DDX23 is a Metabolic Regulator in Hepatocellular Carcinoma

Jianlong Zhou  
Southern Medical University

Xiaoming Wang  
Guangzhou Medical College: Guangzhou Medical University

Jing Liang  
Guangzhou University of Traditional Chinese Medicine: Guangzhou University of Chinese Medicine

Chaohui Tan  
Southern Medical University

Changnan Chen  
Southern Medical University

Yunxiao Lin  
Southern Medical University

Feng Xie  
Southern Medical University

Zhong Chen  
Southern Medical University

Wenhua Huang  
wmwmw2014@126.com  
southern medical university

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**Abstract**

**Background:** Although biochemical activities of RNA helicases have been well-studied, physiological meaning of those factors in both normal and disease condition remained to be clarified.

**Methods:** RNA sequencing (RNA-seq) in HCC cells indicated DDX23 are highly expressed in HCC and high expression of DDX23 is responsible for poor survival of HCC patients. Next, The expression of DDX23 was establish for subsequent investigation. The role of DDX23 in HCC was identified by RNA-seq, RT-qPCR, LC-MS, OCR, ECAR. The effect of DDX23 on proliferative, Cloning information as well as tumorigenicity of transfected cells in mice was examined using loss-of-function experiments.

**Results:** Here, we investigated a new role of RNA helicase in a member of the DEAD box protein family, DDX23 in hepatocellular carcinoma (HCC). RNA level of DDX23 are highly expressed in HCC and high expression of DDX23 is responsible for poor survival of HCC patients. In addition, we demonstrated that DDX23 expression is important for in vitro and in vivo tumorigenesis. RNA sequencing (RNA-seq) in HCC cells indicated that metabolism is the most affected pathway by the DDX23 and most abundant DDX23-interacting RNA are involved in metabolism in HCC, especially glycolysis.

**Conclusions:** These findings provide new insights on the unexpected HCC-related role of DDX23, an opportunities for the development of the therapeutic target which is a master regulator of genes involved in HCC-favorable metabolic reprogram at the RNA level.

**Backgroud**

Hepatocellular carcinoma (HCC) represents the fifth most common cancer of males, and the eighth most common cancer in female candidates. HCC is second leading cause of cancer-related mortality in the world, responsible for approximately 700,000 deaths and more than 250,000 new cases annually. In aspect of etiology, HCC is well-defined disease, attributed by the viral infection and chronic alcohol abuse. While in the United States, Europe, Egypt and Japan, more than 60% of HCC is associated with HCV, about 20% is related to HBV, in Africa and Asia, 60% of HCC is associated with HBV, 20% is related to HCV [1–3]. However, there is no effective cure due to the resistance of HCC to conventional cytotoxic therapies such as radiotherapy and chemotherapy and the difficulties in early diagnosis. Surgical resection and transplantation are therapeutic options for patients with curable tumors at early stage, but relapse happen frequently, which is why it is urgent to find out the specific biomarkers for HCC early diagnosis and promising therapeutic targets. Recently, there were many trials seeking for effective therapeutic targets and diagnostic markers for HCC from metabolic alteration.

Mounting evidence showed metabolic alteration occurs in HCC, elevated in glycolysis, pentose–phosphate pathway (PPP), gluconeogenesis, and β-oxidation and decreased in mitochondrial oxidative phosphorylation, accompanied by the increase of glutamine and glutathione. Especially, in terms of energy metabolism, the increase in glycolysis and the decrease in mitochondrial oxidative phosphorylation in HCC tumors have been shown in several reports [4, 5]. Metabolic alteration in HCC in
part result from HBV and/or HCV infections. HCV enhances glycolysis as a compensatory machinery from impairment of mitochondrial oxidative phosphorylation and the induction of mycophagy [6, 7]. Also, HBV replication up-regulates several glycolytic enzymes and glycolysis associated-mammalian target of rapamycin (MTOR) signaling [8, 9]. However, there is no study to try understanding the metabolic reprogramming occurred in HCC in aspect of fundamental events in this disease, immunity raising from the interaction host cell and virus infection.

While the biochemical, physiological and pathological studies on DDX23 have remained to be followed. So far, no studies have explored potential roles DDX23 in Hepatocellular carcinoma. 80% of HCC is due to hepatitis B virus (HBV) and/or HCV infections, leading to chronic hepatitis and cirrhosis regarded as preneoplastic stages [1–3]. Here, we found that DDX23 is not only highly expressed in HCC tissue but also is responsible for cancer favorable metabolism.

**Method**

**Patients and tissue samples.** After obtaining adequate informed consent, hepatocellular carcinoma (HCC) tissue and adjacent normal tissue (ANT, exceeding the edge of the tumor by at least 2 cm) were obtained from 227 patients who underwent curative resection for HCC in the Department of Hepatobiliary Surgery, Nanfang Hospital Affiliated to Southern Medical University, Guangzhou, China, between November 2010 and May 2015. Patients were monitored for further survival analysis via outpatient follow-up or telephonic interviews. A relapse was diagnosed based on increased post-operative blood alpha-fetoprotein (AFP) levels and imaging tests (ultrasonic examination, computed tomography scan, or magnetic resonance imaging). The follow-up started at the date of surgery and was terminated on 31 August 2016. All patients satisfied the following inclusion criteria: they had not received any other treatments before this surgery; hepatitis B virus (HBV)-related HCC, without HCV) infection, was diagnosed pathologically and serologically; the surgical margins were confirmed to contain no residual carcinoma tissue. This study was approved by the Ethical Committee of Nanfang Hospital at Southern Medical University, and they were performed according to the Declaration of Helsinki (6th revision, 2008).

All samples were immediately frozen in liquid nitrogen and stored at −80°C until the extraction of RNA. For comparison of mRNA and survival rate in samples, total RNA was extracted by homogenizing each tissue in TRIzol reagent (Invitrogen, Carlsbad, CA), according to the manufacturer’s instructions. cDNA was synthesized using PrimeScript cDNA kit (Takara Bio, Kusatsu, Shiga, Japan). Quantitative real-time PCR was performed with an SYBR Premix Ex Taq (Takara Bio). The amplification profile was denatured at 95°C for 2 min, followed by 40 cycles of denaturation at 95°C for 5 seconds, annealing at 60°C for 34 sec. For internal control, 18S was used. The primers for 18S were 5′-GGCCCTGTAATTGGAATGAGTC-3′ (sense) and 5′-CCAAGATCCAACTACGAGCTT-3′ (antisense). The primer for DDX23 were 5′-GCTGTCATTGGTGGCATCTCCA -3′ (sense) and DDX23 5′-CCAGAACCACATAGGTACAGCG-3′ (antisense). The comparative cycle threshold (CT) method was applied to quantify the expression levels of mRNAs. The relative amount of either DDX23 was calculated using the equation $2^{-\Delta CT}$ where $\Delta CT = (CT \text{ either DDX23} - \text{CT 18S})$. All the statistical analyses were performed IBM SPSS Statistics 20.0 (IBM, IL, USA).
Two-sided P-values were calculated, and P<0.05 was considered to indicate a statistically significant result.

**Cell lines, virus, constructs, and transfection.** 293 FT cell line was purchased from Thermo Fisher Scientific. (Waltham, MA). Other cell lines were obtained from ATCC (American Type Culture Collection, USA) and maintained in our laboratory. All cell lines were cultured at 37°C, 5% CO\textsubscript{2} in DMEM (Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum (FBS) (HyClone, South Logan, UT). To establish cells stably silencing gene, lenti-viruses carrying short hairpin RNA (shRNA) in the pLKO.1 puro vector were used. Lenti-viruses were produced by co-transfection into HEK293FT cells with the pLKO.1 vectors, packaging plasmid psPAX2 (Addgene plasmid 12260, Addgene, Cambridge, MA) and envelope pMD2.G (Addgene plasmid 12259), using the PEI. For the doxycycline (Doxy)-inducible DDX23 knock-down, tet-pLKO-puro vector (Addgene #21915) was used. Media were changed 16 h after transfection, and cells were further incubated for 48 h. 72 h post-infection, cell debris in the media were removed by centrifugation at 2000 rpm for 10 min and lenti-viruses were concentrated by Lenti-X concentrator (Clontech, Mountain View, CA). One day before the infection, cells were plated in a six-well plate at a density of 1X10\textsuperscript{5} cells/well and transduced in the presence of 5 μg/ml polybrene for 72 h. The cells were selected for puromycin resistance (2 μg/ml) for 7 days.

**Cell growth assay**

For clonogenic assay, single cell suspensions of indicated cell lines were prepared by trypsinization and the indicated cell lines were seeded in 6-wells plates with a density of 500 cells per well and cultured for 2 weeks. Colonies were stained with crystal violet and counted.

Cell Counting Kit 8 (CCK8) assay was performed in 96-well plates. Briefly, cells were seeded (3× 10\textsuperscript{3} cells/well to 5 × 10\textsuperscript{3} cells/well) and cultured for 24 h-96 h. In each day, the CCK-8 solution was added to each well and cultured for 60 min, then the plates were measured at 450 nm in a microplate reader.

**Animal experiments**

All animal work was approved by Institutional Animal Care and Use Committee (IACUC) at UCSD and Southern Medical University. To establish tumor formation in NODSCID, cells were harvested, resuspended in 0.2 ml serum-free DMEM with Matrigel basement membrane matrix at a 1:1 ratio, and injected on the left and right flank of NODSCID mice subcutaneously (5 × 10\textsuperscript{6} each). For the evaluation of the impact of DDX23 depletion on tumorigenicity, tumors allowed to grow to a size of about 0.5 cm and mice bearing established tumors were into two groups. Measure the tumor size every day. For the PDX model, Subsequently, after injection cell into the liver, siRNA was injected intraperitoneally (i.p.) in every day. The control group was injected with the same volume of Control siRNA.

**Western blotting and co-immunoprecipitation**
Western blot was performed as previously reported [10]. Total protein of cells was extracted in lysis buffer (150 mM NaCl, 0.4 % triton X100, 0.2% NP-40, 10% glycerol 25 mM HEPES pH 7.4, phosphatase inhibitor cocktail, protease inhibitor cocktail) or cells were directly subjected to 1X protein sample buffer. Samples were separated on 8-15% SDS PAGE, and transferred to nitrocellulose membranes. The blots were blocked in blocking buffer (5% skim milk in PBS with 0.05 % Tween 20) then incubated with primary antibodies in the blocking buffer. After washing 3 times with blocking buffer, they were probed using a horseradish peroxidase-conjugated secondary antibody and developed with Supersignal West Pico or Dura (Thermo Fisher Scientific). The following antibodies were used using rabbit polyclonal anti-DDX23, mouse monoclonal anti–α-tubulin (T5168; Sigma-Aldrich), anti–rabbit IgG, HRP-linked antibody (7074S; Cell Signaling Technology), anti–mouse IgG, HRP-linked antibody (7076S; Cell Signaling Technology). The intensity of protein bands was quantified using ImageJ software.

Quantitative PCR analysis

RNA extraction and real time PCR analysis was performed as previously reported[11]. Total RNA was extracted using RNAeasy Mini Kit (Invitrogen) following the manufacturer's instructions. 1 μg of total RNA was reverse-transcribed using high capacity cDNA reverse transcriptase kit (Applied Biosystems, Foster City, CA). Real-time PCR was performed with an ABI Prism 7000 (Applied Biosystems) with FastStart Universal SYBR Green Master (Roche, Indianapolis, IN). The PCR conditions were as follows: 10 min at 95°C, 40 cycles of 15 s at 95°C, and 1 min at 60°C. The average Ct value for each gene was determined from triplicate reactions and normalized with the amount of β-actin.

Extracellular acidification rate (ECAR)

Glycolysis Stress Test (103020-100, Agilent, Santa Clara, CA) was performed with the Seahorse XFe96 Extracellular Flux Analyzer (Agilent, Santa Clara, CA). 2x10^4 cells were plated onto wells of a XF96 Cell Culture Microplate (102416-100, Agilent, Santa Clara, CA) and incubated overnight. Plates were equilibrated in unbuffered XF assay medium supplemented with 2 mM glutamine in the absence of CO₂ for 1 hr. Extracellular acidification rates (ECAR) were assayed by the serial addition of Glucose (10 mM), oligomycin (1 μM) and 2-deoxy-glucose (2-DG, 50 mM) to establish glycolysis (ECAR in response to glucose – ECAR before glucose injection), glycolytic capacity (ECAR in response to oligomycin – ECAR before glucose injection), glycolytic reserve (glycolytic capacity – glycolysis) and non-glycolytic acidification (ECAR before glucose injection). Each plotted value is the mean of at least 3 triplicate wells, and normalized to baseline ECAR and total protein levels. Data were presented as mean ± SD, and significance calculated by two-way ANOVA with Tukey's multiple comparison test.

Oxygen consumption rate (OCR).

Cell Mito Stress Test (103015-100, Agilent, Santa Clara, CA) was measured with the Seahorse XFe96 Extracellular Flux Analyzer (Agilent, Santa Clara, CA). 2x10^4 cells were plated onto each well of a XF96 Cell Culture Microplate (102416-100, Agilent, Santa Clara, CA) and incubated overnight. The plates were equilibrated in unbuffered XF assay medium supplemented with 10 mM glucose and 2 mM glutamine in
the absence of CO\textsubscript{2} for 1 hour. Oxygen consumption rates (OCR) were interrogated by a serial addition of oligomycin (1 \textmu M), carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP, 1.5 \textmu M) and rotenone/antimycin A (Rot/AA, 0.5 \textmu M) to establish basal respiration (OCR before oligomycin injection – OCR in response to Rot/AA), ATP production (OCR before oligomycin injection – OCR in response to oligomycin), maximal respiration (OCR in response to FCCP – OCR in response to Rot/AA), spare capacity (maximal respiration – basal respiration) and proton leak (OCR in response to oligomycin – OCR in response to Rot/AA). Each plotted value is the mean of at least 3 triplicate wells and normalized to baseline OCR and total protein levels. Data are presented as mean ± SD, and significance calculated by two-way ANOVA with Tukey's multiple comparison test.

Results

**DDX23 is up-regulated and corelated to survival in HCC patients.**

Considering HCC is chronic disease in which immunity is chronically activated, there is a possibility that up-regulated RNA helicases as a chronic effect from virus influence on host gene expression and cellular phenotypes since they can modify vast RNA. In supporting this notion, DDX23 were found in the HCC-associated up-regulated gene group, which were identified from the transcriptional dataset newly assembled from the 225 HCC tissues and 220 non-cancerous liver tissues, which obtained from gene expression datasets of Gene Expression Omnibus (GEO) database (available at [http://www.ncbi.nlm.nih.gov/geo/](http://www.ncbi.nlm.nih.gov/geo/)). (Fig. 1a). Comparison of the level of DDX23 revealed HCC have higher expression of DDX23 than non-tumor liver tissues (Fig. 1b). Patients with elevated DDX23 expression had significantly decreased recurrence-free survival (RFS) than the patients in low DDX23 expression group in Kaplan–Meier survival curves (log rank test, p = 0.022) (Fig. 1c). To gain insight into the clinical signature of DDX23 in HCC, we analyzed cohort of patients resected for HCC at Nanfang hospital of southern medical school in Guangzhou, China. Compared to paired ANT tissues, the expression of DDX23 is significantly higher in HCC tissues (Fig. 1d). The increased expression of DDX23 was observed in 83\% (173/227) of patients. These data indicated that DDX23 is not only highly expressed but also independent prognostic marker in HCC.

**DDX23 is necessary for \textit{in vivo} and \textit{in vitro} tumorigenesis in HCC.**

Since DDX23 is upregulated in HCC and corelated to HCC patient survival, we next tested whether DDX23 have impact on the HCC tumorigenicity. DDX23 were silenced in hepatoma cells, PLC/PRF/5 cells by transduction of lenti virus harboring shRNA against either DDX23 (DDX23 KD) or nonspecific scramble shRNA (SC) (Fig. 2a,2d). The depletion of DDX23 expression caused the significant reduction of proliferation and colony-forming in HCC cells (Fig. 2b,2c).

Consistent to the result from in vitro growth assay on DDX23, the level of DDX23 in HCC cells impacted on the xenografted mouse model. To determine the effects of DDX23 expression on HCC tumorigenicity, we made isogenic stable DDX23 KD PLC/PRF/5 cells transduced with lenti virus harboring pLKO-DDX23
shRNA, In vivo xenograft, Hepatoma cells showed consistently result that the knockdown of DDX23 gene impaired tumor growth in vivo (Fig. 2e, 2f). Altogether, it suggested that high level of DDX23 is important for HCC tumorigenicity.

**DDX23 is important for glycolysis in tumor**

To elucidate the molecular mechanism underlying DDX23-mediated tumorigenesis in an unbiased way, we performed RNA-seq on DDX23 inducible knockdown HCC cells which we had confirmed in vivo tumorigenesis model. Significantly 4467 different expressed genes (DEG; fold change (FC) > 2.0) between Control and DDX23 knockdown were significantly enriched in metabolism in KEGG-functional annotation (Fig. 3c). To further clarify the physiological relevance of DDX23 function from metabolism. The decrease of majority of glycolysis genes such as Glut1, PFKL, HK2, GAPDH, GPI, TPI, ENO1, ENO2, PKM2, and LDHA were validated in independent analysis of gene expression by qPCR in HCC cells depleting DDX23 (Fig. 3b). Of note, an alternative splicing form of PKM2, PKM1 was not decreased, implying the post-transcriptional regulation.

**Enhanced glycolysis normalizes the impaired tumor growth by DDX23 knockdown**

Next, we tested if glycolysis regulated by DDX23 are involved in tumorigenesis or not. To do end, we tried to normalize glycolytic metabolism in the DDX23 knockdown HCC cells. Since there were many glycolytic genes were reduced in the DDX23 knockdown HCC cells, Consistent with the finding that the DDX23 knockdown reduces the expression of glycolytic genes, the silence of DDX23 expression decreased glycolytic activity and increases oxidative phosphorylation, indicating that DDX23 drives the metabolic shift from oxidative phosphorylation to glycolysis (Fig. 4c-e). These results suggested that the glycolysis regulated by DDX23 is important for tumorigenicity in HCC. PDX model with induce knockdown DDX23 expression had significantly decreased survival than control group in Kaplan–Meier survival curves (log rank test, p = 0.0343) (Fig. 4f).

**Discussion**

Although the biochemical activity and underlying mechanism of DDX23 have been unveiled in many previous studies, physiological and pathological roles of DDX23 were largely unknow. As the works expanded from the basic role of DDX23, sensing a subset of RNA, anti-viral activity such as detecting foreign viral RNA and permit degradation it was proposed [12]. Here, we for the first time reported that DDX23 known as a RNA helicase on exosome complex is a regulator of metabolism in HCC, especially glycolysis. In HCC patients and multiple HCC cells, DDX23 was up-regulated and the highly expressed DDX23 is responsible for the maintain tumorigenicity and glycolysis. As the underlying mechanism, there were regulation of many glycolysis enzymes.

As metabolic genes additionally to PKM, which potential direct target of DDX23 in HCC, there were PKM and HK2. Due to the lack of effective therapies and late diagnosis, it is rushing to find out new promising target for liver cancer. As such efforts, dysregulated metabolism in HCC is being paid attention on both
remarkable markers and targets. Our studies on the new metabolic regulator, especially by the direct binding to RNA would provide new opportunity to expand the border to seek for therapeutic target and understand cancer metabolism in terms of RNA metabolism.

Conclusions

In summary, our study reveals new insights on the unexpected HCC-related role of DDX23, an opportunities for the development of the therapeutic target which is a master regulator of genes involved in HCC-favorable metabolic reprogram at the RNA level.

Abbreviations


Declarations

Acknowledgments

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Authors’ contributions

W.H. and Z.C. conceived the study. J.Z., X.W., J.L., C.T., and C.C. performed experiments . J.Z., X.W., J.L., wrote the manuscript with contribution from all the authors. All the authors have approved the final version of the manuscript.

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Ethics approval and consent to participate

Ethics Committee in Nanfang Hospital Affiliated to Southern Medical University. Animal experiments were implemented following the ethical standards of animal experiment ratified by Animal Management Committee in Southern Medical University.

Availability of data and material

The datasets used and/or analyzed in the current study are attained fromthe corresponding author on reasonable request.
Competing interests

The authors declare no conflict of interest.

Consent for publication

No applicable

References


Figures

DDX23 is highly expressed and a prognostic factor in HCC patients. (a, b). The analysis of DDX23 in none tumor tissue (n = 220) and hepatocellular carcinoma (HCC) patient tissue (n=225) of dataset from human derived-data base (GSE14520). DDX23 (white line) is one of the enriched gene in HCC. The expression of DDX23 in HCC was compared to the one in none tumor tissue as a log2. Values represent means ± s.d. and Mann-Whitney test used to calculate the statistical significance. P=7.4464e-37. (c). Based on DDX23 expression, postoperative recurrence-free survival (RFS) rate was analyzed with the Kaplan-Meier method, and differences in survival rates were assessed with the log-rank test (Mantel Cox). The survival
probability of the patients with high DDX23 is significantly lower than those with lower DDX23 (p=0.022).

(d) The expression of DDX23 HCC samples (n=227) and ANT samples (n=227) were determined by RT-qPCR assay. 18S was used as an internal control. Fold change (FC) of HCC over ANT in individual patients was displayed in Log2. 83% (54/227) patient have the significant increase of DDX23 expression.

Figure 1

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**Figure 2**

DDX23 is required for tumor growth in vitro and in vivo. (a, b,d) The knockdown of DDX23 gene in PLCR/PRF5 cells reduced the cell growth in vitro. The hepatoma cell PLCR/PRF5 cells was transduced by lentivirus harboring either shRNA targeting DDX23 (DDX23 KD) or non-specific scramble shRNA (Sc) and analyzed for cell growth by CCK8 assay from 24h to 96 hours. The efficiency of knockdown of DDX23 was confirmed by western blotting (WB) for DDX23 and tubulin as an internal control. (c) Knockdown of DDX23 gene inhibited colony formation of PLCR/PRF5 cells. Representative images show colonies stained with crystal violet. The number of colonies in each well was counted. n=3. Values represent the mean±s.d. unpaired t-test was used to calculate the statistical significance. P<0.0001 (d) DDX23 control and knockdown are established in PLC/PRF5 cells by the transduction of lentivirus harboring DDX23 shRNA or DDX23 shControl. DDX23 was confirmed by QPCR for DDX23. (e, f) PLC/PRF5 SC and KD were injected into the both flanks of NSG mice and tumors were allowed to grow to a size of about 0.5 cm. Subsequently, each individual tumor volumes were tracked every day, measures ANOVA, followed by Bonferroni post-tests. PLC/PRF5 cell xenograft showed significant difference. At the end of the treatment,
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DDX23 has a role for glycolysis in HCC. (a, b, c) RNA-seq data of inducible DDX23 KD PLC/PRF/5 cell were cut-off by fold change (2.0) and corrected p values (p<0.05) and applied to enriched pathway analysis. (a) Significantly 4467 different expressed genes (DEG; fold change (FC) > 2.0) between Control and DDX23 knockdown were significantly enriched in metabolism in KEGG-functional annotation. (b) Cut-off genes were applied to metabolism pathway in (c) Validation of the expression of genes involved in glycolysis. The relative expression of glycolysis gene in inducible DDX23 KD PLC/PRF/5 cells with
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**Figure 4**
DDX23 is important for maintaining cancer metabolism in HCC (a,b) ECAR in control cells and DDX23 KD cells in response to glucose (10 mM), oligomycin (1 μM), and 2-DG (50 mM). n = 8 independent experiments. Data are presented as mean value ± s.d., two-way ANOVA with a Tukey’s multiple comparison test. p value is indicated. (c,d) Oxygen consumption rate (OCR) in control cells and DDX23 KD cells in response to oligomycin (1 μM), fluorocarbonyl cyanide phenylhydrazone (FCCP, 1.5 μM), rotenone/antimycin A (Rot/AA, 0.5 μM). n = 8 independent experiments for each group. Data are presented as mean value ± s.d., two-way ANOVA with Tukey’s multiple comparison test. p value is indicated. Source data are provided as a Source Data file. (e) Levels of some metabolites of glycolysis pathways tended to be decrease in Inducible knockdown of DDX23 in PLC/PRF/5. n=4. Values represent the mean ± s.d. Unpaired t-test. ** p< 0.01 (f) HCC tissue is dissociated into cell suspension and subsequently implanted into NSG mice, F3 were used for assay by daily i.p. injection of siRNA–lipid complexes (50 mg/kg body weight) for indicated days. The DDX23 expression levels were inversely correlated with the percent survival. The differences in survival rates were assessed with the log-rank test (Mantel Cox). The survival probability of the treatment with SC RNAi levels (n=6) is significantly lower than those with siDDX23(n=6). * p< 0.05.
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