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Telomerase and mitochondria inhibition promote apoptosis and TET2 and ANMT3a expression in triple negative breast cancer cell lines

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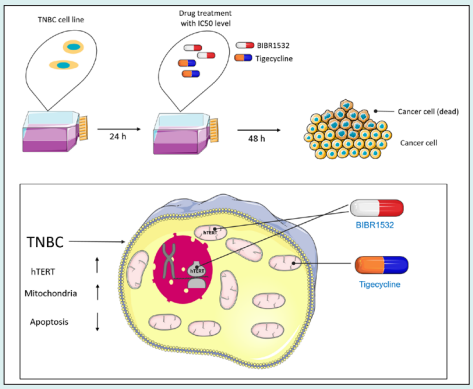
Abstract

Introduction: High metastasis, resistance to common treatments, and high mortality rate, has made triple-negative breast cancer (TNBC) to be the most invasive type of breast cancer. High telomerase activity and mitochondrial biogenesis are involved in breast cancer tumorigenesis. The catalytic subunit of telomerase, telomerase reverse transcriptase (hTERT), plays a role in telomere lengthening and extra-biological functions such as gene expression, mitochondria function, and apoptosis. In this study, it has been aimed to evaluate intrinsic-, extrinsic-apoptosis and DNMT3a and TET2 expression following the inhibition of telomerase and mitochondria respiration in TNBC cell lines.



Methods: TNBC cells were treated with IC_{50} levels of BIBR1532, tigecycline, and also their combination. Then, telomere length, and DNMT3a, TET2, and hTERT expression were evaluated. Finally, apoptosis rate, apoptosis-related proteins, and genes were analyzed.

Results: The present results showed that IC_{50} level of telomerase and inhibition of mitochondria respiration induced apoptosis but did not leave any significant effect on telomere length. The results also indicated that telomerase inhibition induced extrinsic-apoptosis in MDA-MB-231 and caused intrinsic- apoptosis in MDA-MB-468 cells. Furthermore, it was found that the expression of p53 decreased and was ineffective in cell apoptosis. The expressions of DNMT3a and TET2 increased in cells. In addition, combination treatment was better than BIBR1532 and tigecycline alone.

Conclusion: The inhibition of telomerase and mitochondria respiration caused intrinsic- and extrinsic- apoptosis and increased DNMT3a and TET2 expression and it could be utilized in breast cancer treatment.

Introduction

Triple-negative breast cancer (TNBC) is an invasive subtype of breast cancer (containing 15–20%) that do not express Her-2, Estrogen, and Progesterone receptors.[1] TNBC cells have cancer stem cells' (CSCs') origin with less differentiated property and high proliferation levels.[2,3] TNBC patients are resistant to conventional therapy and display higher metastasis, disease recurrence, and

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mortality rate than other subtypes of breast cancer.[4,5] According to the studies, telomerase activity is high in over 90% of breast cancers.[6]

Telomerase is a reverse transcriptase enzyme that protects telomeres (the ends of chromosomes) from erosion and makes genomic stability.[7] Telomerase consists of two subunits including hTERT (catalytic unit) and TERC (RNA template).[8,9] Telomerase activity is associated with the expression of hTERT and causes poor prognosis of breast cancer.[6,10] In addition, hTERT is involved in extra-biological functions such as gene expression and cell proliferation.[8] hTERT translocates to the mitochondria due to exogenous stress, resulting in protecting cells from DNA damage and apoptosis.[8,11] Furthermore, high mitochondrial biogenesis causes stemness, proliferation, tumorigenesis, and metastasis in TNBC, and eventually increases tumor growth and chemotherapy resistance.[12-15]

There are associations between hTERT expression and methylation in breast cancer cells.[16] Methylation, as an epigenetic mechanism, involves cellular functions, the regulation of gene expression, and tumor progression.[17] Demethylation and methylation of DNA is catalyzed by ten-eleven translocation proteins (TET1,2,3) and DNA methyltransferases (DNMT1,3A,3B), respectively.[17,18] Decreased expression of TET and DNMT3a in breast cancer causes metastasis and tumorigenesis.[19,20]

It has been shown that TNBC is resistant to common treatments. In general, telomerase and mitochondria are essential for the progression of cancers and cancer cell survival.[4,14,21] Thus, a better understanding of the effect of their inhibition might help develop effective anti-cancer therapies. In addition, the combination treatment is highly efficient strategy than a single treatment in treating cancer.[22,23]

BIBR1532 is a nonpeptidic, non-nucleoside small molecule that inhibits telomerase activity by binding specifically to the active site of hTERT. It is indicated that BIBR1532 had potential anti-tumor activity in different cancers by triggering replicative senescence and apoptosis.[24,25] BIBR1532 also displays potential efficacy in combination with other anti-cancer agents in breast cancer.[26]

Tigecycline is an antibiotic, with similar structure to Tetracyclines. It inhibits protein translation by strong binding to the 30S ribosomal subunit. Tigecycline has an anti-cancer effect in human acute myeloid leukemia (AML) and various solid tumors including TNBC. In addition, combinations of tigecycline with chemotherapeutic drugs are for cancer treatment.[27,28] Tigecycline has been used in clinical trials for the treatment of acute myeloid leukemia (Clinical Trial No. NCT01332786) and chronic myeloid leukemia (Clinical Trial No. NCT02883036).

In the current study, telomerase and mitochondria respiration in TNBC was inhibited and the expression of apoptosis-related genes, proteins, DNMT3a, and TET 2 were evaluated.

Materials and Methods

Cell lines

MDA-MB-468 and MDA-MB-231 cells (TNBC cell lines) obtained from Pasteur Institute, Tehran, Iran (National Cell Bank). The cells were cultured in Roswell Park Memorial Institute 1640 (RPMI 1640) medium complemented with 1% penicillin/streptomycin and 10% fetal bovine serum (FBS) at 37 °C and 5% CO_2. After 3 passages, MDA-MB-231and MDA-MB-468 cells contained (94.6%) $CD44^+CD24^-$ (Fig. 1A) and (81%) $CD44^+$ $CD24^+$ (Fig. 1B), respectively.

MTT assay

The telomerase inhibitor (BIBR1532) and mitochondria inhibitor (tigecycline) were bought from (Cayman Chemical, MI, USA) and (TCI), respectively. BIBR1532 and tigecycline were prepared in different concentrations during use. As a control group, the cells were cultured without inhibitors.

MDA-MB-231 and MDA-MB-468 cells were seeded in 96-well at 1×10^3 cells/wells and incubated for overnight. After 24 hours, the cells were treated with BIBR1532 (7.5, 15, 30, 60,120) µM and tigecycline (1, 2.5, 5, 10, 20, 40) µM for 24, 48, and 72 hours. After supernatant removal, 30 µL of MTT [5 mg/mL in phosphate-buffered saline (PBS)] (ROTH) solution was added to each well and incubated for 4 hours. Then, the medium was removed and 100 µL dimethyl sulfoxide (DMSO) was added. Finally, the absorbance of each well (OD) was measured at 570 nm by a microplate reader (BioTek ELx808, USA). Half-maximal inhibitory concentration (IC_{50}) of BIBR1532 and tigecycline were calculated using the software GraphPad Prism (6.01).

Apoptosis assay

To evaluate apoptosis in TNBC cells, an Annexin V/7AAD assay was carried out. Briefly, TNBC cells were seeded in a 12-well plate with a density of 5×10^4 and incubated for 24 hours. Then, they were treated with IC_{50} levels of BIBR1532 and tigecycline for 48 hours. The treated and untreated cells were separated by trypsin (Gibco, UK) and washed with PBS. The cells were suspended in 100 µL of 1X binding buffer (BioLegend). 5 µL annexin V antibody plus 5 µL 7AAD were added and incubated in dark and on ice for 25 minutes. Finally, the cells were washed and suspended in 500 µL binding buffer and evaluated using FACS (BD Bioscience). The results were evaluated using FlowJo software (version X.0.7).

RNA extraction and real-time PCR

IC_{50} levels of BIBR1532 and tigecycline were used to treat the cells. Then, the cells were collected and RNA extraction was performed using kit Yekta Tajhiz Azma, Iran. The purity of RNA samples was measured by the Nanodrop instrument (NanoDrop1000; Thermo Fisher Scientific). cDNA synthesis was performed according to

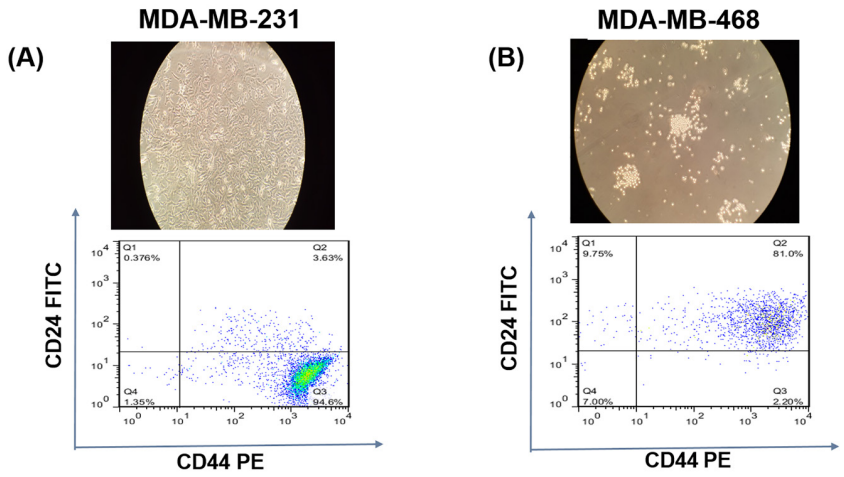

Fig. 1. Characteristics of cells. MDA-MB-231(A) and MDA-MB-468 (B).

the manufacturer's instructions cDNA kit (Yekta Tajhiz Azma, Iran). qPCR was done in 20 µL solution which mixed 10 µL SYBR Green (Yekta Tajhiz Azma, Iran), 2 µL of cDNA, 0.5 µL of each reverse and forward primer, and 7 µL of water without nuclease. The following PCR reactions were performed using a Roche Molecular diagnostic. The PCR reaction started with a 10-minute denaturing step at 94 °C, followed by 40 cycles at 94 °C for 10 seconds, annealing at 56 °C (hTERT, Bax, Bad, Bid, Bcl-2, p53, TET2, DNMT3a and β-actin) and 58 °C (Bcl-xl) for 1 minute, 1 minute at 72 °C, and finally 10 minutes of extension at 72 °C. LightCycler® 96 software was utilized to achieve CT values. For normalization, b-actin was used and the data were analyzed by the $2^{-\Delta\Delta CT}$ method. The sequences of primer are depicted in Table 1.

DNA extraction for telomerase length assay

Genomic DNA was extracted using Yekta Tajhiz Azma (Iran) DNA extraction kit. In brief, the harvested cells were added to 20 µL of proteinase K and 200uL FABG buffer and were incubated for 15 minutes at 60 ˚C. 200 µL of 96% ethanol was added, vortexed, passed to the BG column and then centrifuged at 5000×g for 1 minute. Afterward, 400 µL washing buffer 1 was added and centrifuged for the 30 seconds at 14000×g. Next, 750 µL washing buffer 2 was added, and centrifuged for the 30 seconds at 14000×g. Finally, 50~200 µL elution buffer or ddH2O (pH 7.5~9.0) was added to the BG column and centrifuged for three minutes at 14000×g. Extracted DNA was stored at -20 ˚C until it being used to measure telomere length. Standard curves and quantitative PCR for measuring telomere length before defined by Farahzadi et al.[29,30]

Protein analysis by western blotting

The harvested cells were lysed with lysis buffer (0.01gr SDS, 0.025gr Sodium Deoxycholate, 0.08 g NaCl, 0.003 gr EDTA, 500 µL, PH=8 Tris-HCL, 1 tablet protease inhibitor cocktail, 10 µL Triton 1%) and were centrifuged at 400×g for 10 minutes at 4 °C. The quantity of protein was determined by Bradford assay. First, lysates were resolved on SDS-page gels, transferred to polyvinylidene difluoride (PVDF) membranes and then incubated at room temperature for 90 minutes in blocking buffer, (composed of 5% non-fat milk powder in Tris-buffered saline with 0.1% Tween (TBS-T). The membranes were pre-incubated with primary antibodies for 15 hours, rinsed with TBS-T buffer (Tris-buffered saline with 0.1% Tween), and then incubated with the secondary conjugated antibody for 1h.

Table 1. The details of primers

Gene	Forward primer (5'-3')	Reverse primer (5'-3')	Size (bp)
b-actin	AAACTGGAACGGTGAAGGTG	TATAGAGAAGTGGGGTGGCT	174
hTERT	CAGCAAGTTTGGAAGAACCC	GACATCCCTGCGTTCTTGG	234
Bax	TCACTGAAGCGACTGATGTCC	CTCCCGCCACAAAGATGGTC	194
bad	ACTTCCTCGCCCGAAGAGC	CTTCCCCTGCCCAAGTTCC	198
bcl-2	AGTGAACATTTCGGTGACTT	CTTCCAGACATTCGGAGACC	208
Bcl-xl	ATCCCAGCTCCACATCACC	CGATCCGACTCACCAATACC	202
Bid	AATCAGAGAAGGAACATACCC	ACTTCCCATCATTTGAGTGC	154
P53	TCAGTCTACCTCCCGCCATAA	AGTGGGGAACAAGAAGTGGAG	176
DNMT3a	GAAGACCCCTGGAACTGC	GTCAAATTCCTGGTCGTGG	123
TET2	TGGCTGACAAACTACTGG	CTTCTGGCAAACTTACATCC	190

DNMT3a: DNA methyltransferases 3A, TET2: Ten-eleven translocation proteins.

Finally, the membrane was washed 3 times with TBS-T buffer. The protein was detected with a chemiluminescence (ECL) kit (Roche, UK). The bands were evaluated by NIH ImageJ 1.6 program. For normalization of protein loading, b-actin was utilized.

Statistical analysis

In order to assess data, GraphPad Prism software (6.01) was utilized. The results are reported as mean ± SD. All tests were carried out three times. One-way and two-way analysis of variance (ANOVA) trailed by Bonferroni's and Tukey's multiple comparisons test were used for the significance of experimental variables between different groups. The significance values were defined as $^{*}P \leq 0.05$.

Results

Cytotoxicity assay of telomerase and mitochondria respiration inhibition inTNBC

IC_{50} values of telomerase inhibitor (BIBR1532) and mitochondria inhibitor (tigecycline) were determined in the TNBC cell lines by the MTT assay.[25,31] The IC_{50} rates of the BIBR1532 in MDA-MB-231 and MDA-MB-468 cells were respectively 36 µM and 10 µM at 48 hours (Fig. 2A, B). Furthermore, the IC_{50} level of the tigecycline was 3 µM in MDA-MB-231 cells and 5 µM in MDA-MB-468 cells (Fig. 2C, D). The MDA-MB-231 cells showed higher IC_{50} when compared to BIBR1532- treated MDA-MB-468 cells (Fig. 2A). This may be due to the nature of MDA-MB-231, which contains the highest BCSCs rate and is an aggressive breast cancer cell line.[32]

Telomerase and mitochondria respiration inhibition reduced telomerase activity but not changed telomere length in TNBC cell lines

The expression of hTERT and telomere length in cell lines after treatment with IC_{50} rate (36 µM and10 µM) of BIBR1532 and (3 µM and 5µM) tigecycline was measured in the current. hTERT mRNA levels decreased significantly in cell lines when compared to untreated cells (control) (Fig. 3A, C) and the highest reduction was noted in the combination group. No significant difference in telomere length in both cells (Fig. 3B, D) was noticed. Telomere length in untreated cells of MDA-MB-231 and MDA-MB-468 were 5.9 kbp and 5 kbp, respectively.

Telomerase and mitochondria respiration inhibition increased apoptosis in TNBC cell lines

The rate of apoptosis was assessed with the IC_{50} level of BIBR1532 and tigecycline at the 48 hours in cells using Annexin V (Fig. 4). The results displayed a significant increase in the total percentage of apoptotic cells with BIBR1532, tigecycline, and in combination treatment of MDA-MB-231 (Fig. 4A, B) and MDA-MB-468 (Fig. 4C, D) at 48 hours. The apoptosis rate in combination

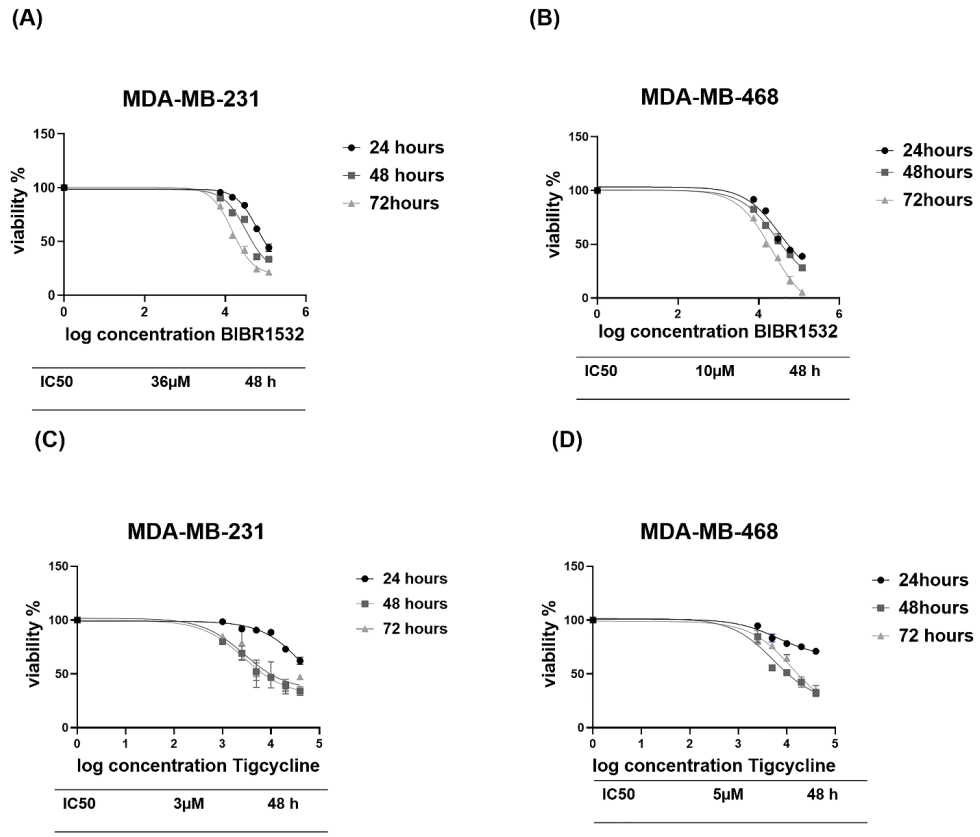

Fig. 2. MTT assay of BIBR1532 and tigecycline in TNBC cell lines. MTT assay of BIBR1532 in MDA-MB-231(A) and MDA-MB-468(b) and for tigecycline in MDA-MB-231(C) and MDA-MB-468 (D).

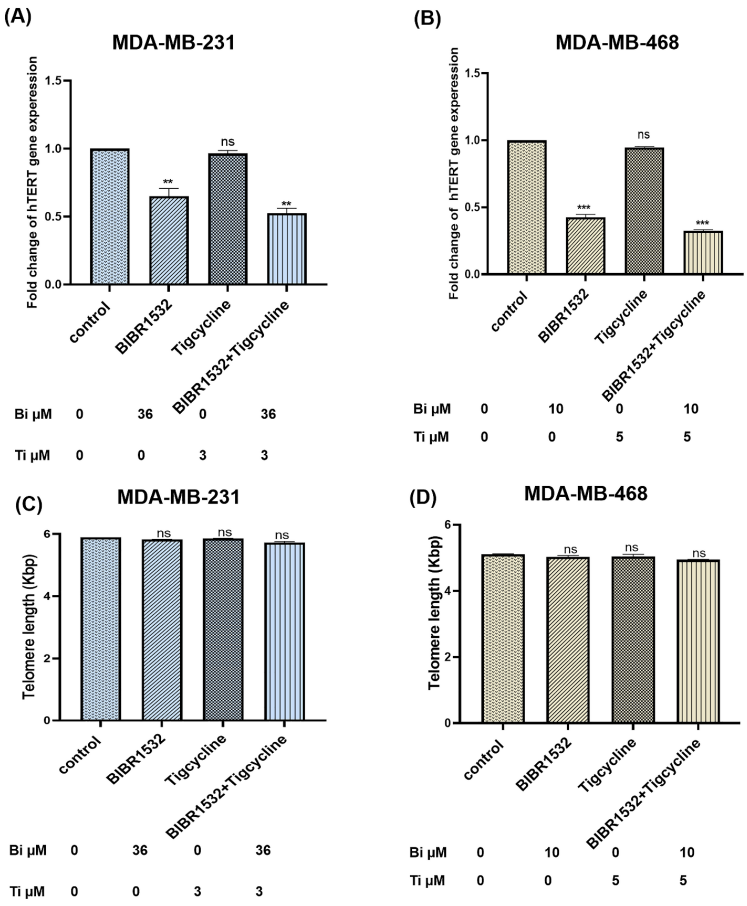

Fig. 3. hTERT expression and telomere length in TNBC cell lines. The expression of hTERT and telomere length is shown in MDA-MB-231(A, B) and MDA-MB-468 (C, D) following BIBR1532, Tigecycline and in combination treatment. The data are normalized against b-actin and indicated as mean ± SD in each group in three independent tests $^{**}P < 0.001$ and $^{***}P < 0.001$ vs. the untreated cells.

treatment was significantly higher than BIBR1532 and tigecycline alone in cells.

The apoptotic genes (Bax, Bad, Bid, Bcl-2, Bcl-xl, and p53) were evaluated following the treatment with IC_{50} level of BIBR1532 and tigecycline in MDA-MB-231(Fig. 5A) and MDA-MB-468 (Fig. 5D) cells. The expressions of Bax, bad, and bid were significantly upregulated in treated group when compared to the control group. The expressions of Bcl-2 and Bcl-xl were significantly downregulated in tigecycline and combination treatment but did not change in response to BIBR1532 treatment in MDA-MB-231 cells (Fig. 5A and D). Furthermore, p53 was significantly reduced in MDA-MB-231 and MDA-MB-468 cells (Fig. 5A, D) and the highest reduction was for combination treatment.

To understand the apoptosis pathway, the protein level of cytochrome C and caspases (9, 8, 3) were measured in MDA-MB-231(Fig. 5B, C) and MDA-MB-468 (Fig. 5E, F). The protein levels of cytochrome C and caspases (9, 8, 3) were significantly increased in combination treatment in cells. It is while, cytochrome C and caspase 9 partially remained unchanged in MDA-MB-231 in response to BIBR1532 treatment and caspase 8 was not changed in MDA-MB-468 with tigecycline treatment.

Telomerase and mitochondria respiration inhibition changed the expression of DNMT3a and TET2

DNMT3a mRNA levels were increased in tigecycline and combination treatment in MDA-MB-231 (Fig. 6A) and MDA-MB-468 (Fig. 6B) cells. However, there was no difference in DNMT3a expression in BIBR1532 treatment in cells. Furthermore, TET2 expression was increased in tigecycline and combination treatment in MDA-MB-468 (Fig. 6B) cells. While no difference in TET2 expression in some treated groups in MDA-MB-231 and MDA-MB-468 cells was found.

Discussion

Considering that, TNBC patients treatment remained a challenge and hTERT involves in the progression of cancer and inhibits mitochondrial apoptosis.[21] In addition, combination therapy is a more effective strategy than monotherapy for improving cancer.[22] Therefore, we inhibited telomerase and mitochondria respiration and evaluated apoptosis and expression of DNMT3a and TET 2.

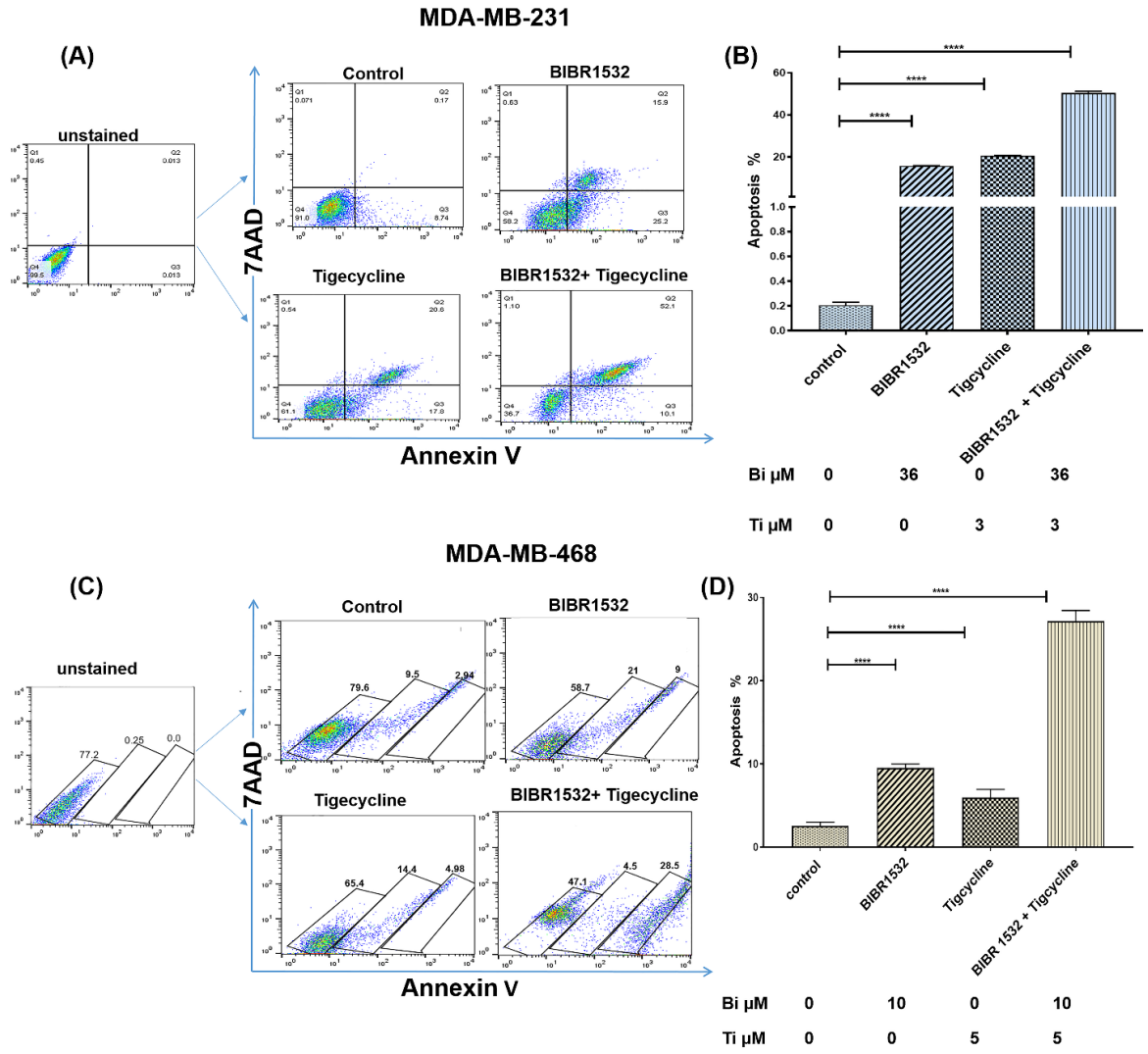

Fig. 4. Apoptosis rate in TNBC cell lines. Flow cytometry results of MDA-MB-231 (A, B) and MDA-MB-468 cells (C, D) cells following treatment with IC50 level of BIBR1532, tigecycline, combination, and untreated control group. The results are indicated as mean ± SD in each group in three independent experiments ****$P < 0.0001$ vs the untreated cells.

According to the former studies, different breast cancer cell lines respond differently to BIBR1532 and tigecycline treatment.[31,33] The present results indicated that BIBR1532 exposure significantly diminished the expression of hTERT but did not leave a significant effect on telomere length. BIBR1532 treatment reduced hTERT expression in breast cancer cell lines.[26,33,34] The inhibition of telomerase often has a long lag phase prior to telomere shortening.[35]A study indicated that long-term exposure with low doses of BIBR 1532 in different malignancy cells induced telomere shortening while, EL-Daly showed that telomere length was not changed with short-term exposure of BIBR 1532 in cancer cells.[25,36,37] Furthermore, hTERT inhibitor did not affect telomere length in breast cancer.[38] Therefore, telomere shortening may be time and dose-dependent with BIBR1532 treatment.

The overexpression of hTERT inhibits mitochondrial apoptosis due to the inhibition of cytochrome c release, the reduction of mitochondrial potential, transmission the Bax to mitochondria.[39-41] Also, hTERT inhibit extrinsic cell death. According to Zhang et al, TERT hampers the induction of TRAIL-mediated cell death.[42,43] Moreover, tigecycline induces intrinsic apoptosis by activating the release of cytochrome c, and cleavage of caspase-9/caspase-3/caspase-7.[27,44] Based on the current findings, the inhibition of telomerase and mitochondria respiration increased apoptosis in TNBC cells. It was found that the inhibition of telomerase and mitochondria respiration up-regulated the expression levels of pro-apoptotic Bax, Bad, Bid genes and caspase 3, 8, 9, and cytochrome c proteins, and it downregulates the expression levels of anti-apoptotic Bcl-2 and Bcl-xl and cause intrinsic-, extrinsic- mediated apoptosis in TNBC cells. These results are consistent with the role of the mitochondrial pathway in apoptosis. Bax accelerated the opening of voltage-dependent anion channels at the outer mitochondrial membrane with the subsequent release of cytochrome c that interacts with the apoptotic protease activating factor-1 (Apaf-1)

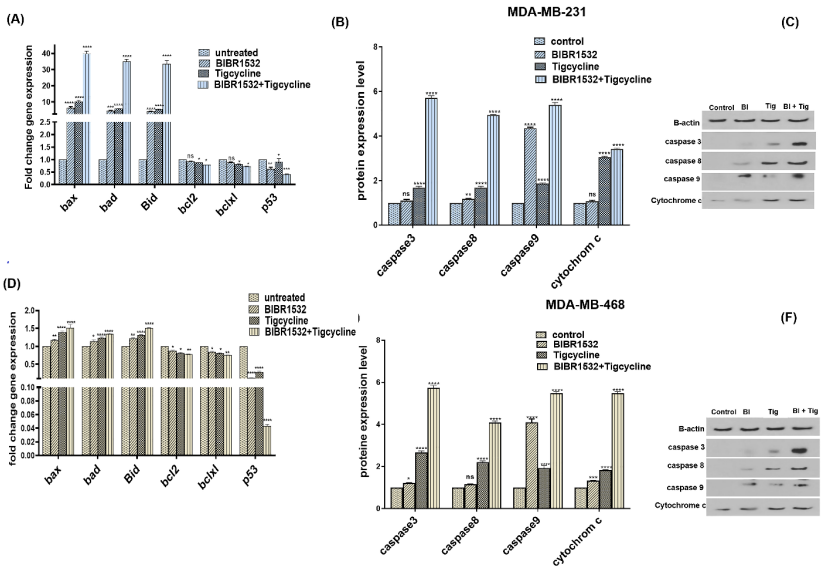

Fig. 5. Expression of apoptotic genes and proteins in TNBC cell lines. The relative mRNA expression of Bax, Bad, Bid Bcl-2, Bcl-xL, and p53 (A) and proteins (B, C) are shown in MDA-MB-231 following IC50 level of BIBR1532, Tigecycline, in combination treatment, and untreated control group. Furthermore, expression of Bax, Bad, Bid Bcl-2, Bcl-xL, and p53 and proteins of MDA-MB-468 are shown in (D) and (E, F) respectively. The data are normalized against b-actin and indicated as mean ± SD in each group in three independent investigations, **P* < 0.05, ***P* < 0.01, ****P* < 0.001 *****P*<0.0001 vs the untreated cells.

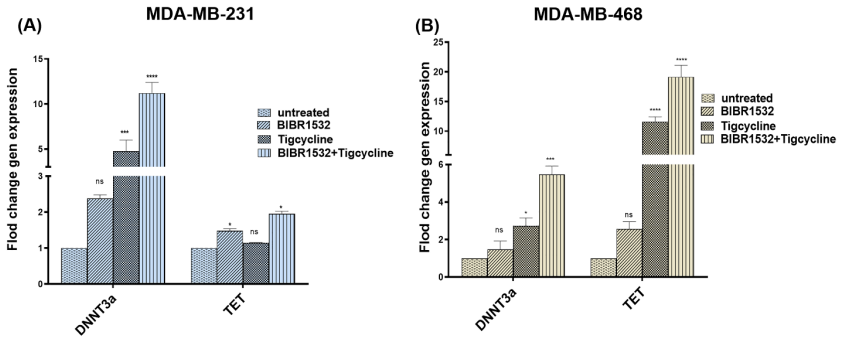

Fig. 6. Expression of DNMT3a and TET2 in TNBC cell lines. DNMT3a and TET2 mRNA expression are shown in MDA-MB-231(a) and MDA-MB-468 (b) after IC50 level of BIBR1532, tigecycline, in combination treatment, and untreated control group. The data are normalized against b-actin and indicated as mean ± SD in each group in three independent investigations, **P* < 0.05, ****P* < 0.001, and *****P* < 0.0001 vs the untreated cells.

and activates the initiator caspase 9. Bad also blocks the anti-apoptotic function of Bcl-xl and Bcl-2 and induces apoptosis.[45-48] Mitochondrial- and receptor-dependent pathways cooperate through Bid to enhance apoptosis. Bid, a pro-apoptotic protein, is cleaved and activated by caspase 8 and led to the mitochondrial permeability transition.[49] The expressions of Bcl-2 and Bcl-xl remain unchanged in hTERT inhibition of MDA-MB-231 cells. It suggests that post-translational modification including methylation modifications can reduce the effect of Bcl-2.[50] Our result showed that telomerase inhibition increased caspase 8 and 3 and induced extrinsic-apoptosis in MDA-MB-231. It is consistent with the result of Rubis et al who showed that the reduction of telomerase activity stimulated the expression of caspase-8, Fas, and FasL genes and activated the death receptor apoptosis in MDA-MB-231 cells.[38] It is while, mitochondria pathway plays a vital role in apoptosis in the inhibition of telomerase in MDA-MB-468 cells. p53 is a DNA-binding transcription factor that is increased in response to cell stress, and plays role in DNA damage repair, apoptosis, and other cellular processes.[51,52] p53 mutant accumulates in TNBC, disturbs DNA repair complex, and causes tumor progression with apoptosis resistance and cancer invasion.[53,54] Based on studies tigecycline acts independently from p53.[55] BIBR1532 and tigecycline therapy down-regulates p53 expression in TNBC cells and induces apoptosis.

The current results suggest that the expressions of DNMT3a and TET2 increased. Epigenetic markers (DNMT3a and TET2) have tumor-suppression functions.[56,57] DNMT3a depletion enhances self-renewal, cancer cell progression, and breast cancer metastasis and

Research Highlights

What is the current knowledge?

√ Triple-negative breast cancers (TNBCs) exhibit the properties of cancer stem cells (CSCs)

√ CSCs are resistant to conventional therapy

√ hTERT, subunit of telomerase, displays several functions such as the effect on mitochondria, gene expression, and apoptosis

√ Mitochondria is involved in breast cancer tumorigenesis.

What is new here?

√ Telomerase and mitochondria respiration inhibition decreased hTERT expression but no change in telomere length in TNBC cell lines.

√ The inhibition of telomerase and mitochondria respiration induced apoptosis in TNBC cell lines.

√ The inhibition of telomerase and mitochondria respiration affected the expression of DNAT3a and TET2 in TNBC cell lines.

decreases TET2 mRNA leading to poor prognosis in breast cancer patients.[19,20,58] DNMT3a and TET deficiency happens in increased telomere length.[59,60]

Conclusion

Inhibition of telomerase and mitochondria respiration caused intrinsic and extrinsic apoptosis and increased DNMT3a and TET2 expression and could be utilized in breast cancer future treatments.

Acknowledgments

This is a report of the database from a PhD thesis registered at Tabriz University of Medical Sciences.

Authors' Contribution

Conceptualization: Mohammad Karimipour, Hojjatollah Nozad Charoudeh.
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Formal analysis: Zeinab Mazloumi, Ali Rafat.
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Project administration: Hojjatollah Nozad Charoudeh.
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Supervision: Hojjatollah Nozad Charoudeh.
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Writing–review & editing: Ali Rafat, Khadijeh Dizaji Asl, Hojjatollah Nozad Charoudeh.

Competing Interests

None of the authors claimed to have any competing interests.

Ethics Statement

This study was approved by the Academic Research Ethics Committee of Tabriz University of Medical Sciences (code of ethics, IR.TBZMED.REC.1398.785).

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