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Original Article

High GPX1 expression promotes esophageal squamous cell carcinoma invasion, migration, proliferation and cisplatin-resistance but can be reduced by vitamin D

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Abstract: Esophageal cancer is one of the most common cancers worldwide. Despite recent progress in the development of novel therapies, esophageal carcinoma remains an aggressive cancer associated with a poor prognosis. The glutathione peroxidase 1 (GPX1) gene located on chromosome 3p21.3 is associated with the cancer of several organs. According to available information, GPX1, a gene downstream of NF-κB, is considered to exert adverse effects on tumour progression and enhance malignancy in some cancers but has not been reported in esophageal cancer. It is also reported that vitamin D (Vit. D), a widely used drug in the clinical setting, could suppress GPX1 expression through the NF-κB pathway. Thus, it is speculated that Vit. D could reduce malignancy in esophageal cancer by altering the NF-κB pathway. In this study, we confirmed our speculation by finding that Vit. D, through the inhibition of GPX1, decreased the migratory, invasive and proliferative capabilities, as well as cisplatin resistance, in esophageal cancer cells. Furthermore, when invasion and migration were reduced in the GPX1-inhibited cells, the expression of urokinase type plasminogen activator (uPA) and matrix metalloproteinase-2 (MMP2) was also suppressed correspondingly. Therefore, we believe that, in esophageal cancer cells, the expression of GPX1 can promote invasion, migration, proliferation and cisplatin resistance, and Vit. D can reduce the associated malignancy through the NF-κB pathway. The Vit. D- and NF-κB-mediated decrease in GPX1 expression resulted in a decrease in MMP2- and uPA-mediated invasion and migration.

Keywords: Esophageal squamous cell carcinoma, glutathione peroxidase 1, NF-κB, urokinase type plasminogen activator, matrix metalloproteinase-2, vitamin D, invasion, migration

Introduction

Esophageal cancer is one of the most commonly diagnosed cancers in Asia and has an increasing incidence worldwide [1]. Most esophageal squamous cell carcinoma (ESCC) cases are diagnosed at the advanced stage [1, 2]. For primary ESCC, surgery is still the most critical treatment. However, the prognosis for these patients is not satisfactory, with a post-surgery 5-year survival rate that is less than 50% [1, 3-5]. In recent clinical practice, auxiliary staging indicators, such as the lymph nodes, have been used to guide treatment. Currently, biological molecules and genes associated with disease progression are the focus of several studies [1, 2]. However, these biological molecules have their limitations and cannot completely and accurately predict whether there is a high or low risk of recurrence and metastasis in patients [1, 5]. Therefore, insight into the molecular alterations associated with esophageal cancer will provide new vision into the fundamental mechanisms underlying ESCC progression and further contribute to improvements in the clinical therapy of ESCC patients [1].

Glutathione peroxidases (GPXs) are a family of antioxidant enzymes with a total of eight family members; their coding genes are located on different chromosomes [6]. Their encoded proteins can efficiently eliminate the reactive oxy-

gen species (ROS) in the body [7, 8]. Different GPXs distributed throughout the body, protect cells from the oxidative DNA damage that occurs in high oxygen conditions, and maintains the body's balance of intracellular redox systems by excess ROS [6, 9]. In the pathological process of cancer development, the expression of GPXs appears obviously aberrant [6]. Many studies confirmed that aberrant expression of GPXs could promote malignant cell proliferation, invasion and metastasis [7-12]. Of the 8 GPXs, GPX1 is the most abundant, which could be responsible for protecting cells from oxidative damage by reducing hydrogen peroxide and other ROS. It has been observed that the overexpression of GPX1 could promote invasion, migration, cisplatin resistance and proliferation in colon, breast, lung and oral adenoid cystic carcinomas [6, 9, 10]. It was also reported that NF-κB, when bound with the promoter region of the GPX1 gene, could be and upstream regulator of GPX1 expression and function [11, 13, 14].

NF-κB, as a ubiquitous nuclear transcription factor in the human body, plays a critical role in cell proliferation, apoptosis and malignant progression [13-16]. In addition, Vitamin D (Vit. D) in the human body acts as a multifunctional lipid hormone-like substance by acting on the Vit. D receptors [17, 18]. In addition to acting as anti-inflammatory and anti-oxidant, it has been widely reported that Vit. D can also inhibit NF-κB expression in B lymphocytes, oral squamous cell carcinoma, prostate cancer and melanoma [18-21]. Accordingly, we speculate that Vit. D could reduce the malignancy of ESCC by down-regulation of GPX1 through the Nf-κb pathway.

Materials and methods

Cell lines and cell culture

A total of 4 ESCC cell lines were used in this study; all cell lines were kindly provided by the Department of Thoracic Surgery, Sun Yat-Sen University Cancer Center (Guangzhou, China). Cells were cultured in RPMI1640 medium (Gibco, Gaithersburg, MD, USA) containing 10% fetal bovine serum (FBS) at 37°C in a humidified atmosphere containing 5% CO_2.

GPX1 overexpression vector and siRNA

Small interfering RNA (siRNA) specific for GPX1 and a control siRNA were constructed by Invitrogen (Carlsbad, California, US). The following target siRNA sequences were used: GPX1 sense, GGUACUACUUAUCGAGAAUTT, anti-sense, AUUCUCGAUAAGUAGUACCTT; scramble, sense UUCUCCGAACGUGUCACGUTT, anti-sense, ACGUGACACGUUCGGAGAATT. Full-length GPX1 coding sequences were PCR amplified and cloned into a pcDNA 3.1 expression vector (Invitrogen, Carlsbad, California, US), following manufacturer's suggested protocol. DNA seuencing was used to verify the constructs. The transfection efficiency of the GPX1 overexpression vector and the siRNA was verified via RT-PCR. Lipofectamine 2000 Transfection Reagent (Invitrogen, Carlsbad, California, US) was used for transfection according to standard protocol.

CCK-8 assay

For proliferation testing, cells were seeded into 96-well plate at 5000/well for culture, and, after 48 hours, optical density (OD value) was measured by the Cell Counting Kit-8 (CCk-8, Dojindo, Japan) assay. 10 μL of CCK-8 solution and 90 μL medium was added to each well and then incubated at 37°C for 1 h. Then the OD value was measured at 450 nm with a Microplate spectrophotometer (Thermo Scientific, Belmont, Massachusetts, US). For cisplatin sensitive testing, cells (3×10^3) were seeded in 96-well plates. After overnight culture, cells were treated with various concentrations of cisplatin (Sigma, Santa Clara, California, US) (0, 2.5, 5, 10, 20 and 40 μmol/L), and CCK-8 assay was applied to examine cytotoxicity of cisplatin after 48 hours of treatment. Relative survival cell (%) was determined by $(OD_{siRNA}/OD_{Scramble})\times100\%$.

Vitamin D and BAY 11-7082 treatment of EC109 and EC9706 cells

EC109 and EC9706 cells were inoculated at 1×106 cells/well in 6-well plates. After overnight culture, 60 nmol/L Vit. D (Sigma, Santa Clara, California, US) was added once a day in fresh medium, for the next 5 days. After overnight culture, ESCC cells were treated with 10 nmol/L BAY 11-7082 (BAY) (Beyotime, Shanghai, China) [22], an inhibitor of NF-κB, for 1 hour.

Reverse transcription polymerase chain reaction (RT-PCR) analysis

RNA extraction was isolated using TRIzol™ reagent (Sigma, Santa Clara, California, US)

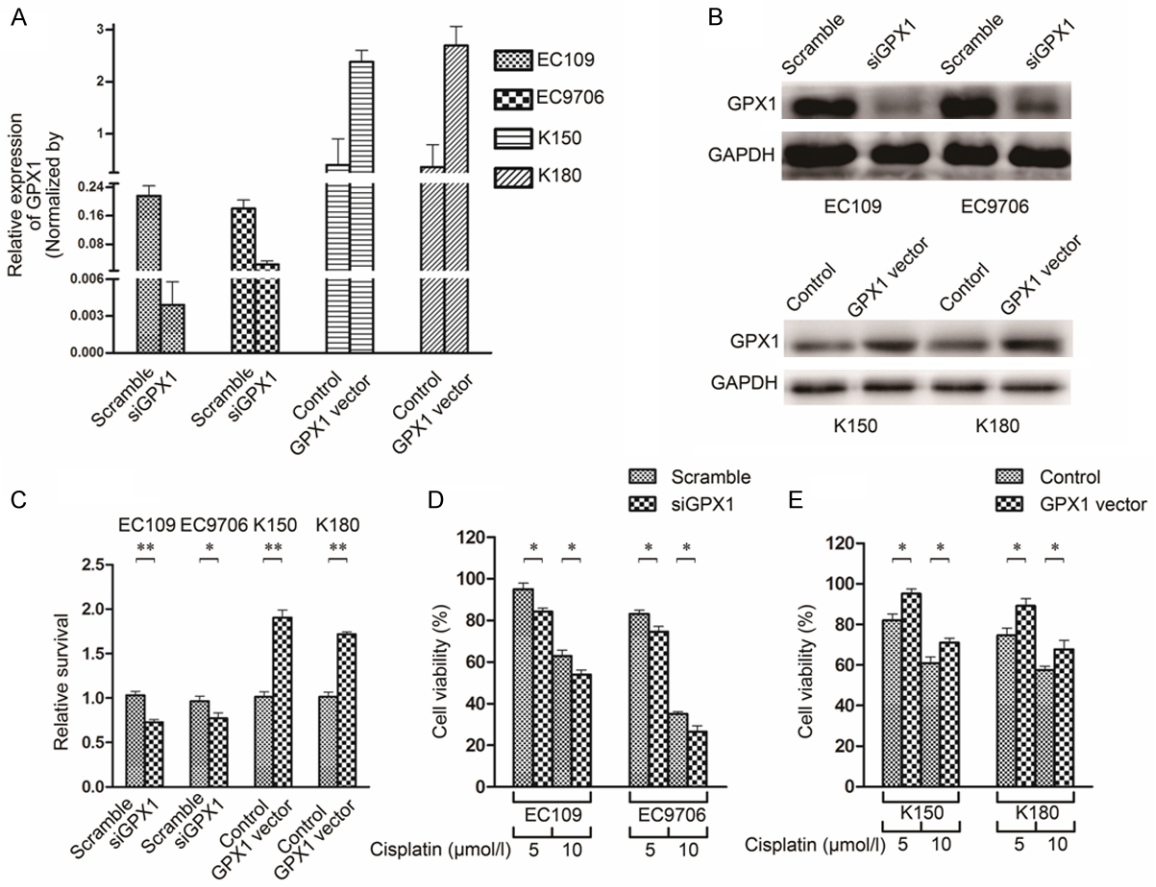

Figure 1. GPX1 expression promotes growth and cisplatin resistance in ESCC cell. (A, B) To confirm the transfection efficiency, we used RT-PCR (A) and western blotting (B). (C) The viability of ESCC cells after transfection with GPX1 siRNA or vector. The percentage of viable cells was evaluated in 48 hours using a CCK-8 assay after transfection. The results are shown as a percentage of the control (*P<0.05; **P<0.01). GPX1 over-expression enhanced the proliferation of ESCC cells. Decreasing (D) or increasing (E) GPX1 expression influenced the cisplatin resistance in ESCC cells. Low GPX1 expression down-regulates cisplatin resistance, and high GPX1 expression enhanced it (*P<0.05).

under standard conditions. Using the Prime Script RT reagent kit (TaKaRa, Otsu, Japan), 2 μg of total RNA was reverse-transcribed into cDNA and amplified by PCR. Quantitative PCR was carried out by the SYBR Green method (TaKaRa, Otsu, Japan) using a CFX96 Real-Time PCR Detection System (Bio-Rad, Hercules, California, US). The primers were purchased from TaKaRa (Otsu, Japan), and GAPDH was used as an endogenous control. The sequences are shown as follows: GAPDH, (sense, 5'-GCACCGTCAAGGCTGAGAAC-3') and (anti-sense, 5'-TGGTGAAGACGCCAGTGGA-3'); GPX1, (sense, 5'-GCGGGGCAAGGTACTACTTA-3') and (anti-sense, 5'-CTCTTCGTTCTTGGCGTTCT-3'). Amplification was performed by a protocol suggested by the manufacturer (TaKaRa, Otsu, Japan).

In vitro migration and invasion assays

For the Transwell migration assays, 5×10^4 cells were plated in the top chamber of each insert (Corning, Corning, New York, US). For the invasion assays, 2×10^5 cells were plated in the upper chamber of each insert, which had previously been coated with 150 mg of Matrigel (BD Biosciences, Canada). The cells in both assays were trypsinised and resuspended in FBS-free RPMI-1640 medium, and 600 μL of medium that had been supplemented with 10% FBS was added to the lower chambers in the migration assay wells and 20% FBS medium in the lower chambers in the invasion assay. After 12 hours or 24 hours of incubation at 37°C for migration or invasion assays, respectively, the cells remaining in the top chambers or on the

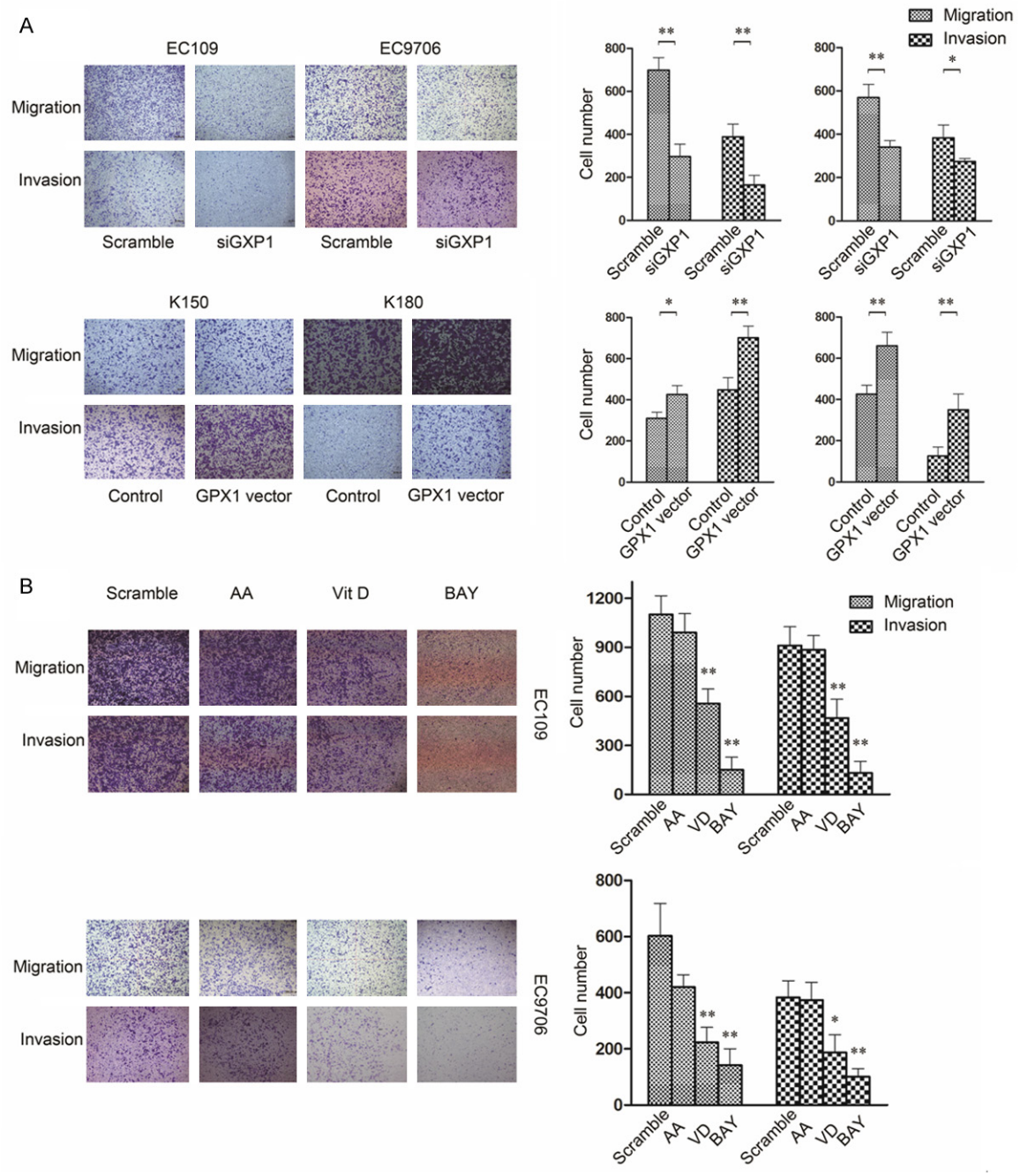

Figure 2. Low GPX1 expression, absolute alcohol (AA), Vit. D and BAY reduced ESCC cells invasion and migration. A. Exogenous over-expression of GPX1 enhanced ESCC cell invasion and migration, whereas the down-regulation of GPX1 suppressed ESCC cell invasion and migration. The differences in cell invasion, and migration were statistically significant (*p<0.05, **p<0.01). B. Correspondingly, invasion and migration were inhibited in Vit. D and BAY treatment groups (P<0.01), in EC109 and EC9706 (*p<0.05, **p<0.01).

upper membranes of the inserts were removed, and the cells that had migrated or invaded into the lower chambers were fixed and stained with a solution of 0.1% crystal violet and 4% paraformaldehyde. The migrating and invading cells were imaged and counted using an IX71 inverted microscope (Olympus, Tokyo, Japan).

Western blot analysis

Following Vit. D, BAY or transfection treatment, the cells were collected and the total protein was extracted from the cells. 30 μg of protein was loaded and separated using a 10% sodium dodecyl sulfate polyacrylamide gel electropho-

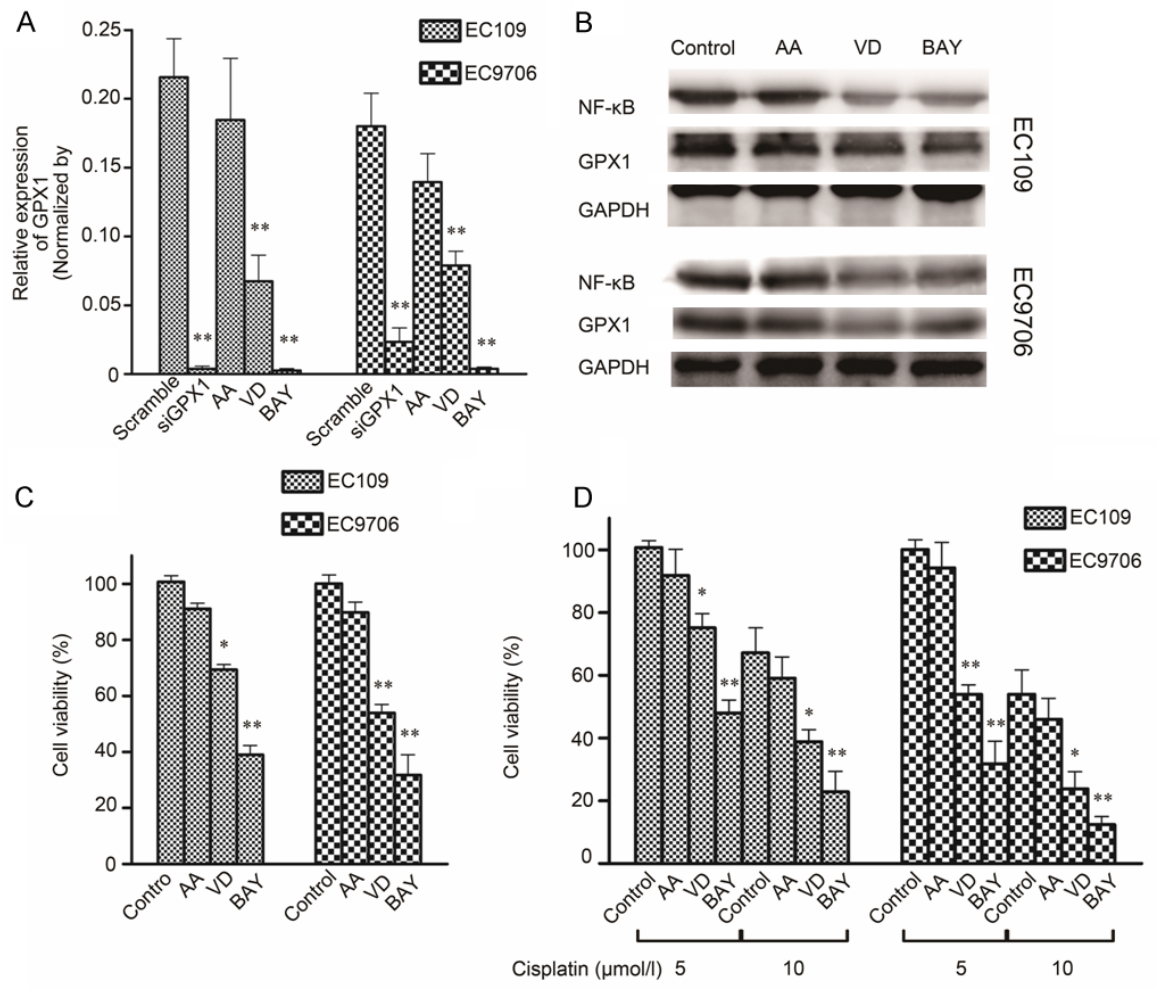

Figure 3. Vit. D reduced ESCC cells NF-κB expression and the capability of growth and cisplatin resistance. To determine GPX1 and NF-κB expression in ESCC cells after Vit. D and BAY treatment, RT-PCR (A) and western blotting (B) were performed. Reduced GPX1 and NF-κB expression was observed in GPX1 siRNA, Vit. D and BAY treated EC109 and EC9706 cells. No decrease was observed after absolute alcohol (AA) and scramble treatment (*P<0.05, **P<0.01). (B) GPX1 and NF-κB expression are reduced after Vit. D and BAY treatment. (C) Correspondingly, the viability of ESCC cells after AA, Vit. D or BAY treatment was determined, and the percentage of viable cells was evaluated by CCK-8 assay as described. The results are shown as a percentage of the control (*P<0.05, **P<0.01). The Vit. D and BAY groups exhibited weaker proliferation; the difference between the control and AA group is not significant (*P<0.05, **P<0.01). (D) EC109 and EC9706 cells were treated with AA, Vit. D and BAY, then cultured with various concentrations of cisplatin, as described. Cisplatin resistance could be reduced by Vit. D and BAY treatment of EC109 and EC9706 cells (*P<0.05, **P<0.01).

resis (SDS-PAGE) (Beyotime, Shanghai, China) gel and transferred to Immobilon-P Transfer Membrane (PVDF) (Beyotime, Shanghai, China). The membranes were blocked with 5% non-fat milk in Tris-buffered saline (TBS) containing 0.1% Tween-20 for 1 h at room temperature. The blots were probed with the relevant primary antibodies overnight at 4°C, washed in TBST, and probe with a species-specific horseradish peroxidase-conjugated secondary antibody (anti-rabbit, ExCell Bio, Shanghai, China). The anti-GPX1 antibody (Abcam, Cambridge, UK), Nf-κB (Gene Tex, Irvine, California, US), anti-uPA antibody (Gene Tex, Irvine, California, US) and anti-MMP2 antibody (Gene Tex, Irvine, California, US) were used to probe the alterations of the protein. GAPDH (Kangcheng Biology, Shanghai, China) was used as a loading control. The western blot process was performed according to standard protocols. The membranes were incubated with secondary antibodies (anti-rabbit) (ExCell Bio, Shanghai, China) for 1 hour at room temperature. An enhanced chemiluminescence detection method (Pierce ECL

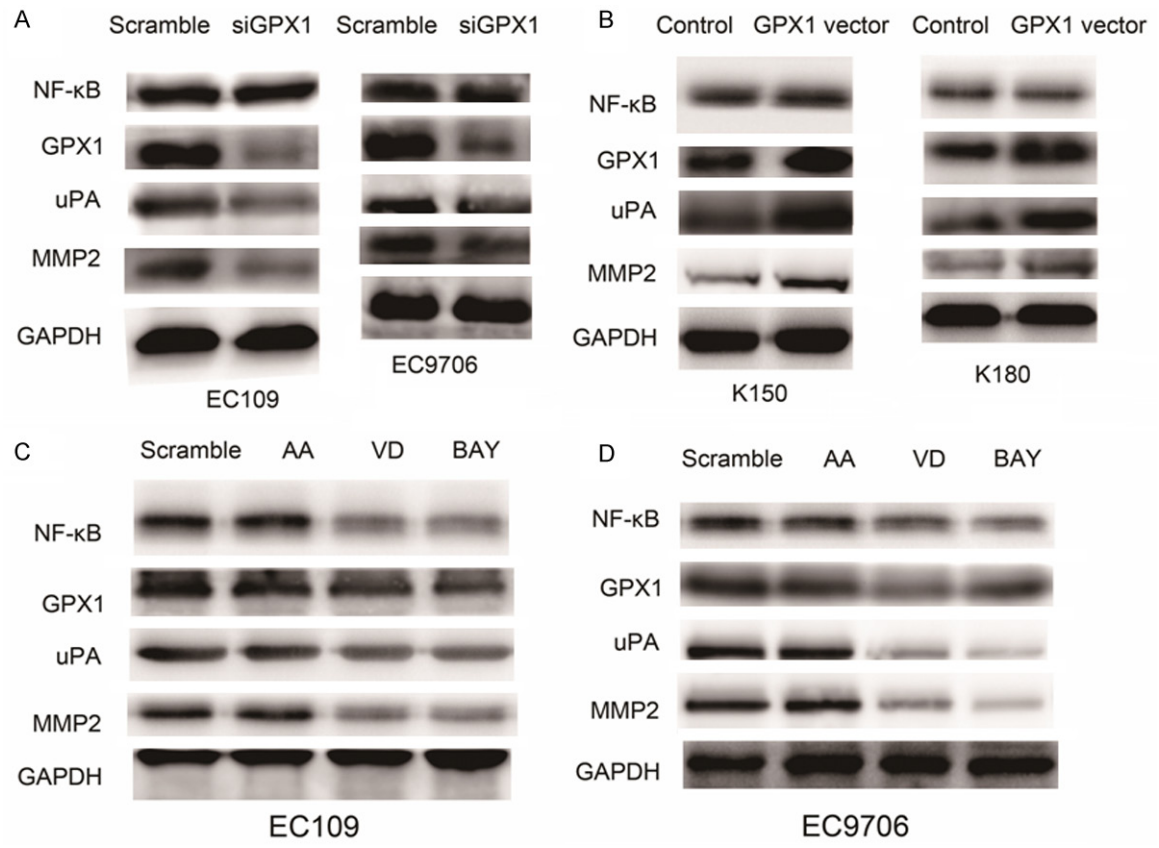

Figure 4. Vit. D regulates GPX1 to reduce malignancy in ESCC cells through the NF-κB pathway, and decreases invasion and migration via uPA and MMP2. After transfection of the GPX1 overexpression vector (B) and GPX1 siRNA (A), invasion, migration, proliferation and cisplatin-resistance was reduced by GPX1 down-regulation and increased by GPX1 up-regulation. Meanwhile, by western blot, GPX1 up-regulation cells also exhibited uPA and MMP2 up-regulation, without an increase in Nf-κb. In the GPX1 down-regulation cells, uPA and MMP2 were reduced without the inhibition of NF-κB. (C, D) BAY and Vit. D were added to ESCC cells at the previously described concentrations. In EC109 (C) and EC9706 (D) cells, treatment with Vit. D and BAY resulted in inhibition of GPX1, uPA, MMP2 and Nf-κb.

Western Blotting Substrate) (Thermo Scientific, Belmont, Massachusetts, US) was used to visualize the blots.

Statistical analysis

All data are presented as the mean ± standard deviation (SD). The data were analyzed with Student's t-test, and *p*-values <0.05 were considered significant. All statistical analyses were performed with the SPSS program package 13.0 (SPSS Inc., Chicago, IL, USA).

Results

GPX1 expression promotes migration, invasion, growth and cisplatin resistance in ESCC cell

We detected the relative expression of GPX1 in 4 ESCC cell lines, and the two highest (EC9706 and EC109) and two lowest (K150 and K180) expressing cell lines were chosen for further study. To determine the functional role of GPX1 in invasion, migration, proliferation and cisplatin resistance, we up-regulated GPX1 expression using an exogenous GPX1 expression vector in the two low relative expression cell lines, K150 and K180, and siRNA (siGPX1) in the two high expressing cell lines, EC109 and EC9706 (**Figure 1A** and **1B**). As shown (**Figure 2A**), enhanced expression of GPX1 promoted ESCC cell invasion and migration in the K150 and K180 cell lines. In contrast, down-regulation of GPX1 attenuated cell invasion and migration in the EC109 and EC9706 cell lines. Forty-eight hours after transfection, we performed a CCK-8 assay to detect the OD values in both GPX1 up- (GPX1 vector) and down-regulation (siGPX1) cell lines. It appeared that GPX1 overexpression could enhance proliferation in the K150

and K180 cell lines, whereas in GPX1 down-regulation cell lines (EC109 and EC9706) the proliferative capacity was reduced (**Figure 1C**). Moreover, 36 hours after transfection with the siRNA or expression vector, the cell lines were cultured in 96-well plates for 48 hours with different concentrations of cisplatin (0, 2.5, 5, 10, 20 and 40 μmol/L). We found that GPX1 overexpression promoted cell viability, while down-regulation reduced viability (**Figure 1D** and **1E**). This indicates that high GPX1 expression could be responsible for the enhanced migration, invasion, proliferation and cisplatin resistance observed in ESCC cell lines.

Vit. D reduces ESCC cells malignancy and NF-κB expression

The correlation between Vit. D and ESCC cell malignancy was further investigated. After 5 days of treatment with Vit. D, the invasive and migratory capacities of EC109 and EC9706 cells were determined with a Transwell assay. Upon Vit. D treatment, EC109 and EC9706 cells exhibited inhibited invasive behavior (**Figure 2B**). Before CCK-8 assay, some of the Vit. D treated cells were cultured in a 96-well plate for 48 hours, in the presence or absence of cisplatin. The results illustrated that Vit. D reduced the proliferation (**Figure 3C**) and cisplatin (**Figure 3D**) resistance of ESCC cells. Moreover, we performed western blots to detect NF-κB expression of the cells after Vit. D treatment (**Figure 3B**). As anticipated (and previously reported in other cancer cells), NF-κB expression was inhibited by Vit. D [18-21] treatment. These results suggest that Vit. D might reduce migration, invasion, proliferation and cisplatin resistance through the NF-κB pathway.

Vit. D down-regulates GPX1 expression through NF-κB pathway

BAY 11-7082, an inhibitor of NF-κB [22], selectively and irreversibly inhibits NF-κB activation. To determine the changes in biological function of the NF-κB down-regulated cells, EC109 and EC9706 were treated with BAY 11-7082. The results suggested that cell invasion and migration were inhibited (**Figure 2B**), and the proliferation (**Figure 3C**) and cisplatin resistance (**Figure 3D**) were reduced. GPX1, as a molecule downstream of NF-κB, was down-regulated after BAY 11-7082 treatment (**Figure 3B**), following the down-regulation of NF-κB, consistent with previous findings in other cancers [11, 13, 14]. In addition, the results also illustrated that GPX1 and NF-κB expression were reduced in the Vit. D treated cells. Therefore, taking these results together illustrates that Vit. D could mimic the activity of BAY 11-7082 to inhibit GPX1 through the NF-κB pathway.

GPX1 overexpression encourages migration and invasion through MMP2 and uPA

It is known that MMP2 and uPA play critical roles in the process of tumour invasion and metastasis [23, 24]. Matrix metalloproteinase-2 (MMP2) is one of the key enzymes involved in maintaining the integrity and turnover of the extracellular matrix (ECM) and can degrade a variety of ECM proteins [23]. Urokinase type plasminogen activator (uPA) is a serine proteolytic enzyme, which is also thought of as a critical accelerant in the process of tumour invasion and metastasis [25, 26]. To identify the underlying mechanisms governing the effects of GPX1 on ESCC cancer cell invasion and migration, we examined the expression of MMP2 and uPA in cells after exogenously modulating the expression of GPX1. In EC109 and EC9706 cells (the relative high GPX1 lines), introduction of GPX1 siRNA resulted in decreased MMP2 and uPA protein expression by western blot (**Figure 4A**). Meanwhile, uPA and MMP2 activity increased in both K150 and K180 cells that had been transfected with the GPX1 vector (**Figure 4B**). It was also appeared that in siGPX1 and GPX1 vector groups the NF-κB expression did not change (**Figure 4A** and **4B**). And the downregulation of GPX1, NF-κB, uPA and MMP2 was observed in the cells which were treated by Vit. D and BAY 11-7082 (**Figure 4C** and **4D**), illustrating the functional importance of MMP2 and uPA in GPX1-mediated signalling for ESCC invasion and migration.

In summary, the results of this study describe a positive correlation between GPX1 expression and cancer cell migration, invasion, proliferation and cisplatin resistance, and that Vit. D, as a widely used medicine, could reduce the associated malignancy.

Discussion

Despite the progress that has been made in the therapy of esophageal cancer, the progno-

sis remains dismal, and the 5-year survival rate is unsatisfactory [1]. The metastasis and local recurrence are responsible for the majority of deaths in esophagus cancer. Although the molecular and genetic mechanisms contributing to esophageal cancer progression are not entirely understood, the altered expression and dysfunction of genes has been proposed as a critical mechanism underlying esophageal cancer invasion, migration, and proliferation [1, 3].

During cell metabolic processes reactive oxygen species (ROS) are continuously generated [6, 27]. Human cancer could be caused by the disruption of genomic integrity led by ROS-induced DNA damage. The findings in tissues (e.g., breast, lung, liver and esophagus) support the idea that altered ROS activity could be a common cause of human cancer [7-12, 27]. Thus antioxidant mechanisms, including GPXs and detoxifying ROS, should play critical roles in protecting cells from ROS-induced DNA damage. Several cancer-related studies found that when intracellular antioxidants such as GPXs and glutathione increase in expression, ROS will be significantly decreased, thus enhancing the degree of malignancy of tumour cells [6, 27-29].

The function and the role of GPX1 in tumours have already been reported in some cancers. By overexpressing MMPs and uPA, GPX1 can promote cell invasion and metastasis [30-33]. Abnormal expression of GPX1 in Barrett's esophagus cases have been reported [27], but the mechanism and significance of this remains unclear. In ESCC, especially in the invasion, migration, cisplatin resistance and proliferation aspects, GPX1 is understudied target. Our study illustrates the effect of GPX1 expression on the biological behavior of esophageal cancer cells. We have proven that GPX1 can promote tumour cell invasion, migration, proliferation and resistance by regulating GPX1 expression.

In ESCC and other carcinomas, NF-κB has a significant influence in the proliferation, apoptosis, metastasis and aromorphosis [16, 34, 35]. Compared to normal tissues, many studies show that there are significant changes in NF-κB expression and activity in many malignant tumour cells, thereby altering downstream protein expression [13, 16, 36]. Vit. D is a fatty hormone-like substance with versatile and indispensable activity in normal human life. It has anti-inflammatory, anti-oxidant effects *in vivo* [18, 19]. The available evidence suggests that it may down-regulate NF-κB, thereby changing the biological functions through the NF-κB pathway in a variety of diseases and tumour cells [17-21, 37]. Moreover, it was reported that GPX1 expression can be reduced through the NF-κB pathway [11, 13]; in our study, using a specific inhibitor of NF-κB, BAY 11-7082 [22], we observe the same phenomenon. Therefore, we speculate that vitamin D can inhibit the NF-κB pathway to further suppress the expression of GPX1, thereby reducing the degree of malignancy of esophageal cancer cells. In this study, the GPX1 relative high expression cells, EC109 and EC9706, were treated with vitamin D, and NF-κB and GPX1 activation decreased in response, while invasion, migration, proliferation and cisplatin resistance also declined, illustrating that vitamin D can decrease GPX1 expression via the NF-κB pathway, thereby reducing the degree of malignancy of tumour cells [13, 17, 18].

MMP2 is a key enzyme involved in maintaining the integrity and turnover of the extracellular matrix (ECM). Studies have demonstrated that the altered GPX1 activity can change the malignancy of breast cancer through MMP2 signaling [33]. Furthermore, uPA is ubiquitously expressed in many normal and malignant cell types and is an important component of the fibrinolytic system, responsible for converting plasminogen into the active enzyme [31, 32, 38]. It can activate downstream signaling pathways, thereby enhancing tumour cell migration and invasion. uPA has been reported to be an critical molecule in GPX1-associated tumour progression and liver fibrosis [30, 32]. With the NF-κB pathway inhibited by BAY and vitamin D, decreased GPX1 expression and reduced tumour cell invasion and migration were observed, while the expression of MMP2 also appeared correspondingly reduced. A similar phenomenon was observed cells transfected with a GPX1-overexpression vector. Thus, we believe that, in ESCC cells, enhanced GPX1 activity can lead to the capability of invasion and migration changes through MMP2 and uPA.

This study confirms the association between vitamin D, GPX1 and ESCC cancer cell invasion and migration. As we have discussed above, GPX1 interacts with other target proteins and

functions in a variety of cellular processes. The mechanism of GPX1 in tumourigenesis remains unclear, and GPX1 biological effect in proliferation and cisplatin resistance is still undefined. Further studies are in progress to examine the role of GPX1 in tumourigenesis.

Conclusions

In our experiments, we found that the overexpression of GPX1, in ESCC cells, can promote invasion, migration, proliferation and cisplatin resistance. Treatment with vitamin D can downregulate GPX1, thus decreasing tumour cell capacity for invasion, migration, proliferation and cisplatin resistance. Moreover, through MMP2 and uPA, invasion and migration were inhibited by decreased GPX1 caused by Vitmin D via the NF-κB pathway. Above all, our findings suggest that in ESCC, GPX1 may have potential as a new biomarker to predict the degree of malignancy. Vitamin D, already commonly used in clinical therapy, could play a greater role in the treatment of esophageal cancer patients.

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Disclosure of conflict of interest

None.

Abbreviations

GPX1, glutathione peroxidase 1; Vit. D, vitamin D; MMP2, matrix metalloproteinase-2; uPA, urokinase type plasminogen activator; ESCC, esophageal squamous cell carcinoma; ROS, reactive oxygen species; FBS, fetal bovine serum; GPX1 siRNA, GPX1 small interfering RNA; PCR, polymerase chain reaction; OD, optical density; BAY, BAY 11-7082; ECM, extracellular matrix.

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