THE ROLE OF AUTOCRINE HUMAN GROWTH HORMONE (hGH) IN HER2+ BREAST CARCINOMA CELLS

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DECLARATION

I hereby declare that the thesis is my original work and it has been written by me in its entirety. I have duly acknowledged all the sources of information which have been used in the thesis.

This thesis has also not been submitted for any degree in any university previously.

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20th August 2014

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TABLE OF CONTENTS

ACKNOWLEDGEMENT ..... **ii**

TABLE OF CONTENTS ..... **iii**

ABSTRACT ..... **vi**

LIST OF FIGURES ..... **viii**

LISTS OF ABBREVIATIONS ..... **xiii**

1 INTRODUCTION **1**

1.1 Cancer 1

1.1.1 Hallmarks of Cancer 2

1.1.1.1 Sustaining Proliferative Signal 3

1.1.1.2 Evading Growth Suppressors 5

1.1.1.3 Resistance to Apoptosis 6

1.1.1.4 Limitless Replicative Potential 8

1.1.1.5 Activate angiogenesis 9

1.1.1.6 Activate Invasion and Metastasis 11

1.1.2 Emerging Hallmarks of Cancer 12

1.1.2.1 Reprogramming of energy metabolism 13

1.1.2.2 Evading Immune Destruction 14

1.2 Mammary Gland 16

1.2.1 Breast Structure and Function 16

1.2.2 Breast Cancer 18

1.2.3 HER2+ breast cancer 23

1.2.3.1 HER2 and its Function 23

1.2.3.2 HER-mediated Signal Transduction 25

1.2.3.3 HER2-mediated mammary carcinoma 27

1.2.3.4 HER2+ Breast Cancer Treatment 29

1.2.3.5 Intrinsic and Acquired Resistance in HER2+ Breast Cancer Treatment 33

1.3 Human Growth Hormone (hGH) 36

1.3.1 Growth Hormone and its Function 36

1.3.2 Growth Hormone Synthesis and Secretion 38

1.3.3 GH-mediated Signal Transduction 39

1.3.4 Growth Hormone-Related Disorder(s) 42

1.3.5 Autocrine hGH in Mammary Neoplasia and Breast Cancer 46

1.4 Autocrine hGH and HER2+ Breast Cancer 54

2 AIMS 56

3 MATERIALS AND METHODS 57

3.1 Breast Carcinoma Cell Lines 57

3.2 Cell Transfection 57

3.3 Drug Treatments 58

3.4 Herceptin-Resistance Cell Lines 59

3.5 Total RNA extraction 59

3.6 Reverse transcription (RT) and Polymerase Chain Reaction (PCR) 60

3.7 Protein Extraction 61

3.8 Protein Quantification 62

3.9 Conditioned Media 62

3.10 Protein Electrophoresis and Western Blot 63

3.11 hGH Enzyme-linked immunosorbent assay (ELISA) 64

3.12 Total Cell Number 64

3.13 Soft Agar Colony Formation (SACF) 65

3.14 Three-Dimensional (3D) Culture of Cells in Matrigel 66

3.15 Apoptosis 66

3.16 Statistics Analysis 67

4 RESULTS 68

4.1 Endogenous hGH expression in HER2+ mammary carcinoma cells 68

4.2 Forced Expression of *hGH* Gene in HER2+ Mammary Carcinoma Cells 70

4.3 Autocrine hGH increases BT474 and SkBR3 HER2+ mammary carcinoma cell number *in vitro* 73

4.4 Forced expression of hGH decreased BT474 and SkBR3 cells from serum deprivation-induced apoptosis 75

4.5 Forced expression of hGH increased BT474 anchorage-independent growth in soft agar and enhanced colony formation in 3D Matrigel™ 76

4.6 Forced expression of hGH in SkBR3 cells enhanced growth in 3D Matrigel™, but has no effect on anchorage-independent growth 78

4.7 Forced expression of hGH increased tyrosine phosphorylation of HER2 (Y1248) in BT474 and SkBR3 mammary carcinoma cells 79

4.8 Forced expression of hGH decreased sensitivity of HER2+ mammary carcinoma cells to Herceptin or Lapatinib treatment 80

4.9 Forced expression of hGH protected BT474 and SkBR3 cells from Herceptin and Lapatinib-induced apoptosis 83

4.10 Autocrine hGH enhanced BT474 anchorage-independent growth in soft agar after Herceptin treatment, but not Lapatinib treatment 85

4.11 Autocrine hGH enhanced BT474 and SkBR3 cell growth capacity in 3D-matrigel after Herceptin or Lapatinib treatment 87

4.12 Expression of hGH in BT474 and SkBR3 Herceptin-resistance mammary carcinoma cells 89

5 DISCUSSIONS 91

6 CONCLUSION 102

7 REFERENCES 103

ABSTRACT

The HER2+ breast cancer is an aggressive subtype of breast cancer arising from frequent amplification of *HER2/neu* gene that result in over-activation of HER pathways and cause uncontrolled cell proliferation and survival. The HER2+ breast cancer recurrence is largely due to resistance towards its therapy, which include the use of HER2-targeted agents such as Herceptin or Lapatinib. It is of importance to understand molecular mechanism towards therapeutic resistance in order to improve overall survival and relapse free survival in HER2+ breast cancer patients. Interestingly, a recent clinical data revealed positive association between HER2 status and tumour human Growth Hormone (hGH) in mammary carcinoma patients. It is thus worth to investigate on the potential role of autocrine expression of hGH and its therapeutic potential in HER2+ subtype breast cancer.

Herein, this project determined the oncogenic capabilities of autocrine hGH in mediating malignant transformation and reducing sensitivity of HER2+ mammary carcinoma cells towards therapeutic agents such as Herceptin or Lapatinib *in vitro*. Functional *in vitro* assays including total cell number, cell viability, soft agar colony formation, growth in 3-dimension (3D) Matrigel^{TM} and apoptosis demonstrated that forced expression of hGH significantly increases total cell number and enhances cell viability in 3D, and reduces apoptotic events in BT474 and SkBR3 HER2-amplified mammary carcinoma cells. Anchorage-independent growth is greatly enhanced in BT474 cells in the presence of autocrine hGH. One mechanism by which autocrine hGH exerts its oncogenic capacity in BT474 and SkBR3 HER2+ mammary carcinoma

cells is through stimulating tyrosine phosphorylation at Y1248 cytoplasmic residue, a major autophosphorylation site of HER2. Besides that, autocrine hGH expression provided selective growth advantage to BT474 and SkBR3 cells from Herceptin- and Lapatinb-induced growth inhibition *in vitro*. Functional inhibition of endogenous hGH using GHR antagonists (B2036 and G120R) was attempted to examine the synergistic effect of blocking GH signalling and inhibiting HER2 activity together with HER2-targeted agents in HER2+ mammary carcinoma cells. Unfortunately, the cell line models used is either unresponsive to B2036 or selected out at early passage in the presence of G120R. Nevertheless, early passage stable cells transfected with G120R exhibited significant reduction in oncogenic capacity in proliferation and anchorage-independent growth, implying that blockage of circulating hGH is effective to decrease oncogenicity of HER2+ mammary carcinoma cells. Based on the experimental evidence, it suggests that hGH may play as additional prognostic marker and possess predictive role in HER2-overexpressing breast cancer. Nevertheless, more work is required to verify the use of GHR antagonists in combination with HER2-targeted therapies in HER2+ tumours. Following that, preclinical and clinical studies can commence to assess feasibility and clinical relevance of introducing GH antagonists in HER2+ breast cancer treatment regimes.

LIST OF FIGURES

Figure 1-1 Hallmarks of cancer (adopted with permission from Hanahan and Weinberg, 2011) [2]. The hallmarks of cancer include: (1) Self-sufficient in growth signals, (2) Insensitivity to anti-growth signals, (3) Evading apoptosis, (4) Sustained angiogenesis, (5) Tissue invasion and metastasis and (6) Limitless replicative potential. 3

Figure 1-2 Schematic diagram depicting the effects of balance between pro- and anti-survival signals on cell homeostasis. (A) In normal healthy cell, cellular mechanisms are tightly controlled to ensure homeostasis in cell number and tissue architecture. (B) In cancer, self-sustainable growth signals and defective in anti-proliferation or cell death signals disrupt tissue homeostasis and drive uncontrolled cell growth to form macroscopic tumours, which may accumulate mutations that lead to malignant transformation to form cancer. 7

Figure 1-3 Standard cancer therapy versus cancer stem cell-targeted therapy. Tumour consists of heterogeneous population of cells that include a small subpopulation of cells known as cancer stem cells (CSC). Standard cancer therapy such as the use of chemotherapeutic drugs usually targets the actively dividing cancer cells rather than the quiescent CSC population, which in turn can initiate tumour re-growth and result in tumour relapse. In contrast, cancer stem cell-targeted therapy aims to directly target the quiescent CSC to eliminate cancer, and the loss of such tumour initiating capacity can greatly improve overall disease-free survival in cancer patients. 9

Figure 1-4 Existing and emerging hallmarks of cancers (adopted with permission from Hanahan and Weinberg, 2011) [2]. Both existing and emerging hallmarks of cancer provided the basis for conceptual understanding of the complex biology of cancer. Besides that, many therapeutic drugs such as the EGFR inhibitors, selective anti-inflammatory drugs and telomerase inhibitors are developed and targeted to these hallmarks of cancer for treatment of various human cancers.....13

Figure 1-5 Transverse section of human mammary gland or breast (adopted with permission from Ali and Charles Coombes, 2002) [38]. Mammary gland consists of a core made up of 15 to 20 lobes surrounded by a layer of adipose tissue. Each lobe is made up of smaller compartments known as lobules, which in turn are made up of milk-producing glands or alveoli. Each alveolus is hollow cavity lined with milk-secreting epithelial cells, which are surrounded by myoepithelial stromal cells and basement membrane. As myoepithelial cells contracts, milk is ejected into each small duct, which join together to form larger terminal ducts that drain milk via the nipple. 16

Figure 1-6 Transverse section of human mammary gland with illustration on the development of neoplastic growth (diagram adopted from breastcancer.org. website). The development of ductal breast carcinoma *in situ* (DCIS) (A) and lobular carcinoma *in situ* (LCIS) (B) towards invasive

cancer as cells break out of the ducts and/or lobules and invade the surrounding stroma. 19

Figure 1-7 Histological images of normal versus carcinoma breast tissue (adopted with permission from Debnath and Brugge, 2005) [42]. Histological image of normal breast tissue revealed a highly organized structure where the hollow lumen is surrounded by luminal secretory cells, which in turn is surrounded by a layer of myoepithelial and basement membrane. Such highly organized structure of normal breast tissue is disrupted in breast carcinoma tissues and is distinct between ductal/lobular non-invasive, ductal/lobular invasive and metastatic cancer tissues samples. For instance, invasive breast tissue samples revealed cells infiltrated into surrounding luminal cells, formed solid nest, poorly formed tubules and filling up of luminal space. 20

Figure 1-8 Members of the human epidermal growth factor receptor (ErbB/HER) family and its phosphotyrosine interacting partners (adopted with permission and modified from Schulze *et al.*, 2005 [59]). All members of ErbB/HER family consist of an extracellular domain, transmembrane domain and intracellular domain. Each member has its distinct phosphotyrosine interacting partners at its intracellular domain. 24

Figure 1-9 Overview of the HER signalling pathway (Illustration reproduced courtesy of Cell Signaling Technology. Inc. (www.cellsignal.om. <http://www.cellsignal.com/>). It illustrated the signalling molecules involved for activation of cellular activities such as cell differentiation, proliferation, growth and chemotaxis. 27

Figure 1-10 Signalling pathways activated in the presence of HER2 overexpression (adopted with permission from Kruser and Wheeler, 2010) [52]. Overexpression of HER2 resulted in increased formation of HER1/HER2 heterodimers, HER2/HER2 homodimers and HER2/HER3 heterodimers, which ultimately enhances activation of proliferation and survival pathways..... 29

Figure 1-11 Schematic diagram illustrating HER2-targeted agents use in the treatment of HER2+ breast cancer. Antibody-based anti-HER2 agents such as Herceptin and Pertuzumab bind to the extracellular domain of HER2 to block its signalling. Trastuzumab-DM1 (TDM-1), an antibody drug conjugate targeting the extracellular domain of HER2 and prevents microtubule polymerization when internalized into cells. Lapatinib is a tyrosine kinase inhibitor (TKI) targeting both EGFR and HER2 by competing with ATP binding site that prevents phosphorylation of cytoplasmic tail and block activation of downstream signalling pathways. 32

Figure 1-12 Schematic diagram representing the human *GH* gene cluster, which consists of GH-N, CS-L, CS-A, GH-V and CS-B (adopted with permission from Kopchick and Andry, 2000) [88]. The *hGH* gene (GH-N) consists of 5 exons (I-V) and four introns (a-d). The GH gene is located on the long arm of chromosome 17. 38

Figure 1-13 Schematic diagram illustrating production of growth hormones and mode of GH action (adopted with permission and modified from Kopchick and Andry, 2000) [88]. Upon receiving appropriate stimuli, pituitary GH will be released in the body. Circulating GH acts on target organs such as liver, muscle, adipose and bone for the release of IGF-1 for growth-stimulatory effects. There are positive and negative regulatory mechanisms that regulate the release of GH.....39

Figure 1-14 Schematic diagram of growth hormone receptor (GHR) (adopted with permission and modified from Zhu *et al.*, 2001 [92]. GHR consists of an extracellular domain for ligand binding, a transmembrane domain and an intracellular domain. Janus Kinase 2 (JAK2) is closely associated to GHR and binds to Box 1 region of this receptor. 40

Figure 1-15 Mode of action of GH versus GHR antagonist, B2036 and PEG-B2036. (adopted with permission from Pradhanaga *et al.*, 2002) [107]. (A) Circulating GH binds to GHR via growth hormone binding protein (GHBP), resulting in GHR dimerization and signal transduction. (B) The B2036 molecule binds to GHR with increase affinity at site 1, but impairs binding at site 2 and block GHR signal transduction. (C) Polyethyl glycol-conjugated B2036 or pegvisomant.....45

Figure 1-16 Autocrine hGH-mediated signal transduction in human mammary epithelial cells (adopted with permission from Perry *et al.*, 2006) [95]. Autocrine hGH is reported to up-regulate telomerase activity, oncogenic transformation, epithelial to mesenchymal transition and chemoresistance, which together provided a platform for oncogenic transformation in breast cancer. 51

Figure 1-17 Oncogenic potential of autocrine hGH acting in autocrine and paracrine (adopted with permission from Perry *et al.*, 2006) [95]. Autocrine hGH upregulate soluble peptide factors, which include insulin-like growth factor-1 (IGF-1) and trefoil factor 1 and 3 (TFF1 and TFF3), bone morphogenic protein-7 (BMP-7) that drives oncogenic transformation.....52

Figure 1-18 Kaplan-Meier analysis examining the significance of tumour hGH expression and/or tumour hPRL expression on both relapse-free survival (RFS) of patients with mammary carcinoma patients (adopted with permission from Wu *et al.*, 2011) [132]......54

Figure 4-1 Endogenous expression of hGH, hGHR and hPRLR in wild-type HER2+ breast carcinoma cells. (A) Endogenous gene expression of *hGH, hGHR* and *hPRLR* in BT474, MDA-MB-361, SkBR3 and MDA-MB-453 HER2+ breast carcinoma cells. (B) Endogenous protein expression of hGH in HER2+ breast carcinoma cells. The loading control used was β-actin. 69

Figure 4-2 hGH ELISA of HER2+ human mammary carcinoma cells, BT474, MDA-MB-361, SkBR3 and MDA-MB-453. (A) Standard curve obtained by plotting concentration of recombinant hGH versus absorbance

(A_{405nm} − A_{490nm}). (B) Quantitative amount of secreted hGH from BT474, MDA-MB-361, SkBR3 and MDA-MB-453 cells. 70

Figure 4-3 Forced expression of hGH in BT474 and SkBR3 HER2+ breast carcinoma cells. (A and C) Expression of hGH in stably transfected BT474 cells and SkBR3 cells respectively. (B and C) Western Blot demonstrated forced expression of hGH in stably transfected BT474 and SkBR3 cells in both whole cell lysate and conditioned media. All experiments used β-actin as loading control. 72

Figure 4-4 hGH ELISA of BT474-Vec, BT474-hGH, SkBR3-Vec and SkBR3-hGH stable cells. (A) Standard curve obtained using recombinant hGH as positive control. (B) Quantitative amount of secreted hGH expressed in BT474-Vec, BT474-hGH, SkBR3-Vec and SkBR3-hGH stable mammary carcinoma cells..... 73

Figure 4-5 Total cell number of BT474-Vec, BT474-hGH, SkBR3-Vec and SkBR3-hGH mammary carcinoma cells over a period of 10 days. (A and B) Total cell number of BT474-Vec and BT474-hGH cells in media supplemented with 10% and 0.5% FBS respectively. (C and D) Total cell number of SkBR3-Vec and SkBR3-hGH cells in media supplemented with 10% and 0.5% FBS respectively. (*$p < 0.05$)..... 74

Figure 4-6 Forced expression of hGH reduced apoptosis in BT474 and SkBR3 cells. (A and B) Caspase 3/7 activity presented as percent control of BT474-Vec/hGH and SkBR3-Vec/hGH respectively after 24 hours serum deprivation. (*$p < 0.05$) 76

Figure 4-7 Forced expression of hGH increased formation of colony and cell viability of BT474 cells in soft agar and 3D Matrigel™. (A and B) Cell viability of BT474-Vec/hGH cells in 0.5% and 10% FBS respectively with representative pictures of colonies formed by BT474-Vec/hGH cells at the indicated conditions. (C) Cell viability and representative picture of BT474-Vec/hGH in 3D Matrigel. Images taken at 4X magnification. (*$p < 0.05$) 77

Figure 4-8 Autocrine hGH increases SkBR3 growth and colony formation in 3D Matrigel. (A) Cell viability and representative pictures of SkBR3-Vec/hGH colonies growth and formation in 3D Matrigel™. Images taken at 10X magnification. (*$p < 0.05$)..... 79

Figure 4-9 Forced expression of hGH increased phosphorylation of HER2 (Y1248) activity in BT474 and SkBR3 mammary carcinoma cells (A and B). The β-actin is used as loading control. Quantitative analysis of the gel bands were also determined using Image J, and the values presented are relative density against β-actin..... 80

Figure 4-10 Dose response curve of Lapatinib on HER2+ breast carcinoma cells plotted as cell viability versus log concentration of lapatinib. (A and B) The IC_{50} of Lapatinib on BT474-Vec/hGH and SkBR3-Vec/hGH stable cells was determined to be 150 nM and approximately 15 nM respectively. 81

Figure 4-11 Autocrine hGH enhanced cell viability of BT474 and SkBR3 cells after treatment with Herceptin or Lapatinib. (A and C) Cell viability of BT474-Vec/hGH and SkBR3-Vec/hGH treated with 50 μg/ml Herceptin or equivalent concentration of BSA (untreated control). (B and D) Cell viability of BT474-Vec/hGH and SkBR3-Vec/hGH treated with 150 nM or 15 nM Lapatinib or equivalent concentration of DMSO (untreated control) respectively. (*$p < 0.05$) 82

Figure 4-12 Autocrine hGH significantly protected HER2+ breast carcinoma cells from Herceptin- and Lapatinib-induced inhibition. (A and B) Caspase 3/7 activity of BT474-Vec/hGH cells in the presence of Herceptin and Lapatinib respectively. (C and D) Caspase 3/7 activity of SkBR3-Vec/hGH cells in the presence of Herceptin and Lapatinib respectively. (* $p < 0.05$) 85

Figure 4-13Autocrine expression of hGH enhanced anchorage-independent growth in BT474 cells in the presence of Herceptin, but not Lapatinib. (A and C) Cell viability BT474-Vec/hGH cells in the presence of Herceptin and Lapatinib respectively. (B and D) Representative pictures of BT474-Vec/hGH colonies formation after Herceptin or Lapatinib treatment respectively. Images taken at 4X magnification. (* $p<0.05$; ns: not significant) 86

Figure 4-14 Autocrine expression of hGH enhanced BT474 cells proliferation and colony formation in 3D Matrigel™ after Herceptin or Lapatinib treatment. (A and C) Cell viability of BT474-Vec/hGH in 3D Matrigel treated with Herceptin or Lapatinib respectively. (B and D) Representative pictures of BT474-Vec/hGH colonies formation in 3D matrigel after Herceptin or Lapatinib treatment respectively. Images taken at 4X magnification. (*$p < 0.05$) 88

Figure 4-15 Autocrine expression of hGH enhanced SkBR3 cells proliferation and colony formation in 3D Matrigel™ after Herceptin or Lapatinib treatment. (A and C) Cell viability of SkBR3-Vec/hGH in 3D Matrigel treated with Herceptin or Lapatinib respectively. (B and D) Representative pictures of SkBR3-Vec/hGH colony cell growth in 3D matrigel after Herceptin or Lapatinib treatment respectively. Images taken at 4X magnification. (*$p < 0.05$) 89

Figure 4-16 Expression level of *hGH* gene in BT474 and SkBR3 Herceptin-resistant mammary carcinoma cells. Both BT474 and SkBR3 cells were treated with Herceptin at 10 μg/ml and 50 μg/ml respectively for a period of 6-9 months. 90

LISTS OF ABBREVIATIONS

αCP1	Poly(C)-binding protein
ADCC	Antibody-dependent cell-mediated cytotoxicity
aPKC	Atypical protein kinase C
ATP	Adenosine Triphosphate
BBB	Blood brain barrier
BCL2	B-cell lymphoma 2
BRCA	Breast cancer susceptibility gene
BMP-7	Bone morphogenic protein-7
CHOP	C/EBP homologous protein
CNS	Central nervous system
CSC	Cancer stem cell
CS-A	Chorionic somatomammotropin-A gene
CS-B	Chorionic somatomammotropin-B gene
CS-L	Chorionic somatomammotropin-like gene
CTL	Cytotoxic T lymphocytes
DCIS	Ductal carcinoma *in situ*
DNA	Deoxyribonucleic acid

ECM	Extracellular matrix
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ER	Estrogen receptor
EMT	Epithelial-mesenchymal transition
FAK	Focal adhesion kinase
GH	Growth hormone
GH-N	GH-normal gene
GH-V	GH-variant gene
GHR	Growth hormone receptor
GHRH	Growth hormone releasing hormone
GHRP	Growth hormone releasing peptides
Grb2	Growth facto receptor bound 2
GRB7	Growth factor-receptor bound protein 7
HMEC	Human mammary epithelial cells
HMEC-1	Human microvascular endothelial cells
HMCC	Human mammary carcinoma cells
hTERT	Human telomerase reverse transcriptase

IGF-1	Insulin growth factor
IGF-1R	Insulin growth factor 1 receptor
IR	Ionising radiation
JAK	Janus Kinase
HER/ErbB	Human epidermal growth factor receptor
LCIS	Lobular carcinoma *in situ*
LNM	Lymph node metastases
MAPK	Mitogen activated protein kinase
MET	Mesenchymal-epithelial transition
MMC	Mitomycin C
MMP	Matrix metalloproteinase
mTOR	Mammalian target of rapamycin
NADPH	Nicotinamide adenine dinucleotide phosphate
NF-κB	Nuclear factor κB
NGR	Neuregulin
NK	Natural killer
ROS	Reactive oxygen species
Rb	Retinoblastoma

rhGH	Recombinant human GH
RFS	Relapse free survival
OS	Overall Survival
Par6	Partition protein 6
PCD	Programmed cell death
PEG	Polyethyl glycol
PI3K-Akt	Phosphatidylinositol-3 kinase-anti-apoptotic serine/threonine kinase
PL	Placental lactogen
PR	Progesterone receptor
PRL	Prolactin
PRLR	Prolactin receptor
PLC	Phospholipase C
PPP	Pentose phosphate pathway
PTB	Phosphotyrosine-binding
Shc	Src homonogy 2/α collagen-related
STAT	Signal transducer and activator of transcription
TDM-1	Trastuzumab emtansine
TKI	Tyrosine kinase inhibitor

TGF-β	Transforming growth factor β
TFF	Trefoil factor
Tsp-1	Thrombospondin-1
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor

1 INTRODUCTION

1.1 Cancer

Cancer development involves multiple processes where normal cell begin to grow uncontrollably and progressively giving rise to new or abnormal growth of cell mass referred to as tumour [1-3]. It is therefore characterized by a loss in normal cell control mechanisms that governs essential cellular functions such as survival, proliferation and differentiation, thereby resulting in disturbed cellular homeostasis [1, 2].

According to GLOBOCAN 2008 database (version 1.2, http://globocan.iarc.fr), the leading cause of mortality worldwide is due to cancer, which accounts for 7.6 million deaths in addition to an approximated 12.7 million new cancer cases were diagnosed. Each year, cancers of the lung, stomach, liver, colorectal and breast cause the most death among all other cancer types. It is projected that cancer mortality to rise to 21 million in 2030 worldwide [4].

In Singapore, an estimate of 1 in 4 Singaporean dies of cancer. In addition, 14 people die from cancer each day and 28 people are diagnosed with cancer every day (Singapore Cancer Registry, Interim Annual Registry Report, Trends in Cancer Incidence in Singapore, 2006-2010). Based on the Singapore Cancer Registry, the top 3 cancers diagnosed in Singapore for female are cancer of the Breast (29%), Colorectal (14%) and Lung (8%). For males, the top 3 cancers are Colorectal (18%), and Lung (16%) followed by Prostate

(11%) cancers (Singapore Cancer Registry, Interim Annual Registry Report, Trends in Cancer Incidence in Singapore, 2006-2010).

In a normal healthy cell, the basic important cellular processes including cell division, motility, differentiation and proliferation are tightly controlled by cell-cell interaction, cell-extracellular matrix (ECM) and other microenvironmental cues (e.g. soluble hormones, cytokines, integrins and growth factors) [1-3, 5]. These interactions transduce many different signals that regulate important cellular functions for gene expression, differentiation, proliferation, motility and even cell death that are necessary to ensure tissue homeostasis [3]. However, in the case of cancer, it is evident that cells acquire biological capabilities or accumulate cellular mutations and/or defects that perturb the tightly controlled cellular regulatory mechanisms [1, 2].

1.1.1 Hallmarks of Cancer

In year 2000, Hanahan and Weinberg proposed six important hallmarks that are required and essential for the development and maintenance of cancer: (1) sustain proliferative signals, (2) evade growth suppressor through insensitivity to inhibitory growth signals, (3) resist cell death via apoptosis or programmed cell death, (4) limitless replication potential, (5) induce angiogenesis, and (6) activate tissue invasion and metastasis. During cancer development, cells begin to acquire some of these functional capabilities at some stage to successfully evade anti-cancer defence mechanisms and transform from normal human cells into a tumorigenic cell mass that can progress and become malignant cancer [1, 2] (Figure 1-1). Each essential cancer hallmark or characteristic is further elaborated in the following sub-headings.

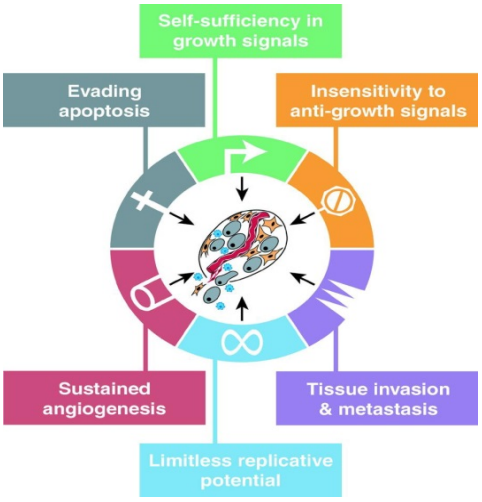


Figure 1-1 Hallmarks of cancer (adopted with permission from Hanahan and Weinberg, 2011) [2]. The hallmarks of cancer include: (1) Self-sufficient in growth signals, (2) Insensitivity to anti-growth signals, (3) Evading apoptosis, (4) Sustained angiogenesis, (5) Tissue invasion and metastasis and (6) Limitless replicative potential.

1.1.1.1 Sustaining Proliferative Signal

In normal physiological condition, cell undergoes active proliferation from quiescent state upon receiving appropriate stimuli or growth signals which include growth factors, components of the extracellular matrix, and cell-to-cell adhesion or interaction molecules [1]. Growth signals are transmitted and relied into the cell by binding to transmembrane receptors displayed on cell surface. Activated transmembrane receptors recruit intracellular adaptors and signalling molecules that rely signals downstream to activate proliferative pathway(s). The availability or release of growth signals are tightly controlled and regulated to ensure homeostasis of cell number for maintenance of normal tissue architecture [2]. However, in cancer, many oncogenes acquire the capability to mimic normal growth signalling that enables self-sufficient growth in sustained manner [2].

Unlike normal cells in culture that depends and utilizes exogenous growth stimulation for maintenance of proper cellular and physiological behaviour,

cancer cells are less dependent on exogenous growth stimuli from their microenvironment for survival by generating their own growth signals [1, 2]. Cancer cells can synthesize growth factors creating a positive feedback signalling loop or autocrine stimulation that allows sustain survival [6]. Alternatively, cancer cells can secrete soluble factors or transmit signals to neighbouring stroma cells, which in turn supply growth factors back to the cancer cells in a paracrine mode [3, 6]. Sustainable proliferative signals are therefore available through these autocrine and/or paracrine stimulation from the cancer cells and/or neighbouring cells that eventually promote uncontrolled cell growth.

Besides supplying themselves the growth signals, deregulation in the transmembrane growth receptors can contribute to sustainable proliferative signals in cancer cells. Deregulation in expression of tyrosine kinase growth receptors can result in over-expression of the receptors, thereby allowing cancer cells to become hyper-responsive to growth signal at low levels [1]. Moreover, cancer cells can utilize ligand-independent signalling through structural alteration of its receptors. Structural alteration such as mutation or truncation may render receptor constitutively active without the need for extracellular stimuli [7, 8]. Hence, growth signals will constantly be available and relied into the cells. Furthermore, mutation or up-regulation of downstream growth effectors such as transcription factors or other intracellular signalling molecules can result in constitutive activation of growth signal pathway(s). Acquisition of such capabilities allows cancer cells to circumvent the needs to stimulate growth pathway(s) via receptor-mediated signalling.

1.1.1.2 Evading Growth Suppressors

Cell and tissue homeostasis are also governed by multiple anti-proliferative signals and negative feedback mechanisms that prevent cells from uncontrolled growth. Many of these anti-proliferation signals can be found to function in the cell cycle.

Cell cycle is a series of an orderly process that allow cells to go through division and replication. It consists of G1 phase, S phase (synthesis), G2 phase and M phase (mitosis). As cells proceed through the cell cycle sequentially from G1 → S → G2 → M phases, checkpoint control mechanisms at the G1, G2 and M phases allow necessary cellular repair before 'defected' cell is allowed to transit to the next phase of its growth cycle. Defects in repair mechanisms at these cell cycle checkpoints or deregulation of molecules involved in cell cycle progression will lead to serious consequences. For instance, tumour suppressors are one mediator that negatively regulates proliferation and play important role in cell cycle checkpoints [9, 10]. The retinoblastoma-associated (Rb) protein is one of the tumour suppressors extensively studied, which function to inhibit cell cycle progression by repressing E2F transcription factors and prevent excessive cell growth [9]. The loss of such tumour suppressor gene function is thus disastrous or detrimental as cells with accumulated cancer-related mutation will be allowed to advance through the cell cycle aberrantly, ultimately leading to cancer [10]. Mutation in another tumour suppressor gene, p53 is also often observed to be associated to cancer [10]. The ability to evade growth suppressors can thus drive uncontrolled growth that is observed in cancer cells.

1.1.1.3 Resistance to Apoptosis

Resistance towards apoptosis or programmed cell death (PCD) is another typical trait observed in almost all types of cancers. Apoptosis is a process where cells undergo death in response to physiological stress such as reactive oxygen species (ROS), DNA damage or injury [5]. Extracellular and intracellular environmental cues will activate anti-apoptotic or pro-apoptotic molecules that determine if a cell should survive or proceed to death pathway.

Adequate apoptotic events are essentially important to maintain cell number and homeostasis. Excessive apoptosis can lead to atrophy while insufficient apoptotic event causes uncontrolled cell proliferation. Defective apoptosis has been shown to be implicated in diseases such as cancer. For instance, mutation or loss of pro-apoptotic regulators like the B-cell lymphoma 2 (BCL-2) have been demonstrated to allow cancer cell to evade apoptosis [11].

Cancer cells not only acquired the ability to evade apoptosis, but also escape anoikis [5, 12, 13]. Anoikis is a normal cell death mechanism or program triggered by cell detachment from surrounding stroma or ECM [5, 12]. This mechanism prevents detached or dying cells from growing and colonizing at secondary site, thereby inhibiting aberrant cell growth or attachment to an inappropriate matrix subsequently [5, 12]. Resistance to anoikis allows anchorage-independent growth and survival during tumour dissemination. The ability to acquire anchorage-independent growth is prominently implicated in malignant transformation observed in many cancer-related studies [14, 15]. Deregulation of anoikis can be achieved via multiple mechanisms, which may include activation of pro-survival pathways through autocrine and paracrine

growth signals, change in integrin expression patterns allowing cells to receive survival signals and growth in different microenvironment, activation of oncogenes, overexpression of growth factor receptor, upregulation of key enzymes involved in integrin or growth factor receptor signalling and activation of epithelial-mesenchymal transformation (EMT) ability [5, 12, 13].

The importance in the balance between cell growth and cell death signals therefore greatly determine tissue homeostasis and dictate the ability of cells to generate a surplus of vast cell population that eventually constitute macroscopic tumours (Figure 1-2).

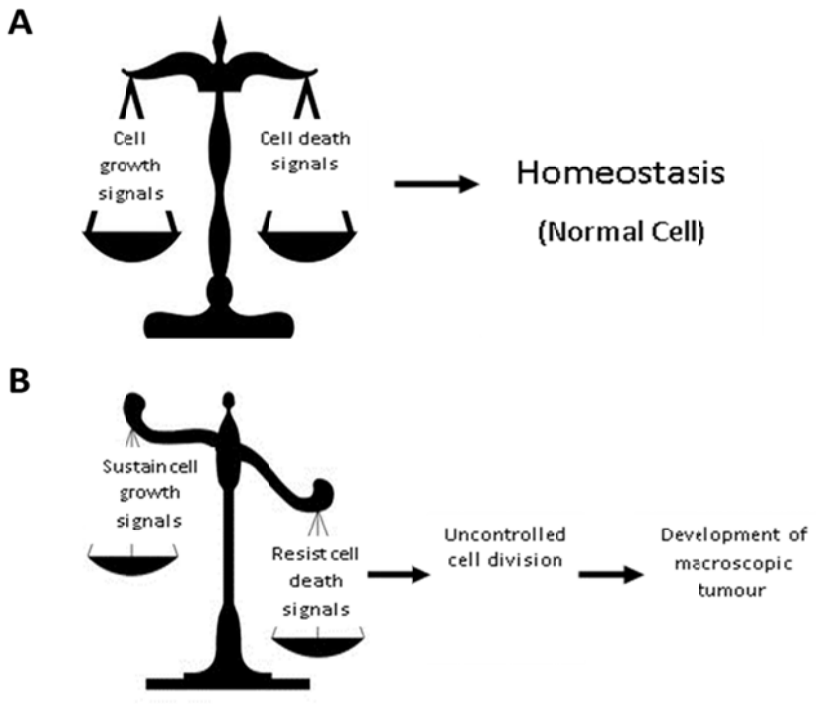


Figure 1-2 Schematic diagram depicting the effects of balance between pro- and anti-survival signals on cell homeostasis. (A) In normal healthy cell, cellular mechanisms are tightly controlled to ensure homeostasis in cell number and tissue architecture. (B) In cancer, self-sustainable growth signals and defective in anti-proliferation or cell death signals disrupt tissue homeostasis and drive uncontrolled cell growth to form macroscopic tumours, which may accumulate mutations that lead to malignant transformation to form cancer.

1.1.1.4 Limitless Replicative Potential

A normal cell has limited lifespan or number of doublings before it enters senescence. As each cell cycle progresses, telomeres located at the end of each chromosome will be shortened until it reach a critical shortened length where DNA damage will be induced to signal cells to enter senescence [16]. Unlike normal cells, telomeres in cancer cells are maintained, which is achieved through upregulation of telomerase enzyme [17]. Therefore, cancers have the ability to divide continuously beyond their replicative potential, and may eventually lead to immortalization.

Recent evidences in cancer studies have suggested presence of a small subpopulation of cells, referred to as cancer stem cells (CSC), within a tumour mass [18]. Like other progenitor stem cells, CSCs possess traits such as quiescent, maintain at G0 phase at cell cycle, self-renewal and able to undergo repeated proliferation through cell cycles. Acquisition of stem cell-like properties allow cancer cells to continuously drive tumour progression when it receive appropriate signals to exit G0, enter the cell cycle to begin replication, synthesize new cancer cells and initiate tumour formation. The existence of CSCs population poses a big challenge and limitations to standard or conventional cancer therapies, which tend to target the actively dividing cancer cells rather than the quiescent CSCs population. Hence, CSCs have been implicated in metastatic dissemination and cancer relapse due to resistance to treatment therapies (Figure 1-3). Currently, cancer research across academia and pharmaceutical industry focus on targeting the CSCs population by looking for specific biomarkers, aiming to eliminate these

quiescent CSC population with tumour-initiating property that are capable of evading standard cancer therapies and therefore overcome multidrug resistance [18] (Figure 1-3). This anti-cancer drug discovery approach, if successful, can greatly improve the overall disease-free survival in cancer patients.

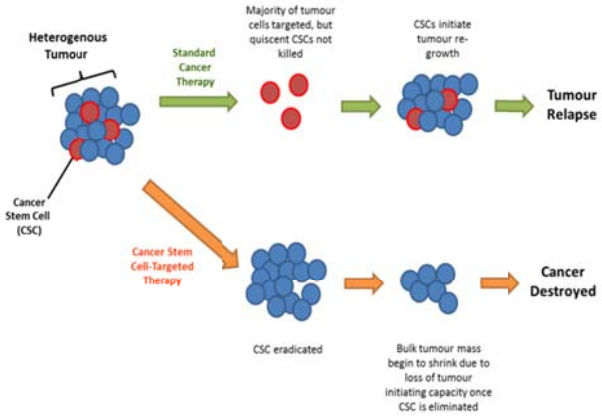


Figure 1-3 Standard cancer therapy versus cancer stem cell-targeted therapy. Tumour consists of heterogeneous population of cells that include a small subpopulation of cells known as cancer stem cells (CSC). Standard cancer therapy such as the use of chemotherapeutic drugs usually targets the actively dividing cancer cells rather than the quiescent CSC population, which in turn can initiate tumour re-growth and result in tumour relapse. In contrast, cancer stem cell-targeted therapy aims to directly target the quiescent CSC to eliminate cancer, and the loss of such tumour initiating capacity can greatly improve overall disease-free survival in cancer patients.

1.1.1.5 Activate angiogenesis

Angiogenesis is a process where new capillary blood vessel is formed from pre-existing vessels to supply oxygen, nutrients and gas exchange in all cells of a tissue for normal cellular function and survival [19, 20]. This process is important during normal physiological processes which include embryo development and placenta implantation. Like normal tissues, tumours require oxygen and nutrients supplies as well as removal of waste. Cancer cells located at the inner tumour mass are often challenged by hypoxic environment. Hence, cancer cells acquire angiogenic capabilities to circumvent this problem to allow tumour growth. Additionally, angiogenesis

plays important role in metastatic development by allowing cancer cells dissemination via the newly-formed vasculatures.

The process of angiogenesis is summarised with the involvement of activating endothelial cells that trigger degradation of both basal membrane and ECM of surrounding cells [21]. Endothelial cells response to pro-angiogenic and anti-angiogenic factors such as vascular endothelial growth factor (VEGF) and Thrombospondins respectively. The balance between pro-angiogenic and anti-angiogenic factors thus control formation of new blood vessel [22].

In cancer, new vasculature is formed via a phenomenon termed angiogenic switch, where there is an imbalance between pro-angiogenic and anti-angiogenic factors [19]. The pro-angiogenic factors are upregulated while angiogenic inhibitors are downregulated in the case of cancer. Tumour cells can secrete pro-angiogenic factors such as VEGF, which can bind to its receptors (VEGFR) on the endothelial cells of pre-existing blood vessels. Such interaction resulted in the secretion of proteolytic enzymes including the matrix metalloproteinases (MMPs) to degrade the basement membrane and ECM. Following degradation, endothelial cells migrate towards the tumour to deposit a new basement membrane. Besides that, endothelial cells will secrete growth factors to the surrounding cells, which in turn support stabilization of new blood vessel at the tumour site [19]. Newly established blood vessel thus enables oxygen and nutrients to be delivered to the hypoxic tumour cells, ultimately allowing tumour growth. Many studies have demonstrated angiogenic factors expressed by cancer cells have an effect on endothelial cell growth *in vitro* [23]. Secreted proteins possessing angiogenic potential such as

the human growth hormone (hGH) [22], trefoil factors (TFFs) [24] and artemin [25] have also been reported to possess angiogenic potentials by enhancing and increasing endothelial cell growth, migration and vascular tube formation both *in vitro* and *in vivo*.

1.1.1.6 Activate Invasion and Metastasis

During the development of human cancer, primary tumour cells can acquire the ability to invade neighbouring tissues and metastasize to a distant site where they may colonize and form secondary tumours [26-28]. Metastatic cancers cause 90% of human cancer death due to resistance to therapeutic treatments and therefore many therapeutic drugs are developed to target metastasis [1, 28].

During the process of invasion, cells undergo epithelial-to-mesenchymal transition (EMT) where epithelial cells downregulate cell-cell interactions, reorganize cytoskeletons, modify cell shape and polarity, and acquire mesenchymal properties. Acquiring invasive capability is the first step that primary tumours begin to disseminate where epithelial markers (e.g. E-CADHERIN and OCCLUDIN) are downregulated and mesenchymal markers (e.g. N-CADHERIN, VIMENTIN, FIBRONECTIN and SNAIL) are upregulated to facilitate metastasis thereafter [26].

Metastasis is a complex multi-step process which involves local invasion, intravasation into lymph and/or blood vessel, survival in circulation, extravasation, recolonization and finally expansion [26, 28]. Upon re-colonization, cancer cells have to undergo reverse EMT or mesenchymal-epithelial transition (MET) to successfully reside and establish tumour growth

at a secondary site [27, 29, 30]. Some studies suggested that therapeutic strategies against EMT alone may be insufficient to target metastatic cancers. It should be combined with MET inhibitors for better eradication of metastatic tumours [26-30].

1.1.2 Emerging Hallmarks of Cancer

Our knowledge and understanding on cancer biology has been enriching as cancer research progresses with more and more scientific findings over the past decades. Many of the new scientific findings have suggested emergence of new hallmarks in addition to the six existing ones, which together, plays crucial role in progression of neoplastic diseases. These emerging hallmarks of cancer include reprogramming of energy metabolism and evading immune destruction (Figure 1-4) [2].

Both the existing and emerging hallmarks of cancer have provided a basic concept for understanding cancer biology. Additionally, it provided a basis for developing therapeutic drugs for treatment against the key hallmarks of cancer.

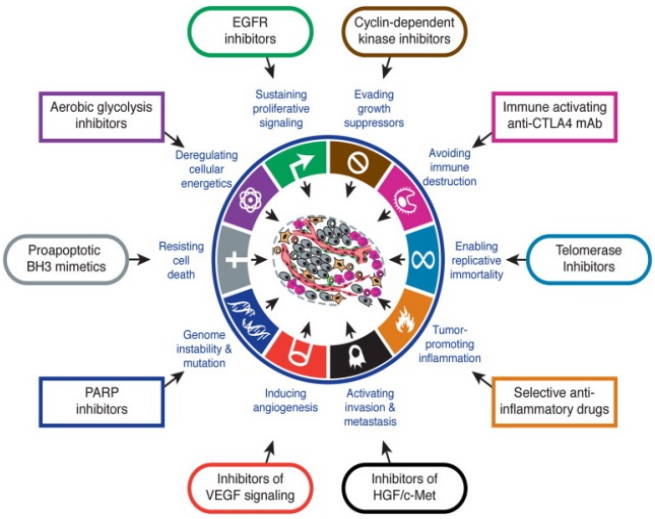


Figure 1-4 Existing and emerging hallmarks of cancers (adopted with permission from Hanahan and Weinberg, 2011) [2]. Both existing and emerging hallmarks of cancer provided the basis for conceptual understanding of the complex biology of cancer. Besides that, many therapeutic drugs such as the EGFR inhibitors, selective anti-inflammatory drugs and telomerase inhibitors are developed and targeted to these hallmarks of cancer for treatment of various human cancers.

1.1.2.1 Reprogramming of energy metabolism

Normal proliferating cells utilises biosynthesis pathway such as glycolysis and oxidative phosphorylation to produce nutrients and energy to fuel cell growth, cell division and replication. Hence, metabolism of proliferating cells differ from cells at resting stage [31]. Similarly, cancer cells do display altered metabolism to meet its bioenergetics and biosynthetic demands due to the high rate of cell division as well as oxygen and nutrients availability.

Most aerobic cellular organisms utilize oxidative phosphorylation for energy supply due to its high efficiency in releasing energy. However, cancer cells utilize aerobic glycolysis, a phenomenon referred as Warburg Effects [32, 33]. In the case of cancer, glucose breakdown from aerobic glycolysis directly provide cancer cells with readily available intermediates such as ribose sugar for generating nucleotides and amino acids. This in turn facilitates

biosynthesis of macromolecules such as nucleic acids, proteins and lipids needed to assemble new cancer cells [31]. The Adenosine Triphosphates (ATPs) generated during aerobic glycolysis serve as bioenergy for cancer cells. It is reported that the oncogene *MYC* can stimulate transcription of genes involved in mediating glycolysis pathway to fuel cancer cells [34]. Other pathways utilized by cancer cells include pentose phosphate pathway (PPP), fatty acid synthesis and glutaminolysis [1, 2, 35]. The use of PPP mainly generates pentoses for nucleic acid synthesis and nicotinamide adenine dinucleotide phosphate (NADPH) as reducing agent to prevent cancer cells from oxidative stress [35].

Cancer cells preferential usage of other alternative metabolic pathways instead of oxidative phosphorylation may be due to the high production of ROS by the latter during the generation of ATP [35]. Oxidative phosphorylation can generate up to a maximum of 36 ATP molecules. The higher number of ATP produced, the more ROS generated. Presence of ROS or by-products such as superoxide, peroxide and hydroxyl radicals are harmful to cell by triggering cellular death. Therefore, cancer cells exhibited differential or altered metabolic profiles so as to strike a balance to obtain sufficient bioenergy and biosynthesis from alternative sources and at the same time reduce oxidative stress that is disadvantage to its survival.

1.1.2.2 Evading Immune Destruction

The immune system consists of an array of cells that play important surveillance role in recognizing and eliminating cancer cells [1, 2]. For successful tumour development, dissemination and metastasis, cancer cells

have acquired different cellular mechanisms to avoid immune clearance. It have been observed that tumour biopsies heavily infiltrated with the cytotoxic T lymphocytes (CTL) and natural killer (NK) cells have better prognosis as compared to those that do not have these killer immune cells. A study has demonstrated cancer cells can paralyze infiltration of immune cells by secreting immunosuppressive factors such as transforming growth factor-beta (TGF-β) to dampen the immune response [36]. By doing so, cancer cells are able to reduce its chances in encountering immune cells and thus evade immune surveillance successfully. Kim *et al*., 2007 has also shown that immune-deficient mice developed carcinogen-induced tumours more frequently and rapidly when compared to its relative immune-competent control mice. In addition, the highly immunogenic cells were observed to be routinely eliminated in the immune-competent hosts, but formed tumours in the immune-deficient host [37]. Based on these studies, it appears that once the immune system is dampened, cancer risk increases significantly and thus cancer incidence becomes more common than expected. Such studies also highlighted the importance of tumour-host immunological interaction as it dictates cancer cells ability to evade immune destruction, clearance efficiency and form tumours. Cancer immunotherapy that uses immune system to fight cancer has since been successful with FDA approval of Provenge for prostate cancer and Herceptin for breast cancer as two of the examples.

1.2 Mammary Gland

1.2.1 Breast Structure and Function

The mammary gland or breast is a highly specialized organ, whose biological function is to produce milk for young mammals [38]. The mammary glands consist of a core made up of 15 to 20 lobes surrounded by a layer of adipose tissue that is covered by the skin. In each lobe are smaller compartment termed lobules, which are made up of milk-producing glands named alveoli. Alveoli are hollow cavities that are lined with milk-secreting cuboidal cells. These milk-secreting cells are surrounded by myoepithelial stromal cells, which in turn contracts and eject milk into the ducts. Small ducts can join together to form larger terminal ducts that drain milk via the nipples (Figure 1-5). [39].

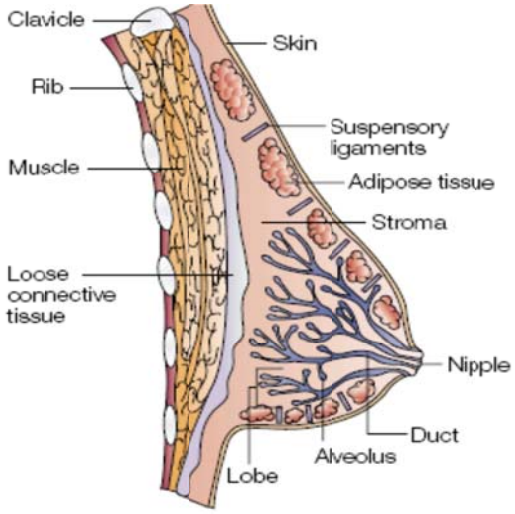


Figure 1-5 Transverse section of human mammary gland or breast (adopted with permission from Ali and Charles Coombes, 2002) [38]. Mammary gland consists of a core made up of 15 to 20 lobes surrounded by a layer of adipose tissue. Each lobe is made up of smaller compartments known as lobules, which in turn are made up of milk-producing glands or alveoli. Each alveolus is hollow cavity lined with milk-secreting epithelial cells, which are surrounded by myoepithelial stromal cells and basement membrane. As myoepithelial cells contracts, milk is ejected into each small duct, which join together to form larger terminal ducts that drain milk via the nipple.

The presence of rudimentary mammary gland at birth in both males and females allows mammary development to begin as early as during

embryogenesis [38]. Subsequent development is initiated during onset of puberty under the influence of estrogen and progesterone for ductal elongation and branching [14, 38]. The level of these steroid hormones varies with the menstrual cycle. Presence of steroid hormones alone is not sufficient to initiate mammary duct differentiation and maturation. Pituitary hormones such as growth hormone (GH) also play an important role in mammary gland development by stimulating the mammary stromal and epithelial cells for ductal elongation and differentiation [14]. This is evident in hypophysectomised and gonadectomised rats where treatment with estrogen and GH has been found to be sufficient to support puberty mammary gland development [40].

During pregnancy, ductal branching and alveoli differentiation increases rapidly together with an increase in adipose tissue and a richer blood flow to prepare the mammary gland for milk production [38]. During this phase, the number of myoepithelial cells also increases under the influence of steroid hormones, growth factors and polypeptide hormones. A few days after birth, lactation begins with increased prolactin (PRL) production. After weaning, the mammary gland undergoes regression or involution with its function and morphology returning to near pre-pregnancy stages where the epithelial cells undergo cell death. Additionally, milk glands and ducts become smaller and are replaced by fibrous and adipose tissue [38].

Breast lumps including cancer develop mostly within the milk ducts and lobules. However, not all breast lumps are cancerous and lead to malignant tumour formation. For instance, presence of fibroid tissue mass or fluid-filled

cyst sacs is usually benign and can be common in women. The following part will focus on breast cancer.

1.2.2 Breast Cancer

Breast cancer is a complex and heterogeneous disease. It is the most common malignant neoplastic disease and the second leading cause of cancer death in women in United States of America (USA). The rate of mortality for breast cancer has deceased steadily due to advance in early detection, diagnosis and treatment. Nevertheless, an estimate of 40,000 women still die as a result of cancer each year due to resistance to wide variety of drugs and metastatic spread of cancer cells to distant organs (World Health Organisation website 2012, http://www.who.int/mediacentre/factsheets/fs297/en/). Each year, more than 1400 women are estimated to be diagnosed with breast cancer, while more than 300 die from breast cancer in Singapore (Singapore Cancer Society website 2012, http://www.singaporecancersociety.org.sg/lac-gci-cancer-facts-n-figures.shtml).

Breast cancer originates from the cells lining the milk ducts and lobules. The presence of abnormal cell layer within the milk ducts and lobules is termed atypical ductal hyperplasia (ADH) that constitute pre-malignant lesion. The pre-malignant lesion stage is known as non-invasive *in-situ* cancer where local surgery to remove the abnormal cell mass is usually sufficient and patient usually has higher survival rate. There are the non-invasive ductal carcinoma *in situ* (DCIS) (Figure 1-6A) or the lobular carcinoma *in situ* (LCIS) (Figure 1-6B), depending on the location that the abnormal cell mass is confined to. Once cancer cells have broken out of the milk ducts and/or lobules and

invaded the surrounding stroma, it becomes an invasive cancer [41]. Invasive mammary carcinoma derived from mammary ducts or lobules are termed the invasive ductal carcinoma or invasive lobular carcinoma respectively. An invasive cancer can thus gain entry into the lymphatic system and/or blood vessels in the stroma and potentially spread to a distant site. The lungs, bones and liver are the most commonly affected secondary organs in terms of breast cancer [41]. Once cancer cells established recolonization at secondary site, it is termed metastatic or advanced late stage cancer.

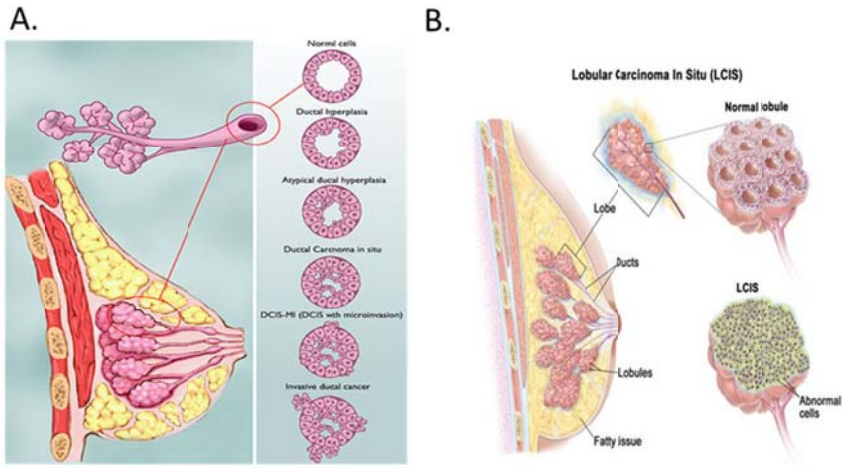


Figure 1-6 Transverse section of human mammary gland with illustration on the development of neoplastic growth (diagram adopted from breastcancer.org. website). The development of ductal breast carcinoma *in situ* (DCIS) (A) and lobular carcinoma *in situ* (LCIS) (B) towards invasive cancer as cells break out of the ducts and/or lobules and invade the surrounding stroma.

Histological images of a normal mammary tissue revealed the hollow lumen is surrounded by luminal secretory cells, which in turn is surrounded by a layer of myoepithelial and basement membrane [41, 42]. Once the myoepithelial layer and basement membrane are disrupted, it indicates an invasive ductal or lobular carcinoma in breast, where the malignant cells infiltrated surrounding tissue, formed solid nest and poorly-formed tubules or filling up of luminal space [42]. As shown in Figure 1-7, the histological images of the highly

organized structure of normal breast tissue is disrupted in carcinoma tissue sample and distinct from non-invasive, invasive and malignant breast cancer tissue [42].

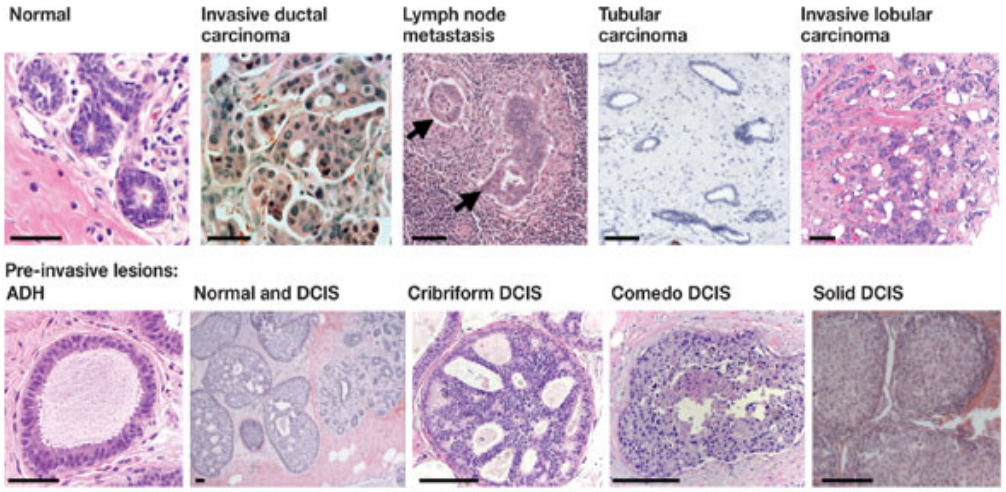


Figure 1-7 Histological images of normal versus carcinoma breast tissue (adopted with permission from Debnath and Brugge, 2005) [42]. Histological image of normal breast tissue revealed a highly organized structure where the hollow lumen is surrounded by luminal secretory cells, which in turn is surrounded by a layer of myoepithelial and basement membrane. Such highly organized structure of normal breast tissue is disrupted in breast carcinoma tissues and is distinct between ductal/lobular non-invasive, ductal/lobular invasive and metastatic cancer tissues samples. For instance, invasive breast tissue samples revealed cells infiltrated into surrounding luminal cells, formed solid nest, poorly formed tubules and filling up of luminal space.

Breast cancer can arise through familial or sporadic mutation. Familial breast cancer is caused by inherited gene mutation, which makes up about 5% to 10% of all breast cancer [41]. For instance, women who have inherited gene mutations in Breast Cancer Susceptibility Gene 1 or 2 (*BRCA1* or *BRCA2*) have an increased risk of the disease [41]. Both BRCA1 and BRCA2 play a role in DNA damage repair and cell cycle regulation pathways. Loss of function mutations of *BRCA1* or *BRCA2* thus greatly increases breast cancer risk. Genetic test is therefore highly advisable for women with strong family history in breast cancer. Besides that, all women are encouraged to have routine self-physical examination and/or mammogram screening for presence

of breast lump. Early detection for presence of breast lumps can greatly increase survival rate. Besides genetics, other risk factors for breast cancer also include gender, age, height, ethnic, age at menarche, age at first full term pregnancy, lactation, obesity and hormonal levels such as estrogens [41].

Taxonomy of breast cancer can be refined based on simple measures such as presence of predictive markers like estrogen receptor (ER), tumour grade, lymph node status and histological type. Based on presence of predictive markers, breast cancer can be divided into two distinct groups which include the ER-positive (ER+) and ER-negative (ER-). This can be further sub-classified into different tumour subtypes such as luminal A (ER+ or progesterone receptor-negative (PR-) and human epidermal growth factor receptor 2-negative (HER2-)), luminal B (ER+ or PR+ and HER2+), HER2-positive (ER-, PR- and HER2+) and basal-like (ER-, PR- and HER2-) [43]. Other than that, there are different breast cancer stages (Stage 0, 1, 2, 3 and 4) depending on the TNM (size of tumour (T), lymph node status (N) and metastatic status (M)). Lymph node and metastatic status refers to whether there is presence of cancer cells in the lymph nodes and whether cancer cells have spread to a distant secondary site respectively [41]. Stage 0 of breast cancer refers to *in-situ* carcinomas such as the LCIS and DCIS where tumours are confined locally at the respective primary lobular and ductal site. At stage 1-3, the tumour cells still reside within the mammary gland or regional lymph nodes. As disease progresses to Stage 4, tumour cells have acquired metastatic capability and spread to other secondary sites. Patients diagnosed with stage 3 or 4 breast cancer have less favourable prognostic outcome. These different classification or taxonomy categorise breast cancer into different criteria and

characteristics that can help doctors dictate the appropriate treatments, predict survival outcome and prognosis for the patients.

Current treatment of breast cancer includes local surgery, radiotherapy, chemotherapy and hormonal therapy. For instance, early stage breast cancer patients diagnosed with small primary tumours are usually treated with local surgery to remove the tumours and subsequently radiotherapy to eradicate residual cancer cells that might not be removed via surgery. Chemotherapeutic drugs can also be used to eliminate tumour cells which have metastasized from the primary tumour or locally advanced stage cancer [44]. Currently, the most common chemotherapeutic drugs used for breast cancer in clinics includes alkylating / crosslinking agents (e.g. cyclophosphamide), anthracyclines (e.g. doxorubicin) and anti-microtubule agents (e.g. paclitaxel) [45]. Hormonal therapy currently available includes anti-ER therapies (e.g. tamoxifen and fulvestrant) and aromatase inhibitors (e.g. letrozole; anastrozole; exemestane). Breast cancer patients diagnosed with lymph node-positive disease where malignant cells have invaded surrounding lymph node and/or blood vessels, usually have a high risk of local recurrence as well as systemic recurrence. A combination of treatment (surgery with radiotherapy, chemotherapy and/or hormonal therapy) will thus be useful for better eradication of the tumour cells and improve overall survival outcome as well as reduce disease relapse.

In some cases, multigene testing is often performed on breast tumours so as to determine combination of therapies (i.e. hormonal and chemotherapy) that might most benefits breast cancer patients [46]. The challenge here will be to administer chemotherapeutic drugs at a dosage that maximizes its efficacy,

and at the same time, minimize its toxicity in patient. Despite the benefits that various therapeutics can offer in targeting cancer cells, *de novo* existence or acquisition of resistance to treatment or relapse still remains an obstacle clinically [46, 47]. It is therefore of importance to investigate and understand pathways responsible for resistance as this will provide clues on mechanisms of resistance to various treatments.

1.2.3 HER2+ breast cancer

The Human Epidermal Growth Factor Receptor 2-positive (HER2+) breast cancer is associated with frequent amplification of the *HER2/neu* gene, which is located at chromosome 17q12-q21.1 [48-51]. It is well studied that over-expression of HER2 plays a critical role in the development of mammary malignancies and is seen in up to 25-30% of breast cancers [48, 49, 52, 53]. It is often associated with an aggressive phenotype where patients diagnosed with HER2+ breast cancer usually have increased risk of disease progression, higher tumour relapse and poorer prognosis as compared to *HER2/neu*-negative breast cancer patients [8, 54, 55] .

1.2.3.1 HER2 and its Function

The HER2 belongs to the human epidermal growth factor receptor (EGFR/ErbB/HER) family. This family of receptor tyrosine kinases (RTKs) have been involved in the development of many types of cancers including breast, ovarian and gastric [7, 53, 56, 57]. In this family, there are four identified members that include the epidermal growth factor receptor (EGFR, also known as HER1), HER2, HER3 and HER4 (Figure 1-8) [53]. The genes encoding the 4 members are located on different chromosomes [53]. All

members are single-pass transmembrane glycoproteins that contain an extracellular ligand-binding domain, a hydrophobic transmembrane domain and an intracellular tyrosine kinase domain [7, 53]. Each receptor has their preferred ligands due to distinct sequences of their extracellular domain that provides specific docking sites [8]. For instance, the epidermal growth factor (EGF) binds preferentially to EGFR/HER1, while neuregulin (NRG) binds preferentially to HER3 and HER4 [53]. Among the HER members, HER2 does not have a known ligand and often acts as a coreceptor or heterodimerization partner for HER1, HER3 and HER4. HER3 have impaired kinase activity, but is able to acquire signalling potential when dimerized with HER2 after ligand binding. Each HER member contains a specific subset of cytoplasmic tyrosine residues that enables transduction of a unique set of signals (Figure 1-8) [8, 52, 57, 58].

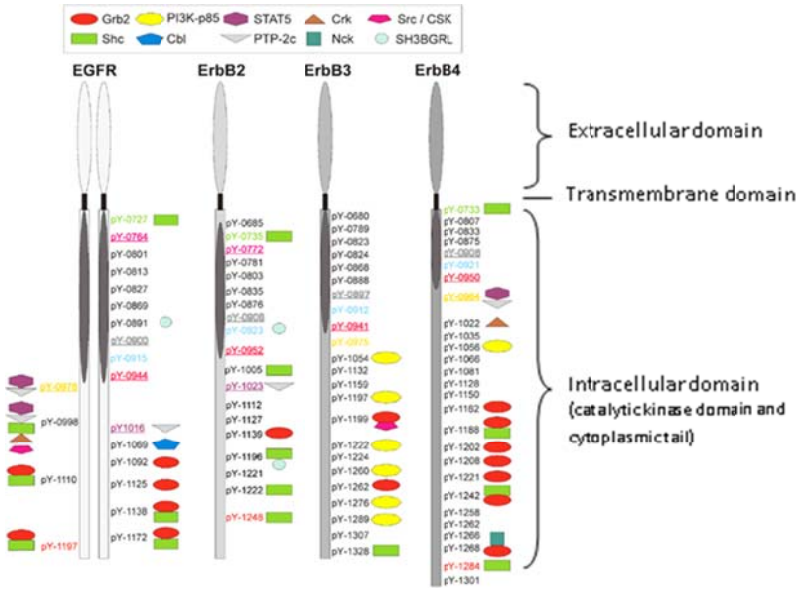


Figure 1-8 Members of the human epidermal growth factor receptor (ErbB/HER) family and its phosphotyrosine interacting partners (adopted with permission and modified from Schulze *et al.*, 2005 [59]). All members of ErbB/HER family consist of an extracellular domain, transmembrane domain and intracellular domain. Each member has its distinct phosphotyrosine interacting partners at its intracellular domain.

All members and its respective ligands are essential and expressed during normal development and maturation of the mammary gland [60]. The EGFR plays a function in promoting ductal growth, while HER2 and HER4 are involved in lobuloalveolar differentiation as well as lactation. Among the members, EGFR and HER2 are commonly deregulated in certain prevalent forms of human cancers where overexpression or mutated forms of these proteins were demonstrated to trigger malignant transformation [58, 60, 61].

1.2.3.2 HER-mediated Signal Transduction

When ligand binds, the receptors undergo conformational changes that allow homo-dimerization (e.g. HER1-HER1; HER2-HER2, HER3-HER3 and HER4-HER4) or hetero-dimerization (e.g. HER1-HER2; HER2-HER3; HER2-HER4) of the receptors [53, 55]. Dimerization results in transactivation and subsequent phosphorylation of its cytoplasmic tyrosine residues, which in turn serves as recognition sites for an array of adaptors and other phosphotyrosine-containing intracellular signalling molecules to rely signals downstream (Figure 1-8) [8, 59]. Signalling molecules such as Src homology 2/α collagen-related (Shc) protein, Signal Transducer and Activator of Transcription 5 (STAT5) and Growth Factor Receptor Bound 2 (Grb2) then relies signal downstream and activate survival or proliferation pathways such as mitogen activated protein kinase (MAPK), phosphatidylinositol-3 kinase-anti-apoptotic serine/threonine kinase (PI3K-Akt) and nuclear factor κB (NFκB) [7, 8, 55, 57]. Among the members, HER1 and HER4 have greater diversity of phosphotyrosine interacting partners than HER2 and HER3 (Figure 1-8). Phosphorylation of different cytoplasmic tyrosine residues is

reportedly related to activation of defined downstream intracellular signalling pathways [7, 59].

The HER signalling network is highly complex, robust and diverse due to the different possible combinations of receptor dimers (e.g. HER1-HER1; HER1-HER2; HER2-HER2; HER2-HER3; HER3-HER3; HER2-HER4 and HER4-HER4) (Figure 1-9). Dimerization pairs that include HER2 have been found to be more stable and cause more potent signalling compared to dimerization pairs without HER2 [53]. Also, dimers formation is highly dependent on the respective expression levels of each HER receptors. Importantly, receptor heterodimerization triggers the intrinsic tyrosine kinase activity of each receptor in the pair that permits the recruitment of different complements of phosphotyrosine-containing signalling molecules at the cytoplasmic tails. Therefore, further diversifies the repertoire of signalling pathways that can be activated [53]. The HER signalling has also been described as a redundant network as the system involves many positive and negative feedback regulatory mechanisms that protects it from various perturbation, a feature which play substantial role in resistance to therapeutic drugs targeting this pathway [62].

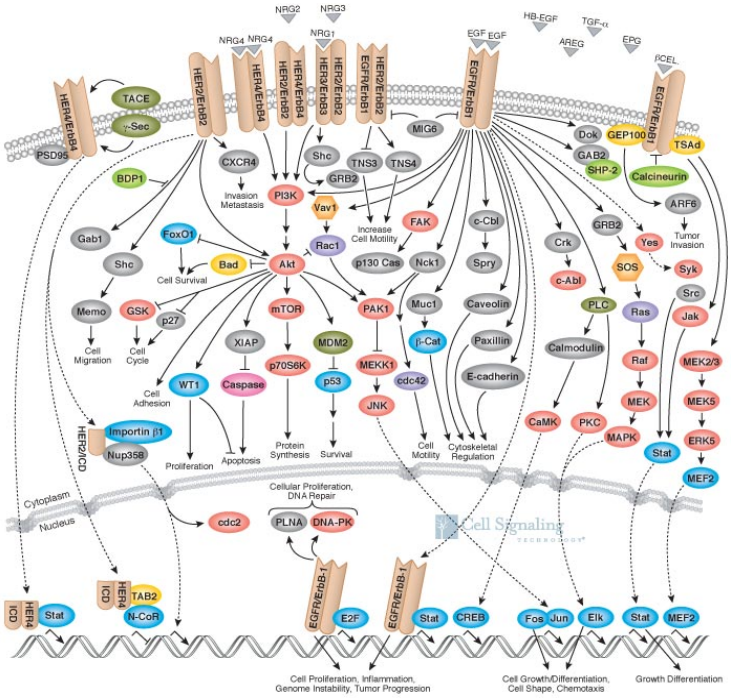


Figure 1-9 Overview of the HER signalling pathway (Illustration reproduced courtesy of Cell Signaling Technology. Inc. (www.cellsignal.om. <http://www.cellsignal.com/>). It illustrated the signalling molecules involved for activation of cellular activities such as cell differentiation, proliferation, growth and chemotaxis.

1.2.3.3 HER2-mediated mammary carcinoma

The HER members can activate a multitude of signalling pathways that govern many aspects of cellular function such as cell growth, proliferation, survival, differentiation and motility. Deregulation and/or overexpression of any HER members can thus potentially contribute to uncontrolled cell growth and promote survival that can lead to formation of tumour. Indeed, studies have shown frequent mutation and/or overexpression of HER family members in a number of human cancers. Among all members, the EGFR and HER2 have been demonstrated to play the most significant role in cancer.

Research studies have consistently observed that amplification of the HER2/*neu* gene promotes proliferation, angiogenesis, enhances invasive and metastatic potential in cancers such as breast, ovarian and colorectal cancers

[57]. Overexpression of HER2 has been reported to correlates with larger tumour size and spread of tumour to lymph nodes. Since HER2 often acts as coreceptor, overexpression of HER2 can result in spontaneous formation and constitutive ligand-independent dimerization of HER2 homodimers, EGFR/HER2 and HER2/HER3 heterodimers with subsequent activation of its receptors cytoplasmic kinase region (Figure 1-10) [52, 63]. Existence of HER2 homodimers can form complex with partition protein 6 (Par6) and atypical protein kinase C (aPKC) resulting in the loss of cell polarity, disruption of tight junction and inhibition of apoptosis that can enhance EMT, cell migratory potential and survival [48, 52]. An increase of EGFR/HER2 heterodimers will result in an increased downstream signalling through Ras/Raf/MAPK pathway, which in turn upregulates Cyclin D1 and subsequently enhances cell cycle progression [52]. The pairing between HER2 and HER3 is thought to trigger the strongest signalling of all receptor combination. Presence of high numbers of HER2/HER3 heterodimers on cell surface enhances cell survival via activation of P13K/Akt pathway [52]. Therefore, enhanced expression of HER2 confers a strong proliferative and survival advantages for tumour cells. It is thus evident that overexpression of HER2 is an oncogenic driver in cancer.

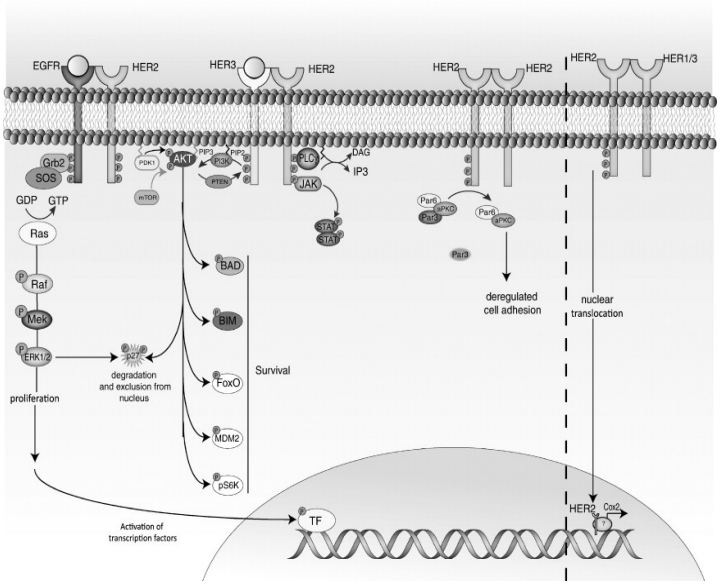


Figure 1-10 Signalling pathways activated in the presence of HER2 overexpression (adopted with permission from Kruser and Wheeler, 2010) [52]. Overexpression of HER2 resulted in increased formation of HER1/HER2 heterodimers, HER2/HER2 homodimers and HER2/HER3 heterodimers, which ultimately enhances activation of proliferation and survival pathways.

1.2.3.4 HER2+ Breast Cancer Treatment

There have been different therapies developed to treat HER2+ breast cancer, all of which involved interfering the HER pathway to stop the cascade of signals that leads to cell growth and survival. Current treatment for HER2+ breast cancer include the use of Trastuzumab (Herceptin), Lapatinib, Pertuzumab and Trastuzumab emtansine (TDM-1), which are therapeutic drugs approved by the US Food and Drug Administration (FDA) (Figure 1-11) [64].

Trastuzumab or Herceptin (Genentech Inc., San Francisco, CA) is the first FDA-approved therapeutic drugs developed for treating HER2+ metastatic breast cancer patients and also in adjuvant setting for HER2+ breast cancer treatment [64, 65]. It is a recombinant monoclonal antibody targeting the extracellular segment (domain 4) of HER2 [52]. Herceptin binding to HER2

induces antibody-dependent cell-mediated cytotoxicity (ADCC), thereby resulting in the disruption of HER [66]. Herceptin-targeted cells exhibited reduction in proliferation and tumour growth *in vitro* and *in vivo* as it undergoes cell cycle arrest at G1 phase [65]. Herceptin can either be administered alone or in combination with chemotherapy after disease progression and resistance have been reported in Herceptin-based therapy [67]. Combination therapy is now often used as a standard first-line treatment for HER2+ metastatic breast cancer patients [52]. The levels of HER2 are either absent or low in most normal tissues except the heart. Therefore, Herceptin-treated breast cancer patients could suffer from cardiovascular side effects [57]. Besides that, this monoclonal antibody lacks action on brain metastases due to blood-brain barrier (BBB).

New or improved therapies have been developed for HER2+ breast cancer patients whose disease progresses and relapses on Herceptin to improve survival and also minimize treatment-related toxicity. Lapatinib, pertuzumab and trastuzumab emtansine (TDM-1) are the new therapeutic options approved by FDA for treating patients facing Herceptin-refractory metastatic breast cancer.

Lapatinib (Tyverb/Tykerb) is a tyrosine kinase inhibitor (TKI) targeting both EGFR/HER1 and HER2 [49, 68]. Lapatinib mode of action is to compete with ATP binding site at the intracellular tyrosine kinase domain of EGFR and HER2, prevent phosphorylation of cytoplasmic tail and thus abrogate activation of downstream signalling pathways that are involved in cell proliferation and survival (Figure 1-11) [64, 65, 67, 69]. It is often used in

combination with either endocrine therapy (e.g. letrozole) or capecitabine for advanced HER2+ breast cancer patients in cases where chemotherapy and Herceptin fails [49]. Unlike Herceptin, Lapatinib is able to carry out its activity for central nervous system (CNS) metastases in HER2+ breast cancer patients due to its small molecular size that may cross the BBB to provide effective action in the cerebrospinal fluid [49]. Besides that, Lapatinib is reportedly to be associated with less cardiotoxicity. HER2-targeted therapy involving the use of Lapatinib have shown high efficacy in HER2+ breast cancer patients, however resistance to this drug is reportedly prevalent [62].

Pertuzumab is a humanized monoclonal antibody that targets the extracellular part of HER2 at Domain 2, which is essential for receptor dimerization. Binding to this domain of the receptor efficiently creates steric hindrance that blocks ligand-induced homo- and heterodimerization in particularly HER2-HER3, thereby inhibiting downstream signalling pathways such as MAPK and PI3K-Akt and ultimately tumour growth [68, 70, 71]. One difference between Pertuzumab and Herceptin is that the latter have minor effect in the presence of ligand as it binds to different extracellular domain on HER2 [70, 71]. Since Pertuzumab and Herceptin binds to HER2 at different domain, the mode of action of Pertuzumab is complementary to that of Herceptin. For instance, preclinical and clinical studies have demonstrated a more comprehensive blockage of HER2-driven signalling pathway when the pertuzumab-mediated HER2 dimerization inhibition and anti-HER2 activity of Herceptin is combined than either agent alone [66, 72, 73] . This synergistic anti-tumour activity resulted in FDA-approval of pertuzumab to be used and combined with Herceptin or docetaxel in treating both local or metastatic unresectable

HER2+ breast cancer patients who have not receive HER2-targeted therapy or chemotherapy previously [70].

Trastuzumab emtansine (DM1) or TDM-1 is an antibody drug conjugate generated to increase potency of antibody-directed therapy. It comprises the anti-HER2 antibody trastuzumab (Herceptin) bound to antimicrotubule cytotoxic agent via thioether linker. Specifically, TDM-1 uses Herceptin to localise the cytotoxic drug to HER2+ breast tumour cells. Upon binding to HER2 on tumour cells, TDM-1 is internalised and subsequently Herceptin and the thioether linker are degraded by lysosomal degradation, releasing DM-1. The liberated DM-1 causes cell cycle arrest and apoptosis through direct inhibition of microtubule assemble and polymerization [68, 70].

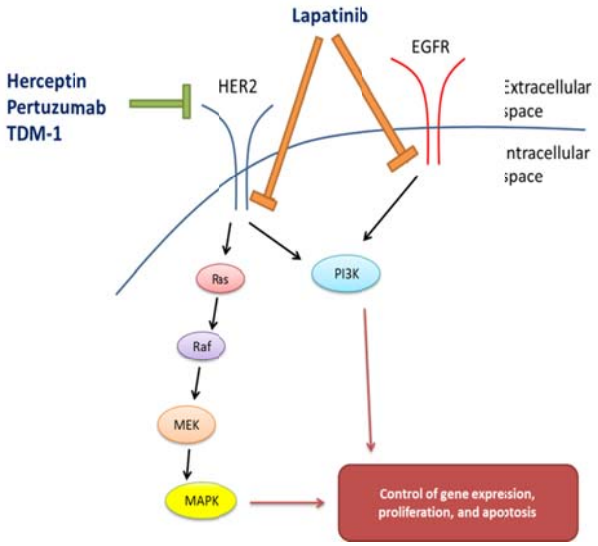


Figure 1-11 Schematic diagram illustrating HER2-targeted agents use in the treatment of HER2+ breast cancer. Antibody-based anti-HER2 agents such as Herceptin and Pertuzumab bind to the extracellular domain of HER2 to block its signalling. Trastuzumab-DM1 (TDM-1), an antibody drug conjugate targeting the extracellular domain of HER2 and prevents microtubule polymerization when internalized into cells. Lapatinib is a tyrosine kinase inhibitor (TKI) targeting both EGFR and HER2 by competing with ATP binding site that prevents phosphorylation of cytoplasmic tail and block activation of downstream signalling pathways.

1.2.3.5 Intrinsic and Acquired Resistance in HER2+ Breast Cancer Treatment

Despite the benefit of Herceptin in treating HER2+ breast cancer patients, intrinsic and acquired resistance have progressively been recognized as a major limitation in current HER2+ breast cancer treatment [52, 71]. The duration of patients' response to Herceptin alone or in combination with other chemotherapeutic drugs have been reported to range from 5 to 9 months, implicating that acquired resistance often develops [50, 52, 74]. Neve *et al.,* 2006 assessed growth inhibition responses of nine HER2-amplified cell lines to Herceptin and found only three out of nine exhibited robust response to the drug as measured by inhibition of BrdU incorporation [75]. This is in line with clinical reports where approximately 35% patients receiving Herceptin alone are responsive to the drug, while others do not respond clinically [50, 60, 75]. Besides that, patients who show primary response were found to develop resistance to Herceptin eventually and disease progresses within one year of treatment [49, 52]. Gene expression analysis suggested that upregulation of genes involved in insulin/MAPK predicts response to Herceptin, whereas the mammalian target of rapamycin (mTOR) and Toll-like receptor pathways associate with Herceptin resistance [75]. However, there are no conclusive biomarker(s) for patient responsiveness to Herceptin. Therefore, there is a need to identify molecular features that may allow more precise identification of HER2+ breast cancer patients' responsiveness to therapeutic protocols containing Herceptin.

One of the mechanisms of resistance to Herceptin is mutation to the target itself particularly at the HER2 domain that contains the binding epitope for Herceptin. This can cause direct steric hindrance or restriction to conformational flexibility that impairs receptor-antibody binding and thus renders Herceptin efficiency to block cell cycle arrest [76].

Another proposed mechanism of resistance to Herceptin is the presence of a truncated form of HER2 (p95-HER2) in the circulation that arises from alternative transcription of HER2. The N-terminal of p95-HER2 retains the constitutively active kinase that can potentially dimerize with other HER members and allow activation of the respective downstream signalling pathways. Unlike HER2, the Herceptin-binding region is absent in p95-HER2, which enables activated signalling even in the presence of Herceptin. Therefore, p95-HER2 is demonstrated to be susceptible to lapatinib inhibition but not Herceptin [63, 74, 76, 77]. It is reported that among the HER2-amplified breast cancers, 30% of which displayed expression of the truncated p95-HER2 and is associated with shorter disease-free survival when compared with tumours that overexpress the full length HER2 [74]. In both *in vitro* and *in vivo* studies, cell lines and xenograft models transfected with the truncated HER2 displayed resistance to Herceptin [78, 79].

The loss of phosphatase and tensin homolog (PTEN) via mutational inactivation or downregulation of expression has also been reported to be strongly correlated with Herceptin resistance. The absence of PTEN results in constitutive activation of PI3K-Akt signalling pathway that drives aberrant cell growth and proliferation in HER2-amplified breast cancer cells, which is also

observed in other tumour types [80]. Furthermore, upregulation of PI3K-Akt signalling through mutational activation of the catalytic subunit of PI3K (PI3KCA) has been implicated in resistance of HER2+ breast cancer to Herceptin [81].

The ability of HER2 to form heterodimers with other extracellular HER members (HER1 and HER3) can result in incomplete inhibition by Herceptin through lateral activation of alternative pathways mediated via HER1 or HER3 [52, 82]. Cross talk between HER2 and other receptors also provides alternative proliferative and survival stimuli for growth-promoting effects to the tumour even in the presence of effective inhibition of the HER pathways. For instance, it is reported that GH is able to stimulate phosphorylation of EGFR/HER1 at Tyr1068 site via Janus Kinase 2 (JAK2) and elicit activation of MAPK pathway that is independent of the intrinsic kinase activity of EGFR/HER1 [83]. Besides that, formation of HER2-insulin growth factor-1 receptor (IGF-1R) heterodimers was observed to phosphorylate and activate HER2 by IGF1-like ligands in Herceptin-resistant cells [84]. Another research group from Japan have reported that in human breast cancer cell line, autocrine secretion of prolactin resulted in constitutive tyrosine phosphorylation of HER2 (Tyr1139) via the JAK2 kinase, thus providing a docking site for stimulation of Ras-MAPK cascade and causing unrestricted proliferation [85].

The findings of these studies suggested additional levels of complexity in Herceptin-acquired resistance and demonstrated that blocking of one molecular target (HER2) may still allow an escape via alternate signalling

cascades. The different mechanisms of resistance to Herceptin also indicated cancer cells ability to continuously adapt to different signalling pathways as a strategy for survival and growth.

Although Lapatinib has been proven to be effective in clinical trials, majority of patients who are Lapatinib-responsive eventually become resistant to this drug. Moreover, most of the patients pre-treated with Herceptin failed to respond to Lapatinib thereafter as well, on an average in less than a year [86]. It has been proposed that the major mechanism of resistance to lapatinib is through activation of compensatory survival pathways [66].

It is becoming clear that HER2 expression status alone is not adequate as predictive marker to predict response to HER2-targeted therapy including Herceptin. There is therefore a need to define the characteristics, identify additional markers and the molecular mechanism of therapeutic resistance in HER2+ mammary carcinoma so as to better design combination therapy, develop more effective HER2 targeting drugs or even tailor the treatment accordingly for HER2+ subtype breast cancer patients.

1.3 Human Growth Hormone (hGH)

1.3.1 Growth Hormone and its Function

The growth hormone (GH) is a secreted peptide hormone whose classically descried biological function is to stimulate longitudinal growth, cell proliferation, differentiation, survival and regeneration [87, 88]. It belongs to a class of evolutionarily related hormonal proteins that include prolactin (PRL) and placental lactogens (PL) [88]. In human, the gene encoding GH (*hGH*) is

located on the long arm of chromosome 17 (17q22-24) [89]. The *hGH* gene belongs to a gene cluster that consists of 5 closely related genes, namely the GH-N (GH-normal gene), the GH-V (GH-variant gene), the CS-L (chorionic somatomammotropin-like gene), the CS-A (chorionic somatomammotropin-A gene), and the CS-B (chorionic somatomammotropin-B gene) (Figure 1-12).

The GH-N (GH1) is the predominant circulating human growth hormone (hGH) in children and adult. Proteolytic processing of GH-N gives rise to a 22 kDa, 191 amino acids peptide that is found predominantly in plasma. Alternative splicing of the GH-N mRNA produces a smaller 20 kDa isoform protein that represents 5-10% of monomeric hGH in the pituitary [88]. The GH-V (GH2) is expressed only in the placenta and present in maternal serum during the late stage of pregnancy. The CS-L, CS-A and CS-B are also referred as placenta lactogen genes [88, 89]. Like GH-V, CS-A and CS-B genes are expressed in human placenta and all play a role during growth of fetus and lactogenesis. The CS-L gene is a pseudogene that does not produce a functional protein [89]. The GH discussed in this thesis referred to the GH-N.

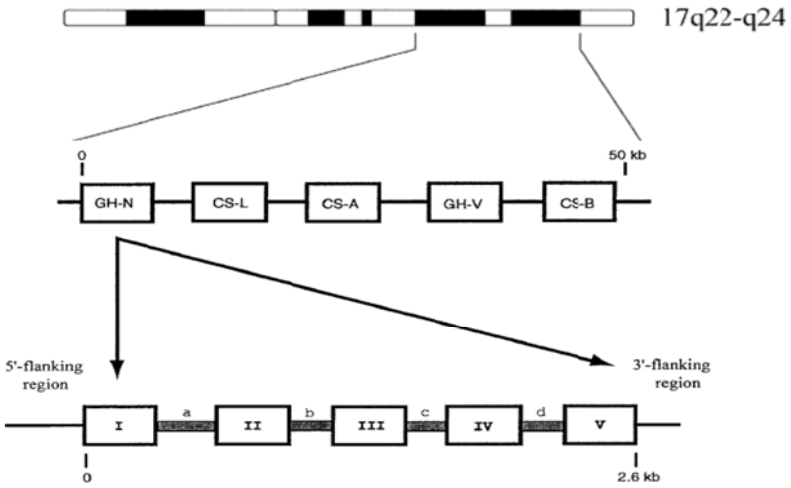


Figure 1-12 Schematic diagram representing the human *GH* gene cluster, which consists of GH-N, CS-L, CS-A, GH-V and CS-B (adopted with permission from Kopchick and Andry, 2000) [88]. The *hGH* gene (GH-N) consists of 5 exons (I-V) and four introns (a-d). The GH gene is located on the long arm of chromosome 17.

1.3.2 Growth Hormone Synthesis and Secretion

GH is synthesized and secreted from somatotropic cells of the anterior pituitary gland in a pulsatile manner upon receiving appropriate stimulation. Synthesis and secretion of GH is regulated by growth hormone releasing hormone (GHRH), growth hormone releasing peptides (GHRPs) and somatostatin [88]. Both GHRH and GHRPs stimulate production of GH while somatostatin inhibits GH synthesis and secretion. Therefore, the balance between these regulatory peptides determines GH release from the pituitary gland. The release of regulatory peptides is primarily affected by physiological stimuli such as exercise, nutrition, sleep and presence of free fatty acid (Figure 2-13) [88].

GH exerts its function either by acting directly on target organs or by binding to its receptor in human liver with high affinity through the induction of insulin growth factor-1 (IGF-1) [89]. The secreted pituitary GH enters the bloodstream and acts on target organs such as the liver, bone and muscles for

production of growth factors, mainly includes IGF-1 (Figure 1-13). IGF-1 has growth-stimulatory effects that stimulate cellular growth, regeneration and repair in a paracrine and endocrine fashion.

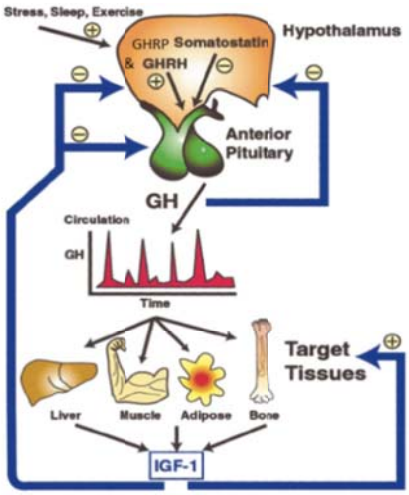


Figure 1-13 Schematic diagram illustrating production of growth hormones and mode of GH action (adopted with permission and modified from Kopchick and Andry, 2000) [88]. Upon receiving appropriate stimuli, pituitary GH will be released in the body. Circulating GH acts on target organs such as liver, muscle, adipose and bone for the release of IGF-1 for growth-stimulatory effects. There are positive and negative regulatory mechanisms that regulate the release of GH.

GH is not exclusively an endocrine hormone produced within somatotrophic cells of the anterior pituitary gland. On tissue-specific level, GH functions on the development and maturation of the tissue and organs. For instance, GH is involved in the maturation of the mammary gland and gonads as well as development of secondary sexual characteristics in boys during puberty [89]. In addition, GH expression has been reported in endothelial cells of blood vessels, fibroblasts, human lymphoid cells and isolated populations of the CNS [89].

1.3.3 GH-mediated Signal Transduction

In human, GH mediates its action through the growth hormone receptor (GHR) and prolactin receptor (PRLR) on target tissues [90, 91]. The GHR

displays 30% amino acid sequence homology with PRLR [89]. Both GHR and PRLP belongs to the Class I cytokine receptors consisting an extracellular region, a single hydrophobic transmembrane domain and an intracellular region (Figure 1-14) [92]. One feature of Class I cytokine receptors is that it lacks intrinsic kinase activity and therefore requires cytoplasmic tyrosine kinases to rely cellular signals downstream into the nucleus for target gene expression [92]. One of the cytoplasmic tyrosine kinases recruited is the Janus family of tyrosine kinases (JAKs) that often associate with cytokine receptors constitutively. The JAKs include JAK1, JAK2, JAK3 and Tyk2, and the predominant JAK utilized by GHR is JAK2. As shown in Figure 1-14, JAK2 binds to Box 1 region of GHR [92].

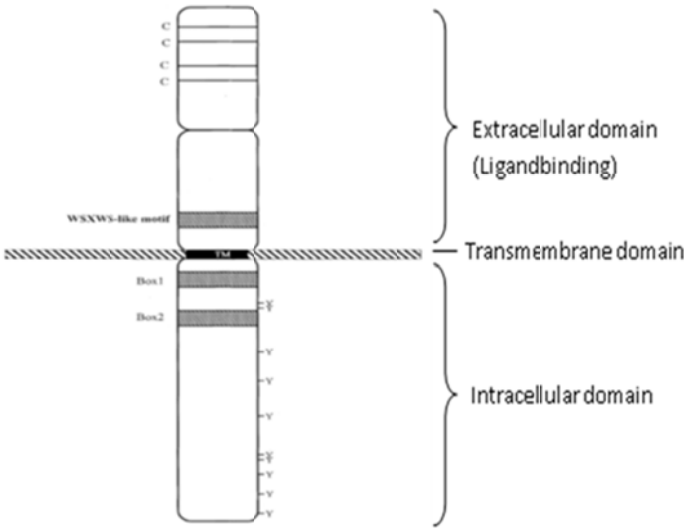


Figure 1-14 Schematic diagram of growth hormone receptor (GHR) (adopted with permission and modified from Zhu *et al.*, 2001 [92]. GHR consists of an extracellular domain for ligand binding, a transmembrane domain and an intracellular domain. Janus Kinase 2 (JAK2) is closely associated to GHR and binds to Box 1 region of this receptor.

There are two receptor-binding sites on GH molecules, namely a high affinity binding site 1 and a low affinity binding site 2 that binds its receptor sequentially [88, 92, 93]. Binding of GH to either GHR and/or PRLR can

cause the receptors to either homodimerize or heterodimerize, leading to its activation [94]. Ligand binding are thought to stabilize GHR-JAK2 complex, allowing two JAK molecules to come closely in proximity to phosphorylate each other [88, 93]. The activated JAK2 in turn phosphorylate multiple intracellular tyrosine residues that serve as docking sites for a variety of cytoplasmic adaptors and other signalling molecules containing SH2 or phosphotyrosine-binding (PTB) motifs to transduce signal downstream [92]. The Shc protein, signal transducer and activator transcription factor 1, 3 and 5 (STAT1, STAT3 and STAT5), phosphoinositide-3 (PI-3) kinase and phospholipase C (PLC), focal adhesion kinase (FAK) are some examples of signalling molecules recruited by GH stimulation [88, 95]. Once bound to GHR, these signalling molecules are rapidly phosphorylated and activated by JAK2. For instance, the activated and phosphorylated STAT1, STAT3 and STAT5 translocate to the nucleus, bind to DNA and activate target gene expression. The phosphorylated Shc protein can interact with and phosphorylate Grb2, which in turn activates Ras-Mitogen Activated Protein Kinase (RAS-RAF-MEK-MAPK) pathway [88]. The MAPK pathway has a pivotal role in regulating of gene transcription, cell proliferation and preventing apoptosis. GH stimulation also activates other signalling pathways such as PI3K-Akt [92].

Predominantly, GH utilizes JAK2 to elicit physiological pathways that stimulates growth, survival and proliferation [88]. Other studies have also shown that GH can recruit JAK1 [96] and other phosphotyrosine kinases (e.g. Src) [97] to rely and transduce GH-mediated signal downstream. Like GHR, PRLR also utilises JAK2 tyrosine kinase to rely GH-mediated signal

transduction into the nucleus for target gene expression [98]. Both PRLR and GHR have been reported to activate very similar signalling pathways such as Ras-Raf-MEK-MAPK and PI3K [98]. Since GH can bind to both GHR and PRLR, crosstalk between the two signalling pathways has been reported during GH stimulation [92].

GH stimulation resembles a bell-shaped curve. In other word, when concentration of GH is high, the higher affinity receptor site 1 will be saturated. This prevents receptor dimerization via the low affinity binding site 2, thereby creating a self-antagonistic effect for GH receptor activation [92]. Also, high concentration of the hormone induces homologous downregulation of receptor expression [99].

1.3.4 Growth Hormone-Related Disorder(s)

Given that GH plays important role in regulating somatic growth, cellular proliferation, differentiation and survival, abnormal GH levels may thus result in pathological conditions.

Hyposecretion of GH from pituitary during childhood can result in dwarfism. One feature that is observed in people suffering from dwarfism or sometimes known as “little people” is short statues or stunted growth due to GH deficiency [100]. The FDA has approved the use of recombinant hGH (rhGH) as the prescription drug to treat dwarfism and other childhood or adult growth deficiencies [101]. Interestingly, there have been studies showing an association between growth hormone deficiencies with protection from cancer. For instance, Guevara-Aquirre J *et al.,* (2011) reported that people who suffered from congenital GHR deficiency known as Laron Syndrome,

exhibited major decline in the incidence of cancer, diabetes as well as pro-aging signalling [102]. In their study, 100 Ecuadorian Laron subjects and 1600 individuals of normal status were monitored over a period of 22 years where no diabetes case and only one non-fatal cancer event in the Laron's subjects was observed. In contrast, the incidence of cancer and diabetes were found to be 17% and 5% respectively in the control subjects. Besides that, an animal model study done using the spontaneous dwarf rat that contains autosomal recessive mutation in the *GH* gene was observed to exhibit significant reduction in chemically-induced carcinogenesis including cancer formation in the breast due to the absence of functional serum GH [103].

Hypersecretion of GH during childhood can lead to gigantism where children display significant height above average. Besides that, excessive GH secretion from anterior pituitary after epiphyseal plate closure at puberty can result in acromegaly in adult [104]. This is evident in animal model study where acromegaly was observed in transgenic rabbits with GH overexpression [105]. Gigantic or acromegalic conditions produce enlarged bones, especially in the face, and also results in organomegaly and is associated with increased morbidity and mortality due to cardiovascular, metabolic and respiratory diseases. Furthermore, epidemiological studies of acromegalic patients indicate an increased in cancer risk such as benign and malignant tumours of the colon, breast, prostate and thyroid [104]. Somatostatin analogs and dopamine agonist have been utilized for gigantism and acromegaly treatment by suppressing GH, thereby controlling its secretion and action. Moreover, a GHR-specific antagonist B2036, has been useful for treating disorders linked to excessive hGH, including gigantism and acromegaly [106]. The molecular

structure of B2036 contains eight amino acid substitutions that are advantageous to its action as the affinity of binding site 1 to GHR is greatly improved. Additionally, it has a single amino acid substitution (glycine → lysine) that impair receptor binding site 2 (G120K), thereby abrogating the necessary conformational change required for functional GHR dimerization and subsequent signal transduction (Figure 1-15B) [107, 108]. Maamra *et al.,* 1999 have demonstrated B2036 prevents GHR-mediated JAK2 phosphorylation and STAT5 signalling [109]. Like the dimerized GH-GHR complex, B2036-GHR complex is rapidly internalised, which reduces its drug efficacy. Nevertheless, rapid internalisation can be overcame by conjugating four to five moieties of polyethyl glycol (PEG) to B2036 that generate a higher molecular weight molecule termed pegvisomant (Figure 1-15C) [107]. The increase in molecular weight also effectively prolonged pegvisomant half-life from body clearance, thereby making it the first clinically efficacious GHR antagonist for acromegaly treatment. Though pegvisomant have reduced antagonistic activity than its unpegylated form due to steric hindrance induced by PEG addition, high concentration is sufficient to completely block GH signalling [107, 108].

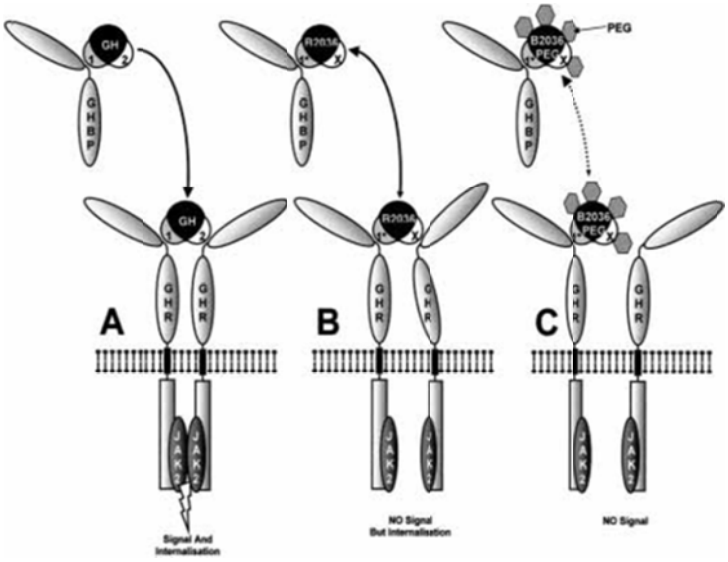


Figure 1-15 Mode of action of GH versus GHR antagonist, B2036 and PEG-B2036. (adopted with permission from Pradhanaga *et al.*, 2002) [107]. (A) Circulating GH binds to GHR via growth hormone binding protein (GHBP), resulting in GHR dimerization and signal transduction. (B) The B2036 molecule binds to GHR with increase affinity at site 1, but impairs binding at site 2 and block GHR signal transduction. (C) Polyethyl glycol-conjugated B2036 or pegvisomant

Experimental and clinical observations on abnormal circulating GH levels or deregulation of GH and/or its receptor evidently reiterated correlation between GH-related disorders or height status with cancer risk. Indeed, epidemiological studies have shown the association between increased cancer risk and height [110]. An epidemiological study done in the USA reported that an 8 cm height increment resulted in a 10% and 30% increase cancer risk in pre- and post-menopausal women respectively [111]. In Sweden, cancer risk is shown to be increased by 10% for every additional height of 5 cm [112]. A similar trend is also observed in a study conducted in Norway population, where 40% increased cancer risk is shown for 15 cm of additional height in pre-menopausal as well as post-menopausal women [113]. Ahlgren *et al.,* (2004) investigated a cohort of 117,415 Danish women on the association between childhood growth and breast cancer risk, and found that women growing faster during adolescence had an estimated 30% and 40% increased risk of pre- and

post-menopausal breast cancer respectively [114]. These few studies described here are among the many epidemiological evidences that highlighted or supported the association between risk of cancer and greater height in both childhood and adulthood. However, the mechanism involved in such epidemiological observation is still unclear.

The next sub-section of this introductory part of the thesis will discuss the role of hGH in mammary neoplasia progression as well as the functional pathways on how deregulation of hGH is linked to breast cancer.

1.3.5 Autocrine hGH in Mammary Neoplasia and Breast Cancer

The involvement of pituitary hormone in pathogenesis of breast cancer is observed where there is dramatic regression of mammary tumour following the removal of pituitary gland or hypophysectomy. Subsequent to this observation, hypophysectomy was employed as therapy for breast cancer in the 1950s [115].

Most recent researches have revealed accumulating numbers of evidence demonstrating the importance of autocrine hGH in development and progression of tumours of the female reproductive system, hence giving rise to cancers of the breast [14], endometrium and ovary observed in cell line models, animal models and clinical settings [116].

Autocrine hGH can produce different growth related effects to endocrine GH. The endocrine GH is secreted from anterior pituitary in a pulsatile fashion. It enters blood circulation and act on numerous target tissues for somatic growth. In contrast, autocrine hGH is secreted continuously and have been indicated as

an oncogene in driving mammary neoplasia development and progression by promoting cell survival, proliferation [95], immortalization, inducing angiogenesis [22], EMT [117] and enhancing metastasis [14, 118, 119]. The studies for these observations are discussed here.

Acquiring the ability to overcome anti-proliferative regulation and resist apoptosis is one of the key step in oncogenic progression, which is observed in the presence of autocrine hGH. Forced expression of the *hGH* gene in non-invasive mammary carcinoma cell line, MCF7 resulted in an upregulation of C/EBP homologues protein (CHOP) in a p38 MAP kinase-dependent manner [119]. Cell proliferation increased significantly when CHOP is upregulated. Concomitantly, the hGH-overexpressing MCF7 cells exhibited decreased apoptotic events. In the same study, usage of specific GHR antagonist B2036 abrogated the increased cell growth and protection against apoptosis. Additionally, B2036 effectively inhibited autocrine hGH-induced activation of JAK2/STAT5, CHOP (p38 MAPK) and Elk-1 (p44/42 MAPK) pathways [119]. Autocrine production of hGH also upregulated expression of homeobox containing gene (homeobox A1 (HOXA1)), a potent mammary oncogene that transcriptionally regulate expression of other genes such as *c-myc*, *cyclin D1* and *Bcl-2*. Together, these genes drive oncogenic transformation of mammary carcinoma cells by increasing proliferation, survival and opposing apoptosis [120].

Limitless replicative potential is achieved by autocrine hGH-mediated upregulation of poly(C)-binding protein (αCP1) that stabilises the telomerase catalytic subunit (human telomerase reverse transcriptase (*hTERT*)) mRNA

resulting in increased hTERT expression at both transcript and protein levels. The increased hTERT expression in turn increases telomerase activity and extends replicative potential of normal human mammary epithelial cells (HMEC) [121]. Additionally, Zhu *et al.*, (2005) demonstrated forced expression of hGH in a normal, but immortalized human mammary epithelial cells (MCF10A) stimulated oncogenic transformation of these cells with subsequent tumour growth and formation in xenograft model through increased cell proliferation and survival [122]. The *in vitro* data shown in the same study revealed autocrine expression of hGH have the ability to enhance anchorage-independent growth in JAK2-dependent manner. Subsequent treatment of hGH-transfected MCF10A and MCF12A immortalized cells with JAK2-specific inhibitor (AG490) abrogated the cells ability to growth and form colony in culture.

The inner environment of tumour mass is often challenged with nutrients and oxygen availability or hypoxia. Therefore, cancer cells exhibit angiogenic switch to create new vasculature that transport nutrients and provide oxygen to the tumour mass. A study conducted by Brunet-Dunand *et al.,* 2009 reported that autocrine production of hGH exhibits oncogenic ability that enhances angiogenic potential of MCF7 mammary carcinoma cells in VEGF-A dependent manner [22]. The increase expression of pro-angiogenic VEGF-A factor in hGH-overexpressing MCF7 cells promoted human microvascular endothelial cell (HMEC-1) tube formation *in vitro*. Induction of tube forming is abrogated in the presence of both the VEGF-A inhibitor (bevacizumab) and GHR-specific antagonist (B2036). Injection of MCF7-hGH overexpression cells into the auxiliary mammary fat pad of nude mice increases microvessel

density and stimulates tumour angiogenesis and lymphangiogenesis as compared to its relative nude mice injected with control cells. The *in vitro* and *in vivo* data clearly indicated autocrine hGH as a potential regulator of tumour neovascularization, angiogenesis and lymphangiogensis, which consequently provides a favourable survival advantage for the cancer cells to progress to the next stage of cancer that is invasion and migration where cancer cells begin to gain entry into blood and lymph node, followed by tumour metastasis to a distant site.

Before metastasis can occur, cancer cells have to undergo morphological changes from a polarized epithelial state to mesenchymal morphology such as loosening of tight junctions in order to be "mobile" for invasion and migration. Autocrine production of hGH in MCF7 cells has been shown to upregulate mesenchymal markers (VIMENTIN), and downregulate epithelial markers and tight-junction molecules (E-CADHERIN, α- and γ-CADHERIN, OCCLUDIN and PLAKGLOBIN) [117]. Also, closing up of artificial wound inflicted on monolayer of hGH-overexpressing MCF7 cells was significantly more rapid than its relative control cells. The increased in cell motility and local invasion of MCF7 cells were due to autocrine hGH-mediated upregulation of matrix metalloproteinases, MMP2 and MMP9, which function to degrade ECM. Tail vein injection of MCF7-Vec and MCF7-hGH cells into immunodeficient mice demonstrated that forced expression of hGH in MCF7 cells promoted lung metastases [22]. Here, it evidently showed the oncogenic role of autocrine hGH in mediating EMT, invasion, migration and metastasis in ER+ mammary carcinoma *in vitro* and *in vivo* [22, 117]. Besides that, autocrine hGH is able to

mediate oncogenesis and increase malignant transformation of normal mammary epithelial cells.

Drug resistance is increasingly recognized as the main cause of chemotherapy failure observed in cancer patients with metastatic disease, including breast cancer. There are increasing evidence indicating that autocrine hGH not only plays a role in oncogenic transformation and progression of breast cancer, but also confers resistance to multiple classes of chemotherapeutic agents used in breast cancer treatment, including doxorubicin [120], tamoxifen [123] and mitomycin C (MMC) [124]. Additionally, Bougen *et al.,* 2012 reported the contribution of autocrine hGH in radioresistance observed in ER+ breast cancer as well as endometrial cancer in their *in vitro* and *in vivo* studies [125]. These chemo- and radioresistance studies demonstrated that following drugs or ionising radiation (IR) exposure, hGH-overexpressing mammary carcinoma cells still exhibited enhancement in cell viability, clonogenic survival, anchorage-independent growth in soft agar and cell transformation in 3D when compared to its relative control-treated and untreated cells. Concordantly, there is a reduction in drugs-induced apoptotic cell death or IR-induced DNA damage in mammary carcinoma cells that are stably transfected with *hGH* gene. Functional inhibition of hGH using B2036 is shown to re-sensitize these mammary carcinoma cells to chemotherapeutic drugs or IR treatment [120, 123-125].

Based on the studies discussed above, Perry *et al.,* 2006 identified and concluded that autocrine and/or paracrine action of hGH is capable of activating an array of signalling pathways (e.g. JAK2-STAT3, JAK2-STAT5,

PI3K, c-Src and ERK) that are involved in cell proliferation, survival, motility and angiogenesis in order to provide pro-survival advantage to drive abnormal growth of tumour mass and tumour malignancy (Figure 1-16). Autocrine and/or paracrine expression of hGH can thus generate a platform for oncogenic manifestation observed in ER+ mammary carcinoma [95]. Also, these observation is exclusively mediated by autocrine production of hGH as addition of exogenous hGH to mammary epithelial cells in culture to mimic pituitary derived hGH, does not exert effects in oncogenic transformation of the cells [117, 120, 122].

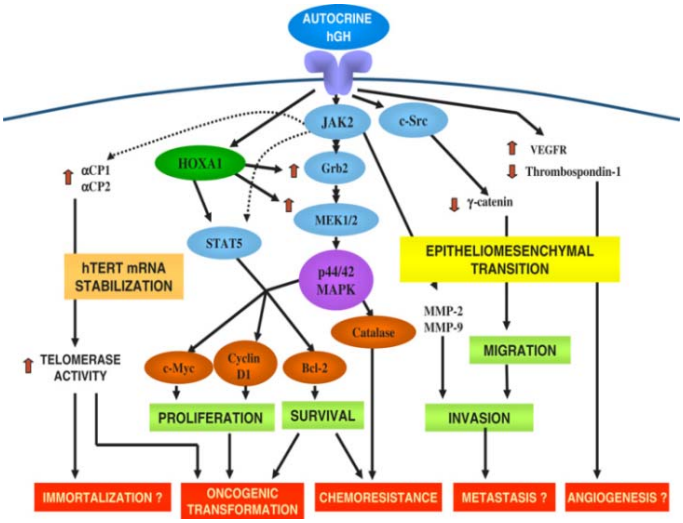


Figure 1-16 Autocrine hGH-mediated signal transduction in human mammary epithelial cells (adopted with permission from Perry *et al.*, 2006) [95]. Autocrine hGH is reported to up-regulate telomerase activity, oncogenic transformation, epithelial to mesenchymal transition and chemoresistance, which together provided a platform for oncogenic transformation in breast cancer.

Subsequent microarray analysis conducted by Xu *et al.,* 2005 identified 305 genes that are differentially expressed in MCF7 cell line in response to autocrine hGH, as well as exogenous hGH [126]. Among these identified genes, IGF-1, trefoil factor 1 and 3 (TFF1 and TFF3), bone morphogenic protein-7 (BMP-7) are some examples of genes found to be upregulated by autocrine hGH. These soluble proteins are capable of enhancing cell survival

and proliferation that eventually generate tumour formation. For instance, elevated IGF-1 level is well reported to induce hyperplasia lesions leading to tumourigenesis and associated with increased risk of breast, colorectal and prostate cancers [127]. Functional characterization of the trefoil factor (TFF1 and TFF3) family revealed that these soluble secretory proteins are capable of increasing cell survival, tumour formation and protection against apoptosis in breast cancer *in vitro* and *in vivo* [128, 129]. The same microarray data also revealed genes that are downregulated in response to autocrine expression of hGH such as the thrombospondin-1 (Tsp1), a known inhibitor of angiogenesis [130]. Therefore, autocrine expression of hGH in human mammary epithelial cells can stimulate upregulation of a number of secreted proteins that are capable of generating multiple autocrine and/or paracrine loops, thereby further promoting oncogenic potentials of autocrine hGH (Figure 1-17).

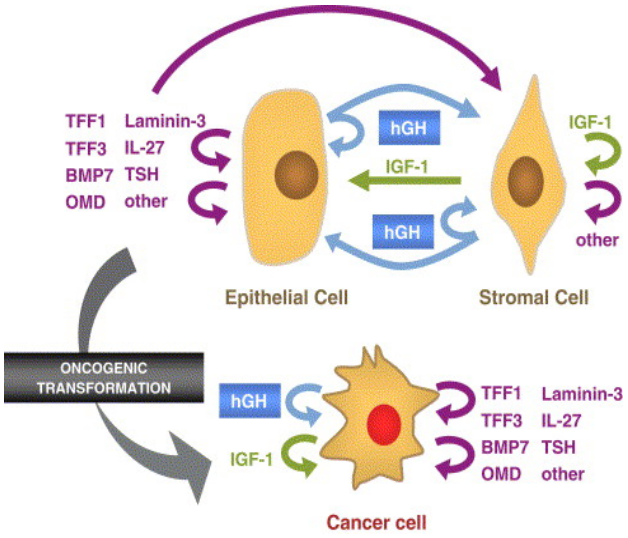


Figure 1-17 Oncogenic potential of autocrine hGH acting in autocrine and paracrine (adopted with permission from Perry *et al.*, 2006) [95]. Autocrine hGH upregulate soluble peptide factors, which include insulin-like growth factor-1 (IGF-1) and trefoil factor 1 and 3 (TFF1 and TFF3), bone morphogenic protein-7 (BMP-7) that drives oncogenic transformation.

All the studies provided a suggestive indication that autocrine expression of hGH to be relevant for disease progression. In accordance, Chiesa J *et al.,* (2011) reported the proliferative effects of autocrine hGH is maintained in primary human mammary carcinoma cells (HMCC) that is derived surgically from biopsies of patients diagnosed with mammary carcinoma [131]. It revealed elevated expression of hGH and its receptor (hGHR) in HMCC at both transcript and protein levels, as well as the extracellularly secreted hGH. Both hGH-positive and negative HMCC proliferated more rapidly in presence of exogenous hGH. However, decreased proliferation is only observed in hGH-positive HMCC when hGH was antagonised by B0236.

A clinical study conducted by Wu *et al.,* 2011 on a cohort of 159 breast cancer patients and 33 benign breast disease patients demonstrated that tumour expression of hGH at both transcript and protein levels were significantly associated with clinicopathologic parameters including lymph node metastases (LNM), higher tumour stage, tumour grade, HER-2 status and proliferative index in mammary and endometrial carcinomas [132]. This study also looked at tumour expression of hPRL in both mammary and endometrial carcinomas. Presence of tumour expression of hPRL at both transcript and protein levels are also reported to be significantly associated with LNM, tumour grade and tumour stage in mammary and endometrial carcinoma. Kaplan-Meier analysis examining the association of tumour expression of hGH and/or hPRL with relapse free survival (RFS) and overall survival (OS) of patients revealed an unfavourable RFS and OS for mammary carcinoma patients with tumour hGH and/or hPRL expression (Figure 1-18) [132]. Similar trend is also observed in endometrial carcinoma where tumour hGH and/or hPRL expression was

positively associated with poorer RFS and OS of patients as compared to patient whose tumours are hGH- and/or hPRL-negative. With these observations, Wu *et al.,* 2001 concluded that tumour hGH and hPRL expression is observed in a variety of tumours such as mammary and endometrial. It predicted significant difference in patient survival outcome, and are therefore useful as predictive markers for prognosis of these cancers. The data suggested that it may also be possible for combinatory inhibition of both hGH and hPRL to produce better survival for mammary and endometrial cancer patients. However, there is a need for studies to verify the applicability or therapeutic potential of inhibiting hGH and/or hPRL as single agent or in combination for mammary and endometrial cancers.

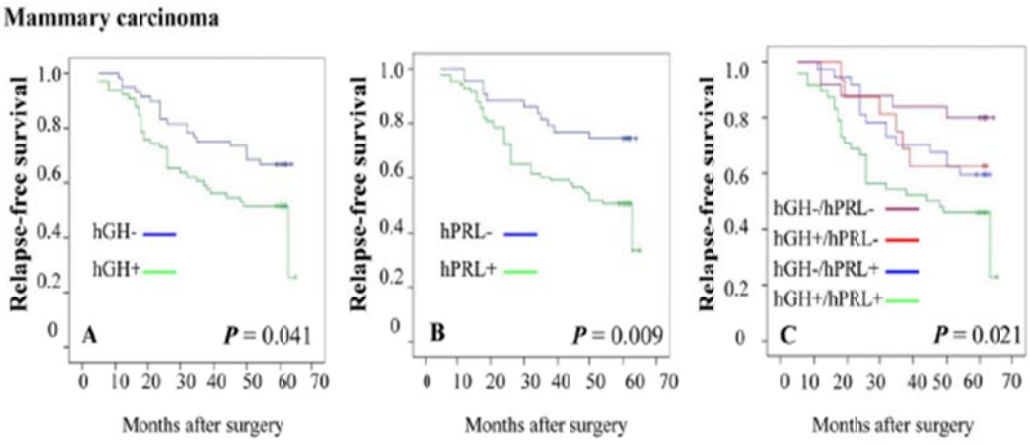


Figure 1-18 Kaplan-Meier analysis examining the significance of tumour hGH expression and/or tumour hPRL expression on both relapse-free survival (RFS) of patients with mammary carcinoma patients (adopted with permission from Wu *et al.,* 2011) [132].

1.4 Autocrine hGH and HER2+ Breast Cancer

The same clinical data from Wu *et al.,* (2011) also revealed an interesting result where there was significant positive correlation between tumour *hGH* mRNA (P = 0.004) and protein (P = 0.001) expression with HER2 status in

mammary carcinoma [132]. Given that autocrine expression of hGH has been shown to possess oncogenic potential in mediating mammary carcinoma progression *in vitro* and correlate with clinicopathological features observed in ER+ mammary carcinoma, it may also contribute to oncogenic manifestation in HER2+ breast cancer. Therefore, determining the role of hGH in HER2+ breast cancer biology will advance the understanding of the oncogenic properties of this molecule. Moreover, it helps to assess the viability of hGH as a potential target of therapies for intervention against HER2+ breast cancer, in particularly those that highly expresses GH in HER2+ tumours.

2 AIMS

The aim of the present study is to:

- Investigate the role of autocrine hGH in HER2+ breast carcinoma cells by forced expression of the *hGH* (GH1) gene in HER2+ breast carcinoma cell lines BT474 and SkBR3;

- Determine if forced expression of hGH abrogated HER2+ breast carcinoma cells to HER2-targeted therapies such as Herceptin or Lapatinib inhibition;

- Investigate synergistic inhibitory effects of GHR-antagonists and HER2-targeted therapy in hGH-overexpressing BT474 and SkBR3 mammary carcinoma cells;

- Examine the role of hGH in Herceptin-resistance HER2+ breast carcinoma cell lines BT474 and SkBR3.

3 MATERIALS AND METHODS

3.1 Breast Carcinoma Cell Lines

The human mammary carcinoma cell lines BT474 and SkBR3 were obtained from the American Type Culture Collection (ATCC). The BT474 cell line was cultured in RPMI 1640 media (Nacalai Tesque, Japan) supplemented with 2 mM L-glutamine, 10% heat-inactivated fetal bovine serum (FBS) (Biowest, France), 100 U/ml penicillin and 100 µg/ml streptomycin (Biowest, France). The SkBR3 cell line was cultured in McCoy's 5A media supplemented with 2 mM L-glutamine, 10% heat-inactivated FBS, 100 U/ml penicillin and 100 µg/ml streptomycin.

Cells were cultured in 75 cm^2 filtered flasks and incubated in 37^oC humidified 5% CO_2 incubator. Media was changed every 2-3 days. Once cells reached 60-70% confluent, cells will be trypsinized and seeded for functional assay.

3.2 Cell Transfection

For generation of hGH expression cell lines, BT474 and SkBR3 cells were stably transfected with an empty pcDNA3.1 vector (pcDNA-vec) as a control or the same pcDNA vector containing the entire *hGH* gene (pcDNA-hGH). These BT474 and SkBR3 transfected cell lines were designated BT474-Vec and BT474-hGH, and SkBR3-Vec and SkBR3-hGH respectively.

For stable transfection, cells were cultured in 25 cm^2 filtered flasks until 70% confluence and transfected using FuGENE 6 Transfection Reagent (Promega) according to the manufacturer's protocol. First, serum free media was mixed with FuGENE 6 transfection reagent at 3:1 ratio for both BT474 and SkBR3

cells in 1.5 ml tubes and incubated at room temperature for 15 minutes. Subsequently, 1.0 µg of plasmid DNA was added into the tube and incubated at room temperature for 30 minutes. During this incubation period, culture media was removed from 25 cm^2 flasks and cells were washed with phosphate buffered saline (PBS). Thereafter, 4 ml of serum-free media was added to the flask. The FuGENE/DNA complex solution was then added directly drop-wise to the cells. Following 6 hours of incubation at 37^oC, 2 ml of complete media was added to each flask.

Pooled stable transfectants were selected with the respective complete media containing 800 µg/ml (BT474) and 1,000 µg/ml (SkBR3) G418 (Sigma-Aldrich) for 3-4 weeks. During the period of selection, the media was changed every 2 days. Subsequently, stable transfectants were expanded and vials of each cell lines were frozen and stored.

3.3 Drug Treatments

Herceptin was kindly provided by Dr Goh Boon Cher from National University Hospital and Cancer Science Institute of Singapore. The working concentration of Herceptin used for functional assay was at 50 µg/ml media. An equivalent concentration of IgG was added to the control wells.

For functional assays involved the use of Lapatinb, drug treatment concentration (IC_{50}) was determined by performing dose response curve. Cells were seeded at 2 x 10^3 cells per 96-well plates in triplicate. Subsequently, 0, 1, 10, 100, 1,000 and 10,000 nM concentration of drugs was added to the cells and incubated for 48 hours. Cell viability was determined using Alarma Blue (Invitrogen) by measuring fluorescence reading. The working concentration of

Lapatinib for functional assay using BT474-Vec/hGH and SkBR3-Vec/hGH stable cells was determined at 150 nM and 15 nM respectively. An equivalent amount of DMSO was added to the control wells.

3.4 Herceptin-Resistance Cell Lines

Both BT474 and SkBR3 Herceptin-acquired resistance clones were generated by constantly treating the parental cell line with 10 μg/ml and 50 μg/ml Herceptin respectively in 25cm^2 filtered flasks for a period of 6 months. Fresh media containing Herceptin was replaced every 3 days.

3.5 Total RNA extraction

Total RNA was isolated from BT474-Vec, BT474-hGH, SkBR3-Vec and SkBR3-hGH cell lines using RNase extraction kit (Qiagen). Cells were lyzed by adding 1 ml Lysis Buffer/10 cm culture dishes, and an equal volume of 70% ethanol were added to the cell lysate in 1.5 ml tube. Subsequently, the lysate was subjected to the columns provided from the kit and centrifuged at 13,000 x g for 15 minutes at 4^{o}C. The columns were washed twice using the Wash Buffers provided and eluted in RNase-free water provided in the kit. The concentration of RNA was determined by Nanodrop. Purity of RNA was also determined using the ratios A_{260}/A_{280nm} and A_{260}/A_{230nm}.

To avoid genomic DNA contamination in subsequent gene expression analysis, the extracted RNA was treated with Dnase I (Invitrogen) according to the protocol provided by the kit. The extracted total RNA was stored at -80^{o}C.

3.6 Reverse transcription (RT) and Polymerase Chain Reaction (PCR)

Concentration of extracted total RNA was determined by Nanadrop prior to RT reaction. Total RNA was converted to cDNA using Superscript VILO cDNA kit (Invitrogen) according to manufacturer's instruction. All reagents were placed and procedure prepared on ice. The cDNA was either stored -20^{o}C or used immediately for PCR.

Polymerase chain reaction (PCR) was performed using the Platinum High Fidelity PCR kit (Invitrogen) based on the protocol provided by manufacturer. The hGH, hPRL, hGHR, hPRLR and β-actin primers with its respective appropriate annealing temperature and cycle number were stated below:

Gene	**Primer Sequence (5'→3')**	**Product Size (bp)**	**Annealing Temperature (^{o}C)**	**PCR cycle**
hGH	F: CCGACACCCTCCAACAGGGA R: CCTTGTCCATGTCCTTCCTG	343	62	35-40
hGHR	F: AGGAAGAGGGGAAACCAGAA R: TTTCCCGGAAGCTTTATCCT	110	55	30
hPRL	F: GTCCCACTACATCCATAACCTC R: CGCTCGGTGAGGATCTTCA	326	60	35

hPRLR	F: ATGATATCGCCGCGCTCG R: CGCTCGGTGAGGATCTTCA	650	60	35
β-actin	F: ATGATATCGCCGCGCTCG R: CGCTCGGTGAGGATCTTCA	581	52	25

For parental or wild-type cells, the PCR cycle for detection of *hGH* gene is 40. For hGH-stably transfected BT474 and SkBR3 cells, 35 cycles was used for detecting *hGH* gene expression.

3.7 Protein Extraction

Cells were seeded on 10 cm tissue culture dishes at 70% confluence, and changed into serum free media the following days for 24 hours. Cells were washed twice with ice cold PBS and harvested in lysis RIPA buffer (2 M NaCl, 10% Na-deoxycholate, 10% SDS, Triton X-100, 1 M Tris-HCl and Water) containing cocktail protease (1^{st} Base Pte Ltd) and phosphatase inhibitors (Calbiochem) by mechanical scraping. Lysates were incubated for 30 minutes on ice with vortexing every 5 minutes. Following incubation, lysates were centrifuged at 13,000 rpm for 10 minutes at 4^{o}C. The cell pellet which consists of cell debris was discarded. The supernatant was transferred into a clean 1.5 ml tubes and stored at -80^{o}C or used immediately for protein analysis.

3.8 Protein Quantification

Protein quantification was measured in triplicate using DC Protein Assay (Bio-Rad Laboratories Pte Ltd). A set of standard solutions (0, 1, 2, 4, 6, 8 and 10 μg/ml) were prepared using Bradford reagent (Bio-Rad Laboratories Pte Ltd). First, 22 μl of DC Reagent A was added into each well of 96-well plate, followed by 1 μl of either protein standards or samples. Subsequently, 176 μl of DC Reagent B was added to each well. The plate was incubated in dark at room temperature for 15 minutes for colourmetric reactions to take place. Protein concentration was determined by reading absorbance at 750 nm using TECAN i-control plate reader. Based on the absorbance of standards, a standard curve was plotted, and concentration of protein sample was determined using equation generated from the standard curve.

3.9 Conditioned Media

Cells were seeded at 3 x 10^4 in 10cm^2 tissue culture dish until 60-70% confluence, and subsequently culture media was changed to serum free for 24 hours. The cell culture supernatant was then collected and subjected to ultracentrifugation at 7,500 x g for 40 minutes using 10 kDa MWCO Ultrafilters concentrators (Millipore). The flow-through was discarded and the section retained in the concentrator was collected for subsequent western blot or ELISA analysis as this fraction was where the secreted hGH (22 kDa) be found. For western blot analysis, equal amount of laemmli loading buffer (Bio-Rad Laboratories Pte Ltd) containing β-mercaptoethanol (Sigma-Aldrich) was added to the supernatant prior to boiling at 99^oC. For subsequent ELISA analysis, the collected conditioned media was stored at -80^oC before use.

3.10 Protein Electrophoresis and Western Blot

Protein extracted from whole cell lysate and conditioned media were resolved by 10% Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) at 80 V for stacking gel (1 M Tris-HCl pH 6.8) and subsequently 120V for resolving gel (1.5 M Tris-HCl pH 8.8) in 1X Tris/Glycine/SDS buffer. For detection of HER2 or pHER2, whole cell protein extract was resolved by 6% SDS-PAGE gel under the same condition. For western blot, the resolved gel was transferred onto polyvinylidene floride (PVDF) membrane (Bio-Rad Laboratories Pte Ltd) using 1X Glycine/Methanol transfer buffer at 100 V at 1 to 1.5 hours. The PVDF membrane was activated in methanol and washed in water prior to use for western blot. Blocking was done using 5% BSA/PBST (Bovine serum albumin in Phosphate-buffered saline with 1% Tween 20) for 2-4 hours at room temperature. Thereafter, the membrane was incubated overnight at 4^{o}C with primary antibodies: rabbit anti-hGH (1:2,000) (Dr Parlow, National Hormone and Peptide Program, Torrance, CA), rabbit anti-HER2 (1:500) (Abcam), rabbit anti-Phospho-HER2 (Y1248) (1:1,000) (Abcam), and mouse anti-β-actin (1:5000) (Santa Cruz) as loading control. Subsequently, the membrane was washed thrice with PBST for 10 minutes. Subsequently, the membrane was incubated with the appropriate secondary anti-rabbit or anti-mouse antibody (Sigma-Aldrich) at 1:10,000 for 1 hour, and again washed thrice with PBST. Lastly, protein bands were detected using horseradish peroxidase western blot detection system (Supersignal West Pico and Femto Chemiluminescence substrate, Pierce, Life Technology) and film (Konica Minolta Fujifilm) was developed using Kodak Medical X-Ray Processor (Carestream Health).

3.11 hGH Enzyme-linked immunosorbent assay (ELISA)

The hGH ELISA kit (Roche) was used to quantitatively measure the expression levels of secreted hGH released into cell culture supernatant of eukaryotic cells transfected with plasmid containing hGH gene. The procedure for ELISA was carried out according to the manufacturer's instructions. For each sample, 100 µl was added into each anti-hGH-precoated well on the ELISA microtitre plate in duplicate. The kit has provided recombinant hGH protein (5 ng) as positive control where a standard curve was plotted at concentration 0, 12.5, 25, 50, 100, 200 and 400 pg/ml against absorbance ($A_{409nm} - A_{490nm}$). The amount of secreted hGH present in each sample was determined from the standard curve generated. Results were expressed as concentration of hGH in pg/ml of the media. Every time an ELISA was performed, a new standard curve was generated.

3.12 Total Cell Number

Cells (BT474-Vec, BT474-hGH, SkBR3-Vec and SkBR3-hGH) were seeded in 6-well plates at a density of 1 x 10^4 cells per well in either 0.5% FBS or 10% FBS medium. For drug treatment, cells were seeded in 24-well plates at a density of 3 x 10^4 cells per well. Total cell number was counted every 2 days for a period of 10 days. Treatment media was changed every third day.

For cell counting, cells were trypsinized using 1X Trypsin/EDTA (Biowest) and subsequently neutralized by adding 1 ml complete media. After that, 10 µl of the cell suspension were mixed with 10 µl of trypan blue (Biowest), and 10 µl of the mixture was loaded on each side of the haemocytometer. Only viable cells were counted. Number of cells contained in 4 quadrants of 16 squares

was counted twice. The counted squares were then added together and averaged. Finally, the total number of cells was determined by the amount of cells/ml using the following formula:

(Number of Cells Counted in all quadrant/4) X 10^4 X dilution factor = cells/ml

Cells/ml X final volume of suspension = total no. of cells

3.13 Soft Agar Colony Formation (SACF)

Cells were cultured to 60-70% confluence in 75 cm^2 flask in complete media. For soft agar colony formation assay, cells were seeded in 96-well microtitre plates at a density of 1 x 10^3 cells per well.

Each well of the 96-well plate was first covered with a base agar layer that is made up of 0.5% agarose in serum free RPMI media (BT474-vec and BT474-hGH) or serum free McCoy's 5A (SkBR3-vec and SkBR3-hGH) media. This base agar layer was allowed to set at room temperature for 30 minutes. Subsequently, 0.7% agarose (Bio-Rad Laboratories Pte Ltd) was prepared in serum free media and incubated in water bath at 42^{o}C to maintain cellular viability and avoid polymerisation of the agar.

Cells were trypsinized and resuspended in complete media to obtain single cell suspension. Equal volume of 0.7% agar was added to equal volume of cell suspension to make a final cell suspension in 5% media with 0.35% agarose. This mixture was added carefully to each well on the top of the base agar to prevent bubbles formation. The plate was left to set at room temperature for 30 minutes and 100 µl of treatment media was then added to each well. Treatment media will diffuse into the semi-solid layer where cells were seeded in

suspension. Experiment was performed in triplicate over a period of 12-14 days. Media was changed every 6-7 days. At day 14, the media was drained and alamar blue was added to determine viability of colonies formed by measuring fluorescence. Alamar blue was incubated for 3-4 hours. Representative pictures of colonies formed were taken using microscope at 10X magnification.

3.14 Three-Dimensional (3D) Culture of Cells in Matrigel

For this assay, cells were plated in 96-well plates at 1 x 10^3 cells per well in 4% growth factor reduced Matrigel^{TM} (BD Biosciences) and 5% serum media and allowed to form colonies.

The plate was first coated with 40 µl of 100% Matrigel and allowed to set at room temperature for 30 minutes. Subsequently, cells were trypsinized and resuspended in 1:1 ratio of serum-free media to complete media to make a final cell suspension in 5% media with 4% matrigel. Cells were allowed to grow and form colony for a period of 14 days. Treatment media was changed every 6-7 days. Cell viability was determined by measuring fluorescence using Alamar blue. Representative pictures of colonies formed were taken using microscope at 10X magnification.

3.15 Apoptosis

Cells were seeded in 96-well black plates at a density of 2 x 10^4 cells per well in either serum-free or 10% FBS or treatment media. After 24 hours of incubation, equal volume of ApoCaspase 3/7 reagent (Promega) was added

and incubated for 30 minutes. Subsequently, apoptosis was determined by measuring caspase 3/7 luminescence activity using TECAN reader.

3.16 Statistics Analysis

Graphical presentations were generated using GraphPad Prism 5. The statistical analysis was done using GraphPad Prism 5 in conjunction with Microsoft Excel. All experiments were performed at least three times and a single representative figure is shown. Data is first analysed using F test to determine equal variance between two comparing samples. If the p value for F test is <0.05, statistical significance will be computed using Student's unpaired two-tailed equal variance t test. However, if p value for F test is >0.05, statistical significance between two comparing samples will then be computed using Student's unpaired two-tailed unequal variance t test. Numerical data were expressed as mean +/- standard error mean (SEM) of triplicate determinants.

4 RESULTS

4.1 Endogenous hGH expression in HER2+ mammary carcinoma cells

Four HER2-overexpressing mammary carcinoma cell lines, BT474, MDA-MB-361, SkBR3 and MDA-MB-453, were selected in this study for screening of endogenous *hGH* mRNA and protein expression. Besides being classified as HER2-amplified cells, BT474 is ER+/PR+, MDA-MB-361 is ER+/PR-, while SkBR3 and MDA-MB-453 are both ER-/PR-. As indicated in Figure 4-1A, it showed that these 4 HER2+ mammary carcinoma cell lines expressed differential endogenous level of *hGH* transcripts. All cell lines expressed the hGHR and hPRLR, which are receptors for hGH ligand [88, 90]. Similarly, all cell lines expressed differential levels of endogenous hGH proteins from the whole cell lysate (Figure 4-1B).

From figure 4-1, the mRNA expression of *hGH* from BT474, MDA-MB-361, SkBR3 and MDA-MB-453 differed from its respective protein expression levels which were obtained from whole cell lysate. One explanation would be hGH being a secretory protein and hence the endogenous hGH produced by the different cell lines may be in the secreted form. As a result, protein extracted from whole cell lysate may not correlate with its expression at the gene level. Therefore, an ELISA was carried out to quantify the amount of secreted hGH from the 4 different HER-amplified cell lines, which may better correlates with its respective mRNA expression levels.

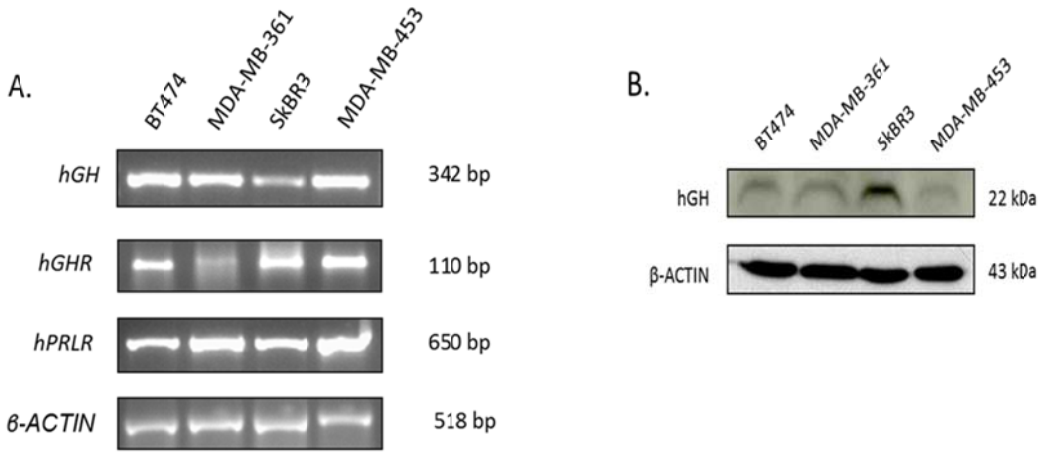


Figure 4-1 Endogenous expression of hGH, hGHR and hPRLR in wild-type HER2+ breast carcinoma cells. (A) Endogenous gene expression of *hGH, hGHR* and *hPRLR* in BT474, MDA-MB-361, SkBR3 and MDA-MB-453 HER2+ breast carcinoma cells. (B) Endogenous protein expression of hGH in HER2+ breast carcinoma cells. The loading control used was β-actin.

An ELISA was performed to quantitatively determine the expression levels of secreted hGH in BT474, MDA-MD-361, SKBR3 and MDA-MB-453 cells. The positive control used for hGH ELISA was the recombinant hGH provided by the kit. Concentration of secreted hGH was determined by calculating the absorbance readings based on the standard curve (Figure 4-2A) obtained from recombinant hGH. The bar chart presented in Figure 4-2B showed the quantitative expression levels of secreted hGH from BT474 (15.8 pg/ml), MDA-MB-361 (6.4 pg/ml), SkBR3 (12.4 pg/ml) and MDA-MB-453 (33.6 pg/ml) cells.

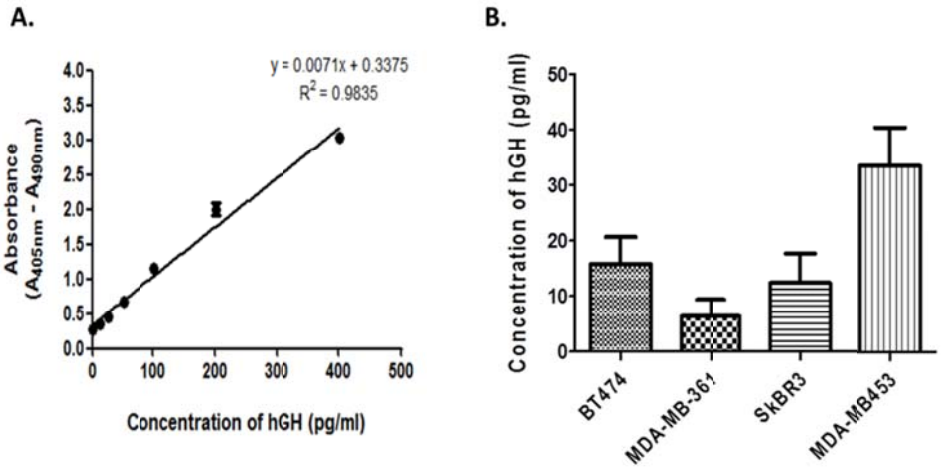


Figure 4-2 hGH ELISA of HER2+ human mammary carcinoma cells, BT474, MDA-MB-361, SkBR3 and MDA-MB-453. (A) Standard curve obtained by plotting concentration of recombinant hGH versus absorbance ($A_{405nm} - A_{490nm}$). (B) Quantitative amount of secreted hGH from BT474, MDA-MB-361, SkBR3 and MDA-MB-453 cells.

Herceptin and Lapatinib will be used in this project to further investigate if autocrine expression of hGH reduces sensitivity of HER2+ mammary carcinoma cells to these conventional drugs used in clinics for treatment of HER2+ breast cancer patients. Therefore, the HER2+ mammary carcinoma cells chosen have to be responsive to Herceptin and Lapatinib. Among the HER2+ mammary carcinoma cell lines, BT474 and SkBR3 are both sensitive to Herceptin and Lapatinib, while MDA-MB-361 and MDA-MB-453 are resistant to Lapatinib and Herceptin respectively [133]. Therefore, the BT474 (ER+/PR+/HER2+) and SkBR3 (ER-/PR-/HER2+) cells were chosen here as the *in vitro* model to investigate the potential role of autocrine hGH in mediating oncogenic behaviour of HER2+ mammary carcinoma cells.

4.2 Forced Expression of *hGH* Gene in HER2+ Mammary Carcinoma Cells

To investigate the role of autocrine hGH in modulating oncogenic potential of HER2+ mammary carcinoma cells, the *hGH (GH1)* gene was stably

transfected in BT474 and SkBR3 cells using FuGene 6 transfection reagent. The empty pcDNA vector was also transfected into both cell lines as control. The resultant transfectants BT474-Vec, BT474-hGH and SkBR3-Vec, SkBR3-hGH stable cell lines were verified by RT-PCR, western blot and ELISA.

As shown in Figure 4-3, the BT474-hGH and SkBR3-hGH cells significantly expressed high levels of *hGH* mRNA and protein as compared to their respective BT474-Vec and SkBR3-Vec control cells, indicating effective forced expression of *hGH* in both cell lines. Concomitantly, western blot analysis on the cell culture supernatants revealed significant elevated levels of secreted hGH protein in BT474-hGH and SkBR3-hGH cells compared to control cells. In addition to the predominant 22 kDa hGH, western blot detected the 20 kDa hGH isoform protein in both BT474-hGH and SkBR3-hGH cells. This smaller molecular weight hGH isoform protein was derived from alterative splicing of *hGH* mRNA.

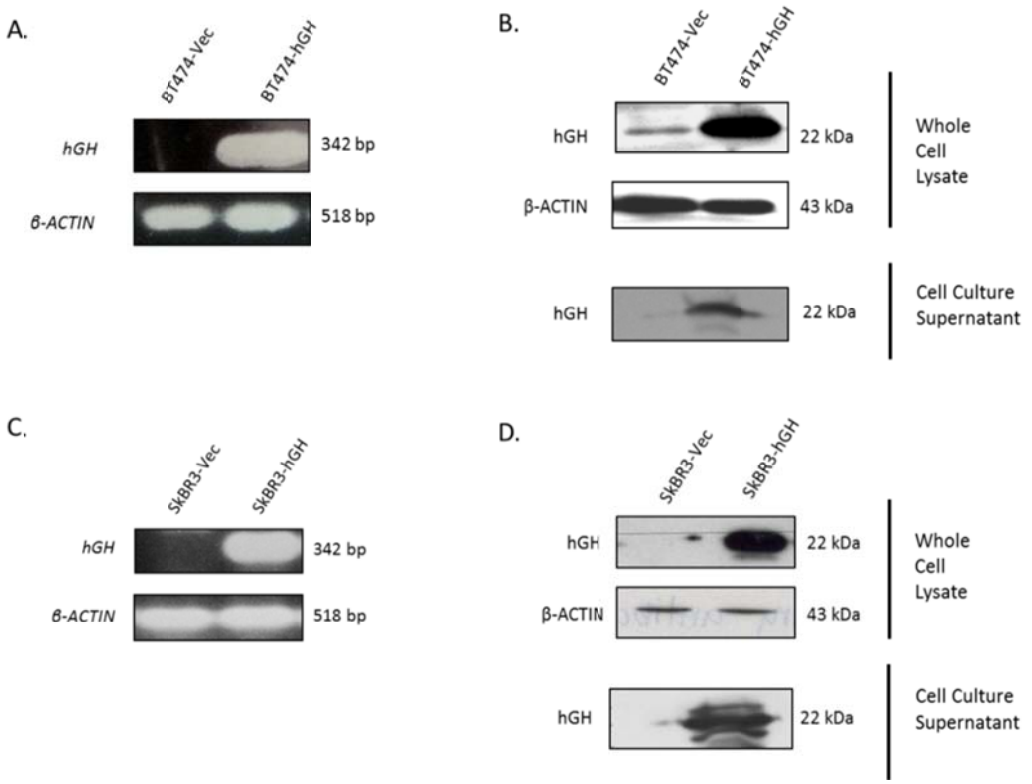


Figure 4-3 Forced expression of hGH in BT474 and SkBR3 HER2+ breast carcinoma cells. (A and C) Expression of hGH in stably transfected BT474 cells and SkBR3 cells respectively. (B and C) Western Blot demonstrated forced expression of hGH in stably transfected BT474 and SkBR3 cells in both whole cell lysate and conditioned media. All experiments used β-actin as loading control.

The protein expression of hGH was not detected in BT474-Vec and SkBR3-Vec via western blot due to the comparatively low amount of hGH levels in the cells. Nevertheless, hGH ELISA is able to quantitatively determine the expression of secreted hGH in both BT474-Vec and SkBR3-Vec cells to be 23.5 pg/ml and 27.5 pg/ml respectively (Figure 4-4B). The BT474-hGH and SkBR3-hGH cells were shown to express significantly higher amounts of secreted hGH at 988.1 pg/ml and 976.4 pg/ml respectively.

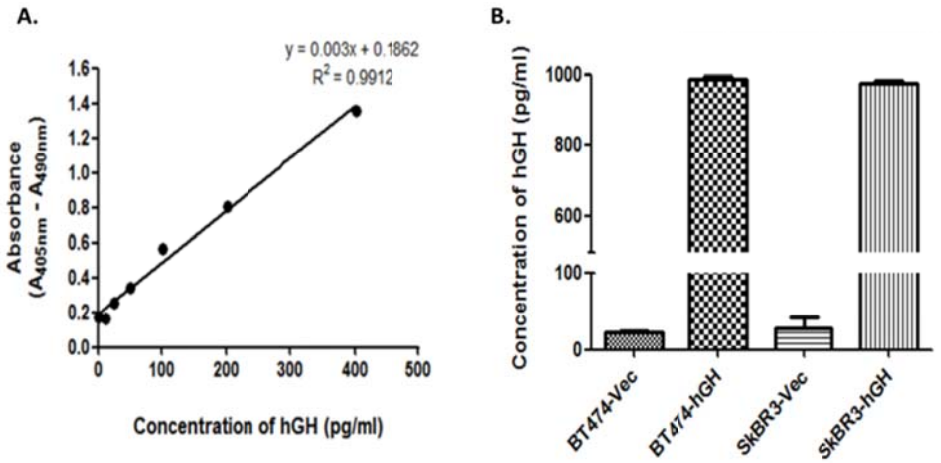


Figure 4-4 hGH ELISA of BT474-Vec, BT474-hGH, SkBR3-Vec and SkBR3-hGH stable cells. (A) Standard curve obtained using recombinant hGH as positive control. (B) Quantitative amount of secreted hGH expressed in BT474-Vec, BT474-hGH, SkBR3-Vec and SkBR3-hGH stable mammary carcinoma cells.

After establishing and verifying stable clones of BT474-Vec, BT474-hGH, SkBR3-Vec and SkBR3-hGH cells, functional assays such as total cell count or cell viability, apoptosis, soft agar colony formation, and growth in 3D matrigel were performed to investigate the contribution of autocrine hGH in promoting oncogenic transformation of HER2+ breast carcinoma cells.

4.3 Autocrine hGH increases BT474 and SkBR3 HER2+ mammary carcinoma cell number *in vitro*

To determine the effect of forced expression of hGH on BT474 and SkBR3 mammary carcinoma cell number, a total cell number assay was performed by determining the number of cells every 2 days, over a period of 10 days.

It was observed that forced expression of hGH in BT474 cells resulted in significant increase in total cell number compared to BT474-Vec control cells in culture conditions supplemented with full serum (10% FBS) over a period of 10 days (Figure 4-5A). One hallmark of cancer cells is their reduced dependence on external growth factors by acquiring self-sufficient growth.

Here, the results indicated that at reduced serum conditions (0.5% FBS), the presence of autocrine hGH is able to provide self-sustaining growth signals in BT474 cells where total cell number was significantly increased when compared to its respective BT474-Vec control cells (Figure 4-5B).

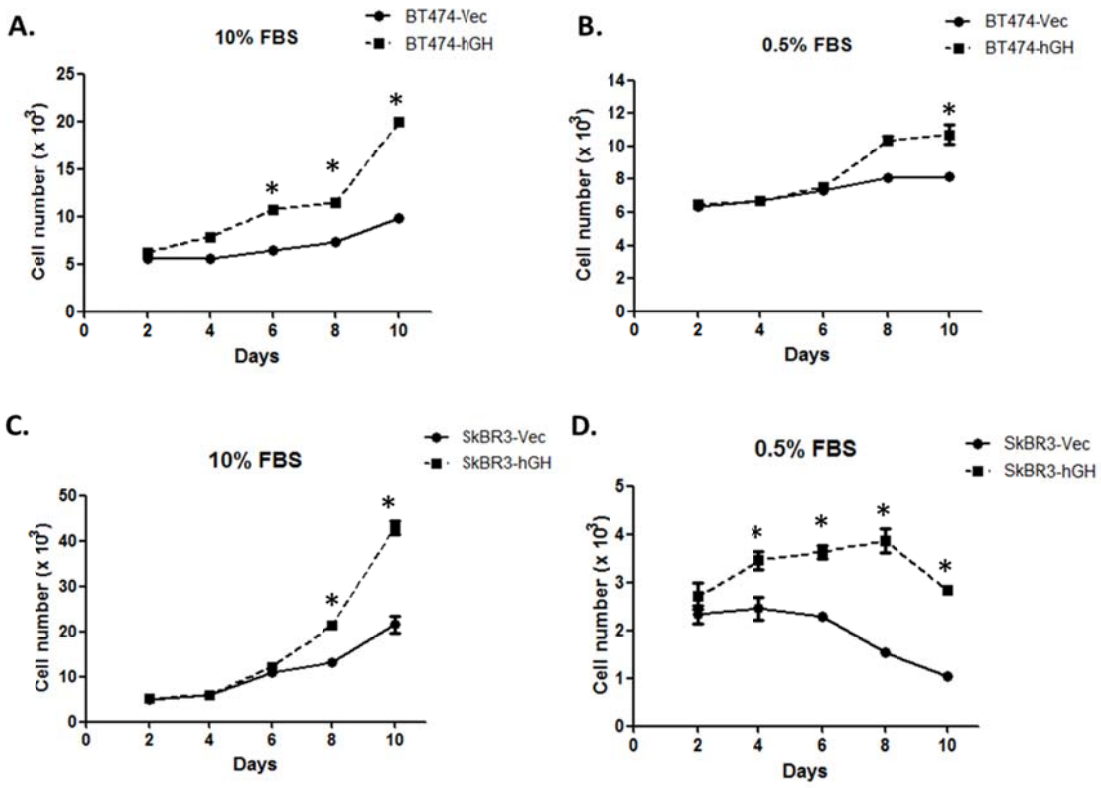


Figure 4-5 Total cell number of BT474-Vec, BT474-hGH, SkBR3-Vec and SkBR3-hGH mammary carcinoma cells over a period of 10 days. (A and B) Total cell number of BT474-Vec and BT474-hGH cells in media supplemented with 10% and 0.5% FBS respectively. (C and D) Total cell number of SkBR3-Vec and SkBR3-hGH cells in media supplemented with 10% and 0.5% FBS respectively. (*$p < 0.05$)

The same trend was also observed in SkBR3-hGH cells where total cell number was significantly higher as compared to SkBR3-Vec cells under 10% and 0.5% serum conditions (Figure 4-5C and D). The SkBR3 cells were unable to tolerate low serum condition, therefore total cell numbers of SkBR3-Vec and SkBR3-hGH cells were observed to decrease at Day 10 (Figure 4-5D). Nevertheless, the trend observed in Figure 4-5D still demonstrated that forced expression of hGH in SkBR3 provided sustainable growth under low

serum condition and promoted significant cell survival and proliferation when compared to its respective SkBR3-Vec cells.

4.4 Forced expression of hGH decreased BT474 and SkBR3 cells from serum deprivation-induced apoptosis

Next, I investigated the effect of forced expression of hGH in BT474 and SkBR3 cells on apoptosis by measuring caspase 3/7 activity. Caspase 3 and caspase 7 are two of the early cell death effectors in apoptosis pathway. Activation of these effector caspases mediate cleavage of regulatory proteins and housekeeping proteins involved in apoptosis, resulting in DNA fragmentation and eventually removal of cells [134]. Therefore, measuring of caspase 3/7 activity will be useful as indicator of apoptotic events in cells. As shown in Figure 4-6, the caspase 3/7 activity of BT474-hGH was observed to be significantly lower as compared to BT474-Vec. A similar trend was observed for SkBR3-Vec/hGH cells where SkBR3-hGH cells exhibited lower induction of apoptosis in comparison with SkBR3-Vec cells. Such observations demonstrated that autocrine expression of hGH protected HER2+ mammary carcinoma cells from serum deprivation-induced apoptosis *in vitro*.

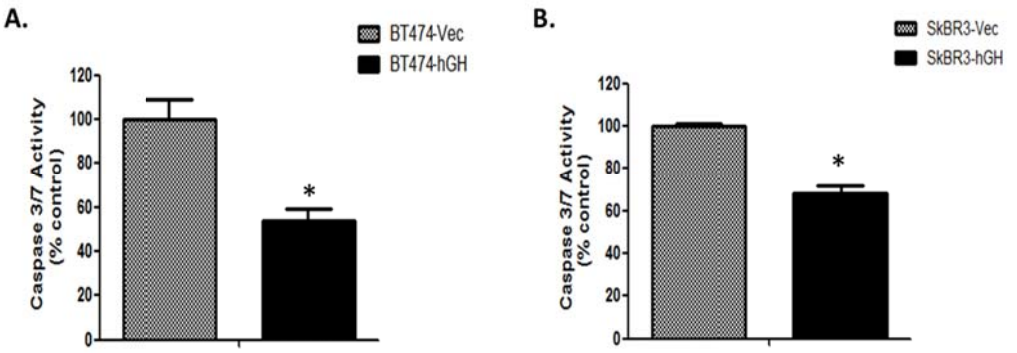


Figure 4-6 Forced expression of hGH reduced apoptosis in BT474 and SkBR3 cells. (A and B) Caspase 3/7 activity presented as percent control of BT474-Vec/hGH and SkBR3-Vec/hGH respectively after 24 hours serum deprivation. (*$p < 0.05$)

4.5 Forced expression of hGH increased BT474 anchorage-independent growth in soft agar and enhanced colony formation in 3D Matrigel™

One phenomenon that cells acquire during the process of oncogenesis is the ability to grow in an anchorage-independent manner and subsequent survival upon detachment from matrix (overcoming anoikis) [5, 12, 13]. This cell transformation feature is essential to ensure cancer cells survival, particularly in the context of metastasis where cells have to migrate in the circulation to distant sites. An inability to acquire the capacity for anchorage-independent growth will render cancer cells success to survive or proliferate. In order to examine and assess this feature of oncogenic transformation *in vitro*, cancer cells were cultured in semi-solid soft agar that prevents cells from attaching to substrate.

Soft agar colony formation assay was performed in both full (10% FBS) and reduced serum (0.5% FBS) conditions, and Alamar blue was used to assess cell viability as marker of total cell number. Based on the images taken from microscope, it was observed that BT474-hGH cells formed larger colonies as

compared to BT474-Vec cells in full and reduced serum conditions (Figure 4-7A and B). Concomitantly, cell viability of BT474-hGH in soft agar was significantly higher when compared to its respective BT474-Vec cells under full and reduced serum conditions. The difference in cell viability between BT474-Vec and BT474-hGH was more significant in the reduced serum (0.5% FBS) condition, where the majority of BT474-Vec cells still remained as single colonies when observed under microscope. In contrast, BT474-hGH cells formed large colonies under the same conditions. Such observation demonstrated that autocrine hGH expression was able to enhance anchorage-independent growth and increase colony forming potential in BT474 cells significantly.

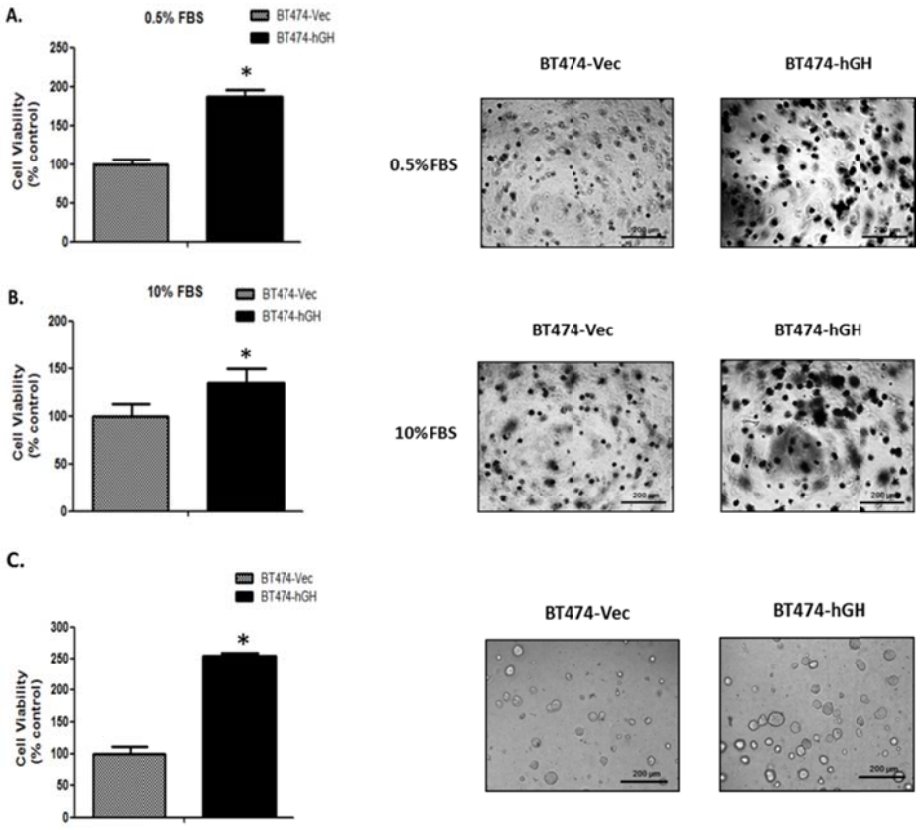


Figure 4-7 Forced expression of hGH increased formation of colony and cell viability of BT474 cells in soft agar and 3D Matrigel™. (A and B) Cell viability of BT474-Vec/hGH cells in 0.5% and 10% FBS respectively with representative pictures of colonies formed by BT474-Vec/hGH cells at the indicated conditions. (C) Cell viability and representative picture of BT474-Vec/hGH in 3D Matrigel. Images taken at 4X magnification. (*$p < 0.05$)

In addition, the 3D growth of BT474-Vec/hGH cells was assessed by culturing cells in growth factor-reduced Matrigel™ in reduced serum (5% FBS) conditions. Forced expression of hGH in BT474 cells markedly increased colonies formation in 3D Matrigel™ over a period of 14 days, indicating enhanced cell growth (Figure 4-7C). The BT474-hGH cells were observed to form large colonies as compared to its respective BT474-Vec control cells (Figure 4-7C image). Therefore, autocrine hGH possessed oncogenic transforming potential that is capable of increasing survival and enhancing anchorage-independent potential and 3D growth of BT474 cells.

4.6 Forced expression of hGH in SkBR3 cells enhanced growth in 3D Matrigel™, but has no effect on anchorage-independent growth

The SkBR3-Vec/hGH cells were cultured in soft agar as well. However, there is no significantly difference between SkBR3-Vec and SkBR3-hGH cells in soft agar, therefore autocrine expression of hGH in SkBR3 has no effect on anchorage-independent growth. The SkBR3-Vec/hGH cells were cultured in 3D growth factor-reduced Matrigel™ to assess oncogenic growth ability that more closely mimics the *in vivo* situation.

In the 3D Matrigel™ assay, SkBR3-hGH cells exhibited significant formation of viable and larger colonies when compared to SkBR3-Vec control cells over a period of 14 days (Figure 4-8). The SkBR3-hGH cells were observed to form larger colonies, while SkBR3-Vec cells either formed few colonies or did not form colonies (remained single cells). Hence, autocrine expression of hGH increased growth potential of SkBR3 cells by enhancing colony formation in 3D.

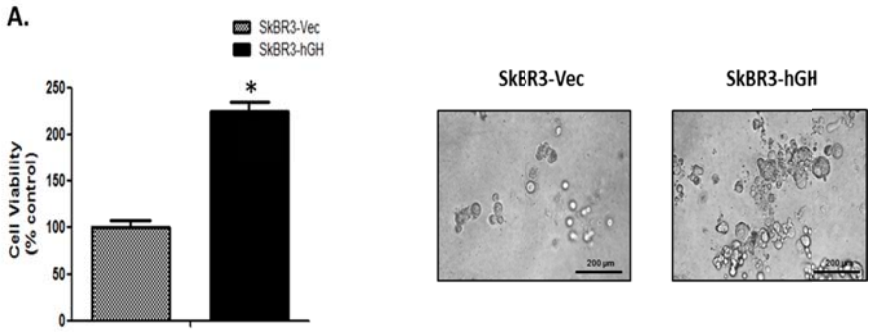
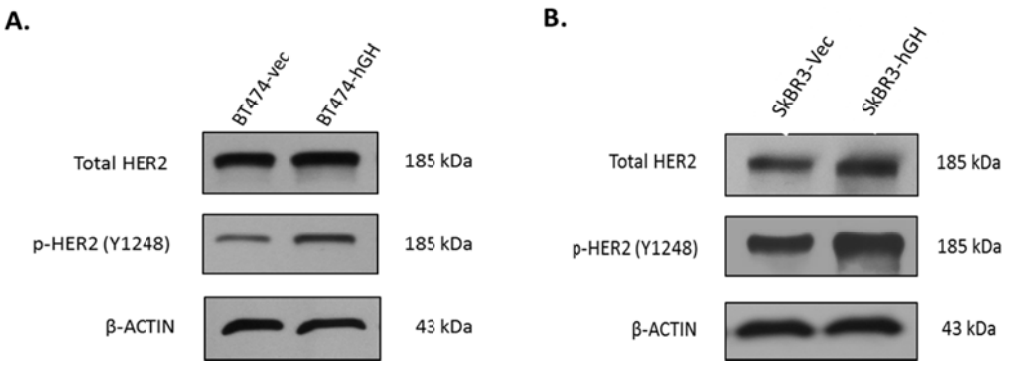


Figure 4-8 Autocrine hGH increases SkBR3 growth and colony formation in 3D Matrigel. (A) Cell viability and representative pictures of SkBR3-Vec/hGH colonies growth and formation in 3D Matrigel™. Images taken at 10X magnification. (*$p < 0.05$)

4.7 Forced expression of hGH increased tyrosine phosphorylation of HER2 (Y1248) in BT474 and SkBR3 mammary carcinoma cells

Based on the above results, forced expression of hGH was able to increase proliferative disorder and enhance oncogenic transformation of HER2+ mammary carcinoma cells. Western blot analysis indicated that forced expression of hGH resulted in increased tyrosine phosphorylation of HER2 (Y1248) in both BT474 and SkBR3 cells as compared to their respective BT474-Vec and SkBR3-Vec control cells (Figure 4-9). The phosphotyrosine Y1248 residue is found to be the major autophosphorylation site of HER2 [135]. The tyrosine phosphorylated HER2 (Y1248) can thus recruit downstream signalling molecules and subsequently transduce signal downstream. The increased HER2 activity in hGH-overexpressing BT474 and SkBR3 cells may thus explain the protection against apoptosis and enhanced viability observed in the total cell count, soft agar and growth factor-reduced matrigel in 3D.



	Relative Density	
	BT474-Vec	BT474-hGH
Total HER2	1.160	1.222
p-HER2 (Y1248)	0.530	0.989
β-ACTIN	1	0.918

	Relative Density	
	SkBR3-Vec	SkBR3-hGH
Total HER2	0.766	0.855
p-HER2 (Y1248)	0.959	1.408
β-ACTIN	1	1

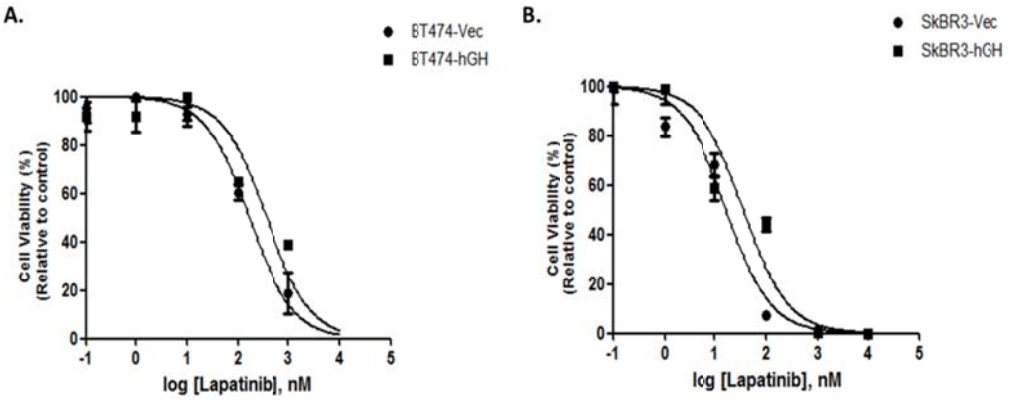
Figure 4-9 Forced expression of hGH increased phosphorylation of HER2 (Y1248) activity in BT474 and SkBR3 mammary carcinoma cells (A and B). The β-actin is used as loading control. Quantitative analysis of the gel bands were also determined using Image J, and the values presented are relative density against β-actin.

4.8 Forced expression of hGH decreased sensitivity of HER2+ mammary carcinoma cells to Herceptin or Lapatinib treatment

Disease relapse or resistance to treatment is one major challenge in treating cancer patients including the HER2+ subtype breast cancer patients [62, 63, 68, 74, 84]. It is interestingly relevant to determine if the oncogenic properties of autocrine hGH reduces sensitivity of BT474 and SkBR3 HER2+ mammary carcinoma cells to HER2-targeted therapy-induced growth inhibition. Herein, cell viability of BT474-Vec/hGH and SkBR3-Vec/hGH were assessed in the presence of either two of the most commonly used HER2-targeted therapeutics, Herceptin or Lapatinib.

Both Herceptin and Lapatinib blocks activation of HER2 pathways by two different modes of actions where the former binds to the extracellular domain of HER2 to initiate ADCC [63, 136], while the latter binds to intracellular

kinase domain of HER2 to abrogate tyrosine phosphorylation and subsequently block HER2 signalling [64, 65, 137]. The concentration of Herceptin used for BT474 and SkBR3 growth inhibition was 50 µg/ml. The dose response curve presented in Figure 4-10A indicated the concentration of Lapatinib to be used on BT474-Vec/hGH cells was 150 nM, while SkBR3-Vec/hGH cells was determined to be 15 nM.



	Lapatinib IC_{50}
BT474-Vec	166.4 nM
BT474-hGH	362.9 nM

	Lapatinib IC_{50}
SkBR3-Vec	16.6 nM
SkBR3-hGH	35.9 nM

Figure 4-10 Dose response curve of Lapatinib on HER2+ breast carcinoma cells plotted as cell viability versus log concentration of lapatinib. (A and B) The IC_{50} of Lapatinib on BT474-Vec/hGH and SkBR3-Vec/hGH stable cells was determined to be 150 nM and approximately 15 nM respectively.

The cell viability assay demonstrated that in the presence of Herceptin, BT474-hGH cells exhibited a gradual increase in cell growth in comparison to its respective treated BT474-Vec cells over a period of 10 days (Figure 4-11A, red line). Such observations indicated that autocrine hGH expression enhanced growth of BT474 cells after Herceptin treatment. Similarly, cell viability of BT474-hGH cells was significantly higher as compared to BT474-Vec cells when treated with Lapatinib (Figure 4-11B blue line). Therefore, the results

demonstrated that autocrine production of hGH also protected BT474 cells from Lapatinib-induced inhibition.

Similar trends were also observed for cell viability assays performed on SkBR3-Vec/hGH cells after Herceptin or Lapatinib treatment. The Herceptin-treated SkBR3-hGH cells significantly increased viability when compared to its respective Herceptin-treated SkBR3-Vec cells (Figure 4-11C). After treatment with Lapatinib, it was observed that SkBR3-hGH cells exhibited higher viability over a period of 10 days in comparison to its respective treated SkBR3-Vec cells.

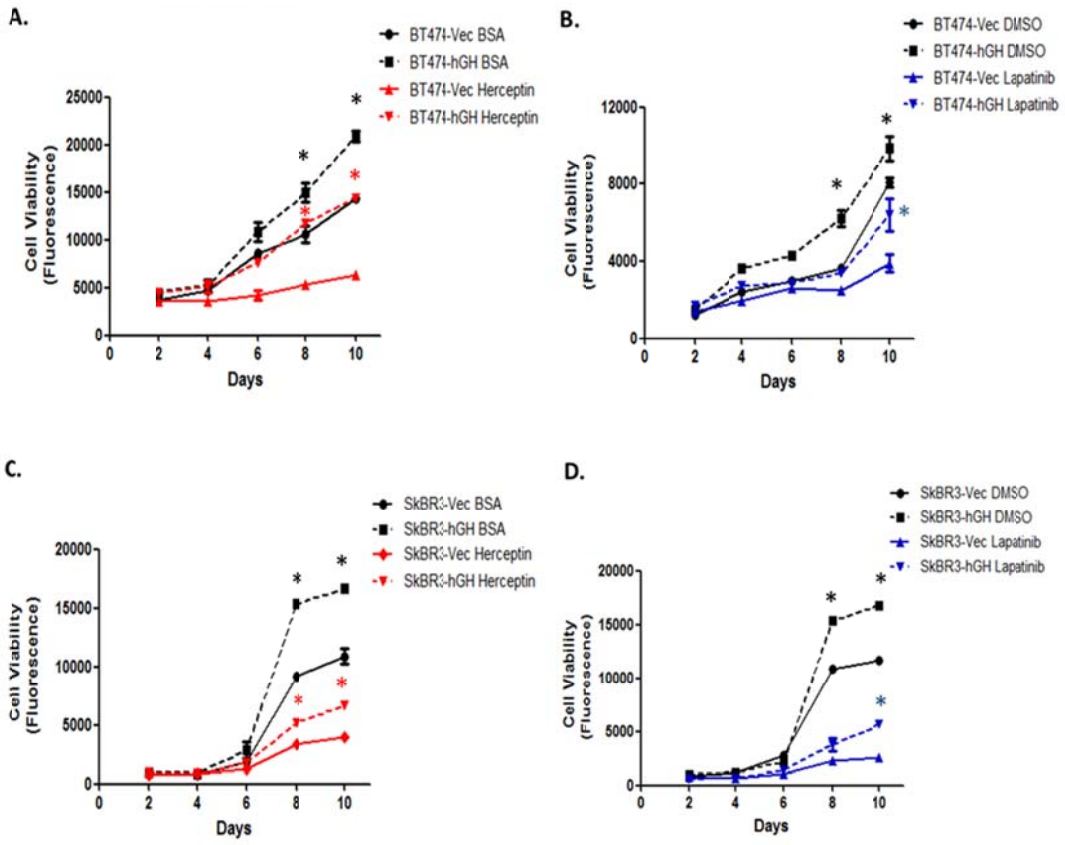


Figure 4-11 Autocrine hGH enhanced cell viability of BT474 and SkBR3 cells after treatment with Herceptin or Lapatinib. (A and C) Cell viability of BT474-Vec/hGH and SkBR3-Vec/hGH treated with 50 µg/ml Herceptin or equivalent concentration of BSA (untreated control). (B and D) Cell viability of BT474-Vec/hGH and SkBR3-Vec/hGH treated with 150 nM or 15 nM Lapatinib or equivalent concentration of DMSO (untreated control) respectively. (*$p < 0.05$)

Here, the cell viability assay demonstrated that autocrine expression of hGH in BT474 and SkBR3 reduced cell sensitivity to Herceptin- or Lapatinib-induced growth inhibition and enhanced cell viability after drug treatment.

4.9 Forced expression of hGH protected BT474 and SkBR3 cells from Herceptin and Lapatinib-induced apoptosis

Next, apoptosis of BT474-Vec/hGH and SkBR3-Vec/hGH cells was assessed by measuring caspase 3/7 activity in the presence of Herceptin or Lapatinib. It was shown that autocrine expression of hGH protected BT474 and SkBR3 cells against apoptosis induced by Herceptin or Lapatinib (Figure 4-12).

As shown in Figure 4-12A and 4-12B, the BSA- and DMSO-treated BT474-hGH exhibited significantly reduced caspase 3/7 activity when compared to its control BSA- and DMSO-treated BT474-Vec cell lines by 1.5 folds and 2.4 folds respectively. Similar trend was observed in SkBR3 cells where forced expression of hGH resulted in marked decreased in caspase 3/7 activity as compared to its respective BSA- and DMSO-treated SkBR3-Vec cells (Figure 4-12C and 4-12D).

Following treatment with Herceptin, BT474-Vec cells exhibited significantly higher caspase 3/7 activity than BT474-hGH cells by 1.7 folds (Figure 4-12A). When expressed as fold difference of BSA-treated BT474-Vec control cells, forced expression of hGH reduced the effect of Herceptin-induced inhibition on BT474 caspase 3/7 activity by 0.8 fold than Herceptin-treated BT474-Vec cells (1.5 folds). Such observation indicated that the presence of autocrine hGH protects BT474 cells from apoptosis.

In Figure 4-12B, a more striking effect was observed with Lapatinib-treated BT474-Vec cells where it exhibited a significant increase in apoptosis by 2.4 folds in comparison to its respective DMSO-treated BT474-Vec control cells ($p < 0.05$). When expressed as fold difference to DMSO-treated BT474-Vec control cells, autocrine hGH reduced the effect of apoptotic inhibition by Lapatinib on BT474 (1.9 folds) as compared to Lapatinib-treated BT474-Vec cells (2.4 folds). Such observations demonstrated that forced expression of hGH also protected BT474 cells from Lapatinib-induced apoptosis.

Upon Herceptin treatment, SkBR3-Vec cells exhibited enhanced significant apoptosis as measured through caspase 3/7 activity by 1.4 folds (Figure 4-12C) when compared to BSA-treated SkBR3-Vec cells. When comparing to the BSA-treated SkBR3-Vec control cells, I observed that Herceptin-treated SkBR3-hGH cells (1 fold) reduced caspase 3/7 activity than Herceptin-treated SkBR3-Vec cells (1.4 fold). Similar trend was observed in the Lapatinib treatment group on SkBR3 stable cells, where caspase 3/7 activity in Lapatinib-treated SkBR3-hGH cells (1.3 fold) was reduced as compared to Lapatinib-treated SkBR3-Vec cells (1.8 fold) relative to DMSO-treated SkBR3-Vec cells (Figure 4-12D). Herein, I observed that SkBR3 cells were protected from apoptosis in the presence of autocrine expression of hGH.

Based on cell viability and apoptosis functional assay done with the use of therapeutic drugs, autocrine expression of hGH enhanced survival of BT474 and SkBR3 HER2+ mammary carcinoma cells by increasing cell growth and concomitantly decreasing apoptosis.

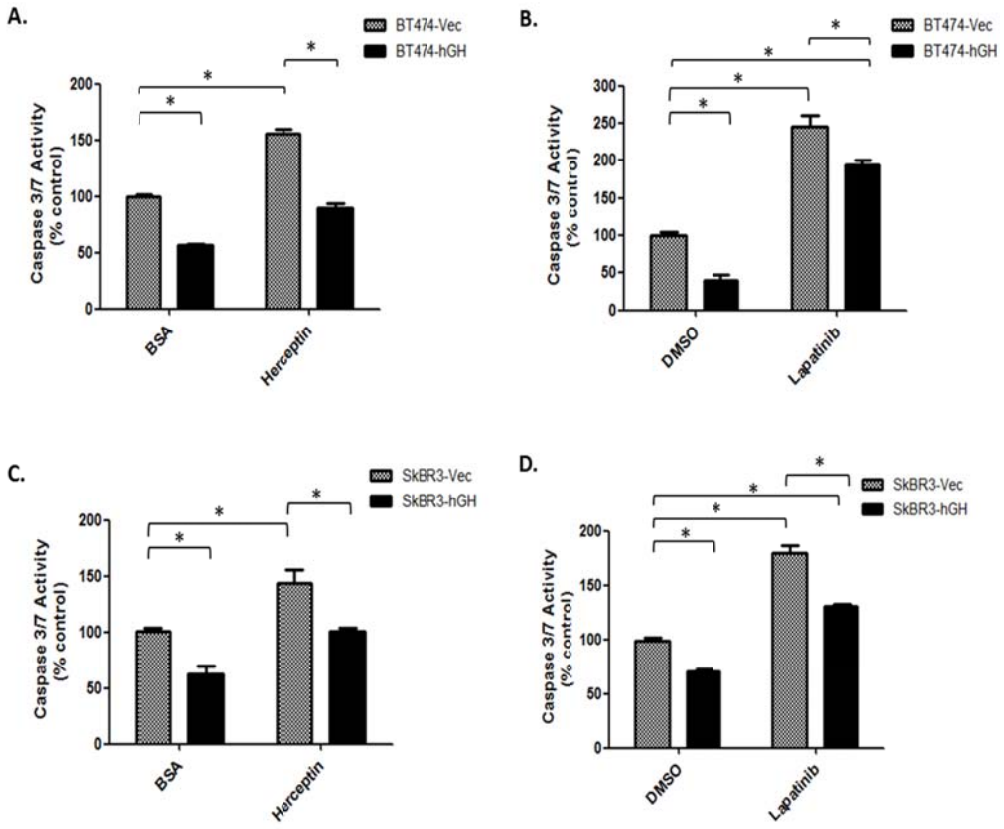


Figure 4-12 Autocrine hGH significantly protected HER2+ breast carcinoma cells from Herceptin- and Lapatinib-induced inhibition. (A and B) Caspase 3/7 activity of BT474-Vec/hGH cells in the presence of Herceptin and Lapatinib respectively. (C and D) Caspase 3/7 activity of SkBR3-Vec/hGH cells in the presence of Herceptin and Lapatinib respectively. (* $p < 0.05$)

4.10 Autocrine hGH enhanced BT474 anchorage-independent growth in soft agar after Herceptin treatment, but not Lapatinib treatment

The ability of BT474-Vec/hGH cells to proliferate and form colonies in soft agar after Herceptin or Lapatinib treatment was subsequently assessed. In the BSA-treated control group, BT474-hGH cells exhibited significant increased cell viability and colony forming abilities in soft agar as compared to its respective BSA-treated BT474-Vec cells (Figure 4-13A and B). The presence of Herceptin effectively inhibited colony formation of BT474-Vec cells in soft agar. Conversely, it was observed that forced expression of hGH abrogated the

inhibition produced by Herceptin in BT474 cells. Therefore, autocrine expression of hGH enhanced anchorage-independent growth ability of BT474 cells in soft agar in the presence of Herceptin treatment.

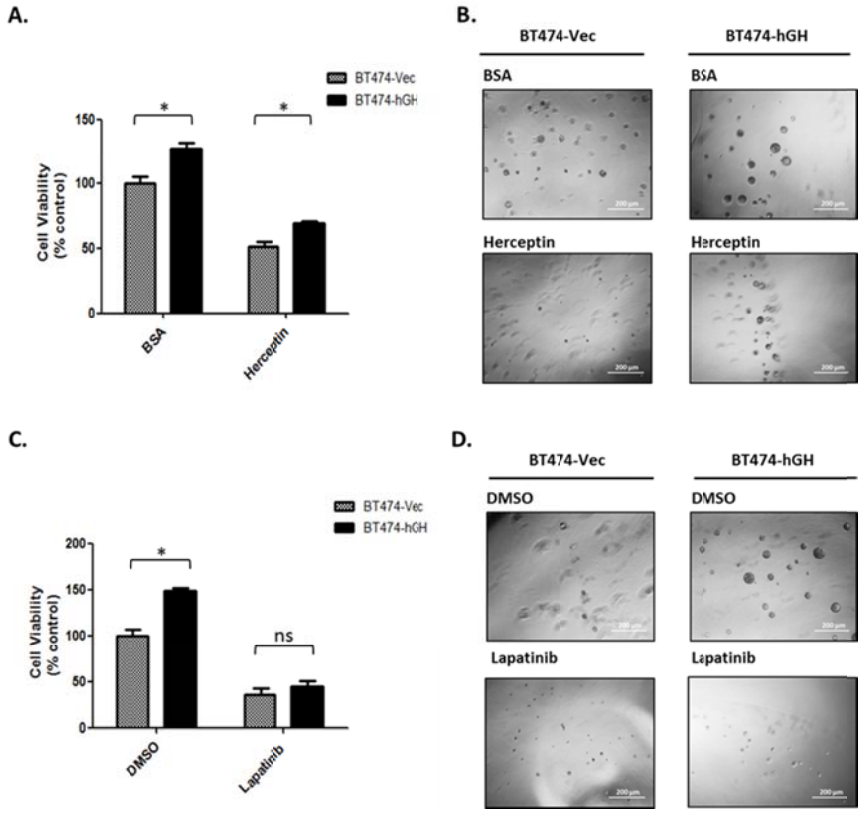


Figure 4-13Autocrine expression of hGH enhanced anchorage-independent growth in BT474 cells in the presence of Herceptin, but not Lapatinib. (A and C) Cell viability BT474-Vec/hGH cells in the presence of Herceptin and Lapatinib respectively. (B and D) Representative pictures of BT474-Vec/hGH colonies formation after Herceptin or Lapatinib treatment respectively. Images taken at 4X magnification. (* $p<0.05$; ns: not significant)

There was no significant difference in anchorage-independent growth ability between BT474-Vec and BT474-hGH cells after Lapatinib treatment, indicating that autocrine expression of hGH in BT474 cells do not provide advantage in the presence of Lapatinib (Figure 4-13C and D). Additionally, Lapatinib effectively inhibited anchorage-independent growth ability of BT474 cells than that of Herceptin.

4.11 Autocrine hGH enhanced BT474 and SkBR3 cell growth capacity in 3D-matrigel after Herceptin or Lapatinib treatment

In 3D growth assays, the BT474-hGH cells exhibited significantly increased cell viability as compared to its respective BT474-Vec cells (Figure 4-14A and B). The presence of Herceptin significantly inhibited viability of BT474-Vec cells. Importantly, Herceptin-treated BT474-hGH cells still exhibited enhanced cell viability in 3D matrigel compared to Herceptin-treated BT474-Vec cells. A similar trend was observed when BT474-Vec and BT474-hGH cells were treated with Lapatinib (Figure 4-14C and D). Taken together, soft agar colony formation and 3D Matrigel growth assays demonstrated that autocrine hGH expression in BT474 cells significantly enhanced anchorage-independent growth and increased viability capacity in 3D after exposure to Herceptin or Lapatinib.

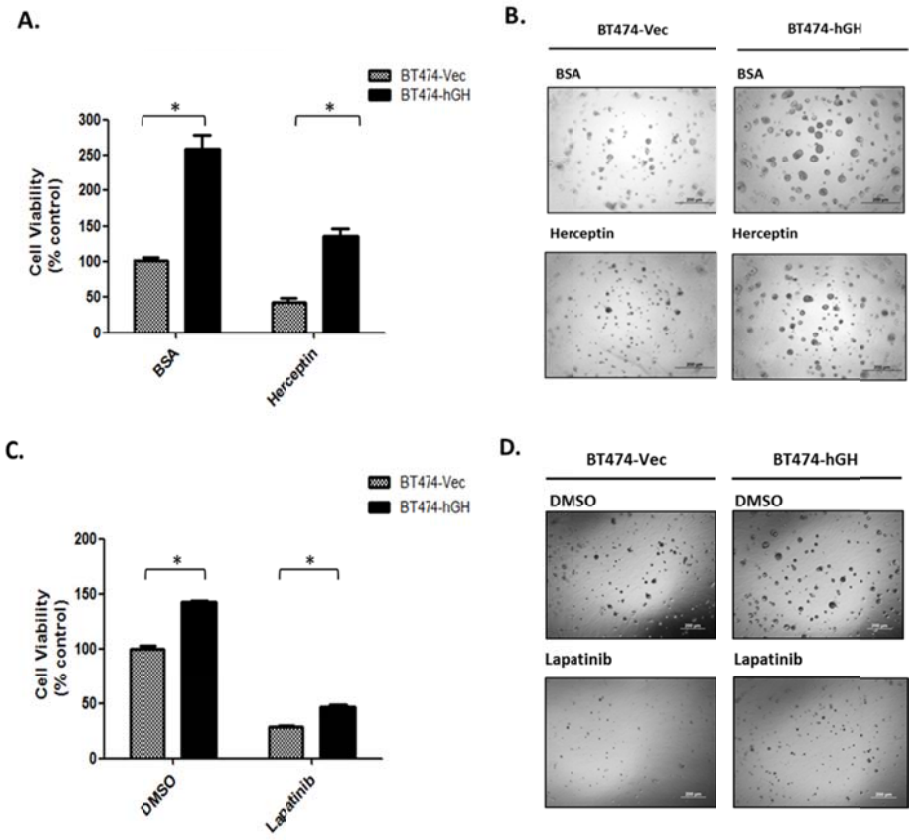


Figure 4-14 Autocrine expression of hGH enhanced BT474 cells proliferation and colony formation in 3D Matrigel™ after Herceptin or Lapatinib treatment. (A and C) Cell viability of BT474-Vec/hGH in 3D Matrigel treated with Herceptin or Lapatinib respectively. (B and D) Representative pictures of BT474-Vec/hGH colonies formation in 3D matrigel after Herceptin or Lapatinib treatment respectively. Images taken at 4X magnification. (*$p < 0.05$)

Importantly, forced expression of hGH in SkBR3 cells also demonstrated significant enhancement of cell viability in 3D matrigel after Herceptin or Lapatinib treatment (Figure 4-15). In both BSA-treated and DMSO-treated groups, SkBR3-hGH cells exhibited marked increased in cell viability as compared to its respective SkBR3-Vec cells. In the presence of Herceptin, cell viability of SkBR3-Vec was significantly inhibited. Conversely, forced expression of hGH in SkBR3 cells abrogated the inhibition produced by Herceptin and SkBR3-hGH cells were observed to exhibit significant enhanced viability in 3D as compared to Herceptin-treated BT474-Vec cells. Treatment of SkBR3-Vec and SkBR3-hGH cells with Lapatinib resulted in

stronger inhibition on cell transformation ability than that of Herceptin-induced inhibition. Nevertheless, Lapatinib-treated SkBR3-hGH cells exhibited increased viability and colonies formation in comparison with its respective Lapatinib-treated SkBR3-Vec cells (Figure 4-15C and D).

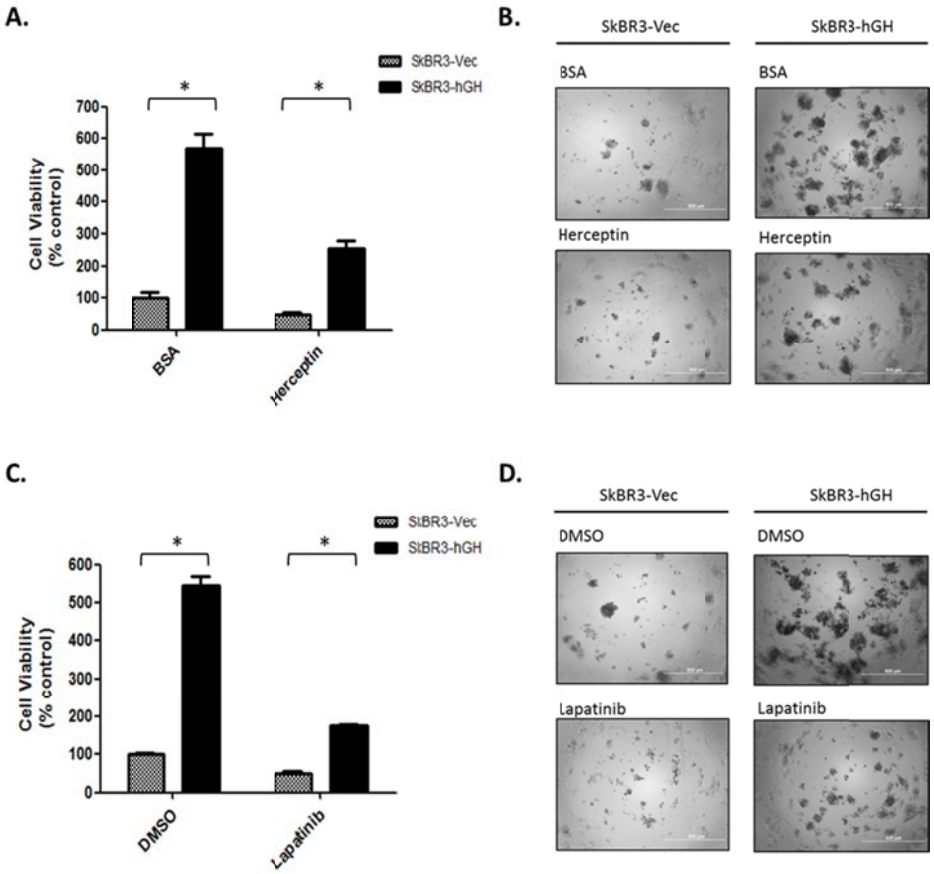


Figure 4-15 Autocrine expression of hGH enhanced SkBR3 cells proliferation and colony formation in 3D Matrigel™ after Herceptin or Lapatinib treatment. (A and C) Cell viability of SkBR3-Vec/hGH in 3D Matrigel treated with Herceptin or Lapatinib respectively. (B and D) Representative pictures of SkBR3-Vec/hGH colony cell growth in 3D matrigel after Herceptin or Lapatinib treatment respectively. Images taken at 4X magnification. (*$p < 0.05$)

4.12 Expression of hGH in BT474 and SkBR3 Herceptin-resistance mammary carcinoma cells

Acquired Herceptin-resistance BT474 and SkBR3 cells were generated by culturing the cells in the presence of 10 μg/ml and 50 μg/ml Herceptin respectively, for a period of 6-9 months to examine the functional role of hGH

in acquired resistance to Herceptin. The respective untreated BT474 and SkBR3 cells were also cultured together as control cells. Subsequently, RNA was extracted from these resistant cells and *hGH* mRNA expression was determined. The RT-PCR analysis showed that there was no significant change in expression of *hGH* gene, thereby indicating that hGH does not play a functional role in acquired Herceptin-resistance in BT474 and SkBR3 cells. In other words, there is no correlation between hGH and Herceptin-acquired resistant in both BT474 and SkBR3 HER2-amplified mammary carcinoma cells (Figure 4-16).

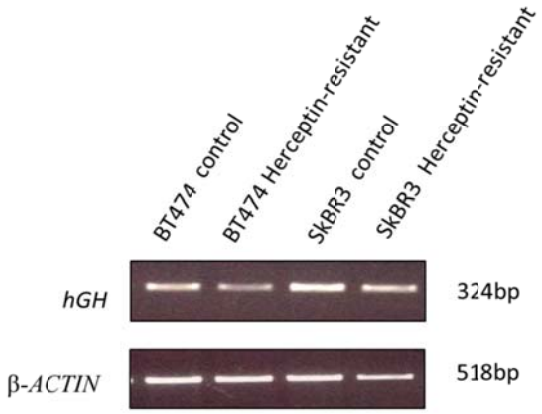


Figure 4-16 Expression level of *hGH* gene in BT474 and SkBR3 Herceptin-resistant mammary carcinoma cells. Both BT474 and SkBR3 cells were treated with Herceptin at 10 μg/ml and 50 μg/ml respectively for a period of 6-9 months.

5 DISCUSSIONS

The HER2+ subtype breast cancer is found in 20-30% of invasive breast cancer and associated with unfavourable prognostic outcome. Advanced and metastatic HER2+ breast cancer patients often exhibit tumour relapse and poor survival outcome [55, 138]. Preclinical studies have shown that in HER2+ breast cancer, the HER pathway which governs growth, survival and proliferation, is overly active [139]. These continuous signals can lead to uncontrolled cell growth and potentially contribute to cancer progression. The development of a recombinant therapeutic drug known as Herceptin that targets HER2 to block its activity has been approved for treatment of patients diagnosed with HER2+ breast cancer [52, 57]. However, *de novo* and acquired resistance to the treatment is still a major challenge faced in clinics when treating this particular subtype of breast cancer patients. For instance, the response rates of Herceptin monotherapy in metastatic breast cancer study is found to be 11-26%, indicating *de novo* resistance [62]. Disease recurrent or relapse is reported in HER2+ breast cancer patients after one year treatment with Herceptin [49, 52]. Treatment using another drug, Lapatinib, for HER2+ breast cancer patients has also been proven to be successful and effective in the blockage of HER2 and EGFR signalling activities, but eventually becomes limited due to emergence of acquired drug resistance as well. Such observation implies that HER2 alone is inadequate as predictive marker to delineate the treatment for HER2+ breast cancer patients. Besides that, it indicates the presence of alternative mechanisms or compensatory pathways in which cancer cells can utilize as survival strategies where blocking of HER2 alone as one molecular target is thus insufficient in therapy. The existence of

disease relapse indicates the need to examine the mechanisms underlying tumour resistance to the treatment. Furthermore, there is the necessity to assess additional biomarkers or alterative combinations therapies that have inhibitory functions different from that of HER2-targeted agents for improved treatment and overall survival for patients with metastatic HER2+ breast cancer.

The association of GH expression has been identified in an increasing number of cancers including breast, endometrial, lung, colon and liver [124, 125, 140-143]. Additionally, autocrine expression of hGH is reportedly correlated with clinicopathological features in ER+ mammary carcinoma malignancies implying that tumour expression of hGH is a potential predictive marker during breast cancer prognosis [132]. Recently, a clinical study performed on a cohort of breast and endometrial carcinomas patients have shown overexpression of tumour hGH mRNA and protein to be positively correlated with HER2 positivity ($P = 0.004$ and $P = 0.001$ respectively) and lymph node metastasis ($P = 0.002$). These patients whose tumour expressed high level of hGH have a significant poorer overall survival and relapse free survival in comparison to those whose tumour are hGH-negative. Therefore, it is worth to investigate the possible functional interaction between hGH and HER2 in order to better understand the oncogenic progression of HER2+ tumours and the mechanisms of resistance in treating HER2+ breast cancer patients. Furthermore, it may allow the intervention of targeted therapy for breast cancer patients whose tumour is HER2+ and hGH+ by blocking GH activity as adjuvant therapy in combination with current HER2-targeting agents.

Herein, this study explored the role of autocrine hGH in HER2+ breast carcinoma cells, and the potential of targeting both hGH and HER2 by combining GHR antagonists and Herceptin or Lapatinib as a possible alternative therapy. It was demonstrated that in the presence of autocrine expression of hGH, the HER2-amplified BT474 and SkBR3 breast carcinoma cells have significantly increased viability and growth potentials in anchorage-independent manner. In the present study, it showed that forced expression of hGH in BT474 and SkBR3 cells increased total cell number *in vitro*. Concomitantly, a decreased in caspase 3/7 activity was observed, thereby indicating autocrine expression of hGH protected BT474 and SkBR3 cells from apoptosis induced by serum deprivation. Additionally, BT474-hGH cells were observed to exhibit increased viability in soft agar colony formation and 3D matrigel assays compared to its respective control cells, which implied that cell growth was significantly enhanced in the presence of autocrine hGH. Similarly, SkBR3-hGH cells exhibited significant enhanced viability in 3D matrigel as compared to its control cells. These observations implied that forced expression of hGH promotes and enhances viability and growth potential of HER2+ breast carcinoma cells. One mechanism by which autocrine hGH increases oncogenic potential and promotes cell survival of HER2+ breast cancer cells was through stimulating tyrosine phosphorylation (Y1248) of HER2, which serve as the major autophosphorylation sites of HER2 [135]. It is reported that HER2 activation of the ERK pathways is primarily dependent on Y1248 phosphotyrosine residues on the intracellular domain of HER2 [144]. The phosphorylation of Y1248 residue resulted in the

recruitment and association of SHC with HER2. Mutation or deletion of this phosphotyrosine residue significantly diminished ERK activation.

HER2+ breast cancer is quite heterogeneous and it can be further sub-divided into hormone receptor-positive (ER+/PR+/HER2+) or hormone receptor-negative (ER-/PR-/HER2+). The BT474 cell line belongs to the hormone receptor-positive, while SkBR3 cell line is hormone receptor-negative. The results observed in this study is thus independent of hormonal receptor status (ER and PR) as forced expression of hGH enhances oncogenic capacity in both BT474 and SkBR3 through increasing total cell number, enhancing anchorage-independent growth and cell growth in 3D, stimulating phosphorylation of HER2 (Y1248) and decreasing apoptosis. Besides that, autocrine hGH was found to reduce BT474 and SkBR3 HER2+ carcinoma cells sensitivity to Herceptin or Lapatinib therapeutic drug treatment with the exception that no significant difference in anchorage-independent growth of BT474 in the presence of Lapatinib.

It has been reported that expression of autocrine hGH is associated with therapeutic resistance including tamoxifen [123], doxorubicin [120], MMC [124] and even ionising radiation [125]. Here, it was further demonstrated that both Herceptin- or Lapatinib-treated hGH-overexpressing BT474 and SkBR3 cells exhibited enhanced total cell number, increased colony formation and survival in 3D and reduced apoptosis. Therefore, autocrine expression of hGH provided a selective growth advantage for BT474 and SkBR3 HER2+ mammary carcinoma cells in the presence of therapeutic protocol containing Herceptin and exerted less oncogenic effect in the presence of Lapatinib.

Treatment with Lapatinib was observed to be more effective than Herceptin in achieving growth inhibition as viability of Lapatinib-treated BT474-Vec/hGH and SkBR3-Vec/hGH cells was significantly lower than that of Herceptin-treated BT474-Vec/hGH and SkBR3-Vec/hGH cells. This also explains why Lapatinib is often used as the preferred agent in combination with other chemotherapeutics for treating HER2+ breast cancer patients when Herceptin fails.

Unpublished work performed by other graduate students in my laboratory working on breast cancer EMT have observed an increase in invasion, migration potential, and enhancement in closing of artificially-inflicted wounds in the presence of autocrine hGH in BT474 and SkBR3 HER2+ breast carcinoma cells. Besides that, autocrine expression of hGH greatly enhanced the mammo-spheroid formation, the Aldehyde Dehydrogenase-positive (ALDH+) and stem cell side population in SkBR3 and MDA-MB-453 (HER2+/ER-) breast carcinoma cells. Additionally, immunodeficient mice injected with hGH-overexpressing MDA-MB-453 cells formed tumours in the mammary fat pad as compared to those injected with control cells. These studies clearly reiterated the oncogenic role of autocrine hGH to increase proliferation, survival, anchorage-independent growth, promote invasion and migration as well as enhance tumour-initiating or cancer stem cells population in HER2+ mammary carcinoma cells *in vitro* and formation of tumours *in vivo*.

Breast cancer is a heterogeneous disease and oncogenesis is driven by multiple signalling pathways. During the process of oncogenesis, cells acquire one, two

or more typical traits of cancer hallmarks as it accumulates mutation. Therefore, a multi-targeted therapeutic approach will greatly offer advantages for breast cancer treatment, in particularly to overcome drug resistance that has become more prevalent. As presented in this study that autocrine hGH is able to increase oncogenic transformation potential of HER2+ breast cancer cells and decrease the cells sensitivity to Herceptin or Lapatinib, blocking or reducing of hGH in circulation may hence decrease proliferative and oncogenic capacities, thereby providing additive effects to Herceptin or Lapatinib inhibition. If synergistic inhibition by combining GHR antagonists and HER2-targeted agents is efficacious, this could be an option of targeted therapy available for the subgroup of breast cancer patients with HER2+/hGH+ tumours. I therefore went on to investigate the effectiveness of using GHR antagonists such as G120R [145] or B2036 [107, 108, 146] to block endogenous hGH in BT474 and SkBR3 cells and hypothesize that a combination of both GHR antagonists and HER2-targeted agent can significantly decrease oncogenic transforming potential of HER2-amplified breast cancer cells compared with either agent alone.

Two approaches were attempted to functionally antagonise or inhibit endogenous GH, which include the use of G120R and B2036. The G120R consists the entire *hGH1* gene with a single point mutation that involves the substitution of an arginine (R) for glycine (G) at residue position 120 [147]. This mutation is introduced within the region where binding site 2 of GH is located which blocks receptor dimerization; thus G120R acts as an antagonist to GH. It is cloned into the pcDNA3.1 plasmid backbone to generate pcDNA-G120R and was stably transfected into BT474 and SkBR3 cells to generate

BT474-G120R and SkBR3-G120R respectively. Monolayer total cell number assay shows that both BT474-G120R and SkBR3-G120R cells exhibited significantly lower total cell number in comparison to its respective BT474-Vec/BT474-hGH and SkBR3-Vec/SkBR3-hGH cells gradually over a period of 10 days (results not shown). In contrast, the hGH-overexpressing BT474 and SkBR3 cells exhibited the highest total cell count, followed by its respective BT474-Vec and SkBR3-Vec control cells. Besides that, BT474-G120R cells have reduced ability to form viable colonies in soft agar, indicating significant decrease in anchorage-independent growth capability (results not shown). Such observations were consistent with our hypothesis where overexpression of hGH will enhance proliferative, survival and cell transforming capacities, while antagonism of hGH will abrogate its oncogenic transforming potential in HER2-amplified cells. However, the pool of G120R-transfected cells selected out after the 3rd passage in culture. The second GHR antagonist used is the B2036 derived from eight amino acid substitution at binding site 1 that enhances its affinity to GHR and a single point mutation of glycine to lysine residue at position 120 (G→K) at binding site 2 that blocks GH dimerization and signalling [146].

Dose-response curve was done to determine the IC_{50} of B2036 to be used on BT474 and SkBR3, but the results I generated indicated that both BT474 and SkBR3 wild-type cell lines do not respond to this antagonist in the assays I utilized (data not shown). Therefore, it is not possible to use B2036 in both BT474 and SkBR3 cell line model generated in our lab. It will be ideal to observe positive outcome where the presence of B2036 will cause BT474 and SkBR3 HER2+ mammary carcinoma cells to exhibit significant reduction in

proliferation and survival. Nevertheless, the use of pcDNA-G120R plasmid in BT474 and SkBR3 do demonstrate that cell viability and growth potential were markedly reduced when GH signalling was blocked.

Like other *in vitro* drug assays or clinical settings, it appeared that cell lines or patients responsiveness to an agent/inhibitor/drug such as B2036 varied. Functional inhibition of GH using B2036 have been performed on MDA-MB-453 (HER2+/ER-) cell by another student in my laboratory who is working on the role of hGH in enhancing cancer stem cells population in ER- mammary carcinoma cells. It is demonstrated that upon treatment with B2036, MDA-MB-453 cells exhibited marked reduction in the ability to form mammo-spheroids in culture. Such observation demonstrated effective blockage of GH action by B2036 and that can greatly reduce viability and growth of MDA-MB-453 HER2+ mammary carcinoma cell. However, MDA-MB-453 cell is not suitable as the *in vitro* model in my study because it is Herceptin-resistance cell line, unlike BT474 and SkBR3 which are both Herceptin- and Lapatinib-responsive.

Other than BT474 and SkBR3, ZR-7530 cell is reported to be one of the three out of nine HER2-amplified mammary carcinoma cell lines that respond to Herceptin [75]. The ZR-7530 cell may thus be an alternative *in vitro* model that is useful to determine synergistic inhibition between GH antagonist and HER2-targeted agent in this project. Having said that, ZR-7530 should be tested for its endogenous hGH level and subsequently its responsiveness to B2036 by performing dose-response experiment.

Despite having shown to be beneficial therapeutically based on previous i*n vitro* studies on mammary and endometrial carcinomas [22, 117, 122, 124, 125], there are still limited *in vivo* studies demonstrating the efficacy of GHR antagonists such as B2036 in cancer treatment. Additionally, GHR antagonists are yet to be tested in cancer-related clinical trials. There is therefore a need for such translational studies to be conducted in order to deduce the feasibility or therapeutic potential of introducing GHR antagonists as adjuvant to sensitize mammary carcinoma cells that are GH-positive to subsequent cancer-related therapy for HER2+ subtype breast cancer in this case. Besides G120R and B2036, other agents capable of inhibiting GH-mediated signalling should also be tested.

In this project, overexpression of hGH were done in already transformed cells, which are BT474 and SkBR3 HER2-amplified breast carcinoma cell lines. Previous study conducted by Zhu *et al, 2005* have revealed oncogenic transformation of normal but immortalized human mammary epithelial cells (MCF10A and MCF12-A) transfected with hGH in a JAK2-dependent manner, demonstrating that autocrine expression of hGH also drive tumorigenesis process in normal cells [122]. Nevertheless, it will be interesting to examine in future study on the sensitivity of these hGH-transfected MCF10A and/or MCF12A normal cells to Herceptin or Lapatinib for a better understanding on the role of autocrine hGH in HER2-targeted therapies.

The emergence of resistance to HER2-targeted therapy has suggested additional escape pathways involved in contributing to resistant growth that

will need to be targeted to fully circumvent such clinical obstacle observed in patients with HER2+-refractory breast cancer. It is challenging to elucidate the molecular mechanisms of Herceptin-resistance in HER2+ breast cancer due to the complexity of signal pathways, which allows cancer cells to utilize alternative compensatory route to strive for survival. Nevertheless, multiple molecular mechanisms contributing to Herceptin-acquired resistance have been described, suggesting that a different targeted-therapeutic approach is required during treatment of HER2+ breast cancer. This also explains why current clinical strategies where all HER2+ patients are treated with a broad similar approach still remains suboptimal. This project aimed to investigate the functional interaction between hGH in Herceptin-acquired resistance *in vitro*. Despite being shown to enhance viability and growth potential in HER2+ breast carcinoma cell lines, it was demonstrated that hGH is not functionally involved in Herceptin-acquired resistance as the expression level of *hGH* in BT474 control/resistance cell and SkBR3 control/resistance cell remained the same.

In summary, this study provided an understanding on the role of autocrine hGH in enhancing cell viability and growth of HER2+ subtype breast carcinoma cells. Forced expression of hGH provided a selective growth advantage for HER2-amplified cells by reducing sensitivity to HER2-targeted agents such as Herceptin or Lapatinib. In addition, tumour expression of hGH may hold significance to being an additional biomarker for treating breast cancer patients whose tumour co-expresses HER2 and hGH. Nevertheless, more work is still needed to verify therapeutic potential or efficiency of combination strategies that functionally inhibits hGH together with

conventional therapies, which together may provide optimal therapeutic regimes in particularly towards HER2+/hGH+ breast cancer.

6 CONCLUSION

In conclusion, HER2+ human breast carcinoma cells BT474 and SkBR3 exhibits significant proliferation, enhances anchorage-independent growth and promotes growth in 3D in the presence of autocrine secretion of hGH. Additionally, autocrine expression of hGH is able to stimulate tyrosine phosphorylation of HER2 (Y1248), thereby activating downstream signalling cascade of HER2 pathway. Furthermore, forced expression of hGH reduces sensitivity of BT474 and SkBR3 HER2+ breast carcinoma cells to HER2-targeted therapies including Herceptin or Lapatinib. Besides BT474 and SkBR3, other HER2+ breast carcinoma cell line such as ZR-7530 should be consider as *in vitro* model to examine synergistic inhibition and the effectiveness of introducing hGH antagonists in combination with HER2-targeted therapeutic agents. This study provided an insight on functional role of autocrine hGH in HER2+ mammary carcinoma progression and oncogenesis. Moreover, together with other experimental evidences performed by other students in my lab, it suggests that autocrine expression of hGH in HER2-overexpressing breast cancer has prognostic and predictive roles, and that hGH can potentially serves as an additional therapeutic marker and target for new therapeutic strategies that specifically target HER2+ breast cancer patients whose tumour biopsies reflect elevated hGH levels.

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