EXPRESSION OF FOXP3 TUMOUR INFILTRATING LYMPHOCYTES IN HER2 POSITIVE BREAST CANCER AND THEIR ASSOCIATION WITH CLINICOPATHOLOGICAL FEATURES

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ADCC: Antibody-dependent cell-mediated cytotoxicity

ASCO: American Society of Clinical Oncology

ASR: Age-standardised incidence rate

CAP: College of American Pathologists

DAB: Diaminobenzidine

DPX: Dibutyl phthalate polystyrene xylene

ECM: Extracellular matrix

ER: Oestrogen receptor

FFPE: Formalin-fixed paraffin-embedded

FOXP3: Forkhead Box Protein P3

FOXP3+: Forkhead Box Protein P3 positive

GPSP: Geran Penerbitan Sarjana Perubatan

H&E: Hematoxylin & Eosin

HER2: Human epidermal growth factor receptor 2

HSNZ: Hospital Sultanah Nur Zahirah

HUSM: Hospital Universiti Sains Malaysia

IBC: Invasive breast cancer

IHC: Immunohistochemistry

ILC: Invasive lobular carcinoma

ISH: In situ hybridisation

JEPeM: Jawatankuasa Etika Penyelidikan Manusia

LIQ: Lower inner quadrant

LOQ: Lower outer quadrant

MDSCs: Myeloid-derived suppressor cells

MREC: Medical Research & Ethics Committee

NCNN: National Comprehensive Cancer Network

NST: No special type

OS: Overall survival

PR: Progesterone receptor

RTK: Receptor tyrosine kinase

SPSS: Statistic package for the social software

TBS: Tris buffer saline

TILs: Tumour infiltrating lymphocytes

TME: Tumour microenvironment

TNBC: Triple-negative breast cancer

TNM: Tumour, node, metastasis

Tregs: Regulatory T cells

UICC: Union for International Cancer Control (UICC)

UIQ: Upper inner quadrant

UOQ: Upper outer quadrant

USM: Universiti Sains Malaysia

WHO: World Health Organization

ABSTRAK

Latar belakang: Faktor transkripsi Forkhead Box Protein P3 (FOXP3) memainkan peranan penting dalam perkembangan dan fungsi sel T pengawalseliaan (Tregs) dan berfungsi sebagai faktor spesifikasi keturunan Treg. FOXP3+ tumor infiltrasi limfosit (TIL) dianggap sebagai penanda prognostik yang penting dalam kanser payudara. Namun begitu, kepentingan prognostik FOXP3+ TIL dalam kanser payudara positif human epidermal growth factor receptor 2 (HER2) masih menjadi kontroversi. Kami menyiasat kadar kanser payudara positif HER2 dan ekspresi FOXP3+ tumor infiltrasi limfosit (TIL) serta menilai hubungan mereka dengan ciri klinikopatologi.

Kaedah: Kajian keratan rentas telah dijalankan melibatkan 167 sampel tisu tertanam parafin diawet formalin (FFPE) bagi kes kanser payudara positif HER2 daripada Jabatan Patologi, Hospital Sultanah Nur Zahirah (HSNZ) dari tahun 2014 hingga 2021. Tisu blok tersebut telah diwarnai dengan antibodi anti-FoxP3 imunohistokimia (IHC). Ekspresi FOXP3+ TIL dikategorikan kepada infiltrasi rendah (gred 0 hingga gred 1) dan infiltrasi tinggi (gred 2 hingga gred 3). Ujian Fischer's Exact digunakan untuk menganalisis perkaitan dengan ciri klinikopatologi. Tahap signifikan kurang daripada 0.05 dianggap signifikan secara statistik.

Keputusan: Jumlah kes kanser payudara dari 2014-2021 adalah 1066 kes. Terdapat 272 (25.5%) kes kanser payudara positif HER2. Antara 167 kes kanser payudara positif HER2 yang terpilih, 164 (98.2%) kes menunjukkan infiltrasi FOXP3+ TIL yang tinggi, dan 3 (1.8%) kes menunjukkan infiltrasi yang rendah. Tidak terdapat perkaitan yang signifikan antara ekspresi FOXP3+ TIL dengan umur diagnosis dibuat ($p > 0.950$), gred tumor ($p=0.091$), saiz tumor ($p=0.235$), status kelenjar limfa ketiak ($p=0.552$), status metastasis ($p > 0.950$), status reseptor estrogen (ER) ($p=0.616$) dan status reseptor

progesteron (PR) ($p > 0.950$).

Kesimpulan: Kanser payudara positif HER2 menunjukkan kadar infiltrasi FOXP3+ TIL yang tinggi walaupun terdapat perbezaan dalam ciri klinikopatologi.

ABSTRACT

Background: The transcription factor Forkhead Box Protein P3 (FOXP3) plays a crucial role in regulatory T cells (Tregs) development and function and serves as a lineage specification factor of Tregs. FOXP3+ tumour infiltrating lymphocytes (TILs) are considered significant prognostic markers in breast cancer. However, the prognostic significance of FOXP3+ TILs in human epidermal growth factor receptor 2 (HER2) positive breast cancers remains controversial. We investigated the proportion of HER2-positive breast cancer and their expression of FOXP3+ TILs and evaluated their association with clinicopathological features.

Methods: A cross-sectional study was conducted involving 167 formalin-fixed paraffin-embedded (FFPE) tissue samples of HER2-positive breast cancer cases from the Department of Pathology, Hospital Sultanah Nur Zahirah (HSNZ) from the year of 2014 to 2021. The tissue sections were stained with immunohistochemistry (IHC) anti-FoxP3 antibodies. FOXP3+ TILs expression was categorised into low infiltration (grade 0 to grade 1) and high infiltration (grade 2 to grade 3). Fischer's Exact test was used to analyse the association with clinicopathological characteristics. A p-value of less than 0.05 was considered statistically significant.

Results: Total breast cancer cases from 2014-2021 were 1066 cases. There were 272 (25.5%) HER2-positive breast cancer cases. Among 167 selected HER2-positive breast cancer cases, 164 (98.2%) cases showed strong FOXP3+ TILs infiltration, and 3 (1.8%) cases showed low infiltration. There was no significant association between the expression of FOXP3+ TILs with the age of diagnosed ($p > 0.950$), tumour grade ($p=0.091$), tumour size ($p=0.235$), axillary nodal status ($p=0.552$), metastasis status ($p > 0.950$), oestrogen receptor (ER) status ($p=0.616$) and progesterone receptor (PR) status

($p > 0.950$).

Conclusion: FOXP3+ TILs were highly infiltrating HER2-positive breast cancer despite variability in clinicopathological characteristics.

CHAPTER 1: INTRODUCTION

1.1 OVERVIEW OF BREAST CANCER

Breast cancer is the most often diagnosed cancer in women and the primary cancer killer among women worldwide, yet patterns and trends range from country to country (Lei *et al.*, 2021). Globally, 2.3 million new instances of breast cancer are thought to be diagnosed each year (Sung *et al.*, 2021). Over the past three decades, breast cancer incidence and mortality rates have grown. Breast cancer incidence has more than doubled between 1990 and 2016 in 60 of 102 nations (including Afghanistan, the Philippines, Brazil, and Argentina), while mortality has doubled in 43 of 102 countries (e.g., Yemen, Paraguay, Libya, Saudi Arabia) (Sharma, 2019). According to the current forecasts, there will be 2.7 million new cases diagnosed annually worldwide by 2030, while there will be 0.87 million fatalities (Ferlay *et al.*, 2021).

According to previous research, the incidence of breast cancer is highest in high-income and high-middle-income countries, ranging from 85.8 to 91.6 cases per 100,000 inhabitants. Meanwhile, the mortality rates are highest in low-middle-income and low-income countries, ranging from 17.4 to 20.1 deaths per 100,000 inhabitants. The economic development, environmental conditions, and racial makeup of the population all affect breast cancer incidence and mortality rates in various nations (DeSantis *et al.*, 2015; Torre *et al.*, 2016). In Malaysia, 34.1% of cancers in females were breast cancer, making it the most prevalent type of cancer. The age-standardised incidence rate (ASR) climbed from 31.1 per 100,000 inhabitants in the previous report to 34.1. Chinese race had the highest occurrence, followed by Indians and Malay (Azizah *et al.*, 2019).

Breast lump was the most prevalent sign of breast cancer, occurring in nearly four out of every five women. Nipple abnormalities, breast pain and abnormalities of the breast skin were the next most often reported presenting symptoms. Smaller groups of women, however, tended to experience symptoms such as non-specific breast abnormalities, back discomfort, musculoskeletal pain, chest pain, weakness or fatigue, and weight loss (Koo *et al.*, 2017). Breast cancer that has developed skin ulcers is regarded as a locally advanced condition (Khoury *et al.*, 2018). The occurrence of tumours according to quadrants of the breast was in reducing order starting from upper outer quadrant (UOQ), upper inner quadrant (UIQ), lower outer quadrant (LOQ), central, and the least was lower inner quadrant (LIQ). Tumours in the centre of the breast were much more likely to have an advanced tumour stage (Rummel *et al.*, 2015).

Bilateral breast cancer is currently more common than ever, accounting for 2%-11% of all breast cancer cases and falling into two categories: synchronous and metachronous bilateral breast cancer (Pan *et al.*, 2019). Age, a family history of breast cancer, early menarche, late menopause, a high body mass index, being obese or overweight, exposure to cigarette smoke, and high consumption of fats or fatty foods in the diet were all linked to an elevated risk of breast cancer in the Asian population. On the other hand, consuming dietary fruits, vegetables, and plant- and soy-based products was linked to a lower risk of breast cancer (Youn and Han, 2020).

It has been shown that several genetic abnormalities are strongly linked to an elevated risk of breast cancer. One of the few known rare but highly penetrant genes, such as BRCA1, BRCA2, PTEN, TP53, CDH1, and STK11, confers up to an 80% lifetime risk of breast cancer and is responsible for up to 25% of hereditary cases. A rare, moderate-

penetrance gene mutation such as CHEK2, BRIP1, ATM, and PALB2, which each carries a two-fold increase in risk, is responsible for an additional 2%-3% of cases (Shiovitz and Korde, 2015). A wide range of morphological characteristics, various immunohistochemical profiles, and distinct histological subtypes with a predetermined clinical course and outcome are all present in invasive breast tumours (Makki, 2015).

Over the past 20 years, breast cancer screening and diagnosis have advanced beyond standard screen-film mammography. Breast cancer screening may soon benefit from new methods in breast imaging. These include digital breast tomosynthesis, contrast material-enhanced spectral mammography, automated three-dimensional breast ultrasonography, ultrasound transmission tomography, elastography, optoacoustic imaging, abbreviated and ultrafast magnetic resonance imaging, diffusion-weighted imaging and molecular breast imaging (Mann *et al.*, 2020). As new information from research is quickly incorporated into clinical practice, the classification of breast tumours continues to change (Tan *et al.*, 2020).

The fifth edition of the World Health Organization (WHO) classification of tumours maintains the familiar systematic approach of earlier volumes, with the content now organised in chronological order starting with benign epithelial proliferations and precursors, progressing through benign neoplasms to in-situ and invasive breast cancer, then moving on to mesenchymal and haematolymphoid neoplasms, tumours of the male breast, and genetic tumour syndromes (Wysocka, 2020). There are many different histological subtypes of invasive breast cancer (IBC) (Makki, 2015). The most common category (40-80%) is invasive breast cancer of no special type (NST), formerly invasive ductal carcinoma. By default, this type is identified as a tumour that cannot be

categorised into special histological categories. Invasive lobular carcinoma (ILC) is the second most frequent histological subtype. Several other subtypes with distinct histological characteristics exist in addition to these two, such as tubular carcinoma, cribriform carcinoma and mucinous carcinoma of the breast (Tan *et al.*, 2020).

In addition to the histological type of the tumour, the results of the histopathological examination should also include the tumour's histological grade, associated carcinoma in situ component, lymphovascular invasion, surgical margin status, and tumour progression stage as determined by the pTNM classification (Fitzgibbons *et al.*, 2021). Histological grading has proved a straightforward and affordable way to evaluate tumour behaviour and invasive breast cancer prognosis, thereby identifying individuals at risk for unfavourable outcomes and perhaps qualifying for (neo) adjuvant therapy. The histologic grade indicates the degree of differentiation, which reveals how much the tumour cells resemble normal breast tissue. The Nottingham grading system is a semiquantitative assessment of three morphological characteristics; tubule and gland formation, nuclear pleomorphism, and mitotic frequency. The three-grade component values are added in total, yielding a score ranging from 3 to 9, and the final grade is then determined based on this score. Grade 1 tumours with scores of 3 to 5 are well differentiated, grade 2 tumours with scores of 6 to 7 are moderately differentiated, and grade 3 tumours with scores of 8 to 9 are poorly differentiated (van Dooijeweert *et al.*, 2022).

The most widely used system for staging breast cancer is the TNM system published by the Union for International Cancer Control (UICC) and the American Joint Committee on Cancer. The TNM acronym suggests that the characteristics of the primary tumour

(often its size, but also other factors like its relationship to nearby structures), characteristics of the regional lymph nodes (often the number and location of involved nodes, but also other factors like the size of the nodal involvement or the presence of extracapsular extension), and the presence or absence of distant metastasis all affect the tumour's prognosis (Cserni *et al.*, 2018). In addition, the expression of oestrogen receptor (ER), progesterone receptor (PR), HER2 and cellular proliferation index Ki67 should be included in the results of the histopathological examination (Fitzgibbons *et al.*, 2021).

1.2 MOLECULAR CLASSIFICATION OF BREAST CANCER

Traditional breast cancer classification, primarily based on clinicopathological characteristics and evaluation of common biomarkers, may not adequately account for the diverse clinical courses of individual breast cancers. Therefore, discoveries from technological advancement added valuable knowledge about the underlying genetic changes and the biological pathways in breast cancer (Eliyatkin *et al.*, 2015). Global gene expression profiling studies classified breast cancers into five intrinsic subtypes by hierarchical clustering: luminal A, luminal B, HER2 overexpression, basal and normal-like subtypes (Dai *et al.*, 2015). Meanwhile, claudin-low is a new intrinsic subtype of breast cancer that has recently been discovered (Pommier *et al.*, 2020).

Luminal A, B and normal-like subtypes are enriched with ER-positive cancers, whereas HER2 overexpression and basal-like breast cancers are ER-negative. Luminal A makes up 40% to 50% of invasive breast cancers, making it the most prevalent molecular subtype. Meanwhile, luminal B represents about 20% of invasive breast cancers (Dai *et al.*, 2015). Luminal A breast cancers typically have the lowest grade and the best

prognosis among the intrinsic subtypes (Hennigs *et al.*, 2016). Compared to luminal A, luminal B breast cancer exhibit lower expression of ER-related genes and higher expression of proliferation-related genes with variable expression of HER2-related genes. Thus, luminal B breast cancers typically have a higher grade and a worse prognosis than luminal A (Li *et al.*, 2016).

Luminal A breast cancers are generally best treated with hormonal therapy alone; however, luminal B tumours might benefit from further chemotherapy (Ahn *et al.*, 2015). Additionally, compared to the luminal A subtype, luminal B revealed a higher mortality risk (Mohammed, 2021). About 15%-20% of invasive breast cancers are of the HER2 overexpression subtype (Godoy-Ortiz *et al.*, 2019). This subtype most likely has a high grade, ER and PR negative and aggressive clinical course (Han *et al.*, 2022). Up to 30%-50% of patients with HER2-positive breast cancer may develop brain metastases (Garcia-Alvarez *et al.*, 2021). Despite the expected course, this subtype responds quite well to anti-HER2-targeted therapy, which leads to a significantly better outcome (Godoy-Ortiz *et al.*, 2019).

Basal-like or triple-negative breast cancer accounts for 15%-20% of invasive breast cancer cases. This subtype shows overexpression of proliferation-related genes but lacks ER, PR, and HER2-related gene expression (Almansour, 2022). Clinical characteristics of this basal-like subtype include high levels of invasiveness, significant metastatic potential, propensity for relapse, and poor prognosis (Haddad *et al.*, 2019). This basal-like subtype of breast cancer continues to be the most challenging to manage. Due to fewer treatment options and a lack of targeted therapies, basal-like breast cancer poses a challenge for both patients and clinicians and is associated with a higher mortality rate

than other breast cancer subtypes (Garrido-Castro *et al.*, 2019). Claudin-low breast cancer subtypes range from 7%-14% of invasive breast cancer. This subtype preferentially exhibits a triple-negative phenotype and is identified by gene expression features. This subtype has also been linked to a low survival rate (Dias *et al.*, 2017).

This molecular classification offers insights into new therapeutic approaches and patient stratifications that impact the management of breast cancer patients. PAM50 (Prediction Analysis of Microarray using 50 classifier genes plus five reference genes), a standardised technique, has been established to categorise breast cancers into luminal A, luminal B, HER2-enriched, and basal-like breast carcinomas. However, gene expression profiling's use in routine clinical practice remained constrained by its high cost and technological complexity. IHC-based surrogate molecular classification has thus been advocated (Tsang and Tse, 2020).

1.3 HORMONE RECEPTORS IN BREAST CANCER

Breast carcinomas from various people exhibit intertumour heterogeneity. Meanwhile, the existence of diverse cell types within a single tumour is what causes intratumoral heterogeneity (Ellsworth *et al.*, 2017). Intertumor and intratumour heterogeneity are influenced by biomarker expression (Turashvili and Brogi, 2017). Over the past few decades, various molecular markers have been investigated to identify potential treatment targets and valuable prognostic tools that predict whether cancer is aggressive or indolent. The most significant molecular markers in the standard treatment for all primary, recurrent, and metastatic breast cancer patients are the ER, PR, and HER2 status. Therefore, every newly diagnosed case of breast cancer must include a standard assessment of the ER/PR/HER2 status (Vivanco, 2018).

About 80% and 60-70% of breast carcinomas, respectively, express the ER and PR (Badowska-Kozakiewicz *et al.,* 2015). In addition, 15%-20% of invasive breast cancers exhibit HER2 gene amplification, which is closely associated with HER2 protein overexpression (Godoy-Ortiz *et al.,* 2019). Meanwhile, over 15-20% of breast tumours are triple-negative breast cancers (TNBC) (Almansour, 2022). The neuroendocrine, circulatory, skeletal, and immunological systems are a few of the multiple biological systems that the hormone oestrogen affects. It also affects both male and female reproduction. As a result, it is also linked to various illnesses and ailments, including obesity, endometriosis, osteoporosis, and other malignancies. Oestrogens are steroidal hormones that serve as the main sex hormone for women. Estrone (E1), estradiol (E2), and estriol (E3) are the three main types of oestrogen. Estrone and estriol are largely produced during pregnancy and after the onset of menopause, respectively. Estradiol (E2) is the main oestrogen in non-pregnant females (Hamilton *et al.*, 2017).

Meanwhile, the primary oestrogen from menarche to menopause is 17-β-estradiol (E2), the most potent estrogen hormone in circulation. Most of the E2 actions are mediated by its two nuclear receptors, ESR1 (which codes for estrogen receptor alpha, ERα) and ESR2 (which codes for estrogen receptor beta, ERβ), which are located on chromosomes 6 and 14, respectively (Yaşar *et al.*, 2017). Oestrogen receptors are nuclear receptors that are a member of the superfamily of steroid hormone receptors. These receptors are primarily found in the cell nucleus but have also been discovered in the cytoplasm and even at the mitochondrial level (Frigo *et al.,* 2021). In the uterus, pituitary, mammary glands, skeletal muscle, adipose tissue, and bone, ERα predominates while ERβ plays a limited role. Contrarily, it has been discovered that the central nervous system, ovary, prostate, lung, and cardiovascular systems depend on the

ERβ to mediate E2 signalling (Hua *et al.*, 2018).

While ERα has five isoforms, ER1-ER5, ERß can be found in three primary protein isoforms that differ in molecular weight: ER-66, ER-46, and ER-36 (Porras *et al.*, 2021). Estrogen tightly controls the formation and growth of the normal breast (Bondesson *et al.*, 2015). The normal human mammary gland and breast express both oestrogen receptors, ERα and ERβ, and it is thought that these receptors cooperate to maintain the regulation of the activities of oestrogen (Hua *et al.*, 2018). Within various regions of breast tissue, there is a high degree of heterogeneity in ERα expression. ERα expression levels throughout life vary, especially after puberty, during menstrual cycles, and pregnancy and lactation (Dall *et al.*, 2018). ERα is expressed solely in luminal epithelial cells. Meanwhile, ERβ is expressed in luminal, myoepithelial and stromal cells (Alferez *et al.*, 2018).

The primary carcinogenesis initiator in breast cancer is ERα; the functions of the ERß isoforms are less apparent, but it has been demonstrated that they can suppress ERα activity (Dall *et al.*, 2018). Although the response rates are much lower for tumours with low expression of ERα, breast cancers have been classified as ERα-positive (ER-positive tumours) if 1% to 100% of epithelial cells exhibit nuclear staining in immunohistochemistry (IHC) with anti-hERα antibodies. Patients are then eligible for hormonal therapies. Meanwhile, if <1% or 0% of the tumour cell nuclei in a sample are immunoreactive, it is considered an ER-negative tumour (Allison *et al.*, 2020).

Progesterone is a major gonadal hormone produced in the corpus luteum of the ovaries and the placenta during pregnancy. It is an endogenous 21-carbon steroid hormone derived from cholesterol via pregnenolone. The Leydig cells in men's testicles, the adrenal cortex, adipose tissue, and other tissues generate progesterone to some extent but in considerably lower amounts (Henderson, 2019). Progesterone has a wide range of biological actions that are either mediated by nuclear receptors or non-nuclear receptor pathways. Progesterone enters the cell through the cell membrane and attaches to the nuclear progesterone receptor's ligand binding domain, which results in a specific conformational change (Taraborrelli, 2015). PR belongs to the group of ligand-dependent transcription factors known as the nuclear steroid receptors. PR has two main isoforms, progesterone receptor A (PR-A, 116 kDa in length) and progesterone receptor B (PR-B, 4 kDa, lacking 164 amino acids at the N-terminus), which are transcribed from the same gene on 11q22-q23 via two different promoters (Fabris *et al.*, 2017).

The ratio of PR-A and PR-B expression may vary among the various reproductive tissues. The PR ratio might vary even within the same organ depending on the hormonal state or developmental stage (Hilton *et al.*, 2018). Normal breast tissue often exhibits 1:1 co-expression of the two isoforms at similar levels. As a lesion progressed from a normal condition to malignancy, there was a discernible rise in the predominant expression of PR-A or PR-B (Rojas *et al.*, 2017). Both basal and luminal epithelial cells in the human breast express PR. A paracrine method is used by luminal PR to simulate the growth of nearby cells. Additionally, PR is expressed in populations of basal epithelial cells and encourages their proliferation (Arendt and Kuperwasser, 2015).

Both types of PR use similar binding abilities to bind the same steroid hormones. However, as ligand-activated transcription factors, these isoforms have distinctive biological and transcriptional properties (Fabris *et al.*, 2017). In general, PR-B is a far more potent activator than PR-A. Therefore, it is likely that PR-A functions mostly as a repressor and PR-B functions mostly as an activator (Lamb *et al.*, 2018). Samples are considered PR-positive tumours when 1% to 100% of the tumour nuclei test positive for PR. Meanwhile, if <1% or 0% of the tumour cell nuclei in a sample are immunoreactive, it is considered as PR negative tumour (Allison *et al.*, 2020). Standard PR antibodies used in IHC do not distinguish between isoforms (Fabris *et al.*, 2017).

The American Society of Clinical Oncology/College of American Pathologists (ASCO/CAP) consistently advises completing an ER, PR, and HER2 evaluation in all invasive breast carcinomas (Allison *et al.*, 2020; Wolff *et al.*, 2018). As mentioned earlier in breast carcinomas, the expression of the biomarkers is crucial for determining patient treatment and establishing prognostic and predictive indicators (Harris *et al.*, 2016).

1.4 OVERVIEW OF HER2

Receptor tyrosine kinases (RTKs) are transmembrane cell-surface proteins that play a key role in mediating cell-to-cell communication and coordinating various intricate biological processes, including cell growth, motility, differentiation, and metabolism (McDonell *et al.*, 2015). A ligand-binding extracellular domain, a transmembrane domain, a juxtamembrane region, a cytosolic tyrosine kinase domain (TKD), and a flexible C-terminal tail make up the structure of the majority of RTK. RTKs are triggered by the formation of intermolecular dimers in the presence of ligands, which

causes kinase activation and phosphorylation of the receptor C-terminal tail (Karpov *et al.*, 2015). Tyrosine kinase activity is a tightly regulated process in healthy cells (Cordover and Minden, 2020).

Many human diseases, particularly cancer, are brought on by the dysregulation of RTK signalling. Gain-of-function mutations, genomic amplification, chromosomal rearrangements, and autocrine activation are the four main pathways that lead to constitutive RTK activation in human malignancies (Du and Lovly, 2018). One of the most significant RTKs involved in the proliferation of cancer cells is the epidermal growth factor receptor (EGFR). ErbB1 (EGFR or HER1), ErbB2 (HER2), ErbB3 (HER3), and ErbB4 (HER4) are the four members that make up the EGFR subfamily. Human epidermal growth factor receptor 2 (HER2/neu, ErbB2) is a 185 kDa transmembrane glycoprotein. It is encoded by HER2/neu oncogene, located on the long arm of chromosome 17 (17q12-21.32) (Wang, 2019).

HER2 does not directly bind to any known ligands, in contrast to other members of the EGFR subfamily. Instead, the activation of HER2-mediated signalling pathways occurs through the ligand-induced formation of heterodimerisation and homodimerisation of the receptors (Ferreira and Pessoa, 2017). The strongest activator of the PI3K/AKT signalling cascade among all ErbB pairings is the HER2/ErbB3 heterodimer, which binds the p85 subunit of PI3K to ErbB3 (Lyu *et al.*, 2018). Activating HER2 in healthy cells opens signalling pathways that regulate normal cell growth, differentiation, motility, and adhesion (Cordover and Minden, 2020). HER2 is often expressed on the cell membranes of epithelial cells in several organs, including the skin, gastrointestinal, respiratory, reproductive, and urinary tracts, as well as the breast (Furrer *et al.*, 2018).

Low quantities of HER2 are expressed in healthy breast epithelial cells (two copies of the HER2 gene and up to 20,000 HER2 receptors) (Zubor *et al.*, 2015).

There are numerous routes through which HER receptors are disrupted. These include mutations that result in constitutive activation of the tyrosine kinase domain, overexpression of agonists and ligands, overexpression of receptors in conjunction with overproduction of ligands, which causes autocrine activation, and endocytosis issues during receptor internalisation (Appert-Collin *et al.*, 2015). HER2 gene amplification is the main cause of HER2 overexpression, which causes the HER2 signalling network to be constitutively activated (Zakrzewski *et al.*, 2019). Amplification or overexpression of HER2 oncoprotein plays an essential role in the pathogenesis of various solid tumours such as cancer of the gastrointestinal tract, bladder, lung, ovary, uterus, cervix and breast (Yan *et al.*, 2015).

Up to 2 million HER2 receptors may be expressed on the surface of tumour cells in HER2-positive breast cancer cells due to an increase in the number of HER2 gene copies (up to 25-50, known as gene amplification) and HER2 receptors (up to 40-100 fold increase, known as protein overexpression) (Peckys *et al.*, 2019). Since there are more HER2 receptors on cell surfaces due to HER2 overexpression, there are more receptor-receptor interactions, which results in prolonged tyrosine phosphorylation of the kinase domain and ongoing activation of the signalling pathways (Du and Lovly, 2018). Also, HER2 overexpression promotes HER2 heterodimerisation with HER1 and HER3, which results in greater activation of the downstream signalling pathways (Jeong *et al.*, 2017). About 15% to 20% of breast cancers are HER2 positive (Vicario *et al.*, 2015).

In breast cancer, HER2 gene amplification as measured by in situ hybridisation (ISH) or protein overexpression as measured by IHC remains the critical predictor of response to HER2-targeted therapy. HER2 positive is defined as the presence of circumferential membrane staining that is complete, intense, and in > 10% of tumour cells as determined by the IHC assay, which results in an IHC score of 3+. ISH assay, which evaluates gene amplification by dual-probe ISH or single-probe ISH depending on whether certain criteria are met, can also be used to determine HER2 positivity (Wolff *et al.*, 2018). HER2-positive breast cancer has historically been linked to a worse prognosis and worse survival rates. However, several therapeutic developments during the past few years have improved the clinical management of HER2-positive disease and its prognosis (Patel *et al.*, 2020).

Generally, the National Comprehensive Cancer Network (NCCN) recommends that patients diagnosed with HER2-positive breast cancer be treated with an anti-HER2-targeted agent (trastuzumab) and chemotherapy in an adjuvant setting. Meanwhile, they recommended adding another anti-HER2-targeted agent (pertuzumab) for patients with node-positive cancer (Gradishar *et al.*, 2020). Trastuzumab inhibits cell growth through a variety of mechanisms, including preventing HER2 dimerisation, downregulating the HER2 receptor through endocytic destruction of the receptor, accumulating the CDK inhibitor p27 and cell cycle arrest, inducing antibody-dependent cell-mediated cytotoxicity (ADCC), and inhibiting constitutive HER2 cleavage/shedding mediated by metalloproteases (Maadi *et al.*, 2021). Trastuzumab has been demonstrated to improve overall survival (OS) in early and advanced breast cancer with HER2 overexpression combined with chemotherapy (Li *et al.*, 2016).

However, anti-HER2 therapy treatment resistance, both primary and acquired, continues to be a significant problem (Vernieri *et al.*, 2019). In addition, several primary and acquired resistance mechanisms to anti-HER2 therapies have been found (Luque-Cabal *et al.*, 2016). These can generally be grouped into three categories. The first category is redundancy within the HER receptor layer, which is the route's capacity to continue signalling despite being partially blocked owing to redundant ligands and receptors that allow for alternative dimerisation patterns. The second category is the ability to reactivate pathway signalling at or downstream of the receptor layer, such as through activating HER or downstream mutations or the disappearance of downstream pathway negative-regulating mechanisms. The third category is escape, which is the employment of alternative pathways that are typically not driving the cancer cell when HER2 is uninhibited but may already exist or be acquired at the time of resistance (Rimawi *et al.*, 2015). Some individuals experience tumour recurrence following adjuvant therapy, and most patients eventually experience disease progression in metastatic settings (Ali *et al.*, 2022). Despite the resistance mechanisms of anti-HER2 therapy, the tumour microenvironment also affects the course of HER2-positive breast cancer (Ji *et al.*, 2021).

1.5 ROLES OF TUMOUR MICROENVIRONMENT IN TUMOUR PROGRESSION

Human cells acquire functional abilities when transitioning from average growth to neoplastic growth. More precisely, these abilities are essential for human cells to create malignant tumours, which are the hallmarks of cancer. The ten hallmarks of cancer currently comprise the acquired capabilities for sustaining proliferative signalling, evading growth suppressors, avoiding immune destruction, enabling replicative

immortality, tumour-promoting inflammation, activating invasion and metastasis, inducing or accessing vasculature, genome instability and mutation, resisting cell death and deregulating cellular metabolism (M. Wang *et al.*, 2017). Two mechanisms, namely genetic/epigenetic changes in the tumour cells and the reorganisation of the tumour microenvironment (TME) components through mutual and dynamic interplay, drive the development and progression of tumours (Baylin and Jones, 2016).

The developing TME is a dynamic, intricate system. Although the TME's make-up varies depending on the type of tumour, immune cells, stromal cells, blood vessels, and extracellular matrix are standard components (Bo *et al.*, 2022). Tumour cells, tumour stromal cells, such as stromal fibroblasts, endothelial cells, immune cells like microglia, macrophages, and lymphocytes, as well as extracellular matrix (ECM) non-cellular components including collagen, fibronectin, hyaluronan, and laminin, among others, are all parts of the TME. These elements are necessary for the neoplastic tissue to communicate and develop properly and to maintain homeostasis (Anderson and Simon, 2018). The cancer cells interact dynamically and bidirectionally with their surroundings through cell-cell or cell-free contacts (including ECM) and the mediators that make these connections possible. The horizontal transfer of genetic information between communicating cellular and non-cellular cells is accomplished through mediators, which are secreted soluble compounds, factors, or vesicles (Baghban *et al.*, 2020).

In order to manipulate the non-malignant cells for their benefit, tumour cells, which serve as the centre of the tumour microenvironment, regulate the activity of cellular and non-cellular components through intricate signalling networks. Dynamic interactions between cancer cells and their microenvironment are crucial for promoting cancer cell

heterogeneity, clonal development, and multidrug resistance, which leads to cancer cell growth and metastasis (Hass *et al.*, 2020). The TME coordinates a programme encouraging angiogenesis to restore oxygen or nutrient supply and remove metabolic waste to combat a hypoxic and acidic microenvironment (Fitzgerald *et al.*, 2018).

As mentioned earlier, immune cells are a crucial component of the TME that is important in cancer development. These immune cells that are linked to tumours have previously been shown to have the ability to either promote or inhibit tumour growth. Immune cells can be broadly divided into two groups: innate immunity cells, which include macrophages, neutrophils, and dendritic cells, and adaptive immune cells, which include T-cells, B-cells, and NK cells (Anderson and Simon, 2018). Basically, during the early stages of carcinogenesis, the tumour-antagonising immune cells in the TME tend to target and kill the cancer cells. However, the cancer cells eventually appear to escape immune monitoring and even suppress the cytotoxic activity of immune cells that are hostile to tumours in various ways (Lei *et al.*, 2020). Meanwhile, several immune cells that support tumorigenesis are being identified, primarily regulatory T cells (Tregs) and myeloid-derived suppressor cells (MDSCs). Tregs that express the FOXP3 marker is essential for maintaining immunological homeostasis and peripheral tolerance (Hatziioannou *et al.*, 2017).

1.6 ROLES OF FOXP3 POSITIVE TUMOUR INFILTRATING LYMPHOCYTES IN BREAST CANCER

All populations of lymphocytic cells that have penetrated the tumour tissue are collectively known as tumour infiltrating lymphocytes (TILs). TILs in the tumour and the surrounding microenvironment are believed to represent a continuous anti-tumour

host immune response (Labani-Motlagh *et al.*, 2020). TILs in breast cancer are predominantly composed of cytotoxic (CD8+) and helper (CD4+) T cells, with a lesser amount of B cells and NK cells (Pruneri *et al.*, 2018). Compared to hormone receptor (HR)-positive tumours, TNBC and HER2-positive breast cancer usually have higher TIL infiltration rates (Stanton and Disis, 2016). Additionally, high TILs expressions are associated with favourable survival and predict pathological complete response rates in these types of breast cancer (Gao *et al.*, 2020).

However, among the various immune cells, Tregs are vital aspects that play a significant part in tumour immunological escape. Tregs, an immunosuppressive subset of CD4+ T cells, are widely recognised as FOXP3 positive (FOXP3+) T cells that are $CD4^{+}CD25^{+}CD127^{low}$ (Rodríguez-Perea *et al.*, 2016). Natural Tregs, developed in the thymus, and induced Tregs derived from naive CD4+ T cells in the periphery, are the two different forms of Tregs (Takeuchi and Nishikawa, 2016). In addition to their crucial responsibilities in preserving immunological homeostasis and self-tolerance, Tregs also regulate autoimmunity, infection, graft-versus-host disease, inflammation, fetal-maternal tolerance, and tumour immunity (Jørgensen *et al.*, 2019). Tregs infiltrate the tumour site from the peripheral circulation, fostering an environment that supports tumour growth. Tregs interfere with innate and acquired immunity by reducing NK and T-cell responses in cancer (Ohue and Nishikawa, 2019).

Tregs are chemo-attracted to the TME. Tregs have many chemokine receptors that react to chemokine generated when the tumour mass develops. In breast cancer, the CCR4 chemokine receptor on Tregs will recognise CCL22 chemokine produced by tumour cells. A signalling cascade results from the chemokine CCL22 with the chemokine

receptor CCR4, changing the shape of Treg cells and enhancing their ability to move, enabling them to infiltrate the TME (Takeuchi and Nishikawa, 2016). By blocking the anti-tumour immune response and contributing to immunosuppression, Treg cells in tumour immunity play a role in the growth and progression of tumours (Cinier *et al.*, 2021). FOXP3+ TILs reduce pro-inflammatory responses inside the TME by suppressing effector function and immune cell migration, production of inhibitory cytokines, metabolic disruption, and metastatic promotion (Scott *et al.*, 2021).

The transcription factor Forkhead Box Protein P3 (FOXP3), also known as scurfin, belongs to the forkhead-winged-helix family of transcription factors. This transcriptional factor plays a crucial role in Treg cell development and function and serves as a lineage specification factor of Treg cells (Pereira *et al.*, 2017). The tumour site, as well as the molecular subtype and tumour stage, significantly impact the prognostic role of FOXP3+ Tregs. In the majority of solid tumours investigated, including cervical, renal, melanomas, and breast malignancies, high FOXP3+ Tregs infiltration is significantly linked to a lower survival rate. FOXP3+ Tregs, on the other hand, are connected to a better survival rate in other tumours, including colorectal, head and neck, and oesophageal malignancies (Shang *et al.*, 2015).

Additionally, increased FOXP3+ TILs levels are strongly associated with nodal metastases, HER2-positive breast cancer, and poor prognosis (Shou *et al.*, 2016). In addition, higher histological grade, ER negativity, and poor recurrence-free survival are all associated with high FOXP3+ Tregs infiltration in breast cancer (Zhou *et al.*, 2017). Meanwhile, complete pathological response after neoadjuvant chemotherapy in TNBC is substantially correlated with reduced Tregs infiltration (Oshi *et al.*, 2020). Other

research shows that HER2-negative tumours, ER and PR-positive tumours, and lower-grade breast cancer exhibit less Tregs infiltration (Papaioannou *et al.*, 2019).

Thus, a few studies, as mentioned, show variable findings and associations. Therefore, this study will investigate the expression of FOXP3+ TILs in HER2-positive breast cancer and their association with clinicopathological features. The ultimate outcomes of this study will provide valuable information in the diagnostic aspect, notably in terms of the possibility of using FOXP3 as a potential prognostic biomarker in HER2-positive breast cancer, as well as helpful in the development of immunotherapy in the future.

CHAPTER 2: OBJECTIVES OF STUDY

2.1 GENERAL OBJECTIVE

To determine the expression of FOXP3 tumour infiltrating lymphocytes in HER2-positive breast cancer and its association with clinicopathological features

2.2 SPECIFIC OBJECTIVES

1. To determine the proportion of HER2-positive breast cancer among invasive breast cancer samples.
2. To determine FOXP3-positive tumour infiltrating lymphocytes in HER2-positive breast cancer.
3. To determine the clinicopathological association of HER2-positive breast cancer with FOXP3-positive tumour infiltrating lymphocytes.

CHAPTER 3: MANUSCRIPT

3.1 TITLE PAGE

Expression of FOXP3 tumour infiltrating lymphocytes in HER2-positive breast cancer and their association with clinicopathological features

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Running title: Association of FOXP3 tumour infiltrating lymphocytes with clinicopathological features in HER2 positive breast cancer

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3.2 ABSTRACT

Background: The transcription factor Forkhead Box Protein P3 (FOXP3) plays a crucial role in regulatory T cells (Tregs) development and function and serves as a lineage specification factor of Tregs. FOXP3+ tumour infiltrating lymphocytes (TILs) are considered significant prognostic markers in breast cancer. However, the prognostic significance of FOXP3+ TILs in human epidermal growth factor receptor 2 (HER2) positive breast cancers remains controversial. Therefore, we investigated the expression of FOXP3+ TILs in HER2-positive breast cancer and its association with clinicopathological features. **Methods:** A cross-sectional study was conducted at the department of pathology, Hospital Sultanah Nur Zahirah (HSNZ), Kuala Terengganu, from the year of 2014 to 2021. One hundred and sixty-seven HER2-positive breast cancer cases that underwent mastectomy or wide local excision were recruited. The tissue sections were stained with immunohistochemistry (IHC) anti-FoxP3 antibodies. FOXP3-positive (FOXP3+) tumour infiltrating lymphocytes (TILs) was categorised into low infiltration (grade 0 to grade 1) and high infiltration (grade 2 to grade 3). Fischer's Exact test was used to analyse the association with clinicopathological characteristics. A p-value of less than 0.05 was considered statistically significant. **Results:** One hundred and sixty-four (98.2%) cases showed high FOXP3+ TILs infiltration, and 3 (1.8%) cases showed low infiltration. There was no significant association between the expression of FOXP3+ TILs with the age of diagnosed ($p > 0.950$), tumour grade ($p=0.091$), tumour size ($p=0.235$), axillary nodal status ($p=0.552$), metastasis status ($p > 0.950$), oestrogen receptor (ER) status ($p=0.616$) and progesterone receptor (PR) status ($p > 0.950$). **Conclusion:** FOXP3+ TILs were highly infiltrating HER2-positive breast cancer despite variability in clinicopathological characteristics.

Keywords: FOXP3, HER2-positive breast cancer, tumour infiltrating lymphocyte

3.3 INTRODUCTION

Breast cancer is the most often diagnosed cancer and the primary contributor to cancer death in women worldwide, yet patterns and trends range from country to country (Lei *et al.*, 2021). Globally, 2.3 million new instances of breast cancer are thought to be diagnosed each year (Sung *et al.*, 2021). According to current forecasts, there will be 2.7 million new cases diagnosed annually worldwide by 2030, while there will be 0.87 million fatalities (Ferlay *et al.*, 2021). Global gene expression profiling studies classified breast cancers into five intrinsic subtypes by hierarchical clustering: luminal A, luminal B, HER2 overexpression, basal and normal-like subtypes (Dai *et al.*, 2015). Meanwhile, claudin-low is a new intrinsic subtype of breast cancer that has recently been discovered (Pommier *et al.*, 2020).

About 80% and 60-70% of breast carcinomas, respectively, express the ER and PR (Badowska-Kozakiewicz *et al.*, 2015). In addition, 15%-20% of invasive breast cancers exhibit human epidermal growth factor receptor 2 (HER2) gene amplification, which is closely associated with HER2 protein overexpression (Godoy-Ortiz *et al.*, 2019). Meanwhile, over 15-20% of breast tumours are triple-negative breast cancers (TNBC) (Almansour, 2022). HER2-positive breast cancer has historically been linked to a worse prognosis and worse survival rates. However, several therapeutic developments during the past few years have improved the clinical management of HER2-positive disease and its prognosis (Patel *et al.*, 2020).

Trastuzumab has been demonstrated to improve overall survival (OS) in early and advanced breast cancer with HER2 overexpression combined with chemotherapy (Li *et al.*, 2016). However, anti-HER2 therapy treatment resistance, both primary and acquired, continues to be a significant problem (Vernieri *et al.*, 2019). Several primary and acquired resistance mechanisms to anti-HER2 therapies have been found (Luque-