MKL1/PINK1-AS/miR-34a-5p Regulates ALDOA to Affect Aerobic Glycolysis in Hepatocellular Carcinoma

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Research

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Abstract

Background

Aldolase A, an important metabolic enzyme in the glycolytic pathway, plays an important role in regulating tumour metabolism. In this study, we investigated the expression pattern of ALDOA in hepatocellular carcinoma and its biological role in tumour progression.

Methods

Bioinformatics analysis, Western Blot and RT-qPCR were performed to detect the relative expression of ALDOA in hepatocellular carcinoma tissues and cell lines. A loss-of-function approach was used to investigate the biological function of ALDOA. The role of ALDOA on glycolysis and apoptosis was assessed by Western Blot, glucose and lactate assay kits, flow cytometry and a nude mouse xenograft model. RNA immunoprecipitation, luciferase reporter experiment, chromatin immunoprecipitation and Western Blot were performed to elucidate the underlying molecular

Results

Elevated ALDOA expression correlated with the prognosis of hepatocellular carcinoma patients. exogenous downregulation of ALDOA expression significantly inhibited cellular glycolysis and promoted apoptosis. Mechanistic studies suggest that ALDOA is a direct target of miR-34a-5p, which can enhance apoptosis and inhibit glycolysis in hepatocellular carcinoma cells by targeting the 3'UTR of ALDOA. PINK1-AS competitively sponged miR-34a-5p to increase ALDOA expression by antagonizing miR-34a-5p-mediated ALDOA inhibition. MKL1 acted as a transcription factor to promote the expression of PINK1-AS and ALDOA, thus promoting the deterioration of hepatocellular carcinoma cells.

Conclusion

This study shows that high expression of ALDOA contributes to the development and poor prognosis of hepatocellular carcinoma and will be a target and potential prognostic biomarker for the treatment of hepatocellular carcinoma.

Background

Hepatocellular carcinoma (HCC) is one of the most common malignant tumours worldwide, with the sixth and fourth highest incidence and mortality rates in the world respectively [1]. With the development of modern science and medical technology, new treatment methods have emerged, but the overall treatment effect of HCC has not been significantly improved [2]. Due to the uncertainty of the tumour location, untimely diagnosis and the ease of metastasis, patients may easily miss the best time for treatment, and the prognosis is often poor [3, 4]. Therefore, research on biomarkers and therapeutic targets for HCC is of great value.

Aerobic glycolysis (Warburg effect) is one of the most important metabolic changes in tumours [5]. In contrast to normal cells, tumours have altered energy production patterns and key enzymes of glycolysis can also act as signalling molecules to regulate important tumour-related signalling pathways, altering the proliferation and invasive capacity of tumour cells [6, 7]. Aldolase A (ALDOA) is an important metabolic enzyme in the glycolytic and gluconeogenic pathways, thereby regulating the body's energy metabolism. Available studies have shown that ALDOA is abnormally expressed in many cancers, such as Lung adenocarcinoma and colorectal cancer [8, 9], and that ALDOA expression is significantly increased in the serum of some tumour patients, suggesting that ALDOA may be a key molecule in tumour development and malignancy. These studies suggest that ALDOA may be a potential marker for the diagnosis of a variety of tumours and a new target for targeted drug therapy, providing an effective approach for the treatment of liver cancer.

MicroRNAs (miRNAs) are a class of non-coding RNAs with regulatory functions and are 20-25 nucleotides in length. miRNAs can cluster with miRNA response elements (MREs) in the 3'UTR region of mRNAs to facilitate degradation or translation of mRNAs into proteins [10]. In recent years, it has been found that miRNA expression is associated with a variety of malignant tumours, and it can directly or indirectly activate or inhibit the expression of proto-oncogenes/oncogenes, thus affecting the proliferation, apoptosis, invasion and metastasis of tumour cells, playing a crucial role in the process of tumour development [11, 12]. MiR-34a-5p has been reported to be dysregulated in gastric, colorectal and breast cancers. Our study suggests that miR-34a-5p has an important role in regulating HCC, however the intrinsic biological function and induction mechanism of miR-34a-5p in HCC remains unknown.

Long-stranded non-coding RNA (lncRNA) refers to RNA with transcripts longer than 200 bases and does not encode proteins [13]. LncRNA has been shown in recent years to regulate gene expression at multiple epigenetic, transcriptional and post-transcriptional levels, affecting a variety of biological and pathological processes in cells [14-16]. PINK1-AS has been shown to be closely associated with the development of various diseases such as glucose metabolism disease, Parkinson's disease and breast cancer [17-19]. Our research shows that PINK1-AS regulated ALDOA signaling by functioning as a competing endogenous RNA (ceRNA), which suppressed the degradation of ALDOA mRNA by competing with miR-34a-5p.

Transcription factors are trans-acting factors involved in the regulation of transcription in eukaryotic cells, capable of binding directly or indirectly to specific cis-acting elements to regulate the transcription of genes [20]. MKL-1 belongs to the MRTF family of transcription factors, which are multifaceted transcriptional regulators involved in the pathogenesis of multiple human diseases [21]. In the current study, we found that ALDOA was upregulated in HCC tissues and acted as an independent predictor of overall survival. Furthermore, the effect on apoptosis was investigated by regulating ALDOA expression in HCC through the PINK1-AS/miR-34a-5p axis. PINK1-AS and ALDOA are direct transcriptional targets of MKL-1, which can directly or indirectly induce the overexpression of ALDOA to inhibit apoptosis in HCC cells. These findings may provide promising biomarkers and valuable therapeutic strategies for the treatment of HCC patients.

Materials And Methods

Cell lines

Human HCC lines Huh-7, HepG2 and normal hepatocyte line (LO2) were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). They were cultured in DMEM or 1640 medium supplemented with 10% fetal bovine serum (FBS) at 37°C in an incubator with 5% CO_2.

Patients and tissue samples

Fresh tumour tissue and paracancerous tissue were collected from 20 patients with HCC surgically removed at Hubei Cancer Hospital from January 2018 to December 2020. Tissue samples were preserved in liquid nitrogen. All patients did not receive preoperative chemotherapy or radiotherapy and signed a written informed consent form. The study was approved by the Ethics Review Committee of Hubei Cancer Hospital.

Western blotting (WB)

Cells were lysed on ice with RIPA lysis buffer (Beyotime) containing PMSF, and protein concentrations were determined using the Enhanced BCA Protein Assay Kit (Meilune). Protein extracts (40 μg) were separated by electrophoresis on 12.5% SDS-polyacrylamide gels, then transferred to nitrocellulose membranes (Sigma-Aldrich) and incubated overnight with antibodies together. Secondary antibodies were added and incubated for 1 h. TBST washes were used for development. The reaction was completed using the Intelligent Chemi-TM Analysis System (Thermo Scientific, USA). The following antibodies were used: Anti-β-actin (1:3000, Abclonal), Anti-ALDOA (1:1000, Abclonal), Anti-MKL-1 (1:1000, Cell Signaling Technology / CST), Anti-Bax (1:1 000, CST), Anti-Bcl2 (1:1000, CST), Anti-GLUT1 (1:1000, Abclonal), Anti-PFKM (1:1000, Abclonal).

qRT-PCR analysis

Expression levels of ALDOA and other genes in HCCr tissues and cells were measured using qRT-PCR according to the manufacturer's instructions (TAKARA). β-actin was used as a control. Primers are listed in Supplementary Table 1. Total RNA was extracted using an ultra-pure RNA kit (CWBIO) and cDNA was synthesized using a HiScript II Q RT SuperMix for qPCR kit (Vazyme, Nanjing, China).

For miRNA quantification, Bulge-loop™ miRNA qRT-PCR primer sets (one RT primer and one pair of qRT-PCR primers per set) were designed by RiboBio (Guangzhou, China) specifically for miR-34a-5p. The cDNA was synthesized using the miRNA 1st Strand cDNA Synthesis Kit (Vazyme).

Transfection

Negative control siRNA (si-NC), siRNA-ALDOA (si-ALDOA), siRNA-PINK1-AS (si-PINK1-AS), siRNA-MKL1 (si-MKL1), Negative control mimic (NC mimic), miR- 34a-5p mimic (mimic), Negative control inhibitor (NC

inhibitor), miR-34a-5p inhibitor (inhibitor) were purchased from RIBOBIO Bio. Primers are listed in Supplementary Table 2. All plasmids were extracted with EndoFree Plasmid Midi Kit (CWBIO), and all fragments and plasmids were transfected with lipofectamine 2000 (Invitrogen, Shanghai, China) according to the manufacturer's instructions.

Glucose and lactate determination

The glucose test kit and the lactic acid test kit (Biovision, USA) were used to measure the concentration of glucose and lactic acid in the cell culture medium according to the manufacturer's protocol. Take the cell culture supernatant after 48 hours of transfection into a 96-well plate, and make 5 replicate wells in each group. Add 10 μL of a certain concentration of working solution to each well and incubate at 37 ℃ for 30 minutes. Detect its *OD* value at the specific wavelength required by the kit.

Lentiviral transfection and stable cell line construction

We purchased a knockdown ALDOA lentivirus from Tsingke (Beijing, China). The lentivirus was transfected into HCC cells with 5 mg/ml polyethylene for 48 hours. Use puromycin (2μg/ml) to select stable cell clones for 1 week. The knockout efficiency was tested by WB and RT-qPCR. Sequences are listed in Supplementary Table 2.

Luciferase reporter experiment

The cells harvested 24 h after transfection were lysed with 1×Passive Lysis Buffer (Promega) for 30 minutes on ice. After centrifugation, 10 μL of the supernatant was taken, and 100 μL of Luciferase Assay Buffer (Promega) was added to it to measure the fluorescence value. Take the same volume of supernatant to measure the protein concentration, and finally calculate the fluorescence value per unit protein concentration.

RNA immunoprecipitation

Use RNA immunoprecipitation (RIP) kit (Boxin Biotech, Guangzhou, China) according to the manufacturer's protocol. The Huh-7 and HepG2 cells were lysed in complete RIP lysis buffer, and the cell extracts were incubated with magnetic beads bound to specific antibodies or control IgG at 4℃ for 6 hours. The beads are washed and incubated with proteinase K to remove protein. Finally, the purified RNA was analyzed by qRT-PCR.

Chromatin immunoprecipitation

The chromatin immunoprecipitation (ChIP) experiment was performed using the EpiQuik ChIP kit (Epigentek) according to the manufacturer's instructions. Huh-7 and HepG2 cells were treated with formaldehyde and incubated for 10 min to produce DNA-protein crosslinks. Cell lysates are then sonicated to produce 200-300 bp chromatin fragments and immunoprecipitated using MKL-1 specific

antibody or IgG as a control. The precipitated chromatin DNA is recovered and analyzed by qRT-PCR. The primers related to ChIP analysis are listed in Supplementary Table 3.

Hematoxylin-eosin staining and immunohistochemistry experiment

The tumor tissues of nude mice were sent to Servicebio Biotech for paraffin-embedded sectioning. Hematoxylin-Eosin staining kit (Solarbio) is used for hematoxylin-eosin staining (H&E)). Use streptavidin alkaline phosphatase (SABC–AP) immunohistochemical staining kit (BOSTER Biological) for immunohistochemistry (IHC) experiments. All the above operations are carried out in strict accordance with the manufacturer's instructions.

Tumor formation assay in nude mouse model

The animal experiment procedure has been approved by the Laboratory Animal Center of Wuhan University of Science and Technology and the Experimental Animal Ethics Review Committee. BALB / c-nu (nude mouse) mice obtained from Wuhan Shulb. Three-week-old nude mouse were randomly divided into two groups, each of which was injected subcutaneously with 1×10^8 Huh-7 cell line that stably knocks down ALDOA and the control group. All nude mouse were euthanized after 30 days. And check the growth of subcutaneous tumors in nude mouse.

Statistical Analysis

All experiments in this article were repeated at least 3 times. These values are expressed as mean ± standard deviation. Kaplan-Meier survival chart was used to draw the survival curve, and the log-rank test was used for testing. A Pearson correlation analysis was performed to analyze the correlation between ALDOA, miR-34a-5p, ALDOA and MKL-1 levels. Two-sided P values <0.05 were considered statistically significant.

Results

Expression of ALDOA is up-regulated in human HCC tissues and correlates with poor prognosis

We assessed ALDOA transcript levels based on the TCGA database in multiple HCC studies (Fig. 1a). Analysis of 15 HCC cohorts in the HCCDB database revealed that ALDOA mRNA expression was significantly higher in HCC tissues than in adjacent normal tissues (Fig. S1).The H&E staining was performed to confirm the morphological characteristics of HCC tissues and paracancerous tissues, IHC was used to detect the distribution of ALDOA in tumour and paraneoplastic tissues, and it was found that ALDOA was mainly expressed in HCC tissues and to a lesser extent in paraneoplastic tissues (Fig. 1b). Subsequently, ALDOA protein was measured by WB analysis and ALDOA mRNA was determined by RT-qPCR. The expression pattern of ALDOA was validated in 20 HCC patients, and ALDOA protein and mRNA showed an overall upward trend (Fig. 1c, d). By analyzing clinicopathological factors, we found that high ALDOA expression is related to the poor prognosis of HCC patients. There is a significant positive

correlation between higher ALDOA levels and cancer stages (Fig. 1e). As shown in Fig. 1f, elevated ALDOA levels predicted a poor prognosis in patients with HCC.

Up-regulation of ALDOA and its prognostic significance in patients with HCC. (a) The box diagram shows the level of ALDOA mRNA in the TCGA database. (b) H&E staining of the HCC tumor tissues and para-carcinoma tissues, Analysis of ALDOA distribution in tumor tissues by using IHC. (c) Validation of ALDOA dys-regulation in HCC by using western blotting analysis (n = 6). (d) Relative levels of ALDOA mRNA in hepatocellular carcinoma tissues (n = 20). (e) Higher ALDOA levels were positively correlated with cancer stage. (f) Kaplan-Meier survival curves showing the effect of ALDOA on overall survival. *, $P< 0.05$; * * , $P< 0.01$; * * * , $P< 0.001$.

ALDOA is required for malignant behaviors in HCC cells

We examined the protein and mRNA expression levels of ALDOA in the normal human hepatocyte line LO2 and two types of HCC cells using WB and RT-qPCR (Fig. 2a, b). To assess the function of ALDOA in HCC, we identified the top 50 genes positively associated with ALDOA in HCC (Fig. S2a), and functional enrichment analysis revealed that ALDOA was significantly associated with glucose metabolic processes (Fig. S2b). We next investigated the effect of ALDOA knockdown on HCC at the cellular level. As shown in Figure 2c, d, e, f, si2-ALDOA exhibited the most pronounced knockdown effect and was therefore selected for subsequent experiments. We found that ALDOA silencing significantly inhibited the expression of aerobic glycolysis-related proteins (Fig. 2g, h). We also analysed the levels of glucose and lactate in the culture medium supernatant and found that ALDOA knockdown also significantly inhibited glucose uptake and lactate production, suggesting that ALDOA silencing significantly inhibited aerobic glycolysis in HCC cells (Fig. 2i, j).

Knockdown of ALDOA inhibits aerobic glycolysis of HCC in vitro. (a) WB assays for protein expression levels of ALDOA in normal hepatocytes and two types of HCC cells. (b) RT-qPCR assays for mRNA expression levels of ALDOA in normal hepatocytes and two types of HCC cells. (c, d, e, f) Validation of the effects of two siRNAs knocking down ALDOA in Huh-7 and HepG2 cells at the mRNA and protein levels. (g, h) WB detection of protein expression of ALDOA after knockdown and aerobic glycolysis-related marker genes in two types of HCC cells. (i, j) Glucose and lactate levels in the culture medium supernatants of two types of HCC cells after knockdown of ALDOA were detected by the kit. *, $P< 0.05$; * * , $P< 0.01$; * * * , $P< 0.001$.

ALDOA promotes HCC cell tumorigenesis in vivo

In a nude mouse xenograft model, Huh7 cells with a lentiviral vector producing shRNA against ALDOA were inoculated subcutaneously into mouse, and Huh-7 cells infected with a lentiviral vector bearing a control shRNA were inoculated subcutaneously into another group of mouse as a control. Through this in vivo experiment, we observed that stable knockdown of ALDOA effectively inhibited tumour growth in nude mouse, as reflected by a significant reduction in tumour size and weight (Fig. 3a, b). We performed histopathological analysis of tumors in nude mouse using H&E staining (Fig. 3c), and then examined the

expression levels of ALDOA, PFKM and GLUT1 in nude mouse tumors by IHC. As shown in Fig. 3d, downregulated of ALDOA, PFKM and GLUT1 expression levels were detected in the sh-ALDOA group. Finally, the protein level of ALDOA in tumor tissues of nude mouse was further detected by WB, and the results showed that the level of ALDOA was reduced in the sh-ALDOA group (Fig. 3e).

ALDOA promotes HCC cell tumorigenesis in vivo. (a) Huh-7 cells transfected with Ctrl shRNA and ALDOA shRNA were injected respectively into nude mouse (n=5), which were killed after 21 days. (b) Tumor weights and volumes were represented as the means of tumor weights ± SD. (c) Representative H&E-stained sections of the tumor tissues isolated from mouse. (d) The expression levels of ALDOA, PFKM, and GLUT1 were determined in nude mouse tumor tissues using IHC. (e) WB results showing the protein expression levels of ALDOA in nude mouse tumour tissues.*, $P< 0.05$; * * , $P< 0.01$.

miR-34a-5p targets ALDOA to exert tumor suppressive effects in HCC

To find miRNAs targeting ALDOA, we performed bioinformatics analysis using four different algorithms including mirDIP, Targetscan, miRDB and TarBase. Fig. 4a shows the overlapping miRNAs targeting ALDOA. Expression analysis showed that miR-34a-5p was more significantly downregulated in HCC cell lines (Fig. 4b). And we confirmed that patients with high miR-34a-5p expression had higher overall survival than those with low miR-34a expression (Fig. 4c). We next overexpress miR-34a-5p in two HCC cell lines, and show that the overexpression of miR-34a-5p can significantly inhibit the expression of ALDOA (Fig. 4c, e), thereby inhibiting the aerobic glycolysis process of HCC cells (Fig. S3a, b, c, d). Correlation analysis suggested a negative correlation between the level of miR-34a-5p and ALDOA expression level in HCC tissue specimens (Fig. S3e). We first found the ALDOA 3'UTR binding site to miR-34a-5p, and then we mutated this binding site to construct the pmirGLO-ALDOA 3'UTR-MUT vector (Fig. 4f, g, h). Luciferase report experiment gene assays showed that miR-34a-5p mimics significantly reduced the luciferase activity of pmirGLO-ALDOA 3'UTR-WT, but had no effect on pmirGLO-ALDOA 3'UTR-MUT activity (Fig. 4i). RIP experiments also proved that miR-34a-5p can bind ALDOA (Fig. 4j, k). Overexpression of ALDOA counteracts the inhibitory effect of miR-34a-5p on ALDOA (Fig. 4l).

MiR-34a-5p targets ALDOA to exert tumor suppressive effects in HCC. (a) Venn diagram showing the 2 miRNAs predicted by four different algorithms to target ALDOA. (b) RT-qPCR analysis showed that miR-34a-5p was more significantly down-regulated in Huh-7 and HepG2 cells. .(c) Kaplan-Meier survival curves showing the effect of miR-34a on overall survival. (d, e) WB detection of ALDOA protein expression levels after overexpression of miR-34a-5p. (f, g, h) Putative binding sequence of miR-34a-5p in the 3'UTR of ALDOA. (i) Dual luciferase reporter experiment revealed that miR-34a-5p could bind to the 3'UTR of ALDOA. (j, k) RIP experimentsproved that miR-34a-5p can bind ALDOA. (l) Luciferase reporter experiment on 293 cells co-transfected with miR-34a-5p mimic or mimic-NC and ALDOA-3′UTR-Luc-WT plasmid, with or without ALDOA. *, $P< 0.05$; * * , $P< 0.01$; * * * , $P< 0.001$.

PINK1-AS targets miR-34a-5p to exert its tumor-promoting effects in HCC

Analysis using miRcode (http://www.mircode.org/) website suggested that PINK1-AS could bind miR-34a-5p (Fig. 5a), Bioinformatics analysis also shows that PINK1-AS is up-regulated in HCC (Fig. 5b), We performed RT-qPCR to quantify the expression levels of PINK1-AS in the normal human hepatocyte line LO2 and two HCC cell lines. As shown in Fig. 5c, PINK1-AS was highly expressed in HCC cell lines, and the overall survival rate of patients with high PINK1-AS expression was poor (Fig. 5d), and the expression of PINK1-AS and miR-34a-5p showed a negative correlation in tumor samples (Fig. 5e). In HCC cells, we found that knockdown the expression of PINK1-AS leads to up-regulation of miR-34a-5p (Fig. 5f, g). Inhibition of miR-34a-5p expression leads to up-regulation of PINK1-AS (Fig. 5h, i). In addition, we generated mutant sequences of PINK1-AS that were unable to bind miR-34a-5p for subsequent fluorophore enzyme reporter gene assays (Fig. 5j). As shown in Fig. 5k, miR-34a-5p mimics significantly reduced luciferase activity in HCC cells transfected with the wild-type PINK1-AS sequence, whereas luciferase activity was not significantly altered in mutant PINK1-AS-transfected HCC cells. After transfection of PINK1-AS expression plasmid, the decreased luciferase activity after transfection of miR-34a-5p was restored (Fig. 5l). Bioinformatics analysis shows that the expression of PINK1-AS and ALDOA is positively correlated in tumor samples (Fig. 5m). Knockdown of PINK1-AS expression will result in down-regulation of ALDOA mRNA and protein levels (Fig. 5n, o, Fig. S4a, b.), and Inhibited the aerobic glycolysis process of tumor cells (Fig. S4c, d, e, f).

PINK1-AS targets miR-34a-5p to exert its tumor-promoting effects in HCC. (a) Analysis of the miRcode (http://www.mircode.org/) website shows that PINK1-AS could bind miR-34a-5p. (b) The box diagram shows the level of PINK1-AS mRNA in the TCGA database. (c) Expression levels of miR-34a-5p were detected by RT-qPCR in PINK1-AS-silenced Huh-7 cells and HepG2 cells. (d) Kaplan-Meier survival curves showing the effect of ALDOA on overall survival. (e) Expression level of PINK1-AS was negative correlated with miR-34a-5p expression level (P<0.001). (f, g) The expression level of miR-34a-5p was detected in HuH-7 cells and HepG2 cells after transfection of si-PINK1-AS by RT-qPC. (h, i) The expression level of PINK1-AS was detected in HuH-7 cells and HepG2 cells after transfection of inhibitor-miR-34a-5p by RT-qPC. (j) Putative binding sequence of miR-34a-5p in the 3'UTR of PINK1-AS. (k) Luciferase reporters containing WT or MUT PINK1-AS transcript as well as blank pmirGLO were co-transfected with miR-34a-5p mimics or miR-control in 293 cells. Luciferase activity was determined using dual luciferase reporter system. (l) Luciferase reporters containing PINK1-AS-Luc-WT were co-transfected with with or without miR-34a-5p and PINK1-AS, Luciferase activity was determined using dual luciferase reporter system. (m) Expression level of PINK1-AS was positively correlated with ALDOA expression level (P<0.001). (n, o) Down-regulation of ALDOA levels in PINK1-AS-silenced Huh-7 cells and HepG2 cells was detected by RT-qPCR. *, P< 0.05; * * , P< 0.01; * * * , P< 0.001.

MKL-1 induces the expression of PINK1-AS and ALDOA by acting as a transcription factor

The promoter regions of PINK1-AS and ALDOA were located and analysed through the UCSC (https://genome.ucsc.edu/) website, the first 2000 bp upstream of the transcription start site was found to contain the MKL-1 binding site.Bioinformatics analysis shows that MKL-1 is up-regulated in HCC (Fig. 6a), The association between MKL-1 expression and survival outcomes of HCC patients was assessed

using Kaplan-Meier survival curves, and analysis showed that patients with high MKL-1 expression had poor elevated overall survival (Fig. 6b). To verify whether MKL-1 mediated the expression of PINK1-AS and ALDOA, we knocked down MKL-1 using siRNA targeting MKL-1 (Fig. 6c, d, e, f). MKL-1 downregulation resulted in a significant decrease in the expression of PINK1-AS and ALDOA in Huh-7 and HepG2 cells (Fig. 6g, h, i, j). Further, we found that silencing of MKL-1 significantly inhibited aerobic glycolysis in HCC cells (Fig. S5a, b). Correlation analysis suggested a positively correlation between the level of MKL-1 and ALDOA expression level in HCC tissue specimens (Fig. 6k). And the expression level of MKL-1 was positively correlated with PINK1-AS expression level (Fig. 6l). To investigate whether MKL-1 directly transcriptionally regulates ALDOA andPINK1-AS expression, we constructed ALDOA and PINK1-AS promoter luciferase reporter plasmids (Fig. 6m, n), and luciferase assays showed that MKL-1 could promote PINK1-AS and ALDOA promoter luciferase activity (Fig. 6o, p). In addition to this, ChIP verified MKL-1 binding to predicted sites in the PINK1-AS and ALDOA promoter regions (Fig. 6q, r).

MKL-1 induces the expression of PINK1-AS and ALDOA as a transcription factor. (a) The box diagram shows the level of PINK1-AS mRNA in the Ualcan database. (b) Kaplan-Meier survival curves showing the effect of MKL-1 on overall survival. (c, d, e, f) WB and RT-qPCR to verify the efficiency of siRNA knockdown of MKL-1. (g, h, i,) WB and RT-qPCR to determine the expression level of ALDOA in Huh-7 and HepG2 cells after knockdown of MKL-1. (j) RT-qPCR determination of PINK1-AS expression levels in Huh-7 and HepG2 cells after knockdown of MKL-1. (k) Correlation analysis showing a positively correlation between MKL-1 and PINK1-AS expression ($P < 0.001$). (l)Expression level of MKL-1 was positively correlated with ALDOA expression level ($P < 0.001$). (m, n) Prediction of MKL-1 binding sites in the human PINK1-AS and ALDOA promoters by gene sequence analysis. (o, p) Luciferase reporter gene test to detect whether MKL-1 can target the PINK1-AS and ALDOA promoters. (q, r) Quantitative ChIP analysis to show direct binding of MKL-1 to the endogenous PINK1-AS and ALDOA promoter regions.

Discussion

The reprogramming of tumour metabolism secures favourable conditions for tumour cells to survive in a new environment [22, 23]. The greater glycolytic capacity in highly malignant or metastatic tumour cells suggests a potential correlation between altered tumour metabolic conditions and their ability to proliferate and migrate invasively. Increasing ALDOA expression accelerates the process of glycolysis in tumour cells, while decreasing ALDOA expression decreases ATP production and reduces cell membrane integrity [24]. In addition to regulating glycolysis and energy metabolism, ALDOA can also be involved in tumourigenesis and development through a variety of pathways. In a hypoxic environment, ALDOA can mediate EMT in tumour cells by regulating hypoxia inducible factor-1α (HIF-1α), which enhances tumour cell migration [25, 26] Caspi M et al. concluded that ALDOA is a new regulator of the wnt signalling pathway that can GSK-3β-dependent mechanism to activate the classical wnt signaling pathway, which is closely related to tumorigenesis and development [27]. In this study, we used TCGA data to determine that ALDOA expression was upregulated in HCC. We then confirmed high ALDOA expression in HCC by RT-qPCR and WB analysis, high ALDOA expression in HCC tissues was associated with poor prognosis and may be an independent prognostic indicator. Through loss-of-function assays, we demonstrated that

ALDOA promoted glycolysis and inhibited apoptosis in HCC in vitro and in vivo. These results suggest that ALDOA may play an important role in the progression of HCC.

Studies have confirmed that dysregulation of miRNAs plays an important role in a variety of diseases, including malignancies and inflammatory diseases [28]. In previous studies, miR-34a-5p was found to have an important role in the pathogenesis of a variety of diseases. Gao J et al. found that miR-34a-5p was lowly expressed in colon cancer patients and could target P53 to promote tumour cell metastasis and disease recurrence [29]. Jiang ZQ et al. showed that garcinia cambogia could target miR-34a-5p through up-regulation of MDM4 gene to inhibit the anti-apoptotic ability of cancer cells in non-small cell lung cancer patients, thereby eliminating or shrinking cancer foci [30]. In addition, miR-34a-5p also has a regulatory role in the pathogenesis of many benign diseases. Cosín-Tomás M et al. found that miR-34a-5p was differentially expressed in the serum of patients with Alzheimer's disease and normal subjects [31]. Li A et al. showed that miR-34a-5p also has an important role in diabetic nephropathy, and high expression of miR-34a-5p inhibited cell proliferation and promote renal fibrosis [32]. In this study, after bioinformatics analysis, we found that ALDOA has a binding region to miR-34a-5p in its 3'UTR, and we hypothesized that ALDOA is likely to be a target gene of miR-34a-5p. Both protein expression assays and luciferase reporter experiment verified that ALDOA is a target gene of miR-34a-5p.

Current studies have shown that lncRNAs play an important role in the regulation of gene expression, embryonic development, species evolution, material metabolism, and tumourigenesis and metastasis [16, 33-35]. PINK1-AS, an antisense RNA of PINK1, is regulated by PINK1-AS in the regulation of mitochondrial function. It has been reported that PINK1-AS expression is elevated in patients with multiple sclerosis [36]. Márki S et al. found that polymorphisms in the PINK1-AS gene were associated with Parkinson's disease [37]. In the present study, we found that PINK1-AS could act as a ceRNA to antagonize miR-34a-5p-mediated degradation of ALDOA and indirectly increase the expression of ALDOA mRNA. In vitro experiments also further demonstrated that PINK1-AS could promote aerobic glycolysis in HCC cells.

MKL-1 is widely expressed in mammals and plays an important role in various physiological and pathological processes in the body [38]. Interfering with MKL-1 expression in breast cancer cells has been reported to attenuate the migration level of tumour cells [39]. The knockdown of MKL-1 in mice significantly reduced the tumorigenic ability of tumor cells and their ability to migrate to the lung with the blood circulation [40]. MKL-1 can also act as a transcription factor to promote cancer development by altering the expression of other genes in cancer cells. MKL-1 can bind directly to the core region of the CAPP1 promoter to regulate the activity of the CAPP1 promoter [41]. MKL-1 mediates TGF-β-induced RhoJ transcription to promote breast cancer cell migration and invasion [42]. In this study, we found that PINK1-AS and ALDOA are direct targets of the transcription factor MKL-1, as confirmed by the binding of MKL-1 to predicted sites in the PINK1-AS and ALDOA promoter regions and the significant induction of PINK1-AS and ALDOA promoter activity induced by MKL-1. Thus, the upregulation of PINK1-AS and ALDOA in HCC is partly attributed to MKL-1 activation during tumour progression.

In this study, we investigated the role and mechanism of ALDOA in HCC. We obtained that ALDOA is a target gene of miR-34a-5p through bioinformatic target analysis, and demonstrated that miR-34a-5p silences the expression of ALDOA by targeting its 3'UTR region, thus affecting glycolysis in HCC cells. PINK1-AS1 could act as a "sponge" for miR-34a-5p and thus inhibit the silencing effect of miR-34a-5p on ALDOA. We also found MKL-1 binding sites at the promoters of ALDOA and PINK1-AS, suggesting that MKL-1 promotes the transcription of ALDOA and PINK1-AS by targeting their promoters. In HCC cells, PINK1-AS and ALDOA levels are consistently parallel, with increased levels promoting aerobic glycolysis.

Conclusion

In conclusion, we comprehensively investigated the functional role and molecular mechanism of ALDOA in HCC. Our results suggest that ALDOA is up-regulated in HCC cell lines and tissues. High ALDOA expression levels are an independent prognostic factor for the overall survival of HCC patients. Our study shows that MKL-1 /PINK1-AS/ miR-34a-5p/ ALDOA form a circuit in cells to co-regulate glycolysis in HCC cells. Our findings support the idea that ALDOA plays a key role in HCC progression and is a potentially effective target for the treatment of HCC.

Abbreviations

HCC: Hepatocellular carcinoma

MREs: MiRNA response elements

ALDOA: Aldolase A

FBS: Fetal bovine serum

WB: Western Blot

IHC: Immunohistochemistry

RIP: RNA immunoprecipitation

ChIP: Chromatin immunoprecipitation

LncRNAs: Long non-coding RNAs

BSA: Bovine serum albumin

CST: Cell Signaling Technologies

qRT-PCR: Quantitative reverse transcription PCR

SDS-PAGE: Sodium dodecyl sulphate-polyacrylamide gel electrophoresis

WT: Wild-type

MUT: Mutant

Declarations

Availability of data and materials

The public datasets analyzed during the current study are available in the repositories listed below:

• UALCAN: http://ualcan.path.uab.edu/analysis.html

• LinkedOmics: http://www.linkedomics.org/login.php

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Conceptualization: Jun Wang and Huimin Zhang; Methodology: Zhoutong Dai, You Huang, Chao Shen and Hui Liu; Statistical analysis: Jiapeng Li, Zitan Peng, Zhen Chen and Yuan Wu; Writing: Jun Wang;

Investigation: Jun Wang, Huimin Zhang, and Zhoutong Dai; Study supervision: Tong-Cun Zhang and Xing-Hua Liao; All authors read and approved the final manuscript.

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Ethics declarations

Ethics approval and consent to participate

The study was approved and reviewed by the Medical Ethics Committee of Wuhan University of Science and Technology and was conducted with the consent of the subjects and in accordance with the Declaration of Helsinki. All mouse experiments were approved by the Animal Protection and Use Committee of the Upper Wuhan University of Science and Technology.

Consent for publication

All authors have given their consent for the publication of this article.

Competing interests

All authors declare that they have no competing interests.

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Figures

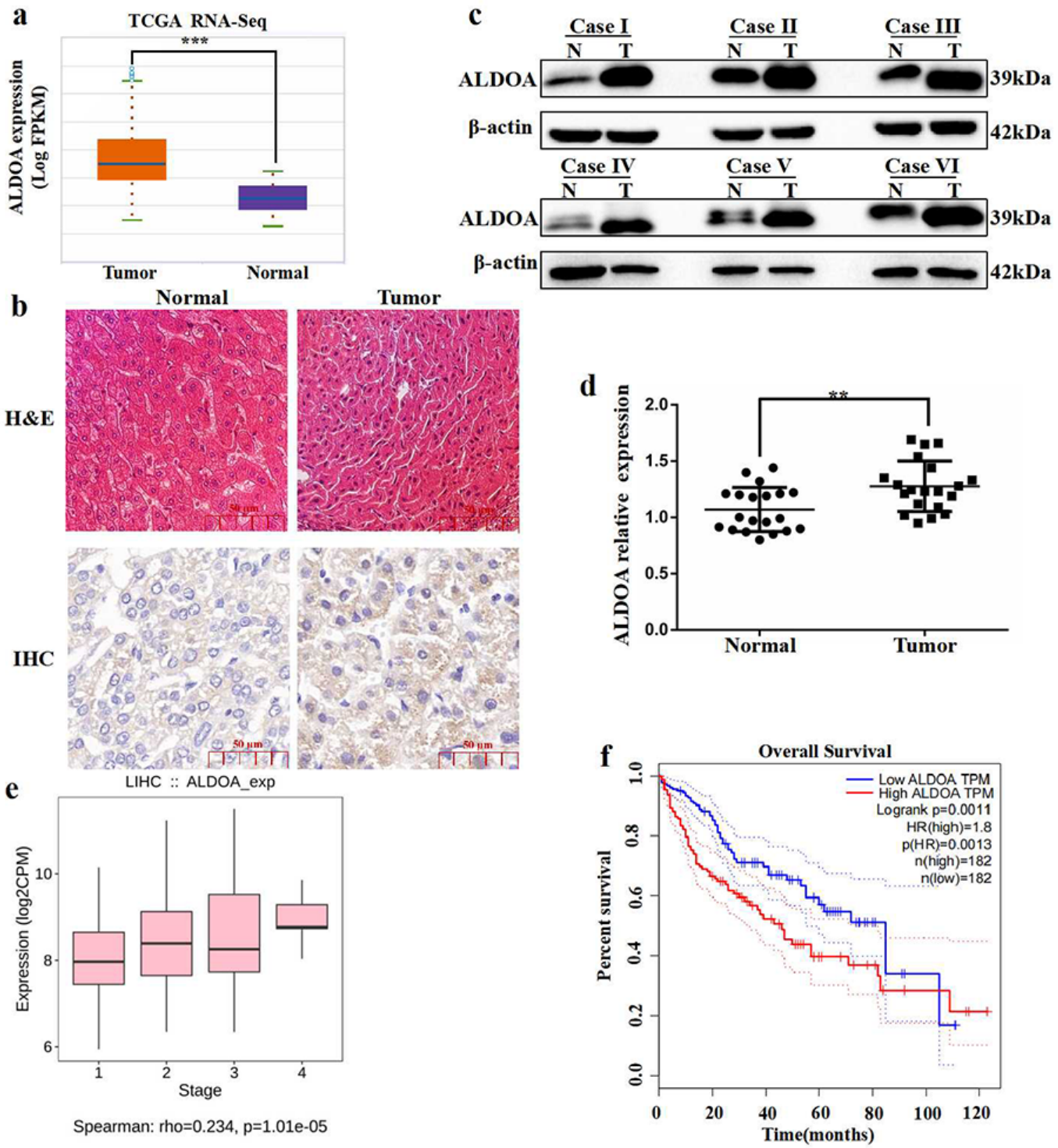

Figure 1

We assessed ALDOA transcript levels based on the TCGA database in multiple HCC studies (Fig. 1a). Analysis of 15 HCC cohorts in the HCCDB database revealed that ALDOA mRNA expression was significantly higher in HCC tissues than in adjacent normal tissues (Fig. S1).The H&E staining was performed to confirm the morphological characteristics of HCC tissues and paracancerous tissues, IHC was used to detect the distribution of ALDOA in tumour and paraneoplastic tissues, and it was found that

ALDOA was mainly expressed in HCC tissues and to a lesser extent in paraneoplastic tissues (Fig. 1b). Subsequently, ALDOA protein was measured by WB analysis and ALDOA mRNA was determined by RT-qPCR. The expression pattern of ALDOA was validated in 20 HCC patients, and ALDOA protein and mRNA showed an overall upward trend (Fig. 1c, d). By analyzing clinicopathological factors, we found that high ALDOA expression is related to the poor prognosis of HCC patients. There is a significant positive correlation between higher ALDOA levels and cancer stages (Fig. 1e). As shown in Fig. 1f, elevated ALDOA levels predicted a poor prognosis in patients with HCC.

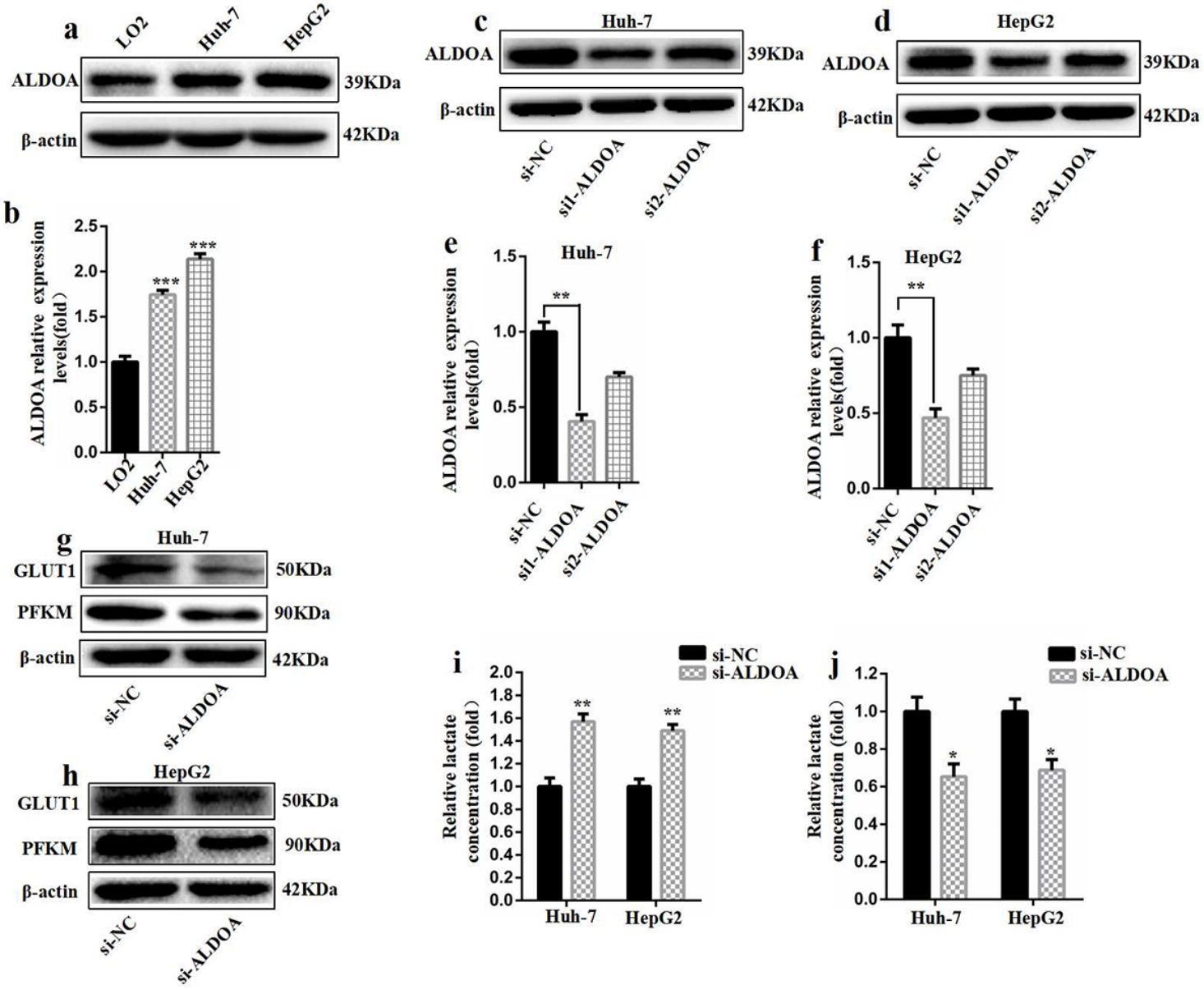

Figure 2

We examined the protein and mRNA expression levels of ALDOA in the normal human hepatocyte line LO2 and two types of HCC cells using WB and RT-qPCR (Fig. 2a, b). To assess the function of ALDOA in HCC, we identified the top 50 genes positively associated with ALDOA in HCC (Fig. S2a), and functional enrichment analysis revealed that ALDOA was significantly associated with glucose metabolic processes

(Fig. S2b). We next investigated the effect of ALDOA knockdown on HCC at the cellular level. As shown in Figure 2c, d, e, f, si2-ALDOA exhibited the most pronounced knockdown effect and was therefore selected for subsequent experiments. We found that ALDOA silencing significantly inhibited the expression of aerobic glycolysis-related proteins (Fig. 2g, h). We also analysed the levels of glucose and lactate in the culture medium supernatant and found that ALDOA knockdown also significantly inhibited glucose uptake and lactate production, suggesting that ALDOA silencing significantly inhibited aerobic glycolysis in HCC cells (Fig. 2i, j).

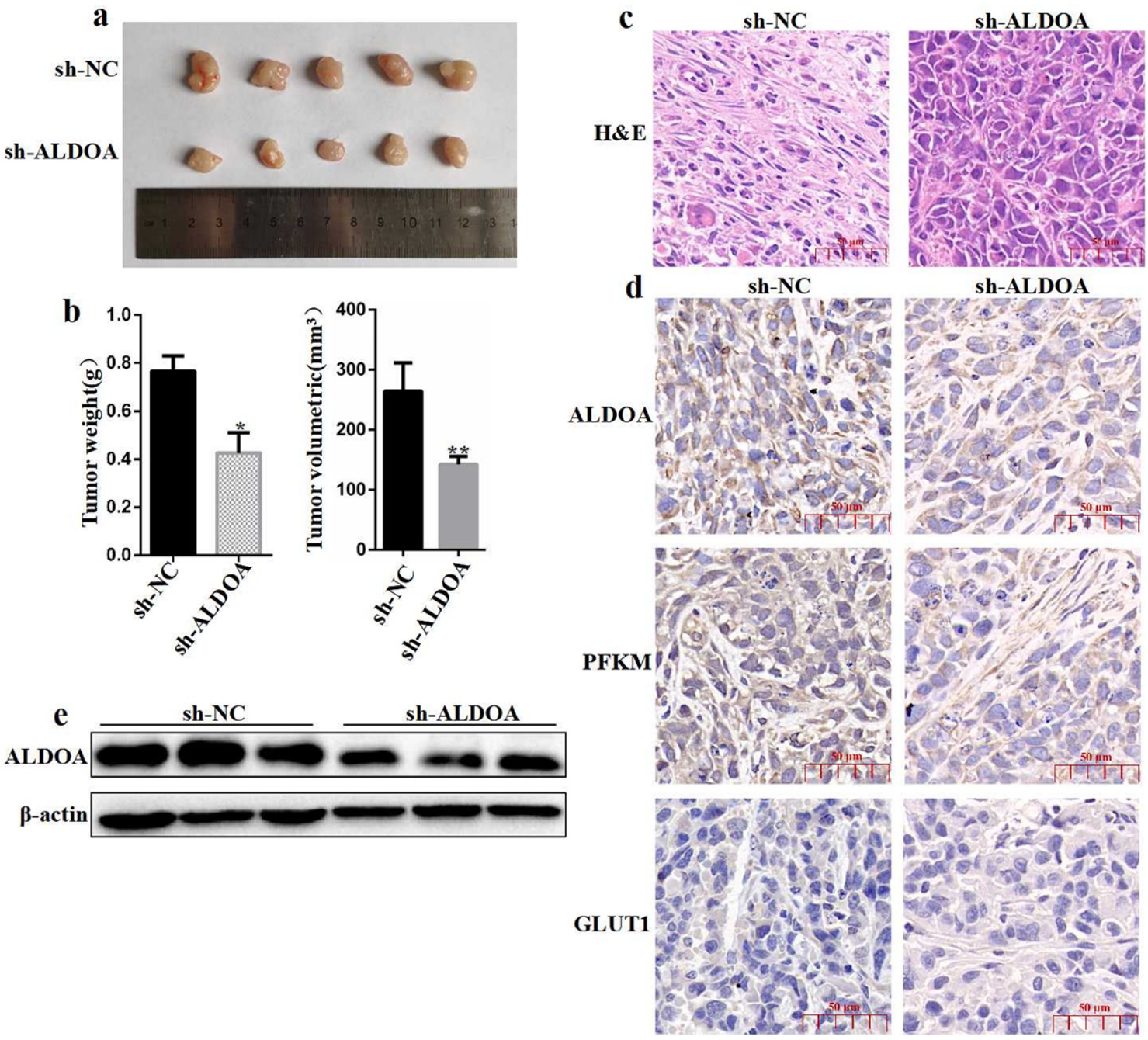

Figure 3

In a nude mouse xenograft model, Huh7 cells with a lentiviral vector producing shRNA against ALDOA were inoculated subcutaneously into mouse, and Huh-7 cells infected with a lentiviral vector bearing a control shRNA were inoculated subcutaneously into another group of mouse as a control. Through this in vivo experiment, we observed that stable knockdown of ALDOA effectively inhibited tumour growth in nude mouse, as reflected by a significant reduction in tumour size and weight (Fig. 3a, b). We performed histopathological analysis of tumors in nude mouse using H&E staining (Fig. 3c), and then examined the expression levels of ALDOA, PFKM and GLUT1 in nude mouse tumors by IHC. As shown in Fig. 3d, downregulated of ALDOA, PFKM and GLUT1 expression levels were detected in the sh-ALDOA group. Finally, the protein level of ALDOA in tumor tissues of nude mouse was further detected by WB, and the results showed that the level of ALDOA was reduced in the sh-ALDOA group (Fig. 3e).

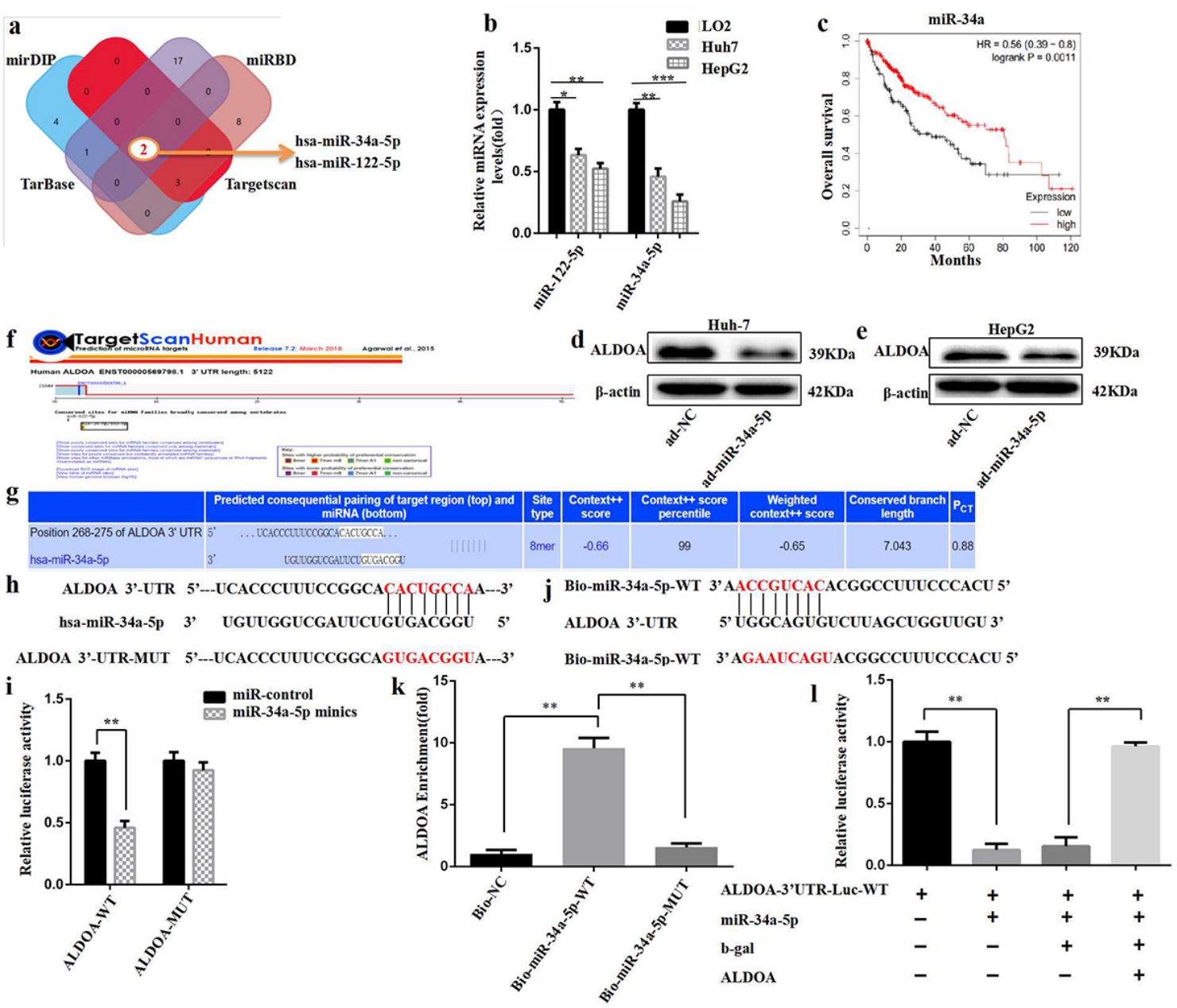

Figure 4

To find miRNAs targeting ALDOA, we performed bioinformatics analysis using four different algorithms including mirDIP, Targetscan, miRDB and TarBase. Fig. 4a shows the overlapping miRNAs targeting ALDOA. Expression analysis showed that miR-34a-5p was more significantly downregulated in HCC cell lines (Fig. 4b). And we confirmed that patients with high miR-34a-5p expression had higher overall survival than those with low miR-34a expression (Fig. 4c). We next overexpress miR-34a-5p in two HCC cell lines, and show that the overexpression of miR-34a-5p can significantly inhibit the expression of ALDOA (Fig. 4c, e), thereby inhibiting the aerobic glycolysis process of HCC cells (Fig. S3a, b, c, d). Correlation analysis suggested a negative correlation between the level of miR-34a-5p and ALDOA expression level in HCC tissue specimens (Fig. S3e). We first found the ALDOA 3'UTR binding site to miR-34a-5p, and then we mutated this binding site to construct the pmirGLO-ALDOA 3'UTR-MUT vector (Fig. 4f, g, h). Luciferase report experiment gene assays showed that miR-34a-5p mimics significantly reduced the luciferase activity of pmirGLO-ALDOA 3'UTR-WT, but had no effect on pmirGLO-ALDOA 3'UTR-MUT activity (Fig. 4i). RIP experiments also proved that miR-34a-5p can bind ALDOA (Fig. 4j, k). Overexpression of ALDOA counteracts the inhibitory effect of miR-34a-5p on ALDOA (Fig. 4l).

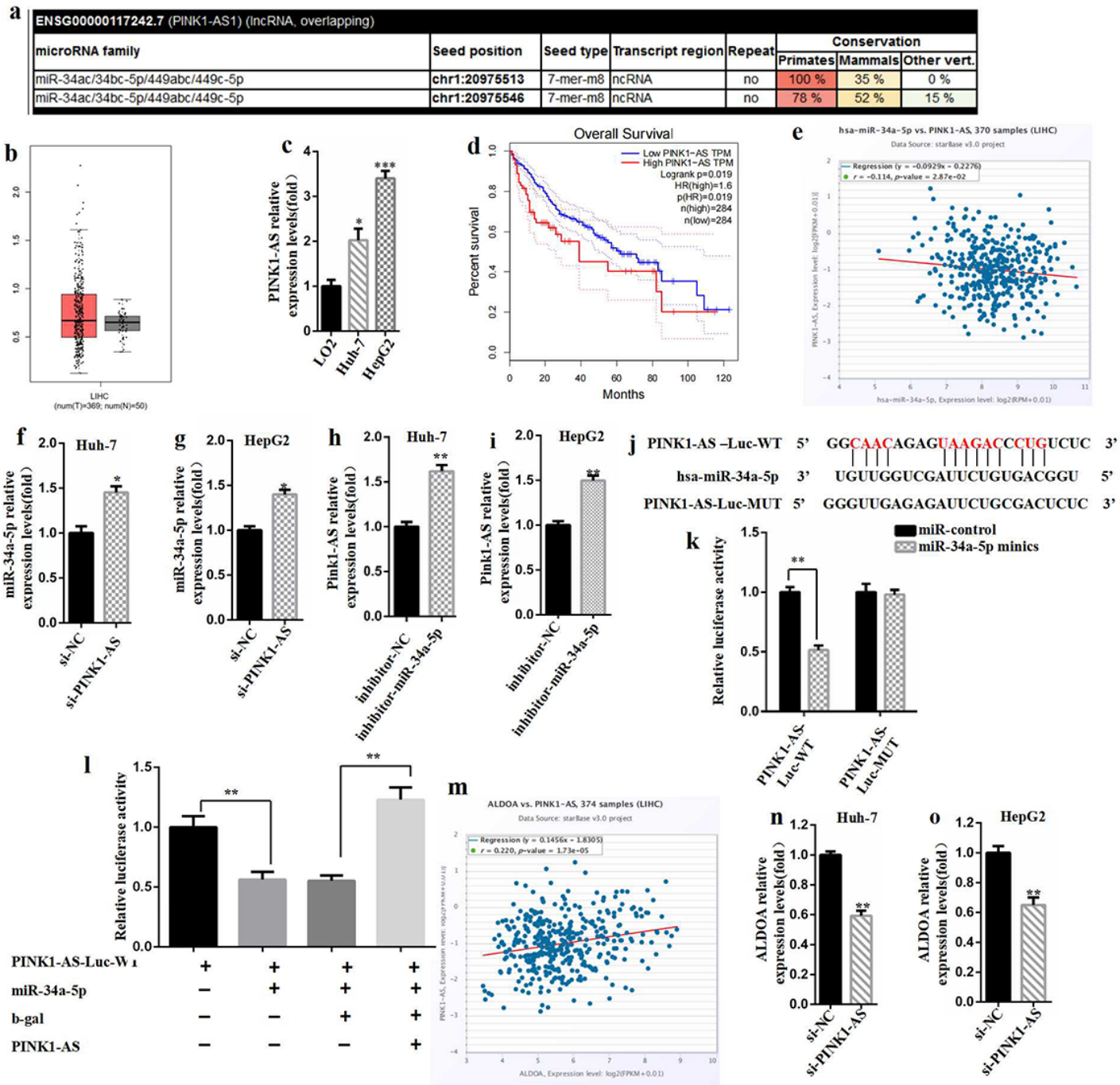

Figure 5

Analysis using miRcode (http://www.mircode.org/) website suggested that PINK1-AS could bind miR-34a-5p (Fig. 5a), Bioinformatics analysis also shows that PINK1-AS is up-regulated in HCC (Fig. 5b), We performed RT-qPCR to quantify the expression levels of PINK1-AS in the normal human hepatocyte line LO2 and two HCC cell lines. As shown in Fig. 5c, PINK1-AS was highly expressed in HCC cell lines, and the overall survival rate of patients with high PINK1-AS expression was poor (Fig. 5d), and the expression of PINK1-AS and miR-34a-5p showed a negative correlation in tumor samples (Fig. 5e). In HCC cells, we found that knockdown the expression of PINK1-AS leads to up-regulation of miR-34a-5p (Fig. 5f, g). Inhibition of miR-34a-5p expression leads to up-regulation of PINK1-AS (Fig. 5h, i). In addition, we

generated mutant sequences of PINK1-AS that were unable to bind miR-34a-5p for subsequent fluorophore enzyme reporter gene assays (Fig. 5j). As shown in Fig. 5k, miR-34a-5p mimics significantly reduced luciferase activity in HCC cells transfected with the wild-type PINK1-AS sequence, whereas luciferase activity was not significantly altered in mutant PINK1-AS-transfected HCC cells. After transfection of PINK1-AS expression plasmid, the decreased luciferase activity after transfection of miR-34a-5p was restored (Fig. 5l). Bioinformatics analysis shows that the expression of PINK1-AS and ALDOA is positively correlated in tumor samples (Fig. 5m). Knockdown of PINK1-AS expression will result in down-regulation of ALDOA mRNA and protein levels (Fig. 5n, o, Fig. S4a, b.), and Inhibited the aerobic glycolysis process of tumor cells (Fig. S4c, d, e, f).

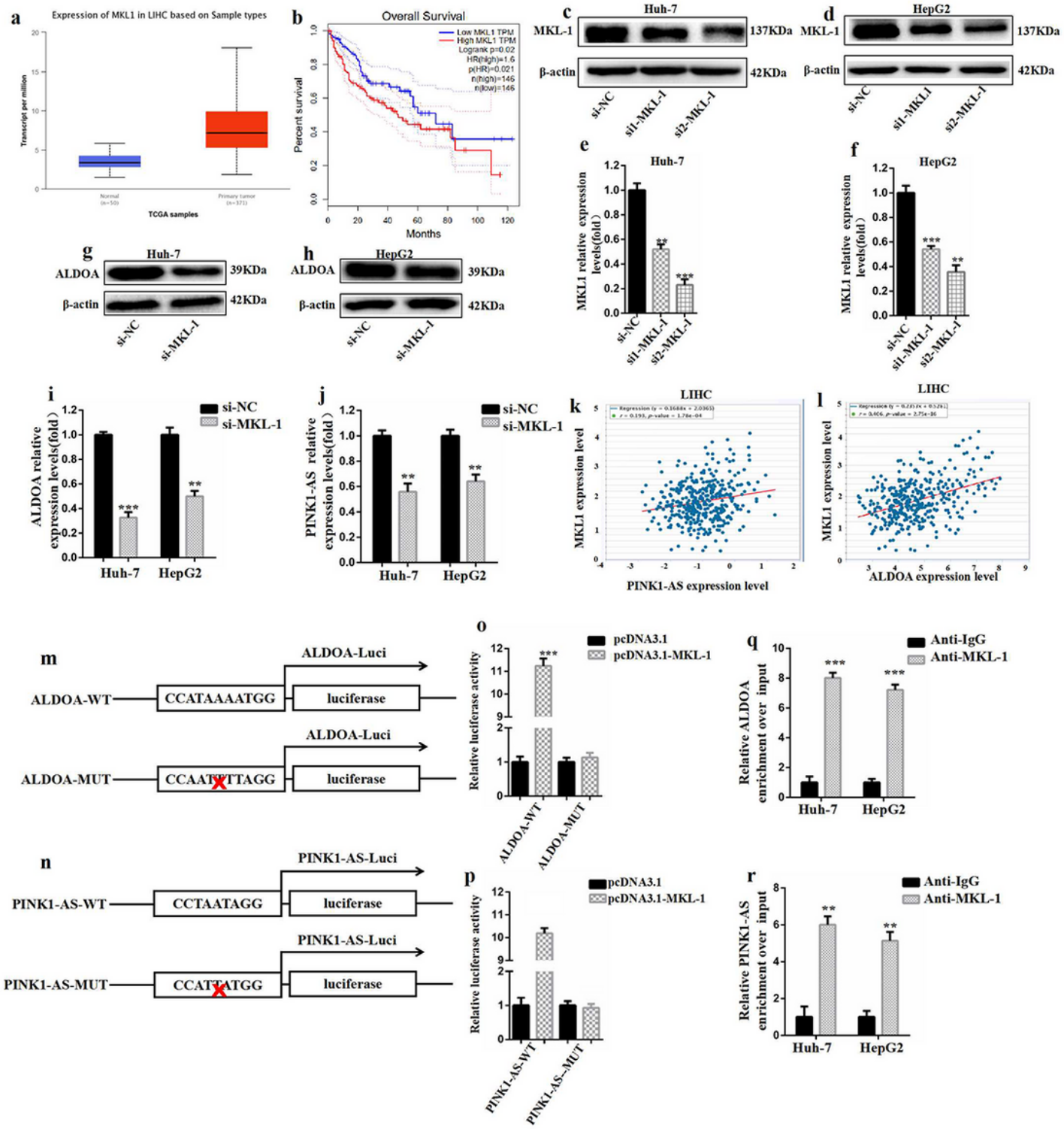

Figure 6

The promoter regions of PINK1-AS and ALDOA were located and analysed through the UCSC (https://genome.ucsc.edu/) website, the first 2000 bp upstream of the transcription start site was found to contain the MKL-1 binding site.Bioinformatics analysis shows that MKL-1 is up-regulated in HCC (Fig. 6a), The association between MKL-1 expression and survival outcomes of HCC patients was assessed using Kaplan-Meier survival curves, and analysis showed that patients with high MKL-1 expression had

poor elevated overall survival (Fig. 6b). To verify whether MKL-1 mediated the expression of PINK1-AS and ALDOA, we knocked down MKL-1 using siRNA targeting MKL-1 (Fig. 6c, d, e, f). MKL-1 downregulation resulted in a significant decrease in the expression of PINK1-AS and ALDOA in Huh-7 and HepG2 cells (Fig. 6g, h, i, j). Further, we found that silencing of MKL-1 significantly inhibited aerobic glycolysis in HCC cells (Fig. S5a, b). Correlation analysis suggested a positively correlation between the level of MKL-1 and ALDOA expression level in HCC tissue specimens (Fig. 6k). And the expression level of MKL-1 was positively correlated with PINK1-AS expression level (Fig. 6l). To investigate whether MKL-1 directly transcriptionally regulates ALDOA andPINK1-AS expression, we constructed ALDOA and PINK1-AS promoter luciferase reporter plasmids (Fig. 6m, n), and luciferase assays showed that MKL-1 could promote PINK1-AS and ALDOA promoter luciferase activity (Fig. 6o, p). In addition to this, ChIP verified MKL-1 binding to predicted sites in the PINK1-AS and ALDOA promoter regions (Fig. 6q, r).

Supplementary Files

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