

MIR-139-3P IS RELATED TO LEFT VENTRICULAR HYPERTROPHY AND CARDIOMYOCYTE APOPTOSIS IN TWO-KIDNEY ONE-CLIP HYPERTENSIVE RATS

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Abstract: MicroRNAs (miRNAs) are important post-transcriptional regulators of gene expression in many physiological and pathological processes. Previous studies have reported the role of miR-139-3p in cancer. However, its specific roles and functions in the heart undergoing hypertrophy have yet to be fully elucidated. In the present study, a significant upregulation of miR-139-3p expression was demonstrated in the left ventricular myocardium of two-kidney one-clip (2K1C) hypertensive rats using microarray and quantitative real-time PCR (qRT-PCR). Based on computational analysis, we observed that miR-139-3p can control the expression of mitogen-activated protein kinase 1 (*MAPK1*) as a target gene, which is essential for the induction of cardiac hypertrophy and cardiomyocyte apoptosis. This study provides first information that the highly expressed miR-139-3p might be closely involved in *MAPK1*-mediated cardiac hypertrophy and cardiomyocyte apoptotic processes in 2K1C rat.

Key words: hypertension; MiR-139-3p; cardiac hypertrophy; cardiomyocyte apoptosis

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INTRODUCTION

Systemic hypertension increases cardiac workload and subsequently induces a hypertrophic response of cardiomyocytes (Barnes et al., 2012). Initially, hypertrophic growth is an adaptive response of the heart to increased workload. In the long term, however, the response of cardiomyocytes may trigger maladaptive growth of the heart muscle and is characterized by various cellular events that are associated with apoptosis, including cytoskeletal reorganization, morphological change and altered expression of apoptosis-regulatory genes (Putinski et al., 2013). Cardiomyocyte apoptosis is common in cardiac hypertrophy and is related to ventricular wall thinning and chamber dilation (Chien et al., 1999).

MicroRNAs (miRNAs) are endogenous, small, non-coding RNAs that have emerged as a new set of modulators of gene expression (Lecellier et al., 2005). Recently, miRNAs have been identified as critical regulators of cardiac hypertrophy (Wang et al., 2005). Additionally, increasing evidence indicates that miRNAs have been implicated in the regulation of apoptosis in cardiovascular disease (Li et al., 2010). It is worth noting that the miRNAs miR-1, miR-21, miR-133, miR-195 and miR-208 that have been shown to regulate hypertrophy also have the potential to regulate apoptosis (Kirchhoff et al., 2002; Cheng et al., 2010; Zhu et al., 2012)

The miR-139 family plays a critical role not only in the regulation of the proliferation and invasion of tumor cells but also in inducing apoptosis (Li et al., 2013; Gu et al., 2014). MiR-139-3p belongs to the miR-139 family. The expression profile of miR-139-3p in tumors has been explored. The results of previous studies showed that miR-139-3p was aberrantly expressed in several tumors, indicating that miR-139-3p is in-

involved in the pathogenesis of tumors (Lin et al., 2011). However, little information is available on the role of the expression of miR-139-3p during the development of left ventricular hypertrophy. Moreover, despite all recent advances in understanding the mechanisms of apoptosis induced by miR-139-3p, the roles of miR-139-3p in myocardial apoptosis are currently not well understood.

Here, we performed miRNA microarray technology to analyze miR-139-3p expression in hypertrophied left ventricular tissue of two-kidney one-clip (2K1C) hypertensive rats, followed by quantitative real-time PCR (qRT-PCR)-based validation. The target genes and biological functions of miR-139-3p were also identified using ingenuity pathway analysis (IPA) for the first time. Finally, we identified the signaling pathways that miR-139-3p and its target gene influences using GeneGo, which helps understand the possible mechanisms of miR-139-3p-mediated cardiac hypertrophy and cardiomyocyte apoptosis.

Table 1. The list of top five relevant diseases and biological functions with their respective p-value obtained from IPA.

Terms	p-value	Molecular Number
Cell Death and Survival	7.88E-8-1.75E-2	4
Tumor Morphology	7.88E-8-6.45E-4	3
Cell-To-Cell Signaling and Interaction	4.41E-7-6.45E-4	3
Tissue Development	4.41E-7-6.45E-4	3
Embryonic Development	4.75E-7-6.45E-4	3

Table 2. The list of top 5 functions relevant to cell death and survival with their respective p-value obtained from IPA.

Terms	p-value	Molecular Number
Cell death	7.88E-8-4.4E-2	3
Apoptosis	4.75E-7-8.04E-3	3
Fragmentation	8.78E-7-3.23E-4	2
Cell viability	8.13E-5-1.75E-2	3
Activation-induced cell death	1.61E-4-1.61E-4	1

MATERIALS AND METHODS

Animals, tissues samples and RNA isolation

Male Wistar rats (weight 120-150 g) were purchased from Vital River Lab Animal Technology Co., Ltd (Beijing, China). All rats were randomly divided into 2 groups: 2K1C group (n=14) and sham group (n=12). The 2K1C hypertension model was then established as described in detail by Sun et al. (2013). Eight weeks after this initial surgery, the rats were anesthetized with sodium pentobarbital after overnight starvation. The left ventricle was immediately removed, blotted dry, weighed and stored at -80°C until RNA extraction. For miRNA microarray, total RNA was extracted as previously described (Sun et al., 2013). For real-time PCR, miRNA extraction was performed with an mirVana miRNA Isolation Kit (Ambion, Austin, TX, USA), according to the manufacturer's instructions. RNA quality and quantity were determined by a NanoDrop 1000

Spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA). Animal studies were performed under conditions approved by the Local Animal Care and Use Committee.

miRNA microarray

miRNA expression profiling was performed with $8 \times 15\text{K}$ Agilent Rat miRNA Microarray V12.0 (Agilent Technologies, Santa Clara, CA, USA) containing probes for 350 miRNAs. 100 ng total RNA was dephosphorylated, denatured, Cyanine3-pCp labeled and hybridized, using the Agilent rat miRNA microarray according to the manufacturer's instructions. After washing, array scanning was performed at a resolution of $5 \mu\text{m}$ using an Agilent DNA Microarray scanner (Agilent Technologies, Santa Clara, CA, USA). Feature extraction and gene signal normalization were performed with Feature Extraction Software 9.5.3 and GeneSpring software 10.1 (Agilent Technologies, Santa Clara, CA, USA).

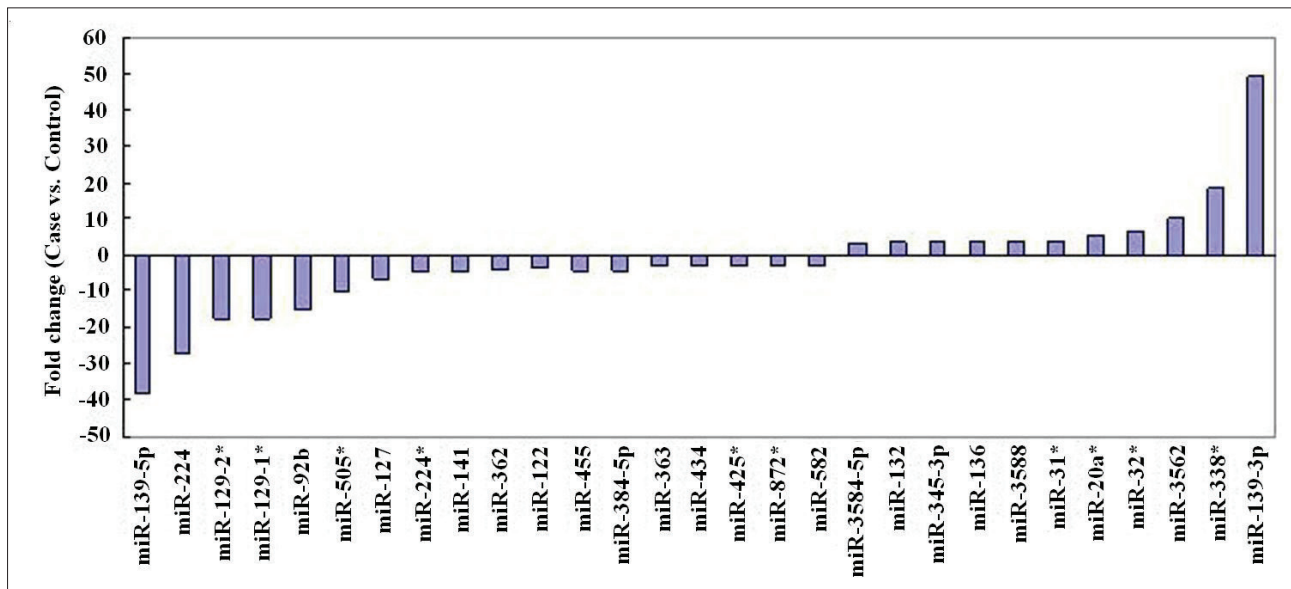


Fig. 1. Differentially expressed miRNAs in 2K1C rats (Case) compared with sham-operated rats (Control). A ± 3.0 -fold change cut-off was applied to all array data sets.

qRT-PCR validation

miRNAs with the highest fold change revealed by miRNA microarray were selected for further validation by qRT-PCR. TaqMan MicroRNA Assays were used as follows: miR-139-3p (002546), miR-21 (000397) and U6 snRNA (001973), which were purchased from Applied Biosystems (Foster City, CA, USA). Total RNA was reverse transcribed by TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). qRT-PCR was performed using TaqMan Universal PCR Master Mix II on a 7500 Fast Real-Time PCR System according to the manufacturer's protocol. A 5-fold serial dilution of pooled cDNA samples was also generated for each assay. All samples were run in triplicate. Based on results of the serial dilution curve and melting curve, a technical quality assessment was performed. Relative expression was then performed using the $\Delta\Delta C_t$ method. Data were standardized by \log_2 transformation.

Bioinformatics analysis

The target genes of the differentially expressed miR-139-3p were identified using the IPA program (<http://www.ingenuity.com>). Meanwhile,

the target genes of rno-mir-139-3p were collected, and subjected to GeneGo pathway annotation.

Statistical analysis

Statistical analysis was performed using SPSS15.0. Differences between groups were compared using the Student's t-test. A *P* value less than 0.05 was considered as statistically significant. All values are expressed as mean \pm SEM.

RESULTS

miRNA expression in left ventricular myocardium from sham-operated and 2K1C rats

We developed a rat model of 2K1C hypertension as previously described (Sun et al., 2013). An miRNA microarray platform containing 350 probes was used to assess miRNA expression profiles in the left ventricular myocardium. Our results demonstrated that miRNAs were differentially expressed between the sham-operated group and 2K1C groups (Fig. 1). The analysis identified that 11 miRNAs were upregulated and 18 were downregulated in the 2K1C group compared to the sham-operated group ($P < 0.05$). A ± 3.0 -fold change cut-off was applied to all array datasets.

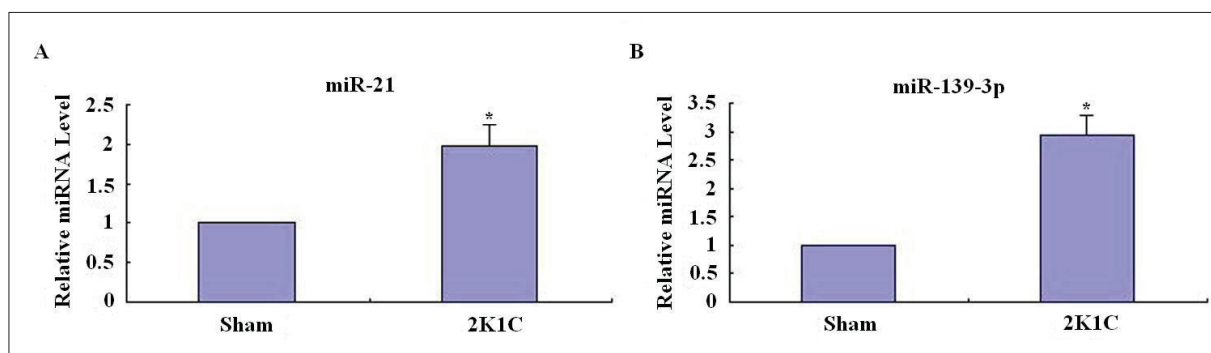


Fig. 2. Relative expression levels of miR-139-3p, miR-21 and U6 snRNA in 2K1C (n=14) and sham-operated rats (n=12). Data derived from qRT-PCR and the fold changes were calculated using the $\Delta\Delta C_t$ comparative quantification method. * $P < 0.05$, vs. sham-operated rats.

Validation of the microarray data by miRNA qRT-PCR analysis

To confirm the results from the microarray analysis, the highest expression miRNA miR-139-3p was selected for further confirmation using qRT-PCR. As expected, a statistically significant upregulation in the expression of miR-139-3p was observed in the 2K1C group compared to the sham-operated group ($P < 0.05$), which was found to be in accordance with the microarray analysis results (Fig. 2). Fig. 2 further revealed that the hypertrophic cardiomyocytes showed a significantly higher expression level of miR-21 compared to the controls ($P < 0.05$), which was in agreement with other studies (Cheng et al., 2010). These results indicated that the results of qRT-PCR analysis were reliable.

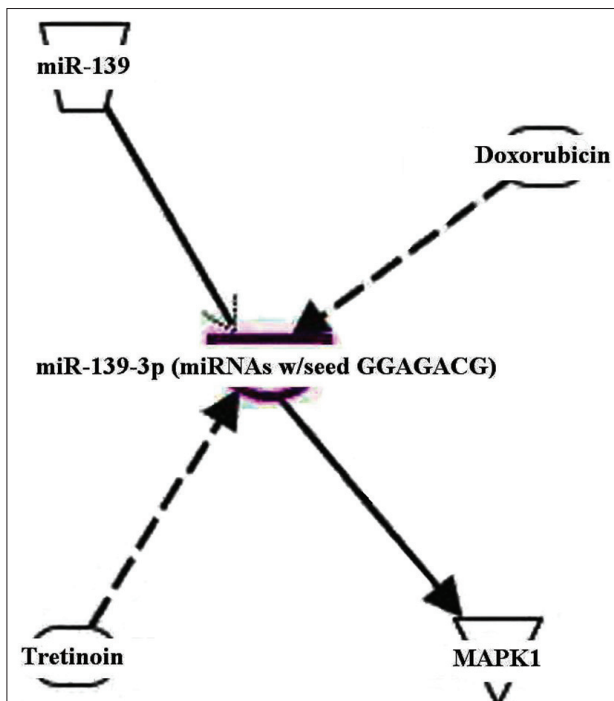


Fig. 3. The target genes of miR-139-3p obtained from IPA. miR-139-3p binds to *MAPK1* and can be regulated by doxorubicin and tretinoin.

The target genes and biological functions of miR-139-3p

The target genes of the differentially expressed miR-139-3p were analyzed using the IPA tool. One target gene, *MAPK1*, was identified after analysis of published data, validated by biological experiments (Fig. 3). In addition, the relevant diseases and biological functions of miR-139-3p were analyzed and the results revealed 5 significant functions (Table 1). Of these functions, *Cell Death and Survival* was the highest rated function with the p-value of $7.88E-8-1.75E-2$. Among *Cell Death and Survival*, *cell death* and *apoptosis* were identified as the top 2 significant functions (Table 2).

Potential mechanisms of miR-139-3p-mediated cardiac hypertrophy and cardiomyocyte apoptosis

Signal transduction pathways associated with the target genes of miR-139-3p were next investigated. IPA analysis showed that the target gene *MAPK1* participates in NFAT-mediated cardiac hypertrophy (Fig. 4). Moreover, GeneGo analysis revealed that *MAPK1* mainly participates in 90 signaling pathways, of which 4 signaling pathways are linked to apoptosis and survival (Table 3). Among these, MEK/Erk/Bad signaling plays an integral role in apoptosis.

DISCUSSION

In the present study, we identified 29 miRNAs that were deregulated in the left ventricular myocardium of 2K1C rats compared with normal tissues, of which 11 miRNAs were upregulated and 18 were downregulated. Additionally, miRNA expressions were further analyzed by transforming

Table 3. MAPK1-mediated apoptosis and survival pathways obtained from GeneGo.

Description	Object list
Anti-apoptotic action of membrane-bound ESR1	Cytochrome c, eNOS, ASK1 (MAP3K5), PDK (PDPK1), MEK1 (MAP2K1), MEK2 (MAP2K2), PKC-epsilon, ESR1 (membrane), PKC-alpha, Nitric Oxide, Bcl-2, 1.14.13.39, 17beta-Estradiol, PI3K cat class IA, c-Src, PtdIns(4,5)P2, Erk (MAPK1/3), Ca(2) cytosol, JNK (MAPK8-10), p90RSK1, Ca(2+) extracellular region, BAD, (L)-arginine, c-Raf-1, PtdIns(3,4,5)P3, PI3K reg class IA (p85), L-type Ca(II) channel, alpha 1C subunit, (S)-citrulline, Bax, Ca(2+) = Ca(2+), 2.7.1.137, Bcl-XL, AKT(PKB), CREB1, MEK4 (MAP2K4)
Anti-apoptotic action of nuclear ESR1 and ESR2	4.6.1.2, MEK3 (MAP2K3), c-Myc, Nitric Oxide, SOD2, p38alpha (MAPK14), O(2)(-), nNOS, Erk (MAPK1/3), Cytochrome c, Bcl-2, ESR2, H(+), ASK1 (MAP3K5), O(2), c-Jun/c-Fos, MEK4 (MAP2K4), 1.14.13.39, c-Raf-1, H(2)O(2) cytoplasm, JNK1 (MAPK8), NF-kB p50/p65, Bax, Thioredoxin, (S)-citrulline, MEK1(MAP2K1), ESR1 (nuclear), 1.15.1.1, GTP, (L)-Arginine, c-Jun, Cyclic GMP cytosol, Protein kinase G1 alpha, Guanylate cyclase 1, soluble, Estradiol cytoplasm, MEK2(MAP2K2), NF-kB1 (p50)
Role of CDK5 in neuronal death and survival	MEK1(MAP2K1), PI3K reg class IA (p85), JNK3(MAPK10), Bcl-2, PDK (PDPK1), Apaf-1, AKT, Erk (MAPK1/3), ErbB3, TrkA, c-Jun, CDK5R1 (p35), PI3K cat class IA, ErbB2, Caspase-3, PtdIns(4,5)P2, NGE, PtdIns(3,4,5)P3, Caspase-9, Cytochrome c, PKC-delta, p53, SOS, Neuregulin 1, Shc, Bax, H-Ras, BAD, NGFR(TNFRSF16), CDK1 (p34), c-Raf-1, 2.7.1.137, CDK5, EGR1
BAD phosphorylation	p90Rsk, MEK2(MAP2K2), IGF-1 receptor, 4.6.1.1, IRS-1, PP2A catalytic, PI3K reg class IA, MEK1(MAP2K1), p70 S6 kinase1, Adenylate cyclase type I, BAD, 14-3-3, p70 S6 kinase2, Shc, PtdIns(4,5)P2, Cytochrome c, Bcl-XL, Erk (MAPK1/3), Calcineurin A (catalytic), PP2C, PI3K cat class IA, PP1-cat alpha, GRB2, AKT(PKB), PDK (PDPK1), G-protein beta/gamma, PtdIns(3,4,5)P3, SOS, ATP cytosol, PKA-cat (cAMP-dependent), Bax, EGFR, PKA-reg (cAMP-dependent), BAD, c-Raf-1, Bcl-2, CDK1 (p34), 2.7.1.137, H-Ras, cAMP, JNK1(MAPK8), G-protein alpha-s

to “fold change”. Out of the 11 miRNAs that were upregulated in our study, 6 miRNAs (rno-miR-3584-5p, rno-miR-132, rno-miR-345-3p, rno-miR-136, rno-miR-3588, and rno-miR-31*) were expressed at least 3-fold, and 5 miRNAs (rno-miR-20a*, rno-miR-32*, rno-miR-3562, rno-miR-338*, and rno-miR-139-3p) were expressed at least 5-fold. Out of 18 miRNAs that showed decreased expressions in our study, 11 miRNAs (rno-miR-582, rno-miR-872*, rno-miR-425*, rno-miR-434, rno-miR-363, rno-miR-122, rno-miR-362, rno-miR-384-5p, rno-miR-455, rno-miR-224*, and rno-miR-141) were downregulated at least 3-fold, and 7 miRNAs (rno-miR-127, rno-miR-505*, rno-miR-92b, rno-miR-129-1*, rno-miR-129-2*, rno-miR-224, and rno-miR-139-5p) were downregulated at least 6-fold. These results demonstrated that aberrant expression of miRNAs is involved in cardiac remodeling. The expression profiles of miRNAs in our study were not entirely consistent with previously

reported results focusing on aberrant miRNAs in 2K1C rats (Sun et al., 2013). In our previous work, a ± 2 -fold change cut-off was applied to all array datasets. However, a ± 3 -fold change cut-off was applied in our current work. We consider this to be due to differences in the screening criteria.

In addition to identifying the aberrant miRNAs in 2K1C rats, certain miRNAs that might have potential clinical significance as reported elsewhere were found to be differentially expressed in the left ventricular myocardium from 2K1C rats. For example, it has been reported that miR-139-3p was differentially expressed in cancers of different stages and grades (Chen et al., 2012). In this study, miR-139-3p was found to be highly expressed in 2K1C rats. These findings indicate that deregulation of miR-139-3p might affect the molecular events in the progression of cardiac hypertrophy.

In order to understand the roles of miR-139-3p in cardiac hypertrophy of hypertensive rats, the target genes and mechanism of regulation were further exploration. With IPA we identified that *MAPK1* was the validated target gene of miR-139-3p. Additionally, miR-139-3p and its target gene *MAPK1* were involved in “Cell Death and

Survival” as suggested by IPA functional analysis. Furthermore, IPA analysis revealed cell death and apoptosis to be the most favored associated functions in cell death and survival. Therefore, miR-139-3p might play a key role in cell death and survival depending on which gene it is targeting during cardiac hypertrophy in hypertensive rats.

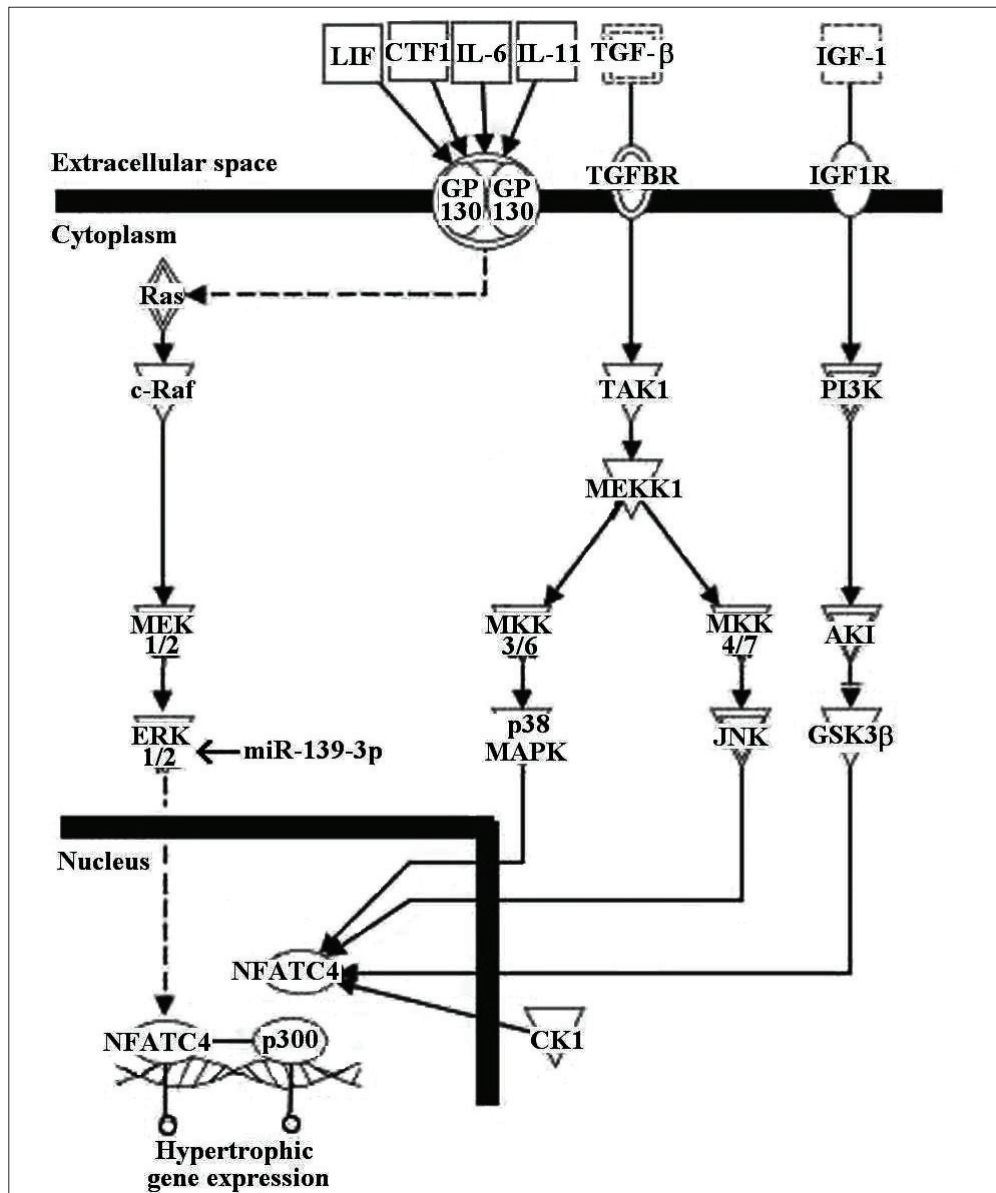


Fig. 4. The cardiac hypertrophy signaling pathway obtained from IPA analysis. *MAPK1* (also known as *ERK*), a target gene of miR-139-3p, participate in NFAT- mediated cardiac hypertrophy.

Moreover, signal transduction pathways associated with miR-139-3p and its target gene *MAPK1* were investigated. Results from the IPA analysis revealed that the target gene participated in the NFATC4-mediated signaling pathway, which is crucial for cardiac hypertrophy as described previously (Mathew et al., 2004). Results from the GeneGo analysis revealed that *MAPK1* could participate in 90 signaling pathways. Since miR-139-3p and its validated target gene *MAPK1* were involved in cell death and survival as suggested by IPA functional analysis, one result to arouse great interest was that miR-139-3p modulated the apoptosis and survival pathways (Table 3). The results showed that MEK/Erk/Bad signaling was a component of these four signaling pathways linked to apoptosis and survival, and regulated apoptosis through anti-apoptotic Bcl-2 family members (Eisenmann et al., 2003). It has been reported that the development of left ventricular hypertrophy and the level of apoptosis in spontaneously hypertensive rats could be modulated by Bcl-2 family proteins (Jiang et al., 2013). Therefore, we have reason to consider that miR-139-3p might modulate cardiomyocyte apoptosis in 2K1C rats through the affected MEK/Erk/Bad pathway.

In conclusion, the results of the present study suggest that miR-139-3p may play a key role in the left ventricular remodeling process. Analysis of its target gene and signaling pathways may add a new mechanism to its roles. However, further studies are required to validate this. The findings of the present study have expanded our knowledge of the molecular alterations involved in heart pathogenesis and the role of miR-139-3p in left ventricular hypertrophy and cardiomyocyte apoptosis.

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Conflict of interest disclosure: The authors declare no conflicts of interest.

REFERENCES

- Barnes, V.A., Kapuku, G.K. and F.A. Treiber (2012). Impact of transcendental meditation on left ventricular mass in African American adolescents. *Evid. Based Complement. Alternat. Med.* **2012**: 923153.
- Chen, W.C., Lin, M.S., Ye, Y.L., Gao, H.J., Song, Z.Y. and X.Y. Shen (2012). microRNA expression pattern and its alteration following celecoxib intervention in human colorectal cancer. *Exp. Ther. Med.* **3**:1039-1048.
- Cheng, Y. and C. Zhang (2010). MicroRNA-21 in cardiovascular disease. *J. Cardiovasc. Transl. Res.* **3**: 251-255.
- Chien, K.R. (1999). Stress pathways and heart failure. *Cell.* **98**: 555-558.
- Eisenmann, K.M., VanBrocklin, M.W., Staffend, N.A., Kitchen, S.M. and H.M. Koo (2003). Mitogen-activated protein kinase pathway-dependent tumor-specific survival signaling in melanoma cells through inactivation of the proapoptotic protein bad. *Cancer Res.* **63**: 8330-8337.
- Gu, W., Li, X. and J. Wang (2014). miR-139 regulates the proliferation and invasion of hepatocellular carcinoma through the WNT/TCF-4 pathway. *Oncol. Rep.* **31**: 397-404.
- Jiang, F.L., Leo, S., Wang, X.G., Li, H., Gong, L.Y., Kuang, Y. and X.F. Xu (2013). Effect of tanshinone IIA on cardiomyocyte hypertrophy and apoptosis in spontaneously hypertensive rats. *Exp. Ther. Med.* **6**: 1517-1521.
- Kirchhoff, S.R., Gupta, S. and A.A. Knowlton (2002). Cytosolic HSP60, apoptosis, and myocardial injury. *Circulation.* **105**: 2899-2904.
- Lecellier, C.H., Dunoyer, P., Arar, K., Lehmann-Che, J., Eyquem, S., Himber, C., Saïb A and O.Voinnet (2005). A cellular microRNA mediates antiviral defense in human cells. *Science.* **308**: 557-560.

- Li, P. (2010). MicroRNAs in cardiac apoptosis. *J. Cardiovasc. Transl. Res.* **3**: 219-224.
- Li, R.Y., Chen, L.C., Zhang, H.Y., Du, W.Z., Feng, Y., Wang, H.B., Wen, J.Q., Liu, X., Li, X.F., Sun, Y., Yang, D.B., Jiang, T., Li, Y.L. and C.L. Jiang (2013). MiR-139 inhibits Mcl-1 expression and potentiates TMZ-induced apoptosis in glioma. *CNS Neurosci. Ther.* **19**: 477-483.
- Lin, M., Chen, W., Huang, J., Gao, H., Ye, Y., Song, Z. and X. Shen (2011). MicroRNA expression profiles in human colorectal cancers with liver metastases. *Oncol. Rep.* **25**: 739-747.
- Mathew, S., Mascareno, E. and M.A. Siddiqui (2004). A ternary complex of transcription factors, Nishéd and NFATc4, and co-activator p300 bound to an intronic sequence, intronic regulatory element, is pivotal for the up-regulation of myosin light chain-2v gene in cardiac hypertrophy. *J. Biol. Chem.* **279**: 41018-41027.
- Putinski, C., Abdul-Ghani, M., Stiles, R., Brunette, S., Dick, S.A., Fernando, P. and L.A. Megeney (2013). Intrinsic-mediated caspase activation is essential for cardiomyocyte hypertrophy. *Proc. Natl. Acad. Sci. U S A.* **110**: E4079-E4087.
- Sun, G., Hu, H., Tian, X., Yue, J., Yu, H., Yang, X. and Z. Wang (2013). Identification and analysis of microRNAs in the left ventricular myocardium of two-kidney one-clip hypertensive rats. *Mol. Med. Rep.* **8**: 339-344.
- Wang, Z., Luo, X., Lu, Y. and B. Yang (2008). miRNAs at the heart of the matter. *J. Mol. Med. (Berl)*. **86**: 771-783.
- Zhu, H., and G.C. Fan (2012). Role of microRNAs in the reperfused myocardium towards post-infarct remodelling. *Cardiovasc. Res.* **94**: 284-292.