ASSESSMENT OF PROLIFERATION RATE IN BREAST CANCER BY KI-67 LABELING INDEX

BY

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‘In the name of Allah, the Most Gracious and the Most Merciful’

All praise and gratitude is to Allah, the Lord to whom every single creature in the heaven and the earth belongs to. Thank Allah for giving me the strength and patience during this challenging time. May peace and blessings be on the leader of all creation, the prophet Muhammad S.A.W, his family, and companions.

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CERTIFICATE

This is to certify that the dissertation entitled "Assessment of Proliferation Rate in Breast Cancer by Ki-67 Labeling Index" is the bona fide record of research work done by Mr Ibrahim Bin Hussin Khan during the period from September 2016 to June 2017 under my supervision.

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ABSTRAK

Pengenalan: Kanser payudara adalah kanser yang paling biasa di kalangan wanita. Salah satu daripada ciri-ciri kanser payudara adalah kaitannya dengan potensi untuk replikasi tanpa had oleh sel-sel malignan. Proliferasi selular ini boleh diukur dengan melihat indeks pelabelan Ki-67, yang merupakan jenis biomarker percambahan. Kajian ini bertujuan untuk membandingkan indeks pelabelan Ki-67 antara manual dan kaedah pengiraan automatik dengan pelbagai nilai pemotongan.

Metodologi: Lapan puluh lapan kanser payudara yang memenuhi kriteria pemasukan dari tahun 2014 hingga 2016 telah direkrut dalam kajian ini. Data klinikopatologi telah diekstrak daripada laporan patologi. Imunohistokimia untuk Ki-67 telah dilakukan pada bahagian-bahagian tisu. Pengiraan manual dan automatik menggunakan perisian ImmunoRatio telah dilakukan untuk menilai indeks pelabelan Ki-67. Dua titik pemotongan yang berbeza, iaitu <14% dan <20% dipilih.

Keputusan: Kebanyakan pesakit yang didiagnosis berumur lebih daripada 40 tahun dan majoriti adalah orang Melayu. Indeks pelabelan ki-67 dengan pengiraan manual dikaitkan dengan kaedah automatik ($r = 0.99$; $p < 0.001$). Apabila mengubahkan nilai pemotongan dari <14% kepada <20%, berbanding dengan kiraan manual, kaedah automatik menghasilkan lebih banyak kanser payudara berisiko Luminal B yang diklasifikasikan semula ke dalam kanser payudara Luminal A. Menggunakan <20% sebagai titik pemotongan, 3 kes yang dinilai sebagai> 20% oleh pengiraan manual diturunkan sebagai <20% oleh kaedah automatik.

Kesimpulan: Kaedah automatik boleh dipercayai dalam menilai kadar percambahan dibandingkan dengan kaedah manual, walaupun ada beberapa kes memperlihatkan perbezaan antara kedua-dua kaedah tersebut

ABSTRACT

Introduction: Breast cancer is the commonest cancer in women. One of the characteristics of breast cancer is its association with the limitless replicative potential of the malignant cells. This cellular proliferation can be gauged by looking at Ki-67 labelling index, which is a type of proliferation biomarker. This study aims to compare the Ki-67 labelling index between the manual and the automated counting methods with various cut-off values.

Methods: Eighty-eight breast cancers that met the inclusion criteria from year 2014 to 2016 were recruited in this study. Clinicopathological data were extracted from the pathology reports. Immunohistochemistry for Ki-67 was performed on tissue sections. Manual counting and automated counting using ImmunoRatio software were performed to assess the Ki-67 labelling index. Two different cut-off points, i.e. <14% and <20% were addressed.

Results: Most of the patients diagnosed were more than 40 years old and majority were Malays. Ki-67 labelling index by manual counting correlated with automated method ($r=0.99$; $p<0.001$). When shifting the cut-off value from <14% to <20%, as compared to manual count, automated method resulted in a larger number of Luminal B breast cancers re-classified into Luminal A breast cancers. Using <20% as cut-off point, 3 cases that were assessed as >20% by manual counting was downgraded as <20% by automated method.

Conclusion: The automated method is reliable in assessing proliferation rate as compared to the manual method, although a minority of cases showed discrepancy between these two methods

CHAPTER 1

1.0 INTRODUCTION

Breast cancer is a type of cancer that develops from breast tissue with several symptoms, which may include a breast lump, deformation of breast shape, skin dimpling, nipple discharge, or erythematous skin changes (Rathnam, 2012). According to MALAYSIAN NATIONAL CANCER REGISTRY REPORT 2007-2011 (MNCRR), a total of 64,275 cancer deaths, either medically certified or non-medically certified, were reported by the National Registration Department in the period of year 2007 to 2011; breast cancer emerged as the most common cancer among female.

Enabling replicative immortality is one of the hallmarks of cancer; it enables the cell to proliferate uncontrollably without any limit. In breast cancer, accumulation of mutations in various genes that control cell proliferation ultimately leads to cell growth and cell division. The resultant cells will no longer respond to the many inhibitory signals for cellular growth and death (Fagagna, 2007). To glimpse into this hallmark of cancer, proliferation rate of breast cancer can be measured via an immunohistochemical staining method to measure the percentage of tumor cells that are positive for Ki-67. Ki-67 is a nuclear protein and represents cellular marker for proliferation: this protein increases as the cells prepare to divide. This protein exits in all active phases of cell cycle (G1, S, G2, and mitosis), however, is not detected from the resting cells (G0) (Ronell Bologna-Molina, 2013).

Measurement of proliferation rate by Ki-67 labeling index can be performed via visual counting of positively stained nuclei under microscope. Recently, image analyzed Ki-67 assessment has also been reported in several studies (Fangfang Zhong, 2016) (Gustav Stålhammar, 2016). Therefore, this study is conducted to compare these two methods of assessment of Ki-67 labelling index, and to assess how the results would impact on further classification of breast cancer in term of molecular subtypes, which would have different prognostic significance.

CHAPTER 2

2.0 LITERATURE REVIEW

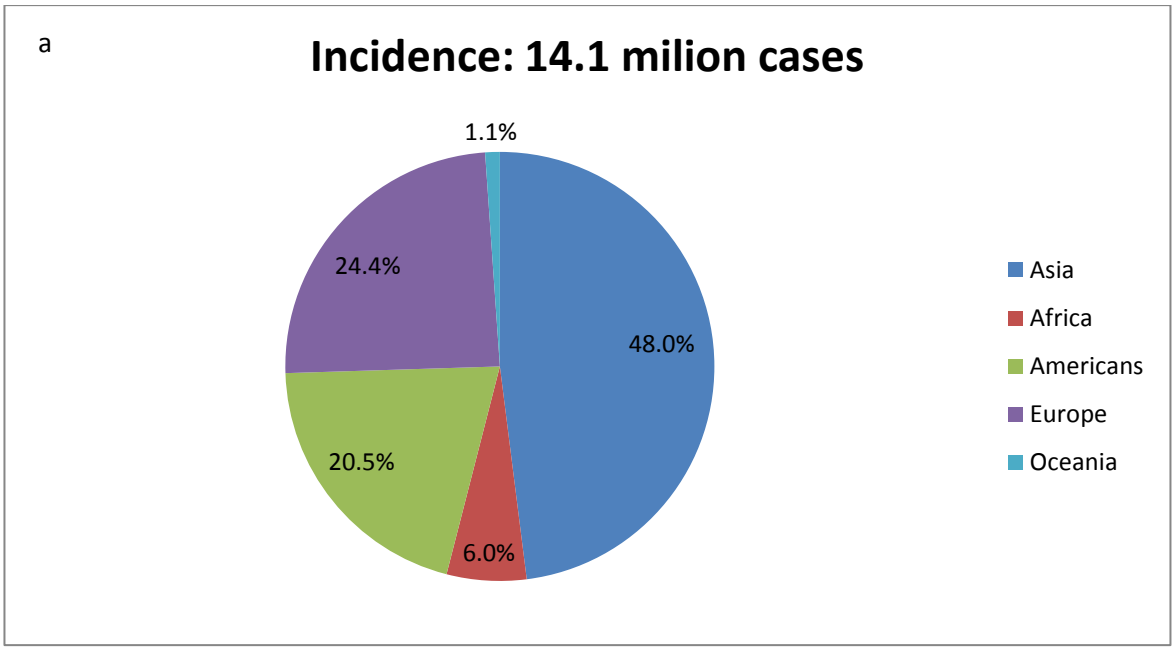
2.1 Breast Cancer

Breast cancer is a type of cancer that develops from breast tissue with several symptoms, which may include a breast lump, deformation of breast shape, skin dimpling, nipple discharge, or erythematous skin changes (Rathnam, 2012). Of those with distant spread, the patients may suffer from bone pain, lymphadenopathy, shortness of breath, or jaundice (Christobel Saunders, 2009). According to world cancer report 2014, risk factors for developing breast cancer are many; well established risk factors include being female, obesity, alcohol consumption, lack of physical exercise, hormone replacement therapy, past exposure to radiation, early age at menarche, having children late or nulliparous, older age, and a past family history of breast cancer (Breast Cancer, 2014). Breast cancer usually refers to malignant transformation of epithelial cells of the lobules and ducts of milk-producing glands and associated passages draining the milk to the nipple; at a much rarer rate, malignant transformation of the stroma tissues such as fatty and fibrous connective tissues of the breast also occurs. (K. Narayanasamy, 2014).

2.2 Epidemiology of Breast Cancer

K McPherson et al. (2000) reported that breast cancer is the commonest cancer in women, comprising 18% of all female cancers (K McPherson, 2000). According to GLOBOCAN 2012, 14.1 million new cancer cases and 8.2 million cancer deaths were estimated in 2012 worldwide (Figure 2.1a and 2.1b), whereby breast cancer appears as the second most common cancer in the world. It is also the most frequent cancer among women; in 2012, an estimated 1.67 million new cancer cases were diagnosed, which comprised about 25% of all cancers. It is the most common cancer diagnosed in both developing and developed regions (Figure 2.2a and 2.2b); more cases were documented in less developed (883,000 cases) than in more developed regions (794,000) (Jacques Ferlay, 2015).

In Malaysia, the census population of Peninsular Malaysia in 2016 was 31.7 million. A total number of 103,507 new cancer cases were diagnosed in Malaysia during the period of year 2007 to 2011 (Table 2.1) whereby 46,794 (45.2%) occurred in males and 56,713 (54.8%) in females (Azizah Ab Manan, 2015). According to MALAYSIAN NATIONAL CANCER REGISTRY REPORT 2007-2011 (MNCRR), a total of 64,275 cancer deaths, either medically certified or non-medically certified, were reported by the National Registration Department of in the period of year 2007 to 2011; breast cancer was the most common cancer among female, recording 18,206 (32.1%) cases (Table 2.2); it was also the most common cancer in the population regardless of sex in Malaysia (Azizah Ab Manan, 2015).



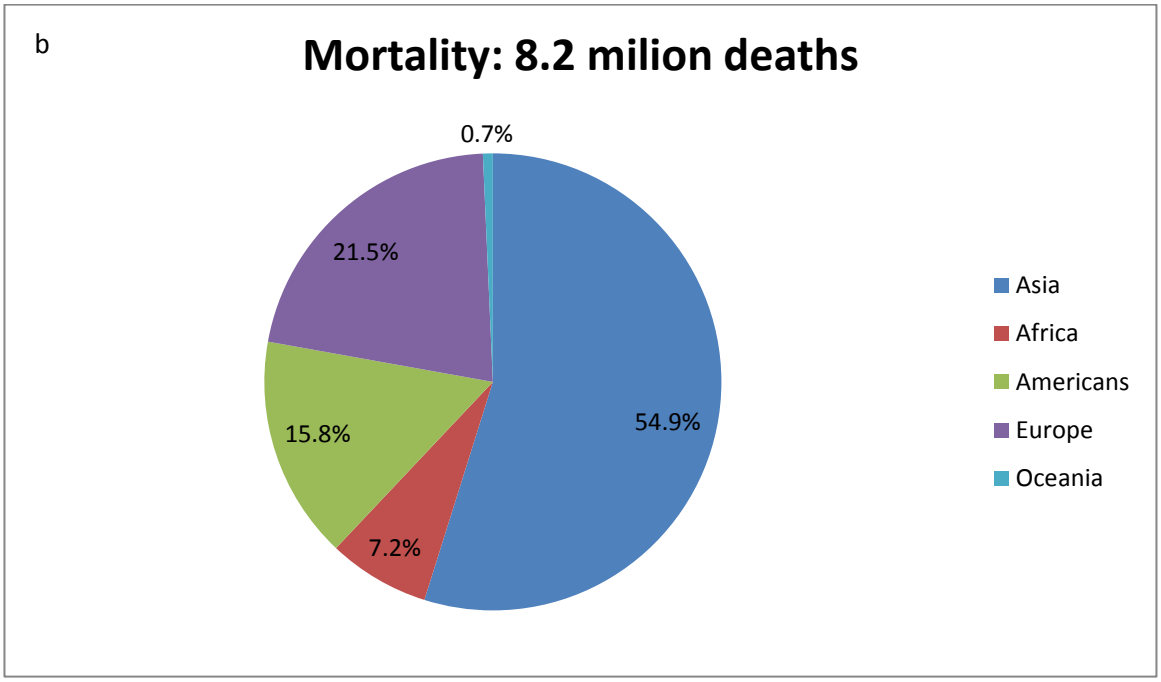
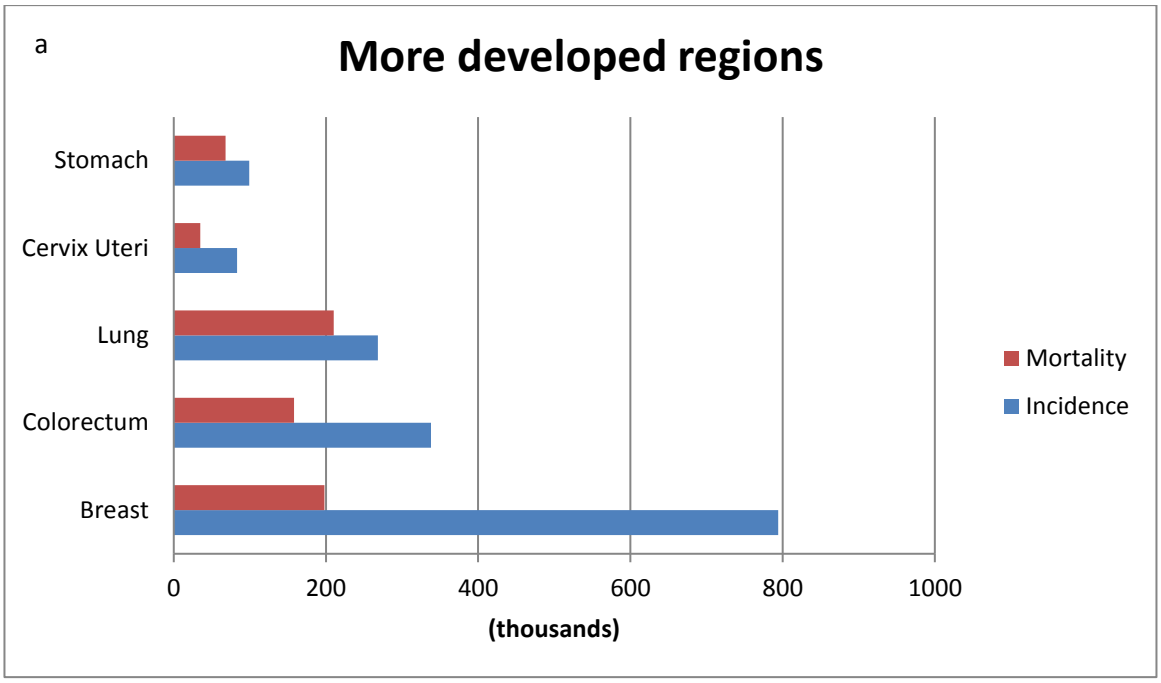


Figure 2.1a and 2.2b: Estimated new cases and deaths worldwide with proportions by world regions for both sexes, 2012. The area of the pie is proportional to the number of new cases or deaths. Adapted from GLOBOCAN 2012



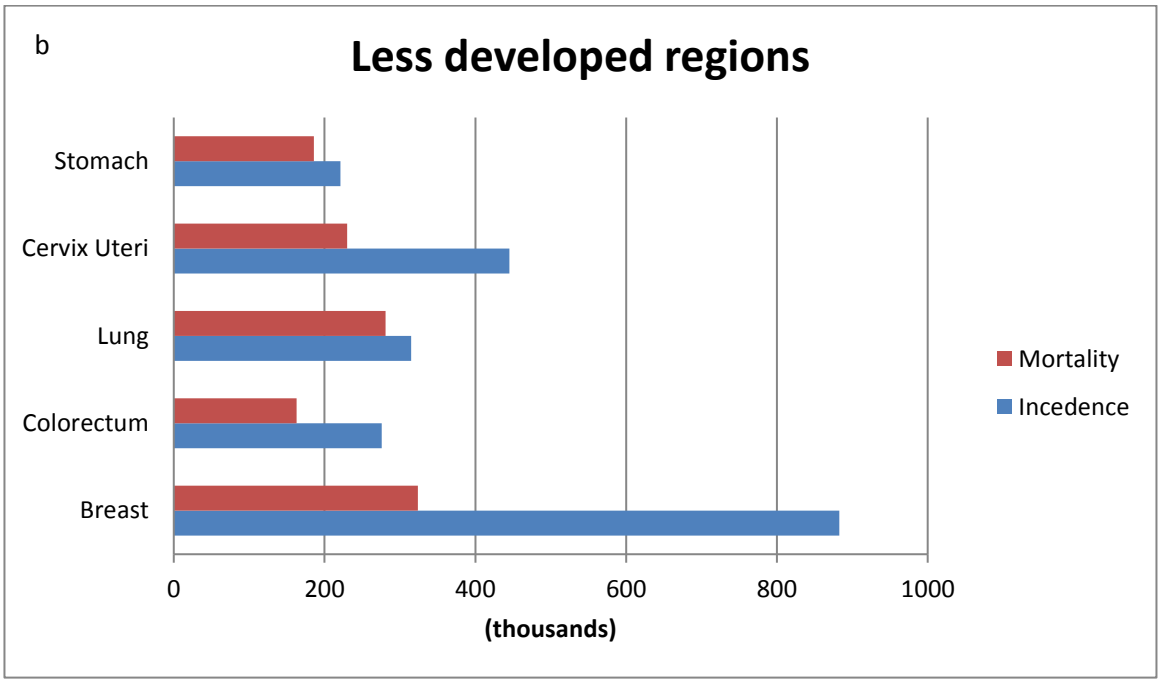


Figure 2.2a and 2.2b: Estimated new cancer cases (incidence) and deaths (mortality) in thousands in more developed and less developed regions of the world among females in 2012. Adapted from GLOBOCAN 2012

Table 2.1: Ten most common cancers among all residents in Malaysia, 2007-2011. Adapted from MNCRR

Icd-10	Sites	Number	%
C50	Breast	18,343	17.7
C18-C21	Colorectal	13,693	13.2
C33-C34	Trachea, Bronchus, Lung	10,608	10.2
C81-C85, C96	Lymphoma	5,374	5.2
C11	Nasopharynx	5,090	4.9
C91-C95	Leukaemia	4,573	4.4
C53	Cervix Uteri	4,352	4.2
C22	Liver	4,128	4.0
C56	Ovary	3,472	3.4
C16	Stomach	3,461	3.3
	Others	30,413	29.4
	Total	**103,507**	**100.0**

Table 2.2: Ten most common cancers in Malaysia among female residents, 2007-2011. Adapted from MNCRR

Icd-10	Sites	Number	%	CR	ASR
C50	Breast	18,206	32.1	28.6	31.1
C18-C21	Colorectal	6,047	10.7	9.5	11.1
C53	Cervix uteri	43,52	7.7	6.8	7.6
C56	ovary	3,472	6.1	5.4	5.9
C33-c34	Trachea, bronchus, lung	3,193	5.6	5.0	6.0
C81-c85, c96	Lymphoma	2,203	3.9	3.5	3.8
C54	Corpus Uteri	2,181	3.8	3.4	3.8
C91-95	Leukaemia	2,024	3.6	3.2	3.4
C73	thyroid	1,723	3.0	2.7	2.9
C16	Stomach	1,447	2.6	2.3	2.6
	Others	11,865	20.9		
	Total	**56,713**	**100.0**	**89.0**	**99.3**

2.2 The Hallmarks of Cancer

In early 2000, Douglas Hanahan and Robert A. Weinberg proposed the concept - the hallmarks of cancer - that comprises six biological capabilities, which are acquired during the multistep carcinogenesis. This proposal constitutes an organized principle to reasonably understand the complexities of neoplastic disease (Figure 2.3). The hallmarks of cancer are summarized as (i) sustaining proliferative signaling, (ii)evading growth suppressors, (iii) resisting cell death, (iv)enabling replicative immortality, (v)inducing angiogenesis, and (vi) activating invasion and metastasis, which are ubiquitous in most and perhaps all types of human tumors (Douglas Hanahan, 2011). Later in 2011, an updated concept by Douglas Hanahan and Robert A. Weinberg (Hallmarks of Cancer: The Next Generation) proposed four additional hallmarks of cancer (Figure 2.4). These four hallmarks are recognized as (vii) deregulation cellular energetics, (viii) avoiding immune destruction, (ix) genome instability and mutation, and (x) tumor-promoting inflammation (Douglas Hanahan, 2011).

Figure 2.3: The hallmarks of cancer: six biological acquired capabilities of cancer as originally proposed. Adapted from (Douglas Hanahan, 2011).

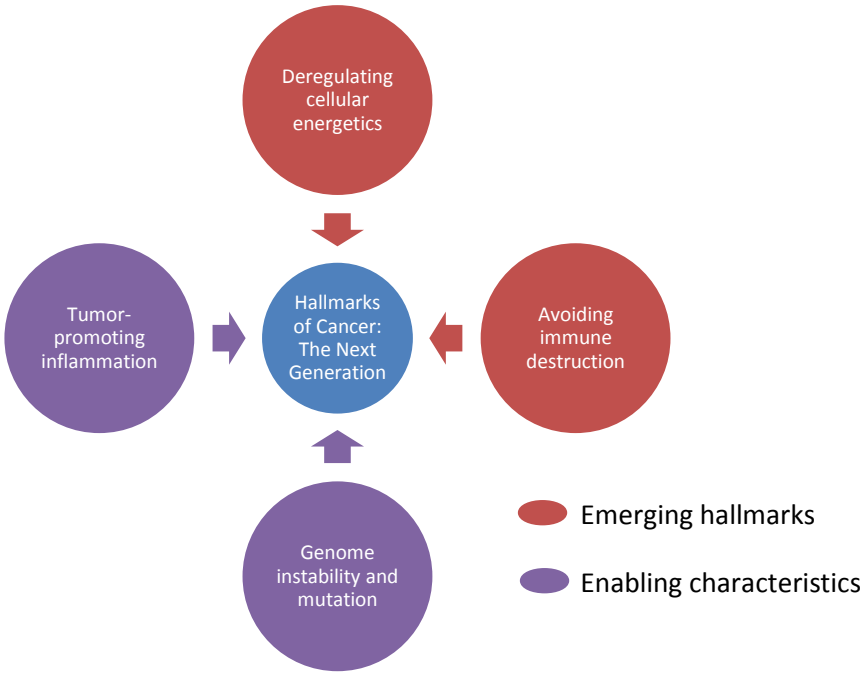


Figure 2.4: The four additional hallmarks of cancer: the emerging hallmarks and enabling characteristics. Adapted from (Douglas Hanahan, 2011).

2.3 Enabling Replicative Immortality

Among the hallmarks of cancer, enabling replicative immortality is probably the most important hallmark. Hayflick in the early works has shown that cells in culture have a finite replicative potential (Hayflick, 1997). After progression through a certain number of doublings, such cell populations will stop growing. This process has been termed as senescence. In cultured human fibroblasts, the process of senescence can be circumvented by inactivation of their pRb and p53 tumor suppressor proteins; this will enable these cells to continue to multiply for additional generations; they will then enter into a second state called crisis (Douglas Hanahan, 2000). According to Wright et al., the crisis state is characterized by massive cell death, karyotypic disarray that is associated with end-to-end chromosomal fusion, and the occasional emergence of an advantageous variant in the rate of 1 in 10^7 cells. This variant of cells has acquired the ability to multiply without limit, the trait termed as immortalization (Wright, 1989).

Hayflick articulated that most types of tumor cells that are propagated in culture appear to be immortalized. This immediately implies that during tumor progression, limitless replicative potential is a trait that is acquired in vivo, thus is basically essential for the development of malignancy. (Hayflick, 1997). Multiple lines of evidence indicate that the capability for unlimited proliferation is made possible by telomeres that protect the ends of chromosomes (Blasco, 2005) (Shay, 2000).

Telomeres are located the end of chromosomes that have a special structure. They protect the ends of chromosomes from fusion, recombination, or being recognized as

uncapped DNA breaks (Harley, 1991) (Blackburn, 2001). However, with each cycle of cell division, telomeres shorten due to the end replication problem in linear chromosomal DNA. Critically shortened telomeres fail to protect the chromosome ends. This will trigger the DNA damage response leading to cellular senescence or apoptosis (Kyotaro Hirashima, 2013). In tumor cells, this problem is resolved as there is a mechanism whereby reactivation of the reverse transcriptase termed telomerase could stabilize the telomere length. Telomerase is unique in structure as it is composed of a RNA component (hTR or hTERC) (Kelland, 2007) and a catalytic protein (hTERT) (Blackburn EH, 2006). In addition, human telomeric DNA is associated with a group of proteins (at least six proteins to date), collectively termed shelterin. TRF1 and TRF2 of shelterin bind to double-stranded telomeric DNA; POT1 in shelterin binds the single-stranded 3′ G-rich overhang; three interconnecting proteins (TIN2, TPP1, and RAP1) act to shape and safeguard telomeres (Wang F, 2007).

2.4 Ki-67

Related to this hallmark of enabling replicative immortality, cell proliferation is one of cellular aspect that could be easily observed and measured. In cancer, cell proliferation constitutes one of the most important prognostic factors and it relates to overall patient survival (Aleskandarany MA, 2010) (Dai H, 2005). There are a number of biomarkers associated with proliferation; Ki-67 is regarded as the most suitable candidate for breast cancer research as this protein is expressed in all the phases of the cell cycle except G0,

either in normal or malignant cells. Ki-67 assessment is an easy and reliable method of assessing the cell cycle pathway (Rumiko Tashima, 2015). Ki-67 has been identified as a nuclear antigen associated with cell proliferation since 1983 (Gerdes J, 1983), and considerable enthusiasm has been poured to employ Ki-67 assessment in studies. Although the protein's structure and properties are now greatly elucidated, its functional role remains elusive (Xue-Qin Yang, 2011).

2.5 Molecular Subtypes of Breast Cancer

With the advent of genomic research, breast cancers have been discovered to constitute several distinct diseases based on gene expression profiling, termed as molecular subtypes of breast cancer. Using a number of immunohistochemically available surrogate markers, these molecular subtypes could be considerably reproduced without more sophisticated gene expression profiling. Cheang et al. classified breast cancers into Luminal A, Luminal B, Her-2-overespressed, and triple negative breast cancer by employing a panel of biomarkers comprised of estrogen receptor, progesterone receptor, Her-2, and Ki-67 labelling index. They proposed a 14% cutoff value for Ki-67 labelling index. Hormone receptor–positive breast cancers having Ki-67 < 14% are classified as Luminal A and those with ≥ 14% as Luminal B breast cancer. This results in comparable molecular subtypes with gene expression profiling method (Cheang MC, 2009). In addition, baseline Ki-67 labelling index has been found to be higher in triple negative breast cancers that do not express ER, PR and HER2 (Bhumsuk Keam, 2011), whereas

ER positive and/or PR positive breast cancers have a lower Ki-67 labelling index (Trihia H, 2003). Furthermore, in the 2011 and in the 2013 St Gallen Consensus Conference, Ki-67 labeling index was recommended for the determination of proliferation. This piece of information forms the criterion to differentiate Luminal A and Luminal B breast cancers (Gnant M, 2011) as pioneered by Perou et al.; the study by Perou et al. was the first in demonstrating the presence of so-called intrinsic molecular breast cancer subtypes (Perou CM, 2000). Table 2.3 shows breast cancer subtypes as recommended by the panel of experts at the St Gallen Consensus (Goldhirsch A, 2013).

Table 2.3: Breast cancer subtypes. Source: (Goldhirsch A, 2013).

Breast cancer	**Definition**
Luminal A	ER positive, PR positive, HER2 negative Ki-67 index low (defined as <14%
Luminal B	Luminal B (HER2 negative) ER positive, HER2 negative and one of: Ki-67 index high (defined as 14% and above) PR negative or low (defined as <20%) Luminal B (HER2 positive) ER positive, HER2 over-expressed or amplified, any ki-67 index, any PR
HER2 over-expressing	HER2 over-expressed or amplified, ER and PR negative
Triple negative	ER and PR negative, HER2 negative

2.6 Assessment of Ki-67

The fundamental of assessment of Ki-67 labelling index is estimation of proportion of positively-stained tumor cell nuclei for Ki-67 protein by immunohistochemistry. Compared to other markers of proliferation, Ki-67 labelling index as such measured is accurate, easy and cost-efficient; its staining and assessment are consistent. Therefore Ki-67 labeling index is deemed an ideal diagnostic tool (Urruticoechea A, 2005). In 2011, the International Ki-67 in Breast Cancer Working Group proposed guidelines for the analysis, reporting, and use of Ki-67. These guidelines aimed to reduce inter-laboratory variability; this would lead to improvement of inter-study comparability of Ki-67 results; however, firm recommendations for best practice could not be put forward due to limited evidence (Mitch Dowsett, 2011).

Visual assessment (VA) at a glance has been widely championed to evaluate Ki-67 labeling index in a considerable number of pathological institutions and laboratories due to its ease; nonetheless there are limitations, for instance, the reproducibility of intermediate Ki-67 labelling index is relatively poor. Of importance, Ki-67 labelling index in such situation, especially in the moderately differentiated (tumor grade 2) breast cancers, is critical for clinical decisions (Vörös A, 2013) (Gudlaugsson E, 2012).

To partly address this problem of reproducibility, recently, image analysis techniques have been introduced; this offers the potential for automated assessment and also better

precision in assessment. Heterogeneity in breast cancer remains one of the unsolved issues in this kind of assessment (Urruticoechea A, 2005).

2.7 Ki-67 labeling index: technicalities

Chung YR et al. in the article 'Interobserver Variability of Ki-67 Measurement in Breast Cancer' has rightly pointed out several methodological issues concerning Ki-67 labeling index interpretation that lead to variability in its measurement (Yul Ri Chung, 2016).

Firstly, the assessment is observer-dependent; the areas selected for counting and the number of tumor cells counted may be different for each observer. Secondly, it is the intrinsic nature of cancer that the breast cancers may exhibit intra-tumoral heterogeneity in cell proliferation with areas showing more intense staining of Ki-67; these intensely stained areas are known as "hot spots". Thirdly, manual counting or digital image analysis of Ki-67 labelling index depends on institutional preference. Lastly, Ki-67 labelling index maybe reported in continuous numbers whereas others may record this index in categorical values with various cutoff values (Yul Ri Chung, 2016). Acknowledging these technicalities, this study aims to address the issue of manual count versus automated count methods. Different cutoff values will be tested to assess the impact of Luminal breast cancer subtyping.

CHAPTER 3

3.0 OBJECTIVE OF STUDY

3.1 General Objective

A retrospective study of a center experience in assessing proliferation index of breast cancer by Ki-67 labeling index

3.2 Specific Objective

The specific objectives of this study are

a) To assess Ki-67 labeling index by (i) manual counting and (ii) automated counting by image analysis software (ImmunoRatio)
b) To compare the results of Ki-67 labeling index between these two methods.
c) To assess the molecular subtyping of breast cancer based on ER, PR, Her-2, CK5/6 and Ki-67 labeling index obtained from these two methods.

CHAPTER 4

4.0 METHODOLOGY

4.1 Study Design and Ethical Approval

This was a retrospective study with review of pathology reports and histopathology slides from the year 2014 to 2016 for breast cancer cases reported in Advanced Medical and Dental Institute (AMDI), Universiti Sains Malaysia. Ethical approval was obtained prior to commencement of this study from Human Research Ethics Committee, USM (HREC) with the approved study protocol code: USM/JEPeM/16120538.

4.2 Study Sample

Archived histopathology slides and corresponding paraffinized tissue blocks from breast cancer mastectomy specimens in the Histopathology and Cytology unit, Advanced Diagnostic Laboratory, Advanced Medical and Dental Institute (AMDI), Universiti Sains Malaysia, from the year 2014 to 2016 were retrieved for this study. The specimens had been examined by pathologists of Histopathology and Cytology unit and formal pathology reports had been issued within the stated period.

4.3 Sample Size

This was a pilot study. Eighty-eight cases were obtained from archive that met the following inclusion criteria.

4.4 Inclusion Criteria

All invasive breast cancer surgical specimens (lumpectomy, wide local excision or mastectomy) for the year 2014 to 2016 were included in this study.

4.5 Exclusion Criteria

Cases that fulfilled the inclusion criteria but the histopathology slides and/or corresponding paraffinized tissue blocks that were unavailable, missing, or inadequate for serial sections were excluded from this study. Pure in situ breast cancer was also excluded.

4.6 Retrieval of Pathology Report and Clinicopathological Data

Clinicopathological data of interest for each case were retrieved from archived formal pathology reports. The data of interest were age, gender, laterality, tumor grade, mitotic rate/10HPF, presence of lymphovascular invasion, tumor pathological stage, pathological stage of lymph node metastasis, tumor size, number of nodal metastasis, number of retrieved lymph nodes, presence of ductal carcinoma in situ (DCIS) component, estrogen and progesterone receptor status, Her-2 expression status, CK 5/6 status and Ki-67 labeling index (manual counting).

4.7 Patient Confidentiality

In order to protect patients' confidentiality, tissue block/slide and clinicopathological data retrieved from each case remained confidential by masking the personal data with the laboratory numbers. Data analysis was referred solely to the laboratory numbers.

4.8 Immunohistochemistry for Ki-67

To obtain Ki-67 labeling index, a section from each case was immunostained with antibody against Ki-67 protein. The immunohistochemistry for Ki-67 is outlined as follows:

(a) Dewax and Hydrate: After tissue was sectioned and mounted, it was placed on hot plate for at least 60 minutes to facilitate adherence. Dewax was performed by immersion in xylene with two changes for 2 minutes each and followed by two changes of absolute alcohol for 2 minutes each. The tissue section was gradually hydrated in 95%, 80%, 70% and 50% alcohol for 2 minutes each and lastly, tissue section was washed thoroughly in distilled water for 2 changes.

(b) Staining: Tissue section was immersed in Target Retrieval Solution (Tris-EDTA buffer, pH 9.0) and heated in pressure cooker for at least 20 minutes. After that, tissue section was cooled in running tap water for 20 minutes. The section was then rinsed with TBS (pH 7.6) before it was treated with 120 µl of Peroxidase Blocking Solution for 5 to 10 minutes. Then, the section was once again rinsed with TBS (pH 7.6) for three times before it was incubated with primary antibody (MIB-1, Dako) of 200 µl per section (dilution of 1: 150) for 1 hour. The section was rinsed with TBS (pH 7.6) for four times or more. It was then incubated with secondary antibody for 30 minutes. Tissue section was rinsed once with TBS (pH 7.6) and distilled water. Excess distilled water around the section was wiped off. It was then incubated with DAB (3, 3′-Diaminobenzidine) substrate 1ml : 1 drop (DAB + substrate : DAB + Chromogen) for 10 minutes. Next, the section was placed under running tap water for a few seconds and counter-stained in Hematoxylin for 1 minute. Lastly it was placed under running tap water for 1 minute.

(c) Dehydrate and Clearing: The tissue section was dehydrated by dipping in 80% alcohol 10 times and followed by dipping in two changes of 95% alcohol 10 times. Next, the section was immersed in absolute alcohol with three changes for 2 minutes each.