

RETRACTED ARTICLE: miR-221 suppression through nanoparticle-based miRNA delivery system for hepatocellular carcinoma therapy and its diagnosis as a potential biomarker

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Background: MicroRNA-221(miR-221) is frequently dysregulated in cancer. The purpose of this study was to explore whether miR-221 can be used as a potential diagnostic marker or therapeutic target for hepatocellular carcinoma (HCC).

Methods: In this study, we investigated whether miR-221 expression was associated with clinicopathological characteristics and prognosis in HCC patients, and we developed a nanoparticle-based miRNA delivery system and detected its therapeutic efficacy in vitro and in vivo.

Results: We found that miR-221 was upregulated in HCC tissues, cell lines and blood of HCC patients. Upregulated miR-221 was associated with clinical TNM stage and tumor capsular infiltration, and showed poor prognosis, suggesting that its suppression could serve as an effective approach for hepatocellular carcinoma therapy. Treatment of HCC cells with nanoparticle/miR-221 inhibitor complex suppressed their growth, colony formation ability, migration and invasion. In vivo, the growth of the tumors treated by the nanoparticle/miR-221 inhibitor complexes were significantly less than those treated by the nanoparticle/miRNA scramble complexes. In addition, circulating miR-221 may be a potential tumor biomarker for early diagnosis of HCC, and combined serum miR-221 and AFP detection gave a better performance than individual detection in early diagnosis of HCC.

Conclusion: These findings suggest that a nanoparticle-based miRNA delivery system could potentially serve as a safe and effective treatment and miR-221 could also be a potential diagnostic marker for HCC.

Keywords: hepatocellular carcinoma, nanoparticle, miR-221, biomarker, therapeutic target

Introduction

Hepatocellular carcinoma (HCC) is one of the common worldwide malignant tumors with high mortality, and early definite diagnosis and appropriate treatment would improve 5-year survival.^{1,2} The pathogenesis of HCC is very complicated with lots of genes and proteins to participate in.³⁻⁵ The advanced clinical stage system is the only accepted method for predicting the prognosis of clinical HCC patients with progressive disease, with surgery being the only major therapeutic option. Diagnosis of HCC is usually dependant on image techniques, blood indicators, and histopathology.^{1,6-9} These methods improve the detection of HCC to some degree, but the sensitivity and specificity for HCC screening remain unsatisfactory. At the same time, identification of the pathogenic mechanisms involved in HCC and development of useful prognostic biomarkers as well as novel targeted therapeutic strategies are the urgent clinical necessities, so we hope to find some new diagnostic biomarkers that can further improve

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the early detection of HCC in at-risk population. Currently, aberrant expression of microRNAs (miRNAs) is a hot topic for various diseases and it is still in its preliminary research stage in HCC diagnosis, treatment, and prognosis in China.

miRNAs are a class of small, single-stranded, and endogenously expressed noncoding RNA molecules with ~18–25 nucleotides. They regulate gene expression that leads to downstream effects on diverse biological processes including early embryonic development and cell proliferation, differentiation, and apoptosis.^{10,11} It has been recognized that the aberrant expression of miRNAs plays essential roles in various biological processes.^{12–15} The miRNAs have different biological functions, such as miRNA-221 (miR-221), one of the miRNAs that be either oncogenes or tumor suppressor genes involved in tumor formation, was confirmed overexpression in HCC tissues while the function and mechanism were not clear at present.¹⁶ At the same time, recent studies showed that the expression level of circulating miR-221 changed with the occurrence and development of tumor, which may act as a potential biomarker and can be applied to the early noninvasive diagnosis of tumor or prognostic assessment.^{17–19}

In the present study, we focused our attention on the expression of miR-221 in HCC tissues and cell lines and affected clinicopathological characteristics and prognosis of liver cancer patients. These results suggest that miR-221 can serve as a potential therapeutic target for HCC. Therefore, the application of miRNAs as potential therapeutic agents requires suitable delivery vectors that effectively protect the miRNAs from nucleases and improve delivery efficiency to the tumor cells. In recent years, nonviral vectors, especially nanoparticle gene vectors, have attracted more and more attention, mainly due to their low toxicity and biodegradability.²⁰ In this study, we investigated poly(lactide-co-glycolic) acid (PLGA)-based nanoparticle for the delivery of miRNA to HCC cells and tested its therapeutic efficacy *in vitro* and *in vivo*. At the same time, we detected serum miR-221 concentration in patients with HCC and healthy controls to analyze the efficiency in the early diagnosis of HCC and made a preliminary exploration on the possibility of miR-221 as a potential biomarker to provide ideas in the early diagnosis and research mechanism of HCC.

Materials and methods

Human tissue samples and cell lines

HCC and adjacent noncancerous tissues were collected and stored from 45 patients who underwent surgical resection between January 2015 and October 2016, of whom 14 cases were well differentiated, 13 cases were moderately differentiated, and 18 cases were poorly differentiated. The corresponding

serum specimens of 45 patients with HCC and the normal controls were collected at the same time. Before surgery, none of the patients received anticancer treatment involving chemotherapy, radiotherapy, or biological treatments. The pathological classification and staging of HCC was in accordance with the AJCC TNM Staging System.²² This study was approved by the Ethics Committee of the Affiliated Hospital of Nantong University, and informed consent was obtained from all participants. The median follow-up time was 23.4 months, which ranged from 3.0 to 60.0 months. Overall survival was defined as the time from the date of operation to the date of death or last contact.

Human liver cancer cell lines (HepG2, Hep3B, Huh7, and HCCLM3) and the normal liver cell line LO-2 were obtained from either the Type Culture Collection of Chinese Academy of Sciences (Shanghai, China) or the American Type Culture Collection. These cells were cultured and stored according to the provider's instructions.

Reagents and apparatus

The following apparatus and reagents were used in this study: miRNA extraction kit (RNAiso for Small RNA), reverse transcription kits (PrimeScript™), and RT-qPCR kits (all from TaKaRa, Tokyo, Japan). Primers were synthesized by Shanghai Yingxin Biotechnology Company (Shanghai, China).

Quantitation of AFP

The concentration of AFP in the serum samples from HCC patients was measured using the ARCHITECT AFP Reagent Kit (Abbott, Chicago, IL, USA), according to the calibrator dose–response curve by the chemiluminescence method and data collected.

miRNA isolation

According to RNAiso for Small RNA instructions, each of 100 mg of liver tissue was homogenized with 2 mL of RNAiso for Small RNA, while 200 μ L of serum sample was extracted with 1 mL of RNAiso, then mixed with 200 μ L of chloroform, standing at room temperature for 5 minutes, and then centrifuged at $12,000\times g$ at 4°C for 15 minutes. The supernatant was transferred to a 1.5 mL RNAase-free centrifuge tube. After that, 500 μ L of isopropanol was added in the tube and placed at room temperature for 10 minutes and was centrifuged at $12,000\times g$ at 4°C for 10 minutes. The supernatant was discarded, and 1 mL of 75% ethanol was added to the precipitate. Then, the mixture was centrifuged at $12,000\times g$ at 4°C for 5 minutes. The supernatant was discarded, and the precipitate was air-dried. Each sample was

eluted with 10 μL of RNase-free water. The concentrations and purity of miRNA were controlled by spectrophotometer. The purity ranged from 1.8 to 2.2.

cDNA synthesis and miRNA quantification

miRNA was subjected to the reverse transcription (RT) reactions using the SYBR PrimeScript™ miRNA RT-PCR Kit (TaKaRa) by PCR instrument (TECHVE TC-412 UK). The transcription reaction mixture was as follows: 20 μL of reverse transcriptase reaction mixture containing 1.5 μL of miRNA sample, 10 μL of reaction buffer mix, 2 μL of 0.1% BSA, 2 μL of PrimeScript RT Enzyme Mix, and 4.5 μL of RNase-free dH_2O . The mixture was incubated at 37°C for 60 minutes and 85°C for 5 seconds. Specific operation methods were used according to the manufacturer's recommendation. The qPCR was run in LineGene Real-Time Detection System as per the manufacturer's instructions, and data were collected at last (Bioer, Hangzhou, China).

The reaction was repeated for 40 cycles. Three parallel wells were set for each sample, and averages were collected for statistical purpose. U6 was selected as the internal normalization control in this study. The relative expression levels of miRNAs were calculated using the comparative $2^{-\Delta\Delta\text{CT}}$ method as described in a previous study.²³ The fold change in miRNAs were calculated by the equation $2^{-\Delta\Delta\text{CT}}$:

$$\Delta\Delta\text{CT} = (\text{CT}_{\text{miRNA}} - \text{CT}_{\text{U6}})_{\text{tumor}} - (\text{CT}_{\text{miRNA}} - \text{CT}_{\text{U6}})_{\text{reference}}$$

where CT is defined as threshold cycle to detect fluorescence and $\Delta\Delta\text{CT}$ is defined as the difference in miR-221 expression between tumor and normal tissue OK group.

Preparation and characterization of nanoparticle/miRNA complexes

PLGA nanoparticles were prepared using a water-in-oil-in-water solvent evaporation technique as described previously with minor modification.²⁴ Briefly, 100 mg of PLGA was dissolved in 1 mL of methylene chloride to form a 10% (w/v) polymer solution. Then, 3 mL of 7% (w/v) poly(vinyl alcohol) aqueous solution was added to the above polymer solution and emulsified by sonification for 120 seconds (Bandelin Electronic, Berlin, Germany). The above emulsion was further added into 50 mL of 1% (w/v) PVA aqueous solution. The resulting mixture was sonicated for 180 seconds and then stirred for 24 hours at room temperature to evaporate the residual methylene chloride. Subsequently, the nanoparticles suspension was washed twice with ddH_2O by centrifugation

at 13,000 rpm for 5 minutes and then resuspended in the ddH_2O . A total of 100 μL of the nanoparticles aqueous solution (10 $\mu\text{g}/\mu\text{L}$) was mixed with 2 μL of polyethylenimine (PEI; Mw \approx 25 kDa) polymer aqueous solution (100 $\mu\text{g}/\mu\text{L}$) to form PEI-modified nanoparticles. A gel retardation assay was used to detect the most appropriate ratio of nanoparticles and miRNA (N/P). Then, PEI-modified nanoparticles suspension was added to the miRNA solution at the 6:1 ratio between nitrogen of polymer and phosphate of RNA (N/P), mixed gently, and then incubated for 15 minutes at room temperature to form nanoparticle/miRNA complexes. The morphology of the nanoparticle/miRNA complexes was characterized by scanning electron microscopy (SEM; S-3400; Hitachi Ltd., Tokyo, Japan). The mean hydrodynamic diameter measurements of nanoparticle/miRNA complexes were performed by dynamic light scattering (Brookhaven Instruments, Holtsville, NY, USA). The zeta potential (surface charge) of the complexes was determined at 25°C with a scattering angle of 90° using Zetaplus (Brookhaven Instruments).

In vitro transfection

In order to detect the transfection efficiency of the nanoparticle/miRNA complexes, cells were seeded in six-well plates at an initial density of 1×10^5 cells/well in completed DMEM. After 24 hours of culture, cells were transfected with nanoparticle/FAM-conjugated miRNA complexes with a dosage of 10 μL of miRNA (20 μM) per well and cultured at 37°C. Lipofectamine/FAM-conjugated miRNA complexes were used as positive controls. At 48 hours post-transfection, cells were washed to get rid of free nanoparticle/FAM-conjugated miRNA complexes, and cells containing FAM-conjugated miRNA were visualized with a fluorescence microscopy (Leica DMR 3000; Leica Microsystems, Wetzlar, Germany). The cells were also collected for the detection of transfection efficiency. The transfection efficiency was assessed using a FACS Calibur™ flow cytometer (BD FACS Calibur; BD Biosciences, San Jose, CA, USA). All transfection data are representative of three independently repeated transfections. In order to explore the biological function of miRNA-221, miRNA-221 mimics, and miR-221 control (miR-con), miRNA-221 inhibitor and corresponding control scramble were designed, synthesized, and then mixed with PEI-modified nanoparticles to form nanoparticle/miRNA complexes for transfection.

Cell proliferation, migration, and invasion assays

Cell viabilities were assessed by performing the 3-(4,5-dimethylthiazole-2-yl)-2,5-biphenyl tetrazoliumbromide

(MTT) assay. The spectrophotometric absorbance at 570 nm was detected for each sample, and the experiments were performed in triplicate and repeated three times.

A colony formation assay was performed as previously described.²¹ Briefly, cells were seeded in a six-well plate at 24 hours after transfection and cultured for 2 weeks in RPMI 1640 medium (Thermo Fisher Scientific, Waltham, MA, USA) containing 10% fetal bovine serum (FBS; Thermo Fisher Scientific). Colonies were fixed and dyed with 0.1% crystal violet (1 mg/mL), and the number of colonies with >50 cells was counted.

Cell invasion was evaluated using a transwell assay with matrigel (8 μ m pore; BD Biosciences). The procedures were performed as previously described.²³ The experiments were repeated three times. Wound healing assays were performed to detect cell migration. The cells were seeded in six-well plates, and an artificial wound was created using a 200 μ L pipette tube. The wound closure was observed after 24 hours and imaged under a microscope. We measured the fraction of cell coverage across the line to assess the migration rate.

Labeling of cells with luciferase

HepG2 and HCCLM3 cells were first infected by lentivirus containing a firefly luciferase gene and a neomycin selection cassette and then exposed to 500 μ g/mL G418 (Thermo Fisher Scientific) for 28 days postinfection. Some luciferase expression clones were further expanded in the culture medium containing 200 μ g/mL G418 to get rid of tumor cells that lose luciferase expression.

Tumorigenesis assay

For the tumorigenesis assay, male BALB/c nude mice (5 weeks old) were selected as animal models. All groups were conditioned and manipulated according to Council on Animal Care guidelines, Nantong University, Nantong, China. This experimental protocol was approved by the Research Ethics Committee of Nantong University. Male BALB/c nude mice were housed four per cage under a 12 hours light–dark cycle and in a temperature-controlled room (25°C \pm 1°C). Mice were allowed access to water and standard laboratory chow ad libitum. Luciferase-expressing cells (1 \times 10⁶ cells/100 μ L per flank) were subcutaneously injected into the dorsal flanks of nude mice (six per group). Tumor-bearing mice were randomized into four different groups for treatments (nanoparticle/miR-con, nanoparticle/miR-221 mimic [miR-221 mimic], nanoparticle/miR-scramble [scramble], nanoparticle/miR-221 inhibitor [miR-221 inhibitor], n=6 per group), when the tumor volume increased to 100 mm³. Prior to administration of each complex, animals were anesthetized and injected with nanoparticle/miRNA complexes (1 nmol of miRNA per

injection) through the tail vein twice per 5 days for 25 days. For bioluminescence imaging, mice were intraperitoneally injected with a dose of 10 μ L luciferin (15 mg/mL) per gram of body weight. Luciferase activity was visualized using the Xenogen IVIS 2000 small-animal In Vivo Imaging System (Xenogen Corp., Alameda, CA, USA).

Statistical analysis

Statistical analysis was conducted using the Statistical Package for the Social Sciences (version 17.0). Quantitative variables were presented as mean \pm SD. Paired samples *t*-test or independent samples *t*-test was used for the analysis of differences between the two groups. Survival was evaluated using the Kaplan–Meier method with the log-rank test. In addition, correlations between miR-122 and AFP were evaluated with Pearson's correlation. The receiver operating characteristic (ROC) curve and area under the curve (AUC) were established to evaluate the diagnostic performance of using serum miRNAs' concentration as diagnostic markers for screening HCC. The Youden's index was used to identify the optimal cutoff point. In all tests, a *P*-value of <0.05 was considered statistically significant.

Results

miR-221 is upregulated in liver cancer cell lines, tissues, and serum samples

miR-221 levels were first determined in the liver cancer cell lines, tissues, and serum samples by real-time PCR. The results showed that they were significantly higher in the liver cancer cell lines (Bel-7402, HepG2, Huh7, and HCCLM3) than in the normal liver cells, LO-2 (*P*<0.05, Figure 1A). In the human tissues, the relative expression amount of miR-221 in HCC tissues was 1.71 \pm 1.05, which was significantly higher than that in the adjacent noncancerous tissues (1.01 \pm 0.45; n=45, *P*<0.05, Figure 1B). At the same time, we detected the serum samples of these patients, and the results showed that the relative level of serum miR-221 ranged from 1.025.579 to 1.277, with a median of 52.305, while in the control group, the relative expression level of miR-221 ranged from 17.860 to 0.004, with a median of 1.091. The box plot statistics of the logarithm of relative expression (lg2^{- $\Delta\Delta$ Ct}) showed that the relative expression level of serum miR-221 was significantly higher than that in the control group (n=45, *P*<0.05, Figure 1C).

Upregulation of miR-221 was associated with clinicopathological characteristics and prognosis in liver cancer patients

To investigate the clinicopathological significance of miR-221 in liver cancer patients, miR-221 levels were measured in the freshly frozen tissues of 45 liver cancer patients.

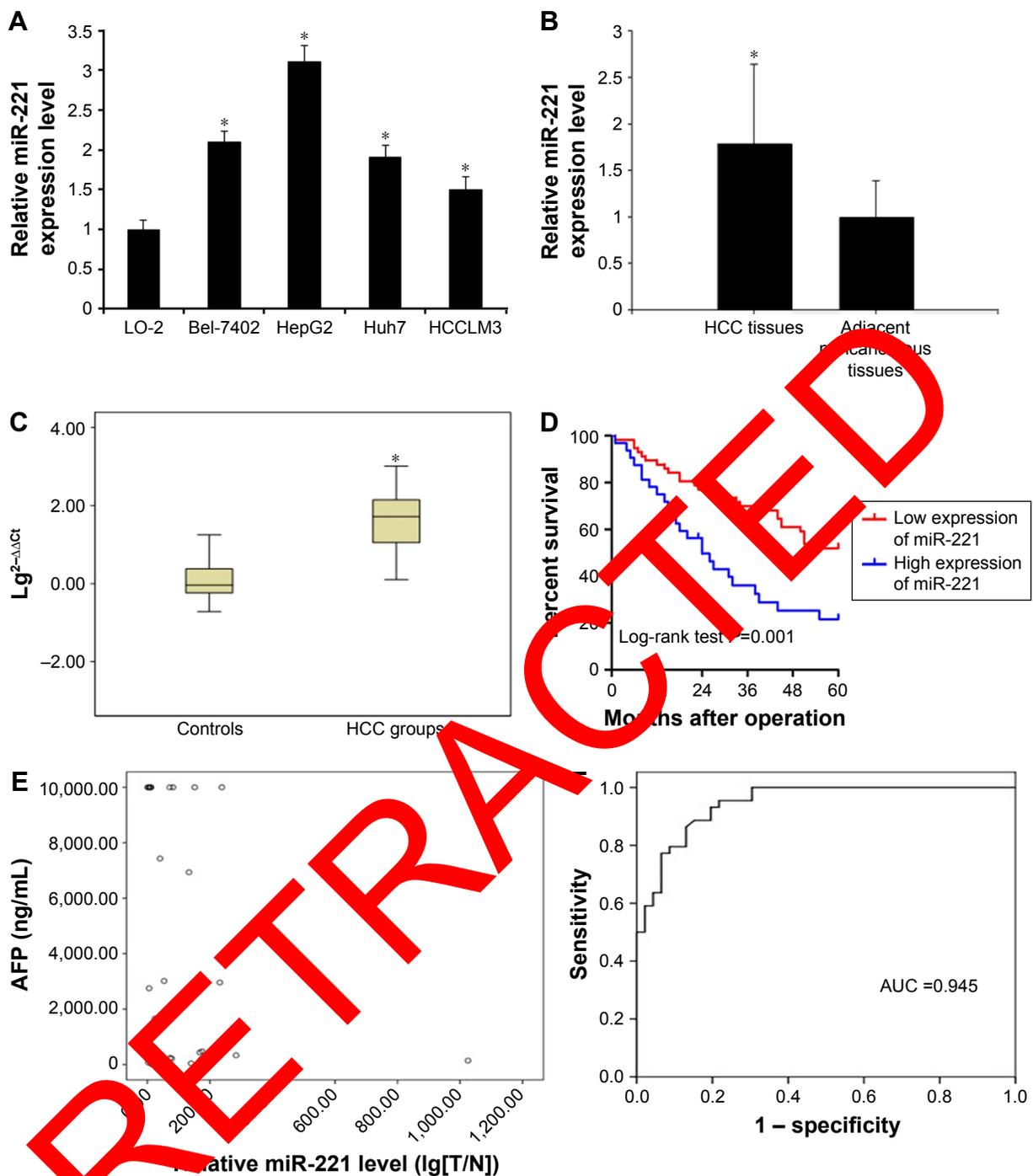


Figure 1 miR-221 is significantly upregulated in liver cancer cell lines, tissues, and serum samples.

Notes: (A) Relative expression levels of miR-221 in liver cancer cell lines and a normal liver cell line, LO-2 (* $P < 0.05$). (B) Relative expression levels of miR-221 in liver cancer tissues ($n=45$) and adjacent normal tissues ($n=45$) (* $P < 0.05$). (C) Serum relative expression of miR-221 in HCC patients and healthy controls ($n=45$) (* $P < 0.05$). The box plot shows the median, 25th, and 75th percentiles of miRNA expressions. (D) Kaplan-Meier curve of overall survival of liver cancer patients with high ($n=45$) and low ($n=45$) miR-221 levels ($P=0.001$). (E) Relative miR-221 level (lg[T/N]). Correlation coefficient: $r=0.198$, $P=0.241$, showing that there was no significant correlation between miR-221 and AFP, indicating that miR-221 can act as an independent predictor in the diagnosis of HCC. (F) ROC curve when choosing the relative level of 2.458 as the optimal cutoff point of miR-221; the Youden's index was maximal, and the area under the curve (AUC) was 0.945 (95% CI: 0.655–0.894, $P < 0.005$). The diagnostic sensitivity, specificity, and accuracy were 93.33, 77.78, and 90.9%, respectively.

Abbreviations: HCC, hepatocellular carcinoma; miR-221, miRNA-221; miRNA, microRNA; ROC, receiver operating characteristic.

The relationship between miR-221 expression levels and clinicopathological parameters is summarized in Table 1.

From the clinicopathological parameters, differences in miR-221 expression were not statistically significant by sex,

age, tumor size, AFP content, HbsAg seropositivity, liver cirrhosis, and differentiation ($P > 0.05$). In contrast, relative expression of miR-221 was correlated with clinical TNM stage ($P=0.009$) and tumor capsular infiltration ($P=0.013$).

Table 1 Correlation between miR-221 expression and clinicopathological parameters in patients with HCC ($\bar{x} \pm s$)

Clinicopathological parameters	n	Relative expression level of miR-221	P-value
Gender			0.501
Male	28	1.95±1.79	
Female	17	1.75±1.26	
Age (years)			0.252
≥60	23	1.98±1.79	
<60	22	1.33±0.89	
Tumor size (cm)			0.195
≥5	24	2.25±1.38	
<5	21	1.75±0.61	
AFP (ng/mL)			0.697
<400	15	2.09±0.81	
≥400	30	1.98±1.12	
HBsAg			0.575
(+)	29	1.66±1.09	
(-)	16	1.72±0.49	
Clinical TNM stage			0.009*
I and II	17	0.77±0.82	
III and IV	28	2.13±0.49	
Tumor capsular infiltration			0.013*
No capsular infiltration	17	0.91±1.09	
Capsular infiltration	28	2.79±1.46	
Cirrhosis			0.723
Yes	25	1.875±1.39	
No	20	1.579±1.44	
Differentiation			0.332
Poorly	18	1.98±1.49	
Moderately well	27	2.11±1.55	

Note: * $P < 0.05$ was considered statistically significant.

Abbreviations: HCC, hepatocellular carcinoma; miR-221, microRNA-221.

Moreover, Kaplan–Meier analysis indicated that patients with high miR-221 expression levels tended to have worse overall survival than those with low levels of miR-221 expression ($P = 0.001$, Figure 1D).

Combined serum miR-221 and AFP in diagnosis of HCC

The scatter point diagram was mapped out to analyze correlation between miR-221 and AFP (Figure 1E). The result showed $r = 0.198$, $P = 0.241 > 0.05$, indicating that miR-221 has no correlation with AFP and it can act as independent predictor of the diagnosis of HCC. According to the ROC curve, when choosing relative level of 2.458 as the optimal cutoff point of miR-221, the Youden's index was maximal, then the diagnostic sensitivity, specificity, and accuracy were 93.33, 77.78, and 90.9%, respectively, and the AUC was 0.945 (95% CI: 0.655–0.894, $P < 0.005$) (Figure 1F). As to serum AFP concentration, we chose 400 $\mu\text{g/L}$ as the cutoff point in the diagnosis of HCC according to criteria made

in Guangzhou by Chinese Society of Liver Cancer (CSLC) in September 2001.²⁴ The result showed that in 19 cases of HCC, the AFP levels were higher than the cutoff point, and the sensitivity was 42.22%; the difference was statistically significant compared to the control group ($P < 0.05$). Combined serum miR-221 and AFP detection gave a better performance with the sensitivity of 96.49% and the accuracy of 93.10% than individual marker in the diagnosis of HCC (Table 2).

Characterization and transfection efficiency of nanoparticle/miRNA complexes

In recent years, nonviral vectors, especially nanoparticle gene vectors, have attracted more and more attention, mainly due to their low toxicity and good degradability.^{20,21}

In this study, PLGA nanoparticle was prepared as described previously²² and used as miRNA transfection vector. The morphology of the nanoparticle/miRNA complexes was observed by SEM. Figure 2 shows typical SEM images of the PLGA nanoparticles (Figure 2A1), PEI-modified nanoparticles (Figure 2A2), and nanoparticle/miRNA complexes (Figure 2A3). It can be seen that the PLGA nanoparticles, PEI-modified nanoparticles, and nanoparticle/miRNA complexes are spherical and uniform in size. Figure 2B presents the hydrodynamic diameter of complexes determined by DLS.

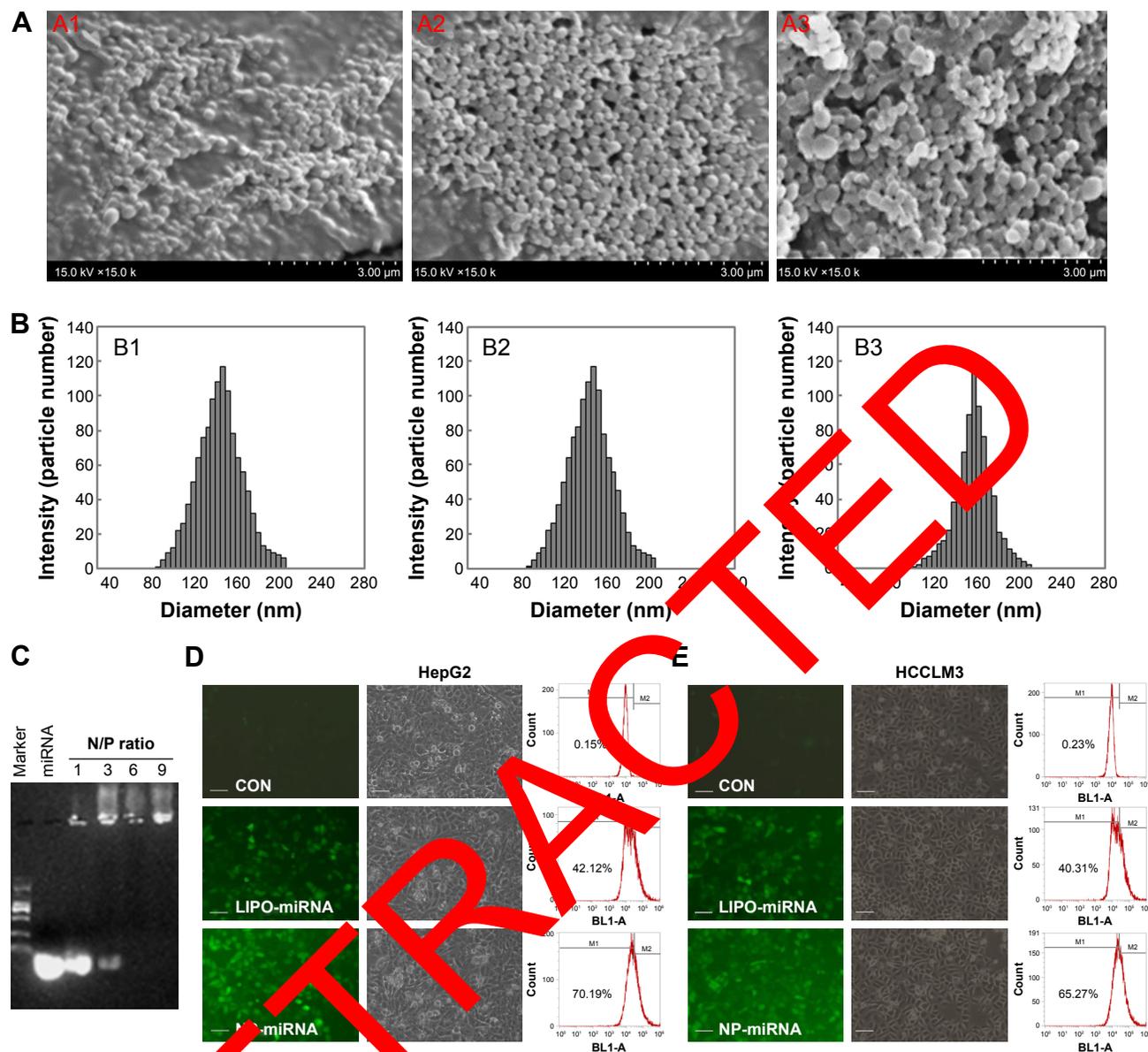
As shown in Figure 2B, it is shown that the mean diameter of the PLGA nanoparticles (Figure 2B1), PEI-modified nanoparticles (Figure 2B2), and nanoparticle/miRNA complexes (Figure 2B3) was ~137, 143, and 150 nm, respectively. The gel retardation assay demonstrated that nanoparticles containing plasmid showed excellent retardation at an N/P ratio of 6:1 (the ratio of moles of the amine groups of polyethyleneimine to moles of the phosphate groups of miRNA) (Figure 2C). The zeta potential of the PLGA nanoparticles, PEI-modified nanoparticles,

Table 2 Predictive performance of miR-221, AFP, and combined detection in the diagnosis of HCC

Statistical parameters	miR-221	AFP	miR-221 + AFP
Sensitivity (%)	93.33	42.22	96.49
Specificity (%)	77.78	91.10	88.00
Accuracy (%)	90.90	73.81	93.10
Positive predictive value (%)	85.71	84.00	86.05
Negative predictive value (%)	95.45	69.50	95.65
Positive likelihood ratio	4.37	6.05	8.21
Negative likelihood ratio	0.03	0.51	0.02

Notes: Combined serum miR-221 and AFP detection gave a better performance than individual in. It may provide a better view for HCC screening.

Abbreviations: HCC, hepatocellular carcinoma; miR-221, microRNA-221.



and nanoparticle/miRNA complexes (N/P ratio of 6:1) was approximately -13.1 , 29.3 , and 21.5 mV, respectively.

Flow cytometry analysis was used to detect the transfection efficiency of nanoparticle/FAM-miRNA complexes. As shown in Figure 2D and E, FAM-miRNA was successfully delivered into the HepG2 and HCCLM3 cells and the transfection efficiency was $\sim 70\%$ and 65% , respectively. It is obvious to observe that the FAM intensity of the nanoparticle/plasmid complexes was higher than lipofectamine/FAM-miRNA complexes. These results showed that the

nanoparticle/miRNA complexes have high transfection efficiency for HepG2 and HCCLM3 cells.

The effect of nanoparticle/miRNA complexes on cell proliferation, migration, and invasion in vitro

Since clinicopathological data indicate that miR-221 expression was significantly associated with clinical TNM stage and tumor capsular infiltration in liver cancer, we speculated that miR-221 may exert promotive effects on cell

proliferation and invasion. Thus, HCCLM3 cell showing relatively lower expression of miR-221 was transfected with nanoparticle/miR-221 mimic complexes to overexpress miR-221. Real-time PCR showed that miR-221 expression level was significantly increased in HCCLM3 cells transfected with nanoparticle/miR-221 mimic complexes ($P<0.05$, Figure 3A). As expected, the ectopic expression of miR-221 markedly promoted HCCLM3 cell proliferation, as demonstrated by the MTT assay ($P<0.05$, Figure 3B) and the colony formation assay ($P<0.05$, Figure 3C). Moreover, the overexpression of miR-221 promoted cell migration and invasion in the liver cancer cells, as indicated by the wound healing and transwell assays (both $P<0.05$, Figure 3D and E). To investigate the relationship between endogenous miR-221 and liver cancer biology, the HepG2 cells presenting with the highest level of miR-221 were transfected with nanoparticle/miR-221 inhibitor complexes to block the endogenous miR-221 expression. Real-time PCR analysis showed that miR-221 was significantly decreased after the treatment with miR-221 inhibitor ($P<0.05$, Figure 4A). Knockdown of miR-221 expression dramatically suppressed cell proliferation, colony formation ability, migration, and invasion (Figure 4B–E, all $P<0.05$).

The effects of nanoparticle/miRNA complexes on tumor growth in vivo

To evaluate the effects of miR-221 in vivo on liver cancer tumor growth, HCC cells were subcutaneously injected into the flanks of nude mice. After treatment with nanoparticle/miR-221 mimic or miR-con complexes for HCCLM3 and nanoparticle/miR-221 inhibitor or scramble for HepG2, the luminescence intensities of the tumors treated with the nanoparticle/miR-221 mimic were significantly more than those treated with the nanoparticle/miR-con complexes ($P<0.05$, Figure 5A and B). In contrast, the luminescence intensities of the tumor treated with the nanoparticle/miR-221 inhibitor were significantly less than those treated with the nanoparticle/scramble complexes ($P<0.05$, Figure 5A and B). Furthermore, we detected the miR-221 expression level from blood serum samples of the mice treated with different complexes at each time point. The results showed that the blood serum expression level of miR-221 in the mice treated with the nanoparticle/miR-221 mimic was significantly higher than those treated with the nanoparticle/miR-con complexes ($P<0.05$, Figure 5C). In contrast, the expression level of miR-221 in the mice treated with the nanoparticle/miR-221 inhibitor was significantly lower than those treated with the nanoparticle/scramble complexes ($P<0.05$, Figure 5C). These results suggest that miR-221,

as a potential therapeutic target and delivery system, can be used as effective therapeutic approach.

Discussion

HCC is a primary neoplasm of the liver with the third highest gastrointestinal tumor incidence worldwide.^{1,25} Many studies have focused on studying genes and proteins underlying the development and progression of HCC.^{4,9,26} However, its underlying molecular mechanism remains unknown. As we all know, the key to prolong the survival of patients with HCC is to improve the early diagnosis of HCC. AFP is the most common tumor biomarker that is used in early diagnosis of HCC, but due to its low diagnostic sensitivity, which in our study was confirmed to be 42.22%, has still a large gap with clinical demand. At present, ideal biomarkers for high sensitivity and specificity are not found in the diagnosis of HCC.

In recent years, investigation of miRNAs as contributors to the initiation and pathogenesis of HCC offers the potential to uncover new therapeutic targets.^{13,14,27} Previous studies have showed that miR-221 was upregulated in HCC tissues, which is involved in the progress of HCC process, and displayed characteristics as oncogene.^{16,28,29} In this study, we found that miR-221 was frequently upregulated in liver cancer cells, and its high expression was significantly associated with clinical TNM stage, tumor capsular infiltration, and poor survival. Our results also showed that the miR-221 expression level was significantly higher in HCC tissues than in the adjacent noncancerous tissues, which was conformed to the previous studies, indicating that miR-221, as a kind of miRNA similar to oncogene, may play a key role in the progression of HCC.

Pineau et al³⁰ in 2010 transferred retroviral vector MSCV-miR-221 into E18 p53^{-/-} liver progenitors and then injected it into mouse body. The mouse model of liver cancer indicated that miR-221 stimulated the onset of tumors when compared with vector-infected cells and promoted tumor progression, significantly shortening the mean time to death as well. Our experimental data showed that relative expression level of miR-221 was significantly higher in clinical TNM stages III and IV than in stages I and II ($P=0.009$), which was correlated with tumor growth and disease progression obviously, suggesting that it not only induced the occurrence of HCC but also promoted the further development of the tumor in its progression, which is similar to those of the previous studies.^{16,31} The results also displayed that the expression level of miR-221 correlated with tumor capsular infiltration ($P=0.013$), which is higher in capsular infiltration group than in no infiltration group, indicating that miR-221 was related to migration,

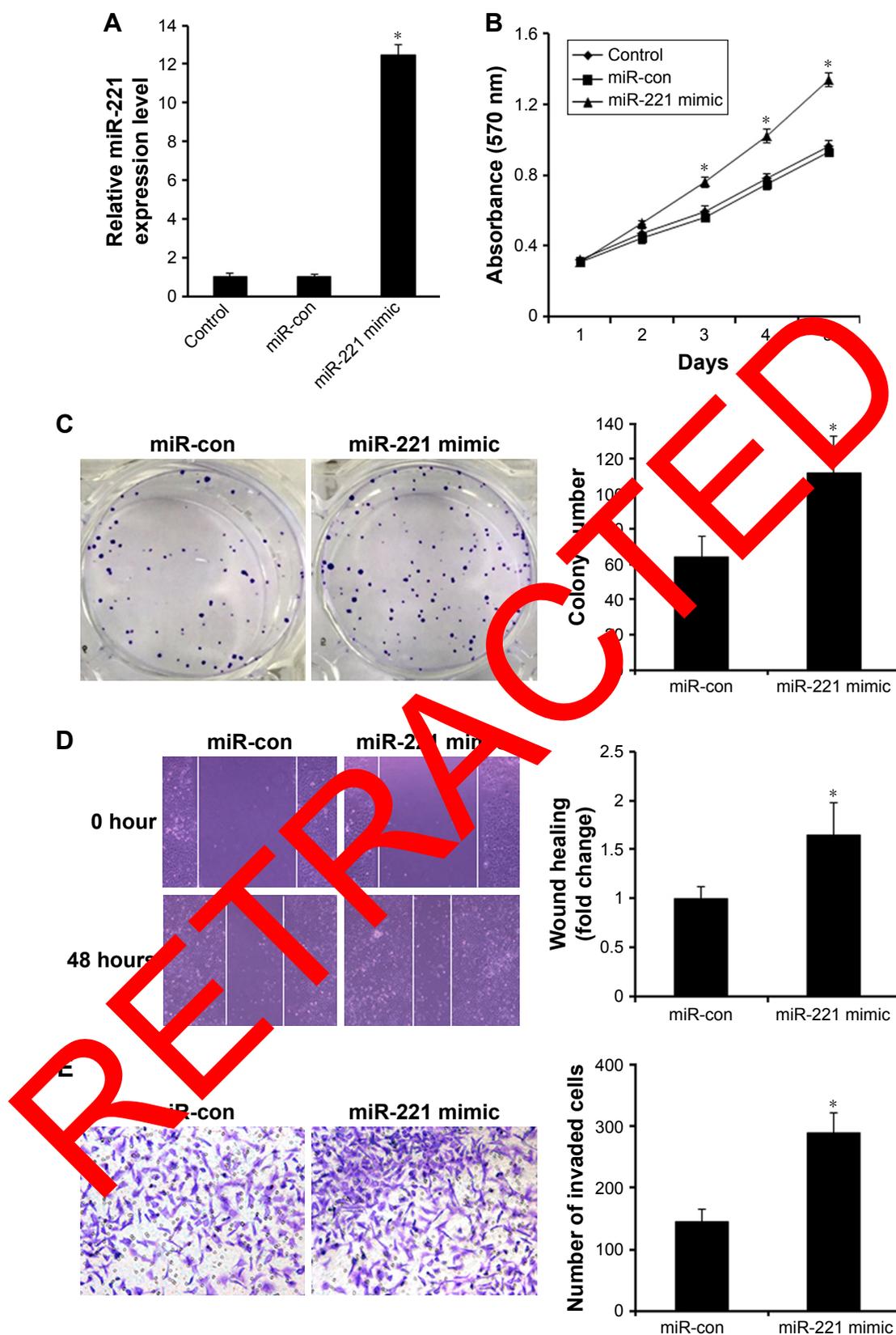


Figure 3 Ectopic miR-221 expression promotes liver cancer cell growth, colony formation, and invasion in vitro.

Notes: (A) Real-time PCR showed that miR-221 expression level was significantly increased in HCCLM3 cells transfected with miR-221 mimic (* $P < 0.05$). (B) Ectopic miR-221 expression significantly promoted cell viability in HCCLM3 cells as demonstrated by the MTT assay (* $P < 0.05$). (C) Ectopic miR-221 significantly increased colony formation numbers in HCCLM3 cells (* $P < 0.05$). (D) Ectopic miR-221 expression promoted the migration of HCCLM3 cells as indicated by the wound healing assay (* $P < 0.05$). (E) Ectopic miR-221 expression significantly promoted the invasiveness of HCCLM3 cells (* $P < 0.05$).

Abbreviations: miR-con, miR-221 control; miR-221, microRNA-221; MTT, 3-(4,5-dimethylthiazole-2-yl)-2,5-biphenyl tetrazoliumbromide.

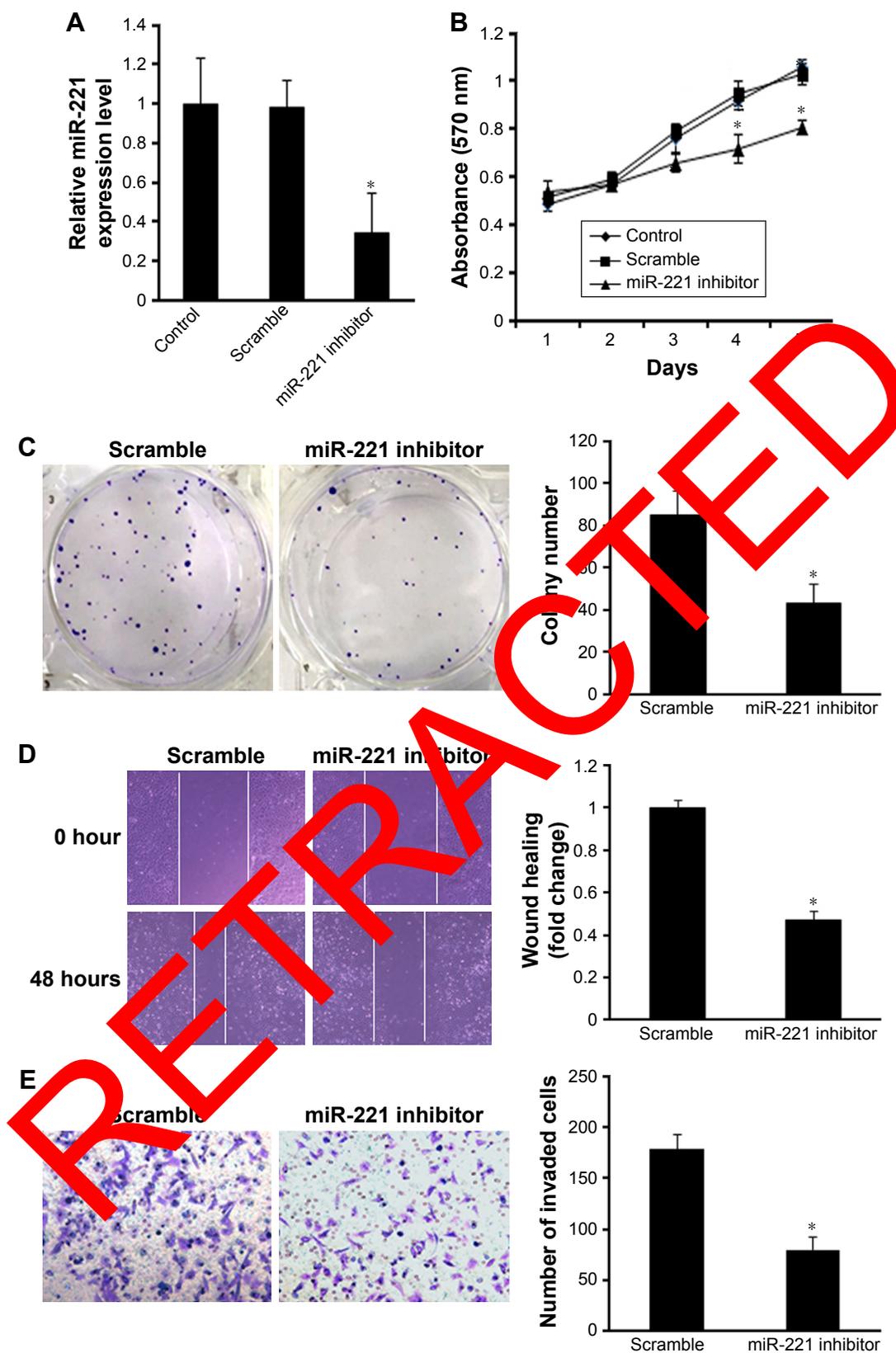


Figure 4 Blocking of endogenous miR-221 inhibits liver cancer proliferation, colony formation, and invasion in vitro.

Notes: (A) Real-time PCR showed that miR-221 expression level was significantly reduced in HepG2 cells transfected with miR-221 inhibitor ($*P < 0.05$). (B) Knockdown of miR-221 significantly suppressed cell proliferation as demonstrated by MTT assay ($*P < 0.05$). (C) Knockdown of miR-221 dramatically inhibited colony formation ability ($*P < 0.05$). (D) Knockdown of miR-221 significantly promoted the migration of HepG2 cells as indicated by the wound healing assay ($*P < 0.05$). (E) Knockdown of miR-221 dramatically inhibited the cell invasion ($*P < 0.05$).

Abbreviations: miR-221, microRNA-221; MTT, 3-(4,5-dimethylthiazole-2-yl)-2,5-biphenyl tetrazoliumbromide.

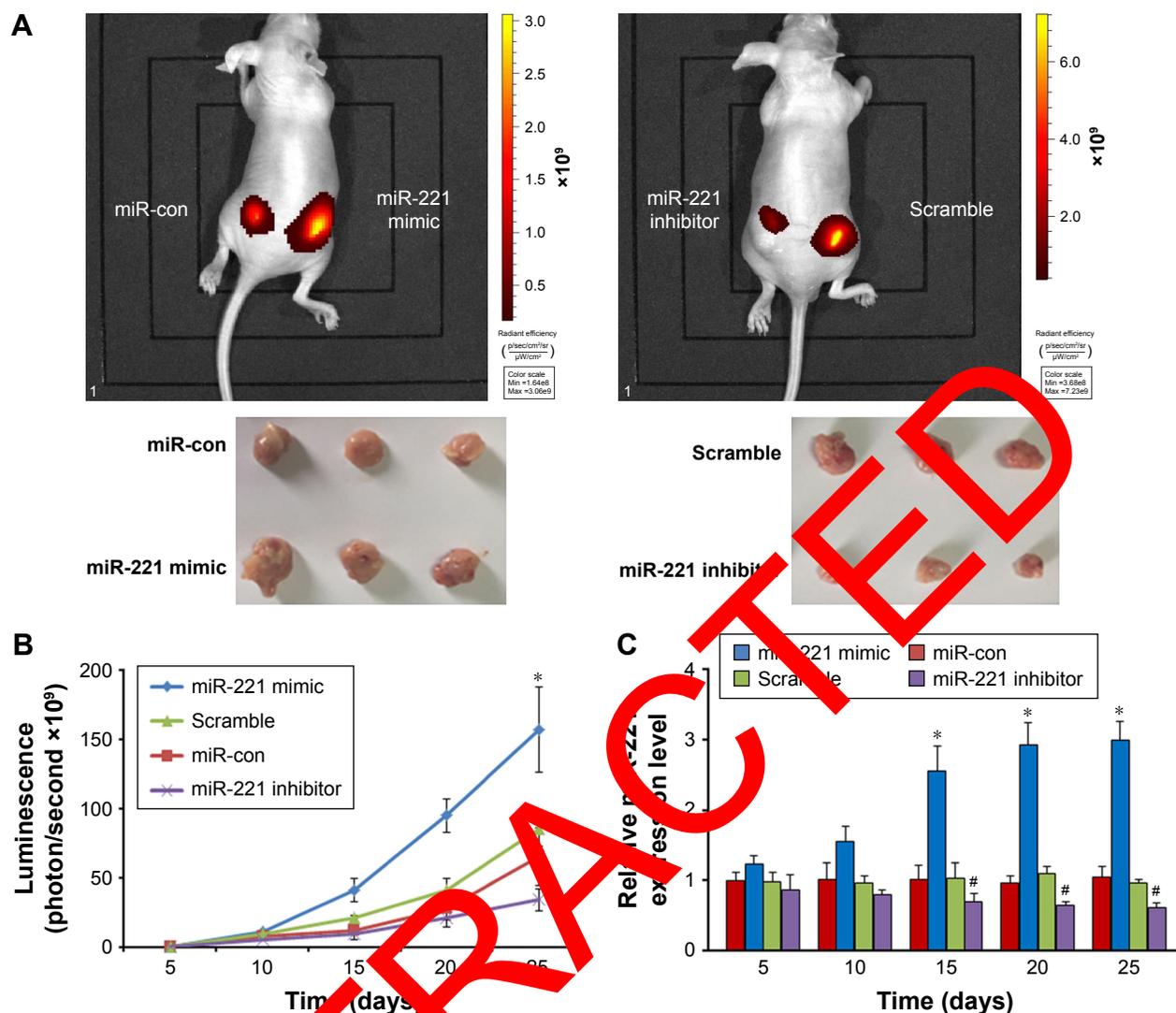


Figure 5 Effect of miR-221 mimic or miR-221 inhibitor on tumor growth in vivo.

Notes: (A) Representative bioluminescence images and xenografts. (B) Tumor growth curves were indicated by bioluminescence (photons/second). * $P < 0.05$ as compared with miR-con groups, # $P < 0.05$ as compared with scramble groups. (C) The miR-221 expression level from blood serum of mice treated with different complexes. * $P < 0.05$ as compared with miR-con groups, # $P < 0.05$ as compared with scramble groups. Data represent the results from three independent experiments.

Abbreviations: miR-con, miR-221 control; miR-221, miRNA-221.

invasion, and metastasis of hepatoma cells. Garofalo et al³² in their experimental results showed that miR-221, by targeting PTEN and TIMP-3 tumor suppressors, induced tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) resistance and enhanced cellular migration through the activation of the AKT pathway and metalloproteinases. AKT occupied the key position of signaling pathways in the body, the activation of which would accelerate cell growth, proliferation, and metastasis; therefore, the activation of PTEN/AKT signaling pathway may be one of the mechanisms in which miR-221 promoted proliferation and metastasis of hepatoma cells.

Recent studies have reported that miRNA profiles change not only in liver tissues but also in blood samples of HCC patients.^{14-16,33} Detecting the levels of miRNAs in serum have

a lot of advantages, such as being noninvasive, widely available, and allowing measurements of very small quantities of miRNAs. The diagnostic potential and prognostic potential of miRNAs as cancer biomarkers rely on their high stability and resistance to the digestion of RNA lysate. In our study, we confirmed that relative expression level of circulating miR-221 was significantly higher in HCC than that in the healthy group. At the same time, from the ROC curve, we determined the cutoff value of serum miR-221 to be 2.458 in the diagnosis of HCC, and the AUC was 0.945 as a result. Combined detection of serum miR-221 and AFP showed better diagnostic sensitivity and accuracy than when using a single marker. Serum miR-221 level showed no correlation with AFP indicating that miR-221 can act as an independent

predictor in the diagnosis of HCC. The fact that high expression of miR-221 is observed in HCC patients adds to the possibility of using this biomarker to precisely monitor the health status of specific organs and also increases the chances of a successful outcome by targeting the at-risk population and clinical diagnosis.

Tumor growth is an important factor for the determination of tumor phenotypes. Therefore, identification of the molecular mechanisms underlying liver cancer growth is of great importance. Recent studies have demonstrated that some miRNAs play critical roles in the initiation and progression of liver cancer.¹³ In the present study, we found that miR-221 inhibitor can inhibit liver cancer cell proliferation and invasion in vitro as well as tumorigenesis in vivo. We believe that this may be an ideal therapeutic strategy in cancer therapies.

Conclusion

miR-221 is upregulated in liver cancer cell lines, tissues, and blood of HCC patients. Upregulated miR-221 was associated with clinical TNM stage, tumor capsular infiltration, and poor prognosis, and the developed nanoparticle-based miRNA delivery system showed good therapeutic efficacy in vitro and in vivo, which may be expected to potentially serve as a safe and effective treatment. Circulating miR-221 may act as a potential tumor biomarker for the diagnosis of HCC, and combined serum miR-221 and AFP detection gave a better performance than individual marker in the diagnosis of HCC. All of these would carry important implications in HCC intervention, prevention, diagnostic, and treatment.

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Disclosure

The authors report no conflicts of interest in this work.

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