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**Research Article** 

# Assessment the of Amplification HER-2/neu Gene by Chromogenic In Situ Hybridization (CISH) Compared to Immunohistochemistry (IHC) Method in Gastric Cancer

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#### Abstract

**Background:** Gastric cancer has a high mortality rate and often has a poor treatment outcome. The HER2/neu gene target therapy has been known as a potential way for treatment.

**Objectives:** The goal of our study was assessment the relation between chromogenic in situ hybridization (CISH) and immunohistochemistry (IHC) methods in determining the best diagnostic method for gastric cancer.

**Methods:** In this historical cohort study, 50 gastric cancer samples were analyzed by CISH and IHC. The relation between clinicalpathological parameters of HER2/neu was also analyzed. Alive patients were followed from 2009 through 2012 for the main outcomes (mortality). The results of these two methods, in terms of sex, age, tumor size, grading, staging, tumor location, metaplasia, presence of necrosis and ulceration, vascular invasion, the TNM system, mucin or signet producing adenocarcinoma cells and patient survival rates were compared.

**Results:** There was no significant difference between IHC and CISH regarding the sex, age, tumor size, grading, staging, tumor location, metaplasia, presence of necrosis and ulceration, vascular invasion, the TNM system, mucin or signet producing adenocarcinoma cells and patient survival rates. Comparison of TNM scores by these two methods showed no significant relationship between IHC and staging, but a statistically significant difference between CISH and different N staging, (P < 0.05) was assessed. **Conclusions:** Comparison between IHC and CISH showed the only significant relationship between CISH and different N staging. Therefore, low amplified CISH was a better diagnostic method for gastric cancer, compared to low expression in IHC.

Keywords: Stomach Neoplasms, Immunohistochemistry, Chromogenic In Situ Hybridization, erbB-2, HER2/neu

## 1. Background

Gastric cancer is the fourth common cancer worldwide. Global incidence of gastric cancer is 10.4 out of every 100,000 people (1). Cancer is the third cause of death and gastric cancer had the most mortality rate during 2004 -2005 in Iran (2). At least 80% of patients in developing countries are in advanced stages at the time of diagnosis, because gastric cancer does not have specific symptoms in early stages but in the past few decades, global mortality of gastric cancer has decreased markedly (3).

The survival of gastric carcinoma is dependent on many factors such as location, grade, stage and invasion of the tumor (4). Gene amplification is a method that can determine the survival of gastric cancer. The HER2/neu overexpression is one of the newly introduced prognostic factors for gastric cancer (5). It is considered, at present, to be an important factor in tumor genesis and especially in tumor progression and metastasis (6). HER2/neu gene expression can be detected by several methods like the Immunohistochemistry (IHC) and chromogenic in situ hybridization (CISH) (7). IHC is demonstrating antigen by means of its binding to an antibody which, in turn, is linked to a label that can be visualized histologically. Thus, the site of the antigen in question is highlighted. In CISH method, samples are evaluated with gene amplification and bright-field microscopy (8).

In this study, we compared CISH and IHC methods regarding the determination of HER2 gene expression in gas-

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tric cancer. These two techniques were studied in largescale of researches but in relation to gastric cancer, few studies have been done on comparing these two methods. CISH and IHC have been developed before but there is no consensus about the best way to determine HER2/neu gene expression up to now (9). Tissue expression of HER-2neu gene amplification by these two methods compared together in several aspects (gender, size and age, grade and stage, location and metaplasia, ulcer and necrosis, mucin or signet producing adenocarcinoma cells, invasion, survival analysis, TNM score) in patients diagnosed with gastric cancer in a historical cohort study.

The goal of our study was assessment the relation between chromogenic in situ hybridization (CISH) and immunohistochemistry (IHC) methods in determining the best diagnostic method for gastric cancer.

#### 2. Methods

#### 2.1. Selection of Patients

In this historical cohort study, 50 samples were collected from patients who underwent surgery because of gastric adenocarcinoma during 2004 - 2008 in Imam-Reza hospital, Mashhad.

All the patients had the intestinal type of adenocarcinoma and none of them showed any evidence of distant metastasis at the time of diagnosis. The absence of distant metastasis was evaluated based on the admission hospital profiles, in which not only the patients' history, physical examination, routine laboratory tests, and peri-operative reports, but also their reports of imaging studies (plain chest x-ray and abdominal/hepatic ultrasonography) were recorded. Moreover, the patients did not receive preoperative adjuvant chemo- or radio-therapy. Thus, the patients who had undergone gastrectomy for non-tumoral conditions (e.g. peptic ulcer) or tumors other than intestinal type adenocarcinoma (e.g. signet-ring cell adenocarcinoma or gastrointestinal stromal tumor), and those with known distant or peritoneal metastasis were excluded. In addition, the patients without suitable tissue for performing CISH or IHC were excluded. The trial was performed in accordance with the declaration of Helsinki and its subsequent revisions and approved by ethics committee of Mashhad University of Medical Sciences.

#### 2.2. Immunohistochemistry Protocol

Four microns thick of specimens that were provided of the intra-tumoral area, cut for IHC staining. Specimens were deparaffinized and immersed in alcohols and then 3% H2O2. Expression of markers was checked by the primary antibody and then specimen were incubated with HRPlabeled secondary antibody and then they are stained and counterstained. In the study, IHC protocol was described in detail.

According to the ASCO/CAP guideline, an IHC staining of +3 was considered a positive HER2/neu result of testing in breast cancer and IHC staining of 0 or +1 was considered the negative result.

IHC protocol was explained with details, in previous studies (9). In briefly, four specimens were prepared for IHC staining. After the deparaffinization of specimens, they immersed in 96%, 80%, and 70% alcohols. Specimens were autoclaved for antigen retrieval after that, marker expression was assessed by using the primary antibody and then HRP-labeled secondary antibody and slides were incubated together. Next, specimens were stained and counterstained with 3, 3'-Diaminobenzidine (DAB) and Hematoxylin, respectively.

The Phosphate-buffered saline (PBS) washing buffer was used in all stages. According to the ASCO/CAP guideline recommendations for HER2/neu a positive HER2/neu result of testing in breast cancer, is an IHC staining of 3+ that is characterized with uniform and intense membrane staining of at least 30 percent of invasive tumor cells and a negative result of testing in breast cancer is an IHC staining of 0 or +1 that is characterized with not observed or the observed staining of membrane is < 10% of the tumor cells (9).

#### 2.3. Chromogenic In Situ Hybridization Protocol

The ZytoDot 2C SPEC HER2/CEN 17 Probe Kit was utilized for the detection of the human HER2 gene and chromosome 17 alpha-satellites. In brief, this protocol was done in 2 days, in the firstday, 4 - 5  $\mu$ m thick tissue dried, then heated at 60°C and washed in 100% ethanol, heated in a covered jar that stands in a boiling water bath for training, then pepsin solution was applied, the probes were added and then the tissue was denatured and hybridized. In day 2, the tissue was washed with buffer SSC and PBS and was counterstained with Mayer's hematoxylin solution, then it was washed, dehydrated, incubated in xylene and cover stained with coverslip and then interpreted (10).

#### 2.4. Follow Up

Alive patients were followed by phone contacts in four years from 2009 to 2012 for the main outcome (mortality). The patients' data including age at diagnosis, date of gastrectomy, sex, history of substance abuse (e.g. nicotine or alcohol) and past medical history were collected from their hospital profiles. Besides, tumor dimensions, the presence of ulcer, depth of invasion, grade of the tumor and the number of involved lymph nodes were gathered by checking archived pathology reports. In addition, other relevant histological variables (e.g. neural, vascular and lymphatic invasions, the status of margins, and the presence of metaplasia) were obtained by reviewing all the patients' archived slides.

#### 2.5. Statistical Methods

Data were analyzed with chi-square fisher exact test using the SPSS software for Windows, version 11.5 (SPSS Inc., Chicago, Illinois, USA). Kolmogorov-Smirnov test was used for checking the normality of all data. Student t-test or Mann-Whitney test was used to compare the continuous data. Numerical data are expressed as a mean  $\pm$  standard deviation or as proportions of the sample size. Univariate survival analyses were performed using the Kaplan-Meier method for analyzing the effect of IHC and CISH on the patients' survival. Besides, differences in observed survival between groups were tested by log ranks tests. Statistically significant P value was considered less than 0.05.

## 3. Results

Among 50 patients, thirty-eight (76%) were male and the mean age was 66 years old. Eight (16.7%) patients were smokers. None of them declared any alcohol consumption, but one patient stated his substance abuse. Twentythree patients had some sort of noteworthy medical history with a wide range of illnesses including hypertension (8 patients), ischemic heart disease (4 patients), diabetes mellitus, dyslipidemia, hyperthyroidism with radioactive iodide ablation therapy, appendectomy, benign prostatic hyperplasia, transient ischemic attack, cataract surgery, degenerative joint disease, bone fracture, cholecystectomy, renal cyst, hysterectomy, asthma, lacrimal gland obstruction, peptic ulcer, renal stone, inguinal hernia, and vitiligo. Nineteen (38.0%) tumors were located in cardia, however 31 (62.0%) were the non-cardia gastric tumor. Mean ( $\pm$  SD) Tumors size were 5.2  $\pm$  1.96 also fortyfive (90%) tumors were ulcerative and most of them were located in lesser curvature (21 tumors, 42%).

Comparison of the number of low and high-grade CISH with different grades of IHC (neg, 1, 2, 3) and investigating the relation of CISH analysis and IHC method by Chi-square showed that negative IHC had low amplification in CISH and all of IHC +3 and most of IHC +2 had high CISH amplification. However, all of IHC +1 had low CISH amplification and high CISH amplification in 2 or 3 IHC score was significantly higher than low (0 or 1) IHC score. While low IHC score (0 or 1) was significantly more than higher IHC (2, 3) score. Pearson Chi-Square analysis was assessed a significant difference between the two methods (P = 0.01) (Table 1) (Figures 1 and 2).



Figure 1. Tumor Cells with Non-Amplified Her 2 Status



Figure 2. Amplification and Chromosome 17 Aneusomie as Indicated by Multiple Green and Red Signals

#### 3.1. Gender

Specimens from male patients showed lower IHC and lower CISH compared to female ones. Male patients have significantly higher IHC and CISH compared to female patients (P = 0.022).

#### 3.2. Size and Age

Comparing the IHC and CISH methods regarding the age and tumor size, revealed no significant difference.

#### 3.3. Grade and Stage

Most of the patients were in grade 1 and stage 3. Pearson Chi-Square test showed that there was no significant difference between different grades and stages of cancer

CISH		ІНС			TOTAL	P Value					
	Neg	+1	+2	+3							
Low	31	5	6	0	42						
High	1	0	2	5	8						
TOTAL	32	5	8	5	50	0.01					

Table 1. Relation of CISH Analysis and Different IHC Grades by Chi Square

in IHC3 (P = 0.61) and CISH (P = 0.27) also between different stages of cancer in CISH (P = 0.24) and IHC (P = 0.47).

# 3.4. Location and Metaplasia

Most of the patients had non-cardiac cancer. Pearson Chi-Square test showed that there was no significant difference between different locations of cancer in IHC3 (P = 0.53) and CISH (P = 0.40) also between metaplasia in CISH (P = 1) and IHC (P = 0.74).

# 3.5. Ulcer and Necrosis

Ulcers were present in most of the patients. Chi-square analysis showed no significant difference regarding ulcer and necrosis between CISH and IHC.

#### 3.6. Mucin or Signet Producing Adenocarcinoma Cells

Chi-square analysis did not show any significant difference between CISH (P = 1) and IHC (P = 0.16) regarding signet and mucin.

#### 3.7. Invasion

There were not any significant difference between CISH (P = 0.24) and IHC (P = 0.23) regarding lymphatic and perineural invasion.

## 3.8. TNM Score

Patients with positive lymph node involvement (LNI) had low CISH compared to others with negative LNI. However, there was no significant difference between Lymph node involvement and CISH analysis (P = 0.66) by Fisher's Exact Test analysis. Patients with positive lymph node had IHC score 0 or 1 compared to others with negative LN. However, Fisher's Exact Test analysis presented no significant difference between Lymph node involvement and IHC analysis (P = 0.70). Patients with N3 staging had low CISH compared to other stages. Fisher's Exact Test analysis showed a significant difference between different N scores and CISH analysis (P = 0.02). In addition, patients with N3 staging had low IHC3 compared to other stages. Fisher's Exact Test analysis showed that there are not statistically significant difference between different N and IHC3 analysis (P = 0.20) so there is not any significant statistical relationship between IHC and different N staging (P > 0.05). However, statistically significant difference between CISH and different N staging (P < 0.05) was assessed. Most of the patients with T3 staging had experienced low CISH compared to other stages. Pearson Chi-Square analysis showed non-significant difference between different tumor depths and CISH analysis (P = 0.74), also most of the patients with T3 staging had experienced low IHC3 compared to other stages and there was not statistically significant difference between different tumor depths in IHC3 analysis (P = 0.44), so there is not any significant statistical relationship between IHC and CISH techniques regarding the different depth of invasion (P > 0.05), (Table 2).

#### 3.9. Survival Analysis

Kaplan-Meier survival curves according to the time of diagnosis by CISH techniques are presented in Figure 3. Mean  $\pm$  standard error for Low-amplification survival time of tumors diagnosis was  $31.9 \pm 5.1$ , 95%CI: 21.9 - 41.8 and median  $\pm$  standard error of survival time were  $16 \pm 5.7$ , 95%CI: 4.9 - 27.1.



Figure 3. Kaplan-Meier Survival Curves According to the Time of Diagnosis by CISH Techniques

Variable		High CISH	Low CISH	P Value	IHC 0 or 1	IHC 2 or 3	P Value
Male		30	8	0.015 <sup>a</sup>	25	13	
Female		12	0		12	0	0.022
Age <sup>b</sup>		63.50 ± 10.78	66 ± 8.98	0.34	$65.62\pm9.06$	$67 \pm 10.33$	0.79
Size <sup>b</sup>		4.43 ± 1.39	$5.3 \pm 2.03$	0.25	5.09 ± 1.91	5.38 ± 2.1	0.65
	1	2	21	0.27	18	5	0.61
Grade (n)	2	2	11		10	3	
	3	4	10		9	5	
	2	5	14	0.24	12	7	0.47
stage(II)	3	3	25		23	5	
Location (n)	Cardia	2	17	0.25	15	2	
Location (II)	Non-cardia	6	25		22	6	0.05
Metanlacia (n)	-	4	21	1.00	19	6	0.74
metapiasia (11)	+	4	21		18	7	
	Absent	0	5	0.57	4	1	0.47
	Peresent	8	37		33	12	
Necrosis(n)	-	4	26	0.69	25	5	0.65
	+	4	16		18	2	
Mucin (n)	-	5	36	0.14	30	11	1.00
	+	3	6		7	2	
Signet(n)	-	7	36	1.00	30	13	0.16
	+	1	6		7	0	
Ivmphatic invasion (n)		3	27	0.24	24	6	0.23
	+	5	15		13	7	
Vascular invasion (n)	-	8	37	0.57	33	12	1.00
	+	0	5		4	1	
Perineural invasion (n)	-	3	26	0.25	22	7	0.72
	+	5	16		15	6	
Lymph Node (LN)	No	1	11	0.66	8	14	0.70
(=)	Yes	7	13		29	9	
	NO	1	n	0.02 <sup>a</sup>	8	4	0.20
$N^{c}(n)$	N1	5	6		6	5	
	N2	0	11		10	1	
	N3	2	14		13	3	
	T2	1	7	0.74	5	3	0.44
$T^{d}(n)$	T3	5	29		27	7	
	T4	2	6		5	3	

Table 2. Comparison of IHC and CISH Methods in Different Aspects

<sup>a</sup> P value < 0.05. <sup>b</sup> Values are expressed as mean  $\pm$  SD.

<sup>c</sup> Values are expressed as mean ± SI <sup>c</sup> N in TNM scoring.

d<sub>T in TNM scoring.</sub>

Also, Mean  $\pm$  standard error for high amplification survival time of tumors diagnosis was (23.3  $\pm$  10.9, 95%CI: 2 - 44.6) and median  $\pm$  standard error of survival time was (9  $\pm$  1.4, 95%CI: 6.2 - 11.8).

Patients in high amplification group were lower survival than low amplification group, but log rank test analysis showed that there was no statistically significant difference between high and low amplification of HER-2 neu by CISH techniques (P = 0.40) (Figure 3).

Mean  $\pm$  standard error of survival time for Low amplification tumors was (29.8  $\pm$  5.2, 95%CI: 19.6 - 39.9) and me-

dian  $\pm$  standard error of survival time was (16  $\pm$  4.4, 95%CI: 7.4 - 24.6).

Also Mean  $\pm$  standard error for high amplification survival time of tumors was (31.1  $\pm$  9.6, 95%CI: 12.2 - 50) and median  $\pm$  standard error of survival time was (10  $\pm$  1.5, 95%CI: 0 - 32.5).

With IHC technique high expression group has lower survival rate than low expression group, but Log Rank Test analysis showed that there was not statistically significant difference between a high and low expression of HER2/neu by IHC techniques (P = 0.88) (Figure 4).



Figure 4. Kaplan-Meier Survival Curves According to the Stage of the Tumor at the Time of Diagnosis by IHC3 Techniques

#### 4. Discussion

In recent years a new treatment modality for gastric cancer is molecular target therapy and a potential therapeutic target is HER-2. However, molecular target therapy for gastric cancer depends on the evaluation of the target gene status. HER-2 status is an important prognostic factor for cancer that plays a key function in initiation, development, and spreading of some cancers (breast and gastric cancer). Patients with positive HER-2 breast cancer have lesser survey than those with HER-2 negative tumors (10-12). Due to high HER-2 over-expression rate in gastric cancer that is about 8.2 - 3.4 %, detecting of HER-2 status can play an important role in the diagnosis of gastric cancer (13).

IHC and CISH have been studied in many researches but few studies have been done on gastric cancer, leaving no consensus to determine the best diagnostic modalities (13). There was poor concordance between local and central or reference IHC testing for HER2 in two previous clinical trials (14, 15). Therefore, it recommends that performing clinical trials with large-scale of patients is needed for determining of HER-2 neu status in patients with gastric cancer. An urgent need to improve the quality control program in laboratories that use IHC testing was suggested by these data (15-19). FISH technique may be used to obtain a successful calibration of the in-house IHC technique (20).

CISH, as a hybridization procedure, is an alternative for FISH (9). Compared to FISH, it has been described as having several advantages (9, 21, 22), for example, analyzing of morphology is easier and it does not require coupled device (CCD) camera to record and fluorescence microscope which are both expensive.

In the study on HER2/neu gene expression in gastric cancer, Gravalos et al. reported an 87% correlation between two methods of IHC and FISH (23). In 2006, Park et al. expressed that among 11 cases of IHC positive, only 54.5% of them exhibited high amplification of CISH and FISH. This study was done to measure gene amplification in gastric cancer patients and the results showed that IHC method was better than CISH method for diagnosis of gastric cancer (24).

Yan et al. evaluated 128 specimens from gastric cancer patients by fluorescence (FISH) and chromogenic in situ hybridization (CISH) and immunohistochemistry (IHC). Although a significant negative correlation was assessed between survival of patient and overexpression of HER2 protein in intestinal-type gastric carcinomas (P < 0.05), any significant difference between the different survey times of two methods might be attributed to the low sample size of the present study (23).

Todorović-Raković et al. compared IHC and CISH in assessing HER2/neu status in breast cancer. They found that IHC method is not enough for measuring HER2/neu gene amplification for breast cancer diagnosis and suggested CISH as a complementary method to be used, especially in patients in stage 1 (25). They also, in another research that was done on 107 patients with metastatic breast cancer, expressed that, there is significant difference in progressionfree interval, between HER2 amplified and non-amplified patients, in HER2 gene expression by CISH method in versus IHC method.

In a study by Jacquemier et al. a concordance was found between FISH and IHC and between FISH and alternative methods like CISH and qPCR, especially in the 2+ cases (26).

In this study, we compared CISH and IHC methods for determining HER2 gene expression in gastric cancer. We considered many parameters and based on these, evaluated the two methods. We found no significant relationship between IHC and CISH in different terms (gender, size and age, grade and stage, location and metaplasia, ulcer and necrosis, signet and mucin-producing adenocarcinoma cells, invasion, survival analysis). Comparison of these two methods regarding the TNM scores showed no significant relationship. However, there was a statistically significant difference between CISH and different N staging (P < 0.05) and low amplified CISH is more capable to detect all these terms compared to low expression in IHC method.

It is accepted that small-scale surveys are not able to add significant information to the currently available knowledge about the association of two studied methods and cancer survival. Large-scale studies are needed to control all the probable prognostic factors.

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## Footnotes

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