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LncRNA DGCR5 promotes non-small cell lung cancer progression *via* sponging miR-218-5p

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Abstract. – OBJECTIVE: Non-small cell lung cancer (NSCLC) is one of the most ordinary malignant tumors worldwide. Recent researches have proved that long noncoding RNAs (lncRNAs) play vital roles in many diseases. The aim of this study was to investigate the exact function of lncRNA DiGeorge syndrome critical region gene 5 (DGCR5) in the development of NSCLC.

PATIENTS AND METHODS: Real Time-quantitative Polymerase Chain Reaction (RT-qPCR) was utilized to detect DGCR5 expression in paired NSCLC patients' tissue samples and cell lines. The function of DGCR5 in NSCLC was detected through wound healing assay and transwell assay *in vitro*. Besides, mechanism assays were conducted to observe the interaction between DGCR5 and microRNA-218-5p (miR-218-5p).

RESULTS: DGCR5 was remarkably highly expressed in NSCLC tissues compared with that of adjacent normal tissues. The migration and invasion of NSCLC cells were significantly promoted *via* overexpression of DGCR5. Further, the silence of DGCR5 significantly inhibited NSCLC cell migration and invasion. Moreover, RT-qPCR revealed that miR-218-5p was down-regulated by overexpression of DGCR5, while miR-218-5p was up-regulated after the knockdown of DGCR5. Further experiments showed that miR-218-5p was a direct target of DGCR5 in NSCLC.

CONCLUSIONS: DGCR5 enhances NSCLC cell migration and invasion *via* targeting miR-218-5p, indicating that DGCR5 may be a potential therapeutic target for NSCLC.

Keywords:
Long noncoding RNA, DGCR5, Non-small cell lung cancer (NSCLC), MiR-218-5p.

Introduction

Lung cancer is one of the most common cancers in the world, seriously threatening human health[1]. Lung cancer mainly originates from the epithelium of the bronchial mucosa, which is also known as bronchogenic carcinoma. Approximately 80% of lung cancer is non-small cell lung cancer (NSCLC)[2]. The primary characteristic of NSCLC includes migration and invasion of neoplasms, which contributes to its high mortality rate and poor survival[3,4]. Thus, understanding the molecular mechanism of its tumorigenesis is fundamental for enhancing the efficacy of NSCLC treatment and improving the poor prognosis of NSCLC patients.

Advances in human genome sequencing have revealed that non-coding RNAs (ncRNAs) account for almost 99% of total transcribed RNAs. Long noncoding RNAs (lncRNAs) are an important subgroup of ncRNAs with more than 200 nucleotides in length. Currently, lncRNAs have been reported as key regulators in several processes of different cancers. For example, lncRNA UCA1 regulates the proliferation, migration, and invasion of human lung cancer cells by modulating microRNA-143 (miR-143) expression[5]. LncRNA SPRY4-IT1 promotes the progression of bladder cancer *via* acting as a sponge for miR-101-3p[6]. LncRNA KCNK15-AS1 regulated by ALKBH5 inhibits cell migration and motility in pancreatic cancer[7]. In addition, lncRNA SPRY4-IT1 is overexpressed in clear cell renal cell carcinoma, which can also be used to predict the prognosis of patients[8].

Previous researches have suggested that lncRNA DiGeorge syndrome critical region gene 5 (DGCR5) plays an important role in tumor biology and development. However, the exact function of DGCR5 in NSCLC has not been fully elucidated so far. Our study demonstrated that DGCR5 was remarkably up-regulated in NSCLC tissues and cell lines. Overexpression

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of DGCR5 significantly promoted the migration and invasion of NSCLC cells. However, the knockdown of DGCR5 remarkably inhibited migration and invasion of NSCLC *in vitro*. Furthermore, our findings demonstrated that the function of DGCR5 in NSCLC was associated with miR-218-5p.

Patients and Methods

Tissue Specimens

54 NSCLC patients who underwent surgery in Jinshan Hospital of Fudan University were enrolled in this study. Subsequently, human tissues were collected from these patients. This investigation was approved by the Ethics Committee of Jinshan Hospital of Fudan University. Signed written informed consents were obtained from all participants before the study. All patients were diagnosed with NSCLC by two independent pathologists without any controversies.

Cell Culture

4 NSCLC cancer cell lines (SPCA1, PC-9, and H358), and 1 normal human bronchial epithelial cell line (16HBE) were purchased from Shanghai Model Cell Bank (Shanghai, China). All cells were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640; Invitrogen, Carlsbad, CA, USA) consisting of 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA) and 1% penicillin/streptomycin. Meanwhile, cells were maintained in an incubator containing 5% CO_2 at 37°C.

Cell Transfection

Lentivirus targeting DGCR5 was first cloned into a pLenti-EF1a-EGFP-F2A-Puro vector (Biosettia, Inc., San Diego, CA, USA). The empty vector was synthesized as well. Subsequently, DGCR5 lentivirus or empty vector were transfected into NSCLC cells according to the instruction of Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA).

shRNA expressing short-hairpin RNA (shRNA) directed against DGCR5 were provided by GenePharma (Shanghai, China). Negative control shRNA was also synthesized. Next, DGCR5 shRNA or negative control shRNA were transfected into NSCLC cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA).

RNA Extraction and Real Time-Quantitative Polymerase Chain Reaction (RT-qPCR)

Total RNA was extracted from NSCLC or tumor tissues using TRIzol Reagent (TaKaRa Bio- technology Co., Ltd., Otsu, Shiga, Japan). The extracted RNA was then reverse transcribed into complementary deoxyribose nucleic acids (cDNAs) through the reverse transcription kit (TaKaRa, Otsu, Shiga, Japan). Primer sequences used for RT-qPCR were as follows: DGCR5 forward: 5′-CACACGC…CCAC-3′ and reverse: 5′-GAG…TGTA…C-3′; Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), forward: 5′-GCA…AAGGCT-GAGAAC…reverse: 5′-TGG…AAGACGC-CAGTGC…3′. The thermal cycle was as follows: pre-denaturation at 95°C for 1 min, followed by 40 cycles of 95°C for 15 s, 60°C for 30 s, and 72°C for 30 s. The relative expression of genes was calculated by the $2^{-\Delta\Delta Ct}$ method.

Wound Healing Assay

Cells were transferred into 6-well plates and cultured in RPMI-1640 medium overnight. Once scratched with a plastic tip, the cells were cultured in serum-free RPMI-1640 medium. Wound and closure was viewed at specific time points. Each assay was independently repeated for three times.

Transwell Assay

5×10^4 cells in 200 µL serum-free RPMI-1640 medium were transformed to the upper chamber of an 8 µm pore size insert (Millipore, Billerica, MA, USA) coated with or without 50 µg Matrigel (BD Biosciences, Franklin Lakes, NJ, USA). Meanwhile, the lower chamber was added with RPMI-1640 and FBS. 48 h later, after wiped by cotton swab, the top surface of chambers was immersed with pre-cooled methanol for 10 min. Then, they were stained with crystal violet for 30 min. The number of migrated cells was counted under a light microscope (Olympus, Tokyo, Japan).

Luciferase Reporter Gene Assay

DIANA LncBase Predicted v.2 was used to predict the potential target gene and fragment sequences containing DGCR5 reaction sites. The luciferase reporter gene assay kit (Promega, Madison, WI, USA) was used to detect luciferase activity of NSCLC cells. A luciferase reporter gene vector was constructed and transfected into NSCLC cells. DGCR5 3′-untranslated region

(3′-UTR) wild-type (WT) sequence named DGCR5-WT was 5′-GGUGAGGAUCUUAUUACUGUA-3′, and mutant sequence of DGCR5 3′-UTR missing the binding site with miR-218-5p named DGCR5-MUT was 5′-GGUGAGGAUCUUAUAUGACAU-3′.

RNA Immunoprecipitation (RIP) Assay

To confirm the endogenous relation between DGCR5 and miR-218-5p, the RNA immunoprecipitation (RIP) assay was carried out in strict accordance with EZMagna RIP RNA-binding protein immunoprecipitation kit (Millipore, Billerica, MA, USA). Transfected NSCLC cells were collected and lysed with RIP lysis buffer containing protease inhibitor and RNase inhibitor. Subsequently, cells were incubated with RIP buffer containing magnetic beads coated with Ago2 antibodies (Millipore, Billerica, MA, USA). IgG was used as a negative control (input group). After incubation for 2 h at 4°C, co-precipitated RNAs were isolated and measured by the RT-qPCR analysis.

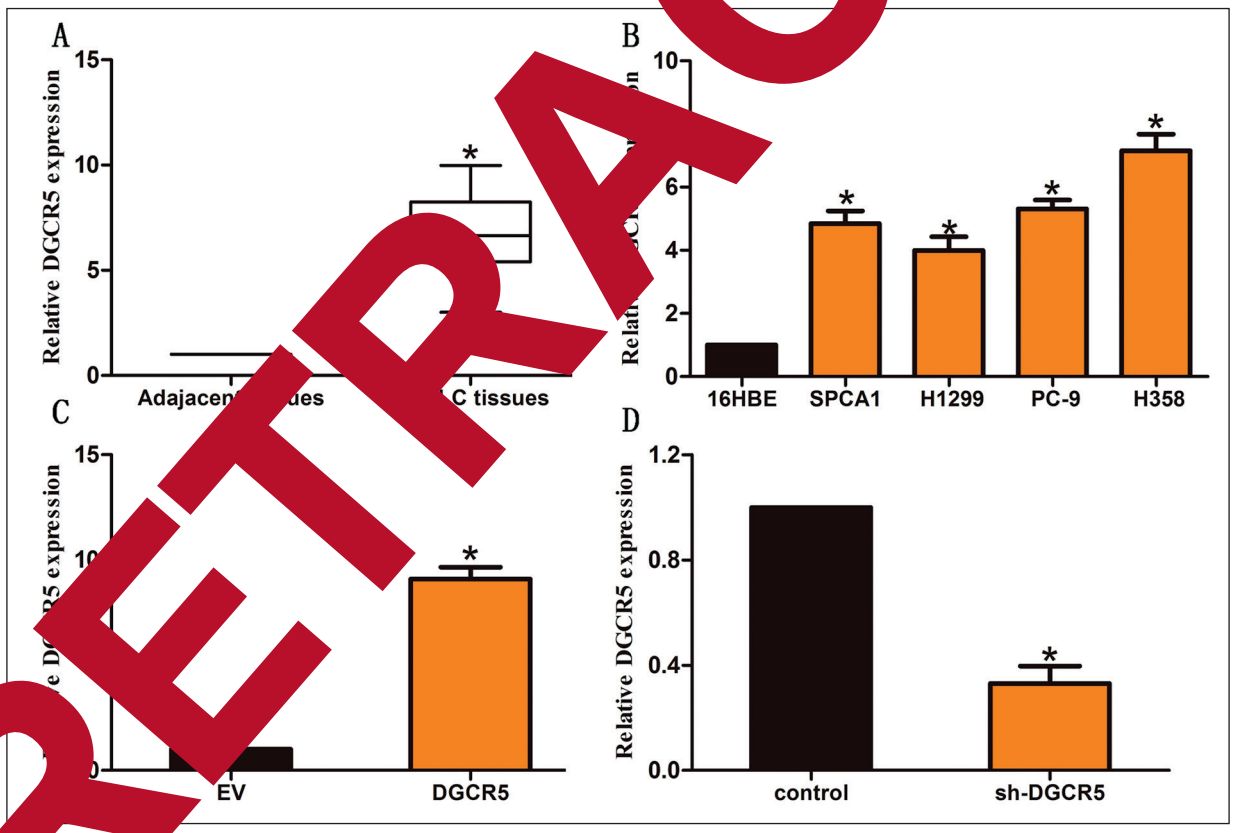
Statistical Analysis

Statistical Product and Ser... Solutions (SPSS) 20.0 (SPSS, Chicago, IL, ... was used for all statistical analysis. Indepe... sample *t*-test was selected when ap... priate. *p* ... was considered statistically s... icant.

Re...

Expression ...el of DGC... NSCLC Ti... d Cell Line...

The R...PCR ... irst conducted to detect DGCR5 expression i... es of 50 patients and 4 N... cell lines. A... esult, DGCR5 was s... icantly up-regulated in NSCLC compared ... hat of adjac... normal tissues (Figure 1A).

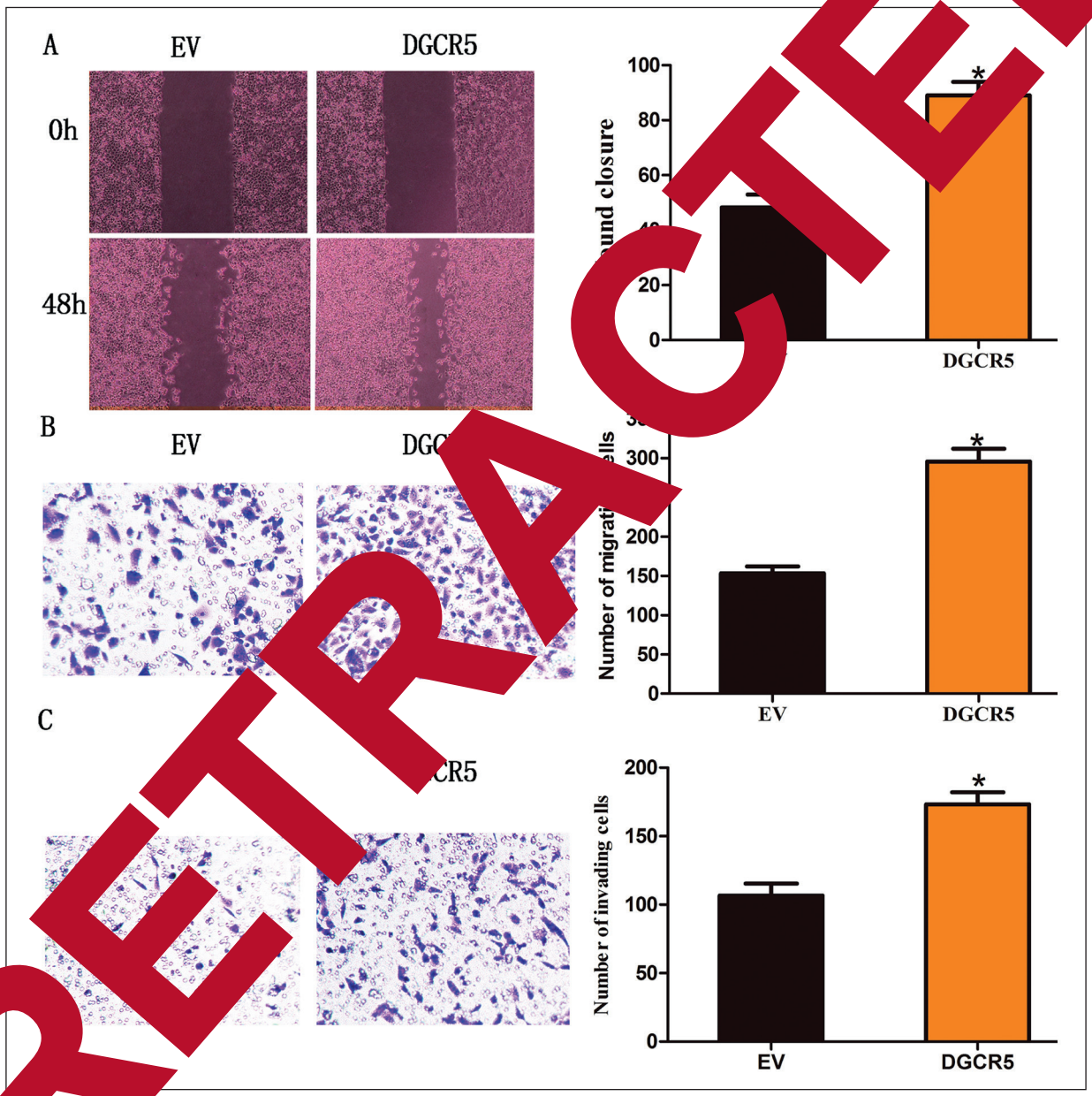


...ure 1. Expression level of DGCR5 increased significantly in NSCLC tissues and cell lines**. A,** DGCR5 expression increased ...cantly in NSCLC tissues compared with adjacent normal tissues. **B,** Expression levels of DGCR5 relative to GAPDH in hu... NSCLC cell lines and normal human bronchial epithelial cell line (16HBE) were determined by RT-qPCR. **C,** DGCR5 expr...sion in NSCLC cells transfected with empty vector (EV) or DGCR5 lentivirus (DGCR5) was detected by RT-qPCR. **D,** DGCR5 expression in NSCLC cells transfected with control shRNA (control) or DGCR5 shRNA (sh-DGCR5) was detected by RT-qPCR. GAPDH was used as an internal control. Data were presented as mean ± standard error of the mean. **p*<0.05.

Compared to 16HBE cells, the DGCR5 expression was significantly higher in NSCLC cells (Figure 1B). In our study, the H1299 cell line was chosen for overexpression of DGCR5, while the H358 cell line was selected for DGCR5 knockdown. Transfection efficiency was verified by RT-qPCR (Figures 1C and 1D).

Overexpression of DGCR5 Promoted Migration and Invasion of H1299 NSCLC Cells

Wound healing assay showed that t... closure of H1299 cells increased signi...tly a... DGCR5 was overexpressed (Figure ...). Transwell assay demonstrated that the num... migrated



...ure 2. Overexpression of DGCR5 promoted H1299 NSCLC cell migration and invasion. **A,** Wound healing assay showed ...e wound closure of NSCLC cells in DGCR5 group increased significantly compared with EV group (magnification: 4... Transwell assay showed that overexpression of DGCR5 significantly enhanced migration of NSCLC cells (mag...fication: 40×). **C,** Transwell assay showed that overexpression of DGCR5 significantly promoted invasion of NSCLC cells (magnification: 40×). Results represented the average of three independent experiments (mean ± standard error of the mean). *p<0.05, as compared with control cells.

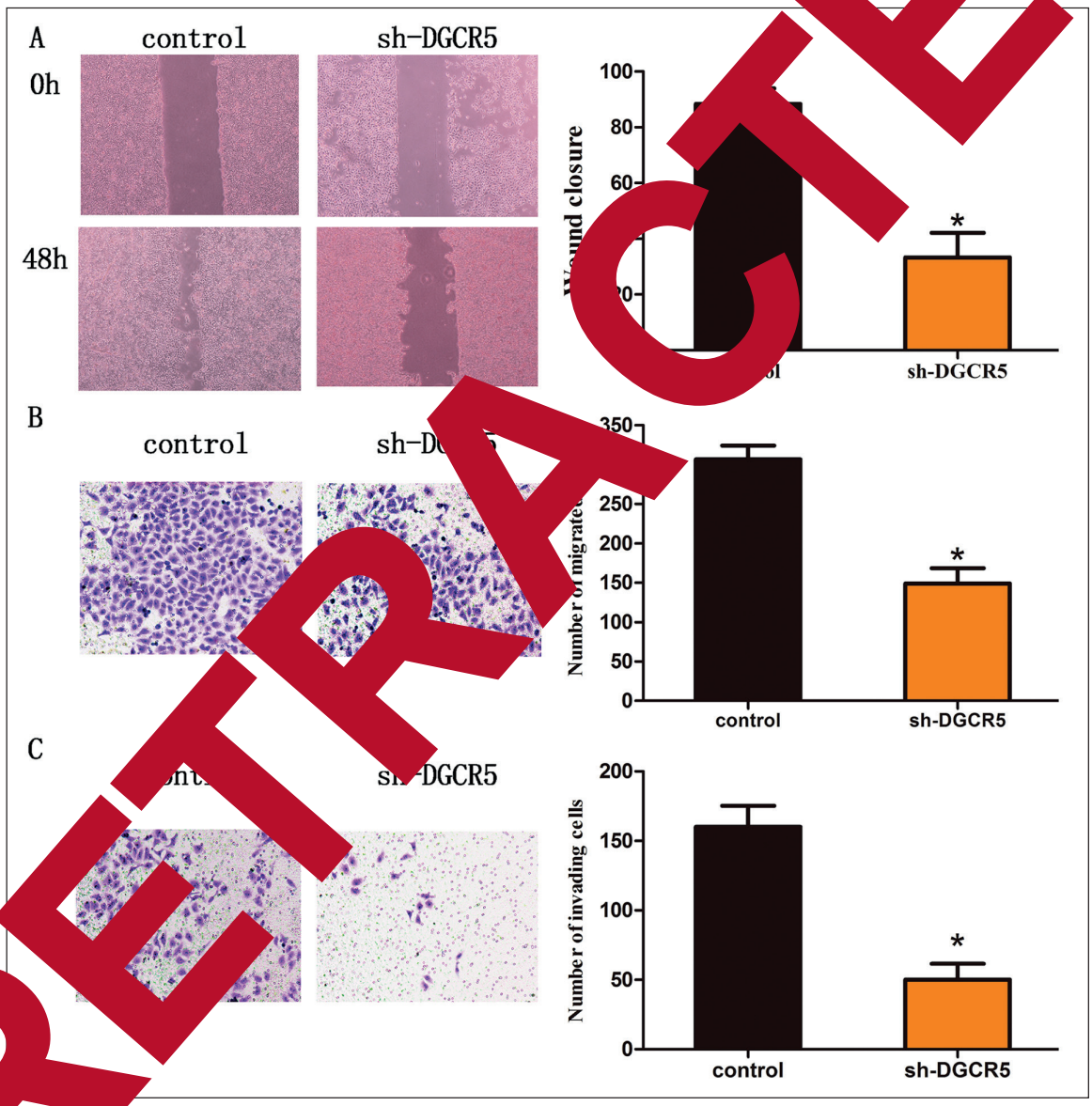
cells markedly increased after DGCR5 overexpression (Figure 2B). Furthermore, the number of invaded cells was significantly elevated after DGCR5 was also overexpressed (Figure 2C).

Silence of DGCR5 Repressed Migration and Invasion of H358 NSCLC Cells

Wound healing assay showed that the wound closure of H358 cells decreased significantly after DGCR5 was silenced (Figure 3A). Transwell assay indicated that the number of migrated cells was markedly reduced after the silence of DGCR5 *in vitro* (Figure 3B). Furthermore, the number of invaded cells decreased r after DGCR5 was silenced (Figure 3

Interaction Between MiR-21 and DGCR5 in NSCLC

DIANA LncBase Predi v.2 wa to search for miRNAs wh contained c



ure 3. Knockdown of DGCR5 inhibited H358 NSCLC cell migration and invasion. **A,** Wound healing assay showed that und closure of NSCLC cells in sh-DGCR5 group decreased remarkably compared with control group (magnification: 40 Transwell assay showed that knockdown of DGCR5 significantly repressed migration of NSCLC cells (magnification: 40×). **C,** Transwell assay showed that knockdown of DGCR5 significantly repressed invasion of NSCLC cells (magnification: 40×). Results represented the average of three independent experiments (mean ± standard error of the mean). *$p<0.05$, as compared with control cells.

mentary base with DGCR5. MiR-218-5p was selected from these miRNAs that were interacted with DGCR5 (Figure 4A). The RT-qPCR assay demonstrated that the expression of miR-218-5p was significantly lower in DGCR5 lentivirus group compared to empty vector group. However, the expression of miR-218-5p was markedly higher in sh-DGCR5 group compared to control group (Figures 4B and 4C). The luciferase reporter gene assay revealed that co-transfection of DGCR5-WT and miR-218-5p significantly decreased luciferase activity, while co-transfection of DGCR5-MUT and miR-218-5p had no effect on the luciferase activity (Figure 4D). In addition, the RIP assay identified that DGCR5 and miR-218-5p were markedly enriched in Ago2-containing beads compared to input group (Figure 4E).

Discussion

Currently, studies have shown th altered expression of lncRNAs is sociated the progression of NSCLC example, ln HEIH facilitates the astasis d proli tion of NSCLC[9], wh ay help to search for novel therape in ns. By ging miR-27b-3p, ln A KC T1 e ces cell proliferation invasion through

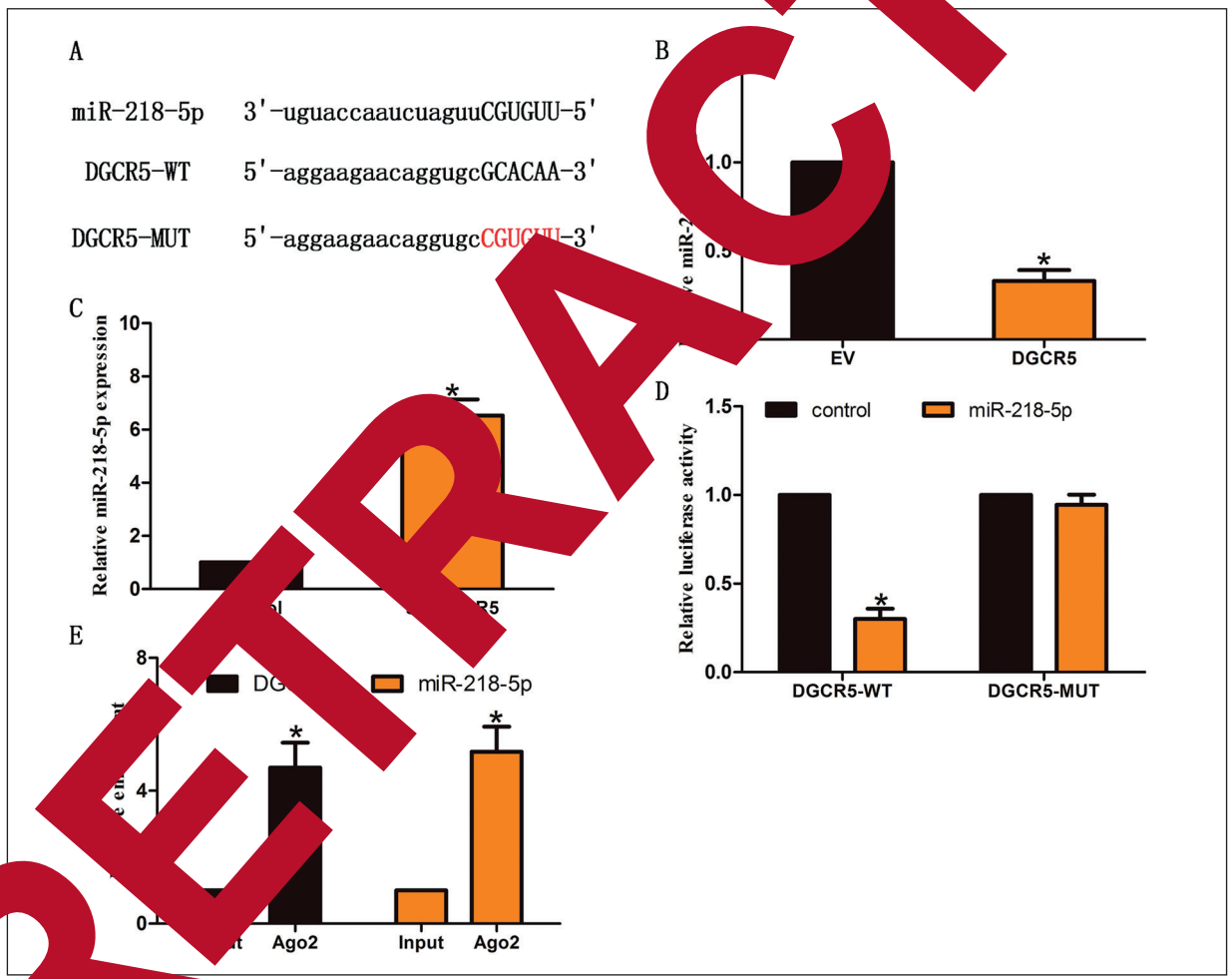


Figure 4. Association between DGCR5 and miR-218-5p in NSCLC. **A,** Binding sites of miR-218-5p on DGCR5. **B,** MiR-218- expression was down-regulated in DGCR5 group compared with EV group. **C,** MiR-218-5p expression was up-regulated DGCR5 group compared with control group. **D,** Co-transfection of miR-218-5p and DGCR5-WT strongly decreased lu e activity, while co-transfection of miR-218-5p and DGCR5-MUT did not change the luciferase activity. **E,** RIP assay iden ed that DGCR5 and miR-218-5p were significantly enriched in Ago2-containing beads compared with input group. Results represented the average of three independent experiments. Data were presented as mean ± standard error of the mean. *$p<0.05$.

the up-regulation of HSP90AA1[10]. Serving as a sponge of miR-497, lncRNA SNHG1 promotes the development of NSCLC *via* the regulation of IGF1-R[11]. By sponging miR-124-3p, lncRNA OGFRP1 participates in the regulation of the cell proliferation in NSCLC[12].

DiGeorge syndrome critical region gene 5 (DGCR5), also known as Linc0037, was first reported as down-regulated in Huntington's disease. Recently, DGCR5 has been confirmed to be a crucial regulator in numerous cancers. For instance, the low expression of DGCR5 is associated with the progression of cervical cancer through the activation of the Wnt signaling pathway[13]. Acting as a competing endogenous RNA of miR-23b, DGCR5[14] induces cell proliferation, invasion, and apoptosis in gastric cancer. Overexpression of DGCR5[15] regulates the progression of bladder cancer through the regulation of P21. Furthermore, Chen et al[16] has shown that DGCR5 is aberrantly expressed in lung cancer and is associated with poor prognosis.

In the present work, DGCR5 was found to be significantly up-regulated in both NSCLC tissues and cell lines. After DGCR5 overexpressio[illegible] migration and invasion of NSCLC were[illegible] cantly promoted. After DGCR5 was kn[illegible]ed down, the migration and invasion of NSCLC [illegible] were markedly suppressed. These results indic[illegible]ed that DGCR5 functioned as [illegible]ogene an[illegible] promoted NSCLC tumorigen[illegible]

In recent years, the int[illegible]tion b[illegible]een lncRNA-miRNA function[illegible]works [illegible]ted a lot of attention. By[illegible]eg[illegible]ulating [illegible]e expression of miR[illegible]b/a/42[illegible]RNA ILF3-AS1 facilitates th[illegible]roliferation[illegible]sion, and migration of m[illegible]ells[17]. UHR[illegible]gulated by dual-strai[illegible]umo[illegible]ressor miR-145, inhibits the [illegible]ressiveness[illegible]ladder cancer[18]. By silencin[illegible] and sponging [illegible]01-3p, lncRNA SNHG[illegible]acilitat[illegible] cell invasion and proliferation[illegible] gastric[illegible]ncer through induction of the epit[illegible]m[illegible]chyma[illegible]ansition[19]. In addition, lncRN[illegible] inhib[illegible]umor growth and metastasis in os[illegible]c[illegible] through regulation of the mi[illegible]1/ARI[illegible]way[20].

M[illegible]-218-5p, k[illegible]own as a tumor suppressor, was f[illegible]wn-regulated in several cancers, [illegible]uding prostate cancer, hepatocellular carcinoma, gastric cancer, and retinoblastoma[21-24]. In the p[illegible]t study, the luciferase reporter gene assay de[illegible]strated that miR-218-5p could be directly targeted by DGCR5. MiR-218-5p expression was significantly down-regulated after the overexpression of DGCR5, whereas it was up-regulated after DGCR5 knockdown. Furthermore, miR-218-5p was significantly enriched by DGCR5 through the RIP assay. All these resul[illegible] that DGCR5 could function as a[illegible]-218-[illegible] sponge in NSCLC.

Conclusions

All together, DGCR[illegible]ould e[illegible]ce NSC[illegible] cell migration and [illegible]on b[illegible]pongin[illegible] miR-218-5p. Our findi[illegible]s i[illegible]at lncR[illegible] DGCR5 acted as a[illegible]spectiv[illegible]peu[illegible]rget for NSCLC.

Conflict of Interest

The authors declare that they have no conflict of interests.

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