RESEARCH ARTICLE

# Association of Human Epidermal Growth Factor Receptor-2 Expression and Clinicopathological Findings in Patients with Colorectal Cancer

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## Abstract

**Background: To determine the frequency of HER-2 overexpression in colorectal cancer (CRC) patients, and to explore the relationship between clinicopathological prognostic factors and their effects on survival, based on immunohistochemistry (IHC) and fluorescent in situ hybridization (FISH) analysis. Materials and Methods: The study included 80 patients with a histologically proven diagnosis of CRC that received adjuvant FOLFOX-4 chemotherapy at our department between March 2006 and September 2010. Patient data were analyzed retrospectively. Results: The median follow-up period and age of the patients were 24 months and 59 years, respectively. In immunohistochemical staining, 3+ staining was found in 2 patients (2.5%) while 2+ was in 13 (16%) . FISH for HER-2 was performed for all of these 15 patients; samples which were 3+ showed positivity but the ones with 2+ were negative. There was no significant correlation between HER-2 expression and age, gender, tumor localization, histological subtype, grade, lymphovascular and perineural invasion, or pTN stage (P>0.05), even when the patients with HER-2 overexpression were analyzed separately. There was also no significant relationship between progression-free survival (PFS) and overall survival (OS), and HER-2 expression, gender, tumor localization, obstruction-perforation, bleeding, histological type, grade, lymphovascular and perineural invasion, or pT staging (P>0.05); however, there was a significant relationship between lymph node involvement, and PFS and OS (P<0.05). Conclusions: Evaluation of HER-2 overexpression in a more comprehensive, multi-center, prospective trial with standardized methods will be an appropriate approach.**

**Keywords:** Adjuvant therapy - colorectal cancer - epidermal growth factor receptor - HER-2

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## Introduction

Worldwide, colorectal cancer (CRC) is the third most common solid tumor, constituting a major health problem (Jemal et al., 2010; Karaca et al., 2011). CRC can be treated with curative intent in early stage patients, but remains a significant cause of death in advanced stage patients. CRC is diagnosed in more than 1 million people worldwide annually and >50% of patients have metastasis (Libutti et al., 2008). Survival in patients with metastatic CRC has improved due the use of targeted therapeutics against vascular endothelial growth factor receptor (VEGFR) and epidermal growth factor receptors (EGFRs), which play a role in the development of CRC (Cunningham et al., 2004; Giantonio et al., 2007).

EGFRs are a member of the ErB tyrosine kinase receptor family and exhibit abnormal activity in many epithelial tumors (Mendelsohn et al., 2006). Four subtypes have been defined: EGFR/ErbB1/HER1, ErbB2 (HER-2/neu), ErbB3 (HER3), and ErbB4 (HER4). Human epidermal growth factor receptor-2 (HER-2) is over expressed in 20-30% of breast cancer patients and is associated with poor prognosis (Ferretti et al., 2007). HER-2-positive breast cancer is an aggressive, high-grade tumor with reduced hormone receptor expression and a tendency for lymph node and distant metastasis. Good response rates and survival were obtained with trastuzumab, which is a humanized monoclonal antibody developed against HER-2 (Landgraf et al., 2007).

In gastric carcinoma patients, especially those with the intestinal type, HER-2 overexpression was reported as 20-35%, which is similar to that in breast cancer (Albarello et al., 2011). The addition of trastuzumab to chemotherapy improved the response rate and survival in these patients, particularly those with the intestinal type (Bang et al., 2010). HER-2 overexpression in CRC patients was reported to be between 10%-83%, based on immunohistochemistry (IHC) and/or fluorescent in situ hybridization (FISH) techniques (Kountourakis et al., 2006). Due to differences in techniques and patient populations, a wide range of results have been reported. To optimize the results, IHC and FISH methods should

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be standardized for CRC as they are for breast cancer, and large-scale studies on CRC must be conducted. The present study aimed to determine the frequency of HER-2 overexpression in CRC patients, and to explore the relationship between clinicopathological prognostic factors and their effects on survival, based on IHC and FISH analysis.

## Materials and Methods

*Study population and protocol*

The study included 80 patients with a histologically proven diagnosis of CRC that received adjuvant FOLFOX-4 chemotherapy (5-FU 400 mg m-2 i.v. bolus on d 1 and 2, 5-FU 22-h infusion of 600 mg m-2 on d 1 and 2, folinic acid 200 mg m-2 i.v. on d 1 and 2, oxaliplatin 85 mg m-2 i.v. bolus on d 1, every 2 weeks) at our department between March 2006 and September 2010. Erciyes University, School of Medicine Ethics Committee approved the study protocol (approval number: 2011/143). Patient data were analyzed retrospectively. Age, gender, histopathological diagnosis, tumor localization, history of obstruction-perforation-bleeding, tumor grade, presence of perineural-lymphovascular invasion, pathological TNM stage at the time of diagnosis, recurrence and metastasis, history of surgery and radiotherapy, adjuvant and metastatic chemotherapy (CT) regimens, follow-up, disease-free survival (DFS), progression-free survival (PFS), and overall survival (OS) data were evaluated.

*Immunohistochemical analysis*

Postsurgical samples (5 $\mu$m thick) were obtained from formalin-fixed paraffin blocks maintained in the pathology archive and placed on poly-l-lysine coated slides in order to evaluate HER-2 overexpression using IHC analysis. The tissue sections were deparaffinized with xylene and dehydrated with alcohol. IHC staining was performed using a Ventana Benchmark Autostainer for HER-2 primary antibody (Thermo Scientific Ab17, e2-4001+3B5, UK) in 1:100 dilution and for a 30-min incubation period. Tissues were counterstained with Mayer's hematoxylin. For HER-2 expression in tumor cells the basolateral membranous staining pattern was evaluated. Membranous staining in ≥10% of cells was considered positive. Staining intensity was scored between 0 and 3, as follows: 0: no membranous staining; 1+: mild membranous staining; 2+: moderate membranous staining; 3+: strong membranous staining (Figure 1).

*FISH analysis*

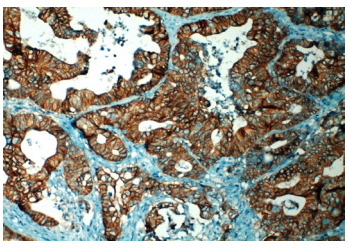

**Figure 1. HER-2 Staining (3+) with IHC and H&E (200×)**

Paraffin blocks for 13 patients with 2+ HER-2 expression and 2 patients with 3+ HER-2 expression based on IHC were sent to a center that was certified to perform FISH examination. Tissue sections prepared from these paraffin blocks underwent FISH analysis using the FDA-approved Vysis PathVysion (IL, USA) HER-2/neu DNA Probe Kit, which uses the HER-2/neu oncogene (17q11.2-q12) region probe (S.Orange) located on chromosome 17 and the centromeric (17p11.1-q11.1) probe (S.Green) of chromosome 17 as an internal control. FISH results were considered positive if the HER-2/neu gene amplification score was ≥2.2 (Figure 2).

*Statistical analysis*

Statistical analysis was performed using SPSS v.15.0. Percentages were calculated for unmeasurable data. Survival was analyzed using the Kaplan-Meier method. The log-rank test was used to determine the significance of the difference between curves. Pearson's correlation coefficients were calculated to determine the relationship between variables. Statistical significance was set at $P<0.05$.

## Results

The median follow-up period and age of the 80 patients in the study were 24 months (range: 1-67 months) and 59 years (range: 33-84 years), respectively. Other patient and tumor characteristics are summarized in Table 1. All patient characteristics were similar in both groups. During follow-up 13 (16%) patients died, 17 (21%) developed recurrence, and median DFS was 55 months (95%CI: 42.84-55.95). In all, 1 (1%) patient had local recurrence, 3 (4%) had local recurrence with distant metastasis, and 13 (16%) had distant metastasis. Among the patients, 8 (10%) had liver, 8 (10%) had pulmonary, 3 (4%) had bone, and 1 (1%) had ovarian metastasis. Additionally, 1 (1%) patient had bladder invasion and 6 (8%) patients had metastasis to multiple regions.

Adjuvant FOLFOX-4 chemotherapy was given to all the patients. The median number of cycles was 12 (range: 4-12) and 20 patients were unable to complete the treatment because of grade 2/3 and and 4 toxicities (diarrhea, mucositis, neuropathy, etc.). Surgery and metastasectomy were not performed in patients with recurrence or metastasis. The first-line metastatic chemotherapy regimen was FOLFIRI + bevacizumab (median PFS: 9 months; 95%CI: 6.19-16.37) in 16 patients and FOLFIRI only in 1 patient due to a history of thrombosis.

IHC and FISH results are summarized in Table 2. In all, 2 patients had HER-2 overexpression with FISH - a 43

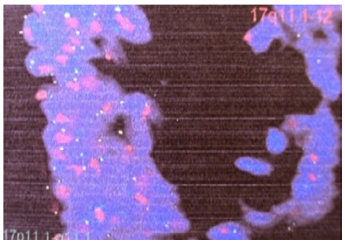

**Figure 2. HER-2 FISH (+)**

**Table 1. The Clinical and Pathological Features of the Patients**

| | | n | % |
|---|---|---|---|
| Gender | Male | 48 | 60 |
| | Female | 32 | 40 |
| Tumor localization | Colon | 52 | 65 |
| | Rectum | 25 | 31 |
| | Rectosigmoid | 3 | 4 |
| Histology | Adenocarcinoma | 70 | 88 |
| | With mucinous component | 9 | 11 |
| | With signet-ring cell | 1 | 1 |
| Grade | 1 | 12 | 15 |
| | 2 | 47 | 59 |
| | 3 | 21 | 26 |
| Obstruction | present | 11 | 14 |
| | absent | 69 | 86 |
| Perforation | present | 3 | 4 |
| | absent | 77 | 96 |
| Bleeding | present | 32 | 40 |
| | absent | 48 | 60 |
| Lymphovascular invasion | present | 29 | 36 |
| | absent | 51 | 64 |
| Perineural invasion | present | 24 | 30 |
| | absent | 56 | 70 |
| Stage | 1 | 0 | 0 |
| | 2A | 14 | 17 |
| | 2B | 12 | 15 |
| | 3A | 6 | 8 |
| | 3B | 25 | 31 |
| | 3C | 23 | 29 |
| | 4 | 0 | 0 |

**Table 2. Results of IHC and FISH Analysis**

| CHER-2 | | n | % |
|---|---|---|---|
| IHC | (-) | 57 | 71.5 |
| | 1+ | 8 | 10.0 |
| | 2+ | 13 | 16.0 |
| | 3+ | 2 | 2.5 |
| FISH | (-) | 13 | 87.0 |
| | (+) | 2 | 13.0 |

year-old male and a 55 year-old female who had 3+ HER-2 expression based on IHC. Both patients had a histological diagnosis of adenocarcinoma. The male patient had a rectal tumor, whereas the female patient had a tumor in the ascending colon. At the time of diagnosis, the male and female patients had T4N1(IIIB) and T3N1(IIIB) disease, respectively. The female patient did not have recurrence during 21 months of follow-up, but the male patient developed recurrence with liver metastases during the 12$^{th}$ month of follow-up and died during the 34$^{th}$ month of follow-up.

There wasn't a significant correlation between HER-2 expression (all degree's with IHC) and age, gender, tumor localization, histologic type, grade, lymphovascular and perineural invasion, or pTN stage (P>0.05), even when the patients with HER-2 overexpression were analyzed separately and the patients with mild-moderate or strong positive IHC staining were evaluated as separate groups. There wasn't a significant relationship between PFS and OS, and HER-2 expression, gender, tumor localization, obstruction-perforation, bleeding, histologic type, grade, lymphovascular and perineural invasion, or pT staging (P>0.05); however, there was a significant relationship between lymph node involvement, and PFS and OS (P<0.05).

## Discussion

Significant improvement in survival has been obtained due to targeted therapeutic agents against VEGFR and EGFR, which play a role in the development of CRC (Cunningham et al., 2004; Giantonio et al., 2007). Today, some biomarkers are being used in routine practice for determining the best and appropriate treatment in metastatic CRC. For example, KRAS and BRAF mutations have predictive importance for cetuximab treatment (Bardelli et al., 2010), and BRAF mutation is associated with poor prognosis (Di et al., 2008). Studies on new biomarkers that may have predictive and/or prognostic importance in CRC are ongoing (Cunningham et al., 2010).

The association between HER-2 overexpression, and aggressive tumor features and poor prognosis in breast cancer patients has been reported in many studies and are now included in oncology guidelines (Wang et al., 2001). Due to the success of anti-HER-2 therapy in breast cancer, the frequency of HER-2 overexpression and potential for anti-HER-2 treatment were investigated in other types of cancers. In a phase III randomized study of ToGA (Bang et al., 2010), the effectiveness of chemotherapy alone versus combination chemotherapy and trastuzumab was investigated in HER-2-positive, advanced stage, gastric or gastro-esophageal junction tumors as a first-line metastatic regimen. HER-2 positivity was observed in 22% of patients based on IHC and FISH screening and this group was included in the study. OS was significantly longer in the trastuzumab arm (13.8 months versus 11.1 months, P=0,0046) and it was suggested that the combination of trastuzumab and chemotherapy should be the standard treatment in HER-2 positive advanced gastric cancer.

Moreover, in CRC cells HER-2 oncogene product HER-2 protein overexpression has been reported (Ramanathan et al., 2004, Baselga et al., 2009). In gastric cancer, especially the intestinal type, HER-2 is highly positive, as in breast cancer, which led to the hypothesis that this receptor could be a potential target in the management and prognostic determination in CRC. For assessment of HER-2 overexpression in the gastrointestinal system, especially the stomach, IHC with a modified scoring system provides highly reliable results. This scoring system is also available for CRC; however, unlike in breast and gastric cancers, in CRC membranous staining of HER-2 and overexpression has been reported at low rates in most studies (Rossi et al., 2002; Kountourakis et al., 2006; Eugene et al., 2011). Kruszewski et al. (2010) used the IHC method to evaluate surgical resection materials obtained from 202 CRC patients, and HER-2 positive membranous reactions were observed in 54 (26.7%) of the patients and staining intensity was 3+ in 31 (15%) cases. In another immunohistochemical study (Ochs et al., 2004) 109 stage II CRC patients were evaluated and HER-2 overexpression was observed in 12 cases (11%). In several other studies the HER-2 positivity

rate was 3.6-11% in CRC patients (McCann et al., 1990; Tsioulias et al.,1990). In the present study only 2 patients (2.5%) had 3+ membranous staining. Although the HER-2 cell growth pathway did not have a major effect on the development of CRC, according to the low expression rate, the response to single-agent trastuzumab therapy was reported in a HER-2-positive CRC patient (Sorscher et al., 2011).

Findings are not consistent in all studies. A study that included 152 CRC patients (Kapitanovic et al., 1997) reported strong positive HER-2 staining in 43% of the patients. Based on the findings the researchers posited that HER-2 was an independent prognostic factor in CRC. Kay et al. studied 164 patients with early-stage CRC (Kay et al., 1994) and reported that the HER-2 positivity rate was 33.5% and that the 5-year survival rate was higher in the HER-2 negative group. HER-2 may be a useful marker in CRC. The above-mentioned studies included more patients than the present study did, but the differences in the findings may have been due to technical differences lack of an appropriate scoring system (neglecting the difference between membranous and cytoplasmic staining), and differences (i.e. geographic and ethnic) in the patient populations. A recent study that included 137 CRC patients that underwent curative surgery evaluated HER-2 overexpression and reported strong (3+) HER-2 staining in 19.7% of the patients based on IHC. Positive FISH results were observed in only 2 patients (1.5%) and HER-2 was suggested to be a prognostic factor in CRC (Park et al., 2007). Interestingly, the lack of correlation between IHC and FISH findings indicates that the study had some technical problems.

Unlike many previous studies, HER-2 overexpression in the present study was measured using both IHC and FISH. Confirmation of IHC findings with FISH increases the reliability of the findings; however, there are some issues associated with FISH analysis. First is the problem of cost. A multi-center study (Saudi Arabia and Switzerland) that included 518 CRC patients reported that the HER-2 overexpression rate was 1.3% based on FISH (Mann et al., 2001). This result and the low HER-2 positivity rate in CRC indicate that FISH is not a cost-effective method for evaluating HER-2 overexpression. FISH may be an appropriate method for evaluating patients only when IHC results are 3+ or 2+. In addition, as FISH analysis was developed for evaluating breast cancer its use in the assessment of CRC, which has different tissue features, could lead to false-positive and false-negative results.

In the present study there wasn't a significant correlation (P>0.05) between HER-2 expression or staining intensity, and age, gender, tumor localization, histologic type, grade, pTN stage, or lymphovascular and perineural invasion when patients were evaluated as follows; 1. Patients with IHC-positive membranous staining (weak [+], moderate [2+], and strong [3+] staining); 2. Patients with moderate (2+) and strong (3+) staining; 3. Patients with strong (3+) positive staining based on IHC and HER-2 overexpression based on FISH. Additionally, there wasn't a significant relationship between HER-2 overexpression (strong [3+] staining) or weak (+) staining based on IHC, and gender, tumor localization, obstruction-perforation-bleeding, histologic type, grade, lymphovascular-perineural invasion, pT stage, or PFS and OS (P>0.05); however, lymph node involvement had a significant relationship with PFS and OS (P<0,05). Similarly, other studies (Rossi et al., 2002 ; Al-Kuraya et al., 2007; Kavanagh et al., 2009; Molaei et al., 2009) reported that there wasn't a correlation between HER-2 positivity and survival or other clinicopathological factors, which, as in the present study, may have been due to small study populations, lack of prospective design, an insufficient follow-up period, low concentrations of HER-2, lack of a standard scoring method, and lack of experience using IHC and FISH in CRC patients. The prognosis of CRC worsens as the number of metastatic regional lymph nodes increases (Wolmark et al., 1986; Blumberg et al., 2002). In the present study lymph node involvement was an indicator of poor prognosis.

Some studies highlighted the prognostic importance of HER-2 expression in CRC and reported that it is correlated with clinicopathological features. Kruszewski et al. (2010) reported a non-significant correlation between positive membranous staining and lymph node involvement (P=0.05). In the present study lymph node involvement was analyzed in patients with all HER-2 immunohistochemical staining intensities and there wasn't a significant correlation (P=0.3). In another study that included 74 CRC patients 51% of those with 2+ or 3+ membranous HER-2 staining based on IHC had a poor prognosis (Molaei et al., 2009). Due to IHC method in these studies, high rate of HER-2 positivity is directly related to experience of pathologist and false-positive reports may have been reported.

Although the results of our study and many other studies which evaluate HER-2 overexpression in CRC patients contradictory, the prognostic and predictive importance of HER-2 known for a long time in breast cancer and shown recently in gastric cancer, is not valid for CRC. Investigation of HER-2 overexpression in more comprehensive, multi-center, prospective trials with standardized methods will be an appropriate approach.

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