GSTpi reduces DNA damage and cell death by regulating the ubiquitination and nuclear translocation of NBS1

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Abstract
Glutathione S-transferase pi (GSTpi) is an important phase II detoxifying enzyme that participates in various physiological processes, such as antioxidant, detoxification, and signal transduction. The high expression level of GSTpi has been reported to be related to drug-resistant and anti-inflammatory and it functioned via its non-catalytic ligandin. However, the previous protection mechanism of GSTpi in DNA damage has not been addressed so far. Nijmegen breakage syndrome 1 (NBS1) is one of the most important sensor proteins to detect damaged DNA. Here, we investigated the interaction between GSTpi and NBS1 in HEK-293 T cells and human breast adenocarcinoma cells during DNA damage. Our results showed that over-expression of GSTpi in cells by transfecting DNA vector decreased the DNA damage level after methyl methanesulfonate (MMS) or adriamycin (ADR) treatment. We found that cytosolic GSTpi could increase NBS1 ubiquitin-mediated degradation in unstimulated cells, which suggested that GSTpi could maintain the basal level of NBS1 during normal conditions. In response to DNA damage, GSTpi can be phosphorylated in Ser184 and inhibit the ubiquitination degradation of NBS1 mediated by Skp2 to recover NBS1 protein level. Phosphorylated GSTpi can further enhance NBS1 nuclear translocation to activate the ATM-Chk2-p53 signaling pathway. Finally, GSTpi blocked the cell cycle in the G2/M phase to allow more time for DNA damage repair. Thus, our finding revealed the novel mechanism of GSTpi via its Ser184 phosphorylation to protect cells from cell death during DNA damage and it enriches the function of GSTpi in drug resistance.

Keywords GSTpi · NBS1 · Ubiquitination · Nuclear translocation · DNA damage response

Introduction
Maintaining genomic stability is critical for the normal functions of living organisms which are constantly exposed to a large number of DNA damaging agents including endogenous and exogenous. Meanwhile, robust and intricate mechanisms such as DNA repair, damage tolerance, cell cycle checkpoints, and cell death pathways have been evolved in response to protect DNA integrity and ensure overall survival [1]. However, the cells will undergo apoptosis or senescence if the DNA damage is too extensive to be repaired. To understand the complex mechanisms of DNA damage repair, identifying the regulatory proteins that participate in the DNA damage repair pathways is essential.

Glutathione S-transferase (GST) is a cytosolic phase II detoxifying enzyme which can catalyze the conjugation of the sulfur atom of glutathione (GSH) to electrophilic groups of substrate molecules [2]. Through enhancing its solubility and making it easier to expel from the cells to maintain the cellular
redox state [3]. According to the sequence similarity, human cytosolic GSTs can be divided into eight classes: α, μ, π, θ, κ, ζ, σ, ω [4]. GSTπi is one of the specific isozymes that express at a high level in the mammalian cells [5]. Generally, GSTπi acts as a catalytic enzyme and inactivates chemotherapeutic substances by conjugating them to GSH [6]. However, GSTπi can also function via its non-catalytic ligand-binding activity since some compounds are not the substrates of GSTπi. Previous reports have demonstrated that GSTπi protects cells against ROS-induced apoptosis by inhibiting the activity of JNK [7]. GSTπi can also interact with TRAF2 and STAT3 to protect cells against different injury stimuli [8, 9]. Recent investigations have suggested that GSTπi specifically interacted with p110α subunit of PI3K to protect breast cancer cells from Adriamycin-induced cell death [10], which explained the mechanism of GSTπi protects cells against anticancer drugs. According to previous research that nuclear GSTπi prevents H2O2-induced DNA damage by scavenging the formation of lipid-peroxide-modified DNA [11], we wondered whether GSTπi could play a critical role in DNA damage response through protein–protein interactions.

Ataxia-telangiectasia mutation (ATM) kinase plays a central role in DNA damage response (DDR) through initiating some cellular processes including cell cycle control, transcription, and DNA repair [12]. Loss of ATM may lead to genome instability and enhance the risk of cancer and other diseases [13]. It has been proposed that the MRN complex, which consists of Mre11, Rad50, and NBS1 is recruited to the DNA repair foci which in turn facilitates ATM to the damage foci and activates ATM kinase activity directly upon DNA double-strand breaks (DSBs) [14–16]. Once mutation of the NBS1 or Mre11 genes, it can cause Nijmegen breakage syndrome (NBS) and ataxia-telangiectasia–like disorder (ATLD), and inactivate ATM activity [17, 18]. Therefore, in our study, we aimed to determine whether GSTπi could interact with NBS1 to protect cells against DNA damage.

In the current research, we investigated the protection mechanism of GSTπi during DNA damage. We found that GSTπi can inhibit the ubiquitination degradation of NBS1 in the cytoplasm, then phosphorylated at Ser184 and combined to NBS1 to activate ATM-Chk2-p53 signaling pathway by enhancing NBS1 nuclear translocation. This finding indicates a novel mechanism of phosphorylated GSTπi entering the nuclear and regulating NBS1 in DNA damage repair, which may be a new therapeutic target to overcome drug resistance in breast cancer cells.

## Materials and methods

### Antibodies, chemicals and plasmids

Antibodies against GAPDH, LaminB, GSTπi, β-actin, ATM, p-ATM (Ser1981) and Skp2 were purchased from Bioworld Technology (Minneapolis, MN, USA). Antibodies against γH2AX, Chk1, p-Chk1 (Ser345), Chk2, p-Chk2 (Thr68), p53, p-p53 (Ser15), NBS1, Mre11, Rad50, Bcl-2, Bax, caspase3 and Cleaved-caspase3 were purchased from Cell Signaling Technology (Beverly, MA, USA). Antibodies against ATR and p-ATR were purchased from ABclonal (Wuhan, Hubei, China). HRP conjugated secondary antibodies against mouse or rabbit IgG were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). DAPI, Alexa Fluor 488 donkey anti-mouse IgG and Alexa Fluor 555 donkey anti-rabbit IgG were purchased from Invitrogen (Carlsbad, CA, USA). HRP conjugated secondary antibodies was obtained from Vazyme Biotech (Nanjing, Jiangsu, China). KU55933, Pifithrin-α, PMA and Gö6983 were purchased from Selleck Chemicals (Houston, TX, USA). Cell Counting Kit-8 Assay Kit was from Dojindo (Shanghai, China). Methyl methanesulfonate, Adriamycin and X-tremeGENE HP DNA Transfection Reagent were purchased from Sigma-Aldrich (St. Louis, MO, USA). DNA Damage Detection Kit was from KeyGEN Biotech (Nanjing, Jiangsu, China). Annexin V-FITC and PI Apoptosis Detection Kit were purchased from Beyotime (Shanghai, China).

Plasmids including pcDNA3.1, Flag-GSTπi, GSTπi-S184A, GSTπi-S184D, GSTπi-Y7F, GSTπi-shRNA and pLKO.1 were constructed in our laboratory. Myc-Skp2 and GSTπi-CRISPR-CAS9 were constructed by Public Protein/Plasmid Library (Nanjing, Jiangsu, China).

### Cell culture and transfection

Human embryonic kidney cell (HEK-293 T, NICR), human breast adenocarcinoma cell (MCF-7, NICR) and its Adriamycin-resistant counterpart cell (MCF-7/ADR, kindly provided by Prof. Zhigang Guo, China) were maintained in DMEM (Wisent, Canada) containing 10% fetal bovine serum (Gibco, USA), 100 IU/mL penicillin, and 10 mg/mL streptomycin (Wisent, Canada) at 37 °C in an atmosphere of 5% CO2. MCF-7/ADR cells were cultured in the presence of a low concentration of ADR (1 μM) every 4 weeks.

Transient transfection was performed as the manufacturer’s instructions using the X-tremeGENE HP DNA Transfection Reagent (Sigma-Aldrich, USA).
**Cell viability assay**

HEK-293 T cells viability assay was performed according to the manufacturer’s instructions (Dojindo, China). The absorption was acquired at 450 nm by a spectrophotometer.

**Co-immunoprecipitation and immunoblotting analysis**

Cells were lysed with radioimmunoprecipitation (RIPA) lysis buffer (Beyotime, China) on ice for 30 min, and the lysates were centrifuged at 12,500 rpm for 15 min. Samples were immunoprecipitated with indicated antibodies at 4 °C overnight, then incubated with protein A/G plus-agarose beads (Santa Cruz Biotechnology) for 3 h and washed with lysis four times. Each of sample was resolved in SDS-PAGE and transferred onto polyvinylidene fluoride membrane. Membranes were incubated in 5% (w/v) TBST with non-fatty milk or bovine serum albumin. Membranes and corresponding primary antibodies were incubated together overnight at 4 °C. Choosing one HRP conjugated secondary antibody to incubate with the primary antibody for one hour at room temperature. The signal bands were visualized via chemiluminescence using the ECL immunoblotting system.

**Immunofluorescence assay**

Cells were fixed in 4% paraformaldehyde for 30 min, then permeabilized with PBS containing 0.1% Triton X-100. After being blocked by 5% bovine serum albumin for 1 h, cells were incubated with primary antibodies overnight at 4 °C, followed by the incubation with Alexa Fluor-488 secondary antibody (Invitrogen) for 1 h in the dark at room temperature. Then the nuclear was stained with DAPI for 5 min. The cells were observed by the Nikon A1 microscope (Nikon, Tokyo, Japan). Using the same fluorescence detection parameters for comparison of different samples.

**Single cell gel electrophoresis**

Cells were digested by 0.05% trypsin and resuspended in PBS. Subsequently, comet assay was performed according to the instructions of the manufacturer. Images were captured using fluorescence microscope. Each sample about 50 cells was analyzed by CASP.

**DNA ladder detection**

After washing with PBS, all cells were harvested and the genomic DNA was extracted using the DNA ladder extraction kit (Beyotime, China). DNA samples were subsequently separated using 1.8% (w/v) agarose gel and visualized by ethidium bromide staining under ultraviolet light.

**Preparation of cytoplasmic and nuclear extracts**

The cells were washed with ice-cold PBS for 3 times, then the supernatant lysed with cytoplasmic lysate RLN on ice for 20 min. Cytoplasmic component was extracted by centrifuging for 10 min at 3000×g. The precipitation lysed with the nuclear lysate RIPA on ice for 20 min and vortexed for 10 s every 5 min to make the cell pellet fully suspend, then centrifuged for 20 min at 12,000×g. The protein concentration was determined by BCA method.

**Cell-cycle analysis by propidium iodide staining**

Cells were plated in 6-well plates, followed by trypsinizing and washing cells with PBS. Then cells were fixed in ice-cold 70% ethanol for 4 °C overnight. After centrifuging, suspending cells in PBS. Next, we stained cells with 500 μL PI for 30 min at 37 °C. Finally, the cells were analyzed with BD flow cytometer.

**Annexin V-FITC and PI dual staining assay**

To determine the effect of GSTpi on the apoptosis in breast cancer cells, the MCF-7/ADR cells were plated in 6-well plates, followed by treating the cells with ADR for 48 h. Then cells were trypsinized and washed with PBS twice, suspended in 200 μL binding buffer. Next, we stained the cells with 5 μL of FITC Annexin V and 10 μL PI for 30 min at 4 °C. Finally, the cells were analyzed with BD flow cytometer.

**Statistical analysis**

Statistical analysis was analyzed by GraphPad Prism 8.0 software. Different cells viabilities were analyzed by one-way analysis of variance (ANOVA) followed by appropriate post hoc tests (Tukey). A p value < 0.05 was considered significant (*p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001). Western blotting
analyses were repeated three times with similar trends and quantified using ImageJ. Statistical analysis was performed using Student’s t test. *p < 0.05; **p < 0.01; ***p < 0.001; n.s.: not significant (p > 0.05). All of the results are presented as mean ± S.D. of at least three independent experiments.

**Results**

**GSTpi protects cells from DNA damage induced by MMS**

To detect the effect of GSTpi on DNA damage repair, we transfected cells with pcDNA3.1 and Flag-GSTpi plasmids, then treated cells with MMS. CCK8 results showed that the viability of forced expression of Flag-GSTpi was significantly evaluated (Fig. 1a). The DNA damage marker γH2AX represents the degree of damage, so we examined the kinetics of DNA repair by monitoring γH2AX signal via immunoblotting and immunofluorescence microscopy. Results from immunoblotting assay showed that the protein level of γH2AX in overexpression of Flag-GSTpi was lower than cells that transfected with pcDNA3.1, however, it had no significant difference, but when the cells were continued to be cultured for 6 h, 12 h, 24 h after withdrawal of the drug, it was significantly decreased. When the recovery time was up to 48 h, the MMS-induced DNA damage seemed to be completely repaired (Fig. 1b). Similarly, the foci of γH2AX were captured when the cells were stimulated, but it reversed when the cells were transfected with Flag-GSTpi (Fig. 1c). These results initially proved that GSTpi can promote DNA damage repair. To further prove the above results, we evaluated the global effect of GSTpi on DNA damage via single cell gel electrophoresis. Overexpression of GSTpi caused the reduction of tails, and the length of tails became short with the recovery time, until 24 h the tails disappeared (Fig. 1d).

We prepared DNA Ladder Extraction Kit to test the level of DNA damage, we saw a DNA ladder when MMS stimulated, and GSTpi attenuated the fragmentation (Fig. 1e). Collectively, these results demonstrated that GSTpi plays a critical role in DNA damage repair.

**GSTpi enters the nucleus to activate the ATM-Chk2-p53 signaling pathway**

To understand the mechanism involved in GSTpi protecting cells from DNA damage, we detected the change of GSTpi protein level after stimulated with MMS. Consistent with previous results that the protein level of GSTpi was increased with recovery time and reached a peak level at 48 h. While, the DNA damage level was maximum when the recovery time was up to 6 h and then declined (Fig. 2a). It has been reported that the protective effect of GSTpi on the tumor was related to its nuclear translocation [19]. We next explored whether the effect of GSTpi on DNA damage repair induced by MMS was dependent on its translocation. Both immunoblotting analysis and confocal microscopy observation showed that GSTpi was predominantly localized in the cytoplasmic in unstimulated cells, but could be detected in the nucleus after MMS treatment. In the end, GSTpi was gradually translocated in the cytoplasmic compartment with the recovery time (Fig. 2b, c). It has also been reported that some proteins were phosphorylated at Ser/Thr-Glu motifs and additional sited in an ATM or ATR-dependent way to respond DNA damage [20, 21]. ATM and ATR also activated a “second wave” of phosphorylation through their
Fig. 2 GSTpi enters the nucleus to activate ATM-Chk2-p53 signal pathway in response to DNA damage. HEK-293 T cells were treated with 0.5 mM MMS or not for 1 h, then cells were allowed to recover in normal medium for various times. a Cell lysate were subjected to immunoblotting using the indicated antibodies. The cells subjected to extraction of their nuclear and cytoplasmic fractions, followed by immunoblotting using the indicated antibodies. Data represent the mean ± SD of at least three independent experiments. b HEK-293 T-WT and HEK-293 T-GSTpi−/− cells were treated with 0.5 mM MMS or not for 1 h, then cells were allowed to recover in normal medium for 6 h, then cell lysates were subjected to immunoblotting using the indicated antibodies. Data represent the mean ± SD of at least three independent experiments. c HEK-293 T-WT cells transfected with pcDNA3.1 (2 μg/mL) or Flag-GSTpi (2 μg/mL) were treated with 0.5 mM MMS or not for 1 h, then cells were allowed to recover in normal medium for 6 h, then cell lysates were subjected to immunoblotting using the indicated antibodies. Data represent the mean ± SD of at least three independent experiments. d, e HEK-293 T-WT cells transfected with pcDNA3.1 (2 μg/mL) or Flag-GSTpi (2 μg/mL) were treated with 0.5 mM MMS or not for 1 h, then cells were allowed to recover in normal medium for 6 h, then cell lysates were subjected to immunoblotting using the indicated antibodies. Data represent the mean ± SD of at least three independent experiments. e HEK-293 T-WT cells transfected with pcDNA3.1 (2 μg/mL), Flag-GSTpi (2 μg/mL), GSTpi-S184A (2 μg/mL) or GSTpi-S184D (2 μg/mL) were treated with 0.5 mM MMS or not for 1 h, then cells were allowed to recover in normal medium for 6 h, then cell lysates were subjected to immunoblotting using the indicated antibodies. f HEK-293 T-WT and HEK-293 T-GSTpi−/− cells were treated with 0.5 mM MMS or not for 1 h, then cells were allowed to recover in normal medium for 6 h, then cell lysates were subjected to immunoblotting using the indicated antibodies. g HEK-293 T-WT cells transfected with pcDNA3.1 (2 μg/mL), Flag-GSTpi (2 μg/mL), GSTpi-S184A (2 μg/mL) or GSTpi-S184D (2 μg/mL) were treated with 0.5 mM MMS or not for 1 h, then cells were allowed to recover in normal medium for 6 h, then cell lysates were subjected to immunoblotting using the indicated antibodies. h HEK-293 T-WT cells were treated the same as in (a), then the cell cycle distribution was measured by BD flow cytometer. Data represent the mean ± SD of at least three independent experiments. Statistics were calculated by unpaired Student’s t test. *p<0.05, **p<0.01, n.s. not significant (p>0.05).

Results from the immunoblotting assay showed that the ratio of phosphorylated Chk2 to Chk1 after MMS treatment when the cells were transected with Flag-GSTpi (Fig. 2d). Then we tested the activation of ATM and ATR, the upstream kinase of Chk2 and Chk1, results showed that GSTpi enhanced the activation of ATM-Chk2 pathway rather than ATR-Chk1 pathway (Fig. 2e). The activated ATM by DNA damage leads to the phosphorylation of several of substrates, such as p53 which mediates the effects of ATM on DNA repair, cell-cycle arrest, apoptosis, and other downstream processes [23]. Subsequently, we explored whether p53 is involved in the pathway. The results showed that the phosphorylation level of p53 was upregulated after MMS stimulation, and GSTpi enhanced the level of phosphorylated p53 (Fig. 2e). To further prove the above results, we constructed a GSTpi−/− cell line using the CRISPR/Cas9 system. Correspondingly, the activation of ATM-Chk2-p53 signaling was significantly reduced in GSTpi−/− cells compared with the WT cells after MMS treatment. As well as, the GSTpi−/− cells showed more severe damage (Fig. 2f).

As we previous experiments showed that Ser184 phosphorylation was necessary for GSTpi to enter the nucleus and associated with HMGB1 [24]. We next transfected cells with pcDNA3.1, wild type GSTpi, GSTpi-S184A (a Ser184 non-phosphorylatable mutant), or the GSTpi-S184D (a Ser184 constant-phosphomimetic mutant), and then performed the immunoblotting assay to detect the activity of ATM-Chk2-p53 signaling pathway. GSTpi-S184A transfection did not have an obvious increase activity of the pathway compared with the WT, and the GSTpi-S184D transfection showed the highest activity (Fig. 2g). These results indicated that the change GSTpi is necessary for promoting...
Fig. 2 (continued)
the activity of the ATM-Chk2-p53 signaling pathway. For further investigation, we treated cells with ATM inhibitor Ku55933 or p53 inhibitor Pifithrin-α (PFTα). Results showed that both Ku55933 and PFTα inhibited GSTpi-induced activation of DNA damage repair signaling pathway (Fig. S1c, d). As p53 performs an important influence on checkpoint functions during the mammalian cell cycle [25–27], our flow-cytometric results showed that overexpression of GSTpi can induce cell cycle arrest in the G2/M phase, this process can be inhibited by PFTα (Fig. 2h). All these results proposed that GSTpi protected cells against DNA damage by entering the nucleus and activating the ATM-Chk2-p53 signaling pathway.

**GSTpi impacts NBS1 in a transferase activity-independent way**

Mre11-Rad50-NBS1(MRN) is not only a sensor of DSBs that activates ATM but also may function in activating ATM to initiate phosphorylation of cellular substrates [28]. To gain further insight into how GSTpi activates ATM, we first assessed whether GSTpi can interact with MRN. We found that GSTpi can promote the protein level of MRN when the cells were stimulated in both HEK-293 T-WT and HEK-293 T-GSTpi−/− cells (Fig. 3a, S2a). The C terminus of NBS1 is necessary for recruiting ATM to DSBs, and the NBS1-ATM interaction mediates ATM checkpoint functions [29]. We next determined whether GSTpi regulates ATM upon DNA damage through the interaction of NBS1. Immunoblotting assay results showed that GSTpi impacted the protein level of NBS1 in a dose-dependent way (Fig. 3b). Then we observed the subcellular location of GSTpi and NBS1 upon/without MMS. In the absence of MMS stimulation, GSTpi and NBS1 were co-localized in the cytoplasmic, and after MMS stimulation, they were rapidly recruited into the nucleus (Fig. 3c). As GSTpi is a phase II detoxifying enzyme, we next analyzed whether the function of GSTpi to promote DNA damage repair was related to its catalytic activity. Cells were transfected with pcDNA3.1, GSTpi-WT, and the catalytically inactive mutant pcDNA3.1-Flag-GSTpi (Y7F, phenylalanine replaced tyrosine in the seventh amino-terminal position), respectively, as shown in Fig. 3d, e, overexpression of GSTpi-Y7F in cells acted as similar as GSTpi-WT in response to MMS. Taken together, these data confirmed that GSTpi regulates NBS1 protein level in a transferase activity-independent manner.

**Phosphorylated GSTpi participates in the nuclear translocation of NBS1**

As a member of the MRN complex, NBS1 can not only recruit ATM to broken DNA molecules but also carry Mre11 and Rad50 into the nucleus. It has been reported that the interaction between KPNA2 and NBS1 promotes the cytoplasmic MRN complex into the nucleus [30]. In our previous experiments, we have proved that GSTpi and NBS1 co-localized in the nucleus after MMS stimulation, then we speculated that there is an interaction between GSTpi and NBS1, this interaction may contribute to the nuclear translocation of NBS1. HEK-293 T-WT cells transfected with pcDNA3.1 or Flag-GSTpi, respectively, were stimulated with MMS or not. The cytoplasmic and nuclear extracts were subjected to immunoblotting assay. We found that NBS1 was distributed in both cytoplasmic and nucleus before MMS treatment; however, GSTpi was mainly distributed in cytoplasmic. When DNA damage occurred, NBS1 entered the nucleus more, and GSTpi transfection increased the distribution of NBS1 in the nucleus (Fig. 4a). As expected, both immunoblotting assay and confocal microscopy in HEK-293 T-GSTpi−/− cells showed the same results (Fig. 4b, c). To further investigate the effect of GSTpi on the nuclear translocation of NBS1, we transfected HEK-293 T-WT or HEK-293 T-GSTpi−/− cells with pcDNA3.1, GSTpi-WT, GSTpi-S184A or GSTpi-S184D plasmids, respectively, the immunoblotting assay demonstrated that both GSTpi-WT and GSTpi-S184D induced the nuclear translocation of NBS1 in MMS-stimulated cells (Fig. 4d, e). Furthermore, GSTpi-S184D plasmid performed a stronger effect on MMS-induced NBS1 nuclear translocation than GSTpi-WT plasmid in both HEK-293 T-WT and HEK-293 T-GSTpi−/− cells, while GSTpi-S184A plasmid almost had no effect on that (Fig. 4d, e). It has been determined in our laboratory that classic protein kinase C (cPKC) acted as the upstream kinase of GSTpi to phosphorylate Ser184 of GSTpi in macrophages [24]. We therefore hypothesized that the phosphorylated Ser184 of GSTpi may be activated by cPKC, and this phosphorylation may mediate the NBS1 nuclear translocation. To test this notion, we treated HEK-293 T-GSTpi−/− cells which transfected with pcDNA3.1 or Flag-GSTpi with cPKC activator or inhibitor. As shown in Fig. 4f, PMA (a cPKC activator) enhanced the NBS1 nuclear translocation, whereas Gö6983 (a broad-spectrum PKC inhibitor) apparently attenuated MMS-stimulated
a) MMS
pcDNA3.1 - - - + - - + - - - -
Flag-GSTpi - - + - - + - - + - -
NBS1 - - - - - - - - - - - -
Mre11 - - - - - - - - - - - -
Rad50 - - - - - - - - - - - -
GSTpi - - - - - - - - - - - -
β-actin - - - - - - - - - - - -

b) MMS
pcDNA3.1 - - - + - - + - - - -
Flag-GSTpi - - + - - + - - + - -
NBS1 - - - - - - - - - - - -
GSTpi - - - - - - - - - - - -
β-actin - - - - - - - - - - - -

b) MMS
pcDNA3.1 - - - + - - + - - - -
Flag-GSTpi - - + - - + - - + - -
NBS1 - - - - - - - - - - - -
GSTpi - - - - - - - - - - - -
β-actin - - - - - - - - - - - -

d) MMS
pcDNA3.1 - - - + - - + - - - -
GSTpi-WT - - + - - + - - + - -
GSTpi-Y7F - - - - - - - - - - - -
p-ATM (Ser1981) - - - - - - - - - - - -
ATM - - - - - - - - - - - -
p-Chk2 (Thr68) - - - - - - - - - - - -
Chk2 - - - - - - - - - - - -
p-p53 (Ser15) - - - - - - - - - - - -
p53 - - - - - - - - - - - -
yH2AX - - - - - - - - - - - -
GSTpi - - - - - - - - - - - -
β-actin - - - - - - - - - - - -

e) MMS
pcDNA3.1 - - - + - - + - - - -
GSTpi-WT - - + - - + - - + - -
GSTpi-Y7F - - - - - - - - - - - -
NBS1 - - - - - - - - - - - -
GSTpi - - - - - - - - - - - -
β-actin - - - - - - - - - - - -

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GSTpi reduces DNA damage and cell death by regulating the ubiquitination and nuclear translocation of NBS1. We also transfected HEK-293 T-WT cells with pcDNA3.1 (2 μg/mL) or Flag-GSTpi (2 μg/mL) were treated with 0.5 mM MMS or not for 1 h, then cells were allowed to recover in normal medium for 6 h, and cell lysates were subjected to immunoblotting using the indicated antibodies. Data represent the mean ± SD of at least three independent experiments. Statistics were calculated by unpaired Student’s t test. *p < 0.05, **p < 0.01. c HEK-293 T-WT cells transfected with pcDNA3.1 (2 μg/mL), GSTpi-WT (2 μg/mL) or GSTpi-S184D (2 μg/mL) were treated with 0.5 mM MMS or not for 1 h, then cells were allowed to recover in normal medium for 6 h, then cell lysates were subjected to immunoblotting using the indicated antibodies. Data represent the mean ± SD of at least three independent experiments. Statistics were calculated by unpaired Student’s t test. **p < 0.01, n.s. not significant (p > 0.05)

NBS1 nuclear translocation. We also transfected HEK-293 T-GSTpi−/− cells with pcDNA3.1, GSTpi-WT, GSTpi-S184A or GSTpi-S184D plasmids, respectively, then stimulated cells with PMA instead of MMS. Interestingly, while GSTpi-WT and GSTpi-S184D significantly increased the NBS1 nuclear translocation, GSTpi-S184A failed to do so (Fig. 3a). These results highlight the critical role of phosphorylated Ser184 of GSTpi by cPKC in regulating the nuclear translocation of NBS1. To further clarify the relationship between GSTpi and NBS1, we examined the combination of GSTpi with NBS1 in the nuclear and cytoplasmic fractions by immunoblotting assay. As shown in Fig. 4g, GSTpi combined with NBS1 in the cytoplasmic without MMS, after MMS stimulation, the association was enhanced in the nucleus. Altogether, preceding results suggested that GSTpi interacted with NBS1 and Ser184 phosphorylation of GSTpi promoted itself and NBS1 nuclear translocation when DNA damage occurred.

**Phosphorylated GSTpi protects NBS1 from ubiquitination degradation by Skp2**

As aforementioned, in unstimulated cells, the amount of NBS1 in the cytoplasmic which transfected with GSTpi-S184A was lowest; after MMS stimulation, the amount of NBS1 in the nucleus which transfected with GSTpi-S184D was highest (Fig. 4d, e). We hypothesized that the phosphorylation and subsequent nuclear localization of GSTpi decreased the ubiquitination of NBS1 in the cytosol and enhanced the nuclear translocation of NBS1. Since Wu et al. have reported that Skp2 E3 ligase integrates ATM activation by ubiquitinating NBS1 [31], we next investigated whether GSTpi could affect the ubiquitination of NBS1 mediated by Skp2. Our results showed that the protein level of NBS1 in WT cells was lower than GSTpi−/− cells. Strikingly, the level of NBS1 was impaired by Myc-Skp2 transfection after MMS treatment in both GSTpi−/− and WT cells. WT cells showed a detectable increase of NBS1 compared to GSTpi−/− cells after MMS treatment, in contrast, WT cells showed a more obvious decrease in the protein level of NBS1 without MMS treatment (Fig. 5a). Furthermore, the GSTpi transfection induced NBS1 ubiquitination, and the proteasome inhibitor MG132 blocked NBS1 degradation in the unstimulated cells. In contrast, after MMS treatment, GSTpi inhibited the ubiquitination of NBS1 (Fig. 5b). Our data indicated that GSTpi performs different functions under different physiological conditions: without external stress, GSTpi played an important role in maintaining the low basal level of NBS1 to keep cellular homeostasis via enhancing the binding and ubiquitination of NBS1 by Skp2; while under genotoxic stress, GSTpi recovered NBS1 level to protect cells from various...
### a

| HEK-293T-WT | Cytoplasm | Nuclear |
|-------------|-----------|---------|
| MMS         | -         | +       |
| pcDNA3.1    | -         | +       |
| Flag-GSTpi  | -         | +       |
| NBS1        | -         | +       |
| GSTpi       | -         | +       |
| GAPDH       | -         | +       |
| Lamin B     | -         | +       |

### b

| HEK-293T-GSTpi | Cytoplasm | Nuclear |
|---------------|-----------|---------|
| MMS           | -         | +       |
| pcDNA3.1      | -         | +       |
| Flag-GSTpi    | -         | +       |
| NBS1          | -         | +       |
| Flag          | -         | +       |
| GSTpi         | -         | +       |
| GAPDH         | -         | +       |
| Lamin B       | -         | +       |

### c

| HEK-293T-GSTpi | Cytoplasm | Nuclear |
|---------------|-----------|---------|
| MMS           | -         | +       |
| pcDNA3.1      | -         | +       |
| Flag-GSTpi    | -         | +       |
| DAPI          | -         | +       |
| GSTpi         | -         | +       |
| NBS1          | -         | +       |
| Merge         | -         | +       |

### d

| HEK-293T-WT | MMS | Cytoplasm | Nuclear |
|-------------|-----|-----------|---------|
| pcDNA3.1    |     | -         | +       |
| GSTpi/S184A |     | -         | +       |
| GSTpi/S194A |     | -         | +       |
| GSTpi/184A  |     | -         | +       |
| GSTpi/S194A |     | -         | +       |

| Cytoplasm   | Nuclear |
|-------------|---------|
| NBS1        | Flag    |
| GapDH       | NBS1    |
| Lamin B     | Flag    |
GSTpi reduces DNA damage and cell death by regulating the ubiquitination and nuclear...
**Fig. 4 (continued)**
nuclear translocation and autophagy. To test our hypothesis, we treated ADR-resistant MCF-7/ADR cells which had a high level of GSTpi compared with ADR-sensitive MCF-7 cells with Gö6983 or/and autophagy inhibitor chloroquine (CQ) to examine cell sensitivity to the chemotherapy drug ADR. As shown in Fig. 6c, ADR-induced apoptosis in MCF-7/ADR cells was more obvious by incubated with Gö6983 or CQ, especially, when MCF-7/ADR cells were treated with Gö6983, CQ, and ADR, the apoptosis increased dramatically. Both optical inverted microscope observation and flow cytometry analysis showed the same results, a combination of Gö6983 and CQ performed a stronger effect on MCF-7/ADR apoptosis (Fig. 6d, e). We further examined NBS1 nuclear translocation, data showed that only Gö6983 treatment could inhibit NBS1 nuclear translocation which indicated that GSTpi promoted breast cancer cells drug-resistance through two individual pathways (Fig. 6f). Taken together, these results demonstrated that targeting GSTpi may induce breast cancer cells apoptosis.

Discussion

Since the living organisms continuously suffer from many stresses such as reactive oxygen species produced by energy reaction, ionizing radiation, ultraviolet radiation, toxic chemical agents, and other environmental factors during normal activities [34], the mechanism that protects cells from various stress remains incompletely understood. These damages may impact nucleic acids, lipids, and proteins and lead to mutations or genome aberrations that result in genetic diseases, developmental defects, cancer, and even death [35]. To handle these serious damages, cells had to develop an intricate network signaling pathway to deal with various stress [36]. Maintaining genomic stability is also especially important for the tumor cells. Losing of one or more DDR pathways has been proved to be part of the stage of cancer development. Whereas, once defects in DDR may lead to more dependence on the remaining pathways [37]. As an important phase II detoxifying enzyme, GSTpi was detected with high expression in many tumors and cancer cell lines [5]. Nevertheless, the high expression level of GSTpi was found to be related to chemoresistance, and studies on this mechanism mainly focused on the detoxification and antioxidant functions of GSTpi [38, 39]. However, most anticancer drugs are not substrates of GSTpi, the reasons for chemoresistance remain unclear. Recently, Dong et al. have reported that GSTpi enhanced the resistance of breast cancer to ADR through autophagy [10], which is independent of its enzyme activity. Consistently, the previous mechanisms involving the non-transferase activity of GSTpi that participates in DNA damage remain unclear [40]. The functional repertoire of GSTpi has broadened significantly beyond its original transferase activity in many biological processes. Our investigation into how GSTpi protects cells from DNA damage provides a physiologically relevant context to understand the reason of the high expression of GSTpi in breast cancer cells.

Based on some previous studies on protecting cells upon the UVC irradiation or MMS treatment [41, 42], we initiated our research to investigate the influence of GSTpi on MMS triggered DNA damage. Our finding revealed that the level of γH2AX was decreased by GSTpi after MMS stimulation. Moreover, both immunofluorescence assay and single-cell gel electrophoresis demonstrated that GSTpi has a vital effect on DDR. Earlier studies have reported that the nuclear GSTpi protects DNA against damage [11, 19]. Recently, Zhou et al. found that GSTpi enters the nuclear and interacts with HMGB1 in response to LPS [24]. Consequently, we conjectured that the capacity of GSTpi to improve DNA damage repair may contribute to its nucleus translocation. Interestingly, our results showed that GSTpi entered the nucleus when DNA damage occurred. This discovery enriches the function of the nuclear GSTpi, which breaks the limitation of previous research on the cytosolic GSTpi [43, 44].

When DNA damage occurred, some specific sensor proteins including ATM, ATR, and DNA-PKcs recruit to damage sites and initiate a DDR [45, 46]. ATM and ATR have similar sequences to lipid kinases of the PI3K family [47]. They can selectively phosphorylate and activate the serine-threonine checkpoint effector kinases which are called Chk2 and Chk1 [48]. Our results demonstrated that GSTpi activated the ATM-Chk2 pathway against MMS-induced DNA damage. Some known substrates of Chk2 including p53, MDMX, Cdc25, and BRCA1 [48–51].
### a. HEK-293T-GSTpi-/- vs HEK-293T-WT

|        | HEK-293T-GSTpi-/- | HEK-293T-WT |
|--------|-------------------|-------------|
| MMS    | -                 | +           |
| pcDNA3.1| +                 | -           |
| Myc-Skp2| -                 | -           |
| NBS1   |                   |             |
| GSTpi  |                   |             |
| Skp2   |                   |             |
| β-actin|                   |             |

### b. HEK-293T-WT vs DMSO vs MG-132

|        | DMSO     | MG-132 |
|--------|----------|--------|
| NBS1   | IB:Ub    | NBS1   |
| GSTpi  | IB:NBS1  | GSTpi  |
| β-actin|_input   | β-actin|

### c. HEK-293T-WT

|        | Cytoplasm | Nuclear |
|--------|-----------|---------|
| MMS    | -         | +       |
| pcDNA3.1| +         | -       |
| Flag-GSTpi| -         | +       |
| IB:NBS1|            |         |
| IB:Skp2|            |         |
| IB:GSTpi|           |         |
| NBS1   |            |         |
| Skp2   |            |         |
| GSTpi  |            |         |
| GAPDH  |            |         |
| Lamin B|            |         |

### d. HEK-293T-WT

|        | Cytoplasm | Nuclear |
|--------|-----------|---------|
| MMS    | -         | +       |
| pcDNA3.1| +         | -       |
| Flag-GSTpi| -         | +       |
| IB:NBS1|            |         |
| IB:Skp2|            |         |
| IB:Flag|            |         |
| NBS1   |            |         |
| Skp2   |            |         |
| Flag   |            |         |
| GAPDH  |            |         |
| Lamin B|            |         |

### e. HEK-293T-WT

|        | Cytoplasm | Nuclear |
|--------|-----------|---------|
| MMS    | -         | +       |
| GSTpi-WT| +         | -       |
| GSTpi-S184A| -     | +       |
| GSTpi-S184D| -      | -       |
| IB:NBS1|            |         |
| IB:Skp2|            |         |
| IB:GSTpi|           |         |
| NBS1   |            |         |
| Skp2   |            |         |
| GSTpi  |            |         |
| GAPDH  |            |         |
| Lamin B|            |         |

### f. HEK-293T-WT

|        | Cytoplasm | Nuclear |
|--------|-----------|---------|
| MMS    | -         | +       |
| GSTpi-WT| +         | -       |
| GSTpi-S184A| -     | +       |
| GSTpi-S184D| -      | -       |
| IB:NBS1|            |         |
| IB:Skp2|            |         |
| IB:Flag|            |         |
| NBS1   |            |         |
| Skp2   |            |         |
| Flag   |            |         |
| GAPDH  |            |         |
| Lamin B|            |         |
GSTpi reduces DNA damage and cell death by regulating the ubiquitination and nuclear...

Phosphorylated GSTpi regulates the ubiquitination of NBS1 mediated by Skp2. a HEK-293 T-WT and HEK-293 T-GSTpi−/− cells transfected with pcDNA3.1 (2 μg/mL) or pcDNA3.1 (2 μg/mL) were treated with 0.5 mM MMS or not for 1 h, then cells were allowed to recover in normal medium for 6 h, and cell lysates were subjected to immunoblotting using the indicated antibodies. Data shown are representative of three independent experiments. b HEK-293 T-WT cells transfected with pcDNA3.1 (2 μg/mL) or Flag-GSTpi (2 μg/mL) were treated with 0.5 mM MMS or not for 1 h, then cells were treated with MG-132 (30 μM) for another 6 h. The cell lysates were immunoprecipitated with anti-NBS1 antibody followed by immunoblotting with anti-ubiquitin (Ub) and anti-NBS1 antibodies. Data shown are representative of three independent experiments. c HEK-293 T-WT cells transfected with pcDNA3.1 (2 μg/mL) or Flag-GSTpi (2 μg/mL) were treated with 0.5 mM MMS or not for 1 h, the nuclear and cytoplasmic fractions were subjected to immunoprecipitation with anti-GSTpi antibody and immunoblotted with anti-NBS1, anti-Skp2 antibody or anti-GSTpi. Data shown are representative of three independent experiments. d HEK-293 T-WT cells transfected with pcDNA3.1 (2 μg/mL) or Flag-GSTpi (2 μg/mL) were treated with 0.5 mM MMS or not for 1 h, the nuclear and cytoplasmic fractions were subjected to immunoprecipitation with anti-NBS1 antibody and immunoblotted with anti-Skp2, anti-NBS1 antibody or anti-Flag. Data shown are representative of three independent experiments. e HEK-293 T-WT cells transfected with pcDNA3.1 (2 μg/mL) or GSTpi-WT (2 μg/mL), GSTpi-S184A (2 μg/mL) or GSTpi-S184D (2 μg/mL), respectively, were treated with 0.5 mM MMS or not for 1 h, the nuclear and cytoplasmic fractions were subjected to immunoprecipitation with anti-GSTpi antibody and immunoblotted with anti-NBS1, anti-Skp2 antibody or anti-GSTpi. Data shown are representative of three independent experiments. f HEK-293 T-WT cells transfected with pcDNA3.1 (2 μg/mL), GSTpi-WT (2 μg/mL), GSTpi-S184A (2 μg/mL) or GSTpi-S184D (2 μg/mL), respectively, were treated with 0.5 mM MMS or not for 1 h, the nuclear and cytoplasmic fractions were subjected to immunoprecipitation with anti-NBS1 antibody and immunoblotted with anti-Skp2, anti-NBS1 antibody or anti-Flag. Data shown are representative of three independent experiments.

Importantly, we found overexpression of GSTpi enhanced the phosphorylated p53, it can also induce the cell cycle arrest in the G2/M phase and gain more time for cells to proceed DNA damage repair.

It has been reported that the MRN complex can not only sense the damage sites but also activate ATM [28, 52]. Particularly, the role of NBS1 in the recruitment of ATM to DNA damage sites is of vital importance [53], so we next explored the relationship between NBS1 and GSTpi. Interestingly, we found for the first time that phosphorylated GSTpi can promote itself and NBS1 into nuclear during DNA damage. What’s more, the apoptotic rate of breast cancer cells was significantly increased when ADR was combined with GSTpi phosphorylated inhibitor and autophagy inhibitor in comparison to treatment with ADR alone; however, NBS1 nuclear translocation could only inhibit by GSTpi phosphorylated inhibitor. These findings displayed a new function of phosphorylated GSTpi that protects breast cancer cells against anti-tumor drugs and resulted in the drug resistance through two individual pathways including autophagy and NBS1 nuclear translocation.

Protein post-translational modification (PTM) is closely related to the activity, stability, localization, interactions, or folding of proteins [54], implying that one or more PTMs to NBS1 may cause its change in protein expression and its translocation to the nucleus. Then we focused on the post-translational modification to NBS1 mediated by GSTpi. It seemed that the increased ubiquitination degradation of NBS1 was induced by cytosolic GSTpi without MMS treatment. On the contrary, phosphorylated GSTpi inhibited the ubiquitination degradation of NBS1 when upon MMS. We further investigated the combination among GSTpi, NBS1, and the ubiquitin-protein ligase (E3 ligase). Our finding showed that cytosolic GSTpi enhanced the interaction of NBS1 and Skp2 in unstimulated cells, conversely, when DNA damage occurred, phosphorylated GSTpi entered the nucleus and make the cytosolic interaction weakened, which indicated that GSTpi can protect NBS1 from ubiquitination degradation mediated by Skp2 when DNA damage occurred. Our research first demonstrated a novel role for GSTpi in regulating the connection of NBS1 and Skp2. However, where the specific binding site is located between GSTpi and NBS1, remains to be elucidated experimentally. Based on the above results, we provided a novel mechanism through which the post-translational modification of NBS1 plays a critical role in sensing DNA damage sites. We also speculated Skp2 may trigger K48-linked ubiquitination of NBS1 and the process was regulated by GSTpi. However, given the capacity of multiple PTMs to regulate NBS1, we cannot completely conclude that the K48-linked...
Fig. 6 GSTpi protects breast cancer cells from anticancer drug-induced apoptosis. a, b MCF-7 cells transfected with pcDNA3.1 (2 μg/mL) or Flag-GSTpi (2 μg/mL) were treated with 1 μM ADR or not for 24 h and cell lysates were subjected to immunoblotting using the indicated antibodies. Data shown are representative of three independent experiments. c MCF-7/ADR cells were pretreated with Gö6983 or CQ for 2 h, then stimulated with 10 μM ADR or not for 24 h, cell lysates were subjected to immunoblotting using the indicated antibodies. Data shown are representative of three independent experiments. d MCF-7/ADR were treated the same as (e), and then use optical inverted microscope to observe cells morphology. Scale bar, 100 μm. Data shown are representative of three independent experiments. e MCF-7/ADR were treated the same as (e), then use optical inverted microscope to observe cells morphology. Scale bar, 100 μm. Data shown are representative of three independent experiments. f MCF-7/ADR were treated the same as (e), western blot analysis was performed with indicated antibody. Data represent the mean ± SD of at least three independent experiments. Statistics were calculated by unpaired Student’s t test. *p < 0.05, **p < 0.01. 

In summary, our present study demonstrated that when DNA damage occurred, GSTpi can be phosphorylated at Ser184, and the phosphorylated GSTpi inhibited the ubiquitination degradation of NBS1 mediated by Skp2 in the cytoplasm, then combined with NBS1 to trigger the G2/M checkpoint arrest through activating the ATM-Chk2-p53 signaling pathway (Fig. 7a). Based on our findings, we explained a novel mechanism of GSTpi protecting cells against DNA damage. This new molecular insight into the new function of GSTpi will offer us a novel strategy for solving the problem of breast cancer drug resistance.

Fig. 7 Schematic representation of GSTpi protecting cells from anticancer drug-induced DNA damage through regulating the ubiquitination of NBS1
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Author contributions Conceptualization: ZY, LL, PC, and JZ; methodology: ZY, LL, PC, and JZ; formal analysis: ZY, LL, PC, and JZ; investigation: JZ, LG, YS, and TH; validation: XF, XB, SL, and JL; writing—original draft preparation: ZY and JZ; writing—review and editing: ZY; funding acquisition: ZY, LL, and LG; resources: SL and LG; visualization: LL and PC; supervision: ZY, LL, and PC.

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Declarations

Conflict of interest The authors declare no competing financial interests.

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