



**UNIVERSITÀ DEGLI STUDI DI SASSARI**  
**SCUOLA DI DOTTORATO IN**  
**SCIENZE BIOMOLECOLARI E BIOTECNOLOGICHE**  
**INDIRIZZO: BIOCHIMICA CLINICA E PROTEOMICA CLINICA.**  
XXVII Ciclo

**IDENTIFICATION OF HER2 PROTEIN OVEREXPRESSION**  
**AND GENE AMPLIFICATION IN GASTRIC CANCER**

**Direttore:**  
**Professor Leonardo A. Sechi**

**Tutor:**  
**Professor Francesco Tanda**  
**Co-Tutor:**  
**Professor Dang Cong Thuan**

**Tesi di dottorato della**  
**Dr. Van Trung Nghia**

## TABLE OF CONTENTS

<b>LIST OF ABBREVIATION.....</b>	<b>2</b>
<b>ABSTRACT .....</b>	<b>3</b>
<b>1. INTRODUCTION.....</b>	<b>4</b>
1.1. GASTRIC CANCER .....	4
1.2. HER2 PROTEIN AND GENE.....	5
1.3. HER2 PROTEIN OVEREXPRESSION AND HER2 GENE AMPLIFICATION IN GASTRIC CANCER.....	8
1.4. TESTING FOR HER2 AND CONCORDANCE BETWEEN HER2 PROTEIN OVEREXPRESSION AND GENE AMPLIFICATION IN GASTRIC CANCER.....	10
1.5. TARGETING HER2 THERAPY: TRASTUZUMAB .....	12
<b>2. AIM OF THE STUDY .....</b>	<b>15</b>
<b>3. MATERIALS AND METHODS .....</b>	<b>16</b>
3.1. STUDY SITES .....	16
3.2. SAMPLE COLLECTION .....	16
3.3. SLIDE PREPARATION .....	16
3.4. SECTIONING AND HEMATOXYLIN AND EOSIN (H&E) STAINING .....	17
3.5. IMMUNOHISTOCHEMICAL STAINING FOR HER2 PROTEIN.....	18
3.6. IMMUNOHISTOCHEMICAL STAINING FOR CD20, CD56, CHROMOGRANIN A, SYNAPTOPHYSIN .....	21
3.7. FLUORESCENCE IN SITU HYBRIDIZATION FOR HER2 GENE.....	21
3.8. STUDY DESIGN.....	25
<b>4. RESULTS AND DISCUSSION .....</b>	<b>27</b>
4.1. CHARACTERISTICS OF SPECIMENS.....	27
4.2. CHARACTERISTICS OF ADENOCARCINOMA SAMPLES .....	29
4.3. HER2 PROTEIN OVEREXPRESSION.....	32
4.4. HER2 GENE AMPLIFICATION .....	34
4.5. CONCORDANCE BETWEEN IHC AND FISH RESULTS .....	36
<b>5. CONCLUSION.....</b>	<b>38</b>
<b>6. BIBLIOGRAPHY .....</b>	<b>39</b>

**LIST OF ABBREVIATION**

ADCC	Antibody dependent cell mediated cytotoxicity
CISH	Colorimetric in situ hybridization
EGFR	Epidermal growth factor receptor
FISH	Fluorescence in situ hybridization
GEJ	Gastroesophageal junction
H&E	Hematoxylin and Eosin
HER2	Human epidermal growth factor receptor 2
IHC	Immunohistochemistry
NCCN	National Comprehensive Cancer Network
SISH	Silver In Situ Hybridization
ToGA	Trastuzumab for Gastric Cancer

## ABSTRACT

Gastric cancer is a leading cause of cancer related mortality, and current treatment outcomes for advanced disease remain poor. HER2 protein overexpression and gene amplification are significant biomarkers for identifying gastric cancer patients who possibly respond to HER2-targeted therapy using trastuzumab.

The aim of this study was to identify prevalence rate of HER2 gene amplification/overexpression in a local population, and determine the concordance rate between IHC and FISH techniques.

Formalin-fixed, paraffin-embedded sections of 43 adenocarcinoma specimens from 61 cases of gastric cancer patients were analyzed. Protein expression was assessed using immunohistochemistry and gene amplification was evaluated by fluorescence in situ hybridization.

11.7% (5/43) and 7,5% (3/40) of gastric adenocarcinomas displayed HER2 protein overexpression (score 3+/2+) and gene amplification, respectively. The concordance between IHC and FISH is high.

## 1. INTRODUCTION

### 1.1. Gastric cancer

Gastric cancer has been described as early as 3000 BC in hieroglyphic inscriptions and papyri manuscripts from ancient Egypt. It is the fourth most common malignancy and one of the leading causes of cancer mortality worldwide [1]. Approximately 21,600 patients are diagnosed annually in the United States, of whom 10,990 are expected to die [2]. In Vietnam, gastric cancer is the fourth most common malignancy and the third leading cause of cancer death in both sexes [3]. The incidence of gastric cancer varies with different geographic regions. Rates are highest in Eastern Asia, Eastern Europe, and South America, while the lowest rates are in North America and parts of Africa. Over 70 percent of gastric cancers occur in developing countries. Gastric cancer is more common in men than in women, in both developed and developing countries [4].

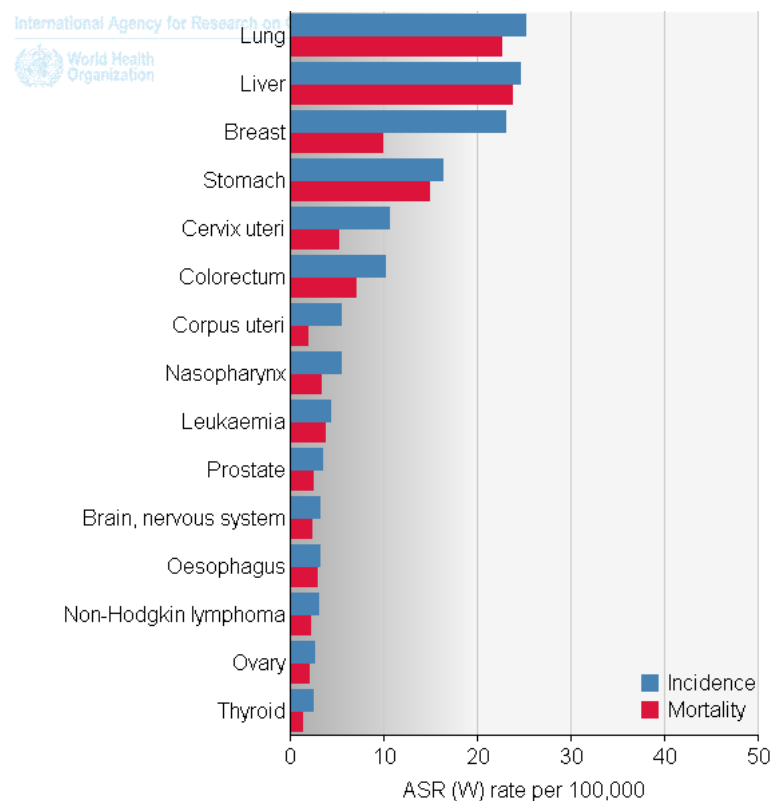


Figure 1.1: *Globocan 2012, Population fact sheets: Vietnam. Estimated age-standardised incidence and mortality rates: both sexes [3]*

Surgical resection is the cornerstone of treatment and can cure patients with early-stage cancer. The survival rate of patients with advanced resectable gastric or gastroesophageal junction (GEJ) cancers, however, remains poor despite new treatment strategies, such as perioperative chemotherapy or adjuvant chemoradiation [5]. In Western countries as well as Vietnam, most gastric cancer patients are diagnosed when the tumor is at an unresectable stage. For these patients, systemic chemotherapy is the main treatment option because it prolongs survival without quality of life compromise. Many single agents and combinations are used in the treatment of metastatic disease. Objective response rates change from 10% to 30% for single-agent therapy and 30% to 60% for combination regimens. Platinum compounds, fluoropyrimidines, anthracyclines, and, recently, taxanes, irinotecan and vinorelbine are the most active drugs. Despite a large number of randomized trials, there is no consensus as to the best agent or regimen. In general, combination chemotherapy regimens provide higher response rates than do single agents, but this translates into only modestly longer durations of disease control and survival that are measured in weeks to a few months [6].

Survival of patients with advanced gastric cancer treated with palliative chemotherapy remains low. New therapies are urgently needed. A better understanding of the molecular basis of cancer has contributed to the development of rationally designed molecular targeted therapies, which interfere with the signalling cascades involved in cell differentiation, proliferation, and survival. Several molecular targeting agents for patients at an unresectable stage are now under evaluation in international randomized studies [7], and trastuzumab, an anti-HER2 monoclonal antibody, has been shown to exhibit antitumor activity against HER2-positive gastric cancers [8]

## **1.2. HER2 protein and gene**

The human epidermal growth factor receptor family of receptors plays a central role in the pathogenesis and treatment of several human cancers. They regulate cell growth, survival, and differentiation by way of multiple signal transduction pathways and play a role in cellular proliferation and differentiation. This family is

composed of four members: HER1 (EGFR), HER2, HER3 (ErbB-3), and HER4 (ErbB-4). All four HER receptors comprise a cysteine-rich extracellular ligand binding site, a transmembrane lipophilic segment, and an intracellular domain with tyrosine kinase catalytic activity. Epidermal growth factor receptor (EGFR, ErbB1, and HER1) was discovered by Carpenter et al at Vanderbilt University, USA, in 1978 [9]. ErbB stands for its origin in the Erb-b gene responsible for avian erythroblastosis virus. The neu oncogene (also known as HER2, ErbB2, or p185) was discovered by a group of scientists at Massachusetts Institute of Technology, Rockefeller, and Harvard University [10]. These 4 receptors share the same molecular structure with an extracellular ligand-binding domain, a short transmembrane domain, and an intracellular domain with TK activity (excepting the HER3). The binding of different ligands to the extracellular domain initiates a signal transduction cascade that can influence many aspects of tumor cell biology, including cell proliferation, apoptosis, adhesion, migration, and differentiation. Ligand binding induces EGFR homodimerization as well as heterodimerization with other types of HER proteins. HER2 does not bind to any known ligand, but it is the preferred heterodimerization partner for other members of the HER family. The HER2 receptor is a 1255 amino acid, 185 kD transmembrane glycoprotein located at the long arm of human chromosome 17 (17q12) [11]. HER2 is expressed in many tissues including the breast, gastrointestinal tract, kidney, heart and its major role in these tissues is to facilitate excessive/uncontrolled cell growth and tumorigenesis [12].

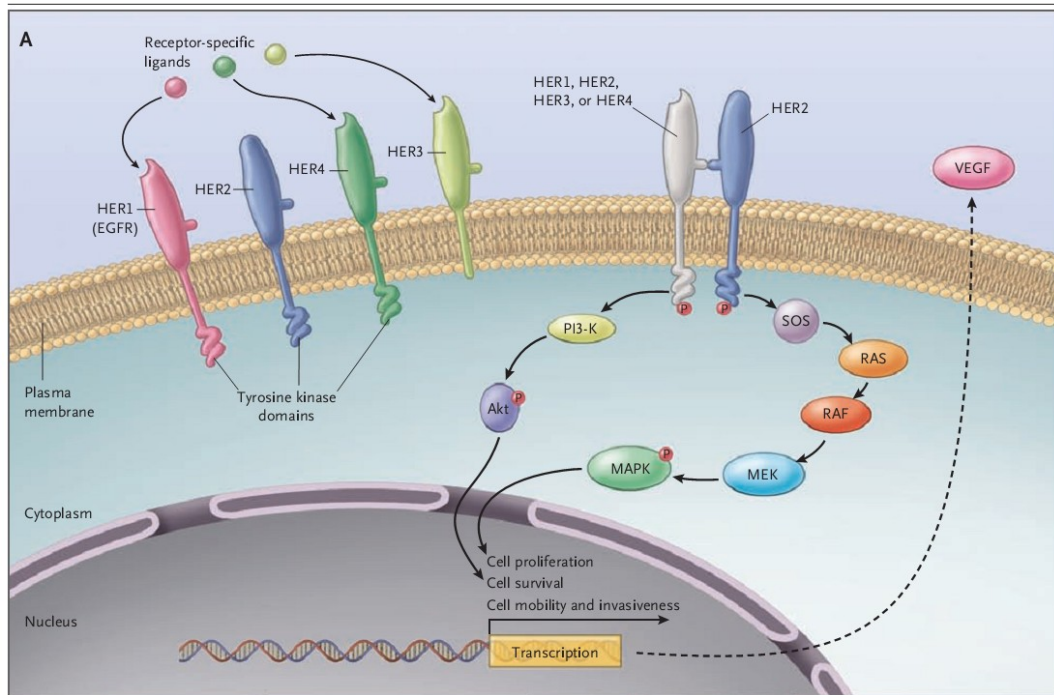


Figure 1.2: Signal Transduction by the HER Family

As shown in Panel A, the four members of the HER family are HER1, HER2, HER3, and HER4. There are receptor-specific ligands for HER1, HER3, and HER4. An intracellular tyrosine kinase domain exists for HER1, HER2, and HER4. Phosphorylation of the tyrosine kinase domain by means of homodimerization or heterodimerization induces both cell proliferation and survival signaling. HER2 is the preferred dimerization partner for the other HER family members. The phosphorylated (activated) tyrosine residues on the intracellular domain of HER2 activate the lipid kinase phosphoinositide 3-kinase (PI3-K), which phosphorylates a phosphatidylinositol that in turn binds and phosphorylates the enzyme Akt transforming factor (Akt), driving cell survival. In parallel, a guanine nucleotide exchange factor, the mammalian homologue of the son of sevenless (SOS), activates the rat sarcoma (RAS) enzyme that, in turn, activates receptor activation factor (RAF) and then the mitogen-activated protein kinase (MAPK) and mitogen extracellular signal kinase (MEK). MEK phosphorylates, among others, the MAPK, driving cellular proliferation. One of many other downstream effects is the production of vascular endothelial growth factor (VEGF) supporting angiogenesis[13]

In carcinomas, HER2 acts as an oncogene, mainly because high-level amplification of the gene induces protein overexpression in the cellular membrane and subsequent acquisition of advantageous properties for a malignant cell [14].



### **1.3. HER2 protein overexpression and HER2 gene amplification in gastric cancer**

Overexpression of HER2 in breast cancer, resulting in HER2-positive subtypes, is associated with very poor prognosis compared with HER2-negative breast cancer [15]. HER2-positive breast cancer is also associated with increased risk of local growth and distant metastasis. Many studies, including several conducted in Japan, have reported that HER2 is also present in other cancers, particularly in gastric cancer [16, 17]. Recent studies on prevalence of HER2 positivity in patients with gastric cancer revealed the frequency of HER2-positive gastric cancer ranging from 6.0 to 36.6 % [18-22] (Table 1.1). Many studies, which determined HER2 overexpression by IHC using monoclonal antibody (HercepTest) and/or gene amplification by FISH, have observed similar rates. In a Japanese series, Yano et al. reported that HER2 overexpression by IHC was found in 23% and gene amplification by FISH in 27% of 200 resected tumors [18]. Bang and coworkers reported that differences in HER2 expression were mainly attributed to the site of the primary tumor (gastric or gastroesophageal junction) and histological type (diffuse or intestinal) [8]. Gravalos and Jimeno in their study of 166 gastric cancer patients observed that HER2 overexpression was most commonly found in gastroesophageal junction (GEJ) tumors and tumors having intestinal type histology [23]. Other studies also confirmed a higher rate of HER2 positivity in GEJ tumors and intestinal subtype [8, 18]. The role of HER2 as a prognostic factor in gastric cancer has been controversial because some of the initial studies failed to find an association with prognosis [24]. Unlike in breast cancer, the studies in gastric cancer to date have yielded inconsistent findings regarding the prognostic relevance of HER2. Some showed that HER2 positivity was associated with a significantly worse prognosis [19, 25, 26], whereas others found no association between HER2 status and prognosis [27, 28]. In a study of 260 gastric cancers, HER2 overexpression was an independent negative prognostic factor and HER2 staining intensity was correlated with tumor size, serosal invasion, and lymph node metastases [29]. In a retrospective study of 108 cases, HER2 overexpression was associated with a poorer 10-year survival [30]. In another study, a significantly

poorer prognosis was observed in HER2-positive early gastric cancer in a series of 226 patients [31]. Nakajima et al. considered that in early-stage tumors, HER2 overexpression is the second poorest prognostic variable after nodal status [32]. In a Korean study, HER2 overexpression and gene amplification with semiquantitative standardized IHC staining, CISH and FISH were determined in 182 gastric cancer patients who underwent curative surgery [33]. Sixteen percent expressed HER2 protein by IHC and HER2 gene amplification was detected in seven patients by CISH and FISH. Intestinal-type cancers exhibited higher rates of HER2 amplification than did diffuse-type cancers ( $P < 0.05$ ). Tumors with HER2 amplification were associated with poor mean survival rates (922 vs 3243 days) and 5-year survival rates (21% vs 63%;  $P < 0.05$ ). Age, TNM stage, and amplification of HER2 were found to be independently related to survival by multivariate analysis.

Table 1.1: *Prevalence of HER2 positivity in patients with gastric cancer*

Study	Country	n	Determination of HER2 status	HER2- positive (%)
Takehana et al. [34]	Japan	352	IHC2+/IHC3+	8,2
Tanner et al. [21]	Finland	231	CISH+	36,6
Yano et al. [18]	Japan	200	IHC2+/IHC3+ FISH	23 27,1
Kim et al. [22]	Korea	248	IHC3+ or IHC2+/FISH+	6
Yan et al. [35]	Singapore	128	FISH+ IHC3+	11,7 9,4
Yan et al. [36]	China	145	IHC3+ or IHC2+/FISH+	10,3
Liu et al. [37]	China	775	IHC3+ or IHC2+/FISH+	12,1
Tsapralis et al. [38]	Greece	120	IHC2+/IHC3+	16,6
Terashima et al. [28]	Japan	829	IHC3+ or IHC2+/DISH+	9
Halon et al. [39]	Poland	78	IHC2+/IHC3+	29,5

#### **1.4. Testing for HER2 and concordance between HER2 protein overexpression and gene amplification in gastric cancer**

Although several methods for HER2 testing have been developed, approximately 20% of current HER2 testing may be inaccurate. Therefore, the American Society of Clinical Oncology (ASCO) and the College of American Pathologists (CAP) have recommended guidelines in HER2 testing to ensure accuracy [40]. The two methods currently approved for HER2 testing are immunohistochemistry (IHC) and fluorescence in situ hybridization (FISH). In gastric cancers, heterogeneity of the HER2 genotype can lead to discrepancies in the results from IHC and FISH testing [41]. Tumor heterogeneity was seen in roughly 4.8% of samples with moderate or strong HER2 staining and was higher than what was experienced in breast cancer (1.4%) [42]. ASCO/CAP guidelines state that intratumoral heterogeneity may contribute to HER2 testing inaccuracy. Incomplete basolateral membrane HER2 IHC staining is also more common in gastric cancer than in breast cancer. This is due to the higher frequency of glandular formations that occur in gastric tissue. In gastric tissue, the basolateral membrane is stained, not the luminal membrane resulting in the heterogeneity. Currently, there are no ASCO/CAP approved HER2 testing guidelines for gastric cancer. Figure 1.3 shows consensus panel recommendations on HER2/IHC scoring for gastric/esophageal cancer.

	Surgical specimen staining pattern	Biopsy specimen staining pattern	HER2 overexpression assessment
0	No reactivity or membranous reactivity in <10% of tumour cells	No reactivity or no membranous reactivity in any tumour cell	Negative
1+	Faint or barely perceptible membranous reactivity in $\geq$ 10% of tumour cells; cells are reactive only in part of their membrane	Tumour cell cluster with a faint or barely perceptible membranous reactivity irrespective of percentage of tumour cells stained	Negative
2+	Weak to moderate complete, basolateral or lateral membranous reactivity in $\geq$ 10% of tumour cells	Tumour cell cluster with a weak to moderate complete, basolateral or lateral membranous reactivity irrespective of percentage of tumour cells stained	Equivocal
3+	Strong complete, basolateral or lateral membranous reactivity in $\geq$ 10% of tumour cells	Tumour cell cluster with a strong complete, basolateral or lateral membranous reactivity irrespective of percentage of tumour cells stained	Positive

HER2=human epidermal growth factor receptor 2 (also known as ERBB2).

Figure 1.3: *Immunohistochemistry scoring for HER2 in gastric and gastro-oesophageal junction cancer, by type of diagnostic specimen [8].*

The National Comprehensive Cancer Network (NCCN) guidelines panel recommended that less than 3+ overexpression of HER2 by IHC should be additionally examined by FISH or other in situ hybridization methods. Gastric cancers with HER2 IHC overexpression of 3+ or FISH positive are considered positive and thus be treated with trastuzumab. Thus, HER2 3+ or FISH+/HER2 IHC 1+, FISH+/HER2 IHC 2+, FISH +/HER2 IHC 3+ gastric cancer patients should be treated with trastuzumab.

In breast cancer, it is generally thought that HER2 overexpression is mainly (95%) achieved via gene amplification (increased copies of the HER2 gene), thereby resulting in increased transcription of the gene, increased HER2 receptors on the cell membrane (protein overexpression), and increased cell proliferation [43]. For breast cancer, a standardization of the FISH and IHC assessments of HER2 protein overexpression and gene amplification has been introduced and the concordance rates between these two methods are ranged from 73% - 98% [44]. However, the concordance of protein expression and gene amplification of HER2 in gastric cancer have been controversial. Studies published in 1990–1991 did not report a high

concordance between these two methods of assessment. In a series of 40 cases analyzed by Lemoine et al. [45], 26% were found to display elevated protein expression, but only 13% evidenced gene amplification. A similar result was also observed by Kameda et al. [46] who detected overexpression without amplification and considered that this may indicate that gene amplification may not be the primary mechanism by which the HER2 protein is overexpressed in gastric cancer. HER2 overexpression may occur by a number of different mechanisms, including transcriptional activation by other genes or post-transcriptional events [47]. Although some studies obtained concordance between HER2-protein overexpression by IHC and HER2 gene amplification by FISH or chromogenic in situ hybridization (CISH) [18, 33, 48, 49], an IHC scoring system for breast cancer was applied in these researches. Hofmann and coworkers noticed that the discrepancies between IHC and FISH were attributed to basolateral membranous immunoreactivity of glandular cells resulting in incomplete membranous reactivity and a higher rate of tumor heterogeneity in gastric cancer compared with breast cancer [41]. In addition, discrepancies in the results for HER2 testing between surgically resected tumors and matched biopsy specimens may also occur due to intratumor heterogeneity in gastric carcinoma tissue [41, 50]. They suggested a modified IHC scoring system for gastric cancer, which was used to pre-ToGA studies [41]. Recent studies report a high concordance between protein overexpression in IHC and amplification by FISH or CISH [35, 36, 51].

### **1.5. Targeting HER2 therapy: Trastuzumab**

HER2 status has become an important biomarker for identifying patients who could respond to HER2 targeting therapy using the humanized monoclonal antibody trastuzumab. Trastuzumab consists of two antigen-specific sites that bind to the juxtamembrane portion of the extracellular domain of the HER2 receptor and that prevent the activation of its intracellular tyrosine kinase. The remainder of the antibody is human IgG with a conserved Fc portion [13]. Antitumor mechanisms suggested for the therapeutic effect of trastuzumab are: (1) a direct antiproliferative effect by blockade of signalling pathways, down-modulation of the HER2 protein,

and activation of apoptotic signals of the tumor cells, and (2) an indirect antitumor effect by antibody dependent cell mediated cytotoxicity (ADCC) activity [52, 53].

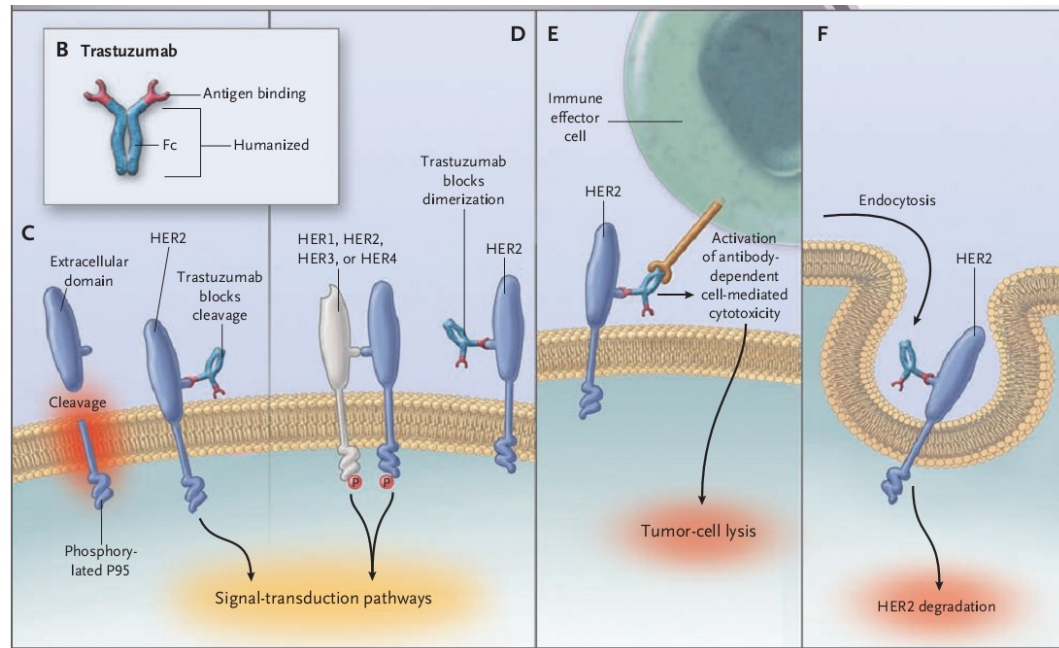


Figure 1.4: *Potential Mechanisms of Action of Trastuzumab.*

*The most well-documented potential mechanisms of action are shown in Panels B through F. Cleavage of the extracellular domain of HER2 leaves a membrane-bound phosphorylated p95, which can activate signal-transduction pathways (Panel B). Binding of trastuzumab to a juxta-membrane domain of HER2 reduces shedding of the extracellular domain, thereby reducing p95 (Panel C). Trastuzumab may reduce HER2 signaling by physically inhibiting either homodimerization, as shown, or heterodimerization (Panel D). Trastuzumab may recruit Fc-competent immune effector cells and the other components of antibody-dependent cell-mediated cytotoxicity, leading to tumor cell death (Panel E). Additional mechanisms such as receptor down regulation through endocytosis have been postulated (Panel F) [13].*

The benefit of trastuzumab in advanced HER2-positive adenocarcinoma of the stomach was addressed in the phase III ToGA trial, which compared standard chemotherapy with and without trastuzumab (8 mg/kg loading dose, then 6 mg/kg every three weeks until disease progression) [8]. The objective response rate was significantly higher with trastuzumab (47 versus 35 percent). At a median follow-up of 17.1 to 18.6 months, median overall survival (the primary endpoint) was significantly better with trastuzumab (13.8 versus 11.1 months). Exploratory analysis in subgroups defined by protein expression proposed that trastuzumab was

most effective in prolonging survival in the subgroup of patients with IHC 3+ tumors, less effective in patients with IHC 2+ tumors, and ineffective in those with HER2 gene-amplified (ie, FISH-positive) but non protein-expressing (IHC 0 or 1+) tumors. Based upon these data, trastuzumab was approved [54], in combination with cisplatin and a fluoropyrimidine, for the treatment of patients with metastatic HER2-overexpressing gastric who have not received prior treatment for metastatic disease. Patients with advanced gastric cancer who are potential candidates for trastuzumab should be screened to determine HER2 status.

## 2. AIM OF THE STUDY

In Vietnam, gastric cancer is the fourth most common malignancy and the third leading cause of cancer death [3]. Most gastric cancer patients are diagnosed when the tumor is at an unresectable stage. For these patients, systemic chemotherapy is the main treatment option because it prolongs survival without quality of life compromise. Now, trastuzumab is approved for the treatment of patients with metastatic HER2-overexpressing gastric who have not received prior treatment for metastatic disease [54]. It is now recommended that all patients with gastric cancer should have their tumors tested for HER2 status at the time of initial diagnosis [55].

In Vietnam, there is little research on HER2 protein overexpression and gene amplification in gastric cancer. The use of trastuzumab for treatment of advanced gastric cancer is limit. Causes mainly due to difficulties in implementing the technique, especially in immunohistochemistry and fluorescence in situ hybridisation. So, we conducted this study with the following aims.

1. Identify HER2 status in Vietnamese gastric cancer patients
2. Evaluate the concordance of HER2 protein overexpression and gene amplification



### 3. MATERIALS AND METHODS

#### 3.1. Study sites

This was a cross-sectional study and was conducted from March 2012 to June 2014, at following settings:

- Operation Theater A of Hue Central Hospital
- Gastrointestinal Endoscopic Center of Hue University Hospital
- Anatomical Pathology Department of Hue University Hospital
- Medical genetic Department of Hue University of Medicine and Pharmacy
- Anatomical Pathology Department of Sassari University Hospital

#### 3.2. Sample collection

Samples was collected from 03/2012 to 04/2014. We had total 61 samplpes include:

- 43 random surgical samples from patients that were diagnosed gastric cancer at Hue Centre Hospital
- 18 biopsy samples that were histopathologically diagnosed gastric cancer at Hue Medical University Hospital
- Patients who received perioperative chemotherapy in the past were excluded

#### 3.3. Slide preparation

##### *a. Fixation of specimens*

- Fixative solution: 10% neutral-buffered formalin (equivalent to 4% formaldehyde solution)
- Fixative volume: 10-20 times greater than specimens volume
- Fixation time: specimens were fixed no later than 20 minutes after taked off from patient's body.

- Fixation duration:

+ biopsy specimens: 6 - 8 hours

+ surgical specimens: 8 - 48 hours

*b. Processing of specimens*

Following fixation, tissue specimens were processed by these protocol

Table 3.1: *Protocol for processing gastric specimens*

Steps	Substances	Time (minutes)	
		Surgical specimen	Biopsy specimen
1	10% neutral-buffered formalin	30	10
2	Distilled water	30	5
3	Alcohol 70%	60	10
4	Alcohol 80%	90	10
5	Alcohol 95%	90	15
6	Alcohol 100%	120	20
7	Alcohol 100%	120	20
8	Alcohol 100 %	120	20
9	Xylene	90	20
10	Xylene	90	20
11	Paraffin	60	20
12	Paraffin	60	20
13	Paraffin	60	20

*c. Embedding in paraffin wax*

- After be processed, specimens were embedded in fresh paraffin wax maintained at 55-60°C.

- Paraffin blocks were stored at room temperature for a long time

**3.4. Sectioning and Hematoxylin and Eosin (H&E) staining**

- Section thickness

+ H&E staining: 2  $\mu$ m

+ IHC: 5  $\mu$ m

+ FISH: 4  $\mu$ m

- Mount on slide and perform H&E staining
- Interpret slides to determine the diagnosis of gastric adenocarcinoma, Lauren's classification (intestinal type vs diffuse type) [56]
- Mark (encircle) the largest possible area of tumor, excluding necrotic, inflammatory, or normal mucosa

### **3.5. Immunohistochemical staining for HER2 protein**

We performed IHC staining for HER2 protein using PATHWAY anti-HER-2/neu (4B5) Rabbit Monoclonal Primary Antibody by Ventana Benchmark Ultra automatic staining system.

#### *a. Principles*

PATHWAY HER2 (4B5) is a rabbit monoclonal antibody, which binds to HER2 in paraffin-embedded tissue sections. The specific antibody can be localized by either a biotin conjugated secondary antibody formulation that recognizes rabbit immunoglobulins followed by the addition of a streptavidin-horseradish peroxidase (HRP) conjugate (iVIEW DAB Detection Kit) or a secondary antibody-HRP conjugate (ultraView Universal DAB Detection Kit). The specific antibody-enzyme complex is then visualized with a precipitating enzyme reaction product. Each step is incubated for a precise time and temperature. At the end of each incubation step, the VENTANA automated slide stainer washes the sections to stop the reaction and to remove unbound material that would hinder the desired reaction in subsequent steps. It also applies Liquid Coverslip, which minimizes evaporation of the aqueous reagents from the specimen slide.

*b. Step by step procedure*

Table 3.2: *Staining protocols for PATHWAY anti-HER2/neu (4B5) with iView DAB detection kit*

<b>Procedure Type</b>	<b>BenchMark ULTRA instrument</b>
Baking	None
Deparaffinization	Selected
Cell Conditioning (Antigen Unmasking)	ULTRA CC1, mild
Enzyme (Protease)	None required
Antibody (Primary)	Approximately 24 minutes, 36°C
A/B Block (Biotin Blocking)	Required
Counterstain (Hematoxylin)	Hematoxylin II, 4 minutes
Post Counterstain	Bluing, 4 minutes

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

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

- Load the slides onto the automated slide stainer.

- Start the staining run.

- At the completion of the run, remove the slides from the automated slide stainer.

- Wash in a mild dishwashing detergent to remove the coverslip solution.

- Dehydrate, clear, and coverslip with permanent mounting media in the usual manner

### c. Criteria for scoring

We scored IHC results based on manufacturer's instruction, Interpretation Guide for VENTANA anti-HER2/neu (4B5) and the criteria for scoring IHC used in ToGA trial [8].

Table 3.3: *Criteria for intensity and pattern of cell membrane staining in gastric carcinoma. Adapted from Interpretation Guide for VENTANA anti-HER2/neu (4B5)*

Staining pattern - resection specimen	Staining pattern - biopsy specimen	Score (report to requesting physician)	HER2 staining assessment
No reactivity or membranous reactivity in <10% of tumour cells	No reactivity or membranous reactivity in any tumour cell	0	Negative
Faint / barely perceptible membranous reactivity in ≥10% of tumour cells; cells are reactive only in part of their membrane	Tumour cell cluster* with a faint / barely perceptible membranous reactivity irrespective of percentage of tumour cells stained	1+	Negative
Weak to moderate complete, basolateral or lateral membranous reactivity in ≥10% of tumour cells	Tumour cell cluster with a weak to moderate complete, basolateral or lateral membranous reactivity irrespective of percentage of tumour cells stained	2+	Equivocal**
Strong complete, basolateral or lateral membranous reactivity in ≥10% of tumour cells	Tumour cell cluster with a strong, complete basolateral or lateral membranous reactivity irrespective of percentage of tumour cells stained	3+	Positive

\* ≥5 cohesive cells

\*\* Recommend reflex to ISH

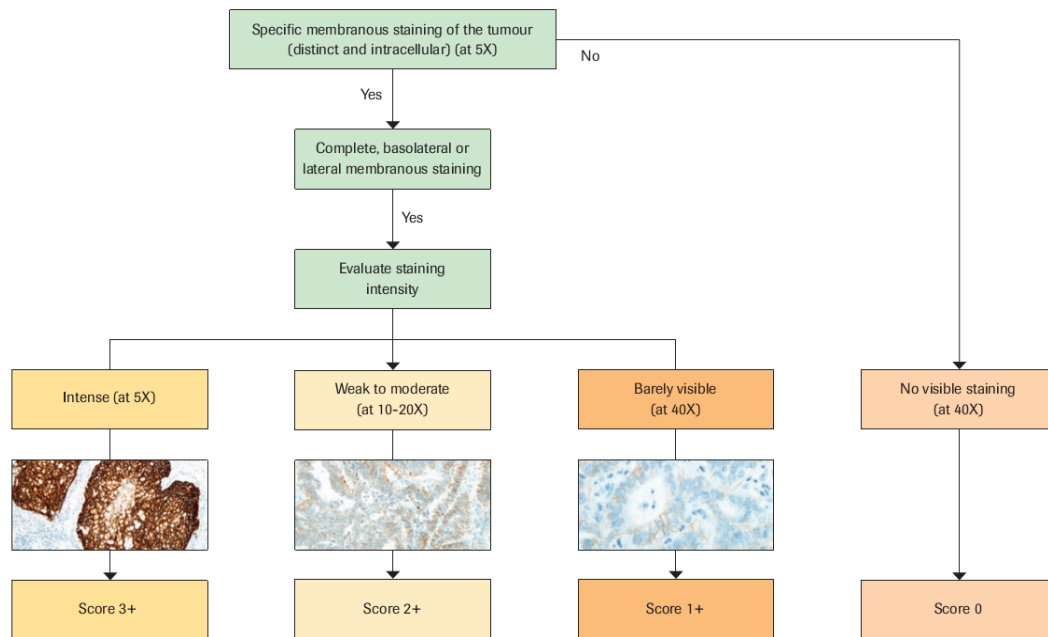


Figure 3.1: *HER2 IHC classification in gastric and oesophageal junction samples.*

*Adapted from Interpretation Guide for VENTANA anti-HER2/neu (4B5)*

Van Trung Nghia - Identification of HER2 protein overexpression and gene amplification in gastric cancer -  
Ph.D thesis in Clinical Biochemistry and Clinical Proteomics of  
Ph.D School in Biomolecular and Biotechnological Science - University of Sassari

### **3.6. Immunohistochemical staining for CD20, CD56, Chromogranin A, Synaptophysin**

We performed these IHC staining on Ventana Benchmark Ultra automatic staining system, similar as with IHC staining for HER2 protein. The results were interpreted by experts in Anatomical Pathological Department of Sassari University Hospital and Hue University Hospital.

### **3.7. Fluorescence in situ hybridization for HER2 gene**

FISH for HER2 gene amplification was conducted using the PathVysion HER2 DNA probe kit (Abbott Molecular Inc).

#### *a. Preparing Specimen slide*

- Cut 4 - 6  $\mu\text{m}$  thick paraffin sections using a microtome
- Float the sections in a protein-free water bath at 40°C
- Mount the section on the positive side of Superfrost slide
- Allow slides to air dry
- Transfer the mark from the H&E slide to the corresponding areas of the unstained slide, marking the glass on the side opposite the tissue section
- Bake the unstained slides 2-24 hours at  $56 \pm 2^\circ\text{C}$
- Process the unstained slides following the procedure below

#### *b. Deparaffinizing slides*

- Immerse slides in Hemo-De for 5 minutes at room temperature
- Repeat twice using new Hemo-De each time
- Dehydrate slides in 100% EtOH for 3 minutes at room temperature. Repeat
- Air dry slides or place slides on a 45-50°C slide warmer for 2-5 minutes

*c. Pretreating slides*

- Immerse slides in Pretreatment Solution at  $80\pm 1^{\circ}\text{C}$  for  $30\pm 5$  minutes
- Immerse slides in purified water for 3 minutes at room temperature
- Remove slides from the jar of purified water. Remove excess water by blotting the edges of the slides on a paper towel

*d. Protease treatment*

- Immerse slides in Protease Solution at  $37\pm 1^{\circ}\text{C}$  for 10-60 minutes
- Immerse slides in purified water for 3 minutes at room temperature
- Remove slides from the jar of purified water. Remove excess water by blotting the edges of the slides on a paper towel

*e. Dehydration*

- Immerse slides in 70% EtOH for 1 minute at room temperature
- Immerse slides in 85% EtOH for 1 minute at room temperature
- Immerse slides in 100% EtOH for 1 minute at room temperature
- Drain the excess EtOH from the slide by blotting on a paper towel, and wipe the underside of the slide
- Dry slides on a  $45\text{-}50^{\circ}\text{C}$  slide warmer for 2-5 minutes

*f. Co-denaturation and hybridization with ThermoBrite*

- Prewarm the probe to room temperature, vortex to mix and spin down
- Apply  $10\mu\text{l}$  of probe to the sample area of the slide
- Place a coverslip ( $22\times 22$  mm) and seal edges with rubber cement
- Program ThermoBrite as follow:

+ Denaturation: 80°C, 5 minutes

+ Hybridization: 37°C, 18 hours

- Close the lid and start the program

*g. Post-hybridization wash*

- Add post-hybridization wash buffer (2X SSC/0.3% NP-40) to a Coplin jar. Prewarm the post-hybridization wash buffer by placing the Coplin jar in the 73±1°C water bath for at least 30 minutes or until solution temperature has reached 73±1°C.

- Add post-hybridization wash buffer to a second Coplin jar and place at room temperature

- Remove the rubber cement seal from the first slide by gently pulling up on the sealant with forceps

- Immerse slide(s) in post-hybridization wash buffer at room temperature and float off coverslip

- After coverslip has been carefully removed, remove excess liquid by wicking off the edge of the slide and immerse slide in post-hybridization wash buffer at 73±1°C for 2-5 minutes

- Remove each slide from the wash bath and air dry in the dark in an upright position

*h. Counterstain*

- Apply 10 µl of DAPI counterstain to the target area of the slide

- Apply a 22x22 mm glass coverslip

- Store the slides in -20°C freezer for at least 30 minutes before viewing

- After removing from the -20°C freezer, allow slides to reach room temperature prior to viewing



*i. Signal enumeration*

- Evaluate slide adequacy using the following criteria:

+ Probe Signal Intensity: The signal should be bright, distinct, and easily evaluable. Signals should be in either bright, compact, oval shapes or stringy, diffuse, oval shapes.

+ Background: The background should appear dark or black and relatively free of fluorescence particles or haziness.

- Recognition of target Signals: Use the prescribed filter. Adjust the depth of the focus, and become familiar with the size and shape of the target signals and noise (debris). Enumerate hybridization signals only among tumor cells. Tumor cells in general are larger than normal cells, lymphocytes, and epithelial cells. Identify target areas by H & E stain on every 10th slide of the same tissue block. Identify these areas on the coverslip after the FISH assay is performed. Use a 25X objective to view the hybridized area and locate the target of interest (tumor cells as identified by H & E stain). Avoid areas of necrosis and where the nuclear borders are ambiguous. Skip those nuclei with signals that require subjective judgment. Skip signals with weak intensity and non-specificity, or with noisy background. Skip nuclei with insufficient counterstain to determine the nuclear border. Enumerate only those nuclei with discrete signals.

- Signal Enumeration:

Using a 40X objective, scan several areas of tumor cells to account for possible heterogeneity. Select an area of good nuclei distribution; avoid areas of the target where hybridization signals are weak. Using a 63X or 100X objective, begin analysis in the upper left quadrant of the selected area and, scanning from left to right, count the number of signals within the nuclear boundary of each evaluable interphase cell according to the guidelines provided.

Focus up and down to find all of the signals present in the nucleus. Count two signals that are the same size and separated by a distance equal or less than the diameter of the signal as one signal. Do not score nuclei with no signals or with signals of only one color. Score only those nuclei with one or more FISH signals of each color. Record counts in a two-way table. Calculate the ratio between "total of HER2 signals" and "total of CEP17 signals"

- Result interpretation:

- + If the ratio is  $<2$ , HER2 gene amplification was not observed
- + If the ratio is  $\geq 2$ , HER2 gene amplification was observed
- + If the ratio is 1,8 - 2,2, count another 20 nuclei, and the slide was enumerated by another technician to verify the results. If still in doubt, the assay was repeated with a fresh specimen slide.

### 3.8. Study design

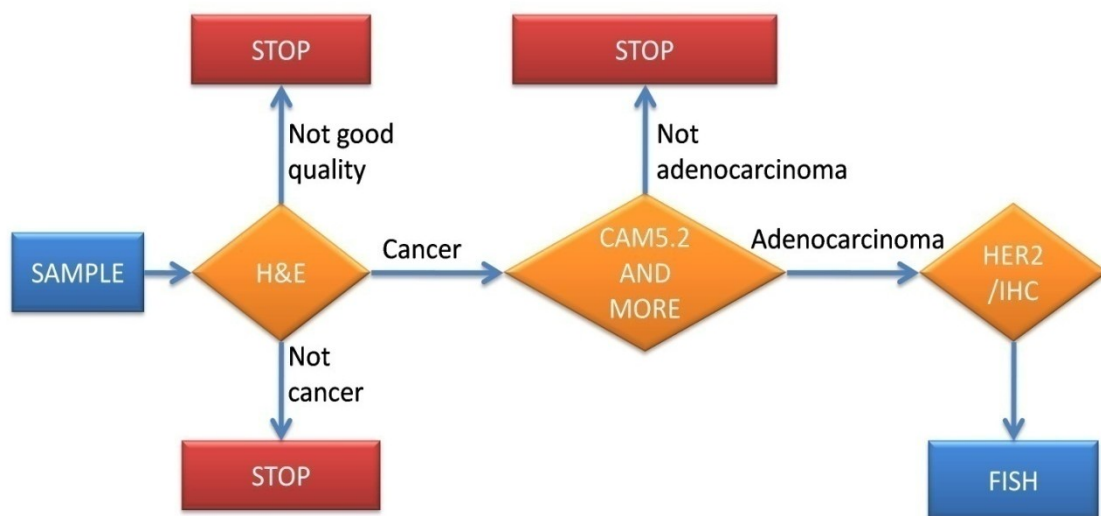


Figure 3.2: *Scheme of study design*

All collected samples were stained H&E and interpreted to determine the diagnosis of gastric cancer. If the slide was not cancer or not good quality (caused mainly by wrong fixation), we excluded the sample.

With gastric cancer samples, we did IHC staining of CAM 5.2, CD20, CD56, Chromogranin A, Synaptophysin depend on initial gastric cancer diagnosis.

All samples which were not adenocarcinoma were excluded. The adenocarcinoma sample was tested IHC HER2 protein and FISH HER2 gene.

## 4. RESULTS AND DISCUSSION

### 4.1. Characteristics of Specimens

We collected total 61 specimens. Characteristics of these specimens are shown in the table 4.1

Table 4.1: *Characteristics of specimens*

<b>Specimen characteristics</b>	<b>Number of cases</b>
Surgical samples	43
Biopsy samples	18
Adenocarcinoma	43
Lymphoma	2
Not good H&E quality	10
Not cancer	6
Total	61

There are 43 specimens were diagnosed adenocarcinoma. We have 10 cases that were diagnosed as "not good quality". The mainly cause due to wrong fixation time (longer 20 minutes after the specimens taked off patient body). We have 6 cases that were not gastric cancer, maybe cause by the surgical specimen are not cut correctly into the cancer areas. 43 adenocarcinoma samples would be done next steps: IHC for HER2 protein and FISH for HER2 gene amplification.

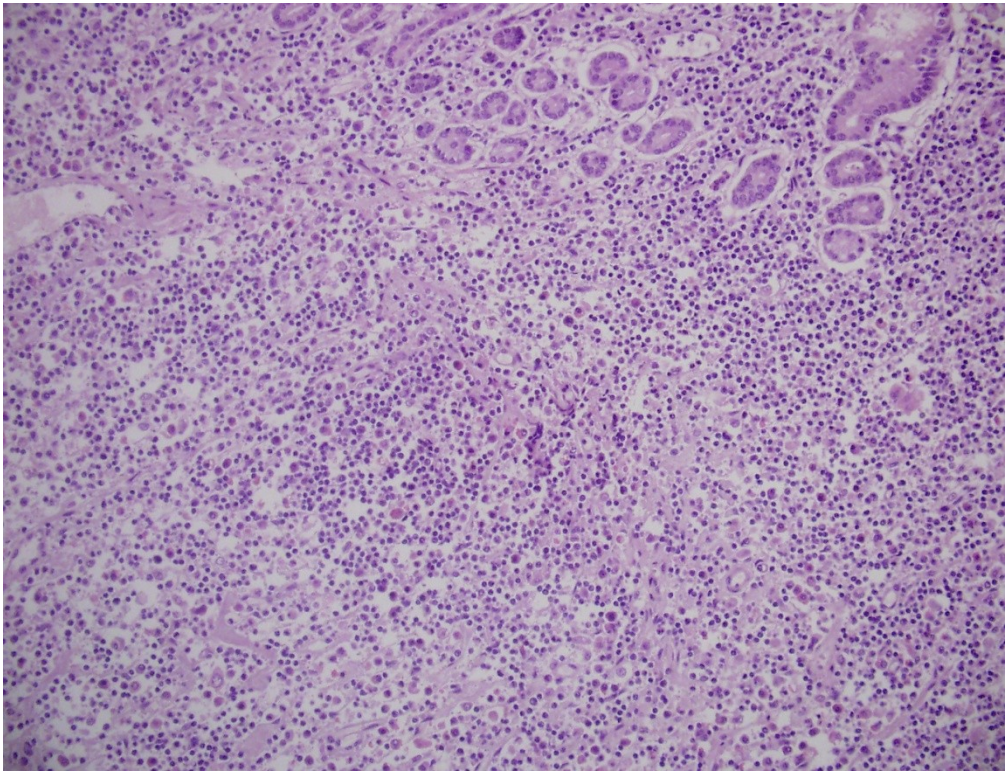


Figure 4.1: *UDD68, lymphoma (H&E, 20X)*

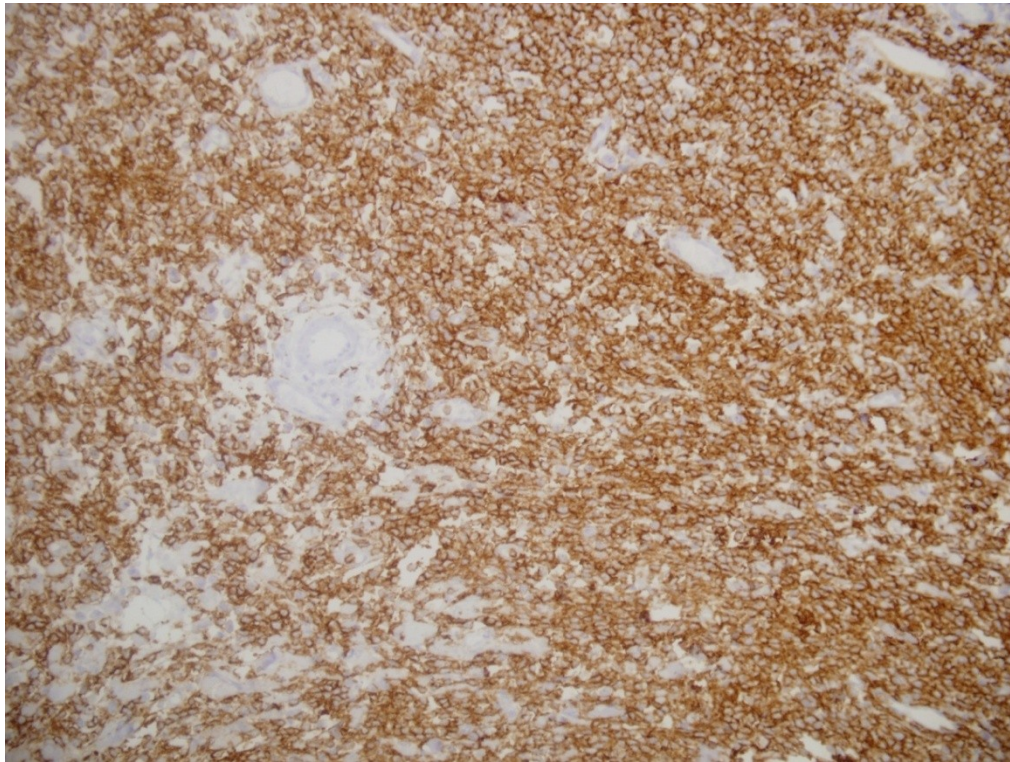


Figure 4.2: *UDD68, lymphoma (CD20/IHC, 20X)*

## 4.2. Characteristics of adenocarcinoma samples

Characteristics of 43 adenocarcinoma samples are shown in the table 4.2.

Median patient age was 60 years (range 31–83 years), and the male/female ratio was 1,71:1 (24 males, 14 females). There were 23 intestinal adenocarcinomas and 20 diffuse adenocarcinomas.

Table 4.2: *Characteristics of adenocarcinoma samples*

<b>Characteristics</b>	<b>Number of cases (%)</b>
Age(years), median (range)	60 (30 – 83)
Sex	
Male	24 (55,8)
Female	14 (32,5)
Not know	5 (11,7)
Sample type	
Surgical	30 (69.7)
Biopsy	13 (30.3)
Histologic type	
Intestinal type	23 (53.4)
Diffuse type	20 (46.6)
Total	43 (100)

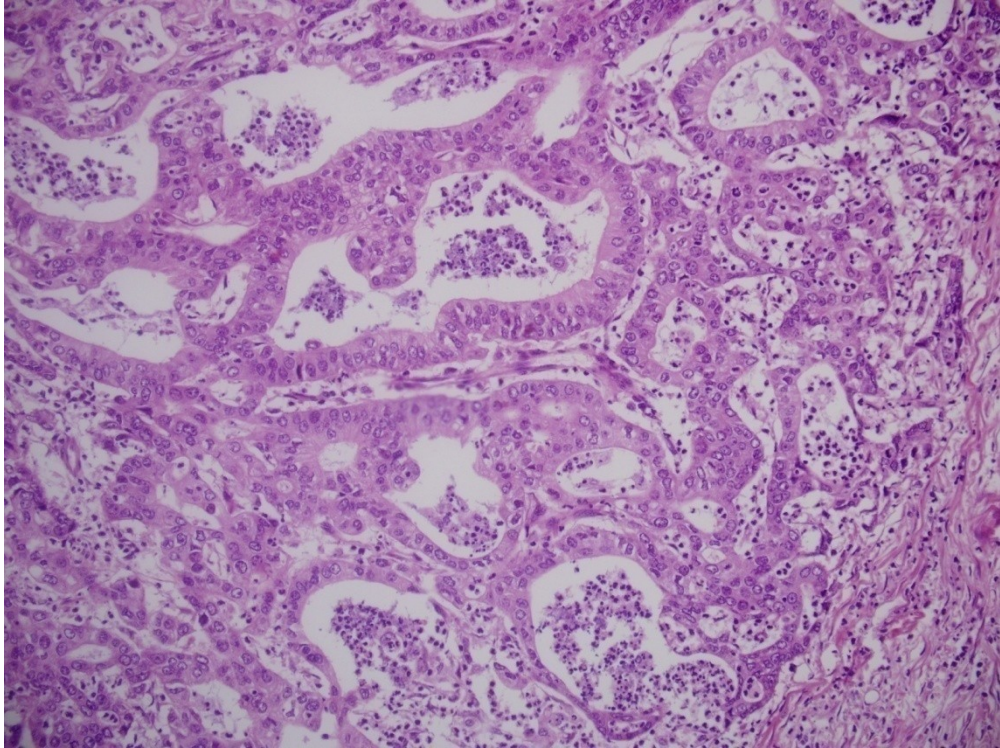


Figure 4.3: *UDD67, adenocarcinoma, intestinal type (H&E, 20X)*

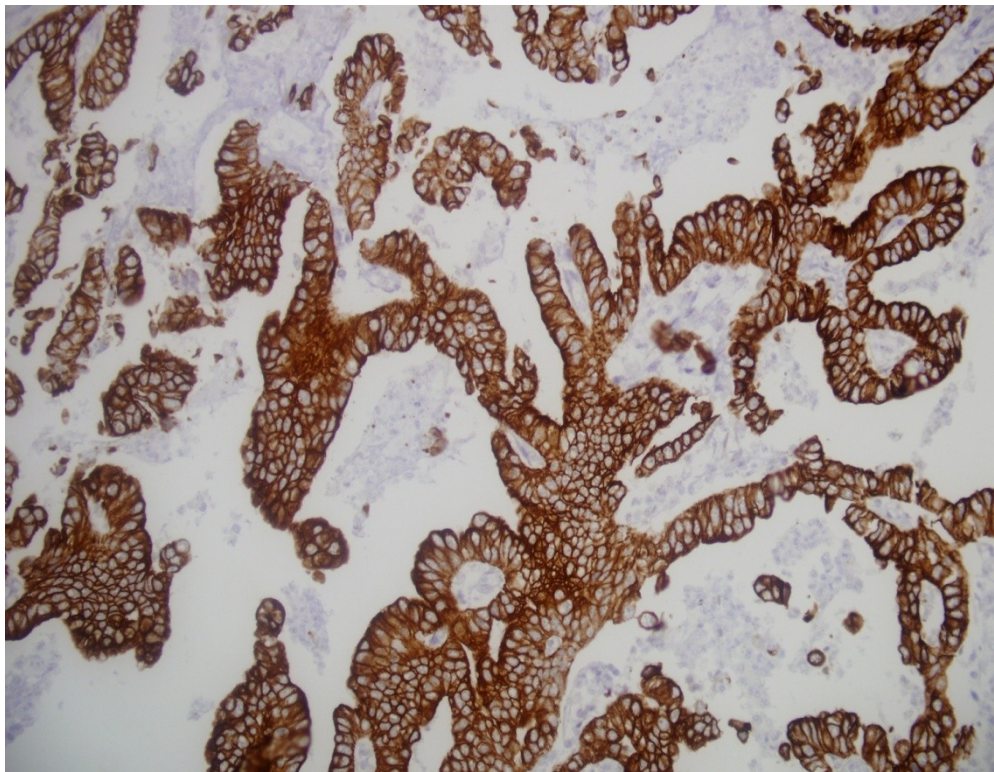


Figure 4.4: *UDD67, adenocarcinoma, intestinal type (CAM 5.2/IHC, 20X)*

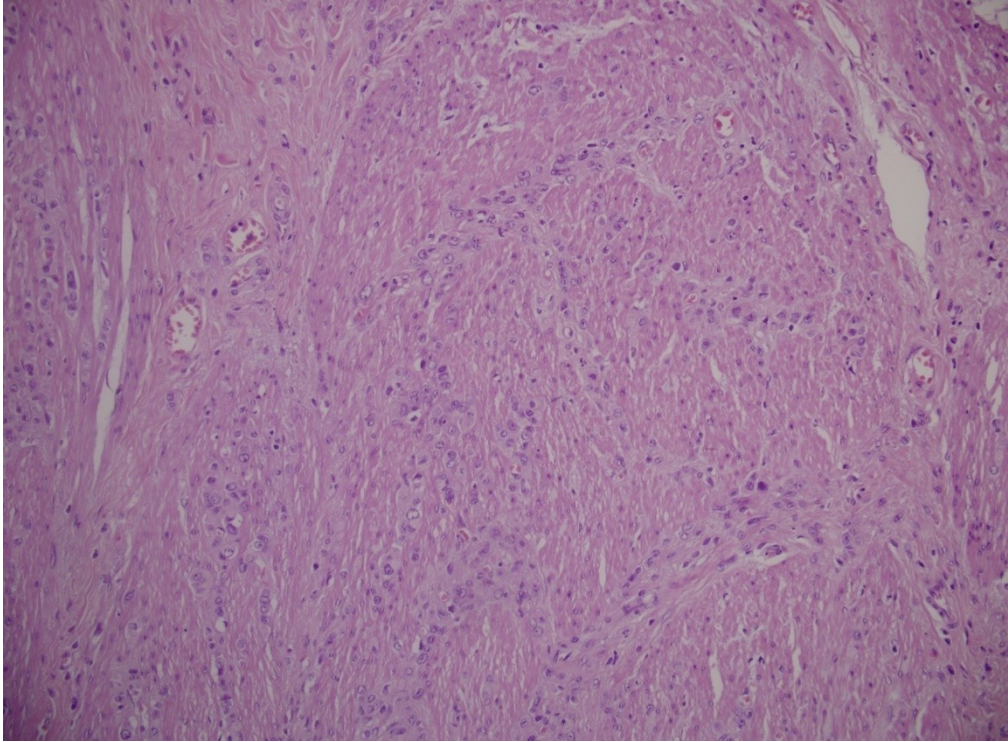


Figure 4.5: *UDD47, adenocarcinoma, diffuse type (H&E, 20X)*

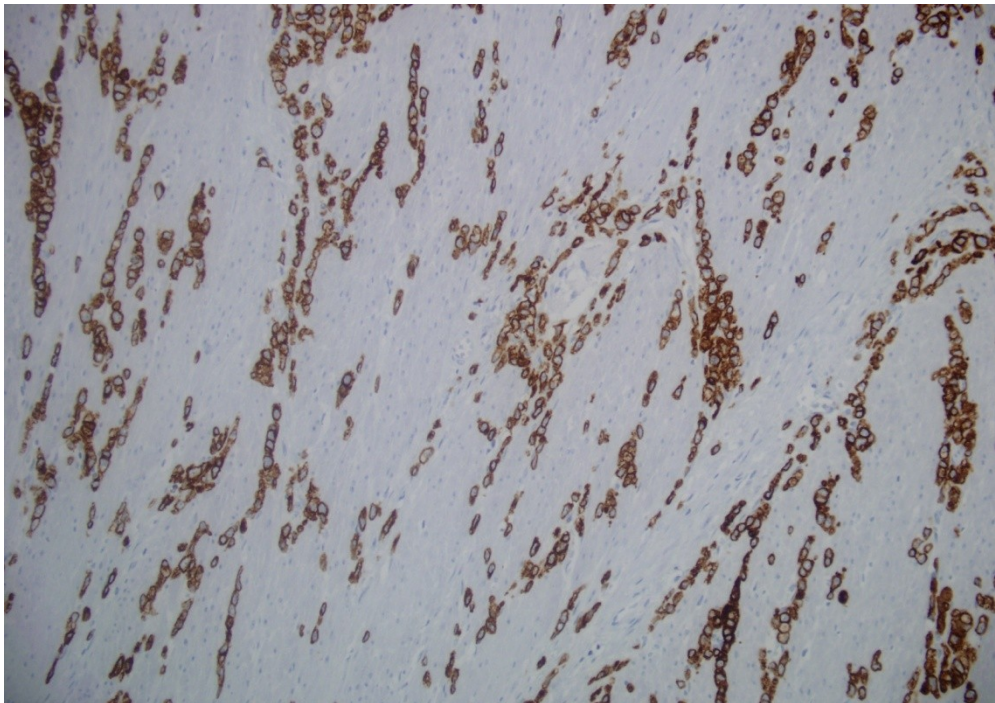


Figure 4.6: *UDD47, adenocarcinoma, diffuse type (CAM 5.2/IHC, 20X)*



### 4.3. HER2 protein overexpression

HER2 is an important marker for identifying patients with advanced gastric cancer who respond to trastuzumab-containing regimens. HER2 tests can be applied not only to surgically resected specimens but also to endoscopic biopsy specimens. Many previous studies that evaluated HER2 status in gastric cancer applied the criteria for assessing breast cancer [18, 30, 48, 57]. Generally, the criteria for HER2 protein overexpression were the same, but there were some differences in the methods used to evaluate HER2 status in breast and gastric cancer [49, 58].

In the present study, HER2 protein status in 43 gastric adenocarcinoma tissue samples was determined with immunohistochemical staining (Figure 4.7)

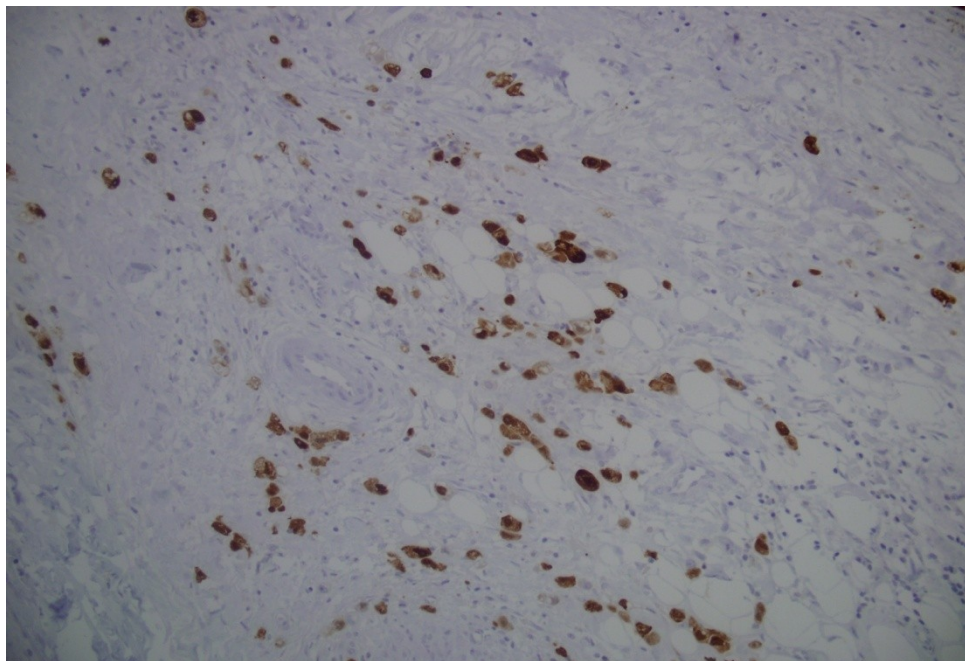


Figure 4.7: *UDD66, adenocarcinoma, HER2 3+ (IHC, 40X)*

Of the 43 gastric adenocarcinoma tissue samples, 29 (67,4%) were scored as 0; 9 (20,9%) as 1+; 3 (6,9%) as 2+, and 2 (4,8%) as 3+. The positive rate (IHC3+/IHC2+) was approximately 11,7% (5/43).

HER2 protein status were shown in the table 4.3.

Table 4.3: *HER2 protein overexpression*

HER2 overexpression	Number of cases (%)		
	Intestinal	Diffuse	Total
IHC score			
0	14 (60.8)	15 (75)	29 (67.4)
1 +	6 (26)	3 (15)	9 (20.9)
2 +	2 (8.6)	1 (5)	3 (6.9)
3 +	1 (4.6)	1 (5)	2 (4.8)
Total	23	20	43 (100)

The proportion of positive HER2 protein overexpression cases was 11,7 %. These results were comparable with those previously reported [41, 59]. Hofmann and coworker made an meta-analysis of 16 IHC studies containing 3,264 samples and reported the mean HER2-positive rate to be 17.6 % (range 6.8–34.0 %) [41]. Takehana et al., Kim et al., Barros-Silva et al. reported the HER2 positive rate to be 8,2%, 6,0%, 9,3%, respectively [22, 34, 60]. In our study, the proportion of positive cases were 11,7% higher than 3 those studies. These differents may be dirived from the variaion of HER2/IHC scoring. Those studies used old scoring that applied for breast cancer, so the proportion is lower.

In our study, the proportion of positive HER2 cases was 13,2% (3/23) and 10% (2/20) in intestinal and diffuse type according to Lauren's classification, respectively. These data are consistant with previous reports that the intestinal type showed a higher rate of HER2 positivity than the diffuse type [35, 36, 58, 61, 62].

#### 4.4. HER2 gene amplification

The HER2/CEP17 ratio was determined using the rate of HER2 signals and CEP17 signals in 20 nuclei. The total number of HER2 signals was divided by the total number of CEP17 signals, with a ratio was  $\geq 2$  according to the indications given by the Abbott-Vysis Company.

In our study, FISH assay was technically successful in 40 (93 %) of the 43 gastric adenocarcinoma cases. The HER2 gene was judged to be amplified in 3 of 40 cases (7,5%) (Figure 4.8). Three FISH failure cases occurred for surgical specimens with IHC 0, 1+ and 2+. Our findings with regard to the prevalence of HER2 gene amplification are similar to figures reported in the literature (generally around 6-30% for gene amplification) [59]. HER2 gene status were showed in the table 4.4.

Table 4.4: *HER2 gene amplification*

HER2 amplification	Number of cases (%)		
	Intestinal	Diffuse	Total
FISH			
positive	2 (9,5)	1 (5,2)	3 (7,5)
negative	19 (91,5)	18 (94,8)	37 (92,5)
Total	21	19	40 (100)

Some genetic alterations are exclusive of given subtypes of gastric carcinomas, as exemplified by CDH1 mutations in diffuse gastric adenocarcinomas [63, 64]. Some authors have stated that HER2 amplification is an exclusive event of intestinal type gastric carcinomas [34, 65]. However, of the 3 adenocarcinoma cases with amplification detected by FISH, one (5%) was diffuse and two were intestinal type according to Lauren's classification. Other authors reported that diffuse gastric

adenocarcinomas account for 5 – 6% of all diffuse adenocarcinomas which is observed by our findings [21, 22, 33].

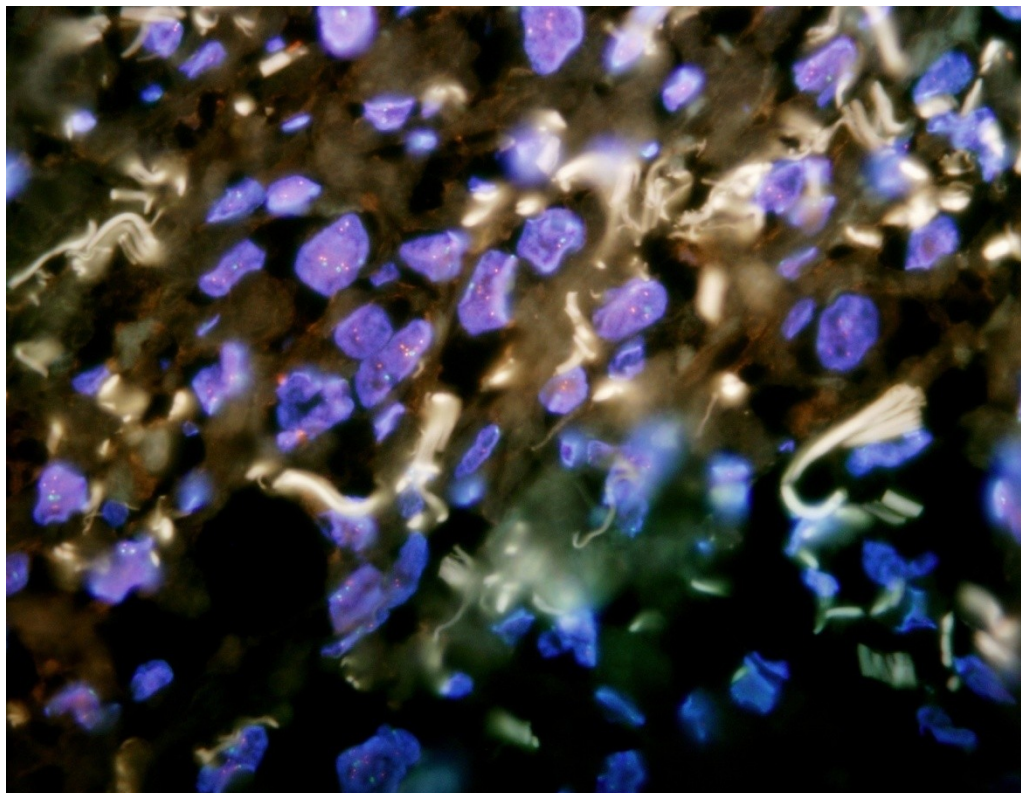


Figure 4.8: *UDD66, adenocarcinoma, FISH (+), 100X*

Conventionally, detecting HER2 gene amplification by FISH is considered the reference standard in breast and gastric cancer [42, 66]. However, FISH suffers from the following drawbacks: the analysis is labour intensive and time consuming, requiring a fluorescence microscope and specific observer training; the photolability of the probes mandates storage of data in the form of archival micrographs, which necessitate the incorporation of a high-resolution digital camera; and the correlation with tumour morphological features, such as tumour heterogeneity, is difficult with FISH. In contrast, the analysis of CISH is less time consuming because it can be performed with a bright field microscope, yields a more long-lasting record than FISH, and allows for correlation of amplification with morphological features [35, 67].

#### 4.5. Concordance between IHC and FISH results

Table 4.5: *Concordance between IHC and FISH*

IHC score	FISH		Concordance rate (%)
	Positive	Negative	
0	1	27	96,4
1 +	0	8	100
2 +	0	2	
3 +	2	0	100

In our study, among 3 FISH positive cases, 1 displayed an HER2 IHC score of 0, two displayed a score of 3+ . One cases which showed HER2 gene amplification but not HER2 IHC 3+ scores was considered discrepant case. The discordances in IHC-negative/FISH-positive cases were mostly attributed to intratumor heterogeneity and low-level amplification. So, with HER2 testing, we suggest that all of gastric adenocarcinomas patients should be tested with IHC and FISH, contemporary.

The concordance rate are 100%, 100% and 96,4% with cases of IHC 3+, 1+ and 0, respectively. These rates are very high and similar with many studies. Yano et al. found a concordance rate between IHC and FISH in the HER2 protein overexpression cases of 87% [18]. In the ToGA trial, the concordance between HER2 positivity by IHC and FISH was 87% and differences were largely due to FISH-positive cases that were IHC 0/1+ [8].

In 2 cases with IHC 2+, one is surgical sample and one is biopsy sample, we have no FISH positive case. The result is different with another previous studies [51]. The different may be derived from a very small number of our patients or from errors of sampling. Warneke et al. evaluated the risk of sampling errors in specimens of biopsy size, which may be caused by heterogeneous overexpression of HER2 in 454 gastric cancer cases [68]. HER2 test (IHC and FISH) results of tissue microarray (TMA) which contained five tissue cylinders were compared with those of matched whole tissue sections of resected gastric cancer specimens. Comparison of whole tissue sections with corresponding TMAs showed a false negative rate of 24 % and a false positive rate of 3 % for TMAs. They concluded that assessment of

the HER2 status in tissue biopsies carries a significant risk of sampling errors rendering patients unsuitable for treatment with trastuzumab. Although their study cohort included 51 % of intestinal-type gastric cancer cases, the high discordant rate for HER2 test results implicated necessity of HER2 testing in whole tissue sections.

## 5. CONCLUSION

In summary, we assessed the HER2 status in 43 samples from surgical and biopsy cases of gastric cancer.

The rates of HER2 protein overexpression are 4,8% of IHC 3+; 6,9% of IHC 2+.

The rate of HER2 gene amplification is 7,5%.

The concordance between IHC and FISH in testing HER2 status of gastric adenocarcinoma is very high but remain 1 discrepant case.

We suggest that we should be performed FISH confirmation of any degree of HER2 postivity with IHC.

## 6. BIBLIOGRAPHY

1. Krejs, G.J., *Gastric cancer: epidemiology and risk factors*. Dig Dis, 2010. **28**(4-5): p. 600-3.
2. Siegel, R., D. Naishadham, and A. Jemal, *Cancer statistics, 2013*. CA Cancer J Clin, 2013. **63**(1): p. 11-30.
3. IARC, *Globocan: Population Fact Sheets: Vietnam 2012*. 2014.
4. Jemal, A., et al., *Global cancer statistics*. CA Cancer J Clin, 2011. **61**(2): p. 69-90.
5. Cunningham, D., et al., *Perioperative Chemotherapy versus Surgery Alone for Resectable Gastroesophageal Cancer*. New England Journal of Medicine, 2006. **355**(1): p. 11-20.
6. Sastre, J., J.A. Garcia-Saenz, and E. Diaz-Rubio, *Chemotherapy for gastric cancer*. World J Gastroenterol, 2006. **12**(2): p. 204-13.
7. Scartozzi, M., et al., *Novel perspectives for the treatment of gastric cancer: from a global approach to a personalized strategy*. Curr Oncol Rep, 2010. **12**(3): p. 175-85.
8. Bang, Y.J., et al., *Trastuzumab in combination with chemotherapy versus chemotherapy alone for treatment of HER2-positive advanced gastric or gastro-oesophageal junction cancer (ToGA): a phase 3, open-label, randomised controlled trial*. Lancet, 2010. **376**(9742): p. 687-97.
9. Carpenter, G., L. King, and S. Cohen, *Epidermal growth factor stimulates phosphorylation in membrane preparations in vitro*. Nature, 1978. **276**(5686): p. 409-410.
10. Padhy, L.C., et al., *Identification of a phosphoprotein specifically induced by the transforming DNA of rat neuroblastomas*. Cell, 1982. **28**(4): p. 865-71.
11. Brandt-Rauf, P.W., M.R. Pincus, and W.P. Carney, *The c-erbB-2 protein in oncogenesis: molecular structure to molecular epidemiology*. Crit Rev Oncog, 1994. **5**(2-3): p. 313-29.
12. Menard, S., et al., *Biologic and therapeutic role of HER2 in cancer*. Oncogene, 2003. **22**(42): p. 6570-8.
13. Hudis, C.A., *Trastuzumab — Mechanism of Action and Use in Clinical Practice*. New England Journal of Medicine, 2007. **357**(1): p. 39-51.
14. Slamon, D.J., et al., *Studies of the HER-2/neu proto-oncogene in human breast and ovarian cancer*. Science, 1989. **244**(4905): p. 707-12.
15. Slamon, D.J., et al., *Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene*. Science, 1987. **235**(4785): p. 177-82.
16. Fukushige, S., et al., *Localization of a novel v-erbB-related gene, c-erbB-2, on human chromosome 17 and its amplification in a gastric cancer cell line*. Mol Cell Biol, 1986. **6**(3): p. 955-8.
17. Semba, K., et al., *A v-erbB-related protooncogene, c-erbB-2, is distinct from the c-erbB-1/epidermal growth factor-receptor gene and is amplified in a human salivary gland adenocarcinoma*. Proc Natl Acad Sci U S A, 1985. **82**(19): p. 6497-501.
18. Yano, T., et al., *Comparison of HER2 gene amplification assessed by fluorescence in situ hybridization and HER2 protein expression assessed by immunohistochemistry in gastric cancer*. Oncol Rep, 2006. **15**(1): p. 65-71.
19. Kim, J.W., et al., *The prognostic significance of HER2 positivity for advanced gastric cancer patients undergoing first-line modified FOLFOX-6 regimen*. Anticancer Res, 2012. **32**(4): p. 1547-53.
20. Shitara, K., et al., *Prognosis of patients with advanced gastric cancer by HER2 status and trastuzumab treatment*. Gastric Cancer, 2013. **16**(2): p. 261-7.

Van Trung Nghia - Identification of HER2 protein overexpression and gene amplification in gastric cancer -  
Ph.D thesis in Clinical Biochemistry and Clinical Proteomics of  
Ph.D School in Biomolecular and Biotechnological Science - University of Sassari



21. Tanner, M., et al., *Amplification of HER-2 in gastric carcinoma: association with Topoisomerase IIalpha gene amplification, intestinal type, poor prognosis and sensitivity to trastuzumab*. Ann Oncol, 2005. **16**(2): p. 273-8.
22. Kim, M.A., et al., *Evaluation of HER-2 gene status in gastric carcinoma using immunohistochemistry, fluorescence in situ hybridization, and real-time quantitative polymerase chain reaction*. Hum Pathol, 2007. **38**(9): p. 1386-93.
23. Gravalos, C. and A. Jimeno, *HER2 in gastric cancer: a new prognostic factor and a novel therapeutic target*. Annals of Oncology, 2008. **19**(9): p. 1523-1529.
24. Tateishi, M., et al., *Clinicopathological significance of c-erbB-2 protein expression in human gastric carcinoma*. J Surg Oncol, 1992. **49**(4): p. 209-12.
25. Dang, H.Z., Y. Yu, and S.C. Jiao, *Prognosis of HER2 over-expressing gastric cancer patients with liver metastasis*. World J Gastroenterol, 2012. **18**(19): p. 2402-7.
26. Jorgensen, J.T. and M. Hersom, *HER2 as a Prognostic Marker in Gastric Cancer - A Systematic Analysis of Data from the Literature*. J Cancer, 2012. **3**: p. 137-44.
27. Janjigian, Y.Y., et al., *Prognosis of metastatic gastric and gastroesophageal junction cancer by HER2 status: a European and USA International collaborative analysis*. Ann Oncol, 2012. **23**(10): p. 2656-62.
28. Terashima, M., et al., *Impact of expression of human epidermal growth factor receptors EGFR and ERBB2 on survival in stage II/III gastric cancer*. Clin Cancer Res, 2012. **18**(21): p. 5992-6000.
29. Yonemura, Y., et al., *Evaluation of immunoreactivity for erbB-2 protein as a marker of poor short term prognosis in gastric cancer*. Cancer Res, 1991. **51**(3): p. 1034-8.
30. Uchino, S., et al., *Overexpression of c-erbB-2 protein in gastric cancer. Its correlation with long-term survival of patients*. Cancer, 1993. **72**(11): p. 3179-84.
31. Mizutani, T., et al., *Relationship of C-erbB-2 protein expression and gene amplification to invasion and metastasis in human gastric cancer*. Cancer, 1993. **72**(7): p. 2083-8.
32. Nakajima, M., et al., *The prognostic significance of amplification and overexpression of c-met and c-erb B-2 in human gastric carcinomas*. Cancer, 1999. **85**(9): p. 1894-902.
33. Park, D.I., et al., *HER-2/neu amplification is an independent prognostic factor in gastric cancer*. Dig Dis Sci, 2006. **51**(8): p. 1371-9.
34. Takehana, T., et al., *Status of c-erbB-2 in gastric adenocarcinoma: a comparative study of immunohistochemistry, fluorescence in situ hybridization and enzyme-linked immuno-sorbent assay*. Int J Cancer, 2002. **98**(6): p. 833-7.
35. Yan, B., et al., *A study of HER2 gene amplification and protein expression in gastric cancer*. J Clin Pathol, 2010. **63**(9): p. 839-42.
36. Yan, S.Y., et al., *Clinicopathologic significance of HER-2/neu protein expression and gene amplification in gastric carcinoma*. World J Gastroenterol, 2011. **17**(11): p. 1501-6.
37. Liu, W., et al., *HER-2/neu overexpression is an independent prognostic factor for intestinal-type and early-stage gastric cancer patients*. J Clin Gastroenterol, 2012. **46**(4): p. e31-7.
38. Tsapralis, D., et al., *Human epidermal growth factor receptor-2 gene amplification in gastric cancer using tissue microarray technology*. World J Gastroenterol, 2012. **18**(2): p. 150-5.
39. Halon, A., et al., *HER-2 expression in immunohistochemistry has no prognostic significance in gastric cancer patients*. ScientificWorldJournal, 2012. **2012**: p. 941259.

40. Wolff, A.C., et al., *Recommendations for human epidermal growth factor receptor 2 testing in breast cancer: American Society of Clinical Oncology/College of American Pathologists clinical practice guideline update*. J Clin Oncol, 2013. **31**(31): p. 3997-4013.
41. Hofmann, M., et al., *Assessment of a HER2 scoring system for gastric cancer: results from a validation study*. Histopathology, 2008. **52**(7): p. 797-805.
42. Ruschoff, J., et al., *HER2 diagnostics in gastric cancer-guideline validation and development of standardized immunohistochemical testing*. Virchows Arch, 2010. **457**(3): p. 299-307.
43. Pauletti, G., et al., *Detection and quantitation of HER-2/neu gene amplification in human breast cancer archival material using fluorescence in situ hybridization*. Oncogene, 1996. **13**(1): p. 63-72.
44. Schnitt, S.J., *Breast cancer in the 21st century: neu opportunities and neu challenges*. Mod Pathol, 2001. **14**(3): p. 213-8.
45. Lemoine, N.R., et al., *Amplification and overexpression of the EGF receptor and c-erbB-2 proto-oncogenes in human stomach cancer*. Br J Cancer, 1991. **64**(1): p. 79-83.
46. Kameda, T., et al., *Expression of ERBB2 in human gastric carcinomas: relationship between p185ERBB2 expression and the gene amplification*. Cancer Res, 1990. **50**(24): p. 8002-9.
47. Hollywood, D.P. and H.C. Hurst, *A novel transcription factor, OB2-1, is required for overexpression of the proto-oncogene c-erbB-2 in mammary tumour lines*. EMBO J, 1993. **12**(6): p. 2369-75.
48. Kimura, M., et al., *A proposal for diagnostically meaningful criteria to classify increased epidermal growth factor receptor and c-erbB-2 gene copy numbers in gastric carcinoma, based on correlation of fluorescence in situ hybridization and immunohistochemical measurements*. Virchows Arch, 2004. **445**(3): p. 255-62.
49. Park, Y.S., et al., *Comprehensive analysis of HER2 expression and gene amplification in gastric cancers using immunohistochemistry and in situ hybridization: which scoring system should we use?* Hum Pathol, 2012. **43**(3): p. 413-22.
50. Grabsch, H., et al., *HER2 expression in gastric cancer: Rare, heterogeneous and of no prognostic value - conclusions from 924 cases of two independent series*. Cell Oncol, 2010. **32**(1-2): p. 57-65.
51. Yoshida, H., et al., *Comparison of HER2 status between surgically resected specimens and matched biopsy specimens of gastric intestinal-type adenocarcinoma*. Virchows Arch, 2014.
52. Sliwkowski, M.X., et al., *Nonclinical studies addressing the mechanism of action of trastuzumab (Herceptin)*. Semin Oncol, 1999. **26**(4 Suppl 12): p. 60-70.
53. Cuello, M., et al., *Down-regulation of the erbB-2 receptor by trastuzumab (herceptin) enhances tumor necrosis factor-related apoptosis-inducing ligand-mediated apoptosis in breast and ovarian cancer cell lines that overexpress erbB-2*. Cancer Res, 2001. **61**(12): p. 4892-900.
54. FDA. *FDA Approval for Trastuzumab*. 2013; Available from: <http://www.cancer.gov/cancertopics/druginfo/fda-trastuzumab>.
55. Ruschoff, J., et al., *HER2 testing in gastric cancer: a practical approach*. Mod Pathol, 2012. **25**(5): p. 637-50.
56. F.T. Bosman, F. and R.H.H. Carneiro, N.D. Theise, *WHO classification of tumours of the digestive system*, in *WHO Classification of Tumours 2010*, International Agency for Research in Cancer: Lyon, France. p. 43.

57. Tafe, L.J., et al., *Human epidermal growth factor receptor 2 testing in gastroesophageal cancer: correlation between immunohistochemistry and fluorescence in situ hybridization*. Arch Pathol Lab Med, 2011. **135**(11): p. 1460-5.
58. Kunz, P.L., et al., *HER2 expression in gastric and gastroesophageal junction adenocarcinoma in a US population: clinicopathologic analysis with proposed approach to HER2 assessment*. Appl Immunohistochem Mol Morphol, 2012. **20**(1): p. 13-24.
59. Boku, N., *HER2-positive gastric cancer*. Gastric Cancer, 2014. **17**(1): p. 1-12.
60. Barros-Silva, J.D., et al., *Association of ERBB2 gene status with histopathological parameters and disease-specific survival in gastric carcinoma patients*. Br J Cancer, 2009. **100**(3): p. 487-93.
61. Moelans, C.B., et al., *Low frequency of HER2 amplification and overexpression in early onset gastric cancer*. Cell Oncol (Dordr), 2011. **34**(2): p. 89-95.
62. Boers, J.E., H. Meeuwissen, and N. Methorst, *HER2 status in gastro-oesophageal adenocarcinomas assessed by two rabbit monoclonal antibodies (SP3 and 4B5) and two in situ hybridization methods (FISH and SISH)*. Histopathology, 2011. **58**(3): p. 383-94.
63. Becker, K.F., et al., *E-cadherin gene mutations provide clues to diffuse type gastric carcinomas*. Cancer Res, 1994. **54**(14): p. 3845-52.
64. Grady, W.M., et al., *Methylation of the CDH1 promoter as the second genetic hit in hereditary diffuse gastric cancer*. Nat Genet, 2000. **26**(1): p. 16-7.
65. Varis, A., et al., *Coamplified and overexpressed genes at ERBB2 locus in gastric cancer*. Int J Cancer, 2004. **109**(4): p. 548-53.
66. Wolff, A.C., et al., *American Society of Clinical Oncology/College of American Pathologists guideline recommendations for human epidermal growth factor receptor 2 testing in breast cancer*. Arch Pathol Lab Med, 2007. **131**(1): p. 18-43.
67. Lambros, M.B., R. Natrajan, and J.S. Reis-Filho, *Chromogenic and fluorescent in situ hybridization in breast cancer*. Hum Pathol, 2007. **38**(8): p. 1105-22.
68. Warneke, V.S., et al., *Her2/neu testing in gastric cancer: evaluating the risk of sampling errors*. Annals of Oncology, 2013. **24**(3): p. 725-733.