Bidirectional interaction of lncRNA AFAP1-AS1 and CRKL accelerates the proliferative and metastatic abilities of hepatocarcinoma cells

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ABSTRACT
Actin filament-associated protein 1 antisense RNA 1 (AFAP1-AS1), a long non-coding RNA transcribed from the antisense strand of protein coding gene AFAP1, has attracted attention in cancer research. Despite, its biological function and regulatory mechanism in hepatocellular carcinoma still unknown. The present study revealed AFAP1-AS1 mediated hepatocarcinoma progression through targeting CRKL. The bidirectional interaction of AFAP1-AS1 and oncogenic protein CRKL, and the deregulation of AFAP1-AS1 effects on Ras, MEK and c-Jun activities were investigated in depth. AFAP1-AS1 was upregulated in surgical tumorous tissues from hepatocarcinoma patients compared with the paired paracancerous non-tumor liver tissues, and in hepatocarcinoma Huh7, HCCLM3 and HepG2 cell lines compared with LO2, a normal liver cell line. AFAP1-AS1 knockdown noticeably suppressed the proliferative, migratory and invasive properties, and the epithelial-mesenchymal transition (EMT) process of HepG2 and HCCLM3 through upregulating E-cadherin and downregulating N-cadherin and vimentin. CRKL knockdown reduced AFAP1-AS1 expression levels in HepG2 and HCCLM3 cells. AFAP1-AS1 suppression impaired CRKL expression in HepG2 and HCCLM3. AFAP1-AS1 level change was positively correlated with the expression level changes of Ras, MEK and c-Jun in mediating the invasiveness of hepatocarcinoma cells. Current work demonstrated AFAP1-AS1 to be an applicable progression indicator of hepatocarcinoma.
hepatocarcinoma. AFAP1-AS1 probably promotes the proliferation, EMT progression and metastasis of hepatocarcinoma cells via CRKL mediated Ras/MEK/c-Jun and cadherin/vimentin signaling pathways. AFAP1-AS1-CRKL bidirectional feedback signaling is worthy of further study on the monitoring, diagnosis and treatment of cancers.

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Introduction

Long non-coding RNAs (lncRNAs) are a group of RNA transcriptional products more than 200 bp long that lacking the potential to encode protein. lncRNA plays critical roles in cell proliferation, invasion, migration, metastasis and apoptosis [1,2]. lncRNA deregulation is involved in a number of cancers, for instance, the notable lncRNAs including H19, HOTAIR, MALAT1 and MEG3 participate in the progresses of gastric, liver, lung, breast and ovarian cancers [3–6].

Actin filament-associated protein 1 antisense RNA 1 (AFAP1-AS1) is composed of 6810 base pairs. It is transcribed from the antisense strand of AFAP1 gene located at 4p16.1. AFAP1 is more reported as an adapter protein participating in various signaling pathways through Src kinase family. There are complementary regions between exon 2 of AFAP1-AS1 with the 14th, 15th and 16th exons of AFAP1 protein coding gene [7–9]. AFAP1-AS1 participates in cell proliferation, migration, invasion and apoptosis. It is commonly reported as an oncogene in the cancers of lung [7], esophagus [10], pancreas [11], stomach [12], colon [13], gall bladder [14] and bile duct [15]. As rarely studied in hepatocarcinoma, the biological function with the underlying molecular mechanism of AFAP1-AS1 in hepatocarcinoma remains elusive.

CRKL (Crk-Like protein), is generally located in various multicellular compartments and maps to chromosome 22q11.21 that encodes a 36 kDa protein [16]. CRKL, comprised of SH2, SH3N and SH3C domains, binds to the upstream molecules e.g. p130CAS, paxillin, CBL, GAB1 via its SH2 domain and mediates protein interaction with downstream signaling molecules like C3G and DOCK180 [17]. CRKL, as an adapter protein, participates in various signaling pathways and contributes in the progression of a variety of cancers [18–21]. CRKL is an important key substrate of BCR-ABL in hematopoietic cancer where constitutively being phosphorylated and promotes abnormal proliferation of blood cells of leukemia patients [22,23]. CRKL is also a significant mediator of EMT of gastric and ovarian carcinoma [24,25]. CRKL is upregulated in many cancers exhibiting various biological processes including cellular proliferation, invasion and metastasis [24–27]. In addition, our laboratory also investigated that CRKL was associated with tumor progression and development in murine hepatocarcinoma Hca-P [26,28], and CRKL upregulation induced the increases of hepatocarcinoma cell malignant behaviours could be suppressed by miR–429, is a direct binding miRNA of CRKL [29]. We also found CRKL upregulation potentially promotes the development and progression of chronic myeloid leukemia (CML) patients (unpublished). More interestingly, CRKL knockdown in K562, a CML cell line, resulted in the downregulation of AFAP1-AS1 (data was provided in the supplementary Table 2). These implicated the correlation of AFAP1-AS1 with CRKL in tumorigenesis. Here, we proposed AFAP1-AS1 playing an important role with CRKL in hepatocarcinoma progression.

Epithelial to mesenchymal transition (EMT) is a common process in cancer development and progression. Throughout EMT, epithelial features are faded and mesenchymal properties are promoted that in turn enhance cell mobility, facilitate metastasis, invasion and tumor progression. Epithelial cells lose their polarity, modify inter-cellular adhesion, reorganize the cytoskeleton, and permit the cells migration. In metastasis, E-cadherin, N-cadherin and vimentin are essential role in promotion of cancer and EMT induction [13]. Our previous work in hepatic cancer also reported the miR-429/CRKL mediated migration invasion through EMT regulators [29]. In present study role of AFAP1-AS1-mediated CRKL was investigated in hepatocarcinoma metastasis via regulating EMT.

The present research exhibited the overexpression of AFAP1-AS1 in hepatocarcinoma tissues and cells in comparison with non-tumor tissues and cell lines. Our research focused the bidirectional positive correlation of AFAP1-AS1 and CRKL in regulating HCC metastasis. The knockdown of AFAP1-AS1 was accompanied by decreasing proliferation, migration and invasion through the regulation mechanism of CRKL.

Material and methods

Cancer cell line culture

Three hepatocarcinoma cell lines Huh7, HepG2 and HCCLM3, and one normal liver cell line LO2 were obtained from Chinese Academy of Sciences (Shanghai, China). LO2, Huh7 (low metastasis) and HCCLM3 (high metastasis) were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Gibco, USA) containing 10% fetal bovine serum (ExCell Bio, China), while RPMI-1640 medium (Gibco, USA) was used to grow HepG2 cell line, containing 15% fetal bovine serum (ExCell Bio, China), in the presence of streptomycin and penicillin 100 U/ml (Gibco, USA), at 37 °C under 5% CO₂.

Transfection of siRNA

The sequence of small interfering RNA (siRNA) 5’-CTATCTGGT CAAACGATT-3’ for AFAP1-AS1 was synthesized by GenePharma (Shanghai, China). 5’TCTCGAACGTGTCAC CT-3’ was designed as the negative control siRNA (siRNA-NC) having no homologous sequence with target gene. The sequence of siRNA was an effective knockdown sequence for AFAP1-AS1. The siRNAs were prepared in diethylpyrocarbonate (DEPEC) H₂O. 1 ml of HepG2 and HCCLM3 cells with density 2×10⁵ per ml were inoculated in six well plates for 12 h. When cells growth reaches 70–75%, then 5 μl siRNA with concentration of 20 μM in serum free medium, was transfected using 5 μl lipofectamine™ 2000 reagent (Invitrogen, USA), incubated at room temperature for 20 min, then 100 μl of mixture used for transfection. Transfected cells were incubated at 37 °C, 5% CO₂ for 24, 48 and 72 h.

Clinical tissue samples

The tumorous tissues with paired paracancerous non-tumour liver tissues from 17 patients (11 male, 6 female; 8 patients ≥ 60 y, 9 patients < 60 y) including 12 of them measured with CRKL level analysis data in our previous work [29], were used for comparative analysis. All human HCC tissue specimens and their corresponding normal liver tissue samples were acquired from the Second Affiliated Hospital of Dalian Medical University, China.
Patients with no history of exposure to anticancer treatment before operation. Tissues were obtained with signed informed consent from patients and approved from Ethical Committee of Dalian Medical University with approval number 2019–014. Patient’s clinical data was collected as hospital’s medical record numbers. Tissue samples were frozen in liquid nitrogen and preserved at −80 °C for further experiments. Analytical protocols were carried out by approved guidelines.

Cell proliferation and clonogenic growth assays

Following transfection of si-NC and si-AFAP1-AS1, 100 µl medium containing 3000 cells of HepG2 and HCCLM3 were incubated in 96 well plates. RPMI-1640 with 15% FBS and DMEM containing 10% FBS was used for HepG2 and HCCLM3. All plates from each cell’s group for 24, 48, 72 and 96 h of independent time period were then incubated at 37 °C, 5% CO2. On respective day, plates were incubated for 4 h after adding 200 µl MTT (5 mg/ml) solutions in darkness. Then, MTT reagent was replaced by 150 µl dimethyl sulphoxide (DMSO) at room temperature, the absorbance at 450 nm was immediately detected using micro plate reader (Thermo, USA). All experiments were confirmed in triplicates and three times independently. Clonogenic growth assay was conducted for determination of AFAP1-AS1 dysregulation effect on colony-forming abilities of HepG2 and HCCLM3. Following the transfection of si-NC and si-AFAP1-AS1 at 48 h, 1000 of each group HepG2 and HCCLM3 cells were inoculated in 2 ml of RPMI-1640 with 15% FBS and DMEM with 10% FBS, loaded into six-well plates incubating at 37 °C under 5% CO2 for 9 d until colonies were visible. Having been fixed in methanol, the colonies were stained with crystal violet (0.5%), and counted manually after taking photographs. Experiment was confirmed in triplicate and three times independently.

Wound healing assay

1 ml of both HepG2 and HCCLM3 cells, with density of 2 × 10^5 cells per ml, were inoculated into each well of six-well plates for each group and transfected with si-NC and si-AFAP1-AS1 as per protocol mentioned earlier. Cells were allowed to grow until they reach 85–90% confluence, then cells monolayer was scratched vertically with 200 µl sterile pipettes. Then 2 ml of respective fresh medium was added after removing detached and floated cells by 1 ml of PBS washing. Once the scratch had been generated then plates were incubated at 37 °C under 5% CO2 for 48 h. Wound recovered gap was measured for si-AFAP1-AS1 transfected cells against si-NC using ImageJ software. Six random fields around wound gap were captured at 0, 24 and 48 h by using microscope (Olympus, Japan) with magnification 10×.

Transwell chamber assays

Transwell chambers (Corning, USA), with 8 µm of pore size, were employed to assess migratory and invasive capabilities of hepatocarcinoma cells. Additionally, ECM (extracellular matrix, Sigma, USA) with dilution 1:50 in serum-free medium was pre-coated in chambers for invasion experiment. After the transfections of si-AFAP1-AS1 and si-NC, 10,000 cells from each group were suspended in 1 ml media with FBS, DMEM for HCCLM3 and RPMI-1640 for HepG2. Then, 200 µl of both calculated cells were charged up into upper chamber of the unit and mounted on top of each well of a 24-well plate. Subsequently, 600 µl medium with 20% FBS, acting as cell’s chemo attractants, was added into each of the respective bottom chamber, then incubated for 48 h at 37 °C with 5% CO2. The migrated or invaded cells on lower surface of filter were subjected for 0.5% crystal violet staining after fixing in absolute methanol. Then five fields of lower sides of chambers were captured using upright light microscope (Olympus, Japan), with 20 × of magnification.

RNA extraction and quantitative real-time-PCR analysis

Total RNAs were extracted from tissues and cells (group of cells include LO2, Huh7, HepG2, HCCLM3, si-AFAP1-AS1-transfected HepG2 and HCCLM3, si-CRKL-transfected HepG2 and HCCLM3, si-NC-transfected HepG2 and HCCLM3, moreover CRKL overexpressed group of cells, utilized in our published work [29], such as HepG2-PCDH-CRKL, HepG2-PCDH-control, HCCLM3-PCDH-CRKL, HCCLM3-PCDH-Control using TrizolTM (Invitrogen, USA) reagent and quantity of RNA measured by NanoDrop 2000 spectrometer (Thermo, USA). Total RNA, using 1 µl of RNA (0.4–1.2 µg), was transcribed into cDNA by PrimeScript™ RT Kit (Takara, Japan), in 20 µl of final volume. qRT-PCR was performed using SYBR Green FastStart ROX (Roche, USA) by StepOne™ 7300 Plus Applied Biosystems (Life, USA). Primers were designed by oligo7 software. The following primers were synthesized: AFAP1-AS1-F, 5'-CCCTGGATATTCTCTCTACCC-3', AFAP1-AS1-R, 5'-TGCTCTTCATGATCTCCG-3'; CRKL-F, 5'-CTTATGACAAGACTGCTT-3'; CRKL-R, 5'-CAGTCTTCTTCCATGCTTT-3'; ACTB-F, 5'-AGGCCAACCCTGACAGG-3'; ACTB-R, 5'-ACGCTTGGATGCAACCTGTA-3'. ACTB (β-actin) was used as endogenous control to compare the expression change of targeted gene. Comparative cycle threshold 2−ΔΔCT method was applied for the calculation of relative levels of the investigated molecules.

Western blotting assay

HepG2 and HCCLM3 transfected cells, including si-AFAP1-AS1 and si-NC, were harvested for total protein extraction. Lysis of cells was carried out by RIPA buffer (50 mM Tris-HCl, 150 mM NaCl, 0.1% Triton X-100, 0.1% SDS, 0.5% sodium deoxycholate), and 0.5 mM PMSF, 1 mM Na2VO4 and 1 µg/ml leupeptin were added supplementarily as cocktail buffer. The supernatant protein samples were collected by centrifuging lysates with 12000 rpm for 15 min at 4 °C. The protein concentration was measured using Bradford assay and adjusted to 3–4 µg/µl. Separation of protein was carried out by 10% SDS-PAGE. Electrophoretic transfer of protein was accomplished on nitrocellulose (Millipore, USA) membrane, and incubated in 5% skim milk (BD, USA) in TBS buffer for 2 h at room temperature. Overnight incubation of nitrocellulose membranes at 4 °C with primary antibodies, including CRKL (1:2000, Genex, USA), p-CRKL (1:1500, cell signal, USA), Cas (1:500, Cell Signal, USA), p-Cas (1:500, Cell Signal, USA), c-Jun (1:500, Abbkine, USA), p-c-Jun (1:500, Abbkine, USA), MEK (1:500, Cell Signal, USA), p-MEK (1:500, Cell Signal, USA), Vimentin (1:1000, Bioss, USA), E-cadherin (1:1000, Bioss, USA), N-cadherin (1:1000, Bioss, USA) and GAPDH (1:4000, Proteintech, USA). After washing for 3 × 10 min with TBST buffer (pH 7.5; 100 mM NaCl, 50 mM Tris, 0.1% Tween-20), membrane was incubated with secondary conjugated antibody at room temperature for 2 h, and washed with TBST for 3 × 10 min. The protein bands were visualized using the enhanced chemiluminescence detection kit (ECL, Advansta, USA) and quantified using the ChemiDoc™ MP system (Bio-Rad, USA).

Immunofluorescence assay

1 ml of HepG2 cells with 2 × 10^5/ml of density, used for si-NC and si-AFAP1-AS1 transfection as per protocol mentioned earlier. After 48 h of siRNA treatment targeting AFAP1-AS1 and si-NC, HepG2 cells were fixed with 200 µl of 4% formaldehyde for 20 min at room temperature, penetrated with 100 µl of 0.1% Triton X-100 for 10 min and blocked with 300 µl of 0.3% BSA for one hour
at room temperature. CRKL primary antibody (1:500, Genex, USA) was incubated in dark at 4 °C overnight. After cells were washed with 500 μl PBS three times, they were incubated with secondary antibody for one hour in dark at room temperature. Finally, 200 μl of 4', 6-diamidino-2-phenylindole (DAPI), with 10 μg/ml of concentration, was subjected to stain HepG2 cells for 10 min at room temperature in dark. Images were captured in upright light microscope (Olympus, Japan) with 20 × magnification.

Data analysis

Graph pad prism 6.0 (La Jolla, USA) was used to analyze the data applying “student t-test”. The data was prepared as mean ± standard deviation for at least triplicate experiments shown in supplementary Table S3. *, **, *** refer p < 0.05, 0.01 and 0.001.

Results

AFAP1-AS1 is overexpressed in hepatocarcinoma patients’ tumorous tissues and cell lines

qRT-PCR assay showed AFAP1-AS1 was overexpressed in hepatocarcinoma patients’ tumorous tissues and hepatocarcinoma cells. In comparison with paired paracancerous non-tumour liver tissues, AFAP1-AS1 was overexpressed in the surgical tumorous tissues from hepatocarcinoma patients (Fig. 1A, p < 0.0001). Our previous work indicated CRKL was also significantly overexpressed in the tumorous tissues of the same cohort of hepatocarcinoma patients as in current work [29]. Data analysis resulted in a strong linear positive correlation between AFAP1-AS1 overexpression and CRKL overexpression in hepatocarcinoma tumorous tissues (Fig. 1B, r = 0.98, n = 13, p < 0.0001). Concordantly, AFAP1-AS1

Fig. 1. AFAP1-AS1 expression levels in hepatocarcinoma patients’ tumorous tissues and cell lines. A. The overall AFAP1-AS1 level was significantly increased in tumorous tissues compared with paired paracancerous non-tumour liver tissues (n = 17). B. AFAP1-AS1 overexpression was positively correlated with CRKL overexpression in patients’ tumorous tissues (p = 0.0001, r = 0.98, n = 13). C. Compared with LO2, AFAP1-AS1 levels were overexpressed in Huh7, HCCLM3 and HepG2 cells. β-Actin was the internal standard. *p < 0.05, **p < 0.01, ***p < 0.001.
was higher expressed by 21% \((p < 0.05)\), 45% \((p < 0.001)\) and 78% \((p < 0.01)\) in Huh7, HCCLM3 and HepG2 cells, comparing with LO2 cells (Fig. 1C). These suggested AFAP1-AS1 upregulation positively correlated with CRKL upregulation probably promoted clinical progression of hepatocarcinoma patients through enhancing the malignant behaviours of hepatocarcinoma cells.

**AFAP1-AS1 knockdown suppressed hepatocarcinoma cells growth**

Knockdown of AFAP1-AS1 decreased the proliferation and colony-forming capacities of HCCLM3 and HepG2. Compared with si-NC-transfected HCCLM3 and HepG2, as in Fig. 2A, AFAP1-AS1 level was decreased by 84% \((p < 0.01)\) and 55% \((p < 0.05)\) in siRNA-transfected HepG2 at 48 and 72 h, and decreased by 18% \((p < 0.05)\), 78% \((p < 0.001)\) and 51% \((p < 0.01)\) in siRNA-transfected HCCLM3 at 24, 48 and 72 h. Compared with corresponding si-NC, proliferation of si-AFAP1-AS1-transduced HepG2 cells was declined by 15% \((p < 0.01)\), 26% \((p < 0.001)\) and 29% \((p < 0.01)\), and that of si-AFAP1-AS1-transfected HCCLM3 was declined by 11% \((p < 0.001)\), 12% \((p < 0.05)\) and 15% \((p < 0.01)\) at 48, 72 and 96 h (Fig. 2B). Consistently, the relative colony forming capacities of si-AFAP1-AS1-transfected HepG2 and HCCLM3 cells

![Fig. 2. The effect AFAP1-AS1 knockdown on the growths of hepatocarcinoma cells. A. Relative expression levels of AFAP1-AS1 in HepG2 and HCCLM3 cells with the si-RNA interference for 24, 48 and 72 h. B. AFAP1-AS1 knockdown on the proliferations of HepG2 and HCCLM3 by MTT assay. C. Clonogenic growth assay of AFAP1-AS1 knockdown on the colony-forming capacities of HepG2 and HCCLM3. *p < 0.05, **p < 0.01, ***p < 0.001.](image-url)
decreased by 45% \((p < 0.01)\) in 55% \((p < 0.01)\) (Fig. 2C) following AFAP1-AS1 knockdown.

**Silencing of AFAP1-AS1 reduced migratory and invasive abilities of hepatocellular carcinoma**

Number of migrated cells in siRNA-transfected HepG2 decreased by 50% \((p < 0.001)\), and in siRNA-transfected HCCLM3 by 39% \((p < 0.001)\) comparing with siRNA control. Similarly, invaded cells of HepG2 and HCCLM3 after siRNA transfections were reduced by 50% \((p < 0.001)\) and 40% \((p < 0.001, \text{Fig. 3A})\). Additional, comparing with si-NC, wound scratch assay disclosed that the motility abilities of siRNA-transfected HepG2 and HCCLM3 cells were suppressed by 50% \((p < 0.01)\) and 61% \((p < 0.001, \text{Fig. 3B})\).

**Bidirectional interaction of AFAP1-AS1 with CRKL**

Our gene microarray data (provided in supplementary Table S2) showed AFAP1-AS1 was downregulated among the deregulated lncRNAs in leukemia K562 cells following CRKL knockdown. RPseq bioinformatics' analysis (http://pridb.gdcb.iastate.edu/RPSeq/references.php) predicated a potential interaction between AFAP1-AS1 and CRKL. These suggested a correlation between the deregulations of AFAP1-AS1 and CRKL in tumorigenesis. Herein, following the knockdown of CRKL by si-CRKL interference, qRT-PCR assay demonstrated AFAP1-AS1 expressions decreased by 17% \((p < 0.001)\) in HepG2 and 33% \((p < 0.01)\) in HCCLM3 (Fig. 4A). Comparing with si-NC, CRKL mRNA levels were reduced by 31% \((p < 0.05)\) and 68% \((p < 0.001)\) in si-AFAP1-AS1-transcted HepG2, and decreased by 13% \((\text{ns})\) and 41% \((p < 0.05)\) in si-AFAP1-AS1-transcted HCCLM3 at 24 and 48 h (Fig. 4B). In comparison with HepG2- and HCCLM3-PCDH-control, AFAP1-AS1 expressions in HepG2-PCDH-CRKL and HCCLM3-PCDH-CRKL were increased by 470% \((p < 0.001)\) and 360% \((p < 0.001)\), respectively (Fig. 4C). Concordantly, CRKL protein levels were decreased in siRNA-transfected HepG2 by 20% \((\text{ns})\) and 48% \((p < 0.01)\) and in siRNA-transfected HCCLM3 by 15% \((\text{ns})\) and 73% \((p < 0.01)\) (Fig. 4D). Moreover, p-CRKL protein levels were suppressed by 52% \((p < 0.01)\) and 88% \((p < 0.001)\) in siRNA-transfected HepG2 and HCCLM3 cells at 48 h (Fig. 4E). Immunofluorescence staining assay also clearly indicated that CRKL was much lower expressed and poorer distributed in siRNA-transfected HepG2 cells than si-NC-transfected cells (Fig. 4F).

**AFAP1-AS1 affects Ras/MEK/c-Jun and regulates epithelial to mesenchymal transition (EMT)**

AFAP1-AS1 silencing by siRNA interference causing decreased levels of Ras, p-Ras, MEK, p-MEK, c-Jun and p-c-Jun by 76% \((p < 0.05)\), 73% \((p < 0.001)\), 72% \((p < 0.05)\), 76% \((p < 0.01)\), 63% \((p < 0.001)\) and 77% \((p < 0.001)\) in HepG2 and by 72% \((p < 0.05)\), 81% \((p < 0.01)\), 42% \((p < 0.05)\), 70% \((p < 0.01)\), 37% \((p < 0.001)\) and 41% \((p < 0.01)\) in HCCLM3 cells (Fig. 5A). The levels of EMT markers, N-cadherin, E-cadherin and vimentin, were measured in siRNA-transfected HepG2 and HCCLM3 cells. Comparing with si-NC group, N-cadherin and vimentin were decreased by 47%

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**Fig. 3.** AFAP1-AS1 knockdown suppressed migration and invasion of hepatocarcinoma cells. A. Transwell chamber assays of AFAP1-AS1 knockdown by si-RNA transfection on the migration and invasion abilities of in HepG2 and HCCLM3. B. Wound healing assays of AFAP1-AS1 knockdown on the motility capacities of HepG2 and HCCLM3. *p < 0.01 and **p < 0.001.
(p < 0.05) and 71% (p < 0.01) in siRNA-transfected HepG2, and decreased by 78% (p < 0.001) and 30% (p < 0.05) in siRNA-transfected HCCLM3 (Fig. 5B). In contrast, although E-cadherin upregulation in siRNA-transfected HepG2 is out of statistical significance, its expression showed a dramatical increase of 1350% (p < 0.01) in siRNA-transfected HCCLM3 (Fig. 5B).
LncRNA AFAP1-AS1 (NR-026892; NCBI database, chromosomal location 4p16.1) is originally derived from the antisense strand of AFAP1 protein [9]. Commonly, AFAP1-AS1 upregulation plays crucial functions in the progressions of esophageal carcinoma [10], stomach carcinoma [12], colon carcinoma [13], gall bladder carcinoma [15] and cholangiocarcinoma [14]. Since very limited number of studies were carried out in HCC progression regarding AFAP1-AS1. The linkage of AFAP1-AS1 in hepatocarcinoma has not been established. Previously, we reported CRKL playing important role in hepatocarcinoma tumorigenesis [29]. While, the association of AFAP1-AS1 with CRKL in hepatocarcinoma was not reported. Using RPSeq tool (http://pridb.gdcb.iastate.edu/RPSeq), we predicted the potential positive interaction between AFAP1-AS1 and CRKL, as their RNA-protein binding sequences provided in supplementary Table S1. Moreover, our unpublished microarray data (supplementary Table S2) showed CRKL knockdown downregulated AFAP1-AS1 in K562 cells. These evidences suggested the potential molecular link of AFAP1-AS1 with CRKL and their regulation mechanism in hepatocarcinoma malignancy. First, we found the overall AFAP1-AS1 levels were both increased in tumorous tissues from hepatocarcinoma patients (Fig. 1A) and in human hepatocarcinoma HepG2, HCCLM3 and Huh7 cell lines (Fig. 1C), which suggested that AFAP1-AS1 overexpression contributed to hepatocarcinoma cell’s malignancy and clinical progression of hepatocarcinoma patients. These findings are consistent with previous study that AFAP1-AS1 acting as significant prognosis biomarker in cholangiocarcinoma progression [15]. Then, our data resulted in a linear positive correlation (r = 0.98, n = 13, Fig. 1B) between the overexpressions of AFAP1-AS1 and CRKL in patients’ tumorous tissues. These suggest that acting as an oncogenic lncRNA, the overexpression of AFAP1-AS1 positively correlated CRKL overexpression contributed to the carcinogenesis in hepatocarcinoma.

AFAP1-AS1 knockdown suppressed the in vitro malignant behaviours of hepatocarcinoma cells significantly. After siRNA transfection targeting AFAP1-AS1 in HepG2 and HCCLM3, the endogenous AFAP1-AS1 level was significantly reduced (Fig. 2A). The relative proliferative (Fig. 2B), colony forming (Fig. 2C), migratory, invasive (Fig. 3A) and motional (Fig. 3B) capacities of HepG2 and HCCLM3 were significantly suppressed in responding to the knockdown of AFAP1-AS1, which were consistent with the studies that AFAP1-AS1 performed oncogenic function by enhancing the proliferation and metastasis of gastric cancer cells through PTEN/p-AKT pathway [30,31]. Our results showed AFAP1-AS1 playing as an oncogenic role in hepatocarcinoma tumorigenesis.

Our lncRNA microarray data (Table S2) showed AFAP1-AS1 was downregulated to CRKL knockdown in leukemia K562 cells. Here, we also found AFAP1-AS1 and CRKL levels were positively

![Figure 5. AFAP1-AS1 mediates the migration and invasion of hepatocarcinoma cells via Ras/MEK/c-Jun-EMT pathway. A. Silencing of AFAP1-AS1 resulted in decreased levels of Ras, MEK and c-Jun, in HepG2 and HCCLM3. B. AFAP1-AS1 knockdown decreased expression levels of N-cadherin and vimentin, and increased expression of E-cadherin in HepG2 and HCCLM3 cells. *p < 0.05, **p < 0.01, ***p < 0.001. ns refers to no statistical significance.](image-url)
correlated in hepatocarcinoma tumorous tissues and cells (Fig. 1). First, AFAP1-AS1 was decreased in si-CRKL-transfected HepG2 and HCCLM3 (Fig. 4A), while was increased in CRKL overexpressing HepG2-PCDH-CRKL and HCCLM3-PCDH-CRKL cells (Fig. 4C). Second, AFAP1-AS1 knockdown resulted in apparent reduced CRKL at both mRNA (Fig. 4B) and protein (Fig. 4D) levels, and decreased protein expression of p-CRKL (Fig. 4E) in HepG2 and HCCLM3 cells. Previously, we reported that CRKL upregulation promoted the in vitro proliferative, migratory and metastatic capacities of HepG2 cell [29]. Therefore, current work further showed a positive correlation and a bidirectional interaction between AFAP1-AS1 and CRKL in hepatocarcinoma.

For the first time, current work showed AFAP1-AS1-CRKL axis influenced the malignant behaviours of hepatocarcinoma cells through Ras/MEK/c-Jun pathway. The levels of Ras, MEK and c-Jun were depleted apparently in si-AFAP1-AS1 transfected HepG2 and HCCLM3 (Fig. 5A). Our previous study showed CRKL promoted HepG2 malignancy through Raf/MEK/ERK [29]. The results suggested a new AFAP1-AS1 upregulation mediated signal transduction in enhancing hepatocarcinoma through activating Ras/MEK/c-Jun pathway.

EMT is a fundamental transformation process in cancer metastasis [32,33]. AFAP1 deregulation was involved in the EMT of gastric and colorectal cancers [13,34]. AFAP1-AS1 affected EMT in hepatocarcinoma progression. Its knockdown resulted in downregulated N-cadherin and vimentin and upregulated E-cadherin in HepG2 and HCCLM3 (Fig. 5B). Previously, we reported CRKL was involved in miR-429 mediated EMT process of HepG2 cells [29]. Clearly, the upregulations of AFAP1-AS1 and CRKL potentially contributed in hepatocarcinoma malignancy by enhancing the EMT of cancer cells.

Current work for the first time linked the crosstalk of Ras-, MEK/ERK- and EMT-related signaling transduction pathways in hepatocarcinoma. AFAP1-AS1 knockdown decreased the migratory and invasive capacities of pancreatic cancer and osteosarcoma by downregulating N-cadherin, vimentin and upregulating E-cadherin [35,36]. AFAP1-AS1 promoted pancreatic cancer progression through IGF1R-mediated MEK/ERK signaling pathway [37]. The deregulation of CRKL played important roles in regulating the progressions of leukemia, lung cancer and ovarian cancer via Ras, MEK and c-Jun signaling pathways [19,25,38,39]. By inhibiting E-cadherin, MEK-ERK signaling transduction regulated the EMT, invasion and metastasis of melanoma carcinoma [40]. c-Jun promoted the invasiveness of nasopharyngeal carcinoma [41] and the proliferation of lung cancer cells by inhibiting E-cadherin [42]. As schemed in Fig. 6, current work disclosed the role of AFAP1-AS1 together with interaction with CRKL and underlying mechanism in hepatocarcinoma malignancy. AFAP1-AS1 together with its interaction with CRKL promotes the proliferation, migration and invasion of cancer cells through CRKL-mediated Ras/MEK/c-Jun-EMT and EMT pathways.

Conclusion

AFAP1-AS1 as a critical oncogenic biomarker, is overexpressed in hepatocarcinoma tissues and cells. AFAP1-AS1 overexpression positively correlates with CRKL overexpression in hepatocarcinoma. AFAP1-AS1 promotes the proliferation, migration and invasion of cancer cells through CRKL-mediated Ras/MEK/c-Jun-EMT pathway. Our research introduces a new molecular path of AFAP1-AS1 via CRKL regulation in carcinogenesis. AFAP1-AS1-CRKL bidirectional interaction is proved to be important in regulating the malignancy, the diagnosis and treatments of hepatocarcinoma.

Declaration of Competing Interest

The authors declared that there is no conflict of interest.

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Author contributions

S. Abdul carried out experiments, data analysis and wrote manuscript draft. J. Wang and A. Majid assisted in experiments. Q. Liu provided hepatocarcinoma tissues. S. Liu and M-Z. Sun designed and guided the experiments, revised and approved the manuscript.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jare.2020.03.010.

References

[1] Yang Z, Li X, Yang Y, He Z, Qu X, Zhang Y. Long noncoding RNAs in the progression, metastasis, and prognosis of osteosarcoma. Cell Death Dis 2016;7 (9):e2389.
[2] Zhou X, Liu S, Cai G, Kong L, Zhang T, Ren Y, et al. Long non coding RNA MALAT1 promotes tumor growth and metastasis by inducing epithelial-mesenchymal transition in oral squamous cell carcinoma. Sci Rep 2015;5:15972.
[3] Zhou X, Ye F, Yin C, Zhuang Y, Yue G, Zhang G. The interaction between miR-141 and IncRNA-H19 in regulating cell proliferation and migration in gastric cancer. Cell Physiol Biochem 2015;36(4):1440–52.
[4] Tang S, Zheng K, Tang Y, Li Z, Zou T, Liu D. Overexpression of serum exosomal HOTAIR is correlated with poor survival and poor response to chemotherapy in breast cancer patients. J Biosci 2019;44(2).
[5] Zhu K, Ren Q, Zhao Y. IncRNA MALAT1 overexpression promotes proliferation, migration and invasion of gastric cancer by activating the PI3K/AKT pathway. Oncology Lett 2019;17(6):5335–42.
[6] Wang L, Yu M. IncRNA MEG3 modified epithelial-mesenchymal transition of ovarian cancer cells by sponging miR-219a-5p and regulating. EGFR 2019,
Baisden JM, Qian Y, Zot HM, Flynn DC. The actin filament-associated protein Crk-like adapter protein regulates CCL19/CCR7-mediated epithelial-to-mesenchymal transition via ERK signaling pathway in epithelial ovarian carcinomas. Med Oncol 2015;32(3):47.

Zeng Z, Bo H, Gong Z, Lian Y, Li X, Li X, et al. AFAP1-AS1, a long noncoding RNA upregulated in lung cancer and promotes invasion and metastasis. Tumour Biol 2016;37(1):729–37.

Bagdeen JM, Qian Y, Zot HM, Flynn DC. The actin filament-associated protein AFAP-110 is an adaptor protein that modulates changes in actin filament integrity. Oncogene 2001;20(44):6435–47.

He J, Wu K, Guo C, Zhou J, Pu W, Deng Y, et al. Long non-coding RNA AFAP1-AS1 plays an oncogenic role in promoting cell migration in non-small cell lung cancer. Cell Mol Life Sci 2018;75(4):4667–81.

Zhou X, Wang W, Zhu W, Yu C, Tao G, Wu Q, et al. High expression of long non-coding RNA AFAP1-AS1 predicts chemoradioresistance and poor prognosis in patients with esophageal squamous cell carcinoma treated with definitive chemoradiotherapy. Mol Carcinog 2016;55(12):2095–105.

Ye Y, Cheng J, Zhou Y, Fu Z, Zhou Q, Wang Y, et al. High expression of AFAP1-AS1 is associated with poor survival and short-term recurrence in pancreatic ductal adenocarcinoma. J Trans Med 2015;13:137.

Feng Y, Zhang Q, Wang J, Liu P. Increased lncRNA AFAP1-AS1 expression predicts poor prognosis and promotes malignant phenotypes in gastric cancer. Eur Rev Med Pharmacol Sci 2017;21(17):3842–5.

Han X, Wang L, Ning Y, Li H, Wang Z. Long non-coding RNA AFAP1-AS1 facilitates tumor growth and promotes metastasis in colorectal cancer. Biol Res 2016;49(1):36.

Ma F, Wang S, Cai Q, Zhang M, Yang Y, Ding J. Overexpression of LncRNA AFAP1-AS1 predicts poor prognosis and promotes cell proliferation and invasion in gallbladder cancer. Biomed Pharmacother 2016;84:1249–55.

Lu X, Zhou C, Li R, Deng Y, Zhao L, Zhai W. Long noncoding RNA AFAP1-AS1 promotes tumor growth and invasion in cholangiocarcinoma. Cell Physiol Biochem 2017;42(1):222–30.

ten Hoeve J, Morris C, Heisterkamp N, Groffen J. Isolation and chromosomal localization of CRKL, a human crrk-like gene. Oncogene 1993;8(9):2469–74.

Feller SM. Crk family adaptors-signalling complex formation and biological roles. Oncogene 2001;20(44):6348–71.

Wang J, Chen X, Li P, Su J, Yu B, Cai Q, et al. CRKL promotes cell proliferation in gastric cancer and is negatively regulated by miR-126. Chem Biol Interact 2013;206(2):230–8.

Cheung HW, Du J, Boehm JS, He F, Weir BA, Wang X, et al. Amplification of CRKL is associated with poor survival and short-term recurrence in pancreatic ductal adenocarcinoma. Med Oncol 2015;47:e165.

Tamura M, Sasaki Y, Kobashi K, Takeda K, Nakagaki T, Idogawa M, et al. CRKL oncogene is downregulated by p53 through miR-200s. Cancer Sci 2015;106(8):1033–40.

Jankowski W, Saleh T, Pai MT, Stram G, Birge RB, Kalodimos CG. Domain organization differences explain Bcr-Abl's preference for CrkL over CrkII. Nat Chem Biol 2012;8(6):590–6.

Ma F, Wang S, Cai Q, Zhang M, Yang Y, Ding J. Overexpression of LncRNA AFAP1-AS1 predicts poor prognosis and promotes cell proliferation and invasion in gallbladder cancer. Biomed Pharmacother 2016;84:1249–55.

Nicholls GL, Raines MA, Vera JC, Lacomas L, Tempst P, Golde DW. Identification of CRKL as the constitutively phosphorylated 39-kD tyrosine phosphoprotein in chronic myelogenous leukemia cells. Blood 1994;84(9):2912–8.

ten Hoeve J, Kaartinen V, Fioretos T, Haataja L, Voncken JW, Heisterkamp N, et al. Cellular interactions of CRKL, and SH2-SH3 adaptor protein. Cancer Res 1994;54(10):2563–7.

Han G, Wu D, Yang Y, Li Z, Zhang J, Li C. Crkl mediates CCL20/CCR6-induced EMT in gastric cancer. Cytokine 2015;76(2):163–9.

Cheng S, Guo J, Yang Q, Yang X. Crk-like adapter protein regulates CCL19/CCR7-mediated epithelial-to-mesenchymal transition via ERK signaling pathway in epithelial ovarian carcinomas. Med Oncol 2015;32(3):47.

Lin Q, Sun M-Z, Guo C, Shi J, Chen X, Liu S. CRKL overexpression suppresses in vitro proliferation, invasion and migration of murine hepatocarcinoma Hca-7 cells. Biomed Pharmacother 2015;69:11–7.

Cuo C, Liu S, Sun M-Z. The role of CT10 regulation of kinase-like in cancer. Future Oncol 2014;10(16):2687–97.

Shi J, Meng L, Sun M-Z, Guo C, Sun X, Lin Q, et al. CRKL knockdown promotes in vitro proliferation, migration and invasion, in vivo tumor malignancy and lymph node metastasis of murine hepatocarcinoma Hca-7 cells. Biomed Pharmacother 2015;71:84–90.

Guo C, Zhao D, Zhang Q, Liu S, Sun M-Z. miR-429 suppresses tumor migration and invasion by targeting CRKL in hepatocellular carcinoma via inhibiting Raf/MEK/ERK pathway and epithelial-mesenchymal transition. Sci Rep 2018;8(1):2375.

Zhou H, Zhang K, Wang T, Cui J, Ji X, Wang Y, et al. Long non-coding RNA AFAP1-antisense RNA 1 promotes the proliferation, migration and invasion of gastric cancer cells and is associated with poor patient survival. Oncol Lett 2018;15(6):8620–6.

Guo J, Li S, Guo G. Long noncoding RNA AFAP1-AS1 promotes cell proliferation and apoptosis of gastric cancer cells via PTEN/p-AKT Pathway. Dig Dis Sci 2017;62(8):2004–10.

Voulgaris A, Pintzas A. Epithelial-mesenchymal transition in cancer metastasis: mechanisms, markers and strategies to overcome drug resistance in the clinic. BBA 2009;1796(2):75–90.

Mathias RA, Simpson RJ. Towards understanding epithelial-mesenchymal transition: a proteomics perspective. BBA 2009;1794(9):1325–31.

Zhou H, Wang F, Chen H, Tan Q, Qiu S, Chen S, et al. Increased expression of long-noncoding RNA ZFAS1 is associated with epithelial-mesenchymal transition of gastric cancer. Aging 2016;8(9):2023–38.

Lou S, Xu J, Wang B, Li S, Ren J, Hu Z, et al. Downregulation of LncRNA AFAP1-AS1 by oridonin inhibits the epithelial-to-mesenchymal transition and proliferation of pancreatic cancer cells. Acta Biochim Biophys Sin 2019;51(8):814–25.

Shi D, Wu F, Mu S, Hu B, Zhong B, Gao F, et al. LncRNA AFAP1-AS1 promotes tumorigenesis and epithelial-mesenchymal transition of osteosarcoma through Rho/JNK/PI3K/Akt/FOXP3 signaling pathway. J Exp Clin Cancer Res 2019;38(1):375.

Chen B, Li Q, Zhou Y, Wang X, Zhang Q, Wang Y, et al. The long coding RNA AFAP1-AS1 promoter regulates tumor cell growth and invasion in pancreatic cancer through upregulating the IGF1R oncogene via sequestration of miR-133a. Cell Cycle 2018;17(16):1949–66.

Senechal K, Halpern J, Sawyer CL. The Crkl adaptor protein transforms fibroblasts and functions in transformation by the BCR-ABL oncogene. J Biol Chem 1996;271(38):32255–61.

Lin F, Xie C, Li Q, Dong Q, Wang E, Wang Y, CRKL promotes lung cancer cell invasion through ERK-MMP9 pathway. Mol Carcinog 2015;54(Suppl 1):E35–44.

Lee DJ, Kang D, Choi M, Choi YJ, Lee JY, Park JH, et al. Peroxiredoxin-2 represses melanoma metastasis by increasing E-Cadherin/beta-Catenin complexes in adhesions junctions. Cancer Res 2013;73(15):4744–57.

Lin C, Yu B, Liang Z, Li L, Qu S, Chen X, et al. Silencing of c-jun decreases cell migration, invasion, and EMT in radiosensitive human nasopharyngeal carcinoma cell line CNE-2R. Oncotargets Therapy 2018;11:3805–15.

Kwon T, Rho JK, Lee JC, Park YH, Shin HJ, Cho S, et al. An important role for peroxiredoxin II in survival of AS49 lung cancer cells resistant to gefitinib. Exp Mol Med 2015;47:e165.