

## To Evaluate the Utility of EGFR and HER-2/NEU Immunoexpression Pattern in Primary Gastrointestinal Tract Malignancies as Surrogate Genetic Prognostic Markers

Prabal Deb\*, Aniruddha Sahu\*\*, Anamika Sinha\*\*\*

\*Associate Professor, Department of Pathology, Armed Forces Medical College, Pune-411040, Maharashtra, India.

\*\*MBBS Student, Department of Pathology, Armed Forces Medical College, Pune-411040, Maharashtra, India.

\*\*\*Graded Specialist (Pathology), Command Hospital, Udhampur, Jammu & Kashmir, India.

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### Abstract

**Background:** Gastrointestinal tract (GIT) cancers have a varied presentation and different prognosis for similarly staged disease. In addition to breast carcinoma, overexpression of components of the HER signalling pathway have been associated with poor outcomes and a more aggressive disease even in primary GIT cancers. To evaluate the utility of EGFR and HER-2/Neu immunoexpression pattern in various primary GIT malignancies as surrogate genetic prognostic markers in comparison to convention histological parameters. **Methods:** A retrospective analysis of formalin fixed, paraffin embedded (FFPE) sections of tumour tissue from 30 GIT resection specimen were done. EGFR and HER2/Neu immunoexpression were evaluated against the histological grade and stage of the tumour. **Result:** In the study population the mean age was 58.4 years. Adenocarcinoma constituted 80% cases, with 73.33% being moderately differentiated. T3 tumours (60% cases) were the most frequently encountered malignancy. Lymphovascular invasion was seen in 13.33% while nodal positivity was noted in 64.29% cases. HER2 overexpression was seen in three cases (10%) of oesophageal squamous cell carcinoma while EGFR overexpression was found in four cases (13.33%). Overall, cases showing EGFR and HER-2 overexpression were mostly of the elderly age group, oesophageal squamous carcinomas, were T2/T3 in size and with nodal involvement. **Conclusion:** EGFR and HER2 have a valuable role as ancillary prognostic markers in GIT malignancies. However standardization of EGFR and HER2 testing procedures along with careful interpretation are essential steps to ensure accurate, reproducible and optimal results.

**Keywords:** EGFR; Genetic prognostic factors; GIT malignancies; HER-2.

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### Introduction

Primary gastrointestinal tract (GIT) carcinomas (involving oesophagus to stomach to colorectal region) account for a large number of cancer-related deaths.[1] Despite the improvement in surgical skills and perioperative management with newer chemotherapeutic and / or radiotherapy regimes, prognosis remains poor.[2] This has led to seeking novel

treatment modalities such as molecular-targeted therapy, which includes small molecule inhibitors of tyrosine kinases (e.g. gefitinib) and humanised monoclonal antibodies, in the form of cetuximab and trastuzumab.

The HER family of receptor tyrosine kinases consists of four members: epidermal growth factor receptor (EGFR: HER-1), HER-2, HER-3, and HER-4. This signalling pathway has an important role in modulating cell proliferation, survival, migration, and differentiation.[3] In the absence of a direct ligand for HER-2, unlike the other members, it is suggested that HER-2 is the preferred heterodimerisation partner for all other HER family members, and that the primary function of HER-2 is as a co-receptor. [3] HER-2 is located on chromosome 17q21 and

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**Corresponding Author:** Colonel Prabal Deb, Associate Professor, Department of Pathology, Armed Forces Medical College, Pune - 411040, Maharashtra, India.

E-mail: debprabal@gmail.com

it encodes a 185kD transmembrane protein that lacks a natural ligand. HER-2 activation initiates signal cascades including the MAPK (mitogen activated protein kinase) and PI3K/AKT (3-kinase) pathways that are essential for cell proliferation and differentiation.[4]

There are several potential strategies for anti-HER family targeting. Two anti-HER family-targeting therapies that have been in clinical development are small-molecule EGFR tyrosine kinase inhibitors such as gefitinib[5] and humanized antibodies against the HER family represented by cetuximab and trastuzumab.[6] The antitumour activity of cetuximab and trastuzumab have been thought to be a resultant of either a direct inhibition of EGFR tyrosine kinase activity[7], the inhibition of cell cycle progression[8], or due to increased levels and activities of pro-apoptotic molecules.[9]

Overexpression of HER family members has been well characterized in breast carcinoma, and has recently been described in variety of cancers including gastrointestinal tract (GIT). Studies have indicated that oesophageal SCC shows a relatively high incidence of EGFR and/or HER-2 overexpression, with Mimura *et al* [10] noting an overexpression of HER-2 in 30.3% oesophageal SCC. In most malignancies overexpression of EGFR and/or HER-2 has been definitively correlated to poor outcomes and a more aggressive disease.[1]

To present study was performed to evaluate the utility of EGFR and HER-2/Neu immunoeexpression pattern in various primary GIT malignancies as surrogate genetic prognostic markers in comparison to convention histological parameters.

## Material and Methods

### Cases

A total of 30 gastrointestinal tract resection specimens (that included cases of oesophageal, gastric or colorectal malignancy) received in a large tertiary care center, were included in the study.

### Sample Processing and Evaluation

All resected specimen of gastrointestinal malignancies incorporated in the study was evaluated for the type and extent of resection, location, size and gross appearance of lesion (ulcerative or proliferative growth), status of apparently uninvolved mucosal lining, and lymph node involvement.

Representative sections were taken as per institutional protocol, which included margins of resection, full thickness of wall at area of maximum induration and from apparently uninvolved mucosa.

### Routine Histological Processing

Specimens were fixed in buffered formalin and paraffin-embedded. Five - seven micron serial sections stained by routine hematoxylin-eosin (H&E) were studied under light microscope (LM). The type of tumour and histologic extent, along with lymph node status were recorded in all cases.

### Immunohistochemistry (IHC)

Representative formalin-fixed paraffin-embedded (FFPE) sections of 4-5µm from tumour areas were stained immunohistochemically using labeled streptavidin biotin (LSAB) technique. After deparaffinisation and rehydration, the sections were autoclaved in 0.01 M citrate buffer (p<sup>H</sup> 6.0) at 121°C for 10 min. Then, the sections were cooled at room temperature for 60 min, immersed in 3% hydrogen peroxidase for 10 min to block endogenous peroxidase activity, and then washed in phosphate-buffered saline (PBS) for 5 min.

**EGFR:** To detect EGFR, mouse anti-human EGFR mAb (DakoCytomation, Denmark) were used. After initial digestion using proteinase K for a period of 40 minutes, the sections were incubated with the antibody (diluted 1 : 100) for 01 h at 4°C in a moist chamber. After washing three times with phosphate buffer saline (PBS) for 5 min, the sections were reacted

with the secondary antibody (biotinylated anti-mouse antibody) for 30 min at room temperature. Then the sections were washed again three times with PBS for 5 min after which they were reacted with peroxidase-conjugated streptavidin for 30 min at room temperature. Finally, the sections were washed three times with PBS for 5 min and then reacted with a solution containing 0.06mM 3,3'-diaminobenzidine and 2mM hydrogen peroxide in 0.05% Tris-HCl buffered at pH 7.6 for 10 min. They were then counterstained with haematoxylin for 30 seconds. After dehydrating with 60-100% isopropyl alcohol, penetrating, and mounting, the sections were observed under light microscope.

**HER-2:** Deparaffinised and rehydrated tissue sections were incubated with the Retrieval Solution in a hot water bath for 40 min at 95-99°C. Then, the sections were cooled to room temperature for 20 min, washed with Tris buffer for 5 min, and endogenous peroxidase was blocked with 3% hydrogen peroxide for 5 min. The anti-HER2 antibody (DakoCytomation, Denmark, prediluted) was used. The sections were washed with Tris buffer for 5 min and incubated with the primary antibody at room temperature for 30 min. After rewashing with Tris buffer for 5 min twice, steps similar to above were followed, till visualisation.

**Evaluation:** EGFR or HER-2 immunopositivity were evaluated by scoring the intensity of reactivity using four categories:

\*0: negative, no discernible staining/background type staining/membranous staining in <10% tumour cells

\*1+: definite cytoplasmic staining/faint, discontinuous membrane staining in >10% tumour cells

\*2+: unequivocal membrane staining with weak to moderate intensity in >10% tumour cells

\*3+: strong and complete plasma membrane staining in >10% tumour cells

A score of 0/1+ was considered negative, scores of 2+ or 3+ was considered positive.

Cytoplasmic staining that may have been present with absence of membrane staining was considered negative.

*Correlation of Results*

Data of relevant clinical and gross examination, along with tumour size, histologic type and lymph node status were correlated with EGFR and HER-2 expression.

**Table 1 : Summary of Clinicopathological Profile of Cases [N = 30]**

<b>Sex Ratio ( M:F)</b>	1.72 : 1
<b>Age at Surgery (Years)</b>	
Range	19 – 87
Mean	58.4
<b>Location</b>	
Oesophagus	5 (16.7%)
Stomach	7 (23.3%)
Small Intestine	1(3.3%)
Large Intestine	17 (56.7%)
<b>Histology</b>	
Squamous cell Carcinoma	5 (16.7%)
Adenocarcinoma	24 (80%)
Neuroendocrine Carcinoma	1(3.3%)
<b>Tumour size</b>	
T1	0
T2	8 (26.7%)
T3	18 (60%)
T4	4 (13.3%)
<b>Metastasis</b>	
Lymphovascular Invasion	4 (13.3%)
Lymph node Metastasis	17/28 (60.7%)
<b>Immunohistochemistry profiles</b>	
<b>HER-2</b>	
0	14
1+	13
2+	1
3+	2
<b>EGFR</b>	
0	20
1+	6
2+	2
3+	2

(N=total number of cases, M= Male, F=Female, T= Tumour, HER2 =Human Epidermal Growth Factor Receptor 2, EGFR= Epidermal Growth Factor Receptor)

**Table 2: Histological Types and Grade of Tumours**

Histological subtypes	Well differentiated	Moderately differentiated	Poorly differentiated	No of Cases
Squamous cell carcinoma	3	2	0	5
Adenocarcinoma	1	20	3	24
Neuroendocrine carcinoma	-	-	-	1

**Table 3: Profile of Cases with HER-2 and EGFR Overexpression Status**

IHC Marker expression	Age	Sex	Site	Histological type & grade	Tumour size	LVI	LN status
HER-2 ++	69	F	Oesophagus	SCC-MD	T2	-	N0
HER-2 +++	68	M	Oesophagus	SCC-MD	T3	-	N1
	74	M	Oesophagus	SCC-WD	T2	-	N0
EGFR ++	69	M	Stomach	NEC	T3	+	N3
	55	M	Ascending colon	ADCA-MD	T3	+	N1
EGFR +++	68	M	Oesophagus	SCC-MD	T3	-	N1
	45	M	Oesophagus	SCC-WD	T3	-	N0

(ADCA-MD: adenocarcinoma – moderately differentiated; EGFR= Epidermal Growth Factor Receptor; F : female; HER-2 =Human Epidermal Growth Factor Receptor 2; LN: lymph node; LVI: lymphovascular invasion; M: male; NEC: Neuroendocrine carcinoma; T: Tumour; SCC -MD: Squamous cell carcinoma – moderately differentiated; SCC -WD: Squamous cell carcinoma – well differentiated)

## Results

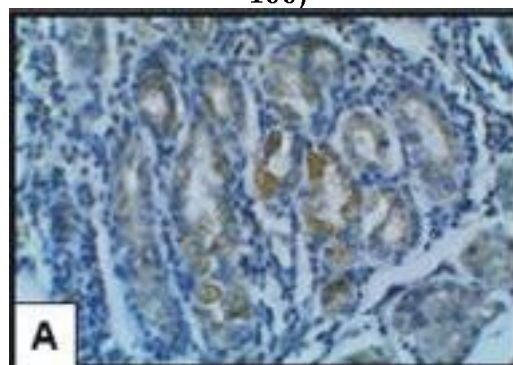
The study population consisted of 19 males and 11 females (M:F::1.72:1). Majority (36.67%) of the cases were in the sixth decade of life. The study data is summarized in Table 1.

The cases consisted of cancers of the oesophagus, stomach, small intestine and large intestine. Majority (56.67%) of the cases were colorectal adenocarcinomas. The esophageal cancers were all squamous cell carcinomas. The seven gastric malignancies constituting 23.33% cases, and only a single case of duodenal carcinoma was seen.

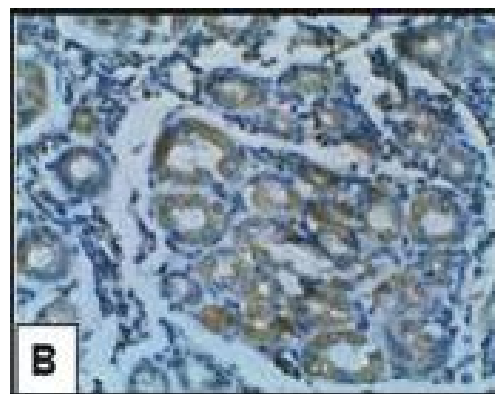
On histology by routine hematoxylin and eosin (H& E stain), 24 cases (80.00%) were adenocarcinomas. Three adenocarcinomas were of the mucinous type, all of which were located in the colorectal region; while two cases were of the signet ring type, one each involving the stomach and colon.

Of the adenocarcinomas, majority (83.33%) were moderately differentiated, while three were poorly differentiated, which included the two cases of signet ring type malignancies. Only

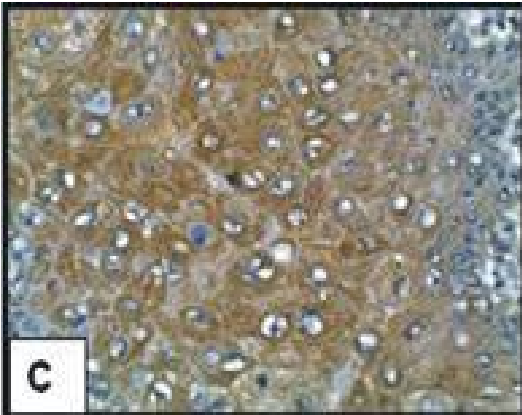
**Figure 1: Immunostaining Patterns Using HER2 Antibody: Showing 1+ (A: HER2 x 100)**



**2+ (B: HER2 x 100)**



3+ (C: HER2 x 100)



one case showed a well-differentiated histology. In contrast, squamous cell carcinomas of the oesophagus consisted of three well differentiated and two moderately differentiated neoplasms. Details of the histological subtypes and grades of differentiation are depicted in Table 2. The T4 tumours consisted of three moderately differentiated adenocarcinomas and one well differentiated squamous cell carcinoma.

Lymphovascular invasion was noted in 4 cases (13.33%), which included two cases of poorly differentiated adenocarcinoma, and one case each of neuroendocrine carcinoma and well differentiated squamous cell carcinoma. Eighteen of the twenty eight cases (64.29%) showed lymph node involvement. Two cases of gastric carcinoma showed extensive nodal involvement, which included a case of moderately differentiated adenocarcinoma and a neuroendocrine tumour, which showed involvement of 16 of 22 nodes and 12 of 16 nodes, respectively. In all but a solitary case of mucinous adenocarcinoma the surgical margin of resection was free of tumour deposits.

#### *Immunohistochemistry Profile*

**HER2 Status:** The overall immunoexpression of HER2 was encountered in 16 cases (53.33%), however it was only weak expression (1+) in 13 cases. Only the esophageal SCC ( 3 cases ) showed 2+/3+ scoring. Interestingly one well differentiated SCC showed a 3+ score.

#### *Epidermal Growth Factor Rreceptor (EGFR)*

**Status:** The overall immunoexpression of EGFR was encountered in 10 cases (33.33%), of which majority (6 out of ten) showed 1+ expression, 2 cases (a moderately differentiated adenocarcinoma and the neuroendocrine tumour), displayed 2+ positivity, and 2 cases (a well differentiated and a moderately differentiated squamous cell carcinoma), displayed 3+ immunoreactivity. Composite profile of cases with HER2 and EGFR overexpression status is presented in Table 3.

### **Discussion**

Tumour grade and TNM stage are the two most important conventional prognostic factors in cases of primary GIT malignancies. However it has been observed that within the same stage there is considerable variation in prognosis. This has served as an impetus to search for additional prognostic markers in order to identify the biologic subsets of this disease and further refine the process of prognostication.

Various biological prognostic factors are often derived from the genetic process, which are thought to represent crucial steps in carcinogenesis of GIT tumours (HER2, EGFR, E-cadherin, DNA copy number changes, microsatellite instability, and changes in expression of several factors including thymidilate synthase, beta-catenin, mucin antigen, p53, COX-2, matrix metalloproteinases, and vascular endothelial growth factor receptor).[11]

The four diffrent receptors of the HER family, viz. HER1 (EGFR or ErbB1), HER2 (ErbB2 or HER-2/Neu), HER3 (ErbB3), and HER4 (ErbB4) are implicated in the development of diffrent kinds of tumors and are now recognized targets for biological therapy in breast, colorectal, lung, head and neck, gastric and gastro-oesophageal junction cancer.[12]

With the availability of trastuzumab (Herceptin), a monoclonal antibody, which specifically targets HER2 protein, and erlotinib

inhibiting EGFR, there is a growing interest to look at the expression of these in various cancers. Similarly cetuximab and panitumumab, which are directed against the EGFR, have proven efficacy in the treatment of metastatic colorectal cancer (mCRC).[13]

An alternative anti-HER2 strategy has been the development of small-molecule tyrosine kinase inhibitors that target not only HER2 but also other proteins of the HER family. The simultaneous inhibition of multiple receptors is an attractive strategy, as interactions between HER2 and EGFR provide a mechanism for signal diversification and augmentation. An example of such a drug is Lapatinib (Tykerb), which is a potent ATP-competitive inhibitor that simultaneously inhibits both EGFR and HER2.[14] Thus there is a need to simultaneously assess both these potential biomarkers, which constituted the basis of the current study in primary cancers of the GIT.

As regards EGFR expression, the present study revealed strong immunopositivity in 40% of the oesophageal SCC. All these cases had more advanced lesions, with higher scores for both pTNM classification and tumor staging. Interestingly detailed analysis of one well differentiated SCC that showed 3+ immunostaining revealed higher TNM stage with lymphovascular invasion. In a study on esophageal SCC by Gotoh *et al* it was noted that only positive immunostaining for EGFR significantly correlated with primary CR for CRT on multivariate analysis.[15] Similarly, Gibault *et al* reported that diffuse EGFR immunostaining showed association with reduced overall survival.[16] As we had only 2 cases and no survival data, it was difficult to draw any specific conclusion other than the fact that the process was validated as per the protocol.

In contrast, most of the adenocarcinomas (29%) in our study showed only weak EGFR immunostaining (1+ to 2+). Sano *et al* had reported EGFR to be overexpressed in anywhere from 25% to 82% of colorectal cancers.[17] Recently Rego *et al* found membranous EGFR expression in 214 (59%) colon carcinomas using

the EGFR PharmDx kit.[18] Analyses performed by immunohistochemistry (IHC) indicate an EGFR protein expression in 60-80% of colorectal tumors. Some recent studies report protein overexpression (defined as 2+ and/or 3+ staining or in >50% of cells) in 35 to 49% of cases.[7-9] Apart from the vagaries of the staining process or the attributes of tumour biology itself, the possible reason for the low weak scores in our study may be attributed to the strict criteria of "membrane staining" that we adopted. Atkins *et al* have concluded that amongst various solid tumors, evaluation of EGFR expression by IHC is dependent on storage time of archived tissue sections. This feature is especially true for colorectal adenocarcinomas, where it is recommended that EGFR immunoreexpression should be tested within the first nine months to avoid false-negative results.[19] However, owing to the small sample size we could not extrapolate the data to analyze the exact cause.

However, despite a high immunoreexpression of EGFR only a fraction of IHC-positive tumors also show EGFR gene amplification.[13] In the study by Tsuchihashi *et al* it was observed that EGFR gene mutations are rare in colorectal cancer and have no clinical relevance with regard to the activity of anti-EGFR therapy.[20] Recently in a meta-analysis Yu *et al* found over-expression of EGFR was significantly correlated with, not only the lymph node status and tumour differentiation grade, but also the poorer OS.[21]

The current study revealed HER2 immunopositivity in all cases of oesophageal SCC, albeit of varying degree, with only 2 cases (40%) displaying strong 3+ immunoreactivity. In the series of Zhan *et al*, 41% esophageal SCC showed HER-2-immunoreexpression with 10.4% showing 3+ scores. They also showed that there was a significant difference in survival rates in cases with and without HER-2/neu overexpression or amplification, and that it was an independent prognostic marker for disease free survival.[22] Similarly Khan *et al* (based on many studies of HER2 and esophageal SCC) concluded that oesophageal SCC showed overexpression of ErbB2 expression, a feature

that was associated with poor prognosis, and suggested that targeting it could be of therapeutic benefit.[23]

In the index study, only 9/25 cases of adenocarcinoma showed weak HER-2-immunopositivity (1+). In a recent study, Gill *et al* found Her-2/neu was positive in 65% of their cases, while considering both cytoplasmic and membranous staining as criteria for positivity, unlike the “membranous positivity” utilized by the current study. On further scrutiny only 7.5 % of their cases showed cytoplasmic-membrane positivity.[24] On the other hand Schuell *et al* in their study found only 26% of their cases showing weak positivity and 3% strong positivity. They also noted that HER-2/neu staining (moderately and strongly positive) was only detected in primary tumours of patients with confirmed metastases.[25] Similar conflicting data exist about the prevalence of HER-2/neu overexpression in colorectal cancer, which ranges from 0 to 83%. [25-26]

Overall, the available literature reflects that the correlating the expression of HER2 and/or EGFR to patient prognosis is fraught by various challenges and continues to be shrouded in controversy that emanates from the variations in the biology of the receptor, its interactions with other members of the family and myriad downstream effects.[13] Further, overamplification of the gene does not always translate into/correlate with protein overexpression.[27] These issues are further complicated by the problems of tissue processing, adoption of different IHC staining protocols, different sources of antibodies, and non-availability of an universally standardized and accepted procedure for staining and evaluation.[28]

## Conclusion

Data from trials like the ToGA and LOGiC [29] for HER-2 in gastric cancer has shown that it is important to understand the evolving tumour biology and judiciously use these markers, in a standardized fashion, as a primary

screening process.[30]

The success of a standardized method of reporting and interpreting HER2 in breast carcinoma is a way forward. Thus standardization of EGFR/HER2 testing procedures and careful interpretation is, therefore, an essential step to ensure accurate and reproducible results. The availability of newer molecular diagnostic tools and plethora of specific monoclonal antibodies makes it imperative that we move ahead cautiously with well validated studies and consensus.

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