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$Kras^{G12D}$-LOH promotes malignant biological behavior and energy metabolism of pancreatic ductal adenocarcinoma cells through the mTOR signaling pathway

X. SHEN[1], L.G. CHANG[1], M.Y. HU[1], D. YAN[2], L.N. ZHOU[1], Y. MA[1], S.K. LING[1], Y.Q. FU[1], S.Y. ZHANG[1], B. KONG[3], P.L. HUANG[1,*]

[1]*Department of Pathology, School of Medicine, Southeast University, Nanjing 210009, China;* [2]*Jiangsu Cancer Hospital, Nanjing 210009, China;* [3]*Department of Gastroenterology, Nanjing Drum Tower Hospital, Nanjing University, Nanjing 210009, China*

**Correspondence: seuhpl@163.com*

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Oncogenic Kras with loss of heterozygosity (LOH) is frequently detected in various tumours. However, the exact function and mechanism by which $Kras^{G12D}$-LOH operates remain unclear. Therefore, the current study investigated the effect of $Kras^{G12D}$-LOH on the malignant phenotype of pancreatic ductal adenocarcinoma (PDAC) cells. Our investigation revealed that $Kras^{G12D}$-LOH is associated with increased proliferation, invasion and reduced apoptosis in PDAC cells. The results also exhibited enhanced glycolytic phenotype of $Kras^{G12D}$-LOH PDAC cells. Hyperactive mTOR plays a significant role in the initiation and maintenance of tumors. To investigate the correlation between $Kras^{G12D}$-LOH and mTOR, the mTOR signaling pathway was detected by western blot analysis. We found that $Kras^{G12D}$-LOH up-regulated Akt, AMPK, REDD1 and mTOR in PDAC cells. In summary, our results demonstrated that $Kras^{G12D}$-LOH promotes oncogenic Kras-induced PDAC by regulating energy metabolism and mTOR signaling pathway. These data may provide novel therapeutic perspectives for PDAC.

Key words: $Kras^{G12D}$, loss of heterozygosity, mTOR pathway, pancreatic cancer, energy metabolism

Pancreatic cancer remains one of the most lethal types of solid malignancies, with increasing rates of morbidity and mortality in recent years [1]. It is estimated that pancreatic cancer will become the second leading cause of cancer-related death by 2030 in the United States [2]. Pancreatic ductal adenocarcinoma (PDAC) is the main pathological type, which accounts for approximately 95% of all pancreatic cancer [3]. The activation of oncogenes and inactivation of tumor suppressor genes can result in the development of tumors. Activated Kras oncogenes, particularly the point mutation in codon 12 of Kras ($Kras^{G12D}$ or $Kras^{G12V}$) are frequently detected in PDAC and pancreatic intraepithelial neoplasia [4, 5]. Moreover, recent studies revealed that the wild-type allele of $Kras^{G12D}$ was progressively lost ($Kras^{G12D}$-LOH) in mice with pancreatic tumors, promoting tumor metastasis [6].

Compared with normal differentiated cells, cancer cells have to adapt their metabolic pathways to meet the high-energy requirements for rapidly proliferating cells [7]. One of the most distinctive phenomena is that cancer cells rely on glycolysis, even under conditions of adequate oxygen (aerobic glycolysis), which is also known as the "Warburg effect" [8]. Although glycolysis seems to be a less efficient pathway for generating adenosine triphosphate (ATP) compared to mitochondrial oxidative phosphorylation (OXPHOS) (2 vs 36 ATPs per metabolised glucose), it provides a survival and growth advantage to tumour cells [9, 10]. Glycolysis provides sufficient ATP and intermediate metabolites by increasing the glycolytic flux [11]. Moreover, lactate is an end product of glycolysis, which produces an acidic microenvironment that promotes the invasive and metastatic potential of tumor cells and aids immune evasion [12, 13]. In PDAC, $Kras^{G12D}$ mediates metabolic reprogramming by up-regulating glycolysis, which is closely correlated with cellular survival and proliferation [14, 15].

Mammalian target of rapamycin (mTOR) is a serine/threonine kinase that regulates a wide range of functions, including cell growth and energy metabolism. There is increasing evidence that mutated Ras promotes mTOR activation, which is significantly related to poor prognosis of pancreatic cancer [16, 17]. The mTOR pathway, which responds to various stresses, is regulated by three major upstream signaling molecules: phosphatidylinositol 3-kinase (PI3K)/Protein kinase B (Akt), AMP-activated protein kinase (AMPK) and

regulated in development and DNA damage responses 1 (REDD1) [18]. However, it is unclear whether there is a novel mechanism through which $Kras^{G12D}$-LOH interacts with mTOR and promotes PDAC tumorigenesis.

Hypoxia is a fundamental characteristic of solid tumours including PDAC [19]. In our study, we investigated the effect of $Kras^{G12D}$-LOH on the malignant phenotype of PDAC cells under normoxic and hypoxic conditions to elucidate its underlying mechanisms.

Materials and methods

Cell lines and cell culture. Mouse pancreatic cancer cell lines 399 ($Kras^{G12D}$ PDAC cell line) and 897 ($Kras^{G12D}$-LOH PDAC cell line) [20] were a kind gift from the pancreas research group of Technical University Munich (Munich, Germany). These cells were cultured in Dulbecco's modified Eagle medium (DMEM, Hyclone, Logan, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Hyclone, Logan, USA), 100 units/ml penicillin and 100 µg/ml streptomycin. Cell lines were maintained in a humidified incubator (95% air and 5% CO_2) at 37 °C. Experiments carried out under hypoxic conditions were performed in a hypoxic incubator (1% O_2, 94% N_2 and 5% CO_2).

Cell proliferation assay. 399 and 897 cells were seeded in 96-well plates in 100 µl of DMEM medium and were cultured under conditions of normoxia (20% O_2) or hypoxia (1% O_2), respectively. After cultivating for 24, 48, 72 or 96 h, 10 µl of reagent from Cell Counting Kit-8 (CCK-8; Dojindo, Kumamoto, Japan) was added to each well. Then, the plate was incubated for additional 2 h in the incubator. The OD at 450 nm was measured using a microplate reader (Multiskan FC, Thermo, Waltham, MA, USA).

Plate colony formation assay. Cells were seeded at a density of 500 cells per well into six-well plates and were cultured for 10 days under normoxic or hypoxic conditions. The colonies were fixed with 4% paraformaldehyde for 30 min at room temperature and then stained with 0.5% crystal violet for 1 h. The number of colonies was counted under a microscope (Nikon Eclipse, Tokyo, Japan) and a cluster of 50 cells was designated as a colony.

Transwell invasion assay. Cells were harvested and resuspended in serum-free medium. Then, 100 µl of cell suspension (5×10^4 cells) was added to the upper chambers of the Transwells (8 µm pore size, Cat. No. 3422, Corning Incorporated, New York, USA), which were covered with 50 µl of Matrigel (1:3 dilutions; BD Biosciences, San Diego, CA). DMEM medium (600 µl) containing 10% FBS was added to the lower chambers. Cells were incubated in a 20% O_2 or 1% O_2 atmosphere for 24 h. Cells on the upper surface membrane were wiped off using a cotton swab. Cells that passed through the membrane were fixed with 4% polyphosphate formaldehyde for 30 min, then stained with 0.5% crystal violet for 30 min and counted under a microscope (Nikon Eclipse, Tokyo, Japan).

Cell cycle assay. Cells were harvested, washed twice with cold phosphate-buffered saline (PBS) and adjusted to a concentration of 1×10^6 cells/ml, then fixed with 70% cold ethanol overnight. After washing with PBS, cells were incubated with RNase for 30 min at 37 °C and stained with propidium iodide (KeyGEN Biotech, Nanjing, China) in dark at 4 °C for 30 min. The proportion of cells in G0/G1, S and G2/M phases was measured by flow cytometry (Becton Dickinson, Mountain View, CA, USA).

Cell apoptosis assay. Cell apoptosis was evaluated by Annexin V-FITC and propidium iodide (PI) labelling kit (KeyGEN Biotech, Nanjing, China) according to the manufacturer's instructions. Treated cells were digested with 0.25% trypsin without EDTA, resuspended in 500 µl of binding buffer and stained with Annexin V-FITC and PI for 15 min in the dark at room temperature. Samples were then analyzed by Accuri C6 flow cytometry (Accuri Cytometers, Ann Arbor, MI, USA).

Measurement of intracellular ATP content and lactate production. 399 and 897 cells were seeded in six-well plates and cultured for 24 h under normoxia or hypoxia. The cells were harvested and ATP levels were measured in the cell lysates using an ATP Assay Kit (Jiancheng, Nanjing, China) according to the manufacturer's instructions. Results were normalised based on the total amount of protein in the cells. The supernatants were collected and lactate concentrations in the conditioned media were determined using a Lactate Assay kit (Jiancheng, Nanjing, China) according to the manufacturer's instructions. Then OD was measured at 530 nm using a microplate reader and the results were analyzed.

Western blot analysis. The expression levels of cellular proteins were determined using western blot analysis. First, total cellular proteins in PDAC cells were extracted using lysis buffer (Beyotime, Shanghai, China) containing 1% protease inhibitor cocktail (KeyGEN Biotech, Nanjing, China). Then, total protein was quantified using a BCA Protein Assay Kit (Beyotime, Shanghai, China), and samples resuspended in loading buffer (Beyotime, Shanghai, China) were treated at 100 °C for 5 min to denature the proteins. Next, protein samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride membranes (Millipore, Massachusetts, USA). The membranes were blocked with 5% bovine serum albumin (BSA) in Tris-Buffered Saline containing 0.1% Tween 20 (TBST) and incubated with the diluted primary antibody (Table 1) at 4 °C overnight. Finally, signals were detected by incubation with secondary antibodies conjugated to horseradish peroxidase and imaging analysis (Tanon-5200, Shanghai, China).

Statistical analysis. All data were expressed as the mean ± SD of at least three independent experiments. Statistical analysis of date was examined by Student's t-test. P-values less than 0.05 were considered to be a statistically significant difference.

Results

Kras^{G12D}-LOH is associated with increased proliferation. Cell growth curves were determined from CCK-8 assay for each treatment at 24, 48, 72 and 96 h. We observed that the proliferation rate of 897 cells was significantly increased compared to 399 cells under normoxic or hypoxic conditions ($p<0.05$ or $p<0.01$, Figure 1A). As shown in Figure 1B, we have observed significantly increased rate of colony formation by 897 cells compared to 399 cells under both normoxic

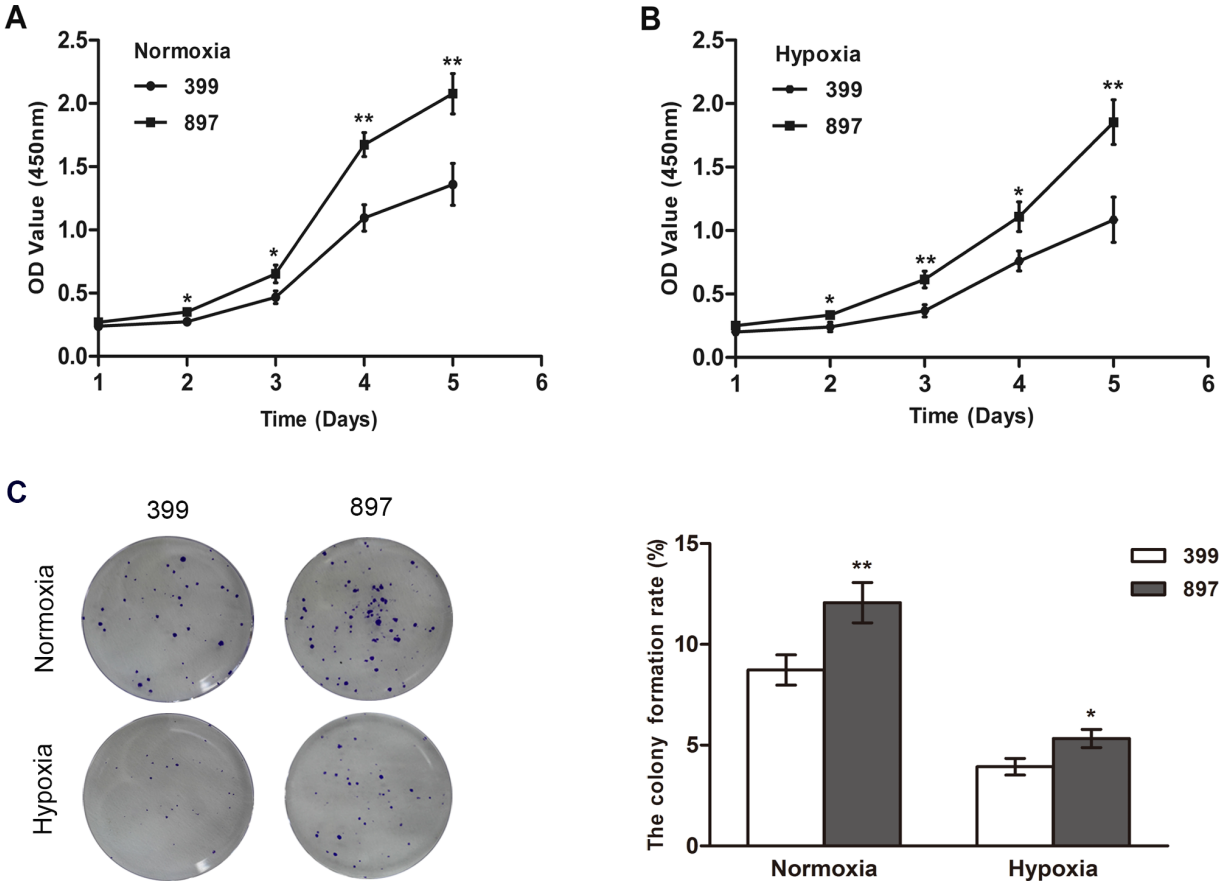

Figure 1. Effect of Kras^{G12D}-LOH on PDAC cell proliferation. A) CCK-8 assay was used to detect proliferation of 399 and 897 cells under normoxic conditions. B) CCK-8 assay was used to detect proliferation of 399 and 897 cells under hypoxic conditions. C) Representative images of the cell plate colony formation assay. 897 cells form significantly more colonies than 399 cells under normoxic and hypoxic conditions. N=3 for 399 cells; n=3 for 897 cells. *p<0.05; **p<0.01.

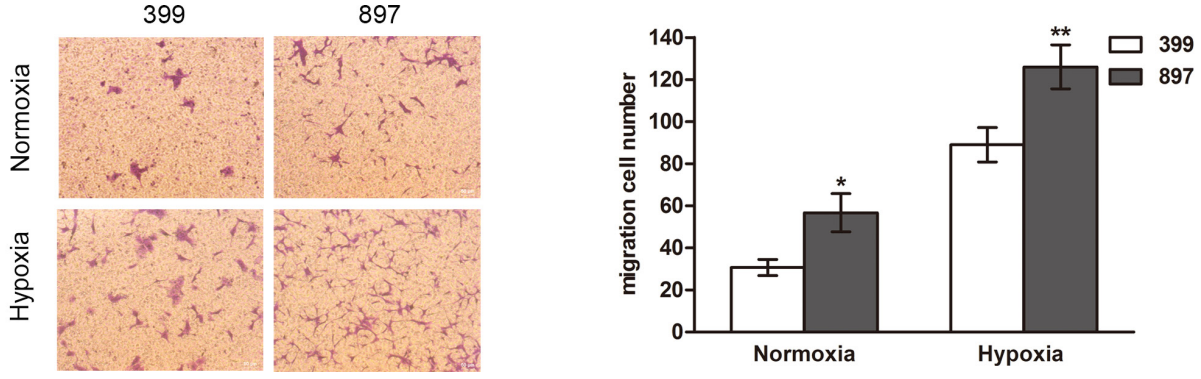

Figure 2. Effect of Kras^{G12D}-LOH on cell invasion. Representative images of the Transwell invasion assay were showed. The cell number of 897 migrating cells was significantly higher than that of 399 cells under normoxic and hypoxic conditions. N=3 for 399 cells; n=3 for 897 cells. *p<0.05; **p<0.01.

Table 1. Primary antibodies in Western blot analysis.

Protein	Antibody	Catalog	Producer	Antibody concentration
p-Akt	Rabbit anti-p-Akt	4060	CST, USA	1:1000
Akt	Rabbit anti-Akt	4691	CST, USA	1:1000
p-AMPK	Rabbit anti-p-AMPK	2535	CST, USA	1:1000
AMPK	Rabbit anti-AMPK	2532	CST, USA	1:1000
p-mTOR	Rabbit anti-p-mTOR	5536	CST, USA	1:1000
mTOR	Rabbit anti-mTOR	2983	CST, USA	1:1000
REDD1	Rabbit anti-REDD1	10638-1-AP	Proteintech, China	1:1000
β-actin	Rabbit anti-β-actin	AP0060	Bioworld, China	1:3000

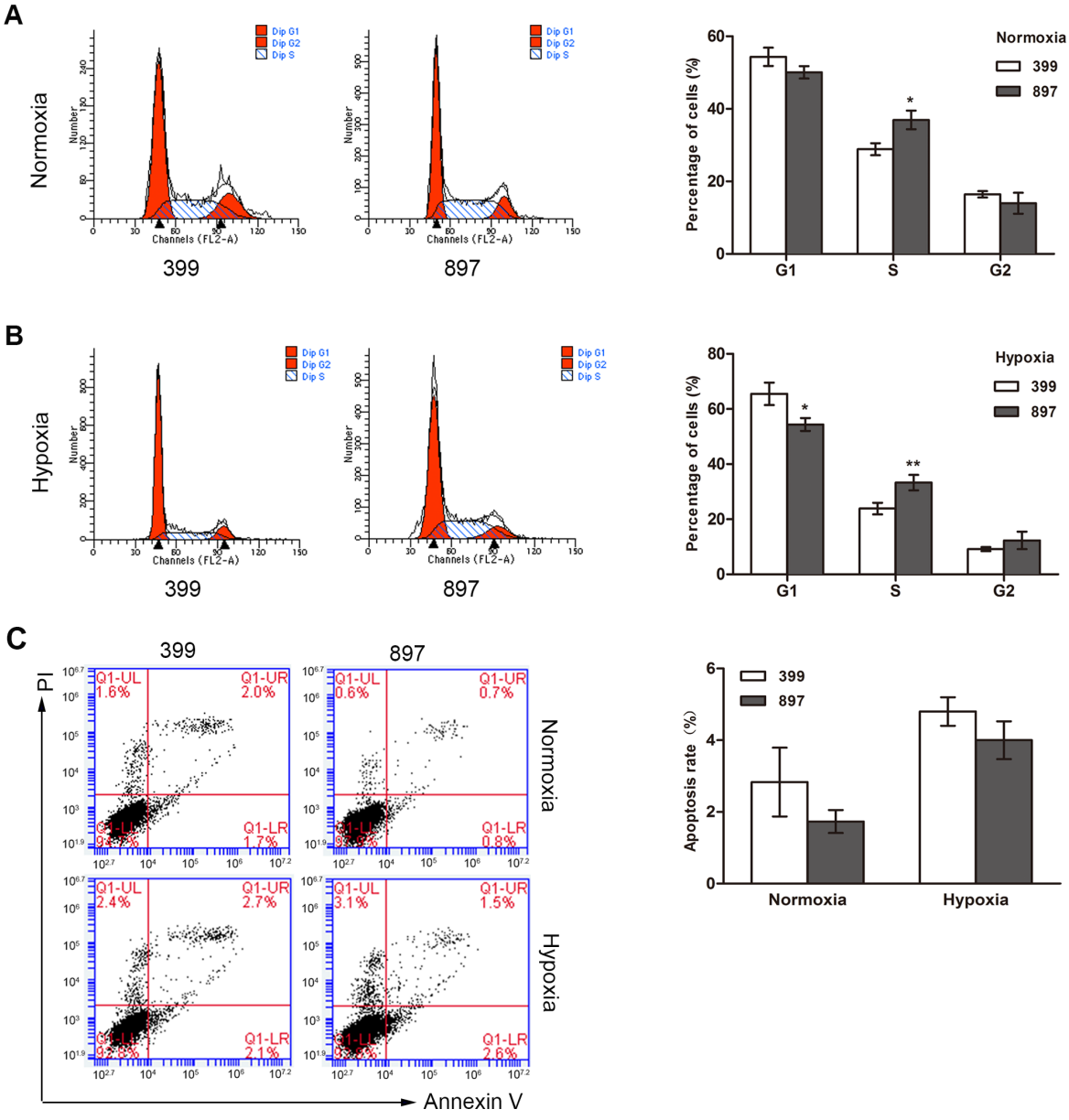

Figure 3. Effect of Kras^{G12D}-LOH on PDAC cells cycle progression and apoptosis. A) Cell cycle progression was analyzed by flow cytometry and the percentage distribution of 399 and 897 cells in G1, G2/M and S-phase under normoxic conditions. B) Cell cycle progression was analyzed by flow cytometry and the percentage distribution of 399 and 897 cells in G1, G2/M and S-phase under hypoxic conditions. C) Cell apoptosis was detected by flow cytometry using FITC-Annexin V/PI staining. There is no obvious difference in apoptosis rate between 399 and 897 cells under normoxic and hypoxic conditions. N=3 for 399 cells; n=3 for 897 cells. *p<0.05; **p<0.01.

and hypoxic conditions ($p<0.01$ or $p<0.05$). Taken together, these results indicate that $Kras^{G12D}$-LOH exerts a significant proliferative effect in PDAC cells.

$Kras^{G12D}$-LOH is linked with enhanced invasion. We examined the effects of $Kras^{G12D}$-LOH on invasion of PDAC cells. Transwell invasion analysis showed that the percentage of invasive cells was significantly higher in 897 cells compared to 399 cells ($p<0.05$ or $p<0.01$) under normoxia and hypoxia (Figure 2). These results demonstrate that $Kras^{G12D}$-LOH might promote metastasis in PDAC cells.

Effect of $Kras^{G12D}$-LOH on cell-cycle progression and apoptosis in PDAC cells. Cell cycle progression and apoptosis of PDAC cells were investigated by flow cytometry. The results show that the S phase ratio of 897 cells was higher than that of 399 cells under normoxia ($p<0.05$) (Figure 3A). Under hypoxia, cell cycle analysis also showed a dramatic increase in the number of cells in S phase ($p<0.01$), which was accompanied by a decrease in the percentage of cells in G1 phase of the cell cycle ($p<0.05$) (Fig. 3B). However, the difference in apoptosis between 399 and 897 cells was not statistically significant under normoxic or hypoxic conditions (Figure 3C).

$Kras^{G12D}$-LOH is correlated with elevated intracellular ATP levels and extracellular lactate production. Intracellular ATP content and lactate production were analyzed to assess the glycolytic activity of 399 and 897 cells. As seen in Figure 4A, the ATP concentration in 897 cells was significantly increased compared to 399 cells under both normoxic and hypoxic conditions ($p<0.05$ or $p<0.01$). As shown in Figure 4B, the secretion of lactate in the supernatant of 897 cells was greater than that of 399 cells ($p<0.05$) under hypoxic conditions, whereas there was no significant difference under normoxia. These results reveal that $Kras^{G12D}$-LOH enhances the glycolytic phenotype of PDAC cells.

$Kras^{G12D}$-LOH activates mTOR activity. One possible explanation for the observed up-regulation of cell growth and energy metabolism is the increased survival ability of $Kras^{G12D}$-LOH PDAC cells. To examine this possibility, we investigated the expression and activation of mTOR signaling, a major regulator of survival signaling (Figure 5). Under normoxia and hypoxia, $Kras^{G12D}$-LOH is linked to the up-regulated REDD1 and increased activation of Akt and mTOR. Furthermore, the phosphorylation level of AMPK was enhanced in $Kras^{G12D}$-LOH cells under normoxia conditions. These findings suggest the possible involvement of mTOR signaling in the oncogenic function of $Kras^{G12D}$-LOH.

Discussion

The occurrence and development of a tumor is a complex multi-step process that involves many genes. Interestingly, in 37% of primary and 80% of metastatic PDAC cell lines, the wild-type Kras allele was lost, resulting in LOH at $Kras^{G12D}$ [6]. Moreover, allelic loss of wild-type Kras is commonly observed in other tumors of human and mice, such as lung tumors [21, 22] and skin tumors [23, 24].

Cell type, gene expression level, and the wild-type allele status affect the response of cells to the activation of oncogenes [25, 26]. Previously, it was reported that a Kras mutant allele-specific imbalance (loss of the Kras wild-type allele or amplification of the Kras mutant allele) is frequently associated with human pancreatic ductal adenocarcinoma and undifferentiated carcinoma, leading to a reduction in the overall survival rate of patients and poor prognosis [27]. In oncogenic Kras-induced leukemogenesis, loss of wild-type Kras can further promote activation of all Ras isoforms [28]. These findings strongly suggest that the wild-type Kras allele could have a suppressive role in the transformation process of the oncogenic allele. Our experimental results support the notion that the wild-type Kras allele may have tumour suppressor function and that $Kras^{G12D}$-LOH promotes malignant properties in PDAC cells under normoxic or hypoxic conditions. It is possible that $Kras^{G12D}$-LOH is caused by a

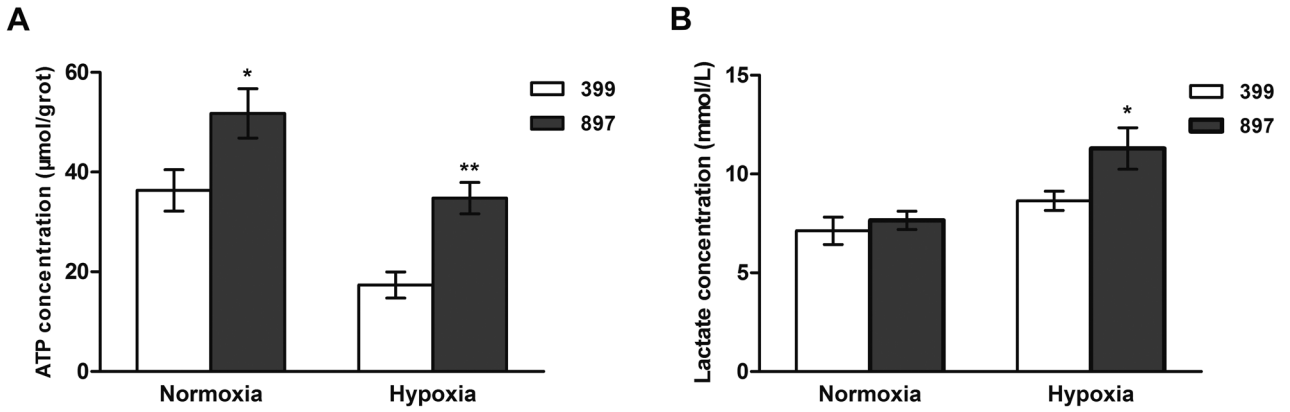

Figure 4. Effect of $Kras^{G12D}$-LOH on the energy metabolism of PDAC cells. The levels of ATP generation and lactate secretion are critical determinants of the cellular energy status. A) Intracellular ATP content of 897 cells was significantly higher than that of 399 cells under normoxic and hypoxic conditions. B) Lactate production by 897 cells was significantly higher than that of 399 cells under hypoxic conditions. N=3 for 399 cells; n=3 for 897 cells. *$p<0.05$; **$p<0.01$.

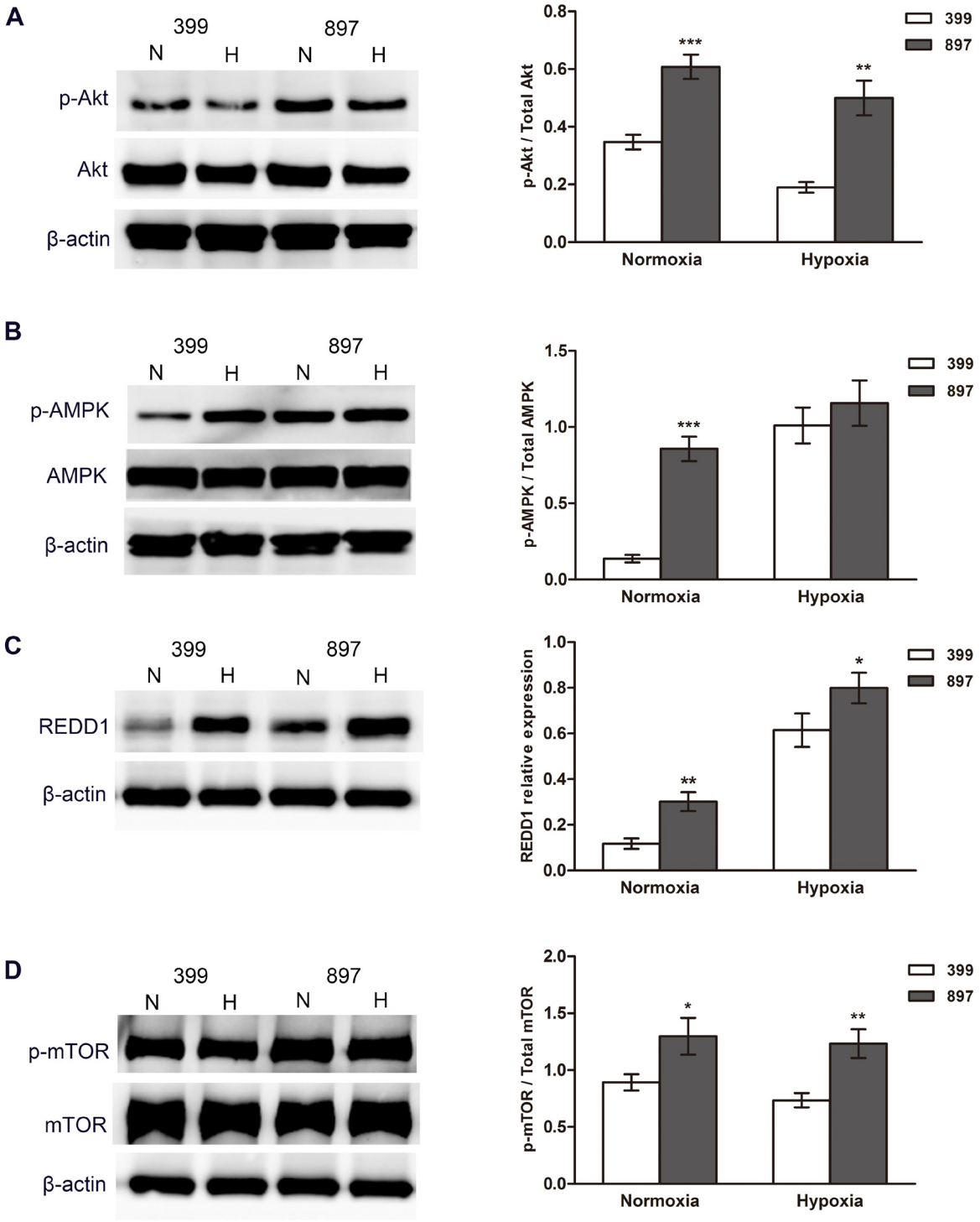

Figure 5. The expression levels of mTOR signaling pathway-related proteins were analyzed by western blotting. A) Western blot analysis shows phosphorylation levels of Akt in 399 and 897 cells under normoxic and hypoxic conditions. B) Western blot analysis shows phosphorylation levels of AMPK in 399 and 897 cells under normoxic and hypoxic conditions. C) Western blot analysis shows expression levels of REDD1 in 399 and 897 cells under normoxic and hypoxic conditions. D) Western blot analysis shows phosphorylation levels of mTOR in 399 and 897 cells under normoxic and hypoxic conditions. N: normoxia, H: hypoxia. N=3 for 399 cells; n=3 for 897 cells. *p<0.05; **p<0.01, ***p<0.001.

"second hit", point mutation and LOH at Kras occurres successively. In addition, the dominant carcinogenic action of $Kras^{G12D}$ plays a major role in the process of tumourigenesis, which may be one of the reasons why oncogenic $Kras^{G12D}$ mask the potentially anticarcinogenic effect of wild-type Kras [29, 30].

Metabolic reprogramming and altered bioenergetics have been regarded as a hallmark of tumor cells [31, 32]. The results of this study show that $Kras^{G12D}$-LOH PDAC cells generated more ATP and drove more lactate production during hypoxia compared to $Kras^{G12D}$ PDAC cells. Hence, it is believed that $Kras^{G12D}$-LOH shifts more readily to glycolysis reprogramming during hypoxia. Lactate, the final product of glycolytic metabolism, plays an important role in tumor cells invasion, metastasis, angiogenesis and evasion of immune surveillance [33, 34]. Overall, $Kras^{G12D}$-LOH can offer proliferative advantages to PDAC cells by increasing the glycolysis rate of cells.

The mTOR signaling pathway is one of the major pathways to regulate cellular survival and energy metabolism. The experimental results indicate that elevated p-Akt levels activate mTOR in $Kras^{G12D}$-LOH PDAC cells. Activated mTOR promotes rapid growth and survival of cancer cells by generating abundant energy and providing substances necessary for biosynthesis. Moreover, some observations have indicated that activation of mTOR can cause a pseudo-hypoxic state in cells, promote lactate production, stimulate the expression of genes encoding glycolytic enzymes, and cause a corresponding increase in the glycolytic flux [35, 36]. Moreover, $Kras^{G12D}$-LOH up-regulated the expression of REDD1 in PDAC cells under normoxic and hypoxic conditions. REDD1 can inhibit mTORC1 in a manner mediated by TSC1/2, which acts as a negative regulator of mTORC1 activity [37]. In addition, previous experiments showed that overexpression of the REDD1 can induce Akt phosphorylation [38, 39]. This suggest that over-expression of REDD1 may synergize with p-Akt expression, leading to PDAC malignant transformation via the PI3K/Akt/mTOR signaling pathway. Interestingly, the basal levels of AMPK phosphorylation were higher in cancer cells with $Kras^{G12D}$-LOH in comparison to $Kras^{G12D}$ PDAC cells. The cellular AMPK is a serine-threonine kinase that regulates energy-producing catabolic processes and energy-consuming anabolic processes. Activated AMPK can increase glycolytic flux by activating key glycolytic enzymes including glucose transporters and PFK-2, to lowered pH [40, 41]. In addition, AMPK exerts dual effects on the PI3K pathway, stimulating PI3K/Akt and inhibiting mTOR [42]. Taken together, these observations indicate that $Kras^{G12D}$-LOH promotes pancreatic cells proliferation and energy metabolism through regulating the mTOR signaling pathway accommodating the metabolic needs of a proliferating cell.

In conclusion, we report that $Kras^{G12D}$-LOH is associated with increased malignant behavior and elevated energy metabolism. Additionally, a positive correlation between $Kras^{G12D}$-LOH and intracellular mTOR activity was observed in these cells. Our findings show that the wild-type Kras allele acts as an antioncogene and that only silencing of the mutant Kras gene could achieve improved treatment efficacy, which may provide novel therapeutic strategies for PDAC.

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