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PRECLINICAL STUDIES

Increased DKC1 expression in glioma and its significance in tumor cell proliferation, migration and invasion

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Summary

The dyskeratosis congenita 1 (DKC1) gene is located on the X chromosome at Xq28. Dyskerin encoded by the DKC1 gene is associated with the formation of certain small RNAs and the telomerase activity. Inherited mutations in DKC1 inactivate the dyskerin and causes dyskeratosis congenital, which is characterized by skin defects, hematopoiesis failure, and increased susceptibility to cancer. DKC1 reportedly up-regulates in several human cancers, including renal cell carcinoma and prostate cancer. Dyskerin is deregulated in B-chronic lymphocytic leukemia and breast carcinomas, but its expression and function in glioma have hardly been investigated. Hence, we were prompted to collect tissue samples and implement cell experiments. Our study reveals that DKC1 expression is significantly increased in the pathological tissues of glioma compared with that in normal tissues. The increased staining of DKC1 is related to the World Health Organization stages of tumors. DKC1 knockdown also significantly inhibits glioma cell growth by altering the expression of cell cycle-relative molecules to arrest at the G1 phase. In the transwell chamber, DKC1 knockdown glioma cells exhibit low motility. Consistent with classic oncogenic pathways, N-cadherin, HIF-1α, and MMP2 expression levels are lower compared with those of the control group. Therefore, DKC1 up-regulation in gliomas is common and necessary for extensive tumor growth. The phenotype of glioma cell lines after DKC1 down-regulation suggests its use as a valuable clinical treatment strategy.

Keywords DKC1 · Glioma · Proliferation · Migration · Invasion

Introduction

Gliomas are the primary brain tumors that account for 50% of primary intracranial tumors [1–3]. As the most malignant type of glioma, glioblastoma multiform (GBM) is regarded as a grade IV glioma according to the WHO [4]. Owing to its resistance to conventional therapies, including drugs and radiotherapy, GBM displays a relentless malignant progression with a median survival of approximately 15 months in patients after treatment [5]. In this study, we reveal the molecular mechanisms underlying the aggressiveness of glioblastomas. These mechanisms might serve as a novel target.

Dyskeratosis congenita 1 (DKC1), the human gene encoding dyskerin, was first identified in dyskeratosis

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congenita (DC), which is a heterogeneous disease characterized by a predisposition to cancer [6]. The mutation in DKC1 in the DC regulates rRNA, modifies telomerase activity, and increases cancer susceptibility [7, 8]. Functions of dyskerin, in combination with the small nucleolar RNAs (snoRNA) to catalyze the isomerization of uridines (U) into pseudouridines (Ψ), is called pseudouridylation. However, the role of deregulated ribosome on tumor initiation and progression remains poorly understood. Dyskerin is also related to telomere activity and mRNA splicing through its association with the RNA component of human telomerase and small Cajal body RNAs (scaRNAs) [9]. In DKC1 mutant mice cells, ribosomal RNA pseudouridylation is first impaired before the onset of DC. Reductions of telomere length has become substantial [10]. Determining which function of dyskerin is primary for the DC remains debatable.

DKC1 expression increases in hepatocellular carcinoma and is correlated with MYC and MKI67 expression [11]. As an oncogene, DKC1 predicts a poor patient prognosis. Consistent with hepatocellular carcinoma, dyskerin is the direct and conserved transcriptional target of c-Myc [12], thereby explaining the tumorigenic process. In breast cancers, low dyskerin expression contains lower levels of telomerase RNA and pseudouridine than those with high expression. Furthermore, cancers with high expression display worse histopathological features and prognosis [13]. However, DKC1 is down-regulated in B-chronic lymphocytic leukemia [14]. Telomerase down-regulation and changes in telomeric protein composition, including dyskerin, are involved in the pathogenesis of cancer. However, the precise role of DKC1 in gliomas cell still remains unclear.

Materials and methods

Patients and samples

This study was approved by the Independent Ethics Committee of the Affiliated Hospital of Xuzhou Medical University. A total of 398 patients were recruited from the time of surgery to the time of death or eventual follow-up. All patients involved in this study provided written informed consent, and the tissue after surgical resection was pathologically verified by the Department of Pathology of the Affiliated Hospital of Xuzhou Medical University. According to the WHO, the tumors were divided according to the pathological grade as follows: 212 cases with grades I–II and 177 cases with grades III–IV. The array dot diameter was 1.5 mm, and each dot represented a tissue spot from an individual specimen that was selected and met the requirement criteria.

Immunohistochemistry

The sections were deparaffinized and rehydrated. The citrate buffer (pH of 6.0) was heated in a microwave oven at 95 °C. Afterward, the sections were placed in the buffer for 15 min in a microwave oven. The buffer was cooled to room temperature to allow handling after antigen retrieval. The sections were washed thrice for 5 min each with phosphate-buffered saline (PBS), and H_2O_2 was added to the slices for 30 min to suppress the endogenous peroxidase activity. After blocking in normal goat serum for 2 h at room temperature, the sections were incubated with rabbit anti-DKC1 antibody (1:200; Abcam, Cambridge, MA, USA) at 4 °C overnight. On the next day, the slices were removed at 4 °C, rewarmed at room temperature for 30 min, and washed thrice with PBS for 5 min each in the dark. Next, the sections were incubated with a secondary antibody at room temperature for 30 min, followed by incubation with streptavidin–peroxidase (both from Zhongshan Biotech, Beijing, China) for an additional 30 min. After rinsing with PBS thrice for 5 min each, the sections were stained using 3, 3′-diaminobenzidine 3, 3′-diaminobenzidine (Zhongshan Biotech), rinsed in distilled water, and counterstained with hematoxylin. Dehydration was performed in 80%, 95%, 95%, and 100% ethanol as well as distilled water sequentially. The sections were sealed with cover slips. Negative controls were stained with non-immune serum to replace the primary antibodies.

Immunohistochemical staining evaluation

The staining result of DKC1 was examined blindly and independently considering the intensity of staining and the proportion of positive tumor cells. The DKC1 staining intensity was scored as follows: 1, negative; 2, weak; 3, moderate; and 4, strong. The proportion of the positively stained cells was defined as follows: 1, 0%–25%; 2, 26%–50%; 3, 51%–75%; and 4, 76%–100%. For statistical analyses, the level of DKC1 staining was evaluated using the immunoreactive score (IRS), which was calculated by multiplying the score of the staining intensity and the proportion of positive cells. According to the IRS, the scores of 1–4 indicate low expression, and 5–16 indicate high expression.

Cell culture and transfection

U251MG and U87MG human glioblastoma cell lines were initially obtained from the Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China). The cells were maintained in high-glucose Dulbecco's modified Eagle's medium (Hyclon, USA) supplemented with 10% fetal bovine serum (FBS) in a 5% CO_2-humidified atmosphere at 37 °C. When cells grew to 30%–50% confluency, they were transfected with control

siRNA, DKC1 siRNA sequence 1, and DKC1 siRNA sequence 2 (GenePharma, Shanghai, China) with siLentFect lipid reagent (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions. The medium was replaced after 24 h. The cells were irrigated twice with PBS and maintained in a fresh medium. At 48 h after transfection, the cells were used for western blot, CCK-8 cell proliferation, cell migration, and Matrigel invasion assays.

Migration and invasion assays

These assays were conducted using a transwell chamber (BD Bioscience, San Jose, CA, USA) with a pore size of 8 μm. The upper chamber was coated with or without Matrigel for cell migration and invasion assays, respectively. A total of 1×10^5 cells were seeded in a serum-free medium in the upper chamber. After incubation for 24 h for migration and 48 h for invasion, the cells in the upper chamber were carefully removed with a cotton swab, the cells that traversed in the membrane were fixed in methanol and stained with crystal violet (0.04% in water, 100 μl), and the permeating cells were calculated and photographed under light microscopy.

Cell proliferation assays

Cell proliferation was evaluated using a Cell Counting Kit-8 (CCK-8). The DKC1 knockdown U251 or U87 and normal cells were seeded in 96-well plates and cultured for 24, 48, 72, and 96 h. At exact time points in 4 days, 10 μl of CCK-8 solution was added to each well containing 100 μl of serum-free medium. After incubation for 2 h at 37 °C, the degree of proliferation was measured based on the increase in absorbance at 450 nm.

Wound healing assay

At 24 h after U251 and U87 glioma cells were transfected with DKC1-siRNA, a rectangular lesion was created using a plastic pipette tip, and the monolayer was irrigated twice with PBS and incubated in serum-free media. At the designated time, five randomly selected fields at the lesion border were determined under an inverted microscope (Olympus).

Western blot analysis

The Cells were harvested and rinsed twice with PBS. The protein concentration was determined by the bicinchoninic acid assay (Pierce, Rockford, IL, USA). All protein samples were denatured and separated by 7.5% SDS-PAGE gels and transferred into a nitrocellulose membrane (Millipore, Billerica, MA, USA). The following antibodies were used for western blot analysis: rabbit anti-DKC1, rabbit anti-N-cadherin, rabbit anti-E-cadherin, rabbit anti-MMP2, rabbit anti-HIF, rabbit anti-cyclin E2 (all obtained from Cell Signaling Technology, Beverly, MA, USA), and mouse anti-GAPDH (Boster Biotechnology, Wuhan, China). The membranes were covered with a horseradish peroxidase-conjugated secondary IgG antibody (Cell Signaling Technology, Beverly, MA, USA) for 2 h at room temperature. Finally, the protein signals were photographed with Tanon™ High-sig ECL Western Blotting Substrate (Tanon, Shanghai, China).

Cell cycle analysis

The U251 and U87 glioma cells were transfected with siRNA. After 48 h, the medium was replaced with a medium without FBS. On the next day, the cell was rinsed with PBS and incubated in a fresh medium containing FBS for 0, 3, and 6 h. The cells were fixed with 70% ethanol at 4 °C overnight. On the next day, the cells were stained with propidium iodide and RNase A. Afterward, the samples were analyzed using a FACS Canto flow cytometer (BD Biosciences). The cell distribution in the different phases of the cell cycle was analyzed using the ModFit LT3.0 software.

Statistical analysis

Statistical analysis was performed with SPSS 16.0 software (SPSS, Chicago, IL). The association between DKC1 staining and the clinicopathological factors of the patients with glioma, including age, gender, WHO grade, and histologic type, was evaluated using the χ^2 test. Student's t test was used for CCK8 cell proliferation, migration, and invasion assays. Survival analysis was implemented using the Kaplan–Meier method. Differences were considered significant when $P < 0.05$.

Results

Increased DKC1 expression was significantly associated with the WHO grade in glioma

To determine whether or not the DKC1 expression is altered in gliomas, we performed immunohistochemical staining in 389 pathologically confirmed glioma tissues. According to the picture in Fig. 1a, DKC1 protein was localized in the nucleolus. The expression was negative or weakly positive in noncancerous cells but was moderate or strongly positive in glioma tissues. In addition, histologic type and WHO grade are important prognostic factors for patients with glioma. We also studied whether or not DKC1 expression is correlated with these factors. Table 1 show that the samples with the IRSs of 1–4 and

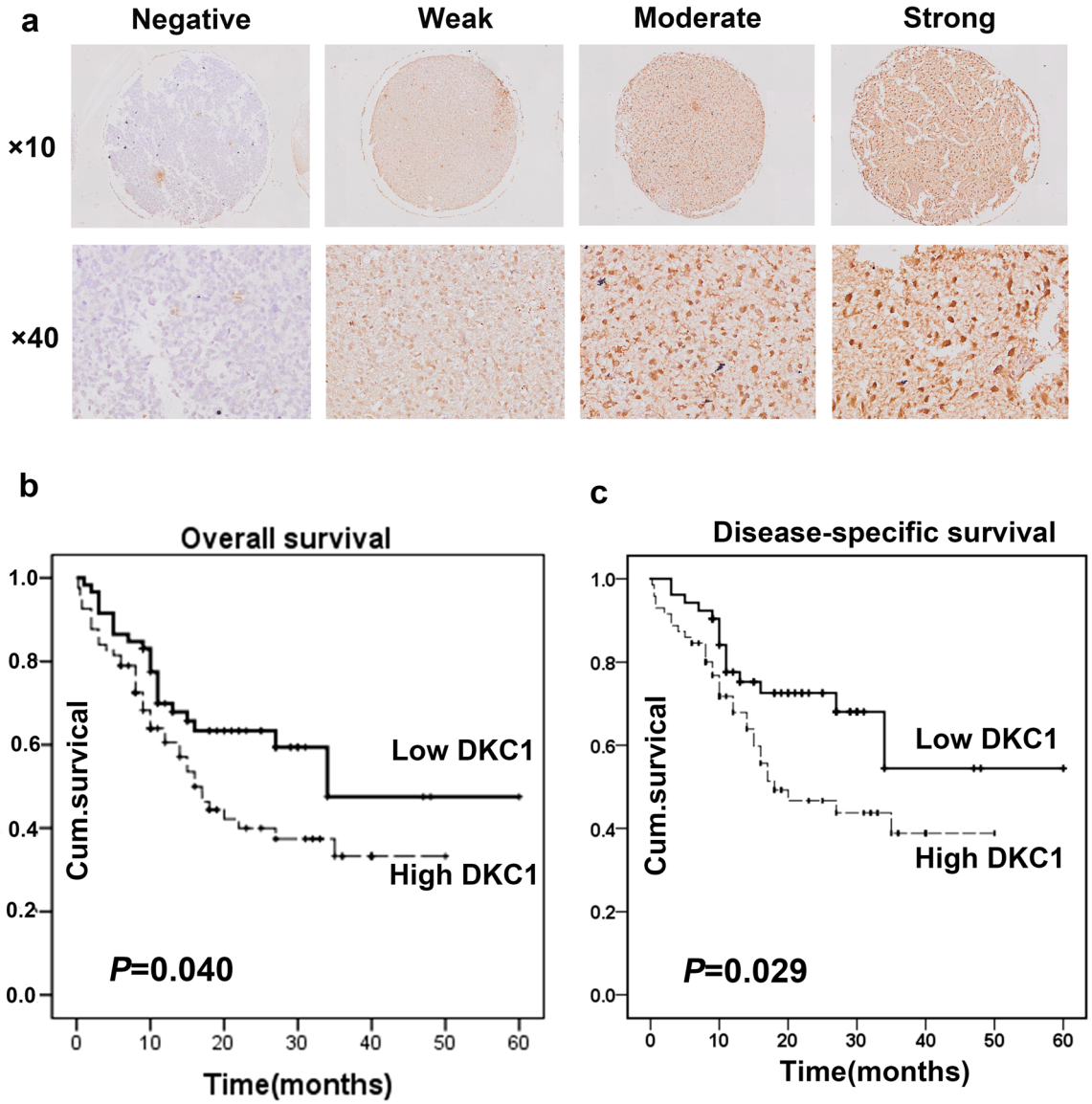

Fig. 1 Expression of DKC1 in human glioma pathological sections. a Representative images DKC1 immunohistochemical staining. From left to right they are: Negative DKC1 staining in glioma tissue; Weak DKC1 staining in glioma tissue; Moderate DKC1 staining in glioma tissue; Strong DKC1 staining in glioma tissue. **b** Overall survival curve of Kaplan–Meier with low and high expression of DKC1 in patients with glioma ($P = 0.040$, log-rank test). **c** Disease-specific survival curve of Kaplan–Meier with low and high expression of DKC1 in patients with glioma ($P = 0.029$, log-rank test)

5–16 were divided into low and high DKC1 dyskerin immunostaining, respectively. Among the 389 histological glioma tissues analyzed, 156 tissues (40.1%) showed low dyskerin immunostaining, and 233 tissues (59.5%) showed high dyskerin immunostaining. Benign samples were observed in 129 of 212 (60.8%) low dyskerin immunostaining group, whereas malignant samples were observed in 150 of 177 (84.7%) high dyskerin immunostaining group. These results indicate that the DKC1 expression in glioma tissues is significantly positively associated with the WHO grade ($P < 0.001$, Table 1) but not with patient's age, gender, and histological type.

Increased DKC1 expression predicted the poor survival of patients

To further study the effect of DKC1 on the clinical outcomes of patients with glioma, we constructed Kaplan–Meier survival curves by using the overall survival and disease-specific survival (Fig. 1b and c). The high expression group (27.363 months, median survival time) had a shorter survival time than the low expression group (35.66 months). The difference in the overall survival rate between patients with low and high dyskerin expression was statistically significant ($P = 0.040$). We obtained a more significant difference in disease-

Table 1 DKC1 staining and clinicopathological characteristics of 389 glioma patients

Variables	DKC1 staining			
	Low (%)	High (%)	Total	*P**
All cases	156 (40.1)	233 (59.5)	389	
Age				
≤42 years	87(42.8)	116(57.2)	203	0.247
>42 years	69(37.0)	117(63.0)	186	
Gender				
Male	88(38.4)	141(61.6)	229	0.420
Female	68(42.5)	92(57.5)	160	
WHO grade				
Benign (I-II)	129(60.8)	83(39.2)	212	<0.001
Malignant (III-IV)	27(15.3)	150(84.7)	177	
Histological type				
Astrocytoma	45(49.5)	46(50.5)	91	0.510
Glioblastoma	9(20.9)	34(79.1)	43	
Oligodendroglioma	3(30.0)	7(70.0)	10	
Ependymoma	1(33.3)	2(66.7)	3	
Pilocyticastrocytoma	2(25.0)	6(75.0)	8	

* Two sided Fisher's exact tests

Some cases were not available for the information

specific survival while excluding non-interference samples who did not die of glioma ($P = 0.029$).

DKC1 knockdown suppressed glioma cell growth

Immunohistochemical assays revealed poor survival in patients with glioma and high DKC1 expression. Thus, we performed cell proliferation assays in vitro. We first transiently transfected the siRNA into U251 and U87 glioma cells, which were then harvested 24 h after transfection for Western blot or cell proliferation. Western blot results confirmed the significant decrease in DKC1 expression in either U251 or U87 compared with that in the control group (Fig. 2a). The transfected cells exhibited decelerated cellular growth in U251 and U87 cells (Fig. 2b).

DKC1 knockdown induced G1 arrest by regulating the cell cycle-related markers

Given that cell proliferation is controlled by the cell cycle, we investigated if the proliferation of DKC1 knockdown cells was due to the change in cell cycle. After cycle synchronization, transfected and control cells were incubated in the fresh medium for 0, 3, and 6 h. The flow cytometer data showed that the G1 population has a slower rate of decline in the two cell lines with DKC1 knockdown compared with the control cells (Fig. 3a–d). Western blot analysis was performed to explore the CDK2 expression and the cell cycle-relative biomarkers. Our results show that DKC1 negatively regulates the CDK2 and cyclin E2 expression (Fig. 6a and b), As a prevention of cyclin-E-CDK2, the down-regulation of p27 is contrary to the retardation of cell cycle at G0/ G1 phase in G1/S checkpoint signal pathway (Fig. 6a and b).

DKC1 knockdown restrained cell migration and invasion in glioma cells

In malignant gliomas, especially glioblastoma, most patients immediately die of distant tumor metastasis. Thus we investigated the role of DKC1 in glioma migration and invasion. The results of wound-healing assays show that compared with the corresponding controls, DKC1 knockdown significantly lowers the speeds of glioma cell migration by 50% in U87 and U251 cells after 48 h of wound healing (Fig. 4a and b). The cell transwell assays were used to continue validating the finding in wound-healing assay. After 24 h, about 600 U251 cells were detected on lower layer, however, the number of in control group was just 200. Our data revealed that the average number of migrations significantly decreased in the U87 and U251 cells with DKC1 knockdown compared with controls (Fig. 5a and b). The results of the cell invasion assays also show that the capabilities of invasion were reduced in the two cell lines with DKC1 knockdown compared with the controls (Fig. 5c and d).

DKC1 knockdown inhibited glioma cell migration and invasion by regulating the cell metastasis-related markers

To further investigate the mechanism underlying migration and invasion, we performed Western blot to examine the typical proteins in oncogenic signaling pathways. MMP2 and N-cadherin play crucial roles in glioma cell migration and invasion. Our results show that MMP2 and N-cadherin decreased in U87 and U251 cells after DKC1 knockdown (Fig. 6a and b). These data are consistent with the Transwell assay results. HIF-1α is a transcription factor that is involved in the angiogenesis required for cancer metastasis [15]. We also observed the down-regulation of HIF-1α after DKC1 knockdown (Fig. 6a and b).

Discussion

Few studies reported the potential role of DKC1 in tumorigenesis, and these results are arguable. In SK-N-BE C and SK-N-SH NB cell lines, the function of DKC1 is

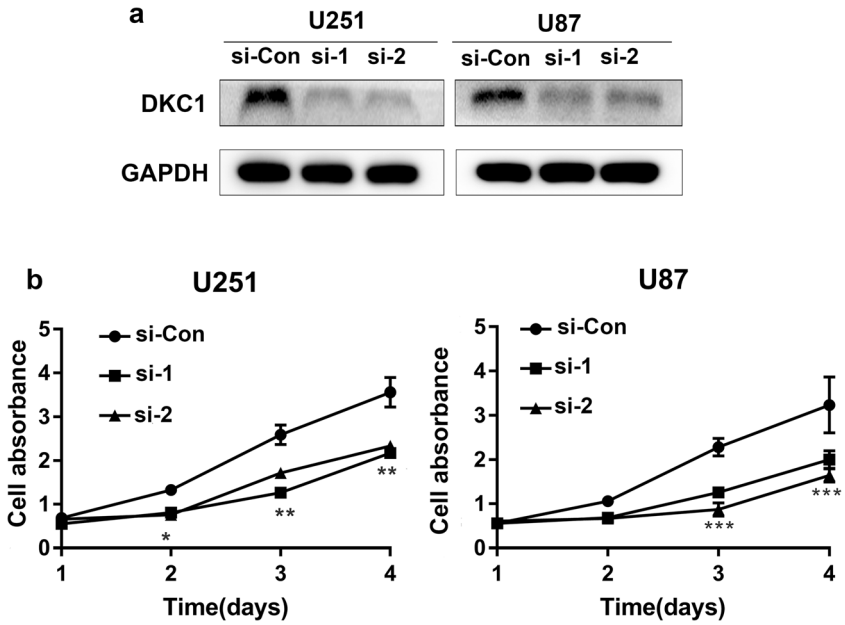

Fig. 2 Knockdown of DKC1 suppresses glioma cell proliferation in vitro. a DKC1 expression at the protein level in U251 and U87 glioma cells was evaluated by Western blot 24 h after transfection. **b** CCK-8 assays revealed that silence of DKC1 suppresses cell proliferation of U251 and U87 glioma cells compared with negative transfected cells. All experiments were conducted in triplicate. Data are shown as mean ± SD. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$

to increase telomerase activity and contribute to advanced tumors, thereby suggesting an oncogenic role. However, another study reported that low DKC1 expression is correlated with tumor progression, thereby revealing that DKC1 serves as a tumor suppressor [16]. Our results reveal the role of DKC1 in glioma from characteristic biological phenotypes, including proliferation, migration, and invasion. The knockdown of DKC1 reduced the capabilities of glioma cell migration, invasion, and proliferation. In the tissues, DKC1 was highly significantly elevated compared with normal tissues, and the increase was significantly stronger in the higher-stage cancers. Therefore, U87 and U251 cells obtained from human primary glioblastoma were applied. Our results suggest that DKC1 may be a potential oncogene in the progression of malignant gliomas.

Cell cycle kinase CDK2-cyclin E controls the passage of cells from the first gap phase (G1) into the DNA

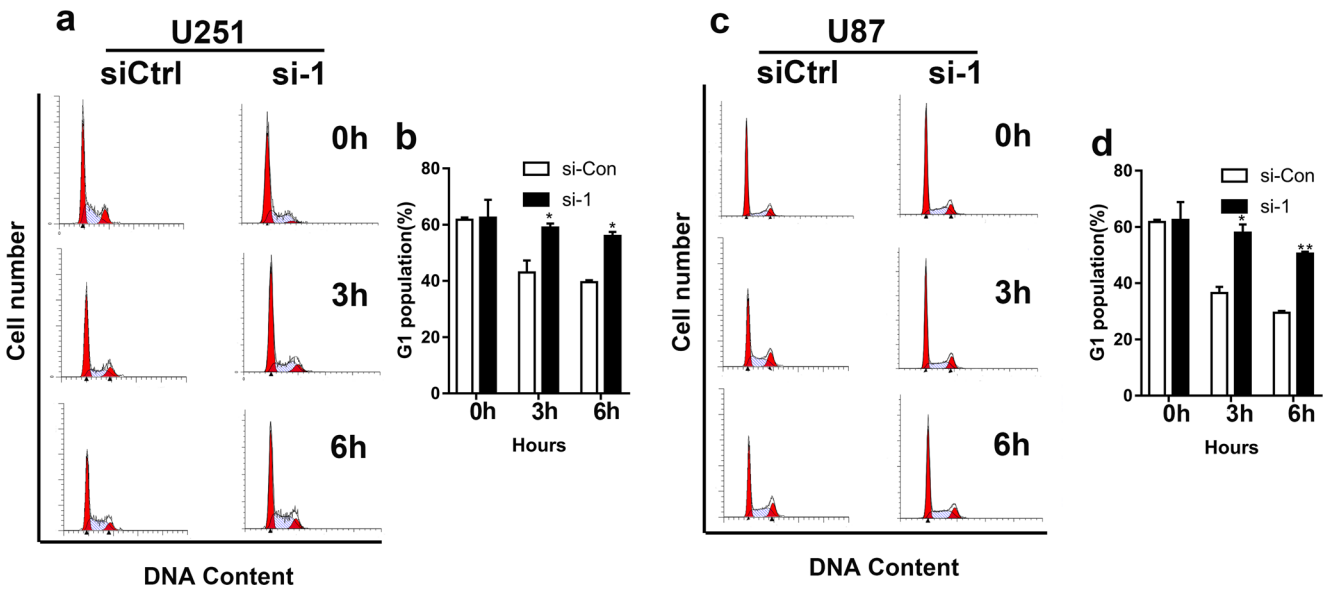

Fig. 3 Silencing of DKC1 blocks cell cycle in G1 phase in U251 and U87 glioma cells. a, **c** Results of flow cytometry in 0, 3, and 6 h after cell cycle synchronization. **b**, **d** Knockdown of DKC1 increased G0/G1 phase cell population, as detected by flow cytometric analysis following Annexin FITC and PI staining. All experiments were carried out in triplicate. Data are shown as mean ± SD.$*P < 0.05$; $**P < 0.01$; $***P < 0.001$

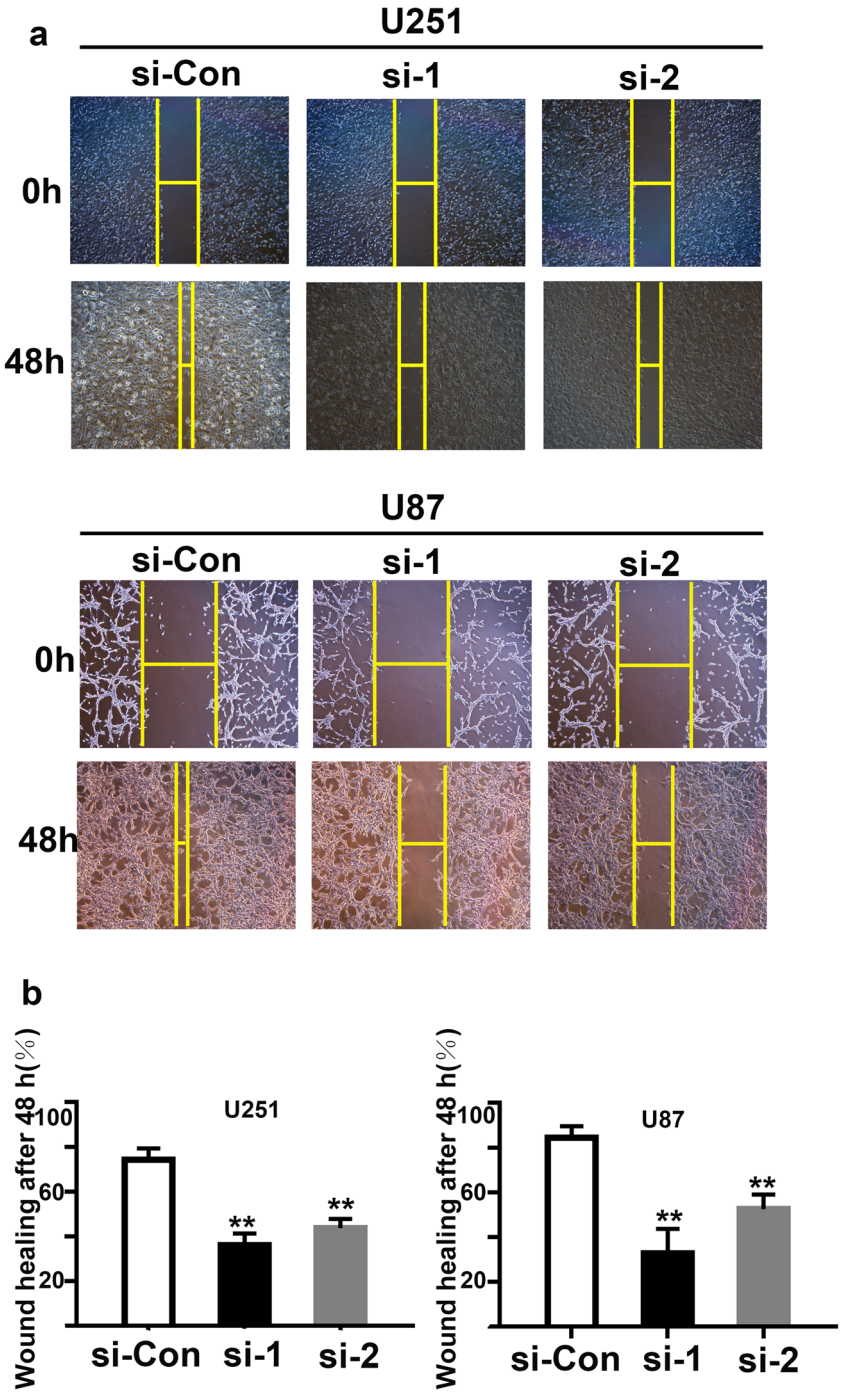

Fig. 4 Knockdown of DKC1 inhibited the speeds of glioma cell wound healing. a Representative pictures of wound healing in U87 and U251 cells with DKC1 knockdown and controls. **b** Percentages of wound healing after 48 h were calculated in U87 and U251 cells with different DKC1 expression levels. Data are shown as mean ± SD.*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$

synthesis phase (S) in the G1/S cell cycle checkpoint [17, 18]. We revealed that DKC1 knockdown significantly inhibits the protein expression of CDK2 and cyclin E, which is consistent with the increase in cell population at G1 phase and the suppression of glioma cell growth. The mechanistic explanation is the robust activation of p53 that arrests cell cycle at G1 in NB69 neuroblastoma cells [19]. Unfortunately, we did not find any change in p53 protein. Thus, we speculate that the amplification of the MYC which contributes to the development of aggressive disease and regulates the cell cycle could be related to DKC1 [20]. In future studies, we will explore the potential molecular mechanisms between DKC1 and G1/S cell cycle checkpoint.

Extensive invasion is another aggressive characteristic of gliomas. DKC1 knockdown significantly inhibits the

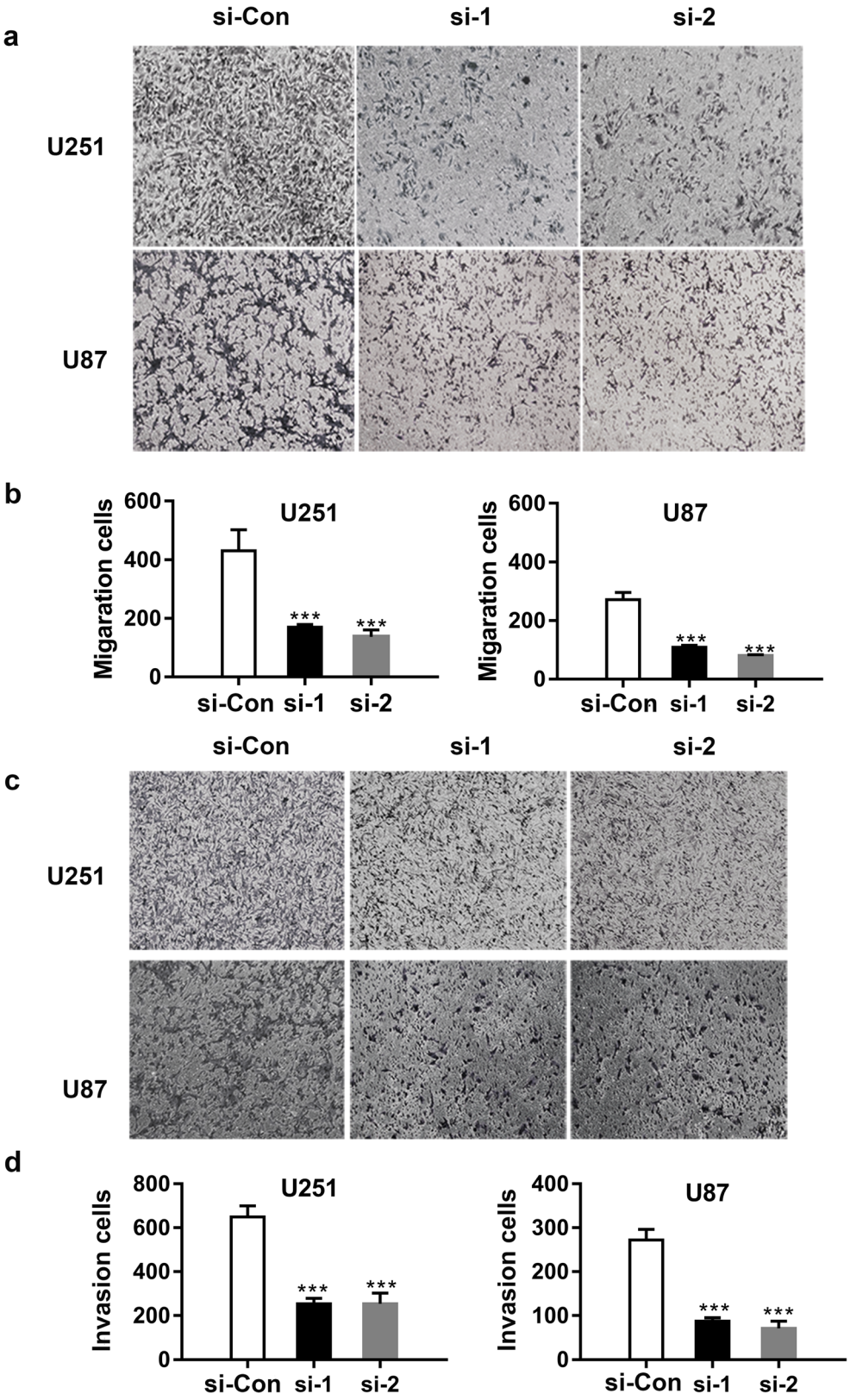

Fig. 5 Knockdown of DKC1 inhibits glioma cell migration and invasion. a, **c** Representative pictures of migration and invasion in U87 and U251 cells with DKC1 knockdown and controls. **b**, **d** Number of cell migration and invasion per field were counted in five random fields for DKC1 knockdown and control groups. Data are shown as mean ± SD. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$

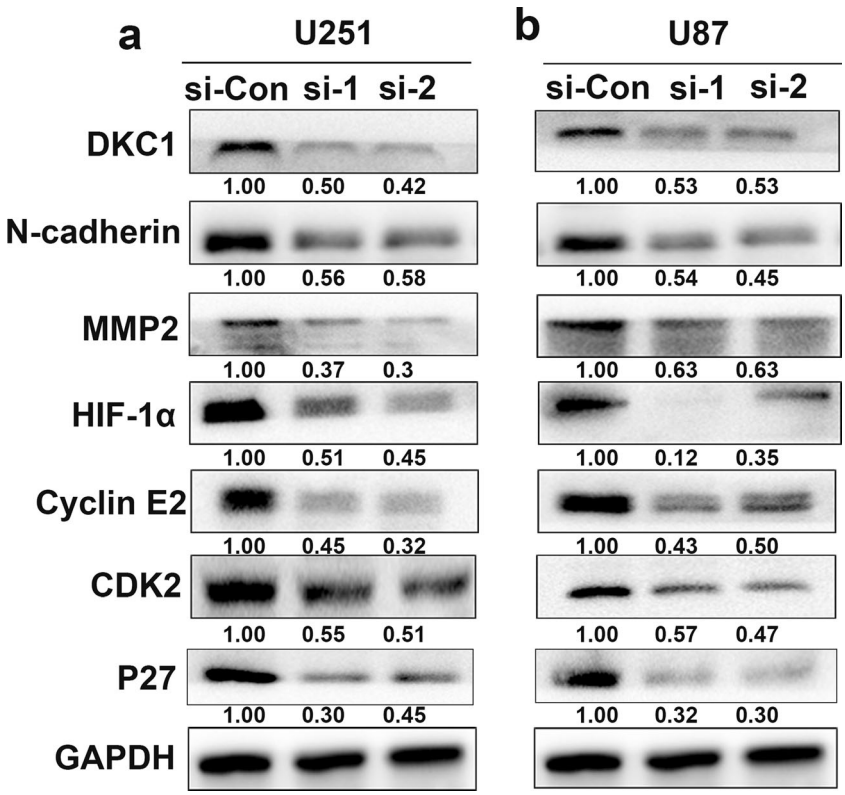

Fig. 6 Silencing of DKC1 alters the related signaling molecules in U251 and U87 cells. a, **b** Western blot analysis of the relative protein levels of N-cadherin, MMP-2, Cyclin E2, CDK2, HIF-1α in DKC1 knockdown, and control groups of U251 and U87 cells. GAPDH was used as a reference control. The numbers blow represent the results of gray level difference analysis. All experiments were conducted in triplicate

wound-healing capabilities of glioma cells. In the migration and invasion assays of glioma cell, various biological processes are involved simultaneously in the progression of glioma cells from the primary site and its metastasis to the paracancerous tissue [21]. Numerous studies showed that MMP2 degrades the extracellular matrix components and plays a key role in tumor cell migration and infiltration of the neighboring tissues [22, 23]. Therefore, we investigated the MMP2 expression with regard to the DKC1 knockdown. Our results show that DKC1 can negatively regulate MMP2.

Epithelial–mesenchymal transition (EMT) is a process wherein epithelial cells lose their cell-to-cell adhesion and gain migratory and invasive properties [24]. In gliomas, EMT is related to enhanced N-cadherin expression and predicts an unfavorable prognostic outcomes [25]. Our study demonstrated that DKC1 knockdown negatively regulates N-cadherin. HIF-1α plays a key role in glioma invasion [26]. The knockdown of HIF-1α in murine and human glioma cells impairs their mobility in vitro and in vivo [27]. Western blot assays showed decreased HIF-1α expression in DKC1 knockdown cells. The relationship between N-cadherin, HIF-1α, and DKC1 needs further investigation.

In conclusion, we showed the association of DKC1 with glioma progression and revealed the inhibitory role of DKC1 in glioma cell proliferation, migration, and invasion. This study may provide a valuable therapeutic strategy to help control the progression of gliomas.

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Compliance with ethical standards

Conflict of interest The authors have declared that no competing interests exist.

Ethical approval This study was performed under a protocol approved by the Institutional Review Boards of the Affiliated Hospital of Xuzhou Medical University.

Informed consent Informed consent was obtained from all individual participants included in the study.

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