



NIRF is frequently upregulated in colorectal cancer and its oncogenicity can be suppressed by let-7a microRNA

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ARTICLE INFO

Article history:

Received 26 May 2011

Received in revised form 16 September 2011

Accepted 26 September 2011

Keywords:

NIRF

Let-7a

CRC

Overall survival

Oncogenicity

ABSTRACT

Np95 ICBP90 RING finger (NIRF) is essential for the regulation of cell proliferation and has been implicated in tumorigenesis. However, the role of NIRF in colorectal cancer (CRC) remains unclear. In this study, we demonstrated that NIRF expression was aberrantly increased in CRC tissues and associated with poor overall survival. Bioinformatics analysis indicated that NIRF was a putative target of the microRNA let-7a, which was confirmed by luciferase reporter assay. We then demonstrated *in vitro* that enforced expression of let-7a, or knockdown of NIRF, led to reduced CRC cell proliferation due to cell cycle arrest at the G0/G1 phase and reduced cell migration. Finally, an *in vivo* tumorigenicity assay in nude mice showed that synthetic let-7a suppressed NIRF expression and reduced tumor growth. Taken together, our results provide new evidence that NIRF has an oncogenic role in CRC. This opens up the possibility of targeting NIRF and let-7a for CRC therapy.

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1. Introduction

Colorectal cancer (CRC) is one of the most common cancers and is a significant contributor to cancer death [1].

Abbreviations: CRC, colorectal cancer; CT, cycle threshold; dsRNA, double-stranded RNA; ICBP90, inverted CCAAT box binding protein of 90 kDa; IHC, immunohistochemistry; mRNA, messenger RNA; miRNA, microRNA; NIRF, Np95 ICBP90 RING finger; PHD, plant homeodomain; qRT-PCR, quantitative real-time reverse-transcription PCR; RING, really interesting new gene; ROC, receiver operating characteristic; RT-qPCR, reverse transcription followed by quantitative real-time PCR; siRNA, small interfering RNA; SRA, set- and ring-associated; MTT, methylthiazolyl-tetrazolium; NC, negative control; PCNA, proliferating cell nuclear antigen; PCNP, PEST-proteolytic signal containing nuclear protein; UHRF, ubiquitin PHD RING finger; UHRF2, ubiquitin-like with plant homeo domain (PHD) and ring finger domains 2; UTR, untranslated region.

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Despite recent advances in the diagnosis and treatment of CRC, the overall prognosis for CRC patients remains poor. Therefore, there is an urgent need to develop novel therapeutic approaches for CRC. To achieve this, a deeper understanding of the molecular and genetic networks that control the initiation and progression of CRC is imperative.

Np95 ICBP90 RING finger (NIRF) protein belongs to the ubiquitin plant homeo domain (PHD) really interesting new gene (RING) finger (UHRF) family and is encoded by a gene located on chromosome 9p23–24 [2]. NIRF protein possesses several diverse structural domains, including an NIRF_N domain, a PHD finger, a set- and ring-associated (SRA) domain, and a RING finger domain, some of which have been shown to be essential for the regulation of cell proliferation [3]. Several studies have investigated the potential role of NIRF in cancers with contradictory results, suggesting the dual potential of NIRF as tumor suppressor or oncogene in malignant cells [2,4–6].

MicroRNAs (miRNAs) are a class of short, highly conserved non-coding RNAs that regulate the expression of

target genes through messenger RNA (mRNA) decay or translation repression [7]. Abnormalities of miRNA expression have been observed in various types of human cancers and are correlated with the prognosis and survival of cancer patients [8,9]. Emerging data suggest that miRNAs may function as oncogenes or tumor suppressor genes and play a critical role in cancer development [10].

Using bioinformatic algorithms and luciferase reporter assay, in this study we found that NIRF is a target of let-7a, a common tumor suppressor miRNA. We wondered which role NIRF played in CRC (tumor suppressor or oncogene) and whether it was regulated by let-7a miRNA. We first compared NIRF expression between CRC tissues and paired normal colorectal tissues to determine if NIRF had potential as a novel prognostic biomarker for CRC. Then we explored the association between let-7a and NIRF in CRC and the implications of this association in the tumorigenesis of CRC.

2. Materials and methods

2.1. Patient samples

The Ethics Committee of the Sixth People's Hospital, Shanghai Jiao Tong University, approved this study. A total of 164 CRC patients were enrolled; all gave written informed consent. The patients underwent surgical resection of tumors at the Sixth People's Hospital, Shanghai Jiao Tong University between 2003 and 2007, during which samples of cancer and adjacent non-tumor tissues were collected. No patients received chemotherapy or radiotherapy before the surgery.

The matched non-tumor adjacent tissue was obtained from a segment of the resected specimens that was the farthest from the tumor (>10 cm). Tissue samples were collected, snap-frozen in liquid nitrogen, and stored at -80°C until used. All tissues were histologically confirmed to be adenocarcinoma of the colon or rectum.

2.2. Tissue microarray construction and immunohistochemistry (IHC)

Formalin-fixed and paraffin-embedded tissue cylinders (diameter 2 mm) were punched from morphologically representative tissue areas and gathered into one recipient paraffin block (3×2.5 cm) using a homemade semi-automated tissue arrayer. Five composite tissue microarray blocks with 328 tissue cores were designed and cut into $5 \mu\text{m}$ sections. To block endogenous peroxidase, dewaxed paraffin sections were treated for 30 min with methanol containing 1% hydrogen peroxide. After washing in Tris buffer, slides were incubated with anti-human NIRF primary antibody (rabbit polyclonal, 1:250 dilution, Abcam, Cambridge, UK). For immunostaining, a peroxidase-conjugated goat anti-rabbit antibody (Zhongshan Goldenbridge Biotechnology, Beijing, China) was used, following the manufacturer's guidelines.

NIRF staining was assessed under a light microscope by two independent investigators. The reading of tissue slides was masked and both assessors were unaware of clinical

outcomes. Staining was considered positive for NIRF when a strong coloration was evident in the epithelial cells, mostly in the nucleus. Tissues were scored semi-quantitatively by counting the positive nuclei of 10 separate fields at $\times 400$ magnification in the areas with the highest density of positive nuclei. The appropriate cutoff score was obtained using receiver operating characteristic (ROC) curve analysis [11]. Briefly, taking the percentage score as the independent variable, the sensitivity and specificity values for tumor or adjacent non-tumor tissue were plotted, generating an ROC curve. The score closest to both maximum sensitivity and specificity, [i.e., the point (0.0, 1.0) on the curve] was selected as the cut-off score. Positive staining above or below the cutoff scores was classified as positive or negative, respectively.

2.3. Cell culture and transfection

The CRC cell lines LoVo, SW480, DLD1, and Caco2 were obtained from the American Type Culture Collection and cultured at a density of 1×10^5 cells/mL in a humidified 5% CO_2 atmosphere at 37°C . LoVo, SW480, DLD1, and Caco2 were maintained in F-12 Ham's nutrient mixture with Kaighn's modification, Leibovitz's L-15, RPMI-1640, and Dulbecco's modified Eagle's medium, respectively. The normal human colon epithelial cell line NCM460 was provided by Incell Corporation (San Antonio, TX) and cultured in M3 base culture media as previously described [12,13]. All media were supplemented with 10% fetal bovine serum and appropriate antibiotics.

The following sequences were designed and synthesized by GenePharma (Shanghai, China): synthetic let-7a (let-7a-mimics) 5'-UGAGGUAGUAGGUUGUAUAGUU-3'; synthetic let-7a inhibitor (let-7a-inhibitor) 5'-AACUAUACAACCUACU ACCUCA-3'; let-7a mimics control (short dsRNAs similar to Dicer-processed miRNAs [14], let-7a-mimics-control) 5'-U UCUCGAACGUGUCACGUTT-3'; let-7a inhibitor control (let-7a-inhibitor-control) 5'-CAGUACUUUUGUGUAGUACA A-3'; the siRNA targeting NIRF (NIRF-siRNA) 5'-GAUCCU GGCUUUGGAAUAUTT-3'; and the negative control siRNA (NC-siRNA) 5'-GGATCATAAGGCCCATAGC-3'.

Transfection was performed with Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol. A final concentration of 50 nM RNA (for mimics) or 100 nM (siRNA) and their respective negative controls were used for each transfection in the following experiments.

2.4. Prediction of candidate miRNAs and luciferase reporter assay

The three most widespread and web-based bioinformatic algorithms (TargetScan, PicTar, and miRanda) were used to predict candidate miRNAs for binding to NIRF, based on complementarities of the nucleotide sequence of the 3'-untranslated region (UTR) of NIRF mRNA. To verify the targeting of endogenous NIRF by miRNA, NIRF 3'-UTR was amplified by PCR, using the primers: forward, 5'-CAAAGGACGATGATCTGCC-3' and reverse, 5'-CTGATCA-TAAAATATTATTAAGGT-3', with HepG2 cell cDNA as a template. The product was purified and cloned into the

pTA2 vector using a TA cloning kit (Toyobo Biotechnology, Shanghai, China), and then subcloned into the psiCHECK vector (Promega, WI, USA) at the XbaI site. DLD1 cells were transfected with NIRF 3'-UTR or a control reporter vector by Lipofectamine 2000 and then cotransfected with miRNA let-7a-mimics or negative control. After 36 h, luciferase assay was performed using the Dual-Luciferase Reporter Assay System (Promega) in triplicate. Firefly luciferase activity was normalized to that of Renilla luciferase for each sample.

2.5. Real-time RT-PCR and Western blot

Total RNA was extracted from the tissues or cultured cells using Trizol (Invitrogen, Carlsbad, CA) for both miRNA and mRNA analyses. For the analysis of mature let-7a, quantitative PCR (RT-qPCR) was performed using the miScript PCR System (Qiagen, Hilden, Germany) according to the manufacturer's instructions, and the following primers: let-7a forward 5'-TGAGGTAGTAGTTGTATAGTT-3', U6 forward 5'-CGCTTCGCGCAGCACATATACTA-3', and the universal reverse primer 5'-GCGAGCACAGAATTAATACGAC-3'. Relative expression was calculated via the comparative cycle threshold (CT) method using the expression of U6 small nuclear RNA as the reference.

NIRF mRNA level was quantified by qRT-PCR using Quantitect SYBR Green PCR Kit (Qiagen) and normalized to β -actin with the following primers: NIRF forward 5'-TTCTACCGG GGCAAGCAGTT-3', and reverse 5'-CCAAAGCCAGGATCAATA AGACG-3'; β -actin forward 5'-CCTGTACGCCAACACAGTGC-3', and reverse 5'-ATACTCTGCTTGCTGATCC-3'. Changes in the expression were calculated using the $2^{-\Delta\Delta Ct}$ method [15].

Total protein was extracted from the specimens or cultured cells using a total protein extraction kit (Millipore, Billerica, MA, USA) according to the manufacturer's instructions. The protein content was determined using a bicinchoninic acid (BCA) protein assay kit (Bio-Rad, Hercules, CA, USA) with bovine serum albumin as the standard. Proteins (20 μ g) were separated by SDS-PAGE and transferred onto polyvinylidene difluoride membranes (Millipore). The membranes were blocked in 5% non-fat milk in Tris-buffered saline with 0.05% Tween-20 at room temperature for 2 h, then probed with antibodies against NIRF (1:3000 dilution, Abcam) or β -actin (1:5000 dilution, Sigma, St. Louis, MO), and finally developed with an enhanced chemiluminescence kit (Amersham Life Science, Little Chalfont, UK).

2.6. Cell proliferation assay and flow cytometry analysis

Cell proliferation was measured with a methyl-thiazolyl-tetrazolium (MTT) assay. Twenty-four hours after transfection, cells were seeded at a density of 5×10^3 /well into 96-well plates and cultured for 24, 48, 72, or 96 h. The cells were then incubated with 20 μ L MTT (5 mg/mL) for 4 h at 37 °C and 150 μ L dimethyl sulfoxide was added to solubilize the crystals for 10 min at room temperature. The optical density was measured at 490 nm using an enzyme-linked immunosorbent assay reader.

For cell cycle analysis, 48 h after transfection the adhered cells were collected by trypsinization and centri-

fuging at 1000 rpm for 5 min. The cells were incubated with propidium iodide (0.05 mg/mL, Sigma) and RNase A (0.1 mg/mL, Sigma) for 30 min at room temperature in the dark and analyzed using a BD FACSCalibur flow cytometer and Cell-Quest software.

2.7. Cell migration and invasion assays

Cell migration and invasion assays were performed using Transwell chambers (8 μ m, Corning Costar, Cambridge, MA). Twenty-four hours after transfection, 5×10^4 cells in 200 μ L serum-free RPMI-1640 medium were placed in the uncoated (migration assay) or 1:10 diluted Matrigel-coated (BD Biosciences, San Jose, CA, USA) upper chamber (invasion assay). The lower chamber was filled with 500 μ L complete RPMI-1640 medium. After cells were incubated for 24 h at 37 °C, cells on the top surface of the membrane were removed by wiping with a cotton swab. The cells that migrated to the bottom surface of the filter membrane were stained with 0.5% crystal violet solution and photographed in five preset fields per insert. After that, the cells were eluted with 33% acetic acid, and the absorbance optical density values for the positively stained cells were measured by microplate reader (Bio Tek) at 570 nm.

2.8. Tumorigenicity assay in nude mice

Six-week-old female BALB/c athymic nude mice (Chinese Academy of Sciences, Shanghai, China) were subcutaneously injected in the right flank with 1.5×10^6 cells in 0.1 mL phosphate buffered saline (PBS). Once cancer cells developed palpable tumors, caliper measurements were performed daily and tumor volume (V) was calculated using the formula $V = (L \times W^2)/2$, where L was the length and W was the width of the tumor. When tumors reached an average volume of 100–150 mm³, the mice were randomly divided into four groups ($n = 5$) for daily intratumoral injection of let-7a-mimics, let-7a-mimics-control, or transfection agent or PBS as controls for 28 days. For each injection, 5 μ g miRNA let-7a-mimics or let-7a-mimics-control was mixed with 8 μ L *in vivo* transfection reagent (Entranster™-in vivo, Engreen, China) and hydrated with PBS at a concentration of 50 μ g/mL to achieve the desired dose.

Growth curves were plotted using average tumor volume within each experimental group at the set time points. The tumor volumes of the mice were recorded for 28 days, after which the mice were euthanized. The dissected tumors were collected and prepared for RNA and protein isolation, and IHC staining. All animal experiments were approved by the animal center of the Sixth People's Hospital affiliated with Shanghai Jiao-Tong University.

2.9. Statistical analyses

Data were presented as mean \pm standard deviation (SD) from at least three independent experiments. Statistical analyses were performed by SPSS software 13.0 (SPSS, Chicago, IL). A paired Student's *t*-test was performed to evaluate the difference in NIRF expression between CRC and corresponding adjacent non-cancer tissues. ROC curve analysis

was used to determine the cut-off points upon which to divide groups to compare different parameters and perform the survival analysis. The χ^2 and Fisher's exact tests were performed to determine the associations between NIRF expression and clinicopathological parameters. Correlations between the expressions of let-7a and NIRF mRNA or protein were calculated using Spearman's rank correlation. The effects of protein expression on overall survival were evaluated by Kaplan–Meier curves, probability (P)-values were calculated using the log rank test. All P -values were two-sided and $P < 0.05$ was considered statistically significant.

3. Results

3.1. NIRF expression is correlated with clinicopathological features of CRC

IHC analysis of 164 cases of CRC and their corresponding non-cancerous tissues showed that positive staining for NIRF was seen in the nuclei of CRC cells (Fig. 1A-1) and corresponding non-cancerous mucosa cells (Fig. 1A-3), although no staining was observed in some samples (Fig. 1A-2 and A-4). The mean percentages of cells stained positive for NIRF in cancerous and corresponding non-cancerous mucosa were 71.0% and 40.9%, respectively. By comparing the percentage of positive cells, it was determined that NIRF expression in colorectal carcinoma was statistically higher than the adjacent non-cancerous mucosa ($P < 0.001$; Fig. 1B).

Correlation analysis revealed that NIRF expression was positively correlated with tumor size, depth of invasion, positive lymph node and metastasis to other organs, and negatively correlated with Dukes staging ($P < 0.05$), but was not correlated with patient age, gender, tumor location, differentiation or venous invasion ($P > 0.05$; Table 1). Furthermore, according to the Kaplan–Meier test the patients with positive NIRF staining had shorter survival than those with negative NIRF staining ($P = 0.024$; Fig. 1C).

3.2. Let-7a miRNA binds to the 3'-UTR of NIRF

Using three web-based bioinformatic algorithms (TargetScan, PicTar and miRanda), we identified 16, 20, and 48 candidate miRNAs, respectively, that bind to the NIRF 3'-UTR (Supplementary Table S1 and Supplementary Fig. S1). Among these miRNAs, let-7a attracted our attention due to its tumor suppressor characteristics. We then performed a luciferase reporter assay to verify that let-7a directly targets NIRF. As shown in Fig. 2A, let-7a-mimics suppressed luciferase activity ($P < 0.05$). In contrast, the let-7a-mimics-control had no significant effect on luciferase activity ($P > 0.05$). These results suggest that the 3'-UTR of the NIRF gene contained regulatory elements by which let-7a regulates NIRF expression. Furthermore, the introduction of let-7a-mimics or let-7a-inhibitor into DLD1 cells caused downregulation and upregulation of NIRF expression, respectively, as demonstrated by qRT-PCR and Western blot analysis (Fig. 2B and C). Taken together, these data indicate that let-7a may regulate NIRF expression through let-7a-binding sites at the 3'-UTR of NIRF.

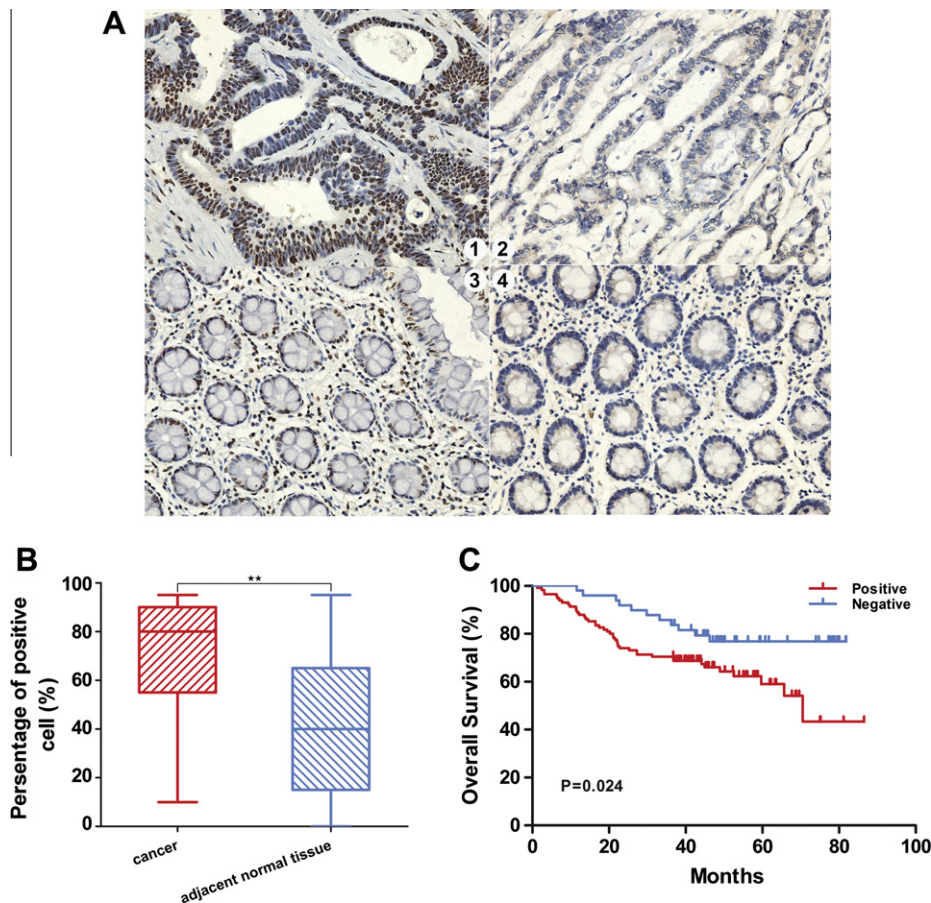


Fig. 1. IHC staining of NIRF in CRC. (A-1) Nuclear staining of NIRF in CRC cells. (A-2) No NIRF staining in CRC cells. (A-3) Expression of NIRF in normal colonic epithelial cells. (A-4) Lack of NIRF expression in normal colonic epithelial cells. (B) The percentage of positively stained cells in cancer and adjacent normal tissues ($\star P < 0.05$, $\star\star P < 0.01$). (C) Kaplan–Meier analysis of overall survival in CRC patients according to NIRF expression level. The NIRF positive group ($n = 115$) showed significantly shorter survival compared with the negative group ($n = 49$; $P = 0.024$; log-rank test).

Table 1
Association between patients' characteristics and NIRF expression in 164 CRC cases.

Clinicopathologic variable	NIRF expression		Total (n = 164)	χ^2	P
	Positive (n = 115)	Negative (n = 49)			
<i>Age (years)</i>					
≥60	84	39	123	0.786	0.375
<60	31	10	41		
<i>Gender</i>				0.077	0.781
Male	63	28	91		
Female	52	21	73		
<i>Tumor size (cm)</i>				7.188	0.007
≥5	73	20	93		
<5	42	29	71		
<i>Tumor location</i>				2.302	0.316
Right colon	41	14	55		
Left colon	43	16	59		
Rectum	31	19	50		
<i>Histology differentiation</i>				2.326	0.127
Well and moderate	78	39	117		
Poor	37	10	47		
<i>Depth of invasion</i>				15.558	<0.001
Tis-T2	7	14	21		
T3-T4	108	35	143		
<i>Venous invasion</i>				1.287	0.271
Yes	83	31	114		
No	32	18	50		
<i>Positive lymph nodes</i>				13.107	<0.001
Yes	66	13	79		
No	49	36	85		
<i>Metastases to other organs</i>				4.052	0.044
Present	21	3	24		
Absent	94	46	140		
<i>Duke's staging</i>				6.926	0.01
A + B	59	36	95		
C + D	56	13	69		

Well and moderate: well and moderately differentiated; poor: poorly differentiated.

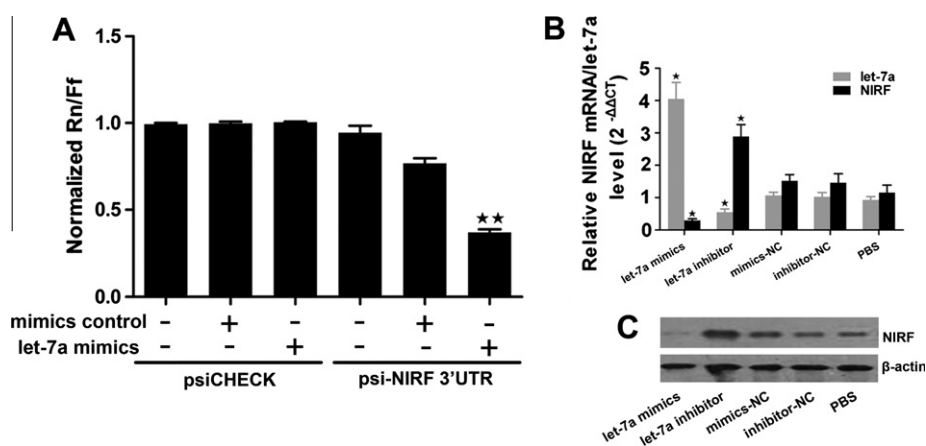


Fig. 2. Let-7a negatively regulates NIRF expression via the NIRF 3'-UTR. (A) Luciferase activity of various reporters in the presence or absence of let-7a-mimics in DLD1 cells. The normalized luciferase activity in the control group was set to one. $P < 0.01$, let-7a-mimics plus psi-NIRF 3'-UTR vs. the other five groups. (B) qRT-PCR analysis of NIRF mRNA in cells transfected with let-7a-mimics, -inhibitor or corresponding negative controls, $P < 0.05$ let-7a mimics vs. the other three groups; $P < 0.05$ let-7a-inhibitor vs. the other three groups. (C) Representative Western blot analysis of NIRF protein level in cells transfected with let-7a-mimics, -inhibitor or corresponding negative controls were also detected by Western blot. All data were representative of three independent experiments, $\star P < 0.05$, $\star\star P < 0.01$; mimics/inhibitor-NC, the mimics/inhibitor negative control oligonucleotide strands.

3.3. NIRF upregulation is correlated with reduced let-7a levels in CRC tissues and human colon cancer cells

To reveal the relationship between NIRF and let-7a expression levels in CRC, we performed RT-qPCR to detect let-7a miRNA, and qRT-PCR for NIRF mRNA levels, and Western blot analysis for NIRF protein levels, in human CRC tumor tissues, adjacent non-tumor tissues, and cell lines. The results showed that let-7a expression was significantly reduced in 15 of 20 tumor samples and all four colon cancer cell lines, compared to matched non-tumor tissues and the normal colon epithelial cell line, respectively (Fig. 3A and B). In contrast, NIRF expression at both the mRNA and protein levels was elevated in 9 of 15 corresponding tumor samples and the four colon cancer cell lines (Fig. 3C–F). Moreover, a significant negative correlation between let-7a and NIRF mRNA and protein levels was revealed in both CRC tissues (Fig. 3G) and colon cancer cells (Fig. 3H).

3.4. Induction of let-7a or knockdown of NIRF by siRNA leads to reduced cell proliferation and migration in CRC cells

The significant inverse association between let-7a and NIRF in CRC samples and cells prompted us to explore the possible antagonistic effects of let-7a and NIRF in CRC development. Both overexpression of let-7a and siRNA-mediated NIRF silencing significantly restrained the proliferation and migration of CRC cells (Fig. 4A, D and E), although they had no significant effect on the invasion of CRC cells (Supplementary Fig. S2). To

further examine whether the decreased proliferation of DLD1 cells was due to cell cycle arrest, cell cycle progression was analyzed by propidium iodide staining and flow cytometry. The results showed that DLD1 cells that had been transfected with let-7a-mimics or NIRF-siRNA were obviously arrested at the G0/G1 phase (Fig. 4B and C). Collectively, these data suggest that let-7a suppresses NIRF expression, which induces cell cycle arrest and leads to reduced cell proliferation and migration.

3.5. Ectopic let-7a expression suppresses NIRF expression and inhibits tumor growth *in vivo*

To confirm the *in vitro* findings shown above, an *in vivo* tumor model was established. We observed that the mouse group with let-7a-mimics had a lower proliferation rate, and formed substantially smaller tumors, than the other three groups (Fig. 5A). The tumor volume at the time of death in mice injected with let-7a-mimics was $491.0 \pm 94.27 \text{ mm}^3$, which was significantly less than that in mice injected with mimics-NC ($768.3 \pm 203.0 \text{ mm}^3$), Entranster transfection reagent ($701.3 \pm 201.3 \text{ mm}^3$), or PBS ($982.8 \pm 34.31 \text{ mm}^3$; Fig. 5B). Moreover, the mean tumor weight at the end of the experiment was markedly lower in the group with let-7a-mimics ($0.266 \pm 0.100 \text{ g}$) compared to the mimics-NC ($0.447 \pm 0.219 \text{ g}$), Entranster transfection reagent ($0.572 \pm 0.127 \text{ g}$), and PBS ($0.726 \pm 0.162 \text{ g}$) groups (Fig. 5C).

To confirm that synthetic let-7a was successfully delivered into the DLD1 tumor cells and suppressed the expression of NIRF, we evaluated the let-7a and NIRF mRNA and protein levels in tumor tissues 24 h after

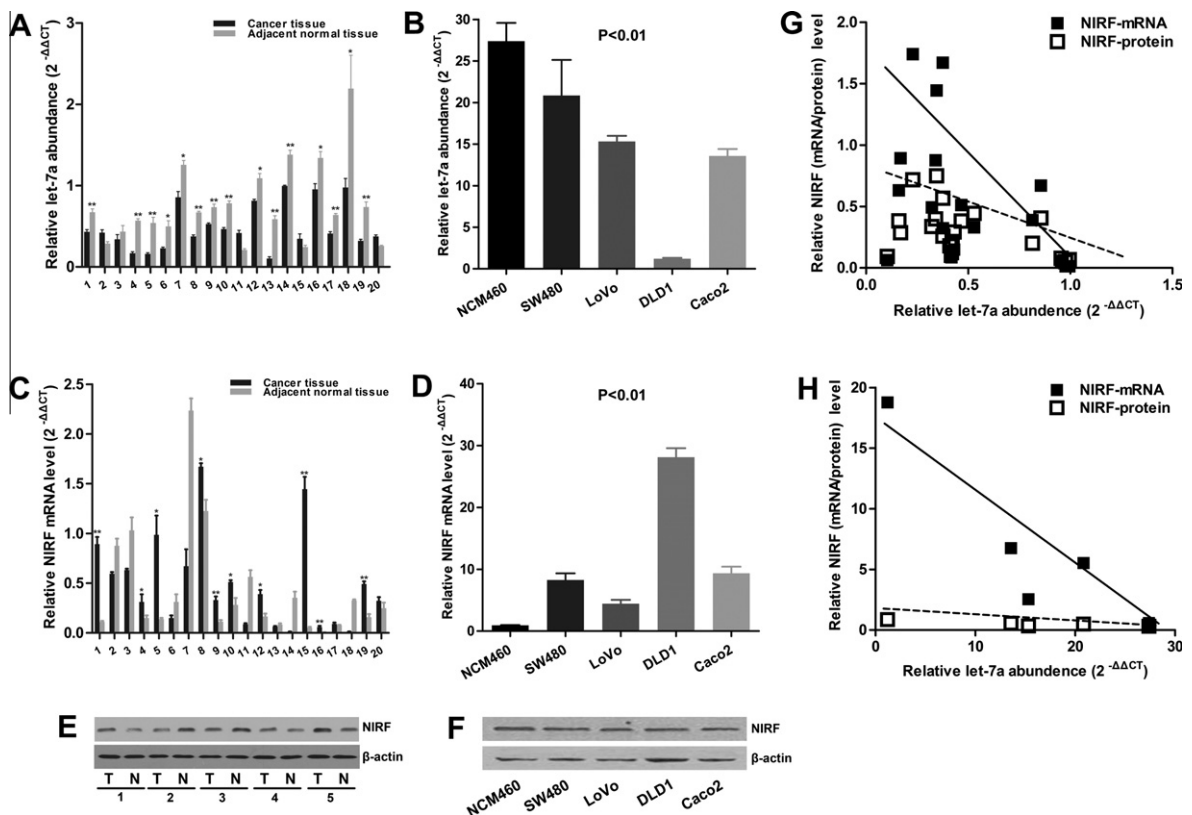


Fig. 3. Inverse correlation between the expression of let-7a and NIRF in CRC tissues and cell lines. (A–D) Relative expression of let-7a, NIRF mRNA, and NIRF protein levels in 20 paired CRC tissues and adjacent normal (A and C; *P*-value represented the comparison between paired CRC tissues and adjacent normal). Relative expression of let-7a, NIRF mRNA, and NIRF protein levels in four colon cancer cell lines and a normal colon epithelial cell line (B and D). $P < 0.01$, NCM460 (normal) vs. the other four CRC cell lines. Relative expression data were analyzed by using the $2^{-\Delta\Delta CT}$ method. All assays were performed in triplicate. β -Actin was used as an internal control, $\star P < 0.05$, $\star\star P < 0.01$. (E and F) Representative Western blot analysis of NIRF protein level in corresponding tissues and cell lines. (G and H) The scatter plot of correlation between let-7a and NIRF mRNA/protein levels in 20 CRC tissues; (G) inverse correlation was obtained by Spearman's correlation, $r = -0.523$, $P = 0.018$ for NIRF mRNA levels and $r = -0.397$, $P = 0.043$ for NIRF protein levels, respectively; (H) the scatter plot of correlation between let-7a and NIRF mRNA/protein levels in four colon cancer cell lines and a normal colon epithelial cell line. Inverse correlation was obtained by Spearman's correlation, $r = -0.9$, $P = 0.037$ for NIRF mRNA levels and protein levels.

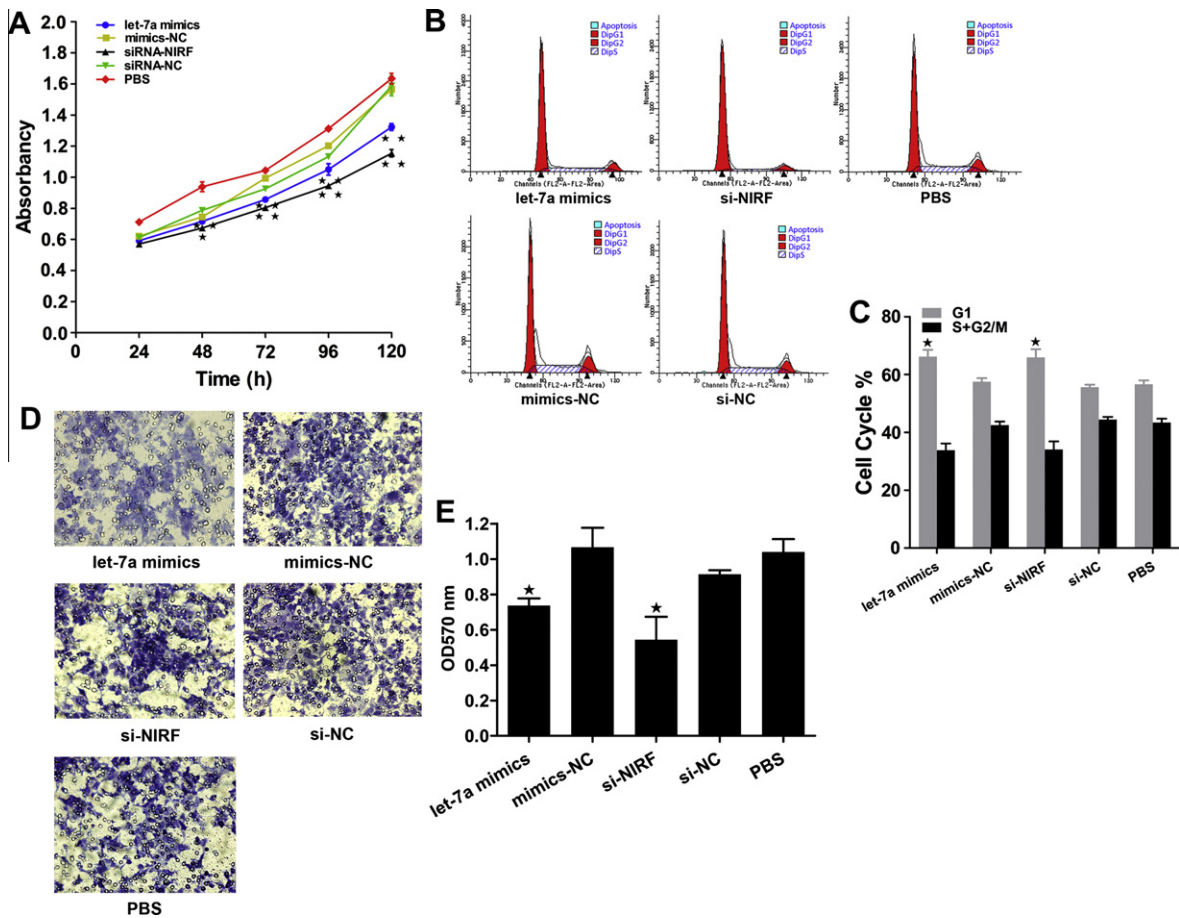


Fig. 4. Let-7a overexpression or NIRF silencing inhibits aggressive behaviors of CRC cell line DLD1 *in vitro*. (A) Transfection with let-7a-mimics or NIRF-siRNA but not negative controls (mimics-NC/si-NC/PBS) inhibited cell growth at the indicated time points ($n = 3$). (B and C) Representative cytograms (B) and the results of three independent experiments (C) show that transfection with let-7a-mimics or NIRF-siRNA but not negative controls (mimics-NC/si-NC/PBS) suppressed cell cycle progression. $\star P < 0.05$, $\star\star P < 0.01$; mimics/si-NC, the mimics/siRNA negative control oligonucleotide strands. (D and E) Transwell migration assays showed that DLD1 cells transfected with let-7a-mimics or NIRF-siRNA had lower migratory potentials. Representative images were shown at the left (D). Graphs indicated the average number of cells per field 24 h after transfection (E). Data represented the mean \pm SD of at least three independent experiments. The P -value represented the comparison among let-7a-mimics or siRNA-NIRF and the other three control groups at the indicated time points. $\star P < 0.05$, $\star\star P < 0.01$. Magnification in C and D, $\times 200$.

the final administration. The results showed that NIRF mRNA and protein levels were inversely correlated with increased accumulation of let-7a in let-7a-treated groups, compared to tumors that received mimics-NC, Entranster transfection reagent, or PBS (Fig. 5D and E).

Furthermore, we performed IHC on the xenografts using antibodies specific to NIRF or proliferating cell nuclear antigen (PCNA), a standard marker for cellular proliferation. As shown in Fig. 5F, DLD1 cells displayed reduced levels of NIRF and PCNA in response to let-7a treatment. This observation is in accordance with our previous observations that let-7a suppressed the expression of NIRF. Based on these data we conclude that synthetic let-7a miRNA successfully entered DLD1 tumor cells to suppress NIRF expression, leading to reduced tumor growth.

4. Discussion

UHRF is a nuclear protein family identified recently, and its members mainly function as transcription factors involved in the regulation of cell proliferation. ICBP90 (inverted CCAAT box binding protein of 90 kDa) is the most studied member of this family, and has been suggested as a potential oncoprotein in many malignant tumors [3]. NIRF

possesses several diverse structural domains which include an NIRF_N domain, a PHD finger, an SRA domain, and a RING finger domain, some of which are shared by ICBP90 and confirmed to play a critical role in cell proliferation [16–18].

In the present study, we found that NIRF expression at both the mRNA and protein levels was significantly higher in CRC tumor tissues than in matched non-tumor tissues. Furthermore, its overexpression was associated with malignant clinicopathological features and short survival of CRC patients. In CRC cells *in vitro*, knockdown of NIRF by siRNA led to reduced cell proliferation and migration. These results reveal the oncogenic features of NIRF in CRC. Indeed, it has been reported that the tumor cells HT-1080 (human fibrosarcoma) and HepG2 (human hepatocellular carcinoma) express constitutively high levels of NIRF mRNA. A study by Mori et al. [2] found that proliferating normal human fetal lung fibroblasts expressed high levels of NIRF mRNA, which was largely repressed upon

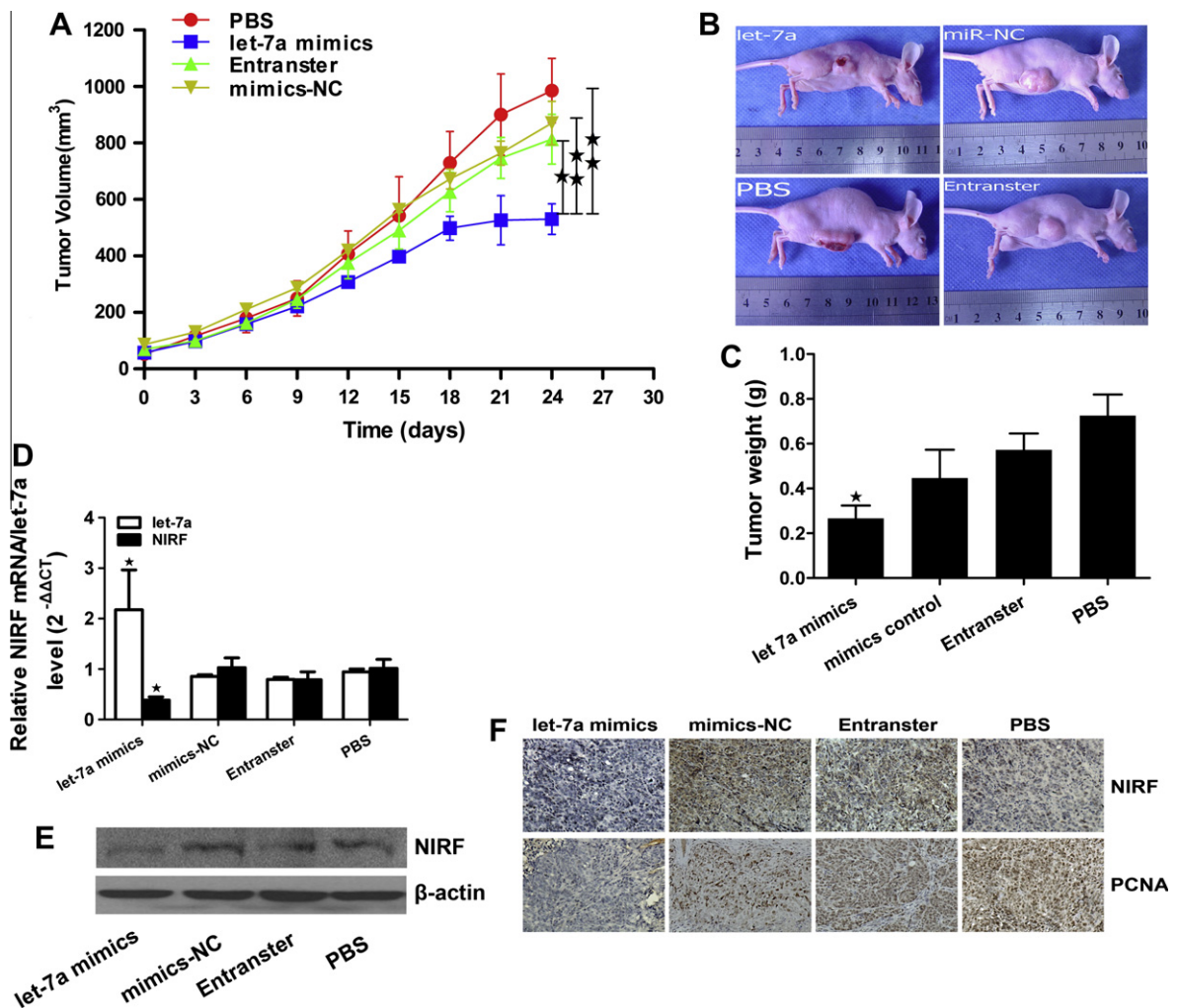


Fig. 5. Synthetic let-7a-mimics inhibits the growth of colon tumor xenografts. (A) Tumor volume growth curve after intra-tumor injection of let-7a-mimics, mimics control (mimics-NC), transfection reagent (Entranster) or PBS. Over 19 days, let-7a-mimics treatment resulted in significantly decreased tumor growth, compared to mimics-NC, Entranster, or PBS groups (Dunnett's *t*-test, $P < 0.05$). (B) Images of mice carrying DLD1 tumors after being killed on day 28. (C) Tumor weights of four groups measured when the mice were killed. Tumors in let-7a-mimics group were on average smaller than the tumors in the other three groups (Dunnett's *t*-test, $P < 0.05$). (D) Relative let-7a or NIRF mRNA levels in DLD1 tumors were examined by qRT-PCR when the tumors were harvested 1 day after the final treatment. The averages and standard deviations of five animals for each group are shown. (E) Representative Western blot analysis demonstrating a significant reduction of NIRF protein level in the let-7a-mimics treatment group compared with the other three groups. (F) IHC staining for NIRF and PCNA in xenografts. Significantly decreased NIRF and PCNA expressions were observed in the tumors treated with let-7a-mimics compared to the other three groups. The *P*-value represents the comparison among let-7a-mimics and the other three control groups. ★ $P < 0.05$, ★★ $P < 0.01$. Magnification $\times 200$ in F.

growth arrest. However, Mori et al. [19] reported that NIRF has the capability of being a ubiquitin ligase for PEST-proteolytic signal containing nuclear protein (PCNP) ubiquitination, therefore modulating cellular homeostasis. In another study they found that NIRF was involved in cell cycle control as a negative regulator [4]. These discrepant findings suggest that NIRF may play dual roles as a tumor suppressor or an oncogene depending on the cellular context, similar to transforming growth factor β (TGFB) [20].

There is accumulating evidence that the aberrant expression of miRNAs is linked to the development of CRC [21,22]. Sophisticated computer-based approaches for miRNA

identification and target prediction, combined with validation techniques to confirm these predictions, have contributed greatly to the discovery of new miRNAs and their functional characterization. Consequently, small molecule mediated dysregulation of miRNA emerges as a potential new therapeutic approach for human diseases including cancer [23]. Among human cancer-related miRNAs, the let-7 family has attracted significant attention because its family members are expressed aberrantly in a variety of cancers. As a member of the let-7 miRNA family, let-7a has been reported to be expressed at lower than normal levels in many cancers including CRC [24–26], and also to play a role in the

inhibition of tumor growth by targeting oncogenes such as Ras [27], HMGA2 [24], c-Myc, and several genes related to the cell cycle [25,26].

The current study provides several lines of evidence that NIRF is a novel target of let-7a and their antagonistic interaction plays an important role in the development of CRC. First, the luciferase reporter assay demonstrated that luciferase activity, under the control of the NIRF 3'-UTR, could be regulated by let-7a. Moreover, this downregulation was mediated by the direct binding of let-7a miRNA to the NIRF 3'-UTR, because the deletion of this region abrogated this effect. Secondly, an inverse correlation between NIRF and let-7a levels was observed in CRC tissues and cell lines. Thirdly, overexpression of let-7a suppressed NIRF levels and led to reduced cell proliferation and migration in CRC cells. Finally, the results of the *in vivo* tumorigenicity assay in nude mice showed that synthetic let-7a suppressed NIRF expression and reduced tumor growth.

Interestingly, a recent study in lung cancer showed that let-7a negatively regulated the expression of NIRF, and NIRF was required for let-7a-mediated elevation of p21^{WAF1} [6]. Our results are consistent with this report, and support the contention that the antagonistic association between NIRF and let-7a may be a phenomenon commonly occurring during the development of different cancers.

In summary, in this study we demonstrated that NIRF is frequently upregulated in CRC and is a potential oncogene for CRC development. A further large-scale survey is needed to evaluate the potential of NIRF as a novel biomarker for CRC. In addition, our results show that let-7a negatively regulates NIRF expression and inhibits CRC tumorigenesis in nude mice. These findings open up the possibility of applying let-7a miRNA toward clinical CRC treatments.

Acknowledgments

This study was supported by Grants from the Ph.D. Grant of Shanghai Jiao Tong University School of Medicine (No. BXJ201137), the Major Basic Research Program of Shanghai (No. 07DZ19505), and the National 973 Basic Research Program of China (No. 2008CB517403).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.canlet.2011.09.033.

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