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Original Article

Biological functional role of miR-146a-5p on cell growth and caspase-3/7 activity via targeting TRAF6 in hepatocellular carcinoma

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Abstract: Background: We have previously reported that miR-146a-5p (previous ID: miR-146a) level was significantly reduced in hepatocellular carcinoma (HCC) tissues and its biological function could be partially exerted via targeting epidermal growth factor receptor (EGFR). However, miR-146a-5p could play its biological role via other targets. The relationships between miR-146a-5p and tumor necrosis factor (TNF) receptor associated factor 6 (TRAF6) have been investigated in several diseases, but, their associations remain unclarified in HCC. Thus, the objective of the present study was to assess the biological function of miR-146a-5p via targeting TRAF6 in HCC. Methods: *In silico*, the prediction analysis of the complementary sequences between miR-146a-5p and the 3'-untranslated region of TRAF6 was performed. Further experiments *in vitro* on HepG2 and Hep3B cell lines were carried out to validate the biological function of miR-146a-5p, as well as TRAF6 with the assays of cell viability, proliferation, apoptosis and caspase-3/7 activity. Finally, the clinical role of miR-146a-5p and TRAF6 in HCC tissues was validated using the data downloaded from the cancer genome atlas (TCGA). Results: Complementary sequences between miR-146a-5p and the 3'-untranslated regions of TRAF6 were confirmed *in silico*. In both HepG2 and Hep3B cell lines, miR-146a-5p mimic and TRAF6 silencing repressed the cell growth as demonstrated by different approaches. Furthermore, miR-146a-5p mimic and TRAF6 knock-down induced cell apoptosis and caspase-3/7 activity. The biological effect was more potent when generated from TRAF6 siRNA as compared to that from miR-146a-5p mimic. Additionally, significantly down-regulated miR-146a was observed in 354 cases of HCC cases in TCGA database ($P<0.001$). The area under the curve (AUC) in receiver operating characteristic (ROC) curve reached 0.686 (95% CI: 0.628-0.744, $P<0.001$) for miR-146a to diagnose HCC. Conclusions: MiR-146a-5p might play its vital role in the carcinogenesis and progression of HCC partly via targeting TRAF6.

Keywords: MiR-146a-5p, TRAF6, HCC, cell growth, apoptosis

Introduction

Hepatocellular carcinoma (HCC) is the second leading cause of cancer death. Its incidence as well as mortality rate will be still on the increase over the forthcoming decades [1-4]. The carcinogenesis and progression of HCC is a multistep process. Major etiological factors for HCC include HBV and HCV infection, long-term exposure to chemical agents (aflatoxin B1 or alcohol), etc. [5-8]. Surgical resection has been regarded as the standard treatment for HCC [9-11]. However, most HCC patients are not appropriate for curative surgical resection when being diagnosed because of their large tumor size, vascular involvement, multifocal disease, and extrahepatic spread [12, 13]. Thus, the majority of HCC patients do not have the opportunity for radical surgery and end up with poor survival [1, 2, 6, 14, 15]. Hence, it is

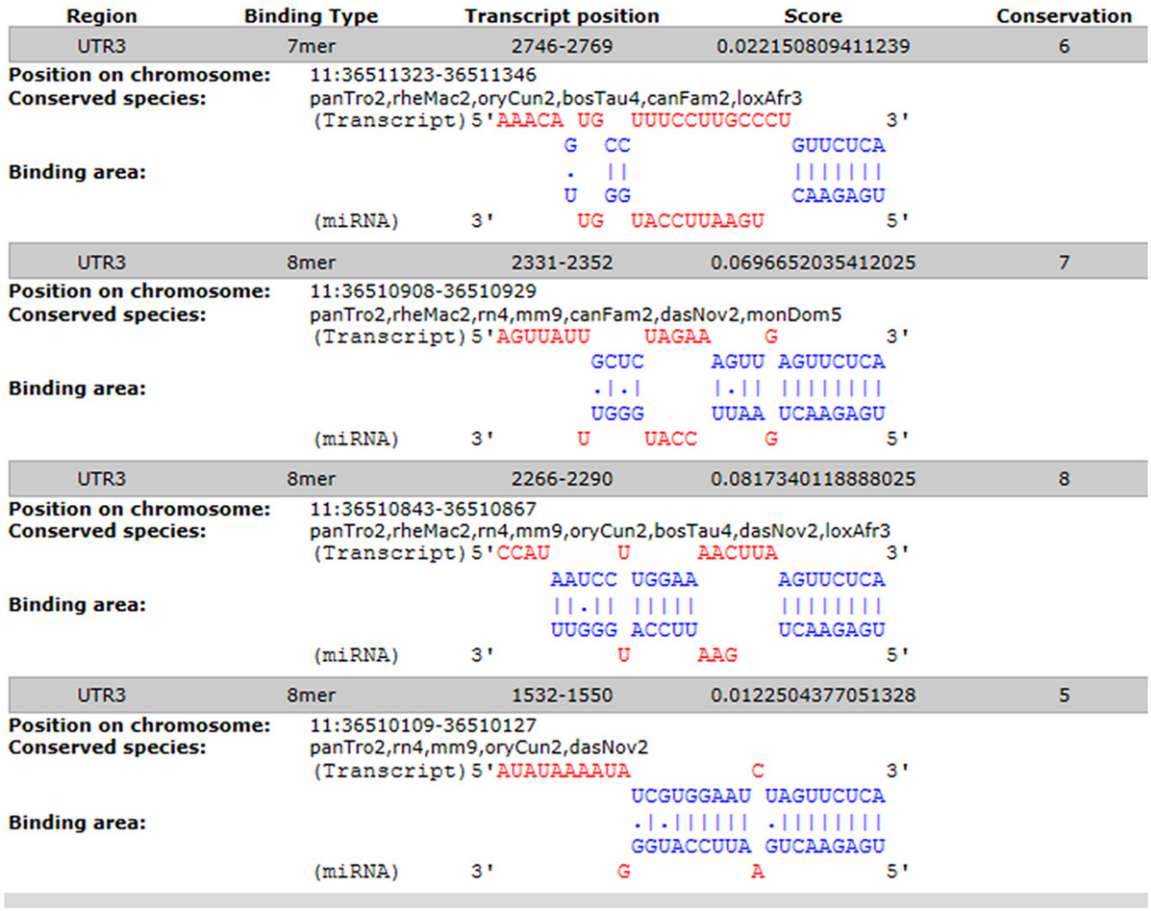

Figure 1. Complementary sequences between miR-146a-5p and the 3'-untranslated regions of TRAF6. The schematic was provided from Tarbase (http://diana.imis.athena-innovation.gr/DianaTools/index.php?r=tarbase/index & mirnas=hsa-miR-146a-5p).

of urgent need to clarify the molecular mechanisms involved in the HCC aggressiveness and novel biomarkers for early diagnosis of HCC, as well as the underlying molecular mechanism of HCC development, to improve the survival of HCC patients.

MicroRNAs (miRNAs), an abundant class of endogenous, small, non-coding RNAs, have been identified as gene expression regulators [16-19]. Emerging evidence indicates that miRNAs play a critical role in the regulation of various biological and pathological processes including cellular differentiation, proliferation, metastasis and apoptosis [20-24]. MiRNAs elicit their regulatory effects by binding to the fully or partially complementary sites within the 3'-untranslated region (UTR) of target messenger RNAs (mRNAs), causing the degradation, deadenylation or the translational repression of mRNA. Additionally, recent studies have suggested that dysregulation of specific miRNAs was correlated with tumor development and progression, including HCC [25-30].

Among all the miRNAs related to HCC, miR-146a-5p level was reported, by other groups and ours, to be reduced in HCC patients compared to the non-tumor controls [31-33]. It is also found that miR-146a-5p could suppress cell proliferation and enhance cell apoptosis via different targets [33-35]. However, more possible targets for miR-146a-5p could exist. The relationships between miR-146a-5p and TNF receptor associated factor 6 (TRAF6) have been explored in other diseases [36-40]. Moreover, our previous work found that TRAF6 protein was over-expressed in HCC tissue. On the contrary, miR-146a-5p was under-expressed [41]. However, the correlation and potential

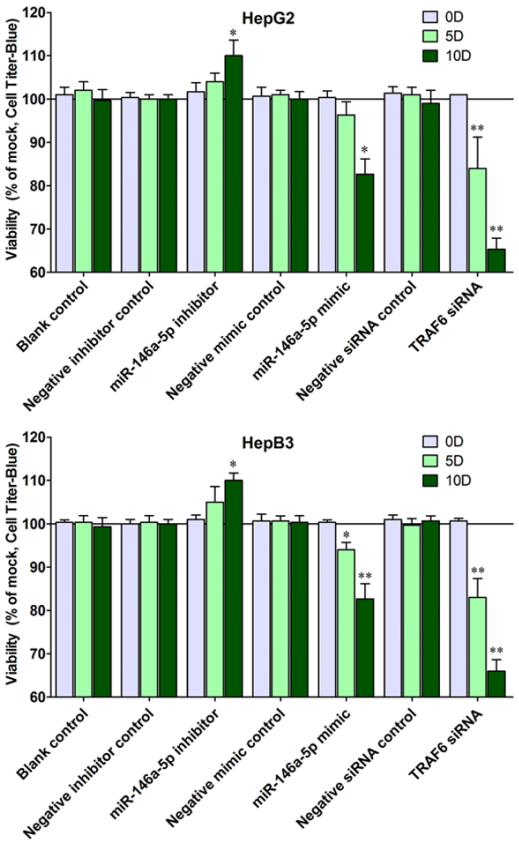

Figure 2. Detection of the effect of miR-146a-5p and TRAF6 on cell viability using cell viability kits. HepG2 and Hep3B cell lines were transfected with negative inhibitors, miR-146a-5p inhibitors, negative mimic, miR-146a-5p mimic, negative siRNA and TRAF6 siRNA, respectively. The cell viability was detected on 0, 5th, 10th day after the transfection. (*P<0.05, **P<0.01).

molecular mechanism between miR-146a-5p and TRAF6 still remain unknown in HCC. Based on the above information, we carried out this current *in vitro* study to unravel the prospective relationships between miR-146a-5p and the new target, TRAF6.

Material and methods

In silico confirmation

The complementary sequences between miR-146a-5p and the 3'-untranslated region of TRAF6 were investigated by the miRWalk, mirtarbase, Tarbase, PITA, MicroT4, miRMap, RNA-22, TargetScan, miRanda, miRNAMap, RNAhybrid, miRBridge, MiRDB and PICTAR2 databases.

In vitro experiments

Transfection of miR-146a-5p mimic, inhibitor and TRAF6 siRNA: The human HCC-derived cell lines HepG2 (HB-8065) and Hep3B (HB-8064) were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). All the experiments *in vitro* were performed in triplicate. HepG2 and Hep3B cells were incubated in 24-well-plates (2.5×10^4 cells per well) or 96-well-plates (2.5×10^3 cells per well) and cultured in the RPMI1640 medium with 10% fetal bovine serum at 37°C in a 5% CO_2 incubator for 24 hours before the transfection, so that the cell confluence could reach upwards of 50% to 60% when being transfected. Lipofectamine™ 2000 reagent (Invitrogen) was mixed with miRNA inhibitor negative control, miR-146a-5p inhibitor, miRNA mimic negative control and miR-146a-5p mimic (Ambion, Life Technologies, Europe B.V., Gent, Belgium), as well as TRAF6 siRNA (*5'-CCCAGUCACACAUGAGAAU-3'*), respectively. The final concentration was 60 nmol/L and the standing time for the mixture was 20 minutes at room temperature. Then, the mixture was added to the 24- or 96-well-plates and cultured at 37°C in a 5% CO_2 incubator. After the transfection, we chose 0, 5 and 10 days as time points to detect the cell growth and apoptosis.

Cellular growth: Cell proliferation was measured with MTS kit based on manufacturer's instructions. After being treated with different agents for 0, 5 and 10 days, the cells were washed with phosphate-buffered saline (PBS) twice. Solutions including 100 μL 10% fetal bovine serum and 20 μL MTS reagents were added to subset of plates. After the cells were incubated for two hours, absorbance at 490 nm was measured with a microplate reader (Thermo). Meanwhile, the cell viability was conducted with Cell Titer-Blue kit (G8080, Promega, USA) according to manufacturer's instructions on 0, 5 and 10 days after transfection. The cell viability was determined with FL600 fluorescent detector (Bio-Tek, USA) at 560 nm or 590 nm. Furthermore, Hoechst 33342/propidium iodide (PI) double florescent chromatin staining was performed to evaluate the number of viable cells [26, 35, 42, 43].

Cellular caspase-3/7 activity and apoptosis: After the cell viability assay was finished, exper-

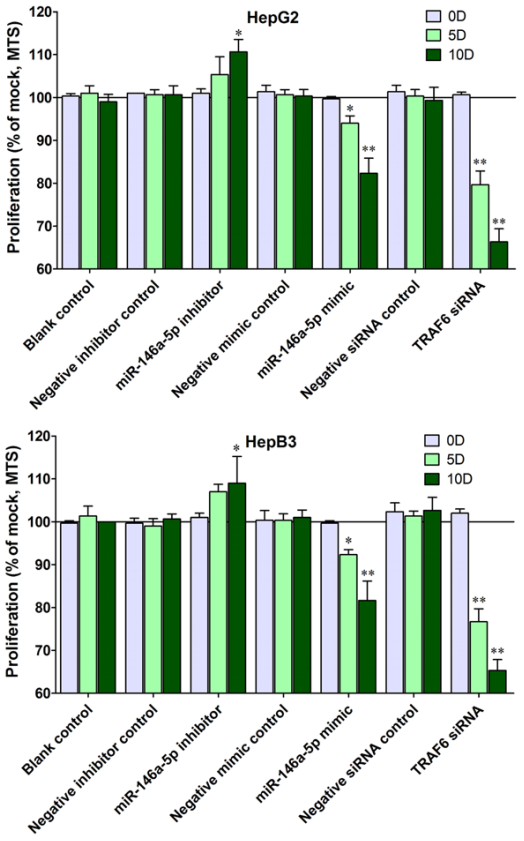

Figure 3. Detection of the effect of miR-146a-5p and TRAF6 on cell proliferation using MTS kits. HepG2 and Hep3B cell lines were transfected with negative inhibitors, miR-146a-5p inhibitors, negative mimic, miR-146a-5p mimic, negative siRNA and TRAF6 siRNA, respectively. The cell proliferation was detected on 0, 5th, 10th day after the transfection. (*P<0.05, **P<0.01).

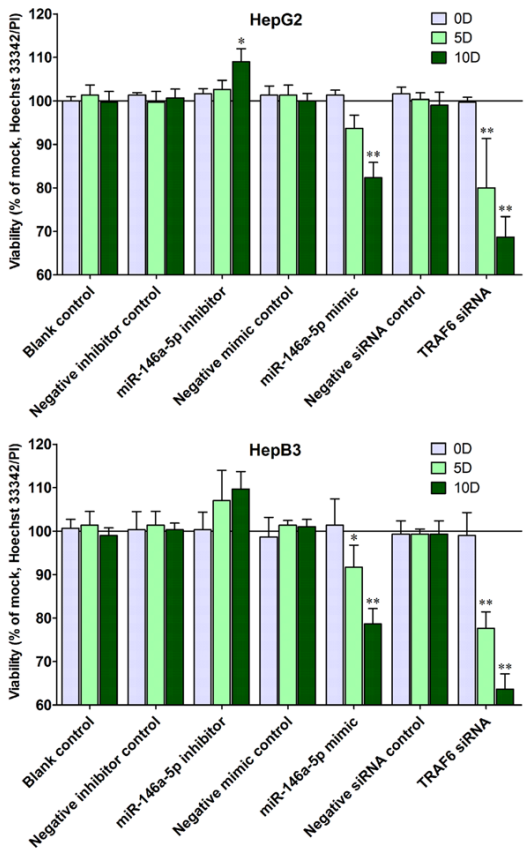

Figure 4. Detection of the effect of miR-146a-5p and TRAF6 on cell growth using Hoechst 33342/PI fluorescent staining. HepG2 and Hep3B cell lines were transfected with negative inhibitors, miR-146a-5p inhibitors, negative mimic, miR-146a-5p mimic, negative siRNA and TRAF6 siRNA, respectively. The cell growth was detected on 0, 5th, 10th day after the transfection. (*P<0.05, **P<0.01).

iments on cells of the same 96-well-plates were carried out to detect caspase-3/7 activity. Caspase-3/7 reagents were added into each well, and then the cells were incubated for an hour. Fluorescence intensity was measured at 499 nm or 512 nm with FL600 fluorescent detector (Bio-Tek, USA). Additionally, Hoechst 33342/PI double florescent chromatin staining was performed to evaluate the number of apoptotic and necrotic cells as previously reported [26, 35, 42, 43].

Western blot and dual-luciferase assay: The process of western blot was shown in previous studies [42], In brief, 25 μg proteins obtained from the cell lines were added in 8% SDS-PAGE separation gel. Then the mixture was transferred to nitrocellulose membranes and probed with primary antibodies against the primary antibodies of TRAF6 ((D-10): sc-8409, 60 kDa, dilution: 1:500, Santa Cruz Biotechnology) and internal reference β-actin ((C4): sc-47778, 43 kDa, dilution: 1:1000, Santa Cruz Biotechnology). After the membranes were incubated with secondary antibodies, enhanced chemiluminescence detection was performed to identify the expression levels of each protein. For dual-luciferase assay, the 3'-UTR of TRAF6 with putative miR-146a-5p-binding site was amplified and cloned into pmirGLO vector (Promega, Madison, WI, USA). Mutated versions of TRAF6 (TRAF6-mut-UTR) containing 4-bp substitutions in the miR-146a-5p target sites were constructed by site-directed mutagenesis. Cells were cultured in 96-well-plates and then co-transfected with 100 ng of wide-type (wt) or mutated TRAF6 3'-UTR constructs, or 50 mM of negative control or miR-146a-5p mimics up to 96 hours [44]. Luciferase activity was monitored as rela-

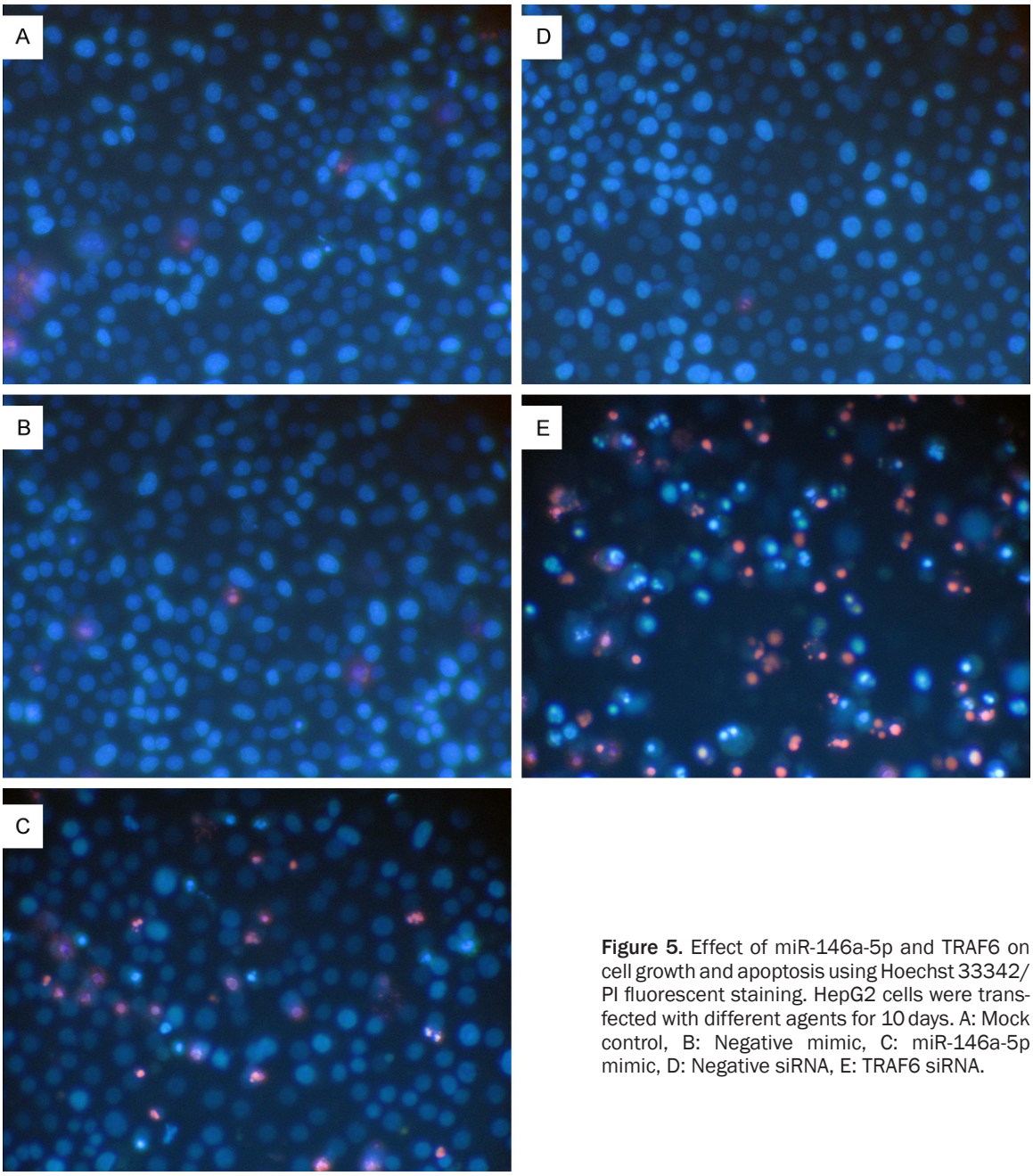

Figure 5. Effect of miR-146a-5p and TRAF6 on cell growth and apoptosis using Hoechst 33342/PI fluorescent staining. HepG2 cells were transfected with different agents for 10 days. A: Mock control, B: Negative mimic, C: miR-146a-5p mimic, D: Negative siRNA, E: TRAF6 siRNA.

tive light unit with the dual luciferase reporter assay kit (Promega) based on the manufacturer [42].

Clinical validation in TCGA database

The level three data of miR-146a, as well as TRAF6 expression in HCC and adjacent non-tumor liver tissues were extracted from the cancer genome atlas (TCGA) database on October 1, 2016. We also downloaded relevant clinicopathological parameters, including the information of survival (http://cancergenome.nih.gov/).

Statistical analysis

SPSS 22.0 was used for all statistical analyses in the study. The quantitative data were displayed as mean ± standard deviation. With regard to the significance between different groups *in vitro*, one-way analysis of variance (ANOVA) test was selected. If the *P* value of ANOVA was less than 0.05, the least significant difference (LSD) method would be conducted to compare the alteration between two groups. The ROC curve was drawn to identify the diagnostic significance of miR-146a and TRAF6 in

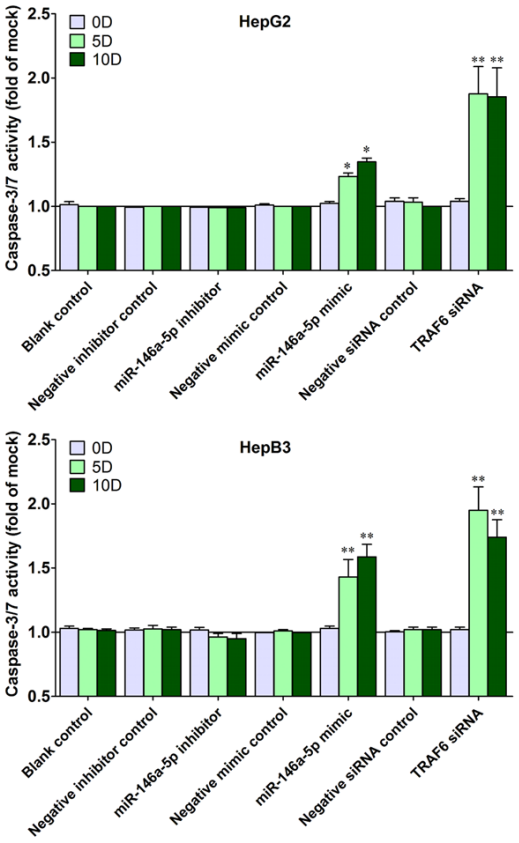

Figure 6. Detection of the effect of miR-146a-5p and TRAF6 on caspase-3/7 activity. HepG2 and Hep3B cell lines were transfected with negative inhibitors, miR-146a-5p inhibitors, negative mimic, miR-146a-5p mimic, negative siRNA and TRAF6 siRNA, respectively. The cell caspase-3/7 activity was detected on 0, 5th, 10th day after the transfection. (*P<0.05, **P<0.01).

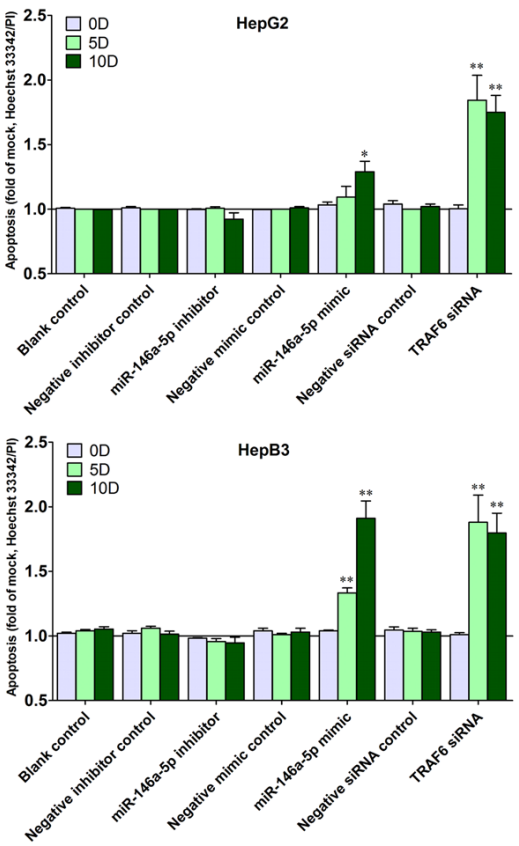

Figure 7. Detection of the effect of miR-146a-5p and TRAF6 on apoptosis using Hoechst 33342/PI fluorescent staining. HepG2 and Hep3B cell lines were transfected with negative inhibitors, miR-146a-5p inhibitors, negative mimic, miR-146a-5p mimic, negative siRNA and TRAF6 siRNA, respectively. The cellular apoptosis was detected on 0, 5th, 10th day after the transfection. (*P<0.05, **P<0.01).

HCC based on TCGA data. Significance of difference between HCC and para-HCC non-cancerous liver tissues, as well as various clinicopathological characteristics, was analyzed by Student's t test. The Spearman analysis was utilized to evaluate the correlations between miR-146a and TRAF6. Cox regression and K-M curve were performed to evaluate the prognostic value of miR-146a and TRAF6 in HCC patients. *P* value less than 0.05 indicated a statistical difference.

Results

Complementary sequences confirmed in silico

Based on the different target prediction and validation databases, we confirmed the complementary sequences between miR-146a-5p and the 3'-UTRs of TRAF6 (**Figure 1**).

The influence of miR-146a-5p and TRAF6 on cell growth in HepG2 and Hep3B cell lines

The transfection efficiency of miR-146a-5p inhibitor and mimic, as well as TRAF6 siRNA was confirmed by real-time RT-qPCR or western blot as previously reported (data not shown) [35, 42, 45, 46]. By using cell viability kit, we identified that on the 10th day after the transfection of miR-146a-5p inhibitor, cell viability was improved slightly in both HepG2 (110.00±3.61 vs 99.67±2.52, P=0.049) and Hep3B (110.00±1.73 vs 99.33±2.08, P=0.003) cells as compared to the control group. On the 10th day after the transfection of miR-146a-5p mimic, cell growth was significantly inhibited in both cells (HepG2: 82.67±3.51 vs 100.00±1.73, P=0.006; Hep3B: 82.67±3.51 vs 100.33±1.53, P=0.005, **Figure 2**). Moreover, much str-

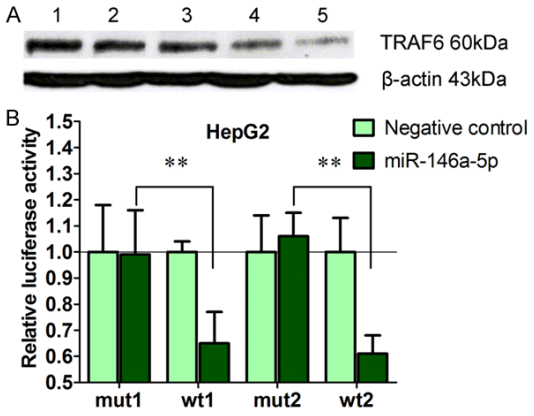

Figure 8. TRAF6 as a target gene of miR-146a-5p in HCC cells. A: Western blot. HepG2 cells were transfected with different agents for 10 days. 1: Mock control, 2: Negative mimic, 3: Negative siRNA, 4: miR-146a-5p mimic, 5: TRAF6 siRNA. B: Luciferase reporter assay. Relative luciferase activity of HepG2 cells after transfection with the wt or mut 3'-UTR of TRAF6 was presented. Luciferase reporter assay showed that miR-146a-5p blocked TRAF6 3'-UTR luciferase activity of both wt constructs, but not mut constructs. wt: wide type; mut: mutated. (*P<0.05, **P<0.01).

onger effect on the inhibition of cell growth via TRAF6 siRNA (HepG2: 65.33±2.52 vs 99.00±3.00, P=0.003; Hep3B: 66.00±2.65 vs 100.67±1.16, P=0.004) was noted compared with the control group (**Figure 2**). To confirm the finding from cell viability test, we repeated the experiments with MTS assay and Hoechst 33342/PI double staining assay, which rendered accordant results (**Figures 3**-**5**).

The impact of miR-146a-5p and TRAF6 on apoptosis in HepG2 and Hep3B cell lines

Experiments using caspase-3/7 were carried out to verify the impact of miR-146a-5p and TRAF6 on cell apoptosis in HCC cells. The effect of miR-146a-5p inhibitor on caspase-3/7 activity was not obvious in either HepG2 or Hep3B cells (data not shown). However, in HepG2 cell lines, miR-146a-5p mimic improved caspase-3/7 activity on the 5th day (1.23±0.05 vs 1.00±0.03, P=0.013) and 10th day after the transfection (1.35±0.05 vs 1.01±0.03, P=0.007). In Hep3B cell lines, on the 10th day after the transfection, miR-146a-5p mimic induced more apparent caspase-3/7 activity (1.59±0.17 vs 1.00±0.01, P=0.025, **Figure 6**). Again, knock-down of TRAF6 (HepG2: 1.35±0.05 vs 1.00±0.01, P=0.007; Hep3B: 1.74±0.24 vs 1.02±0.03, P=0.03) on 10th day gained more powerful effect on apoptosis induction than in control group in both two cell lines (**Figure 6**). In order to further verify the impact of miR-146-5p and TRAF6 on apoptosis, we carried out Hoechst 33342/PI fluorescent staining, which was in line with the results from caspase-3/7 assay (**Figures 5**, **7**).

TRAF6 as a target gene of miR-146a-5p in HCC with Western blot and luciferase reporter assay

Western blot demonstrated that after 10 days post miR-146a-5p mimic transfection, the TRAF6 protein level was downregulated to 50%-60%, which suggested that TRAF6 expression was influenced by the increased amount of miR-146a-5p in the HCC cells (**Figure 8A**). Furthermore, it was certain that TRAF6 is a direct target gene of miR-146a-5p in HCC cells, as verified by luciferase reporter assay (**Figure 8B**).

MiR-146a-5p and TRAF6 expression in HCC from TCGA data

Lower miR-146a expression was noted in HCC based on TCGA data as compared to non-neoplastic liver tissues (8.03±1.68 vs 8.97±0.85, P<0.001, **Figure 9A**). The AUC of lower miR-146a was 0.686 (95% CI: 0.628-0.744, P< 0.001, **Figure 9B**). However, we failed to observe significant relationships between miR-146a-5p level and any clinicopathological features, including survival (data not shown). Then, we also detected the clinical value of TRAF6. Unexpectedly, lower TRAF6 mRNA level was found in HCC tissues based on TCGA data (data not shown). We also performed a matched analysis with 49 pairs of HCC and non-tumor tissues, and gained the consistent expression trends of miR-146a and TRAF6, as compared to the total cohort of 354 cases (data not shown). Thus, TCGA data failed to reveal an inverse relationship between miR-146a and TRAF6 mRNA level in HCC.

Discussion

In the research, we confirmed that miR-146a-5p could significantly inhibit cell growth and induce apoptosis in HCC, which was consistent with our previous report [35]. In addition, we demonstrated that TRAF6 could act as a target

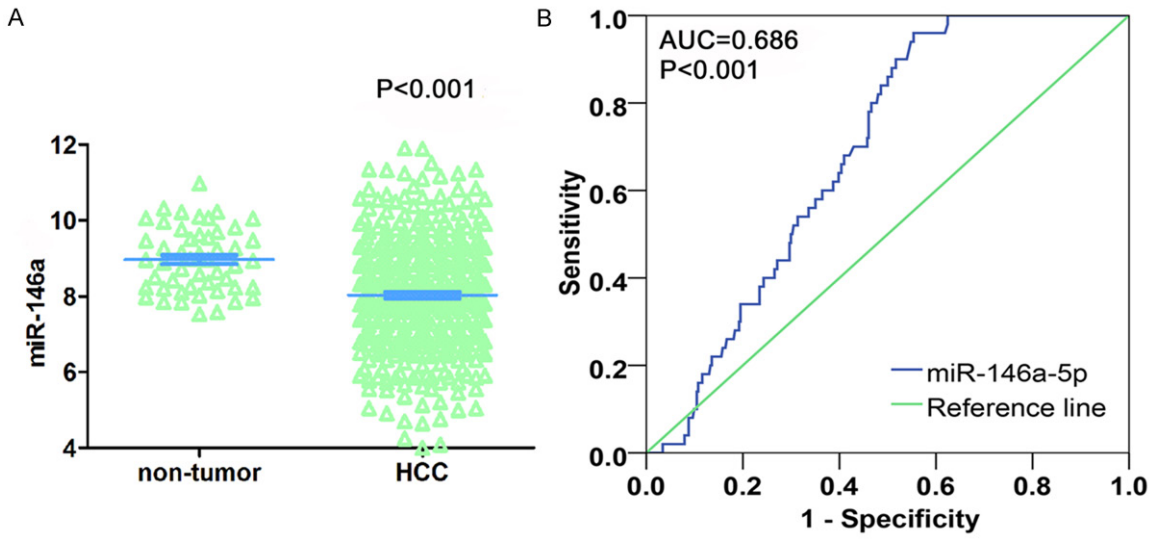

Figure 9. Clinical role of miR-146a in HCC tissues based on TCGA data. A: Difference of miR-146a between HCC and non-cancerous liver tissues. B: ROC curve.

gene of miR-146a-5p in HCC cell line as proved by functional experiments, western blot and luciferase reporter assay. Based on the aforementioned evidence, we revealed that miR-146a-5p could restrain cell growth and induce apoptosis in HCC partially via targeting TRAF6.

With regard to the role of miR-146a-5p in cancer cells, Wang et al clarified that miR-146a-5p inhibited proliferation and induced apoptosis in lung cancer cell by miR-146a-5p mimics transfection [16]. A similar trend was reported by Li et al in non-small cell lung cancer [47]. In breast cancer, Liu et al unveiled that miR-146a-5p could inhibit cell migration and invasion [48]. In addition, Cui et al clarified that miR-146a-5p inhibited cell proliferation and enhanced chemosensitivity in epithelial ovarian cancer [49]. Zhang et al and our previous work both reported that miR-146a-5p could suppress cell invasion and metastasis *in vitro* and *in vivo* in HCC [33, 35]. The above investigation for various cancers, including HCC, was consistent with our current findings and they confirmed the role of miR-146a-5p as an antineoplastic miRNA in HCC, which made the conclusion of this current study more convincing.

After the investigation for the function of miR-146a-5p in HCC cell, we further focused on the regulative mechanisms of miR-146a-5p and its possible target genes in HCC. In the previous studies, several genes targeted by miR-146a-5p were revealed in diverse diseases. In NSCLC, Wang et al and Li et al described that miR-146a-5p could target macrophage migration inhibitory factor (MIF), cyclin D1 (CCND1) and cyclin D2 (CCND2) directly [16, 47]. In breast cancer, Ras homolog family member A (RhoA) were demonstrated to be directly targeted by miR-146a-5p [48]. Moreover, Cui et al reported superoxide dismutase 2 (SOD2) as a novel target gene of miR-146a-5p in epithelial ovarian cancer [49]. TRAF6 and IRAK1 as direct target genes of miR-146a-5p were also reported in polymicrobial sepsis, oral carcinoma and liver ischemia/reperfusion injury [36, 39, 40]. However, the relationship between miR-146a-5p and TRAF6 in HCC remained obscure.

TRAF6, not only takes part in inflammation and immunity, but also is involved in the IL1 receptor signaling pathway and activates NF-KB signal pathway. Known as an oncogene, TRAF6 has been reported to be involved in the carcinogenesis of breast cancer, esophagus cancer and colorectal cancer [50-53]. We previously also found that the expression of TRAF6 protein was remarkably higher in HCC tissues than that of normal liver, cirrhosis and adjacent non-cancerous liver tissues by immunohistochemistry. Additionally, the expression of TRAF6 was positively related to the distant metastasis of HCC [41]. Herein, we confirmed that silencing TRAF6 expression could inhibit cell proliferation in HepG2 and Hep3B cell lines. The caspase-3/7

experiments and Hoechst 33342/PI fluorescent staining revealed that after the silencing of TRAF6, HCC cells underwent apoptosis, which was similar to the results after the transfection with miR-146a-5p mimic. Afterwards, the binding sites among TRAF6 and miR-146a-5p were predicted in several databases and consistent results were confirmed by western blot and luciferase reporter assay. Taken together, all these results suggested that TRAF6 might be oncogenes targeted by miR-146a-5p which could promote the development and progression of HCC.

Although we elucidated that miR-146a-5p could inhibit cell growth and induce apoptosis via targeting TRAF6 in HCC *in vitro*, several limitations could not be ignored. Firstly, only two HCC cell lines were involved in this *in vitro* study which may lack representativeness; Secondly, in this study, the corresponding experiments *in vivo* have not been performed to confirm the roles of miR-146a-5p and its target, TRAF6 in HCC; Thirdly, we failed to validate the relationship between the expression of miR-146a and TRAF6 in HCC tissues based on TCGA database, which may result from the detection approach of high throughput RNA sequencing and different patient population. Regarding the above limitations, further investigation should be carried out in our future work as follows: Firstly, several other HCC lines will be applied to explore the roles of miR-146a-5p and TRAF6; Secondly, future *in vivo* experiments will cover to verify the roles of miR-146a-5p and TRAF6 in HCC to support the current conclusion in the study; Thirdly, expression of miR-146a-5p and TRAF6, as well as their correlation will be validated by qRT-PCR or immunohistochemistry with a larger sample size to increase the accuracy of detection.

Conclusion

By restraining cell growth and inducing cell apoptosis, miR-146a-5p could inhibit HCC progression partially via targeting TRAF6. However, the clinical relationship between miR-146a-5p and TRAF6 needs further verification.

Acknowledgements

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Disclosure of conflict of interest

None.

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