Ral GTPases targeted by miR-215-5p promote lung cancer proliferation and migration

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Research

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Abstract

Background

The Ras-like guanosine triphosphatases (Ral GTPases) belongs to the Ras superfamily of small GTPases. Ras mutations occur in more than one in three human tumors. However, treatments acting directly on Ras post-translational modifications were developed and have been manufactured for many years, although they have demonstrated poor clinical performance. Ral GTPases include RalA and RalB, seem to be a new potential pathway downstream of mutant Ras.

Methods

In this study, we examined protein and mRNA level of Ral GTPases in lung specimens from 12 lung cancer patients using Western Blot and RT-PCR. The effects of RalA and RalB on the proliferation and migration were examined by functional tests in vitro and in vivo. The binding site in miR-215-5p and RalA or RalB was predicted using bioinformatics software and proved by Western Blot, RT-PCR and luciferase assay. The effect of miR-215-5p on RalA and RalB were examined in cell lines and xenograft mice.

Results

Here, we reported that miR-215-5p was downregulated in human lung cancer tissues compared with noncancerous tissues, whereas the expression level of Ral GTPases was higher. We further verified that the negative regulation of Ral GTPases by miR-215-5p could inhibit the proliferation and migration of lung cancer in vitro and in vivo.

Conclusion

In this study, we reported that RalA and RalB promote lung cancer proliferation and migration. Moreover, we identified miR-215-5p as a tumor suppressor that targets Ral GTPases. Our results may offer therapeutic opportunities in lung cancer.

Background

The Ras-like guanosine triphosphatases (Ral GTPases) are members of the Ras superfamily of small GTPases. Ras mutations occur in more than one in three human tumors, a finding that has drawn much attention to Ras-inhibiting cancer treatments for more than 25 years [1]. Unfortunately, impactful Ras-inhibiting chemicals have not been reported. Ral GTPases include RalA and RalB, which share 82% homology with RalA [2]. Ral GTPases seem to be a new potential pathway downstream of Ras that has not been targeted to date. In addition, numerous clinical and experimental data suggest that RalA and RalB are significant drivers of diverse biological processes in various human cancers, including skin, lung,

pancreatic, colon, prostate, and bladder [3]. Thus, it is credible that regulation of Ral GTPases downstream of the oncogenic Ras could contribute to lung cancer treatments.

Small noncoding RNA molecules (19–22 nucleotides in length) known as microRNAs (miRNAs) were found to act as endogenous suppressors of gene expression by binding to the 3’ untranslated region (UTR) of mRNAs [4]. Many studies have shown that miRNAs play an important role in many biological processes of tumors, such as proliferation, differentiation, apoptosis and invasion [5]. miR-215-5p is a promising miRNA for cancer therapy. Dysregulation of miR-215-5p has been reported in various cancers, such as hepatocellular carcinoma [6], gastric cancer [7], breast cancer [8], glioma [9], and epithelial ovarian cancer [10]. miR-215-5p can act as an oncogene by targeting tumor suppressor genes. For example, miR-215 promotes migration and invasion by targeting retinoblastoma tumor suppressor gene 1 in gastric cancer and advances high-grade glioma by regulating retinoblastoma 1 [7]. miR-215-5p can also work as a tumor suppressor by inhibiting oncogenes. miR-215-5p inhibits growth and invasion by targeting AKT serine/threonine kinase 1 in breast cancer and NOB1 in epithelial ovarian cancer [10]. Thus, miR-215-5p may be identified as a target for lung cancer treatments.

In our study, we measured the expression levels of RalA and RalB in fresh human lung cancer tissue samples and in appropriate adjacent noncancerous tissue samples. We detected a negative correlation between miR-215-5p and Ral GTPase protein levels but not between mRNA levels. The direct inhibition of RalA and RalB translation by miR-215-5p and the potential role of miR-215-5p as a tumor suppressor in nonsmall cell lung cancer (NSCLC) were experimentally validated in vitro.

Methods And Materials

Cell culture and human tissue samples

The human lung cancer cell lines A549 and H358 were purchased from the Shanghai Institute of Cell Biology, Chinese Academy of Sciences (Shanghai, China). A549 and H358 cells were cultured in DMEM supplemented with 10% fetal bovine serum (Gibco) and incubated at 37 ℃ in a humidified air atmosphere containing 5% carbon dioxide. Fresh human lung cancer tissue samples were obtained from patients undergoing surgical procedures at the Drum Tower Hospital affiliated with the Medical School of Nanjing University (Nanjing, China). All patients in the study, or their guardians, provided written consent, and the Ethics Committee from the Affiliated Hospital of Nanjing University of Chinese Medicine approved all aspects of this study.

Transfections and luciferase reporter assay

Synthetic miR-215-5p mimic was purchased from Gene Script and transfected into lung cancer cells to overexpress miR-215. Scrambled negative control RNA transfected into A549 and H358 cells was used as a negative control. A mammalian expression plasmid encoding the human RalA or RalB open reading frame with an empty plasmid, which served as a negative control, was also ordered from Gene Script. To transfect the miR-215-5p mimic, negative control RNA or plasmid into A549 cells, Lipofectamine 3000

(Invitrogen) was applied when the lung cancer cells were 70% to 90% confluent. The cells were harvested at 24 h after transfection, or when the container was full of cells.

To validate the direct binding of Ral mRNA and miR-215-5p, the Ral GTPase 3’-UTRs containing the sequences that interacted with the miR-215-5p seed sequence were mutated (RalA binding site from AGGTCAA to TCCAGTT and RalB binding site from TAGGTCAA to ATCCAGTT) and inserted into an equivalent luciferase reporter. A β-galactosidase (β-gal) expression plasmid (Invitrogen) was used as a transfection efficiency control.

Protein extraction and Western blot analysis

The lysis buffer used was radioimmune precipitation assay lysis buffer (Beyotime, Haimen, China) with a protease and phosphatase inhibitor mixture (Thermo Scientific). Tissue samples were frozen in liquid nitrogen, ground into powder and lysed in lysis buffer on ice for 30 min. Then, the lysates were centrifuged for 12 min (12,000 g, 4 °C), and the supernatant was collected. Protein concentration was measured using the Pierce BCA Protein Assay Kit (Thermo Scientific). The RalA and RalB protein levels were analyzed by Western blotting with monoclonal antibodies (Cell Signaling Technology, 3526 and 3523). The protein levels were normalized by probing the same blots with a GAPDH antibody (Santa Cruz Biotechnology, sc-25778). The Western blot bands were analyzed using ImageJ software.

RNA isolation and quantitative RT-PCR

We extracted total RNA from cells and tissue samples for further study using Trizol reagent (Invitrogen). To measure the RNA concentration, a Microplate reader was used.

To quantify RalA and RalB mRNAs, 1 μg total RNA was reverse-transcribed to cDNA using avian myeloblastosis virus reverse transcriptase and OligoDT (TaKaRa). The reaction was incubated as follows: 16 °C for 5 min, 42 °C for 60 min and 70 °C for 10 min. Then, the real-time PCR mixture, consisting of specific primers for RalA or RalB and GAPDH and the RT product SYBR Green dye (Invitrogen), was incubated under the following conditions: 95 °C for 5 min, followed by 40 cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 1 min. The primer sequences used were as follows: RalA (sense: 5'-ATGGCTGCAAATAAGCCCAAG-3'); RalA (antisense: 5'-TGTCTGCTTTGGTAGGCTCATA-3'); RalB (sense: 5'-AGCCCTGACGCTTCAGTTC-3'); RalB (antisense: 5'-AGCGGTGTCCAGAATATCTATCT-3'); GAPDH (sense: 5’-CGAGCCACATCGCTCAGACA-3’); and GAPDH (antisense: 5’-GTGGTGAAGACGCCAGTGGA-3’). The amounts of RalA and RalB mRNA were normalized relative to GAPDH, and the $2^{-\Delta\Delta C}{}_{T}$ method was used to analyze the data.

To quantify miRNA, 1 μg total RNA was reverse-transcribed to cDNA using avian myeloblastosis virus reverse transcriptase (TaKaRa) and a stem-loop RT primer (Thermo Scientific) under the following conditions: 16 °C for 30 min, 42 °C for 30 min, and 85 °C for 5 min. Next, the real-time PCR mixture was incubated on an Applied Biosystems 7500 sequence detection system with a TaqMan PCR kit as follows:

95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The relative amount of miRNA was normalized to U6, and the $2^{-\Delta\Delta CT}$ method was used to analyze the data.

Cell proliferation assay

To estimate the proliferation ability of lung cancer cells, we used the EdU Cell Proliferation Assay Kit (Takara) to incorporate 5-ethynyl-2'-deoxyuridine (EdU). The EdU assay data were analyzed with Image Pro Plus software.

Cell migration assay

The migration ability of A549 lung cancer cells was tested using cell culture inserts (Millicell, 12-Well Hanging Inserts, Germany). The inserts were pretreated by coating the outboard of the polycarbonate membranes on the bottom of the upper compartment of the chamber with 0.1% gelatin. Then, A549 cells and DMEM culture medium containing 1% FBS were added to the upper chamber for 24 h after transfection of cells. Next, DMEM culture medium containing 10% FBS was added to the lower compartment, and the Transwell-containing plates were incubated for 20 h in a 5% CO2 atmosphere. After a 20 h incubation, the lung cancer cells would be apt to invade and penetrate the gelatin-coated polycarbonate membranes to reach the upper chamber containing the more nutritious DMEM with higher FBS content. The cells on the outboard surface of inserts were washed, fixed and dyed. Finally, images of the outboard surfaces (with migrant cells) were captured by a photomicroscope (5 fields/chamber) (BX51 Olympus, Japan).

Establishment of tumor xenografts in mice

Six-week-old male SCID mice (nu/nu) were purchased from the Model Animal Research Center of Nanjing University (Nanjing, China) and maintained under specific pathogen-free conditions at Nanjing University. Lentivirus for miR-215-5p overexpression was purchased from GenePharma (Shanghai, China). Puromycin (Sigma-Aldrich, USA) was used to successfully obtain stably infected cells. A549 cells that were injected with this lentivirus or a control lentivirus and transfected with RalA and RalB overexpression plasmid or cotransfected with RalA and RalB overexpression plasmids and miR-215-5p lentivirus were injected subcutaneously into SCID mice (2×10^6 cells per mouse, five mice per group). Mice were sacrificed 24 days after injection to remove the xenografted tumors. Tumor volumes were calculated by the formula: Tumor volume = [length * $(\text{width})^2$]/2. A portion of tumor tissues was fixed in 4% paraformaldehyde for 24h and then further processed for H&E and immunocytochemical staining for RalA, RalB and Ki67. The remaining tissue was used for protein and total RNA extraction. The experiment was repeated three times. All animal handing and care were approved by the Institutional Review Board of Nanjing University (Nanjing, China) and performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Statistical analysis

All of the images from the Western blot assay, the EdU assay, the migration assay, the immunofluorescence experiments, the colony formation experiments or the animal experiments were representative of at least three independent experiments or staining results. Quantitative RT-PCR and luciferase reporter assays were performed in triplicate, and each experiment was repeated several times. The results are presented as the means ± SE of at least three independent experiments. Observed differences were considered statistically significant at $p < 0.05$ by using Student's t-test.

Results

Upregulation of Ral GTPase protein but not mRNA in lung cancer

First, we examined the expression levels of Ral GTPases in lung specimens from 12 lung cancer patients. Compared with corresponding noncancerous tissues, RalA and RalB protein levels were obviously higher in lung cancer tissues (Fig. 1, A-C). Interestingly, RT-PCR results showed that there was no significant difference in RalA and RalB mRNA levels between these paired tissues (Fig. 1, D and E). This disparity between Ral GTPase protein and mRNA levels in lung cancer tissues strongly suggests that a post-transcriptional mechanism is involved in the regulation of RalA and RalB.

Ral GTPases promote lung cancer proliferation and migration

Next, we examined the effect of Ral GTPases on lung cancer proliferation and migration to assess the role of Ral GTPases in lung cancer. RalA and RalB were overexpressed with an expression plasmid designed to specifically express the full-length open reading frame (ORF) of RalA or RalB without the 3'-UTR. The control vector and the RalA or RalB overexpression plasmids were transfected into A549 cells. Then, we used the EdU assay to evaluate the effects of Ral GTPases on proliferation (Fig. 1, H and I). We found that transfection of the overexpression plasmid of Ral GTPases could enhance the viability of lung cancer cells. We also performed CCK8 assay to investigate the role of RalA and RalB in proliferation of lung cancer and got the same conclusion (Additional file 1: Figure.S1, A and B). Similarly, to evaluate the effects of RalA and RalB on the migration ability of lung cancer cells in vitro, we used Transwell migration assays. The Transwell assay showed that the percentage of migrating cells was significantly higher in A549 cells transfected with the overexpression plasmid of Ral GTPases (Fig. 1, F and G). All together, these results indicate that overexpression of Ral GTPases might heighten the proliferation and migration ability of lung cancer cells in vitro.

Identification of Ral GTPases as a target of miR-215-5p

miRNAs have been found to play a significant role in almost all biological processes pertaining to cancer development and have been used in the diagnosis, prognosis and treatment of multiple cancers. It seems that identification of efficacious miRNAs that could regulate expression of Ral GTPases in NSCLC is a potential method to treat NSCLC. Therefore, three algorithms, PicTar [11], TargetScan [12] and miRanda (http://www.microrna.org/microrna/ home.do), were used jointly. By this method, four miRNAs (miR-1-3p, miR-206, miR-192-5p, and miR-215-5p) were identified as regulators of RalA, and three miRNAs (miR-98-

5p, miR-192-5p, and miR-215-5p) were identified as regulators of RalB. In addition, the minimum free energy values between these miRNAs and the Ral GTPases are well within the range of miRNA-target pairs. miR-215-5p was predicted to bind not only to RalA but also to RalB (Fig. 2, A and B). Thus, we chose pre-215-5p for further study.

Detection of an inverse correlation between miR-215-5p and Ral GTPase levels in NSCLC tissues

Our previous work showed that miR-215-5p can bind to the 3'-UTR of Ral GTPase mRNA to inhibit RalA and RalB expression, but there is no evidence to prove that dysregulation of miR-215-5p participates in the upregulation of Ral GTPases in lung cancer. Thus, we examined miR-215-5p levels in lung cancer tissues and corresponding noncancerous tissues. As expected, miR-215-5p levels were significantly lower in NSCLC tissues than in corresponding noncancerous tissues (Fig. 2C). Furthermore, the inverse correlation between miR-215-5p and Ral GTPase protein levels (Fig. 2, D and E) and the disparity between the miR-215-5p and Ral GTPase mRNA levels (Fig. 2, F and G) were further illustrated using Pearson's correlation scatter plots. This result suggested that miR-215-5p regulates RalA and RalB post-transcriptionally, as demonstrated not only by computational prediction but also by noting the inverse correlation between miR-215-5p and Ral GTPase levels in NSCLC tissues.

miR-215-5p directly regulates RalA and RalB expression at the posttranscriptional level

To determine whether miR-215-5p could regulate Ral GTPases in lung cancer, we examined the protein levels of RalA and RalB in A549 and H358 cells overexpressing miR-215-5p. The overexpression of miR-215-5p was achieved by transiently transfecting lung cancer cells with a miR-215-5p mimic. Subsequently, we determined the efficiency of transfection to verify that mimics were transfected into A549 and H358 cells (Fig. 2H). Western blot analysis results showed that A549 and H358 cells overexpressing miR-215-5p have lower levels of Ral GTPase protein (Fig. 2, I and J) and similar levels of Ral GTPase mRNA (Fig. 2K).

To determine whether miR-215-5p regulates Ral GTPase expression through binding directly to the 3'-UTR of Ral GTPase mRNA, the entire 3'-UTR of Ral GTPase mRNA containing the presumed candidate miRNA-binding sites was fused downstream of the firefly luciferase gene in a reporter plasmid. Then, we transfected the resulting plasmid into A549 cells along with either pre-miR-control (a miRNA negative control) or a miR-215-5p mimic. As expected, the luciferase reporter activity in A549 cells transfected with pre-miRNAs was reduced compared with that in lung cancer cells transfected with pre-miR-control (Fig. 2, L and M). Moreover, to confirm the specificity of miR-215-5p targeting to the putative binding sequence on the Ral GTPase 3'-UTR, point mutations were designed into the corresponding binding sites in the RalA or RalB 3'-UTRs (Fig. 2, L and M). We found that the luciferase mutant gene reporter activity could not be affected by the miR-215-5p mimic.

Thus, we conclude that miR-215-5p specifically regulates Ral GTPase protein expression post-transcriptionally and directly.

miR-215-5p inhibits lung cancer proliferation and migration

Ral GTPases are known to participate in the proliferation, invasion, apoptosis, and migration in various cancer cells. To identify the role of miR-215-5p in lung cancer, we first evaluated the effects of miR-215-5p on the proliferation of A549 cells by EdU assay. As expected, A549 cells transfected with the miR-215-5p mimic showed decreased proliferation compared with A549 cells transfected with the negative control RNA (Fig. 3, C and D). Moreover, when we used the RalA or RalB overexpression plasmids to overexpress RalA or RalB in A549 cells, cells transfected with both miR-215-5p mimic and the RalA or RalB overexpression plasmid showed significantly higher proliferation rates compared with the cells transfected with miR-215-5p mimic and negative control plasmid together (Fig. 3, G and H). This finding suggested that miR-215-5p inhibits lung cancer proliferation by silencing Ral GTPases. CCK8 assay that we performed leads to the same conclusion (Additional file 1: Figure.S2, A-C).

We also tested the effect of miR-215-5p on the migration ability of lung cancer using Transwell migration assays. The Transwell migration assay showed that the percentage of migratory cells in the chamber containing the A549 cells transfected with miR-215-5p was lower compared with those chambers containing A549 cells transfected with the negative control RNA (Fig. 3, A and B). Additionally, when miR-215-5p mimic and RalA or RalB were transfected into A549 cells jointly, the percentage of migratory lung cancer cells in the inserts was lower than those cells transfected with miR-215-5p and the negative control plasmid (Fig. 3, E and F). This phenomenon suggested that overexpression RalA or RalB could reverse the effect of miR-215-5p on lung cancer cells. This result indicated that miR-215-5p inhibits lung cancer migration by silencing Ral GTPases.

Taken together, our results suggest that RalA and RalB are crucial to the proliferation and migration of lung cancer cells and that miR-215-5p might inhibit proliferation and migration in lung cancer cells by silencing Ral GTPases.

miR-215-5p inhibits lung cancer growth in vivo by targeting Ral GTPases

Finally, we investigated the effects of miR-215-5p and Ral GTPases on the growth of lung cancer xenografts in nude mice. To establish tumor xenografts in mice, we first infected A549 cells with a miR-215-5p overexpression lentivirus, transfected A549 cells with RalA and RalB overexpression plasmid. Then, we implanted the infected or transfected A549 cells into 4-week-old nude mice subcutaneously. We evaluated tumor growth 24 days after cell implantation. The xenografted tumors are larger in the control group than in the miR-215-5p-overexpressing group but smaller than in the RalA and RalB overexpressing group obviously. At the same time, the joint of miR-215-5p overexpressing lentivirus and Ral GTPases can make the xenografted tumors smaller compared with the RalA and RalB overexpressing group (Fig.4A). As expected, the volumes and weights of the xenografted tumors show the same results (Fig.4, B and C). The overexpression of miR-215-5p attenuated inhibits lung cancer growth by silencing Ral GTPases. Then, we embedded xenografted tumors in paraffin and performed H&E staining or immunohistochemical asssys. H&E staining showed less cell mitosis in tumors of the miR-215-5p-overexpressing group than in tumors of the control group. Xenografts tumors with both miR-215-5p and

Ral GTPases overexpression emerged reduced cell mitosis compared to xenografts tumors with Ral GTPases overexpression alone (Fig.4, D). This also suggesting that miR-215-5p can attenuate the growth-promoting effect of RalA and RalB. Immunohitochemical staining revealed lower RalA, RalB and Ki67 levels in tumors from the miR-215-5p-overexpressing group, when tumors from the Ral GTPases overexpressing group showed increased RalA, RalB and Ki67 level in tumors transfected with Ral GTPases (Fig.4, D-G). We also examined the protein expression levels of Ral GTPases of xenografts tumors by Western blot. Tumors from the miR-215-5p-overexpressing group have lower RalA and RalB levels compared to the control group (Fig. 4, H and I). At the same time, the joint of miR-215-5p overexpressing lentivirus and Ral GTPases can make the protein expression levels of Ral GTPases of xenografted tumors lower compared with the RalA and RalB overexpressing group (Fig. 4, H and I). These results are consistent with the in vitro findings, which firmly validated the tumor suppressor role of miR-215-5p in lung cancer tumorigenesis through targeting RalA and RalB.

Discussion

Lung cancer is the most prevalent tumor type, with the highest morbidity and mortality worldwide [13–15]. The predominant type of lung cancer is NSCLC, which accounts for 75%-80% of all cases [16]. Unfortunately, the five-year overall survival rate for most advanced lung cancer patients remains 15%, despite the developments of early diagnoses and multimodality treatments for lung cancer [17].

The Ras-like GTPases RalA and RalB, which are known as oncogenes regulating proliferation, migration, and apoptosis, play an important role as drivers of tumorigenesis in various cancers. Chemicals that inhibit their functions are useful therapies for cancer. In our study, we found that overexpression of RalA or RalB could enhance the proliferation and migration of lung cancer cells, proving that Ral is an essential oncogene and a potential target of NSCLC therapy.

Interestingly, miRNAs are abnormally expressed in various cancers and were proven to play an important role as oncogenes or tumor suppressor genes through post-transcriptional regulation of mRNA. In our study, we found that the level of miR-215-5p was lower in lung cancer tissues compared with that in normal noncancerous tissues, which suggested that miR-215-5p may participate in the regulation of tumor related genes as an anti-oncogene. Downregulation of miR-215 has been reported in other cancers, such as gastric cancer and breast cancer. These studies have shown that miR-215 is capable of inhibiting cancer by targeting specific oncogenes, inhibiting the transcription of their mRNAs, and suppressing these oncogenes. In our study, we found that lung cancer cells transfected with miR-215-5p had lower levels of proliferation and migration than cells transfected with a negative control. At the same time, overexpression of RalA and RalB with plasmids could attenuate the influence of miR-215-5p. All these results suggested that miR-215-5p functions as a tumor suppressor mainly by targeting RalA and RalB in lung cancer. These results also partially explain the relationship between the downregulation of miR-215-5p in lung cancer tissues and the extreme proliferation and migration of lung cancer.

Ras mutations play an important role in various tumors and are especially common in pancreatic, lung, and colorectal cancer [6]. Consequently, treatments acting directly on Ras post-translational modifications were developed and have been manufactured for many years, although they have demonstrated poor clinical performance. Then, attention was paid to signaling components downstream of Ras such as the phosphoinositide 3-kinase-AKT-mTOR pathway [18] and the Raf-MEK-ERK mitogen-activated protein kinases pathway [19]. A new downstream pathway, a third pathway that leads to the activation of the Ras-like small GTPases, has received much attention and seems to be a potential target for cancer therapy. For example, one study reported the use of a structure-based approach to inhibit Ral GTPases [8]. A stapled peptide approach was also reported to regulate RalA and RalB [20]. However, these approaches have not shown considerable clinical promise. On the other hand, Ral GTPases serving as a molecular target of Ras have shown high potential, since there is no better alternative for inhibiting Ras mutation. At the same time, the dysregulation of miRNAs in cancer suggests that altering miRNA expression is a potential therapeutic strategy. Currently, we are able to compensate for lost miRNAs by overexpressing miRNA mimics and to silence the overexpression of miRNAs using antagomirs. In this study, miR-215-5p showed an anti-tumor effect in vitro through the negative regulation of Ral GTPases in humans. We hypothesize that a replacement treatment with miR-215-5p mimics may be a promising strategy for carcinomas characterized by miR-215-5p downregulation. Future studies are needed to develop an effective delivery system for the use of miR-215-5p in lung cancer therapy.

Conclusion

Our study indicates that RalA and RalB were increased in lung cancer tissues and cell lines. High expression level of RalA and RalB promotes lung cancer proliferation and migration. Moreover, miR-215-5p targets to RalA and RalB directly and inhibits lung cancer growth in vitro and vivo. Altogether, our study explains the role of miR-215-5p as a tumor suppressor in lung cancer through disrupted translation of Ral GTPases.

Abbreviations

Ral GTPases: Ras-like guanosine triphosphatases; RalA, RAS like proto-oncogene A; RalB, RAS like proto-oncogene B; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; UTR: Untranslated region.

Declarations

Ethics approval and consent to participate

All patients in the study, or their guardians, provided written consent, and the Ethics Committee from the Affiliated Hospital of Nanjing University of Chinese Medicine approved all aspects of this study.

Availability of data and materials

All data related to this study are included in this paper and its supplementary information files.

Authors' contributions

Guangxin Zhou designed this study. Hao Zhu and Shufang Cui contributed equally to this work, and both should be considered as the first authors. Gentao Fan collected fresh human lung cancer tissue samples.

Consent for publication

All authors who have contributed to the study agree to publish it.

Competing interests

The authors declare no conflict of interest.

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Figures

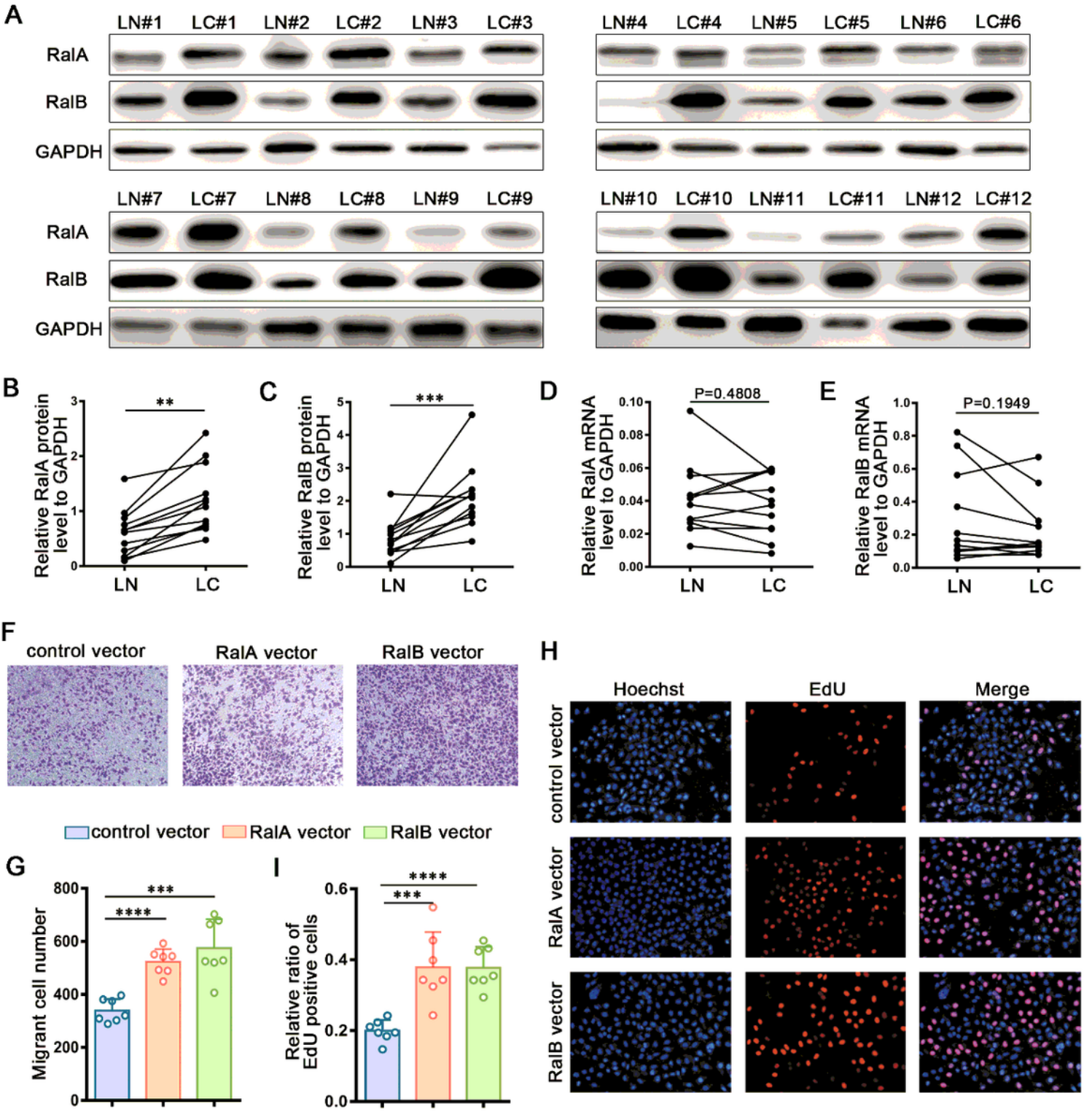

Figure 1

Upregulated RalA and RalB functioned as an oncogene in lung cancer. (A~C) Western blotting analysis of the expression of RalA and RalB protein in 12 pairs of noncancerous tissue (NC) and lung cancer (LC) samples. (A) representative image; (B) quantitative analysis of RalA protein; (C) quantitative analysis of RalA protein. (D and E) Quantitative RT-PCR analysis was used to evaluate the expression levels of RalA (A) and RalB (B) mRNA in NC and LC samples. (F and G) Transwell analysis of migranted A549 cells

treated with control vector, RalA overexpression plamid or RalB overexpression plamid. (F) representative image; (G) quantitative analysis. (H and I) EdU assay was performed in A549 cells treated with control vector, RalA overexpression plamid or RalB overexpression plamid. (H) representative image; (I) quantitative analysis. **, p<0.01; ***, p< 0.001; ****, p<0.0001.

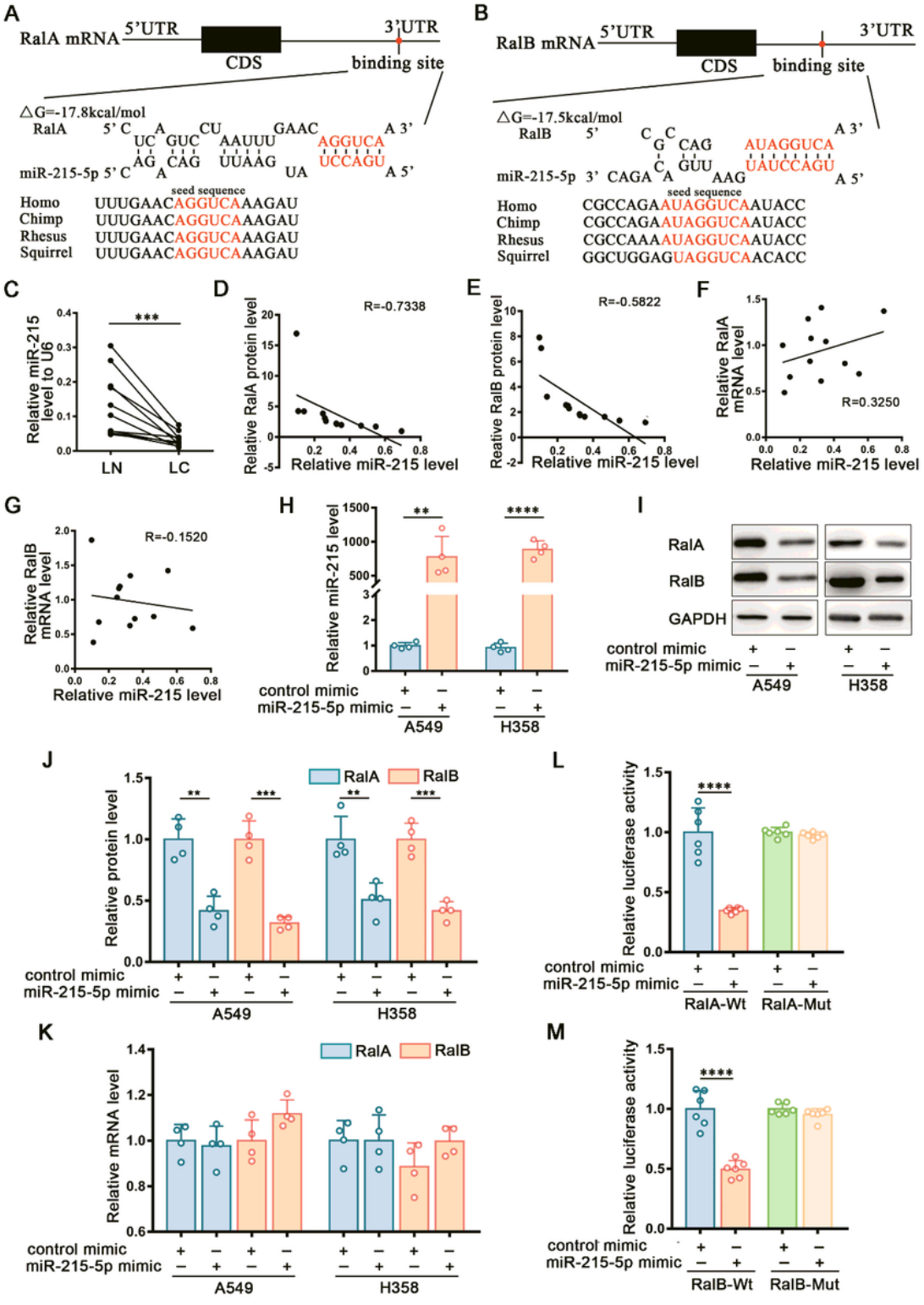

Figure 2

Prediction of RalA and RalB as targets of miR-215-5p simultaneously. (A and B) Schematic description of the hypothetical duplex formed by the interactions between the binding site in the miR-215-5p and RalA 3′-UTR (A), or RalB 3′-UTR (B). All nucleotides in these regions are highly conserved across species, including human, mouse and rat; the predicted free energy values of the hybrids are indicated. (C) Quantitative RT-PCR analysis was used to evaluate the expression levels of miR-215-5p in 12 pairs of NC and LC samples. (D~G) Pearson's correlation scatter plot of the fold changes of miR-215-5p and RalA protein (D), RalB protein (E), RalA mRNA (F) or RalB mRNA (G). (H) Quantitative RT-PCR analysis of miR-215-5p levels in A549 and H358 cells transfected with control mimic or miR-215-5p mimic. (I and J) Western blotting analysis of the expression of RalA and RalB protein in A549 and H358 cells treated with control mimic or miR-215-5p mimic. (I) representative image; (J) quantitative analysis of RalA and RalB protein. (K) Quantitative RT-PCR analysis of RalA and RalB mRNA level in A549 and H358 cells transfected with control mimic and miR-215-5p mimic. (L and M) The relative luciferase activities in A549 cells transfected with wild type or mutant Ral GTPases 3′-UTR. (L) RalA; (M) RalB. **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$.

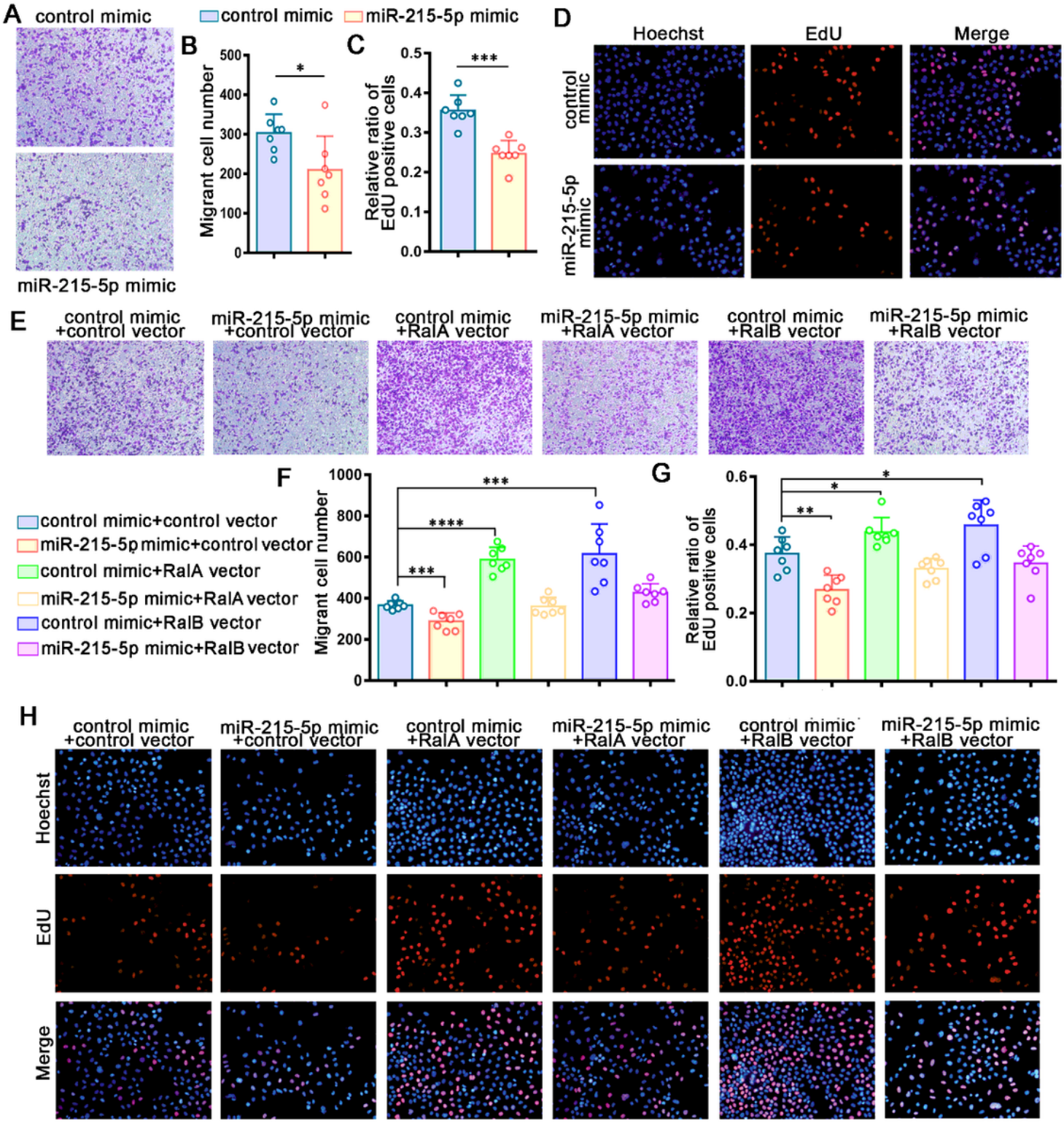

Figure 3

miR-215-5p inhibits lung cancer proliferation and migration by down-regulating RalA and RalB. (A and B) Transwell analysis of migranted A549 cells treated with control mimic or miR-215-5p mimic. (A) representative image; (B) quantitative analysis. (C and D) EdU assay was performed in A549 cells treated with control mimic or miR-215-5p mimic. (C) quantitative analysis; (D) representative image. (E and F) Transwell analysis of migranted A549 cells treated with equal doses of control mimic plus control vector,

miR-215-5p plus control vector, control mimic plus RalA overexpression plasmid, miR-215-5p mimic plus RalA overexpression plasmid, control mimic plus /RalB overexpression plasmid, or control mimic plus RalB overexpression plasmid. (E) representative image; (F) quantitative analysis. (G and H) EdU assay was performed in A549 cells treated with equal doses of control mimic plus control vector, miR-215-5p plus control vector, control mimic plus RalA overexpression plasmid, miR-215-5p mimic plus RalA overexpression plasmid, control mimic plus RalB overexpression plasmid, or control mimic plus RalB overexpression plasmid. (G) quantitative analysis; (H) representative image. *, P<0.05; **, P<0.01; ***, p<0.001, ****, p<0.0001.

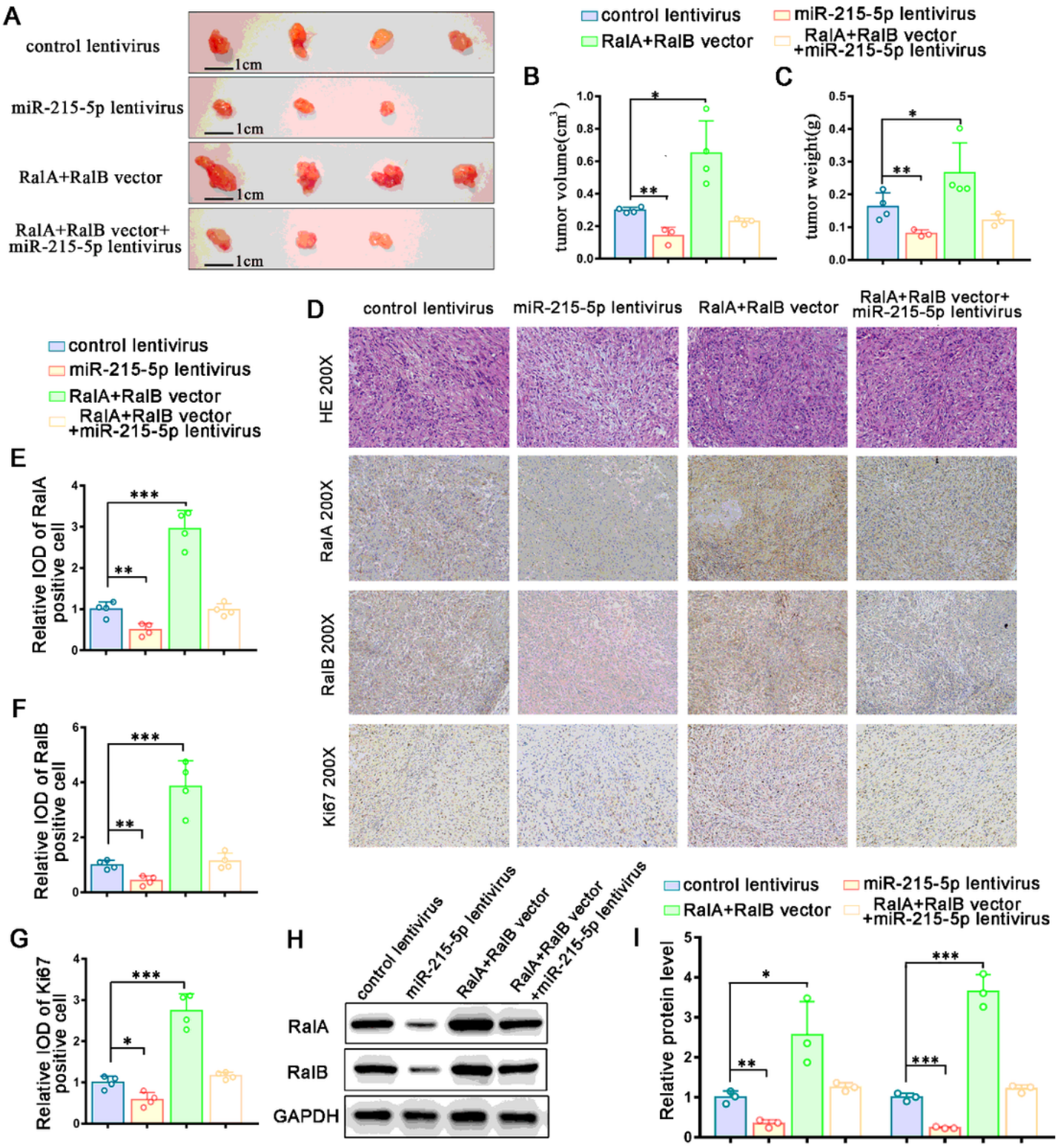

Figure 4

Effects of RalA, RalB and miR-215-5p overexpression on the growth of lung cancer cell xenografts in mice. (A) Representative images of tumors from mice implanted with control A549 cells, miR-215-5p-overexpressing A549 cells, RalA and RalB vector-overexpressing A549 cells and RalA, RalB and miR-215-5p co-overexpressing A549 cells. A549 cells (2 × 106 cells per mouse) with different treatments were implanted subcutaneously into 6-week-old SCID mice (5 mice per group). (B)Tumor volume; (C) Tumor

weight. (D~G) H&E-stained sections and immunohistochemical staining for RalA, RalB and Ki-67 in tumors from implanted mice. (D) representative image; (E) quantitative analysis of RalA; (F) quantitative analysis of RalB; (G) quantitative analysis of Ki67. (H and I) Western blotting analysis of the expression of RalA and RalB protein in tumor from the implanted mice. (H) the implanted mice; (I) quantitative analysis of RalA and RalB protein. *, P<0.05; **, P<0.01; *** , p<0.001.

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