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Original Article

MicroRNA 218 modulates PKC/AKT pathway to protect lidocaine-induced neurotoxicity in ganglia *in vitro*

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Abstract: Objectives: Application of local anesthetics such as lidocaine may induce neurotoxicity in peripheral sensory neurons of dorsal root ganglia (DRG). The present study examined the effect of microRNA 218 (miR-218) expression in the process of local anesthetic-induced neurotoxicity. Methods: DRG neurons from 8-week-old adult C57BL/6 mice were cultured in vitro. Lidocaine was applied to induce apoptosis. The expression level of miR-218 was assessed by quantitative real-time polymerase chain reaction (qRT-PCR). Lentiviral vector was applied to silence miR-218 in DRG neurons. The protective effect of miR-218 knockdown against lidocaine-induced neurotoxicity was examined by TUNEL assay. Western blotting analysis was used to assess the activation of PKC/AKT signaling pathway. Results: The application of lidocaine significantly induced the apoptosis and increased the expression of miR-218 in DRG neurons, both in concentration-dependent manners. Inhibition of miR-218 reduced lidocaine-induced apoptosis in DRG neurons. Western blotting analysis demonstrated that PKC/AKT signaling pathway was activated after inhibiton of miR-218 during the process of lidocaine-induced neurotoxicity. Furthermore, siRNA-mediated AKT inhibition reversed the protective effect of miR-218 downregulation on lidocaine-induced apoptosis of DRG neurons. Conclusions: Our study clearly demonstrated that miR-218 played an active role in regulating lidocaine-induced neurotoxicity in DRG neurons, partly through the regulation of AKT. Inhibition of miR-128 may provide a potential therapeutic target to protect local anesthetic-induced neurotoxicity.

Keywords: Lidocaine, miR-128, local anesthesia, neurotoxicity, AKT

Introduction

In recent decades, growing evidence, both clinically and experimentally, has shown that local administration of anesthetics could induce severe or even irreversible neurotoxicity in peripheral neurons including dorsal root ganglion (DRG) neurons [1-4]. Especially while local anesthetics were applied near growing neurons under the circumstance of chemical or mechanical injury in patients, their long-term neurotoxic effect on those growing neurons could not be ignored in clinical practice. Thus, it is of great interest toto elucidate the underlying mechanisms of local anesthetic-induced neurotoxicity, as well as to develop potential clinical strategies to rescue or protect peripheral neurons from neurotoxicity induced by local application of anesthetics.

MicroRNAs (miRNAs) are groups of endogenous, non-coding small RNA typically containing 21-23 nucleotides that modulate gene expression by binding to the 3'-untranslated region (3'-UTR) of their target mRNAs, resulting in their post-translational inhibition or cleavage [5, 6]. Various miRNAs have been found in the peripheral nervous system, including eye, ear, spinal cord and DRG, where they play critical roles in regulating neurogenesis, neural development and neural pathology [7-11]. Recently, several studies have shown that miRNA plays important roles in development and local injury of DRGs [12, 13]. However, the involvement of miRNAs during the process of anesthesia-induced neurotoxicity in DRG remains poorly understood and requires further study.

Thus, To further explore the underlying mechanisms of miRNAs in regulating neurotoxicity in DRG induced by local anesthetics, one of the important neuronal miRNAs, microRNA 218 (mR-218), was investigated for its endogenous

regulatory role during DRG neurotoxicity. Also, we set to investigate whether miR-218 is capable of modulating lidocaine-induced neural apoptosis in DRG and the molecular pathways associated with miR-218 regulation during the process of local anesthesia induced DRG neurotoxicity.

Materials and methods

In vitro culture of DRG cells

DRG were extracted from adult male C57BL/6 mice (8 weeks old), and immediately immersed in F-12 culture medium (Invitrogen, Carlsbad, CA, USA) containing 2 mg/ml collagenase incubated in a humidified chamber at 37°C and 5% CO_2 for 2 hours. Ganglia were treated with 0.05% trypsin (Invitrogen, Carlsbad, CA, USA) for 15 min at 37°C, and then triturated with a glass pipette. The dissociated DRGs were seeded into 6-well plates in F-12 medium supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin (P/S, 100 μg/ml). All experiments followed the National Institute of Health Guidelines for the Care and Use of Laboratory Animals and were approved by the committee for Animal Experiments of Sir Run Run Shaw Hospital, School of Medicine, Zhejiang University.

Lidocaine treatment

To introduce toxicity in DRGs, various concentrations of lidocaine solutions (1-100 μM), were added into each well after seeding for 2 hours. After quick wash of HBSS medium (Gibco, Grand Island, NY, USA). DRGs were cultured in fresh F-12 medium with FBS and P/S for another 24 hours before further examination.

TUNEL staining assay

DRGs were fixed by 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS), mixed with 0.3% Triton X-100 for 30 min. Apoptosis was measured by a terminal deoxyribonucleotidyl transferase (TdT)-mediated biotin-16-dUTP nick-end labeling (TUNEL) assay using an In-Situ Apoptosis Detection Kit (Chemicon, Carlsbad, CA, USA). A neuronal specific nuclei antibody NeuN (1:1000, Cell Signaling, USA) was used to identify DRGs in the culture. Imaging was taken on an Olympus BH51 fluorescence microscope (Olympus, Japan). To quantify DRG apoptosis, 5 visual areas (100 × 100 μM) were randomly selected for each well and the percentage of TUNEL-positive DRGs (TUNEL against NeuN) were averaged for each experimental condition.

Quantitative real-time reverse transcription-PCR (qRT-PCR)

DRGs in culture were collected and RNAs were extracted using a Trizol RNA purification Kit (Qiagen, Carlsbad, CA, USA) according to the manufacturer's protocol. Total RNA was examined by a NanoDrop ND-3000 spectrophotometer (NanoDrop, Carlsbad, CA, USA) at 260 and 280 nm (A260/280), and analyzed with an Agilent Bioanalyzer 4100 system (Agilent, UK). QRT-PCR to probe miR-218 was conducted by a TaqMan miRNA Assay with Mm_miR-218 miScript Primers according to the manufacturer's protocol (Applied Biosystems, Foster City, CA, USA).

MicroRNA 218 downregulation in DRG

In order to down-regulate endogenous murine miR-218 in DRGs, synthetic mouse miRNA inhibitor oligonucleotide against mmu-miR-218 (miR218-I) (BiboBio, Guangzhou, China) were transfected into cells using Lipofectamine™ 2000 (Invitrogen) following the manufacturer's instructions., along with a non-specific control miRNA oligonucleotide (miR-C). On the experimental day, 1 μM miR218-I or miR-C was added into DRG culture 6 hours before lidocaine application.

Western blot analysis

DRGs were collected from 6-well plates. Cell lysates were prepared with a lysis buffer containing 50 mM Tris (pH 7.6), 150 mM NaCl, 1 mM EDTA, 10% glycerol, and 0.5% NP-40 and protease inhibitor cocktail (Invitrogen, Carlsbad, CA, USA). Total proteins extracted from each group of cells were determined by a BCA protein assay (Pierce, USA) and then separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a PVDF membrane (Bio-Rad, USA). After the application of a block buffer for 1 hour at room temperature, the membranes were incubated with primary antibodies against PI3k3/serine-threonine kinase (AKT, 1:2000 dilution, Cell Signaling Technology, USA), phosphor-AKT

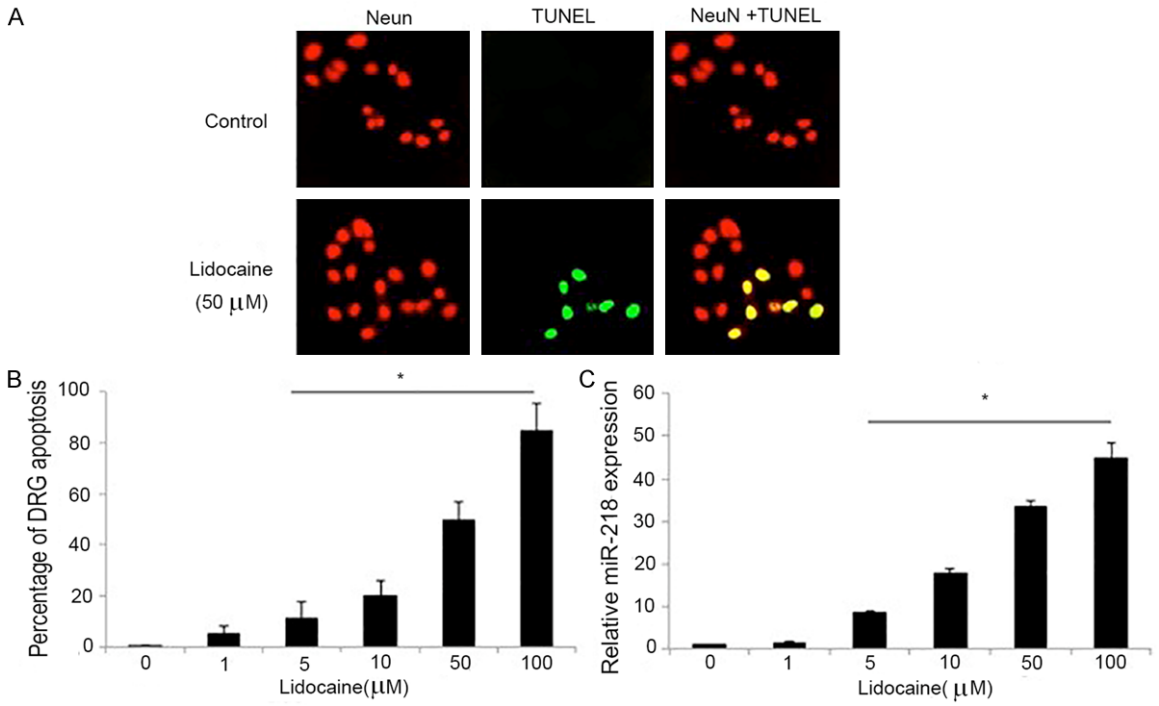

Figure 1. Lidocaine induced apoptosis and upregulated miR-218 in mouse DRG neurons *in vitro*. DRG neurons were extracted from 8-week-old C57BL/6 mice and cultured *in vitro*. Various concentrations of lidocaine were added into culture for 2 hours. After that, DRG neurons were cultured with fresh medium for another 24 hours, followed by TUNEL assay. A. Representative fluorescent images were shown for the untreated (control) and lidocaine-treated (lidocaine, 50 mM) DRG neurons. A neuronal nuclei marker (NeuN, Red) was used to identify DRG neurons in the culture, along with TUNEL staining (Green) to identify apoptotic DRG neurons. B. The percentage of apoptotic DRG neurons in response to various concentrations of lidocaine was evaluated (*$P < 0.05$, compared to 0 mM lidocaine). C. The mRNA expression levels of miR-218 in response to various concentrations of lidocaine were evaluated by quantitative RT-PCR (*$P < 0.05$, compared to 0 mM lidocaine).

(pAKT, 1:200 dilution, Cell Signaling Technology, USA), protein kinase C (PKC, 1:2000 dilution, Cell Signaling Technology, USA), phosphorylated PKC (pPKC, 1:200 dilution, Cell Signaling Technology, USA) and β-actin (1:2000 dilution; Cell Signaling Technology, USA) overnight at 4°C. The blots were then washed and incubated with horseradish peroxidase conjugated secondary antibody (Cell Signaling Technology, USA) overnight at 4°C. The immune complex was detected by an enhanced chemiluminesence system (Amersham Biosciences, USA). The band intensities on the film were quantitatively analyzed by densitometry with a computer-assisted imaging analysis system (ImageJ, NIH, USA).

SiRNA transfection

The gene-silencing siRNA to knock down AKT (AKT-siRNA), along its non-specific control siRNA (C-siRNA) were purchased from RiboBio (BiboBio, Guangzhou, China). The transfection of siRNAs (100 nM) in DRG culture was applied along with a Lipofectamine 2000 reagent (Sigma Aldrich, USA) according to the manufacturer's protocol. The efficiency of siRNA was examined by Western blotting 24 hours after transfection.

Statistical analysis

All data in the present study were shown as the mean ± S.E.M. Statistical comparison was made by one-way ANOVA followed by student's t-test using SPSS software 12.0 (SPSS, Chicago, Illinois, USA). At the same time, we used a two-way ANOVA as statistical analysis method to analyse the effects of lidocaine concentration and exposure time on DRG cell viability. Statistical significance was characterized if $P < 0.05$. All experiments were repeated at least three times.

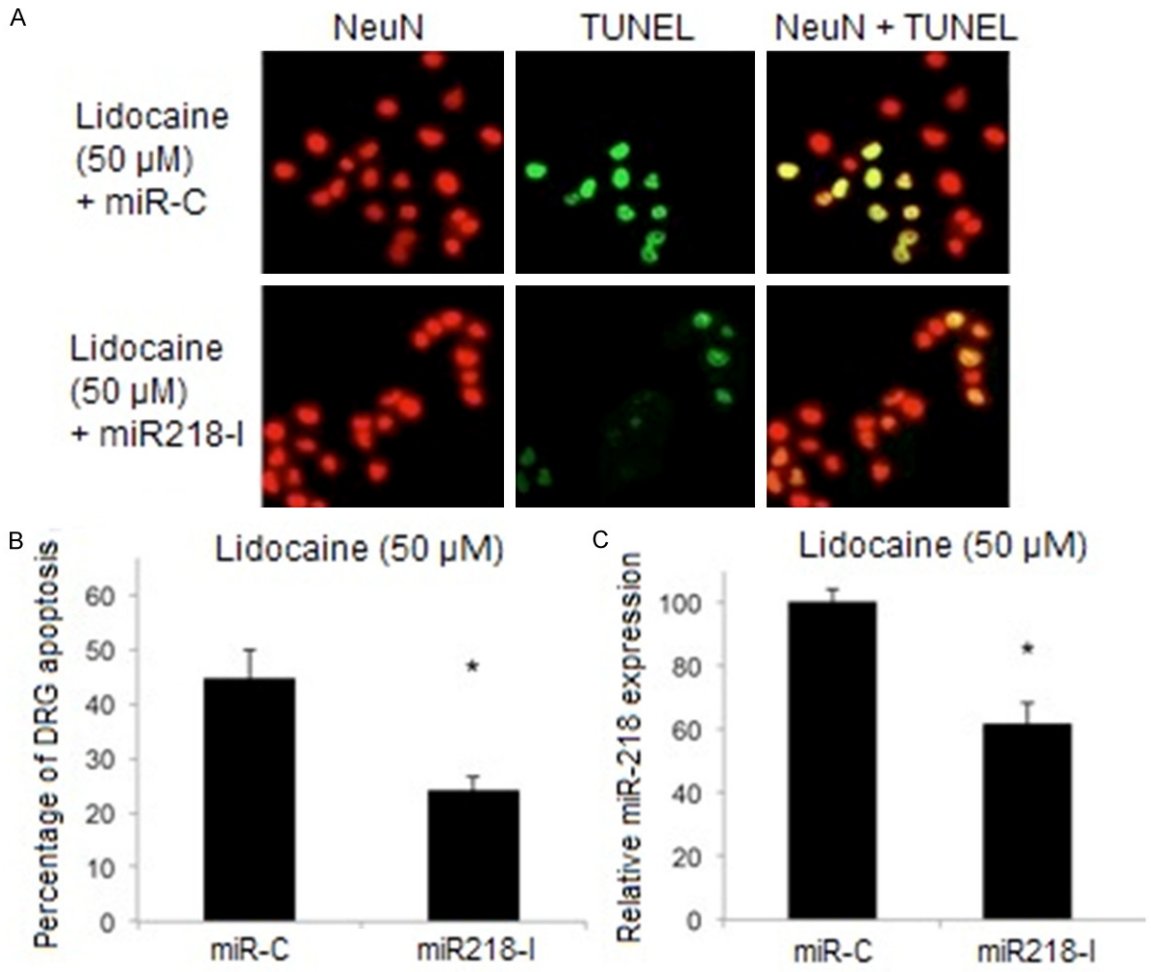

Figure 2. MiR-218 Downregulation protected lidocaine-induced apoptosis in mouse DRG neurons *in vitro*. Cultured mouse DRG neurons were pre-treated with miR218-inhibitor oligonucleotide (miR218-I, 1 mM), or a non-specific miRNA oligonucleotide (miR-NC, 1 mM) for 6 hours, followed by 2-hour treatment of lidocaine (50 mM). The neurons were then washed and cultured with fresh medium for another 24 hours. A. TUNEL staining (Green), along with NeuN staining (Red), was performed for the DRG neurons pre-treated with miR-NC, or miR218-I. B. The percentages of apoptotic DRG neurons were compared between the neurons treated with miR-C and those treated with miR218-I (*$P < 0.05$). C. The specificity of lentivirus on down-regulating endogenous miR-218 was measured by qRT-PCR (*$P < 0.05$).

Results

Lidocaine induced apoptosis and miR-218 upregulation in mouse DRG cells in vitro

Lidocaine has been shown to induce neurotoxicity during local anesthesia [2, 4]. Specifically, lidocaine inhibited neurite growth and collapsed growth cones of DRG neurons in culture [14, 15]. Thus, we firstly examined whether lidocaine could induce cellular apoptosis in cultured adult mouse DRG neurons and affect the miR-218 expression.

We cultured DRG neurons from adult C57BL/6 mice and treated them with different concentrations of lidocaine (0~100 μM) for 2 hours. We found that lidocaine (50 μM) induced significant apoptosis (TUNEL staining, green) in DRG neurons (NeuN staining, red) (**Figure 1A**). A quantitative measurement with various concentrations demonstrated that significant apoptosis in DRG neurons was induced by lidocaine in a concentration-dependent manner from 5 μM to 100 μM (**Figure 1B**, *$P < 0.05$). Additionally, we found that miR-218 expression was significantly upregulated, also in a lido-

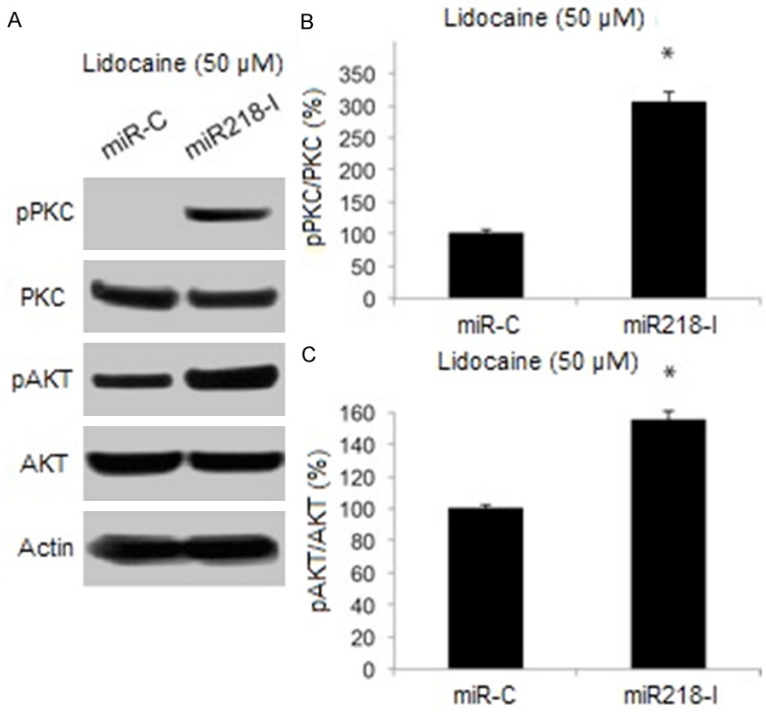

Figure 3. MiR-218 Downregulation activated PKC/AKT pathways in mouse DRG neurons *in vitro*. DRG neurons were pre-treated with miR218-I (1 mM), or miR-C (1 mM) for 6 hours, followed by lidocaine (50 mM) treatment for 2 hours and additional culture with fresh medium for 24 hours. (A) Western blot was conducted to evaluate the protein expression levels of AKT, phospho-AKT (pAKT), PKC and phosphorylated-TrkB (pPKC), in DRG neurons while they were pre-treated with miR218-I or miR-C before lidocaine treatment. Also, quantitative measurement was conducted to compare the phosphorylation levels of PKC (B) and AKT (C) (*$P < 0.05$).

caine-concentration dependent manner in DRG neurons *in vitro* (**Figure 1C**, *$P < 0.05$).

MiR-218 downregulation rescued lidocaine-induced apoptosis

The effect of miR-218 on lidocaine-induced apoptosis was investigated by transfection of the miR-218 inhibitor into DRG neurons. We found that, lidocaine-induced apoptosis was reduced by miR218 down-regulation (**Figure 2A**). Quantitative measurement of the percentage of apoptotic DRG neurons confirmed this observation by showing significantly less apoptosis in DRG neurons pre-treated with miR218-I, as compared to the neurons pre-treated with miR-C (**Figure 2B**, *$P < 0.05$).

To evaluate the specificity of miR-218 inhibitor, we performed qRT-PCR to measure the mRNA expression levels of miR-218. We found that miR218-I specifically and efficiently knocked down the expression of endogenous miRNA-218 in DRG neurons (**Figure 2C**, *$P < 0.05$), which suggested that miR-218 played a critical role in lidocaine-induced apoptosis in DRG neurons.

Inhibition of miR-218 up-regulated PKC/AKT signaling pathways

As we discovered that miR-218 had a functional role in regulating lidocaine-induced apoptosis in mouse DRG neurons, we further explored the underlying molecular signaling pathways involved in this process. Western blotting assay was applied to evaluate the activation of PKC/AKT signaling pathways in DRG neurons treated with miR-C or miR218-I. We found no significant changes of total protein expression levels of PKC or AKT after inhibition of miR-218 (**Figure 3A**). However, a significant increase of the phosphorylation of PKC and AKT was found (**Figure 3B** and **3C**, *$P < 0.05$). Thus, our results suggested that miR-218 downregulation activated PKC/AKT pathways during the process of lidocaine-induced apoptosis in mouse DRG neurons.

AKT signaling pathway was involved in miR-218 regulation in mouse DRG neurons in vitro

Finally, we explored whether AKT pathway was directly involved in miR-218 induced protection against apoptosis in DRG neurons. To test this hypothesis, we applied AKT-specific siRNA to down-regulate endogenous AKT expression in DRG neurons. We found that AKT protein was specifically down-regulated by AKT-siRNA (**Figure 4A**). We then examined the effect of AKT downregulation on miR-218 protection on lidocaine-induced apoptosis in DRG neurons. First, we treated DRG neurons with either 100 nM C_siRNA or 100 nM AKT_siRNA for 24 hours. Then, we treated DRG neurons with 1 μM miR218-I for 6 hours, followed by 2-hour

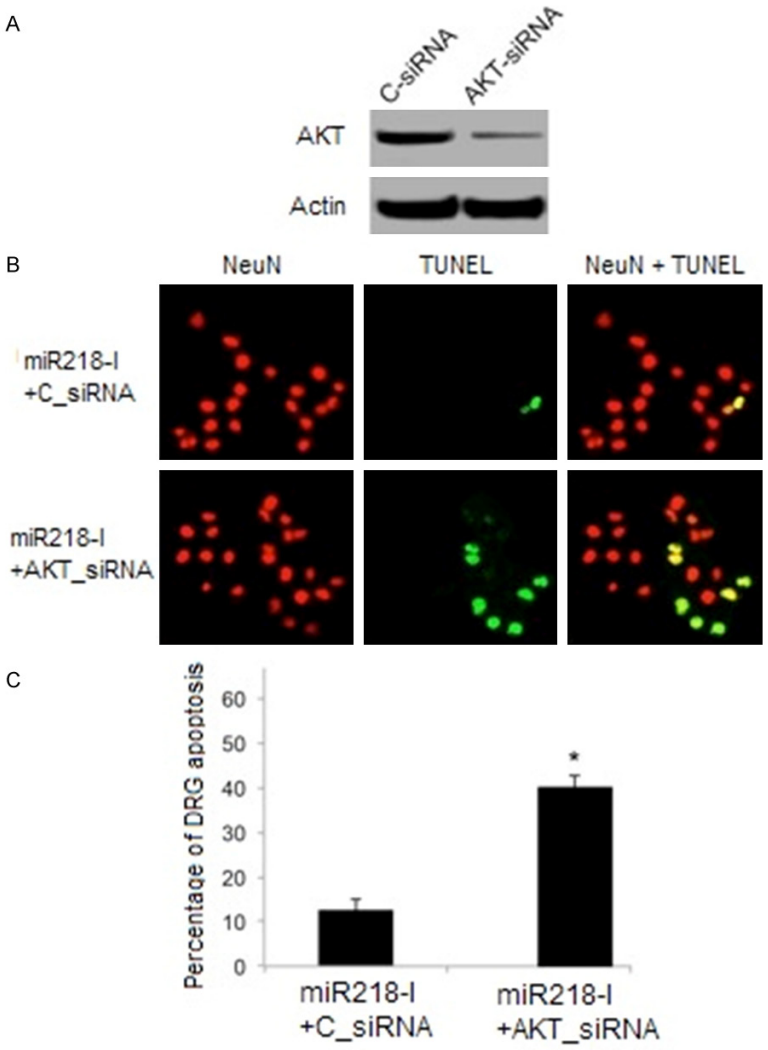

Figure 4. AKT pathway was involved in miR-218 regulation in mouse DRG neurons *in vitro*. A. DRG neurons were treated with AKT specific siRNA (AKT_siRNA, 100 nM) or non-specific siRNA (C_siRNA, 100 nM) for 24 hours, followed by western blotting to examine the efficiency of siRNA assay. B. DRG neurons were pre-treated with AKT_siRNA (100 nM) or C_siRNA (100 nM) for 24 hours, followed by 6-hour treatment of miR218-I (1 mM) and 2-hour treatment of lidocaine (50 mM). TUNEL assay was conducted after DRG neurons were cultured for another 24 hours with fresh medium. C. The percentage of apoptotic DRG neurons were compared between those pre-treated with C_siRNA or AKT_siRNA (*$P < 0.05$).

lidocaine (50 μM) treatment and additional 24-hour culture with fresh medium. Finally, we found that AKT down-regulation reversed the protective effect of miR-218 on lidocaine induced apoptosis (**Figure 4B**). This observation was then confirmed by quantitative measurement showing that the percentage of apoptotic DRG neurons was significantly increased after AKT down-regulation in DRG neurons (**Figure 4C**, *$P < 0.05$).

Thus, our results suggested that miR-218 protect against lidocaine induced apoptosis via AKT signaling pathway in mouse DRG neurons *in vitro*.

Discussions

Local application of anesthetics, such as lidocaine, near the site of DRG may lead to permanent and irrepressible neurotoxicity [2]. In the present study, we cultured adult mouse DRGs and induced neuronal apoptosis by lidocaine *in vitro*. We demonstrated that both the percentage of apoptotic DRG neurons and endogenous miR-218 expressions were up-regulated by lidocaine in dose-dependent manner. MiR-218 has been shown to be an active tumor suppressor in regulating carcinoma apoptosis in many forms of human cancers [16-18]. Also, miR-218 was suggested to be involved in sciatic nerve injury in the spinal cord [18]. Up to date, however, little is known about whether miR-218 is directly involved in the neural or toxic injury to the spinal cord nerve population, including DRG. Thus, our results showed that miR-218 expression was modulated by anesthesia-induce neurotoxicity in DRG. Most importantly, we demonstrated that, miR-218 down-regulation rescued DRGs from lidocaine-induced neural apoptosis, thus revealing a functional role of miR-218 during the process of anesthesia-induced neurotoxicity. In human cancers, miR-218 is normally acting as a tumor suppressor and its overexpression targets many of the apoptosis associated pathways, such as mTOR in oral cancer [18] and NF-KappB in malignant glioma [19], to induce cancer cell apoptosis. Thus, the findings of our results showing miR-218 was up-regulated during apoptosis, and its

subsequent down-regulation rescued apoptosis in DRG are consistent with the mechanism of miR-218 in cancer regulation, suggesting that miR-218 is an apoptosis-associated miRNA in both DRG and cancer cells.

Also in the present study, we demonstrated that miR-218 downregulation activated PKC and AKT singling pathways. Further evidence showed that siRNA-mediated AKT inhibition was directly involved in the protection on apoptosis by miR-218 down-regulation. This is also consistent with other studies showing that miR-218 directly regulation AKT pathways to affect apoptosis in cervical and oral cancers [18, 20]. Thus, it is very likely that similar down-stream molecular pathways are involved in miR-218 regulation in both DRG and cancer.

Overall, our study identified a novel regulator, miR-218 in modulating anesthesia-induced neurotoxicity in DRG neurons. Down regulation of miR-218 may help to further our understanding on the underlying mechanisms of miRNA regulation in anesthesia-induced neurotoxicity in peripheral nervous system.

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Disclosure of conflict of interest

None.

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References

[1] Sakura S, Kirihara Y, Muguruma T, Kishimoto T and Saito Y. The comparative neurotoxicity of intrathecal lidocaine and bupivacaine in rats. Anesth Analg 2005; 101: 541-547.

[2] Radwan IA, Saito S and Goto F. The neurotoxicity of local anesthetics on growing neurons: a comparative study of lidocaine, bupivacaine, mepivacaine, and ropivacaine. Anesth Analg 2002; 94: 319-324.

[3] Johnson ME. Potential neurotoxicity of spinal anesthesia with lidocaine. Mayo Clin Proc 2000; 75: 921-932.

[4] Snoeck M. Articaine: a review of its use for local and regional anesthesia. Local Reg Anesth 2012; 5: 23-33.

[5] Kosik KS. The neuronal microRNA system. Nat Rev Neurosci 2006; 7: 911-920.

[6] Pillai RS. MicroRNA function: multiple mechanisms for a tiny RNA? RNA 2005; 11: 1753-1761.

[7] Hua YJ, Tang ZY, Tu K, Zhu L, Li YX, Xie L and Xiao HS. Identification and target prediction of miRNAs specifically expressed in rat neural tissue. BMC Genomics 2009; 10: 214.

[8] Bali KK, Hackenberg M, Lubin A, Kuner R and Devor M. Sources of individual variability: miRNAs that predispose to neuropathic pain identified using genome-wide sequencing. Mol Pain 2014; 10: 22.

[9] Cvekl A and Mitton KP. Epigenetic regulatory mechanisms in vertebrate eye development and disease. Heredity (Edinb) 2010; 105: 135-151.

[10] Friedman LM, Dror AA, Mor E, Tenne T, Toren G, Satoh T, Biesemeier DJ, Shomron N, Fekete DM, Hornstein E and Avraham KB. MicroRNAs are essential for development and function of inner ear hair cells in vertebrates. Proc Natl Acad Sci U S A 2009; 106: 7915-7920.

[11] Lu A, Huang Z, Zhang C, Zhang X, Zhao J, Zhang H, Zhang Q, Wu S and Yi X. Differential expression of microRNAs in dorsal root ganglia after sciatic nerve injury. Neural Regen Res 2014; 9: 1031-1040.

[12] Zhou S, Shen D, Wang Y, Gong L, Tang X, Yu B, Gu X and Ding F. microRNA-222 targeting PTEN promotes neurite outgrowth from adult dorsal root ganglion neurons following sciatic nerve transection. PLoS One 2012; 7: e44768.

[13] Tam Tam S, Bastian I, Zhou XF, Vander Hoek M, Michael MZ, Gibbins IL and Haberberger RV. MicroRNA-143 expression in dorsal root ganglion neurons. Cell Tissue Res 2011; 346: 163-173.

[14] Hiruma H, Maruyama H, Simada ZB, Katakura T, Hoka S, Takenaka T and Kawakami T. Lidocaine inhibits neurite growth in mouse dorsal root ganglion cells in culture. Acta Neurobiol Exp (Wars) 1999; 59: 323-327.

[15] Radwan IA, Saito S and Goto F. Growth cone collapsing effect of lidocaine on DRG neurons is partially reversed by several neurotrophic factors. Anesthesiology 2002; 97: 630-635.

[16] Alajez NM, Lenarduzzi M, Ito E, Hui AB, Shi W, Bruce J, Yue S, Huang SH, Xu W, Waldron J, O'Sullivan B and Liu FF. MiR-218 suppresses nasopharyngeal cancer progression through downregulation of survivin and the SLIT2-ROBO1 pathway. Cancer Res 2011; 71: 2381-2391.

[17] Gao C, Zhang Z, Liu W, Xiao S, Gu W and Lu H. Reduced microRNA-218 expression is associated with high nuclear factor kappa B activation in gastric cancer. Cancer 2010; 116: 41-49.

[18] Uesugi A, Kozaki K, Tsuruta T, Furuta M, Morita K, Imoto I, Omura K and Inazawa J. The tumor suppressive microRNA miR-218 targets the mTOR component Rictor and inhibits AKT phosphorylation in oral cancer. Cancer Res 2011; 71: 5765-5778.

[19] Xia H, Yan Y, Hu M, Wang Y, Wang Y, Dai Y, Chen J, Di G, Chen X and Jiang X. MiR-218 sensitizes glioma cells to apoptosis and inhibits tumorigenicity by regulating ECOP-mediated suppression of NF-kappaB activity. Neuro Oncol 2013; 15: 413-422.

[20] Li J, Ping Z and Ning H. MiR-218 impairs tumor growth and increases chemo-sensitivity to cisplatin in cervical cancer. Int J Mol Sci 2012; 13: 16053-16064.