The Effect of Treatment on HIF-1 Alpha Gene Expression, Cell Growth and Apoptosis in Breast Cancer MCF-7 Cell Line

Doaa S. Soliman¹, Salwa Elsayed Mohamed², Wael H. Roshy³, Wael S. Abdel-Mageed⁴ Ibrahim Helmy⁵ and Khalil Halfawy⁶

¹Molecular Biology Department, Faculty of Sciences, Alexandria University, Egypt.
²Molecular Cell Biology and Molecular Biology Department, Genetic Engineering and Biotechnology Research Institute, (GEBRI) University of Sadat City, Egypt.
³Central Public Health Laboratories, Ministry of Health and Population, Cairo, Egypt.
⁴Genetics Department, Faculty of Agriculture, Beni-Suif University, Egypt.
⁵Biochemistry Department, Faculty of Science, Kafr El sheikh University, Egypt.
⁶Molecular Genetics and Molecular Biology Department, Genetic engineering and Biotechnology Research Institute, Sadat City University, Egypt.

Received: 04 May 2023       Accepted: 05 June 2023       Published: 20 July 2023

ABSTRACT

Background and Objectives: Breast cancer is the most frequent cancer in women globally, the second greatest cause of cancer mortality in women after lung cancer, and the fifth highest cause of death from cancer illnesses overall. Per the World Health Organization (WHO) estimates from 2012, accounting for 23% of all cancer deaths. Breast Cancer was the top tumor among women in all cancer incidences in the Middle East, accounting for 27.7 percent to 38.2 percent of all recorded tumors. Breast cancer is mostly caused by hereditary mutations in the BRCA1 and BRCA2 breast cancer susceptibility genes. Methodology: To achieve this aim we conducted the study on MCF-7 cell lines and evaluated the cytotoxicity of different concentration of Curcuma Longa Extract, on the expression of genes (HIF-1α, HIF-1β, BAX and BCL-2). Results: The results showed that the different concentration of Curcuma Longa Extract inhibited the proliferation of the Breast cancer cells (MCF-7) through upregulation Pro-Apoptotic gene BAX and downregulation of gene Anti-Apoptotic BCL-2 and also downregulate HIF-1α and HIF-1β as a result cancer pathways have been arrested. We investigated the ability of Curcuma Longa Extract to inhibit MCF-7 cell lines growth by cytotoxicity assay after the MCF-7 cell lines were treated with various concentrations of Curcuma Longa Extract (25-50 µM) for 24 and 48h. In the viability of MCF-7 cell lines for 24 and 48 h. All concentrations were able to inhibit the proliferation of the cancer cells.

Keywords: Breast cancer, hereditary mutations, cytotoxicity, Curcuma Longa Extract

1. Introduction

Breast cancer is a common cause of malignancy in women and has a high risk regardless of a patient's race or ethnicity. According to recent statistics, breast cancer which accounts for 25% of all malignancies diagnosed each year is the leading cause of death for women globally (Wang and Xu, 2019).

Cancer is a genetic disorder that can arise from a combination of external factors acting in concert with internal genetic changes. This cancerous condition is brought on by a somatic DNA mutation at the cellular level, which is followed by exposure to carcinogens (Gupta and Prakash, 2014). Observational studies have shown that hormone replacement medication, food, lifestyle, and chemical exposure can all have an impact on normal "epigenetic and endocrine signalling," which can then result in the development and progression of breast cancer (Pan et al., 2014).

Corresponding Author: Doaa S. Soliman, Molecular Biology Department, Faculty of Sciences, Alexandria University, Egypt.
A common symptom of solid tumours is hypoxia, or a lack of oxygen in the cells. This is because solid tumours outgrow their pre-existing vasculature. While normal tissues have an oxygen tension of about 7%, solid tumours have an oxygen tension of about 1.3%. Tumour cells may adapt to low oxygen settings or produce vascularization to survive in hypoxic situations (Muz et al., 2015). Vascular endothelial growth factor (VEGF), which has been the subject of the most in-depth research into cancer angiogenesis, is currently being treated using the anti-VEGF antibody AvastinTM (Loizzi et al., 2017).

The transcriptional regulator hypoxia-inducible factor-1 (HIF-1) is a key mechanism by which cancer cells adapt to hypoxia. The two components that make up HIF-1 are HIF-1α and HIF-1β. HIF-1 is not impacted by oxygen levels since it is created constitutively, in contrast to HIF-1 subunits that are oxygen-regulated. In a number of cell types, the HIF-1 component with the highest degree of expression controls oxygen homeostasis (Choi et al., 2011).

A rising body of evidence points to the importance of the low oxygen microenvironment in regulating the growth and spread of breast cancer, which is crucial throughout cancer formation. The (HIF-1), a key player in the control of the hypoxia response, has been extensively studied during these processes (Liu et al., 2015).

The majority of human solid tumours respond to decreased O2 availability primarily through controlling the expression of over 1000 target genes through the actions of "two hypoxia-inducible factors (HIF-1 and HIF-2)" (Semenza, 2012). According to Zhong et al. (1999), HIF-1 overexpression has been confirmed in a number of solid tumours, including brain, ovarian, bladder, lung, uterine, breast, colon, pancreatic, prostate, and renal cancer. HIF-1 is a heterodimeric transcription factor made up of a precisely regulated HIF-1 subunit and a constitutively expressed HIF-1 component, commonly referred to as "the aryl hydrocarbon receptor nuclear translocator [ARNT]" (Knowles, 2022).

The majority of solid tumors include hypoxic zones where greater cell proliferation promotes higher oxygen demand. The situation is made worse when cancer cells are isolated and far from a working blood artery, further limiting oxygen supply (Bader et al., 2020). The transcription of hypoxia-inducible factors is an essential mechanism that facilitates cell adaptation to hypoxic environments (HIFs). Many genes involved in breast cancer cell invasion and metastasis are transcriptionally regulated by hypoxia-inducible factors (Jun et al., 2017).

Patients are more likely to get metastases if HIF expression is highly expressed in their primary tumour biopsies. The current study will highlight the potential function of hypoxia in breast cancer metastasis to bone by taking into account the regulation of several metastatic process stages, including invasion, migration, margination, and extravasation (Gilkes, 2016).

A crucial part of the Curcuma Longa Extract oids family is curcuma longa. It is made from the "rhizome of Curcuma longa L." and goes by the name diferuloylmethane. It was discovered in 1815, but Roughley and Whiting defined its chemical structure in 1973. Its melting point ranges from "176 °C to 177" °C (Mbese et al., 2019). Curcuma longa extract, one of turmeric's most potent, secure, benign, and substantial bioactive components, has a variety of biological functions. Low bioavailability and absorption rates are the main issues with curcuma longa extract (Segneanu et al., 2022).

Due to this, numerous scientists are working to improve Curcuma longa's bioavailability, therapeutic effectiveness, and pharmacological qualities for the treatment of human illnesses utilising a variety of techniques, such as the development of innovative Curcuma longa Extract (Sohn et al., 2021). Curcuma longa extract underwent structural change, resulting in molecules with a wide range of biological activities that can be used to treat a variety of diseases, including cardiovascular disease, diabetes, neurological disorders, and other maladies. As anticancer, anti-inflammatory, antibacterial, and antioxidant agents, curcuma longa extract and its ingredients are frequently used by medical professionals (Mbese et al., 2019).

2. Material and Methods

Preparation of Curcuma Longa

Curcuma longa (Turmeric) root was selected based on their ethnomedical importance. Healthy disease-free roots and seeds were purchased from local market in Egypt. The plant materials were dried and pulverized. A weight of 40 mg of well air-dried powder of Curcuma longa roots was infused
in distal water (100ml) until complete exhaustion. The infusion was filtered through four-layered muslin cloth. Total concentration of obtained extract was 40 mg/L that was stored at 4°C till further use.

**Cell line and Cell culture**

Human breast cancer cell line, MCF7, was obtained from central public health laboratories in Egypt (CPHL). The cells were cultivated in T75 tissue culture flasks in low glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 μg/mL penicillin, 100 μg/mL streptomycin, 2 mM/L-glutamine and incubated in a 95%humidified incubator containing 5% CO2 at 37°C. Now cells ready for treatment with curcumin.

**MTT Assays**

To evaluate the in vitro Cytotoxicity of the Curcuma Longa extract, the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay was performed as previously described. cells were seeded in 96-well plates in DMEM supplemented with 10% fetal bovine serum, and 1% antibiotic antymycotic mixture. cell cultured overnight, the growth medium was removed from each well and the cells washed with 1X phosphate buffered saline (PBS). Different concentrations of Curcuma Long extract starting from 0.4 μg/ml were two-fold serially diluted in DMEM then added to cultured cells in 96-well plate in triplicate and incubated for 24 h post treatment to determine the cytotoxic concentration 50 (CC50). The medium was then discarded, and the cells washed with 1X PBS adding MTT solution (20 µL/well of 5 mg/ml stock solution) and incubated at 37 °C for 4 h till formulation of formazan crystals. Crystals were dissolved using a volume of 200 µL of dimethyl sulfoxide (DMSO) and the absorbance measured at λ max 540 nm using an ELISA microplate reader. Finally, the percentage of cytotoxicity compared to the untreated cells was determined.

\[
\% \text{ Cytotoxicity} = \frac{\text{Absorbance of cell without treatment} - \text{Absorbance of cell with treatment}}{\text{Absorbance of cell without treatment}} \times 100
\]

The total RNA was then extracted from cells using Qiagen extraction kit according to the manufacturer’s protocol (Qiagen, Hilden, Germany). Subsequently, 500 ng of the purified RNA were used to synthesize the complementary DNA (cDNA) with random hexamer primers (Thermo Scientific) and (HERA SYBR® green RT-qPCR kit) Reverse Transcriptase (Thermo Scientific) according to the manufacturer’s protocol. The quantitative real-time PCR (qRT-PCR) reaction mixture (25 μl) comprises the following: 0.5 μl of cDNA template, 12.5 μl of Maxima SYBR green PCR master mix (Thermo Scientific) and 1 μl of each primer (100 μM forward and reverse primers). Reactions were run in triplicate on Applied Biosystems 7500 real-time PCR system (Applied Biosystems, Foster City, California, USA). The cycling conditions were as follows: 2 min at 50 °C, 2 min at 95 °C, cDNA were amplified by 45 cycles of PCR, with each cycle consisting of 30 s at 94 °C, 30 s at 52 °C, and 30 s at 72 °C.

The primer sequences were as follows:

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIf-1α (Forward)</td>
<td>(5'-GACAAGCCACCTGAGGAG-3')</td>
</tr>
<tr>
<td>HIf-1α (Reverse)</td>
<td>(5'-CCA TTA ACG CCG AGGC-3')</td>
</tr>
<tr>
<td>HIf-1β (Forward)</td>
<td>(5'-CCCAGTCACCTTTCTCCAC-3')</td>
</tr>
<tr>
<td>HIf-1β (Reverse)</td>
<td>(5'-GAAGACCTCTCAGCCTCCTC-3')</td>
</tr>
<tr>
<td>BAX (Forward)</td>
<td>(5'-GTT TCA TCC AGG ATC GAG CAG-3')</td>
</tr>
<tr>
<td>BAX(Reverse)</td>
<td>(5'-CAT CTT CTT CCAGATGCT GA-3')</td>
</tr>
<tr>
<td>BCL-2 (Forward)</td>
<td>(5'-CCT GTG GAT GAC TGA GTACC-3')</td>
</tr>
<tr>
<td>BCL-2 (Reverse)</td>
<td>(5'-GAG ACA GCC AGG AGA AAT CA-3')</td>
</tr>
</tbody>
</table>

Ct values were normalized to the values of the control β-actin housekeeping transcripts and log fold change were calculated according to the equation of $2^{-\Delta\Delta ct}$
3. Results
In this study the effect of Curcuma Longa extract was detected on (MCF7) cell line as models of human Breast cancer cell line. B. Actin was used as housekeeping gene (positive control), (HIF-1α), (HIF-1β), BAX and BCL-2.

1-Cytotoxicity of Curcuma Longa extract against MCF-7 Cell Lines Using MTT assay.
Cytotoxicity tests are often designed to evaluate damage to cellular membranes, cell viability, cell death, or cell growth. To assess the cytotoxic effect of two different doses of Curcuma Longa Extract against human breast cancer cells (MCF7), the cells were treated with various concentrations of Curcuma Longa Extract (25 to 50 µM). The MTT test was used to measure cell viability after 24 hours of incubation. The cytotoxicity assay results are shown in (fig. 1).

Curcuma Longa Extract’s capacity to suppress the proliferation of MCF-7 cell lines was studied using a cytotoxicity test after the MCF-7 cell lines were exposed with various doses of Curcuma Longa Extract (25-50 µM). Figure 1 depicts the concentrations and time-dependent effect of Curcuma on the viability of MCF-7 cell lines treated with various doses of Curcuma (25-50 µM) for 24 and 48 hours. MCF-7 cell lines were tested for vitality after 24 and 48 hours. All amounts inhibited the multiplication of cancer cells (MCF-7) and demonstrated viability (fig.1).

Fig. 1: Effects of different concentrations (25–50 µM) of curcuma on the viability of MCF-7 cell lines treated for 24 h and 48 h, as measured by MT assay.

Molecular Evaluation of HIF-1α under Normoxic Condition (21 % oxygen, 5% CO2) treated with Curcumin 25 µM

Fig. 2: The effect of Curcuma Longa Extract 50 µM on the gene expression of the HIF-1α Gene in MCF-7 Cell Line. The data represent a significant difference (P < 0.05) from control group across
different exposure hours to Curcuma Longa Extract as the time increase the down regulation of the HIF-1α gene was increase.

Molecular Evaluation of HIF-1β under Normoxic Condition (21 % oxygen, 5% CO2) treated with Curcumin 25 µM

![Graph](image1)

**Fig. 3:** The effect of Curcuma Longa Extract 25 µM on the gene expression of the HIF-1α Gene in MCF-7 Cell Line. The data represent a significant difference (P < 0.05) from control group across different exposure hours to Curcuma Longa Extract as the time increase the down regulation of the HIF-1α gene was increase.

Molecular Evaluation of HIF-1α under Normoxic Condition (21 % oxygen, 5% CO2) treated with Curcumin 50 µM

![Graph](image2)

**Fig. 4:** The effect of Curcuma Longa Extract 50 µM on the gene expression of the HIF-1α Gene in MCF-7 Cell Line. The data represent a significant difference from control group across different exposure hours to Curcuma Longa Extract as the time increase the down regulation of the HIF-1α gene was increase.
Molecular Evaluation of HIF-β under Normoxic Condition (21% oxygen, 5% CO2) treated with Curcumin 50 µM

![Molecular Evaluation of HIF-β](image)

**Fig. 5:** The effect of Curcuma Longa Extract 50 µM on the gene expression of the HIF-β Gene in MCF-7 Cell Line. The data represent a significant difference from control group across different exposure hours to Curcuma Longa Extract as the time increase the down regulation of the HIF-β gene was increase.

Molecular Evaluation of HIF-1α under Hypoxic Condition Hypoxic Condition (2% Oxygen, 5% CO2, 3.1 L Nitrogen) with curcuma longa 25 µM

![Molecular Evaluation of HIF-1α](image)

**Fig. 6:** The effect of Curcuma Longa Extract 25µM on the gene expression of the HIF-1α Gene in MCF-7 Cell Line. The data represent a significant difference from control group across different exposure hours to Curcuma Longa Extract as the time increase the down regulation of the HIF-1α gene was increase.
Molecular Evaluation of HIF-β under Hypoxic Condition, Hypoxic Condition (2 % Oxygen, 5% CO2, 3.1 L Nitrogen) treated with curcuma longa extract 25 µM

Fig. 7: the effect of Curcuma Longa Extract 25 µM on the gene expression of the HIF-1α Gene in MCF-7 Cell Line. The data represent a significant difference from control group across different exposure hours to Curcuma Longa Extract as the time increase the down regulation of the HIF-β gene was increase.

Molecular Evaluation of HIF-1α under Hypoxic Condition, Hypoxic Condition (2 % Oxygen, 5% CO2, 3.1 L Nitrogen) treated with curcuma longa extract 50 µM

Fig. 8: The effect of Curcuma Longa Extract 50 µM on the gene expression of the HIF-1α Gene in MCF-7 Cell Line. The data represent a significant difference from control group across different exposure hours to Curcuma Longa Extract as the time increase the down regulation of the HIF-1α gene was increase.
Molecular Evaluation of HIF-1α under Hypoxic Condition, Hypoxic Condition (2 % Oxygen, 5% CO2, 3.1 L Nitrogen) treated with curcuma longa extract 50 µM

![Graph showing Log Fold Change vs Number of Hours for HIF-1β gene expression in MCF-7 Cell Line.](image)

**Fig. 9:** The effect of Curcuma Longa Extract 50 µM on the gene expression of the HIF-β Gene in MCF-7 Cell Line. The data represent a significant difference from control group across different exposure hours to Curcuma Longa Extract as the time increase the down regulation of the HIF-β gene was increased.

4. Discussion

Breast cancer is the most frequent cancer in women globally, the second greatest cause of cancer mortality in women after lung cancer, and the fifth highest cause of death from cancer illnesses overall. Per the World Health Organization (WHO) estimates from 2012, accounting for 23% of all cancer deaths. Breast Cancer was the top tumor among women in all cancer incidences in the Middle East, accounting for 27.7 percent to 38.2 percent of all recorded tumors. Breast cancer is mostly caused by hereditary mutations in the BRCA1 and BRCA2 breast cancer susceptibility genes. Gene expression mutations account for around 5-10% of all breast cancer cases. Other recognized risk factors for breast cancer include obesity and the use of hormone treatments (progestin and estrogen). Breast cancer is treated with surgery, radiation treatment, and chemotherapy, however radiation and chemotherapy can harm healthy cells. "Gene therapy, monoclonal antibodies, Adjuvant therapy, and enzyme inhibitors", and are some of the innovative treatments for breast cancer that have recently been studied. Understanding how oncogenes affect cell signaling and metabolism to allow cell change and target them has recently aroused the scientific community's interest.

The hypoxia-inducible factor-1 (HIF-1) transcription factor is a heterodimeric transcription factor. The primary molecule directing the cellular response to hypoxia is HIF-1. It assists hypoxic tumor cells in shifting "glucose metabolism from the more efficient oxidative phosphorylation to the less efficient glycolytic route" in order to sustain energy production (the Warburg effect) (Koppenol Bounds and Dang 2011). Activated HIF-1 plays a critical role in tumor cell adaptation to oxygen fluctuations via transcriptional activation of over 100 downstream genes that regulate important biological processes necessary for tumor survival and growth. These genes are involved in the metabolism of glucose, cell proliferation, migration, and angiogenesis. Chemotherapy resistance and greater patient mortality are related with increased transcription of HIF target genes in breast tumor (Samanta et al., 2014).

Breast cancer is distinguished by a sequence of genetic alterations, making it an attractive candidate for gene therapy administration. Cancer gene therapy has recently expanded to include the introduction of correcting genes, cytotoxic genes, siRNA or shRNA interference treatments, microRNA up- or downregulation, and DNA immunization towards overexpressed tumor antigens (McCrudden and McCarthy, 2014). Loss of HIF-1 activity in many cancers inhibits angiogenesis, resulting in decreased tumor bulk and metastasis. Sunitinib, in conjunction with an HIF-1 dimerization inhibitor (acriflavine), was shown to effectively limit tumor growth (Yin et al., 2015).
"Apoptosis" is the process through which abnormal cells die. The capacity of a chemopreventive drug to elicit apoptosis as a strategy for tumor suppression is a watershed moment in determining its efficacy. The mitochondrial apoptotic cascade is begun within the cell by the release of pro-apoptotic proteins from the mitochondria, which activate caspase proteases, resulting in apoptosis. During p53-mediated apoptosis, the pro-apoptotic gene Bax is up-regulated in various systems. During apoptosis, the Bax gene is up-regulated while the Bcl-2 gene is down-regulated (Elmore, 2007).

In conclusion, it was found that curcuma Longa extract had cytotoxic effect on MCF-7 cells. MCF-7 Cell line was treated with two different concentrations of Curcuma Longa Extract 25 µM and 50 µM targeting HIF-1α and HIF-1β under Hypoxic and Normoxic Conditions, in conclusion this study found that Curcuma Longa Extract down regulates the HIFs genes under hypoxic and normoxic conditions. This make curcuma a strong anti-cancer Agent and induced upregulation of Bax (proapoptotic genes) and downregulation (anti-Apoptotic genes) of the BCL-2 gene.

References


