

ORIGINAL ARTICLE

GPX8 promotes migration and invasion by regulating epithelial characteristics in non-small cell lung cancer

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Abstract

Background: Non-small cell lung cancer (NSCLC) is the most common cause of cancer-related death worldwide. The family of glutathione peroxidase (GPX), an important antioxidant enzyme in human tissues, has been discovered to play a key role in the development of cancers. GPX8 is the most promising molecule of the family in a therapeutic strategy against a variety of cancers.

The main purpose of this study was to examine and analyze the function and clinical value of GPX8 in NSCLC.

Methods: Immunohistochemistry (IHC), western blot analysis and quantitative real-time polymerase chain reaction (qPCR) were used to assess GPX8 expression and its clinical significance in NSCLC. A series of cell biology experiments and bioinformatic analysis tools were further used to study the function of GPX8.

Results: GPX8 expression in tumor tissues was much higher than that in normal lung tissues. High expression of GPX8 in NSCLC was correlated with a worse clinical outcome and prognosis. Furthermore, GPX8 could inhibit the apoptosis of tumor cells and promote its migration and invasion.

Conclusions: Our results conclusively demonstrated that GPX8 could affect the oncogenesis and prognosis of NSCLC via regulating epithelial characteristics. The study also illustrated that GPX8 could serve as a prognostic predictor and potential therapeutic target for NSCLC.

Introduction

For several decades, lung cancer has been the most common cancer with high morbidity and mortality in the world.¹ Non-small cell lung cancer (NSCLC) is the most frequent type of lung cancer. Even with the current advances achieved in the research of NSCLC, the metastasis and recurrence are still a huge challenge for treatment. One of the reasons is the lack of effective molecular mechanisms. It is crucial to discover more cancer biomarkers with high sensitivity and strong specificity in the process of tumor occurrence and development.²

Glutathione peroxidase (GPX) family including 8 subtypes plays a key role in antioxidant reaction and an important scavenger of reactive oxygen species (ROS). In addition to antagonizing hydrogen peroxide *in vivo*, it also has significant effects in substance metabolism, signal

transduction and tumor development. Furthermore, each of the GPX subtype has unique characteristics in distribution, subcellular localization, substrate specificity, and epigenetic function.^{3–7} Nowadays, GPX family has been found to play an important role during tumor development.⁸ Mounting evidence demonstrates that GPX1–6 are highly correlated with the oncogenesis, progression and treatment of various tumors, such as head and neck cancer,^{9,10} lung cancer,¹¹ hepatocellular carcinoma,¹² digestive tumor,^{13,14} ovarian cancer.¹⁵ And family members in cancer also have different roles and influences.

GPX8 is a member of the final confirmation of the family,¹⁶ GPX8 is deemed to carry a crucial functional area in the GPX family, which is the vital characteristics compared with other members. As a result, the intense attention of scientific researchers on GPX8 has reached a new height

and became the top priority of the GPX family. It can be seen that GPX8 has a very powerful function in the body, which needs to be further studied in the future. Especially in cancer researches, it locates in a preliminary phase. Few studies have been showed the implications of GPX8 in the research of cancers, especially in lung cancer. Our current study is to investigate the role of GPX8 in NSCLC.

Methods

Specimens and clinical information

We randomly selected paraffin-embedded samples and corresponding clinical details of a cohort of patients ($n = 219$) who were diagnosed with NSCLC at Tianjin Medical University Cancer Institute and Hospital. Seven pairs of frozen lung tissues were used for western blot and quantitative real-time polymerase chain reaction (qPCR). All patients gave their written informed consent before participation in this study.

Cell lines

Human lung cell lines (H520, A549, H1299, PC9, H460 and BEAS-2B) were obtained from the American Type Culture Collection and maintained in our laboratory. All cell lines were cultured at 37°C in a 5% CO₂ humidified atmosphere in RPMI 1640 (Gibco) with 10% fetal bovine serum (HyClone).

Tissue microarray (TMA) and immunohistochemical analysis

A total of 219 paraffin-embedded tissue blocks and tissue microarray (TMA) sections were constructed from patients with NSCLC. Representative tumor regions on sections were identified and marked by pathologists. Tissue cores of TMA blocks with a diameter of 2 mm were punched from marked donor blocks. The specific process of TMA construction is shown in Fig 1a. GPX8 antibody (Abcam, ab183664) was used to detect its expression in TMA. Envision two-step method was mainly used to perform immunohistochemical staining according to the manufacturer's instructions. Staining intensity of GPX8 was classified into 1 (weak), 2 (moderate), and 3 (strong). Staining frequency of each sample was as follows: 0 for negative sample or <5% stained tumor tissue; 1 for samples stained between 6% and 25%; 2 for samples stained between 26% and 50%; 3 for samples stained between 56% and 75%; and 4 for samples stained >75%, and each TMA result was determined by two pathologists using light microscopy.

Western blot and qPCR

Tissue proteins were extracted using the lysis buffer containing a protease inhibitor cocktail. Equivalent protein samples were then separated by SDS-PAGE and transferred to PVDF membranes. The house keeping gene GAPDH was used as an endogenous control. Total RNA was extracted from NSCLC tissues using TRIzol according to the manufacturer's instructions (Thermo Fisher Scientific). The cDNA fragment was synthesized by a reverse transcription kit (Applied TakaRa, Dalian, China). The primer sequences of GPX8 were 5'-TGAGGGTCAAGTTGTGAA-3' and 5'-CAGAGCTGCTATGTCAGG-3'. GAPDH was applied as the calibration control in all specimens. Relative expression levels of GPX8 were quantified with the 2- $\Delta\Delta$ Ct method. GAPDH was applied as the calibration control.

Lentivirus packaging and cell infection

The lentiviral short hairpin RNA (shRNA) in hU6-MCS-Ubiquitin-EGFP-IRES-puromycin and the control sequences were synthesized by Shanghai Genechem Co., Ltd. (Shanghai, China) in the study.

Lentivirus was conducted in HEK293T cells and used to construct stable NSCLC cell lines of downregulated GPX8. Finally, the transfected cells were verified using qPCR and western blot.

Cell viability and colony formation assay

Cell viability was assessed using cell-counting kit-8 CCK-8 assay. Cells were seeded into 96-well culture plates. At the indicated time point (24, 48, 72, 96 hours), CCK-8 reagent was added into each well and incubated at 37°C for three hours. The optical density (OD) value was then measured at 450 nm. The indicated cells were plated into six-well plates and incubated for two weeks at 37°C. The cells were then washed with PBS, fixed and stained for 25 minutes. The number of colonies were counted using a microscope.

Cell apoptosis and cycle assay

Cells were seeded into six-well plates and collected after 48 hours. A total of 1×10^6 cells were harvested and double-stained with FITC Annexin V and 7-AAD following the manufacturer's instructions.

Cells were washed twice with cold PBS, and fixed with ice-cold methanol overnight at 4°C. The cells were then stained with propidium iodide, RNase A and Triton X-100 for 25 minutes. Cell apoptosis and cell cycle were performed using flow cytometry.

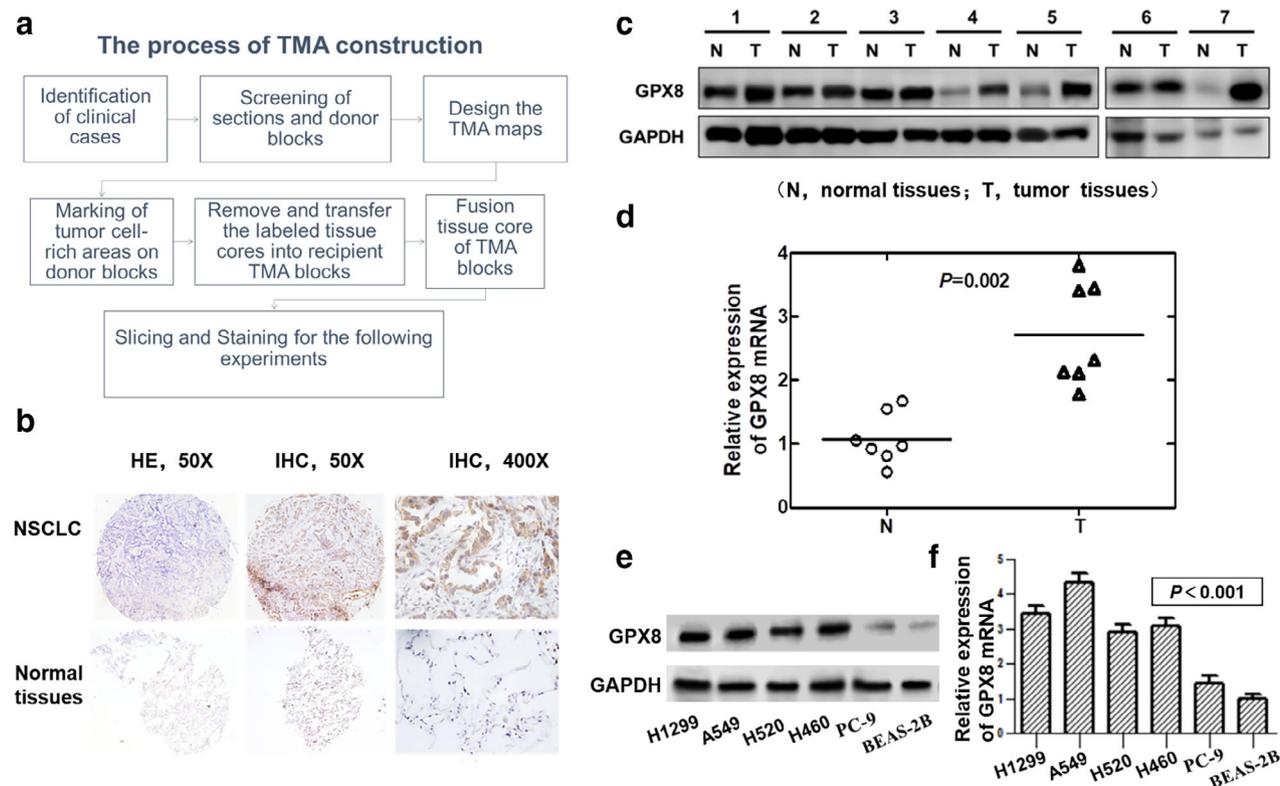


Figure 1 GPX8 expression in NSCLC tissues and cell lines. (a) The process of TMA construction. (b) Representative hematoxylin and eosin (H&E) and immunohistochemical (IHC) staining of GPX8 expression in NSCLC and corresponding normal lung tissues. GPX8 expression was localized in the cytoplasm and weakly detectable in normal lung tissues. (c) GPX8 expression in seven pairs of fresh NSCLC and adjacent tissues was detected by western blot. (d) GPX8 mRNA expression from seven paired patients was analyzed using qPCR. Relative mRNA levels were normalized to GAPDH. (e) GPX8 expression in NSCLC cell lines. (f) Endogenous GPX8 mRNA levels determined by qPCR in NSCLC cell lines. Statistically significant differential expression of mRNA levels of GPX8 in five NSCLC cell lines as revealed by single factor ANOVA analysis ($P < 0.001$).

Wound healing assay and transwell migration assay

Cells were plated in six-well plates at a density of 1×10^6 cells per well. Two parallel staining lines were scratched on each well with a plastic pipette tip. Two parallel staining lines were painted on each well. The wound width was measured at 0, 12, and 24 hours and digital images were taken with an inverted microscope. Cells (2×10^5) in serum-free 1640 were added into the upper well of each migration chamber, and the lower chamber was filled with 10% FBS medium. After 36 hours incubation, cells were fixed with 4% paraformaldehyde and stained with crystal violet. The number of migrated cells were counted under a microscope.

Analyses of gene ontology (GO) term enrichment and KEGG pathway

The RNA of A549-shGPX8 cells and A549-shcontrol cells were extracted, and then sent directly to Jing Neng biotechnology company (Shanghai, China) for transcriptome sequencing and term enrichment analysis. The requirement

of differential expression gene screening was to meet the requirement of a P -value less than 0.05, and more than two times the differential expression range.

Statistical analysis

Statistical analysis was performed using SPSS 24.0 software and GraphPad Prism 5.0 software. Categorical and continuous variables were evaluated using Chi-square test, Student's t -test, variance analysis and Wilcoxon test. Survival analysis was established using the Kaplan–Meier method. Survival curves were compared by the log-rank test. $P < 0.05$ was considered statistically significant.

Results

GPX8 expression in NSCLC tissues and cell lines

In this study, paraffin-embedded tissues from 60 NSCLC patients were tested by immunohistochemistry (IHC) to

clarify the GPX8 expression. The results revealed that GPX8 staining was predominantly located in the cytoplasm, but weakly detectable in normal lung tissues (Fig 1b). The positive rate of GPX8 in tumor tissues was also significantly higher than that in normal lung tissues (66.7% vs. 26.7%, $P = 0.002$). Western blot and qPCR methods were used to assess GPX8 expression in tissues from seven NSCLC cases and related normal lung tissues, respectively. The results indicated that the GPX8 expression level in tumor tissues of NSCLC patients was dramatically higher than that in normal lung tissues (Fig 1c,d). We also analyzed GPX8 expression in a series of NSCLC cell lines. The results showed that GPX8 expression was upregulated in lung cancer cell lines, especially in A549, H520 and H1299 cells (Fig 1e,f).

GPX8 was correlated with clinicopathological features of NSCLC

To analyze the relationship between GPX8 and clinicopathological characteristics, IHA was performed on 219 TMA samples. GPX8 tended to be highly expressed in NSCLC patients with large tumor diameter, pathological type, poor differentiation and advanced TNM stage (Table 1, Fig 2a,b). We also observed a correlation between GPX8 expression and common clinical biomarkers from patients with NSCLC (Fig 2c). The results showed that patients with positive GPX8 expression always had higher levels of CEA, SCCA and CYFRA21-1. There was no significant difference in other clinical parameters. In summary, the expression of GPX8 in patients has a crucial impact on clinical outcome.

Prognostic significance of GPX8 in NSCLC

Following determination that GPX8 expression was significantly associated with several clinicopathological parameters, often indicative of a better or worse prognosis in NSCLC, we further investigated the relationship between GPX8 and the survival time of patients with NSCLC using Kaplan–Meier survival analysis. The survival curves demonstrated that there was a significant correlation between GPX8 and the survival time of patients with NSCLC using Kaplan–Meier survival analysis. The survival curves demonstrated that the overall survival of patients with negative GPX8 expression was statistically significantly longer than that of patients with positive GPX8 expression (Fig 2d). Furthermore, we achieved the same results in both adenocarcinoma and squamous cell carcinoma. GPX8 expression is independently associated with worse overall survival in NSCLC patients. Collectively, our study indicated that GPX8 may serve as a candidate prognostic biomarker.

Table 1 Relationship between GPX8 expression and clinical characteristics

| Characteristics | GPX8 expression | | | P-value |
|--------------------|-----------------|----------|------|---------|
| | Low | Moderate | High | |
| Gender | | | | |
| Male | 44 | 29 | 43 | 0.895 |
| Female | 41 | 23 | 39 | |
| Age | | | | |
| ≥60 | 44 | 27 | 40 | 0.909 |
| <60 | 41 | 25 | 42 | |
| Smoking history | | | | |
| Yes | 41 | 22 | 42 | 0.601 |
| No | 44 | 30 | 40 | |
| Pulmonary disease | | | | |
| Yes | 8 | 6 | 4 | 0.344 |
| No | 77 | 46 | 78 | |
| TNM stage | | | | |
| I | 32 | 17 | 32 | 0.026 |
| II | 27 | 20 | 15 | |
| IIIA | 16 | 15 | 24 | |
| IIIB | 10 | 0 | 11 | |
| Tumor diameter | | | | |
| >3 cm | 62 | 41 | 49 | 0.043 |
| ≤3 cm | 23 | 11 | 33 | |
| Pathological type | | | | |
| SCC | 51 | 31 | 12 | <0.001 |
| Adenocarcinoma | 25 | 11 | 61 | |
| Others | 9 | 9 | 9 | |
| Histological grade | | | | |
| Well | 24 | 4 | 11 | 0.012 |
| Moderately | 52 | 39 | 54 | |
| Poorly | 9 | 9 | 17 | |

Effects of GPX8 on proliferation and apoptosis

For exploration of the activity of GPX8 in NSCLC cell proliferation, we first evaluated the effect of downregulation of GPX8 on cell growth using the CCK8 assay and plate clone formation assay. We discovered that there was no significant difference in cell proliferation between.

A549-shGPX8 cells and vector-transfected control cells (Fig 3a), although there were differences between the two groups at each fixed time point (Fig 3b). In addition, flow cytometry analysis showed that there was no correlation with cell cycle distribution in A549-shGPX8 cells compared with the negative control (Fig 3c), whereas, cell apoptosis was prominently elicited in A549-shGPX8 ($P = 0.002$) cells compared with control cells (Fig 3d). In Fig 3e, we directly observed the difference of A549 cells in early apoptosis and total apoptosis after interfering with GPX8 expression. ShGPX8 treatment of A549 cells decreased the number of sizable colonies, and as a result suppressed the cell colony formation capacity (Fig 3f).

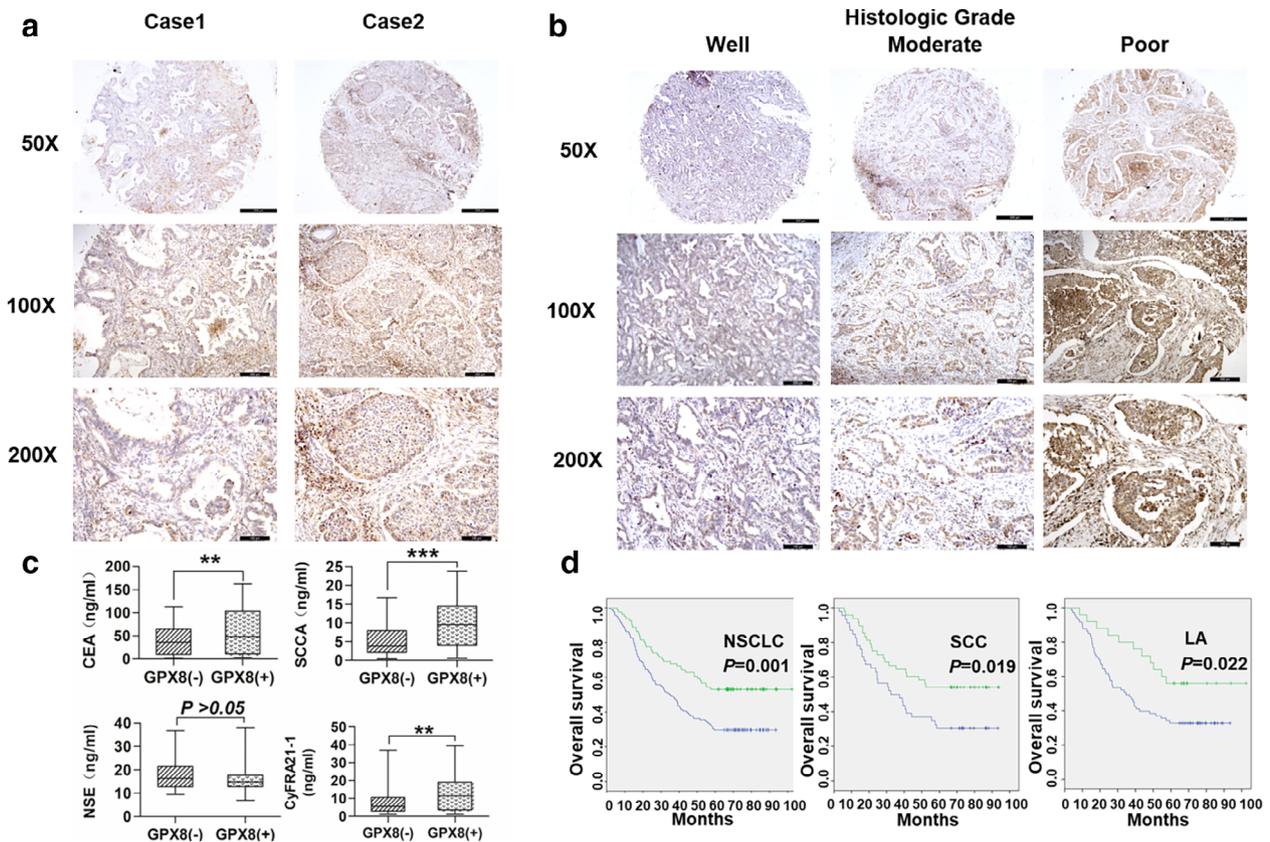


Figure 2 Correlation between GPX8 expression and clinicopathological features in NSCLC. (a) Representative images of GPX8 IHC staining in lung adenocarcinoma and squamous cell carcinoma, respectively. (b) IHC estimation of GPX8 in NSCLC tissues with different histological grades. (c) The relationship of GPX8 expression and common clinical biomarkers (** $P < 0.01$, *** $P < 0.001$). (d) Analyses of overall survival based on the expression of GPX8 in patients with NSCLC —, Positive; —, Negative; —, Positive; —, Negative; —, Positive; —, Negative. LAC, lung adenocarcinoma; NSCLC, non-small cell lung cancer; SCC, squamous cell carcinoma.

GPX8 had no discernible effect on proliferation and cell cycle but induced apoptosis and inhibited cell colony formation in NSCLC cells.

Downregulation of GPX8 suppressed cell migration and invasion

The effect of potential metastatic ability of GPX8 was performed via classic scratch wound assay and tumor invasion assay. In contrast, downexpression of GPX8 resulted in a significant inhibition of wound closure, since the migrated cell number was significantly lower than that of the control, and took a longer time to fill the wound area in A549 (Fig 4a). The results demonstrated that downexpression of GPX8 could suppress cell migration and indicate a defect in migration of NSCLC cells. Transwell assay was also used to detect migration. Downregulation of GPX8 reduced the number of crystal violet staining cells, and only a few tumor cells migrated from the upper to the lower chamber. The number

of migrated cells was counted (Fig 4b) and downregulation of GPX8 distinctly reduced cell migration and invasion. In addition, we evaluated the metastatic ability of GPX8 using paraffin-embedded tissues from 60 NSCLC patients, which were divided into three groups as shown in Fig 4c. GPX8 expression increased gradually in normal lung tissues, NSCLC tissues without lymph node metastasis and NSCLC tissues with lymph node metastasis (Fig 4d).

Discovery of potential key genes and pathways associated with NSCLC via bioinformatic analysis

Currently, few studies have reported a correlation between GPX8 expression and tumorigenesis. The relevant molecular mechanism is also unclear. GO term analysis (Fig 5a) using A549 cells revealed that downexpression of GPX8 induced upregulation of 211 genes and downregulation of 202 genes (fold change >2 , $P < 0.05$). In Fig 5a, the scatter

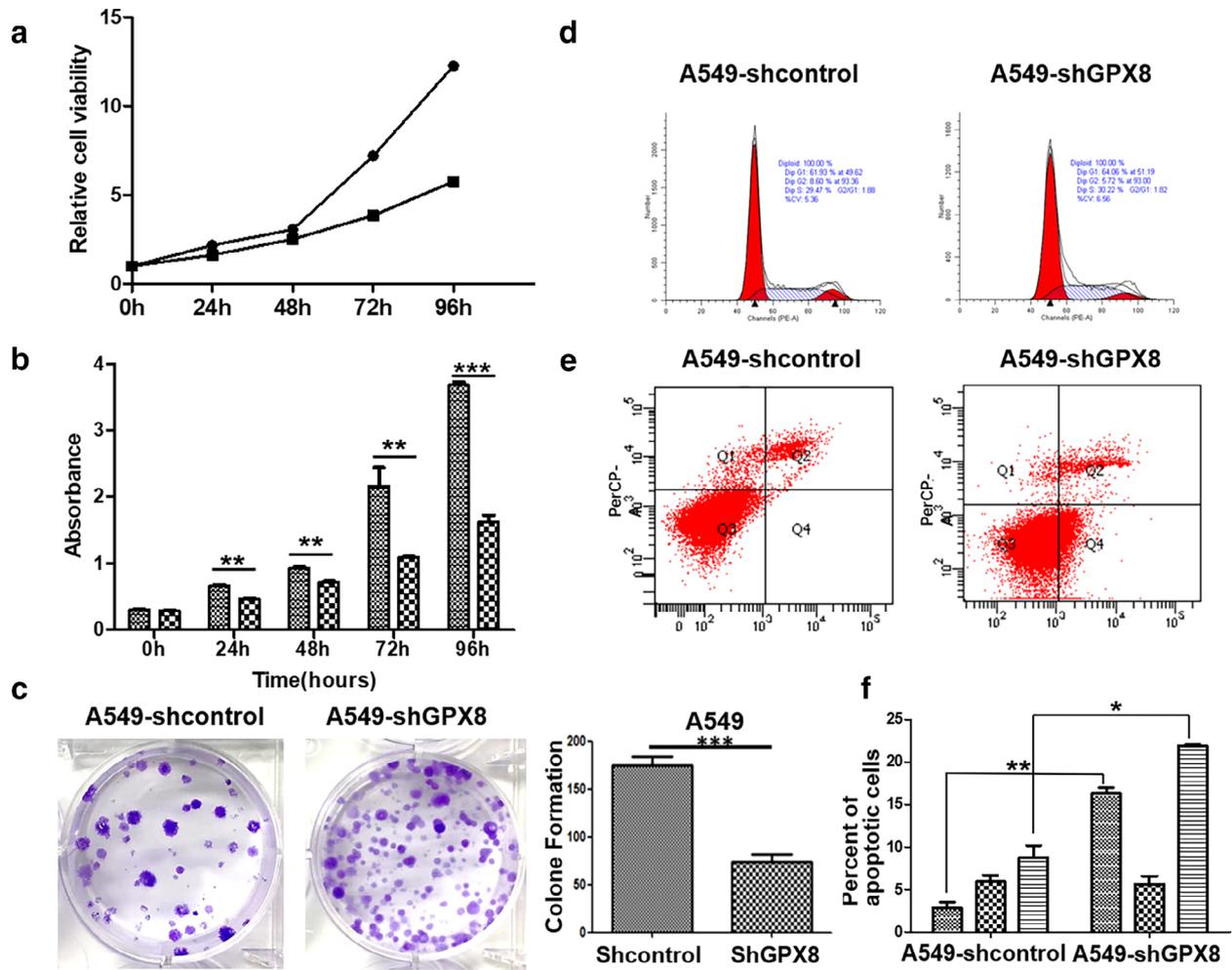


Figure 3 Effects of GPX8 on cell proliferation and apoptosis in vitro (a) CCK-8 assay analysis of cell proliferation in A549-shGPX8 and control cells ($P > 0.05$) —●—, A549-shControl; —■—, A549-shGPX8. (b) Cell proliferation of the two groups at each fixed time point (** $P < 0.01$, *** $P < 0.001$) (▨, A549-shControl; ▩, A549-shGPX8). (c) Colony formation assay of A549 cells (*** $P < 0.001$). (d) Cell cycle analysis of A549 cells by flow cytometry A549-shcontrol: ■, Dip G1; ■, Dip G2; □, Dip S. A549-shGPX8: ■, Dip G1; ■, Dip G2; □, Dip S. (e) Cell apoptosis analysis by flow cytometry. Q4 and Q2 regions represent the rate of early and late apoptotic cells, respectively. (f) The difference of A549 cells in early apoptosis and total apoptosis (* $P < 0.05$, ** $P < 0.01$) (▨, REA; ▩, RLA; ▭, RTA. REA, the rate of early apoptotic cells; RLA, the rate of late apoptotic cells; RTA, the rate of total apoptotic cells.

plot demonstrates the distribution trend of different genes which were classified into three functional categories using the GO term pathway: biological process, cellular component and molecular function. It was mainly enriched in various vesicles and junction structures of cells involved in anatomical structure morphogenesis and regulation of cell localization, molecular function, and intracellular signal transduction (Fig 5b). KEGG analysis was performed and showed that differential expression genes were mainly enriched in pathways in cancer, PI3K-Akt signaling pathway, hepatitis B, focal adhesion and MAPK signaling pathway (Fig 5c,d).

Correlation of GPX8 expression with protein indicators of epithelial characteristics

Based on the above bioinformatic analysis, we further confirmed the correlation of cell morphogenesis with GPX8 in A549 cells. E-cadherin and Vimentin were the two most common and vital molecules, maintaining cell morphology and mediating migration and invasion of cancer cells.

We chose two NSCLC cell lines (A549 and H520), and demonstrated there was a relationship by decreasing GPX8 expression. Western blot showed that downregulation of GPX8 was associated with the recovery of E-cadherin and

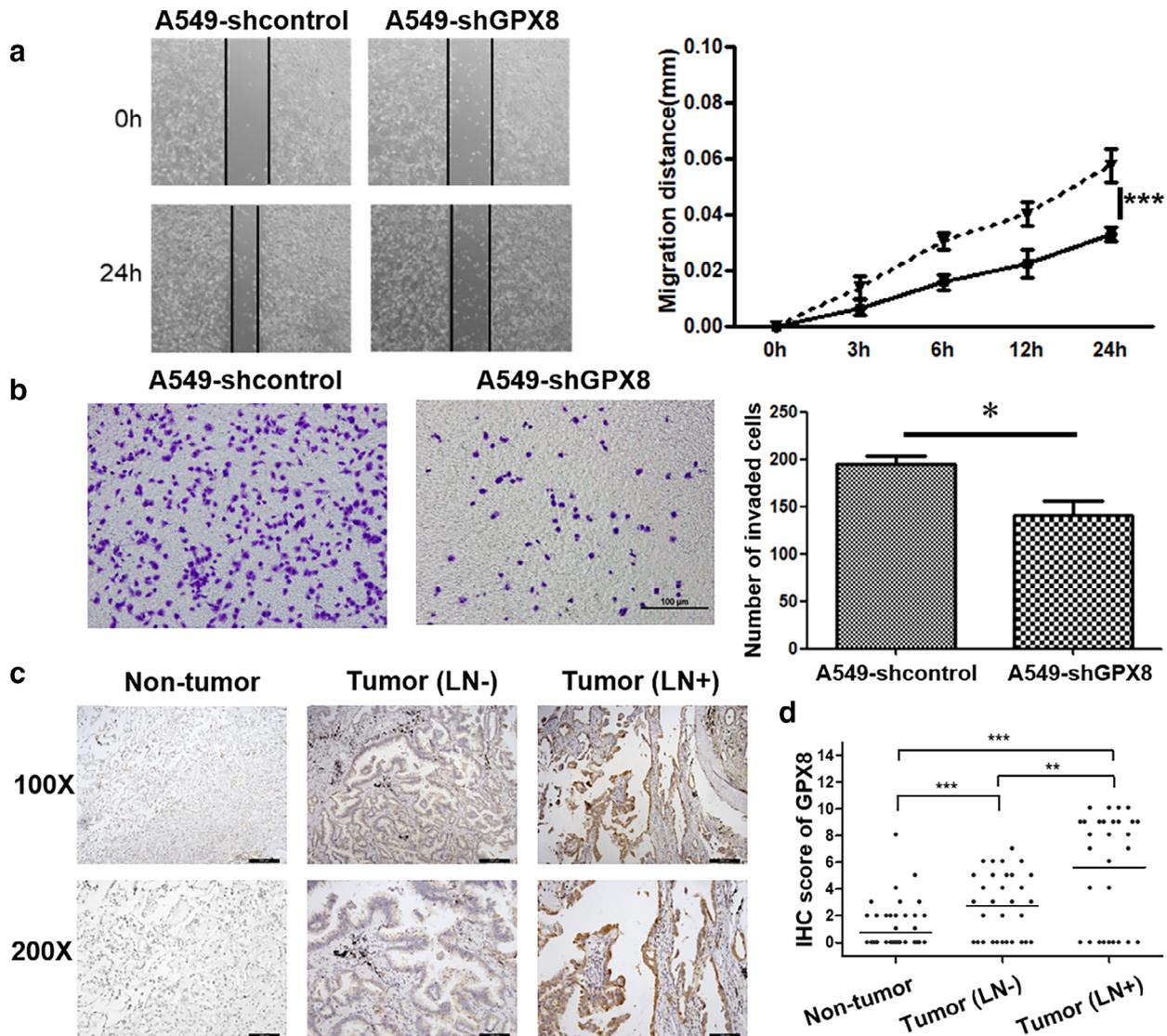


Figure 4 Effects of GPX8 on cell migration and invasion in NSCLC (a) Scratch assay comparing the migration in A549-shControl and A549-shGPX8 cells ($***P < 0.05$) —▼—, Shcontrol; —●—, ShGPX8. (b) Invasion of A549 cells using transwell assay ($*P < 0.05$). All experiments were repeated at least three times. (c) Representative images of GPX8 expression in normal lung tissues, NSCLC tissues with or without lymph nodes. (d) Statistical analysis of GPX8 scores in the three groups ($**P < 0.01$, $***P < 0.001$).

decrease of Vimentin (Fig 5e). Loss of E-cadherin is closely linked to tumor metastasis. Consequently, we also detected MMP9 as a widely used marker for metastasis which confirmed our expectations. The results showed that GPX8 expression could affect cell morphology, thereby regulating the migration and invasion of NSCLC cells.

Discussion

This article focused on GPX8 in NSCLC with emphasis on the clinical implications and preliminary mechanism exploration. In this retrospective study, the results showed

that GPX8 expression was markedly increased in NSCLC tissues compared with normal lung tissues. High expression of GPX8 in NSCLC patients was often accompanied by poor differentiation, and progressive stage. Furthermore, our findings indicated that GPX8 might be an independent biomarker for the prognosis of NSCLC patients. In order to better study and determine the main role of GPX8 in NSCLC, we conducted a series of cellular functional studies in vitro. We found that GPX8 down-expression promoted apoptosis, inhibited cell clonality, and restrained cell proliferation and invasion. All these results revealed that GPX8 could be a novel cancer-promoting

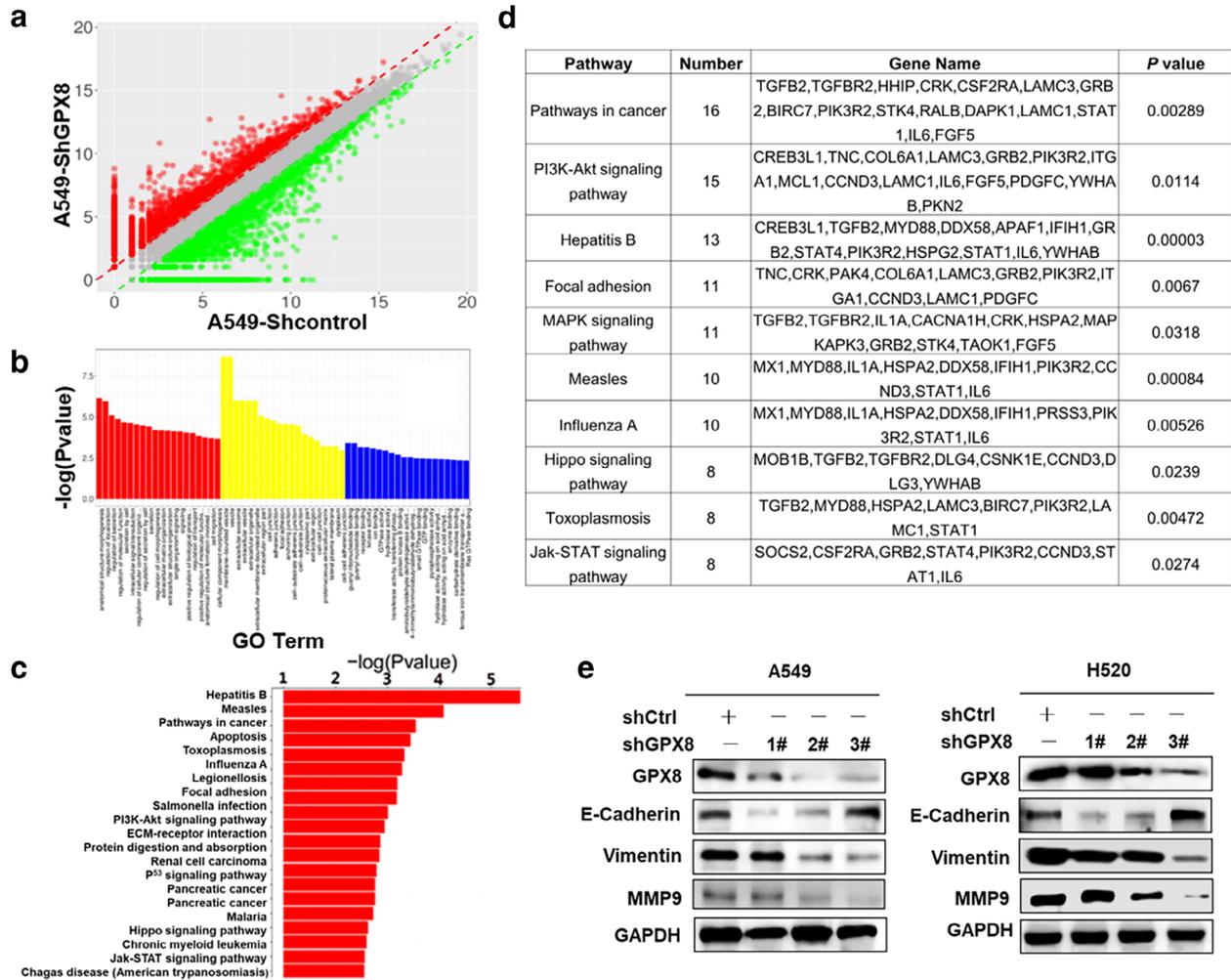


Figure 5 Preliminary study on the mechanism of GPX8 in NSCLC (a) The scatter plot indicated the distribution trend of different genes through GO biology process enrichment analysis ●, Unchanged; ●, Up; ●, Down. (b) GO enrichment analysis of differential expression genes ■, BP; ■, CC; ■, MF. BP biological process; CC, cellular component; MF, molecular function. (c) KEGG pathway analysis of differential expression genes involved in the identified pathways. (d) List of differential expression genes enriched in the KEGG pathway. We selected the top 10 for display based on the number of differential genes in the pathway. (e) Correlation of GPX8 expression with epithelial characteristics proteins in A549 and H520 cells.

gene, and has a close association with NSCLC development and involvement.

In the GPX family, many studies have previously reported that there is a relationship between various GPX subtypes and tumor proliferation and apoptosis.⁷ Huang *et al.* found that YAP activation led to excessive accumulation of ROS by downregulating the antioxidant enzyme GPX2 in a manner related to p63 blockade in lung squamous cell carcinoma.¹¹ Oxidation of signaling proteins induced by ROS have been reported to enhance the development of inflammation and apoptosis. The GPX family have also been reported to be able to stabilize the signaling cascades speed and prevent the vicious circle of oxidative stress and apoptosis.¹⁷ Suzuki *et al.* also found that the

expression of GPX2 in rat hepatoma cells was not only related to cell proliferation, but also related to tumor invasion and metastasis.¹⁸ Our previous results coincided with those mentioned in these studies. We found that the migration and invasion ability of A549 cells decreased significantly after the interference of GPX8 expression. These findings suggest that GPX8 plays a key role in NSCLC, which can inhibit the apoptosis of tumor cells and promote migration and invasion.

It is a known fact that the GPX family protects normal cells in the body from DNA damage caused by oxygen free radicals.^{19,20} It has also been suggested that the GPX family in tumor cells is regulated by epigenetics.²¹ More importantly, GPX8 has been found to be involved in the

development of tumorigenesis in lung cancer.^{22,23} In particular, GPX8 is regarded as a promising molecule of the GPX family, and researchers have discovered it could contribute to the development of potential therapeutic strategies against a variety of cancers. However, its exact mechanism, especially in NSCLC, has not been previously reported. We used high-throughput sequencing technology to explore the possible molecular regulatory pathway of GPX8 in NSCLC. We found and further confirmed that GPX8 expression could affect cell morphology and regulate migration and invasion of NSCLC. Although there is still much to be explored in depth, we now have a direction in which to lead our research based on the above results. It is reasonable to presume that epithelial stromal transformation and the PI3K-Akt signaling pathway are most likely to be involved.

In this study, the results provide a detailed outlook of the value of GPX8 for diagnosis and prognosis in NSCLC patients. We verified that GPX8 is aberrant in NSCLC and associated with certain clinicopathological parameters. Our results demonstrate that GPX8 could play a critical role in the oncogenesis and prognosis of NSCLC, and the key role of GPX8 in NSCLC is closely related to its regulation of cell morphology, inhibition of apoptosis and promotion of cell metastasis.

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Disclosure

The authors report no conflicts of interest in this work.

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