Original paper

Prognostic importance of molecular-like classification in gastroesophageal junction tumors (Izmir Oncology Group {IZOG} study)

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A comprehensive molecular classification was published in 2014 within The Cancer Genome Atlas (TCGA) to guide clinical approaches and treatment strategies. This study aimed to investigate the clinicopathological and prognostic importance of the classification using immunohistochemistry (IHC) and chromogenic in situ hybridization (CISH) to identify potential surrogate markers of molecular changes in gastroesophageal junction (GEJ) adenocarcinomas.
A total of 52 GEJ adenocarcinomas were divided into five groups using IHC with MLH-1, E-cadherin, p53 and CISH with EBER: 1) microsatellite unstable (MSI: negative with MLH-1), 2) genomically stable tumors (GS: positive with p53), 3) chromosomally unstable tumors (CUN: negative with e-cadherin), 4) EBV+ tumors (EBV+: positive with EBER) and 5) unclassifiable (G-NOS: MLH-1 and e-cadherin positive with p53 and negative with EBER).
The largest group consisted of 24 (46.2%) cases of CUN tumors. This group was followed by groups of GS with 14 (26.9%) cases, MSI with 7 (13.5%) cases, and EBV + with 3 (5.8%) cases, respectively. Although this classification was not associated with pathological features, it was found to be closely related to prognosis ($p = 0.029$). Patients with EBV+ tumors had the longest overall survival, followed by the G-NOS, MSI, CUN, GS groups.

Key words: gastroesophageal junction adenocarcinoma, molecular classification, The Cancer Genome Atlas, immunohistochemical analysis, chromogenic *in situ* hybridization.

Introduction

The etiology, clinical and pathological features of gastroesophageal carcinoma differ from those of distal gastric cancers (GCs). In addition, the classification of GEJ tumors is controversial in the literature and has been defined under different names including distal esophageal cancer, proximal gastric cancer, and cardia cancer [1]. In the 2018 classification of the World Health Organization, GEJ ad-

enocarcinomas appear under the title of esophageal tumors [2]. By definition, GEJ tumors are morphologically adenocarcinomas and have the gastroesophageal junction within 2 cm of their epicenter. Since 1976, GEJ tumor rates have been increasing by 4-10% each year [3, 4]. It is known that GEJ tumors display aggressive behavior by early local and systemic invasion. Advanced stage [5, 6], and tumor size [7], presence of lymphatic invasion [1], and presence of perineural invasion [9, 10] are correlated with poor prognosis. Because the esophagus does not have a serosa and is in close proximity to multiple organs and structures, direct extension is common. In addition, the rich submucosal lymphatic network of the esophagus and the GEJ is responsible for high lymph node involvement risk. Treatment strategies include radiotherapy and neoadjuvant or perioperative multimodal chemotherapy, and both are treatment options that have emerged over the past decade to eradicate micrometastatic disease and improve both survival rates and surgical outcomes [11, 12]. However, the lack of significant prolongation in survival despite all these treatments has prompted researchers to seek more specific targets. However, with targeted therapy, in recent years significant progress has been made in examining the genomic structure of the GEJ to identify molecular subtypes. Based on histopathological classifications, studies have intensified to define prognostic and predictive molecular biomarkers, although the heterogeneous malignancies of the GEJ make it difficult to discover biomarkers and characterize genomic features of GEJ adenocarcinomas. With two new genomic classifications by The Cancer Genome Atlas (TCGA) Research Network [13] and Asian Cancer Research Group (ACRG) [14], significant steps have been taken for gastric cancer (GC) and there has been hope for GEJ tumors as well. The Cancer Genome Atlas (TCGA) research network reported the most comprehensive identification of genetic alterations associated with GC, combining data from six different platforms: whole-exome and genome sequencing, messenger RNA-sequencing, microRNA sequencing, array-based somatic copy number analysis and reverse-phase protein array profiling, plus evaluation of microsatellite instability [13]. This analysis put forth a molecular classification for GC, dividing it into four subtypes: EBV-positive tumors (EBV+), microsatellite unstable tumors (MSI), genomically stable tumors (GS) and tumors with chromosomal instability (CUN). Although TCGA does not correlate with prognosis, it has been suggested by some authors that it may be useful in choosing treatment [15, 16]. The advantage of the TCGA molecular classification strategy over other morphological classifications is that it paves the way for targeted treatment studies, especially in terms of subgroups that show EBV positivity and the presence of MSI. The ACRG study used four major molecular signatures for recognition of the GC subtypes. These molecular signatures include p53 status as MSI, microsatellite stable with epithelial-to-mesenchymal transition phenotype (MSS/EMT), MSS/TP53+(intact TP53 activity), and MSS/ TP53 (functional loss of TP53) [11]. The main purpose of all these classifications is actually to guide clinical approaches and treatment strategies. Difficult and expensive techniques used in these classifications, whose prognostic importance has not been sufficiently demonstrated, create difficulties in the application of classifications. For this reason, classifications do not take place sufficiently in clinical practice.

In this study, we aimed to investigate the clinicopathological and prognostic importance of classification using immunohistochemistry (IHC) and chromogenic in situ hybridization (CISH) to identify potential surrogate markers of molecular changes.

Material and methods

Patient selection

Between 2010 and 2018, patients who were operated on for gastroesophageal junction (GEJ) tumors were screened via the electronic archive system. Patients with a history of either perioperative chemoradiation or neoadjuvant chemotherapy were excluded from the study. The slides and macroscopic findings of the cases were re-evaluated and 52 cases that match the definition of GEJ adenocarcinoma in the WHO 2018 classification were included in the study. The demographic features of the patients (age, sex) and the macroscopic features of the tumors (the distance of the tumor center to the junction, size) were obtained from the electronic archive system.

Histopathological features

Hematoxylin-eosin-stained sections of all patients were re-evaluated in terms of differentiation, lymphovascular (LVI) and perineurial (PNI) invasion, surgical margin positivity, presence of Barrett's metaplasia, primary tumor (pT), regional lymph nodes (pN), pathological stage, and clinical stage. World Health Organization 2018 Classification of Tumors of the Digestive System was used for the histological differentiation grade, pT, pN, pathological stage, and clinical stage [2]. The presence of macroscopic and/or microscopic tumors at the surgical margin was considered surgical margin positivity. The other parameters were classified as present or absent.

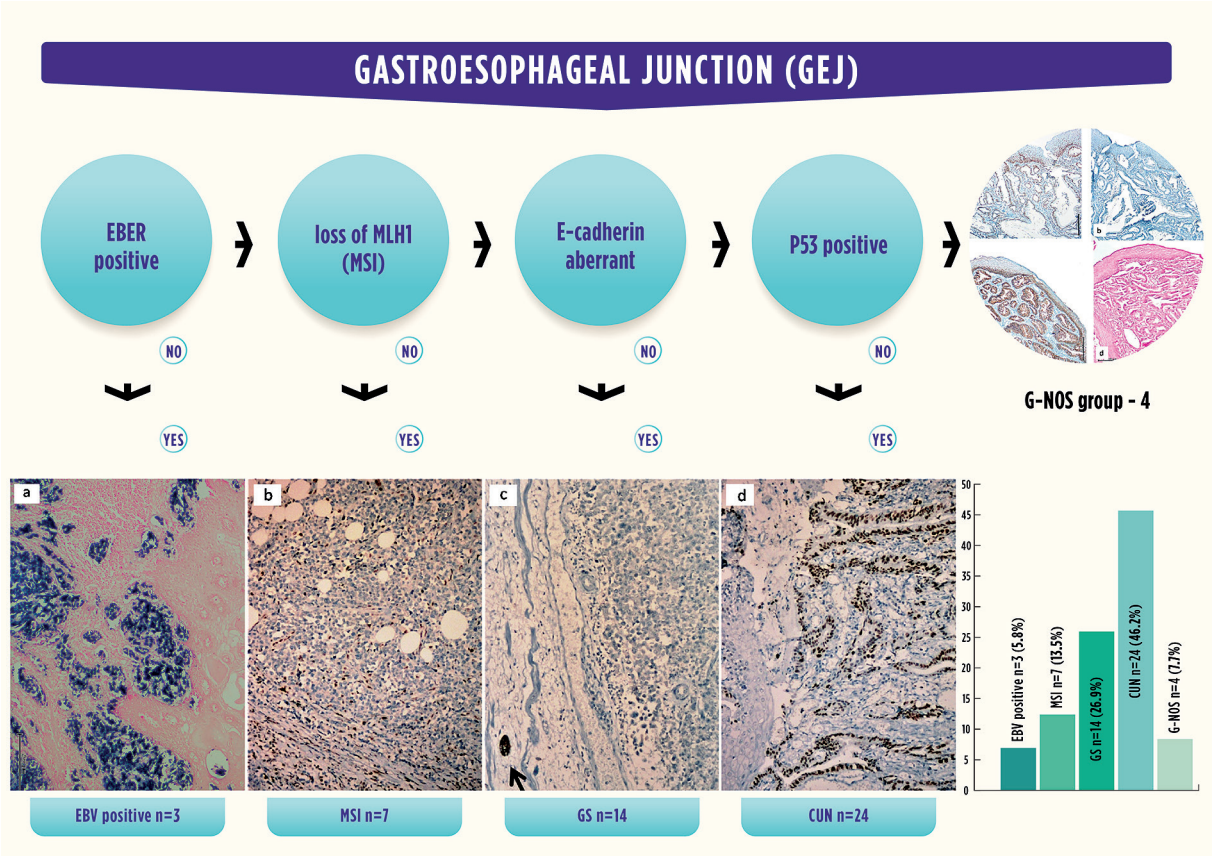

Fig.1. The algorithm used in tumor classification and the distribution of cases according to groups. EBV – Epstein-Barr virus; MSI – microsatellite instable; GS – genomically stable; CUN – chromosomal instability; G-NOS – non-classified tumor

Immunohistochemistry and chromogenic in situ hybridization

One of the blocks that best reflected the characteristics of the tumor was chosen and sections of five micron thickness were taken. Immunohistochemical staining was performed using the following antibodies in an automated stainer (Ventana BenchMark XT, Ventana Medical Systems, Tucson, AZ): anti-MLH1 (Catalog number: 790-5091, Ventana Medical Systems, Tucson, AZ. Primary Antibody was incubated for 40 minutes at 37°C), anti-p53 (DO-7) (Catalog number 800-2912, Ventana Medical Systems, Tucson, AZ. Primary Antibody was incubated for 16 minutes at 37°C), anti-E-cadherin (Catalog number 790-4497, Ventana Medical Systems, Tucson, AZ. Primary Antibody was incubated for 24 minutes at 37°C). Background staining for all slides was done with hematoxylin. The analysis of immunohistochemical staining was conducted using the criteria described below. Loss of expression of MLH1 was designated as complete loss of nuclear staining in all tumor cells with a positive background reaction in benign epithelium and lymphocytes. Abnormal expression of E-cadherin was defined as complete loss of membranous expression or apparently reduced membranous staining (> 30%) or nuclear or cytoplasmic staining [17]. For p53, strong staining in more than 70% of tumor cell nuclei was considered positivity, similar to previous studies [18].

For EBV, a ready-to-use Epstein-Barr Virus Early RNA (EBER) probe (Catalog number: 800-2842, Ventana Medical Systems, Tucson, AZ) was used together with ISH-Protease 3 pretreatment for 28 min and 1-h probe incubation. The signal was detected with the ISH iVIEW Blue Detection Kit. Finally, the slides were counterstained with Red stain II. Staining with blue in tumor cell nuclei was considered positive.

Classification of tumors

Since there is no consensus on the classification of gastroesophageal adenocarcinomas, the molecular classification determined by TCGA(10) for gastric cancers was adapted and used in this study according to immunohistochemical data. The tumors were classified into five types: 1) EBV+ tumors, if tumor was EBER positive, 2) microsatellite instable tumors (MSI), if tumor had loss of MLH1, 3) genomically stable tumor (GS), if tumor had abnormal expres-

sion of E-cadherin, 4) chromosomal unstable tumor (CUN) if tumor was p53 positive. 5) However, in the immunohistochemical evaluation, we observed that a group of tumors was not suitable for this classification. These tumors had E-cadherin and MLH1 expression, but no p53 expression, and they were also EBV negative with EBER. We classified these tumors as the NOS group (G-NOS).

The algorithm used in tumor classification and the distribution of cases according to groups are shown in Fig. 1.

Survival time and statistics

The prognostic information was obtained from the archive records of the Local Cancer Monitoring and Follow-up Center. Overall survival time (OS) was calculated from the date of resection to the date of death or to the date of the latest follow-up. The follow-up data of the patients were updated in January 2021. In statistical analysis, the χ^2 test, Fisher's exact test, and Kaplan-Meier test were performed with the SPSS software version 21.0. A p-value ≤ 0.05 was considered statistically significant.

This study followed the Declaration of Helsinki on medical protocol and ethics and the Regional Ethical Review Board approved the study. Before the study, approval was obtained from the clinical research ethics committee of our hospital and there is no conflict of interest between the authors. To protect personal privacy, identifying information in the electronic database was encrypted. Informed consent was waived by the ethics committee because no intervention was involved and no patient-identifying information was included.

Results

Demographic and histopathological features

There were 41 men (78.8%) and 11 women (21.2%). The mean age was 63.00 ±13.45 years (range, 32-87 years). The mean size of tumors was 6.18 ±2.84 cm (min: 1.4, max: 12.5 cm). In all cases, at least two of the tubular, papillary, mucinous or signet ring cell patterns were observed mixed. The mean time of OS was 24.019 ±3.778 months and the median time of OS was 11.000 ±2.704 months (min.: 1 month – max.: 110 months). The cumulative proportion surviving in the first year was 75.0%. This rate decreased to 53.8% in the 2nd year and 3.8% in the 4th year. A significant relationship between the overall survival time and sex, age, and size was not found ($p > 0.05$).

The number of cases according to histomorphological features and the mean survival times of the patients, and their statistical relationships, are shown in Table I.

Tumor categories

The largest group consisted of 24 (46.2%) cases with CUN. This group was followed by GS with 14 (26.9%) cases, MSI with 7 (13.5%) cases, and EBV + with 3 (5.8%) cases. The number of cases classified as G-NOS because there was expression of E-cadherin and MLH1 but no p53 expression was 4 (7.7%).

The specific staining patterns of the samples from the EBV positive, MSI, CUN and GS groups are shown in Fig. 2.

In the statistical analyses, the molecular characteristics were not correlated with histopathological features: differentiation ($p = 0.075$), LVI ($p = 0.505$), PNI ($p = 0.602$), surgical margin positivity ($p = 0.441$), presence of Barrett metaplasia ($p = 0.101$), pT ($p = 0.369$), pN ($p = 0.308$), pathological stage ($p = 0.289$) and clinical stage ($p = 0.726$).

As seen in Table I, the longest survival time was 62.333 months in patients with EBV+ tumor, while the shortest survival time was in patients with GS tumor with 9.214 months. A statistically significant relationship was found between molecular characteristics and survival time ($p = 0.029$).

The graph of Kaplan-Meier survival analysis according to the categories of tumors is shown in Fig. 3.

Discussion

Unfortunately, there is considerable confusion in the definition of gastroesophageal carcinomas in the literature. While proximal gastric carcinomas are included in this group by some authors, adenocarcinomas of the stomach and esophagus are defined collectively in this group by some authors [1, 15]. However, we know that these tumors, which are located at the junction of two different anatomical and histological regions, can often behave differently from gastric tumors and sometimes from esophageal tumors. In the book published by the World Health Organization in 2018, GEJ adenocarcinomas were included with esophageal adenocarcinomas and it was clearly explained which tumors should be defined as GEJ adenocarcinoma [2]. In order to be defined as GEJ adenocarcinoma, the esophagogastric junction must be located within the area 2 cm away from the center of the tumor in adenocarcinoma morphology and the tumor must have extended towards the esophagus.

To the best of our knowledge, our study is a pioneering study in a homogeneous group based on this definition in the literature. For this reason, it makes an important contribution to the literature in terms of revealing the characteristics of GEJ adenocarcinomas. In our study, the prognostic significance of the presence of PNI, positivity of surgical margins, lymph node metastasis and advanced tumor stage was determined. These findings are prognostic

Table I. Number of cases according to histomorphological features and mean survival times of patients, and their statistical relationships

Parameter	Number of cases (%)	Overall survival time	P-value
Sex			
Female	11(21.2%)	31.273 ±8.214	0.418
Male	41 (78.8%)	22.073 ±4.255	
Type of tumor			
EBV+	3 (5.8%)	62.333 ±17.910	0.029
MSI	7 (13.5%)	25.429 ±12.750	
CUN	24 (46.2%)	24.708 ±5.459	
GS	14 (26.9%)	9.214 ±2.358	
G-NOS	4 (7.7%)	40.500 ±15.930	
Differentiation			
Well/moderately differentiated	27 (51.9%)	28.407 ±5.936	0.185
Poorly differentiated	25 (48.1%)	19.280 ±4.477	
Lymphovascular invasion			
Absent	11 (21.2%)	32.545 ±11.475	0.286
Present	41 (78.8%)	21.732 ±3.691	
Perineural invasion			
Absent	12 (23.1)	48.917 ±9.891	0.001
Present	40 (76.9%)	16.550 ±3.128	
Surgical margin			
Negative	40 (76.9%)	28.550 ±4.628	0.003
Positive	12 (23.1%)	8.917 ±2.575	
Barrett's metaplasia			
Absent	31 (59.6%)	18.419 ±4.068	0.099
Present	21 (40.4%)	32.286 ±6.909	
Primary tumor (pT)			
pT1+pT2	7 (13.5%)	42.571 ±12.592	0.324
pT3	40 (76.9%)	21.700 ±4.230	
pT4	5 (9.6%)	16.600 ±4.331	
Regional lymph nodes (pN)			
N0	4 (7.7%)	58.750 ±23.228	0.013
N1	10 (19.2%)	40.300 ±9.091	
N2	3 (5.8%)	19.333 ±13.333	
N3	35 (67.3%)	15.800 ±3.300	
Pathological stage			
Stage 2	4 (7.7%)	58.750 ±23.228	0.009
Stage 3	14 (26.9%)	33.643 ±7.457	
Stage 4	34 (65.4%)	15.971 ±3.394	
Clinical stage			
Stage 2	3 (5.8%)	53.333 ±25.667	0.006
Stage 3	11 (21.2%)	43.455 ±9.864	
Stage 4	38 (73.1%)	16.079 ±3.162	

MSI – microsatellite instable tumors; GS – genomically stable tumors; CUN – chromosomal unstable tumors; G-NOS – non-classified tumor

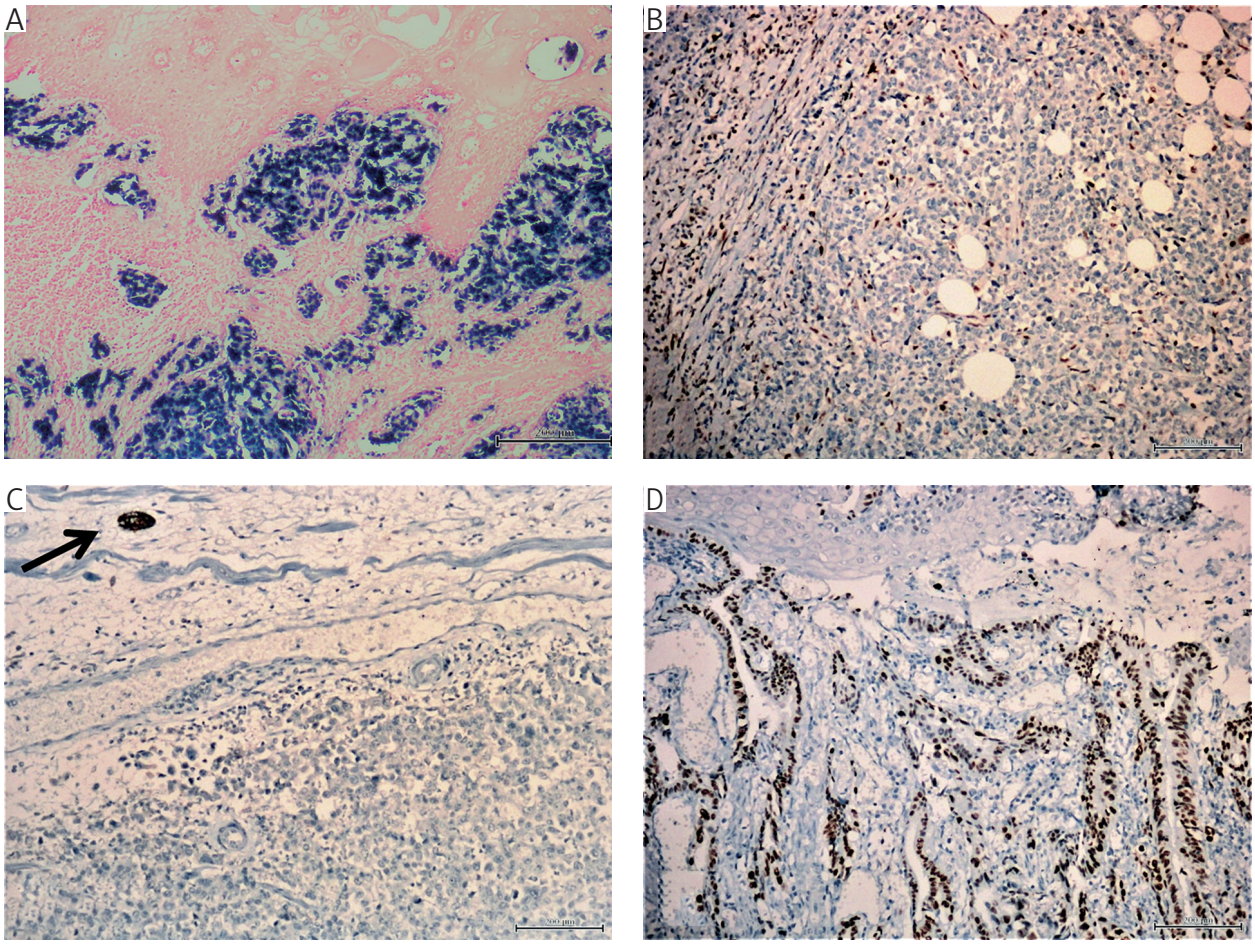

Fig. 2. A) EBER positive case: Under the squamous epithelium, tumor cells have nuclear blue staining positive with CISH. B) Case in MSI category: Significant brown staining in inflammatory cells shows positivity internal control. Staining due to loss of expression is not observed in tumor cells. C) Case in GS category: the black arrow shows the internal control of the subepithelial gland with membranous staining with E-cadherin. No staining is observed in tumor cells. D) Case in CUN category: p53 immunohistochemical stain shows strong nuclear positivity in more than 70% of tumor cells

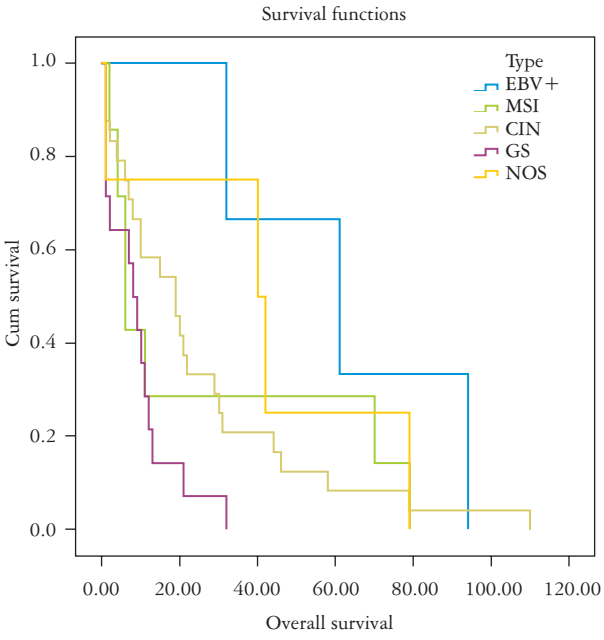

Fig. 3. Kaplan-Meier survival analysis chart by tumor categories

features defined for both gastric tumors and esophageal tumors. It is seen that there are similar results in studies reported on GEJ tumors [5, 6, 7, 8, 9, 10].

Today, we realize that the classification made according to anatomical localization for many tumors is not sufficient to determine the prognostic importance of the characteristics of the tumor. Classifications based on the molecular characteristics of the tumor have started to appear in the literature at an increasing rate [19, 20, 21]. After TCGA announced it in 2014, the molecular classification was used in many studies on gastric tumors, followed by GEJ adenocarcinoma studies, albeit few [13, 15]. However, the techniques used in molecular classification are not methods that can be easily applied in every pathology laboratory. Cost is also a major issue. For this reason, there is a need for classifications using the immunohistochemical equivalents of the molecules used in molecular classification. Because the morphological appearance of GEJ adenocarcinomas is similar

to gastric tumors and the molecular classifications specific to esophageal carcinomas are not clear, we used the immunohistochemical responses of molecular markers defined for gastric tumors in TCGA in our study. As is known, gastric carcinomas represent a very large group in terms of their origins, molecular pathways, histopathological diversity, and biological behavior. The Cancer Genome Atlas (TCGA) and the ACRG cohort molecular classifications contribute to what is known of the pathogenesis and etiology of GC, and these molecular classifications are promising for targeted therapy studies [13, 14]. TCGA study opened the way for new treatment models and clinical studies by identifying the problematic pathways and driver mutations involved in carcinogenesis. However, it is not feasible to widely use TCGA molecular classification for routine clinical diagnostics or in pathology laboratories. Therefore, some studies did use chromogenic in situ hybridization (FISH) and immunohistochemistry (IHC) in order to define the potential markers of molecular alterations [22, 23, 24]. In these studies with immunophenotypic classification, as in our study, TCGA such as EBV (for EBV subtype), MLH1 (for MSI subtype), p53 (for CUN subtype) and epithelial cadherin (E-cadherin) (for GS subtype) derived from molecular classification were used. However, the relationships of the groups formed in these studies with histopathological features have not been adequately examined, and their correlation with clinical data in terms of prognosis and biological behavior has not been made in detail. Moreover, these studies were conducted in case series that included either gastric cancers alone or gastric cancers with GEJ tumors. In conclusion, as in our study, it has been shown that molecular-like classification can be performed using immunohistochemical analysis and *in situ* hybridization in these studies.

In most immunohistochemical-based studies, tumors were in one of the four main groups. However, in rare studies, a fifth group has been defined as in our study. In the study of Di Pinto *et al*., it was stated that all immunomarkers were negative in this group, which was defined as "normal pattern" [25]. In our study, a fifth subgroup was determined, different from the TCGA classification. These tumors had E-cadherin and MLH1 expression, but no p53 expression, and they also were EBV negative. In the literature, it is also stated that gastric cancers associated with the MSI subtype and EBV have a better prognosis than others [26]. In the survival analyses of these tumors, it was seen that they were between EBV+ tumors and MSI tumors. Microsatellite instability is a genetic change in DNA mismatch repair genes (MMR) that results from genetic or epigenetic inactivation [27, 28]. As it is known, the most commonly used markers among MSI immunohistochemical tests are MLH-1, PMS-2, MSH-2 and MSH-6. Loss of expression can be observed in one or more of these markers, which is suspicious for MSI and should be confirmed by further molecular studies if clinical data support it. We think that the use of MLH-1 antibody alone in our study creates a deficiency in the detection of MSI tumors. As previously shown in the study of Zhao *et al*., tumors with loss of expression of other markers and included in the MSI group may exist even if MLH-1 is present [29]. We think that it is important to use the necessary markers for MSI as a panel in immunohistochemical classifications.

Another striking finding in studies is the frequent use of multiple blocks (TMAs). Considering tumor heterogeneity, this situation requires us to approach the results cautiously. To our knowledge, this is the first study to carry out an immunophenotypic classification exclusively based on the entire section obtained from a tumor block in gastroesophageal junction adenocarcinoma. In our study, the limitation of evaluation due to tumor heterogeneity was minimized. Therefore, our data have been made more reliable for the literature.

EBV has not yet been reported in esophageal adenocarcinomas; however, its prevalence in GC is approximately 10% and it has been reported as 2.7% in gastroesophageal junction cancers [30]. TCGA showed increased expression of PD-L1 and PD-L2 in this subtype in mRNA evaluation. Therefore, the EBV subtype is considered a promising candidate for anti-PD-1/PD-L1 therapy in gastroesophageal cancers. Based on the positive results of the KEYNOTE-059 study, the Food and Drug Administration (FDA) has approved pembrolizumab, an anti-PD-1 monoclonal antibody, for the treatment of patients with advanced GC or GEJ adenocarcinoma if programmed cell death-ligand 1 (PD-L1)-positive. This was followed by approval by the Japanese Ministry of Health, Labor and Welfare (MHLW) of nivolumab (anti-PD-1 monoclonal IgG4 antibody) in patients with advanced unresectable advanced or recurrent GC after two previous chemotherapy treatments [31, 32]. Similar to the EBV subtype, MSI also displays overexpression of PD-L1 [33]. Overexpressed PDL1 expression in the EBV subtype and high mutation and neoantigen load in the MSI subtype increase the immune response [34, 35]. PD-1 and PDL-1 antibodies were not used in our study. However, in the light of these promising results, we can say that patients with GEJ adenocarcinoma in the EBV positive group may be candidates for investigation of PD-1 and PDL-1 expression and possibly for immunotherapy.

When TCGA classified tumors according to the number of somatic copy number changes, the group characterized by low mutation rates and low copy number changes was classified as a genomically stable (GS) subtype [13]. Prognostically, the GS

subtype has been reported to be associated with the worst overall survival and relapse-free survival among the four TCGA subtypes and has been shown to be resistant to chemotherapy [36]. In our study, GS tumors constituted the second largest group and had a significantly worse prognosis. Considering that this tumor group can be detected by immunohistochemical E-cadherin negativity, it was concluded that it would be beneficial to include it in the routine pathological reporting of GEJ tumors.

It has been reported that CUN tumors, which form the largest group in our study, constitute approximately 50% of GC and are most frequently located in the gastroesophageal junction/cardia [13]. This group of tumors is most similar to esophageal adenocarcinoma in terms of molecular characteristics [37]. It is reported in the literature that the prognosis of CUN is similar to the MSI subtype, similar to the results of our study [36]. It would not be surprising if a mutation in this molecule, which is considered to be the "gatekeeper", results in the accumulation of many mutations. Detection of additional genomic anomalies of this tumor group, which can be easily detected by immunohistochemical p53 positivity, will provide hope for targeted therapies.

In conclusion, the fact that many studies, including TCGA and ACGR, evaluated GEJ carcinomas together with gastric carcinomas causes us to have very limited information about GEJ tumors. The diversity of the panels used in molecular classification and the use of difficult and expensive techniques have prevented the molecular classification of these tumors from being used in daily practice. In the literature, our study is the first to apply the TCGA panel with IHC counterparts, which is a cost-effective and easy-to-apply method, in homogeneous GEJ tumors defined according to the WHO (2018). In addition, the statistically significant difference between the groups in terms of survival and the detection of a fifth group in addition to the TCGA groups are results that will shed light on the literature. We expect these results to be supported by studies in large series.

The authors declare no conflict of interest.

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