ANGPTL4 AND IGF-1 EXPRESSIONS IN RELATION TO MOLECULAR SUBTYPES IN YOUNG INVASIVE BREAST CARCINOMA

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DISSERTATION SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF PATHOLOGY (ANATOMICAL PATHOLOGY)

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LIST OF ABBREVIATIONS

ANGPTL4	Angiopoietin-like protein 4
ASCO/CAP	American Society of Clinical Oncology/ College American of Pathology
cm	Centimetre
CNB	Core Needle Biopsy
DAB	3,3'diaminobenzidine
DDISH	Dual-colour dual-hapten brightfield in situ hybridization
ER	Estrogen receptor
FFPE	Formalin fixed paraffinized embedded
FISH	Fluorescence in situ hybridization
H&E	Haematoxylin and eosin
HER2	Human epidermal growth factor receptor 2
HPE	Histopathological examination
HUSM	Hospital Universiti Sains Malaysia
IBC	Invasive breast carcinoma
IGF-1	Insulin growth factor-1
IHC	Immunohistochemical

IRS	Immunoreactivity scoring system
ISH	In situ hybridization
NST	No special type
PR	Progesterone receptor
WLE	Wide local excision
IDC	Invasive ductal carcinoma
Non-IDC	Non- invasive ductal carcinoma
WHO	World Health Organization

ABSTRAK

Latar belakang: Kanser payudara dalam kalangan pesakit muda dilaporkan semakin agresif. *Angiopoietin-like protein 4* (ANGPTL4) adalah sebahagian daripada keluarga protein angiopoietin. Protein ini memainkan peranan penting dalam pertumbuhan sel kanser yang menyebabkan kanser merebak dengan cepat. Sementara itu *insulin growth factor-1* (IGF-1) adalah agen mitosis yang mampu memulakan pembentukan kanser payudara dan menyebabkan ia merebak. Matlamat kajian ini adalah untuk menyiasat hubung kait antara kanser payudara dalam kalangan pesakit muda dengan ekpresi protein ANGPTL4 dan IGF-1.

Metodologi: Kajian *cross-sectional* telah dijalankan dengan menggunakan 75 blok tisu kanser payudara usia muda berumur kurang daripada 45 tahun. Subjenis molekular kanser payudara dibahagikan kepada *luminal A, luminal B, HER2 overexpression* dan *triple negative* berdasarkan pada panel pemangku immunohistokimia ER, PR dan HER2. Semua tisu sampel melalui ujian immunohistokimia bagi protein ANGPTL4 dan IGF-1.

Keputusan: Julat umur pesakit muda adalah antara 23-44 tahun dan purata umur adalah 37(SD 5.48). Majoriti subjenis molekular adalah *luminal A* 28 (37.7%), diikuti oleh *triple negative* 20(26.7%), *luminal B* 15 (20%) dan *HER2 overexpression* 12 (16%). Dalam kalangan 75 pesakit ini, 51 (68%) daripada mereka adalah immunopositif bagi ANGPTL4. Sementara itu 67 (89%) kes menunjukkan ekpresi positif terhadap IGF-1. Kedua- dua protein ini juga mununjukkan ekpresi dalam kanser gred tinggi, kanser bersaiz lebih besar dan positif nodal limfa. Manakala, dalam kalangan subjenis molekular 19 (37%) daripada *luminal A* adalah positif terhadap ANGPTL4 diikuti oleh *triple*

negative 14 (28%), *luminal B* 9(18%) dan *HER2 overexpression 8* (16%). Ekpresi IGF-1 juga tinggi dalam *luminal A* 27(40.3%) tetapi rendah dalam *HER2 overexpression* 10(14.9%). Bagaimanapun, tiada hubung kait yang penting antara ANGPTL4 (p=0.897) dan IGF-1 (p=0.091) terhadap subjenis molekular.

Kesimpulan: Majoriti tisu pesakit kanser payudara usia muda menunjukkan ekpresi positif terhadap ANGPTL4 dan IGF-1 adalah subjenis *luminal A*. Tambahan lagi kebanyakan ekpresi kedua-dua protein ini wujud dalam kanser gred tinggi, kanser bersaiz lebih besar dan positif nodus limfa. Walau bagaimanapun, tiada hubung kait yang penting antara ANGPTL4 dan IGF-1 protein dengan subjenis molekular. Penemuan ini menunjukkan kedua-dua protein ini berpotensi digunakan untuk memahami bagaimana kanser merebak. Adalah diharapkan penemuan ini dapat membantu menambah baik rawatan bagi pesakit kanser payudara. Bagaimanapun, kajian lanjut menggunakan skala jumlah pesakit yang lebih besar diperlukan unutk mengkaji peranan ANGPTL4 dan IGF-1 dalam kanser payudara.

ABSTRACT

Background: Invasive breast carcinoma (IBC) in a young patient has been reported to be more aggressive. Angiopoietin-like protein 4 (ANGPTL4) is part of angiopoietin family proteins and plays a critical role in cancer growth and contributes to metastasis. Meanwhile, insulin growth factor-1 (IGF-1) is a potent mitogen that can stimulate breast cancer development with metastatic potential. This study aimed to investigate the possible association of young IBC with the expression of IGF-1 and ANGPTL4.

Methodology: A cross-sectional study was conducted using 75 archived formalin-fixed paraffin-embedded tissue blocks of young IBC with age <45 years old. The molecular IBC subtype was classified into luminal A, luminal B, HER2 overexpression, and triple negative based on the surrogate markers of ER, PR and HER2. All samples were stained for ANGPTL4 and IGF-1 by immunohistochemistry method.

Results: The age of patients ranged from 23-44 years old, with the mean age of 37 (SD 5.48). Luminal A 28(37.7%), were the highest molecular subtype in young IBC followed by triple negative 20(26.7%), luminal B 15 (20%) and HER2 overexpression 12 (16%). Among 75 cases of young IBC patient, 51(68%) of them were immunopositive for ANGPTL4, meanwhile, 67(89%) were positive towards IGF-1. Both proteins were highly expressed in high-grade IBC, larger tumour size, and lymph node metastasis. 19(37%) of luminal A subtype were positive for ANGPTL4 followed by triple negative 14(28%), luminal B 9(18%) and HER2 overexpression 8 (16%). The expression of IGF-1 was also high in luminal A 27(40.3%) but lowest in HER2 overexpression 10(14.9%). However, there was no significant association of ANGPTL4 (p=0.897) and IGF-1 (p=0.091) to molecular subtypes.

Conclusion: Majority of young IBC shows ANGPTL4 and IGF-1 proteins expression towards luminal A. Moreover, these proteins were also highly expressed in high-grade IBC, larger tumour size, and lymph node metastasis. However, there were no significant association of molecular subtypes with both proteins. These findings may emphasise the use of these markers as a potential tool for understanding the tumour progression and metastatic behaviour. It is hoping these findings would assist in the development of new breast cancer management strategies that will improve the clinical outcomes. Thus, further study with a larger sample might be conducted to explore the role of ANGPTL4 and IGF-1 in invasive breast carcinomas.

Keywords: ANGPTL4, IGF-1, molecular *subtypes, young invasive breast cancer*

CHAPTER 1

INTRODUCTION

1.1 Overview of young Invasive breast carcinoma

Invasive breast carcinoma, (IBC) is a malignant epithelial neoplasm of the breast. The IBC occur when the malignant cells breach the myoepithelial and basement membrane with evidence of stromal invasion (Goldblum *et al.,* 2018). The 2019 World Health Organization (WHO) Classification of Tumours of the Breast, categorized IBC into histology defined subtypes as well as molecular subtypes. Among the histology subtypes, invasive carcinoma, no special type (NST) accounting for the largest category (Hoon Tan *et al*., 2020). Meanwhile, the special IBC subtype includes microinvasive carcinoma, invasive lobular carcinoma, tubular carcinoma, cribriform carcinoma, mucinous carcinoma, metaplastic carcinoma, invasive micropapillary carcinoma, carcinoma with apocrine differentiation and salivary gland type tumours (Lakhani *et al.,* 2012).

Histology defined subtypes of IBC provide clinical significance in prognostication. For example, metaplastic carcinoma had a worse prognosis as compared with invasive lobular carcinoma (Lakhani *et al.,* 2012). IBC are also grouped into biomarker defined subtypes base on oestrogen receptor (ER) and human epidermal growth factor receptor 2 (HER2) which give a value in term of treatment (Hoon Tan *et al*., 2020). Majority of IBC patients are diagnosed during postmenopausal years. However, IBC can develop at any years (Goldblum *et al.,* 2018). Young age is an independent risk factor for death from breast cancer (Livi *et al*., 2010). Young IBC are more-likely to be hereditary than IBC in older women. Young IBC patients are also diagnosed at the advanced stage and often more aggressive and difficult to treat (Centres for Disease Control and Prevention, 2019).

In Malaysia, IBC is the leading of the top three cancers, with 32.1% of all cancer among females. The incidence was highest among Chinese ethnicity (Azizah AM *et al*., 2016). The median age of IBC in Malaysia is 26.1 years old (Yip *et al*.,2014). Moreover, in Malaysia IBC are also affected younger age as reported by (Abdullah *et al*., 2013), with the youngest age of 12 years old. In Kelantan, the youngest patient diagnosed with IBC is 20 years old (Norsa'adah *et al.,* 2005). *Centres for Disease Control and Prevention* also listed breast carcinoma as the most common malignant tumour in the United States with 11% of new cases affect women under age of 45 years old (Centers for Disease Control and Prevention, 2019). Similar findings are also observed in Saudi Arabia (Colak *et al*., 2013).

Breast cancers are unifocal and can occur in any quadrant of the breast, with the higher frequency in the upper-outer quadrant (Goldblum *et al.,* 2018). The most common clinical sign in IBC is palpable mass, followed by skin retraction, nipple inversion, nipple discharge, skin colour and texture changes (Lakhani *et al*., 2012). The aetiology of IBC is multifactorial, includes hormones, reproductive factors, high fat and protein diet combine with lack of physical exercise as well as genetic factors (Lakhani *et al*., 2012).

It is well-known that breast cancer *(BRCA) 1* and *2* gene mutations may increase the risk of developing hereditary breast and ovarian cancer (Mersch *et al*., 2015). Genetic factors may contribute to early onset of breast cancer. In the UK, about 3% of all breast cancers are associated with mutations in *BRCA1* or *BRCA2* (Assi HA *et al*.,2013). However, the percentage is increases in Ashkenazi Jews to up to 40% (Assi HA *et al*., 2013). Another potential mutation in IBC is *TP53* mutation. Although it is rare, this gene mutation contributed to young breast cancer in Li-Fraumeni syndrome, which affects women between 20 and 40 years of age (Assi HA *et al*., 2013).

The pathological prognostic parameters provide an informative information on progression of the disease. The parameters include histology type, histology grade, tumour size, lymph node status, lymphovascular invasion and tumour stage (Hoon Tan *et al*., 2020). Meanwhile, the predictive markers of ER, PR and HER2 expression allow selection of therapy in IBC patients. IBC is also classified based on molecular subtypes according to a gene expression profile-validated immunohistochemical surrogate panel. There are 4 well established molecular subtypes which include luminal A, luminal B, HER2 overexpression and triple negative. Among these subtypes, luminal A is associated with the better prognosis (Goldblum *et al.,* 2018).

Many articles reported young IBC experience poor outcome with aggressive molecular subtypes as compared to the older women (Assi HA *et al.,* 2013; Johnson *et al.,* 2015). Young IBC tends to present with higher histology grade and demonstrated basal and HER2- enriched subtypes as compared to the elderly. Similar findings had been elaborated by Cancello *et al.,* (2010) who also identified young IBC women tend to have high histology grade. However, in contradiction to Azim *et al.,* (2012) who recognized the young IBC is associated with luminal A which indicated a better outcome. Although his study also reported there is a significant higher risk of recurrence amongst young IBC.

1.2 Molecular subtype

IBC is a heterogeneous disease and composed of many biologically distinct entities. The molecular subtypes of IBC most widely recognized by their gene expression signature. The molecular subtypes exhibit specific characteristics with more specific targeted forms of therapy (Goldblum *et al.,* 2018). Efforts have been made to use immunohistochemistry as a surrogate panel. The panels include estrogen receptor (ER), progesterone receptor

(PR) and human epidermal growth factor receptor 2 (HER2). There are 4 well established molecular subtypes as simplified in Table in 1.1 (Assi HA *et al*., 2013; El Chediak *et al.,* 2017; Lian *et al*., 2017). Among these subtypes, luminal A has the best prognosis as compared to HER2 overexpression and triple negative (Goldblum *et al.,* 2018).

Table 1.1: Molecular subtypes classification according to gene expression profile-validated immunohistochemical surrogate panel. Source: Assi HA *et al.* (2013).

Molecular subtype	**ER**	**PR**	**HER2**
Luminal A	Positive and/or	Positive	Negative
Luminal B	Positive and/or	Positive	Positive
Her2	Negative	Negative	Positive
Triple negative	Negative	Negative	Negative

The development of the hormonal receptor markers of oestrogen and progesterone give a good impact on breast cancer treatment. The ER and PR positivity are accounted for at least 1% of nuclear positivity by immunohistochemistry (Goldblum *et al.,* 2018). Meanwhile, *HER2* (c-*erb*B-2) is an oncogene which belongs to the family of epidermal growth factor receptors. IBC with HER2 positivity showed high-grade tumours morphology but responded very well with HER2 targeted therapy such as Trastuzumab (Goldblum *et al.,* 2018).

HER2 overexpression and amplification can be measured by immunohistochemistry (IHC) or fluorescent in situ hybridization (FISH) (Zoppoli *et al*., 2017). The HER2 expression can be divided into positive, negative and equivocal as recommended by the American Society of Clinical Oncology/College of American Pathologists guidelines (Goldblum *et al.,* 2018). A positive HER2 by IHC is defined by staining 3+ (uniform,

intense membrane staining of greater than 10% of invasive tumour cells), or a FISH result of > six *HER2* gene copies, or a FISH ratio *(HER2* gene signals to chromosome 17 signals) of > 2.0. Meanwhile negative HER2 is by IHC staining of 0 or 1+ or, a FISH result of < 4.0 *HER2* copies per nucleus or a FISH ratio of < 2. For equivocal HER2 result by IHC staining of 2+ (weak and moderate membrane staining in > 10% of tumour cells), or and a FISH result of ≥4.0 and less than 6.0 copies per nucleus. When the HER2 by IHC is 2+ (equivocal), FISH is then performed (Goldblum *et al.,* 2018).

1.3 Angiopoietin-like protein 4 and invasive breast carcinoma

Angiopoietin-like protein 4 (ANGPTL4) is a secretory glycoprotein produce in human and encoded by the *ANGPTL4* gene. It is also known as peroxisome proliferator-activated receptor γ-induced angiopoietin-related protein (Kubo *et al*., 2016). ANGPTL4 protein is highly expressed in adipocyte, liver, placental and ischemic tissues (Santulli, 2014). In normal physiology, ANGPTL4 protein involved in lipid and glucose metabolism, vascular permeability, angiogenesis and wound healing (Georgiadi *et al.,* 2013; Santulli, 2014). There are many tumours associated with ANGPTL4 protein expression, include cancer of the breast, oesophageal, stomach, colorectal, hepatocellular, prostate and renal cell carcinoma (Kim *et al*., 2011; Kubo *et al*., 2016; La Paglia *et al.,* 2017). In tumour, ANGPTL4 protein inhibit regulation of apoptosis, promote angiogenesis and tumour proliferation (Tan *et al*., 2012; Johnson *et al*., 2015; La Paglia *et al*., 2017). Study by Johnson *et al.,* (2015) reveal that the *ANGPLT-4* gene expression may be associated with a poorer outcome amongst the younger IBC patients with aggressive molecular subtypes. The same finding was also reported by (Zhang *et al*., 2012; Yotsumoto *et al*., 2013) who demonstrate higher expression of ANGPTL4 protein were seen in more aggressive

molecular subtypes. The elevated expression of ANGPTL4 protein in IBC were also correlate with metastasis to lung and brain (Johnson *et al*., 2015).

However, Galaup *et al.,* (2006) and Okochi-Takada *et al*., (2014) identified ANGPTL4 protein act as a tumour suppressor, inhibits angiogenesis and prevents metastasis. These studies findings are contradicted to the effect of ANGPTL4 protein on tumour metastasis. Interestingly, study by Johnson *et al.,* (2015) also suggested the used of general angiogenesis inhibitors or specific agents against ANGPTL4 may be useful for treatment IBC among young patients with aggressive molecular subtypes. This indicates another alternative choice of IBC treatment. However, the research on ANGPTL4 protein in young IBC is limited.

1.4 Insulin-like growth factor-1 and invasive breast carcinoma.

Insulin-like growth factor (IGF) system is a group of peptides composed of a complex network of ligands (IGF-I and IGF-II), receptors (IGF-IR and IGF-IIR), and at least six IGF-binding proteins (IGFBP-1 to 6) and IGFBP proteases (Chong *et al*., 2007; Hudelist *et al.,* 2007). IGF system plays a significant role in human physiology particularly in the development of the mammary glands. Insulin-like growth factor-1 (IGF-1) is a polypeptide encoded by chromosome 12 and produced mainly in the liver under the direct stimulation of Growth Hormone (Christopoulos *et al*., 2015).

IGF-1 protein acts as a stimulator of mitosis and inhibits apoptosis in normal physiology, growth and development (Chong *et al*., 2007; Hudelist *et al.,* 2007). Meanwhile, in the development of breast formation, IGF-1 act as a key mediator between mammary terminal end bud and ductal formation (Christopoulos *et al*., 2015). In IBC, IGF-1 contributed to

tumour progression as a potent mitogen, increased the metastatic potential and resistance to apoptosis in the tumour cells. Increased of IGF-1 protein expression are in favours of a more aggressive cancer and indicate poorer prognosis (Chong *et al*., 2007). Study by Choi *et al.* (2013) recognized the IGF-1 protein expression was higher in HER-2 overexpression and lowest in luminal A subtypes of IBC (Choi *et al*., 2013). This may indicate that IBC with IGF-1 protein expression associated with poor prognosis. The role of IGF-1 were also seen in non-small cell lung carcinoma, which high expression of IGF-1 expression were seen and associated with lymph nodes metastases (Wang *et al.,* 2013)

Young IBC is increasing worldwide. In view of the increasing trend of young IBC, we select age less than 45 years old as a designation of young based on common clinical practices and previous researches (Colak *et al*., 2013; Donnelly *et al*., 2013; Thewes *et al*., 2013). The correlation of molecular subtype in young IBC in term of prognosis give valuable impact in treatment. Therefore, looking for potential biomarkers, especially in young women with breast cancer and determine their association with known molecular subtypes, will give an important added value. Our aims were to determine the expression of ANGPTL4 and IGF-1, in young invasive breast carcinoma. Thus, through this study, we would to see the association of ANGPTL4 and IGF-1 with molecular breast subtypes in young IBC patients.

CHAPTER 2

STUDY PROTOCOL

2.1 Study protocol

Title: ANGPTL4 AND IGF-1 EXPRESSIONS IN RELATION TO MOLECULAR SUBTYPES IN YOUNG INVASIVE BREAST CARCINOMA.

Introduction

Breast cancer is the most common type of cancer among women worldwide with an estimated 1,300,000 new cases and 465,000 deaths annually (Colak *et al*., 2013). In Malaysia, breast cancer is accounted for 32.1% of all cancer among females (Azizah AM *et al*., 2016). Young women with invasive breast cancer (IBC) have an inferior outcome and commonly manifested with aggressive biological subtypes. The data regarding biological differences between breast carcinoma in young (diagnosed at <40 years of age) versus older women is controversial (Johnson *et al.,* 2015). Meanwhile, study from Assi HA *et al*. (2013) documented young breast carcinoma is more likely to be triple negative or HER2 overexpress subtypes, and present at an advanced stage.

Angiopoietin-like protein 4 (ANGPTL4)

Angiopoietin-like protein 4 (ANGPTL4) is a protein in human encoded by the *ANGPTL4* gene. It is highly expressed in adipose tissue, liver, placental tissue, and ischemic tissues (Santulli, 2014). In cancer, ANGPTL4 plays a critical role in tumour growth, tumour progression and specifically contributes to cancer metastasis by protecting endothelial cells from apoptosis and facilitating cell migration (Santulli, 2014). High expression of ANGPTL4 in primary breast tumour is strongly associated with metastasis to the lung and brain (Johnson *et al.*, 2015). Overexpression of ANGPTL4 is associated with an inferior outcome for triple negative subtypes but not HER2 overexpression tumours in

young IBC (Johnson *et al.*, 2015). Yi *et al.* (2013) reported, significantly higher protein expressions of ANGPTL4 in triple negative subtype. Meanwhile Johnson *et al.* (2015) suggested the use of general angiogenesis inhibitors or specific agents against ANGPTL4 may be particularly beneficial for young IBC. However not many study ANGPTL4 in young IBC has been conducted.

Insulin-like growth factor 1 (IGF-1)

Insulin-like growth factor 1 (IGF-1) is a peptide which stimulates mitosis and inhibits apoptosis. In normal physiology, IGF-1 system plays an important role in normal growth and development. The IGF-1 system has been shown to promote malignant transformation of normal breast cells. It is also able to maintain malignant phenotype, increase metastatic potential, resistance to apoptosis and cytotoxic drugs (Chong *et al.*, 2007; Hudelist *et al.*, 2007). In IBC, IGF-1 contributed to tumour progression as a potent mitogen, increased the metastatic potential and resistance to apoptosis in the tumour cells. Increased of IGF-1 protein expression are in favours of a more aggressive cancer and indicate poorer prognosis (Chong *et al.*, 2007).

Molecular subtypes of breast carcinoma.

Breast cancer is a highly heterogeneous disease that encompassing numbers of biologically distinct entities. It is well established that there are at least 4 main subtypes of breast cancer based on different patterns of gene expression. Breast cancer molecular subtypes were classified according to a gene expression profile-validated immunohistochemical surrogate panel as luminal A (ER+ and/or PR+ and HER2-), luminal B (ER+ and/or PR+ and HER2+), HER2 overexpression (ER-, PR-, and HER2+) and triple-negative (ER-, PR- and HER2-) (Assi HA *et al.*, 2013). Luminal A subtype

with grade 1 or 2 tumours, tend to have the best prognosis. Meanwhile HER2 overexpression tumours and the triple negative subtype confer bad prognosis. Many studies have confirmed the increased proportion of ER/PR-negativity, HER2 overexpression, and high grade were observed in young women with breast cancer (Assi HA *et al.*, 2013).

Rationale of study & Justification

The molecular subtypes of breast carcinoma give useful information in prognosis, planning treatment and important in developing new therapies. Thus, identification of new biomarkers especially in young women with breast cancer and determine their association with known molecular subtypes will give important additional value.

Research Objectives

General objective

To study the expression of ANGPTL4 and IGF-1 proteins in young patient (<45-year-old) with invasive breast carcinoma and their association with molecular subtypes.

Specific objective

1. To determine ANGPTL4 and IGF-1 protein expressions in young invasive breast carcinoma (<45 years old).

2. To determine the association between the expressions of ANGPTL4 and IGF-1 protein in young invasive breast carcinoma.

3. To determine the association of ANGPTL4 and IGF-1 protein expressions in young invasive breast carcinoma with molecular subtypes, respectively.

Research hypothesis

- There is an association between ANGPTL4 and IGF-1 protein expressions in young invasive breast carcinoma.
- There is an association between ANGPTL4 and IGF-1 protein expressions with molecular subtypes in young invasive breast carcinoma.