# Expression of CHAC1 in Breast Cancer Cell Lines

**RESEARCH PROJECT**

***Submitted to the***

**Department of Human Genetics and Molecular Medicine**

**Central University of Punjab**


***Submitted by***

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Bathinda, 151001

2018

## CERTIFICATE

I declare that the dissertation entitled **"Expression of CHAC1 in Breast Cancer Cell Lines''** has been prepared by me under the guidance of Dr. Harish Chander, Assistant Professor, Department of Human Genetics and Molecular Medicine, School of Health Sciences, Central University of Punjab. No part of this dissertation has formed the basis for the award of any degree or fellowship previously.

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## ABSTRACT

**Keywords:** Breast Cancer, CHAC1, Unfolded Protein Response

Breast cancer is the commonly diagnosed type of cancer in women and is a major cause of deaths in women. Unfolded Protein Response Pathway is a signaling pathway induced in endoplasmic reticulum as a stress response. This type of stress signaling has been seen to be activated in many tumors including breast cancer. CHAC1 (Glutathione specific gamma- glutamylcyclotransferas-1) is a member of UPR Pathway. It was first discovered as a component of the ATF4 arm of the UPR pathway in a co-regulated group of genes. CHAC1 expression is necessary and sufficient to induce well-characterized markers of apoptosis. CHAC1 is involved in the inhibition of TNFRS6B via ATF4-ATF3-CHOP signaling. This sensitizes cells to commit to apoptosis following induction of UPR pathway. Although CHAC1 is a pro-apoptotic component of the UPR pathway, its expression in breast cancers have been noted to be remarkably high. To study the role of CHAC1 in breast cancer, we analyzed the expression of CHAC1 at mRNA level by using Real-Time PCR and further checked its' protein expression by Western-blotting. Its expression was found to be higher in ER positive cells as compared to the ER negative cells. Further investigations were performed by transfecting MDA-MB-231 cells by ER-alpha, which confirmed that presence of ER-alpha leads to the higher expression cHAC1 in breast cancer cells.

Ankita Sharma Dr. Harish Chander

## ACKNOWLEDGEMENT

First and foremost I extend gratitude to my project supervisor **Dr. Harish Chander**, Assistant Professor, Centre for Human genetics and Molecular Medicine, for instilling in me the confidence and drive for doing an arduous task and having faith in my abilities to accomplish any assignment which I set out to do. I thank him also for being a guardian, a friend as well as an erudite scientist to troubleshoot the problems encountered during the research work.

I am extremely thankful to **Prof.Anjana Munshi** Dean and COC, Centre for Human Genetics and Molecular Medicine, School of Health Sciences, Central University of Punjab, Bathinda for her constant encouragement. I am grateful to honourable Vice Chancellor, **Prof. R.K. Kohli &** Dean Academic Affairs **Prof. P Rama Rao,** Central University of Punjab, Bathinda.

I extend my heartfelt thanks to **Dr. Sandeep Singh** and other faculty members of the Centre for their mature and scholarly guidance in making this study fruitful.

I would like to thank **Mr. Vikas Jindal, Mr. Pawan Poonia, Dr. Gajendra S Vishawakarma, Mr. Prabhat Suman, Mr. Vikrant Mehta, Mr. Sarthak Mishra** and my lab-mates for their kind cooperation and support while performing my research work.

I would like to express my very profound gratitude to **Garima, Bharti, Divya, Shouvik, Amit, Ritu** and **Riya** for their unfailing support and encouragement throughout the project.

Lastly, I would like to thank source of energy, **my parents** for caring and giving me the right strength and guidance with right people to complete my work.

Ankita Sharma
Date:

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# LIST OF ABBREVIATIONS

| S. No | Full Form | Abbreviation |
|---|---|---|
| 1 | Sodium Dodecyle Sulfate | SDS |
| 2 | Glutathione specific gamma-glutamylcyclotransferas-1 | CHAC1 |
| 3 | Dulbecco's Modified Eagle Medium | DMEM |
| 4 | Unfolded Protein Response | UPR |
| 5 | Dimethylsulfoxide | DMSO |
| 6 | Bovine Serum Albumin | BSA |
| 7 | DNA Binding Domain | DBD |
| 8 | Ethylenediaminetetraacetic acid | EDTA |
| 9 | Ammonium per Sulphate | APS |
| 10 | Tetramethylethylenediamine | TEMED |
| 11 | Phosphate Buffer Saline and Tween 20 | PBST |
| 12 | Phosphate Buffer Saline | PBS |
| 13 | Enhanced Chemiluminescence | ECL |
| 14 | Nitrocellulose Membrane | NCP |
| 15 | Estrogen receptor | ER |

# CHAPTER-I

# INTRODUCTION

# 1.0. INTRODUCTION

Cancer is a complex, multifactorial disease. Breast cancer is the most frequently diagnosed type of cancer in women and is a leading cause of deaths worldwide. The incidence of breast cancer is more common in North American and European population whereas African and Asian populations show relatively lower rates of incidence (Ingvarsson. S., 2001). Breast cancers can roughly be divided into three subtypes: Hormone Receptor Positive (65-75%), HER2 Positive (15-20%) and Triple Negative Breast Cancer (15%).

Unfolded protein response is a specific type of stress signaling or cellular response, which is induced when there is an accumulation of unfolded/misfolded proteins in the endoplasmic reticulum. As a result of this unfolded protein response, the protein-folding capacity of the endoplasmic reticulum is increased, global protein synthesis is reduced, and the removal of unfolded or misfolded proteins from the endoplasmic reticulum is increased (Cao, S. S. et al., 2012). It is already known that hypoxia and glucose deprivation factors are the activators of UPR pathway, which are also enhancers of the metastasis and are linked to poor differentiation (Le et al, 2004).

CHAC1 (Glutathione specific gamma- glutamylcyclotransferas-1) is a member of unfolded protein response pathway. It encodes a member of the gamma-glutamylcyclotransferase protein family. This gene is regulated by ox-PAPC (oxidized 1-palmitoyl-2-arachidonyl-*sn*-3-glycero-phosphorylcholine). It was identified as a component of the ATF4 (activating transcription factor 4) arm of the unfolded protein response pathway (Gargalovic et al, 2006).

CHAC1 expression is necessary and sufficient to induce well-characterized markers of apoptosis. CHAC1 is involved in the inhibition of TNFRS6B via ATF4-ATF3-CHOP signaling. This sensitizes cells to commit to apoptosis following induction of UPR pathway. Although CHAC1 is a pro-apoptotic component of the UPR pathway, its expression in breast cancers have been noted to be remarkably high (Mungrue et al, 2009).

### 1.1. Objectives:

To determine the expression of CHAC1 mRNA in breast cancer cell lines.

To analyze CHAC1 protein expression in ER negative and ER positive cell lines.

To analyze CHAC1 expression in ER-alpha transfected MDA-MB-231 cells.

# CHAPTER-II

# REVIEW OF LITERATURE

## 2.0. REVIEW OF LITERATURE

One of the leading causes for cancer-related deaths in females is breast cancer. 15-20% of breast cancer is originated from triple-negative breast cancer (TNBC) subgroup that is ER negative (ER= estrogen-receptor), PR negative (PR= progesterone-receptor) and HER- 2 negative (Hung *et al.,* 2014). Frequent metabolic changes are exhibited by cancer cells towards metabolism and maintenance of redox balance. This leads to promotion of cancer cell proliferation and help the cancer cells to adapt to overcome stresses (Galluzzi *et al.,* 2013). These metabolic changes linked to cancer are relate to the activation of proto-oncogenes and in the inactivation of tumour suppressor genes (Galluzzi *et al.,* 2013). Also, cancer cells can raise the uptake of nutrients such as glucose (Vander *et al., 2009).* In nutrient deprived conditions cancer cells become susceptible to death. Thus, in developing anti-cancer therapy targeting cancer metabolism has been proposed as an emerging and promising strategy (Galluzzi *et al.,* 2013).

Different types of cell deaths have been reported that are induced by nutrient starvation. These include apoptosis (Shin *et al*., 2015), non-apoptotic, iron-dependent, oxidative death (ferroptosis) (Dixon *et al*., 2012), autophagy-mediated cell death (Changou *et al*., 2014). In TNBC cells cysteine starvation can leads to development of programed necrosis but not apoptosis or autophagy-mediated cell death *(Chen et al., 2017).* In Humans liver tissues can synthesize cysteine from L-methionine by the trans-sulfuration pathway (Rosado *et al*., 2006). In one-third of TNBC tumors (*in vivo) expression* of the Xc- cystine/glutamate antiporter was found (Timmerman *et al*., 2013). Recent studies shows depicts that breast cancer can be suppressed by Inhibition of the Xc- cystine/glutamate antiporter (Tang *et al*., 2016).

In spite of the fact that integrated stress response plays a crucial role in cellular survival and homeostasis in response to various types of stress, but if cell is exposed to severe stress then it can lead to cell death (Pakos-Zebrucka et al., 2016). Phosphorylation of eIF2α at serine 51 by one of the four eIF2α kinases: GCN2, PERK, dsRNA-activated protein kinase R (PKR), and heme-regulated inhibitor eIF2α kinase (HRI) are the major events in the integrated stress response pathway (Pakos- Zebrucka et al., 2016).The two most common eIF2α kinases, which are

activated by amino acid starvation are GCN2 and PERK (Pakos-Zebrucka et al., 2016). Studies depicted that HAEC and can induce CHAC1 in them by tunicamycin and its induction is inhibited by ATF4 small interfering RNA (siRNA) (Gargalovic *et al*., 2006).UPR activation leads to the induction of yeast homolog of CHAC1 (YER163c) (Travers *et al*., 2000).

During normal growth and differentiation, cells encounter conditions that activate a range of cellular stress-response pathways, including the unfolded protein response (UPR), which in turn is activated when the function of the Endoplasmic Reticulum (ER) is altered and disrupts the proper folding and maturation of secretory-pathway proteins (Lee, 1992). Several studies indicate that activation of the UPR might have a crucial role in tumor growth. But, the prolonged activation can initiate apoptosis, which could serve to protect the host (Ma and Hendershot, 2004).

Ferlay *et al* in 2010 stated that nearly one-third of all cancers in women worldwide are breast and ovarian cancer. The unfolded protein response (UPR) pathway has been shown to be activated in several of tumors including breast cancer (Fernandez et al, 2000; Scriven et al, 2009).

Activation of UPR pathway as well as the overexpression of UPR components have been shown to contribute to human tumors such as breast tumors (Fernandez et al, 2000), hepatocellular carcinomas (Shuda et al, 2003), gastric tumors (Song et al, 2001) and esophageal adenocarcinomas (Chen et al, 2001).

In recent times, CHAC1 a novel pro-apoptotic component of UPR pathway has been identified (Mungrue et al, 2009). Until now, two alternatively spliced transcript variants of this molecule have been described, out of which Transcript variant 1 (GenBank: NM_024111.3) encodes the longer isoform a whereas, the transcript variant 2 (GenBank: NM_001142776.1) lacks an alternate in-frame segment and hence results in a shorter protein isoform b (Goebel *et al.,*2012).

A positive correlation between poor tumor differentiation and higher CHAC1 mRNA expression levels in human breast carcinoma has been recently identified (Goebel *et al.,* 2012).

Goebel *et al* also suggested that CHAC1 may have a role in cell migration and proliferation as they found significantly reduced migration and proliferation in vitro, in CHAC1 knockdown Hs578T breast cancer cells. Increased migration and proliferation was seen in CHAC1-overexpressing cells. Similar results were

obtained in HOC-7 ovarian cancer cells. However, no association with apoptosis was observed in CHAC1-knockdown and overexpression experiments (Goebel *et al.,* 2012). However, it is still not clear that how UPR activation plays a role in cell survival and cell death, as UPR pathway activation in cancer results either in apoptosis and disease resolution or in an anti-apoptotic and pro-angiogenic effect, which results in disease progression. (Ma and Hendershot, 2004; Scriven *et al.,* 2007).

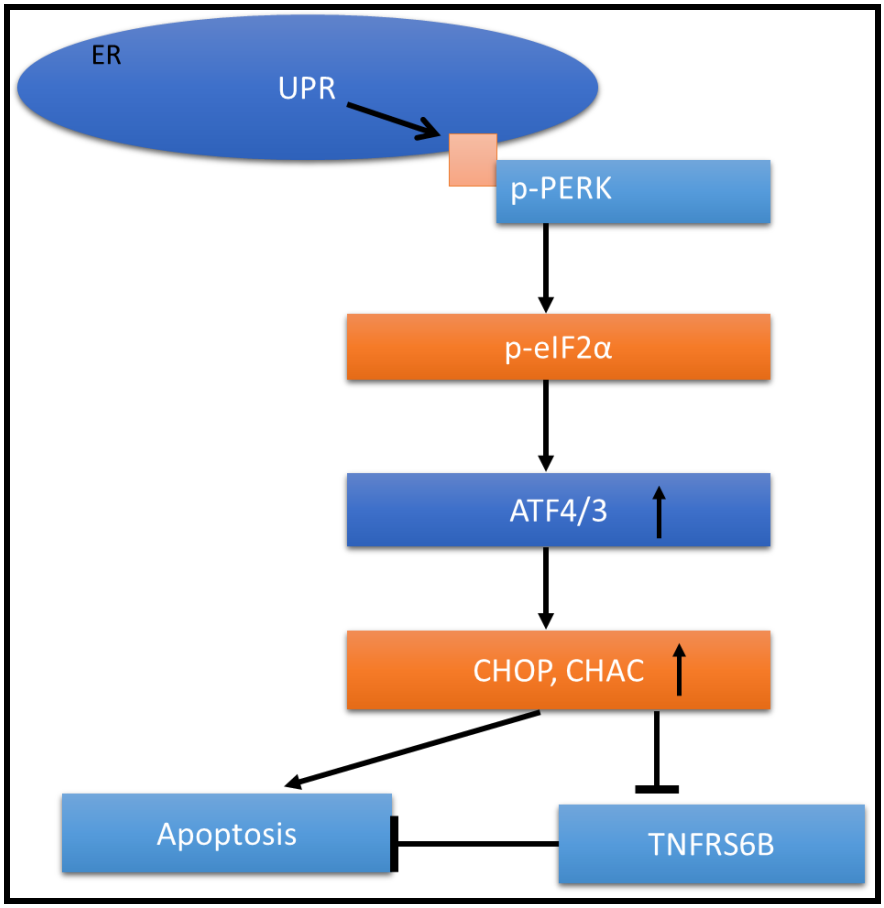



**Figure: 1 Activation of CHAC1 via Unfolded Protein Response Signaling.**

# CHAPTER- III

# METHODOLOGY

# 3.0. METHODOLOGY

## 3.1. Mammalian Cell Culture

### 3.1.1 Cells

A total of five cell lines were used for the study. HBL100 () cells were received as a gift from Dr. Andrew Craig, Queen's University, Canada. The human breast cancer cell line T47D was obtained from National Centre for Cell Science (NCCS), Pune, India. MCF-7 (Michigan Cancer Foundation 7), MDA-MB-231 (M.D. Anderson Metastatic Breast Cancer 231), MDA-MB-453 (M.D. Anderson Metastatic Breast Cancer 453) were also obtained from NCCS, Pune.

All the cell lines were cultured in suitable mediums, In addition to it, the supplementation of 10% FBS and 1% penicillin and Streptomycin solution was also provided and incubation was done in a humidified environment containing 5% $CO_2$ and $37^0$C temperature. Cell lines used and their origin and ER status is as follows:

| Cells | Type | ER Status | Media |
|---|---|---|---|
| **HBL 100** | Human Normal Mammary Gland Cells | Positive | DMEM |
| **MCF7** | Invasive Ductal Carcinoma Cells | Positive | DMEM |
| **T47D** | Invasive Ductal Carcinoma Cells | Positive | RPMI |
| **MDAMB 231** | Invasive Ductal Carcinoma Cells | Negative | DMEM |
| **MDAMB453** | Invasive Ductal Carcinoma Cells | Negative | DMEM |

**Table: 1 List of Cell Lines and Their Properties**

### 3.1.2. Passaging of Cells:

Cells were passaged routinely. For this, adherent cell lines were first washed with Phosphate Buffer Saline, this was followed by Trypsin/EDTA treatment for a short

period of time (approximately 5 to 7 minutes). The detached cells were resuspended in their respective media 10% FBS and 1% penicillin and Streptomycin solution (complete media) and plated in a new culture flask.

### 3.1.3. Freezing and Thawing of Cells:

For the long-term storage, approximately 8 – 9 million cells were trypsinized and were then transferred into a falcon, and then diluted using complete media. The cells were then pelleted down and resuspended in their respective media (DMEM/RPMI) containing 10% DMSO (Cryo-medium). These pelleted cells were now stored in cryo-vials at $80^0$C.

For thawing, the cryopreserved cells were quickly thawed in a $37^0$C water bath. The cell suspension was then transferred to the falcon, diluted with their respective complete medium and finally centrifuged at 1100 rpm. The pellet was then resuspended in the medium and seeded into the flask.

### 3.1.4. Cell Counting:

Cell Counting was performed by using Neubauer's Chamber method. The number of cells in per mL of suspension was calculated by using the formula:

| Cells per mL= Mean cell Count per Large Square X $10^4$ |
|---|

### 3.2. ER Transfection:

Transfection of ER-α was done in triple negative breast cancer cells (MDA-MB-231) in variable concentrations (2µg and 4µg) by using the reverse transfection method. pcDNA vector transfected cells were as a control for transfection.

For transfection, three 100 mm dishes were plated with a large amount of triple negative breast cancer cells (MDA-MB-231 cells) (approximately 3.5 x $10^6$) and transfection mixture was added to it.

| Content | Control | ER- GFP (2µg) | ER- GFP (4µg) |
|---|---|---|---|
| **Serum Free Media** | 500 µl | 500 µl | 500 µl |
| **LTx Reagent** | 12.5 µl | 12.5 µl | 12.5 µl |
| **Plus Reagent** | 2.5 µl | 2.5 µl | 2.5 µl |
| **Plasmid** | 2 µl (1 µg/µl) | 2 µl (1 µg/µl) | 4 µl (1 µg/µl) |

*****Mixture was incubated for 25 minutes at room temperature.

**Table: 2 Reaction Mixture for Transfection**

The media was changed at an interval of 9-10 hours and at 30 hours, the cells were harvested and pelleted. The pellets were further used for RNA isolation and Protein estimation.

### 3.3. RNA Isolation and cDNA Synthesis

Total RNA isolation for both the transfected and non-transfected cells was performed using RNeasy® Powerlyzer® Tissue & Cells Kit (Qiagen) according to the manufacturer's instruction manual. The isolated RNA was then eluted in 50-100 µl of RNase free water. The concentration and quality of RNA was then estimated by the use of NanoDrop and the RNA was stored at -80 ºC until further use.

cDNA was then synthesized using kit method (iScript cDNA synthesis Kit-Bio-Rad). For this, variable amount of RNA template was mixed with 4 µg 5x iScript Reaction Mix and 1 µg of iScript Reverse Transcriptase. The mixture was then brought to a total of 20 µg by adding up nuclease free water. The incubation of the complete reaction mix was then done in dry-bath. The incubation timings for every stage were as follows:

| | |
|---|---|
| **Priming** | **5 min at 25 ºC** |
| **Reverse Transcription** | 20 min at 46 ºC |
| **RT Inactivation** | 1 min at 95 ºC |
| **Optional Step** | Hold at 4 ºC |

**Table: 3 Incubation time at different stages for cDNA synthesis**

The newly synthesized cDNA was then stored at -20 ºC until further use.

### 3.4. Quantitative Real Time PCR

To determine the mRNA expression of CHAC1 both in non-transfected cells (HBL100, T47D, MCF-7, MDA-MB-231, MDA-MB-453) and ER-alpha transfected MDA-MB-231 cells, compared to the house-keeping gene GAPDH mRNA, following reaction mixture was prepared:

| **Reagent** | **Volume** |
|---|---|
| **SYBR Green JumpStart Taq Ready Mix (2x)** | 10 |
| **cDNA** | 1 |
| **Forward Primer (10)** | 1 |
| **Reverse Primer (10)** | 1 |
| **Double Distilled $H_2O$** | 7 |
| **Total Volume** | **20** |

**Table: 4 Reaction Mix for qRT-PCR**

*The PCR program was set on standard (40 cycles).

The temperature settings for the reaction was as follows:

94 ºC for 10 min

94 ºC for 15 sec

58 ºC for 1 min

70 ºC for 1 min

60 ºC for 15 sec

93.9 ºC for 15 sec

After the completion of reaction, the data was then analyzed and the relative expression of CHAC1 as compared to that of house-keeping gene GAPDH was calculated using the following formula:

$$2^{-\Delta\Delta Ct}$$

Where, ΔΔCt= ΔCT of sample – ΔCT of reference

ΔCT= Ct of gene of interest – Ct of housekeeping gene

Further, the standard deviation was calculated by using the following formula:

$$S= \sqrt{(s1^2+s2^2)}$$

Where, s1= Standard Deviation of the gene of interest

s2= Standard Deviation of the housekeeping gene

The Error margin was calculated by the following formula:

$$2^{-\Delta\Delta Ct+/-s}$$

The error used for putting error bars was calculated using the following formula:

$$2^{-\Delta\Delta Ct+/-s} - 2^{-\Delta\Delta Ct}$$

### 3.5. Protein Estimation and Quantification:

#### 3.5.1. Lysate Preparation:

Cells were harvested from T75 flasks by adding chilled PBS and centrifuging the cells at 3000 rpm for 5 minutes. The pellet was then re-suspended in NP-40 lysis buffer (including protease inhibitor cocktail (PI) in 1:1000 ratio), vortexed for small

intervals (10 minutes) and incubated on ice for 30 minutes. The suspension was then centrifuged for 10 minutes at 12000 rpm at 4ºC to remove the cell debris. The supernatant was carefully transferred to a fresh Eppendorf tube and stored at -4ºC for future use.

### 3.5.2. Protein Quantification by Bradford Assay:

After the preparation of lysates, protein concentration was determined using Bradford Assay. It was done using a 96 well plate. For this, bovine serum albumin was used as a standard, at a concentration of 1mg/ml and lysis buffer was used as a blank. Sample lysates and Bradford reagent (Bio-Rad) was added and readings were taken on a micro-plate reader. Readings were compared to a standard BSA dilution series.

| Name of Standards | For BSA Standard (mg/ml) | Bradford reagent |
|---|---|---|
| S1 | 0.5µl | 200µl |
| S2 | 1µl | 200µl |
| S3 | 2µl | 200µl |
| S4 | 3µl | 200µl |
| S5 | 4µl & so on | 200µl |

| Name of Samples | Quantity | Bradford reagent |
|---|---|---|
| A | 1µl | 200µl |
| B | 1µl | 200µl |
| C | 1µl | 200µl |
| D | 1µl | 200µl |
| E | 1µl | 200µl |

**Table: 5 Dilution Series Used for Bradford Assay**

### 3.5.3. SDS-PAGE

The very first step of this process was gel preparation. 1 mm, 12% resolving gel (10% acrylamide gel) was prepared. After polymerization of resolving gel, stacking gel was prepared.

| 30% Acrylamide | 12% Gel | |
|---|---|---|
| **Quantity** | **5ml** | **10 ml** |
| **Water** | 1.5 ml | 3ml |
| **30% acrylamide** | 2.0 ml | 4.0ml |
| **1.5M Tris (pH= 8.8)** | 1.25ml | 2.5ml |
| **10% SDS** | 50 µl | 100µl |
| **10% APS** | 50 µl | 100 µl |
| **TEMED** | 5 µl | 10 µl |

**Table: 6 Resolving Gel Components**

| 30% Acrylamide | 4% |
|---|---|
| **Water** | 2.975 ml |
| **30% Acrylamide** | 670 µl |
| **0.5 M Tris-HCl (pH=6.8)** | 1.25 ml |
| **10% SDS** | 50 µl |
| **10% APS** | 50 µl |
| **TEMED** | 5 µl |

**Table: 7 Stacking Gel Components**

The gel preparation was followed by sample preparation and loading of prepared sample into the gel. The electrophoresis was carried out in 1XSDS at a voltage of 70V for 20 minutes followed by a voltage of 200 V for 45 minutes.

### 3.5.4. WESTERN BLOTTING

The primary step involved in western blotting or immunoblotting is the transfer of protein onto nitrocellulose membrane by using BioRad Tans-Blot Turbo Blotting

System. For transferring the gel is taken out of the electrophoresis system and transferred into a container having transfer buffer in it. The gel is then sandwiched between two paper towels in manner that gel is placed above the nitrocellulose membrane in the transfer assembly. The transfer buffer was poured to keep the setup wet. Then the turbo blot was set at 25V for voltage and 1.3 Amperes of current for 10 minutes. After time completes the gel from the setup is discarded and membrane is used for further processing.

After transferring the protein onto the membrane, the NCP was incubated in 20 ml of 5% milk (Prepared in 1X PBST) for one hour on the rocker. The significance of this step is to block the non-specific binding sites. After blocking, the membrane was washed with 1X PBST buffer for 3 times with an interval of 10 minutes.

Primary antibody was added to a solution of 3% milk (prepared in 1X pbst). Dilutions for antibodies were as follows:

| **Name of Primary Antibody** | **Dilution** |
|---|---|
| **CHAC1** | 1:1000 |
| **GAPDH** | 1:6000 |
| **ER-** | 1:1000 |

**Table: 8 Primary Antibody Dilutions Used**

The membrane was then incubated in primary antibody overnight on a rota-spinner at 4ºC. After overnight incubation, the membrane was washed with 1X PBST for 3 times at an interval of 10 min. After this, secondary antibody was added to a solution of 3% milk (prepared in 1X pbst) (Dilution- 1:5000).

Further, membrane was incubated in this solution for 2 hours on a rocker at room temperature followed by washing with 1X PBST for 3 times with 10 min interval. The membrane was then developed. ECL was used as a substrate. ChemiDoc (Bio-Rad) machine was used to visualize the bands. Software used was Image lab 3.0. Further analysis was done.

### 3.6. STATISTICAL ANALYSIS

For quantification of bands, densitometry analysis of western-blot results was done using ImageJ software (developed by NIH).

# CHAPTER- IV

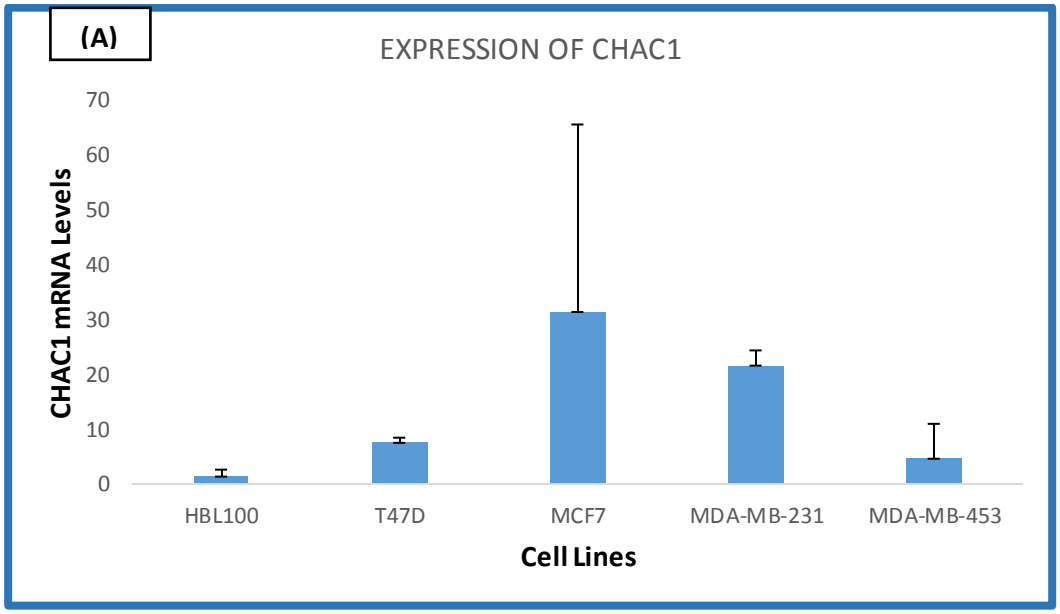
# RESULTS

# 4.0. RESULTS

## 4.1. Expression Level of CHAC1 in Different Breast Cancer Cell Lines

Real Time PCR was performed to check the mRNA expression of CHAC1 on basal level. The results showed that the expression of CHAC1 mRNA was higher in T47D and MCF-7 cells, which are both ER positive cell lines. The expression of CHAC1 was also found to be high in ER negative MDA-MB-231 and MDA-MB-453 cells, but it was less as compared to the ER positive cells. From this, we hypothesized that there might be a correlation between ER-alpha and CHAC1.

To further confirm the above results, we checked the basal level expression of CHAC1 at protein level by carrying out western blotting. The results of western blotting was also similar to that of Real Time PCR. The expression was found to be high in MCF7 and T47D cells as compared to MDA-MB-231 and MDA-MB-453 cells. Densitometry analysis also showed the similar results.



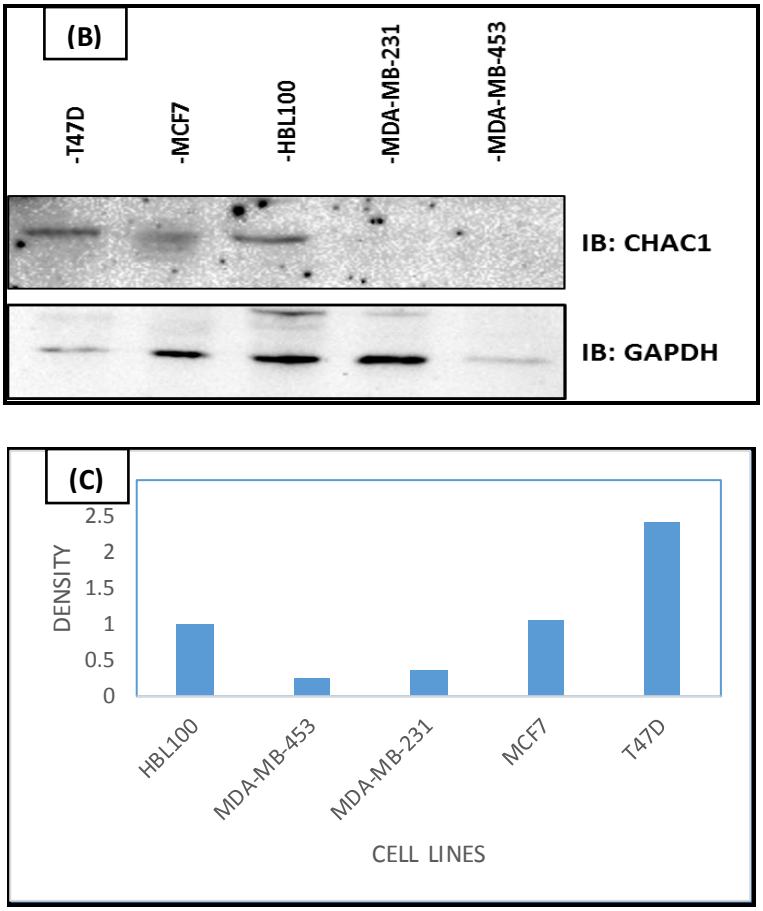



**Figure: 2 Expression Level of CHAC1 in Different Breast Cancer Cell Lines**

A. mRNA levels of CHAC1 normalized to GAPDH was analyzed using $2^{-\Delta\Delta CT}$
B. Lysates from the above mentioned breast cancer cell lines were subjected to western-blotting with CHAC1 and GAPDH antibodies. Samples were loaded in equal total protein concentration.
C. Quantification of bands was done using densitometry analysis.

## 4.2. Expression Levels of CHAC1 in ER-alpha transfected MDA-MB-231 cells

To further check the correlation between the presence of ER-alpha and the expression of CHAC1, MDA-MB-231 cells (Triple negative breast cancer cells) were transfected with ER-alpha. The expression of CHAC1 mRNA was then checked by real time PCR. The results showed that the expression of CHAC1 was higher in the cells where ER-alpha concentration was high.

To further confirm this, we performed western blotting to check the CHAC1 protein expression. No bands were obtained in the control and lower ER-alpha

concentration samples and very faint bands were observed in the higher ER-alpha concentration samples. However, the results obtained were not promising since the CHAC1 antibody did not work properly due to unknown reasons.

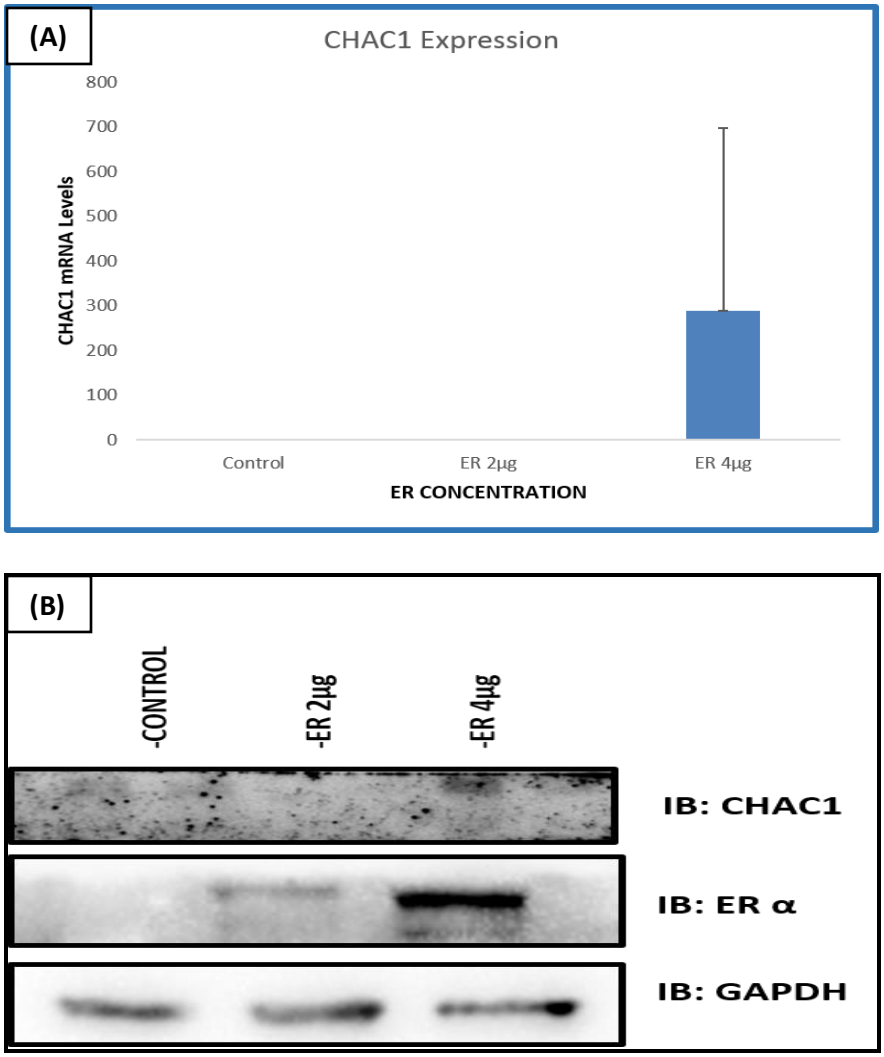



**Figure: 3 Expression Levels of CHAC1 in ER-alpha transfected MDA-MB-231 cells**

A. mRNA levels of CHAC1 normalized to GAPDH was analyzed using $2^{-\Delta\Delta CT}$

B. Lysates from the transfected MDA-MB-231 breast cancer cell lines at variable concentrations were subjected to western-blotting using ER-alpha, CHAC1 and GAPDH antibodies.

# CHAPTER- V

# DISCUSSION

## 5.0. DISCUSSIONS

The higher expression of CHAC1 in breast cancer cells as compared to the normal cells indicates that the CHAC1 is a pro-survival component.

The basal level of CHAC, both at mRNA and protein level were found to be very high in MCF7 and T47D cells and MDA-MB-231 cells. The expression of CHAC1 was found to be highest in MCF-7 and T47D which are ER positive cell lines. From this we hypothesized that there could be a possible correlation between the presence of ER-alpha and CHAC1 expression. So, to further explicate the molecular mechanism involved in CHAC1 expression, the MDA-MB-231 cells, which are originally triple negative in nature, were transfected with variable amount of ER-alpha. The transfection of ER-alpha was first confirmed by western-blotting technique. The results showed higher expression of ER- alpha at 4 µg concentration as compared to that of 2 µg concentration. This indicated the successful transfection of ER-alpha.

The expression levels of CHAC1 in these transfected cells were checked by using immunoblotting technique. The bands of CHAC1 for 4 µg concentration of ER-alpha were more visible than that of 2 µg concentration of ER-alpha, which indicates that the expression of CHAC1 is affected by the presence of ER-alpha and this could be the reason of higher expression of CHAC1 in MCF-7 and T47D.

However, the role of ER-alpha in CHAC1 expression could not be clearly defined as the antibody being used was not working due to unknown reasons. But the other results confirmed that there is a possibility that CHAC1 expression has a correlation with the presence of ER-alpha.

## 5.1. Conclusion and Future Perspectives:

The expression of CHAC1 was found to be high in different breast cancer cell lines. The expression was much higher in ER positive cells lines as compared to the ER negative cell lines. This shows a correlation between the expression of ER alpha and CHAC1. Higher expression of pro-apoptotic CHAC1 is co-related with breast cancer progression and poor prognosis. Further studies could reveal the potential of CHAC1 as an independent prognostic indicator and therapeutic target of breast cancers.

## SUMMARY

**Introduction:** Cancer is a complex, multifactorial disease. Breast cancer is the most frequently diagnosed type of cancer in women and is a leading cause of deaths worldwide. The incidence of breast cancer is more common in North American and European population whereas African and Asian populations show relatively lower rates of incidence. Unfolded Protein Response Pathway is a stress signaling pathway in the endoplasmic reticulum. This signaling is induced in many human solid tumors including breast cancer. CHAC1 (Glutathione specific gamma-glutamylcyclotransferas-1) is a member of UPR Pathway. CHAC1 expression is necessary and sufficient to induce well-characterized markers of apoptosis. CHAC1 is involved in the inhibition of TNFRS6B via ATF4-ATF3-CHOP signaling. This sensitizes cells to commit to apoptosis following induction of UPR pathway. Although CHAC1 is a pro-apoptotic component of the UPR pathway, its expression in breast cancers have been noted to be remarkably high.

**Methodology:** To check the expression of CHAC1 both at m-RNA and protein level, western-blotting and real time PCR techniques were performed.

**Results:** The expression of CHAC1, both at mRNA and protein level were found to be high in ER positive cell lines. The ER alpha transfected MDA-MB-231 cells showed the similar results, which confirms that the presence of ER-alpha contributes to the expression of CHAC1.

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# APPENDIX I

## List of Materials

The following instruments used for all the experimental purposes.

| S. No. | Name of Instruments | Manufacturing company |
|---|---|---|
| 1 | Weighing balance | Mettler Tolendo |
| 2 | Autoclave | Narang Scientific Works |
| 3 | Hotplate | Tarsons |
| 4 | CO2 incubator | New Brunswick, UK |
| 5 | Inverted microscope | Olympus Magnus |
| 6 | Laminar air flow | NSW (Narang Scientific Works) |
| 7 | Microplate reader | Synergy H1 |
| 8 | Oven | Samsung |
| 9 | ProFlex™ PCR System | Thermo Fisher Scientific |
| 10 | Refrigerated Centrifuge 5430R | Eppendorf, Germany |
| 11 | Trans-Blot Turbo™ Blotting System | Bio-Rad |
| 12 | chemi Doc™ XR+ Gel Documentation system | Bio-Rad |
| 13 | Vortex Shaker | Tarsons |
| 14 | Bench top Centrifuge | Tarsons |
| 15 | Deep Freezer (-20°C) | REM 600 |
| 16 | Refrigerator | Sharp |
| 17 | SDS-PAGE Electrophoresis Apparatus | Bio-Rad |
| 18 | Real Time PCR | Applied Biosystems |
| 19 | NanoDrop 2000 Spectrophotometer | Thermo Scientific |
| 20 | Laminar Air Flow | Esco |
| 21 | Ultra-Centrifuge | Thermo Scientific |

# APPENDIX II

## List of Chemicals and Reagents

The following are biological chemicals used for conducting biological work.

| S.No. | Chemicals | Manufacturing Company |
|---|---|---|
| 1 | Dulbecco's Modified Eagle Medium | Gibco |
| 2 | Roswell Park Memorial Institute-1640 | Gibco |
| 3 | Antibiotics(100X) with 10,000 units Penicillin and 10 mg streptomycin per ml | HiMedia |
| 4 | Fetal bovine serum | Gibco |
| 5 | DMSO | Sigma |
| 6 | Methanol | SRL |
| 7 | BSA | Amberesco |
| 8 | PBS | HiMedia |
| 9 | Ethanol | SRL |
| 10 | TAE buffer | Bio-Rad |
| 11 | Protein Biomarker | Bio-Rad |
| 12 | Enhanced bioluminescence | Invetrogen |
| 13 | Tween 20 | Amresco |
| 14 | GAPDH Antibody (ZG003) | Thermo Fisher |
| 15 | KIBRA Antibody | Cell Signaling |
| 16 | iScript™ cDNA Synthesis Kit | BIO RAD |
| 17 | RNA isolation Kit | Qiagen |
| 18 | GAPDH Primer | SIGMA-ALDRICH |
| 19 | KIBRA Primer | Eurofins |
| 20 | iTaq™ Universal SYBER Green Supermix | BIO RAD |

| | | |
|---|---|---|
| 21 | Peroxidase- conjugated AffiniPure Goat Anti-Rabbit IgG (H+L) | Jackson immunoresearch |
| 22 | Peroxidase- conjugated AffiniPure Mouse Anti-Rabbit IgG (H+L) | Jackson immunoresearch |
| 23 | Lipofectamine LTX &PLUS™ Reagent | Invitrogen |
| 24 | HiPerFect Transfection Reagent | Qiagen |
| 25 | Plasmocin™ Treatment | InvivoGen |
| 26 | pCDNA vector (710) | Gifted by Andrew Craig (Queens University) |
| 27 | ER-alpha GFP (712) | Gifted by Andrew Craig (Queens University) |
| 28 | Nitrocellulose Membrane | Invitrogen |
| 29 | Ethylenediaminetetraacetic acid | SBL |
| 30 | Ammonium per Sulphate | Ambion |
| 31 | Tetramethylethylenediamine | Invitrogen |
| 32 | Phosphate Buffer Saline | Gibco |

## Student Approval Form

| | |
|---|---|
| **Name of the Author** | Ankita Sharma |
| **Department** | Human Genetics and Molecular Medicine |
| **Degree** | M.Sc. Life Sciences with specialization in Molecular Medicine |
| **University** | Central University of Punjab |
| **Guide** | Dr. Harish Chander |
| **Project Title** | Expression of CHAC1 in Breast Cancer Cell Lines |
| **Year of Award** | 2018 |

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| View only (No downloads) (Worldwide) | |

Signature of the Candidate

Signature of the Supervisor

Place:

Date:

**Central University of Punjab**

## Declaration

I declare that all the changes suggested by the VC nominee examiner in the Research Project entitles **"Expression of CHAC1 in Breast Cancer Cell Lines"** submitted by me for the award of degree of Masters in Science in life Sciences with specialization in Molecular Medicine in the Department of Human Genetics and Molecular Medicine has been incorporated in the Research Project.

(Ankita Sharma)

Department of Human Genetics and Molecular Medicine

School of Health Sciences

Central University of Punjab

Date:

(Dr. Harish Chander)

Department of Human Genetics and Molecular Medicine

School of Health Sciences

Central University of Punjab

Date:

URKUND

# Urkund Analysis Result

| | |
|---|---|
| **Analysed Document:** | CHAC_New.docx (D39325384) |
| **Submitted:** | 5/28/2018 12:35:00 PM |
| **Submitted By:** | uradandapraveen@gmail.com |
| **Significance:** | 1 % |

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https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3251857/

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