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BIBR1532 inhibits proliferation and metastasis of esophageal squamous cancer cells by inducing telomere dysregulation

Wang Q *et al*. BIBR1532 induces telomere dysregulation in ESCC

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Abstract

BACKGROUND

Esophageal squamous cell carcinoma (ESCC) is a malignant tumor with high morbidity and mortality, and easy to develop resistance to chemotherapeutic agents. Telomeres are DNA-protein complexes located at the termini of chromosomes in eukaryotic cells, which are unreplaceable in maintaining the stability and integrity of genome. Telomerase, an RNA-dependent DNA polymerase, play vital role in telomere length maintain, targeting telomerase is a promising therapeutic strategy for cancer.

AIM

To investigate the efficacy and underlying mechanisms of BIBR1532, a telomerase inhibitor, in ESCC.

METHODS

KYSE150 and KYSE410 cells were exposed to various concentrations of BIBR1532. Cell viability was assessed at 48 hours and 72 hours to determine the IC_{50} values. The effects of BIBR1532 on ESCC cell proliferation, migration, cell cycle progression, and cellular senescence were evaluated using the cell counting kit-8 assay, plate colony formation assay, scratch assay, transwell assay, flow cytometry, and β-galactosidase staining, respectively. Western blotting was performed to detect the expression of human telomerase reverse transcriptase (hTERT), ataxia-telangiectasia mutated gene (ATM), Rad3-related protein (ATR), check point kinase 1 (CHK-1), CHK2, phosphorylated histone H2AX (γ-H2AX), and telomere-binding proteins in BIBR1532-treated ESCC cells. Additionally, a tumor-bearing nude mouse model was established to evaluate the anti-cancer effect of BIBR1532 *in vivo*.

RESULTS

The IC_{50} values for KYSE150 and KYSE410 cells after 48 hours of BIBR1532 exposure were 48.53 μM and 39.59 μM, respectively. These values decreased to 37.22 μM and 22.71 μM, respectively, following a longer exposure of 72 hours. BIBR1532 exhibited dose-dependent effects on KYSE150 and KYSE410 cells, including decreased hTERT expression, inhibition of proliferation and metastasis, and induction of cellular senescence. Mechanistically, BIBR1532 upregulated the expression of the DNA damage response (DDR) protein, γ-H2AX, and activated the ATR/CHK1 and ATM/CHK2 pathways. BIBR1532 downregulated the expression of telomere-binding proteins, including TRF1, TRF2, POT1, and TPP1. In a nude mouse xenograft model, BIBR1532 significantly suppressed tumor growth, reduced hTERT expression, and increased γ-H2AX protein levels. Hematoxylin and eosin staining of various organs, including the heart, liver, spleen, lungs, and kidneys, revealed no apparent adverse effects.

CONCLUSION

BIBR1532 exerts anti-cancer effects on ESCC by inducing DDR through the ATR/CHK1 and ATM/CHK2 pathways and downregulating the expression of telomere-binding proteins.

Key Words: Esophageal squamous cell carcinoma; BIBR1532; Human telomerase reverse transcriptase; DNA damage response; Telomere-binding proteins

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Core Tip: BIBR1532 inhibited proliferation and metastasis of esophageal squamous cell carcinoma (ESCC) cells in a dose-dependent manner, which also effectively blocked the growth of ESCC in tumor-bearing nude mouse model. Mechanistically, BIBR1532 downregulated the expression of telomere-binding proteins, upregulated the expression of phosphorylated histone H2AX, and activated the Rad3-related protein/check point kinase 1 (CHK-1) and ataxia-telangiectasia mutated gene/CHK2 pathways. The *in vitro* and *in vivo* studies showed that BIBR1532 is a potential chemotherapeutic drug for ESCC.

INTRODUCTION

Esophageal cancer (EC) is one of the most frequently occurring malignant tumors worldwide, ranking seventh in terms of incidence and sixth in terms of mortality[1]. Approximately 600000 new cases of EC are reported globally each year, and over half of these occur in China. Esophageal squamous cell carcinoma (ESCC) is the predominant histological variety, constituting more than 90% of cases[1-3]. The incidence of ESCC has been correlated with various dietary and lifestyle factors, including the consumption of very hot food and drinks, alcohol intake, and cigarette smoking. Additional risk factors include the consumption of mold-contaminated food, food preparation methods involving charring or smoking, drinking water quality, soil composition, and environmental microbial communities[4]. According to the EC Diagnosis and Treatment Guidelines (version 2022), ESCC treatment modalities include surgery, radiotherapy, chemotherapy, targeted therapy, immunotherapy, and combination therapies[4]. However, the initial stages of EC are often asymptomatic and a significant number of patients present with advanced local disease or distant metastases upon diagnosis. This precludes surgical intervention and necessitates systemic drug therapy to manage the disease[4]. ESCC has been documented to develop resistance to radiotherapy and chemotherapy due to heterogeneity in histology, molecular characteristics, and etiology[5]. Immunotherapy and targeted therapy, although promising, may present with severe side effects or offer uncertain efficacy[6,7]. The current standard first-line chemotherapy regimen typically comprises paclitaxel combined with cisplatin, or cisplatin in combination with fluorouracil or capecitabine. However, side effects of these treatments are often undesirable. Despite advances in ESCC treatment, the prognosis remains poor, with an overall five-year survival rate of < 30%[8]. This highlights the urgent need to identify novel and safe anti-EC agents.

Telomeres are DNA-protein complexes located at the termini of chromosomes in eukaryotic cells. They consist of short repetitive sequences of telomeric DNA (5’-TTAGGG-3’) and associated proteins (TRF1, TRF2, TPP1, POT1, TIN2, and RAP1). The primary role of telomeres is to shield chromosome ends from degradation or aberrant recombination[9,10]. Telomere length homeostasis depends on cellular division and telomerase activity. Telomerase is an RNA-dependent DNA polymerase comprising RNA molecules with telomeric template sequences and a protein catalytic subunit telomerase reverse transcriptase (TERT)[11]. Several studies have identified human TERT (hTERT) as a key regulator of telomerase activity[12]. Although telomerase remains transcriptionally inactive in most somatic cells, it is reactivated in over 85% of human cancers, primarily through upregulation of the typically silent hTERT gene[13]. Our previous study demonstrated that the expression of hTERT mRNA in ESCC tissues is significantly upregulated and that hTERT overexpression substantially enhances the proliferation and metastatic potential of cancer cells[14]. These findings suggest that hTERT contributes to ESCC progression and is a potential therapeutic target in afflicted patients.

For nearly two decades, targeting telomerase has been the focus of cancer research. Therapeutic strategies include immunotherapy, small-molecule telomerase inhibitors, oligonucleotide inhibitors, and plant compounds[15]. Among these, the small molecule inhibitor BIBR1532 is a non-nucleoside, non-competitive antagonist that selectively inhibits hTERT[16]. Lavanya *et al*[17] demonstrated that BIBR1532 dose-dependently curtails telomerase activity in human glioblastoma LN18 cells, inducing apoptosis through downregulation of telomerase at both the transcriptional and translational levels. Ding *et al*[18] revealed that lower concentrations of BIBR1532 effectively suppress telomerase activity and exacerbate radiation-induced telomeric dysfunction, compromising chromosome stability and inhibiting the ataxia-telangiectasia mutated gene (ATM)/check point kinase 1 (CHK-1) pathway, thereby impeding DNA damage response (DDR). Another study indicated that telomerase inhibition could regulate epithelial-mesenchymal transition (EMT) in breast cancer, suggesting that telomerase inhibitors primarily eradicate breast cancer stem cells, constrain cellular migration and invasiveness, and ultimately prevent breast cancer cell metastasis[19]. Collectively, these studies imply that BIBR1532 may function as a broad-spectrum anti-cancer agent. However, the effects of BIBR1532 on ESCC have yet to be reported. This study aimed to decipher the biological impact and molecular mechanisms of the telomerase inhibitor BIBR1532 in ESCC cells and provide data for its future clinical use as a potential therapeutic agent for ESCC.

MATERIALS AND METHODS

Reagents and antibodies

RPMI 1640 medium was obtained from Gibco (Grand Island, NY, United States). Fetal bovine serum (FBS) was purchased from OPCEL (Inner Mongolia, China). Penicillin and Streptomycin were procured from SolarBio (Beijing, China). BIBR 1532 was obtained from Pottery (Shanghai, China). Cell counting kit-8 (CCK-8) and β-galactosidase (β-gal) detection kits were obtained from Beyotime Biotechnology (Shanghai, China). The cell cycle detection kit was provided by KeyGen Biotech (Nanjing, Jiangsu Province, China). Immunohistochemistry (IHC) kit was purchased from BOSTER (Wuhan, Hubei Province, China). Antibodies against GAPDH, CHK1, CHK2, phosphorylated histone H2AX (γ-H2AX), P-Rad3-related protein (P-ATR), P-

ATM, hTERT, and goat anti-rabbit IgG were purchased from Cell Signaling Technology (Beverly, CA, United States). Goat anti-rabbit IgG antibody was purchased from BOSTER (Wuhan, Hubei Province, China).

Cell culture

The human ESCC cell lines KYSE150 and KYSE410 were obtained from the Translational Medicine Research Center of the North Sichuan Medical College. All the cells were cultured in DMEM supplemented with 10% FBS at 37 °C in a humidified atmosphere containing 5% CO_2.

CCK-8 assay

CCK-8 assay was performed to assess the viability of KYSE150 and KYSE410 cells. Briefly, the cells were seeded in 96-well plates at a density of 3×10^3 cells/well and incubated at 37 °C in 5% CO_2. After 24 hours, cells were treated with DMSO (vehicle control) or a series of BIBR1532 concentrations (25 μM, 50 μM, 75 μM, and 100 μM) for 48 hours or 72 hours. At the indicated time points, 10 μL of the CCK-8 reagent was added to each well. The plates were incubated for 2 hours in the dark, and the absorbance (A) was measured at 450 nm using a microplate reader.

Plate colony formation assay

KYSE150 and KYSE410 cells were seeded in six-well plates at a density of 2×10^3 cells/well. After 24 hours of incubation, cells were treated with either DMSO or different concentrations of BIBR1532 (25 μM, 50 μM, and 75 μM). Cells were cultured for 1-2 weeks until visible clones emerged in control wells. Subsequently, cells were fixed with methanol for 10 minutes and stained with crystal violet for 10 minutes. Finally, plates were washed, air-dried, and imaged.

Wound healing assay

KYSE150 and KYSE410 cells were seeded in six-well plates at a density of 5 × 10^5 cells/well. When the cell monolayer reached approximately 90% confluence, two parallel scratches were created across the cell layer by using a sterile pipette tip. The plates were washed three times with PBS and fresh culture medium was added. Initial images (0 hour) were captured, and the cells were treated with either DMSO or a series of BIBR1532 concentrations (25, 50, and 75). After 24 hours, images of the same locations were obtained using an optical microscope to assess the wound closure.

Transwell assay

KYSE150 and KYSE410 cells were cultured, trypsinized (0.25% w/v), and resuspended in serum-free RPMI 1640. The cell density was adjusted to 5 × 10^5 cells/mL. Subsequently, 500 µL of RPMI 1640 medium containing 10% FBS was added to the lower chamber of a 12-well plate, and 200 µL of the cell suspension was added to the upper chamber of the Transwell insert. The cells were then treated with DMSO or different concentrations of BIBR1532 (25 µM, 50 µM, and 75 µM). After 24 hours, the non-migrated cells on the upper surface of the membrane were removed, and the migrated cells on the lower surface were fixed with methanol for 10 minutes and stained with crystal violet for 20 minutes. Images were captured using an optical microscope.

Cell senescence assay

KYSE150 and KYSE410 cells were seeded into six-well plates at a density of 1 × 10^5 cells/well and treated with either DMSO or different concentrations of BIBR1532 (25 µM, 50 µM, and 75 µM). After treatment, cells were washed twice with PBS and fixed at room temperature for 15 minutes. Subsequently, the cells were washed three times with PBS and incubated overnight at 37 °C in β-gal staining solution. The plates were sealed with plastic wrap to prevent evaporation, and the senescent cells were observed under an optical microscope.

Tumor-bearing nude mice model construction and treatment

Male 6-week-old BALB/c nude mice (approximately 20 g) were purchased from Beijing Laboratory Animal Research Center (Beijing, China). Animal care and experiments were performed with the approval of the Animal Ethics Committee of North Sichuan Medical College. KYSE150 cells in the logarithmic phase of growth were harvested and adjusted to a density of 1×10^7 cells/mL. Subsequently, 100 μL of the cell suspension was subcutaneously implanted into the upper right flank of each mouse. When the tumor volume reached approximately 100 mm^3, mice were randomly divided into two groups (n = 4 per group). The treatment group received intraperitoneal injections of BIBR1532 (50 mg/kg), whereas the control group received an equal volume of DMSO. Injections were administered every two days. Tumor dimensions were measured using a Vernier caliper every two days, and tumor volumes were calculated using the following formula: Volume (mm^3) = length × $width^2/2$. On day 20, all mice were euthanized and tumor tissues were collected and fixed in 4% paraformaldehyde solution for 24 hours. Fixed tissues were embedded in paraffin for further analysis.

Immunohistochemical staining

Paraffin-embedded xenograft tissues were sectioned and subjected to immunohistochemical staining. Briefly, sections were deparaffinized, rehydrated, and subjected to antigen retrieval using sodium citrate buffer. The sections were incubated with primary antibodies against hTERT, Ki-67, and γ-H2AX at 4 °C overnight. Subsequently, the sections were stained using an immunostaining kit (BOSTER, Wuhan, China), according to the manufacturer's instructions.

Hematoxylin and eosin staining of the tissue specimens

Paraffin-embedded tissue sections were deparaffinized and rehydrated. The nuclei were stained with hematoxylin, and the cytoplasm was counterstained with eosin. Finally, the sections were dehydrated, cleared, mounted, and observed under a microscope.

Western blotting

Tissue samples were homogenized, and total proteins were extracted using RIPA buffer. Proteins were separated using SDS-PAGE and electrophoretically transferred to polyvinylidene difluoride (PVDF) membranes. The PVDF membranes were washed and blocked with 5% skimmed milk for 1 hour at room temperature. Subsequently, the membranes were incubated with the primary antibodies overnight at 4 °C. After washing, membranes were incubated with secondary antibodies for 1 hour at room temperature. Finally, the protein bands were detected using an enhanced chemiluminescence system (VILBER FUSION FX7, France).

Statistical analysis

The experimental data were analyzed using the GraphPad Prism 8 software. Data are presented as mean ± SD of three independent experiments. Comparisons between two groups were performed using the Student's *t*-test, and comparisons between multiple groups were performed using analysis of variance (ANOVA). Differences were considered statistically significant at $P < 0.05$.

RESULTS

IC50 of BIBR1532 in ESCC Cells

To assess the potential toxicity of BIBR1532 in ESCC cells, KYSE150 and KYSE410 cells were treated with various concentrations of BIBR1532 (25 μM, 50 μM, 75 μM, 100 μM, 125 μM, and 150 μM) for 48 hours or 72 hours. Cell viability was evaluated, and the half-maximal inhibitory concentration (IC50) values were calculated. Our results indicated a significant dose-dependent decrease in the viability of both KYSE150 and KYSE410 cells post-treatment at both time points (Figure 1). Based on these findings, BIBR1532 concentrations of 25 μM, 50 μM, and 75 μM and a 48-hour treatment duration were selected for subsequent experiments.

BIBR1532 inhibits ESCC proliferation

Since BIBR1532 is a telomerase inhibitor, we first evaluated whether it regulates the expression of hTERT, a core catalytic subunit of telomerase. The results showed that BIBR1532 significantly downregulated the expression of hTERT in KYSE150 and KYSE410 cells in a dose-dependent manner (Figure 2A). Subsequently, we evaluated the effect of BIBR1532 on the proliferation of KYSE150 and KYSE410 cells. As shown in Figure 2B, BIBR1532 significantly inhibited the viability of both the cell lines. The inhibitory effect on cell viability increased gradually with higher concentrations of BIBR1532 and longer incubation times, in a time- and dose-dependent manner. Plate colony formation assays further confirmed the ability of BIBR1532 to inhibit proliferation of KYSE150 and KYSE410 cells (Figure 2C).

BIBR1532 inhibits the migration ability of ESCC

To explore whether BIBR1532 regulates the migration capacity of EC cells, scratch and Transwell assays were used to evaluate the migration of KYSE150 and KYSE410 cells after exposure to BIBR1532. The results showed that the migration distance of KYSE150 and KYSE410 cells in the BIBR1532-treated group was significantly shorter than that in the DMSO control group, in a dose-dependent manner (Figure 3A). Similarly, Transwell assays revealed that the number of migrated cells in the BIBR1532-treated group was significantly lower than that in the DMSO group (Figure 3B). These results suggested that BIBR1532 significantly inhibits the migration of EC cells.

BIBR1532 induces senescence in ESCC

Because BIBR1532 is a telomerase inhibitor, we investigated whether it induced cellular senescence. β-gal staining assay was employed to detect the effect of BIBR1532 on the senescence of KYSE150 and KYSE410 cells. The number of senescent cells in the BIBR1532-treated group was significantly higher than that in the D"> BIBR1532 inhibits the proliferation and migration of ESCC through downregulation of hTERT.

To determine whether BIBR1532 exerted its anti-cancer effect on ESCC cells by downregulating hTERT expression, we treated the cells with BIBR1532 (50 μM) combined with hTERT overexpression. The results showed that BIBR1532 inhibited the proliferation and migration of KYSE150 cells. Moreover, hTERT overexpression in KYSE150 cells promoted ESCC cell proliferation and migration. Importantly, hTERT overexpression alleviated the tumor-suppressive effects of BIBR1532 in KYSE150 cells (Figure 5). These results indicated that BIBR1532 exerts anti-cancer effects on ESCC by downregulating hTERT expression.

BIBR1532 downregulates the expression of telomere protective proteins in EC cells

Studies have demonstrated that BIBR1532 is cytotoxic and can cause direct damage to telomere structure, leading to the loss of TRF2 binding, thereby inducing telomere dysfunction[20]. To investigate whether BIBR1532 regulated the expression of other telomere protection proteins, the expression of shelterin proteins (TRF1, TRF2, TPP1, POT1, TIN2, and RAP1) was evaluated in KYSE150 and KYSE410 cells treated with BIBR1532. The results showed that BIBR1532 significantly downregulated the expression of TRF1, TRF2, TPP1, and POT1 in both cell lines, but had no significant effect on the expression of TIN2 and RAP1 (Figure 6A and B). These results indicated that BIBR1532 may lead to the dysregulation of telomere structure and function.

BIBR1532 regulates the DDR of EC cells through the ATM/ATR signaling pathway

The main function of telomere protection proteins is to protect DNA ends from being recognized as double-strand breaks that trigger DDR[15]. Excessive lengthening or shortening of telomeres results in an imbalance in genome homeostasis, ultimately inducing DDR. Given that BIBR1532 inhibits the expression of hTERT and the telomere protection proteins TRF1, TRF2, TPP1, and POT1, we hypothesized that BIBR1532 can induce DDR in ESCC cells. As shown in Figure 6C and D, the expression of γ-H2AX in KYSE150 and KYSE410 cells was significantly upregulated after treatment with the indicated concentrations of BIBR1532. ATM- and ATR-mediated protein expression levels of CHK2 and CHK1, which are important downstream regulators of the DNA damage repair pathway, were also upregulated. These results suggested that BIBR1532 may induce DDR in ESCC cells through the ATM-CHK2 and ATR-CHK1 pathways.

BIBR1532 inhibits tumor growth in ESCC xenograft mouse model

To further investigate the anti-cancer effect of BIBR1532 in vivo, nude mouse xenograft models were constructed by subscapular injection of KYSE150 cells. The results showed that tumor growth was inhibited in xenograft mice after treatment with BIBR1532 (Figure 7A and B). Moreover, the tumor weight was significantly lower in BIBR1532-treated mice than in DMSO-treated controls (Figure 7C). Furthermore, IHC results showed that the expression of hTERT and Ki-67 was reduced, whereas that of γ-H2AX was increased in tumor tissues treated with BIBR1532 (Figure 7D). Consistent with the IHC results, western blot analysis confirmed that the expression of hTERT was significantly downregulated, whereas that of γ-H2AX was upregulated in tumors treated with BIBR1532 (Figure 7E). Importantly, macroscopic images and hematoxylin and eosin (H&E) staining of the heart, liver, spleen, lungs, and kidneys of mice showed no obvious differences between the BIBR1532-treated group and the DMSO group (Figure 7F and G). Taken together, these data demonstrated that BIBR1532 inhibits ESCC growth *in vivo* without apparent toxicity.

DISCUSSION

Telomerase, an enzyme comprising catalytic proteins and RNA templates, is responsible for telomere elongation in cells. Normally, telomerase expression is suppressed in somatic cells, resulting in telomere shortening after cell division[21]. During the malignant transformation of cells, telomerase is reactivated, promoting uncontrolled cell growth. Studies have shown that telomerase maintains tumor cell proliferation capacity and promotes tumor development by regulating telomere length[22]. Previous research has demonstrated that hTERT is highly expressed in various tumors, including ESCC[23-25]. Our previous studies also confirmed that hTERT overexpression promotes the proliferation and metastasis of EC cells[14]. Therefore, targeting hTERT holds great potential for EC treatment.

Various telomerase inhibitors have been developed as potential therapeutic agents. Among them, BIBR1532 is a selective non-competitive inhibitor that primarily impairs DNA substrate extension after extending to the 5′ end of the template, reducing the number of TTAGGG repeats, and inhibiting long reactant formation[26]. BIBR1532 inhibits the proliferation, migration, and invasion of endometrial cells in patients with endometriosis by reducing the cascade reaction of telomerase activity and the MAPK signaling pathway[27]. In addition to benign diseases, BIBR1532 is primarily used in cancer therapy. For instance, it inhibits proliferation and migration, while inducing apoptosis in anaplastic thyroid cancer SW1736 cells[28]. Consistent with previous studies, we found that BIBR5132 downregulated hTERT expression and inhibited ESCC cell proliferation in a dose-dependent manner. Moreover, BIBR1532 significantly inhibited EC cell migration in a dose-dependent manner. Next, we explored the *in vivo* anti-EC effect of BIBR1532. These results demonstrated that BIBR1532 inhibited the growth of transplanted tumors in nude mice. H&E staining of the heart, liver, spleen, lung, and kidney tissues showed that BIBR1532 did not cause obvious microscopic changes in the key organs of nude mice, indicating its low toxicity in vivo. These findings further demonstrated that BIBR1532 is a potential therapeutic drug for ESCC.

Telomeres play a crucial role in the maintenance of chromosomal integrity. In eukaryotic cells, telomeres are protected by the telomere protection protein complex to prevent chromosome end DNA molecules from being recognized as broken double-stranded DNA, which induces ATM- or ataxia-telangiectasia mutated gene and ATR-dependent DDR[29,30]. When DNA is damaged, upstream ATM and/or ATR protein kinases are activated, subsequently activating downstream signal transduction kinases, CHK1 and/or CHK2, as well as a series of receptors (BRCA1, MDC1, and 53BP1). These kinase cascades enhance DNA damage signals and initiate different response systems through the effectors CDC25, p53, and SMC1, ultimately leading to cell cycle arrest, activation of DNA damage repair pathways, and cell apoptosis and/or senescence[31].

In this study, we found that the expression of telomere protection proteins (TRF1, TRF2, TPP1, and POT1) was downregulated in BIBR1532 treated EC cells KYSE150 and KYSE410. Concurrently, the expression of the DNA damage-related response proteins γ-H2AX, p-ATM, CHK1, p-ATR, and CHK2 was significantly upregulated. These findings indicate that BIBR1532 inhibits telomerase activity and downregulates the expression of telomere protection proteins, inducing DDR and activating the ATM/ATR signaling pathway in EC cells. Our findings are consistent with those of a previous study showing that the knockdown of TPP1 induces DDR in EC cells[32].

Studies have reported that cell cycle arrest is involved in the inhibitory effect of BIBR1532[18,33,34]; however, its impact on ESCC cell cycle distribution was previously unknown. In this study, we found that BIBR1532 induced cell cycle arrest in the G2/M phase in a dose-dependent manner. Additionally, we observed that BIBR1532 induced cellular senescence in a concentration-dependent manner, which is consistent with a series of reactions following telomere attrition, such as triggering the DDR pathway, leading to cell cycle arrest, cellular senescence, and apoptosis[35,36].

CONCLUSION

In summary, we found that BIBR1532 inhibited the proliferation and migration of ESCC cells, which is likely associated with the activation of DDR and the ATM/ATR signaling pathway, ultimately leading to telomere dysregulation and cellular senescence. Although this study presents novel findings, it has several limitations. First, we were unable to uncover the detailed mechanisms by which BIBR1532 downregulates hTERT expression, or whether it affects telomerase activity. Second, the mechanism by which BIBR1532 downregulates the expression of the four telomere protective proteins remains unclear. In the near future, we will further investigate the role of BIBR1532 in ESCC to provide more information regarding its potential therapeutic application. This may include exploring the direct effects of BIBR1532 on telomerase activity, elucidating the molecular pathways involved in the downregulation of telomere-protective proteins, and investigating potential combination therapies to enhance its anti-cancer effects.

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Figure 1 IC_{50} determination of BIBR1532 in esophageal squamous cell carcinoma cells. A and B: Viability of KYSE150 (A) and KYSE410 (B) cells after treatment with the indicated concentrations of BIBR1532 for 48 hours; C and D: Viability of KYSE150 (C) and KYSE410 (D) cells after treatment with the indicated concentrations of BIBR1532 for 72 hours.

Figure 2 BIBR1532 inhibits the proliferation of KYSE150 and KYSE410 cells. A: Human telomerase reverse transcriptase expression in KYSE150 and KYSE410 cells after exposure to a series of BIBR1532 concentrations; B: Viability of KYSE150 and KYSE410 cells after treatment with various concentrations of BIBR1532 at the indicated time points; C: Plate colony formation by KYSE150 and KYSE410 cells after treatment with various concentrations of BIBR1532 at the indicated time points. $^aP < 0.05$ was compared with the control group respectively. $P < 0.05$ was considered statistically significant. hTERT: Human telomerase reverse transcriptase.

Figure 3 Effect of BIBR1532 on the migration of KYSE150 and KYSE410 cells. A: Migration of KYSE150 and KYSE410 cells after treatment with various concentrations of BIBR1532 for 24 hours, as evaluated by scratch assay; B: Migration of KYSE150 and

KYSE410 cells after treatment with various concentrations of BIBR1532 for 24 hours, as evaluated by the transwell assay.

Figure 4 Effect of BIBR1532 on the senescence of KYSE150 and KYSE410 cells. A: Senescence of KYSE150 and KYSE410 cells after treatment with various concentrations of BIBR1532 for 48 hours; B: Expression of the cell senescence biomarker p53 in KYSE150 and KYSE410 cells after treatment with various BIBR1532 concentrations for 48 hours.

Figure 5 Proliferation and migration of KYSE150 and KYSE410 cells after treatment with BIBR1532 combined with human telomerase reverse transcriptase overexpression. A: Plate colony formation by KYSE150 cells after treatment with BIBR1532 (50 μM) and/or human telomerase reverse transcriptase (hTERT) overexpression for 48 hours; B: Migration evaluated by transwell assay of KYSE150 cells after treatment with BIBR1532 (50 μM) and/or hTERT overexpression for 48 hours; C: Migration evaluated by the scratch assay of KYSE150 cells after treatment with BIBR1532 (50 μM) and/or hTERT overexpression for 48 hours. hTERT: Human telomerase reverse transcriptase.

Figure 6 Effect of BIBR1532 on shelterin protein and key proteins in the DNA damage response pathway expression in KYSE150 and KYSE410 cells. A and B: Expression of TRF1, TRF2, TPP1, POT1, TIN2, and RAP1 in KYSE150 (A) and KYSE410 (B) cells after treatment with indicated concentrations of BIBR1532 for 48 hours; C and D: Expression of γ-H2AX, p-ATM, CHK2, p-ATR, and CHK1 in KYSE150 (C) and KYSE410 (D) cells after treatment with the indicated concentrations of BIBR1532 for 48 hours. TRF1, TRF2, TPP1, POT1, TIN2, and RAP1γ-H2AX, p-ATM, CHK2, p-ATR, and CHK1

Figure 7 Anti-cancer effect of BIBR1532 in an esophageal squamous cell carcinoma xenograft mouse model. A-C: Tumor images (A), growth curves (B), and tumor weights (C) obtained from xenograft tumors derived from KYSE150 cells treated with BIBR1532 (50 mg/kg); D and E: Expression of human telomerase reverse transcriptase, Ki-67, and γ-H2AX in xenografted tumor tissues evaluated by immunohistochemistry (D) and Western blotting (E); F: Macroscopic images of the heart, liver, spleen, lungs, and kidneys of nude mice treated with BIBR1532 or DMSO; G: Hematoxylin and eosin staining of the heart, liver, spleen, lung, and kidney tissues from nude mice treated with BIBR1532 or DMSO. [a]$P < 0.05$ was compared with the control group respectively. $P < 0.05$ was considered statistically significant. hTERT: Human telomerase reverse transcriptase; γ-H2AX: Phosphorylated histone H2AX.

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