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MiR-874-3p Promotes Onset and Progression of Hodgkin's Lymphoma by Affecting P-MNK1 and PDHA1 Proteins

Qingfang Zeng*, Qinghua Lan, Tao Zhan, Jianming Zhong and Xiaojian Wang

Internal Medicine Area Three, Ganzhou Cancer Hospital, Ganzhou 341000, Jiangsi Province, China

KEYWORDS Hodgkin's Lymphoma. miR-874-3p. PDHA1. p-Mnk1. Prognosis. Survival

ABSTRACT The researchers aimed to investigate the associations among miR-874-3p, phosphorylated mitogen-activated protein kinase-interacting kinase 1 (p-Mnk1) and pyruvate dehydrogenase E1 subunit alpha 1 (PDHA1), as well as to explore their roles in the onset and progression of Hodgkin's lymphoma (HL). Flow cytometry, Western blotting, quantitative reverse transcription-polymerase chain reaction, and assays of luciferase, immunofluorescence and cell counting kit-8 were employed to assess the functions of miR-874-3p in affecting the severity and prognosis of HL patients. HL patients presented notably lowered miR-874-3p expression. Besides, with respect to nucleus pulposus (NP) cells, miR-874-3p was negatively correlated with p-Mnk1, and down-regulated PDHA1 expression. Following miR-874-3p overexpression, NP cells exhibited increased aggregin expression. MiR-874-3p hardly affected NP cell apoptosis, whereas down-regulating miR-874-3p not only promoted extracellular matrix degradation but also suppressed NP cell proliferation. The miR-874-3p/p-Mnk1/PDHA1 axis may work as a promising marker in diagnosing and treating HL.

INTRODUCTION

Hodgkin's lymphoma (HL), a distinctive lymphoma subtype, is regarded as one of the malignant tumors that occur most frequently in young people. HL usually spreads from a group of lymph nodes, mainly involving cervical and supraclavicular lymph nodes, to other lymph nodes, and even invades blood vessels and affects the spleen, liver, bone marrow and digestive tract in the late stage (Tiacci et al. 2018). It is pathologically characterized by nucleus pulposus (NP) cell apoptosis and excessive proliferation of abnormal lymphocytes (Malik et al. 2022). As a key player in maintaining intervertebral disc (IVD), NP cells can modulate the expressions of catabolic, inflammatory, anabolic and anti-catabolic cytokines. Moreover, NP cells in human beings were previously taken as targets of gene therapeutic approaches by virtue of adenoviral or lentiviral systems (Hosseinkhani et al. 2023).

The phosphorylation of pyruvate dehydrogenase E1 subunit alpha 1 (PDHA1) triggered by phosphorylated mitogen-activated protein (MAP) kinase-interacting kinase 1 (p-Mnk1) on Ser-209 is able to facilitate cellular survival, proliferation, metastasis, and malignant transformation (Koc et al. 2023). The positive protein expression percentages of p-Mnk1 and PDHA1 in HL (83.5% and 75.4%, respectively) rose apparently in contrast to those in the normal tissues of control group (40.0% and 32.9%, respectively) (Kumar et al. 2023). Metastatic HL has markedly up-regulated protein expression of PDHA1 in comparison to corresponding primary HL. Moreover, there are significant inverse relationships between the increased expressions of PDHA1 and p-Mnk1 and overall survival rate (Chen et al. 2022). Furthermore, p-Mnk1 expression is significantly positively correlated with PDHA1 expression in HL (Park and Baek 2022). According to multivariate analysis, p-Mnk1 and PDHA1 expressions are independent factors influencing HL prognosis despite clinical stage, lymph node metastasis, radio-chemotherapy combination, tissue type, age and gender ratio (Wang et al. 2022). Taken together, highly expressed p-Mnk1 and PDHA1 may function as novel biomarkers that may be utilized to predict the poor prognosis as well as treatment outcomes for HL patients, contributing to the formulation of effective treatment strategies.

Considering that the phosphorylation of PDHA1 can be triggered by p-Mnk1, targeting the Mnk/PDHA1 pathway is potentially applicable to HL treatment (Bou-Petit et al. 2022). Highly expressed p-Mnk1 may be an independent predictive biomarker for such patients (Ghorbian et al. 2016). Meanwhile, repressing p-Mnk1 expression *via* Mnk inhibitor (CGP57380) probably di-

*Address for correspondence:
Qingfang Zeng
E-mail: zengqfgch@csc-edu.cn

minishes PDHA1 phosphorylation triggered by Rapalogs (RAD001) and Akt activation (Bowles and Setton 2017). Furthermore, the binding of CGP57380 to RAD001 can induce NP cell apoptosis in HL through activating the internal mitochondrial pathway while performing synergistic antitumor functions both inside and outside organisms (Newell et al. 2017; Desmoulin et al. 2020). In short, targeting the PDHA1 and p-Mnk1 signaling pathway to enhance the efficacy of miR-874-3p may be suitable for the personalized treatment of HL patients.

Objectives

Therefore, the researchers investigated the associations among miR-874-3p, p-Mnk1 and PDHA1 expressions, and explored their roles in the onset and progression of HL, aiming to provide valuable experimental data for clinical treatment.

METHODOLOGY

Collection of Clinical Samples

The ethics committee of the hospital issued approval to the protocol of this study, and all patients provided the informed consent. A total of 54 patients with HL were selected from our hospital for the collection of clinical samples. Among them, 28 patients who had obvious symptoms of HL and underwent puncture biopsy were included as an HL group, and the other 26 patients with inflammatory lymphadenopathy without the signs of HL were set as a control group.

RNA Isolation Plus Quantitative Reverse Transcription-polymerase Chain Reaction (qRT-PCR)

TRIzol reagent provided by Invitrogen, USA, or miRNeasy kit purchased from Qiagen, USA (Cat. No. 217004), was adopted for extraction of total RNA. Reverse transcription kit (Promega, USA) and oligo-dT primers were utilized to synthesize cDNA (D'Este et al. 2018). Besides, SYBR Green offered by Applied Biosystems, USA) was applied to conduct qRT-PCR, following the standard protocol of kits and using GAPDH as the endogenous control (Liu et al. 2020). The $^{\Delta\Delta}$CT method was utilized for calculation. In addition, stem-loop primers specially designed for miRNAs were employed to perform reverse transcription, with U6 RNA being the endogenous control of miRNA.

NP Cell Separation and Incubation

The patients who received treatment for HL offered human IVD tissues. F-12 medium (Gibco, Invitrogen, USA) mixed with 5 percent fetal bovine serum, streptomycin (Gibco, USA, 100 mg/mL) as well as penicillin (100 U/mL, Gibco, USA) was utilized to incubate NP cells (Chen et al. 2020). The NP cells with the characteristics of immunofluorescence from a patient were used for subsequent experiments. As for each test, the cells were provided by the same patient. The incubated NP cells which were separated from patients generally kept proliferating in the 6th generation. The 3rd-generation cells were mainly utilized and incubated.

Lentivirus (LV) Infection of NP Cells

LV containing expression sequences was offered by Gene Chemical Technology Co., Ltd. (China). The H1 promoter was used to stimulate miR-874-overexpressed lentivirus containing pre-miRNA sequence of miR-874. MiR-874-inhibited lentivirus and negative control (NC) lentivirus contained target sequence (TCGGTCCGGGGCAG) against miR-874-3p and random sequence, respectively, both of which were stimulated by the H1 promoter. The lentiviruses that were all driven by CMV promoter were employed as the reporter genes to test the infection efficiency (Wu et al. 2018). The 6-well plates were utilized for overnight NP cell inoculation, with each well supplemented with 2 mL of medium. Then culture medium (2 mL, Sigma-Aldrich, USA) was added into the sample, which was concentrated to 100 ìL. After 72 h, the cells were collected for subsequent experiments.

Vector Establishment Together With Dual Luciferase Assay

Amplification and cloning of PDHA1 gene into pMIR-Report vector (the lower reach of luciferase gene) bought from Applied Biosystems, USA. The construction was referred to as PDHA1-Luc. Then

the mutation of the binding site of miR-874-3p predicted by PDHA1 was implemented using two-step PCR, and the new structure was referred to as PDHA1-Mut-Luc. Later, HEK293 cells underwent inoculation into 96-well plates, followed by transfection with miRNA mimics, reporter plasmid (100 ng, pMIR-Report constructed control vector), internal control plasmid (pRL-TK, 10 ng) and Lipofectamine 2000 as per the instructions provided by the manufacturer. Finally, the dual-luciferase reporter assay system developed by Promega, USA, was applied to determine the activity of reporter gene 48 h later.

Apoptosis and Cell Counting Kit-8 (CCK-8) Assays

On the basis of Annexin V/PI apoptosis kit purchased from Invitrogen, USA, flow cytometry was also utilized to examine cell apoptosis. CCK-8 assay kit (Beyotime, China) was employed to determine cell proliferation or viability. All the experiments were accomplished in triplicates.

Immunofluorescence Assay and Western Blotting (WB)

After isolation and incubation, the cells were subjected to 15 min of fixation with paraformaldehyde (4%), which were sealed in PBS added with 2% BSA, processed with 0.2% Triton-X100 (lasting 15 min), and incubated using anti-ACAN (Abcam, USA; 1:200 diluted), anti-collagen II (Millipore, USA; 1:100 diluted), anti-Sox9 (Millipore, USA; 1:100 diluted) plus anti-FOXF1 (Santa Cruz Biotechnology, Inc., USA; 1:50 diluted) primary antibodies, and then with secondary antibodies (1:500, Invitrogen, USA). Subsequent to DAPI (Sigma, USA) staining, observation by virtue of a fluorescence microscope was conducted for the cells. WB according to the standard protocol was exerted. In short, sodium dodecyl sulfate buffer was adopted for the preparation of total cell lysates. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was carried out on each protein measured in an equal amount, after which the protein underwent transfer onto a PVDF membrane subjected to incubation with primary antibodies against p-Mnk1 (Abcam, USA; dilution ratio 1:2,000), PDHA1 (Abcam, USA; dilution ratio 1:2,000), gel (Santa Cruz Biotechnology, Inc., USA; dilution ratio 1: 500), collagen II (Millipore, USA; dilution ratio 1: 1,000), collagen I (Millipore, USA; dilution ratio 1: 1,000) and GAPDH (Abcam, dilution ratio 1: 5,000).

Statistical Analysis

Mean ± standard deviation was adopted as the expression format of most quantitative data. The *t*-test or analysis of variance was performed for statistical comparative analysis of qRT-PCR, and Ty analysis for CCK-8 as well as luciferase activator. The correlations of miR-874-3p with p-Mnk1 and PDHA1 mRNA expression levels in HL patients were explored by means of Pearson's correlation analysis. $P<0.05$ signified a statistically significant difference.

RESULTS

MiR-874-3p Expression Declined in HL Patients and Exhibited a Negative Correlation with p-Mnk1

According to the results of qRT-PCR, the HL group had an overtly lower miR-874-3p expression level than the control group, as shown in Figure 1A. Considering that p-Mnk1 was a crucial gene associated with HL and the gene downstream of miR-874-3p in cancers, HL patients and controls were chosen for further analysis of p-Mnk1 expression levels. Compared to the control group, HL patients had a higher level of p-Mnk1 (Fig. 1B). PDHA1, as another key gene involving ECM degradation in IVD, also had significantly increased expression (Fig. 1C). In the case of miR-874-3p with a higher expression level, both p-Mnk1 and PDHA1 displayed lowered expression levels in the HL group (Fig. 1D and E).

p-Mnk1 was Down-regulated by miR-874-3p in NP Cells

In vitro isolation and culture of human NP cells were accomplished for the purpose of further evaluating the impact of miR-874-3p on HL. To demonstrate that the incubated cells were NP cells, immunofluorescence assay was conducted. The markers related to NP cells (collagen II, ACAN, Sox9, FOXF1, etc.) were found in the incubated cells (Fig. 2A). MiR-874-3p is competent

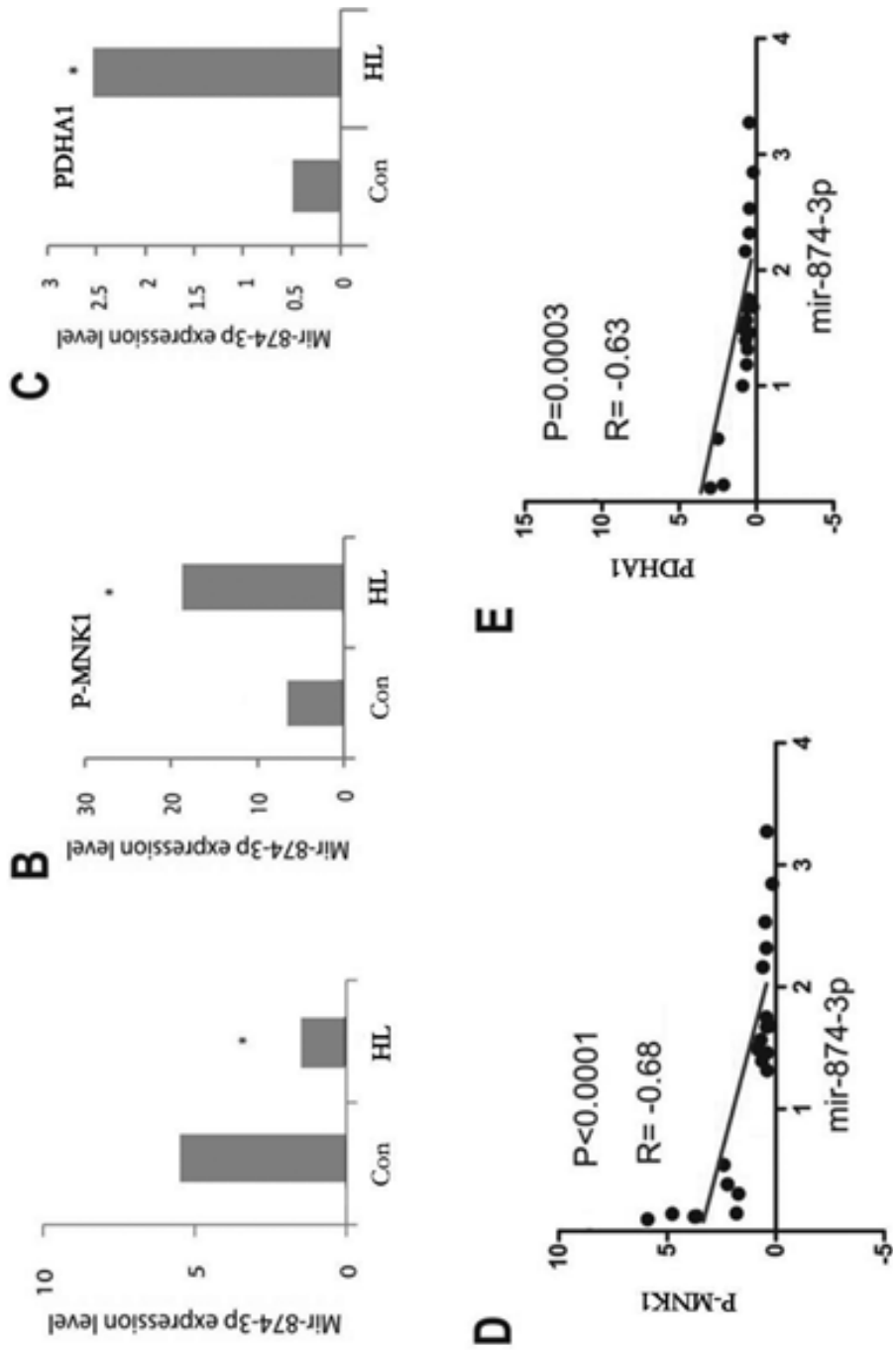

Fig. 1. MiR-874-3p expression significantly declined in tissue samples of HL patients and was negatively correlated with p-Mnk1. A: Relative expression levels of miR-874-3p in HL group (n=28) and control group (n=26); B and C: relative expression levels of p-Mnk1 and PDHA1 in HL group (n=28) and control group (n=26); D and E: miR-874-3p and p-Mnk1 and PDHA1 mRNA expression levels in HL patients (n=28) detected by qRT-PCR. HL: Hodgkin's lymphoma; p-Mnk1: phosphorylated mitogen-activated protein kinase-interacting kinase 1; PDHA1: pyruvate dehydrogenase E1 subunit alpha 1; qRT-PCR: quantitative reverse transcription-polymerase chain reaction
Source: Authors

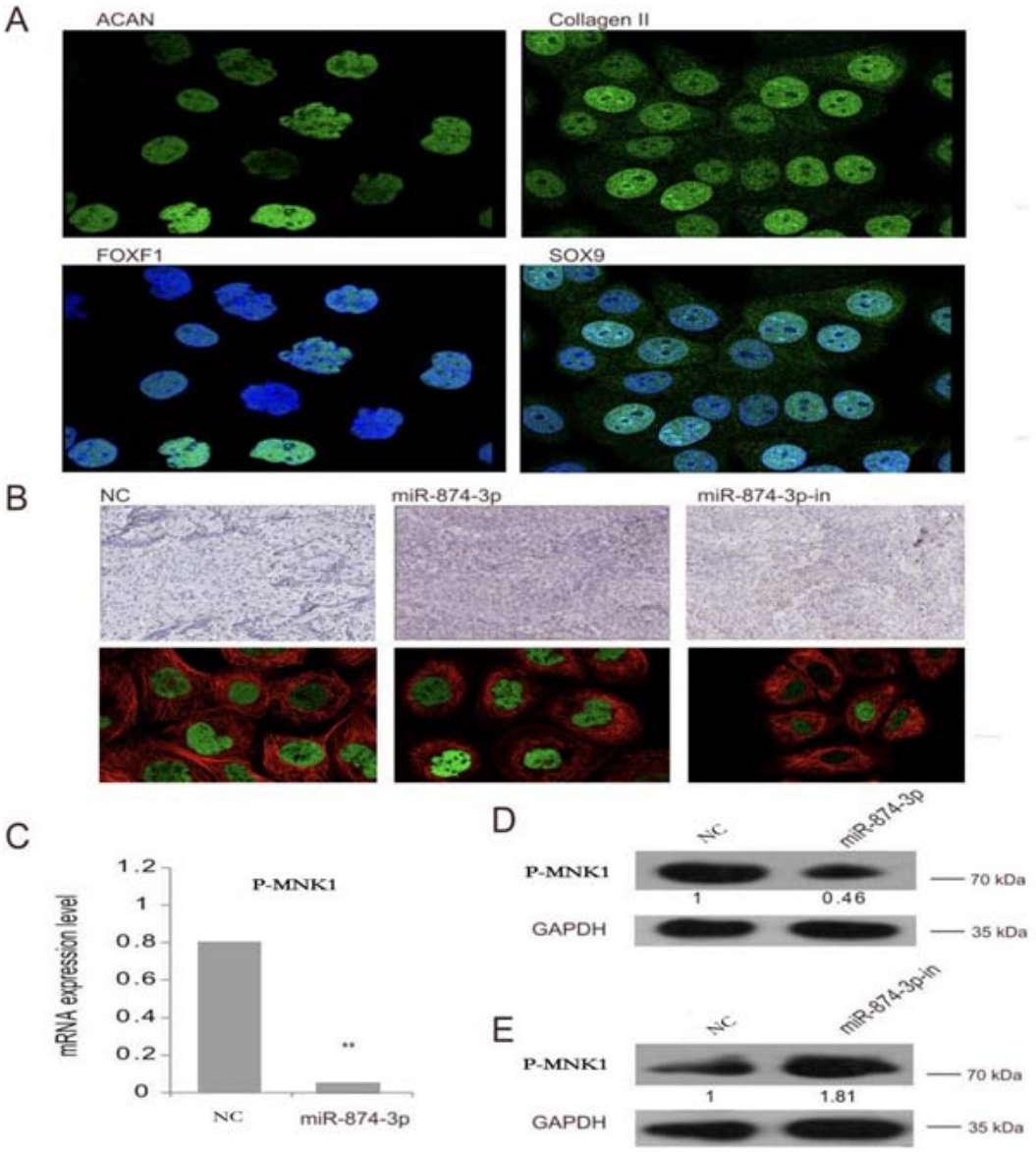

Fig. 2. MiR-874-3p down-regulated p-Mnk1 in NP cells. A: Immunofluorescence assay: incubated cells expressing NP cell markers; B: Characteristics of incubated NP cells and infection efficiency (over 80%) of NC, miR-874-3-expressed and miR-874-3p-inhibited lentiviruses; C: qRT-PCR: down-regulated expression level of p-Mnk1 after infection with lentivirus expressing miR-874-3p; D and E: WB: p-Mnk1 expression level after overexpression or inhibition of miR-874-3p. NC: Negative control; NP: nucleus pulposus; p-Mnk1: phosphorylated mitogen-activated protein kinase-interacting kinase 1; qRT-PCR: quantitative reverse transcription-polymerase chain reaction
Source: Authors

in down-regulating p-Mnk1 in cancer cells. Hence, the NP cells infected were used for the activation of the produced lentiviruses expressing and inhibiting miR-874-3p, as well as NC lentivirus, therein. The efficiency of infection was ensured to be high enough (over 80%) (Fig. 2B). Compared with NC, p-Mnk1 displayed a significantly reduced expression level subsequent to infection with lentivirus expressing miR-874-3p (Fig. 2C). In addition, WB and in-depth research confirmed down-regulation of p-Mnk1 expression by miR-874-3p in NP cells (Fig. 2D). Moreover, the p-Mnk1 expression in NP cells was increased by suppressing miR-874-3p, according to Figure 2E.

MiR-874-3p Down-regulated PDHA1 Expression in NP Cells

A negative association was found between miR-874-3p and PDHA1 expressions in NP tissues obtained from human beings (Fig. 1E). Hence, the ability of miR-874-3p to target PDHA1 in cells obtained from NP was further investigated. Firstly, miR-874-3p and PDHA1 mRNA sequences were analyzed by RNA22, an online miRNA target prediction tool. MiR-874-3p possessed sequences binding to PDHA1 mRNA (Fig. 3A). In contrast to that in the control group, down-regulated PDHA1 expression was detected following infection with lentivirus expressing miR-874-3p (Fig. 3B). Additionally, a luciferase reporter gene containing downstream PDHA1 sequence was constructed for the purpose of further exploring if miR-874-3p repressed PDHA1 by the classical miRNA target mechanism (Fig. 3C). Compared with the control group, merely the cells with PDHA1-Luc and miR-874-3p transfected concurrently had weakened luciferase activity. Moreover, the binding site of miR-874-3p predicted by PDHA1 in the luciferase reporter gene structure was mutated, and the new structure was referred to as PDHA1-Mut-Luc. The inhibiting function of miR-874-3p was partially reversed by means of mutating the binding site of miR-874-3p predicted (Fig. 3D), suggesting the binding of miR-874-3p to the site predicted by directly down-regulating PDHA1. As demonstrated by WB, as for NP cells, miR-874-3p was capable of lowering PDHA1 protein level (Fig. 3E). Besides, a larger amount of PDHA1 protein was expressed in NP cells in the case that miR-874-3p inhibitor was used for transfection, which may benefit the treatment of HL.

Importance of Abnormally Expressed miR-874-3p to HL Pathology

The result of down-regulating miR-874-3p in affecting HL occurrence was explored. WB exhibited that NP cells had an increased level of aggregin with miR-874-3p overexpressed (Fig. 4A), whereas its expression decreased as miR-874-3p was restrained (Fig. 4B-C). No evident degradation of collagen was found in the aforementioned cells with repressed miR-874-3p (Fig. 4D). The findings mentioned above demonstrated the ability of miR-874-3p to mediate the carcinogenesis of B lymphocytes by specifically targeting p-Mnk1/PDHA1. However, further research regarding the mechanism is still in need. NP cell apoptosis with lentivirus expressing miR-874-3p adopted for infection was analyzed. Compared with NC, miR-874-3p did not exert a prominent effect on NP cell apoptosis (Fig. 4E and F). Furthermore, CCK-8 assay showed that inhibiting miR-874-3p could restrain NP cells from the aspect of proliferation (Fig. 4G). Taken together, miR-874-3p was not capable of remarkably influencing NP cell apoptosis, but down-regulating its expression induced the carcinogenesis of B lymphocytes.

DISCUSSION

MiR-874-3p has been reported to have clinical implications (Song et al. 2021; Yang 2022). The dysregulation of miR-874-3p interacting with p-Mnk1 and PDHA1 has a significant function in affecting HL progression (Wang et al. 2018; Han et al. 2020; Ren et al. 2020). Although miR-874-3p possesses the ability to regulate p-Mnk1 in certain cancers (Zhu et al. 2019), no report on whether such interaction exists in NP cells has been issued. Therefore, miR-874-3p inhibited p-Mnk1 expression in NP cells according to the present study, which indicated significant inhibitory effect of miR-874-3p on HL, being consistent with a previous literature (Gaál 2022). Another important gene, PDHA1, can also induce lymphoma, and its overexpression serves as a prominent player in HL progression (Ma et al. 2020). However, the specific mechanisms of PDHA1 overexpression and PDHA1 regulatory factors in HL remain unclear. Herein, miR-874-3p was found to regulate PDHA1 in HL, while increased PDHA1 expression can be attributed to the reduction of

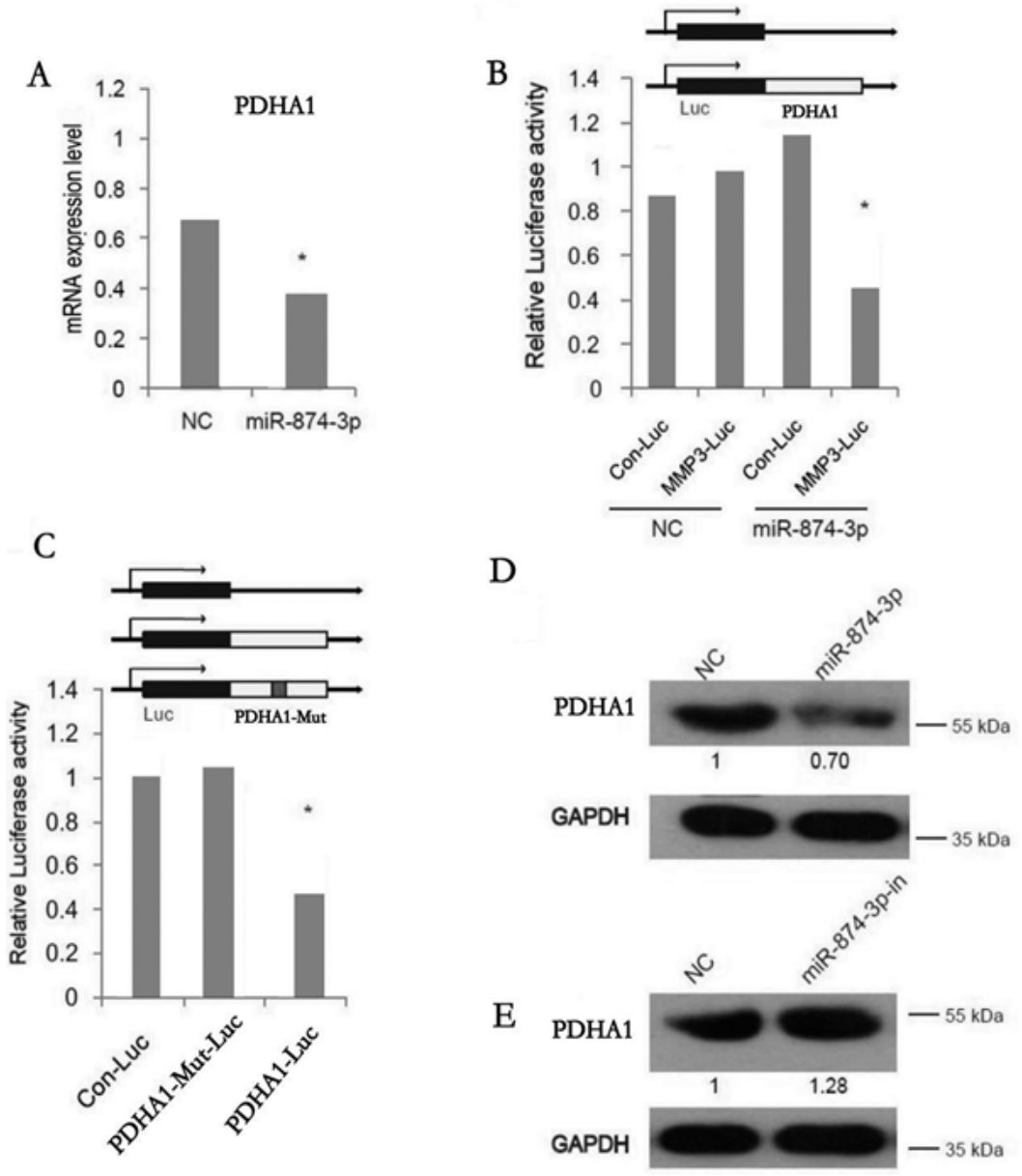

Fig. 3. MiR-874-3p down-regulated PDHA1 expression in NP cells. A: qRT-PCR results of PDHA1 mRNA expression level in NP cells after infection with lentivirus expressing miR-874-3p; B: Dual-luciferase reporter gene assay: inhibition of PDHA1 by miR-874-3p; C: Dual-luciferase reporter gene assay: suppression of PDHA1 by miR-874-3p through the predicted binding sites; D and E: WB: PDHA1 protein level in NP cells after overexpression or inhibition of miR-874-3p. NC: Negative control; NP: nucleus pulposus; PDHA1: pyruvate dehydrogenase E1 subunit alpha 1; WB: Western blotting
Source: Authors

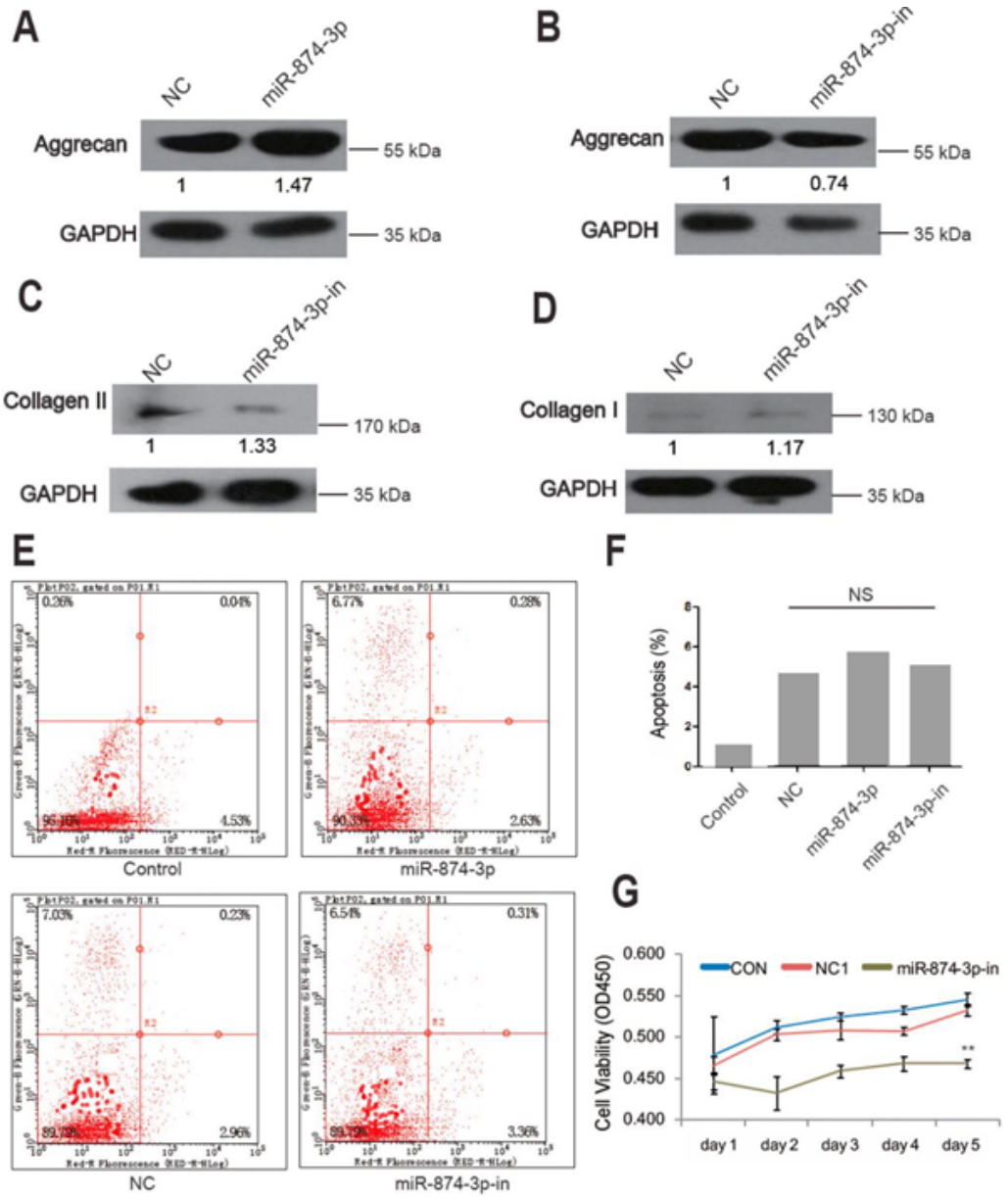

Fig. 4. Important diagnostic role of miR-874-3p in HL pathology. A: WB results of polymeric glycoprotein level in NP cells after overexpression of miR-874-3p; B-D: WB results of polymeric gel (B), collagen II (C) and collagen I (D) protein expressions in NP cells after inhibition of miR-874-3p; E: Representative scatter diagram of NP cell apoptosis after miR-874-3p overexpression or inhibition detected by flow cytometry; F: Statistical results of apoptosis detection by flow cytometry; G: Determination of NP cell viability by CCK-8 assay. HL: Hodgkin's lymphoma; NC: negative control; NP: nucleus pulposus; WB: Western blotting
Source: Authors

miR-874-3p expression in HL. However, no reports on these issues have been published so far.

In addition, miR-874-3p also facilitates NP cell proliferation (Kim et al. 2017). Collectively, miR-874-3p inhibits p-Mnk1 and PDHA1 in cells collected from NP. Besides, lowly expressed miR-874-3p increases p-Mnk1 and PDHA1 expressions, eventually resulting in the occurrence of HL (Gaál 2022). It was verified through the present study that miR-874-3p acted as a potential biomarker for HL diagnosis, being in accordance with a previous study (Noorbakhsh et al. 2021). MiR-874-3p is also crucial for the *in vivo* function of p-Mnk1/PDHA1 and HL-related phenotypes. Nevertheless, p-Mnk1/PDHA1 protein level could not be analyzed from the samples of enrolled patients because of small sample size (Ruiz-Gómez et al. 2019). Herein, miR-874-3p exerted no remarkable influence on HL, while the apoptosis of lymphocytes was an alternative classical type of pathological change of HL. Nevertheless, regarding the apoptosis attributable to such specific triggers as pro-inflammatory cytokines and other HL-associated mediators (Rosina et al. 2019), the influencing role of miR-874-3p is worthy of further research.

Targeting the NP tissues provided by HL patients in this study, miR-874-3p expression reduced dramatically, which had a pivotal function in HL, which was consistent with a previous literature (Song et al. 2019). In previous literatures, the regulation of miRNA expression has been reported to be mediated by transcriptional regulation, histone modification and application (Liu et al. 2019; Xu et al. 2019).

Both p-Mnk1 and PDHA1 exert crucial effects on cancers and inflammation besides influencing HL progression (Liu et al. 2018). Mnk-mediated B lymphocyte mutation is able to stimulate lymphoma to metastasize from the primary site (Huang et al. 2019). Notably, Mnks can degrade the main components of basement membrane, as a vital step in cell metastasis. Correlations also exist between Mnks and several chronic diseases (idiopathic interstitial pneumonia, bronchiectasis, etc.) (Miki et al. 2017). Furthermore, Mnks is involved in tumor occurrence and progression, atherosclerosis advancement as well as wound recovery (Yeh et al. 2020). Likewise, this study revealed that p-Mnk1 and PDHA1 worked as the genes targeting miR-874-3p, whereas miR-874-3p down-regulated the expressions of these two proteins.

CONCLUSION

In summary, novel regulatory factors p-Mnk1 and PDHA1 contribute to the understanding of tumor precursors. The miR-874-3p/p-Mnk1/PDHA1 axis can be taken as a latent marker concerning HL diagnosis and treatment. Although whether miR-874-3p participates in HL progression needs further validation, it indeed shows an antitumor effect.

RECOMMENDATIONS

Further studies using other cell lines, animals and clinical samples are still in need to validate that miR-874-3p, p-Mnk1 and PDHA1 are potentially eligible targets for curing other tumors.

ABBREVIATIONS

CCK-8: Cell counting kit-8
HL: Hodgkin's lymphoma
IVD: intervertebral disc
LV: lentivirus
NC: negative control
NP: nucleus pulposus
p-Mnk1: phosphorylated mitogen-activated protein kinase-interacting kinase 1
PDHA1: pyruvate dehydrogenase E1 subunit alpha 1
qRT-PCR: quantitative reverse transcription-polymerase chain reaction
WB: Western blotting

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