EXPRESSION OF TUMOUR MARKERS AND DETERMINATION OF MICROVESSEL DENSITY IN MNU-INDUCED BREAST TUMOUR

by

SITI NORASIKIN BINTI MOHD NAFI

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Thesis submitted in fulfillment of the requirements for the degree of Master of Science (Molecular Pathology)

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LIST OF ABBREVIATION

%	Percent
µg	Microgram
bFGF	Basic fibroblast growth factor
Bp	Base pair
cDNA	complementary deoxyribonucleic acid
Cm	Centimeter
CO2	Carbon dioxide
CT value	threshold cycle value
DCIS	Ductal carcinoma insitu
DEPC	Diethylpyrocarbonate
DMBA	7,12-dimethylbenz (a) antracene
E-cadherin	epithelial cadherin
EDTA	Ethylene-diamine-tetracetic acid
EGFR	epidermal growth factor receptor
ER	Estrogen receptor
H&E	Haematoxylin and Eosin (stain)
IDP	Intraductal proliferation
IHC	Immunohistochemistry
Kg	Kilograms
L	Litre
LR	Laminin receptor
LSAB	Labeled streptavidin biotin
Mg	Milligram
Min	Minute
Mmol	Milimole
MNU	1-methyl-1-nitrosourea
mRNA	messenger ribonucleic acid
MVD	microvessel density
Ng	Nanogram
ºC	Celcius
PBS	Phosphate buffer saline
PCR	Polimerase Chain Reaction

PF4	Platelet factor 4
PR	progesterone receptor
RNase	Ribonuclease
rpm	revolutions per minute
RT PCR	Real Time PCR
SEM	Standard Error Mean
TBE	Tris Boric EDTA
TDLU	Terminal ductal lobular unit
TE	Tris-EDTA
TEB	Terminal End Buds
Δ	Delta
PPAR	Peroxisome proliferators activated receptor

LIST OF PUBLICATIONS & SEMINARS

Journal (submitted for publication)

Hasnan Jaafar, Fauziah Mohamad Idris, Siti Norasikin Mohd Nafi (2009) The association between phenotype and size of breast tumors induced by 1-methyl-1-nitrosourea (MNU) injection in rats.*Med Sci Monit* ,15(5), 129-134.

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Mohd Nafi SN & Jaafar H. Histological features of MNU-induced breast tumour in animal model following bFGF and PF4 intervention. Biomedical Imaging Intervention Journal (2005).
(Abstract of the Asian Breast Diseases Association (ABDA) 3rd Teaching Course: Advances in the Management of Breast Diseases, Kuantan, 28-30 May 2005)

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3. **SN Mohd Nafi,** H Jaafar. The Immuno-Expression of ER and cerbB2 in the MNU Model Following bFGF and PF4 Intervention, 1st Health and Medical Sciences Conference, Universiti Sains Malaysia. 25-27 May 2006. *Oral presentation.*

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PENGEKSPRESIAN PENANDA TUMOR DAN PENENTUAN DENSITI SALURAN MIKRO DARAH PADA TUMOR PAYUDARA DIBAWAH ARUHAN MNU

ABSTRAK

Angiogenesis memainkan peranan penting dalam perkembangan tumor payudara. Perkaitan fenomena ini dengan pengekspresian penanda-penanda tumor payudara masih belum dihuraikan secara terperinci. Untuk kajian ini, pengaruhan tumor payudara dilakukan melalui suntikan karsinogen MNU (1-methyl-1-nitrosurea) pada tikus berumur 21 hari. Suntikan dilakukan secara intraperitoneal pada dos 70 mg/kg berat badan selama 3 hari berterusan. Tumour yang terhasil kemudiannya diinterven dengan faktor angiogenesis; bFGF dan PF4 pada dos 10μg/tumor. Saiz tumbesaran tumor dipantau. Pengekspresian gen penanda seperti ER, PR, EGFR, cerbB2, E-cadherin dan LR pada sampel tumor telah dikaji berdasarkan analisis immunohistokimia dan RTPCR. Aktiviti angiogenesis pula telah dikaji berdasarkan perkiraan densiti saluran darah mikro, hasil daripada penandaan FVIII-RA secara teknik immunohistokimia. Keputusan histologi menunjukkan kebanyakan tumour payudara aruhan MNU adalah malignan yang terdiri daripada jenis cribriform berbanding jenis papilari. Kebanyakan ketumbuhan jenis benin dan pre-neoplastik aadalah pada tumour saiz kurang daripada 1.2cm. Tumor di bawah aruhan angiogenesis mempunyai jenis histologi yang sama dengan kontrol. Penyekatan angiogenesis menunjukkan peningkatan tumor nekrosis dan bilngan tumor jenis 'NST'. Tumbesaran tumor pada kontrol menunjukkan kebergantungan terhadap salur darah peritumoral dan intratumoral. Peningkatan bilangan salur darah di intratumoral adalah lebih signifikan daripada di peritumoral. Tumour payudara yang dirawat oleh PF4 mempunyai penurunan salur darah yang significant manakala tumour payudara yang dirawat oleh bFGF tidak menunjukkan sebarang peningkatan salur darah yang signifikan. Semakin tumor membesar, terdapat

peningkatan ekspresi ER, PR, EGFR dan cerbB2. Pengekspresian EGFR dan cerbB2 berterusan meningkat walaupun pada keadaan dibawah aruhan ataupun penyekatan angiogenesis. Kami juga mendapati peningkatan tumour invasif pada kumpulan PF4 disebabkan oleh penurunan ekspresi ER dan PR, dan peningkatan LR. Kami juga menjumpai perkaitan yang signifikan di antara peningkatan MVD dengan pengekspresian berlebihan ER, PR, EGFR, cerbB2 dan LR mRNA, dan penurunan E-cadherin mRNA pada kontrol. Perkaitan ini dilihat berlaku samada di kawasan peritumoral mahupun intratumoral. Terdapat juga perkaitan yang signifikan di antara peritumoral MVD dengan ekspresi ER, PR dan LR pada kumpulan bFGF. Perkaitan yang signifikan turut dikesan di antara intratumoral MVD dengan ekspresi ER dan PR pada kumpulan PF4. Penutupnya, penemuan kami menunjukkan penurunan bekalan darah kepada tumor payudara menyebabkan peningkatan keagresifan tumor yang berdasarkan kehadiran fenotip agresif pada kumpulan PF4 dengan penurunan ER dan PR di kawasan intratumoral. Walaupun fenotip tumor pada kumpulan di bawah aruhan angiogenesis adalah sama dengan control, kadar pertumbuhan tumor yang cepat pada kumpulan ini di sokong oleh perkaitan yang signifikan dengan peningkatan ekspresi ER, PR dan LR pada kawasan peritumoral. Kami juga mendapati ekspresi EGFR dan cerbB2 dalam mempengaruhi tumbesaran tumor dan penurunan E-cadherin tidak menunjukkan kebergantungan terhadap angiogenesis.

EXPRESSION OF TUMOUR MARKERS AND DETERMINATION OF MICROVESSEL DENSITY IN MNU-INDUCED BREAST TUMOUR

ABSTRACT

Angiogenesis plays an important role in breast tumour development. The association of this phenomenon with the expression of breast tumour markers has not been fully elucidated. In this study, breast tumours were induced by injecting rats intraperitoneally with 1-methyl-1-nitrosurea (MNU) at dose of 70 mg/kg body weight in 3 consecutive days into 21-days old rats. The breast tumours were then subjected to intratumoural angiogenesis promotion using basic Fibroblast Growth Factor (bFGF) or inhibition using Platelet Factor 4 (PF4) at dose 10µg/tumour. The size of the tumours was monitored. The tumour tissues taken were subjected to quantitative-RTPCR for ER, PR, EGFR, cerbB2, E-cadherin and LR expression. Angiogenesis was determined by microvessel density with immunohistochemical staining for FVIII-RA. Histological findings showed most of the MNU-induced breast tumours were malignant where cribriform type predominated papillary type. Benign and preneoplastic lesions were seen mainly in tumours of size less than 1.2 cm. Promoting angiogenesis appear to have similar histological features with the control group. We noted that suppression of angiogenesis appeared to increase tumour necrosis and the number of diffuse infiltrating ductal carcinoma, not specified type (NST). It was clearly seen that tumour growth in control group shows dependency on peritumoural and intratumoural blood vessels. There was significant increase in the number of intratumoural blood vessels compared to peritumoural blood vessels. PF4-treated breast tumours have significant reduction of tumour blood vessels and bFGF-treated breast tumours do not show any significant increase of tumour blood vessel. As tumour grew, there was significant increase of ER, PR, EGFR and cerbB2 expressions. The expressions of EGFR and cerbB2 continue to

increase irrespective of whether angiogenesis is increased or suppressed. We also noted that the invasiveness of breast tumours was increased in PF4-treated group by decrease expressions of ER and PR; and increase expression of LR. We found a significant association between increased MVD and overexpressions of ER, PR, EGFR, c-erbB2 and LR mRNA, and downregulation of E-cadherin mRNA expression in the control group. This association occurs irrespective whether the MVD is in peritumoural or intratumoural regions. There was also significant association between peritumoural MVD with the expressions of ER, PR and LR seen in bFGF-treated group. There was significant association between intratumoural MVD with the expressions of ER and PR seen in PF4-treated group. In conclusion, our findings have shown that reduction of blood supply to breast tumour causes increase aggressiveness of the tumour as illustrated by the presence of more aggressive phenotypes in the PF4-treated group with down regulations of ER and PR in the intratumoural region. Eventhough tumour phenotype in the angiogenic promoted group was similar to the control group, the growth rate was faster and this was supported by significant association with increased expressions of ER, PR and LR in the peritumoural region. We also noted that expressions of EGFR and cerbB2 in promoting tumour growth and down regulation of E-cadherin were independent of angiogenesis.

CHAPTER ONE

INTRODUCTION

1.1 Introduction

In the present world, breast cancer is the most common threats, and predominantly being diagnosed among women. Sasco indicated that about 1, 050, 000 cases of breast cancer are reported worldwide in year 2000 with mortality cases of 372,000 (Sasco, 2003). The number is increasing year by year.

It has been noted that the developed countries had the highest rate of breast cancer incidence in contrast to developing countries (Parkin *et al.*, 2005, Lacey *et al.*, 2002, Parkin *et al.*, 2001). United States is estimated to have approximately 212,920 of new breast cancer cases with 40,970 deaths among women in 2006 (Smigal *et al.*, 2006). In European countries, the breast cancer is even higher with an estimate of 370, 100 new cases and 129 900 deaths were diagnosed in year 2004 (Boyle and Ferlay, 2005 a). However, the breast cancer rate is also seen to increase in the Asia countries that commonly have low incidence of breast cancer. The same phenomenon is also noted in Malaysia. Data from the National Cancer Registry of Malaysia for 2004 showed an age-standardised incidence rate (ASR) of 46.2 per 100,000 women (Yip *et al.,* 2006)

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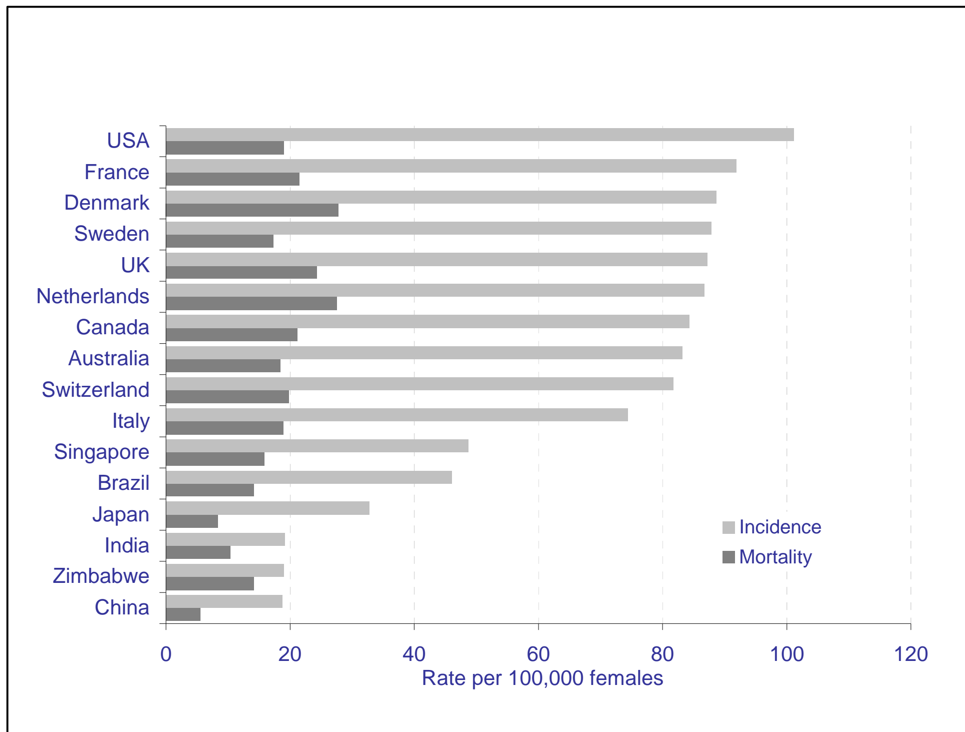

Figure 1.1. Age standardized (world) incidence and mortality rates, female breast cancer in selected countries, 2002 estimates (Parkin *et al.*, 2005).

Delay in seeking diagnosis among women with breast cancer has been found to be the major factor for the difficulty in providing better treatment to the patient. Many of them had advance breast cancer by the time diagnosis was made. Distant metastasis progression of the breast cancer is commonly associated with the increasing cases of morbidity and mortality cases in women.

Many great efforts have been done in order to improve the early detection of breast cancer as well as treatment strategies directed towards this malignancy. More

attention needs to be given to the investigation on biological progressions of the breast cancer. Controlling the mechanisms of tumour growth at the beginning of the process is important apart from treating the solid tumour itself. There are many strategic pathways that can be manipulated including initial and sustain of tumour growth, loss of cell-cell adhesions, increase cell-stromal interaction and further growth with the final event of angiogenesis.

Angiogenesis is a growth process of new blood vessels from existing vasculature, which enable tumour cells to increase their blood supply. This process is an important step for tumour growth, progression and metastasis (Pandya *et al.*, 2006, Sledge, 2000). Realizing this has encouraged researchers to pursue further to investigate on how tumours send out signals to recruit new blood vessels. This hopefully will help us in controlling and probably eradicate the ability of the tumour in manipulating the angiogenic switch. Angiogenic processes in breast tumour samples can be assessed by measuring the molecules involved in the establishment of the tumour vasculature, including angiogenic growth factors and their receptors, cell adhesion molecules, proteases and markers of activated, proliferating, cytokine stimulated or angiogenic vessels.

Previous studies describe the association between angiogenesis and tumour metastases and prognosis (Gasparini and Harris, 1995, Gasparini *et al.,* 1995, Bosari *et al.,* 1992, Weidner *et al.,* 1991, Liotta *et al.,* 1991). Figure 1.2 shows a diagram of this association which was illustrated by Folkman, 1995. However, very few studies have been done, especially at experimental level to look at the role of angiogenesis at the early

phase of tumour development. It is important to investigate to see whether angiogenesis is dependent or not on the early events of breast tumour development.

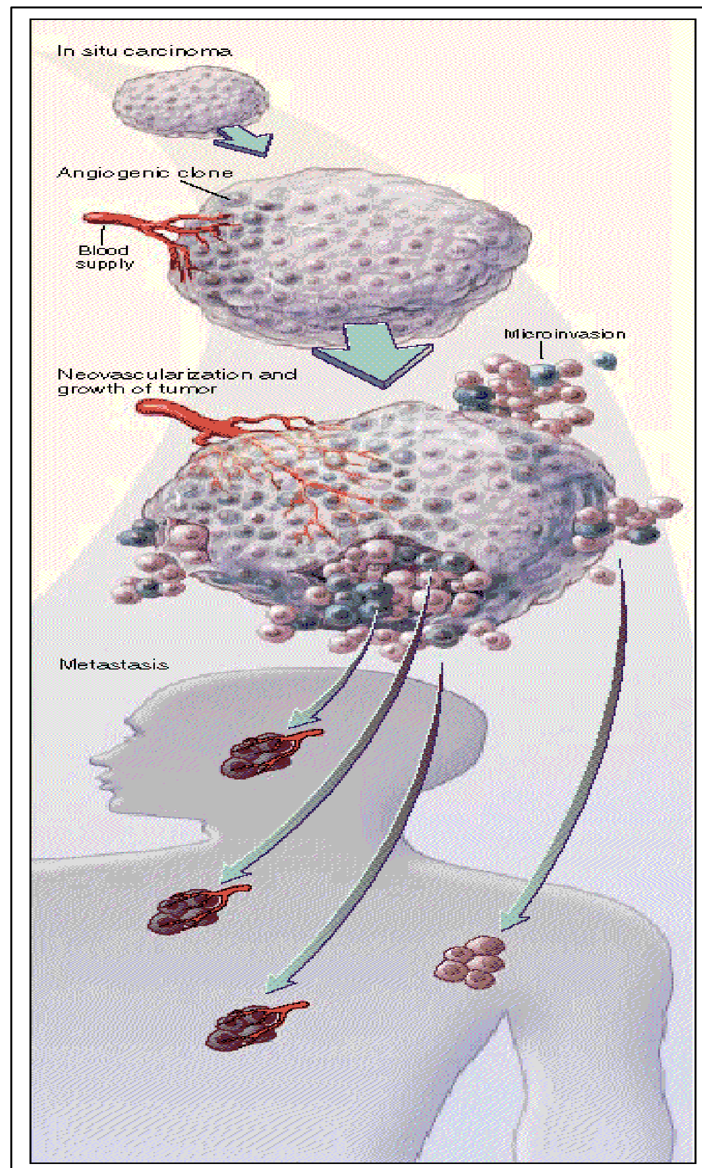

Figure 1.2. The development of heterogeneity in the angiogenic phenotypes in a primary tumour and its relation to metastasis potential (Folkman, 1995).

The mechanism of breast tumourigenesis entails multiple events that are mediated under several cellular interactions between cancer cells, growth factors and components of extracellular matrix. In the current study, Estrogen Receptor (ER), Progesterone Receptor (PR), Epidermal Growth Factor Receptor (EGFR), cerbB2, E-cadherin and Laminin Receptor (LR) are the selected markers to observe such interactions in breast tumourigenesis.

Early event in tumourigenesis such as dysregulated/ autoregulated proliferation involves over expression of ovarian hormone receptors i.e. ER and PR (Russo and Russo, 2006). Further growth of tumour cells is aided by regulation of growth factor receptors such as EGFR and cerbB2. The overexpression of both receptors has been identified in primary breast tumour and is shown to be maintained throughout tumour progression (Tsutsui *et al.*, 2003). E-cadherin is shown to be the principle adhesion molecule that promotes dedifferentiation of breast cancer cells (Heinmann *et al.,* 2000). Decrease levels of E-cadherin causes the loss of cell-cell attachment in breast cancer. Other important breast tumourigenesis event is the cell to stromal interaction. The event has been shown to be regulated by the overexpression of LR in breast cancer (Givant-Horwitz *et al.,* 2005).

Being able to understand the interactions between the above receptors' expressions associated with angiogenic activity may help us to design further the study on how to stop certain important tumour signals or to prevent the tumour from getting more aggressive or to destroy it completely.

CHAPTER TWO

LITERATURE REVIEW

2.1 Human breast development

The breast is the most frequently diagnosed malignancy in the female population (Boyle and Ferlay, 2005 b). Changes in human breast associated with infantile growth, puberty, pregnancy, lactation and post-menopausal regression have been studied (Russo and Russo, 2004). Further study need to be carried out to understand how human breast development is influenced by those endocrinological and reproductive events. This will help to identify the risks of developing breast cancer.

2.1.1 Mammary gland morphogenesis

Normal breast tissue is comprised of epithelial and stromal elements. The epithelial part consists of branching ducts which connect to the lobules and to the nipples (Figure 2.1). The stroma part is composed of connective tissues that are surrounded by fat. In normal breast structure, the stroma makes up the majority of the breast volume.

Human mammary gland morphogenesis occurs in a lifelong process from embryonic life to senescence. The process can be divided into two different stages; the developmental stage and the differentiation stage. The developmental stage involves initial steps of mammary gland transformation which include the progressive processes of ductal elongation, branching and sprouting of ductules, and formation of lobules structure (Russo *et al.,* 2005). Lobules are the structural and functional milk-producing units of the

breast. Russo *et al.* (1992) termed the lobule unit as terminal ductal lobular unit (TDLU). An average of 4-11 ductules that cluster around a terminal duct is said to form TDLU. Cell proliferation and differentiation occur almost simultaneously in the formation of lobules.

The changes in lobule transition are most marked during the time of puberty (Russo *et al.,* 2005, Russo *et al*., 2002). Less differentiated structures of terminal ducts and lobules type 1(Lob 1) are found in the breasts of young women. Progressions of lobules type 2(Lob2) is also seen. Only minimal formation of lobules type 3(Lob 3) is observed. In contrast to young women, adult women have completed the cycle of lobular development with the formation of lobules type 4 (Lob4). This occurs particularly during pregnancy and lactation. The presence of Lob4 indicates maximum expression of development and differentiation of the adult glands.

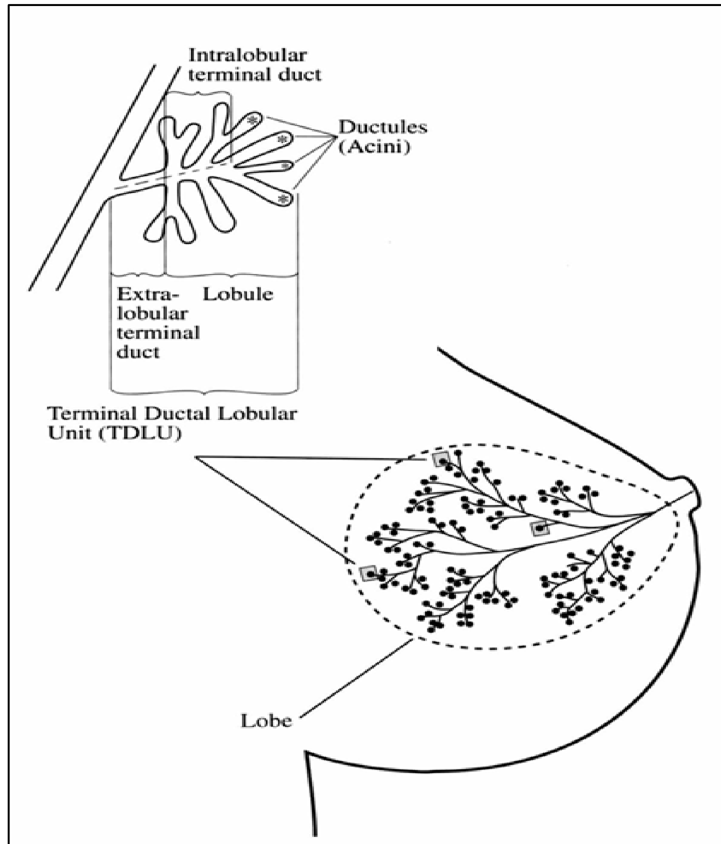

Figure 2.1. Anatomy of the human breast, a lobe and a TDLU. (Tabár *et al*., 1996)

2.1.2 Epithelial cell types of the breast

Histologically, the ductal and lobular structures are lined by continuous layer of luminal epithelial cells (ECs). The luminal epithelial in turn is surrounded by a basal, layer composed of myoepithelial cell and the basement membrane. Similar type of luminal epithelial is seen in both human and animal model (Thompson *et al.,* 2000). Differences between the myo- and luminal phenotype have been described based on the type of protein expression (Russo and Russo, 2004). It has been noted that the luminal cells express cytokeratins and nuclear receptors for ovarian hormone receptors more prominently compared to myoepithelial cells. In young rats, more than 90% of the steroid-induced epithelial cell proliferation occurs in the luminal cells (Perusinghe *et al.,* 1992).

2.1.3 Hormonal influences on the development of the breast

In both human and animal experimental studies, it is well-established that the breast is a hormone-responsive organ. The mechanism of breast development occurs in a synchronous manner involving specific hormonal and growth factor stimuli. The responses to hormonal influences are to regulate cell proliferation, cell differentiation and apoptosis.

Estrogen hormone plays a major role in promoting human breast development. The initial report on the estrogen influences has been carried out by Beatson *et al.* in 1869 (Russo and Russo, 2004). They have shown that removal of ovaries is known to

induce the risk for developing breast cancer. The findings promote further studies on using anti-estrogen agents to suppress breast tumour.

It has been shown in animal studies that estrogen stimulation through estrogen receptor-α (ER-α) induce elongation and branching of the ducts. The activities reach a maximum limit at prepubertal age for rats. Gene-knockout studies have demonstrated that mammary glands fail to develop beyond the prepubertal stage in mice lacking ER-α (Korach *et al.,* 1996). The estrogen receptor-β (ER-β) is also required for normal lobule-alveolar development as suggested by the discovery of larger alveoli and less epithelial component in ER-β -/- mice compared to wild type controls (Foster *et al.,* 1991).

Progesterone and prolactin activities during the onset of sexual maturity increase the branching and size of alveolar buds. The activities occur in every estrous cycle until the rat reaches the age of 63 days. During pregnancy, progesterone has been shown to contribute to ductal branching and lobule-alveolar development that promote milk production (Conneely *et al.,* 2003, Conneely and Lydon, 2000). Prolactin also acts directly on the mammary epithelium to induce lobule-alveolar development in pregnant animals (Brisken *et al.,* 1999). A prolactin receptor-knockout mouse model revealed that a functional prolactin receptor (PRLR) is required for mammary gland development and milk production during pregnancy (Kelly *et al.,* 2002).

2.2 Breast tumour

2.2.1 The site origin of breast tumour

Studies have shown that TDLU is the most naturally target site of malignancy in human breast (Russo *et al.,* 2005, Russo *et al,* 1992). The structure has been shown to have the highest proliferation index, concentration of estrogen receptors and number of blood vessels per lobular structure (Russo and Russo, 2004). The peak proliferation activities of TDLU are commonly associated with human development in the early adulthood. This supports the fact that the breast is more susceptible to carcinogen and prone to undergo neoplastic transformation at the early maturity phase.

In rats, the terminal end bud (TEB) is considered the structure comparable to TDLU. It has been found that the highest proliferation activity and rate of carcinogen binding to the DNA of TEBs are in pre-pubertal rats (Russo *et al.,* 1992). In addition, higher incidence of breast cancer is usually seen when rats are exposed to carcinogens at a young age. Thus, this may supports that the susceptibility on breast to be cancerous occurs at the early age of human development.

2.2.2 Breast tumour pathogenesis

The important feature of breast malignancy is the loss of tubular-alveolar pattern of the mammary gland. This pattern is usually maintained in benign tumours i.e. the adenomas and fibroadenomas. The characteristics of benign lesions in human and rat have been previously described (Thompson *et al.,* 2000). A wide spectrum of benign lesions has been observed in human while a very limited number of benign lesions are

seen in rats. The subtypes of benign lesions in rat model include fibroadenoma, mammary gland adenoma, papillary cyst adenoma, intraductal papilloma and epidermal inclusion cyst.

The histopathology characteristics that are used to sub-classify breast cancer into its categories depend on where in the glandular or ductal unit of the breast the cancer arises. Ductal carcinomas begin in the ducts, while lobular carcinomas have a pattern involving the lobules or glands. It is well understood that all invasive ductal and invasive lobular carcinomas originate either in the terminal duct or the lobule. From Russo *et al.* (2005), it is noticed that both invasive ductal and invasive lobular carcinomas originate from the intralobular ducts of the TDLU.

Cancer cells that remain confined to the lobule and the ducts are called 'in situ' or 'non-invasive'. They are usually referred to as pre-malignant lesions. This is due to the fact that cancer cells have not yet gained the ability to spread to other parts of the body. The types of premalignant lesions have been identified in human as well in animal models (Thompson and Singh, 2000, Thompson *et al.,* 2000). Higher occurrences of premalignant of breast lesions are found in animal model. The subtypes were cribriform and papillary DCIS (ductal carcinoma in situ) and hyperplasia of the epithelium which is also known by the term Intraductal Proliferation (IDP). The high incidence of pre-malignant lesions encourages researchers to use rat model in studying the mechanisms of breast tumourigenesis in early stages of development.

The premalignant lesions are precursors of invasive ductal or lobular breast tumours. The occurrence of premalignancy lesions is associated with the risk of

developing breast cancer (Page *et al.,* 2000). The increasing relative risk (RR) of breast cancer associated with such premalignant lesions has been found to correlate the increased proliferation and accumulation of genetic damage (Allred *et al.,* 2001, Allred and Mohsin, 2000). However, there are also tumours that arise from a site with no observed precursor lesion. Anderson and Miller supported this by reporting that invasive tumours are not always associated with local DCIS or LCIS (Anderson and Miller, 1994).

An invasive cancer is one in which the cells have moved outside the ducts and lobules into the surrounding breast tissue. This type of cancer is seen mostly in humans and has been classified into the 'special' and 'no special' type (NST). Invasive carcinoma of NST is the commonest type of breast cancer in human which accounts for up to 85% of cases. Similar findings were also observed in rats that exhibited high frequency of NST adenocarcinomas (Thompson *et al.,* 2000). Special types of carcinomas that have been observed in human are invasive lobular carcinoma, invasive tubular, cribriform, medullary and mucinous cancers. Many of the special type cancers have better prognosis, which in other words, gave a higher chance of survival among patients compared to the NST type. Majority of the invasive carcinoma subtypes that have been observed in rat model were cribriform, papillary and NST (Thompson *et al.,* 2000, Thompson *et al.*, 1995). Other subtypes of human breast carcinomas were uncommonly observed in rats.

2.3 Angiogenesis

2.3.1 Biological background

2.3.1.1 Mechanism of angiogenesis

Angiogenesis is a process of new blood vessel formation from existing vasculature (Folkman, 2003, Kerbel, 2000, Fokman, 1995). This phenomenon plays an important role in a variety of physiological and pathological processes. In physiological conditions, angiogenesis occurs in embryo development, wound healing and response to ovulation.

All tissues develop a vascular network that provide cells with nutrients and oxygen and enable them to eliminate metabolic wastes. This process is inert in normal tissues and becomes active in rapidly growing tissues. Angiogenesis requires cooperation and interaction between a variety of cells, growth factors, and components of the extracellular matrix. The angiogenic process is summarized as follows:

1. A cell that is activated by lack of oxygen releases angiogenic molecules. This event promotes the proliferation of inflammatory and endothelial cells.

2. During migration, inflammatory cells also secrete molecules that intensify the angiogenic stimuli.

3. Endothelial cells play a major role the formation of blood vessels. The cells responded to the angiogenic call by differentiating and secreting matrix metalloproteases (MMP). The MMPs digest the blood-vessel walls and enable them to escape and migrate towards the site of the angiogenic stimuli.

4 The digestion of blood vessel walls supports the proliferative and migratory activity of endothelial cells. Subsequently, the endothelial cells form a capillary tube by altering the arrangement of their adherence-membrane proteins.

5 Finally, the capillaries emanating from the arterioles and venules joined together. This process is defined as anastomosis which results in a continuous blood flow.

Angiogenesis exerts its normal physiological activity through a series of 'on' and 'off' regulatory switches (Pandya *et al.,* 2006). This angiogenic switches are maintained through a fine balance of angiogenesis modulators that induce the formation of blood vessels (the 'on' switches) and those that inhibit the process (the 'off' switches). When this balance is disturbed, it causes unrestrained blood-vessel formation in diseases that depends on angiogenesis. Downregulation of angiogenesis is seen in physiological diseases such as coronary artery disease (CAD), cardiac failure and tissue injury. Up regulation angiogenesis in turn is seen in some pathological conditions such as arthrosclerosis, diabetic retinopathy and cancer.

2.3.1.2 Measurement of blood vessel

Microvessel measurement is clinically important in cancer studies as well as in other angiogenic-dependent diseases. Studies of various tumours revealed the significant correlation of microvessel density (MVD) with tumour growth and metastasis (Uzzan *et al.,* 2004, Nisato *et al.,* 2003). MVD has been shown to be an important prognostic indicator in disease.

Recently, encourages researchers put their efforts to find ways to optimize the method measuring MVD. The most common counting technique that has been widely used is known as the 'hot-spot' technique (McGinley *et al.,* 2002, Kerbel, 2000). The technique focuses on quantifying blood vessels as a reflection of angiogenesis in highly stained regions. The immunohistochemistry staining method is used for this purpose. A variety of blood vessel endothelium markers for the identification of microvessels in histological sections have been developed for routine purposes. The markers include Factor VIII-related antigen (FVIII-RA), CD31 or CD34 (Uzzan *et al.,* 2004).

FVIII-RA has been identified as the coagulation protein expressed by the endothelium of the blood vessels (Folkman*,* 1995). This marker has been widely used in immunohistochemical study for endothelial vascular's staining. A study by Gasparini *et al.* (1995) has shown that high vessels count using FVIII-RA marker correlated well with the occurrence of nodal and distant metastases. Besides, microvessel counts stained by FVIII-RA are significantly related with stage II and stage III breast cancer (Bhatavdekar *et al.,* 2000). Other endothelial markers i.e, CD31 and CD34 are also commonly described in previous studies but both markers show no association with breast cancer prognosis. Goulding *et al.* (1995) showed no correlation between positive CD31 staining with lymph nodes status, recurrence, distant metastases or overall survival. Paradiso *et al.* (2001) observed no correlation between positive CD34 with clinical or pathological features of the breast tumours

2.3.2 Modulation of angiogenic activity

Angiogenesis is tightly controlled by a set of positive and negative modulators that are regulated in a fine balance condition. These molecules are produced by stromal and infiltrating tumour cells (Carmelliet and Jain, 2000). A study by Relf *et al.,* (1997) noted that tumour cells express a variety of angiogenic modulators which relative production varies with time.

Two important positive modulators that have been described are basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF). The angiogenic activities of these molecules are found to be in synergistic manner. The angiogenic effects of these positive modulators are counterbalanced by inhibitory molecules such as angiostatin, endostatin, thrombospondin-1 (TSP-1), the 16-kD human prolactin fragment (16-kD human PRL), or platelet factor 4 (PF4). Table 2.1 shows multiple angiogenic factors and anti-angiogenic factors listed by Folkman, 1995.

Table 2.1 List of angiogenic factors and anti-angiogenic factors (Fokman, 1995).

Endogenous angiogenic factors	Endogenous negative regulators of endothelial-cell proliferation
Basic Fibroblast Growth factors	Platelet factor 4
Acidic Fibroblast Growth factors	Thrombospondin-1
Transforming growth factor-alpha	Tissue inhibitors of metalloproteinase (TIMP)-1
Transforming growth factor-beta	TIMP-2
Vascular endothelial growth factor	TIMP-3
Platelet-derived endothelial cell growth factor	Prolactin (16 kd fragment)
Granulocyte colony-stimulating factor	Angiostatin (38-kd fragment of plasminogen
Placental growth factor	bFGF soluble receptor
Interleukin-8	Transforming growth factor-beta
Hepatocyte growth factor	Interferon Alfa
Proliferin	Placental proliferin-related protein

2.3.2.1 bFGF as the angiogenic promoter

The most potent known angiogenic peptide is the bFGF (Folkman, 1995). This important pro-angiogenic factor has been found to be produced by tumour, stromal and endothelial cells. The factor stimulates endothelial proliferation, protease production, chemotaxis, and vascular tube formation (Gross *et al.,* 1993).

The role of bFGF in tumour angiogenesis has been extensively studied. It is suggested that triggering angiogenesis switch through upregulating pro-angiogenic gene and downregulating anti-angiogenic gene expression is one of the major mechanisms of bFGF-induced angiogenesis (Yue *et al.,* 2006). Compagni *et al.* (2000) used the AdsFGFR (the recombinant adenovirus that expressing soluble fibroblast growth factor (FGF) receptor) to assess the FGF functions in tumour angiogenesis. They found that FGF receptors i.e. FGF-1 and FGF-2 exert highly pleiotropic functions during multiple stages of tumour development. Giavazzi *et al.,* (2001) found that the expression of bFGF affects the initial growth and neovascularization of HEC-1-B human endometrial adenocarcinoma in nude mice. In addition, bFGF has been found to stimulate tumour vascularization in a synergistic action with VEGF although both growth factors are found to have separable role on vessel formation and tumour growth (Giavazzi *et al.,* 2003)

It is known that bFGF is a strong mitogen and chemotatic factor for vascular endothelial cells. During wound healing of normal tissues and in tumor development, the action of heparin sulfate proteoglycans (HSPGs) activates bFGF, thus mediating the formation of new blood vessels. HSPGs modulate the binding of bFGF and VEGF to their respective receptors. A study by Javerzat *et al.* (2002) noted that the HSPGs

stabilize the bFGF/FGF receptor complex, protect bFGF from degradation and facilitate bFGF dimerization. They proposed that inhibitors may possibly disturb the bFGF activity in two ways; either by blocking the interaction of bFGF with its receptors or by disrupting the by-stander effect of HSPGs for efficient growth factor binding.

It has been discovered that the anti-angiogenic factor acts directly against the action of bFGF. It is known that PF4 complexes with bFGF and inhibits heparin-induced bFGF dimerization (Perollet *et al.,* 1998). Hegedorn *et al.* (2001) had characterized a peptide from C-terminus of PF4 which is thought to inhibit the angiogenic activity induced by bFGF.

2.3.2.2 Platelet factor 4 (PF4) as the angiogenic inhibitor

PF4 is an anti-angiogenic factor that inhibits angiogenesis and growth factor-stimulated endothelial cell proliferation (Raouva *et al.,* 2006). This blood coagulation factor is synthesized by megakaryocytes and seized normally in platelets. PF4 exerts the anti-angiogenic properties by binding to heparin and thereby neutralized the heparin activity. It is established that PF4/heparin complexes are highly immunogenic and obtain self-reacting anti-PF4/heparin antibodies in a T cell-dependent manner (Survana *et al.,* 2005).

It has been noted that PF4 regulates angiogenesis in a negative manner by interfering bFGF and VEGF activity (Bikfalvi, 2004, Bikfalvi and Bicknell, 2002). The anti-angiogenic properties of PF4 were also described by Perrolet *et al.* (1998), where

they found that PF4 inhibited the bFGF-dimer formation or heparin-induced bFGF dimer formation.

The PF4 displays an anti-angiogenic activity *in vivo* and inhibits tumour growth without affecting the proliferation rate of the cancerous cells (Yamaguchi *et al.,* 2005). Li *et al.* (2003) found that viral vector-mediated cDNA transfer of PF4 resulted in inhibition of solid tumors through anti-angiogenic action *in vivo*.

Based on those facts, it can be seen why PF4 is an important angiogenesis inhibitor *in vitro* and *in vivo* models. PF4 may also be developed into a therapeutic agent. One example is the study on C-terminal fragment of PF4. This peptide has been noted to inhibit angiogenic events induced by bFGF (Hegedorn *et al.,* 2001).

2.3.3 Tumour angiogenesis

Angiogenesis performs a critical role in the development of cancer (Pandya *et al.,* 2006, Carmeliet and Jain, 2000). The development of new blood vessels is necessary both at the beginning and at the end of distant metastasis development. It has been found that solid tumors could potentially survive without stimulating angiogenesis. However, this activity is very limited to tumour size smaller than 1 to 2 cubic millimeters (Kerbel, 2000, Folkman, 1995). For further growth and spread, tumour cells need blood supply that will bring oxygen and nutrients and remove metabolic wastes. Oxygen and nutrients have difficulty diffusing into cells at the center of the tumor when the tumour has reached beyond the critical volume of 2 cubic millimeters. This causes a state of cellular hypoxia

that marks the onset of tumor angiogenesis. The latter mechanism describes the growth of blood supply from existing vessels were induced by the tumour itself.

Albo *et al.* (2004) summarize four potential mechanisms by which tumours can stimulate angiogenesis:

1 Tumours stimulate the sprouting of new blood vessels by secreting pro-angiogenic factors (Folkman, 2003). Those pro-angiogenic factors are vascular endothelial growth factor (VEGF), basic fibroblast growth factor, transforming growth factor beta and others.

2 Tumour angiogenesis is stimulated by co-opting the existing vasculature. This is evidenced in Holash study, as they found that the angiogenic antagonist angiopoietin-2 and pro-angiogenic vascular endothelial growth factor (VEGF) play critical role of regulators of balance between vascular regression and growth (Holash *et al.,* 1999).

3 Angiogenesis is said to be contributed by circulating hematopoietic precursor cells from bone marrow (Lyden *et al.,* 2001). The EC (endothelial cell) progenitors can supply the growth of blood vessels to ischemic areas and can deliver the anti- or pro-angiogenic agents to unrestrained angiogenesis sites.

4 The fourth potential mechanism is 'vascular-mimicry' process. The process

involves the formation of vasculogenic-like networks by the tumour cells with simultaneous expression of vascular-associated cell markers (Sood *et al.,* 2002).

Understanding angiogenesis and its unique characteristics in tumour growth has provided insights into a number of ways to interrupt this process. Therapies targeting tumour angiogenesis are rapidly emerging and hold great promise. As a result, a number of anti-angiogenic agents are currently in pre-clinical and clinical studies. Thalidomide, endostatin, the anti-VEGF receptor SU5416 and recombinant humanized anti-VEGF monoclonal antibody are among the compounds for anti-angiogenic therapy (Gasparini *et al.,* 2001, Eder *et al.,* 2000, Mendel *et al.,* 2000, Sledge, 2000). The therapy shows great potential when combine with chemotherapy and radiation therapy.

2.3.3.1 Breast tumour angiogenesis

The importance of angiogenesis in the growth of human breast cancer and their value as a marker of metastasis potential of tumour are well emphasized in previous study (Folkman, 2003). Weidner *et al.* (1991) demonstrated the significance correlation between tumour MVD in invasive breast cancer and the presence of axillary lymph nodes and distant metastases. This finding has also been confirmed by several experimental studies (Nisato *et al.,* 2003, Gasparini and Harris, 1995, Gasparini *et al.,* 1995, Weidner *et al.,* 1992, Bosari *et al.,* 1992, Liotta *et al.,* 1991). Increased MVD is also associated with the transition from low grade to high grade in situ lesions (Engel *et al.,* 1997).

It has been suggested that tumour MVD in breast cancer is an independent prognostic factor for disease free and overall survival. However, there appears to be arguments on assessing MVD in breast cancer patients due to the considerable variation in nodal status, duration of follow-up as well as the methods and criteria used for counting MVD (Gasparini *et al.,* 1995, Simpson and Page, 1995).

Previous studies have also shown the association between the tumour MVD and preinvasive lesions of the breast (Thompson *et al*., 2002, Vartanian and Weidner, 1995). The angiogenesis in *in situ* stage differs from that of invasive tumours. It was observed that there were increased blood vessels in the normal stroma around and beneath the in situ composition of tumour cells (Mc Ginley *et al.,* 2002). This was in contrast to those seen in tumour cells of the invasive phase. The structure found in breast *in situ* tumours suggests that the angiogenic factors are strongly influenced the induction of tumour growth at the early steps. It is believed that those angiogenic factors are produced by the inflammatory and stromal cells rather than those produced from tumour cells themselves (Rosen, 2002, Linderholm *et al.,* 2000).