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Original Article

Human haptoglobin contributes to breast cancer oncogenesis through glycolytic activity modulation

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Abstract: Cellular metabolism reprogramming is a hallmark in cancers including breast cancer. Switching off the glycolytic energy in cancer has been indicated as one of the anti-cancer strategies. Aberrant haptoglobin (*HP*) expression has been shown to cause metabolic dysfunction and implicated in different malignancies. However, its roles in breast cancer and glycolysis remain elusive. Here, we reported *HP* was upregulated in breast cancer tissues and the circulation. *HP* conferred oncogenic roles by regulating cell cycle progression and apoptosis in breast cancer cells. Further analysis identified the correlation between *HP* and glycolytic enzymes such as glucose-6-phosphate isomerase (*GPI*) and hexokinase (*HK*). Glycolytic activities were altered upon *HP* knockdown which were confirmed by glucose uptake and LDH activity assays. *GPI* was found to be downstream effector of *HP* while knockdown of *GPI* led to decreased glycolytic activity and restored oxygen consumption. *GPI* silencing decreased cell migration/invasion ability and sensitized breast cancer cells to chemo-drug. Moreover, animal study suggested inhibition of both *HP* and *GPI* significantly impeded tumor growth in mice. Collectively, we report for the first time the oncogenic roles of *HP*, at least partially, through regulating glycolysis and its downstream effector, *GPI*, contributes in maintaining EMT and chemoresistance in breast cancer.

Keywords: Haptoglobin, breast cancer, glycolysis, *GPI*

Introduction

Breast cancer is one of the most common malignancies among women. According to the Globocan data from the World Health Organization, there were approximately 2.1 million new cases diagnosed in 2018 (Globocan 2018, http://gco.iarc.fr/today/fact-sheets-cancers). It was the second most common cancer accounting for about 12% of all new cancer cases in women and caused around 63,000 cancer-related mortalities worldwide. The 5-year survival rates for localized, regional and distant breast cancer were 99%, 85% and 27%, respectively [1].

It is well-known that mitochondria to utilising energy from alternative sources including glucose, fatty acids and amino acids to generate ATP through the Krebs cycle in the presence of oxygen in normal cells [2]. In the absence of oxygen, however, glucose is converted into lactate instead [3]. In cancer cells, a large amount and rapid production of ATP are essential to fuel up and maintain the uncontrolled proliferation. A great proportion of ATP (as much as 60%) is thus generated even lack of oxygen through glycolysis. Numerous human cancers such as colorectal [4], breast [5], lung cancer [6] and glioblastoma [7] display high rate of glycolysis under aerobic conditions, which was first described as Warburg effect in the early 1920s [8]. Although this mitochondrial bypass is energy inefficient, it is compensated by increased glycolytic glucose flux to provide additional biosynthetic precursors to support the rapid energy demand [9]. The rate of glucose metabolism through aerobic glycolysis is 100 times faster than the complete oxidation of glucose in the mitochondria [10]. One of the explanations for glycolysis dominance in cancer cells is to prevent DNA damage from oxygen radicals generated during oxidative phosphorylation [11].

Table 1. Clinical characteristics of breast cancer patients

	Breast Cancer (n=58)
Age [years; mean (SD)]	44.4 (8.3)
Histological type	
DCIS	3
IDC	54
ILC	1
Bilateral Cancer	4
Histology grade	
1	5
2	28
3	15
Unknown	10
Stage	
0	4
I	29
II	17
III	5
IV	3
Subtypes	
Luminal	53
HER2	5

Abbreviation: DCIS, ductal carcinoma in situ; IDC, invasive ductal carcinoma; ILC, invasive lobular carcinoma; HER2, human epidermal growth factor receptor 2.

Acute phase reactant proteins (APRPs) are produced in the liver during inflammation, tissue injury or cancer development. Studies have discovered a connection between APRPs and cancer, which was suggested as a cancer biomarker [12]. Haptoglobin (*HP)* is a serum α2-sialoglycoprotein that functions as an anti-oxidative APRP by binding with free haemoglobin. *HP* is involved in the pathogenesis of tumors and infections through both innate and acquired immunity [13]. Evidence supported that expression of *HP* was elevated in pancreatic, prostate, colon and liver cancer, suggesting its potential role as a biomarker for cancer diagnosis [14]. It binds to free plasma haemoglobin to prevent iron loss and renal damage [15]. Some studies reported that *HP* was involved in angiogenesis and cell migration [16, 17]. Recent investigation unveiled that circulating *HP* level not only correlated with insulinemia in obese individuals [18], but also contributed to glucose and lipid metabolic dysfunction in liver cancer patients such as insulin resistance and hepatosteatosis [19]. The well-established *HP*-associated glucose metabolic dysfunction inspired us to study the linkage between *HP* and glycolysis in breast cancer.

Methods

Ethics approval and consent to participate

This study was reviewed and approved by the Institutional Review Boards (IRB) of the University of Hong Kong and IRB of the collaborating centres. The details of the study information was explained to each participants and signed consent forms were obtained from all recruited individuals.

Patients and specimens

We included 30 normal healthy individuals and 58 breast cancer patients with informed consent through the Hong Kong Hereditary Breast Cancer Family Registry, Queen Mary Hospital and other hospitals in Hong Kong. Sample collection protocols were approved by the Institutional Review Boards (IRB) of the University of Hong Kong and IRB of the collaborating centres. For all participants, we collected details on pathological and clinical factors associated with breast cancer risk and prognosis such as age, staging, subtypes, etc. Clinico-pathological data of breast cancer patients was listed in **Table 1**.

Cell culture and transfection

MDA-MB-231 (triple negative breast cancer, TNBC), MDA-MB-468 (TNBC), MCF-7 (estrogen receptor positive, ER+) and T47D (estrogen receptor positive, ER+) were used. MDA-MB-231 (ATCC no. HTB-26) and MDA-MB-468 (ATCC no. HTB-132) cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS). MCF-7 (ATCC no. HTB-22) and T47D (ATCC no. HTB-133) cells were cultured in DMEM medium supplemented with 10% FBS. The glucose-free RPMI-1640 medium supplemented with 10% FBS was used to enhance glycolytic activity. For transient transfection, cells were transfected with Allstar Negative Control siRNA or target gene siRNA for 3 days. For stable knockdown cells development, the expression vectors containing shRNA constructs against target genes or scrambled sequences were purchased from GenePharma. After transfection for 3 days, cells were select-

Table 2. qRT-PCR primers

Gene	Forward primer (5'- to 3')	Reverse primer (5'- to 3')
HP	CAGCCAGAAACATAACCC	TCTACACCCTAACTACTCCC
GPI	CCTGGGCTCCAGTGATCC	GATATGCCCATGGTTGGTGT
HK1	CGAGGTTTATGACACCCCAG	TGGCATCATAGTCCTCATCTATT
HK2	AAAGTAACGGACAATGGGCT	ACTGGACTTGAATCCCTTGG
GLUT1	TTGGCTACAACACTGGAGTC	TGGACCCATGTCTGGTTGTA
PDK1	AAGCAGTTCCTGGACTTCGG	TCTTGCAGGCCATACAGCAT
Beta-ACTIN	AGAGCTACGAGCTGCCTGAC	AGCACTGTGTTGGCGTACAG

ed by geneticin (G418) for 4 weeks and the knockdown efficiency was confirmed by qRT-PCR and Western blot.

Cell proliferation assay

Transient transfected cells (5×10^3 for MDA-MB-231, 8×10^3 for MDA-MB-468) were seeded in a 96-well microtiter plate with triplicates. After 72 h of incubation, cell viability was measured by MTT assay, intracellular purple formazan was solubilized in DMSO followed by the colorimetric product quantified at absorbance 570 nm by microplate reader (Thermo Fisher Scientific).

ELISA

Serum samples from 30 normal healthy individuals, 58 breast cancer patients, 44 pre-operation and post-operation serum samples from breast cancer patients were used to quantify the haptoglobin levels. The 96-well ELISA plate was pre-coated with antibody specific for haptoglobin. All the steps were performed according to the manufacturer's instruction. Briefly, 25 µl of serum or culture medium and 25 µl of biotinylated haptoglobin was added into each well and incubated for 1 h at 37°C. After washing with 200 µl of wash buffer, 50 µl of SP conjugate was added and incubated for 30 min. Then 50 µl of chromogen substrate was added and incubated for 10 min followed by adding 50 µl of stop solution. The optical density of each well was determined at 450 nm wavelength within immediately by microplate photometer (Thermo Fisher Scientific).

Cell cycle analysis

Cells were fixed in iced-cold 70% ethanol at -20°C overnight. Then cells were washed with PBS and stained with 20 µg/ml propidium iodide (PI) and 0.2 mg/ml of RNase A for 30 min and subjected to flow cytometric analysis. Flow cytometric study was performed by BD FACSCalibur using CellQuest software (BD Biosciences). The average values of G0/G1, S and G2/M phases calculated from at least three independent experiments.

Apoptosis assay

Cellular apoptosis was detected using FITC Annexin V Apoptosis Detection Kit according to manufacturer's instructions. Briefly, cells were suspended in binding buffer. FITC Annexin V together with PI were added into the resuspended cells and incubated 15 min at room temperature in the dark. After incubation, binding buffer was added to suspend the cells and analysed using BD FACSCalibur.

Glucose uptake assay

Cells were seeded in 6-well plates at 1×10^4 cells/well for 24 h. Briefly, the culture medium was removed from each well and replaced with 2 ml of culture medium in the absence or presence of 10 µM fluorescent 2-NBDG. Cells were incubated for 10 min prior to flow cytometry analysis.

qRT-PCR

Total RNA was reversely transcribed into cDNA with High Capacity cDNA Reverse Transcription kit (Thermo Fisher Scientific). Real-time qPCR was performed using QuantiTect SYBR Green PCR Kit in Roche LightCycler480 II system (Roche). Primers sequences were listed in **Table 2**. The expression level of housekeeping gene was used for normalization. The reaction for each sample was performed in triplicates.

Lactate dehydrogenase (LDH) activity assay

Serum or cell culture medium were added to a final volume of 50 µl with LDH assay buffer in 96-well plate. Then 50 µl of the Master Reaction Mix was added to each of the wells. After mix well, plate was incubated at dark for 2-3 min and measured the absorbance at 450 nm as initial reading. After taking the final reading at 30 min, calculated the enzymatic activity by the standard curve as described in standard protocol.

Flow cytometry analysis of SOX2-positive cells

2×10^5 cells/well were incubated with primary antibody (isotype IgG control or *SOX2*) at 4°C overnight. After washed with PBS for 3 times, cells were incubated with secondary antibody conjugated with fluorescence signal for 60 min at room temperature and subjected to flow cytometry analysis.

Extracellular oxygen consumption assay

Cells were seeded at a density of 8×10^4 cells/well in a 96-well plate. After 24 h, culture media were replaced with 150 µl of fresh culture media and 10 µl of reconstituted extracellular O_2 consumption reagent (Abcam) was added into each well. Each well was sealed immediately with 100 µl high-sensitivity mineral oil to avoid diffusion of oxygen into the assay medium and measured the signal using fluorescence plate reader (Molecular Devices) at 3 min intervals for 100 min at Ex/Em=380/650 nm.

Western blotting

Cells lysate were prepared in 100 µl of lysis buffer (Cell Signaling Technology). After centrifuged at 14,000 g for 15 min, supernatant was subjected to Bradford assay (Bio-Rad) for protein quantification. 80 µg of protein was separated by 10% SDS-PAGE and transferred to a polyvinylidene difluoride membrane using a transfer system. The unspecific binding sites were blocked with 5% non-fat milk in tris-buffered saline with Tween-20 (TBST) for 60 min at room temperature. The membranes were washed with TBST and incubated with primary antibody at 4°C overnight with gentle shaking. Afterwards, secondary antibody (Cell Signaling Technology) was added and incubated for 60 min at room temperature. The membrane was washed for 3 times, 5 min each time in TBST. Chemiluminescence was determined using the enhanced chemiluminescence system (Amersham Biosciences).

In vivo xenograft animal model

The animal experiment ethics (CULATR 4409-17) was approved by the Committee on the Use of Live Animals in Teaching and Research in the University of Hong Kong. 6-week old female NOD-SCID mice were used for animal model. Briefly, 2×10^6 MDA-MB-231 cells with shControl or sh*HP* suspended in 100 µl of PBS were injected into the mammary fat pad of the mice. Mice were randomly divided into: i) shControl; ii) sh*HP*; iii) shControl+E4P (*GPI* antagonist); iv) sh*HP*+E4P. shControl+E4P, sh*HP*/sh*HP*+E4P group, respectively. Tumor volumes were measured from two weeks onwards with palpable mass by using formula [1/2(length × width^2)] every 7 days. After 2 weeks of post-injection, E4P was administered by intraperitoneal injection at a dose of 25 mg/kg twice a week for 6 weeks. Mice were sacrificed and tumors were harvested at week 8.

Immunohistochemistry (IHC) staining

Briefly, antigen unmasking was performed after deparaffinization. The sections were incubated with primary antibodies (Cell Signaling Technology: Ki67, and E-cadherin; Thermo Fisher Scientific: HP and GPI; Abcam: SOX2) at 4°C overnight. The detection was performed according to the manufacturer's instructions using Signal Stain Boost Detection Reagent (Cell Signaling Technology) and incubated in a humidified chamber for 30 min at room temperature followed by washing. Then Signal Stain DAB was added to each section and subjected to the dehydration of the sections by using 95% and 100% ethanol respectively before mounting.

Statistical analysis

The differences between groups were estimated by Student's t-test, non-parametric Mann-Whitney U test as appropriate. $P < 0.05$ was considered as statistically significant. All statistical analyses were performed using GraphPad Prism 6.0.

Results

HP expression is higher in breast cancer patients

HP expression was evaluated in normal tissues (NC), paired tumor tissues (T) and adjacent non-tumor tissues (TN) by qRT-PCR. Result showed that *HP* mRNA level was significantly higher in T when compared to TN and NC, while there was no significant difference between NC and TN (**Figure 1A**). Furthermore, *HP* mRNA level was significantly higher in the plasma of

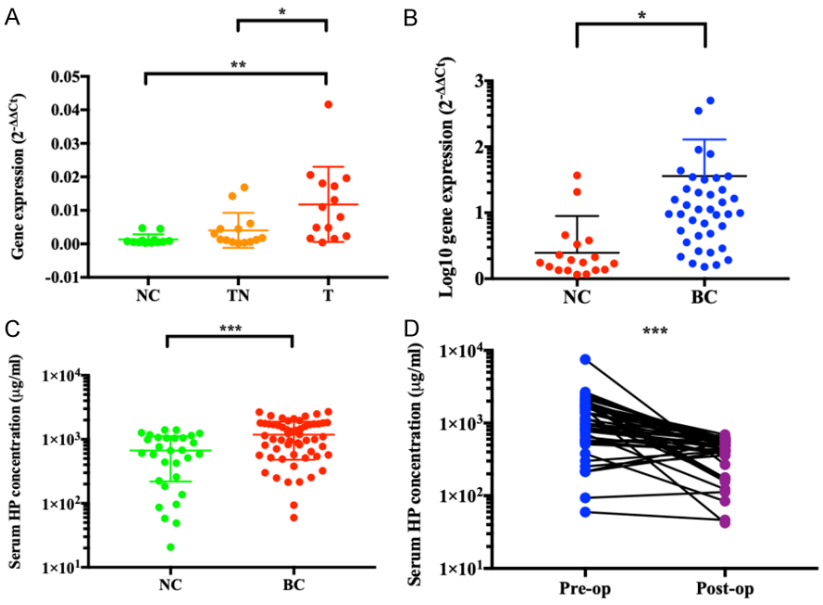

Figure 1. *HP* expression in breast cancer tissues and circulation. A. *HP* mRNA levels in breast cancer tissues (T), tumor adjacent normal tissues (TN) and normal controls' (NC) tissues; B. *HP* mRNA expressions in breast cancer (BC) plasma and normal controls; C. ELISA of haptoglobin concentration in breast cancers and normal controls' serum samples; D. Haptoglobin levels in pre-operation and post-operation serum samples. *P < 0.05, **P < 0.01, ***P < 0.001 indicates statistically different.

breast cancer (BC) patients when compared with NC (**Figure 1B**). Similarly, circulating *HP* peptide concentration was also higher in BC than NC (**Figure 1C**). *HP* level in post-operative patients' serum were remarkedly lower when compared with the pre-operative serum (**Figure 1D**).

HP conferred tumorigenic role by modulating G1-phase cell cycle arrest and apoptosis

The expression of *HP* was highest in TNBC cells, namely, MDA-MB-231 and MDA-MB-468 (**Figure 2A**), cell proliferation was inhibited after knockdown of *HP* by siRNA (**Figure 2B**). Functional experiments indicated that knockdown of *HP* led to G0/G1 phase cell cycle arrest (**Figure 2C**) and decreased *Cyclin-D1* protein expression (**Figure 2D**). Apoptosis analysis identified increased late apoptotic cell population in both cell lines (**Figure 2E**).

HP is required for glycolysis activity in breast cancer

To elucidate the relationship between *HP* and glycolysis, we first compared the expression of glycolysis-related genes in full glucose and glucose-free conditions. In glucose-free condition, the expression level of *HP* as well as some key enzymes in glycolysis pathway, for instance *HK1* and *GLUT1,* were significantly upregulated when compared with the full glucose condition, indicating glycolytic activity was enhanced in the absence of glucose (**Figure 3A**). Knockdown of *HP* decreased the expressions of glycolysis-related key enzymes (*GPI*, *GLUT1*, *HK1*, *HK2*, *PDK1* etc), which provided more solid evidence to support our hypothesis that *HP* is closely related to cancer glycolysis (**Figure 3B**, **3C**). The increased glucose influx via glycolytic process is one of the signatures in Warburg effect, we evaluated the glucose influx by 2-NBDG uptake assay in cells transfected with *HP* siRNA. A decreased uptake of glucose analog, 2-NBDG, was observed in si*HP* when compared with siControl, suggesting an inhibition of glycolytic activity upon knockdown of *HP* (**Figure 3D**). Besides, we further measured the lactate dehydrogenase (LDH) activity, which is an enzyme in anaerobic glycolysis, in normal and patients' serum samples. A significant higher LDH activity level was seen in BC (**Figure 3E**). Also, LDH activity was decreased in the cell culture medium after knockdown of *HP* (**Figure 3F**), implicating that *HP* is partly related to glycolysis.

GPI, a downstream effector of HP, contributed to invasion and migration

As shown in **Figure 3B**, knockdown of *HP* led to downregulation of *GPI*, we further investigated the functional role of this potential *HP* downstream effector in breast cancer cells. In stable *GPI* knockdown cells (**Figure 4A**), we observed a steady inhibition of glycolytic activity in *GPI*-deficient cells, which was reflected by reduced 2-NBDG uptake (**Figure 4B**) and LDH activity in the cell culture medium (**Figure 4C**). Based on

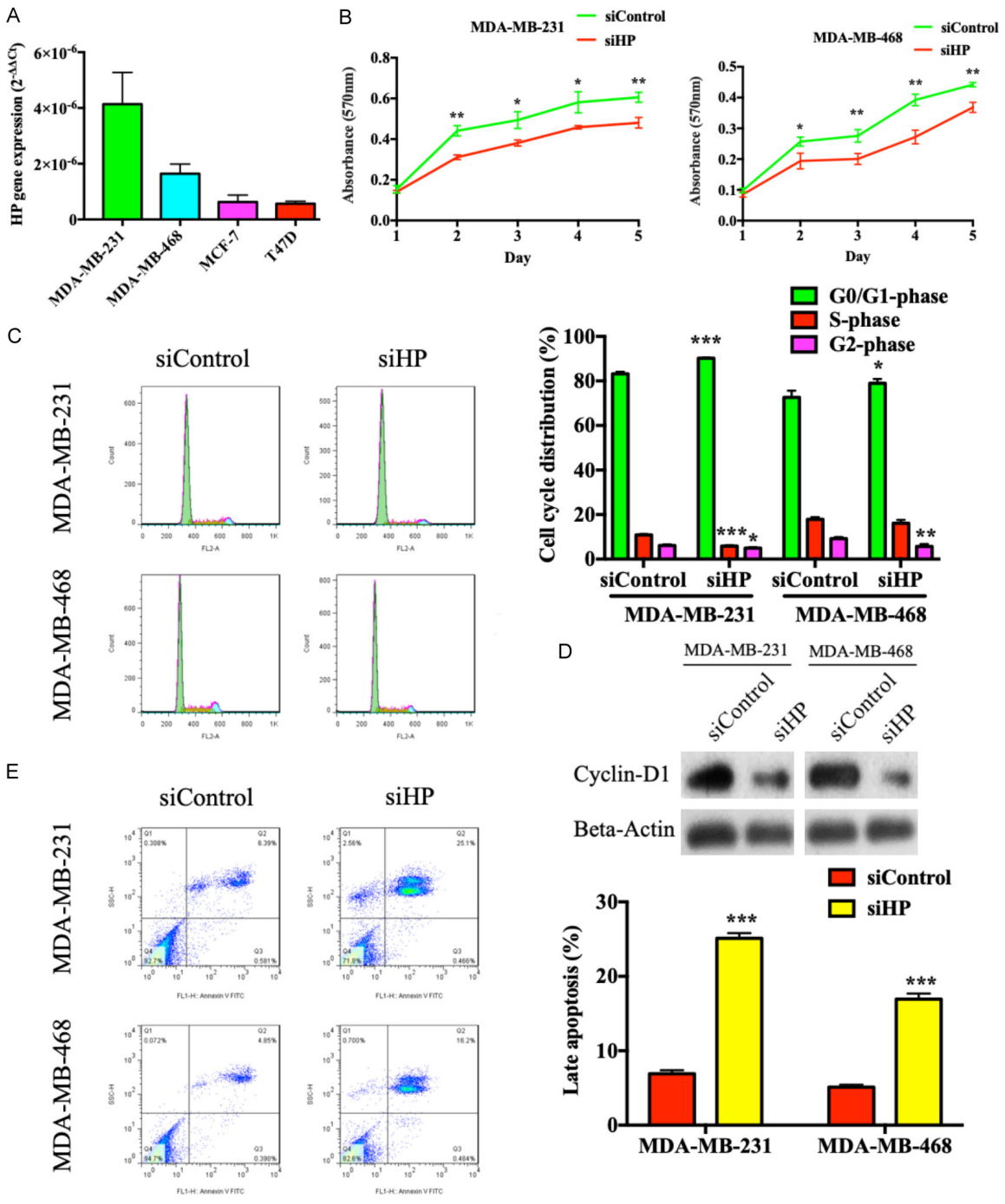

Figure 2. *HP* expressions in breast cancer cell lines and its effect on cell proliferation. (A) qRT-PCR assay was performed to detect the expression of *HP* in breast cancer cell lines; (B) MTT assay in breast cancer cells upon *HP* siRNA knockdown; (C) Cell cycle analysis and (D) Western blotting of Cyclin-D1 expression in siControl and si*HP* cells; (E) Apoptotic assay were performed in breast cancer cells after *HP* siRNA knockdown. Results are presented as mean ± SD. *P < 0.05, **P < 0.01, ***P < 0.001 indicates statistically different.

the fact that cells consume less oxygen than the mitochondrial oxidation during glycolysis, the oxygen consumption rate could be used as an indicator for glycolysis. Result revealed that *GPI* knockdown with taxol treatment consumed the most amount of oxygen among all groups, indicating an enhanced inhibition on glycolysis when compared with sh*GPI* alone (**Figure 4D**). *GPI*

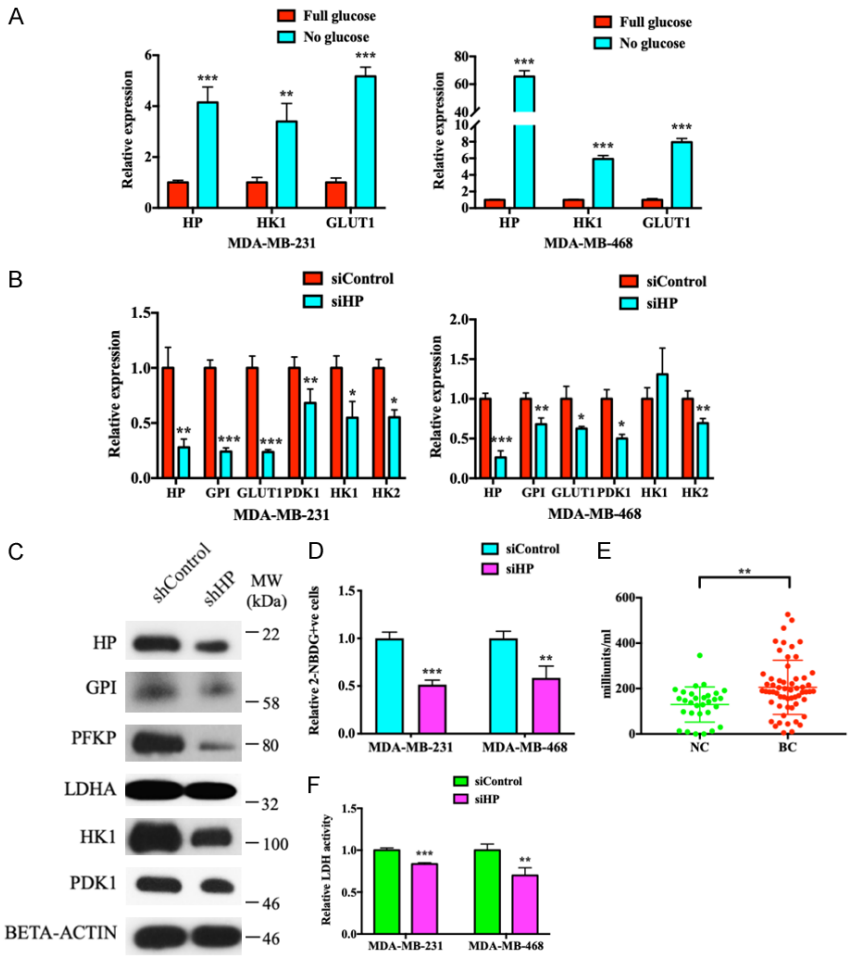

Figure 3. *HP* is closely related to glycolytic activity in breast cancer cells. (A) *HP* and glycolytic enzymes' expressions in breast cancer cells treated with glucose-free medium; (B) qRT-PCR was introduced to determine the expression levels of key glycolytic enzymes in breast cancer cells after knockdown of *HP*; (C) Protein levels of glycolytic enzymes after *HP* siRNA treatment were detected by Western blot analysis ; (D) 2-NBDG assay was performed to determine the glucose uptake rate in breast cancer cells with *HP* silencing; (E) LDH activity levels were determined in the serum of breast cancer patients and normal control and (F) culture medium from breast cancer cells after knockdown of *HP*. Results are presented as mean ± SD. *P < 0.05, **P < 0.01, ***P < 0.001 indicates statistically different.

knockdown with taxol treatment showed more prominent effect on cell proliferation than sh*GPI* or taxol alone (**Figure 4E**). In addition, *GPI* knockdown decreased cell invasion (**Figure 4F**) and migration ability (**Figure 4G**). Furthermore, flow analysis revealed that *GPI* knockdown significantly decreased *SOX2*-positive cells, suggesting a relationship between *GPI* and cancer stemness (**Figure 4H**).

HP silencing inhibited tumor growth in vivo

To evaluate the roles of *HP* and *GPI* in tumor growth, mice with shControl or sh*HP* cells were administered with *GPI* antagonist (E4P). Tumor volume in sh*HP*+ E4P mice was smaller than sh*HP* mice (**Figure 5A**, **5B**). IHC staining and Western blotting results showed that *Ki67, HP, GPI* and *SOX2* expressions were decreased while *E-Cadherin* expressions were increased in tumors with sh*HP*/ E4P treatment (**Figure 5C**, **5D**). All these data showed that knockdown of *HP* caused glycolytic inhibition and subsequently impeded tumor growth in breast cancer.

Discussion

The link between aerobic glycolysis and tumorigenesis has been well-established for a century since the discovery by Otto Warburg [8]. High glycolytic activity and its generated lactate level significantly correlated with tumor recurrence and metastasis, resulting in poor prognosis in cervical cancer [20]. Aerobic glycolysis is an unique signature of cancer cells, therefore, a number of potential candidate drugs have thus developed and tested [21]. Although these potent antagonists or inhibitors for glycolysis have demonstrated promising anticancer effects, most of them are still in preclinical phase [22, 23].

One of the most undesirable side-effects of anti-glycolytic agents is systemic toxicity, which due to the disruption of metabolic function of glycolytic enzymes in normal cells. The significance of *HP* in glucose metabolism and its well-established correlation with various cancers, however, investigation on the functional role of

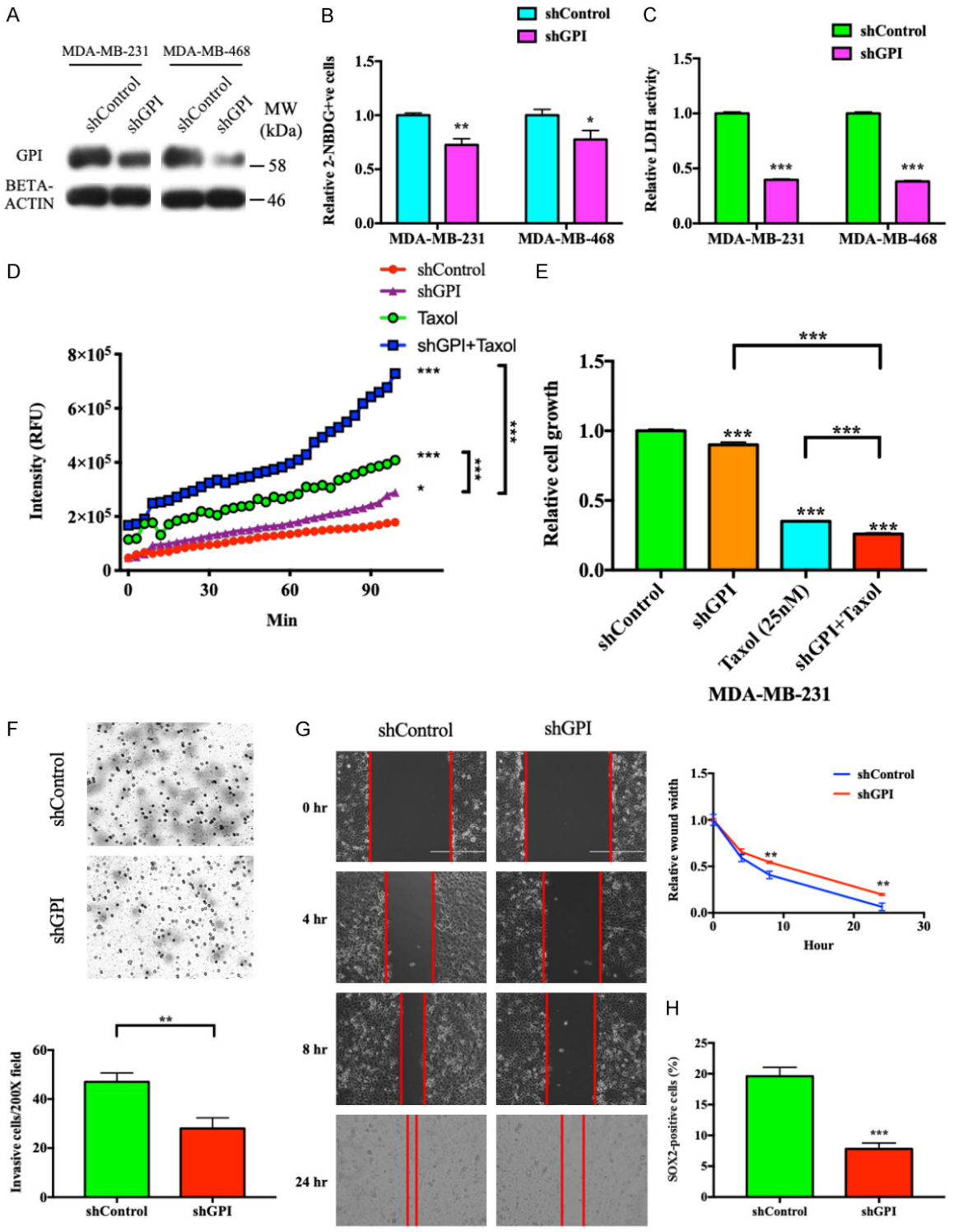

Figure 4. *HP* downstream effector *GPI* contributes to invasion and migration and sensitizes breast cancer cells to taxol agents. (A) Western blotting of GPI protein expression in shControl and sh*HP* cells; (B) Glucose uptake rate was investigated by 2-NBDG uptake assay in breast cancer cells with *GPI* silencing; (C) LDH activity in cell culture medium after knockdown of *GPI*; (D) Oxygen levels in cell culture medium were assessed by oxygen consumption rate assay in breast cancer cells followed by different treatments; (E) Cell viability was determined by MTT assay after *GPI* silencing or taxol treatment; (F) Invasion ability and (G) migratory ability of breast cancer cells with knockdown of *GPI*; (H) Flow cytometry analysis revealed the *SOX2* positive population in breast cancer cells with *GPI* silencing. Results are presented as mean ± SD. *P < 0.05, **P < 0.01, ***P < 0.001 indicates statistically different.

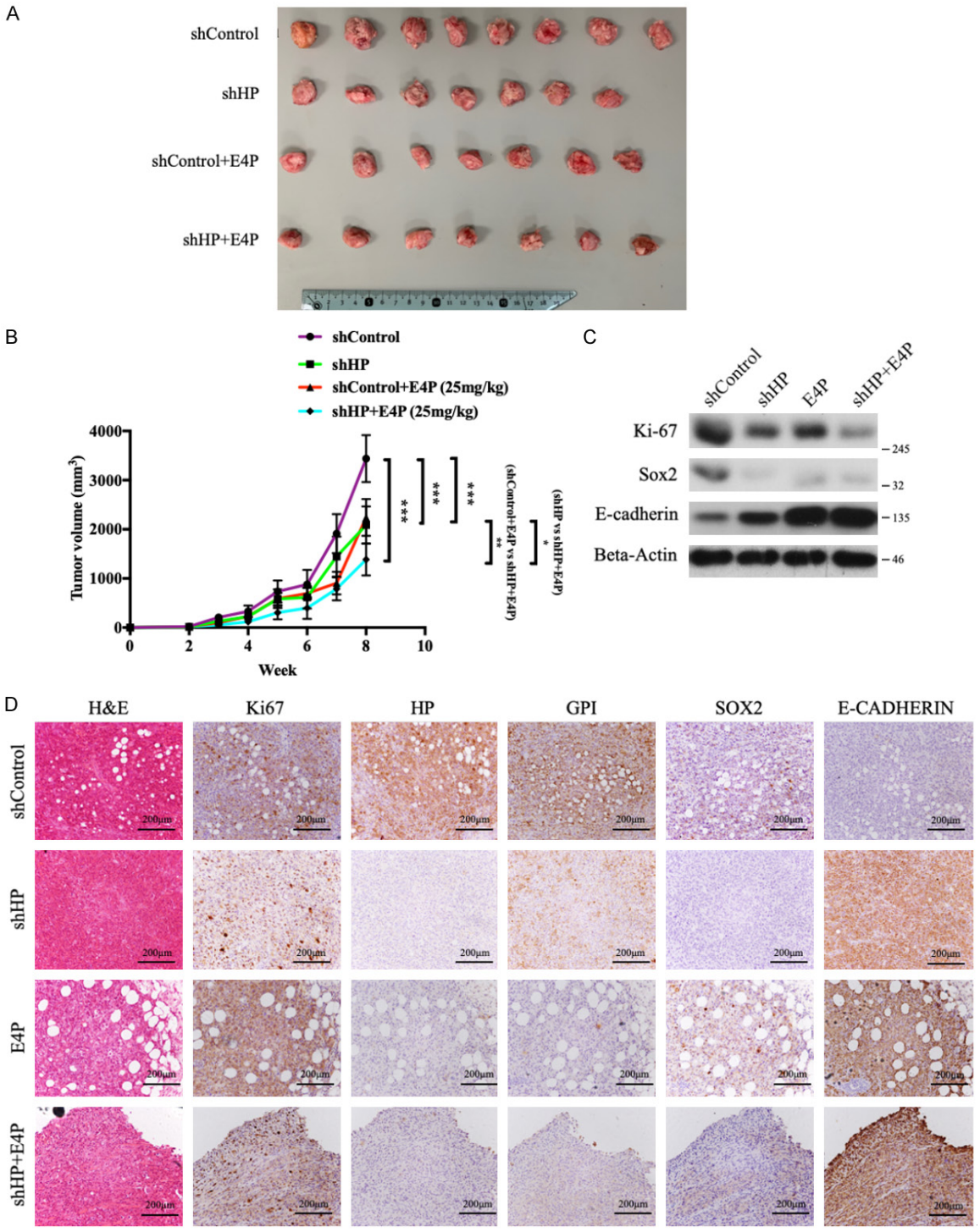

Figure 5. *HP* silencing inhibited tumor growth *in vivo*. (A) Gross appearance of xenograft tumors in NOD-SCID mice injected of breast cancer cells with different treatment groups; (B) Volumes of tumors from week 0 to week 8 and (C) Western blotting results in mice tumors; (D) Representative IHC images of mice tumors in different treatment groups. Results are presented as mean ± SD. **P < 0.01, ***P < 0.001 indicates statistically different.

HP in breast cancer is lacking. Elevated serum *HP* level was confirmed as a prognostic marker in a number of solid tumors including HCC [24] and ovarian cancer [25]. Thus far, only one study reported the elevated serum *HP* was associated with poor clinical outcome in TNBC

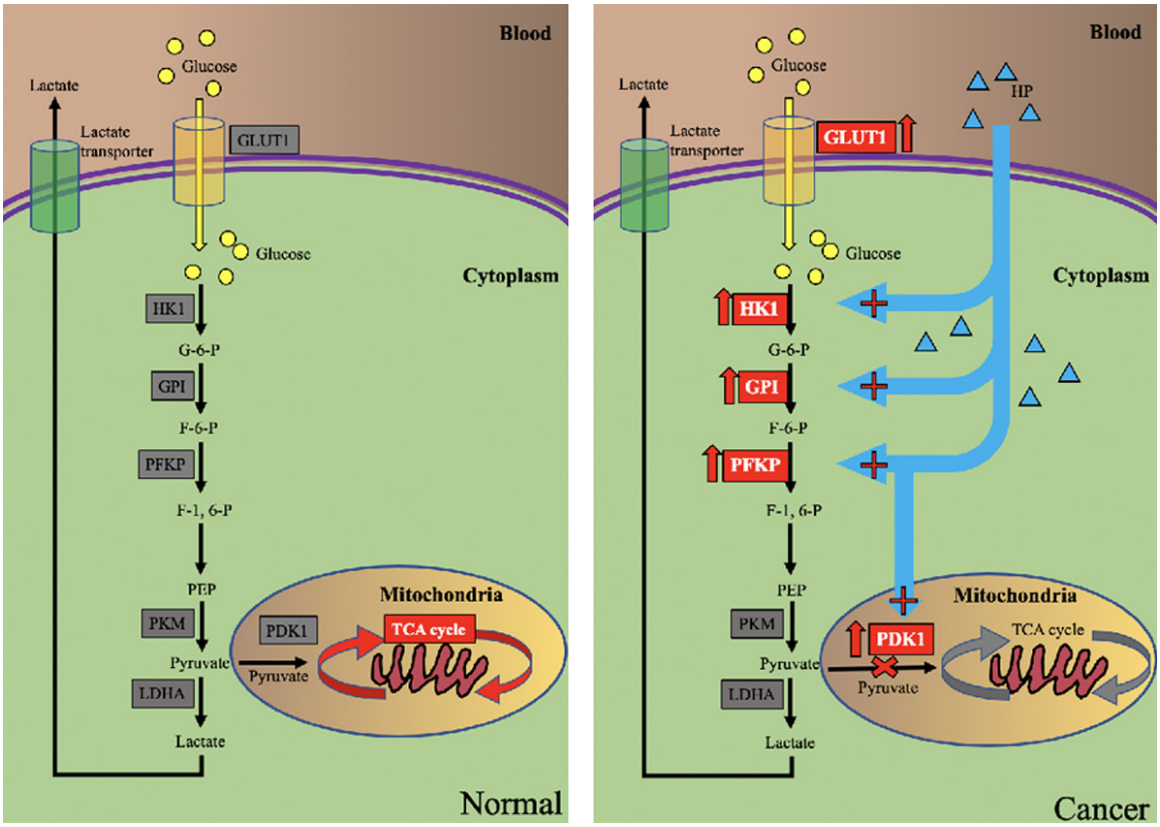

Figure 6. A schematic diagram of *HP*-based regulation in breast cancer glycolytic signaling pathway. In breast cancer cells, circulating *HP* increased with enhanced glycolytic activity and upregulation of key enzymes *GLUT1*, *HK1*, *GPI* and *PFKP*. This metabolic reprogramming forces cells to generate energy through glycolysis rather than TCA cycle which in turns favors tumor growth.

patients [26]. In this study, *HP* expression was upregulated in tumor tissues and serum of breast cancer patients, particular in TNBC when compared to other molecular subtypes. Previous studies have suggested that *HP* is a metastatic facilitator in cancer due to its involvement in both migration and angiogenesis [17]. However, the functional role of HP in breast cancer remains unclear. We demonstrated an inhibited cell growth in *HP*-deficient breast cancer cells, which may ascribe to alter cell cycle arrest and apoptosis. *HP* is implicated in various glucose disorders such as obesity-associated hepatosteatosis [19], insulinemia [18], diabetes mellitus [27] and metabolic dysfunctional syndrome PCOS [28]. In recent years, studies on the glycolysis addiction and the therapeutic benefit of using specific glycolytic inhibition in cancers have been carried out. Using glucose-free medium to enhance the glycolytic activity in breast cancers cells, increased expressions of glycolytic key enzymes and significant *HP* upregulation were observed in this study. Knockdown of *HP* significantly decreased expression of key enzymes such as *GLUT1*, an ubiquitous glucose transporter carries glucose from the extracellular matrix into cells which is essential for cell viability [29]. *GLUT1* regulates the first rate-limiting step of glycolysis in glucose metabolism. Moreover, numerous studies have shown *GLUT1* promoted proliferation and metastasis by inhibiting apoptosis in HCC, breast cancer and renal cancer [30-32]. The hexokinases *HK2*, is a key mediator of aerobic glycolysis in glioblastoma multiforme and HCC [33, 34]. *GPI* is a member of glucose phosphate isomerase protein family, interconverting glucose-6-phosphate and fructose-6-phosphate in the cytoplasm. Recently, higher *GPI* expression has been associated with gastric cancer tumorigenesis [35]. Moreover, Warburg effect could be fully suppressed by disrupting the upstream glycolytic enzyme *GPI*, which forced the reprogramming of the cancer cells to rely on oxidative phosphorylation rather than fermentative

glycolysis [36]. Meanwhile, our result also demonstrated decreased *PDK1* expression in *HP*-deficient cells, thereby enhancing the use of pyruvate in the tricarboxylic acid (TCA) cycle. This result provides evidence that knockdown of *HP* led to strengthened TCA cycle and oxidative phosphorylation. For example, in gastric cancer, high *PDK1* expression was reported to correlate with *HIF-1α* and poor survival [37]. *PDK1* was essential for tumor maintenance and progression in melanoma [38]. Elevated serum LDH levels have been indicated as prognostic biomarkers for poor survival in multiple cancers including lymphoma, prostate cancer and NSCLC. Elevated serum LDH levels have shown to promote tumor initiation, metastatic potential, recurrence and chemotherapy resistance [39]. In this study, we also revealed a significant reduction in glucose uptake and LDH activity, suggesting *HP* silencing was able to block glycolysis in breast cancer.

The above data suggested that *HP* plays critical role in breast cancer glycolysis through regulation of key enzymes. With decreased glucose uptake, LDH activity and reversal of oxygen consumption after knockdown of *GPI* in breast cancer cells, a significant inhibition of glycolysis was confirmed. Cell invasion and migration ability were significantly impeded in *GPI*-deficiency. In previous study, *GPI* was also reported to be upregulated in many cancers such as liver cancer, lung cancer and ovarian cancer [28]. In addition, *GPI* plays important roles in cell migration, angiogenesis, metastasis, proliferation and drug resistance [29-33]. In spite of the toxicity of anti-glycolytic agents, combined chemotherapeutic drugs and glycolytic inhibitors have already been demonstrated to be promising strategy to overcome drug resistance in cancer [40]. This metabolic shift may confer inverse effect in *in vitro* and *in vivo* tumor cell growth and warrants more investigation to treat against cancer.

In summary, we concluded that under normal condition, the glycolytic pathway is not the preferred metabolic pathway for energy production. Pyruvate in normal cells is transported into mitochondria for efficient energy production by the TCA cycle. In contrast, increased circulating *HP* level and the glycolytic activity in malignant cells enhance upregulation of key enzymes such as *GLUT1*, *HK1*, *GPI* and *PFKP*. With the upregulation of *PDK1*, pyruvate is forced to go through the glycolysis pathway rather the TCA cycle, which in turns favors the tumor growth (**Figure 6**).

Conclusion

Taken together, our findings demonstrate for the first time that *HP* acts as an oncogenic player and also a critical member in regulating glycolysis pathway in breast cancer. *HP* inhibition caused glycolytic pathway disruption and impaired tumor growth. By inhibiting both *HP* and its downstream effector, *GPI*, they enhanced the sensitivity of breast cancer cells to chemotherapy. This study provides new insight into the novel potential therapeutic strategy to improve breast cancer treatment outcome in the future.

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Disclosure of conflict of interest

None.

Abbreviations

E4P, erythrose 4-phosphate; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; 2-NBDG, 2-deoxy-2-[(7-nitro-2,1,3-benzoxadiazol-4-yl) amino]-D-glucose; LDH, lactate dehydrogenase; ELISA, enzyme-linked immunosorbent assay; EMT, epithelial-mesenchymal transition; HCC, hepatocellular carcinoma; HNSCC, head and neck squamous cell carcinoma; CRC, colorectal cancer; PCOS, polycystic ovary syndrome; GLUT1, glucose transporter 1; HK, hexokinase; GPI, glucose-6-phosphate isomerase; NSCLC, non-small-cell lung carcinoma; TCA, tricarboxylic acid cycle.

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