Translational Oncology 20 (2022) 101421

Contents lists available at ScienceDirect

Translational Oncology

journal homepage: www.elsevier.com/locate/tranon

Original Research

AKR1C1 promotes non-small cell lung cancer proliferation via crosstalk between HIF-1α and metabolic reprogramming

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ARTICLE INFO

Keywords:
HIF-1α
AKR1C1
Metabolic reprogramming
Proliferation

ABSTRACT

Non-small cell lung cancer (NSCLC) ranks first among cancer death worldwide. Despite efficacy and safety priority, targeted therapy only benefits ~30% patients, leading to the unchanged survival rates for whole NSCLC patients. Metabolic reprogramming occurs to offer energy and intermediates for fuelling cancer cells proliferation. Thus, mechanistic insights into metabolic reprogramming may shed light upon NSCLC proliferation and find new proper targets for NSCLC treatment. Herein, we used loss- and gain-of-function experiments to uncover that highly expressed aldo-keto reductase family1 member C1 (AKR1C1) accelerated NSCLC cells proliferation via metabolic reprogramming. Further molecular profiling analyses demonstrated that AKR1C1 augmented the expression of hypoxia-inducible factor 1-alpha (HIF-1α), which could drive tumour metabolic reprogramming. What's more, AKR1C1 significantly correlated with HIF-1α signaling, which predicted poor prognosis for NSCLC patients. Collectively, our data display that AKR1C1 reprograms tumour metabolism to promote NSCLC cells proliferation by activating HIF-1α. These newly acquired data not only establish the specific role for AKR1C1 in metabolic reprogramming, but also hint to the possibility that AKR1C1 may be a new therapeutic target for NSCLC treatment.

Introduction

Non-small cell lung cancer (NSCLC) accounts for ~85% lung cancer, ranking leading death among cancer worldwide [1]. Although remarkable advances in NSCLC treatment, tumour growth and proliferation still account for treatment failure and high mortality rates in NSCLC.

Abbreviations: AKR1C1, aldo-keto reductase family 1 member C1; AKR1C2, aldo-keto reductase family 1 member C2; AKR1C3, aldo-keto reductase family 1 member C3; AML, acute myeloid leukemia; CHX, cycloheximide; DMSO, dimethylsulfoxide; GFPT1, glutamine-fructose-6-phosphate transaminase 1; GOT1, glutamic-oxaloacetic transaminase 1; GOT1L1, glutamic-oxaloacetic transaminase 1 like 1; GRIK2, glutamate ionotropic receptor kainate type subunit 2; HIF-1α, hypoxia-inducible factor 1-alpha; HK1, hexokinase 1; HK2, hexokinase 2; H&E, hematoxylin and eosin staining; IHC, immunohistochemistry; KEGG, Kyoto Encyclopedia of Genes and Genomes; LDHA, lactate dehydrogenase A; LDHB, lactate dehydrogenase B; LDHD, lactate dehydrogenase D; MSCs, mesenchymal stromal cells; NSCLC, non-small cell lung cancer; PFS, progression-free survival; PVDF, polyvinylidene fluoride; qRT-PCR, quantitative real-time polymerase chain reaction; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; SQSTM1, sequestosome 1; SRB, Sulforhodamine B.

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https://doi.org/10.1016/j.tranon.2022.101421
Received 12 January 2022; Received in revised form 8 March 2022; Accepted 5 April 2022

Therefore, it is urgent to uncover the molecular mechanisms underlying NSCLC proliferation and find new better therapeutic targets.

Aberrantly expressed or activated genes have long been the center for finding therapeutic targets in NSCLC, leading to advances in NSCLC treatment [2–4]. In our previous works, we focused on aldo-keto reductase 1, family member, C1 (AKR1C1), highly expressed in NSCLC [5–7]. Among human aldo-keto reductase family, AKR1C1-AKR1C3 share high homology [8]. Mounting evidence reveals that AKR1C1 upregulates in many types of cancers, especially in NSCLC, predicting the overall survival of patients [5,9,10]. Reductase endows AKR1C1 with resistance to cisplatin or methotrexate in human colon cancers and to daunorubicin in leukemic cells [11,12]. By interfering with tyrosine protein kinase signaling, AKR1C1 possesses the ability to promote metastasis of bladder cancer and NSCLC cells [5,13]. For further research, AKR1C1 also participates in autophagy modulation, which may be attributed to directly binding with sequestosome 1 (SQSTM1) [6]. Interestingly, AKR1C1 ectopic expression could lead to malignant transformations and proliferation in NIH-3T3 cells whereas the underlying mechanisms remain enigmatic [14].

Proliferation is one of the hallmarks of cancer [15]. With the development of cancer research, genomic alterations are not sufficient to explain how cancer cells rapidly divide. Recently, metabolic reprogramming emerges as an important piece during the process of cancer cell proliferation [16]. Metabolic reprogramming is mainly featured by aberrant glucose metabolism and glutaminolysis, to maintain cellular bioenergetics and macromolecular biosynthesis, creating a perfect nest for tumour cell proliferation [17–19]. Among accumulated metabolites in the tumour microenvironment, lactate functions as not only a byproduct of metabolic remodeling, but also a signaling molecule involved in cancer cell survival, proliferation and metastasis [20–23]. However, whether AKR1C1 could interfere lactate production to modulate the metabolic reprogramming for NSCLC is unknown.

As an important signaling molecular, lactate is capable of stabilizing hypoxia-inducing factor 1 (HIF1) [21,24]. The HIF1 transcription factors account for primary transcriptional responses to hypoxia (< 2% O_2) or hypoxia-mimic stress (Warburg effect, gene mutation...) [24]. The activity of HIF1 requires HIF-1α, which is essential for tumour growth and progression [25]. Higher levels of HIF-1α protein are observed in various kinds of human cancers compared to their normal tissues, correlating with relapse in cervical cancer, endometrial carcinoma, ovarian cancer and NSCLC [25,26]. Once activated in tumours, HIF-1α drives dysregulation of metabolism via targeting glucose transporters and glycolytic enzymes, resulting in augmented lactate production to fertilize cancer cells [27]. Consistent with this, glucose transporters and glycolytic enzymes are continuously overexpressed in >70% of cancers [23]. Therefore, HIF-1α plays a vital role during metabolic reprogramming, which attracts attention to the modulation of HIF-1α signaling cascade, shedding light upon new better therapeutics to target metabolic reprogramming.

In this current study, we found that AKR1C1 promoted the proliferation of NSCLC cells. Further mechanism studies revealed that AKR1C1 knockdown decreased lactate production and the expressions of HIF-1α target genes by modulating HIF-1α protein level in NSCLC cells, highlighting a candidate strategy for AKR1C1-dominated NSCLC to target metabolic reprogramming.

Materials and methods

Chemicals

Cycloheximide (CHX, HY-12320, CAS No. 66-81-9) and Sulforhodamine B (SRB, S1402-25G, CAS No. 3520-42-1) were purchased from Sigma-Aldrich Trading Co.Ltd. (Shanghai, China). PX478 2HCl (T6961, CAS No. 685898-44-6) and IDF-11774 (T5537, CAS No. 1429054-28-3) were bought from TOPSCIENCE (Shanghai, China). CHX, PX478 2HCl and IDF-11774 were dissolved in DMSO and stored at −80°C.

Cell lines and culture conditions

Cell lines were from Shanghai Institutes for Biological Sciences (Cell Bank of China Science Academy, Shanghai, China). All these cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (RPMI 1640, Gibco, Grand Island, New York, USA) supplied with 10% fetal bovine serum (FBS, Gibco, Grand Island, New York, USA). Hypoxic (1% O_2) or normoxic (20% O_2) culture conditions were established in an incubator (Forma Scientific, Inc., Marietta, Ohio, USA) at 37 °C. The hypoxic conditions (1% O_2) contained a humidified mixture of 1% O_2, 5% CO_2 and 94% N_2. The normoxic conditions (20% O_2) were in a humidified atmosphere with 5% CO_2.

Microarray analysis

Cells (AKR1C1 siRNA and NC siRNA samples) were lysed in TRIzol reagent (15596026, Invitrogen, Carlsbad, California, USA) and frozen at −80°C. Two days later, samples were analyzed by microarray technology (SurePrint G3 Human Gene Expression 8× 60K v2, Agilent Array®). Cluster 3.0 and TreeView were the software to analyze gene expression profiles.

Immunohistochemical assay

Paraffin-embedded tumour tissues were dewaxed and rehydrated. The antigen retrieval for the tumour tissues was achieved by using a microwave oven in sodium citrate buffer (pH 6.0) for 30 min. According to the manufacturer's protocol, a SP Kit (KIT 9710, MAIXIN, Fuzhou, Fujian, China) was applied to the pretreated tumour tissues. AKR1C1 antibody (GTX105620, GeneTex, Irvine, California, USA) was diluted in 1:2000. Images were photographed by an inverted microscope (Leica DMI 4000 B, Wetzlar, Germany). Further analyses were conducted by Image-Pro Plus software. (Normal lung tissue: n = 8; Cancerous lung tissue: n = 56, 46 cases of NSCLC and 10 cases of small cell lung cancer). The tissue microarrays were from US Biomax (Maryland, USA).

Western blotting

Cells were immediately lysed in 1.25× Loading buffer (5× loading: 62.5mM Tris-HCl, 0.1% bromophenol blue, 5% mercaptoethanol, 10% SDS, 50% glycerol, pH 6.8), then boiled at 95°C for 15 min and stored at −80°C. Samples were subjected to wells of sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, Massachusetts, USA). Membrane was blocked with 5% skimmed milk for 30 min and washed with T-PBS (including 1‰ Tween-20 in phosphate-buffered saline) for 3 times (15 min, 5 min, 5 min). Then membrane was incubated in primary antibodies overnight. Primary antibodies: β-Actin (1:1000, sc-1615, Santa Cruz), AKR1C1 (1:5000, GTX105620, GeneTex), LDHA (1:1000, sc-137244, Santa Cruz), HIF-1α (1:1000, 610958, BD Biosciences). Secondary antibodies were incubated for 1 h at room temperature. Washed for three times (15 min, 5 min, 5 min), the membrane was detected according to ECL detection reagent instructions. Images were photographed using Amersham Imager 600 (GE Healthcare, Fairfield, Connecticut, USA) or GelView 6000M (Biolight Biotechnology, Guangzhou, China). The samples used for HIF-1α detection were divided into several tubes and were stored at −80 °C avoiding thaw.

Colony formation

Cells were infected with lentivirus and incubated for three days. Then cells were seeded into 6-well or 24-well plates (500 cells/well or 1000 cells/well) and were also harvested to confirm the overexpression efficiency. Fresh medium was replenished every three days. 18-29 days later, medium was removed and cells were fixed with 10% (wt/vol)

trichloroacetic acid for 2 h. Washed by double distilled water (DDW) for three times, plates were dried out at 60°C. Then cells were stained by SRB for 30 min and washing with 1% (vol/vol) acetic acid for five times. After dried out, the plates were photographed.

Plasmid transfection and RNA interference

Cell were seeded into 6-well plates at the confluency of 50% for transient overexpression and of 30% for transient knockdown. After 24-36 h, cells were transfected according to the manufacturer's instructions of Oligofectamine (12252011, Thermo Fisher Scientific, Rochester, New York, USA) or X-treme GENE HP DNA Transfection Reagent (6366546001, Roche, Mannheim, Germany). Additionally, all siRNA was obtained from GenePharma Co. Ltd (Shanghai, China). Table 1 contains the human short RNA target sequences.

Stable AKR1C1 silencing and overexpression were achieved by lentivirus-delivered technology. When cells were infected with lentivirus, the culture medium contain 5% FBS and 5 μg/mL polybrene (Sigma-Aldrich Trading Co.Ltd., Shanghai, China). After cultured for 24 h, cells were replenished with fresh medium.

Lactate measurements

NCI-H460 were seeded into 96-well plate at 4×10^3 cells/well. About 36 h later, cells were transfected with siAKR1C1/2/3 RNA and NC RNA for twice. Thencells were changed with fresh medium without FBS (RPMI 1640). Cultured overnight, cells were fixed with 10% (wt/vol) trichloroacetic acid followed by SRB assay. The culture medium was collected and subjected to lactate detection according to the lactate colorimetric/fluorometric assay kit (K607-100, Biovision, Milpitas, California, USA). The OD was determined at 570 nm for lactate colorimetric assay.

Quantitative reverse-transcriptase polymerase chain reaction

Total mRNA was extracted according to TRIzol reagent protocol (15596026, Invitrogen, Carlsbad, California, USA). 500 ng RNA was reversely transcribed into cDNA with oligo (dT) primers. The final dilution of cDNA used for quantitative real-time polymerase chain reaction (qRT-PCR) is 1/10. The process of qRT-PCR: Denaturation (95°C for 3 min); Amplification (40 Ct: 95°C for 3 s, 60°C for 31 s); Melting curve (95°C for 15 s, 60°C for 1 min); End (4°C). Oligonucleotide primers were synthesized by Shangya (Hangzhou, Zhejiang, China). The sequence of primers is provided in Table 2.

Proliferation assay

Cells were fixed by 10% trichloroacetic acid at 4℃ for at least 1 h. The plates were washed by DDW for 5 times. Then the plates were dried at 60℃ for 2 h. After that, cells were stained by SRB (2.0 g SRB was dissolved in 500 mL 1% acetic acid). After 30 min, cells in plates were washed by 1% acetic acid for 5 times followed by dehydration at 60℃ for 2 h. Finally, SRB were dissolved in tris base (0.6 g tris base was dissolved in 500 mL DDW). The OD were determined at 515 nm.

Table 1
The human short RNA target sequences.

Gene	Sequences
siAKR1C1#1	5'-AAGCTTTAGAGGCCACCAAAT-3'
siAKR1C1#2	5'-GCCACCAAATTGGCAATTGAA-3'
siAKR1C2#1	5'-CTGCTTGGCGACTTCAGTAAG-3'
siAKR1C2#2	5'-AGCTCTAGAGGCCGTCAAATT-3'
siAKR1C3#1	5'-CCAGAGGTTCCGAGAAGTAAA-3'
siAKR1C3#2	5'-CCTAGACAGAAATCTCCACTA-3'

Table 2
The oligonucleotide primers for qRT-PCR.

Gene	Sequences
AKR1C1	Sense: 5'-GTAAAGCTTTAGAGGCCAC-3'
	Antisense: 5'-GAGGTCAACATAATCCAATTG-3'
AKR1C2	Sense: 5'-CCTAAAAGTAAAGCTCTAGAGGCCGT-3'
	Antisense: 5'-GAAAATGAATAAGATAGAGGTCAACATAG-3'
AKR1C3	Sense: 5'-GAGAAGTAAAGCTTTGGAGGTCACA-3'
	Antisense: 5'-CAACCTGCTCCTCATTATTGTATAAATGA-3'
GOT1L1	Sense: 5'-CCACCCTTTCAGTGTTCATGG-3'
	Antisense: 5'-GCCTTCATTTGTCATGCAGACT-3'
GFPT1	Sense: 5'-AACTACCATGTTCCTCGAACGA-3'
	Antisense: 5'-CTCCATCAAATCCCACACCAG-3'
GRIK2	Sense: 5'-TTCAGGCGCACCGTTAAACT-3'
	Antisense: 5'-GCTCCCATTGGGCCAGATT-3'
GOT1	Sense: 5'-ATTTCTTAGCGCGTTGGTACA-3'
	Antisense: 5'-ACACAGCATTGTGATTCTCCC-3'
HK1	Sense: 5'-GCTCTCCGATGAAACTCTCATAG-3'
	Antisense: 5'-GGACCTTACGAATGTTGGCAA-3'
HK2	Sense: 5'-GAGCCACCACTCACCCTACT -3'
	Antisense: 5'-CCAGGCATTCGGCAATGTG-3'
LDHA	Sense: 5'-TTGACCTACGTGGCTTGGAAG-3'
	Antisense: 5'-GGTAACGGAATCGGGCTGAAT-3'
LDHB	Sense: 5'-CCTCAGATCGTCAAGTACAGTCC-3'
	Antisense: 5'-ATCACGCGGTGTTTGGGTAAT-3'
LDHD	Sense: 5'-AGGTGCGAACCTCCTGATG-3'
	Antisense: 5'-CGGTGCCGAATGGGATGAT-3'

Statistical analysis

Significance was determined by two-tailed Student's t-test, n = 3. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ns, $p \geq 0.05$. For nonlinear relationships, the significance was determined by logistic regression analysis, ns, $p \geq 0.05$. For Kaplan-Meier curves, the significance and hazard ratio (HR, High risk *vs.* Low risk) with 95% confidence interval (CI) were generated by log-rank tests and univariate cox proportional hazards regression, ns, Log-rank $p \geq 0.05$.

Results

High AKR1C1 is closely related to poor prognosis in NSCLC

By analyzing the Bioinformatica database (*http://bioinformatica.mty.itesm.mx:8080/Biomatec/SurvivaX.jsp*), including Botling Lung, Raponi Beer Lung, Hou Philipsen Lung and Larsen Lung, we found that the mRNA levels of *AKR1C1* significantly upregulated in tumour tissues with high risk for patient recurrence (high risk tumours *vs.* low risk tumours: p = 4.96e-69 for Botling Lung, p = 1.49e-42 for Raponi Beer Lung, p = 1.2e-18 for Hou Philipsen Lung, p = 3.84e-13 for Larsen Lung) (Fig. 1a). Considering the fact that NSCLC accounts for ~85% lung cancer [1], we speculated that AKR1C1 may play a vital role in NSCLC progression and associated with cancer risk, which encouraged us to explore the protein levels of AKR1C1 in NSCLC. As demonstrated by the hematoxylin and eosin staining (H&E) and immunohistochemistry (IHC) data, NSCLC tumours harbored higher AKR1C1 expression than normal lung tissues (Normal lung tissues: Log IOD = 3.105 ± 0.430, n = 8; NSCLC cancerous lung tissues: Log IOD = 4.338 ± 0.768, n = 46; p = 5.39e-05) (Fig. 1b). However, the protein of AKR1C1 was barely detectable in normal lung tissues (Fig. 1c). The demographic features and clinicopathological characteristics for all patients in this study were also shown in Table 3, including patient age, patient sex and tumour status. To analyze a possible influence of these parameters in AKR1C1 protein expression, we employed a logistic regression analysis. The results in Table 3 showed that the stage of lung cancer patients may has effects on AKR1C1 protein expression (p = 0.045), underscoring the important role of AKR1C1 during tumour progression. Collectively, these findings indicate that AKR1C1 overexpression is associated with NSCLC progression.

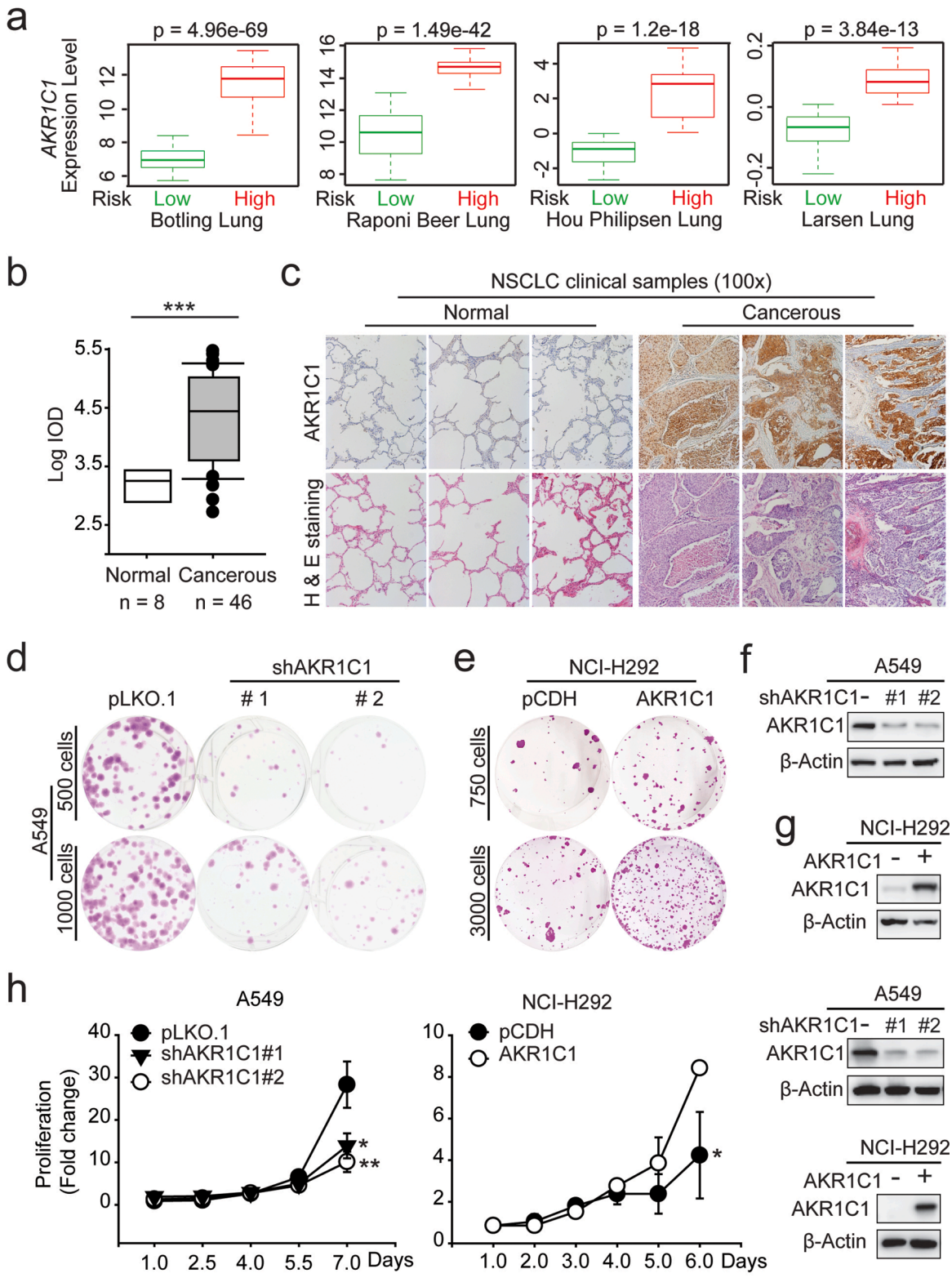

Fig. 1. AKR1C1 promoted NSCLC cell proliferation. a. AKR1C1 upregulated in high risk for lung cancer patient recurrence from several datasets. b. NSCLC tumour tissues displayed higher protein levels of AKR1C1 compared to normal lung tissues demonstrated by IHC staining. The expression levels were analyzed by Image-Pro Plus. (Normal lung tissues: n = 8; Cancerous lung tissues: n = 46) c. Representative images were photographed by a microscope (100×). d. A549 cells were infected with shAKR1C1 or pLKO.1 lentivirus. Three days later, cells were seeded into 6-well plate. Cultured for 3 weeks, A549 cells were subjected to SRB staining. e. NCI-H292 cells were infected with pCDH-AKR1C1 or pCDH lentivirus. Three days later, cells were seeded into 6-well plate. Cultured for 29 days, NCI-H292 cells were subjected to SRB staining. f. Knockdown efficiency of shAKR1C1 in A549. g. Overexpression efficiency of pCDH-AKR1C1 in NCI-H292. h. SRB staining was conducted to evaluated the proliferation abilities of NSCLC cells. Cells were infected with pCDH/AKR1C1 or pLKO.1/shAKR1C1 lentivirus. Two days later, 500/well cells were plated into 96-well plates. Cells were fixed by trichloroacetic acid at the indicated time followed by SRB assays. Statistical significance was determined by Student's t-test. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

Table 3
The demographic features and clinicopathological characteristics for lung cancer patients.

Items	p value of logistic regression analysis		Normal	Cancerous
Total		Number	n = 8	n = 56
Age	0.261, ns	Years old, mean ± SD	21.00 ± 0.00	57.07 ± 9.63
Sex	0.014, *	Female, n (%)	8 (100%)	12 (21.43%)
		Male, n (%)	0 (0.00%)	44 (78.57%)
Stage	0.045, *	I, n (%)	—	4 (7.14%)
		II, n (%)	—	32 (57.14%)
		III, n (%)	—	20 (35.71%)
		IV, n (%)	—	0 (0.00%)
Metastasis	—	Distant, n (%)	—	0 (0.00%)
		Lymph node, n (%)	—	52 (92.86%)
		Primary, n (%)	—	4 (7.14%)
Pathology	0.810, ns	Squamous cell carcinoma	—	21 (37.50%)
		Adenocarcinoma	—	21 (37.50%)
		Large cell carcinoma	—	4 (7.14%)
		Small cell lung cancer	—	10 (17.86%)

AKR1C1 accelerates tumour growth

Proliferation is a hallmark of cancer, including NSCLC [15]. It is thus pertinent to explore gateways to prevent cancer cell proliferation. Although recent work reported that AKR1C1 upregulated in mesenchymal stromal cells (MSCs) to promote the survival of acute myeloid leukaemia (AML) cells, still it remains an enigma for the role of AKR1C1 in NSCLC proliferation [28].

In the present study, we conducted colony formation assay and SRB staining to evaluate the role of AKR1C1 in NSCLC proliferation. Interestingly, AKR1C1 shRNA obviously impeded the colony formation ability of A549 cells (Fig. 1d and 1f). AKR1C1 overexpression conferred a growth advantage to NCI-H292 cells as shown in Fig. 1e and 1g. In SRB staining, A549 cells in shAKR1C1 groups grew slower than the pLKO.1 group at the 7.0th day (13.899 ± 2.933 for shAKR1C1#1 group, p=0.015690; 10.130 ± 2.416 for shAKR1C1#2 group, p=0.006160; 28.304 ± 5.447 for pLKO.1 group) and NCI-H292 cells in AKR1C1 groups grew faster than the pCDH group at the 6.0th day (8.429 ± 0.286 for AKR1C1 group, p=0.025952; 4.238 ± 2.082 for pCDH group) (Fig. 1h). In conclusion, these results suggest that AKR1C1 could promote the proliferation of NSCLC and the underlying mechanisms need further research.

AKR1C1 involves in metabolic homeostasis in NSCLC cells

In order to unveil the underlying mechanisms of AKR1C1-induced proliferation in NSCLC, we implemented the microarray analyses to gain insights into this process. As shown in Fig. 2a, Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses indicated that AKR1C1 knockdown interfered several metabolic signaling pathways, including pyruvate metabolism (p=0.009469), cysteine and methionine metabolism (p=0.013447), fatty acid metabolism (p=0.032059) and HIF-1 signaling pathway (p=0.012342). Consistent with these data, a large number of metabolic-related genes were downregulated by siAKR1C1 in NCI-H460 cells (Fig. 2b).

For further study, qRT-PCR analyses were employed to confirm the transcription profile of these genes on the condition of AKR1C1 knockdown. Compared to the control group, mRNA levels were decreased to (0.996±0.461)-fold for glutamic-oxaloacetic transaminase 1 like 1 (*GOT1L1*) (p=0.683036), (0.201±0.026)-fold for glutamine-fructose-6-phosphate transaminase 1 (*GFPT1*) (p=0.002348), (0.449 ±0.097)-fold for glutamate ionotropic receptor kainate type subunit 2 (*GRIK2*) (p=0.014897), (0.135±0.001)-fold for glutamic-oxaloacetic transaminase 1 (*GOT1*) (p=0.001024), (0.322±0.024)-fold for hexokinase 1 (*HK1*) (p=0.008521), (0.194±0.062)-fold for hexokinase 2 (*HK2*) (p=0.024705), (0.187±0.010)-fold for lactate dehydrogenase A (*LDHA*) (p=0.000636), (0.231±0.015)-fold for lactate dehydrogenase B (*LDHB*) (p=0.000344) and increased to (2.950±0.434)-fold for lactate dehydrogenase D (*LDHD*) (p=0.024131) (Fig. 2c).

Collectively, our data reveal that AKR1C1 might steer NSCLC cells toward metabolic reprogramming.

AKR1C1 knockdown diminishes lactate production of NSCLC cells

Metabolic reprogramming is one of the hallmarks of cancer, accompanied with increased lactate production stemming from glucose metabolism and glutaminolysis, also with generated intermediates and energy to fuel cancer cells growth and proliferation [17,19,29]. Thus, lactate production is postulated as the indicator to monitor metabolism dysregulation [21,30,31].

To verify whether AKR1C1 is essential for metabolic reprogramming, we measured extracellular lactate production. As depicted in Fig. 3a, siRNA targeting AKR1C1 diminished extracellular lactate to (0.711 ± 0.070)-fold for siAKR1C1#1 (p=0.029683, *vs.* the control) and (0.692 ± 0.010)-fold for siAKR1C1#2 (p=0.001506, *vs.* the control), while both of the sequences specifically targeted AKR1C1 (Fig. 3b). Although sharing high homology with AKR1C1 [8], the knockdowns of AKR1C2 and AKR1C3 imposed little effects on extracellular lactate production in NCI-H460, which were displayed in Fig. 3c and 3d (0.896 ± 0.087 for siAKR1C2#1, p=0.238217, *vs.* the control; 1.240 ± 0.090 for siAKR1C2#2, p=0.064478, *vs.* the control; 0.825 ± 0.116 for siAKR1C3#1, p=0.168486, *vs.* the control; 0.912 ± 0.269 for siAKR1C3#2, p=0.692340, *vs.* the control).

Taken together, these data suggest that siAKR1C1 leads to the restraint of lactate production in NSCLC cells.

AKR1C1 enhances HIF-1α expression

Glucose transporters and glycolytic enzymes function as HIF-1α target genes to modulate lactate production [23,27]. In the above-mentioned findings (Fig. 2), KEGG analyses from the microarray data dictated the role of AKR1C1 in HIF1 signaling pathway (p=0.012342); further qRT-PCR analyses confirmed the effects of AKR1C1 knockdown on HIF-1α target genes (*HK2* and *LDHA*). Thus, HIF-1α may play a central role for AKR1C1-involved metabolic reprogramming in NSCLC.

We next asked whether HIF-1α protein level was affected by AKR1C1, because HIF-1α transcriptional activity is controlled primarily through protein stabilization and post-translational modification [32]. The canonical molecular mechanisms for HIF-1α protein stability underlie O_2-lability [32]. Thus, we first evaluated the effects of AKR1C1 on exogenous HIF-1α protein accumulated by hypoxia (1% O_2). As shown by western blotting in HIF-1α overexpressed NSCLC cells, AKR1C1 hindered exogenous HIF-1α protein degradation at the time of 6 min after 20% O_2 exposure, compared to the control group, (AKR1C1+HIF-1α)-group *vs.* (HIF-1α)-group, (Fig. 4a). This data indicated that AKR1C1 might prolong the half-life of exogenous HIF-1α protein. Then, we were encouraged to explore the effects of AKR1C1 on endogenous HIF-1α protein accumulated by hypoxia (1% O_2). At the time of 10 min after CHX treatment (a popular inhibitor of protein synthesis), hypoxia accumulated endogenous HIF-1α protein was totally degraded in the control group, whereas AKR1C1 hindered this process in the AKR1C1 overexpressed group, which may be attributed to enhanced HIF-1α expression (Fig. 4b). These data were consistent with that in

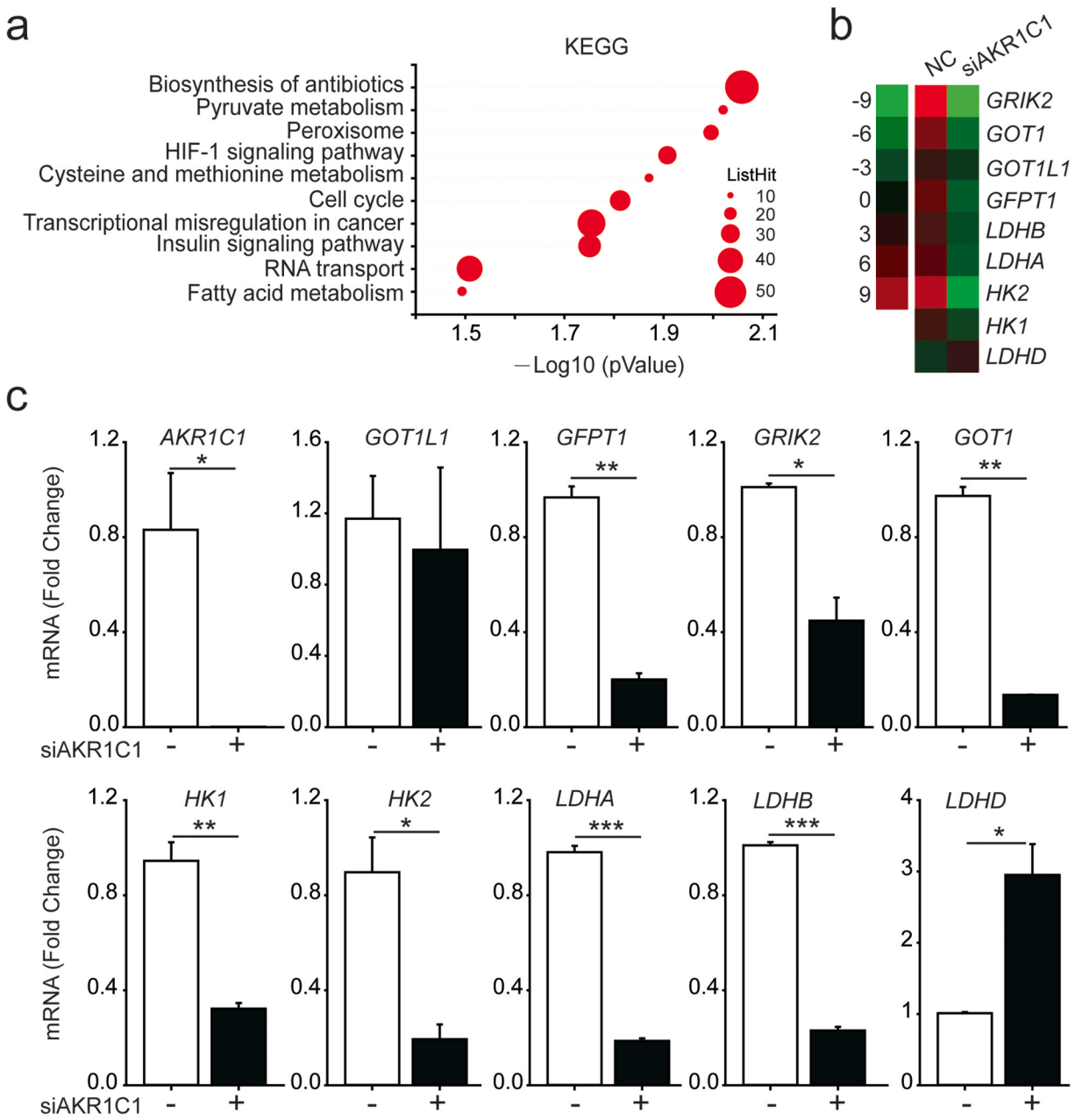

Fig. 2. AKR1C1 knockdown remodeled the expression profiles of metabolic genes. AKR1C1 siRNA was introduced into NCI-H460 cells for twice at the confluency of 60%. 36 h later, cells were lysed in TRIzol reagent followed by microarray analysis. KEGG pathway (a) and Heat map (b) from microarray data were shown. Genes with a fold-change ≥2 were used for KEGG analysis, including the most downregulated and upregulated genes (siAKR1C1 *vs.* NC). The ListHit for Biosynthesis of antibiotics was 53, for Pyruvate metabolism was 15, for Peroxisome was 25, for HIF-1 signaling pathway was 29, for Cysteine and methionine metabolism was 14, for Cell cycle was 33, for Transcriptional misregulation in cancer was 44, for Insulin signaling pathway was 36, for RNA transport was 41, for Fatty acid metabolism was 15. c. qRT-PCR technology was conducted to evaluate the levels of metabolic genes. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

Fig. 4c and 4d, blockade of AKR1C1 mitigated HIF-1α protein and AKR1C1 overexpression augmented HIF-1α protein under both normoxia and hypoxia.

We further found that expressions of AKR1C1 were correlated with several HIF-1α target genes in NSCLC from Wachi Lung (*www.oncomine.com, Gene: AKR1C1; Analysis type: cancer vs. normal analysis; data type: mRNA; sample type: clinical specimen*), including NDUFA4 mitochondrial complex associated like 2 (*NDUFA4L2*) (Correlation=0.891), pyruvate kinase M2 (*PKM2*) (Correlation=0.851) and *LDHA* (Correlation=0.851), which function as important elements in metabolic reprogramming [33, 34]. Therefore, AKR1C1 plays an important role in HIF-1α pathway (Fig. 4e). Meanwhile, AKR1C1 knockdown resulted in decreased transcriptional activity of HIF-1α as shown by impaired LDHA expression, the latter of which is one important glycolytic enzyme (Fig. 4f). To determine clinical relevance of AKR1C1-HIF-1α signaling in NSCLC patients, we analyzed the expression levels of *AKR1C1* and *LDHA* in NSCLC tumours divided by high or low expression groups (*www.aclbi.com*) . The progression-free survival (PFS) curves showed that lung patients with high expression of *AKR1C1-LDHA* possessed a significantly shorter PFS time compared to low expression group (Log-rank p=0.000162) (Fig. 4g). What's more, two HIF-1α inhibitors (IDF-11774 and PX-478) could impaired the growth advantage to NCI-H292 cells conferred by AKR1C1 transfection (Fig. 4h and 4i), which indicated that the HIF-1α inhibitor may phenocopy the effect of AKR1C1 silencing on NSCLC cells growth.

These observations collectively identify that AKR1C1 enhances HIF-

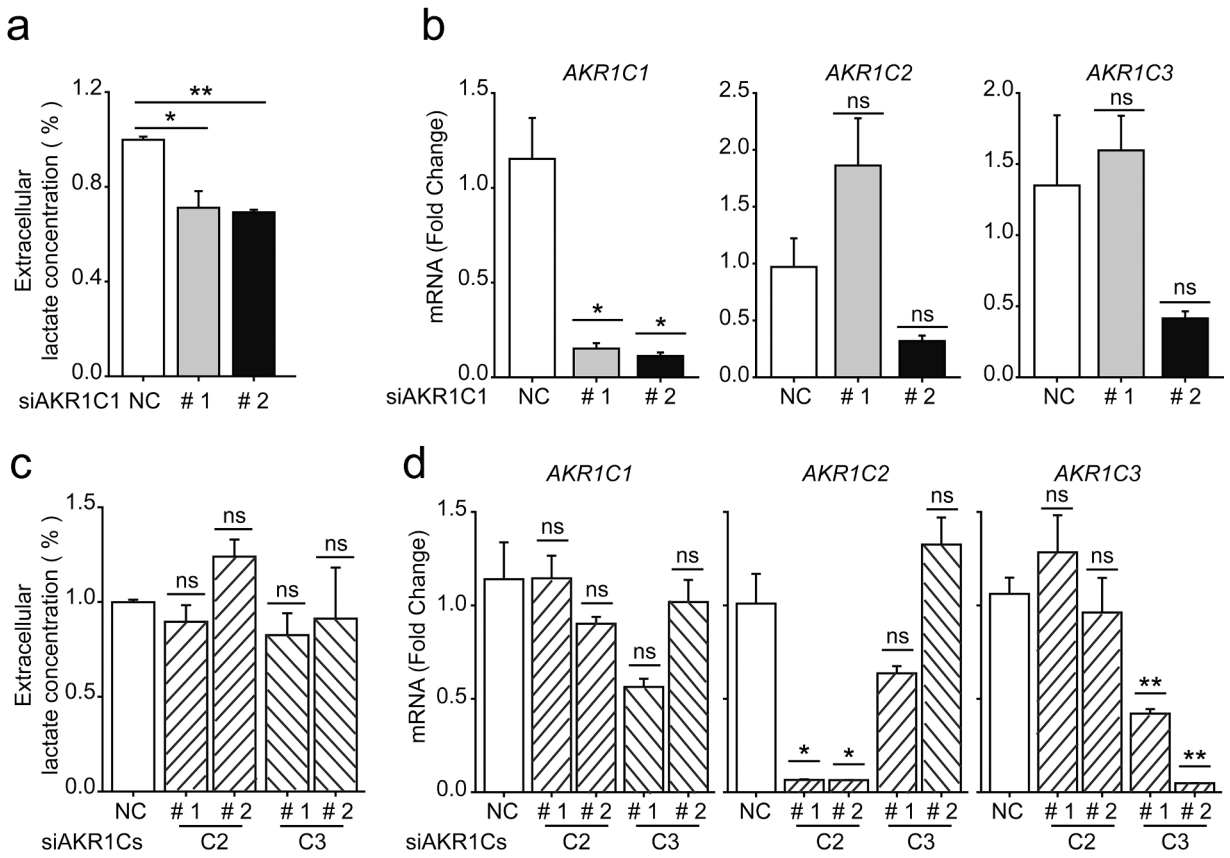

Fig. 3. Deletion of AKR1C1 impaired lactate production in NSCLC cells. (a and c) Lactate production in siAKR1C1, siAKR1C2, siAKR1C3 and NC cells. (b and d) AKR1C1, AKR1C2 and AKR1C3 expression levels were assessed by qRT-PCR. NCI-H460 were seeded into 96-well plate at 4×10^3 cells/well or into 6-well plate at 2×10^5 cells/well at the same time. 36 h later, siRNA targeting AKR1C1 were introduced into NCI-H460 for twice followed by 24 h incubation. The cells in 96-well plate were changed with fresh medium (RPMI-1640) overnight. Then cells were subjected to SRB assay and their culture medium was subjected to lactate detection. The cells in 6-well plate were subjected to qRT-PCR.

1α expression and functions as an important role in HIF-1α signaling pathway.

Discussion

Cancer cells are well-known for rapid proliferation, which is fuelled by biomass from metabolic reprogramming [15–19]. However, drugs for targeting metabolic reprogramming are limited, which emphasizes the mechanisms underlying cancer metabolic reprogramming. In this study, we conducted a microarray analysis to identify the role of highly expressed AKR1C1 in NSCLC and found that AKR1C1 rewired metabolic fluxes and modulated HIF-1α signalling to accelerate NSCLC cells proliferation.

Metabolic reprogramming has long been the focus of cancer precision treatment [17–19,29,35]. A successful case in point is the isocitrate dehydrogenase (IDH) inhibitors. Mutations in *IDH* genes occur in ~30% of all AML patients, leading to epigenetic dysregulation and differentiation blockade [36]. Several IDH inhibitors have been approved by Food and Drug Administration (FDA) for relapsed or refractory AML [36,37]. In addition, the novel IDH inhibitors have entered clinical trials in China as well. Another case is the development of inhibitors to target indoleamine 2,3 dioxygenase (IDO), which mediates kynurenine pathway of tryptophan degradation resulting in immune escape [38]. Due to promising results of epacadostat (an inhibitor of IDO) in Phase I/II clinical trials, Phase III clinical trial was sponsored to evaluate the regimen of epacadostat and pembrolizumab in melanoma [38,39]. However, targeting metabolic reprogramming seems to have its own dilemma. The Phase III clinical trial showed no additional benefits for IDO inhibitor, and IDH inhibitors monotherapy failed in solid tumours [37,39,40]. Recent evidence also showed that metabolic reprogramming is not always dependent on mutations in metabolic enzymes [41,42]. Further mechanistic insights into cancer metabolic reprogramming will reveal new therapeutic strategies to target metabolic reprogramming. Here, we uncover the new role of AKR1C1 to remodel metabolism in NSCLC cells to promote tumour growth via HIF-1α signalling pathway. These newly acquired data may help in the design of better therapeutics targeting metabolic reprogramming for the treatment of AKR1C1-dominated NSCLC.

As the central role of metabolic reprogramming is to promote cancer proliferation, it is thus pertinent to ask how metabolic reprogramming is harnessed by cancer cells [16–19,29]. Judging from accumulative evidence over the past years, the answer is oriented to a major hypoxia induced transcription factor HIF-1α [24,25,27]. Higher HIF-1α levels are observed in several solid cancers companied by strong association with poor prognosis [26]. In normoxia, HIF-1α is degraded in proteasome via its E3 ubiquitin ligase, the von Hippel-Lindau (VHL) protein, which could recognize hydroxylated HIF-1α [32]. With decreased hydroxylation by proline hydroxylase domains (PHDs) in hypoxia, HIF-1α accumulates and responses to hypoxia [32]. In fact, there exists spatial and temporal diversity within tumours and hyperactivation of HIF-1α is also observed in normoxic condition. Previous work has established that RAS proto-oncogene, GTPase (RAS) possesses the ability to regulate HIF-1α activity independent of the hypoxic condition [43–45]. A recent study suggested that NAD(P)H quinone dehydrogenase 1 (NQO1) hinders HIF-1α degradation under normoxic condition [46]. In line with these findings, our data reveal that overexpressed AKR1C1 in NSCLC elevates HIF-1α protein levels under both normoxic and hypoxic conditions (Fig. 4). All these results underscore the heterogeneity in tumour during

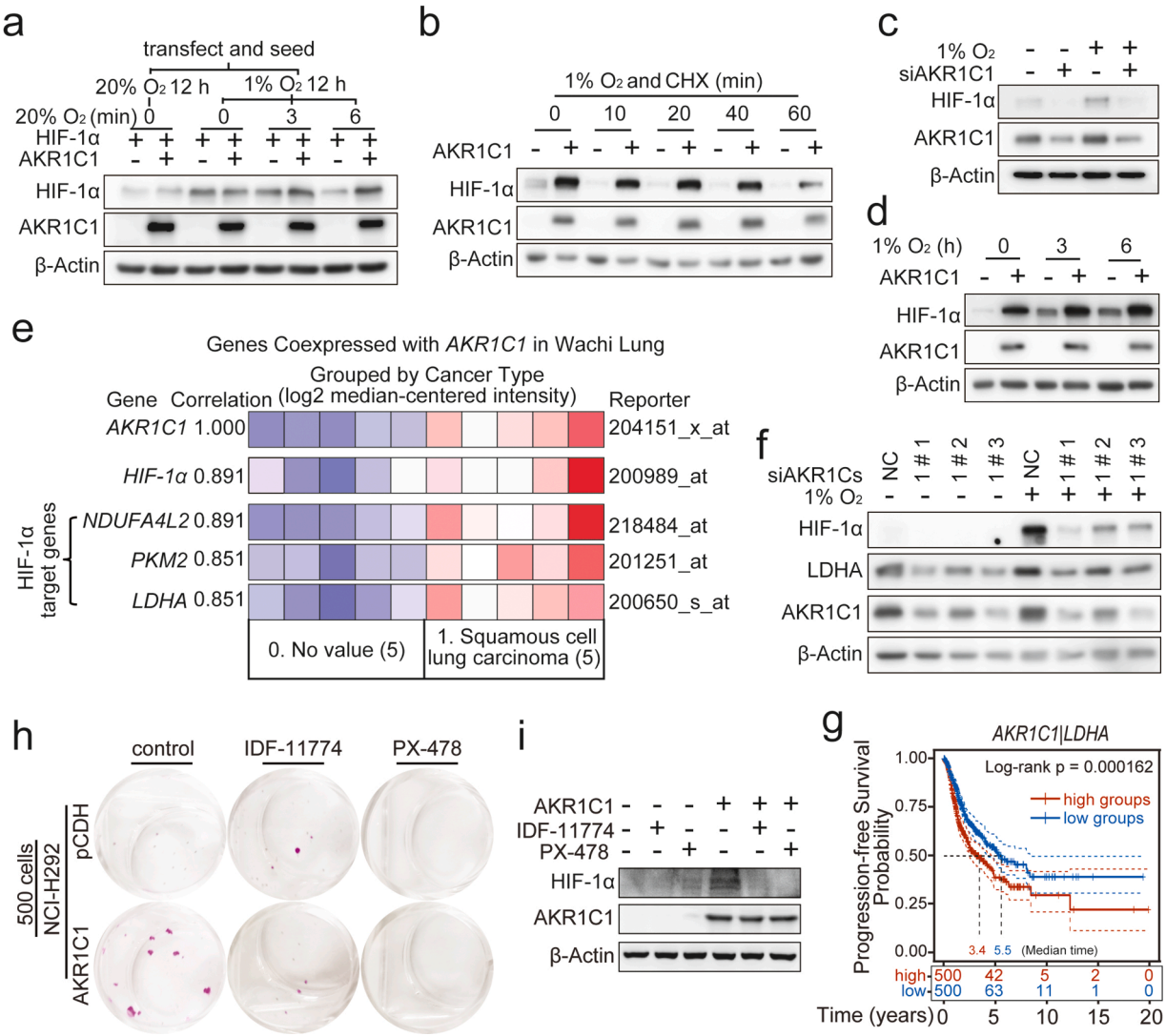

Fig. 4. AKR1C1 regulated glycolysis by enhancing HIF-1α signaling. a. AKR1C1 prolonged the half-life of exogenous HIF-1α protein. Cells were transfected with HIF-1α plasmid and AKR1C1 plasmid. After 36 h, a (AKR1C1+HIF-1α)-group and a (HIF-1α)-group were exposed to 20% O_2 for 12 h, and other groups were exposed to 1% O_2 for 12 h. Then the two groups, previously exposed to 20% O2 for 12 h, were harvested immediately. Other groups, previously exposed to 1% O2 for 12 h, were exposed to 20% O_2 for another indicated time (0 min, 3 min and 6 min) followed by quick harvest. b. AKR1C1 hindered the degradation of endogenous HIF-1α protein under normoxia. Cells were transfected with AKR1C1 plasmid. After 36 h, cells were treated with 50 μg/mL CHX and exposed to 1% O_2 for the indicated time (0 min, 10 min, 20 min, 40 min and 60 min) followed by quick harvest. c. AKR1C1 knockdown decreased the expression of HIF-1α. Cells were transfected with siAKR1C1 for twice. Then cells were exposed to 1% O_2 overnight followed by quick harvest. d. AKR1C1 augmented HIF-1α protein expression. Cells were transfected with AKR1C1 plasmid. After 36 h, cells were exposed to 1% O_2 for indicated time (0 h, 3 h and 6 h) followed by quick harvest. e. AKR1C1 closely correlated with the expression of HIF-1α-target genes in Wachi Lung dataset. f. The deletion of AKR1C1 attenuated protein levels of HIF-1α target genes. Cells were transfected with siAKR1Cs for twice. Then cells were exposed to 1% O_2 overnight followed by quick harvest. a, b and d. NCI-H1299 cells were seeded into 6-well plate at 50% confluency overnight. c and f. NCI-H460 cells were seeded into 6-well plate at 30% confluency and cultured for 36 h. g. The PFS curve of lung cancer patients with high and low expression of *AKR1C1-LDHA* in tumour tissues. High expression groups *vs.* low expression groups: Log-rank p=0.000162, HR = 1.50 (1.22~1.85). According to risk score (the prognostic index) determined by beta coefficients multiplied by gene expression values, high and low expression groups were split into the same size. h. 500/well cells in different groups were seeded into 24-well plate and cells were also harvested to confirm the overexpression efficiency. Cultured for 18 days, NCI-H292 cells were subjected to colony formation assay. i. The inhibitors of HIF-1α impared the accumulation of HIF-1α. 30-50% cells were plated into 24-well plated. After 24 h, cells were treated with IDF-11774 (10 μM) or PX-478 (10 μM) for 36 h followed by quick harvest.

metabolic adaptation.

It is reported that reductase endows AKR1C1 with resistance towards cisplatin, methotrexate, daunorubicin and progesterone, indicating functional catalytic roles of AKR1C1 in tumour progression [5,11,12,47,48]. Although progesterone is the main substrate for AKR1C1, higher AKR1C1 is observed in nonhormone-related cancers, especially NSCLC and blockade of catalytic functions for AKR1C1 fails to efficiently prevent cancer progression, underlying the non-catalytic roles of AKR1C1 [5,10]. Our previous work uncovered the catalytically independent roles of AKR1C1 in NSCLC metastasis and autophagy process [5–7]. Despite of possessing similar enzymatic activities, AKR1C2 and AKR1C3 do not participate in the aforementioned processes [5–7]. This is also reminiscent of lactate measurement data in our present study, in which knockdown of AKR1C1 not AKR1C2/3 diminished lactate production of NSCLC cells (Fig. 3). Similarly, recent studies also focused on the non-catalytic roles of enzymes in cancer metabolic reprogramming [42]. Nonetheless, it remains an enigma for the catalytic role of AKR1C1 in metabolic reprogramming, which deserves further research. Collectively, higher expressed AKR1C1 actually exerts catalytic-dependent and catalytic-independent activity in NSCLC, which may orient future

therapeutics to proteolysis-targeting chimeras (PROTAC)-based AKR1C1 degradation.

Taken together, we revealed the important role of AKR1C1 during proliferation process of NSCLC cells. Mechanistic and functional analyses showed that AKR1C1 could reshape diverse metabolic pathways, which may be attributed to the modulation of HIF-1α signalling in NSCLC. These newly acquired findings not only identify a previously unknown biological function for AKR1C1 in NSCLC proliferation and the possible underlying mechanism, but also shed light upon precision treatment for AKR1C1-dominated NSCLC.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and material

Not applicable.

Funding

This work was supported by the National Natural Science Foundation of China (no. 82102741), the Natural Science Foundation of Zhejiang Province (no. LY21H160056), the Science and Technology Research Project of Henan Province (no. 222102310560), the Henan Provincial Medical Science and Technology Project (no. LHGJ20210178).

CRediT authorship contribution statement

Lin-Lin Chang: Conceptualization, Data curation, Funding acquisition, Investigation, Methodology, Writing – original draft. **Pei-Hua Lu:** Data curation, Investigation, Writing – original draft. **Wei Yang:** Data curation, Investigation, Methodology, Validation. **Yan Hu:** Investigation, Methodology, Validation. **Lin Zheng:** Methodology, Validation. **Qiong Zhao:** Funding acquisition, Methodology. **Neng-Ming Lin:** Resources, Supervision, Writing – review & editing. **Wen-Zhou Zhang:** Resources, Supervision, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

Not applicable.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.tranon.2022.101421.

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