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Overexpression of Her1 (EGFR) in Gastric Cancer: A Saudi Regional Population Based Study*

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ABSTRACT

Background: Gastric cancer is one of the commonest malignant tumor worldwide. Its treatment remains a challenge for physicians. Epidermal growth factor receptor (EGFR) inhibitors have played a significant role in the management of solid malignancies including colorectal cancer. In this study we aimed to determine EGFR expression in gastric adenocarcinoma by standardized immunohistochemistry in a Saudi regional population based cohort and also to evaluate Ki-67 proliferating index and p-53 mutation status. **Materials and Methods:** Gastric carcinoma (GC) cases comprising surgical resection specimens and endoscopic biopsies, were selected, from the pathology archives of King Fahd Hospital of the University of Dammam (KFHU), spanning a time period of 6 years. The histological GC type was delineated according to Laurens classification and immunohistochemical (IHC) protein analysis for EGFR, Ki-67 and p-53 was carried out. **Results:** 42 cases of gastric GC were analyzed and EGFR overexpression was demonstrated in 4.76% of cases. Out of these 2.38% had membranous and the remaining demonstrated predominantly cytoplasmic along with focal membranous positivity. Ki-67 proliferation index ranged from moderate to high and and p-53 mutation status was negative in these cases. **Conclusion:** Low EGFR expressivity could be reflective of regional variation in cancer characteristics. The study also highlights the inadequacy of the currently employed gastric EGFR interpretation criterions and stresses on development of standardized and uniform EGFR evaluation protocols tailored for gastric needs.

Keywords: Gastric Cancer; EGFR Overexpression; Immunocytochemistry; Protein Analysis

1. Introduction

Gastric cancer is one of the commonest malignant tumor worldwide and [1-3]. It ranks fourth amongst the most commonly diagnosed cancer, with more than 1,300,000 cases diagnosed yearly [4,5]. The estimated incidence of gastric cancer in the United States was 21,500 in 2008 [6] and a cumulative mortality of esophageal and gastric cancer being approximately 1,100,000 [5], which emphasizes the global challenge in dealing with these diseases. This cancer epitomizes the concept of geographical variation in prevalence distribution with an incidence wise changing and shifting epidemiological trends, and currently East Asia makes up for a significant proportion of new cases [7]. Japan ranks the highest with increasing incidence also seen in many developing countries in Asia, Africa and Latin America [8].

Gastric cancer treatment remains a challenge for physicians [9]. Advanced gastric cancer is associated with poor prognosis, with the resultant, post diagnostic mean survival rate being approximately 10 to 11 months [4]. Targeted therapies based on the evaluation and analysis of the status of target genes [10,11] have been the recent addition in gastric cancer treatment. Molecular therapies constitute monoclonal antibodies or small molecule inhibitors targeting either growth factors or growth factor receptor kinases. Anti-HER2 humanized monoclonal antibody Trastuzumab has been proved to improve patients prognostics in HER2-positive gastric carcinoma [12]. Epidermal growth factor receptor (EGFR) inhibitors have played a significant role in the management of solid malignancies including colorectal cancer. Currently, there are four EGFR inhibitors approved by the FDA including two small molecule tyrosine kinase inhibitors (erlotinib and gefitinib) and two monoclonal antibodies (cetuximab and panitumumab) [13-17]. In gastric carcinoma EGFR is a new field, unraveling new therapeutic options for patients. Recapitulating the pattern of drug-diagnostic Herceptin/HercepTest, patients trialed and evaluated for anti EGFR drugs need to fulfill the prerequisite of IHC evidence of EGFR positive expression. This not only augments the pathologist role in therapy selection but also stresses on their responsibility of providing accurate and reproducible EGFR analysis results [18]. Standardiza-

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tion of EGFR immunoreactivity expression criteria in gastric cancer is undergoing trials and tribulations and requires to be tailored precisely for the endogenous gastric needs as has been accomplished for Her-2 in gastric cancer.

In our study we aimed to determine EGFR expression in gastric adenocarcinoma by standardized immunohistochemistry in a Saudi regional population based cohort and simultaneous evaluation of Ki-67 proliferating index and p-53 mutation status.

2. Materials and Methods

2.1. Specimen Selection and Clinicopathological Parameters

Gastric carcinoma cases comprising surgical resection specimens and endoscopic biopsies, were selected, from the pathology archives of King Fahd Hospital of the University (KFHU), spanning a time period of 6 years, under the approved protocols of the research and ethical committee of University of Dammam. Patient consent was waived due to loss of follow up for old cases. Only gastric adenocarcinomas with availability of representative blocks and sufficient tissue material to perform the required histopatholgical procedures were selected. Gastric neoplasia besides adenocarcinoma, metastatic cancer of the stomach from another organ and metastatic tumor of gastric origin without concomitant histological material from primary tumor in the stomach were excluded from the study. The current study comprises 42 cases of gastric adenocarcinoma

2.2. Histological Classification of Gastric Cancer

The histological GC type was delineated according to Laurens classification, this classification divides adenocarcinomas into two types: ***intestinal*** (consisting of well formed tubules) and ***diffuse*** (diffuse tumor infiltration without well formed tubules, frequently with signet ring cells) [19].

2.3. Immunohistochemistry

Immunohistochemical staining using the labeled streptavidin-biotin (LSAB) method with 3,3'-diaminobenzidine (DAB) as a chromagen was performed for EGFR (HER1), p-53 and Ki-67 on 4 µm thick paraffin sections cut from conventional blocks. The staining was performed concurrently in a Ventana Benchmark automated immunostainer according to the manufacturer's instructtions (Ventana Medical Systems Inc., Strasbourg). Sources and dilutions of the primary antibodies used in the study are listed in **Table 1**. The immunostained sections were examined under a light microscope and evaluated manually by 2 pathologists (AA and DT). Any interpretational discrepancies were resolved under a double-headed microscope.

Table 1. Sources and dilutions of primary antibodies used in the study.

Antibody	Clone	Manufacturer	Dilution
EGFR	5B7	Ventana	Prediluted
Ki-67	MIB-1	Dako	Prediluted
p-53	DO-7	Dako	Prediluted

EGFR = epidermal growth factor receptor.

2.4. Evaluation of Immunostaining

EGFR: Both membranous and cytoplasmic staining were considered for evaluation. The membranous positivity for EGFR (mEGFR) was evaluated in the following manner: 0, no discernible staining or background type staining; 1+, equivocal discontinuous membrane staining; 2+, unequivocal membrane staining with moderate intensity; and 3+, strong and complete plasma membrane staining. More than 10% of the cells were required to meet the criteria for EGFR analysis. Scores of 2+ and 3+ staining levels were considered to be EGFR overexpression [9]. For positive cytoplasmic EGFR (cEGFR) staining, an intense homogenous staining of the cytoplasm was necessary. There was no cEGFR without membrane stainin [20].

Ki-67: Positive staining was defined as positive nuclear staining. Cytoplasmic staining was considered negative. The percentage of positive nuclei was expressed as a "Ki-67 labeling index" which is the percent of cells expressing Ki-67 determined by counting 1000 cells/slide. The percentage of positive cells was scored as follows: less than 10% = low proliferative activity, 10% - 40% = moderate proliferative activity, and more than 40% = high proliferative activity [21].

P53: Positive staining was defined as positive nuclear staining. Cytoplasmic staining was considered negative. Tumors were considered focally positive when unequivocal staining was present in 10% - 50% of tumor cells, and as diffusely positive when more than 50% of the tumor cells were positive [22].

2.5. Statistical Analysis

Data was entered into SPSS windows. Frequencies were calculated using descriptive statistics for categorical variables.

3. Results

Out of a total of 42 cases of gastric cancer retrieved, 38 specimens were biopsies and 4 were partial gastrectomies. Male to female ratio was 25:9 and median age of the pa-

tients was 67 years (extreme 88 - 42 years). Diffuse type gastric cancer was seen in 23 (54.76%) and Intestinal type in 19 (45.23%) of cases. EGFR expression pattern is shown in **Table 2** and **Figure 1**. Ki-67 proliferation index and p-53 mutation status is given in **Tables 3** and **4** respectively. In cases showing EGFR overexpression (n = 2) p-53 mutation status was negative and Ki-67 ranged from moderate to low.

The **Figure 2** shows H & E staining in gastric carcinoma cases while **Figure 3** shows the respective immunohistochemical panels.

4. Discussion

We demonstrated a low EGFR overexpression by protein analysis, comprising a mere 4.76% of 2+ intensity and none with 3+ immunoreactivity. Out of these 2.38% had membranous and the remaining demonstrated predominantly cytoplasmic along with focal membranous positivity. This low positivity and pattern of expression arouses several discussable issues. Firstly whether this expressivity could be part of the concept of ethnic variation in cancer distribution or could it be merely reflective of the marked variations in the intrinsic protein accentuating potential by various commercially available kits. Also they grey zone of moderate cytoplasmic positivity needs to be stressed on as regards whether these tumors be trialed for further genetic evaluation and selected for anti-EGFR therapy, the sensitivity and long term prognostic benefits of which are currently being debated. Both of the cases were endoscopic biopsies and 10% of tumor showing positivity was the cut off point. Is this criterion applicable only for the gastrectomy specimens or needs to be downgraded as in Her-2neu positivity, where presence of just 5 positive cells now justifies institution of targeted therapy in small biopsies?

Regarding tumor related ethnic variation a tremendous heterogeneity is seen in terms of epidemiology, tumor histology and expression of molecular markers in gastric cancer [23]. EGFR overexpression has been documented to show marked ethnic variations. Mammano *et al*. detected EGFR protein expression in only 6% of the cases, and concluded in their study that EGFR protein expression is low and specific EGFR gene mutations are very rare or absent in gastric adenocarcinoma [24]. A some what similar expression pattern is also reported by Takehana *et al.*, who, in their series of 413 gastric carcinomas, found negative EGFR protein expression in 89.6%, low level in 8.2% and high level in 2.2% of cases [25], Lee *et al*., who found no EGFR gene mutations in 185 gastric adenocarcinomas in a series of Korean patients [26], and by Mimori *et al*., who found a silent mutation in exon 20 in only 5 of 39 (5.1%) Japanese patients [27]. These studies are in agreement with our study that also shows a very low EGFR expressivity but in contrast with multiple studies documenting a much higher prevalence pattern.

Table 2. EGFR overexpression in gastric cancer (n = 42).

Staining intensity	N	% age
0	34	80.95
1+	6	14.28
2+	2	4.76
3+	0	0

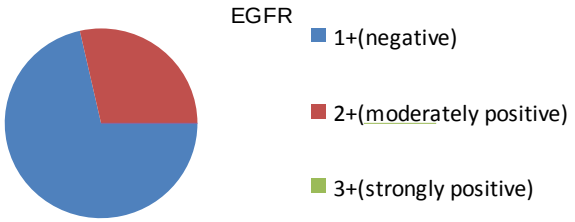

Figure 1. EGFR overexpression in gastric cancer (n = 42).

Table 3. Ki-67 Proliferation Index in gastric cancer (n = 42).

Proliferative activity	N	% age
Low	6	14.28
Moderate	14	33.33
High	22	52.38

Table 4. p-53 mutation status in gastric cancer (n = 42).

p-53 status	N	% age
Negative	22	52.38
Focal	10	23.80
Diffuse	10	23.80

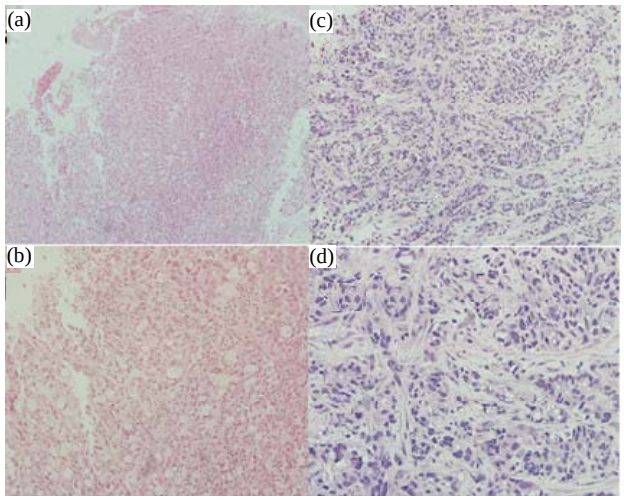

Figure 2. Gastric Carcinoma H & E. (a) Diffuse gastric carcinoma H & E ×20; (b) Diffuse gastric carcinoma H & E ×40; (c) Intestinal type gastric carcinoma H & E ×20; (d) Intestinal type gastric carcinoma H & E ×40.

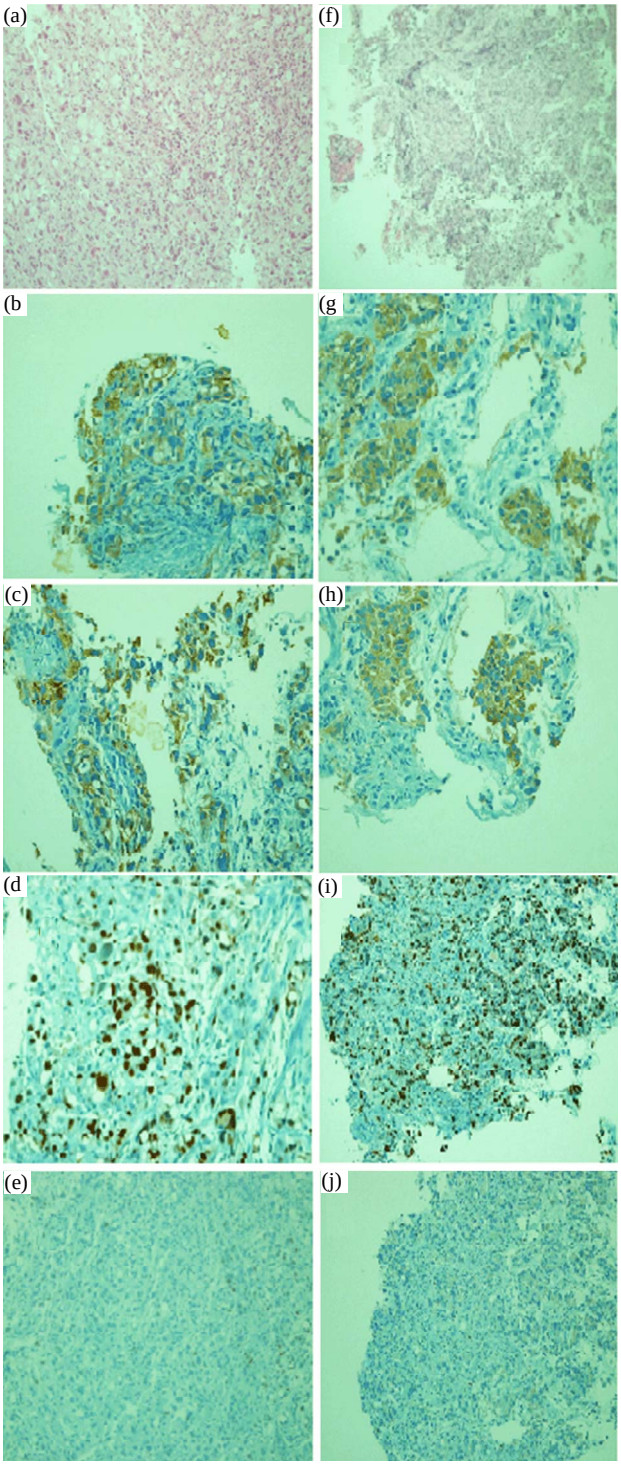

Figure 3. Gastric carcinomas with panel of IHC. (a) Diffuse gastric cancer ×40 H & E; (b) EGFR ×20 2+ (Cyto + memb); (c) EGFR ×40; (d) Ki-67 moderate; (e) p-53 negative; (f) Intestinal Type gastric cancer ×40 H & E; (g) EGFR ×20 2+ (cyto); (h) EGFR ×40; (i) Ki-67 high; (j) p-53 negative.

Liang *et al*. documented 41% of EGFR expression by IHC in a subset of Chinese patients; with 16% also revealing FISH positivity [9]. In a study by Galizia *et al*. in an Italian population with an aim to correlate EGFR positivity by IHC with disease recurrence and survival in univariate and multivariate analyses, forty-four percent (36 cases) of gastric cancers were EGFR positive [28]. These multiple studies highlight the concept that specific EGFR mutations show a wide regional and ethnic variation in gastric adenocarcinoma, a fact substantiated in other tumors also, as demonstrated by Paez *et al.*, who found a significant difference between the mutation rate of EGFR gene in non small cell lung carcinoma(NSCLC) from USA (2%) and from Japan (26%) [29].

The low EGFR expressivity pattern may also be attributed the type of kit employed for immunostaining. In a comparative study conducted by Lee *et al*. in NSCLC different intensities of staining were discerned by different commercially available kits. EGFR protein overexpression was observed in 56% of tumors with Zymed EGFR kit, in 51% with Dako EGFR pharmDx kit, in 5% with Dako and in 18% with Novocastra. Both Zymed and Dako pharmDx kit were more sensitive than the Dako test (clone H11) and Novocastra clone EGFR 113. They concluded that EGFR protein overexpression rate varied from 4% to 72% according to different antibody clones and histologic subtypes, and EGFR protein expression detected by Zymed and Dako pharmDx was significantly associated with a high EGFR gene copy number [30]. We had employed Ventanna kit for our study. Our pattern of expression could be attributed to the different intrinsic sensitivity of the protein highlighting potential of the kit. More extensive comparative studies regarding standardization and harmonization of the different methodologies employed for EGFR protein expression, need to be carried out so such marked variations, if existent with our logistics, may not get manifested.

Regarding membranous pattern of immunoreactivity, in our study 2.38% of cases had a heterogenous pattern of membranous staining, with mostly 2+ pattern but with scattered individual cells showing intense complete membranous 3+ pattern of expression. These cells however did not fulfil the 10% criteria to accord 3+ to the case as a whole. The EGFR IHC interpretation criteria in gastric cancer is essentially the same as in breast malignancies. Does it need to be questioned, modified and downgraded as in Her-2 neu testing in gastric cancer where after validation in TOGA trial [31], the criterion is less strict and 10% cut-off rule applies to resection specimen whereas in biopsies any group of at least 5 tumour cells showing immunoreactivity satisfies the positivity standard and in addition [31] the completeness of membrane staining is not a “condition sine qua non” [12]. In EGFR IHC interpreting protocol, no such downgrdation and revision has been implemented. The intratumoral heterogeniety, more significant in small endoscopic biopsies mandates modification of expressivity

criterions and need them to be divergent from those accepted and validated for breast cancers.

As far as cytoplasmic pattern of expression is concerned, 2.38% of cases in our study revealed, diffuse moderate cytoplasmic staining with focal membranous accentuation. Although marked, diffuse cytoplasmic staining has been considered by some studies [20] but no well defined protocols have been delineated and inclusion of these cases for further genetic evaluation and incytoplasmic positivity needs to be standardized and validated as recent studies have proved EGFR to act as a cytoplasmic/nuclear shuttling transcription factor [32] with its activation and subsequent nuclear translocalization, leading to regulation of gene expression and mediation of specific cellular processes [33,34]. This is distinct from EGFR mediated transduction of mitogenic signals through activating multiple signaling cascades [35]. Could tracking into this cytoplasmic positivity unmask a potential valid anti EGFR therapy candidate, yet needs to be ascertained. EGFR inspite of being a promising target in cancer therapy, yet failed to acquire definitive, sensitive potential accurate molecular predictors of sensitivity to EGFR inhibitors for patients having gastro-esophageal cancers [36]. The dichotomous cytoplasmic and membranous staining in other tumors holds prognostic significance as reported in renal cell carcinoma [37] with its association with worsening progression and prognosis [38]. In gastric cancer, however, such confirmation yet remains to be done.

Ki-67 proliferation index in our cases with EGFR immunoreactivity ranged from moderate to high, implicating these tumors to be aggressive. EGFR expression correlates with disease recurrence and poorer survival [28]. This is substantiated by other studies as well in which EGFR overexpression is associated with an aggressive tumor and also with lymph node metastasis [39]. EGFR dimerization and activation leads to downstream upregulation of multiple processes that can result in cancer cell proliferation, reduced apoptosis, tumor-induced angiogenesis, and activation of invasion, dedifferentiation of cancer cells and enhanced metastatic potential [40,41].

The role of p-53 as a prognostic factor in gastric cancer is controversial. One school of thought describes expression of EGFR and p-53 to have a negative correlation with patients prognostics [42-44]. In a study on an arab population the expression of p53 was found to correlate with aggressive gastric cancer characteristics [45]. On the other hand, studies have reported p53 to have no influence on prognosis [46,47]. Liu *et al*. demonstrated p53 nuclear reactivity in association with bax and c-myc and with well-differentiated histology and with no prognostic significance [48]. In our study the overall p-53 reactivity was found to be in 47.60% of cases with diffuse positivity in 23.80% and focal positivity also in 23.52% but in both the EGFR expressing cases, it was non reactive. The cases comprised equal percentage of diffuse and intestinal types. Multiple genetic and epigenetic alterations have been found to underlie gastric carcinogenesis with different combinations becoming apparent in the two histological types of gastric cancer. p-53 mutation, reduced p27 expression, cyclin E expression etc in the intestinal-type and LOH at chromosome 17p, mutation of p53 and mutation or loss of E-cadherin to name some in the poorly differentiated gastric cancers [49]. Although p-53 mutation is seen to formulate an integral overlapping component in both the types the lack of expression in our cases could imply other molecular alterations besides p-53 mutation to be more operative.

5. Conclusion

Low EGFR expressivity could be reflective of regional variation in cancer characteristics. The study also highlights the inadequacy of the currently employed gastric EGFR interpretation criterions and stresses on development of standardized and uniform EGFR evaluation protocols tailored for gastric needs.

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