EVALUATING THE EXPRESSION OF *HMGCR* IN HER2 NEGATIVE AND HER2 POSITIVE BREAST CANCER

ROSMAIZAN BINTI DRAHMAN

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EVALUATING THE EXPRESSION OF *HMGCR* IN HER2 NEGATIVE AND HER2 POSITIVE BREAST CANCER

by

ROSMAIZAN BINTI DRAHMAN

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATION

HMGCR	3-hydroxy-3-methylglutaryl Coenzyme A reductase
HER	Human epidermal growth factor receptor
EGFR	Epidermal growth factor receptor
PR	Progesterone receptor
ER	Estrogen receptor
TNBC	Triple negative breast cancer
RNA	Ribonucleic acid
MVA	Mevalonate
FISH	Fluorescence in situ hybridization
c-Cbl	Casitas B-lineage lymphoma
ADCC	Antibody dependent cellular cytotoxicity
PI3K	Phosphatidylinositol 3-kinase
MAPK	Mitogen activated protein kinase
mTOR	Mammalian target of rapamycin
MUC 4	Mucin 4
CD44	Cluster of differentiation 44

Akt	Protein kinase B
PTEN	Phosphatase and tensin homolog
PI3KR1	Phosphoinositide-3-kinase regulatory subunit 1
PI3KCA	Phosphatidylinositol-4,5-biphosphate 3-kinasen catalytic subunit alpha
IGF-1R	Insulin-Like Growth Factor-1 Receptor
SREBP-2	Sterol regulatory elements binding protein
AACT	Acetyl coenzyme A acetyltransferase
HMGS	3-hydroxy-3-methylglutaryl Coenzyme A synthase
MK	Mevalonate kinase
PMK	Phosphomevalonate kinase
MVD	Mevalonate diphosphate decarboxylase
NADPH	Nicotinamide diphosphate
IPP	Isopentenyl diphosphate
DMAPP	Dimethylallyl diphosphate
IPPI	Isopentenyl diphosphate isomerase
FPP	Farnesyl pyrophosphate
tRNA	Transfer RNA

kDa	Kilodalton
BMI	Body mass index
LXR	Liver X Receptor
27HC	27-Hydroxycholesterol
qPCR	Real time polymerase chain reaction
mRNA	Messenger RNA
DMEM	Dulbecco's modified eagle medium
PenStrep	Penicillin/Streptomycin
U/mL	Unit per millilitre
FBS	Fetal bovine serum
EDTA	Ethylenediaminetetraacetic acid
RIPA	Radioimmunoprecipitation assay
TBS	Tris buffered saline
BSA	Bovine serum albumin
ECL	Enhanced chemiluminescent
cDNA	Complementary deoxyribonucleic acid
β-mercaptoethanol	Beta-mercaptoethanol
DAB	3,3'-Diaminobenzidine

HRP	Hydrogen peroxidase
IgG	Immunoglobulin G
CO_2	Carbon dioxide
BSC	Biosafety cabinet
TG SDS	Tris glycine sodium dodecyl sulphate
TBS-T	Tris buffered saline-Tween 20
M	Molar
L	Litre
g	g force
µL	Microlitre
PVDF	Polivinylidene fluoride
V	Volt
EGF	Epidermal growth factor
RT	Reverse transcription
CT value	Cycle threshold value
ΔCT	Delta cycle threshold
IRS	Immunoreactive scoring
IHC	Immunohistochemistry

β actin	Beta actin
cm	Centimetres
FFPE	Formalin fixed paraffin embedded

PENILAIAN PENGEKSPRESAN *HMGCR* DALAM KANSER PAYUDARA HER2 POSITIF DAN HER2 NEGATIF

ABSTRAK

3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGCR) adalah enzim penentu laju dalam proses biosintesis kolesterol yang dikaitkan dengan perkembangan kanser. Hubungan antara HMGCR dan HER2 sudah dibuktikan, namun, fungsi HMGCR dalam perkembangan kanser payudara HER2 positif dan kerintangan rawatan anti HER2 masih belum diketahui. Kajian ini bertujuan untuk menentukan ekspresi HMGCR dalam kanser payudara HER2 positif dan HER2 negatif dan perhubungan antara ekspresi HMGCR dan ciri-ciri klinikopatologi pesakit. Titisan sel MDA-MB-453 dan MDA-MB-361 (HER2 positif), MDA-MB-231 dan MCF-7 (HER2 negatif) telah dikultur di dalam medium DMEM dengan 4% FBS dan 1% antibiotik. Sel disubkultur dalam piring Petri dan protein diekstrak bila sel konfluen. Selain itu, paras mRNA HMGCR juga dikaji menggunakan sampel blok tisu pesakit dengan skor IHC HER2 0, HER2 1+, HER2 2+ dan HER2 3+. Kemudian, ujian pewarnaan immunohistokimia bagi HMGCR juga dijalankan ke atas sampel blok tisu HER2 positif dan HER2 negatif. Kadar ekspresi dasar untuk protein HMGCR didapati tinggi dalam MDA-MB-453 berbanding sel-sel kanser payudara yang lain (p=0.011). Ekspresi mRNA HMGCR didapati lebih tinggi dalam sampel kanser payudara HER2 IHC 3+ berbanding sampel kanser payudara jenis lain,namun perbezaan ekspresi ini tidak terbukti secara statistic (p=0.118). Berdasarkan penemuan ujian IHC, peratusan HER2 IHC 3+ dengan tinggi ekspresi protein HMGCR adalah 88.9 % (32 dari 36 kes), lebih tinggi berbanding peratusan kes tinggi ekspresi protein HMGCR dalam sampel HER2 IHC 0 dan HER2 IHC 1+, iaitu 69.4% (25 dari 36

kes) (p=0.042). Analisis hubung kait mendapati majoriti kes kanser payudara dengan tinggi ekspresi HMGCR ditemui dalam sampel HER2 positif, saiz tumor yang lebih besar (> 2cm), skor tinggi (Skor 2 and Skor 3) untuk parameter gred tumor iaitu pleomorfisme nukleus, pembentukan tubul, dan kiraan mitosis, gred tumor yang lebih tinggi (Gred 2 dan Gred 3), ER positif, PR negatif, dan pembabitan nodus limfa. Namun, perhubungan ini tidak terbukti secara statistik. Keseluruhannya, penemuan kajian ini menyumbang kepada pengetahuan sedia ada berkenaan kaitan antara ekspresi HMGCR dan kanser payudara HER2 positif. Kajian lanjutan perlu dijalankan untuk menentukan penglibatan antara molekul HMGCR dan kanser payudara HER2 positif berhubung pertumbuhan tumor dan kerintangan terhadap rawatan anti-HER2 dalam kanser payudara HER2 positif.

Kata kunci: HMGCR, status HER2, kanser payudara, biosintesis kolesterol

EVALUATING THE EXPRESSION OF *HMGCR* IN HER2 POSITIVE AND HER2 NEGATIVE BREAST CANCER

ABSTRACT

3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGCR) is the rate-limiting enzyme in cholesterol biosynthesis pathway that has been associated with cancer development. Although the connection between HMGCR and HER2 has been established, the function of HMGCR in the advanced progression of HER2 positive breast cancer and anti HER2 therapy resistance remains unknown. The purpose of this research is to study the expression of HMGCR in HER2 positive and HER2 negative breast cancer and to determine the association between HMGCR expression and clinicopathological characteristics of breast cancer patients. Breast cancer cell lines MDA-MB-453 and MDA-MB-361 (HER2 positive), MDA-MB-231 and MCF-7 (HER2 negative) were cultured in DMEM media supplemented with 4% FBS and 1% antibiotic. When the cells reached confluence, they were subcultured in Petri dishes for protein extraction and western blotting. In addition, the levels of HMGCR mRNA were determined by qPCR analysis on FFPE (formalin fixed paraffin embedded) breast cancer tissues with IHC scores of HER2 0, HER2 1+, HER2 2+ and HER2 3+. Moreover, HMGCR immunohistochemistry (IHC) staining was conducted on FFPE HER2 positive and HER2 negative breast cancer tissues. In comparison to other breast cancer cell lines, the HER2 positive cell line MDA-MB-453 had a high level of basal HMGCR protein ($p=0.011$). Furthermore, HMGCR mRNA expression was much greater in HER2 IHC 3+ breast cancer samples than in other HER2 breast cancer subtypes, but this difference was not statistically significant ($p=0.118$). According to the IHC study, the percentage of HER2

IHC 3+ samples with high HMGCR protein expression was 88.9 % (32 out of 36 cases), which was considerably greater than the percentage of HER2 IHC 0 & 1+ samples with high HMGCR protein expression of 69.4 % (25 out of 36 cases) (p=0.042). However, the association analysis revealed that the majority of breast cancer cases with high HMGCR expression were among samples with HER2 positivity, a larger tumour size (> 2cm), high scores (Score 2 and Score 3) on tumour grade parameters such as nuclear pleomorphism, tubular formation, and mitotic count, a higher tumour grade (Grade 2 and Grade 3), ER positivity, PR negativity, and the presence of lymph node involvement. The correlations, however, were not statistically significant. Our results contribute to the existing body of knowledge on the link between HMGCR expression and HER2 positive breast cancer. Further research should be conducted to determine the molecular connections between HMGCR and HER2 positive breast cancer in relation to tumour development and resistance to anti-HER2 treatment in HER2 positive breast cancer.

Keywords: HMGCR, HER2 status, breast cancer, cholesterol biosynthesis

CHAPTER 1

INTRODUCTION

1.1 Background of Study

Globally, millions of deaths linked to cancer have been reported each year and the figure is expected to increase due to lifestyle factors, among others (Grosso et al., 2017; Kispert & McHowat, 2017). Breast cancer is the most common cancer among females. In 2018, the number of new breast cancer cases among females worldwide was 47, 500 (Ferlay et al., 2019). 7, 593 breast cancer cases were registered in Malaysia in 2018, accounting for 32.7% of female cancer cases in the same year (Ferlay et al., 2019). A study reported that Malaysian women with breast cancer have poor survival rates and usually presented themselves at a later stage of cancer (Lim et al., 2015).

HER2 positive breast cancer is one of the breast cancer molecular subtypes that makes up a significant proportion, about 20-25% of breast cancer cases (Wang & Xu, 2019). This molecular subtype of breast cancer arises from overexpression of Human Epidermal Growth Factor Receptor-2 (HER2). HER2 positive breast cancer is associated with poorly differentiated tumor cell characteristics and lower overall survival rate compared to other subtypes (Zuo et al., 2017). In 2014, HER2 positive breast cancer was recorded in 28% of all breast cancer cases in Malaysia (Hanin et al., 2018). Several treatments are available for breast cancer patients in local hospitals such as surgery, chemotherapy, radiotherapy, hormonal therapy and targeted therapy (M. S. Lee et al., 2019). As a prognostic marker in breast cancer, advancement in the medical field has seen various targeted therapy designed for HER2 positive breast cancer in order to solve the

problem. Trastuzumab is one of the first targeted drug treatments approved by the Food and Drugs Administration (FDA). This drug is a recombinant monoclonal antibody that targets extracellular domains of the HER2 receptors (Nahta et al., 2004). Despite this promising development, 40% to 50% of patients receiving the treatment showed resistance to trastuzumab (Gschwantler-kaulich et al., 2017). More studies are needed to improve anti-HER2 treatment to solve the issue.

Over the past three decades, global obesity rate has continuously increased, resulting in nearly 10% of the world population being obese (Friedrich, 2017). In local setting, Malaysia became the country with the highest prevalence of obesity and overweight in Southeast Asia, recorded at 46.3% (Helble, 2017). Obesity is a condition defined by accumulation of excessive body fat which leads to impairment of physical functions and increase in the risks of certain illnesses (Helble, 2017). Recent studies suggested link between obesity and cancer-related death, whereby mortality rate was also the lowest among those with normal range of BMI (Bhaskaran et al., 2018). Studies has also found that obesity has a positive correlation to increased breast cancer risk, 2% for each 5 kg/m2 increase in BMI (Liu et al., 2018). Obesity is also very closely related to cholesterol level which accumulates in our body through food intake or naturally produced by the mevalonate pathway (MVA pathway).

Cholesterol is a vital molecule produced in the endoplasmic reticulum of cells that carry out important functions in our body such as cell structure component and in intracellular trafficking (Zhang et al., 2019). Despite playing important roles in the body, cholesterol has been recognized as one of the risk factors for cancer (Ding et al., 2019; Garcia-Estevez & Moreno-Bueno, 2019; Murai, 2015; Radišauskas et al., 2016). In

breast cancer, it was demonstrated that cholesterol accelerated tumor growth, increased aggressiveness of the tumor and promoted angiogenesis (Blücher & Stadler, 2017). Furthermore, adipocytes *in vitro* culture was found to promote breast cancer cell proliferation (Blücher & Stadler, 2017). Studies have also suggested that tumor cells tend to overexpress Scavenger Receptor Type B1 (SR-B1) which is responsible in cellular uptake of cholesterol, a receptor which was suspected to cause cancer aggressiveness and poor survival among breast cancer patients (Mooberry et al., 2016).

The MVA pathway is the biosynthetic pathway responsible for producing cholesterol using Acetyl-Coenzyme A as the starting molecule (Mullen et al., 2016). The rate-limiting step of the complex pathway is controlled by an enzyme called HMGCR which reduces HMG Coenzyme A into mevalonate (Mullen et al., 2016). Due to its prominent involvement in cholesterol production, this enzyme is therefore necessary for multiple cellular functions associated with cholesterol such as proliferation, signaling molecule and cell structure component (Aguilar-Ballester et al., 2020; Chimento et al., 2019). Given the importance of HMGCR in the pathway, targeting this enzyme is potentially useful in studying the relationship between cholesterol production and cancer (Munir et al., 2018).

This study focused on the association between breast cancer and cholesterol synthesis, specifically the possible link between HER2 positive breast cancer and HMGCR. The study on HER2 positive and HER2 negative breast cancer was conducted in two phases: cell lines study and study on breast cancer FFPE tissue samples. Cell lines used were MDA-MB-453, MDA-MB-361, MDA-MB-231, and MCF-7. For human tissue study, the samples of various HER2 status were selected among invasive ductal

carcinoma- No Special Type (NST) cases.

Previously, it was reported that the upregulation of HMGCR was found to be modulated by HER2 overexpression (Asslan et al., 1999). HER2 positive cell lines showed high expression of HMGCR whereas HER2 negative cells did not exhibit similar reaction (Asslan et al., 1999). In addition, HMGCR inhibition via statin treatment also downregulated HER2 expression and induced cell death in HER2 positive breast cancer cells (Zhao et al., 2013). The above studies highlighted the association between HMGCR and HER2 positive breast cancer. However, the expression of HMGCR has not been studied in comparison between HER2 positive and HER2 negative breast cancer. Based on current literature, the role of HMGCR expression in the advanced progression of HER2 positive breast cancer and anti HER2 therapy resistance remains unclear.

1.2 Study Rationale

HER2 positive breast cancer is associated with poorly differentiated tumour cell characteristics and lower overall survival rate (Zuo et al., 2017). HER2 positive breast cancer rate was 28% of all breast cancer cases in Malaysia (Hanin et al., 2018). Several treatments are available for breast cancer patients in local hospitals such as surgery, chemotherapy, radiotherapy, hormonal therapy and targeted therapy (M. S. Lee et al., 2019).

In local setting, trastuzumab targeted therapy is used as adjuvant treatment for HER2 positive breast cancer but only accessible to 19% of the patients (S. W. Lee, 2016). Therefore, unresponsiveness to trastuzumab treatment could pose a significant socioeconomic impact to the patients and the community.

Both cholesterol and HMGCR have been implicated in breast cancer progression. The involvement of HMGCR as one of the most important enzyme and biomarker in the pathway was commonly studied in Estrogen Receptor (ER) positive tumour. Despite the findings that HMGCR expression plays a role in HER2 positive breast cancer progression, it's prognostic or predictive role should be further investigated in HER2 positive breast cancer patients. Given the association between HMGCR and HER2 positive breast cancer, this study on HMGCR expression in HER2 positive and HER2 negative breast cancer may provide better understanding on involvement of HMGCR in HER2 positive breast cancer progression.

1.3 Study Objectives

1. General Objective
    - To investigate the expression of HMGCR in HER2 positive and HER2 negative breast cancer
2. Specific Objectives
    - To determine the HMGCR protein expression in MDA-MB-231, MCF-7, MDA-MB-453, and MDA-MB-361 cell lines by western blotting
    - To determine HMGCR mRNA expression in HER2 IHC 0, HER2 IHC 1+, HER2 IHC 2+ and HER2 IHC 3+ FFPE tissue breast cancer samples using qPCR.
    - To study the association between HMGCR expression with clinicopathological features of HER2 positive and HER2 negative breast cancer patients

CHAPTER 2

LITERATURE REVIEW

2.1 Breast Cancer Subtypes

Previously, breast cancer was classified based on the tissue origin of the tumour into carcinoma and sarcoma. Breast cancer that originated from epithelial cells are categorized as carcinoma whereas sarcoma consisted of cancer cells that grew from connective tissue of the breast. Breast carcinoma were further classified into *in situ* carcinoma (localized to the lobules or ducts where the cancer cells originated) or invasive carcinoma that penetrated surrounding tissues (Zubair et al., 2021). Invasive breast carcinoma was further categorized as morphologically identifiable types and no special types (NST).

At molecular level, breast cancer subtypes are distinguished from each other based on the expressions of PR, ER and HER2. Luminal type breast cancer is characterized by positive expression of hormone receptors (Wang & Xu, 2019). Luminal A and Luminal B share positive expression for ER but differ in proliferation-related gene expression such as Ki-67 which is higher in Luminal B (Prat et al., 2015). Luminal type breast cancer, Luminal A and Luminal B have better prognostic outcome compared to other subtypes (Dai et al., 2017). Another subtype of breast cancer is HER2 positive breast cancer, characterized by overexpression of HER2 receptors (Dai et al., 2017).

This subtype of breast cancer is more aggressive than Luminal type and have poor prognosis (Dai et al., 2017). Lastly, triple negative breast cancer (TNBC) subtype is a group of breast cancer which lacks expression of all the receptors (Dai et al., 2017). TNBC

represents the worst prognosis and most advanced stage at diagnosis (Dai et al., 2017). Due to the lack of molecular target expression, this subtype is also the most challenging to treat (Garrido-Castro et al., 2019).

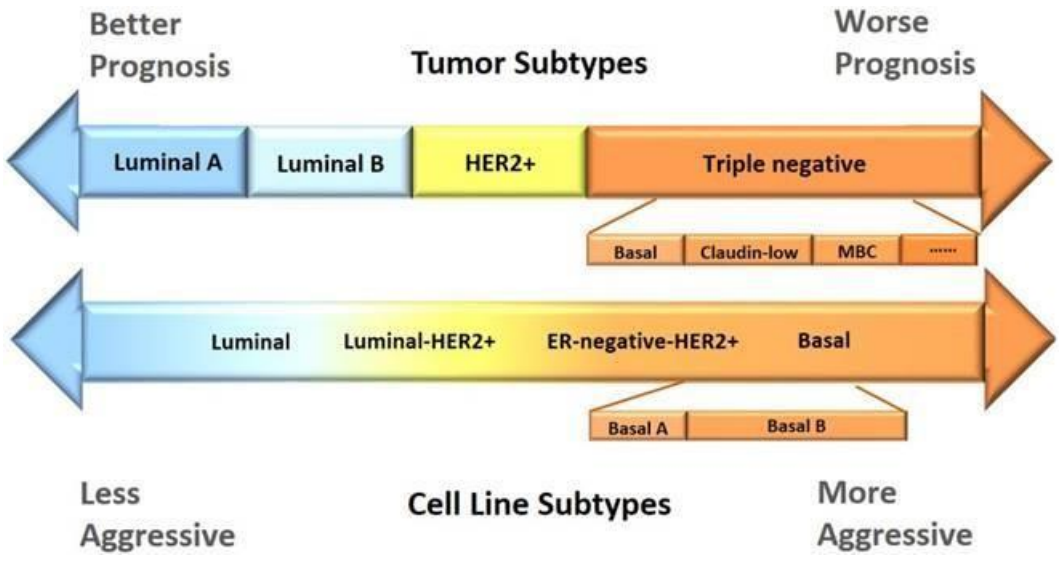

Figure 2.1: Breast cancer prognosis according to molecular subtypes (Dai et al., 2017)

2.1.1 The Incidence of HER2 Positive Breast Cancer

Globally, recent data suggested that HER2 positive breast cancer accounts for 20-25% of breast cancer cases (Wang & Xu, 2019). As reported in Table 2.1, rate of HER2 positive breast cancer was studied in a number of Asian countries (Pathmanathan et al., 2016). A total of 96 studies participated in this research, involving 30, 179 patients (Pathmanathan et al., 2016). HER2 positive incidence among the countries ranged from 19.7% as reported in China to 44.2% in Thailand. Overall rate across the participant countries was 23.5%. Majority of the cases were either HER2 negative or HER2 positive, while smaller proportion of the patients were HER2 equivocal. The minimum HER2 positive rate was reported in China, 4.4% whereas the maximum rate was 51.6% as reported in Malaysia.

Table 2.1: Incidence of HER2 positive breast cancer in Asian countries

Country	Number of laboratories	Number of patients	HER2-positive, *n* (%) [95% CI]	HER2-equivocal, *n* (%) [95% CI]	HER2-negative, *n* (%) [95% CI]	Minimum HER2 positivity rate (%)	Maximum HER2 positivity rate (%)
China	49	10,903	2,154 (19.7) [17.91–21.40]	2,061 (19.8) [16.58–23.06]	6,688 (60.3) [57.40–63.20]	4.4	34.3
Hong Kong	2	652	179 (26.9) [22.01–31.69]	84 (9.0) [0.0–24.27]	389 (64.6) [44.31–84.95]	23.6	28.7
Indonesia	7	3,042	745 (22.8) [15.64–30.01]	407 (15.6) [9.88–21.31]	1,890 (60.7) [52.27–69.18]	7.9	34.7
Malaysia	17	1,841	643 (32.6) [26.65–38.46]	173 (12.2) [7.94–16.47]	1,025 (56.7) [49.70–63.80]	16.7	51.6
Philippines	17	6,985	1,733 (24.4) [21.67–27.15]	1,455 (24.7) [20.87–28.56]	3,797 (49.5) [43.53–55.47]	14.9	34.7
Taiwan	1	2,062	553 (26.8) [24.93–28.74]	169 (8.2) [7.01–9.38]	1,340 (65.0) [62.93–67.04]	NA†	NA†
Thailand	1	77	34 (44.2) [33.12–55.23]	5 (6.5) [0.99–12.00]	38 (49.4) [38.18–60.52]	NA†	NA†
Vietnam	2	4,617	1,435 (31.1) [29.74–32.41]	697 (13.2) [8.02–18.45]	2,485 (56.2) [49.37–62.93]	29.7	31.3
Overall	96	30,179	7,476 (23.5) [21.83–25.16]	5,051 (18.2) [16.43–20.00]	17,652 (57.8) [55.57–59.9]	4.4	51.6

Note. From "Human epidermal growth factor receptor 2 status of breast cancer patients in Asia: Results from a large, multicountry study" by Pathmanathan, N., Geng, J. S., Li, W., Nie, X., Veloso, J., Hill, J., McCloud, P., & Bilous, M. , 2016, *Asia-Pacific Journal of Clinical Oncology*, *12*(4), 369–379. https://doi.org/10.1111/ajco.12514

In local clinical practice, HER2 status is routinely determined by IHC and fluorescence in situ hybridization (FISH) (Albagoush & Limaiem, 2021; Nitta et al., 2016). Figure 2.2 shows HER2 status determination by both methods. Based on the report, IHC was used to detect HER2 protein overexpression whereas FISH was used to detect gene amplification (Beach & Royce, 2016). Primarily, breast cancer specimens are tested using IHC staining for HER2 expression based on membrane staining (Albagoush & Limaiem, 2021; Nitta et al., 2016). Intensity and positive cell fraction are taken into account for scoring. The scores are recorded on a scale of 0 to 3+ whereby Score 0 to 1+ are considered as HER2 negative, Score 2+ as HER2 equivocal and Score 3+ as HER2 positive as described in Figure 2.2. Specimens with equivocal scores will further be tested using FISH to determine positive or negative status of HER2 (Albagoush & Limaiem, 2021). For FISH analysis, the case will be considered as HER2 amplified if the specimen

was reported with any of the following observation: 1) HER2/CEP17 ratio $\leq$ 2 and mean HER2 signal/cell<4, or 2), HER2/CEP17 ratio <2 and mean HER2 signal/cell $\leq$6, or 3) HER2/CEP17 ratio <2 and mean HER2 signal/cell is $\geq$4 but <6 (Albagoush & Limaiem, 2021).

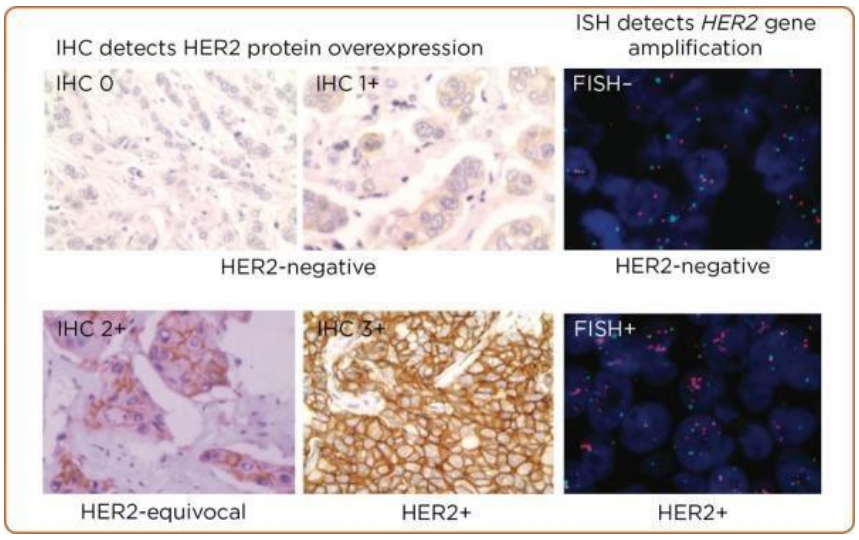

Figure 2.2: The determination of HER2 status by IHC and FISH (Beach & Royce, 2016

Overexpression of HER2 receptors in breast cancer cells occurs when the cancer cells carry amplified HER2 genes of up to 25 to 50 copies to produce a maximum of 100 times more HER2 protein than normal cells (Albagoush & Limaiem, 2021). As a result, 2 million HER2 receptors will be expressed on the cell surfaces (Albagoush & Limaiem, 2021).

2.1.2 HER2 and EGF Family

HER2 receptor is a member of Epidermal Growth Factor (EGF) family of tyrosine kinase receptors which also include HER1, HER3 and HER4. This group of receptors carry out important functions involving cell proliferation, differentiation and survival (Albagoush & Limaiem, 2021). The receptors are activated by ligand binding at the outer domain, followed by dimerization between the receptors and phosphorylation of cytoplasmic domain to activate downstream pathways (Albagoush & Limaiem, 2021). HER2 has a distinctive characteristic of being activated without ligand binding and preferred as dimerization partner by the other receptors (Albagoush & Limaiem, 2021). Therefore, overexpression of HER2 receptor causes constitutive activation of their downstream pathways such as phosphatidylinositol 3-kinase (PI3K pathway) and mitogen activated protein kinase pathway (MAPK pathway) (Figure 1) (Albagoush & Limaiem, 2021; Daniela et al., 2018). Since these pathways are responsible for cell division and survival, continuous activation will ultimately result in carcinogenesis (Albagoush & Limaiem, 2021).

2.1.3 Anti HER2 Targeted Therapy

Due to the limitations of target specificity in conventional treatments, targeted treatment was developed. Targeted treatment affects only cancer cells while healthy cells are uninterrupted. HER2 overexpression in breast cancer leads to continuous activation of many downstream pathways, increasing mortality rate among patients (Daniela et al., 2018). Advancement in the medical field has seen various targeted therapy designed for HER2 positive breast cancer in order to solve the problem.

Trastuzumab is one of the first targeted drug treatments approved by the FDA. This drug is a recombinant monoclonal antibody that targets extracellular domains of the HER2 receptors (Nahta et al., 2004). Another targeted therapy for HER2 positive breast cancer patients is pertuzumab, a monoclonal antibody which blocks HER2/HER3 heterodimerization. This drug is effective when used alone, but previous study found that there is also synergistic potential between pertuzumab and trastuzumab (Chen et al., 2019). TD-M1 (trastuzumab emtansine) is another approved drug for HER2 positive breast cancer treatment that consist of antibody conjugated with cytotoxic drugs that specifically targets HER2 positive cancer cells, increasing treatment efficiency due to delivery of the drug into the cells (Montemurro et al., 2020).

Lapatinib is a reversible tyrosine kinase inhibitor of EGFR/HER2 that is capable of blocking both EGFR and HER2. Lapatinib is an inhibitor of PI3K/Akt and MAPK/Erk1/2 pathways (X. Zhu & Verma, 2015). Researchers found synergistic effect of combined treatment of lapatinib and capecitabine, which gives better outcome for patients with prior trastuzumab exposure (Yang et al., 2020). Another study has demonstrated that lapatinib could act against HER2 positive cancer cells by inducing

apoptotic activity (Eustace et al., 2018). Furthermore, combination of trastuzumab and lapatinib in treatment also gives better results compared to monotherapy of either drug alone (Xin et al., 2016). Afatinib and neratinib are two second-generation tyrosine kinase inhibitors that irreversibly inhibit more than one HER family member, which both inhibitors have shown their efficacy in clinical trials either as monotherapy or in combination (Feldinger & Kong, 2015).

Combination of anti-HER2 therapy a by covalently binding to the receptors and prevent autophosphorylation and endocrine therapy such as letrozole and anastrozole is also one of the targeted therapies particularly in postmenopausal women. The combinations of these endocrine treatments with trastuzumab or lapatinib were associated with significantly improved progression-free survival rates (X. Zhu & Verma, 2015). mTOR inhibitors is another type of targeted therapy against HER2- amplified breast cancer, which may even be beneficial against trastuzumab resistance, as demonstrated by combination of taxane and trastuzumab in the presence of everolimus, which results in significant improvement of survival rates (X. Zhu & Verma, 2015).

2.1.4 Trastuzumab Resistance

The mechanisms of trastuzumab resistance involves steric effect, whereby trastuzumab binding to HER2 is blocked due to proteolysis of the receptors by metalloprotease ADAM10 (a disintegrin and metalloproteinase domain-containing protein 10) or by post translational modification (Luque-Cabal et al., 2016). In this process, HER2 receptors become mutated, forming truncated p95HER2 isoform with no extracellular domain (ECD) (Luque-Cabal et al., 2016).

Trastuzumab resistance may also occur as a result of intrinsic alterations-interference of PTEN (phosphatase and tensin homolog expression), increased PI3K/Akt activity, and the modulation of $p27^{KAIP1}$(Luque-Cabal et al., 2016). PTEN inhibits the activation of PI3K pathway, one of the downstream signaling pathway that contributes to tumor progression, which is a target for trastuzumab action. Reduction in PTEN level increases phosphorylation of PI3K/Akt, thus overcoming the effect of trastuzumab-mediated growth inhibition in HER2 positive cancer cells (Luque-Cabal et al., 2016).

PI3KR1 and PI3KCA genes encode the subunit of p85 of PI3K/Akt pathway and subunit of p100 of PI3K pathway. Both genes are related to trastuzumab resistance by increasing the activity of the PI3K/Akt pathway. These genes are frequently mutated and overexpressed in breast cancer cases, resulting in constitutive activation of PI3K/Akt pathway (Luque-Cabal et al., 2016). Growth inhibitory properties of trastuzumab depends on the cyclin-dependent kinase-inhibiting protein $p27^{KAIP1}$ because this inhibitor is effective in decreasing cell proliferation and enhance apoptosis, whereas decreased expression of this protein can lead to trastuzumab resistance (Luque-Cabal et al., 2016).

Resistance to trastuzumab also occurs by increased signaling from other EGFR family members. Despite the known effectiveness of trastuzumab action against HER2 in breast cancer, dimerization among other receptors in the family can still activate downstream pathways although in the presence of trastuzumab (Luque-Cabal et al., 2016). Increased signaling from other receptors such as IGF-1R (Insulin-Like Growth Factor-1 Receptor) overexpression can also cause trastuzumab resistance. IGF-1R is a transmembrane tyrosine kinase receptor that shares a common downstream signaling pathway with HER2. Therefore, IGF-1R downstream effectors promote cell proliferation

and metastasis. IGF-1R that interacts with HER2 can mediate trastuzumab resistance. This binding may involve in PI3K/Akt pathway and lead to degradation of p27 (Kispert & McHowat, 2017).

2.1.5 Trastuzumab Mechanism of Action

The FDA defined trastuzumab as a recombinant humanized monoclonal antibody, directed against extracellular domain IV of HER2 receptors (Chen et al., 2019). This drug act specifically against HER2 positive cells by various mechanisms such as blocking HER2 dimerization, HER2 internalization by endocytosis and triggering antibody dependent cellular cytotoxicity (ADCC) (Luque- Cabal et al., 2016; Wang & Xu, 2019; Yang et al., 2020). At intracellular environment, trastuzumab triggers HER2 endocytosis by high affinity binding to the receptor and subsequently forming crosslinked complex with HER2 (Cheng et al., 2020). Ultimately, the receptor is internalized by the cell (Cheng et al., 2020). In addition, trastuzumab blocks EGF receptor dimerization in HER2-overexpressing cells, preventing downstream signaling of Akt/PI3K and MAPK thus affecting the inhibition of cell proliferation, growth and survival (Daniela et al., 2018; Maadi et al., 2018). Following trastuzumab binding, PTEN becomes activated and further increases anti-tumor effect of trastuzumab (Daniela et al., 2018). Another mechanism of action of trastuzumab is HER2 endocytosis which leads to HER2 downregulation (Luque-Cabal et al., 2016). ADCC is an example of extracellular mechanism of trastuzumab action, mainly involving natural killer cells (NK cells) (Luque-Cabal et al., 2016; Wang & Xu, 2019; Yang et al., 2020). Trastuzumab binding to HER2- amplified cancer cells attracts NK cells to bind to trastuzumab and lyse the tumor cells (Luque-Cabal et al., 2016).

2.2 Cholesterol Synthesis

2.2.1 MVA Pathway

The MVA pathway is an important metabolic pathway that produces the precursor for isoprenoids in most eukaryotes, archaea and a number of eubacteria with Acetyl Coenzyme A as a precursor (Mullen et al., 2016). Examples of important enzymes at the start of this pathway are acetoacetyl CoA thiolase, HMG-CoA synthase, HMG-CoA reductase, phosphomevalonate kinase and mevalonate diphosphate decarboxylase as shown in Figure 2.3. These enzymes react in the reaction sequence to produce the precursor of isoprenoids, farnesyl pyrophosphate, FPP (Mullen et al., 2016). After the production of FPP, MVA pathway diverges into different reactions to ultimately yield the many types of isoprenoids such as coenzyme Q, cholesterol, isopentenyl adenosine and dolichol (Mullen et al., 2016). These metabolites play vital roles in promoting membrane association, tRNA (transfer RNA) modification, protein glycosylation, and in mitochondria, forming the electron transport chain (Zhang et al., 2019).

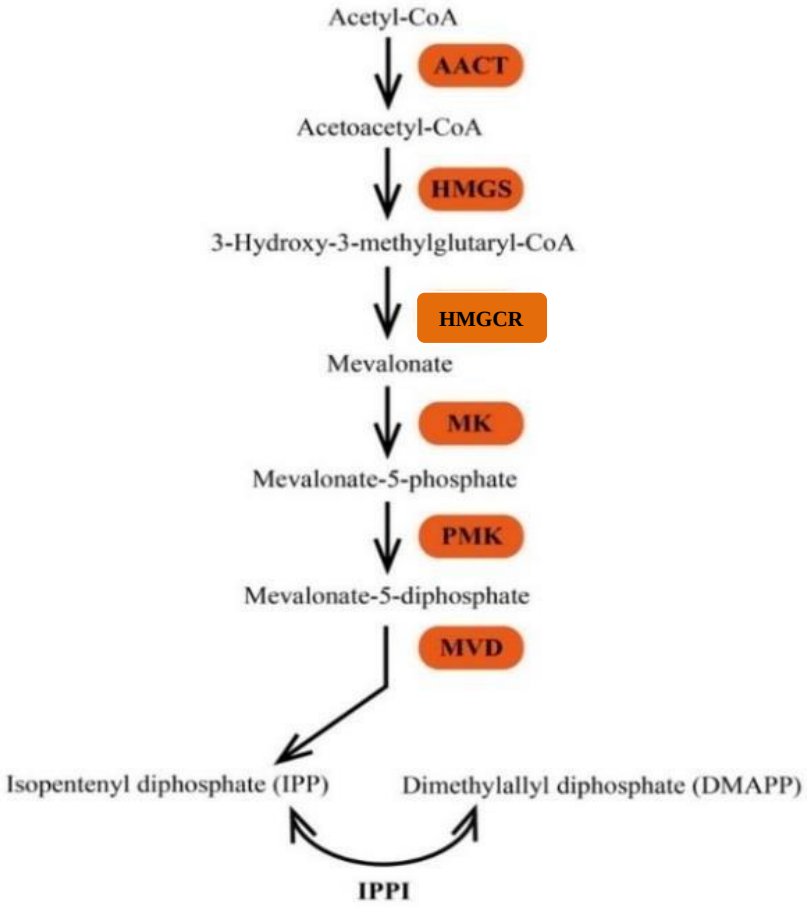

Figure 2.3: MVA pathway components involved in cholesterol synthesis (Zhang et al., 2019).

Recent study reported that the MVA pathway is such a vital metabolic process that the inhibition of this pathway in cancer cells affects epigenetic activity of the cells. MVA pathway inhibition reduce the level of NADPH (nicotinamide adenine dinucleotide phosphate), a product of glycolysis and fatty acid oxidation that regulates DNA repair genes in the cells (Karlic et al., 2015). Another importance of MVA pathway is that the precursors and metabolites of cholesterol also function as signaling molecules in the cells (Mullen et al., 2016). This biosynthetic pathway of cholesterol production has also been discovered to play significant role in replication of mammalian cells (Mullen et al., 2016;

Zhang et al., 2019).

2.2.2 The Role of HMGCR in Cholesterol Synthesis

Cholesterol biosynthesis is catalyzed by a number of enzymes including HMGCR, an enzyme with the size of 97 kDa found in the endoplasmic reticulum (Nathan, 2020). This enzyme functions at the rate limiting step of the biosynthetic process, by reducing HMG CoA to mevalonate (Beckwitt et al., 2018). The conversion of HMG CoA to mevalonate occurs in two steps; the first reduction produces bound mevadyl-CoA, followed by further reduction to yield mevalonate (Gesto et al., 2020). This part of the reaction requires the use of two molecules of NADPH. HMGCR is an enzyme mainly found in eukaryotes, archaea and a number of eubacteria (Gesto et al., 2020). Analysis of HMGCR structure revealed that the enzyme consists of N-terminal domain, eight transmembrane segments, short linker and C-terminal with some parts of the membrane-embedded protein acting as the sterol-sensing domain (Gesto et al., 2020). The active form of the enzyme occurs as tetramers; the monomers are arranged as dimers in one subunit and then the dimers pair up to form the tetramer (Gesto et al., 2020). The active site of the enzyme is found to be located at the monomer-monomer interface whereas the dimer-dimer interface is a hydrophobic region (Gesto et al., 2020). HMGCR is a strictly regulated enzyme, with its activity modulated by the amount of its own target product, cholesterol, and can also be inhibited by statin drugs (Beckwitt et al., 2018; Nathan, 2020). In cells, the importance of this enzyme is mainly associated to its role in production of cholesterol, a molecule that carries out various tasks such as in signaling, membrane component and in controlling the progression of cell cycle (Nathan, 2020). The authors also reported that supply of cholesterol is crucial in cell cycle whereby cholesterol level

is found to be elevated at S phase and absence of cholesterol causes the cycle to halt at G1 phase and the cycle resumes once the cholesterol supply is restored (Singh et al., 2013). Furthermore, cholesterol is also known as one of the risk factors of cancer (Garcia-Estevez & Moreno-Bueno, 2019). Recent study observed that serum cholesterol level promotes growth and progression of prostate cancer in transgenic mice models (Allott et al., 2018). In breast cancer, it was demonstrated that cholesterol metastatic effect in mice subjects through the involvement of 27HC (Baek et al., 2017).

2.3 Obesity and Cholesterol

Over the past three decades, global obesity rate has continuously increased, resulting in nearly 10% of the world population being obese (Friedrich, 2017). In local setting, Malaysia became the country with the highest prevalence of obesity and overweight in Southeast Asia, recorded at 46.3% (Helble, 2017). Obesity is a condition defined by accumulation of excessive body fat which leads to impairment of physical functions and increase in the risks of certain illnesses (Helble, 2017).

Under normal circumstances, cholesterol and free fatty acid (FFA) are processed together by enterocytes in the intestine into chylomicrons (Rahmany & Jialal, 2020). This molecule is then secreted into lymphatic vessels before entering circulation (Qaid & Abdelrahman, 2016). Chylomicrons and VLDL (Very Low Density Lipoprotein) then delivers FFA to the heart, skeletal muscle and adipose tissue for energy production and storage function (Qaid & Abdelrahman, 2016). The release of FFA from VLDL and chylomicrons is primarily mediated by lipoprotein lipase (LPL), a process known as lipolysis (Qaid & Abdelrahman, 2016). This enzyme is in turn controlled by insulin which also facilitates the uptake of FFA by adipocytes (Feingold & Grunfeld, 2000). The FFA

taken up by cells are later re-assembled into fat in cytoplasm whereas chylomicrons remnants and VLDL are transported back to the liver (Feingold & Grunfeld, 2000). During energy starvation, FFA will be released again from the cells, with reduced level of insulin (Qaid & Abdelrahman, 2016). Due to these functions of insulin, it is suggested that insulin does play a major role in lipoprotein metabolism. One of the cellular events that indicates dyslipidemia in obesity is when triglycerides accumulates in the liver, leading to increased production of VLDL by the liver (Højland Ipsen et al., 2016). Since VLDL competes with chylomicrons for FFA uptake by myocytes and adipocytes, high level of VLDL could impair the lipolysis which causes high level of fat and cholesterol being transported back into the liver (Højland Ipsen et al., 2016). Alternatively, VLDL and chylomicrons may be transported to tissues for removal of cholesterol, a process that usually takes place in the liver. In other tissues, cholesterol may not be processed efficiently, leading to cholesterol accumulation in the tissues (Højland Ipsen et al., 2016).

Association between cholesterol and cancer progression remains controversial. Studies have found that cholesterol and cholesterol regulator levels were reportedly higher in breast cancer (Munir et al., 2018). Therefore, it was concluded that cholesterol did correlate with cancer progression. Further studies will be required to confirm the causative relationship between cholesterol and cancer progression.

2.3.1 The Role of Cholesterol in Supporting Breast Cancer Growth

In breast cancer, it was demonstrated that cholesterol accelerated tumor growth, increased aggressiveness of the tumor and promoted angiogenesis (Blücher & Stadler, 2017). Furthermore, adipocytes *in vitro* culture was found to promote breast cancer cell proliferation (Blücher & Stadler, 2017). Studies have also suggested that tumor cells tend to overexpress SR-B1 which is responsible in cellular uptake of cholesterol, a receptor which was suspected to cause cancer aggressiveness and poor survival among breast cancer patients (Mooberry et al., 2016). Blood cholesterol as an indicator of obesity has been implicated as a risk factor for breast cancer (Garcia-Estevez and Moreno-Bueno, 2019). Regardless of BMI and hormone receptor status, serum lipid was found to be directly associated with breast cancer risks (His et al., 2017). Dietary cholesterol was found to associate with breast cancer risk and the finding became more evident with increased cholesterol intake (Li et al., 2017). Another study also mentioned such association, but it was only true among postmenopausal women and not in premenopausal women (Nelson, 2018). Physiologically, cholesterol level in the body is strictly regulated mainly by sterol regulatory element binding protein (SREBP) and liver X receptor (LXR) (Nelson, 2018). Given the tight control over cholesterol synthesis, it was difficult to understand involvement of cholesterol in breast cancer progression. The role of cholesterol in breast cancer can be explained partly by understanding the function of cholesterol as cell membrane component and its interaction with other membrane components that can trigger reaction cascades of lipid accumulation (Nelson, 2018; Sezgin et al., 2017). As part of the membrane, high level of cholesterol might stimulate signaling pathways such as PI3K. This was demonstrated in mice fed with high

cholesterol Western diet and wild type mice. When treated with PI3K inhibitor, tumor growth in the high- cholesterol group mice decreased (Nelson, 2018). Cholesterol metabolite, 27- Hydroxycholesterol (27HC) was suggested to mediate ER positive breast cancer proliferation (Baek and Nelson, 2016).

2.3.2 HMGCR Expression in Cancer

As an important enzyme in cholesterol synthesis pathway, inhibition of HMGCR activity was used to reduce blood cholesterol level using statin drug (Beckwitt et al., 2018). Statin drug not only affect HMGCR activity and cholesterol level, but also cellular activity such as proliferation. Blocking MVA pathway activity also leads to reduced level of other important components for cancer cell progression such as those involved in cell migration and survival (Beckwitt et al., 2018). Statin competitively inhibit HMGCR by binding at the enzyme active site, thus preventing conversion of HMG-CoA into mevalonate (Beckwitt et al., 2018). In breast cancer, use of lipophilic statin reduces the rate of recurrence and mortality among the study population (Beckwitt et al., 2018). However, the authors noted that such effects were only observed with lipophilic statin whereas hydrophilic statins did not have any effect on breast cancer incidence, recurrence and mortality. High expression levels of cholesterol biosynthesis genes that including HMGCR were also associated with shorter recurrence-free survival (RFS) and overall survival in ER positive breast cancer (Kimbung et al., 2016). An evaluation from the Malmö Diet and Cancer Study breast cancer cohort demonstrated that HMGCR moderate/strong expression was associated with worse prognosis such as higher histological grade, high Ki67, and ER negativity (Bjarnadottir et al., 2020). Another study found that HMGCR staining was associated with triple negative breast cancer in Korean

patients (Kim et al., 2019). Additionally, ER positive patients with positive expression of HMGCR were shown to have an improved response to tamoxifen treatment, indicating the role of HMGCR as a predictive biomarker for tamoxifen sensitivity in breast cancer (Gustbée et al., 2015).

2.3.3 The Association of HMGCR and HER2

Preclinical studies showed that in HER2-overexpressing cell lines, the upregulation of HMGCR was found to be modulated by HER2 overexpression (Asslan et al., 1999). HER2 positive cell lines showed high expression of HMGCR whereas HER2 negative cells did not exhibit similar reaction (Asslan et al., 1999). In addition, HMGCR inhibition via statin treatment also downregulates HER2 expression and induce cell death in HER2 positive breast cancer cells (Zhao et al., 2013). HMGCR was found to promote transformation in cells and xenograft model (Clendening et al., 2010).

HER2 overexpression and upregulation of HMGCR has both been linked to adverse tumour characteristics and poor prognosis (Bjarnadottir et al., 2020; Clendening et al., 2010; Dutra et al., 2004). HMGCR was found to be associated with high histological grade, high Ki67 and ER negativity (Bjarnadottir et al., 2020). HER2 overexpression in breast cancer was also linked to poor disease outcome such as lower rate of survival and high rate of recurrence (Ahn et al., 2020; Criscitiello et al., 2013).

The above studies highlight the association between HMGCR and HER2 positive breast cancer. However, the function of HMGCR in progression of HER2 positive breast cancer is still unknown. The expression of HMGCR has not been studied in comparison between HER2 positive and HER2 negative breast cancer. Based on current

literature, the role of HMGCR expression in the advanced progression of HER2 positive breast cancer and anti HER2 therapy resistance remains unclear.