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Original Article

Overexpression of BRCA1 attenuates the sensitivity of PC9 cells to gefitinib

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Abstract: Gefitinib is an orally active antitumor agent which inhibits uncontrolled cell proliferation by interrupting epidermal growth factor receptor (EGFR) signaling pathways. Various in vitro and in vivo studies have revealed that the upregulated expression of breast cancer susceptibility gene 1 (BRCA1) is associated with chemoresistance and reduced survival following chemotherapies. In this study, a gefitinib-highly-sensitive cell line, PC-9, was used to investigate the effect of BRCA1 expression on the sensitivity of PC-9 cells to gefitinib. PC-9 cells were stably transfected with BRCA-1 (HA-tagged). Transfected and untransfected PC-9 cells were treated with gefitinib, phosphorylated γH2AX was examined by western blot to determine the DNA damages. Following the treatment of gefitinib, the inhibition of proliferation of the PC-9 cells, PC-9-pcDNA3.1 cells, and BRCA1-transfected PC-9 cells were determined. Also, a comet assay was performed to determine the DNA damage caused by gefitinib. The treatment of geftinib for 6 hr, 12 h, and 24 hr significantly increased the cellular expression of phosphorylated γH2AX. With the treatment of gefitinib, the inhibition of proliferation of BRCA-1 overexpressed PC-9 cells was significantly lower than that of the non-transfected PC-9 cells, indicating the overexpression of BRCA1 plays a role in attenuating the sensitivity of PC-9 cells to gefitinib. The comet assay revealed that BRCA1 transfected cells showed a shorter comet tail, indicating the overexpression of BRCA1 attenuated the DNA damages caused by gefitinib. The overexpression of BRCA1 reduced the DNA damages, and enhanced DNA repair mechanisms. Also, gefitinib-mediated inhibition of cell proliferation is attenuated by the expression of BRCA1.

Keywords: BRCA1, gefitinib, cell proliferation

Introduction

Breast cancer early onset gene 1 (*BRCA1*) was revealed to be the genetic basis for the development of hereditary breast and ovarian cancers in early 1990s [1]. The *BRCA1* gene encodes the breast cancer type 1 susceptibility protein (BRCA1), which contains three domains, the RING domain at the N terminus, a central part with a coiled-coil domain, and tandem BRCA1 carboxyl terminal repeats (BRCT) at the C terminus [2]. BRCA1 is one of the essential cellular proteins that contributes to the DNA repair mechanism by mediating homologous recombination [3]. The molecular mechanism of DNA repair involves the resection of double-strand breaks at 5' and 3' ends by BRCA1, and loading of the RAD51 recombinases onto the damage sites to initiate DNA repair [4]. Recent studies discovered that BRCA1 interacts with other proteins to form complexes which then translocate to the DNA damage sites and repair the damaged DNAs [5-7].

Besides the contribution of BRCA1 to maintain genomic integrity, histone protein H2AX is also an essential component in DNA repair. H2AX is a member of the histone H2A family, and can get a rapid serine-phosphorylation to form γH2AX at the damage sites [8]. A double strand break can lead to accumulation of γH2AX, which recruits cellular proteins involved in DNA repair.

Gefitinib is an orally active anticancer drug, which acts as a tyrosine kinase inhibitor (TKI), and is widely used for patients with non-small cell lung cancer. Gefitinib has been reported to interfere with cancer metastasis by targeting the epidermal growth factor receptor (EGFR)

tyrosine kinase [9], although the EGFR mutations have been suggested to restrict the effectiveness of gefitinib [10]. Despite of the EGFR mutations, other genetic mutations have been discussed to influence the sensitivity of cancerous cells against EGFR TKIs. High expression of BRCA1 detected by qRT-PCR indicated its role as a prognostic biomarker in resected NSCLC [11]. Therefore, in this study, we utilized the gefitinib-highly sensitive PC-9 cell line, and conducted PC-9-BRCA1 cells to evaluate the effect of the overexpression of BRCA1 on the sensitivity of the PC-9 cells against gefitinib treatment.

Materials and methods

Cell culture

Human lung cancer PC-9 (adenocarcinoma) cells were obtained from MeiXuan Biological Science and Technology Co., Ltd. (Shanghai, China). Cells were grown with DMEM (Gibco™, USA) supplemented with 10% Fetal Bovine Serum (Gibco™, USA), 1% penicillin/streptomycin (Gibco™, USA), and 1% HEPES buffer (Gibco™, USA).

Transfection

PC-9 cells were cultured in a 6-well tray in the DMEM supplemented with 10% FBS and 1% HEPES buffer but without penicillin/streptomycin, and transfected with 10 μl of Lipofectamine® 2000 reagent (Invitrogen, USA) and 2.5 μg of pcDNA3.1-BRCA1 (HA-tagged), or pcDNA3.1 as a control for 24 hours at 37°C with 5% CO_2. After the transfection, PC-9 cells were cultured with DMEM with 10% FBS, 1% penicillin/streptomycin, and 1% HEPES buffer.

Cytotoxicity assay

Non-transfected PC-9 cells were cultured in a 6-well tray for 24 hours (10^6 cell/well), and treated with 5 μmol/L of gefitinib, or DMSO for the control groups. PC-9 cells from both groups were exposed to the drugs for 6hours, 12 hours and 24 hours. Transfected PC-9-BRCA1 and PC-9-pcDNA3.1 cells were also cultured in a 6-well tray for 24 hours (10^6 cell/well), and treated with 5 μmol/L of gefitinib, or DMSO. PC-9-BRCA1 and PC-9-pcDNA3.1 cells were exposed to the drugs for 24 hours, 48 hours, and 72 hours. Cell growth inhibition was indicated as the percentage of the absorbance of cell cultures measured at 630 nm with the Multiskan reader (Multiskan MK3, Thermo, USA).

Western blotting

Transfected and non-transfected PC-9 cells were collected and lysed in the cell lysis buffer (Cell Signaling Technology, Inc), and sonicated for three cycles of 30 seconds on, and 30 seconds off. Followed by cell lysis, protein assays were performed, and 35 μg of total protein from each sample were loaded for SDS-PAGE. The transferred membranes were blocked with 5% skim milk overnight at 4°C, and incubated with primary antibodies for 2 hours. Followed by three PBS washes, the membranes were incubated with secondary antibodies for 2 hours. All the primary antibodies, including rabbit polyclonal anti-HA antibody, anti-actin antibody, anti-p-γH2AX antibody, and anti-pRAD51 antibody, as well as goat-anti-rabbit secondary antibodies (HRP-conjugated) were purchased from Abcam®. The membranes were then reacted with ECL reagents (Sigma-Aldrich, USA) for western blotting detection.

Single cell gel electrophoresis assay (SCGE)

A SCGE kit was purchased from Trevigen®, and the assay was performed as per the protocol provided by the manufacturer. 10^5 cells were combined with molten LMAgarose at 1:10 (v/v), and 50 μl of the mixture was pipetted onto CometSlide™. The slides were placed flat at 4°C in the dark for 30 min for complete gelling and improved adhesion of the samples, followed by an overnight incubation in the Lysis Solution at 4°C. The slides were then removed from the Lysis Solution, and immersed into the Neutral Electrophoresis Buffer for 30 min. The slides and 850 ml of the Neutral Electrophoresis Buffer were added into the electrophoresis tray, and run at 21 volts, 4°C for 45 min. Following the electrophoresis, DNAs were precipitated with the DNA Precipitation Solution, and 70% ethanol. The samples were dyed with SYBR® Gold, and dried for visualization at 496 nm.

Results

Gefitinib induced DNA damage of non-transfected PC-9 cells

As an orally active anticancer drug, gefitinib targets EGFR inside the cells to inhibit cell prolif-

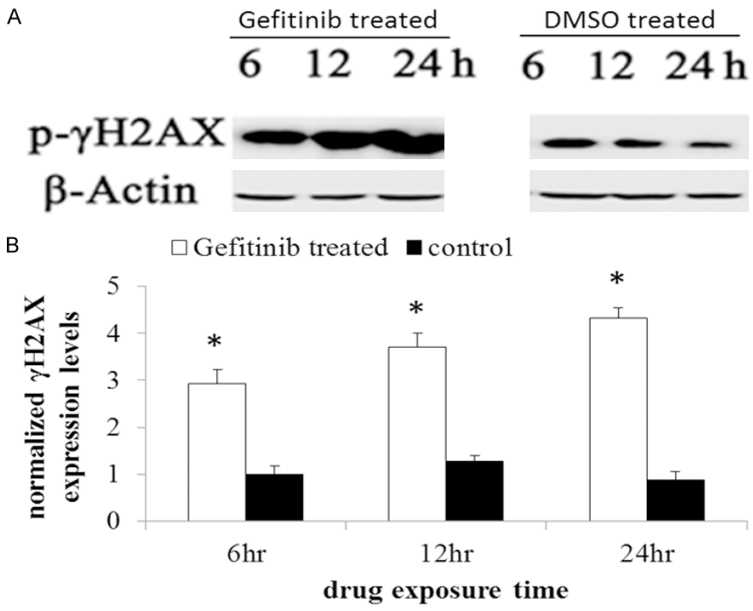

Figure 1. The expression of pγH2AX in PC-9 cells in response to gefitinib or control treatment. A. Immunoblotting detected increased expression of pγH2AX in response to gefitinib treatment in PC-9 cells. B. Gefitinib treated PC-9 cells had a significantly higher expression level of pγH2AX than the control groups. The expression level of pγH2AX increased in an exposure time-dependent manner. *Gefitinib treatment vs. control, $P<0.05$.

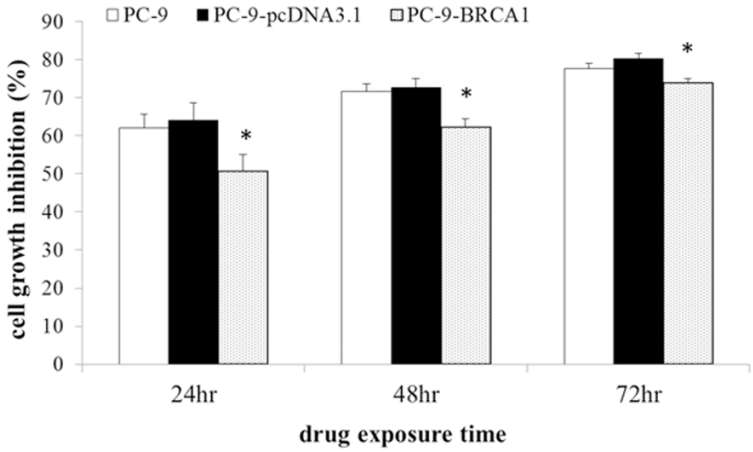

Figure 2. PC-9, PC-9-pcDNA3.1, and PC-9-BRCA1 cell growth inhibition induced by gefitinib at various exposure time. Treatment with gefitinib resulted in cell growth inhibition, which was significantly attenuated by the overexpression of BRCA1. *PC-9-BRCA1 vs. PC-9 cells, $P<0.05$.

eration. To evaluate the effect of gefitinib on DNA damages, non-transfected PC-9 cells were exposed to gefitinib for 6 hours, 12 hours, or 24 hours, followed by western blotting detection of phosphorylated γH2AX (pγH2AX), which is an indication of DNA damage. Compared with the DMSO-treated control groups, significantly higher expression of pγH2AX were detected (**Figure 1**) in response to gefitinib. Also, the detected cellular level of pγH2AX increased in an exposure time-dependent manner. These results confirm that gefitinib treatment can induce DNA damages.

Effect of overexpression of BRCA1 on gefitinib-induced cell growth inhibition

Besides the induction of DNA damage, gefitinib also inhibits cell growth, as shown in **Figure 2**. To evaluate the effect of BRCA1 on gefitinib-induced cell growth inhibition, PC-9 cells, and transfected PC-9-pcDNA3.1 cells, and PC-9-BRCA1 cells were exposed to gefitinib for 24 hours, 48 hours, or 72 hours. Normalized to DMSO-treated PC-9 cells, gefitinib inhibited cell growth for all three cell lines. As the drug exposure time prolonged, PC-9 cells and PC-9-pcDNA3.1 cells exhibited similar and the highest cell growth inhibition, whereas PC-9-BRCA1 had significantly lower cell growth inhibition, suggesting the overexpression of BRCA1 attenuates the induction of cell growth inhibition by gefitinib.

Effect of overexpression of BRCA1 on gefitinib-induced DNA damage

The results above show a reduction in cell growth inhibition by the overexpression of BRCA1, following the gefitinib treatment. To investigate whether this is due to BRCA1-induced reduction in DNA damage, single-cell gel electrophoresis (comet) assays were performed to examine the DNA damage. PC-9, PC-9-pcDNA3.1 and PC-9-

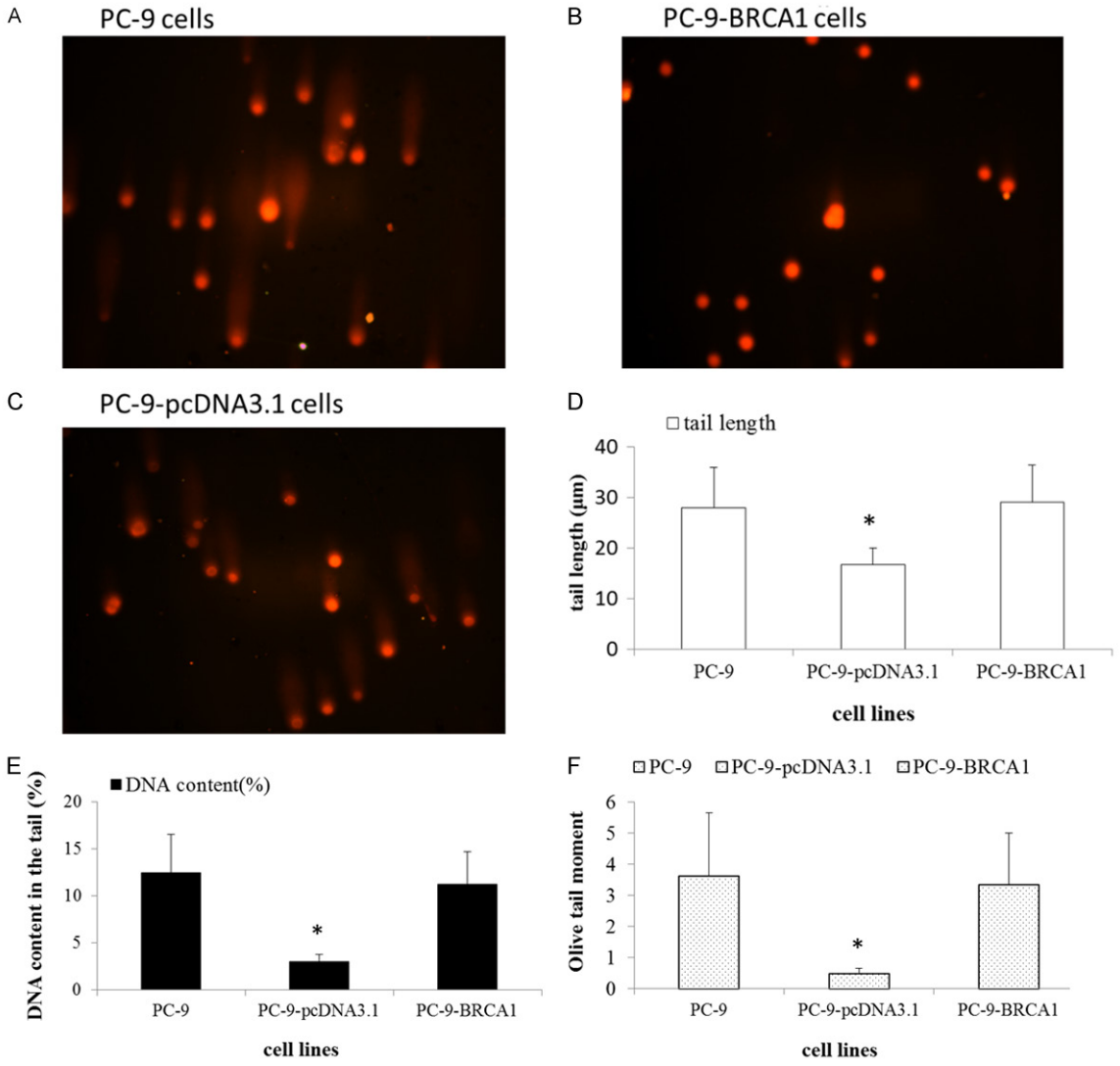

Figure 3. Gefitinib-induced DNA breaks examined by single-cell gel electrophoresis (comet) assays. (A-C) show the gefitinib-induced DNA breaks in PC-9, PC-9-BRCA1, and PC-9-pcDNA3.1 cells, respectively. With BRCA1 overexpression, the cells exhibited the shortest tail length (D), the lowest DNA content in the tail (E), and the shortest Olive tail moment (F). *PC-9-BRCA1 vs. PC-9 cells, $P<0.05$.

BRCA1 cells were treated with gefitinib for 48 hours, and were examined by the comet assay. **Figure 3** shows that gefitinib induced similar levels of DNA damage for the PC-9 and PC-9-pcDNA3.1 cells, whereas the overexpression of BRCA1 significantly reduced the numbers of DNA strand breaks induced by gefitinib.

BRCA1 promotes DNA repair and reduces sensitivity of PC-9 cells against gefitinib

To study the effect of BRCA1 on the sensitivity of PC-9 cells against gefitinib, western blotting was performed to examine the expression of pγH2AX, which indicates DNA breaks, and pRAD51, which indicates DNA repair. Compared with the treatment with DMSO, gefitinib induces increased expression of pγH2AX for PC-9, PC-9-pcDNA3.1, and PC-9-BRCA1 cells ($P>0.05$). In addition, all three cell lines exhibit the increased expression of pγH2AX in an exposure time-dependent manner. In contrast, the expression of pRAD51 was increased in response to the gefitinib treatment, however, the overexpression of BRCA1 results in the reduction of pRAD51 when the gefitinib exposure prolonged ($P<0.05$). Non-reduced pRAD51 expression in PC-9 and PC-9-pcDNA3.1 cells

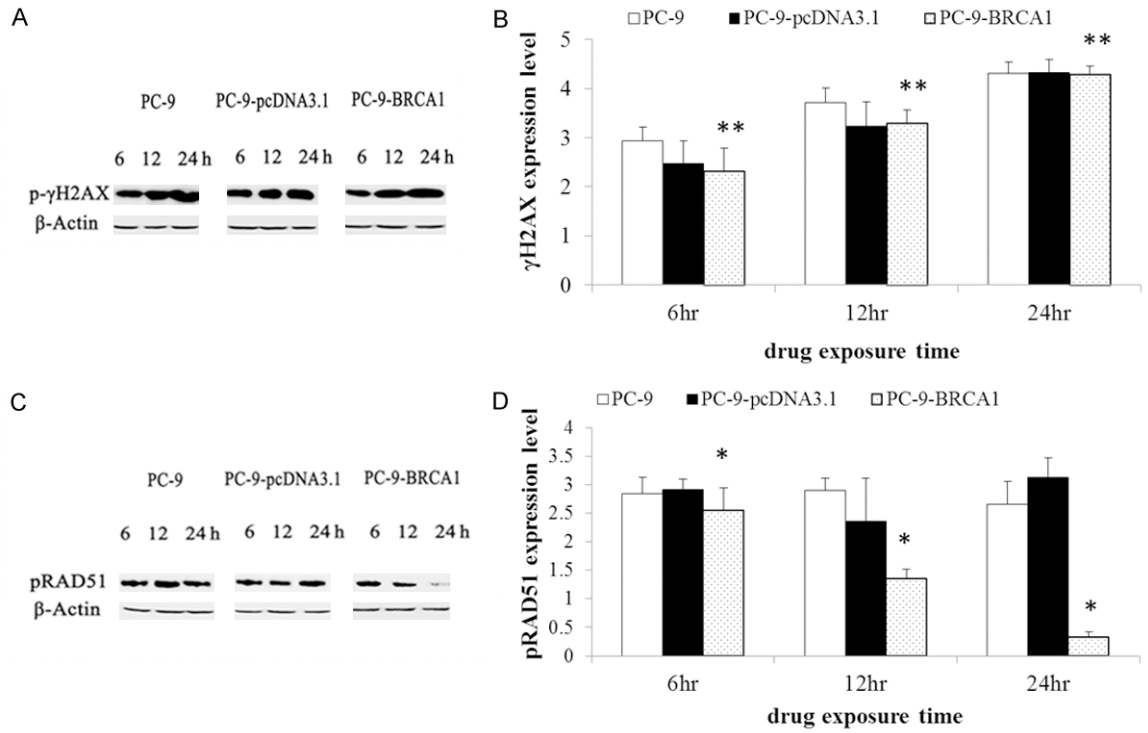

Figure 4. The expression of pγH2AX and pRAD51 in PC-9, PC-9-pcDNA3.1, and PC-9-BRCA1 cells treated with gefitinib at various exposure times. A. Immunoblotting detected increased expression of pγH2AX in all three cell lines, and the expression level increased in a drug exposure time-dependent manner. B. The expression level of pγH2AX in the PC-9-BRCA1 cells were compared with that in the PC-9 cells, however, no significant difference was detected. $^{**}P>0.05$. C. Immunoblotting detected increased expression of pRAD51 in PC-9 and PC-9-pcDNA3.1 cells, whereas the increased expression of pRAD51 was significantly attenuated by the overexpression of BRCA1. $^{*}P<0.05$. D. QPCR detected relative expression level of pRAD51 in PC-9 and PC-9-pcDNA3.1 cells in different exposure time.

indicates the DNA damages and triggers of DNA repair. However, the DNA repair mechanism was incomplete without the overexpression of BRCA1. Therefore, the expression of BRCA1 promotes the DNA repair mechanism, and reduces the sensitivity of PC-9 cells against gefitinib (**Figure 4**).

Discussion

Lung cancer was reported as the leading cause of cancer-related deaths, with 1.59 million deaths in 2012. As a type of lung cancer, NSCLC accounts for over 80% of all lung cancer cases, and exhibits poor prognosis and worse survival [12]. Activation and overexpression of EGFR has been detected in various cancer types, and suggested to facilitate tumorigenesis, cancer progression and metastasis [13]. Therefore, EGFR is one of the important targets for cancer treatments, including the small molecule TKI gefitinib, which blocks the activation of EGFR. Studies showed that treatment with gefitinib reduced the levels of pEGFR, pAKT, and pERK [14, 15], which involve in DNA synthesis and DNA repair. The DNA repair mechanism mediated by EGFR is shown to require the association of catalytic subunits of DNA-protein kinases (DNA-PKcs), which form complexes with EGFRs, modulating non-homologous end joining to repair the DNA strand breaks [16]. Treatment with gefitinib can inhibit the activation of EGFR, and blocks the interaction between DNA-PKcs and EGFRs.

The presence of pγH2AX indicates DNA damages, including double-strand breaks and single-strand lesions. The DNA damages could be caused by exogenous factors, such as irradiation, or endogenous factors, such as oncogenetic mutations, and normal DNA replications [17]. Studies on A549 and H1299 cells suggested anti-cancer agent gefitinib treatment interfered with DNA repair mechanisms, and therefore, prolonged the presence of DSBs, which induced the accumulation of pγH2AX

[18]. Our study also confirmed increased cellular level of pγH2AX in response to gefitinib in the PC-9 cells. Although the PC-9 cells in our study were not exposed to radiation for generating DNA damages, the major source of DNA damage in our model is the normal DNA replication. The accumulated pγH2AX is essential for recruiting various complexes involved in DNA repair, including BRCA1.

As an important mechanism for DNA repair, homologous recombination (HR) requires BRCA1 to regulate transcription, and activate cell-cycle checkpoints, and thus, repair the DNA damages [3]. The essential role of BRCA1 in DNA repair involves interactions between BRCA1 and various cellular proteins, such as BARD1, PALB, and RAD51 [19-21]. In our study, we detected an increased cellular level of pRAD51 in response to the DNA lesions caused by gefitinib. The colocalization of BRCA1 and RAD51 was detected in nuclear foci by Scully et al [22]. Instead of direct interactions between BRCA1 and RAD51, BRCA1 recruits BRCA2, which facilitates the loading of RAD51 onto the DNA damage sites [23]. In this complex, BRCA1 plays a role in recognizing DSB sites, as the knockdown of BRCA1 was shown to reduce the formation of RAD51 foci on the DNA strands. An important domain of this complex is the coiled-coil domain of BRCA1. Missense mutations of the coiled-coil domain have been shown to disrupt the interactions between BRCA1 and PALB2, reducing the HR efficacy [24, 25]. After the recognition of the DNA damage sites by BRCA1, the recruited RAD51 is the protein facilitating DNA repair directly. Depletion of RAD51C in Hela cells revealed a lower nuclear RAD51 level, indicating that, besides the involvement of BRCA2, RAD51C is another important protein which facilitates the entry of RAD51 into the nucleus [26]. Therefore, in this study, the overexpression of BRCA1 significantly increased pRAD51 with an early response to gefitinib. However, the cellular level of pRAD51 started reducing after 12 hours gefitinib treatment in the BRCA1 overexpressed PC-9 cells. This might be due to reduced activities of those proteins facilitating the entry of RAD51 into the nucleus, resulting in reduced DNA repair. This also explains the increased pγH2AX after 12 hour gefitinib treatment, as well as increased cell growth inhibition after 24 hour gefitinib treatment in the BRCA1 overexpressed PC-9 cells. Despite mediating the activity of RAD51 for DNA strand invasion, BRCA1 can also interact with other proteins, including BACH1 for the S-phase checkpoint, and Rap80 for ubiquitinated chromatin targeting, which also plays a role in regulating cell proliferation [4].

The role of BRCA1 in DNA repair as well as cell-cycle regulation has been studied in various cancer types. In the cell lines from breast and ovarian cancers, knockdown of BRCA1 facilitated the growth of malignant cells, whereas the proliferation of tumor cells was inhibited by the introduction of BRCA1 into the cells [27]. These results indicated the growth-inhibitory activity of BRCA1 in the breast and ovarian cancers. However, BRCA1 exhibits an opposite effect in NSCLC. In our study, the overexpression of BRCA1 attenuates the cell-growth inhibition induced by gefitinib, although the cell-growth inhibition increased as gefitinib exposure prolonged.

It is reported that 44% of NSCLC showed reduced BRCA1 mRNA and BRCA1 protein expression. In addition, a study on the BRCA1-deficient subgroup of NSCLC revealed an effective treatment with olaparib, which inhibits the progression of NSCLC by targeting Poly (ADP-ribose) polymerase (PARP). A phase IB study of olaparib noted that EGFR-mutated patients, who had a high expression level of BRCA1, showed a shorter progression-free survival when treated with TKIs, such as gefitinib [28]. This indicated the overexpressed BRCA1 reduced the effectiveness of TKI treatments, which is consistent with our findings. Our study revealed that the overexpression of BRCA1 significantly reduced DNA damage, and attenuated the gefitinib-induced cell growth inhibition.

The effect of gefitinib on cell growth inhibition and DNA damages were evaluated in the PC-9 cell lines. The DNA damages, as well as the cell growth inhibition, induced by gefitinib treatment were significantly attenuated by the overexpression of BRCA1, although the attenuation could not prolong over 24 hours. Our findings revealed the significant role of BRCA1 in the survival of NSCLC cells in response to the anticancer TKI drugs. Therefore, we believe that BRCA1 can be treated as a target for drug development.

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Disclosure of conflict of interest

None.

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