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HER2 STATUS AND MOLECULAR SUBTYPES OF BREAST CARCINOMA IN CENTRAL VIETNAM

Director: Prof. LEONARDO A. SECHI

Tutor: Prof. FRANCESCO TANDA

Co-tutor: A/Prof. DANG CONG THUAN

PhD thesis of

Dr. NGUYEN PHUONG THAO TIEN

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ATTESTATION OF AUTHORSHIP

I hereby declare that this submission is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person except that which appears in the citations and acknowledgements. Nor does it contain material, which to a substantial extent I have submitted for the qualification for any other degree of another university or other institution of higher learning.

Name: Nguyen Phuong Thao Tien Signed:

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LIST OF ABBREVIATIONS

AJCC	American Joint Committee on Cancer
ASCO	American Society of Clinical Oncology
ASR	Age-standardized incidence rate
CAP	College of American Pathologists
CISH	Chromogenic in situ hybridization
DCIS	Ductal carcinoma in situ
DBA	Diaminobenzidine tetrahydrochloride
DISH	Dual colour in situ hybridisation
DNA	Deoxyribonucleic acid
ER	Estrogen receptor
ErbB	Epidermal growth factor receptor
FDA	US Food and Drug Administration
FISH	Fluorescence in situ hybridisation
HER2	Human epidermal growth factor receptor 2
HR	Hormone receptor
IDC	Invasive ductal carcinoma
IHC	Immunohistochemistry
ILC	Invasive lobular carcinoma
ISH	In situ hybridization
LCIS	Lobular carcinoma in situ
LSI	Locus Specific Identifier
PR	Progesterone receptor
SISH	Silver-enhanced in situ hybridization
TNBC	Triple negative breast cancer
WHO	World Health Organization

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ABSTRACT

Background. Breast cancer is the most commonly occurring cancer in the females and the leading cause of cancer deaths in Vietnamese women. Beside the traditional histopathological classification according to the WHO, the genetic and molecular findings in the last decades have introduced a new nomenclature to identify of breast cancers oriented mostly to the functional characteristics of the neoplastic cells and the needs of therapy. Based on these criteria, breast cancers are divided into five main groups according to the expression levels of biomarkers such as estrogen (ER), progesterone receptors (PR), proliferation index (Ki67) and human epidermal growth factor receptor 2 (HER2). Among them, HER2 protein overexpression and gene amplification have a particularly important role to play in the classification of molecular subtypes. It is also a significant biomarker which has prognostic and predictive value and is a goal of targeted therapy.

The aim of this study was to identify the prevalence rate of HER2 gene amplification or overexpression in the local Vietnamese population, and determine the molecular subtypes of breast cancer.

Materials and methods. Paraffin tissue blocks from 88 Vietnamese women diagnosed consecutively with invasive primary breast carcinoma during a period of 12 months, from April 2016 to April 2017. These blocks underwent immunohistochemistry (IHC) and fluorescence in situ hybridization (FISH) and dual in situ hybridization (DISH) for the assessment of HER2 status. HER2 positive includes the HER2 (3+) score in IHC or the HER2 gene amplification in FISH. The IHC for ER, PR and Ki67 were also evaluated to determine molecular subtypes. The analyses were based on the guideline of ASCO/CAP 2013.

Results. The median age of patients was 52.5. By using IHC, 30.7 % of tumours were strongly expressed in (3+) score the HER2 protein. The HER2 equivocal results occupied 9.1%. Gene amplification by FISH was found in 25% of tumours with an equivocal score in IHC. The prevalence rate of HER2 positive was 32.9%. The concordance between IHC and DISH assay were 100% in case of IHC

positive or negative. In case HER2 results by using immunohistochemistry technique were equivocal, DISH was able to identify 2 cases (25% of cases) as positive. The concordance between FISH and DISH in the equivocal cases (8 cases) was 100%. Luminal B subtype accounted for the highest proportion, at 37.5% whereas luminal A was the lowest, at 18.2%. The HER2 enriched and triple negative subtype occupied 22.7% and 21.6%, respectively. A significant relationship was found between HER2 status as well as molecular subtypes and some clinicopathological characteristics and biomarkers.

Conclusion. HER2 gene amplification was found in 32.9% of Vietnamese breast cancers. This prevalence was considerably higher than in published studies on women from Western countries. Luminal B subtype was the most frequent, at 37.5% while the uncommon belonged to luminal A, at 18.2%. The HER2 enrich and triple negative subtypes were fairly higher than that in literature. HER2 status as well as molecular classification had prognostic and therapeutical significance.

1. INTRODUCTION

1.1. Breast Cancer

Breast cancer (BC) is currently the most common cancer in women worldwide. The incidence and the mortality of breast cancer continues to increase rapidly in most countries, with over 2 million new cancer cases diagnosed and approximately 600,000 cancer deaths in 2018 [1]. However, in the USA, the last decade (2005-2014) witnessed a stability in the incidence rate and a decline in the mortality rate (2006-2015) as a result of the earlier diagnosis following the implementation of screening programs and the access to the new therapies [2]. The estimated incidence and the mortality rates of breast cancer vary across communities and countries. The global age-standardized incidence rate (ASR) of breast cancer is 46.3 per 100,000 women and the mortality incidence rate is 13.0 per 100,000 women [1].

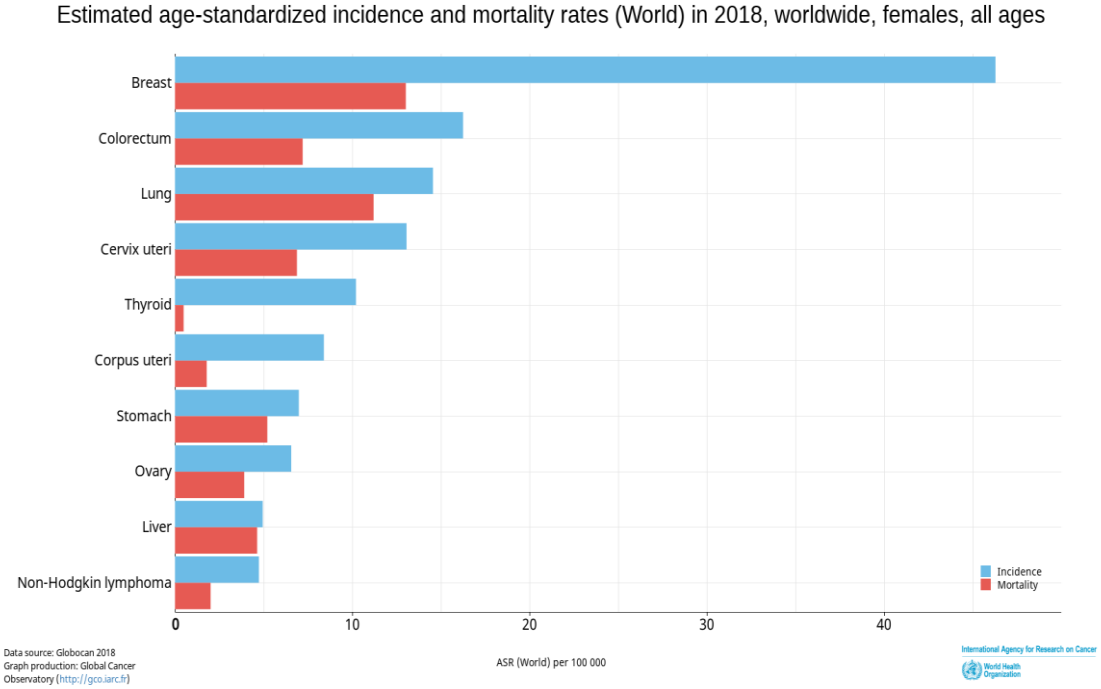


Figure 1.1. Estimated age -standardized incidence and mortality rate in 2018, worldwide, females [1].

In Vietnam, breast cancer ranks the first in the list of common cancers in women. The last decade has witnessed an increase in the incidence of breast

cancer, with 15,229 newly-diagnosed cases having been reported, occupying 20.6% of all cancers in women. The ASR of breast cancer in Vietnam is around 26.4 per 100,000 women in 2018 compared to 23.0 in 2012 [3]. However, breast cancer has a high mortality rate, accounting for 6,103 deaths in 2018 and occupying approximately 14% of all deaths in women and the first-leading cause of death for cancer among Vietnamese women. [1].

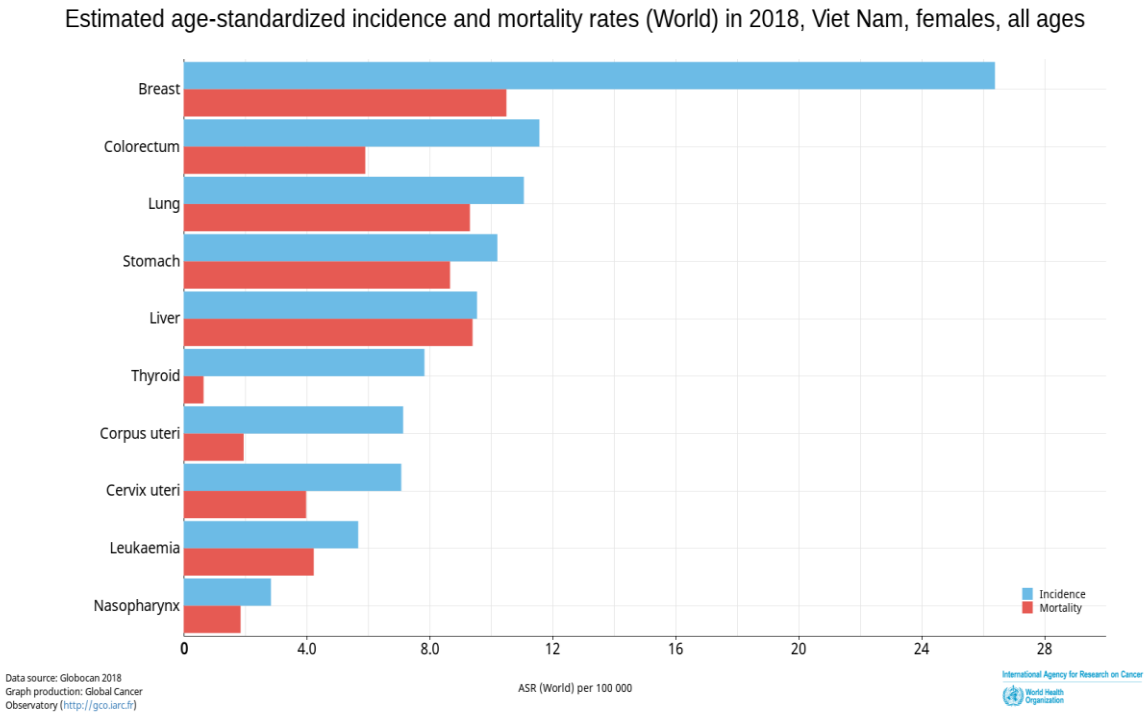


Figure 1.2. Estimated age -standardized incidence and mortality rate in 2018, Vietnam, females [1]

Nowadays, the effectiveness of treatment and screening program generally aid in improving breast cancer survival time and its prognosis. However, this type of cancer is a clinically heterogeneous disease. In clinical settings, therefore, although patient might have the same histopathological characteristics or histologic stages, there are variations in their clinical manifestations, progresses, responses to treatments and prognoses [4]. Various molecular studies have been performed during the last decades to enhance a better understanding of tumor carcinogenesis and to determine the biological properties of tumor cells. These studies have shown that although breast cancer has the same histologic types and grades, it is not the unique group. A number of researchers have found that breast carcinoma is

composed of multiple molecular subtypes [5]. These subtypes of breast cancer might differ in risk-factor epidemiology, in the progression of the disease and in the response to therapy [6]. The classification of molecular subtypes is very useful in terms of deciding optimal adjuvant treatment and determining accurate prognosis for breast cancer. According to the 13th St Gallen International Breast Cancer Conference (2013), BC was classified into five main subtypes including luminal A, luminal B-HER2 negative, luminal B-HER2 positive, HER2 enrich and triple negative. This classification was made based on biomarkers expressions such as HER2, ER, PR, Ki67 in immunohistochemistry (IHC) and the amplification of HER2 gene in in situ hybridization (ISH). The treatment and prognosis is absolutely different among the subtypes.

Many studies have shown that the amplification of the HER2/neu proto-oncogene or HER2 protein overexpression has an important role to play in this disease. It is also an important biomarker which has prognostic and predictive value and is a goal of targeted therapy [7]. HER2 gene shows amplification in about 15-20% of patients with breast cancers in Western countries. HER2-positive carcinomas were known to be very aggressive tumors with very poor prognosis until few years ago. New targeted therapies (trastuzumab) have been recently developed, which have improved the prognosis of these patients. It is very important to recognize these tumors to be treated properly [7], [8]. Beside HER2 gene amplification and molecular subtypes, those prognostic factors such as patient's age, the type of tumors, lymph node status, tumors size, histologic grade, estrogen receptor, progesterone receptor and proliferation index Ki67 also play an important role in the management and treatment of patients with breast cancer.

1.2. Histopathological Characteristics

1.2.1. Histologic Type

Histopathological features have so far become important factors to diagnose cancers in general and breast cancers in particular [9]. Comprehensive knowledge of pathologists about normal histology of mammary glands is absolutely necessary for the accuracy of pathologic features evaluation. The alterations of mammary glands on X rays examination depend on genders, ages, menstrual cycles, pregnancy, breast-feeding

and menopause status. Thus, when deciding whether the mammogram result is normal or abnormal, it is necessary to consider all the above factors.

Most breast tumors are carcinomas [10]. These malignant tumors are classified into in situ and invasive lesions. The most frequent breast cancers (occupied approximately 75%) belong to the group of invasive ductal carcinomas and the remaining ones have been divided into 17 distinct types. They include invasive lobular carcinomas, invasive papillary carcinomas, metaplastic carcinomas, tubular carcinomas and others [11].

According WHO histological classification of tumors of the breast 2012 [12], the histologic types is included:

- Epithelial Tumors
  - Micro-invasive carcinoma
  - Invasive breast carcinoma**:** Invasive carcinoma of no special type (NST), Invasive lobular carcinoma, Tubular carcinoma, Cribriform carcinoma, Mucinous carcinoma, Carcinoma with medullary features, Carcinoma with apocrine differentiation, Carcinoma with signet-ring-cell differentiation, Invasive micro-papillary carcinoma, Metaplastic carcinoma of no special type and Rare types
  - Epithelial- myoepithelial tumors
  - Precursor lesions
  - Intraductal proliferative lesions
  - Papillary lesions
  - Benign epithelial proliferations
- Mesenchymal tumors
- Fibroepithelial tumors
- Tumors of the nipple
- Malignant lymphoma
- Metastatic tumors
- Tumors of the male breast
- Clinical patterns

1.2.2. Histologic grade [13]

To determine the grade of breast cancer, different available "scoring systems" have been used. Nowadays, the Nottingham Histologic Score system (the Elston-Ellis modification of Scarff-Bloom-Richardson grading system) has a great tendency to be applied. According to this scoring system, there are three factors that the pathologists take into consideration, including the amount of gland formation, the nuclear features and the mitotic activity. Each of these features is scored from 1-3, and then each score is added to give a final total score ranging from 3-9.

The score is assessed following:

- *Glandular (acinar)/tubular differentiation*
  - Score 1: >75% of tumor area forming glandular/tubular structures
  - Score 2: 10% to 75% of tumor area forming glandular/tubular structures
  - Score 3: <10% of tumor area forming glandular/tubular structures
- *Nuclear Pleomorphism*
  - Score 1: Nuclei small with little increase in size in comparison with normal breast epithelial cells, regular outlines, uniform nuclear chromatin, little variation in size
  - Score 2: Cells larger than normal with open vesicular nuclei, visible nucleoli, and moderate variability in both size and shape
  - Score 3: Vesicular nuclei, often with prominent nucleoli, exhibiting marked variation in size and shape, occasionally with very large and bizarre forms
- *Mitotic Count*

The mitotic count score criteria vary depending on the field diameter of the microscope used by the pathologist. The pathologist will count how many mitotic figures are seen in 10 high power fields. Using a high power field diameter of 0.50 mm, the criteria are as follows:

- Score 1: less than or equal to 7 mitoses per 10 high power fields
- Score 2: 8-14 mitoses per 10 high power fields
- Score 3: equal to or greater than 15 mitoses per 10 high power fields

The final total score is used to determine the grade (Nottingham Histologic Score) in the following way:

1. Grade 1 tumors have a score of 3-5
2. Grade 2 tumors have a score of 6-7
3. Grade 3 tumors have a score of 8-9

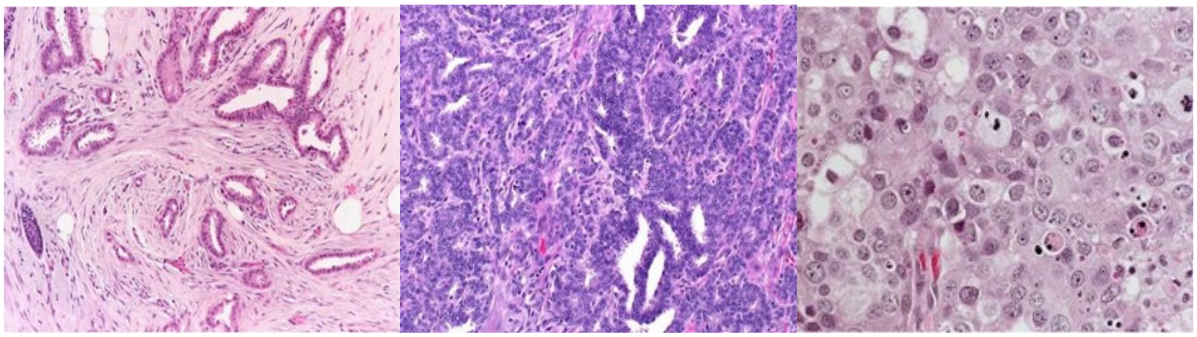


Figure 1.3: Invasive Ductal Carcinoma [13]

1.2.3. Tumour size

Tumour size is an important prognostic factor of breast cancer. Tumour sizes are inversely proportional to survival rates and are directly proportional to distant recurrence rates [14]. An increase in tumour size will result in a decrease in survival rate regardless of lymph node status and as lympho nodes involvement increased, survival rates also drop without reference to tumor size. Tumor size and positive-lympho node status have a linear relationship. Patients with a tumors size <2 cm have a higher 5-year survival rate than those with tumor size >5cm without consideration of lymph node status [15]. Besides, increased tumor size leads to rapid metastasis. Tumor size is also the strongest prognostic factor and has an important role in adjuvant treatment decisions when the lymph node involvement is absent [16].

The classification of primary tumor (T) size according WHO 2012 [12]

TX Primary tumor cannot be assessed

T0 No evidence of primary tumor

Tis Carcinoma in situ

Tis (DCIS) Ductal carcinoma in situ

Tis (LCIS) Lobular carcinoma in situ

Tis (Paget) Paget disease of the nipple with not associated with invasive carcinoma and/or carcinoma in situ (DCIS and/or LCIS) in the underlying breast parachyma

T1 Tumor 2 cm or less in greatest dimension

T2 Tumor more than 2 cm but not more than 5 cm in greatest dimension

T3 Tumor more than 5 cm in greatest dimension

T4 Tumor of any size with direct extension to the chest wall and/or to the skin (ulceration or skin nodules)

1.2.4. Lymph node status

Status of axillary lymph node metastasis is a significant prognostic factor. The number of positive axillary lymph nodes have a correlation with the risk for distant recurrence [17]. Nowadays, the number of metastatic nodes is used to access for stage of disease to decide the adjuvant therapy. In case of lymph node-negative patients and hormone receptor-positive tumors, endocrine therapy alone is often considered as the first choice of the treatment.

1.2.5. Stage of disease

Breast cancer is staged using the World Health Organization Classification of Tumours [12], which is based on:

- The size of the breast tumor (T) and if it has grown into nearby areas
- Whether the cancer has reached nearby lymph nodes (N)
- Whether the cancer has metastasized (spread to other parts of the body) (M)

Stage 0	Tis	N0	M0
Stage I	T1	N0	M0
Stage IIA	T0	N1	M0
	T1	N1	M0
	T2	N0	M0
Stage IIB	T2	N1	M0
	T3	N0	M0
Stage IIIA	T0	N2	M0
	T1	N2	M0
	T2	N2	M0
	T3	N1	M0
	T3	N2	M0
Stage IIIB	T4	N0	M0
	T4	N1	M0
	T4	N2	M0
Stage IIIC	Any T	N3	M0
Stage IV	Any T	Any N	M1

Table 1.1: Stage grouping

1.3. Biomarker status in breast cancer

1.3.1. Hormone receptors (Estrogen and Progesterone receptors)

Estrogen and progesterone are steroid hormones which make a contribution to the development of normal breast. Both estrogen and progesterone are members of nuclear hormone receptor (HR) superfamily [18]. They can interact with hormone receptors in nuclear cell. Estrogen and progesterone regulate gene transcription through binding to DNA response factors directly or via other transcription elements and recruiting co-regulators. In addition, they can cross-talk with another signaling pathways through non-genomic mechanisms [18]. Estrogen and progesterone are main hormones involved in the regulation of tumor growth in breast cancer [19].

An experience consisting of 302 breast cancer patients conducted in Ivory Coast showed that ER and PR positivity was in 169 cases (56%) and 148 cases (50%), respectively [20]. The research implemented in 5,993 breast cancers by Nadji and colleagues, from USA, among 5,497 infiltrating breast carcinomas, 75% were ER-positive, 55% of tumors reacted positively to PR and 55% of cases expressed both ER and PR [21]. Expression of ER and PR is a very strongly and useful predictor. Thus, the determination of ER status has significant clinical values and is widely applied in routine histopathological diagnoses. The presence of ER is a good prognostic marker because it is an indicator of less aggressive tumors. The breast cancer patients with ER-positive have an overall longer survival and disease-free time in comparison with ER-negative ones [22]. The evaluation of ER, PR aids in predicting the response to hormone therapy. Therefore, this is a routine test for breast cancer patients.

1.3.2. Proliferation marker Ki-67

Ki67 gene is located on the long arm of chromosome 10 (10q25) and encode Ki-67 protein in nuclear cell [23]. Ki67 protein expresses very low level in normal breast tissue (<3%) [24], [25].

In breast cancer, the evaluation of proliferation is one of the important factors for diagnosis, prognosis and treatment decisions. Many studies demonstrated that

Ki-67 is associated with the common histopathological parameters, as well as the biomarker. In a study with 12,155 breast cancer cases involved, De Azambuja and colleagues showed that the positive Ki-67 confers a higher risk of relapse and a lower survival rate. These authors have reported that high expression of Ki-67 is related with worse prognoses [26]. Bouzubar and co-workers have confirmed that there is a correlation between the high level of Ki-67 immunostaining and the early relapse of breast cancer after mastectomy [27]. In a research performed in Sweden the sample of which is collected from Vietnamese and Swedish breast carcinomas patients, Thang and colleagues have shown the correlation between Ki-67 and clinicopathological parameters. Using 15% as a cut- off value for Ki-67, the rate of Ki-67>15% was 76% and 63% in Vietnamese and Swedish population, respectively [28]. Another study in Torino (Italy) with 1,688 luminal breast cancers involved, Bustreo et al. reported that the rate of Ki67≥14% is 51.3% of all cases [29]. The research conducted by the Tumor Centre Regensburg (Bavaria, Germany) with the participation of 4,692 invasive breast cancer patients showed that the higher Ki-67 status was associated with higher grade, higher tumor stage and more aggressive tumor [30].

1.4. Human epidermal growth factor receptor 2 (HER2)

1.4.1. HER2 protein and HER2 gene

Human epidermal growth factor receptor 2 (HER2) is one of four members of the epidermal growth factor receptor (ErbB) family, encoded by the gene located on the long arm chromosome 17 (17q12). This is a tyrosine kinase transmembrane growth factor receptor involved in signal transduction pathways that regulate cell growth and differentiation. The ErbB/HER protein-tyrosine kinases consist of a growth-factor-binding extracellular domain, a single transmembrane part, an intracellular protein-tyrosine kinase catalytic domain and a tyrosine-containing cytoplasmic tail [7], [31].

Until now, many studies showed that HER2 has no identified ligand. Therefore, they believe that the activation have been occurring through heterodimerization with another HER family member, although HER2 homodimers

can also form when overexpressing. Heterodimers could make more powerful signals than homodimers. Heterodimers containing HER2 might have an extremely high ligand binding and signaling potency compared to hetero-and homodimers without HER2. Among all members of HER family, HER3 is the preferred dimerization partner of HER2. The combination HER2-HER3 is the most potent heterodimer in carcinoma cells, causing the most mitogenic index [32], [33]. Heterodimers of HER2-HER3 cause increased stability and prolonged activation, leading to enhanced downstream outcomes such as proliferation, migration, differentiation and survival [7], [33], [34], [35].

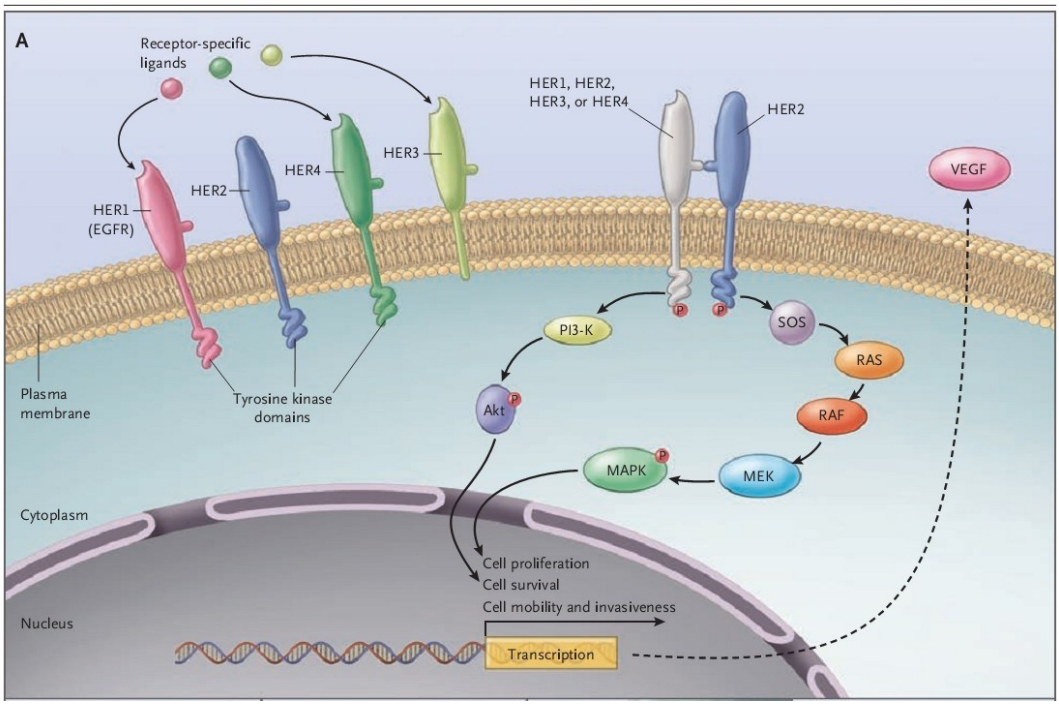


Figure 1.4: Signal Transduction by the HER Family [36]

1.4.2. HER2 overexpression and HER2 gene amplification

HER2 protein overexpression or HER2 gene amplification has an important role to play in breast carcinoma and highly correlated with cell proliferation, survival, differentiation, invasion, metastasis and bad prognosis [7], [31], [37], [38].

Approximately 15-20% of breast cancer patients in Western countries have HER2 gene amplification [37]. This rate is higher in Asian countries such as Malaysia (32.6%), ThaiLan (44.2%), HongKong (26.9%) than that in Western countries according to some published studies on women from the latter countries [39].

Nº	Name	HER2 gene no amplified (%)	HER2 gene amplified (%)	Country
1	Pathmanathan [39]	68.9	31.1	South Vietnam
2	Thang V.H [40]	59	41	North Vietnam
3	Pathmanathan [39]	67.4	32.6	Malaysia
4	Choi [41]	62.5	47.5	Korea
6	Hadi [42]	81.4	18.6	USA
7	Rasmussen [43]	76.9	23.1	Denmark
8	Thang V.H [40]	86	14	Sweden
9	ASCO-CAP 2013[37]		**15-20**	Western

Table 1.2: Prevalence of HER2 positivity in patients with breast cancer

Overexpression of HER2 protein may occur in either the presence or absence of HER2 gene amplification. Many studies have showed that the correlation between HER2 and breast cancer outcome. The patients with HER2-positive tumors have a poorer prognosis than others with HER2-negative tumors [44], [45], [46], [47]. In 1987, Slamon and colleagues showed the correlation between HER2 status and the prediction of overall survival and time to relapse in node-positive patients [48]. Many studies have the similar result to Slamon's study [35], [49], [50], [51]. Another publication in 2015 suggested that there was no association between HER2 expression and age, histological type but a correlation with grade, size and nodal involvement [52]. In 2005, Ariga and co-workers found a correlation between HER2/neu amplification and tumor type, high histological grade, and high proliferative MIB-1 index whereas there was no correlation between the size of tumors and the age of patients with HER2 positive [53]. Shokouh and colleagues, in their study conducted in 566 breast cancers, have recently showed a significant correlation between the tumor grade and HER2 overexpression [52], [54]. A similar result was found in the study on women in Asian countries presented with breast cancer at higher histological grade [39], [55]. In addition, many studies showed that there was an inverse relation between HER2 expression and steroid hormone receptor status [53], [56], [57].

The tumors with higher Ki-67 expression are also associated with a higher HER2 overexpression. High proliferation was correlated with poor prognostic factors such as high grade, negative estrogen receptor and HER2 amplification [28], [52].

1.4.3. Testing for HER2 protein overexpression and HER2 gene amplification in breast cancer

Nowadays, many methods for HER2 testing have been developed, however, about 20% of current HER2 testing is not correct [58]. Therefore, the American Society of Clinical Oncology (ASCO) and the College of American Pathologists (CAP) have recommended guidelines in HER2 testing to ensure accuracy [37]. The two methods currently approved for HER2 testing are immunohistochemistry (IHC) and in situ hybridization (ISH). Beside the standard fluorescence in situ hybridization (FISH), there is a growing number of new ISH techniques which are applied, such as chromogenic in situ hybridization (CISH), silver-enhanced in situ hybridization (SISH) and dual in situ hybridization (DISH). The IHC has been used to detect the HER2 overexpression and the ISH identifying the HER2 gene amplification. The concordance between IHC and FISH assessing of HER2 protein overexpression and gene amplification ranged from 73% to 98% [59], [60]. Fluorescence in situ hybridization (FISH) is a powerful technique used in the detection of chromosomal abnormalities. Fluorescence labeled DNA probes complementary to regions of individual chromosomes is used in FISH. These labeled DNA segments were hybridized with the cytological targets in the sample. Until now, HER2 fluorescence in situ hybridization is a gold standard to detect HER2 gene amplification in breast cancer. Recent studies report a high concordance between protein overexpression in IHC and amplification by FISH or CISH [61]. A new ISH technique with a fully automated, called the Dual ISH, detecting HER2 gene amplification was approved by U.S. Food and Drug Administration (FDA) in June 2011. The Dual-ISH test detects both HER2 gene and chromosome 17 on a single slide by a light microscope. This new technique is increasingly applied at many laboratories. Mansfield has recent shown that the concordance between FISH and DISH is 92% [62].

1.4.4. HER2 and targeting therapy

HER2- positive carcinomas were known to be very aggressive tumours with very bad prognosis until few years ago. New target therapies (trastuzumab) have been recently developed, which have changed the prognosis of these patients. Therefore, it is very important to recognize these tumours to be treated properly. Targeted therapies for breast cancer are new therapies using substances or drugs which block the evolution of cancer. Those therapies interfere the function of specific molecules responsible for tumor cell proliferation and survival. Recently, trastuzumab has been approved for the treatment of HER2 adjuvant breast cancer. This is the humanized monoclonal antibody consisting of two antigen-specific sites that can bind to the extracellular domain of the HER2 receptor and that prevent the activation of its intracellular tyrosine kinase [63]. In 2007, Hudis et co-workers reported that trastuzumab improved overall survival in late-stage HER2-positive breast cancer from 20.3 to 25.1 months [36]. Using trastuzumab alone or in combination with chemotherapy was a significant regimen in neoadjuvant therapies of the breast cancer treatment.

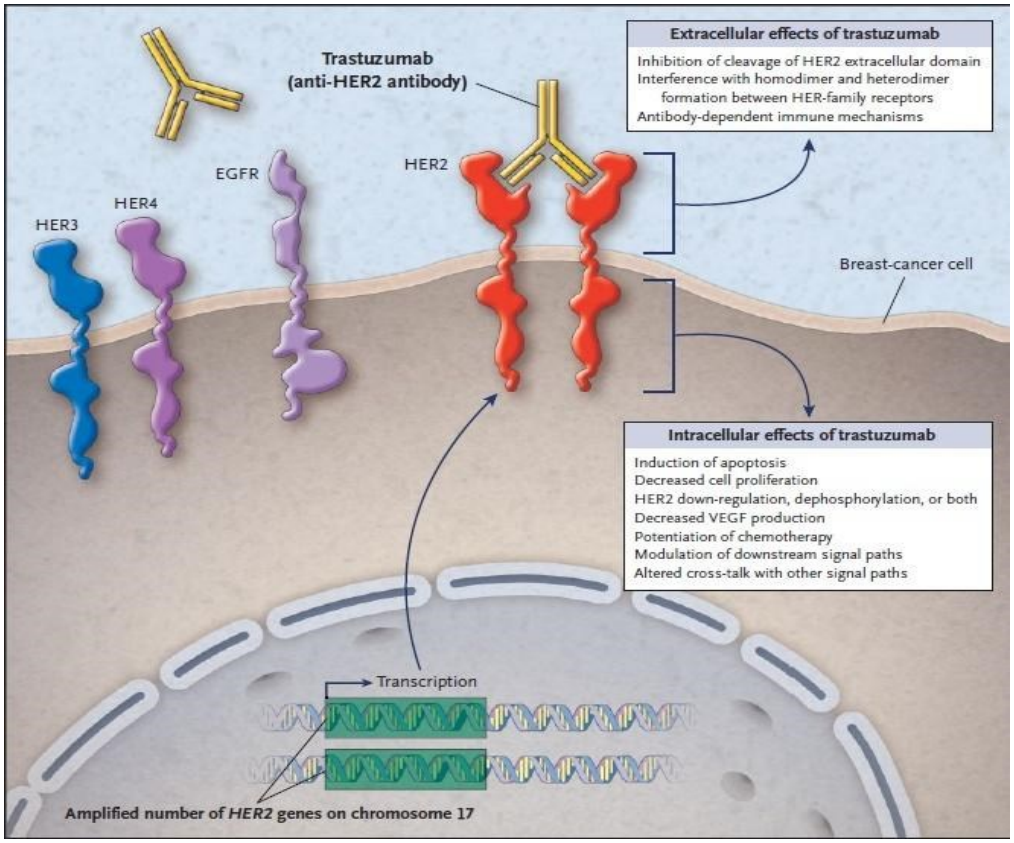


Figure 1.5: Interactions between Trastuzumab and Tumor Cells [64].

1.5. Classification of molecular subtype breast cancer

Many researches have shown that breast carcinoma includes many groups of molecules. The treatment and prognosis differ among the groups. There is a variation among the studies in the way how molecular subtypes breast cancer were classified, but basically it was based on the expression of biomarkers (HER2, ER, PR, Ki67) in IHC and the amplification of HER2 gene in ISH. There are five main subtypes, including luminal A, luminal B-HER2 negative with high Ki67, luminal B-HER2 positive, HER2 enrich and triple negative breast cancer (TNBC). Luminal A subtype is the best survival one while the triple negative breast cancer subtype has the poorest survival. The 13th St Gallen International Breast Cancer Conference added the Ki67 index to distinguish between luminal A and luminal B. Many authors have used the 14% such as optimal cut off point of Ki67 in their studies. Luminal A is characterized by positive hormone receptor, negative HER2 status and Ki67<14%. Luminal B also has HR+, HER2-, but Ki67≥14%. The subtype with HR+ and HER2+ is classified to luminal B-HER2 positive [65], [66], [67]. HER2 enrich subtype has HR-negative and HER2-positive markers. The triple negative is a subtype lacking the expression of hormone receptor as well as HER2 gene.

Study	Luminal A%	Luminal B-HER2 negative%	Luminal B-HER2 positive%	HER2 enrich %	Triple negative %	Country
Sun [68]	24	22	17	18	19	China (2015)
Park [69]	53.1	5.2	16.4	9.1	16.2	Korea (2011)
De Macêdo Andrade [70]	23.8	10	34.6	14.5	17.1	Brazil (2014)
El Fatemi [71]	30.5	41.8		9.2	18.5	Morocco (2012)
Kondov [72]	26.6	31.4	24.1	8.6	9.3	Macedonia (2018)
Caldarella [9]	34	25	11	11	19	Italy (2012)
Chaeng [73]	36	19		17	28	Canada (2009)

Table 1.3: Distribution of molecular subtypes

The study of Sadek and colleagues (2017) also demonstrated that the distribution of molecular subtypes differ considerably in race, risk factors, prognosis and response to treatment [74]. TNBCs account for 10–16% of all breast cancers [75]. The incidence of TNBC varied by region as well as ethnicity with the highest rates in African-Americans (23.7%), and lowest in Filipino patients (8.9%) [76]. Triple negative had more high grade cancer and mortality compared to HER2 subtype for black populations. [74]. The relapse rate of triple negative is the highest among the others [77], [78], [79]. The HER2 subtype is an aggressive tumour with bad prognosis, only responding to trastuzumab-targeted therapy. HER2 subtype had more late cancer stage compared to luminal [74]. In the Asians, the HER2 subtype is dominant compared to luminal A subtype in Western countries [9], [80]. In the study conducted in Canada, Fallahpour and co-workers showed that luminal A cancers accounted for 59.0% of all breast cancers. This is higher than the proportion found in other Canadian studies, 41%–44% [66], [81], [82]. Many studies have shown the significant correlation between molecular subtypes and the prognostic factors [83], [84], [85].

2. AIMS OF THE STUDY

Breast cancer is the most commonly occurring cancer and the leading cause of cancer deaths in Vietnamese women. Nowadays, the effectiveness of treatment aids in improving breast cancer survival time and its prognosis. However, the vast majority of breast cancer cases are diagnosed in late stage of disease with large size of tumors and metastasis. In addition, some advanced techniques like fluorescence in situ hybridization, dual in situ hybridization techniques which evaluate the HER2 gene amplification are still not widely applied. Therefore, many cases are not properly diagnosed, classified and treated. The use of targeting therapy (trastuzumab) for treatment of breast cancer is very limited.

Many studies have shown that breast cancer includes many molecular subtypes which have different treatments and prognoses. Therefore, the classification always has an important role to play in the choice of treatment therapies and the evaluation of response to therapy.

So, we conducted this study with the following aims:

1. To identify HER2 status of breast carcinoma patients in Central Vietnam

2. To determine the molecular subtypes of breast carcinoma.

3. To assess the relationship between HER2, molecular subtypes of breast cancer and prognostic factors.

4. To evaluate the concordance of HER2 protein overexpression and gene amplification

3. MATERIALS AND METHODS

3.1. Study sites

This was a descriptive cross-sectional study, conducted from November 2015 to October 2018, at following settings:

- Anatomical Pathology Institute of Sassari University Hospital
- Anatomical Pathology Department of Hue University Hospital
- Anatomical Pathology Department of Hue Central Hospital

3.2. Samples collection

- The study included 88 surgical samples from patients that were diagnosed such as breast carcinomas at Hue University Hospital and Hue Central Hospital, Hue city, Vietnam from 04/2016 to 04/2017
- The samples from the breast carcinoma patients who received perioperative chemotherapy in the past were excluded.
- Specimens were fixed with 10% neutral-buffered formalin within 30 minutes after taking off from patient's body.
- All samples were re-examined histopathology to confirm the breast carcinoma at Anatomical Pathology Institute of Sassari University Hospital**.**

3.3. Samples preparation

3.3.1. Fixation of specimens

- The 10% neutral-buffered formalin (equivalent to 4% formaldehyde solution) was used to fix the specimens. Fixative volume was 10- 20 times as much as specimen volume. The optimal time for fixation is in 24 - 48 hours.

3.3.2. Processing of specimens

Following fixation, tissue specimens were processed by protocol for the surgical specimens

3.3.3. Embedding in paraffin wax

Following processing, specimens were embedded in fresh paraffin wax at 55-60°C, then, stored at room temperature.

3.3.4. Sectioning paraffin block

Section thickness for hematoxylin and eosin staining is about 2-3µm. Section thickness is also about 2-3µm for immunohistochemistry and 4-5µm for fluorescence in situ hybridization.

3.4. Hematoxylin and Eosin (H&E) staining

The slides containing paraffin sections were placed in a slide holder (glass or metal). The implementation of Hematoxylin and Eosin (H&E) stainstrictly followed the protocol which accompanied the staining machine. After then, the slides were coverslipped with Permount (xylene based).

3.5. Immunohistochemical staining (IHC) for Her2-protein

The HER2 protein was identified by immunohistochemistry by using PATHWAY anti-HER-2/*neu*(4B5) Rabbit Monoclonal Primary Antibody 5ml (~6µg/ml) by Ventana Benchmark Ultra automatic staining system [86].

3.5.1. Principles

PATHWAY HER2 (4B5) is a rabbit monoclonal antibody binding to HER2 in paraffin embedded tissue sections. The focalization of the specific antibody could be implemented by ultraView. The ultraView Universal DAB Detection Kit detects specific mouse and rabbit primary antibodies bound to an antigen in paraffin-embedded tissue sections. The specific antibody is located by a cocktail of enzyme labeled secondary antibodies (HRP Multimer). The complex is then visualized with hydrogen peroxide substrate and 3, 3'diaminobenzidine tetrahydrochloride (DAB) chromogen, which produces a brown precipitate that is readily observed by light microscopy [87]. Every step requires accurate time and temperature. The reaction, at the end of every incubation step, is terminated by using the Ventana automated slide stainer which might aid in the sections washing to remove unbound material. This procedure could also be advantageous to the enhancement of desired reaction in following steps. In addition, the application of Lipid Coverslip™ could limit the reagents which are in water-based specimens to evaporate.

3.5.2. Step by Step Procedure

We applied the procedures for staining on the Ventana automated slide stainer

Procedure	BenchMark ULTRA
Deparaffinization	Selected
Cell Conditioning (Antigen Unmasking)	Cell Conditioning 1, Mild
Enzyme (Protease)	None required
Antibody (Primary)	Approximately 16 minutes, 37°C
Detection Kit (UltraView Universal DAB Detection Kit)	Automated
Counterstain (Hematoxylin)	Hematoxylin II, 4minutes
Post Counterstain	Bluing, 4 minutes

Table 3.1: Recommended Staining Protocols for PATHWAY anti-HER-2/neu (4B5) with ultraView Universal DAB Detection Kit [86].

The Ventana Medical Systems, Inc. UltraView Universal DAB Detection Kit is an indirect, biotin-free system for detecting mouse IgG, mouse IgM and rabbit primary antibodies. The kit is expected to identify targets by immunohistochemistry in section of formalin-fixed, paraffin-embedded and frozen tissue that are stained on the BenchMark Ultra systems [88].

- Apply slide bar code label which corresponds to the antibody protocol be performed.

- Load the primary antibody, appropriate detection kit dispensers, and required accessory reagents onto the reagent tray and put them on the automated slide stainer. Check bulk fluids and waste.

- Load the slides into the automated slide stainer.

- Start the staining runs.

- After finishing the run, remove the slides from the automated slide stainer.

- Using a mild dishwashing detergent wash the slide to remove the coverslip solution, then dehydrate, clear, and coverslip with permanent mounting media in the usual manner.

3.5.3. Scoring Conventions for the Interpretation of PATHWAY HER2 (4B5)

We scored IHC results based on manufacturer's instruction, Interpretation Guide for VENTANA anti-HER2/*neu* (4B5) and Recommendation for HER2 testing of ASCO- CAP 2013.

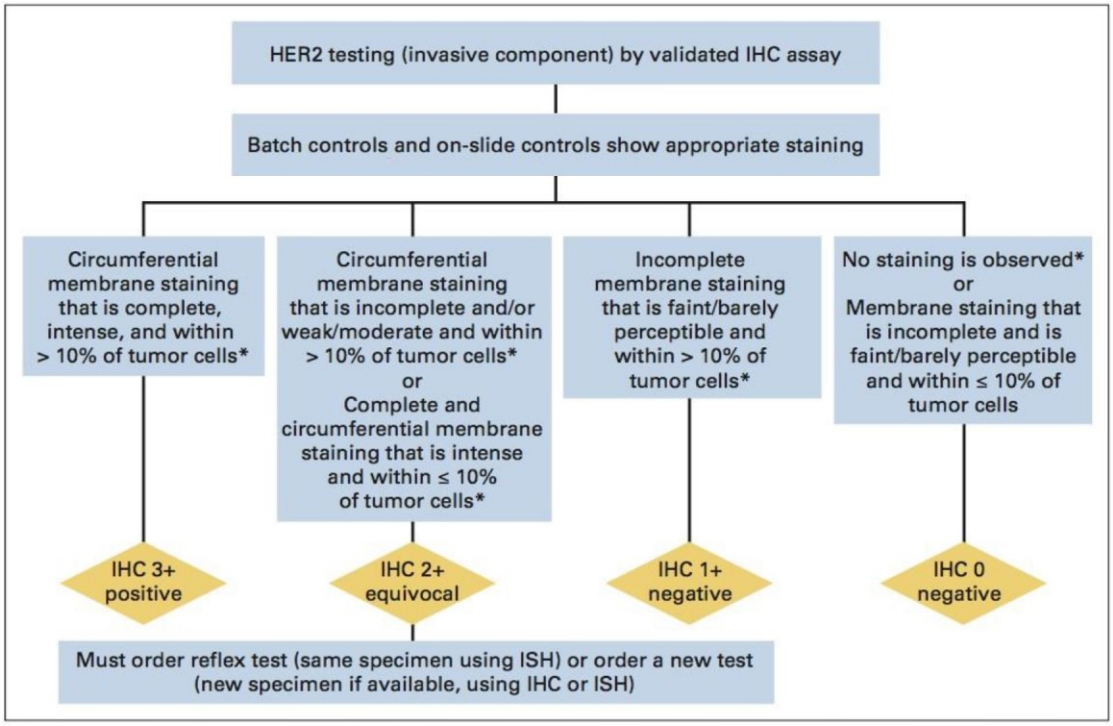


Figure 3.1: Evaluation of human epidermal growth factor receptor 2 (HER2) protein expression by immunohistochemistry (IHC) assay [37]

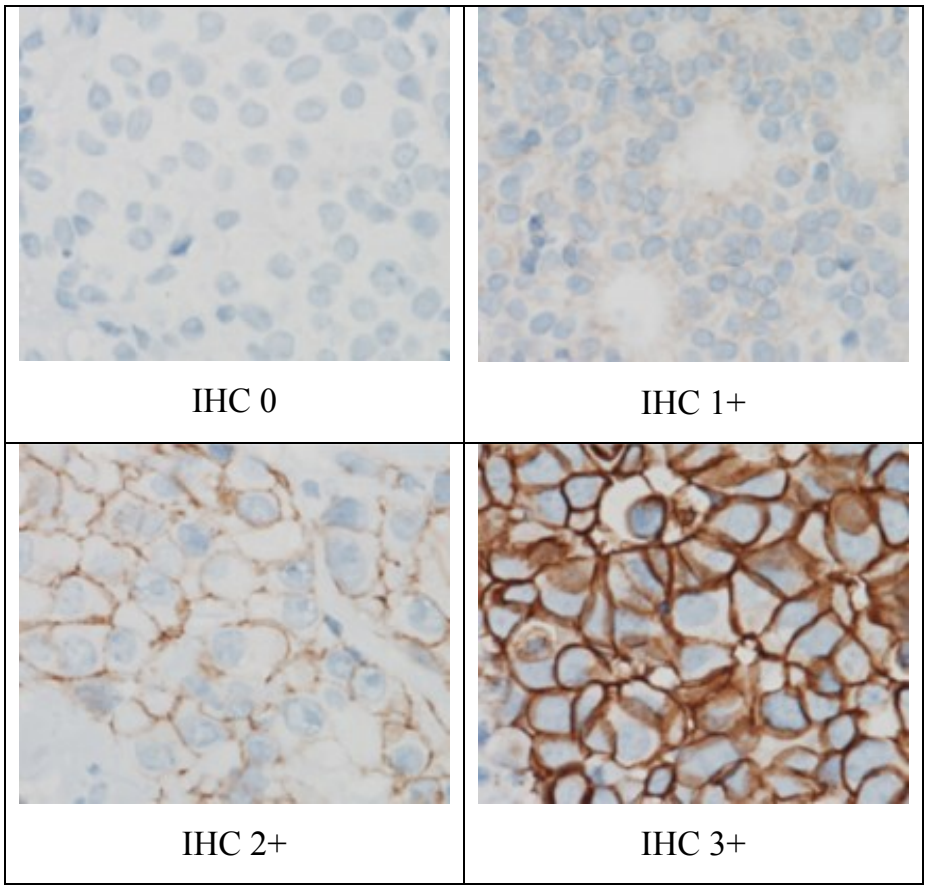


Figure 3.2: IHC Test Measures HER2 Protein Overexpression [86]

3.6. Immunohistochemical staining (IHC) for ER, PR, Ki-67 and E-cadherin

We have performed Estrogen receptor (ER), Progesterone receptor (PR), Ki-67, E-cadherin assay by using respectively VENTANA anti-Estrogen Receptor (SP1) Rabbit Monoclonal Primary Antibody, anti-Progesterone Receptor (1E2) Rabbit Monoclonal Primary Antibody, anti-Ki-67 (30-9) Rabbit Monoclonal Primary Antibody, anti-E-cadherin (36) Mouse Monoclonal Primary Antibody with Ventana Benchmark Ultra automatic staining system. The process was conducted similar to IHC staining for HER2 protein by Ventana Benchmark Ultra automatic staining system.

We selected a Ki67 index of 14% as the cut point for evaluation, the cells stained ≥ 14% were considered as high. ER and PR are scored using ≥1% of tumor staining as the positive.

The results were confirmed by experts in Anatomical Pathological Institute of Sassari University Hospital.

3.7. Fluorescence in situ hybridization (FISH) for HER2 gene [89]

FISH for HER2 gene amplification

At the Institute of Pathological Anatomy of Sassari, the samples have been analyzed for HER2 gene amplification with Fluorescence In Situ Hybridization (FISH) method. The currently recommended methodology for the identification of HER2 gene amplification is the FISH analysis using PathVysion HER2 DNA probe kit (Abbott Molecular Inc) that has been optimized only for identifying and quantifying amplification of the HER-2 gene. The kit is intended for in vitro diagnostic (IVD) [89], [90], [91], [92], [93], [94], [95]. Formalin-fixed and paraffin-embedded 4μm sections, after deparaffinization with Hemo-de reagent, have been immersed in 0.2N HCl for 20 minutes; after, they have been washed in purified water first and wash buffer then. The pretreatment stage has been performed at 80 ± 1°C for 30 minutes. After sequential washes, the slides are subjected to a Protease Treatment at 37 ± 1°C for 10 to 60 minutes. In general, the time is around 30 minutes but depends on the size of the slide. For the slide of 3μ, the time is 30 minutes. The 3μ is the best size of the slide that should be analyzed by FISH assay. After fixing the slides with formalin for 10 minutes

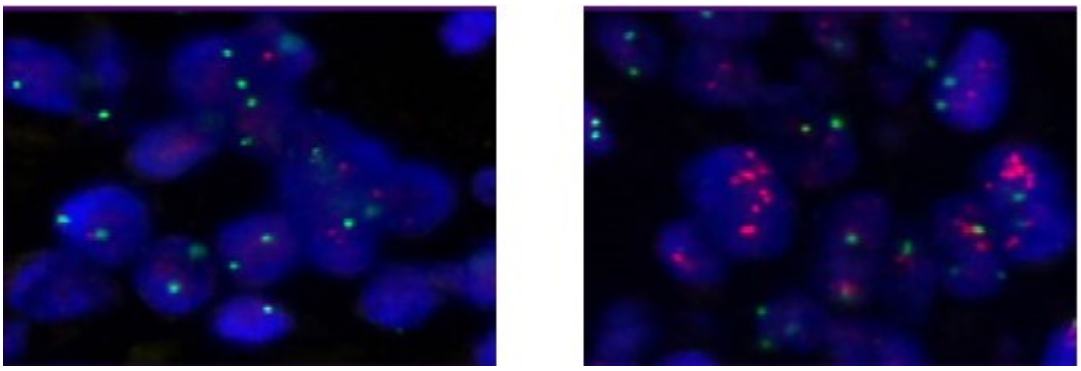
at room temperature and after the dehydration, the DNA has been denatured at 72°C for 5 minutes and then hybridized at 37°C overnight into the Hy-Brite, after the addition of the PathVysion HER2 DNA probe. Following the hybridization, the slides are washed with the wash buffer and colored with 4', 6-diamidino-2-phenylindole (DAPI) solution and cover-slipped. The hybridized slides are stored at -20°C and protected from light until their reading on the microscope. The evaluation of HER-2 amplification gene has been analyzed by direct detection using fluorescence microscope by Olympus BX41. The first step has been the localization of the tumor area using the 4X and 10X objectives, avoiding the areas of necrosis and where the nuclear borders are ambiguous (tumor cells as identified by H & E stain).

After locating the area and positioning the immersion oil, using the 100X objective, the slides have been evaluated using the prescribed filters to analyze the quality of HER2 signals and quality of tissue morphology. It was used 4 different filters to analyze the slides: first of all, DAPI filter to distinguish the nuclear boundaries and the integrity of the nuclei and then a triple filter (DAPI/red/green) to distinguish the nuclear boundaries (DAPI filter) and the hybridization of the probe, with red and green signals (RED/GREEN filter); for each nucleus, has count the number of LSI (Locus specific identifier) HER-2/*neu* signals and the number of CEP 17 signals.

LSI HER-2 to CEP 17 Ratio Determination

After scanning several areas of tumor cells to account for possible heterogeneity, it was been selected an area of good nuclei distribution. Using 100X objective, the analysis has been started in the upper left quadrant of the selected area and, scanning from left to right, has been count the number of signals within the nuclear boundary of each evaluable interphase cell according to the worldwide guidelines. The recommended method for LSI HER-2/*neu* to CEP 17 ratio determination is by dividing the total number of LSI HER-2/*neu* signals by the total number of CEP 17 signals in counting the same 20 nuclei. For each nucleus, must be count the number of LSI HER-2/neu signals and the number of CEP 17 signals. To calculate the final result, has been used the following ratio: total LSI HER-2/*neu* signals/total CEP 17 signals.

We based on the ASCO/CAP Guideline Update 2013 for recommendations for human epidermal growth factor receptor 2 testing in breast cancer to analyse the final results [37]. If the LSI HER-2/*neu* to CEP17 ratio is ≥2, HER-2/*neu* gene amplification was observed. In case of the ratio is <2, should be count the average HER2 copy number. If the average HER2 copy number ≥6.0 signals/cell, the HER-2/*neu* gene amplification was reported. If the average HER2 copy number <4.0 signals/cell, HER-2/*neu* gene amplification was not observed. In case of the borderline (the average HER2 copy number ≥4.0 signals/cell and <6.0 signals/cell), should be count an additional 20 nuclei and recalculate the ratio based on the total of 40 nuclei. If still in doubt, the assay was repeated with a fresh specimen slide.



HER2 Not Amplified HER2 Amplified

Figure 3.3: HER2 gene amplification by FISH

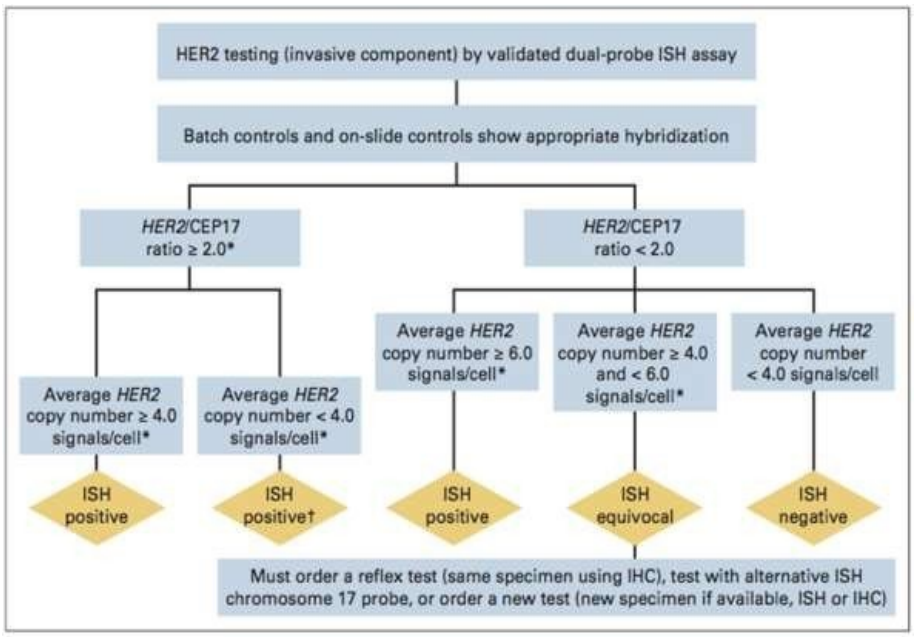


Figure 3.4: Evaluation HER2 gene amplification by In Situ Hybridization (ISH) [37]

3.8. Dual color in situ hybridization (DISH) for HER2 gene [96]

DISH for HER2 gene amplification

The DISH was performed at Anatomical Pathology Department of Hue University Hospital, Vietnam for 52 selected cases, included 27 negative HER2 cases, 8 equivocal HER2 cases and 17 positive HER2 cases in IHC.

The HER2 gene amplification was defined by Dual In situ Hybridization using the INFORM HER2 Dual In Situ Hybridization DNA probe cocktail assay by Ventana Medical Systems. This technique identifies HER2 gene status by detecting the HER2 copies via silver in situ hybridization (SISH) and the Chromosome 17 (Chr17) copies via chromogenic red in situ hybridization (Red ISH) on a single slide.

Routinely processed, formalin-fixed and paraffin-embedded tissues were applied to this technique. The section should be cut approximately 3-4μm of thickness. Then, we have performed DISH assay following the protocol setting on Ventana BenchMark Series automated slide stainers

Procedure	**BenchMark**
Deparaffinization	Selected
Extended Depar	Not Selected
Cell Conditioning	Selected Cell Conditioning CC2 Mild CC2 - 8 min Standard CC2 - 12 min Extended CC2 - 8 min
ISH-Protease 3	16 min
Denaturation	20 min
Hybridization	6 hours
Stringency wash	72°C
SISH Multimer	16 min
Silver Chromogen	4 min
Red ISH Multimer	24 min
Red Chromogen	8 min
Counterstain	Hematoxylin II, 8minutes
Post Counterstain	Bluing Reagent, 4 minutes

Table 3.2: Recommended Staining Protocol for INFORM HER2 Dual ISH DNA Probe Cocktail on Ventana BenchMark Series automated slide stainers

We have applied the slide bar code label that corresponds to the probe protocol to be performed. After then loading the INFORM HER2 Dual ISH DNA Probe Cocktail, reagents from UltraView Red ISH DIG and UltraView SISH DNP Detection Kits, and requiring accessory reagents into the reagent tray(s). Reagent tray(s) were placed on automated slide stainer. Loading slides into the automated slide stainer and starting the staining run. At the run has finished, remove slides from the automated slide stainer and start proceeding with dehydration procedure. Removing liquid coverslip solution and washing the slides in 2 sequential solutions of a mild dishwashing detergent (do not use detergent designed for automatic dishwashers). After then, the slides were rinsed well with distilled water, about 1 minute. Shake off excess water. Then put the slides in an oven (45-60°C) to dry or air dry at ambient temperature. In an oven, drying times range from 10 minutes to one hour until completely dry. After then, the slides were cover-slipped and transferred into xylene bath for approximately 30 seconds. Then, place coverslip on slide.

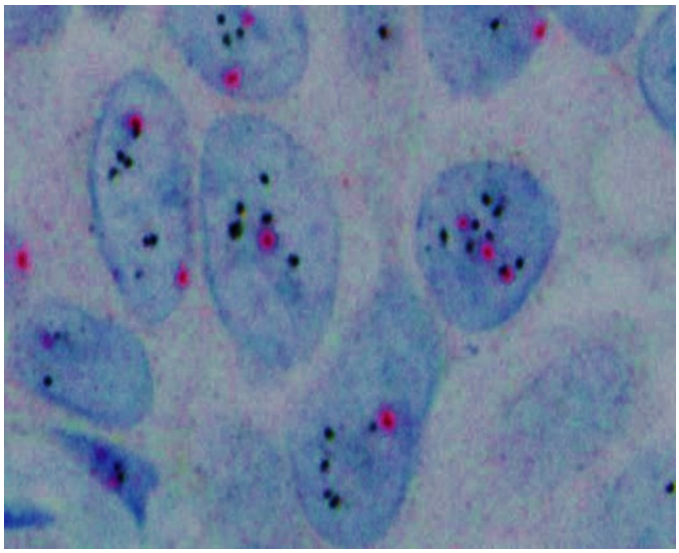


Figure 3.5: HER2 gene amplification by Dual ISH (DISH)

Interpret the result of DISH:

Using objectives as high as 40X to 60X of light microscopy to enumerate the black signals of HER2 gene amplification and the red signals of Chromosome 17 for all 20 nuclei in the invasive breast carcinoma, basing on the guideline of Ventana. To calculate the final result, has been used the following ratio: Total HER2/*neu* signals/ Total CEP 17 signals.

After then, we have analysed final results basing on the ASCO/CAP Guideline Update 2013 for the recommendations for human epidermal growth factor receptor 2 testing in breast cancer [37]. This evaluated way is absolutely similar to that in FISH technique.

If the LSI HER-2/*neu* to CEP 17 ratio is ≥ 2, HER-2/*neu* gene amplification was observed. In case of the ratio is < 2, should be count the average HER2 copy number. If the average HER2 copy number ≥ 6.0 signals/cell, the HER-2/*neu* gene amplification was reported. If the average HER2 copy number <4.0 signals/cell, HER-2/*neu* gene amplification was not observed. In case of the borderline (the average HER2 copy number ≥ 4.0 signals/cell and < 6.0 signals/cell), should be count an additional 20 nuclei and recalculate the ratio based on the total of 40 nuclei. If still in doubt, the assay was repeated with a fresh specimen slide.

3.9. Study design

All specimens were re-examined on Hematoxylin-Eosin staining to confirm, at Institute of Pathological Anatomy of Sassari University Hospital, the histopathological diagnosis of the breast carcinoma**.** Classification of tumors based on WHO histological classification of tumors of the breast, TNM classification of carcinomas of the breast 2012 and the Nottingham Histologic Score system (the Elston-Ellis modification of Scarff-Bloom-Richardson grading system). The IHC was performed on all formalin-fixed paraffin embedded tissue sections of patients. FISH technique was performed on all HER2 equivocal (2+) cases in IHC. The Dual-ISH assay was applied to 52 selected cases.

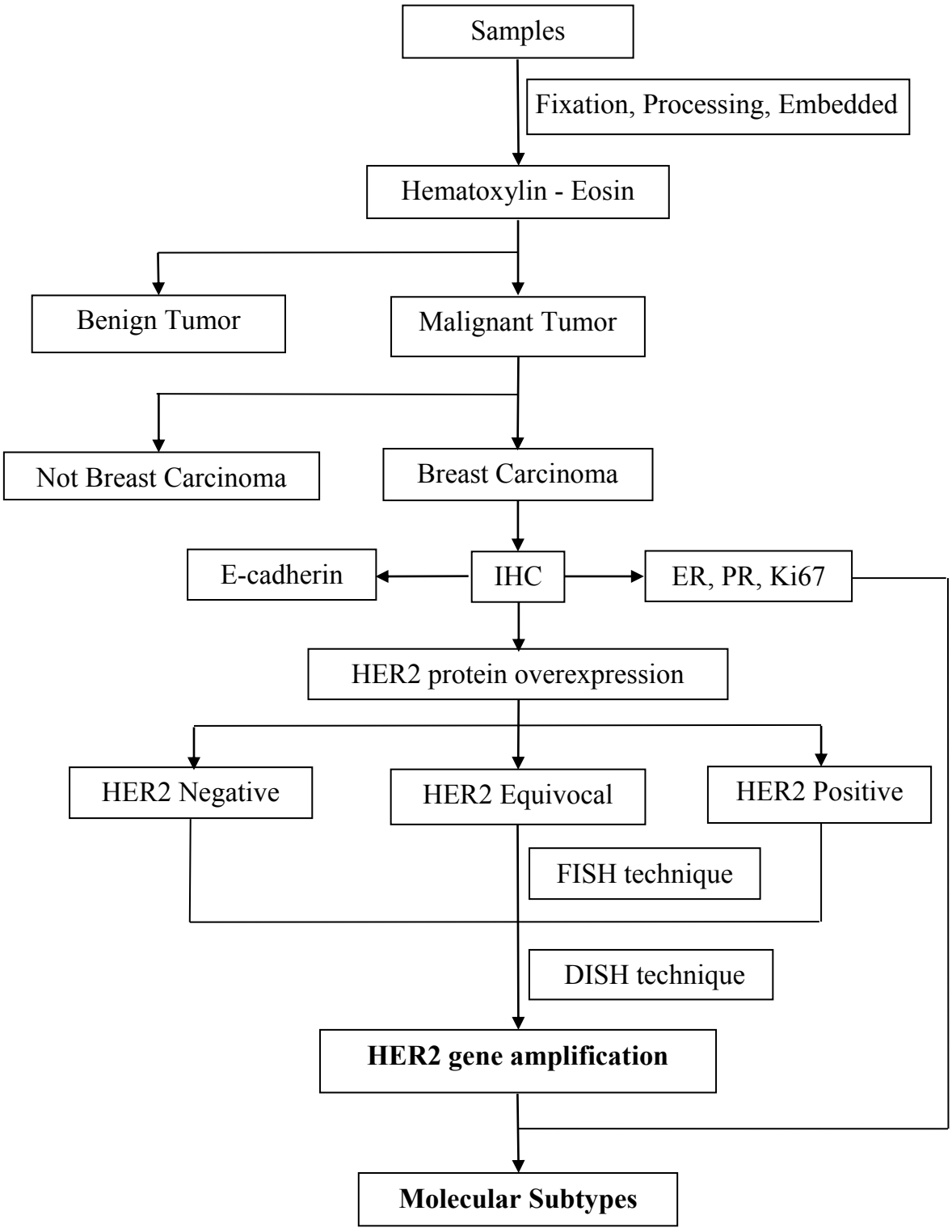


Figure 3.6: Scheme of study design

3.10. Statistical analysis

The data has been collected and stored using Excel, then has been analyzed with SPSS 16.0

4. RESULTS

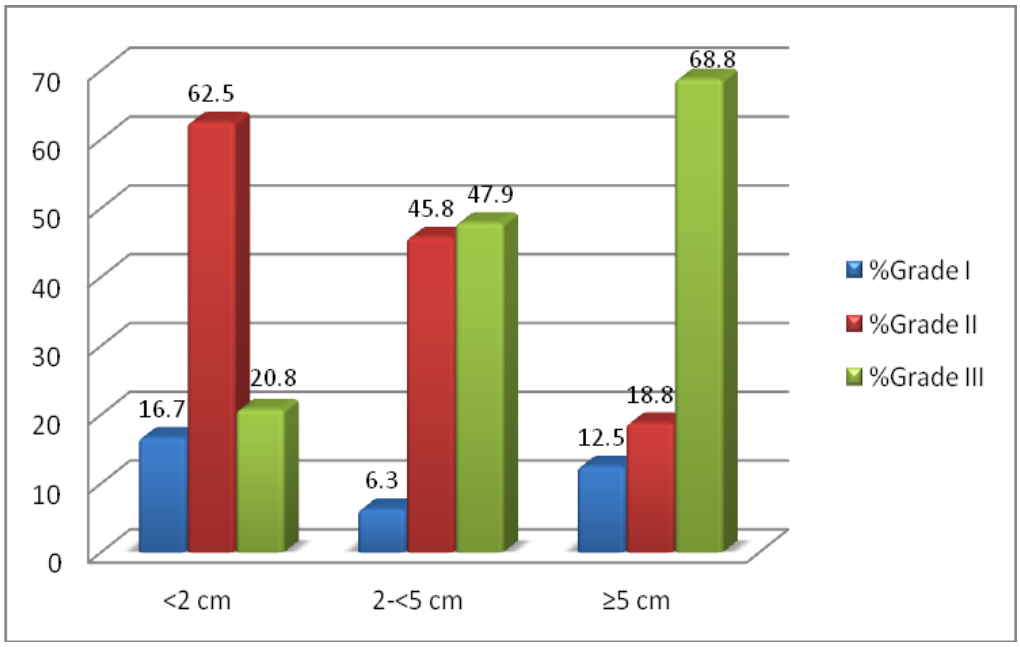
4.1. Clinicopathological characteristics of specimens

We collected 88 specimens of invasive breast carcinomas in women. The clinicopathological characteristics of those specimens include age, tumour size, histological type, histological grade, lymph node involvement and stage of disease which are shown in the table below:

Parameters	Number (%)
Age	
Range	29-91
Median ± standard deviation (X ± SD)	52.5 ± 12.8
< 50	29 (33)
≥50 - <70	49 (55.7)
≥ 70	10 (11.4)
Tumor size	
≤2	24 (27.3)
>2- ≤5cm	48 (54.5)
>5cm	16 (18.2)
Histological type	
Ductal	82 (93.2)
Others	6 (6.8)
Histological grade	
I	9 (10.2)
II	40 (45.5)
III	39 (44.3)
Lymph node status	
No metastasis	38 (43.2)
Metastasis	48 (54.5)
Unknown	2 (2.3)
Stage of disease	
I	13 (14.8)
II	47 (53.4)
III	26 (29.5)
Unknown	2 (2.3)

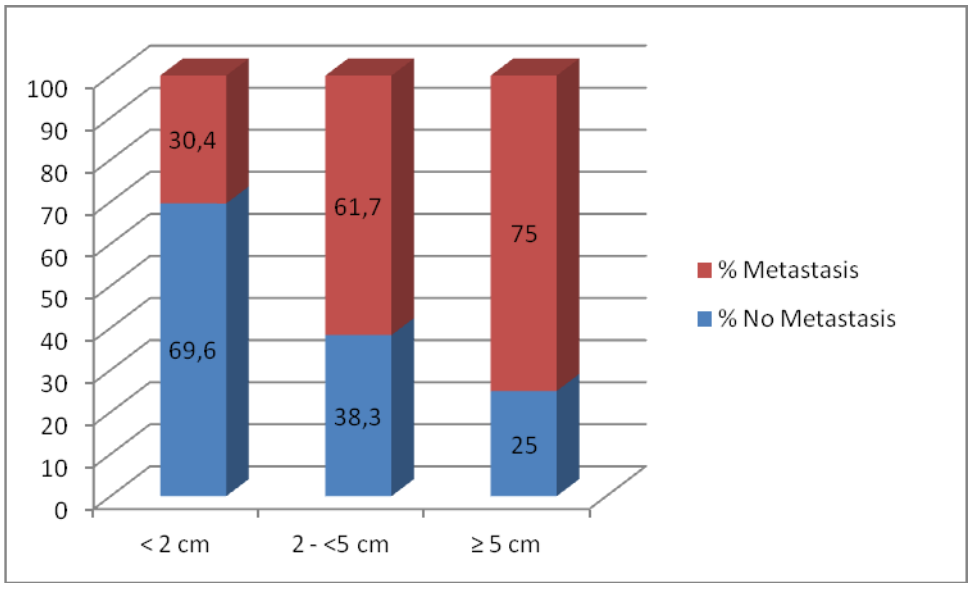
Table 4.1: Clinicopathological characteristic of specimens

In our study, the median age was around 52 years (range between 29 and 31). The group of women aged 50-79 occupied the highest proportion, at 55.7%. The most common tumor size was 2 – 5cm, with a percentage of 54.5%. The invasive ductal carcinoma accounted for the vast majority of samples, at about 93.2%. The classification of histologic grade was applied based on Nottingham Histologic Score system (the Elston-Ellis modification of Scarff-Bloom-Richardson grading system). The tumors which were of histologic grade II and III was found in 89.8% of cases while the figure for grade I is lower, at 10.2%. The cases which had lymph node metastasis accounted for 54.5%, higher than those with no metastasis. The stage of diseases was assessed based on TNM classification of breast carcinoma 2012. While the highest rate is stage II, at 53.4%, the figure for early stage (stage I) is the lowest, at 14.8%.



p=0.014

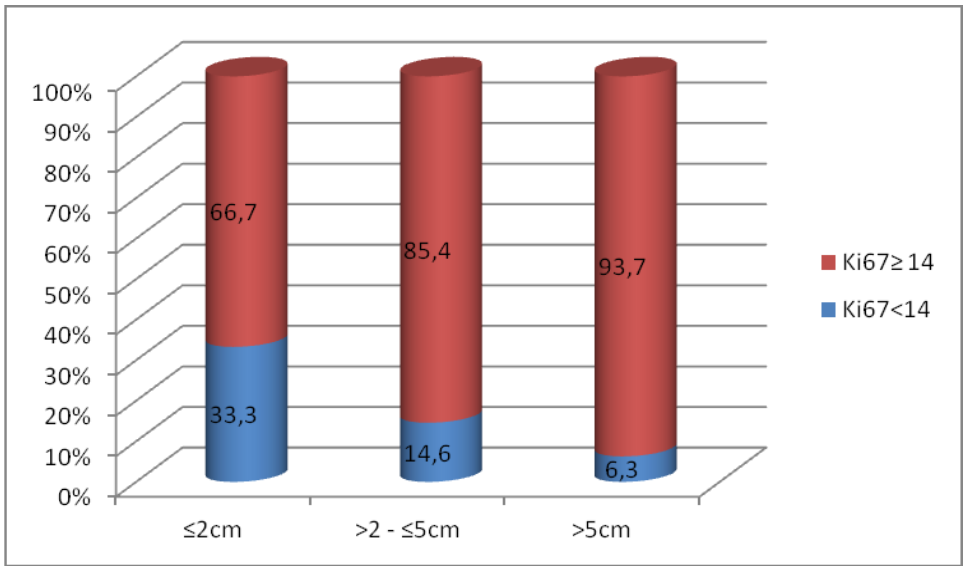
Chart 4.1: Relationship between tumour size and histologic grade



p<0.05

Chart 4.2: Relationship between tumour size and lymph node status

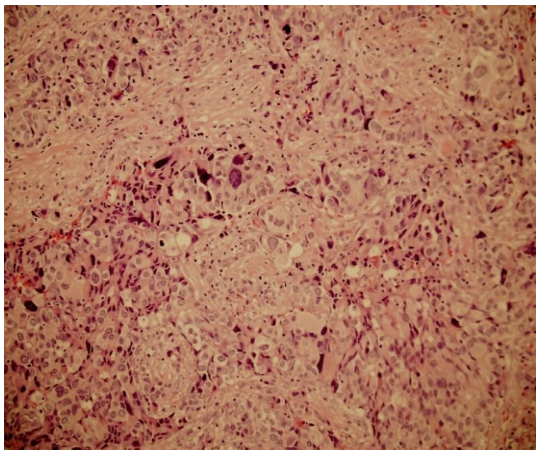
The chart 4.1 and chart 4.2 showed that there was a significant difference between the tumour size and histological grade as well as metastasis, with p<0.05. Most of the tumours with more than 5cm in size had grade III (68.8%) and the figure for tumours with metastasis was 75%.



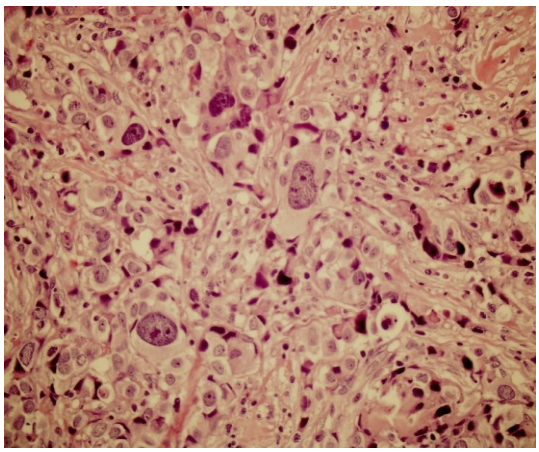
p<0.001

Chart 4.3: Relationship between the tumour size and Ki67

Our data demonstrated that the vast majority of tumors larger than 5cm in size expressed a high level of Ki67 (93.7%) compared to that of tumors smaller than 2cm in size (66.7%). The difference is statistical significance.



Grade III (x10)



Grade III (x40)

Figure 4.1: Code 6497. Invasive Ductal Carcinoma

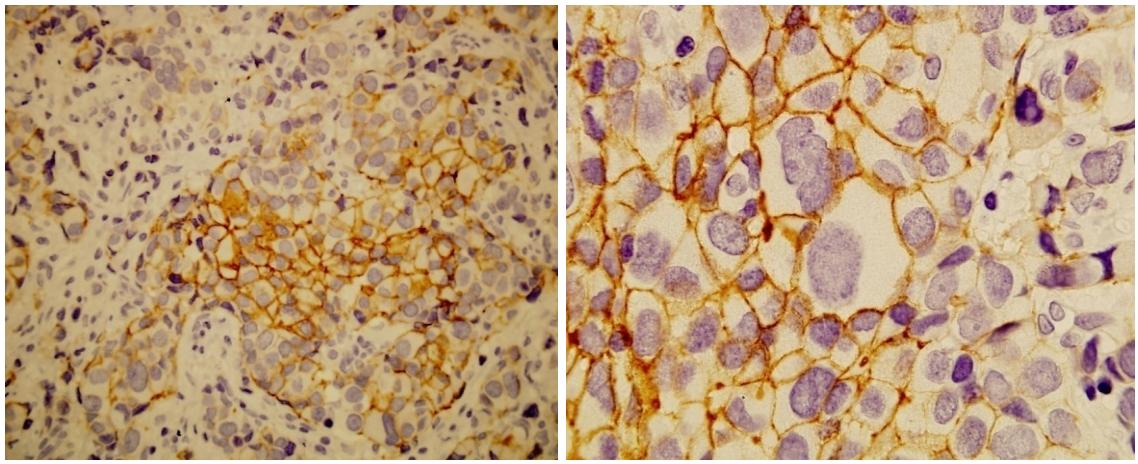
4.2. Human epidermal growth factor receptor 2 (HER2) status

4.2.1. HER2 protein overexpression in immunohistochemistry

HER2 protein overexpression		**Total**		
		Number	**Percentage (%)**	
Negative	0	50	56.8	60.2
	1+	3	3.4	
Equivocal	2+	8	9.1	
Positive	3+	27	30.7	
Total		**88**	**100%**	

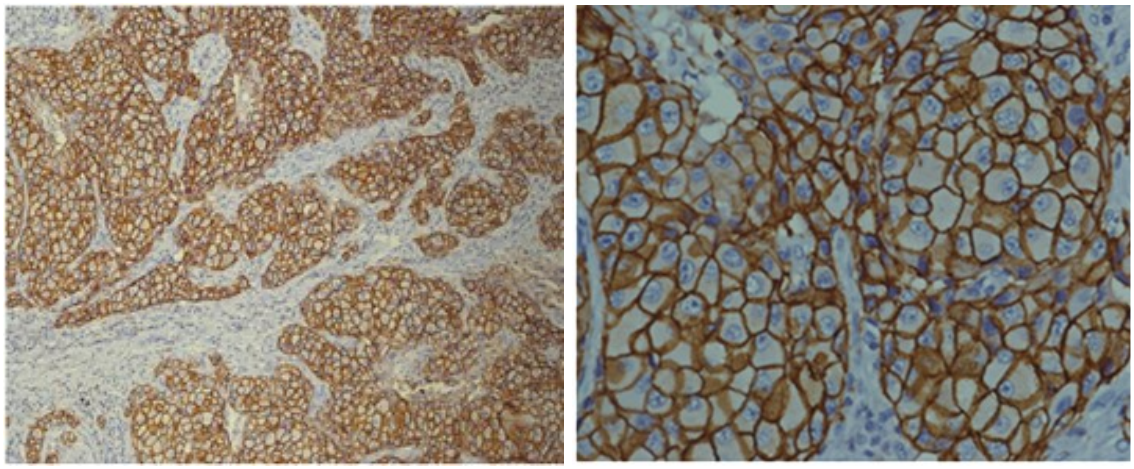
Table 4.2: HER2 protein overexpression in immunohistochemistry

We performed HER2 immunohistochemical staining in 88 samples. The results of HER2 protein over-expression were interpreted as 0 and 1+ (negative); 2+ (equivocal) and 3+ (positive) score according ASCO-CAP guideline 2013. We had 27 breast cancer samples (30.7%) which were defined as positive for HER2 protein over-expression, 8 cases with 2+ score (9.1%) as equivocal and 53 negative cases, accounting for 60.2%.



HER2 (2+) x 10 HER2 (2+) x 40

Figure 4.2: Code 6497. HER2 protein over-expression 2+ score (equivocal)



HER2 (3+) x 10 HER2 (3+) x 40

Figure 4.3: Code 2813. HER2 protein over-expression 3+ score (positive)

4.2.2. HER2 gene amplification by Fluorescence In Situ Hybridization

We used FISH assay to identify HER2 gene amplification with 8 cases having (2+) score in IHC. The results were presented in the table below:

HER2 gene amplification	**Total**	
	Number	**Percentage (%)**
Negative	6	75.0
Equivocal	0	0
Positive	2	25.0
Total	**8**	**100%**

Table 4.3: HER2 gene amplification by FISH

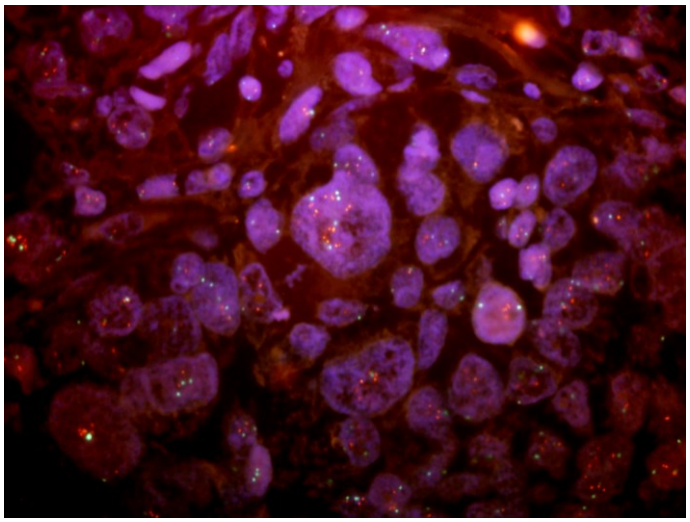


Figure 4.4: Code 6497. HER2 gene amplification by FISH

Based on the IHC and FISH results for HER2, HER2 positivity was defined as either an HER2 (3+) in IHC or an amplification of the HER2 gene in FISH. Therefore, we had 29 cases with HER2-positive (approximately 32.9%) and those with negative results were 59 cases, collectively accounting for about 67.1% in total.

HER2 status		**Number**		**Percentage (%)**
Negative		59		67.1
Positive	3+/ IHC	27	29	32.9
	Amplification/FISH	2		
Total		**88**		**100**

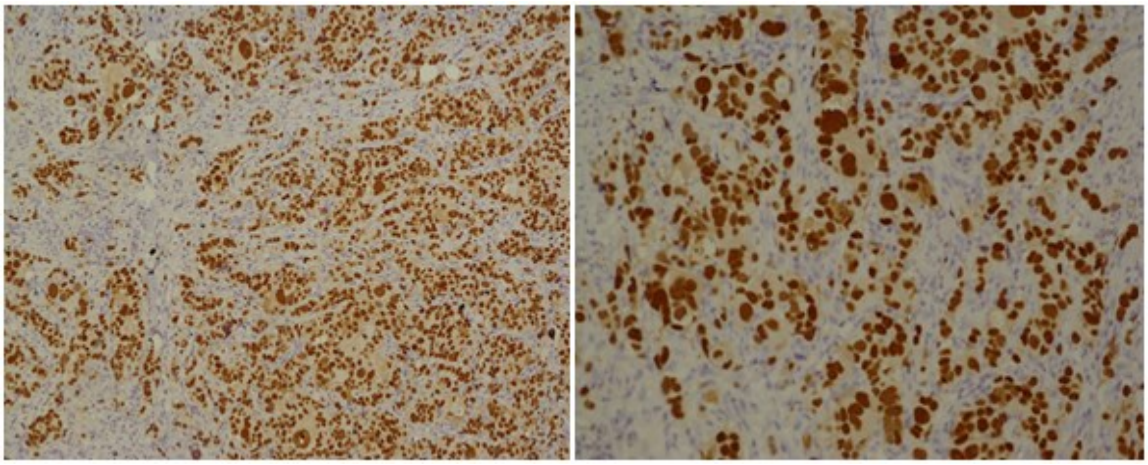
Table 4.4: HER2 status on breast cancer

4.3. Biomarker status

Parameters	**Number (%)**
ER status	
Negative	39 (44.3)
Positive	49 (55.7)
PR status	
Negative	52 (59.1)
Positive	36 (40.9)
Ki67	
< 14%	16 (18.2)
≥ 14%	72 (81.8)

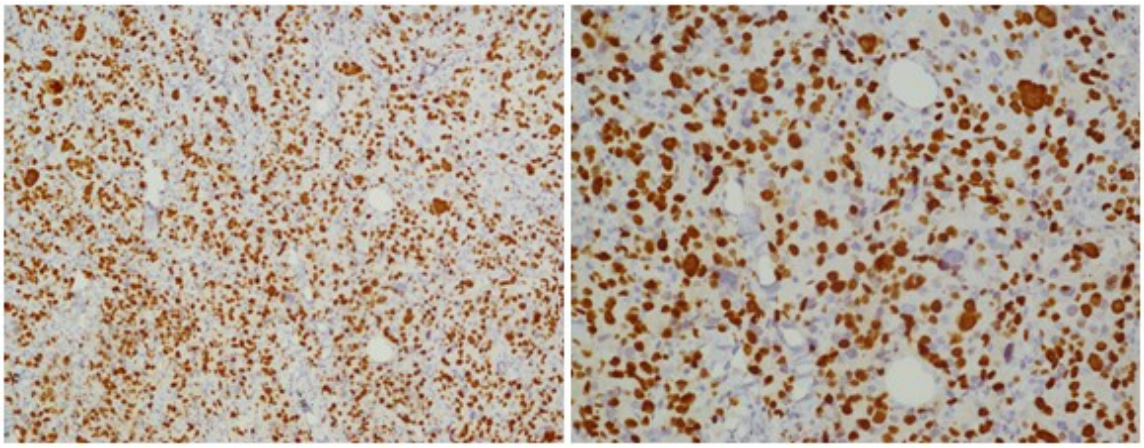
Table 4.5: Biomarker status of breast cancer

We obtained IHC results of ER, PR and Ki-67 in 88 cases. The ER positive was responsible for approximately 55.7% of cases, followed by the ER negative, at 44.3%. The figure for PR positive and negative was 40.9% and 59.1%, respectively. For the proliferation index Ki67, 14% was chosen as an optimal cut off for the division of 2 groups of Ki67 in this study. The proportion of group with high Ki67 (≥14%) was 81.8%, much higher than the group of low Ki67 (at 18.2%)



ER (+)90% x10 ER (+)90% x20

Figure 4.5: Code 6497. Estrogen receptor positive



Ki67 (+)60% x10 Ki67 (+)60% x20

Figure 4.6: Code 6497. Proliferation index Ki67

4.4. Molecular Subtypes Breast Cancer

Molecular subtypes of breast carcinoma were classified based on the biomarkers status such as ER, PR, Ki67, HER2 in accordance with the 13th Saint Gallen conference 2013 and using 14% as an optimal cut off point of Ki67. There were five groups including luminal A, luminal B-HER2 negative, luminal B-HER2 positive, HER2 enrich and triple negative breast cancer. The luminal B was the most frequent in this study, at 37.5% while the uncommon belonged to luminal A, at 18.2%. The HER2 enrich and triple negative breast cancer were 22.7% and 21.6%, respectively (chart 4.4).

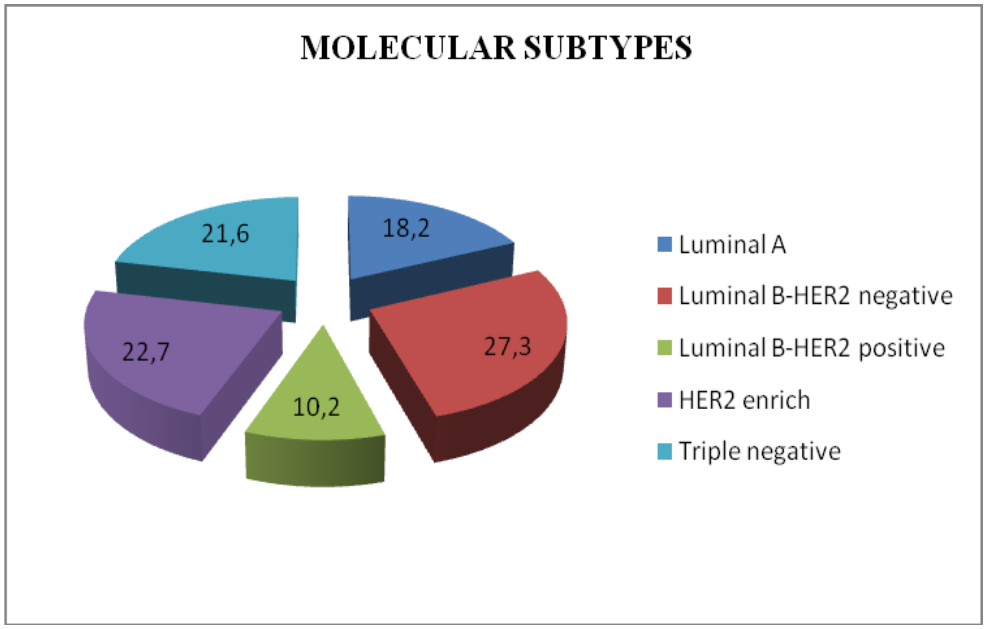


Chart 4.4: Distribution of molecular subtype of breast cancer

4.5. Relationship between HER2 and prognostic factors

4.5.1. Relationship between HER2 and clinicopathological characteristics

A significant difference between HER2 status breast cancer and histological grade was found with $p < 0.001$. We also observed the differences between the HER2 status and tumor size, stage of disease, lymph node status, however, not reaching statistical significance.

Parameters	HER2 status		p value
	Negative	Positive	
	Number (%)	Number (%)	
	59 (67.1)	29 (32.9)	
Age			
< 50	17 (28.8)	12 (41.8)	p= 0.395
≥50 - <70	34 (57.6)	15 (51.7)	
≥ 70	8 (13.6)	2 (6.5)	
Tumor size			
≤2 cm	19 (32.2)	5 (17.2)	p=0.061
>2- ≤5cm	33 (55.9)	15 (51.7)	
>5cm	7 (11.9)	9 (31.1)	
Stage of disease			
I	12 (21.1)	1 (3.4)	p=0.057
II	31 (54.4)	16(55.2)	
III	14 (24.5)	12 (41.4)	
Histological type			
Ductal	53 (89.8)	29 (100.0)	p=0.172
Others	6 (10.2)	0 (0.0)	
Histological grade			
I	9 (15.3)	0 (0.0)	**p<0.001**
II	34 (57.6)	6 (20.7)	
III	16 (27.1)	23 (79.3)	
Lymph node status			
No metastasis	29 (50.9)	9 (31.1)	p= 0.08
Metastasis	28 (49.1)	20 (68.9)	

Table 4.6: Relationship between HER2 and clinicopathological characteristics

4.5.2. Relationship between HER2 and biomarkers

There was a significant difference between HER2 status breast cancer and ER status ($p< 0.001$). The majority of samples in HER2-positive group (69%) had a negative ER status. A statistically significant difference was seen between HER2 status and proliferation index Ki67 ($p<0.005$). All the samples of positive HER2 group have a high Ki67 ≥ 14%.

Parameters	HER2 status		P value
	Negative	Positive	
	Number (%) 59 (67.1)	Number (%) 29 (32.9)	
ER status			p<0.001
Negative	19 (32.8)	20 (69.0)	
Positive	40 (67.2)	9 (31.0)	
PR status			p=0.187
Negative	32 (54.2)	20 (69.0)	
Positive	27 (45.8)	9 (31.0)	
Ki 67			p=0.002
< 14	16 (27.1)	0 (0.0)	
≥ 14	43 (72.9)	29 (100.0)	

Table 4.7: Relationship between HER2 and biomarkers

4.6. Relationship between molecular subtypes and prognostic factors

In our study, we have found statistically significant differences between molecular subtype and histological type, histological grade, biomarkers (ER, PR, Ki67, HER2) with $p <0.001$. There was also a significant difference between molecular subtype and stage of disease with $p<0.05$. Most of the luminal A cases had stage I (43.7%), no metastasis (62%) and low Ki67 (100%). In contrast, the TNBCs and also HER2 enrich subtype were often in grade III, higher metastasis and higher Ki67.

Parameters	Molecular Subtype				P value
	Luminal A	Luminal B	HER2enrich	TNBC	
	Number (%) 16 (18.2)	Number (%) 33(37.5)	Number (%) 20 (22.7)	Number (%) 19 (21.6)	
Age					
<50	4 (25)	11 (33.4)	7 (35%)	7 (36.8)	p= 0.89
≥50	12 (75%)	22 (36.6%)	13 (75%)	12 (63.2)	
Tumor size					
≤2	8 (50%)	8 (24.2)	4 (20)	4 (21.1)	
>2- ≤5cm	7 (43.8%)	17 (51.6)	12 (60)	12 (63.3)	p= 0.438
>5cm	1 (6,2%)	8 (24.2)	4 (20)	3 (15.6)	
Stage of disease					
I	7 (43.7)	3 (9.1)	1 (5)	2 (10.6)	
II	6 (37.5)	19 (57.6)	12 (60)	10 (52.6)	**p= 0.029**
III	1 (6.3%)	11 (33.3)	7 (35)	7 (36.8)	
Unknown	2 (12.5)	0 (0)	0 (0)	0 (0)	
Histologic type					
Ductal	11 (68.8)	32 (66.9)	20 (100)	19 (100)	**p< 0.001**
Others	5 (31.2)	1 (3.1)	0 (0)	0 (0)	
Histologic grade					
I	6 (37.5)	2 (6.1)	0 (0)	1 (5.3)	
II	10 (62.5)	19 (57.6)	4 (20)	7 (36.8)	**p< 0.001**
III	0 (0)	12 (36.4)	16 (80)	11 (57.9)	
Lymph node status					
No metastasis	10 (62.5)	12 (36.4)	9 (45)	7 (36.8)	p= 0.141
Metastasis	4 (25)	21 (65.6)	11 (55)	12 (63.2)	
Unknown	2(12.5)	0 (0)	0 (0)	0 (0)	
Ki67					
<14%	16 (100)	0 (0)	0 (0)	0 (0)	**p< 0.001**
≥ 14%	0 (0)	33 (100)	20 (100)	19 (100)	

Table 4.8: Relationship between molecular subtype and prognostic factors

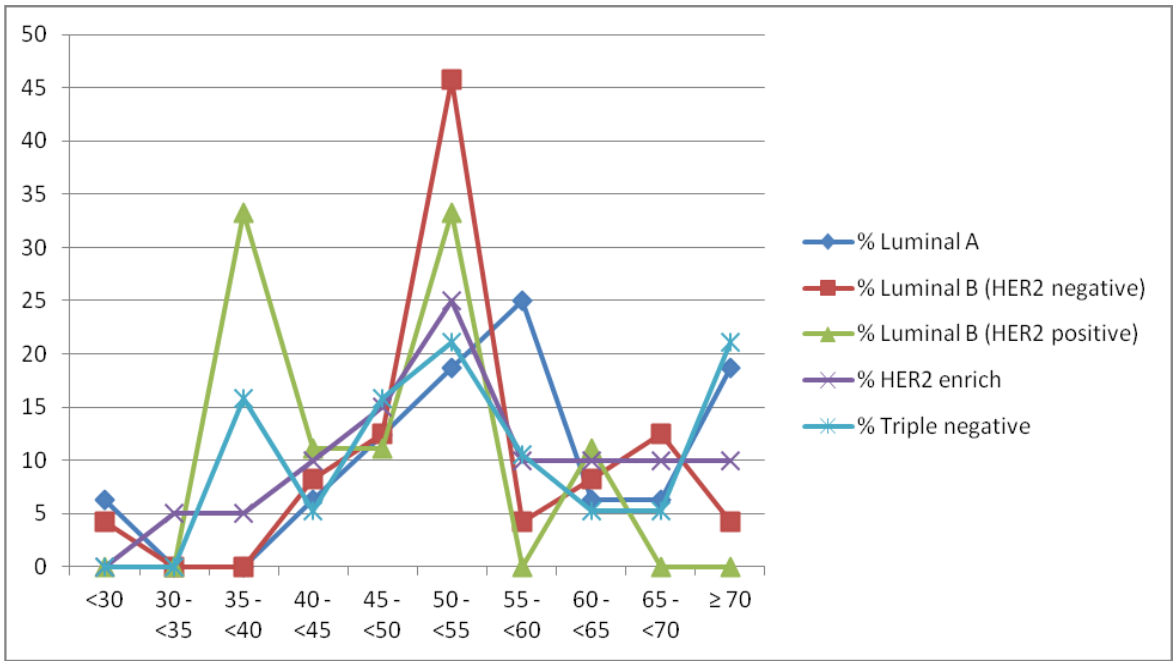


Chart 4.5: Distribution of molecular subtype breast cancer by age

The chart 4.5 showed that the luminal A subtype was common in women aged 55-60 years. The prevalence of luminal B-HER2 negative and HER2 enrich subtype reached its peak at the age of 50-55. Interestingly, triple negative subtypes were common in all three age groups: from 35 to 40, 50-55 and over 70 years old. Luminal B-HER2 positive was extremely prevalent at 2 age groups: very young women aged between 35 and 40 like triple negative subtype and middle aged 50-55, like other subtypes.

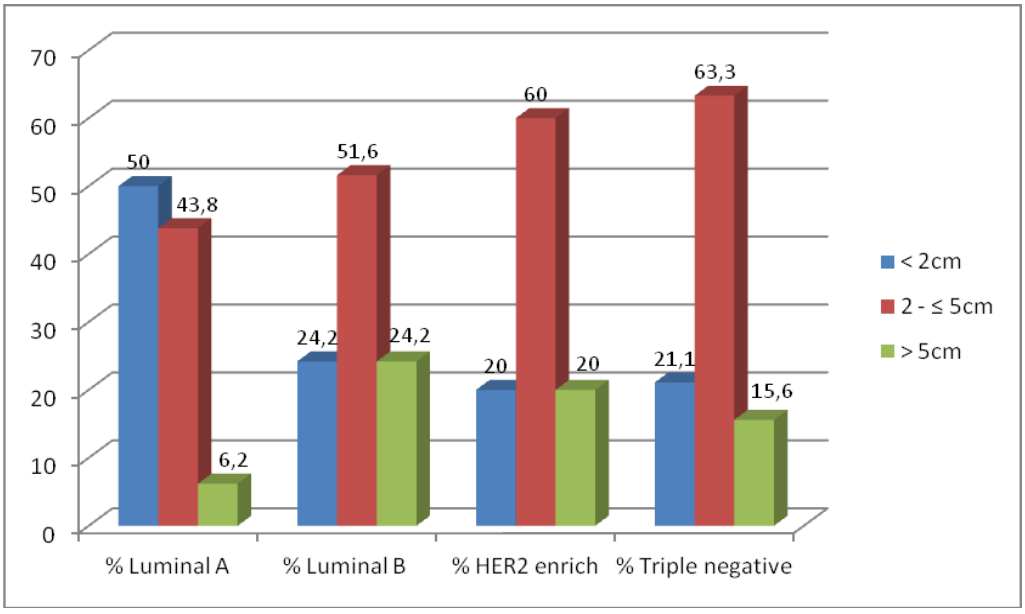


Chart 4.6: Relationship between molecular subtypes and tumour size

The chart above demonstrated that the majority of luminal A cases were the small-sized tumours (≤ 2cm), accounting for 50%. The tumors with a large size (≥5cm) just occupied 6.2% of luminal A cases. The proportion of tumours ≤ 2cm in size accounted for 24.2%, 20% and 21.1% of the total in the group of luminal B, HER2 enrich and triple negative subtype, respectively. Most of the tumours in luminal B, HER2 enrich and triple negative subtype group were larger than 2cm.

4.7. Concordance between IHC and DISH results

We performed DISH technique to identify HER2 gene amplification for 52 samples selected from our original ones. Those samples underwent IHC, which included 26 samples in 0 score, 1 sample in (1+) score, 8 samples in (2+) score and 17 samples in (3+) score. The results of DISH are shown in the table 4.9.

HER2 gene amplification	**Total**	
	Number	Percentage (%)
Negative	33	63.5
Equivocal	0	0
Amplified	19	36.5
Total	52	100%

Table 4.9: HER2 gene amplification by DISH

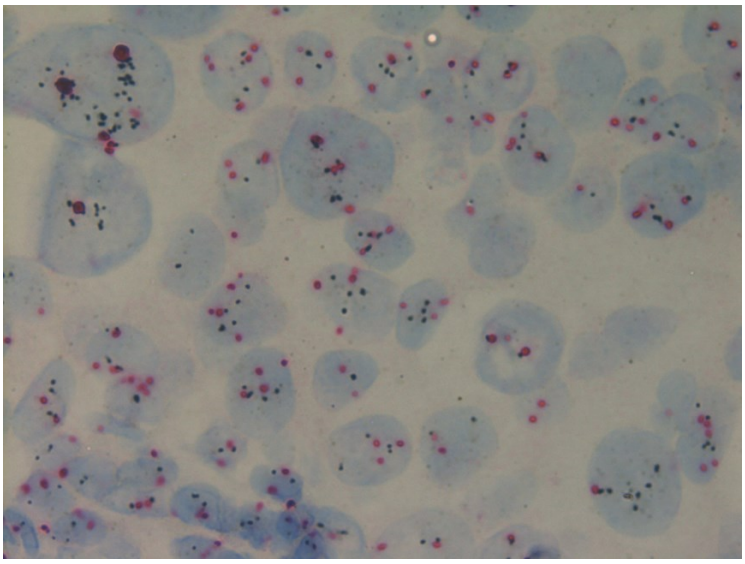


Figure 4.7: Code 6497. HER2 gene amplification by DISH

Comparing the results of DISH to that of IHC assay, we have found the concordance between two techniques:

IHC Score	Dual-ISH		Concordance rate (%)
	Amplified	Not Amplified	
0	0	26	100
1+	0	1	100
2+	2	6	25
3+	17	0	100

Table 4.10: Concordance between IHC and DISH

In this study, all of the samples which had negative HER2 protein overexpression in IHC (score 0 and 1+) were also not amplified of HER2 gene in DISH. The concordance rate between IHC and DISH was 100% regardless of the positivity of HER2 status. Table 4.10 showed that all cases of HER2-positive in IHC had an amplification of gene in DISH. However, for 8 equivocal cases in IHC, we had 2 HER2 gene amplified cases and 6 cases without amplification.

The concordance rate between IHC and DISH assay were 100% and 25% with cases of IHC positive or negative and equivocal, respectively.

5. DISCUSSION

5.1. Clinicopathological characteristic of specimens

The results from our study have shown that the median age at diagnosis of breast cancer was 52.5. The youngest patient was 29 years old and the oldest patient was 91 years old. The median age in our results is similar to that in other reports of many Asian countries, such as China and Japan (around 53.9) [97], Korea (around 51) [98] but is lower than that of Western countries (Sweden, Italia), namely 63 years old [97], [99]. In a study conducted by Onitilo et al. in the USA, the mean age is higher, at 62.7 years [100]. The 50- to 70-year–old group of our data accounted for the highest proportion, at 55.7%. Another study in Italy, 36% of patients diagnosed with breast cancer were in the group of age between 50 to 69 years old. The patients with over 70 years old occupied 21% [101].

The histological type of this study is shown to be similar to that of many other studies with 93.2% invasive ductal carcinoma [84], [97], [100]. Tumour size from 2 to 5 cm is the most prevalent, which is different from studies of Onitilo (2009) and Colleoni (2011). Their studies reported that the tumours less than 2cm in size occupied the highest proportion [100], [102]. Most of the tumours in our samples were in grade II and grade III, collectively accounting for 89.8% while the early grade (grade I) was just a minority 10.2%. This is untrue for the study of Onitilo (from USA) as well as the study of Colleoni (from Italy), which showed a higher percentage of grade I (about 23%) compared to our study [100], [102].

These dissimilarities between our study and others may occur as a result of the differences in races, lifestyles, living environments and age at menarche, menopause status, number of previous pregnancies [103], [104] or expression of late diagnosis.

Our study have shown that the stage II and stage III were the majority with a proportion of 82.9 % whereas stage I occupied a lower percentage. This data is similar to study of Vu Hong, which was also carried out in Vietnamese women with 70% for stage II and 19% for stage III [105]. However, those figures followed an

opposite pattern compared to a study of Leong et al., from Sweden, with 56% of the invasive cancers belonging to stage I, 37% for stage II, 5 % for stage III, and 2% for stage IV [97]. The study of Onitilo and colleagues, from USA, also showed that the most common breast cancer was classified as stage I (56.4%) while stage II and III occupied 36% and 7.7%, respectively [100].

Similar to staging, most of our samples showed nodal metastasis, at 54.5%, which is higher than in some studies of foreign authors such as Carey (USA) at 39%, Spitale (Switzerland), at 37.1% and Onitilo (USA), at 33.5% [5], [79], [100]. In our study, statistically significant differences were found between the size of the tumour and histological grade as well as the tumour size and metastasis with $p<0.05$. The rate of grade III was approximately 68.8% of tumours more than 5cm in size compared to 20.8% of tumours less than 2cm in size (chart 4.1). In addition, most of cases with a large tumour in size (≥5cm) had metastasis (chart 4.2).

The differences about clinicopathological characteristic between our result and other studies may be due to the varying characteristics among populations and the late-stage diagnosis. It is mainly because breast cancer screening programs have not widely applied in general population. Information about screening processes and the integration of screening services into other areas of the health system are not sufficiently provided. Therefore, many breast carcinoma patients present to hospital with large tumour size, late stage of disease, high grade and metastasis.

5.2. HER2 protein overexpression and HER2 gene amplification

Immunohistochemistry and fluorescence in situ hybridization have been applied into HER2 testing. Although both methods of IHC and FISH have been approved by FDA, FISH assay proves to have higher sensitivity and specificity. However, the cost of FISH technique is much higher, so FISH should be used only in case of HER2 (2+) in IHC and when trastuzumab monotherapy is indicated. Until now, FISH technique is generally known as the gold standard.

We performed IHC to detect HER2 protein overexpression by using PATHWAY anti-HER2/*neu* (4B5) Rabbit Monoclonal Primary Antibody, on Ventana Benchmark Ultra automatic staining system at the Institute of Pathological

Anatomy of Sassari. The results of the HER2 receptor status assessment using the IHC method are shown in table 4.2. We had 30.7% for score (3+) and 9.1% for score (2+). In case of HER2 (2+) in IHC, we performed the FISH technique to identify the gene amplification. We had 25% of cases with amplification and 75% of cases no amplification by FISH. Our result showed that, the prevalence rate of HER2 positive was 32.9% in total (table 4.4).

Regarding the results of HER2 gene amplification, when making a comparison between our study and other two previous studies on Vietnamese women, one of which was conducted in Vietnam and the remaining was in Sweden; it is seen that there is a similarity with a high incidence of HER2 positive [39], [40].

The comparison between HER2 gene amplification results in this study and other studies are shown in chart 5.1

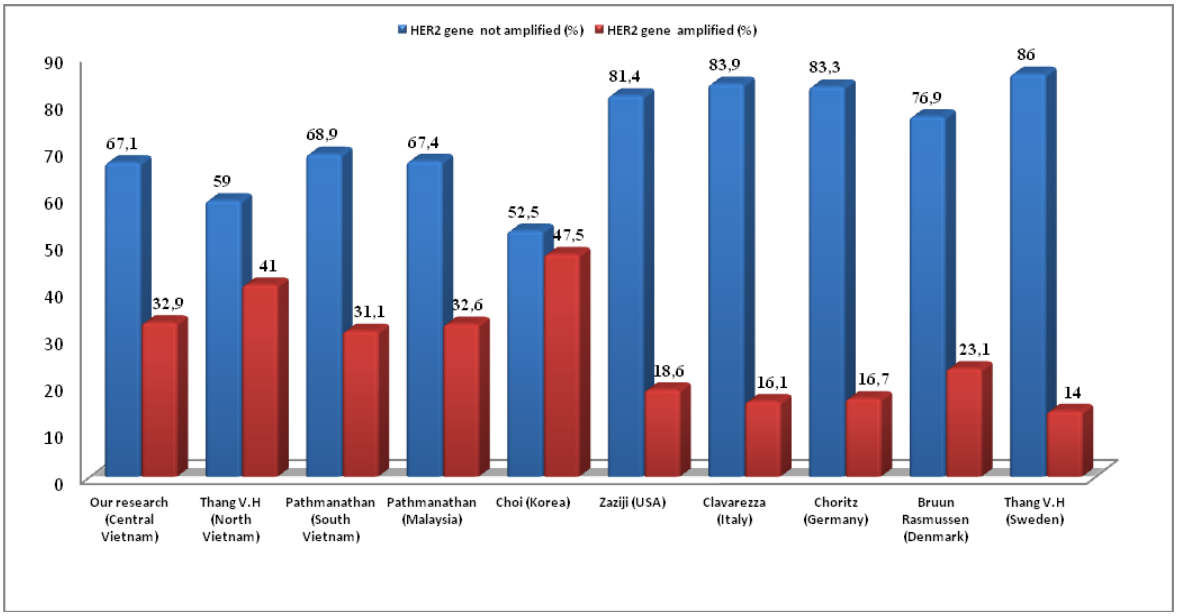


Chart 5.1: Comparison the rate of HER2 gene amplification

According ASCO-CAP 2013, the incidence rates of HER2-positive breast cancers account for approximately 15% -20% of total in Western countries [37]. Interestingly, the result of our study was different from the previous one conducted on Western women. The rate of HER2-positive breast carcinoma diagnosed in Vietnamese women is generally higher than in published studies on women from Western countries, such as study of Clavarezza in Italy [106], Bruun Rasmussen in Denmark [43], Choritz in Germany [107], and Thang in Sweden

[40]. Another study of Yaziji and co-workers performed in USA demonstrated a much lower rate of HER2 positive than my result did in Vietnam [42]. However, many studies in Asian women observed a similar rate of HER2-positive to our data [39]. Our study is also similar to the study of Pathmanathan which was conducted in Southern Vietnam [39]. Especially, the study of Thang which assessed HER2 status in Northern Vietnamese women and whose techniques was performed in Sweden showed that the rate of HER2 positive is 41%, a little bit higher than in our research [40].

Although the results of studies are inconsistent between authors, many recent studies have shown that the women population from Asian countries, including Vietnam have a substantially higher incidence of HER2-positive breast cancers compared to Western ones [39], [108]. These differences may be due to the variations in population characteristics such as biological tumours, ethnicity, geography and environment [39], [40], [109]. However, according to ASCO-CAP 2013, the differences in the rate of HER2 receptor expression are mainly attributed to the distinctive biology of the breast cancer patient population [37].

5.3. The classification molecular subtypes breast carcinoma

5.3.1. Biomarkers status

Hormone receptor and proliferation index were analysed by immunohistochemistry (IHC) using an automated slide stainer (Bench Mark Ultra, Ventana). Of 88 samples, we have 55.7% positive for ER, 40.9% positive for PR and 81.8% for Ki67≥14% (table 4.5). Our result corresponds to Thang's research which was conducted in Sweden and whose sample was from Northern Vietnamese women [110]. Another study conducted in China, Sun et al. showed that the ER-, PR- and HER2-positive rates in IHC were 60% (758/1,259), 51% (642/1,259) and 35% (439/1,259), respectively [68]. In a study from Brasil, De Macêdo Andrade and colleagues reported a positive proportion of 66.5% and 56.5% for ER and PR, respectively [70]. Park et al. found that there is 72.4% for ER-positive and 62.6% for PR-positive and 77.7% for Ki67≥14% [69]. In 2009, the study of Onitilo et co-workers conducted in Wisconsin (USA) showed that the positive ER, PR were 77.9% and 59.1 %, respectively [100]. Also, hormonal receptors were positive in

83.5% of the study population in Italy, compared to the expectation of approximately 75% [111] and 77% ER-positive [112]. Similar to studies of Onitilo and Clavarezza [111], the result of Nadji demonstrated the rate of positive ER and PR are much higher than those in our study [21]. However, many studies have shown that the ER-positive in breast cancer is very different between various ethnicities [113], [114]. In addition, the different biological features can be due to various races and regions. There are many reports about the estrogen receptor of Vietnamese patients in breast cancer which were similar to that of Asian patients and were lower than that in some previous studies for Caucasian patients [110], [115].

Hormonal receptor status of primary breast cancer is a predicting treatment response to postoperative adjuvant systemic endocrine therapy. Therefore, it is very important to determine positive ER patients who have good prognoses and will receive most benefits from endocrine therapy.

With regards to the proliferation index, we used 14% as an optimal cut off point to divide Ki67 into 2 groups [73]. In this study, the Ki67≥ 14% group occupied 81.8% and the figure for Ki67< 14% was 18.2%. Ki67 is an important biomarker which has been applied into routine clinical pathological practice. This is a prognosis factor which predicts response or resistance to chemotherapy and endocrine therapy. The 2013 Saint Gallen Conference indicated that a Ki67 level of ≥14% could distinguish between luminal A and luminal B-HER2 negative tumours in breast carcinoma molecular subtyping [37].

5.3.2. Molecular Subtype Breast cancer

According the Saint Gallen Conference 2013, basing on the expression levels of ER, PR, HER2 and the Ki67 proliferation index, the breast carcinoma was divided into five subtypes as follows:

Molecular Subtypes	ER and/or PgR	HER2	Ki67 Expression
Luminal A	Positive	Negative	<14%
Luminal B-HER2 negative	Positive	Negative	≥14%
Luminal B-HER2 positive	Positive	Positive	Any
HER2 overexpression	Negative	Positive	Any
Triple negative	Negative	Negative	Any

In our research, we have determined the molecular subtypes basing on this classification. We found that the luminal A, luminal B-HER2 negative, luminal B-HER2 positive, HER2 enriched and triple negative breast cancer were present in 16 (at 18.2%), in 24 (at 27.3%), in 9 (at 10.2%), in 20 (at 22.7%) and in 19 cases (at 21.6%) patients, respectively (Chart 4.4). The luminal B was the most common, occupied 38% of all samples.

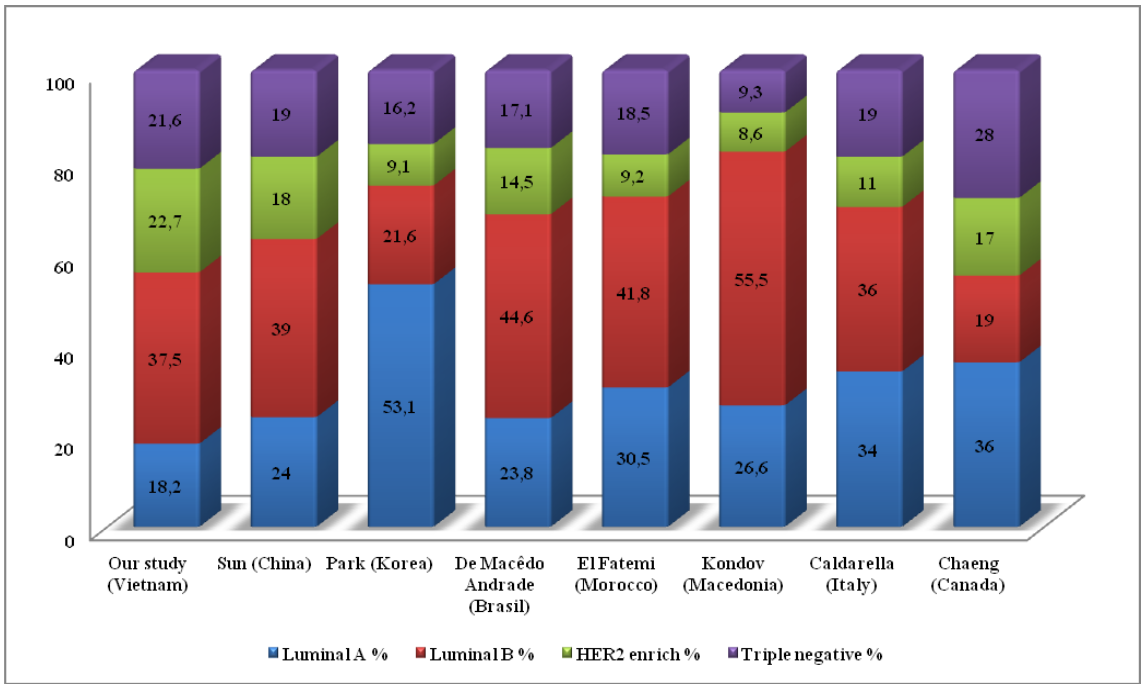


Chart 5.2: Comparison the distribution of molecular subtypes

Our result is similar to studies of Sun and co-workers which was conducted on Chinese patients in 2015 [68]. El. Fatemi and colleagues also had the same result studied on North African women [71]. In another analysis about molecular breast cancer subtypes in North-Eastern Brazil, De Macêdo Andrade et al. reported that the luminal B subtype was more prevalent (44.6%), followed by luminal A (23.8%). Their result is also corresponded to our analysis [70]. However, several studies about molecular subtypes from different countries have shown different results [9], [69], [72], [73]. The study of Wang and co-workers, in 2016 also showed the distribution of subtypes is not absolutely similar to our result [116]. The luminal A in our study is lower than those in many previous studies from Western countries and Canada [9], [69], [73]. In literature, the luminal A is found in 50-72% of breast cancer patients. This subtype has the best

prognosis with low proliferative index, good differentiation and lowest risk of local recurrence and relapse [85], [117], [118]. However, there are different results reported in literature regarding this subtype with 34% of Italy, 3.9% of Saudi Arabia, 71% of Japan [9], [119], [120].

Nowadays, luminal B subtype is divided into 2 groups, included luminal B with positive estrogen receptor, negative HER2 receptor, higher Ki-67 value of over 14% and luminal B with HER2 positive, estrogen negative and progesterone negative. Both types of luminal B have worse prognosis than luminal A subtype. Many previous studies showed that luminal B subtype is responsible for 10-20% of breast cancer patients [121], [122], [123]. In our data, luminal B is found in 37.5%, being higher than previous studies but similar to studies on Southern China and on North African women [71], [124]. The increase in the prevalence of luminal B might be as a result of the high number of HER2-positive cases in this study (Chart 4.4). In addition, the chart 4.3 showed that the majority of our breast cancer patients are detected in late stage of disease with a large tumour which has a positive relationship with proliferation index ($p<0.001$). The vast majority of the large tumours in size express a high level of Ki67 (≥14%). Therefore, the application of the Ki67 index ≥14% as a cut-off point to distinguish between luminal A and luminal B molecular subtypes could increase the rate of luminal B breast cancer and could be expression of the bigger size of the tumour when diagnosed and expression of late diagnosis.

In our study, we have accounted 22.7% and 21.6% in HER2 enriched subtype and triple negative breast cancer, respectively. Both subtypes are very aggressive and have the worst prognostic. These subtypes are always associated with a reduction in survival rates. The proportion of HER2 enriched subtype in our data is much higher than that in Italy (11%), in USA (7.4%) and in France (4.3%) [9], [80], [100]. Our figure for HER2 enriched subtype is also slightly higher than that in China (18%) and in South Asia (17.8%) [68], [85].

The prevalence of triple negative in our study (21.6%) is similar to another studies in Korean (19.9%), in Morocco (23.4%), in Algeria (20.8%) and

in black population of the USA (20%) [71], [125], [126]. However, our figure for triple negative is higher than that in white population of the USA (9%) in studies of Clarke and Howlader [126], [127]. Our result is also higher than studies of Kondov (9.3%), Cortet (10.7%), Park (16.2%) and Caldarella (19%) which conducted in France, Macedonia, Korea and Italy, respectively [9], [69], [72], [80]. The triple negative has a shorter survival time and a higher recurrence rate compared to other subtypes.

We observed the difference in prevalence among our subtypes and those reported in the previous studies because breast cancer is known as a heterogeneous disease. Therefore, the differences in data may explain the disparities in biological features between tumours of patients in many countries. In addition, the geography and the ethnicity and environment also play an important role in different distribution of molecular subtypes. Even in large countries, the regions having different races will have a different distribution of molecular subtypes [127], [128]. The HER2-positive tumours are dominant in Asian countries while triple negative is frequent in African and black USA population. Our figure for triple negative is slightly higher in comparison with that for some Asian countries. The luminal A and luminal B are more common in Western countries and white USA population [9], [127].

Our data have shown that approximately a half of cases in the sample are aggressive tumours with bad prognoses.

5.4. Relationship between HER2 status and prognostic factors

We have found out the statistically significant difference between the HER2 status and histologic grade ($p < 0.001$), between the HER2 status and proliferation index ($p < 0.005$). Most of the cases in positive HER2 group have a high histologic grade and high Ki67. A lot of studies have a similar results to our study [39], [40], [43, 53], [55], [129], [130]. In addition, our data showed that there were also significantly negative correlation between the HER2 status and the estrogen receptor with $p < 0.001$ (Table 4.7). The ER-negative is more frequent in positive

HER2 group. Many previous studies reported the correlation between HER2 status and the other prognosis [40], [52], [56], [131].

In our study, we have found out the difference between HER2 status and size of tumour (p=0.061), between HER2 status and stage at disease (p=0.057), as well as lymph node status (p=0.08). However, those differences are not statistically significant. Similar to our results, in the study conducted in Chicago (Illinois, USA), Ariga and co-workers showed that there was no correlation between the size of the tumour and the age of patients with HER2 positive [53].

5.5. Relationship between molecular subtypes and prognostic factors

Our data showed that there are significant differences between molecular subtype and histologic type, histologic grade and proliferation index with $p < 0.001$ (table 4.8). The frequent histopathological grade in luminal A was grade II (62.5%) while most of the HER2 enriched cases had higher grade (grade III). Besides, the study showed a significant difference between subtype and stage at disease with $p<0.05$. The vast majority of the luminal A subtype were in early stage of disease. In addition, the chart 4.6 also showed that 50% of tumours less than 2cm in size were luminal A subtype and the large tumours (≥5cm) just occupied 6.2% of this subtype. Therefore, the characteristics of luminal A in our study were absolutely similar to that in previous studies, with small tumour size, low grade, low Ki67 and less metastasis [132], [133]. The luminal A subtype has the best prognosis, in contrast, luminal B-HER2 positive, triple negative and HER2 enrich subtype have the worst prognosis. Even HER2 enriched tumours are more aggressive and have poorer prognosis than triple negative [121], [134]. However, the specific therapy has changed a lot in recent years. The target therapy has considerable improved the prognosis of this subtype. Besides the HER2 subtype, we accounted for a high proportion of triple negative breast cancer (Chart 4.4). The table 4.8 showed that the majority of our TNBC tumours were invasive ductal carcinoma with high histological grade and high Ki-67 expression. Many authors have demonstrated the similar results to our data [9], [83], [100], [135].

The HER2 positive subtype was more higher in Vietnamese population than Western populations [28]. Therefore, the development of standardized HER2 assay is very necessary to identify HER2 gene amplification and using the anti-HER2 therapy.

The distribution of molecular subtypes by age at diagnosis is presented by chart 4.5. While luminal A subtype was the most common in women aged 55-60 years, most cases of luminal B-HER2 negative and HER2 enrich are detected in the earlier age group (50-55). Those results correspond with many studies which showed that the luminal A occurs in older women than others [72], [85], [123], [136], [137]. In particular, in our data, the triple negative subtypes are found in all three profiles by age: young adults (35-40), middle-aged (50-55) and older adults (aged over 70). This distribution is not similar among the studies [123], [138], [139]. This might due to triple negative breast cancer is a heterogeneous disease. The TNBC has been shown to be represented according to gene expression profiles by different entities. Some of them are able to identify four subtypes and others suggest six different groups [67], [123], [140].

The luminal B-HER2 positive in our result was extremely prevalent in very young women aged between 35-40 like triple subtypes and in middle-aged women (50-55) like other subtypes. The study of Li and co-workers conducted in China showed that the luminal B-HER2 positive was common at age <50 [141]. However, in another study with population-based study from SEER program, Wu et al. reported that the luminal B-HER2 enrich occurs at the period from 50 to 64 years old [142]. The different results of studies show the diversity of biological tumour cells.

The incidence of breast cancer in Vietnam is lower than United States and European countries. However, the majority of Vietnamese patients are diagnosed at late stage of disease. Therefore, the treatments are more difficult. With the hope to detect the breast cancer in earlier stage, we suggest a screening program should be done in a more young age as usually done in Western countries, particularly in woman with familiar history of breast cancer.

5.6. Concordance between IHC and DISH

The results shown in table 4.10 have demonstrated the high concordance between DISH and IHC in evaluating the HER2 gene status in invasive breast cancer. The concordances between IHC and DISH in testing HER2 status were 100% in case of IHC positive or negative. DISH assay is a new technique which is fully automatic and whose results could be interpreted by light microscopy. Many studies showed that there is a high concordance between DISH and other techniques in assessing the HER2 gene amplification [143]. In 2015, Chivukula and colleagues observed a concordance rate of 98% between FISH and DISH [144]. A similar to Chivukula's result was obtained in the study of Horii and co-workers, which also reported the same concordance of 96.2% [145]. In another experiment, Papouchado et al. showed that there is a concordance between FISH and SISH which is similar to DISH technique with a result of 98.9% [146].

In our data, in case of HER2 equivocal in IHC, we found that the concordance between IHC and DISH was 25%. The rate of gene amplification of HER2 equivocal cases in IHC varied among studies because the overexpression of HER2 protein may occur both in the presence or absence of gene amplification [147]. Many studies had a similar result to our data. Owens and colleagues, in their study, found that 23.3% HER2 gene amplification in FISH seen in IHC (2+) cases [148] while in another study, SISH assay was applied in case of HER2 equivocal in IHC, Musa et al. reported that the concordance between SISH and IHC is 36.5% [87]. Until now, FISH technique always remains a "gold standard" to identify the HER2 gene amplification. Many laboratories prefer to use FISH assay to diagnose. However, FISH methodology requires a longer time for staining, specialized training and fluorescence microscopy analysis. DISH technique has more advantages, being recommended as a new assay for evaluating HER2 status. However, according to many studies, the concordance between FISH and DISH technique is not completely the same. Therefore, in some particular cases, FISH technique should be applied in order to have the most precise assessment. It is also a very important tool to decide the targeted therapy.

6. CONCLUSION

In summary, we assessed the HER2 status and the molecular subtypes in 88 samples from surgically removed breast cancer in Central Vietnam.

- The rates of HER2 protein overexpression were 30.7% for immunohistochemistry (IHC) 3+ score and 9.1% for IHC 2+ score.
- Gene amplification by fluorescence in situ hybridization (FISH) was found in 25% of tumours with an equivocal score of immunohistochemistry.
- The prevalence rate of HER2 positive was 32.9%.
- The concordance between immunohistochemistry and dual in situ hybridization (DISH) in testing HER2 status were 100% in case of IHC positive or negative and 25% in case of IHC equivocal.
- The concordance between FISH and DISH in case of equivocal IHC was 100%
- The luminal B subtype was the most frequent (37.5%) while the uncommon belonged to luminal A, at 18.2%. The HER2 enriched and triple negative subtype occupied 22.7% and 21.6%, respectively.
- A significant correlation was found between HER2 status and histological grade, estrogen receptor (ER) status and Ki67 proliferation index.
- A significant correlation was found between molecular subtypes and histological stage, type, grade and Ki67.

We suggest that further investigations should be done with more samples (all Vietnamese breast cancer patients, possible within an organized national cancer registry) as well as further studies for subtypes of triple negative breast cancer.

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