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Role and mechanism of miR-548-3p/ DAG1 in the occurrence and malignant transformation of laryngeal carcinoma.

Jia Chen, Yu Lin Zhou, Ke Wen, Shi Huang, Nan Hou, Ling Wang and Yi Wang

Otorhinolaryngology Head and Neck Surgery, The First Affiliated Hospital of Chengdu Medical College, Chengdu, 610500, China.

Key words: laryngeal cancer; hsa-miR-548-3p; DAG1.

Abstract. The AMC-HN-8 cell line and the primary human laryngeal epithelial cell lines were utilized in this work to explore the molecular mechanism of miR-548-3p regulating the gene DAG1 to induce the occurrence and malignant transformation of laryngeal carcinoma. Non-coding RNA miR-548-3p overexpression plasmid, interference plasmid and blank plasmid were constructed, and the plasmids were transfected into AMC-HN-8 cells, respectively. Meanwhile, a non-transfected plasmid group and a human laryngeal epithelial primary cell group were set up. Five groups of cells were named as NC (Normal control), Model, Ov-miR-548-3p, Sh-miR-548-3p and Blank-plasmid group. The luciferase reporter experiment was used to analyze the regulation characteristics of hsa-miR-548-3p on dystrophin-associated glycoprotein 1 (DAG1). Immunofluorescence was used to analyze the relative expression characteristics of the protein DAG1. The cell cloning experiment was used to analyze the proliferation characteristics of AMC-HN-8. The scratch healing test was used to analyze the migration ability of AMC-HN-8. The transwell test was used to analyze the invasion ability of AMC-HN-8. The RT-PCR was used to analyze the expression level of miR-548-3p. Western blot experiments were used to analyze the expression of protein DAG1, laminin α2 (LAMA2) and utrophin (UTRN). The luciferase report experiment and immunofluorescence test found that the expression of DAG1 and miR-548-3p are positively correlated. Cell cloning, scratching and migration experiments identified that the activity of laryngeal cancer cells was positively correlated with the expression of DAG1. The results of Western blot analysis further strengthened the above conclusions. Through carrying out research on the cellular levels, our work has demonstrated that miR-548-3p regulated the content of protein DAG1, and then further induced malignant transformation of laryngeal carcinoma.

Corresponding author. Yi Wang, Otorhinolaryngology Head and Neck Surgery, The First Affiliated Hospital of Chengdu Medical College, No. 278, Middle Section of Baoguang Avenue, Xindu District, Chengdu, 610500, China. Tel: +86-8301-6131. Fax: +86-8301-6131. E-mail: y_wang5566@126.com

Papel y mecanismo de miR-548-3p/DAG1 en la aparición y transformación maligna del carcinoma de laringe.

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Palabras clave: cáncer de laringe; hsa-miR-548-3p; DAG1.

Resumen. En este trabajo se utilizaron la línea celular AMC-HN-8 y la línea celular epitelial laríngea humana primaria, para explorar el mecanismo molecular regulador del miR-548-3p sobre el gen DAG1 para inducir la aparición y la transformación maligna del carcinoma laríngeo. Se construyeron el plásmido de sobreexpresión de miR-548-3p de ARN no codificante, el plásmido de interferencia y el plásmido en blanco, y los plásmidos se transfectaron en células AMC-HN-8 respectivamente. Mientras tanto, se establecieron un grupo de plásmidos no transfectados y un grupo de células primarias epiteliales laríngeas humanas. Se nombraron cinco grupos de células como NC (control normal), modelo, Ov-miR-548-3p, Sh-miR-548-3p y grupo de plásmido en blanco. El experimento indicador de luciferasa se utilizó para analizar las características de regulación de hsa-miR-548-3p en la glicoproteína 1 asociada a distrofina (DAG1). Se utilizó inmunofluorescencia para analizar las características de expresión relativa de la proteína DAG1. El experimento de clonación celular se utilizó para analizar las características de proliferación de AMC-HN-8. Se utilizó la prueba de cicatrización por rascado para analizar la capacidad de migración de AMC-HN-8. La prueba de transwell se utilizó para analizar la capacidad de invasión de AMC-HN-8. Se utilizó RT-PCR para analizar el nivel de expresión de miR-548-3p. Se usó un experimento de transferencia Western (Western blot) para analizar las expresiones de la proteína DAG1, laminina α2 (LAMA2) y utrofina (UTRN). El experimento de reporte de luciferasa y la prueba de inmunofluorescencia encontraron que la expresión de DAG1 y miR-548-3p están positivamente correlacionadas. Los experimentos de clonación celular, rascado y migración, identificaron que la actividad de las células cancerosas de laringe se correlacionó positivamente con la expresión de DAG1. Los resultados del análisis de transferencia Western fortalecieron aún más las conclusiones anteriores. A través de la investigación a nivel celular, nuestro proyecto ha demostrado que miR-548-3p regula el contenido de la proteína DAG1 y luego induce la transformación maligna del carcinoma de laringe.

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INTRODUCTION

Laryngeal cancer is a common malignant tumor among head and neck tumors. According to statistics from various places in China, it accounts for 7.9% to 35% of malignant tumors in the ear, nose and throat, and ranks third in head and neck malignancies. The treatment of laryngeal cancer in China is still based on surgery [1]. Surgical resection combined with radiotherapy and chemotherapy is mainly used. Laryngeal cancer is di-

vided into primary and secondary [2]. Primary laryngeal carcinoma refers to tumors whose primary site is in the larynx, and squamous cell carcinoma is the most common. Secondary laryngeal cancer refers to the metastasis of malignant tumors from other parts to the larynx, which is relatively rare [3]. Symptoms of laryngeal cancer are mainly hoarseness, dyspnea, cough, dysphagia, neck lymph node metastasis, etc.[4].

The function of DAG1 is to participate in the assembly of laminin and basement membrane, muscle membrane stability, cell survival, peripheral nerve myelination, lymph node structure, cell migration and epithelial polarization [5,6].

miRNA is a type of endogenous small RNA with a length of about 20-24 nucleotides, which has a variety of important regulatory effects in cells. Each miRNA can have multiple target genes, and several miRNAs can also regulate the same gene. The high degree of conservation of miRNA is closely related to the importance of its function. miRNA is closely related to the evolution of its target genes, and studying its evolutionary history helps to further understand its mechanism and function [7]. miR-548 is a larger, poorly conserved primate-specific miRNA gene family, consists of 69 human miR-548 genes located in almost all human chromosomes, and the miR-548 gene family enrichment pathway analysis showed they played important roles in various human diseases [8]. miR-548c is a member of miR-548 and the mature miR-548c-3p is obtained from it.

miR-548c-3p is low expressed in hypopharyngeal carcinoma tissues and cell lines, inhibits the proliferation, cloning, migration and invasion of FaDu cells, and promotes cell apoptosis [9]. Its expression pattern is consistent with the expression pattern of tumor suppressor genes [10]. miR-548c-3p targets *TP53BP2*, and the molecular axis of miR-548-3p/*TP53BP2* affects the biological functions of hypopharyngeal carcinoma cells such as proliferation, colonization, migration, invasion, cycle and apoptosis [11].

To explore the role and mechanism of miR-548-3p/DAG1 in the occurrence and malignant transformation of laryngeal carcinoma, the human laryngeal carcinoma cell line AMC-HN-8 and the primary human laryngeal epithelial cell line were utilized here. We found that the non-coding RNA miR-548-3p can target and regulate the gene DAG1, and then further induce malignant transformation of laryngeal carcinoma.

MATERIALS AND METHODS

Experiment design

The culture the human laryngeal carcinoma cell line AMC-HN-8 and the primary human laryngeal epithelial cell line were strictly in accordance with the requirements of aseptic cultures. The non-coding RNA miR-548-3p overexpression plasmid, interference plasmid and blank control plasmid were constructed, and the plasmids were transfected into AMC-HN-8 cells respectively. At the same time, a group of non-transfected plasmid group and a human laryngeal epithelial primary cell group were set up. Five groups of cells were named as NC group, Model group, Ov-miR-548-3p group, Sh-miR-548-3p group and Blank-plasmid group. The luciferase reporter experiment was used to analyze the regulation characteristics of miR-548-3p on gene DAG1. Immunofluorescence was used to analyze the relative expression characteristics of the protein DAG1. The cell cloning experiment was used to analyze the proliferation characteristics of laryngeal carcinoma cell lines. The scratch healing test was used to analyze the migration ability of laryngeal cancer cell lines. The transwell test was used to analyze the invasion ability of laryngeal cancer cell lines. RT-PCR was used to analyze the expression level of miR-548-3p. A Western blot was used to analyze the expression of protein DAG1, LAMA2 and UTRN.

Luciferase reporter experiment

Recombinant plasmid preparation: a recombinant plasmid containing the gene to be

tested DAG1/miR-548-3p was prepared. The reporter gene with DAG1/ miR-548-3p label and the target gene were co-transfected for 48h. Cell processing: the dual luciferase detection kit was operated according to the protocol. Fluorescence detection: a microplate reader was used for fluorescence intensity detection, and finally data analysis was performed.

Immunofluorescence analysis

In each group of cells, 1% BSA was applied for blocking at room temperature for 30 min to block non-specific epitopes. The specific primary antibody was incubated according to the recommended instructions for the antibody and let it stand overnight in a humidified box at 4°C. The slices were taken out the next day and rewarmed at room temperature for 30 min. The corresponding immunofluorescence secondary antibody was selected and then incubated at 37°C for 30 min in the dark. The nucleus was stained with DAPI under dark conditions. Anti-fluorescence quencher was added for mounting. Finally, a fluorescence microscope was used to observe and take pictures.

Cell cloning experiment

Each group of cells were taken in the logarithmic growth phase, digested with 0.25% trypsin and pipetted into single cells, and suspended in a culture medium of 10% fetal bovine serum for later use. The cell suspension was diluted in gradient multiples, and each group of cells was inoculated into a dish containing 10 mL of pre-warmed culture medium at 37°C at a gradient density of 50, 100, and 200 cells per dish, and gently rotated to make the cells uniformly dispersed. Placed it in a cell incubator at 37°C, 5% CO_2 and saturated humidity for 2-3 weeks. It was frequently observed that when there were visible clones in the petri dish, the culture were stopped. The supernatant was discarded and washed carefully with PBS twice. To fix the cells 5 mL of 4% paraformaldehyde was added for 15 minutes. The fixative solution was then removed, an appropriate amount of GIMSA was added and the dye solution was applied to dye for 10-30 minutes, then slowly washed off with running water.

Cell scratch test

After the cells of each group were digested and counted, 8×10^5 cells were divided into 35mm^2 culture dishes. A marker was used to draw a line on the bottom of the dish as a mark, the culture medium was aspirated, and a 10 μL pipette tip was used to mark the cells in the dish perpendicularly to the marker. A rinse with PBS was used to remove the marked cells, and serum-free culture medium was added to continue culturing. Pictures were taken at 24h, and the intersection of the line drawn by the marker was selected and the cell scratch as the observation point, and then observed at a fixed point.

Cell migration test

Twenty-four hours before the experiment, the cells of different groups were replaced with serum-free medium, and the culture was continued. Before inoculation, the 24-well plate and transwell chamber were soaked with 1×PBS for 5 min to moisten the chamber. The cells were digested, washed with serum-free medium, resuspended in serum-free medium, counted and diluted to adjust the cell density to 5×10^5/ mL. Then 0.2 mL cell suspension (5×10^4 cells) were inoculated into the transwell chamber, and 0.7 mL of RPMI-1640 medium containing 10% PBS was added to the lower 24-well plate, 3 replicate holes per group, and placed in a 37°C incubator for 24 hours to finish the culture. To each well of the above cells 1 mL of 4% formaldehyde solution was added, and fixed at room temperature for 10 min. The fixative solution aspirated, washed once with 1x PBS. To each well, 1mL 0.5% crystal violet solution was added, washed with 1×PBS three times after dyeing for 30 min. A cotton swab was used to carefully wipe off the cells that have not migrated in the transwell, and placed under a 200× microscope for observation.

RT-PCR analysis

The AMC-HN-8 cell and primary human laryngeal epithelial cell were treated with TRIzol reagent to extract the total RNA in the cells. Revert Aid TW first Strand cDNA Synthesis Kit was used to synthesize the first chain of DNA. QuantiNova SyBr Green PCR Kit was used to perform PCR analysis. Reaction conditions: pre-denaturation at 95°C for 1 minute, denaturation at 95°C for 30 seconds, annealing at 60°C for 30 seconds, and elongation at 72°C for 30 seconds, running 40 cycles.

Western blot analysis

Cells were collected from each group, and 200 μL of cell lysate was added to each six-well plate. After sonication, the cells were lysed on ice for 1 hour. The lysed cell sample was centrifuged at 12,500 rpm for 15 minutes at 4°C. Then, the supernatant was transfered in the centrifuge tube to a clean centrifuge tube. β-actin protein quantification kit was used to quantify protein concentration. The measured protein samples were stored at -80°C. In Western blot electrophoresis, the protein loading concentration was 50 μg per well. After SDS-PAGE electrophoresis, the membrane was transferred and blocked. Proteins DAG1, LAMA2 and UTRN primary antibody (1: 500, anti-human, Thermo-Fisher, USA) were diluted to use concentration. The samples were incubated overnight on a shaker at 4°C. After washing with PBS, the samples were incubated with the secondary antibody (1: 1000, anti-human, Thermo-Fisher, USA) for 30 minutes at room temperature in the dark. Finally, a developer was used for development and photography.

Statistical analysis

The experimental results are expressed as means ± standard deviations. Quantitative variables between two groups were compared by the Student's *t* test (normal distribution) or Mann-Whitney *U* test (non-normal distribution), and one-way or two-way ANOVA was used for comparing multiple groups. Pearson $\chi 2$ test or Fisher's exact test were used to compare qualitative variables. Pearman's analysis was conducted for correlation analysis. Statistical analysis was performed using the SPSS 22.0 software. The figures were produced with Origin 2021 and Adobe Illustrator 2020 software.

RESULTS

miR-548-3p had a significant regulatory effect on gene DAG1

The results of this experiment are shown in Fig. 1. It can be seen from the figure that miR-548-3p was significantly increased with the expression of DAG1-wild type (WT) ($p=0.0003$) and was remarkably decreased with the expression of DAG-mutant type (MUT) ($p=0.008$), indicating that miR-548-3p has a significant regulatory effect on the gene DAG1.

The results of immunofluorescence analysis are shown in Fig. 2. The green fluorescence in the figure was emitted by the DAG1 protein A. This experiment visually indicated the difference in *DAG1* protein expression between the groups, and the experimental results were consistent with the results of the luciferase report experiment B.

Protein DAG1 induced malignant transformation of laryngeal carcinoma

The cell clone formation experiment is an important technical method used to detect cell proliferation, invasiveness, and sensitivity to killing factors. The clone formation rate reflects the two important traits of cell population dependence and proliferation ability. The results of the cell cloning experiment are shown in Fig. 3. It can be seen from the figure that when miR-548-3p was overexpressed, the number of cell clones increased significantly. When miR-548-3p was interfered, cell cloning was also inhibited. This indicated that the expression of miR-548-3p was positively correlated with the malignant degree of laryngeal cancer cells.

The results of the cell scratch test are shown in Fig. 4. Cell scratch is a typical meth-

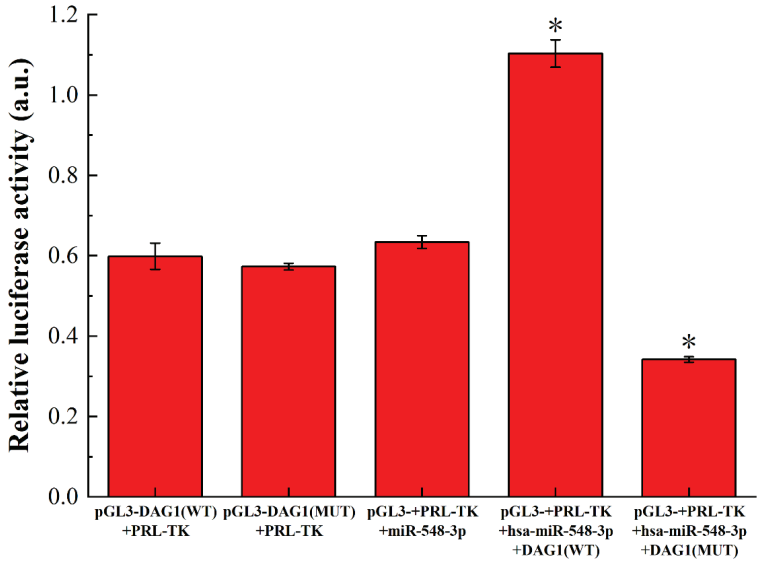

Fig. 1. The results of the dual luciferase detection experiment. miR-548-3p has a significant regulatory effect on gene DAG1 ($p<0.05$).

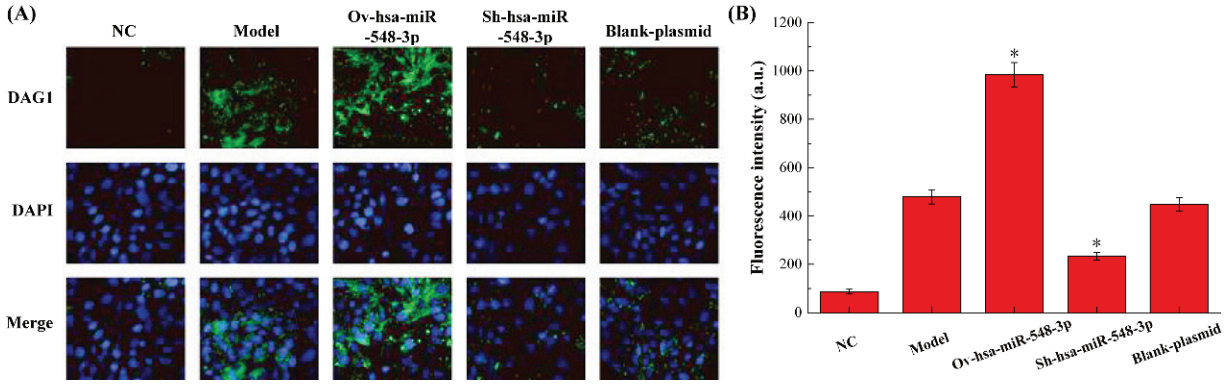

Fig. 2. The results of immunofluorescence analysis of the expression characteristics of DAG1. The expression of DAG1 protein in the Ov-miR-548-3p group and Sh-miR-548-3p group changed significantly ($p< 0.05$).

od used to detect the invasion ability of tumor cells. After 24 hours, the narrower the scratch, the stronger the cell invasion ability. From the results, we can know that overexpression of miR-548-3p significantly enhanced the migration ability of laryngeal cancer cells, while interference with the expression of miR-548-3p could inhibit the migration of laryngeal cancer cells. The blank plasmid had no effect on the expression of miR-548-3p, so the cell scratch of Model group and Blank-plasmid group was very serious.

The cell migration test is similar to the cell scratch test, which detects the strength of cell invasion. The more cells, the stronger the tumor cell's malignant metastasis and invasion ability. The results of the cell migration test are shown in Fig. 5. Comparing the results of Fig. 4 and Fig. 5, we can find that the results of the cell migration test are consistent with the results of the cell scratch test. The three experiments of cell cloning, scratching and migration all reached a common conclusion: the overexpression of miR-548-3p enhanced the malignant transformation of laryngeal carcinoma, whereas miR-548-3p knockdown lead to inhibition of malignant transformation.

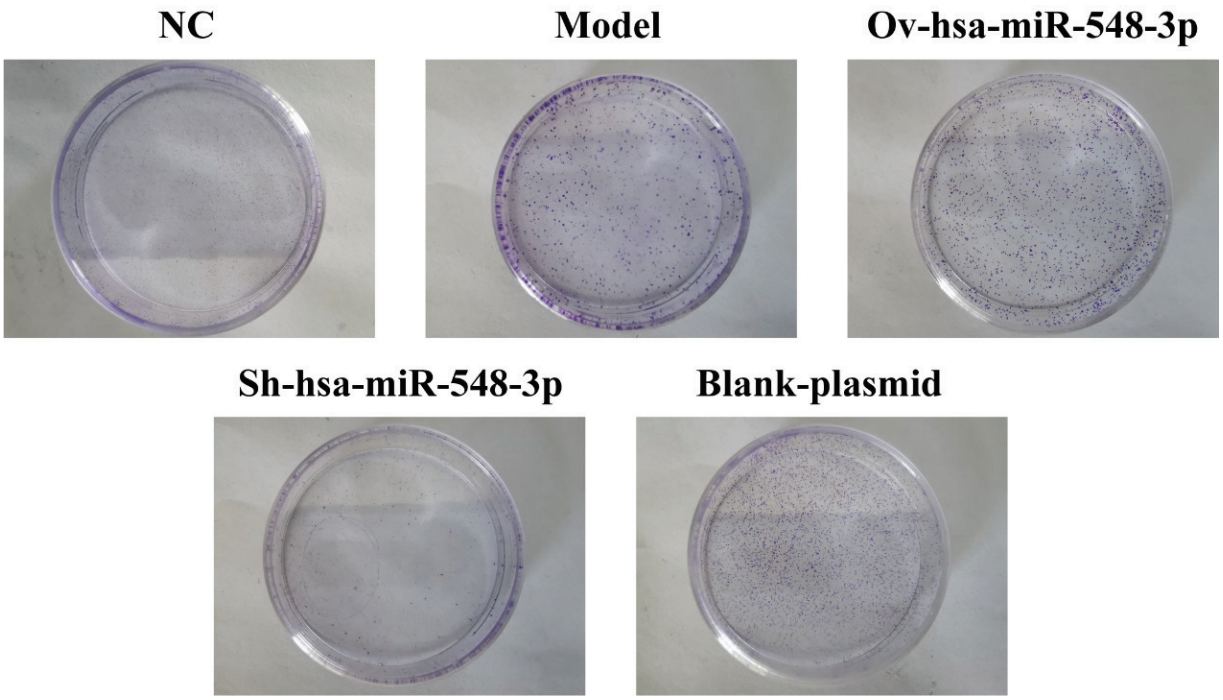

Fig. 3. The results of cell migration experiment. There was no cloning phenomenon in the NC group. The number of cell clones in the Ov-miR-548-3p group and Sh-miR-548-3p group was significantly different from that in the Model group ($p<0.05$).

Plasmids had clear effects on the expression of miR-548-3p

The results of RT-PCR analysis are shown in Fig. 6. miR-548-3p is expressed in normal cells, so there was a certain amount of miR-548-3p in the NC group. The expression of miR-548-3p in the Model group increased significantly, and the expression of miR-548-3p increased and decreased greatly through the influence of overexpression plasmid and interference plasmid. This was positively correlated with DAG1 expression and the malignant degree of laryngeal cancer cells, confirming the correlation of the three.

Expression of the protein to be analyzed showed the same trend

The results of Western blot analysis are shown in Fig. 7. The protein DAG1 is involved in the assembly of laminin and basement membrane, muscle membrane stability and cell survival. Its overexpression will improve the viability of cancer cells. The protein LAMA2 is the main component of the basement membrane and interacts closely with other extracellular matrix components. Its overexpression will increase the migration and invasion ability of cancer cells. UTRN is also a typical oncogene expressed protein. Its overexpression will increase the activity of cancer cells. Compared with model group, the expression of DAG1, LAMA2 and UTRN were significantly increased through overexpression of miR-548-3p plasmid, thus the expression of these proteins was obviously decreased with the treatment of interference plasmid. The expression of the above-mentioned proteins is positively correlated with malignant degree of laryngeal cancer cells, and the results of this experiment also show this.

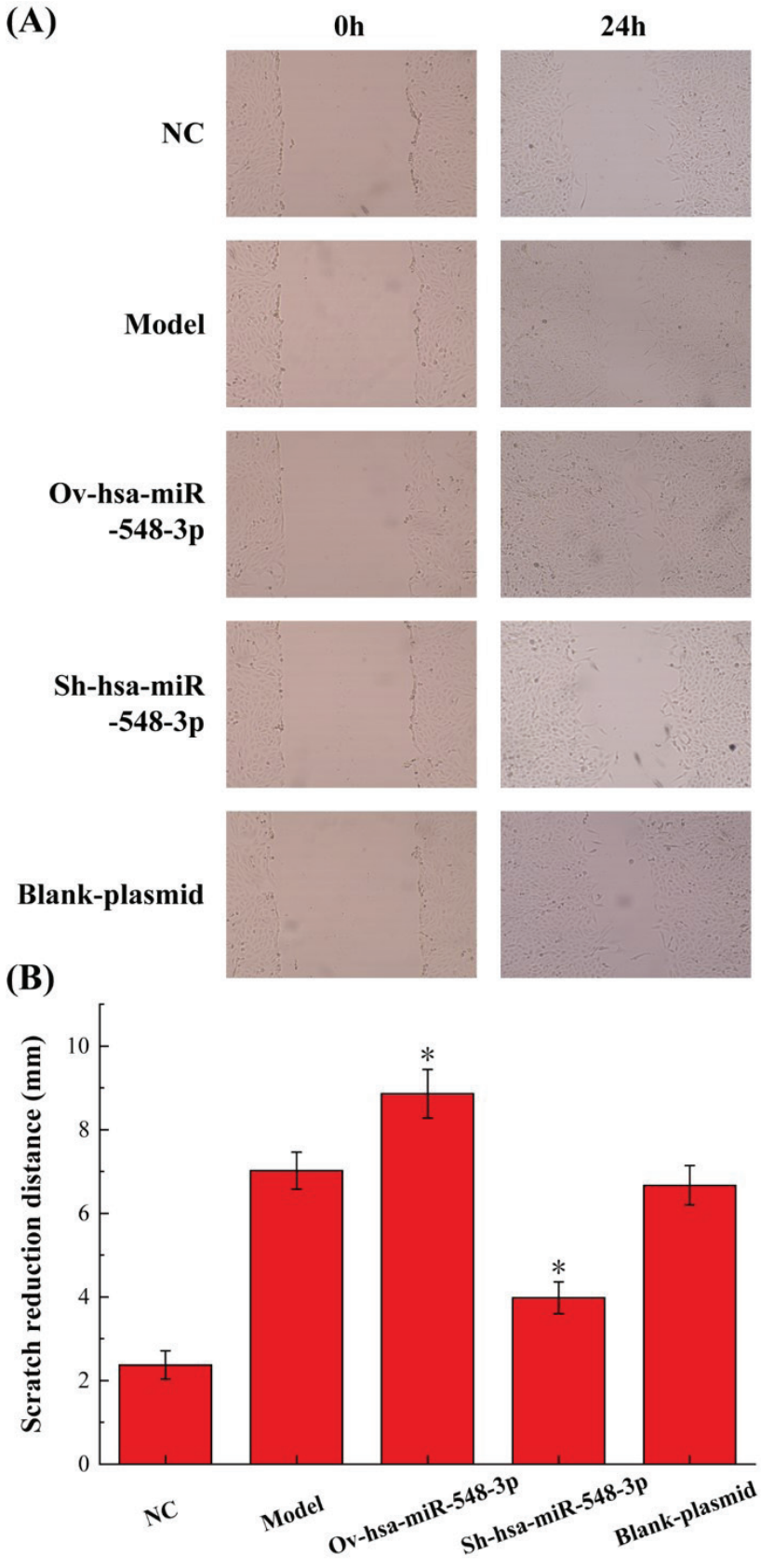

Fig. 4. The results of cell scratch test. The data of NC and Model group are consistent with the normal value.

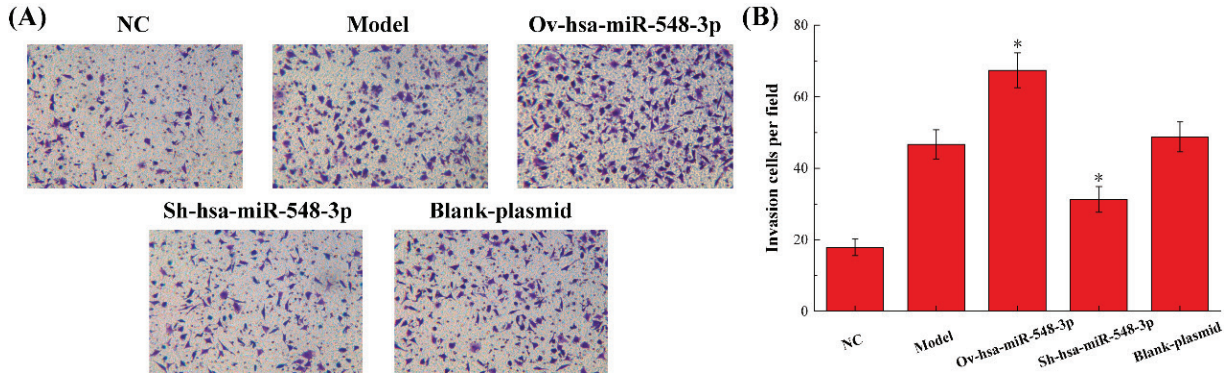

Fig. 5. The results of cell migration test. The data of NC and Model group are consistent with the normal value. The results of this experiment are consistent with the results of the cell scratch test.

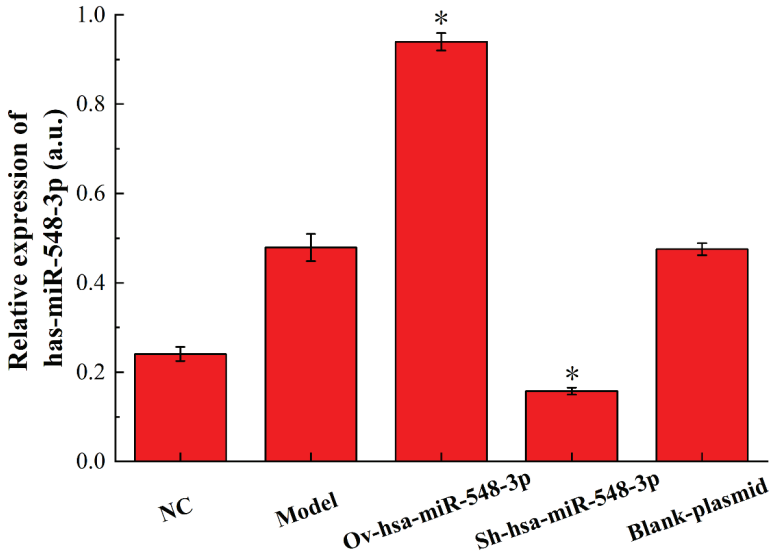

Fig. 6. The results of RT-PCR analysis. The results of this experiment are consistent with the results of the dual luciferase detection experiment and immunofluorescence analysis.

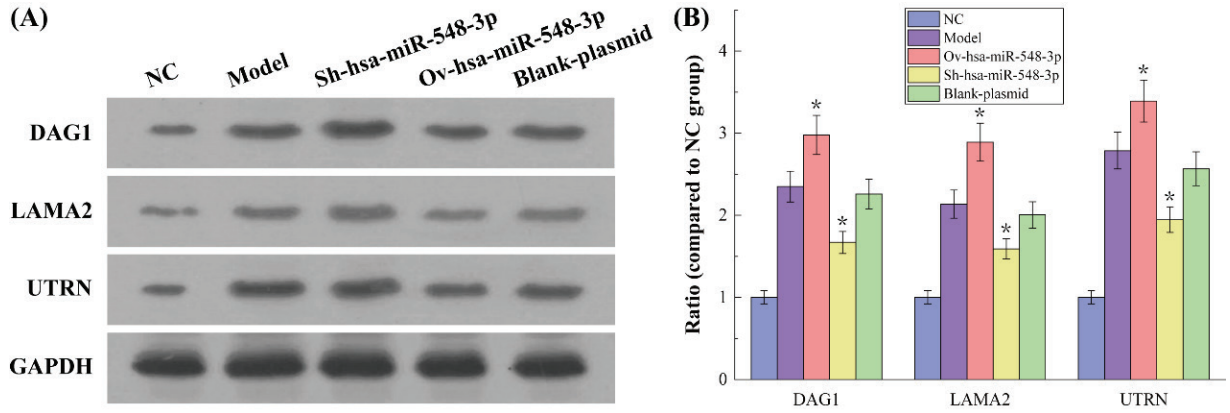

Fig. 7. The results of Western blot analysis. The data of NC and Model group are all consistent with the normal value. All the results of Ov-miR-548-3p group and Sh-miR-548-3p group were statistically different from the results of Model group ($p<0.05$). (A) Original gel electrophoresis image. (B) The ratio of protein DAG1, LAMA2 and UTRN expression compared to NC group.

DISCUSSION

The essence of malignant tumors is abnormal cell proliferation, which locally invades surrounding normal tissues, and even metastasizes to other parts of the body through the circulatory system. From the perspective of molecular pathology, the root cause of malignant tumors is the accumulation of DNA mutations. The accumulation of mutations leads to the large expression of proteins that promote cell growth, making cell cycle control abnormal [12-14]. Primary laryngeal carcinoma is a kind of squamous cell carcinoma, and its malignancy ranks in the forefront of all cancers [15-16]. This project aims to promote the development of diagnosis and treatment of laryngeal cancer by identifying the underlying molecular mechanism of laryngeal cancer exacerbation.

miR-548-3p has been shown to be widely involved in the regulation of cancer genes. Luo et al pointed out that decrease of miR-548c-3p could upregulate the expression of ITGAV and contribute to tumor progression [9]. Observation of samples from oral squamous cell carcinoma patients found that miR-548-3p was increased in the OSCC group compared with the control group [17]. This work has indicated that miR-548-3p indirectly participates in the various processes of malignant transformation of laryngeal cancer by regulating the expression of DAG1.

In skeletal muscle, the DAG1 complex works as a transmembrane linkage between the extracellular matrix and the cytoskeleton. α-DAG1 is extracellular and binds to merosin α-2 laminin in the basement membrane, while β-DAG1 is a transmembrane protein and binds to dystrophin, which is a large rod-like cytoskeletal protein, absent in Duchenne muscular dystrophy patients. Dystrophin binds to intracellular actin cables. In this way, the DAG1 complex, which links the extracellular matrix to the intracellular actin cables, is thought to provide structural integrity in muscle tissues [18,19]. The DAG1 complex is also known to serve as an agrin receptor in muscle, where it may regulate agrin-induced acetylcholine receptor clustering at the neuromuscular junction. There is also evidence which suggests the function of DAG1 as a part of the signal transduction pathway because it is shown that Grb2, a mediator of the Ras-related signal pathway, can interact with the cytoplasmic domain of DAG1 [20]. DAG1 also plays an important role in the development and progression of cancer, and the regulation of DAG1 by miR-548-3p is particularly important in the progression of laryngeal cancer.

α-DAG1 is extracellular peripheral glycoprotein that acts as a receptor for extracellular matrix proteins containing laminin-G domains and receptor for laminin-2 (LAMA2) and agrin in peripheral nerve Schwann cells. It also acts as a receptor for laminin LAMA5 [21,22]. β-DAG1 is transmembrane protein that plays important roles in connecting the extracellular matrix to the cytoskeleton, which acts as a cell adhesion receptor in both muscle and non-muscle tissues. It is a receptor for both DMD and UTRN and, through these interactions, scaffolds axin to the cytoskeleton. β-DAG1 also functions in cell adhesion-mediated signaling and implicated in cell polarity [23,24]. The direct action of DAG1 with the proteins LAMA2 and UTRN enhances the proliferation, migration and invasion ability of laryngeal cancer cells, thereby increasing the malignant degree of laryngeal cancer in all directions.

In summary, this experiment controlled the expression of miR-548-3p by adding different plasmids, and then controlled the expression of protein DAG1, and finally achieved the effect of controlling the malignant degree of laryngeal cancer. Cell cloning, scratching and migration experiments were used to determine the viability of laryngeal cancer cells. At the same time, by measuring the expression of cancer-related proteins, the regulatory effect of protein DAG1 on laryngeal cancer was further investigated. We reported the novel function of miR-548-3p in regulating laryngeal carcinoma cell mi-

gration and invasion though modulating the expression of DAG1. This provides a potential strategy for the diagnosis and treatment of laryngeal cancer. Nevertheless, more in-depth research is still needed to sterenght the conclusions of this experiment and laryngeal cancer samples are also needed to truly promote the conclusions of this work for the future diagnosis and treatment of lung cancer.

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Ethics approval and consent to participate

The research protocol has been reviewed and approved by the Ethical Committee and Institutional Review Board of The First Affiliated Hospital of Chengdu Medical College and written informed consent was obtained from all patients.

Competing interests

The authors declared that they have no competing interests.

Author's ORCID numbers

- Jia Chen: 0000-0002-9678-2523
- Yu Lin Zhou: 0000-0003-0034-093X
- Ke Wen: 0000-0002-0354-9985
- Shi Huang: 0000-0001-9811-4265
- Nan Hou: 0000-0001-9875-107X
- Ling Wang: 0000-0001-6367-2988
- Yi Wang: 0000-0001-5350-207X

Authors' contributions

Each author has made an important scientific contribution to the study and has assisted with the drafting or revising of the manuscript.

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