International Journal of Biochemistry and Cell Biology 110 (2019) 1–8

Contents lists available at ScienceDirect

International Journal of Biochemistry and Cell Biology

journal homepage: www.elsevier.com/locate/biocel

Repression of lncRNA NEAT1 enhances the antitumor activity of CD8$^+$T cells against hepatocellular carcinoma via regulating miR-155/Tim-3

Kai Yan[a,1], Yong Fu[a,1], Nan Zhu[a], Zhuo Wang[a], Jin-ling Hong[b], Yao Li[c], Wei-jing Li[c], Hai-bin Zhang[a,*], Jing-hai Song[c,**]

[a] Fifth Department of Liver Surgery, Eastern Hepatobiliary Surgery Hospital, The Second Military Medical University, Shanghai 200438, China
[b] Department of Hepatology, Shanghai Putuo District Central Hospital, Shanghai 200062, China
[c] Department of General Surgery, Beijing Hospital, National Center of Gerontology, Beijing 100730, China

ARTICLE INFO

Keywords:
Hepatocellular carcinoma
NEAT1
miR-155
Tim-3
CD8$^+$T cells

ABSTRACT

Background: Immunotherapy is a promising method for the treatment of hepatocellular carcinoma (HCC), in which CD8$^+$T cells play a key role. The influence of long noncoding RNA (lncRNA) nuclear-enriched autosomal transcript 1(NEAT1) on the antitumor activity of CD8$^+$T cells was clarified in this study.

Methods: Peripheral blood mononuclear cells (PBMCs) were isolated from HCC patients, and the expressions of NEAT1 and Tim-3 were determined by qRT-PCR and western blot, respectively. CD8$^+$T cell apoptosis and cell percentage were analyzed via flow cytometry. The cytolysis activity of CD8$^+$T cells against HCC cells was examined. RNA immunoprecipitation (RIP) and RNA pull-down assay were performed to explore the interaction between NEAT1 and miR-155.

Results: NEAT1 and Tim-3 were up-regulated in the PBMCs of patients with HCC (n = 20) compared with healthy subjects (n = 20). Down-regulation of NEAT1 restrained CD8$^+$T cell apoptosis and enhanced the cytolysis activity, while interference of miR-155 showed the opposite effects by up-regulating Tim-3. Binding and interaction between NEAT1 and miR-155 were validated in CD8$^+$T cells. Down-regulation of NEAT1 restrained CD8$^+$T cell apoptosis and enhanced the cytolysis activity through the miR-155/Tim-3 pathway. Repression of NEAT1 suppressed tumor growth in HCC mice.

Conclusion: Via modulating the miR-155/Tim-3 pathway, repression of NEAT1 restrained CD8$^+$T cell apoptosis and enhanced the cytolysis activity against HCC, implying an effective target for improving the outcome of immunotherapy.

1. Introduction

In terms of prevalence and mortality, hepatocellular carcinoma (HCC) is considered the fifth most common malignancy worldwide and it is characterized by its poor prognosis and progressive development (Shi et al., 2011). Immunotherapy is a promising method for the treatment of HCC, and plenty of immune cells have been implicated in such treatment (Flecken et al., 2014). However, when tumors escape from immunological surveillance, this significantly contributes to tumor progression and hinders the outcome of immune-based treatment (Guo et al., 2014). Hence, the mechanisms responsible for regulating immune cell function are strongly focused on and widely studied at present.

Effector CD8$^+$T cell-mediated tumor-associated antigens recognition and cytotoxic killing play an essential role in the control of cancer development (Fu et al., 2007). The amount of CD8$^+$T cells has been claimed to be a good indicator for predicting the HCC prognosis (Sun et al., 2015). CD8$^+$T cell apoptosis usually results in a decreased cell number in the tumor microenvironment and reduced activity of tumor inhibition, which is linked with colon cancer progression and post-operative recurrence of HCC (Shi et al., 2011). T-cell immunoglobulin and mucin domain protein 3 (Tim-3) functions in enforcing CD8$^+$T cell

* Corresponding author at: Fifth Department of Liver Surgery, Eastern Hepatobiliary Surgery Hospital, Second Military Medical University, No. 225 Changhai Road, Yangpu District, Shanghai 200438, China.
** Corresponding author at: Department of General Surgery, Beijing Hospital, National Center of Gerontology, No. 1 Dahua Road, Dongdan, Dongcheng District, Beijing 100730, China.
E-mail addresses: drzhanghaibin@hotmail.com (H.-b. Zhang), jhaisong2003@163.com (J.-h. Song).
[1] Equal contributors.

https://doi.org/10.1016/j.biocel.2019.01.019
Received 25 July 2018; Received in revised form 7 January 2019; Accepted 29 January 2019
Available online 30 January 2019
1357-2725/

exhaustion and participates in the apoptosis of $CD8^+$T cells (Kang et al., 2015). In addition, Tim-3^+ Tregs from human HCC repressed autologous $CD8^+$T cells proliferation and cytokine production in vitro (Yan et al., 2013), implying the key regulatory role of Tim-3 in tumor immunity through promoting $CD8^+$T cell apoptosis.

MicroRNAs (miRNAs) are a class of noncoding RNAs that negatively regulate genes expression by binding with the 3′ untranslated region (UTR) of their mRNAs, and they widely participate in various biological processes. MiR-155 is one of the miRNAs involved in the pathogenesis of cancers, including breast cancer (Kim et al., 2015), rectal cancer (Yang et al., 2015), liver cancer (Liu et al., 2015), lung cancer (Xie et al., 2015), and gastric cancer (Han et al., 2015). In HCC, miR-155 promoted epithelial mesenchymal transition, cell invasion, metastasis, proliferation, and HCC formation, contributing to the malignant behaviors of this tumor (Kong et al., 2016; Li et al., 2017; Tang et al., 2016; Song et al., 2018). Moreover, it has been revealed that miR-155 augments $CD8^+$T cell antitumor activity (Ji et al., 2015) and miR-155-modulated Tim-3 expression regulates natural killer cell function (Cheng et al., 2015). It can be inferred that miR-155 may affect the antitumor activity of $CD8^+$T cells through targeting Tim-3 in HCC.

Long noncoding RNA (lncRNA) nuclear-enriched autosomal transcript 1 (NEAT1) has been proven to have high expression in HCC tissues and promote HCC cell invasion and metastasis (Guo et al., 2015; Zheng et al., 2018). In the plasma of human immunodeficiency virus 1 (HIV-1)-infected patients, NEAT1 was down-regulated, and its expression was positively correlated with CD4 T cell counts (Jin et al., 2016). Via bioinformatics analysis (DIANA), we found the complementary base pairs between NEAT1 and miR-155, suggesting a potential interaction between them. Although the role of NEAT1 in $CD8^+$T cells has not been clarified, it can be speculated that NEAT1 may contribute to the immune escape of HCC by affecting the antitumor function of $CD8^+$T cells, and its regulatory effect on the miR-155/Tim-3 signaling pathway deserves exploration. This study was carried out to clarify the effect of NEAT1 on $CD8^+$T cell apoptosis and cytolysis activity, aiming to improve the curative effect of HCC immunotherapy.

2. Materials and methods

2.1. Clinical samples

This study was approved by the ethics committee of the Eastern Hepatobiliary Surgery Hospital and performed in accordance with the Helsinki Declaration. Fresh peripheral blood specimens from HCC patients (n = 20) and healthy subjects (n = 20) were collected for peripheral blood mononuclear cell (PBMC) isolation. HCC patients (including 15 males and 5 females, with 13 cases more than 55 years old) and controls (including 13 males and 7 females, with 16 cases more than 55 years old) were age and gender-matched. No treatment was adopted for the HCC patients before the study. Before blood collection, all the participants agreed to and signed the informed consent forms for sample donation for the study. PBMCs were isolated by Ficoll-Paque (TBD Science, China) density gradient centrifugation. After centrifugation at 2500 rpm/min for 15 min, the supernatant was discarded, and the lymphocyte layer was collected.

2.2. Western blot

PBMCs were treated in Radioimmunoprecipitation assay (RIPA) buffer (Beyotime, China) containing protease and phosphatase inhibitors, and protein was obtained after centrifugation. Protein concentration was measured using a Pierce BCA Assay Kit (Thermo Scientific), followed by separation by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and electro-blotting onto a nitrocellulose filter. The filter was blocked overnight at room temperature in 5% non-fat milk dissolved in Tris-buffered saline with Tween-20 (TBST) and incubated with primary antibodies against Tim-3 and β-actin (Abcam) at 4℃ overnight. The membrane was washed in 0.1% TBST and incubated with horseradish-peroxidase-bound secondary antibodies for 2 h at room temperature. Finally, the membrane was visualized using an enhanced chemiluminescence (ECL) system (Thermo Fisher).

2.3. Quantitative real-time PCR (qRT-PCR)

PBMCs were homogenized with 1 ml Trizol reagent to extract total RNA, of which the concentration and purity were examined spectrophotometrically. The reverse reaction was performed for cDNA synthesis using a SuperScript Reverse Transcription Kit (Invitrogen). QRT-PCR was performed using a SYBR Green Master Mix (Applied Biosystems, USA) on a 7500 Fast RT-PCR System (Applied Biosystems). The design and synthesis of oligonucleotide primers of specific genes were completed by Sangon Biotech (Shanghai, China). The Ct values were examined using the default threshold settings, and the $2^{-\Delta\Delta Ct}$ method was applied for calculating the relative quantification.

2.4. Flow cytometry

For Tim-3^+CD8^+ T cells percentage analysis, PBMCs were diluted to 1×10^6 and washed twice with phosphate-buffered saline (PBS). Then, the cells were stained by fluorescein isothiocyanate (FITC) or PE-conjugated antibodies (BD Biosciences) at 4 °C for 30 min in PBS containing 1% fetal bovine system (FBS). Samples were evaluated using a BD FACSCalibur cytometer (BD Biosciences) and data were analyzed via Flow Jo V10 software (Tree Star Inc.).

2.5. Cell culture and cell transfection

$CD8^+$ T cells were isolated from PBMCs of two healthy donors using a magnetic bead cell sorting (MACS) negative selection kit (Miltenyi Biotech), and they were activated with anti-CD3ε (2 μg/ml, BD Biosciences) and anti-CD28 (1 μg/ml, BD Biosciences) in RPMI-1640 medium (Sigma-Aldrich) supplemented with rhIL-2 (120IU/ml, Chiron) for 24 h. Small interference RNA (siRNA) targeting NEAT1 (si-NEAT1) or its negative control (si-control) was purchased from GenePharma Co. Ltd (Shanghai, China) and transfected into $CD8^+$ T cells using Lipofectamine2000 (Invitrogen, USA) according to the manufacturer's instructions.

2.6. $CD8^+$ T cell apoptosis analysis

After transfection with si-NEAT1 or si-control, $CD8^+$ T cell apoptosis was measured using an Annexin V-FITC/propidium iodide (PI) cell apoptosis detection kit (Sigma, USA) followed by flow cytometric analysis. $CD8^+$ T cells (1×10^6/well) were seeded in the 6-well plates and then stained with 200 μl Annexin V-FITC and 10 μl of PI, followed by flow cytometric analysis with a flow cytometer (FACSCalibur, BD Biosciences) and Flow Jo V10 software (Tree Star Inc.).

2.7. Cytolysis activity assay

For the cytotoxicity assay, cells from HepG2.2.15, a human hepatoma cell line stably transfected with the hepatitis B virus (HBV) genome, were used as the target cells. Before cytotoxicity assay, HepG2.2.15 cells were radiolabeled with [methyl-^{3}H] thymidine to a concentration of 5μ Ci/ml for 24 h at 37 °C. $CD8^+$ T cells transfected with si-NEAT1 or si-control were mixed and incubated with labeled HepG2.2.15 cells in 96-well culture plates overnight. The culture plate was harvested and examined with beta radiation counting, and the % cytotoxicity was calculated using the formula: % cytotoxicity = $[(S - E)/S] \times 100$ (E: number of cells in experimental wells; S: number of cells in spontaneous release wells) (Kang et al., 2015).

2.8. RNA immunoprecipitation (RIP)

RIP assay was carried out using the Magna RIP RNA-Binding Protein Immunoprecipitation Kit (Millipore). Argonaute2 (AGO2) protein is often used in the RIP assay, as it is the key component of the RNA-induced silencing complex (RISC), which can bind with miRNAs. $CD8^+$ T cells were lysed with RIP buffer, and then 100 µl of cell extract was incubated with RIP buffer containing magnetic beads conjugated with anti-AGO2 antibody (Millipore) or normal mouse IgG as a negative control. RNA-protein complexes were immunoprecipitated with protein A agarose beads and qRT-PCR was performed for detecting the NEAT1 and miR-155 levels.

2.9. RNA pull-down assay

RNA pull-down assay was conducted to investigate whether NEAT1 interacted with miR-155, using synthesized NEAT1 as a probe, and lncRNA loc285194 (Loc) was used as the positive control. A DNA fragment including the whole NEAT1 sequence was synthesized and biotinylated (GenePharma Co., Ltd.), and biotin-labeled RNAs were reversely transcribed using Biotin RNA Labeling Mix and T7 RNA polymerase (Roche). The reaction product was treated with RNase-free DNase I (Roche) and extracted with the RNeasy Mini Kit (Qiagen) for western blot or qRT-PCR analysis.

2.10. Mouse tumor model establishment

Following the Guide for the Care and Use of Laboratory Animals proposed by the Chinese National Institutes of Health, experiments on C57BL/6J mice (8 weeks old) were completed under anesthesia. For the orthotopic tumor graft, 2×10^6 mouse H22 cells were injected subcutaneously in the right flanks of the mice (Wang et al., 2009). Short hairpin RNA (shRNA) targeting human lncRNA NEAT1 or its negative control oligonucleotide was ligated into the pGC-LV lentivirus vector (GenePharma, Shanghai), and the virus particles were harvested after lentivirus was packaged in HEK293T cells for 72 h. The packaged lentivirus (5 µl) marked Lv-sh-NEAT1 (n = 8) or its negative control Lv-shRNA (n = 8) was transfected into $CD8^+$T cells, which were re-suspended in 0.2 ml of Hanks' Balanced Salt Solution (HBSS) and subsequently introduced into the mouse models via tail vein injection (Kang et al., 2015). On the 7th, 14th, 21st, and 28th day following $CD8^+$T cell injection, tumor diameters were measured with a Vernier caliper, and tumor size was calculated using the following formula: $V = [(\text{length} \times \text{width}^2)/2]$.

2.11. Statistical analysis

All the experiments were repeated at least three times, and all the data were presented as mean ± standard deviation. Statistical analysis was performed using the Student's *t*-test in GraphPad Prism 6 (GraphPad Software, Inc., San Diego, CA, USA) for two-group comparisons, while one-way analysis of variance (ANOVA) followed by the Newman–Keuls post hoc test was used for multi-group comparisons. A value of $P < 0.05$ was considered significant.

3. Results

3.1. NEAT1 and Tim-3 were up-regulated in PBMCs of patients with HCC

To investigate the expression levels of NEAT1 and Tim-3 in HCC, PBMCs were separately isolated from the blood samples of HCC patients (n = 20) and healthy subjects (control, n = 20). The protein level detected by western blot indicated a higher Tim-3 expression in HCC PBMCs than that in control group (Fig. 1A, left panel). A dot plot including all 20 samples was also generated (Fig. 1A, right panel). Compared with the control group, the expression of NEAT1 was up-regulated in HCC PBMCs, but the expression of miR-155 was lower (Fig. 1B). Subsequently, the percentage of $Tim\text{-}3^+CD8^+$ cells was analyzed by flow cytometry, which demonstrated that the $Tim\text{-}3^+CD8^+$ cells proportion was increased in HCC PBMCs compared with that in control PBMCs (Fig. 1C). Our results illustrated that NEAT1 and Tim-3 were up-regulated in the PBMCs of patients with HCC, while miR-155 was down-regulated.

3.2. Down-regulation of NEAT1 restrained $CD8^+$T cell apoptosis and enhanced the cytolysis activity

We next assessed the influence of NEAT1 repression on $CD8^+$T cell apoptosis and cytolysis activity. $CD8^+$T cells were isolated from PBMCs of two healthy donors and activated, followed by si-control or si-NEAT1 transfection. Fig. 2A shows that the NEAT1 expression was significantly reduced after si-NEAT1 transfection. The results of flow cytometric analysis indicated that down-regulation of NEAT1 with si-NEAT1 transfection inhibited $CD8^+$T cell apoptosis, and the cleaved caspase 3 was also decreased (Fig. 2B), suggesting that NEAT1 enhanced $CD8^+$T cell apoptosis via a caspase-dependent pathway. For the cytotoxicity assay, HepG2.2.15 cells were used as the target cells. After transfection with si-concrol or si-NEAT1 and then co-culturing with HepG2.2.15 cells, the cytolysis activity of $CD8^+$T cells against the tumor cells was evaluated. The result demonstrated that interference of NEAT1 with si-NEAT1 markedly augmented the cytolysis activity of $CD8^+$T cells (Fig. 2C). The above results validated that down-regulation of NEAT1 restrained $CD8^+$T cell apoptosis and enhanced the cytolysis activity, while the underlying mechanism was still uncertain.

3.3. Interference of miR-155 induced $CD8^+$T cell apoptosis and decreased the cytolysis activity by up-regulating Tim-3

As the effect of miR-155 on strengthening the antitumor effect of $CD8^+$T cells and its negative regulation of Tim-3 have been claimed, the effect of miR-155 expression on $CD8^+$T cell apoptosis and cytolysis activity was assessed. $CD8^+$T cells were activated and divided into four groups: NC (negative control of miR-155 inhibitor), miR-155 inhibitor, miR-155 inhibitor + anti-control, and miR-155 inhibitor + anti-Tim-3. For Tim-3 blockage, $CD8^+$T cells were incubated with anti-human Tim-3 antibody (anti-Tim-3) for an additional 48 h after 48 h of cell transfection, with IgG acting as the control (anti-control) (Cheng et al., 2015). Compared with the NC group, transfection with miR-155 inhibitor dramatically reduced miR-155 expression in $CD8^+$T cells (Fig. 3A). However, the Tim-3 protein level was augmented by miR-155 inhibitor, which was inverted by Tim-3 blockage (Fig. 3B). The $CD8^+$T cell apoptosis was also promoted by the miR-155 inhibitor but suppressed after Tim-3 blockage (Fig. 3C). In contrast, cytolysis activity was inhibited by miR-155 inhibitor but reversed after Tim-3 blockage (Fig. 3D). These findings indicated that interference of miR-155 induced $CD8^+$T cell apoptosis and decreased the cytolysis activity via targeting Tim-3.

3.4. Interaction between NEAT1 and miR-155 in $CD8^+$T cells

Fig. 4A displays the complementary base pairs between NEAT1 and miR-155, predicted using a bioinformatics method (DIANA). Herein, the binding and interaction between NEAT1 and miR-155 in $CD8^+$T cells were determined by RIP and RNA pull-down assay. AGO2 antibody was used in the RIP assay, and the level of NEAT1 and miR-155 was determined. As shown in Fig. 4B with the RIP result, the levels of NEAT1 and miR-155 were significantly higher in the AGO2 antibody group compared with the IgG group (Fig. 4B). For RNA pull-down assay, western blot was performed to examine AGO2 level in the pull-down complex of NEAT1. Extensive miR-155 expression was detected in the pull-down complex of NEAT1, with the Loc group serving as the positive control and antisense RNA as the negative control (Fig. 4C).

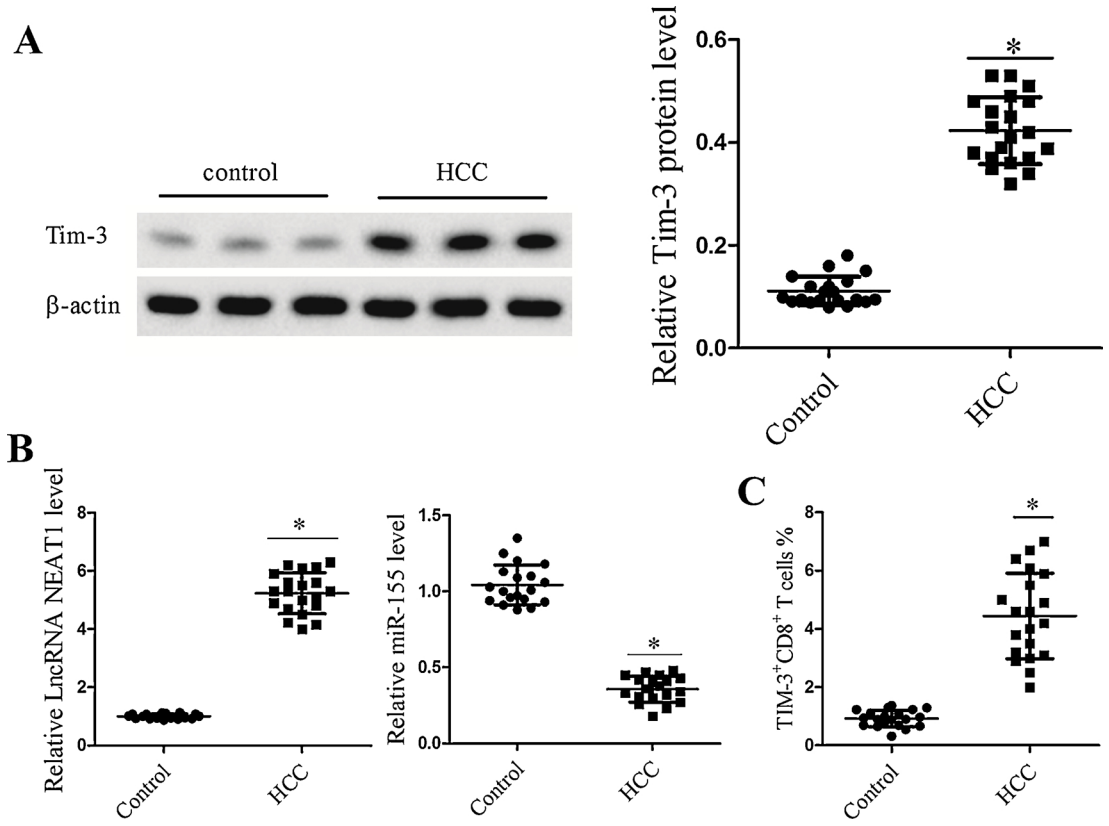

Fig. 1. NEAT1 and Tim-3 were up-regulated in PBMCs of patients with HCC. PBMCs were separately isolated from the blood samples of HCC patients (n = 20) and healthy subjects (control, n = 20). (A) The Tim-3 protein level was determined by western blot. (B) qRT-PCR was performed for examining the expression of NEAT1 and miR-155. (C) The percentage of Tim-3 + CD8 + T cells was analyzed by flow cytometry. All experiments were repeated at least three times, and all the data are presented as mean ± standard deviation. Statistical analysis was performed using the Student's *t*-test. A value of $P < 0.05$ was considered significant. *$P < 0.05$ compared with control.

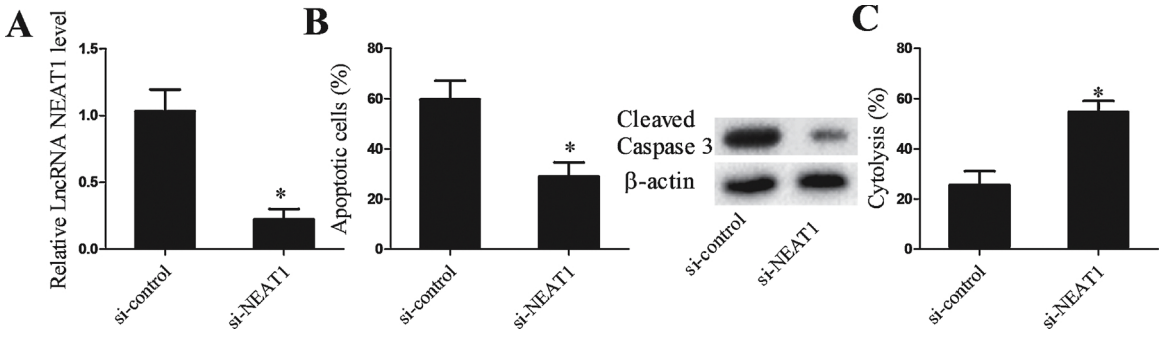

Fig. 2. Down-regulation of NEAT1 restrained $CD8^+$T cell apoptosis and enhanced the cytolysis activity. $CD8^+$T cells were isolated from PBMCs of two healthy donors using the magnetic bead cell sorting (MACS) method and activated and followed by si-control or si-NEAT1 transfection. (A) The expression of NEAT1 in $CD8^+$T cells was measured with qRT-PCR after cell transfection. (B) $CD8^+$T cell apoptosis was evaluated by flow cytometry after cell transfection. (C) Cytolysis activity of $CD8^+$T cells against HCC cells was detected after cell transfection. All experiments were repeated at least three times, and all the data are presented as mean ± standard deviation. Statistical analysis was performed by using the Student's *t*-test. A value of $P < 0.05$ was considered significant. *$P < 0.05$ compared with si-control.

3.5. Down-regulation of NEAT1 restrained $CD8^+$T cell apoptosis and enhanced the cytolysis activity through the miR-155/Tim-3 pathway

To evaluate the regulatory relationship between NEAT1 and miR-155 and their influence on $CD8^+$T cell apoptosis and cytolysis activity, $CD8^+$T cells were activated and allocated into four groups, as follows: si-control, si-NEAT1, si-NEAT1 + NC, and si-NEAT1 + miR-155 inhibitor. It was shown that si-NEAT1 transfection remarkably promoted miR-155 expression, which was then repressed by the miR-155 inhibitor (Fig. 5A). Conversely, the Tim-3 protein expression was inhibited with si-NEAT1 transfection but elevated after miR-155 inhibitor transfection (Fig. 5B). In addition, si-NEAT1 transfection clearly inhibited $CD8^+$T cell apoptosis, which was further reversed by the miR-155 inhibitor (Fig. 5C). The cytolysis activity of $CD8^+$T cells was increased by si-NEAT1 but restrained after miR-155 inhibitor transfection (Fig. 5D). It could be summarized that interference of NEAT1 inhibited $CD8^+$T cells apoptosis and enhanced the cytolysis activity through the miR-155/Tim-3 pathway.

3.6. Interference of NEAT1 suppressed tumor growth in HCC mice

To estimate the effect of NEAT1 expression on HCC progression in vivo, the mouse HCC models were constructed and injected with $CD8^+$T cells with Lv-sh-NEAT1 (n = 8) or Lv-shRNA (n = 8). The

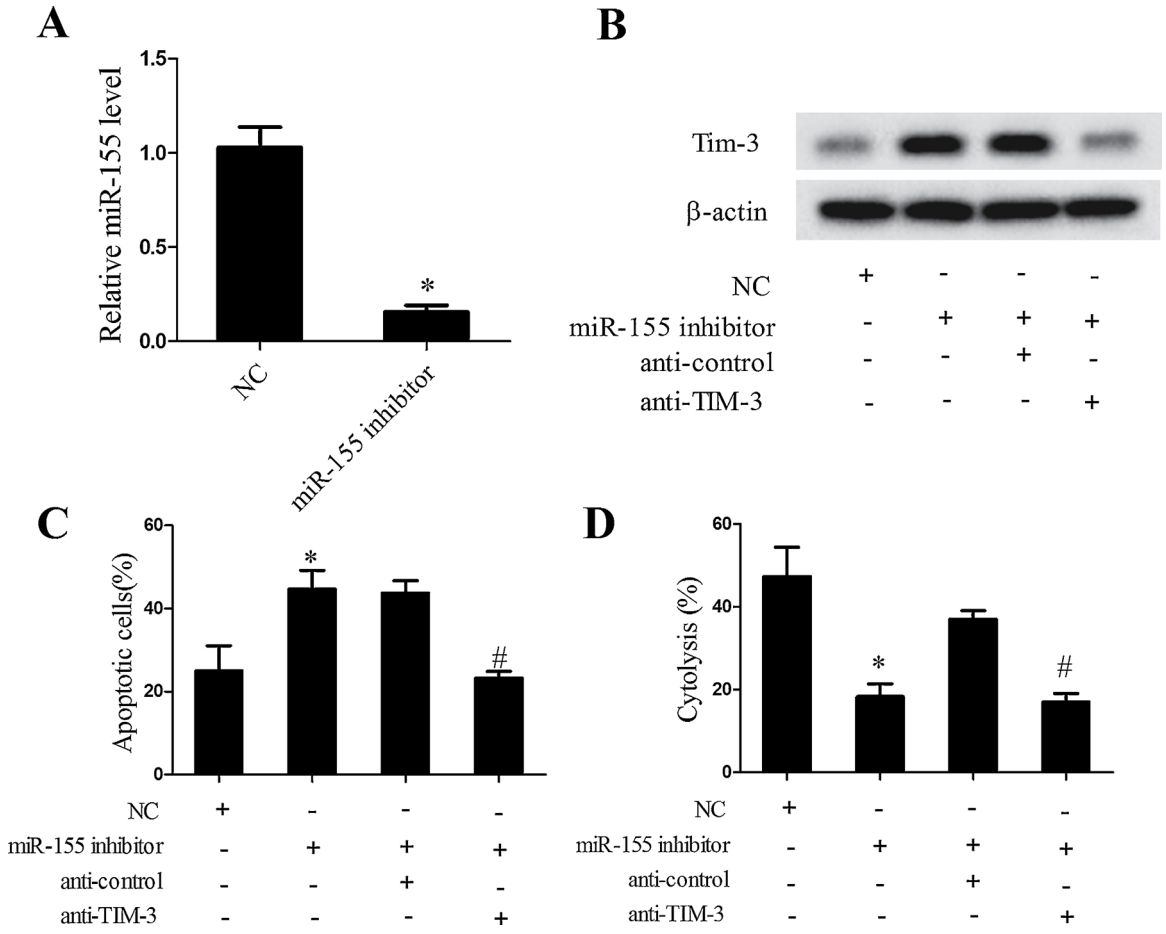

Fig. 3. Interference of miR-155 induced CD8$^+$T cells apoptosis and decreased the cytolysis activity by up-regulating Tim-3. CD8$^+$T cells were isolated from PBMCs of two healthy donors using the magnetic bead cell sorting (MACS) method. CD8$^+$T cells were activated and divided into four groups, as follows: NC (the negative control of miR-155 inhibitor), miR-155 inhibitor, miR-155 inhibitor + anti-control, and miR-155 inhibitor + anti-Tim-3. (A) qRT-PCR was performed for quantifying the expression of miR-155 in CD8$^+$T cells. (B) Tim-3 protein expression was determined by western blot in CD8$^+$T cells. (C) CD8$^+$T cell apoptosis was evaluated by flow cytometry. (D) Cytolysis activity of CD8$^+$T cells against HCC cells was detected. *P < 0.05 compared with NC; All experiments were repeated at least three times, and all the data were presented as mean ± standard deviation. Statistical analysis was performed using the Student's *t*-test. A value of P < 0.05 was considered significant. #P < 0.05 compared with anti-control.

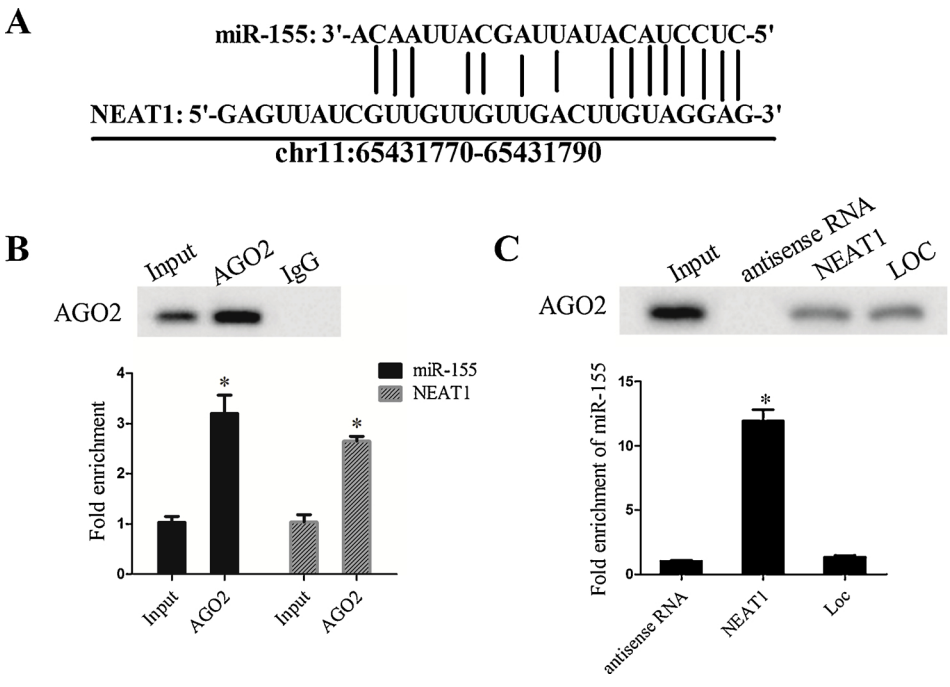

Fig. 4. Interaction between NEAT1 and miR-155 in CD8$^+$T cells. CD8$^+$T cells were isolated from PBMCs of two healthy donors with the magnetic bead cell sorting (MACS) method. Interaction between NEAT1 and miR-155 in CD8$^+$T cells was predicted with the bioinformatics method and verified using RNA immunoprecipitation (RIP) and RNA pull-down assay. (A) Bioinformatics analysis software (DIANA) predicted the binding sites between NEAT1 and miR-155. (B) RIP assay was carried out to assess the binding condition between NEAT1 and miR-155, and IP-western was performed to determine whether NEAT1 and miR-155 were present in the AGO2 protein complex. (C) RNA pull-down assay was conducted for examining the interaction between NEAT1 and miR-155. All experiments were repeated at least three times, and all the data are presented as mean ± standard deviation. Statistical analysis was performed by using the Student's *t*-test. A value of P < 0.05 was considered significant. *P < 0.05 compared with Input or antisense RNA.

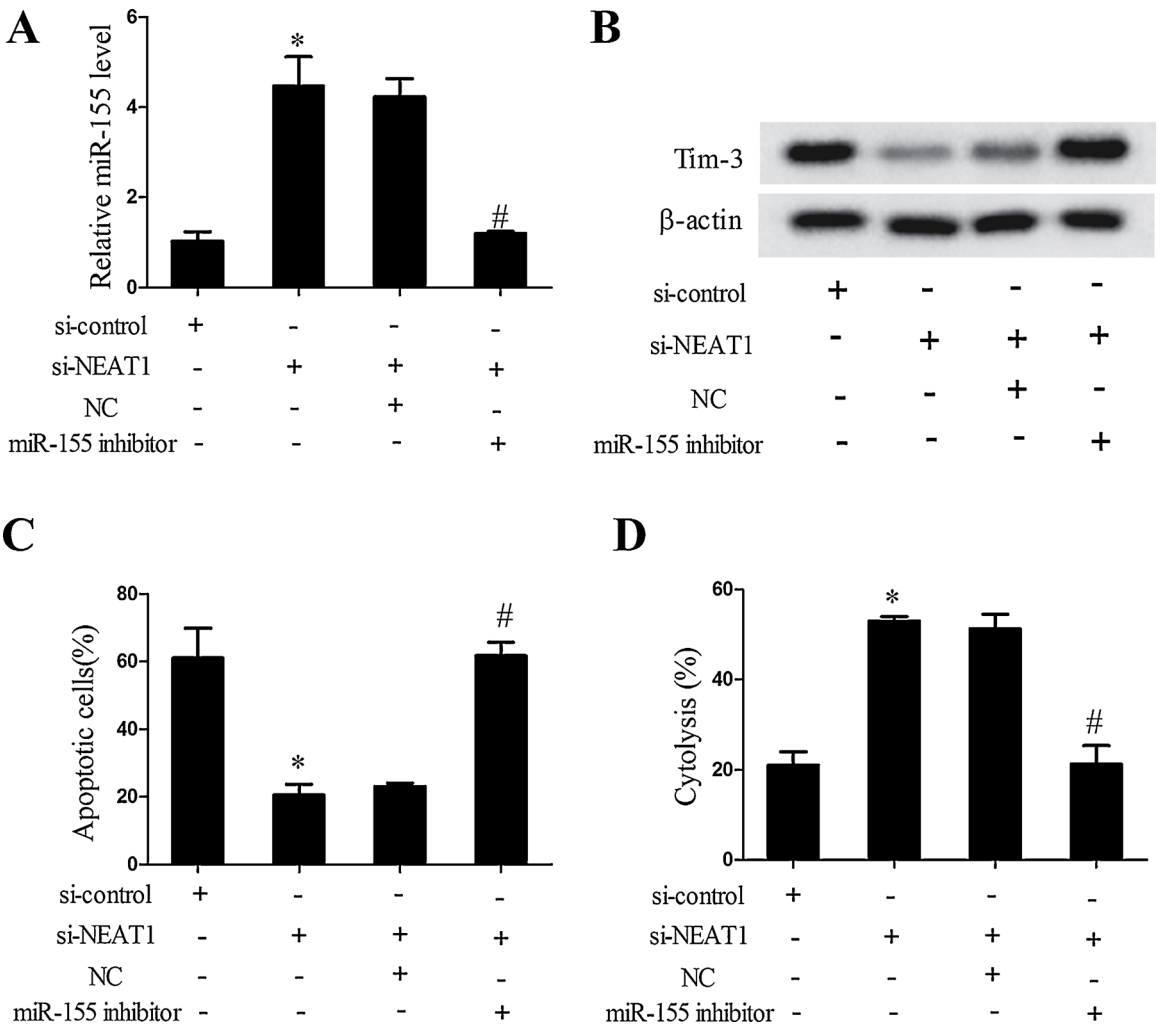

Fig. 5. Down-regulation of NEAT1 restrained CD8$^+$T cell apoptosis and enhanced the cytolysis activity through the miR-155/Tim-3 pathway. CD8$^+$T cells were isolated from PBMCs of two healthy donors using the magnetic bead cell sorting (MACS) method. CD8$^+$T cells were activated and allocated into four groups via cell transfection, as follows: si-control, si-NEAT1, si-NEAT1 + NC, and si-NEAT1 + miR-155 inhibitor. (A) The expression of miR-155 in CD8$^+$T cells was quantified by qRT-PCR. (B) Tim-3 protein expression was determined by western blot in CD8$^+$T cells. (C) CD8$^+$T cell apoptosis was evaluated by flow cytometry. (D) Cytolysis activity of CD8$^+$T cells against HCC cells was detected. *P $<$ 0.05 compared with si-control; All experiments were repeated at least three times, and all the data are presented as mean ± standard deviation. Statistical analysis was performed using the Student's *t*-test. A value of P $<$ 0.05 was considered significant. $^{\#}$P $<$ 0.05 compared with NC.

tumor sizes were determined after 7, 14, 21, and 28 days. Fig. 6A revealed that the tumor volume was smaller in the mice with Lv-sh-NEAT1 compared with the Lv-shRNA group from the 14th day. Correspondingly, the expression of NEAT1 in tumor tissue was lower in the Lv-sh-NEAT1 group compared with the Lv-shRNA group, while the miR-155 level was augmented after NEAT1 down-regulation (Fig. 6B). When compared with the Lv-shRNA transfection group, the Tim-3 protein expression was down-regulated after NEAT1 interference (Fig. 6C).

4. Discussion

Due to its roles in inducing the epithelial-mesenchymal transition and modulating cell growth, invasion, and migration, NEAT1 has been considered as a general tumor promoter (Yu et al., 2017; Cao et al., 2016). In this study, the effect of NEAT1 on the antitumor function of immune cells was initially highlighted. We illuminated that repression of NEAT1 restrained CD8$^+$T cell apoptosis and enhanced the cytolysis activity against HCC via controlling the miR-155/Tim-3 pathway. These findings revealed an effective therapeutic target for HCC treatment and provided novel insight into improving the outcome of immunotherapy.

Despite the increasing incidence of HCC, the therapeutic options still remain limited, and effective therapies are urgently needed. Based on the sufficient investigation of immune response during tumor development, the efficacy and safety of immunotherapy have been evaluated in cancer clinical treatment (Li et al., 2015). Diverse molecules were found to have essential roles in immune evasion of tumor cells, acting in immunomodulation and serving as therapeutic targets in immunotherapy (Prieto et al., 2015).

As a crucial lncRNA for the formation of nuclear body paraspeckles, NEAT1 has been identified to be involved in immune responses, serving as an inducible lncRNA in mouse brains infected with the Japanese encephalitis or rabies virus (Imamura and Akimitsu, 2014). HIV-1 infection also induced NEAT1 in mice, in which NEAT1 modulated HIV-1 replication by affecting the export of HIV-1 mRNAs from the nucleus to cytoplasm (Zhang et al., 2013). NEAT1 also facilitated the expression of antiviral genes including cytokines like interleukin-8 (IL8) (Imamura et al., 2014), and NEAT1 participated in the DNA-mediated innate response by forming a multi-subunit complex (Morchikh et al., 2017). In this study, we further highlighted that the low expression of NEAT1 contributed to strengthening the antitumor function of CD8$^+$T cells, implying the capacity of NEAT1 in inducing the immune escape of HCC.

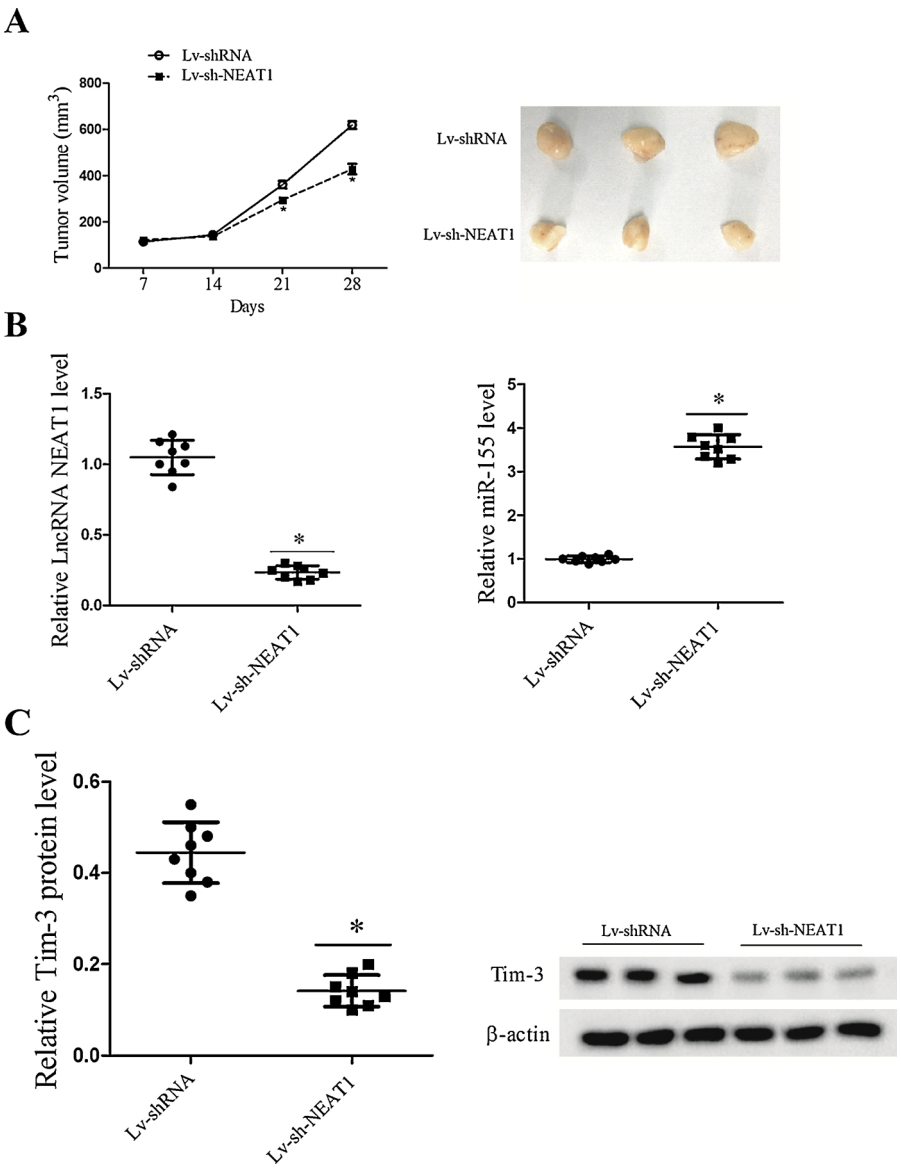

Fig. 6. Interference of NEAT1 suppressed tumor growth in HCC mice. Mouse hepatoma tumor models were constructed and injected with $CD8^+$T cells with Lv-sh-NEAT1 (n = 8) or Lv-shRNA (n = 8). (A) The tumor volumes were detected at 7, 14, 21, and 28 days after lentivirus injection. (B) The expression of NEAT1 and miR-155 was measured using qRT-PCR. (C) The Tim-3 protein level was determined with western blot. All experiments were repeated at least three times, and all the data are presented as mean ± standard deviation. Statistical analysis was performed using the Student's *t*-test. A value of $P < 0.05$ was considered significant. *$P < 0.05$ compared with Lv-shRNA.

In view of the general role of $CD8^+$T cells in controlling cancer development, NEAT1 may be implicated in many other cancers' immune reactions, exhibiting a bright future as an immune checkpoint in immunotherapy.

The interaction between NEAT1 and miR-155 was initially clarified in our study, unraveling a novel regulatory system influencing the antitumor activity of $CD8^+$T cells against HCC. Emerging evidence has indicated that miR-155 is associated with the tumorigenesis of breast cancer, rectal cancer, liver cancer, lung cancer, and bladder cancer (Kim et al., 2015; Yang et al., 2015; Liu et al., 2015; Xie et al., 2015; Zhang et al., 2016), as a well-known oncogenic miRNA. In addition, miR-155 also contributed to malignant behaviors of HCC by stimulating epithelial-mesenchymal transition, cell invasion and metastasis (Li et al., 2017). Apart from its involvement in cancers, miR-155 also functions as a regulator of the immune system and T lymphocyte function (Xie et al., 2014; Song and Lee, 2015). A previous study reported that miR-155 augmented $CD8^+$T cells' antitumor activity by enhancing their responsiveness to homeostatic cytokines (Ji et al., 2015). In the present research, we proved that the interference of miR-155 induced CD8 + T cell apoptosis and decreased the cytolysis activity by up-regulating Tim-3, thereby demonstrating its role in improving $CD8^+$T cells' antitumor activity once again and indicating its antineoplastic function in HCC. We attribute this distinct role of miR-155 to the diversity of miRNAs' targets, which has been discussed in terms of cancer pathogenesis and immune activities (Xie et al., 2014).

By targeting Tim-3, a member of the T-cell immunoglobulin and mucin domain proteins (Tim), miR-155 exerted its immune regulation effect in HCC. Tim-3 is emerging as a key participant in cancer immunotherapy for its pivotal role in regulating antitumor immunity, especially in promoting the development of a severe dysfunctional phenotype in $CD8^+$T cells in cancer (Anderson, 2014). The current study illustrated that up-regulation of Tim-3 induced CD8 + T cell apoptosis and decreased the cytolysis activity, thereby contributing to HCC progression. These findings reconfirmed the general function of Tim-3 in modulating $CD8^+$T cell apoptosis and proliferation (Kang et al., 2015; Yan et al., 2013), as well as its tumor-promoting action in HCC (Yan et al., 2015). This study not only discovered the regulatory role of lncRNA NEAT1 in $CD8^+$T cell function but also emphasized its effect on the immune escape of HCC, offering a theoretical basis for developing effective immunotherapy and improving its outcomes in HCC treatment.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgement

This study was supported by a grant from the National Natural Science Foundation (No. 81671581).

References

Anderson, A.C., 2014. Tim-3: an emerging target in the cancer immunotherapy landscape. Cancer Immunol. Res. 2 (5), 393–398.
Cao, J., Zhang, Y., Yang, J., He, S., Li, M., Yan, S., Chen, Y., Qu, C., Xu, L., 2016. NEAT1 regulates pancreatic cancer cell growth, invasion and migration though mircroRNA-335-5p/c-met axis. Am. J. Cancer Res. 6 (10), 2361–2374.
Cheng, Y.Q., Ren, J.P., Zhao, J., Wang, J.M., Zhou, Y., Li, G.Y., Moorman, J.P., Yao, Z.Q., 2015. MicroRNA-155 regulates interferon-gamma production in natural killer cells via Tim-3 signalling in chronic hepatitis C virus infection. Immunology 145 (4), 485–497.
Flecken, T., Schmidt, N., Hild, S., Gostick, E., Drognitz, O., Zeiser, R., Schemmer, P., Bruns, H., Eiermann, T., Price, D.A., et al., 2014. Immunodominance and functional alterations of tumor-associated antigen-specific CD8+ T-cell responses in hepatocellular carcinoma. Hepatology 59 (4), 1415–1426.
Fu, J., Xu, D., Liu, Z., Shi, M., Zhao, P., Fu, B., Zhang, Z., Yang, H., Zhang, H., Zhou, C., et al., 2007. Increased regulatory T cells correlate with CD8 T-cell impairment and poor survival in hepatocellular carcinoma patients. Gastroenterology 132 (7), 2328–2339.
Guo, C.L., Yang, X.H., Cheng, W., Xu, Y., Li, J.B., Sun, Y.X., Bi, Y.M., Zhang, L., Wang, Q.C., 2014. Expression of Fas/FasL in CD8+ T and CD3+ Foxp3+ Treg cells—relationship with apoptosis of circulating CD8+ T cells in hepatocellular carcinoma patients. Asian Pac. J. Cancer Prev. 15 (6), 2613–2618.
Guo, S., Chen, W., Luo, Y., Ren, F., Zhong, T., Rong, M., Dang, Y., Feng, Z., Chen, G., 2015. Clinical implication of long non-coding RNA NEAT1 expression in hepatocellular carcinoma patients. Int. J. Clin. Exp. Pathol. 8 (5), 5395–5402.
Han, S., Yang, S., Cai, Z., Pan, D., Li, Z., Huang, Z., Zhang, P., Zhu, H., Lei, L., Wang, W., 2015. Anti-Warburg effect of rosmarinic acid via miR-155 in gastric cancer cells. Drug Des. Dev. Ther. 9, 2695–2703.
Imamura, K., Akimitsu, N., 2014. Long non-coding RNAs involved in immune responses. Front. Immunol. 5, 573.
Imamura, K., Imamachi, N., Akizuki, G., Kumakura, M., Kawaguchi, A., Nagata, K., Kato, A., Kawaguchi, Y., Sato, H., Yoneda, M., et al., 2014. Long noncoding RNA NEAT1-dependent SFPQ relocation from promoter region to paraspeckle mediates IL8 expression upon immune stimuli. Mol. Cell 53 (3), 393–406.
Ji, Y., Wrzesinski, C., Yu, Z., Hu, J., Gautam, S., Hawk, N.V., Telford, W.G., Palmer, D.C., Franco, Z., Sukumar, M., et al., 2015. miR-155 augments CD8+ T-cell antitumor activity in lymphoreplete hosts by enhancing responsiveness to homeostatic gammac cytokines. Proc. Natl. Acad. Sci. U. S. A. 112 (2), 476–481.
Jin, C., Peng, X., Xie, T., Lu, X., Liu, F., Wu, H., Yang, Z., Wang, J., Cheng, L., Wu, N., 2016. Detection of the long noncoding RNAs nuclear-enriched autosomal transcript 1 (NEAT1) and metastasis associated lung adenocarcinoma transcript 1 in the peripheral blood of HIV-1-infected patients. HIV Med. 17 (1), 68–72.
Kang, C.W., Dutta, A., Chang, L.Y., Mahalingam, J., Lin, Y.C., Chiang, J.M., Hsu, C.Y., Huang, C.T., Su, W.T., Chu, Y.Y., et al., 2015. Apoptosis of tumor infiltrating effector TIM-3+CD8+ T cells in colon cancer. Sci. Rep. 5, 15659.
Kim, S., Rhee, J.K., Yoo, H.J., Lee, H.J., Lee, E.J., Lee, J.W., Yu JH, Son B.H., Gong, G., Kim, S.B., et al., 2015. Bioinformatic and metabolomic analysis reveals miR-155 regulates thiamine level in breast cancer. Cancer Lett. 357 (2), 488–497.
Kong, X., Liu, F., Gao, J., 2016. MiR-155 promotes epithelial-mesenchymal transition in hepatocellular carcinoma cells through the activation of PI3K/SGK3/beta-catenin signaling pathways. Oncotarget 7 (40), 66051–66060.
Li, S., Yang, F., Ren, X., 2015. Immunotherapy for hepatocellular carcinoma. Drug Discov. Ther. 9 (5), 363–371.
Li, D.P., Fan, J., Wu, Y.J., Xie, Y.F., Zha, J.M., Zhou, X.M., 2017. MiR-155 up-regulated by TGF-beta promotes epithelial-mesenchymal transition, invasion and metastasis of human hepatocellular carcinoma cells in vitro. Am. J. Transl. Res. 9 (6), 2956–2965.
Liu, F., Kong, X., Lv, L., Gao, J., 2015. MiR-155 targets TP53INP1 to regulate liver cancer stem cell acquisition and self-renewal. FEBS Lett. 589 (4), 500–506.
Morchikh, M., Cribier, A., Raffel, R., Amraoui, S., Cau, J., Severac, D., Dubois, E., Schwartz, O., Bennasser, Y., Benkirane, M., 2017. HEXIM1 and NEAT1 long non-coding RNA form a multi-subunit complex that regulates DNA-Mediated innate immune response. Mol. Cell 67 (3), 387–399 e385.
Prieto, J., Melero, I., Sangro, B., 2015. Immunological landscape and immunotherapy of hepatocellular carcinoma. Nat. Rev. Gastroenterol. Hepatol. 12 (12), 681–700.
Shi, F., Shi, M., Zeng, Z., Qi, R.Z., Liu, Z.W., Zhang, J.Y., Yang, Y.P., Tien, P., Wang, F.S., 2011. PD-1 and PD-L1 upregulation promotes CD8(+) T-cell apoptosis and postoperative recurrence in hepatocellular carcinoma patients. Int. J. Cancer 128 (4), 887–896.
Song, J., Lee, J.E., 2015. miR-155 is involved in Alzheimer's disease by regulating T lymphocyte function. Front. Aging Neurosci. 7, 61.
Song, X., Tan, S., Wu, Z., Xu, L., Wang, Z., Xu, Y., Wang, T., Gao, C., Gong, Y., Liang, X., et al., 2018. HBV suppresses ZHX2 expression to promote proliferation of HCC through miR-155 activation. Int. J. Cancer 143 (12), 3120–3130.
Sun, C., Xu, J., Song, J., Liu, C., Wang, J., Weng, C., Sun, H., Wei, H., Xiao, W., Sun, R., et al., 2015. The predictive value of centre tumour CD8(+) T cells in patients with hepatocellular carcinoma: comparison with Immunoscore. Oncotarget 6 (34), 35602–35615.
Tang, B., Lei, B., Qi, G., Liang, X., Tang, F., Yuan, S., Wang, Z., Yu, S., He, S., 2016. MicroRNA-155-3p promotes hepatocellular carcinoma formation by suppressing FBXW7 expression. J. Exp. Clin. Cancer Res. 35 (1), 93.
Wang, S., Jia, L., Zhou, H., Shi, W., Zhang, J., 2009. Knockdown of caveolin-1 by siRNA inhibits the transformation of mouse hepatoma H22 cells in vitro and in vivo. Oligonucleotides 19 (1), 81–88.
Xie, G.B., Liu, W.J., Pan, Z.J., Cheng, T.Y., Luo, C., 2014. Evolution of the mir-155 family and possible targets in cancers and the immune system. Asian Pac. J. Cancer Prev. 15 (18), 7547–7552.
Xie, K., Ma, H., Liang, C., Wang, C., Qin, N., Shen, W., Gu, Y., Yan, C., Zhang, K., Dai, N., et al., 2015. A functional variant in miR-155 regulation region contributes to lung cancer risk and survival. Oncotarget 6 (40), 42781–42792.
Yan, J., Zhang, Y., Zhang, J.P., Liang, J., Li, L., Zheng, L., 2013. Tim-3 expression defines regulatory T cells in human tumors. PLoS One 8 (3), e58006.
Yan, W., Liu, X., Ma, H., Zhang, H., Song, X., Gao, L., Liang, X., Ma, C., 2015. Tim-3 fosters HCC development by enhancing TGF-beta-mediated alternative activation of macrophages. Gut 64 (10), 1593–1604.
Yang, Y., Tang, T., Peng, W., Xia, L., Wang, X., Duan, B., Shu, Y., 2015. The comparison of miR-155 with computed tomography and computed tomography plus serum amyloid A protein in staging rectal cancer. J. Surg. Res. 193 (2), 764–771.
Yu, X., Li, Z., Zheng, H.Y., Chan, M.T., Wu, W.K., 2017. NEAT1: A novel cancer-related long non-coding RNA. Cell Prolif . 50 (2), e12329.
Zhang, Q., Chen, C.Y., Yedavalli, V.S., Jeang, K.T., 2013. NEAT1 long noncoding RNA and paraspeckle bodies modulate HIV-1 posttranscriptional expression. MBio 4 (1), e00596–00512.
Zhang, X., Zhang, Y., Liu, X., Fang, A., Wang, J., Yang, Y., Wang, L., Du, L., Wang, C., 2016. Direct quantitative detection for cell-free miR-155 in urine: a potential role in diagnosis and prognosis for non-muscle invasive bladder cancer. Oncotarget 7 (3), 3255–3266.
Zheng, X., Zhang, Y., Liu, Y., Fang, L., Li, L., Sun, J., Pan, Z., Xin, W., Huang, P., 2018. HIF-2alpha activated lncRNA NEAT1 promotes hepatocellular carcinoma cell invasion and metastasis by affecting the epithelial-mesenchymal transition. J. Cell. Biochem. 119 (4), 3247–3256.