EXPRESSION OF HER4 ISOFORMS AND HER4 LOCALISATION IN HER2 NEGATIVE BREAST CANCER CELL LINES

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EXPRESSION OF HER4 ISOFORMS AND HER4 LOCALISATION IN HER2 NEGATIVE BREAST CANCER CELL LINES

by

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LIST OF SYMBOLS

S_{80}	Soluble 80 kDa intracellular domains
CT	Threshold cycle
ΔCT	delta CT value
Tm	Melting temperature

LIST OF ABBREVIATIONS

HER	Human Epidermal growth factor Receptor
EGFR	Epidermal Growth Factor Receptor
TACE	Tumor necrosis factor Alpha-Converting Enzyme
PI3K	Phosphatidylinositol 3-kinase
EGF	Epidermal Growth Factor
HB-EGF	Heparin Binding- Epidermal Growth Factor
IHC	Immunohistochemistry
CEN 17	Chromosome 17 centromere
ER	Estrogen Receptor
PR	Progesterone receptor
MAPK	Mitogen activated protein kinase
PTEN	Phosphatase and tensin homology
RTK	Receptor tyrosine kinases
EGFRs	Epidermal growth factor receptor
TGF-α	Transforming growth factor -α
NRG	Neuregulins
AR	Amphiregulin
RIP	regulated intramembrane proteolysis
DMEM	Dulbecco's Modified Eagle Medium
PenStrep	Penicillin / Streptomycin
FBS	Fetal bovine Serum
BSC	Biological safety cabinet
TNBC	Triple negative breast cancer
PVDF	Polyvinimylidene difluoride
TBS	Tris-buffered saline
ECL	Enhanced chemiluminesence
q RT-PCR	quantitative Real Time-Polymerase Chain Reaction
NTC	Non- template control
GAPDH	Glyceraldehyde-3-phospahte dehydrogenase
BSA	Bovine serum albumin
EPR	Epiregulin

MES	2-morpholinoethanesulfonic acid
SDS	Sodium dodecyl sulfate

EKSPRESI HER4 ISOFORM DAN HER4 PENSETEMPATAN DI DALAM HER2 NEGATIF KANSER PAYUDARA SEL LINE

ABSTRAK

Kanser payudara merupakan antara kanser yang lazim dalam kalangan wanita di mana jenis kanser payudara paling banyak didapati adalah HER2 positif. Pesakit dengan kanser payudara jenis HER2 positif biasanya dirawat dengan antibodi monoklonal Herceptin yang terbukti mampu menambah baik kelangsungan hidup pesakit. Namun begitu, kerintangan terhadap Herceptin secara primer atau perolehan menjadi cabaran terhadap keberkesanan rawatan ini. Peningkatan ekspresi yang berlebihan pada reseptor– reseptor HER1, HER2 dan HER4 merupakan antara mekanisma rintangan kepada Herceptin. Berbanding dengan ahli HER yang lain, HER4 mempunyai fungsi yang tidak jelas terhadap kanser payudara di mana sesetengah kajian mengaitkan HER4 dengan fungsi onkogen manakala sesetengah kajian yang lain mengaitkan HER4 dengan fungsi penghalang tumbesaran tumor. Fungsi HER4 yang bertentangan adalah disebabkan oleh kehadiran isofom dan pensetempatan HER4 yang berbeza. Dalam kajian ini, kami menentukan ekspresi HER4 isofom dan pesetempatan HER4 di dalam dua jenis titisan sel HER2 negatif iaitu MCF-7 dan MDA-MB 231. Ekspresi HER4 isoform (CYT-1, CYT-2, JM-a dan JM-b) ditentukan mengunakan teknik *'qRT-PCR'* manakala pesetempatan HER4 ditentukan mengunakan teknik *'western blot'*. Hasil kajian mendapati terdapat perbezaan ekspresi HER4 isofom di antara MDA-MB-231 (HER2- / ER + / PR +) dan MCF-7 (HER2- / ER + / PR +). HER4 nuklear dan HER4 sitoplasmik lebih banyak diekspreskan dalam sel MCF-7 berbanding dengan MDA-MB 231. Ekspresi isoform HER4 dan pesetempatan HER4 yang lebih tinggi pada sel MCF-7 yang

mempunyai ekspresi ER positif mencadangkan hubungan HER4 dan ER dalam mengaruhkan mekanisme rintangan anti-HER2. Pemahaman mengenai peranan interaksi HER4-ER dalam rintangan anti-HER2 adalah diperlukan pada masa hadapan.

EXPRESSION OF HER4 ISOFORMS AND HER4 LOCALISATION IN HER2 NEGATIVE BREAST CANCER CELL LINES

ABSTRACT

Breast cancer is the most common cancer-affecting women with the most common subtype is HER2 positive breast cancer. Patients with HER2 positive breast cancer are treated with Herceptin, a monoclonal anti-HER2 antibody that is proven to improve survival. Unfortunately, the incidence of primary or acquired resistance to Herceptin remains a challenge for an effective treatment. The overexpressions of HER family receptors such as HER1, HER3 and HER4 are one of the mechanisms of Herceptin resistance. Among the HER family receptors, the role of HER4 in breast cancer is uncertain where some reports suggest a tumour suppressive function while others suggest an oncogenic function. The conflicting role of HER4 is due to the presence of different isoforms and nuclear translocation of HER4. In this study, we investigated the expression of HER4 isoforms and its localisation in HER2-negative cell lines, MCF-7 and MDA-MB 231. The expression of HER4 isoforms (CYT-1, CYT-2, JM-a and JM-b) was determined by qRT-PCR, while nuclear and cytoplasmic localisation of HER4 was determined by western blot. There is a variability of HER4 isoforms expressions in between the MDA-MB-231 (HER2-/ER+/PR+) and MCF-7 (HER2-/ER+/PR+). Nuclear and cytoplasmic HER4 were highly expressed in MCF-7 cells than MDA-MB 231. High expressions of HER4 isoforms and HER4 localisations in MCF-7 that have positive ER expression may suggest a possible association of HER4 and ER in mediating the mechanism of anti-

HER2 resistance. In future, understanding on the role of HER4-ER interaction in anti-HER2 resistance is warranted.

CHAPTER 1

INTRODUCTION

1.1 Background of study

Breast cancer is the most common cancer and leading causes of cancer related death amongst women worldwide (Azamjah et al., 2019). Treatments for breast cancer depend on different subtype of the cancer (Perou *et al.*, 2000). For instance, breast cancer with a positive hormone receptor is eligible for targeted hormone therapy. The most commonly overexpress hormones detected in breast cancer are estrogen and progesterone. In addition, amplification of HER2 gene was detected in 10-40% of breast cancer patient (Menard *et al.*, 2000).

HER2 positive is one of the molecular subtypes of the breast cancer. HER2 is a member of human epidermal growth factor receptor of tyrosine kinase family. Overexpression of HER2 in breast cancer is associated with aggressive behavior and poor outcomes (Sarode *et al.*, 2015). Patient with positive HER2 overexpression are treated with Herceptin, a monoclonal antibody that binds to juxtamembrane region of HER2 receptor (Vogel *et al.*, 2001). Herceptin given as part of treatment regimen together with chemotherapy had been proven to be effective by increasing relapse free survival for HER2 positive breast cancer patient (Eiermann, 2001). However, Herceptin resistance had given a new challenge to the treatment. Patients who develop resistance to Herceptin show disease progression within one year of treatment with Herceptin (Lavaud and Andre, 2014). HER2 positive breast cancer patient might developed primary or acquired resistance to Herceptin. Primary resistance can be defining as patients shows no response after initial treatments while acquired resistance can be defining as patient initially responding to treatments but

after prolonged exposure, the disease relapse. (Simasi *et al.*, 2014). The mechanism of resistance to Herceptin can be summarized and classified to three categories, which is the steric effects or masking of Herceptin binding site which occurs with formation of truncated HER2 receptor. The second mechanism is alternative elevations of other tyrosine kinase receptor which also can induce similar cellular response with HER2 receptor. Lastly, the mechanism for Herceptin resistance is alterations in HER2 downstream signaling such as mutation of PTEN (Vu and Claret, 2012).

HER2 belongs to human epidermal growth factor receptors that also include other members, HER1, HER3 and HER4. In contrast with HER2 and HER1, the role of HER4 expression in breast cancer remains unclear. Some studies such as study by Han et al. (2014) shows HER 4 can regulates estrogen stimulated genes and associated with breast tumor cell proliferation and cell cycle progression indicate oncogenic function of HER4. Meanwhile in other different study shows reactivation of HER4 expression by treating HER4-negative cells lines with the demethylation agent and stimulation with heregulin results in increase cell death (Das et al., 2010).

The contradicting role of HER4 is due to the presence of different HER4 isoforms and localisation of the receptor. HER4 mRNA splicing occurs in two regions. The first region is the juxtamembrane producing two isoforms, JM-a and JM-b (Elenius *et al.*, 1997). These two isoforms differ from each other by their susceptibility to the cleavage. Only JM-a isoforms which include exon 16 is susceptible to cleavage while JM-b is not. Cleavage of JM-a isoforms is mediated by metalloprotease known as tumor necrosis factor alpha converting enzyme (TACE) or ADAM 17 (Rio *et al.*, 2000). The first cleavage releases a 120kDa fragment of

HER4 into extracellular cells leaving attached 80 kDa fragments in cell membrane. The JM-a isoforms undergo secondary proteolytic cleavage catalyzed by the gamma (γ)-secretase to release soluble 80kDa intracellular domain (S80) which can translocate to the nucleus and can regulate transcriptional activity (Muraoka-Cook *et al.*, 2006).

The second mRNA splicing occurs in the cytoplasmic domain (CYT) producing two isoforms, CYT-1 and CYT-2. The CYT-1 isoform differ from CYT-2 by the presence of exon 26, which contains the binding site for phosphatidyl inositol 3 – kinase (PI3K) (Elenius *et al.*, 1999). The biological response for both isoforms are different from each other. Both isoforms can mediate proliferation (Kainulainen *et al.*, 2000) but only CYT-1 isoforms can mediate cell differentiation (Muraoka-Cook *et al.*, 2009). In addition, CYT-1 isoforms also include PPXY motif that causes the isoforms to be highly susceptible to degradation compare to CYT-2 (Sundvall *et al.*, 2008). Different expression of isoforms and localization of HER4 in breast cancer shows different prognosis. The presence of nuclear HER4 in breast cancer is associated with poor prognosis (Junttila *et al.*, 2005). Inhibitions of HER4 phosphorylation and cleavage by a monoclonal antibody results in reduced proliferation and colony formation in MCF-7 and T47D breast cancer cell lines (Hollmen *et al.*, 2009). The cytoplasmic isoforms of HER4 also shows varied outcome. In breast cancer, expression of full length CYT-1 isoform results in the formation of neoplastic lesion wheres expression of CYT-2 isoform only result in a low incidence of hyperplasia (Wali *et al.*, 2014). In addition, the splice switching oligonucleotide that converts CYT-1 to CYT-2 isoform can inhibit growth of breast cancer cell (Nielsen *et al.*, 2013).

Previous studies had also reviewed the role of HER4 in mediating resistance to treatment against HER2. Although Herceptin is developed to reduce HER2 effects, it is unable to reduce HER2 phosphorylation (Scaltriti *et al.*, 2009). The phosphorylation of HER2 is maintained by activation of other HER family receptor including HER4, which form dimerization partner with HER2 (Gijsen *et al.*, 2010). HER4 receptor activation occurs due to the release of the ligand in a Herceptin treated cell (Kong *et al.*, 2008) mediated by ADAM 10 (Ebbing *et al.*, 2016) and ADAM 17 (Gijsen *et al.*, 2010). Increase level of ADAM 17 induce release of TGF-α, amphiregullin, heparin binding(HB)-EGF and epiregullin while ADAM 10 induce release of EGF and neuregulins (Duffy *et al.*, 2011). Prolonged Herceptin treatment had been shown to increase the level of ADAM protease (Feldinger *et al.*, 2014).

Acquired resistance to Herceptin treatment in HER2 breast cancer occurs when the resistance cell shifts their dependency to HER4. Knockdown of HER4 expression by siRNA in lapatinib-resistance breast cancer cell will increase cell apoptosis (Canfield *et al.*, 2015). The role of HER4 in mediating resistance in other cancer is also well studied. In gastric cancer, HER4 together with YAP1 increased Herceptin resistance (Shi *et al.*, 2018). In lung cancer, inhibition of a ligand-mediated HER4 signalling enhance the duration of treatment (Hegde *et al.*, 2013). Herceptin also induce HER4 cleavage and nuclear translocation. Preventing nuclear translocation by γ-secretase inhibitor(GSi) in cell treated with Herceptin can prevents their cleavage and enhanced Herceptin response (Nafi *et al.*, 2014)

Nevertheless, no studies mentioned above included investigation in a panel of primary Herceptin-resistant breast cancer. About 30% of patients selected for Herceptin therapy shows improvement in disease free survival while other shows

primary resistance despite overexpression of HER2 receptor (Narayan *et al.*, 2009). The underlying mechanism for primary drug resistance usually develops before treatment is given. Most of the previous studies used two most common HER2-positive cell lines that develop parental and acquired Herceptin-resistant, SKBR3 and BT474 cells. It would be essential to consider whether HER4 expression could predict response in relation to the primary Herceptin resistant. Example of cell lines that have negative response to anti- HER2 treatments are MCF-7and MDA-MB-231(Wilken and Maihle, 2010). MCF-7 cell lines are HER2 negative/ER positive (luminal A) while MDA-MB-231 cell lines are HER2 negative/ER negative (Basal type) (Holliday and Speirs, 2011). A study by Orzechowska *et al.* (2017) shows that the differences in hormone receptor express by luminal A and basal type breast cancer contributes to distinct profiles of both cancer types. Therefore, it was concluding that the differences in characteristic and response among different breast cancer subtypes may influence the expression of HER4 isoforms and HER4 cellular localisation. Variation in expression of HER4 isoforms together with the localisation might contribute to the Herceptin resistance.

1.2 Rationale of study

Development of Herceptin that blocks HER2 receptor has helped reverse the severe prognostic effect related to HER2 overexpression in breast cancer patients. However, Herceptin treatment also has drawbacks. Prolonged treatment with Herceptin had been associated with cardiac toxicity (Lamot *et al.*, 2010). In addition, the cost of treatment is also high (Wolff *et al.*, 2007). Therefore, it is important to accurately choose the patients who will gain most benefits from the treatment. Less

than 35% of patients show response to Herceptin treatment despite the overexpression of HER2 receptor (Vu and Claret, 2012).

Previous studies had shown that HER4 expression and activation play a role in mediating the mechanisms of acquired resistance to Herceptin. In this study, the role of HER4 in a luminal A and basal type breast cancer cell was investigated. Both luminal A and basal breast cancer were not responsive to Herceptin due to the absence of HER2 overexpression. Different subtypes of breast cancer cells have different molecular profiles which contribute to differing characteristics and responsiveness to the therapy. Therefore, it is believed that different subtypes of breast cancer may have different patterns of HER4 expression. In addition, this study also focuses on the expressions of different HER4 isoforms and their localisations in HER2-negative cell lines. The expressions of different HER4 isoforms and their localisations indicate different signalling pathway in breast cancer and hence might contribute to varying role to mediate resistance to the treatment.

In clinical practice, there is currently no conclusive biomarker for Herceptin treatment where they just depend on HER2 overexpression. Therefore, understanding the mechanism of action and development of resistance for Herceptin is necessary. Such knowledge will guide the design of better combination therapy and selection of patients for better respond to the treatment. HER4 expression and activation could be a new molecular mediator in understanding mechanism of resistance to anti-HER2 therapies. Therefore, this current study focuses on investigating the expression of HER4 isoforms and their localisation in various HER2-negative cancer cell models in vitro. This study aims to elucidate the mechanism of HER4 expression and their activation in the HER2- negative cell lines. The finding of this study might assist the

understanding of some fundamental aspects for molecular mechanisms of HER4 signalling in mediating Herceptin resistance.

1.3 Objective of study

1.3.1 General Objective

- To study the expression of HER4 isoforms and its localisation in a HER2-negative breast cancer cell lines.

1.3.2 Specific Objective

- To determine the mRNA expression of HER4 isoforms in MCF-7 and MDA-MB 231 cell lines by using RT-PCR.
- To analyse HER4 cellular localisation by protein expression in MCF-7 and MDA-MB 231 cell lines using western blot.

CHAPTER 2

LITERATURE REVIEW

2.1 Breast cancer incidence

Breast cancer is the most common female malignancy in both developing and developed country. It is estimated that 1 in 8 women will be affected with breast cancer in their lifetimes (Hsu and Hung, 2018). In 2012, there were an estimated 1.7 million new breast cancer cases reported worldwide (Stewart and Wild, 2017). It is the most common cause of death among women which account for 14% of death related to cancer worldwide (Lahart *et al.*, 2015). It was recorded that there were 252,710 new cases of invasive breast cancer and 63,410 new cases of in situ breast carcinoma among US women in 2017 (DeSantis *et al.*, 2017).

The incidence of breast cancer was higher in developed country compared to less developed country (Torre *et al.*, 2015). However, the survival rate for women with breast cancer is higher in developed country since women in more developed country received a better care and treatment (Downing *et al.*, 2007). The top five country with highest recorded standard mortality rates were Fiji, Bahamas, Macedonia and Pakistan (Ghoncheh *et al.*, 2016).

In Malaysia, 18, 343 breast cancer cases were recorded during the period of 2007 to 2011, make up to 17.7% of all cancer types as shown in Figure 2.1. Most effected women were in the group age 25 to 75 years.

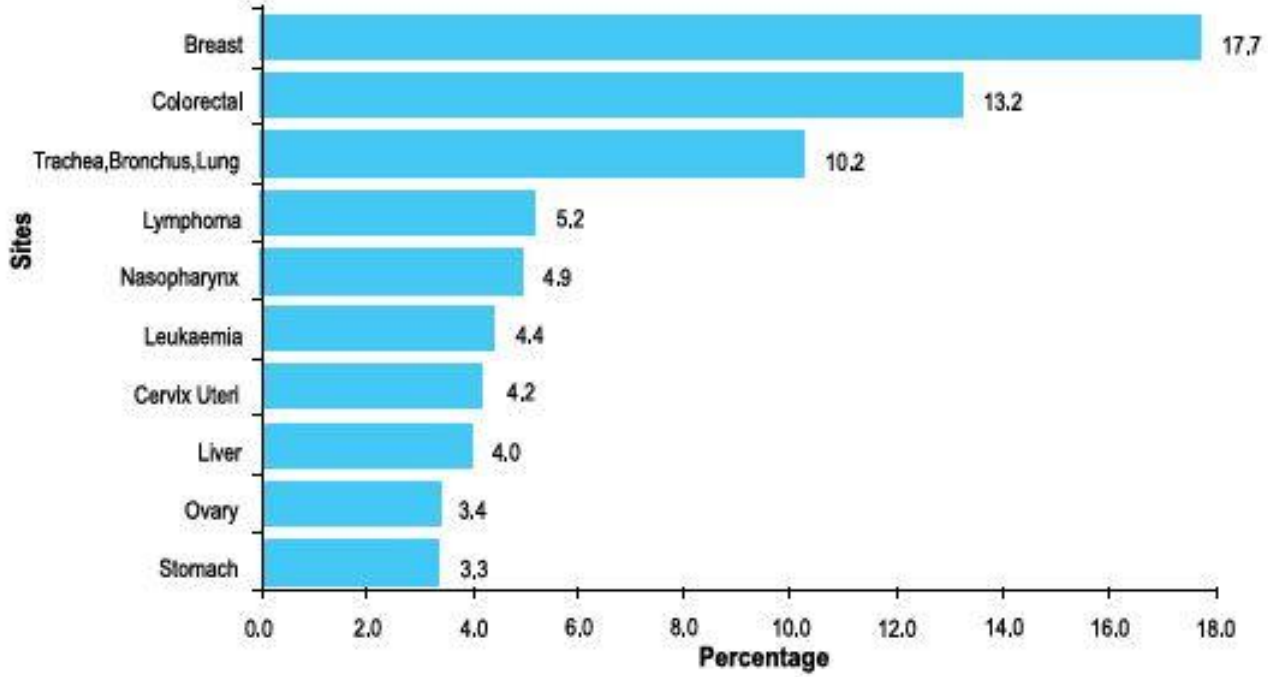

Figure 2.1 Percentage of cancer incidence according to the primary sites among Malaysian residence from 2011 to 2015 (Ministry of Health Malaysia, 2017).

Figure 2.2 shows the percentage of the five highest causes of mortality among women in Malaysia. According to the Department of Statistic Malaysia, in 2018 the five most common causes of mortality among female in Malaysia were pneumonia, ischemic heart disease, cerebrovascular disease, malignant neoplasm of the breast and diabetes mellitus (Department of Statistic Malaysia, 2019). The percentage of death due to malignant neoplasm of the breast was 4.2% (1 249 cases) in 2018 which decreased by 0.2% compare to 2017.

The Ministry of Health Malaysia had been promoting breast self-examination since 1995 (Dahlui *et al.*, 2011). Breast self-examination is a patient centred, less invasive and inexpensive (Hackshaw and Paul, 2003). An early detection of breast cancer can prolong survival rate of breast cancer patients (Rahimzadeh *et al.*, 2014). Treatments for breast cancer include surgery, chemotherapy and hormonal therapy. A study conducted among Malaysia and Singapore population shows 70% of patients had mastectomy, 30% of patients had breast conserving surgery, 56% of patients had chemotherapy and 60% had hormonal therapy (Bhoo Pathy *et al.*, 2011).

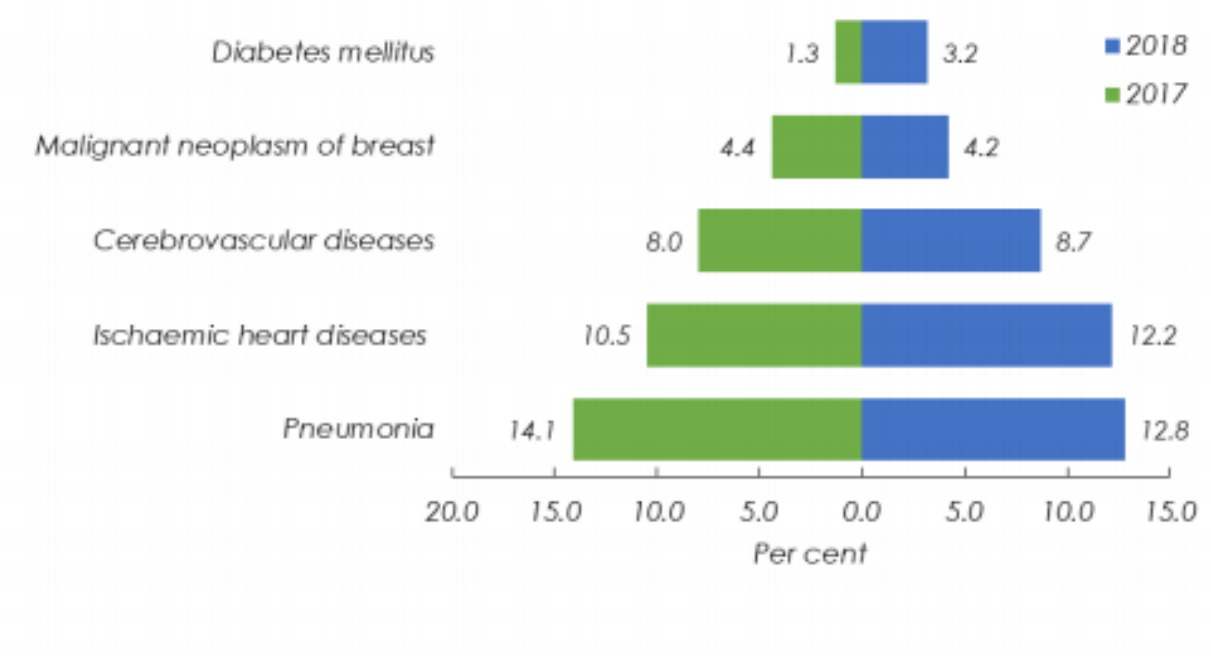

Figure 2.2 Percentage of five common causes of death among female in Malaysia (Department of Statistic Malaysia, 2019)

2.2 Breast cancer subtype

Breast cancer is highly heterogeneous disease that can be divided into several subtypes. Each subtypes of breast cancer have different pathological characteristic and clinical implication. Conventionally, breast cancer can be divided based on morphology. Breast cancer is classifying to different morphology subtypes based on degree of tumor differentiation using hematoxylin-eosin staining (Rakha et al., 2010). Classification using morphology is semi-quantitative, simple and low-cost method. One of the method used to classified breast cancer by morphology appearance is Nottingham grading system, which are used to classify breast cancer based on tumour tubule formation, number of mitotic figure and nuclear polymorphism (Chang et al., 2015).

With the advanced of the high throughput platform for gene expression analysis, the breast cancer can further classify based on molecular profiling. The pioneer scientist, Sorlie classified breast cancer into five different subtypes as shown in Figure 2.3 which are luminal A, luminal B, HER2 overexpression, and basal-like subtype. A study done to validate the subtyping of breast cancer using molecular profiling shows difference in relapse free and overall survival for each group (Hu *et al.*, 2006). In addition, tissue microarray was also used to validate molecular profiling at the protein expression level showing different clinical outcomes for each group (Abd El-Rehim *et al.*, 2005). The molecular subtypes are also associated with morphology characteristic of breast cancer as different molecular pathways show specific tumor morphologies (Bombonati and Sgroi, 2011).

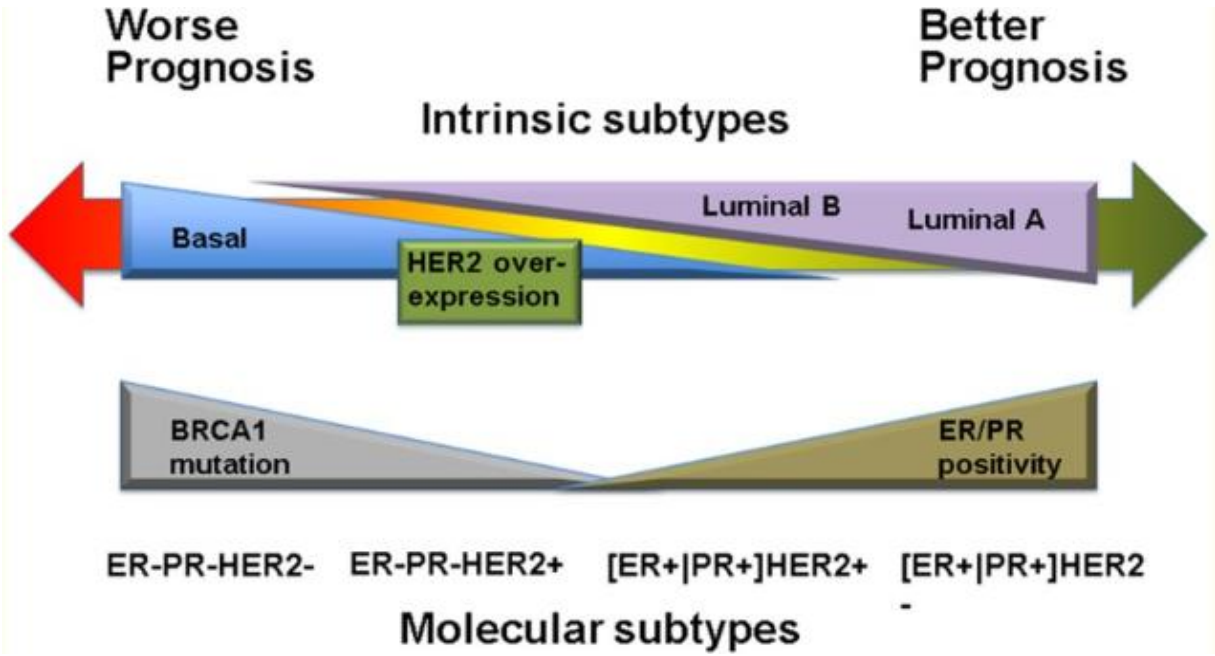

Figure 2.3 Association of cancer molecular subtypes with the clinical outcome (Dai *et al.*, 2015).

The luminal breast cancer can be classified into two subtypes, luminal A and luminal B (Sotiriou *et al.*, 2003). The luminal A can be define as tumor with ER+, PR+, HER2- while luminal B is a tumor with ER+, PR+, HER2+ (Nielsen *et al.*, 2004).The prevalence of luminal subtype depends on several factor. A study conducted among Pakistani community shows luminal B is more prevalent compared to luminal A (Hashmi *et al.*, 2018). However, in other study conducted among Japanese women shows luminal A is more prevalent compared to luminal B (Shibuta *et al.*, 2011). In overall, luminal breast cancer has a good prognosis with luminal A tends to have better prognosis compared to luminal B (Cancello *et al.*, 2013).

HER2 overexpress define as breast cancer with overexpress HER2 gene. It is highly aggressive types compared to luminal type and are classify as grade III breast cancer (Vallejos *et al.*, 2010). The presence of amplified HER2 gene in breast cancer is an indicator for a targeted therapy (Neve *et al.*, 2001). The details for HER2 positive breast cancer types are explain in the next subtopics 2.6.

The last subtype for breast cancer classified based on molecular profiling is basal type or also known as triple negative breast cancer. This class of breast cancer has negative expression of HER2, ER and PR (Kreike *et al.*, 2007). The expression profile of basal type mimic the normal epithelial cell with positive expression of EGFR and cytokeratin (Cheang *et al.*, 2008). In addition, basal type of breast cancer also confers a TP53 mutation (Kriegsmann *et al.*, 2014) and BRCA1 mutation (Abd El-Rehim *et al.*, 2005). BRCA1 is tumor suppressor gene that plays an importance role in controlling DNA repair (Liu *et al.*, 2008). The frequency of TP53 mutation lower in luminal subtypes follow by HER2 positive and highly found in triple negative subtypes (Dumay *et al.*, 2013). Mutation of TP53 and BRCA1 gene had

been linked to aggressive behavior of triple negative breast cancer such as lower survival prognosis with higher chance of disease relapse (Ho-Yen *et al.*, 2012). The basal breast cancer was not amenable for targeted therapy as it lacks of hormone expression (Brenton *et al.*, 2005).

2.3 Receptor tyrosine kinase

Receptor tyrosine kinases (RTK) are cell surface receptors that bind with high affinity to specific ligands such as growth factors and hormone. Activation of receptor leads to phosphorylation of tyrosine kinase in the intracellular domain which activate cellular pathway including RAS/MAPK signaling pathway, PI3K/AKT signaling and Jak2/STAT signaling among others (Schlessinger, 2014). Important cellular activity such as cell growth, differentiation, adhesion, migration and death are transmitted through tyrosine kinase (Robinson *et al.*, 2000). The subfamily of RTK includes epidermal growth factor receptor (EGFRs/ ErbB), fibroblast growth factor (FGFRs), insulin-like growth factor (IGFR), platelet derived growth factor (PDGFRs), vascular endothelial growth factor receptor (VEGFRs), hepatocyte growth factor receptor (HGFRs) and nerve growth factor (NGFRs) (Li and Hristova, 2006). Ligands binding to the RTK induce receptor dimerisation and phosphorylation of intracellular tyrosine kinase domain (Lemmon and Schlessinger, 2010). Phosphorylate RTK become a docking site for secondary messenger proteins and lead to induction of downstream signal.

Unregulated signaling by RTK is critically involves in malignant transformation, diabetes, inflammation, arteriosclerosis and angiogenesis (Lemmon and Schlessinger, 2010). For instance, overexpression of EGFRs was detected in lung cancer (Hirsch *et al.*, 2009), bladder cancer, cervical cancer, breast cancer and pancreatic cancer (Hong and Ullrich, 2000). Meanwhile, overexpression of VEGF receptor leads to the formation of new blood vessel and tumor invasion (Karkkainen and Petrova, 2000).

2.4 Human epidermal growth factor receptor (HER)

Human epidermal growth factor receptor (HER) is one of the tyrosine kinase receptor. The discovery of HER family receptor initiated from the discovery of ligand known as epidermal growth factor (EGF) by Stanley Cohen isolated from a mouse salivary gland and cause a mice eyes to open earlier than normal (Cohen, 1962). Isolation and characterisation of EGF brings to discovery of the receptor HER1 (Lipsick, 2019). There are also another ligands that can bind to HER known as EGF-like growth factors which include transforming growth factor (TGF)-α , neuregulins (NRG) , amphiregulin (AR), epiregulin (EPR) and Heparin binding-epidermal growth factor (HB- EGF) (Lacal, 2006).Other family members of HER are HER2, HER3 and HER4 were discovering later and the members share 40-45% sequence alike (Stein and Staros, 2000).

All family member of HER receptors are made up of extracellular ligand-binding domain, a transmembrane domain and intracellular domain (Bazley and Gullick, 2005). The extracellular domain consists of two homologous leucine-rich domains (L1 and L2) and two homologous cysteine-rich domains (CR1 and CR2) (Rajaram *et al.*, 2017). The domains are arranging in a tandem repeat where two homologous domains are separate by another homologous domain. (Leahy, 2004). In the inactive form, without ligand binding the extracellular domain exist in a tethered form that hide the dimerisation loop (Ferguson *et al.*, 2003). When the ligand binds between the domain L1 and L2 the formation of extracellular domain changes to an extended confirmation that exposed the dimerization loop (Lu *et al.*, 2010). Active receptor can form dimer with the same class of receptor (homodimer) or with different classes of receptor (heterodimer) (Duneau *et al.*, 2007). Receptor

hetrodimerisation is able to induce a more potent signal (Zaczek *et al.*, 2005). Figure 2.4 shows specific ligands binding to each receptors and formation of dimerization after receptor activated.

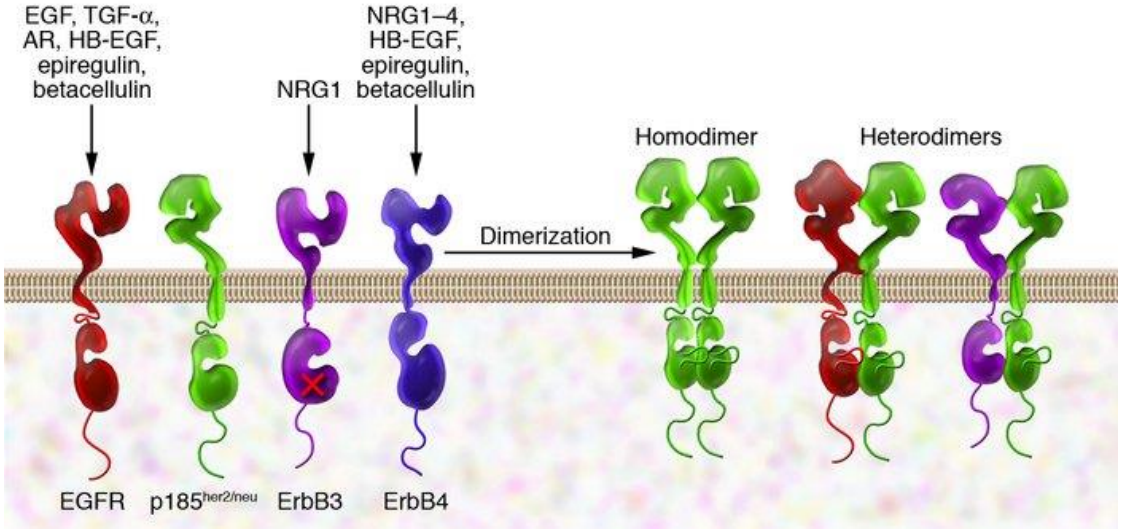

Figure 2.4 ErbB family receptor and the ligands bind to the receptor (Zhang et al. (2007).

The second part for ErbB receptor is transmembrane domain which consist of a single membrane spanning α-helix that function in stabilising the receptor dimerization structure (Mendrola *et al.*, 2002).

Lastly, intracellular domain consists of tyrosine kinase domain flanked by juxtamembrane and C-terminal regulatory protein (Burgess *et al.*, 2003). The juxtamembrane is located next to transmembrane region. The juxtamembrane region contains a nuclear localisation sequence (NLS) which conserved in all HER family. The NLS plays an important role in mediating nuclear localisation of the receptor (Hsu and Hung, 2007).

Next, tyrosine kinase in intracellular domain is divide into cytoplasmic domain that consists of NH2-terminal lobe (N-lobe) and COOH-terminal lobe (C-lobe). The N-lobe is composed of β-sheet and α-helix while C-lobe only composed of α-helix and the lobe was separated by a cleft where ATP, ATP analogues and ATP inhibitor can bind (Stamos *et al.*, 2002). A study by Zhang *et al.* (2006) shows that HER receptor was activated by allosteric mechanism similar to activation of cyclin-dependent kinase(CDK-2). When the receptor dimerize, the N-lobe of one receptor will interact with the C-lobe of another receptor resulting in kinase activation (Zhang *et al.*, 2006). Another part in intracellular domain is C-terminal regulatory protein, which consist of various tyrosine residues, which phosphorylates when the receptor is activate (Jones *et al.*, 2006). The phosphorylate tyrosine then become a docking sites for various adapter protein and enzymes possessing Src homology (SH2) or phosphotyrosine-binding (PTB) domains (Hubbard and Miller, 2007). The protein with Src homology 2 (SH2) domains such as GrB protein bind to phosphorylated protein which then activate RAS protein (Haley and Gullick, 2009). Upon

phosphorylation, RAS activate RAF kinase. Phosphorylated RAF then activate MEK1 and MEK2. The activated MEK kinase in turn phosphorylate and activate ERK protein (Karoulia *et al.*, 2017). Lastly, activated ERK then induce proliferation of many cell types (Matallanas *et al.*, 2011).

Other signalling cascade activate by HER receptor family is PI3K kinase pathway. After receptor activation, the phosptidylinositol 4, 5-biphosphate (PIP2) is phosphorylate forming phosphatidylinositol 3, 4, 5-triphospahte (PIP3). The PIP3 recruit AKT and then mTOR (Sarbassov *et al.*, 2005). The activated mTOR provides cell with survival signal by avoiding apoptosis (Song *et al.*, 2005).

2.4.1 Human epidermal growth factor receptor 1 (HER1)

The first EGF receptor known as HER1 was characterised by Cohen *et al.* (1982). HER1 is a 170-kDA transmembrane tyrosine kinase receptor (Shostak and Chariot, 2015). The EGF, TGF-α, HB-EGF and betacellulin (BTC) are classify as high-affinity ligands while AR and EPR are classify as low-affinity ligands (Singh *et al.*, 2016). After binding to the ligands, HER1 is able to form hetrodimers and hemodimers, which results in activation of intracellular signals. Two significance pathways activated by HER1 are RAS-Raf-MEK-ERK and the PI3K-PDK1-AKT pathway (Yarden and Sliwkowski, 2001). The activity of HER1 is crucial in embryonic development in which the receptors involve in organogenesis for organ such as brain, heart and lung (Janmaat and Giaccone, 2003).

Previous studies show aberrant expression of HER1 was associated with the development of cancer. The expression of HER1 are alter in cancer cells due to gene amplification and mutation of the receptor (Roskoski, 2014). A mutation of intracellular HER1 had been observed in 48-89% of lung cancer (Gupta *et al.*, 2009). Meanwhile, in breast cancer the mutation of HER1 receptor was rarely detected (Bemanian *et al.*, 2015). Aberrant changes of HER1 in breast cancer are overexpression of the receptors compared to normal cells. The overexpression of HER1 in mammary glands results in epithelial-mesenchymal transition, migration and tumor invasion (Masuda *et al.*, 2012). The epithelial-mesenchymal transition lead to resistance to chemotherapeutic for breast cancer (Sarrió *et al.*, 2008).

2.4.2 Human epidermal growth factor receptor 2 (HER2)

The HER2 oncogene was discovered in a rat exposed to carcinogen by Padhy *et al.* (1982). HER2 gene was located on short arm of chromosome 17 and encode

185-kDa protein (Sato *et al*., 1983). Molecular cloning had revealed that both HER2 and EGFR share a similar tyrosine kinase domain but differ in the extracellular domain (Schechter *et al.,* 1984). Compared to HER1, HER2 does not bind to ligand but has an active confirmation (Garrett *et al.*, 2003). HER2 is the most preferred partner for hetrodimerisation because the dimerisation loop is constitutively exposed (Novotny *et al.*, 2016). The expression of HER2 receptor is crucial for normal growth and development of mammary gland (Yarden, 2001a). A study shows null expression of HER2 receptors in mammary glands of mice cause delay in growth of ductal during puberty and adolescence (Jackson-Fisher *et al.*, 2004). The unregulated expression of HER2 was associated with the development of tumours. Overexpression of HER2 mostly detected in malignant of epithelial origin rather than mesenchyme derived tumour (Yan *et al.*, 2015). Amplification and overexpression of HER2 was detected in breast, gastric, bladder and lung cancer (Nida Iqbal *et al*., 2014). A study by Pahuja et al., (2018) identifies somatic mutation of HER2 receptors in multiple cancers. Breast cancer patients with HER2 somatic mutation shows a poor survival (Toghui *et al*., 2017). Apart from having mutation, HER2 receptor also exist as truncated form known as p95HER2 fragments. The p95HER2 fragment arise from proteolytic cleavage mediated by metalloprotease removing extracellular domain of HER2 (Yuan et al., 2003). The fragments also exist through translation of alternative initial codons producing 90-95 kDA fragment (Pederson et al. 2009). A study shows that patient expressing p95HER2 did not respond to Herceptin treatment (Chumsri et al., 2011).

2.4.3 Human epidermal growth factor receptor 3 (HER 3)

HER3 was first isolated by Kraus *et al.* (1989). The receptors were express in normal epithelial tissue (Shi *et al.*, 2010) and located on the long arm of chromosome 12 and encoded by 23,651 nucleotides which is then translated to 1,342 amino acids (Mishra *et al.*, 2018). Only one ligand that bind to HER3 receptor which is NRG (Yarden, 2001b). The unique characteristic of HER3 receptor is it has impaired intrinsic tyrosine kinase activity due the altered amino acid residues in the catalytic domain (Citri *et al.*, 2003). HER3 receptors plays an important role in normal cell proliferation and survival (Yarden and Pines, 2012). Uncontrolled expression of HER3 receptors was observed in cancers such as breast, ovarian, prostate, colon, stomach, oral cavity and lung (Sithanandam and Anderson, 2008). Co-expression of HER3 and HER2 was detected in 50-70% of human breast cancer (Mujoo *et al.*, 2014).

2.4.4 Human epidermal growth factor receptor 4 (HER 4)

HER4 is 180kDa glycoprotein and in human is located on chromosome 2 in the q33.3 regions (Zimonjic *et al.*, 1995). The receptor was first isolated from human by Plowman *et al.* (1993) and rat by Zhao *et al.* (1999). The structure of HER4 receptor is similar with other family members with the extracellular domain has highest homology with HER3 while the kinase domain has highest homology with HER2 and HER1 (Plowman *et al.*, 1993).

In the absence of the ligand bind, HER4 exist in tethered formation similar to EGFR and HER3 (Bouyain *et al.*, 2005). Ligands bind to HER4 include NRG, BTC, HB-EGF and EPR (Normanno *et al.*, 2005). The gene for Neuregulin (NRG) consists of four members, which are NRG-1, NRG-2, NRG-3 and NRG-4 (Breuleux,