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Prognostic Significance of *HER2* and *MLH1* Protein Expression in Colorectal Cancer

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Abstract

In colorectal cancer (CRC), numerous studies showed *HER2* overexpression and amplification as a therapeutic and prognostic target. Further, it was suggested that genetic instability of mismatch repair gene *MLH1* might increase negative predictive value of *HER2* overexpression. Genetic or epigenetic inactivation of *MLH1* gene is generally associated with loss of immunohistochemical expression of the corresponding protein. Hence, present study aimed to investigate the prevalence of *HER2* and *MLH1* protein expression by immunohistochemistry in untreated CRC patients (N=82) and further to examine their association with various clinicopathological variables and prognosis. Cytoplasmic *HER2* and *MLH1* protein expression was found in 31% and 29% of patients, respectively. With regard to clinicopathological parameters, incidence of *MLH1* positivity was significantly higher in patients with adenocarcinomas as compared to mucinous adenocarcinomas (p=0.021). In relation to survival analysis, *HER2* negative expression was associated with unfavorable RFS (p=0.074) and OS (p=0.027) as compared to *HER2* positive expression in total patients. Further a trend of reduced OS was observed with *HER2* negative expression in subgroups of patients with early stage (p=0.082), colon cancer (p=0.099) and rectal cancer (p=0.098). Similar trend of reduced RFS (p=0.072) and OS (p=0.083) was noted with *HER2* negative expression in patients treated with adjuvant therapy. However, *MLH1* protein expression failed to show any prognostic or predictive value. Further, significant positive correlation was observed between *HER2* and *MLH1* protein expression (p<0.001). In conclusion, absence of cytoplasmic *HER2* protein expression could be a useful biomarker to identify high risk group of CRC patients with poor clinical outcome. Further, coexpression of *HER2* and *MLH1* protein may impart additional information on disease outcome in CRC patients.

Keywords: *HER2* protein expression; *MLH1* protein expression; Colorectal cancer; Immunohistochemistry; Prognosis

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Introduction

Colorectal cancer (CRC) ranks the third most common cancer and the fourth leading cause of cancer death worldwide, accounting for about 1.4 million new cases and nearly 700 000 deaths in 2012 [1]. Despite advances in chemotherapeutic treatment in CRC, it is still not capable of preventing recurrence in all patients [2]. Therefore, identification of new therapeutic targets is essential which can improve efficacy of treatment in CRC patients.

Compounds targeted against specific tumor proteins are under trial for many cancers. One of these targets is Human Epidermal Growth Factor Receptor 2 (*HER2*), which is primarily associated with breast cancer [2]. In CRC, recent research efforts have focused on the *HER2* expression due to its proven importance in breast cancer. The *HER2* receptor is a 1255 amino acid, 185 kD transmembrane glycoprotein located at the long arm of human chromosome 17 (17q12). The HER receptors exist as monomers on cell surface and may be activated upon dimerization with *HER2* or other family members. Homo or heterodimerization results in the autophosphorylation of tyrosine residues within the cytoplasmic domain of the receptors and initiates a variety of signaling pathways, resulting in cell proliferation, survival, differentiation, angiogenesis, and invasion [3]. Further, in patients with breast and gastric cancer having *HER2* positive tumors, *HER2*-targeting

agents such as trastuzumab, pertuzumab, and lapatinib are part of the standard therapy. Besides, in many epithelial malignancies such as cancer of the lung, prostate, bladder, pancreas, esophagus and stomach, over expression of *HER2* has been reported. However, overexpression of *HER2* in CRC shows a wide range of variability between 0-84% in different studies [4]. Further, conflicting reports exist about the prevalence of *HER2* over expression in CRC as well as the relationship between *HER2* over expression and the clinicopathological features. Moreover, a very scarce data is available for *HER2* testing particularly in colorectal adenocarcinomas and use of *HER2* therapy in the treatment of CRC patients has been less extensively investigated. Therefore, correlations of *HER2* expression with clinicopathological features and its therapeutic and prognostic implications in CRC urgently require clarification.

Furthermore, recently, Sanguedolce et al. suggested that genetic instability of mismatch repair gene *MLH1* (MutL homolog 1) and MSH2 (MutS homolog 2) might increase negative predictive value of *HER2* overexpression [5]. Moreover, genetic or epigenetic inactivation of *MLH1* gene is generally associated with loss of immunohistochemical expression of the corresponding protein. *MLH1* is a protein in humans that is encoded by *MLH1* gene located on short arm of chromosome 3 (3p22.2). One report suggested that immunohistochemical analysis of *MLH1* and MSH2 expression is a rapid and accurate method for identifying large bowel tumors of MSI-H phenotype [6]. They observed loss of *MLH1* in 90.9% of MSI-H carcinomas whereas all MSI-L and MSS (microsatellite stable) showed normal expression of *MLH1*. Recent studies suggest that immunohistochemical analysis of *MLH1* gene product expression may identify MSI-H colorectal adenocarcinomas [7]. Moreover, several studies have demonstrated a better prognosis for patients with colorectal MSI-H carcinomas with respect to patients with MSI-L/MSS tumors [7,8]. Therefore, studying *MLH1* protein expression along with *HER2* expression may give additional information with regard to prediction of treatment response and prognosis in CRC patients. Hence, present study aimed to study the prevalence of *HER2* and *MLH1* protein expression and their association with clinicopathological parameters in CRC patients. Further, prognostic and predictive value of *HER2* and *MLH1* was evaluated.

Materials and Methods

Patients

A total 82 untreated histologically confirmed CRC patients at The Gujarat Cancer and Research Institute, Ahmedabad, India, between 2015 and 2017, were enrolled in this study. A detailed clinical history including tumor size, lymph node status, histological grade, disease stage, treatment given, etc. during follow up was obtained from case files maintained at the Medical Record Department of the institute. TNM classification with World Health Organization (WHO) Grading System was taken into consideration for pathological staging. Primary treatment offered to all patients was surgery or surgery followed by adjuvant chemotherapy and/or radiotherapy. The main chemotherapeutic treatment included were 5-FU and leucovorin, oral Capecitabine, or in combination with Oxaliplatin. Out of 82 patients enrolled, 78 patients who followed up for the period of 24 months or until death within that period, were included for Over-all survival (OS) analysis. For OS, 3 patients with stage IV cancer and one patient who lost to follow-up were excluded. Out of 78 patients, 15 patients with persistent disease were excluded from relapse–free survival (RFS) analysis. Thus, 63 patients were included for RFS analysis. Survival analysis was also performed in the subgroups of patients with early stage and advanced stage disease; as well as in colon cancer and rectal cancer patients after sub-grouping them according to tumor site. Further, to investigate the predictive value of *HER2* and *MLH1*, survival analysis was also performed in patients treated with adjuvant therapy. The patient and tumor characteristics are shown in **Table 1**.

HER2 and *MLH1* protein expression by Immunohistochemistry (IHC)

For the study of *HER2* and *MLH1* protein expression, paraffin embedded tumor tissue blocks were retrieved from the histopathology department of the institute. Briefly, 4 µm thick sections were cut from the formalin fixed paraffin embedded tumor tissue blocks and were mounted on 3-aminopropyletriethoxy silane coated glass slides. The immunohistochemical staining was carried out using Anti-c-erbB-2 (*HER2*/neu) mouse monoclonal antibody IgG1 (1:20 dilution, clone-CB11, Biogenex, Fremont, CA) and *MLH1* mouse monoclonal antibody IgG1 (1:20 dilution, clone- G168-15, BD Biosciences Pharmingen) along with Mouse and Rabbit specific HRP/DAB (ABC) Detection IHC kit from Abcam, as per manufacturer's protocol recommendations. Antigenicity was retrieved by heating the tissue sections in 10 mM tri-sodium citrate buffer (pH-6.0) solution for 20 minutes in a pressure cooker prior to application of the primary antibody. For antigen retrieval, the pressure cooker was filled with 1500 ml of distilled water and the cooker was placed on the conventional electric stove, covered with an unlocked lid, and heated to a boil. Then the IHC jar, having tissue sections in 10mM tri-sodium citrate buffer (pH-6.0) solution, was placed in the pressure cooker and tissues were boiled for 20 minutes at temperature ranging from 95 to 100°C. All sections were scored independently by two independent researchers in a blinded fashion. A semi-quantitative scoring method ranging from negative (no staining or, 10% of cells stained) to 3+ (1+staining in 11-30% of the cells: weak, 2+ staining in 31-50% of cells: moderate and 3+ staining in >50% of cells: intense) was used.

Ethics statement

Written consent of the patients who underwent surgery at the Department of Surgical Oncology was obtained, prior to primary tumor tissue collection. The study was approved by the Institutional Scientific and Ethical Review Committees.

Statistical analysis

The statistical data analysis was performed with the help of SPSS (Stastistical Package for the Social Sciences) software. Two tailed chi square $\left(\chi^2\right)$ test was used to determine the association of *HER2* and *MLH1* protein expression with clinicopathological

Table 1 Patient and tumor characteristics.

Characteristics	N (%)
Age (years) (Range: 20-80 years) Median age: 56 years	
<56	41 (50)
≥56	41 (50)
Gender	
Female	37 (45)
Male	45 (55)
Tumor site	
Colon	48 (59)
Rectum	34 (41)
Tumor size	
T2	14 (17)
T3	62 (76)
T4	06 (07)
TNM stage	
I	08 (10)
II	39 (48)
III	32 (39)
IV	03 (03)
Tumor differentiation	
Well	13 (16)
Moderate	59 (72)
Poor	10 (12)
Histological type	
Adenocarcinoma	61 (74)
Mucinous adenocarcinoma	21 (26)
Necrosis	
Absent	75 (92)
Present	07 (08)
Lymphatic permeation	
Absent	66 (80)
Present	16 (20)
Vascular permeation	
Absent	78 (95)
Present	04 (05)
Recurrence/Metastasis (N=63)	
Absent	57 (90)
Present	06 (10)
Disease outcome (N=78)	
Alive	62 (79)
Dead	16 (21)

variables. RFS and OS were calculated using Kaplan-Meier method and Log rank test. Correlation between two parameters was calculated using Spearman's correlation coefficient (r) method. P value ≤ 0.05 was considered significant.

Results

Incidence of *HER2* and *MLH1* protein expression

The expression of *HER2* and *MLH1* protein was localized in cytoplasm of the epithelial cells of colon and rectal tumors. *HER2* positive protein expression was observed in 31% (25/82) of patients whereas 69% (57/82) of patients showed *HER2* negative protein expression. Further, there was a preponderance of negative expression of *MLH1* in 71% (58/82) of the patients and 29% (24/82) showed positive *MLH1* protein expression. Representative pattern of *HER2* and *MLH1* protein expression in CRC patients is shown in **Figure 1**.

Correlation of *HER2* and *MLH1* protein expression with clinicopathological parameters

A trend of higher *HER2* positive expression was observed in ≥ 56 years age group patients (39%) as compared to <56 years age group patients $\left(22\%, \chi^2 = 2.820,\ r = +0.185,\ p = 0.095\right)$. Further, when the patients were sub grouped in four different age groups with a 15 years gap period (20-35, 36-50, 51-65 and 66-80), no such trend was noted. Moreover, a trend of higher *HER2* positive expression was observed in patients having absence of necrosis (33%) as compared to those having presence of necrosis $\left(0\%, \chi^2 = 3.357, r = -0.202, p = 0.068\right)$. Further, a significant higher *MLH1* positivity was bserved in patients with adenocarcinoma (36%) as compared to those with mucinous adenocarcinoma $\left(9\%; \chi^2 = 5.316, r = -0.255, p = 0.021\right)$ and a trend of higher incidence of *MLH1* positivity was observed in males (37%) as compared to females $\left(\chi^2 = 5.223, r = +0.198, p = 0.074\right)$. In addition, when *MLH1* protein expression was correlated with four different age groups, an increasing trend of *MLH1* positivity was noted with the increase in the age $\left(\chi^2 = 5.223, r = +0.198, p = 0.074\right)$. The observed *MLH1* positivity was 0% (20-35 years), 30% (36-50 years), 32% (51-65 years), and 42% (66-80 years).

Intercorrelation between *HER2* and *MLH1* protein expression

A significant positive correlation was observed between *HER2* and *MLH1* protein expression $\left(r = +0.389, p < 0.001\right)$.

Survival analysis for RFS (N=63) and OS (N=78) in relation to *HER2* and *MLH1* protein expression in CRC patients

In total patients, Kaplan-Meier univariate survival analysis showed that patients having negative *HER2* expression was associated with a trend of reduced RFS $\left(14\%, 6/42; Log\ rank = 3.195, \mathrm{df} = 1, \mathrm{p} = 0.074\right)$ **Figure 2a** and a significant reduced OS $(27\%,\ 15/55;\ \text{Log rank}=4.882,\ \text{df}=1,\ \text{p}=0.027)$ **Figure 2b** as compared to those with positive *HER2* expression $\left(RFS: 0\%, 0/21; OS: 4\%, 1/23\right)$. Further, according to disease stage, in early stage patients, a trend of high incidence of death was noted in *HER2* negative patients (21%,7/33) as compared to those with *HER2* positive patients $\left(0\%, 0/13; Log\ rank = 3.021, df = 1, p = 0.082\right)$. Moreover, according to tumor site, a trend of unfavorable OS was observed in colon cancer patients with negative *HER2* expression $\left(21\%, 7/34\right)$ as compared to those with positive *HER2* expression $\left(0\%, 0/12; \text{Log rank} = 2.726, \mathrm{df} = 1, \mathrm{p} = 0.099\right)$. Further, a trend of unfavorable OS was also noted in rectal cancer patients with negative *HER2* expression $\left(32\%; 8/21\right)$ as compared to those with positive *HER2* expression $\left(9\%, 8/21; Log\ rank = 2.724, df = 1, p = 0.098\right)$. However, *MLH1* protein expression showed no significant association with RFS and OS in total patients or in any of the subgroups.

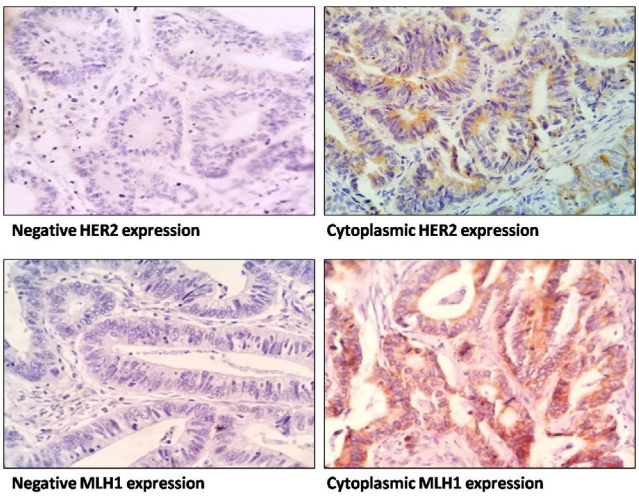

Figure 1 Immuno-histochemical staining of HER2 and MLH1 in tumor tissues (40x).

Survival analysis for RFS (N=54) and OS (N=64) in relation to *HER2* and *MLH1* protein expression in CRC patients treated with adjuvant therapy

In total CRC patients treated with adjuvant therapy, Kaplan-Meier univariate survival analysis demonstrated that patients with negative *HER2* expression showed a trend of reduced RFS $(17\%, 6/36; Log\ rank = 3.231, df = 1, p = 0.072)$ and a trend of reduced OS $(24\%, 11/45;$ Log rank=2.999, df=1, p=0.083) as compared to those with positive *HER2* expression $(RFS: 0\%, 0/18; OS: 5\%, 1/19)$. Further, *MLH1* expression showed no significant association with RFS or OS in patients treated with adjuvant therapy.

Discussion

HER2 has been shown to be an effective target for adjuvant therapy for breast cancer and gastric cancer patients. Currently the value of *HER2* status in colorectal cancer is also being explored and a great degree of variation has been reported on detection of *HER2* over expression in CRC by IHC [9]. In present study, we investigated the prevalence of *HER2* over expression using Biogenex CB11 monoclonal antibody in cohort of 82 CRC patients. Only cytoplasmic staining of *HER2* protein was found in 31% of the studied patients. In contrast, Dursun et al, using Biogenex CB11 reported 14% membranous staining and no cytoplasmic staining in colorectal neoplasia [10]. Kavanagh et al. detected HER-2 overexpression in 11% of CRC patients and showed membranous staining [11]. Further, Kountoutakis et al. and Gill et al. observed cytoplasmic staining along with membranous staining using NCL-CB11 monoclonal antibody, where the former found 22.64% *HER2* positivity in colorectal adenocarcinoma and the latter found 65% *HER2* positivity in colon carcinoma [12,13]. Schuell et al. using different *HER2* antibody also reported similar incidence with 29.9% of 77 CRC patients showing *HER2* immunopositivity [14]. Kruszewski et al. observed 24% of *HER2* positive protein expression in a cohort of 50 CRC patients [15]. In addition, some studies in CRC have also reported incidence of *HER2* positivity as low as 0.5% [16] to 11% [11]. The debate about such a wide range of *HER2* expression might be attributed to several causes, such as primary antibody difference, a difference in scoring systems, a difference of sample size, racial differences, and heterogeneity of study population. The unique finding of the present study is that it showed only cytoplasmic immunostaining for *HER2* expression as compared to other studies showing *HER2* membranous and/or cytoplasmic staining. This result of the current study might suggest that probably the membrane bound *HER2* was internalized into the cytoplasm upon activation or *HER2* was activated internally via homodimerization [17]. Further, half et al. found that cytoplasmic *HER2* was localized near rough endoplasmic reticulum (rER) [18], which led them to hypothesize that *HER2* is likely to be derived from original peptide synthesis and thus cytoplasmic *HER2* expression would be of genomic origin.

In present study, a trend of higher incidence of *HER2* positivity was observed in older age group as compared to younger age group. Gill et al. and Kountourakis et al. also observed significantly higher immunopositivity of *HER2* in older age patients in colon and colorectal carcinoma, respectively [13,12]. Additionally, although not significant, present study showed association between *HER2* positive expression and absence of necrosis. One study in CRC showed that *HER2* positivity was correlated with perineural invasion, vascular invasion, lymph node metastases, and higher TNM stage [19]. Tavangar et al. and Antonacopoulou et al. observed higher incidence of *HER2* positivity in advanced stage patients as compared to early stage CRC patients [20,21]. On the other side, Schuell et al. and Sayadnejad et al. showed no significant association of *HER2* expression with clinicopathological parameters in CRC patients [14,22].

Assuming that cytoplasmic *HER2* has a prognostic role in CRC, it might also be involved in tumor pathogenesis like membranous *HER2* in breast cancer. Hence, the current study evaluated the prognostic significance and observed that *HER2* positive patients had a significantly improved overall survival (p=0.027) and recurrence free survival (p=0.074) time than their respective counter parts. Additionally, the subgroups of early stage, colon cancer and rectal cancer patients with *HER2* positive tumors, displayed a trend towards better disease outcome. In accordance, although not significant, Tu et al. also observed higher survival rates of *HER2* positive patients as compared to *HER2* negative patients in CRC [23]. These results are however in contrast with other studies in which a negative correlation of *HER2* positivity was observed with survival [24]. However, Schuell et al. and Kruszewski et al. observed no correlation between *HER2* expression and survival in CRC patients [13,14]. Kavanagh et al. also demonstrated that overexpression of *HER2* was not a predictor of disease-free and overall survival in CRC patients [11].

Presence of intracellular *HER2* might have therapeutic implications in treatment with targeted therapy. Hence, present study evaluated the utility of *HER2* in patients treated with adjuvant therapy, and, observed a trend of better RFS and OS with positive *HER2* expression suggesting that *HER2* immunopositivity could be a useful prognosticator in predicting better treatment response in CRC patients. There are reports that if cytoplasmic *HER2* is

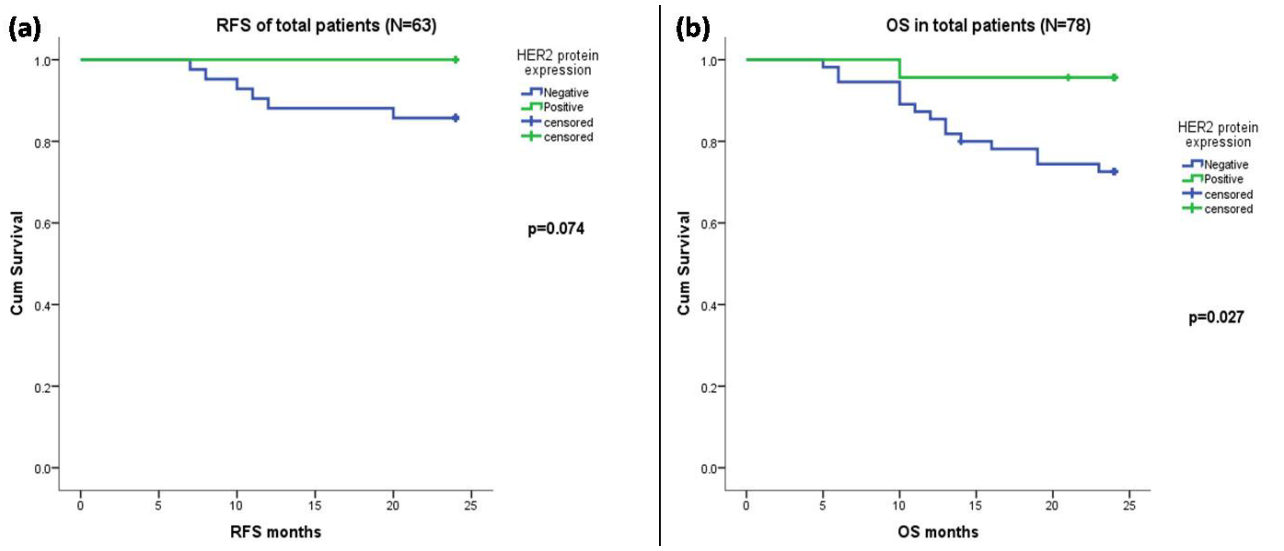

Figure 2 Survival analysis in total CRC patients (a) A trend of reduced RFS and (b) A significant reduced OS in patients with negative HER2 expression as compared to those with positive HER2 expression.

actively involved in colorectal carcinogenesis, the administration of lapatinib or other intracellular *HER2*-targeting compounds could be a breakthrough in the treatment of especially CRC those who have failed to respond to adjuvant chemotherapy [17]. However, a major limitation of the current study is that mutational results of KRAS and BRAF have not been included and that *HER2* is responsible for cell survival and proliferation via signaling through the RAS–RAF–ERK and PI3K–PTEN–AKT pathways [25]. So, present results were unable to elucidate the exact relationship of *HER2* expression with therapeutic response in CRC. Moreover, we found that *HER2* protein was indeed present in patients with CRC but very few cases showed strong expression enough to consider for its therapeutic target.

Now, it is an established fact that investigation of MSI status in CRC is warranted for three reasons: first, as a potential screening tool for HNPCC, second as a prognostic marker and, finally, as a potential predictor of response to chemotherapy. Therefore, to improve understanding of CRC further, investigation of mismatch repair gene *MLH1* could be important, which is most often known to arise from epigenetic silencing in sporadic cancer. Hence, present study focused on exploring the prevalence of *MLH1* protein expression and its prognostic value in CRC patients.

In current study, only 29% of 82 cases showed positive cytoplasmic *MLH1* expression while 71% of the patients lacked *MLH1* expression. Lack of protein expression as a surrogate for MSI is clinically important to patients. Cytoplasmic staining in the absence of nuclear staining for *MLH1* has been previously described in only 1 CRC case among the studied patients [26]. Moreover, in a study conducted on endometrial cancer patients by Berends et al, immunohistochemical staining in three patients showed clear cytoplasmic, but no nuclear staining for *MLH1* in tumor cells while in normal cells, a normal nuclear staining pattern was observed. Apart from the possibility of a technical artifact, it would be hypothesized that the *MLH1* protein was incompletely synthesized in the cytoplasmic ribosomes due to a mutation (as yet undetected) in the *MLH1* gene that prevents transport to the nucleus [27].

In relation to clinical parameters, in current study a trend of higher incidence of negative *MLH1* expression was observed in females as compared to male patients. Similar correlation of *MLH1* expression with gender was obtained in a study on CRC patients conducted by Soreide et al. [28]. However, Kishi et al, in 97 surgical specimens after chemotherapy, observed that *MLH1* expression did not show any correlation with clinicopathological features, such as gender, age, tumor location, histological grade, tumor length, tumor depth, and lymph node metastasis in esophageal cancer [29]. Further, present study showed that negative *MLH1* expression was significantly higher in mucinous adenocarcinoma as compared to adenocarcinoma (p=0.021). It is known that mucinous histological types of tumors are usually of aggressive phenotype compared that of adenocarcinomas. Hence, the finding probably indicates that loss of *MLH1* protein expression could be useful in identifying patients with aggressive phenotype.

Few studies are available regarding prognostic value of *MLH1* expression in CRC. Present study reported better OS and RFS in patients with negative *MLH1* expression; however, the data was not statistically significant. In accordance, recently Huang et al. demonstrated that the 5-year-survival rate was significantly lower for *MLH1* overexpression (77.5% versus 94.4%, p=0.039) in colon cancer patients [30]. Kruschewski et al. showed that significant differences for overall and recurrence-free survival were not seen for patients with *MLH1*-negative carcinomas, although there was a tendency for longer overall survival (72 vs. 63 months) [31]. Moreover, aberrant MMRP expression may have an effect on the prognosis of stage III CRC patients [32]. Further, Wilczak et al. described that MMR (MSH6, *MLH1* and PMS2) overexpression was linked to poor outcome in prostate cancer. It is interesting that MSI positive (*MLH1* negative) CRCs seem to have a better prognosis, because they are often less differentiated and larger than MSI negative cases (features high proliferation and low

survival) [33]. The reasons for the putative survival advantage are not known, but plausible explanations include a self-destructive effect of numerous mutations accumulating in the cell genome, possibly mutating genes necessary for the viability of the malignant clone. Resulting mutant proteins may also be transferred to the cell membrane to evoke an immune reaction against the tumor [34].

In a recent report, it was observed that simultaneous genetic instability of *MLH1* and MSH2 might increase the negative predictive value of *HER2* overexpression [5]. Hence, present study for the first time evaluated intercorrelation between *HER2* and *MLH1* protein expression in CRC and observed significant positive correlation between them. Additionally, patients coexpressing both *HER2* and *MLH1* proteins had significantly better disease outcome as compared to those with absence of both the proteins (data not shown). Thus, it suggests that coexpression of *HER2* and *MLH1* could be useful in discriminating high/low risk group of CRC patients.

Conclusion

In conclusion, absence of cytoplasmic *HER2* protein expression could be useful biomarker to classify high risk group of CRC patients with poor clinical outcome. Although *MLH1* protein expression alone had no prognostic value in studied patients, combine use of *HER2* and *MLH1* protein expression may probably emerge as a biomarker to predict clinical outcome in CRC patients.

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