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Glucose metabolism provide distinct prosurvival benefits to non-small cell lung carcinomas

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ABSTRACT

Heterogeneity within the same tumor type has been described to be complex and occur at multiple levels. Less is known about the heterogeneity at the level of metabolism, within a tumor set, yet metabolic pathways are highly relevant to survival signaling in tumors. In this study, we profiled the glucose metabolism of several non-small cell lung carcinoma (NSCLC) cell lines and could show that, NSCLC display distinct glycolytic metabolism. Genetic and pharmacological perturbation of glycolysis was selectively toxic to NSCLCs with high rates of glycolysis. Furthermore, high expression of hexokinase-2, localized at the mitochondria, was a feature of the NSCLCs dependent on glucose catabolism. Our study provides evidence for quantitative metabolic diversity in NSCLCs and indicates that glucose metabolism provide differential prosurvival benefits to NSCLCs.

1. Introduction

Altered metabolism was one of the first biomarkers for cancer [1]. In particular, enhanced glucose metabolism under aerobic conditions is a common feature of many tumors, known as the Warburg effect. Tumor cells rewire their metabolism to meet the high demand of rapidly dividing cells, to generate new biomass and adenosine triphosphate (ATP). Cells have two pathways to generate ATP, oxidative phosphorylation or glycolysis. Glucose is converted into pyruvate, producing NADH from NAD+ and ATP from ADP during glycolysis. The glycolytic pathway continues if pyruvate is reduced to lactate, as NAD+ will be regenerated [2]. Glycolysis is a very rapid process although inefficient in generating ATP, as the majority of energy is lost when the cell secretes lactate. Oxidative phosphorylation is the most efficient cellular machinery in producing ATP and generates beyond 20× as many ATP molecules [3]. Oxidative phosphorylation occurs when mitochondrial carbon substrates like pyruvate or succinate are being oxidized in the mitochondria. Reducing equivalents are subsequently delivered to the mitochondrial respiratory chain, forming a proton gradient that is coupled to ATP biosynthesis [4,5]. The dependency on enhanced glucose metabolism distinguishes many cancer cells from their normal counterparts. This predicts that perturbation of glucose metabolism might display selectivity towards the cancer cells [2,6,7].

Hexokinases (HK) regulate the first committed step in the catabolism of glucose. There are four (HK1-4) isoforms of hexokinase expressed in mammalian cells. The isoforms differ in their expression profile, biochemical properties as well as their intracellular localization. HK2 has been shown to be the predominant isoform that is highly expressed in cancer cells, a feature distinguishing them from their normal counterparts [8–10].

Heterogeneity in the utilization of glycolysis has been reported in physiological processes including the activation of the immune system [11], stem cell growth [12], during the cell cycle [13] as well as in angiogenesis [14]. This type of metabolic heterogeneity represents a control step that allows certain cell populations to be highly proliferative, when required.

To date, tumor heterogeneity represents one of the major problems with unsuccessful treatment. Heterogeneity within the same tumor type has been described to be complex and occur at multiple levels. Histological analysis is still the most common method to differentiate tumor from a normal tissue. As for NSCLC, it comprises of three histological groups, adenocarcinoma, squamous cell carcinoma and large cell lung carcinoma. However, molecular subtyping of NSCLC have revealed additional layer of the complexity of this tumor heterogeneity. In particular, oncogenic mutations in KRAS (Kristen rat sarcoma viral oncogene homolog), EGFR (Epidermal Growth Factor Receptor), or fusions of EML4 (Echinoderm Microtubule-associated protein-Like 4) with -ALK

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(Anaplastic Lymphoma Kinase) which are the three most common biomarkers used in the clinics [15–17].

Metabolic heterogeneity within the same tumor type, that share similar initial diagnosis, has been less investigated [3,18]. We have previously uncovered quantitative metabolic heterogeneity within the Diffuse Large B Cell Lymphomas (DLBCL). In particular, we observed that DLBCL consist of metabolic subgroups, where some subtypes is dependent on fatty acid oxidation, a powerful antioxidant capacity and increased mitochondrial oxidative phosphorylation and other DLBCLs was more dependent on catabolism of glucose for energy production and generated more lactate [19]. In this study, we focused on the interrogation of glucose metabolism in NSCLCs.

2. Materials and methods

2.1. Cell lines and culture conditions

The non-small cell lung carcinoma cell lines NCI-H1792, NCI-H838, NCI-H1563 and NCI-H1573 (ATCC) were all grown in RPMI-1640 medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin and 2% (w/v) glutamine. Cells were grown in a humidified 5% CO_2 atmosphere at 37 °C allowing exponential cell growth.

2.2. Mitochondrial respirometry

Mitochondrial Oxygen Consumption (OCR) analysis in real-time was measured using the XFp Extracellular Flux Analyzer Seahorse Bioscience. Cells were seeded in an XFp miniplate at a concentration of 15,000 cells/well in RPMI medium. On the following day, cell were washed twice with 180 μl of XF Base medium (Seahorse Bioscience) supplemented with 10 mM glucose followed by the incubation with 180 μl of the same medium. After baseline measurement, 1 μM Oligomycin (a mitochondrial ATP synthase inhibitor) was injected to analyze the ATP-coupled mitochondrial oxygen consumption rate.

2.3. Assessment of glycolytic function

The Extracellular Acidification Rate (ECAR) was measured in real-time using the XFp Extracellular Flux Analyzer (Seahorse Bioscience). Cells were seeded in an XFp miniplate at a concentration of 15,000 cells/well in RPMI medium. On the following day, cell were washed twice with 180 μl of XF Base medium (Seahorse Bioscience) followed by the incubation with 180 μl of the same medium. Cells were analyzed using XFp Glycolysis Stress Test Kit (Seahorse Bioscience) following the manufacturer's instruction. Briefly, after baseline measurement, the following injections were made 10 mM Glucose, 1 μM Oligomycin and 50 mM 2-DG.

2.4. Western blot

Immunoblotting was performed as previously described [20]. Briefly, equal amount of the samples were mixed with loading buffer, boiled for 5 min, and subjected to 15% SDS–PAGE at 40 mA followed by transfer to nitrocellulose membranes for 90 min at 120 V. Membranes were blocked for 30 min with 5% non-fat milk in TBS at room temperature and subsequently probed with rabbit anti-hexokinase-2 (Cell Signaling) and mouse anti-Tubulin (Sigma Aldrich). The primary antibodies were diluted in TBS containing 1% (Bovine serum albumin) BSA, and 0.1% NaN_3. Horseradish peroxidase-conjugated secondary antibodies (Pierce) were diluted in 2.5% blocking buffer. Clarity™ Western ECL (BioRad) was used for revealing the blots.

2.5. Immunocytochemistry

Immunocytochemistry was performed as previously described [21]. Briefly, cells were seeded on glass coverslips, fixed 20 min in 4% formaldehyde at 4 °C and then washed twice with 1×PBS. Incubations with primary antibodies diluted (1:400) in PBS containing 0.3% Triton X-100 and 0.5% BSA were performed at 4 °C overnight in a humidified chamber. The slides were washed the following day with PBS before incubation with secondary antibodies (1:200) at room temperature for 1 h. Nuclei were counterstained with DAPI (10 μg/ml) by 5 min incubation at room temperature. The following primary antibodies were used: rabbit anti-HK2 (Cell Signaling) and mouse anti-cytochrome c (BD Bioscience). Secondary FITC-conjugated antibodies (Molecular probes) were directed to donkey anti-mouse (Alexa 488) and goat anti-rabbit (Alexa 563). Stained slides were mounted using Vectashield H-1000 (Vector Laboratories Inc.) and examined under a Zeiss LSM 510 META confocal laser scanner microscope (Zeiss).

2.6. Starvation assays

Cells were grown in RPMI medium supplemented with 10% dialyzed FBS in the presence or absence of 10 mM D-glucose. Cell death was analyzed using Toxilight Cell Death kit (Lonza) after 24 h of starvation.

2.7. RNAi studies

Genetic silencing of HK2 was achieved by transfection of two independent siRNAs targeting HK2 (GenePharma) using Interferin (Polyplus transfection). The effect of knockdown was assessed by western blot and Cell death assays 24 h after transfection.

2.8. Statistical analysis

In all cases, mean ± SEM are presented. The number of independent experiments is indicated in the figure legends and statistical significance was determined using two-tailed Student's *t*-test.

3. Results

Enhanced glucose metabolism is a common feature of many tumors that distinguish them from their normal counterparts.

To assess the glycolytic function of NSCLC cell lines, the extracellular acidification rate (ECAR) was analyzed, in real-time, using an Extracellular Flux Analyzer. Upon injection of glucose, the non-small cell lung carcinomas (NSCLC) NCI-H1792 and NCI-H838 displayed a significantly enhanced glucose metabolism than NCI-H1563 and NCI-H1573 (Fig. 1A). In addition, glycolysis (Fig. 1B) as well as the glycolytic capacity (Fig. 1C) was elevated in NCI-H1792 and NCI-H838 cells compared to NCI-H1563 and NCI-H1573 cell lines, revealing distinct glycolytic demand in NSCLCs.

To understand whether glycolysis was used to fuel bioenergetics in the form of ATP, we analyzed mitochondrial oxygen consumption rates (OCR) on glucose, as the only carbon substrate, with a central focus on the ATP associated OCR (oligomycin sensitive). The results revealed that the NSCLCs with higher capacity for glucose catabolism also use glucose to generate ATP to a higher extent than cells less glycolytic NSCLCs (Fig. 1D). Furthermore, a phenogram analysis of OCR and ECAR on glucose displayed that NCI-H1792 and NCI-H838 are significantly more metabolically active than NCI-H1563 and NCI-H1573, as glucose stimulates both mitochondrial oxygen consumption rates as well as ECAR (Fig. 1E). These observations are suggestive of quantitative metabolic heterogeneity in the catabolism of glucose in NSCLC.

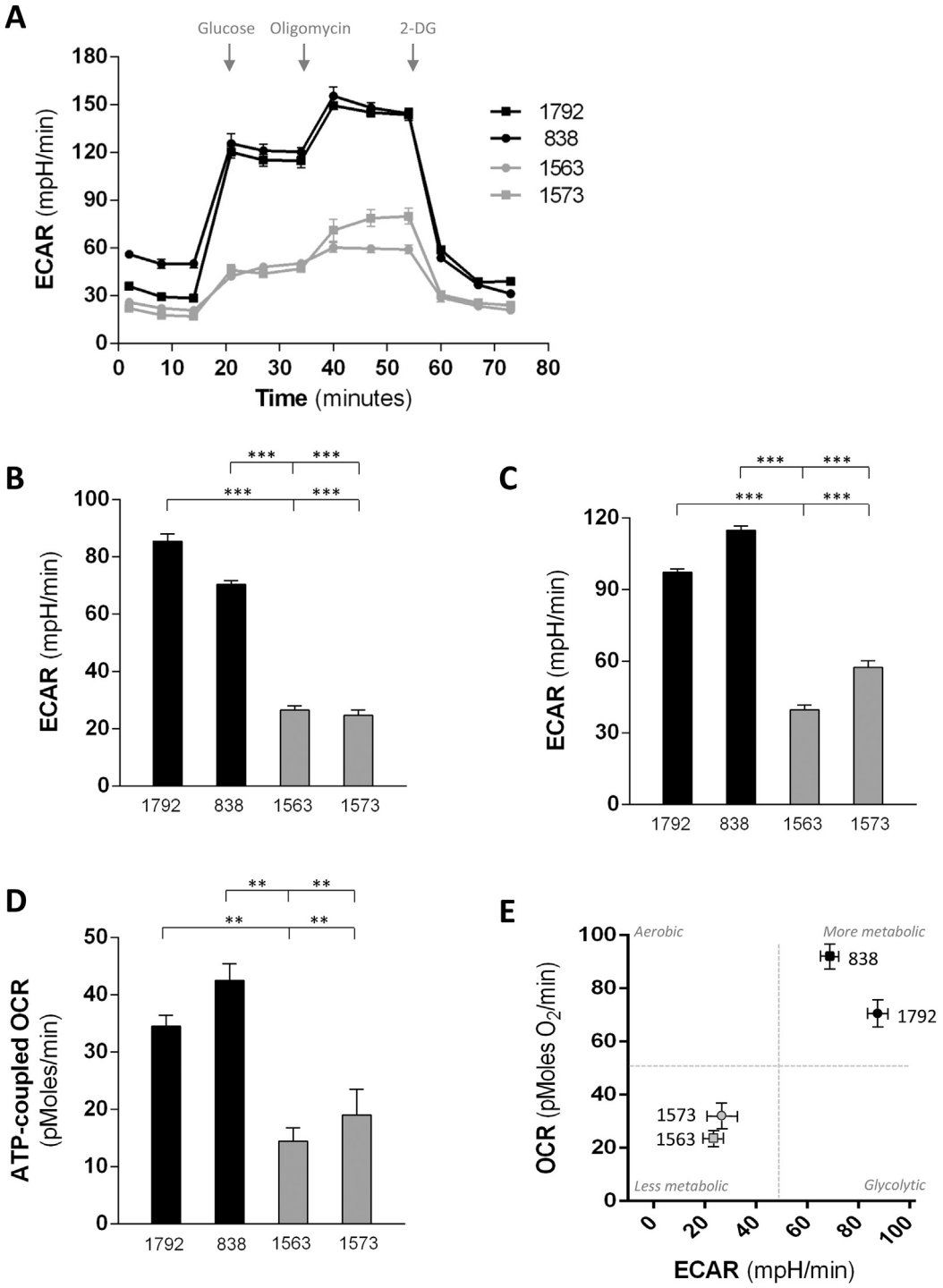

Fig. 1. Distinct utilization of glucose in NSCLCs. Assessment of the (A) glycolytic function, (B) glycolysis and (C) glycolytic capacity (D) ATP-coupled mitochondrial oxygen consumption rates (OCR) on glucose (E) phenogram analysis comparing OCR and ECAR in NSCLC cells using an extracellular flux analyzer. 6–9 independent OCR and ECAR measurements for each cell line. Error bars, ±SEM. $^{**}p < 0.01$; $^{***}p < 0.001$; two-tailed student's t-test.

The distinct capacity to utilize glucose predicts differences in sensitivity to depletion of the glycolytic pathway. To provide a genetic approach, two independent siRNAs targeting hexokinase-2 (HK2) was used. The results revealed that genetic depletion of HK2 (Fig. 2A) was selectively toxic to the glycolytic NSCLCs (Fig. 2B). All together, these observations suggest that glucose catabolism provides differential prosurvival benefits to NSCLCs.

To probe the physiological relevance of a distinct capacity to do glycolysis, the cells were exposed to glucose withdrawal and the effect on survival was analyzed (Fig. 3A). The highly glycolytic NSCLC cell lines (NCI-H1792 and NCI-H838) were significantly more sensitive to such metabolic perturbation (Fig. 3A). These observations predict a selective sensitivity to 2-deoxy-glucose (a glucose-analog that inhibits glycolysis) to the highly glycolytic NSCLCs compared to the less glycolytic NSCLCs. Pharmacologic inhibition of glycolysis by low levels of 2-deoxy-glucose proved substantial and selective toxicity to the glycolytic NSCLCs (Fig. 3B). Combined, these results provide evidence that NSCLC is

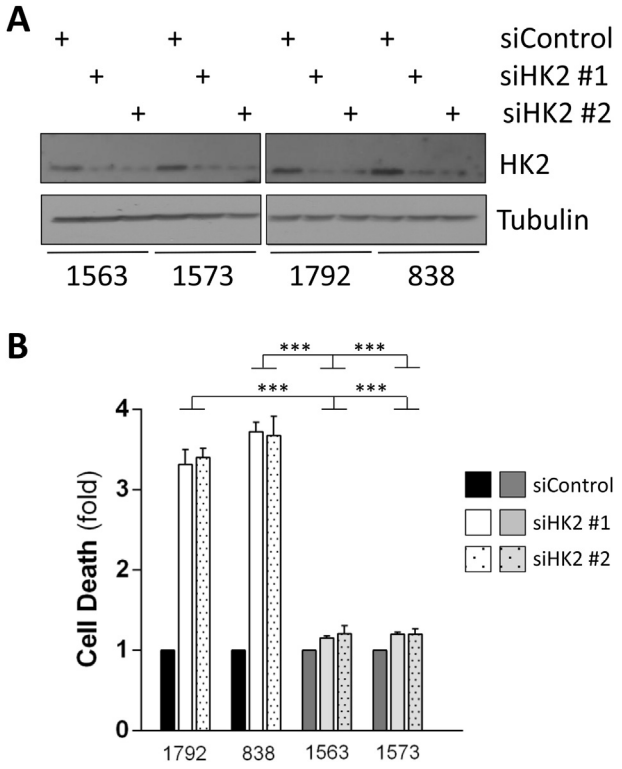

Fig. 2. Differential contribution of glycolysis to the survival of NSCLCs. Cells were depleted of HK2 by two independent siRNAs and subsequently (A) the protein level was analyzed using western blot (B) the effect on cell death. Tubulin was used as a loading control on western blot.

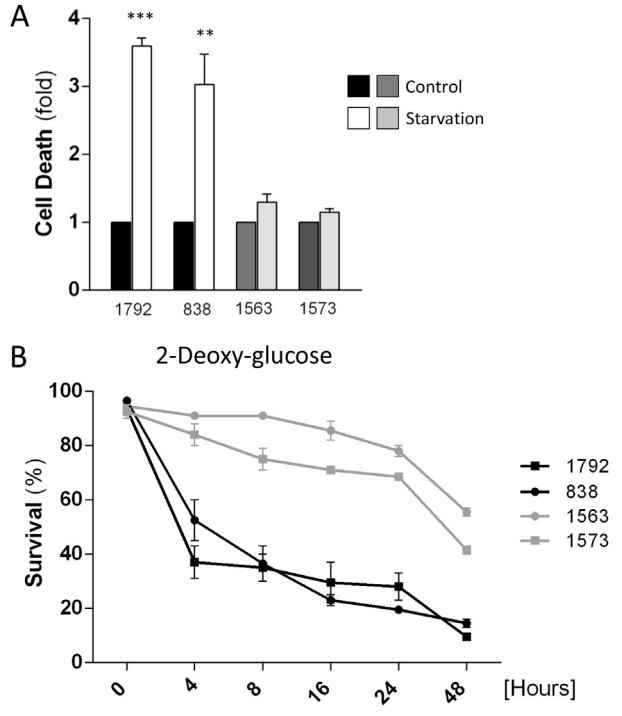

Fig. 3. Perturbations of glycolysis have distinct effects on the survival of NSCLCs. (A) The NSCLC cells were starved of glucose for 24 h and the effect on cell toxicity was analyzed. (B) The effect of pharmacological inhibition of glycolysis, by 1 mM 2-deoxy-glucose, to cell survival was analyzed from 0 to 48 h of exposure. Error bars, ±SEM. $^{**}p < 0.01$; $^{***}p < 0.001$; two-tailed student's t-test.

metabolically diverse with a differential capacity to perform glycolysis that is tightly associated with survival signaling.

Hexokinase-2 (HK2) has been reported to be localized in the cytosol or attached to the mitochondria and the subcellular localization appears to be associated with different functions of the enzyme. While the cytosolic HK2 preferentially promote the pentose phosphate pathway, the mitochondrial HK2 promotes glycolysis and oxidative phosphorylation [8]. To investigate whether the observed difference in glycolysis in NSCLCs could be explained by the expression level and activity of HK2, or by the enzymes physical association with mitochondria, its subcellular localization was investigated by immunocytochemistry. The results revealed that the NSCLC cells exhibiting high glycolytic function also express higher level of HK2, which was largely associated with mitochondria (Fig. 4A, B). Thus, we concluded that the expression level and subcellular localization seem to be important for the metabolic plasticity in NSCLC.

4. Discussion

There is accumulating evidence that tumor cells exhibit metabolic adaptations that distinguishes them from normal cells. The metabolic adaptations have prognostic and therapeutic value.

Elevated glucose metabolism is a common feature of rapidly proliferating cells like many tumors. Therefore to suppress glycolysis or the activity of glycolytic enzymes has been suggested to offer a potential therapeutic target, with potentially low toxic effects on normal cells. Suppressing glycolysis can be done at multiple levels including systemic inhibition (blocking gluconeogenesis) by anti-diabetic agents such as metformin [22], which force cells to rely on fatty acid oxidation for energy supply, or by supplying a ketogenic diet [23]. Additional ways of targeting glycolysis involves direct targeting of glycolytic enzymes. Both strategies of targeting glucose metabolism are based on the elevated cell autonomous dependence on glucose metabolism, which are currently being evaluated in the clinics.

Although several isoforms of hexokinases exists, HK2 are primarily used by tumors [8]. It was recently demonstrated that genetic deletion of HK2 inhibits tumor growth in preclinical cancer mouse models. Importantly, systemic deletion of HK2 displayed no toxic effects on the animals [24]. This implies that, in particular, high expressing HK2 tumors might potentially be targeted with limited side effects. This is consistent with our observations that genetic depletion HK2 in low expressing HK2 cancer cells appears to be well tolerated and non-toxic, while it exerts strong and selective toxicity to high HK2 expressing cells.

Metabolic heterogeneity within the same tumor type has been less investigated although multiple examples exist [3,18,25] where cancer cells express different isoforms of metabolic enzymes. The enzymes activities are further controlled by posttranslational modifications [26]. Our results provide a functional validation of this metabolic diversity within the same tumor type, as high HK2 expressing NSCLCs are highly glycolytic and display selective sensitivity to perturbations of glycolysis. These NSCLCs were more metabolically active and used glucose for a multiple cellular functions, including oxidation of glucose in mitochondria, contributing to the cellular ATP pool (OCR) and producing lactate (ECAR) as an end product of glycolysis.

Beyond NSCLCs, we have previously observed metabolic heterogeneity in DLBCLs [19], and it was demonstrated that differential expression of monocarboxylate transporter 4 (MCT 4) was associated with a distinct metabolism in pancreatic ductal adenocarcinomas [27]. MCT1-4 transporters were reported to be highly expressed in NSCLC tumors, and inhibition of lactate export was found to sensitize cells to phenformin [28]. In all cases, the metabolic heterogeneity

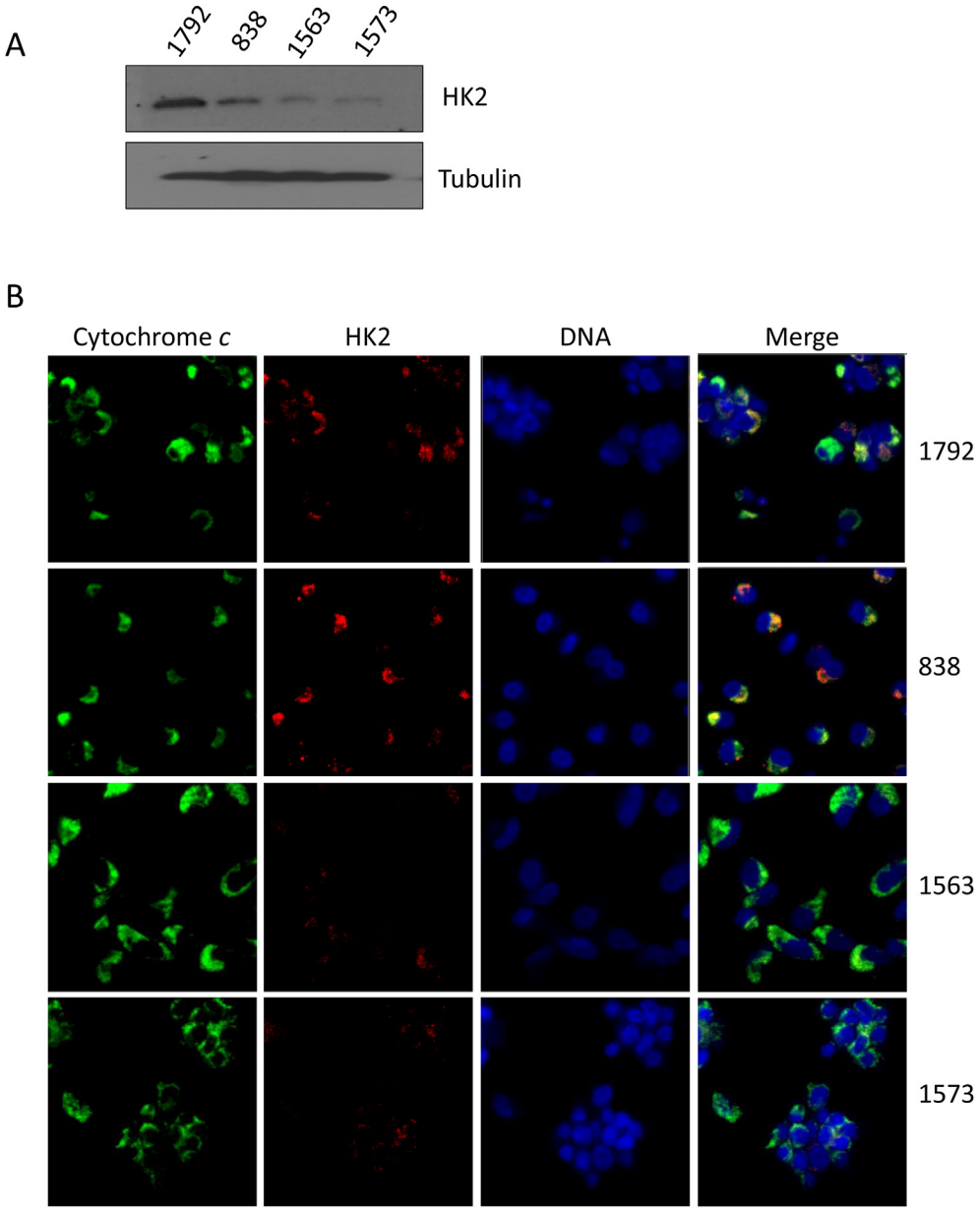

Fig. 4. Expression and subcellular localization of hexokinase-2 in NSCLCs. (A) The expression level and (B) subcellular localization of HK2 was analyzed by western blot. Tubulin was used as a loading control on western blot. Cytochrome *c* (green) was used as a marker of mitochondria, DAPI a marker of DNA (blue) and HK2 (red).

was strongly associated with survival signaling and is likely a way for tumors adapt to various biosynthetic and bioenergetic needs. Metabolic profiling could beyond uncover 'metabolic subtypes', also increase our understanding on the nutritional preference of these 'subtypes'. This could be important for the molecular pathogenesis and may have therapeutic benefits.

Conflict of interest

All authors declare no conflict of interest.

Acknowledgments

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