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ORIGINAL RESEARCH

RETRACTED ARTICLE: Matrine Regulates Proliferation, Apoptosis, Cell Cycle, Migration, and Invasion of Non-Small Cell Lung Cancer Cells Through the circFUT8/miR-944/YES1 Axis

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Background: Non-small cell lung carcinoma (NSCLC) is the major histological subtype of cancer cases. In the present study, we investigated the association between Matrine, an active component of Chinese medicine, and circFUT8 in NSCLC cells.

Methods: The proliferation ability of NSCLC cells was assessed by MTT and colony-forming assays. Flow cytometry assay was performed to show the apoptosis and cell cycle distribution in NSCLC cells. The protein expression levels of Bcl-2, Cleaved Caspase-3 (C-Caspase3), and YES proto-oncogene 1 (YES1) were measured by Western blot assay. Migration and invasion of NSCLC cells were determined by transwell assay. The expression levels of circFUT8, miR-944 and YES1 were quantified by real-time quantitative polymerase chain reaction (RT-qPCR) assay. The interaction relationship between miR-944 and circFUT8/YES1 was confirmed by dual-luciferase reporter assay. The anti-tumor role of Matrine in vivo was explored by a xenograft experiment.

Results: Matrine functioned as a carcinoma inhibitor by repressing proliferation, cell cycle progression, migration, and invasion while inducing apoptosis in NSCLC cells. Importantly, overexpression of circFUT8 counteracted Matrine-induced effects on NSCLC cells. MiR-944, interacted with YES1, was a target of circFUT8. Under Matrine condition, overexpression of circFUT8 increased proliferation, migration, and invasion while inhibited apoptosis, which was abolished by the upregulation of miR-944. Whereas the silencing of YES1 counteracted miR-944 inhibitor-induced effects on NSCLC cells. Eventually, we also confirmed that Matrine impeded NSCLC tumor growth in vivo.

Conclusion: Matrine regulated proliferation, apoptosis, cell cycle, migration, and invasion of NSCLC cells through the circFUT8/miR-944/YES1 axis, which provided novel information for Matrine in NSCLC.

Keywords: Matrine, circFUT8, miR-944, YES1, NSCLC

Background

Non-small cell lung cancer (NSCLC) is a universal histological subtype in all lung cancers, occupying nearly 85% proportion of all lung cancers.[1] What is more, the clinical outcomes of NSCLC patients remain worse, and the 5-year survival rate of NSCLC patients is only 15%.[2] In the recent study, it was reported that circular RNAs (circRNAs) were identified as vital regulators in NSCLC.[3]

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CircRNAs are endogenous RNA molecules with closed loops and cannot code proteins, which are generated with back-splicing manner.[4] CircFUT8 (circ_0001998) is derived from the fucosylation 8 (FUT8) gene and located on chr14 (65922338–66028484). The upregulation of circFUT8 was highly associated with colorectal cancer, which functioned as an important regulator by regulation of miRNA-mRNA network.[5] However, the functions and underlying mechanisms of circFUT8 in NSCLC are still largely obscure.

Micro RNAs (miRNAs) are small RNAs without coding protein abilities that modulate target mRNA expression involved in tumor progression by targeting the 3′untranslated region (UTR) of mRNAs, resulting in degradation and inhibiting translation.[6] Previous researches found that miR-944 was frequently dysregulated in a variety of human cancers.[7,8] Furthermore, the high-expression of miR-944 was a reliable prognostic index for pancreatic cancer patients.[9] Consistently, the diagnostic value of miR-944 was found in operable squamous lung cell carcinoma.[10] Nevertheless, the role of miR-944 has not been investigated thoroughly in NSCLC.

YES proto-oncogene 1 (YES1) is a member of Src family tyrosine kinases (SFKs) family, which was involved in the development of human cancers.[11] For example, Liu et al revealed that activating YES1 strengthened the malignant phenotype of melanoma cells.[12] Moreover, YES1 has been demonstrated to contribute tumorigeneses such as pancreatic cancer,[13] gastric cancer,-[14] and thyroid cancer.[15] Suppression of YES1 could inhibit rhabdomyosarcoma cell growth in vitro and in vivo, identifying YES1 as a critical signal for tumor growth.[16] Interestingly, YES1 also induced chemotherapeutic resistance to several cancers including NSCLC.[17] Therefore, the functional role of YES1 was investigated in NSCLC.

In the current research, we investigated the function of Matrine treatment on NSCLC cells and association with circFUT8/miR-944/YES1 axis.

Methods

Cell Lines and Cell Culture

The normal human lung epithelial cell line (BEAS-2B) and NSCLC cell lines (H522 and H1975) were purchased from the American Type Culture Collection (Rockville, MD, USA). All cells were maintained in RPMI 1640 medium (Biochrom KG, Berlin, Germany) supplemented with 10% fetal bovine serum (Sigma, San Francisco, CA, USA), 1% penicillin/streptomycin (Sigma) at the condition of 37°C and 5% CO_2. In addition, Matrine (SM8130-20mg; Solarbio, Beijing, China) was dissolved in ddH_2O (40 mg/mL) and placed at 4°C as stock solution.

Cell Proliferation Assay

In brief, 200 μL of H522 and H1975 cells (1.5×10^4 cells/mL) were seeded into 96-well plates. After 24 h, 20 μL of 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-2H-tetrazol-3-ium bromide (MTT; Sigma) was added into cells and then incubated for 4 h. Dimethyl sulfoxide (DMSO) reagents were added to dissolve formazan crystals. The cell viability was measured under a microplate reader (BioTek, Winooski, VT, USA). Furthermore, colony-forming assay was performed as described by Ge et al.[18] In short, H522 and H1975 cells were dispersed into a single cell suspension. Approximately 600 cells were seeded into a 12-well plate preheated at 37°C. Following incubation at the condition of 37°C and 5% CO_2 for 14 days, the clones were observed under a microscope (40×, Leica, Wetzlar, Germany).

Flow Cytometry Assay

Apoptotic cells were monitored by the annexin V-fluorescein isothiocyanate (FITC) Apoptosis Detection Kit (Sigma) in compliance with the references. In brief, H522 and H1975 cells were diluted into the density of 10^6 cells/mL and then incubated with the staining buffer supplemented with FITC and propidium iodine (PI). After 10 min of incubation at 4°C for 30 min in dark condition, mixtures were analyzed under a flow cytometry (Applied Biosystems, Foster City, CA, USA). Cell cycle detection CycleTEST PLUS DNA Reagent Kit (BD Biosciences, San Jose, CA, USA) was used to show cell cycle distribution in compliance with the instructions of manufacturer.

Western Blot Assay

Briefly, H522 and H1975 cells were lysed by RIPA lysis (Cell Signaling Technology, Danvers, MA, USA) on ice for 30 min. After centrifuging and quantifying, 40 μg of total protein was segregated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and then electrophoretically transferred nitrocellulose filter membranes (GE Healthcare, Piscataway, NJ, USA). After blocking with 3% Albumin Bovine V (Amyjet scientific, Wuhan, China), the membranes were incubated with anti-Cleaved Caspase-3 (C-Caspase3; ab2302; 1:1500 dilution; Abcam, Cambridge, MA, USA), anti-B-cell lymphoma-2 (Bcl-2;

ab59348; 1:1500 dilution; Abcam), or anti-YES1 (ab109744; 1:1500 dilution; Abcam) overnight at 4°C, with anti-GAPDH (ab181602; 1:1500 dilution; Abcam) as control. After that, secondary antibody (ab1500771; 1:3000 dilution; Abcam) was added into membranes and incubated for 2 h at room temperature. The immunoreactive bands were shown on Alpha Innotech Imaging System (Protein Simple, Santa Clara, CA, USA) with ECL Western Blotting Detection Kit (Solarbio).

Migration and Invasion Assay

The migration assay of H522 and H1975 cells was conducted using 24-well transwell chambers (Millipore, Billerica, MA, USA) with 8-μm polycarbonate membrane. In brief, H522 or H1975 cells (5×10^5 cells/well) were seeded into the upper chamber with 200 μL of serum free-medium while complete medium was injected into the bottom chamber as a chemoattractant. 24 h later, cells that migrated to the underside of the membrane were fixed by 95% ethanol for 30 min, stained with crystal violet (Beyotime, Shanghai, China), and then imaged under a microscope (Leica). Image Lab software 5.2 (Bio-Rad, Hercules, CA, USA) was used for data analysis. 24-well transwell was covered with Matrigel (BD Biosciences) in advance for invasion assay, while other procedures were not changed.

Real-Time Quantitative Polymerase Chain Reaction (RT-qPCR) Assay

Trizol reagent (Sigma) was applied to isolate the total RNA following the manufacturer's protocol. The complementary DNA was acquired using a reverse transcription kit (Takara, Dalian, China) or microRNA Reverse Transcription Kit (Qiagen, Hilden, Germany). To compare expression levels between groups, RT-qPCR was performed with a QuantiTect SYBR Green RT-PCR Kit (Qiagen) on thermal CFX6 System (Bio-Rad). The RT-qPCR results were analyzed by $2^{-\Delta\Delta Ct}$ method, with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or endogenous small nuclear RNA U6 as reference. Furthermore, PARIS™ Kit was purchased from Abcam for subcellular localization of circFUT8 according to the manufacturer's instructions. The RNase R was purchased from Epicentre Technologies (Madison, WI, USA) for RNase R treatment assay. Total RNA was treated with or without 3U/mg RNase R for 15 min at 37°C, following by RT-qPCR assay.

The sequences of primers:

circFUT8 (sense-5′-CACTCTAGCCGAGAACTGTCC-3′; antisense-5′-TTGTCCTGTACTTCATGCGCT-3′);

linearFUT8 (sense-5′-AACTGGTTCAGCGGAGAATAAC-3′; antisense-5′-TGAGATTCCAAGATGAGTGTTCG-3′);

miR-944 (sense-5′-GCCGAGAAATTATTGTACAT-3′; antisense-5′-CTCAACTGGTGTCGTGGA-3′);

GAPDH (sense-5′-CATCCATGACAACTTTGGTA-3′; antisense-5′-CGTTGGCAGTGGGGACACGG-3′);

U6 (sense-5′-CTCGCTTCGGCAGCACA-3′; antisense-5′-AACGCTTCACGAATTTGCGT-3′).

Transfection Assay

CircFUT8-overexpressing vector (circFUT8), and negative control (vector), small interfering RNA (siRNA) objecting to YES1 (si-YES1) and si-NC were acquired from RiboBio (Guangzhou, China). MiR-944 mimic (miR-944), miR-944 inhibitor (anti-miR-944), anti-miR-NC were designed by GeneCopoeia (Rockville, MD, USA). Transient transfection with the aforementioned oligonucleotides or plasmids into H522 and H1975 cells was conducted with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) under the producer's instructions.

Dual-Luciferase Reporter Assay

The possible target of circFUT8 and miR-944 were shown by starBase v2.0 (http://starbase.sysu.edu.cn/). Partial sequences of circFUT8 or 3′ UTR of YES1 mRNA containing interacted sites with miR-944 were synthesized and cloned into pGL3-basic vectors (Realgene, Nanjing, China), named as circFUT8 WT and YES1 3′ UTR WT, with circFUT8 MUT and YES1 3′ UTR MUT as controls. When reached 60–70% confluence in 24-well plates, H522 and H1975 cells were co-transfected with 0.4 μg of vectors and 20 pmol of miR-944 mimic or miR-NC. After 48 h, H522 and H1975 cells were collected for luciferase activity assay under the VICTOR2 fluorometry (PerkinElmer, Waltham, MA, USA), with Renilla luciferase activity as reference.

In vivo Experiment

All animal experiments were performed with the instruction of the National Institutes of Health guide for the care and use of laboratory animals (NIH Publications No. 8023), which was permitted by the Institutional Animal Care and Use Committee of Jingmen NO.1 people's hospital. In animal experiments, 6 nude mice per group were

used and nude mice were randomly assigned to two groups before injection of H522 cells. H522 cells (5×10^6) in 100 μL of phosphate buffer saline were implanted subcutaneously into the right flank of nude mice (Vital River Laboratory, Beijing, China) under specific pathogen-free conditions. The mice were treated with or without 5 g/kg of Matrine in saline once daily by intraperitoneal injection. Tumor growth was monitored based on volume=1/2 (length×width^2). 35 d after injection, mice were killed by cervical dislocation after deep anesthesia with 2% isoflurane for subsequent experiments. Immunohistochemical assay was performed as previous description.[19]

Statistical Analysis

The statistical analyses were conducted with statistical software GraphPad Prism 7 (GraphPad, La Jolla, CA, USA). Statistically significant of two treatment groups or multiple groups was assessed using Student's *t*-test or one-way analysis of variance, respectively. All data were shown as mean ± standard deviation from three independent experiments.

Results

Matrine Inhibited Proliferation, Cell Cycle Process, Migration, and Invasion While Increased the Apoptotic Response in NSCLC Cells

To assess the effects of Matrine on NSCLC cells, H522 and H1975 cells were cultured with normal media supplemented Matrine at different concentrations (0, 0.5, 1, and 2 mg/mL) for 24 h. As shown in Figure 1A and B, cell viability was reduced in H522 and H1975 cells exposed to Matrine, especially in 2 mg/mL of Matrine group (over 50% decrease). Likewise, colony numbers were reduced after treatment with Matrine, suggesting colony-forming inhibition of NSCLC cells (Figure 1C and D). Furthermore, the treatment of Matrine induced nearly 4-fold increase in apoptosis rate in H522 and H1975 cells compared with control, having a dose-dependent method (Figure 1E and F). As Bcl-2 and C-Caspase3 were important apoptosis-related proteins, their expression was assessed by Western blot assay. Bcl-2 was downregulated by 50% while C-Caspase3 was upregulated by 3-fold in H522 and H1975 cells after treatment with 2 mg/mL of Matrine (Figure 1G and H). Matrine inhibited cell cycle process by increasing G0/G1 phase and decreasing S phase cells (Figure 1I and J). The results of the transwell assay suggested that migration and invasion of H522 and H1975 cells were downregulated by 50% in 2 mg/mL of Matrine compared with control (Figure 1K and L). Together, these results revealed that Matrine played a key role in NSCLC cells.

Matrine Inhibited circFUT8 Expression in NSCLC Cells

As presented in Figure 2A, GEO accession (https://www.ncbi.nlm.nih.gov/geo/geo2r/?acc=GSE112214) suggested that circFUT8 was upregulated in NSCLC tissues when compared with normal tissues. We also confirmed that circFUT8 was increased by 2 to 3-fold in H522 and H1975 cells than that in BEAS-2B (Figure 2B). After performing RNase R experiments, linearFUT8 was obviously downregulated over 50%, while circFUT8 was not changed, implying that circFUT8 was resistant to RNase R (Figure 2C and D). As described by Figure 2E and F, the results of RT-qPCR assay showed the predominant cytoplasmic distribution of circFUT8. What is more, we found that circFUT8 expression was decreased by as much as 50% in H522 and H1975 cells after treatment with Matrine (Figure 2G and H). Therefore, the association between Matrine and circFUT8 was investigated in the next experiments.

Matrine Functioned as an Anti-Carcinogenesis Role Through Regulation of circFUT8 in NSCLC Cells

As Matrine can inhibit circFUT8 expression in H522 and H1975 cells, it is necessary to assess the relationship between Matrine and circFUT8 in NSCLC cells. The downregulation of circFUT8 (over 50%) induced by Matrine was overturned in H522 and H1975 cells by transfection with circFUT8 (Figure 3A and B). Matrine could repress the proliferation ability of H522 and H1975 cells by over 50% decrease, whereas co-transfected with circFUT8 counteracted the effects of Matrine (Figure 3C–F). The flow cytometry assay uncovered that overexpression of circFUT8 could partly neutralize the enhancement effects of Matrine on cell apoptosis (Figure 3G and H). As verified in Figure 3I and J, Matrine inhibited Bcl-2 while enhanced C-Caspase3 expression in H522 and H1975 cells, which were overturned by the upregulation of circFUT8. Furthermore, overexpression of circFUT8 abolished the suppressive function of Matrine on cell cycle progress (Figure 3K and L). The analysis results of transwell assay indicated that migration and invasion were restored by circFUT8 in H522 and H1975 cells

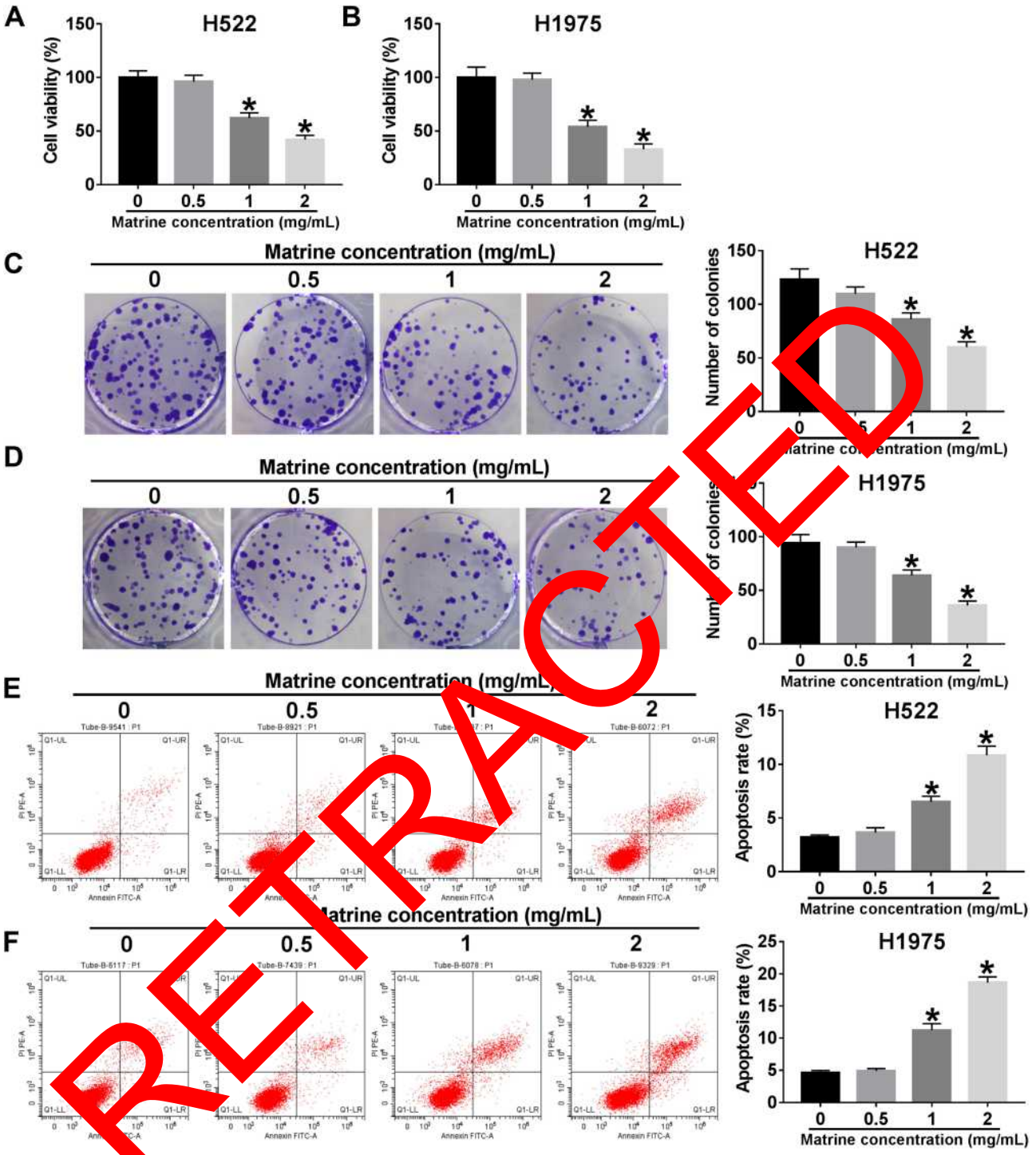

Figure 1 continued.

exposed to Matrine (Figure 3M and N). Considering space, the representative pictures of colony-forming, flow cytometry, and transwell assays were presented in Supplementary Figure S1. Conclusively, Matrine functioned as a carcinoma inhibitor by decreasing circFUT8 expression in H522 and H1975 cells.

CircFUT8 Regulated miR-944 Expression in NSCLC Cells

By bioinformatics analysis, we identified miR-944 as a potential target miRNA for circFUT8 (Figure 4A). The predicted results were confirmed by dual-luciferase reporter assay; miR-944 mimic lead a 50% decrease in

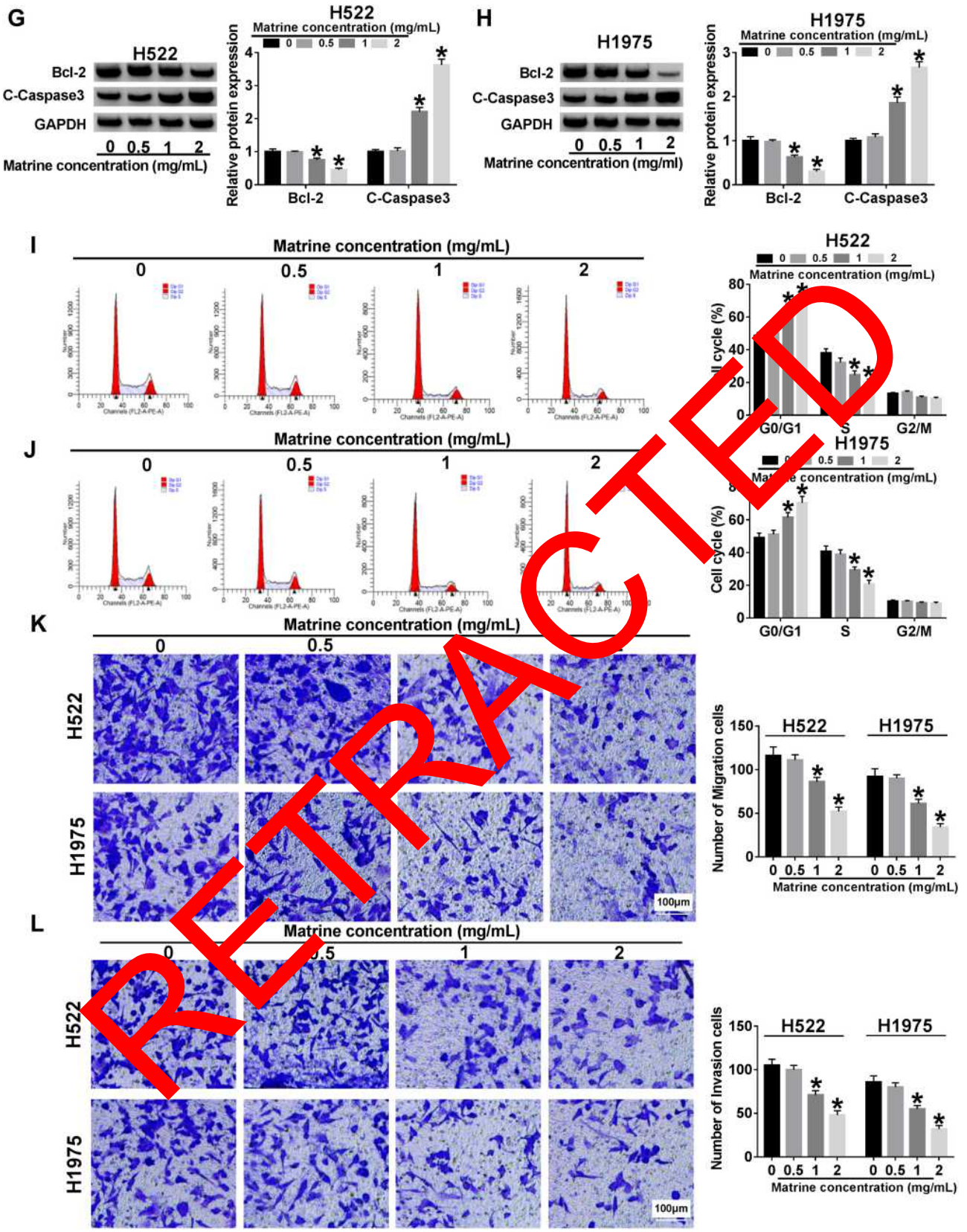

Figure 1 The effects of Matrine on proliferation, apoptosis, cell cycle, migration, and invasion in NSCLC cells. (**A**–**L**) H522 and H1975 cells were treated with Matrine with different concentrations (0, 0.5, 1, and 2 mg/mL) for 24 h. (**A**–**D**) The proliferation ability of H522 and H1975 cells were measured by MTT and colony-forming assays. (**E** and **F**) The flow cytometry assay was performed to monitor apoptotic cells. (**G** and **H**) The protein expression levels of Bcl-2 and C-Caspase3 were detected by Western blot assay. (**I** and **J**) The cell cycle distribution was shown by flow cytometry assay. (**K** and **L**) The migration and invasion of H522 and H1975 cells were determined by transwell assay. *$P < 0.05$.

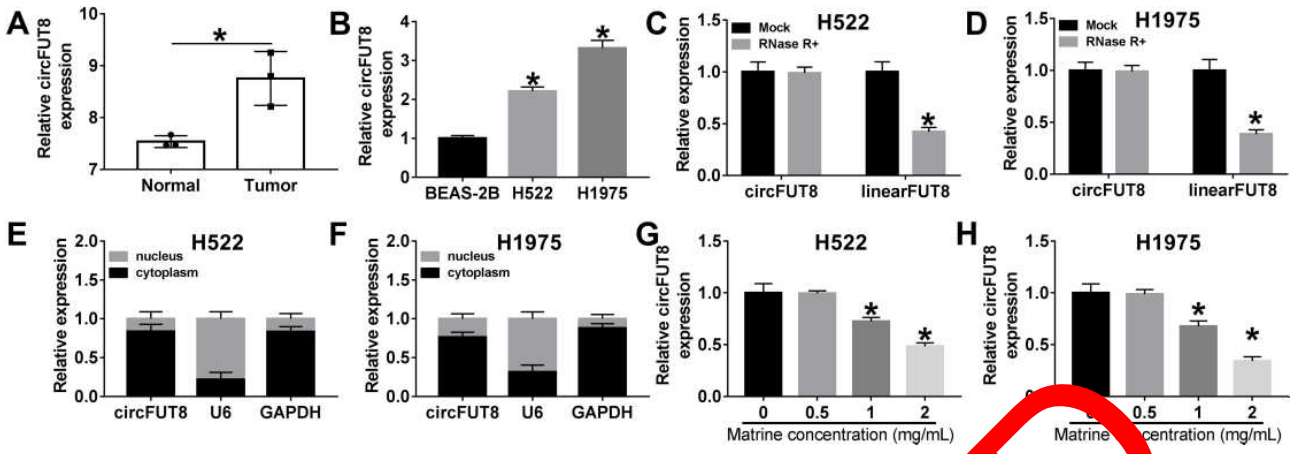

Figure 2 The expression level of circFUT8 in NSCLC tissues and cells. (**A**) The expression level of circFUT8 was shown from GEO accession. (**B**) CircFUT8 level was measured by RT-qPCR assay in NSCLC cells and control group. (**C** and **D**) RT-qPCR assay was conducted to show expression of circFUT8 and linearFUT8 in H522 and H1975 cells after treatment with RNase R. (**E** and **F**) The abundant of circFUT8 in the cytoplasm and nucleus was quantified by RT-qPCR assay. (**G** and **H**) After being treated by Matrine, the level of circFUT8 was shown by RT-qPCR assay in H522 and H1975 cells. *$P < 0.05$.

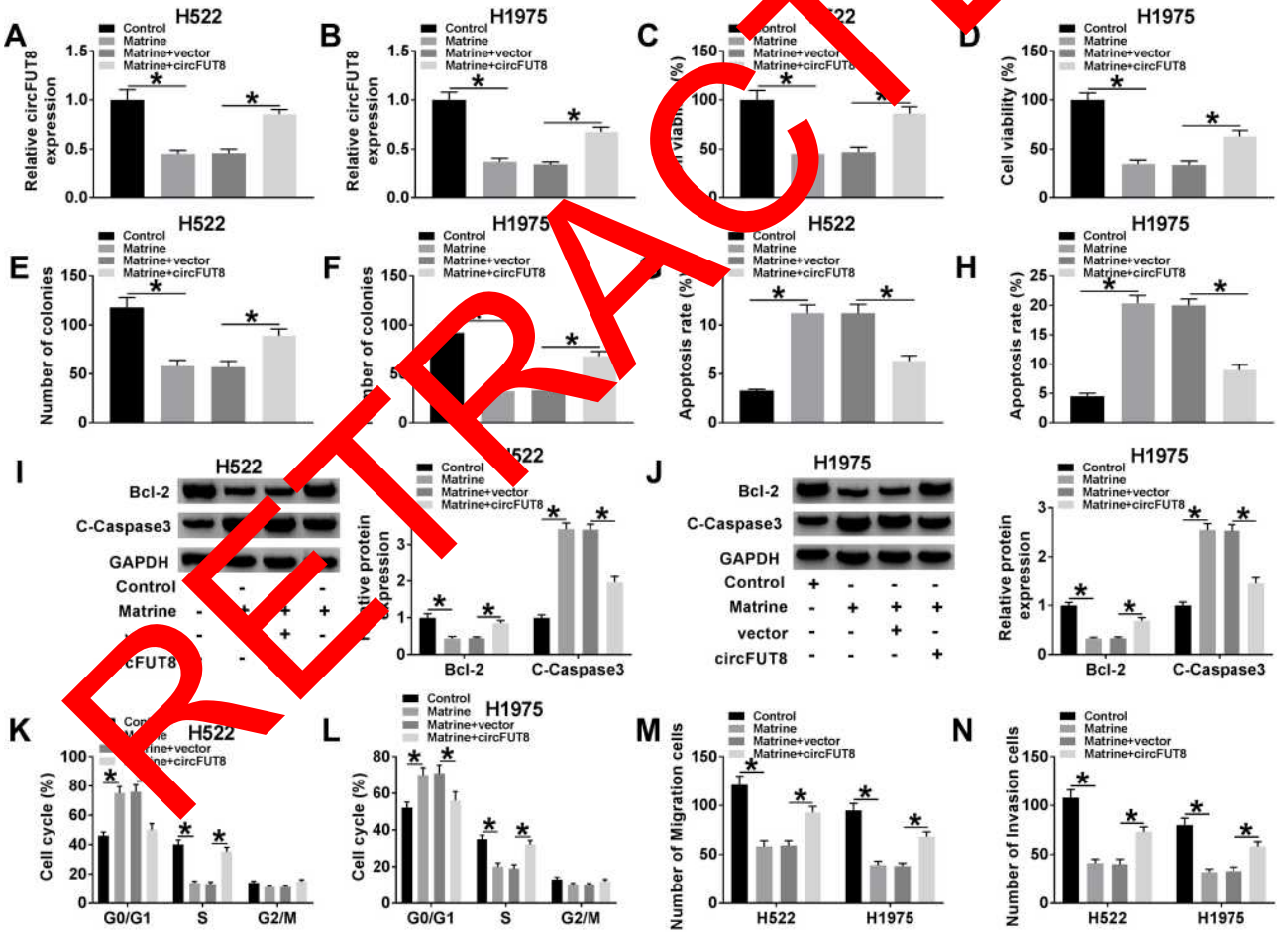

Figure 3 Effects of circFUT8 overexpression on proliferation, apoptosis, cell cycle, migration, and invasion in NSCLC cells treated with Matrine. (**A**–**N**) H522 and H1975 cells were divided into four groups: Control, Matrine, Matrine+vector, and Matrine+circFUT8. (**A** and **B**) The expression level of circFUT8 was examined by RT-qPCR assay after transfection. (**C**–**F**) MTT and colony-forming assays were used to measuring cell proliferation. (**G** and **H**) The apoptosis rate of H522 and H1975 cells was shown by flow cytometry assay. (**I** and **J**) The Western blot assay was performed to assess the expression of Bcl-2 and C-Caspase3 in H522 and H1975 cells. (**K** and **L**) The cell cycle distribution was shown by flow cytometry assay. (**M** and **N**) The transwell assay was conducted in H522 and H1975 cells post-transfection. *$P < 0.05$.

luciferase activity of cells transfected with circFUT8 WT, while there was no change in circFUT8 MUT group (Figure 4B and C). Moreover, overexpression of circFUT8 suppressed miR-944 expression in NSCLC cells by 50% decrease (Figure 4D and E). After treatment of Matrine, H522 and H1975 cells showed a 2-fold increase in miR-944 level compared with control (Figure 4F and G). In summary, miR-944 was a direct target of circFUT8 and was negatively regulated by circFUT8 in NSCLC cells.

CircFUT8/miR-944 Regulated Proliferation, Apoptosis, Cell Cycle, Migration, and Invasion of NSCLC Cells Treated with Matrine

It had been confirmed that miR-944 was a target of circFUT8 in NSCLC cells, and the effects of miR-944 overexpression were explored in NSCLC cells. Under Matrine stimulus conditions, transfection of miR-944 mimic reversed the low-expression of miR-944 caused by circFUT8 (Figure 5A and B). The upregulation of miR-944 obviously restrained circFUT8-induced enhancement effects on cell proliferation in H522 and H1975 cells treated with Matrine (Figure 5C–F). Additionally, miR-944 mimic increased the apoptosis of H522 and H1975 cells by almost 4 times in the presence of circFUT8 and Matrine (Figure 5G and H). The results of Western blot assay suggested that the upregulation of Bcl-2 and downregulation of C-Caspase3 induced by circFUT8 were overturned by miR-944 mimic in H522 and H1975 cells exposed to Matrine (Figure 5I and J). Interestingly, the enhancement effects on cell cycle p[illegible]s in Matrine-induced H522 and H1975 cells ca[illegible]ed by ci[illegible]UT8 were abolished by overexpression of [illegible]R-944 (Figu[illegible] 5K and L). Co-transfection of circF[illegible]T8 and [illegible]iR-944 [illegible]uld counteract the circFUT8-indu[illegible]ed upre[illegible]ulatio[illegible]migration and invasion of H522 and [illegible]1975 cells exposed to Matrine (Figure 5M a[illegible] N). I[illegible]additio[illegible], Supplementary Figure S2 sh[illegible]v[illegible] the repres[illegible]tive pictures of colony-forming, flow cytom[illegible]y, and transwell assays. Therefore, Matrin[illegible]gulated pro[illegible]feration, apoptosis, cell cycle, mig[illegible]tion, and invasion of NSCLC cells by regulating circ[illegible]UT8/miR-9[illegible] axis.

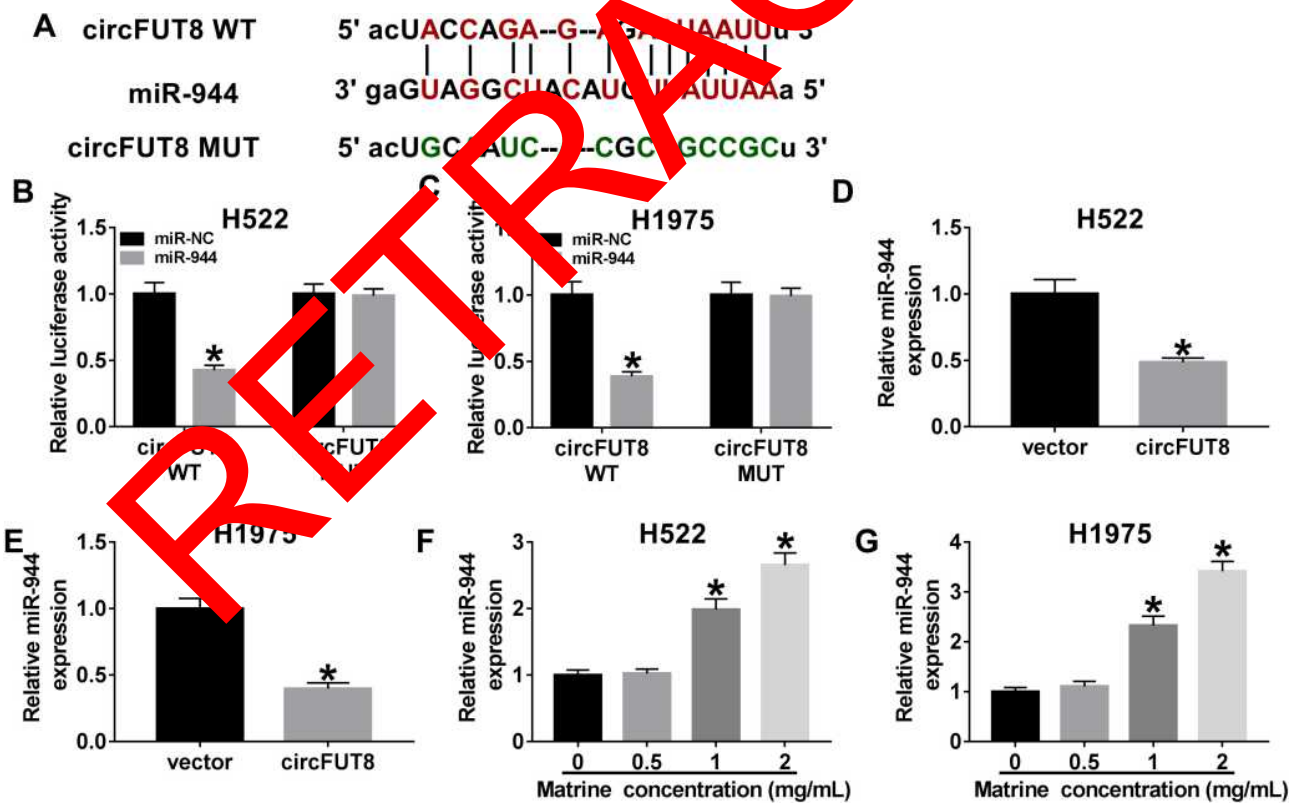

Figure 4 MiR-944 was a direct target of circFUT8 in NSCLC cells. (**A**) Binding regions between miR-944 and circFUT8 were shown. (**B** and **C**) The relative luciferase activity was analyzed in H522 and H1975 cells co-transfected with reports and miR-944 mimic or miR-NC. (**D** and **E**) RT-qPCR assay was conducted to evaluate miR-944 level in H522 and H1975 cells transfected with vector or circFUT8. (**F** and **G**) The expression level of miR-944 was assessed by RT-qPCR assay in H522 and H1975 cells exposed to Matrine with different concentrations (0, 0.5, 1, and 2 mg/mL) for 24 h. *$P < 0.05$.

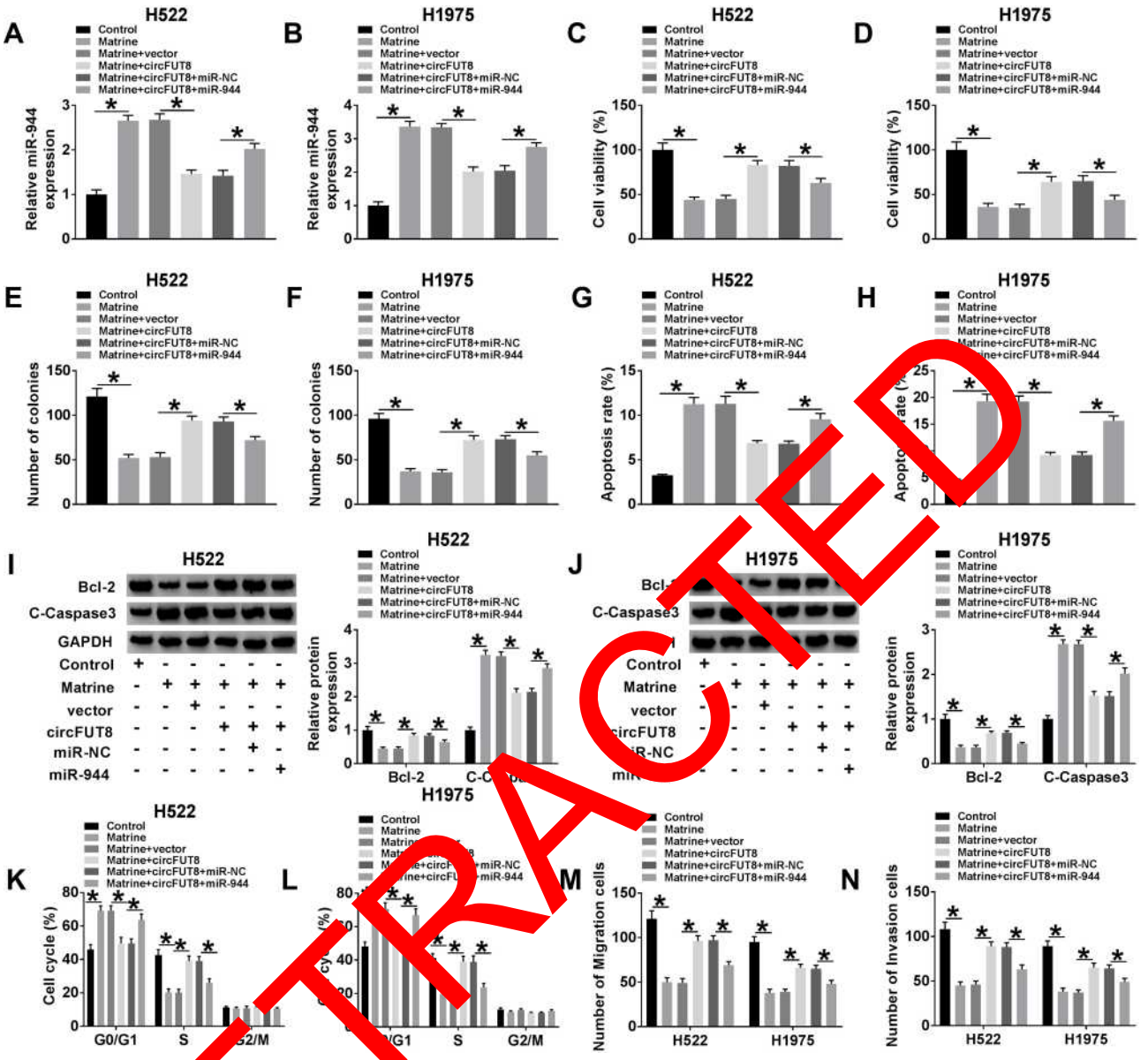

Figure 5 Matrine regula[te] proliferati[on], apoptosis, cell cycle, migration, and invasion of NSCLC cells through circFUT8/miR-944 axis. (**A**–**N**) H522 and H1975 cells were divided into six groups: Co[ntr]ol, M[atr]ine, Matrin[e]+vector, Matrine+circFUT8, Matrine+circFUT8+miR-NC, and Matrine+circFUT8+miR-944. (**A** and **B**) The expression level of miR-944 was detected [by] qPCR assa[y]. (**C**–**F**) The proliferation capability of H522 and H1975 cells was analyzed by MTT and colony-forming assays. (**G** and **H**) The flow cytometry [a]ssay w[as] used to [s]how the [a]poptosis rate of H522 and H1975 cells. (**I** and **J**) The expression levels of Bcl-2 and C-Caspase3 were calculated with Western blot assay. (**K** and **L**) The [c]ell cycle di[s]tribution was shown by flow cytometry assay. (**M** and **N**) The transwell assay was performed to assess migration and invasion of H522 and H197[5] cells post-t[r]ansfection. *$P < 0.05$.

YES1 Was a Target mRNA of miR-944 in NSCLC Cells

The possible target mRNA of miR-944 was predicted by bioinformatics tools. As displayed in Figure 6A, miR-944 had the binding region in 3′UTR of YES1 mRNA. The results of dual-luciferase reporter assay suggested that miR-944 mimic inhibited the luciferase activity of the YES1 3′ UTR WT group by 50% compared with miR-NC group, and this reduction was disappeared in YES1 3′ UTR MUT group (Figure 6B and C). The expression level of YES1 was increased over 2 times in H522 and H1975 cells after the silencing of miR-944 (Figure 6D and E). In addition, Matrine inhibited YES1 expression in H522 and H1975 cells with a dose-dependent method (Figure 6F and G). Interestingly, the upregulation of YES1 in H522 and H1975 cells (over 2-fold) induced by circFUT8 was abolished by overexpression of miR-944 (Figure 6H and I). These data suggest that circFUT8/

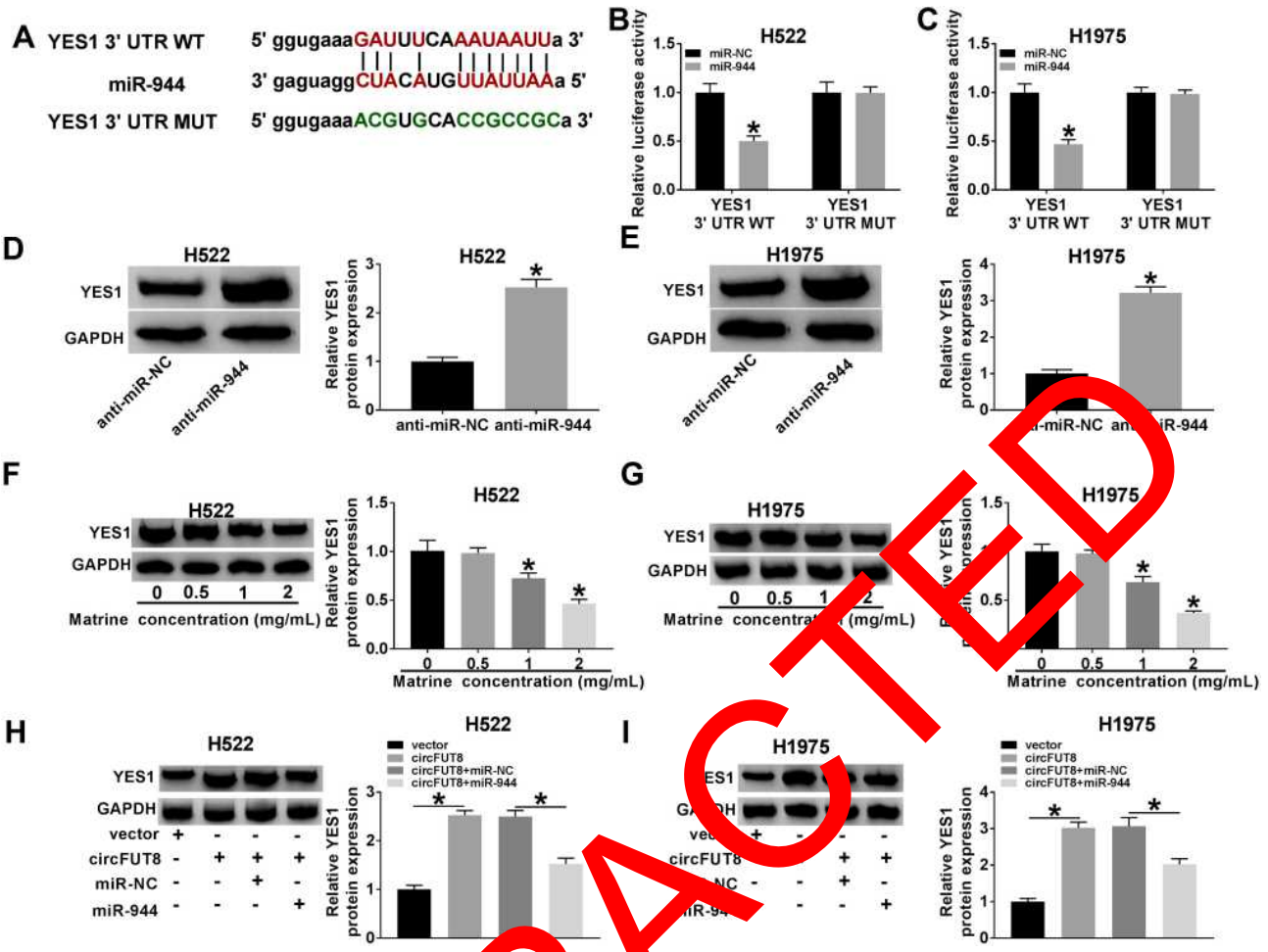

Figure 6 CircFUT8/miR-944 axis regulated YES1 expression in NSCLC cells. (**A**) MiR-944 had the binding region in 3'UTR of YES1 mRNA. (**B** and **C**) The luciferase activities of the wild-type YES1 3' UTR WT and mutant YES1 3' UTR MUT were measured. (**D** and **E**) Western blot assay was performed to assess YES1 level in H522 and H1975 cells transfected with anti-miR-NC or anti-miR-944. (**F** and **G**) After treatment with Matrine with different concentrations (0, 0.5, 1, and 2 mg/mL) for 24 h, the expression of YES1 was shown by Western blot assay. (**H** and **I**) The protein expression level of YES1 was determined by Western blot assay in H522 and H1975 cells transfected with vector, circFUT8, circFUT8+miR-NC or circFUT8+miR-944. *$P < 0.05$.

miR-944 axis regulated YES1 expression in NSCLC cells.

MiR-944/YES1 Axis Participated in the Regulatory Function of Matrine in NSCLC Cells

As presented in Figure 7A and B, YES1 was significantly increased in miR-944-depleted H522 and H1975 cells, while co-transfected with si-YES1 inhibited YES1 expression under Matrine stimulus conditions. Furthermore, the silencing of YES1 significantly abolished anti-miR-944-induced increase in cell proliferation in NSCLC cells exposed to Matrine (Figure 7C–F). In flow cytometry assay, knockdown of YES1 impeded anti-miR-944-induced inhibitory effects on apoptosis in H522 and H1975 cells treated with Matrine (Figure 7G and H). Moreover, the silencing of miR-944 increased Bcl-2 and decreased C-Caspase3 expression in Matrine-induced H522 and H1975 cells, which were abolished by knockdown of YES1 (Figure 7I and J). Co-transfection of anti-miR-944 and si-YES1 could offset the anti-miR-944-induced effects on cell cycle process in Matrine-induced H522 and H1975 cells (Figure 7K and L). The results of transwell assay implied that the enhancement effects of anti-miR-944 on migration and invasion were eliminated by si-YES1 in H522 and H1975 cells exposed to Matrine (Figure 7M and N). The representative pictures of functional experiments were presented in Supplementary Figure S3. These results suggested that Matrine-induced effects on proliferation, apoptosis, cell cycle, migration, and invasion of NSCLC cells were dependent on miR-944/YES1 axis.

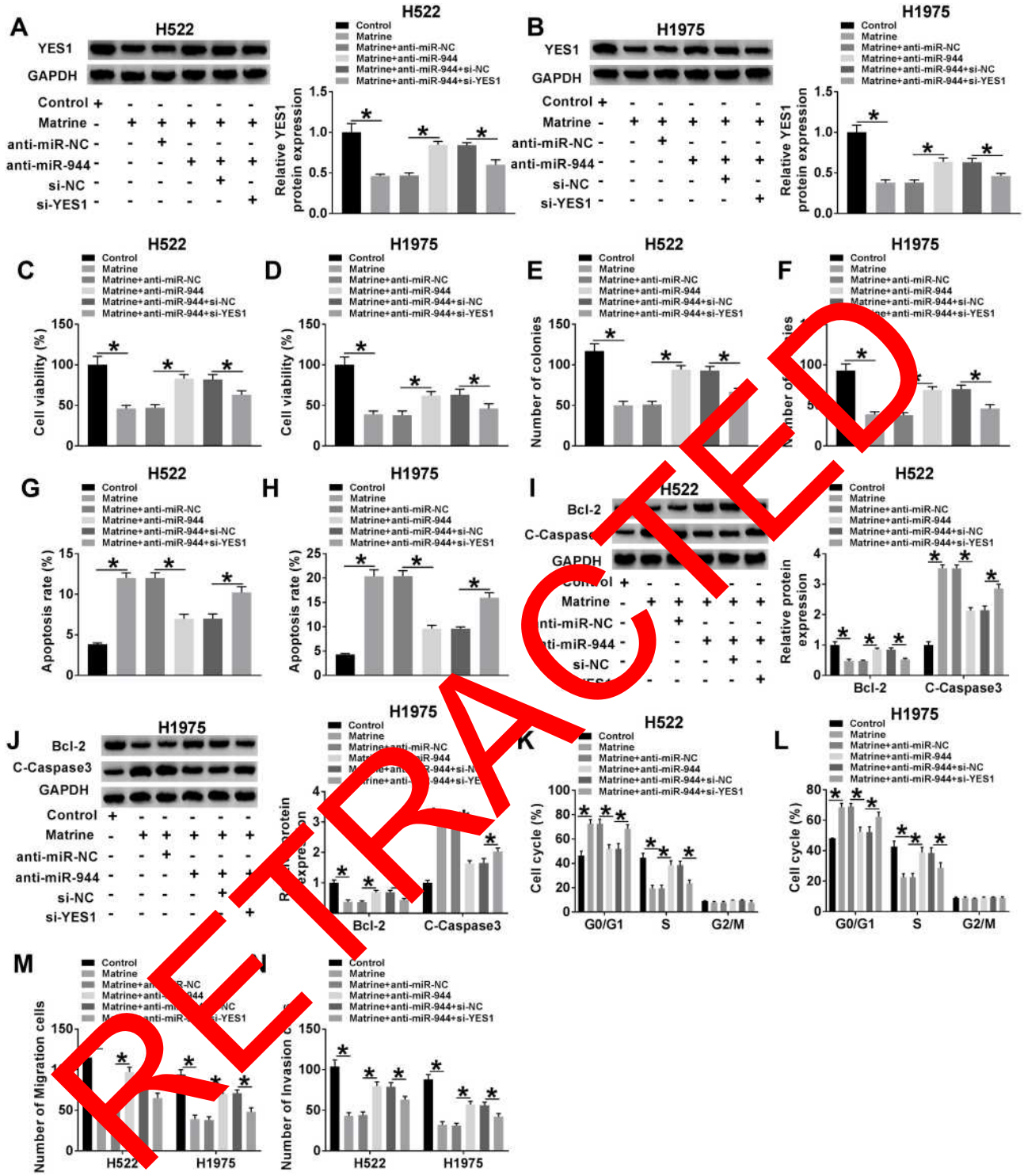

Figure 7 MiR-944/YES1 regulated proliferation, apoptosis, cell cycle, migration, and invasion of NSCLC cells exposed to Matrine. (**A**–**N**) H522 and H1975 cells were divided into six groups: Control, Matrine, Matrine+anti-miR-NC, Matrine+anti-miR-944, Matrine+anti-miR-944+si-NC, or Matrine+anti-miR-944+si-YES1. (**A** and **B**) The protein expression level of YES1 was evaluated by Western blot assay. (**C**–**F**) MTT and colony-forming assays were conducted to assess cell proliferation. (**G** and **H**) The apoptosis rate was determined by flow cytometry assay. (**I** and **J**) The expression levels of Bcl-2 and C-Caspase3 were quantified with Western blot assay. (**K** and **L**) The cell cycle distribution was displayed by flow cytometry assay. (**M** and **N**) The migration and invasion were measured by transwell assay in H522 and H1975 cells. *$P < 0.05$.

Matrine Suppressed Tumor Growth in vivo

The regulatory roles of Matrine were explored in vivo by a xenograft experiment. As shown in Figure 8A and B, when compared with control group, Matrine group showed significant decrease in volume; besides, it also showed a 2-fold decrease xenograft weight compared with control group. CircFUT8 was decreased by 50%, and miR-944 was increased by 3-fold in Matrine group compared with control group (Figure 8C and D). The results of Western blot assay revealed that YES1 was downregulated by 50% in Matrine group (Figure 8E). Additionally, immunohistochemical assay revealed the increased C-Caspase 3-positive cells and decreased ki-67-positive cells in Matrine group (Figure 8F). Therefore, Matrine could impede tumor growth by regulation of circFUT8/miR-944/YES1 axis partially.

Discussion

Matrine, an active component isolated from the roots of Sophora species, has various pharmacological activities, including anti-cancer.[20] Functionally, Matrine suppressed proliferation, while induced apoptosis in breast carcinoma cells through Bcl-2 signal path.[21] Similarly, Tan et al also confirmed that Matrine induced-apoptosis was contributed to the regulation of caspase activity in NSCLC cells.[22] Not surprisingly, Matrine promoted apoptosis via affecting apoptosis-related protein expression, including Bcl-2 and Caspase3; importantly, Matrine could inhibit drug-resistance of NSCLC cells for chemotherapeutic drugs with a dosage-dependent manner, implying the anti-cancer potentials of Matrine in NSCLC.[23] Analogous tumor inhibition impacts by Matrine in NSCLC were also confirmed by other researchers.[24,25]

As we all know, it was widely reported that circRNAs exhibited their effects through functioning as miRNA sponges through competing endogenous RNAs network.[26] A previous report revealed that circFUT8 was a differentially expressed circRNA in liver cancer, and performed function by circRNA-miRNA-mRNA network.[27] Not surprisingly, the significance of circFUT8/miR-570-3p/Krüppel-like Factor 10 network was reported in bladder

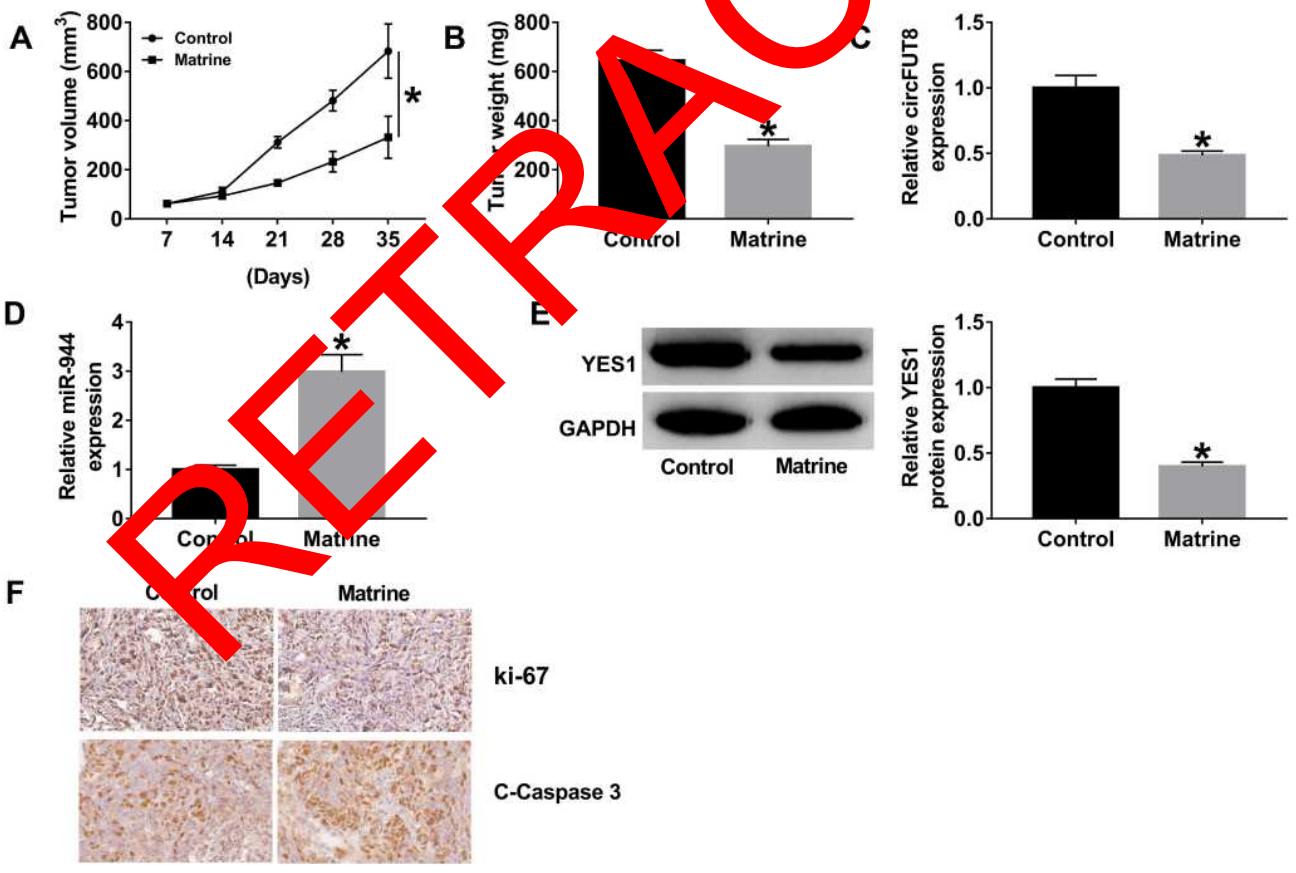

Figure 8 Matrine repressed NSCLC tumor growth in vivo. (**A** and **B**) The volume and weight of xenograft tumors were shown. (**C** and **D**) The expression levels of circFUT8 and miR-944 were estimated with RT-qPCR assay. (**E**) Western blot assay was used to show the expression level of YES1 in tumor tissues, with GAPDH as control. (**F**) Immunohistochemistry for ki-67 and C-Caspase3 was performed in tumor tissues. *$P < 0.05$.

cancer.[28] We confirmed that miR-944 was a target of circFUT8 in NSCLC. Additionally, it has been reported that deregulation of miR-944 play either tumor suppressive or oncogenic function in human malignancies,[8,29] which may contribute to the different regulatory patterns in different diverse of tumors. In NSCLC, Liu et al revealed that the upregulation of miR-944 could suppress NSCLC cell proliferation in vitro.[30] The tumor-suppressive function of miR-944 was dependent on inhibiting expression of oncogenes, such as transcription 1.[31]

The silencing of YES1 triggered the inhibitory effects on proliferation and cell cycle process in ovarian cancer cells.[32] The target gene array analyses by Lee et al revealed that miR-203 enhanced apoptosis of human oral cancer cells by binding to YES1.[33] Similar to previous results,[34,35] the cancerogenic function of YES1 was confirmed in NSCLC. Although previous researches had reported that YES1 was targeted by miRNAs in NSCLC cells, we investigated other miRNAs involved in YES1 in NSCLC. YES1 was involved in the pathogenesis of NSCLC by acting as a target of miR-133a, overexpression of YES1 eliminated the suppressive effect of miR-133a on NSCLC cell growth.[36] In this paper, YES1 was predicted as a targ of miR-944 in NSCLC cells, and the cancerogenic function of YES1 was inhibited by miR-944 exclusive, our findings illuminated how Matrine plays a carcinom inhibitor in NSCLC by interaction with circ miR-944/YES1 axis, which might offer novel therapeutic strategies for NSCLC in the future.

Conclusion

Matrine suppresse circFUT8 expression in NSCLC cells. The anti-cancer effe of Matrine in NSCLC showed a dosage-dependent manner. In summary, we investigated the contributions of Matrine in NSCLC and its interaction with circFUT8/miR-944/YES1 axis, which indicated a new perspective of the roles of Matrine in NSCLC.

Data Sharing Statement

Please contact the corresponding author for the data request.

Ethics Approval

This study was reviewed and approved by the Ethics Committee of Jingmen NO.1 people's hospital.

Consent to Publish

Not applicable.

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Disclosure

The authors declare that they have no financial or non-financial conflicts of interest for this work.

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