

CYTOPLASMIC EXPRESSION OF HER2 IN GASTRIC ADENOCARCINOMA: AN UNUSUAL FINDING.

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Abstract

Aim: To study the prevalence of HER-2 expression in gastric adenocarcinoma and its correlation with *Helicobacter pylori*.

Method: 50 gastric cancer biopsies were included in the study. Sections were stained with H&E for morphological details and classified into intestinal and diffuse type gastric adenocarcinoma. Loeffler's methylene blue was used for identification and grading of *Helicobacter pylori*. Immunohistochemistry for expression of HER2 protein was done. Pattern of staining was noted and scoring was done. Monoclonal mouse Anti-HER2/neu (c-erbB2) clone CB11 was used.

Statistical analysis was done using chi square test and spearman rank correlation coefficient. $P \leq 0.05$ was taken as critical level of significance.

Result: Out of 50 cases 9(18%) were intestinal and 41(82%) were diffuse type adenocarcinoma. *Helicobacter pylori* were seen in 8/9 (88.89%) and 35/41(85.36%) cases of intestinal and diffuse type adenocarcinoma respectively. HER2 positivity was recorded as membranous, membranous + cytoplasmic and cytoplasmic. 55.56% of cases in intestinal and 73.14% in diffuse type adenocarcinoma showed cytoplasmic ± membranous staining. Expression of HER2 scoring was compared with increasing grades of *Helicobacter pylori* infection, a significant association ($P < 0.05$) but weak correlation was found between *Helicobacter pylori* and HER2 expression.

Conclusion: HER2 overexpression is significantly associated with *Helicobacter pylori* however correlation is weak. Predominant cytoplasmic pattern of expression of HER2 in diffuse gastric adenocarcinoma is unusual and needs further investigation.

Introduction

Stomach cancer is the fifth leading cause of cancer and the third leading cause of death from cancer making up 7% of cases and 9% of deaths (World Cancer Report 2014). In India, gastric cancer is ranked the 5th and 7th most common cancer in males and females, respectively [1] and is second leading cause of death from cancer in both men and women [2].

Role of HER-2 protein in treatment of breast cancer is well established [3]. Some of the studies have shown its importance in treatment of other malignancies including GI malignancies [4]. HER 2 overexpression has been shown in 21.4 % of gastric cancer [5] and was related to short survival [6].

Gastric cancer develops in a multistep progression of precancerous lesions and is primarily determined by environmental factors. Prevalence of *Helicobacter pylori* has been described in precancerous lesions in isolation [7] and is classified as a group I carcinogen [8], especially for intestinal type gastric carcinoma [9].

Pathogenic strains of *Helicobacter pylori* have been shown to activate the epidermal growth factor receptor (EGFR) by altered signal transduction and gene expression that may contribute to pathogenesis. A C-terminal region of the CagA protein has also been suggested to be able to regulate host cell gene transcription, independent of protein tyrosine phosphorylation [10].

As prevalence of gastric cancer is high in some part of India and there are only few studies describing expression of HER2 in gastric cancer, [5, 11, 12] the present study was taken with an aim to study the pattern of HER-2 expression in gastric adenocarcinoma and its correlation with *Helicobacter pylori*.

Material and methods

50 gastric cancer biopsies were included in the study. Sections were stained with H&E for morphological details and classified into intestinal and diffuse type gastric adenocarcinoma according to Lauren classification [13] (Figure 1a,b). Loeffler's methylene blue [14] was used for identification and grading of *Helicobacter pylori* that were semiquantitative graded as Grade 0 - no Bacteria, Grade 1 - occasional bacteria seen in some high power field, Grade 2 - bacteria intermediate between grade 1 and grade 3 and grade 3 - numerous bacteria in most of the fields (Figure 2). Immunohistochemistry for expression of HER2 protein was done and was scored according to Ru'schoff et al; 2012 [15] [Table 1]. As many sections showed prominent cytoplasmic staining, pattern of staining was noted as membranous, membranous + cytoplasmic and cytoplasmic staining (Figure 3a, b, c), [Table 2]. Monoclonal mouse Anti-HER2/neu (c-erbB2) clone CB11 (BioGenex, Fremont CA) were used.

Statistical analysis

Done using chi square test and spearman rank correlation coefficient. P value ≤ 0.05 was taken as critical level of significance.

Result

Nine out of 50 (18%) showed intestinal and 41/50 (82%) showed diffuse adenocarcinoma. 77.8% and 41.5% of intestinal and diffuse type adenocarcinoma were considered to be true positive for HER2 expression due to membranous (\pm cytoplasmic stain) positivity (Table 3). The difference was statistically significant (p value <0.05). *Helicobacter pylori* were seen in 8/9 (88.89%) and 35/41(85.36%) cases of intestinal and diffuse type adenocarcinoma respectively. Most of the cases had Grade 3 positivity. The percentages and grades of *Helicobacter pylori* in intestinal and diffuse type adenocarcinoma are shown in table 4.

When expression of HER2 scoring was compared with increasing grades of *Helicobacter pylori* infection, a significant association ($P <0.05$) was found between the two (Figure 4). However when true correlation was assessed by spearman rank correlation between two parameters, it was found to be weak ($r = 0.169$).

Discussion

In present study 44.44% and 14.63% intestinal and diffuse adenocarcinoma showed only membranous positivity (true positive) for HER2. It was in accordance with earlier reports from India [5]. However when cytoplasmic staining was also included it came to be 77.78% and 41.46% in intestinal and diffuse adenocarcinoma respectively. This was much higher as compared to earlier studies from India and abroad [11, 12, 16, 17]. In most of the earlier studies from India HER2 positivity was graded as per the guidelines for breast tissue [18]. In present study the grading and scoring of HER2 positivity in gastric tissue was done as per guidelines by Ru'schoff et al; 2012 [15], who have separately scored the endoscopic biopsy or resected specimen. According to them even a single cluster of positive tumor cells in endoscopic biopsy should be taken as positive and tumor cell cluster with a strong complete, basolateral, or lateral membranous reactivity irrespective of percentage of tumor cells stained were taken as 3+ve. Ru'schoff et al in their study of ToGA trial for treatment of gastric cancer observed that false negative may be as high as 50%, when HER2 scoring criteria and testing principles for breast cancer is used in gastric cancer [15]

Bang *et al* observed, HER2 expression and amplification in gastric cancer is much more heterogeneous. Ring-shaped staining was found more an exception than the rule^[16]. Lateral / basolateral membranous positivity was taken as positive^[15]. Due to small sized biopsy, a cut off $\geq 10\%$ was discarded and membranous reactivity irrespective of percentage of tumor cells were taken as positive. It is also important to be aware of focal staining that was commonly reported in gastric cancer^[19, 20]. Consequently, gastric cancer- specific protocols for HER2 testing were developed and standardized which had improved the concordance level between IHC & FISH tests to 93.5%^[15, 19].

Membranous HER2 over-expression has been observed predominantly in intestinal type gastric cancer. It was much less in diffuse type gastric cancer where HER2 expression was mainly seen as cytoplasmic staining. Cytoplasmic expression is unusual and taken as negative for HER2 overexpression in breast cancer. Cytoplasmic staining has been reported earlier also in gastric and colonic adenocarcinoma^[21, 22, 23]. In present study 55.56% of cases in intestinal and 73.14% in diffuse type adenocarcinoma showed cytoplasmic + membranous staining or cytoplasmic only stain. Table 5 summarizes various studies from colon where membranous and cytoplasmic positivity was scored separately. As shown 66 % of colon adenocarcinoma by Osako *et al* and 64% by Half *et al* showed cytoplasmic stain only^[21, 26]. In some other studies from gastrointestinal tract authors have observed cytoplasmic staining along with membranous but did not report it separately. Therefore they found very high percentage of HER2 positivity in these cases as compared to those who have taken only membranous positivity^[29, 30]. Studies from gastric cancer are few^[5, 11, 12, 16, 17].

HER2 staining in breast cancer also shows cytoplasmic staining, but there are strict guidelines by FDA to ignore cytoplasmic overexpression^[31] as trastuzumab act only on extracellular domain of membranous HER2. As intracellular HER2 cannot be targeted by trastuzumab, it does not correlate with HER2 mRNA levels, have no correlation with any clinical outcome, and it may even be a different protein than membranous HER2^[32].

Various theories had been put forwarded by earlier workers for this unusual pattern of cytoplasmic staining in gastrointestinal cancer. Blok *et al* attributed the cytoplasmic overexpression in 30 % of colorectal tumors to older tissue samples, but it was not clear why it was seen in diffuse adenocarcinoma predominantly^[32]. Other workers attributed this to variation in the type and clone of antibody^[25]. Table 6 shows various types of antibodies used for identifying the cytoplasmic positivity in some earlier reports and it has been noted that monoclonal antibodies are associated with increased cytoplasmic overexpression^[21] and polyclonal antibodies showed increased membranous overexpression^[34, 35]. Half *et al* observed a cytoplasmic staining in 64% cases of colonic adenocarcinoma using monoclonal antibodies CB11 and 3B5^[21]. Schrohl *et al* showed that monoclonal antibody clones 4B5 and CB11 cross-reacted with HER4 and result in cytoplasmic expression^[22]. In the present study also antibody used for HER2 was CB11.

Earlier workers also tried to find out its association with actual gene amplification by FISH and PCR method. Half *et al* reported that 80%(4/5) membranous HER2-overexpressing tumors showed amplification with FISH, while no cytoplasmic HER2-overexpressing tumors were confirmed with this technique^[21]. Moreover, RT-PCR was performed and showed a 12-fold higher mRNA expression in membranous overexpressing tumors compared to cytoplasmic overexpressing tumors. Kavanagh *et al* confirmed 100% (2/2) and 11.11%(1/9) positivity by FISH in cases showing 3+ and 2+ membranous positivity respectively in colonic biopsy^[36]. Similarly Nathanson *et al* also reported 100% (3/3) and 50 % (1/2) confirmation by both FISH and PCR in cases showing 3+ and 2+ membranous positivity respectively^[37]. However both of them failed to observe any such amplification in cases with cytoplasmic positivity. These results clearly indicate that membranous overexpression is associated with gene amplification, while cytoplasmic overexpression is due to other mechanisms.

Osako *et al* performed a western blot analysis on one membranous overexpressing specimen, five membranous + cytoplasmic specimens, and one negative sample^[26]. They observed 185 kD and 155 kD peptides in the membranous overexpressing specimen, but only 155 kD peptides in the cytoplasmic overexpressing specimens.

A western blot using a P-Tyr antibody, detecting phosphorylated (activated) tyrosine kinase domains showed a 185 kD peptide in the intracellular fraction, indicating an intracellular activated HER2-receptor^[29]. Thus it is suggested that cytoplasmic HER2 form homodimers that leads to an intracellular activation of the tyrosine kinase domain and

tumors with cytoplasmic staining will not be benefited by trastuzumab and other compound which act on intracellular tyrosine kinase could be a treatment option in these patients.

When gene amplification was not proved to be the actual cause, some authors also tried to find other causes of cytoplasmic expression. Kameda *et al* in gastric cancer showed cytoplasmic overexpression of HER2 and suggested variable mechanism for it, eg, via increased levels of promoter-binding proteins to the TATA-box located in the promoter-region of the HER2-gene^[23]. Theoretically, mutations in downstream targets of HER2, as in KRAS, might influence the expression of HER2 via affected feedback processes. These mechanisms could play a role in cytoplasmic HER2 overexpression without gene amplification. Although, as a transmembrane receptor, why does HER2 not migrate to the cell membrane is still unknown^[32].

There are some studies from colorectal carcinoma which showed clinical correlation and split there scoring criterion into membranous and cytoplasmic expression^[26, 28]. Cytoplasmic expression in some studies showed correlation with differentiation of tumor. Thus in colorectal carcinoma, poorly differentiated carcinoma similar to diffuse carcinoma stomach showed increased cytoplasmic expression^[21, 38]. There is evidence that in colorectal cancer cytoplasmic HER2 could in fact be associated with survival prognosis. Of different publications that analyzed clinical prognostic parameters, few showed a worse patient survival with cytoplasmic tumor overexpression of HER2^[28, 46] while other showed no correlation with any clinical parameter^[27, 39].

As trastuzumab act on membranous HER2, it has no role on cytoplasmic HER2 expression. It has been suggested that drugs like lapatinib that acts on intracellular HER2-targeting compounds, might be a new treatment option for the patients having cytoplasmic HER2 overexpression^[40]. Trials had already been started to study role of different drugs in intracellular HER2-targeting compounds.

Helicobacter pylori infection has been defined as an etiological agent in gastric carcinoma, 95% cases with duodenal ulcers and 70-80% cases with gastric ulcers. Concomitance of *Helicobacter pylori* and gastric cancer has been reported at different rates from different studies. Studies in Asian countries such as Thailand, India, Bangladesh, Pakistan, Iran, Saudi Arabian countries, Israel and Malaysia, have reported a high frequency of *Helicobacter pylori* infection co-existing with a low incidence of gastric cancer^[41]. The prevalence of *Helicobacter pylori* was reported to be more in controls than in patients group (80% vs 78%)^[7].

Helicobacter pylori is known to play a major role in EGF receptor expression in gastric mucosal cells^[42]. There are a few studies reporting an association between *Helicobacter pylori* infection and the alteration of EGFR products. Changes like growth, proliferation and differentiation are observed with EGF receptor expression and may lead to gastric cancer development^[42]. Coyle *et al* showed a decrease in HER2 protein overexpression after bacterial eradication^[43]. Wong *et al* measured EGF and EGFR mRNA levels by using ELISA and observed that both EGF and EGFR mRNA levels were increased by *Helicobacter pylori* infection, and decreased after eradication^[44]. However, few other studies showed no relationship between *Helicobacter pylori* and HER2 overexpression^[45, 46].

In the present study a significant association was found between grades of *Helicobacter pylori* and HER2 expression grades. However correlation was weak. Effect of eradication could not be studied due to short duration of study.

The main limitation of the present study is smaller number of intestinal type adenocarcinoma as compared to diffuse type. In earlier studies also from northern India, prevalence of diffuse type gastric cancer was more as compared to intestinal type. These findings were different from southern Indian reports where intestinal adenocarcinoma was high. These variations had been attributed to variation in diets in different parts of country^[47].

Conclusion

Thus it can be concluded that predominant cytoplasmic expression of HER2 in diffuse type gastric adenocarcinoma is unusual and needs further investigation. HER2 overexpression is significantly associated with *Helicobacter pylori* however correlation is weak.

This was a preliminary study highlighting the different pattern of HER2 expression by IHC in gastric adenocarcinoma as compared to breast. Other larger projects to find the pathogenesis of this pattern of staining may further help in deciding the treatment modalities for these patients.

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Table1: Scoring of HER2 overexpression [Ru'schoff et al; 2012]

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

Table 2: Pattern of HER2 expression in Intestinal and Diffuse type Adenocarcinoma

Type of Cancer	Total number of cases	Membranous	Membranous+ Cytoplasmic	Cytoplasmic	No staining
INTESTINAL TYPE	9	4 (44.44%)	5 (55.56%)	0 (0%)	0 (0%)
DIFFUSE TYPE	41	6 (14.63%)	17 (41.43%)	13 (31%)	5 (10%)

Table 3: Scoring of HER2 expression in intestinal and diffuse type adenocarcinoma

Score	Intestinal type	Diffuse type
0 (Negative)	0(0%)	10 (24.4%)
1+ (Negative)	0(0%)	8(19.51%)
2+ (Equivocal)	2 (22.22%)	6 (14.63%)
3+ (Positive)	7 (77.78%)	17 (41.46%)

Table 4: Grading of Helicobacter pylori

Helicobacter pylori	Intestinal Type	Diffuse Type
Grade 0	1 (11.1%)	6 (14.63%)
Grade 1	1 (11.1%)	4 (9.75%)
Grade 2	2 (22.2%)	14 (34.15%)
Grade 3	5 (55.5%)	17 (41.46%)

Table 5: Studies in colon carcinoma who have split their findings in membranous and cytoplasmic HER2 expression.

Authors	Year	Antibody	Patients (n)	% membrane	% cytoplasm
Kay et al ^[24]	1994	NCL-CB11	164	0	34
Arnaout et al ^[25]	1992	4D5, 21N, HER14	70	0	7-34
Osako et al ^[26]	1998	Nichirei	146	2	66
Half et al ^[21]	2004	e2-4001 & 3B5	96	5	64
Kountourakis et al ^[27]	2006	NCL-CB11	106	6	17
Gill et al ^[28]	2011	NCL-CB11	40	8	58

Table 6: Studies using different antibodies by authors in colon carcinoma who have split their finding in cytoplasmic and membranous expression

Antibody	Company	Monoclonal/ polyclonal	Patients(n)	%membrane	%cytoplasmic
4D5 ^[25]	Genentech	Monoclonal	70	0.00	0.00
HER14 ^[25]	Genentech	Polyclonal	70	0.00	34.29
21N ^[25, 33]	Polyclonal	93	1.08	5.38	5.38
NCL-CB11 ^[24, 28, 31]	Novocastra	Monoclonal	310	2.58	30.97
e2-4001 ^[21]	Neomarkers	Monoclonal	96	5.21	63.54
3B5 ^[21]	Oncogene science	Monoclonal	96	5.21	63.54
4881 ^[34]	Zymed	Polyclonal	137	47.45	
A0485 ^[35]	Dako	Polyclonal	74	51.35	

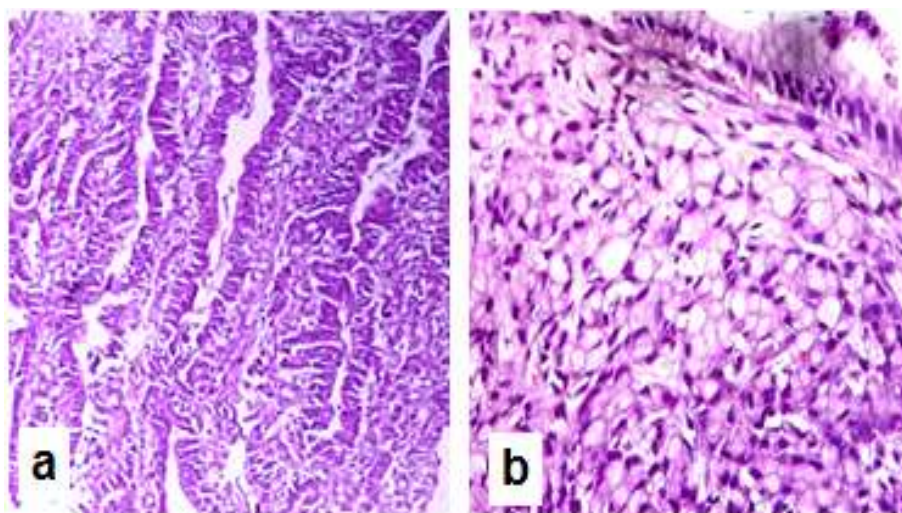


Figure 1: Intestinal (a) and diffuse (b) adenocarcinoma (H&E x 400)

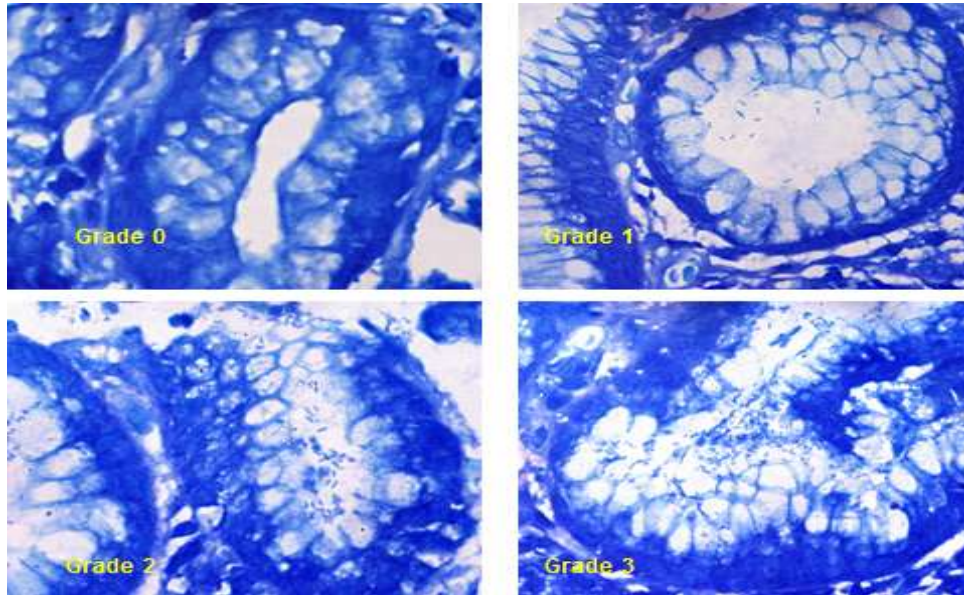


Figure 2: Different grades of *Helicobacter pylori* (Loeffler's Methylene blue x 1000)

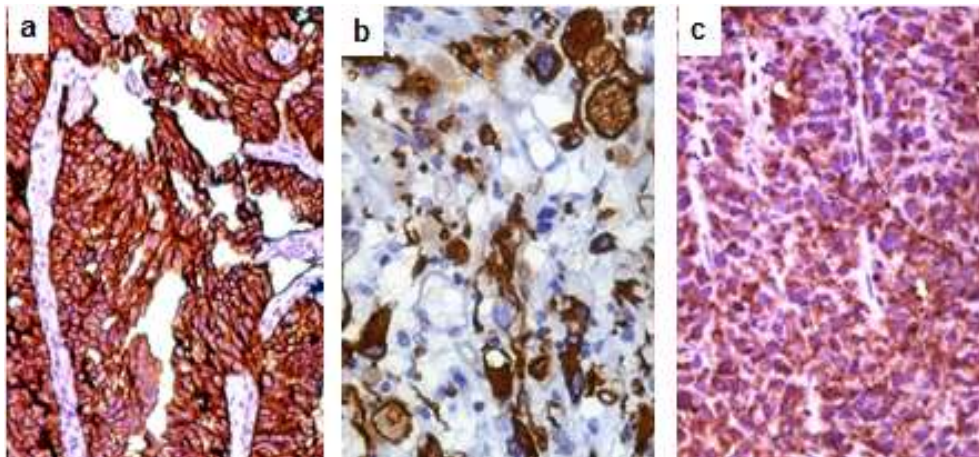


Figure 3: Pattern of HER2 staining as membranous (a), membranous + cytoplasmic (b) and cytoplasmic (c) in gastric adenocarcinoma (IHC x 400)

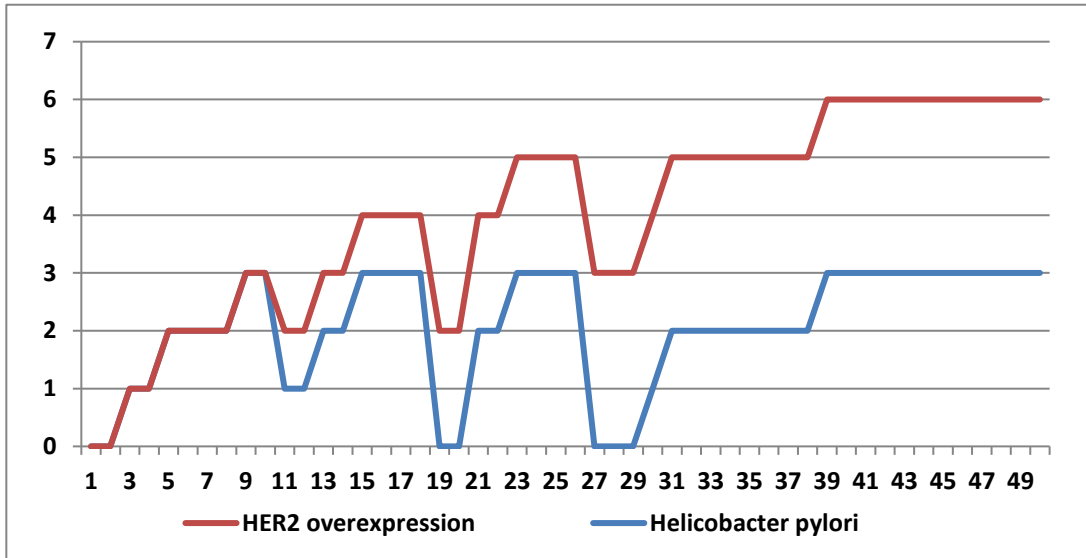


Figure 4: Correlation of Helicobacter pylori with Her 2 in gastric cancer