

## ORIGINAL ARTICLE



Converging Healthcare &amp; Technology

## INTERNATIONAL JOURNAL OF CONVERGENCE IN HEALTHCARE

Published by  
IJCIH & Pratyaksh Medicare LLP

www.ijcih.com

## Effects of Reactive Oxygen Species in p16 Expression and its Modulation by Curcumin in MCF-7 Breast Cancer Cell Line

**Soma Bhattacharjee***Associate Editor, International Journal of Physiology, Noida (UP)*

### Abstract

Cancer is a group disease involving abnormal cell growth with the potential spread to other parts of the body. Cancer is also known as a malignant tumor (National cancer institute 2014). A neoplasm or tumor is a group of cells that have undergone unregulated growth, and will often form a mass or lump, but may be distributed diffusely. The genetic changes that contribute to cancer tend to affect three main types of genes- proto-oncogenes, tumor suppressor genes and DNA repair genes

**Objectives:** To investigate the role of p16 protein expression and to see the effect of curcumin in ROS affected MCF-7 cells by western blotting.

Keeping the background in mind, the present study were designed to analysis the role of p16 protein expression and to see the effect of curcumin in ROS affected MCF-7 cells by western blotting. Also see the possible cellular mechanisms by evaluating cell cycle distribution and intracellular ROS generation.

**Keywords:** *Reactive Oxygen Species, Breast Cancer, Cell line, Circumin, p16 gene.*

### Introduction

Cancer is a group disease involving abnormal cell growth with the potential spread to other parts of the body. Cancer is also known as a malignant tumor (National cancer institute 2014). A neoplasm or tumor is a group of cells that have undergone unregulated growth, and will often form a mass or lump, but may be distributed diffusely.

The genetic changes that contribute to cancer tend to affect three main types of genes- proto-oncogenes, tumor

suppressor genes and DNA repair genes. These changes are sometimes called “drivers of cancer”. Proto-oncogene is involved in normal cell growth and division. However, when these genes are altered in certain ways or are more active than normal, they may become cancer-causing genes (or oncogenes), allowing cells to grow and survive when they should not (Vlahopoulos SA et al., 2008)<sup>1</sup>.

Breast cancer is most commonly diagnosed cancer and leading cause of cancer death in female worldwide [Jemal A et al., 2011]<sup>2</sup>. In India, breast cancer is the leading cancer in women, showing rising trend, especially in urban India. Among Indian women, breast cancer and uterine cervix cancer together account for about 50% of all the other cancers. According to the recent nationwide figures, there were about 1,44,937 new cases and approximately 70,218 deaths due to breast cancer [WHO, 2012]<sup>3</sup>. The incidence of breast cancer is increasing in the

---

**Corresponding Author:****Soma Bhattacharjee**Associate Editor, International Journal of  
Physiology, Noida (UP)

email id: soma.s.bhattacharya03@gmail.com

developing world due to increase life expectancy, increase urbanization and adoption of western lifestyles. Although some risk reduction might be achieved with prevention, these strategies cannot eliminate the majority of breast cancers that develop in low- and middle-income countries where breast cancer is diagnosed in very late stages. Therefore, early detection in order to improve breast cancer outcome and survival remains the cornerstone of breast cancer control (WHO 2013)<sup>3</sup>.

p16 (also known as cyclin-dependent kinase inhibitor 2A, multiple tumor suppressor 1 and as several other synonyms), is a tumor suppressor protein, that in humans is encoded by the CDKN2A gene.[ Nobori T et al., 1994; Stone S et al., 1995]<sup>4</sup> p16 plays an important role in cell cycle regulation by decelerating cells progression from G1 phase to S phase, and therefore acts as a tumor suppressor that is implicated in the prevention of cancers, notably melanoma, oropharyngeal squamous cell carcinoma, cervical cancer, esophageal and breast cancer. p16 can be used to improve the histological diagnostic accuracy of CIN3. The CDKN2A gene is frequently mutated or deleted in a wide variety of tumors.

Reactive oxygen species (ROS) are chemically reactive chemical species containing oxygen. Examples include peroxides, superoxide, hydroxyl radical, and singlet oxygen [Hayyan M et al., 2016]. In a biological context, ROS are formed as a natural byproduct of the normal metabolism of oxygen and have important roles in cell signaling and homeostasis [Devasagayam T et al., 2004]. However, during times of environmental stress (e.g., UV or heat exposure), ROS levels can increase dramatically. This may result in significant damage to cell structures. Cumulatively, this is known as oxidative

stress. ROS are also generated by exogenous sources such as ionizing radiation [Sosa Torres ME et al., 2015]<sup>5</sup>.

## Review of Literature

The term cancer refers to a group of diseases which share similar characteristics. Cancer can affect all living cells in the body, at all ages and in both genders. The causation is multifactorial and the disease process differs at different sites. Tobacco is the single most important identified risk factor for cancer. The control of cancer requires the effective implementation of knowledge derived from more than two decades of successful research. It is now known that over one-third of cancers are preventable, and one-third potentially curable provided they are diagnosed early in their course. The quality of life of patients with incurable disease can be improved with palliative care. A rational concept to put science into practice has to be formulated to counter this disease. In cancer, even with limited resources, an impact can be achieved if the right priorities and strategies are established and implemented. The carcinogenic agents that people breathe, eat, drink and are otherwise exposed to, largely determine the occurrence of the disease [Piepkorn M et al., 2000]<sup>7</sup>.

**Current Scenario:** India has a National Cancer Control Programmes which was established in 1975–76. This has contributed to the development of Regional Cancer Centers (RCCs), oncology wings in medical colleges and support for purchase of teletherapy machines. The District Cancer Control Programmes was initiated but did not result in sustainable and productive activity [Imran Ali et al., 2011]<sup>8</sup>.

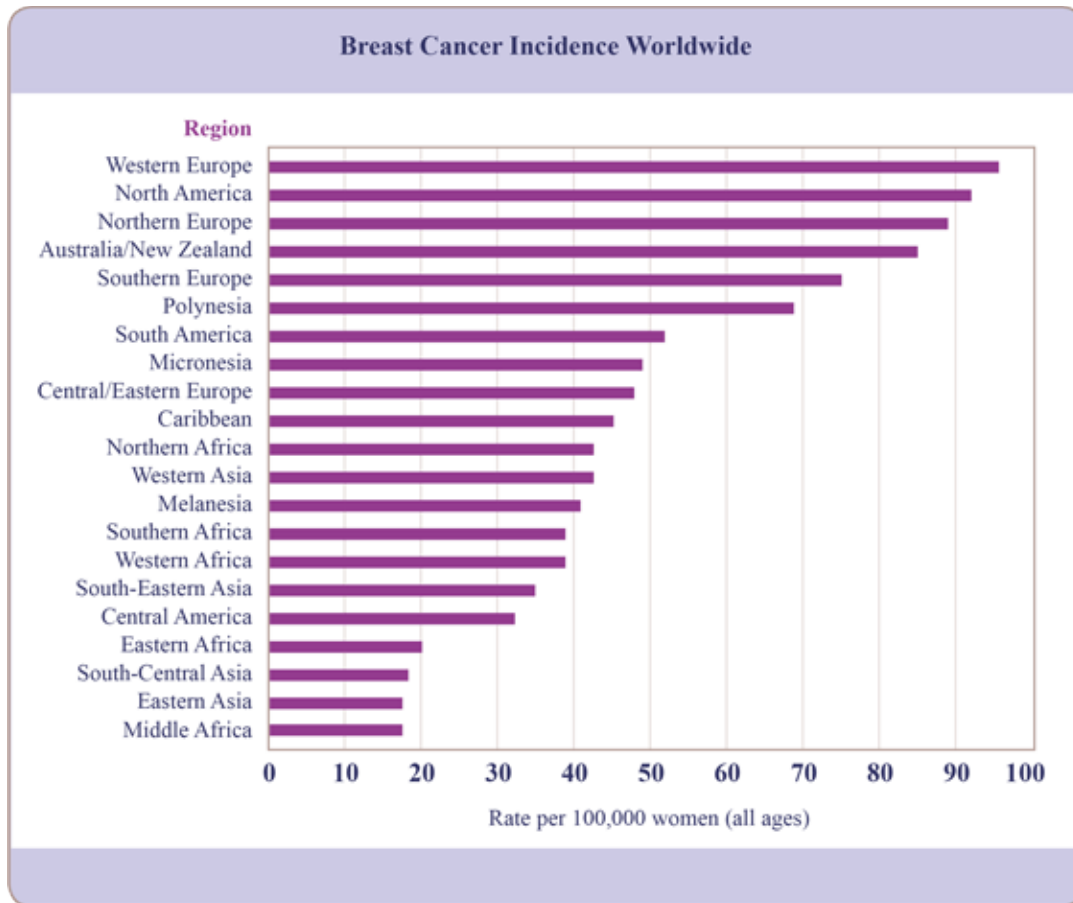


Figure 1: Prevalence of cancer worldwide

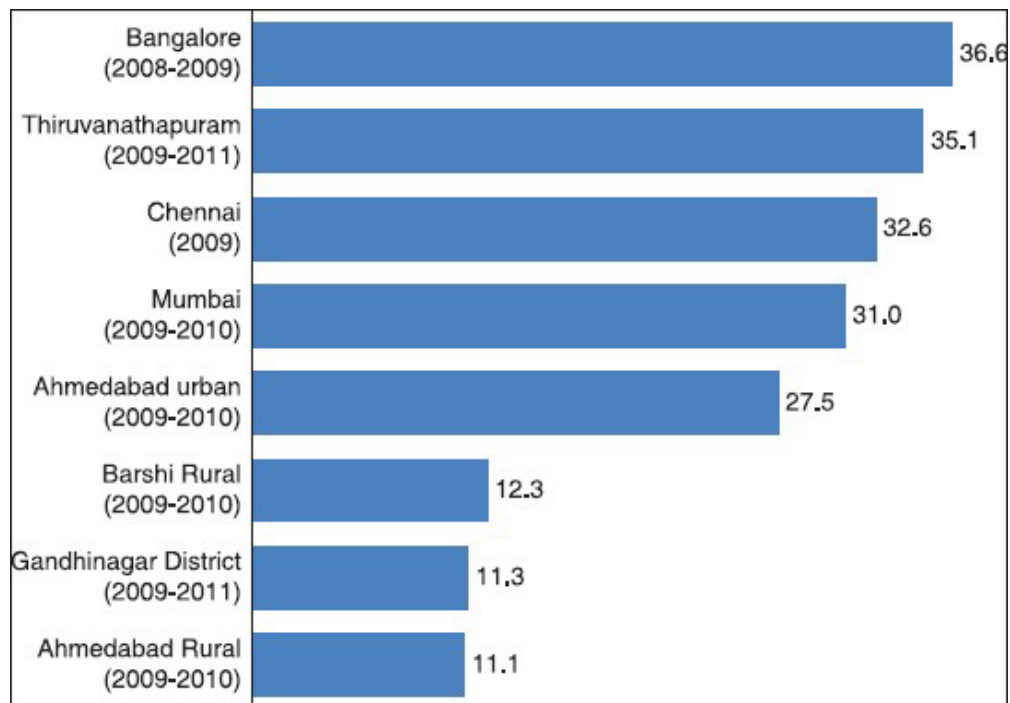


Figure 2: Prevalence of cancer in India

**Cancer Prevention:** There is no uniform cancer prevention strategy for the entire country. Awareness programmes have been undertaken in a few places, but there is no uniform standardized information, education and communication (IEC) strategy for cancer prevention. There is no education on risk factors, early warning signals and their management. Cancer screening is not practiced in an organized fashion in any part of India. There are sporadic attempts at opportunistic interventions and small-scale research studies for field interventions.

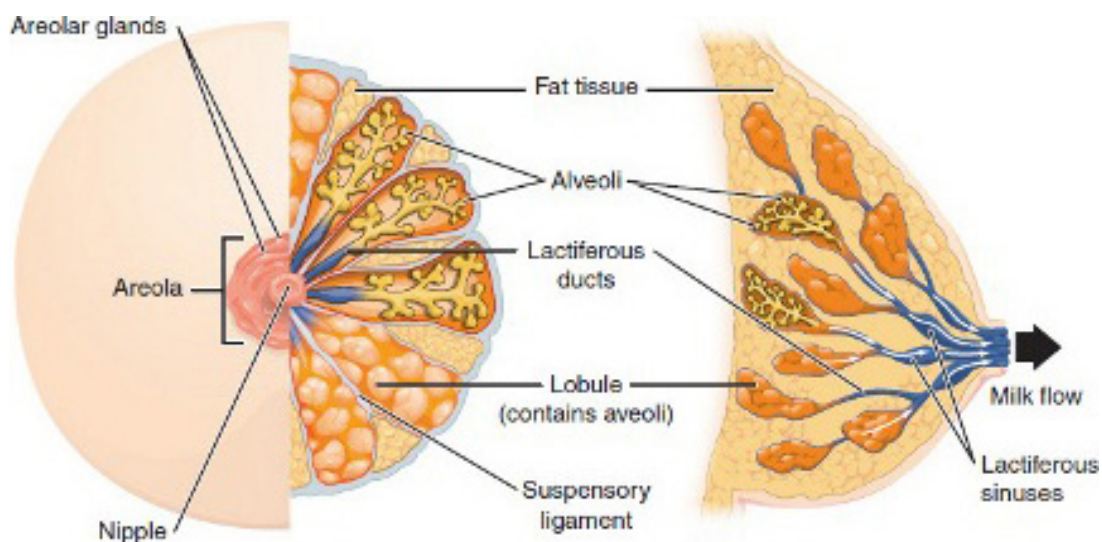
**Cancer Treatment:** Treatment facilities are also mostly limited to urban areas of the country. There are no uniform protocols for management and the availability and affordability of cancer treatment shows wide disparities. The majority of patients with cancer present to a cancer treatment centre in late stages of the disease (80% are advanced) and this adds to the already high morbidity, mortality and expenditure. Treatment results are about 20% less than what is observed for similar conditions in more developed countries, mostly due to late diagnosis and inappropriate treatment. Paediatric cancers are highly curable but this has not been achieved in India due to lack of access to quality care and lack of support systems.

**Anatomy of Normal Breast:** Female's breasts serve as mammary glands which produce and secrete milk. Mature mammary glands are composed of the alveoli lined with milk-secreting cuboidal cells which are surrounded by myoepithelial cells. These alveoli combine to form *lobules*. Each lobule consists of lactiferous duct that drains into nipple. The myoepithelial cells contract

because of oxytocin hormone, excreting the milk secreted by alveolar units into the lobule lumen toward the nipple. Terminal duct lobular units (TDLUs) are the primary unit of the gland, which produce the fatty breast milk. The terminal lactiferous ducts excrete the milk from TDLUs into 4–18 lactiferous ducts, which drain to the nipple; the milk-glands-to-fat ratio is 2:1 in lactating women and 1:1 in non lactating women [Silvrstein M (ed)., 1997]<sup>9</sup>.

**Lymphatic drainage to breast:** Over 75% of the lymph from the breast reaches to the axillary lymph nodes on the same side of the body, whereas 25% of the lymph travels to the parasternal knobs. Some amount of remaining lymph transit to other breast, and to the abdominal lymph knobs. The axillary lymph nodes inclusive of pectoral, subscapular and humeral lymph nodes groups, that conduit to the central axillary lymph nodes.

Most breast tumors' arise from the human mammary epithelium. Estrogen and progesterone receptors play important role in development of mammary gland. Histological studies have shown that most human breast tumors' appear to be arising from terminal duct lobular units and presents morphological characteristics of luminal epithelial cells. Moreover, most human breast tumours show the biochemical features of luminal cells. Human tumors also contain estrogen receptors and progesterone receptors in the normal breast, which are expressed only in the luminal epithelial cell. Luminal epithelial cells are considered the primary targets for malignant transformation and tumor formation



**Figure 3: Structure of normal breast**

**Receptors responsible for the development of mammary glands:** The Estrogen Receptor (ER) is a type of nuclear receptors which acts as ligand-inducible transcription factors. These nuclear receptor proteins, including the ER, are composed of distinct functional domains. The ER binds via the DNA-binding domain to cognate DNA estrogen response in the promoter region of the genes. Transcriptional activation of ER is likely to acts through a conformational change in AF-2 upon ligand binding, while ligand-independent activation by phosphorylation from other signaling pathways occurs through AF-1. The ER influences gene expression by interacting directly with transcriptional components and/or by acting in concert with specific receptor-associated coactivator or co-repressor proteins that likely recognize certain ER conformations<sup>10</sup>. Estradiol and progesterone are major regulators of mammary epithelial cell proliferation. At puberty, the epithelial cells segregate and emigrate into the stromal fat pad as terminal end buds (TEBs) in a process called ductal elongation. This process appears to be induced by estradiol and growth hormone, which may signal through its local mediator, insulin-like growth factor-1 (IGF-1). Estradiol and progesterone can stimulate the expression of growth factor genes including EGF, transforming growth factor- $\alpha$  (TGF- $\alpha$ ), amphiregulin and IGFs, needed for mammary gland development. As a result, estradiol can indirectly activate tyrosine kinase receptors by inducing expression of their ligands, thereby amplifying the mitogenic signal on the epithelial cap cells. Epidermal growth factor receptor (EGFR) and erbB2 are also present on ER-containing stromal cells; therefore

EGF could enhance the mitogenic signal coming from the stroma. Growth factors acting through their tyrosine kinase receptors have been shown to activate the ER in a ligand-independent manner, and activate the ER in a synergistic fashion with estradiol using the MAP kinase pathway. In addition, activation of protein kinase C pathways can indirectly affect ER activity by altering the stability of ER mRNA.

**Molecular Subtype Classification of Breast Cancer:** The commonly recognized molecular subtypes with distinct molecular features of mammary cells in breast carcinomas are Luminal A, Luminal B, Basal like and HER2/neu positive. HER2/neu is a proto-oncogene that encodes a 185-kDa protein which is transmembrane tyrosine kinase receptors. When activated, further activates different signaling pathways that increase a various cellular functions that include promoting cell division and inhibiting apoptosis. Tumors from mutant BRCA1 carriers are the basal-like tumor subtype. Basal-like tumors represents the dysfunction in the BRCA1 pathway.

The ER-positive luminal tumours previously had shown expression patterns similar to the normal luminal epithelial cells of the mammary gland, to ER as well as genes in association with an active ER pathway. Luminal A tumours have increased expression of ER-activated genes and low proliferation of related genes; whereas luminal B associated malignancies presents with higher histological grade, shows increased proliferation rates with significant worse prognosis than luminal A tumours.

Subtypes	Characteristics
Luminal A	ER (+) and/or PR(+), HER2 (-)
Luminal B	ER (+) and/or PR(+), HER2 (+)
HER2 overexpressing	ER (-), PR(-), HER2 (+)
Basal-like	ER (-), PR(-), HER2 (-), cytokeratin 5/6 (+) and/or EGFR (+)
Normal breast-like	Expressing genes characteristic of adipose tissue

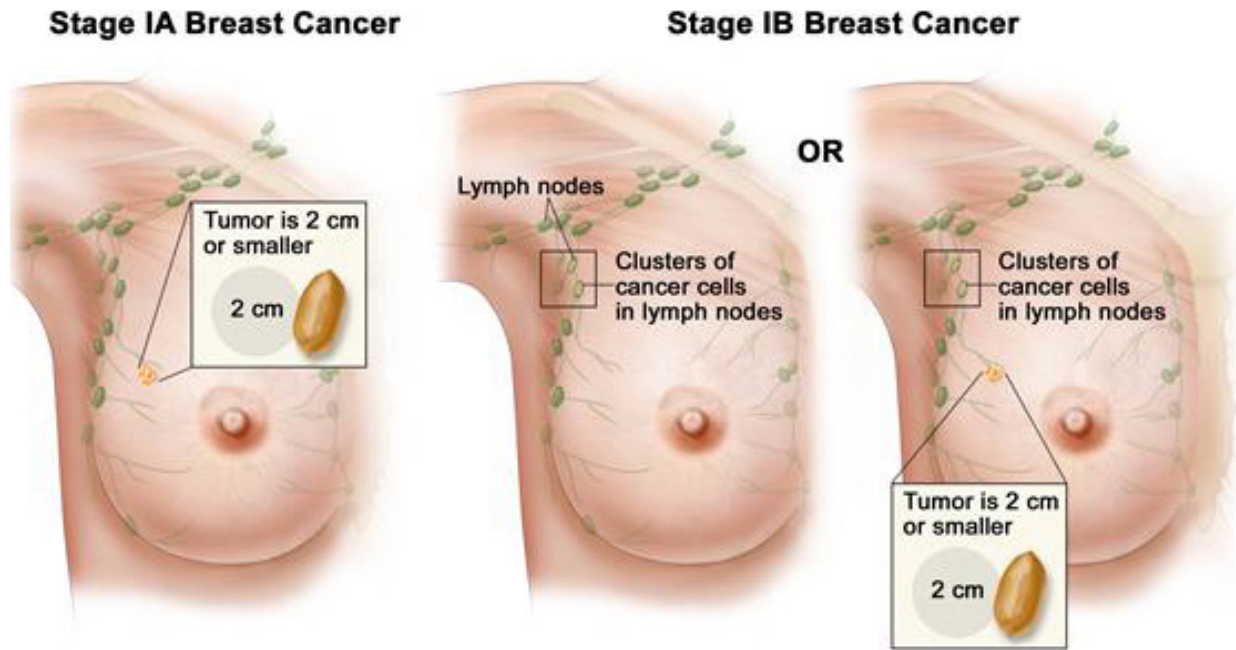
**Table 1: Subtypes and characteristics**



**Histological Type of Breast Cancer:** Histological type refers to the growth pattern of tumours. These may include malignancies of invasive ductal, invasive lobular, tubular, invasive cribriform, malignancies of neuroendocrine, and apocrine, adenoid malignancy, medullary, and malignancies of micro papillary, and metaplastic type. Approximately 25% of invasive breast

cancers are ‘special types’, and distinguished by distinctive growth patterns and cytological features.

**Stages of Breast Cancer:** Staging is the process of finding out how widespread the cancer is when it is found. The stage is the most important factor in deciding how to treat the cancer and determining how successful treatment might be.



**Figure 4: Breast cancer stages (1 A & B).**

**Stage 0 (Tis, N0, and M0):** This is *ductal carcinoma in situ (DCIS)*, a pre-cancer of the breast. Many consider DCIS the earliest form of breast cancer. In DCIS, cancer cells are still within a duct and have not invaded deeper into the surrounding fatty breast tissue.

*Lobular carcinoma in situ (LCIS)* sometimes also is classified as stage 0 breast cancers, but most oncologists believe it is not a true cancer or pre-cancer. Paget disease of the nipple (without an underlying tumor mass) is also stage 0. In all cases the cancer has not spread to lymph nodes or distant sites.

**Stage IA (T1, N0, M0):** The tumor is 2 cm (about 3/4 of an inch) or less across (T1) and has not spread to lymph nodes (N0) or distant sites (M0).

**Stage IB (T0 or T1, N1mi, M0):** The tumor is 2 cm or less across (or is not found) (T0 or T1) with micro metastases in 1 to 3 axillary lymph nodes (the cancer in

the underarm lymph nodes is greater than 0.2mm across and/or more than 200 cells but is not larger than 2 mm (N1mi). The cancer has not spread to distant sites (M0).

**Genetic Cause of Breast Cancer: p16 Gene:** In humans, p16 is encoded by the CDKN2A gene, located on chromosome 9 (9p21.3) p16 is a cyclin-dependent kinase (CDK) inhibitor that slows down the cell cycle by prohibiting progression from G1 phase to S phase. Normally, CDK4/6 binds cyclin D and forms an active protein complex that phosphorylates retinoblastoma protein (pRB). Once phosphorylated, pRB disassociates from the transcription factor E2F1, liberating E2F1 from its cytoplasm bound state allowing it to enter the nucleus. Once in the nucleus, E2F1 promotes the transcription of target genes that are essential for transition from G1 to S phase.

p16 acts as a tumor suppressor by binding to CDK4/6 and preventing its interaction with cyclin D. This

interaction ultimately inhibits the downstream activities of transcription factors, such as E2F1, and arrests cell proliferation. This pathway connects the processes of tumor oncogenesis and senescence, fixing them on opposite ends of a spectrum. On one end, the hypermethylation, mutation, or deletion of p16 leads to downregulation of the gene and can lead to cancer through the dysregulation of cell cycle progression. Conversely, activation of p16 through the ROS pathway, DNA damage, or senescence leads to the buildup of p16 in tissues and is implicated in aging of cells.

Regulation of p16 is complex and involves the interaction of several transcription factors, as well as several proteins involved in epigenetic modification through methylation and repression of the promoter region.

PRC1 and PRC2 are two protein complexes that modify the expression of p16 through the interaction of various transcription factors that execute methylation patterns that can repress transcription of p16. These pathways are activated in cellular response to reduce senescence.

**Reactive oxygen species:** ROS is generated in various metabolic pathways the NADPH oxidation and oxidative-phosphorylation conjugate respiration. Elevated ROS levels causes increased macromolecular damages, specifically damages to DNA. Estrogen contributes in the development of ROS (reactive oxygen species). The primary reason for estrogen promoted ROS is because of mitogenic functions of estrogen is most likely ER dependent. E-ER increases the cellular anabolic metabolism by up-regulating c-Myc. p16 gene is also a regulator of ROS. p16 regulates ROS through regulating NRF2, a critical transcriptional factor of several important anti-oxidant genes. A recent study discovered the activation of PI3K-AKT in promoting a novel function of NRF2 which in turn averts the cell metabolism from aerobic to anaerobic respiration. E-ER apparently activates NRF2 function in averting the cell metabolism pathways as well, leading to alteration in the ROS pressure produced by the mitogenic effect of estrogen In p16 mutant cancer cells, P13K-AT activates NRF2 and perhaps leads to the invariable degradation and regeneration of NRF2.

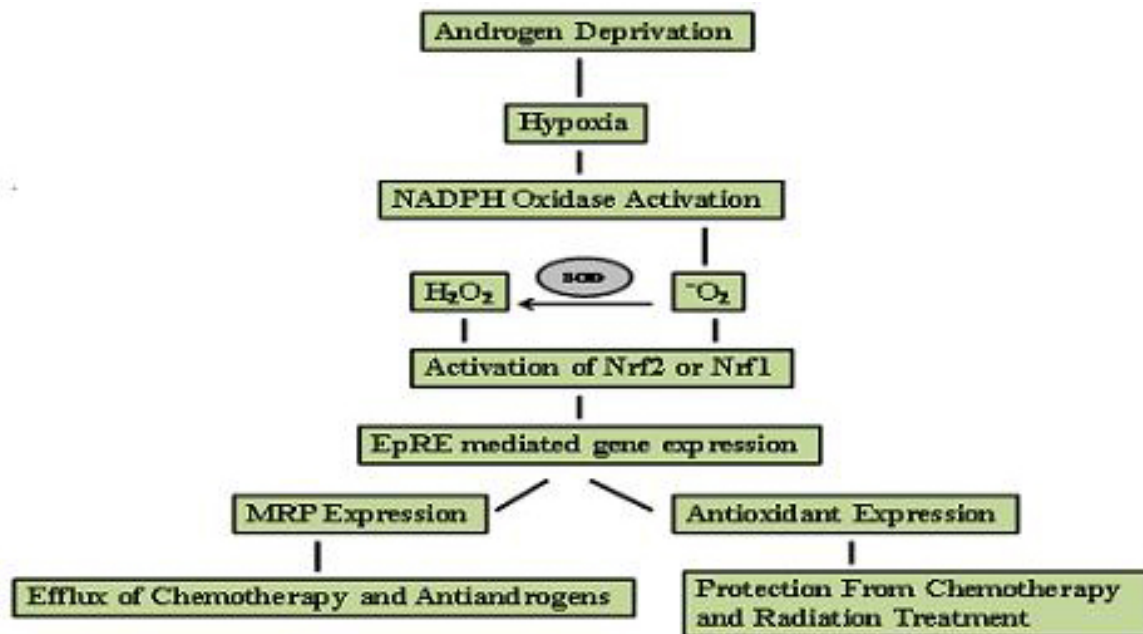


Figure 5: Reactive oxygen species in cancer

**Curcumin:** Curcumin is a symmetric molecule, also known as diferuloyl methane. The IUPAC name of curcumin is (1*E*, 6*E*)-1, 7-bis (4-hydroxy-3-methoxyphenyl)-1, 6 heptadiene-3, 5-dione, with chemical formula  $C_{21}H_{20}O_6$ , and molecular weight of 368.38. It has three chemical entities in its structure: two aromatic ring systems containing o-methoxy phenolic groups, connected by a seven carbon linker consisting of an,  $\alpha$   $\beta$ -unsaturated  $\beta$ -diketone moiety. Curcumin has three reactive functional groups: one diketone moiety, and two phenolic groups. Important chemical reactions associated with the biological activity of curcumin are the hydrogen donation reactions leading to oxidation of curcumin, reversible and irreversible nucleophilic addition (Michael reaction) reactions, hydrolysis, degradation and enzymatic reactions.



Figure 6- Curcumin(*Curcuma Longa*)

## Materials and Method

### Cell Culture

**Cell Revival:-** Prepared 10% DMEM media (45ml DMEM + 5ml FBS + 50 unit penicillin/streptomycin). Thaw the preserved cells in water bath at 37°C. Add 1ml of fresh medium & mix it and transfer it in falcon tube contain 1ml of media. Centrifuged at 400g for 5 min. Discard the supernatant and add 1ml of fresh media and mix it. Transfer 3ml of 10% DMEM in culture flask and

add cell line in flask and keep in  $CO_2$  incubator for overnight at 5%  $CO_2$  at 37°C.

**Cell Passaging:-** Observed the cells attached to the surface of flask and removed the media and added fresh media and kept in  $CO_2$  incubator until use. Cells were splits from one flask to six different flasks for ultra violet exposure. Each flask contained cell count 20,000 cells per flask and allows growing till 70% of cell confluences. Removed the media and washed with PBS and Added 1ml of PBS to each flask and exposed to ultra violet radiation of  $1300\mu J/cm^2$  exposure to each flask for 10min, 20min, 30min, 40min & 50min for 5 flasks respectively and 6<sup>th</sup> left for Control.

**MTT Assay:** MTT assay was done in 96 well plates with  $10^4$  cells per well. Cells were incubated for 24hrs. For loading of curcumin, cells were (in 96 well plate) incubated with curcumin at different concentration of 10,20,30,40, 50 ( $\mu M$ ). After 24hrs of incubation MTT solution (5 mg/mL) was added to each well, and incubated for 4hr in incubator then formazan precipitate formed was dissolved in 100  $\mu L$  MTT lysis buffer and incubated for 1hr, then the absorbance was measured in an 96 well plate reader at 570nm. The cell viability ratio was calculated by the following formula: Cell viability ratio (%) = control treated OD  $\times$  100%. The  $IC_{50}$  (the concentration reducing cell viability by 50%) was calculated from the concentration-response and expressed as  $\mu M$ .

**Preparation of Diacetate Dichloro Fluorescein (DYE):** Prepare 20 $\mu l$  of 1M in DMSO (dimethyl sulfoxide) & then make final concentration of 20mM (20 $\mu l$  of stock solution + 980 $\mu l$  DMSO) (freshly prepared).

**UV Exposure and ROS Generation and quantification:** Cells were treated with UV radiation at  $1300\mu J/cm^2$  for different time interval of 10, 20,30,40,50 mins before the drug treatment. Two groups of flasks were taken for without curcumin treated and with curcumin treated. After ultra violet exposure fluorescent dye DCFDA was added to the each flask and allowed to incubate for 45 min. cells were washed in 1x PBS. Observation was made under fluorescent microscope and microscopic image is taken under 20 xs with excitation wavelength 485nm & emission wavelength 535nm.

Quantification of ROS levels was done in both treated and untreated group flasks with curcumin by 96 wells plate at absorbance of 570nm at Microplate reader.



**Drug Treatment:** After UV exposure to the mcf7 cells at different time interval, curcumin treatment was given at different concentration given below and kept the entire flask for incubation for 24 hrs.

**Table 2: curcumin treatment with different concentration**

Flask	Time Interval	Curcumin (Conc. in $\mu\text{M}$ )
Flask A	Control	0 $\mu\text{M}$
Flask B	10 min	10 $\mu\text{M}$
Flask C	20 min	20 $\mu\text{M}$
Flask D	30 min	30 $\mu\text{M}$
Flask E	40 min	40 $\mu\text{M}$
Flask F	50 min	50 $\mu\text{M}$

**Protein Isolation:** Cells were taken in the eppendroff from each flask and centrifuged at 6000rpm for 1min to

obtain pellet. Pellet is washed in 1xPBS n centrifuged again at 6000rpm for 1min. Discard the PBS and add WHOLE CELL LYSATE (50 $\mu\text{L}$  each) and keep it for incubation for 1 hr at 4 $^{\circ}\text{C}$  with interval tapping at every 10 min. centrifuge it at 12000rpm for 20 min at 4 $^{\circ}\text{C}$  and store at 20 $^{\circ}\text{C}$ .

**SDS-PAGE Western blotting:** It is sodium dodecyl sulfate- polyacrylamide gel electrophoresis technique used to separate biological macromolecules, usually protein, according to their electrophoretic mobility. For proteins, SDS is an anionic detergent applied to protein samples to linearize proteins and to impart a negative charge to linearized proteins.

The protein concentration of the extract was determined by Bradford protein estimation method. The total protein was estimated by 1 $\mu\text{l}$  protein + 49  $\mu\text{l}$  of distilled water + 200 $\mu\text{l}$  Bradford reagent and read at 595nm at Microplate reader.

**Table 3: Concentration of resolving and stacking gel**

Resolving GEL (6%)	Stacking GEL (5%)
30% acrylamide stock(29:1) 1.0ml	30% acrylamide stock(29:1) 0.5ml
1.5nM Tris-cl(ph 8.8) 1.3ml	1.5nM Tris-cl(ph 6.8) 0.38ml
10%SDS 0.050ml	10% SDS 0.03ml
10% Ammonium persulfate(APS) 0.050ml	10% Ammonium persulfate(APS) 0.050ml
N,N,N,N Tetra methylene diamine(TEMED) 0.004ml	N,N,N,N Tetra methylene diamine (TEMED) 0.003ml
Distilled water 2.6ml	Distilled water 2.1ml
Total volume 5ml	Total volume 3ml

**Semi dry transfer:** For semi dry transfer of proteins to a PVDF membrane the following transfer sandwich was made in a semi dry transfer apparatus. Briefly, PVDF membrane was cut in appropriate size (that is size equal to gel)and was kept in methanol for 3min. given a brief rinse in distilled water and finally transferred to 1X transfer buffer (25mM Tris-cl, 250mM glycine, 0.1% SDS , 20% methanol) for 15min. appropriately sized 3mm Watman paper were cut and soaked in 1X transfer buffer. Similarly gel was also transferred to 1X transfer buffer for 15min and the semi dry electroblotting was done by applying a constant current of 1mA/cm<sup>2</sup> in a cold room (4 $^{\circ}\text{C}$ ) for 1hr. while making the transfer sandwich care was taken that no air bubble are trapped in.

**Detection of specific protein:** This is done for detection of a specific protein on the membrane and blocking of all the non-specific sites. It was incubated in a solution of 10% fat-free milk in PBS-T (PBS containing 0.055% Tween 20) at 4 $^{\circ}\text{C}$  for 30 min at a low speed on a rocker platform. The membrane was then washed three times for 5 min each with fresh changes of PBS-T, incubated overnight at 4 $^{\circ}\text{C}$  in primary antibody (p16(sigma Aldrich) and  $\beta$  actin (sigma Aldrich)) specific for the respective protein (1:5000 dilution of 100 $\mu\text{g}/\text{ml}$  solution) (in 5% fat-free milk in PBST-T and kept for incubation in the horseradish peroxide labeled anti-rabbit- IgG (1:1000) (in 5% fat-free milk in PBST-T) for 2hrs at 4 $^{\circ}\text{C}$ . After final washing of the membrane four times each for 5 min

with PBS-T, it was processed for luminol detection system (Santa Cruz biotechnology, Inc, California, and USA).

**Luminol detection:** For luminol detection of protein, the processed membrane was briefly dried on a piece of 3mM whatman paper. In the meantime, equal volume of luminol solution 1 was mixed with an equal volume of luminol solution 2 (Santa Cruz biotechnology, Inc, California, USA). The final volume of the mixture was so made that it was sufficient to cover the membrane completely. Poured the solution on the entire surface of the membrane and immediately proceeded for autoradiograph after covering the membrane with saran wrap. The specific protein bands were identified and a comparison was made between controls and sample.

## Results

**Cell culture:** MCF-7 breast cancer cell lines were cultured using DMEM medium (Figure 14) which contains 10% Fetal Bovine Serum (FBS) and antibiotics penicillin/streptomycin (50units/ml) in cell culture incubator with 5% CO<sub>2</sub>.

**MTT assay:** MTT assay was performed in MCF-7 breast cancer cell lines to see number of viable cells present. MCF-7 cell lines was cultured and maintained in DMEM media at 37°C which is supplemented with 5% CO<sub>2</sub>. MTT assay was performed in 96-well plate; cells were counted in haemocytometer by adding equal volume of tryphan blue dye and cultured cells. 10,000 cells seeded in each wells of 96 well plates and kept in incubator for 24 hours, next day MTT dye (5mg/ml) added to each well and incubated for 4hr then added lysis buffer and incubated for 1hr and read at 570nm wavelength.

**Effects of Reactive Oxygen Species in MCF-7 Breast cancer cell line without curcumin:** In this study we cultured the MCF-7 breast cancer cells and ROS was generated by exposure of cells to UV radiations at 1300 $\mu$ J/m<sup>2</sup> for different time intervals. The levels of ROS were examined under fluorescence microscope after staining with DCFDA dye.

**Western Blotting analysis:** Western blotting was done for the expression analysis of p16 proteins and compare with internal control  $\beta$ -Actin. Whole cell protein was isolated from UV exposed MCF-7 cells these treated cells and quantified by Bradford method. 30 $\mu$ g proteins of each sample were loaded in wells of SDS-PAGE gel and

run on low voltage. Protein bands were transferred in PVDF membrane and expose on x-ray film by using luminal.

**Effect of ROS expression of p16 protein in MCF-7 breast cancer cells:** Further, we checked the expression profile of p16 proteins in UV exposed cells by western blotting at different time interval and showed that the expression of p16 protein is increased in higher exposure time as compared to lower time interval and ROS expression is decreased. The same membrane was re-probed for  $\beta$ -actin antibody as internal loading control and showed that the expression of  $\beta$ -actin is normal.

**[B] Effects of Reactive Oxygen Species in MCF-7 Breast cancer cell line with curcumin:** The different concentration of curcumin treatment cells were exposed to UV radiations at different time intervals. Cells were kept for incubation for overnight at 37°C. Then cells were washed with 1xPBS and DCFDA dye was added and examined under fluorescence microscope.

**Western Blotting Analysis:** Western blotting analysis was done to check the expression of p16 with ROS induced UV exposed MCF-7 cells with curcumin treatment at different concentration. Whole cell protein was isolated from UV exposed MCF-7 cells these treated cells and quantified by Bradford method. 30 $\mu$ g proteins of each sample were loaded in each wells for SDS-PAGE gel and run on low voltage. Protein bands were transferred in PVDF membrane and expose on x-ray film by using luminal.

**Effect of ROS on p16 protein expression in MCF-7 breast cancer cells:** We checked the expression profile of p16 proteins in UV exposed cells by western blotting at different time interval and treated with curcumin anti cancer drug and showed that the expression of p16 protein is increasing in the lower time interval as compared to untreated cells and ROS expression is increased. The same membrane was re-probed by  $\beta$ -actin antibody as internal loading control.

**Comparison between curcumin treated and untreated MCF-7 cells followed byUV exposure:** Further comparative analysis was done of UV induced ROS levels in MCF-7 cells with curcumin treatment and untreated cells. ROS assay was quantified by 96 well plates at Microplate reader at absorbance of 535nm. Curcumin treated cells showed that the expression of p16 protein is increasing in the lower concentration of curcumin as compared to untreated cells and ROS level is

decreased. But without curcumin treated cells showed that the expression of p16 protein is increased in higher time interval and ROS level is decreased.

### Statistical Analysis

Statistical analysis was done by using unpaired t-test. For untreated MCF-7 cells analysis was done between levels of ROS and expression of p16. The set value taken was  $p < 0.05$  and the result was found significant with p value  $< 0.001$ . Similarly statistical analysis done between cells treated with curcumin and p16 expression with set value of  $p < 0.005$  and result was significant with p value  $< 0.001$ .

### Discussion and Conclusion

Breast cancer is most commonly diagnosed cancer and leading cause of cancer death in female worldwide. In India, breast cancer is the leading cancer in women, showing rising trend, especially in urban India. Among Indian women, breast cancer and uterine cervix cancer together account for about 50% of all the other cancers. According to the recent nationwide figures, there were about 1,44,937 new cases and approximately 70,218 deaths due to breast cancer. The incidence of breast cancer is increasing in the developing world due to increase life expectancy, increase urbanization and adoption of western lifestyles.

**Effect of Curcumin levels of ROS generated by UV exposure in MCF7 Breast cancer cells:** ROS are involved in the regulation of many signal transduction pathways as well as cell proliferation and differentiation. In normal cells, cellular redox homeostasis is tightly regulated by the balance between ROS generation and elimination. However, higher levels of ROS were observed in a wide spectrum of cancer cells, which in part is due to the activation of oncogenes, aberrant metabolism, mitochondrial dysfunction and the absence of functional p53. Intriguingly, it is believed that on one hand, ROS contribute to the initiation and progression of cancer, but on the other hand, excessive amounts of ROS act as cellular toxicants which lead to cancer cell growth arrest, apoptosis, senescence or necrosis. It is speculated that the malignant cells which are under increased level of oxidative stress would be more vulnerable to further ROS attack.

The present study the effects of curcumin level on ROS generated by UV exposure in MCF-7 breast cancer cell line. UV radiations elicit the intracellular signaling pathways causing the various functional changes in cells. Studies have evidences that shows UV radiations induced ROS generation plays vital role in the metabolic transformations in cells. Anti tumor effects of curcumin is seen in both breast cancer estrogen receptor positive and negative cells and estrogen dependent and independent gene suppression mechanism.

**Analysis of p16 expression by ROS levels in curcumin treated UV exposed MCF7 cells:** A well-known tumor suppressor protein is the cyclin-dependent kinase inhibitor p16 which functions by negatively regulating cell cycle and is involved in ROS-induced premature cellular senescence. It was reported that p16 knockdown increased intracellular ROS level and oxidative DNA damage which were further enhanced by oxidative stress.

Further we also examined the expression of p16 protein in regulation of intracellular ROS levels in MCF-7 breast cancer cell lines by western blotting method. The expression levels of p16 were found to be increase with decrease levels of ROS induced by UV radiations with curcumin treated. p16 is considered to be regulator of ROS, the underlying mechanism for this regulation is by regulating NRF2, a transcriptional factor for various antioxidant genes like *Are*, *Nqo1*, and *Hmox1*. According to studies done in this regard, P13K-AKT activation promotes a novel function of nrf2 that redirects the cell metabolism from aerobic to anaerobic respiration which further leads to a possible conclusion that ROS management and cell metabolism is interconnected by NRF1 in proliferating cells. Also explains the possibility that increased P13K-Akt compromises the risk of damage brought by ROS in p16 mutant cancer cells via NRF2 mediated anti oxidant pathways. The statistical analysis between the p16 expression and levels of ROS without curcumin treated and with curcumin treated found to be significant showing the correlations between increasing expression of p16 with decreasing levels of ROS with curcumin treatment.

The potential effects of curcumin in breast cancer cell line have been identified recently. Nonetheless the molecular mechanism of action underlying in anti tumor activity of curcumin is still need further investigation.

Moreover the molecular mechanism of curcumin on tumor cells in vivo needs further investigation.

**Ethical Clearance:** Not required

**Conflict of Interest:** Nil

**Source of Funding:** Self

### References

1. Vlahopoulos SA, Logotheti S, Mikas D, Giarika A, Gorgoulis V, Zoumpourlis V. Bioessays. The role of ATF-2 in oncogenesis 2008 Apr;30(4):314-27.
2. Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global cancer statistics. CA Cancer J Clin. 2011 Mar-Apr;61(2):69-90.
3. WHO International Agency for Research on Cancer, WHO 2013.
4. Nobori T, Miura K, Wu DJ, Lois A, Takabayashi K, Carson DA. Deletions of the cyclin-dependent kinase-4 inhibitor gene in multiple human cancers. Nature. 1994 Apr 21;368(6473):753-6.
5. Sosa Torres ME, Saucedo-Vázquez JP, Kroneck PM The magic of dioxygen. Met Ions LifeSci. 2015;15:1-12.
6. Kunnumakkara AB, Anand P, Aggarwal BB. Curcumin inhibits proliferation, invasion, angiogenesis and metastasis of different cancers through interaction with multiple cell signaling proteins. Cancer Lett. 2008 Oct 8;269(2):199-225.
7. Piepkorn M. Melanoma genetics: an update with focus on the CDKN2A(p16)/ARF tumor suppressors. J Am Acad Dermatol 2000; 42: 705–722.
8. Imran Ali, Waseem A. Wani and Kishwar Saleem Cancer Scenario in India with Future Perspectives Cancer Therapy 2011, Vol 8, 56-70.
9. Silverstein, M. (ed.). Ductal Carcinoma in Situ of the Breast. Baltimore, MD: Williams & Wilkins, 1997.
10. Cheng AL, Hsu CH, Lin JK, Hsu MM, Ho YF, Shen TS, Ko JY, Lin JT, Lin BR, Ming-Shiang W, Yu HS, Jee SH, Chen GS, Chen TM, Chen CA, Lai MK, Pu YS, Pan MH, Wang YJ, Tsai CC, Hsieh CY Phase I clinical trial of curcumin, a chemopreventive agent, in patients with high-risk or pre-malignant lesions. Anticancer Res. 2001 Jul-Aug;21(4B):2895-900.