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Original Article

Immunohistochemical analysis of Sonic Hedgehog in gastric adenocarcinoma and the relationship between mucin and HER2 expressions

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Abstract

Objective: Most of the gastric adenocarcinomas are still recognized in advanced stages despite the improvements in diagnostic methods. Inhibition of the SHH pathway is predicted to be a targeted therapy in advanced gastric adenocarcinomas. Determining the relationship of the SHH pathway with other prognostic markers in gastric cancers is being investigated in terms of patient management. This study aimed to investigate the relationship between Sonic Hedgehog (SHH) and mucin core proteins (MUCs) antibody expressions with prognosis and Human epidermal growth factor receptor-2 (HER2) levels in gastric adenocarcinomas.

Methods: Eighty-six patients diagnosed with gastric adenocarcinoma in surgical resection material between 2008-2014 were included in the study. The clinicopathological findings of the cases were recorded from hospital documents. Pathologic diagnoses were reclassified based on WHO (2019) classifications. An examination of MUC1, MUC2, MUC5AC, MUC6, SHH, and HER2 antibodies was performed immunohistochemically. The tissue microarray technique was used in the study. The clinicopathological findings were statistically evaluated by comparing them with the immunohistochemical findings and survival.

Results: The study found that tumor diameter, lymphovascular embolus, perineural invasion, lymph node metastasis, and tumor depth had a statistically significant impact on survival. Additionally, the immunohistochemical examination showed no correlation between SHH, mucin antibodies, and HER2 scores with survival. According to our results, HER2 overexpression was associated with MUC1 to luminal staining (p=0.0001). When HER2 expression and SHH expressions were compared, all cases with HER2 overexpression were found to be positive with SHH (p=0.03). This is the first study to determine the relationship between SHH, MUC1, and HER2 immunohistochemical expressions in gastric adenocarcinomas.

Conclusion: Examining the HER2 relationship between SHH and MUC1 expressions we have shown, with future genetic and molecular studies, will provide an understanding of different malignancy pathways in gastric adenocarcinomas.

Keywords: Gastric adenocarcinoma, mucins, Sonic Hedgehog, human epidermal growth factor receptor-2.

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INTRODUCTION

Gastric cancer accounts for approximately 7-8% of all cancers worldwide (1). Although its incidence has decreased in the last 15 years, it is still one of the most common causes of cancer deaths (1). Mucin core proteins (MUCs) are high molecular weight glycoproteins that include many chains of surrounding carbohydrates attached to a central polypeptide (2). Mucus core protein 1 (MUC1) (episialin) is a membrane-associated mucin (2). MUC1 is highly expressed in intestinal-type carcinoma, especially in well and intermediately differentiated adenocarcinomas (2). MUC5AC is highly expressed in well-differentiated tumors, and its expression has been reported to decrease with differentiation. (2). Sonic Hedgehog (SHH) has first been defined in the Drosophila melanogaster (vinegar fly) homologue (3). It controls cell division in adult cells and affects the development of some cancers (4). SHH pathway regulates the expression of transcription factors and target genes, impacting cell growth, survival, and differentiation (5,6). In addition to stomach cancers, the SHH pathway has been extensively researched in other types of cancer, including basal cell carcinoma, medulloblastoma, pancreatic cancer, and colon cancer (7-10). No SHH expression is observed in normal gastric mucosa; however, its expression gradually increases compared to that of normal mucosa in some cases, such as intestinal metaplasia and gastritis and some neoplastic conditions (11). The Hedgehog pathway plays a significant role in the proliferation and growth of stomach cancer cells. It is worth noting that this pathway is activated in approximately one-third of stomach cancer cases (12). The human epidermal growth factor receptor-2 (HER-2) gene is a member of the HER family and is a protooncogene (13). Overexpression of HER2 has been found to be associated with serosal invasion, lymph node metastasis, stage, and distant metastasis in many studies (6). However, some studies have failed to establish a relationship between HER2 status and clinicopathological features and survival (14). Other studies have found that HER2 is more likely to be positive in proximal localization, intestinal type, and advanced stage tumors with clinicopathological features (15-17).

Most of the stomach adenocarcinomas are diagnosed in advanced stages in spite of the developments in diagnostic methods. In studies using some SHH pathway inhibitor agents, it has been shown to stop the proliferation of tumor cells. HER2 is utilized in the treatment of selected patients with gastric adenocarcinomas. Studies have shown variable results regarding the relationship between SHH, HER2, and mucin antibodies and survival. There are few studies in the literature investigating the relationship between these therapeutic agents. Knowing the relationship between this pathway and other prognostic features in gastric cancers may offer new treatment options in advanced patients.

This study aims to investigate immunohistochemistry of Sonic Hedgehog (SHH) and mucin core proteins (MUCs) antibody expressions in gastric adenocarcinomas in relation to prognosis and human epidermal growth factor receptor-2 (HER-2) levels.

MATERIALS AND METHODS

The present study received ethics committee approval from Pamukkale University non-invasive clinical research ethics committee at the board meeting numbered 07 dated 04-29-2014. The study was conducted retrospectively at our institute. All patients (86 patients) who were diagnosed with gastric adenocarcinoma in surgical resection material between 2008 and 2014 and whose materials were in the archive were included in the study. The blocks best representing the tumor were selected; histopathological findings such as the histopathological type, tumor size, tumor depth, number of lymph nodes, and number of metastatic lymph nodes were noted by a reevaluation of the pathology samples and reports. Preparations of the selected cases were removed from the archives of the Department of Pathology and were evaluated by two observers at the same time on a multi-headed microscope. Pathological diagnoses were again classified according to WHO (2019) classifications (1). For survival evaluation, cases were followed from the time of diagnosis until the end date of the study (November 31, 2015) or until death. The clinicopathological parameters, immunohistochemical findings, and their relationship with survival were evaluated statistically.

The tissue microarray technique (TMA) was used to evaluate multiple cases in a single section. The areas

most indicative of the tumor were collectively determined through consensus by two pathologists. Three tissues with a diameter of 2 mm were taken from the original tumor blocks and embedded in the recipient blocks. Then, 3-4 micron thick sections were taken from the recipient blocks for immunohistochemical and hematoxylin-eosin staining.

For deparaffinization, the isolated sections were dried in the oven at 60 °C for at least two hours. The entire staining process, including deparaffinization and antigen retrieval, was performed in fully automated BenchMark XT immunohistochemistry-staining equipment (Ventana Inc., USA). The used antibodies are MUC1 (Spring, SPM492), MUC2 (Spring, SPM512), MUC5AC (Dako, CLH2), SHH (Spring, AB73958), HER2 (Cell Marque, SP3) 1/100 dilution, MUC6 (Cell Marque, MRQ-20) 1/250 dilution. Immunohistochemical score was used: negative: non-staining, +: 0-5% staining, ++: 5-25% staining, +++: 25% and higher staining (18). Cases luminally stained for MUC1 were noted. The Hercep-TestTM scoring system for breast cancer was used for HER2 scoring with a modified form of it for stomach cancer (19). Cases with HER-2 and SHH scores of 2-3 were grouped as overexpression.

Statistical analysis

All analyses were performed using the SPSS program (version 21.0, SPSS Inc., Chicago, IL, USA). Continuous variables were expressed as mean±standard deviation and categorical variables were expressed as numbers and percentages. When the parametric test assumptions are provided, the significance test of the difference between the two averages and the One-Way Variance analysis were used to compare the independent group differences. Mann Whitney U test and Kruskal Wallis Variance Analysis were used in the comparison of independent group differences when parametric test hypotheses were unmet. Differences between categorical variables were analyzed using the Chi-Square test. For survival analysis, Kaplan Meier survival analysis and log-rank method were used to analyze survival differences between independent groups. Also, the Cox regression method was used to define the factors affecting survival. A value of $p<0.05$ was accepted as significant for the results obtained.

RESULTS

Clinicopathologic features

The distributions of clinicopathological features and their relationship with survival are presented in Table 1. Table 1 shows that among the clinicopathological features, tumor diameter, tumor depth, presence of lymphovascular embolus, presence of perineural invasion, presence of lymph node metastasis, and nodal stage have a statistically significant impact on survival. When factors significantly associated with survival were evaluated by multivariate Cox regression analysis, large tumor diameter, and perineural invasion were found to be independent risk factors for survival. Large tumor diameter negatively affects survival by 1.98 times and the presence of perineural invasion by 2.98 times.

Immunohistochemical findings

The results of immunohistochemical scoring are given in Table 2. No significant relationship was found between survival and immunohistochemical examinations of MUC1, MUC2, MUC5AC, MUC6, and SHH. Among the cases with MUC1 luminal staining, the rate of HER2 score ++/+++ was found to be 50%, and it was found to be 17.1% among the cases with no luminal staining ($p=0.0001$) (Figure 3A, 3B). When HER2 and SHH expressions were compared, all 19 cases with a HER2 score of 2-3 (100%) were found to be SHH positive (greater than 5%) ($p=0.03$).

Survival analysis

For survival assessment, cases were followed from the time of diagnosis until the end date of the study (November 31, 2015) or until death. Survival data were obtained from the hospital information system. Of

Table 1. Clinicopathological features distribution.

Clinicopathological features		Average (Month)	Median (Month)	P
Survival	General	28.8±3.2	17±3	
Gender	Male	25.9±3.4	17±2.09	0.18
	Female	37.5±7.03	31±10.8	
Age	Under 62 years old	28.17±4.6	18±3.3	0.98
	Over 62 years old	26.95±3.7	17±2.6	
Tumor diameter	Below 5 cm	35±4.4	27±6.6	**0.01**
	Over 5 cm	19.9±3.8	13±1.5	
Tumor localization	Proximal Distal	28.1±4.5 31.7±5.1	17±2.4 31.7±5.1	0.11
	Linitis plastica	13.6±4.1	11±4.08	
Histopathological grade	Low grade Intermediate grade	55.3±15.2 25.03±4.03	54.3±14.2 24±5.05	0.27
	High grade	19.6±4.4	14±2.3	
Lymphovascular embolus	+ -	24±3.2 41.4±7.7	14±2.07 42±11.6	**0.02**
Perineural invasion	+ -	22.12±3.1 43.6±6.6	13±1.4 42±14.5	**0.01**
Distant metastasis	+ -	16.6±3.9 31.2±3.6	17±1.4 18±5.1	0.11
Lymph node metastasis	+	24.1±3.2	37±5.8	**0.01**
	-	40.06±6.8	14±2.2	
Nodal stage	N0 N1 N2	40.06±6.8 34.7±5.8 24.2±3.8	37±5.8 23±15.08 20±7.2	**0.01**
	N3	19.32±3.7	12±1.7	
Tumor depth	T1 T2 T3	64.6±8.5 6.3±3.3 35.9±6.5	61.4±7.4 4±1.6 33±13.5	**0.008**
	T4	18.8±2.07	14±2.2	

Table 2. Immunohistochemical scoring distribution.

Immunohistochemical distributions	Negative n (%)	+ n (%)	++ n (%)	+++ n (%)
MUC1	13 (15.5)	9 (10.7)	23 (26.2)	40 (47.6)
MUC2	63 (74.1)	12 (14.1)	3 (3.5)	7 (8.2)
MUC5AC	33 (38.8)	11 (12.9)	15 (17.6)	26 (30.6)
MUC6	32 (37.6)	14 (16.5)	10 (11.8)	29 (34.1)
SHH	3 (3.5)	5 (5.99)	21 (24.7)	56 (65.1)
HER2	58 (67.4)	8 (9.3)	7 (8.1)	13 (15.1)

Abbreviations: *MUC: Mucin Core Protein, SHH: Sonic Hedgehog, HER2: Human epidermal growth factor receptor-2.*

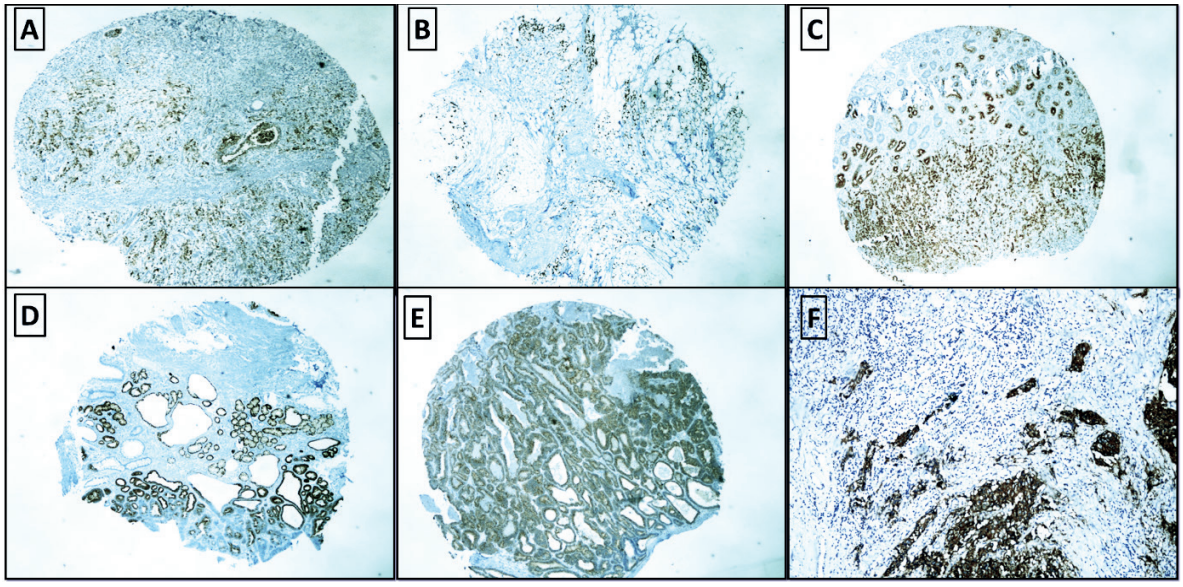

Figure 1. Gastric adenocarcinoma staining positive for mucin, SHH and HER2: A) MUC1 score +++ (40X), B) MUC2 score +++ (40X), C) MUC5AC score +++ (40X), D) MUC6 score +++ (40X), E) SHH score +++ (40X), F) HER2 score 3(40X).

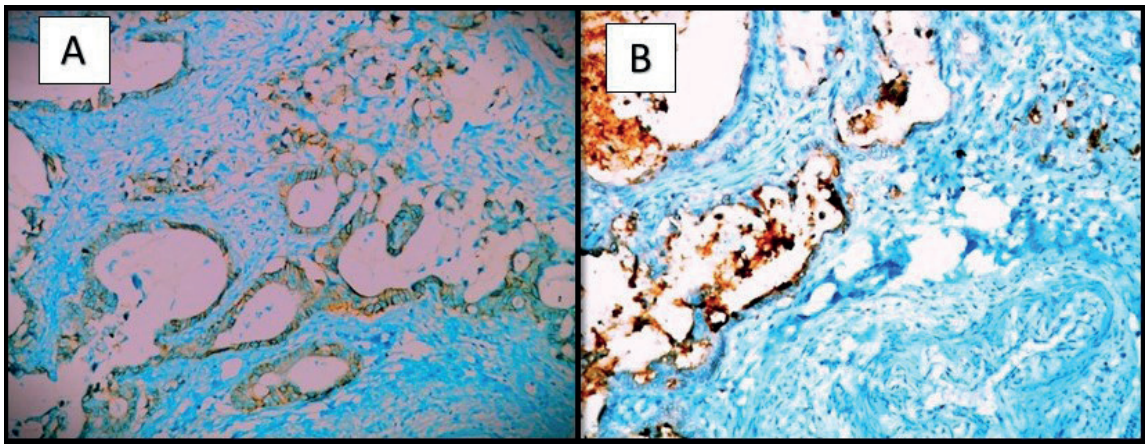

Figure 2. A: Immunohistochemical HER2 score 3 staining (20X) B: MUC1 score +++ luminal staining in the same area (20X)

the 86 cases included in the study, 31 (36%) were alive. The mean overall survival was 28.8±3.2 months, and the median survival was 17±3 months. Thirty-one out of 86 cases included in the study are alive (36%). Mean overall survival was 28.8±3.2 months, and median survival was 17±3 months.

DISCUSSION

Gastric cancer is one of the most common causes of death due to cancer (1). The incidence and mortality of gastric cancer have been gradually decreasing worldwide (1). Therefore, it is important to diagnose the disease early and to develop new agents for treatment. Although gastric cancer is seen rarely under the age of 30 years, its incidence gradually increases with increasing age (1). The study group in this study is compatible with the literature in terms of age distribution. No significant association was found between age and survival in this study, although there have been studies in the literature reporting that advanced age is a negative prognostic factor (15).

Stomach cancer is seen twice more frequently in men compared to women (1). This ratio has been reported to be around 1.8 -2.6/1 in the literature (14). The male/female ratio was found to be higher in this study compared to the literature findings (3.3/1).The mean tumor diameter in this study was compatible with the findings of the literature and was found to be associated with poor prognostic factors (20). Tumor diameter was found to be an independent risk factor by univariate and multivariate analyses. The rate of LVE varies between 7.2% and 86% in gastric cancer in the studies performed (21,22). The rate of lymphovascular embolus was 80.2% in this study, and LVE was found to affect survival by 2.23-fold in multivariate analysis negatively. PNI was found to be an independent risk factor in this study and negatively affected survival by 2.98-fold. As indicated in this study, the presence of lymphovascular embolus and perineural invasion are important prognostic factors that should be stated in pathology reports. The depth of tumor invasion is one of the most important prognostic factors (23,24). Currently, gastric cancers are diagnosed when the depth of invasion exceeds submucosa (25). The rate of early and advanced gastric cancers in this study was 8.1% and 91.9%, respectively. Mean survival was 64.6 and 24.7 months in early and advanced gastric cancers, respectively (p=0.008). The findings in this study were compatible with the literature; however, no association was found between other clinicopathological parameters. The effects of mucin expressions on survival are still controversial (26-28). No significant association was found between mucin expressions and survival in this study. There is no standard method of investigation of mucin expressions in terms of scoring and the studied antibodies in the literature. The main reason for incoherence in the studies might be the absence of a standard method. Also, the TMA method was used in this study, and thus, staining could not be evaluated in the whole tumor tissue, which might be a factor affecting the results. On the contrary, significant results were obtained in a study in which mucin expressions were evaluated using the TMA method (18). The cytoplasmic tail of MUC1, was demonstrated to be associated with the virulence factor CagA of HP, and wnt-β catenin was demonstrated to be the main stimulator of the intracellular signal cascade (29). MUC1 expression was reported to be increased in HP infection, and MUC1 has been reported to undertake the role of an intracellular signal pathway in HP-related gastric cancers through the Wnt-β catenin pathway (29). The association of MUC1 and HP +++ cases in this study supports this opinion. In studies on MUC1 genes, it was found that it mediated the resistance to recombinant HER2/neu antibody, trastuzumab, and therefore it is important to shut up the MUC1 gene in targeted therapy (30). HER2 overexpression was found in 50% of the cases with MUC1 luminal staining in this study. There is no study in the literature reporting the association of MUC1 immunohistochemical expression and HER2, and this study is the first to detect this association. We propose an examination of the correlation between MUC1 luminal expression and HER2 overexpression concerning targeted therapies. Studies have demonstrated significant associations between MUC2 expression and non-gastric mucinous carcinomas observed in other organs, such as the colon and pancreas (2,11). Although it was not found to be statistically significant in this study, a high rate of MUC2 expression was noted in WHO mucinous adenocarcinoma. In a study by Ilhan et al, MUC5AC expression was detected to be decreased indirectly proportional to the depth of invasion, tumor differentiation, and number of metastatic lymph nodes (2). MUC5AC expression loss was observed in cases with only PNI positivity among the prognostic factors in this study. This series is the first that detects

a higher rate of MUC5AC expression at a young age. MUC6 has been reported to be a good prognostic factor in gastric adenocarcinomas (18). MUC6 expression was found to be high in cases only with no lymph node metastasis among the clinicopathological parameters in this series, and this was interpreted in favor of a good prognostic factor. Sonic Hedgehog has been highly expressed in gastric adenocarcinomas compared to normal gastric mucosa in the studies performed (31). Overexpression of SHH has been found to be associated with advanced stage, increased tumor invasion, well differentiation, and poor prognosis (32-34). Lee et al. found a higher rate of SHH in intestinal cancers and tubular adenocarcinomas compared to mucinous and signet-ring cell cancers (34). The only study indicating that the overexpression of SHH served as a favorable prognostic factor was conducted by Kim et al (35). In studies using the SMO-specific inhibitor (cyclopamine) to investigate the role of the SHH pathway in therapy, it has been found that the proliferation of tumor cells has stopped (35,36). Inhibition of the SHH pathway in advanced-stage gastric adenocarcinomas has been predicted to be a targeted therapy. In this series, Lauren intestinal type overexpression of SHH was noted and it was compatible with the previous studies. There is no study reporting an association between SHH and HER2 expression. SHH was found to be ++/+++ in all cases with overexpression with HER2 in this study. In light of this information, an association might be found between SHH and HER2 development pathways, and new opinions might be presented regarding the studies on targeted therapies through the hedgehog pathway. In a study on breast cancer, resistance was detected against neoadjuvant treatment with trastuzumab in Gli1 positive cases through hedgehog pathway in HER2 positive cases, and thus it has been reported that it could be a new treatment agent in HER2 positive cases (38). Since inhibition of a new pathway in tumor development will increase the rate of treatment, such treatment methods should be investigated. HER2 condition has been detected to be associated with serosal invasion, lymph node metastasis, stage, and distant metastasis in most studies (16,39). The studies performed revealed a higher rate of positivity of HER2 in proximal localization, intestinal type, and advanced age tumors, and it is associated with shorter survival; however, the addition of anti-HER2 agents in the treatment protocols increases the efficacy of targeting the treatment (16,40). Mean survival has been increased from 11.1 months to 13.8 months with trastuzumab treatment in addition to the chemotherapy applied (39). In this study, which is compatible with the findings of the literature, a significantly higher rate of HER2 expression was found in the intestinal type. Seventeen out of 20 cases (85%) with overexpression of HER2 were found to have tumors exceeding the serosa. In this study, although we observed a significant difference in survival with different levels of HER2 expressions, no statistical significance was found due to the low number of cases. Although HER2 expression is related to poor prognosis in gastric cancers, it presents a chance of treatment in advanced cases and increases survival; thus, HER2 expression is both clinically and prognostically important.

Limitations:

Our study has several limitations. It is important to note that this was a single-center study. While we believe that our number of patients was statistically sufficient, we acknowledge that analysis with a larger number of patients may lead to different results. Additionally, we were unable to carry out molecular research due to cost constraints.

CONCLUSION

No classification was found as a possible independent prognostic factor among the histopathological classifications used in gastric adenocarcinomas in this study. Tumor diameter, depth of invasion, LVE, PNI, the number of total and metastatic lymph nodes, nodal stage, and tumor grade are found as prognostic factors that have to be stated in gastric cancer reporting. This is the first study to detect the association of cases with MUC1 luminal expression with HER2 overexpression. We suggest that investigating the relationship between MUC1 luminal expression and HER2 overexpression may shed light on understanding the mechanisms of carcinogenesis. Detection of SHH overexpression in all cases with HER2 overexpression demonstrates the association between SHH and HER2 development pathways. We suggest that this relationship should also be evaluated in targeted treatments in advanced HER2-resistant cancers. In conclusion, investigation of SHH and MUC1 expressions, which we have shown to be associated with HER2,

may provide an understanding of the different pathways of the malignancy process in gastric cancers with many different developmental mechanisms and may increase the rate of treatment in advanced stages.

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