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Doctoral Thesis

Functional Characterisation of Glycosyltransferase 1 Domain Containing 1 gene in Mammary Carcinoma Cells

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A thesis submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Biomedical Sciences, The University of Auckland, 2013.

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&

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Abstract

Breast cancer remains the most prevalent form of cancer among women throughout the world. In the post-genomic research era many gene families involved in mammmary carcinoma as well as in other carcinomas have been identified. High-throughput technology “omics” platforms such as gene expression microarrays, genome sequencing and tandem mass spectrometry have also led to the discovery of many novel molecules and pathways. However, the biological relevance of these novel molecules is often unknown. In this study, I have investigated the role of a hypothetical gene called Glycosyltransferase 1 Domain Containing 1 (GLT1D1) in mammary carcinoma cells.

Differential expression of GLT1D1 in variety of cancers as indicated by microarray studies and its prediction as secreted protein made it an attractive candidate for the study. Preliminary studies to confirm gene expression by immunoblot analysis using multiple human tissues and sera revealed that GLT1D1 is highly expressed in liver, moderately in kidney, intestine and stomach and secreted into sera.

Initially, attempts were made to establish GLT1D1 stable cell lines to functionally characterise the gene. However, GLT1D1 transfection followed by stable selection resulted in cell death of mammmary carcinoma cell lines, MCF-7 and BT549. Cell viability assay results showed that GLT1D1 differentially reduced MCF-7 and BT-549 cells viability when grown in low serum and full serum media. It was found that while, GLT1D1 reduced MCF-7 cell viability under both full serum and low serum growth conditions, it only reduced BT-549 cell viability under low serum growth condition. Further analysis showed that GLT1D1 transfected MCF-7 and BT-549 cells undergo apoptotic cell death when grown in low serum condition. Experiments using the general caspase inhibitor, Z-VAD-FMK, demonstrated that GLT1D1 caused caspase independent apoptotic cell death in MCF-7 cells and caspase dependent apoptotic cell death in BT-549 cells. Further investigation addressed the GLT1D1 induced reduction in MCF-7 cell viability under full serum growth conditions. The microscopy and immunoblotting analysis of the autophagy signature molecule LC3 confirmed the induction of autophagy in MCF-7. Additionally, autophagy inhibitor 3-MA abrogated GLT1D1 mediated autophagy induction and cell death effect.

In addition, this study explored the mechanism of action for the observed GLT1D1 mediated apoptotic cell death and cell death with autophagy. It was found that GLT1D1 downregulated BCL2 mRNA and protein expression. Furthermore, the study demonstrated that BCL2 down-regulation increased the BAX/BCL2 ratio and autophagic molecule Beclin-1 expression in cells causing apoptotic cell death and cell death with autophagy. Since cell death related molecules are often localised to mitochondria, studies were performed to verify GLT1D1 localisation to mitochondria. Experimental results suggest probable mitochondrial localisation of GLT1D1 in certain tissue types.
In summary, this study reveals the functional characteristics of the GLT1D1 gene in causing apoptotic cell death and cell death with autophagy. These results together suggest a potential tumour suppressor function for GLT1D1.
Dedicated to

Aayi, Appa

and

Vasudha chikki, Dyumani kaka
Acknowledgements

First of all, I like to thank my supervisors Dr Dong-Xu Liu and Professor Peter E. Lobie for providing me with this opportunity to accomplish the study and for their administrative and technical support to carry out this study. I also like to thank Dr Jo Perry, who in the capacity of an advisor helped me to resolve technical and administrative issues. Dr Michael Steiner has been a great advisor and I like to thank him for all the technical help he has lent me during the study.

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# Table of Contents

ABSTRACT........................................................................................................................................................................II

ACKNOWLEDGEMENTS ...............................................................................................................................................................IV

LIST OF FIGURES...........................................................................................................................................................................IX

LIST OF TABLES ...........................................................................................................................................................................XI

CHAPTER 1. GENERAL INTRODUCTION ................................................................................................................................. 1

1.1. INTRODUCTION ................................................................................................................................................................. 1

1.2. CANCER .................................................................................................................................................................................... 1

   1.2.1. An account of cancer characteristics .......................................................................................................................... 2

   1.2.2. Hallmarks of cancer ......................................................................................................................................................... 3

1.3. BREAST CANCER ...................................................................................................................................................................... 8

   1.3.1. Mammary gland structure and development .................................................................................................................. 8

   1.3.2. Breast cancer histopathology ........................................................................................................................................ 10

   1.3.3. Evolution of breast cancer ........................................................................................................................................ 11

   1.3.4. Precursor lesions and risk indicators .......................................................................................................................... 12

   1.3.5. Cell origin of breast cancer .......................................................................................................................................... 12

   1.3.6. Tumour progression ......................................................................................................................................................... 13

   1.3.7. Role of tumour microenvironment in tumour progression .......................................................................................... 13

   1.3.8. Key molecules involved in breast cancer .................................................................................................................... 14

   1.3.9. Breast cancer treatment ................................................................................................................................................ 15

1.4. THE CELL DEATH ................................................................................................................................................................... 16

   1.4.1. Types of cell death ......................................................................................................................................................... 16

   1.4.2. Apoptosis .......................................................................................................................................................................... 17

      1.4.2.1. Historical perspective ............................................................................................................................................... 17

      1.4.2.2. Apoptotic pathways ................................................................................................................................................. 17

      1.4.2.3. Extrinsic pathway ...................................................................................................................................................... 18

      1.4.2.4. Intrinsic pathway ................................................................................................................................................... 20

      1.4.2.5. Role of apoptosis in growth and development .................................................................................................. 22

      1.4.2.6. Apoptosis in cancer ............................................................................................................................................... 23

      1.4.2.7. Methods for monitoring apoptosis ...................................................................................................................... 25

      1.4.2.8. Microscopic and flow cytometric methods ....................................................................................................... 25

   1.4.3. Autophagy ........................................................................................................................................................................ 27

      1.4.3.1. Historical perspective ............................................................................................................................................. 27

      1.4.3.2. Autophagic pathway ............................................................................................................................................... 28

      1.4.3.3. Autophagy in development and differentiation .................................................................................................. 30

      1.4.3.4. Autophagy in cancer .............................................................................................................................................. 31

      1.4.3.5. Methods for monitoring autophagy .................................................................................................................... 33

      1.4.3.6. Measuring the number of autophagosomes ......................................................................................................... 33

      1.4.3.7. Electron microscopy .............................................................................................................................................. 33

      1.4.3.8. Fluorescence microscopy ...................................................................................................................................... 34

      1.4.3.9. Biochemical assay ......................................................................................................................................................... 34

      1.4.3.10. Measuring autophagic flux ................................................................................................................................ 34

   1.4.4. Regulated Necrosis .......................................................................................................................................................... 35

   1.4.5. Other types of cell death ............................................................................................................................................. 35

1.5. RATIONALE OF THE PhD STUDY ........................................................................................................................................ 36

1.6. INTRODUCTION TO GLT1D1 .............................................................................................................................................. 38
CHAPTER 2. MATERIALS AND METHODS

2.1. MATERIALS

2.1.1. Chemicals, Reagents, Antibodies and Primers

2.2. METHODS

2.2.1. Molecular biology

2.2.1.1. Gene cloning

2.2.1.2. DNA and RNA isolation

2.2.1.3. One-step RT-PCR

2.2.2. Recombinant protein expression and purification

2.2.2.1. Protein induction

2.2.2.2. Protein purification

2.2.2.3. Immunisation

2.2.2.4. Antibody purification

2.2.2.5. Immunoblotting

2.2.2.6. Protein quantification

2.2.3. Cell culture

2.2.3.1. Human cell lines

2.2.3.2. Passaging human cell lines

2.2.4. Cell biology methods

2.2.4.1. Generating stable cell lines

2.2.4.2. Transient transfection

2.2.4.3. Cytochemistry

2.2.4.4. MTT assay for cell viability

2.2.4.5. Apoptosis assay

2.2.4.6. Autophagy assay

2.2.4.7. Luciferase assay

2.2.4.8. GLT1D1 localisation

2.2.4.9. Statistical analysis

CHAPTER 3. RESULTS

3.1. INTRODUCTION

3.2. EXPRESSION AND PURIFICATION OF GST-GLT1D1 AND GST-GLT1D1-2 ANTIGENS

3.3. ANTIBODY PRODUCTION AND PURIFICATION

3.3.1. Purification of anti-GLT1D1 and anti-GLT1D1-2 antibody

3.4. GLT1D1 EXPRESSION IN HUMAN TISSUES AND CANCER CELLS

3.4.1. Human cancer cell line screening for GLT1D1 expression

3.5. FUNCTIONAL CHARACTERISATION OF GLT1D1

3.5.1. The GLT1D1 cells undergo cell death during stable selection

3.5.2. GLT1D1 mediates cell death

3.5.3. GLT1D1 promotes apoptotic cell death in BT-549 and MCF-7 cells

3.5.4. GLT1D1 promotes cell death with autophagy in MCF-7 cells

3.5.4.1. LC3 Immunoblotting

3.5.4.2. LC3 fluorescence assay

3.5.4.3. GLT1D1 increases autophagic flux in MCF-7 cells

3.5.4.4. Autophagy inhibitor 3-MA abrogates GLT1D1 mediated autophagy in MCF-7 cells

3.5.4.5. 3-MA abrogates GLT1D1 mediated cell death with autophagy
List of Figures

Figure 1 : Six hallmarks of cancer summarised by Hanahan and Weinberg. ................................................................. 3
Figure 2 : Structure of normal breast and a lobule ........................................................................................................... 9
Figure 3 : (A) Stages of mammary gland development at puberty, adult life, pregnancy and lactation (B) 
Dichotomous, lobular branching of ducts .................................................................................................................. 10
Figure 4 : Breast cancer histopathology through different stages ...................................................................................... 11
Figure 5 : Multi-step model proposed for breast cancer progression .................................................................................. 12
Figure 6 : The extrinsic apoptotic pathway. .................................................................................................................... 19
Figure 7 : Intrinsic apoptotic pathway. .......................................................................................................................... 21
Figure 8 : Apoptosis during development ........................................................................................................................ 23
Figure 9 : Diagrammatic representation of the autophagic pathway. .................................................................................... 29
Figure 10 : Microarray analysis showing differential expression of GLT1D1 in breast cancer. ......................................................... 37
Figure 11 : Microarray analysis showing differential expression of GLT1D1 in oesophageal cancer. .................................................. 37
Figure 12 : – Amino acid sequence of GLT1D1 isoforms and the recognized peptide sequences ............................................. 40
Figure 13 : Recombinant GST-GLT1D1 and GST-GLT1D1-2 expression and purification. ....................................................... 56
Figure 14 : Qualitative screening of the anti-sera obtained from rabbits. .................................................................................. 58
Figure 15 : Confirmation of covalent cross linking of GST, GST-GLT1D1-2 and GST-GLT1D1 recombinant proteins 
to the GSH sepharose. ......................................................................................................................................................... 60
Figure 16 : Confirmation of GLT1D1 expression in human liver tissue. ..................................................................................... 62
Figure 17 : GLT1D1 is present in sera. ............................................................................................................................... 63
Figure 18 : Cancer cells’ screening for GLT1D1 (A) mRNA and (B) protein expression. .......................................................... 64
Figure 19 : GLT1D1 (A) mRNA and (B) protein expression in human breast cancer cell lines. ..................................................... 65
Figure 20 : Transient and stable forced-expression of GLT1D1 in different cancer cell lines. .................................................. 68
Figure 21 : Determination of GLT1D1 mediated cell viability effects on MCF-7 and BT-549 under full serum 
(10%) growth condition. .................................................................................................................................................. 69
Figure 22 : Determination of the cell viability effects of GLT1D1 on MCF-7 and BT-549 under low (0.2%) serum 
growth condition. .............................................................................................................................................................. 70
Figure 23 : Determination of the apoptotic effects of GLT1D1 on MCF-7 cells under full serum growth condition. .............. 71
Figure 24 : Determination of the apoptotic effects of GLT1D1 on MCF-7 cells under full serum growth condition. .............. 72
Figure 25 : Determination of the apoptotic effects of GLT1D1 on MCF-7 (A) and BT-549 (B) cells under low 
serum growth condition. .................................................................................................................................................. 73
Figure 26 : Determination of the caspase inhibitor effects on GLT1D1 mediated apoptosis in MCF-7 (A) and BT- 
549 (B) cells under low serum growth condition. ............................................................................................................. 74
Figure 27 : Determination of GLT1D1 mediated autophagy in MCF-7 cells by LC3 immunoblotting assay ............... 76
Figure 28 : Validation of GLT1D1 mediated autophagosome formation in BT-549 cells by LC3 immunoblotting 
assay. ......................................................................................................................................................................................... 77
Figure 29 : Quantitative measurement of GLT1D1 mediated autophagosome formation in .......................................................... 78
Figure 30 : Confirmation of GLT1D1 mediated autophagy in MCF-7 cells by electron microscopy. ................................. 80
Figure 31 : Confirmation of GLT1D1 mediated increase in autophagic flux in MCF-7 cells. ..................................................... 82
Figure 32 : Inhibition of GLT1D1 mediated autophagy in MCF-7 cells using autophagy ......................................................... 83
Figure 37: Inhibition of GLT1D1 mediated MCF-7 cell death with autophagy, using autophagy inhibitor 3-MA.
Figure 38: Measurement of GLT1D1 mediated decrease in BCL2 promoter activity.
Figure 39: Evaluation of GLT1D1 mediated BCL2 down regulation in MCF7 and BT549 cells.
Figure 40: Evaluation of GLT1D1 mediated perturbed BAX/BCL2 ratio in MCF7 and BT549 cells.
Figure 41: Evaluation of GLT1D1 mediated up-regulation of Beclin-1 expression in MCF7 cells.
Figure 42: The GLT1D1’s mitochondrial localisation determination in MCF7 cells using mitochondrial targeting element (MTE) of Cytochrome Oxidase-8 (COX-8).
Figure 43: The GLT1D1’s mitochondrial localisation determination in MCF7 cells using mitotracker dye.
Figure 44: The GLT1D1’s mitochondrial localisation determination in MCF7 cells using mitochondrial targeting element (MTE) of Methionine Sulfoxide Reductase (MSRA).
Figure 45: Determination of GLT1D1 localisation in MCF7 cells by sub-fractionation method.
Figure 46: Diagramatic representation of a possible scheme of GLT1D1 action in cells.
List of Tables

Table 1 : The programmed cell deaths and their characteristics ................................................................. 17
Table 2 : List of chemicals and reagents ....................................................................................................... 42
Table 3 : List of antibodies .......................................................................................................................... 42
Table 4 : List of primers ................................................................................................................................. 42
Table 5 : The rabbit immunisation and bleeding details ............................................................................... 46
Table 6 : List of wild-type cell lines .............................................................................................................. 48
Table 7 : List of stable cell lines .................................................................................................................... 50
### LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>ADH</td>
<td>Atypical ductal hyperplasia</td>
</tr>
<tr>
<td>Akt</td>
<td>V-akt murine thymoma viral oncogene homolog</td>
</tr>
<tr>
<td>ATCC</td>
<td>American type culture collection</td>
</tr>
<tr>
<td>APS</td>
<td>Ammonium persulfate</td>
</tr>
<tr>
<td>ARTN</td>
<td>Artemin</td>
</tr>
<tr>
<td>BAK</td>
<td>BCL2-antagonist/Killer</td>
</tr>
<tr>
<td>BAX</td>
<td>BCL2-associated X protein</td>
</tr>
<tr>
<td>BCL2</td>
<td>B-cell CLL/lymphoma 2</td>
</tr>
<tr>
<td>BCL-XL</td>
<td>BCL-X long arm</td>
</tr>
<tr>
<td>BMP</td>
<td>Bone morphogenetic protein</td>
</tr>
<tr>
<td>bp</td>
<td>Base pair</td>
</tr>
<tr>
<td>BRCA1</td>
<td>Breast cancer gene 1</td>
</tr>
<tr>
<td>BRCA2</td>
<td>Breast cancer gene 2</td>
</tr>
<tr>
<td>BrdU</td>
<td>Bromodeoxyuridine</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>DCIS</td>
<td>Ductal carcinoma in situ</td>
</tr>
<tr>
<td>DEPC</td>
<td>Diethyl pyrocarbonate</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>ECL</td>
<td>Enhanced chemoluminescence</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EGF-R</td>
<td>Epidermal growth factor-receptor</td>
</tr>
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<td>EMT</td>
<td>Epithelial mesenchymal transition</td>
</tr>
<tr>
<td>ER</td>
<td>Estrogen receptor</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular-regulated kinase</td>
</tr>
<tr>
<td>FasL</td>
<td>Fas Ligand</td>
</tr>
<tr>
<td>FAK</td>
<td>Focal adhesion kinase</td>
</tr>
<tr>
<td>FBS</td>
<td>Foetal bovine serum</td>
</tr>
<tr>
<td>FGFR2</td>
<td>Fibroblast growth factor receptor 2</td>
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<td>G418</td>
<td>Geneticin</td>
</tr>
<tr>
<td>GADPH</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GLT1D1</td>
<td>Glycosyltransferase 1 Domain Containing 1</td>
</tr>
<tr>
<td>GSH</td>
<td>Glutathione</td>
</tr>
<tr>
<td>GST</td>
<td>Glutathione S-transferase</td>
</tr>
<tr>
<td>H2O2</td>
<td>Hydrogen peroxide</td>
</tr>
<tr>
<td>HCl</td>
<td>Hydrochloric acid</td>
</tr>
<tr>
<td>HER2</td>
<td>Human epidermal growth factor 2</td>
</tr>
<tr>
<td>hGH</td>
<td>Human growth hormone</td>
</tr>
<tr>
<td>HIF-1</td>
<td>Hypoxia Inducible Factor 1</td>
</tr>
<tr>
<td>IBC</td>
<td>Invasive breast carcinoma</td>
</tr>
<tr>
<td>IDC</td>
<td>Invasive ductal carcinoma</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>IGF-1</td>
<td>Insulin-like growth factor-1</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>ILC</td>
<td>Invasive lobular carcinoma</td>
</tr>
<tr>
<td>kb</td>
<td>Kilo base</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilo Daltons</td>
</tr>
<tr>
<td>LCIS</td>
<td>Lobular carcinoma in situ</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen activated protein kinase</td>
</tr>
<tr>
<td>MEK</td>
<td>MAPK kinase-ERK kinase</td>
</tr>
<tr>
<td>MET</td>
<td>Mesenchymal epithelial transition</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloproteinases</td>
</tr>
<tr>
<td>Myc</td>
<td>Myelocytomatosis viral oncogene homolog</td>
</tr>
<tr>
<td>mTOR</td>
<td>Mammalian target of rapamycin</td>
</tr>
<tr>
<td>MTT</td>
<td>3-[4, 5-dimethyl-thiazol-2-yl]-2,5-diphenyltetrazolium bromide</td>
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<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Name</td>
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<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphatidylinositol-3 kinase</td>
</tr>
<tr>
<td>pBR</td>
<td>Retinoblastoma protein</td>
</tr>
<tr>
<td>p53</td>
<td>Tumour protein 53</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet-derived growth factor</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphatidylinositol-3 kinase</td>
</tr>
<tr>
<td>PIP3</td>
<td>Phosphatidylinositol (3, 4, 5) triphosphate</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PTEN</td>
<td>Phosphatase and tensin homolog</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidene fluoride</td>
</tr>
<tr>
<td>RCF = g</td>
<td>Relative centrifuge force</td>
</tr>
<tr>
<td>RE</td>
<td>Restriction enzyme</td>
</tr>
<tr>
<td>RET</td>
<td>Receptor tyrosine kinase</td>
</tr>
<tr>
<td>RPM</td>
<td>Revolutions per minutes</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>Src</td>
<td>Rous sarcoma virus</td>
</tr>
<tr>
<td>SSC</td>
<td>Spermatogonial stem cell</td>
</tr>
<tr>
<td>TAE</td>
<td>Tris-Acetate-EDTA</td>
</tr>
<tr>
<td>TDLU</td>
<td>Terminal duct lobulo-alveolar units</td>
</tr>
<tr>
<td>TEB</td>
<td>Terminal end buds</td>
</tr>
<tr>
<td>TEMED</td>
<td>Tetramethyl ethylenediamin</td>
</tr>
<tr>
<td>TERT</td>
<td>Telomerase reverse transcriptase</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor Beta</td>
</tr>
<tr>
<td>TNFα</td>
<td>Tumour Necrosis Factor α</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular Endothelial Growth Factor</td>
</tr>
<tr>
<td>VEGFR</td>
<td>Vascular Endothelial Growth Factor Receptor</td>
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</table>
CHAPTER 1. GENERAL INTRODUCTION

1.1. INTRODUCTION

Humanity has known of the disease of cancer for centuries. For the first time in the 4th century BCE, Hippocrates coined the term carcinoma derived from word “karkinos”, meaning crab in Greek. He probably coined the term observing the disease characteristics similar to the crab. Although, Hippocrates was the first individual to coin the term the disease and its characteristics were known prior to his time [1]. The oldest available documented case of cancer is from 1500 BCE in Egypt [2]. The Edwin Smith surgical papyrus describes 8 cases of male breast tumours [3]. The symptoms of malignancy were also described in Chinese and Arabic medical writings [4]. Recent palaeopathological studies on Egyptian mummy samples of 905 individuals spanning 3200-500 BCE and ancient German samples of 2547 individuals spanning 1400 BCE to 800 CE have proven the occurrences of cancer in different populations over 4000 years [5]. Though the prevalence of the disease in ancient time is elusive, its occurrence is certainly proved.

Until the end of 18th century, cancer disease was not systematically studied although Hippocrates had coined the term much earlier. Bichat in the 18th century studied and described different types of carcinomas. Later in 19th century Muller and Virchow microscopically established that the cancer tumour was made up of cells [4]. A significant progress on understanding and management of the disease has been made in the 20th century.

In this review of the literature, I will summarise the general principles of cancer, followed by a discussion on breast cancer initiation, molecular evolution, diagnosis and treatment. I will also outline the types of cell death and focus on apoptotic cell death and cell death with autophagy. Later in the chapter, I will give an introduction to the gene, Glycosyltransferase1 Domain Containing 1 (GLT1D1) and discuss the aim and rationale of conducting doctoral studies on this gene.

1.2. Cancer

Cancer is a disorder in which the cellular control over cell proliferation and cell death is lost. It is broadly classified based on the cellular origin of the tumour. Tumours originating from epithelial cells are called carcinomas. Examples of carcinoma include breast, prostate, lung and colon carcinomas. Cancers involving connective tissues such as bone, cartilage and neurons originating from mesenchymal cells are termed sarcomas. Hematopoietically originating cancers are referred to as lymphoma and leukaemia. The precursor or embryonic cells transform into malignant phenotype to be known as blastoma. The germ-line cells also become cancerous and they are categorised as germ cell tumours [6].

The process of normal growth regulated cells turning into unregulated cancerous cells is often termed as transformation. Important genes regulating cell growth and development are altered during

1
transformation. These genes are frequently referred as oncogenes and tumour suppressor genes. Group of genes that favour transformation is termed as oncogenes and the group of genes that inhibit transformation is called as tumour suppressor genes. The two sets of genes function antagonistically and yet complementarily to preserve cellular homeostasis. The equilibrium existing between these two sets of genes in cells is perturbed leading to the genesis of cancer [7] [8].

A cancer is highly heterogeneous and complex in nature. There are several types of cancer and several subtypes within one particular type. Above-mentioned cancer types are only general categories of cancer. Closer observation of a malignant tumour reveals its heterogeneity and complexity.

1.2.1. An account of cancer characteristics

The complexity and heterogeneity of tumour raises questions about its origin and genesis. Several theories have been proposed to explain tumorigenesis. The majority of them theorise that the tumorigenesis is a multistep process. Hanahan and Weinberg stated that the multistep process is driven by genetic or epigenetic modifications within cells. These genetic alterations could be as small as a point mutation or as severe as changes in chromosomal components which gradually drive the transformation of normal cells into a malignant tumour [9]. Conversely, Green and Evans theorised that in cancer there is a deregulation of proliferation together with reduction in apoptosis [10].

Mutation driven multi-step tumorigenesis could be best explained by considering colorectal cancer. Numbers of studies from different laboratories have established the sequence of normal colonic epithelium transforming into malignant derivatives with sequential accumulation of genetic and epigenetic mutations [11]. The colorectal patient derived samples revealed genetic mutation leading to the activation of Wnt signalling [12, 13]. Concomitantly they revealed inactivation of p53 and transforming growth factor β (TGFβ) pathway due to mutations in the tumour suppressor genes TP53, APC and SMAD4 [14-17]. Activation of the other oncogene, KRAS is often encountered in these samples that in turn activates mitogen activated protein kinase (MAPK) signalling pathway [18, 19]. These genetic and epigenetic changes correlate with the clinical stages of the colorectal cancer. While the benign tumours harbour fewer mutations, the advanced tumours harbour most of the mutations if not all. Overall, the understanding of colorectal cancer serves as a good example for the multi-step tumorigenesis.

Hanahan and Weinberg summarised the factors that drive tumorigenesis as hallmarks of cancer in their seminal paper. They described the hallmark capabilities as self-sufficiency in growth signals, insensitivity to antigrowth signals, evasion of programmed cell death, limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis (Figure 1) [9]. In the subsequent edition of hallmarks of cancer in the year 2011, they gave an account of emerging hallmarks and enabling capabilities in cancer. The six hallmarks of cancer are discussed in detail in the following section, as they comprehensively describe the general principles of cancer initiation, promotion and progression.
Six capabilities, namely, self-sufficiency in growth signals, insensitivity to antigrowth signals, evasion of programmed cell death, limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis acquired during tumorigenesis through genetic and epigenetic alterations categorised as hallmarks of cancer by Hanahan and Weinberg.

1.2.2. Hallmarks of cancer

**Self-sufficiency in growth signals**: External growth factors are required for the normal cells to grow and divide. Growth receptors present on the cell membrane capture these growth factors and transmit the signalling into the cells through cell signalling machinery. Many known oncogenes play the role of growth factors and help tumour cells to become independent of normal external growth factors.

Tumour cells achieve self-sufficiency in growth signals mainly by (a) autocrine growth factor secretion \[20\] (b) over-expressing growth factor receptors or retaining the mutated or structurally altered forms of that helps to achieve hyper-responsive signalling \[20, 21\] (c) attaining mutations in the downstream signalling pathway that facilitates ligand free signalling \[22\].

Tumour cells secrete their own growth factors to which they have evolved to respond to. An autocrine response mechanism promotes the tumour development. Tumour cells also possess the altered extracellular receptors and transducer elements that are effective in carrying the growth signals from the autocrine growth factors. The altered signal transduction machinery is thus able to respond to lower levels of autocrine growth signals \[20\].

The altered cell surface receptor molecules are mutated or over-expressed to carry signalling at lower levels of ligand or in some cases in absence of the ligand. Many receptors carrying tyrosine kinase
activity are over-expressed in various types of cancer. This hyper-sensitises cancer cells to respond to the ambient levels of growth signals [20, 21].

High-throughput cancer cell genome sequencing studies indicate constitutive signalling activation triggered by activated growth factor receptor due to somatic mutation. It is found that EGF receptors lacking the cytoplasmic domains are known to signal constitutively in glioblastoma. Cancer cells express modified integrins or extra cellular matrix receptors that modulate the way the cell interacts with its surroundings. The modified integrins are able to link the cancer cells to the extra cellular matrix. This linking affects the ability of the cell to undergo apoptosis, cell motility and cell cycle stages [22].

**Insensitivity to antigrowth signals:** Antigrowth signals co-exist along with the proliferative signals in cells. Tumour suppressor genes regulate uncontrolled cell growth. Important genes that mainly check uncontrolled proliferation are retinoblastoma proteins (pRB) and tumour protein 53 (p53). They determine whether the cell enters mitogenic phase or not [23-25].

The RB protein checks cell proliferation by sensing external cell signals. Phosphorylation status of the RB protein is the deciding factor that determines whether or not the cell enters into S phase from G1 phase. RB protein inhibits the E2F transcription factor that transcribes genes responsible for driving cell proliferation when hypophosphorylated. TGFβ family of ligands indirectly converge on the RB protein phosphorylation and thus checks cell proliferation. TGFβ regulates p21 and p15INK4B which inhibit cyclin:CDK complex that is responsible for the phosphorylation of the RB protein. TGFβ is also known to inhibit the cell proliferation by suppressing c-myc expression, which regulates G1 cell cycle machinery [26-28].

One of the ways cancer cells survives proliferation check is through disruption of RB protein and TGFβ pathways. In certain types of tumours the TGFβ pathway is ineffective to keep RB protein hypophosphorylated due to fewer numbers of TGFβ receptors on the cell surface. And in few other types of tumours the pathway is ineffective due to mutation of the TGFβ receptors [29-31]. Studies have established that in few other types of tumours, the TGFβ downstream elements such as Smad4 protein and p15INK4B are either mutated or eliminated leading to loss of function [32]. Although TGFβ is known to check proliferation in the initial phase of tumour growth, its paradoxical tumour promoting function in the advanced stages of cancer is well established [30].

Unlike RB protein, p53 principally responds to the intracellular stress. Elements such as cellular energy level, growth promoting-signals and oxygenation status is sensed by p53. It seizes cell proliferation until these critical cellular elements are completely restored. In the events of severe genomic damage, p53 causes apoptotic cell death and thus prevents the proliferation of a possible malignant cell. Many tumours possess either mutated p53 or mutated upstream or downstream elements of p53 pathway to escape from the gatekeeper of the cell cycle progression [23, 33].

Overall, in tumorigenesis cells elude antigrowth signals either by disruption or mutation of key tumour
suppressor genes or their components and thus manage to escape the cellular regulation.

Evading cell death: There is a balance in tissues for production of new cells and elimination of damaged cells. Along with the loss of regulation in proliferation, there is also a loss of regulation in cell death in cancer tumours. Increase in the tumour size not only depends on the rate of proliferation but also on the rate of cell elimination mainly by apoptosis [34] and cell death by autophagy and necrosis. Studies have revealed that tumour cells evade cell death. Recent studies have also emphasized the roles of autophagy and necrotic cell death in tumorigenesis, which are circumstantial [35, 36].

Cells possess apoptotic machinery and are evolved to respond to intrinsic and extrinsic cell death factors. In the event of apoptosis cellular chromosomes are degraded, membranes are disrupted and cytoskeletal structures are disrupted. Neighbouring cells in the normal tissues engulf and clear these dissociated structure [37].

Cells respond to apoptotic signals both intrinsically and extrinsically. Intrinsic stress factors such as low levels of nucleotide or energy pool of low levels of oxygen are sensed within the cells and an intrinsic pathway involving mitochondria is activated [38]. The extrinsic factors such as Tumour Necrosis Factor α (TNFα) and Fas Ligand (FasL) can activate extrinsic apoptotic pathway by binding onto their respective receptors and transducing death signals. Both intrinsic and extrinsic pathways eventually execute cell death through effector caspase enzymes [37]. One of the common ways cancer cells have evolved to evade apoptosis is through disruption of TP53 gene function [33]. Cancer cells also escape apoptosis by overexpressing anti-apoptotic proteins such as BCL2, BCL-XL and down regulating the pro-apoptotic proteins such as BAX, BAD [34, 39].

Cells respond to starvation by a physiologic means called autophagy. In autophagy the cytoplasmic components are sequestered into double membrane structures and are subsequently degraded by lysosomes. The contents of the degraded lysosomes are released back into the cytoplasm for cell’s own utilisation. This serves as a source of energy for the starving cell [40]. Autophagy can be either a cell survival factor or a factor to cause the cell death. The exact role of autophagy in cancer is yet to be explored. Studies have indicated that autophagy is operational in cancer cells at basal levels to help tumour cells to survive under poor nourishment. However, the autophagy can engulf the tumour cell when there is a severe autophagic induction [35]. Tumour cells evoke elevated levels of autophagy under the influence of certain cytotoxic drugs, nutrient starvation and radiotherapy as a survival strategy.

Necrosis represents another type of cell death. Like autophagy, the role of necrosis in cancer is subjective. Studies have indicated that the cells swell and outpour the cellular contents in necrotic cell death. Important molecules, including interleukin-1α, which is an essential component of the inflammatory reaction is outpoured into the cellular surrounding [41]. Although the inflammatory cells of the immune system are known to harm the growing tumour cells, studies have established that the inflammatory cells nourish the advanced stages of cancer cells by supplying the growth factors. So,
the necrotic cell death harms the tumour cells in the beginning but it also provides a favourable condition for tumour growth and development [36, 41].

**Enabling replicative potential:** Cells have limited replicative potential. After certain number of cell divisions, they seize to divide and enter a stage known as cell senescence. The replicative potential of the cells is determined by hexameric repeats called telomere. With every division the normal cell loses 50-100 bp of telomere. Sequential loss of telomere creates unstable chromosome [42] [43].

One type of DNA polymerase called telomerase is able to compensate for the lost telomeric segment by adding DNA bases. Tumour cells are able to attain their replicative potential by overexpressing the telomerase enzyme [44, 45]. Overexpressed telomerases are able to compensate for the lost telomeric segment in the transformed cells [46].

Recent evidences have implicated telomerases in activation of Wnt signalling pathway. A subunit of telomerase called TERT has been associated in tumorigenesis. The novel functions of TERT include amplification of the Wnt signalling pathway by acting as a co-factor. The other telomerase independent activity of TERT includes resistance to apoptosis and proliferation enhancement by involving in DNA damage repair and RNA dependent RNA polymerase activity. Thus, by all these means the tumour cell achieves limitless replicative potential [47, 48].

**Sustained angiogenesis:** Blood vessels nourish the cells of tissues by supplying nutrients and oxygen. Early during embryogenesis, there is formation of vasculature and from this vasculature new blood vessels are sprouted which is termed as angiogenesis. Angiogenesis is a highly regulated process, which is usually activated in response to wound healing and reproductive changes in females. This highly regulated physiologic process is compromised during tumorigenesis. There is a continuous vascularisation in tumour to satisfy the nutrition and oxygen demand [49].

One of the ways angiogenesis regulated in tissues is through inducers and inhibitors. The angiogenesis inducer and inhibitory molecules bind to the cell surface receptors on the vascular endothelial cells and elicit respective functions. While the vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF) family of growth factors stimulate angiogenesis, the thrombospondin -1 (TSP-1) inhibits angiogenesis in endothelial cells [50]. Other than these growth factors, different class of integrins expressed in quiescent and sprouting vessels regulate angiogenesis. Along with extracellular proteases, the pro-angiogenic integrins facilitate endothelial cells tumour invasion [51, 52]. Tumour angiogenesis, unlike normal tissue angiogenesis is erratic since the angiogenesis is stimulated as result of imbalance between pro-angiogenic and anti-angiogenic factors. Due to the irregularity tumour vessels are characterised by uneven sprouting and micro haemorrhaging [53].

Studies have indicated that angiogenesis is essential for the rapid expansion of tumour. Angiogenesis is stimulated in the early stages of invasive tumour development [54]. Tumour cells reveal upregulated expression levels of the angiogenic stimulators such as VEGF and FGFs. These growth
factors intern stimulate angiogenesis in quiescent blood vessels. Yet, in other types of tumours the expression levels of angiogenic inhibitors TSP-1 and β-interferon are downregulated [53].

There are also other means by which tumours sustain angiogenesis. The pericytes and bone marrow derived cells such as macrophages, mast cells, and neutrophils play critical role in tumour angiogenesis. The pericytes lie adjacent to the endothelial cells in blood vessels and provide mechanical and physiological support. Studies have revealed that in tumours the pericyte covers vasculature and help in blood vessel maintenance. Other group of bone marrow derived macrophages, mast cells and neutrophils are shown to surround the lesion and help tumour cells to maintain angiogenesis. By adapting these mechanisms, the tumour achieves sustained angiogenesis [55, 56].

*Tissue invasion and metastasis:* Cancer deaths in the category of solid tumour are accounted for tissue invasion and migration. The tumour cells infiltrate the local tissues and manage to metastasise to distant organs and establish to form new tumours in the site. The tissue invasion and migration can be subdivided into local tissue invasion, intravestation through lymph nodes and extravestation from the lymph nodes to the distant organs. The process in which the newly migrated cells eventually form tumour mass is known as colonisation [57, 58]. Although the biochemical determinants of the process are not fully understood, both the processes seem to adopt similar strategies such as activation of extracellular matrix (ECM) and changes in local microenvironment [59].

Tumour cells are found to have altered ECM molecules, namely E-cadherin, N-cadherin and integrin. E-cadherin is frequently expressed in non-migratory cells to form the gap-junctions of the cells that hold the cells by means of binding to substratum or to the ECM of other cells. On the contrary, N-cadherin is expressed in migrating neurons and mesenchymal cells. Tumour cells exhibit low E-cadherin expression levels and high N-cadherin expression levels to embark migration [60]. The other important molecule that undergoes change during tumour cell metastasis is integrin. The α and β subunits of integrin are modified in the cells leading to change in substrate specificity. Change of substrate specificity allows tumour cells to bind to the protease degraded stromal cells [61, 62].

The other important way in which tumour cells achieve tissue invasion and migration is through a transition called epithelial mesenchymal transition (EMT) [63]. The EMT is observed during embryonic development where epithelial cells transform into mesenchymal or fibroblast like cells through altered transcriptional regulation. EMT transcriptional factors such as Snail, Slug, Twist and Zeb1/2 that are activated during embryogenesis are also activated in various types of cancers in different combinations [64, 65]. The cancer cells mainly acquire the ability to invade and migrate by means of adopting EMT.

Evidences have established that stromal cells [66, 67] and macrophages [68, 69] also play important role in tumour cell invasion and migration. While the stromal cells secrete cytokines and nourish tumour cells [70], the macrophages residing in the margins of the tumour secrete metalloprotein excesses and cysteine proteases and facilitate invasion and migration [71].
Since the publication of hallmarks of cancer in the year 2000, additional hallmarks have been proposed. Significant of them have been genomic instability [72] and cancer related inflammation [73]. Hanahan and Weinberg described these hallmarks as enabling characteristics [74]. The neoplastic cells acquire cancer characteristics with progressive genomic alteration. For achieving the hallmarks, the neoplastic cells develop higher rates of mutation [72], often by mutating the caretakers of the genome. Mutation of these caretaker genes acts as an enabling characteristic in acquiring the hallmarks. The other enabling characteristic that provides the growth advantage is tumour-promoting inflammation. Chronic inflammation predisposes individuals to different types of cancers. The inflammatory bowel disease is often related to increased incidence of colon cancer. Oncogenic events leading to transformation also turn on inflammatory reactions to further propagate neoplasm [75].

1.3. Breast Cancer

Uncontrolled tissue growth originating from breast tissue could be termed as breast cancer. Worldwide, the breast cancer is considered as a major health issue. It ranks the highest amongst the cancers in women [76, 77]. Although majority of women are affected by breast cancer, men too can develop breast cancer. The disease incidence is increasing in the developed countries as well as in the developing countries [78]. This rise in incidence is often rationalised to be the increase in the life expectancy and urbanisation and adoption of western lifestyle [79].

High incidence of breast cancer in New Zealand women is well recognised. In 2009, it accounted for more than 2500 new cases and 600 deaths. The government figures have shown that 1 in 9 women are developing breast cancer in their lifetime [80]. Since 1997, breast cancer has accounted for 15% of all cancer deaths and 26% of all cancer registrations [81]. Due to BreastScreen Aotearoa, an excellent nationwide breast screening programme, the cancer survival rates have increased. Early diagnosis of breast cancer has decreased cancer deaths by nearly 24% between 1995-2005 [82].

Breast cancer could be originating either from ducts or lobules of the breast [83]. The breast tissue undergoes several morphological changes throughout the growth and development [84]. Understanding the structure and mammary gland development will thus help in understanding the breast cancer genesis.

1.3.1. Mammary gland structure and development

The mammary gland is made-up of several ducts and lobules invading the underlying alveolar adipose tissue. The ducts and lobules branch from the nipple into the adipose tissue resembling the tree branching. The ducts and lobules are composed of layers epithelial cells covered by a layer of contractile myoepithelial cells (Figure 2). Club-shaped lobules are the end of structures of mammary gland. There is a hollow lumen in the centre of the lobule to store the milk from the glands, which is eventually secreted into nipples under the stimulation of oxytocin hormone [84].
Figure 2: Structure of normal breast and a lobule
(The figures are reproduced from Nature Review Cancer. 2002 Feb; 2(2):101-12, with permission)

Human mammary gland has 15-30 lobes that drain into the nipple, branches into series of ducts in
the stroma of the breast. Functional unit of mammary gland, the lobule is showing layers of
epithelial cells wrapped by myoepithelial cells with central lumen. Epithelial cells produce milk and
upon the myoepithelial contraction eject milk into the central lumen.

The mammary gland undergoes major morphological changes throughout life, unlike other organs.
Development of human breast takes place over prenatal, infantile growth, puberty, pregnancy and
lactation and post menopause periods [85]. The embryonic mammary gland is comparable in all
mammals and it is derived from ectodermal bud [86]. It is a primitive structure with ducts and ductules
lined with one or two layers of epithelial cells surrounded by a layer of myoepithelial cells are found in
the newborn breast. There is hardly any mammary gland development until puberty.

The main development of human breast occurs at puberty characterised by proliferation and
dichotomous differentiation of ducts into club-shaped buds, which in turn differentiate into alveolar
buds, ducts or branches. The levels of branching of the alveolar buds into lobules 1, 2 and 3 occur
through the puberty and adult life. Approximately 11 alveolar buds are clustered around the ends of
terminal duct are differentiated into approximately 47 and 80 alveolar buds to form lobule 2 and 3,
respectively (Figure 3B). The terminal alveolar bud along with the surrounding stroma is termed as
terminal duct-lobular unit (TDLU). The ducts and alveolar buds are lined by double-layered epithelium.
The lobule formation in female breast occurs within 1-2 years of first menstrual cycle [84]. However,
the terminal differentiation takes place with successive menstrual cycles spanning years of
development. The branching, elongation and proliferation of alveolar buds mainly require growth and
estrogen hormones secreted from pituitary and ovary [87].
At puberty club-shaped structures called terminal end buds appear at the tip of the ducts (Aa). In mature virgin the ducts appear adequately branched (Ab). During pregnancy alveolar buds differentiate into milk producing structures (Ac). At the time of lactation luminal cells fully mature and synthesise and secrete milk into lumen. (B) Dichotomous branching of the ducts into primary, secondary and tertiary lobes.

During pregnancy, the female breast rapidly expands and differentiates under the influence of pregnancy hormones (Figure 3A). The pregnancy steroids such as estrogen, progesterone, prolactin and placental lactogens influence the terminal alveolar bud differentiation. Estrogen influences the ductal system and progesterone mainly influences alveolar development and tertiary side branching [88]. Prolactin is also known to contribute to the tertiary branching [89]. By the end of second trimester the terminal tertiary differentiation of female breast is completed with the development of milk secretory capability. The myoepithelial cells around epithelial layer contract under the influence of oxytocin hormone after parturition and during lactation to secrete the milk [90]. After pregnancy and lactation, the female breast returns to adult state capable of entering the active state again. Finally at menopause the glandular portion of the breast considerably shrinks and the stromal layer becomes dense.

1.3.2. Breast cancer histopathology

Histopathological examination of breast tumour suggests multi-stage progression of breast cancer development (Figure 4) [91]. In situ carcinoma is broadly categorised as Ductal Carcinoma In Situ (DCIS) and Lobular Carcinoma In Situ (LCIS) [92]. Morphologically, DCIS originates from ducts of mammary gland accounting for about 85-90% of all In Situ cases and LCIS originates from milk secreting lobules of mammary gland accounting for about 8-10 % of all cases [93, 94]. Subsequently, while almost all of LCIS develop into invasive lobular carcinoma (ILC), only about 30% of DCIS develop into invasive ductal carcinoma (IDC). The IDC accounts for about 75% of Invasive Breast Carcinoma (IBC) cases and ILC accounts for about 10% of IBC cases [95, 96]. The remaining
percentages of breast carcinoma are categorized as medullary, neuroendocrine, tubular, apocrine, metaplastic, mucinous, inflammatory, comedo, adenoid cystic, and micro papillary types [97-99].

1.3.3. Evolution of breast cancer

The molecular analyses of normal mammary precursor cells and invasive breast cancer cells have revealed the transition to be multi-step process accompanied by radical genetic and epigenetic changes [107]. Historically, the multi-stage transition was theorised to be similar to the Vogelstein’s model of colon carcinogenesis [108]. This model proposed a simplistic carcinogenesis progression (Figure 5), where normal breast epithelial cells from TDLUs transform into premalignant lesion called hyperplasia of usual type (HUT) then develops into Atypical Ductal Hyperplasia (ADH) and subsequently gives rise to DCIS. DCIS acquires more genetic and epigenetic changes to advance to the higher forms of IBC. A similar pathway of progression was also proposed for ILC [109].

![Figure 4: Breast cancer histopathology through different stages.](image)

*Figure 4: Breast cancer histopathology through different stages.*

(The figure is reproduced from Nat Rev Cancer 2007, 7(9):659-672, with permission)

Terminal ductal lobular unit (TDLU) contains lobules and ducts with luminal epithelium and myoepithelial cells. Atypical ductal hyperplasia (ADH) is the precursor of ductal carcinoma in situ (DCIS) with irregular cells. DCIS is a lesion without invasive characteristics. By local tissue invasion DCIS develops into invasive breast cancer (IBC). IBC drains into the lymph nodes to become advanced metastatic tumour.

The IDC and ILC histologically differ and they reveal different patterns of invasion in mammary stroma. IDC consists of a mass of ill-defined large cells broken free from ductolobular system to invade the mammary fat pad of the breast. This infiltration is detected by the body and reacted by a desmoplastic response. Inflammatory cells are accumulated in desmoplastic response followed by a layer of fibrous tissue. The tumour cells invade the fibrous connective tissue to form ill-defined masses [100]. On the other hand ILC lacks desmoplastic response. In ILC, the tumour cells continue to grow and invade the normal glandular structures and mammary stroma. Infiltrating tumour cells follow a strict pattern of “single-file” or strands of cells in ILC [100, 101]. The two IBCs are mainly distinguished by the presence or absence of cell surface marker E-cadherin. The marker is only detectable in IDC and not in ILC [102, 103].

With disease progression, tumour cells invade larger areas of mammary gland and eventually find their way into lymphatic system. The sentinel lymph nodes that directly drain cancerous mammary gland are the ones to receive metastatic tumour cells [104]. Additionally, metastasis is seen in other distant organs including lungs, bones, CNS and liver in advanced stages of breast cancer [105, 106].
The multi-step of breast cancer progression model based on the histopathological observations. The model describes the pathways for invasive ductal carcinoma (IDC) and invasive lobular carcinoma (ILC) which are now considered over-simplistic.

While the improvement in immunohistochemistry and molecular genetics confirmed the premalignant lesion theories, they questioned the role and validity of some lesions along the above pathway (Figure 5) [102, 110]. The reliable techniques such as precise laser micro dissection [111], comparative genomic hybridisation (CGH) [112], loss of heterozygosity (LOH), DNA amplification and genome wide transcription analysis microarray platforms [113, 114] have helped in revisiting the above pathway. The resultant gene-expression profile studies have helped to categorise breast cancer into luminal A, luminal B, HER2-like basal-like and normal breast-like sub types [115, 116].

1.3.4. Precursor lesions and risk indicators

The early premalignant risk factors or lesions such as HUT and ADH lesions can be detected by mammography. The complex histopathological classification of these risk factors is found to be very useful in assessing the risk and patient management [117, 118]. The risk factor assessment includes hereditary factor also as it is one of the main risk factors. At genetic level the cancer susceptibility genes can be categorised as high, moderate and low penetrance genes. Along with TP53, Breast Cancer 1 and 2 (BRCA1 and 2) account for less than 25% of excess risk [119, 120]. The high throughput studies have indicated the SNPs and allelic variants of thymocyte selection–associated high mobility group box 9 (TNRC9), fibroblast growth factor receptor 2 (FGFR2), lymphocyte-specific protein (LSP), caspase 8 (CASP8), mitogen-activated kinase 1 (MAP3K1), and transforming growth factor β1 (TGFB1) as risk factors [121-125].

1.3.5. Cell origin of breast cancer

Two leading models are proposed for explaining the cell origin of breast cancer. One is sporadic clonal evolution model and the other is cancer stem cells (CSC) model. The sporadic clonal evolution hypothesis suggests random mutation of any mammary epithelial cells that continues to epigenetically and genetically evolve to form tumour [126, 127]. According to CSC hypothesis, the stem and progenitor cells maintain tumour progression [126, 128]. According to a third proposal for cellular origin, neither the clonal evolution nor the cancer stem cells are mutually exclusive. It suggests that
the cancer stem cells might undergo clonal evolution to form the advanced stages of tumour [129-131]. The normal breast stem cells are long-lived cells capable of giving rise to multi-lineage cells to maintain the tubulo-lobular architecture [132, 133]. Due to their longevity, it is proposed that they tend to accumulate genetic and epigenetic mutations [134].

1.3.6. Tumour progression

Tumour progression in breast tissue could be best understood by considering histopathological and genomic and transcriptomic studies, together. In recent times, the molecular tumour grading system involving genomic and transcriptomic studies is also adopted along with the histopathological typing in clinics, to understand the tumour characteristics and to adopt appropriate line of treatment. While the histological tumour typing gives the clinical picture and the origin of the tumour, the molecular tumour grading indicates the extent, complexity and genomic instability of the tumour [135, 136].

The clinically recognised low and high-grade IDCs represent the different extents of chromosomal aberrations. The low-grade tumours are frequently shown to have chromosomal loss of 16q and gain of 1q, 16p and 8q. On the contrary, the high-grade tumours are shown to have chromosomal loss of 8p, 11q, 13q, 1p and 18q and gains of 8q, 17q, 20q and 16p. Additionally, the high-grade tumours display high-level amplifications of 17q12 and 11q13 [136-139]. The analysis of intermediate grade IDCs suggests the presence of a combination of low-grade-like and high-grade-like IDCs [135]. However, the frequent loss of 16q in low-grade IDCs and infrequent chromosomal loss of 16q in high-grade IDCs suggests that the low-grade IDCs hardly evolve into high-grade IDCs [140, 141].

The ILC that represents minor portions of invasive breast carcinomas shares tumour grade progression genetic pathway with that of IDC in a few ways. The CGH analysis has established that the low-grade ILCs reveal frequent chromosomal loss of 16q [142, 143] and gain of 1q and amplification of 17q12 in the high-grade ILC [144, 145]. This shows the similarity in evolutionary pathways of IDC and ILC. However, the grade and molecular comparisons between ILC and IDC suggests unique transcriptional profile for ILC. The genes associated with cell-adhesion and cytoskeletal signalling pathways are poorly expressed in ILC resulting in loss of E-cadherin expression [146].

1.3.7. Role of tumour microenvironment in tumour progression

The focus of breast cancer research has been breast epithelial cells for past few decades ignoring the non-neoplastic cells of tumour microenvironment. Recent evidences have established that the tumour microenvironment comprising of myoepithelial cells, fibroblasts, myofibroblasts, adipocytes, inflammatory cells and endothelial cells play an important role in tumour progression [147-149]. These cells were considered as bystander cells earlier. On the contrary, cells forming tumour microenvironment are found to be active through cell signalling loop with the transformed neoplastic cells [150]. The stromal cells show higher expression levels of genes coding for the extracellular matrix proteins and matrix metalloproteinases (MMPs) [150, 151]. The myoepithelial cells associated
with DCIS, show differential expression of the genes coding for secreted proteins and cell surface receptors that are connected to autocrine/paracrine signalling, invasion and migration [152].

1.3.8. Key molecules involved in breast cancer

A number of molecules and signalling pathways are involved in breast cancer. Not any one major pathway is predominantly mutated in breast cancer like bowel or pancreatic cancer. There are a number of genes and molecules involved in the development of breast cancer. Some of the major ones include estrogen, BRCA1 and 2, TP53, cyclin-D1, epidermal growth factor receptor (ERBB1/HER1), epidermal growth factor 2 receptor (ERBB2/HER2), Vascular Endothelial Growth Factor (VEGF), Transforming Growth Factor (TGF-β). These molecules have been the therapeutic targets due to their important roles in breast tumour progression.

The female sex hormone estrogen secreted by the ovaries plays an important role in the growth and development of the female breast during puberty. The breast cancer risk is associated with levels of estrogen exposure throughout life [153, 154]. Estrogen is known to stimulate mitogenic activity in the breast and it is mediated by ERα and ERβ receptors. Apparently, about 70% of breast tumours are ERα positive. The binding of estrogen hormone on its receptor activates the estrogen responsive element (ERE), which in turn activates the target genes such as IGF-1, Cyclin D1 and myc [155]. In breast cancer context, estrogen is capable of activating oncogenes, producing autocrine/paracrine factors including VEGF, inducing genotoxicity and stimulating nucleic acid synthesis [156, 157].

BRCA1 and 2 genes code for single and double stranded DNA breaks repairing protein and thus act as tumour suppressor gene. They are used as genetic markers for assessing familial breast and ovary cancer. While 95% of familial breast cancers carry BRCA1 mutation, it only accounts for about 5% of total breast cancer incidences [158].

TP53 is one more important tumour suppressor gene frequently mutated in up to 30% of breast cancers. When the tumour becomes invasive, the rate of TP53 mutation is also increased up to 3 or more times. TP53 is found to be poor prognostic factor for chemotherapy [159, 160]. However, in combination with other markers it has been found to be a good diagnostic marker for familial breast cancer [161].

The Cyclin D1 is a cell cycle molecule. Along with CDK4/6, it phosphorylates the retinoblastoma protein to facilitate the G1 to S-Phase transition of cell cycle [162, 163]. Cyclin D1 is amplified in up to 50% of breast tumours, due to the amplification of the gene locus. It is more often associated with the ER+ breast tumours than any other particular types of breast tumours [164].

Erb family of receptors consisting of HER1, HER2, HER3 and HER4 are members of tyrosine kinase receptors. These receptors bind to the growth factors and send mitogenic signals into the cells. Among these receptors HER1 and HER2 have gained considerable amount of attention due to their important role in breast cancer and other cancer progression [165, 166]. These receptors are overexpressed during cancer progression and drive the cell cycle progression by activating signalling
pathways Raf/MEK/ERK and PI3K/PDK1/Akt pathways [167, 168]. HER2 is been target of popular humanised monoclonal antibody Trastuzumab therapy and has been found to reduce metastatic breast cancer deaths by 20% [169].

Tumour cells are known to drive angiogenic processes to meet their nutritional needs. New blood vessel branching in tumours is activated by factors such as VEGF. The hypoxic tumour cells secrete VEGF to promote angiogenesis [170]. The VEGF expression is deregulated in breast tumours by a number of factors including hormones, mutated proteins and HER2 [171, 172].

TGF-β is a multifunctional cytokine, basically inhibits mammary epithelial cells growth by suppressing cell division or causing apoptosis. As the tumour growth is on progress, the genetic and epigenetic factors transpire TGF-β into a cytokine promoting angiogenesis, invasion, metastasis and evasion from immune system[173, 174]. This duality of TGF- β is called as TGF- β paradox [175].

1.3.9. Breast cancer treatment

Before adopting any particular breast cancer therapeutic module, the type and the grade of cancer is assessed. In clinics, five main sub-types of breast cancers namely luminal A, luminal B, HER2-enriched, basal-like and normal breast-like cancers are recognised [115]. The immunohistopathology testing of a tumour assesses the expression levels of estrogen receptor (ER), Progesterone Receptor PgR, HER2 and Ki67 and helps clinicians to understand the type and grade of the breast cancer [176]. After the breast cancer is diagnosed, the tests are done to assess the spread of cancer within the breast and other parts of the body including lungs, bone and liver by chest X-ray, bones scan, CT scan and PET scan [177, 178]. Other factors such as tumour size, grade, nodal involvement, age, menopausal status, are also considered to assess the relapse chances before initiating treatment [179].

Based on the grade of the tumour, the line of treatment is initiated. Treatment modules include surgery, hormone therapy, chemotherapy, radiotherapy and targeted therapy. The treatment modules are adopted either in isolation or in combination, depending on the type and stage of the cancer. Therefore, the line of treatment differs from patient to patient.

In surgery, breast-conserving surgery is the most preferred form. An early stage breast cancer is treated with chemo or targeted therapy and partial mastectomy [180, 181]. Chemotherapy shrinks the size of the tumour, and reduces the volume of the tissue to be removed during surgical treatment. After the operation, adjuvant therapy, radiotherapy and/or chemotherapy is undertaken to prevent the chance of relapse [181-183]. These measures are taken to prevent ipsilateral breast or distant organ metastasis. However, for patients with more severe breast cancer, surgical intervention is usually, mastectomy (total removal of the whole breast) or modified radical mastectomy where the whole breast along with the draining sentinel and other lymph nodes are removed [184].

Hormone therapy involves reducing the availability estrogen to the growing cancer cells in the event of their dependency on the hormone. Tamoxifen, a popular drug used in hormone therapy acts as an
antagonist to the estrogen receptor in the cancerous breast tissue. Along with tamoxifen treatment, an aromatase inhibitor Leterozole that inhibits the conversion of androgen to estrogen, by liver and fat tissues is prescribed for postmenopausal women with breast cancer [185, 186].

Targeted therapy includes treatment aimed at the cancer cells and spares the normal cells. Tyrosine kinase receptor (TKR) inhibitors and monoclonal antibodies are two types of targeted therapies. A third type, targeting Poly ADP Ribose Polymerase (PARP) is used in the treatment of triple negative breast cancer [187]. The TKR from Erb family of receptors are frequently targeted with inhibitory drugs and monoclonal antibody. HER2 is frequently targeted with monoclonal antibodies [188] and drugs that blocks the action of HER2 [189].

1.4. The cell death

Dysfunctional and irreversible state of the cell is often termed as cell death. In cell death, the cell reaches ‘point of no return’ from which it cannot return back and become a functional cell again [190]. The cells undergo cell death prior to reaching their end stage in instances such as severe physical or physiological insult. However, there are also instances where the cells continue to exist in situations such as cancer. Both early and delayed cell death results in pathophysiology. Therefore, a proper cell death function is customary for the tissue homeostasis [191].

1.4.1. Types of cell death

Cell death is attained in different ways. The nomenclature committee on cell death (NCCD), 2005, made recommendations to identify and distinguish different types of cell deaths [192]. The pace of cell death research in the past decade necessitated an update of this recommendation. Therefore, the NCCD committee in 2009 and recently in 2012, made several recommendations to classify cell death based on the biochemical and physiological changes taking place in the cell during cell death [190, 193]. The recent classification and the recommendations of the committee are discussed below.

First and foremost, according to the NCCD recommendations a cell can be categorized as dead cell, if one of the three following criteria is satisfied: (1) In in vitro, the plasma membrane integrity of the cell is lost and it is able to uptake the vital dye into the cell (e.g., Trypan blue, PI) (2) The cell components including the nucleus is fragmented into discrete bodies (3) The corpse or the fragments of the dead cell is engulfed by the adjacent cell in vivo.

The NCCD has broadly classified cell death into programmed (typical) and non-programmed (atypical) cell death. An account of typical cell deaths is given below in Table 1.
### 1.4.2. Apoptosis

#### 1.4.2.1. Historical perspective

Apoptosis is one of the well-studied cell death pathways. Historically, cell death was thought to be a passive process, although the cell death phenomenon was observed many years ago [194]. The view started changing with studies in developmental changes in tadpole and silkworm [195, 196]. The early study showed that the cell death could be postponed by adding inhibitors of protein or RNA synthesis. Kerr, Wylie, and Currie (1972) made a significant contribution to the early apoptosis research. In their seminal work on apoptosis they presented illustrious morphological images of apoptotic cells. Their work implicated, the role of apoptosis in normal embryonic development and the apoptosis induction resulting in tumour regression upon therapy [197]. This work formed the basis to define apoptosis as a cell death process where the cell shrinks in size and the organelles including nuclei are fragmented into smaller units and phagocytised by the adjacent or neighbouring cells. The genetic basis of apoptosis came to light through genetic studies of *C. elegans* [198, 199]. The specific mutational effects affecting programmed cell death demonstrated that the cell death is part of a normal developmental process. Subsequent characterisation of the molecules involved in the pathway gave an understanding of cell death machinery involving cysteine proteases called caspases [200-202].

#### 1.4.2.2. Apoptotic pathways

Apoptosis is a multi-step process involving multiple pathways. The multiple pathways can be broadly categorised as extrinsic (death receptor pathway) and intrinsic (mitochondrial pathway) pathways. As the name suggests the extrinsic pathway becomes operational with external cell death triggering molecules and the intrinsic pathway becomes operational with internal cell signals [203]. The apoptotic cell death execution is carried out by a group of caspases. However, there are pathways in which the apoptotic cell death is achieved independent of these caspases.

Sequence of events systematically progresses during apoptosis. Either an internal or an external trigger factor induces the apoptotic process. The caspases bring about series of cell breakdown effects, once the process is activated. There are two different classes of caspases, namely, initiator

<table>
<thead>
<tr>
<th>Types of cell death</th>
<th>Characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apoptosis</td>
<td>Cell shrinkage, chromatin condensation, loss of mitochondrial membrane potential and permeability of cell membrane with the display of phosphotidyl serine groups outside the cell membrane.</td>
</tr>
<tr>
<td>Autophagy</td>
<td>Cells full of autophagosomes and autolysosomes within the cytoplasm of the cell.</td>
</tr>
<tr>
<td>Necrosis</td>
<td>Early rupture of plasma membrane, dilated mitochondria and other cytoplasmic organelles.</td>
</tr>
</tbody>
</table>

Table 1: The programmed cell deaths and their characteristics
and effector caspases [204]. The initiator caspases (caspase 2, 8, 9 and 10) cleave pro-effector caspases (caspase 3, 6 and 7) to activate them in cells. The effector caspases upon activation cleave other molecular substrates [205]. The most important effector caspase-3 along with caspase 6 and 7 cleave large number of nuclear proteins resulting in chromatin margination, DNA fragmentation and chromatin condensation [206].

1.4.2.3. Extrinsic pathway

The external cell death ligands bind to cell death receptor located on the cell surface and induce extrinsic apoptotic pathway to cause cell death. The cell death ligands such as tumour necrosis factor α (TNFα), FAS ligand (FASL) and tumour necrosis factor superfamily 10 (TNFSF10) (also known as TNF related apoptosis inducing ligand - TRAIL) bind to various death receptors FAS, TNFα receptor (TNFR) and TRAIL receptor (TRAILR) to induce extrinsic cell death [207, 208]. A class of receptors known as ‘dependence receptors’ are known to induce extrinsic apoptotic pathway when their ligands concentration drop below the critical threshold [209].

The FASL mediated apoptosis represents the prototype of extrinsic apoptosis pathway (Figure 6). The FAS subunits oligomerise to form a trimer, which is called pre-ligand assembly domain (PLAD) in absence of FASL [210]. The trimer structure is stabilised upon FASL binding and the death signal is conveyed into the cytoplasm through death domain (DD) [211]. The death domain recruits other proteins such as FAS associated protein with a death domain (FADD) [212], receptor interacting protein kinase 1 (RIPK1 or also known as RIP1), E3 ubiquitin ligases, isoforms of FLICE like inhibitory protein (FLIP) and cellular inhibitor of apoptosis proteins (c-IAPs) [213]. This complex of proteins is called death inducing signalling complex (DISC). The DISC in turn activates caspase-8 or caspase 10 [214]. The DISC contains both pro-survival and pro-death signals. However, when the pro-death signals last longer, the death signal compounds to cell death. The DISC activated caspase-8 could either directly activate caspase-3 through proteolytic maturation or elicit mitochondrial outer membrane permeabilisation (MOMP) [215].
When FAS Ligand (FASL) binds to its receptor, FAS, the cytoplasmic domain of the trimeric receptor recruits cellular inhibitor of apoptosis proteins (cIAPs), cytoplasmic FLICE like inhibitor protein (c-FLIPs), FAS associated Death Domain (FADD) and pro-caspase 8 or 10 to form a complex called death inducing signalling complex (DISC). With persistent death signalling the caspase 8 is activated that in turn activates caspase-3 or elicits mitochondrial outer membrane potential (MOMP). Dependence receptors like DCC or UNC5B also elicit extrinsic apoptosis when the concentration of their ligands reduces below a critical concentration. A pro-apoptotic signalling is conveyed by the receptors through DRAL and TUCAN, an assembly that activates caspase-9. DRAL and TUCAN bring about apoptotic effect by dephosphorylating and activating death associated protein kinase 1 (DAPK1). DAPK-1 brings about apoptotic effect either by direct caspase activation or by means of MOMP.

Unlike FASL, the TNFα induces apoptosis by recruiting TNFR associated DD (TRADD), FADD and caspase-8 to the cytoplasmic death domain of the TNFR [208, 216]. This indicates the presence of different subclasses of death receptors. The association variance of death domain with pro-death and pro-survival molecules also enables the DISC to recruit the growth factors such as transforming growth factor-β (TGFβ) activated kinase-1 (TAK-1), TAK-1 binding protein 2 (TAB2) and TAB3 which in turn activate nuclear factor κB (NF-κB) pathway [217].

The proteolytic activation of caspase-3 takes place in a mitochondrion independent and mitochondrion dependent manner. In lymphocytes, which are dubbed, as type I cells [218]; the caspase-8 directly activates caspase-3 by proteolytic cleavage [219]. In cells such as hepatocytes, which are dubbed as type II cells [220]; the capase-8 mediates the proteolytic cleavage of BH-3 interacting domain death agonist (BID). The truncated BID (tBID) permeabilises mitochondria by releasing cytochrome C (CYTC) from the inner membrane of mitochondria. This leads to the
dissipation of mitochondrial membrane potential ($\Delta \Psi_m$) and MOMP [221]. The freed CYTC forms dATP dependent apoptosome complex with apoptotic protease activating factor 1 (APAF-1) and caspase -9 in the cytoplasm [222]. The apoptosome complex in turn activates caspase-3 and brings about the further apoptotic changes in the cells. Thus the type I cells undergo apoptosis without any contribution from mitochondrion (tBID and MOMP are dispensable, though these occur in type I cells) and type II cells succumb with loss of $\Delta \Psi_m$ and signs of MOMP.

A new extrinsic mechanism of caspase-3 activation by dependence receptors has begun to emerge. The dependence receptors such as Patched and DCC, in absence of their respective ligands appear to interact with cytoplasmic protein DRAL to activate the caspase-3 [223, 224]. Thus the dependence receptor extrinsic pathway reveals the existence of a new form of extrinsic pathway.

**1.4.2.4. Intrinsic pathway**

The intrinsic apoptotic pathway is triggered in response to range types of intracellular stress factors. The intracellular includes oxidative damage, DNA damage, severe nutrient deprivation, toxicity due to Ca$^{2+}$ overload and accumulation of unfolded proteins in endoplasmic reticulum etc. The apoptosis response could either be due to one of these factors or combinations of these factors. The source of stimulation of the pathway is heterogeneous in origin. However, all of them have the mitochondrion-centric control system in common [225].

Often, when the cells are stressed they have both propagating and pro-apoptotic signals in operation at the same time. This allows the cells to either recover from the stress or succumb to the fatal apoptosis. The two differing signals converge on mitochondria, and the cellular machinery allows the stronger signal to take precedence. The intrinsic apoptotic signal is triggered by mitochondrial permeabilisation when the pro-apoptotic signal takes precedence over propagation signals [226]. The BCL2 family proteins consisting pro-survival (BCL2, BCL-XL and BCL-W) and pro-apoptotic (BAX, BAD, BAK and BOK) play an important role in preventing and facilitating permeabilisation of the mitochondrion, respectively. The mitochondrion permeabilisation either originates from the outer membrane facilitated by the pro-apoptotic BCL2 protein or from the inner membrane facilitated through a phenomenon called mitochondrial permeability transition due to the opening of a multiprotein complex [227].
Under cellular stress such as high intracellular Ca\textsuperscript{2+} levels, DNA damage pro-death signals are generated and it converges onto mitochondria. The pro-death signal causes mitochondrial outer membrane permeabilisation (MOMP) leading to the dissipation of mitochondrial transmembrane potential ($\Delta\Psi_m$). The respiratory pathways are disrupted resulting in the accumulation of reactive oxygen species (ROS). Proteins from mitochondrial intermembrane space (IMS) are released into the cytoplasm. The cytochrome c (CYTC) along with apoptotic protease activator factor 1 (APAF1) and dATP forms apoptosome that activates caspase-9 which in turn activates caspase-3. Direct IAP-binding protein with low pI (DIABLO) and high temperature requirement protein A2 (HTRA2) inhibit IAPs and facilitate pro-death signal. Apoptosis inducing factor (AIF) and Endonuclease G (ENDOG) get relocated to nucleus and carry-out caspase independent DNA fragmentation.

Though the caspases are the main cell executioner molecules, the intrinsic apoptotic pathway could also be caspase independent. Upon MOMP, there are two different paths by which the cell reaches its endpoint either in a caspase dependent or independent manner (Figure 7). In caspase dependent path, the CYTC released from the mitochondria forms apoptosome complex in the cytoplasm to activate executioner caspase-3. The CYTC forms apoptosome complex with APAF-1 and caspase-9 in a dATP dependent manner [222]. The caspase-9 proteolytically cleave and activate caspase-3. Activated caspase-3 carries out the chromatin condensation and DNA fragmentation [228]. In caspase independent path, the cytotoxic proteins, apoptosis-inducing factor (AIF) and endonuclease G (ENDOG) are released from the inner membrane of mitochondria into cytoplasm and subsequently relocate to nucleus. In the nucleus they mediate large-scale chromatin condensation and DNA fragmentation [229]. Apart from this, there is also a report of occurrence of alternative caspase independent cell death where the AIF mediates poly-ADP-ribose (PAR) polymer inducing caspase independent cell death [230].

When there is MOMP in most mitochondria of the cell through internal apoptotic pathway as a response to internal stress factor, three important changes occurring in the cellular environment could
be observed. (1) Cessation of $\Delta \Psi_m$ activity and ATP synthesis due to dissipation of $\Delta \Psi_m$. (2) The toxic proteins such as CYTC, AIF and ENDOG release from the internal mitochondrial membrane into the cytoplasm (3) The respiratory chain inhibition due to CYTC release, increases the ROS accumulation in the cell and thus stimulates a positive apoptotic feedback [226].

1.4.2.5. Role of apoptosis in growth and development

Apoptosis plays an important role in normal growth and development of organisms. Biochemical and genetic studies have provided useful information about its role, regulation and mechanisms in normal development. Apoptotic cell death in development is essential for eliminating defective cells, regulating cell number and limiting organ size, removing superfluous structures and tissue sculpting [231, 232] (Figure 8).

Physiological role of apoptosis in mammalian development was understood by studies using nematode (Cenorhabditis elegans), insect (Drosophila melanogaster) and murine models. Although, C. elegans [199, 233] and Drosophila melanogaster [234] study models are primitive, they were very useful in understanding the basic aspects of apoptosis since murine knockout and transgenic models often become complicated due to genetic redundancy [235]. Less complexity and the ease of genetic manipulation facilitated C. elegans to be the model system to understand the genetic regulation of apoptosis. The fruitfly Drosophila melanogaster served as an intermediate model in between nematode and murine model systems.

During the development of organisms there is regulated apoptotic cell death and cell division process (Figure 8). In C. elegans there is a precise pattern of embryonic cell death and cell division. During its development, out of 1090 somatic cells 131 cells are predetermined to undergo cell death [236]. Similarly the excess cells of nervous system in vertebrates and invertebrates are removed by apoptosis during the development. They participate in stage specific developments facilitating appropriate cell migration patterns and morphogenesis and are not required in later stages of development. Around 50% of neuronal cells get eliminated once the axons reach their targets. The neuronal cells death are not predetermined like C. elegans, but are regulated by a limiting neurotrophic factor [237, 238].
During development apoptosis is required for the formation of structures such as digits of hand (a) deletion of structures as in the metamorphosis of insect (b) controlling cell numbers in systems such as nervous system (c) and elimination of abnormal cells that have mutations (d).

Many larval tissues and embryonic tissues that are not required in the adult life are removed by apoptosis and thus the role of apoptosis becomes significant in tissue sculpting (Figure 8). For example, apoptosis is important for the proper finger and toe digits formation. During the developmental stages, inter digital cells are removed by means of apoptosis, the deficiency of which leads to the formation of webbed fingers in infants [239]. In frog’s transition from tadpole to adult life, major structural remodelling such as loss of tail, notochord and intestine takes place through apoptosis [240]. The insect metamorphosis involves major tissue rearrangement wherein the insect cells undergo apoptotic cell death or differentiate into other tissue cells [241].

Elimination of defective cells for tissue homeostasis is the other essential function of apoptosis. The cells, which have defective DNA fidelity and improper functioning, are removed from the tissues by programmed cell death. One example for this is the apoptotic elimination of lymphocytes from immune system that produce self-reactive receptors [242].

1.4.2.6. Apoptosis in cancer

In normal tissues the cell proliferation is governed by physiological factors that ensure homeostasis. Many of these physiological factors control growth at inappropriate sites by inducing apoptosis. In
cancer, this control mechanism is lost allowing tumours to proliferate beyond growth limit. Cancer cells within tumours evolve by developing resistance to apoptosis along with other hallmarks [9, 74].

Uncontrolled proliferation of cells in tumour is often found to be associated with inactivation of pro-apoptotic proteins and expression of anti-apoptotic proteins. Tumour cell evades apoptosis by harbouring inactivated tumour suppressor proteins. The tumour suppressor proteins such as p53, ARF and pRB, are mutated in more than 50% of the cancers [243]. On the other hand the anti-apoptotic proteins such as BCL2, BAG-3 [244] and survivin [245] are overexpressed in cancer, helping the cancer cells to live longer and proliferate.

While the tumour cells evolve to survive by evading apoptosis through above molecules, they depend on survival signals to further their existence. Survival signals include growth factors, hormones, cytokines and cell adhesion molecules. These signals are mediated in to the cancer cells through PI3K/AKT pathway. The PI3K/AKT pathway is known to transduce growth signals from external molecules such as IGF1/2, IL-3 [246] and internally by molecules originating from Ras oncogene [247], or by loss of tumour suppressor pTEN [248].

A normal cell has definite replicative potential and after certain cell divisions it ceases to replicate and reaches senescence. With every replication the cell loses part of its telomere. Accumulated loss of telomere over generations induces p53 independent apoptotic cell death [249]. However, in tumour cells telomere restoration takes place through elevated levels of telomerase activity.

Cytotoxic T lymphocytes (CTL) of adaptive immune system together with natural killer (NK) cells of innate immune system form immune surveillance against tumour cells. These cells use calcium dependent granule exocytosis and calcium independent CD95L pathway to eliminate tumour cells. In granule exocytosis, lymphocytes secrete cytotoxic granules containing membrane permeability protein called perforin and proteolytic enzymes containing granules called granzymes [250]. Perforin permeabilises the cell membrane for granzymes, which enter the cells and activate caspases of the cell to induce apoptosis [251]. Additionally, the granzymes can activate intrinsic apoptotic pathway by cleaving the BCL2 family protein BID [252]. In calcium independent CD95L pathway, the lymphocytes elicit apoptosis by activating CD95 receptor on the target cell [253, 254]. Tumour cells escape immune surveillance by secreting immunosuppressive factors and TGF-β [255, 256]. The escape mechanism also includes recruitment of inflammatory regulatory T cells [257] and myeloid derived suppressor cells [258] that are immunosuppressive.

Resistance to apoptosis in tumours poses serious challenge in cancer treatment. Chemotherapy and irradiation depend on apoptosis process to kill tumour cells. Due to the apoptotic resistance of tumour cells, this treatment module remains ineffective, especially in cases of cancer recurrences. The anti-cancer drugs are classified as antimetabolites, DNA-damaging agents, topoisomerases, mitotic inhibitors and nucleotide analogues [259]. The therapy may involve a blend of these anti-cancer drugs along with irradiation or in isolation, which cause cellular stress and cell death. Most forms these cellular stresses induce p53 expression which intern induce the expression of intrinsic apoptotic
pathway molecules BAX [260], NOXA [261] and PUMA [262] and extrinsic apoptotic pathway molecules such as CD95 [263], TRAIL-R1 [264] and TRAIL-R2 [265]. Clearly, apoptosis is not only one way of cell death in response to cancer therapy. However, tumour resistance to apoptosis remains to be one of the major ways by which the tumour cells develop therapeutic resistance.

1.4.2.7. Methods for monitoring apoptosis

Several methods are used to monitor apoptosis in in vitro and in vivo. Historically, a good account of apoptotic knowledge was obtained using electron microscopic studies [197]. This study was conducted when there was barely any knowledge about cell death and the steps involved in it. However, our knowledge on types of cell death and apoptosis, in particular, in normal health and pathogenesis has remarkably improved [191, 204]. Accordingly so have the techniques of detecting apoptosis. With the better understanding of the molecular changes occurring during apoptosis and with the growth of instrumental sophistication, we have been able to detect apoptosis with more accuracy and precision than ever before.

The apoptosis detection methods are based on the changes in morphology of the cell and effector and target molecules involved in the process. The apoptotic changes such as cell shrinkage, changes on the cell membrane, chromatin condensation can be detected by sophisticated microscopy and flow cytometry techniques [266]. The other molecular changes occurring in the effector and target molecules in the apoptotic cell can be assessed by immunoblotting technique.

1.4.2.8. Microscopic and flow cytometric methods

The microscopic and flow cytometric methods are very sensitive techniques that detect the morphological and molecular changes in the apoptotic cells [267]. In vitro, the apoptotic changes such as activation of caspases, changes in plasma membrane, changes in mitochondrial membrane potential, chromatin condensation and DNA fragmentation can be detected by flow cytometry and fluorescent microscopy [267, 268]. The cell population under apoptotic investigation are chemically fixed and/or labelled with a fluorescent probe and analysed using fluorescent microscopy or flow cytometry [268]. Flow cytometry remains the preferred method of analysis of the two as it provides high precision and speed. The ability of flow cytometer to detect and screen small size objects such as cells with great accuracy and speed is exploited to precisely determine the percentage of apoptotic cells in a large pool of cells [269, 270]. However, due to the nature of the experiment, this technique can only be used to determine the apoptotic percentage of blood cells and cultured cells in vitro. Optimally a probed pool of cells can easily be categorised as apoptotic and non-apoptotic cells using this technology.

The apoptosis detection methods are based on the characteristic morphological and molecular changes during the fatal cellular process. Due to the Caspase-3 and/or AIF and ENDOG activity, the nuclear condensation takes place and features as one of the early morphological changes in apoptosis [192]. The nuclear condensation in the cells can be detected by fixing the cells and labelling
them with the fluorescent dye Hoescht 33342 and subsequent analysis [269]. The cells show condensed chromatin or fragmented nuclei under the microscope and in the flow cytometer the cells are sorted as apoptotic cells based on their low DNA content. The fragmented DNA bases inside the apoptotic nuclei aids in the detection of apoptosis. The terminal deoxynucleotidyl transferase (TdT), transfers deoxy uridine triphosphate (dUTP) to the nicked ends of the DNA and this addition in turn is secondarily detected and analysed in an assay called terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) assay [271]. The TUNEL assay can be used to detect the apoptotic cells in tissues. However, in order to accurately determine apoptotic cells, the TUNEL assay has to be performed at optimum conditions otherwise, it is difficult to distinguish between apoptotic, necrotic and autophagic types of cell deaths by the TUNEL assay [272].

In an incompletely understood Ca\(^{2+}\) mediated aminophospholipid translocase reversal of the phosphotidylserine (PS) to the outer membrane of the plasma membrane remains to be a characteristic feature of the apoptotic cell [273]. This morphological change is even in the necrotic cells. However, the necrotic cells loose the integrity of their plasma membrane quite early, therefore a simultaneous examination of cell membrane integrity distinguishes apoptosis from necrosis [268]. The annexin-V is found to be binding to PS in a Ca\(^{2+}\) dependent way. So, annexin-V conjugated to a fluorescent dye called fluorescein isothiocyanate (FITC) is used to detect the apoptotic PS display [274-276]. To check the integrity of the plasma membrane simultaneously, the cells are treated with fluorescent dye propidium iodide (PI), which is capable of staining the DNA of the permeability-compromised cells. The cultured cells are directly treated with these fluorescent reagents and analysed immediately.

The mitochondrial membrane potential, \(\Delta \Psi_m\), is compromised with the release of CYTC from the inner mitochondrial membrane during the intrinsic apoptotic pathway [221, 226]. This can be detected by labelling the cells with 5,5’,6,6’-tetrachloro-1,1’,3,3’-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) [277, 278]. When the mitochondria are intact and the \(\Delta \Psi_m\) is higher, the JC-1 accumulates in mitochondria and forms J-aggregates that are fluorescent at 590nm for an excitation at 488nm. The flow cytometer is able to sort and analyse the cell population as low and high \(\Delta \Psi_m\) cells based on this factor. Under the microscope, a healthy cell bearing healthy mitochondria shows red J-aggregates, and an apoptotic cell with loss of \(\Delta \Psi_m\), forms green fluorescent monomers in the cytoplasm [279, 280]. Thus the apoptotic cells with the loss \(\Delta \Psi_m\), can be distinguished from non-apoptotic cells.

The molecular changes such as caspase activation and poly ADP ribosyl polymerase (PARP) cleavage are used as apoptosis detection method. The proteolytic activation of executioner caspases 3 and 7 are most commonly determined along with the PARP cleavage, by immunoblotting. After getting cleaved by their activation caspases, the active caspases 3 and 7 are detected as 17 and 19 kDa proteins, respectively [281, 282]. Depending on the cell type, other caspases activation is also used as one of the tools to determine apoptosis [282]. Upon activation the caspase-3 and caspase-7 cleave PARP in the nucleus. The PARP binds to single strand DNA breaks and signals to DNA ligase III and DNA polymerase beta for repairing. Full-length active 113 kDa PARP protein is cleaved into inactive 89 and 24 kDa fragments [283, 284]. The caspase activation [281] and PARP cleavage are
generally determined [284] by immunoblotting technique. However, flow cytometry and ELISA techniques could also be used to determine caspase activation [285, 286].

The techniques discussed above are capable of determining apoptosis accurately. However, any one particular technique cannot be employed in isolation for determining the apoptosis. Both biochemical and morphological examinations are essential, especially, for in situ apoptosis determination [287].

1.4.3. Autophagy

The autophagy literally means self-eating. In this type of cell death, the cells own lysosomal digestive enzymes engulf the cells. The autophagy is generally induced in the cells under certain stress conditions such as cell starvation, microorganism invasion, removal of damaged organelles and missfolded proteins. Induced autophagy need not necessarily be causing cell death in these situations; instead, it could be promoting cell survival. Autophagy is characterised by the formation of double-layered autophagosomes pinching a part of the cytoplasm. The autophagosome may consist of dysfunctional organelle, miss-folded protein or invaded microorganism. These autophagosomes fuse with the lysosomes of the cell and form autolysosomes. The autolysosome digest the contents of autophagosome and release the digested components back into cytoplasm for cell’s own use [288-290].

1.4.3.1. Historical perspective

In 1963, Christian de Duve coined the term autophagy and he is justifiably considered to be the pioneer of autophagy research. In 1955, Christian de Duve discovered the lysosome in the cell. Along with other workers, he visualized the lysosome and autophagy process under electron microscope [291-293]. The initial sequestering and phagophore formation and the other intermediate steps of autophagy was discovered using electro injected radioactive probing [294].

The progress of autophagy research in yeast helped to extrapolate the study to the mammalian cells [295]. The time-lapse studies showed clear sequential progress of double membrane structure to form double membrane autophagosome [296]. The discovery of target of rapamycin (TOR) gene and its characterization was important in the development of autophagy research. Initially discovered in yeast and subsequently in mammalians, the TOR is identified with cell cycle progression and protein synthesis [297]. It was shown that the rapamycin induced autophagy by inhibiting the mammalian target of rapamycin (mTOR) [298].

The discoveries of autophagy genes in higher eukaryotes led to the rapid expansion of our understanding on autophagy. The first autophagy related gene – 1 (Atg-1) was discovered by genetic screening of autophagy mutants in yeast [299], followed by a series of Atg gene discoveries raising the number to 31 [300]. This approach inspired researchers to study mammalian autophagy genes Atg-5 and Atg-12, which are conserved from yeast to human [301]. Yet in another study, Tamotsu Yoshimori and Noboru Mizushima characterized another important autophagy gene Atg-8, which
transformed the autophagy detection methods in mammals and other higher eukaryotes [302]. All these important discoveries have helped to gain greater understanding of autophagy process.

1.4.3.2. Autophagic pathway

At least three different forms of autophagy have been described. They are chaperone mediated autophagy [303], micro [304] and macroautophagy [305]. All three types differ in terms of their mode of cargo delivery to the lysosome and physiological function. While the chaperone mediated autophagy involves the selective degradation of proteins with “KFERQ” motif, the micro and macroautophagy involve indiscriminate degradation of organelle, protein, and lipids. The macroautophagy is discussed in the below sections as this mode of catabolic processing is involved in the higher eukaryotes and conserved from yeast to mammals.

The autophagic pathway operates through a double membrane structure called autophagosome. Cells under starvation or stress form a small vesicular intracellular structure called phagophores (also known as isolation membrane). The phagophore gradually grows into a double membrane vesicle like structure, engulfing part of cytoplasm that may include damaged organelles such as mitochondria and ribosomes (Figure 9). This double membrane functional unit is called as autophagosome. Through electron tomography technique, the endoplasmic reticulum is shown to be the membrane source of the autophagosome within the cells [306]. The outer membrane of the autophagosome fuses with lysosome to form autolysosome. Contents inside the autolysosomes are digested by the lysosome enzymes acid hydrolases. Subsequently, the digested contents are then released into the cytoplasm for cell’s own use [307, 308].
Autophagic pathway involving the formation of phagophore, autophagosome and autolysosome. A double membrane structure from endoplasmic reticulum is sequestered to form phagophore. Phagophore engulfs part of the cytoplasm including organelles such as ribosomes and mitochondria forms autophagosome. The autophagosome fuses with lysosome to form autolysosome. The contents inside the autolysosome including LC3 is degraded in autophagy. Contents of the digest is released into the cytoplasm for cells own utilisation.

The autophagosome formation involves nucleation and elongation steps to form the isolation membrane. Certain evolutionarily conserved gene products called Atg proteins are involved in orchestrating the above two steps. The Atg1/ULK1 protein-kinase complex (composed of ULK1, Atg13, FIP200, Beclin-1, Atg14, LC3, Atg12, and Atg16L1) plays an important role in the nucleation of isolation membrane in mammals [309, 310]. Amongst these, Beclin-1 is shown to be negatively regulated and directly interacting with BCL2 protein with BH-3 domain [311]. Over-expression of Beclin-1 is also established to be fatal for the cells [312]. Two ubiquitin like conjugation systems, the Atg12 and Atg8/microtubule associated protein light chain 3 (LC3) contribute to the elongation of the isolation membrane [313].

Other Atg proteins and factors are required for the fusion of lysosome to the autophagosome to form autolysosome. Except LC3 (a mammalian analogue of yeast Atg8), all these proteins are found to be associated either with nucleation or elongation of the isolation membrane and not throughout [302, 314]. The LC3 is conjugated to Phosphotidylethanolamine (PE) through C terminal glycine and amino group of PE, in the process [315]. These two important distinctions make LC3 a marker protein for detecting autophagy in cells [316].
1.4.3.3. Autophagy in development and differentiation

Autophagy (macroautophagy) plays an important physiological role in mammalian development and differentiation [317]. Initial studies on autophagy in lower eukaryotes such as yeast have provided information about the functioning of autophagic process both in physiology and pathology [40]. The discovery of autophagic related (ATG) genes in yeast, lead to the deeper understanding of the process even in higher organisms such as mammals as the pathway and genes involved are highly conserved in eukaryotes. Autophagy plays an important physiological role by removing defective proteins and organelles [318], helping the cells in adapting to starvation [319, 320] suppressing tumour formation, eliminating intracellular microbes and antigen presentation [321].

Autophagy’s role in pre-implantation development [322], survival during neonatal starvation [323], cell differentiation during adipogenesis [324], erythropoiesis [325] and lymphopoiesis [326] are recognised in recent times. Apart from these remodelling roles of autophagy, it’s importance in maintaining tissue homeostasis in terminally differentiated cells such as neurons is also well recognised.

One of the earliest autophagic process observed in mammalian development is in fertilised oocytes. A massive induction of autophagy after 4 hours of fertilisation is seen in the oocyte, which otherwise seems to be at minimum in unfertilised and highly differentiated oocyte. The ATG5-/- mice only survive only early embryogenesis proves that ATG5 is required for the early phase of pre-implantation development [322].

During the early neonatal period of mice another massive induction of autophagy is observed. One or two days after the birth, severe autophagy is induced in all neonatal tissues except brain [323]. In the womb the foetus is supplied with nutrients through placenta and at birth this supply is terminated exposing the neonates to severe starvation. Despite normal appearances of ATG3, ATG5, ATG7, ATG9 and ATG16L1 knockout mice at birth, succumbed to death in one day's time. Amino acid pool in the plasma and tissue of these knockout neonates are decreased. However, it is unknown whether the decrease in amino acid pool in the plasma is the sole reason for premature death of the neonates [327].

Adipocytes are differentiated from preadipocytes undergoing marked intracellular remodelling. The adipocytes differentiate from fibroblasts into round cells containing lipid droplets. Autophagy is induced in these cells during differentiation from round preadipocyte cells into adipocytes. In presence of adipogenic factors primary mouse embryonic fibroblasts (MEFs) can be differentiated into mature adipocytes. But, the ATG5-/- MEF fails to differentiate in to matured fibroblasts shows the requirement of autophagy in the differentiation [328]. Like the adipocytes, even erythrocytes undergo considerable intracellular remodelling during their differentiation from erythroblasts. Erythroblasts have nuclei and other organelles in them, which are removed and replaced by heme molecules in the matured erythrocytes. Although, electron microscopy results have implicated autphagic role in the removal of organelle [325], the ATG5-/- neonates have organelle eliminated in their erythrocytes makes the argument uncertain [329]. However, the Nix mediated mitophagy resulting in the selective removal of mitochondria in erythrocytes proves the essential role of autophagy in erythropoiesis [330].
The B-lymphocyte and T-lymphocyte specific ATG gene deletion experiments have emphasised the role of autophagy in lymphocyte differentiation. The gene specific deletion resulted in severe anaemia and considerable decrease in B and T-lymphocytes [331]. The survival of mature T-lymphocyte depends on appropriate population of mitochondria within the cell, which is ensured by mitophagy. Autophagy defective T cells lack this mechanism to undergo cell death [332].

Tissue specific ATG knockouts have helped in understanding the importance of autophagy in maintaining the tissue homeostasis by controlling the quality of intracellular proteins and organelles. The tissue specific ATG knockouts display accumulation of ubiquitin positive protein aggregates. The protein aggregates include cytosolic, ubiquitylated and p62 proteins. The accumulation of proteins leads to the cellular toxicity and formation of dysfunctional organs underscores the critical role of autophagy in maintaining the tissue homeostasis.

Along with controlling the quality of cellular proteins, autophagy controls the quality of organelles, especially of mitochondria. In the neuronal cells, the dysfunctional mitochondria are cleared in the cell by autophagy [333]. An ubiquitin ligase, Parkin is translocated to dysfunctional mitochondria from cytoplasm, by interacting with PINK1 protein. After locating to mitochondria Parkin induces mitophagy. In familial Parkinson’s disease, the Parkin protein is mutated obstructing the clearance of the dysfunctional mitochondria [334].

1.4.3.4. Autophagy in cancer

The role of autophagy in cancer is been a great subject of debate in scientific community due to the paradoxical role of autophagy in cancer. There are evidences to suggest that autophagy serves as a potent oncogenic factor [335] and at the same time there are evidences to suggest tumour-suppressor roles of autophagy [336]. Additionally, autophagy induced by tissues hypoxia is also reported to have important roles in cancer therapeutic resistance [337, 338]. Hence, the role of autophagy in cancer and its treatment or in normal tissue is context dependent.

Cancer cells are known to rely on basal levels of autophagy to maintain the cellular homeostasis and genomic integrity. Due to lack of vasculature and poor nutrient and oxygen supply, tumour cells continually strive to maintain homeostatic integrity. Autophagy is one of the processes that serve to support these demands [335]. The catabolic process not only helps the cells to remove damaged organelles and proteins, but also, ensures supply of metabolic intermediates for the production of molecules such as adenosine triphosphate (ATP) [339]. In addition to maintaining the cellular homeostasis, autophagy is also reported to promote later stages of tumour progression. For example, autophagy plays a critical role in epithelial carcinoma cell survival during metastasis. When these epithelial carcinoma cells are detached from the extra cellular matrix for metastasis, autophagy protects them from anoikis [340]. Furthermore, in ovarian cancer autophagy is induced by aplasia Ras homolog 1 tumour suppressor gene is shown to protect the dormant tumour cells in the context of tumour microenvironment [341]. Many studies have also shown role of autophagy in therapeutic resistance.
Oncogenes and tumour suppressor genes manipulate autophagy levels in tumour cells through intermediary key complexes mammalian target of Rapamycin complex 1 (mTORC1) and Beclin-1-hVps34 complex. The mTORC1 senses energy levels, nutrient availability and mitogenic signals in cells. Activity of mTORC1 is inversely correlated with the induction of autophagy. Rather, mTORC1 complex is pivotal in determining whether the net effect of the signalling is pro or anti-autophagic. The mTORC1 complex is deregulated in most cancers leading to the autophagy induction [342]. The Beclin-1-hVps34 complex is operational downstream of mTORC1 to regulate autophagy levels in cells [343]. Beclin-1 is known to form heterodimer and get stabilised by interacting with the anti-apoptotic members of the BCL2 family (BCL2, BCL-XL and MCL1) through its BH-3 domain [344]. For the formation of autophagosome, monomeric form of Beclin-1 associates and activates PI3K-III hVps34. Hence, any perturbations in BCL2 family proteins affect the Beclin-1 functioning in autophagy [311].

Apart from Becli-1’s BH-3 domain interaction with the BCL2 family proteins, three different regulatory proteins are known to bind to Beclin-1 through domains other than BH-3 domain. The core complex Beclin-1-hVps34-p150 is known to be involved in these three protein mediated autophagy regulation. The regulatory proteins are ATG14/BARKOR, UV irradiation Associated-Gene (UVRAG) and Rubicon [345]. ATG14/BARKOR positively regulates autophagy by binding to beclin-1 and translocating it to autophagosomes competing with UVRAG. On the contrary UVRAG and Rubicon negatively regulate autophagy by suppressing the formation of autophagosome and endosome [346]. Although, these proteins are known to regulate autophagy by binding to Beclin-1, their direct role in cancer is not yet fully understood.

Tumour suppressor function of autophagy was first ascribed through genetic studies of Beclin-1 in cancer. In high percentage of cancers of human prostate, breast and ovary, the Beclin-1 is monoallelically deleted from the tumour susceptibility locus of the cells. Furthermore, the Beclin-1 protein expression in breast cancer tissues found to be downregulated. Ectopic over-expression of the gene reduced cancer cell proliferation in vitro and its ability form tumour in vivo [347]. The heterozygous Beclin-1 disruption in mice exposed its susceptibility to spontaneous carcinogenesis. The knockout mice was prone to spontaneous tumour formation including B cell lymphoma, lung adenocarcinoma and hepatocellular carcinoma. This proved that Beclin-1 acts as a haploinsufficient tumour suppressor [348, 349].

Other autophagy related genes mutations have illustrated the importance of autophagy as a tumour-suppressor. The ATG5 deletion in NK cells is known to cause cell malignancy [350]. A Beclin-1 binding protein and a positive regulator of autophagy, UVRAG nonsense mutations are known to be the factors in colon [351] and gastric carcinomas [352]. Down regulation of another positive regulator of autophagy Bif-1, known to be related to colon adenocarcinoma [353]. The Atg4c knockout mice also found susceptible to fibrosarcomas. These facts yet again highlight the tumour suppressor function of autophagy [354].

The cell protective function of autophagy under cellular stress poses challenge in cancer treatment. The treatment efficacy also lies in the ability of the approach to modulate autophagy for causing cell death. Recent reports have shown that the pharmacological inhibition of autophagy using chloroquine
may help in tumour therapy [355]. Another study on the other hand has shown the reverse, which has exploited the ability of autophagy to cause cell death. In this xenograft study, the tumour suppressor ARHI is expressed using Doxycycline (Dox) as inducer. Autophagy was induced along with induction of the ARHI led to the reduction in tumour size in mice. In addition to this, when chloroquine was included in the treatment, it further reduced the tumour growth. On the contrary, the tumour size dramatically increased when Dox was withdrawn resulting in the reduction of autophagy and ARHI expression [341]. However, while inhibiting autophagy for cancer treatment, the most critical function of autophagy in maintaining tissue homeostasis should be taken into consideration.

1.4.3.5. Methods for monitoring autophagy

A number of methods have been in practice to monitor autophagy in mammalian cells since the discovery of this cellular process. With our increased understanding of the process, the existing methods have been redefined and other reliable new methods have been proposed time to time.

The detection methods assess the turnover of the autophagic functional unit, autophagosome. Since autophagosome is an intermediate of a dynamic cellular process, measurement of its number in cells directly or indirectly doesn’t actually indicate the induction of autophagy. The formation and clearance of the autophagosome depends on its upstream and downstream pathways. Blockade in either of the pathways results in decrease or increase of the autophagosome numbers. Therefore, the increase in autophagosome number could indicate induction of autophagy or blockade in the downstream processing of the autophagosome. Apart from measuring the autophagosome numbers, methods are employed to measure the rate of clearance of autophagosome, which is known as “autophagic flux” [356].

1.4.3.6. Measuring the number of autophagosomes

The autophagosome numbers in cells can be directly measured by electron microscopy and fluorescence microscopy or indirectly measured by biochemical methods. The LC3, mammalian analogue of yeast Atg8, serves as a reference molecule to determine autophagy number within the cell due to its association with the isolation membrane, autophagosome and autolysosome.

1.4.3.7. Electron microscopy

The autophagy research history began with the identification of autophagosome in cells using electron microscopy. The osmium tetroxide staining reveals the electron density and thus the ultrastructure of the cell under electron microscope. Autophagosome is visualized as a double membrane structure containing the undigested contents of the cytoplasm including other organelles such as peroxisomes, ribosomes and mitochondria. The autolysosomes are revealed as double membrane structures with partially or completely digested cellular contents owing to the fusion with lysosome [357]. These autophagic structures can be visualized with great accuracy using electron microscopy. However, precautions need to be taken in accurately identifying autophagosome and autolysosome structures and distinguishing intracellular pathogenesis.

33
1.4.3.8. Fluorescence microscopy

The autophagosome count uses fluorescence microscopy techniques and exploits the association of LC3 molecule with the autophagic process. To assess the autophagosome numbers, the LC3 is visualized under the microscope either by immunocytochemistry or tagging to a fluorescent molecule such as green fluorescent protein (GFP) or red fluorescent protein (RFP). The LC3 forms punctate structure in the cytoplasm of the autophagic cells. Numbers of punctae correspond to the numbers of autophagosomes within the cells [315, 316]. Basal levels of autophagy are observed in certain cell types such as cancer cells. This basal level of autophagy has to be compensated in order to determine the induction of autophagy. The number of punctae in at least 200 cells should be counted to determine the basal levels of autophagy in any particular cell type. Certain precautions have to be adopted while employing fluorescence microscopic method to determine the number of autophagosomes. Factors such as accurate counting of punctae, compensation for basal levels of autophagy and aggregation due to LC3-GFP overexpression have to be considered [356, 358].

1.4.3.9. Biochemical assay

The LC3 turnover during the autophagic process serves as a basis for biochemical determination of induction of autophagy. LC3 is processed at C terminus by protease Atg4 to become LC3-I. LC3-I is subsequently conjugated with PE to become LC3-II. While the mobility of PE conjugated LC3-II increases on SDS-PAGE, the mobility of unconjugated LC3-I is retarded. The LC3-I band appears around 18 kDa and LC3-II band appears around 16 kDa on SDS-PAGE, which allows distinction between the two on an immunoblot. The LC3-II is associated with autophagosome increases and the onset of autophagy is reflected on the immunoblot. The amount of LC3-II detected on immunoblot is reported usually correlates well with the number of autophagosomes making it a reliable biochemical technique to detect induction of autophagy [302].

1.4.3.10. Measuring autophagic flux

The assays described above to determine the autophagosome number cannot be exclusively used as a measure for induction of autophagy. As noted above, the increase in the autophagosome number may reflect induction of autophagy and blockade in the downstream processing of autophagosome. In order to prove induction of autophagy, it is important to rule-out that the increase in autophagosome number is not due to its blockade. The complete digestion of autophagosomal contents in autolysosome reflects the completion or turnover of autophagic process. The digestive process thus serves as a basis for autophagic flux measurement [356, 359].

The LC3 processing yet again proves useful in measuring autophagic flux. The LC3 is also digestively degraded during autophagic process. So, there is a constant making and breaking of LC3-II molecule in autophagy. This turnover of LC3 in autophagy can be experimentally proven using lysosome protease inhibitors E-64d and Pepstatin-A. The use of protease inhibitors results in the accumulation of LC3-II band on immunoblot and increases in punctae number in the cytoplasm of the autophagic cell [316].
Other reliable assays to measure the autophagic flux are p62 degradation assay and long-lived protein degradation assay. These two assay principles are based on the protein degradation process of autophagy. It is known that p62 binds to LC3 to get incorporated into autophagosome and is degraded in autolysosome. So, the p62 concentration in cells is inversely related with the autophagic activity [360]. The long-lived protein assay is measured using $^{14}$C or $^{3}$H valine or leucine. This assay is generally conducted in presence of autophagic inhibitor 3-MA to accurately measure the long-lived proteins degradation in autophagy [361].

The induction of autophagy has to be confirmed by performing multiple relevant assays, as there is no single "gold standard" assay to monitor autophagy. Additionally, it is recommended that autophagic activity is confirmed by using either pharmacological or si-RNA inhibition of autophagy [356, 359].

1.4.4. Regulated Necrosis

For a long time, necrotic cell death was thought to be accidental random cell death due to lack of morphological traits. However, recent studies have shown that the necrosis is a regulated process and it has an important role in many physiological and pathological contexts [362]. The factors such as excitotoxins, ligation of death receptors [363] and alkylating DNA damage [364] are known to trigger regulated necrosis. When the caspases are functionally inhibited either genetically or pharmacologically, the RIP1 and RIP3 still remain active and indulge in functional interactions that ultimately cause necrotic cell death [365, 366].

The regulated necrosis can further classified into RIP1 dependent and RIP-3 dependent regulated necrosis. The RIP1 is activated in the first type, and it can be measured by monitoring RIP1 phosphorylation on S161 or by enzymatic activity assays. Such regulated necrosis can be inhibited using RIP1 inhibitor such as necrostatin-1 [367]. The RIP3 dependent regulated necrosis are reported but they are insensitive to necrostatins [368].

1.4.5. Other types of cell death

Other types of recognised and the recommended types of cell death include mitotic catastrophe, anoikis, entosis, parthanatos, pyroptosis, netosis, and cornification [193]. Mitotic catastrophe is a term often used to describe cell death occurring during mitotic process. Cell death reported to be triggered due to aberrant mitosis and executed during the subsequent interphase [369]. The term anoikis literally mean the state of being homeless. This type of cell death description is given when the cells undergo apoptotic cell death due to the absence of cell to matrix interactions [370]. Cell survival depends on the signals conveyed from growth factors such as epidermal growth factor upon interaction with extracellular matrix. This interaction seizes once the cells are detached from the substratum causing anoikis [371]. Entosis is a term used to describe “cell in cell” phenomenon that are frequently seen in the non-phagocytic cells in tumour samples. It is also reported in the lymphoblasts of Huntington’s patients. Due to the nature of this cell death it is often dubbed as cell cannibalism [372]. Entosis is also induced when there is a loss of extracellular matrix interaction. However, it differs from anoikis by not activating the apoptotic executioners [372, 373]. Parthanatos is
referred to a particular type of cell death involving DNA damage response form PARPs. Particularly PARP-1 is accounted for more than 90% of such catastrophic cellular activity. PARP-1 is involved in DNA repair machinery [374, 375]. However, the hyper activation of PARP-1 leads to depletion of NAD⁺ and ATP resulting in ΔΨᵢₐ₅ dissipation and AIF release [230]. Pyroptotic type of cell death is referred to the apoptotic cell death caused by bacterial infection of the cell. The bacteria such as *Salmonella typhimurium* and *Shigella flexneri* are known to cause this type of cell death. The immunological cells neutrophils and eosinophils release something called as neutrophil extracellular traps (NETs). The NETs are micro structures consisting of histones, granular antimicrobial proteins and nuclear chromatin are known to kill bacteria [376, 377]. In the bargain the releasing neutrophils undergo atypical type of cell degeneration, which is called as netosis [378]. Cornification is associated with terminal differentiation of the anucleated cells of the body including skin cells, lens and RBCs. Lens and RBCs escape this atypical type of apoptotic or necrotic cell death [379, 380]. Whereas, in the skin the cells undergo cornification to form a layer of dead keratinocytes, containing a mixture of proteins such as keratin and loricrin and lipids such as fatty acids and ceramides [381]. These layers of cornified keratinocytes provide structural rigidity and mechanical protection to the skin [382].

It is to be noted that different types of cell deaths are neither isolated nor mutually exclusive in their signalling cascades. The pro-survival pathways are also operational along with the cell death pathways. Moreover, when there is cellular stress, multiple lethal mechanisms become operational in the cell. Ultimately, the cell fate is dependent on the pathway that overtakes the other.

### 1.5. Rationale of the PhD study

The understanding of cancer disease and its management has grown enormously over the last few decades. The data from the human genome-sequencing project has opened up new avenues for cancer researchers. New molecules and new understanding about the known molecular players are emerging at an astonishing rate ever since the completion of the project. Our understanding of the disease of cancer is becoming clearer with time. However, there are still areas where the actual molecular mechanisms and the roles of the newer unknown molecules are yet to be defined. In this study, an attempt is made to characterise a new molecule that has differential expression in different cancers.

Sensing the importance of cell surface receptors and secreted proteins in cancer treatment, we used genomic tools to identify GLT1D1. Oncomine is a genomic platform that has vast cancer microarray data collection and an integrated data-mining platform [383]. The microarray analysis suggests differential expression of GLT1D1 in different types of cancer including breast cancer (Figure 10).
Figure 10: Microarray analysis showing differential expression of GLT1D1 in breast cancer.

Graph adopted from Oncomine™ (Compendia Bioscience, Ann Arbor, MI).

Microarray data showing the differential expression of GLT1D1 in normal breast (0) and invasive ductal breast carcinoma (1). For differential analysis, the raw values obtained from microarray experiments are normalised by Log2 transformation and median centring in the oncomine database. The analysis revealed an increase in GLT1D1 expression (1.443 fold, \( p \text{value} = 8.10E-4 \)) in invasive ductal breast carcinoma relative to normal breast.

Figure 11: Microarray analysis showing differential expression of GLT1D1 in oesophageal cancer.

Graph adopted from Oncomine™ (Compendia Bioscience, Ann Arbor, MI).

Microarray data showing the differential expression of GLT1D1 in oesophagus (0) and Barrett’s oesophagus (1) and oesophageal carcinoma (2). For differential analysis, the raw values obtained from microarray experiments are normalised by Log2 transformation and median centring in the oncomine database. The analysis revealed an increase in GLT1D1 expression in Barrett’s oesophagus (1.867 fold, \( p \text{value} = 0.002 \)) and oesophageal carcinoma (4.207 fold, \( p \text{value} = 4.06E-9 \)) relative to normal oesophagus.
We performed systematic search of Oncomine and another genomics tool UniProtKB [384] to identify the GLT1D1 gene. The novelty, differential expression in cancer, signal peptide and membrane spanning region prediction of GLT1D1 made it an interesting molecule to study the gene as a possible therapeutic target.

The Oncomine database analysis revealed differential expression of GLT1D1 when compared expression in cancer versus normal tissues. For example, in the above (Figure 10) and (Figure 11) GLT1D1 gene expression analysis of breast cancer and oesophageal cancer is compared with that of their normal tissues. In the above figures the raw values are normalised by Log2 transformation and median centring. GLT1D1 has increased in expression (1.443 fold, pvalue = 8.10E-4) in invasive ductal breast carcinoma relative to normal breast (Figure 10) [70]. In oesophageal cancer, the GLT1D1 has increased in expression in Barrett’s oesophagus (1.867 fold, pvalue = 0.002) and oesophageal carcinoma (4.207 fold, pvalue = 4.06E-9) relative to normal oesophagus (Figure 11) [385].

GLT1D1 protein sequence analysis using Pfam protein prediction database indicates a signal peptide and a membrane spanning region [386] for the protein. This indication of protein being a secreted or a membrane protein prompted us to select GLT1D1 for the study from therapeutic intervention point of view.

1.6. Introduction to GLT1D1

The GLT1D1 gene is annotated as a hypothetical protein-coding gene in the non-redundant database UniProtKB. The sequence analysis of GLT1D1 shows that it is highly conserved protein in mammals and may exist in different isoforms. Three major isoforms of human GLT1D1 are annotated in UniProtKB, with accession numbers Q96MS3-1, 2 and 3. All these isoforms have first 108 N-terminal amino acids in common and differ in their C-terminus as shown in Figure 1. The Q96MS3-1 is the largest isoform of the three, with 346 amino acids. In Q96MS3-2, 79 amino acids of Q96MS3-1 from 126 to 205 are missing, whereas, the shortest Q96MS3-3 has a unique C-terminus. The protein domain prediction indicates the presence of a glycosyltransferase domain in the c-terminus of Q96MS3-1 and 2 (Figure 12). Thus the gene derives its name.
Figure 12: Graphical representation of GLT1D1 (Q96MS3) protein and its isoforms as annotated in UniProt-KB

The PRoteomics IDEntifications (PRIDE) [387], a mass-spectrometry database has five different peptide submissions for GLT1D1. Previous studies have shown that GLT1D1 is transcribed into mRNA [388-390]. But there are no published studies available yet to show that GLT1D1 is actually translated. The protein identification by mass-spectrometry proves useful in this circumstance [391]. Using the UniProtKB accession number Q96MS3, five different peptide submissions for GLT1D1 was found in PRIDE database (Figure 13). The identified peptides correspond to different regions of the GLT1D1 protein, mostly to the Q96MS3-1 and Q96MS3-2 isoforms (Figure 13). Only one of the identified peptides corresponds to the Q96MS3-3 isoform. Therefore the study was limited to the Q96MS3-1 and Q96MS3-2 isoforms. The protein regions represented in bold (Figure 13A) corresponds to the peptides identified and submitted to the database. These identified peptides are reported to be from human plasma, platelet and hepatocytes [392, 393]. Thus the peptide submissions in the PRIDE database prove that GLT1D1 gene is actually translated into protein.
A

Sequence of the identified peptides of GLT1D1 (Q96MS3).
1. LLFLAVLRPHTGNAVTAQR
2. NCFAYNSSVSEGMSAAILEAMDLQFPV
3. NPGNAAVVKEHTGFFSNPQEFVHLAK
4. EYVRMYHSWQVER
5. MYHSWQVERDTYQQLIR

B

Isoform Q96MS3-1
MRLLFLAVLRPHTGNAVTAQRVFAHLEAGHVC/LKDAFDSESIEANL/LAENCEAAL/LHYRGGRLQGHRIPFGVIF
GGTDVNECANKEKTVMGRLVEAEARFAFTEHKMAAQOVP+HAGKVKVYQSQGIAATTPAANFNTFLQERSEINGS
ADNLHIFLICGLGRLCVKDPYLVDAFSWCHCEPNVHLVIVGPEVDPVFTREVKAVKRAAGVRIGEHPQEDLHAVVNCF
AVVNSSVSEGMSAAILEAMDLQFPVLRNYQGNAAVVKEHTGFFSNPQEFVHLAKRLLVSDEPAKEIVVNGREYVRMY
HSWQVERDTYQQLIRKLEGSTED

Isoform Q96MS3-2
MRLLFLAVLRPHTGNAVTAQRVFAHLEAGHVC/LKDAFDSESIEANL/LAENCEAAL/LHYRGGRLQGHRIPFGVIF
GGTDVNECANKEKTVMGRLVEAEARFAFTEHKMAAQOVP+HAGKVKVYQSQGIAATTPAANFNTFLQERSEINGS
ADNLHIFLICGLGRLCVKDPYLVDAFSWCHCEPNVHLVIVGPEVDPVFTREVKAVKRAAGVRIGEHPQEDLHAVVNCF
AVVNSSVSEGMSAAILEAMDLQFPVLRNYQGNAAVVKEHTGFFSNPQEFVHLAKRLLVSDEPAKEIVVNGREYVRMY
HSWQVERDTYQQLIRKLEGSTED

Isoform Q96MS3-3
MRLLFLAVLRPHTGNAVTAQRVFAHLEAGHVC/LKDAFDSESIEANL/LAENCEAAL/LHYRGGRLQGHRIPFGVIF
GGTDVNECANKEKTVMGRLVEAEARFAFTEHKMAAQOVP+HAGKVKVYQSQGIAATTPAANFNTFLQERSEINGS
ADNLHIFLICGLGRLCVKDPYLVDAFSWCHCEPNVHLVIVGPEVDPVFTREVKAVKRAAGVRIGEHPQEDLHAVVNCF
AVVNSSVSEGMSAAILEAMDLQFPVLRNYQGNAAVVKEHTGFFSNPQEFVHLAKRLLVSDEPAKEIVVNGREYVRMY
HSWQVERDTYQQLIRKLEGSTED

Figure 13. – Amino acid sequence of GLT1D1 isoforms and the recognized peptide sequences
(A) Sequence of the identified peptides of GLT1D1. (B) Sequences of all the three isoforms of GLT1D1
with the identified peptide regions of the protein depicted in bold.

1.7. Study Hypothesis

The following hypothesis was made, based on the above information available about the gene.

I. GLT1D1 has a role in oncogenesis

To examine this hypothesis, forced-expression study model (gain-of-function) was used. The results
obtained by this method revealed that the gene has a role in cell death under the study conditions.
Therefore, it was further hypothesised that

II. Forced-expression of GLT1D1 causes cell death

This hypothesis was examined by employing different cell death assays. The breast cancer cell lines
MCF-7 and BT-549 were chosen as cell models for the study. Cell, molecular and biochemistry-based
assays were used to characterise the type and mechanism of GLT1D1 mediated cell death.
CHAPTER 2. MATERIALS AND METHODS

2.1. MATERIALS

2.1.1. Chemicals, Reagents, Antibodies and Primers

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<th>Material</th>
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<tr>
<td>1Kb plus DNA ladder</td>
<td>Invitrogen Life Technologies, Carlsbad, CA, USA</td>
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<td>3-[4,5-Dimethyl-thiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT)</td>
<td>Sigma Chemical Company, St Louis, MO, USA</td>
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<td>Acrylamide/Bis solution (40%)</td>
<td>Bio-rad laboratories, Inc., Hercules, CA, USA</td>
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<td>Agarose (ultra pure)</td>
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<td>Anti-β-ACTIN mouse monoclonal Ab</td>
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<td>Complete Minlab protease inhibitor</td>
<td>Roche Diagnostics GmbH, Mannheim, Germany</td>
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<td>EDTA</td>
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<td>Ethanol (absolute, analytical grade)</td>
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<td>Freund's adjuvant (complete)</td>
<td>Sigma Chemical Company, St Louis, MO, USA</td>
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<tr>
<td>Freund's adjuvant (incomplete)</td>
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<td>FuGENE HD Transfection reagent</td>
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<td>Genetone (G418)</td>
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<td>Glutathione Spharosell 4B</td>
<td>GE Healthcare, USA</td>
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<td>Glutathione (reduced)</td>
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<td>Glycerol</td>
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<tr>
<td>Glycine</td>
<td>Applichem GmbH, Darmstadt, Germany</td>
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<tr>
<td>HBSS</td>
<td>Sigma Chemical Company, St Louis, MO, USA</td>
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<tr>
<td>Hoescht 333/342</td>
<td>Sigma Chemical Company, St Louis, MO, USA</td>
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<tr>
<td>Hydrochloric acid</td>
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<td>Isopropanol</td>
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<td>KpnI restriction enzyme</td>
<td>New England Biolabs, USA</td>
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<td>L-glutamine</td>
<td>Gibco New Zealand Ltd., Auckland, New Zealand</td>
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<td>Magnesium Chloride</td>
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<tr>
<td>Matrigel</td>
<td>BD Biosciences, Franklin lakes, NJ, USA</td>
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<td>Methanol</td>
<td>Scientific Supplies Ltd, Auckland, New Zealand</td>
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<td>Mercaptoethanol</td>
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<td>Mitotracker-red CMXRos</td>
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<tr>
<td>NheI restriction enzyme</td>
<td>New England Biolabs, USA</td>
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<td>Nef restriction enzyme</td>
<td>New England Biolabs, USA</td>
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<td>Penicillin/ Streptomycin</td>
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<td>PVDF membrane</td>
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<td>Qiagen OneStep RT-PCR kit</td>
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</tr>
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<td>Qiagen plasmid maxi kit</td>
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<td>Seeblue plus2 protein marker</td>
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<td>Sodium dodecyl sulfate (SDS)</td>
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<td>Sodium Phosphate</td>
<td>Scientific Supplies Ltd, Auckland, New Zealand</td>
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<tr>
<td>Supersignal west Dura extended duration substrates</td>
<td>Pierce Biotechnology, Inc., Rockford, Illinois, USA</td>
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Table 2: List of chemicals and reagents

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<th>Antibody</th>
<th>Company</th>
<th>Concentration for Western Blot</th>
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<tr>
<td>Anti-BCL2</td>
<td>BD Biosciences, Franklin lakes, NJ, USA</td>
<td>1:500</td>
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<tr>
<td>Anti-BAX</td>
<td>Santa Cruz Biotechnologies, CA, USA</td>
<td>1:10,000</td>
</tr>
<tr>
<td>Anti-LC3</td>
<td>Abcam, Australia</td>
<td>1:2,000</td>
</tr>
<tr>
<td>Anti-Bclin-1</td>
<td>Abcam, Australia</td>
<td>1:5,000</td>
</tr>
<tr>
<td>Anti-β-Actin</td>
<td>Sigma, NZ</td>
<td>1:20,000</td>
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<tr>
<td>Anti-c-myc</td>
<td>Roche Diagnostics GmbH, Mannheim, Germany</td>
<td>1:5,000</td>
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Table 3: List of antibodies

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<thead>
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<th>Gene Target</th>
<th>F or R</th>
<th>Sequence 5' - 3'</th>
</tr>
</thead>
<tbody>
<tr>
<td>GLT1DNA F</td>
<td>Glycosyltransferase-1 domain containing-1</td>
<td>Forward</td>
<td>CACGAATTCATGCGGCTCCT</td>
</tr>
<tr>
<td>GLT1DNA R</td>
<td>Glycosyltransferase-1 domain containing-1</td>
<td>Reverse</td>
<td>GTGCTCGAGATCTCAGTCGGCTTC</td>
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<tr>
<td>GLT5</td>
<td>Glycosyltransferase-1 domain containing-1</td>
<td>Forward</td>
<td>ATAGGATCATGCGGCTCCTGTTCCTCCTGCGGCG</td>
</tr>
<tr>
<td>GLTX3</td>
<td>Glycosyltransferase-1 domain containing-1</td>
<td>Reverse</td>
<td>TATGAATTCATCCTGAGTCGGCTTCCTGCG</td>
</tr>
<tr>
<td>GLTID1 F</td>
<td>Glycosyltransferase-1 domain containing-1</td>
<td>Forward</td>
<td>ATAGCTAGCATGCGGCTCCTGTTCCTGCG</td>
</tr>
<tr>
<td>GLTID1 R</td>
<td>Glycosyltransferase-1 domain containing-1</td>
<td>Reverse</td>
<td>TATGCGGCCGCAATCTGAGTCGGCTTCCTGCG</td>
</tr>
<tr>
<td>LC3B F</td>
<td>Microtubule associated light chain 3-B (LC3B)</td>
<td>Forward</td>
<td>ATTTGAAATCTGAGTCGGCTTCCTGACCTT</td>
</tr>
<tr>
<td>LC3B R</td>
<td>Microtubule associated light chain 3-B (LC3B)</td>
<td>Reverse</td>
<td>ATTTGACCCACAGTACGCTACGCTTC</td>
</tr>
<tr>
<td>COX8F</td>
<td>Cytochrome oxidase-8 (COX8)</td>
<td>Forward</td>
<td>ATTTGAGTCGGCCGAGTCGGCTTC</td>
</tr>
<tr>
<td>COX8R</td>
<td>Cytochrome oxidase-8 (COX8)</td>
<td>Reverse</td>
<td>ATTTGACCCACAGTACGCTACGCTTC</td>
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</tbody>
</table>

Semi quantitative (one-step) RT-PCR PRIMERS

<table>
<thead>
<tr>
<th>Name</th>
<th>Gene Target</th>
<th>F or R</th>
<th>Sequence 5' - 3'</th>
</tr>
</thead>
<tbody>
<tr>
<td>GLTID1</td>
<td>Glycosyltransferase-1 domain containing-1</td>
<td>Forward</td>
<td>CCTCATCTGAGTCGGCTT</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse</td>
<td>TCCCTGTAAAGATCTGAGTGGG</td>
</tr>
<tr>
<td>Beta-Actin</td>
<td>ACTIN, Beta</td>
<td>Forward</td>
<td>ATGATATGCGGCTGCCTG</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse</td>
<td>CGCTCTGAGTCTCAGTGC</td>
</tr>
<tr>
<td>BAX</td>
<td>BCL2-associated X protein</td>
<td>Forward</td>
<td>GGGTGCTGAGTCGGCTG</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse</td>
<td>AGACGGTACGGCGAAGTC</td>
</tr>
<tr>
<td>BCL2</td>
<td>B-cell CLL/lymphoma 2</td>
<td>Forward</td>
<td>TTCGACATGAGTCGGCTG</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse</td>
<td>AGACGGTACGGCGAAGTC</td>
</tr>
</tbody>
</table>

Table 4: List of primers

- T4 DNA ligase: New England Biolabs, USA
- Tetramethyl-ethylendiamin (TEMED): Sigma Chemical Company, St Louis, MO, USA
- TRIzol reagent: Invitrogen Life Technologies, Carlsbad, CA, USA
- Trypsin: Serva Electrophoresis GmbH, Heidelberg, Germany
- Triton x 100: Sigma Chemical Company, St Louis, MO, USA
- Triethanolamine (TEA): Sigma Chemical Company, St Louis, MO, USA
- Trypan Blue: Gibco New Zealand Ltd., Auckland, New Zealand
- Tween-20: Sigma Chemical Company, St Louis, MO, USA
- Vectashield mounting media: Vector Laboratories, Burlingame, USA
- General caspase inhibitor Z-VAD-FMK: BD Biosciences, Franklin lakes, NJ, USA
2.2. METHODS

2.2.1. Molecular biology

2.2.1.1. Gene cloning

The GLT1D1 and GLT1D1-2 genes were amplified using GLTcDNA F and GLTcDNA R primers (Table 4) from (RZPD - German Science Centre for Genome Research) open reading frame clones. The genes were further cloned into pIRESneo3, a mammalian expression vector and pGEX4T1, a bacterial expression vector. For cloning the genes between BamHI and EcoRI site of the pGEX4T1 vector, the genes were amplified using GLT5 and GLTx3 primers (Table 4) from the above cDNA amplicons. For cloning the genes between NheI and NotI site of the pIRESneo3 vector, the genes were amplified using GLT1D1F and GLT1D1R primers (Table 4) from the above cDNA amplicons. The vectors and amplicons were digested with appropriate restriction enzymes (from New England Biolabs) at 37°C for forty minutes and for four hours, respectively. After digestion the vectors and amplicons were ligated using T4DNA ligase (from New England Biolabs) for 2 hours at 25°C. The ligated vectors were transformed into high efficiency DH5α competent cells and screened for positive clones by insert release. Two positive clones from each gene constructs were sequence confirmed and one of them was used in the following experiments. Each of these gene constructs resulted from above clones were named as pIRES-GLT1D1, pIRES-GLT1D1-2 and pGEX-GLT1D1, pGEX-GLT1D1-2. The GLT1D1 gene was sub cloned into pEGFP-N1 vector by releasing the insert from the above pIRES-GLT1D1 construct and following the same cloning protocol as above. The resultant construct was named as pEGFP-GLT1D1.

Light chain microtubule associated protein-B (LC3B), was cloned into pDsRed2 vector to produce an N-term fusion protein with red fluorescent protein (RFP). In mammalian cells, first 22 C-term amino acids of nascent LC3 are proteolytically processed to form mature LC3-I protein. To obtain a functional LC3-RFP fusion, an N-term fusion protein was produced. LC3B gene was amplified using LC3B-F and LC3B-R (Table 4) primers on the cDNA of the total RNA of DU145 cell line. The amplicon was cloned into the KpnI and EcoRI cloning sites of the pDsRed2 vector using the same cloning protocol as above. The resultant construct was named as pDsRed-LC3B.

Cytochrome oxidase-8 (COX8) and MSRA mitochondrial signal coding nucleotides were also cloned into pDsRed2 vector to produce a RFP fusion protein. The COX8 and MSRA nucleotides were amplified using the COX8F and COX8R, MSRAF and MSRAR primers (Table 4), respectively. MCF-7 total RNA converted into cDNA was used as template. The COX8 gene was cloned into the KpnI and EcoRI cloning sites of the pDsRed2 vector using the same cloning protocol as above. The resulted construct was named as pDsRed-COX8 and pDsRed-MSRA.
2.2.1.2. DNA and RNA isolation

The plasmid DNA constructs required for the study were isolated after transforming and amplifying them in DH5α bacteria. Transformed bacteria were selected on antibiotic plates depending upon the antibiotic resistance of the plasmid. A single colony from the selection plate was inoculated and grown in 400 ml liquid broth (LB) media containing selection antibiotic for 16 hours. Bacteria was pelleted down at 4000g for 10 minutes at 4°C. The pellet was stored at -20°C until the extraction of the plasmid DNA.

Plasmid DNA from the bacterial pellet was extracted using Qiagen plasmid maxi kit. The plasmid DNA was isolated according to the manufacturer’s protocol. The plasmid DNA pellet was dissolved in 1ml DNAse free water and the DNA was stored at -20°C for long-term use.

RNA from the cancer cell lines was isolated using TRIzol reagent. The cell lines were grown or transfected as per the experimental design and the total RNA from the cells were extracted as per the manufactures protocol. The RNA pellet was dissolved in RNAse free water. The RNA was treated with DNase enzyme (1U/ug RNA) to get rid of contaminating cellular genomic DNA. The RNA was re-precipitated in ethanol after seizing the DNase enzyme activity using 2mM EDTA. This RNA pellet was dissolved in RNAse free water. Thus extracted RNA was assessed for its purity and concentration by spectrophotometry using a nanometer. The purity of RNA was assessed calculating A260/A280 ratio. The RNA was considered as good quality RNA and it was stored at -80°C for long-term use.

2.2.1.3. One-step RT-PCR

One step RT-PCR was set-up using One-step RT-PCR kit (Qiagen). The total RNA isolated from cell lines as described in the previous step was used as template. The PCR reaction was set-up according to the manufacturer’s protocol. The gene specific primers were also included in the reaction and an annealing temperature of 58°C was used. In semi-quantitative RT-PCR reactions, total number of reaction cycles varied from 30 to 40 cycles to analyse gene expression at log phase. In end-point one-step RT-PCR analyses, the number of cycles was increased to 45 cycles. Upon the completion of PCR, the amplicons were analysed either on 1% or 1.5% agarose DNA gel.

2.2.2. Recombinant protein expression and purification

2.2.2.1. Protein induction

The recombinant GLT1D1 and GLT1D1-2 proteins were expressed and purified as GST fusion proteins. The pGEX-GLT1D1 and pGEX-GLT1D1-2 constructs were transformed into BL21DE3/pLysS strain and selected on an ampicillin (100 μg/ml) and chloramphenicol (20 μg/ml) antibiotic LB plates. A single colony from the plate was picked and grown overnight in 50 ml LB media containing the above two antibiotics (same concentration as above). Next day morning, the 50 ml pre-inoculum was spun down (6000g for 10 min at room temperature) and the media was discarded
retaining the bacterial pellet. The bacterial pellet was gently resuspended in 10 ml LB media. This was added to 1 litre LB media containing the above antibiotics in the same concentration. The bacteria was allowed to grow at 37°C for about two hours till the culture reached the mid log phase (0.6 optical density). Induction was carried out using 0.1 mM of IPTG (isopropyl-β-D-thiogalactopyranoside). The culture was induced at 25°C for 4 hours. The cells were pelleted at 6000g for 10 minutes at 4°C (Servall RC refrigerated centrifuge) and were stored at -80°C until purification.

2.2.2.2. Protein purification

The cells were taken out from the freezer and 50 ml of pre-chilled lysis buffer (1% Triton X-100 in PBS + 1 tablet of Roche protease inhibitor tablet + 1 mM PMSF) was added. The pellet was thoroughly resuspended in the buffer. The protein was carried out at 4°C during the course of purification either by keeping the protein purification containers on ice or by using cold room for purification procedure. The bacterial lysate appeared very slimy at this stage and 0.1% of sodium lauryl sarcosine was added to the lysate. The lysate was resuspended once again. The lysate was sonicated (80T SONICLEAN bench top ultrasonic cleaners) for about 1 minute (30 % power). The cell lysis using sonication was continued with intermittent breaks till the complete lysis of the cells, which was marked by the disappearance of the viscosity of the lysate. The lysate was centrifuged at 15000g, 4°C for 30 minutes (using SS-34 rotor of the Servall RC refrigerated centrifuge). The lysate supernatant was separated from the pellet and 10 mM DTT was added to the supernatant and mixed. One ml glutathione Sepharose® 4B matrix (Amersham Biosciences) pre-equilibrated with lysis buffer was added to this lysate supernatant. The lysate along with the beads in a 50 ml falcon was incubated on a rocker for 2 hours in cold room. After the incubation the beads were packed into a 20 ml column allowing the flow through to drain. The lysate supernatant pre and post incubation samples, corresponding to 100 μl bacterial culture was saved for SDS-PAGE analysis. Three washes of 10 ml of 1% Triton X-100 in PBS was carried out followed by three washes of 10 ml of PBS. Fusion proteins bound to the beads were eluted using 5 ml elution buffer (10 mM reduced glutathione, 150 mM NaCl and 100 mM Tris pH 9.0), twice. The second elution was carried out incubating 5 ml elution buffer overnight, due to the strong affinity of proteins to the beads. The eluted protein was buffer exchanged to PBS by extensive dialysis (against 25 litre PBS for 3 days). Final concentration of the protein was adjusted to 1mg/ml after the buffer exchange and the proteins were stored at -80°C.

2.2.2.3. Immunisation

The immunisation and antibody raising was carried out using above purified fusion proteins (used as antigens) and the New Zealand white rabbits (Ethical approval no – AEC R763). The immunisation was carried out at regular intervals as mentioned in Table 5. At first, the pre-immunisation sera were collected and then the rabbits were injected with 500 μg proteins and Freund’s complete adjuvant. In subsequent booster immunisations, the rabbits were injected with 200 μg proteins and Freund’s incomplete adjuvant. Each antigen was injected into two rabbits to increase the chances of obtaining the antibody against the proteins of interest. The test bleeding of rabbits were carried out after 30 and
45 days of first immunisation. These antisera were tested for protein specific antibodies by immunoblotting. After 45 days of immunisation, the rabbits were bled once a month for fours times. As per the ethically approved protocols, the rabbits were culled after 180 days to obtain maximum antibody.

<table>
<thead>
<tr>
<th>Immunisation period</th>
<th>Immunization</th>
<th>Antigen</th>
<th>Adjuvant</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 Days</td>
<td>1st Immunisation</td>
<td>500 μg Antigen</td>
<td>1 ml Freund’s complete adjuvant</td>
</tr>
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<td>15 Days</td>
<td>1st Booster</td>
<td>200 μg Antigen</td>
<td>1 ml Freund’s Incomplete adjuvant</td>
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<td>30 Days</td>
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<td>60 Days</td>
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<td>135 Days</td>
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<td>7th Booster</td>
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<tr>
<td>180 Days</td>
<td>Maximum bleeding</td>
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</tr>
</tbody>
</table>

Table 5: The rabbit immunisation and bleeding details

2.2.2.4. Antibody purification

The antibody from the rabbit anti-sera was purified by means of affinity purification. The recombinant antigens immobilised on GST Sepharose beads were used as probes to extract the proteins’ specific antibody from the antisera. Recombinant antigens were immobilised on the GST Sepharose beads by cross-linking the two using dimethyl pimelimidate-HCl (DMP) ([394]. The recombinant proteins were purified (previously explained) and they were left on beads in the purification columns without eluting from the beads. Along with these proteins, GST proteins was also over-expressed and purified
following the previously explained protocol. The GST and GST-fusion proteins bound to the Sepharose beads were washed twice using 0.2 M borate-NaOH, pH 8.6 buffers. The DMP solution (2 ml 0.2 M triethanolamine buffered to pH 8.3 and 15.5 mg of dimethyl pimelimidate-HCl) was added to the drained resin. The beads were incubated in DMP solution with intermittent mixing for 30 minutes at room temperature to allow cross-linking. The DMP solution was drained and in the following step, the beads with 10 ml 0.2 M ethanolamine-HCl, pH 8.2 for 60 minutes terminated the cross-linking. The resin was washed twice with 2 ml of 0.1 M glycine, pH 2.5 buffer to remove non-covalently linked proteins. The beads were washed with PBS buffer and then stored at 4°C until the next use. To confirm the covalent linking, the samples before and after the linking was analysed on SDS-PAGE.

Anti-sera containing anti-GST and anti-GLT1D1, anti-GLT1D1-2 were passed through the above GST and GST-GLT1D1, GST-GLT1D1-2 immobilised columns. In the first instance, 1 ml anti-sera was brought to 1X PBS and then it was centrifuged at 12000g for 15 min at 4°C. The supernatant was incubated with the GST immobilised beads for four hours at 4°C. The antisera from GST beads were checked for the presence of anti-GST antibody at this stage by immunoblotting. This process of incubation was continued until the anti-GST antibody was cleared from the antisera. The antisera free of anti-GST antibody were incubated with GST-GLT1D1 or GST-GLT1D1-2 immobilised beads for 4 hours. The beads were washed with 10 ml PBS twice and the bound antibody was eluted in 2 ml elution buffer (0.1 M glycine, pH 2.5). The eluate was collected 200 μl 100mM Tris pH 8.0 to neutralise the acidic elution buffer. The antibody was subsequently dialysed against 10 litre PBS for two days. The antibody was concentrated to 1 mg/ml concentration and stored at -80°C for long-term usage. 1:10,000 dilution of this stock of the antibody was used in the following GLT1D1 immunoblotting experiments.

2.2.2.5. Immunoblotting

The immunoblot experiments involving cell-lines was carried out after lysing the cells and analysing the protein concentration. The cells grown in a 6 well plates were briefly washed with 1X PBS and 150 μl lysis buffer (10 mM Tris pH 7.2, 150 mM NaCl, 1% SDS, 0.1% sodium deoxycholate, 0.1% Triton X-100, 0.5 mM EDTA and one complete mini-tab protease inhibitor for 50 ml lysis buffer) was added to each well. The cells were lysed in the lysis buffer by slow retropipetting. The lysate was spun at 14000g for 20 minutes at 4°C to separate lysate and the cell debris. The lysate supernatant was separated and the protein concentration was determined as described in section on Protein quantification section.

After normalising all the samples for protein concentration, the samples were taken to immunoblotting analysis. A total of 50 μg of protein was loaded into each lanes of 12% SDS-PAGE gel and proteins were separated at 120 V for 90 minutes using Bio-Rad mini gel apparatus. Separated proteins were transferred onto the PVDF membrane using the wet-transfer method at 100V for 90 minutes. The PVDF membrane was blocked in 5% fat free milk solution (casein dissolved in 0.1% PBS Tween-20) for 1 hour at room temperature. The membrane was probed with the primary antibody appropriately
diluted in 5% milk solution over night. Non-specifically bound primary antibody was washed using 50 ml 0.1% PBS Tween-20 (PBST) for 5 minutes and three times on a rocker. After washing the membrane, it was probed with appropriate secondary antibody that was diluted in 5% milk solution for 1 hour at room temperature. Horseradish peroxidase substrate that emits luminescence on conversion to product was added on to the membrane and incubated for 5 minutes. Luminescence emitting from the protein bands were captured on the X-ray film in the dark room. The membranes were stripped using 100 mM glycine buffer pH 2.5 before reprobing and detecting other proteins on the same membrane.

2.2.2.6. Protein quantification

The proteins concentrations were measured using DC protein assay kit (Bio-Rad Laboratories, CA, USA). The working reagent A was prepared by adding 20 μl of reagent S to one ml of reagent A. BSA with the concentration of 1 mg/ml was diluted with the lysis buffer (previously described) to a series of standards containing from 0 to 5 mg/ml protein. Unknown protein sample (20 μl) was also diluted five or ten times with lysis buffer. Standards and test samples (5 μl) were transferred into a 96-well plate. Reagent A+S (25 μl) was added into each well, followed by 200 μl reagent B. The 96-well plate was incubated for 15 min with gentle agitation in dark at room temperature. Absorbance measures were read at 750 nm using Synergy2 multi-mode micro plate reader. Based on the absorbance measures of standards, the standard curve was plotted and the unknown concentration of the proteins was calculated.

2.2.3. Cell culture

2.2.3.1. Human cell lines

The human cell lines utilised in this body of research, their culturing conditions and their origin are outlined in Table 6.

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Description</th>
<th>Culture Conditions</th>
<th>Obtained from</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCF-7</td>
<td>Breast cancer cell line from a pleural effusion of an infiltrating ductal carcinoma (Engel and Young 1978). The cell line is well differentiated, epithelioid, estrogen receptor positive and non-invasive. Isolated by CM McGrath. Breast cancer cell line derived from a pleural effusion of an infiltrating ductal carcinoma. The cell line is well differentiated, epithelial, ER positive and non-invasive.</td>
<td>Culture in RPMI 1640 medium (Invitrogen CAT #: 31800-105) supplemented with 10% heat in-activated fetal bovine serum, 2 g/litre NaHCO₃, 100IU/ml penicillin, 100μg/ml streptomycin (Invitrogen CAT #: 15140-122) and 2mM L-Glutamine (Invitrogen CAT #: 35050-061).</td>
<td>ATCC</td>
</tr>
<tr>
<td>BT-549</td>
<td>The BT-549 line was isolated in 1978 by W.G. Coutinho and E.Y. Lasfargues. Source tissue consisted of a papillary, invasive ductal tumor which had metastasized to 3 of 7 regional lymph nodes. The established population was polymorphic with epithelial like components and multinucleated giant cells. A mucin-like material was secreted into the medium. The cell line is ER negative and highly invasive.</td>
<td></td>
<td>ATCC</td>
</tr>
</tbody>
</table>

Table 6 : List of wild-type cell lines

48
Other cell lines that were also used in the course of the study include AN-3, ED-27, RL-95, HESC, ECC-1, PC-3, DU-145, BeWo, BT-474, BT-549, MCF-10a, MDA-MB-231, T47D, ZR-751, A-549, H-460, H-2199, H-1975, AGS, Keto-III and MKN-45. All cell lines were cultured under the supplier's (ATCC) culture conditions.

2.2.3.2. Passaging human cell lines

The cells were grown in 75 cm² culture flasks (T75) with approximately 15 ml of ATCC recommended culture medium and were incubated at 37°C in a 5% CO₂ incubator. The passaging of the cell lines was done once the cells reached 70-80% confluence. The growth media was removed from the flask and the cells were gently washed twice with 1X PBS. Three ml 1X trypsin (i.e. trypsin/EDTA) was added to the side of the flask and slowly rolled over the cell surface. The cells were incubated at 37°C for 3-5 minutes. After the cells got detached, 10 ml of full growth media containing 10% sera was added to the flask. The cells were then spun at 350g for 5 minutes and the supernatant was discarded. Fresh 20 ml full growth media was added to the cells and the cells were resuspended by retropipetting to obtain single cells. 3.5 ml to 5 ml of this cell suspension was transferred into new flasks containing 15 ml growth media.

For long-term storage of the cells, approximately, 1.5 million cells were frozen in 1.8 ml freezing vials in liquid nitrogen. The cells were trypsinised as described above and the cell pellet free of trypsin were resuspended in 40% low serum growth media, 50% heat inactivated foetal bovine serum (FBS) and 10% DMSO. The cryo mix was aliquoted into 1.8 ml NUNC cryovials (Nalgene, Rochester, NY, USA). These vials were placed into isopropanol containing freezing chamber (Nalgene, Rochester, NY, USA) that ensured gradual freezing of the cells when they were placed in -80°C freezer. The vials were left in the freezer for eight hours. Finally, the frozen cells were transferred and stored in the vapour phase of the liquid nitrogen.

The cells were revived from liquid nitrogen by rapidly thawing them in 37°C. The cell vials from liquid nitrogen were brought out on dry ice and they were thawed rapidly in 37°C water bath. The cells were immediately transferred into falcon tube containing 10 ml of pre-warmed growth media. The cells were spun at 350g for 5 minutes. Supernatant containing DMSO were removed and the cells were resuspended in 8 ml growth media for transferring into a T25 flask and incubated at 37°C in a 5% CO₂ incubator.

2.2.4. Cell biology methods

2.2.4.1. Generating stable cell lines

The stable cell lines were generated using SAINT-MIX (Synvolux Therapeutics B.V., Groningen, The Netherlands). The plasmids (pIRESneo3 constructs) were linearized using AhdI restriction enzyme and 10 μg linearized plasmid DNA was used for generating stable cells. Ten μg DNA was diluted and mixed in HBS component of SAINT-MIX transfection reagent and 100 μl of SAINT-MIX reagent was added to the HBS/DNA mix. The transfection mix was incubated for 15 minutes at room temperature.
In the meantime, the growth media from the parental cell line grown at 70-80% confluence in T75 flask was removed. The cells were gently washed using serum and antibiotic free growth media. After the incubation period the transfection mix was slowly added on top of the cells. Ten ml serum and antibiotic free growth media was added to the flask. The cells were returned to the CO₂ incubator. After four hours of incubation the serum and antibiotic free media was replaced with full growth media. The selection of the cells with G418 antibiotic with desired antibiotic concentration (Table 7) was carried out after 48 hours of transfection. The cells were selected for 3 weeks in full serum media containing appropriate concentration of G418, until single visible transfectected cell colonies were visible. Individual clones were pooled and further propagated for making freezing stocks and experimental purpose.

<table>
<thead>
<tr>
<th>Cell Line Designation</th>
<th>Parent Cell Line</th>
<th>Transfected Construct</th>
<th>Antibiotic concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCF7-Vec</td>
<td>MCF-7</td>
<td>pRF5neo3</td>
<td>800µg/ml Geneticin (G418)</td>
</tr>
<tr>
<td>MCF7-GLT1D1</td>
<td>MCF-7</td>
<td>pRF5neo3-GLT1D1</td>
<td>700µg/ml Geneticin (G418)</td>
</tr>
<tr>
<td>BT549-Vec</td>
<td>BT549</td>
<td>pRF5neo3</td>
<td>700µg/ml Geneticin (G418)</td>
</tr>
<tr>
<td>BT549-GLT1D1</td>
<td>BT549</td>
<td>pRF5neo3-GLT1D1</td>
<td>700µg/ml Geneticin (G418)</td>
</tr>
<tr>
<td>PC3-Vec</td>
<td>PC3</td>
<td>pRF5neo3</td>
<td>400µg/ml Geneticin (G418)</td>
</tr>
<tr>
<td>PC3-GLT1D1</td>
<td>PC3</td>
<td>pRF5neo3-GLT1D1</td>
<td>400µg/ml Geneticin (G418)</td>
</tr>
<tr>
<td>DU145-Vec</td>
<td>DU145</td>
<td>pRF5neo3</td>
<td>400µg/ml Geneticin (G418)</td>
</tr>
<tr>
<td>DU145-GLT1D1</td>
<td>DU145</td>
<td>pRF5neo3-GLT1D1</td>
<td>400µg/ml Geneticin (G418)</td>
</tr>
</tbody>
</table>

Table 7: List of stable cell lines

2.2.4.2. Transient transfection

The transient transfections were carried out using Fugene HD (Roche, diagnostics) reagent. The cells were seeded into the culture plates according to the manufacturer's recommendation. In a 6 well (9.4 cm²) plate 350,000 healthy cells/well were seeded and incubated with growth medium overnight before transfection. One µg plasmid DNA was diluted in 100 µl antibiotic and low serum growth media and 3 µl Fugene HD transfection reagent was added to this and mixed. The transfection mix was incubated for 15 minutes and added on top of the above cells in 6 well plates. The cells were left with the transfection reagent for up to 24 to 72 hours depending on the nature of the experiments.

2.2.4.3. Cytochemistry

For the cytochemistry studies, the cells were grown in 4 well culture slides (BD Biosciences). Each chamber received 50,000 cells and was grown overnight in the incubator. The transient transfection with the desired constructs was carried out as described in the Transient transfection section. After the time point, the cells were gently washed with 1 ml PBS and fixed using 4% paraformaldehyde (prepared in PBS) for fifteen minutes at room temperature. The cells were permeabilised and blocked by incubating in 2% BBX (0.1 % Triton X-100, 2% BSA, 150mM NaCl, prepared in PBS) for 1 hour at room temperature. The cells were gently washed once with 2ml PBS and the nuclei were labelled using Hoescht 33258 (1µg/ml) in PBS. The cells were gently washed twice with 2 ml PBS to get rid of excess Hoescht dye. The cells were mounted using the vectashield mounting media and examined under a Carl Zeiss (Jena, Germany) Axioplan microscope equipped with epifluorescence optics microscope and a Bio-Rad MRC1024 confocal laser system.
2.2.4.4. MTT assay for cell viability

The cell viability was measured using 3-[4,5-dimethyl-thiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) method. The cells were transiently transfected using the control or test plasmids as described in the above section. After trypsinising the cells and 5,000 cells per well were seeded into 96 well plates either with 200 μl full or low serum growth media. At each time point (0 to 7 days) 20 μl MTT (0.2 μm filtered, 5 mg/ml MTT in PBS) was added to each well. After 4 hours incubation at 37°C, 100 μl of acidified 10% SDS solution was added to each well to induce cell lysis and solubilise formazan crystals. The cell viability was measured at 590nm (minus background at 695nm) using a micro-plate reader (Synergy2 multi mode micro-plate reader).

2.2.4.5. Apoptosis assay

Cells were grown and transiently transfected with control and test plasmid DNA as mentioned above. The cells were transfected for 24 hours and then the media was changed to low serum media. The cells were incubated with low serum media for either a period of 24, 48 or 72 hours. An additional well grown in similar conditions (without transfection) was treated with 5 mM hydrogen peroxide for 2 hours and used as positive control cells. The cells were then trypsinised and harvested along with the culture media. The cells were centrifuged at 350g for 5 minutes to form a pellet. The growth media was carefully aspirated and the cell pellet was slowly resuspended in 100 μl of Annexin V assay buffer (containing 20 μl of Annexin V-fluos labelling reagent and 20 μl of propidium iodide) as per the manufactures protocol. Using Neuber's haemocytometer, the cells were counted under the microscope. A 10 μl sample from the above Annexin V and PI treated cell suspension was slowly introduced into the haemocytometer. The cells that fell into the four large corner squares of the haemocytometer were counted. The average number of cells in each corner multiplied by 10 represented the number of cells per one μl in the cell suspension. The total number of cells was counted under phase contrast and that of the apoptotic cells under fluorescence microscope using blue (for detecting Annexin-V) and green (for detecting PI) filters. The total cell and apoptotic cell counts from each sample was carried out for six times. The percentage of apoptotic cells was established from these counts and the graphs were plotted following the method described in the Statistical analysis section.

The apoptosis inhibition studies were carried out using the same method in presence or absence of the general caspase inhibitor Z-VAD-FMK. The inhibitor was dissolved in 100% DMSO. During control and test cells treatment, the final concentration of DMSO was limited to 0.2%. After 24 hours of transfection the control cells were treated with the vehicle and the test cells were treated with 20 μM Z-VAD-FMK in full growth media for one hour. After one hour, the growth medium of both control and test cells was replaced with low serum media containing vehicle and 20 μM Z-VAD-FMK, respectively. The cells were incubated until they were assessed by apoptosis assay. The effect of caspase inhibitor in BT-549 cells was assessed after 48 hours in low serum media and that in MCF-7 cells was assessed after 72 hours in low serum media.
2.2.4.6. Autophagy assay

To assess the induction of autophagy, the LC3 immunoblotting was carried out following the Immunoblotting protocol. However, the composition of the lysis buffer differed in LC3 immunoblotting. The lysis buffer for LC3 immunoblotting comprised 25 mM Tris pH 8.0, 10% SDS and protease inhibitor (one complete mini-tab protease inhibitor for 50ml lysis buffer). Cells were transiently transfected with the control and GLT1D1 constructs as described earlier. The cells were analysed for LC3 processing by immunoblotting at 24 to 72 hours time intervals depending on the experiment. The cells grown in serum free media served as positive control.

The pDsRed-LC3B plasmid along with pIRESneo3 or pIRES-GLT1D1 plasmids were transiently transfected into the cells and the punctate structures were counted under florescent microscope in LC3 fluorescence assay. The transient transfections were carried out as described in the Transient transfection section. After 72 hours after transient transfection, the cells were fixed and processed for microscopic examination as described in the Cytochemistry section. The cellular cytoplasmic regions were examined for punctate structures. Number of punctae formed in a cell due to basal autophagic activity was determined by counting and averaging number of punctae in 200 control cells in random areas of assay plate. While, an MCF-7 cell having more than 5 punctae was considered as autophagic cell, a BT-549 cell having more than 20 punctae was considered as autophagic cell. 400 cells in random fields of the control and test assay plates were examined for induction of autophagy. The graphs were plotted following the method described in the Statistical analysis section.

The ultrastructure of the autophagic cell cytoplasm was visualised using electron microscopy technique. The MCF-7 cells were transiently transfected with control or GLT1D1 plasmids. After 72 hours of transfection, the cells were trypsinised and fixed in 1ml, 2.5% glutaraldehyde for 3 hours at room temperature. The cells are washed with 1 ml PBS for three times for 10 minutes. Then the cells were stained with 1% osmium tetroxide for one hour. The cells were dehydrated in gradient levels of ethanol starting from 30%, 50%, 70%, 90% to 100% leaving in each gradient for ten minutes followed by 100% acetone treatment for two times. Dehydrated cells were then infiltrated in type 812-epoxy resin and embedded in moulds and cured at 60°C for 48 hours. After curing the ultrathin sectioning (approximately 70 nm thick) was carried-out and the sections were placed on copper 400 mesh grids. The sections were then stained with uranyl acetate and lead citrate. The images of cytoplasmic ultrastructure were captured using FEI/Philips Tecnai CM12 transmission electron microscope.

Autophagic flux was measured using the lysosomal inhibitors Pepstatin-A and E64d. 10 µg/ml of each inhibitor was added into the growth media. The control cells were treated with the same concentration of vehicle as the testing reactions. The autophagic flux was analysed for LC3 processing by LC3 immunoblotting and LC3 fluorescence assay. These two assays were carried-out as previously described.

Autophagy inhibition study was carried out using the 3-Methyladenine (3-MA) inhibitor. The cells were treated with or without 10mM 3-MA before two hours of transfection. After 72 hours, the cells were
analysed for LC3 processing by LC3 immunoblotting and LC3 fluorescence assay as mentioned in the previous section.

2.2.4.7. Luciferase assay

Cells were transiently transfected with 0.5μg of plasmid containing gene promoter for BCL2 and 0.2 μg of β-galactosidase reporter vector along with the control plasmid or GLT1D1 plasmid, in triplicate. The Luciferase reporter assay was performed using Luciferase Assay System (Promega Co., Madison, WI, USA) after 48-72 hours of transfection. The cells were gently washed with 1 ml PBS and 200 μl of 1x reporter lysis buffer was added to each well of six-well plates and plates were stored at -80°C for two hours. Cells were scraped from wells after two hours and lysed by retropipetting and cell lysate were centrifuged at 12000g, 4°C for ten minute. 20 μl cell lysate supernatant from each sample was aliquoted into a luciferase 96-well microtiter plate (Greiner Bio-One, Frickenhausen, Germany). 100 μl luciferase assay reagent was dispensed into each well and read for 10 seconds with a two second measurement delay using Victor2TM1420 Multilabel Counter (Wallac–PerkinElmer Life Sciences, Turku, Finland).

The luciferase activity reading was recorded and normalized to protein quantity and transfection efficiency determined by β-Galactosidase reporter assay. β-Galactosidase enzyme assay was carried out in parallel to the luciferase reporter assay by employing a β- galactosidase enzyme assay system (Promega Co., Madison, WI, USA). Cell lysate (40 μl) from each sample was mixed with equal volumes of assay 2x buffer in a 96-well plate. The plate was covered and incubated at 37°C for 30 minutes. Finally, the absorbance of the samples at 420nm was measured on a micro-plate reader (Synergy2 multi mode micro-plate reader).

2.2.4.8. GLT1D1 localisation

Mitochondrial co-localisation examination was carried out by two different methods. In the first method the GLT1D1 localisation was observed with respect to a known mitochondrial protein COX8. In this method, 0.5 μg of pDsRed-COX8 was transfected along with pEGFP or pEGFP-GLT1D1 for 48 hours. After the time point, the cells were processed for microscopic examination as described in the Cytochemistry section.

In the second method, the cells were transiently transfected with pEGFP or pEGFP-GLT1D1 plasmids. After 48 hours the cellular mitochondria were labelled using Mitotracker-red CMXRos (Invitrogen, USA). The labelling was carried out according to the manufacturer’s protocol with a slight modification. The cells were incubated with 25 nM Mitotracker red dye in PBS at 37°C for 10 minutes instead of low serum growth media as described by the manufacturer. The cells were then taken out of the incubator and processed for microscopic examination as described in the Cytochemistry section above.

Cellular sub-fractionation method was used to determine the GLT1D1 localisation. In this, experiment, the cells were transected with pIRES-GLT1D1 plasmid for 48 hours. After 48 hours the cells were
trypsinised and resuspended in 200 μl homogenisation buffer (10 mM HEPES pH 7.5, 200 mM mannitol, 70 mM sucrose and 1 mM EGTA). Cells were incubated for 30 minutes in homogenization buffer keeping the container on ice. After the incubation the cells were transferred and homogenized in a glass homogenizer with 30 strokes. Low speed (600g) centrifugation at 4°C for 10 minutes separated the nucleus and whole cells from the cytoplasmic fraction containing mitochondria. The nuclear fraction was homogenized again to effectively lyse the unlysed cells. The homogenate samples were examined under the microscope intermittently and the strokes were contained to prevent over lysis of the cells. The homogenate was centrifuged at 4°C for 10 minutes and the separated nuclear pellet was lysed in 50 μl lysis buffer (25 mM Tris pH8.0, 10% SDS and 1 complete mini-protease inhibitor tablet). The cytoplasmic fraction containing mitochondria was subjected to higher speed centrifugation at 11000g at 4°C for 30 minutes. The supernatant and the pellet were separated and the mitochondrial fraction was washed with 1 ml homogenisation buffer twice to avoid cytoplasmic fraction contamination. The mitochondrial pellet was resuspended in 50 μl lysis buffer. The protein concentration of all the fractions was determined using DC protein assay (Bio-Rad Laboratories, CA, USA) as described in the Protein quantification section. A total 50 μg protein was loaded on SDS-PAGE and further analysed by immunoblotting.

2.2.4.9. Statistical analysis

The statistics software program Sigma Stat 3.1 was used in conjunction with Microsoft Excel for statistical calculations. The graphical presentations were generated using Microsoft Excel. All data are expressed as means ± SE of triplicate determinants. All experiments were performed at least three times. Data were analysed using an unpaired two-tailed t test or analysis of variance (ANOVA). * corresponds to P values less than 0.05. ** corresponds to p values less than 0.01. *** corresponds to p values less than 0.001.
CHAPTER 3. RESULTS

3.1. INTRODUCTION

Based on bioinformatics prediction tools and the peptide submissions in the mass-spectrometric database PRIDE, GLT1D1 was deemed to be translated into the protein. A specific antibody to the protein of interest is essential to study its function. The recombinant GLT1D1 proteins were therefore produced to raise the antibody against them. In the first section of my results chapter, I present the data obtained from GLT1D1 recombinant antigen and antibody purification.

I present the GLT1D1 protein expression data in different human tissues in the second section of results chapter.

The GLT1D1 gene function study was performed by gain-of-function approach using a forced-expression model system in mammary carcinoma cells. The results obtained by the forced expression of GLT1D1 in mammary carcinoma cells are presented in the third section of results chapter.

After establishing the gene function, the possibility of GLT1D1 localisation to mitochondria was explored. Data obtained from these experiments are presented in the fourth section of my results chapter.

The two isoforms Q96MS3-1 (GLT1D1), Q96MS3-2 (GLT1D1-2) of the GLT1D1 gene were considered for recombinant protein production. GLT1D1 and GLT1D1-2 were cloned into the bacterial expression vector pGEX-4T1 as glutathione-S-transferase (GST) fusions and resulted constructs were designated as pGEX4T1-GLT1D1 and pGEX4T1-GLT1D1-2, respectively. To achieve gene expression in mammalian cells, the gene was cloned into the mammalian expression vector pIRESneo3. The resulting clones from GLT1D1 and GLT1D1-2 isoforms were termed as pIRES-GLT1D1 and pIRES-GLT1D1-2, respectively.

3.2. Expression and purification of GST-GLT1D1 and GST-GLT1D1-2 antigens

At first, recombinant GLT1D1 was produced to raise antibody against the protein. Bacterial expression system was used to produce the protein. The GLT1D1 and GLT1D1-2 were expressed as GST fusion proteins in bacteria. The fusion proteins were designated GST-GLT1D1 and GST-GLT1D1-2 and subsequently the fusion proteins were purified using GST-affinity chromatography. Figure 14 represents the results of recombinant GLT1D1 and GLT1D1-2 antigen expression and purification. The Figure 14A reveals the expression and solubility characteristics and the Figure 14B represents the purity and integrity of the recombinant antigens.
Figure 14: Recombinant GST-GLT1D1 and GST-GLT1D1-2 expression and purification.

GST-GLT1D1 and GST-GLT1D1-2 were expressed in BL21DE3pLysS bacterial strain, using 50 μM IPTG for 3 hours at 25°C. The samples from pre and post-induction were collected and the post-induction sample was separated into soluble and insoluble fractions. These samples were analysed on a 10% SDS-PAGE gel (A). The soluble fractions of induced GST-GLT1D1 and GST-GLT1D1-2 samples were applied onto GST beads and affinity purified. Purified proteins were eluted from the beads and analysed on 12% SDS-PAGE gel (B).

The recombinant GLT1D1 and GLT1D1-2 proteins expressed in BL21DE3 pLysS were insoluble and found as inclusion bodies as seen in the lane 5 and lane 9 of Figure 14A. To improve the solubility of the protein, the inducer isopropyl-β-D-thio-galactopyranoside (IPTG) concentration, induction time and temperature was optimised. It was found that 50 μM IPTG induction for 3 hours at 25°C in the log phase of bacterial growth (0.6 optical density at 600nm) yielded soluble protein. The soluble protein obtained from the above optimisation yielded recombinant antigens in the range of 1.5mg~2mg/10g wet weight of bacterial pellet. The proteins were purified to near homogeneity by glutathione affinity chromatography (Figure 14B). The purity of the GST-GLT1D1 and GST-GLT1D1-2 antigens were assessed on a reducing SDS-PAGE gel and it was found to be 95% and 92%, respectively by densitometry. Observed discrepancy in the apparent molecular weight of GST-GLT1D1-2 in Figure 14A and B is likely due to the difference in percentage of the gel. The antigens were found to be stable without any degradation after purification.
3.3. Antibody production and purification

To raise antibodies against the GLT1D1 and GLT1D1-2 proteins, the purified fusion antigens were injected into rabbits for immunisation. An immunisation schedule, as mentioned in Table 5, was followed. Each antigen was injected into two rabbits to increase the chances of obtaining an antibody against the proteins of interest. The rabbits injected with GLT1D1 and GLT1D1-2 antigens were numbered as 265 or 266 and 242 or 245, respectively. The antibodies against the proteins of interest were found to be present in the antiserum of the rabbits after 45 days of the first immunisation (Figure 4).

Figure 15 shows the detection of GLT1D1 protein using antisera obtained from different rabbits after 180 days of immunisation. The rabbits were bled every month after 45 days of initial immunisation until 180 days to obtain maximum yield of antibody, although the antibody was detected at low levels after 45 days of immunisation. GLT1D1 was force-expressed in MCF-7 cells and it was used along with its vector control for testing the quality of the antisera. Myc-tagged GLT1D1 (GLT1D1-M1) force-expressed in MCF-7 was detected using anti-myc antibody and it served as a positive control in the experiment. The antisera from rabbits 265 and 245 detected the forced expression of GLT1D1 very well, whereas the antisera from rabbits 266 and 242 poorly detected GLT1D1. As observed in the immunoblot image (Figure 15), antisera from rabbit 245 detected single and specific GLT1D1 protein band on the blot at an expected 38.5 kDa matching the positive control GLT1D1-M1 in size. Antisera from rabbit 265 detected GLT1D1 along with other non-specific proteins. Conversely, antisera from rabbit 266 completely failed to detect GLT1D1. Even the affinity purified antibody from rabbit 245 and 265 showed a similar specificity trend (data not shown). The antibody from rabbit 245 was therefore used in the later studies due to its higher specificity to GLT1D1.
The anti-sera collected from rabbits after 180 days of immunisation were screened for anti-GLT1D1 antibodies generation. For testing, GLT1D1 was force-expressed in the MCF-7 and used in the experiment along with the vector control. MCF-7 cells were transiently transfected with empty vector plasmid (MCF7-Vec) or plasmids expressing GLT1D1 (MCF7-GLT1D1) or GLT1D1-M1 (MCF7-GLT1D1-M1). After 48 hours after transfection, cells were harvested and processed for immunoblotting. A total of 50 μg protein from GLT1D1 and vector samples was loaded in two adjacent lanes on SDS-PAGE in four repeats followed by a lane with GLT1D1-M1 sample. After the blocking step, the membrane was cut to separate four repeats and the single GLT1D1-M1 lane. Each of the four membranes was probed with 25 μg/ml of anti-sera from rabbits and the GLT1D1-M1 lane was probed with anti-myc antibody that served as a positive control.

To purify the anti-GLT1D1 and anti-GLT1D1-2 antibodies, an affinity purification approach was followed that involved two steps. The antigen used for antibody production was a GST fusion protein, therefore the antisera contained antibody against GST. In the first step, this anti-GST antibody was cleared from the anti-sera and in the second step; isolation of specific antibodies raised against the proteins of interest was achieved. Anti-GST antibody was extracted from the antisera by applying the anti-sera onto GST immobilised affinity beads. The unbound antisera cleared of anti-GST antibody was then applied onto the respective GST-GLT1D1 and GST-GLT1D1-2 immobilised affinity beads to isolate anti-GLT1D1 and anti-GLT1D1-2 specific antibodies, respectively [394].

Immobilisation of the recombinant proteins GST, GST-GLT1D1 and GST-GLT1D1-2 onto glutathione (GSH) Sepharose beads involved two steps. In first step of immobilisation, the recombinant proteins were purified to near homogeneity by affinity chromatography using GSH Sepharose beads. After the final step of purification, the affinity-purified recombinant proteins were left on the beads without elution. Subsequently in the second step, the proteins were covalently linked to the GSH Sepharose beads. Before covalent linking a small portion of the beads were tested on SDS-PAGE to assess the purity and integrity of the proteins (Figure 16). As seen in lane numbers 1, 5 and 8 of Figure 16, the recombinant proteins were purified to near homogeneity. Protein quantification revealed 15 mg of purified GST protein, 11 mg of purified GST-GLT1D1 and 10 mg of GST-GLT1D1-2 per 1 ml of GSH sepharose bead. A negligible amount of degradation of GLT1D1 and GLT1D1-2 proteins was seen in the lanes 5 and 6, respectively, which did not cause any interference in the downstream processing.

![Figure 15: Qualitative screening of the anti-sera obtained from rabbits.](image.png)
In the second step of immobilisation, above purified recombinant proteins were chemically cross-linked to the GSH Sepharose beads. The covalent linking was verified by running a small portion of the beads on SDS-PAGE before and after cross-linking. Figure 17 shows the confirmation of covalent linking of recombinant proteins to the GSH-Sepharose beads. Samples before cross-linking, loaded in the lanes 3, 7 and 10 of the proteins GST, GST-GLT1D1-2 and GST-GLT1D1, respectively, revealed a conspicuous protein band around the expected molecular weight. The samples after cross-linking, loaded in the lanes 1, 5 and 8 of the proteins GST, GST-GLT1D1-2 and GST-GLT1D1 remained in the wells of the gel and confirmed cross-linking of the proteins to the beads. When the protein samples are processed for SDS-PAGE analysis, they were boiled with 1X SDS-PAGE sample buffer. In this process, the proteins attached to the GSH Sepharose beads by affinity interactions leached into the sample buffer. In contrast, the proteins cross-linked to the GSH Sepharose beads by covalent interaction remained on the beads without leaching into the sample buffer, hence, appeared in the wells of the gel along with the beads. This result indicated effective cross-linking of the proteins to the GSH Sepharose beads, making them suitable for the antibody purification. The GST, GST-GLT1D1 and GST-GLT1D1-2 proteins cross-linked to GSH Sepharose beads were termed GSH-GST, GSH-GLT1D1 and GSH-GLT1D1-2, respectively.
3.3.1. Purification of anti-GLT1D1 and anti-GLT1D1-2 antibody

In the first step of antibody purification, the antibody raised against GST was separated from the antisera using the above cross-linked GSH-GST beads. One ml GSH-GST bead was used to separate anti-GST antibody in 10 ml of rabbit antisera. Both the anti-GLT1D1 and anti-GLT1D1-2 antisera were passed through GSH-GST beads twice to clear anti-GST antibody.

In the second step, the antibody from anti-GLT1D1 and anti-GLT1D1-2 antisera was extracted by affinity purifying them on GSH-GLT1D1 and GSH-GLT1D1-2 beads, respectively. The anti-GST cleared antisera from the previous step, was passed through GSH-GLT1D1 and GSH-GLT1D1-2 beads. Two rounds of purification similar to the previous step were performed to obtain the maximum yield of antibody.

The anti-GLT1D1-2 antibody from rabbit 245 showed higher specificity than the anti-GLT1D1 antibody from rabbit 265 (Figure 15). Additionally, anti-GLT1D1-2 antibody specifically recognised both GLT1D1 and GLT1D1-2 at expected sizes (Figure 18). Therefore, the affinity-purified antibody from rabbit 245 was used further in the study and was termed anti-GLT1D1 antibody.
Affinity purified anti-GLT1D1 antibody from rabbit 245 was tested for specificity. The MCF-7 cells were transiently transfected with plasmids expressing myc tagged human growth hormone (MCF7-hGH), or GLT1D1 (MCF7-GLT1D1-M1), or GLT1D1-2 (MCF7-GLT1D1-2-M1) or the empty vector plasmid (MCF7-Vec). After 48 hours of transfection cells were harvested and analysed by immunoblotting. A total protein of 50 μg from of each of these hGH, GLT1D1, GLT1D1-2, and vector samples were loaded on SDS-PAGE in two repeats. After blocking step, the membrane was cut to separate two repeats. One of the membranes was probed with 1 μg/ml anti-myc antibody and the other was probed with 1 μg/ml anti-GLT1D1 antibody. The specificity of anti-GLT1D1 was examined after the affinity purification. For this purpose, myc tagged human growth hormone (hGH), GLT1D1 and GLT1D1-2 were transiently expressed in MCF-7. The myc tagged hGH was used as positive control for the anti-myc probed blot and it was used as negative control in the anti-GLT1D1 probed blot. The samples were loaded on SDS-PAGE in two sets for the immunoblotting analysis. One of them was probed with anti-myc antibody and the other was probed with anti-GLT1D1 antibody. The immunoblot results (Figure 18) revealed specific bands corresponding to GLT1D1 and GLT1D1-2 proteins around expected molecular weight of 38.5 kDa and 29 kDa, respectively, both by anti-myc and anti-GLT1D1 antibody. Anti-GLT1D1 antibody recognised GLT1D1 isoforms without any other background bands. While myc tagged hGH lane tested positive with anti-myc antibody, it tested negative with anti-GLT1D1 antibody. Hence, from Figure 18 results, it was concluded that GLT1D1 and GLT1D1-2 isoforms detected by anti-GLT1D1 antibody and anti-myc antibody are the same and anti-GLT1D1 antibody is specific to GLT1D1 and GLT1D1-2.
3.4. GLT1D1 expression in human tissues and cancer cells

Since the access to human tissues was limited, at first, the extent and different forms of GLT1D1 protein expression in human tissues was examined using the affinity purified anti-GLT1D1 antibody. A commercially available ready-to-use PVDF membrane called INSTA-blot (from Imgenex) that contained denatured and resolved protein, extracted from different human tissues was used for protein expression screening. The blot included human tissue samples from brain, heart, small intestine, kidney, liver, lung, skeletal muscle, stomach, spleen, ovary and testis. Each lane of the blot contained 20 μg total protein from the tissues. Expression level of GLT1D1 in these human tissues was determined by probing the membrane with anti-GLT1D1 antibody. Immunoblotting result presented in Figure 19 reveals GLT1D1 expression. A specific protein band around 40kDa was seen in the lanes corresponding to small intestine, kidney and liver tissues. This size matched with the predicted size of GLT1D1 isoform of the gene. No detectable expression corresponding to the other predicted isoforms namely GLT1D1-2 and GLT1D1-3 was seen on the blot. The liver tissue showed higher levels of GLT1D1 expression in comparison to kidney and small intestine. This result suggested that GLT1D1 isoform of the GLT1D1 gene is actually expressed as protein in human tissues.

Figure 19: Immunoblot showing the expression of GLT1D1 in human tissues.
Commercially available INSTA-blot was used for screening GLT1D1 expression in different human tissues. The blot was probed with 1 μg/ml anti-GLT1D1 antibody. Each lane contained 20 μg total protein from human tissues including human brain, heart, small intestine, kidney, liver, lung, skeletal muscle, stomach, spleen, ovary and testis.

The above immunoblotting experiment suggested a predominant GLT1D1 expression in human liver, small intestine and kidney. However, using the INSTA-blot, it was not possible to verify the exact size of GLT1D1 expressed in human tissues. Hence, GLT1D1 was transiently expressed in the liver cancer cell line HepG2 and this was used as positive control to verify GLT1D1 protein size expressed in human liver. Human liver sample was used in this experiment to compare the molecular weight of GLT1D1 in the other two tissues kidney and small intestine, since, it was possible to procure only the human liver sample.
The HepG2 cells were transiently transfected with plasmid expressing GLT1D1 (GLT1D1) for 48 hours. The GLT1D1 sample with 10 μg total protein (served as positive control) was loaded next to human liver lysate containing 20 μg total protein and analysed by immunoblotting using anti-GLT1D1 antibody.

The immunoblotting result (Figure 20) revealed that GLT1D1 expressed in liver exactly matched with the transiently expressed GLT1D1 in HepG2 cell line, in size. The expected molecular weight of GLT1D1 is 38.5 kDa. Both GLT1D1 expressed in liver cancer cell line and liver tissue revealed a specific protein band around this size. From the earlier experiment (Figure 19), it was clear that GLT1D1 expressed in liver, kidney and small intestine have the same molecular weight. These two results (Figure 19 and Figure 20) together confirmed that GLT1D1 is predominantly expressed in liver, small intestine and kidney.

The peptide submissions for GLT1D1 in PRIDE database suggest that GLT1D1 protein is present in sera. Therefore, human sera was also analysed by immunoblotting along with the transiently expressed GLT1D1, as positive control (Figure 21). Results of this experiment, revealed two distinct protein bands. One matching the size of transiently expressed 38.5 kDa positive control and another major band above 50 kDa band. To determine the identity of the 50 kDa protein band, samples were loaded in duplicates. One of the portions was probed with anti-GLT1D1 antibody followed by secondary antibody (Figure 21A) and the second portion was probed with anti-rabbit IgG secondary antibody (Figure 21B). The 50 KDa band detected in (Figure 21B) verified that it was a band specifically detected by the anti-rabbit IgG antibody. This immunoblot analysis also supported the previous observation that GLT1D1 is the predominant isoform expressed in human tissues. This result led us to focus on the predominant GLT1D1 isoform rather than the other predicted GLT1D1-2 isoform of the gene.
Figure 21: GLT1D1 is present in sera.
Sera from a male and a female individual were examined for the presence of GLT1D1 by immunoblotting. MCF-7 cells transfected with plasmid expressing GLT1D1 (MCF7-GLT1D1) was included in the experiment to serve as positive control. A total protein of 50 μg was used in the analysis and loaded in two sets of repeats. The samples loaded in duplicates were separated during antibody probing. The blots were probed with (A) anti-GLT1D1 antibody followed by secondary anti-rabbit antibody and (B) anti-rabbit secondary antibody to differentiate the non-specific band appearing due to non-specific secondary anti-rabbit antibody binding.

3.4.1. Human cancer cell line screening for GLT1D1 expression

Prior to the functional characterization of GLT1D1 gene, mRNA and protein expression screening of the gene in various cancer cells was carried out to assess the endogenous expression levels of the gene. For this purpose cancer cell lines of various tissue origins were selected for GLT1D1 screening.
Figure 22: Cancer cells’ screening for GLT1D1 (A) mRNA and (B) protein expression.

Total RNA and total protein from primary and immortalised breast cell line MCF10A and other cancer cell lines including MCF-7, BT-549, PC-3, AGS, Colo-320, HEK-293 and HepG2 were extracted. MCF-7 cells transfected with plasmid expressing GLT1D1 (MCF7-GLT1D1) was included in the experiment to use as positive control. (A) Using 1 μg total RNA as template, GLT1D1 was amplified in 45 cycles by reverse transcription PCR. β–ACTIN was used as loading control. (B) A total of 70 μg of protein from each cancer cell lines was analysed by immunoblotting along with 25 μg of MCF7-GLT1D1 sample. The membrane was first probed for GLT1D1 expression followed by β–ACTIN probing that served as loading control.

The range of screened cancer cells included MCF-7, BT-549 from breast cancer, PC-3 from prostate cancer, AGS from gastric cancer, Colo-320 from colorectal cancer, HEK-293 from kidney and HepG-2 from liver cancer. A non-transformed immortalized primary cell line MCF-10a from breast tissue was also included in the screening panel. As observed in the Figure 22A, screening for GLT1D1 mRNA expression using isoform specific primers revealed high levels of GLT1D1 expression in MCF-10a and MCF7 followed by BT-549, Colo-320 and HepG2. GLT1D1 was not expressed in AGS and PC-3 cancer cells. Surprisingly, these cancer cells tested negative for GLT1D1 protein expression (Figure 22B). No detectable expression was found even at higher exposure of the immunoblot.

Since the study focused on breast cancer, a panel of breast cancer cell lines MCF-7, MDA-MB 231, BT-549, BT-474, T47D, ZR-751 and MCF-10A were screened to assess GLT1D1 mRNA and protein expression. The screening results (Figure 23A) of GLT1D1 mRNA expression revealed varied expression levels of the gene between the cell lines. The MCF10a, MCF-7 and BT-474 cell lines showed high levels of mRNA expression followed by BT-549 cell line. GLT1D1 was not expressed in MDA-MB 231, T47D and ZR-751 cell lines. Similar to the cancer cells protein expression results, no detectable protein expression was to be seen in any of these breast cancer cell lines (Figure 23B).
The lack of detectable levels of protein expression suggests that there is no or undetectable levels of GLT1D1 protein expression.

**Figure 23:** GLT1D1 (A) mRNA and (B) protein expression in human breast cancer cell lines. Total RNA and total protein from primary and immortalised breast cell line MCF10A and other breast cancer cell lines including MCF-7, BT-549, BT-474, T47D and ZR-751 were extracted. MCF-7 cells transfected with plasmid expressing GLT1D1 (MCF7-GLT1D1) was included in the experiment to serve as positive control. (A) Using 1.5 μg total RNA as template, GLT1D1 was amplified in 45 cycles by reverse transcription PCR. β–ACTIN was used as loading control. (B) A total of 70 μg of protein from each cancer cell lines was analysed by immunoblotting. The membrane was first probed for GLT1D1 expression followed by β–ACTIN probing that served as loading control.

Further, due to the absence of GLT1D1 protein expression in the above cancer cells more cancer cell lines were screened for GLT1D1 protein expression. The endometrial cancer cells AN-3, ED-27, RL-95, HESC, ECC-1, BeWo, breast cancer cells BT-474, BT-549, MCF-7, MDA-MB-231, T47D, ZR-751, lung cancer cells A-549, H-460, H-2199, H-1975 and gastric cancer cells AGS, Keto-III, MKN-45 were included in the screening panel. However, similar to the previous protein expression results, all cancer cells tested negative for GLT1D1 protein expression (data not shown). These experimental results (Figure 22 and Figure 23) suggested that in the above cancer cells GLT1D1 is transcribed into mRNA but failed to translate into the protein. Alternatively, it also suggested that the protein expression was lower than the immunoblotting detection threshold.

### 3.5. Functional characterisation of GLT1D1

#### 3.5.1. The GLT1D1 cells undergo cell death during stable selection

Information on GLT1D1 function or its role in cancer is sparse. With a focus to determine the functional aspects of GLT1D1 in mammary carcinoma, establishing a cell model system stably
expressing GLT1D1 was desired. The triple negative mammary carcinoma cell line BT-549 and estrogen receptor positive cell line MCF-7 were chosen for this purpose.

Mammary carcinoma cells MCF-7 and BT-549 cells were transfected with empty vector plasmid pIRESneo3 or plasmid expressing GLT1D1 to establish cell model system for the study. Surprisingly, three weeks’ stable selection resulted in false colonies devoid of GLT1D1 expression both in MCF-7 and BT-549 cells. This was an unexpected result. Immunoblotting analysis revealed that GLT1D1 got transfected and transiently expressed as protein in the cells. However, GLT1D1 expression was not observed after the stable selection. To verify this loss of expression, the cells before and after stable selection were analysed for mRNA and protein expression.

The MCF-7 and BT-549 cells were transfected with empty vector plasmid pIRESneo3 (termed as MCF7-Vector) and (termed as BT549-Vector) or plasmid expressing pIRESneo3GLT1D1 (termed as MCF7-GLT1D1) and (termed as BT549-GLT1D1). After 72 hours of transfection, the cells were trypsinised and 200,000 cells were processed for mRNA and protein expression (termed as transient) analysis. Remaining transfected cells were selected using G418 antibiotic for three weeks. Fewer colonies were observed in the MCF7-GLT1D1 and BT549-GLT1D1 flasks as compared to their respective vector controls. The colonies were further propagated and screened for GLT1D1 mRNA and protein expression (termed as stable) analysis. Figure 24, depicts the result obtained by analysing transient and stable samples. The analysis revealed that GLT1D1 expressed as mRNA and protein after 72 hours of transfection in MCF7-GLT1D1 and BT549-GLT1D1 cells. However, the selection with G418 antibiotic for three weeks resulted in colonies without GLT1D1 forced-expression. Stable MCF7-GLT1D1 and BT549-GLT1D1 cells revealed mRNA expression levels that matched the expression levels of their respective vector controls and absence of protein expression clearly indicating lack of GLT1D1 forced-expression. This result implied that the colonies appeared after three weeks’ G418 antibiotic selection were false-positive colonies and MCF7-GLT1D1 and BT549-GLT1D1 cells were eliminated in the process.
Mammary carcinoma cells MCF-7 and BT-549 were transfected with empty vector plasmid (Vector) as control or plasmid expressing GLT1D1 (GLT1D1). After 48 hours of transfection, the cells were trypsinised and 200,000 cells were processed for mRNA and protein expression analysis and the resulted samples (transient) were stored at -80°C. Remaining of the transfected cells were selected on antibiotic for three weeks and the colonies appeared were further propagated. Thus stably selected cells were processed for mRNA and protein expression (stable) analysis. Transient and Stable samples were analysed by one step RT-PCR and immunoblotting analysis. A total of 1 μg RNA and 70 μg of protein from each samples were used for analysis. The β–ACTIN was used as loading control.

Lack of GLT1D1 protein expression in cancer cells (Figure 22 and Figure 23) and elimination of cells expressing the gene during stable selection (Figure 24) suggested a possible role for GLT1D1 in cell death. Considering these results, it was hypothesised that GLT1D1 mediates cell death.

### 3.5.2. GLT1D1 mediates cell death

To verify the hypothesis, earlier chosen mammary carcinoma cells MCF-7 and BT-549 were used as cell models. These two cell models were used in the cell death studies considering the difference in their cell death pathway. MCF-7 cells are known to carry mutation in the caspase-3 molecule that makes the cell line resistant to caspase dependent apoptotic pathway. On the other hand BT-549 readily undergoes apoptotic cell death with no known insufficiency in the apoptotic pathway. The combination of these two cell lines was therefore thought to be good model systems for determining the characteristics and types of GLT1D1 mediated cell death.

At first, the above hypothesis was examined by measuring the cell viability in GLT1D1 transfected cells. MCF-7 and BT-549 cells were transfected with empty vector plasmid (Vector) or plasmid expressing GLT1D1 (GLT1D1). After 24 hours of transfection, the cells were trypsinised and seeded...
into 96-well plates. Cell viability of these cells were determined by 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay under full (10%) serum growth conditions. Figure 25 represents seven days cell viability data of Vector or GLT1D1 transfected MCF-7 and BT-549 cells. GLT1D1 reduced 10% cell viability in MCF-7 cells. Under full (10%) serum growth conditions, GLT1D1 did not exhibit any effect on BT-549 cells' viability.

GLT1D1 reduced 10% cell viability in MCF-7 cells. Under full (10%) serum growth conditions, GLT1D1 did not exhibit any effect on BT-549 cells' viability. However, the failure to obtain stable expression of GLT1D1 in BT-549 cells (Figure 24), implicated GLT1D1 in cell death. Considering the possibility of BT549-GLT1D1 cells death under G418 selection pressure, it was hypothesised that GLT1D1 mediated cell death under stress conditions.

To analyse this hypothesis, the above cell viability assay was performed under low (0.2%) serum growth condition that worked as stress factor in the experiment. Low serum MTT assay result (Figure 26) revealed that GLT1D1 promoted cell death both in MCF-7 and BT-549 cells. Cell viability of MCF7-GLT1D1 and BT549-GLT1D1 samples were significantly reduced by 18% and 14.66 % compared with MCF7-Vector and BT549-Vector cells, respectively. Interestingly, GLT1D1 reduced additional 8% cell viability under low serum growth condition compared to full serum growth condition. This implied that GLT1D1 mediated higher levels of cell death under low serum growth condition compared to cell death under full serum growth condition.
Figure 26: Determination of the cell viability effects of GLT1D1 on MCF-7 and BT-549 under low (0.2%) serum growth condition.

The breast cancer cells MCF-7 (A) and BT-549 (B) were transiently transfected with empty vector plasmid (Vector) or plasmid expressing GLT1D1 (GLT1D1). After 24 hours of transfection, the cells were trypsinised and five thousand cells were seeded into each well of 96-well plates in triplicate. In the 96 well plates, cells were grown deprived (0.2%) of serum. Viability of the transfected cells was assessed by MTT assay from 0 to 7 days. * P<0.05, ** P<0.01.

The above results suggested that GLT1D1 promoted cell death in MCF-7 and BT-549 cells under stress conditions such as low serum growth condition. Further studies, hence, focused on understanding GLT1D1 mediated cell death type.

3.5.3. GLT1D1 promotes apoptotic cell death in BT-549 and MCF-7 cells

The Nomenclature Committee on Cell Death (NCCD), 2012 has classified cell death into two major types, namely, the programmed and non-programmed types of cell deaths and several other subtypes under these two types. To identify the type of GLT1D1 mediated cell death, at first, the most common and well-characterised type of cell death, apoptosis, was taken into account.

The Annexin-V assay was used to ascertain, if GLT1D1 caused apoptotic cell death in MCF-7 and BT-549. The assay determined both early and late apoptotic cells. Annexin-V bound to the reversed phosphotidyl serine (PS) groups on the apoptotic cell membranes in a Ca²⁺ dependent manner. Annexin-V tagged to a fluorescent molecule, fluorescein isothiocyanate, was used in the assay to enable apoptotic cell detection. The cell membrane permeability in late apoptotic cells were identified using Propidium Iodide (PI) stain.
3.5.3.1. Apoptosis assay under full serum growth condition

First, the annexin-V apoptosis assay was performed to determine if GLT1D1 mediated apoptotic cell death of MCF-7 cells under full serum growth condition. To do this, MCF-7 cells were transiently transfected with vector control plasmid (Vector) or plasmid expressing GLT1D1 (GLT1D1) under full serum growth conditions. After 24, 48 and 72 hours of transfection, the cells were processed for the annexin-V assay. The assay results revealed that there was no difference in the rate of apoptosis in MCF7-GLT1D1 cells compared with MCF7-Vector control cells (Figure 27). The positive control (500 μM H₂O₂) included in the experiment revealed 9%, 13.2% and 17.5% increase in the rate of apoptosis over 24, 48 and 72 hours, respectively.

![MCF-7 Annexin-V assay (10%)](image)

**Figure 27 : Determination of the apoptotic effects of GLT1D1 on MCF-7 cells under full serum growth condition.**

The MCF-7 cells were transiently transfected with empty vector (Vector) or plasmid expressing GLT1D1 (GLT1D1). After 24, 48 and 72 hours of transfection, the cells were assessed for apoptotic cell death using fluorescein isothiocyanate-conjugated annexin V (annexin V) and propidium iodide (PI). The annexin V positive cells were counted as early apoptotic cells (early apoptosis) and the PI and annexin V cells were counted as late apoptotic cells (late apoptosis). Cells treated with 500 μM H₂O₂ for the same number of hours as vector and GLT1D1 cells were used as positive control. **P<0.01, ***P<0.001.**
The MCF-7 cells were transiently transfected with plasmids expressing GLT1D1 or empty vector (Vector). After 48 hours of transfection cells were trypsinised and seeded into 24 well plate in triplicate and cells were incubated in full growth medium. After seven days of seeding, the cells were assessed for apoptotic cell death using fluorescein isothiocyanate-conjugated annexin V (annexin V) and propidium iodide (PI). The annexin V positive cells were counted as early apoptotic cells (early apoptosis) and the PI and annexin V cells were counted as late apoptotic cells (late apoptosis). Cells grown in growth media containing low (0.2%) serum were used as positive control. *** P<0.001.

The previous MCF-7 cell viability experiment under full serum growth condition result (Figure 25) revealed that over seven days GLT1D1 reduced 10% of MCF7-GLT1D1 cell viability compared with its vector control. This result implied GLT1D1 mediated cell death in MCF-7 cells under full serum growth condition. Annexin-V apoptosis assay was therefore performed to determine if GLT1D1 mediated apoptotic cell death of MCF-7 cells in seven days' period.

MCF-7 and BT-549 cells were transfected with empty vector plasmid (Vector) or plasmid expressing GLT1D1 (GLT1D1). After 48 hours of transfection cells were trypsinised and seeded into 24 well plates. Figure 28 shows the apoptosis assay results after 7 days of plating of the cells into 24 well plate. No difference in the rate of apoptosis was observed in MCF7-GLT1D1 cells compared with its vector control. The positive control grown under low (0.2%) serum growth condition over seven days’ period on the other hand revealed significant 33.5% increase in apoptosis compared with the vector control. This result clearly suggested that GLT1D1 mediated mode of cell death in MCF-7 under full serum growth condition is not apoptotic.

The rate of apoptosis under full serum growth condition in BT-549 cells was not determined since GLT1D1 did not have any effect on BT-549 cell viability under full serum growth condition.
3.5.3.2. GLT1D1 mediated apoptotic cell death in MCF-7 and BT-549 under low serum growth condition

The cell viability assay result under low serum condition (Figure 26) suggested GLT1D1 mediated cell death both in MCF-7 and BT-549 cells. Annexin-V assay was used to examine if GLT1D1 mediated apoptotic cell death in these mammary carcinoma cells under low serum growth condition.

Figure 29, shows GLT1D1 mediated MCF-7 and BT-549 cell apoptosis assay results under low serum conditions. Under low serum growth conditions, the rate of apoptosis in MCF7-GLT1D1 and BT549-GLT1D1 cells was significantly higher than their respective vector controls. There was 31.78%, 25% and 29.39% increase in early, late and total apoptosis of MCF7-GLT1D1 cells compared with that of MCF7-Vector cells, respectively, over 24 hours. After 48 hours, there was 25.84%, 22.22% and 24.15% increase in early, late and total apoptosis of MCF7-GLT1D1 cells compared with that of MCF7-Vector cells, respectively. Similarly, there was 29.39%, 33.25% and 30.98% increase in early, late and total apoptosis of BT549-GLT1D1 cells compared with that of BT549-Vector cells, respectively, over 24 hours. After 48 hours, there was 21.31%, 32.22% and 28.23% increase in early, late and total apoptosis of BT549-GLT1D1 cells compared with that of BT549-Vector cells, respectively. This result confirmed that GLT1D1 produced apoptotic cell death in MCF-7 and BT-549 cells under low serum growth condition. Thus, the earlier observed cell death under low serum growth condition (Figure 26) was mediated by GLT1D1 through apoptotic pathway.

![Figure 29](image-url)

**Figure 29 : Determination of the apoptotic effects of GLT1D1 on MCF-7 (A) and BT-549 (B) cells under low serum growth condition.**

The MCF-7 (A) and BT-549 (B) cells were transiently transfected with empty vector (Vector) or plasmids expressing GLT1D1 (GLT1D1). After 24 hours of transfection, the cells were deprived of serum. The cells were assessed for apoptosis using fluorescein isothiocyanate-conjugated annexin V (annexin V) and propidium iodide (PI) after 24 and 48 hours of serum depletion. The annexin V positive cells were counted as early apoptotic cells (early apoptosis) and the PI and annexin V cells were counted as late apoptotic cells (late apoptosis). *P<0.05, **P<0.01.

The above result suggested that GLT1D1 promoted apoptotic cell death under serum depletion. The ability of GLT1D1 to promote apoptotic cell death differed in the two experimental cell lines. GLT1D1
caused higher percentage of apoptosis in BT-549 cells compared to MCF-7 cells. It suggested that GLT1D1 readily promoted apoptosis in BT-549 cells compared to MCF-7 cells.

GLT1D1 mediated apoptosis was further characterised using general caspase inhibitor Z-VAD-FMK. Rate of apoptosis in the mammary carcinoma cells were assessed in presence and absence of general caspase inhibitor Z-VAD-FMK. MCF-7 and BT-549 cells were transfected with empty vector plasmid (Vector) or plasmid expressing GLT1D1 (GLT1D1) and treated with or without 20 μM Z-VAD-FMK. The cells were serum deprived after 24 hours of transfection. Rate of apoptosis of the cells was assessed after 48 hours of serum deprivation.

Figure 30: Determination of the caspase inhibitor effects on GLT1D1 mediated apoptosis in MCF-7 (A) and BT-549 (B) cells under low serum growth condition.

MCF-7 (A) and BT-549 (B) cells were transiently transfected with plasmids expressing GLT1D1 or empty vector (Vector) and treated with or without 20 μM general caspase inhibitor Z-VAD-FMK. After 24 hours of transfection, cells were deprived of serum. 48 hours after serum deprivation, the apoptotic cell death was assessed using fluorescein isothiocyanate-conjugated annexin V (annexin V) and propidium iodide (PI). The annexin V positive cells were counted as early apoptotic cells (early apoptosis) and the PI and annexin V positive and PI positive cells were counted as late apoptotic cells (late apoptosis).

* P<0.05, ** P<0.01.

In presence of the caspase inhibitor, GLT1D1 mediated apoptosis of BT-549 cells was significantly reduced (Figure 30A). However, in presence of the caspase inhibitor GLT1D1 mediated apoptosis of MCF-7 cells remained unchanged (Figure 30B). While there was significant 22.78%, 26% and 19.41% reduction in the early, late and total apoptosis of inhibitor treated BT549-Vector cells compared with that of the untreated BT549-Vector cells, respectively, there was highly significant 39.13%, 39.36% and 28.17% reduction in the early, late and total apoptosis of inhibitor treated BT549-GLT1D1 cells compared with that of the untreated BT549-GLT1D1 cells, respectively. There was significant 27.27%, 21.80% and 22.25% reduction in the early, late and total apoptosis of inhibitor treated MCF7-Vector cells compared with that of the untreated MCF7-Vector cells, respectively. However, the inhibitory effect was insignificant in the inhibitor treated MCF7-GLT1D1 cells compared with the untreated MCF7-GLT1D1 cells.
The above results suggested that GLT1D1 mediated caspase independent apoptosis in MCF-7 cells and caspase dependent apoptosis in BT-549 cells. An insignificant reduction in the rate of apoptosis between inhibitor treated and untreated MCF7-GLT1D1 suggesting GLT1D1 mediated caspase independent apoptosis. On the contrary, there was a clear reduction in the rate of apoptosis between inhibitor treated and untreated BT-549-GLT1D1 cells suggesting GLT1D1 mediated caspase dependent apoptosis in BT-549 cells.

The above experimental results (Figure 29) clearly demonstrated that GLT1D1 mediated apoptotic cell death in MCF-7 and BT-549 cells under serum deprivation. Furthermore, the experimental results (Figure 30) illustrated that GLT1D1 mediated caspase independent cell death and caspase dependent cell death in MCF-7 and BT-549 cells, respectively.

### 3.5.4. GLT1D1 promotes cell death with autophagy in MCF-7 cells

The above experiments have established the apoptotic effect of GLT1D1 expression under low serum growth condition in MCF-7 and BT-549. However, these experiments are not able to explain why there is 10% reduction in MCF7-GLT1D1 cell viability compared with MCF-Vector cells under full serum growth condition (Figure 25). Furthermore, the annexin V assay result (Figure 28) revealed that the mode of GLT1D1 mediated MCF-7 cell death under full serum growth condition is not apoptotic. It therefore required further analysis to explain the observed cell death. To determine the mode of cell death, the next category of programmed cell death, namely cell death with autophagy, was taken into account.

In autophagy, part of the cytoplasm is sequestered into a double-layered membrane to form the autophagosome. The lysosome of the cell fuses with autophagosome and digests its contents. The digested contents are released into the cell cytoplasm for cell’s own utilization [307]. It is shown that a signature molecule called, microtubule associated protein light chain 3 (LC3) is associated with autophagic process. The LC3 protein is proven to be useful in quantifying the autophagosome number by biochemical and microscopic assays and thus detecting autophagy induction in cells. LC3 is processed at C-terminus to form a 16kDa molecule, which is designated as LC3-I. LC3-I is subsequently lipidated by conjugating with phosphotidylethanolamine to form LC3-II, which is eventually assembled into the autophagosome membrane. The lipidated LC3-II acquires greater mobility on SDS-PAGE and resolves as a 14kDa molecule [302]. Thus, the biochemical and microscopic determination of LC3-II formation serves as an indicator of autophagosome formation and autophagy induction.

#### 3.5.4.1. LC3 Immunoblotting

To determine if GLT1D1 induced autophagy, the formation of LC3-II molecule was first examined by immunoblotting analysis. LC3-II levels in MCF-7 cells transiently transfected with empty vector (MCF7-Vector) or the plasmid expressing GLT1D1 (MCF7-GLT1D1), were assessed under full serum growth condition by immunoblotting. LC3-II levels in these cells were assessed after 24, 48 and 72
hours of transfection using anti-LC3 antibody that detected LC3-I and LC3-II protein bands at 16 kDa and 14 kDa, respectively. The immunoblot analysis (Figure 31A) revealed that GLT1D1 induced autophagy in MCF-7 cells. LC3-II formation increased in MCF7-GLT1D1 cells at 24, 48 and 72 hours indicating the induction of autophagy. Serum starvation is known to induce autophagy, so the serum-starved cells were used as positive control in the experiment. A conspicuous increase in the level of LC3-II formation after 72 hours of GLT1D1 transfection signified distinct induction of autophagy (Figure 31A and Figure 31B). Therefore, this time point was chosen as optimal time point for further analysis. Low levels of LC3-II formation were also observed in the MCF7-Vector cells. This indicated basal levels of autophagy commonly observed in the cancer cells including MCF-7 cells. This result suggested that GLT1D1 mediated induction of autophagy in MCF-7 cells.

Figure 31: Determination of GLT1D1 mediated autophagy in MCF-7 cells by LC3 immunoblotting assay.
Plasmids expressing GLT1D1 (MCF7-GLT1D1) or empty vector (MCF7-Vector) were transfected into MCF-7 cells. After 24, 48 or 72 hours of transfection (A), the cells were processed and a total of 50 μg of protein was analysed for LC3 and GLT1D1 protein expression by immunoblotting. Subsequently, the membrane was probed for β–ACTIN that was used as loading control. Cells grown in low serum media for same number of hours was used as positive control. (B) Samples from 72 hours were re-analysed in adjacent lanes for comparison.
The above observation highlighted GLT1D1 mediated autophagy in MCF7 cells. This raised a question if GLT1D1 induced autophagy in BT-549 cells under full serum growth condition.

**Figure 32 : Validation of GLT1D1 mediated autophagosome formation in BT-549 cells by LC3 immunoblotting assay.**

BT-549 cells were transfected with plasmids expressing GLT1D1 or empty vector. After 72 hours of transfection, the cells were processed and a total of 50 \( \mu \text{g} \) of protein was analysed for LC3 and GLT1D1 protein expression by immunoblotting. Subsequently, the membrane was probed for \( \beta-\text{ACTIN} \) that was used as loading control. Cells grown in low serum media for same number of hours was used as positive control.

To determine if GLT1D1 mediated induction of autophagy in BT-549 cells, the LC3 immunoblotting assay was performed after 72 hours of transient transfection of BT-549 cells with empty vector plasmid or plasmid expressing GLT1D1. Figure 32 reveals the immunoblot analysis result. The LC3-II formation levels in BT549-Vector cells matched with that of BT549-GLT1D1 cells indicating absence of autophagy induction in BT549-GLT1D1 cells. Basal levels of autophagy differ between cancer cell types. A high level of basal autophagy was seen in BT-549 cells. This result suggested lack of GLT1D1 mediated autophagy in BT-549 cells.

### 3.5.4.2. LC3 fluorescence assay

To further confirm GLT1D1 mediated autophagy in MCF-7 cells (Figure 31) and lack of GLT1D1 mediated autophagy in BT-549 cells (Figure 32), the LC3 fluorescence assay was performed. The fluorescence assay quantitatively determined induction of autophagy. Studies have established that LC3-II is associated with autophagosomes and it can be microscopically detected by using a fluorescent protein tagged microtubule associated protein light chain 3 isoform B (LC3B). With the induction of autophagy, fluorescent protein tagged LC3 are targeted to the autophagosomes and the accumulation appear as punctate structures in the cytoplasm of the cells when visualised under the fluorescence microscope [302, 316]. Number of punctae within the cells reflected autophagosome numbers. The red fluorescent protein (RFP) was tagged to LC3B (RFP-LC3) to use in the analysis.

To confirm previous results (Figure 31 and Figure 32) and quantitatively determine autophagy induction, MCF-7 and BT-549 cells were co-transfected with plasmid expressing RFP-LC3 and empty vector plasmid (Vector) or plasmid expressing RFP-LC3 and GLT1D1 (GLT1D1). After 72 hours of co-
transfection, the cells were processed and RFP-LC3 fluorescent punctae in the cells were counted under fluorescence microscope. To compensate for the basal levels of autophagy, a threshold of 5 punctae per MCF-7 cell and 20 punctae per BT-549 cell was determined. Cells revealing number of punctae above the threshold were counted as autophagy positive cells. As observed in Figure 33, the BT-549 cells typically showed high levels of basal autophagy reflected as higher number of punctae compared to MCF7. Assay result revealed 36.79% higher autophagic cells in MCF7-GLT1D1 sample compared with MCF7-Vector sample (Figure 33A). On the contrary, there was an insignificant increase in the percentage of autophagic cells in BT549-GLT1D1 sample compared with BT549-Vector sample (Figure 33B). This assay result thereby verified the previously observed GLT1D1 mediated autophagy in MCF7 cells as well as lack of GLT1D1 mediated autophagy in BT-549 cells. Therefore, further GLT1D1 mediated autophagy characterisation was carried out in MCF-7 cells alone.

**Figure 33: Quantitative measurement of GLT1D1 mediated autophagosome formation in MCF-7 cells by LC3 fluorescence assay.**

The MCF-7 (A) and BT-549 (B) cells were co-transfected with plasmid expressing RFP-LC3 and empty vector (Vector) or plasmid expressing RFP-LC3 and GLT1D1 (GLT1D1). After 72 hours of transfection, the cells were processed and analysed for fluorescent LC3 punctate structure in the cytoplasm of the cells. Cells grown in low serum media for same number of hours was used as positive control. *P<0.05, **P<0.01
1.1.1.1. Electron microscopy

Additionally, GLT1D1 mediated autophagy in MCF-7 cells was confirmed visualizing the ultrastructure of cytoplasm and identifying autophagosomes using electron microscopy. To achieve this, MCF-7 cells were transfected with empty vector plasmid (Vector) or plasmid expressing GLT1D1 (GLT1D1). 72 hours after transfection, the cells were processed for electron microscopy and the ultrastructure of the cells was visualized using transmission electron microscope (TEM). A serum starved positive control was also included in the experiment. The electron micrographs of these cells are represented in Figure 34. While, the ultrastructure of MCF7-Vector cell revealed a healthy cytoplasm, the ultrastructure of MCF7-GLT1D1 revealed double membrane autophagosomes and autolysosomes resembling the double membrane structures of positive control cells as indicated by the arrow marks in the figure. Electron microscopy analysis thus directly confirmed GLT1D1 mediated autophagosome and autolysosome formation in MCF-7 cells.
Figure 34: Confirmation of GLT1D1 mediated autophagy in MCF-7 cells by electron microscopy.

MCF-7 cells were transfected with empty vector (A) or plasmid expressing GLT1D1 (B). After 72 hours of transfection, the cells were processed and analysed under electron microscope. Ultrastructure of the cells revealing characteristic autophagosomes and autolysosomes were micrographed. Cells grown in low serum media for same number of hours was used as positive control (C).
3.5.4.3. GLT1D1 increases autophagic flux in MCF-7 cells

Autophagy is a dynamic process. In this dynamic process the lysosomal enzymes degrade the cytoplasmic content inside the autophagosome and releases the degraded contents into the cytoplasm of the cells. The above experiments have measured autophagosomes and implied GLT1D1 mediated autophagy induction in MCF-7 cells. However, the autophagosome number is not always the indicator of autophagy induction. The increase in autophagosome number could be due to the increase in generation of autophagosomes, which may not be necessarily degraded or the increase in autophagosome number could due to the inhibition of autophagosomal downstream processing. Mere quantification of autophagosomes therefore does not give a complete picture of the autophagic turnover and thereby autophagy induction. Instead, increase in autophagic flux can only fully confirm autophagy induction [302, 316]. Therefore, GLT1D1 mediated autophagic flux in MCF-7 cells was investigated.

The autophagic flux can be measured by monitoring LC3 turnover. The LC3-II is known to be present on the inner and outer membrane of autophagosomes. During lysosomal degradation, LC3-II is also processed and degraded along with autophagosomes. Cellular treatment with lysosomal inhibitors, Pepstatin-A and E64d, leads to the blockade of autophagic flux and the accumulation of undigested LC3-II. This is reflected as a major band of LC3-II on the immunoblot indicating blockade of high turnover of LC3-II and thus high autophagic flux [302, 359].

To determine GLT1D1 mediated autophagic flux in MCF7 cells, the cells were transiently transfected with empty vector plasmid (MCF7-Vector) or plasmid expressing GLT1D1 (MCF7-GLT1D1) for LC3 immunoblotting analysis and the cells were transiently transfected with empty vector plasmid (MCF7-Vector) or plasmid expressing GLT1D1 (MCF7-GLT1D1) along with the plasmid expressing LC3-RFP for LC3 fluorescence assay. The cells were treated with or without Pepstatin A and E64d inhibitors during transfection. After 72 hours, LC3-II level was monitored by immunoblotting and the autophagic cell percentage was measured by LC3 fluorescence assay. The immunoblotting assay result (Figure 35A) revealed higher levels of LC3-II accumulation in the untreated MCF7-GLT1D1 cells compared with the untreated MCF7-Vector cells suggesting increase in the formation of autophagosomes in MCF7-GLT1D1 cells and thus indication of autophagy induction. The assay result also revealed higher levels of LC3-II accumulation in the inhibitor treated MCF7-GLT1D1 cells compared with the inhibitor treated MCF7-Vector cells suggesting higher levels of autophagic flux in MCF7-GLT1D1 cells and thus providing confirmation of the induction of autophagy.
Figure 35: Confirmation of GLT1D1 mediated increase in autophagic flux in MCF-7 cells. MCF-7 cells were transfected with empty vector (Vector) or plasmid expressing GLT1D1 (GLT1D1). The transfected cells were simultaneously treated with or without 10 μg/ml of Pepstatin A (PepA) and E64d, lysosomal inhibitors. After 72 hours of transfection, the cells were subjected to LC3 immunoblotting (A) and LC3 fluorescence (B) assays.

The LC3-fluorescence assay result (Figure 35B) was in agreement with the LC3 immunoblotting assay result (Figure 35A). There was a low 8% increase in the percentage of autophagic cells between untreated MCF7-GLT1D1 and MCF7-Vector group and there was a high 18% increase in the percentage of autophagic cells between inhibitor treated MCF7-GLT1D1 and MCF7-Vector group. The assay reflected 125% increase in the percentage of autophagic cells between inhibitor treated MCF7-GLT1D1 and MCF7-Vector groups and untreated MCF7-GLT1D1 and MCF7-Vector group. The low and high increase in the percentage of autophagic cells between the treated and the untreated group and the percentage increase between the two groups reflected GLT1D1 mediated increase in autophagic flux.

The inhibition of LC3 turnover using lysosomal inhibitors thus demonstrated that GLT1D1 increased autophagic flux in MCF-7 cells and confirmed GLT1D1 mediated autophagy in MCF-7 cells.

3.5.4.4. Autophagy inhibitor 3-MA abrogates GLT1D1 mediated autophagy in MCF-7 cells

Finally, GLT1D1 mediated autophagy was characterized using a pharmacological inhibitor, 3-methyladenince (3-MA). The PI3-kinase inhibitor 3-MA, which also inhibits autophagy, was used for modulating the autophagy process. 3-MA inhibits autophagy at the double membrane autophagosome sequestration stage [302, 359, 395]. The inhibitory effect of 3-MA was assessed by LC3 immunoblotting and LC3 fluorescence assay.

To determine if 3-MA is able to inhibit GLT1D1 mediated autophagy, MCF-7 cells were pre-incubated with or without 3-MA for two hours. After two hours of incubation, the cells were transiently transfected with empty vector plasmid (MCF7-Vector) or plasmid expressing GLT1D1 (MCF7-GLT1D1) for LC3
immunoblotting analysis and the cells were transiently transfected with empty vector plasmid (MCF7-Vector) or plasmid expressing GLT1D1 (MCF7-GLT1D1) along with the plasmid expressing LC3-RFP for LC3 fluorescence assay. After 72 hours of transfection, the cells were processed for LC3 immunoblotting assay and LC3 fluorescence assay. The immunoblotting assay result (Figure 36A) revealed reduced levels of LC3-II accumulation in inhibitor treated MCF7-Vector cells compared with untreated MCF7-Vector cells suggested that 3-MA inhibited of basal levels of autophagy in MCF7-Vector cells. The assay also revealed reduced levels of LC3-II accumulation in inhibitor treated MCF7-GLT1D1 cells compared with untreated MCF7-GLT1D1 cells suggested that 3-MA abrogated GLT1D1 mediated autophagy in MCF7 cells.

![Figure 36](image)

**Figure 36 : Inhibition of GLT1D1 mediated autophagy in MCF-7 cells using autophagy inhibitor 3-MA.**

MCF-7 cells were pre-incubated with or without 10 mM 3-MA for two hours. Then the cells were transfected with empty vector (Vector) or plasmid expressing GLT1D1 (GLT1D1). The transfected cells were pre-treated with or without 10 mM 3MA, the autophagic inhibitor. After 72 hours of transfection, the cells were subjected to LC3 immunoblotting (A) and LC3 fluorescence (B) assays. *P<0.05, ** P<0.01

The LC3-fluorescence assay result (Figure 36B) was in agreement with the LC3 immunoblotting assay result (Figure 36A). The fluorescence assay result revealed that 3-MA reduced 23.1% of autophagic MCF7-Vector cells, which indicated inhibition of basal levels of autophagy in MCF7-Vector cells. On the other hand, the assay result revealed that 3-MA reduced 42.2 % of autophagic MCF7-GLT1D1 cells, which indicated abrogation of GLT1D1 mediated autophagy in MCF7 cells. The reduction in the percentage of autophagic cells in 3-MA treated MCF7-GLT1D1 cells implied abrogation of GLT1D1 mediated autophagy in MCF-7 cells.

This result conclusively demonstrated that GLT1D1 mediated autophagy in MCF-7 cells and it can be pharmacologically inhibited using 3-MA.
3.5.4.5. 3-MA abrogates GLT1D1 mediated cell death with autophagy

Above experiments aptly demonstrated the GLT1D1 mediated autophagy in MCF-7 cells. However, the effect of autophagy on cell viability remained unanswered. Therefore, GLT1D1 mediated autophagic effect on MCF-7 cells’ viability was next investigated using the autophagy inhibitor 3-MA.

To determine GLT1D1 mediated autophagic effects on MCF-7 cells viability, the cells were pre-incubated with or without 3-MA for two hours. After two hours of pre-incubation, the cells were transiently transfected with empty vector plasmid (MCF7-Vector) or plasmid expressing GLT1D1 (MCF7-GLT1D1). Viability of the cells was determined by trypan blue dye exclusion method after 7 days’ of transfection. Seven days’ period was chosen for analysis since the earlier MCF-7 cell viability assay under full serum growth condition result (Figure 25) revealed GLT1D1 mediated reduction in cell viability after seven days of the transfection.

**Figure 37 : Inhibition of GLT1D1 mediated MCF-7 cell death with autophagy, using autophagy inhibitor 3-MA.**

MCF-7 cells were pre-treated with 10 mM 3-MA and transfected with empty vector (Vector) or plasmid expressing GLT1D1 (GLT1D1). After 72 hours of transfection, the cell viability of the cells was determined by trypan blue dye exclusion method. **P<0.01**

Figure 37 represents GLT1D1 mediated autophagic effect on MCF-7 cells’ viability. The assay revealed that in absence of 3-MA there was a significant 70.75% increase in MCF7-GLT1D1 non-viable cells compared MCF7-Vector non-viable cells indicating GLT1D1 mediated cell death. In presence of 3-MA there was only 10.01% increase in MCF7-GLT1D1 non-viable cells compared with MCF7-Vector non-viable cells suggesting abrogation of GLT1D1 mediated cell death. Autophagy inhibitor 3-MA reduced 32.23% non-viable cells in MCF7-GLT1D1. This reduction in the non-viable cells clearly demonstrated abrogation of GLT1D1 mediated MCF-7 cell death with autophagy. Thus the experiment proved that GLT1D1 mediated reduction in MCF-7 cell viability under full serum growth condition is due to cell death with autophagy.
3.5.5. Mechanism of action

GLT1D1 mediated cell death mechanism was next examined after characterising the cell death. From the above experiments, it is clear that GLT1D1 mediated apoptotic cell death in MCF-7 and BT-549 cells under low serum growth conditions and mediated cell death with autophagy in MCF-7 cells under full serum growth condition.

3.5.5.1. Effect of GLT1D1 expression on BCL2 expression levels in MCF-7 and BT-549 cells

BCL2 is one of the important cell survival molecules connected to both apoptotic cell death and cell death with autophagy pathways [396]. A number of studies have established the role of BCL2 in protecting cell against apoptosis [397-399]. BCL2 and the BCL-XL proteins have also been shown to directly interact with the autophagic protein Beclin-1 through their BH-3 domain [344, 400]. Reduced expression levels of BCL2 are implicated both in autophagy and apoptosis. The expression levels of BCL2 were therefore assessed to understand GLT1D1 mediated cell death mechanism.

BCL2 promoter activity assay

To understand GLT1D1 mediated cell death mechanism, at first, the BCL2 promoter activity was measured by luciferase promoter activity assay. Mammary carcinoma cells MCF-7 and BT-549 were transiently transfected with empty vector plasmid (Vector) or plasmid expressing GLT1D1 (GLT1D1) along with plasmid containing the BCL2 gene promoter and plasmid expressing β-galactosidase. After 24 hours of transfection, the cells incubated in low serum growth media for 48 hours. The promoter activity was measured under low serum condition, since the apoptotic cell death effect was observed under low serum condition. The cells were processed and BCL2 promoter activity was measured.

![Figure 38: Measurement of GLT1D1 mediated decrease in BCL2 promoter activity.](image)

MCF-7 (A) and BT-549 (B) cells were transfected with empty vector (Vector) or plasmid expressing GLT1D1 (GLT1D1) along with plasmid containing the BCL2 gene promoter and plasmid expressing β-galactosidase. After 48 hours of transfection, the cells were processed and luciferase activity was measured. *** P<0.001

85
**BCL2** promoter activity assay result (Figure 38), revealed that there was highly significant 303.16% and 204.02% down-regulation of **BCL2** promoter activity in MCF7-GLT1D1 and BT549-GLT1D1 cells compared with MCF7-Vector and BT549-Vector cells, respectively. This promoter activity assay result suggested that GLT1D1 downregulated BCL2 promoter activity in MCF-7 and BT-549 cells.

**BCL2** protein expression levels

Furthermore, the **BCL2** protein expression levels were assessed to understand if the observed down-regulation of **BCL2** promoter activity is actually reflected at protein expression levels. To assess the effect of GLT1D1 expression on **BCL2** expression levels in MCF-7 and BT-549, the cells were transiently transfected with empty vector plasmid (Vector) or plasmid expressing GLT1D1 (GLT1D1). After 24 hours of transfection, the cells incubated in low serum growth media for 48 hours. 48 hours after low serum growth media incubation, the cells were processed for immunoblotting analysis.

![Figure 39: Evaluation of GLT1D1 mediated BCL2 down regulation in MCF7 and BT549 cells.](image)

**Figure 39**: Evaluation of GLT1D1 mediated BCL2 down regulation in MCF7 and BT549 cells. MCF-7 (A) and BT-549 (B) cells were transfected with empty vector (Vector) or plasmid expressing GLT1D1 (GLT1D1). After 24 hours of transfection, the cells incubated in low serum growth media for 48 hours. 48 hours after low serum growth media incubation, the cells were processed and BCL2 expression levels along with GLT1D1 and β–ACTIN protein levels were assessed by immunoblotting. β–ACTIN was used as loading control.

As observed in Figure 39, there was a reduction in BCL2 protein expression levels both in MCF7-GLT1D1 and BT549-GLT1D1 cells compared with MCF7-Vector and BT-549-Vector cells, respectively. This result clearly illustrated that GLT1D1 down-regulated the BCL2 protein expression levels in MCF-7 and BT-549 cells.

**3.5.5.2. GLT1D1 expression increases BAX/BCL2 ratio in MCF-7 and BT-549 cells**

The BAX protein is antagonistic to BCL2 protein in function. These antagonistic proteins are maintained at a definite ratio in cells for preserving the cellular homeostasis. Any imbalance in BAX/BCL2 ratio perturbs the cellular homeostasis resulting in either anti-apoptotic or pro-apoptotic consequences [401, 402]. To further understand the above observed BCL2 down-regulation consequences and to unravel the mechanism behind the observed apoptotic cell death, BCL2 and BAX expression levels were simultaneously assessed.
MCF-7 and BT-549 cells were transfected with empty vector (Vector) or plasmid expressing GLT1D1 (GLT1D1). After 24 hours of transfection, the cells incubated in low serum growth media for 48 hours. 48 hours after low serum growth media incubation, cells were processed for mRNA and protein expression analysis by semi-quantitative RT-PCR and immunoblotting analysis. The analysis (Figure 40) revealed down-regulation of BCL2 mRNA and protein expression in MCF7-GLT1D1 and BT549-GLT1D1 cells compared with MCF7-Vector and BT549-Vector cells, respectively. On the contrary, BAX mRNA and protein expression levels in the cells remained unchanged. The densitometric analysis of ratio of BAX and BCL2 revealed that there was 47.58% and 59.11% increase in the BAX/BCL2 mRNA and protein ratio, respectively, in MCF7-GLT1D1 cells compared with MCF7-Vector cells. Furthermore, there was 44.68% and 43.89% increase in the BAX/BCL2 mRNA and protein ratio, respectively, in BT549-GLT1D1 cells compared with BT549-Vector cells. The increase in BAX/BCL2 ratio is known to yield pro-apoptotic consequences. Thus the increased BAX/BCL2 ratio explained one of the mechanisms behind the observed apoptotic cell death under low serum condition.
3.5.5.3. GLT1D1 expression increases autophagic protein Beclin-1 expression levels in MCF-7 cells

Studies have shown that the elevated levels of Beclin-1 expression in cells induces the cell death with autophagy [347, 403]. There are studies that have proven interaction of Beclin-1 protein with BCL2 through its BH3 domain [311, 396, 404]. Reduced levels of BCL2 leading to the increased expression levels of Beclin-1 resulting in MCF-7 cell death with autophagy [405] is also established. The above results have established GLT1D1 mediated BCL2 down-regulation in MCF-7 cells. To further unravel the mechanism behind the observed cell death with autophagy in MCF-7 cells, the effect of GLT1D1 expression on BCL2 and Beclin-1 expression levels under full serum growth condition was next determined.

To examine the effect of GLT1D1 expression on BCL2 and Beclin-1 expression levels, MCF-7 were transiently transfected with empty vector plasmid (Vector) or plasmid expressing GLT1D1 (GLT1D1). After 72 hours of transfection, the cells were processed for mRNA and protein expression analysis by semi-quantitative RT-PCR and immunoblotting analysis. As observed in Figure 41, there was a marked reduction in the mRNA and protein expression of BCL2 with concomitant increase of that of Beclin-1 in MCF7-GLT1D1 cells compared with MCF7-Vector cells.

![Figure 41](image)

**Figure 41**: Evaluation of GLT1D1 mediated up-regulation of Beclin-1 expression in MCF7 cells. The cells were transfected with empty vector (Vector) or plasmid expressing GLT1D1 (GLT1D1). After 48 hours of transfection, Beclin-1 mRNA (A) and protein (B) expression was analysed by semi-quantitative RT-PCR and immunoblotting assays along with BCL2, GLT1D1 and β-ACTIN expression levels. β-ACTIN was used as loading control.

This result clearly demonstrated that GLT1D1 increased the expression levels of autophagic protein Beclin-1. Thus the elevated levels of Beclin-1 expression explained the mechanism behind the observed cell death with autophagy in MCF-7 cells under full serum growth condition.
3.5.6. GLT1D1 and Mitochondria

The powerhouse of the cell, the mitochondria, is involved in apoptosis and autophagy [406]. Apoptosis related molecules such as BCL2 family proteins [407-409], cytochrome-C [410] and autophagy related molecule such as p19ARF [411] are localised to mitochondria. These proteins perform various physiological functions and also play active roles in the cell death pathways. These cell death proteins execute cell death function by converging onto mitochondria. My earlier study results have established that GLT1D1 mediated apoptotic cell death in MCF-7 and BT-549 cells and cell death with autophagy in MCF-7 cells. In view of the ability of GLT1D1 to mediate both the types of cell death, the possibility of GLT1D1 localisation to mitochondria was explored.

To examine mitochondrial localisation, a fusion construct GLT1D1-GFP was made by fusing GLT1D1 with the Green Fluorescent Protein (GFP). To label mitochondria, mitochondrial targeting element (MTE) of cytochrome oxidase 8 (COX8) and Methionine Sulfoxide Reductase (MSRA) [412, 413] and a commercially available mitotracker dye were used in the experiments. Fusion constructs COX8-RFP and MSRA-RFP were made fusing the MTE of COX8 and MSRA with red fluorescent protein (RFP), respectively. The GFP and RFP molecules enabled visualisation of the molecules under the fluorescence microscope and their localisation inside cells.

3.5.6.1. GLT1D1 mitochondrial localisation determination using COX8

To examine if GLT1D1 localised to mitochondria, MCF-7 cells were transfected with plasmid expressing GFP (GFP) or GLT1D1-GFP (GLT1D1-GFP) along with the plasmid expressing COX8-RFP. The cells were processed for cytochemical examination after 48 hours of transfection.

![Figure 42: The GLT1D1’s mitochondrial localisation determination in MCF7 cells using mitochondrial targeting element (MTE) of Cytochrome Oxidase-8 (COX-8). MCF-7 cells were transfected with the plasmid expressing (A) Green Fluorescent Protein (GFP) or (B) GLT1D1-GFP (GLT1-GFP) along with plasmid expressing MTE of COX8 (COX8-RFP). After 48 hours of transfection, cells were processed for cytochemical analysis under fluorescence microscope. Cells’ nuclei were stained using Hoescht 33258.](image-url)
Figure 42 reveals the localisation results of GFP and GFP-GLT1D1 when expressed along with the COX8-RFP in MCF-7 cells. The COX8-RFP fusion protein labelled mitochondria and it appeared as red fluorescent punctae in the cytoplasm of the cells. In the vector control GFP cells (Figure 42A), green fluorescence was observed throughout the cells without suggesting any specific localisation of GFP protein within the cell. The green fluorescence of GFP did not merge with the red fluorescence of mitochondrial COX8-RFP suggestive of the absence of GFP localisation to mitochondria. Whereas, in GLT1D1-GFP cells (Figure 42B), green fluorescence punctae distributed throughout the cytoplasm of the cells suggesting a particular localisation pattern. The green fluorescence of GLT1D1-GFP perfectly merged with the red fluorescence of mitochondrial COX8-RFP observed as yellow colour clearly suggested GLT1D1 co-localisation to mitochondria.

3.5.6.2. GLT1D1 mitochondrial localisation determination using mitotracker dye

To validate the above observed GLT1D1 mitochondrial localisation by a second method, the localisation was determined using a commercially available mitochondrial dye called mitotracker. The mitotracker selectively labels mitochondria of live cells in a controlled environment.

![Figure 43](image)

**Figure 43 : The GLT1D1’s mitochondrial localisation determination in MCF7 cells using mitotracker dye.**

The cells were transfected with plasmids expressing (A) Green Fluorescent Protein (GFP) or (B) GLT1D1-GFP (GLT1D1-GFP). After 48 hours of transfection, cells' mitochondria were stained using commercial mitochondrial dye, mitotracker. Subsequently cells were processed for cytochemical analysis under fluorescence microscope. Cells’ nuclei were stained using Hoescht 33258.

To determine GLT1D1 localisation, MCF-7 cells were transfected with plasmid expressing GFP (GFP) or GLT1D1-GFP (GLT1D1-GFP). After 48 hours of transfection, the cellular mitochondria were labelled using mitotracker dye followed by processing of the cells for cytochemical analysis.

Surprisingly, the mitochondrial localisation of GLT1D1 as observed using mitotracker (Figure 43) contradicted the localisation result that was previously observed using MTE of COX8 (Figure 42). The punctate appearance of GLT1D1-GFP in MCF-7 cells transfected with GLT1D1-GFP and COX8-RFP was completely absent in MCF-7 cells transfected only with GLT1D1-GFP. Instead, the GLT1D1-GFP
cellular distribution pattern in MCF-7 cells transfected only with GLT1D1-GFP resembled the cellular distribution pattern of MCF-7 cells transfected with vector control GFP. The mitotracker dye labelled mitochondria of the cells and it appeared as red fluorescent punctae in the cytoplasm of the cells. In the vector control GFP cells (Figure 43A), green fluorescence of GFP did not merge with the red fluorescence of mitotracker dye suggesting the absence of GFP localisation to mitochondria. Similarly, in GLT1D1-GFP cells (Figure 43B) also green fluorescence of GLT1D1-GFP did not merge with the red fluorescence of mitotracker dye suggesting absence of GLT1D1 localisation to mitochondria.
3.5.6.3. GLT1D1 localisation study using MSRA

The above microscopic experimental results revealed contradicting results for GLT1D1 mitochondrial localisation. To re-examine these results, GLT1D1 mitochondrial localisation was determined using another mitochondrial marker MSRA.

Figure 44: The GLT1D1’s mitochondrial localisation determination in MCF7 cells using mitochondrial targeting element (MTE) of Methionine Sulfoxide Reductase (MSRA). The cells were transfected with plasmids expressing (A) Green Fluorescent Protein (GFP) or (B) & (C) GLT1D1-GFP (GLT1D1-GFP) along with plasmid expressing MTE of MSRA (MSRA-RFP). After 48 hours of transfection, cells were processed for cytochemical examination and analysed under fluorescence microscope. Cells’ nuclei were stained using Hoescht 33258.

To determine GLT1D1 localisation to mitochondria, MCF-7 cells were transfected with plasmid expressing GFP (GFP) or GLT1D1-GFP (GLT1D1-GFP) along with the plasmid expressing MSRA-RFP. The cells were processed for cytochemical examination after 48 hours of transfection.
Interestingly, the mitochondrial localisation result obtained by the above experiment (Figure 44 A, B and C) revealed a pattern, which was a combination of results obtained by COX8 (Figure 42) and mitotracker (Figure 43) experiments. Cell population with distinct punctate appearance of GLT1D1-GFP as seen in MCF-7 cells transfected with GLT1D1-GFP and COX8-RFP and cell population with scattering pattern of GLT1D1-GFP in MCF-7 cells transfected only with GLT1D1-GFP was observed in the MSRA-RFP experiment. The MSRA-RFP appeared as red fluorescent punctae in the cytoplasm and labelled mitochondria of the cells. In the vector control GFP cells (Figure 44A), green fluorescence of GFP did not merge with the red fluorescence of MSRA-RFP suggesting the absence of GFP localisation to mitochondria. Whereas, in GLT1D1-GFP cells, 25% of the cells exhibited scattered green fluorescence throughout the cells (Figure 44B), resembling vector control GFP cells suggesting absence of GLT1D1 mitochondrial localisation. Remaining 75% of the cells exhibited punctate green fluorescence in the cytoplasm of the cell (Figure 44C) merging with the red fluorescence of MSRA-RFP evidenced as yellow fluorescence in the merged image suggesting GLT1D1 localisation to mitochondria.

### 3.5.6.4. GLT1D1 localisation study by subcellular fractionation method

Finally, subcellular fractionation technique was employed to conclusively determine whether GLT1D1 localised to mitochondria or not. MCF-7 cells were transfected with plasmid expressing GLT1D1-GFP for 48 hours. The cells were processed for cellular fractionation and separated into nuclear, mitochondrial and cytoplasmic fractions. The GLT1D1 distribution in nuclear, mitochondrial and cytoplasmic fractions was assessed using the fraction marker proteins Histone H3, COX IV and α-tubulin, respectively.

![Figure 45: Determination of GLT1D1 localisation in MCF7 cells by sub-fractionation method.](image)

The MCF7 cells were transiently transfected with plasmid expressing GLT1D1-GFP. After 48 hours of transfection, cells were harvested and sub-fractionated into nuclear, mitochondrial and cytoplasmic fractions. The fractions were analysed for the presence of GFP-GLT1D1 protein along with the marker proteins of the fractions.
The Figure 45 represents the subcellular fractionation result where the immunoblot was probed with marker protein and GLT1D1 antibodies. The analysis revealed that GLT1D1-GFP is present in the cytoplasmic and nuclear fraction. There was no GLT1D1 protein band in the mitochondrial fraction suggesting the absence of GLT1D1 localisation to mitochondria. This result re-confirmed the earlier microscopic observation (Figure 43) where GLT1D1-GFP was found to be not localised to mitochondria where plasmids expressing GLT1D1-GFP is transfected into MCF-7 cells alone without plasmids expressing either COX8-RFP or MSRA-RFP.

From the above experiments, it could be concluded that GLT1D1 localised to mitochondria in MCF-7 cells only in the presence of either COX-8 or MSRA. From the mitotracker dye (Figure 43) and subcellular fractionation (Figure 45) experiments, it was found that GLT1D1 did not particularly localise to mitochondria.
CHAPTER 4. DISCUSSION

4.1. INTRODUCTION

There has been an outpour of large pool of genetic information in the post-genomic era that has opened new avenues to researchers for investigating the causes of diseases [414]. Cancer research is one of the research areas, which has seen extensive development ever since the completion of the human genome project. Post-genomic era has witnessed the identification of significant number of genes involved in oncogenesis. Advent of high-throughput technology platforms such as microarray based gene expression analysis has helped to rapidly discover new genes and new molecular players. A census in 2004 by Andrew Futreal et al reported 291 human genes that are known to cause spontaneous or familial cancer development when mutated. This accounts for more than 1% of total number of genes [415]. A recent update of cancer implicated genes increased to 384, which accounts for almost 2% of the total number of human genes. Number of genes implicated in cancer continues to grow over the years. However, the biological significance or the functional aspects of the genes identified in such studies often remain unclear [416].

In this study, I characterised the function of one such novel human gene called GLT1D1. By microarray studies (Figure 10 and Figure 11), it was observed that GLT1D1 is overexpressed in different types of cancers. Overexpression of GLT1D1 in cancer and its molecular characteristics made it an interesting candidate for the study. GLT1D1 shared the distinctive overexpression feature of oncogenes [417]. Furthermore, the protein structure prediction databases, UniProtKB and Pfam revealed the presence of a signal peptide for GLT1D1 indicating it to be a secreted protein. Secreted oncogenic proteins such as human growth hormone [418, 419] and Artemin [420] are proven to be useful therapeutic targets for monoclonal antibody therapy in cancer. Considering the expression characteristics of the gene in cancer, it was hypothesised that GLT1D1 may possess a role in breast cancer.

Overexpression or forced-expression study model through gain of function of a gene is a useful tool to determine the function of that gene [421]. The functioning of genes involved in embryonic patterning in Xenopus [422] and conversion of fibroblasts to myoblasts [423] was discovered using forced-expression study model. Subsequently, the effect of the gene expression was assessed by the functional assays that suggested the possible role of the gene [422, 424]. Similarly, in the present study, the gene of interest was cloned into the mammalian expression vector pIRES so that it could be overexpressed in the desired mammalian cell model. Mammary carcinoma cells, MCF-7 and BT-549, were used as cell models to assess the above hypothesis. The gene was transiently force-expressed and functional assays were performed to characterise its function.

Herein, for the first time, I report the cell death effect of GLT1D1 in mammary carcinoma cells. I have demonstrated that GLT1D1 mediates apoptotic cell death in MCF-7 and BT-549 cells and cell death
with autophagy in MCF-7 cells. I have also illustrated that GLT1D1 mediates cell death by down regulating cell survival protein BCL2. Furthermore, my study confirmed that GLT1D1 mediates apoptotic cell death by altering BAX/BCL2 ratio and cell death with autophagy by upregulating the expression levels of autophagic protein, Beclin-1. The pro-apoptotic and pro-autophagic proteins are mostly associated with tumour-suppressor function. Based on the above GLT1D1 mediated cell death results, it could be advocated that GLT1D1 has a tumour-suppressor function.

4.1.1. GLT1D1 expression analysis

It was important to establish that the GLT1D1 gene is actually expressed as a protein although mass-spectrometry based peptide submissions in the PRIDE database, proved that the gene is expressed as a protein and that the protein is present in sera. An antibody with high specificity against the protein of interest is a useful tool to confirm the expression of that protein. GLT1D1 specific polyclonal antibody was hence raised to examine the protein expression in tissues and cell lines (Figure 18).

The affinity purified, GLT1D1 specific antibody was used to screen the GLT1D1 and GLT1D1-2 protein expression in human tissues and cancer cell lines. Our experimental results (Figure 19) confirmed that GLT1D1 is the predominant isoform, which is expressed in small intestine, kidney and liver. It was therefore decided to keep the study focus limited to GLT1D1 isoform rather than other two isoforms of the gene. Although, the immunoblot revealed an expected size band around 38.5kDa, the exact size of the protein band could not be determined by this experiment, as the experiment was done using a commercially available blot. The total protein from human liver was therefore immunoblotted along with the force-expressed GLT1D1 in adjacent lanes to compare and confirm the GLT1D1 isoform expression. The immunoblotting result (Figure 20) validated and confirmed that 38.5 kDa GLT1D1 is the predominant isoform expressed in human tissues. Furthermore, the experimental results (Figure 21) revealed the presence of GLT1D1 protein in human sera, corroborates with the PRIDE GLT1D1 peptide submission.

GLT1D1 mRNA and protein expression levels in cancer cells were examined prior to its functional characterisation (Figure 22 and Figure 23). Reverse transcription PCR using GLT1D1 isoform specific primers revealed transcription of the gene into mRNA in different cancer cell lines. Interestingly, none of the screened cancer cell lines revealed detectable levels of GLT1D1 protein expression on immunoblot. The breast cancer cell lines, in which the function of the gene was probed, also displayed this pattern. The absence of protein expression even in liver and kidney cancer cell lines HepG2 and HEK-293 cells, respectively, is noteworthy to be mentioned here. The tissue screening results have shown high levels of protein expression in these organs whereas the transformed cell lines from these tissues lack or have undetectable levels of the protein expression. It is interesting to observe that while there is detectable level of mRNA expression, there is lack or undetectable levels of GLT1D1 protein expression, in cancer cells. This could be due to non-sense or frameshift mutation in the upstream region of the gene that could have resulted into lack of expression or partial expression of GLT1D1 protein. It is also possible that the cancer cells would have lost GLT1D1
function during the course of transformation. This possibility strongly suggests a tumour suppressor function for the protein. However, mutational analysis upstream of GLT1D1 gene in cancer cells would help to understand this better. This analysis could be carried-out in a future course of study.

A high-throughput pilot study by Laurent Barb et al, which screened 466 proteins for their cellular localisation, also studied GLT1D1 protein [425]. The data obtained for the proteins are available in the human protein atlas website. The study claims to have detected GLT1D1 expression in normal and cancer tissues. However, no information is available about the antibody characterisation in this study. In addition to this, the study does not confirm any particular isoform of GLT1D1. Two antibodies commercially available with Sigma-Aldrich refer to this data in human protein atlas website. The information available from Sigma-Aldrich, hints that the antibodies are raised against two regions of GLT1D1-2 protein. But, the immunoblotting results of these two antibodies have two different results on same set of samples, suggesting the possibility of non-specific or other isoform protein detection. Moreover, there is no reference recombinant protein loaded on the immunoblot for molecular weight comparison. On the contrary, I have used GLT1D1 antibody in this study, which detected the protein from force-expressed cells as well as from human tissues (Figure 19) with high specificity. Furthermore, I have compared the molecular weight of the protein expressed in liver and recombinant GLT1D1 by running them in adjacent lanes on an immunoblot (Figure 20). The confirmed molecular weight is in agreement with the predicted size of GLT1D1 protein and the PRIDE peptide submissions for the protein, thus confirming the identity of the protein.

Figure 21 result, revealed GLT1D1 protein being present in human sera. As previously mentioned, GLT1D1 is predominantly expressed in human liver and kidney. So, like other major serum and plasma proteins, GLT1D1 could also be synthesised and secreted from human liver [426] and kidney [427]. GLT1D1 in sera could be transporting molecules such as minerals, hormones or vitamins. With the present study it is difficult to speculate any particular transportation function for the protein. However, determining serum GLT1D1 levels in liver and kidney carcinomas could prove to be useful for diagnostic or prognostic purposes.

4.1.2. GLT1D1 functional analysis

To study the functional role of GLT1D1 in mammary carcinoma, gain of function approach was adopted by force-expressing the gene. For achieving stable forced-expression, the plasmid expression GLT1D1 was transfected into MCF-7 and BT-549 cells and grown in media containing selection antibiotic. However, stable GLT1D1 expression in these cells could not be achieved since the cells expressing GLT1D1 got eliminated during the selection process as evidenced in the RT-PCR and immunoblotting results (Figure 24) of transient and stable cells. After confirming these unexpected experimental results, we were led to re-hypothesise that GLT1D1 mediates cell-death. Cell death due to excessive protein expression and cell signalling was taken into consideration while forming the second hypothesis (GLT1D1 mediates cell death), as the cell senescence and cell death due to excessive cell signalling from oncogenes such as KRAS, MYC and RAF [428, 429] are
reported. The transfection of linearized plRES-GLT1D1 plasmid facilitates random integration of the gene into the cancer cell genome. This should have resulted in both low and high concentrations of GLT1D1 expressing colonies. Even, if we assume that high expression levels of GLT1D1 caused cell death, there should have been stable colonies with low GLT1D1 expression. On all three different occasions of experimental repetition, I obtained colonies without GLT1D1 expression. This clearly suggested that GLT1D1 has a role in cell death rather than in cell proliferation.

4.1.3. GLT1D1 mediated cell death analysis

To examine, if GLT1D1 expression caused cell death, we continued to use MCF-7 and BT-549 cells as cell models. The MCF-7 cells have mutated caspase-3 enzyme [430], so they are deficient in apoptotic pathway. Whereas, the BT-549 cells with no known mutations in the intrinsic apoptotic pathway, readily undergo apoptosis. While choosing the cells, the mutational status of the other important molecule in cell death namely p53 was also considered. MCF-7 is known to carry a wild-type TP53 gene whereas the BT-549 cells are known to carry a mutation in TP53 gene [431]. These important differences in the status of cell death molecules in MCF-7 and BT-549 cells were thought to be useful in determining the type of cell death and its pathway and thought to give a picture that would enable us to further probe the molecular functioning of the gene. These two molecularly different mammary carcinoma cells were therefore chosen in the study to probe the cell death function of the gene.

While the cell viability study under full serum (10%) growth condition highlighted GLT1D1 mediated cell death in MCF-7 and not in BT-549 cells, the low serum (0.2%) growth condition (serum depletion) highlighted the GLT1D1 mediated cell death both in MCF-7 and BT-549 (Figure 25 and Figure 26). The experimental results (Figure 29) demonstrated that GLT1D1 caused apoptotic cell death in MCF-7 and BT-549 under low serum growth condition. The serum depletion worked as stress factor in the experiment. BT-549 cell death and 80% increase in the percentage of MCF-7 cell death under serum deprivation (Figure 25 and Figure 26) also implied that GLT1D1 required a the stress factor such as low serum deprivation to mediate cell death and could not induce cell death in the cells on its own. Additionally, using general caspase inhibitor Z-VAD-FMK, we illustrated that GLT1D1 mediated caspase independent cell death in MCF-7 and caspase dependent cell death in BT-549 (Figure 30) under serum deprivation. GLT1D1 mediated caspase independent apoptotic pathway in MCF-7 could be due to the caspase-3 deficiency in the cells.

However, it was interesting to see that under full serum growth condition there was 10% reduction in cell viability in MCF7-GLT1D1 than MCF7-Vector cells (Figure 25). The apoptosis assay conducted after seven days of transfection (Figure 28) confirmed that the observed cell death was not due to apoptosis. This result prompted us to investigate further to understand GLT1D1 mediated MCF-7 cell death under full serum growth condition.
To understand GLT1D1 mediated MCF-7 cell death under full serum growth condition, we examined the other common type of programmed cell death pathway namely, autophagy. The autophagy assays performed using biochemical and microscopic techniques, proved that the observed reduction in cell viability in MCF7-GLT1D1 is due to cell death with autophagy. The LC3 immunoblotting and fluorescence microscopy assays confirmed the GLT1D1 mediated induction of autophagy in MCF-7 cells, while its absence in BT-549 cells (Figure 33). Electron microscopy results confirmed GLT1D1 mediated autophagy by revealing double membrane autophagosome and autolysosome structures (Figure 34). Finally, GLT1D1 mediated increase in autophagic flux measured using the lysosomal inhibitor Pepstatin-A and E-64d conclusively confirmed the autophagy induction (Figure 35). Furthermore, the increase in autophagic flux was confirmed using autophagic inhibitor 3-MA (Figure 36). The GLT1D1 mediated cell death with autophagy was conclusively confirmed by abrogating the cell death using the autophagic inhibitor 3-MA (Figure 37).

Interestingly, GLT1D1 differentially mediated apoptotic cell death and cell death with autophagy in MCF-7 and BT-549 cells. While GLT1D1 caused caspase independent cell death in MCF-7, it caused caspase dependent apoptotic cell death in BT-549 cells under low serum growth condition. Additionally, GLT1D1 mediated cell death with autophagy in MCF-7 and this effect is absent in BT-549 cells. The differential cell death effect between the two cell lines could be because of their caspase-3 mutation status [430]. GLT1D1 could be therefore operating in a caspase independent pathway in MCF-7. Further study with caspase-3 function restored MCF-7 would resolve the caspase independent cell death in MCF-7. As compared to MCF-7, high levels of basal autophagy was observed in BT-549 cells (Figure 33). Hence, GLT1D1 could have failed to elicit autophagy in BT-549 that has high levels of basal autophagy. The difference in the basal level of autophagy between the two cell lines could be due to different origins of the cell lines MCF-7 (adenocarcinoma) and BT-549 (ductal carcinoma). More studies with cell lines having low and high basal autophagy levels would answer the observed cell death with autophagy in MCF-7 and its absence in BT-549.

4.1.4. Mechanism of Action

The expression analysis revealed GLT1D1 mediated BCL2 down regulation. This helped to understand the molecular mechanism behind GLT1D1 mediated apoptosis and autophagy. BCL2 is an important cell survival molecule involved in protecting cells against both apoptotic cell death and cell death with autophagy [40, 400]. BAX is antagonistic to BCL2 in function and is known to induce apoptotic cell death. A definite ratio of the two antagonistic proteins is a pre-requisite for cellular homeostasis [401]. Our results confirmed that due to BCL2 down-regulation, the BAX/BCL2 ratio is increased in MCF7-GLT1D1 and BT549-GLT1D1 cells when compared with MCF7-Vector and BT549-Vector respectively (Figure 40). This result established the molecular basis for GLT1D1 mediated apoptotic cell death. Furthermore, we assessed the effect of BCL2 down-regulation in the context of autophagy in MCF-7 cells. BCL2 is known to directly interact with the autophagic molecule Beclin-1 through BH-3 domain and thus preventing cell death with autophagy [396]. Our results
(Figure 41) clearly illustrate that Beclin-1 mRNA and protein expression level is elevated due to the low expression levels of BCL2. This result is in agreement with the previous work where the authors have shown that the silencing of BCL2 by si-RNA in MCF-7 cells elevated Beclin-1 expression level and induced cell death with autophagy in MCF-7 cells [405].

From the above discussion, it is possible to schematically derive the action of GLT1D1 protein in cells as depicted in Figure 46. The experimental results have illustrated that GLT1D1 downregulated cell survival protein BCL2. How exactly GLT1D1 achieves this in the cell is a matter of further study. It could be postulated that GLT1D1 down-regulates BCL2 expression levels by directly or indirectly moderating the expression. As demonstrated in the study, the BAX/BCL2 ratio got increased and the autophagic protein Beclin-1 expression levels also got elevated as a consequence of BCL2 down-regulation causing apoptotic cell death [432, 433] and cell death with autophagy [405], respectively. Our study thus comprehensively explains the mechanism behind GLT1D1 mediated autophagic cell death and cell death with autophagy.
4.1.5. Localisation to mitochondria

We further examined whether GLT1D1 localised to mitochondria, since the molecules related to cell death are often localised to mitochondria. The biological relevance of a protein is frequently closely linked to its compartmentalisation within the cell [434]. We used microscopic [435] and sub-cellular fractionation [436] methods to verify mitochondrial localisation of GLT1D1. The GLT1D1 mitochondrial localisation study yielded interesting results. First, we used the mitochondrial localisation signal peptide of COX8 as reference molecule [412, 413] to determine GLT1D1’s localisation. The COX8 experimental results (Figure 42) clearly revealed that GLT1D1 is localised to mitochondria. However, when we attempted to confirm this result using mitotracker dye, a contradicting result emerged that did not support our earlier GLT1D1’s mitochondrial localisation observation. To obtain further clarity, we used MTE of one more mitochondrial protein, MSRA, as reference molecule [437]. In this experiment, there were two distinct populations of cells, (i) a population of cells without GLT1D1 localisation to mitochondria (Figure 44B) and (ii) a population of cells with GLT1D1 localisation to mitochondria (Figure 44C). Furthermore, we resolved GLT1D1 mitochondrial localisation by performing sub-cellular fractionation of MCF7-GLT1D1-GFP cells. The cellular sub-fractionation results illustrated that GLT1D1 localised to nucleus and cytoplasm (Figure 45) and not to the mitochondria. This result was in agreement with microscopic experimental results that used Mitotracker dye.

Microscopic experiments using the MTE of COX8 and MSRA suggested that GLT1D1 localised to mitochondria; whereas, the mitotracker and sub-fractionation results suggested GLT1D1 localised to nuclei and cytoplasm. It can be noted that GLT1D1 localises to mitochondria when it is co-expressed with MTE of COX8 and MSRA. In the absence of these peptides GLT1D1 localises to nuclei and cytoplasm, as evidenced by the mitotracker dye and sub-cellular fractionation experiments. This difference in GLT1D1 compartmentalisation suggests differential functioning of the molecule in different tissues or organs. For example, GLT1D1 may have different localisation and function in liver, heart and skeletal muscle cells, which possess a high number of mitochondria and high mitochondrial protein turnover as against skin tissue that has less mitochondria and less mitochondrial protein turnover. This can be the subject of a future course of investigation, where GLT1D1 localisation could be determined in different human tissues using the GLT1D1 specific antibody.

4.2. Conclusion

My study highlights GLT1D1 mediated breast cancer cell death. The study also includes protein expression levels in normal human tissues as well as cancer cell lines. The absence or undetectable levels of GLT1D1 protein expression in cancer cell lines and breast cancer cell death mediation is in accord with the characteristics of a tumour suppressor molecule. These results put-together suggest a possible tumour suppressor function for GLT1D1 protein. GLT1D1 screening studies using cancer tissues and gene knockout studies using mouse models would provide more definite answers to this theory.
This study was commenced based on microarray data that suggested GLT1D1’s over-expression characteristics in different carcinomas (Figure 10 and Figure 11). However, my study results confirm cell death effect of GLT1D1 in cancer cells suggesting a possible tumour suppressor function and are in contradiction with the microarray over-expression data. It has to be noted here that microarray data only gives a picture of gene expression at mRNA level and not at protein level. There could be loss of GLT1D1 protein expression due to frameshift mutations in cancer tissues although high levels of GLT1D1 mRNA are produced. It is also possible that GLT1D1 protein is expressed in cancer tissues with other components of its cell death pathway mutated, aborting GLT1D1 cancer cell death effect. To find a definite answer in this regard, cancer tissue screening for GLT1D1 protein expression and mutational analysis of the gene needs to be performed.

4.3. Future direction

The above sequence of experimental results clearly demonstrated that forced expression of GLT1D1 elicited apoptotic cell death in MCF-7 and BT-549 cells and cell death with autophagy in MCF-7 cells. My study has also established the molecular mechanisms behind GLT1D1 mediated cell deaths. Thus the study provides a strong basis for future studies. As this is a novel gene, there is lot of scope for further studies that can unravel the complete picture of the molecule.

Further functional study of GLT1D1 in other transformed and non-transformed cell types from organs such as liver and kidney where GLT1D1 is highly expressed would further strengthen the present study results. Studying the gene function in the primary and transformed cells derived from these organs would also give a greater understanding of the gene functioning. It would be a good idea to clone GLT1D1 with an inducible promoter in the future course of the study, since the gene expression under inducible promoter provides more defined experimental conditions.

Biochemical studies to examine the glycosyltransferase activity of GLT1D1 would be an important experiment that will help to understand more about the observed cell death. In vitro studies to understand the substrates used and products formed in the biochemical activity of the glycosyltransferase could provide useful information about the mechanism of BCL2 down regulation. The other molecules involved in GLT1D1 mediated cell death pathway could be identified by co-immunoprecipitation approach.

GLT1D1 protein localisation and expression levels in normal and cancer tissues should be studied to further explore the possibility of tumour suppressor function. Efforts should also be made to correlate the gene and protein expression levels with clinical stages of cancer. Since the protein is present in sera, studying its clinical correlation with different cancer stages by ELISA would provide a greater opportunity to exploit the molecule as a diagnostic or prognostic indicator.

The future course of study should also be designed to understand the normal physiological function of GLT1D1. The gene knockout and transgenic studies using mouse model could provide greater insight of the normal and pathological function of the gene. The GLT1D1 transgenic and knockout mouse
models could also answer the tumour suppressor function of the protein, which is implicated in the present study.
REFERENCE


How shall I eat thee?


Cis1/Atg31 is required for autophagosome formation in Saccharomyces cerevisiae.

Apg1p, a novel protein kinase required for the autophagic process in Saccharomyces cerevisiae.


The LC3 conjugation system in mammalian autophagy.

A ubiquitin-like system mediates protein lipidation.

Methods for monitoring autophagy.


Autophagy: a lysosomal degradation pathway with a central role in health and disease.


Fine structure of the autophagosome.


The role of autophagy in mammalian development: cell makeover rather than cell death.


The role of autophagy in mammalian development: cell makeover rather than cell death.


Autophagy in embryonic erythroid cells: its role in maturation.


Autophagy regulates adipose mass and differentiation in mice.


Autophagy in embryonic erythroid cells: its role in maturation.


## APPENDIX – A

### DNA Loading dye 6X
- 0.25% Bromophenol blue
- 0.25% Xylene cyanol
- 30% glycerol

### 50X TAE stock buffer
<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris</td>
<td>242g/L</td>
</tr>
<tr>
<td>Glacial acetic acid</td>
<td>57.1 ml/L</td>
</tr>
<tr>
<td>Na&lt;sub&gt;2&lt;/sub&gt;EDTA.2H&lt;sub&gt;2&lt;/sub&gt;O</td>
<td>37.2g/L</td>
</tr>
</tbody>
</table>

### DEPC water
Add 0.1% DEPC to MilliQ water
Mix for over night
Autoclave for 20 min to remove DEPC
Store at RT

### LB broth
<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>10g</td>
</tr>
<tr>
<td>Tryptone</td>
<td>10g</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>5g</td>
</tr>
</tbody>
</table>
Add MilliQ water to a final volume of 1L
Adjust pH to 7.0 with 10M NaOH
Autoclave

### LB agar
<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>10g</td>
</tr>
<tr>
<td>Tryptone</td>
<td>10g</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>5g</td>
</tr>
<tr>
<td>Agar</td>
<td>20g</td>
</tr>
</tbody>
</table>
Add MilliQ water to a final volume of 1L
Adjust pH to 7.0 with 10M NaOH
Autoclave, Pour into petri dishes (~ 25ml/one plate)
**Cell lysis Buffer (western blot)**

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris</td>
<td>10 mM pH 7.2</td>
</tr>
<tr>
<td>NaCl</td>
<td>150 mM</td>
</tr>
<tr>
<td>SDS</td>
<td>1%</td>
</tr>
<tr>
<td>Sodium deoxycholate</td>
<td>0.1%</td>
</tr>
<tr>
<td>Triton X-100</td>
<td>0.1%</td>
</tr>
<tr>
<td>EDTA</td>
<td>0.5 mM</td>
</tr>
</tbody>
</table>

One complete mini-tab protease inhibitor for 50 ml lysis buffer

**Cell lysis Buffer (western blot)**

<table>
<thead>
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<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris</td>
<td>25 mM pH 8.0</td>
</tr>
<tr>
<td>SDS</td>
<td>10%</td>
</tr>
<tr>
<td>Protease inhibitor</td>
<td>One complete mini-tab</td>
</tr>
</tbody>
</table>

One complete mini-tab protease inhibitor for 50 ml lysis buffer

**4X Laemmlli sample buffer**

<table>
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<th>Concentration</th>
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<tbody>
<tr>
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<td>50 mM pH 6.8</td>
</tr>
<tr>
<td>SDS</td>
<td>2%</td>
</tr>
<tr>
<td>Glycerol</td>
<td>10%</td>
</tr>
<tr>
<td>EDTA</td>
<td>10 mM</td>
</tr>
<tr>
<td>BPB</td>
<td>0.02%</td>
</tr>
<tr>
<td>β-Mercaptoethanol</td>
<td>5% 12.5 M</td>
</tr>
</tbody>
</table>

**0.5 M Tris-HCl, pH 6.8**

<table>
<thead>
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<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris</td>
<td>6 g</td>
</tr>
<tr>
<td>MilliQ Water</td>
<td>60.0 ml</td>
</tr>
</tbody>
</table>

Adjust pH to 6.8 with 2 N HCl and made up to 100 ml with MilliQ Water

**1.5 M Tris-HCl, pH 8.8**

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
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<tbody>
<tr>
<td>Tris</td>
<td>27.3 g</td>
</tr>
<tr>
<td>MilliQ Water</td>
<td>80.0 ml</td>
</tr>
</tbody>
</table>

Adjust pH to 8.8 with 2 N HCl and made up to 150 ml with MilliQ Water
### Resolving Gel (pH 8.8)

<table>
<thead>
<tr>
<th>Percentage of gel</th>
<th>8%</th>
<th>10%</th>
<th>12.5%</th>
</tr>
</thead>
<tbody>
<tr>
<td>30% Acrylamide</td>
<td>2 ml</td>
<td>2.5 ml</td>
<td>3.1 ml</td>
</tr>
<tr>
<td>1.0M Tris-HCl pH 8.8</td>
<td>3 ml</td>
<td>3 ml</td>
<td>3 ml</td>
</tr>
<tr>
<td>20% (w/v) SDS</td>
<td>38 µl</td>
<td>38 µl</td>
<td>38 µl</td>
</tr>
<tr>
<td>MilliQ-H₂O</td>
<td>2.43 ml</td>
<td>1.9 ml</td>
<td>1.3 ml</td>
</tr>
</tbody>
</table>

Mixed together, add APS and TEMED just before pouring.

<table>
<thead>
<tr>
<th></th>
<th>8%</th>
<th>10%</th>
<th>12.5%</th>
</tr>
</thead>
<tbody>
<tr>
<td>10% APS</td>
<td>36 µl</td>
<td>36 µl</td>
<td>36 µl</td>
</tr>
<tr>
<td>TEMED</td>
<td>5 µl</td>
<td>5 µl</td>
<td>5 µl</td>
</tr>
<tr>
<td>Final Volume</td>
<td>7.5 ml</td>
<td>7.5 ml</td>
<td>7.5 ml</td>
</tr>
</tbody>
</table>

### Stacking Gel (pH 6.8)

<table>
<thead>
<tr>
<th>Percentage of gel</th>
<th>4%</th>
<th>6%</th>
</tr>
</thead>
<tbody>
<tr>
<td>30% Acrylamide</td>
<td>660 µl</td>
<td>1 ml</td>
</tr>
<tr>
<td>1 M Tris-HCl pH 6.8</td>
<td>630 µl</td>
<td>630 µl</td>
</tr>
<tr>
<td>20% (w/v) SDS</td>
<td>25 µl</td>
<td>25 µl</td>
</tr>
<tr>
<td>MilliQ H₂O</td>
<td>3.6 ml</td>
<td>3.6 ml</td>
</tr>
</tbody>
</table>

Mixed together, add APS and TEMED just before pouring.

<table>
<thead>
<tr>
<th></th>
<th>4%</th>
<th>6%</th>
</tr>
</thead>
<tbody>
<tr>
<td>10% APS</td>
<td>25 µl</td>
<td>25 µl</td>
</tr>
<tr>
<td>TEMED</td>
<td>5 µl</td>
<td>5 µl</td>
</tr>
<tr>
<td>Final volume</td>
<td>5 ml</td>
<td>5 ml</td>
</tr>
</tbody>
</table>

### 4X Electrophoresis stock buffer

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycine</td>
<td>57.4 g</td>
</tr>
<tr>
<td>Tris</td>
<td>7 g</td>
</tr>
<tr>
<td>SDS</td>
<td>4 g</td>
</tr>
</tbody>
</table>

Add MilliQ Water to a final volume 1 L.

### Tris-glycine Electrophoretic Transfer Buffer

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycine</td>
<td>14.4 g</td>
</tr>
<tr>
<td>Tris</td>
<td>3 g</td>
</tr>
<tr>
<td>Methanol</td>
<td>200 ml</td>
</tr>
</tbody>
</table>

Add MilliQ Water to a final volume 1 L.

Make it freshly each time and chilled to 4°C.
### Blocking Buffer

Non-fat milk powder – 5 g in 0.1% PBST to a final volume of 100 ml

### 0.1% PBST

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>1X PBS</td>
<td>999 ml</td>
</tr>
<tr>
<td>Tween 20</td>
<td>1 ml</td>
</tr>
</tbody>
</table>

### Blocking solution (BrdU labelling and staining)

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 X PBS</td>
<td>10 ml</td>
</tr>
<tr>
<td>Horse serum</td>
<td>3 drops</td>
</tr>
</tbody>
</table>

### Primary Antibody solution (BrdU labelling and staining)

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-BrdU antibody</td>
<td>50 µl</td>
</tr>
<tr>
<td>1 X PBS</td>
<td>10 ml</td>
</tr>
<tr>
<td>BSA</td>
<td>200 µg</td>
</tr>
</tbody>
</table>

### Secondary Antibody solution (BrdU labelling and staining)

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Secondary antibody</td>
<td>1 drop</td>
</tr>
<tr>
<td>1 X PBS</td>
<td>10 ml</td>
</tr>
<tr>
<td>Horse serum</td>
<td>2 drops</td>
</tr>
<tr>
<td>Tween 20</td>
<td>2 µl</td>
</tr>
</tbody>
</table>

### Tertiary Antibody solution (BrdU labelling and staining)

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reagent A</td>
<td>1 drop</td>
</tr>
<tr>
<td>Reagent B</td>
<td>1 drop</td>
</tr>
<tr>
<td>1 X PBS</td>
<td>10 ml</td>
</tr>
</tbody>
</table>

### Substrate solution (BrdU labelling and staining)

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>DAB tablet</td>
<td>1</td>
</tr>
<tr>
<td>H₂O₂ tablet</td>
<td>1</td>
</tr>
<tr>
<td>MilliQ H₂O</td>
<td>1ml</td>
</tr>
</tbody>
</table>
### Comassie destain

Methanol – 95 ml  
Glacial acetic acid – 75 ml  
Made up to 1 L with MilliQ H₂O

### 10X PBS

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>80 g</td>
</tr>
<tr>
<td>Na₂HPO₄</td>
<td>14.4 g</td>
</tr>
<tr>
<td>KCl</td>
<td>2 g</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>2.4 g</td>
</tr>
</tbody>
</table>

Make up to volume 1L with MilliQ H₂O  
Adjust pH 7.2 with HCl

### 10X Trypsin (0.25%)  

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trypsin</td>
<td>2.5 g</td>
</tr>
<tr>
<td>EDTA</td>
<td>0.372 g</td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>0.35 g</td>
</tr>
<tr>
<td>HBSS (Sigma H-4891)</td>
<td>1 bottle</td>
</tr>
</tbody>
</table>

Make up to volume 1L with MilliQ H₂O  
Adjust pH 7.2 with HCl  
Store at –20°C

### HBSS

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>KCl</td>
<td>0.4 g/L</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>0.06 g/L</td>
</tr>
<tr>
<td>NaCl</td>
<td>8.0 g/L</td>
</tr>
<tr>
<td>Na₂HPO₄</td>
<td>0.04788 g/L</td>
</tr>
<tr>
<td>D-glucose</td>
<td>1.0 g/L</td>
</tr>
</tbody>
</table>

### 0.4% Trypan blue

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trypan blue</td>
<td>0.4 g</td>
</tr>
</tbody>
</table>

Bring to a slow boil and cool to room temperature  
Make up to 100 ml with PBS
# APPENDIX – B

<table>
<thead>
<tr>
<th>Date</th>
<th>License number</th>
<th>Type of use</th>
<th>Publication</th>
<th>Title</th>
</tr>
</thead>
</table>