BRCA1 mutated cells are less likely to undergo ROS-mediated apoptosis after exposure to eribulin and paclitaxel

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Abstract
Triple negative breast cancer has a high frequency of BRCA1 gene mutations. In this experiment, we examined whether there are cells that are not led to apoptosis in different subtypes of breast cancer with poor prognosis with BRCA1 mutation and wild type BRCA cells. Cells with BRCA1 wild-type (MDA-MB-231 and BT-549) or mutated (MDA-MB-436) BRCA1 were exposed to anticancer drugs, and the levels of reactive oxygen species (ROS) produced by oxidative stress and Annexin V (an index of apoptosis) were examined. The wild-type MDA-MB-231 cells showed increased ROS levels and Annexin V after exposure to eribulin and paclitaxel. Hence, the pathway leading to apoptosis may be activated by oxidative stress. ROS levels in BT-549 cells were significantly increased after exposure to eribulin and paclitaxel. However, there was no change in Annexin V. BRCA1-mutated MDA-MB-436 cells showed significantly increased ROS levels after exposure to eribulin and paclitaxel and no change in the Annexin V levels. This suggests that BRCA1 wild-type BT-549 cells and BRCA1-mutated MDA-MB-436 cells were resistant to ROS-mediated apoptosis. These results indicate that BRCA1 mutation and cell subtypes should be investigated prior to selecting the chemotherapy combination to enable appropriate selection in clinical practice.

Key words: breast cancer, BRCA1 mutation, reactive oxygen species, eribulin, paclitaxel

Introduction
A relationship between gene mutation and breast cancer treatment has been reported for the prevention and treatment of this disease1. Approximately 5%–10% of breast cancer cases are hereditary. Of those, 25% are associated with germ cell gene mutations2-5. The BRCA1 and BRCA2 genes, which are involved in repairing damaged DNA, are tumor suppressor proteins. Mutations in BRCA1 and BRCA2 are present in breast and ovarian cancers, which are unique to women. This cause is involved in hereditary breast cancer and ovarian cancer syndrome, which are autosomal dominant genetic traits. Approximately 12% of women develop breast cancer in their lifetime. It is estimated that approximately 72% and 69% of women who inherit the BRCA1 and BRCA2 mutations, respectively, in the HBOC family will develop breast cancer by the age of 80 years6-8. There are six subtypes of triple negative, which is considered to have a particularly poor prognosis among breast cancers9: BL1 (basal-like 1), BL2 (basal-like 2), IM (immunomodulatory), M (mesenchymal), MSL (mesenchymal stem-like), LAR (Luminal androgen receptor). There is a high frequency of BRCA1 gene mutations in triple negative. BT-549 cells, like MDA-MB-231, do not have mutations in BRCA. Drug sensitivity has not been examined according to the presence or absence of BRCA1 mutation. In this experiment, we aimed to investigate whether both drugs are effective against breast cancer with poor prognosis associated with BRCA1 mutation, and whether there are cells without BRCA1 mutation that are not led to apoptosis by different subtypes.

Therefore, this study was conducted using triple-negative breast cancer (TNBC) cells with BRCA1 wild-type and BRCA1 mutation. Eribulin is a non-

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taxane resulting in apoptosis in tumor cells through a mechanism that prevents the formation of normal mitotic spindles of microtubule dynamics\textsuperscript{10}. Paclitaxel is taxane-based, promoting microtubule protein polymerization through stabilization and hyperformation of microtubules. Paclitaxel inhibits cell division by impairing the function of the mitotic spindle, leading to apoptosis in tumor cells\textsuperscript{11}. Cancer cells are known to be resistant to anti-cancer drugs. It has been reported that cancer cells survive by producing carbon monoxide (CO), a biological gas, as one of their resistance mechanisms. Oxidative stress (ROS) and carbon monoxide (CO) are closely related. In this study, we investigated the relationship between ROS and cells that survive by producing CO, which is a mechanism of resistance to anticancer drugs\textsuperscript{12}. The purpose of this study was to expand the therapeutic options for HBOC patients with BRCA1 mutation. For this purpose, we investigated the characteristics of BRCA1 wild-type and BRCA1 mutation cells.

**Materials and methods**

**Cells and culture conditions**

Human breast cancer cell lines MDA-MB-231 and BT-549 cells (TNBC cells with BRCA1 wild-type) and MDA-MB-436 (Japanese Cancer Resources Bank, Osaka, Japan) having a BRCA1 mutation were used. MDA-MB-436 cells were cultured in RPMI-1640 culture medium (Sigma–Aldrich, Oberhaching, Germany). MDA-MB-231 and BT-549 cells were grown in Dulbecco’s modified Eagle’s medium Ham/F12 (Sigma–Aldrich, Oberhaching, Germany) culture medium. Immobilized fetal bovine serum (10%; Gibco Life Technologies, CA, USA) and 100 units/ml of penicillin and 100 mg/ml of streptomycin (Gibco penicillin–streptomycin liquid; Invitrogen, Carlsbad, CA, USA) were added to the culture medium. RPMI-1640 was further supplemented with 1 mM of pyruvic acid (Sigma–Aldrich). The CO\textsubscript{2} incubator was set at a temperature of 37°C and an oxygen concentration of 5%.

**Anticancer drugs**

Cells were exposed to eribulin mesylate (1 µg/ml; Eisai Co., Ltd, Tokyo, Japan) or paclitaxel (0.05 ng/ml; SANDOZ, Tokyo, Japan) through exposure infusion in a 10-cm Petri dish.

**ROS level measurement**

MDA-MB-231, BT-549, and MDA-MB-436 cells (1 × 10\textsuperscript{5} cells) were prepared in 12 ml medium. Petri dish collagen-coated microplate six-well with lid collagen type I (#4810-010; IWAKI), 2 ml/well was sprinkled on each sheet. Subsequently, the cells were cultured for 48 hr. Following exposure to eribulin (1 µg/ml) or paclitaxel (0.05 ng/ml), the cells were cultured for 24 hr. The cells seeded in a six-well Petri dish were detached with trypsin-5.3 mmol/l ethylenediaminetetraacetic acid (EDTA-4Na solution, #208-17251; WAKO, Osaka, Japan). Cells were collected in a 15-ml tube and centrifuged at 1,500 rpm for 5 min. The liquid in the tube was removed, and the cells were resuspended in PBS (10 ml). The cells (10 µl) were injected into the Counting Slides (#145-0011; BIO-RAD, CA, USA) and measured using a TC20\textsuperscript{TM} Automated Cell Counter (BIO-RAD). Next, 100 µl of 1 × 10\textsuperscript{5} cells/ml extracellular fluid and 100 µl of Annexin V solution (Muse\textsuperscript{TM} Annexin V & Dead Cell Reagent, Part No. 4700-1485; MERCK, NJ, USA) in an L5-ml tube were added and mixed. The cells were allowed to react for 20 min, and apoptosis was measured with the Muse\textsuperscript{TM} Cell Analyzer (MERCK).

**Significance test**

Two-way analysis of variance using Bonferroni’s method was performed to determine the significance of results. A $p < 0.05$ denoted statistically significant difference.

**English proofreading**

The English of the manuscript was edited by Enago (www.enago.jp).
**Results**

**ROS levels in BRCA1 mutated cells and BRCA1 wild-type cells exposed to eribulin and paclitaxel**

In control BRCA1 wild-type MDA-MB-231 cells, the ROS levels were $474 \pm 81 \text{ Fl}/\mu\text{g/TP}$. Following exposure to eribulin and paclitaxel, these levels were $961 \pm 109 \text{ Fl}/\mu\text{g/TP}$ and $924 \pm 16 \text{ Fl}/\mu\text{g}$, respectively. It was measured to be significantly higher than the control. In control BRCA1 wild-type BT-549 cells, the ROS levels were $647 \pm 56 \text{ Fl}/\mu\text{g/TP}$ and $573 \pm 50 \text{ Fl}/\mu\text{g/TP}$, respectively. It was measured to be significantly higher than the control. In control MDA-MB-436 cells with BRCA1 mutation, the ROS levels were $350 \pm 18 \text{ Fl}/\mu\text{g/TP}$. After exposure to eribulin and paclitaxel, these levels were $847 \pm 141 \text{ Fl}/\mu\text{g/TP}$ and $647 \pm 31 \text{ Fl}/\mu\text{g/TP}$, respectively. It was measured to be significantly higher than the control ($\text{Fig. 1}$).

**Apoptosis detections by Annexin V in BRCA1 mutated cells and BRCA1 wild type cells exposed to eribulin and paclitaxel**

In control BRCA1 wild-type MDA-MB-231 cells, the rate of apoptosis (detected by Annexin V) was 4.58%. After exposure to eribulin and paclitaxel, the apoptotic rate was 9.98% and 9.11%, respectively. It was measured to be significantly higher than the control. In control BRCA1 wild-type BT-549 cells, the rate of apoptosis was 3.6% ± 0.2%. Following exposure to eribulin and paclitaxel, the apoptotic rate was 3.1% ± 0.4% and 3.3% ± 1%, respectively. No significant difference was observed compared to the control. In control MDA-MB-436 cells with BRCA1 mutation, the rate of apoptosis was 2.1% ± 0.1%. After exposure eribulin and paclitaxel, the apoptotic rate was 2.3% ± 0.1% and 1.9% ± 0.2%, respectively. No significant difference was observed compared to the control ($\text{Fig. 2}$).

**Discussion**

The levels of ROS and Annexin V were measured to determine if the pathway leading to apoptosis in cells with BRCA1 mutation was activated by oxidative stress. Anticancer drugs, such as eribulin and paclitaxel, inhibit microtubule elongation. This causes cell cycle arrest in the G2/M phase and inhibits cell division through hyperformation and stabilization of microtubules. ROS production was significantly
increased after exposure of MDA-MB-231 cells to eribulin and paclitaxel, demonstrating the occurrence of ROS-mediated apoptosis. BRCA1 wild-type BT-549 cells showed a significant increase in ROS production, but no change in Annexin V. Hence, it was considered that apoptosis was induced by oxidative stress in only few cells. An explanation for this observation is that BT-549 cells have mutations in the tumor suppressor gene PTEN. This gene inhibits AKT by suppressing the activity of PI3K-mediated signal transduction[14]. Because PTEN acts as a regulator of PI3K/AKT/mTOR signaling, cancer cells proliferate when PTEN is deficient. If so, apoptosis was not induced in BT-549 cells with mutations in PTEN even after exposure to eribulin and paclitaxel, and the cancer cells would have survived.

The BRCA1 gene, which is involved in repairing DNA, is a tumor suppressor protein located on chromosome 17q21[15]. Mutations in the BRCA gene impair its DNA damage repair function. MDA-MB-436 cells with a BRCA1 mutation did not undergo apoptosis even following the production of ROS by exposure to eribulin or paclitaxel. The reason for this is thought to be that poly (ADP-ribose) polymerase (PARP) repairs the DNA of cells that have lost BRCA1/2 function, keeping the cells alive. If treated with anticancer drugs that act on microtubules, cell death is not likely to occur. PARP is an enzyme involved in the repair of DNA single-strand breaks. It recognizes the single-strand break and repairs the DNA through base excision repair[16]. Homologous recombination, which repairs DNA damage, occurs in the late S phase and G2 phase of the cell cycle[17]. Thus, in cells with BRCA1/2 mutations, PARP repairs DNA instead of BRCA1/2. For this reason, eribulin and paclitaxel are considered ineffective in patients with these mutations[18].

Anticancer drugs are less effective in patients with a BRCA1 mutation than in those with BRCA1 wild-type. For this reason, therapeutic agents that inhibit PARP and induce cell death have developed for the treatment of ovarian cancer with BRCA1 mutation. In 2014, PARP inhibitors were clinically introduced in the United States of America and Europe. In Japan, the PARP inhibitor olaparib (Limpaza®) was approved in July 2018. It was indicated for the treatment of BRCA1 mutation-positive and HER2-negative inoperable or recurrent breast cancer with a history of chemotherapy[19]. In addition to olaparib, other PARP inhibitors (e.g., niraparib, rucaparib, talazoparib, and
veliparib) are currently being developed\(^2\). Eribulin is approved for the treatment of inoperable or recurrent breast cancer. In particular, it has been reported to be effective against TNBC with a poor prognosis. It is expected that the combination of PARP inhibitors and eribulin will generate evidence that may provide new treatment options for TNBC. In April 2020, health insurance coverage was applied to HBOC BRCA tests, risk-reducing mastectomy, breast reconstruction, and risk-reducing salpingo-oophorectomy for patients receiving treatment with olaparib and those with pre-existing conditions in Japan.

In recent years, our understanding of the BRCA1/2 gene has deepened. In the future, in addition to traditional platinum and taxanes or angiogenesis inhibitors, a wider range of drugs that match the subtype of breast cancer will be available. It is possible that apoptosis can be induced even in subtypes of breast cancer with BRCA1 mutation. Further clarification of the characteristics of BRCA1 wild-type and mutation cells will lead to the development of new therapeutic options. This study revealed that cells with a BRCA1 mutation are less likely to undergo ROS-mediated apoptosis after exposure to eribulin and paclitaxel. These results indicate that BRCA1 mutation and cell subtypes should be investigated prior to chemotherapy selection, so that appropriate chemotherapy treatment selection can be made in clinical practice.

Conflict of interest disclosure
The authors declare that they have no conflict of interest.

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