HEREGULIN-ENHANCED TYROSINE PHOSPHORYLATION IN BREAST CANCER CELLS: ROLE OF PP2A IN HER-2/*NEU* ONCOGENIC SIGNALLING

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SUMMARY

HER-2/*neu* is an established adverse prognostic factor of breast cancer. Patients with tumours overexpressing HER-2/*neu* have significantly shortened overall survival. Studying the mechanisms by which HER-2/*neu* overexpression translate into the more aggressive biological phenotype would not only provide a better understanding of the increased virulence of breast cancers overexpressing this oncogene but may also lead to rational targeted therapeutic strategies to arrest cancer growth.

Activation of HER-2/*neu* leads to activation or suppression of multiple signalling cascades and plays a vital role in cell survival and growth. A signal transduction antibody array was used in this study to characterize the tyrosine phosphorylation profiles in heregulin (HRG)-treated BT474 breast cancer cells.A group of 80 molecules in which tyrosine phosphorylation was highly regulated by HRG-enhanced HER-2/*neu* signalling was identified. These phosphoproteins included many known HER-2/*neu*-regulated molecules (e.g., Shc, AKT, Syk and Stat1) and proteins that had not been previously linked to HER-2/*neu* signalling, such as Fas-associated death domain protein (FADD), apoptosis repressor with CARD domain (ARC), and protein phosphatase type 2A (PP2A).

Pharmacological inhibition with the HER-2/*neu* inhibitor AG825, PI3K inhibitor LY294002, MEK1/2 inhibitor PD98095, and p38 MAPK inhibitor SB203580, confirmed that PP2A phosphorylation was modulated by the complicated, HER-2/*neu*-driven downstream signalling network, with the PI3K and MEK1/2 positively, while the p38 MAPK negatively, regulating its tyrosine phosphorylation.

In breast tumour specimens and cell lines, expression of tyrosine^{307}-phosphorylated PP2A (pY307-PP2A) was highly increased in the HER-2/*neu*-positive breast tumours and cell lines, and significantly correlated to tumour progression, thus enhancing its potential prognostic value. The data in this thesis provides meaningful information in the elucidation of the HER-2/*neu*-driven tyrosine phosphorylation network, and in the development of phosphopeptide-related targets as prognostication indicators.

PP2A, in its activated form as a phosphatase, is a tumour suppressor. However, when PP2A is phosphorylated at the tyrosine residue (pY307), it loses its phosphatase activity and becomes inactivated. A higher expression of pY307-PP2A in HER-2/*neu*- positive breast tumour samples, which was significantly correlated to tumour progression was reported here, and in this context, PP2A could function as a proto-oncogene.

The above and subsequent findings led us to postulate that the critical role of PP2A in maintaining the balance between cell survival and cell death may be linked to its phosphorylation status at its Y307 residue. Hence, further investigatation on the effects of knocking down the PP2A catalytic subunit which contains the Y307 amino acid residue in two HER-2/*neu*-positive breast cancer cell lines, BT474 and SKBR3 were carried out. The results showed that this caused the silenced HER-2/*neu* breast cancer cells to undergo apoptosis and furthermore, that such apoptosis was mediated by p38 MAPK-caspase 3/ PARP activation. Understanding the role of PP2A in HER-2/*neu*-positive cells might thus provide insight into new targets for breast cancer therapy.

PUBLICATIONS

Publications related to this thesis:

1. **Wong L.L.**, Zhang D., Chang C.F., Koay E.S. (2010) Silencing of the PP2A catalytic subunit causes HER-2/*neu* positive breast cancer cells to undergo apoptosis. Experimental Cell Research. In press (DOI number: 10.1016/j.yexcr.2010.06.007), PMID: 20558158. Impact factor: 3.589

2. **Wong L.L.**, Chang C.F., Koay E.S., Zhang D. (2009) Tyrosine phosphorylation of PP2A is regulated by HER-2 signalling and correlates with breast cancer progression. International Journal of Oncology. 34(5):1291-1301. Impact factor: 2.447

Other publications:

1. Zhang D., **Wong L.L.,** Koay E.S. (2007) Phosphorylation of Ser78 of Hsp27 correlated with HER-2/*neu* status and lymph node positivity in breast cancer. Molecular Cancer. 6:52. Impact factor: 4.160

2. Zhang D., Tai L.K., **Wong L.L.**, Chiu L.L., Sethi S.K., Koay E.S. (2005) Proteomics study reveals that proteins involved in metabolic and detoxification pathways are highly expressed in HER-2/*neu*-positive breast cancer. Mol. Cell. Proteomics. 4(11):1686-96. Impact factor: 8.791

3. Zhang D.H., Tai L.K., **Wong L.L.**, Sethi S.K., Koay E.S. (2005) Proteomics of breast cancer: enhanced expression of cytokeratin19 in human epidermal growth factor receptor type 2 positive breast tumours. Proteomics. (7):1797-805. Impact factor: 4.426

4. Zhang D.H.*, **Wong L.L.***, Tai L.K., Koay E.S., Hewitt R.E. (2005) Overexpression of CC3/TIP30 is associated with HER-2/*neu* status in breast cancer. J. Cancer Res. Clin. Oncol. 131(9):603-8. Impact factor: 2.261
   **Equal Contribution*

Meeting Proceedings:

1. **Wong L.L.**, Zhang D., Chang C.F., Koay E.S. Deciphering the PP2A-mediated signalling modulation in breast cancer. Keystone Symposia Conference. 25-30 Jan 2009. Taos, New Mexico, USA

2. **Wong L.L.**, Zhang D., Chang C.F., Koay E.S. Tyrosine-phosphorylated signal modulators regulated by Heregulin-enhanced HER-2/*neu* signalling. HUPO 6th Annual World Congress. 06-10 Oct 2007. Seoul, Korea.

3. **Wong L.L.**, Zhang D., Chang C.F., Koay E.S. Dissecting the heregulin-regulated tyrosine phosphoproteome in breast cancer using antibody arrays. Joint Third AOHUPO and Fourth Structural Biology and Functional Genomics Conference. 4-7 Dec 2006. Singapore.

4. **Wong L.L.**, Boo X.L., Koay E.S., Zhang D. Hsp27 phosphorylation at residue Ser^{78} was regulated by HER-2/*neu*-p38 MAPK pathway and strongly correlated with HER-2/*neu* status and lymph node positivity in breast cancer. National Health Group Annual Scientific Congress. 30 Sep-1 Oct 2006. Singapore

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LIST OF ABBREVIATIONS

2-DE	2-dimensional electrophoresis
5-HT	5-hydroxytryptamine
ADH	Atypical ductal hyperplasia
Ago	Argonaute
AKT	Protein kinase B
Alg-2	Alpha-1, 3-mannosyltransferase
ALH	Atypical lobular hyperplasia
APC	Adenomatosis polyposis coli
ARC	Apoptosis repressor with CARD domain protein
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
BAD	BCL-2 associated death promoter
BCL-2	B-cell lymphoma 2
BPE	Bovine pituitary extract
BRCA	Breast cancer susceptibility protein
BSA	Bovine serum albumin
CDC25A	Cell division cycle 25 homolog A
Cdk	Cyclin-dependent kinase
cDNA	Complementary DNA
CEP17	Chromosome 17 centromere
CHAPS	3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate
CISH	Chromogenic *in situ* hybridization
CLA	Conjugated linoleic acid
COX-2	Cyclooxygenase-2
CXCR4	Chemokine receptor
DAB	3, 3' Diaminobenzidine
DCIS	Ductal carcinoma *in situ*
Dicer	dsRNA-specific RNAse III family ribonuclease
DMEM	Dulbecco's modified Eagle medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
dUTP	2´-Deoxyuridine, 5´-Triphosphate
dsRNA	Double stranded RNA
EDTA	Ethylenediaminetetraacetic acid
EGFR	Epidermal growth factor receptor
ELISA	Enzyme-linked immunosorbent assay
ER	Estrogen receptor
ETS	E26 transformation specific
FADD	FAS-associated death domain protein
FAK	Focal adhesion kinase-1
FBS	Fetal bovine serum

FDA	Food and Drug Administration (USA)
FISH	Fluorescence *in situ* hybridization
GTPase	Guanosine triphosphate hydrolase enzyme
HUT	Hyperplasia of usual type
H&E	Hematoxylin and eosin
HER-2/*neu*	Human epidermal growth factor receptor type 2
hpRNAs	Hairpin RNAs
HRG	Heregulin
HRP	Horseradish peroxidase
Hsp	Heat shock protein
hTERT	Telomerase reverse transcriptase
IDC	Invasive ductal carcinoma
IHC	Immunohistochemistry
IgG	Immunoglobulin G
ILC	Invasive lobular carcinoma
IMAC	Immobilized metal ion/metal chelate affinity chromatography
IPG	Immobilized pH gradient
LCIS	Lobular carcinoma *in situ*
LCMS	Liquid chromatography mass spectrometry
MAPK	Mitogen-activated protein kinase
MEGM	Mammary epithelial cell complete medium
miRNA	MicroRNA
mRNA	messenger ribonucleic acid
MUC4	Mucin-4
Myc	Myelocytomatosis viral oncogene homolog (avian)
OA	Okadaic acid
PAR	Partition protein 6
PARP	Poly (ADP-ribose) polymerase
PDCD-6	Programmed cell death protein 6
PDGFR	Platelet-derived growth factor receptor
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PI3K	Phosphatidylinositol 3-kinase
PKC	Protein kinase C
PLC	Phospholipase C
PPP	Phosphoprotein phosphatases
PP1	Protein phosphatase 1
PP2A	Protein phosphatase type 2A
PTP1B	Protein tyrosine phosphatase 1B
PTEN	Phosphatase and tensin homolog
PTM	Protein post-translational modifications
PVDF	Hybond-P polyvinylidene fluoride
pYMT	Polyoma middle T
RISC	RNA-induced silencing complex
RNAi	RNA interference
RTK	Receptor tyrosine kinase

rTDT	Terminal deoxynucleotidyl transferase
RT-PCR	Reverse transcription-polymerase chain reaction
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
Shc	Src homology 2 domain-containing transforming protein
SILAC	Stable isotope labeling with amino acids in cell culture
SISH	Silver *in situ* hybridization
siRNA	Small RNA
Src	Sarcoma
ST	Small tumour antigen
SV40	Simian virus 40
Syk	Src-related protein tyrosine kinases
TBE	Tris/Borate/EDTA
TBS-T	Tris-buffered saline containing 0.1% Tween-20
TMA	Tissue microarray
TNF	Tumour necrosis factor
TUNEL	Terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling
TP53	Gene coding for p53
VEGF	Vascular endothelial growth factor

CHAPTER 1

INTRODUCTION

1.1 Breast cancer

1.1.1 Incidence of breast cancer

Breast cancer is the second leading cause of cancer deaths in women today after lung cancer and is the most common cancer among women globally (American Cancer Society. *Cancer Facts & Figures 2009*. Atlanta: American Cancer Society; 2009). According to the American Cancer Society, about 1.3 million women will be diagnosed with breast cancer annually and about 465,000 women will die from this disease. In general, the incidence of breast cancer has risen about 30% in the past 25 years in western countries. Due to the increased early detection screening, the breast cancer rates are reported to be on a rising trend in many countries, on the contrary, the deaths caused by breast cancer is on a decreasing trajectory, presumably as a result of improved screening and treatment. Based on the GLOBOCAN 2002 worldwide statistics on breast cancer incidence, mortality and prevalence, Singapore ranked as having the highest breast cancer incidence and mortality rates in Asia, after the western countries (Table 1-1). From 1968 to 2002, Singapore experienced an almost 3-fold increase in breast cancer incidence and the observed dissimilarity among the different ethnic groups suggested ethic differences in exposure or response to certain risk factors (Sim *et al.*, 2006). According to the Singapore Cancer Registry, breast cancer is the top cancer type affecting the women in Singapore (Table 1-2). In the past 35 years, breast cancer has remained the most frequent cancer among the women and an upward trend in incidence has continued (Figure 1-1).

Breast Cancer Worldwide		
Breast (All ages)	Incidence	Deaths
United States	101.1	19
France	91.9	21.5
Denmark	88.7	27.8
Sweden	87.7	17.3
United Kingdom	87.2	24.3
Netherlands	86.7	27.5
Canada	84.3	21.1
Australia	83.2	18.4
Switzerland	81.7	19.8
Italy	74.4	18.9
Singapore	**48.7**	**15.8**
Brazil	46	14.1
Japan	32.7	8.3
India	19.1	10.4
Zimbabwe	19	14.1
China	18.7	5.5

Table 1-1. Breast cancer incidence and mortality worldwide. Note: numbers are per 100,000. Data adapted from *J. Ferlay, F. Bray, P. Pisani and D.M. Parkin. GLOBOCAN 2002. Cancer Incidence, Mortality and Prevalence Worldwide. IARC Cancer Base No. 5, version 2.0. IARC Press, Lyon, 2004, with modification.*

Site	Ranking	No. of Cases	% of all cancers
Breast	**1**	**6798**	**36.2**
Colo-rectum	2	3375	18.0
Lung	3	1868	9.9
Corpus Uteri	4	1332	7.1
Ovary	5	1327	7.1
Cervix Uteri	6	1009	5.4
Stomach	7	885	4.7
Skin (including melanoma)	8	840	4.5
Lymphoma	9	698	3.7
Thyroid	10	645	3.4

Table 1-2. Ten most frequent cancers affecting Singapore women, 2003-2007. Breast cancer is the top cancer type affecting the women in Singapore. Data extracted from *Singapore Cancer Registry, Interim Report: Trends in Cancer Incidence in Singapore 2003-2007, with modification.*

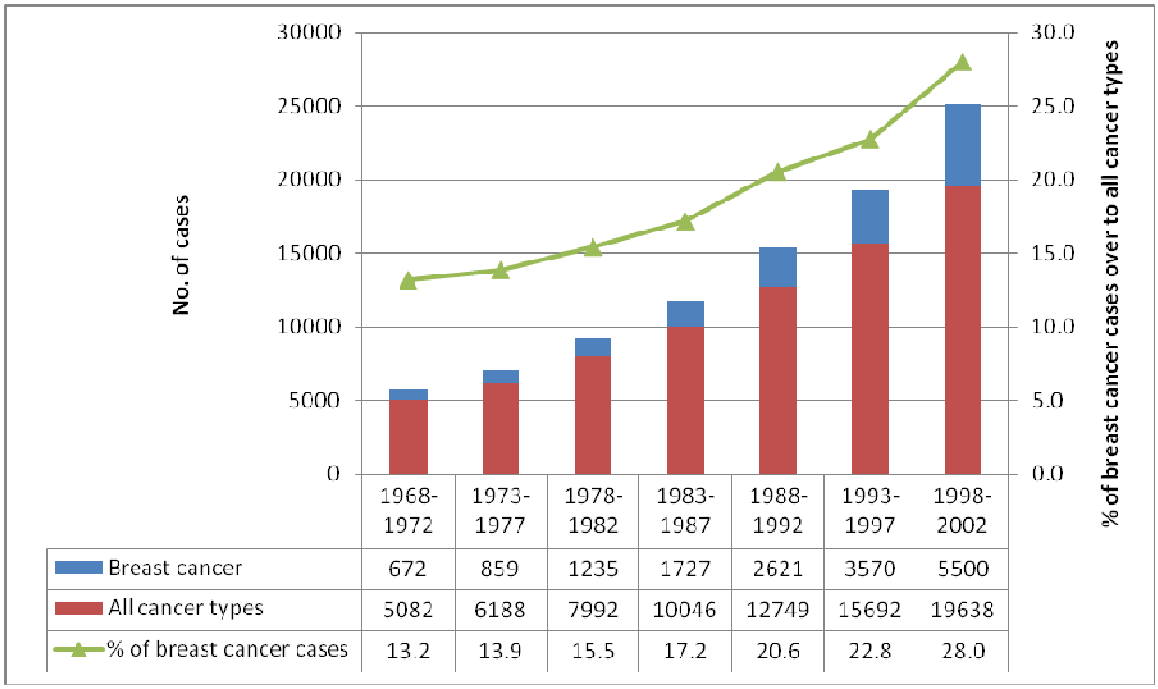


Figure 1-1. Number of cases with breast cancer and all cancer types in Singapore females 1968-2002, by 5-year period. Breast cancer has remained the most frequent cancer among the women and an upward trend in incidence has continued over the past 35 years. Data extracted from the *Singapore Cancer Registry Report No. 6: Trends in Cancer Incidence in Singapore 1968-2002, with modification.*

1.1.2 Classification of breast cancer

The breasts are fascinating organs that are composed of fatty tissue that contains the glands responsible for milk production in late pregnancy and after childbirth. Within each breast, there are about 15 to 25 lobes formed by groups of lobules which are the milk glands. Each lobule is composed of grape-like clusters of acini (also called alveoli), the hollow sacs that make and hold breast milk. The lobules are arranged around the ducts that funnel milk to the nipples. About 15 to 20 ducts come together near the areola (dark, circular area around the nipple) to form ampullae – dilated ducts where milk accumulate before it reaches the nipple surface (Figure 1-2).

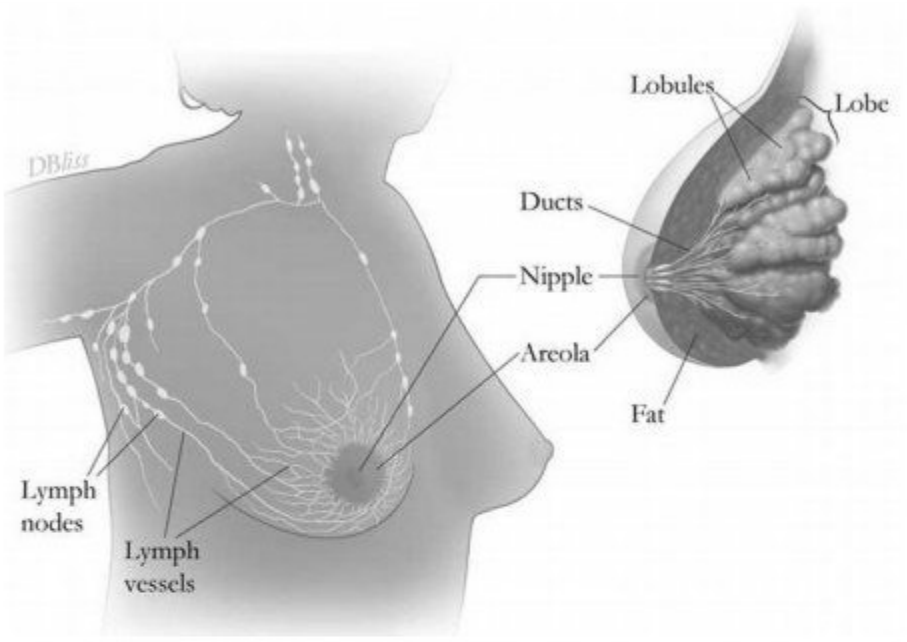


Figure 1-2. Gross anatomy of the human breast. Figure was adapted with permission from *http://www.cancer.gov/cancertopics/wyntk/breast/page2, last accessed on 10 June 2010.*

Breast cancer occurs when the cells start to grow abnormally. It is a complex and heterogeneous disease, comprising a myriad of tumour entities associated with distinctive histological patterns and different biological features and clinical behaviours. Histological aspects of breast cancer taxonomy have been described in the literature. In general, the tumour progression occurs in a sequence of defined stages. The primary lesion of neoplastic breast disease could arise from either the ducts or the lobular region of the breast. The precursor for ductal breast carcinoma which is named 'hyperplasia of usual type' (HUT) will then develop into atypical ductal hyperplasia (ADH), whereas, the initial stage of lobular breast carcinoma is defined by atypical lobular hyperplasia (ALH). This could then progress into ductal carcinoma *in situ* or lobular carcinoma *in situ* (DCIS or LCIS). The malignant epithelial cells are enclosed in the normal ducts for DCIS and

lobules for LCIS whose basal membrane persists intact. If undetected or untreated, DCIS or LCIS could advance to ductal or lobular invasive carcinoma (IDC or ILC). At this stage the cancer cells infiltrate through the basal membrane into the stroma (Pinder and Ellis, 2003; Guinebretière *et al.*, 2005) (Figure 1-3). This ultimately leads to metastasis of the tumour cells to other parts of the body such as bone, liver, lung (Hasebe *et al.*, 2008) and recently breast cancer metastasis to the stomach (rare case) was also reported (Eo, 2008).

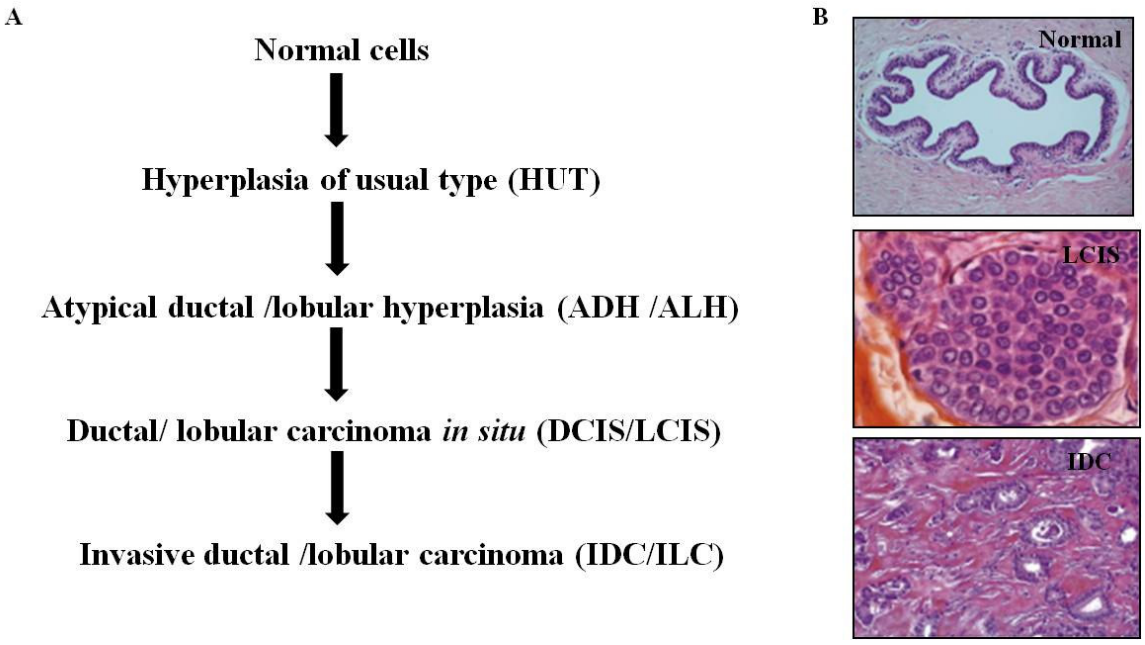


Figure 1-3. Stages of breast tumour progression. A. Range of normal cells to invasive ductal cancer cells. B Upper panel: Normal large duct. H&E Staining x100. Its lumen is irregular with pseudo papillary projections, lined with a flat epithelium. Middle panel: Lobular carcinoma *in situ*. H&E Staining x400. The tumour cells have small round and regular nuclei. They have distended into the lumen of the acini where they are enclosed, without breaking through the basal membrane. Lower panel: Invasive ductal carcinoma. H&E Staining x200. This tumour associates an invasive component, which is responsible for lymph node and distant metastasis, a fibrous stroma and an *in situ* component where the largest microcalcifications are located. H&E staining inset figures were adapted with permission from *Guinebretière, J.M., Menet, E., Tardivon, A., Cherel, P., and Vanel, D: Normal and pathological breast, the histological basis. Eur J Radiol 2005, 54: 6-14, with modification.*

1.1.3 Aetiology of breast cancer

When a patient is diagnosed of breast cancer, it is natural to ask the doctor what may have caused the disease. Like any other forms of cancer, breast cancer is a multifactorial disease. Breast cancer results from molecular alterations that are genetically and/or environmentally induced which later on led to uncontrolled abnormal cell proliferation. A number of risk factors listed in Table 1-3 are allegedly associated with the development of breast cancer.

Risk factors for breast cancer	
High (relatively)	**Low (relatively)**
Increasing age	Early menarche/late menopause
Breast cancer in a first-degree relative	Elevated levels of oestrogens and androgens
Atypical ductal or lobular hyperplasia	Age at first live birth over 30 years or nulliparity
Lobular carcinoma *in-situ*	Diet and alcohol consumption
Prior history of breast cancer	Postmenopausal obesity
BRCA1 and BRCA2 mutations	Exercise
Increasing mammographic breast density	

Table 1-3. Risk factors for breast cancer. Data extracted and summarized from *Higa, G.M. Breast cancer: beyond the cutting edge. Expert Opin Pharmacother 2009, 10, 2479-2498, with modification.*

Aside from being female, age is probably the most important risk factor of breast cancer. According to the data on age-specific incidence of breast cancer in Singapore 1998-2002, there was a notable shift of peak age-specific incidence from premenopausal (45-49 years) in the period 1993-1997, to postmenopausal (50-55 years) in the period 1998-1999 (Jara-Lazaro *et al.*, 2010). The age pattern suggests that the highest age-specific incidence rate is occurring progressively later in life. The incidence rate continues to increase after the menopause and 45% of all cases occurred of women 50 years of age and older (Figure 1-4).

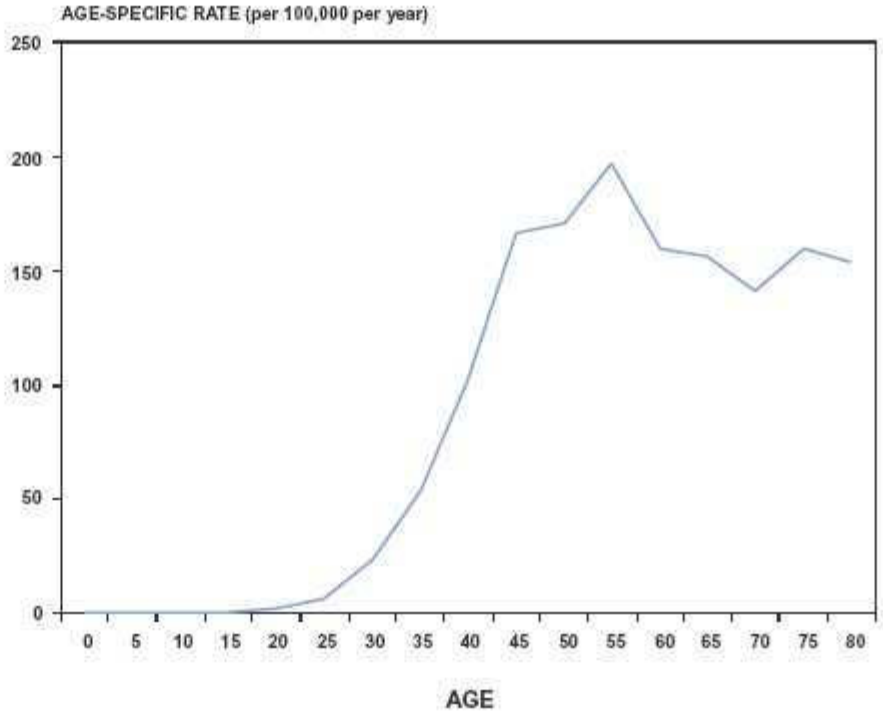


Figure 1-4. Female breast cancer: age-specific incidence, 1998-2002. Data extracted from the *Singapore Cancer Registry Report No. 6: Trends in Cancer Incidence in Singapore 1968-2002.*

Risk is also enhanced by a personal or family history of breast cancer and inherited genetic mutations in the breast cancer susceptibility genes BRCA1 and BRCA2 (Miki *et al.*, 1994; Wooster *et al.*, 1995). Most studies on familial risk of breast cancer have found about two-fold relative risks for first degree relatives of affected patients. With affected second-degree relatives, there is a lesser increase in risk (Pharoah *et al.*, 1997). In the early 1990, specific mutations were identified in both the tumour suppressor genes *BRCA1* and *BRCA2*, which are responsible for familial breast and ovarian cancers. Although these mutations cause approximately 5-10% of all breast cancer cases, they are rare in the general population. Another two genes that are also known to confer high risk of breast cancer are *TP53* and *PTEN*. Despite all these genes conferring a high risk, they

account for a relatively small proportion of inherited breast cancer. HER-2/*neu* which encodes for the epidermal growth factor receptor type-2, c-erbB2, expressed on the surface of breast cells, is another commonly affected gene in breast cancer. Amplification of the HER-2/*neu* gene or overexpression of the HER-2/*neu* encoded receptor, c-erbB2, occurs in 20-30% of breast cancer and is associated with the more aggressive phenotype of breast cancer (Schnitt, 2001; Vernimmen *et al.*, 2003).

Epidemiological studies of breast cancer have established a few risk factors playing a key role in the causation of this disease (Key *et al.*, 2001). Other evidences such as increased exposure to oestrogens, early menarche, late menopause, obesity in postmenopausal women, oral contraceptives, hormonal therapy and alcohol consumption have also been shown to increase the risk of breast cancer. Breastfeeding, childbearing, regular exercise and low fat diet are associated with a lower risk of breast cancer.

1.2 HER-2/*neu*-positive breast cancer

1.2.1 Definition

The human epidermal growth factor receptor 2 (c-erbB2) and its encoding gene (HER-2, HER-2/*neu*) had been functionally implicated in the pathogenesis of human breast cancer for more than two decades and more than 10,000 publications are in print to study the role and significance of this receptor. The HER-2/*neu* gene was first originally identified in rat neuroectodermal tumours (Shih *et al.*, 1981) and later its close human relative was isolated (Schechter *et al.*, 1984). It is located on chromosome 17q21 and encodes a 185-kDa transmembrane tyrosine kinase receptor protein that is a member of the epidermal

growth factor receptor (EGFR) family. The HER-2/*neu* encoded protein, also known as c-erbB2, contains a 95-110 kDa cysteine-rich extracellular ligand binding ectodomain, a hydrophobic membrane-spanning domain (3 kDa) and a short juxtamembrane segment, and an intracellular tyrosine kinase domain (70-90 kDa) linked to a carboxyl (C)-terminal tail (Tommasi *et al.*, 2004) as shown in Figure 1-5.

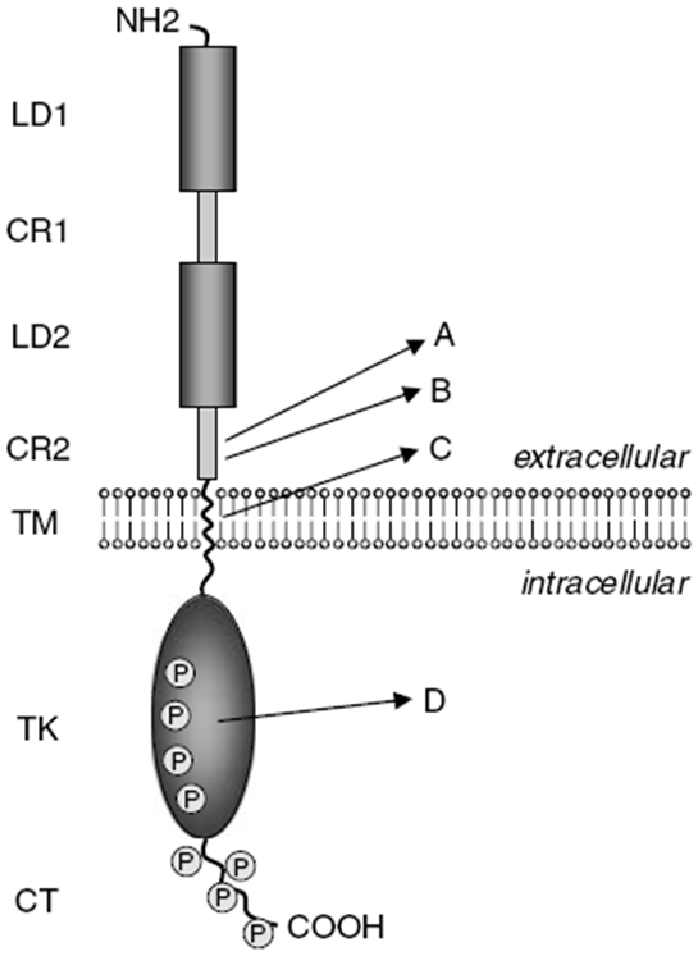


Figure 1-5. Structure of the c-erbB2 receptor, encoded by the HER-2/*neu* gene. The c-erbB2 receptor consists of extracellular ligand-binding domain with two cysteine-rich regions, a hydrophobic short transmembrane domain, and an intracellular domain that contains a catalytic tyrosine kinase domain and a carboxyl terminal tail. Numerous sites of tyrosine phosphorylation within the tyrosine kinase and carboxyl terminal domains are indicated by circled P. The letters on the right point to specific areas that are altered or mutated in certain naturally occurring or experimentally induced cancers. (A) Site of somatic mutations found in tumours arising in MMTV-*neu* mice. (B) Site of the 48 bp deletion in the naturally occurring human ΔHER2 isoform. (C) Site of the mutation in the neuT oncogene initially discovered in a rat carcinogen-induced tumour model and subsequently used in numerous *in vitro* and transgenic experimental models. (D) Site of mutations found in rare cases of human lung cancers. Figure was adapted with permission from *MM Moasser: The oncogene HER2: its signalling and transforming functions and its role in human cancer pathogenesis. Oncogene 2007, 26 (45): 6469-87, with modification.*

To date, no known ligand(s) for the c-erbB2 receptor has yet been identified. Like other RTKs, upon ligand binding to their extracellular domain, the receptor undergoes heterodimerization with either HER-1 (Pinkas-Kramarski *et al.*, 1996; Pinkas-Kramarski *et al.*, 1997) or HER-3 or HER-4 (Tzahar *et al.*, 1996; Burden and Yarden, 1997; Pinkas-Kramarski *et al.*, 1998) that lead to autophosphorylation at its carboxyl terminal. These phosphorylation sites will then become docking sites responsible in translating the activated signals initiated into downstream physiological actions. In normal cells, the HER-2/*neu* plays a role in cell growth and differentiation. However, amplification of the gene that leads to overexpression of the receptor caused the development of many types of cancers including the breast, ovarian (Slamon *et al.*, 1989), small subset of lung (Hirsch *et al.*, 2002), certain gastrointestinal tract tumours (Khan *et al.*, 2002, Yano *et al.*, 2006) and bladder (Latif *et al.*, 2003). These patients have poor response rates as well as short overall and disease-free survival compared to patients whose tumours do not overexpress this receptor.

1.2.2 Clinical significance of HER-2/*neu* as a prognostic indicator in breast cancer

HER-2/*neu* gene amplification and/or receptor overexpression has/have been identified in 10-34% of breast carcinoma (Schechter *et al.*, 1984). HER-2/*neu* gene amplification is associated with increased cell proliferation, cell motility, tumour invasiveness, progressive regional and distant metastases, accelerated angiogenesis and reduced apoptosis (Moasser, 2007). As a clinicopathological parameter, HER-2/*neu*-positive breast cancer is scored as intermediate or high histological grade, usually with the features of lacking estrogen receptors and progesterone receptors and exhibiting positive

lymph node metastases. The association of HER-2/*neu*-positive status with specific pathologic conditions is summarized in Table 1-4. HER-2/*neu*-positive cases are usually associated with the higher-grade and extensive forms of DCIS. The HER-2/*neu*-positive tumour cells usually expressed more than 10- to 100- fold with up to 2 million receptors (Burstein, 2005) compared to the normal breast epithelial cells that have only 2 copies of the HER-2/*neu* gene and express between 20,000 and 50,000 HER-2/*neu* receptors (Lohrisch and Piccart, 2001) on the cell surface.

Pathologic condition	HER-2 status
DCIS	High-grade subtype, multifocal disease, and cases with comedonecrosis are positive
ILC versus IDC	ILC lower HER-2-positive rate (<10%) restricted to the pleomorphic ILC subtype
Tumour grade in IDC and ILC	HER-2-positive status in low-grade tumours extremely rare
Paget's disease	Virtually all are HER-2-positive
Inflammatory carcinoma	No association between HER-2 status and inflammatory carcinoma confirmed to date
Mucinous (colloid) carcinoma	Rare HER-2-positive mucinous carcinomas pursue an aggressive clinical course
Medullary carcinoma	Typical are HER-2-positive
Primary versus metastatic carcinoma	A near uniform consensus of multiple published studies states that HER-2 status of matched primary and metastatic breast cancer samples maintain the same HER-2 status throughout the course of the disease in (at least) 70% to 80% of cases
***BRCA1/BRCA2* mutation-associated carcinomas**	Hereditary breast cancer consistently features a lower incidence of HER-2-positivity than sporadic disease
Breast sarcomas	These tumours are HER-2 negative
Male breast carcinoma	No consistent association of HER-2 status or response to anti-HER-2 targeted therapy fpr male breast cancer, the low number of cases limits confidence in these observations
Benign breast conditions	A near significant association with low-level HER-2 expression in benign breast biopsies with subsequent development of invasive breast cancer has been reported

Table 1-4. HER-2/*neu* status and breast pathology. Table extracted from *Ross JS, Slodkowska EA, Symmans WF, Pusztai L, Ravdin PM, Hortobagyi GN: The HER-2 receptor and breast cancer: ten years of targeted anti-HER-2 therapy and personalized medicine. The Oncologist 2009, 14 (4):320-68, with modification.*

1.2.3 Diagnosis of HER-2/*neu*-positive breast cancer

There are a series of morphology-driven, slide-based assays employed to detect the HER-2/*neu* gene amplification and HER-2/*neu* protein overexpression. The *in vitro* laboratory techniques used to diagnose HER-2/*neu*-positive breast tumours are categorized in Table 1-5. Slide-based assays include immunohistochemistry (IHC), fluorescence *in situ* hybridization (FISH), chromogenic *in situ* hybridization (CISH) and silver *in situ* hybridization (SISH). The non slide-based assays include Southern and slot blotting, reverse transcription-polymerase chain reaction (RT-PCR), mRNA microarray and enzyme-linked immunosorbent assay (ELISA). Among all the techniques, IHC and FISH are the most widely used to evaluate HER-2/*neu* status.

Table 1-5. Summary of HER-2/*neu* tests for breast cancer. Table extracted from *Ross JS, Slodkowska EA, Symmans WF, Pusztai L, Ravdin PM, Hortobagyi GN: The HER-2 receptor and breast cancer: ten years of targeted anti-HER-2 therapy and personalized medicine. The Oncologist 2009, 14 (4):320-68, with modification.*

Test	Manufacturer	Year introduced	FDA status	Current commercial status	Included in drug label	Method	Test Target	Test format	FDA test results	ASCO-CAP recommended test result
IHC CTA	Genetech	1995	Not submiited	NA	X	IHC	Protein	Clinical trial	NA	NA
IHC HercepTest™	Ventana Medical Systems	1998	Premarket approved	On the market	√	IHC	Protein	Decentralized in clinical trial and labs	2+, 3+ , positive	0,1+, negative 2+, equivocal 3+, positive
IHC Pahway™	Ventana Medical Systems	2002	Premarket approved	On the market	X	IHC	Protein	Decentralized in clinical trial and labs	2+, 3+, positive	0,1+, negative 2+, equivocal 3+, positive
FISH Inform™	Ventana Medical Systems	1997	Premarket approved	On the market	X	FISH	Gene	Decentralized in clinical trial and labs	>4.0 HER-2 gene signals/nucleus, positive	<4.0, negative 4.0-6.0, equivocal >6.0, positive
FISH PathVysion™	Abbott Molecular	2002	Premarket approved	On the market	√	FISH	Gene	Decentralized in clinical trial and labs	>2.0 HER-2 gene signals/CEP17, positive	<2.0, negative 2.0-2.2, equivocal >2.2, positive
FISH HER-2 PharmDx™	Dako	2005	Premarket approved	On the market	X	FISH	Gene	Decentralized in clinical trial and labs	>2.0 HER-2 gene signals/CEP17, positive	Not included
CISH SpotLight™	Invitrogen	2008	Premarket approved	On the market	X	FISH	Gene	Decentralized in clinical trial and labs	>5.0 HER-2 gene signals/nucleus, positive	<5.0, negative 5.0-10.0, low positive >10.0, high positive
SISH EnzMet™	Ventana Medical Systems	2008	Pending	In development	X	FISH	Gene	Pending	NA	Two-probe system pending; <6.0, negative >6.0, positive
mRNA OncotypeDX™	Genomic Health	2005	Not approved	On the market	X	RT-PCR	mRNA	Centralized at company	NA	NA
mRNA	Various	2005	Not approved	Homebrews	X	RT-PCR	mRNA	Decentralized in clinical trial and labs	NA	NA
Dimerization HERmark™	Monogram Biosciences	2008	Not approved	On the market	X	Capillary Electrophoresis	Protein dimers	Decentralized in clinical trial and labs	NA	NA
ELISA serum HER-2 Advia Centaur™	Siemens Healthcare Diagnostics	2002	Premarket approved	On the market	X	Sandwich immunoassay	Protein (serum)	Decentralized in clinical trial and labs	15 ng/ml	NA

IHC is performed on formalin-fixed-paraffin-embedded tissue and occasionally on frozen samples. Specimens are scored as 0, 1+, 2+ and 3+, based on the staining intensity and 3+ depicts the strongest staining intensity. In order to have a better interpretation of the immunostain, it is beneficial to establish a relationship between the number of receptors on a cell surface and the distribution and intensity of the immunostaining. This is achievable by using cell lines to establish a standardized IHC scoring system (Figure 1-6). This method is fast, widely available and relatively inexpensive. However, the results can vary significantly between different laboratories due to the different antibodies used or to subjective judgment criteria in the scoring system. The IHC tests that are approved by U.S. Food and Drug Administration (FDA) to identify patients with HER-2/*neu*-overexpressing breast cancer are the Dako Herceptest™ IHC assay (DAKO, Carpinteria, CA) and the Ventana Pathway™ IHC assay (Ventana, Tucson, Az) (Perez and Baweja, 2008).

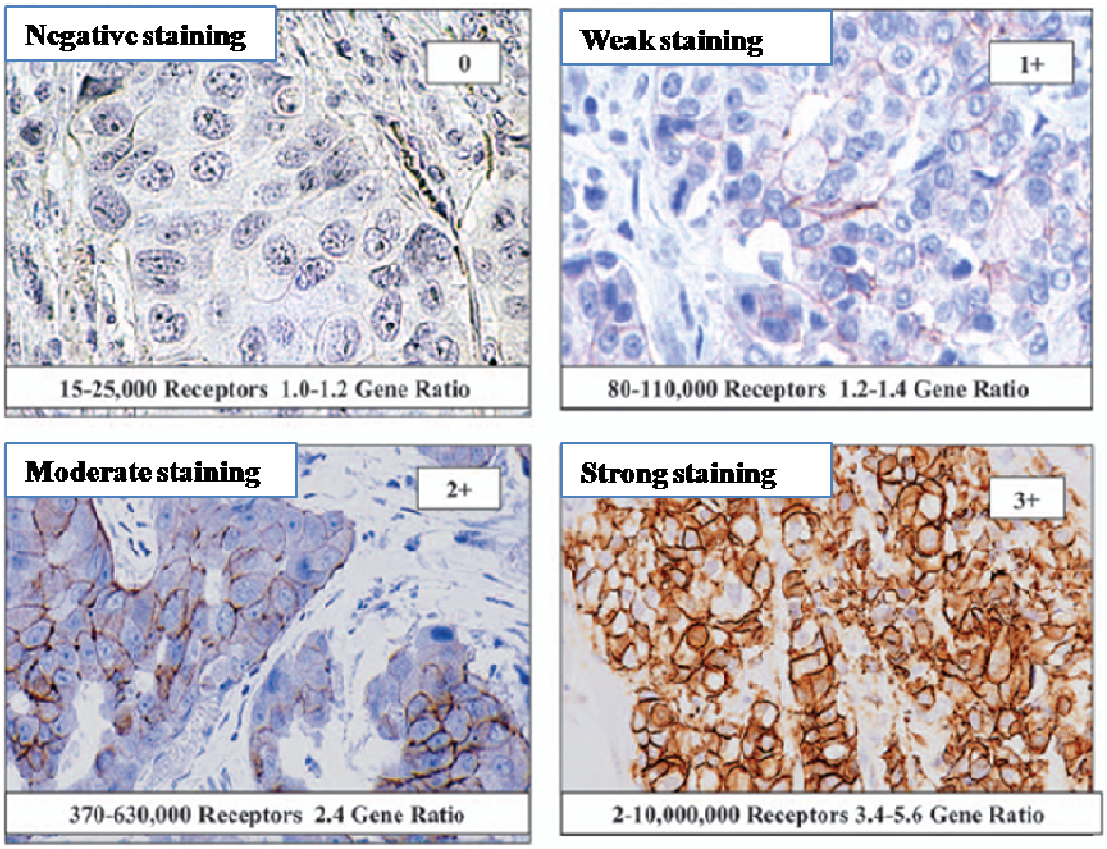


Figure 1-6. IHC staining of HER-2/*neu*-encoded c-erbB2 receptors and the scoring system. There are four categories of IHC staining scores of HER-2/*neu*. Top panels depict 0, in which cells containing <20,000 receptors would show no staining; and 1+, in which cells containing approximately 100,000 receptors would show partial membrane staining. Bottom panels indicate 2+, in which cells containing approximately 500,000 receptors would show moderately complete membrane staining; and 3+, in cases with cells containing approximately 2,300,000 receptors would show strong and complete membrane staining. Scoring of positives is based on the American Society Clinical Oncology-College of American Pathologists guidelines for HER-2/*neu* IHC scoring. Figure was extracted with permission from *Ross JS, Slodkowska EA, Symmans WF, Pusztai L, Ravdin PM, Hortobagyi GN: The HER-2 receptor and breast cancer: ten years of targeted anti-HER-2 therapy and personalized medicine. The Oncologist 2009, 14 (4):320-68, with modification.*

The FISH technique is a morphology-driven slide-based DNA hybridization assay using fluorescent probes. It tests the gene copies in tumour cells using fluorescent probes on formalin-fixed-paraffin-embedded tissue (Figure 1-7). HER-2/*neu*-positive breast cancers may express as many as 50-100-fold of the gene copies, compared to breast cancers

without HER-2/*neu* gene amplification. FISH has a better scoring system than IHC and incorporates an internal control in its protocol. However, the cost of performing FISH is higher and it is more time-consuming as compared to IHC. Currently, most laboratories performing HER-2/*neu* testing use IHC as a screening test, with results of 0 and 1+ considered as negative, and 3+ scores as positive; those with 2+ scores will be considered as equivocal and will be further subjected to FISH for confirmation, (Tubbs *et al.*, 2001; Kobayashi *et al.*, 2002). The two FISH assays that have been approved by FDA for diagnostic use to identify HER-2/*neu* gene amplification are the Oncor/Ventana Inform™ FISH test and the Abbott/ Vysis PathVysion™ FISH assay (Perez and Baweja, 2008).

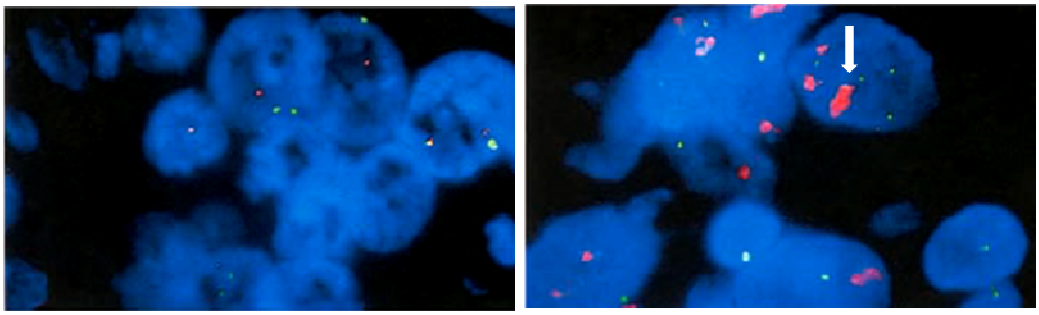


Figure 1-7. FISH staining of HER-2/*neu* gene copies. Left panel shows a negative HER-2/*neu* gene amplification. Right panel shows a positive HER-2/*neu* gene amplification with fluorescent detection in nucleus (arrow). Figures were extracted with permission from *Zhang D, Salto-Tellez M, Do E, Putti TC, Koay ES: Evaluation of HER-2/neu oncogene status in breast tumours on tissue microarrays. Hum Pathol. 2003, 34(4):362-8, with modification.*

CISH is a new method for detection of HER-2/*neu* expression. It uses the technology of FISH *in situ* hybridization and the chromogenic signal detection of IHC to detect HER-2/*neu* gene amplification as shown in Figure 1-8. This method has emerged as a more practical and cost effective option compared to FISH.

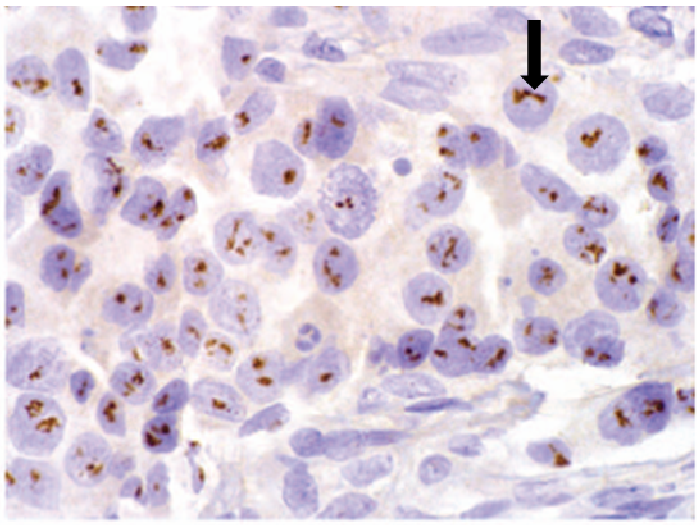


Figure 1-8. CISH staining of HER-2/*neu* gene copies. This image depicts an invasive duct carcinoma with significant HER-2/*neu* gene amplification (arrow) determined by the Invitrogen Spot-Light™ CISH assay. Figure was extracted with permission from *Ross JS, Slodkowska EA, Symmans WF, Pusztai L, Ravdin PM, Hortobagyi GN: The HER-2 receptor and breast cancer: ten years of targeted anti-HER-2 therapy and personalized medicine. The Oncologist 2009, 14 (4):320-68, with modification.*

Studies have confirmed a very high concordance between CISH and FISH, typically in the 97-99% range (Wixom *et al.*, 2004; Li-Ning-T *et al.*, 2005; Bilous *et al.*, 2006; Pothos *et al.*, 2008). In 2008, FDA approved the Spot-Light™ CISH assay (Invitrogen, Inc., Carlsbad, CA) for diagnostic use in identifying patients with HER-2/*neu* gene amplification. Another method that is currently under FDA review is Silver *in situ* hybridization (SISH) which employs both HER-2/*neu* and chromosome 17 centromere (CEP17) probes hybridized on separate slides (Ross *et al.*, 2009). A comparison between IHC, FISH, CISH and SISH techniques is summarized as in Table 1-6.

Techniques	IHC	FISH	CISH	SISH
Identification	HER-2/*neu* protein expression	Number of HER-2/*neu* gene amplification	Number of HER-2/*neu* gene amplification	Number of HER-2/*neu* gene copy number
Probes used	NA	Fluorescent probe	HER-2/*neu* probe or both HER-2/*neu* and CEP17 probe	HER-2/*neu* and CEP17 probe
Scoring System	Semiquantitative and subjective score interpretation based on staining intensity	Objective and quantitative	Fast interpretation of staining result, but subjective score interpretation	Similar to CISH and relatively easy to interpret
Type of microscope	Standard light	Fluorescent microscope	Standard light	Standard light
Cost and performance	Quick, cheap and easy to perform. Success rate is higher than ISH	Specific and sensitive but time consuming and costly	Lower cost and faster compared to FISH	Fully automated and rapidly performed, complete within 6 hours (faster than FISH)
Stability of staining/ signal	Stained slides can be stored for long periods	Signals decay over time	Staining remains stable for long periods	Staining remains stable for long periods
Morphologic features	Morphologic features of cell can be determined	Area of invasive carcinoma may be difficult to identify	Evaluates gene copy number and tissue histopathologic features simultaneously	Evaluates gene copy number and tissue histopathologic features simultaneously
Accessibility	Established technology, perform in most pathology laboratories	Established technology	Relatively new technology and less established, need more experience	New technology, need more experience
Results variation/ Control	Standardization and validation are required due to the variation in testing protocols. Result analysis is susceptible to	Internal control is incorporated in the protocol	No intrinsic control for the chromosome 17 copy number. Result analysis is susceptible to interobserver	

	interobserver variability	variability

Table 1-6. Comparison of IHC, FISH, CISH and SISH. Data extracted and summarized from *Penault-Llorca F, Bilous M, Dowsett M, Hanna W, Osamura RY, Rüschoff J, van de Vijver M: Emerging technologies for assessing HER2 amplification.Am J Clin Pathol. 2009 ;132(4):539-48, with modification.*

1.2.4 Signalling networks regulated by HER-2/*neu*

HER-2/*neu* activation triggers a plethora of downstream second messenger signalling cascades and resultant crosstalk with other transmembrane signalling pathways, leading to diverse biological effects (Figure 1-9). Overexpression of HER-2/*neu* causes increased HER-2/*neu* heterodimerization with EGFR (HER-1) or HER-3. Stimulation of EGFR-HER-2 heterodimerization, in turn, causes the HER-2/*neu* overexpressing cells to exhibit significantly prolonged activation of downstream mitogen-activated protein kinase (MAPK) and c-jun (Figure 1-9 I) (Karunagaran *et al.*, 1996). Activation of HER-2/*neu* also interrupts apical-basal polarity by associating with PAR6-aPKC, and thus disrupts the normal epithelial organization, resulting in cell proliferation as well as protecting the cells against apoptosis (Figure 1-9 II) (Aranda *et al.*, 2006; Nolan *et al.*, 2008).

Upon binding of ligands such as Heregulin (HRG) (Wallasch *et al.*, 1995), HER-3 heterodimerizes with HER-2/*neu* and induces cell transformation via activation of the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT) pathway, which subsequently induces tumorigenesis (Figure 1-9 III) (Alimandi *et al.*, 1995; Wallasch *et*

al., 1995; Ram and Ethier, 1996). Evidence from the above studies shows that HER-2/*neu* and HER-3 dimers are the most active and potent signalling heterodimers. In addition, large numbers of the ΔHER-2 transcript, a normal byproduct of HER-2/*neu* transcription, have been detected in breast tumours, and it has been proposed that the increased ΔHER-2 transcript levels found in HER-2/*neu*-positive breast tumours could be one of the driving factors towards tumorigenesis (Figure 1-9 IV) (Siegel *et al.*, 1999; Castiglioni *et al.*, 2006). Several other transcription factors that are activated by HER-2/*neu* overexpression will generate gene expression profiles that are involved in cell proliferation and survival (Figure 1-9 V). Transcription factors that have been reported as direct targets of HER-2/*neu* are cyclooxygenase-2 (COX-2) (Subbaramaiah *et al.*, 2002), E26 transformation specific transcription factor (ETS) (Shepherd *et al.*, 2001; Goel and Janknecht, 2003) and the cheomokine receptor (CXCR4) (Li *et al.*, 2004). These transcription factors are involved in mammary tumorigenesis and metastasis. In addition, heterodimers containing HER-2/*neu* undergo slower dissociation and endocytosis, and thus are more frequently recycled back to cell surface and prolonging the potent downstream signals (Citri and Yarden, 2006).

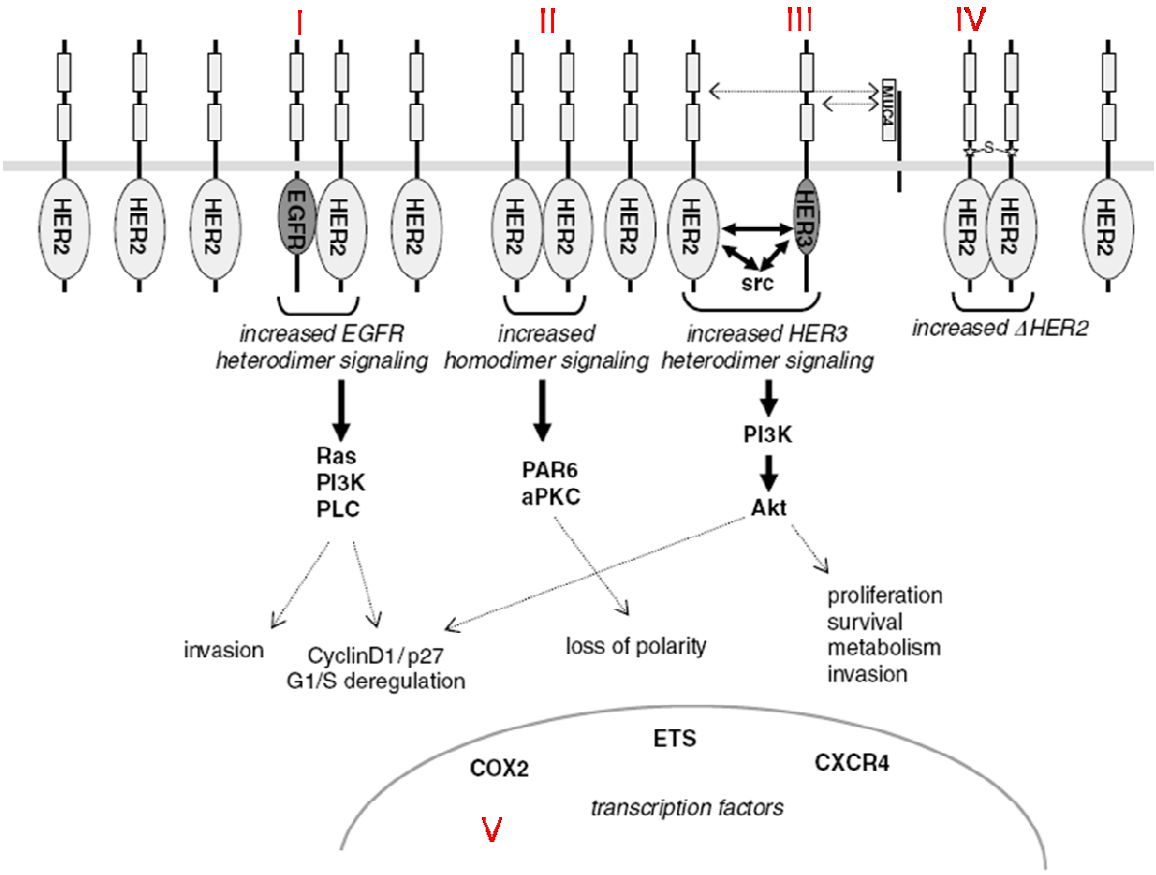


Figure 1-9. Schematic diagram of HER-2/*neu* signalling pathways that contribute to tumorigenesis. I. HER-2/*neu* overexpression enhances the HER-2/EGFR dimerization and drives the cells to proliferate and invade. II. Homodimerization of HER-2 disrupts the cell polarity. III. Heterodimerization of HER-2/HER-3 enhances the cell proliferation, survival, invasion and increases intracellular metabolism. IV. Augmented HER-2/*neu* expression also results in an increase of the rare ΔHER-2/*neu* isoforms with more potent signalling characteristic. V. Several transcription factors that are induced by HER-2 overexpressing cells result in a plethora of gene expression changes. Figure was adapted with permission from *MM Moasser: The oncogene HER2: its signalling and transforming functions and its role in human cancer pathogenesis. Oncogene 2007, 26 (45): 6469-87, with modification.*

1.2.5 Therapeutic interventions in HER-2/*neu*-positive breast cancer patients

The intense research work on HER-2/*neu* in relation to breast cancer has yielded at least two drugs that have successfully passed clinical trials and secured FDA approval for treatment of patients with metastatic breast cancer that overexpress HER-2/*neu*.

Trastuzumab (Herceptin®, Genentech, Inc., South San Francisco, CA) was approved by FDA in 1998. It is a monoclonal IgG_1 class humanized murine antibody that has been widely used in combination with chemotherapy in patients with metastatic breast cancer. More recently, in 2007, Lapatinib (Tykerb®, GlaxoSmith Kline) was approved by FDA for use in combination with capecitabine (chemotherapy drug) for treatment of HER-2/*neu*-positive metastatic breast cancer. Lapatinib is an orally available small molecule with dual inhibitory effects on EGFR and HER-2/*neu* tyrosine kinases.

Trastuzumab recognizes and binds with high affinity to an epitope on the extracellular domain of HER-2/*neu* receptor (Vogel *et al.*, 2002; Burstein *et al.*, 2003). This antibody therapy has become an important therapeutic option for patients with HER-2/*neu*-positive breast cancer and is widely used for its approved indications in both the adjuvant and metastatic settings (Hortobagyi, 2001; Perez and Baweja, 2008; Dahabreh *et al.*, 2008; Whenham *et al.*, 2008). However, there are evidences showing that use of trastuzumab combined with anthracyclines (chemotherapy drug) may cause congestive heart failure (Tan-Chiu *et al.*, 2005). Therefore, the use of this drug should be given only to patients with a low risk for cardiovascular morbidity.

Lapatinib causes prolonged downregulation of tyrosine phosphorylation of EGFR and HER-2/*neu* in tumour cells (Wood *et al.*, 2004). It binds to the intracellular domains of EGFR and HER-2/*neu*, blocking the activation of downstream MAPK signalling (Nahta *et al.*, 2007). A recent study in evaluating Lapatinib as a first-line therapy in HER-2/*neu*-positive metastatic breast cancer showed that this drug is effective in halting the disease

progression. Approximately 24% of 138 patients treated with Lapatinib for 17.6 weeks showed an overall response with 31% deriving some clinical benefit. Furthermore, progression-free rates were 63 and 43% at 4 and 6 months, respectively (Gomez *et al.*, 2008). Treatment with Lapatinib has proven clinically active and tolerable, warranting its further investigation as a first-line therapy in HER-2/*neu*-positive breast cancer.

Other drugs that are in clinical trials are perutuzumab, a monoclonal antibody that sterically blocks dimerization of HER-2/*neu* with EGFR and HER3 at different epitopes as compared to trastuzumab (Bernard-Marty *et al.*, 2006); and Neratinib, an irreversible pan-HER inhibitor that inhibits EGFR, HER-2/*neu* and HER-4 (Rabindran *et al.*, 2004; Wong *et al.*, 2009). So far, all the drugs being developed are promising despite some showing major adverse events such as nausea and myelosuppression. There are cases of patients who were not responsive to trastuzumab due to the presence of other glycoproteins such as mucin-4 (MUC4) that might mask the binding of trastuzumab to HER-2/*neu* (Nagy *et al.*, 2005). Overall, the anti-cancer drugs are expensive due to the enormous research expenditure spent on their development. Multidrug therapy based on the targeted drug approach is definitely an attractive advancement in breast cancer treatment but it is important to ensure it is affordable, in order to benefit all patients.

1.2.6 Current findings of HER-2/*neu*-linked studies

Research focused on HER-2/*neu* has a history of more than two decades since it was first functionally implicated in the pathogenesis of a subtype of human breast cancer. However, understanding of the precise mechanism(s) by which HER-2/*neu* signalling

induces tumorigenesis remains as a challenge to researchers.

HER-2/*neu* signalling has been reported to crosslink with many other pathways. In a mouse model with inactive serotonin receptors (5-hydroxytryptamine, 5-HT), that normally regulate cardiovascular functions during embryogenesis and adulthood, embryonic and neonatal death were reported to be accompanied by reduction in the expression levels of HER-2/*neu*, which may have led to midgestation lethality (Nebigil *et al.*, 2000). This model suggested that 5-HT receptors use the HER-2/*neu* signalling pathway for cardiac differentiation. Loss of HER-2/*neu* signalling in cardiac tissues was shown to cause dilation in cardiomyopathy in adult mice and may thus explain the clinical implication for the use of HER-2/*neu* targeted therapy (Garratt *et al.*, 2003; Negro *et al.*, 2004). Another observation by Andrechek *et al.* indicated that HER-2/*neu* plays a critical role in the initial stages of mammary gland morphogenesis. The *in vivo* studies showed that ablation of HER-2/*neu* led to a striking ductal elongation defect and reduced branching in the adult mammary gland. However, targeted disruption of HER-2/*neu* had little impact on the ability of these animals to lactate (Andrechek *et al.*, 2005).

Heat shock protein 90 (Hsp90) is a ubiquitously expressed molecular chaperone that controls the folding, assembly, intracellular disposition, and proteolytic turnover of many proteins. Hsp90 interacts with HER-2/*neu* and promotes the maturation of HER-2/*neu* to a state of competent readiness for dimerization and activation (Citri *et al.*, 2004). Sidera *et al.* demonstrated a novel interaction between surface Hsp90 and the extracellular domain of HER-2/*neu* and further suggested the involvement of Hsp90 in heregulin-

induced HER-2/*neu* activation and signalling, leading to cytoskeletal rearrangement, essential for cell invasion (Sidera *et al.*, 2008).

The role of HER-2/*neu* is well known in oncogenesis due to its overexpression in tumour cells. Overexpression of unaltered HER-2/*neu* coding sequences in NIH/3T3 cells was shown to cause cellular transformation and tumorigenesis (Hudziak *et al.*, 1987). In addition, when HER-2/*neu* complementary DNA was expressed in NIH/3T3 cells under the control of the SV40 promoter, high levels of the HER-2/*neu* product associated with malignant transformation of NIH/3T3 cells were observed in human mammary tumour cells that overexpressed this gene (Di Fiore *et al.*, 1987). Overexpression of HER-2/*neu* engineered into the EGF receptor-negative cell line, NR6, displayed a highly transformed and tumorigenic phenotype as compared with the control cells (Chazin *et al.*, 1992). These findings demonstrate the effect of overexpression of HER-2/*neu* in the development of several human malignancies. In breast cancer, overexpression of HER-2/*neu* in MCF7 breast cancer cell lines resulted in enhanced HER-2/*neu* signalling through the PI3K pathway and led to tumorigenesis (Benz *et al.*, 1992). In another study using the non-tumorigenic human breast cell line MCF10A, overexpression of HER-2/*neu* increased cell proliferation, reduced cell apoptosis and promoted the formation of solid multi-acinar structures resembling early epithelial transformation (Muthuswamy *et al.*, 2001). Furthermore, HER-2/*neu* has also been implicated in cell invasion in HER-2/*neu* overexpressing human mammary epithelial (HME) cells via PI3K-dependent and p38 MAP kinase-dependent pathways which lead to activation of AKT, and activation of PKC-delta, via PI3K, and result in mediating cell invasion (Woods Ignatoski *et al.*,

2003).

Overexpression and/or activity of src kinases are associated with breast cancer progression. Src kinases are important second messengers of HER-2/*neu* and increases in their activated forms correlate with HER-2/*neu* positivity, high tumour grade, necrosis and elevated epithelial proliferation (Wilson *et al.*, 2006). Reported evidence showed that src kinases may function upstream (Ishizawar *et al.*, 2007) and downstream (Muthuswamy and Muller, 1995; Vadlamudi *et al.*, 2003; Kim *et al.*, 2005; Xu *et al.*, 2007) of the transforming functions of HER-2/*neu* and making them critical enhancers of HER-2/*neu*-driven tumorigenesis.

The polarized glandular organization of epithelial cells is frequently lost during development of carcinomas. Homodimerization and activation of HER-2/*neu* also disrupt the apical-basal polarity by associating with Par6-aPKC, components of the Par polarity complex (Aranda *et al.*, 2006). The heterodimerization of EGFR and HER-2/*neu* was found to induce cell invasion by activating PI3K, Ras/MAPK and PLCγ signalling pathways in HER-2/*neu*-positive breast cancer (Zhan *et al.*, 2006). Numerous downstream signals have been reported to associate with the invasive phenotype of HER-2/*neu*-positive breast cancers. These downstream signal molecules comprise of PI3K (Ignatoski *et al.*, 2000), PKCα, src, focal adhesion kinase (Benlimame *et al.*, 2005), α4 integrin, β4 integrin (Gambaletta *et al.*, 2000) and transforming growth factor-β (Seton-Rogers *et al.*, 2004). In the cell cycle, deregulation of the G1/S phase can lead to uncontrolled cell proliferation. HER-2/*neu* overexpression in breast epithelial cells

deregulates the G1/S phase control through upregulation of cyclin D1, E and cdk6 and enhance degradation of p27 (Timms *et al.*, 2002).

In addition to the crosstalk with other cell signalling pathways, HER-2/*neu* gene amplification in breast cancer tissues also leads to various genomic alterations. For instance, protein tyrosine phosphatase 1B (PTP1B) is often amplified in HER-2/*neu* amplified breast cancers (Tanner *et al.*, 1996) and is required for HER-2/*neu* transformation in human breast epithelial cell. Inhibition of PTP1B delays HER-2/*neu*-induced mammary tumorigenesis (Julien *et al*., 2007; Bentires-Alj and Neel, 2007; Arias-Romero *et al.*, 2009). Although the tyrosine phosphatases frequently play a negative feedback role in growth factor receptor signalling, the overexpression of PTP1B has led to a hypothesis that phosphatases play an important role in regulating cancer cell activity. A plausible functional link between the HER-2/*neu* and phosphatases may yet represent an additional signalling pathway.

All these findings have added valuable information to the complexity of HER-2/*ne*-mediated breast cancer progression. However, there are yet more to be unraveled in order to have a detailed understanding of the mechanisms underpinning HER-2/*neu*-mediated signalling. This will aid in the design and development of targeted drug therapies and may help overcome the effects of drugs resistance currently encountered by HER-2/*neu*-positive breast cancer patients.

1.3 Protein post-translational modifications

1.3.1 Definition

Proteins are the building blocks from which cells are built; they also execute all cell functions. Proteins are translated from mRNA and undergo chemical modifications before becoming functional in different body cellular activities, from DNA replication and protein synthesis to sensory perception or the acquisition of nutrients. These modifications are collectively termed as post-translational modifications (PTM). It is a crucial process in generating the heterogeneity in proteins, which is required in the utilization of the same proteins for different cellular functions in different cell types.

PTM occurs in two broad categories:

1. Covalent modification of a nucleophilic amino acid side chain by an electrophilic fragment of a cosubstrate.

2. Cleavage of a protein backbone at a specific peptide bond.

The first category is by enzyme-catalyzed covalent additions of a chemical group to a side chain residue such as phosphorylation, methylation, glycosylation, etc. The second category is covalent cleavage of peptide backbones by proteases or autocatalytic cleavage (Walsh *et al.*, 2005). The modified proteins coordinate with other discrete proteins and form small complexes that are involved in various metabolic routes. To date, there are at least 100 functional pathways annotated, classified and stored (Kanehisa *et al.*, 2008; Matthews *et al.*, 2009).

1.3.2 Protein phosphorylation and the role of protein kinases

Protein phosphorylations play an important role in signal transduction pathways and control many cell biological processes. The concept was proposed by Fishcer and Krebs in 1955, when they biochemically reconstituted the conversion of phosphorylase *b* to phosphorylase *a* in a cell-free muscle extract and showed that such reaction required phosphorylase kinase and ATP (Fischer and Krebs, 1955). The mammalian phosphoproteomes consist of phosphorSer (pS), phosphorThr (pT) and phosphoTyr (pY) residues. The enzymes dedicated to protein phosphorylation are among the largest class of PTM enzymes which is the superfamily of protein kinases termed kinome, with over 500 members (Manning *et al.*, 2002). Protein kinases can be categorized into two big groups: Ser/Thr-specific kinases and Tyr-specific kinases (Hornbeck *et al.*, 2004; Ubersax and Ferrell, 2007). All kinases have a common catalytic domain that catalyses the transfer of the γ-phosphate group of ATP to the hydroxyl oxygen of the Ser, Thr or Tyr residue of the substrate (Ubersax and Ferrell, 2007; Turk, 2008). Most kinases work within complex networks with phosphatases and other effector proteins. For example, receptor tyrosine kinases such as HER-2/*neu*-encoded receptors dimerize upon binding of ligand and phosphorylate each other's multiple Tyr sites, as described in 1.2.4. The downstream effectors then specifically recognize these phosphorylated sites through SH2 domains and trigger the subsequent signal amplifications. In cancer cells, overexpression of kinases cause the unabated cell proliferation and thus phosphoproteomics is necessary to fully unveil the phosphorylation patterns and regulation of signalling networks.

1.3.3 Protein phosphorylation and the role of phosphatases

The reversible phosphorylation of protein is accomplished by the opposing activities of kinases and phosphatases and is an essential mechanism of cell regulation. To provide a tight reversible and adjustable control of protein phosphorylation, there exists a similar complexity in the number of phosphatases (Virshup and Shenolikar, 2009). Phosphatases control the specific dephosphorylation of thousands of phosphoprotein substrates. Protein phosphatases also demonstrate a strict specificity for phosphorSer (pS), phosphorThr (pT) or phosphoTyr (pY) residues (Charbonneau and Tonks, 1992). The two most abundant and extensively studied phosphoprotein phosphatases (PPPs) are members of the protein phosphatase 1 (PP1) and protein phosphatase type 2A (PP2A) family. The catalytic subunit of the phosphatases is able to associate with a myriad of regulatory subunit components that determine their functional specificity. The cellular functions of protein phosphatases have been increasingly studied and documented over the past decade. A few phosphatases have been directly implicated in the aetiology of tumours. For example, the protein phosphatase, PTEN, was reported to be mutated or deleted in various cancers (Di Cristofano and Pandolfi, 2000); the dual specificities of the protein phosphatases, CDC25A and CDC25B, are important regulators of cell cycle progression and both exhibit oncogenic potential (Galaktionov *et al.*, 1995); and PP2A, a phosphatase that functions to reverse the action of kinases in many signalling cascades, might be a potential tumour suppressor (Westermarck and Hahn, 2008).

1.4 Protein phosphatase type 2A (PP2A)

1.4.1 PP2A structure and function

PP2A is a serine/threonine phosphatase that plays a crucial role in cellular physiology. It is a heterotrimeric holoenzyme consisting of a structural or scaffolding A subunit (PP2A/A or PR65 subunit), a regulatory B subunit (PP2A/B) and a catalytic C subunit (PP2A/C). Each subunit plays a different role: the 36-kDa C subunit is the enzymatically active component; the 54-, or 55-, or 72-kDa B subunit acts as the targeting module that directs the enzyme to various intracellular locations and also provides distinct substrate specificity, and the 60-kDa A subunit appears to function primarily as a scaffolding protein that serves to assemble the different subunits into one holoenzyme (Figure 1-11) (Shi, 2009). All of these subunits come in various isoforms, so that the ABC holoenzyme is a structurally diverse enzyme in which a single catalytic C subunit can associate with a wide array of regulatory B subunits (Schönthal, 2001). The regulatory B subunit comprises four families: B (also known as B55 or PR55), B' (B56 or PR61), B" (PR48/PR72/PR130) and B"' (PR93/PR110). Each family consists of two to five isoforms: α, β, γ, δ, and ε (Shi, 2009). The finding of tyrosine phosphorylation of PP2A/C in response to growth stimulation and v-src transformation of fibroblasts provided strong evidence of PP2A activity being acutely regulated by tyrosine phosphorylation (Chen *et al.*, 1992; Chen *et al.*, 1994; Brautigan, 1995) . Phosphorylation at the tyrosine 307 residue (pY307-PP2A) inhibits the recruitment of the regulatory B subunits to the core enzyme (Longin *et al.*, 2007).

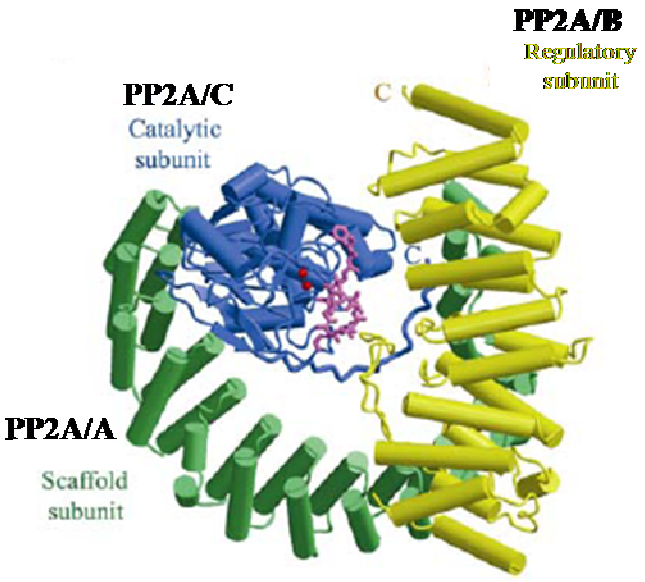


Figure 1-10. Structure of PP2A. Structure of the PP2A holoenzyme consists of a B regulatory subunit that binds to the scaffolding A subunit and C catalytic subunit. The highly conserved ridge is highlighted in magenta. Figure was adapted with permission from *Shi et al.: Assembly and structure of protein phosphatase 2A. Science in China, Seriec C, Life Sciences 2009, 52 (2): 135-46, with modification.*

PP2A is highly abundant, ubiquitous and remarkably conserved. It plays an important role in development, cell proliferation and death, cell motility, cytoskeleton dynamics, cell cycle and numerous signalling pathways (Janssens and Goris, 2001; Sontag, 2001). The multiple ways of PP2A regulation responsible for PP2A functional specificity is summarized in Figure 1-12 (Sontag, 2001).

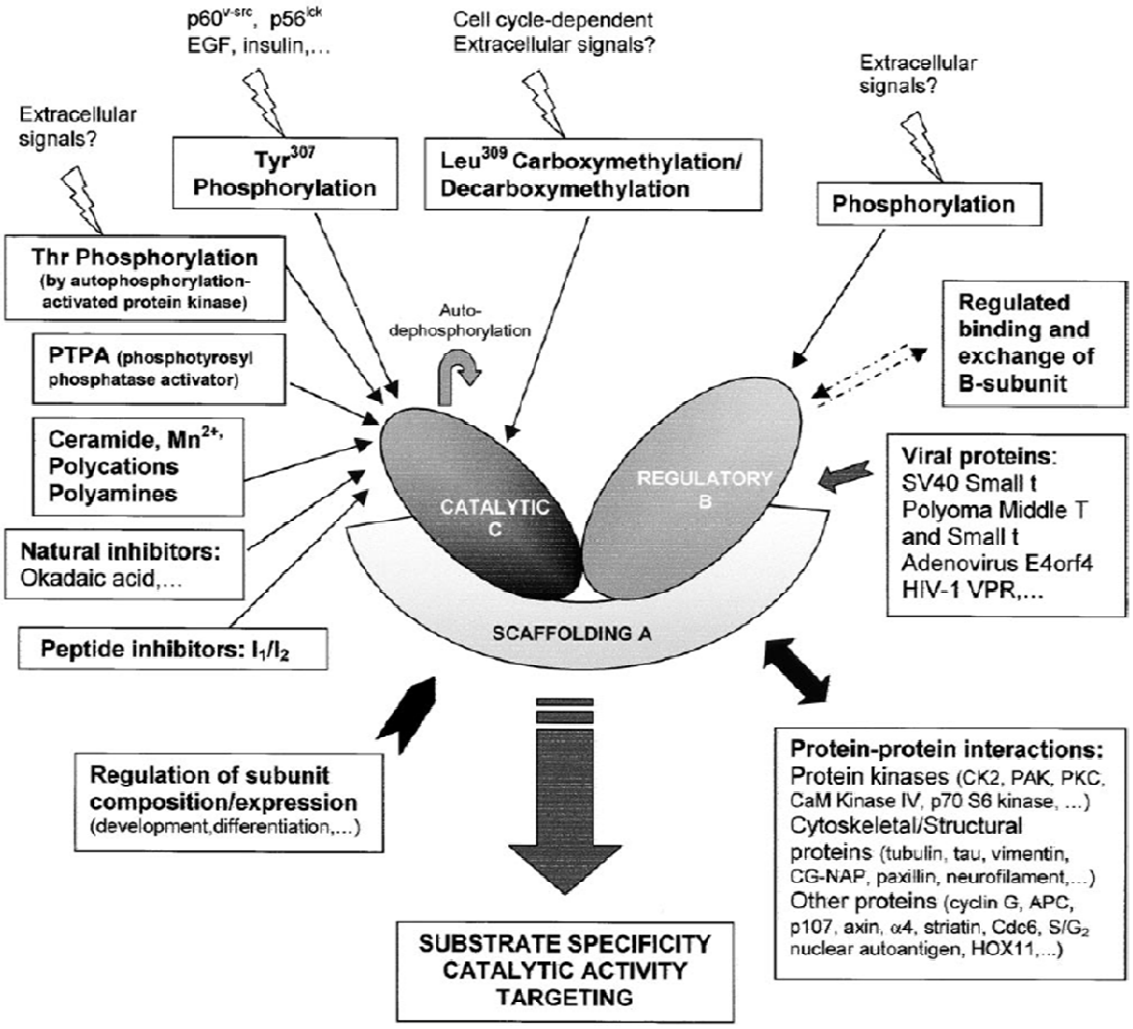


Figure 1-11. Summary of the multiple ways of intracellular PP2A regulation. Figure was adapted with permission from *Sontag E: Protein phosphatase 2A: the Trojan Horse of cellular signalling. Cellular Signalling, 2001, 13 (1): 7-16.*

1.4.2 PP2A and cancer

PP2A was first suggested as a tumour suppressor based on the study using okadaic acid (OA), a PP2A inhibitor. Treatment of mice using OA resulted in tumour formation (Fujiki and Suganuma, 1993) by activating cancer promoting pathways (Schönthal, 2001). SV40 small tumour antigen (ST) and polyoma middle T (pyMT) induce cellular transformation in human cells by altering PP2A activity and displacing the regulatory B

subunit from the holoenzyme complex (Campbell *et al.*, 1995). pyMT appears to activate the MAP kinase pathway while ST stimulate AKT phosphorylation in a PP2A-dependent pathway and thus promoting cell survival (Rodriguez-Viciana *et al.*, 2006; Andrabi *et al.*, 2007). Different PP2A subunits are found to be either mutated or aberrantly expressed in a number of malignancies as shown in Table 1-7 (Perrotti and Neviani, 2008).

Subunit	Genetic and/or functional alteration	Malignancy
PP2A$_{B56\gamma}$	Aberrant expression	Metastaic melanoma cells
PP2A$_{A\beta}$	Somatic mutations	Lung tumor; Colorectal carcinoma; Breast cancer
PP2A$_{A\beta}$	Deletion; alternative splicing; downregulation	B-CLL
PP2A$_{ABC}$	Loss of phosphatase activity through the inhibitor SET	CML-BC
PP2A$_{ABC}$	Loss of phosphatase activity through the inhibitor SET	Ph(+)B-ALL
PP2A$_{ABC}$	Loss of phosphatase activity through Pml	Prostate tumour

Table 1-7. Mutations or abnormal expression of PP2A subunits found in human cancers. Figure was adapted with permission from *Perrotti et al.: Protein phosphatase 2A (PP2A), a druggable tumour suppressor in Ph1 (+) leukemias. Cancer Metastasis Rev, 2008, 27 (2): 159-68, with modification.*

The PP2A/B has been found to be overexpressed or truncated in metastatic melanoma and due to the impaired PP2A in dephosphorylating focal adhesion protein, this led to cell invasion (Francia *et al.*, 1999; Ito *et al.*, 2000). Recent findings also reported the involvement of PP2A/B in Wnt/β-catenin signalling and MAPK pathways (Eichhorn *et al.*, 2009), that elicited caspase-dependent apoptosis. Due to the diversified PP2A/B interactions with many targets such as c-myc (Arnold and Sears, 2006), anti-apoptotic protein BCL-2 (Ruvolo *et al.*, 2002), pro-apoptotic protein BAD (Chiang *et al.*, 2003) and p53 (Li *et al.*, 2007), it is therefore not surprising that abnormal expression of PP2A/B can cause cell metastasis and transformation and that PP2A is suggested as a

putative tumour suppressor.

Mutation of PP2A/A isoforms were reported in lung, colorectal and breast cancers (Wang *et al.*, 1998; Ruediger *et al.*, 2001; Esplin *et al.*, 2006). The loss of function of PP2A/A cooperates with the expression of the large-T antigens, hTERT and H-RAS, to transform human cells (Sablina *et al.*, 2007). PP2A/A also interacts with GTPase RalA, a regulator of membrane transport, apoptosis, transcription cell migration and cell proliferation. Loss of PP2A/A causes hyperphosphorylation of RalA that leads to tumour formation (Sablina *et al.*, 2007).

Due to the complexity of the role of PP2A in cellular behaviour, deregulation of any of its subunits can affect its holoenzyme functions. The individual roles of PP2A subunits in cancer and their specific roles in signalling pathways can be further dissected using gene knockdown technology.

1.5 Gene silencing

1.5.1 Definition

RNA interference (RNAi) is a process of sequence-specific, post-translational gene silencing induced by double-stranded RNA (dsRNA), resulting in null or hypomorphic phenotypes. This phenomenon was first observed by Fire and Mello *et al.* in the nematode *Caenorhabditis elegans* (*C. elegans*). They proposed that it is the presence of dsRNA that is responsible for producing the interfering activity (Fire *et al.*, 1998) and a Nobel prize in medicine was awarded to them in 2006 for this discovery. Methods that

exploit RNAi mechanisms have since been developing rapidly and scientists employ it as a research tool to elucidate the functions and interactions of mammalian genes.

The characteristics of RNAi are as follows:

- it is highly specific to the targeted gene of interest
- it is remarkably potent as only a few dsRNA molecules per cell can induce a robust response (Mello and Conte, 2004)
- the interfering activity (and presumably the dsRNA) can cause interference in cells and tissues far removed from the site of introduction

The canonical inducer dsRNA is introduced directly into the cytoplasm or taken up exogenously from the environment (Mello and Conte, 2004) before being processed into siRNAs that directly silence the gene. siRNAs were originally observed in transgene- and virus-induced silencing plants that play a role in natural genome defense (Mello and Conte, 2004). Shortly thereafter, functional studies of siRNA in plants led to the discovery of *trans*-acting siRNAs that are diced from specific genomic transcripts and function to regulate discrete sets of genes (Vazquez *et al.*, 2004; Allen *et al.*, 2005). Recently, endogenous sources of siRNA such as convergent mRNA transcripts and other natural sense-antisense pairs, duplexes involving pseudogene-derived antisense transcripts and the sense mRNA from their cognate genes as well as hairpin RNAs (hpRNAs) were identified (Golden *et al.*, 2008).

1.5.2 Mechanisms of RNAi

There are two subgroups of post-translational gene-silencing small RNAs: siRNAs and microRNAs (miRNAs). siRNAs exist in both mammalians (Svoboda *et al*., 2004; Watanabe *et al.*, 2006) and lower eukaryotes (Ketting *et al.*, 1999; Tabara *et al*., 1999), while miRNAs have been found only in mammalians but not in lower eukaryotes. RNAi processes occur at two stages: initiation and effector stages (Ashihara *et al.*, 2010). In the initiation stage, dsRNA is introduced exogenously or endogenously into the target cells and is processed into shorter-length dsRNAs (of 21-23 nucleotides), termed siRNAs, by the ribonuclease activity of the dsDNA-specific RNAse III family ribonuclease, Dicer (Bernstein *et al.*, 2001). In the effector stage, the siRNAs then enter into an RNA-induced silencing complex (RISC) assembly pathway (Hammond *et al.*, 2000) that involves duplex unwinding, culminating in the stable association of only one of the two strands with the Argonaute (Ago) effector protein (Meister and Tuschl, 2004; Meister *et al*., 2005; Tomari and Zamore, 2005; Carthew and Sontheimer, 2009). The antisense strand, also called the guide strand, is selected by the Ago2 protein and will bind to the sequence-specific base pairs of its mRNA target and will direct the silencing. The sense strand or also known as the passenger strand, is then degraded (Matranga *et al.*, 2005). The antisense strand will then cleave the targeted mRNA, resulting in the decreased expression of the target gene (Figure 1-13).

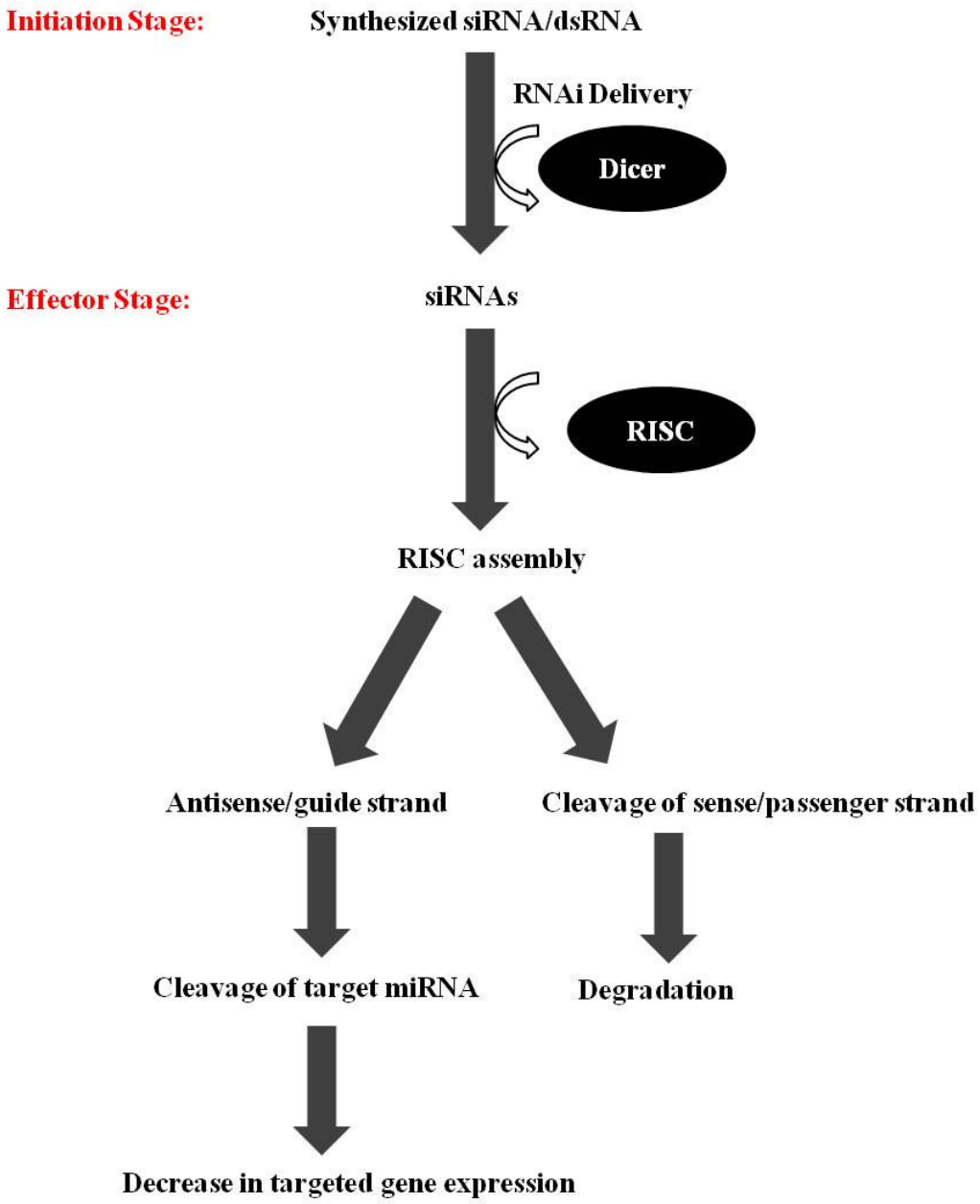


Figure 1-12. Mechanism of RNAi. The dsRNA is introduced into the cells and processed into siRNA lengths of 21-23 nucleotides by Dicer. siRNA then enters the RISC assembly pathway and unwinds into two single strands of RNA. The sense or passenger strand is rapidly degraded whereas the antisense or guide strand binds to the target mRNA and causes the target mRNA to degrade. Figure was sourced from *Ashihara E et al.: Future prospects of RNA interference for cancer therapies. Current Drug Targets 2010, 11 (3): 345-60, with modification.*

1.5.3 RNAi as a tool of analysis

The discovery of RNAi proffers the power of performing loss-of-function testing in mammalian cells and leads to elucidatation of gene functions on an unprecedented scale. Several libraries of siRNA are available commercially, through collaboration or in-house production. Even limited scale RNAi screenings in druggable targets (Lee *et al.*, 2009) or in protein kinases and oncology targets (Quon and Kassner, 2009) are yielding important information regarding intermediary pathways and cellular responses to therapeutics and other treatments.

Due to the high specificity and efficiency of the RNAi technology, it has been recently evaluated as a potential therapeutic strategy for cancer treatment. Targeted genes such as antiapoptotic proteins, cell cycle regulators, transcription factors, signal transduction proteins and oncogenes are all targets of RNAi technology as many of them are associated with poor prognosis in cancer patients. A few research groups have recently demonstrated the success of using siRNA treatment as cancer therapy in a xenograft mouse model (Yano *et al.*, 2004; Fu *et al.*, 2005; Sonoke *et al.*, 2008). These studies have demonstrated that siRNA treatment against antiapoptotic Bcl-2 proteins inhibited the tumour cell proliferation. Synthetic Bcl-2 siRNA was administered using a cationic liposome and shown to suppress tumour progression in the xenograft mouse model (Yano *et al.*, 2004; Fu *et al.*, 2005; Sonoke *et al.*, 2008). As overexpression of β-catenin was detected in many types of cancer, others have used siRNA against β-catenin and showed that this suppressed the proliferation of colon cancer cells and melanoma cells by activating caspase-dependent apoptosis (Verma *et al.*, 2003; Ashihara *et al.*, 2009).

Likewise, similar results were obtained against the vascular endothelial growth factor (VEGF) receptor, which plays an important role in angiogenesis and lymphangiogenesis and promotes cancer cells motility and metastasis. Treatment with siRNA against VEGF successfully halted metastasis in breast cancer in a mouse xenograft model (Chen *et al.*, 2005).

RNAi remains as a powerful strategy not only to aid the researchers in understanding the functions of specific genes and their interacting partners, it also may offer attractive therapeutic options for physicians on patient treatments.

1.6 Rationale and objectives of this research project

The HER-2/*neu* proto-oncogene is an established adverse prognostic factor of breast cancer. Studying the mechanisms by which HER2/*neu* overexpression translate into the more aggressive biological behaviour manifested would not only provide a better understanding of the biological basis of the increased virulence of breast cancers overexpressing this oncogene but may also provide rational, target-based therapeutic strategies to stop the accelerated cancer growth and metastasis in this sub-group of patients. Despite numerous proteomics techniques having been employed to study its mechanisms of action, the challenges of understanding HER-2/*neu* activation are still to be met and further work is fuelled by the emergence of new biomarkers.

Hypothesis: The hypothesis in this study is that HER-2/*neu* activation causes multiple phosphorylation of downstream signaling modulators that are involved in cell

proliferation and growth, as such, in this research, the focus is to study the phosphoproteomic profiles of the tyrosine phosphorylation changes initiated by HRG-mediated HER-2/*neu* activation. HER2/*neu* activation was found to be able to cause the downstream phosphorylation of PP2A phosphatase, a putative tumour suppressor protein that is involved in many signalling pathways in mammalian cells. The PP2A/C subunit (more specifically, phosphorylation at its tyrosine residue pY307 residue and/or methylation) is known to be critical in regulating its activity in cancer cells. Although much work on the association of PP2A/A and PP2A/B subunits with cancer had been reported (Francia *et al.*, 1999; Ito *et al.*, 2000; Ruvolo *et al.*, 2002; Chiang *et al.*, 2003; Arnold and Sears, 2006; Li *et al.*, 2007; Sablina *et al.*, 2007), the precise role of PP2A/C in breast cancer remained to be studied in detail. We aimed to fill in some of the gaps in this area.

The bigger aims of the ongoing PP2A-related research are to elucidate the HER-2/*neu*-driven tyrosine phosphorylation network and to identify some putative phosphopeptide targets as new prognostic markers. Thus, the aim of this thesis project is to further to test on the hypothesis that one of the identified proteins, PP2A/C, plays a critical role in HER-2/*neu*-mediated breast tumorigenesis. An interactive functional relationship between HER2/*neu* and PP2A/C in several breast cancer cell-lines was investigated, and its findings were reported herein.

The **specific aims of this thesis** are:

1. To identify the differential tyrosine phosphorylation of various signal modulators

activated by HRG mediated HER-2/*neu* signalling using an antibody array.

2. To verify the selected identified protein using 1D and 2D immunoblotting methods. This will confirm the antibody array results.

3. To study the role of HER-2/*neu* signalling in the regulation of PP2A phosphorylation.

4. To further examine if there is any differential expression of PP2A and pY307-PP2A in both breast non-tumorigenic cancer cell lines as well as clinical samples.

5. To investigate the resultant consequences of PP2A/C knockdown on cellular behaviour in HER-2/*neu*-positive breast cancer cells and its downstream signalling cascade.

Understanding the mechanisms underlying HER-2/*neu* tumorigenesis and metastasis, and whether there is differential involvement of various critical pathways in the more aggressive HER-2/*neu* positive breast cancer patients, will provide significant insights into the biology of breast cancer progression. More importantly, these insights may inform on chemo-preventive measures.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

2.1.1 Ligand and inhibitors

Heregulin α1 (HRG), a ligand of HER-3 and HER-2/*neu*, was from Neomarkers (Fremont, CA, USA). LY294002, a specific inhibitor of the p110 catalytic subunit of PI3K; AG825, a specific inhibitor of HER-2/*neu* tyrosine kinase, and SB203580, a specific inhibitor of p38 MAPK, were purchased from Calbiochem (San Diego, CA, USA). PD98059, a specific inhibitor of MEK, was from USBiological (Swampscott, MA, USA). All the inhibitors were dissolved in dimethyl sulfoxide (DMSO) and stored at -20^0C in the dark as stock solutions until use.

2.1.2 Antibodies

The following antibodies were used for immunoblot analysis: rabbit anti-phospho-HER-2/*neu* (Tyr1248) (1:1000), mouse anti-ERK1/2 (1:1000), rabbit anti-phospho-ERK1/2 (1:500), rabbit anti-ARC (1:200), rabbit anti-FADD (1:200), rabbit anti-N-Shc (1:1000), rabbit anti-phospho-Shc (Tyr239/240) (1:200), rabbit anti-PP2A (1:200), rabbit anti-phospho-PP2A (Tyr307) (1:1000), mouse anti-Stat1 (1:200), and rabbit anti-phospho-Stat1 (Tyr701) (1:200), mouse anti-Hsp27(1:5000) and rabbit anti-Hsp27 Ser78 (1:200) were obtained from Santa Cruz Biotechnology, Inc. (CA, USA). Rabbit anti-PP2A (1:1000) and rabbit anti-PP2A (Tyr307) (1:1000) were procured from Epitomics Inc. (CA, USA). Mouse anti-phosphotyrosine 4G10 (1:1000) was purchased from Upstate Biotechnology Inc. (Lake Placid, NY, USA). Mouse anti-HER-2/*neu* (1:1000) was

obtained from Neomarkers (Fremont, CA, USA). Mouse anti-Ezrin (1:100) was bought from BD Biosciences PharMingen (San Diego, CA, USA) and horseradish peroxidase (HRP)-conjugated phosphotyrosine (1:1000) was procured from Hypromatrix (Worcester, MA, USA). Mouse anti β-actin was purchased from Sigma-Aldrich (MO, USA) and rabbit anti-caspase 3, rabbit anti-PARP, rabbit anti-AKT, rabbit anti-AKT (Ser473), rabbit anti-p38 MAPK, rabbit anti-p38 MAPK (Tyr182/Thr180) were obtained from Cell Signalling Technology (MA, USA).

2.1.3 Cell-lines

All cell lines used in this research project were purchased from the American Type Culture Collection (ATCC, VA, USA). The human HER-2/*neu*-positive BT474 and SKBR3 breast cancer cell lines were grown in Dulbecco's modified eagle medium (DMEM) and McCoy's 5A medium respectively supplemented with 10% fetal bovine serum (FBS, GIBCO); the human non-tumorigenic MCF12A mammary epithelial cell lines were grown in mammary epithelial cell complete medium (MEGM), supplemented with bovine pituitary extract (BPE). All the cells lines were grown at 37^{o}C in a humidified 5% CO_2 incubator.

2.1.4 Fresh frozen clinical specimens

Six HER-2/*neu*-positive, and five HER-2/*neu*-negative frozen breast tumours and nine frozen non-tumour tissues were obtained from the Tissue Repository, National University Hospital (Singapore). All the breast tumours and matched normal tissues were obtained with patients' consent and complied with the regulations set by the Institutional Review

Board, such as de-identification of patients' identity from the samples studied to protect their confidentiality.

2.2 Methods

2.2.1 Cell culture treatment

I. Treatment with HRG and optimization

For HRG stimulation, BT474 cells (70~80% confluence) were serum-starved for 20 h and the amount of HRG used was optimized (final concentration: 3 nM) and added into the culture medium. Cells were then incubated for the indicated time periods (5, 10, 30 and 60 min). Cells treated with an equal volume of DMSO were used as control. Each experiment was carried out in triplicates and the optimization for HRG used was accessed by Western blotting analysis.

II. Treatment with inhibitors

For the inhibitor treatment (with AG825, SB203580, PD98059 and LY294002), the BT474 cells (at 70~80% confluence) were fed with fresh medium, supplemented with different individual inhibitors at the indicated concentrations in Table 2-1, for 10 and 30 min. Cells were then harvested for Western blotting. For the cell treatment with both HRG and inhibitors, serum-starved cells were induced with HRG for 30 min, followed by treating with different inhibitors in the presence of HRG, or followed by inhibitor treatment after removing HRG, for 10 and 30 min. Cells treated with an equal volume of DMSO were used as control. Each treatment was performed in triplicates and each treatment was repeated thrice.

Inhibitors	Targeted Pathway	Amount used
AG825	HER-2/*neu* Tyrosine Kinase	100 µM
SB203580	p38 MAPK	40 µM
PD98059	MEK	10 µM
LY294002	PI3K	10 µM

Table 2-1. Amount of inhibitors used for different treatments.

III. Treatment with siRNA

The siGENOME SMARTpool® PP2A (M-003598), alpha isoform (siPP2A/Cα) (NM_002715) from Dharmacon, Inc. (Thermo Fisher Scientific, CO, USA), was used to specifically knock down the PP2A/C alpha gene in the breast cancer cell lines. Non-specific control pool from Dharmacon, Inc. was used as the scrambled siRNA control. 2 X 10^5 cells were seeded in a 6-well plate one day prior to transfection. For each well, the siRNA stock was diluted in the appropriate amount of Opti-MEM®I reduced serum medium and the cells were transfected with LipofectAMINE™ 2000 (Invitrogen, CA, USA), according to the manufacturer's instructions. Cells were incubated at 37°C in a CO_2 incubator for 24-72 h and the effect of gene silencing was analysed by immunoblots.

2.2.2 Protein extraction

I. Total cell lysates

To prepare cell lysates, cells were collected after trypsin treatment and washed three times with ice-cold phosphate-buffered saline (PBS, pH 7.4). Cells were then resuspended in cold RIPA buffer (1% Igepal, 1% sodium deoxycholate, 0.15 M sodium

chloride, 0.01 M sodium phosphate, pH 7.2, and 2 mM EDTA) supplemented with protease inhibitors (Roche Diagnostics, Indianapolis, IN) and phosphatase cocktail inhibitors I and II (1:100; Sigma-Aldrich, Steinheim, Germany), and kept on ice for 30 min. Cell lysates were then centrifuged at 4^{o}C and the supernatants were collected. Protein concentrations were determined using the Coomassie Plus Bradford assay (Pierce, Rockford, IL).

II. Fresh frozen tumour tissues

Frozen aliquots of pre-selected tumour tissues were immersed in liquid nitrogen and ground into powder form with a pestle and mortar. The cells were lysed in 250–350 µl of lysis buffer (Reagent 1) from the Sequential Extraction Kit (Bio-Rad, Hercules, CA), supplemented with 1U of DNase and protease inhibitor cocktails (Complete Mini, Roche). The sample mixtures were incubated on ice, vortexed vigorously every 10 min for 1 h and centrifuged for 20 min at 16 000 x g, 4°C to remove the cell debris. The supernatant was transferred into a new tube and 100% acetone was added to the supernatant at four times sample volume and incubated overnight at -20°C for protein precipitation. The precipitated proteins were washed two times with acetone: water (4:1) before 80-150 µl of sample solution containing 8 M Urea, 4% CHAPS, 60 mM DTT and 2% IPG buffer (pH 3-10) was added. The actual volume added being adjusted according to the pellet size, to dissolve the protein. The protein samples were kept at -80ºC until further use.

2.2.3 Immunoblotting /Western blotting

Proteins were separated on 10-12% SDS-PAGE gels using Mini-PROTEAN 3 Electrophoresis Cells (Bio-Rad, Hercules, CA, USA) at 70 V for 30 min and 85 V until the dye front reached the edge of the gel. The proteins were then transferred onto Hybond-P Polyvinylidene Fluoride (PVDF) membrane (GE Healthcare, Uppsala, Sweden) using the wet transfer apparatus (Bio-Rad, Hercules, CA) at 110V for 1hr. The membrane was blocked with 5% non-fat milk (Santa Cruz Biotechnology, Inc., CA, USA) in Tris-buffered saline containing 0.1% Tween-20 (TBS-T) and incubated overnight with respective antibodies to be tested at 4ºC. Secondary antibodies – the HRP-conjugated goat anti-mouse IgG (Molecular Probes, Invitrogen, Oregon, USA) at 1:5000 dilutions in TBS-T or HRP-conjugated goat anti-rabbit IgG (ZYMED® Laboratories Inc. San Francisco, CA, USA) at 1: 10 000 dilutions in TBS-T were applied for 1 h after washing off any unbound primary antibodies. The chemiluminescent signals were detected using SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL, USA). Signals were then captured with the MULTI GENIUS BioImaging System (Syngene, Frederick, MD, USA) and the signal intensities were analyzed using the GeneTools software (Syngene, Frederick, MD, USA). The same membrane was reprobed with antibody for β-actin to control for protein loading and transfer.

2.2.4 Human phospho-receptor tyrosine kinase (Phospho-RTK) array analysis

The serum-starved BT474 cells treated with HRG or with DMSO were rinsed with PBS before adding the NP-40 Lysis buffer. The lysates were gently rock at 2-8^{o}C for 30 min and microfuged at 14,000 x g for 5 min. The extracted proteins concentrations were

determined using the Coomassie Plus Bradford assay (Pierce, Rockford, IL, USA). 300 µg of extracted protein was used for the human phospho-receptor tyrosine kinase (Phospho-RTK) array (R&D Systems, Inc., Minneapolis, MN, USA) analysis according to the manufacturer's protocol. The array was blocked with Array Buffer 1 for 1 h on a rocking platform shaker followed by overnight incubation with the diluted lysates at 4^{o}C overnight. After washing, the anti-phospho-tyrosine-HRP conjugated antibody was applied on the array for 2 h at room temperature with gentle agitation. The chemiluminescent signals were then visualized using the SuperSignal® West Pico Chemiluminescent Substrate (Pierce, Rockford, IL, USA) and captured with the MULTI GENIUS Bio Imaging System (Syngene, Frederick, MD, USA). The signal intensity of each spot was analyzed using the GeneTools software (Syngene, Frederick, MD, USA).

2.2.5 Signal transduction antibody array analysis

The serum-starved BT474 cells were treated with HRG (3 nM) or with DMSO for 30 min and the total proteins were extracted. After the array was blocked with 5% non-fat milk in TBS-T for 1 h, equal amounts of proteins from both the HRG-treated and the DMSO-treated (control) cells were diluted to 1 mg/ml and then probed with the signal transduction antibody array (Hypromatrix, Inc., Worcester, MA, USA), respectively, for 2 h at room temperature, according to the manufacturer's instructions. The arrays were washed with TBS-T buffer and further incubated with HRP-conjugated anti-phosphotyrosine antibody (Hypromatrix, Inc., Worcester, MA, USA) for 2 h with gentle agitation. The chemiluminescent signals were then visualized using the SuperSignal® West Pico Chemiluminescent Substrate (Pierce, Rockford, IL, USA) and captured with

the MULTI GENIUS Bio Imaging System (Syngene, Frederick, MD, USA). The signal intensity of each spot was analyzed using the GeneTools software (Syngene, Frederick, MD, USA). The relative tyrosine phosphorylation level of each protein was interpreted as the ratio of the spot intensity in the HRG-treated sample to the spot intensity in the control sample.

2.2.6 Immunoprecipitation

For immunoprecipitation, 500 µg of proteins was immunoprecipitated with selective antibodies (anti-FADD, anti-ARC, and anti-Ezrin) at 4^0C and gently agitated overnight. Protein A-Sepharose or protein G-Sepharose (GE Healthcare, Uppsala, Sweden) was then added and the mixture incubated at 4^0C for 2 h. The agarose beads were collected, washed and resuspended in 50 µl of sample buffer containing 50 mM Tris-HCl, pH 7.6, 2% SDS, 10% glycerol, 10 mM DTT and 0.2% bromophenol blue. After boiling for 5 min, samples were cooled down on ice and analyzed by Western blotting as described in 2.2.3.

2.2.7 Two-dimensional electrophoresis and immunoblotting

Protein separation by 2-dimensional electrophoresis (2-DE) was carried out to examine the phosphorylation profile of protein of interest. Briefly, 50 µg of protein was mixed with 125 µl of rehydration buffer (7 M urea, 2% CHAPS, 3.5 mg DTT and 1% IPG buffer, pH 3-10 or pH 4-7) and separated using precast Immobiline IPG DryStrip (70 mm, pH 3-10 or pH 4-7, GE Healthcare, Uppsala, Sweden) with the Ettan™ IPGphor™ Isoelectric Focusing System (GE Healthcare, Uppsala, Sweden). Prior to SDS-PAGE, the

strips were then equilibrated for 20 min in equilibration buffer (50 mM Tris-HCl, pH 8.8, 30% glycerol, 7 M Urea, 2% SDS) containing DTT (100 mg per strip), followed by further equilibration with the same buffer with iodoacetamide (250 mg per strip). Protein transfer and immunoblotting were performed and signals were detected, as described in 2.2.3.

2.2.8 Immunohistochemistry and tissue microarray

A breast disease spectrum tissue array including 206 cases was purchased from US Biomax (Rockville, MD, USA). The array contained 32 metastatic carcinomas, 68 invasive ductal carcinomas, 22 each of lobular and intraductal carcinomas, 16 normal tissues, and 44 other non-malignant tissues. Immunohistochemical (IHC) staining of pY307-PP2A was conducted using the DAKO Envision system. Briefly, sections were deparaffinised, rehydrated, and the antigens unmasked using the DAKO® Target Retrieval Solution. Endogenous peroxidases were removed using 3% hydrogen peroxide, followed by incubating with anti-pY307-PP2A (1:25) antibody for 1 h at room temperature. The sections were further treated with labelled dextran polymer conjugated with HRP for 30 min and incubated with DAB$^+$-substrate chromogen solution for 5-10 min. Sections were counterstained with Mayer's hematoxylin and mounted. The degree of staining of pY307-PP2A was scored as 0 (negative to weak), 1 (moderate) and 2 (strong), according to the staining intensity of the tumour cells. Scoring was performed by two pathologists and supervisor, independently. Cases with discrepant scores were re-scored by the same or additional scorers, to obtain a consensus score, failing which the scores were excluded from the data analysis.

2.2.9 RNA extraction

RNA from both cell lines and tissue were extracted using NucleoSpin® RNA II (Macherey-Nagel GmbH & Co. KG, Germany) kit according to the manufacturer's protocol. 20 mg of frozen aliquots of pre-selected normal and tumour paired tissues were ground into powder form using a pestle and mortar in the presence of liquid nitrogen. $5x10^6$ cultured cells trypsinized from the culture flask were washed and pelleted. Lysis buffer RA1 containing β-mercaptoethanol was added to both tissue and cell lines sample followed by filtration. Cleared lysates were passed through the provided NucleoSpin® RNA II Column for RNA binding followed by DNA digestion. Purified RNA was eluted and its concentration was determined using Nanodrop Spectrophotometer ND-1000 (Thermo Fisher Scientific, Lafayette, CO, USA).

2.2.10 Reverse transcription polymerase chain reaction (RT-PCR) amplification

0.5 µg purified RNA template was used for first strand cDNA synthesis using RT-PCR System kit (Promega, WI, USA). Reverse transcription was performed at 42°C for 1 h, followed by 70°C for 15 min. Second strand cDNA synthesis and PCR amplification was carried out as showed in Table 2-2 using Thermal Cycler GeneAmp®PCR System 9600 (Applied Biosystems, CA, USA).

Steps	Temperature (°C)	Time
1. Initializing	94	5 min
2. Denaturation	94*	30 s
3. Annealing	60*	30 s
4. Extension/Elongation	72*	30 s

5. Final Elongation	72	7 min
6. Final Hold	4	∞

Table 2-2. PCR amplification steps. *30 cycles (Steps 2-4)

Primers used in the PCR were designed based on the Homo sapiens protein phosphatase 2A, catalytic subunit, alpha isoform, mRNA (PubMed accession no: NM_002715) using online Primer Quest Software (Integrated DNA Technologies, Inc.). The sequence for both forward and reverse primers were as followed:

Forward primer: 5'-TTG GTG GAT GGG CAG ATC TTC TGT-3'

Reverse primer: 5'-GGT GCT GGG TCA AAC TGC AAG AAA-3'

2.2.11 DNA agarose gel electrophoresis and DNA purification

1.5% DNA agarose gel (Seakem®LE Agarose, Cambrex Bio Science Rockland, Inc., Rockland, ME, USA) in TBE containing 1 µl of Ethidium Bromide (10 mg/ml) was casted and the DNA gel electrophoresis was carried out at 90 V for 40 min to visualize the full length of PP2A catalytic subunit cDNA. The cDNA gel band was excised and purified using QIAquick Gel Extraction Kit (QIAGEN, CA, USA) according to the manufacturer's protocol.

2.2.12 DNA sequencing

Purified cDNA was sequenced with the BigDye® Terminator v3.1 Cycle Sequencing Kit

(Applied Biosystems, CA, USA). Insertions, deletions and single base mutations causing amino acid replacement were analyzed by comparing the sequence on the Locus ID NM-002715 database.

2.2.13 Immunofluorescence and confocal microscopy

The cells were grown on glass coverslips and treated with siPP2A/Cα before fixation with 4% paraformaldehyde (Sigma-Aldrich, Steinheim, Germany) in PBS for 15 min. They were then washed with PBS and permeabilized with 0.1% Triton X-100 for 10 min. After a wash with 0.02% PBST, the cells were blocked with 3% BSA in PBST for 1 h followed by incubation with polyclonal rabbit-anti PP2A primary antibody (1 :50, Cell Signalling Technology®). The cells were then washed with 0.02% PBST followed by incubation with Alexa Fluor® 532 (1:1000, Invitrogen, Inc., Carlsbad, CA) for 1 h. Slides were mounted using the antifade mounting fluid containing DAPI and the images were visualized and captured using Olympus Fluoview FV500 (Olympus, Japan). Raw images were analyzed using the Olympus FV10-ASW Viewer Software (Olympus, Japan).

2.2.14 Biological assays

I. Tyrosine phosphatase assay

Protein tyrosine phosphatase activity was analyzed using the Tyrosine Phosphatase Assay System (Promega, WI, USA), according to the manufacturer's instructions. Both control and siRNA-treated cells were harvested and cell lysates were then passed through the Sephadex® G-25 resin spin column to remove endogenous phosphatase. The sample lysates collected were then incubated with or without the substrates, Tyrosine

Phosphopeptide-1 (PP-1) and -2 (PP-2). The reaction was carried out at 30^{o}C for 5 min, and then stopped by adding 50 ul Molybdate Dye/Additive mixture, and the tyrosine phosphatase activity was determined from its optical density at 620 nm.

II. Cell apoptosis TUNEL (terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling) assay

The effect of PP2A/C silencing on cell apoptosis was performed using the TUNEL assay according to the manufacturer's instructions. Both the control and siRNA-treated cells were suspended in PBS and adjusted to 3x10^{5} cells/ml for each slide preparation. The cells were then fixed in 4% paraformaldehyde (Sigma-Aldrich, MO, USA) for 25 min at 4^{o}C and permeabilized in 0.2% Triton X-100 for 10 min. The cells were then equilibrated at room temperature for 10 min followed by incubation with the Terminal Deoxynucleotidyl Transferase (rTdT) enzyme incorporated with Fluorescein-12-dUTP for 60 min at 37^{o}C. The reaction was terminated using 2xSSC for 15 min at room temperature. After the washing with PBS, the samples were mounted in VECTASHIELD®+DAPI (Vector Laboratories, Inc, CA, USA). The samples were then analyzed under a fluorescence microscope (Olympus Fluoview FV500 confocal microscope, Japan) with the filter set to view the green rTdT-labeled apoptotic cells at 531 nm and the blue DAPI-stained nuclei at 358 nm. Raw images were analyzed using the Olympus FV10-ASW 1.7 Viewer software (Olympus, Japan). The number of apoptotic fluorescent cells was calculated based on five randomly selected fields per sample.

III. Caspase 3 activity assay

Caspase 3 activity was assessed using the Caspase-Glo® 3/7 assay system (Promega, WI, USA), a homogenous, luminescent assay that measures both caspase 3 and 7 activities. Briefly, the cells were seeded in a white-walled 96-well plate and treated with siRNA. For each well, 100 µl of Caspase- Glo® 3/7 assay reagent was added and the mixture was gently mixed using a plate shaker at 400 rpm for 30 s and incubated at room temperature for 2 h. The wells containing culture medium served as assay blanks. Emitted luminescence from each sample was measured with the Tecan Infinite F200 plate-reading luminometer (Tecan Trading AG, Switzerland).

IV. Flow cytometric analysis

The siRNA treated cells were trypsinized and collected, followed by washing with ice-cold PBS (pH 7.4) prior to fixation with 70% ice-cold ethanol for 1 h at 4^{o}C. The cells were then suspended with 1 ml of Propidium Iodide/RNAase for 1 h at room temperature in the dark. The DNA content and distribution of cells in the various phases of cell cycle were analyzed using a flow cytometer (CyAn ADP Analyzer, Dako, Glostrup, Denmark). Cell cycle data analysis was performed by using the Summit™ software (Dako, Glostrup, Denmark).

2.2.15 Statistical analysis

The Student's *t*-test was used to compare the significance of relative phosphorylation of each signalling molecules between controls and treated samples, as well as between breast cancer cell lines and non-tumorigenic cell line; and the efficiency of siRNA

knockdown between the control and siRNA-treated cells. The difference of tyrosine-phosphorylated PP2A (pY307-PP2A) between different types of tissues (lymph node metastasis, malignant, non-malignant and normal tissues) was analyzed with Fisher's exact test. Two-sided $p < 0.05$ was considered as of significance. All the cell culture experiments were carried out in triplicate and repeated at least twice. All the cell-based functional assays were carried out at least 3 times and data were interpreted as mean ±SD as indicated. One-way analysis of variance or Student *t*-test was used to analyze the significance of differences. Two-tailed $p<0.05$ was considered significant and data are represented as means ± SD.

CHAPTER 3

HRG-ENHANCED HER-2/*neu* SIGNALLING

3.1 Overview

The HER-2/*neu*-encoded transmembrane protein is a member of the family of receptor tyrosine kinases (RTKs) (Reese and Slamon, 1997). The RTKs command central roles in several signal transduction pathways regulating cell proliferation, differentiation, and survival (Holbro *et al.*, 2003). The ligandless HER-2 receptor has an extracellular domain, a transmembrane domain and a C-terminal intracellular domain. Receptor dimerization of HER-2 with other RTK members (EGFR/HER-1, or HER-3) induces the activation of its downstream signalling by autophosphorylation of tyrosine residues at its C-terminal sites and recruitment of other docking molecules, such as Grb2, Stat and Shc. The key HER-2-regulated signalling pathways include the Ras/Raf/mitogen-activated protein kinase (MAPK) and phosphatidylinositol-3 kinase (PI3K) pathways, which are essential for cell proliferation and transformation (Grant *et al.*, 2002). However, the details of the signalling network regulated by HER-2/*neu*, remain largely unclear. Dissecting the exceedingly complex protein phosphorylation and dephosphorylation events involved within the tumour cell microenvironment remains an area of intense research.

Recently, a group of tyrosine-phosphorylated proteins regulated by HER-2/*neu* signalling have been identified using proteomics strategies (Mukherji *et al.*, 2006; Bose *et al*., 2006; Friedman *et al.*, 2007; Wolf-Yadlin *et al.*, 2006; Kumar *et al.*, 2007; Löbke *et al.*, 2008). By profiling the tyrosine-phosphorylated proteins in HER-2/*neu*-overexpressing breast

and ovarian cancer cell lines, Mukherji *et al.* found that the adaptor/docking proteins, kinases and other proteins involved in cell proliferation and migration were highly tyrosine-phosphorylated and treatment with Herceptin attenuated their phosphorylation (Mukherji *et al.*, 2006). In addition, several uncharacterized RNA binding proteins, e.g., SRRM2 and SFRS9, were also highly phosphorylated in the HER-2/*neu*-overexpressing cells (Mukherji *et al.*, 2006). When these cells were treated with HRG α1, an EGF-like growth factor, proteins such as paxillin, FAK and GIT1 were highly tyrosine-phosphorylated and the signal network involved in cell migration was amplified (Kumar *et al*., 2007). Bose *et al.* reported that inhibition of HER-2/*neu* signalling with the specific tyrosine kinase inhibitor, PD168393, led to the identification of several previously uncharacterized phosphoproteins, including Axl tyrosine kinase, the adaptor protein Fybs and the calcium-binding protein PDCD-6/Alg-2 (Bose *et al.*, 2006). Löbke *et al.* used protein microarrays to analyze the pathway activation after stimulation with epidermal growth factor and HRG, or its inhibition after blocking with Gefitinib or Herceptin, to characterize the distinct signalling properties of the different ErbB receptor subtypes (Löbke *et al.*, 2008). Thus, it is possible, with appropriate use of different complementary phosphoproteomics investigative methods, to dissect, identify and characterize the tyrosine-phosphorylated proteins in the HER-2-driven signal network and achieve a better understanding of the mechanisms underlying the cellular response. Profiling the tyrosine-phosphorylated proteome is the logical first step to elucidate the dynamic phosphorylation and dephosphorylation of a variety of proteins which constitute the complicated network controlling cellular behaviour under the influence of receptor tyrosine kinase signalling.

In this study, HRG as an activator of the HER-2/*neu* signalling pathway was used. HRG is a member of the epidermal growth factor (EGF)-like growth factor family. It modulates the cell proliferation and differentiation by stimulating the activation of the ErbB tyrosine kinase receptor (TKR) family, and thus, enhances the HER-2/*neu*-related signalling network. This HRG-induced heterodimerization of HER-2 with HER-3 is an example of the involvement of TKR heterodimerization in the development of human malignancies, at the molecular level (Wallasch *et al.*, 1995). Li Li *et al.* created and used the HRG-null mice to demonstrate that HRG is an important mammary gland mitogen regulating alveolar development and lactogenesis (Li *et al.*, 2002). To further understand the complex tyrosine-phosphorylation profiles of the interactive downstream proteins involved in the HER-2/*neu* signalling cascade, HER-2/*neu*-expressing BT474 cells were treated with HRG. Using an antibody array, a group of 80 signal modulators which are differentially tyrosine-phosphorylated by HRG-enhanced HER-2/*neu* signalling were identified. After undergoing post-translational modifications, these signal modulators are involved in multiple cellular functions such as apoptosis, signal transduction, transcription, cell cycle regulation and cell proliferation in the transformed malignant cells.

3.2 Results

3.2.1 HRG enhanced HER-2/*neu* phosphorylation in a dose- and time-dependent manner

Breast cancer cells from the HER-2/*neu*-overexpressing BT474 cell-line were treated with DMSO (to serve as controls) or HRG at 3 different concentrations of HRG at 0.3

nM, 3.0 nM, and 5.0 nM at 2 time points (10 and 30 minutes). Activation of HER-2/*neu* signalling was measured by the extent of its phosphorylation level via Western blotting. As shown in Figure 3-1 A, BT474 cells responded to HRG treatment at 3.0 nM at 30 min with a significant increase in the phosphorylation of HER-2/*neu*, compared to the control. The cells were further treated with 3.0 nM HRG for various longer time periods, to observe the prolonged pattern of HER-2/*neu* activation by HRG. In Figure 3-1 B, HER-2/*neu* activation was detected at 5 min post-HRG treatment, showing a gradual increase at 10 min and 30 min, when it peaked, followed by a drop of phosphorylation in 60 min. Therefore, for the subsequent experiments, the optimized condition of treating the cells at 3.0 nM HRG for 30 minutes was applied.

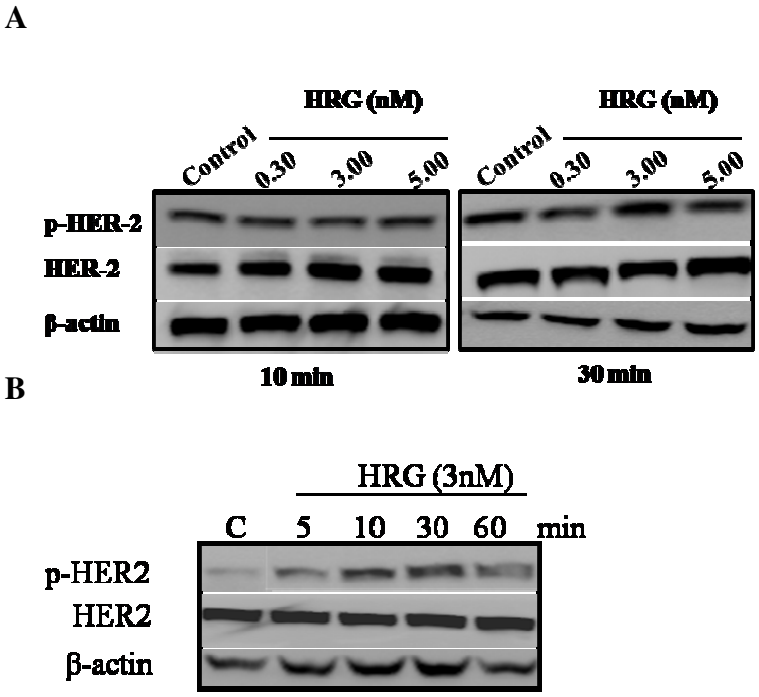


Figure 3-1. HRG stimulation of HER-2/*neu* activation. A. BT474 breast cancer cells at 70-80% confluence were serum-starved for 20 h and then treated with different amount of HRG or DMSO (control) for 10 minutes and 30 minutes. Cells were harvested and phosphorylation levels were determined by Western blotting with their respective antibodies as described in Materials and Methods. HER-2/*neu* was activated by HRG in a dose- and time-dependent manner. B. Activation of HER-2/*neu* increased from 5 min onwards and peaked at 30 min, followed by attenuation in phosphorylation after 60 min.

3.2.2 HRG is a specific activator of EGFR, HER-2 and HER-3 tyrosine phosphoryation but not other receptor tyrosine kinases

A human phospho-RTK array was employed to identify the relative levels of phosphorylation of 42 different RTKs (see Appendices: Table 1). In this array, capture and control antibodies had been spotted, in duplicate, on the nitrocellulose membrane. Cell lysates were first diluted and then incubated with the array. After binding the extracellular domains of both phosphorylated and unphosphorylated RTKs, unbound materials were washed away. A pan anti-phospho-tyrosine antibody conjugated with horseradish peroxidase was then used to detect the chemiluminescence signals released by the phosphorylated tyrosine residues of specific, activated receptors. As shown in Figure 3-2, BT474 cells were treated with HRG to induce the tyrosine phosphorylation of the ErbB receptors and the phosphorylation pattern was analyzed using the above-described human phospho-RTK array. Phosphorylation of EGFR (or ErbB1) and ErbB3 (or HER-3) were slightly increased upon HRG treatment compared to the control, whereas phosphorylation of HER-2 (or ErbB2) was significantly enhanced (more than 2-fold) upon HRG treatment as compared to the control. No other RTKs included in the array were phosphorylated by HRG treatment. Therefore, HRG is a specific ligand for the ErbB family RTKs, involved in the specific activation of the EGFR/HER-1, HER-2 and HER-3 receptors only.

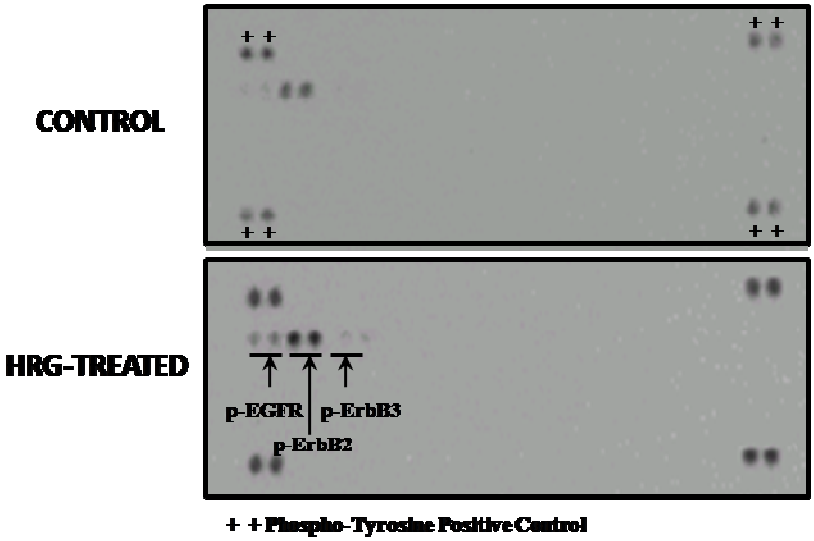


Figure 3-2. HRG is specific in its activation of EGFR, HER-2 and HER-3, as shown by the Human Phospho-RTK array. BT474 breast cancer cells at 70-80% confluence were serum-starved for 20 h and then treated with HRG (3 nM) or DMSO (control) for 30 minutes. Cells were harvested and subjected to human phospho-RTK array analysis as described in Materials and Methods. HRG specifically stimulated the tyrosine phosphorylation of EGFR/HER-1, HER-2/ErbB2 and HER-3/ErbB3 only.

3.2.3 HRG stimulates phosphorylation of the HER-2 receptor/ErbB2 and its downstream interacting signalling molecules

To examine whether the downstream interactive partners of the HER-2/*neu* signalling cascade were also activated by HRG, Western blot analysis on the known and well studied downstream signalling molecules of the HER-2/*neu*-linked signalling cascade such as the AKT and ERK1/2 pathways were performed. Treatment of BT474 breast cancer cells with HRG showed that phosphorylation of HER-2 as well as its key downstream modulators, AKT and ERK ½ were time dependent. As shown in Figure 3-3, when the cells were treated with HRG for 30 min, phosphorylation of HER-2, ERK1/2 and AKT were highly enhanced, as determined by Western blotting with their respective phospho-specific antibodies.

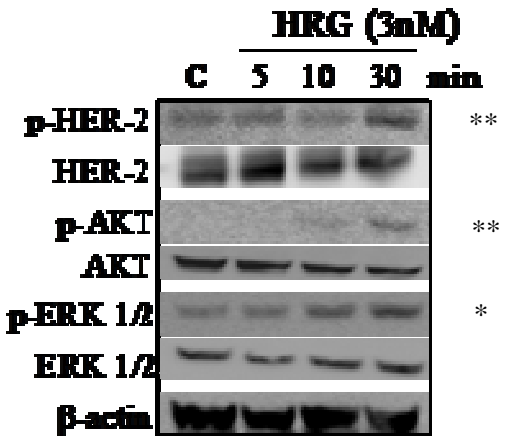


Figure 3-3. HRG stimulated phosphorylation of HER-2 and its downstream interacting signalling molecules. BT474 breast cancer cells at 70-80% confluence were serum-starved for 20 h and then treated with HRG (3 nM) or DMSO (control) for 5 minutes, 10 minutes and 30 minutes. Cells were harvested and phosphorylation of HER-2, AKT and ERK1/2 were determined by Western blotting with their respective antibodies, as described in Materials and Methods. The relative phosphorylation levels of HER-2, ERK1/2 and AKT were normalized to their respective basal levels. Significant differences of treatment effects with respective controls at different time intervals were analyzed using the Student t-test. * $p<0.05$, ** $p<0.01$. Data represent average of triplicate experiments.

3.2.4 Differential tyrosine phosphorylation profiles between the HRG-treated and DMSO-treated (control) BT474 cells, derived using signal transduction antibody arrays

To investigate the tyrosine-phosphorylated molecules regulated by HER-2/*neu* signalling, the BT474 cells were treated with HRG or DMSO (as control) for 30 min and signal transduction antibody arrays were used to study the changes of tyrosine phosphorylation of different signalling modulators. The signal transduction antibody array contains 400 antibodies (see Appendices: Table 2) that are immobilized on a membrane, each at a predetermined position. These antibodies recognize and capture the corresponding specific antigen-associated proteins. The proteins captured are then detected by immunoblotting. This array is especially suited for screening protein-protein interactions

and detecting protein tyrosine phosphorylation. Figure 3-4 A shows the differential tyrosine phosphorylation patterns on the filter between the HRG-treated and control BT474 cells. 80 of the 400 arrayed molecules (20%) were found to show significant differences in the spot intensities of treated samples *vs.* controls. The tyrosine-phosphorylated and dephosphorylated proteins identified are listed in Table 3-1.

Functional analysis (Gene Ontology) showed that these proteins are mainly involved in cell apoptosis (25%), signal transduction (25%), and transcriptional activity (16%) (Figure 3-4. B). Not surprisingly, several known HER-2/*neu*-regulated proteins, e.g. Src homology 2 domain-containing transforming protein (SHC), Stat-1 transcription factor, Src-related protein tyrosine kinases (Syk), ErbB2, and AKT, were found to be highly tyrosine-phosphorylated after HRG treatment, which further confirms the efficiency of the antibody array in dissecting the tyrosine-phosphorylated protein profiles. Most importantly, a group of proteins whose tyrosine phosphorylations have not been previously linked to HER-2/*neu* signalling was also reported. For example, cell apoptosis-related proteins such as apoptosis repressor with CARD domain protein (ARC) and FAS-associated death domain protein (FADD), the cell motility-associated protein villin 2 (Ezrin), and protein phosphatase type 2A (PP2A), were highly tyrosine-phosphorylated in the HRG-treated BT474 cells. Interestingly, only 2 proteins, p-EGFR and CD3 epsilon (Figure 3-4 A, located at K4 and C7) were found to be highly dephosphorylated under the HRG-enhanced HER-2/*neu* signalling. Collectively, a large number of HER-2-regulated tyrosine-phosphorylated and -dephosphorylated signal proteins, including several novel signal modulators, as listed in Table 3-1, were

identified.

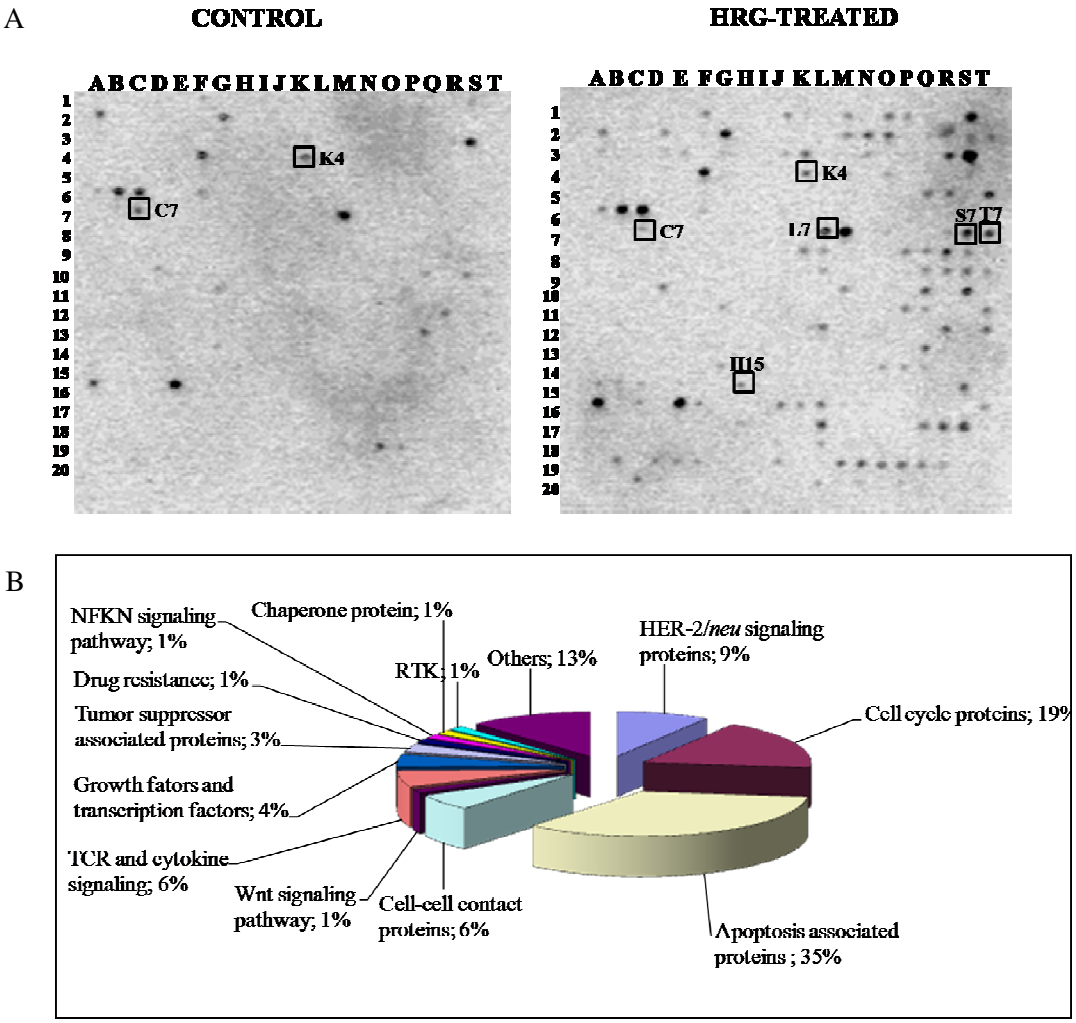


Figure 3-4. Differential tyrosine phosphorylation profiles between the HRG-treated and DMSO-treated (control) BT474 cells, detected on signal transduction antibody arrays. A: Representative filters showing the differential tyrosine phosphorylation profiles. Serum-starved BT474 cells (70-80% confluence) were treated with HRG or DMSO for 30 min and equal amounts of proteins were separately incubated with two signal transduction arrays. An anti-phosphotyrosine antibody conjugated to horseradish peroxidase (HRP) was used to detect phosphorylated tyrosine residues of specific proteins. Signals were captured with the MULTI GENIUS Bio Imaging System (Syngene) and the signal intensity of each spot was analyzed using the Syngene GeneTools software. The relative tyrosine phosphorylation level of each protein was interpreted as the ratio of spot intensity in the HRG-treated sample to the corresponding spot intensity in the control sample. Spots K4 and C7 represent p-EGFR and CD3 epsilon with reduced phosphotyrosine signals in the HRG-treated BT474 cells. Other spots (L7: HER2; S7: Ezrin; T7: FADD and H15: PP2A) show the increased phosphotyrosine signals in the HRG-treated BT474 cells. Underlined spots represent the proteins involved

in cell apoptosis. B: Grouping of identified tyrosine phosphorylated and –dephosphorylated molecules. Only the proteins with a ratio of at least 1.5-fold difference or only detected in either of the two filters were selected and functionally grouped in the pie chart.

Table 3-1. Identified tyrosine-phosphorylated proteins from HRG-treated BT474 cells. Relative tyrosine phosphorylation was expressed as the ratio of spot intensity on filter probed with the HRG-treated samples to that on the filter treated with control sample. Only the proteins with a ratio of >1.5-fold increase (or decrease) or only those detected on the filter probed with HRG-treated samples (or on the filter probed with control sample) were considered as increased (or decreased) in their tyrosine phosphorylations.

Functional groups	Proteins	Location on arrays	Tyrosine phosphorylation level	Functional groups	Proteins	Location on arrays	Tyrosine phosphorylation level
Known HER2 signalling proteins	erbB2	L7	**Increased**	**Cell cycle and cell proliferation**	CUL-1	T5	**Increased**
	PI3K p85	E15	**Increased**		Cyclin D3	C6	**Increased**
	AKT 1/2	F1	**Increased**		GADD45	R8	**Increased**
	SHC	L17	**Increased**		GADD153	S8	**Increased**
	Stat1	L18	**Increased**		P45 skp2	G14	**Increased**
	Syk	B19	**Increased**	**Signal transduction-related proteins**	HCAM	K3	**Increased**
	Crk	R5	**Increased**		CD3 epsilon	K4	**Decreased**
Apoptosis and anti-apoptosis related proteins	BOK	D2	**Increased**		CD28	M4	**Increased**
	Bcl-2	G2	**Increased**		Crk	R5	**Increased**
	BID	N2	**Increased**		EphA4	I7	**Increased**
	Bim	O2	**Increased**		Eps8	K7	**Increased**
	BAP1	R2	**Increased**		p-EGFR	C7	**Decreased**
	Caspase3	R3	**Increased**		Frizzled	P8	**Increased**
	Caspase4	S3	**Increased**		HS1	Q9	**Increased**
	CD40	O4	**Increased**		IRAK	Q10	**Increased**
	FADD	T7	**Increased**		JNK1,2,3	E11	**Increased**
	FLASH	K8	**Increased**		p-c-Raf-1	E16	**Increased**
	FLIPs/1	L8	**Increased**		Rap2	J16	**Increased**
	GADD34	Q8	**Increased**		RXR a,b,r	L16	**Increased**
	raidd	F16	**Increased**		TCR α	E19	**Increased**
	PAR-4	P14	**Increased**		TCR β	F19	**Increased**
	SODD	S17	**Increased**		Patched	O14	**Increased**

	Apaf1	M1	**Increased**		PDGF R	A15	**Increased**
	TNFR1	M19	**Increased**		Rab1	S15	**Increased**
	TNFR2	N19	**Increased**		TANK	D19	**Increased**
	TRADD	P19	**Increased**		Throid R a1	J19	**Increased**
	ARC	O1	**Increased**				
	TRAF1	Q19	**Increased**	**Cell adhesion-related proteins**	VCAM-1	N3	**Increased**
	TRAF2	R19	**Increased**		Bcl-6	M2	**Increased**
	TRAIL	C20	**Increased**		Intergrin α5	M10	**Increased**
Transcription-related proteins	ATF-2	P1	**Increased**	**Immune response and cytokine regulation**	IL1R1	D10	**Increased**
	Elongin A	G7	**Increased**		IRF2	S10	**Increased**
	HDAC1	L9	**Increased**		SOCS-1	R17	**Increased**
	ICSBP	S9	**Increased**	**Tumour suppressors**	Maspin	P11	**Increased**
	Id1	T9	**Increased**		PP2A,2B	H15	**Increased**
	Max	Q11	**Increased**	**DNA repair and drug resistant proteins**	Rad52	C16	**Increased**
	MyoD	L12	**Increased**		Mdr	T11	**Increased**
	NF kappaB p65	T12	**Increased**	**Cytoskeleton reorganization proteins**			
	RACK1	A16	**Increased**		Ezrin	S7	**Increased**
	Rar R	K16	**Increased**		B7-1	S1	**Increased**
	c-Rel	T16	**Increased**				
	Smad4	Q17	**Increased**				
	VDR	L20	**Increased**				

3.2.5 Validation of antibody array results

To confirm the HRG-enhanced tyrosine phosphorylation of the signal proteins identified by the antibody array, the BT474 cells were treated with HRG for 30 min and the tyrosine phosphorylated levels of 2 proteins (Shc and Stat1) that are previously known to be directly regulated by HER-2/*neu* signalling, and 4 other proteins (FADD, ARC, Ezrin and PP2A) that had not been previously reported to be associated with HER-2/*neu* signalling, were analysed by immunoprecitation and Western blotting. As shown in Figure 3-5 A, HRG significantly enhanced phosphorylation of Shc and Stat1, two important mediators in the HER-2/*neu* signalling pathway (Dankort *et al.*, 2001) , relative to that observed in the control cells treated with DMSO. Similarly, tyrosine phosphorylation of PP2A, Ezrin, ARC and FADD were highly increased after HRG treatment (Figure 3-5 B). 2-dimensional electrophoresis (2-DE) was employed to examine the post-modification patterns of these signal modulators upon treatment. Immunoblot analysis of these 4 proteins after 2-DE separation revealed additional multi-spots with increased intensities, appearing on the acidic side of the main spots of PP2A, Ezrin, FADD and ARC in the HRG-treated cells (Figure 3-5 C), giving further evidence of phosphorylation modification of these proteins under HRG stimulation.

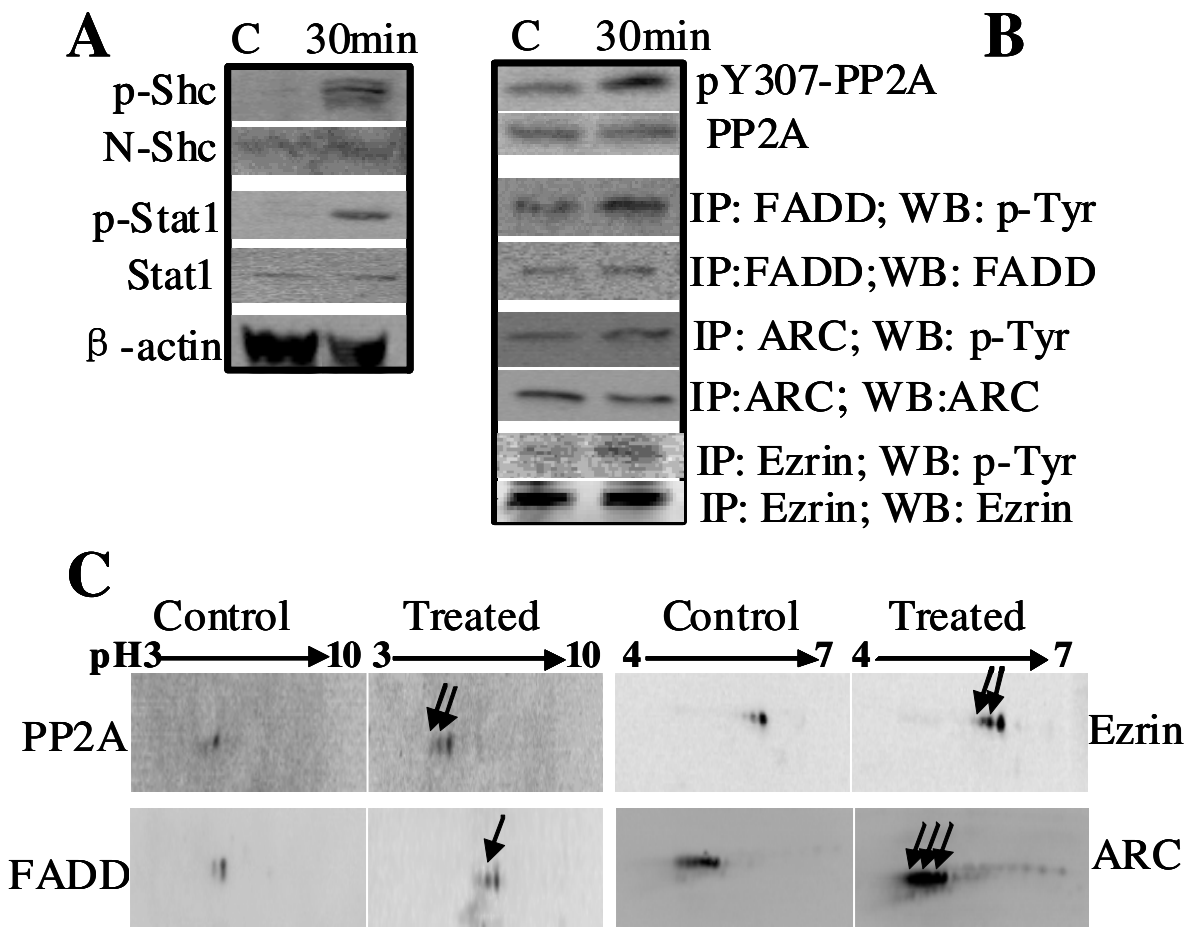


Figure 3-5. Validation of differential tyrosine phosphorylation of identified proteins by Western blotting. A: Known proteins associated with HER2/*neu* signalling. B: Novel tyrosine-phosphorylated proteins under HER2/*neu* signalling. BT474 breast cancer cells (70-80% confluence) were treated with HRG (3 nM) or DMSO for 30 minutes, and equal amount of proteins were used for Western blotting, as described in Materials and Methods. The levels of proteins and their tyrosine-phosphorylated forms were detected with their respective specific antibodies. For proteins (FADD, ARC and Ezrin) without specific phosphotyrosine antibodies, immunoprecipitation (IP) was carried out with the primary antibody and protein A-Sepharose or protein G-Sepharose. The protein complexes were separated and the tyrosine phosphorylation was detected with an anti-phosphotyrosine antibody. C: 2-dimensional electrophoresis and immunoblot. Equal amounts of proteins (50 μg) from both HRG-treated and control BT474 cells were separated with IPG strips (70 mm, pH 3 to 10 or pH 4 to 7), followed by SDS-PAGE. Protein spots were detected with their respective antibodies and visualized with a chemiluminescent substrate, as described in Materials and Methods. Arrows indicate the enhancement of multi-spots in HRG-treat BT474 cells. *IP*: immunoprecipitation; *WB*, Western blotting.

3.3 Discussion

Tyrosine phosphorylation is one of the central regulatory mechanisms of signal transduction in cancer progression. Proteins transiently or stably phosphorylated at tyrosine residues constitute the tyrosine phosphoproteome, a complex network leading to malignant transformation and disease. In breast cancer, the HER-2/*neu* oncogene modulates cell survival and apoptosis by regulating its downstream signalling cascade of interlinked pathways, including those involving PI3K/AKT, Raf/MEK1/ERK and p38 MAPK. To fully inventorize the tyrosine phosphoproteins involved in the HER-2/*neu* signalling network, phosphoproteomic profiles have been compiled using a variety of methods based on the SILAC, IMAC/Nano-LC-MS/MS and antibody microarray platforms and novel tyrosine phosphoproteins have been identified (Mukherji *et al*., 2006; Bose *et al.*, 2006; Gembitsky *et al.*, 2004). These investigations were designed to support the concept that dissecting the changes in tyrosine phosphorylation of signal molecules would represent a fundamental task to elucidate the biologic/biochemical basis of disease progression and to develop therapeutic drugs targeting specific tyrosine-phosphoproteins. Findings from some of these studies have provided the proof-of-concept sought.

In this study, the HER-2-driven tyrosine phosphorylation profiles of the key signal modulators involved in various cellular functions were investigated using the TransSignalling antibody array (Hypromatrix), and 78 tyrosine-phosphorylated proteins and 2 tyrosine-dephosphorylated proteins, tyrosine phosphorylation of which were stimulated or suppressed following HRG-enhanced HER-2/*neu* signalling were

identified. These differentially phosphorylated molecules are involved in diverse essential cell functions (apoptosis, transcription, cell adhesion, etc., as listed in Table 3-1). In addition to specific downstream proteins (e.g., Shc, Stat1 and AKT) that were previously known to be upregulated by HER-2/*neu* signalling, most of the apoptosis-related proteins were also found to be tyrosine phosphorylated when the BT474 cells were treated with HRG. For example, the HER-2-driven tyrosine phosphorylation of Fas-associated Death Domain (FADD) showed significantly enhanced HER-2-linked phosphorylation, as verified by Western blotting (Figure 3-5 B), a first-time finding. FADD was originally identified as a mediator of apoptosis upon activation of the death receptor protein Fas. In addition to this well-characterized proapoptotic signalling cascade, the nonapoptotic function of FADD had been reported earlier (Park *et al.*, 2005). Matsuyoshi *et al*. found that FADD phosphorylation at its Ser194 residue was regulated by the c-jun N-terminal kinase (JNK), affected cell cycle arrest and inhibited cell invasion (Matsuyoshi *et al.*, 2006), but whether tyrosine phosphorylation of FADD inactivated its apoptotic activity remains unclear. More recently, Shackleton *et al.* reported an unexpected role for FADD in modulating mammary epithelial cell survival after the establishment of lactation in transgenic mice that overexpressed the truncated FADD protein (Shackleton *et al.*, 2009), however, the mechanism of how FADD regulates the cell survival was not worked out. Mapping the tyrosine phosphorylation sites and elucidating its role in cancer cells would be critical to understanding the cross-talk between HER-2/*neu* signalling and related intracellular apoptotic signals.

The Apoptosis Repressor with CARD Domain (ARC) and ezrin were first time reported

to be tyrosine-phosphorylated by the HER-2-driven signalling in BT474 breast cancer cells (Figure 3-5 B). ARC is reported to be highly expressed primarily in brain and myogenic tissues (Koseki *et al.*, 1998). When cells are triggered by stimuli such as hydrogen peroxide, hypoxia and the Fas ligand, ARC appears to assume a protective role, inhibiting the cells from undergoing apoptosis (Ekhterae *et al.*, 1999; Hong *et al.*, 2003). Mercier *et al.* reported the presence of ARC in multiple colon cancer cell lines as well as primary human colon adenocarcinomas, which indicates that ARC may be a novel marker of human colon cancer (Mercier *et al.*, 2008). An abundance of ARC protein was also detected in the epithelium of primary human breast cancers compared to that of benign breast tissue and conferred chemo- and radio-resistance (Mercier *et al.*, 2005; Wang *et al.*, 2005). However, little is known about the mechanism by which HER-2/*neu* signalling regulates tyrosine phophorylation of ARC in cancer cells.

Ezrin, an important member of the ezrin-radixin-moesin family involved in cell surface structures (Bretscher, 1999), has been shown to regulate the PI3K/AKT pathway and promote cell survival (Gautreau *et al.*, 1999). Li *et al.* demonstrated that ezrin shRNA specifically inhibits ezrin expression of MDA-MB231 cells with high metastasis potential and deregulates several functions such as cell motility and invasion indicating that ezrin may play a role in the development of human breast cancer cells through regulating the activation of Src kinases (Li *et al.*, 2008). A recent immunohistochemical study showed that phosphorylated ezrin was mostly found in HER-2/*neu*-positive breast tumours (Sarrió *et al.*, 2006). Data from this study provide further support that HER-2-driven phosphorylation of ezrin may contribute to the metastatic potential of breast cancer.

Most intriguingly, PP2A, a major protein serine/threonine phosphatase that participates in many signalling pathways in mammalian cells (Janssens and Goris, 2001), was demonstrated to be intrinsically involved in the HER-2 signalling cascade. PP2A, which appears to be critically involved in cellular growth control and potentially in the development of cancer (Schönthal, 2001), was found to be highly phosphorylated at the tyrosine 307 residue of its catalytic subunit in the HRG-treated BT474 cells (Figure 3-5 B). PP2A modulates cell survival and apoptosis and tyrosine phosphorylation of PP2A results in inactivation of its phosphatase activity (Chen *et al.*, 1992; Chen *et al.*, 1994) and loss of its function as a tumour suppressor (Ruediger *et al.*, 2001). Despite the full recognition of the importance of tyrosine phosphorylation in regulating its activity, the molecular mechanism of HER-2-triggered tyrosine phosphorylation of PP2A remains unknown.

In summary, the tyrosine phosphoproteomics study with the antibody arrays identified numerous novel signal modulators that are highly phosphorylated (78 of 80) or dephosphorylated (2 of 80), in response to HER-2/*neu* signalling activation. These molecules constitute a group of potential targets in the development of novel treatment regimes to arrest tumour progression in HER-2/*neu*-positive breast cancer cells by modulating their tyrosine phosphorylation status.

CHAPTER 4

REGULATION OF PP2A BY HER-2/*neu*

4.1 Overview

Many cellular processes, including cell cycle progression, growth factor signalling and cell transformation, is regulated by protein phosphorylation. Reversible phosphorylation of proteins by kinases and phosphatases is a key regulatory mechanism in the control of cell survival to cell death. PP2A has been shown to have a tumour suppressive function as a mutation deletion was found in human lung, breast, colon cancers as well as melanoma (Ruediger *et al.*, 2001). It is a heterotrimer consisting of a 36-kDa catalytic subunit (PP2A/C), a 65-kDa structural A subunit (PP2A/A), and a variable regulatory subunit ranging in size from 50 to 130 kDa (PP2A/B). The dynamic combinations of regulatory B subunit assembly determine the specificity and regulation of PP2A (Janssens and Goris, 2001). The findings of tyrosine phosphorylation of PP2A in response to growth stimulation and v-scr transformation of fibroblasts provide strong evidence of PP2A activity being acutely regulated by tyrosine phosphorylation (Chen *et al.*, 1994). However, much remains to be discovered about the modulation and physiological significance of PP2A regulation and functions *in vivo.*

In 1992, D.L. Brautigan's group published their findings in *Science* showing that an *in vitro* study, several receptor and non-receptor tyrosine kinases phosphorylated the catalytic subunit of PP2A in a stoichiometric reaction at pY307 (Chen *et al.*, 1992). Thus, the phosphorylation of the catalytic subunit seems to be under the regulation of PP2A. In 1994, the same group analyzed phosphorylation of PP2A in whole cell lysates using two-

dimensional gel electrophoresis and immunoblotting. This data further support their hypothesis that the activity of PP2A is regulated by tyrosine phosphorylation in intact cells. PP2A thus appears to function as a growth suppressor and its activity is negatively regulated by oncogenic signals (Chen *et al.*, 1994). It was then reported that PP2A activity was higher in ER-positive breast cancer cell lines than in ER-negative cell-lines (Gopalakrishna *et al.*, 1999). In their experiments, PKC-phosphorylated histone H1 was used as a substrate for the PP2A assay. The authors also showed that the higher levels of PP2A in ER-positive cell lines inversely correlated with the PKC activity profiles.

A new role of PP2A was proposed in a study of adenovirus type 5 E1A-mediated sensitization to anticancer drug-induced apoptosis, which showed that upregulation of PP2A/C expression results in repression of AKT activation and upregulation of p38 activation (Liao and Hung, 2004). The study was well designed and used both siRNA technology and commercially available inhibitors of PP2A as tools to study the mechanism of action of PP2A. Interestingly, PP2A may also be involved in TNF-α-induced PARP cleavage and apoptosis (Liao and Hung, 2004). Another kinase that plays a pivotal role in breast cancer progression is the mitogen-activated protein kinase (MAPK). It was reported that increased PP2A and reduced phospho-ERK1/2 were detected when the anti-carcinogenic dietary fatty acid, conjugated linoleic acid (CLA), was used on breast cancer cells (Miglietta *et al.*, 2006). This suggested that PP2A may play a role in the deactivation of ERK1/2, a cell survival signal modulator.

Protein phosphorylation of PP2A at pY307 regulates its phosphatase activity. Thus far,

there is little data linking the phosphorylation status of PP2A with breast cancer progression. Although oncogenes such as HER2/*neu* are detected in 20-30% of breast cancer patients, the causal relationship between PP2A and oncogenic HER2/*neu* has, however, not yet been reported and thus, it is important to focus on investigating the interactive roles between these two signal transducers.

As mentioned in Chapter 3 (page 62), PP2A was found to be highly tyrosine-phosphorylated at pY307 in a time-dependent manner when HER-2/*neu* signalling was enhanced in the HRG-treated BT474 breast cancer cells. To further verify the role of HER-2/*neu* signalling in the regulation of PP2A phosphorylation, the HER-2/*neu* signalling was blocked by treating BT474 cells with tyrphostin AG825, a specific HER-2/*neu* kinase inhibitor (Tsai *et al.*, 1996). The conceptual role of HER-2/*neu* signalling in modulating tyrosine phosphorylation of PP2A was supported by the finding of PP2A as one of the identified signal modulators linked to HER-2/*neu* activation, from the signal transduction array results (see Chapter 3, page 67). As reported in the next section, further inhibition studies with various specific inhibitors showed that the PI3K/AKT and Raf/Ras/MEK pathways positively regulate, whereas the p38 MAPK pathway negatively regulates, the tyrosine phosphorylation of PP2A.

4.2 Results

4.2.1 Inhibition of HER-2/*neu* signalling using AG825

BT474 cells were treated with AG825, a selective ATP-competitive inhibitor of the tyrosine kinase activity of HER-2/*neu*. In cell-free assays, AG825 was shown to inhibit HER-2/*neu*, EGFR, and PDGFR autophosphorylation, with IC_{50} values of 0.35, 19, and 40 μM, respectively (Reese and Slamon, 1997). In this study, optimization of the AG825 concentration and optimal time treatment to obtain maximal inhibition of the tyrosine kinase activity was carried out with three different concentrations of AG825 at 5 μM, 50 μM and 100 μM, and at three different time points of 5, 10 and 30 minutes, as shown in Figure 4-1. The tyrosine kinase activity was measured by its (tyrosine-) phosphorylation level using Western blotting. These results showed that the phosphorylation level of HER-2/*neu* was significantly decreased with 100 μM of AG825 treatment after 10 min ($p<0.01$) and 30 min ($p<0.05$) of incubation. Thereafter, this optimized condition was used to examine the effects of HER-2/*neu* signalling blockage on PP2A phosphorylation.

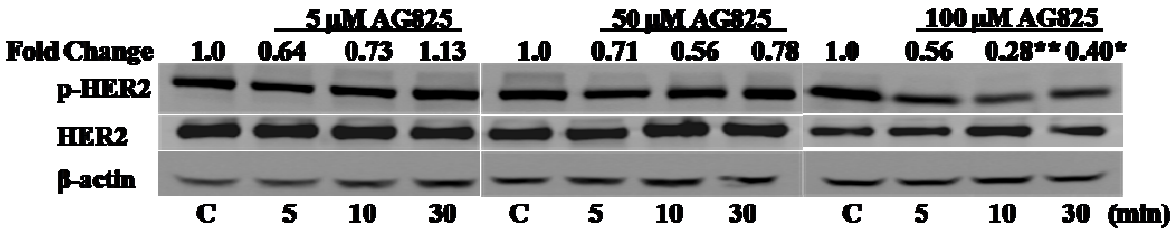


Figure 4-1. Inhibition of HER-2/*neu* activation by AG825. BT474 breast cancer cells at 70-80% confluence were serum-starved for 20 h and then treated with different amounts of AG825 or DMSO (control) for 5 min, 10 min and 30 min. Cells were harvested and phosphorylation levels were determined by Western blotting with the respective antibody, as described in Materials and Methods (page 49). HER-2/*neu* phosphorylation was inhibited by AG825 in a dose- and time-dependent manner. Maximal suppression of HER-2/*neu* phosphorylation, which should have been observed upon HER-2/*neu* activation in the absence of inhibitors, was observed after addition of 100 μM of AG825, and after 10 min and 30 min treatment. Analysis of the significant differences of treatment *versus* control results at different time intervals was by the Student t-test. * $p<0.05$, ** $p<0.01$. Data shown represent the average of triplicate experiments.

4.2.2 HER2/*neu* signalling regulates tyrosine phosphorylation of PP2A

To further verify the role of HER-2/*neu* signalling in the regulation of PP2A phosphorylation, the phosphorylation of PP2A was examined by blocking the HER-2/*neu* signalling using AG825. BT474 cells were treated with both HRG to stimulate the activation of HER-2/*neu* signalling and AG825 to inhibit its activation, and examined the phosphorylation at pY307 of PP2A by Western blotting. As shown in Figure 4-2 A, activation of HER-2/*neu* by HRG stimulated the activation of PP2A phosphorylation. When the HER-2/*neu* phosphorylation was inhibited using AG825, just as the phosphorylation of HER-2/*neu* was reduced in cells treated with AG825, pY307 of PP2A was decreased in the same way (Figure 4-2 B). Following that, the cells were treated with HRG and followed by AG825 to examine whether phosphorylation of pY307-PP2A was regulated by HER-2/*neu* activation. When the serum-starved BT474 cells were treated

with HRG for 30 min, followed by treatment with AG825 with HRG (Figure 4-2 C, lanes 2-5) or without HRG (lanes 6-7), the HRG-enhanced phosphorylation of PP2A was significantly suppressed by AG825, after 10 min (lane 3) and after 30 min (lane 4) and the effect was reversed at the 60 min treatment time (lane 5). These results demonstrated the regulatory role of HER-2/*neu* signalling in tyrosine phosphorylation of PP2A.

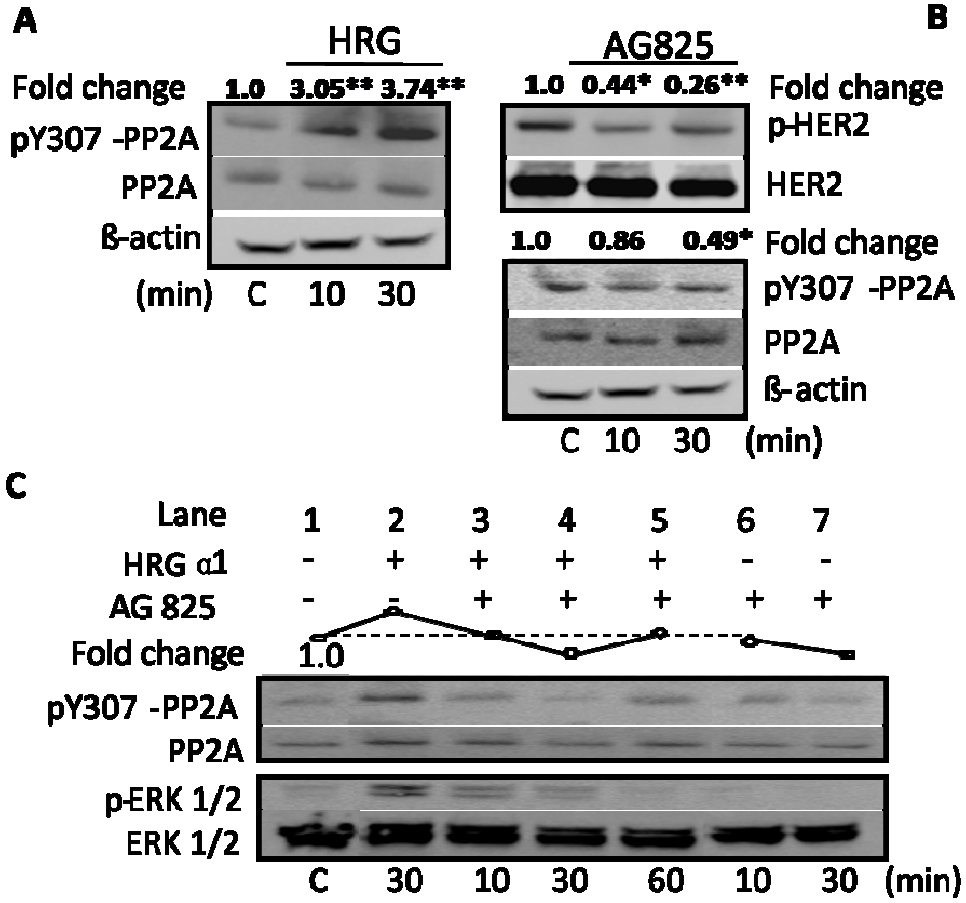


Figure 4-2. Regulation of tyrosine phosphorylation (pY307) of PP2A by HER2/*neu* signalling. A: Time-dependent tyrosine phosphorylation of PP2A by HRG treatment. Serum-starved BT474 cells were treated with HRG for 10 and 30 min and the levels of PP2A and its tyrosine-phosphorylated form pY307-PP2A were detected by Western blotting. B: Reduction of pY307 of PP2A by HER-2/*neu* kinase inhibitor AG825. BT474 cells at 70-80% confluence were treated with AG825 (100 μM) for 10 and 30 min and the phospho-HER-2, HER-2, PP2A and pY307-PP2A were detected with their respective antibodies by Western blotting. C: Attenuation of pY307 of PP2A by AG825 in the HRG-treated BT474 cells. Serum-starved BT474 cells were treated with HRG α1 for 30 min, and inhibitor AG825 (100 μM) was added to the cultures for further treatment for

10, 30 and 60 min (lanes 3-5), or the HRG was removed and fresh medium with AG825 (100 μM) was added to treat the cells for 10 and 30 min (lanes 6-7). Cells were harvested and both PP2A and pY307-PP2A were detected by Western blotting. The levels of ERK1/2 and phosphor-ERK were assayed to indicate the AG825-inhibited HER-2/*neu* signalling. The relative tyrosine phosphorylation levels of HER-2/*neu* and PP2A were normalized with its basal level (p-HER-2/HER-2, pY307-PP2A/PP2A), respectively. The fold change of relative phosphorylation was interpreted as the ratio of the relative phosphorylation level of the experimental cultures *vs* that in the control culture which is arbitrarily assigned as 1. Data shown represent the average of triplicate experiments. The Student *t*-test was used for the analysis of significance. * $p<0.05$, ** $p<0.01$

4.2.3 Inhibition of PI3K/AKT, MEK/ERK and p38 MAPK pathways

HER-2/*neu* signalling activates several downstream signalling pathways, such as PI3K/AKT, thus leading to dysregulated cell proliferation and enhanced cell survival (Jorissen *et al.*, 2003). To further understand the regulatory mechanism of PP2A phosphorylation as driven by HER-2/*neu* signalling, different inhibitors targeting PI3K/AKT, MEK/ERK, and p38 MAPK were used to block the respective pathway. As shown in Figure 4-3, LY294002 a specific inhibitor of the p110 catalytic subunit of PI3K targeting PI3K/AKT (Kataoka *et al.*, 2010; Nelson and Fry, 2001), PD98059 a specific inhibitor targeting MEK/ERK (Dudley *et al.*, 1995; Pang *et al.*, 1995), and SB203580, a specific inhibitor targeting p38 MAPK (Wen *et al.*, 2006), were used to target their respective pathways. The deactivation of each of these three individual signal modulators was assessed by measuring their respective phosphorylation level versus those recorded for their respective controls, by Western blotting. LY294002 inhibited the activation of AKT significantly at 30 min ($p<0.01$) (Figure 4-3 A); similarly, PD98059 also inhibited the activation of MEK/ERK significantly at 30 min ($p<0.01$) (Figure 4-3 B); and SB203580 inhibited the activation of p38 MAPK, albeit slightly less significantly at 30 min ($p<0.05$) (Figure 4-3 C).

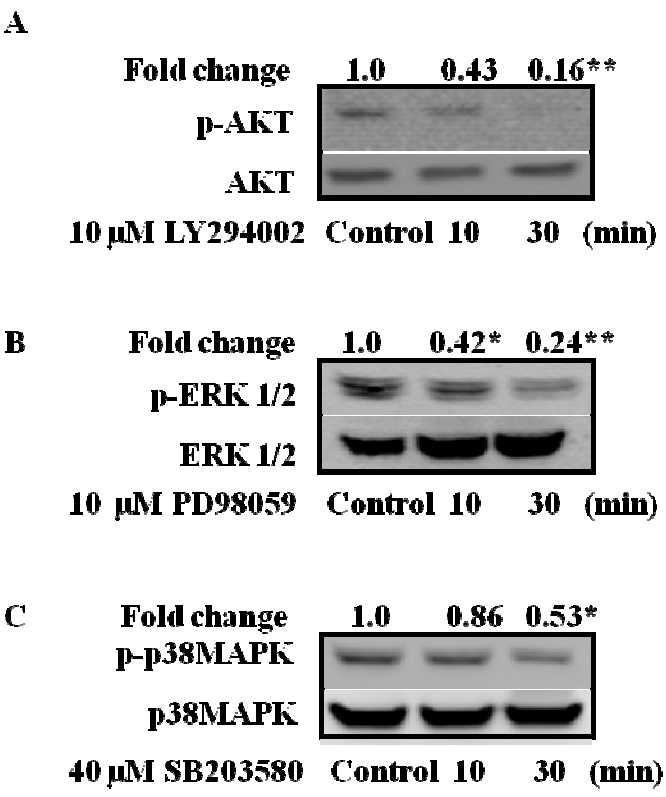


Figure 4-3. Inhibition of PI3K/AKT, MEK/ERK, and p38 MAPK pathway activation using their respective inhibitors. BT474 breast cancer cells at 70-80% confluence were serum-starved for 20 h and then treated with different amounts of the respective specific inhibitors or DMSO (control) for 10 min and 30 min. Cells were harvested and phosphorylation levels were determined by Western blotting with their respective antibodies, as described in Materials and Methods (see page 49). A. The PI3K/AKT pathway was deactivated by 10 μM of LY294002. B. The MEK/ERK pathway was inhibited by 10 μM of PD98059. C. The phosphorylation level of p38 MAPK decreased upon treatment of 40 μM of SB203580. Significant differences of treatment *versus* control at different time intervals were analyzed using the Student *t*-test. * $p<0.05$, ** $p<0.01$. Data shown represent the average of triplicate experiments.

4.2.4 PI3K/AKT and MEK/ERK positively regulate, whereas p38 MAPK negatively regulates tyrosine phosphorylation of PP2A

To investigate the effect of specific inhibitors blocking the activation of HER-2/*neu* downstream signal modulators on PP2A phosphorylation, the level of phosphorylated

PP2A at its tyrosine 307 residue (pY307-PP2A) in the BT474 cells at different time points post-treatment, were analyzed by Western blotting. When the intrinsic PI3K activity in the BT474 cells was blocked with the specific PI3K inhibitor, LY294002, the amount of pY307-PP2A formed was significantly reduced ($p<0.05$) (Figure 4-4 A). Similarly, a significant reduction of the pY307-PP2A levels ($p<0.05$) also occurred when the intrinsic MEK/ERK pathway was interrupted with the MEK I specific inhibitor, PD98059, which targets the MEK/ERK pathway (Figure 4-4 B). However, the cells treated with SB203580, a specific inhibitor of p38 MAPK, showed a notably increased level of pY307-PP2A ($p<0.05$) (Figure 4-4 C), implying that activation of p38 MAPK reduces tyrosine phosphorylation of PP2A. This is consistent with the function of p38 as a tumour suppressor: activation of p38α/β causes cell growth arrest, senescence and/or apoptosis while inhibition of p38α/β interrupts their growth arrest and resumes cell proliferation (Bulavin and Fornace, 2004; Aguirre-Ghiso *et al.*, 2003). The enhanced level of pY307-PP2A at the inhibitory state of p38 MAPK (as shown in Figure 4-4 C) could be attributed to inactivation of PP2A activity as a tumour suppressor, thereby facilitating cell survival.

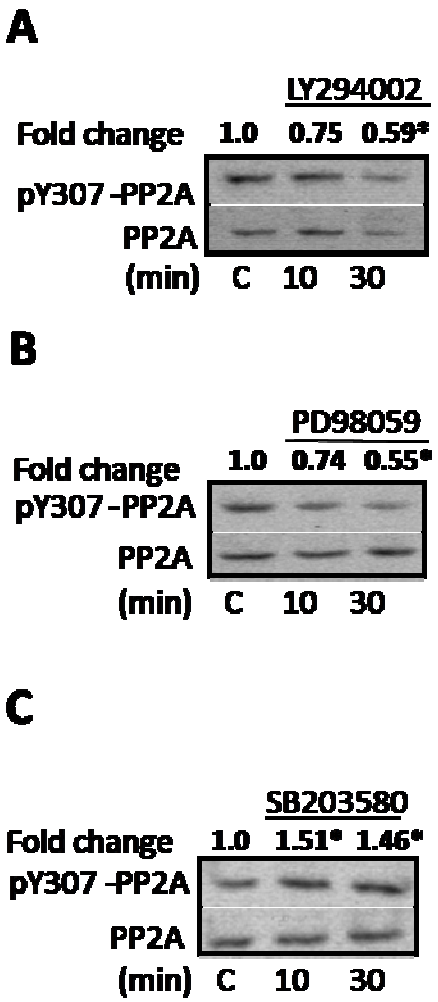


Figure 4-4. Effects of different inhibitors on tyrosine phosphorylation of PP2A. BT474 cells at 70-80% confluence were incubated with fresh medium containing either the PI3K inhibitor LY294002 (10 μM), or the MEK 1 inhibitor PD98059 (10 μM), or the p38 MAPK inhibitor SB 203580 (40 μM), respectively, for 10 and 30 min. Cells were harvested and the levels of PP2A and pY307-PP2A were detected by Western blotting. A: Inhibition of PI3K specific inhibitor LY294002 on PP2A phosphorylation in BT474 cells. B: Inhibition of MEK I inhibitor, PD98059, on the PP2A phosphorylation in BT474 cells. C: Enhancement of p38 MAPK inhibitor, SB203580, on the PP2A phosphorylation in BT474 cells. The relative tyrosine phosphorylation of PP2A was normalized to its basal level (pY307-PP2A/PP2A), and interpreted as the fold change. Data shown represent the average from triplicate experiments. * $p<0.05$, (Student *t*-test)

4.2.5 Stimulation of PP2A by HRG and the effects of inhibitors on tyrosine phosphorylation of PP2A

To further investigate the regulatory role of these inhibitors on pY307 of PP2A in BT474 cells subjected to the HRG-enhanced HER-2/*neu* signalling, the serum-starved BT474 cells were induced with HRG for 30 min, followed by treatment with the respective inhibitors, with or without HRG in the medium. As indicated in the western blotting results in Figure 4-5, the HRG-enhanced pY307 of PP2A was significantly decreased by both the PI3K inhibitor, LY294002 (Figure 4-5 A) and the MEK 1 inhibitor, PD98059 (Figure 4-5 B), in a time-dependent manner. In contrast, addition of the p38 MAPK inhibitor, SB203580, to the HRG-treated cells continued to increase the level of pY307 of PP2A (Figure 4-5 C)). Taken together, these results clearly demonstrated that the tyrosine phosphorylation of PP2A in the cells was regulated by a complex multi-signalling network, with PI3K/AKT and MEK/ERK exerting a positive effect, whereas p38 MAPK has a negative effect, on this essential process. Activation of PI3K or MEK could inactivate PP2A activity by enhancing its tyrosine phosphorylation. In contrast, activation of p38 MAPK could activate PP2A by inhibiting its tyrosine phosphorylation.

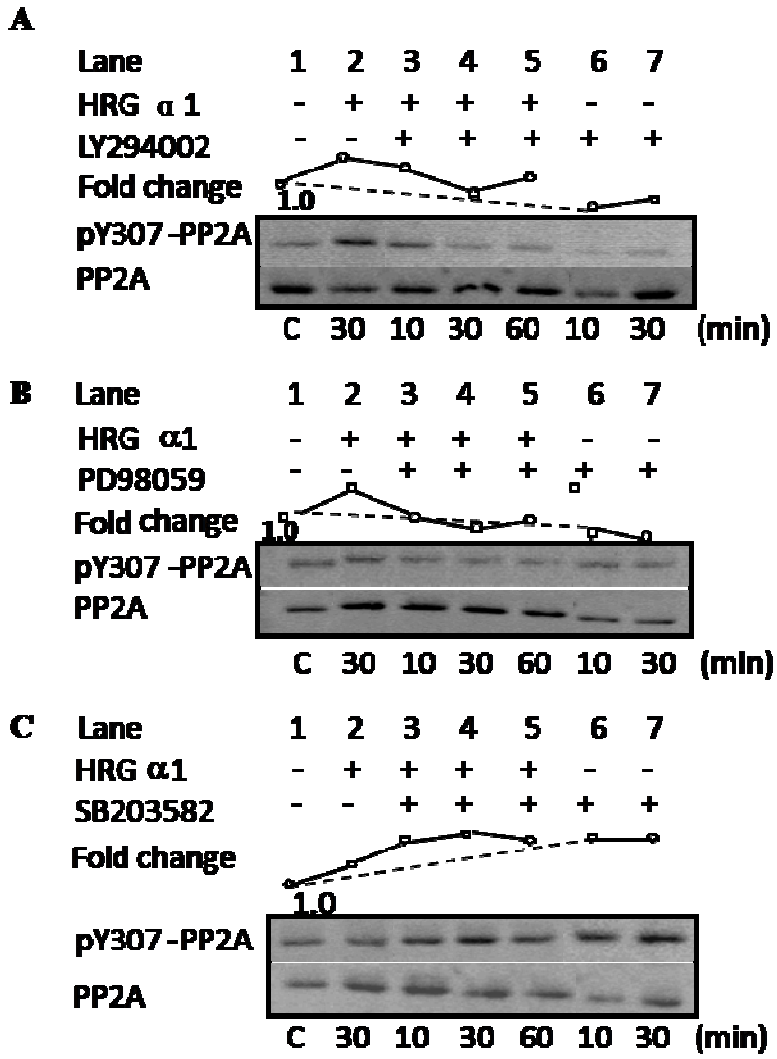


Figure 4-5. Stimulation of HRG and the effects of different inhibitors on tyrosine phosphorylation of PP2A. To test the role of different inhibitors on the HRG-enhanced PP2A phosphorylation, BT474 cells were serum-starved for 20 h and then treated for 30 min with HRG. Different inhibitors were directly added to the HRG-treated cultures (lanes 3-5) or added with fresh medium after removing HRG (lanes 6-7). HRG-treated BT474 cells were further treated with the inhibitors at the indicated time intervals and the PP2A phosphorylation levels were detected by Western blotting. A: Attenuation of pY307-PP2A by LY294002 in the HRG-treated BT474 cells. Serum-starved BT474 cells were treated with HRG α1 for 30 min, and inhibitor LY294002 (10 μM) was added to the cultures for further treatment for 10, 30 and 60 min (lanes 3-5), or the HRG was removed and fresh medium with LY294002 (10 μM) was added to treat the cells for 10 and 30 min (lanes 6-7). Cells were harvested and both PP2A and pY307-PP2A were detected by Western blotting. B: Attenuation of pY307-PP2A by PD98059 in the HRG-treated BT474 cells. Serum-starved BT474 cells were treated with HRG α1 for 30 min, and inhibitor PD98059 (10 μM) was added to the cultures for further treatment for 10, 30 and 60 min (lanes 3-5), or the HRG was removed and fresh medium with PD98059 (10 μM) was added to treat the cells for 10 and 30 min (lanes 6-7). Cells were harvested and both

PP2A and pY307-PP2A were detected by Western blotting. C: Enhanced expression of pY307-PP2A by SB203580 in the HRG-treated BT474 cells. Serum-starved BT474 cells were treated with HRG α1 for 30 min, and inhibitor SB203580 (40 μM) was added to the cultures for further treatment for 10, 30 and 60 min (lanes 3-5), or the HRG was removed and fresh medium with SB203580 (40 μM) was added to treat the cells for 10 and 30 min (lanes 6-7). Cells were harvested and both PP2A and pY307-PP2A were detected by Western blotting. In all three experimental groups, the relative tyrosine phosphorylation levels of PP2A were normalized to the corresponding basal levels (pY307-PP2A/PP2A), and interpreted as the fold change observed. The fold change of relative phosphorylation was interpreted as the ratio of the relative phosphorylation level of the experimental cultures *vs* that in the control culture which is arbitrarily assigned as 1. Data shown represent the average from triplicate experiments.

4.3 Discussion

It has been found that phosphorylation at tyrosine 307 and/or methylation of the catalytic C subunit (PP2A/C) are the crucial steps in regulating PP2A activity (Chen *et al*., 1994; Favre *et al.*, 1994). Thus the posttranslational modification of PP2A is a critical regulatory step in exerting its role in mammalian cells, especially in cancer cells where it regulates the intricate balance between cell survival and apoptosis by manipulating the activities of the key signal molecules, such as AKT, Bax, Calpains, *etc*. (Andrabi *et al.*, 2007; Kong *et al.*, 2004; Grethe and Pörn-Ares, 2006; Xin and Deng, 2006). Given the importance of PP2A as a multifunctional modulator, the molecular regulation of PP2A activity by the intrinsic and/or extrinsic signal network will decide the cell's destination.

An earlier study showed that the Simian virus 40 small T antigen (SV40 ST) directly inhibits the activity of PP2A in promoting cell transformation (Sontag and Sontag, 2006). On the other hand, oncogenic signals enhanced by non-receptor- and receptor-linked tyrosine kinases, including the EGFR and the insulin receptor, stimulate tyrosine phosphorylation of PP2A and thereby inhibit its activity (Tsai *et al.*, 1996). Considering

the fact that EGF and the related HER-2/*neu* RTKs are the most prevalent oncogenes in breast cancer, therefore, the hypothesis in this study is that HER-2/*neu* signalling may also play a key role in "tuning" PP2A activity by regulation of tyrosine phosphorylation as well.

As hypothesised, tyrosine phosphorylation of PP2A was found significantly affected either by switching on HER-2/*neu* signalling with HRG or by switching off HER-2 signalling with its specific inhibitor AG825 (Figure 4-2). This data further emphasized the importance of oncogenic signalling in regulating PP2A activity. Furthermore, the inhibitor experiments targeting PI3K, MEK1 and p38 MAPK demonstrated their respective contributions to the tyrosine phosphorylation of PP2A (Figure 4-3 and Figure 4-4). Tyrosine phosphorylation of PP2A was highly inhibited in both HRG-treated and untreated BT474 cells by PI3K (Figure 4-5 A) or MEK1 (Figure 4-5 B) inhibitors (LY294002 and PD98059, respectively), but was markedly increased in these same cells upon addition of the p38 MAPK inhibitor, SB203580 (Figure 4-5 C). These results indicated that the PI3K/AKT and Raf/MEK1/ERK pathways positively regulate, whereas the p38 MAPK pathway negatively regulates, PP2A activity in breast cancer cells. These observations are consistent with the cell survival role of activated PI3K/AKT (Park and Kim, 2007) (Zhou *et al.,* 2004) and Raf/MEK1/ERK and with the cell pro-apoptotic role of activated p38 MAPK in HER-2/*neu* breast cancer progression (Neve *et al.*, 2002).

This data address and add credence to the concept that the key HER-2-linked downstream pathways constitute an interactive network to regulate the phosphorylation status of

PP2A. This tyrosine phosphorylation-based modification of PP2A activity by the cross-signalling pathways in cancer cells further highlights its central role in regulating cell survival and apoptotic behaviour.

In summary, the complicated regulatory mechanism of PP2A phosphorylation by the downstream pathways of HER-2/*neu* signalling reveals its essential role, at least in part, in deciding the destiny of these cells: the activated PI3K- and Raf/MEK pathways stimulate PP2A phosphorylation and attenuate its activity to facilitate cell survival, whereas the activated p38 MAPK pathway reduces PP2A phosphorylation and increases its activity to accelerate cell apoptotic activity.

CHAPTER 5

CORRELATION OF PP2A AND BREAST CANCER

5.1 Overview

PP2A is a heterotrimer holoenzyme with different activities and diverse substrate specifications (Janssens and Goris, 2001). The three distinct functional components (structural, catalytic and regulatory subunits) give rise to the holoenzyme complex which plays an important role as a serine/threonine phosphatase. The catalytic subunit is encoded by two distinct ubiquitously expressed gene, the Cα and Cβ subunits which share 97% sequence identity (Fellner *et al.*, 2003). Phosphophorylation occurs at either Tyrosine 307 or Threonine 304 (Longin *et al.*, 2007). Phosphorylation at Tyrosine 307 inhibits the recruitment of the regulatory B subunits to the core enzyme (Longin *et al.*, 2004). There is very little data available about the correlation of pY307-PP2A in relation to cancer. Thus far, pY307-PP2A has been reported to be highly expressed in parallel with tau hyperphosphorylation in Alzheimer's disease (Liu *et al.*, 2008).

The PP2A/A subunit has two isoforms- the α- and β-isoforms, which are encoded by PPP2R1A and PPP2R1B, respectively (Hemmings *et al.*, 1990). Somatic alterations in 15% (5 out of 33) of primary lung tumours, 6% (4 out of 70) of lung tumour-derived cell lines, and 15% (2 out of 13) of primary colon tumours were detected by sequencing the PPP2R1B gene, located on human chromosome 11q22-24. One deletional mutation was revealed, forming a truncated PP2A-Aβ protein that was unable to bind to the catalytic subunit. This clearly showed that the PPP2R1B gene is altered in human lung and colorectal carcinomas, suggesting a role in human tumourigenesis (Wang *et al.*, 1998).

Another report from Calin *et al.* described low frequency mutations being detected in breast and lung carcinoma, and in melanomas, involving both PPP2R1A and PPP2R1B. An exon deletion affecting PPP2R1B was found in four breast carcinomas while nucleotide substitutions changing highly conserved amino acids and frame-shift mutations were detected in PPP2R1A (Calin *et al.*, 2000). However, it still remains unclear whether these low frequency mutations are causal or accidental. Nevertheless, the mutation detected in the PP2A/A subunit strengthens the potential role of PP2A in human tumourigenesis. The most recent study by Dupont *et al.* revealed that in a cohort study of 450 women with history of benign breast disease, specific haplotypes of genes that encode PP2A subunits are associated with breast cancer and interact with benign proliferative breast disease to modify risk (Dupont *et al.*, 2009).

PP2A phosphorylation is clearly regulated by HER-2/*neu* in the *in vitro* study (Chapter 4). To further examine if there is any differential expression of PP2A and pY307-PP2A in breast non-tumorigenic and breast cancer cell lines, as well as in clinical (tumour) samples, total proteins from mammary epithelial cell lines, breast cancer cell lines as well as frozen tissue samples were extracted and subjected them to Western blotting analysis using the appropriate respective antibodies. The level of tyrosine-phosphorylated PP2A at the tyrosine307 residue (pY307-PP2A) was found to be increased in the HER-2-positive breast cancer tissue samples and in related cell lines. The clinical significance of this as a prognostication biomarker of cancer progression was further assessed in a panel of 206 clinical specimens in a tissue microarray. Enigmatically, the immunohistochemical staining on the tissue microarray showed that expression of pY307-PP2A was highly

correlated with breast cancer progression. DNA sequencing of the full length of the PP2A/C subunit gene sequence extracted from both the breast cancer cell lines and breast tumour tissues were performed subsequently. Howvever, no mutations were found.

5.2 Results

5.2.1 pY307-PP2A was highly expressed in HER-2/*neu*-positive breast cancer cell lines

The expression of PP2A and its phosphorylated derivative, pY307-PP2A, in a non-tumorigenic MCF12A cell line, and two HER-2/*neu*-positive breast cancer cell lines, BT474 and SKBR3 were analysed. As shown in Figure 5-1 A, the expression of the basal PP2A was similar in the three cell lines. However, the expressed levels of both pY307-PP2A (normalized to β-actin, Figure 5-1 B) as well as the relative phosphorylation of PP2A (pY307-PP2A/PP2A, normalized to basal PP2A, Figure 5-1 D) were significantly higher in both the BT474 and SKBR3 HER-2/*neu*-positive breast cancer cells compared to the MCF12A cells ($p<0.05$). No significant difference in PP2A expression (normalized to β-actin, Figure 5-1 B) was observed in the 3 cell lines.

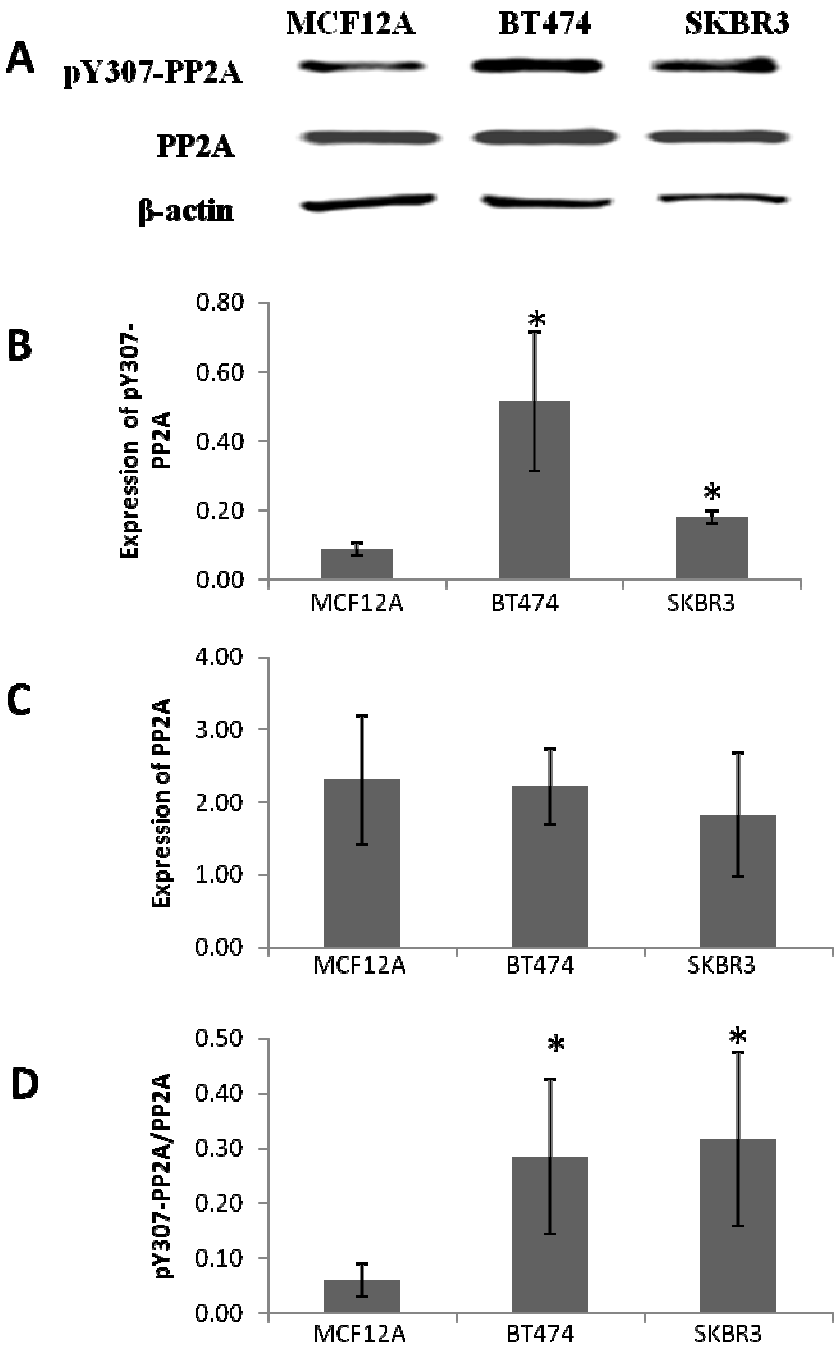


Figure 5-1. Expression of PP2A and tyrosine-phosphorylated PP2A (pY307-PP2A) in breast non-tumorigenic and cancer cell lines. A: Western blotting of PP2A and pY307-PP2A in the human non-tumorigenic mammary epithelial cell line, MCF12A and the HER-2/*neu*-positive breast cancer cell lines, BT474 and SKBR3. Total protein lysates were extracted from the cell lines and subjected to 10% SDS-PAGE, transferred to PVDF membranes, and immunodetected using anti-PP2A and anti-pY307-PP2A antibodies, followed by conjugated secondary antibody. The fluorescence signals were captured using the MULTI GENIUS Bio Imaging System and signal intensities analysed by the GeneTools software (Syngene). B: Expression levels of pY307-PP2A normalized

to β-actin. C: Expression levels of PP2A normalized to β-actin. D: Relative phosphorylation of PP2A normalized to total PP2A (pY307-PP2A/PP2A) in the three cell lines. Data shown represent the mean ±SD of triplicate experiment. Bars, SD; *p<0.05

5.2.2 pY307-PP2A was highly expressed in HER-2/*neu*-positive breast tumours

The expression of PP2A and pY307-PP2A in a panel of clinical specimens were investigated. As indicated in Figure 5-2 A, PP2A was expressed in both the tumour and non-tumour tissues. However, PP2A (normalized to β-actin) was highly expressed in the non-tumour tissues and downregulated in the tumour tissues (Figure 5-2 B), further supporting the role of PP2A as a tumour suppressor in breast cancer progression. pY307-PP2A is found only detected in the tumour tissues (Figure 5-1 A, right panel) and that the level of pY307-PP2A (normalized to β-actin) is significantly increased in the HER-2/*neu*-positive tumours, compared to the corresponding levels observed in the HER-2/*neu*-negative tumours (Figure 5-2 D). However, the relative phosphorylation level of PP2A (pY307-PP2A/PP2A) was not markedly enhanced in the HER-2/*neu*-positive tumours (Figure 5-2 C). Taking into account that phosphorylation of PP2A is a transient process under growth factor stimulation, as reported by Chen *et al.* (Chen *et al.*, 1994), and as demonstrated in this present study in an *in vitro* cell system, this transient phenomenon may not be readily observed in the tumour specimens.

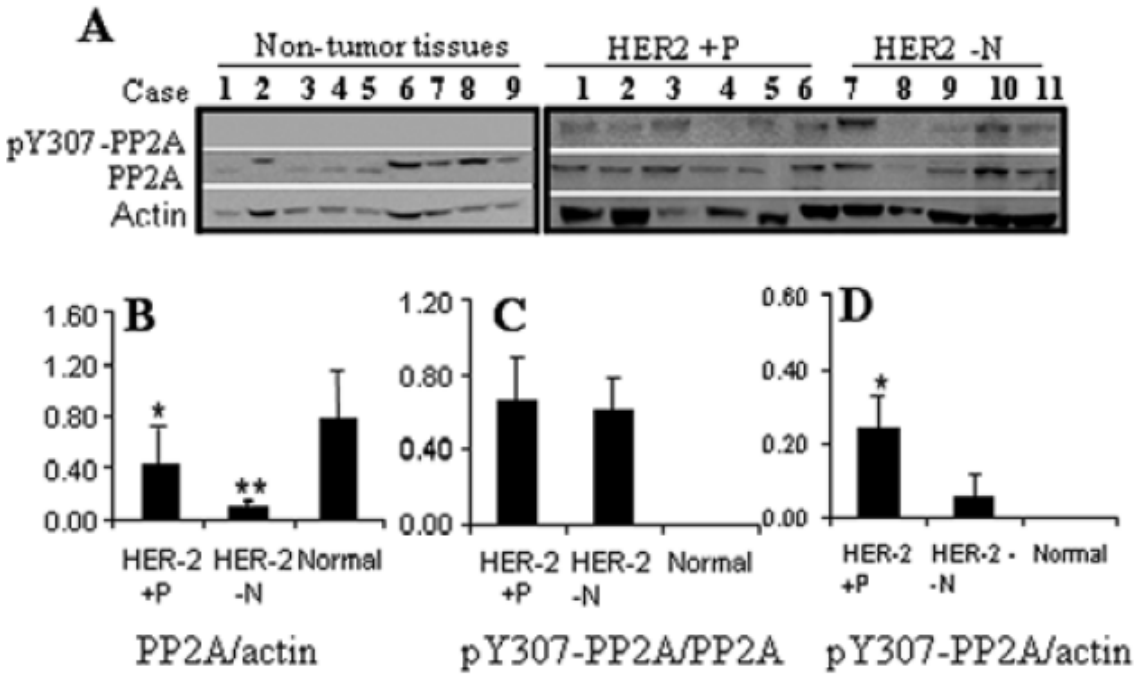


Figure 5-2. Expression of PP2A and tyrosine-phosphorylated PP2A (pY307-PP2A) in clinical non-tumour and tumour breast specimens. A: Western blotting of PP2A and pY307-PP2A in tumour and non-tumour tissues. Total soluble proteins from both frozen breast tumour and non-tumour tissues were solubilised in Tissue Protein Extraction Reagent® (Pierce, Rockford, IL), after grounded to powder in liquid nitrogen. Proteins (40 μg) were separated by SDS-PAGE (10%), transferred to PVDF membranes, and immunodetected using anti-pY307-PP2A and anti-PP2A antibodies, followed by HRP-conjugated secondary antibody. Signals were captured with the MULTI GENIUS Bio Imaging System and the signal intensities were analyzed by the GeneTools software (Syngene). B: Level of PP2A normalised to β-actin in breast tissues. C: Level of pY307-PP2A normalized to β-actin in breast tissues. D: Relative phosphorylation of PP2A normalized to total PP2A (pY307-PP2A/PP2A) in breast tissues. Data shown represent the mean ± SD of triplicate experiments. * $p < 0.05$, ** $p < 0.01$ (Students' *t*-test).

5.2.3 Expression levels of pY307-PP2A correlated with breast cancer progression

To investigate whether pY307-PP2A expression is associated with breast cancer progression, the level of pY307-PP2A was further analyzed by immunohistochemistry with the specific antibody to pY307-PP2A (Epitomics Inc., CA, USA) in all 206 tissue cores included in the tissue microarray (TMA) purchased from Biomax (Rockville, MD, USA). The TMA included 206 different cases, with 32 cases of metastatic breast

carcinomas, 116 malignant breast carcinomas, 44 non-malignant tissues and 16 non-tumours (see Appendices: Table 3). Scoring of the staining intensity of each case was made by two independent pathologists and one principal investigator. The staining intensity was classified as negative and weak (score 0), moderate (score 1) and strong (score 2) to indicate the expression of pY307-PP2A in the different tissue specimens. In cases in which a discrepancy was noted, a subsequent review carried out to reach a consensus was mostly but not always successful; discordant results were excluded from statistical data analysis. Representative cases with different scores are shown in Figure 5-3 (A, B, C). Of the 34 non-malignant tissues including benign growths, hyperplasia and inflammation, only 2 cases (6%) showed strong staining intensity (score 2) and 12 (35%) moderate staining intensity (score 1), whereas in the 106 malignant carcinomas, 21 cases (20%) were strongly stained and 57 moderately stained (54%) ($p < 0.01$). Noticeably, pY307-PP2A was strongly stained in 8 of the 30 (27%) metastatic carcinomas, and with a higher frequency than that observed in the malignant carcinomas (20%), although not at a significant level ($p = 0.15$). None of the 14 normal tissues showed high expression of pY307-PP2A. Collectively, expression of pY307-PP2A was found to be progressively increased with the tumour stage progression of the patients being assessed (Figure 5-3 D).

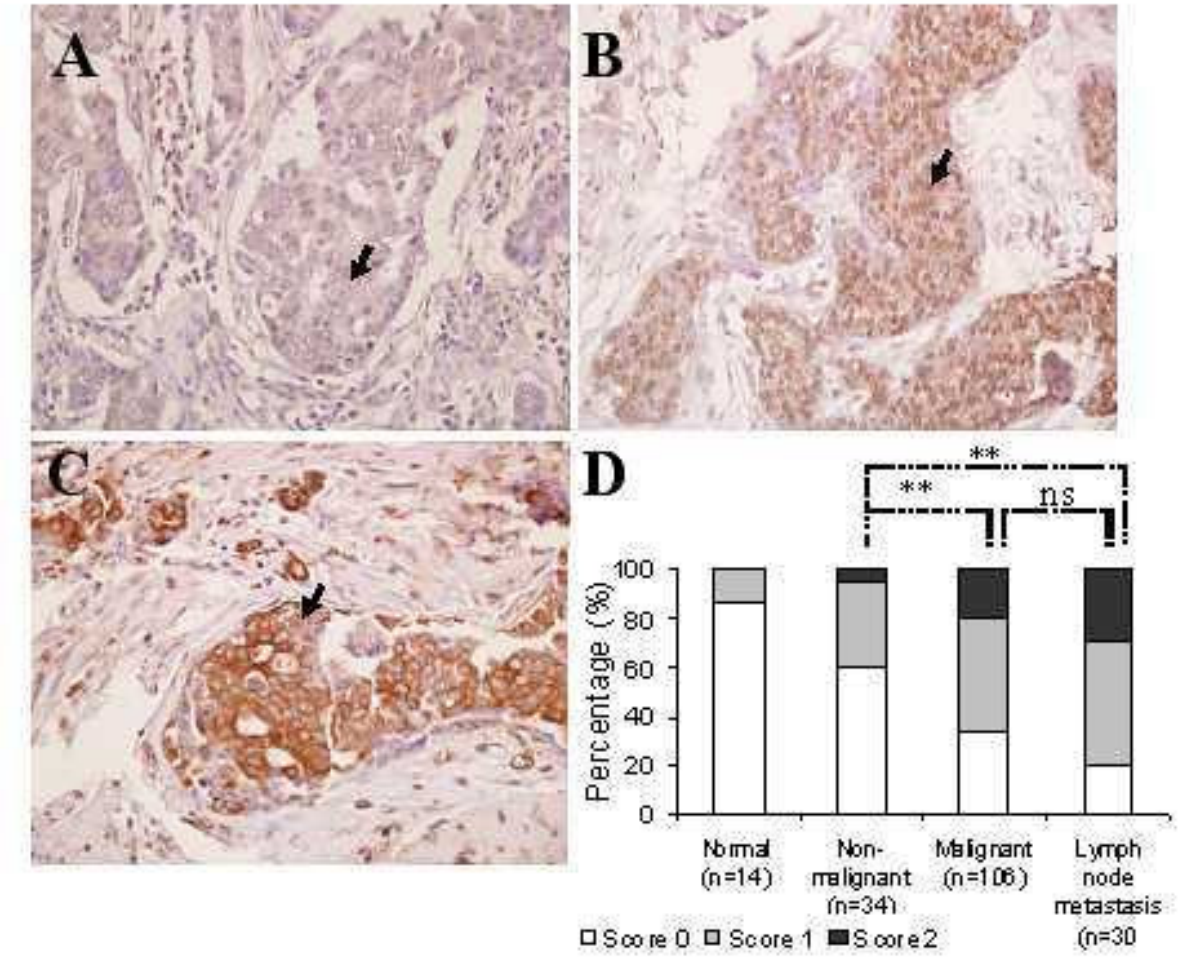


Figure 5-3. Representative immunohistochemical staining of pY307-PP2A on tissue cores in a breast tissue microarray. A-C showed increasing staining levels of score 0 to score 2. Sections were deparaffinised in xylene and rehydrated in graded alcohols. After unmasking antigen and removing endogenous peroxidases, sections were incubated for 1 h with antibodies against pY307-PP2A at room temperature, followed by detection with labelled dextran polymer conjugated with peroxidase and DAB+-substrate chromagen solution. Nuclei were lightly stained with Mayer's haematoxylin. The extent of staining was scored as 0, 1 and 2, according to the staining intensity of the tumour cells. D: Distribution of pY307-PP2A expression in breast tissues. Breast tissues on the microarrays were classified as normal, non-malignant, malignant and lymph node metastasis (malignant cases). Data were expressed as percentage of cases with various scores in each subgroup. $p < 0.05$ was considered as significant (Fisher's exact test). * $p<0.05$, ** $p<0.01$, *ns*: no significant difference. Arrows indicate the tumour cells.

5.2.4 DNA sequencing of the PP2A catalytic subunit

Given that the PP2A/A mutations were detected in breast, lung, and colon carcinomas and in melanomas (Wang *et al.*, 1998; Calin *et al.*, 2000), whether there were any mutations in the PP2A/C subunits of those samples that demonstrated strong pY307-PP2A expression and staining, were investigated in both breast cancer cell lines and tumour tissues. Five human breast cell lines - one human non-tumourigenic breast cell line MCF12A; two HER-2/*neu*-negative breast cancer cell lines, MDA-MB231 and BT549, and two HER-2/*neu*-positive breast cancer cell lines, BT474 and SKBR3, were processed and analyzed for the presence of mutations in PP2A/C by DNA sequencing. Seven breast cancers together with their respective normal paired tissue samples from the same patients' biopsies obtained from the Tissue Repository of the National University Hospital (Singapore) were also subjected to DNA sequencing. However, there were no mutations found in any of the processed samples (Figure 5-4) (see Appendices: Table 4) amongst both the cancer cell lines and tumour samples.

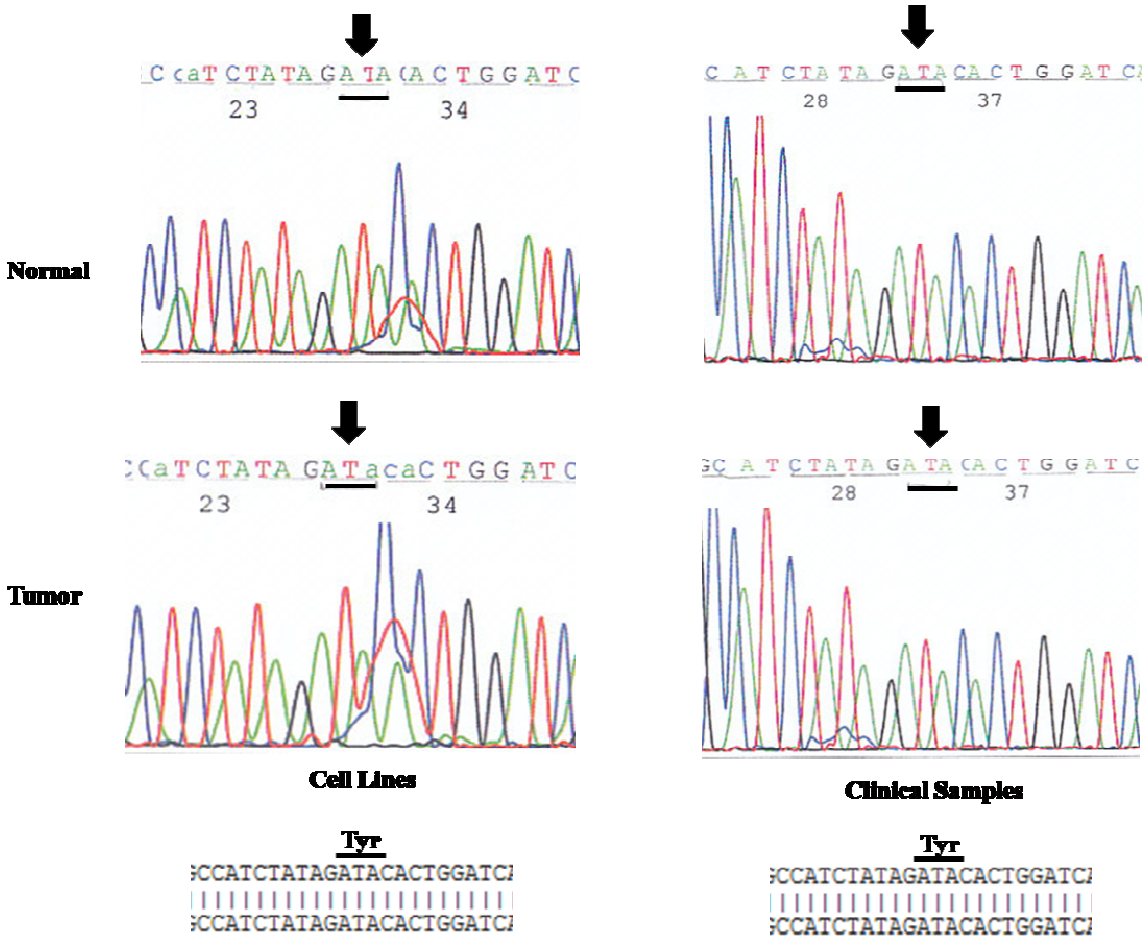


Figure 5-4. DNA Sequencing of the PP2A catalytic subunit (PP2A/C). Representatives of sequence chromatograms showed that there was no mutation found in both tumour cell lines and clinical sample as compared to their non-tumourigenic cell lines and normal clinical tissue samples.

5.3 Discussion

PP2A activity was shown to be required for cells to progress through the early phase of the cell cycle to enter the S phase (Yan and Mumby, 1999), and to exert an anti-apoptotic effect on malignant testicular germ cell tumours (Schweyer *et al.*, 2007). The contrasting functions of PP2A may be attributed to its existence as a multitasking, enzyme complex rather than as a single enzyme entity (Janssens and Goris, 2001). Whilst it had been

reported that PP2A activity was higher in ER-positive breast cancer cell lines than in the ER-negative cell lines (Gopalakrishna *et al.*, 1999), there is scanty information of pY307-PP2A expression in human breast tissues. Recently, Liu *et al.* reported a consistent increase in the levels of pY307-PP2A in parallel with tau hyperphosphorylation in Alzheimer's disease. Their immunohistochemistry and immunofluorescence confocal microscopy showed an accumulation of pY307-PP2A in pretangled neurons preceding PHF-tau accumulation. This suggests that the post-translational modification of pY307-PP2A could be an early event in the pathogenesis of Alzheimer's disease-related tau pathology (Liu *et al.*, 2008).

To further investigate the distribution and expression of PP2A and pY307-PP2A in breast cancer specimens, and to determine whether the level of pY307-PP2A is associated with cancer progression, the levels of both PP2A and pY307-PP2A expression in a panel of resected breast tumours as well as numerous breast cell lines were tested. The relative expressions of the PP2A and pY307-PP2A in HER-2/*neu*-positive breast cancer cells (BT474 and SKBR3) and non-tumorigenic mammary epithelial cells (MCF12A) were analyzed by western blots (Figure 5-1). The pY307-PP2A expression in the BT474 and SKBR3 cells was found to be higher (with statistically significant differences) compared to that in the MCF12A cells. This suggested that PP2A phosphatase activity is inactivated in both the HER-2/*neu*-positive cell lines, and that the loss of function of PP2A as a tumour suppressor arguably contributes to the aggressiveness of the HER-2/*neu*-positive breast tumours.

A comparison of the PP2A expression between tumour and non-tumour clinical samples (Figure 5-2) further supported the role of PP2A as a tumour suppressor in breast cancer. For the tumour samples, notably, the observation that pY307-PP2A was only detected in all the tumour samples studied (n=11), but not in any of the non-tumour samples (n=9) (Figure 5-2 A), strongly suggests the inactivation of PP2A in tumours. Interestingly, the relative tyrosine phosphorylation of PP2A (pY307-PP2A/PP2A) was not significantly different in the HER-2-positive and –negative tumours (Figure 5-2 C). This is not surprising when one considers that as tyrosine phosphorylation induced by the oncogenic HER-2/*neu* signalling is a transient response, as verified by this study and supported by data from other studies using an *in vitro* cell system (Chen *et al.*, 1994). These transient signals may not be readily observed or captured in the tumour samples, without the use of real-time bioimaging techniques. The increased expression of pY307-PP2A in the HER-2/*neu*-positive breast tumours is consistent with the subsequent tyrosine phosphorylation and inactivation of PP2A expressed in this subtype of breast tumours. Moreover, tumours with higher positive staining of pY307-PP2A were associated with patients in the later stages of breast cancer progression, most notably in those with malignancy and lymph node metastasis (Figure 5-3). This tissue microarray study supported the strong association of pY307-PP2A with tumour progression, and further highlighted the potential of pY307-PP2A as a viable targeted therapy candidate to block off tumorigenesis by its inherent property of reactivating PP2A activity. The results of this study also indicate that pY307-PP2A has a potential prognostic value in determining breast cancer progression.

DNA sequencing was performed to examine whether there were any inherent mutations that could have caused the differential pY307 expression in any or all of the breast tumour cell lines and/or resected tumour tissues studied. No mutation in both breast cancer cell lines and tumour tissue samples (Figure 5-4) was found and this may imply that the overexpression of pY307 could be caused by its upstream oncogenic mechanisms arising from other oncogenic signal modulators such as HER-2/*neu* or Src proteins.

In summary, higher expression level of pY307-PP2A in tumour cell lines and tissues specimens may indicate that the loss of function of phosphatases in cancerous cells contribute to their malignant transformation and disease progression. As reported in this findings, in both the breast cancer cell lines and clinical tumour specimens, pY307-PP2A is highly increased in the more aggressive HER-2-positive breast tumours and also markedly associated with tumour progression. In this context, cells that express high expression of inactivated PP2A could be prone to be more oncogenic. This investigation further indicates pY307-PP2A as an important prognostic factor in breast cancer development.

CHAPTER 6

FUNCTIONAL ROLE OF PP2A IN HER-2/*neu* BREAST CANCER CELL LINES

6.1 Overview

PP2A is highly conserved and ubiquitously expressed. It is very much involved in a multitude of cellular processes by dephosphorylating specific substrates, and thus providing a negative feedback to signals initiated by upstream protein kinases. PP2A phosphatase activity is involved in regulating cell cycle, signal transduction, DNA replication, transcription and translation (Schönthal, 2001; Janssens and Goris, 2001). Several studies had shown that PP2A can potently restrict migration and invasion of various cell types (Xu and Deng, 2006; Pullar *et al.*, 2003; Young *et al.*, 2003; Young *et al.*, 2002). Dysregulation of PP2A has been reported to lead to potentially debilitating diseases such as cancer (Schönthal, 2001) and Alzheimer's disease (Liu *et al.*, 2008; Qian *et al.*, 2009).

The HER-2/*neu* proto-oncogene-encoded receptor has been found to be overexpressed in about one-third of breast cancer tumours (Rubin and Yarden, 2001). This ligandless receptor is able to heterodimerize with itself or with other members of the receptor tyrosine kinase family and causes autophosphorylation, wherein the signal transduction events are triggered (Warren and Landgraf, 2006). Thus, in cancer cells numerous heterodimers are formed, resulting in unabated aggressive tumour growth and death. However, relatively little is known about the cellular phosphatase(s) responsible in the activation/deactivation of the HER-2/*neu* signalling cascade. Three recent studies reported that protein tyrosine phosphatase 1B (PTP1B) is required for HER-2/*neu*

transformation in human breast epithelial cells and inhibition of PTP1B delays HER-2/*neu*-induced mammary tumorigenesis (Arias-Romero *et al.*, 2009; Julien *et al.*, 2007; Bentires-Alj and Neel, 2007). Overexpression of wild-type PTP1B has been reported to enhance PP2A activity, accompanied by dephosphorylation of PP2A/C and thus, it was suggested that PTP1B is associated with PP2A in the regulation of sterol regulatory element-binding protein-1 gene expression in the insulin resistance/metabolic syndrome (Shi *et al.*, 2007; Ugi *et al.*, 2009). The results in Chapter 3demonstratedthe use of breast cancer cell lines as an *in vitro* model, and that PP2A was phosphorylated upon HRG-enhanced-HER-2/*neu* signalling. In addition, evidences were provided to emphasis that pY307-PP2A was highly increased in HER-2/*neu*-positive breast tumours and significantly related to breast cancer progression (Chapter 5). Therefore, both PTP1B- and PP2A-related interactions may play a role in HER-2/*neu*-driven tumorigenesis. Furthermore, PP2A phosphatase activity could therefore also be inactivated by phosphorylation in HER-2/*neu*-positive cells. This has been supported by the studies showing that receptor tyrosine kinases and non-protein receptor tyrosine kinases inactivate PP2A phosphatase activity by phosphorylation of the PP2A/C at the Y307 site (Chen *et al.*, 1992; Chen *et al.*, 1994; Brautigan, 1995).

Given the importance of the role of PP2A in cancer progression, the molecular regulation of PP2A was hypothesized may contribute significantly to the cell survival or cell death in HER-2/*neu*-overexpressed breast cancer cells. Although the role of pY307-PP2A is well documented, it remains to be determined whether the HER-2/*neu*-encoded receptor can phosphorylate the carboxy-terminal region of the C subunit of PP2A (Y307),

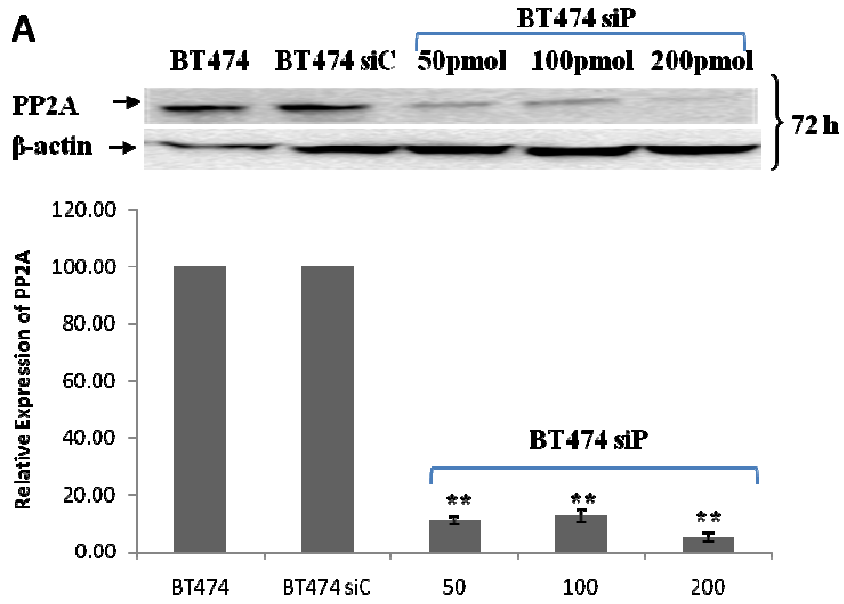
resulting in inhibition of phosphatase activity in a similar manner as Src, p56lck, the epidermal growth factor (EGF) receptor and the insulin receptor (Chen *et al.*, 1992; Chen *et al.*, 1994). To investigate the resultant consequences of PP2A knockdown on cellular behaviour in HER-2/*neu*-positive breast cancer cells and its downstream signalling cascade, namely the PI3K/AKT, Ras/Raf/ERK and p38 MAP kinase pathways, the PP2A/C subunit in the HER-2/*neu*-positive breast cancer cell lines, BT474 and SKBR3 was silenced. The silencing of PP2A/C was demonstrated to cause the HER-2/*neu*-positive breast cancer cells to undergo apoptosis via the p38 MAPK-related pathway. These results further supported the essential role of pY307-PP2A in cell survival in HER-2/*neu*-positive breast cancer cells.

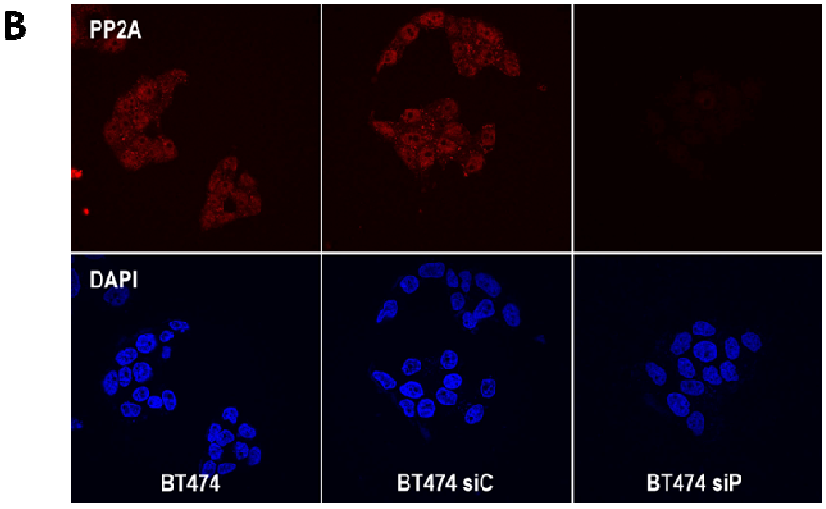
6.2 Results

6.2.1 Silencing of PP2A/C using siRNA

To determine the effect of silencing PP2A/C in HER-2/*neu*-positive breast cancer cell lines, BT474 and SKBR3 were transfected with siPP2A/Cα and the scrambled siRNA negative control. After a 48- or 72-h transfection (depending on cell type), total proteins from each control and transfectant were extracted to examine the knock-down effects by western blotting and immunofluorescence staining. The morphology of all the cells was not altered after silencing. As indicated in Figure 6-1, siPP2A/Cα-treated BT474 (BT474 siP, Figure 6-1 A) and SKBR3 (SKBR3 siP, Figure 6-1 C) cells showed a significant reduction (up to 70%) of PP2A as compared to the siRNA negative control (BT474 siC and SKBR3 siC) and their parental cells ($p<0.01$). The immunofluorescence staining analysis further substantiated the efficiency of silencing using siRNA, with both BT474

siP and SKBR3 siP showing weak/negligible PP2A staining (Figure 6-1 B and D).





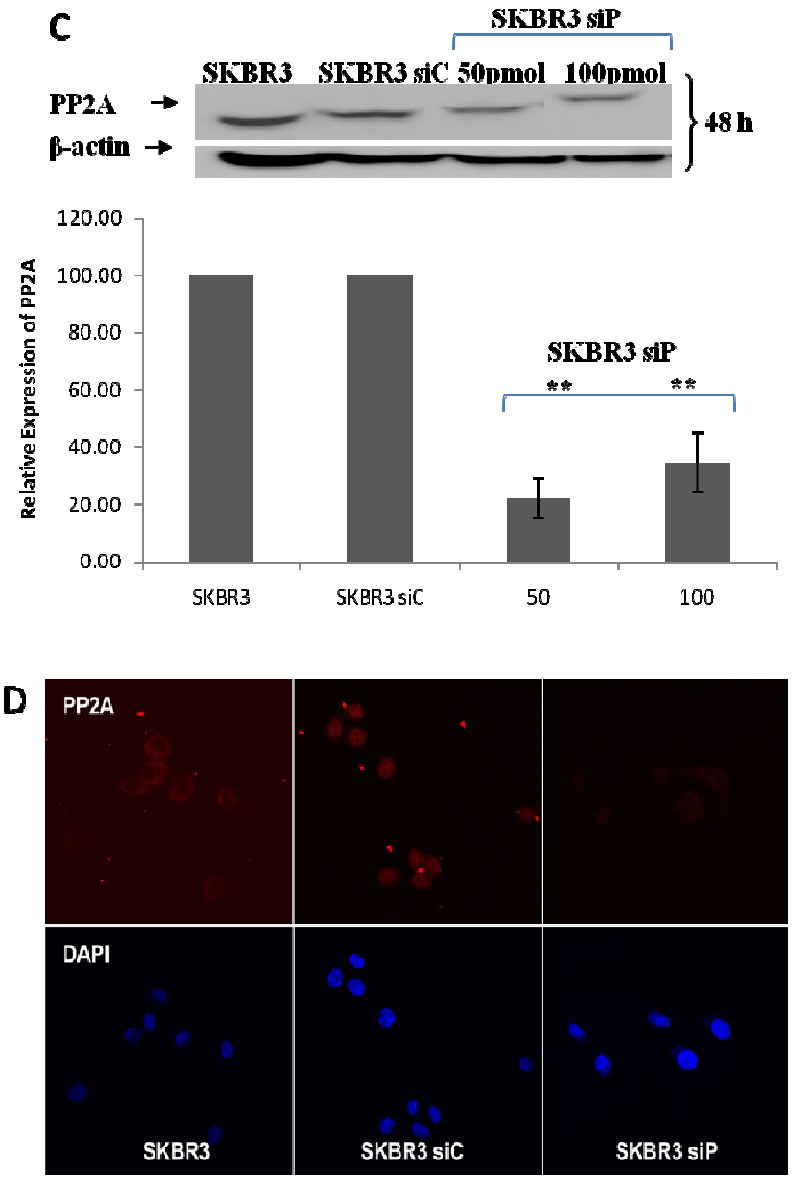


Figure 6-1. Silencing of PP2A/C using siRNA from siGENOME SMARTpool® PP2A. A: Western blotting of PP2A expression levels and graphical representations of silencing efficiency after 72-h transfection with siRNA on BT474 cell line compared to the negative control siC, at different concentrations of siRNA. B: Immunofluorescent detection of PP2A expression in parental BT474, BT474 siC and BT474 siP cells. Cells on the cover slip were fixed with 4% paraformaldehyde for 15 min and permeabilized with 1% Triton-X for 10 min. After washing, cells were incubated with anti-PP2A antibody, followed by incubation with Alexa Fluor® 532 for 1 h. Cells were then counterstained with DAPI and signals were captured by confocal microscopy (X60 magnifications, oil lens). C: Western blotting of PP2A expression levels and graphical representations of silencing efficiency after 48-h transfection with siRNA on SKBR3 cell line compared to the negative control siC, at different concentrations of siRNA. B: Immunofluorescent detection of PP2A expression in parental SKBR3, SKBR3 siC and

SKBR3 siP cells. Data shown represent the mean ±SD of triplicate experiments. Bars, SD; **p<0.01 with respect to siC.

6.2.2 Silencing of PP2A/C caused a decrease in pY307-PP2A expression and attenuated tyrosine phosphatase activities

Upon silencing of PP2A/C as described above in section 6.2.1, the expression of pY307-PP2A was also significantly reduced in both BT474 siP and SKBR3 siP as compared to the controls BT474 siC and SKBR3 siC ($p<0.05$), as shown in the immunoblots (Figure 6-2 A).

The effect of silencing on cellular tyrosine phosphatase activity was further assessed. Knocking down of the PP2A/C subunit in both BT474 and SKBR3 cells highly decreased cellular protein tyrosine phosphatase activity when compared to the BT474 siC and SKBR3 siC ($p<0.05$). Noticeably, in comparison with SKBR3 cells, silencing of PP2A/C in BT474 cells showed a higher efficiency in the reduction of tyrosine phosphatase activity with both PP-1 and PP-2 substrates as compared to SKBR3 (Figure 6-2 B).

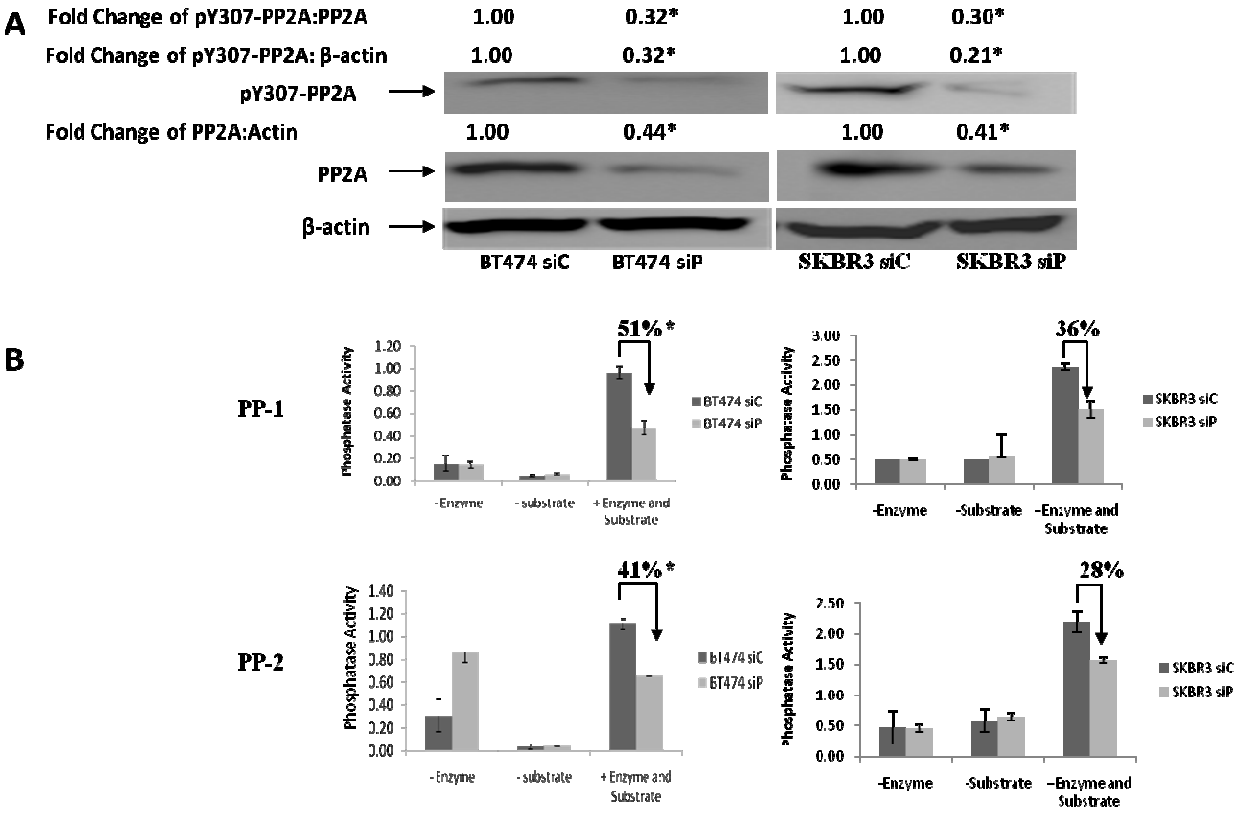


Figure 6-2. Silencing of PP2A/C reduced pY307-PP2A expression and its tyrosine phosphatase activity. A: The expression levels of pY307-PP2A were analyzed in BT474 siC, BT474 siP and SKBR3 siC**,** SKBR3 siP, upon treatment with siRNA for 72 h and 48 h respectively, and subjected to Western blotting analysis. β-actin was used as the loading (baseline) control. The expression of both pY307-PP2A normalized with β-actin expression level and its relative phosphorylation of PP2A normalized to total PP2A (pY307-PP2A/PP2A) were expressed as fold-change relative to the siC controls. B: Tyrosine phosphatase assay was performed, as described in Materials and Methods (section 2.2.14.I) to measure the protein tyrosine phosphatase activity. Purified enzymes were reacted for 5 min at 30^{o}C with Tyr-phosphopeptide-1 (PP-1) and -2 (PP-2). Reactions carried out without addition of enzyme and substrates served as negative controls. Data showed that both PP-1 and PP-2 activities were reduced upon silencing of PP2A/C catalytic subunit in both BT474 and SKBR3 cells. Bars, SD; *p<0.05

6.2.3 Silencing of PP2A/C led to a slight increase of the sub G1 phase in the cell cycle

The phosphorylated PP2A/C, pY307-PP2A, were investigated to check whether they are involved in cell cycle regulation in the HER-2/*neu*-positive cancer cells. As shown in Figure 6-3, knockdown of PP2A/C in both BT474 and SKBR3 cells did not result in any significant changes to the percentage of cells in G0/G1, S and G2/M phases when compared to the scrambled siRNA control cells. However, both siPP2A/Cα-treated BT474 (Figure 6-3 A) and SKBR3 cells (Figure 6-3 B) showed an increased cell population (5-fold and 2-fold increase, respectively) in the sub G1 phase, indicating that the siPP2A/Cα-treated cells had undergone apoptosis.

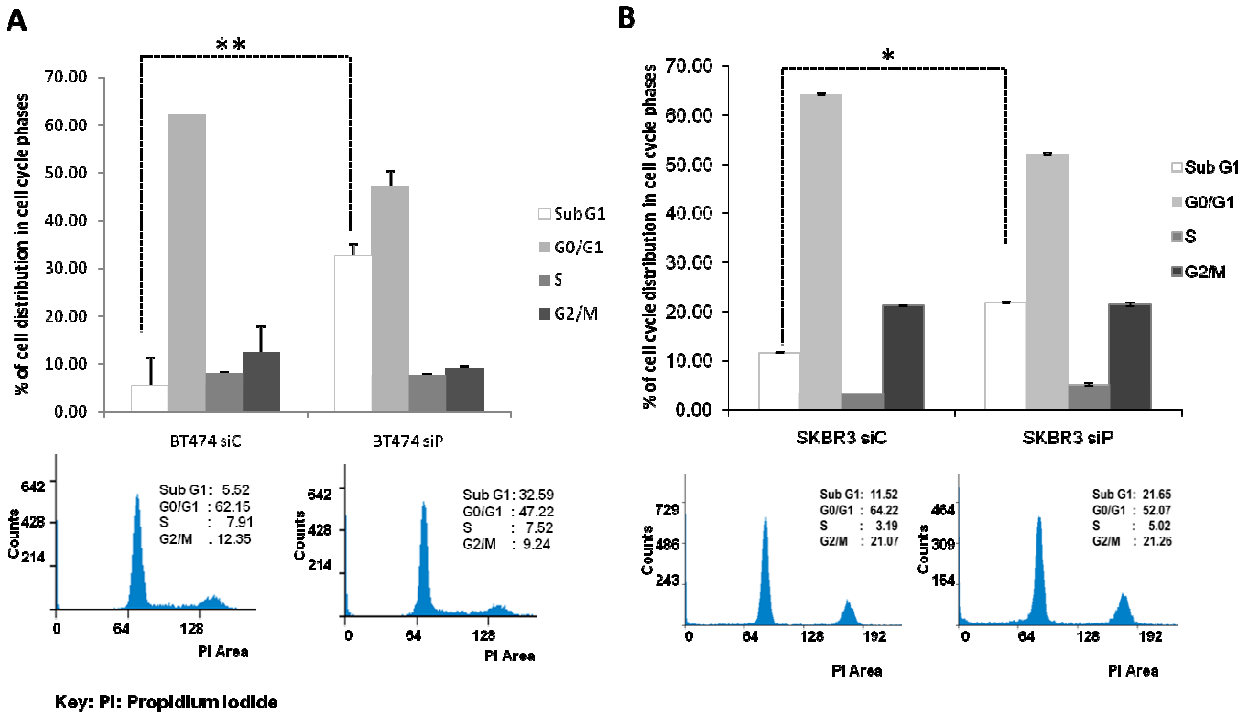
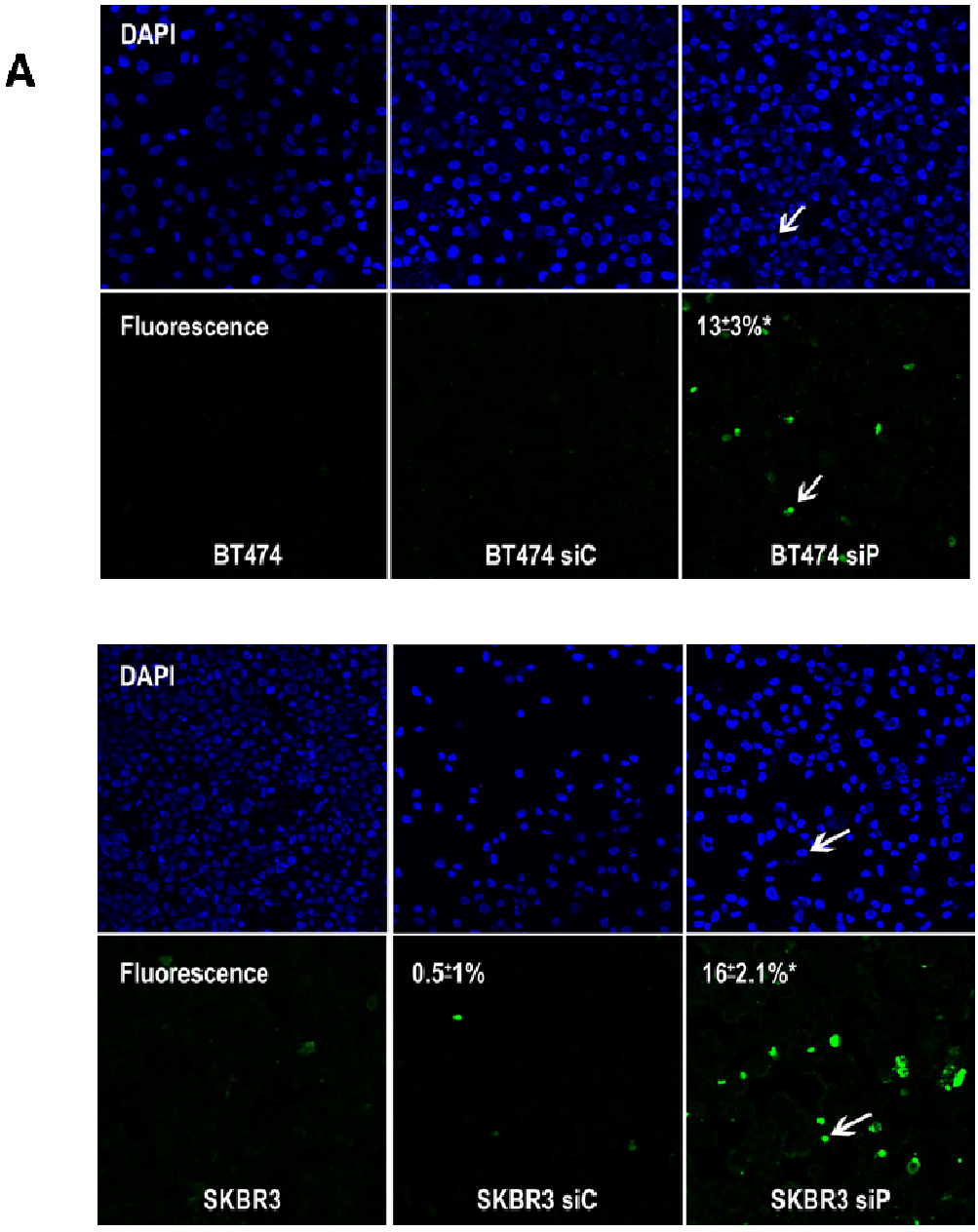


Figure 6-3. Silencing of PP2A/C caused a slight increase of the sub G1 phase in cell cycle. For cell cycle analysis, cells were treated with siRNA, as described in Materials and Methods (section 2.2.14.IV). Cells were washed with PBS and harvested by trypsinization and fixed with ice-cold 70% ethanol. Cells were then stained with 1 ml of propidium iodide/RNAase stain for 1 h and analyzed by flow cytometry. Cell distribution at various phases of cell cycle was expressed as percentage in bar charts (upper panels). Representative of cell cycle diagram of BT474 siC and BT474 siP (lower panels, left) as well as SKBR3 siC and SKBR3 siP (lower panels, right). Note that a more prominent sub G1 peak, suggestive of apoptosis, was evident in both the BT474 (Fig. 5A) and SKBR3 (Fig. 5B) cells, after treatment with siPP2Acα, compared to the negative siC controls. Bars, SD; *p<0.05, **p<0.01

6.2.4 Silencing of PP2A/C facilitated cell apoptosis

To further verify the results presented above in section 6.2.3, the apoptotic activities of both the siPP2A/Cα and siC treated cells were assessed using the TUNEL assay. Silencing of PP2A/Cα in both BT474 and SKBR3 cells significantly enhanced cell apoptosis (Figure 6-4 A). Furthermore, the caspase 3 and its targeted substrate, poly (ADP-ribose) polymerase (PARP), were cleaved in both siPP2A/Cα-treated BT474 and SKBR3 cells, suggesting the involvement of caspase 3 in siPP2A/Cα-driven apoptosis

(Figure 6-4 B). This was also supported by the elevated caspase 3 activities as assessed with the Caspase-Glo®3/7 assay in the siPP2A/Cα-treated BT474 and SKBR3 cells (Figure 6-4 C).



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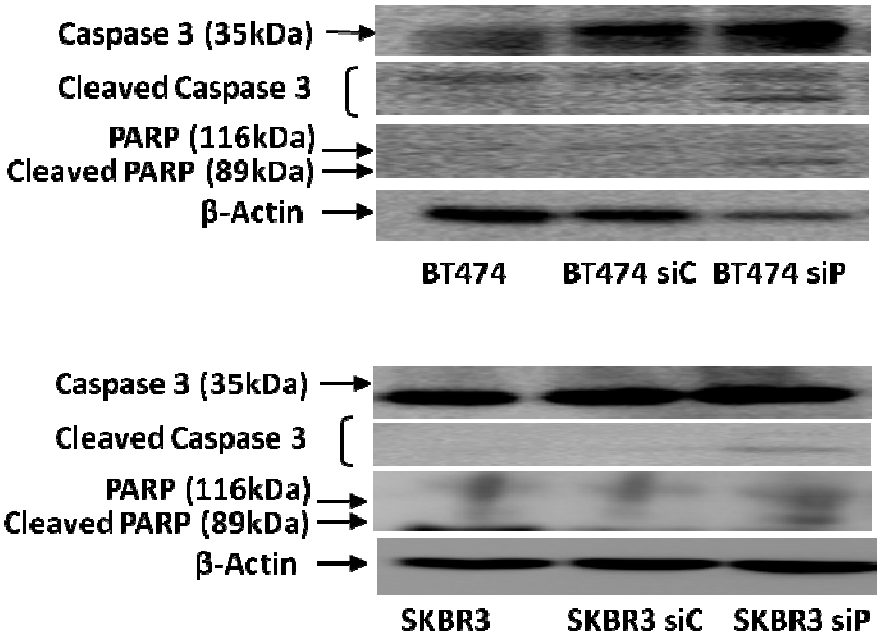
Caspase 3 (35kDa)
Cleaved Caspase 3
PARP (116kDa)
Cleaved PARP (89kDa)
β-Actin
BT474
BT474 siC
BT474 siP
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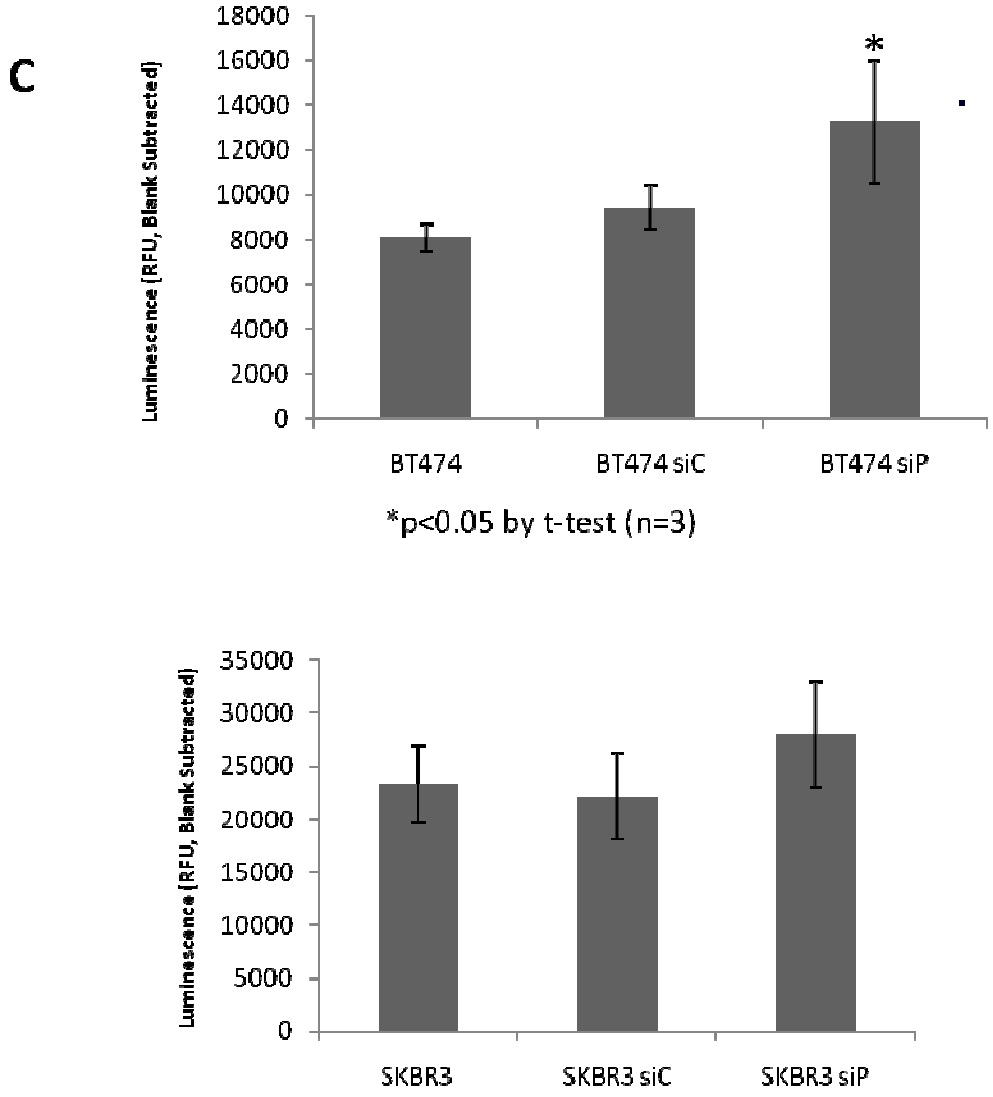

Figure 6-4. Silencing of PP2A/C caused caspase 3-dependent apoptosis. A: The Dapi and fluorescence images are representative of the siPP2A/Cα-driven cell apoptosis results, using the TUNEL assay. DNA of apoptotic cells was labeled with fluorescein-12-dUTP by Terminal Deoxynucleotidyl Transferase (rTdT) and visualized using fluorescence microscopy. The number of apoptotic fluorescent cells (green, as indicated by arrows) was calculated for each sample (five randomly selected fields per sample) and the mean percentage of apoptotic cells ±SD is indicated in the BT474 siP and SKBR3 siP panels. The arrows in the DAPI staining panels indicate nucleus staining. B: Western blotting of caspase 3 and cleaved caspase 3 as well as its target PARP. Cleaved caspase 3 and PARP were detected in both BT474 siP and SKBR3 siP indicating the apoptosis is caspase 3-dependent. C: Cells were plated in 96-well plates and treated with siRNA as described in Materials and Methods (section 2.2.14.III). Caspase-Glo®3/7 reagent was added to the wells and luminescence was measured after 2 h of incubation. Results were

subtracted from those from wells containing culture medium without cells. Both BT474 siP and SKBR3 siP showed enhanced caspase 3 activities compared with the respective siC controls and parental cells. Bars, SD; *p<0.05

6.2.5 Silencing of PP2A/C caused cell apoptosis via the p38 MAPK/Hsp27 signalling pathway

The HER-2/*neu* downstream signalling pathways of PI3K/AKT, Ras/Raf/ERK and p38 MAPK form an essential part of the central network within cells that regulate cell survival and apoptosis. Activation of these pathways is modulated by the phosphorylation status of key modulators such as AKT, ERK and p38 MAPK. The effect of silencing PP2A/C on the phosphorylation status of AKT, ERK 1/2 and p38 MAPK in BT474 siP and SKBR3 siP cells was further examined. As shown in Figure 6-5 A, expression of phosphorylated ERK 1/2 were significantly decreased upon silencing of PP2A/C in both BT474 siP and SKBR3 siP ($p<0.05$). In contrast, expression of the phosphorylated form of AKT was significantly enhanced in BT474 siP by 2-fold compared to the BT474 siC ($p<0.05$). However, this was not observed in SKBR 3 siP (Figure 6-5 B). This may further indicate that silencing of the PP2A/C and its phosphorylation could lead to a decrease in its phosphatase activity that then may possibly result in constitutive activation of kinases such as AKT, especially in the case of BT474 siP-treated cells.

Considering the fact that activation of p38 MAPK leads to cell growth arrest, senescence and/or apoptosis (Tanaka *et al.*, 2003; Aburai *et al.*, 2010; Segreto *et al.*, 2010), further investigation was carried out to determine whether silencing PP2A/C causes any changes in p38 MAPK phosphorylation. The phosphorylation of p38 MAPK was found to be remarkably stimulated in both BT474 siP and SKBR3 siP as compared to BT474 siC

(p<0.01) and SKBR3 siC (p<0.05), as shown in Figure 6-5 C. Activation of p38 signalling was further supported by enhanced phosphorylation of Hsp27 Ser78, a downstream target of p38 MAPK, by silencing of PP2A/Cα in both BT474 (p<0.01) and SKBR3 cells (p<0.05) (Figure 6-5 D). Clearly, these results indicated that siPP2A/Cα-mediated cell apoptosis in HER-2/*neu*-positive cells is involved in a multi-faceted activation/inactivation of the central signalling network and the p38 MAPK pathway could be the pathway mediating apoptosis upon PP2A/Cα silencing.

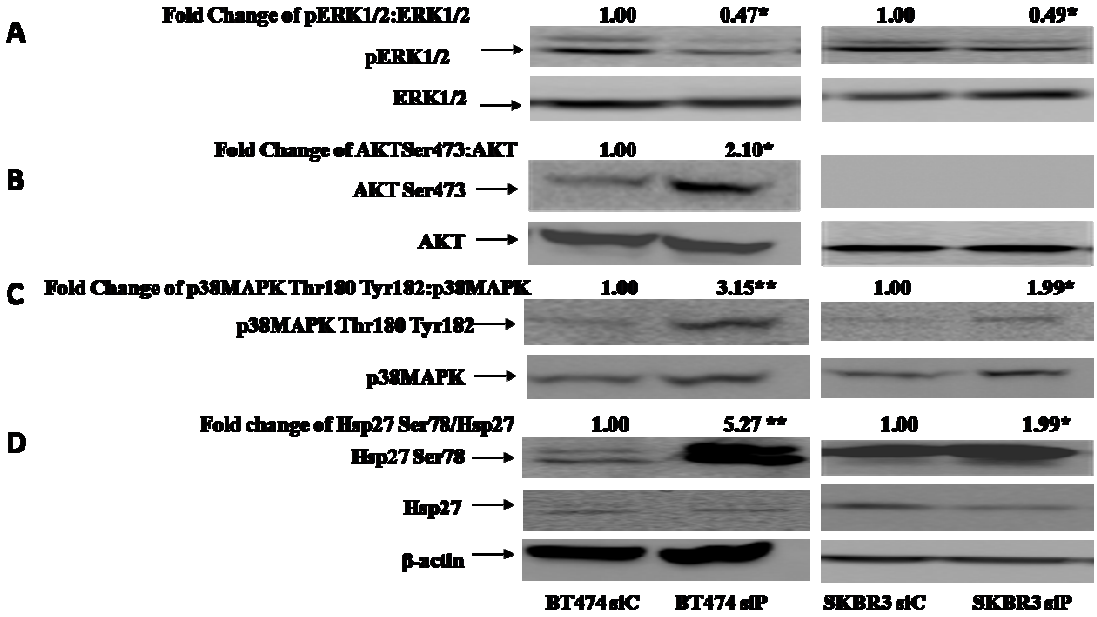

Figure 6-5. Silencing of PP2A/C reduced phospho-ERK 1/2 expression and enhanced phospho-AKT, phospho-p38 MAPK and phospho-Hsp27 Ser 78 expression. Western blotting was performed to compare the downstream modulators of the HER-2/*neu* signalling pathway. A: Relative phosphorylation of ERK1/2 normalized to total ERK1/2. B: Relative phosphorylation of pAKT Ser473 normalized to total AKT. C: Relative phosphorylation of p38 MAPK Thr180 Tyr182 normalized to total p38 MAPK. D: Relative phosphorylation of Hsp27 Ser78 normalized to total Hsp27. β-actin was used as the loading (baseline) control. Relative phosphorylation of each signal modulator was normalized to total basal level and expressed as fold-change of the respective siC controls. Data shown represent the mean ±SD of triplicate experiments. *p<0.05, **p<0.01 (Student *t*-test).

6.3 Discussion

HER-2/*neu* receptors activate a multiplicity of intracellular pathways via their ability to interact with numerous signal transducers. A wide array of antibodies and inhibitors have been invented to exploit them as therapeutic targets (Davoli *et al.*, 2010). Many kinases are involved in the activation of this signalling cascade. However, the roles of phosphatases that facilitate these effects are generally not well defined, although recently there is growing evidence of the importance of PTP1B in HER-2/*neu* tumorigenicity (Arias-Romero *et al.*, 2009; Julien *et al.*, 2007; Bentires-Alj and Neel, 2007; Brown-Shimer *et al.*, 1992; Zhai *et al.*, 1993; Wiener *et al.*, 1994).

Breast cancer cells that overexpress HER-2/*neu*-encoded receptors are dependent on this oncogene for their proliferation and survival. In previous study (Chapter 5), the high expression of pY307-PP2A was detected in HER-2/*neu*-positive tumour samples. In this functional study, BT474 and SKBR3, the two HER-2/*neu*-positive breast cancer cell lines were used as an *in vitro* model to decipher the role of PP2A/C in the HER-2/*neu*-positive breast cancer subgroup. Upon silencing the PP2A/C in both BT474 and SKBR3 cells using siRNA against PP2A/C, the pY307-PP2A expression level was significantly decreased relative to the decrease in the PP2A expression level. This further implied that the phosphatase activity of PP2A had been reduced. The tyrosine phosphatase assay also showed a markedly decreased activity in both BT474 and SKBR3 cells. These observations are consistent with the cellular pro-oncogenic role of activated protein tyrosine phosphatase such as PTP1B. PTP1B expression is linked to promotion of aberrant cell division and survival in human breast epithelial cells (Arias-Romero *et al.*,

2009). Given that PTP1B is required for HER-2/*neu* transformation and its association with PP2A is critical for PP2A activation, it is postulated that the decrease in tyrosine phosphatase activity could be contributed by PTP1B. However, the association of PTP1B-PP2A and HER-2/*neu* remains to be further elucidated. These results suggested that silencing of the PP2A/C leads to reduced tyrosine phosphatase activity that could be partly mediated by PTP1B, and thus, contribute to the deceleration of the cell proliferation and survival rate in HER-2/*neu* breast cancer cells.

Removal of PP2A/C has been proven to be fatal to cells. Deletion of the PP2A/Cα in homozygous null mutant mice, was embryonically lethal (Götz *et al.*, 1998). Knocking down of PP2A/A or /C or both B56-targeting subunits led to development of the morphological and biochemical changes characteristic of programmed cell death (Li *et al.*, 2002). He *et al* found that silencing of PP2A/C greatly decreased PP2A activity and developed localized death in plant stems and cells (He *et al.*, 2004). In this study, a sub-G1 peak which is an indicator of early apoptosis was observed when the PP2A/C was silenced. In addition, the induction of siPP2A/Cα caused a noticeably accelerated cell death in both BT474 and SKBR3 cells. This finding was further supported by both enhanced caspase 3 activities and evidence of cleaved caspase 3 and PARP in Western and immunoblots in siPP2A/Cα treated cells. The apoptosis induced by silencing PP2A is therefore caspase 3-dependent. Altogether, these findings clearly supported the role of PP2A in regulating HER-2/*neu* positive breast cancer signalling.

PP2A was reported to promote apoptosis by dephosphorylating p38 MAPK in

neutrophils. Dephosphorylation of p38 MAPK in turn was reported to reconstitute the impaired caspase 3 activity and enabling the activated caspase 3 to initiate the apoptosis in the neutrophils (Alvarado-Kristensson and Andersson, 2005). However, the above results was intriguing as silencing of PP2A/C was shown to cause enhanced phosphorylation of p38 MAPK which later on initiated apoptosis via caspase 3 activity. This finding indicates that PP2A plays a role in regulating apoptosis via modulating p38 MAPK phosphorylation in a cell-type dependent manner. These results agreed with the recent report by Boudreau *et al.* showing a direct association between PP2A and p38 MAPK in human T leukemia cells (Boudreau *et al.*, 2007). They found that PP2A was involved in maintaining the equilibrium that exists between cell survival and cell death in cultures of T leukemia cells and also demonstrated the contribution of p38 MAPK to cellular homeostasis in leukemia cells.

Interestingly, in another study (Cairns *et al.*, 1994), PP2A had been reported as primarily responsible for the dephosphorylation/phosphorylation of Hsp27 in MRC-5 fibroblasts cells. Cairns J *et al*. showed that when the PP2A/C was phosphorylated at its tyrosine residue, it was no longer able to dephosphorylate Hsp27 (Cairns *et al.*, 1994). Our group had earlier shown that the phosphorylation of Ser78 of Hsp27 was correlated with HER-2/*neu* status and lymph node positivity (Zhang *et al.*, 2007), which corroborated with the suggestion that phosphorylation of Hsp27Ser78 in the HER-2/*neu* cells was predominantly mediated by the p38 MAPK pathway, although the specific role of Hsp27 Ser78 has yet to be fully elucidated. Here, Hsp27 Ser78, regulated by p38 MAPK, was found to be highly phosphorylated upon silencing of PP2A/C. This further demonstrated

the significant correlation of p38 MAPK/Hsp27 with PP2A.

Knocking down PP2A/C or its regulatory subunits results in apoptosis (Götz *et al.*, 1998; Li *et al.*, 2002; Strack *et al.*, 2004; Kong *et al.*, 2004). However, we cannot completely exclude the possibility that silencing PP2A/C also changes the binding of regulatory B subunits and this may cause the aberrant response of cell apoptosis. It is therefore necessary to identify the variable B subunit complexes that may play a role or are responsible in directing the phosphorylation cascade in HER-2/*neu* activated signalling pathway.

This model summarized the findings of this theses (Figure 6-6):

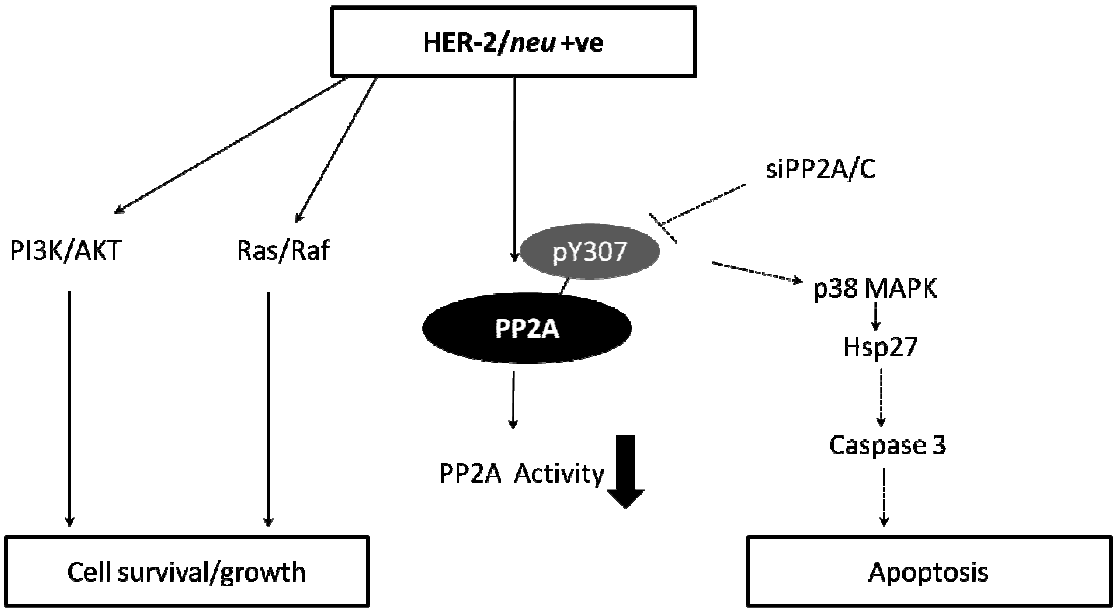

Figure 6-6. Proposed role of PP2A in HER-2/*neu* signalling pathway. The schematic diagram proposes that inhibition of the PP2A/C and its phosphorylation lead to a decrease in its phosphatase activity that could possibly result in constitutive activation of kinases such as PI3K/AKT that play a contributory role in aggressive tumour growth. The

counteracting signal modulator, p38 MAPK, however, is therein activated, by enhanced phosphorylation of p38 MAPK and Hsp27 that trigger the cells to undergo apoptosis in a caspase-3 dependent manner in HER-2/*neu* positive breast cancer cell lines.

In this model, we propose that inhibition of the PP2A/C and its phosphorylation lead to a decrease in its phosphatase activity, that could possibly result in constitutive activation of kinases such as AKT that play a role in aggressive tumour growth. The counteracting signal modulator, p38 MAPK, however, is therein activated, triggering the cells to undergo apoptosis via the caspase 3-dependent pathway and it would appear from this study that the latter predominates, at least in the BT474 and SKBR3 breast cancer cell lines.

In summary, these results suggest that inhibition of PP2A/C-induced apoptosis in HER-2/*neu* breast cancer cells occurs via p38 MAPK/Hsp27 mediation, in a caspase 3-dependent manner. Therefore, by targeting the PP2A/C in HER-2/*neu*-positive cancer cells, it may reduce the aggressiveness of the cancerous cell proliferation rate. These findings imply that patients with HER-2/*neu*-positive breast cancers may benefit from the inhibition of pY307-PP2A as a form of targeted therapy.

CHAPTER 7

GENERAL DISCUSSION AND CONCLUSION

7.1 The significance of deciphering the role of tyrosine phosphorylation in HER-2/*neu* breast cancer cells

Phosphorylation, in particular tyrosine phosphorylation, is featured prominently in the biology of cancer. The tyrosine kinase and their substrates are potential molecular cancer markers that aid in predicting clinical outcome and responses to drug (Lim, 2005). Understanding of the tyrosine phosphorylation in HER-2/*neu* positive breast cancer could help in discovering attractive drug targets. For example, trastuzumab was the first biological agent developed and approved for the use in HER-2/*neu* breast cancer treatment. Trastuzumab inhibits HER-2/*neu* activation and subsequently interrupts its downstream pathways. Other tyrosine kinase inhibitors such as Lapatinib, Neratinib, *etc.* are currently being developed to treat HER-2/*neu*-positive breast cancer patients by inhibiting the tyrosine phosphorylation that leads to activation of downstream growth promoting pathways (Roy and Perez, 2009).

This study showed that activation of HRG-mediated HER-2/*neu* signalling causes tyrosine phosphorylation in a group of signal transductors in HER-2/*neu*-positive breast cancer cells. HRG, a 45kDa protein existing in multiple secreted isoforms derived from alternatively spliced genes (Tan *et al.*, 1999), was used to trigger the activation of HER-2/*neu* signalling. Studies elsewhere had shown that tyrosine phosphorylation was expressed in response to HRG in NIH 3T3 cells expressing both HER-2 and HER-3 or HER-2 and HER-4 (Reese and Slamon, 1997). The effects of activation of HER-2/*neu* on

its phosphorylation status as well as those of key downstream modulators such as PI3K/AKT and Ras/Raf/ERK were examined and confirmed by western blotting. In addition, an antibody array was employed to study the tyrosine phosphorylation profiles of key metabolites involved in the HER-2/*neu* activating pathway. The results showed that HER-2/*neu* activation is able to trigger many other signal modulators. Of special interest to us was the finding that pY307-PP2A is highly activated upon HRG activation and that it is related to breast cancer progression. Pharmacological inhibition with the HER-2/*neu* inhibitor AG825, PI3K inhibitor LY294002, MEK1/2 inhibitor PD98095, and p38 MAPK inhibitor SB203580, further confirmed that PP2A phosphorylation was modulated by the complicated, HER-2/*neu*-driven downstream signal network, with the PI3K and MEK1/2 positively, while the p38 MAPK negatively, regulating its tyrosine phosphorylation. These data confirmed the correlation of HER-2/*neu* activation and PP2A tyrosine phosphorylation, which led us to further study the functional role of PP2A.

The correlation of PP2A tyrosine phosphorylation with HER-2/*neu* and its association with tumour progression was first reported by our group (Wong *et al.*, 2009). To substantiate that finding, much effort was further needed to study the role of pY307-PP2A in HER-2/*neu* signalling and to translate the basic research knowledge to clinical usage. The work presented in this thesis represent our approaches in understanding the tyrosine phosphorylation regulated by HER-2/*neu* signalling, which hopefully has contributed to the discovery of a potential phosphoprotein biomarker of breast cancer biology.

7.2 Antibody array-based technologies for cancer protein profiling and functional proteomics analyses

A signal transduction antibody array was used in this study to decipher the tyrosine phosphorylation profile of the key signalling modulators that are regulated by HRG-mediated HER-2/*neu* signalling pathways. Previous work by many other research groups had unraveled the different pathways that are related to HER-2/*neu* signalling such as the MAPK pathway, PI3K/AKT signalling pathway, phospholipase C-γ (PLC)-associated pathway, cell cycle regulation, wnt signalling pathways, NF-kappa B pathways, *etc* (Reese and Slamon, 1997; Zhou and Hung, 2003). From this recent work, other additional signal modulators that had not been previously reported to be related to HER-2/*neu* were unveiled. 78 tyrosine-phosphorylated proteins and 2 tyrosine-dephosphorylated proteins, tyrosine phosphorylation of which were stimulated or suppressed following HRG-enhanced HER-2/*neu* signalling were identified. Four of them, namely, Ezrin, PP2A, FADD and ARC, were validated as reported in Chapter 3. Each of these signal molecules wields an impact in controlling the cellular function and fate. This work further emphasized the complexity of HER-2/*neu*-driven biology within the cancer cells, which contribute to their high proliferation rate and aggressive behaviour.

Antibody arrays have become a prominent tool for gathering information required to better understanding of disease biology. Comparing to cDNA analysis by microarray used in gene expression profiling, antibody arrays can provide additional information on protein interactions, localization and correlation. It is widely known that the mRNA

transcript abundance level might not directly correlate with the protein abundance level as not all mRNA is translated into protein. Thus the use of antibody arrays in the characterization of signalling networks and in cancer biomarker discovery (Alhamdani *et al.*, 2009; Sanchez-Carbayo, 2010). Hudelist *et al.* identified a number of proteins that showed increased expression levels in malignant breast tissues, such as casein kinase Ie, p53, annexin XI, CDC25C, eIF-4E and MAP kinase 7, using a protein microarray system which contains 378 well characterized monoclonal antibodies. They were investigating the differential gene expression patterns of malignant and adjacent normal breast tissues in a patient with primary breast cancer (Hudelist *et al.*, 2004). Other reports on bladder cancer (Sanchez-Carbayo *et al.*, 2006), colorectal cancer (Sreekumar *et al.*, 2001; Hao *et al.*, 2008; Madoz-Gúrpide *et al.*, 2007), prostate cancer (Miller *et al.*, 2003; Shafer *et al.*, 2007; Fujita *et al.*, 2008), pancreatic cancer (Orchekowski *et al.*, 2005; Chen *et al.*, 2007; Ingvarsson *et al.*, 2008) and lung cancer (Kullmann *et al.*, 2008; Gao *et al.*, 2005) have showed the power of antibody array in providing useful, clinically relevant data.

Precautionary steps were carried out when using the antibody array. The specific downstream proteins such as Shc, Stat1 and AKT, which were previously known to be upregulated by HER-2/*neu* signalling, were identified by the array were checked and validatedby Western blot analysis. In each case, the results obtained by Western blots matched the data obtained by the antibody array system. This is important so as to avoid any misinterpretation of the array results. The vast majority of antibody arrays have been generated using monoclonal and polyclonal antibodies that are commercially available. There are legitimate concerns about the performance of these antibodies. In this study,

antibodies bought from other companies and not from the antibody array provider were used for validation by Western blots. This measure assured the authenticity of the findings.

7.3 PP2A as a tumour suppressor or proto-oncogene?

A few phosphatases have been shown to have dual specificity. Some phosphatases, such as CDC25, have been shown to be potential human oncogenes. Both CDC25A and CDC25B are important regulators of cell cycle progression and are able to transform cell in culture and therefore are oncogenic. CDC25B was overexpressed in 32% of human primary breast cancers tested (Galaktionov *et al.*, 1995). On the other hand, PTEN, the phosphatase of the PI3K and AKT pathways, was found to be a tumour suppressor as mutation and deletion of this gene was found in various cancer cell types (Simpson and Parsons, 2001).

There are evidences showing that PP2A is a negative regulator of cell growth and as a potential tumour suppressor (Guy *et al.*, 1992; Schönthal, 2001). However, there are a couple of observations contradicting this view and proposing that PP2A exerts both positive and negative effects in cell growth regulation and tumorigenesis.

The two main evidences for PP2A as a tumour suppressor are its potential contributions to cellular growth and its involvement in cellular transformation. Okadaic acid (OA) has been widely used to bind the PP2A/C and shown to efficiently block its enzymatic activities (Bialojan and Takai, 1988). OA is a potent tumour promoter; hence inhibition

of OA decreases the phosphatase activity and hence contributes to tumour formation. Cells that are treated with OA demonstrated enhanced phosphorylation in an array of cellular proteins (Guy *et al.*, 1992). PP2A is inactivated by this compound and causes elevated expression of c-fos and c-jun, two proto-oncogenes regulating cell proliferation (Schönthal, 2001). Furthermore, treatment of OA activates the MAP kinase pathway and thus acts as a negative regulator of cell growth (Sontag *et al.*, 1993). In addition, PP2A plays a role in inhibition of transformation. For example, PP2A can be inactivated in response to cell growth stimulation by receptor tyrosine kinase or non-receptor tyrosine kinase (Chen *et al.*, 1994). PP2A also inhibits telomerase, an enzyme that is found to be elevated in cancer cells (Li *et al.*, 1997). In colorectal cancers in which the APC tumour suppressor is mutated, PP2A rapidly dephosphorylates β-catenin and prevents it from entering into the downstream ubiquitination machinery, leading to its stabilization and high levels of accumulation and the development of cancer (Su *et al.*, 2008). In another study, increased PP2A/C was found to reduce the rate of transformation of mouse fibroblasts by H-Ras and the polyoma virus medium T oncogenes *in vitro* (Baharians and Schönthal, 1999).

Studies also revealed that mice lacking the PP2A/C alpha isoforms are not viable (Götz *et al.*, 1998), and impaired function of PP2A may contribute to tumorigenesis. PP2A has been shown to be a regulator of Raf-1, a (proto)oncogene product -- PP2A is able to form a stable complex with Raf-1 and thereby facilitate kinase activation (Abraham *et al.*, 2000). This finding is indeed surprising as PP2A inhibits other modulators further downstream of MAP kinase pathway (Schönthal, 2001). PP2A may have both a positive

and a negative role in cell cycle regulation. In the G1 phase, inhibition of PP2A activity results in the decreased expression of cyclin D2, cyclin E and cyclin A, which are essential regulatory subunits of the CDK complexes and results in G1 phase arrest (Yan and Mumby, 1999; Schönthal and Feramisco, 1993). Thus, PP2A is required for cells to progress through the early phases of the cell cycle to enter S phase. On the contrary, during the G2/M phase transition, PP2A inhibits the activation of M-phase-specific CDKs, such as cdk-1 cyclin B complex, which are essential for entry and progression through this phase of the cell cycle (Kawabe *et al.*, 1997; Kinoshita *et al.*, 1993; Félix *et al.*, 1990; Clarke *et al.*, 1993; Lee *et al.*, 1994).

In this study, siRNA was chosen and not OA, which has been widely used by other groups, to study the role of PP2A. OA has been shown to inhibit other phosphatases such as serine/threonine phosphatase 1, PP1 (Dawson and Holmes, 1999). Therefore, an experiment design that uses solely OA may have to be interpreted carefully since there is a potential that other than PP2A, the other phosphatases are inhibited as well. Alternatively, a combination of OA, siRNA and other drugs can be tried out and used, if they yield improved results.

From the above, there are prevailing evidences to support PP2A as a potent tumour suppressor. However, unlike other classical tumour suppressors such as p53 and RB, mice with deficient p53 and RB are viable though prone to tumour development (Sharan and Bradley, 1995), whereas mice with deficient PP2A are lethal (Götz *et al.*, 1998). Whilst p53 and RB inactivation or inhibition promotes proliferation, inactivation of PP2A

or deletion, in contrast, promotes cell death. In this study, PP2A is associated with tumorigenesis and loss of PP2A/C does not cause cell proliferation but causes apoptosis instead. Hence, PP2A cannot be classified as a tumour suppressor in this case but more as a proto-oncogene. In the light of the diversity of PP2A subunits and their ability to form a large repertoire of different holoenzyemes, PP2A has been described as a multitasking enzyme and characterization of the relevant combinations and assigned roles will be an important and challenging study.

7.4 The role of PP2A in HER-2/*neu*-overexpressing breast cancer

Treatment of HER-2/*neu*-positive breast cancer has undergone advanced improvement with the current focus on targeted therapy, from use of Trastuzumab in chemotherapy to the imminent introduction of Lapatinib as well as other tyrosine kinase inhibitors. Hence, it is important to understand the mechanisms of action of HER-2/*neu* and to further develop new therapeutic options in controlling the aggressiveness of the cancer cells.

From literature reviews, PP2A has been shown to play an important role in control of cell cycle, apoptosis and cell fate determination. The PP2A/C makes up to 0.1% of total cell protein and is encoded by two genes, the Cα and Cβ subunits. The levels of PP2A/C are tightly controlled in the cell at the translational or post-translational levels. From this study, targeting the expression of PP2A/C in HER-2/*neu*-positive breast cancer cells could result in the cancer cells undergoing apoptosis. This is an important finding since HER-2/*neu* gene amplification is associated with increased cell proliferation, cell motility, tumour invasiveness, progressive regional and distant metastases, accelerated

angiogenesis, and reduced apoptosis (Moasser, 2007). This present study revealed that following HER-2/*neu* activation, the activated PI3K- and Raf/MEK pathways stimulate PP2A phosphorylation and attenuate its activity to facilitate cell survival, whereas the activated p38 MAPK pathway reduces PP2A phosphorylation and increases its activity to accelerate cell apoptotic activity. This data showed the important role of PP2A in HER-2/*neu* oncogenic signalling. This is first time reported that the inhibition of the PP2A/C and its phosphorylation not only lead to a decrease in its phosphatase activity that could possibly result in constitutive activation of kinases such as AKT that play a role in aggressive tumour growth, but also at the same time trigger the counteracting signal modulator p38 MAPK. Thus, the cells are committed to undergo apoptosis via the caspase 3-dependent pathway. These findings provide an understanding of the role of PP2A in HER2/*neu*-positive breast cancer cells and thus provide insight into new targets for breast cancer therapy.

7.5 Use of siRNA in cancer therapy

The tissue microarray study supported the strong association of pY307-PP2A with breast tumour progression and postulated that pY307-PP2A has a potential prognostic value in determining breast cancer progression. In HER-2/*neu*-positive breast cancer cell lines and breast tumour samples, the higher expression of pY307-PP2A showed that pY307-PP2A could serve as a target therapy to block off tumorigenesis by its inherent property of reactivating PP2A activity. To reactivate the PP2A activity, expression of pY307 needs to be decreased since the phosphatase activity is regulated by pY307. Alternatively, the phosphoryation of pY307-PP2A could be reduced or inactivated by inhibitors or siRNA.

In the functional studies of PP2A/C in HER-2/*neu*-positive cells, siRNA was used to target the catalytic subunit where the pY307 resides and knock down its expression. The results showed that these cells undergo apoptosis. Therefore, it will be exciting if either an activator of PP2A activity or an inhibitor of its phosphorylation could be developed to treat the aggressive tumours cells in HER-2/*neu*-positive breast cancer patients, in combination of other forms of chemotherapy.

Recently, a nanomedicine-based approach for the delivery of siRNA in cancer has become a hot topic, in relation to achieving personalized cancer treatment. The first targeted delivery of siRNA in a patient was administered with four doses of Calando's CALAA-01 for the treatment of cancer in a clinical trial. This development showed another milestone for siRNA technology, which many proponents have high hopes for in the treatment of a diverse range of illness (Davis, 2009). Ongoing clinical trials using siRNA involve chemically modified or naked siRNA and locally administered siRNA to topical diseases such as age-related macular degeneration, diabetic macular oedema and respiratory virus infection and pachyonychia congenital (Fattal and Bochot, 2006; Bitko *et al.*, 2005). Recently, systemic administrations of siRNA in ongoing clinical cancer trials are gaining popular. Nanoliposomal siRNA was shown to be more effective for systemic delivery of siRNA into tumour tissue and a Phase 1 clinical trial using this nanoliposomal delivery mode is ongoing (Ozpolat *et al.*, 2010). Eguchi *et al.* created a peptide-transduction domain-double stranded RNA binding domain (PTD-DRBD) fusion protein to bind the siRNA and deliver it into primary tumour cells where it specifically targets mRNA from cancer-promoting genes without being toxic to the cells (Eguchi *et*

al., 2009; Eguchi and Dowdy, 2010). In glioblastoma treatment, combinatorial targeting of EGFR and AKT2 (but not AKT1 or AKT3), by PTD-DRBD delivered siRNAs synergized to induce tumour cell-specific apoptosis. *In vivo* PTD-DRBD delivery of EGFR and AKT2 siRNAs induced tumour-specific apoptosis and significantly increased survival in intracerebral glioblastoma mouse models ($p < 0.0005$), whereas delivery of irrelevant control siRNAs did not alter longevity. Thus, siRNA-induced synthetic lethal RNAi responses have great potential for personalized treatment of cancer (Michiue *et al.*, 2009).

With the aids of biotechnology, development of appropriately designed siRNAs targeting the pY307-PP2A to turn off the insistent growth switch in HER-2/*neu*-positive breast cancer cells to grow and proliferate, will be feasible. Both conventional cancer therapy using chemotherapy and radiotherapy cause complications to the cancer patients. The advent of nanomedicine combined with siRNA therapy, which appears to have less cytotoxicity and minimal side effects, offer promising options for cancer treatment.

7.6 Conclusion

Cancer research is a challenging field to work on. Each time when a particular gene/protein function is unravelled, it helps the reseachers to have a better understanding of the disease pathways and hopefully offer a better clinical treatment to the affected patients.

Deciphering the signalling pathway in cancer initiation and progression has paved the

way for the development of numerous therapeutic leads. To this end, PP2A appears to be a new candidate in regulating the oncogenic role of HER-2/*neu*. Recent studies on a few potent and selective PP2A inhibitors such as cantharidin and its derivatives have shown therapeutic effects against various cancers by attenuating the growth of cancer cell through G2/M cell cycle arrest and apoptosis (Huh *et al.*, 2004) (Li *et al.*, 2010). Here, the study demonstrated that silencing of PP2A/C caused HER-2/*neu*-positive breast cancer cell lines to undergo apoptosis via the p38 MAPK-mediated caspase 3/PARP pathway.

7.7 Future works

This study provides another platform for understanding the role of tyrosine phosphorylation in HER-2/*neu*-positive breast cancer. The novel findings of correlation of HER-2/*neu* and PP2A will provide the groundwork for better understanding of the aggressiveness of HER-2/*neu* phenotype. As only the functional study of PP2A was examined in this study, it will be beneficial if the other identified proteins could be further analyzed. These proteins include those involved in cell cycle regulation, cell apoptosis and cell growth.

In vitro system was used to examine the functional role of PP2A/C in HER-2/*neu*-positive breast cancer cell lines in this study. *In vivo* studies should be followed up next to assess the functions of PP2A/C in a knock-out model, employing both genetic and pharmacological approaches, before the knowledge can be translated into drug development. Transgenic mice (NDL2 mice) with a deletion mutation in the region of

ErbB2 encoding its extracellular domain have been generated by Siegel *et al*. These transgenic NDL2 female mice develop multiple mammary tumours with elevated levels of ErbB2 and ErbB3 with phosphorylated tyrosine. Furthermore this mouse model mimics human breast cancer as the alternative spliced form of ErbB2 that is analogous to the ErbB2 deletion mutant in NDL2 has been found in human breast cancer (Siegel *et al.*, 1999). Deletion of the PP2A activity in the NDL2 transgenic mice by either breeding with PP2A/C-deficient mice or by treatment with PP2A siRNA or specific inhibitor is proposed and followed by examining the results of the mammary gland and tumorigenesis. With this approach, it will enable us to understand the role of PP2A/C in tumorigenesis and investigate whether the development of PP2A inhibitor or siRNA therapy can be used as a new targeted treatment on HER-2/*neu*-overexpressing breast cancer patients.

Furthermore, the TMA result demonstrated that PP2A is associated with breast cancer progression and was highly expressed in HER-2/*neu*-positive breast cancer. It will be important to conduct an extended study with a bigger number of patient samples, and correlate the results with other clinicopathological parameters, such as tumour stage and AJCC staging. This will enhance our understanding of the role of PP2A in HER-2/*neu*-positive breast cancer in Asian women.

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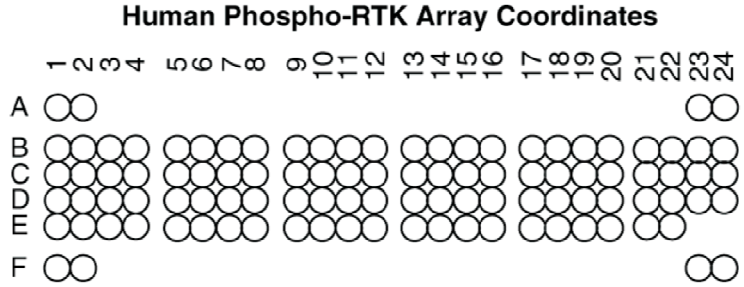
APPENDICES

Table 1: Human phospho-RTK array. Refer to the table below for the human phospho-RTK array coordinates.



Coordinate	Receptor Family	RTK/Control		Coordinate	Receptor Family	RTK/Control
A1, A2	Control (+)	PY-Control*		D1, D2	Tie	Tie-2
A23, A24	Control (+)	PY-Control*		D3, D4	NGF R	TrkA
B1, B2	EGF R	EGF R		D5, D6	NGF R	TrkB
B3, B4	EGF R	ErbB2		D7, D8	NGF R	TrkC
B5, B6	EGF R	ErbB3		D9, D10	VEGF R	VEGF R1
B7, B8	EGF R	ErbB4		D11, D12	VEGF R	VEGF R2
B9, B10	FGF R	FGF R1		D13, D14	VEGF R	VEGF R3
B11, B12	FGF R	FGF R2α		D15, D16	MuSK	MuSK
B13, B14	FGF R	FGF R3		D17, D18	Eph R	EphA1
B15, B16	FGF R	FGF R4		D19, D20	Eph R	EphA2
B17, B18	Insulin R	Insulin R		D21, D22	Eph R	EphA3
B19, B20	Insulin R	IGF-I R		D23, D24	Eph R	EphA4
B21, B22	Axl	Axl		E1, E2	Eph R	EphA6
B23, B24	Axl	Dtk		E3, E4	Eph R	EphA7
C1, C2	Axl	Mer		E5, E6	Eph R	EphB1
C3, C4	HGF R	HGF R		E7, E8	Eph R	EphB2
C5, C6	HGF R	MSP R		E9, E10	Eph R	EphB4
C7, C8	PDGF R	PDGF Rα		E11, E12	Eph R	EphB6
C9, C10	PDGF R	PDGF Rβ		E13, E14	Control (-)	Mouse IgG_1
C11, C12	PDGF R	SCF R		E15, E16	Control (-)	Mouse IgG_{2A}
C13, C14	PDGF R	Flt-3		E17, E18	Control (-)	Mouse IgG_{2B}
C15, C16	PDGF R	M-CSF R		E19, E20	Control (-)	Goat IgG
C17, C18	RET	c-Ret		E21, E22	Control (-)	PBS
C19, C20	ROR	ROR1		F1, F2	Control (+)	PY-Control*
C21, C22	ROR	ROR2		F23, F24	Control (+)	PY-Control*
C23, C24	Tie	Tie-1				

*Phospho-Tyrosine Positive Control

Table 2: The signal transduction antiboday array. Refer to table below for the antibody list by position on the array.

	A	B	C	D	E	F	G	H	I	J
1	14-3-3	c-Abl	ACINUS	ACINUS-p23	AIF	Akt 1/2	ALK	Amphiphysin	Bin 1	Ankyrin
2	Bad	Bak	Bax	BOK	NBK	Bag-1	Bcl-2	Bcl-w	Bcl-xS/L	A1
3	BRCA2	Brk	Brm	Btk	C/EBP beta	E-Cadherin	N-Cadherin	VE-Cadherin	Pan-Cadherin	BLCAM
4	Caspase6	Caspase7	Caspase8	Caspase9	Caspase10	a-Catenin	b-Catenin	r-catenin	c-Cbl	CBP
5	Cdk1 / Cdc2	Cdk2	Cdk4	Cdk6	Chk	C-IAP1	C-IAP2	CIDE-A	CIDE-B	Clathrin
6	Cyclin A	Cyclin B	Cyclin D3	Cyclin E	Cyclin H	cytochrome	DAXX	DCC	Desmoglein	DFF45 / ICAD
7	E2F1	EGFR	p-EGFR	Egr-1	Egr-2	Egr-3	Elongin A	EphA1	EphA4	EphB1
8	FAF-1	FAK	Fas	FasL	FAST	FGFR1	FGFR2	FGFR3	FGFR4	FHIT
9	GATA-1	GATA-2	GATA-3	G-CSF R	gp130	Granzyme	GRB2	GRB7	GRB14	GRK 2
10	IEXs/1	IFN-a R a	IFN-g R a	IL 1 R1	IL 2 R alpha	IL 2 R, beta	IL 2 gamma	IL 3	IL4R a	ING1-p33
11	ISGF3 gamma	Jak1	Jak2	Jak3	JNK1,2,3	p-JNK1,2,3	c-Jun	p-c-Jun	KAP	c-Kit
12	MEF2	MEK1	MEKK1	MEKK2	Menin	Met	MGMT	MMp-3	MMp-9	Mos
13	Ikappa B-a	p-Ikappa B-a	Ikappa B-b	Ikappa B-r	Ikappa B-e	Ikappa B kinase a	Ikappa B kinase b	Nibrin	NIK	nip1
14	p19Skp1	p21WAF1 / CIP1	p27	p35	p38 MAPK	p-p38	p45 skp2	p53	p55 CDC	p57 (Kip2)
15	PDGF	PDGF Receptor b	Phospholipase Cr	Phospholipase D	PI3kinase	PKC α,β,χ	Polo-like kinase	PP1,2A,2B,PPX	pp120	PSD-95
16	RACK1	Rad51	Rad52	c-Raf-1	p-c-Raf-1	RAIDD	RalA	Ran BP-1	Rap1	Rap2
17	Rel B	Ret	Rho A	RICK	RIP	Ron alpha	Rsk-1	Sam68	E-selectin	L-Selectin
18	sp1	sp2	Blk	c-Fgr	fyn	Lck	Lyn	c-Src	Yes	SRF
19	SURVIVIN	Syk	Syntaxin6	TANK	TCR alpha	TCR beta	TDAG51	TGFb R1	TGFb R2	Thyroid R a1
20	TRAF5	TRAF6	TRAIL	TrkA, B, C	TSG101	TTK	Tuberin	Tyk2	VASP	Vav

Table 2: The signal transduction antiboday array (continued)

	K	L	M	N	O	P	Q	R	S	T
1	Annexin VI	ANT	Apaf1	APC	ARC	ATF-2	p-ATF-2	Axl	B7-1	B7-2
2	Mcl-1	Bcl-3	Bcl-6	BID	Bim	BM28	BMX	BAP1	BARD1	BRCA1
3	HCAM	ICAM-1	PECAM-1	VCAM-1	CAS	Caspase1	Caspase2	Caspase3	Caspase4	Caspase5
4	CD 3 epsilon	CD27	CD28	CD30	CD40	CD45	Cdc6	Cdc25A	Cdc34	CDC42GAP
5	Clusterin	Connexin 32	Connexin 43	Cortactin	CPAN	CREB	CREM-1	Crk	Csk	CUL-1
6	DIVA	DMBT1	DMC1	DcR1	DcR2	DR3	DR4	DR5	Dynamin	Dynamin II
7	eps8	erbB2	erbB3	erbB4	ERK1	ERK2	Estrogen R a	Ets-1/2	Ezrin	FADD
8	FLASH	FLIPs/l	Flt-3/2	Flt-4	c-Fos	Frizzled	GADD34	GADD45	GADD153	GAK
9	GSK-3 alpha	HDAC1	hdlg	Ne-dlg	hILP	Hrk	HS1	HSP-70	ICSBP	Id1
10	Insulin R b	Integrin-alphal	Integrin a 5	Integrin a V	Integrin b 1	Integrin b 3	IRAK	IRF1	IRF2	IRS-1
11	L1	LGI1	LIFR	Mad-1	MAD2	Maspin	Max	MDA-7	MDM2	Mdr
12	Myc (c-Myc)	MyoD	NCK	NF-1	NF1GRP	NF2	NFATC	NF-kappa B 50	NF-kappa B 52	NF-kappa B p65
13	nip2	nip3	eNOS	iNOS	nNOS	Notch	Ntk	Numr 1	Ob Receptor	p16
14	P63	P73	p130Cas	p300	Patched	PAR-4	PARP	Pax-5	Paxillin	PCNA
15	PTEN	SH-PTP	PTP1B	SH-PTP2	PYK2	Rab1A	Rab3	Rab5	Rab11	Rac1
16	RAR r	RXR a,b,r	Ras	Ras-GAP	Rb (p107)	Rb (p110)	Rb2 (130)	RBBP	Rbx1 & 2	c-Rel
17	P-Selectin	SHC	SHIP	Sik	SIVA	Smad (1/2/3)	Smad 4	SOCS-1	SODD	Sos1/2
18	STAM	Stat1	p-Stat1	Stat2	Stat3	p-Stat3	Stat4	Stat5a	Stat5b	Stat6
19	TIA-1	TIAR	TNFR1	TNFR2	TOSO	TRADD	TRAF1	TRAF2	TRAF3	TRAF4
20	VDAC1	VDR	VEGFR1	VEGFR2	VHL	WT	XRCC4	YY1	ZAP70 Kinase	ZO-1

Table 3: Tissue Microarray (US Biomax, Rockville, MD, USA). Refer to the table below for the histopathological parameters of tissue microarray used.

Catalogue	Position	Sex	Age	Organ	Pathology	Grade	Type
'BR2082	'A1	'F	'40	'Lymph node	'Metastatic invasive ductal carcinoma	'2	'Metastasis
'BR2082	'A2	'F	'55	'Lymph node	'Metastatic invasive ductal carcinoma	'1	'Metastasis
'BR2082	'A3	'F	'56	'Lymph node	'Metastatic invasive ductal carcinoma	'2	'Metastasis
'BR2082	'A4	'F	'48	'Lymph node	'Metastatic invasive ductal carcinoma (adipose tissue)	'-	'Metastasis
'BR2082	'A5	'F	'57	'Lymph node	'Metastatic invasive ductal carcinoma	'2	'Metastasis
'BR2082	'A6	'F	'56	'Lymph node	'Metastatic invasive ductal carcinoma	'2	'Metastasis
'BR2082	'A7	'F	'42	'Lymph node	'Metastatic invasive ductal carcinoma	'2	'Metastasis
'BR2082	'A8	'F	'49	'Lymph node	'Metastatic invasive ductal carcinoma	'2	'Metastasis
'BR2082	'A9	'F	'66	'Lymph node	'Metastatic invasive ductal carcinoma	'2	'Metastasis
'BR2082	'A10	'F	'38	'Lymph node	'Metastatic invasive ductal carcinoma	'1	'Metastasis
'BR2082	'A11	'F	'51	'Lymph node	'Metastatic invasive ductal carcinoma	'2	'Metastasis
'BR2082	'A12	'F	'39	'Lymph node	'Metastatic invasive ductal carcinoma	'2	'Metastasis
'BR2082	'A13	'F	'53	'Lymph node	'Metastatic invasive ductal carcinoma	'2	'Metastasis

Table 3: Tissue Microarray (continued)

'BR2082	'A14	'F	'56	'Lymph node	'Metastatic invasive ductal carcinoma (adipose tissue and blood vessel)	'-	'Metastasis
'BR2082	'A15	'F	'44	'Lymph node	'Metastatic invasive ductal carcinoma	'2	'Metastasis
'BR2082	'A16	'F	'55	'Lymph node	'Metastatic invasive ductal carcinoma	'2	'Metastasis
'BR2082	'B1	'F	'47	'Lymph node	'Metastatic invasive ductal carcinoma	'2	'Metastasis
'BR2082	'B2	'F	'39	'Lymph node	'Metastatic invasive ductal carcinoma	'1	'Metastasis
'BR2082	'B3	'F	'52	'Lymph node	'Metastatic invasive ductal carcinoma of No. 81	'2	'Metastasis
'BR2082	'B4	'F	'60	'Lymph node	'Metastatic invasive ductal carcinoma	'2	'Metastasis
'BR2082	'B5	'F	'52	'Lymph node	'Metastatic invasive ductal carcinoma	'2	'Metastasis
'BR2082	'B6	'F	'28	'Lymph node	'Metastatic invasive ductal carcinoma	'3	'Metastasis
'BR2082	'B7	'F	'58	'Lymph node	'Metastatic invasive ductal carcinoma	'2	'Metastasis
'BR2082	'B8	'F	'42	'Lymph node	'Metastatic invasive ductal carcinoma	'2	'Metastasis
'BR2082	'B9	'F	'42	'Lymph node	'Metastatic invasive ductal carcinoma	'2	'Metastasis
'BR2082	'B10	'F	'48	'Lymph node	'Metastatic invasive ductal carcinoma	'2	'Metastasis
'BR2082	'B11	'F	'80	'Lymph node	'Metastatic invasive ductal carcinoma	'2	'Metastasis
'BR2082	'B12	'F	'59	'Lymph node	'Metastatic invasive ductal carcinoma	'2	'Metastasis

Table 3: Tissue Microarray (continued)

'BR2082	'B13	'F	'60	'Lymph node	'Metastatic invasive ductal carcinoma	'2	'Metastasis
'BR2082	'B14	'F	'28	'Lymph node	'Metastatic invasive ductal carcinoma of No. 79	'2	'Metastasis
'BR2082	'B15	'F	'55	'Lymph node	'Metastatic invasive ductal carcinoma	'2	'Metastasis
'BR2082	'B16	'F	'62	'Lymph node	'Metastatic invasive ductal carcinoma	'2	'Metastasis
'BR2082	'C1	'F	'30	'Breast	'Invasive ductal carcinoma	'1	'Malignant
'BR2082	'C2	'F	'44	'Breast	'Invasive ductal carcinoma (sparse)	'1	'Malignant
'BR2082	'C3	'F	'37	'Breast	'Invasive ductal carcinoma	'1	'Malignant
'BR2082	'C4	'F	'61	'Breast	'Invasive ductal carcinoma	'2	'Malignant
'BR2082	'C5	'F	'50	'Breast	'Invasive ductal carcinoma	'2	'Malignant
'BR2082	'C6	'F	'39	'Breast	'Invasive ductal carcinoma	'2	'Malignant
'BR2082	'C7	'F	'42	'Breast	'Invasive ductal carcinoma	'1	'Malignant
'BR2082	'C8	'F	'41	'Breast	'Invasive ductal carcinoma	'2	'Malignant
'BR2082	'C9	'F	'65	'Breast	'Invasive ductal carcinoma	'2	'Malignant
'BR2082	'C10	'F	'63	'Breast	'Invasive ductal carcinoma	'2	'Malignant
'BR2082	'C11	'F	'71	'Breast	'Invasive ductal carcinoma	'2	'Malignant
'BR2082	'C12	'F	'43	'Breast	'Invasive ductal carcinoma	'2	'Malignant
'BR2082	'C13	'F	'54	'Breast	'Invasive ductal carcinoma	'2	'Malignant
'BR2082	'C14	'F	'38	'Breast	'Invasive ductal carcinoma	'2	'Malignant
'BR2082	'C15	'F	'47	'Breast	'Invasive ductal carcinoma	'2	'Malignant
'BR2082	'C16	'F	'44	'Breast	'Invasive ductal carcinoma	'2	'Malignant
'BR2082	'D1	'F	'53	'Breast	'Invasive ductal carcinoma	'2	'Malignant
'BR2082	'D2	'F	'42	'Breast	'Invasive ductal carcinoma	'2	'Malignant
'BR2082	'D3	'F	'59	'Breast	'Invasive ductal carcinoma	'2	'Malignant
'BR2082	'D4	'F	'38	'Breast	'Invasive ductal carcinoma	'2	'Malignant

Table 3: Tissue Microarray (continued)

'BR2082	'D5	'F	'43	'Breast	'Invasive ductal carcinoma	'2	'Malignant
'BR2082	'D6	'F	'48	'Breast	'Invasive ductal carcinoma	'2	'Malignant
'BR2082	'D7	'F	'41	'Breast	'Invasive ductal carcinoma	'2	'Malignant
'BR2082	'D8	'F	'38	'Breast	'Invasive ductal carcinoma	'2	'Malignant
'BR2082	'D9	'F	'45	'Breast	'Invasive ductal carcinoma	'2	'Malignant
'BR2082	'D10	'F	'42	'Breast	'Invasive ductal carcinoma (fibrofatty tissue)	'-	'Malignant
'BR2082	'D11	'F	'41	'Breast	'Invasive ductal carcinoma	'2	'Malignant
'BR2082	'D12	'F	'32	'Breast	'Invasive ductal carcinoma	'2	'Malignant
'BR2082	'D13	'F	'62	'Breast	'Invasive ductal carcinoma	'2	'Malignant
'BR2082	'D14	'F	'50	'Breast	'Invasive ductal carcinoma	'2	'Malignant
'BR2082	'D15	'F	'68	'Breast	'Invasive ductal carcinoma	'2	'Malignant
'BR2082	'D16	'F	'44	'Breast	'Invasive ductal carcinoma	'2	'Malignant
'BR2082	'E1	'F	'45	'Breast	'Invasive ductal carcinoma	'2	'Malignant
'BR2082	'E2	'F	'29	'Breast	'Invasive ductal carcinoma	'2	'Malignant
'BR2082	'E3	'F	'67	'Breast	'Invasive ductal carcinoma	'1	'Malignant
'BR2082	'E4	'F	'50	'Breast	'Invasive ductal carcinoma (breast tissue)	'-	'Malignant
'BR2082	'E5	'F	'54	'Breast	'Invasive ductal carcinoma	'2	'Malignant
'BR2082	'E6	'F	'35	'Breast	'Invasive ductal carcinoma	'2	'Malignant
'BR2082	'E7	'F	'43	'Breast	'Invasive ductal carcinoma (fibrofatty tissue)	'-	'Malignant
'BR2082	'E8	'F	'29	'Breast	'Invasive ductal carcinoma	'2	'Malignant
'BR2082	'E9	'F	'55	'Breast	'Invasive ductal carcinoma	'2	'Malignant
'BR2082	'E10	'F	'50	'Breast	'Invasive ductal carcinoma (sparse)	'2	'Malignant
'BR2082	'E11	'F	'43	'Breast	'Invasive ductal carcinoma	'2	'Malignant
'BR2082	'E12	'F	'43	'Breast	'Invasive ductal carcinoma	'2	'Malignant
'BR2082	'E13	'F	'45	'Breast	'Invasive ductal carcinoma	'2	'Malignant

Table 3: Tissue Microarray (continued)

'BR2082	'E14	'F	'50	'Breast	'Invasive ductal carcinoma	'2	'Malignant
'BR2082	'E15	'F	'28	'Breast	'Invasive ductal carcinoma	'2	'Malignant
'BR2082	'E16	'F	'55	'Breast	'Invasive ductal carcinoma	'2	'Malignant
'BR2082	'F1	'F	'52	'Breast	'Invasive ductal carcinoma	'2	'Malignant
'BR2082	'F2	'F	'38	'Breast	'Invasive ductal carcinoma	'2	'Malignant
'BR2082	'F3	'F	'49	'Breast	'Invasive ductal carcinoma (sparse)	'-	'Malignant
'BR2082	'F4	'F	'46	'Breast	'Invasive ductal carcinoma	'2	'Malignant
'BR2082	'F5	'F	'62	'Breast	'Invasive ductal carcinoma	'2	'Malignant
'BR2082	'F6	'F	'42	'Breast	'Invasive ductal carcinoma	'2	'Malignant
'BR2082	'F7	'F	'53	'Breast	'Invasive ductal carcinoma	'2	'Malignant
'BR2082	'F8	'F	'45	'Breast	'Invasive ductal carcinoma	'2	'Malignant
'BR2082	'F9	'F	'32	'Breast	'Invasive ductal carcinoma	'2	'Malignant
'BR2082	'F10	'F	'29	'Breast	'Invasive ductal carcinoma	'2	'Malignant
'BR2082	'F11	'F	'65	'Breast	'Invasive ductal carcinoma (fibrofatty tissue)	'-	'Malignant
'BR2082	'F12	'F	'64	'Breast	'Invasive ductal carcinoma (sparse)	'2	'Malignant
'BR2082	'F13	'F	'63	'Breast	'Invasive ductal carcinoma	'2	'Malignant
'BR2082	'F14	'F	'51	'Breast	'Invasive ductal carcinoma	'3	'Malignant
'BR2082	'F15	'F	'56	'Breast	'Invasive ductal carcinoma	'2	'Malignant
'BR2082	'F16	'F	'62	'Breast	'Invasive ductal carcinoma	'2	'Malignant
'BR2082	'G1	'F	'37	'Breast	'Invasive ductal carcinoma (skeletal muscle and blood vessel)	'-	'Malignant
'BR2082	'G2	'F	'32	'Breast	'Invasive ductal carcinoma	'2	'Malignant
'BR2082	'G3	'F	'55	'Breast	'Invasive ductal carcinoma	'2	'Malignant
'BR2082	'G4	'F	'53	'Breast	'Invasive ductal carcinoma	'2	'Malignant
'BR2082	'G5	'F	'50	'Breast	'Invasive lobular carcinoma	'-	'Malignant
'BR2082	'G6	'F	'38	'Breast	'Invasive lobular carcinoma (breast tissue)	'-	'Malignant

Table 3: Tissue Microarray (continued)

'BR2082	'G7	'F	'47	'Breast	'Invasive lobular carcinoma	'-	'Malignant
'BR2082	'G8	'F	'51	'Breast	'Invasive lobular carcinoma (sparse)	'-	'Malignant
'BR2082	'G9	'F	'69	'Breast	'Invasive lobular carcinoma	'-	'Malignant
'BR2082	'G10	'F	'70	'Breast	'Invasive lobular carcinoma	'-	'Malignant
'BR2082	'G11	'F	'59	'Breast	'Invasive lobular carcinoma	'-	'Malignant
'BR2082	'G12	'F	'35	'Breast	'Invasive lobular carcinoma	'-	'Malignant
'BR2082	'G13	'F	'38	'Breast	'Invasive lobular carcinoma	'-	'Malignant
'BR2082	'G14	'F	'42	'Breast	'Invasive lobular carcinoma	'-	'Malignant
'BR2082	'G15	'F	'52	'Breast	'Invasive lobular carcinoma	'-	'Malignant
'BR2082	'G16	'F	'41	'Breast	'Invasive lobular carcinoma	'-	'Malignant
'BR2082	'H1	'F	'47	'Breast	'Invasive lobular carcinoma	'-	'Malignant
'BR2082	'H2	'F	'50	'Breast	'Invasive lobular carcinoma	'-	'Malignant
'BR2082	'H3	'F	'66	'Breast	'Invasive lobular carcinoma (sparse)	'-	'Malignant
'BR2082	'H4	'F	'46	'Breast	'Invasive lobular carcinoma	'-	'Malignant
'BR2082	'H5	'F	'48	'Breast	'Invasive lobular carcinoma	'-	'Malignant
'BR2082	'H6	'F	'38	'Breast	'Invasive lobular carcinoma (fibrofatty tissue)	'-	'Malignant
'BR2082	'H7	'F	'45	'Breast	'Invasive lobular carcinoma (sparse)	'-	'Malignant
'BR2082	'H8	'F	'52	'Breast	'Invasive lobular carcinoma	'-	'Malignant
'BR2082	'H9	'F	'45	'Breast	'Invasive lobular carcinoma	'-	'Malignant
'BR2082	'H10	'F	'52	'Breast	'Invasive lobular carcinoma (sparse)	'-	'Malignant
'BR2082	'H11	'F	'41	'Breast	'Squamous cell carcinoma	'1–2	'Malignant
'BR2082	'H12	'F	'37	'Breast	'Squamous cell carcinoma	'2	'Malignant
'BR2082	'H13	'F	'48	'Breast	'Squamous cell carcinoma	'2	'Malignant
'BR2082	'H14	'F	'47	'Breast	'Squamous cell carcinoma	'2	'Malignant
'BR2082	'H15	'F	'49	'Breast	'Intraductal carcinoma	'-	'Malignant
'BR2082	'H16	'F	'46	'Breast	'Intraductal carcinoma (sparse)	'-	'Malignant
'BR2082	'I1	'F	'38	'Breast	'Intraductal carcinoma	'-	'Malignant

Table 3: Tissue Microarray (continued)

'BR2082	'I2	'F	'42	'Breast	'Intraductal carcinoma	'-	'Malignant
'BR2082	'I3	'F	'27	'Breast	'Intraductal carcinoma (fibrous tissue)	'-	'Malignant
'BR2082	'I4	'F	'30	'Breast	'Intraductal carcinoma (breast tissue)	'-	'Malignant
'BR2082	'I5	'F	'41	'Breast	'Intraductal carcinoma (hyperplasia of breast duct)	'-	'Malignant
'BR2082	'I6	'F	'43	'Breast	'Intraductal carcinoma	'-	'Malignant
'BR2082	'I7	'F	'32	'Breast	'Intraductal carcinoma	'-	'Malignant
'BR2082	'I8	'F	'58	'Breast	'Intraductal carcinoma	'-	'Malignant
'BR2082	'I9	'F	'67	'Breast	'Intraductal carcinoma	'-	'Malignant
'BR2082	'I10	'F	'30	'Breast	'Intraductal carcinoma (breast tissue)	'-	'Malignant
'BR2082	'I11	'F	'49	'Breast	'Intraductal carcinoma with early infiltration	'-	'Malignant
'BR2082	'I12	'F	'32	'Breast	'Intraductal carcinoma	'-	'Malignant
'BR2082	'I13	'F	'81	'Breast	'Intraductal carcinoma	'-	'Malignant
'BR2082	'I14	'F	'39	'Breast	'Intraductal carcinoma (breast tissue)	'-	'Malignant
'BR2082	'I15	'F	'43	'Breast	'Intraductal carcinoma	'-	'Malignant
'BR2082	'I16	'F	'35	'Breast	'Intraductal carcinoma (fibrous tissue)	'-	'Malignant
'BR2082	'J1	'F	'46	'Breast	'Intraductal carcinoma (sparse)	'-	'Malignant
'BR2082	'J2	'F	'60	'Breast	'Intraductal carcinoma	'-	'Malignant
'BR2082	'J3	'F	'64	'Breast	'Intraductal carcinoma	'-	'Malignant
'BR2082	'J4	'F	'40	'Breast	'Intraductal carcinoma	'-	'Malignant
'BR2082	'J5	'F	'48	'Breast	'Lobular carcinoma in situ (breast tissue)	'-	'In Situ
'BR2082	'J6	'F	'54	'Breast	'Lobular carcinoma in situ	'-	'In Situ

Table 3: Tissue Microarray (continued)

'BR2082	'J7	'F	'49	'Breast	'Lobular carcinoma in situ	'-	'In Situ
'BR2082	'J8	'F	'62	'Breast	'Lobular carcinoma in situ (fibrous tissue)	'-	'In Situ
'BR2082	'J9	'F	'44	'Breast	'Fibroadenoma	'-	'Benign
'BR2082	'J10	'F	'27	'Breast	'Fibroadenoma	'-	'Benign
'BR2082	'J11	'F	'23	'Breast	'Fibroadenoma	'-	'Benign
'BR2082	'J12	'F	'23	'Breast	'Fibroadenoma	'-	'Benign
'BR2082	'J13	'F	'19	'Breast	'Fibroadenoma	'-	'Benign
'BR2082	'J14	'F	'23	'Breast	'Fibroadenoma	'-	'Benign
'BR2082	'J15	'F	'42	'Breast	'Fibroadenoma	'-	'Benign
'BR2082	'J16	'F	'25	'Breast	'Fibroadenoma	'-	'Benign
'BR2082	'K1	'F	'48	'Breast	'Mild hyperplasia of breast duct	'-	'Hyperplasia
'BR2082	'K2	'F	'43	'Breast	'Adenosis with hyperplasia of duct	'-	'Hyperplasia
'BR2082	'K3	'F	'41	'Breast	'Adenosis with hyperplasia of duct	'-	'Hyperplasia
'BR2082	'K4	'F	'34	'Breast	'Mild hyperplasia of breast duct	'-	'Hyperplasia
'BR2082	'K5	'F	'40	'Breast	'Atypical hyperplasia in duct (grade II)	'-	'Hyperplasia
'BR2082	'K6	'F	'76	'Breast	'Hyperplasia (fibrofatty tissue)	'-	'Hyperplasia
'BR2082	'K7	'F	'70	'Breast	'Hyperplasia (fibrous tissue)	'-	'Hyperplasia
'BR2082	'K8	'F	'37	'Breast	'Adenosis with mild hyperplasia of duct	'-	'Hyperplasia
'BR2082	'K9	'F	'45	'Breast	'Adenosis with mild hyperplasia of duct	'-	'Hyperplasia
'BR2082	'K10	'F	'32	'Breast	'Hyperplasia (fibrofatty tissue)	'-	'Hyperplasia
'BR2082	'K11	'F	'48	'Breast	'Hyperplasia (fibrous tissue)	'-	'Hyperplasia
'BR2082	'K12	'F	'45	'Breast	'Atypical hyperplasia of duct (grade II-III)	'-	'Hyperplasia
'BR2082	'K13	'F	'40	'Breast	'Cyclomastopathy	'-	'Hyperplasia
'BR2082	'K14	'F	'22	'Breast	'Cyclomastopathy	'-	'Hyperplasia

Table 3: Tissue Microarray (continued)

'BR2082	'K15	'F	'28	'Breast	'Cyclomastopathy (sparse)	'-	'Hyperplasia
'BR2082	'K16	'F	'35	'Breast	'Hyperplasia (fibrofatty tissue)	'-	'Hyperplasia
'BR2082	'L1	'F	'61	'Breast	'Plasma cell mastitis	'-	'Inflammation
'BR2082	'L2	'F	'28	'Breast	'Plasma cell mastitis	'-	'Inflammation
'BR2082	'L3	'F	'55	'Breast	'Plasma cell mastitis	'-	'Inflammation
'BR2082	'L4	'F	'26	'Breast	'Plasma cell mastitis	'-	'Inflammation
'BR2082	'L5	'F	'78	'Breast	'Plasma cell mastitis	'-	'Inflammation
'BR2082	'L6	'F	'46	'Breast	'Plasma cell mastitis	'-	'Inflammation
'BR2082	'L7	'F	'28	'Breast	'Chronic inflammation	'-	'Inflammation
'BR2082	'L8	'F	'10	'Breast	'Acute mastitis	'-	'Inflammation
'BR2082	'L9	'F	'45	'Breast	'Mild chronic inflammation	'-	'Inflammation
'BR2082	'L10	'F	'57	'Breast	'Mild chronic inflammation	'-	'Inflammation
'BR2082	'L11	'F	'33	'Breast	'Mild chronic inflammation	'-	'Inflammation
'BR2082	'L12	'F	'39	'Breast	'Chronic inflammation of fibrous tissue	'-	'Inflammation
'BR2082	'L13	'F	'67	'Breast	'Granulomatous mastitis	'-	'Inflammation
'BR2082	'L14	'F	'40	'Breast	'Chronic inflammation	'-	'Inflammation
'BR2082	'L15	'F	'36	'Breast	'Chronic inflammation	'-	'Inflammation
'BR2082	'L16	'F	'44	'Breast	'Plasma cell mastitis with ductal ectasia	'-	'Inflammation
'BR2082	'M1	'F	'61	'Breast	'Cancer adjacent normal breast tissue	'-	'NAT
'BR2082	'M2	'F	'31	'Breast	'Cancer adjacent normal breast tissue (cyclomastopathy)	'-	'NAT
'BR2082	'M3	'F	'45	'Breast	'Cancer adjacent normal breast tissue	'-	'NAT
'BR2082	'M4	'F	'53	'Breast	'Cancer adjacent normal breast tissue	'-	'NAT
'BR2082	'M5	'F	'36	'Breast	'Cancer adjacent normal breast	'-	'NAT

Table 3: Tissue Microarray (continued)

					tissue (sparse)		
'BR2082	'M6	'F	'35	'Breast	'Cancer adjacent normal breast tissue	'-	'NAT
'BR2082	'M7	'F	'44	'Breast	'Cancer adjacent normal breast tissue (fibrous tissue)	'-	'NAT
'BR2082	'M8	'F	'33	'Breast	'Cancer adjacent normal breast tissue (fibrous tissue)	'-	'NAT
'BR2082	'M9	'F	'58	'Breast	'Cancer adjacent normal breast tissue (adipose tissue)	'-	'NAT
'BR2082	'M10	'F	'46	'Breast	'Cancer adjacent normal breast tissue	'-	'NAT
'BR2082	'M11	'F	'15	'Breast	'Normal breast tissue	'-	'Normal
'BR2082	'M12	'F	'21	'Breast	'Normal breast tissue (sparse)	'-	'Normal
'BR2082	'M13	'F	'21	'Breast	'Normal breast tissue	'-	'Normal
'BR2082	'M14	'F	'35	'Breast	'Normal breast tissue (with mild ductal ectasia)	'-	'Normal
'BR2082	'M15	'F	'19	'Breast	'Normal breast tissue (fibrous tissue)	'-	'Normal
'BR2082	'M16	'F	'27	'Breast	'Normal breast tissue (fibrous tissue)	'-	'Normal

Table 4: DNA Sequencing. Refer to the sequence chromatogram for both normal (N) and tumor (T) tissues; and breast cancer non tumorigenic and tumour cell lines

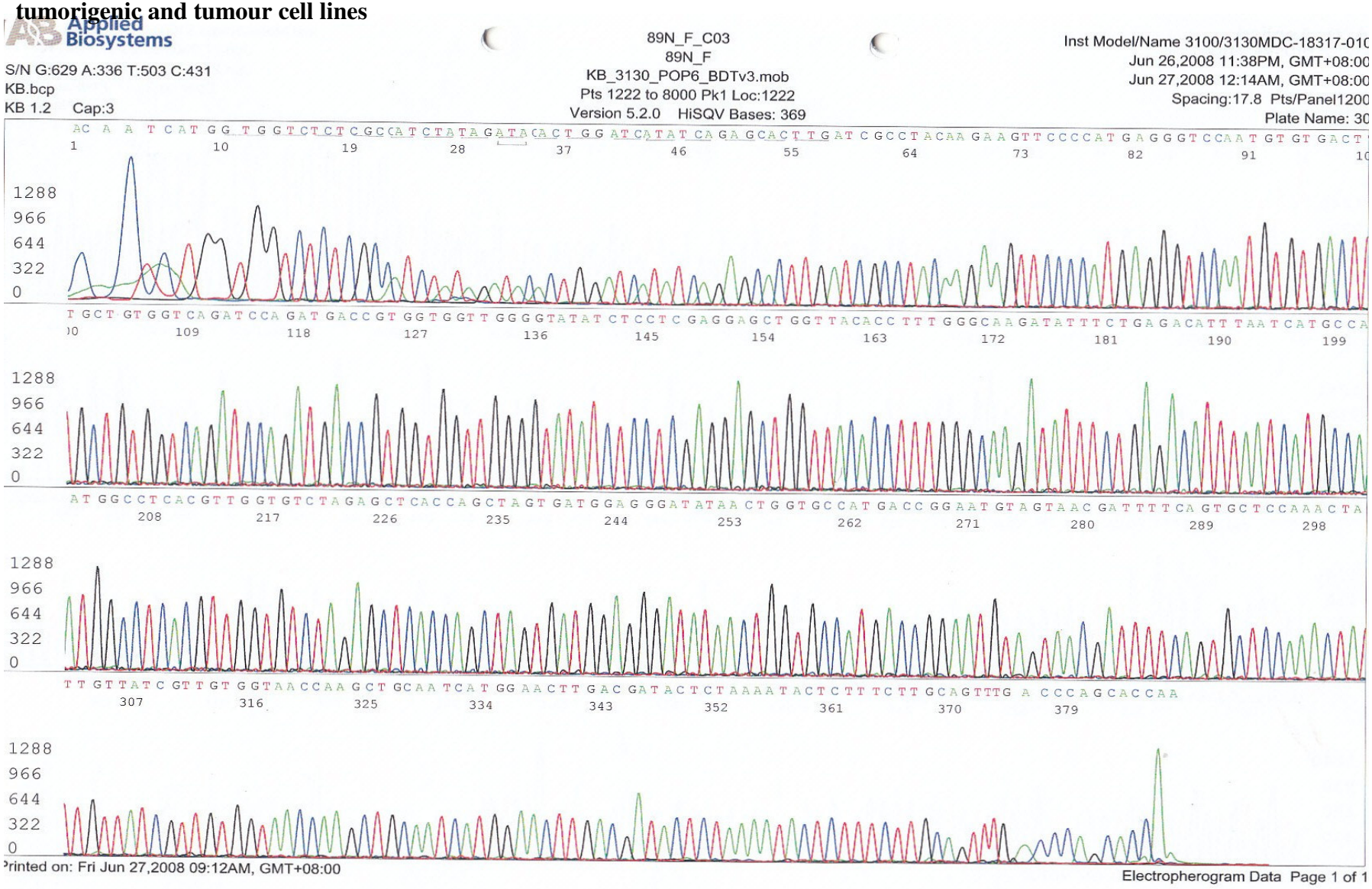


Table 4: DNA Sequencing (continued)

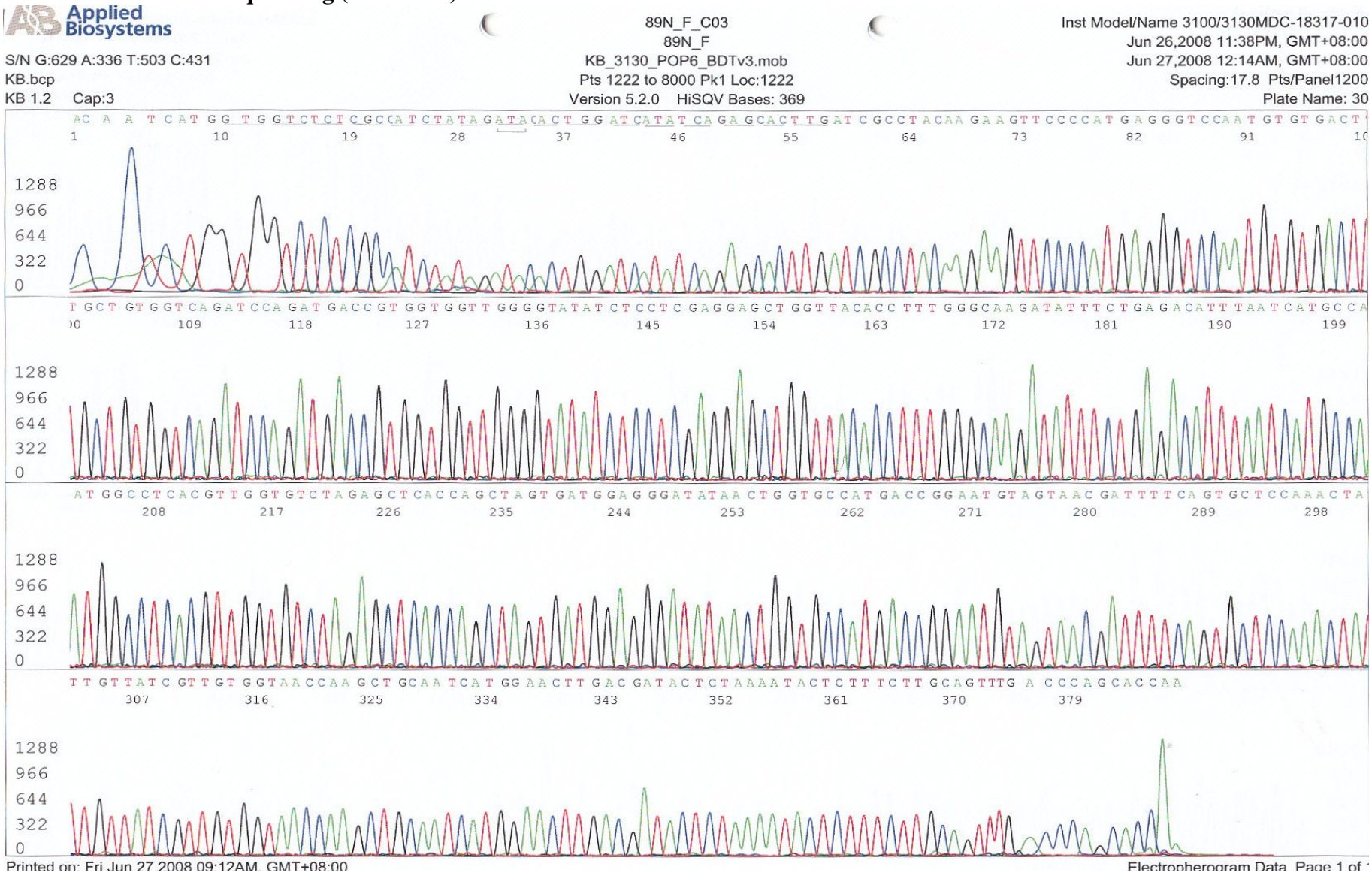


Table 4: DNA Sequencing (continued)

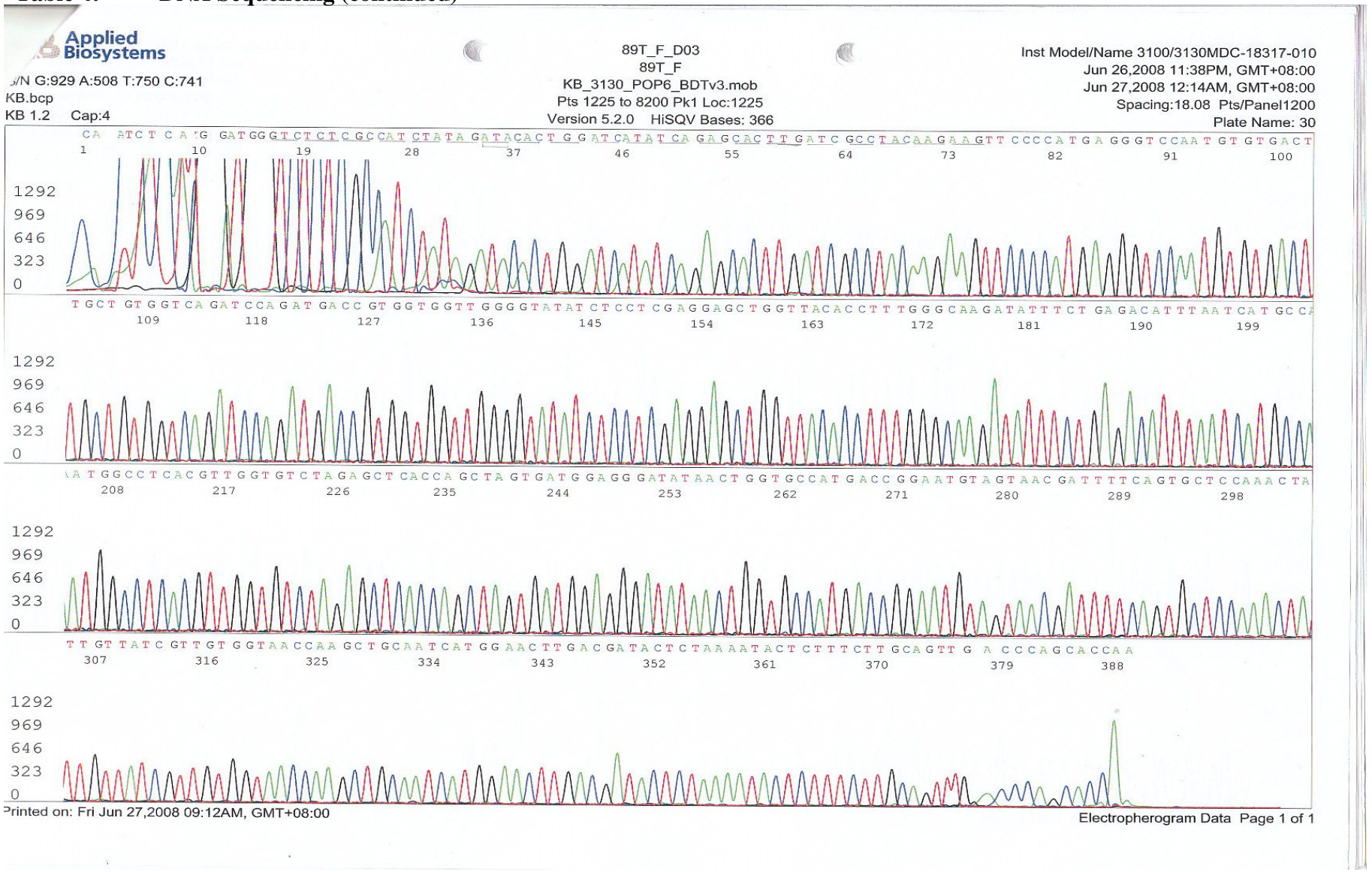


Table 4: DNA Sequencing (continued)

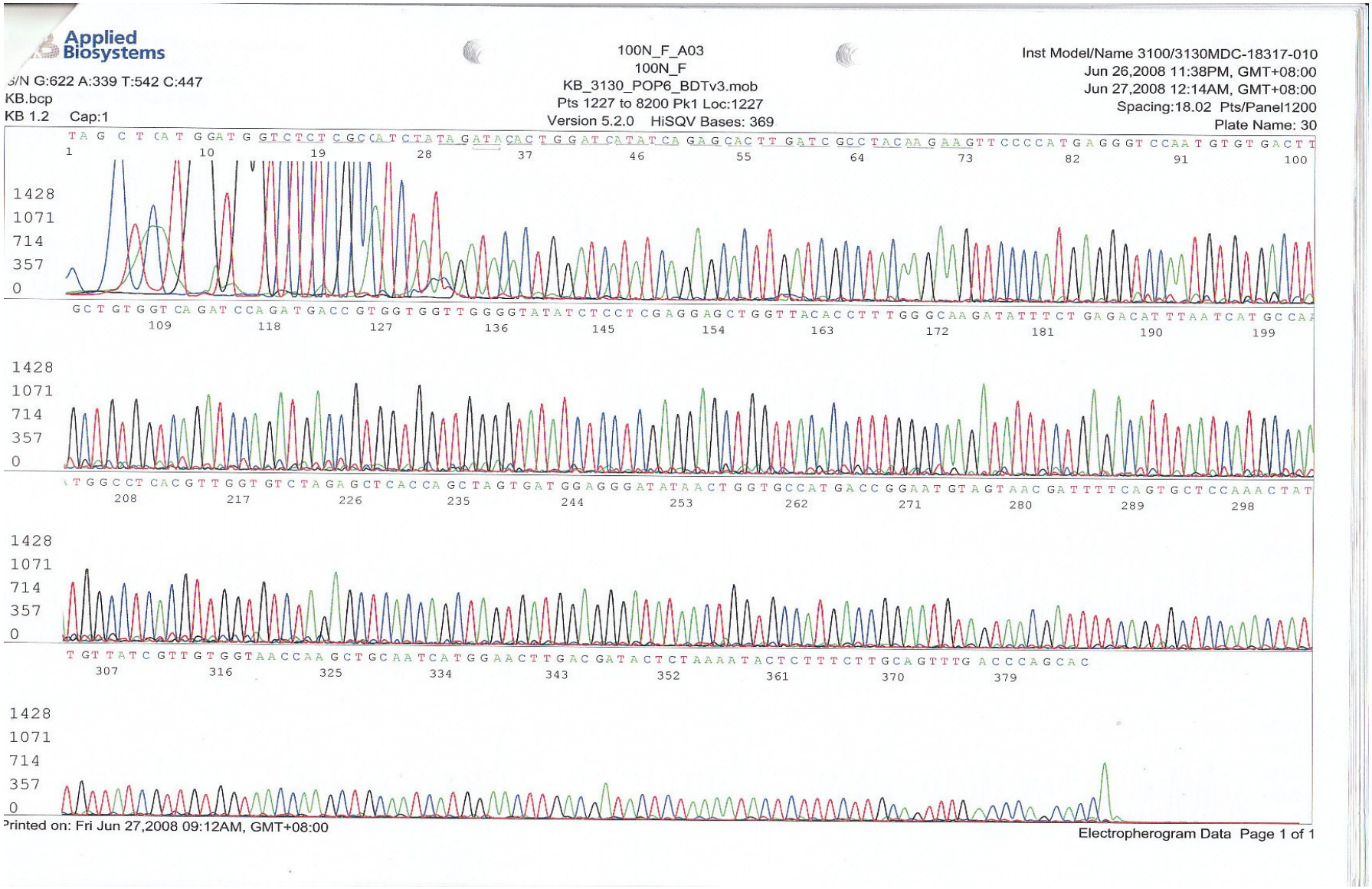


Table 4: DNA Sequencing (continued)

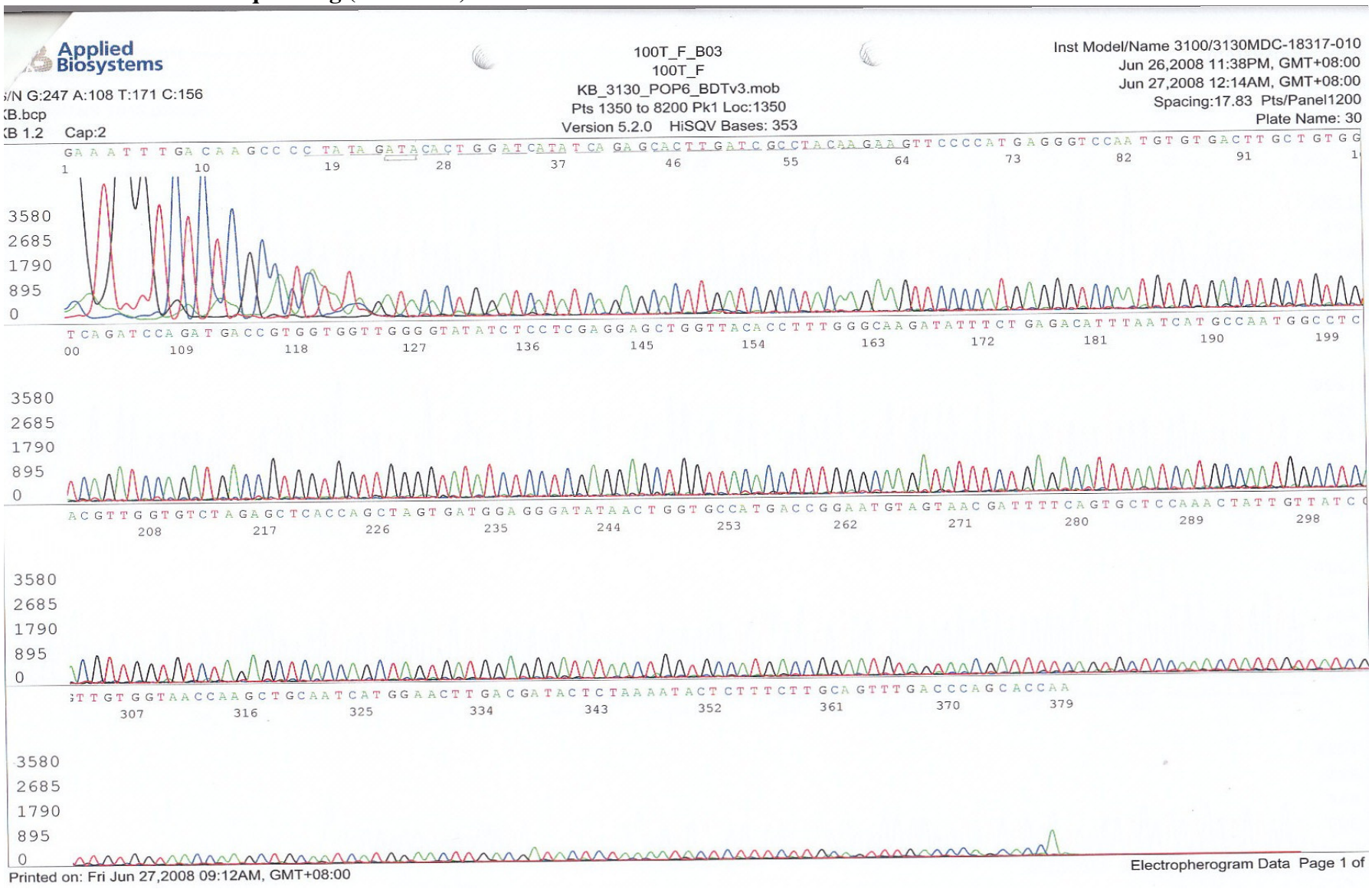


Table 4: DNA Sequencing (continued)

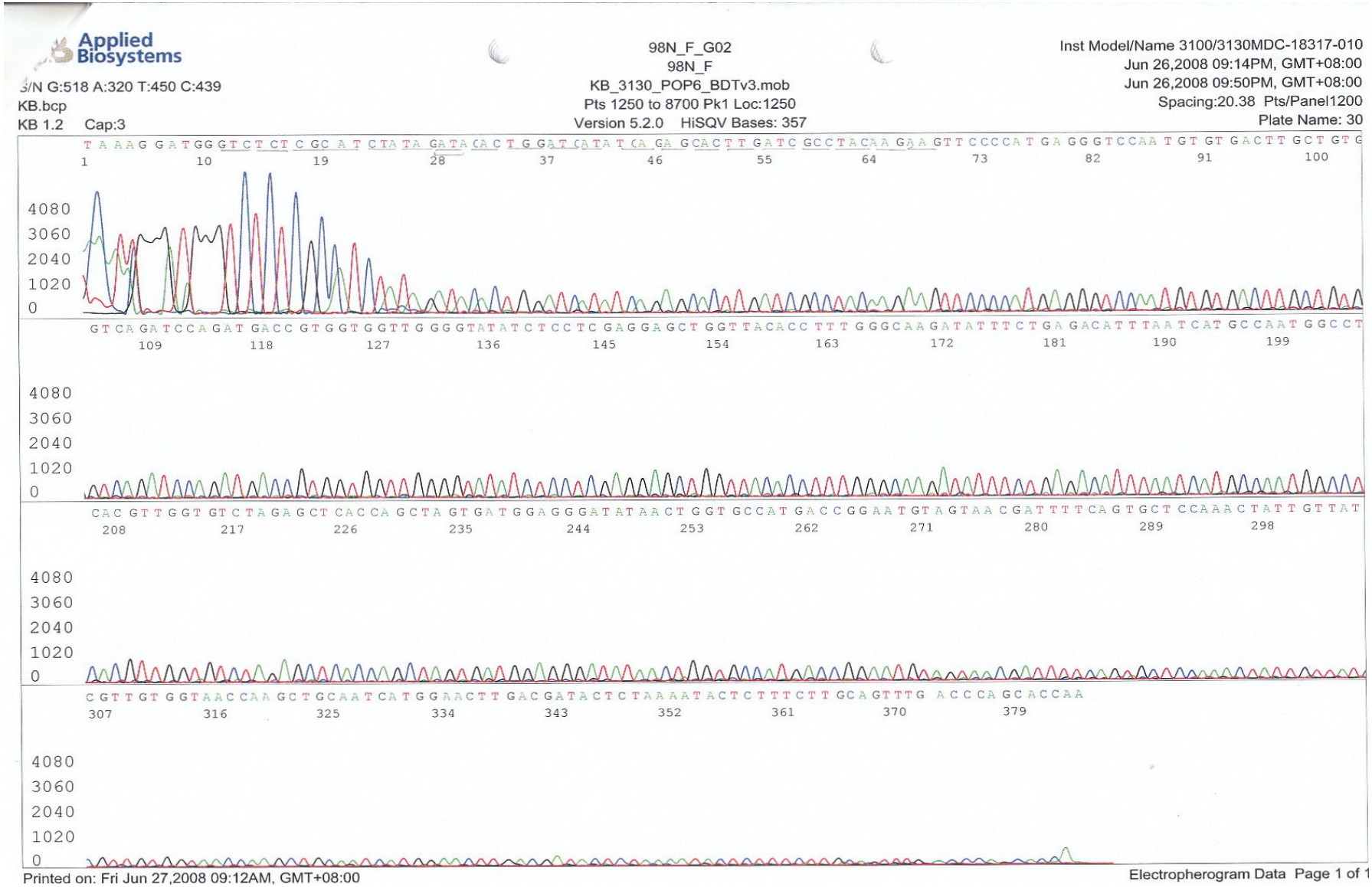


Table 4: DNA Sequencing (continued)

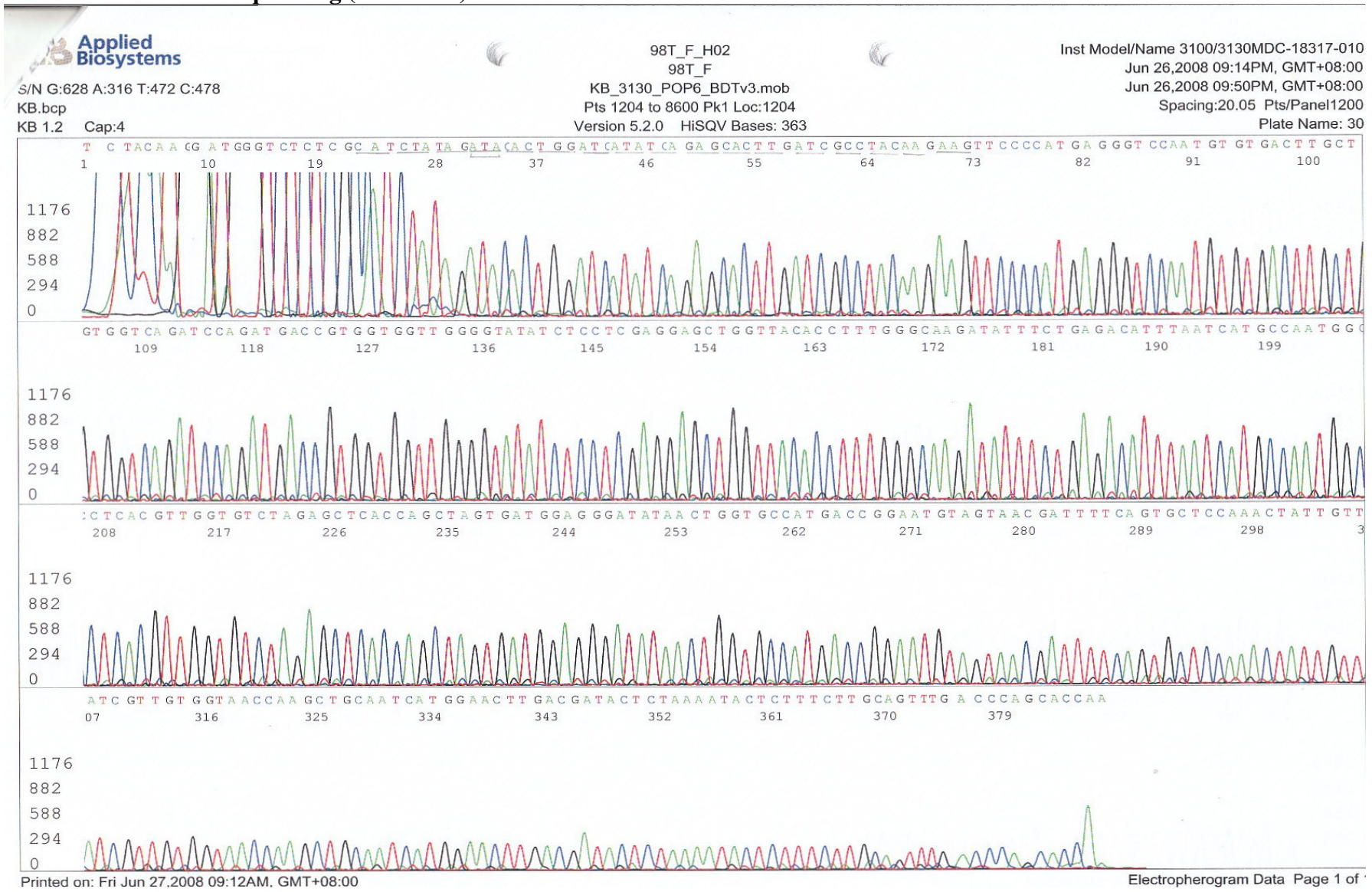
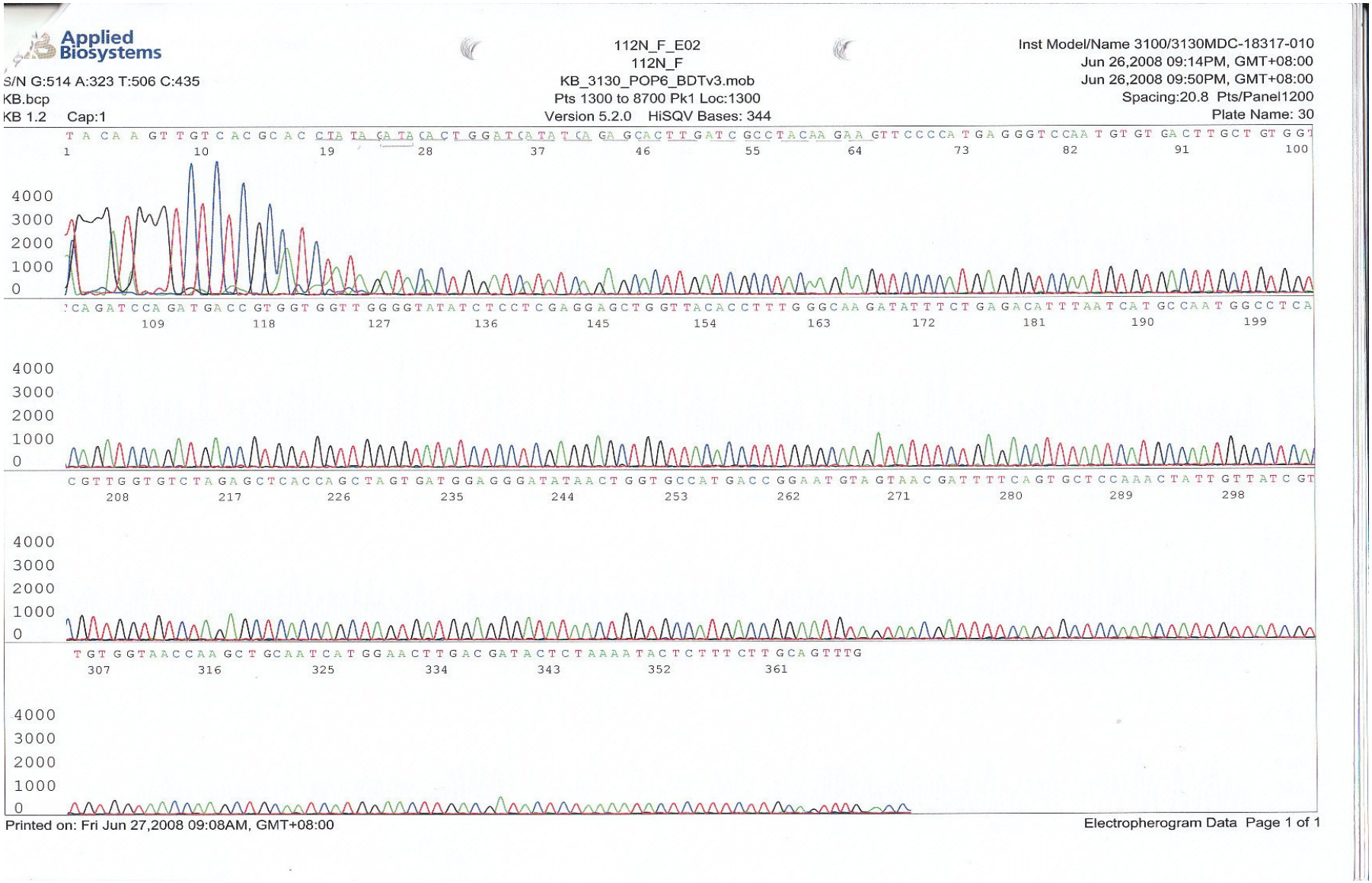


Table 4: DNA Sequencing (continued)



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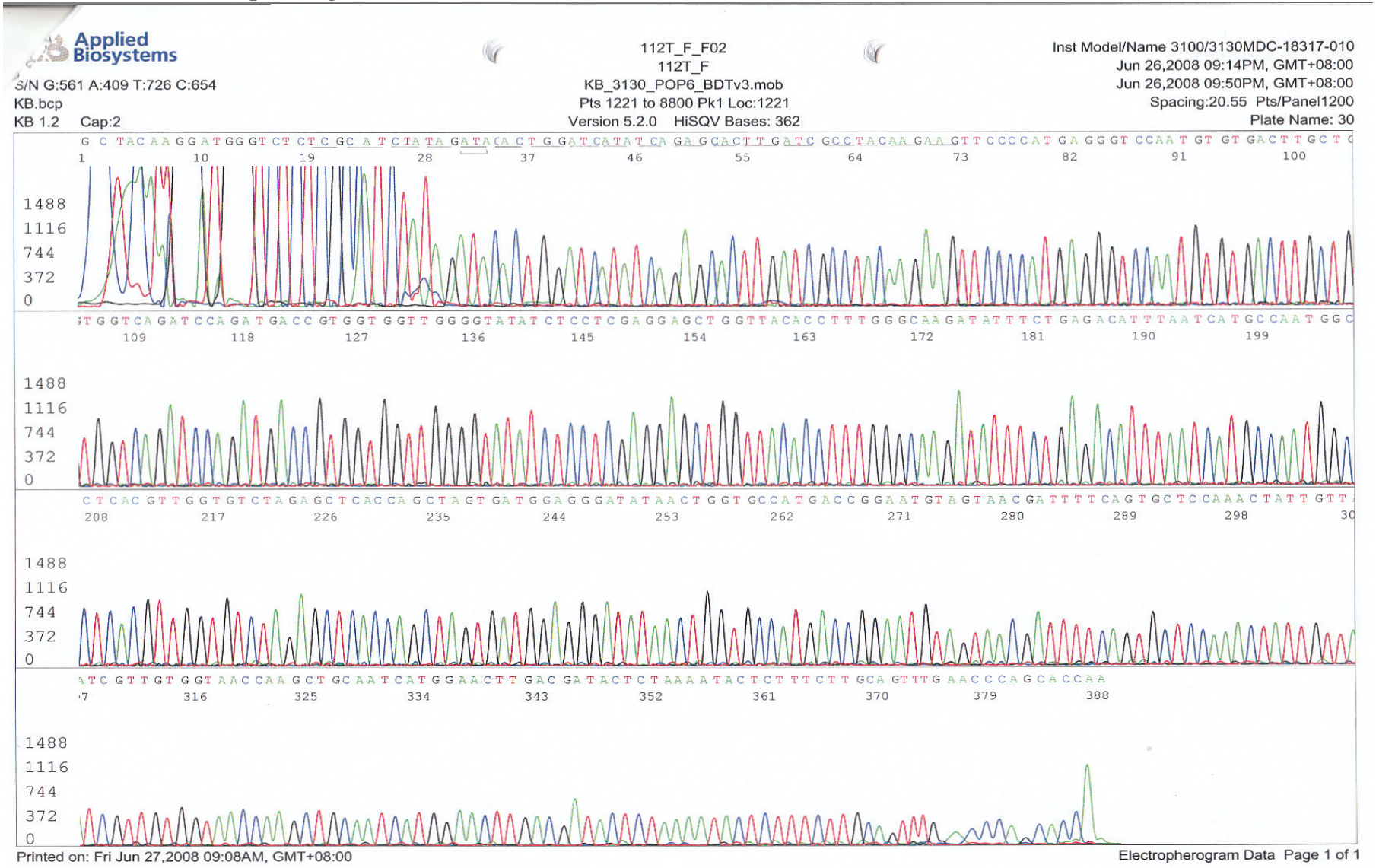

Table 4: DNA Sequencing (continued)

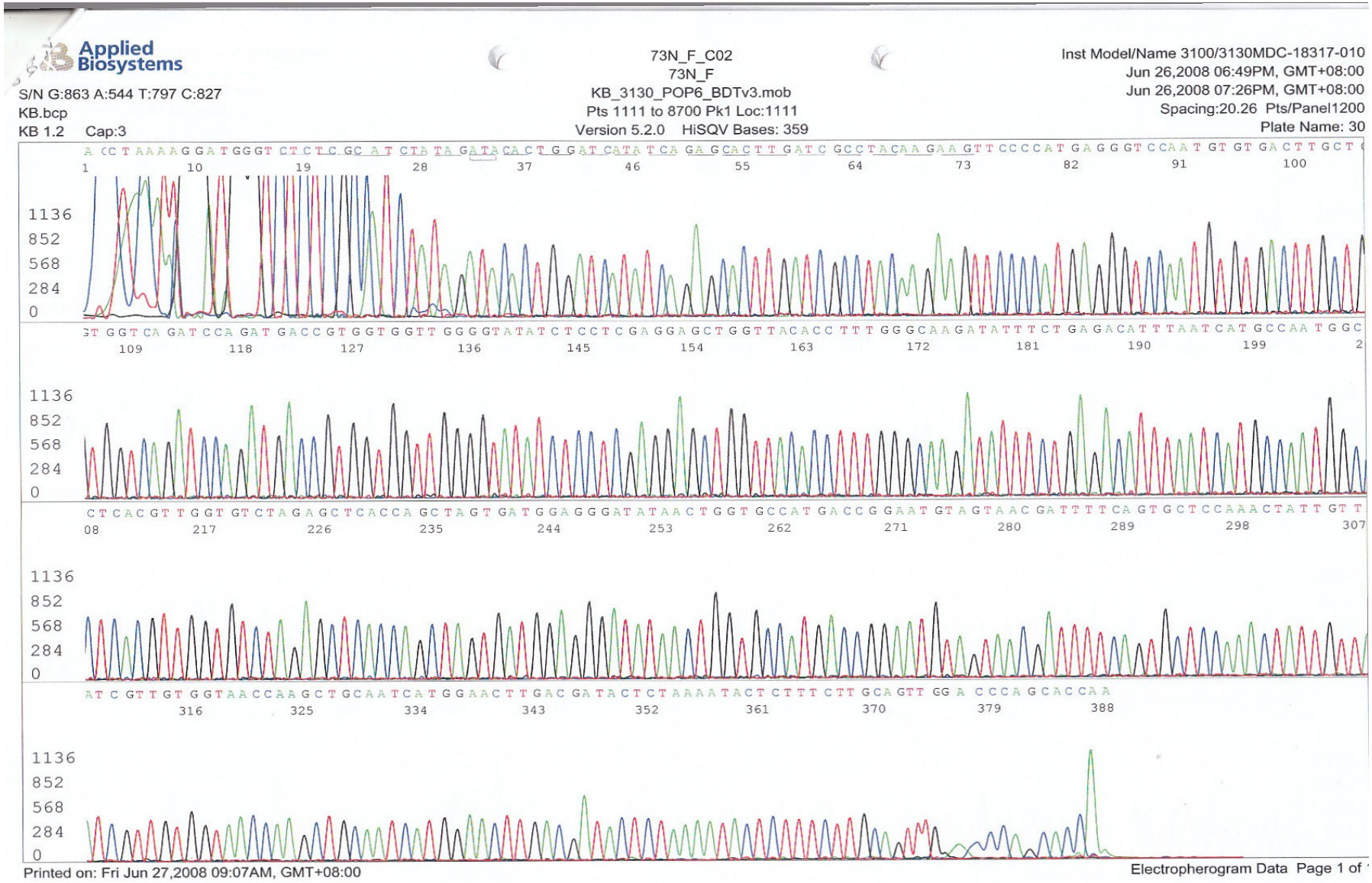

Table 4: DNA Sequencing (continued)

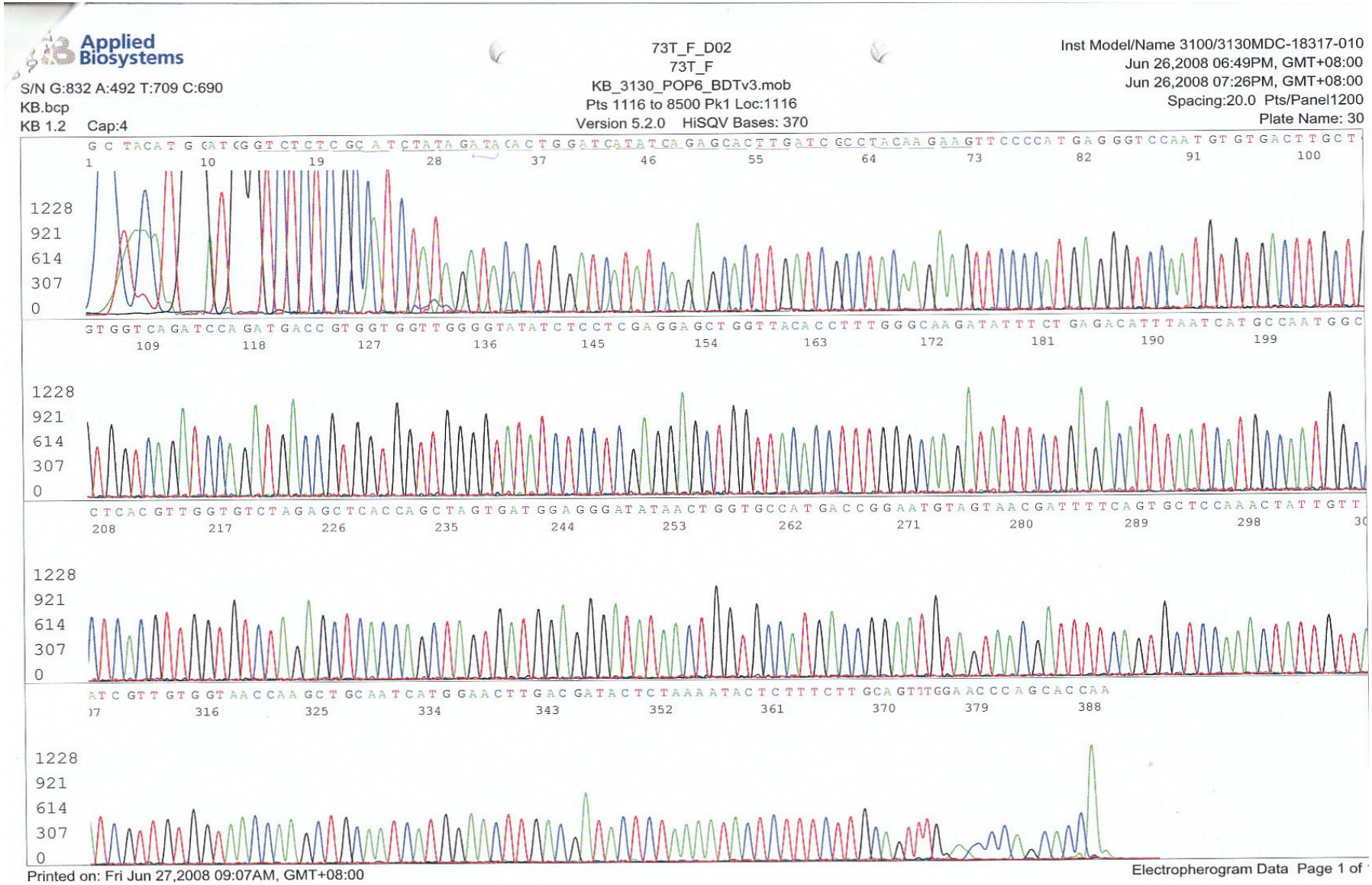

Table 4: DNA Sequencing (continued)

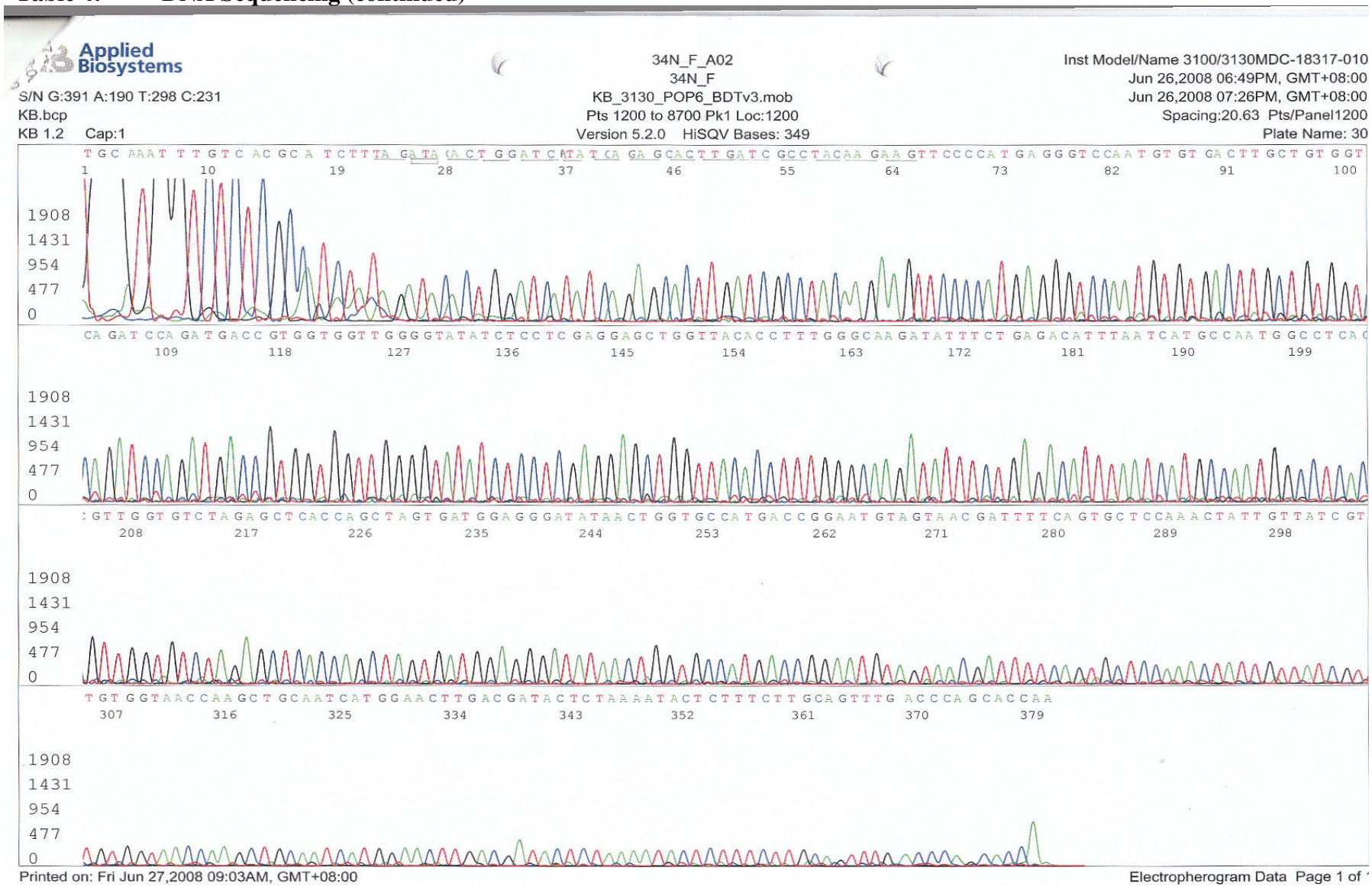

Table 4: DNA Sequencing (continued)

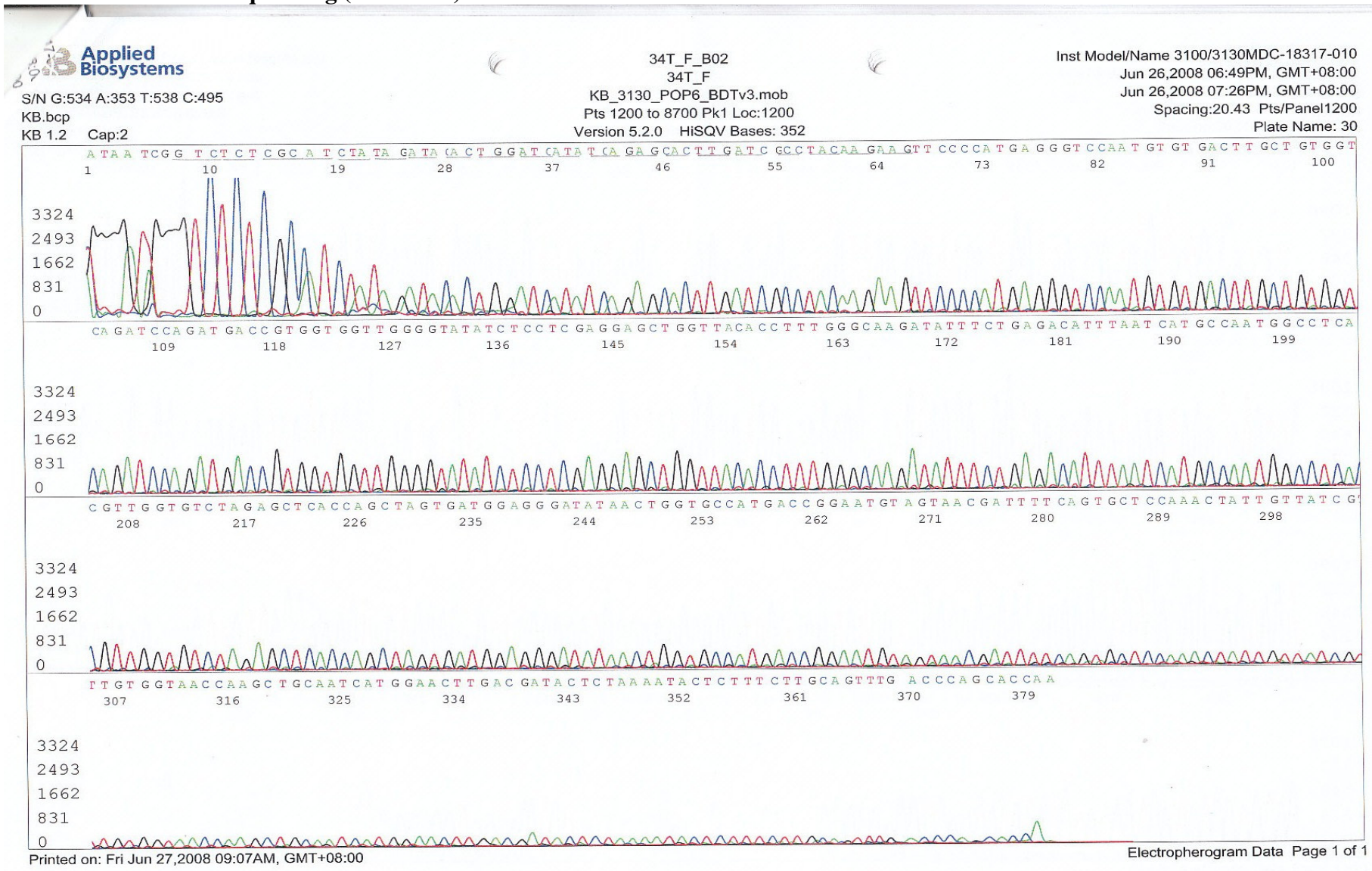

Table 4: DNA Sequencing (continued)

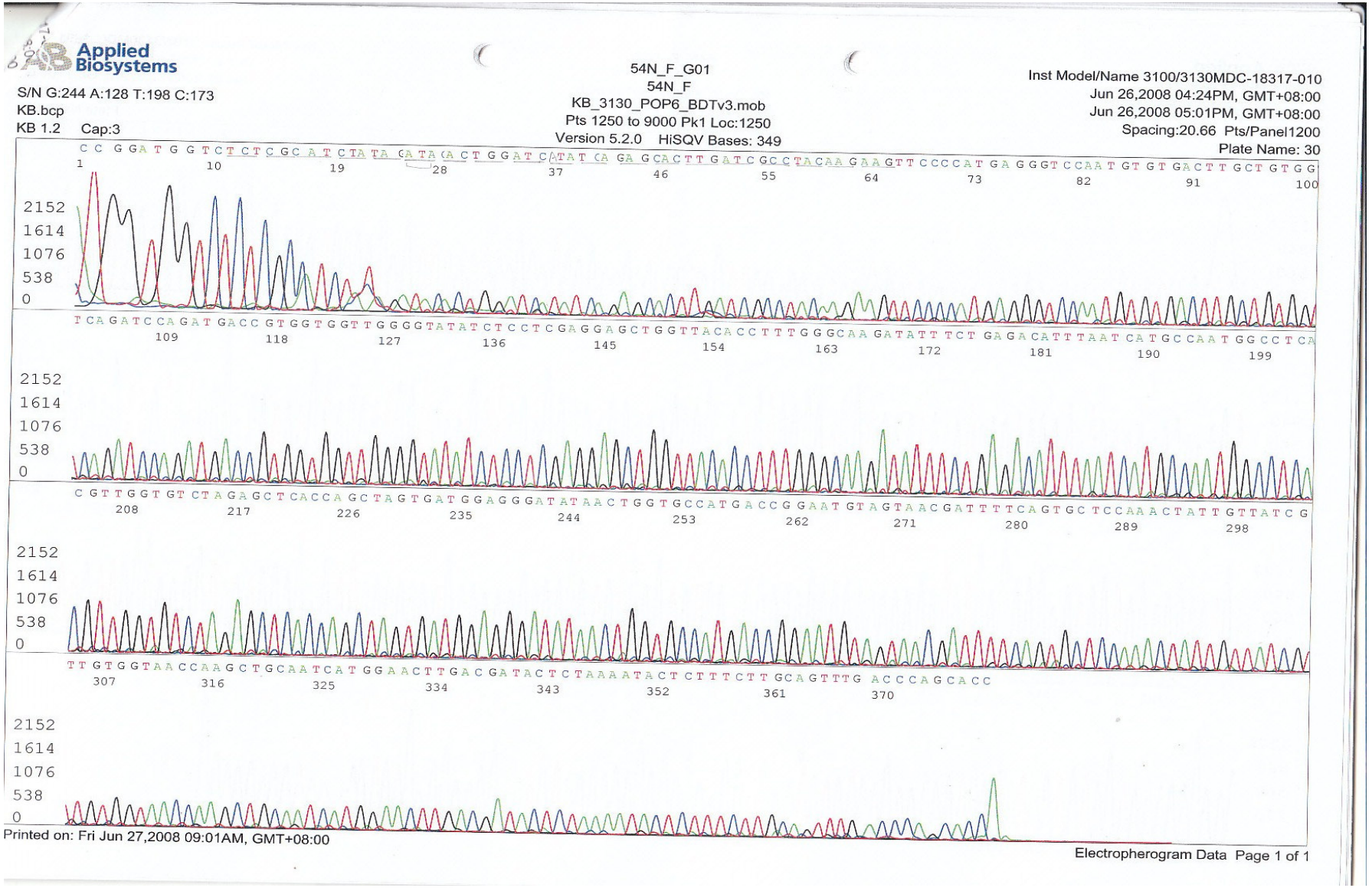

Table 4: DNA Sequencing (continued)

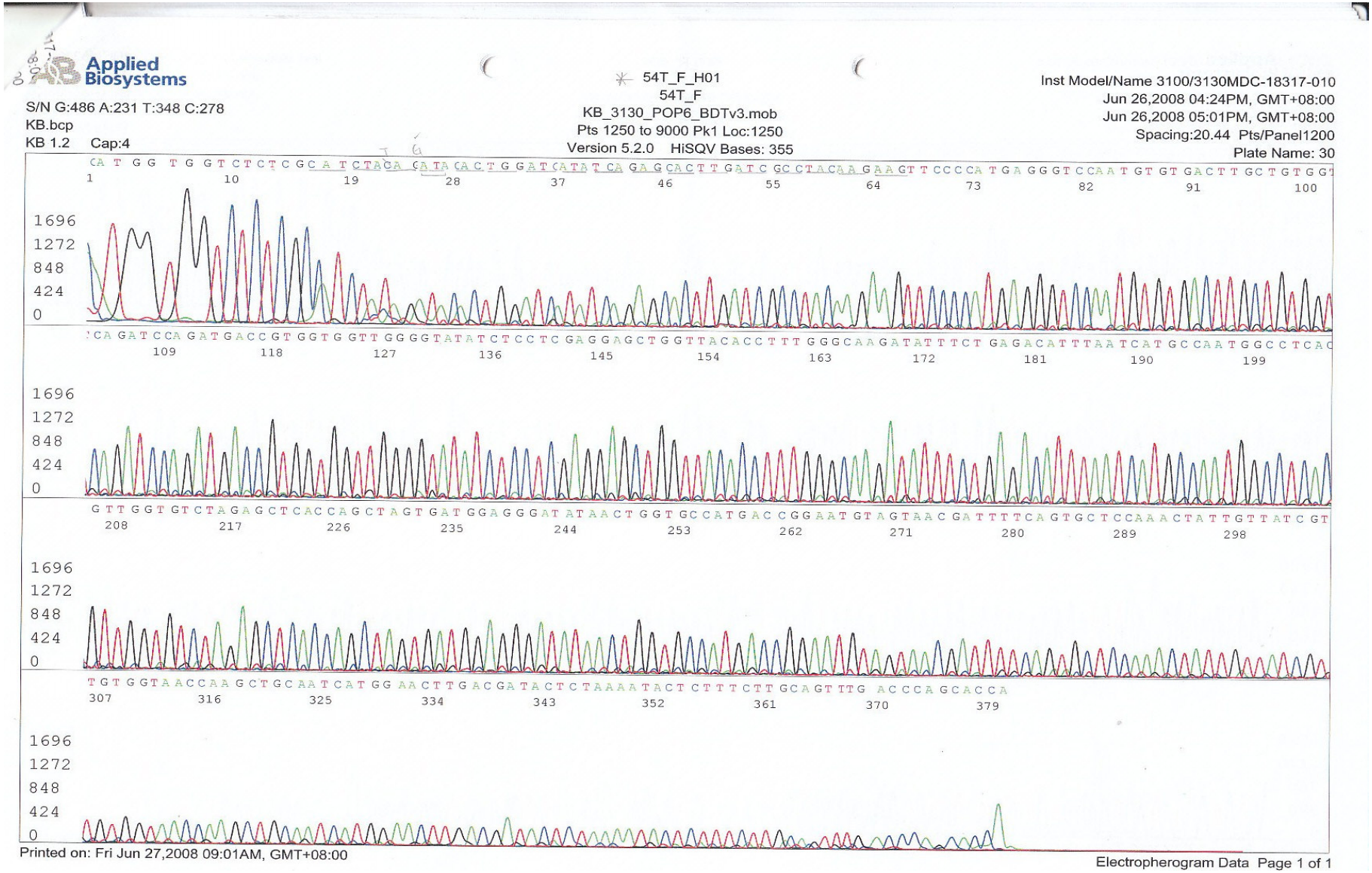

Table 4: DNA Sequencing (continued)

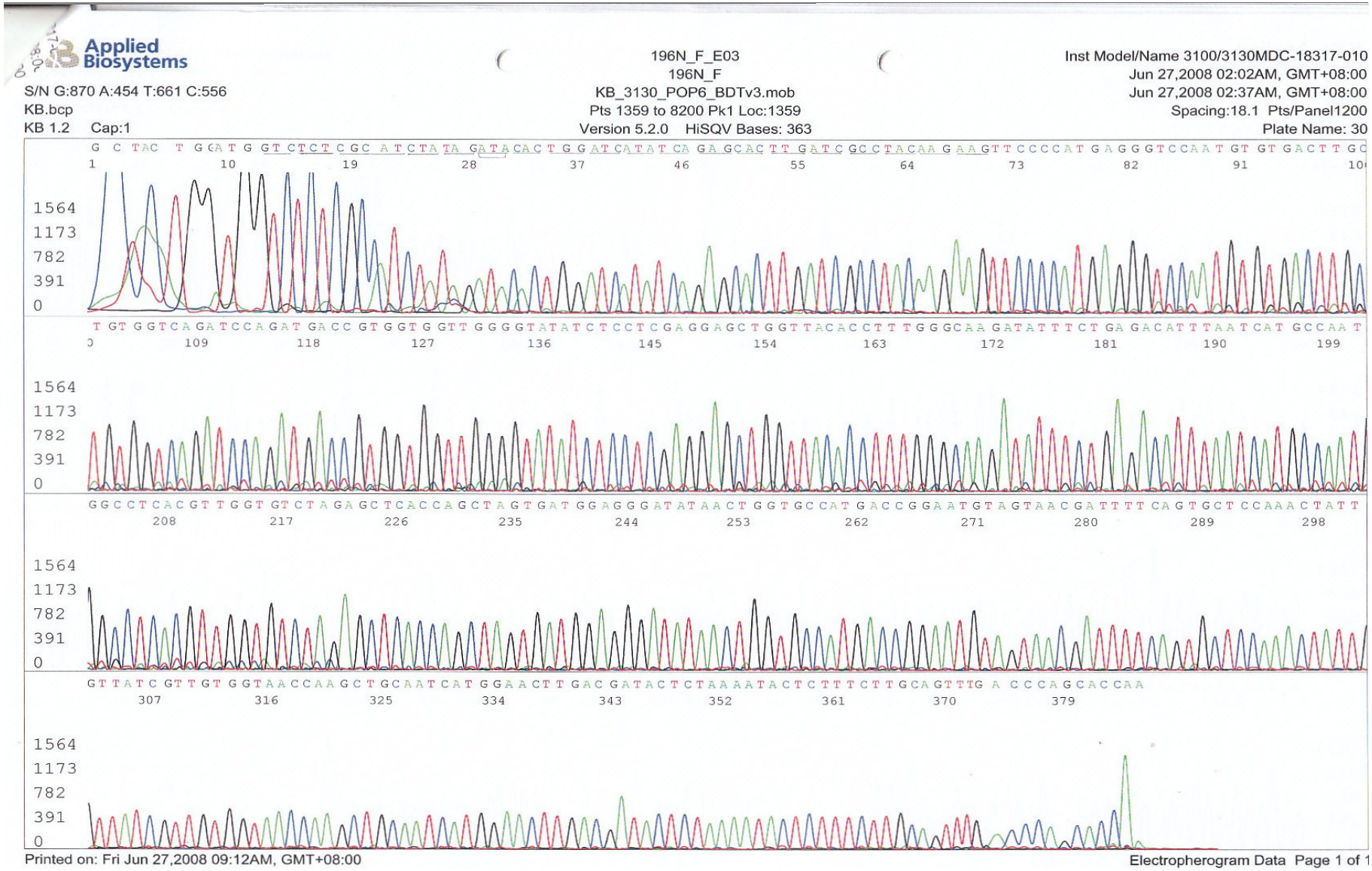

Table 4: DNA Sequencing (continued)

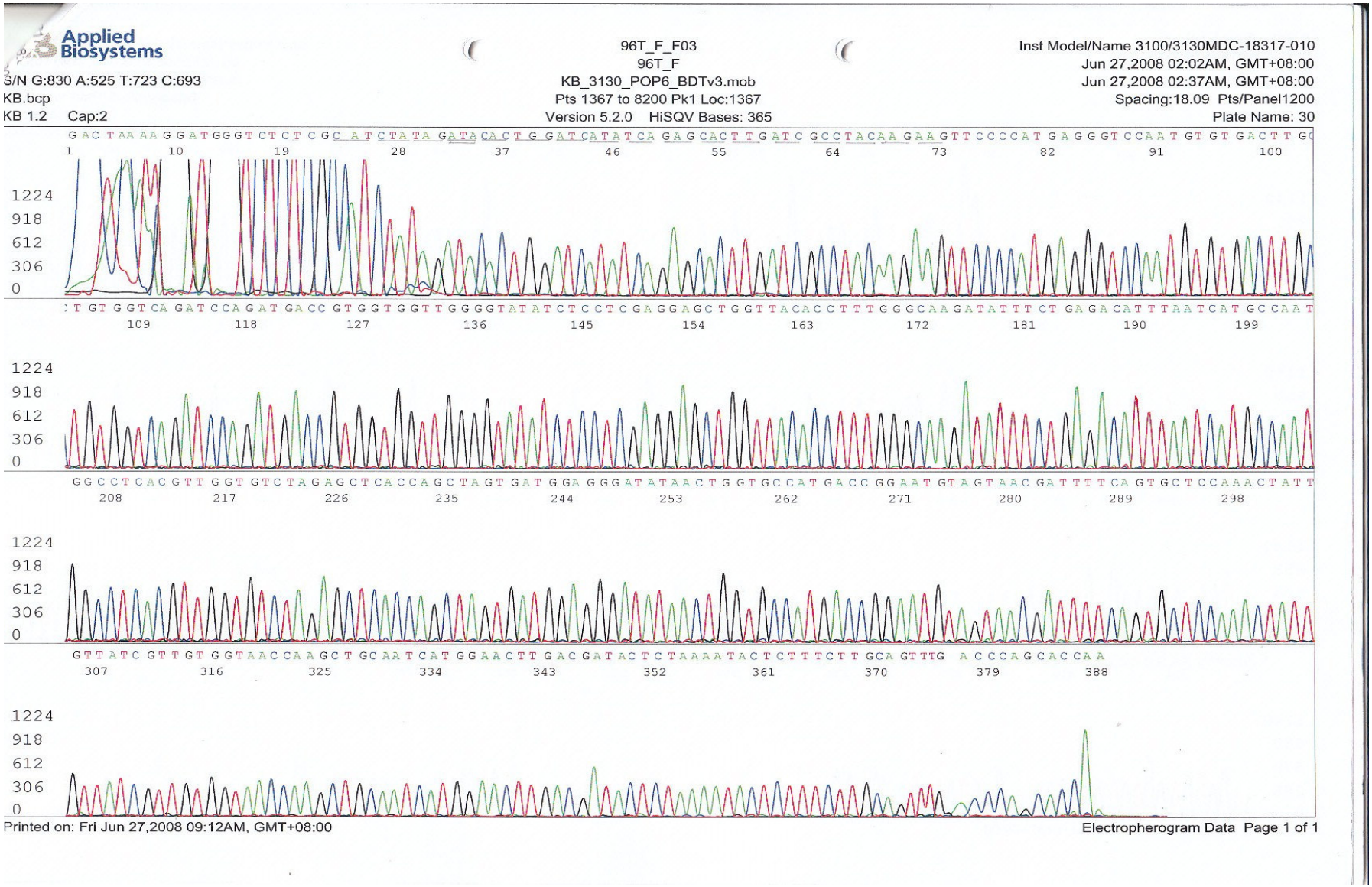

Table 4: DNA Sequencing (continued)

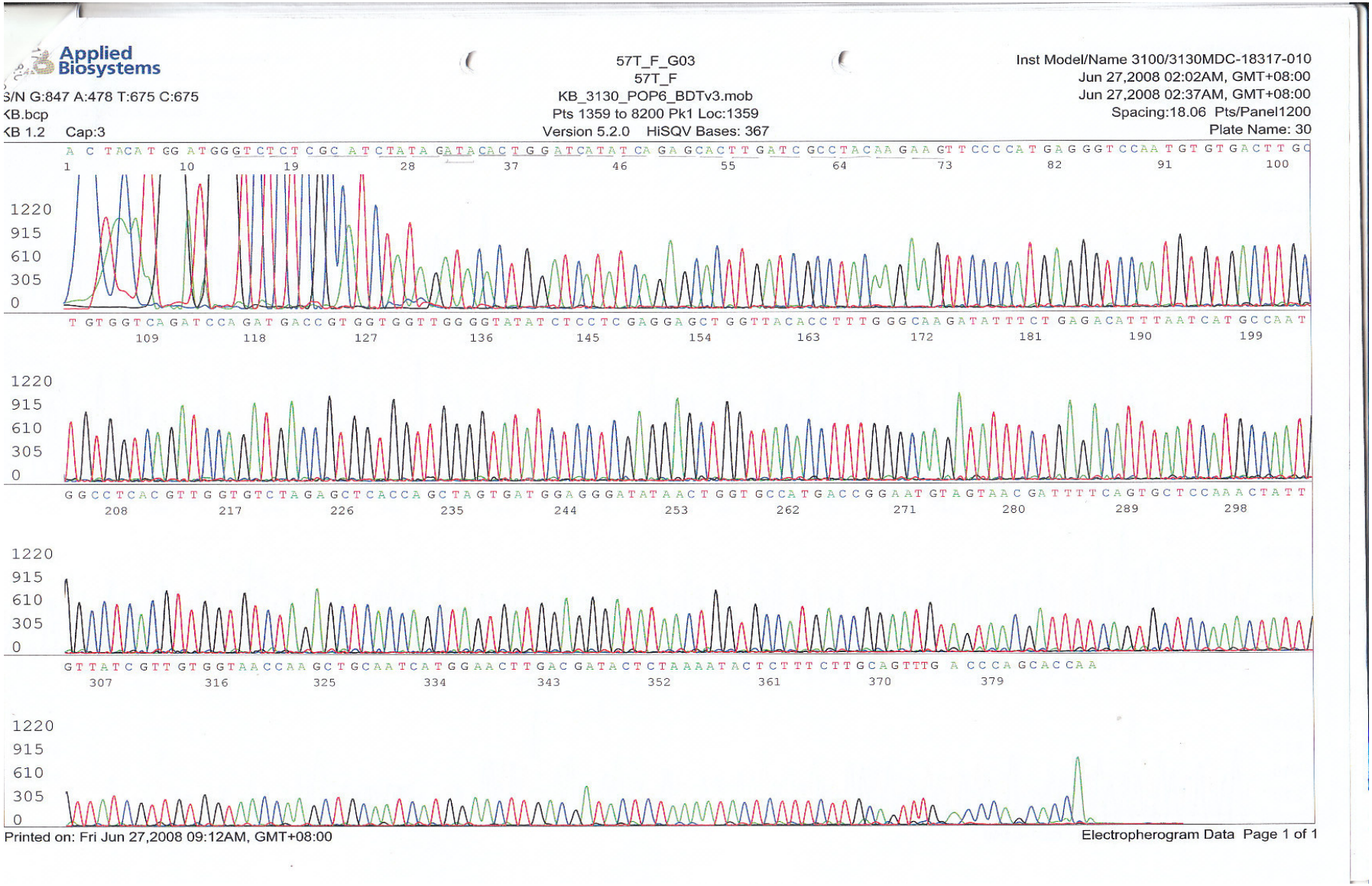

Table 4: DNA Sequencing (continued)

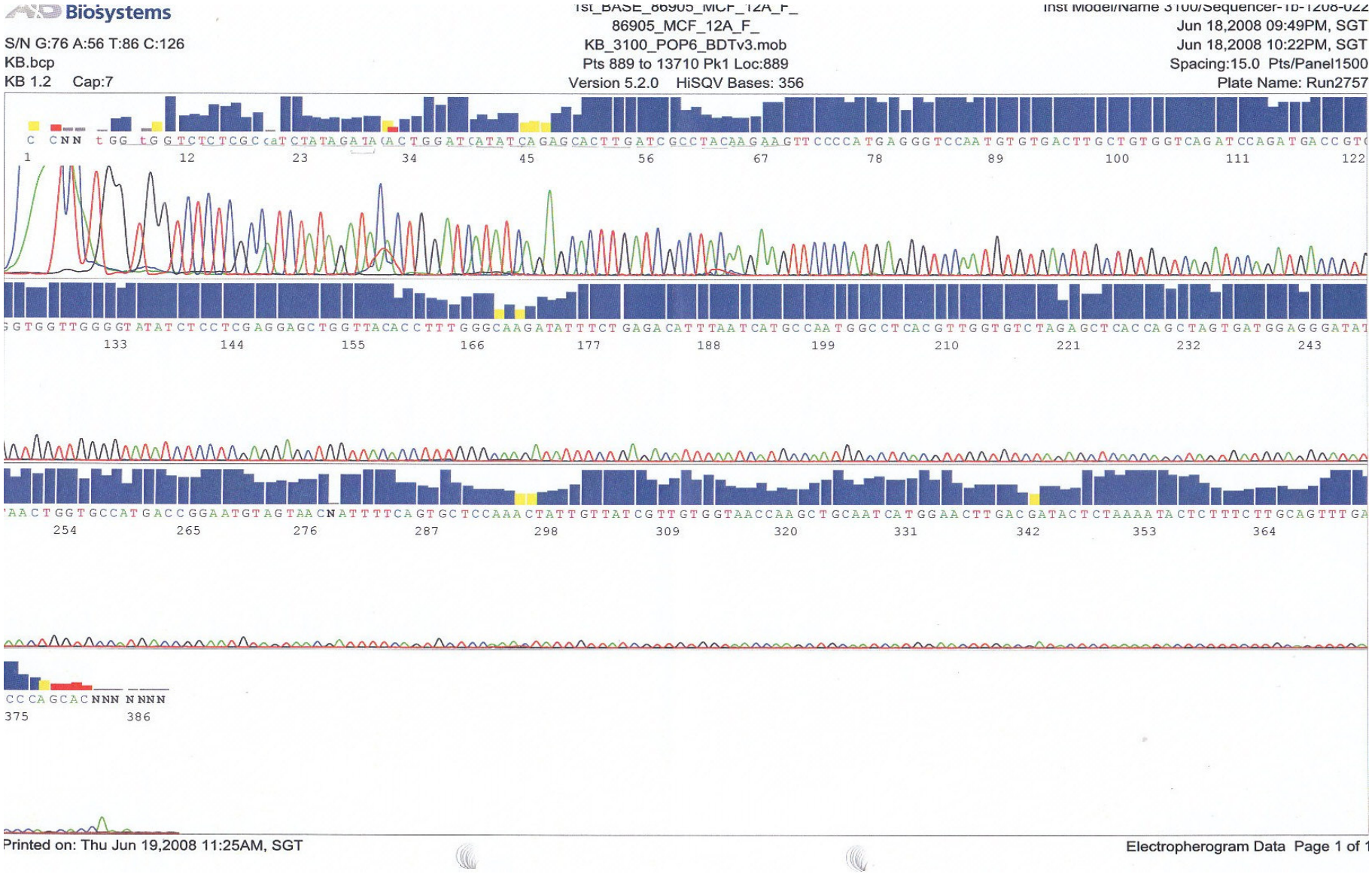

Table 4: DNA Sequencing (continued)

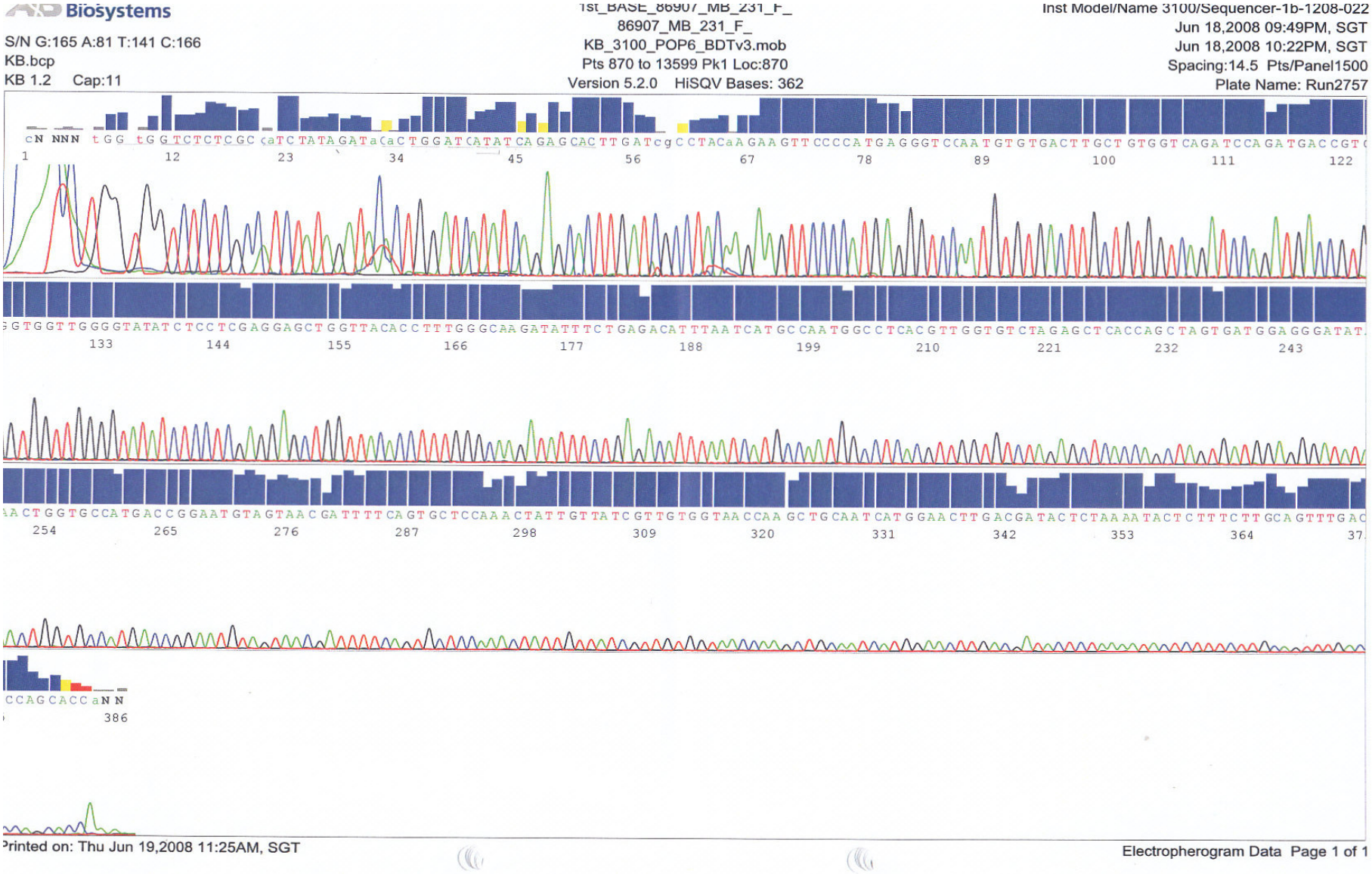

Table 4: DNA Sequencing (continued)

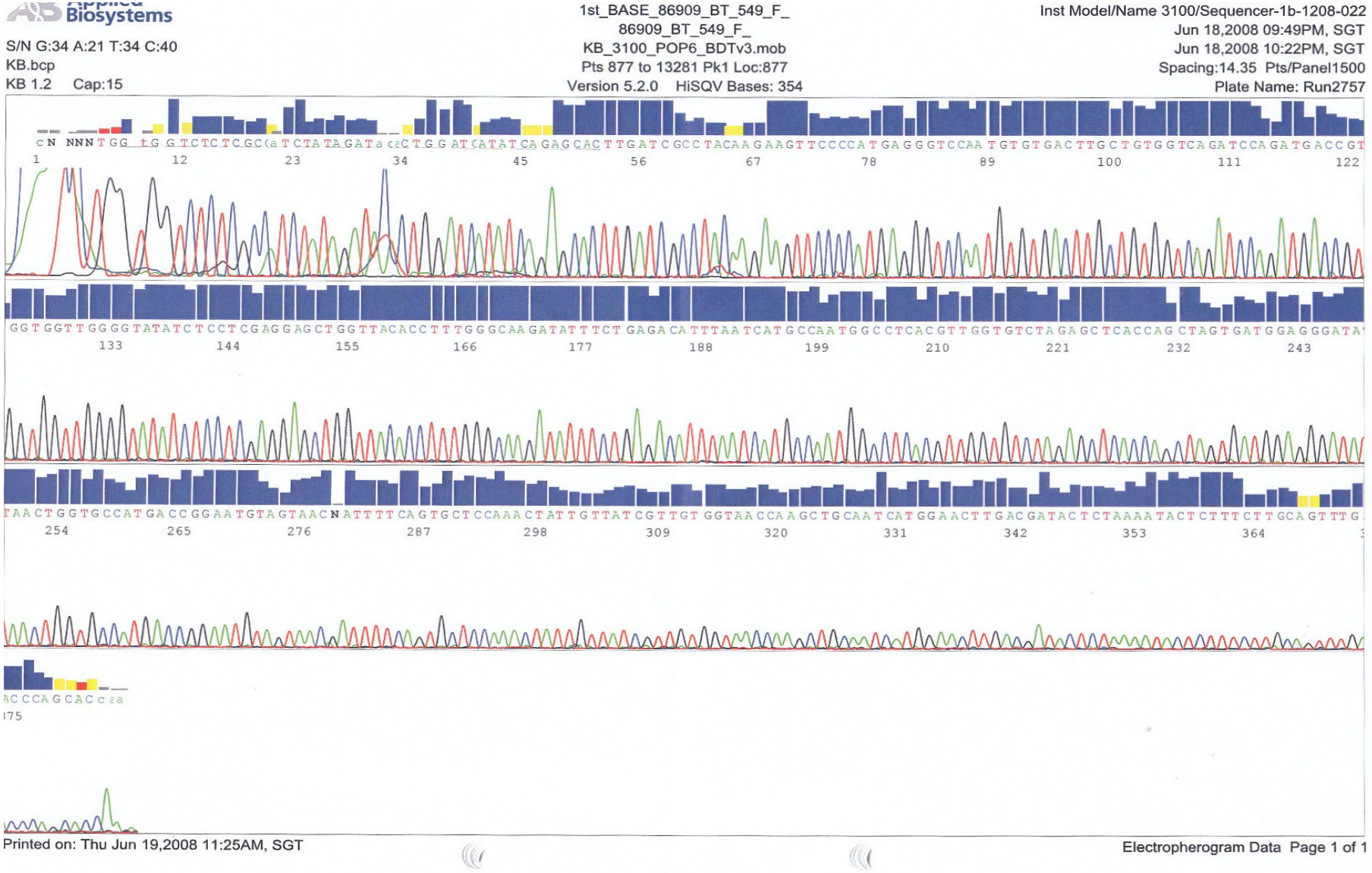

Table 4: DNA Sequencing (continued)

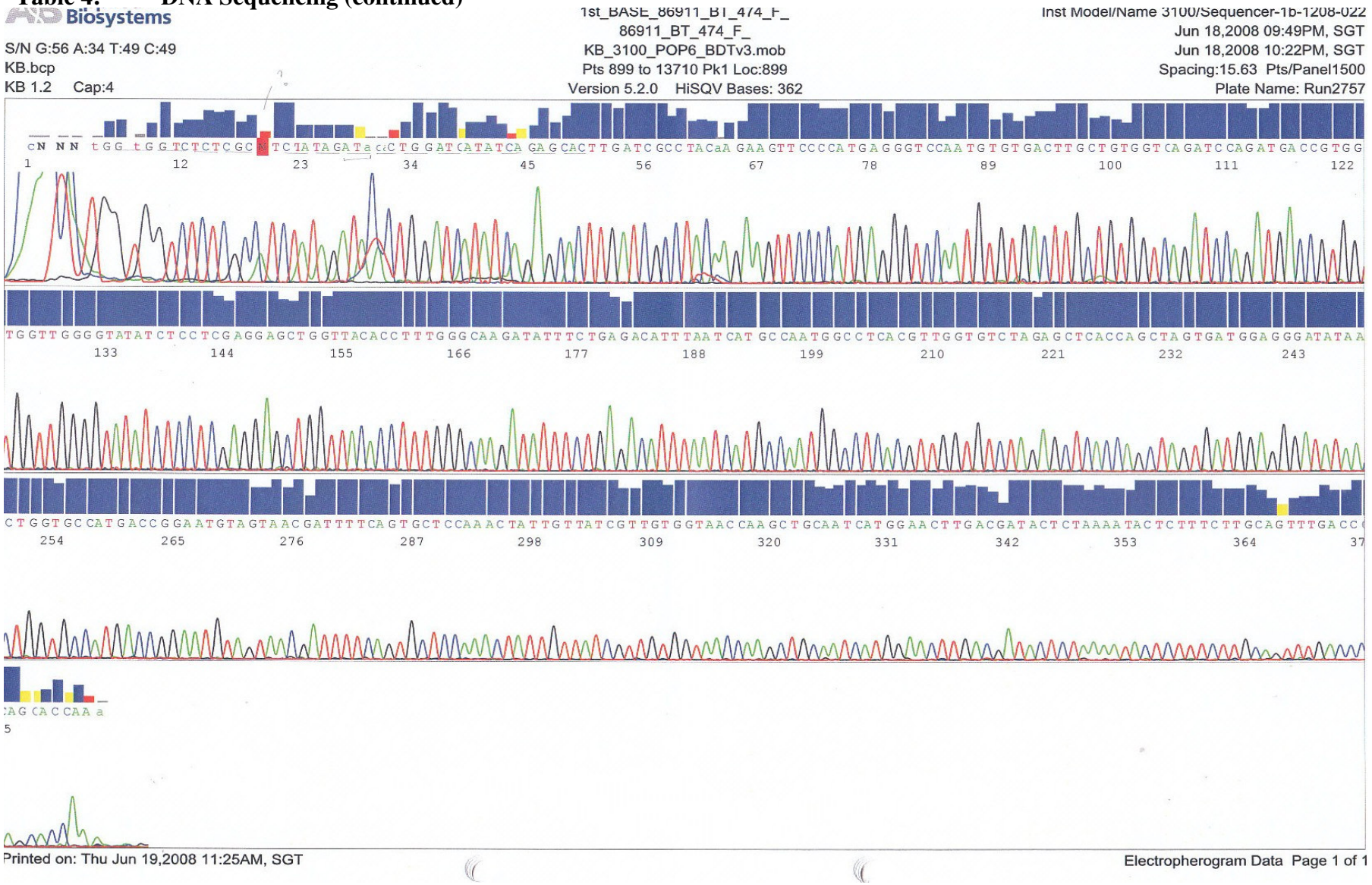

Table 4: DNA Sequencing (continued)