Prevalence of microsatellite instable and Epstein-Barr Virus-driven gastro-esophageal cancer in a large Belgian cohort

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Abstract

Introduction: **Patients with gastroesophageal adenocarcinoma (GEC) with microsatellite instability-high (MSI-H) or Epstein Barr Virus positivity (EBV+) might be good candidates for immunotherapy. Incidences of about 10% have been reported for both features, but are dependent on geographical region and disease stage.**

Aim: **The aim is to study the prevalence of MSI-H and EBV+ in a Belgian single center cohort of patients with GEC.**

Methods: **We retrospectively assessed the files of all patients with a newly diagnosed GEC between August, 1st 2018 and February, 29th 2020 at the University Hospitals Leuven, Belgium. Microsatellite instability (MSI) status was determined using immunohistochemistry (IHC) and polymerase chain reaction (PCR). EBV+ was assessed using in situ hybridization (ISH). A case report is provided to illustrate the importance of testing for MSI in GEC.**

Results: **247 gastroesophageal adenocarcinomas were included in this analysis. 62 (56% stage IV) of those were tested for EBV, but only 1 turned out to be EBV positive (1.6%). 116 patients (44.0% stage IV) were tested for MSI, of which 11 were MSI-H (9.5%). Half of the MSI-H tumors identified were at the gastroesophageal junction (GEJ). A patient with MSI-H metastatic GEC obtained a complete response with nivolumab, which persisted after discontinuation of treatment.**

Conclusion: **While we confirm that about 10% of GECs are MSI-H, the incidence of EBV+ in our cohort (1.6%) is clearly lower than expected. Given the important prognostic and predictive implications, every gastroesophageal cancer should be tested for MSI.** (Acta gastroenterol. belg., **2022,** 85, **1-5**).

Keywords: real world incidence, checkpoint inhibitors, molecular subtyping, upper gastro-intestinal tumors.

Introduction

Gastroesophageal cancer (GEC) is the 12th most common cancer type in Belgium, accounting for about 1500 new diagnoses each year (1,2). Predictions estimate an increase in incidence of 35% by 2040 (1). Despite advancements in gastroesophageal cancer diagnosis and treatment, 5-year survival remains poor (2). Worldwide, GEC was the fifth leading cause of cancer related death in 2020 (1).

The majority of GEC are adenocarcinomas, which are subdivided in histological or molecular subtypes. The Lauren classification distinguishes an intestinal cancer from a diffuse type gastric cancer based on histology and has prognostic implications (3). More recently, molecular subclasses of GEC have been proposed based on the integration of data from several omics platforms. The Cancer Genome Atlas (TCGA) proposed four molecular subtypes: (1) an Epstein-Barr-virus positive subtype (EBV+); (2) a microsatellite instable subtype with high mutational burden (MSI-H); (3) genomically stable (GS) tumors; and (4) tumors with chromosomal instability (CIN) (4).

In recent years, the prognostic and predictive impact of this classification has become increasingly clear. EBV+ disease tends to have a better prognosis, while patients with genomically stable tumors, enriched for diffuse type gastric cancer, have worse outcomes. Moreover, both retrospective and prospective studies have shown an impressive clinical benefit using checkpoint inhibitors in the EBV+ and especially the MSI-H subtype (5). These findings have increased awareness among clinicians to perform molecular testing on tissue specimens for clinical decision-making.

In this study, we retrospectively assessed the prevalence of EBV+ and MSI-H tumors in a large Belgian single center cohort. The clinical importance of molecular testing in gastric cancer is further illustrated with a case report of a patient with MSI-H metastatic GEC treated with immunotherapy.

Methods

Study design and data collection

This single center retrospective cohort study evaluated patient data collected at the University Hospitals Leuven, Belgium. The Research Ethics Committee UZ/KU Leuven review approved the design and deemed formal patient consent unnecessary due to the retrospective, anonymous and observational nature of this study.

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Patients

All patients with a newly diagnosed adenocarcinoma of the esophagus, gastroesophageal junction or stomach between August, 1st 2018 and February, 29th 2020 were included. The data were extracted in October 2019 and in May 2020 from patient records and collected in an electronic database system, Microsoft Excel, in a pseudonymized manner. The analyzed patient and disease characteristics included date of birth, sex, EBV, MSI, TNM classification, stage, tumor location, mechanism of mismatch repair protein deficiency (MMR-D), histology and *BRAF* p.V600E mutation.

Patients with insufficient data were included in this study, with the lacking data reported as missing.

MSI, EBV and BRAF testing

All samples were assessed for microsatellite instability using an immune histochemistry (IHC) assay. Parrafin sections were immunostained for MLH1, MSH2 and MSH6 (mouse monoclonal anti-human antibodies; DakoCytomation, Glostrup, Denmark) and PMS2 (BD Pharmingen™, San Diego, CA). In brief, 5 µM paraffin sections were placed on silanized slides (DakoCytomation), dewaxed in xylene and rehydrated in decreasing concentrations of ethanol. While immersed in citrate buffer (pH 6.0), slides were placed in a calibrated warm water bath (95-99 °C) for 3 minutes to perform epitope retrieval. Incubation with, respectively, the FLEX Monoclonal Mouse Anti-Human MutL Protein Homolog 1, Clone ES05 Ready-to-Use, the MSH2 antibody (1:50) was carried out at room temperature. Staining was performed using the EnVision system (DakoCytomation) according to the manufacturer's recommendations. All incubation steps were followed by a wash in three changes of phosphate-buffered saline (pH 7.6). Loss of mismatch repair (MMR) protein expression was recorded when the neoplastic cells demonstrated absence of nuclear staining, while surrounding stromal cells and/or reactive lymphocytes showed a moderate to strong nuclear staining reaction.

All samples with loss of MMR protein expression were confirmed using polymerase chain reaction (PCR). In this test, a panel of DNA sequences containing nucleotide repeats is amplified. If 40% or more of the markers show expansion or contraction of the microsatellites in the tumor compared with the control samples of the same patient (e.g. blood sample), the tumor is reported to have a high level of MSI (6).

All tumors and corresponding normal tissue of patients with abnormal MMR IHC were tested with PCR with the MSI kit (MSI analysis system, Promega). Hypermethylation of MLH1 promotor was tested with methylation specific multiplex ligation dependent probe amplification ME011 kit (MRC Holland). With the same probe mix the *BRAF* p.V600E mutation (LRG299_t1: c.1799T>A, p.Val600Glu) was detected. Patients with MMR-D on PCR without hypermethylation (<30%) were referred for germline (Lynch) testing.

Samples were assessed for EBV positivity with in situ hybridization (ISH) using a 30-mer-digoxigenin-labeled oligonucleotide probe (Research Genetics, Huntsville, AL), according to manufacturer's instructions. A control poly-A-probe (Ventana Roche, Arizona, USA) was used to check for RNA integrity and a proven EBV-driven lymphoma was used as a positive control. Cases were defined as EBV+ if EBER was expressed in all tumor cells in which RNA was preserved. Since August, 1st 2019, reflex testing has been applied for all gastric adenocarcinomas (7).

All molecular testing was performed before the study data collection.

Results:

Patient cohort

Between August, 1st 2018 and February, 29th 2020 there were 247 newly diagnosed adenocarcinomas of the

Table 1. — **Patient and disease characteristics**

	n	%
Age in years; median (range)	67 (29-94)	
Sex		
Female	46	18.6
Male	201	81.4
Molecular Testing		
EBV ISH		
Not Tested	185	74.9
Tested	62	25.1
Negative	61	24.7
Positive	1	0.4
MSI		
Not Tested	131	53
Tested	116	47
Failed	2	1.7
MSS	103	88.8
MSI-H	11	9.5
Disease Stage (in tested population)		
I	23	19.8
II	24	20.7
III	11	9.5
IV	52	44.8
Unknown	6	5.2
Tumor Location		
Distal Esophagus	103	41.7
GEJ	70	28.3
Cardia	31	12.6
Fundus	3	1.2
Corpus	13	5.3
Antrum	21	8.5
Stomach NOS	6	2.4

EBV ISH, EBV in situ hybridization; MSI, microsatellite instability; MSS, microsatellite stable; MSI-H, microsatellite instability-high; GEJ, gastro-esophageal junction; NOS, not otherwise specified.

esophagus, GEJ and stomach (Table 1). Median age at diagnosis was 67 years and 81.3% of patients were male. The majority of tumors (70%) had a proximal location, either in the distal esophagus or at the GEJ.

MSI testing

116 of 247 tumor samples were tested for MSI using IHC (Figure 1). 11 were found to be MSI-H (9.5%). Median age at diagnosis was 81 years and 64.6% of patients were male. Of the MSI-H patients, 2 had stage I cancer, 3 stage II, 1 stage III and 2 stage IV. The disease stage of 3 patients was unknown. Half of the MSI-H tumors identified were located at the GEJ. All MSI-H cases were confirmed by PCR. In 10 out of the 11 cases, hypermethylation of the MLH1 promotor was the underlying mechanism of mismatch repair deficiency (MMR-D). In 1 case, we found no hypermethylation. Germline testing was performed but did not show any mismatch repair gene mutation. In our samples, MSI-H was associated with poorly differentiated histology, tumor infiltrating lymphocytes and mucinous differentiation. The most common subtype was the diffuse type. No *BRAF* p.V600E mutation was found in our MSI-H tumor samples. Only 3 patients with MSI-H tumors received checkpoint inhibition (nivolumab): 1 with stage II and 2 with stage IV cancer (Table 2).

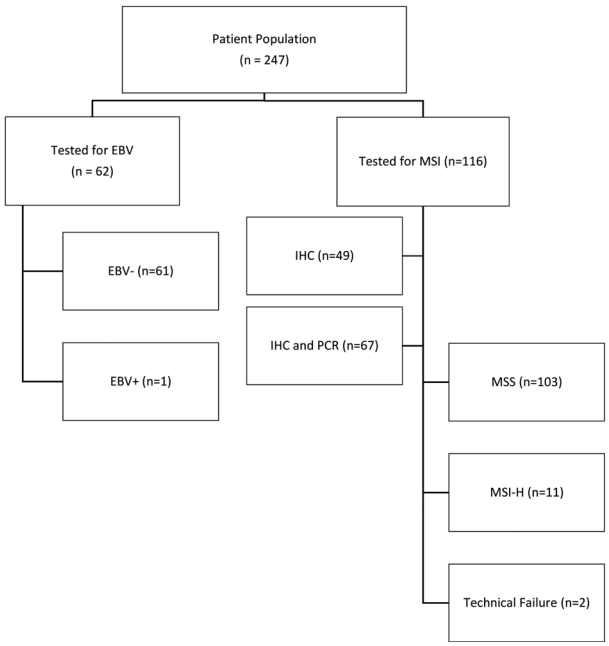

Fig. 1 — Study flow diagram.

Table 2. — **Features MSI-H Tumors**

	n=11	%
Age in years; median (range)	81 (60-88)	
Sex		
Female	4	36.4
Male	7	64.6
TNM stage		
IA	0	0
IB	2	18.2
II	3	27.3
III	1	9.1
IV	2	1.,2
Unknown	3	2.3
Location		
Distal esophagus - GEJ	6	54.6
Cardia	1	9.1
Corpus	2	18.2
Antrum	2	18.2
Mechanism MMR-D		
Hypermethylation MLH1 promotor		
<15%	1	9.1
>15%	10	90.9
Lynch Syndrome	0	0
Histology		
Diffuse	6	54.5
Intestinal	2	18.2
Intestinal/Medullary	1	9.1
NOS	2	18.2
***BRAF* p.V600E Mutation**		
Negative	11	100
Positive	0	0
Treatment with immunotherapy	3	27.3
Nivolumab		
Stage II	1	9.1
Stage IV	2	18.2

MSI-H, microsatellite instability-high; GEJ, gastro-esophageal junction; MMR-D, mismatch repair deficiency; NOS, not otherwise specified.

EBV testing

62 (56% stage IV) samples were tested for EBV, but only 1 turned out to be EBV positive (1.6%).

The EBV positive patient had stage IV disease at presentation.

Case report

A 61-year-old man with a diagnosis of stage IIIC gastric adenocarcinoma (cT4bN3aM0) was treated with neo-adjuvant chemotherapy with Cisplatinum-5-Fluorouracil, followed by surgery including total omentectomy, gastric resection and extended lymphadenectomy, followed by adjuvant chemotherapy with Docetaxel-Oxaliplatinum-5-Fluorouracil (FLOT). Pathology report confirmed the presence of poorly differentiated adenocarcinoma, ypG3T4N3aM1 (due to the presence of peritoneal implants), compatible with stage IV disease. Further molecular testing revealed EBV- (CISH), HER 2- (IHC) and MSI-H (PCR) status based on hypermethylation of MLH1-promotor (MS-MLPA). Unfortunately, after 14 months of follow-up, local recurrence was seen in the epigastric region (3.4 x 2.9 x 3.7 cm) with invasion of the left liver lobe and possibly of the adjacent pancreas, as well as millimetric adjacent

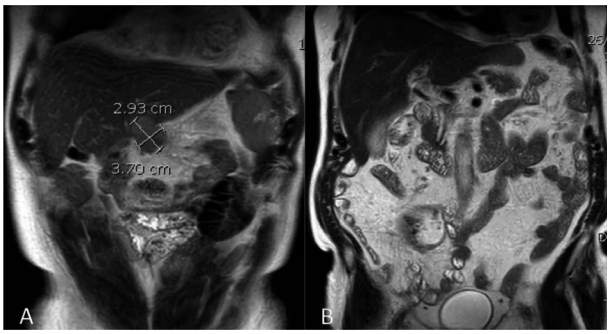

Fig. 2. — Case report. A: local recurrence after 14 months of follow-up. B: persisting complete remission 1 year after stopping PD-1 inhibition.

glands (Figure 2a). Nivolumab 3mg/kg q2w (anti-PD1 inhibition) was started in compassionate use, with partial response after 4 administrations and complete remission after 9 months of treatment. After 2 years of anti-PD1 inhibition and persistent complete response on MRI, checkpoint inhibition was discontinued. One year later, a persistent complete response was still observed on MRI (Figure 2b).

Discussion

In this study, we describe a relatively large cohort of patients with gastroesophageal cancer tested for MSI and/or EBV. To our knowledge, no other studies have reported these subtypes of GEC in a Belgian context.

Due to the prognostic and predictive implications, molecular classification of GEC has found its way into clinical practice. Most is known about the clinical consequences of microsatellite instability in GEC. Retrospective analyses of prospective trials have shown that MSI-H gastric cancers have a better prognosis after curative-intent surgery compared to microsatellite stable disease (8). Moreover, a pooled analysis of the MAGIC, CLASSIC, ITACA-S and ARTIST trial, suggests that patients with MSI-H gastric cancer might not derive any benefit from standard of care perioperative chemotherapy (8). Whether chemotherapy should be omitted in such case is a matter of ongoing scientific debate (9). Perhaps, the platelet-to-lymphocyte ratio (PLR) can guide us in this decision, as PLR is an independent predictor for overall survival (OS) in stage IV GC (10). Finally, it has become clear that the general sensitivity of metastatic MSI-H cancers to immune checkpoint inhibitors, regardless of histology, also applies to adenocarcinomas of the upper gastrointestinal tract. In this manuscript, we describe a case of a patient with complete remission of metastatic MSI-H gastric cancer using PD-1 inhibition. The effect persisted one year after discontinuation of the immunotherapy.

In a recent analysis of 84 patients with MSI-H GEC included in three KEYNOTE studies, treatment with pembrolizumab was associated with prolonged overall survival (median not reached in all trials), progression free survival and superior response rate compared to chemotherapy (11). Despite these convincing data in a disease with limited treatment options, no checkpoint inhibitor is approved for MSI-H metastatic GEC by the European Medicines Agency, denying a possible curative treatment to hundreds of European patients each year.

Data on immune checkpoint inhibition in patients with EBV driven GEC is scarce. However, the viral involvement in these tumors could confer a similar sensitivity as MSI-H disease. In a retrospective cohort study where patients with metastatic gastric cancer received treatment with pembrolizumab (12), responses were enriched in patients with EBV+ disease.

In the literature, incidences of +/-10% have been reported for both MSI-H and EBV+ tumors. While the incidence of MSI-H tumors is in line with these findings, the incidence of EBV+ in our cohort (1.6%) is clearly lower than what would be expected (12,13). A number of factors could explain this observation. Due to the presence of therapeutic implications, testing was mostly performed in stage IV disease which might entail a selection bias. The lower incidence of EBV+ may also be due to geographical factors (4). In the TCGA study, a significant variation in prevalence across countries was observed, with a more than fivefold variation for MSI, EBV and GS subgroups (14). The CIN subgroup was the most stable across populations. The cause of this variation is unclear and needs to be further investigated. The incidence of EBV+ in our Belgian population is comparable to the German population (2.6%).

The TCGA reported that MSI-H tumors are mostly located in the gastric body or antrum (4). We found a remarkably high incidence of MSI-high tumors at proximal sites such as the esophagus or GEJ. This is largely caused by the fact that this cohort contained a majority of esophageal cancers. Nevertheless, it proves that testing for MSI should not be restricted to distally located tumors. Other known independent predictive factors for MSI-H GEC are older age (>70y) and sex (female), this was also the case in our cohort (15). Similar to colon cancer, MSI-H GEC is also characterized by increased tumor-infiltrating lymphocytes (16). Remarkable is the fact that we mostly found a diffuse subtype in our MSI-H cohort, in contrast to findings in literature, where an intestinal type was more common (17). Contrary to colorectal cancer, *BRAF* p.V600E mutation appears to be rare in MSI-H GEC. Therefore, there does not seem to be a relationship between MMR-D and *BRAF* p.V600E mutation in gastric carcinoma (18,19). Our study confirms this finding, as none of the tumor samples harbored a *BRAF* p.V600E mutation. Nearly all MSI-H cases (90.9%) were based on hypermethylation of the MLH1 promotor, which is in line with literature (20).

The major limitations of this study are its retrospective design and the lack of MSI and/or EBV result in a large fraction of patients. A possible explanation for the latter is the lack of reimbursement for immunotherapy in these subtypes, resulting in a lesser incentive to test. Because

of the low availability, only 3 of the MSI-H patients were treated with checkpoint inhibition. Due to the low number of MSI-H and EBV+ cases, no conclusions could be drawn on their prognostic or predictive value in this cohort.

Conclusion

In this Belgian cohort, 9.5% of GEC tested for MSI were MSI-H. Given the important implications for prognosis and treatment, and the possibility to identify patients with Lynch syndrome, every upper GI adenocarcinoma should be tested for MSI, regardless of disease location, stage at diagnosis and histology. Testing for EBV on the other hand should be limited to the context of clinical studies given the low incidence in Belgium and the absence of prospective studies supporting the use of checkpoint inhibitors.

Conflict of interest statement

None.

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