



Her2/neu Protein Expression and Oncogene Amplification in Gastric Carcinoma with Clinico-Pathological Correlation in Egyptian Patients

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Abstract

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AIM: Amplification of the *Her2/neu* gene and overexpression of the Her2/neu protein in gastric carcinoma (GC) is a golden criterion for target therapy with trastuzumab (Herceptin). We aim to evaluate the immunohistochemical protein expression and amplification of the oncogene Her2/neu by FISH technique in the epithelial gastric carcinoma and to compare their association with different clinicopathological parameters aiming at identifying positive cases that may benefit from targeted therapy.

MATERIALS AND METHODS: This study was done on eighty-five tumour tissue samples from patients with GC as well as thirty non-malignant lesions (Gastritis, intestinal metaplasia, adenoma with low-grade dysplasia, adenoma with high-grade dysplasia). All were immunohistochemically stained with Her2/neu antibody.

RESULTS: All equivocal and some selected GC cases were submitted for FISH technique to detect *Her2/neu* gene amplification. By immunohistochemistry twenty-three cases (27%) were defined as positive for *Her2/neu* gene amplification and/or protein overexpression. The levels of Her2/neu positive (3+), Her2/neu equivocal (2+) and Her2/neu negative (1+/0) were measurable in 14.2%, 32.9% and 52.9% of the samples, respectively. FISH showed that *Her2/neu* gene was amplified in 22 cases, 10 Her2/neu positive (3+), 11 (39.3%) Her2/neu equivocal (2+) and 1 Her2/neu negative (1+) cases with IHC staining those who can benefit from anti Her2/neu target therapy. *Her2/neu* was overexpressed positivity (3+) more in intestinal type and mixed carcinoma, and moderately differentiated tumours. None of gastritis, intestinal metaplasia or adenoma with low-grade dysplasia cases showed positivity for Her2/neu (3+). The Her2/neu positivity (3+) was associated with both adenocarcinoma cases and high-grade dysplasia (P = 0.002).

CONCLUSIONS: The results highlight the necessity of FISH test for further categorization when gastric cancer cases are equivocal (2+) by IHC to determine eligibility for the targeted therapy. Stepwise increase in the expression of Her2/neu was seen in low-grade dysplasia, high-grade dysplasia and carcinoma cases implying its role in cancer evolution. Overexpression of Her 2/neu in GC patients can be promising in selecting those who can get benefit from anti-Her2/neu target therapy.

Introduction

Gastric cancer (GC) is a major cause of cancer death worldwide, especially in developing countries. The incidence of GC varies from country to country, probably as a result of genetic and environmental factors [1]. In Egypt, GC is the 12th most common cancer in both sexes representing

1.6% of the total cancers. It is the 12th leading cause of cancer death representing 2.2% of the total cancer mortality [2]. This is shown in many Egyptian populations- based cancer registries [3]. At the Egyptian National Cancer Institute hospital-based registry 2002–2010, GC is the 14th most common cancer representing 1.8% of cases in both sexes [4].

There is a need to stratify patients with GC into the appropriate screening, surveillance, or

treatment programs. Although histopathology remains the most reliable and less expensive method, numerous efforts have been made to identify and validate novel biomarkers to accomplish the above goals. In recent years, several molecules have been identified and tested for their clinical relevance in GC management, with the exception of Her2/neu, none of the biomarkers is currently used in clinical practice, and some of them were described in single studies [5].

Despite ongoing advances in the treatment of gastroesophageal cancer, prognosis remains poor. The best promise to improve this poor survival is provided by new targeted agents. Of these, human epidermal growth factor receptor 2 (Her2/neu) is currently in the spotlight [6].

Her2/neu is a proto-oncogene located on chromosome 17q21 and a member of the human epidermal growth factor receptor (EGFR) family. It encodes an 185 kD transmembrane tyrosine kinase receptor protein that, through dimerization with other family members, regulates signal transduction in cellular processes including proliferation, differentiation, and cellular survival [7]. Many studies have indicated a role of Her2/neu in the development of various types of human cancer. Her2/neu is amplified, in about 10–20 % of breast carcinomas [6], in GC have been shown in studies. Her2/neu positivity can be detected in approximately 20% of patients, which is a characteristic associated with poor prognosis [8]. Since the costs for trastuzumab therapy are high and side effects are significant, accurate selection of eligible patients for this therapy is crucial. Her2/neu status is mainly assessed by immunohistochemistry (IHC) and chromogenic (CISH) or fluorescence *in situ* hybridization (FISH) [6]. To eliminate discrepancies observed between IHC and FISH, Hofmann et al. [9] established an IHC scoring system specific for GC. In an international consensus meeting, modifications to the breast scoring system were made mainly based on the more frequent basolateral (incomplete) membrane staining and heterogeneity in GC.

The aim is to evaluate the immunohistochemical protein expression and amplification of the oncogene Her2/neu by FISH technique in the epithelial gastric carcinoma and to compare and detect their association with different clinicopathologic parameters aiming at identifying positive cases that may benefit from targeted therapy.

Material and Methods

Eighty-five cases of carcinoma of the stomach were collected from archived tissue samples from the Surgical Pathology Department of Theodor Bilharz

Research Institute (TBRI) for patients who had undergone total, subtotal or partial gastrectomy was evaluated for Her2/neu status using IHC analysis. In addition to this, 9 endoscopic gastric biopsies for patients with gastritis were also included in the study. None of the patients had undergone prior preoperative radiation, chemotherapy or targeted therapy.

Of the 85 cases, 40 cases were selected with intestinal-type gastric adenocarcinoma with different grades and stages and were compared using immunohistochemical analysis to 30 other tissue samples in the form of 4 cases of intestinal metaplasia, 10 cases of adenoma with low grade dysplasia, and 7 cases of adenoma with high grade dysplasia that were isolated from juxta tumoral tissues in addition to the 9 endoscopic gastric biopsies with gastritis. Based on the outcome of the immunohistochemical analysis of Her2/neu protein in the 85 cases of gastric carcinoma, 40 cases were evaluated for the Her2/neu gene status using the FISH technique, all cases with equivocal Her2/neu protein expression (score 2+) to detect gene status and document eligibility for targeted therapy (28 samples), 10 randomly selected cases with positive Her2 protein expression (score 3+) and only 2 randomly selected cases negative for protein expression (score 1+).

Histopathological study

Paraffin blocks containing representative samples of the tumours were selected by reviewing all of the available H&E stained slides. The Lauren classification of gastric cancer was followed to subgroup all cases in histopathology according to Lauren [10].

The patient demographics and pathologic information were retrieved from each pathology report including Personal data, Clinical data; Tumor localisation, type of surgery, tumour type and grade, depth of invasion, the number of lymph nodes resected and the number of lymph nodes with metastases and final pathological diagnosis. All stomach cancers with a midpoint in the stomach lying more than 5 cm distal to the EGJ, or those within 5 cm of the EGJ but not extending into the EGJ or esophagus, are staged using the gastric (not-EGJ) cancer staging system set out in the 7th edition of the American Joint Committee on Cancer staging, and cancers whose midpoint is in the lower thoracic esophagus, EGJ, or within the proximal 5 cm of the stomach (cardia) that extend into the EGJ or esophagus are staged as adenocarcinoma of the esophagus [11].

Tumours are graded according to WHO grading system into well differentiated, moderately differentiated and poorly differentiated [12].

Immunohistochemical technique

Immunohistochemistry for Her2/neu was performed on sections cut from the paraffin blocks with a commercially available rabbit monoclonal Anti-human Her2/neu antibody (CELL MARQUE SP3, USA). Briefly, samples were sectioned at 4 μ m onto positively charged slides (Superfrost plus, Menzel-Glaser, Germany) and the slides were stained on an automated platform the Dako autostainer Link 48. Heat-induced antigen retrieval was used for 30 min at 97°C in the high-PH EnVision™ FLEX Target Retrieval Solution and the primary antibody was used at a dilution of 1 in 100.

Interpretation of immunostaining

Immunostaining of Her2/neu was performed and scored according to the revised scoring criteria of Hofmann et al. (9) used in the ToGA trial, which was based on the intensity of membrane staining and quantity of positive neoplastic cells on a scale of 0-3. A score of 0 or 1+ was considered negative while scores 2+ was considered equivocal and 3+ was positive. Heterogeneous Her2/neu expression was considered if the tumour shows the presence of both Her2/neu-positive and Her2/neu-negative subpopulations of carcinoma cells in a single tumour [13]. All immunostained slides were analysed using Zeiss microscope with high resolution (Axioscope, Germany) at different powers (x 50, x 100, x 200, x 400).

Gene expression of Her2 by fluorescence in situ hybridization

FISH analysis was used on a representative proportion of the tissue, using the Path Vision kit (Abbott Laboratories, Abbott Park, IL, USA). All samples with positive HER2 protein expression were evaluated using labelled probes for both fluorophores, i.e., Vysis CEP 17 17p11.1-q11.1 Alpha Satellite DNA Spectrum Green, and Vysis LSI HER-2/neu 17q11.2-12 Spectrum Orange.

Paraffin was removed from 4 μ m tissue sections by washing the slides in xylene for ten min, then in 100% alcohol for five min twice, then air drying them. Slides were then immersed in pretreatment solution at 80°C for 15 minutes, and then tissue sections were digested with protease solution by immersing slides in solution at 37°C for five minutes. Slides were air dried for 2–5 minutes. Tissue sections were post-fixed in 10% neutral buffered formalin at room temperature for 10min before dehydration in ascending grades of alcohol and air drying, Tissue sections were denatured in the denaturation solution at 72°C for five min. Then in ascending grades of alcohol 70%, 95%, 100% alcohol for one min. each, air dried for 2–5 minutes. Probes for the pericentromeric region of chromosome 17 (Spectrum Green™) and the locus-

specific probe for Her2 (Spectrum Orange™) were used. For each section, one μ l of each probe was added to seven μ l hybridization mix (50% formamide, 2 × SSC, 10% dextran sulphate) and one μ l deionized water and denatured in a water bath at 72°C for five min and then hybridised overnight at 37°C. Post-hybridization washes were done by immersing slides in pre-warmed 2X SSC/0.3% NP-40 at 73.1°C, for two mins. Slides were air dried in darkness. Slides were mounted in 10 μ l diaminido phenyl indole (DAPI)/antifade. Control sections of normal stomach and Her2 amplified breast tumours were included in each run.

Interpretation of FISH

Slides were viewed with a fluorescence microscope (BX51 upright, Olympus Corp, Japan), where three areas were identified and in each area, 20 nuclei were assessed. Chromosome 17 copy number and HER2 copy number were assessed for each of the 20 nuclei at 1000 magnification. A ratio of chromosome 17 copy number over HER2/neu copy number was obtained from the 60 nuclei. HER2/neu was classified based on the value used in breast cancer diagnostics into 'amplified' (Her2/neu/centromere 17 ratios >2) 'not amplified' (Her2/neu/centromere17 ratio <2) or polysomy (more than 2 green signals).

Statistical analysis

SPSS for Windows, version 18 was used for statistical analysis (IBM corporation, Armonk, new York, USA). Means of different groups were compared using one-way ANOVA. Comparison between percent positive cases was calculated by Chi-Square test. A p-value of 0.05 was considered of statistical significance.

Results

Her2/neu expression by IHC

The patients' clinicopathological characteristics of the 85 cases gastric carcinoma are listed in (Fig. 1; Table 1). Twelve cases (14.2%) were scored as positive for Her2/neu membrane staining (3+), 32.9% were equivocal (2+), and 52.9% were Her2/neu negative (0/1+) (Table2). Her2/neu protein expression by IHC in adjacent non-tumor normal appearing mucosa was detected in 11/85 (12.9%) of gastrectomy specimens. The Her2/neu expression was membranous (score 1+ or 2+) (Fig. 2; Tables 2-3).

There was a higher Her2/neu positivity (3+) in the cases with intestinal type adenocarcinoma and

mixed type in relation to diffuse type carcinoma. The positivity (3+) in the mixed type was detected in intestinal differentiation, not in diffuse areas. The mixed type was 20% showed positivity (3+), followed by the intestinal type (13%) with no statistical difference between different types.

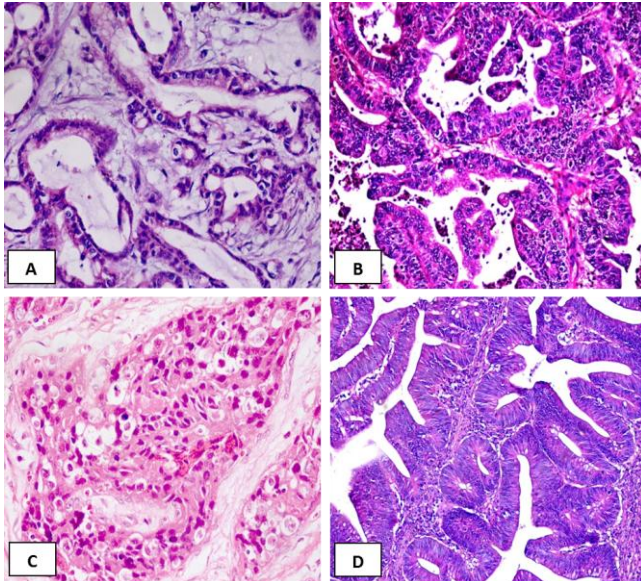


Figure 1: H&E stained gastric adenocarcinoma sections. A) case of well differentiated gastric carcinoma; malignant cells show mild nuclear anaplasia and arranged in well-formed glands (H&E x 400); B), case shows moderately differentiated carcinoma, shows papillary architecture with fibrovascular cores covered by malignant epithelial cells show moderate nuclear anaplasia (H&E x 200); C) case of poorly differentiated gastric carcinoma, malignant cells show moderate nuclear anaplasia (H&E x 400); D) case of adenoma with high-grade dysplasia, showed irregular glands with marked nuclear stratification (H&E x 200)

Her2/neu positivity (3+) was 18% in the moderately differentiated tumours and 9.7 % of the poorly differentiated tumours with no significance difference between them.

Table 1: The relation between the pathologic parameters and Her2/neu protein positivity by IHC in carcinoma cases

Pathologic data	Number (%)	Her2/neu (IHC) (score 3+)		P value
		Positive	%	
Histologic type				
Adenocarcinoma; intestinal type	54 (63.5%)	7	(13%)	0.567 (insignificant)
Mixed	20 (23.5%)	4	(20%)	
Diffuse	11 (13%)	1	(9%)	
Histopathological Grade				
Well differentiated	4 (4.7%)	0	(0 %)	0.272 (insignificant)
Moderately differentiated	50 (58.9%)	9	(18%)	
Poorly differentiated	31 (36.4%)		(9.7%)	
Depth of invasion				
T1	0	3	(0%)	0.499 (insignificant)
T2	14 (16.5%)	4	(28.6%)	
T3	45 (52.9%)	5	(11.1%)	
T4	26 (30.6%)	3	(11.5%)	
Lymph node status				
N0	21 (24.7%)	3	(14.2%)	0.724 (insignificant)
N1	11 (12.9%)	2	(18.2%)	
N2	25 (29.4%)	4	(16%)	
N3	28 (33%)	3	(10.7%)	
Location of the tumour*				
GEJ	14 (16.6%)	1	(7.1%)	0.575 (insignificant)
Gastric	37 (43.5%)	6	(16.2%)	

*40% of samples the location of the tumor was not specified.

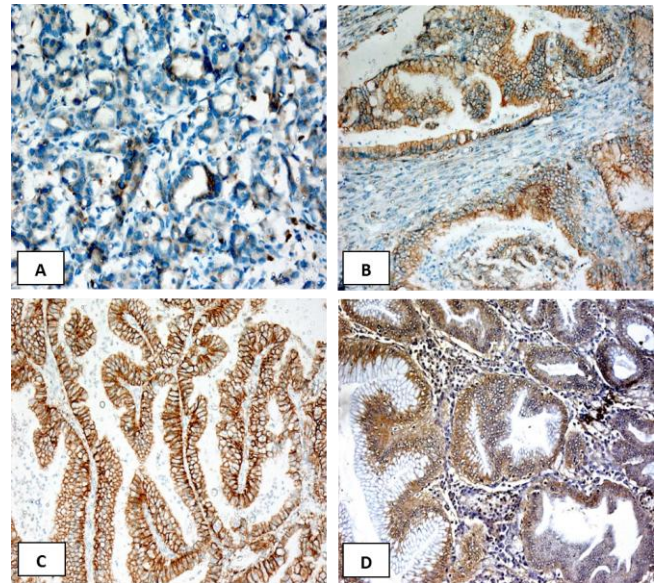


Figure 2: Immunohistochemistry for Her2/neu in gastric sections expressed as a brown membranous stain. A) case of moderately differentiated gastric carcinoma, malignant glands show visible weak Her2/neu protein expression at high power (Score 1+) (IHC, DAB, x 400); B) case of moderately differentiated gastric carcinoma, malignant glands show moderate membranous basolateral and complete Her2/neu protein expression (Score 2+) (IHC, DAB, x 200); C) case of moderately differentiated gastric carcinoma with papillary pattern shows strong membranous staining for Her2/neu (Score 3+) (x 200); D) Dysplastic acini with moderate Her2/neu expression (Score 2+) in a case of gastric carcinoma (IHC, DAB x 200)

The positive expression of Her2/neu (3+) was associated with moderately differentiated tumours, but this correlation is not statistically significant (P = 0.272). Her2/neu positivity (3+) was found more in the tumours with T2 stage (28.6%).

Table 2: Immunohistochemistry - Fluorescence in-situ hybridization concordance in gastric carcinoma cases

IHC	FISH		Total
	Negative	Positive	
Negative	1 (50%)	1 (50%)	2 (100%)
Equivocal	17 (60.7)	11 (39.3%)	28 (100%)
Positive	0	10 (100%)	10 (100%)
Total	18 (45%)	22 (55%)	40 (100%)

Cross tables; Pearson Chi- Square test; p value = 0.004 (significant).

Her2/neu expression in gastric non-malignant lesions were nine cases of endoscopic biopsies diagnosed as gastritis, and lesions in adjacent non-tumorous mucosa in gastrectomy specimens for GC cases (four cases of intestinal metaplasia, ten cases of adenoma with low-grade dysplasia, and seven cases of adenoma with high-grade dysplasia). The results of these non-malignant gastric lesions were compared to the results of forty adenocarcinoma cases (intestinal type), selected from the 85 carcinoma cases.

None of gastritis, intestinal metaplasia or adenoma with low-grade dysplasia samples showed strong cellular membranous positivity of Her2/neu

protein (3+). The positivity of Her2/neu (3+) was associated with both adenocarcinoma cases (17.5%) and a case of high-grade dysplasia (14.3%), this correlation was highly significant ($P < 0.002$) (Table 3).

Table 3: Her2/neu protein positivity in cases of gastritis, intestinal metaplasia, both low and high-grade dysplasia and adenocarcinoma

Item	Negative	Her2/neu Equivocal	Positive	Total
Gastritis	9 (100%)	0 (0%)	0 (0%)	9
Intestinal Metaplasia	0 (0%)	4 (100%)	0 (0%)	4
Adenoma with low grade dysplasia	7 (70%)	3 (30%)	0 (0%)	10
Adenoma with high grade dysplasia	3 (42.85%)	3 (42.85%)	1 (14.3%)	7
Adenocarcinoma	19 (47.5%)	14 (35%)	7 (17.5%)	40
TOTAL	38 (54.3%)	24 (34.3%)	8 (11.4%)	70

Cross tables; Pearson Chi- Square test; p value = 0.002 (highly significant).

Her2/neu expression by FISH

Within the group of cases showed equivocal Her2/neu protein expression (2+), 17 cases did not display Her2/neu gene amplification, and 11 cases (39.3%) showed gene amplification. All cases with Her2/neu protein positive expression (3+) displayed gene amplification. A negative Her2/neu protein expression case (0/1+) showed gene amplification. The correlation was statistically significant (Fig. 3).

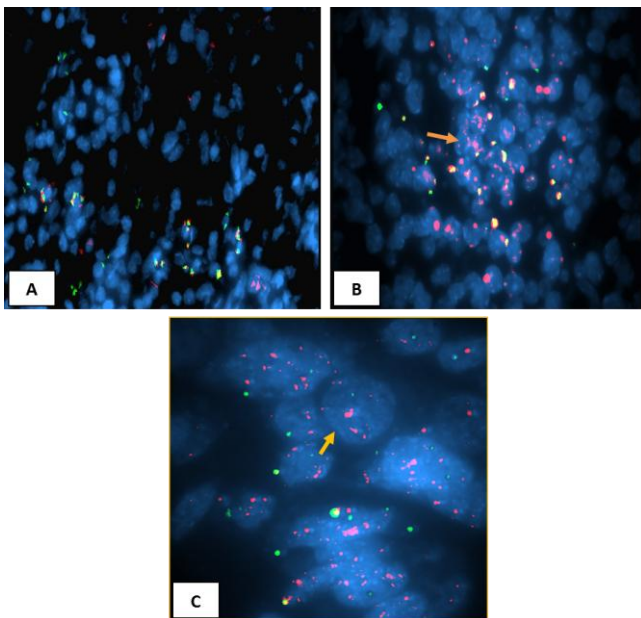


Figure 3: Fluorescent in situ Hybridization using probes directed against Her2/neu in gastric carcinoma (FISH, Her2/neu gene, x1000). A) case of moderately differentiated gastric carcinoma, negative for Her2/neu gene amplification, IHC score 2+, Red signals (Her2/neu gene), green signals [chromosome enumeration probe 17 (CEP17)], blue signals (nuclei stain with DAPI); B) case of moderately differentiated gastric carcinoma, positive for Her2/neu gene amplification, showing red clusters (orange arrow) and was, IHC score 2+; C) case of moderately differentiated gastric carcinoma, positive for Her2/neu gene amplification, showing red clusters (orange arrow), and was IHC score 3+

After IHC and FISH examination, cases with IHC score 3+ or IHC score 2+ with positive gene amplification by FISH positive were considered positive cases, while cases with IHC score 0, 1+ or 2+ without gene amplification were negative cases. Considering both IHC and FISH results, there were 23 of 85 cases included in the study positive for Her2/neu (27%), while 62 cases were negative (73%).

Discussion

Gastric cancer is not a single disease, but a conglomerate of histologically, biologically and genetically heterogeneous diseases, conditioned by the gradual accumulation of various genetic and epigenetic alterations leading to the activation of several molecular pathways resulting in markedly different responses to the same treatment. In the wake of increased molecular pathways underlying the breast cancer, more is actually known about the biological behaviour of gastric cancer and its intrinsic subtypes, particularly the identification of the Her2/neu amplified gastric cancer subtype [14]. The first randomised Phase III trial (ToGA) showed that trastuzumab in combination with conventional chemotherapy is superior to conventional chemotherapy alone in Her2/neu-positive advanced gastric cancer. Therefore, an accurate evaluation of Her2/neu status in gastric cancer has become increasingly important [15]. We found that intestinal-type adenocarcinoma accounts 63.5% of our cases, which is consistent with the reported data by Zeeneldin et al. [16], was 47.6%. Most of our cases belong to moderately differentiated tumours (58.9%), which is in contrast to the other study, that they reported 58.1% of cases belong to tumours with G-III / IV [16].

In our study, 14.2% of GC and GEJ carcinoma cases were Her2/neu-positive (score +3) by IHC. Her2/neu positivity after FISH examination (IHC +3 or IHC +2/FISH positive) was 27%. In the ToGA trial, the percentage of Her2/neu-positive (IHC 3+ or IHC 2+/FISH positive) GC or GEJ cancer patients was 22.1% overall and around 10.4% of IHC 3+ in resected samples [17], Jørgensen [8], found 19% Her2/neu positivity, Chen et al. (18) and Otsu et al. (19) reported a range of 5.1 % to 15.6% for Her2/neu overexpression.

In our research, 13% of intestinal type had an expression (3+). In mixed type, 20% were strongly positive at intestinal type foci, while only one case of diffuse type showed strong positivity [19-23], find a prevalence in intestinal type ranging from 6.1% to 28.57%. The percentage of poorly differentiated tumours with Her2/neu overexpression is about half the percentage of moderately differentiated tumours.

None of the well-differentiated tumours shows expression of Her2/neu, while Shan and his colleagues [24] found that Her2/neu overexpression in the Chinese patient was in 11.1%, 37.5% and 0% in well, moderately and poorly differentiated tumours, respectively. Similarly, Her2/neu overexpression was 16%, 20% and 6% of well, moderate and poor differentiation respectively in the Indian study [25]. Many types of research demonstrate the significant association between Her2/neu overexpression and tumour differentiation [18]. Others do not find this association [26]. These conflicting data may be due to different sample sizes and the low prevalence of Her2/neu in GC and GEJ adenocarcinoma.

In the present study, there is a higher Her2/neu overexpression in T2 category and N1 category with no statistically significant correlation between them this with an agreement with Kataoka et al. [27]- Kunz et al. [28]. In contrast to data reported by Liang et al. [29] that Her2/neu positivity significantly related to TNM stage of gastric cancer.

We found 39.3% of equivocal cases have Her2/neu gene amplification by FISH, while all studied cases score 3+ by IHC showed gene amplification. One case showed negative IHC expression for Her2/neu protein (Score 1+) revealed gene amplification by FISH. These are in agreement with Yan et al. [26] and Shan et al. [30]. In the current study, Her2/neu gene was amplified in all samples with oncoprotein overexpression at 3+ level, which is consistent with the reported data by Yan and his colleagues [26] and reported in 97.5%, 87.5% and 73.9 of the score +3 samples in studies conducted by Shan et al.[30], Gordon et al. [13] and He et al. [31] respectively.

Coping with our findings, many studies enrolled Her2/neu gene amplification in Her2/neu IHC-negative cases in 1.3%- 22.58% (Yan et al. [26] and Gordon et al. [13] and He et al. [31] and Shan et al.[30]. Discrepancy between protein expression and gene amplification indicates that overexpression of Her2/neu in GC, differently from breast cancer, may be regulated by several mechanisms, including transcriptional activation of other genes or post transcriptional events [14], these data highlights the need and importance of further clarifying the relationship between Her2/neu gene amplification and protein overexpression in GC.

We observed Her2/neu immunoreactivity in normal-appearing gastric acini adjacent to a tumour in about 13% of the studied gastrectomy specimens. This matches with the study done by Zhang et al. [32] in Chinese patients samples, which reported Her2/neu overexpression in adjacent non-tumorous tissues in 4 of the 72 gastrectomy specimens of GC. A significant difference for Her2/neu overexpression between the GC and the non-tumor group was observed (18.1 vs. 5.6%, $P < 0.05$). Wang et al. [33] showed that patients with advanced gastric cancer had Her2/neu

expression on the adjacent non-tumor stomach tissues.

We found a significant association between Her2/neu expression and high-grade dysplasia and adenocarcinoma. Thus, a stepwise increase in the expression of Her2/neu was seen in low-grade dysplasia, high-grade dysplasia and carcinoma. In our study, four cases of intestinal metaplasia (100%), three cases of adenoma with low-grade dysplasia (30%) and three cases of adenoma with high-grade dysplasia (42.85%) showed equivocal Her2/neu immunostaining (score 2+). Slesak et al. [34] reported a high frequency of expression of Her2/neu in the chronic gastritis area with intestinal metaplasia in the territory of gastric carcinoma.

Rossi et al. [35] and Hu et al. [36] and Fassan et al. [37] described the upregulation of Her2/neu in gastric dysplastic lesions. Her2/neu overexpression is not an event acquired at a later moment by malignant gastric cells, but represents the initial timing of this process probably occurring in early stages of cancerogenesis with subsequent increase of expression proportionally to disease progression, making possible the Her2/neu test on either the primary tumor or a metastatic tumor with similar results of Marano and Roviello [14], that is explain this finding.

In conclusion, Her2/neu expression in Egyptian patients was comparable to that in other populations; 27% of Egyptian patients with primary GC and GEJ adenocarcinoma were Her2/neu-positive on IHC and FISH. Her2/neu positivity (3+) was common in the mixed, intestinal type and moderately differentiated carcinoma. The results highlight the necessity of FISH test for further categorization when gastric cancer cases are equivocal (2+) by IHC to determine eligibility for the targeted therapy. Stepwise increase in the expression of Her2/neu was seen in low-grade dysplasia, high-grade dysplasia and carcinoma cases implying its role in cancer evolution.

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