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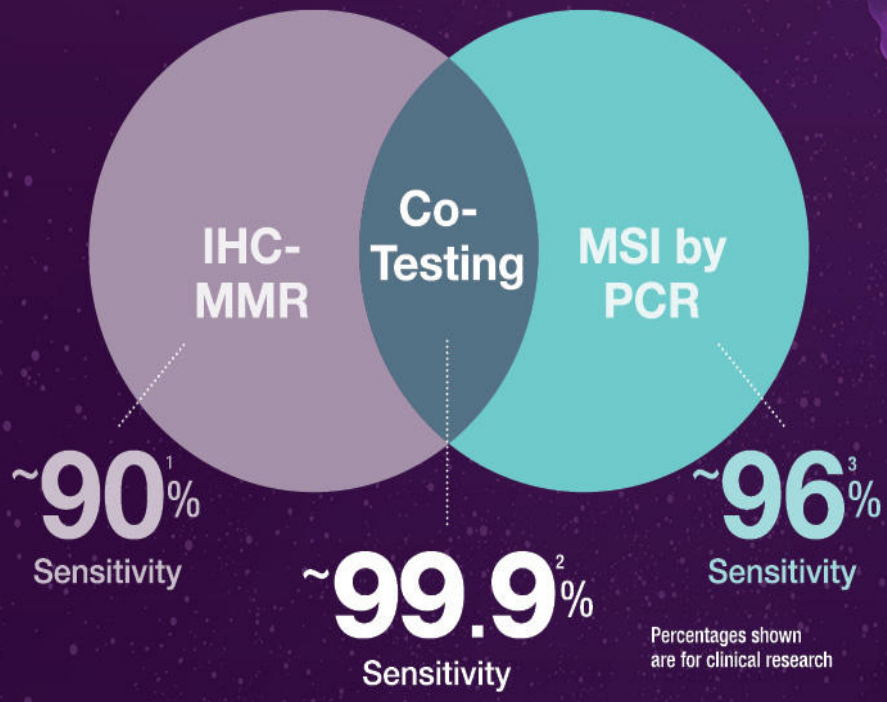

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Title Preservation of Epstein-Barr-Virus Status and Mismatch Repair Protein Status along the Metastatic Course of Gastric Cancer

Running Title EBV and MMR Status in metastasis of gastric cancer

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Conflicts of interest

The authors have declared no conflicts of interest.

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Abstract

(1) Background: EBV in-situ hybridization and mismatch repair (MMR) protein immunohistochemistry identifies two subgroups of gastric cancer (GC) with high immunogenicity and likelihood for response to immune checkpoint inhibition. As tumor biology may change during the metastatic course which can negatively influence the success of therapeutic decisions made on primary tissue, we investigated the consistency of GC EBV and MMR status within primary tumors and metastases. (2) Patients and Methods: We investigated a cohort of 415 primary resected GC, including 111 cases with corresponding distant metastases and 297 cases with lymph node metastases. Tumors were analyzed by EBV in-situ hybridization and MLH1, PMS2, MSH2, and MSH6 immunohistochemistry using tissue microarray technique. (3) Results: Primary tumors were grouped EBV-positive MMR-proficient, EBV-negative MMR-deficient and EBV-negative MMR-proficient. 11/415 (2.7%) of primary tumors were EBV-positive MMR-proficient whereas 49/415 (11.8%) of tumors were EBV-negative MMR-deficient. EBV and MMR protein status showed full concordance with that of the primary tumors. MMR-deficient tumors were of lower pT-category ($p<0.001$), had fewer lymph node metastases (24/49 (49%) versus 273/361 (75.6%) cases; $p<0.001$) and a lower rate of distant metastases (6/49 (12.2%) versus 105/366 (28.7%) cases; $p=0.015$). (4) Conclusion: We demonstrate a strong correlation of EBV and MMR status between primary tumors, lymph node and distant metastases in a large series of primary resected GC. The cases showed the expected frequency of EBV-positive MMR-deficient and EBV-negative MMR-proficient tumors. We conclude that tissue testing for molecular subtyping for therapeutic decision-making can be reliably performed on primary tumors and metastases in GC.

Keywords mismatch repair, microsatellite instability, Epstein-Barr virus, gastric cancer, metastases, molecular subtype

Introduction

Gastric cancer (GC) currently is the fifth most common cancer and the third most common cause of cancer related death worldwide[1,2]. Despite advances in therapy including neoadjuvant chemotherapy and HER2-targeted therapy, the overall 5-year

survival rate remains below 40%[3]. A high rate of resistance towards conventional chemotherapeutics and the presence of locally advanced or metastatic disease at the time of initial diagnosis appear to be the major factors that contribute to the poor outcome[4]. In the past, GC has been classified according to gross features, the predominant histological growth pattern or the cohesiveness of tumor cells. However, these classification systems have a limited use for patient management in individual cases and correspond only partially to underlying molecular events driving tumorigenesis[5,6].

In 2014 and 2015 the Cancer Genome Atlas (TCGA) project and the Asian Cancer Research Group (ACRG) provided a molecular classification of GC based on multimodal molecular and gene expression analysis of ~300 cases each[5,7]. Both studies proposed four molecular subgroups that were defined by key molecular events provide a basis for targeted therapies and correlate to overall survival in case of the ACRG study. The TCGA study defines four mutually exclusive subtypes that partially overlap with the ACRG subtypes: (1) Epstein-Barr-Virus (EBV)-positive, (2) microsatellite instability (MSI), (3) genomically stable (GS) and (4) chromosomal instability (CIN). These molecular subtypes can be detected using routine immunohistochemistry and in-situ hybridization techniques as demonstrated by previous studies. In particular, identification of EBV-positive and MSI tumors can be accomplished by using EBER in-situ hybridization and Mismatch repair (MMR) protein (MLH1, PMS2, MSH2, and MSH6) immunohistochemistry[8-10]. The concordance rate of MMR expression profiles by immunohistochemistry and microsatellite instability testing has been shown to be as high as 99% for GC [11]. Thus, these techniques may represent a convenient screening tool for patient stratification in the clinical setting and to study cohorts of GC that differ from the TCGA and ACRG cohorts regarding patient ethnicity, geographic distribution, risk factors and tumor stage.

EBV-positive and MMR-deficient tumors are promising candidates for PD1 (programmed cell death protein 1)/PD-L1 (programmed death-ligand 1) based immune checkpoint inhibition. This is due to the amplification of the PD-L1 gene in EBV-positive tumors and a hypermutated phenotype with a high tumor mutational burden in MMR-deficient tumors[12,13]. As immune checkpoint inhibition has become a promising therapeutic option

in metastatic gastroesophageal malignancies, reliable tissue testing for molecular subtyping has major impact on therapeutic decision making. However, data about the concordance between the molecular subtype of GC in primary tumors and metastases are scarce. We systematically investigated the EBV and MMR status in a well-characterized western European cohort of 415 primary resected GC with a special focus on the comparison between primary tumors and corresponding lymph node and distant metastases[12].

Materials and Methods

Patients

Buffered formalin-fixed paraffin-embedded tumor tissue from patients with gastric adenocarcinoma treated at the Department of Surgery, Inselspital Bern, University of Bern, Switzerland, was used for this study. We selected those patients from a consecutive series between 1993 and 2013 who did not undergo neoadjuvant therapy and with enough material and histopathological data and basic clinical information. We excluded patients with gastric stump and carcinomas of the gastroesophageal junction. First, we included all cases with distant metastasis that where biopsy proven e either upon initial diagnosis or during follow-up (n=111). Second, we randomly selected additional 304 cases without distant metastasis in order to increase statistical power and to include cancers of all stages. The final cohort consisted of 415 primary resected chemotherapy-naïve gastric carcinomas, including 111 cases with distant and 297 cases with lymph node metastasis. TNM categories and staging were reclassified for all cases according to the eighth edition of the UICC TNM classification of malignant tumors. An overview of the clinicopathological features of the cohort is illustrated in Table 1.

Tissue microarray

A next-generation tissue microarray (ngTMA) containing all cases was constructed as described before, with robot assisted digital annotation of the selected slides for placing the TMA cores[14,15]. The TMA consists of three tissue cores (core size 0.6 mm) each of the tumor center and the tumor front of the resection specimen as well as corresponding lymph node and distant metastases. Full slide sections were obtained for selected cases where immunohistochemistry results were heterogeneous or differed between primary

tumor and corresponding metastases. Approval by the local ethics commission granted the use of archival tissue for molecular and immunohistochemical analysis as well as TMA construction (University of Bern, Switzerland, No. 200/14).

Immunohistochemistry and in-situ hybridization

Immunohistochemical staining was performed on an automated immunostainer (Ventana BenchMark ULTRA, Roche Diagnostics, Oro Valley, AZ, USA) using the following antibodies: MLH1 (clone M1), MSH2 (antibody clone G219-1129), MSH6 (clone SP93) and PMS2 (clone A16-4). Pretreatment with Cell Conditioning 1 solution was carried out for 64, 40, 64, 92 min and primary antibodies were incubated for 24, 12, 12, min respectively. Signal was detected with OptiView Universal DAB Detection Kit and Amplification Kit (all antibodies and reagents Ventana, Roche Diagnostics). EBER in-situ hybridization was performed on an automated immunostainer (Bond III, Leica Biosystems, Newcastle, UK). A 15 min pretreatment with pyruvate dehydrogenase E1 (Leica Biosystems) was followed by a 2-h incubation with a ready-to-use EBER probe (Bond Ready-to-Use ISH EBER Probe, Leica Biosystems). Immunodetection was performed with the Bond Polymer Refine Detection Kit with 3-3′-diaminobenzidine-DAB as chromogen (Leica Biosystems). Finally, all samples were counterstained with hematoxylin and mounted in Aquatex (Merck, Darmstadt, Germany). Examples of the staining are shown in Figure 1. EBER in-situ hybridization was scored as either positive or negative according to the presence or absence of a strong intranuclear staining. MMR protein expression was scored as retained in the presence of a strong intranuclear staining. MMR protein expression was scored based on the presence or absence of nuclear staining in tumor cells. Only cores containing non-neoplastic stroma or immune cells with strong intranuclear staining for MMR proteins serving as an internal positive control were classified as valid for the analysis of MMR protein status. MMR deficiency was defined as absence of the expression of MLH1 and PMS2, MSH2 and MSH6, PMS2 or MSH6. All cases were scored by two independent reviewers (including N.B., B.D. and R.L.). Consensus for divergent cases was reached by reviewing the slides on a multi-headed scope.

Statistical analysis

Statistical analysis was carried out using the IBM SPSS 24.0 Statistics software (IBM, Chicago, IL, USA). Correlations between categorical variables were conducted using χ2-square and Fisher's exact tests. *p* values were two-sided and regarded as significant if $p < 0.05$.

Results

EBV status and MMR protein status in primary tumors: EBER in-situ hybridization as well as MLH1, PMS2, MSH2 and MSH6 immunohistochemistry was analyzed in the tissue of the primary tumor in all 415 cases. Primary tumors were grouped into three subtypes based on EBV and MMR protein status: (1) EBV-positive MMR-proficient (2) EBV-negative MMR-deficient and (3) EBV-negative MMR-proficient. Only 11/415 (2.7%) of primary tumors were EBV-positive MMR-proficient whereas 49/415 (11.8%) of tumors were EBV-negative MMR-deficient. All MMR-deficient tumors showed loss of MLH1 expression with concordant loss of PMS2 expression. A loss of MSH2 and MSH6 expression was not observed. We did not identify a single tumor that was both EBV-positive as well as MMR-deficient, a rare phenomenon that has been described previously in one of 799 cases of a western GC cohort[11]. Intratumoral heterogeneity of EBV or MMR status between tumor front and tumor center was not observed. Representative images of all subtypes are shown in Figure 1.

Relationship of EBV status and MMR protein status with clinical and histopathological parameters: Patients with EBV-positive tumors were more likely to be male (10/11 (90.9%) versus 247/404 (61.1%) cases; p=0.045), all other relationships with clinicopathological parameters were not significant. Patients with MMR-deficient tumors were older (median age 75 years (45-92 years) versus median age 69 years (31-93 years); p<0.001), tumors were of lower pT-category (p<0.001), had fewer lymph node metastases (24/49 (49%) versus 273/361 (75.6%) cases; p<0.001) and a lower rate of distant metastases (6/49 (12.2%) versus 105/366 (28.7%) cases; p=0.015). In addition, MMR-deficient tumors were of lower histological grade (p=0.025), predominantly of intestinal type morphology (41/49 (83.7%) versus 171/366 (46.7%) cases; p<0.001) and more likely to be localized to the antrum of the stomach (34/49 (69.4%) versus 164/366 (44.8%) cases; p=0.047).

EBV status and MMR protein status along the metastatic course: MLH1 immunohistochemistry was analyzed in 269/297 (90.6%) lymph node and 98/111 (88.3%) distant metastases. EBER in-situ hybridization was analyzed in 284/297 (95.6%) lymph node and 103/111 (92.8%) distant metastases. In all investigated metastatic cases EBV and MMR protein status showed complete concordance with that of the primary tumors. Representative images of primary tumors and their metastases are shown in Figure 2.

Discussion

The molecular classification of GC into four subtypes as proposed by TCGA allows the stratification of GC patients into different prognostic and predictive groups[5,7]. Among the four subtypes, MSI and EBV-positive tumors have gained attention, as they are candidates for PD1/PD-L1 based immune checkpoint inhibition, which has become a promising therapeutic option in advanced GC[10,12]. We therefore analyzed EBV and MMR protein status in the primary tumors and corresponding metastases in a large western cohort of primary resected GC. The frequency of EBV-positive and MMR-deficient tumors in our cohort is slightly lower in comparison to GC cohorts of previous publications, where EBV-positive tumors occurred in a range between 4-14% and MMR-deficient tumors between 8-26%[5,7-11,16]. We hypothesize that the observed differences of EBV frequency are mainly due to different patient populations under study, and reflect the known geographical variance of EBV positivity; with both ethnicity and lifestyle as well as environmental risk factors and co-infections as contributing factors[17]. We exclude intratumoral heterogeneity as a potential source of declaring a case as false negative, as all EBV-positive cases showed uniform positivity in all TMA tissue cores where tumoral tissue was present. In addition, we selectively analyzed full slide sections of EBV-positive cases that also demonstrated a uniform positivity. Our data supports the previous observation that MSI in GC is due to loss of MLH1 expression in the vast majority of cases, as none of our MMR deficient cases showed a loss of MSH2, MS6H or isolated loss of PMS2 expression[18]. In addition, this is to our knowledge the first study that systematically investigated the EBV and MMR protein status in corresponding lymph node and distant metastasis. Our comparative analysis of lymph node and distant metastases showed full concordance of EBV and MMR protein status along the metastatic course. The observed full concordance suggests the following conclusions.

First, the evaluation of EBV or MMR protein status for immunotherapy eligibility testing either on tissue of the primary tumor or the metastasis is sufficient and that there is no need to reevaluate metachrone metastases. Second, the primary tumor and its metastases likely have a similar putative response towards immune checkpoint inhibition, as the molecular key events that predict response are preserved along the metastatic course.

One major limitation of our study is the low number of EBV-positive GCs. All EBV-positive GCs show concordant EBV-positive metastases, but we cannot rule out that EBV expression might potentially be lost during the metastatic course of disease in a larger study population. The second major limitation of our study is the lack of a long-term follow up and thus missing overall survival data. However, the detailed pathological parameters available show a prognostic association of patients with MMR-deficient tumors and a more favorable course of disease, with a lower frequency of lymph node and distant metastases, lower histological grade and tumor stage, which is in line with previous studies [5,16,19,20]. Since the basic pathological characterization of our cohort is comparable to data from literature and our focus was set on the correlation of EBV and MMR status in primary tumors and metastases we consider the lack of clinical follow up as an acceptable weakness. The third major limitation of our study is the retrospective study design. Our cohort consist of primary resected tumors encompassing cases from a historical pre-neoadjuvant therapy era, as the initial diagnosis for the vast majority of our cases was before the publication of the MAGIC trial in 2006[21]. However, this allows us to study molecular alterations without the biological interference of preoperative chemotherapy. Most of the tumors would have now been treated by neoadjuvant therapy, and the clinical course may be influenced by the local and systemic response to this treatment as well[12,22]. Moreover, several multimodal treatment concepts exist which also may have different impact on the clinical course of patients with different molecular subtypes of GC.

In conclusion, we demonstrate a strong correlation of EBV and MMR status between primary tumors, lymph node and distant metastases, using in situ hybridization and immunohistochemistry in a large series of GC. In addition, we speculate that the concordance of EBV and MMR status between primary tumor and metachronous

metastases remains unchanged irrespective of adjuvant therapy, although we lack detailed clinical data regarding the administration of postoperative chemotherapy. The investigated cases showed the expected frequency of EBV-positive MMR-deficient and EBV-negative MMR-proficient tumors. We conclude that tissue testing for molecular subtyping for potential therapeutic decision-making can be reliably performed on both primary tumors and metastases in GC.

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BD, RL and NB designed experiments, carried out the experiments and wrote the manuscript. BG and MDB assisted in acquiring the samples and reviewed the manuscript. This work was supported by the Swiss Cancer League (Grant Number: KFS-3700-08-2015)

Ethical approval and consent to participate

Approval by the local ethics commission granted the use of archival tissue for molecular and immunohistochemical analysis as well as TMA construction (University of Bern, Switzerland, No. 200/14). The study was performed in accordance with the Declaration of Helsinki.

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Table 1

Clinicopathological characteristics of the patient cohort in relationship to EBV status and mismatch repair protein status

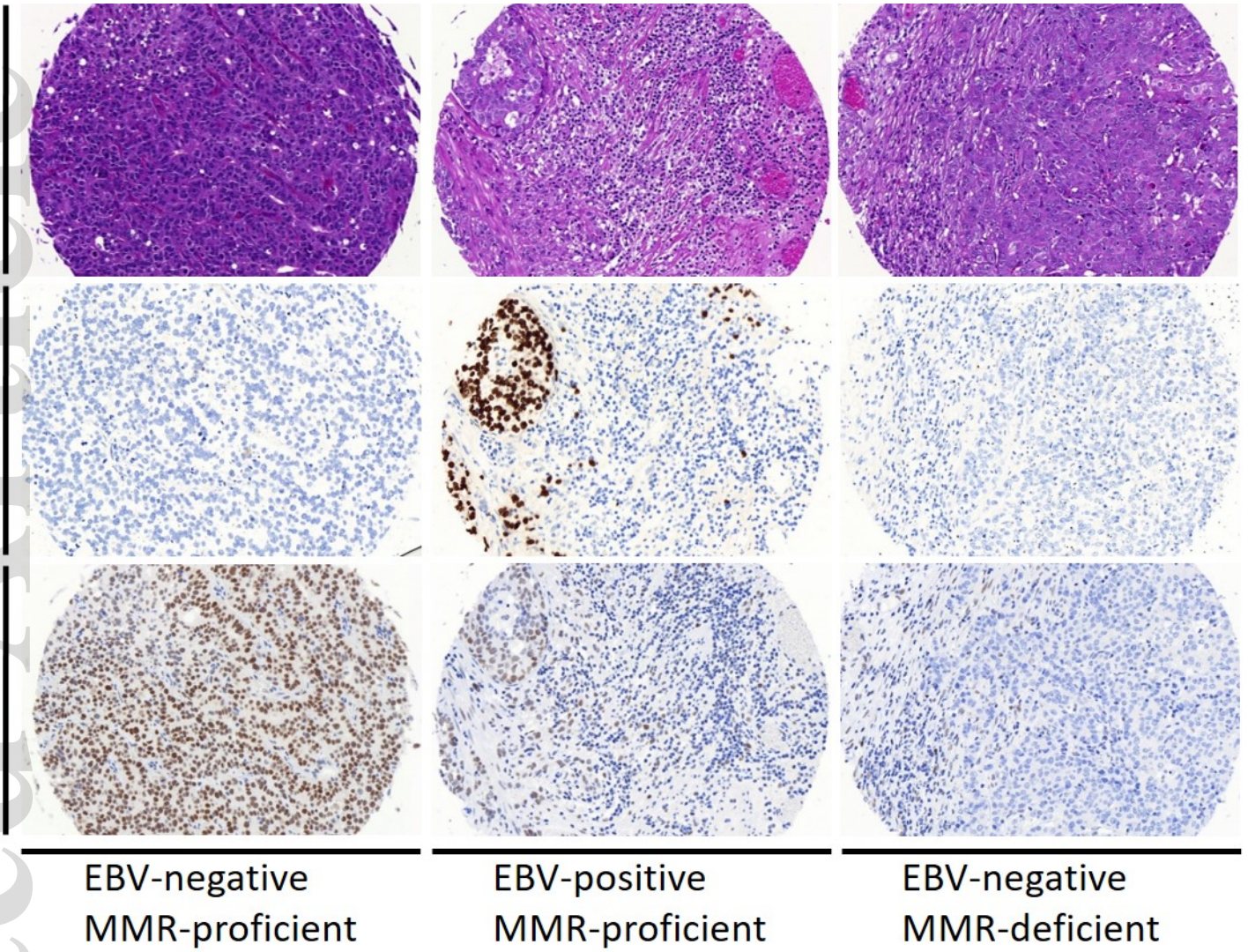
Factors	*Number of patients*	*EBV-positive MMR-proficient*	*EBV-negative MMR-deficient*	*EBV-negative MMR-proficient*	*p value*
Total	415	11 (2.7%)	49 (11.8%)	355 (85.5%)	
Gender					0.087
Male	257 (61.9%)	10 (3.9%)	27 (10.5%)	220 (85.6%)	
Female	158 (38.1%)	1 (0.6%)	22 (13.9%)	135 (85.4%)	
Age, median (min-max)	71 (31-93)	69 (58-87)	75 (45-91)	70 (31-93)	

pT category					0.006
T1	49 (11.8%)	0	8 (16%)	41 (83.7%)	
T2	54 (13%)	1 (1.9%)	14 (31.1%)	39 (72.2%)	
T3	149 (35.9%)	5 (3.4%)	17 (11.4%)	127 (85.2%)	
T4	163 (39.3%)	5 (3.1%)	10 (6.1%)	148 (90.8%)	
pN stage					<0.001
N0	113 (27.6%)	3 (2.7%)	25 (22.1%)	85 (75.2%)	
N1-3	297 (72.4%)	8 (2.7%)	24 (8.1%)	265 (89.2%)	
M					0.05
M0	304 (73.2%)	8 (2.6%)	43 (14.1%)	253 (83.2%)	
M1	111 (26.7%)	3 (2.7%)	6 (5.4%)	102 (91.9%)	
Grading					0.084
G1	23 (5.5%)	0	2 (8.7%)	21 (91.3%)	
G2	97 (23.4%)	2 (2.1%)	19 (19.6%)	76 (78.4%)	
G3	295 (70.6%)	9 (3.1%)	28 (9.5%)	258 (87.5%)	
Laurén classification					<0.001
Intestinal	212 (51.1%)	6 (2.8%)	41 (19.3%)	165 (77.8%)	
Diffuse	134 (32.3%)	2 (1.5%)	3 (2.2%)	129 (96.3%)	
Mixed	65 (15.7%)	3 (4.6%)	5 (7.7%)	57 (87.7%)	
Indeterminate	4 (0.9%)	0	0	4 (100%)	
Tumor localization					0.118
Body/Fundus	99 (23.8%)	4 (4%)	8 (8.1%)	87 (87.9%)	
Antrum	198 (47.7%)	2 (1%)	34 (17.2%)	162 (81.8%)	
Body/Fundus/Antrum	33 (8%)	2 (6.1%)	3 (9.1%)	28 (84.8%)	
Cardia	58 (14%)	2 (3.4%)	3 (5.2%)	53 (91.4%)	
Entire stomach	4 (0.9%)	0	0	4 (100%)	

Figure legends

Figure 1: Representative images of TMA cores of primary tumors stained with an antibody directed against MLH1 and with an EBER in-situ hybridization probe.

Figure 2: Preservation of EBV status and Mismatch Repair Protein status along the metastatic course of gastric cancer A: Representative images of an EBV-positive MMR-proficient primary tumor and the corresponding distant metastasis. B: Representative images of an EBV-negative MMR-deficient primary tumor and the corresponding lymph node metastasis.



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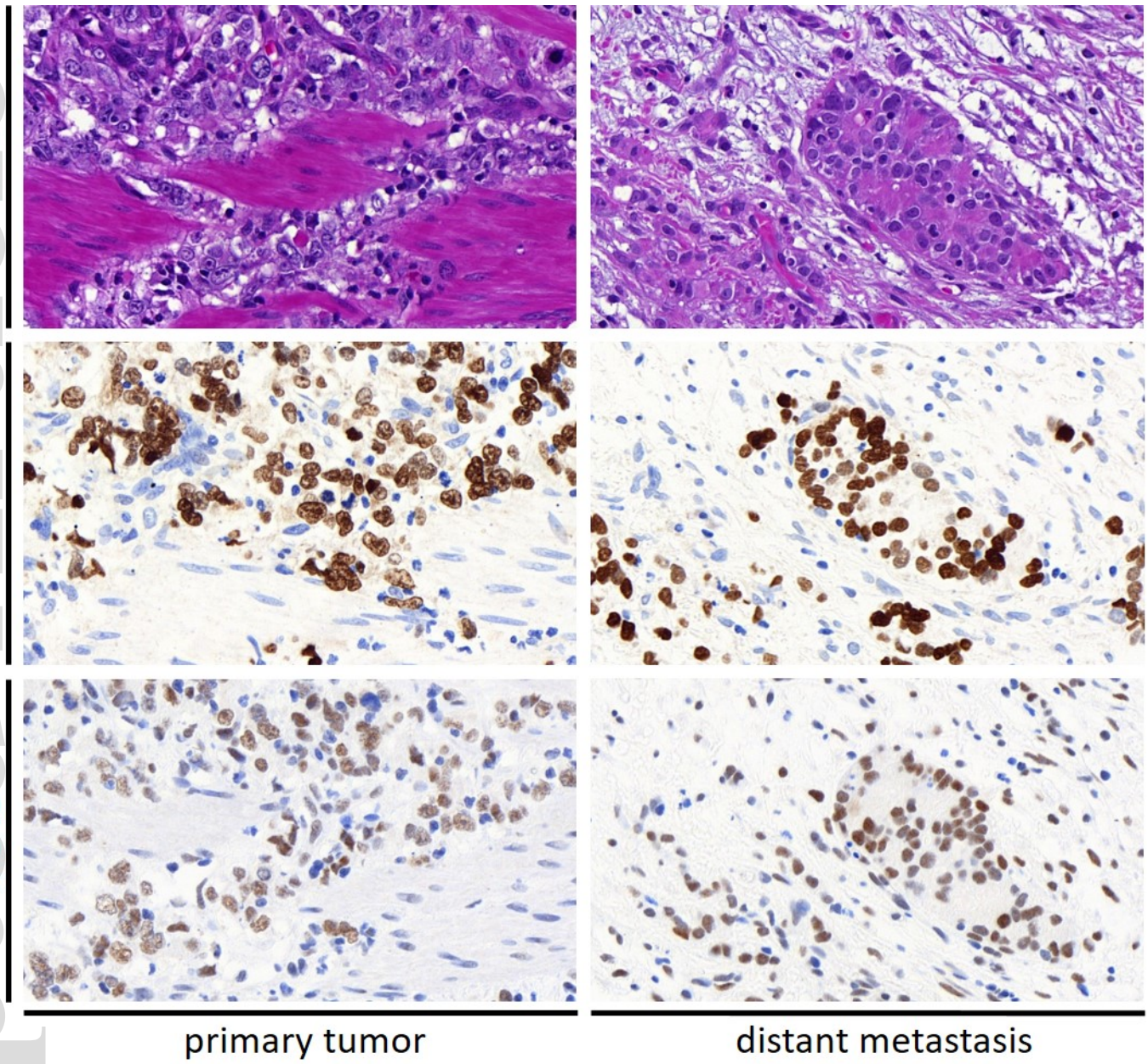

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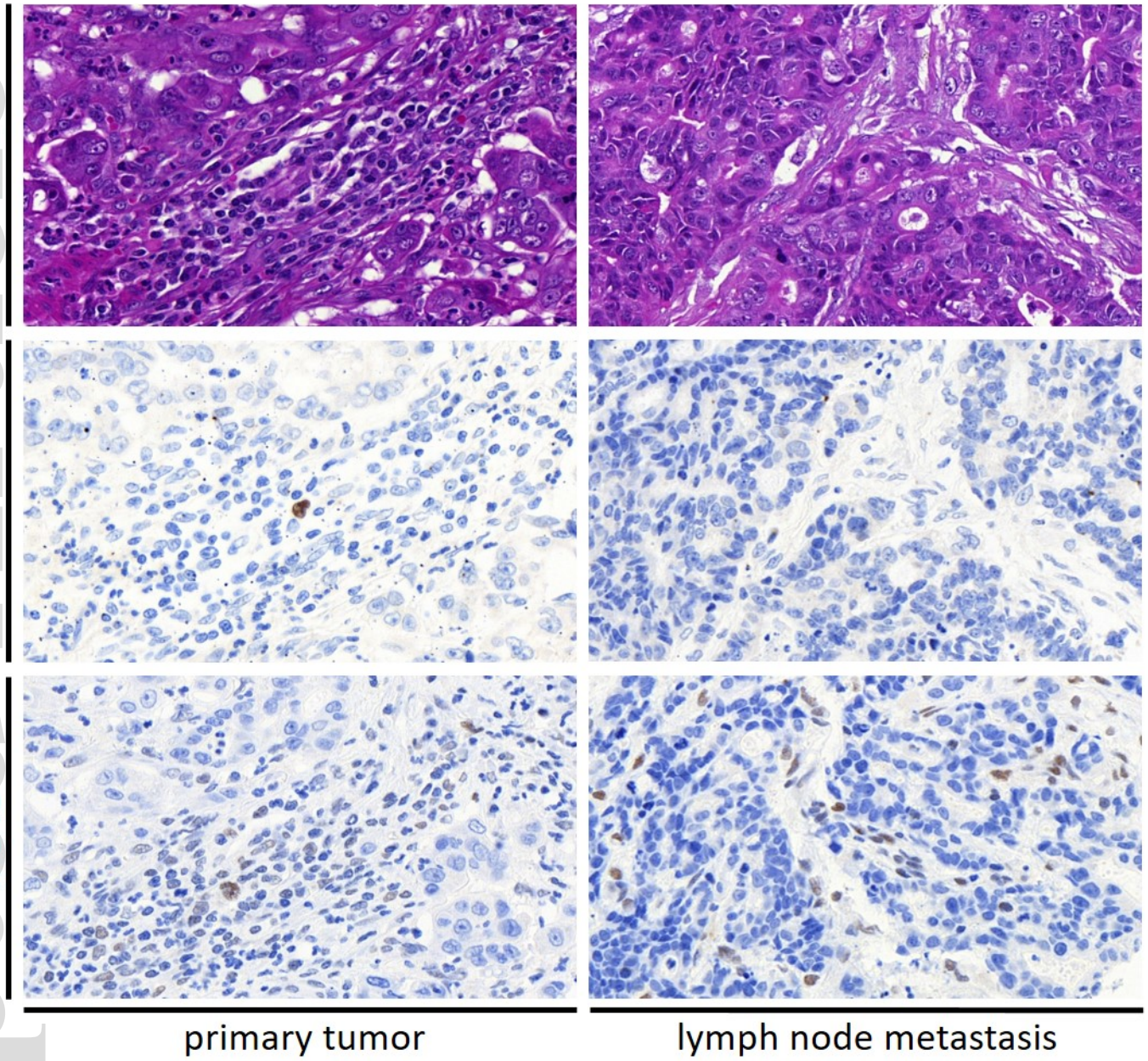

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