LYMPHOVASCULAR INVASION IN BREAST CARCINOMA

By

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'In the name of Allah, the Most Gracious and the Most Merciful'

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LIST OF SYMBOLS, ABBREVIATIONS AND ACRONYMS

%	Percentage
<	Less than
α	Alpha
β	Beta
≤	Less or equal to
≥	More or equal to
AD	Anno Domini
Ang-1	Angiopoietin-1
Ang-2	Angiopoietin-2
ASR	Age-standardized incidence rate
BC	Before Christ
BVI	Blood vascular invasion
DAB	3,3'-diaminobenzidine
DPX	Distrene, Plasticiser, Xylene solution
EGF	Epidermal growth factor
Eph-2A	Ephrin type-A receptor 2
Eph-B4	Ephrin type B receptor 4
ER	Estrogen receptor

FFPE	Formalin-fixed paraffin embedded
FGF	Fibroblast growth factor
g	gram
H&E	Hematoxylin and eosin
HCl	Hydrochloric acid
HREC	Human Research Ethics Comittee
ICC	Intraclass cerrelation coefficient
IHC	Immunohistochemistry
IL-8	Interleukin-8
IT	Intratumoral
L	Liter
LN	Lymph node
LVD	Lymphatic vessel density
LVI	Lymphatic vessel invasion
LYVE-1	Lymphatic vessel endothelial hyaluronan receptor 1
µm	Micrometer
mL	Milliliter
mm	Millimeter
mm^2	Millimeter squared

MVD	Microvessel density
n	Frequency
N	Number of samples
PBS	Phosphate buffer saline
PDGF	Platelet-derived growth factor
PPSK	Pusat Pengajian Sains Kesihatan
PR	Progesterone receptor
Prox-1	Prospero related homeobox-1
PT	Peritumoral
SD	Standard deviation
SPSS	Statistical Package for the Social Sciences
TGF-β	Transforming growth factor beta
Tie2	Tyrosine kinasewith immunoglobulin (Ig) and EGF homology domain
TNF-α	Tumor necrosis factor alpha
VE cadherin	Vascular endothelial-cadherin
VEGF	Vascular endothelial growth factor
VEGFR2	Vascular endothelial growth factor receptor 2
VEGFR3	Vascular endothelial growth factor receptor 3
W	Watt

ABSTRAK

Penyerangan limfovaskular yang terdiri daripada penyerangan vaskular darah dan penyerangan salur limfa telah menjadi salah satu kewujudan yang penting dalam karsinoma payudara. Walau bagaimanapun, masih tidak dapat dijelaskan jenis serangan vaskular yang mana memainkan peranan utama dalam metastasis. Tujuan kajian ini adalah untuk mengkaji peranan penyerangan saluran darah dan limfa, dan kepadatan saluran mikro sebagai penanda biologi ramalan dalam 19 pesakit karsinoma payudara. Kaedah imunohistokimia telah digunakan di mana bahagian karsinoma payudara telah dilumurkan dengan anti-D2-40 dan anti-CD34 antibodi untuk menilai kedua-dua penyerangan saluran limfa dan darah. Kedua-dua serangan vaskular dengan mengunakan kaedah ini dibandingkan dengan penyerangan vaskular yang ditentukan oleh pewarnaan hematoksilin & eosin (H&E). Penyerangan vaskular juga dinilai melalui hubungkaitan dengan kriteria klinikopatologi. Penyerangan vaskular dikesan pada 16 (84.2%) kes; 5/16 (31.3%) adalah penyerangan salur limfa, 2/16 (72.5%) adalah penyerangan saluran darah dan 9/16 (56.3%) menunjukkan kedua-dua penyerangan saluran limfa dan darah. Penyerangan salur limfa dalam tumor nyata sekali berkaitan dengan saiz tumor (p=0,029), manakala penyerangan salur limfa di luar tumor dikaitkan dengan metastasis jauh (p=0,018) dan penyerangan saluran darah (p=0.024). Keputusan ini menunjukkan bahawa penyerangan vaskular dalam karsinoma payudara kebanyakannya berlaku melalui salur limfa dan perkaitan antara penyerangan limfa dengan metastasis jauh memberi bukti tentang kepentingan biologi mereka dalam karsinoma payudara. Oleh itu, cadangan bahawa penyerangan limfa diterima dalam amalan klinikal sebagai salah satu potensi penanda biologi ramalan karsinoma payudara.

ABSTRACT

Lymphovascular invasion comprising of blood vascular invasion and lymphatic vessel invasion has become one of the important existence in breast carcinoma. However, it is not clear which type of vascular invasion plays a major role in metastasis. The aims of this study were to investigate the role of lymphatic and blood vessel invasion, and lymphatic and microvessel density as prognostic biomarkers in 19 breast carcinoma patients. Immunohistochemical method was used where sections of breast carcinoma were stained with anti-D2-40 and anti-CD34 antibodies to evaluate lymphatic and blood vessel invasion respectively. Vascular invasion by immunohistochemical method was compared with vascular invasion determined by hematoxylin & eosin (H&E) staining. Vascular invasion was also assessed by correlating with clinicopathological criteria. Vascular invasion was detected in 16 (84.2%) cases; 5/16 (31.3%) were lymphatic vessel invasion, 2/16 (72.5%) were blood vessel invasion and 9/16 (56.3%) showed both lymphatic and blood vessel invasion. Intratumoral lymphatic vessel invasion was significantly associated with tumor size ($p=0.029$), while peritumoral lymphatic vessel invasion was associated with distant metastasis ($p=0.018$) and blood vessel invasion ($p=0.024$). These results suggest that vascular invasion in breast carcinoma predominantly occurs via lymphatic vessels and the association of lymphatic invasion with distant metastasis provides evidence for their biological importance in breast carcinoma. Hence, it is suggested that lymphatic invasion be included in clinical practices as one of the potential prognostic biomarkers of breast carcinoma.

CHAPTER 1

1.0 INTRODUCTION

1.1 Background of Study

Cancer can be defined as a disease in which cells grow in an abnormal way leading to uncontrolled growth and proliferation of the cells (Hejmadi, 2010). There are more than 100 distinct types of cancer (American Cancer Society, 2014). Among all types of cancer present, breast cancer is the most common type of cancer occuring in adult women worldwide (Jemal et al., 2011). Breast cancer is a malignant tumour which begins to develop in the cells that line the lobules or ducts of the breast (American Cancer Society, 2014).

In 2007, the National Cancer Registry reported 3,242 cases of females diagnosed with breast cancer in Malaysia. The highest incidence occured between 50-59 age groups. The age-standardized incidence rate (ASR) differed between the three major races present in Malaysia which are the Malay, Chinese and Indian. Chinese women had the highest incidence rate with 38.1 per 100,000 population followed by Indian at 33.7 per 100,000 population. Malay women had the lowest incidence of breast cancer which is at 25.4 per 100,000 population (Zainal Ariffin and Nor Saleha, 2011). This cancer occurs more frequently in developing countries compared to the occurrence in the less developed countries due to the rapid aging of populations caused by diet, tobacco and infectious disease (Kanavos, 2006).

Metastasis is a process involving spread of cancer cells from the primary sites to other parts of the body. The cancer cells need to be able to leave the primary site of the tumour, invade and migrate through the extracellular matrix, enter the circulation, arrest at vascular bed, extravasate into new tissue and undergo cell division to become new

tumour (Liotta & Stetler-Stevenson, 1991). There are four pathways of metastasis including blood vessels, lymphatic vessels, local invasion and direct seeding of body cavities (Rubin & Reisner, 2009). Angiogenesis is important for cancer cell metastasis. The growth of new blood capillaries provide new sites for the cancer cells to enter into the circulation. Angiogenesis provides new vessels, which are highly permeable with less intact basement membrane (Zetter, 1998).

Besides angiogenesis, metastasis of breast cancer cells could also occur through the lymphatic system. The presence of cancer cells in the lymph node shows the extent of the carcinoma cell dissemination into the circulation, which gives a significant risk for metastatic spread to other parts of the body (Gurleyik et al., 2007). Lymphangiogenesis, the formation of new lymph vessels, has become one of the important existence in breast carcinoma. However, compared to blood vessels spread, less is known about lymphatic vessels spread because less markers present for lymphatic vessel detection and it is difficult to differentiate between both vessels (Nakamura et al., 2005).

Because the invasion of tumor cells through the lymphatic vessels has been less studied, therefore it is of interest to determine whether this metastatic route will show significant prognostic factor of breast cancer. This study aimed to investigate the incidence of lymphatic vessel invasion and lymphatic vessel density in comparison with blood vessel invasion and blood vessel density as prognostic biomarkers in breast cancer patients.

1.2 Objectives of the Study

1.2.1 General Objective

- To study lymphovascular invasion in breast carcinoma patients

1.2.2 Specific objectives

1) To study the topography and characteristics of lymphatic vessels in breast carcinoma and to examine their associations with clinicopathological criteria
2) To distinguish between lymphatic vessel invasion and blood vessel invasion in breast carcinoma by comparing the differential expression of blood vascular marker, CD34 and lymphatic marker, D2-40 in formalin-fixed paraffin embedded (FFPE) samples using immunohistochemistry (IHC)
3) To compare between conventional assessments of vascular invasion using H&E with that of IHC
4) To investigate the role of lymphatic and blood vessel invasion, and lymphatic and microvessel density as prognostic biomarkers in breast carcinoma patients

CHAPTER 2

2.0 LITERATURE REVIEW

2.1 History of Breast Cancer

Breast cancer was discovered since ancient time during the pyramid age of Egypt (300-2500 before century, BC) where some cases described the presence of protrusive tumor in the breast of injured men. The discovery continued during the Greek and Roman period. Physician such as Hippocrates (460-375 BC) believed that this disease arises from natural causes instead of gods' or spirits' actions. He also described the characteristic of breast tumor which will spread to other part of the body. During the Greek and Roman period, the stages of breast carcinoma was described and the removal of breast cancers through surgery was conducted (Donegan, 2006).

In the Middle ages (476-1500 anno domini, AD), although there were treatments for breast cancer, there was no record that this disease was cured by that treatment. The development of medical education increases the knowledge on breast anatomy and declines the previous thought by Henri de Mondeville in late Middle Age time regarding the development of breast carcinoma by distinguishing between causal factor of hard tumor in the breast due to black bile from the liver and the existence of true cancer by formation of combusted black bile from the other three body fluids (blood, phlegm and yellow bile). During Middle ages, removal of breast or mastectomy was conducted. Mastectomy was done with no anesthesia or antisepsis that led to painful experience of the patient (Donegan, 2006).

There are many improvement and progression in the pathology of human and the safety of surgery in the 19^{th} century. The invention of microscope has helped in the

discovery of the morphology of cancer cells and their metastasis to other parts of the body. From the 20th century until today, many treatment for breast cancer has been developed and used to reduce the prevalence and recurrence of this cancer, for example radiotherapy which acts by killing the cancerous cells using radiation. However, this technique has given side effects that are hardly endured by the patient and it is also very expensive.

Surgery is another type of treatment for breast cancer. This method is used to remove tumor that rest in the breast and also to determine whether cancer cells have metastasize to the lymph nodes under the arm i.e the axillary lymph nodes (American Cancer Society, 2014). Surgery can be conducted in two ways, breast-conserving surgery and mastectomy. Breast-conserving surgery is performed by removing the tumor and the surounding tissue. This surgery is also known as lumpectomy or quadrantectomy. Mastectomy is an act of removing the entire breast that was affected by the tumor. Surgery is the most chosen treatment of breast cancer. Although mastectomy is successfully performed, the patient still needs to undergo radiation therapy because not all cancer cells or tissues of the breast were removed during the surgery (American Cancer Society, 2014).

Chemotherapy is a treatment whereby drugs act by killing the cancer cells. These drugs are taken through oral or intravenous administration (American Cancer Society, 2014). These drugs are administered before and after surgery. Drugs given after surgery is to prevent the recurrence of breast cancer. Unfortunately, this therapy may cause side effect because the drugs do not only kill cancer cells but also some normal cells. This may cause side effects such as nausea, vomiting, pain, mood disturbance and fatigue (Hoffman, 2007).

2.2 Metastasis of Tumour

Discovery of cancer cells metastasis has enlighten researchers to study the mechanisms on how cancer cells spread over the human body. In order to determine the extent of the tumor cells spread, one must know the steps of metastasis which consists a series of steps for the tumor cells to clonize new sites. If the tumor cells miss one of the steps, this will lead to failure in colonization and proliferation of tumor cells to other part of the body.

Figure 2.1 shows the metastatic process of cancer. Metastasis occurs when the primary tumor undergo proliferation. The proliferated tumor will invade the adjacent tissues and the basement membrane. Individual tumor cells continue to enter the blood vessels or lymphatic vessels after detachment from the primary tumor. As they reach to other organs, tumor cells will arrest in the small vessels. Extravasation then occurs and tumor cells proliferate in the organ (Hunter et al., 2008). Cancer cells may reach to the lung and liver, which are the frequent sites of metastasis. Some of the cancer cells will invade microcirculations (Rubin & Reisner, 2009).

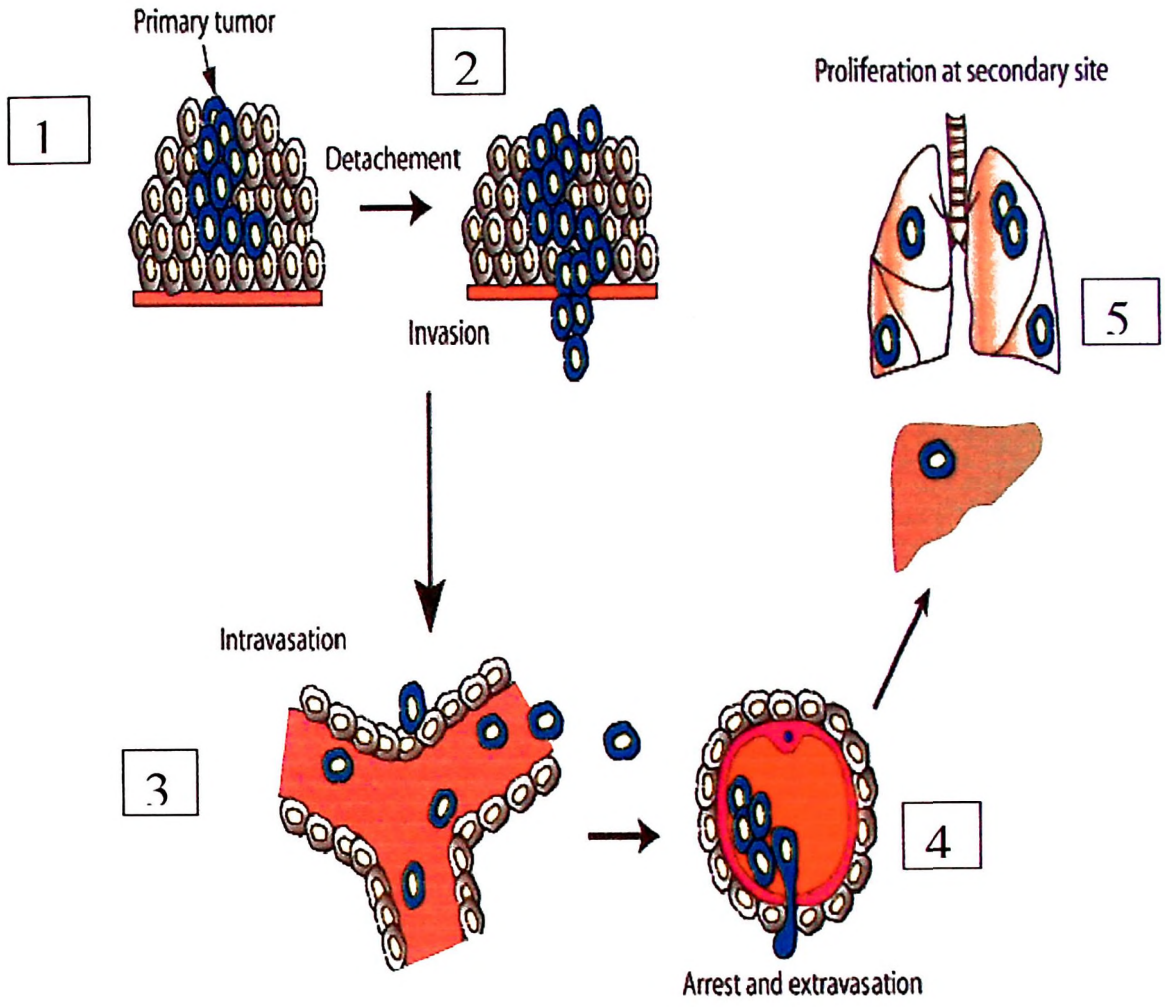

Figure 2.1 : The metastasis process

Metastasis process started with (1) proliferation of primary tumor, (2) invasion through adjacent tissue and basement membrane, (3) intravasation to blood and/or lymphatic vessels, (4) arrest and extravasation through vessels into basement membrane and tissue , and (5) proliferation at secondary site or organ (Hunter et al., 2008).

Tumour cells may spread to blood vessels indirectly via lymphatic vessels (Wong & Hynes, 2006). Tumor cells may readily penetrate the lymphatic vessels because of the lack of basement membrane in the lymphatic capillaries. The cells are carried to the regional draining lymph nodes and broaden throughout the nodes. Breast cancer mostly presents with the regional lymphatic pattern of metastatic spread (Rubin & Reisner, 2009).

Tumour cells may also spread to the organs adjacent to the body cavities. Organs that are mostly involved are ovaries, gastrointestinal tract and lungs. Peritoneal and pleural cavities are sites that are commonly invaded through direct seeding. Other body cavities such as pericardial cavity, joint space and subarachnoid space are also involved (Rubin & Reisner, 2009). Local invasion occurs when cancer cells invade surrounding normal tissues and form new tumour in the local area (Boundless, 2014).

2.3 Angiogenesis and Lymphangiogenesis

In order for tumor cells to localize to the distant sites or organs, metastasis must occur and it can be accomplished through lymphovascular invasion. Lymphovascular invasion consists of invasion through the blood vessels and also the lymphatic vessels (Rubin & Reisner, 2009).

Blood vessels are the most important site for the metastasis of tumor cells. Tumor expansion occurs through the building of new blood vessels which is also known as angiogenesis. Normal angiogenesis mostly occurs in the embryo by vaculogenesis process (Papetti & Herman, 2002). Figure 2.2 shows the mechanism of angiogenesis. The mechanism of normal angiogenesis starts by removing the pericytes or the supporting cell structure from the endothelium and vessel destabilization by

angiopoietin-2 (Ang2) shift endothelial cells. Vessel hyperpermeability will be induced by vascular endothelial growth factor (VEGF). This factor permits local extravasation of proteases and components of matrix from the bloodstream.

Then, endothelial cells proliferate and migrate through the modified matrix. Tube formation allows the blood to flow through the tubes. Proliferation and migration of mesenchymal cells occur along the new vessels. Mesenchymal cells are then differentiated into mature pericytes. The new vessels undergone stabilization process such as establishment of endothelial cells inactivation, fortifying of cell-cell contacts, and refinement of new matrix (Papetti & Herman, 2002).

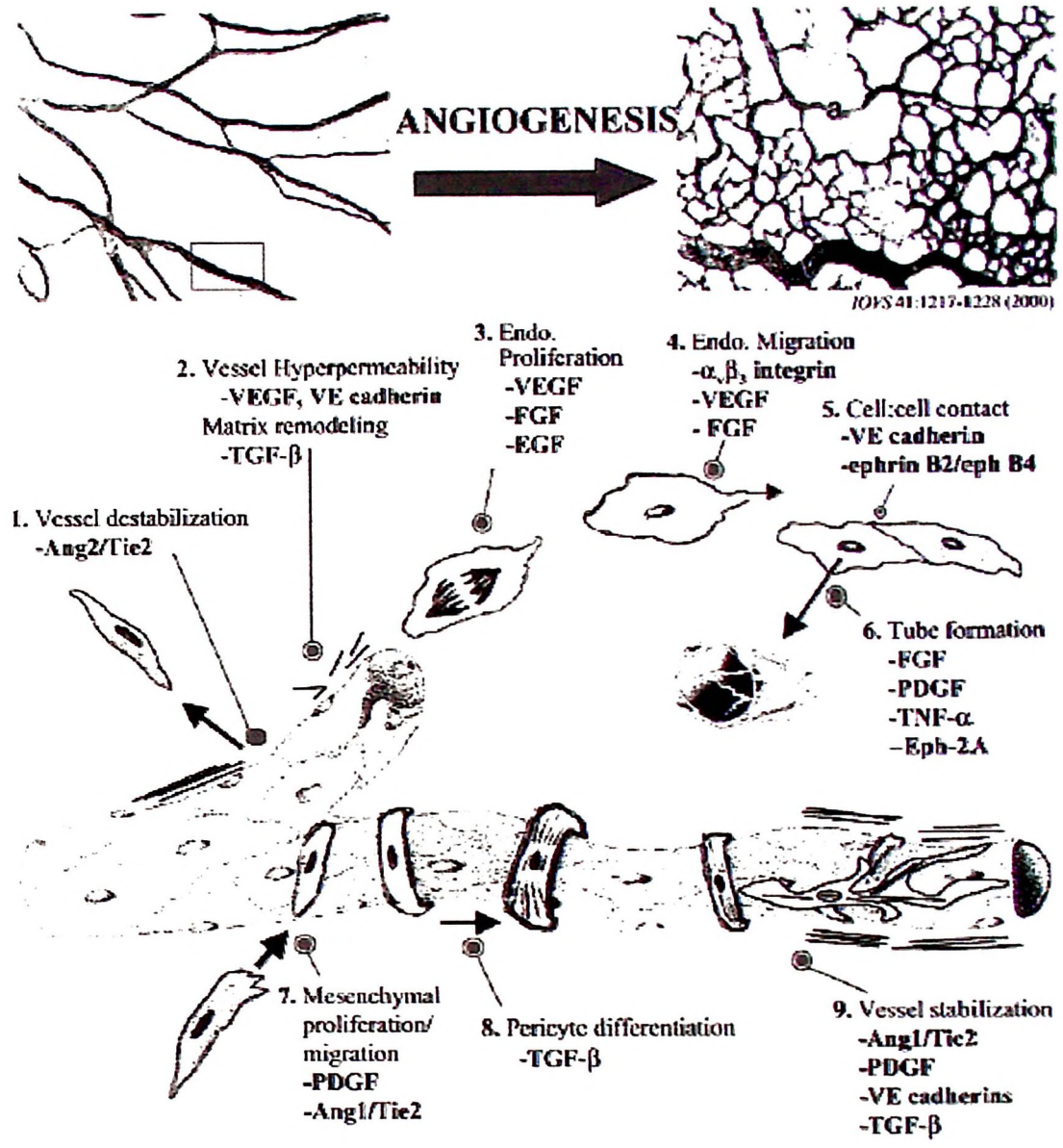

Figure 2.2 : Mechanisms of normal angiogenesis

Angiogenesis started with removal of pericytes from endothelium and destabilization (1) of the vessels by angiopoietin-2 shift endothelial cells. Vessel hyperpermeability (2) induces by permiting local extravasation of proteases and matrix components from the bloodstream. Endothelial cells proliferated (3) and migrated (4) through modified matrix (5), followed by tube formation which allow flow of the blood (6). Mesenchymal cells proliferate, migrate (7) and differentiated into pericytes (8). The new vessels undergone stabilization process such as establishment of endothelial cells inactivatio, fortifying of cell-cell contacts,and refinement of matrix (9) (Papetti & Herman, 2002).

Zetter (1998) stated that early study on angiogenesis was focused on the enlargement of tumor. The new blood vessels actually function to provide a route for the tumor cells to invade the bloodstream. Weidner et al. (1991) wanted to identify the association between angiogenesis and the occurence of metastasis. In this study, they counted the density of microvessels and found that there was a correlation between angiogenesis and metastatic spread.

Papetti and Herman (2002) have illustrated the mechanism of tumor angiogenesis as shown in Figure 2.3. When a tumor grows, the inner region of the tumor becomes avascular and hypoxic. Hypoxia leads to the production of angiogenic factors such as VEGF, fibroblast growth factor (FGF), and interleukin-8. These factors are upregulated for the formation of new blood vessels through angiogenesis.

Solid tumor needs oxygen and nutrients to maintain its vitality as low oxygen may lead to hypoxia which will result in the death of tumor (Karamysheva, 2008). A study conducted by Algive and Chalkey in 1945 (Eichhorn, 2007) proved a connection between the building of intrinsic network of vasculature with the solid tumor development. The new blood vessels that form from the process of angiogenesis has fragmented basement membrane and leaky compared to older vessels. Thus, it will be easier for tumor cells to infiltrate the new blood vessels compared to the more mature vessels. Angiogenesis is mostly induced when the metastatic tumor cells undergo proliferation at the metastatic sites. A highly angiogenic primary tumor will release angiogenic tumor cells. When angiogenic tumor cells reach the site of metastasis, they will induce angiogenesis and continue to metastasize to other part of the body or within the same site (Rubin & Reisner, 2009).

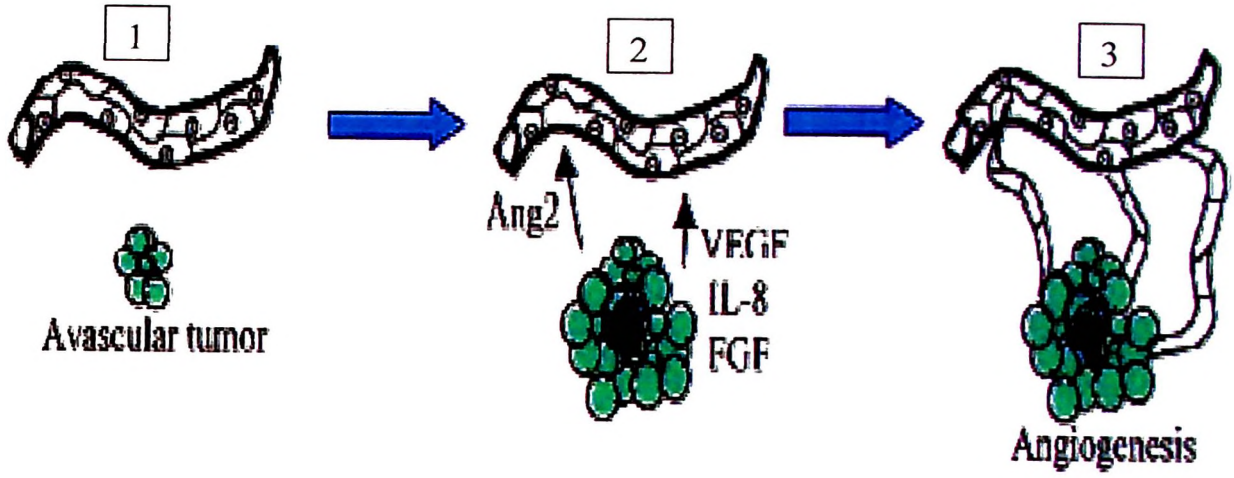

Figure 2.3: Mechanisms of tumor angiogenesis

Avascular tumor (1) grows until become hypoxic and angiogenic factors such as VEGF, FGF and interleukin (IL)-8 become upregulate(2) for angiogenesis process (3) (Papetti & Herman, 2002).

As blood vessels become a part of tumor expansion, several studies have identified the technique to determine the presence of tumor emboli in the blood vessels. Hematoxylin & eosin (H&E) staining has been used to identify the invasion of tumor cells through vasculatures. Unfortunately, H&E staining unefficiently differentiates between the blood vascular invasion and lymphatic invasion.

Lymphatic invasion involves lymphatic vessels and lymphatic tissues. Vascular of lymphatic contain the fluid called lymph that is actually absorbed into the capillaries of lymphatic from the interstitial fluids (Tanis et al., 2001). Other substances that are absorbed are macromolecules, microorganisms, toxins, waste products and foreign substances (Chikly, 2003). The lymphatic capillaries consist of a single layer of endothelial cells with diameter ranging between 10 to 50 μm. The lymph fluid will be drained into the collecting lymphatic vessels from the lymphatic capillaries. From the collecting lymphatic vessels, the fluid will be drained into the lymph nodes.

The lymphatic system of breast was originally described by a Dutch physician named Camper in 1770 (Tanis et al., 2001). Figure 2.4 shows the lymphatic circulation of the breast. He identified the lymphatic drainage to lymph nodes along the internal mammary vessels. The lymph nodes were found to extend from the fifth intercostal space to the retroclavicular glands. Cancerous cells may spread to other part of the body not only through the blood vessels but also through the lymphatic vessels. Lymphatic vessels can be characterized by thin-walled vessels having low-pressure, lymph nodes, lymphoid tissue and lymphocytes circulating in the vessels of lymphatic (Stacker et al., 2002).

Similar with blood vessels, new lymphatic vessels can also be formed through the process of lymphangiogenesis. However, the metastatic spread of tumor cells is less

known as there is still no clear evidences on whether this process of developing new lymphatic vessels could be one of the causal of metastasis of tumor cells to other part of the body (Stacker et al., 2002). Previous study has identified tumor cells in the lymphatic vessels and also the lymph nodes (Stacker et al., 2002). Unfortunately, earlier studies could not differentiate between blood vessels and lymphatic vessels when stained with hematoxylin & eosin (H&E) (O'Donnell et al., 2008). Thus, in order to differentiate between these two vessels, many markers have been determined to detect the blood vessels and lymphatic vessels.

Angiogenesis and lymphangiogenesis occur primarily due to the release of angiogenic and lymphangiogenic molecular mediators. When these mediators are released, endothelial cells for both vascular and lymphatic will undergo proliferation and migrate to the site of metastasis where extracellular matrix is degraded. This is followed by the assembly of endothelial cells to form a tube-like structures (Stacker et al., 2002). Some of the molecular mediators for this process were used as markers

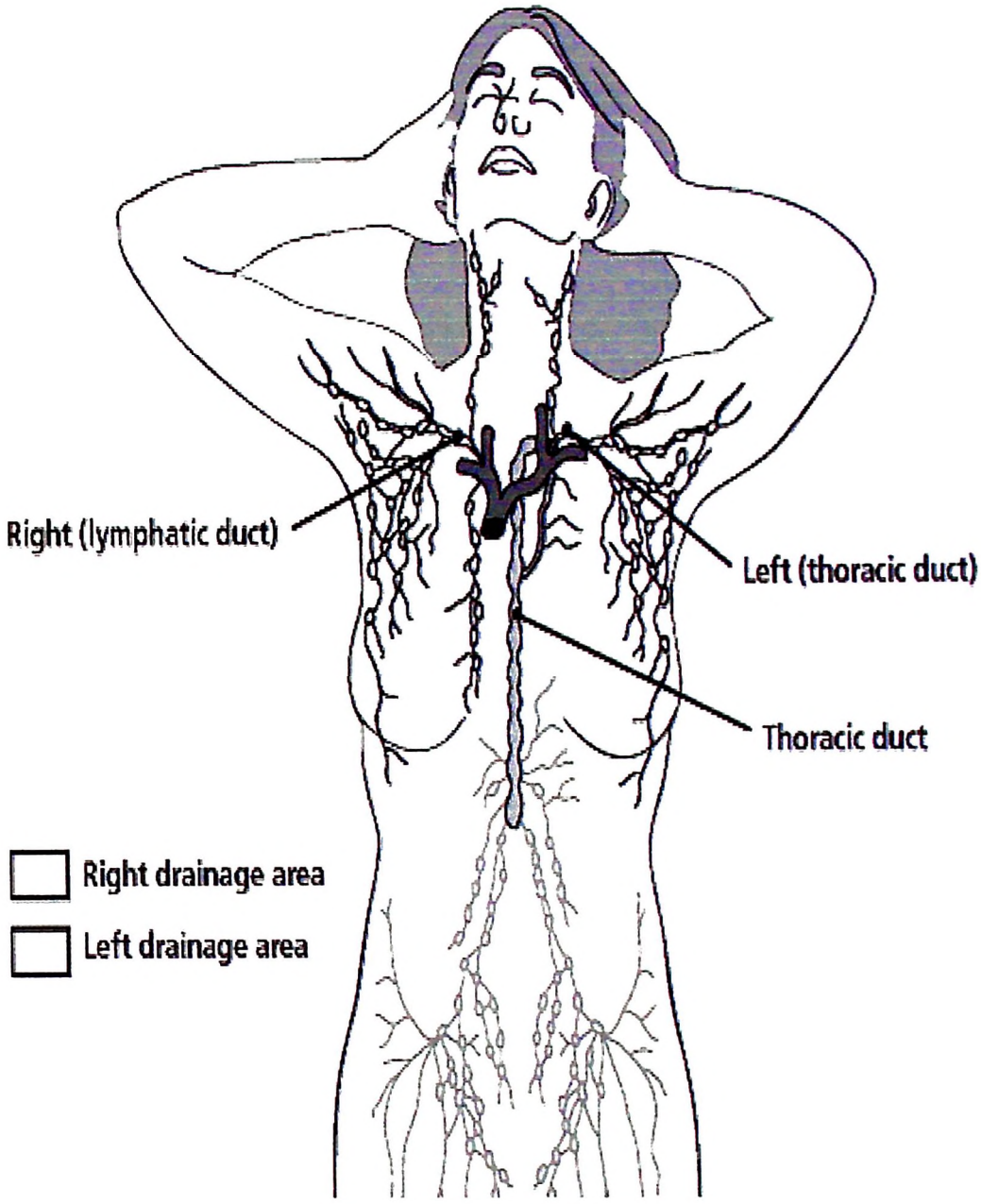

Figure 2.4 : The lymphatic system of breast

Lymphatic drainage to lymph nodes where lymph nodes extend from the fifth intercostal space to retroclavicular glands (American Cancer Society, 2013).

to detect and differentiate between the blood vessels and the lymphatic vessels through immunohistochemical identification.

2.4 Molecular Markers

The use of molecular markers has been shown to increase the detection rate of blood vascular vessels (Lee et al., 2011; Van den Eynden et al., 2006; Kamlesh Verma et al., 2013) and lymphatic vessels (Van den Eynden et al., 2009; El-Gohary et al.,2008; Nakamura et al., 2005; Kato et al., 2005; Schoppmann et al., 2004; Williams et al., 2003; Stacker et al., 2002). The preferential markers that are commonly used to detect blood vessels include CD31, CD34 and factor VII-related antigen. The markers for detecting lymphatic vessels was found later and some of the markers present are vascular endothelial growth factor, lymphatic vessel endothelial hyaluronan receptor 1, Prox1 and D2-40.

2.4.1 Vascular Vessel Molecular Markers

2.4.1.1 CD31

CD31 is a 130-kDa transmembrane glycoprotein platelet endothelial cell adhesion molecule-1 (PECAM-1). This marker is expressed on monocytes, platelets, selected T-cell subsets and endothelial cells. CD31 is mostly found on blood vascular endothelial cells but less on lymphatic endothelial cells. Lee and collegues (2011) studied the association of CD31 with the prognostic factors and recurrence of breast

carcinoma. In their study, CD31 had no significant relationship with prognostic factors and it also had no association with the recurrence of breast carcinoma.

2.4.1.2 CD34

CD34 is a hematopoietic progenitor cell antigen and the most common marker used for the hematopoietic progenitor cells and endothelial cells (Stuart, 2013). Fina et al., as cited in Martin et al., 1997, believed that CD34 is involved in the adhesion of leucocyte and migration of endothelial cells during angiogenesis. Study conducted by Martin et al. in 1997 reported a high detection of microvessel density when using CD34 in immunohistochemical staining compared to factor VIII-related antigen and CD31 (Kamlesh Verma et al., 2013). CD34 did not stain any tumor or inflammatory cells which made it easier to count the microvessels.

2.4.2 Lymphatic Vessels Molecular Markers

2.4.2.1 VEGF

Vascular endothelial growth factor (VEGF) family is one of the lymphangiogenic factorS for detection of lymphatic vessels. VEGF and its receptor are associated with the level of tumour vascularization (Papetti & Herman, 2002). High expression of VEGF also correlates with increased intratumoural microvessel density and also low prognosis in breast cancer patients. Low oxygen level leads to hypoxia and increases the expression of VEGF. Oxygen tension leads to neovascularization which form though the release of VEGF. Thus, VEGF expression is highly associated with angiogenesis and also lymphangiogenesis by cancer cells.

VEGFC, one of the factors from VEGD family, promotes lymphangiogenesis (Williams et al., 2003). VEGFC binds to VEGFR3, a primary tyrosine kinase-linked receptor. This receptor is expressed on lymphatic endothelium. Previous studies used VEGFR3 as the marker for the presence of lymphatic vessels (Arnaout-Alkarain et al., 2007). However, VEGFR3 expression was reported to express in blood vessels, thus decreasing in specificity for lymphatic vessels in tumor.

2.4.2.2 LYVE-1

LYVE-1 or known as lymphatic receptor for hyaluronan is a homologue of the CD44 hyaluronan receptor (Kato et al., 2005). LYVE-1 is thought to act by transfering hyaluronan from the tissue to the lymph (Stacker et al., 2002). Inspite of the ability to specifically stained the lymphatic vessels, LYVE-1, also stain blood vessels of the lungs (Arnaout-Alkarain, 2007). Therefore, this marker is less used.

2.4.2.3 Prox1

Prox1, a homeobox gene product, is expressed on the endothelial cells of the lymphatic. It is required for lymphatic vessel development. However, Prox1 is also expressed on other cell types and tissues thus making its use in analysis of immunohistochemical technique become limited to identify the lymphatic vessels (Stacker et al., 2002).

2.4.2.4 D2-40

D2-40 was reported as the best marker to detect the lymphatic vessels (Lee et al. 2011). D2-40 is a monoclonal antibody which showed sensitivity to the endothelial cells of lymphatics and was able to specifically differentiate between lymphatic vessels and blood vessels (Van den Eynden et al., 2009). D2-40 antibodies has high percentages of sensitivity and specificity to lymphatic endothelium.

CHAPTER 3

3.0 MATERIALS AND METHODOLOGY

3.1 Materials

3.1.1 Formalin Fixed Paraffin Embedded (FFPE) Tissue Blocks

Nineteen FFPE tissue blocks were obtained from Department of Pathology, Universiti Sains Malaysia Hospital.

3.1.2 Chemicals and Reagents

The chemicals and reagents used in this study are listed in Table 3.1.

3.1.3 Commercial Kit and Consumable Items

Commercial kit and consumable items are listed in Table 3.2 and 3.3

3.1.4 Laboratory Equipment

Laboratory equipmet used this study are listed in Table 3.4

3.1.5 Software

List of sofware used in this study are listed in Table 3.5

Table 3.1: List of chemicals and reagents used in this study.

Chemicals	**Supplier**
Harris hematoxylin	Sigma-Aldrich, USA
Eosin Y	Sigma-Aldrich, USA
Ethanol	EMSURE, Germany
Xylene	BDH Chemicals, UK
Trisodium citrate dehydrate	EMSURE, Germany
30% hydrogen peroxidase	Merck KgaA, Germany
Methanol	LiChrosolv, Germany
Phosphate buffer saline (PBS)	Bioland Scientific, USA
Antibody diluent	Dako, Germany
Hydrochloric acid	Sigma-Aldrich, USA

*USA: United States of America

*UK: United Kindom

Table 3.2 Commercial kit used in this study.

Commercial Kit	**Supplier**
Dako REAL™ EnVision™ Detection System, Peroxidase/DAB+, Rabbit/Mouse	Dako, Germany

Table 3.3 List of consumable items used in this study

Consumable items	**Supplier**
Glass slides	Sail Brand, China
Silane coated slides	Muto Pure Chemicals Co., Japan
Coverslips	Favorit, Malaysia