

miR-99b-5p Targets AKT1 to Modulate Microglial Polarization: A Prognostic Biomarker for Spinal Cord Injury

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Article

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Abstract

Study design:

Cross-sectional study.

Objectives

This study aimed to investigate the mechanism by which miR-99b-5p regulates microglial polarization via targeting AKT1, and to evaluate its potential as a prognostic biomarker and therapeutic target for spinal cord injury (SCI).

Setting:

The study was conducted at Wuhan Hankou Hospital and Nedong District People's Hospital, China.

Methods

A total of 90 individuals with SCI and 70 healthy controls were enrolled. Serum levels of miR-99b-5p, inflammatory cytokines, and microglial polarization markers were quantified. Functional outcomes were assessed using the AIS, FIM, SCIM-III, and WISCI-II scales. *In vitro* investigations included cell transfection, LPS-induced polarization, co-culture, and dual-luciferase reporter assays.

Results

Serum miR-99b-5p was significantly elevated in SCI individuals (AUC = 0.872) and positively correlated with AIS grade and functional outcomes (FIM, SCIM-III, WISCI-II). The miR-99b-5p mimic selectively inhibited M1 polarization in microglia, downregulating markers such as iNOS and TNF- α , while exhibiting no significant effect on macrophages. AKT1 was identified as a direct target of miR-99b-5p, and its overexpression reversed the inhibitory effect of miR-99b-5p on microglial polarization. Furthermore, miR-99b-5p alleviated neuronal inflammation and apoptosis, as evidenced by decreased Bax and increased Bcl-2 expression, through its regulatory action on microglia.

Conclusion

miR-99b-5p targets AKT1 to suppress microglial M1 polarization, thereby alleviating neuroinflammation and neuronal damage in SCI. Its serum level serves as a promising prognostic biomarker and potential therapeutic target.

Introduction

Following spinal cord injury (SCI), the complex pathological cascade is central to the development of long-term neurological deficits, with immune and inflammatory responses driving secondary injury being particularly critical [1, 2]. In this process, the dynamic polarization of microglia and infiltrating macrophages profoundly influences the homeostasis of the local microenvironment and the outcomes of neural repair [3, 4]. Therefore, elucidating the key molecular mechanisms that regulate the fate of these immune cells is an important direction for exploring novel therapeutic strategies for SCI.

In recent years, microRNAs (miRNAs), as key post-transcriptional regulators of gene expression, have garnered increasing attention for their roles in the pathological progression of SCI [5]. miR-99b-5p has emerged as a molecule of particular interest due to its diverse regulatory roles across multiple disease contexts. Current research highlights its core function in modulating immune-inflammatory pathways and cell survival, with distinct roles reported in different pathological settings. In cancer, for example, miR-99b-5p acts as a tumor suppressor by targeting the mTOR/AR axis, thereby inhibiting the proliferation of prostate cancer cells and inducing autophagy [6]. miR-99b-5p can alleviate apoptosis and pyroptosis of neurons after paclitaxel chemotherapy by inhibiting the activation of NLRP3 and improving oxidative stress [7]. Beyond these, it participates in immune regulation in osteoarthritis and schizophrenia-like models, where it modulates macrophage polarization or microglial inflammatory responses via pathway-specific mechanisms [8, 9]. Collectively, these findings establish miR-99b-5p as a key regulator of cell fate and immune homeostasis, with potential as a biomarker and therapeutic target across various diseases.

Studies have shown that miR-99b-5p expression is upregulated in mice after SCI and that it can target the mTOR signaling pathway [10]. Inhibition of miR-99b-5p can reverse the injury-induced downregulation of mTOR, thereby reducing neuronal apoptosis and promoting neurite outgrowth [11]. However, despite these findings, the specific role and mechanisms of miR-99b-5p in the macrophage/microglia-centered immune microenvironment following SCI remain unclear. In particular, there is a lack of direct evidence regarding its cell type-specific regulatory functions, especially its ability to intervene in the polarization of macrophages/microglia directly. This knowledge gap limits the potential for developing this molecule into a precise therapeutic target for SCI.

To address this gap, this study aims to systematically elucidate the specific role and molecular mechanisms of miR-99b-5p in remodeling the immune microenvironment after SCI, with a focus on its regulatory effects on macrophage/microglia polarization. The findings are expected to provide new insights into the immunopathological mechanisms of SCI and offer a potential therapeutic target for developing immune modulation-based neural repair strategies.

Materials and methods

2.1 Study participants

In this study, 90 individuals with SCI admitted to Hankou Hospital of Wuhan City from June 2022 to June 2024 were enrolled as the study group. Additionally, 70 healthy individuals with routine physical examinations during the same period were selected as controls, excluding those with neurological disorders, inflammatory conditions, or organic lesions. This study was approved by the Hospital Ethics Committee, adhering to the Declaration of Helsinki.

2.2 Main Reagents and Instruments

Cell lines (HMC3, THP-1, SH-SY5Y, HEK293T) were from the Cell Bank of the Chinese Academy of Sciences. miR-99b-5p mimics/inhibitors (RiboBio), AKT1 overexpression plasmid (GeneChem), ELISA kits (Boster), qRT-PCR kits (TaKaRa), and the Dual-Luciferase® System (Promega) were used. Key instruments included an ABI 7500 qPCR system and a ChemiDoc XRS + imager.

2.3 Clinical Sample Detection and Functional Evaluation

2.3.1 Serum Analysis

Serum was isolated and stored at -80°C. Inflammatory cytokines (TNF- α , IL-1 β , IL-6) and polarization markers (M1 markers: TNF- α , iNOS; M2 markers: CD206, Arg-1) were quantified by ELISA. Serum miR-99b-5p levels were quantified by qRT-PCR.

2.3.2 Neurological and Functional Recovery Assessment

Evaluations were conducted using standardized scales. The severity of neurological impairment was graded using the American Spinal Injury Association Impairment Scale (AIS), while functional recovery was comprehensively assessed through the Functional Independence Measure (FIM), the Spinal Cord Independence Measure III (SCIM-III), and the Walking Index for SCI II (WISCI-II). All assessments were independently conducted by two experienced clinicians who were blinded to the laboratory results.

2.4 In Vitro Cell Experiments

2.4.1 Cell Culture and Group Treatment

All cell lines were cultured in DMEM supplemented with 10% fetal bovine serum. THP-1 cells were differentiated into macrophages using 100 nM PMA for 24 hours. For functional studies, cells were treated with LPS (5 μ g/mL, 24 h) to induce M1 polarization, following transfection with miR-99b-5p mimics, inhibitors, or the AKT1 overexpression plasmid.

2.4.2 CCK-8 Assay for Cytotoxicity

HMC3 and THP-1 cells were seeded in 96-well plates, treated with miR-99b-5p mimic for 24 h, and 10 μ L of CCK-8 reagent was added. Absorbance at 450 nm was measured, and cell viability was calculated to determine a non-cytotoxic working concentration.

2.4.3 qRT-PCR for Polarization Marker Expression

Total RNA from cells was reverse-transcribed. mRNA levels of M1 markers (iNOS, TNF- α) and M2 markers (CD206, Arg-1) were measured by qRT-PCR, with *GAPDH* as the endogenous control. Relative expression was calculated using the $2^{(-\Delta\Delta Ct)}$ method.

2.4.4 Microglia-Neuron Co-culture

A non-contact co-culture system was established using Transwell chambers. HMC3 microglia (upper chamber) were stimulated with LPS and transfected. SH-SY5Y neurons (lower chamber) were cultured separately. Conditioned media and cells from both compartments were collected after 48 hours for downstream analysis.

2.4.5 Western Blot for Protein Expression

Proteins from SH-SY5Y cells were extracted, separated by SDS-PAGE, and transferred to PVDF membranes. Membranes were incubated with primary antibodies against Bax, Bcl-2, and β -actin, followed by HRP-conjugated secondary antibodies. Protein bands were visualized using an ECL substrate.

2.4.6 Dual-Luciferase Reporter Assay

AKT1 wild-type (WT) and mutant (MUT) 3'-UTR fragments were cloned into luciferase reporter plasmids. HEK293T cells were co-transfected with reporter plasmids and NC mimic/miR-99b-5p mimic. Luciferase activities were measured 48 h post-transfection, with relative activity normalized to Renilla luciferase luminescence.

2.5 Bioinformatics Analysis

The genes associated with SCI were retrieved from the GeneCards database (<https://www.genecards.org/>), using a relevance score cutoff of ≥ 20 . This threshold is commonly applied to prioritize genes that have direct functional links to SCI pathophysiology [12]. Predicted targets of miR-99b-5p were obtained from the miRWalk database (<http://mirwalk.umm.uni-heidelberg.de/>), with the following filtering criteria: (a) predicted by at least 3 of the 12 integrated algorithms (including TargetScan, miRanda, RNAhybrid, etc.) to ensure reliability; (b) conserved binding sites between miR-99b-5p and the 3'-UTR of target genes (as defined by TargetScan conservation score ≥ 0.5); (c) excluding genes with predicted binding free energy > -20 kcal/mol (indicating unstable binding interactions).

2.6 Statistical Analysis

Statistical analyses were performed using SPSS 26.0, and graphs were generated with GraphPad Prism 8.0. Continuous data were expressed as mean $\pm$ standard deviation ($\bar{x} \pm s$). Between-group comparisons for continuous data were analyzed by Student's t-test (two groups) or one-way analysis of variance (ANOVA) with LSD post-hoc test (multiple groups). Categorical data were compared using the chi-square test. Correlations between serum miR-99b-5p levels and neurological/clinical variables were evaluated by Spearman's rank correlation analysis. A two-tailed *P* value < 0.05 was considered statistically significant.

Results

High miR-99b-5p Expression Correlates with Improved Functional Recovery in Individuals with SCI

Baseline demographic characteristics, including gender, age, and body mass index (BMI), were comparable between the SCI group and healthy controls (Table 1), which helped minimize potential confounding effects.

Table 1
Comparison of Baseline Data between Healthy Control Individuals and SCI Individuals

	Control group n = 70	SCI group n = 90	P value
Basic information			
Age (years, mean ± SD)	39.77 ± 12.58	41.77 ± 12.29	0.366
BMI (kg/m², mean ± SD)	22.47 ± 0.93	22.47 ± 0.84	0.925
Gender (Male/Female, n)	34/36	47/43	0.647
Cause of damage			
Traffic accidents (n, %)	-	31/33.3	-
Falls from height (n, %)	-	21/22.50	-
Injury from a heavy object (n, %)	-	22/23.6	-
Other (n, %)	-	16/17.20	-
Injured segments			
Cervical segment (n, %)	-	27/29.0	-
Thoracolumbar segment (n, %)	-	30/32.2	-
Waist section (n, %)	-	33/35.4	-
Note: SCI, spinal cord injury; BMI, body mass index (unit: kg/m²). "-" indicates data not applicable to the corresponding group.			

While miR-99b-5p upregulation has been documented in murine SCI models, its serum expression profile in humans with SCI remains largely uncharacterized [13]. Given the well-recognized role of miRNAs in regulating the pathophysiological processes of SCI [14], serum miR-99b-5p levels were quantified initially. Consistent with trends reported in preclinical animal studies, serum miR-99b-5p expression was significantly higher in the SCI group than in the healthy control group (Fig. 1A). Receiver operating characteristic (ROC) curve analysis was further performed to evaluate its discriminative ability, showing an area under the curve (AUC) of 0.872 (95% CI, 0.820 to 0.923, Fig. 1B), indicating that serum miR-99b-5p has promising diagnostic potential for SCI.

To evaluate the associations between serum miR-99b-5p and clinical characteristics in individuals with SCI, Spearman correlation analysis was conducted (Fig. 1C). The results showed no significant correlations between miR-99b-5p levels and non-neurological variables (age, BMI, gender, cause of damage, injured segments; all $P > 0.05$). In contrast, miR-99b-5p was positively correlated with key neurological indices: the correlation coefficient (Spearman's ρ) was 0.344 ($P < 0.001$) for AIS grade, 0.551 ($P < 0.001$) for FIM score, 0.527 ($P < 0.001$) for SCIM-III score, and 0.724 ($P < 0.001$) for WISCI-II score. To further explore the diagnostic value of serum miR-99b-5p for neurological outcomes in SCI, ROC curve analysis was performed (Fig. 1D). The results revealed that miR-99b-5p had good discriminatory ability: the AUC was 0.817 (95% CI, 0.731 to 0.902, $P < 0.001$) for AIS grade, 0.789 (95% CI, 0.694 to 0.884, $P < 0.001$) for FIM score, 0.853 (95% CI, 0.769 to 0.937, $P < 0.001$) for SCIM-III score, and 0.639 (95% CI, 0.524 to 0.754, $P = 0.018$) for WISCI-II score.

In summary, the findings demonstrate that elevated serum miR-99b-5p levels are strongly correlated with favorable functional recovery in individuals with SCI.

High Expression of miR-99b-5p Significantly Inhibits M1 Polarization of Microglia

Within the complex microenvironment of SCI, the polarization states of macrophages and microglia have been established as pivotal regulators of secondary injury and repair processes, whose dynamic balance critically influences neuronal survival, axonal regeneration, and functional recovery outcomes [15, 16]. To investigate whether miR-99b-5p is involved in regulating this balance, serum levels of polarization markers were measured initially. Individuals with SCI were stratified into low expression and high expression groups based on the median serum miR-99b-5p level. Compared with the low expression group, individuals in the high miR-99b-5p group exhibited significantly lower levels of M1 markers (iNOS and TNF- α) and higher levels of M2 markers (CD206 and Arg-1) (Fig. 2A), suggesting a potential role for miR-99b-5p in suppressing M1 polarization.

To further verify this regulatory effect and assess its cell-type specificity, *in vitro* M1 polarization models were established using HMC3 microglia and THP-1 macrophages. The miR-99b-5p mimic showed no significant cytotoxicity in either cell line as determined by the CCK-8 assay (Fig. 2B-C), and a concentration of 10 μ M was therefore selected for subsequent experiments. In LPS-stimulated HMC3 microglia, the miR-99b-5p mimic significantly suppressed the upregulation of M1 markers (iNOS and TNF- α) (Fig. 2D). In contrast, in THP-1 macrophages, the miR-99b-5p mimic did not consistently reverse LPS-induced M1 polarization but significantly attenuated the LPS-mediated downregulation of the M2 marker Arg-1 (Fig. 2E), highlighting the complexity and cell-context dependency of macrophage polarization regulation. Together, these results indicate that miR-99b-5p selectively inhibits M1 polarization in microglia while exerting a distinct regulatory effect on macrophages.

miR-99b-5p Attenuates Microglial M1 Polarization by Directly Targeting AKT1

To systematically identify the downstream targets and signaling pathways of miR-99b-5p in SCI, two gene sets were integrated for analysis: (1) SCI-associated genes with high relevance (GeneCards score $\geq$

20) to ensure functional relevance to SCI; (2) high-confidence targets of miR-99b-5p (predicted by ≥ 3 algorithms, conserved binding sites, and binding free energy < -20 kcal/mol) to reduce false-positive predictions. This integration yielded 933 overlapping candidate genes (Fig. 3A), which were hypothesized to mediate the biological effects of miR-99b-5p in SCI.

KEGG pathway enrichment analysis identified 15 significantly enriched pathways. The top 10 pathways (Fig. 3B) included multiple signaling cascades and biological processes closely related to SCI pathophysiology: (1) Pathways in cancer; (2) MAPK signaling pathway; (3) Pathways of neurodegeneration – multiple diseases; (4) Neuroactive ligand-receptor interaction; (5) AGE-RAGE signaling pathway in diabetic complications; (6) Colorectal cancer; (7) cAMP signaling pathway; (8) Cell adhesion molecules; (9) Apoptosis; (10) Cholinergic synapse (Fig. 3B). Among these, the MAPK signaling pathway was prioritized for further validation. This pathway is a well-characterized core regulator of microglial polarization, neuroinflammation, and neuronal survival—key pathological processes driving secondary injury after SCI [17, 18]. Consistent with this prediction, LPS stimulation significantly upregulated the expression of key MAPK members (p38 MAPK, ERK1/2, and JNK), and miR-99b-5p mimic transfection significantly reversed this upregulation (Fig. 3C), confirming that miR-99b-5p suppresses microglial M1 polarization at least partially via modulating the MAPK pathway.

To identify the direct upstream target of miR-99b-5p mediating this effect, a protein-protein interaction (PPI) network was constructed from the intersecting genes. Among top-ranked hub genes, AKT serine/threonine kinase 1 (AKT1) was a promising candidate. Although its pivotal role in modulating microglial polarization through the PI3K/AKT pathway is well-recognized [19], whether it serves as a functional target for miR-99b-5p in the context of SCI remained unknown. In silico analysis predicted a high-probability binding site for miR-99b-5p within the 3'-UTR of AKT1, featuring canonical seed region complementarity (Fig. 3D). This interaction was experimentally confirmed by dual-luciferase reporter assay, where the miR-99b-5p mimic significantly suppressed luciferase activity of the wild-type (WT) AKT1 3'-UTR reporter but not the mutant (MUT) construct (Fig. 3E), confirming AKT1 as a direct target of miR-99b-5p.

Rescue experiments verified the functional relevance of the miR-99b-5p-AKT1 interaction. miR-99b-5p mimic effectively inhibited LPS-induced upregulation of M1 markers (iNOS, TNF- α), while AKT1 co-overexpression partially but significantly reversed this suppression (Figs. 3F-G).

Collectively, these findings demonstrate that miR-99b-5p directly targets AKT1, modulates downstream pathways including MAPK, and thereby attenuates microglial M1 polarization, providing novel insights into the molecular mechanism of miR-99b-5p in regulating microglial polarization during SCI.

miR-99b-5p Alleviates Neuroinflammation and Neuronal Apoptosis by Suppressing Microglial M1 Polarization

To link the observed clinical associations with the proposed cellular mechanisms, the correlation between serum miR-99b-5p levels and systemic inflammation was investigated. The results showed that

individuals with high serum miR-99b-5p expression exhibited significantly lower levels of key pro-inflammatory cytokines (TNF- α , IL-1 β , IL-6) compared with those with low expression (Fig. 4A).

To verify whether miR-99b-5p exerts neuroprotection by regulating microglial paracrine signaling, a controlled *in vitro* model was established using a non-contact Transwell co-culture system to isolate the effects of soluble factors secreted by HMC3 microglia on SH-SY5Y neurons. Transfection of miR-99b-5p mimic into LPS-stimulated microglia significantly downregulated M1 marker secretion (iNOS, TNF- α) and reduced neuroinflammatory factors (IL-6, IL-1 β) in the neuronal compartment compared to the LPS control. Conversely, the miR-99b-5p inhibitor exacerbated these inflammatory responses (Figs. 4B-E). These results confirm that miR-99b-5p mitigates neuroinflammation via a microglia-dependent paracrine mechanism.

The impact of attenuated inflammation on neuronal survival was subsequently explored. KEGG analysis implicated the apoptosis pathway, prompting an examination of key apoptotic regulators. In neurons co-cultured with miR-99b-5p-overexpressing microglia, pro-apoptotic Bax protein expression was significantly decreased, while anti-apoptotic Bcl-2 levels were increased (Figs. 4F-H). This anti-apoptotic effect was validated using an independent pre-miR-99b-5p overexpression system with consistent results.

Collectively, data from the clinical cohort and reductionist co-culture model demonstrate that miR-99b-5p dually attenuates neuroinflammation and inhibits neuronal apoptosis, dependent on its ability to suppress microglial M1 polarization.

Discussion

This study delineates a neuroprotective role for miR-99b-5p in SCI by demonstrating its capacity to mitigate neuroinflammation and neuronal apoptosis through the specific suppression of microglial M1 polarization via the AKT1 pathway. These findings integrate miR-99b-5p into the immunoregulatory network of the injured spinal cord, underscore the significance of miRNA-mediated regulation in neural injury, and offer novel insights into the underlying pathophysiological mechanisms.

Clinically, serum levels of miR-99b-5p were found to be significantly elevated in individuals following SCI. Notably, higher circulating levels of this microRNA correlated with less severe neurological impairment and more favorable functional recovery, reinforcing the concept that the upregulation of specific protective microRNAs post-injury is associated with improved clinical outcomes [20, 21]. While prior investigations have largely centered on other microRNAs such as miR-21 and miR-124, the present study is the first to establish a direct link between miR-99b-5p and clinical prognosis in human SCI, thereby expanding the scope of potential prognostic biomarkers. Collectively, the results suggest that miR-99b-5p functions not merely as a passive injury marker but as an endogenous protective factor that actively mitigates secondary pathological processes. This aligns with the broader theoretical framework wherein microRNAs are recognized as key regulators of cellular homeostasis within the injury microenvironment, critically influencing the ultimate course of tissue repair and recovery [22].

A pivotal mechanistic finding is the specific inhibition of microglial M1 polarization by miR-99b-5p, supported by both clinical biomarker correlations and direct *in vitro* evidence. In serum from individuals with SCI, elevated miR-99b-5p levels were inversely correlated with classic M1 polarization markers, including iNOS and TNF- α , reinforcing the well-established link between balanced microglial polarization and functional recovery [23, 24]. Notably, this regulatory effect exhibited distinct cell-type specificity, being pronounced in HMC3 microglia but minimal in THP-1-derived macrophages. This distinction advances the understanding of lineage-selective miRNA actions in immune regulation and highlights a potential avenue for precise therapeutic intervention in SCI that may spare systemic immunity [25, 26]. Bioinformatic analysis implicated the MAPK pathway, a central regulator of microglial activation and SCI progression. As AKT1 is a known upstream modulator of MAPK signaling, a coherent regulatory axis is proposed whereby miR-99b-5p targets AKT1 to modulate MAPK activity, thereby suppressing microglial M1 polarization.

The translational potential of miR-99b-5p is underscored by its dual role, serving both as a quantifiable serum biomarker and as a functional regulator of the local spinal cord microenvironment. Its serum levels correlate with both the extent of systemic inflammation and the degree of neurological recovery, suggesting its potential utility as an auxiliary, non-invasive prognostic tool for the early clinical assessment of SCI. To investigate the direct neuroimmune interaction underlying this clinical correlation, an *in vitro* co-culture model was employed to isolate and examine the paracrine axis between microglia and neurons [27]. The experimental results established a clear causal relationship: overexpression of miR-99b-5p in microglia directly suppressed their polarization toward the M1 phenotype and the secretion of pro-inflammatory factors, which was sufficient to attenuate neuronal apoptosis. This finding provides direct mechanistic evidence for the therapeutic strategy of achieving neuroprotection by targeting the regulation of microglial polarization [28].

Nevertheless, the limitations inherent in this simplified *in vitro* model are acknowledged. While invaluable for dissecting specific cellular interactions, it cannot replicate the full pathophysiological complexity of the SCI microenvironment, which includes dynamic crosstalk among astrocytes, oligodendrocytes, infiltrating peripheral immune cells, and the evolving glial scar, all critical factors influencing repair and regeneration [29]. Most importantly, the anti-inflammatory and neuroprotective efficacy observed *in vitro* requires validation in a living organism. Therefore, future investigations utilizing established *in vivo* rodent spinal SCI models are essential for verifying therapeutic efficacy, defining pharmacokinetic profiles and optimal delivery strategies, and evaluating the long-term functional impact of miR-99b-5p modulation within an integrated physiological context [30].

In summary, this work establishes miR-99b-5p as a multifaceted candidate in SCI: a serologically accessible prognostic biomarker and a cell-type-specific molecular regulator that alleviates neuroinflammation and neuronal apoptosis by targeting AKT1-mediated microglial polarization. These insights offer a novel therapeutic target and a refined conceptual framework for mitigating the detrimental neuroimmune cascade following SCI.

Declarations

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CRediT authorship contribution statement

Deming Chen: Data curation, Methodology, Writing – review & editing. Shibo Feng: Formal analysis, Writing – review & editing.

Chenge Xian: Investigation, Writing – review & editing.

Ruye Li: Resources, Conceptualization, Project administration, Supervision, Writing – review & editing.

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Declaration of competing interest

No known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper exist.

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Ethics declarations

Competing interests

No known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper exist.

Ethical approval

The study was carried out following the Declaration of Helsinki and applicable national and international guidelines for research involving human participants. It received ethical approval from the Ethics Committee of Hankou Hospital in Wuhan City (Ethics Approval Number: Hanyilunshen [2022]-RC-012).

Before starting the study, the research protocol, informed consent form, and other relevant materials were reviewed and approved by the ethics committee to ensure participants' safety and adherence to research ethics. Informed consent was obtained from all participants involved in the study.

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Figures

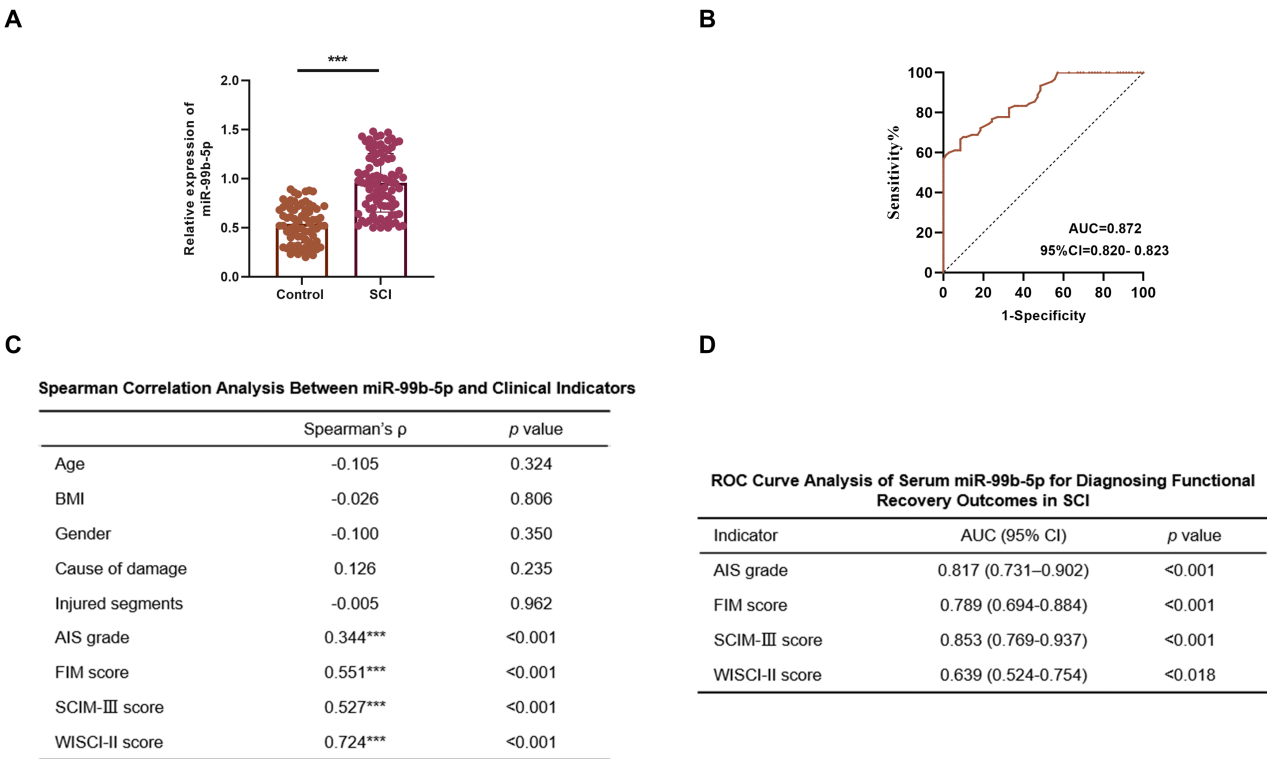


Figure 1

Associations of serum miR-99b-5p with functional recovery in individuals with SCI. (A) Relative expression of serum miR-99b-5p was measured by qRT-PCR in healthy controls (n=70) and in individuals with SCI (n=90). Compared with the control group, *** $P < 0.001$. (B) The discriminative ability of serum miR-99b-5p between the two groups was evaluated by ROC curve analysis. The diagonal reference line (AUC = 0.5) is shown, with the calculated AUC and 95% CI indicated. (C) Spearman rank correlation analysis between serum miR-99b-5p expression and clinical indicators. (D) ROC curve analysis of serum miR-99b-5p for predicting neurological outcomes in individuals with SCI, including neurological impairment severity (assessed by AIS grade) and functional recovery (evaluated by FIM, SCIM-III, and WISCI-II scores).

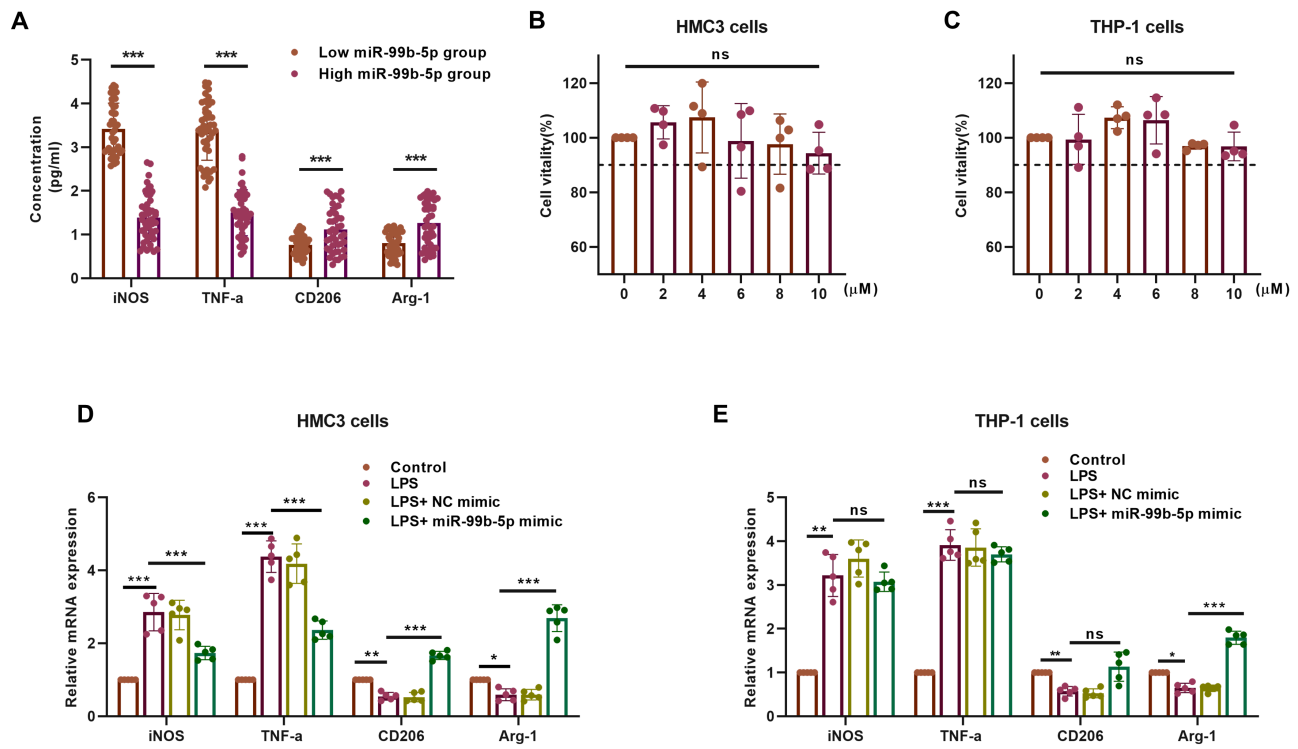


Figure 2

miR-99b-5p specifically inhibits microglial M1 polarization. (A) Serum concentrations of M1 markers (iNOS, TNF-α) and M2 markers (CD206, Arg-1) were measured by ELISA in individuals with SCI, who were stratified into low and high miR-99b-5p expression groups according to the median serum miR-99b-5p level of the SCI cohort. (B-C) Cytotoxicity of the miR-99b-5p mimic on HMC3 microglia and PMA-differentiated THP-1 macrophages was assessed using the CCK-8 assay after 24 hours of treatment. (D-E) The mRNA expression levels of M1 markers (iNOS, TNF-α) and M2 markers (CD206, Arg-1) in LPS-stimulated HMC3 cells and PMA- and LPS-co-stimulated THP-1 cells were determined by qRT-PCR following transfection with miR-99b-5p mimic or NC mimic. Data are presented as mean ± SD. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; ns, not significant.

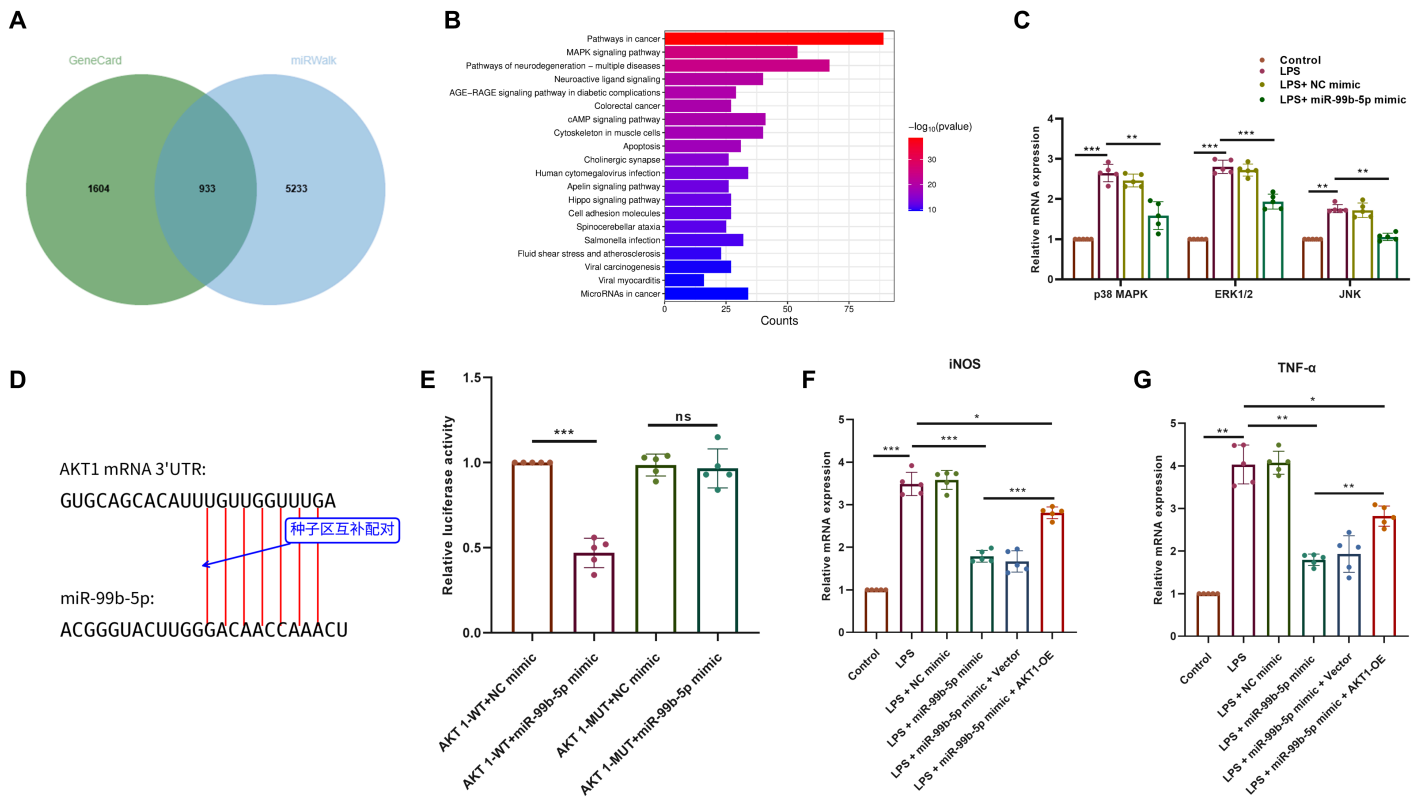


Figure 3

miR-99b-5p targets AKT1 to inhibit microglial M1 polarization. (A) Venn diagram showing the intersection of SCI-associated genes (from GeneCards) and predicted target genes of miR-99b-5p (from miRWalk). (B) KEGG pathway enrichment analysis of the intersecting genes. (C) mRNA expression levels of key MAPK pathway genes (p38 MAPK, ERK1/2, JNK) in HMC3 cells, measured by qRT-PCR. (D) Schematic of the predicted binding site of miR-99b-5p within the 3'-UTR of AKT1. (E) Validation of direct targeting of AKT1 by miR-99b-5p via dual-luciferase reporter assay. Luciferase activity was measured in HEK293T cells co-transfected with miR-99b-5p mimic and a reporter plasmid containing either the wild-type (WT) or mutant (MUT) AKT1 3'-UTR. (F-G) The mRNA expression levels of M1 markers (iNOS and TNF-α) in HMC3 cells were determined by qRT-PCR after treatment with LPS, miR-99b-5p mimic, and AKT1 overexpression (AKT1-OE). Data are presented as mean ± SD. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; ns, not significant.

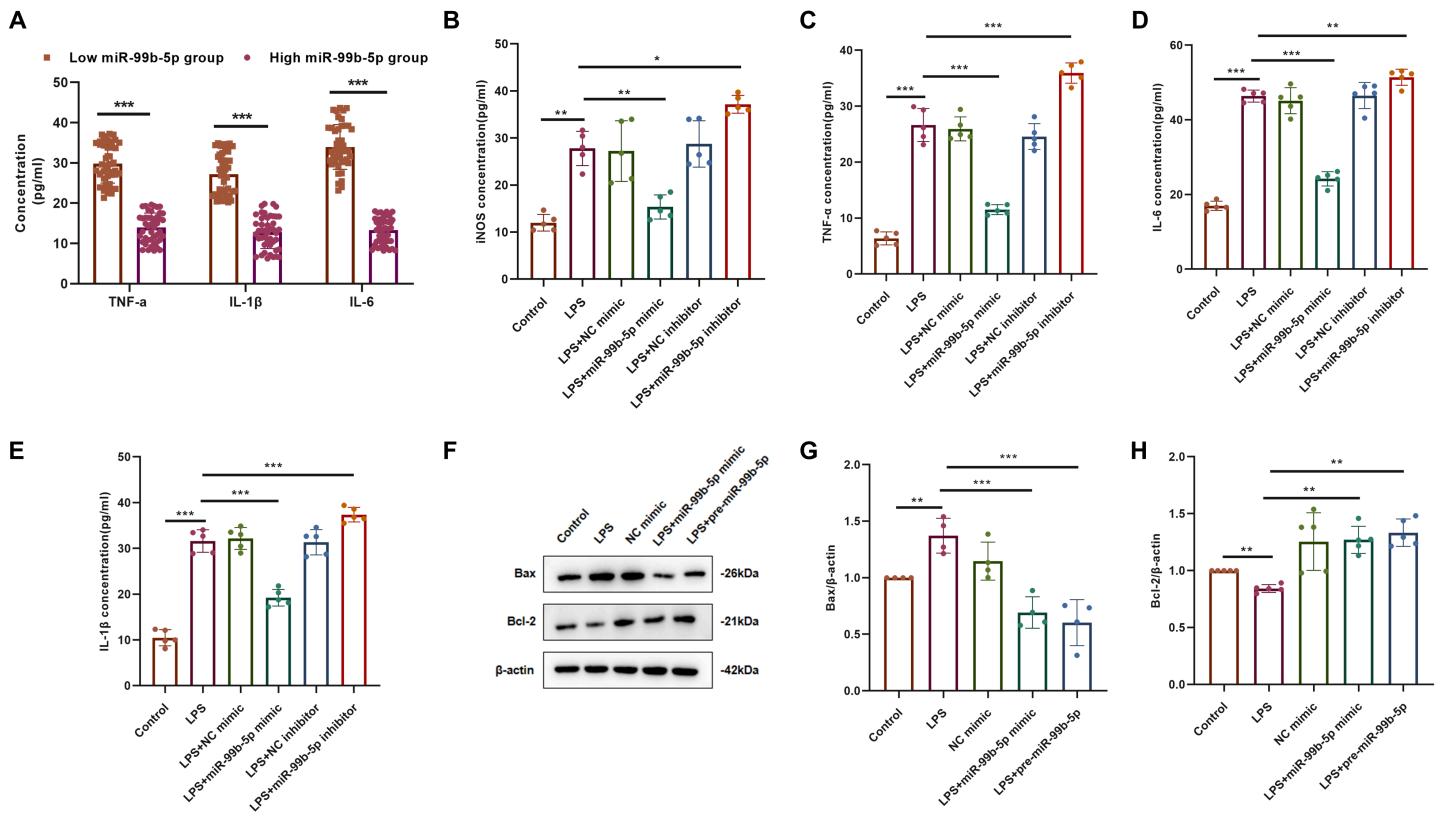


Figure 4

miR-99b-5p attenuates neuroinflammation and neuronal apoptosis by suppressing microglial M1 polarization in a co-culture system. (A) Serum levels of the pro-inflammatory cytokines TNF-α, IL-1β, and IL-6 were measured by ELISA in SCI individuals stratified by low and high serum miR-99b-5p expression. (B-C) Concentrations of the M1 polarization markers iNOS and TNF-α in the upper chamber (microglial compartment) were quantified by ELISA. (D-E) Levels of the neuroinflammatory factors IL-6 and IL-1β in the lower chamber (neuronal compartment) were determined by ELISA. (F-H) Expression of the pro-apoptotic protein Bax and the anti-apoptotic protein Bcl-2 in SH-SY5Y cells from the lower chamber was analyzed by Western Blot. Representative blots (F) and quantitative analysis (G, H) are shown. Data are presented as mean ± SD. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.