SURVIVAL OF BREAST CANCER PATIENTS AND THE POTENTIAL FOR BETTER PROGNOSTICATION

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DECLARATION

I hereby declare that the thesis is my original work and it has been written by me in its entirety. I have duly acknowledged all the sources of information which have been used in the thesis.

This thesis has also not been submitted for any degree in any university previously.

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Contents

Summary

Many factors influence the survival of patients with breast cancer. Tumour biology is likely the most important; secondly, the treatment options and the response to the treatment. Individuals with the same stage and similar pathological diagnoses can experience different clinical courses. There are prognostic tools using clinical and tumour indicators, but most are not individualised. Molecular platforms for personalised medicine has since evolved and perhaps will provide better prognostication.

In this thesis, we study prognostic factors and survival of Singaporean women with breast cancer and the potential for better prognostication in Singaporean women:

1. To investigate the ethnic difference in survival between an Asian population and a Western population: Singapore and Stockholm.
2. To investigate the ethnic difference in survival between different ethnic groups in Singapore: Chinese, Malays and Indians.
3. To investigate the effects of biological factors of tumours on survival amongst the different ethnic groups in Singapore: effect of receptor status.
4. To design and investigate the use of molecular tools to prognosticate breast cancer in Singapore:
    a. To validate a series of gene expression signatures that we had previously described in our local Asian breast cancer population.
    b. To design and validate the use of a custom molecular array as an adjunct tool in the prognostication of breast cancer in addition to standard clinical tests.

Paper published

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Clinical Validation of a Customized Multiple Signature Microarray for Breast Cancer Clinical Cancer Research 2008 Jan 15;14(2):461-9.

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Gek-Hsiang Lim, Benita Tan, Per Hall, Kamila Czene, Kee-Seng Chia. Do Asian breast cancer patients have poorer survival than their Western counterparts? American Association for Cancer Research Annual Meeting. 12 - 16 April 2008. San Diego, California, USA.

List of tables

List of figures and illustrations

Abbreviations

Abrreviations

AI	Aromatase inhibitors
ASCO	American Society of Clinical Oncology
ASR	Age-standardised rate / ratio
BMI	Body mass index
BTB	Breast tumour board
CAP	College of American Pathologists
CI	Confidence Interval
CISH	Chromogenic in situ hybridization
DFS	Disease free survival
DNA	Deoxyribonucleic acid
EBCTCG	Early Breast Cancer Trialists' Collaborative Group
EBTCG	Early Breast Cancer Trialists' Collaborative Group
EGRF	Epidermal growth factor receptor
ELISA	Enzyme-linked immonosorbent assay
ER	Estrogen receptor
FISH	Fluorescence in situ hybridisation
H&E	Haematoxylin and eosin
HDB	Housing Development Board
HER2	Epidermal growth factor receptor 2, previously called HER2/neu, or ERBB-2
HRT	Hormone replacement therapy
IDC	Invasive ductal carcinoma
IHC	Immunohistochemistry
IR	Incidence rate
LHRH	luteinizing hormone-releasing hormone
LVI	Lymphovascular invasion
MR	Mortality rate
MSA	Multiple signature assay
NCC	National cancer centre
NCCN	National Comprehensive Cancer Network
NIH	National Institutes of Health
NPI	Nottingham Prognostic Index
OECD	Organisation for Economic Cooperation and Development
OR	Odds ratio
PCR	Differential polymerase chain reaction
PR	Progesterone receptor
RNA	Ribonucleic acid
RR	Relative ratio
RSR	Relative survival ratio
RT-PCR	Reverse transcription polymerase chain reaction
SD	Standard deviation
SEER	Surveillance, Epidemiology and End Results
SES	Social economic status
SGH	Singapore General Hospital
SISH	Silver-enhanced in situ hybridization
SOP	Standard Operating Protocol

MAIN BODY OF THESIS

CHAPTER 1 INTRODUCTION & LITERATURE REVIEW

1.1 Review of risk factors of breast cancer: prognostic and predictive factors

Carcinoma of the breast is a major cause of worldwide morbidity and mortality in females. Incidence of breast cancer is driven by many well reported risk factors (Table 1) and some of these factors in addition have prognostic implications.

Table 1 Risk factors of breast cancer

Factors that cannot be changed	
Gender	Family history
Age	Personal history of breast cancer
Genetic risk factors	Ethnicity
BRCA1 and BRCA2	Dense breast tissue
Other genes	Benign proliferative lesions with atypia
ATM in ataxia-telangiectasia	Atypical ductal hyperplasia (ADH)
p53 and CHEK2 in Li-Fraumeni syndrome	Atypical lobular hyperplasia (ALH)
PTEN in Cowden syndrome	Lobular carcinoma in situ
CDH1	Menstrual periods: early menarche, late menopause
STK11 in Peutz-Jeghers syndrome	Previous chest radiation
	Diethylstilbestrol exposure

Lifestyle-related factors and breast cancer risk	
Child bearing	Alcohol
Oral contraceptive use	Obesity
Hormonal replacement therapy	Physical activity
Breast feeding	Smoking

Many factors influence the survival of patients with breast cancer; they include patient factors, stage of disease, tumour biology, and cancer treatment. However,

there is much variability as individuals with the same stage and similar pathological diagnoses can experience different clinical courses. Tumour biology is likely the most important; secondly, the treatment options and the response to the treatment. Breast cancer survival is driven by the variability of the patients and their tumours.

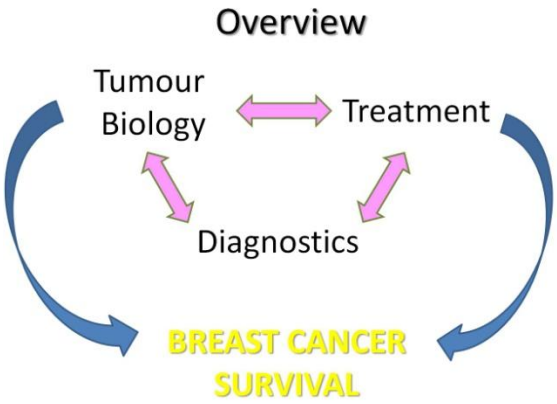


Figure 1 Overview of the factors that affect breast cancer survival

Axillary nodal status, age, tumour size, pathologic grade, and hormone receptor status are the established prognostic and/or predictive factors for selection of adjuvant treatments(1). Some factors, like a person's age and ethnicity, cannot be changed; while others such as lifestyle behaviours: smoking, drinking, exercise, diet, obesity and pregnancies are modifiable; and these may influence the risk of breast cancer, the tumour biology and hence influence survival (Table 2).

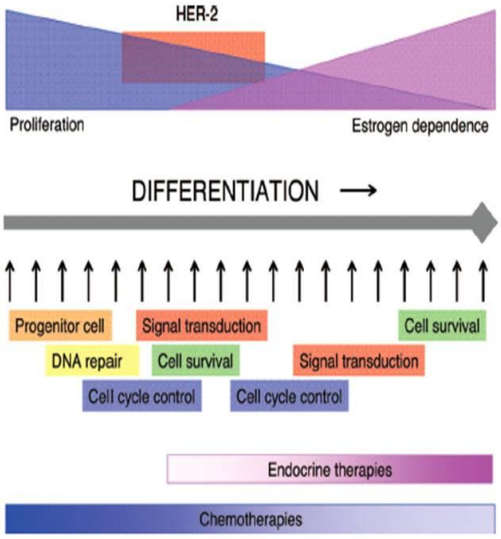
Breast cancer is commonly treated by various combinations of surgery, chemotherapy, radiation therapy, hormone therapy and targeted therapy via a multimodality approach. Selection of therapy is influenced by clinical and pathologic features that could predict their response to these therapies.

Table 2 Factors influencing breast cancer biology and response to treatment

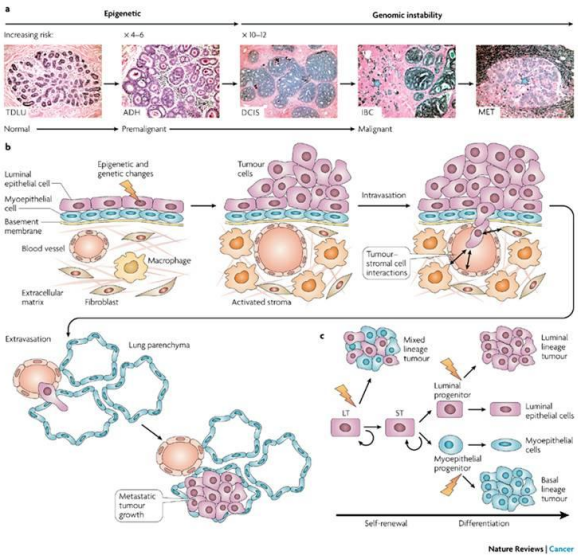
Patient Characteristics	**Steroid Receptors and regulated Factors**
Age at diagnosis	ER and PR
Ethnicity	pS2
Hormomal Risk Factors	Heat shock proteins
Histopathologic Features	**Growth Factors and receptors**
Histologic subtype	EGFR
Axillary lymph node metastasis	HER/neu
Tumour Size	
Histological Grade	**Tumour Suppressor Genes**
Lymphovascular invasion	p53
	nm23
Cell Proliferation	
Mitotic index	**Measure of invasiveness**
Thymidine-labelling index	Cathepsin D
S-phase by flow cytometry	Plasminogen activators
Ki-67/MIB-1	Laminin receptors
	Angiogenesis
	Microvessel density
	VEGF

1.2 Tumour Biology

There is much genetic variation in breast cancer tumours resulting in a heterogeneous group of tumours. Different risk factors are associated with different tumour characteristics and they in turn influence and predict the response to certain therapy and hence the difference in outcome. Some of these factors are well documented, many others just being investigated.



Schema of tumour differentiation. This figure shows tumoral differentiation as a continuum that results from the extent of molecular oncogenic aberration and influences tumour phenotype and treatment sensitivity. From Synmmans WF. A pathologist's perspective on emerging genomic tests for breast cancer. Semin Oncol 2007;34(suppl 3):S4-S9.



Modelling breast cancer: one size does not fit all. Tracy Vargo-Gogola & Jeffrey M. Rosen. Nature Reviews Cancer 7, 659-672

Figure 2 Tumour differentiation resulting in tumour phenotype and treatment heterogeneity

Hormonal receptor status

The importance of steroid hormone receptors to the biology of breast cancer was recognised more than 40 years ago. Human breast cancers are dependent upon oestrogen and /or progesterone for growth and that is mediated through the ERs and PRs. ER and PR are both members of the nuclear hormone receptor family that includes the androgen and retinoid receptors. They are located in the cytoplasm and operate as ligand-dependent transcription factors. Attachment of a lipid-soluble hormone to the ligand-binding domain results in unmasking of the DNA-binding sites on the receptor, followed by migration into the nucleus, and

binding to specific hormone-responsive elements near the genes that are responsible for the physiologic actions of the hormone.

Tumour expression of ER and/or PR can identify those most likely to benefit from endocrine therapy and due to this predictive value, measurement of these receptors has become routine in breast cancer management. While the predictive value of hormone receptor expression is well accepted, its prognostic importance has been a matter of debate for many years.

The independent contribution of PR expression has been debated(2) but recent reports indicate that patients with ER-positive but PR-negative breast cancers have a worse prognosis than those with both ER and PR positive tumours (3; 4). However, the predictive value of PR expression remains controversial, but as PR positivity may be helpful in selecting patients with ER-negative breast cancer who might benefit from a highly effective and low toxicity treatment, endocrine therapy is usually recommended. ER-negative/PR-positive tumours are uncommon (2; 5; 6).

Population based studies have shown that compared to women with ER+/PR+ tumours, women with ER+/PR-, ER-/PR+, or ER-/PR- tumours experienced higher risks of mortality, which were largely independent of the various demographic and clinical tumour characteristics (Figure 3) (7; 8).

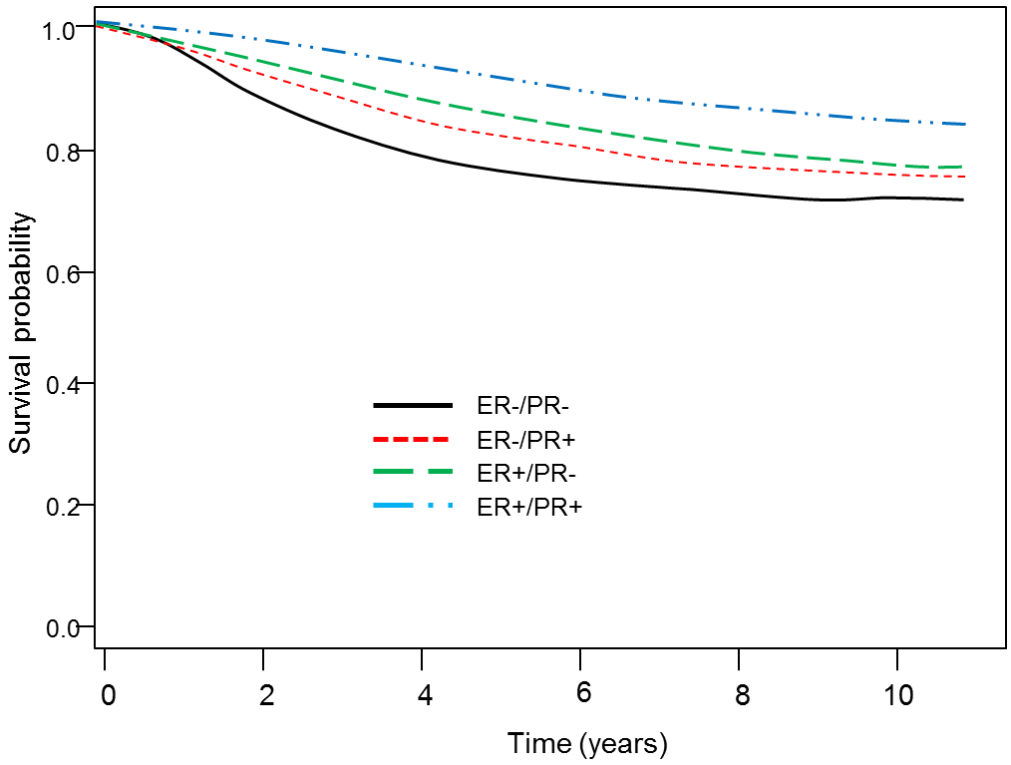


Figure 3 Breast carcinoma-specific survival is illustrated according to oestrogen receptor (ER) and progesterone receptor (PR) status.
+: Positive; -: negative. Adapted from Grann VR, et al. Cancer. 2005 Jun 1;103(11):2241-51**(7).**

Endocrine therapy

The goal of endocrine therapy is to prevent breast cancer cells from receiving stimulation from endogenous oestrogen. Ovarian ablation by radiation or surgery was standard therapy in the 1950s but over time, this has been replaced with pharmacologic endocrine therapy. This include blockade of the ER by selective ER modulators such as tamoxifen or suppression of oestrogen synthesis by luteinizing hormone-releasing hormone (LHRH) agonists and by aromatase inhibitors (AIs, e.g., anastrozole). Tamoxifen is used in premenopausal women and the benefits of adjuvant tamoxifen have been most clearly demonstrated in the EBTCG meta-analysis (9). While tamoxifen remains an option for

postmenopausal women, AIs are generally preferred as they are more effective in preventing cancer recurrence in the first two years after surgery (10) or An adjuvant treatment strategy incorporating an aromatase inhibitor (AI) as primary (initial endocrine therapy), sequential (using both tamoxifen and an AI in either order) or extended (AI after five years of tamoxifen) therapy reduces the risk of breast cancer recurrence compared to five years of tamoxifen alone (11).

HER2 receptor status

The HER2 receptor belongs to the epidermal growth factor receptor (EGFR) family of receptors controlling epithelial cell growth and differentiation (12; 13) and possibly angiogenesis (14; 15). Previously called *HER2/neu*, or *ERBB2*, amplification of *HER2* gene or expression of its protein is seen in 18 to 20 per cent of human breast cancers (16; 17). *HER2* overexpression is associated with high rates of disease recurrence and death in the absence of adjuvant systemic therapy (18; 19). It has been correlated with higher Scarff-Bloom-Richardson grade, younger age at diagnosis, and a comedo ductal carcinoma in situ component (20).

Guidelines from the ASCO expert panel recommend against using HER2 positivity purely as a prognostic factor, but strongly recommended its use as a predictive factor for response to specific therapies such as trastuzumab and lapatinib (2) as outcomes are heavily influenced by subsequent therapy; sensitivity to different types of chemotherapeutic agents and preferential use of anthracycline-based chemotherapy. HER2 positivity is associated with relative

resistance to endocrine therapies but this is not used to withhold endocrine therapy (2).

Although HER2 positive tumours are associated with high grade and LVI positivity where prognostic effects had been seen in advanced cancer, in low-grade, node-negative patients, HER2 significantly affects the survival in this otherwise very good prognostic group(21).

'Triple negative' breast cancer

The triple-negative phenotype where the tumours did not exhibit the ER, PR and HER2 receptor became important because of its relation to the basal-like subtype of breast cancer. Triple-negative breast cancers affect younger, non-Hispanic black and Hispanic women in areas of low SES. The tumours were diagnosed at later stage and were more aggressive, and these women had poorer survival regardless of stage (22).

Grade

Poorly differentiated tumours have long been known to pursue a more aggressive course than their well differentiated counterparts (23; 24). Until recently histological grading has not been accepted as a routine procedure, mainly because of perceived problems with reproducibility and consistency.

Together with tumour size and nodal status, grade has been found to be the most important prognostic factors for long-term survival(19). The higher failure rate for patients with high-grade tumours was due to a larger number of failures in regional and visceral sites.(25)

Lymphovascular invasion

Lymphovascular invasion (LVI) is a widely recognized prognostic factor in lymph node-negative breast cancers (26). However, there are controversial data about its prognostic significance in lymph node-positive patients (27; 28). The presence of LVI was a stronger predictor of early recurrence than tumour grade in postmenopausal patients who received no adjuvant therapy (25). LVI should be considered in the therapeutic strategy as a decision making tool in the adjuvant chemotherapy setting

1.3 Ethnic differences in breast cancer

Factors that may differ between ethnic groups that need to be considered include genetic differences such as effects of ethnic differences on tumour biology and ethnic difference in response to treatment; as well as psychosocial differences between ethnic groups in seeking and accepting medical treatment. Prognostic and predictive tools are just as important for recommendations of treatment.

Population based study of ethnic differences in breast cancer survival is limited except for those between Afro-American women, Asian-American women and

Caucasian American women. In the United States, Afro-American women are known to have a worse outcome compared with Caucasian American women. This has been attributed to increased diagnosis of late-stage breast cancers, which could be explained by delayed diagnosis reflecting the socioeconomic status, cultural beliefs, access to healthcare (29), and the proportion of oestrogen receptor (ER)-negative tumours in the Afro-American women (30). However, observed differences between tumour specimens obtained from Afro-American women and tumour specimens obtained from white women, independent of stage and age at diagnosis, indicated that race may be a determinant, or a surrogate for other determinants, of aggressive breast carcinoma and specific cell cycle defects (31).

Hormonal factors and tumour biology

The ethnic disparity in incidence, mortality and survival is evident in the United States, where breast cancer is predominantly a postmenopausal disease in the Caucasian American population (30; 32) but a premenopausal disease in Asian or non-Caucasian populations (33–35). ER and/or PR negativity has been correlated with premenopausal disease, black race, and poor prognostic factor groups while oestrogen receptor positivity has been associated with postmenopausal disease, white race, and favourable tumour characteristics (7).

In US women diagnosed with breast cancer from 1992 to 1998, a positive ER was found in 81% of non-Hispanic Whites and in 66% of Afro-Americans (36). ER-negative breast cancer, however, seems to be influenced by parity and age

at first birth. Multiparity and early age at first birth were associated with reduced relative risk of ER + PR + tumours in many studies (37–41) although there were also reports that did not show significant differences in association of parity, age at first live birth, breastfeeding history, age at menopause, or synthetic hormonal use between molecular subtypes of breast cancer (42). It has been reported that increased parity at an early age is associated with ER-negative breast cancer (43). Early age at first birth could also have an adverse effect on the prognosis of breast cancer (40; 44) due to the interplay of hormonal factors in tumour biology and prognosis. More Afro-American women have children early (<20 years old) (45; 46) and tend to have higher parity compared with the non-Hispanic White women (47). Interethnic relations, including the influx of other migrant populations causing possible genetic complexities would not explain the rising cancer trend, because large-scale genetic mutations would manifest only after two to three generations (48).

Socioeconomic status

Socioeconomic deprivation may be responsible for the increased risk of breast cancer mortality in African American and Hispanic patients, as they are more likely than white American patients to be diagnosed with advanced disease. Racial differences in breast cancer incidence can largely be accounted for by ethnic differences in SES among white, Hispanic and Asian/Pacific-Islander women, but not between these populations and black women (49). Stage at diagnosis, first course treatment and race explained most of the socioeconomic disparity in breast cancer survival (50; 51). Among white women, social

deprivation is related to poor breast cancer prognosis, with increased prevalence rates of high-grade, oestrogen receptor (ER)-negative tumours, similar to that of triple-negative breast cancers observed in African American and Hispanic women. Triple-negative breast cancers affect younger, non-Hispanic black and Hispanic women in areas of low SES(52). In a Detroit study population, race was not statistically significantly associated with unfavourable breast cancer outcomes. However, low SES was associated with late-stage breast cancer at diagnosis, type of treatment received, and death(53). A positive family history of breast cancer may reduce SES differences in access to screening and optimal treatment(54).

However, the relationship of SES with breast cancer is not a simple one as it is intertwined with the risk of developing breast cancer. A large population study in Wisconsin showed that after controlling for individual education and other individual-level risk factors (age, mammography use, family history of breast cancer, parity, age at first birth, alcohol intake, body mass index, hormone replacement use, oral contraceptive use, and menopausal status), women living in the highest SES communities had greater odds of having breast cancer than women living in the lowest SES communities (55); there is a decrease in mortality rates of localized breast cancer as SES declined, whereas regional breast cancer mortality rates tended to increase (56).

Others factors

Other factors such as diet, obesity, other behavioural, cultural and environmental known to affect incidence (38), has effects on prognosis that is less known (57); and these differences exist between ethnic groups. Diet for instance, soy intake amongst Chinese (58; 59) have been shown to decrease risk of death in Chinese women; but soy is not present in the western diet. Obesity is associated with advanced breast cancer at diagnosis, high tumour proliferation rates, and more triple-negative phenotypes, indicating that it may adversely contribute to prognosis (60).

1.4 Relative survival ratio

The most common measures in cancer are incidence, mortality, and survival. A statistical measure can be considered desirable if it reflects the underlying quantity of interest. A public health goal is to prevent the occurrence of cancer and doctors play a large part in these endeavours. The goal of the clinician is to reduce morbidity and mortality amongst those individuals who will experience morbidity and mortality due to cancer. To assess progress towards the goal of reducing cancer mortality we would ideally like to be able to measure cancer mortality amongst individuals who are destined to experience increased mortality due to cancer. From a clinical perspective, mortality amongst the patients is of greater interest.

Relative survival ratios were computed by taking the ratio of observed survival to expected survival, accounting for the competing causes of death.

$$\frac{\text{Relative survival}}{\text{Expected survival}} = \text{observed survival}$$

Population mortality rates do not serve our purpose as they measure mortality in the entire population during a specified period of time. The published cancer mortality rates for a particular year are calculated by counting the number of deaths due to cancer amongst patients diagnosed over a period of many years, for some malignancies during several decades. The denominator includes the entire population, irrespective of whether or not they have been diagnosed with cancer. Mortality rates will therefore be subject not only to trends in cancer patient survival but also to trends in cancer incidence. As such, they are not an ideal measure of the progress in diagnosis and treatment of cancer.

Estimates of patient survival are based upon 'patients diagnosed with cancer'; not identical to 'individuals who will experience increased mortality due to cancer' but close. This difference between the desired study base and the actual study base should be kept in mind when interpreting the estimates(61).

1.4 Descriptive epidemiology in Singapore

Breast cancer trend

Singapore is characterised by three main ethnic groups: Chinese, Malays and Indians. These three ethnic groups have remarkable differences in breast cancer incidence in spite of relatively similar changes in reproductive and socioeconomic changes. The overall incidence of breast cancer almost tripled from 22.0 per

100,000 in 1973-77 to 60.0 per 100,000 person years in 2005–2009(48; 62). It has continued to be the most common cancer among females; thus, it also comprised the greatest proportion of cancer deaths (63; 64).

The increase in breast cancer incidence was different among the Chinese, Malays and Indians, as was previously reported (48). Singaporean Malay women in the earlier years had the lowest risk of developing breast cancer. However, Chinese and Malay women born in later birth cohorts had higher risks of developing breast cancer compared to their counterparts born in 1926–1930; the risk increase was sharper in the pre-menopausal Malays than in the Chinese. In the last decade, the increase in breast cancer incidence was highest among the Malays; the age-standardised rate (ASR) increased from 44.8 to 58.7 per 100,000 from 1998–2002 to 2005–2009(62). Among the Chinese, the ASR of breast cancer increased from 57.4 to 60.8 per 100,000 and 47.4 to 53.8 among the Indians in the same periods. However, the age-standardised mortality rate had remained fairly constant, at about 14 per 100,000 person-years in the period 1998–2007(62).

Although breast cancer was the most common cancer found among Singapore women, the prognosis was relatively optimistic. The five-year age-standardised RSR has remained at about 76% in the period of 1998–2002 to 2005–2009(62). Data from the Singapore breast screening pilot project in 1994–1997 as well as the nation-wide breast screening programme (BreastScreen Singapore) launched in Singapore in January 2002 (65) showed that breast screening is effective in allowing for cancers in less advanced stages to be detected and treated early, thereby improving the survival of breast cancer patients. More than 30% of pre-

invasive ductal carcinoma in situ was detected among pre-menopausal women through the programme (66; 67).

Socioeconomic Status

Singapore underwent much economic restructuring in the 1980s and 1990s, resulting in marked economic improvements over these two decades (68) with an increase in Gross Domestic Product was 725% from 1980 to 1999 (International Monetary Fund statistics). This resulted in improved living standards, improved education and presumably better awareness of the disease, and in better healthcare including breast screening – albeit opportunistic. Singapore is a small island and enjoys a large network of affordable and easily accessible primary healthcare services that with heavily subsidized hospital and specialist care services. Healthcare indicators such as life expectancy, the infant mortality rate and the hospital to population ratio are comparable to developed countries like Sweden (69; 70).

Using education and housing type as a surrogate for SES, Chinese have the highest SES; more Malays were living in public housing (HDB, Housing Development Board), especially in the smaller 1-2-room flats, compared to the Chinese and Indians; and the Chinese and Indians tended to have higher education. Younger Singaporeans received higher education than older Singaporeans and this improved over the decade in all groups, signifying overall improvement in education. Although the proportion of Malays receiving higher education more than doubled over the decade, this was still less compared to the Chinese and Indians (Table 3).

Fertility

Total fertility by calendar year declined across the three ethnic groups, from over 4,000 births per 1,000 women in the 1960s to around or less than 2,000 births per 1,000 women in the late 1990s. The Malays tend to have the highest total fertility rate, followed by the Indians and lastly the Chinese, with more than a third of Malay women having 3 or more children (Table 3).

There was also an increasing trend in the age at first birth for the three ethnic groups. The median age at first birth in 1970 ranged from 21.6 to 24.9, while in 2000, it ranged from 25.6 to 29.2. In 1970, the median age of first birth for Malay women was 21.6, compared to 24.9 for the Indians and 23.8 for the Chinese. By the year 2000, the median age had increased to 25.6 for the Malays, 29.2 for the Indians and 28.9 for the Chinese. The Chinese had the highest rate of increase over the 30 years period while the Malays consistently had the median youngest age at first birth (48). The impact of multiparity on premenopausal breast cancer risk differs across ethnic groups in Singapore. Increasing parity reduces the risk of premenopausal breast cancer in Malay, but not in Chinese and Indian women. Uniparous Malay women have twice the risk of premenopausal breast cancer compared to uniparous Chinese. This excess risk disappears after giving birth to >or=3 children. Indian women have lower premenopausal breast cancer risks than Chinese, regardless of their parity status (71).

Table 3 Distribution of housing types, education and fertility by ethnicity

	Chinese		Malays		Indians	
	2000	2010	2000	2010	2000	2010
House type						
HDB	86.4	81.3	98.2	96.8	89.1	82.7
1-2-Room	4.4	4.1	6.5	8.7	8.1	4.9
3-Room	25.6	19.9	28.4	22.0	24.4	21.0
4-Room	32.2	31.2	41.0	39.2	31.8	32.0
5-Room & Executive	23.7	25.7	22.3	26.9	24.5	24.6
Private property	12.6	18.0	1.5	2.8	9.6	16.3
Private flats	6.9	11.7	0.8	1.9	5.2	11.9
Landed	5.7	6.3	0.7	0.9	4.4	4.4
Others*	1.0	0.7	0.3	0.4	1.3	1.0
Education						
Highest Qualification Attained						
No Qualification	32.0	20.2	30.5	20.0	27.7	13.9
Primary	25.6	21.9	33.1	30.1	31.4	24.5
Secondary	33.3	38.2	34.9	45.0	35.5	42.0
Polytechnic & University	9.2	19.6	1.5	4.9	5.3	19.6
Proportion with at least post-secondary qualifications						
25 - 34 years	60.0	83.7	31.5	62.9	55.6	84.0
35 - 44 years	34.4	64.8	14.8	41.0	36.2	74.5
45 - 54 years	22.3	36.2	8.7	20.2	24.7	42.5
Fertility						
Number of children**						
0	6.3	9.8	5.6	6.1	7.0	7.7
1	16.1	20.6	9.7	9.5	13.9	17.8
2	44.8	45.2	24.9	25.5	43.5	45.5
3	26.6	20.4	33.8	33.2	26.4	22.1
4 & over	6.2	4.0	26.0	25.7	9.3	7.0
Average number of children**	2.2	1.9	2.8	2.7	2.2	2.1

Census of population 2010 Singstat Singapore

** includes other public flats, non-HDB shophouses and attap/zinc-roofed houses.*

**in ever-married Females Aged 40-49 Years

Culture and religion

Cultural beliefs and attitudes in conjunction with SES could largely account for effect of race on breast cancer stage (72). Religion and culture are closely related and there is a difference between the ethnic groups. In 2010, 57 per cent of the Chinese identified themselves as Buddhists or Taoists, 20 per cent as Christians and 22 per cent as persons with no religion. Among the Indians, Hindus comprised 59 per cent, 22 per cent were Muslims and 13 per cent were Christians; while 99 per cent of Malays were Muslims (73).

Diet and lifestyle

Singapore offers a wide variety of culinary delights and much of this a direct consequence of the difference in cuisine between ethnic groups. Chinese women have a high intake of soy and consume the lowest amount of dietary fat (74) while Malay women are less likely to consume alcohol. Prevalence of obesity (BMI>/=30kg/m2) is highest in Malay women (23.1%), compared to the Chinese (3%) and Indians (18.3%) as reported in the National Health Survey in 1998 (75).

1.5 Comparison with other populations

The ASR of breast cancer in Singapore is amongst the highest compared to the Asian counterparts, but lower than that in the USA and Europe(76). While the five-year ASR of breast cancer in Singapore was 60.0 per 100,000 person-years in 2005–2009, the corresponding ASR in Malaysia, China and India in 2008 were

37.0, 21.6 and 22.9 per 100,000 person-years, respectively; this was 76.0 per 100,000 person years in USA correspondingly. In 2008, the age-standardised mortality rate of breast cancer in Singapore was comparable to that in Malaysia and the USA, but higher than that in China and India. The age-standardised mortality rates were 14.7, 5.7, 11.1 and 14.7 per 100,000 person-years for Malaysia, China, India and the USA, respectively(77). The five-year breast cancer survival in Singapore was lower than that in the USA (as represented by SEER) and China, but higher than that in India.(6) The five-year age-standardised RSR of 76% in Singapore was slightly lower than that of countries in the Organisation for Economic Cooperation and Development (OECD) (81.2% in 2002–2007)(78).

1.6 Prognostic tools

The 'ideal' prognostic test

Accurate prediction of survival is important in treatment recommendation following surgery for breast cancer. It allows clinicians to determine benefit from adjuvant therapy. A useful prognostic factor in breast cancer should have the following characteristics according to an NIH Consensus Conference (79):

- Provide significant and independent prognostic value, validated by clinical testing
- Determination must be feasible, reproducible and widely available, with quality control

- Results should be readily interpretable by the clinician
- Measurement of the marker must not consume tissue needed for other tests, particularly routine histopathologic evaluation

The current standard of care utilises a battery of assessments to prognosticate breast cancer, predict response to guide treatment. These include pathological factors that retain independent significance on multivariate analysis such as tumour size, tumour grade and lymph node status in addition to the efficacy of any adjuvant therapy; as well as predictive markers to treatment. However, these markers are associated with limits, including significant inter-observer variability and their reliance on measuring single biomarkers and hence the need to carry out multiple independent tests on the same tumour.

Histopathology

Tumour histological subtype, grade and LVI are standard histological features considered in the management of breast cancer. Each of these are subject to inter-observer variability(80) which results in inconsistencies in a proportion of cases.

Assays for ER and PR status

However, the methods of measurement have been fraught with variations that resulted in inaccurate test results due to variety of factors such as specimen handling, tissue fixation, antigen retrieval, and antibody type. In addition,

interpretation of the assay results is variable due to variable threshold values as well as inter-observer variations. These resulted in serious issues with ER reliability. A joint committee representing ASCO and the College of American Pathologists (CAP) published guidelines that specifically address causes of variation related to measurement of ER and PR by IHC in 2010 (5; 6), which has become the predominant method of measuring ER and PR in clinical practice. The guideline recommendations include definition of appropriate specimen handling, fixation, analytical testing methods, thresholds for interpretation of positive and negative results, quality assurance methods, and monitoring strategies for individual laboratories.

Assays for HER2 expression

There are many ways to measure the activity of the HER2 oncogene; but the best method, in terms of the type of assay and the optimal method to perform each assay, is controversial. The available assays are:

- HER2 gene amplification- Fluorescence in situ hybridisation (FISH), chromogenic in situ hybridization (CISH), silver-enhanced in situ hybridization (SISH) or differential polymerase chain reaction (PCR)
- Overexpression of the HER2 protein product- Western blotting, enzyme-linked immonosorbent assay (ELISA), or immunohistochemistry (IHC)
- Overexpression of HER2 RNA- Northern blotting or reverse transcription PCR (RT-PCR)

However, many of these methods have not been well standardized. Prospective sub-studies from adjuvant trastuzumab trials indicate that at least 20 per cent of

the HER2 assays performed locally were incorrect when the same specimen was re-evaluated in a high volume central laboratory (81–83). However, a high concordance was seen in central and reference laboratories (83). These highlight the importance of using high volume, experienced laboratories for HER2 testing to optimise the selection of patients most likely to benefit from trastuzumab. The joint committee representing ASCO and the College of American Pathologists (CAP) published guidelines that specifically address the technical and analytical aspects of HER2 testing (84). This committee recommended strict accreditation for laboratories providing HER2 testing and periodic proficiency testing.

Clinical prediction models

The current available clinical prediction systems include the Nottingham Prognostic Index (NPI) (85; 86), a prognostic scoring system using tumour size, grade and lymph node status; the web-based prognostication and treatment benefit tool Adjuvant! Online (87; 88) as well as the more recent Predict Tool (89; 90) which uses a combination of clinical and pathological markers. However, these are based on the Surveillance, Epidemiology and End Results (SEER) registry and the Eastern Cancer Registration and Information Centre (ECRIC) respectively, which may limit their generalizability, especially in Asia. Adjuvant!Online's prognostic performance validated in high income Caucasian populations, has recently been found to be over optimistic in middle income Asian breast cancer patients (91).

Molecular classification and prediction models

Breast cancer profiling i.e., characterisation has significantly advanced over the past decade due to the development of sophisticated high throughput technologies, such as gene expression arrays that permit the simultaneous measurement of thousands of genes to create a molecular portrait of a tumour. Breast cancer tumour subtypes (92–95), expression signatures for clinical prognosis (96–98), ER/HER2 receptor status (99), and response to chemotherapy (100–102) have been established. Reassuringly, broadly similar molecular classes have been discovered (103) that are largely conserved across microarray platforms (104) and ethnic populations (105), and the reproducibility of gene expression signature–based predictions has been confirmed in replicate experiments (106; 107). Compared with conventional assays, molecular profiling platforms offer the potential advantage of measuring multiple biomarkers and signatures in a single test. These have been enthusiastically embraced by the scientific community and hailed as a major breakthrough on the way to individually tailored therapies.

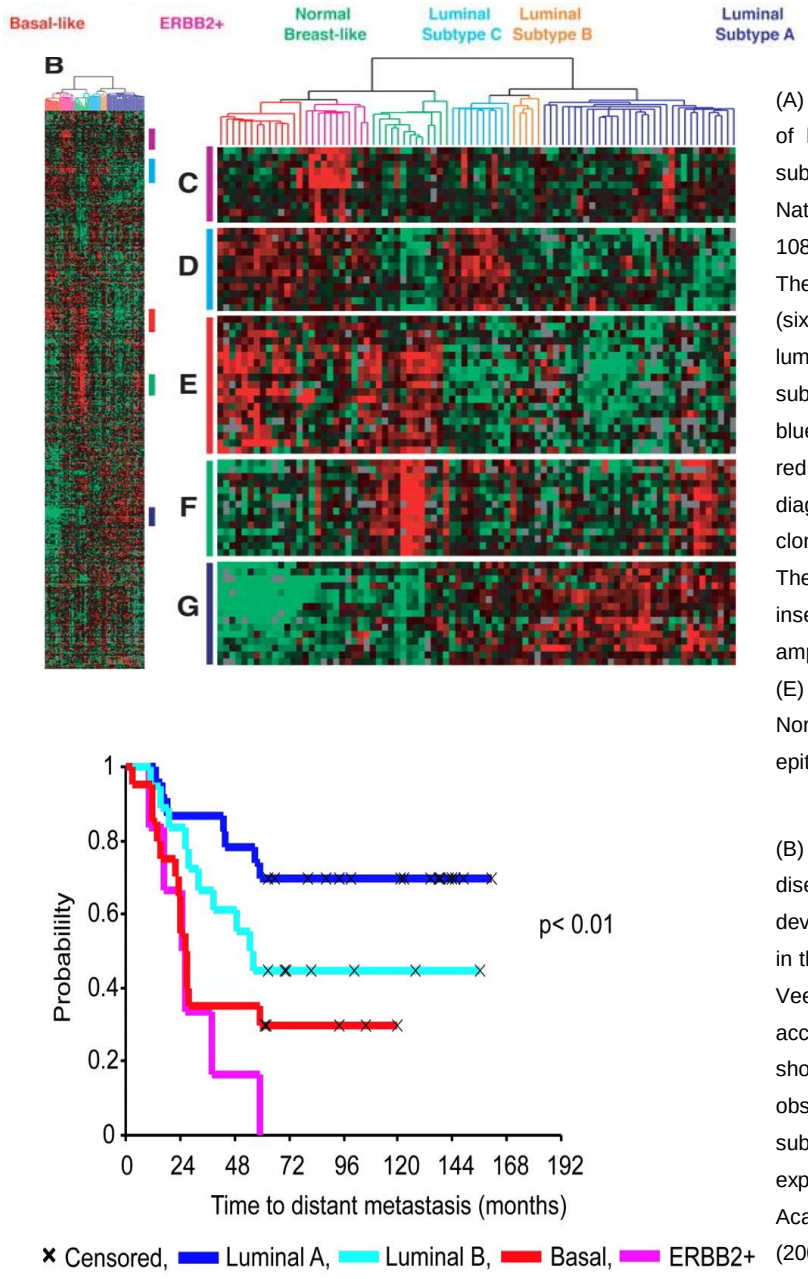
Molecular profiling has led to classification of breast cancer into the following five subtypes(93; 103):

- Basal-like.
- HER2+.
- Normal.
- Luminal A.
- Luminal B.

The normal-like subtype was one of the initial subtypes identified and consistently appears in breast cancer clusters. It remains unclear whether it represents a separate subtype or a technical artefact due to a low tumour cell composition of the sampled specimen. Nevertheless, to establish the true clinical utility of molecular profiling, it is essential to validate such signatures in independent, prospectively defined patient cohorts (108) before accepting these new technologies in daily clinical practice. Although some profiling assays have recently undergone clinical validation (109; 110), these studies have been mostly done on U.S. and European cohorts (109; 111), and there is currently no similar project in Asia. Various clinical, epidemiologic, and molecular differences in breast cancer have been reported between different ethnic groups (32; 33; 105; 112–114), thus raising the need to assess the reliability of these signatures in an Asian cohort.

There are several multi-gene signatures in breast cancer available commercially, Table 4 (115), two such molecular tests merit specific comment. OncotypeDX (Genomic Health) is a RT-PCR-based assay that can be performed on formalin-fixed tissue from paraffin blocks. It is based on the analysis of the expression of 21 genes and provides a 'recurrence score' that correlates with outcome, as well

Figure 4 Gene expression patterns of breast carcinoma



(A) Sorlie T, et al. Gene expression patterns of breast carcinomas distinguishes tumour subclasses with clinical implications. Proc Natl Acad Sci USA 2001, 98:10869-10874(91).

The cluster dendrogram showing the five (six) subtypes of tumors are colored as: luminal subtype A, dark blue; luminal subtype B, yellow; luminal subtype C, light blue; normal breast-like, green; basal-like, red; and ERBB2+, pink. (B) The full cluster diagram scaled down (the complete 456-clone cluster diagram is available as Fig. 4). The colored bars on the right represent the inserts presented in C–G. (C) ERBB2 amplicon cluster. (D) Novel unknown cluster. (E) Basal epithelial cell-enriched cluster. (F) Normal breast-like cluster. (G) Luminal epithelial gene cluster containing ER

(B) Kaplan–Meier analysis of disease outcome: time to development of distant metastasis in the 97 sporadic cases from van't Veer et al. Patients were stratified according to the subtypes as shown. Sorlie, T et al. Repeated observation of breast tumour subtypes in independent gene expression data sets. Proc Natl Acad Sca. USA 100, 8414-8423 (2003) (103)

as likelihood of response to endocrine therapy and likelihood of response to chemotherapy(110; 111; 116; 117). Mammaprint (Agendia) uses expression array analysis of 70 genes to identify patients with good and poor prognostic signatures(109; 118; 119). This assay requires fresh frozen tumour tissue. It should be noted that, although both of these tests are already being used in patient management, their ultimate value will be determined by the results of prospective clinical trials that are currently underway, namely, the TAILORx trial to study OncotypeDX (117) and the MINDACT trial to study Mammaprint (119).

Table 4 Main features of the commercially available multi-gene signatures in breast cancer.

Assay name	Genes (*n*)	Source	Platform	Training dataset	Output data
Biology driven					
PAM50 subtype-predictor	55*	FFPE/FF	qRT-PCR/microarray/nCounter	Breast cancer-based cohort	Luminal A Luminal B HER2-E Basal-like Normal-like
Survival driven					
PAM50 risk of relapse (ROR)	55*	FFPE/FF	qRT-PCR/microarray/nCounter	RFS; ER+/ER−/node− breast cancer patients receiving no adjuvant systemic treatment	Continuous Variable Low–medium–high
Oncotype DX®	21	FFPE	qRT-PCR	Survival: largely ER+/node− breast cancer patients receiving tamoxifen-only adjuvant treatment	Continuous Variable Low–intermediate–high
MammaPrint®	70	FF	Microarray	DRFS: largely ER+/node− breast cancer patients receiving no adjuvant systemic treatment	Continuous Variable Good–bad
Pathology driven					
MapQuant Dx™	97	FF	Microarray	Grade 1 vs 3 in ER+ patients with breast cancer	Continuous Variable Low–high
Survival and pathology driven					
Breast Cancer Index	7	FFPE	qRT-PCR	MGI: grade 1 vs 3, HOXB13/IL17BR ratio DRFS: ER+ patients with breast cancer receiving tamoxifen-only treatment	Continuous Variable Low–intermediate–high

*5 genes included for expression normalization.

Abbreviations: DRFS, distant relapse-free survival; FF, fresh-frozen; FFPE, formalin-fixed paraffin-embedded; MGI, molecular grade index; qRT-PCR, quantitative reverse transcription PCR; RFS, relapse-free survival.

Practical implications of gene-expression-based assays for breast oncologists. Nat. Rev. Clin. Oncol. (115)

CHAPTER 2 AIMS

The aim of this thesis is to study the survival of breast cancer patients and the potential for better prognostication in Singaporean women

Specific aims:

5. To investigate the ethnic difference in survival between an Asian population and a Western population: Singapore and Stockholm.

6. To investigate the ethnic difference in survival between different ethnic groups in Singapore: Chinese, Malays and Indians.

7. To investigate the effects of biological factors of tumours on survival amongst the different ethnic groups in Singapore: effect of receptor status.

8. To design and investigate the use of molecular tools to prognosticate breast cancer in Singapore:
    a. To validate a series of gene expression signatures that we had previously described in our local Asian breast cancer population.
    b. To design and validate the use of a custom molecular array as an adjunct tool in the prognostication of breast cancer in addition to standard clinical tests.

CHAPTER 3 STUDY 1: ETHNIC DIFFERENCE IN SURVIVAL BETWEEN STOCKHOLM AND SINGAPORE

3.1 Subjects and Methods

All cases of invasive breast cancer diagnosed from 1 January 1980 to 31 December 1999 were obtained from the Singapore and Stockholm cancer registries (ICD 9: 174; ICD 10: C50). Patients with a previous malignancy, including contralateral breast cancer, and those diagnosed with breast cancer at autopsy (death certificate only) were excluded from the study. Follow-up was performed until 31 December 2005 by matching with the national death register. The cause of death was coded in accordance with the International Classification of Diseases and Causes of Death ICD9. The ethical committees at the National University of Singapore and Karolinska Institutet accepted the study without any restrictions. This is normal when de-identified material is used.

Singapore Cancer Registry

The Singapore Cancer Registry is a population-based registry that was started in 1968. It receives voluntary notifications of incident cancers from all medical practitioners and pathology laboratories, as well as reviews, death certificates and hospital discharges for all patients. Staff of the Registry also reviewed cancer patient hospital discharges and death certificates. The completeness of reporting is high: 96% in the 1970s and close to 100% in the 1990s. The proportion of death-certificate-only notifications was 4.2% for the period 1968 to 1977, 1.0% for 1993 to 1997 and 0.9% for 1998 to 2002 (120). Between 1980 and 1999, 10,287

female Singapore residents (citizens and permanent residents) diagnosed with breast cancer were identified and included in the study (120).

Stockholm Breast Cancer Registry

The Stockholm Breast Cancer Registry was started in 1977 and receives notification of newly diagnosed cases of breast cancer at all departments of oncology and surgery in Stockholm County, which is inhabited by 1.7 million individuals. There were 17,090 women diagnosed with breast cancer recorded in the calendar period 1980 to 1999. None of them were death-certificate-only diagnoses.

Stage information

The stage of the breast cancer in the Singapore Cancer Registry was classified as localized cancer, regional spread and distant metastases based on the notification forms before 2001. Cancers are staged as local if they are confined entirely to the breast. Regional cancers are those that have extended beyond the limits of the breast directly into surrounding tissues or organs, or into lymph nodes in the region. Distant cancers are those that have spread beyond these locations. No attempt was made to access the extent of localized invasion or the number of regional lymph nodes involved.

Stage information in the Stockholm registry was available according to the TNM staging system: tumour stage, lymph node stage and metastatic spread. This information was reclassified, using the Surveillance, Epidemiology and End Results Comparative Staging Guide for Cancer (121), to be comparable with the Singapore cohort – as localized disease where breast cancer was only identified

in the mammary gland; regional disease when there is direct extension to surrounding tissues or organs, or axillary lymph nodes are affected; and distant metastasis.

The stage information from the cancer registry is a combination of clinical and pathological staging reported by the respective hospitals, but the proportion is unknown.

Statistical analysis

Age at diagnosis was categorized into six age groups (<35 years, 35 to 44 years, 45 to 54 years, 55 to 64 years, 65 to 74 years, 75+ years) and the period of diagnosis divided into two 10-year periods (1980 to 1989 and 1990 to 1999) to identify change over time; 1990 was selected because the early 1990s were when the use of tamoxifen became widely accepted and adjuvant treatment was more standardized in Singapore.

Association of clinical variables between study groups were performed using chi2 test, descriptive prognostic comparisons between Singaporean women and Swedish women were performed by relative survival analyses. Relative survival ratios were computed by taking the ratio of observed survival to expected survival, accounting for the competing causes of death.

$$\frac{\text{Relative survival}}{\text{Expected survival}} = \text{observed survival}$$

The expected survival probabilities were calculated using Ederer II method (122) derived from the general female population from Singapore and Stockholm,

respectively, similar to the breast cancer patients in terms of attained age and calendar period of diagnosis. In order to compare the survival between the two countries, cumulative relative survival ratios were age-standardized to the world standard cancer population (122). A 3-year central moving average for the 5-year relative survival ratios at each calendar year of diagnosis was used to depict the trend across the calendar periods. Joinpoint regression analysis was used to estimate the annual mortality trends from the five-yearly rates available for the Singaporean general population(123).

A Poisson regression model was also used to calculate the excess hazards of death, taking into account the age, disease stage, period of diagnosis, country and years of follow-up. Interactions between country and the age of diagnosis, and between the calendar period and the age of diagnosis, were also analysed. Two age groups (</= 50 years old and >50 years old) were used to represent the premenopausal and postmenopausal age groups in this analysis. The incidence rates were calculated using the number of invasive breast cancer cases out of the total female population of each country for each time period. The cause of death information was used only to calculate the cause-specific mortality rate, which is the total of breast cancer deaths divided by the total female population of each country. Five-year incidence and mortality rates were reported and were age-standardized to the world standard population (123). STATA 8.2 (StataCorp. College Station, TX: Stata Corporation) was used for the statistical analyses.

3.2 Results

Study population

Table 5 presents the characteristics of the women in Singapore and Stockholm, diagnosed with breast cancer from 1980 to 1999. At the end of 2005, 9,330 (55%) of the Swedish women and 4,782 (46%) of the Singaporean women had died. As Chinese made up the majority of the Singaporean cohort, Chinese women were also studied as a separate group. The median age at diagnosis was stable within each country over the years of diagnosis; however, the Singaporean women were more than a decade younger than those in Stockholm.

Stage information was only available for two-thirds of the Singapore cohort. The Swedish women were followed up for a median of 8.7 years (range 0.003 to 26.0 years) while the Singaporean women were followed up for 7.7 years (range 0.003 to 26.9 years).

Table 5 Characteristics of the Stockholm and Singapore breast cancer cohorts

Characteristics	Stockholm Breast Cancer Registry	Singapore Cancer Registry	Singapore Cancer Registry (Chinese only)
Period of diagnosis	1980-1999	1980-1999	1980-1999
Number of breast cancer cases	17090	10287	8663
1980-1989 (%)	7932 (46)	3135 (30)	2661 (31)
1990-1999 (%)	9148 (53)	7152 (70)	6002 (69)
Median age at diagnosis	62	52	52
Standard deviation	14	13	13
Range(years)	18 - 101	12 - 98	17 – 98
Diagnosed < or = 50 years (%)	4247 (25)	5360 (52)	4472 (52)
Diagnosed >50 years (%)	12843 (75)	4927 (48)	4191 (48)
Number of deaths (%)	9330 (55)	4782 (46)	3896 (45)
Number of breast cancer deaths (%)	4050 (24)	2684 (26)	2159 (25)
Number of women with information on stage* (%)	16869 (99)	6569 (64)	5544 (64)
1980-1989			
Local cancer (%)	6047 (77)	1090 (48)	953 (50)
Regional cancer (%)	1667 (21)	961 (43)	804 (42)
Distant cancer (%)	165 (2)	196 (9)	155 (8)
1990-1999			
Local cancer (%)	7387 (82)	2283 (53)	1977 (55)
Regional cancer (%)	1358 (15)	1692 (39)	1393 (38)
Distant cancer (%)	245 (3)	347 (8)	262 (7)
Estrogen receptor status			
Positive	9874 (77)	not available	not available
Negative	2984 (33)	not available	not available

* Invasive cancers are local stage if they are confined entirely to the breast. Regional cancers are those that have extended beyond the limits of the breast directly into surrounding tissues or organs, or into lymph nodes in the region. Distant cancers are those that have spread beyond these localizations

Overall survival

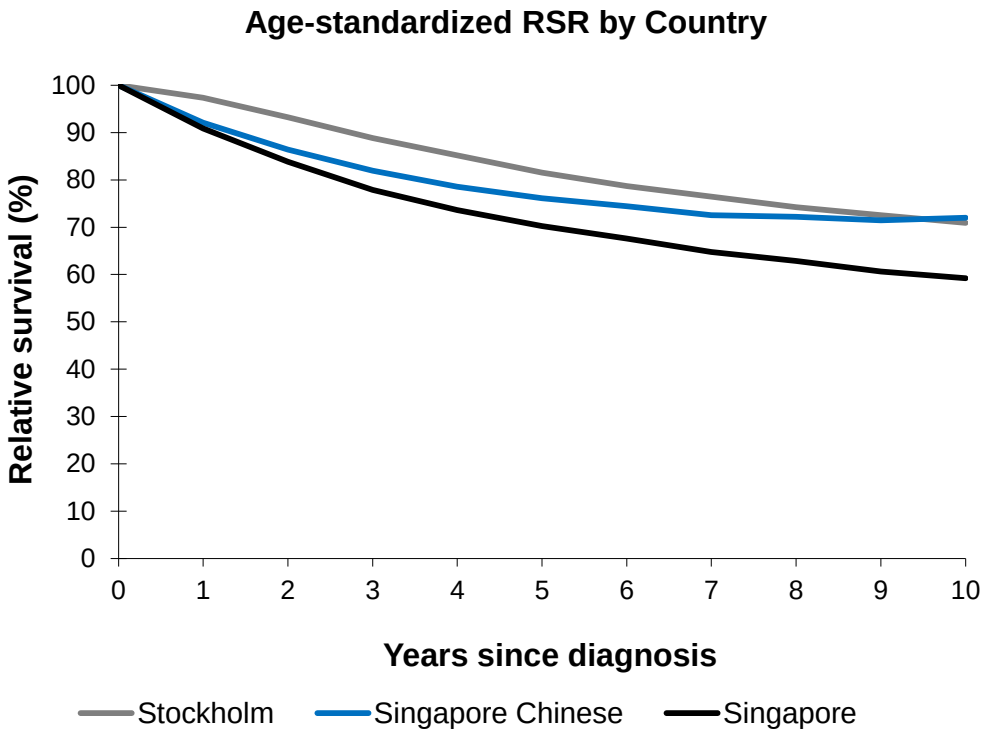


Figure 5 Overall age-standardized relative survival of women diagnosed with breast cancer from 1980 to 1999 in Singapore and in Stockholm in relation to time since diagnosis

The overall relative survival for women diagnosed with breast cancer from 1980 to 1999 appeared better for the Swedish cohort, where the Swedish women constantly outperformed the Singaporean women at each year of follow-up (Figure 5). The overall age-standardized 5-year relative survival for Singaporean women and for Swedish women was significantly different at 70% and 82%, respectively. The Singaporean Chinese has an overall age-standardized 5-year relative survival in between at 76% (Figure 5). However, the overall age-standardized 10-year relative survival of the Singapore Chinese women approaches that of the Swedish women (Figure 5).

Table 6 Overall and 5-year age standardized relative survival of women with breast cancer in

Characteristic	Stockholm Breast Cancer Registry		Singapore Cancer Registry		Singapore Cancer Registry (Chinese)	
5-year survival rates	%	95% CI	%	95% CI	%	95% CI
Overall observed survival	74	72 - 75	64	62 - 67	66	63 - 68
Overall relative survival	82	80 - 83	70	67 - 73	76	73 - 79
Specific 5-year relative survival						
Local						
1980-1989	88	85 - 91	81	71 - 89	86	75 - 94
1990-1999	88	85 - 90	90	85 - 95	99	92 - 105
Regional						
1980-1989	58	51 - 65	47	37 - 57	50	38 - 61
1990-1999	64	57 - 71	68	61 - 75	74	65 - 82
Distant						
1980-1989	19	6 - 37	21	8 - 39	26	10 - 49
1990-1999	21	9 - 37	28	17 - 42	33	18 - 49

Survival by stage

The majority of Swedish patients were diagnosed with a localized cancer while only about one-half of the Singaporean women had localized cancer (Table 5). Over the past 20 years there has been a small increase in women presenting with localized disease with a larger corresponding decrease in regional disease in the Singaporean women; the proportion of metastatic cases remained fairly constant (Table 5). When all of the women diagnosed in 1980 to 1999 were stratified by the stage of breast cancer, there was no difference in the relative survival between the two countries, though the Singaporean Chinese appear to have slightly better relative survival in the earlier stages (Figure 6).

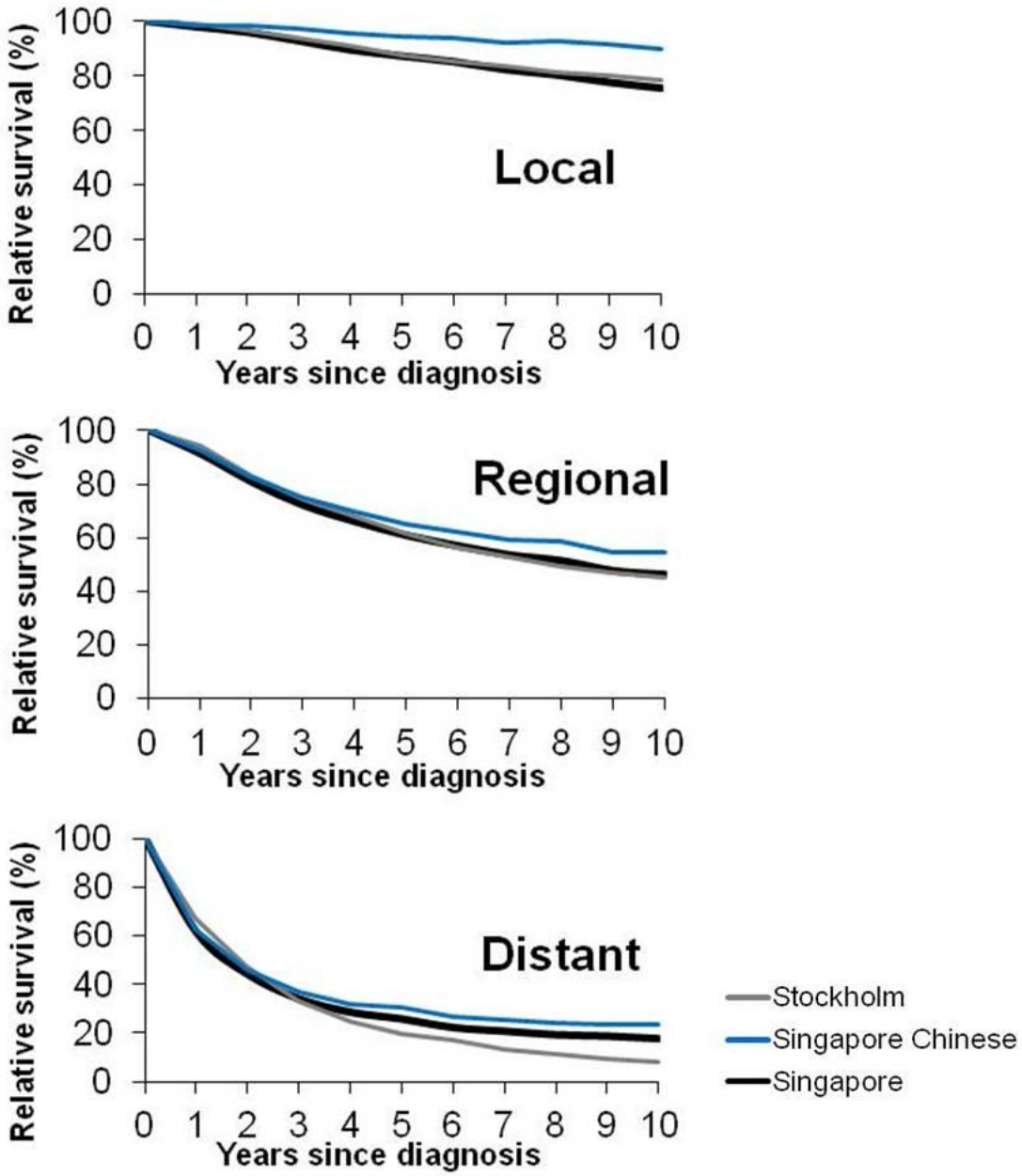


Figure 6 Age-standardized relative survival of women diagnosed with breast cancer from 1980 to 1999 in Singapore and in Stockholm by the stage of cancer in relation to time since diagnosis

Survival by period of diagnosis

The 5-year age-standardized relative survival ratio was used to compare the survival by the period of diagnosis between the two cohorts. In the 1980s the Singaporean women showed an improvement in survival across all stages, most marked in those with regional disease (with improvement of almost 20%); a smaller improvement was seen in the Swedish women with the same stage. Singaporean women diagnosed with local cancer and regional cancer had poorer survival than the Swedish women (Table 6 and Figure 8). In the 1990s survival among the Singaporean women with localized cancer and regional cancer improved and was comparable with the Swedish women. In addition, these Singaporean women with local cancer showed a marginal survival advantage over the Swedish cohort in the later period.

Survival by age group

Singaporean women were diagnosed with breast cancer earlier in life than the Swedish women; they were on average 10 years younger. While 52% of the Singaporean women diagnosed with breast cancer were 50 years old or younger, only 25% of the Swedish women were 50 years old or younger (Table 5). Over the two decades, the median age in the Stockholm women decreased from 64 to 61 years while it remained stable at 50 years old for the Singaporean women. The prognostic outlook was more optimistic for women with breast cancer when they were between 35 and 75 years old. This was consistent in both countries (Table 7).

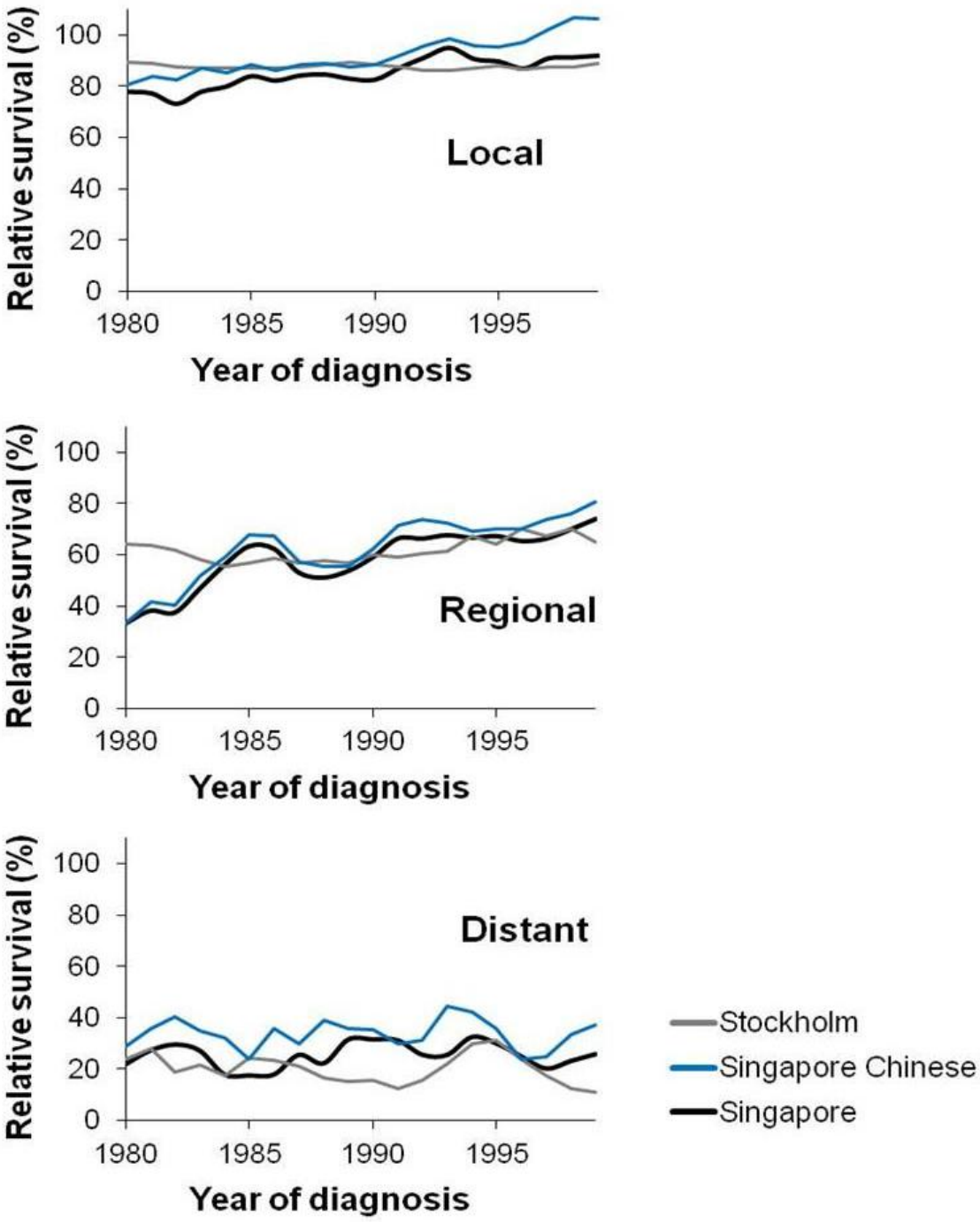


Figure 7 Five-year age-standardized relative survival of women with breast cancer in Singapore and in Stockholm by the stage of breast cancer and the calendar year of diagnosis (3-year centred moving average).

Poisson regression

Figure 8 presents the risk of death of the women taking age, disease stage, and period of diagnosis and years of follow-up into account in each country. As expected, the stage of cancer is an important predictor of survival. The risk of death was decreased in the later period for both countries and improvement was greater in the Singaporean women. As there was a significant interaction between the age at diagnosis and the period of diagnosis ($p = 0.008$), comparison between the two populations was performed stratified by the age and period of diagnosis.

Table 7 Poisson regression: excess risk of death by stratified by country

	Singapore				Singapore (Chinese)				Stockholm			
	RR	95% CI		*p value*	RR	95% CI		*p value*	RR	95% CI		*p value*
Year of follow-up												
1	1.00	(reference)			1.00	(reference)			1.00	(reference)		
2	1.32	1.14	1.51	<0.0001	1.36	1.15	1.60	<0.0001	1.69	1.47	1.94	<0.0001
3	1.21	1.04	1.40	0.013	1.25	1.05	1.49	0.014	1.90	1.65	2.18	<0.0001
4	1.12	0.95	1.31	0.178	1.17	0.97	1.41	0.106	1.71	1.48	1.98	<0.0001
5	0.96	0.81	1.14	0.631	0.98	0.80	1.21	0.859	1.84	1.59	2.14	<0.0001
Age group												
<35	1.00	(reference)			1.00	(reference)			1.00	(reference)		
35-54	0.65	0.54	0.78	<0.0001	0.67	0.54	0.84	<0.0001	0.71	0.57	0.88	0.002
45-54	0.66	0.55	0.79	<0.0001	0.70	0.57	0.87	0.001	0.56	0.45	0.69	<0.0001
55-64	0.91	0.75	1.10	0.314	0.95	0.76	1.19	0.675	0.60	0.49	0.74	<0.0001
65-74	0.77	0.62	0.96	0.022	0.71	0.54	0.94	0.016	0.78	0.64	0.96	0.019
75+	1.06	0.81	1.39	0.673	0.79	0.54	1.14	0.201	0.89	0.71	1.10	0.281
Stage												
Local	1.00	(reference)			1.00	(reference)			1.00	(reference)		
Regional	3.66	3.22	4.17	<0.0001	4.18	3.57	4.89	<0.0001	4.02	3.66	4.41	<0.0001
Distant	13.29	11.41	15.48	<0.0001	15.19	12.64	18.26	<0.0001	17.25	15.09	19.71	<0.0001
Period of diagnosis												
1980-89	1.00	(reference)			1.00	(reference)			1.00	(reference)		
1990-99	0.53	0.48	0.58	<0.0001	0.50	0.45	0.56	<0.0001	0.91	0.83	0.99	0.027

Table 8 Poisson regression: excess risk of death between countries stratified by age and period of diagnosis

	RR	95% CI		*p value*	RR	95% CI		*p value*
	1980-89				**1990-99**			
Age <=50								
Country*								
Stockholm	1.00	(reference)			1.00	(reference)		
Singapore	1.26	1.08	1.47	0.003	0.81	0.70	0.94	0.007
Age >50								
Country*								
Stockholm	1.00	(reference)			1.00	(reference)		
Singapore	1.48	1.29	1.69	<0.0001	0.78	0.68	0.89	<0.0001
	1980-89				**1990-99**			
Age <=50								
Country*								
Stockholm	1.00	(reference)			1.00	(reference)		
Singapore (Ch)	1.15	0.98	1.36	0.085	0.70	0.60	0.83	<0.0001
Age >50								
Country*								
Stockholm	1.00	(reference)			1.00	(reference)		
Singapore (Ch)	1.30	1.12	1.51	0.001	0.64	0.55	0.75	<0.0001

*Adjusted for year of follow up and stage
Ch: Chinese

Interaction and effect modification

As there was a significant interaction between the age at diagnosis and the period of diagnosis (p = 0.008), comparison between the two populations was performed stratified by the age and period of diagnosis (Table 8) to show the difference in effects.

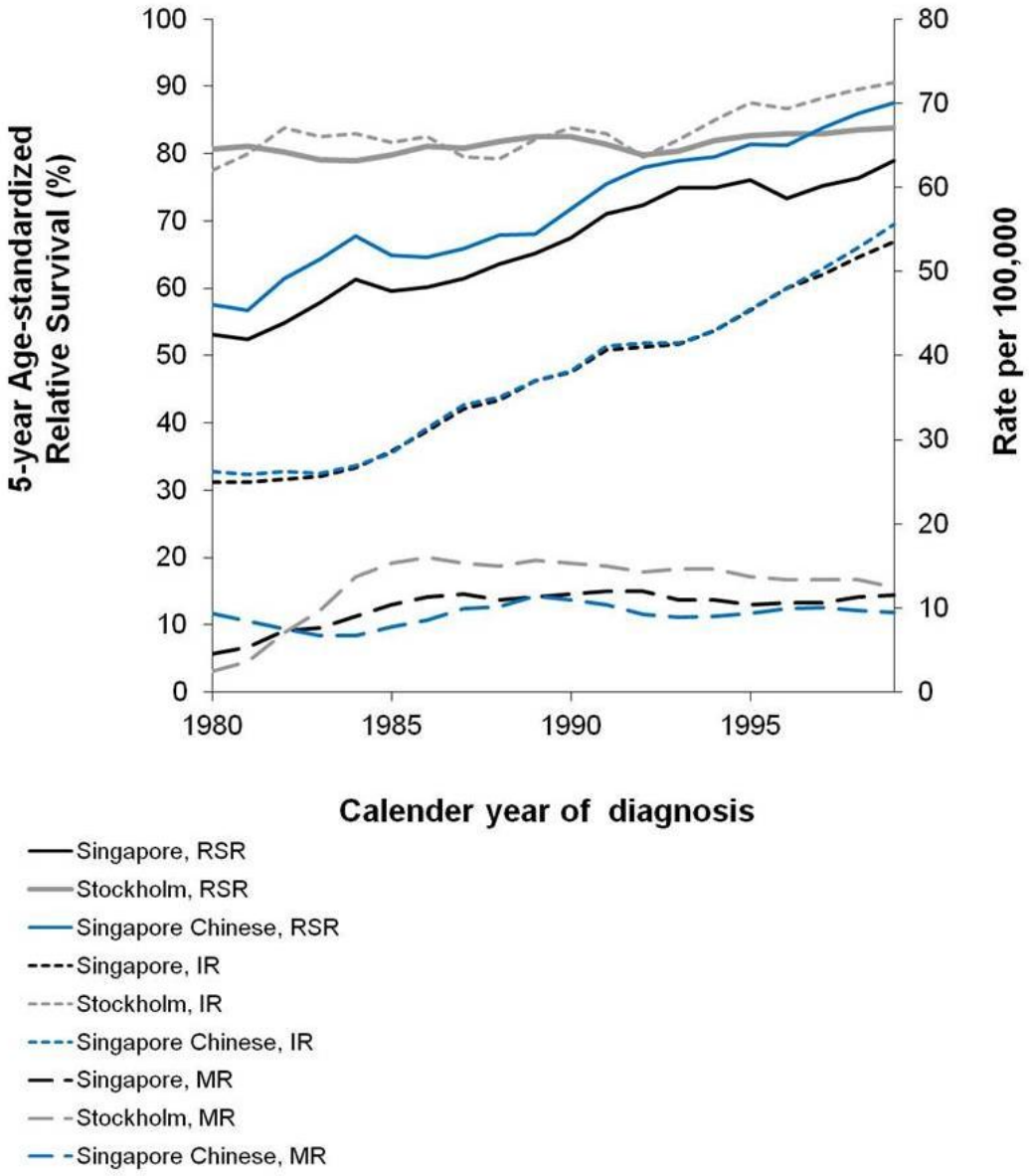
In 1980 to 1989 premenopausal Singaporean women had 26% increased risk of death compared with the women in Stockholm, adjusted for stage and year of follow-up, while the postmenopausal women had 48% increased risk. In 1990 to 1999 the Singaporean women experienced a decreased risk of death of 19% and 22%, respectively, for the premenopausal women and the postmenopausal women compared with the Swedish women; Comparison with the Singapore Chinese women showed a decreased risk of death of 30% and 36% (Table 8).

In Stockholm, survival improved only in the premenopausal women over the two decades (hazard ratio = 0.74, $P < 0.001$). In Singapore, the improvement was for all women over the same period (hazard ratio = 0.51 and hazard ratio = 0.54 for premenopausal women and postmenopausal women, $P <$ and $P < 0.001$, respectively; data not shown).

Survival in comparison with incidence and mortality rates

The incidence rate of breast cancer in Singaporean women has more than doubled during the period of study (1980 to 1999). In contrast, the increase in incidence in the Swedish women was only modest. The 5-year cause-specific mortality rate was constant in the Singaporean women from 1985 to 1999 (Figure 8). The mortality rate for the Swedish women also remained relatively constant over the period. The marked improvement in relative survival in the Singaporean women over the two decades is consistent with the discrepancies of the incidence and mortality rates described; the survival in the Swedish women was stable.

Figure 8 Trends in incidence, 5-year cause specific mortality and 5-year age-standardized relative survival rates in patients with breast cancer in Singapore and Stockholm across calendar year of diagnosis
IR, Incidence Rate; MR, Mortality Rate; RSR, Relative Survival ratio



The effect of ER status in the Stockholm population

The ER status of the Singaporean population was not available for comparison. Study of the effect of ER-positive tumours within the Stockholm population showed the survival advantage (Table 9 and Figure 9)

Table 9 Poisson regression: excess risk of death for women in Stockholm

	RR	95% CI		*p value*
Year of follow-up				
1	1.0	(reference)		
2	3.2	2.6	4.1	<0.005
3	3.8	3.0	4.9	<0.005
4	3.4	2.7	4.3	<0.005
5	3.7	2.9	4.7	<0.005
Age group				
<35	1.0	(reference)		
35-54	0.7	0.5	0.9	0.006
45-54	0.6	0.4	0.7	<0.005
55-64	0.6	0.5	0.8	<0.005
65-74	0.8	0.7	1.1	0.174
75+	0.8	0.6	1.1	0.145
Stage				
Local	1.0	(reference)		
Regional	3.1	2.8	3.5	<0.005
Distant	20.8	15.8	27.5	<0.005
Period of diagnosis				
1980-89	1.0	(reference)		
1990-99	0.9	0.8	1.0	0.208
ER status				
ER positive	1.0	(reference)		
ER negative	2.6	2.4	2.9	<0.005

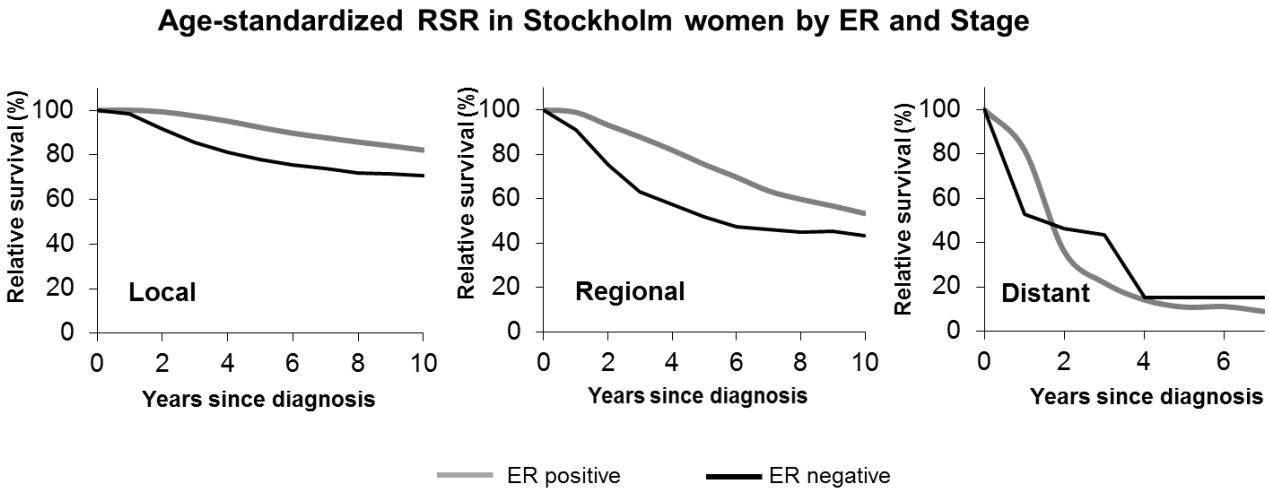


Figure 9 Age-standardized relative survival of women diagnosed with breast cancer from 1980 to 1999 in Stockholm by ER status and stage of cancer in relation to time since diagnosis.

3.3 Discussion

Methodological considerations

Internal validity can be defined as the absence of systematic errors within the study. The classification of systematic errors is not clear cut, but may be classified as bias and confounding. Systematic errors are not affected by sample size, which is different from random variable, which is reduced with increased sample size.

Intermediate factors are factors caused by the exposure and causing the outcome and are not confounders. Tumour characteristics are considered intermediate factors between exposure and breast cancer deaths.

Interaction refers to factors acting together (synergistic or antagonistic) on the outcome. Compared to the main effects in a study, interaction between two variables always have a much lower power (124).

Precision is a measure of random errors, the influence of chance that leads to uncertainty of the estimates found in a study. Precision is improved with increase in sample size.

External validity is the possibility to generalise the finding to other populations than the one under study. The first requirement is internal validity and enough precision; it depends on the knowledge on or assumptions of biological interactions with other exposures that might vary between populations.

Study design

This is a population based observational or cohort study. Generally, cohort studies are expensive and time-consuming for rare-outcomes requiring exposure information on a large number of individuals to obtain an adequate number of persons with the outcome of interest. Population register based studies however overcome this drawback of cohort studies; being rather cheap and covering significantly more patients (e.g. over 10,000 for a single site) followed for a long period. It gives good power to address the question of prognosis in relation to the various factors of interest.

Bias

Bias may be defined as any systematic error that results in an invalid estimate of the association between exposure and outcome of the disease. It is caused by faulty design and/or deficient execution of sampling process.

Selection bias: Study Population and Cancer Registries

The strengths of the study 1 include the large number of cases, from population-based registries that report high levels of reliability (120; 125). This reduces the issues of selection bias in smaller cohort studies. Women in both countries have individual unique national registration numbers as citizens and permanent residents that allow for accurate personal data collection. Women with bilateral breast cancers or multiple cancers can be excluded from the study. The study also extends over two decades, which was probably long enough to observe differences and allow the study of trends. Women with a previous malignancy, including contralateral breast cancer, and those diagnosed with breast cancer at autopsy (death certificate only) were excluded from the study to remove the bias these factors may cause.

Time bias with Breast screening

Survival time is measured from the date of diagnosis to the date of death; however, diagnosis does not occur at the same point in the natural history for every patient, so care is required when interpreting survival estimates. Earlier diagnosis by screening can cause ***lead-time bias*** and falsely depict better survival, where the time of diagnosis was earlier while the death from breast cancer was not delayed or avoided. Without a nationwide breast screening program in Singapore, the lead-time bias could not be an advantage for

Singapore to explain the decreased risk of death for 1990 to 1999. Opportunistic screening is available and widely used in clinics, however, as healthcare and health awareness improved with the socioeconomic status of Singapore in the 1990s. ***Length-time bias*** may exist and is a limitation in this comparison since screening is probably more extensive, having been implemented early in Stockholm. We would expect a more favourable outcome in the Swedish population when a greater proportion of slow-growing breast cancer with good prognosis is being diagnosed (126). This is difficult to quantify without a randomized trial. Cultural differences amongst ethnic groups in Singapore may also contribute towards response to breast screening and hence result in differences in stage distribution of breast cancer amongst the ethnic groups (see discussion on stage distribution).

Confounding

Associations between a variable and outcome can be affected by a third variable, a confounder. The confounder must be a risk factor of the outcome associated with the exposure variable, and not caused by the exposure or outcome. Confounding factors such as registry completeness, stage migration and distribution and diagnostic improvements have to be considered when analysing trends in cancer survival. As ethnicity can be an important confounder for population comparisons, this was addressed in the study.

Stage and clinical information

Incomplete disease stage information for one-third of women in Singapore is a limitation of the study. To our knowledge, every effort has been put in to ensure the completeness of cancer reporting over the years. Clinical staging information, which used to be reported voluntarily, could contribute to the lack of information. There is now a follow-up mechanism by the registry, however, to obtain detailed clinical information from the clinical case notes. The proportion of unknown disease stage was hence worst in the early 1990s, only 52% with complete clinical staging for cases in 1990 to 1994 ($p < 0.001$), and this improved in the last 5 years of the study. This incomplete staging is probably random; however, as the age-standardized survival for the Singaporean women with unknown disease stage was comparable with the overall survival of those with stage information (data not shown). The ethnic distribution between the two groups was not different ($p = 0.576$), and the age distribution by the period of diagnosis was also similar except for fewer women between 35 and 54 years old being diagnosed in 1985 to 1989 ($p = 0.032$).

The ***accuracy of staging*** could have affected the stage distribution and should be regarded with caution. It is possible that, in the earlier years, node-positive tumours were underdiagnosed and falsely classified as being localized, and hence appeared to have poorer survival. The axillary dissection and histologic assessment of specimens may have been less thorough. The proportion of such cases is unknown in this study, but is probably small. Active screening for distant metastases at the time of initial diagnosis, a practice routinely adopted in Singapore but not in Sweden, can induce stage migration and increase the stage-dependent survival in all stages to the benefit of the Singaporean women;

Swedish women with clinically occult distant metastasis may have been under-staged.

Ethnic Differences

Sweden has a relatively homogenous in its ethnicity of white Europeans while in Singapore, Chinese form a majority of about 80 per cent of the population. As ethnicity can be an important confounder for population comparisons, a comparison with Singaporean Chinese was made with the Stockholm population. This showed a slight improved survival amongst the Chinese population compared to the general population.

Others factors

Treatment information is not available in the study 1. This is also a limiting factor as confounding due to differences in treatment between the groups may impact the survival outcome. Other factors such as body mass index, diet, and other behavioural, cultural, environmental, or genetic differences are factors that have been known to affect incidence (38), but the effect on prognosis is less known (57), and is not available in these studies. Singapore, however, reported fewer women who smoke 3.2% (2004 National Health Survey statistics) (127) compared with 29% of Swedish women in 1980, and 18% in 2005 (Sweden statistics). Only about 6% of postmenopausal women in Singapore are on hormonal replacement therapy for menopause (128), compared with 21% of women in Sweden (129). Obesity (BMI > 30kg/m2) was approximately 10 % of women in Sweden in a 2006 report (130) and 6 % of the population in Singapore (2004 National Health Survey statistics) (127). Alcohol consumption reported to be 0.3% in women in Singapore (2007 National Health Survey statistics).

Study of survival trends

Relative survival has become the preferred measure for the analysis of patient survival based on data from population-based cancer registries. The relative survival ratio (RSR) is defined as the observed survival in the patient group divided by the expected survival of a comparable group from the general population, matched to the patients with respect to the main factors affecting patient survival and assumed to be practically free of the cancer of interest.

The strength of the relative survival ratio is that it provides a measure of the excess mortality experienced by patients diagnosed with cancer, irrespective of whether the excess mortality is directly or indirectly attributable to the cancer. An advantage of this measure is that information on cause of death is not required; thereby circumventing problems with the inaccuracy or non-availability of death certificates. This method is preferable when cause of death classification is unreliable, but this was not a likely issue with our studies. Similar to all-cause mortality, this is inferior compared to cause-specific death when the cases are expected to have a different overall mortality than the base population. However, most women in the studies died from breast cancer, hence competing risks are not likely to influence the results.

As advocated by Dickman and Adami, the trends in survival were interpreted in context with the incidence and mortality rates to evaluate the progress against cancer (61). The marked improvement in relative survival in the Singaporean women over the two decades is consistent with the discrepancies of the incidence and mortality rates described.

Effects of Stage distribution

The observation of the overall survival advantage in Stockholm (Figure 1.1) occurs because the proportion of women diagnosed with a local cancer (80%) was larger than that of Singaporean women (51%), (Table 1.1). This larger diagnosis is a consequence of established organized mammographic screening in Sweden since the late 1980s, which became nationwide in the mid-1990s. This advantage was present throughout the study period as more Swedish women were consistently being diagnosed with localized disease (Table 1.1). The effect of stage distribution being a key reason for the difference in survival of the Singaporean population compared with the Stockholm population is reminiscent of a study from Stockholm in the period 1961 to 1973 (131), and is again reflected in a later comparison of screened and non-screened Danish and Swedish populations (132). This survival advantage of the women in Stockholm disappeared when the observations are stratified by stage, with the Singaporean women enjoying better survival in the later years of diagnosis.

Effects of Period of diagnosis

When compared across the period of diagnosis in the study, there was a consistent overall decreased risk of death. There was decreased risk of death in the premenopausal women in both populations and in the postmenopausal women in Singapore, signifying improvements in both countries. In the later period where nationwide screening was still not present in Singapore, the Singaporean women were performing no worse than the Swedish women (Figure 1.3). Interestingly, after adjusting for potential confounders, there was a mean 19%

(CI 6% to 30%) decrease in risk of death in the premenopausal and a mean 22% (CI 11% to 32%) decrease in the postmenopausal Singaporean women as compared with Swedish women during the period 1990 to 1999 (Table 1.4).

Improved health care services

A significant change in Singapore during the study period is that Singapore underwent much economic restructuring in the 1980s and 1990s, resulting in marked *economic improvements* over these two decades (68). The increase in Gross Domestic Product was 725% in Singapore and 190% in Sweden from 1980 to 1999 (International Monetary Fund statistics). This resulted in improved living standards, improved education and presumably better awareness of the disease and better healthcare including breast screening – albeit opportunistic – in Singapore.

Like Sweden, Singapore enjoys a large network of affordable primary healthcare services that refer to government-funded (Sweden) or heavily subsidized (Singapore) hospital and specialist care services. Healthcare indicators such as life expectancy, the infant mortality rate and the hospital to population ratio are comparable (69; 70). This coincided with a time trend towards less advanced tumours being diagnosed, where a small but definite increase in women with localized disease and a corresponding decrease in women with regional disease over the study period was observed in Singapore (Table 1.1). This is also supported by the finding of an increase in incidence of ductal carcinoma in situ from 0.4% in 1983 to 1989 to 8.1% in 1999 (Singapore Cancer Registry statistics), an indicator of increased mammographic screening.

As compared with the Swedish women who had enjoyed a more stable economy during this period, the small increase in incidence of localised breast cancer in Singapore was probably the result of initiation of screening practices that came with increased awareness, better education, and better healthcare facilities as our economy improved, to a greater benefit for the Singaporean women, even without a national screening program.

Better breast cancer therapies

Anti-oestrogen treatment has been well established since the 1990s. Seventy-seven per cent of the Swedish women had tumours that were positive for the ER. This is in contrast to a report of 55% Singaporean women from a local institution (133) in 2007. The Swedish women would be expected to perform better in each stage if the receptor status was indeed stage independent. Although the survival advantage for the Swedish women with ER-positive tumours, the present study could not demonstrate the postulated survival advantage of ER-positive tumours between the two populations, or perhaps the effect of this factor is small. Nevertheless, the improvement of survival only in the premenopausal Swedish women across the period is probably the result of increased aggressiveness in treating this group of women, with tamoxifen (134) as well as chemotherapy.

In addition, the improvement in survival in each stage across the periods – including for those with distant metastasis, albeit small (Figure 1.3 and Table 1.2) – would suggest improvement in treatment options with the use of adjuvant therapy with anti-oestrogen and chemotherapy that had become more standardized in the 1990s in Singapore. The quality of the healthcare and

treatment routines probably did not differ to a larger extent or to the benefit of Swedish women in the later part of the follow-up period. Treatment details are not available for these patients and are a study limitation. Treatment of breast cancer, however, was standardized under institutional practice in both Stockholm and Singapore in the 1990s, and this was comparable (personal communication with P Hall, Stockholm, and CY Wong, Singapore). The survival equivalence after stratification by stage (Figure 1.2) suggests that both Singaporean women and Swedish women respond similarly to the treatments given.

The relatively stable mortality and the corresponding marked increase in the survival ratios in the Singaporean women probably represented improved treatment options. The more superior survival ratios in the earlier years of diagnosis in the Swedish women remained relatively stable, while the increase in incidence coincided with a less dramatic change in the survival compared with the Singaporean women (Figure 1.4).

Ethnic differences

Sweden has a relatively homogenous in its ethnicity of white Europeans while in Singapore, Chinese form a majority of about 80 per cent of the population. Until the 1970s Singaporean women had a mean fertility rate of approximately five children compared with two children for Swedish women (44). This higher fertility in the earlier Singaporean birth cohort contributed to the low incidence of breast cancer, especially postmenopausal breast cancer. Unfortunately, this may have resulted in a higher proportion of premenopausal breast cancer (Table 1.1), which is more likely to be ER-negative with poorer survival. Parity may therefore

not only affect risk (43; 135–137), but also the prognosis of breast cancer. This study is limited by the absence of information of the tumour receptor status as well as the unavailability of complete parity information in the Singaporean cohort. Although after adjusting for stage, age and period of diagnosis, the survival between this two ethnically different populations were not largely different. It remains unsettled whether risk factors of breast cancer that influence the cancer incidence and malignant phenotype will affect the prognosis.

Information of risk factors such as BMI, other behavioural habits such as smoking and alcohol, HRT, environmental factors and menstrual history were not available in these studies. However, the incidence of obesity, HRT, alcohol consumption and smoking were known to be low in Singapore and less likely to influence the tumour biology and survival.

Future studies could include using a more recent cohort where both populations are relatively stable in the economy, and when therapy for breast cancer is standardized, as well as inclusion of other risk factors of interest such as tumour biology, treatment, parity and effect of the SES.

3.4 Conclusion

The survival of women with breast cancer in each disease stage was comparable between the Singapore and Stockholm cohorts. Better economic status associated with increased awareness of the disease, better access to screening routines or healthcare quality and options, seen in Stockholm and in Singapore

only in the later decade, offer the main explanation for the prognostic differences and similarities.

CHAPTER 4 STUDY 2: SURVIVAL OF SINGAPOREAN WOMEN WITH BREAST CANCER: ETHNIC DIFFERENCES

4.1 Subjects and Methods

All cases of invasive breast cancer diagnosed from 1 January 1968 to 31 December 2006 were obtained from the Singapore Cancer Registry, National Registry of Diseases Office (NDRO). Patients with a previous malignancy, including contralateral breast cancer, and those diagnosed with breast cancer at autopsy (death certificate only) were excluded from the study. Follow-up was performed until 31 December 2008 by matching with the national death register. The cause of death was coded in accordance with the International Classification of Diseases and Causes of Death ICD9. The ethical committee at the National University of Singapore accepted the study without any restrictions. This is normal when de-identified material is used.

For the comparison between ethnic Chinese, Malays and Indians in Singapore, 20517 women diagnosed between 1968 and 2006 were included in the study. The methods were similar to study 1 and have been described in 3.1.

4.2 Results

Study population

Table 10 presents the characteristics of the Singaporean women diagnosed with breast cancer from 1968 to 2006 with follow up till 31 December 2008. At the end

of 2008, 7937 (39%) women had died. The Malay women tended to be younger and were diagnosed in a later stage compared to the Chinese and Indians.

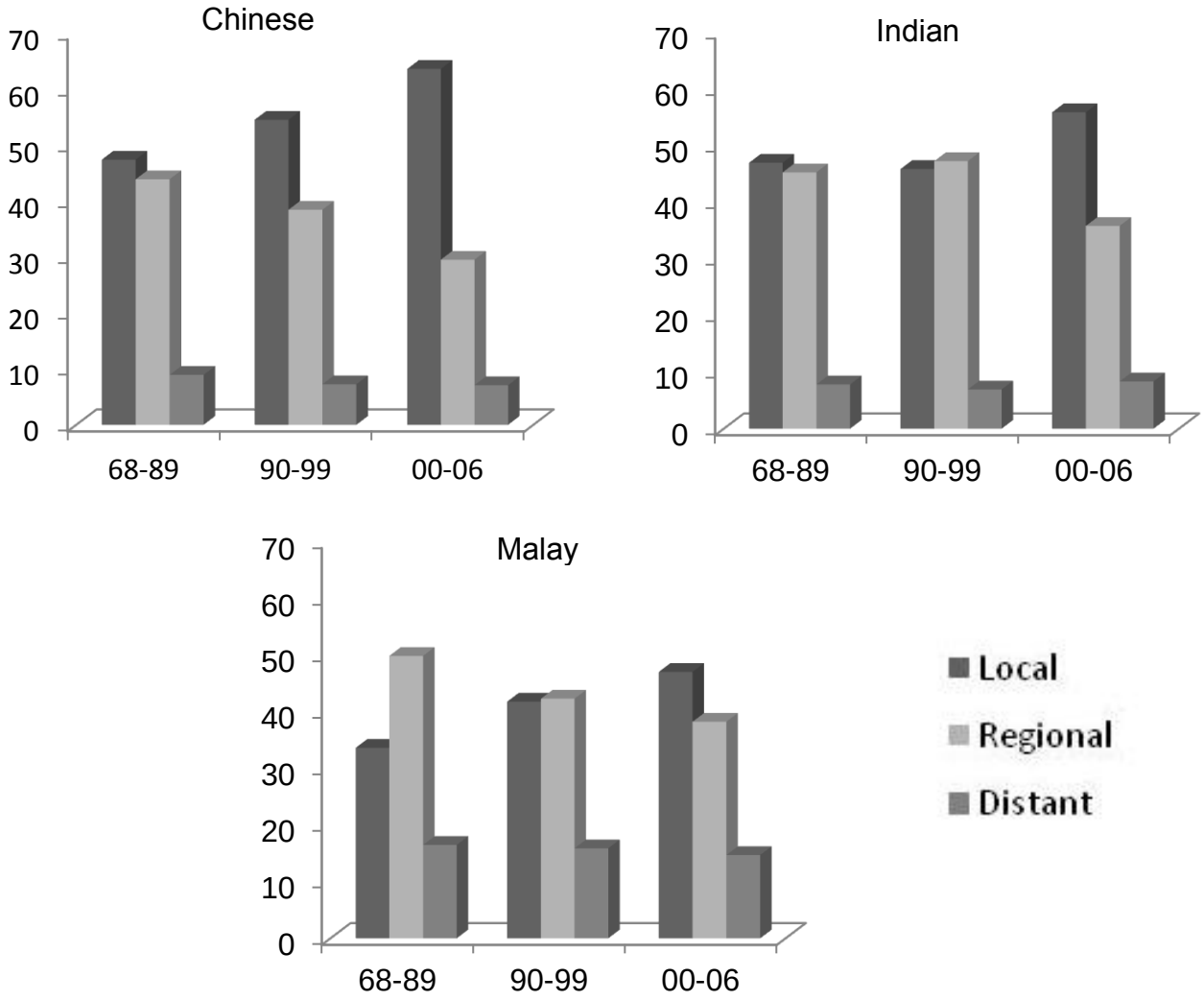
Table 10 Characteristics of Singaporean breast cancer cohort by ethnicity

Characteristic	Chinese	Malays	Indians	Total	p value
Frequency (%)	17499 (85)	2007 (10)	1011 (5)	20517 (100)	
Age					
</= 50 (%)	8470 (48)	1203 (60)	464 (46)	10137 (49)	<0.005
>50 (%)	9029 (52)	804 (40)	547 (54)	10380 (51)	
Stage (% known)	12411 (71)	1389 (69)	730 (72)	14530 (71)	
Local	7041 (57)	590 (42)	370 (51)	8001 (55)	<0.005
Regional	4431 (36)	584 (42)	303 (42)	5318 (37)	
Distant	939 (8)	215 (15)	57 (8)	1211 (8)	
Deaths	6518 (37)	993 (49)	426 (42)	7937 (39)	<0.005
Breast cancer deaths	4736 (27)	795 (40)	311 (31)	5842 (28)	<0.005

Stage distribution over calendar period

Over the calendar years, there is an increase in the proportion of women diagnosed with local stage cancer and a corresponding decrease in those with regional disease. This trend is consistent across the ethnic groups, with the Chinese showing most improvement. Amongst the Malays, the higher proportion of those with regional disease reversed in the latest calendar period (Figure 10).

Figure 10 Stage distributions of women with breast cancer by ethnicity and calendar period.



Overall survival by ethnicity

The overall relative survival for women diagnosed with breast cancer from 1968 to 2006 appeared better for the Chinese, with the Malays showing the poorest survival (Figure 11). The overall age-standardized 5-year relative survival for the

Chinese, Malays and Indians was significantly different at 79%, 59% and 72%, respectively (Table 11).

Figure 11 Age standardised relative survival rate by ethnicity and in relation to years since diagnosis

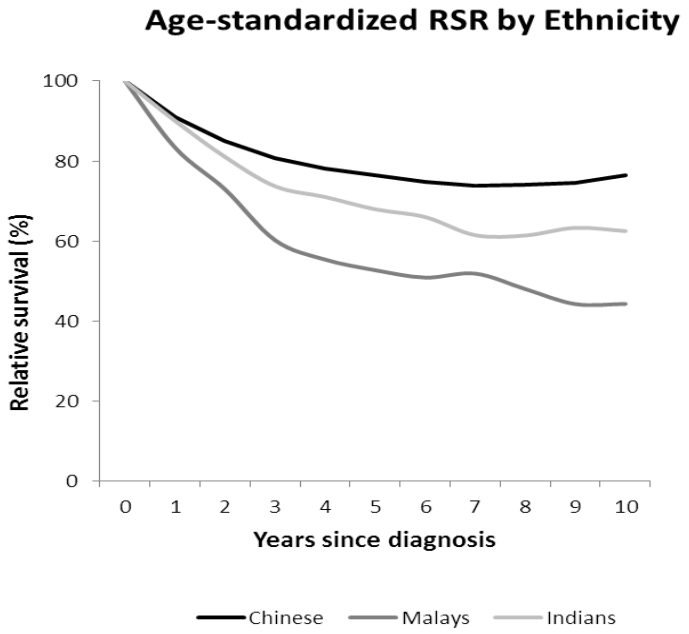


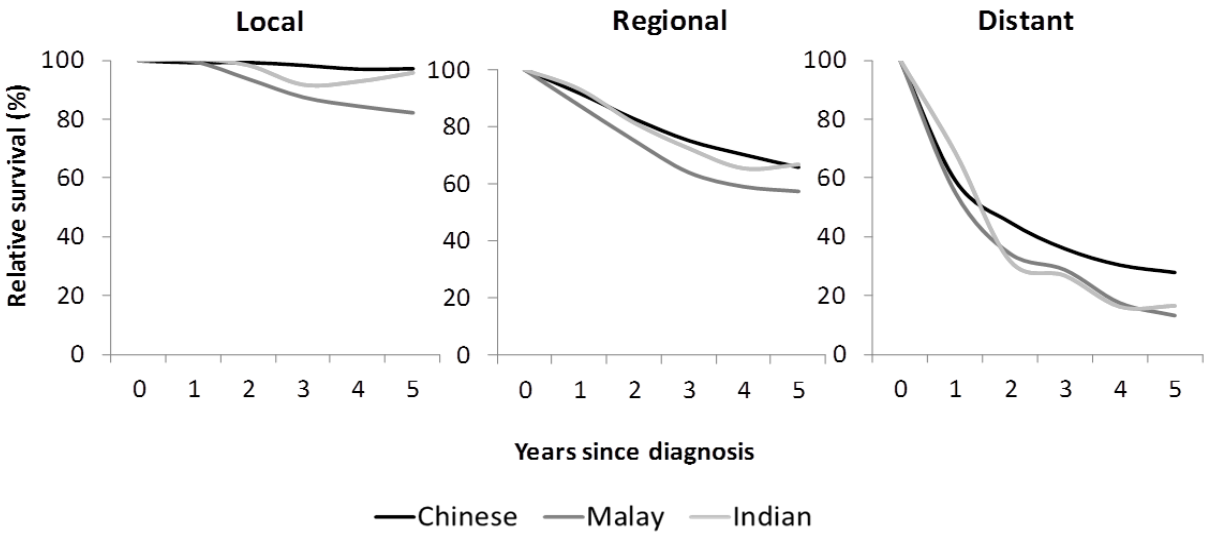
Table 11 Relative 2, 5 and 10-year survival ratios by ethnicity

	Chinese		Malays		Indians	
Characteristic	95% CI		95% CI		95% CI	
2-year survival rates						
Overall relative survival	90	88 - 92	76	67 - 83	87	76 - 95
Overall observed survival	84	83 - 86	72	64 - 78	82	72 - 89
5-year survival rates						
Overall relative survival	79	76 - 82	59	48 - 68	72	59 - 84
Overall observed survival	66	64 - 68	51	43 - 58	62	52 - 70
10-year survival rates						
Overall relative survival	77	71 - 83	44	31 - 63	63	43 - 87
Overall observed survival	47	45 - 50	32	25 - 41	45	34 - 56

Survival by ethnicity and stage

When the women were stratified by ethnicity, there was a survival advantage seen in the Chinese compared to the Malays. This trend was less consistent amongst the Indians (Figure 12).

Figure 12 Five-year relative survival ratios by ethnicity and stage in relation to years since diagnosis.



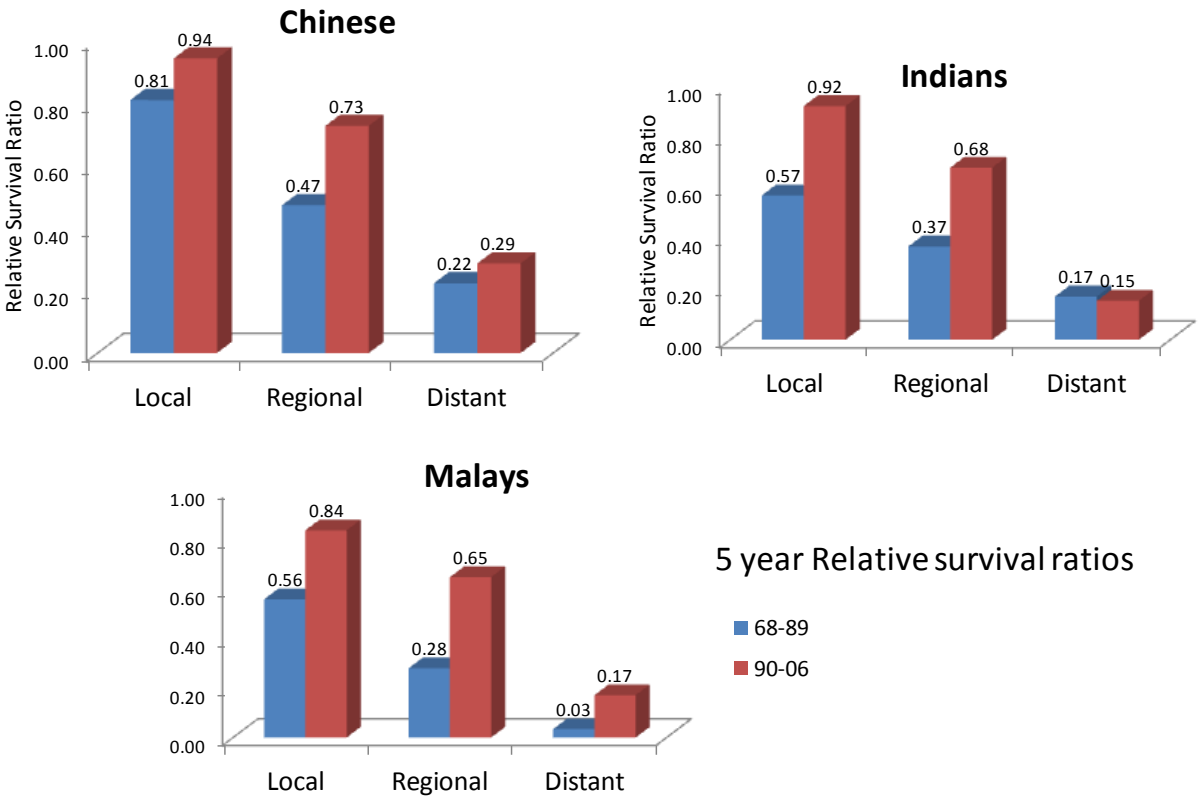
Survival by ethnicity, stage and calendar period

Table 12 and Figure 13 show the improved 5-year relative survival rates by ethnicity and stage over the calendar period of diagnosis. The Malays with regional disease diagnosed in the later calendar period made the largest improvement.

Table 12 Five-year relative survival ratios by ethnicity and stage over the calendar period of diagnosis

		Relative survival rate (CI)				Observed survival rate (CI)			
					1968-1989				
Chinese	Local	83	81	-	86	75	73	-	77
Malays		62	52	-	72	58	48	-	67
Indians		73	61	-	82	68	57	-	76
Chinese	Regional	50	47	-	53	44	42	-	47
Malays		35	27	-	43	33	26	-	40
Indians		45	34	-	57	42	31	-	53
Chinese	Distant	22	17	-	28	19	14	-	24
Malays		8	3	-	18	8	2	-	17
Indians		23	6	-	48	21	5	-	45
					1990-2003				
Chinese	Local	97	96	-	98	90	89	-	91
Malays		86	81	-	90	82	77	-	86
Indians		94	88	-	99	87	81	-	91
Chinese	Regional	76	73	-	78	70	68	-	72
Malays		64	57	-	69	60	54	-	66
Indians		72	63	-	79	67	59	-	73
Chinese	Distant	32	27	-	37	28	24	-	33
Malays		22	14	-	31	20	13	-	29
Indians		21	9	-	37	20	8	-	36

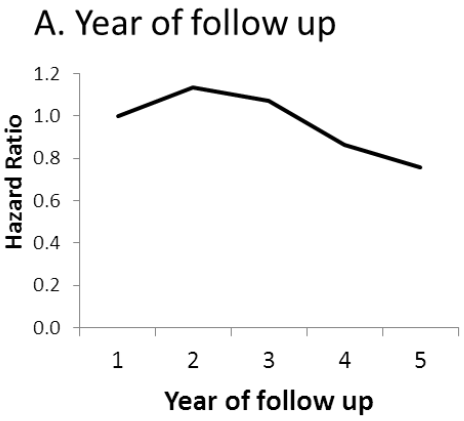
Figure 13 Five-year relative survival ratios by ethnicity, stage and calendar period of diagnosis.

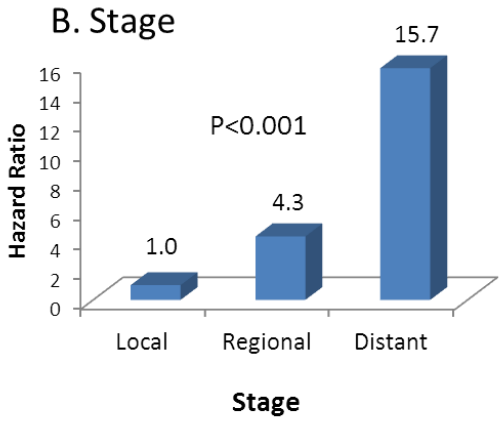


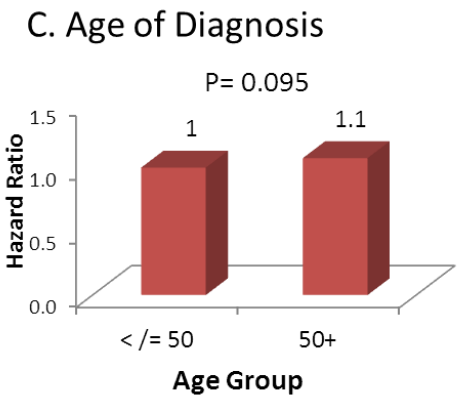
Poisson regression: excess risk of death

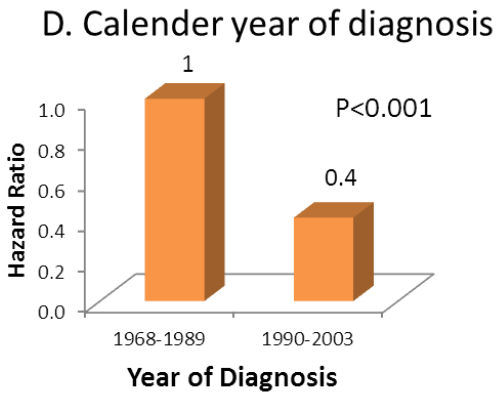
Figure 14 presents the risk of death of the women taking age, disease stage, period of diagnosis, years of follow-up and ethnicity. As expected, the stage of cancer is an important predictor of survival. The risk of death was decreased in the later period but the risk of death remained the highest amongst the Malays with some reduction in the later period (Table 13).

Figure 14 Poisson regression: adjusted excess risk of death by (A) Year of follow up; (B) Stage; (C) Age of diagnosis; (D) Calendar year of diagnosis; (E) Stage.









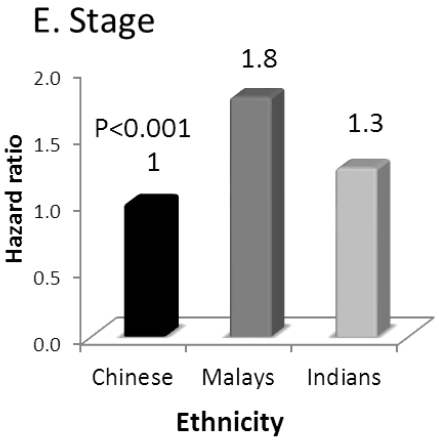


Table 13 Poisson regression: adjusted excess risk of death stratified by period of diagnosis

	RR	95% CI		*p-value*		RR	95% CI		*p-value*
Period of diagnosis		**1968-1989**					**1990-2003**		
Year of follow-up					**Year of follow-up**				
1	1.0	(reference)			1	1.0	(reference)		
2	1.1	1.0	1.3	0.162	2	1.3	1.1	1.6	0.001
3	1.0	0.9	1.2	0.979	3	1.3	1.1	1.6	0.002
4	0.7	0.6	0.9	0.002	4	1.2	1.0	1.5	0.028
5	0.7	0.5	0.8	<0.005	5	1.0	0.8	1.3	0.785
Age group					**Age group**				
<35	1.0	(reference)			<35	1.0	(reference)		
35-54	0.7	0.6	0.8	<0.005	35-54	0.8	0.6	1.0	0.072
45-54	0.8	0.7	1.0	0.035	45-54	0.7	0.6	0.9	0.013
55-64	0.9	0.8	1.1	0.49	55-64	0.9	0.7	1.2	0.63
65-74	0.8	0.6	1.0	0.026	65-74	0.9	0.7	1.2	0.475
75+	0.9	0.6	1.3	0.451	75+	1.0	0.7	1.4	0.94
Stage					**Stage**				
Local	1.0	(reference)			Local	1.0	(reference)		
Regional	3.2	2.8	3.7	<0.005	Regional	5.1	4.2	6.0	<0.005
Distant	9.5	8.0	11.2	<0.005	Distant	23.3	19.3	28.2	<0.005
Ethnicity					**Ethnicity**				
Chinese	1.0	(reference)			Chinese	1.0	(reference)		
Malay	1.7	1.5	2.0	<0.005	Malay	1.6	1.3	1.8	<0.005
Indian	1.1	0.8	1.4	0.623	Indian	1.3	1.1	1.7	0.015

4.3 Discussion

Methodical considerations

The methodical considerations are similar to study 1 and have been described in chapter 3.3. The number of Indian and Malay women was small compared to the

Chinese and this has potential sample size bias with decreased predictive potential with less than steady trends when associations with various covariates were studied.

Effects of Period of diagnosis and Treatment

When compared across the period of diagnosis in the study, there was a consistent overall decreased risk of death in all ethnic groups. However, treatment information is not available in the study; this is also a limiting factor as confounding due to differences in treatment between the groups may impact the survival outcome. In addition, women in this study were treated by various institutions over a long period of time. Treatment of breast cancer was standardized under institutional practice in Singapore in the 1990s. Variability of treatment between institutions over the period could have contributed systematic as well as random measurement errors. Nevertheless, comparable survival between Singapore and Stockholm (study 1) when the cancer is stratified by stage suggest that treatment in Singapore in the later years was comparable and of good standard.

Ethnic differences

Singaporean Malay women were younger and presented in a more advanced stage of breast cancer. This was associated with poorer prognosis compared to the other ethnic counterparts. Singaporean Chinese had the best prognosis compared to the Indians and Malays. This is similar to a recent report using a

Singapore-Malaysia hospital based cancer registry for women diagnosed with invasive breast cancer from 1995 to 2007(138).

As the Malays fared the poorest even after adjusting for disease stage, age, follow up, period of diagnosis, other reasons must be considered.

Tumour biology

In Asian populations, breast cancers in premenopausal young women were associated with higher grade, lymph node involvement and LVI even when majority of tumours were ER and PR positive, and associated with a higher proportion of cerbB2-positive tumours compared to SEER data (34). Multiparty contributed to a decrease in post-menopausal breast cancer amongst the Malays, reducing the median age of women with breast cancer in Malays; this itself could have been the reason of higher grade cancers amongst the Malays. In addition, earlier age of the first birth and multiparty at a young age possibly contributed to the development of tumours with poorer biology of higher grade and presence of LVI.

However, differences in tumour biology and characteristics were not available for comparison as a probable cause for the differences seen in this study. Similar to study 1, this is again a limiting factor as confounding because differences in tumour biology between the ethnic groups may impact the survival outcome.

Socioeconomic status and cultural differences

Malays in Singapore tended to have poorer SES (Chapter 1) and this is also associated with lower education and together, these could affect their awareness and understanding of the disease, seeking of medical attention and their choice of accepting recommended treatment. This is also supported by the higher proportion of advanced stage cancer in this study, likely synonymous with delay in seeking treatment. Cultural and religious beliefs affect relationships with men, perceived risk, and beliefs in traditional or alternative treatments for breast cancer, may affect disease awareness, access to early detection and thus stage at presentation of breast cancer and treatment (70). Cultural differences in coping mechanisms that our women have when faced with the fear of being diagnosed with breast cancer, may affect their attitude to the disease, and hence delay diagnosis and treatment.

Several studies from our neighbouring Malaysia showed that fear of surgery, influence by friends, belief that alternative therapy works, bad experience in hospital, financial problems, fear of inability to work after the mastectomy, lack of time, having young children, believing that prayer was sufficient, were reasons for delaying medical attention and treatment, and choosing alternative therapy; this was especially prevalent amongst the Malays (139; 140). A report in 2007 studying women who present with late disease revealed that a fatalistic view of cancer may be a reason for women not wanting to have treatment (141). Use of alternative therapy was another observation in this group, which included oral preparations, applications and spiritual prayers. Similarly, psychosocial factors due to the similar cultural and religious beliefs in each of the ethnic groups in Singapore and Malaysia may be the reason for the disparity seen in Singapore.

Other factors

Other factors such as BMI, diet, and other behavioural, or environmental differences are not available in this study. But Singapore have few women who smoke 3.2% (2004 National Health Survey statistics) (127). Only about 6% of postmenopausal women in Singapore are on hormonal replacement therapy for menopause (128); Chinese women have a high intake of soy and consume the lowest amount of dietary fat (74) while Malay women are less likely to consume alcohol. Prevalence of obesity (BMI>/=30kg/m2) is highest in Malay women (23.1%), compared to the Chinese (3%) and Indians (18.3%) as reported in the National Health Survey in 1998 (75) and this may also be contributory.

4.4 Conclusion

Trends in breast cancer survival between ethnic groups in Singapore: Chinese, Malays and Indians were different. There were more premenopausal Malays presenting with breast cancer compared to the Chinese and Indians. They presented with a more advanced stage of disease and have poorer prognosis even after adjusting for disease stage, age, follow up, period of diagnosis. Tumour biology and treatment information is not available in this study, but perhaps socioeconomic status, cultural and lifestyle differences between the ethnic groups could explain some of the survival difference. The effect of tumour biology will be studied in study 3.

CHAPTER 5 Study 3: Effect of Tumour Biology on the Survival of Breast cancer amongst different ethnic groups in Singapore: an institutional study

5.1 Subjects and Methods

Singapore Hospital Based Cancer Registry

The Singapore General Hospital-National Cancer Centre Breast Tumour Board (BTB) was started in 2001. It collects prospectively voluntary reports of patients diagnosed and treated for breast cancer mainly in the Department of General Surgery, Singapore General Hospital and Department of Surgical Oncology, National Cancer Centre, Singapore. The database records almost 700 new breast cancers a year, and consists of breast surgeons, medical and radiation oncologists, and pathologists. This is convened weekly to review all cases treated. Patient demographics, history of cancer, cancer histology and characteristic, staging details, surgery and neoadjuvant treatment details are recorded in a computer database, maintained by an informatics team. Adjuvant treatment recommendations made at the meeting are also recorded. The completeness of reporting is not known but it has been expected to improve over the years. Patients less likely to be recorded would include those who were not seen by the two surgical departments and did not have surgical treatment.

A total of 2245 Chinese, Malay and Indian female Singaporean residents with unilateral primary invasive breast carcinoma recorded from 2001 to 2007 in the BTB were included. Women with a previous malignancy, including contralateral

breast cancer were excluded from the study. Follow-up to 31 December 2010 with death information was obtained by matching with the national death register and case note reviews. The methods were similar to study 1 and 2, and have been described in 3.1.

The clinical stage was determined from the tumour, lymph node involvement and metastatic disease at diagnosis and classified as local, regional and distant (metastasis). Classifications into clinical subtypes were done according to the ER, PR and cerbB2/HER2 status on IHC and or FISH (Table 14):

Table 14 Classification of clinical subtypes by receptor status

Subtype classification	**Oestrogen receptor**	**Progesterone receptor**	**cerbB2/HER2-neu**
ER/PR positive			
HER2-	either or both (+)		(-)
HER2+			(+)
Triple negative	(-)	(-)	(-)
HER2 positive (ER/PR+, HER2+)	(-)	(-)	(+)

5.2 Results

Study population

Table 15 shows the characteristics of the Singaporean Chinese, Malay and Indian women treated for breast cancer in a local institution for breast cancer from 2001 to 2007, with follow up till 31 December 2010. The ethnic distribution is comparable to the population study (study 2); similarly, the Malays were younger and presented in more advanced stage compared to the Chinese and Indians.

Staging is based on histopathology except for 7% of cases with neoadjuvant chemotherapy and 4% with metastatic disease. The all cause and breast cancer death was highest amongst the Malays during the follow-up period.

Eighty-three per cent received complete loco regional surgery, with either a mastectomy or breast conservation; and axillary surgery. Two per cent did not have any operation while just 9 women had either excision of the tumour only or axillary clearance only (others). Eight per cent received chemotherapy as 1st line treatment, of which 97% were neoadjuvant chemotherapy; the rest were palliative in nature. Detailed information on adjuvant therapy such as chemotherapy, hormonal therapy, targeted therapy (Herceptin) and radiotherapy were not available in the database, but were available as Tumour Board recommendations, as intention to treat. There were no differences between the ethnic groups.

Table 15 Characteristics and treatment of Singaporean women with breast cancer by ethnicity

Characteristic (%)	Chinese	Malays	Indians	Total	*p*
Frequency	1940 (86)	192 (9)	113 (5)	2245 (100)	
Age					
</= 50	753 (39)	105 (55)	54 (48)	912 (41)	< 0.005
>50	1187 (61)	87 (45)	59 (52)	1333 (59)	
Stage* (% complete)	1925 (99)	191 (99)	113 (100)	2229 (99)	< 0.005
Local	1085 (56)	73 (38)	58 (51)	1216 (55)	
Regional	768 (40)	101 (53)	51 (45)	920 (41)	
Distant	72 (4)	17 (9)	4 (4)	93 (4)	
Deaths	324 (17)	54 (28)	21 (19)	399 (18)	< 0.005
Breast cancer deaths	283 (15)	48 (25)	19 (17)	350 (16)	
Treatment modality					
Breast and axillary surgery					
Local and regional disease (n=2136)					
Complete surgery**	1817 (98)	171 (98)	105 (86)	2093 (98)	0.169
Incomplete surgery	27 (1)	1 (0.6)	4 (4)	32 (92)	
None	9 (0.5)	2 (1)	0	11 (0.5)	
Metastatic or incomplete staging (n=109)					
Complete surgery**	53 (61)	14 (78)	4 (100)	71 (65)	0.228
Incomplete surgery	6 (7)	2 (14)	0	8 (65)	
None	28 (32)	2 (11)	0	30 (28)	
Type of breast surgery (n=2245)					
None	37 (1.9)	4 (2.1)	0	41 (1.8)	0.828
Mastectomy	1357 (69.9)	131 (68.2)	78 (69)	1566 (69.8)	
Breast conservation	539 (27.8)	56 (29.2)	34 (30.1)	629 (28)	
Others	7 (0.4)	1 (0.5)	1 (0.9)	9 (0.4)	
Neoadjuvant chemotherapy (n=2136)	123 (7)	21 (12)	9 (8)	153 (7)	0.444

**Staging is based on histopathology except for 7% of cases with neoadjuvant chemotherapy and 4% with metastatic disease*

*** Complete surgery refers to complete breast and axillary surgery; incomplete surgery refers to either breast surgery without or axillary surgery*

Tumour characteristics

Characteristics by ethnicity

The differences in tumour histopathological characteristics by ethnicity are described in Table 16. The proportions of ER, PR and HER2 status of tumours are similar across the ethnic groups; but Chinese have fewer grade 3 tumours and tumours which are less likely to have lymphovascular invasion. When the tumours were classified into subtypes based on the receptor status, there appears to be more triple negative tumours amongst the Indians (p=0.298).

Within each ethnic group, there was no significant change in ER and PR positivity and LVI by age groups; but in the Chinese, proportion of grade 3 tumours decreased with increasing age (p=0.042), this trend was also present in the Malays, though not significant (p=0.234), while the numbers were too small amongst the Indians. ER positivity was similar across age groups by ethnicity Figure 15.

Table 17 and Table 18 show the correlation between LVI and grade with the receptor status and clinical subtypes, where ER and PR negative tumours are more likely high grade; HER2 positive tumours are more likely LVI positive.

Characteristics by tumour subtype

Table 18 demonstrates association of the clinico-pathologic variables with the clinical tumour subtypes. Although the duration of study is short, there was a shift in the later 3 years towards more ER/PR+ tumours compared to the initial 4 years.

Figure 15 Oestrogen positivity by ethnicity and age group

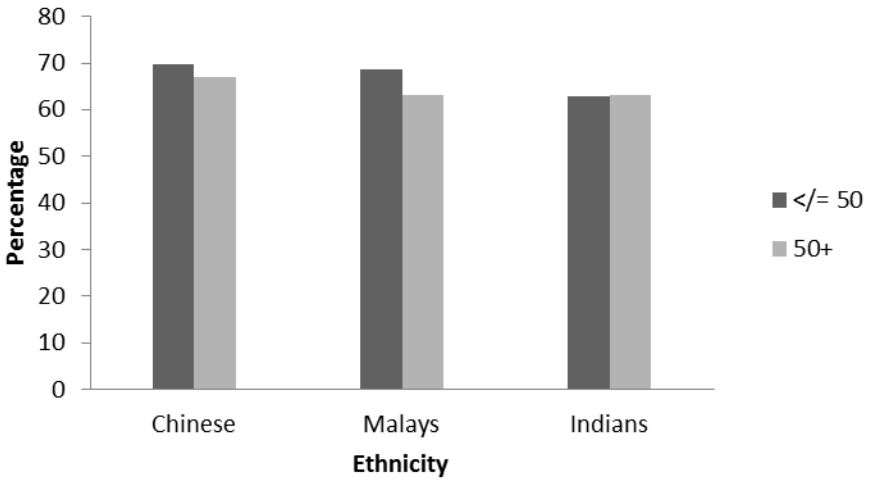


Table 16 Tumour characteristics by ethnicity

Characteristic (%)	Chinese	Malays	Indians	Total	p
Receptor status					
ER (% available)	1910 (98)	192 (100)	111 (98)	2213 (99)	
Positive	1300 (68)	127 (66)	70 (63)	1497 (68)	0.493
Negative	610 (32)	65 (34)	41 (37)	716 (32)	
PR (% available)	1901 (98)	191 (99)	111 (98)	2203 (98)	
Positive	1108 (58)	110 (58)	66 (59)	1284 (58)	0.951
Negative	793 (48)	81 (42)	45 (41)	919 (42)	
HER2 (% available)	1624 (84)	167 (87)	100 (88)	1891 (84)	
Positive	390 (24)	47 (28)	20 (20)	457 (24)	0.3
Negative	1234 (76)	120 (72)	80 (80)	1434 (76)	
Grade of tumour (% available)	1837 (95)	187 (97)	109 (96)	2133 (95)	
1	329 (18)	27 (14)	12 (11)	368 (17)	0.015
2	701 (38)	57 (30)	40 (37)	798 (37)	
3	807 (44)	103 (55)	57 (52)	967 (45)	
LVI (% available)	1695 (87)	170 (89)	100 (58)	1965 (88)	
No	1205 (71)	102 (60)	65 (65)	1372 (80)	0.006
Yes	412 (29)	59 (40)	27 (35)	498 (30)	
Subtype classification					
Frequency (% available)	1604 (83)	165 (86)	100 (88)	1869 (83)	0.298
ER/PR positive	1162 (72)	120 (73)	68 (68)	1350 (72)	
HER2-	959 (60)	92 (56)	56 (56)	1107 (59)	
HER2+	203 (12)	28 (17)	12 (12)	243 (13)	
Triple negative	258 (16)	26 (16)	24 (24)	308 (16)	
HER2 positive (ER/PR-, HER2+)	184 (11)	19 (12)	8 (8)	211 (11)	

ER/PR positivity is based on 10% or more of invasive tumour cells staining with an intensity of at least 2+.HER2 positivity is based on cerbB2 by IHC: 65% had raw scores of intensity of 3+, 35% were recorded as positive without details of raw scores; IHC 0/1+: negative: IHC 2+: equivoval.

Table 17 Tumour characteristics by receptor status

	ER		PR		HER2	
Tumour characteristics	Positive	Negative	Positive	Negative	Positive	Negative
Grade						
1	346 (24)	18 (3)	293 (24)	71 (8)	16 (4)	303 (22)
2	638 (45)	151 (22)	529 (43)	256 (29)	146 (33)	544 (39)
3	451 (31)	509 (75)	412 (33)	544 (63)	275 (63)	536 (39)
	p<0.005*		p<0.005*		p<0.005*	
LVI						
Positive	386 (29)	200 (32)	332 (29)	252 (31)	146 (37)	345 (27)
Negative	944 (71)	417 (68)	801 (71)	557 (69)	244 (63)	930 (73)
	p=0.129		p=0.382		p<0.005*	

Table 18 Clinical and histopathological features by tumour subtype classification

Subtype	**ER+ and/or PR+, HER2-**	**ER+ and/or PR+, HER2+**	**ER-, PR-, HER2-**	**ER-, PR-, HER2+**	**Total**	***p***
Characteristic						
Age						0.085
< or = 50	473 (42)	105 (43)	110 (35)	78 (37)	766 (41)	
50+	641 (58)	140 (57)	200 (65)	133 (63)	1114 (59)	
Period						
2001-2004	552 (55)	148 (15)	187 (18)	125 (12)	1012 (100)	< 0.005
2005-2007	562 (65)	97 (11)	123 (14)	86 (10)	868 (100)	
Stage						0.001
Local	641 (58)	122 (50)	166 (54)	90 (43)	1019 (54)	
Regional	431 (39)	111 (45)	123 (40)	105 (50)	770 (41)	
Distant	35 (3)	10 (4)	19 (6)	16 (8)	80 (4)	
Incomplete	7 (1)	2 (1)	2 (1)	0 (0)	11 (1)	
Grade						< 0.005
1	292 (27)	15 (6)	10 (3)	1 (1)	318 (18)	
2	485 (45)	89 (37)	54 (18)	56 (28)	684 (38)	
3	300 (28)	134 (56)	232 (78)	141 (71)	807 (45)	
LVI						0.001
No	735 (66)	134 (55)	192 (62)	110 (52)	1171 (62)	
Yes	266 (24)	73 (30)	75 (24)	73 (35)	487 (26)	
Not available	113 (10)	38 (16)	43 (14)	28 (13)	222 (12)	

Overall survival by ethnicity

The overall survival is similar to the population study in study 2, where the Chinese constantly outperformed the Malays and the Indians. The overall age-standardized 5-year relative survival for the Chinese, Malay and Indian women was significantly different at 83%, 64% and 72% respectively (Figure 16 and Table 19). The trend is less stable in the Indians due to the small population size.

Figure 16 Overall 5-year age-standardized relative survival of women with breast cancer by ethnicity in relation to time since diagnosis.

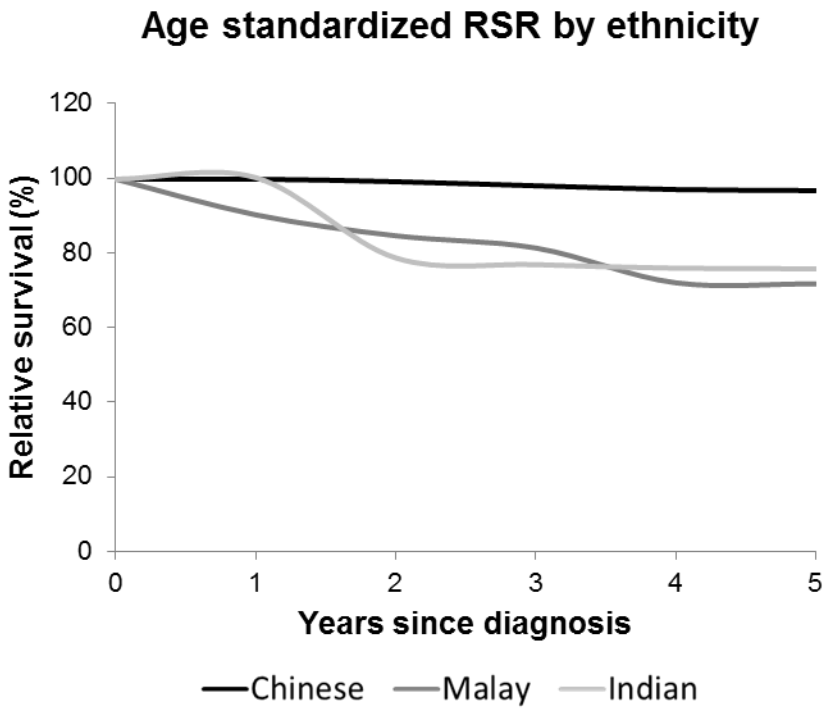


Table 19 Overall and stage specific 5-year age-standardised relative survival of Singaporean women

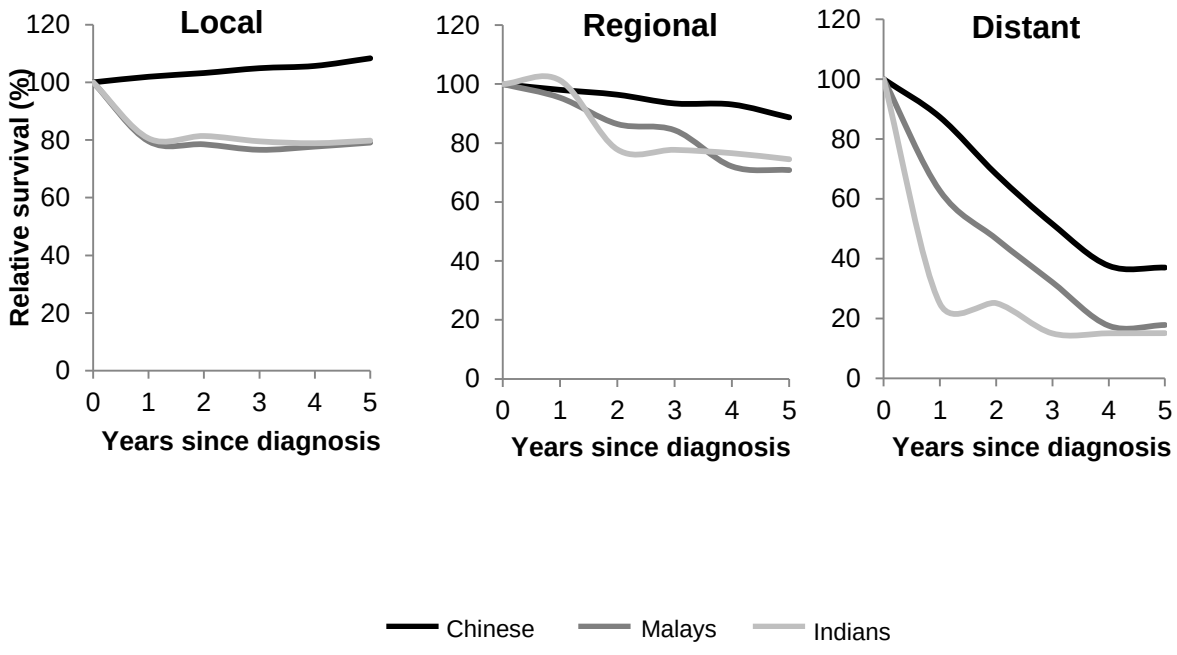
Characteristic	Chinese		Malays		Indians	
Overall 5-year survival rates	%	95% CI	%	95% CI	%	95% CI
Overall observed survival	97	88 - 103	72	45 - 97	76	65 - 80
Overall relative survival	83	77 - 87	64	42 - 83	72	61 - 76
Specific 5-year relative survival						
Local	108	98 - 113	79	59 - 85	80	73 - 83
Regional	89	73 - 100	71	32 - 100	74	52 - 81
Distant	37	15 - 57	18	1 - 42	15	15 - 15

Survival by ethnicity and stage

The survival trend by ethnicity and stage in this institutional cohort is consistent with the Singapore population in study 2, but the trend is less stable as the number of Malay and Indian women in this study cohort is small (Figure 17). The 5-year RSR amongst the Chinese women with localised cancer was 108%. A relative survival greater than 100% indicates better survival among the Chinese women with localized breast cancer than in the general population. This is usually observed when statistics are based on small numbers of cases, unlikely in this study; or competing mortality is lower in these women as compared with the general population. This may be due selection bias of Chinese women with lower comorbidity with tumours that are indolent, nonlethal and do not limit their survival. This may also be due to a 'healthy patient effect', whereby these patients experience lower mortality due to other causes as a result of having greater than average contact with the health system, change in lifestyle and health habits after breast cancer diagnosis which alters death rates from other diseases.

This was also seen in a study where a relative survival >100% was seen in men with low-grade prostate cancer, regardless of treatment, at least during the first 5 years. This paradoxically high relative survival rate is probably explained by a selection of men with lower comorbidity and tumours that do not limit the survival of their hosts, in whom tumours are revealed despite few or minor symptoms. Men with similar tumours but more concurrent disease are less likely to undergo an examination in which indolent, nonlethal tumours would be detected. Consequently, men with low-grade prostate cancer appear to have better survival than the general population (142).

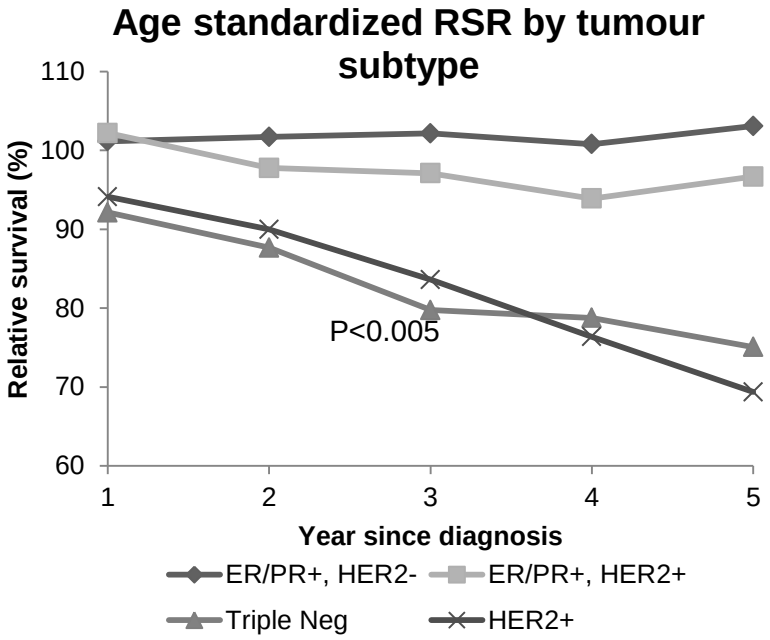
Figure 17 Five-year age-standardized relative survival of women with breast cancer by ethnicity and stage of cancer in relation to time since diagnosis



Survival by tumour subtypes

Overall prognosis by subtype classification based on tumour receptor: ER, PR and HER2 in Figure 18 showed that women with ER and/or PR positive tumours have similar survival, but those whose tumours were ER/PR positive, HER2 negative performed better compared to those who were ER/PR positive, positive. Those with triple negative (ER/PR/HER2-) and ER/PR-, HER2+ tumours fared poorer and the survival was similar in these 2 groups ($p<0.05$).

Figure 18 Five-year age-standardized relative survival of women with breast cancer by tumour subtype classification in relation to time since diagnosis

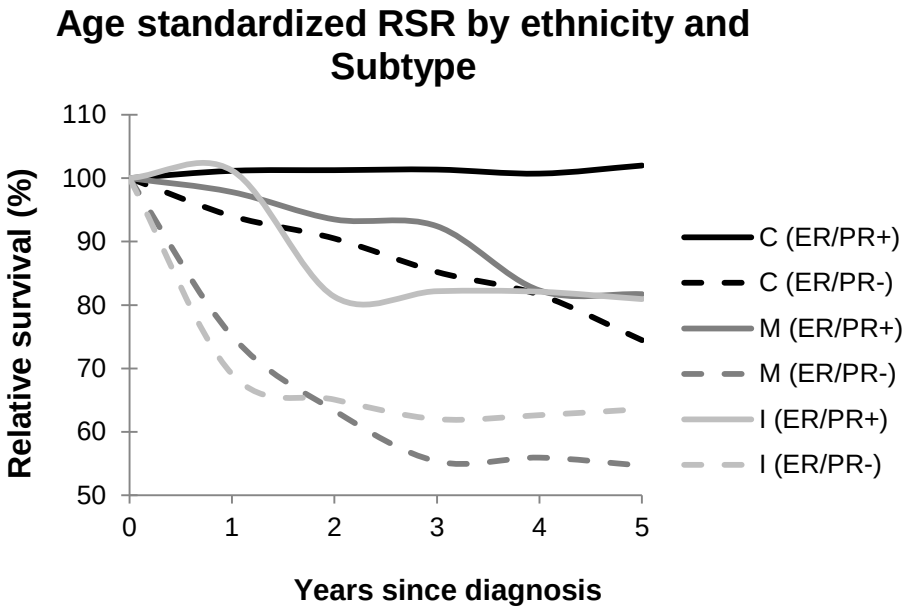


**unadjusted*

Survival by ethnicity and tumour subtypes

Figure 19 shows the 5-year age-standardised relative survival by ethnicity and subtype. As the numbers amongst the Malays and Indians were small, the subtype classification was regrouped into 2 groups: ER/PR+ (ER+ and/or PR+, HER2- and ER+ and/or PR+, HER2+) and ER/PR- (ER-, PR-, HER2- and ER-, PR-, HER2+). The number of events was still too few amongst the Indians to show any discernible estimates.

Figure 19 Five-year age-standardised relative survivals by ER/PR status and ethnicity



Ethnicity	RR	95% CI		p
Chinese	4.3	2.6	7.2	<0.005
Malay	2.4	1.0	5.7	0.056

C: Chinese, M: Malays, I: Indians; ER/PR+ (ER+ and/or PR+, HER2- and ER+ and/or PR+, HER2+) and ER/PR- (ER-, PR-, HER2- and ER-, PR-, HER2+); RR: relative risk of mortality by ER/PR- to ER/PR+ status within each ethnic group; unadjusted.

Poisson regression: excess risk of death

Table 20 presents the risk of death of the women by ethnicity, disease stage, tumour subtype, tumour grade, LVI with years of follow-up taken into account. The survival for ER/PR+/HER2+ and ER/PR+/HER2- were similar and hence were grouped as ER/PR+ in the univariate and multivariate analysis because of the small numbers of Malay and Indian women in the study. Age was not a significant factor. On multivariate analysis, as expected, the stage of cancer is an important predictor of survival. Malay women have twice the risk of death compared to the Chinese women. Tumour characteristics: tumour subtype, grade and LVI independently predicted prognosis.

Table 20 Poisson regression analysis: univariate and multivariate analysis

Variable	HR	p	95% CI		Variable	HR	p	95% CI	
Univariate*					**Multivariate**				
Clinical					**Clinical**				
Ethnicity					Year of follow up				
Chinese	1.0	(reference)			1	1.0	(reference)		
Malay	2.5	<0.005	1.6	6.3	2	2.2	0.011	1.2	4.0
Indian	0.9	0.891	0.3	2.5	3	2.4	0.004	1.3	4.4
					4	1.8	0.083	0.9	3.5
Age					5	2.2	0.027	1.1	4.3
</= 50	1.0	(reference)							
>50	0.9	0.461	0.6	1.3	Ethnicity				
					Chinese	1.0	(reference)		
Stage					Malay	1.7	0.023	1.1	2.7
Local & Regional	1.0	(reference)			Indian	0.7	0.488	0.3	1.8
Distant	12.3	<0.005	7.9	19.1					
					Stage				
Tumour					Local & Regional	1.0	(reference)		
Subtype					Distant	6.9	<0.005	3.5	13.6
ER/PR positive	1.0	(reference)							
Triple negative	4.4	<0.005	2.7	7.0	**Tumour**				
HER2 positive	3.5	<0.005	2.0	6.0	Subtype				
					ER/PR positive	1.0	(reference)		
Grade					Triple negative	3.4	<0.005	2.2	5.3
1 and 2	1.0	(reference)			HER2 positive	2.1	0.003	1.3	3.4
3	1.6	<0.005	1.0	2.2					
					Grade				
LVI					1 and 2	1.0	(reference)		
Yes	1.0	(reference)			3	1.7	0.028	1.1	2.6
No	0.2	<0.005	0.1	0.3					
					LVI				
					Yes	1.0	(reference)		
					No	0.4	<0.005	0.3	0.6

**Adjusted for years of follow up*

5.3 Discussion

Methodical considerations

This is a single institution based study and has limitations related to selection bias, being a single tertiary centre which may treat patients with different medical attention seeking behaviour and treatment preferences; completeness of the database; as well as sample size. However, being the largest institution in Singapore, it still recorded a large number of cases, treating about 700 cases of breast cancer a year. The clinic-pathological information in the database enabled the study of factors otherwise not available in the population database.

Malays and Indians form a small proportion of the study population and the ability to analyse the different subgroups by age, subtype, stage, grade and LVI is limited especially amongst the Indians. Women with distant metastatic disease in this database were likely under reported; as compared with the population study (study 4), however, the proportion of those with distant disease is small even at the population level of about 8% compared to 4% in this series. Although this is not reflective of the distribution in the general population, the aim of this study was to study the effects of the receptor status of the tumours between the ethnic groups; the contribution of the difference in distribution may not have a significant consequence. However, it does limit the ability to assess differences in women with metastatic breast cancer especially amongst the Malays and Indians where the population numbers were small.

A relative survival greater than 100% indicates better survival among the Chinese women with localized breast cancer than in the general population. This may be observed when statistics are based on small numbers of cases, unlikely in this study; or competing mortality is lower in these women as compared with the general population. This may be due selection bias of Chinese women with lower comorbidity with tumours that are indolent, nonlethal and do not limit their survival. This may also be due to a 'healthy patient effect', whereby these patients experience lower mortality due to other causes as a result of having greater than average contact with the health system, change in lifestyle and health habits after breast cancer diagnosis which alters death rates from other diseases.

This is similar to a study where a relative survival >100% was seen in men with low-grade prostate cancer, regardless of treatment, at least during the first 5 years. This paradoxically high relative survival rate is probably explained by a selection of men with lower comorbidity and tumours that do not limit the survival of their hosts, in whom tumours are revealed despite few or minor symptoms. Men with similar tumours but more concurrent disease are less likely to undergo an examination in which indolent, nonlethal tumours would be detected. Consequently, men with low-grade prostate cancer appear to have better survival than the general population (142).

The ***completeness of tests*** on tumour characteristics such as receptor status, LVI and grade were not available in 5 to 17% of the cases in the study. This was inevitable as some of these tumours were too small to assess these parameters or the IHC tests failed on the sample. Study of these early cancers in relation to

their receptor status would be diminished, but fortunately these did not contribute a large number.

Exclusion of unknown cases e.g., on the HER2 may introduce bias for complete case analysis in a review by Bhoo PN *et al* (143). However, in this reference, there was a selection bias of when HER2 status was assessed: in the group of patients where the lowest predicted mortality risk and patients with highest predicted mortality risk were significantly less likely to be tested for HER2 status, whereas those with intermediate predicted mortality risk were more likely to be tested.

In our series, all cases were assessed for cerbB2, with no selection based on expected mortality or on other clinical parameters. The cases where complete receptor status, i.e., all 3: ER, PR and cerbB2 were not available for the subtyping and were grouped as unknown were mainly due to the indeterminate or unknown HER2 status, where the cerbB2 was 2+/equivoval/indeterminate (74%), while the rest were either not assessable because of a small invasive focus, in post-neoadjuvant cases or not done. As use of Herceptin as adjuvant therapy was not yet standard therapy, HER2 by FISH was not performed. This by itself would introduce a similar selection bias as referenced, but perhaps to a lesser degree as more than 80% of the tumours had known HER2 status, either positive or negative.

In this study, the relative survival for the unknown group was very similar to the ER+/PR+/HER2- group i.e., of good prognosis and account for about 16% of all the cases, of which two thirds were ER/PR positive. When these cases were

reviewed and compared against the various parameters, they were comparable by stage, ethnicity and age, hence in risk stratification against these variables, the bias if present is unlikely significant.

Histology and immunohistochemistry was used to assess the tumour and tumour receptor status. Like grade and LVI, these variables are subject to measurement bias as there are inter-observer as well as inter-assay variability; However, as a large tertiary centre with a high clinical load and experienced pathologists; as well as that the laboratories receive rigorous assessment and accreditation from the College of American Pathologists (CAP) and follow the American Society of Clinical Oncology/College of American Pathologists guidelines of sample handling, this possible systematic observer bias resulting in misclassification is likely reduced. The cut-off for determining positivity of receptor status has changed over the years, but for the duration of the study, there was no change within our laboratory; a semi-quantitative method defined ER positivity as 10% or more of invasive tumour cells staining with an intensity of at least 2+.

Tumour biology

The proportion of ER positive tumours are higher in this study when compared to previous reports (144; 145). This could be due to the selection criteria differences; this study comprised only of women with no prior or bilateral breast cancer. Prior or bilateral breast cancer has been associated with higher ER positive tumours. Treatment with anti-oestrogen in patients with ER-positive tumours decreased the risk of second ER-positive tumours but not ER-negative tumours (146). The

risk of second ER-negative breast cancer is very high after a first ER-negative tumour, in particular among women with strong family history (147). In addition, ER negativity as a risk factor for developing metachronous tumours (148) with ER-negative first cancer have a specifically increased risk of ER-negative contralateral breast cancer (149). While there was no significant difference in survival for patients with bilateral compared to unilateral tumour, synchronous tumour has been associated with poorer survival in comparison to metachronous tumour (150).

Overall, a shift towards more ER-positive disease was found during the latter part of the study period; this could reflect screening practices as our national breast screening program started in 2002, but this could still influence survival (151), as also seen in study 2.

The ER and PR status and clinical subtype was similar between the ethnic groups suggesting that the proportion of ER and PR status was not affected or not in the same manner by ethnicity or ethnic related factors between them. Chinese with ER/PR positive tumours showed better survival compared to those with ER/PR negative tumours, the unadjusted relative risk of 4.3, $p<0.005$, while the relative risk was 2.4, $p=0.056$ amongst the Malays; the numbers were too small amongst the Indians. The HER2 receptor positivity was more common amongst the Malays although this was not statistically significant. Similar to the African American, when the tumours were classified into subtypes based on the receptor status, Indians too seem to have a higher proportion of triple negative cancers (52) though not statistically significant, and this had been associated with an increased risk of death.

The Chinese have the better tumour characteristics such as lower grade and LVI negative tumours explaining the better prognosis, while high grade tumours and tumours with LVI was seen in larger proportions in the Malays and Indians contributing to the poorer survival amongst the Malays. Consistent with known reports on tumour biology (152), correlation between LVI and grade with the receptor status, where ER and PR negative, and HER2 positive tumours are more likely high grade; HER2 positive tumours are more likely LVI positive were also seen in the study.

Hence, receptor status is not responsible for the poorer survival in Malays; grade and LVI were likely reasons for the survival difference. However, ethnicity is still a significant risk of death where Malays have twice the risk of death in Chinese after adjusting for disease stage, tumour subtype, tumour grade, LVI and years of follow-up. Either genetic difference that are not seen by the ER, PR or HER2 phenotype affect the prognosis, or other factors such as psychosocial factors associated with ethnicity contribute to the survival difference as discussed in chapter 4.

5.4 Conclusion

It is interesting to find that ER status was comparable between the Chinese, Malays and Indians, as well as by menopausal status (using age 50 years old as the cut-off); similarly for PR and HER2 status resulting in similar clinical subtypes distribution between the ethnic groups. Women with different tumour subtypes

showed different survival amongst the Chinese, but this was not significant amongst the Malays. Although tumour grade and LVI were correlated with ethnicity, they were independent predictors of risk of death. Ethnicity was also an independent risk of death; hence, tumour biology alone does not explain the difference in survival difference between ethnicity.

CHAPTER 6 STUDY 4: CUSTOM MULTI-SIGNATURE ARRAY (MSA) FOR BREAST CANCER IN SINGAPORE

6.1 Subjects and Methods

Tissue Repository

In study 4, human breast tissues from the period 2000-2004 were obtained from the National Cancer Centre (NCC) Tissue Repository, after appropriate approvals from the National Cancer Centre Repository and Ethics Committees and the Singapore General Hospital Ethics Committee. Samples were selected using the following predefined inclusion criteria: histological diagnosis of invasive ductal carcinoma (IDC) of the breast, newly diagnosed non-metastatic breast cancer with no prior treatment, no history of other cancers or the presence of synchronous contra-lateral breast cancer, availability of frozen tissue and complete clinical data. Exclusion criteria were: tumours with no detectable IDC on haematoxylin and eosin (H&E) sections of the frozen sample, and degraded RNA. All patients underwent surgical treatment, mastectomy or breast conservation surgery, and were given adjuvant therapy: chemotherapy, hormonal therapy and chest wall radiotherapy according to institutional practice guidelines. Tissue harvesting, preparation for storage, storage and release of tissue was performed by the NCC Tissue Repository and under Repository Protocols. Follow-up was done by matching with the national death register for all studies, as well as clinical case records.

Clinical and histopathology variables

Clinical variables age, ethnicity and histological variables including tumour size, nodal status, tumour subtype, histologic grade (modified Bloom-Richardson grading), lymphovascular invasion (LVI), and ER, PR and HER2 status were collected from the formal histological report.

Repeat assessment by immunohistochemistry (IHC) were done for study 4 by two pathologists. Immunohistochemistry antibodies were SP1 for ER and SP3 for HER2 (Labvision); ER/PR positivity is 10% or more of invasive tumour cells staining with an intensity of at least 2+. HER2 IHC staining scored according to Herceptest specifications (DAKO). HER2 positivity was based on FISH, which was done on all the frozen tissue samples using the PathVysion kit from Vysis, Inc., in which a HER2/CEP ratio of 2.2 was deemed as amplified according to the 2007 American Society of Clinical Oncology/College of American Pathologists guidelines (84). Immunohistochemistry and FISH were done in the Department of Pathology, Singapore General Hospital, a College of American Pathologists accredited laboratory. Concordance between HER2 FISH and immunohistochemistry obtained by our laboratory reported 100% for immunohistochemistry 3+ and 85% for immunohistochemistry 2+ in the recent review of consecutive routine cases of invasive breast cancer done on paraffin sections. This percentage is well within the variables recommended for HER2 testing by Wolff et al. (84). FISH is not routinely done for immunohistochemistry negative and 1+ cases in our institution, and hence these data are not available.

Multiple signature assay (MSA)

MSA Chip Design

The breast cancer MSA was fabricated using CombimatrixTM electrochemical in-situ synthesis technology, and contains probes for 188 genes representing five previously identified breast cancer molecular signatures. These include signatures for the Luminal/ER+, HER2, and basaloid subtypes of breast cancer, which we had previously shown to be present in the Asian breast cancer population (95; 105); and 2 expression signatures previously identified by the research group in NCC using local breast cancer tumours: NPI-ES, a molecular signature of the Nottingham Prognostic Index, a pathologic stratification staging system for breast cancer prognostication(96); and TuM1, a signature for low histologic grade that may also serve as a potential predictive biomarker for tamoxifen response(153). Notably, both the NPI-ES and TuM1 signatures are specific to Luminal/ER+ tumours. A complete list of MSA genes is provided in

Table **21**. For each gene, we used vendor-provided software to design five independent (35mer) probes, and each probe was replicated eight times across the MSA at random locations. We also included forty-five control genes from different cellular pathways as internal standards for normalizing RNA expression levels (38). Finally, 25mer sequences (5 replicates per probe) designed against the 20x Eukaryotic Hybridization Control Kit (Affymetrix) was included to facilitate monitoring of the hybridisation process.

Table 21 List of genes on the MSA. a) Luminal/Oestrogen Receptor positive (ER+), b) HER2, and c) 'Basaloid', d) Nottingham Prognostic Index (NPI-ES), and e) low histologic grade (TuM1).

GenBank ID	Gene Name
a) Luminal/Oestrogen Receptor (ER+)	
NM_005080	X-box binding protein 1
NM_004374	cytochrome c oxidase subunit Vic
AL572542	CGI-49 protein
AI635449	solute carrier family 39 (zinc transporter), member 6
NM_021173	polymerase (DNA-directed), delta 4
NM_004636	sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3B
AI435828	stanniocalcin 2
H05812	insulin-like growth factor 1 receptor
NM_000633	B-cell CLL/lymphoma 2
NM_001218	carbonic anhydrase XII
AI857639	phorbol-12-myristate-13-acetate-induced protein 1
BC001012	carbonic anhydrase XII
NM_005375	v-myb myeloblastosis viral oncogene homolog (avian)
NM_003225	trefoil factor 1 (breast cancer, estrogen-inducible sequence expressed in)
NM_003462	dynein, axonemal, light intermediate polypeptide 1
NM_000125	estrogen receptor 1
NM_000156	guanidinoacetate N-methyltransferase
NM_014668	GREB1 protein
NM_003834	regulator of G-protein signalling 11
J03778	microtubule-associated protein tau
NM_000767	cytochrome P450, family 2, subfamily B, polypeptide 6
NM_002570	proprotein convertase subtilisin/kexin type 6
J02639	serine (or cysteine) proteinase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 5
AF237813	4-aminobutyrate aminotransferase
BC003070	GATA binding protein 3
AF230929	annexin A9
M29873	cytochrome P450, family 2, subfamily B, polypeptide 6
BC002915	discs, large homolog 5 (Drosophila)
AB015706	interleukin 6 signal transducer (gp130, oncostatin M receptor)
AK023837	thyroid hormone receptor associated protein 2
BE256900	jumonji domain containing 2B
BF131791	WW domain containing E3 ubiquitin protein ligase 1
AI348094	KIAA0882 protein
BF508639	elongation of very long chain fatty acids (FEN1/Elo2, SUR4/Elo3, yeast)-like 2
L48722	protein tyrosine phosphatase type IVA, member 2
NM_014019	HSPC009 protein
NM_018478	chromosome 20 open reading frame 35
NM_024573	chromosome 6 open reading frame 211
NM_022131	calsyntenin 2
AI378044	UDP-glucose ceramide glucosyltransferase
BC000580	hypoxia-inducible factor prolyl 4-hydroxylase
AK001105	LAG1 longevity assurance homolog 2 (S. cerevisiae)
D87469	cadherin, EGF LAG seven-pass G-type receptor 2 (flamingo homolog, Drosophila)

NM_005264	GDNF family receptor alpha 1

b) HER2

NM_000126	electron-transfer-flavoprotein, alpha polypeptide (glutaric aciduria II)
NM_021122	acyl-CoA synthetase long-chain family member 1
NM_002964	S100 calcium binding protein A8 (calgranulin A)
NM_006804	START domain containing 3
NM_003064	secretory leukocyte protease inhibitor (antileukoproteinase)
AI758763	transducin-like enhancer of split 1 (E(sp1) homolog, Drosophila)
NM_002965	S100 calcium binding protein A9 (calgranulin B)
NM_005923	mitogen-activated protein kinase kinase kinase 5
AW157202	SRY (sex determining region Y)-box 11
NM_014817	KIAA0644 gene product
NM_006741	protein phosphatase 1, regulatory (inhibitor) subunit 1A
NM_014274	transient receptor potential cation channel, subfamily V, member 6
BC005047	dual specificity phosphatase 6
AF289489	aspartate beta-hydroxylase
AB008790	growth factor receptor-bound protein 7
BC005297	kynurenine 3-monooxygenase (kynurenine 3-hydroxylase)
AF202063	fibroblast growth factor receptor 4
AB029025	KIAA1102 protein
NM_003221	transcription factor AP-2 beta (activating enhancer binding protein 2 beta)
X03363	v-erb-b2 erythroblastic leukemia viral oncogene homolog 2, neuro/glioblastoma derived oncogene homolog (avian)
BF003134	chloride channel, calcium activated, family member 2
BF589529	DBCCR1-like
NM_024306	fatty acid 2-hydroxylase
NM_024861	hypothetical protein FLJ22671
BF033007	per1-like domain containing 1

c) 'Basaloid'

NM_000424	keratin 5 (epidermolysis bullosa simplex, Dowling-Meara/Kobner/Weber-Cockayne types)
NM_005228	epidermal growth factor receptor (erythroblastic leukemia viral (v-erb-b) oncogene homolog, avian)
NM_015271	tripartite motif-containing 2
NM_001444	atty acid binding protein 5 (psoriasis-associated)
NM_012101	tripartite motif-containing 29
NM_002423	matrix metalloproteinase 7 (matrilysin, uterine)
NM_004949	desmocollin 2
NM_002639	serine (or cysteine) proteinase inhibitor, clade B (ovalbumin), member 5
NM_014211	gamma-aminobutyric acid (GABA) A receptor, pi
NM_006533	melanoma inhibitory activity
NM_030920	acidic (leucine-rich) nuclear phosphoprotein 32 family, member E /// acidic (leucine-rich) nuclear phosphoprotein 32 family, member E
AF007162	crystallin, alpha B
AF061812	keratin 16 (focal non-epidermolytic palmoplantar keratoderma)
AI367319	SRY (sex determining region Y)-box 10
AK026420	Desmuslin
AU145890	forkhead box C1
AI831452	keratin 6B

BE542323	vestigial like 1 (Drosophila)
NM_022893	B-cell CLL/lymphoma 11A (zinc finger protein)
NM_003740	potassium channel, subfamily K, member 5
NM_007231	solute carrier family 6 (neurotransmitter transporter), member 14
NM_017422	calmodulin-like skin protein
NM_017578	ropporin, rhophilin associated protein 1
NM_001426	engrailed homolog 1
Z19574	keratin 17

d) Nottingham Prognostic Index (NPI-ES)

NM_002106	H2A histone family, member Z
NM_006763	BTG family, member 2
BC002802	suppressor of Ty 4 homolog 1 (S. cerevisiae)
NM_001814	cathepsin C
BE966236	ribonucleotide reductase M2 polypeptide
NM_001168	baculoviral IAP repeat-containing 5 (survivin)
NM_014669	nucleoporin 93kDa
NM_021953	forkhead box M1
NM_000295	serine (or cysteine) proteinase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 1
NM_002358	MAD2 mitotic arrest deficient-like 1 (yeast)
BG170541	met proto-oncogene (hepatocyte growth factor receptor)
NM_002996	chemokine (C-X3-C motif) ligand 1
NM_014750	discs, large homolog 7 (Drosophila)
NM_004523	kinesin family member 11
NM_003686	exonuclease 1
NM_003226	trefoil factor 3 (intestinal)
NM_002452	nudix (nucleoside diphosphate linked moiety X)-type motif 1
NM_013296	G-protein signalling modulator 2 (AGS3-like, C. elegans)
NM_003536	histone 1, H3h
NM_005951	metallothionein 1H
NM_017522	low density lipoprotein receptor-related protein 8, apolipoprotein e receptor
NM_003524	H2B histone family, member J
NM_005952	metallothionein 1X
AW149681	lysosomal associated protein transmembrane 4 beta
U17496	proteasome (prosome, macropain) subunit, beta type, 8 (large multifunctional protease 7)
AF133425	tetraspan 1
BC002649	histone 1, H1c
BC000893	histone 1, H2bk
AF321125	DNA replication factor
AB000221	chemokine (C-C motif) ligand 18 (pulmonary and activation-regulated)
AF098158	TPX2, microtubule-associated protein homolog (Xenopus laevis)
D88357	cell division cycle 2, G1 to S and G2 to M
AF133298	cytochrome P450, family 4, subfamily F, polypeptide 8
AF033111	Homo sapiens Siva-2 mRNA, complete cds
AF333388	Homo sapiens metallothionein 1H-like protein mRNA, complete cds
AL582836	paternally expressed 10
AA604621	MCM4 minichromosome maintenance deficient 4 (S. cerevisiae)
NM_005953	metallothionein 2A
BF974389	Mouse Mammary Turmor Virus Receptor homolog 1

AI991252	butyrophilin, subfamily 3, member A2
AL120173	adenylate cyclase 1 (brain)
AA927724	adenine phosphoribosyltransferase
NM_003530	histone 1, H3d
AI738662	homeo box HB9
BG540628	Human active IgK chain from GM 607, V-kappa-2 region
H53689	immunoglobulin lambda constant 2 (Kern-Oz- marker)
NM_016185	hematological and neurological expressed 1
NM_015895	geminin, DNA replication inhibitor
NM_020188	DC13 protein
NM_018131	chromosome 10 open reading frame 3
NM_012177	F-box protein 5
NM_006014	DNA segment on chromosome X (unique) 9879 expressed sequence
NM_024745	SHC SH2-domain binding protein 1
NM_018455	uncharacterized bone marrow protein BM039
NM_017669	hypothetical protein FLJ20105
NM_018063	helicase, lymphoid-specific
NM_018846	kelch-like 7 (Drosophila)
NM_031299	cell division cycle associated 3 /// cell division cycle associated 3
BC003186	DNA replication complex GINS protein PSF2
AB044548	eukaryotic translation initiation factor 4E binding protein 1
AI859865	MCM4 minichromosome maintenance deficient 4 (S. cerevisiae)

e) Low histologic grade (TuM1)

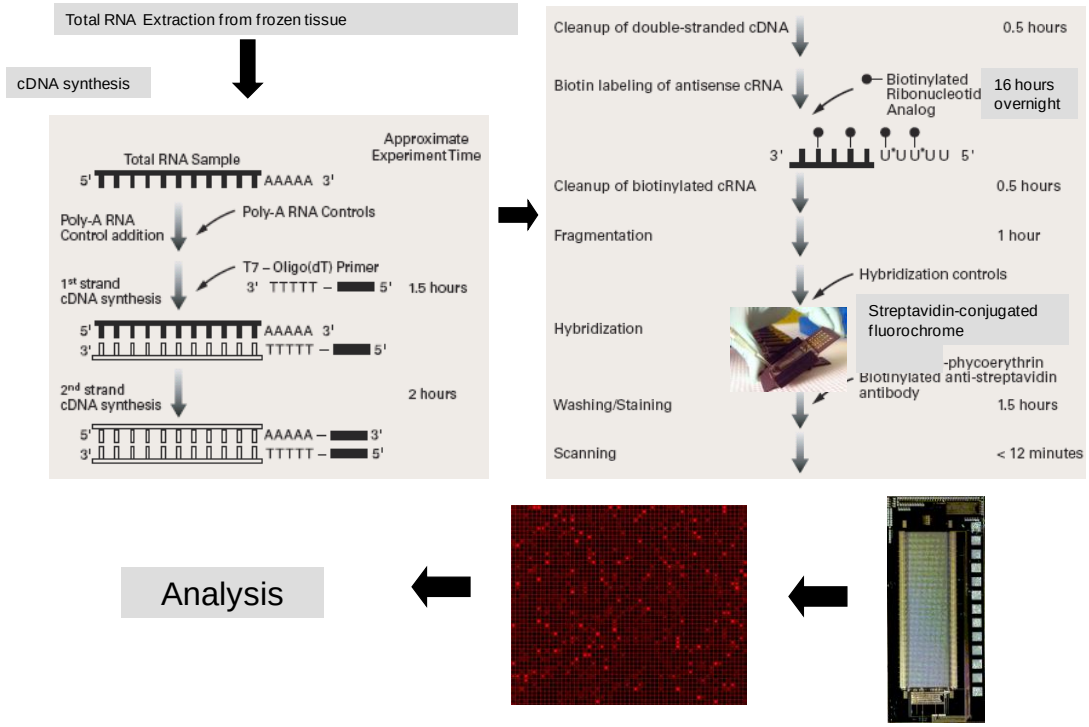
NM_018422	pleckstrin and Sec7 domain containing 3
NM_015310	pleckstrin and Sec7 domain containing 3
NM_014456	programmed cell death 4 (neoplastic transformation inhibitor)
NM_000662	N-acetyltransferase 1 (arylamine N-acetyltransferase)
NM_014782	armadillo repeat containing, X-linked 2
NM_006197	pericentriolar material 1
NM_016337	Enah/Vasp-like
NM_024788	hypothetical protein FLJ21062
AU160041	chromosome 9 open reading frame 116
AW970881	Clone IMAGE:5759947, mRNA
AB050468	leucine-rich repeats and immunoglobulin-like domains 1 /// leucine-rich repeats and immunoglobulin-like domains 1
U56725	heat shock 70kD protein 2
AJ001306	InaD-like protein
NM_016640	mitochondrial ribosomal protein S30
NM_000165	gap junction protein, alpha 1, 43kDa (connexin 43)
AK022172	flavin containing monooxygenase 5
AF047033	solute carrier family 4, sodium bicarbonate cotransporter, member 7
AW242916	interleukin 6 signal transducer (gp130, oncostatin M receptor)
NM_002065	glutamate-ammonia ligase (glutamine synthase)
NM_005059	relaxin 2 (H2)
NM_016210	g20 protein
NM_004727	solute carrier family 24 (sodium/potassium/calcium exchanger), member 1
NM_000169	galactosidase, alpha
AF083108	sirtuin (silent mating type information regulation 2 homolog) 3 (S. cerevisiae)
NM_017606	zinc finger protein 395
BF593509	myosin binding protein C, slow type

AW242315	MRNA; cDNA DKFZp586M0723 (from clone DKFZp586M0723)
AL512727	MRNA; cDNA DKFZp547P042 (from clone DKFZp547P042)
T79953	KIAA0040 gene product
AA824369	beta-transducin repeat containing
BC000576	quinoid dihydropteridine reductase
AA530995	Clone 24405 mRNA sequence
NM_001045	solute carrier family 6 (neurotransmitter transporter, serotonin), member 4

MSA Standard Operating Protocol (SOP)

RNA was extracted from frozen tissue using Trizol (Invitrogen, Carlsbad, CA), and RNA quality was assessed using an Agilent Bio Analyser (Agilent Technologies, Palo Alto, CA). Samples were processed for MSA profiling if there were clear 18S and 28S peaks with no minor peaks present, while samples without the 18S/28S peaks were regarded as degraded and excluded. Figure 20 is a flowchart illustrating the standard operating protocol of the assay.

Figure 20 Standard operating protocol for tumour sample preparation, RNA extraction and MSA



MSA Data Analysis

Microarray Imager software (Combimatrix) was used to generate probe-level intensities. Individual MSAs were normalized by median-scaling the expression of control genes to the same value. To calibrate the MSAs, we generated MSA profiles for a training set of 16 tumours that had been previously profiled using Affymetrix U133plus Genechips (96). For each gene, we weighted the MSA probes by their strength of correlation to the Affymetrix expression data. Average-linkage hierarchical clustering using Pearson correlation distance metrics was performed using CLUSTER and displayed using TREEVIEW software. Individual MSA profiles in the validation set were classified using Support Vector Machine algorithms (Genedata AG, Basel, Switzerland). The MSA gene expression data has been deposited into the Gene Expression Omnibus (accession number GSE7422).

Quantitative RT-PCR for HER2gene expression.

Total RNA was reverse transcribed using Superscript II Reverse Transcriptase (Invitrogen) and quantitative PCR was done using HER2/ERBB2 TaqMan probes (Hs00170433_m1) on a 7500 Real-time system (Applied Biosystems). TaqMan glyceraldehyde-3-phosphate dehydrogenase probes (Hs99999905_m1) were used as internal controls. All tumor samples were run in triplicates and compared against negative controls, which were either water controls or tissue samples from reduction mammoplasty for benign breast hypertrophy.

Statistical analysis

Association of clinical variables between study groups were performed using chi2 test, t test, one-way ANOVA, and Fisher's exact tests. Kappa test was used to assess the agreement of the MSA with standard clinical tests such as IHC; logistic regression was used to assess the association of individual NPI components (tumour size, histologic grade, and lymph node status) with the MSA NPI-ES signature.

Survival differences were plotted using Kaplan-Meier curves and Cox regression was used for the univariate analysis. Cox regression with stepwise forward hierarchical selection was used for the multivariate analysis assess if the expression signatures would contribute to the risk prediction in conjunction the clinically important variables, without any assumption on any underlying theory to base the model selection. The probability of inclusion into the multivariate model was 0.1. STATA10 (StataCorp.College Station, TX: Stata Corporation) was used for the statistical analyses.

6.2 Results

Optimization of breast cancer MSA

Using a standard operating protocol, we carried out several assays to assess the technical variability of the MSA, including replicate sample assays by the same operator, replicate sample assays by different operators, and replicate assays by different operators at different times. The assays were done both using RNA from breast cancer cell lines (MCF7) and primary tumours (Figure 21A). As shown in Table 22A, we attained good reproducibility in the MSA standard operating protocol with correlation coefficients ranging from 0.92 to 0.96 across the different assays (Table 22B).

To assess long-term MSA reproducibility, a set of RNA samples was extracted from primary tumours, and four pairs of replicate hybridizations were done in which each member of a replicate pair was spaced 12 months apart. The replicate profiles also showed a high degree of concordance [correlation coefficients, 0.94 (0.85-0.99)], indicating that the MSA is likely to have good long-term technical reproducibility. This level of consistency is similar to that achieved by other microarray platforms (25, 39) and suggests that the technical performance of the MSA is likely to be robust toward different operators and sample types. To calibrate the MSA against primary tumour samples, we profiled 16 breast tumours (the ‘‘training set’’) previously assayed using standard Affymetrix GeneChip technology. Using a hierarchical clustering algorithm, we computed weights for each MSA probe such that the 16 training set tumours segregated into similar sample clusters (luminal/ER+, HER2, and basaloid) as the Affymetrix arrays (Figure 21B).

Table 22 Optimization of SOP and consistency of MSA

(A)	Day 1	Day 2	Day 3	Day 3a	Operator B
Day 1 - Operator A	1				
Day 2 - Operator A	0.94	1			
Day 3 - Operator A	0.96	0.94	1		0.9
Day 3[a] - Operator A	0.94	0.92	0.94	1	0.92
Day 1 - Operator B	0.93	0.93	N.A.	N.A.	1

(B)	20020386 Operator A	20020386 Operator B	20020418 Operator A	20020418 Operator B	20021003 Operator A	20021003 Operator B
20020386 Operator A	1					
20020386 Operator B	0.94	1				
20020418 Operator A	0.8	0.74	1			
20020418 Operator B	0.81	0.74	0.98	1		
20021003 Operator A	0.87	0.79	0.9	0.94	1	
20021003 Operator B	0.87	0.78	0.89	0.94	1	1

A) Correlation table comparing protocol consistency, inter-array and technical reproducibility from the MSA hybridizations performed by Operator A on day 1, 2 and 3. Inter-operator consistency was also demonstrated by comparison of hybridizations performed by both operator A and B on day 1.
B) Correlation table comparing MSA hybridizations of three breast tumour samples performed by Operator A and Operator B. Inter-operator consistency was observed for all the hybridizations.

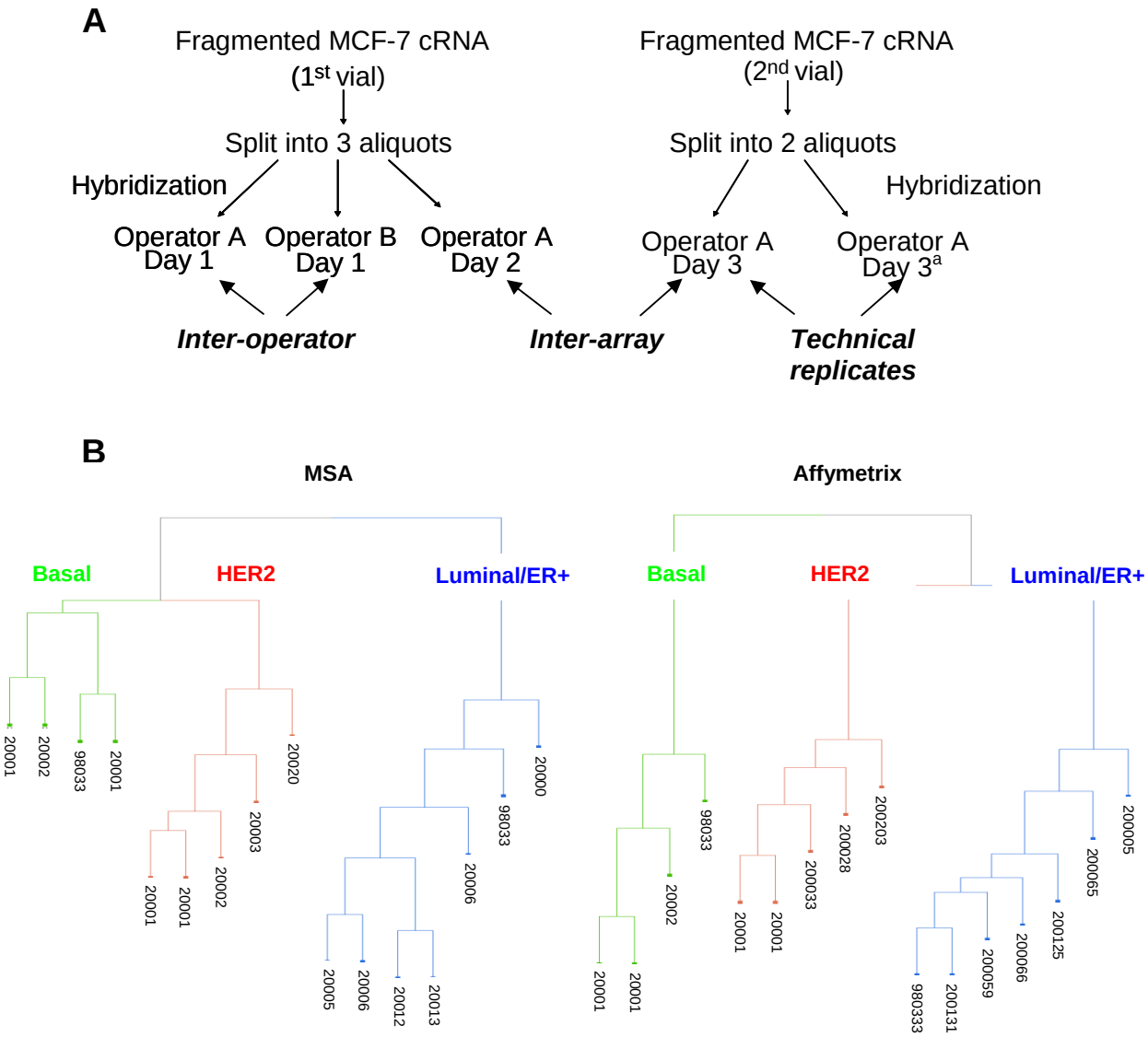


Figure 21 Optimization of MSA standard operating protocol

(A) Methods to assess MSA reproducibility using MCF-7 cell lines. Left, hybridization on two MSAs by operator A on day 1 and day 2; hybridization by operator B on day 1. Right: hybridization on two MSAs (3 and 3a) by operator A on day 3.
(B) Hierarchical clustering showing molecular subtype classification (92 genes) of 16 tumours (e.g., 980333 and 2000196) in the training set on MSAs (left) and Affymetrix U133-Plus arrays (right). The coloured lines represent the subtype classification: green, basal; red, HER2; blue, luminal/ER+. cRNA, complementary RNA.

Clinical features of patients and tumours

Two hundred sixty-seven cases met our predefined inclusion criteria. All patients underwent surgical treatment, mastectomy, or breast conservation surgery and were given adjuvant therapy (chemotherapy, hormonal therapy, and chest wall radiotherapy) according to institutional practice guidelines. Pathological staging was available for all cases. After surgery, two thirds of patients had adjuvant chemotherapy and two thirds had adjuvant hormonal therapy based on the ER and/or progesterone immunohistochemical status at the time of surgery.

On histopathologic assessment of tumor content, 38 tumors with no invasive ductal carcinoma were excluded, and a further 61 tumors were excluded for degraded RNA. There was insufficient RNA in three cases and, hence, these were also excluded. In total, 165 (62%) tumors were finally selected as the validation test set. The mean presence of invasive tumor was 72% (SD, 22%) and the RNA Bioanalyzer ratio was 1.5 (SD, 0.3) in the validation test set of 165 cases. The clinicopathologic variables of the breast cancer patients and their tumors are summarized in Table 23. Importantly, there were no significant differences in these clinical variables between the training set, the ‘‘high-quality’’ validation test set, and the excluded cases ($P > 0.05$), suggesting that the populations are comparable. The mean age of patients in the validation set was 56 +/- 12 (SD) years with 40% presenting with localized disease, which is similar to national demographic data for the local breast cancer population (120; 154). These results suggest that the validation set is likely to serve as a reasonably representative sampling of breast cancer cases in Singapore.

Table 23 Clinical features of the patients and tumours

	Training	Test	Excluded	*p*
Number of cases	16	165	102	
Age (years)				
Mean	52	56	59	0.953
Range	30 - 79	33 - 83	35 – 87	
SD	11	12	11	
Age group (%)				
< or = 54 years old	11 (69)	82 (50)	44 (43)	0.143
> 54 years old	5 (31)	83 (50)	58 (57)	
Tumour size (cm)				
Mean	3.7	3.6	3.7	*0.545
Range	1.6-6.0	0.6 - 12.5	0.3-12.0	
SD	1.3	1.9	2	
T stage** (%)				
T1	4 (25)	36 (22)	13 (13)	0.452
T2	10 (62)	102 (61)	72 (70)	
T3	2 (13)	23 (14)	16 (16)	
T4	0 (0)	5 (3)	1 (1)	
Grade				
1	3 (19)	18 (11)	10 (10)	0.597
2	3 (19)	54 (32)	37 (36)	
3	10 (62)	94 (57)	55 (54)	
Lymph node status (%)				
Negative	6 (38)	66 (40)	51 (50)	0.245
Positive	10 (62)	100 (60)	51 (50)	

All Invasive ductal carcinoma of the breast.

* comparison bewteen test and excluded cases only

** T stage: TNM classification based on American Joint Committee on Cancer (AJCC) Cancer Staging Manual, Sixth Edition

Validation of MSA expression signatures

MSA classification and validation against Affymetrix Gene-Chip technology

We generated MSA profiles for all 165 validation set tumors. Eighty-nine (54%) of the cases were luminal/ER+, 28 (17%) were HER2, and 29 (18%) were basaloid; 19 (11%) were indeterminate. To compare the robustness of the MSA profiles to a different technology platform, RNA from a subset of the validation set tumors

(83 cases, 50%) was also applied to standard Affymetrix genome-wide arrays. Of the 83 cases, 9 (10%) were associated with indeterminate calls on either the MSA or Affymetrix platforms. Of the remaining 74 cases, there was a high concordance of 95% between both technologies, with the MSA classifying 51 tumors as luminal/ER+, 12 as HER2, and 11 as basaloid, and the Affymetrix platform predicting 51 as luminal/ER+, 14 HER2, and 9 basaloid (Table 24). The k test confirmed that the level of observed concordance was highly significant (k = 0.89, P < 0.001). This result suggests that the signature genes on the MSA are likely to have good cross-platform transportability.

Table 24 Concordance of molecular subtype classification between array platforms

		Affymetrix	
MSA	Luminal(ER+)	HER2	Basaloid
Luminal(ER+)	50	1	
HER2	1	11	
Basaloid		2	9

kappa test 0.89, p<0.001

Molecular subtype prediction (Luminal/ER+, HER2 and Basaloid) of 74 tumors by MSA and Affymetrix arrays, 9 cases of indeterminate calls were not available for comparison.

MSA validation against ER status

Eighty-nine of 146 (61%) validation cases were classified as luminal/ER positive by the MSA and 57 cases as ER negative. A correlation to ER immunohistochemistry revealed a 90% concordance between the MSA and immunohistochemistry classifications (P < 0.001, k test; Table 4.3A), indicating a good correlation between gene expression and immunohistochemistry. Similar associations about breast tumor ER status have previously been reported (11).

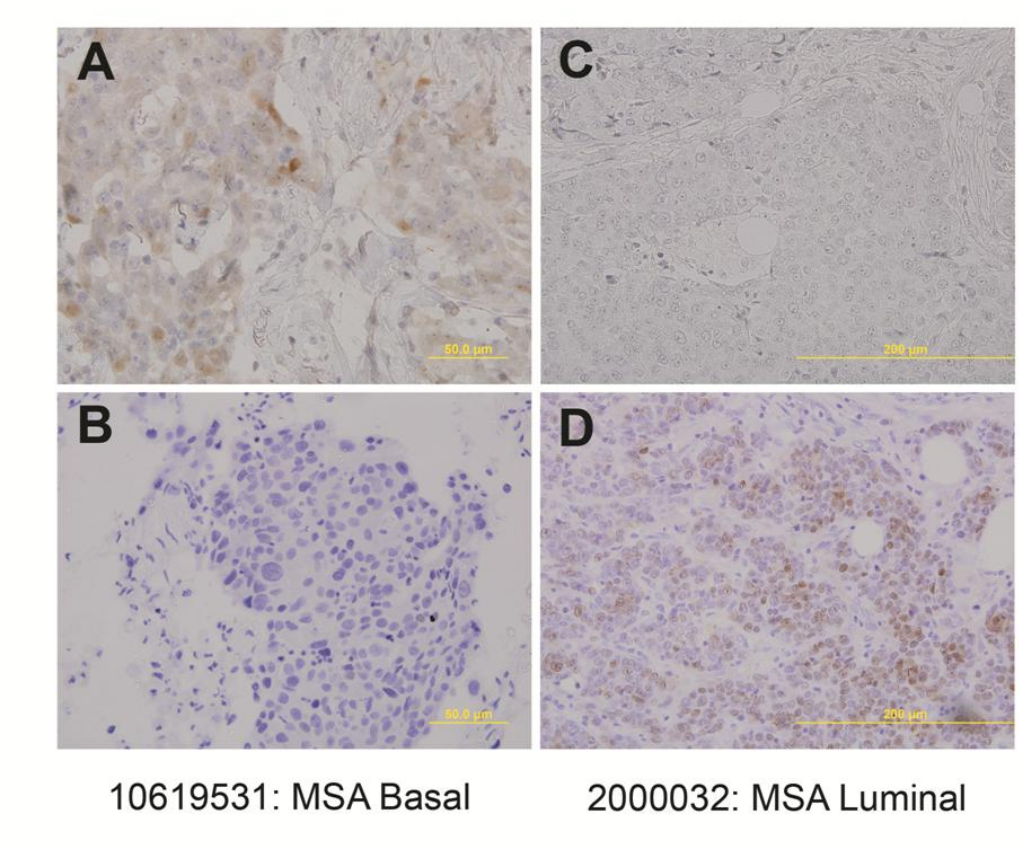
To investigate the discrepant classifications, we retrieved paraffin-embedded tumor blocks for all 165 validation set cases from the hospital pathology archives and subjected these tissues to a repeat ER immunohistochemistry procedure; only 2 cases did not have remaining tissue for reassessment. The correlation of the repeat immunohistochemistry with the ER MSA remained good at 89% ($P < 0.001$, k test). Of the 14 cases with discrepant immunohistochemistry and ER MSA classifications, 6 reassessments supported the ER MSA classification, with some tumors originally classified as ER+ by immunohistochemistry, now supporting an ER-negative immunohistochemistry classification and vice versa (Figure 22; Table 25).

Table 25 Validation of our gene signatures (ER, ERBB2, NPI-ES and TuM1) on 165 breast tumors using the customized microarrays.

(A) ER status by Immunohistochemistry (IHC)				
MSA Signature	*ER+*		*ER-*	
ER+	85		4	kappa=0.795
ER-	10		47	p<0.001
(B) HER2 status by FISH	*FISH ratio*		*FISH ratio*	
MSA Signature	*ratio < 2.2*		*> or =2.2*	
HER2+	4		24	kappa=0.433
HER2-	86		31	p<0.001
(C) HER2 status by FISH*	*FISH ratio*		*FISH ratio*	
MSA Signature	*ratio < 5*		*> or = 5*	
HER2+	8		20	kappa=0.646
HER2-	109		8	p<0.001
(D) Nottingham Prognostic Index (NPI)				
NPI-ES	*NPI < 4*		*NPI = or > 4*	
Low	20		17	kappa=0.316
High	12		40	p=0.001
(E) Tumour Grade				
TuM1	*1*	*2*	*3*	
High	15	16	14	Fisher's
Low	2	22	20	0.002

***FISH ratio of >5 for medium and high positive HER2

Figure 22 Reassessment of ER status by immunohistochemistry.



Repeat immunohistochemistry for ER status in two immunohistochemistry and MSA discrepant cases. A and C, original immunohistochemistry; B and D, repeat immunohistochemistry. A and B, sample10619531: MSA Basal. C and D, sample 2000032: MSA luminal. A,original immunohistochemistry: 3+, 20%
(positive); B, repeat immunohistochemistry:1+, 5%(negative); C, originalimmunohistochemistry: negative; D, repeat immunohistochemistry: 2+,15% (positive). Images produced using light microscopy with Olympus BX51 fitted with camera DP70 (magnification,X 400).

Table 26 Summary of discrepant cases between MSA classification and IHC for ER status.

ER discrepant cases	ER Original IHC	ER MSA Classification	ER Rescore intensity	ER Rescore %	ER Rescore Call
(n=4)					
2000388	Neg	Luminal	0	0	Neg
2000032	Neg	**Luminal**	2+	15%	**Pos**
2001196	Neg	**Luminal**	3+	90%	**Pos**
20020163	Neg	**Luminal**	3+	30%	**Pos**
(n=10)					
11401764	Pos	HER2	1-2+	10%	Borderline
2000174	Pos	**Basal**	2+	5%	**Neg**
10619531	Pos	**Basal**	1+	5%	**Neg**
10860931	Pos	**Basal**	2+	5%	**Neg**
2000191	Pos	Basal	2+	25%	Pos
20020443	Pos	Basal	2-3+	90%	Pos
20020896	Pos	Basal	3+	90%	Pos
2000149	Pos	HER2	2+	50%	Pos
20020207	Pos	HER2	3+	80%	Pos
20020386	Pos	HER2	3+	50%	Pos

Pos: positive; Neg: negative. The original ER status was obtained from the histological report; positivity is defined by a combination of intensity and percentage of staining.

Repeat IHC of 14 discrepant cases between ER MSA and IHC classifications. Repeat IHC corresponding to MSA predictions are in bold.

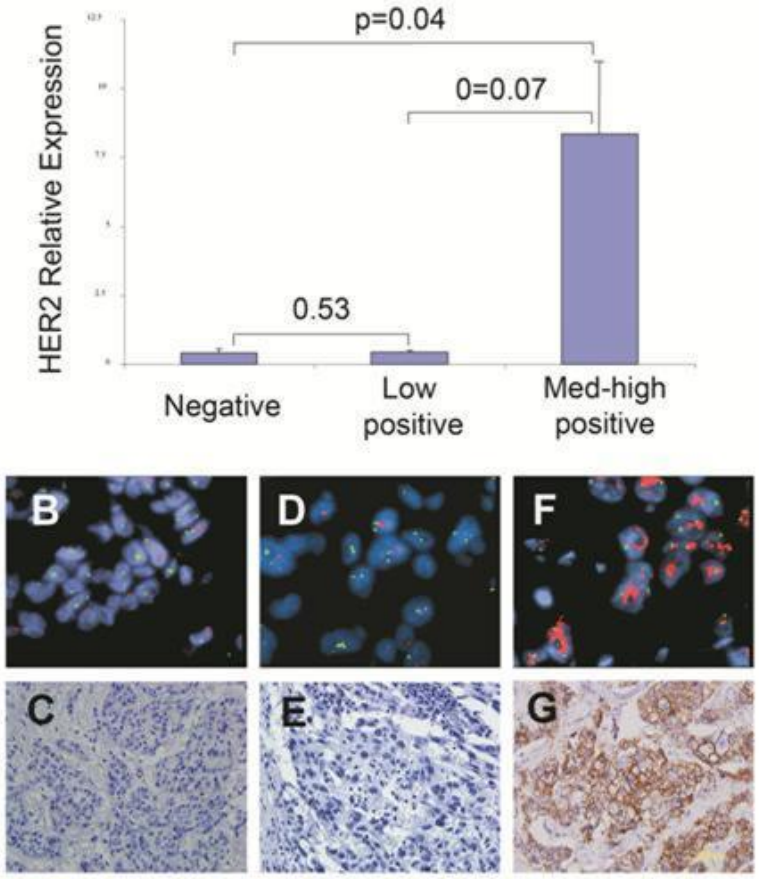
MSA validation against HER2 receptor status

Twenty-eight of 146 (19%) of cases were classified as HER2 positive by the MSA and the rest as HER2 negative. There was a good agreement of 76% between HER2 status by MSA and FISH using the established HER2/CEP FISH ratio cutoff of 2.2 recommended by the American Society of Clinical Oncology/ College of American Pathologists guidelines ($p < 0.001$, $k = 0.433$; Table 25B). Only one

case did not have sufficient tissue for reassessment by FISH. Thus, there seems to be a strong positive correlation between MSA HER2 signature expression and high-level HER2 FISH positivity. Interestingly, when the tumours were divided into 2 groups: negative or low-level HER2 amplification (FISH ratio <5); and tumours with medium (FISH ratio 5-10) or high-level (FISH ratio >10) HER2 amplification based on categories from the PathVysion kit (155), we observed a further improvement in concordance between the HER2 FISH and MSA results from (76% to 89%) ($k = 0.646$, $p < 0.001$; Table 25C). To ask if this could be due to poor MSA sensitivity, we independently measured HER2 gene and protein expression in a subset of FISH-negative, low-level amplified, and high-level amplified samples by quantitative RT-PCR, a more sensitive method for assaying gene expression. We found that HER2 gene expression in the FISH-negative and low-level amplified samples were highly similar even by quantitative RT-PCR ($p = 0.53$), whereas high-level amplified samples exhibited significantly elevated HER2 gene expression ($p = 0.04$ and $p = 0.007$, compared with FISH-negative and low-level amplified samples, respectively; Table 27A). At the protein level, > 90% of low-level amplified cases showed absent or marginal (immunohistochemistry 1+) HER2 protein expression similar to FISHnegative samples (Figure 23B; Table 4.5). In contrast, two thirds of high-level amplified cases showed moderate (immunohistochemistry 2+) to strong (immunohistochemistry 3+) HER2 protein overexpression (Figure 23B; Table 27B), chi2 test, $P < 0.001$). To confirm this finding, we subjected all available cases in the validation set to a repeat HER2 immunohistochemical assessment. The slides were scored and reviewed by two pathologists under current criteria and compared with FISH. Even with the increase in the sample size, our observation remained unchanged. It is noted that HER2 amplification in

immunohistochemistry-negative and immunohistochemistry 1+ cases has previously been reported in other series (17). These results suggest that the apparent bias of MSA HER2 predictions for high-level FISH amplifications is unlikely to be simply due to a lackof MSA sensitivity but may be due to certain low-level amplification tumors behaving more like FISH-negative samples.

Figure 23 HER2 status by FISH, quantitative RT-PCR, and immunohistochemistry in non- amplified, low-level amplified, and high-amplified tumours.



A, quantitative RT-PCR of 23 FISH-positive samples using HER2 TaqMan probes. P values were calculated using t test to compare mean HER2 expression. B, D, and F, HER2 gene amplification by FISH (magnification X1,000); orange signals represent copies of HER-2 gene and green signals represent copies of chromosome 17. C, E, and G, HER2 protein expression by immunohistochemistry; images produced by light microscopy with the Olympus BX51 fitted with camera DP70 (magnification, X 400). B and C, sample 2000327, FISH ratio 1.1, immunohistochemistry-negative; D and E, sample 2001099, FISH ratio 2.8, immunohistochemistry-negative; F and G, sample 2002015, FISH ratio 8.7, immunohistochemistry 3+.

Table 27 Correlation of HER2 expression by FISH with MSA and IHC

		Fluorescent In-Situ Hybridization (FISH)					
		Negative	Positive		Negative-low	Medium-high	
A. Gene Signature		ratio <2.2	ratio ≥2.2	concordance	ratio <5	ratio ≥5	concordance
	HER2-	86	31	76%	109	8	89%
	HER2+	4	24	<0.001	8	20	<0.001
B. IHC Intensity							
0	(Negative)	84	28	<0.001	107	5	<0.001
1	(weak positive)	0	3		1	2	
2	(borderline)	4	7		6	5	
3	(strong positive)	1	15		1	15	

(A) HER2 expression signature correlation to FISH negative and FISH positive (cut-off 2.2). FISH medium-high level and low- and negative- level samples (cut-off 5). (n=145, FISH was not performed for 1 case with insufficient tissue); B) Correlation of HER2 protein expression by IHC to HER2 FISH negative and FISH positive (cut-off 2.2); FISH medium-high level and low- and negative- level samples (cut-off 5). (n=142, IHC was not performed for 3 cases with insufficient tissue).

MSA validation against the NPI and low tumor grade

The NPI is a clinicopathologic staging system that incorporates tumor size, tumor grade, and lymph node status for breast cancer prognostication. Fifty-two of 89 (58%) luminal/ER+ cases were classified by the MSA as expressing high levels of the NPI-ES expression signature, whereas 37 cases were classified as NPI-ES negative. Using a previously defined NPI cutoff value of 4 (96). with lower NPI values indicating good prognosis and high NPI values indicating poor prognosis, a concordance of 67% between NPI-ES expression and NPI status (k = 0.316, P = 0.001; Table 4.3D) was observed. Of the individual NPI components, only histologic grade (P = 0.004) was significantly associated with NPI-ES expression in this set of tumors, whereas lymph node status (P = 0.103) and tumor size (P = 0.096) were not (Table 4.6).

Table 28 Association of the components of Nottingham Prognostic Index (NPI) with NPIES

Characteristic	***p***
Tumor size	0.096
Lymph node status (positive or negative)	0.103
Histological grade (1, 2, or 3)	0.004

* Univariate logistic regression

This result validates the association between the NPI and NPI-ES in an independent patient population. The TuM1 expression signature was previously identified by our group as a potential biomarker for low histologic grade and predictor of tamoxifen response (153). Forty-five of 89 (51%) luminal/ER+ cases were classified as expressing high levels of the TuM1 expression signature, whereas 44 cases were classified as TuM1 negative. A comparison with histologic grade (modified Bloom-Richardson grading) revealed that 15 of 17 (88%) grade 1 tumors exhibited high levels of TuM1 expression compared with 30 of 72 grade 2 and 3 tumors ($p = 0.002$, Fisher's exact test). These figures are consistent with our previous findings (153) and validate the association between TuM1 expression and tumor grade.

The effect of archival protocol affects sample tissue quality

Of the 267 cases that were initially evaluated for this study, 165 (62%) met our inclusion criteria and were profiled on the MSA, whereas the remaining cases were excluded. We examined the excluded samples and found a striking association between the likelihood of a sample being excluded and the year of archival. Specifically, whereas the percentage of excluded cases for either degraded RNA or lack of tumor content was 56% for samples archived in 2000,

this number was only 5% for samples archived from 2004, where only one sample (5%) was excluded for degraded RNA (Table 29). This finding is consistent with an improvement of archival protocol over the years. Moreover, a review of the 19 cases wherein the MSA produced indeterminate calls showed that the tissue had good tumor content (72%; SD, 25%) and good quality RNA (Bioanalyzer ratio, 1.5; SD, 0.3), indicating that the inability to classify these samples is likely not to be a result of poor tissue quality. Thus, while not ignoring the strong necessity for improving and standardizing protocols for tissue handling and preservation, these results suggest that our standard operating protocol for the MSA platform may have general applicability to the current clinical setting with a low frequency of sample loss. One obvious way to increase number of cases suitable for MSA profiling would be to inspect the tissue blockat the time of accrual to ensure adequacy of sampling.

Table 29 Review of the Excluded Samples

	Excluded cases				Test	Total
	No. (% by Yr)				No. (% by Yr)	No.
Year	No invasive tumour	Totally degraded	Insufficient RNA	Total		
2000	18 (29)	16 (26)	1 (2)	35 (56)	27 (44)	62
2001	4 (9)	8 (18)	0 (0)	12 (27)	32 (73)	44
2002	10 (13)	24 (30)	1 (1)	35 (44)	44 (56)	79
2003	6 (10)	12 (19)	1 (2)	19 (31)	43 (69)	62
2004	0 (0)	1 (5)	0 (0)	1 (5)	19 (95)	20
Overall	38 (14)	61 (23)	3 (1)	102 (38)	165 (62)	267

Total number of cases and the reasons of exclusion from the validation test set of cases accrued, showing the trend over the years 2000 to 2004.

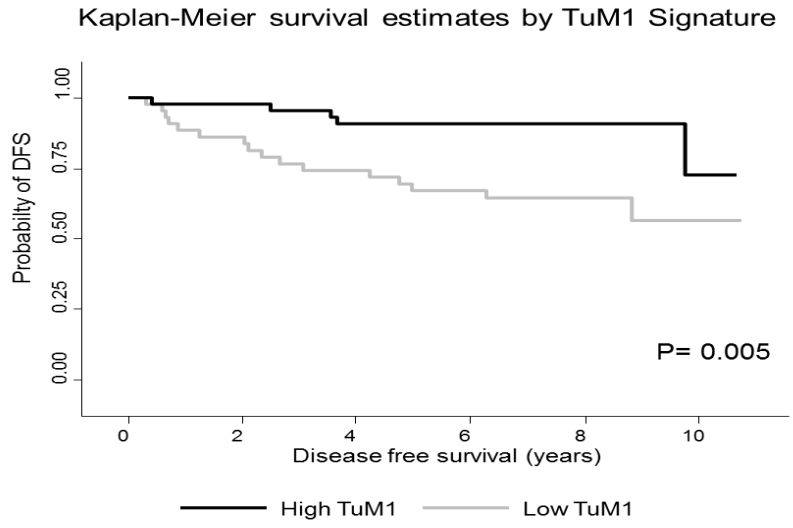
Kaplan Meier Graphs for survival

The patient follow-up in our validation series is a median of 7.3 years (SD, 2.6 years) and disease-free survival period of 7.1 years (SD, 3.0 years).

TuM1 signature

Patients with luminal/ER+ tumors expressing high levels of the TuM1 signature were associated with significantly improved disease-free survival compared with patients with low-TuM1 expressing tumors (hazard ratio (HR), 0.24; p = 0.005; Figure 24).

Figure 24 Kaplan-Meier survival graphs byTuM1gene signature.



		Recurrence		
TuM1 signature		No (%)	Yes (%)	Total
Low		27 (61)	17 (39)	44
High		38(84)	7 (16)	45
	Total	65	24	89

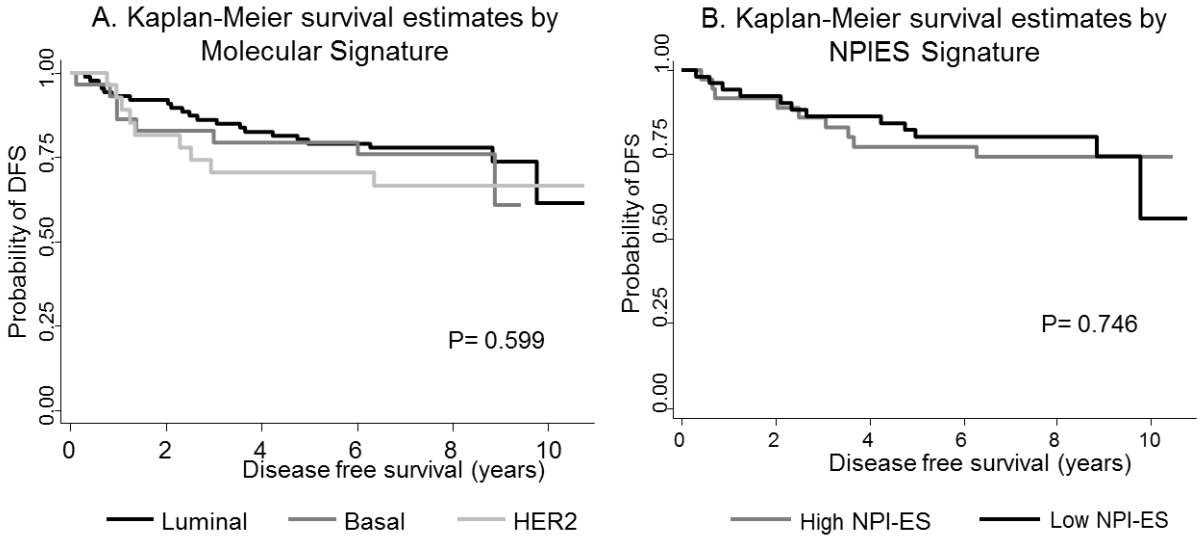
Kaplan-Meier analysis of the probability of remaining free of distant metastasis in patients with luminal/ ER+ subtype tumours only (n = 89). High expression ofTuM1 is represented by the black line and low expression of TuM1by the grey line. The HR of highTuM1to low TuM1and p values was calculated with the Cox-regression, with stepwise forward hierarchical selection in the multivariate analysis.

By Molecular signature and NPIES signature

However, molecular and NPI-ES signature could not predict any survival difference in this validation set (

).

Figure 25 Kaplan-Meier survival graphs by NPIES gene signature.



	Recurrence		
Molecular signature	No (%)	Yes (%)	Total
Luminal	65 (73)	24 (27)	89
Basal	20 (69)	9 (31)	29
HER2	19 (68)	9 (32)	28
Total	104	42	146

	Recurrence		
NPIES signature	No (%)	Yes (%)	Total
Low	26 (70)	11 (30)	37
High	39 (75)	13 (25)	52
Total	65	24	89

Univariate and multivariate analysis

In a univariate analysis involving patient age (</=54 or >54 years, representing premenopausal/perimenopausal and postmenopausal categories, respectively), tumor stage, lymph node status, histologic grade, lymphovascular invasion, NPI, TuM1, and NPI-ES, age (p = 0.046), tumor stage (p = 0.015), grade (p = 0.083), and TuM1 (p = 0.01) were significant predictors of disease-free survival. However, a subsequent multivariate analysis of these variables with a probability of inclusion of 0.1, which also included the use of adjuvant chemotherapy (p= 0.088), showed that only TuM1 (HR, 0.20; p = 0.019) remained as the only independent prognostic factor (Table 30). Notably, the use of adjuvant chemotherapy (HR, 0.7; p = 0.225) in this cohort did not significantly affect disease-free survival. Because patients in this validation set who had ER+ tumors were the recipients of antihormonal therapy, this result is consistent with our initial hypothesis that TuM1 may predict response to antihormonal therapy.

Table 30 Univariate and multivariate analysis associating clinicopathologic factors with disease-free survival in Luminal/ER+ tumours (n=89).

UNIVARIATE:				
Variable	**HR**	***p***	**95% CI**	
Clinical				
Tumour stage (TNM)	2.0	0.015*	1.1	3.6
Age group				
< or = 54 years	1	reference		
> 54 years	2.0	0.046*	1.0	3.8
Nottingham Prognostic Index (NPI)				
NPI < 4	1	reference		
NPI > or = 4	2.0	0.15	0.8	5.1
Lymph nodes status (positive to negative)	2.0	0.188	0.7	5.5
Histological Grade				
Grade 1	1	reference		
Grade 2	3.3	0.27	0.4	26.5
Grage 3	6.1	0.083*	0.8	48.0
LVI (pos vs neg)	1.8	0.201	0.7	4.6
Molecular				
TuM1				
low expression	1	reference		
high expression	0.2	0.01*	0.1	0.7
NPIES				
high expression	1	reference		
low expression	0.6	0.273	0.3	1.5
Treatment				
Chemotherapy	0.5	0.088*	0.2	1.1
Radiotherapy	1.2	0.648	0.5	3.1
Hormonal therapy	0.5	0.25	0.2	1.6
MULTIVARIATE:				
Model 1: stepwise forward hierachical selection				
TuM1	0.2	0.019**	0.1	0.8
Age > 54 years	1.8	0.212	0.7	4.6
Model 2: including adjuvant chemotherapy				
Tum1	0.3	0.029**	0.1	0.9
Age > 54 years	1.6	0.347	0.6	4.3
Chemotherapy	0.7	0.382	0.3	1.7

*p value < 0.1 selected into the multivariate model

**p < 0.05

6.3 Discussion

Methodical considerations

Study 4 is a cross sectional type of design to produce a custom microarray to validate a set of microarray signatures previously discovered on a selected set of local subjects. Important factors in such studies include sensitivity and specificity of the platform, both inter and intra-assay reproducibility. Also important is knowledge of the degree of cross-platform agreement.

Age at 54 years was used at the cut off for the menopausal status to exclude the peri-menopausal group from the menopausal group in this study. This categorization is different from the previous studies, and is the decision of the research groups, although ideally, it is preferable to use the same age threshold in the same thesis.

Selection bias

The cases selected were based on the availability of frozen tumour samples in the tissue repository. These tumours tended to be larger hence in a more advanced stage, but this is unlikely to contribute significantly to the internal validation of this study. The clinical characteristics were random, the training set was found to be comparable with the validation set.

Misclassification

The ability to classify the tumours accurately using the custom array is dependent on several factors:

- Robustness of the signature
- Quality of the tissue samples
- Design of the custom array
- Reproducibility and consistency of the assay
- Specificity and sensitivity of assay

The set of signatures used to design this custom array was based on prior discoveries. It important however, to note that this knowledge was based on a reliable microarray technology in the initial discovery experiments. These were validated using external datasets in prior studies but not in a local dataset. The cases were also selected based on strict selection criteria where there must be adequate tumour cells with full histological information as well as good quality RNA as this may result in misclassification.

Design of the custom array was based on replicate gene probes for the selected gene in multiple replicates and control genes to improve specificity and sensitivity. This was also calibrated and correlated using the Affymetrix, an established technology which was used to discover the gene signatures. Replicate experiments using biological and experimental replicates were performed for protocol consistency, intra-array, inter-array and technical reproducibility by technologists specialized in each particular microarray labelling and hybridization protocol to ensure the MSA protocol was good. Correlation with standard clinical

tests such as IHC for ER and PR, as well as FISH and RT-PCR for HER2 was performed with the MSA. Additional efforts were made to reassess the ER and study the discrepancies

.

Confounding

Ethnicity was not considered in this study, but unlike experiments to discover sets of genes associated with the biological phenomena of interest, the effect of ethnicity on the tumour biology is likely small for a validation study to assess tumour classification, with established subtypes (92–95), based on the microarray platforms (104) and ethnic populations (105), and the reproducibility of gene expression signature–based predictions has been confirmed in replicate experiments (106; 107).

Custom multi-signature assay

In the current treatment of breast cancer, lymph node status, tumour size and histologic grade, histologic tumour type, and lymphatic vascular invasion are the most prognostic factors available (156). The most widely prognostic tool used clinically is the TNM staging, where grade is still not included in the revision of the TNM staging system of breast cancer despite its prognostic value (157). Histopathological methods of classification such as immunohistochemistry (IHC) and fluorescent in situ hybridization (FISH) are routine for its predictive value for therapy with survival benefits. However, assessment of these parameters is associated with limits, including significant inter-observer variability and their reliance on measuring single biomarkers and hence the need to carry out multiple independent tests on the same tumour. Thus, there is a need to improve

existing breast tumour classification methods in terms of robustness, comprehensiveness, and efficiency for better prognostication.

The primary objective of study 4 was to validate a series of gene expression signatures that we had previously described in our local Asian breast cancer population. These include signatures for molecular subtype classification (luminal/ER+, HER2, and basaloid), the NPI (NPI-ES), and low histologic grade (TuM1). The MSA was highly reliable with respect to several clinical variables, supporting the use of custom arrays to function as a potential adjunct to standard immunohistochemistry and FISH in clinical practice. One notable element was the decision to validate these signatures using a customized microarray platform as opposed to a generic genome-wide array as custom designed arrays have only been used in a few validation studies (100; 158; 159), and most of these have examined a single expression signature. In concept, the use of a focused array may be advantageous for the actual implementation of such devices in the clinical setting. First, limiting the array to informative genes may increase their cost effectiveness (e.g., by establishing multiplex arrays in which multiple patient samples are profiled on a single array). Second, having limited gene content may also prove useful in transporting these informative gene sets across other technology platforms that are superior to current solid-substrate arrays in terms of sensitivity, assay speed, and sample quantity (e.g., quantitative RT-PCR). Third, compared with standard assays, the MSA is able to assess multiple biomarkers in a single test. Currently, at least two or three independent assays (IHC for ER and HER2 and FISH for HER2) are required with each assay requiring different sets of technical protocols and reagents. The ability to achieve comparable accuracies of ER and HER2 classification using a single test and

technical protocol, coupled with the ability to also discern additional signatures (i.e., NPI-ES and TuM1), highlights the potential for such array platforms in the molecular diagnostic arena.

The signatures and validation series were derived from a population of predominantly Chinese patients. Reports have described intriguing epidemiologic, clinical, and molecular differences in breast cancer between different ethnic groups (160). For example, whereas the peak of breast cancer incidence in Western populations occurs in the postmenopausal age group and is relatively rare among those <40 years of age, many Asian populations display a striking premenopausal incidence peak (33; 161; 162). This may be due to age-related differences in breast cancer risk factors like parity and body size for women of different ethnicities. At the metabolic and histopathologic level, ethnic differences in the levels of circulating sex steroid hormones (163) and differing frequencies of breast cancer histologic subtypes have also been reported (22). Furthermore, at least one report has observed distinct gene expression patterns associated with African American breast cancer (164), and recent discoveries have shown that lung cancers in Asian women are frequently mutated in the EGFR gene (165). Taken collectively, these observations support the need for considering how ethnicity might influence the performance of such molecular diagnostic assays; and to use a local gene signature instead of commercially available assays developed from tumours from Caucasian populations.

One interesting subpopulation in this validation series involved tumours possessing low levels of HER2 FISH amplification. These cases tended to be immunohistochemistry negative for HER2 and the majority were luminal/ER+ by

the MSA. At present, trastuzumab (Herceptin) is clinically recommended in the adjuvant setting for HER2 FISH–positive samples. In this study, we observed a good concordance of 76% between HER2 MSA and FISH at the 2.2 threshold. However, the concordance further improved to 89% when the tumours were divided into negative/low positive (ratio <5) and medium-high level amplified categories (ratio >5). One possible explanation is that certain tumours with low-level FISH positivity (ratio, 2.2-5) may behave more similarly to FISH-negative samples than to tumours with high-level amplification (ratio >5), with no protein significant expression. Supporting this, it was reported that in a large series of 6,556 tissues analysed with HER2 immunohistochemistry and FISH, FISH amplification could be observed in HER2 immunohistochemistry-negative and immunohistochemistry 1+ cases (166). This observation as confirmed using three different independent assays: MSA HER2 expression signature, HER2 quantitative RT-PCR, and HER2 protein immunohistochemistry. These results suggest that it may prove valuable for future studies to investigate the validity of raising the FISH ratio to >2.2, the threshold that is currently recommended.

In the background where many current standard parameters used have limitations that there is a constant need to improve existing breast tumour classification methods in terms of robustness, comprehensiveness, and efficiency for better prognostication, that research such as this is undertaken. There are limitations of the MSA in terms of the technology as fresh frozen tissues were used instead of paraffin embedded samples, making the test cumbersome. We can overcome this as tissue can be collected fresh at surgery or biopsy, as we do for cancers such as lymphomas. Indeed if the signatures were merely equivalent, with no additional signatures, in addition to the cumbersome nature and high cost

for the gene expression analysis, the test is indeed not attractive and will be a significant limitation for clinical use. However, I would still argue on the potential benefits of:

- The ability to achieve comparable accuracies of ER and HER2 classification.
- Be an adjunct for ER classification as reassessments by IHC supported the ER MSA classification providing a more accurate ER assessment.
- Using a single test and technical protocol in replacement of separate tests with potential cost savings as the cost decreases
- Coupling with the ability to also discern additional signatures (i.e., NPI-ES and TuM1)

Future work could include looking at the use of paraffin-embedded tissue, refinement of the assay, use of other platforms as well as multiplex assays to address the current limitations.

6.4 Conclusion

The results demonstrate the reproducibility and robustness of the gene signatures on the MSA, with a simple operating protocol that can measure multiple biomarkers simultaneously (i.e., the standard ER and HER2 markers). In addition, the TuM1, a low-grade marker has prognostic implications. One potential weakness of this study is that the clinical follow-up is still in the early years and immature, and hence the long-term prognostic power of some

signatures cannot be conclusively assessed. Nevertheless, at least one signature (TuM1) was associated with improved survival, even in this interim analysis. Future work will involve assessing the long-term outcome of disease in our breast cancer patients and comparing them to similar outcome metrics in the United States and Europe.

CHAPTER 7 CONCLUSIONS

In this thesis, we demonstrated the following:

1. Trends in breast cancer survival in Singapore compared to a western population of Stockholm which showed that the main difference in overall survival difference was due to the presentation of more advanced stage of breast cancer in Singapore. This advantage disappeared when survival was stratified by stage.
2. We hypothesize that better economic status associated with increased awareness of the disease, better access to screening routines or healthcare quality and options, seen in Stockholm and in Singapore only in the later decade, offer the main explanation for the prognostic differences and similarities.
3. Trends in breast cancer survival between ethnic groups in Singapore: Chinese, Malays and Indians were demonstrated. There were more premenopausal Malays presenting with breast cancer compared to the Chinese and Indians. They presented with a more advanced stage of disease and have poorer prognosis. Within the ethnic groups, there is a difference in stage distribution; stratified by stage, there is a difference in survival between the ethnic groups; and when taken together, stage and ethnicity were independent predictors. Ethnic difference and possibly factors related to ethnicity such as SES were likely reasons for this overall difference.
4. The tumour biology in terms of ER, PR and HER2 were not different by ethnicity and hence is not a likely reason for survival difference between ethnicity; however, there was some difference in grade and LVI between ethnicity. Grade and LVI were correlated with ethnicity and likely contributed

to the survival difference. Nevertheless, tumour subtype, grade, LVI, stage and ethnicity were independent risk factors of death. Hence, other factors associated with ethnicity such as tumour biology other than subtype, response to treatment as well as cultural difference in seeking treatment could be contributory.

5. There is a drive for a development of a single ideal prognostic test to provide the necessary information for treatment; and advances in the molecular high throughput technology has led to an explosion of its utility to discover such a test, especially one which could possibly decipher the genetic and ethnic differences. This journey to design and validate a custom multi-signature assay (MSA) is a significant step forward as a proof of point in a local Singaporean context, but further work is needed.

In this era of personalized medicine (also termed personalized genomics, genomic medicine, or theranostics), the application of patient-specific profiles, incorporating genetic and genomic data as well as clinical and environmental factors, to assess individual risks and tailor prevention and disease management strategies has gained popularity and significance. Translating these results to public health would include the following:

- Scientific discovery of ethnic driven differences in breast cancer biology in Singapore e.g., tumours in Malay women
- Customize treatment plans that could encompass targeted pharmacotherapy to improve drug response and reduce toxicity and expense

- Targeted health education and planning, development of support and financial help groups for those most at risk, e.g., amongst the Malays with lower SES
- Targeted recommendations for lifestyle modifications for the modifiable risk factors such as adolescent pregnancies which could affect breast cancer biology and prognosis
- Develop prevention strategies and enhance patient satisfaction with healthcare

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