Original Article

NFKB1 Signalling Activation Contributes to TRPV1 Over-expression via Repressing MiR-375 and MiR-455: a Study on Neuropathic Low Back Pain

(neuropathic pain / transient receptor potential cation channel subfamily V member 1 / chronic LBP / NFKB1)

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Abstract. Transient receptor potential cation channel subfamily V member 1 (TRPV1) has been found over-expressed in low back pain (LBP) patients with neuropathic pain (NP), but the underlying mechanism is still unclear. In the present study, the up-regulation of the TRPV1 protein level in sinuvertebral nerve biopsies from patients with NP was verified by immunoblotting, but the TRPV1 mRNA level was not significantly changed. MiRNAs targeting TRPV1 mRNA were predicted by a bioinformatic tool, and the interactions between the miRNAs and TRPV1 were confirmed by dual luciferase assay. The correlation between NFKB1 signalling and TRPV1 expression was analysed and confirmed by using sNF96.2 cells after lipopolysaccharide stimulation. We found that five out of 18 miRNAs repressed TRPV1 expression, and the levels of miR-375 and miR-455 were negatively correlated with the protein level of TRPV1 in patients with NP. MiR-375 and miR-455 were identified to repress TRPV1 expression via targeting the 3'UTR of TRPV1 mRNA. NFKB1 signalling activation down-regulated the expression of miR-375 and miR-455, and thus up-regulated the TRPV1 protein level. In conclusion, we partially unveiled the mechanism of how TRPV1 is over-expressed in chronic LBP patients with NP and provided two potential candidate miRNAs for NP treatment.

Introduction

Chronic low back pain (LBP) is regarded as LBP that persists over 12 weeks without a significant pain improvement (Baron et al., 2016). Neuropathic pain (NP) is a kind of chronic pain caused by damage or disease affecting the somatosensory nervous system (Costigan et al., 2009). NP is one of the reasons of chronic LBP. Some reports suggest that about 16–55 % of patients with chronic LBP possibly have NP (Hassan et al., 2004; Kaki et al., 2005; Freynhagen et al., 2006; Beith et al., 2011). Unfortunately, NP often responds poorly to standard pain treatments and remains extremely difficult to cure. Thus, unveiling the pathogenesis and identifying suitable molecular targets are emergency for developing efficient methods to treat NP.

Transient receptor potential cation channel subfamily V member 1 (TRPV1) was firstly identified by Caterina et al. as a receptor of capsaicin in 1997 (Caterina et al., 1997). Subsequently, the activation of TRPV1 was also found after exposure to high temperature and bacterial infection (Aneiros et al., 2011; Boonen et al., 2018). TRPV1 is expressed in a wide range of tissues and its functions are related to neurogenic inflammation, neuropathic pain, autoimmune disorders, cancer, and immune cell functioning (Birder et al., 2002; Rehman et al., 2013; Bertin et al., 2014; Han et al., 2016; Malenczyk et al., 2017). Han et al. observed over-expressed TRPV1 in epidermal keratinocytes of patients with postherpetic neuralgia, but the key factors controlling TRPV1 expression are still unknown.

It has been reported that the neuro-immune imbalance plays a role in the pathophysiology of NP. As a key regulator for inflammation and pain, activation of the signalling complex nuclear factor κB (NFKB1) signal-

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Abbreviations: 3'UTR – 3' untranslated region, LBP – low back pain, LPS – lipopolysaccharide, NFKB1 – nuclear factor kappa B subunit 1, NP – neuropathic pain, TRPV1 – transient receptor potential cation channel subfamily V member 1.

ling pathway is related to the pathogenesis of NP. However, whether the activation of NFKB1 signalling pathway is related to TRPV1 over-expression is unknown (Ledeboer et al., 2005; Miyoshi et al., 2008).

MicroRNAs are a group of short non-coding RNAs (ncRNAs) usually repressing gene expression at the post-transcriptional level. MiRNAs are key regulators for maintaining normal human body physiologic conditions, and abnormal miRNA levels have been found to be related to multiple human diseases spanning psychiatric disorders to malignant cancers (Maes et al., 2009; Farazi et al., 2013; Xu et al., 2013). Researchers found that dysregulated miRNAs, such as miR-132, miR-183, miR-96 and miR-30, play important roles in different states of the NP in rat models (Aldrich et al., 2009; Chen et al., 2014; Leinders et al., 2016). Meanwhile, down-regulated miR-199 was identified to augment visceral pain through up-regulating TRPV1 (Zhou et al., 2016).

In the present study, the TRPV1 protein level in 23 chronic LBP patients with NP and 10 no chronic LBP controls was examined. The candidate miRNAs that may directly target TRPV1 were predicted by a bioinformatic tool and confirmed by dual luciferase assay. Two microRNAs were identified to target the 3' untranslated region (UTR) of TRPV1 and were negatively correlated with the protein level of TRPV1. Activated NFKB1 signalling was found to control TRPV1 expression via repressing the level of the miRNAs.

Material and Methods

Patients with chronic LBP were recruited at the Orthopaedics and Plastic Surgery, Wuhan Hanyang Hospital, between June 2015 and February 2021, where they underwent herniated disc surgery, including sinuvertebral nerve biopsy. The study was approved by the Wuhan Hanyang Hospital Ethics Committee, and written informed consent was obtained from all the participants before recruitment. The diagnosis of neuropathy was based on characteristic symptoms and signs in the neurological examination and typical findings in the electrophysiological assessment with standard nerve conduction studies in motor and sensory nerves (Kimura, 1989). Patients with autoimmune, chronic, inflammatory, neoplastic, or psychiatric diseases were excluded.

Neuropathies were classified as painful if the patients reported pain with an intensity of 3 or more on a numeric rating scale (NRS) ranging from 0 to 10; score 0 meaning "no pain". The control group consisted of 10 patients without NP who underwent herniated disc surgery.

RNA extraction and quality determination

Sinuvertebral nerve biopsy was performed in 23 patients with NP and 10 control patients without NP who underwent disc surgery at the Orthopaedics and Plastic Surgery, Wuhan Hanyang Hospital.

Total RNA was extracted from tissue samples using Trizol Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. RNA concentration and purity were determined using a model ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE). Only samples with the absorbance ratios 260 nm/280 nm of ~2.0 and 260 nm/230 nm of 1.9–2.2 were used in the study. Quantitative RT-PCR analysis was used to determine the relative level of the six selected miRNAs. The method of $-\Delta\Delta Ct$ was used to determine the relative level of target genes. U6 small nuclear RNA was used for normalization. Each sample in each group was measured in triplicate and the quantitative RT-PCR was repeated at least three times.

Western blotting

Protein was extracted from tissue or cell samples by RIPA buffer, and then boiled in sodium dodecyl sulphate/β-mercaptoethanol sample buffer for 5 min at 95 °C. Ten micrograms of each sample was loaded into the lanes of 10% polyacrylamide gel separately. Proteins were separated by electrophoresis and then blotted onto a polyvinylidene fluoride membrane (Amersham Pharmacia Biotech, St. Albans, Herts, UK) by electrophoretic transfer. The membrane was firstly blocked by 5 % BSA and then incubated with the particular primary antibody for 2 h at 37 °C. After incubation with the horseradish peroxidase-conjugated secondary antibody, the specific protein-antibody complex was detected by using the ECL kit (Pierce, Appleton, WI). The β-actin signal was used as a total cell lysate loading control. Lamin B1 was used as the loading control for nuclear proteins and α-tubulin was used as the control for cytosol proteins.

Antibody information: rabbit anti-TRPV1 polyclonal antibody (1 : 1000 dilution, ab6166, Abcam, Cambridge, MA), rabbit anti-NFKB1 polyclonal antibody (1 : 3000 dilution, AV38413, Sigma-Aldrich, St. Louis, MO), rabbit anti-α-tubulin polyclonal antibody (1 : 5000 dilution, ab15246, Abcam), mouse anti-lamin B1 monoclonal antibody (1 : 5000 dilution, sc-56144, Santa Cruz Biotechnology Inc., Santa Cruz, CA), mouse anti-β-actin monoclonal antibody (1 : 5000 dilution, sc-8432, Santa Cruz Biotechnology).

Cell culture

HEK293T human embryonic kidney cells and sNF96.2 human malignant peripheral nerve sheath tumour cells were obtained from the American Type Culture Collection (ATCC) and cultured in Dulbecco's modified Eagle's medium (Hyclone, Logan, UT) containing 10 % foetal bovine serum (Hyclone), 100 IU/ml penicillin and 10 mg/ml streptomycin. All cells were maintained at 37 °C under an atmosphere of 5 % CO_2.

Dual luciferase assays

The full length of 1368 bp TRPV1 3'UTR was cloned into a pmirGLO vector (Promega, Madison, WI) to generate a reporter vector, inserting the TRPV1 3'UTR of firefly luciferase. MiRNA mimics and inhibitors were the product of the GenePharma Co., Ltd. (Shanghai,

Table 1. Sequences of synthesized oligonucleotides

Name	Sequence (5' to 3')
miR-7 mimic	UGGAAGACUAGUGAUUUUGUUGUU
miR-17 mimic	CAAAGUGCUUACAGUGCAGGUAG
miR-20 mimic	UAAAGUGCUUAUAGUGCAGGUAG
miR-93 mimic	CAAAGUGCUGUUCGUGCAGGUAG
miR-106 mimic	AAAAGUGCUUACAGUGCAGGUAG
miR-122 mimic	UGGAGUGUGACAAUGGUGUUUG
miR-124 mimic	UAAGGCACGCGGUGAAUGCCAA
miR-186 mimic	CAAAGAAUUCUCCUUUUGGGCU
miR-199 mimic	ACAGUAGUCUGCACAUUGGUUA
miR-214 mimic	ACAGCAGGCACAGACAGGCAGU
miR-217 mimic	UACUGCAUCAGGAACUGAUUGGA
miR-338 mimic	UCCAGCAUCAGUGAUUUUGUUG
miR-342 mimic	UCUCACACAGAAAUCGCACCCGU
miR-375 mimic	UUUGUUCGUUCGGCUCGCGUGA
miR-455 mimic	GCAGUCCAUGGGCAUAUACAC
miR-493 mimic	UUGUACAUGGUAGGCUUUCAUU
miR-543 mimic	AAACAUUCGCGGUGCACUUCUU
miR-655 mimic	AUAAUACAUGGUUAACCUCUUU
NC	UUUGUACUACACAAAAGUACUG
miR-93 inhibitor	CUACCUGCACGAACAGCACUUUG
miR-186 inhibitor	AGCCCAAAAGGAGAAUUCUUUG
miR-214 inhibitor	ACUGCCUGUCUGUGCCUGCUGU
miR-375 inhibitor	UCACGCGAGCCGAACGAACAAA
miR-455 inhibitor	GUGUAUAUGCCCAUGGACUGC
Anti-control	GUGUAUUCGUAUGUUAGCGGU

China; see Table 1). HEK293T cells were seeded in 48-well plates with a density of 5×10^4 per well. Luciferase reporter vectors were co-transfected with the miRNA mimic or inhibitor into HEK293T cells using lipofectamine 2000 (Invitrogen). Forty-eight hours post transfection, cells were harvested and subjected to dual luciferase assay. Each treatment was performed in triplicate in three independent experiments. The results are expressed as relative luciferase activity (firefly luciferase/Renilla luciferase).

Statistical analysis

Statistical analysis was performed using SPSS software version 19.0 (IBM Corp., Armonk, NY) and data are presented as the mean ± standard. Data between two different groups were analysed by the Student's *t*-test. The correlation between miRNAs and TRPV1 protein level was analysed by the χ^2 test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Over-expression of TRPV1 has been found to increase patient sensitivity to pain. To understand whether TRPV1 is over-expressed in chronic LBP patients with NP, the protein level of TRPV1 was firstly detected in the sinuvertebral nerve biopsies from 23 patients with lumbar intervertebral disc herniation and NP (Fig. 1A–D) and 10 controls. We found that 73.9 % (17 out of 23) patients had the TRPV1 protein level above the mean value in controls (Fig. 1E and F), and 56.6 % (13 out of 23) patients had up-regulated TRPV1 mRNA (Fig. 1G). Since the TRPV1 mRNA level was not significantly up-regulated in chronic LBP patients with NP ($P = 0.11$), while the TRPV1 protein level was significantly increased ($P = 0.0062$), we proposed that the TRPV1 up-regulation may be induced by dysregulated miRNAs. To verify our hypothesis, the miRNAs that may target TRPV1 mRNA were firstly predicted by using the online bioinformatic tool TargetScan (https://www.targetscan.org). We found that 18 miRNAs had the potential of targeting TRPV1 mRNA 3'UTR directly.

To confirm which miRNA represses TRPV1 expression directly, a reporter vector containing the full-length TRPV1 3'UTR was constructed. The reporter vector was co-transfected with one of the candidate miRNAs into HEK293T cells, and then luciferase activities were examined 48 hours post transfection. As shown in Fig. 2A, five (miR-93, miR-186, miR-214, miR-375 and miR-455) out of 18 miRNAs significantly repressed the luciferase activity. Meanwhile, the inhibitor of each one of the five miRNAs could up-regulate the luciferase activity (Fig. 2B), indicating that these miRNAs repressed luciferase expression through targeting TRPV1 3'UTR. Subsequently, these five miRNAs were transiently transfected into sNF96.2 cells, and immunoblotting was then used to examine the endogenous TRPV1 level. As shown in Fig. 2C, all of these five miRNAs repressed endogenous TRPV1 significantly. To explore which miRNA repressed TRPV1 in chronic LBP patients, the level of these five candidate miRNAs was firstly investigated in tissue samples. As shown in Fig. 3A, the level of miR-455 was significantly reduced in these patients with NP. Subsequent correlation analysis showed a negative correlation between TRPV1 and miR-455 or miR-375, suggesting that TRPV1 over-expression in LBP NP patients may be induced by the decreased level of miRNA (Fig. 3B).

To further identify the binding sites of miR-455 and miR-375 in the 3'UTR of TRPV1 mRNA, two mutant TRPV1 reporter vectors were generated (Fig. 4A). Dual luciferase assay results indicated that when five nucleotides of the 3'UTR of TRPV1 were mutated, the luciferase activity was not repressed by miR-455 (Fig. 4B). Meanwhile, miR-455 inhibitor and miR-375 inhibitor could up-regulate the endogenous TRPV1 expression, indicating that miR-455 and miR-375 repressed TRPV1 expression via targeting these two target sites of TRPV1 mRNA 3'UTR (Fig. 4C).

It has been reported that NFKB1 signalling is activated in NP patients (Ahmed et al., 2019), while several miRNAs, including miR-375, were found to be up-regulated by NFKB1 inhibitor (Doukas et al., 2018). To

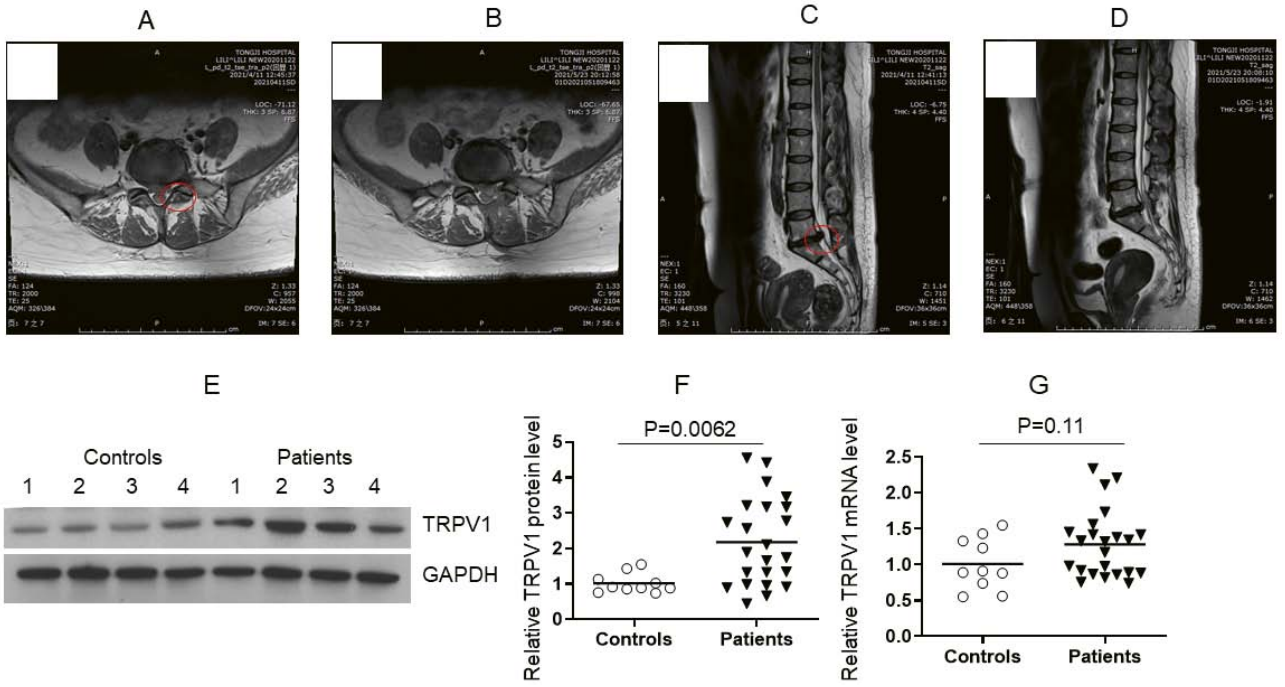

Fig. 1. TRPV1 is over-expressed in chronic LBP patients with NP.
(A) CT image of cross-section of a lumbar vertebra. The herniated disk was circled. The patient's information was masked. **(B)** The same patient's CT image of cross-section of the lumbar vertebra after surgery. The patient's information was masked. **(C)** CT image of sagittal scan of the lumbar vertebra. The herniated disk was circled. The patient's information was masked. **(D)** The same patient's CT image of sagittal scan of the lumbar vertebra after surgery. The patient's information was masked. **(E and F)** Total protein was extracted from tissue biopsies of 23 chronic LBP patients with NP and 10 controls. The TRPV1 protein level was examined by immunoblotting. **(G)** Total RNA was extracted from tissue biopsies of 23 chronic LBP patients with NP and 10 controls. The TRPV1 mRNA level was examined by qRT-PCR. Results were analysed by unpaired *t*-test and $P < 0.05$ was considered significant.

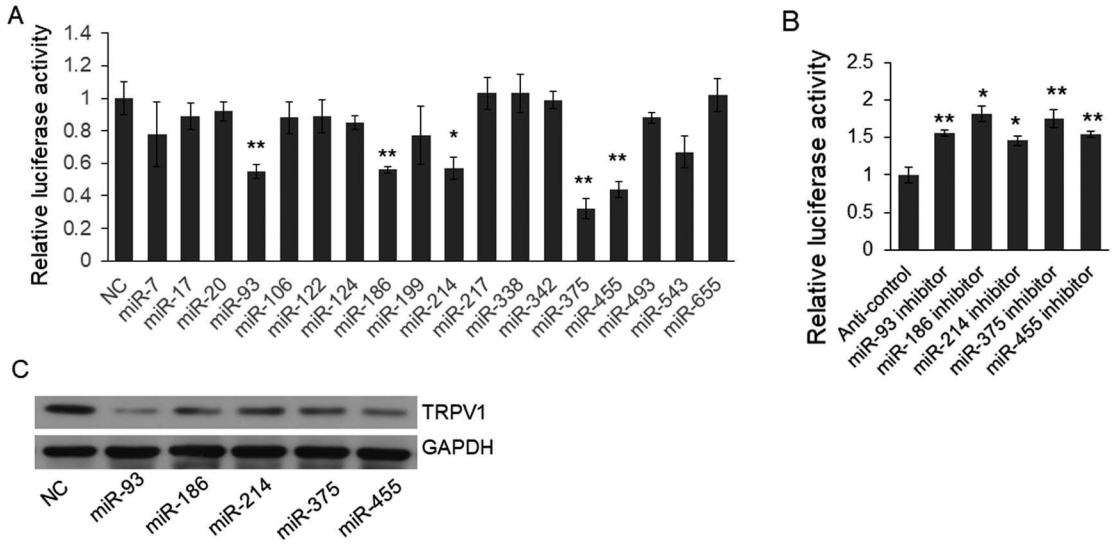

Fig. 2. Five miRNAs target the 3'UTR of TRPV1 mRNA and repress TRPV1.
(**A**, **B**) A reporter vector containing the full length of TRPV1 3'UTR was constructed. HEK293T cells were transfected with a TRPV1 reporter vector and one of the candidate miRNA mimics or inhibitors for 48 hours, with cel-miR-239 as mimic control (NC) and a double-stranded RNA oligo with no identifiable effects as anti-control. The cells were then subjected to dual luciferase assay. Results were analysed by paired *t*-test against NC control and $P < 0.05$ was considered significant. (**C**) sNF96.2 cells were transfected with one of the miRNA mimics with cel-miR-239 as mimic control (NC). Forty-eight hours after transfection, the cells were lysed and subjected to immunoblotting to detect TRPV1 expression.

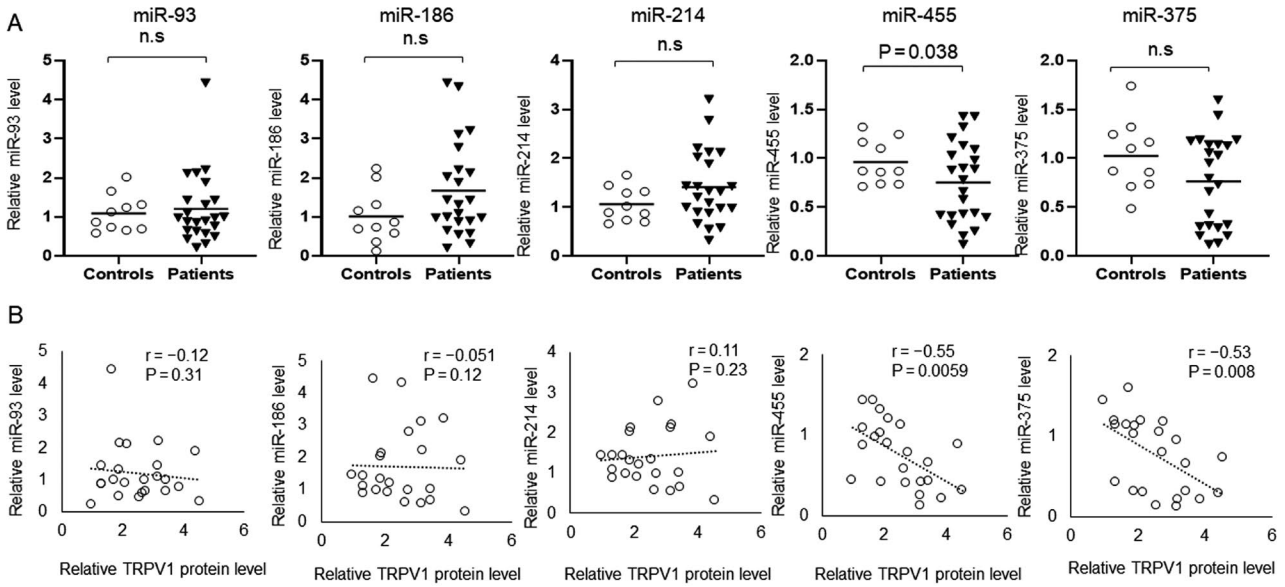

Fig. 3. MiR-375 and miR-455 negatively correlated with the TRPV1 protein level.
(**A**) The levels of five candidate miRNAs were examined by qRT-PCR in the biopsies from NP patients and controls. Results were analysed by unpaired *t*-test and $P < 0.05$ was considered significant. (**B**) The correlation between miRNAs and TRPV1 protein level was analysed by χ^2 test and $P < 0.05$ was considered significant.

understand whether the down-regulation of miR-375 and miR-455 was induced by NFKB1 activation, the level of NFKB1 in the biopsies of chronic LBP NP patients was investigated. As shown in Fig. 5A and B, the mRNA and protein levels of NFKB1 were both up-regulated in chronic LBP patients with NP. Significant negative correlations were found between NFKB1 and miR-375 or miR-455 (Fig. 5C), suggesting that the expression of miR-375 and miR-455 may be repressed by NFKB1 signalling.

To verify the above-mentioned hypothesis, sNF96.2 cells were treated with LPS to activate NFKB1 signalling (Fig. 5D), and then the expression of miR-375 and miR-455 was investigated. As shown in Fig. 5E, the levels of miR-375 and miR-455 were gradually down-regulated after LPS treatment, indicating that NFKB1 signalling repressed the miR-375 and miR-455 expression.

Discussion

It has been reported that TRPV1 sensitization is enhanced and contributes to the development of neuropathic pain in a rat chronic constriction injury model (CCI) (Kanai et al., 2005; Malek et al., 2015). The modulation of TRPV1 in neuropathic pain was therefore under attention, and several drugs relieved neuropathic pain by inhibiting TRPV1 (Luo et al., 2018; Sun et al., 2018). In the present study, we observed an increased

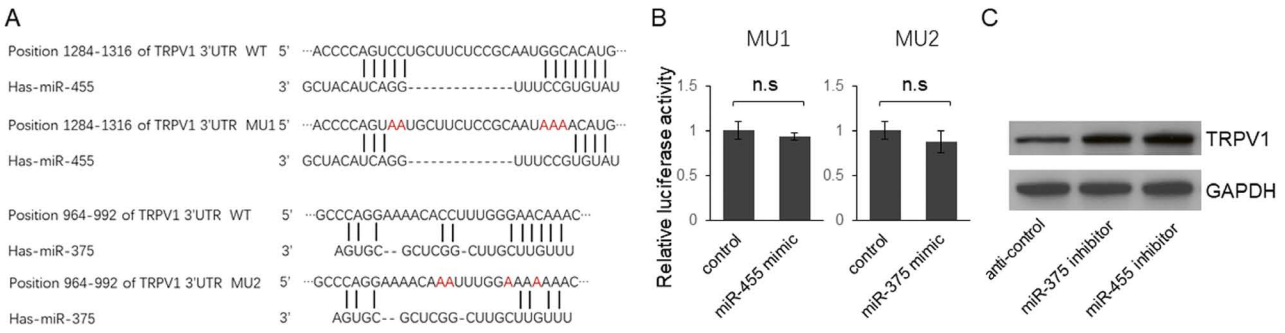

Fig. 4. Identification of the target sites of miR-375 and miR-455 in TRPV1 mRNA
(**A**) Schematic diagram of the predicted interaction between mutant TRPV1 mRNA and miR-375 or miR-455, with cel-miR-239 as mimic control (NC). (**B**) Dual luciferase assay. (**C**) sNF96.2 cells were transfected with one of the miRNA inhibitors for 48 hours, with a double-stranded RNA oligo with no identifiable effects as anti-control. The protein level of TRPV1 was examined by immunoblotting.

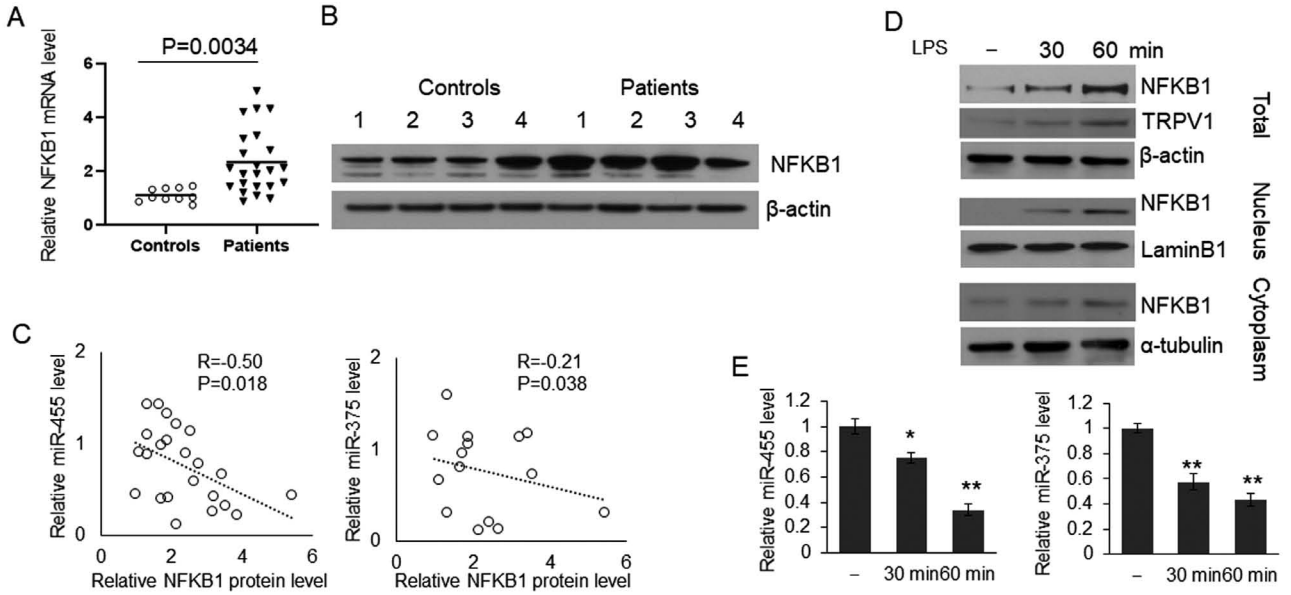

Fig. 5. NFKB1 signalling activation contributes to TRPV1 over-expression via repressing miR-375 and miR-455 expression.

(**A**) The mRNA level of NFKB1 was detected by qRT-PCR. (**B**) The protein level of NFKB1 was detected by immunoblotting. (**C**) Correlation analysis. (**D**) sNF96.2 cells were treated with 1 μg/ml LPS for 30 min or 60 min and then subjected to cytoplasm and nucleus protein extraction. The level of TRPV1 in the total cell lysate, cytoplasm and nucleus was detected by immunoblotting.

TRPV1 protein level in chronic LBP patients with NP. This finding is consistent with the findings reported in the CCI model (Kanai et al., 2005; Malek et al., 2015) and patients with the herpes zoster-induced acute pain (Han et al., 2016). The TRPV1 mRNA level was not increased in chronic LBP patients with NP, which may be caused by the cohort variations and different aetiology.

Since the TRPV1 mRNA level was not significantly up-regulated, our research focused on miRNAs, a group of post-transcriptional regulators repressing gene expression without changing their mRNA level. We confirmed that miR-375 and miR-455 repress TRPV1 expression through binding with 3'UTR of its mRNA. We were also the first to identify a negative correlation between TRPV1 and miR-375 or miR-455 in chronic LBP patients with NP.

The role of NFKB1 and its downstream pro-inflammatory cytokines in the mechanisms underlying inflammatory and neuropathic pain processes has been reported (Kim et al., 2004; Hartung et al., 2015). A positive correlation between the level of NFKB1 and TRPV1 was reported by Ahmed et al. (2019) in patients with a degenerative disc disease, but the underlying mechanism was not revealed. In the present study, we identified the regulatory axis of NFKB1-specific miRNAs-TRPV1 in patients with chronic LBP with NP, which partially unveiled the mechanism how NFKB1 regulates TRPV1 and contributes to LBP NP.

There are still limits of this study such as: (1) the source of RNA in the biopsy samples may be complex, and precise methods for sample collection or post-collection processing are needed to prevent contamination from other tissues such as ganglions; (2) the mechanism of how NFKB1 signalling regulates the miRNA expression is not known, and (3) these findings need to be confirmed in larger cohorts.

In conclusion, this study partially unveils the mechanism of how TRPV1 is over-expressed in chronic LBP patients with NP and provides two candidate miRNAs for NP treatment.

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