has not yet ascertained. Cav-3, as the cytoskeleton protein of signal molecule on cell membrane and negative regulating protein in myocardial cells and skeletal muscle cells, can bind with the downstream signal molecules to regulate the responses of cells to external stimuli. Many scholars[19,20] believe that the development of VMC is closely associated with the myocardial fibrosis. In this study, the results revealed that at 7 d after model establishment of BALB/C mice in the virus group, we observed pathological changes accompanied with the inflammatory responses, infiltration of inflammatory cells, swelling and vague transverse striation in myocardial cells and necrosis of myocardial cells with minor fibroplasia in local parts; since the 14th day, infiltration of inflammatory cells was gradually absorbed, and the myocardial fibers were in malalignment with obvious fibroplasia, which was coincident with the symptoms of VMC. Through detecting the content of CK-MB in serum that was associated with myocardial injuries in the virus group and the Shenmai group, we found that the contents in the virus group and the Shenmai group were significantly higher than that in the control group. Compared with the virus group, evident recovery was observed in pathological condition of BALB/C mice in the Shenmai group, and

the difference in pathological scores between the Shenmai group and the virus group had statistical significance. Expressions of Cav-3 and Smad3 in the virus group and Shenmai group were significantly higher than those in the control group, and the differences had statistical significance; however, higher mRNA expressions were identified in the virus group. Survival analysis performed by statistics software showed that in the virus group, the survival rate in 30 days was about 60%, while in the Shenmai group, the survival rate in 30 days reached 85%, indicating that the survival rate of BALB/C mice is much higher when the protein expressions of Cav-3 and Smad3 sustain at a low level.

Conclusions

The results of this study suggested that there is a correlation between the expressions of Cav-3 and Smad3 and the occurrence and development of VMC, and that the expressions of Cav-3 and Smad3 in prognosis are closely associated with the survival rate, which can be served as the theoretical evidence for further research into the pathogenesis of VMC and development of relevant clinical drugs.

Conflict of interest

The authors declare no conflicts of interest.

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