Research Article

HOXA-AS3 Promotes Proliferation and Migration of Hepatocellular Carcinoma Cells via the miR-455-5p/PD-L1 Axis

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Hepatocellular carcinoma (HCC) is the most prevalent type of hepatic carcinoma. Long noncoding RNAs (lncRNAs) are considered crucial regulators of gene expression; however, their functions in HCC are not well understood. Thus, the present study is aimed at elucidating the functions of the lncRNA HOXA-AS3 in HCC. The functions of the HOXA-AS3/miR-455-5p/programmed death-ligand 1 (PD-L1) axis were investigated in vitro via qRT-PCR and dual-luciferase reporter assays. The effect of HOXA-AS3 expression on tumor growth and metastasis was assessed using a mouse xenograft model. High HOXA-AS3 expression was observed in the HCC cell lines. Furthermore, overexpression of HOXA-AS3 in HCC cells enhanced proliferation, migration, and invasion, regulated the cell cycle, and retarded apoptosis. We also identified an miR-455-5p binding site in HOXA-AS3. By sponging miR-455-5p, HOXA-AS3 increased the expression of PD-L1. Additionally, both the inhibition of PD-L1 and overexpression of miR-455-5p reversed the effects on cell proliferation and invasion triggered by the overexpression of HOXA-AS3. In conclusion, HOXA-AS3 modulated the functions of HCC cells through the miR-455-5p/PD-L1 axis. Therefore, HOXA-AS3 may be a novel therapeutic target for HCC.

1. Introduction

Among hepatic carcinomas, hepatocellular carcinoma (HCC) is the most prevalent worldwide [1, 2]. The incidence of HCC is relatively low in the Western world, while there is a high prevalence in Asia. However, during the past 30 years, incidence has increased twofold in America and onefold in Britain [3, 4]. The 5-year survival of HCC patients remains low, and HCC causes approximately 600,000 annual deaths [5]. There has been little progress in developing effective treatments for HCC over the last 20 to 30 years. Therefore, there is an urgent need for new, reliable treatments for patients with HCC.

Long noncoding RNAs (lncRNAs) are a class of noncoding RNAs (ncRNAs) that exhibit a limited capacity for protein encoding [6]. Cumulative evidence has shown that lncRNAs can affect various biological processes and that they are involved in the generation and development of tumors [7, 8]. The most commonly explored role of lncRNAs is that of a major regulator of gene expression, which is performed by sequestering or “sponging” other regulators (e.g., miRNAs) [9]. For instance, IncRNA NR_027471 has been shown to inhibit the progression of osteogenic sarcoma as a competing endogenous RNA (ceRNA) of miRNA-8055 [10]. LINC01436 functions as a ceRNA that...
contributes to the progression of gastric cancer by sponging miR-513a-5p [11]. In other studies, various lncRNAs, including MALAT1 [12], LINC01189 [13], ST8SIA6-AS1 [14], and SNAI3-AS1 [15], have been shown to contribute to the pathophysiological aspects of HCC through competitive microRNA (miRNA) binding. Nevertheless, the functions of HOXA-AS3 in HCC remain to be elucidated.

The aim of this study was to measure HOXA-AS3 expression in HCC cell lines and analyze its effects. We hypothesized that HOXA-AS3 promotes PD-L1 expression by sponging miR-455-5p, thereby modulating HCC pathogenesis. Thus, HOXA-AS3 may be a candidate therapeutic target for HCC.

2. Materials and Methods

2.1. Bioinformatics Analysis. The TCGA database (https://portal.gdc.cancer.gov/) was used for identifying differences in gene expression and overall survival. Lists of differentially expressed genes (P value < 0.05, |log2FC| > 1) were prepared by using the limma package of R. Overall survival of HCC patients was prepared by using survival package and survminer package of R. The downloaded data from TCGA database can be found in Table S1. starBase 3.0 (http://starbase.sysu.edu.cn/) was used to predict miRNAs which have putative binding sites for HOXA-AS3 and PD-L1.

2.2. Culture of HCC Cells. HCC cell lines (Hep3B, SNU-387, Li-7, and HuH-7) and a normal human liver cell line (L-02) were purchased from the Shanghai Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Cells were cultured at 37°C in an incubator with 5% CO2 in Dulbecco’s modified Eagle’s medium (DMEM) (Gibco BRL, Grand Island, NY, USA) with 10% fetal bovine serum (Gibco, Carlsbad, CA, USA).

2.3. Real-Time PCR, Cell Transfection, and Lentivirus Production and Transduction. Total RNA was extracted from HCC cells using TRIzol reagent (Invitrogen), following the manufacturer’s instructions. To generate cDNA, the extracted total RNA was reverse transcribed using Takara’s Reverse Transcription Kit. Then, a SYBR Green PCR kit (Takara, Dalian, China) was used for qPCR. GAPDH was used to normalize mRNA and lncRNA, and U6 was used to normalize miRNA. The primers used are listed in Table 1. Anti-miR-455-5p, miR-455-5p mimics, anti-miR-NC, miR-NC, HOXA-AS3 shRNA, and HOXA-AS3-expressing vectors for cell transfection were synthesized by Ruibo (Guangzhou, China). Cell transfection was performed using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer’s instructions.

HEK293T cells were used to generate lentiviral particles with scrambled HOXA-AS3 shRNA and HOXA-AS3-expressing vectors. Subsequently, recombinant lentiviruses were used to infect HCC cells, while 2 μg/mL puromycin was used for cell selection.

2.4. Determination of Cell Proliferation. The CCK-8 (Beijing, China) was used to assess cell activity. Before adding the CCK-8 reagent, transfected cells in each well of a 96-well plate were subjected to overnight culture. A microplate reader was used to measure the optical density values at 450 nm.

Subsequently, a Cell-Light EdU Cell Proliferation/DNA Kit (RiboBio Co., Ltd., Guangzhou, China) was used to assess cell proliferation. The CCK-8 (Beyotime, Beijing, China) was used to assess cell activity. Before adding the CCK-8 reagent, transfected cells in each well of a 96-well plate were subjected to overnight culture. A microplate reader was used to measure the optical density values at 450 nm.

To assess the colony formation of HCC cells, monoplasm suspensions of HuH-7 and Hep3B cells were seeded into each well of a 12-well plate at equal concentrations and incubated in DMEM containing 10% fetal bovine serum. After 12 days, visible colonies were stained and photographed for counting.

2.5. Transwell, Cell Cycle, and Apoptosis Assays. The upper transwell chamber was precoated with Matrigel for 30 min.

| Name     | Sequence                |
|----------|-------------------------|
| HOXA-AS3 | 5’-AGGAAACATCACGGGCTACA-3’  |
|          | 5’-ATCCCTAAGTGCTTGCACCCCT-3’  |
| miR-455-5p | 5’-ACACTCCAGTTGGATGCTCTTTTGGACT-3’  |
|          | 5’-CTCAACTGTTGTCGAGTGCTGAGTGATG-3’  |
| GAPDH    | 5’-AACGGTGTCAGTGGTGGCACCTG-3’  |
|          | 5’-AGTGGGGTTCGCTGTTGAAGT-3’  |
| U6       | 5’-ACGGTTCAGAAATTGGGTGCT-3’  |
| PD-L1    | 5’-TGCAGCCAGGCTAATTGTTT-3’  |

Table 1: Sequences of primers for qRT-PCR.
Figure 1: HOXA-AS3 expression is increased in HCC cells. (a, b) TCGA heat map (a) and volcano map (b) of IncRNA expression in HCC patients. (c) HOXA-AS3 expression in HCC tissues and controls in TCGA dataset. (d) Association of high HOXA-AS3 expression with shorter overall survival in TCGA dataset. (e) HOXA-AS3 expression in HCC cell lines. Data are reported as the mean ± SD of three separate experiments; **P < 0.01 and ***P < 0.001.
Figure 2: HOXA-AS3 promotes HCC cell proliferation and cell cycle. (a) HOXA-AS3 expression in transfected Hep3B and HuH-7 cells. (b) CCK-8 assay, (c) EdU (bar = 100 μm), and (d) colony formation assays to determine the effects of HOXA-AS3 on HCC cell proliferation. (e) Cell cycle of transfected Hep3B and HuH-7 cells. Data are reported as the mean ± SD of three separate experiments; *P < 0.05, **P < 0.01, and ***P < 0.001.
and HOXA-AS3 incubation for 40 min at room temperature. Finally, the specimens were used for cell suspension, followed by 100 μg/mL RNase A (KeyGen BioTECH) and 50 μg/mL pronidium iodide were used for cell suspension, followed by rinsing twice with ice-cold PBS and immobilization with 4% paraformaldehyde, stained with crystal violet, and imaged under a microscope. Cell analysis was performed three times for each group.

Cell trypsinization was then conducted for separation, followed by rinsing twice with ice-cold PBS and immobilization with 70% ethanol overnight at -20°C. The next day, 100 μg/mL RNase A (KeyGen BioTECH) and 50 μg/mL propidium iodide were used for cell suspension, followed by incubation for 40 min at room temperature. Finally, the specific stages of the cell cycle were detected by flow cytometry.

For apoptosis assays, cells were rinsed with PBS and stained using the Annexin V-FITC Apoptosis Detection Kit (Affymetrix eBioscience) according to the manufacturer’s instructions. Apoptosis was assessed using a FACS flow cytometer (BD Biosciences).

2.6. Dual-Luciferase Reporter Assay. The 3′-UTRs of PD-L1 and HOXA-AS3 were amplified and separately cloned downstream of the firefly luciferase gene in the pGL3 vector (Promega). These were called wild-type (WT) 3′-UTRs. Following the manufacturer’s instructions, a QuickChange site-directed mutagenesis kit (Stratagene, Cedar Creek, USA) was employed for mutation induction, and mutant miR-455-5p binding sites were identified in the 3′-UTRs of both PD-L1 and HOXA-AS3. The mutant 3′-UTRs were called MUT 3′-UTRs. WT or MUT 3′-UTRs of PD-L1 and HOXA-AS3 and miR-NC or miR-455-5p were used to transfect HCC cells. After 48 h, luciferase assays were performed using a dual-luciferase reporter assay system (Promega). The analysis was repeated three times per group.

2.7. Immunohistochemistry (IHC). Using formalin-fixed and paraffin-embedded xenograft tumor sections, anti-Ki-67 (ab16667; Abcam, Shanghai, China) and anti-PD-L1 antibodies (ab205921; Abcam) were used for IHC as reported previously [16, 17].

2.8. Tumor Formation In Vivo. After stable transfection, BALB/c (nu/nu) mice (5 weeks old) were administered 2 × 10⁶ Hep3B cells (HOXA-AS3 or Lv-NC) by subcutaneous flank injection. Before euthanasia, the tumor volume (V) was determined weekly for four weeks, calculated as V = 0.5 × length × width². After approximately 28 days, the mice were sacrificed by cervical dislocation to harvest the tumors surgically. Subsequently, the tumor tissues were photographed, weighed, and stored in liquid nitrogen before use. The in vivo experiments were performed at the SPF Animal Laboratory at Tongji Medical College, Huazhong University of Science and Technology. Experimental procedures involving animals obtained approval from the Ethics Committee for Experimental Animals of Hubei Cancer Hospital, Tongji Medical College, Huazhong University of Science and Technology.

An advanced-stage pulmonary metastasis model was constructed by inoculating each mouse with Hep3B cells (1 × 10⁷) through stable injection into the tail vein. After 4 weeks, mice were euthanized for lung collection and hematoxylin-eosin staining.

2.9. Bioinformatics Analysis and Statistical Analysis. The TCGA (https://portal.gdc.cancer.gov/) was used for identifying differences in gene expression. Lists of differentially expressed genes (Pvalue < 0.05, |log2FC| > 1) were prepared by using the limma package of R. Data are reported as mean ± standard deviation (SD). A two-tailed Student’s t-test
Figure 4: Continued.
was used for comparisons between two groups, and one-way ANOVA was used for comparisons among multiple groups. The statistical significance threshold was set at \( P < 0.05 \).

3. Results

3.1. HOXA-AS3 Expression Was Increased in HCC Cells. TCGA database was used to select lncRNAs associated with HCC. We observed upregulated HOXA-AS3 expression in HCC tissues (Figures 1(a)–1(c)), which was associated with unfavorable prognosis in HCC patients (Figure 1(d)). qRT-PCR was used to measure the HOXA-AS3 expression level in a normal human liver cell line (L-02) and HCC cell lines (Hep3B, SNU-387, Li-7, and HuH-7). The findings revealed that HCC cells had considerably higher HOXA-AS3 expression levels than L-02 cells (Figure 1(e)).

3.2. HOXA-AS3 Promoted HCC Proliferation and Invasion In Vitro. To determine how HOXA-AS3 functions in HCC cells, we performed a variety of in vitro assays in Hep3B and HuH-7 cells. We overexpressed HOXA-AS3 in Hep3B cells and downregulated HOXA-AS3 expression in HuH-7 cells (Figure 2(a)). CCK-8, EdU, and colony formation assays showed that higher HOXA-AS3 expression remarkably enhanced colony formation and cell proliferation (Figures 2(b)–2(d)). Furthermore, flow cytometry revealed that the S phase in the HOXA-AS3 overexpression group was longer than that in the Lv-NC group (Figure 2(e)). Subsequently, apoptosis and transwell invasion assays, respectively, suggested that HOXA-AS3 retarded HCC cell apoptosis (Figure 3(a)) and promoted HCC cell invasion (Figure 3(b)).

3.3. HOXA-AS3 Promoted PD-L1 Expression by Binding to miR-455-5p. First, the subcellular localization of HOXA-AS3 was detected using RNA-FISH. Most HOXA-AS3 was localized in the cytoplasm, with very little HOXA-AS3 in the nucleus (Figure 4(a)). In previous studies, PD-L1 was associated with unfavorable prognosis in HCC patients [18] and shown to contribute to HCC proliferation and metastasis [19]. Here, a positive correlation was observed between HOXA-AS3 and PD-L1 expression in HCC cells (Figure 4(b)). Furthermore, enhanced HOXA-AS3 expression increased PD-L1 expression in Hep3B cells (Figure 4(c)), and downregulation of PD-L1 was observed after knockdown of HOXA-AS3 in HuH-7 cells (Figure 4(d)).

Subsequently, bioinformatics analysis was conducted using starBase 3.0 (http://starbase.sysu.edu.cn/). Only miR-455-5p was found to have putative binding sites for both HOXA-AS3 and PD-L1 (Figure 4(e)). The qRTP-PCR results showed that compared with the L-02 cell line, HCC cell lines exhibited notably lower miR-455-5p expression and higher PD-L1 expression (Figure 4(f)). Bioinformatics analysis revealed a complementary relationship between the miR-455-5p sequence and sequences in the 3'-UTRs of both PD-L1 and HOXA-AS3 (Figure 4(g)).

Luciferase activity in HEK293T cells cotransfected with PD-L1 WT 3'-UTR and miR-455-5p mimic was notably

![Figure 4: HOXA-AS3 enhances PD-L1 expression via binding to the 3'-UTR of miR-455-5p. (a) Typical images of the subcellular localization of HOXA-AS3 in Hep3B cells as shown by RNA-FISH. (b) Association between HOXA-AS3 expression and PD-L1 expression in HCC cells evaluated by Pearson’s correlation analysis. (c, d) Effects of HOXA-AS3 overexpression or knockdown on PD-L1 expression in HCC cells. (e) Bioinformatics analysis by using starBase. (f) Levels of miR-455-5p and PD-L1 in HCC cells. (f–i) Dual-luciferase reporter assay with binding sites. (j) Effects of miR-455-5p mimics or inhibitor on miR-455-5p expression in HCC cells. (k) Effects of anti-miR-455-5p and HOXA-AS3 shRNA on PD-L1 expression in HCC cells. (l) Overexpression of a fragment of HOXA-AS3 carrying the MRE of miR-455-5p could reverse the inhibition of PD-L1 expression induced by overexpressed miR-455-5p. Data are reported as the mean ± SD of three separate experiments. *\( P < 0.05 \), **\( P < 0.01 \), and ***\( P < 0.001 \).]
**Figure 5:** HOXA-AS3 overexpression promotes the tumor growth and metastasis in vivo. (a) Xenograft tumors. (b) Faster growth of xenograft tumors from HOXA-AS3 cells vs. tumors from Lv-NC cells. (c) Xenograft tumor weight. (d) Detection of HOXA-AS3 expression in xenograft tumors. (e) Overexpressed HOXA-AS3 notably contributes to PD-L1 and Ki-67 levels in tumors vs. the negative control group (bar = 50 μm). (f) Upregulation of HOXA-AS3 contributes to tumor metastasis in vivo. Typical microscopic and macroscopic images of lungs (H&E staining). (g) Upregulation of HOXA-AS3 represses apoptosis (bar = 20 μm). **P < 0.01 and ***P < 0.001.
Figure 6: HOXA-AS3 modulates HCC cell proliferation and invasion via miR-455-5p/PD-L1 axis. (a) EdU (bar = 100 μm), and (b) colony formation assays assessing the proliferation and colony formation of HuH-7 and Hep3B cells. (c) Images of HuH-7 and Hep3B cells by invasion assay (bar = 100 μm). Data are reported as the mean ± SD of three separate experiments; **P < 0.01 and ***P < 0.001.
suppressed compared to that in cells cotransfected with PD-L1 MUT 3′-UTR and miR-455-5p mimic (Figure 4(h)). HEK293T cells cotransfected with HOXA-AS3 WT 3′-UTR and miR-455-5p mimic significantly inhibited luciferase activity, while cotransfecting mutant-type PD-L1 and miR-455-5p mimics did not affect the luciferase activity (Figure 4(i)).

Subsequently, we explored whether the modulation of PD-L1 expression by HOXA-AS3 in HCC cells was dependent on miR-455-5p expression. The transfection efficiency of miR-455-5p mimics and inhibitors is shown in Figure 4(j). In HCC cells, PD-L1 expression was upregulated by downregulating miR-455-5p (Figure 4(k)). Additionally, the inhibition of PD-L1 expression induced by HOXA-AS3 shRNA was reversed by the inhibition of miR-455-5p expression (Figure 4(k)). Thereafter, we synthesized a plasmid (HOXA-AS3 WT) that overexpressed a fragment of HOXA-AS3 containing the MRE for miR-455-5p to analyze the effects of HOXA-AS3 on PD-L1 expression in cells with or without miR-455-5p. HOXA-AS3 WT reversed the miR-455-5p mimic-induced suppression of PD-L1 expression (Figure 4(l)), indicating that HOXA-AS3 served as a sponge for miR-455-5p. In other words, when HOXA-AS3 binds to the 3′-UTR of miR-455-5p, less miR-455-5p can bind to PD-L1, thus suppressing its translation. It is assumed that this is the mechanism underlying the effect of HOXA-AS3 on PD-L1 expression.

3.4. Increase in HOXA-AS3 Expression Promoted Tumor Growth and Metastasis. We further explored whether higher HOXA-AS3 expression contributed to tumor growth in vivo. Xenograft tumor growth increased with the overexpression of HOXA-AS3 (Figure 5(a)). Furthermore, the mean weight and volume of xenograft tumors in the HOXA-AS3 overexpression group were greater than that in the Lv-NC group (Figures 5(b) and 5(c)). Next, HOXA-AS3 expression in xenograft tissues was analyzed (Figure 5(d)). IHC analysis indicated that the HOXA-AS3 overexpression group also had higher Ki-67 and PD-L1 expression than the Lv-NC group (Figure 5(e)). Additionally, in the in vivo lung metastasis model, overexpression of HOXA-AS3 considerably enhanced lung metastasis (Figure 5(f)). Finally, TUNEL staining revealed that cell apoptosis was remarkably repressed by overexpression of HOXA-AS3 (Figure 5(g)).

3.5. HOXA-AS3 Regulated the Proliferation, Invasion, Apoptosis, and Cell Cycle of HCC Cells via the miR-455-5p/PD-L1 Axis. To confirm the roles of the HOXA-AS3/miR-455-5p/PD-L1 axis in HCC, rescue experiments were performed in HuH-7 and Hep3B cells. HuH-7 cells were transfected with the NC vector or PD-L1 overexpression vector, and Hep3B cells were transfected with si-PD-L1 or si-NC (Figure S1A). We then conducted colony formation, EdU, transwell invasion, apoptosis, and cell cycle assays, which suggested that overexpression of both anti-miR-455-5p and PD-L1 reversed the effects of sh-HOXA-AS3 in HuH-7 cells (Figures 6(a)–6(c), Figure S1B, 1C). Furthermore, miR-455-5p mimics and si-PD-L1 reversed the impact of HOXA-AS3 overexpression in Hep3B cells (Figures 6(a)–6(c), Figure S1B, 1C).

4. Discussion

Despite rapid development in strategies for the early diagnosis and treatment of HCC, the majority of patients develop metastasis and chemical resistance [20, 21]. To improve the prognosis of HCC patients, it is crucial to discover new therapeutic targets and improve our understanding of the pathways associated with cancer occurrence and progression [22, 23]. IncRNAs have been recently shown to have vital effects on the progression of various tumors, including HCC [24, 25]. Therefore, in this study, a dataset from TCGA was used to analyze HCC-related IncRNAs. HOXA-AS3 was chosen as the subject, and its expression was evaluated in HCC cells.

Our assays indicated that HOXA-AS3 contributed to cell proliferation and invasion and repressed apoptosis to a certain extent. In addition, overexpressed HOXA-AS3 was shown to enhance the cell cycle transition from G1 to S phase. Moreover, the high HOXA-AS3 expression group had shorter overall survival than the low HOXA-AS3 expression group. These results suggest that HOXA-AS3 is a candidate biomarker for the prognosis of patients with HCC and that it may promote HCC progression.

Tumor cells are known to alter T cell activities to avoid antitumor immune responses and ensure their survival [26, 27]. Importantly, tumor cells interact with and induce apoptosis of CD8+ T cells to contribute to tumor growth and metastasis, as shown in previous studies [28, 29]. Additionally, strong evidence from several studies has revealed that blocking PD-1/PD-L1 by neutralizing PD-1 or PD-L1 can abrogate the immune evasion of tumor cells and activate CD8+ T cells [30–32], indicating that antibodies against PD-1 and PD-L1 are effective clinical immunotherapies for cancers [33–35]. Studies have been conducted on the addition of anti-PD-L1 and anti-PD-1 antibodies to lymphoma immunotherapy [36, 37]. In other studies, PD-L1 knockdown was shown to suppress cell proliferation and invasion in head and neck cancer [38], ovarian cancer [39], and breast cancer [40]. Zhou et al. [41] revealed that LINC00473, acting as a sponge of miRNA-195-5p, drove the development of pancreatic cancer by increasing PD-L1 expression. In this study, PD-L1 expression was higher in HCC cells than in normal liver cells. Overexpression of PD-L1 reversed the effects on HCC cell proliferation, invasion, and colony formation caused by HOXA-AS3 shRNA.

Nevertheless, this study had several limitations. Future studies should investigate whether HOXA-AS3/miR-455-5p alters the activity of CD8+ T cells via the PD-1/PD-L1 axis and whether targeting HOXA-AS3 can increase the effectiveness of HCC immunotherapies based on PD-1/PD-L1 blockade.

5. Conclusions

Collectively, the results of this study revealed that HOXA-AS3 expression was strikingly increased in HCC cells and that HOXA-AS3 regulated PD-L1 expression by sponging miR-455-5p. Additionally, overexpressed HOXA-AS3
contributed to HCC cell invasion and proliferation by targeting the miR-455-5p/PD-L1 axis. Therefore, HOXA-AS3 may serve as a new target for HCC treatment and as a candidate biomarker for prognosis.

Data Availability

The data used to support the findings of this study are included within the article.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

Authors’ Contributions

Cheng Zeng, Shaojun Ye, and Yu Chen contributed equally to this work.

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Supplementary Materials

See Figure S1 in the Supplementary Material for comprehensive image analysis. (Supplementary Materials)

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