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MicroRNA-584-5p Restrains the Viability of Lung Cancer Cells through Targeting DPY30 Directly

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KEYWORDS Cell Viability. Dpy-30 Histone Methyltransferase Complex Regulatory Subunit. Ki67. MicroRNA-584-5p. Proliferating Cell Nuclear Antigen

ABSTRACT Dysregulation of microRNAs (miRNAs) in lung cancer participates in lung cancer progression. This study was focused on investigating regulatory mechanism of miR-584-5p in lung cancer cells. Differential miR-584-5p and DPY30 expressions in samples of lung carcinoma were evaluated using ENCORI and GEPIA (http://gepia.cancer-pku.cn/). Using RT-qPCR, miR-584-5p was detected to be downregulated in lung cancer cells. MiR-584-5p overexpression inhibited H460 cell viability and suppressing Ki67 and PCNA protein expressions. Moreover, DPY30 was found to be targeted by miR-584-5p using luciferase reporter test, which was upregulated in lung cancer cells. Furthermore, overexpressed DPY30 restrained effects of miR-584-5p mimics, causing accelerated H460 cell viabilities and upregulated Ki67 and PCNA protein expressions. In H460 cells, miR-594-5p suppressed cell viability and inhibited protein expressions of Ki67 and PCNA through binding DPY30.

INTRODUCTION

Lung cancer, a malignant tumor that is diagnosed most frequently, causes the most of cancer-related deaths (Nasim et al. 2019). It presents huge threats to human life and health and concerns are shown in fighting against the disease. Many studies have shown that smoking habits, polluted environment, and obstructive pulmonary diseases are key risk factors of this malignancy (Dai et al. 2017; Schabath and Cote 2019; Wang et al. 2019). Surgery is currently the most effective treatment in dealing with this disease in early stage, but the biggest challenge is that most cases of lung cancer are normally found at critical stages (Hoy et al. 2019). Additionally, radical radiotherapy, genetically targeted treatments and immunotherapy of lung cancer have been improved to treat lung cancer, in which radiotherapy has been used for all stages of this disease (Jones and Baldwin 2018; Vinod and Hau 2020). With the development of genetic technology, dysregulated noncoding RNAs have been discovered to participate in lung cancer progression (Ghafouri-Fard et al. 2020).

MicroRNAs (miRNAs) are endogenously expressed noncoding RNAs that are about 19-23 nucleotides (Li et al. 2018). Evidence has verified that miRNAs participated in biological progressions of disorders through targeting mRNAs (Du et al. 2018). Altered miRNA expressions have also been discovered in malignancies including lung cancer (Iqbal et al. 2019). MiR-584-5p was discovered to express differentially in several human malignancies such as human glioblastoma (Cao et al. 2021), medulloblastoma (Abdelfattah et al. 2018) and neuroblastoma (Xiang et al. 2015). MiR-584-5p acts differentially in different kinds of tumors. MiR-584-5p upregulation inhibited gastric cancer cell growth but facilitated apoptosis by targeting WWP1 (Li et al. 2017). MiR-584-5p was shown to restrain neuroblastoma cell viability, invasion and migratory ability by interplaying with matrix metalloproteinase 14 (Xiang et al. 2015). In medulloblastoma, miR-584-5p was shown to suppress cell growth and to increase the survival rate of mice, which also caused DNA damage and arrested cell cycle through binding eIF4E3 and HDAC1 (Abdelfattah et al. 2018). MiR 584 5p was detected to

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be suppressed in tissues of lung cancer (Zhou et al. 2017), which gives us an urge to explore its impacts on lung cancer.

DPY30 is a complex of SET domain containing 1 (SET1) and mixed-lineage leukemia (MLL), which was mainly located in cell nucleuses (Lee YT et al. 2021). It was promoted in gastric cancer samples and cells and facilitated proliferation and migratory and invasive abilities (Lee et al. 2015). Besides that, DPY30 expression was increased in cervical squamous cell carcinoma samples, which facilitated proliferation, invasiveness, migratory ability and EMT via Wnt/â-catenin signaling pathway (He et al. 2019). Nevertheless, no study has reported roles of DPY30 in lung cancer. Based on ENCORI (https://starbase.sysu.edu.cn/), putative binding spots were displayed of DPY30 with miR-584-5p. Hence, the researchers hypothesized that miR-584-5p might modulate lung cancer cell progressions via binding DPY30 directly.

Objective

This study aimed at probing impacts of miR-584-5p and DPY30 on modulating viabilities of H460 cells and their effects on Ki67 and PCNA protein expressions.

Experimental

Main Reagents

DMEM medium (Gibco, USA), FBS (Gibco), penicillin/streptomycin (Gibco), pcDNA3.1 vector (RiboBio, China), NC mimics (GenePharma, Shanghai, China), miR-584-5p mimics (GenePharma), Lipofectamine 3000 (Invitrogen, USA), Trizol reagent (Beyotime, Shanghai, China), SYBR Green (Beyotime), TaqMan MicroRNA Assay kit (Applied Biosystems, USA), Transcriptor One-Step RT-PCR Kit (Sigma Aldrich, USA), CCK-8 (Beyotime), pmiRGLO vector (Promega, USA), RIPA buffer (Beyotime), SDS-PAGE (Beyotime), anti-Ki67 (ab92742, Abcam, UK), anti-PCNA (ab92552, Abcam), anti-GAPDH (ab181603, Abcam), TBST (Beyotime), goat anti-rabbit IgG H&L (HRP) (ab6721, Abcam).

METHODOLOGY

Cell Culture

Lung cancer cell lines (H522, A549 and H460) and BEAS-2B (human normal lung epithelial cells) were obtained from ATCC (USA). Afterwards, DMEM medium added with 10 percent FBS, 1 percent penicillin/streptomycin (Gibco, USA) were applied for cultivation of cells at 37°C with 5 percent CO_2.

Cell Transfection

NC mimics and miR-584-5p mimics were offered by GenePharma (Shanghai, China). Overexpression plasmid of DPY30 (oeDPY30) were synthesized using pcDNA3.1 vector (RiboBio, China). Before transfection, 6-well plates were used to incubate H460 cells (5×10^5 cells/well). When cell confluence reached 85 percent, transfection was performed with Lipofectamine 3000 (Invitrogen, USA) based on protocols from manufacturers. RT-qPCR assay was done to assess RNA expressions after transfection for 48h.

RT-Qpcr

To isolate total RNA, Trizol reagent (Beyotime, Shanghai, China) was used for lysing cultured cells. The cDNA of miR-584-5p was synthesized using the TaqMan MicroRNA Assay kit (Applied Biosystems, USA). Meanwhile, cDNA of DPY30 was performed using Transcriptor One-Step RT-PCR Kit (Sigma Aldrich, USA). Thereafter, CFX Opus Real-Time PCR System (Bio-Rad, USA) with SYBR Green (Beyotime) was applied for PCR. U6 and GAPDH were applied for normalizing miR-584-5p and DPY30, respectively. Sequences about primers were shown: miR-584-5p, F 5'-TTATGGTTTGCCTGGGACTGAG-3' and R 5'-GCGAGCACAGAATTAATACGAC-3'(Li et al. 2017); U6, F 5'-ATTGGAACGATACAGAGAAGATT-3' and R 5'-GGAACGCTTCACGAATTTG-3'(Xu et al. 2019); DPY30, F 5'-AACGCAGGTTGCAGAAAATCCT-32 and R 5'-TCTGATCCAGGTAGGCACGAG-3'(Hong et al. 2020) and GAPDH, F 5'- CATCACCATCTTCCAGGAGCG-3' and R 5'-TGACCTTGCCCACAGCCTT-3'(Zhou et al. 2020). MiR-584-5p and DPY30 relative RNA expressions were checked using the $2^{-\Delta\Delta Ct}$ method.

CCK-8 Assay

Cells were harvested followed by inoculation in a 96-well plate (4×10^3 cells/well). Cells were kept culturing at 37^0C and 5 percent CO_2 for 24h, 48h

and 72h. 10µl CCK-8 (Beyotime) was used for cultivation of cells in wells for another 1h. The OD (optical density) value was evaluated at 450nm wavelength by EnSight Multimode Microplate Reader (PerkinElmer, USA).

Luciferase Reporter Test

To analyze interplay of DPY30 with miR-584-5p, ENCORI (https://starbase.sysu.edu.cn/) offered putative binding spots between them. Cells were cultivated in a 24-well plate (1×10^5 cells per well). Meanwhile, pmiRGLO vector (Promega, USA) were used for the insertion of wild or mutated types of DPY30 (DPY30-wt/mut). Thereafter, in H460 cells, co-transfection of NC/miR-583-5p mimics or DPY30-wt/mut were performed through Lipofectamine 3000. Then, dual-Luciferase Reporter Assay system (Promega) evaluated fluorescence.

Western Blot

Proteins were segregated by RIPA buffer (Beyotime) followed by isolation with SDS-PAGE (10%, Beyotime). Thereafter, protein was shifted onto PVDF membranes and covered using 5 percent non-fat milk powder for 1h at 25^0C. Anti-Ki67 (ab92742, 1: 1000, Abcam, UK), anti-PCNA (ab92552, 1:1000) and anti-GAPDH (ab181603, 1:1000) were cultured with membranes at 4°C overnight. After rinsed by TBST (Beyotime), goat anti-rabbit IgG H&L (HRP) (ab6721; 1:800) was applied for cultivation with membranes at 25! for 1h. GAPDH was served for the normalization of DPY30. Image J (NIH, USA) was applied for the detection of protein expressions.

Statistical Analysis

Data is given as the mean ± SD. Analysis was done by SPSS 19.0 (IBM, USA) and GraphPad Prism 9 (GraphPad, USA). A Student's t-test analyzed differences in two groups. One-way ANOVA compared differences among three or more groups. $P < 0.05$ was meaningful in statistics.

RESULTS

MiR-584-5p Was Suppressed in Lung Cancer Tissue Samples and Cells

According to the ENCORI Pan-Cancer analysis, lung cancer samples had lower miR-584-5p expressions than normal ones (Fig. 1A). RT-qPCR then detected miR-584-5p expressions in A549, H460 and H522 and normal BEAS-2B cells. Results indicated decreased miR-584-5p expressions in those three cancer cells (Fig. 1B, $^{**}P<0.05$). Therefore, miR-584-5p was suppressed in both tissue samples and cells of lung cancer.

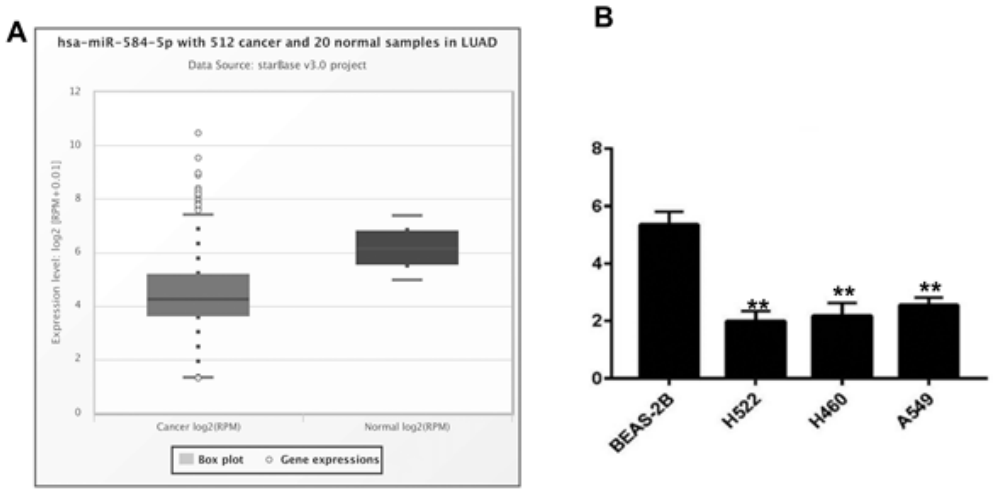

Fig. 1. MiR-584-5p low expressed in lung cancer samples and cells.
A: Relative miR-584-5p expression in lung cancer samples and normal ones.
B: MiR-584-5p expressions in BEAS-2B, A549, H522 and H460 cells, $^{**}P<0.05$

Overexpressed miR-584-5p Restricted Lung Cancer Proliferation

After miR-584-5p expressions were evaluated, NC/miR-584-5p mimics were used to analyze its effects, which revealed a higher miR-584-5p expression after overexpression (Fig. 2A, $^{**}P<0.05$). Furthermore, CCK-8 assay examined lung cell viability after transfection, showing that the viability of H460 cells was hampered by miR-584-5p mimics (Fig. 2B, $^{**}P<0.05$). Researchers further validated protein expressions related to proliferation by western blot, indicating that Ki67 and PCNA were inhibited with the upregulation of miR-584-5p (Fig. 2C, D, E, $^{**}P<0.05$). Results implied a repressive impact of miR-584-5p on regulating proliferative capacity of lung cancer cells.

DPY30 Was Targeted Directly by MiR-584-5p

After effects of miR-584-5p were examined, underlying mechanism of this miRNA was analyzed. ENCORI revealed a mRNA DPY30, which had putative binding spots with miR-584-5p (Fig. 3A). Thereafter, luciferase reporter test was used to confirm their binding, showing a lower fluorescence in DPY30-wt with miR-584-5p mimics group than others (Fig. 3B, $^{**}P<0.05$). Based on the Pan-Cancer analysis of ENCORI, the Co-Expression Analysis revealed a negative interaction between

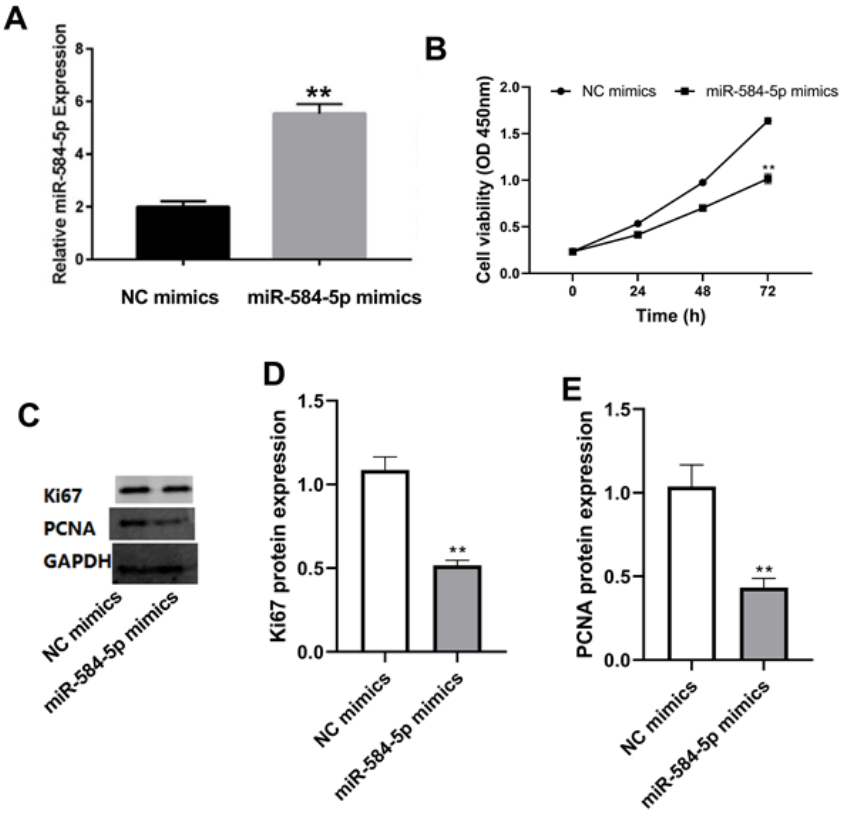

Fig. 2. MiR-584-5p upregulation reduced H460 cell proliferation
A: MiR-584-5p expression after upregulation were evaluated through RT-qPCR, $^{**}P<0.05$
B: MiR-584-5p overexpression regulating H460 cell viability were validated using CCK-8, $^{**}P<0.05$
C, D, E: Ki67 and PCNA protein expressions in H460 cells with miR-584-5p mimics, $^{**}P<0.05$

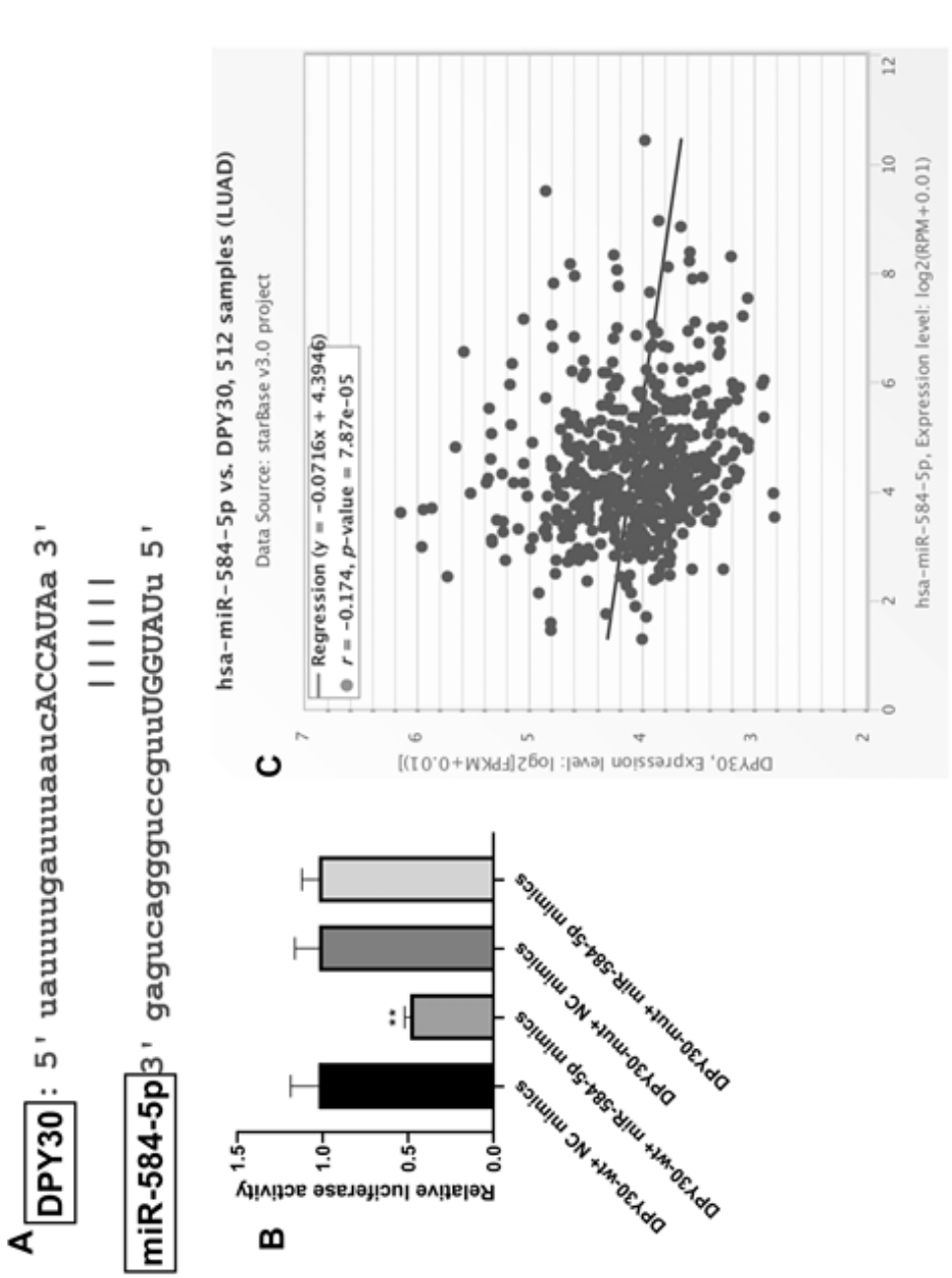

Fig. 3. MiR-584-5p bound DPY30 in lung cancer cells
A: Binding spots between DPY30 and miR-584-5p were provided by ENCORI (https://starbase.sysu.edu.cn/)
B: Luciferase reporter test evaluated fluorescence in NC/miR-584-5p mimics with DPY30-wt/mut in H460 cells, $^{**}P<0.05$
C: Pan-Cancer analysis of ENCORI offered miR-584-5p and DPY30 co-expression

these two (Fig. 3C). Hence, miR-584-5p might interplay with DPY30 in lung cancer cells.

DPY30 Expression is Higher in Lung Cancer Samples and Cells

After correlation of these two genes were examined in lung cancer samples, DPY30 expression was evaluated later. According to data of GEPIA (http://gepia.cancer-pku.cn/), DPY30 mRNA expression was upregulated in lung cancers (Fig. 4A). Thereafter, its mRNA expression in cancer cells and normal cells were detected, indicating that H460, H522 and A549 cells had higher DPY30 expressions than the BEAS-2B cells (Fig. 4B, $^{**}P<0.05$). Therefore, DPY30 was upregulated in lung cancer tissue samples and cells.

Upregulated DPY30 Restrained Impacts of miR-584-5p

The researchers then evaluated the interplay between DPY30 and miR-584-5p in H460 cells, showing that miR-584-5p overexpression downregulated DPY30 expression, which was then reversed by the overexpressed DPY30 (Fig. 5A, $^{**}P<0.05$). Meanwhile, miR-584-5p mimics reduced H460 cell viability, which was then restored to be accelerated by DPY30 overexpression (Fig. 5B, $^{**}P<0.05$). Beyond that, low expressions of Ki67 and PCNA caused by miR-584-5p mimics were both reversed after transfected with DPY30 overexpression (Fig. 5C, D, E, $^{**}P<0.05$).

DISCUSSION

Using bioinformatic tools and cellular approaches, the researchers have examined that miR-584-5p was suppressed while DPY30 expressions were promoted in lung cancer tissue samples and cells. The researchers also found that miR-584-5p restrained cell viability and inhibited Ki67 and PCNA protein expressions through binding DPY30 directly.

Several studies have linked the development of lung cancer to abnormal behavior of miRNAs (Jiang et al. 2018; Yang et al. 2018). MiRNAs can be connected to being the source of cancer by affecting several facets of cancer biology, which means that studies on cancer-associated miRNAs are necessary to provide new ways about investigating their roles in progressions of lung cancer. MiRNAs have been discovered to modulate continuance and growth of lung cancer (Li X et al. 2019;

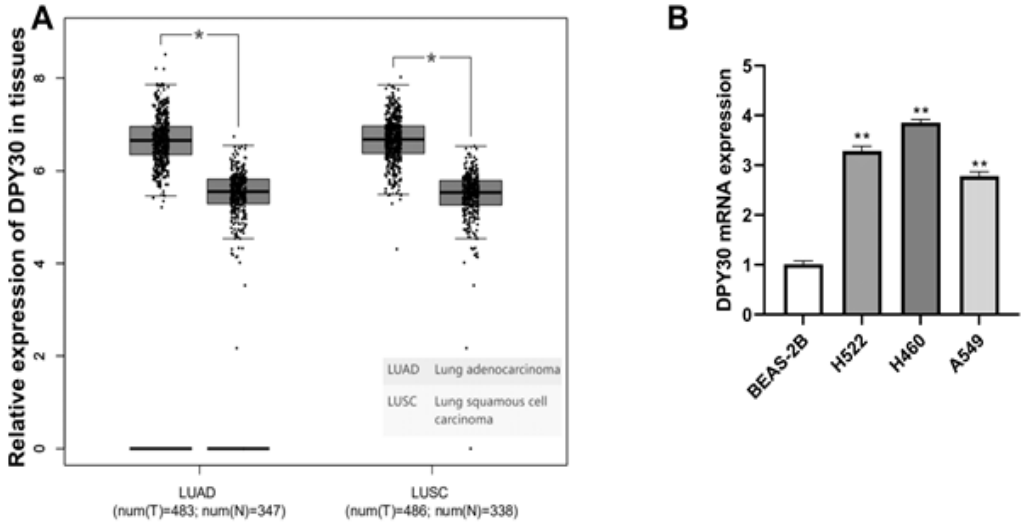

Fig. 4. DPY30 was promoted in lung cancer samples and cells
A: DPY30 mRNA expression in lung cancer samples were shown by GEPIA
B: DPY30 mRNA expression in BEAS-2B, H460, H522 and A549 cells were validated by RT-qPCR, $^{**}P<0.05$

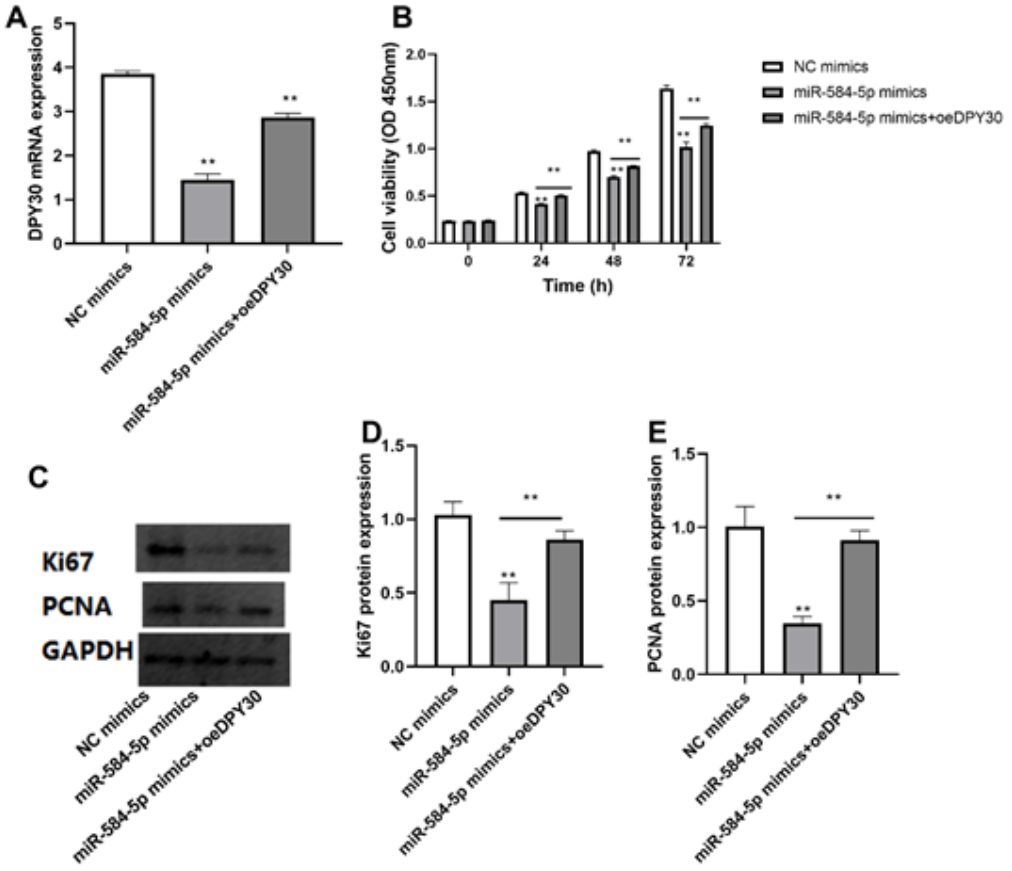

Fig. 5. DPY30 overexpression hampered impacts of miR-584-5p
A: DPY30 mRNA expression were assessed by RT-qPCR after transfection of overexpressed miR-584-5p or oeDPY30 with miR-584-5p mimics, $^{**}P<0.05$
B: CCK-8 evaluated cell viability with upregulated miR-584-5p and DPY30, $^{**}P<0.05$
C, D, E: Ki67 and PCNA protein expressions were validated by western blot in miR-584-5p mimics group and oeDPY30 with miR-584-5p mimics group, $^{**}P<0.05$

Zhang et al. 2019). MiR-1246 overexpression restrained the invasive ability and inhibited EMT of A549 cells by binding CXCR4 (Xu et al. 2018). MiR-584-5p was prompted in hepatocellular carcinoma (Wei et al. 2019), but it hindered progressions of gastric cancer (Li et al. 2017). Additionally, miR-584-5p expression was decreased in smoking-induced lung cancer and also decreased gradually with the development of lung cancer, whose overexpression hampered invasiveness and migratory ability of lung cancer cells through targeting YKT6 (Lee SB et al. 2021). Ma et al. have discovered that miR-584-5p was sponged by circRNAABCB10, causing upregulation of E2F5, which led to accelerated progression of lung cancer (Ma et al. 2020). Researchers first examined miR-584-5p expressions in lung cancer samples and Pan-Cancer analysis in ENCORI revealed that it was low expressed in tumor tissues. Moreover, RT-qPCR also indicated low miR-584-5p expressions in lung cancer cells lines. Furthermore, its overexpression promoted miR-584-5p expression but suppressed the H460 cell proliferation.

Functions of DPY30 in lung cancer have not been elucidated but it accelerated progressions of

gastric cancer (Lee et al. 2015) and cholangiocarcinoma (Hong et al. 2020) were previously reported. Moreover, DPY30 accelerated epithelial ovarian cancer cell proliferation, invasiveness and migration, whose high expression also has positive correlation with poor prognosis of patients (Zhang et al. 2018). Researchers have demonstrated impacts of DPY30 on lung cancer cells and its interaction with miR-584-5p. GEPIA and RT-qPCR revealed that DPY30 expressed higher in lung cancer tissue samples and cells. Furthermore, miR-584-5p overexpression caused low DPY30 mRNA expression, low cell viability and downregulated Ki67 and PCNA protein expression, which were all restored by DPY30 overexpression. The researchers found this new target of miR-584-5p in lung cancer cells and clarified its oncogenic role, which might be a novel discovery.

CONCLUSION

In summary, miR-584-5p expression was low in lung cancer tissue samples and cells, which could restrain lung cancer cell viabilities via targeting DPY30 and suppressing Ki67 and PCNA. All results implied that miR-584-5p a promising biomarker to hamper lung cancer cell progression. However, the researchers still need evidence in vivo and clinical stage to support the researchers' finding.

RECOMMENDATIONS

MiR-584-5p was suppressed in lung cancer tissue samples and cells while its overexpression upregulated miR-584-5p expression but suppressed H460 cell viabilities and downregulated Ki67 and PCNA protein expressions. Then, DPY30 was detected to be targeted by miR-584-5p in H460 cells, which was also elevated in lung cancer tissue samples and cells. Moreover, DPY30 overexpression reversed effects of miR-584-5p, leading to facilitated H460 cell viabilities and promoted Ki67 and PCNA protein expressions.

ABBREVIATION LIST

miR-584-5p: MicroRNA-584-5p;
DPY30: Dpy-30 histone methyltransferase complex regulatory subunit;
PCNA: Proliferating cell nuclear antigen;
WWP1: WW domain containing E3 ubiquitin protein ligase 1;
eIF4E3: eukaryotic translation initiation factor 4E3;
HDAC1: histone deacetylase 1;
SET1: SET domain containing 1;
EMT: Epithelial-Mesenchymal Transition;
DMEM: Dulbecco's Modified Eagle Medium;
SDS-PAGE: sodium dodecyl sulfate – polyacrylamide gel electrophoresis;
RIPA: Radio Immunoprecipitation Assay;
YKT6: YKT6 v-SNARE homolog;
E2F5: E2F transcription factor 5.

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