

# Expression of KIBRA in Breast Cancer Cell Lines

**RESEARCH PROJECT**

*Submitted to the*

**Department of Human Genetics and Molecular Medicine  
Central University of Punjab**



*Submitted by*

**Garima Singh**

(Registration No: 16mslsmm03)

M.Sc. Life Sciences with Specialization in Molecular Medicine

*Under the Supervision of*

**Dr. Harish Chander**

Centre for Human Genetics and Molecular Medicine

Central University of Punjab,

Bathinda, 151001

2018

## CERTIFICATE

I declare that the dissertation entitled “**Expression of KIBRA 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.

Garima Singh  
Centre for Human Genetics and Molecular Medicine,  
School of Health Sciences,  
Central University of Punjab, Bathinda- 151001.  
Date:

## CERTIFICATE

I declare that the dissertation entitled “**Expression of KIBRA in Breast Cancer Cell Lines**” has been prepared by me under the guidance of Dr. Harish Chander, Assistant Professor, Centre for 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.

Dr. Harish Chander  
Assistant Professor,  
Department of Human Genetics and Molecular Medicine,  
School of Health Sciences,  
Central University of Punjab, Bathinda- 151001.  
Date:

## Expression of KIBRA in Breast Cancer Cell Lines

Name of the student : Garima Singh  
Registration Number : 16mslsmm03  
Degree for which submitted : Masters of Science  
Supervisor : Dr. Harish Chander.  
Department : Human Genetics and Molecular Medicine  
School : Health Sciences

### ABSTRACT

**Keywords:** Breast Cancer, KIBRA, Metastasis, DDR1, DLC1 & PKC $\zeta$ , ER

Breast cancer the most frequently diagnosed cancer and its metastasis to distant organs accounts the majority of deaths. Numerous genes and proteins are involved in the cause of metastasis. Though KIBRA is one of the component of Hippo Pathway and is reported as tumor suppressor but this scaffolding protein has also been found to be an emerging and important player in the process of metastasis. It has been reported that KIBRA protein interacts with various proteins through its domain and leads cytoskeleton arrangement, cell polarity and migration. N terminal and C terminal of the protein contains the WW, Internal C<sub>2</sub> & putative class III PDZ domain that interacts with DDR1, DLC1 & PKC $\zeta$  and helps the breast cancer cells to metastasize. To study whether KIBRA is involved in breast cancer metastasis, we checked its expression at both protein and mRNA level by Immunoblotting and Real Time PCR which showed increased KIBRA expression in ER positive cells. Further investigation to elucidate the role of KIBRA in ER positive cells, ER transfection and immunoblotting in triple negative breast cancer cell lines were performed, which indicated that ER leads to enhanced KIBRA expression in breast cancer cells.

Garima Singh

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 **Dr. Gajendra S Vishwakarma, Mr. Vikas Jindal, Mr. Pawan Poonia** for providing technical support in the laboratory. I am also grateful to **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 am indebted to my friends **Ankita, Bharti, Divya, Shouvik, Amit, Vijeta** and **Usha** for their round the clock support and endless help throughout the project.

I am at dearth of words to express my gratitude to **my parents**, who have been patient, supportive and caring. To them I dedicate this thesis.

Garima Singh  
Date:

## TABLE OF CONTENTS

<b>Sr. No</b>	<b>Content</b>	<b>Page No.</b>
<b>1</b>	<b>Introduction (Chapter 1)</b>	<b>1</b>
<b>2</b>	<b>Review of Literature (Chapter 2)</b>	<b>4</b>
<b>3</b>	<b>Methodology (Chapter 3)</b>	<b>11</b>
<b>4</b>	<b>Results (Chapter 4)</b>	<b>20</b>
<b>5</b>	<b>Discussion, Conclusion and future perspectives(Chapter 5)</b>	<b>24</b>
<b>6</b>	<b>References</b>	<b>28</b>

## LIST OF FIGURES

<b>Figure No.</b>	<b>Description</b>	<b>Page No.</b>
1	Structure of KIBRA: different domain and their function	5
2	Interacting Partners of KIBRA Involved in Metastasis	8
3	Temperature Condition for Real Time PCR	15
4	Expression on KIBRA in different Breast Cancer Cells	22
5	ER Transfection and Immunoblotting	23
6	The Synergistic Mode of Action of KIBRA And ER For Metastasis	25

## LIST OF TABLES

<b>TABLE.NO.</b>	<b>DESCRIPTION</b>	<b>PAGE NO.</b>
<b>1</b>	<b>List of Cell Lines and Their Properties</b>	<b>12</b>
<b>2</b>	<b>Reaction Mixture for Transfection</b>	<b>14</b>
<b>3</b>	<b>Incubation time at different stages for cDNA synthesis</b>	<b>14</b>
<b>4</b>	<b>Reaction Mix for qRT-PCR</b>	<b>15</b>
<b>5</b>	<b>Bradford Assay Estimation Layout</b>	<b>17</b>
<b>6</b>	<b>Resolving Gel Components</b>	<b>17</b>
<b>7</b>	<b>Stocking Gel Components</b>	<b>18</b>
<b>8</b>	<b>List of Antibody Dilution</b>	<b>19</b>

## LIST OF ABBREVIATIONS

S. No	Full Form	Abbreviation
1	Sodium Dodecyle Sulfate	SDS
2	Polyacrylamide Gel Electrophoresis	PAGE
3	Dulbecco's Modified Eagle Medium	DMEM
4	Fetal Bovine Serum	FBS
5	Dimethylsulfoxide	DMSO
6	Bovine Serum Albumin	BSA
7	Estrogen Receptor $\alpha$	ER $\alpha$
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	Protease Inhibitor	PI
16	Phenylmethylsulfonyl Fluoride	PMSF
17	Phenoxypolyethoxyethanol	NP 40

# **CHAPTER I**

# **INTRODUCTION**

## 1.0. INTRODUCTION

Breast cancer is one of the most common invasive malignancies diagnosed and is the second leading cause of death among women globally (Friedenreich, 2011). It is a heterogeneous disease which is characterized by different molecular drivers. Several studies conducted led to scientific advancements and progress in breast cancer research and therapy, still most patients with breast cancer are prone to recurrence, chemoresistance and metastasis. And since the outcome of treatment are drastically different for different cancer types, especially in the case of triple negative breast cancer (TNBC), patient having aggressive clinical course, the chances of early relapse is high and that of survival rate is low (X. Zhang et al., 2016).

Coping with the challenges like recurrence, chemo-resistance and metastasis is onerous. The increased propensity of motility and invasiveness, chemo-resistance and radio-resistance among epithelial malignant tumour is endowed by epithelial-mesenchymal transition (EMT), a critical biological process during embryonic development (Kang & Massagué, 2004; Thiery & Morgan, 2004; Thiery & Sleeman, 2006). Therefore, EMT is considered as the elementary step of chemo-resistance, local recurrence and metastasis. The mechanism of EMT has been widely studied over decades, and a number of hypotheses have been proposed such as signalling pathways (transforming growth factor- $\beta$ /Wnt/Notch) (Ahmad et al., 2012; Debies et al., 2008; Li, Wicha, Schwartz, & Sun, 2011), cancer stem cells (Bao et al., 2012), miRNA (Wang et al., 2010), oncogenic events. Proto-oncogene activation (ras) (Hanahan & Weinberg, 2011), cancer stem cells, miRNA and inflammation are associated with the induction of EMT, but the EMT mechanism and the genes involved have not been explored completely. Thus, the extensive understanding of the molecular mechanisms and identification of the genes responsible for breast cancer recurrence, chemo-resistance and metastasis are necessary for precision medicine (X. Zhang et al., 2016).

KIBRA (KIdney/BRAin protein), also known as WWC1 (WW and C2 domain containing 1), is a multi-domain phosphor-protein and is predominantly found in brain and kidney. It interacts with several signalling molecules and regulates cell polarity, cell migration and cell cycle (Duning et al., 2008a; Kremerskothen et al., 2003; Schneider et al., 2010;

Yoshihama, Chida, & Ohno, 2012). It was initially cloned and characterized by Kremerskothenetal (Kremerskothen et al., 2003) as a molecule which interacts with postsynaptic dendrin protein (human dendrin KIAA0749) (Herb et al., 1996; Neuner-Jehle, Denizot, Borbély, & Mallet, 1996). Since then it has been a subject of interest in the field of cognitive neuropsychology. However, the role of KIBRA in breast cancer came into light only after the discovery of regulation of oestrogen receptor activity by binding to the dynein light chain 1(DLC1) molecule (Suresh K. Rayala et al., 2006). Later on, it was also reported that the KIBRA interacts with discoidin domain receptor 1(DDR1) and modulates collagen-induced MAPK kinase signalling in breast cells (Hilton et al., 2008). Several studies have shown that KIBRA promotes oncogenic signalling, however, despite all these studies, the molecular mechanism involved and its oncogenic potential remains unclear.

### **1.1. OBJECTIVES**

- To determine the expression of KIBRA in breast cancer cell lines.
- To compare the expression of KIBRA in receptor positive and receptor negative breast cancer cell lines.

**CHAPTER II**

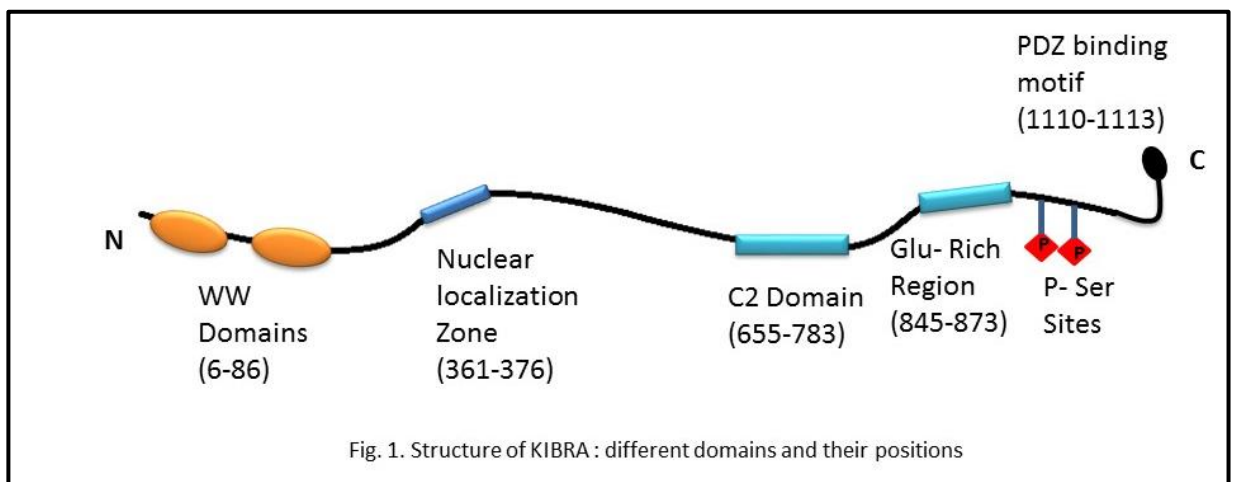
**REVIEW OF**

**LITERATURE**

## 2.0. REVIEW OF LITERATURE

### 2.1. Structure of KIBRA And Regulation

Human protein KIBRA is a scaffolding protein and is encoded by the WWC1 gene located at chromosome 5q35.1. It consists of 1113 amino acids and has an approximate size of 125.3 kDa. KIBRA consists of two N terminal WW domains (positions 6-39 and 54-86 respectively) covering a stretch of 35-40 amino acids (Suresh K. Rayala et al., 2006). Both of the domains consists of two conserved tryptophan residue, an internal block of aromatic amino acids and a conserved proline residue (Dobrosotskaya, Guy, & James, 1997). These domains interact with the protein consisting of the proline rich region (PPXY). A 15 amino acid long zone responsible for nuclear localization has been identified between amino acid 361 and 376 (Suresh K. Rayala et al., 2006). An internal C2, Ca<sup>2+</sup> sensitive, (Büther, Plaas, Barnekow, & Kremerskothen, 2004) domain is composed of two four stranded  $\beta$  sheets and is located in between 655 and 783. This 128 stretches long amino acid residue is involved in phospholipid binding in a calcium-dependent manner. Calcium binding induces a change in electrostatic potential which plays a role in the enhancement of phospholipid binding (Rizo & Südhof, 1998). Apart from these a glutamic acid-rich region is located in between 845 and 873 (Kremerskothen et al., 2003). At C terminal a class III PDZ binding sequence is located in between 1110 and 1113 (Duning et al., 2008a), and has a major role in the formation and function of signal transduction complexes (Fig.1) (Fanning & Anderson, 1999).



Previous studies has revealed that KIBRA is a major regulator of Hippo signaling pathway and is involved in inhibiting cell proliferation and apoptosis (Xiao et al., 2011) but further studies has reported that phosphorylation regulation of KIBRA by mitotic kinases (Aurora and CDK1) during mitosis (Xiao et al., 2011), ERK (extracellular signal-related kinases) at Ser<sup>548</sup> and RSK (p90 ribosomal S6 kinases)at Thr<sup>929</sup> and Ser<sup>947</sup> leads to cell migration and proliferation (Yang et al., 2014).

## **2.2. Interacting Partners of KIBRA.**

Several studies have reported the role of KIBRA in numerous cellular functions such as cell polarity and migration, transcriptional regulation, vesicle transport and synaptogenesis. These functions were acknowledged after a study identified the interacting partners of KIBRA via yeast two-hybrid screening.

KIAA0749, a postsynaptic dendrin protein, was the first interacting partner identified (Kremerskothen et al., 2003). This dendrin protein interacts with the WW domain of KIBRA through its PPxY motif and is found to be localized in the dendritic region. It plays a major role in cytoskeleton organisation (Kremerskothen, Plaas, Kindler, Frotscher, & Barnekow, 2005). KIAA0749 also interact with  $\alpha$ -actinin and synaptic scaffolding molecule S-SCAM (Kremerskothen, Kindler, Finger, Veltel, & Barnekow, 2006), and is responsible for sleep deprivation (Neuner-Jehle et al., 1996).

Further studies have identified synaptopodin and PKC $\zeta$  as the interacting partners of KIBRA which has asserted its role in the process of postsynaptic density (PSD) (Büther et al., 2004; Duning et al., 2008a). Similar to dendrin the PPxY motif of synaptopodin interacts with the WW domain of KIBRA and help in cytoskeleton arrangement (Duning et al., 2008a; Kremerskothen et al., 2005). KIAA0513 is also an interacting partner of KIBRA which has a potential link with cognition and is found to be upregulated in schizophrenic patients (Lauriat et al., 2006).

PATJ (PALS1- associated tight junction protein) is another interacting partner of KIBRA which has asserted its role in cell polarity. It is a component of the evolutionarily conserved multiprotein complex and interacts with the putative class III PDZ binding site of KIBRA (Shin, Straight, & Margolis, 2005; Shin, Wang, & Margolis, 2007). Apart from PATJ, another link of KIBRA with cytoskeleton was acknowledged after

identification of binding of dynein-complex with it (Traer et al., 2007). This interaction was substantiated by the study describing the simultaneous interaction of KIBRA with Dynein light chain 1 (DLC1) and histone H3. The binding of KIBRA with H3 is mediated via the glutamic acid-rich region of KIBRA, located near the C terminus (Fig.2) (Suresh K. Rayala et al., 2006).

The concept of involvement of KIBRA in transcriptional regulation is further supported by various studies suggesting the upregulation of KIBRA expression upon the application of progesterone and its binding with discoidin domain receptor1 (DDR1). DDR1 is a tyrosine kinase, important for the development of mammary gland and in a molecular complex with KIBRA and PKC $\zeta$  is, involved in the collagen-regulated stimulation of MAPK cascade (Hilton et al., 2008).

### **2.3.Interaction Partners Involved in Metastasis**

#### **2.3.1. DDR1**

DDR1 is epithelial-specific and highly expressed during pregnancy and several primary breast cancers (Barker et al., 1995). In an experiment performed on female mice DDR1 knockout shows defects in blastocyst implantation together with hyper-proliferation and abnormal branching of the mammary ducts and an increased amount of collagenous extracellular matrix surrounding the mammary epithelium (Vogel, Aszódi, Alves, & Pawson, 2001). This suggests that DDR1 has a role in mediating extracellular matrix (ECM) signalling within the mammary gland and this signalling plays a role in alveolar morphogenesis and regulation of cell motility and adhesion (Fata, Werb, & Bissell, 2004). Deregulation of this signalling interaction may cause migration, invasion, metastasis and leads to tumour progression.

DDR1 interacts with KIBRA in the process of tumor progression. Its PPxy motif binds to the binds to the WW binding motif of KIBRA and regulates ERK MAPK pathway in the ligand-dependent response of DDR1. (Heidi N. Hilton et.al). DDR1 get activated when its ligand (collagen I or IV) comes and bind to it, this leads to dissociation of the KIBRA-DDR1 complex which indicates that KIBRA plays a role in the downstream signalling pathways induced by the extracellular matrix. E. Faraci-Orf et.al., showed that forced activation and expression of DDR1 in mouse mammary epithelial HC11 cells

with collagen results in increased activation of Stat5, a downstream target of Prlr and increased  $\beta$ -casein gene expression (Faraci-Orf, McFadden, & Vogel, 2006).

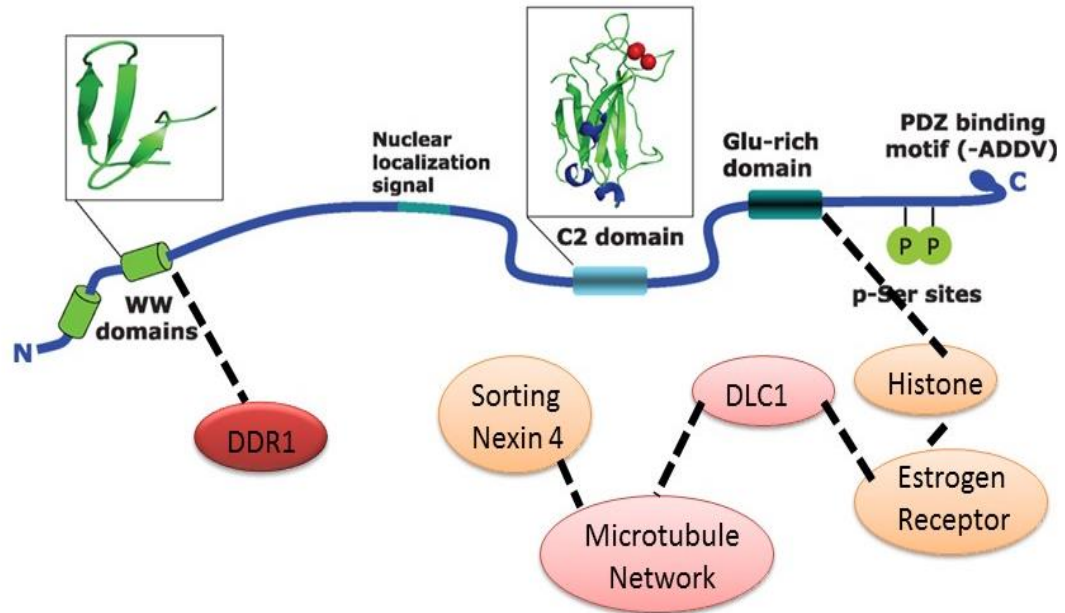


Figure 2: Interacting Partners of KIBRA Involved in Metastasis

### 2.3.2. Protein kinase C $\zeta$

Protein kinase C $\zeta$ , a member of PKC family of serine/threonine kinases, is another interacting partner of KIBRA as well as of DDR1 involved in the process of metastasis. It interacts with a small KIBRA fragment of 44aa fragment (953 to 996) containing four potential PKC phosphorylation sites (S967, S975, S978 and S981) through its complete catalytic domain (Büther et al., 2004) and is involved in multiple signal transduction pathways and modulate the processes like cell proliferation, adhesion, invasion and chemokine- triggered migration in breast cancer (Sun et al., 2005; Urtreger, C Grossoni, B Falbo, G Kazanietz, & Bal, 2005; Wu et al., 2010; F. Zhang et al., 2010). The interaction of PKC $\zeta$  with DDR1 and KIBRA in the presence of collagen forms a complex which leads to ERK MAPK cascade activation (Curat & Vogel, 2002). Collagen stimulate the DDR1 which lead to dissociation of complex and allow either PKC $\zeta$ -KIBRA complex for downstream signaling or stimulated DDR1 to participate in Ras/ ERK signaling (Xie & Haslam, 1997).

PKC $\zeta$  is a major player of PAR polarity complex, responsible for the establishment of the cell polarity, but the PAR polarity complex independent function of PKC $\zeta$  has been observed in the invasive progression of breast cancer. PKC $\zeta$  depletion promotes EMT in absence of functional PAR polarity complex. An oncogenic PKC $\zeta$  - NF $\kappa$ B-p65 signalling suppresses E-cadherin and ZO-1 expression and promotes epithelial-mesenchymal transition (EMT) and cause invasion in breast cancer. In a study conducted on experimental animal models by Arindam Paul et.al. PKC $\zeta$  was found to be highly active in invasive and metastatic breast cancers rather than non-invasive ductal carcinomas and the depletion if PKC $\zeta$  inhibits invasion and metastasis in breast cancer cells (Paul, Danley, Saha, Tawfik, & Paul, 2015).

### **2.3.3. Dynein Light Chain 1**

DLC1 is a cytoplasmic protein which is encoded by DYNLL1 gene in a human being (Pfister et al., 2005). It is an 8 kDa highly conserved protein component of cytoplasmic dynein complex and is expressed in numerous tissues. Along with its role in dynein motor function, it also interacts with Pak1 (p21- Activated Kinase 1) which phosphorylates and upregulates DLC1 expression and promotes the growth of ER-positive breast cancer cells. In addition, conditional upregulation of DLC1 facilitates recruitment of DLC1-ER complex to the ER target gene pS2 which facilitates estrogen-induced ER transactivation growth stimulation, and anchorage-independent growth of breast cancer cells (Suresh K Rayala et al., 2005). Rayala et.al in a study revealed that KIBRA interacts with DLC1 and potentiates ER transactivation by getting recruited at ER-responsive element (ERE) sites in ER-responsive genes in a ligand-induced manner through the underlying mechanism. The glutamic acid-rich region of KIBRA interacts with histone H3 which lead to the opening of chromatin with the subsequent recruitment of KIBRA-DLC1 and DLC1-ER complexes to chromatin of ER-targeted genes (Suresh K. Rayala et al., 2006).

SNX 4 (sorting nexin 4) interacts with the dynein (microtubule motor protein) and KIBRA and forms a complex which sort out the transferrin receptor (TfnR), a component involved in proliferation and cell survival (Daniels, Delgado, Rodriguez, Helguera, & Penichet, 2006), from lysosomal-mediated degradation and guide towards the juxtannuclear endocytic recycling pathway (Traer et al., 2007).

#### **2.3.4. PALS1-associated tight junction protein (PATJ)**

PATJ has been identified as another interacting partner of KIBRA where last four amino-acids of KIBRA interact with the eight PDZ domain of PATJ. Additionally, KIBRA also interact directly with synaptopodin (involved in actin based cytoskeleton organization) and regulate directional migration (Duning et al., 2008b).

PATJ is a member of an evolutionary conserved system the Pals1-PATJ-Crb complex (Protein-associated with Lin seven1-Pals1 associated tight junction protein-Crums3 complex) which regulates apicobasal polarity, tight junction formation, signaling, and directional migration of eukaryotic cells (Margolis & Borg, 2005; Shin, Fogg, & Margolis, 2006) by regulating reorientation of the MTOC (microtubule-organizing centre) and localization of PKC and PAR3 to the leading edge in direction of migration (Shin et al., 2007) .

# **CHAPTER III**

# **METHODOLOGY**

### 3.0. METHODOLOGY

#### 3.1. Mammalian Cell Culture

##### 3.1.1. Cells:

The human breast cancer cell line T47D was acquired from National Centre for Cell Science (NCCS), Pune, India. HBL100 cells were received as a gift from Dr. Andrew Craig, Queen's University, Canada. MCF 7 (Michigan Cancer Foundation 7), MDAMB 231 (M.D. Anderson Metastatic Breast Cancer 231), MDAMB 453 (M.D. Anderson Metastatic Breast Cancer 453) were also purchased from NCCS, Pune, India.

All the cell lines were cultured in their respective mediums along with the supplementation of 10% FBS and 1% penicillin and Streptomycin solution and incubated in the humidified environment containing 5% CO<sub>2</sub> and 37°C temperature. Different cell lines and their origin and ER status is mentioned in Table1.

<b>Cells</b>	<b>Type</b>	<b>ER Status</b>	<b>Media</b>
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:

In cell passaging adherent cell lines were first washed with PBS which was followed by Trypsin/EDTA treatment for a short span of time (approximately 5 to 7 minutes) to avoid cell damage. 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 transferred into a falcon which is then diluted by the complete media. The cells were pelleted down and resuspended in their respective media containing 10% DMSO (Cryomedium). These cells were stored in cryovials at 80°C.

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

### **3.1.4. Cell Counting:**

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

$$\text{Cells per mL} = \text{Mean cell Count per Large Square} \times 10^4$$

### **3.2. ER Transfection:**

ER  $\alpha$  was transfected to triple negative breast cancer cells in variable concentration (2 $\mu$ g and 4 $\mu$ g) via reverse transfection method. For control pcDNA vector transfected cells were used.

For transfection three 100 mm dishes were plated with a large amount of triple negative breast cancer cells (approximately  $3.5 \times 10^6$ ) and transfection mixture (Table 2.) was added to it.

<b>Content</b>	<b>Control</b>	<b>ER- GFP (2µg)</b>	<b>ER- GFP (4µg)</b>
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 is incubated at room temperature for 25 minutes.

**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 pelleted down and used for RNA isolation and Protein estimation.

### **3.3. RNA Isolation and cDNA Synthesis**

For both the transfected and non-transfected cells total RNA isolation was performed using RNeasy® Powerlyzer® Tissue & Cells Kit (Qiagen) followed by elution of isolated RNA in 50-100 µl of RNase free water. Before storing the RNA at -80 °C until further use the purity and concentration of isolated RNA is need to be estimated by using Nanodrop at 260 nm (260/280 ratio for pure RNA is~2.0).

This isolated RNA is then used to synthesize cDNA by using iScript cDNA synthesis Kit (Bio-Rad). For this, variable amount of RNA mixed with 5x iScript Reaction Mix (4 µg) and iScript Reverse Transcriptase (1 µg). After making up the volume to 20 µg by adding up nuclease free water. The mixture is incubated at different temperatures for different time as mentioned in Table 3. The newly synthesized cDNA is then stored at -20 °C until further use.

Priming	5 min at 25 °C
Reverse Transcription	20 min at 46 °C
RT Inactivation	1 min at 95 °C
Optional Step	Hold at 4 °C

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

### 3.4. Quantitative Real Time PCR

To determine the mRNA expression of KIBRA in T47D, MCF-7, MDA-MB-231, MDA-MB-453) compared to the house-keeping gene GAPDH mRNA, following reaction mixture (Table 4) 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 <sub>2</sub> O	7
<b>Total Volume</b>	<b>20</b>

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:

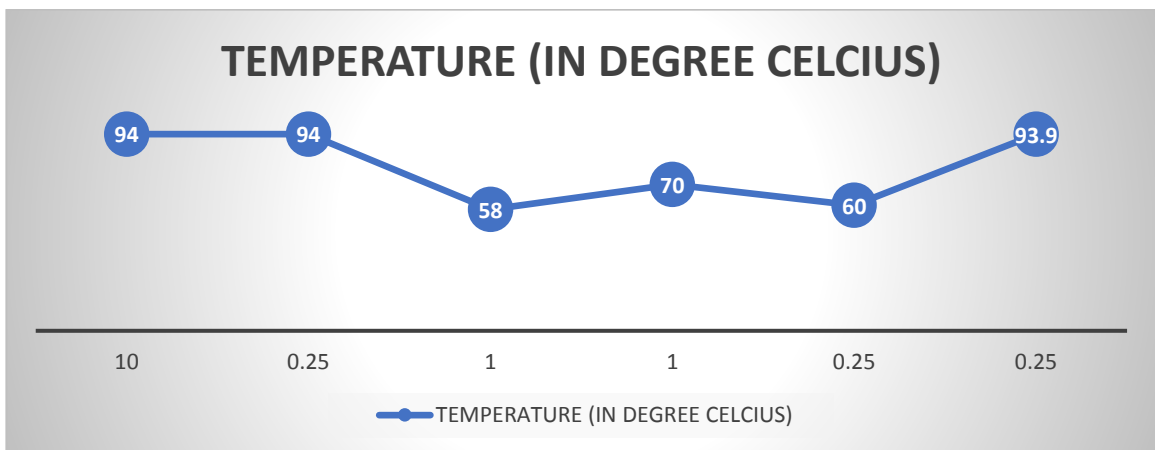


Figure 3: Temperature Condition for Real Time PCR

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

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

Where,  $\Delta\Delta Ct = \Delta Ct$  of sample –  $\Delta Ct$  of reference

$\Delta Ct = Ct$  of gene of interest –  $Ct$  of housekeeping gene

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

$$S = \sqrt{(s_1^2 + s_2^2)}$$

Where,  $s_1 =$  Standard Deviation of the gene of interest

$s_2 =$  Standard Deviation of the housekeeping gene

The Error margin was calculated by the following formula:

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

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

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

### **3.5. Protein Estimation and Quantification:**

#### **3.5.1. Lysate Preparation:**

For lysate preparation the cells were harvested by using PBS followed by centrifuging it at 3000 rpm for 5 minutes. The pellets were resuspended into NP-40 lysis buffer (including protease inhibitor cocktail (PI) in 1:1000 ratio and PMSF), vortexed at every 10 minutes and incubated at ice for 30 minutes. This mixture was then centrifuged for 10 minutes at 12000 rpm at 4°C to extract the total protein. The pellet was discarded and supernatant was transferred to an Eppendorf tube and stored at -4°C for future use.

### 3.5.2. Protein Quantification by Bradford Assay:

Cell lysate preparation was followed by protein quantification to determine protein concentration using Bradford Assay. For this, bovine serum albumin 1mg/ml was used as a standard and lysis buffer was used as a blank. Sample lysates (1 $\mu$ l each) and Bradford reagent (Bio-Rad) were added and readings were taken on a 96 well micro-plate reader. Readings were compared to a standard BSA dilution series.

Name of Standards	For BSA Standard (mg/ml)	Bradford reagent
S1	0.5 $\mu$ l	200 $\mu$ l
S2	1 $\mu$ l	200 $\mu$ l
S3	2 $\mu$ l	200 $\mu$ l
S4	3 $\mu$ l	200 $\mu$ l
S5	4 $\mu$ l & so on	200 $\mu$ l

Name of Samples	Quantity	Bradford reagent
A	1 $\mu$ l	200 $\mu$ l
B	1 $\mu$ l	200 $\mu$ l
C	1 $\mu$ l	200 $\mu$ l
D	1 $\mu$ l	200 $\mu$ l
E	1 $\mu$ l	200 $\mu$ l

Table 5: Bradford Assay Estimation Layout

### 3.5.3 SDS PAGE

SDS-PAGE is a discontinuous system and consist of resolving gel and stacking gel. 1 mm gel having 10% resolving gel (10% acrylamide gel) was prepared post polymerization to resolving system stacking gel was prepared.

30% Acrylamide	10%	
	5ml	10 ml
Quantity		
Water	2 ml	4ml
30% Acrylamide	1.5 ml	3.0ml
1.5M Tris (pH= 8.8)	1.25ml	2.5ml
10% SDS	50 $\mu$ l	100 $\mu$ l
10% APS	50 $\mu$ l	100 $\mu$ l
TEMED	5 $\mu$ l	10 $\mu$ l

Table 6(a): Resolving Gel Components

<b>30% Acrylamide</b>	<b>4%</b>
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 6 (b): Stocking 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 blocking of the membrane using 5% fat-free milk is done to block the non-specific binding sites. For blocking the NCP was incubated in 20 ml of 5% milk (Prepared in 1X PBST) for one hour on the rocker. After this, the membrane was washed 3 times with an interval of 10 minutes using 1X PBST buffer. The membrane is then cut into different parts according to the molecular weight of different proteins of interest and incubated in their respective primary antibody

(prepared in 3% of milk or 5% of BSA) overnight. Dilutions for antibodies were as follows (Table 7).

<b>Name of Primary Antibody</b>	<b>Dilution</b>
KIBRA (in 5% BSA)	1:1000
GAPDH	1:6000
ER- $\alpha$	1:1000

**Table 7: List of Antibody Dilution**

After overnight incubation, the membrane was washed again with 1X PBST for 3 times at an interval of 10 min. After washing the membrane was incubated in the secondary antibody (prepared in 3% of milk, Dilution- 1:5000) 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 to visualize the bands for protein expression using ECL solution on ChemiDoc (Bio-Rad) machine (Software Image lab 3.0) Further analysis was done.

### **3.6. Statistical Analysis**

For the densitometric analysis of the Immunoblotting results ImageJ software, developed by NIH was used. It displays and analyze the expression level of protein by quantifying the bands.

# **CHAPTER IV**

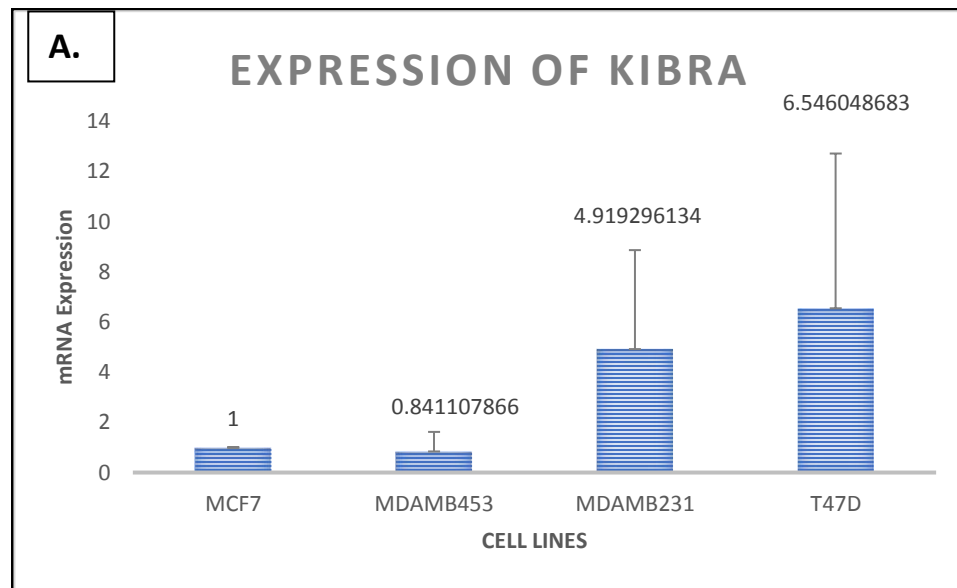
## **RESULTS**

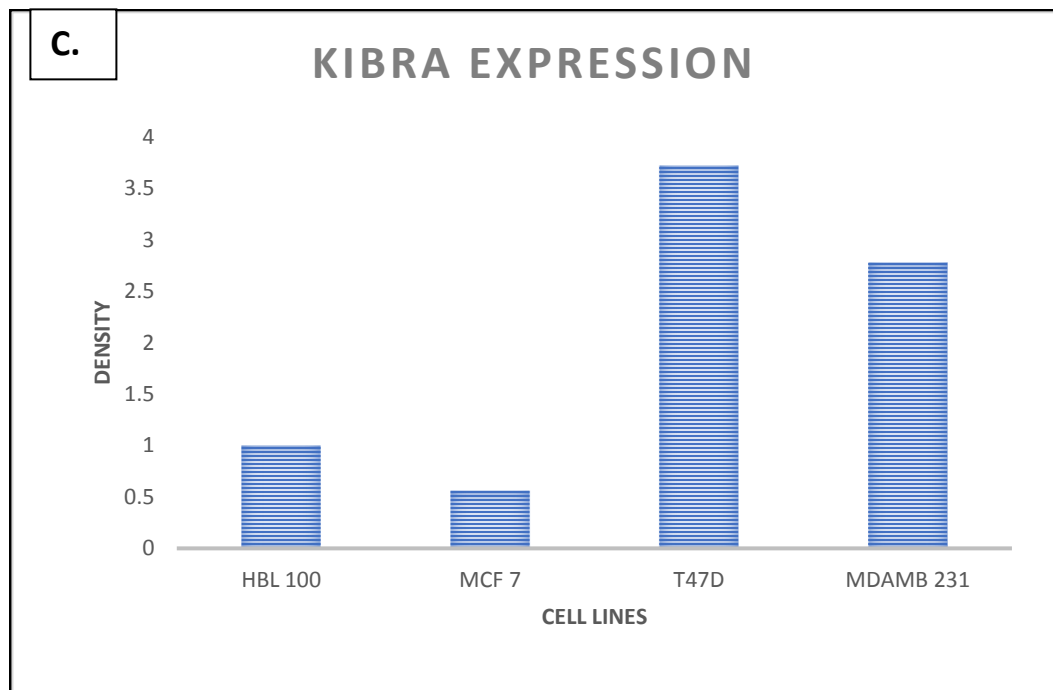
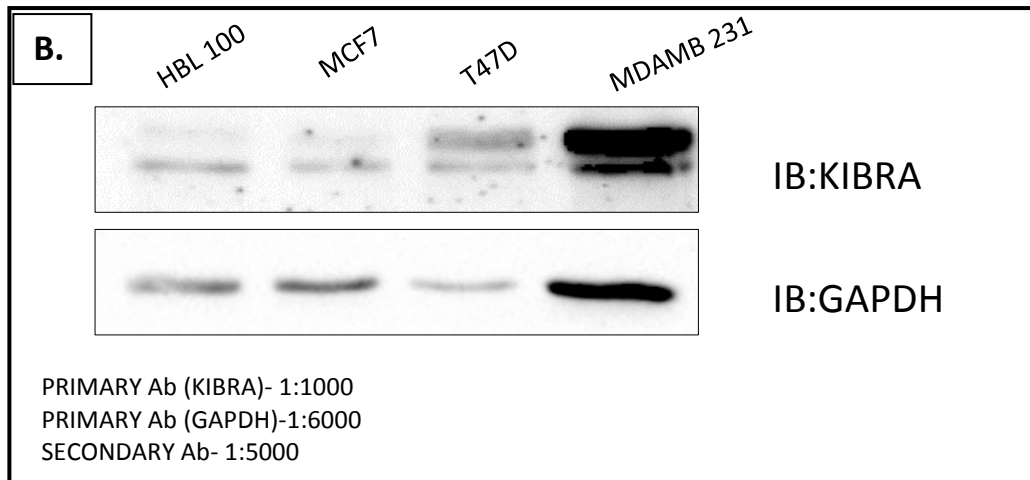
## 4.0. RESULTS

### 4.1. Expression level of KIBRA in Different Breast Cancer Cells

The basal level expression of KIBRA at mRNA level was checked by using Real Time PCR and was found to be high in T47D and MDAMB 231 cells, both of which are metastatic breast cancer cell lines, rather than any other cell lines. Also, the expression level in T47D cells, which was ER positive was higher than MDAMB231 cells which was ER negative. This shows that KIBRA expression is involved in metastasis.

To confirm this result and to check the role of KIBRA at protein level immunoblotting was performed which showed the same results as that of mRNA expression. The protein expression of KIBRA was higher in T47D cells.



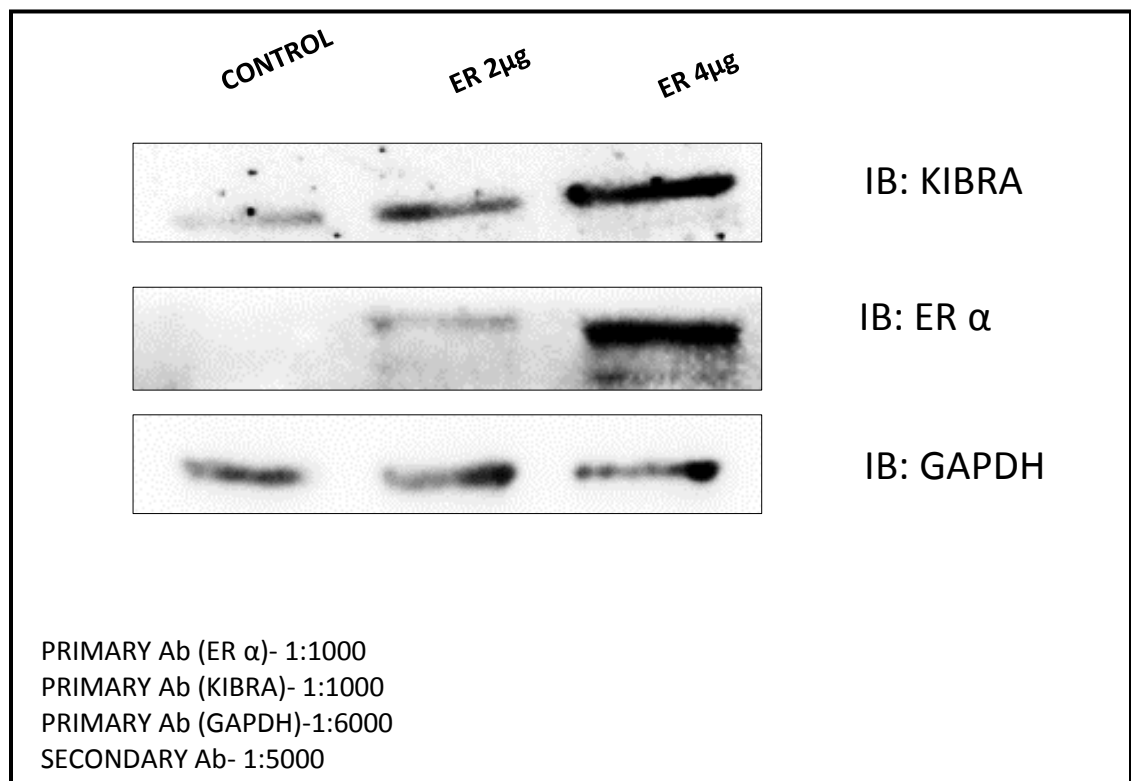


**Figure 4: Expression on KIBRA in different Breast Cancer Cells**

- A. mRNA level of KIBRA was normalized to GAPDH was analyzed using  $2^{-\Delta\Delta CT}$
- B. Lysates from indicated breast cancer cell lines were subjected to immunoblotting with KIBRA and GAPDH antibodies.
- C. Densitometric quantification of bands was performed to analyze and equalize the protein loaded

## 4.2. ER Transfection and Immunoblotting

The KIBRA expression was found to be higher in ER positive cells. To confirm any connection between ER and KIBRA expression the MDAMB 231, ER negative cell lines, were transfected with ER  $\alpha$  vector at variable concentration. The expression level for ER  $\alpha$ , to confirm transfection and KIBRA was checked by immunoblotting. The results had shown the higher expression of ER $\alpha$  as well that of KIBRA in cells transfected with higher concentration.



**Figure 5: ER Transfection and Immunoblotting**

Lysates from the transfected MDAMB 231 cell lines at variable concentration were subjected to immunoblotting using GAPDH, ER  $\alpha$  and KIBRA antibodies.

# **CHAPTER V**

# **DISCUSSION**

## 5.0. DISCUSSION

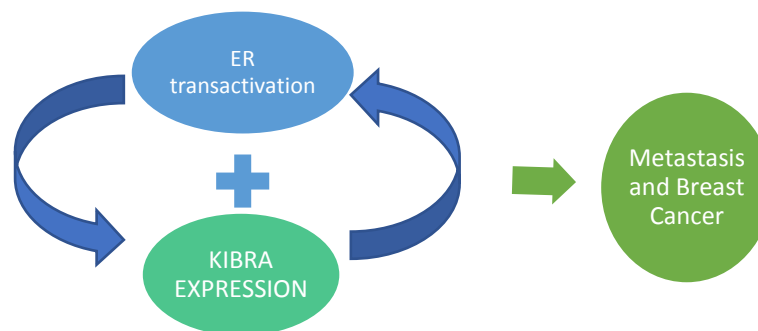
The higher expression of KIBRA in breast cancer cells rather than the normal cells indicates that the KIBRA could be a possible oncogene.

The Basal level expression of KIBRA both at mRNA and protein level were found to be high in T47D cell lines and MDAMB 231 cell lines. The expression of KIBRA in T47D was highest which is an ER positive cell line. From this we hypothesized that there could be a connection of ER presence and KIBRA expression. So, to elucidate the molecular mechanism involved the MDAMB 231 cells, ER negative in nature, were transfected with variable amount of ER alpha. The transfection of ER alpha is first confirmed by immunoblotting technique which showed higher expression level of ER in 4 µg concentration rather than 2 µg concentration. This indicates the successful transfection of ER alpha.

The expression level of KIBRA in these transfected cells was checked by using immunoblotting technique the bands of KIBRA for 4 µg concentration of ER were more intense than that of 2 µg concentration of ER which indicates that expression of KIBRA is affected by presence of ER alpha which could be a reason of higher expression of KIBRA in T47D cells.

## 5.1. CONCLUSION AND FUTURE PROSPECTS

The first conclusion which this study draws is that, KIBRA is an oncogene since its expression is higher in breast cancer cell lines. Apart from this this study has also concluded that the presence of ER alpha synergize the metastasis caused by the KIBRA in breast cancer cell lines.



**Figure 6: The Synergistic Mode of Action of KIBRA and ER for Metastasis**

Since KIBRA is the less researched scaffolding protein in field of breast cancer and most of the mechanism involved are still unknown so more research could be performed to unveil the underlying mechanisms involved. Knowing the mode of action of KIBRA could make it as a potential therapeutic target for modulating chemo-resistance in cancer cells.

## **SUMMARY**

**INTRODUCTION:** Breast cancer is one of the most common invasive malignancies diagnosed and is the second leading cause of death among women globally. It is a heterogeneous disease which is characterized by different molecular drivers. The increased propensity of motility and invasiveness, chemo-resistance and radio-resistance among epithelial malignant tumour is endowed by epithelial-mesenchymal transition (EMT), a critical biological process during embryonic development. Therefore, EMT is considered as the elementary step of chemo-resistance, local recurrence and metastasis. KIBRA is one such protein which interacts with several signalling molecules and regulates cell polarity, cell migration and cell cycle. The role of KIBRA in breast cancer came into light only after the discovery of regulation of oestrogen receptor activity by binding to the dynein light chain 1(DLC1) molecule. Later on, it was also reported that the KIBRA interacts with discoidin domain receptor 1(DDR1) and modulates collagen-induced MAPK kinase signalling in breast cells.

**METHODOLOGY:** To study the expression level of KIBRA in breast cancer cells and to investigate the correlation between KIBRA expression and ER, Immunoblotting and Real Time PCR were used.

**RESULTS AND DISCUSSION:** By studying the expression of KIBRA it was observed that its expression is higher in breast cancer cells. Further investigation by transfecting ER  $\alpha$  in ER negative cells showed that the expression of KIBRA varies directly with expression of ER. Based on these results we can conclude that presence of ER synergizes with KIBRA and leads to metastasis.

## REFERENCES

- Ahmad, A., Sarkar, S. H., Bitar, B., Ali, S., Aboukameel, A., Sethi, S., . . . Sarkar, F. H. (2012). Garcinol Regulates EMT and Wnt Signaling Pathways *In Vitro* and *In Vivo*, Leading to Anticancer Activity against Breast Cancer Cells. *Molecular Cancer Therapeutics*, 11(10), 2193-2201. doi: 10.1158/1535-7163.mct-12-0232-t
- Anuj, Arivazhagan, L., Surabhi, R. P., Kanakarajan, A., Sundaram, S., Pitani, R. S., . . . Rayala, S. K. (2017). KIBRA attains oncogenic activity by repressing RASSF1A. *British Journal Of Cancer*, 117, 553. doi: 10.1038/bjc.2017.192
- <https://www.nature.com/articles/bjc2017192#supplementary-information>
- Bao, B., Ahmad, A., Li, Y., Azmi, A. S., Ali, S., Banerjee, S., . . . Sarkar, F. H. (2012). Targeting CSCs within the tumor microenvironment for cancer therapy: a potential role of mesenchymal stem cells. *Expert Opinion on Therapeutic Targets*, 16(10), 1041-1054. doi: 10.1517/14728222.2012.714774
- Barker, K. T., Martindale, J. E., Mitchell, P. J., Kamalati, T., Page, M. J., Phippard, D. J., . . . Crompton, M. R. (1995). Expression patterns of the novel receptor-like tyrosine kinase, DDR, in human breast tumours. *Oncogene*, 10(3), 569-575.
- Büther, K., Plaas, C., Barnekow, A., & Kremerskothen, J. (2004). KIBRA is a novel substrate for protein kinase C $\zeta$ . *Biochemical and Biophysical Research Communications*, 317(3), 703-707. doi: 10.1016/j.bbrc.2004.03.107
- Curat, C. A., & Vogel, W. F. (2002). Discoidin domain receptor 1 controls growth and adhesion of mesangial cells. *Journal of the American Society of Nephrology*, 13(11), 2648-2656. doi: 10.1097/01.ASN.0000032419.13208.0C
- Daniels, T. R., Delgado, T., Rodriguez, J. A., Helguera, G., & Penichet, M. L. (2006). The transferrin receptor part I: Biology and targeting with cytotoxic antibodies for the treatment of cancer. *Clinical Immunology*, 121(2), 144-158. doi: <https://doi.org/10.1016/j.clim.2006.06.010>
- Debies, M. T., Gestl, S. A., Mathers, J. L., Mikse, O. R., Leonard, T. L., Moody, S. E., . . . Gunther, E. J. (2008). Tumor escape in a Wnt1-dependent mouse breast cancer model is enabled by p19(Arf)/p53 pathway lesions but not p16(Ink4a) loss. *The Journal of Clinical Investigation*, 118(1), 51-63. doi: 10.1172/JCI33320
- Dobrosotskaya, I., Guy, R. K., & James, G. L. (1997). MAGI-1, a membrane-associated guanylate kinase with a unique arrangement of protein-protein interaction domains. *Journal of Biological Chemistry*, 272(50), 31589-31597. doi: 10.1074/jbc.272.50.31589
- Duning, K., Schurek, E.-M., Schlüter, M., Bayer, M., Reinhardt, H.-C., Schwab, A., . . . Pavenstädt, H. (2008a). KIBRA Modulates Directional Migration of Podocytes. *Journal of the American Society of Nephrology*, 19(10), 1891-1903. doi: 10.1681/asn.2007080916
- Duning, K., Schurek, E.-M., Schlüter, M., Bayer, M., Reinhardt, H.-C., Schwab, A., . . . Pavenstädt, H. (2008b). KIBRA Modulates Directional Migration of Podocytes. *Journal of the American Society of Nephrology : JASN*, 19(10), 1891-1903. doi: 10.1681/ASN.2007080916

- Fanning, A. S., & Anderson, J. M. (1999). PDZ domains: fundamental building blocks in the organization of protein complexes at the plasma membrane. *Journal of Clinical Investigation*, *103*(6), 767-772.
- Faraci-Orf, E., McFadden, C., & Vogel, W. F. (2006). DDR1 signaling is essential to sustain Stat5 function during lactogenesis. *Journal of Cellular Biochemistry*, *97*(1), 109-121. doi: doi:10.1002/jcb.20618
- Fata, J. E., Werb, Z., & Bissell, M. J. (2004). Regulation of mammary gland branching morphogenesis by the extracellular matrix and its remodeling enzymes. *Breast Cancer Research*, *6*(1), 1-11.
- Friedenreich, C. M. (2011). Physical Activity and Breast Cancer: Review of the Epidemiologic Evidence and Biologic Mechanisms. In H.-J. Senn & F. Otto (Eds.), *Clinical Cancer Prevention* (pp. 125-139). Berlin, Heidelberg: Springer Berlin Heidelberg.
- Hanahan, D., & Weinberg, R. A. (2011). Hallmarks of cancer: The next generation. *Cell*, *144*(5), 646-674. doi: 10.1016/j.cell.2011.02.013
- Herb, A., Wisden, W., Catania, M. V., Maréchal, D., Dresse, A., & Seeburg, P. H. (1996). Prominent dendritic localization in forebrain neurons of a novel mRNA and its product, dendrin. *Molecular and Cellular Neurosciences*, *8*(5), 367-374. doi: 10.1006/mcne.1996.0594
- Hilton, H. N., Stanford, P. M., Harris, J., Oakes, S. R., Kaplan, W., Daly, R. J., & Ormandy, C. J. (2008). KIBRA interacts with discoidin domain receptor 1 to modulate collagen-induced signalling. *Biochimica et Biophysica Acta - Molecular Cell Research*, *1783*(3), 383-393. doi: 10.1016/j.bbamcr.2007.12.007
- Kang, Y., & Massagué, J. (2004). Epithelial-mesenchymal transitions: Twist in development and metastasis. *Cell*, *118*(3), 277-279. doi: 10.1016/j.cell.2004.07.011
- Knight, J. F., Sung, V. Y. C., Kuzmin, E., Couzens, A. L., de Verteuil, D. A., Ratcliffe, C. D. H., . . . Park, M. (2018). KIBRA (WWC1) Is a Metastasis Suppressor Gene Affected by Chromosome 5q Loss in Triple-Negative Breast Cancer. *Cell Reports*, *22*(12), 3191-3205. doi: <https://doi.org/10.1016/j.celrep.2018.02.095>
- Kremerskothen, J., Kindler, S., Finger, I., Veltel, S., & Barnekow, A. (2006). Postsynaptic recruitment of Dendrin depends on both dendritic mRNA transport and synaptic anchoring. *Journal of Neurochemistry*, *96*(6), 1659-1666. doi: doi:10.1111/j.1471-4159.2006.03679.x
- Kremerskothen, J., Plaas, C., Büther, K., Finger, I., Veltel, S., Matanis, T., . . . Barnekow, A. (2003). Characterization of KIBRA, a novel WW domain-containing protein. *Biochemical and biophysical research communications*, *300*(4), 862-867. doi: [https://doi.org/10.1016/S0006-291X\(02\)02945-5](https://doi.org/10.1016/S0006-291X(02)02945-5)
- Kremerskothen, J., Plaas, C., Kindler, S., Frotscher, M., & Barnekow, A. (2005). Synaptopodin, a molecule involved in the formation of the dendritic spine apparatus, is a dual actin/ $\alpha$ -actinin binding protein. *Journal of Neurochemistry*, *92*(3), 597-606. doi: doi:10.1111/j.1471-4159.2004.02888.x
- Lauriat, T. L., Dracheva, S., Kremerskothen, J., Duning, K., Haroutunian, V., Buxbaum, J. D., . . . Alison McInnes, L. (2006). Characterization of KIAA0513, a novel signaling molecule that interacts with modulators of neuroplasticity, apoptosis, and the cytoskeleton. *Brain Research*, *1121*(1), 1-11. doi: <https://doi.org/10.1016/j.brainres.2006.08.099>

- Li, Y., Wicha, M. S., Schwartz, S. J., & Sun, D. (2011). Implications of cancer stem cell theory for cancer chemoprevention by natural dietary compounds. *Journal of Nutritional Biochemistry*, 22(9), 799-806. doi: 10.1016/j.jnutbio.2010.11.001
- Margolis, B., & Borg, J.-P. (2005). Apicobasal polarity complexes. *Journal of Cell Science*, 118(22), 5157-5159. doi: 10.1242/jcs.02597
- Neuner-Jehle, M., Denizot, J. P., Borbély, A. A., & Mallet, J. (1996). Characterization and sleep deprivation-induced expression modulation of dendrin, a novel dendritic protein in rat brain neurons. *Journal of Neuroscience Research*, 46(2), 138-151. doi: doi:10.1002/(SICI)1097-4547(19961015)46:2<138::AID-JNR2>3.0.CO;2-I
- Paul, A., Danley, M., Saha, B., Tawfik, O., & Paul, S. (2015). PKC $\zeta$  Promotes Breast Cancer Invasion by Regulating Expression of E-cadherin and Zonula Occludens-1 (ZO-1) via NF $\kappa$ B-p65. *Scientific Reports*, 5, 12520. doi: 10.1038/srep12520
- <https://www.nature.com/articles/srep12520#supplementary-information>
- Pfister, K. K., Fisher, E. M. C., Gibbons, I. R., Hays, T. S., Holzbaaur, E. L. F., McIntosh, J. R., . . . Vallee, R. B. (2005). Cytoplasmic dynein nomenclature. *The Journal of Cell Biology*, 171(3), 411-413. doi: 10.1083/jcb.200508078
- Rayala, S. K., den Hollander, P., Balasenthil, S., Yang, Z., Broaddus, R. R., & Kumar, R. (2005). Functional regulation of oestrogen receptor pathway by the dynein light chain 1. *EMBO reports*, 6(6), 538-544. doi: 10.1038/sj.embor.7400417
- Rayala, S. K., den Hollander, P., Manavathi, B., Talukder, A. H., Song, C., Peng, S., . . . Kumar, R. (2006). Essential Role of KIBRA in Co-activator Function of Dynein Light Chain 1 in Mammalian Cells. *Journal of Biological Chemistry*, 281(28), 19092-19099. doi: 10.1074/jbc.M600021200
- Rizo, J., & Südhof, T. C. (1998). C2-domains, Structure and Function of a Universal Ca<sup>2+</sup>-binding Domain. *Journal of Biological Chemistry*, 273(26), 15879-15882. doi: 10.1074/jbc.273.26.15879
- Schneider, A., Huentelman, M., Kremerskothen, J., Duning, K., Spoelgen, R., & Nikolic, K. (2010). KIBRA: a new gateway to learning and memory? *Frontiers in Aging Neuroscience*, 2(4). doi: 10.3389/neuro.24.004.2010
- Shin, K., Fogg, V. C., & Margolis, B. (2006). Tight Junctions and Cell Polarity. *Annual Review of Cell and Developmental Biology*, 22(1), 207-235. doi: 10.1146/annurev.cellbio.22.010305.104219
- Shin, K., Straight, S., & Margolis, B. (2005). PATJ regulates tight junction formation and polarity in mammalian epithelial cells. *The Journal of Cell Biology*, 168(5), 705-711. doi: 10.1083/jcb.200408064
- Shin, K., Wang, Q., & Margolis, B. (2007). PATJ regulates directional migration of mammalian epithelial cells. *EMBO reports*, 8(2), 158-164. doi: 10.1038/sj.embor.7400890
- Sun, R., Gao, P., Chen, L., Ma, D., Wang, J., Oppenheim, J. J., & Zhang, N. (2005). Protein Kinase C  $\zeta$  Is Required for Epidermal Growth Factor-Induced Chemotaxis of Human Breast Cancer Cells. *Cancer Research*, 65(4), 1433-1441. doi: 10.1158/0008-5472.can-04-1163
- Thiery, J. P., & Morgan, M. (2004). Breast cancer progression with a Twist. *Nature Medicine*, 10, 777. doi: 10.1038/nm0804-777

- Thiery, J. P., & Sleeman, J. P. (2006). Complex networks orchestrate epithelial–mesenchymal transitions. *Nature Reviews Molecular Cell Biology*, 7, 131. doi: 10.1038/nrm1835
- Traer, C. J., Rutherford, A. C., Palmer, K. J., Wassmer, T., Oakley, J., Attar, N., . . . Cullen, P. J. (2007). SNX4 coordinates endosomal sorting of TfnR with dynein-mediated transport into the endocytic recycling compartment. *Nature Cell Biology*, 9, 1370. doi: 10.1038/ncb1656
- <https://www.nature.com/articles/ncb1656#supplementary-information>
- Urtreger, A., C Grossoni, V., B Falbo, K., G Kazanietz, M., & Bal, D. (2005). *Atypical protein kinase C-zeta modulates clonogenicity, motility, and secretion of proteolytic enzymes in murine mammary cells* (Vol. 42).
- Vogel, W. F., Aszódi, A., Alves, F., & Pawson, T. (2001). Discoidin domain receptor 1 tyrosine kinase has an essential role in mammary gland development. *Molecular and Cellular Biology*, 21(8), 2906-2917. doi: 10.1128/MCB.21.8.2906-2917.2001
- Wang, Z., Li, Y., Ahmad, A., Azmi, A. S., Kong, D., Banerjee, S., & Sarkar, F. H. (2010). Targeting miRNAs involved in cancer stem cell and EMT regulation: An emerging concept in overcoming drug resistance. *Drug Resistance Updates*, 13(4-5), 109-118. doi: 10.1016/j.drug.2010.07.001
- Wu, J., Zhang, B., Wu, M., Li, H., Niu, R., Ying, G., & Zhang, N. (2010). Screening of a PKC  $\zeta$ -specific kinase inhibitor PKC $\zeta$ 1257.3 which inhibits EGF-induced breast cancer cell chemotaxis. *Investigational New Drugs*, 28(3), 268-275. doi: 10.1007/s10637-009-9242-8
- Xiao, L., Chen, Y., Ji, M., Volle, D. J., Lewis, R. E., Tsai, M.-Y., & Dong, J. (2011). KIBRA Protein Phosphorylation Is Regulated by Mitotic Kinase Aurora and Protein Phosphatase 1. *Journal of Biological Chemistry*, 286(42), 36304-36315. doi: 10.1074/jbc.M111.246850
- Xie, J., & Haslam, S. Z. (1997). Extracellular matrix regulates ovarian hormone-dependent proliferation of mouse mammary epithelial cells. *Endocrinology*, 138(6), 2466-2473. doi: 10.1210/en.138.6.2466
- Yang, S., Ji, M., Zhang, L., Chen, Y., Wennmann, D. O., Kremerskothen, J., & Dong, J. (2014). Phosphorylation of KIBRA by the extracellular signal-regulated kinase (ERK)–ribosomal S6 kinase (RSK) cascade modulates cell proliferation and migration. *Cellular Signalling*, 26(2), 343-351. doi: <https://doi.org/10.1016/j.cellsig.2013.11.012>
- Yoshihama, Y., Chida, K., & Ohno, S. (2012). The KIBRA–aPKC connection. *Communicative & Integrative Biology*, 5(2), 146-151. doi: 10.4161/cib.18849
- Zhang, F., Zhang, X., Li, M., Chen, P., Zhang, B., Guo, H., . . . Zhang, N. (2010). mTOR Complex Component Rictor Interacts with PKC $\zeta$  and Regulates Cancer Cell Metastasis. *Cancer Research*, 70(22), 9360-9370. doi: 10.1158/0008-5472.can-10-0207
- Zhang, X., Liu, X., Luo, J., Xiao, W., Ye, X., Chen, M., . . . Zhang, G. J. (2016). Notch3 inhibits epithelial–mesenchymal transition by activating Kibra-mediated Hippo/YAP signaling in breast cancer epithelial cells. *Oncogenesis*, 5(11), e269. doi: 10.1038/oncsis.2016.67

## 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

<b>Name of the Author</b>	Garima Singh
<b>Department</b>	Human Genetics and Molecular Medicine
<b>Degree</b>	M.Sc. Life Sciences with specialization in Molecular Medicine
<b>University</b>	Central University of Punjab
<b>Guide</b>	Dr. Harish Chander
<b>Project Title</b>	Expression of KIBRA in Breast Cancer Cell Lines
<b>Year of Award</b>	2018

### Agreement

1. I hereby certify that, if appropriate, I have obtained and attached hereto a written permission/ statement from the owner(s) of each third party copyrighted matter to be included in my Research Project allowing distribution as specified below.
2. I hereby grant to the university and its agents the non-exclusive license to archive and make accessible, under the conditions specified below, my Research Project in whole or in part in all forms of media, now or hereafter known. I retain all other ownership rights to the copyright of the Research Project. I also retain the right to use in future works (such as articles or books) all or part of this Project Report.

**Conditions:**

Release the entire work for access worldwide	
Release the entire work for Central University of Punjab only for: 1 year 2 years 3 years and after this time release the work for access worldwide.	
Release the entire work for Central University of Punjab only, while at the same time releasing the following parts of the work (e.g. because other parts relate to publications for worldwide access: a) Bibliographic details and synopsis only b) Bibliographic details, synopsis and the following chapters only c) Preview/ Table of contents/ Page only	
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 KIBRA 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.

(Garima Singh)

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 Analysis Result

Analysed Document: plag check.docx (D39280342)

Submitted: 5/27/2018 12:54:00 PM

Submitted By: uradandapraveen@gmail.com

Significance: 4 %

### Sources included in the report:

<https://bmccancer.biomedcentral.com/articles/10.1186/s12885-018-4491-6> <https://bio-protocol.org/e850>

### Instances where selected sources appear:

2