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## Breast Cancer Cell Lines, HER2/Neu Phenotype, and a Higher Propensity to Reactive Oxygen Species Production

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### ABSTRACT

**Background:** The reactive oxygen species (ROS) generated in the biological systems play an important role in pathological conditions and specific normal cellular processes, such as signaling pathways and drug sensitivity.

**Methods:** Intracellular ROS was determined using (2'-7'dichlorofluorescein diacetate) DCFH-DA fluorimetric probe, malondialdehyde as lipid peroxidation index detected by the Thiobarbituric acid reactive substances method. Cells' protein carbonyl contents were assessed with 2,4-Dinitrophenylhydrazine (DNPH) derivatization spectrophotometrically at 360-385 nm.

**Results:** The oxidative stress induces ROS ( $p < 0.05$ ), MDA (Malondialdehyde) ( $p < 0.05$ ), and protein carbonylation ( $p < 0.01$ ) was significantly higher in HER2-positive BT-474, SK-BR-3, and MDA-MB-453 compared to the HER2-negative MDA-MB-231 and MCF-7 cell lines.

**Conclusion:** We hypothesized that increased oxidative stress in HER2-positive cell lines is due to the oncogenic function of the HER2 and PI3K/Akt signaling activation, resulting in glycolysis induction. It is assumed that HER2-positive cell lines with high ROS levels are more vulnerable to further damage by increased ROS levels induced by pro-oxidant anticancer agents.

### Introduction

Caused by the reactive oxygen species (ROS), oxidative stress is defined as an imbalance between generation and elimination of the ROS<sup>1</sup>. ROS is commonly produced in the cells as a consequence of aerobic respiration as well as intermediates in the various enzymatic and non-enzymatic processes<sup>2,3</sup>. ROS damages the number of macromolecules such as nucleic acids, proteins, and fatty acids, resulting in a change in their structure and function<sup>3</sup>. Cells apply

both enzymatic and non-enzymatic mechanisms for neutralizing the produced free radicals<sup>4</sup>. The imbalance between ROS generation and antioxidant scavenging systems induced by oxidative stress<sup>5</sup> is involved in the etiology of various diseases and conditions such as neurodegenerative disorder (Alzheimer, Parkinson), cardiovascular disease (atherosclerosis), inflammatory disease (arthritis, lupus erythematosus), diabetes mellitus, aging, and carcinogenesis<sup>6</sup>. ROS damages cell membrane lipids such as polyunsaturated fatty acids (PUFAs) and initiate lipid peroxidation<sup>7</sup>. Malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE) are important markers of the lipids oxidative damage<sup>8</sup>. ROS's most important destructive effects on DNA are double-strand breaks and purine/pyrimidine modification resulting in the mutation, genomic instability, and an altered gene expression<sup>9</sup>. The

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accumulation of 8-hydroxy guanosine (8-OHdG) is an index of the DNA oxidative damage<sup>10</sup>. The altered redox imbalance toward peroxidants also affects proteins, causing protein fragmentation and production of the carbonyl (CO) groups in lysine, arginine, threonine, and proline residues proteins. Other oxidative changes include converting the cysteine residues in the proteins to disulfide bonds, methionine to methionine sulphoxide, tryptophan to kynurenine formyl kynurenine<sup>11</sup>.

Despite such destructive effects, ROS has many biological functions in normal cells<sup>12</sup>. Recent studies have suggested that ROS act as the second messenger in numerous cellular processes<sup>13</sup>. Disturbance in the balance between ROS concentration and antioxidant systems, resulting in oxidized redox-sensitive proteins activation and triggering signaling pathways<sup>14</sup>. It is also well known that a modest increase in the ROS is involved in the multiple specific signaling pathways in cancer cells, such as survival, proliferation, angiogenesis, and metastasis. At the same time, higher levels of ROS can trigger cell death signaling pathways such as death receptor – and mitochondria-mediated apoptosis, necrosis, and autophagy<sup>15, 16</sup>. The target proteins for ROS in signaling pathways have thiol groups that act as redox sensors<sup>17</sup>. The ROS targeted signaling proteins are receptor tyrosine kinases (RTKs) such as PDGF- $\alpha$ , PDGF- $\beta$ , VEGF, and EGF receptors, non-receptor tyrosine Kinases (NRTKs) such as Src- kinase and Janus kinase (JAK), protein tyrosine phosphatases (PTPs), serine/threonine kinases such as Akt and MAPK (mitogen-activated protein kinase) family, NF- $\kappa$ B, AP-1 transcription factor, p21 and p53<sup>13,18</sup>.

Because of the double-edged sword property of the ROS and its role in the survival or cell death, ROS manipulating therapy may be a useful strategy for eliminating cancer cells<sup>18</sup>. This study aims to measure the oxidative stress markers in the breast cancer cell lines (BT-474, SK-BR-3, MDA-MB-453, MDA-MB-231, and MCF-7) as the first step in order to study the drug sensitivity in HER2 positive breast cancer cells.

## Methods

### Materials

The human breast cancer cell lines (BT-474, SK-BR-3, MDA-MB-453, MDA-MB-231, and MCF-7) were obtained from the Iranian Biological Resource Centre (IBRC, Tehran, Iran). DMEM and RPMI-1640 cell culture media, fetal bovine serum (FBS), and penicillin-streptomycin were purchased from Gibco, BRL (Life technology, Paisley, Scotland). Trichloroacetic acids (TCA) and phenylmethylsulfonyl fluoride (PMSF) were obtained from Sigma Chem. Co (Germany). Also, 2,4-Dinitrophenylhydrazine (DCFH-DA) was obtained from a Molecular probe (Eugene, Oregon, USA). Dimethyl sulfoxide (DMSO) and thiobarbituric acid (TBA) were obtained from Merck (Darmstadt, Germany).

### Cell culture

BT-474, MDA-MB-453, and MDA-MB-231 cells were maintained in RPMI-1640 medium with 10% fetal bovine serum (FBS). SK-BR-3 and MCF-7 cells were maintained in the Dulbecco's minimum Eagle's media (DMEM) with 10% FBS. BT-474 and SKBR3 media were supplemented with 2mM L-Glutamine, as well. Penicillin (100U/ml) and streptomycin (100 $\mu$ g/ml) were used to control bacterial contamination in all cell lines culture media. The cells cultured in the T-25 flask were maintained at 37°C in the humidified atmosphere with 5% CO<sub>2</sub> and passaged every two days.

### Cell lysate preparation

For preparation of the cell lysate, the cells were collected, washed with cold PBS (pH 7.4), and were lysed in the ice-cold lysis buffer [50 mM Tris-HCl, pH 7.4, 0.5% (v/v) Triton X-100, 0.1% (w/v) SDS, 150 mM NaCl, 1 mM EDTA] containing 1mM PMSF as protease inhibitor. Cells were kept on ice for 30 min while vortexed every 10 min. The resulting mixture was centrifuged at 14,000  $\times$ g for 15 min at 4°C. The obtained supernatant protein concentration was assayed using Bradford protein assay.

### Determination of intracellular ROS

Generation of ROS was detected with 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) fluorimetric nonpolar probe, which was taken up by the cells and deacetylated by cellular esterases to the polar 2',7'-dichloro-dihydro fluorescein (DCFH) which was oxidized by ROS and other peroxides to highly fluorescent 2',7'- dichlorofluorescein (DCF). Therefore, the intensity of the fluorescence was correlated with the amount of ROS. The experiments were carried out using a stock solution of DCFH-DA (20mM w/v) prepared in DMSO and stored in the dark at -20 °C. For the determination of ROS,  $2 \times 10^4$  cells were seeded per 96-well plate. After 24 h, 100  $\mu$ L DCFH-DA solution (final concentration of 10  $\mu$ M) was added to each well and incubated at 37 °C for 45 min in the dark. The fluorescence readings were taken at excitation and emission wavelengths of 480 and 530 nm, respectively, in a BioTek Synergy HT multi-detection microplate reader.

### Determination of malondialdehyde

Malondialdehyde (MDA) is the end product of polyunsaturated fatty acids (PUFAs) peroxidation in the cells. This reactive aldehyde is among the important indicators of oxidative stress. MDA production was quantified by the thiobarbituric acid reactive substances (TBARS) method. Briefly, cells were seeded in  $3 \times 10^5$  per well of a 6-well plate, and 500  $\mu$ L of cell lysates were mixed with 1 mL of 10% (w/v) cold TCA to precipitate proteins. 1 ml 0.67 % (w/v) thiobarbituric acid (TBA) was added to the supernatant and was heated at 95°C for one hour. The pink-colored



product (MDA– TBA complex) absorbance was determined at 532nm spectrophotometrically. The lysates' protein concentration was determined by the Bradford method, and the MDA concentration of the samples was calculated using the following formula: Absorbance at 532nm /  $1.56 \times 10^5 \text{ cm}^{-1}$  and expressed as nmol/mg protein.

#### Determination of the protein carbonylation

The protein carbonyl (PCO) content is a common index of oxidative modification of the proteins. Protein carbonyl contents were quantified through evaluating the reaction between 2,4-dinitrophenylhydrazine (DNPH) and protein carbonyls producing Schiff base to convert hydrazone derivatives as determined by applying spectrophotometer at 360-385 nm. Briefly,  $3 \times 10^5$  cells were seeded per well in a 6-well plate, trypsinized, and lysed with proper lysis buffer at 4°C. A volume of 900  $\mu\text{L}$  of the cell lysate was mixed with 100  $\mu\text{L}$  10%(w/v) streptomycin solution. Following 15 min incubation, centrifugation was carried out at 5000 rpm  $\times$  10 min, and the supernatant was used to assay protein content. 0.2 ml of dinitrophenylhydrazine (10 mM in 2N HCl) was added to the supernatant. After 1 hr. of incubation at room temperature and vortexing every 10 min, 2 ml of TCA (10 % w/v) was added and centrifuged (3000 rpm, 10 min). The precipitate was washed twice with 4 ml of ethanol/ethyl acetate (1:1, v/v), then dissolved in 1 ml of guanidine hydrochloride (6M, pH = 2.3) and vortexed. Protein carbonyl content was determined at 360nm, and results were reported as nmol applying the Beer-Lambert formula ( $\epsilon_{\text{DNPH}} = 2.29 \times 10^4 \text{ cm}^{-1} \text{ M}^{-1}$ ).

#### Statistical analysis

Each experiment was carried out in triplicate, and

all experiments were performed three times. Statistical analysis was performed using SPSS (version 16) and the results are expressed as means  $\pm$  standard deviation (SD). The differences between the two groups were tested using Student's t-test, and a P-value = 0.05 was considered statistically significant.

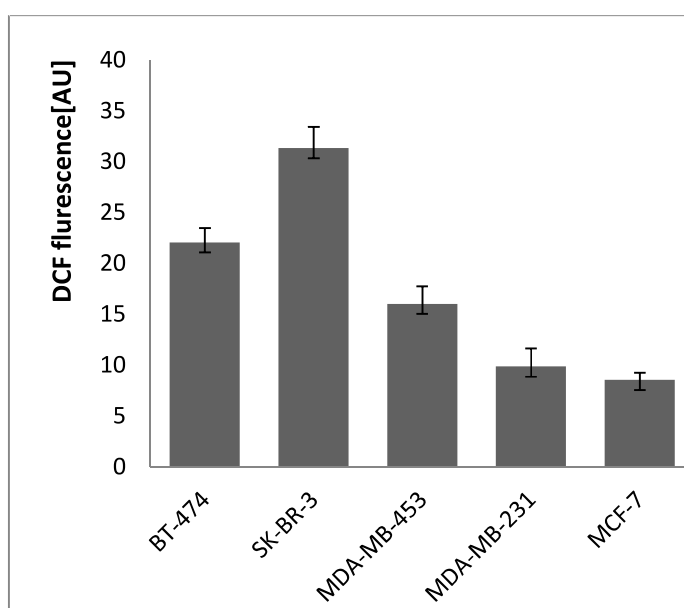
## Results

### HER2-positive cells and reactive oxygen species production

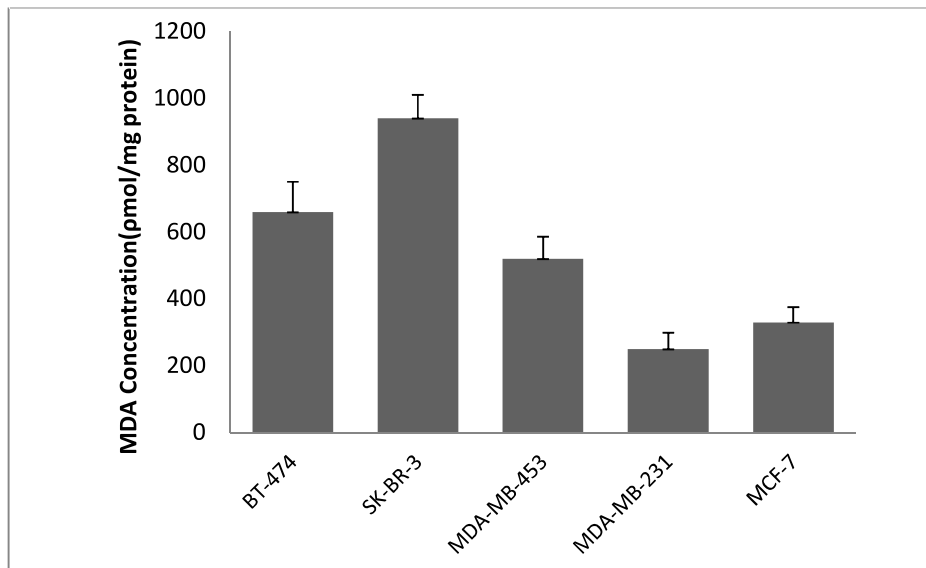
The DCFH-DA probe determined the concentration of intracellular reactive oxygen species (ROS), and the fluorescence intensity was measured in a 96 well plate using a spectrofluorometer. DCF fluorescence intensity correlated with ROS concentration of the cells. Figure 1 shows the relative DCF-fluorescence intensity (mean  $\pm$  SD, N=3) in BT-474, SK-BR-3, MDA-MB-453, MDA-MB-231, and MCF-7 breast cancer cell lines, respectively. A significant difference was detected between ROS levels in HER2-positive BT-474, SK-BR-3, and MDA-MB-453 cells compared with the HER2-negative MDA-MB-231 MCF-7 cells ( $p < 0.05$ ).

### Evaluation of lipid peroxidation

Malondialdehyde, as a major degradation product of the lipid peroxidation, was determined by the thiobarbituric acid (TBA) test. This spectrophotometric method is based on the reaction between MDA and the two TBA molecules, resulting in a pink-colored complex formation (TBA2-MDA) with maximum absorption at 535nm. The level of malondialdehyde in the HER2-positive BT-474, SK-BR-3, and MDA-MB-453 was significantly higher ( $p < 0.01$ ) than that in HER2-negative MDA-MB-231 and MCF-7 cells, correspondingly ( $p < 0.05$ ). (Figure 2)



**Figure 1.** ROS level of HER2-positive (BT-474, SK-BR-3, and MDA-MB-453) and HER2-negative (MDA-MB-231 and MCF-7) breast cancer cell lines. Each column represents the mean value  $\pm$  standard deviation of the three independent experiments. ( $p < 0.01$ ). As control, DCFH-DA solution without cells was used in this experiment. The ROS level in the HER2-positive cells was significantly higher as compared with the HER2-negative cells. ( $p < 0.05$ )



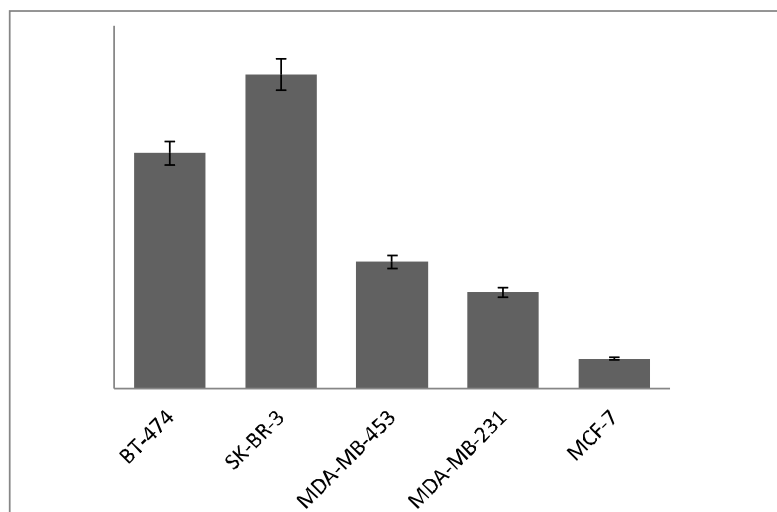
**Figure 2.** Malondialdehyde level of HER2 positive (BT-474, SK-BR-3, and MDA-MB-453) and HER2 negative (MDA-MB-231 and MCF7) breast cancer cell lines. Each column represents the mean  $\pm$  standard deviation of three individual experiments. ( $p < 0.01$ ). The malondialdehyde level in the HER2 positive-cells was significantly higher as compared with the HER2-negative. ( $p < 0.05$ )

#### Determination of protein carbonyl content of cells

As a consequence of reactive oxygen species production, cellular protein oxidation results in the inactivation of protein functions. Direct oxidation of lysine, arginine, proline, and threonine (i.e., the primary protein oxidation) amino acids or addition of the reactive aldehyde to the amino acids side chains (secondary protein oxidation) causes the formation of reactive ketones that react with 2,4-dinitrophenylhydrazine (DNPH) to form detectable hydrazones. The obtained results indicate that protein carbonyl levels in HER2-positive cells, BT-474, SK-BR-3, and MDA-MB-453 are significantly higher ( $p < 0.01$ ) than HER2-negative cells, including MDA-MB-231 and MCF-7 cells ( $p < 0.05$ ). (Figure 3)

#### Discussion

Breast cancer is the most common type of cancer among women worldwide and the second leading cause of cancer mortality among women in developing countries, and a leading cause of death even in the developed countries<sup>19,21</sup> Almost one among the eight women in the US will develop invasive breast cancer during their lifetime<sup>22</sup> The HER2 transmembrane receptor tyrosine kinase (RTK) gene has been found to be amplified in approximately 15–30% of invasive breast cancers. Breast tumors with HER2 overexpression are more invasive and resistant to anticancer therapies than breast tumors negative for this gene.<sup>23</sup>



**Figure 3.** Protein carbonyls levels of BT-474, SK-BR-3, MDA-MB-453, MDA-MB-231, and MCF7 breast cancer cell lines. Each column represents the mean value  $\pm$  standard deviation of three individual experiments ( $p < 0.01$ ). The protein carbonyl level in the HER2 positive cells (BT-474, SK-BR-3, and MDA-MB-453) was significantly higher than HER2 negative (MDA-MB-231 and MCF7) cells. ( $p < 0.05$ )





While there is considerable evidence suggesting that cancer cells have elevated levels of reactive oxygen species, ROS's status in HER2 positive cells has not received much research attention. The elevated levels of ROS in cancer cells act as a second messenger involved in the induction of signaling pathways, resulting in many biological responses, such as proliferation, apoptosis, cell survival, etc.<sup>24</sup>. In the present study, we evaluated the level of ROS, malondialdehyde, and protein carbonyl content as the markers of the oxidative stress in the HER2 positive cells (i.e., BT-474, SK-BR-3, and MDA-MB-453) as well as HER2 negative breast cancer cell lines (i.e., MDA-MB-231, MCF-7). Our findings indicate that as the oxidative stress markers increase, the levels of ROS (Figure 1), malondialdehyde (Figure 2), and protein carbonylation (Figure 3) are significantly increased in the HER2 positive breast cancer cells (i.e., BT-474, SK-BR-3, and MDA-MB-453) as compared with the levels of such markers in the HER2 negative cells including MDA-MB-231 and MCF-7 cells.

There is evidence indicating an association between the HER2 receptor and oxidative stress signaling. Several ROS, in particular, hydrogen peroxide can act as the modulator of PI3K/Akt and p38 MAPK pathways. These pathways are activated by the HER2 receptor<sup>25</sup>. Akt or protein kinase B is one of the important downstream signaling proteins of the HER2 receptor. Up-regulation and aberrant activation of the Akt have been identified in many cancerous cells, such as breast cancer cells<sup>26,27</sup>. Moreover, recent studies have shown that Akt is involved in regulating intracellular reactive oxygen species by stimulating glycolysis and oxidative phosphorylation in the mitochondria via phosphorylation of FoxO transcription factor, ERK1/2, and Rac signaling pathway<sup>28,29</sup>. This evidence supports our finding of the increasing levels of ROS in the HER2 positive breast cancer cells.

Gupta et al., in 2012, showed the positive roles of ROS in tumorigenesis, prevention, and therapy, and by comparing our results with them, we may assume that at lower ROS levels, cell growth and antioxidant production are stimulated. However, at the threshold and higher ROS levels, the cells' antioxidant defense would be exhausted and lead the cells to programmed cell death. In HER2 positive cell lines with high ROS levels, the threshold levels are lower than those in the HER2 negative breast cancer cells<sup>30</sup>.

In conclusion, the findings of this study would predict that HER2 positive breast cancer cell lines including HER2-positive BT-474, SK-BR-3, and MDA-MB-453 with high ROS levels should be more sensitive to the induction of apoptosis by ROS generative agents and may also provide a novel chemotherapeutic means for developing drugs to eliminate HER2 positive breast cancer cells ( $p < 0.05$ ).

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### Conflict of Interest

None.

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