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Gastric cancer molecular classification based on immunohistochemistry and *in-situ* hybridisation and mortality

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Background and aims: Gastric cancers (GC) are divided into subtypes based on molecular profile: Epstein–Barr virus (EBV)-positive, microsatellite instability (MSI), chromosomal instability (CIN) and genomically stable (GS) tumours. The prognostic impact of this classification is unclear. The aim was to evaluate whether the molecular subtypes determined using *in-situ* hybridisation (ISH) and immunohistochemistry (IHC) are associated with clinicopathological parameters and prognosis.

Methods and results: The study included 503 GC patients. Based on ISH (EBV) and IHC (MSI and TP53), tumours were divided into EBV-positive, MSI, CIN (EBVneg/MSS/TP53aberrant) and GS (EBVneg/MSS/TP53wild-type) subgroups. Survival analyses with intestinal- and diffuse-type tumours were examined separately. EBV-positive tumours associated with male sex. Both EBV-positive and MSI tumours associated with intestinal type. CIN tumours associated with intestinal-type and positive lymph node status. GS tumours associated with diffuse-type and negative lymph node status. In the total cohort, no significant differences in the 5-year survival were observed. In intestinal tumours, the 5-year survival was better in EBV-positive tumours compared with GS tumours [hazard ratio (HR) = 0.57, 95% confidence interval (CI) = 0.33–0.99]. In diffuse tumours, the 5-year survival was worse in CIN tumours compared with GS tumours (HR = 1.57, 95% CI = 1.14–2.18). In radically resected diffuse tumours, the 5-year survival was worse in MSI tumours compared with GS tumours (HR = 3.26, 95% CI = 1.20–8.82).

Conclusions: The molecular classification is associated with histological type but not prognosis in GC. As the prognostic effects of molecular subtypes in intestinal- and diffuse-type cancers may differ, combining histological and molecular information is recommended for future studies.

Keywords: gastric cancer, immunohistochemistry, *in situ* hybridisation, molecular classification

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Abbreviations: ACRG, the Asian Cancer Research Group; AJCC, the American Joint Committee on Cancer; CIN, chromosomal instability; EBER, Epstein-Barr encoded RNA; EBV, Epstein-Barr virus; GC, gastric cancer; GS, genomically stable; IHC, immunohistochemistry; ISH, in-situ hybridisation; MSI, microsatellite instability; MSS, microsatellite stable; TCGA, The Cancer Genome Atlas; TMA, tissue microarray; TP53, tumor protein p53.

Introduction

Gastric cancer (GC) is the fifth most common cancer globally.[1] Due to its frequently advanced stage at diagnosis, it has a poor prognosis with an estimated 5-year survival rate of < 20%.[2] In clinical practice, GC prognosis and treatment guidelines are estimated using the American Joint Committee on Cancer tumour, node, metastasis (AJCC TNM) staging. However, GC is a heterogeneous disease, where even similar clinicopathological parameters lead to different outcomes. Both genetic and environmental factors are involved in the initiation and progression of GC, leading to classifications based on the molecular profile of GC. The Cancer Genome Atlas (TCGA) research network has classified GC into four subtypes: Epstein–Barr virus-positive (EBV), microsatellite instability (MSI), chromosomal instability (CIN) and genomically stable (GS).[3] In addition, the Asian Cancer Research Group (ACRG) network team has classified GC into four molecular subtypes: tumours with MSI, microsatellite stable tumours showing epithelial–mesenchymal transition (MSS/EMT), MSS tumours with intact tumour protein p53 (MSS/TP53^{+}) and MSS tumours with loss of TP53 (MSS/TP53^{-}).[4] These subtypes may have different characteristics, prognosis and potential for targeted therapies.[5–10] However, sequencing technologies are not suitable for routine diagnostics; therefore, more simple methods are needed and have already been evaluated in previous smaller GC studies.[11–17] However, there are slight differences between the results. In addition, only one previous study included a subgroup analysis by histological subtype, but in that study none of the diffuse tumours had EBV-positive or MSI phenotype, limiting further analyses. Further studies with larger sample sizes based on the histological subtype of the tumours are needed.

The aim of this study is to evaluate whether the GC molecular subtype determined using tissue microarray (TMA), immunohistochemistry (IHC) and *in-situ* hybridisation (ISH) is associated with clinicopathological parameters and prognosis, and to compare the results between intestinal and diffuse histological subtypes.

Material and methods

STUDY DESIGN AND DATA COLLECTION

This study was a retrospective cohort study in a single tertiary care hospital in northern Finland. A total of 601 histologically confirmed GC patients underwent gastrectomy for gastric cancer between the years 1983 and 2016 in Oulu University Hospital. Of these, the final series consisted of 503 patients with available representative tissue material. The patients were originally identified from the archives of the Department of Pathology at the Oulu University Hospital. Clinical data and pathology reports were collected from the patient records. The 100% complete follow-up data were acquired from the Cause of Death Registry at Statistics Finland, available until the end of 2019. The immutable national personal numbers assigned to each resident were used to combine data from the patient records.

TISSUE MICROARRAY

The most representative tumour areas with the deepest tumour invasion were selected from the diagnostic haematoxylin and eosin slides, which were scanned using Aperio AT2 (Leica Biosystems, Wetzlar, Germany). A total of four cores with a diameter of 1.0 mm were extracted from each patient. Two cores from the tumour front and two cores from the tumour centre were included to achieve representative samplings from different parts of the tumour. The cores were punched from paraffin-embedded tissue blocks and transferred to a receiver block. Tissue microarrays (TMAs) were constructed with computer-driven TMA-device Galileo TMA CK4500 program (Integrated Systems Engineering, Milan, Italy).

IMMUNOHISTOCHEMISTRY

The TMA samples were cut in 4 μm sections for immunohistochemical staining (IHC), placed on glass slides, deparaffinised in xylene and rehydrated through graded alcohols. Rehydrated samples were put into a microwave oven for antigen retrieval with Tris-ethylenediamine tetraacetic acid (EDTA)-buffer (pH 9) 800 W for 2 min and 150 W for 15 min and then cooled to room temperature for 20 min. Samples were rinsed in distilled water and phosphate-buffered saline with Tween (PBS-T) and endogenous peroxidase was then neutralised in peroxidase blocking solution (Dako S2023; Dako, Glostrup, Denmark) for 5 min. After a wash in PBS-T, sections were incubated with antibodies; MLH1 [diluted 1:50 60 min; Leica Novocastra, Espoo, Finland (NCL-LMLH1), ER2 40 min; Leica Bond Polymer Refine Detection System (DS9800)], MSH2 [diluted 1:800 60 min; BD Pharmingen, Ventaa, Finland (556349), ER2 40 min; Bond Polymer Refine Detection System (DS9800)],

MSH6 [diluted 1:200 60 min, BD Pharmingen (610919), ER2 40 min; Bond Polymer Refine Detection System (DS9800)], PMS2 [diluted 1:100 60 min; BD Pharmingen (556415), ER2 40 min] and TP53 [diluted 1:2400 30 min (DO-7); Dako code M7001, ER2 30 min]. EBV status was determined by ISH, with probes against Epstein–Barr encoded RNA (pb058; Leica Microsystems), according to the manufacturer's instructions (protocol A, digestion 15 min, enzyme prep 1, hybridisation 2 h).

HISTOLOGICAL ANALYSIS

TMA slides were digitised using Aperio AT2 (Leica Biosystems, Wetzlar, Germany). QuPath software was used to analyse the cores from scanned slides.[18] It was decided a priori that the cores for MLH1, MSH2, MSH6, PMS2 and EBER would be analysed by one researcher (M.E.). Staining was scored either positive or negative depending upon nuclear reaction. A positive score was given if at least one core was positively stained. Negative score was given if markers showed complete loss of nuclear reactivity with positive background reaction in benign tissue. If the background was negative together with negative nuclear reactivity, staining was considered unreliable, and it was excluded from the analysis. A tumour was classified as MSI if at least one of the markers (MLH1, MSH2, MSH6 or PMS2) was negative, and tumours that maintained the expressions of all markers were defined as MSS. EBV status was determined by ISH, and tumour was classified either EBV-positive or EBV-negative, according to the nuclear reaction. In the TP53 staining, we decided a priori that M.E. would analyse all TP53 staining, but all unclear heterogeneous TP53 cases ($n = 26$) would be analysed together with a gastrointestinal pathologist (E.-M.B.). Aberrant expression (TP53 aberr) was defined as a complete loss of or strong TP53 nuclear positivity. Heterogeneous expression (TP53 wt) was defined as a pattern ranging from a few positive cells to almost all cell-staining, but with variable intensity. Examples of EBER *in-situ* hybridisation and MLH1 and TP53 immunohistochemistry are shown in Figure 1.

STATISTICAL ANALYSIS

The primary outcome of this study is 5-year survival, defined as death for any cause during 5 years after surgery. IBM SPSS Statistics version 26.0 (IBM Corp., Armonk, NY, USA) was used for all statistical analyses. The χ^2 test was used to compare categorical variables and analysis of variance was used to compare continuous variables. The Kaplan–Meier method was used to compare mortality between groups and to obtain Kaplan–Meier curves, and the log-rank test was used to determine statistical significance of differences between groups. A Cox regression model was used to perform univariable and multivariable analysis, providing hazard ratios (HR) with 95% confidence intervals (CI). Cox regression was adjusted for potential confounding variables: year of surgery (continuous variable), age at diagnosis (continuous variable); sex (male or female); administration of perioperative chemotherapy (yes or no), tumour stage (stage I + II or stage III + IV), Laurén classification (intestinal, diffuse or mixed) and radical resection (R0 or R1/2). R0 resection was defined as no cancer cells seen microscopically within 1 mm of the tumour border. Subgroup analyses were conducted in intestinal and diffuse histological types, adjusted for other confounders as listed above, and also for histological grade (I–II or III) in the intestinal-type subgroup. *P*-values < 0.05 were accepted as statistically significant.

Results

PATIENTS

The final series consisted of 503 adenocarcinoma patients whose tissue samples were available for further analyses. The median age of the patients was 69 years (range = 27–90), and 60.6% of patients were male. Of these 503 patients, 372 (74.0%) underwent microscopically confirmed R0 resection and 131 (26.0%) had R1/2 resection, including patients treated with palliative intent, as well as 32 (6.4%) patients who had distant metastases discovered at the time of surgery. Only 21 (4.2%) patients underwent perioperative chemotherapy (Table 1).

EBV *IN-SITU* HYBRIDISATION AND MSI AND TP53 IMMUNOHISTOCHEMISTRY

EBV RNA was found to be present in 37 of 503 (7.4%) of all tumour samples. EBV positivity was found in 29 of 263 (11.0%) of the intestinal tumours and in six of 222 (2.7%) of the diffuse tumours. MSI subtype was observed in 35 of 503 (7.0%) of all tumour samples, in 28 of 263 (10.6%) of the intestinal tumours and in seven of 222 (3.2%) of the diffuse tumours. Aberrant TP53 expression was found in 256 of 503 (50.9%) of all tumour samples, in 145 of 263 (55.1%) of the intestinal tumours and in 104 of 222 (46.8%) of the diffuse

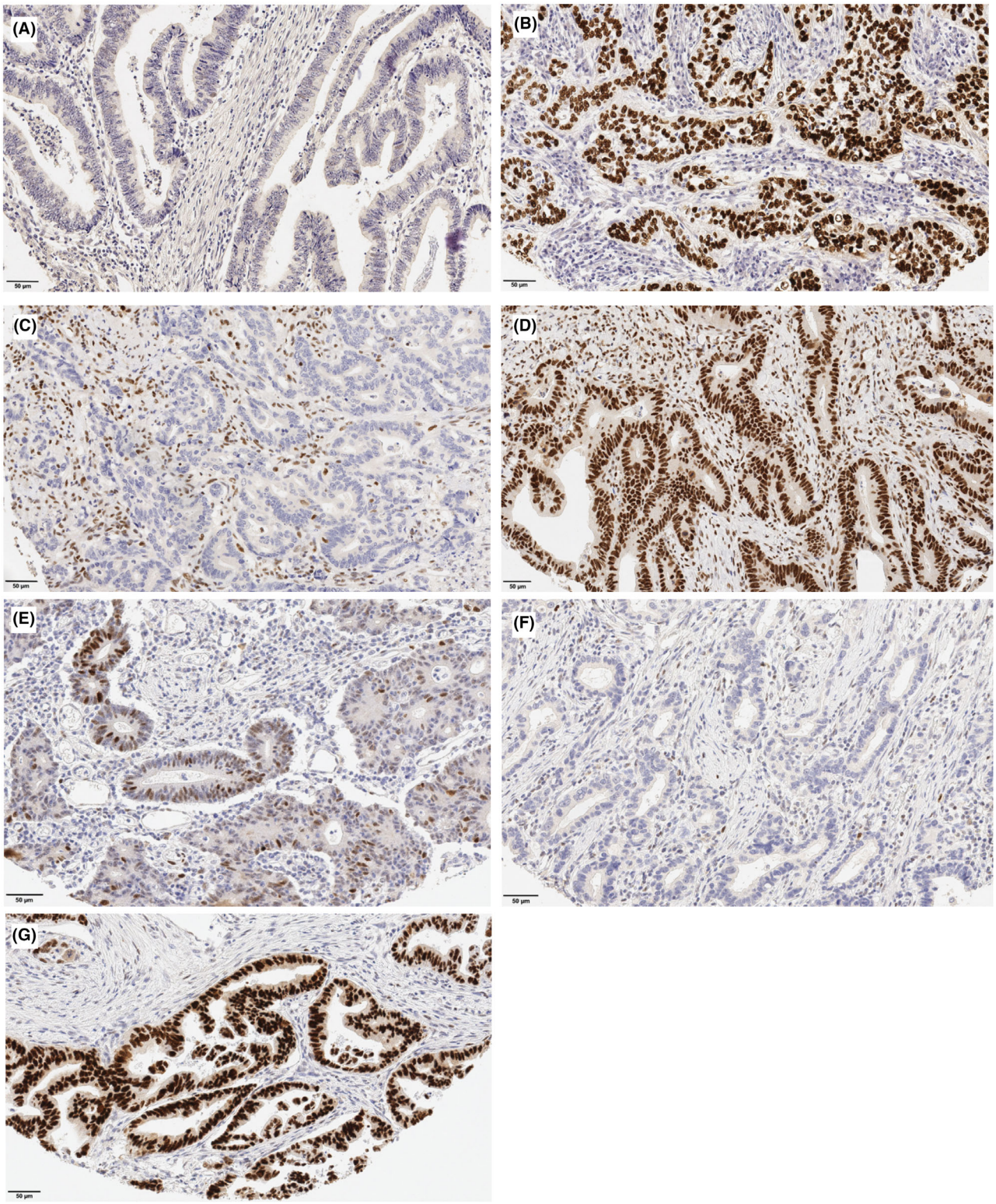

Figure 1. Representative images of EBER *in-situ* hybridisation and MLH1 and TP53 immunohistochemistry in gastric adenocarcinoma. **A,** EBV negative, **B,** EBV-positive, **C,** MLH1 negative (MSI), **D,** MLH1 positive (MSS), **E,** heterogeneous TP53 expression (wild-type), **F,** a complete loss of TP53 expression (aberrant) and **G,** strong TP53 expression (aberrant).

Table 1. Patient characteristics of the 503 patients with gastric adenocarcinoma

Characteristics	No.
Year of surgery, median (IQR) years	1999 (1991–2008)
Age (years)	
< 69	252 (50.1%)
≥ 69	251 (49.9%)
Sex	
Male	305 (60.6%)
Female	198 (39.4%)
Radicality of resection	
R0	372 (74.0%)
R1 or R2	131 (26.0%)
Perioperative chemotherapy	
Yes	21 (4.2%)
No	482 (95.8%)
Metastases	
Yes	32 (6.4%)
No	471 (93.6%)
Laurén classification	
Intestinal	263 (52.3%)
Diffuse	222 (44.1%)
Mixed/not classified	18 (3.6%)
Stage	
I + II	294 (58.4%)
III + IV	209 (41.6%)

tumours (Figure 2, Supporting information, Table S1). Among the intestinal-type tumours, aberrant TP53 was more common in EBV-negative than EBV-positive tumours and were more often MSS than MSI ($P < 0.001$, Supporting information, Table S2).

The EBV-negative/MSS/TP53 aberration group was considered as chromosomal instable (CIN) subtype. CIN subtype was found in 238 of 503 (47.3%) of all tumours, in 137 of 263 (52.1%) of the intestinal tumours and in 101 of 222 (45.5%) of the diffuse tumours. EBV-negative/MSS/TP53 wild-type was considered genomically stable (GS) subtype (Figure 2). GS subtype was detected in 177 of 503 (35.1%) of all tumours, in 69 of 263 (26.2%) of the intestinal tumours and in 108 of 222 (48.6%) of the diffuse tumours (Supporting information, Table S1).

ASSOCIATION OF EBV, MSI, CIN AND GS STATUS WITH CLINICOPATHOLOGICAL VARIABLES

EBV-positive tumours were more common among male than female patients ($P = 0.024$). Thirty of the 305 (9.8%) male patients and seven of the 198 (3.5%) female patients had EBV-positive tumours. Both EBV-positive and MSI subtype were observed to be significantly more common among intestinal-type histology ($P < 0.001$) and the majority of patients were radically resected ($P = 0.008$). MSI subtype was seemingly more common in older patients, but the difference was not statistically significant ($P = 0.054$). CIN tumours associated with intestinal-type histology and with positive lymph node status, while GS tumours more often exhibited diffuse histology ($P < 0.001$) and associated with negative lymph node status ($P = 0.046$) (Table 2).

ASSOCIATION OF EBV, MSI, CIN AND GS STATUS WITH SURVIVAL

The 5-year survival was 40.5% in the EBV-positive group, 31.4% in the MSI group, 24.6% in the CIN group and 29.9% in the GS group (log-rank $P = 0.223$, Figure 3). In the subgroup analysis with radically resected patients, the 5-year survival was 39.4% in the EBV-positive group, 31.3% in the MSI group, 31.4% in the CIN group and 37.1% in the GS subgroup ($P = 0.676$, Figure 3). No statistically significant association was observed between subgroup status and survival in the total cohort of 503 patients (Table 3).

In the subgroup analysis with intestinal-type histology, the 5-year survival was 41.4% in the EBV-positive group, 35.7% in the MSI group, 27.7% in the CIN subgroup and 20.3% in the GS group ($P = 0.251$, Figure 3). The 5-year survival was significantly better in the EBV-positive group compared to the GS group in the multivariable analysis (HR = 0.57, 95% CI = 0.33–0.99, Table 3). In the subgroup analysis with R0 resected intestinal-type tumours, the 5-year survival was 40.7% in the EBV-positive group, 37.0% in the MSI group, 31.4% in the CIN subgroup and 25.5% in the GS group ($P = 0.762$). No statistically significant association was observed between subgroup status and survival (Table 3).

In the subgroup analysis with diffuse-type histology, the 5-year survival was 50.0% in the

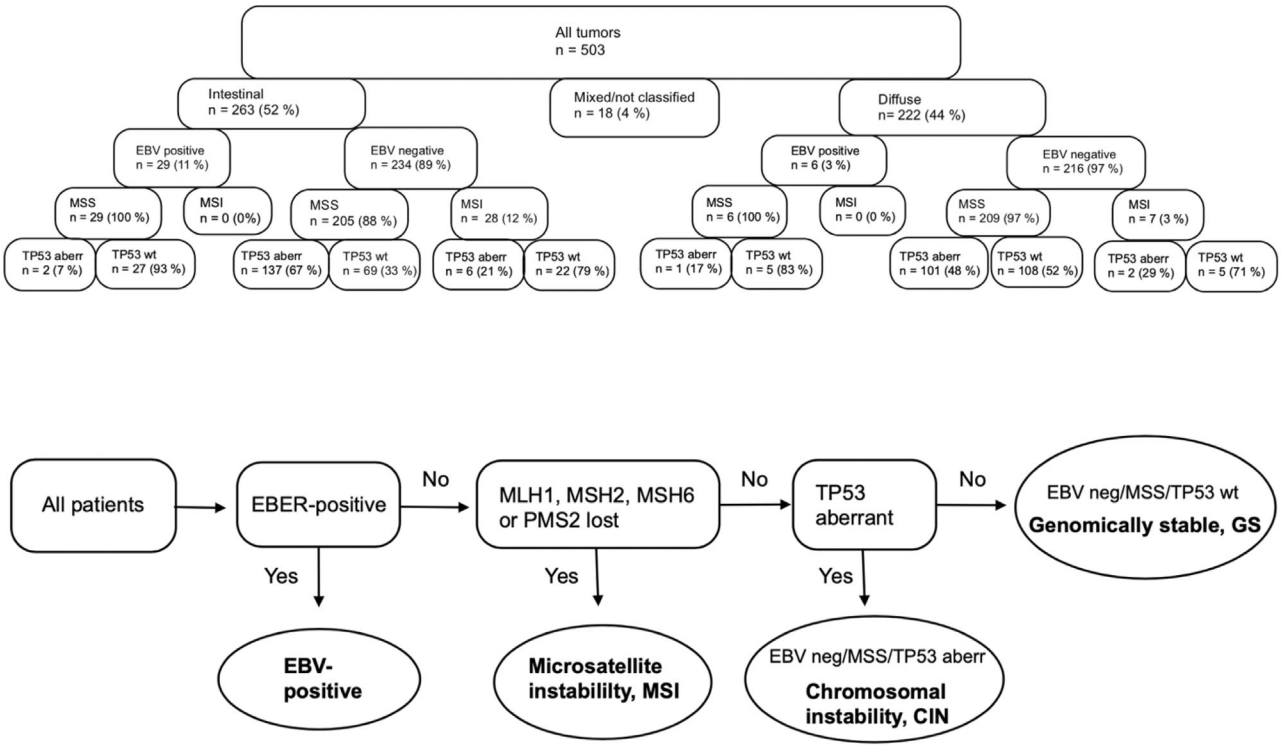

Figure 2. Classification of gastric adenocarcinomas based on *in-situ* hybridisation and immunohistochemistry.

EBV-positive group, 14.3% in the MSI group, 18.8% in the CIN subgroup and 35.2% in the GS group ($P = 0.027$, Figure 3). The 5-year survival was significantly worse in the CIN group compared to the GS group in the multivariable analysis (HR = 1.57, 95% CI = 1.14–2.18). In the subgroup analysis with R0 resected diffuse-type tumours, the 5-year survival was 40.0% in the EBV-positive group, 0% in the MSI group, 29.7% in the CIN subgroup and 47.9% in the GS group ($P = 0.013$, Figure 3). The 5-year survival was significantly worse in the MSI group compared to the GS group in the multivariable analysis (HR = 3.26, 95% CI = 1.20–8.82, Table 3).

Discussion

IHC and ISH were used in the present study to define molecular subgroups of gastric adenocarcinomas, and the association between the subgroups and patient survival was explored.

The strengths of the study are the large study size, with reliable 100% complete follow-up information and relatively low selection bias. The diagnosis and treatment occurred in the same hospital, minimising the selection bias, although the retrospective single-institution design and the unavailability of some samples in the source cohort might also limit its applicability for larger populations. Patients with non-radical resections were also included to minimise selection bias and maximise the statistical power of this study. However, sensitivity analyses, excluding palliative and non-radically resected patients, were also performed to evaluate the molecular subtypes in a more homogenous patient population. While immunohistochemistry is a commonly used technique in clinical settings to assess protein expression, it is important to recognise that different laboratory processing methods may have a significant impact on immunostaining results.[19] However, all immunostainings are well-validated and in daily clinical use in the hospital laboratory. In the current study, the lack of a validation cohort could be considered one of the primary limitations. The long study period may cause some confounding due to improvements in GC treatment and varying staging methods, which is why the year of surgery was taken into account in the adjusted analyses. While the TMA technique is an accurate method for analysis of protein expression, it may be limited in evaluating heterogenous tumours.[14,20] This limitation was mitigated by examining four tissue cores from each tumour. The analysis of MSI status using IHC has proved to be feasible,

Table 2. Associations between EBV, MSI, CIN and GS status and clinicopathological variables in 503 surgically resected patients with gastric adenocarcinoma

Variable	EBV-pos	MSI	CIN (EBV-neg MSS, TP53 aberr)	GS (EBV- neg MSS, TP53 wt)	Total	*P*-value
Year of surgery, median (IQR) years	1997 (1987–2004)	2001 (1990–2010)	1998 (1991–2009)	1999 (1991–2007)		0.348
Age (years)						
< 69	17 (45.9%)	10 (28.6%)	126 (51.6%)	99 (52.9%)	252/503	0.055
≥ 69	20 (54.1%)	25 (71.4%)	118 (48.4%)	88 (47.1%)	251/503	
Sex						
Male	30 (81.1%)	20 (57.1%)	152 (62.3%)	103 (55.1%)	305/503	**0.024**
Female	7 (18.9%)	15 (42.9%)	92 (37.7%)	84 (44.9%)	198/503	
T						
T_{1+2}	11 (29.7%)	9 (25.7%)	70 (28.7%)	51 (27.3%)	141/503	0.974
T_{3+4}	26 (70.3%)	26 (74.3%)	174 (71.3%)	176 (72.7%)	362/503	
Lymph nodes						
Negative	23 (62.2%)	20 (57.1%)	106 (43.4%)	100 (53.5%)	249/503	**0.046**
Positive	14 (37.8%)	15 (42.9%)	138 (56.6%)	87 (46.5%)	254/503	
Organ metastases						
Negative	36 (97.3%)	34 (97.1%)	223 (91.4%)	178 (95.2%)	471/503	0.204
Positive	1 (2.7%)	1 (2.9%)	21 (8.6%)	9 (4.8%)	32/503	
Stage						
I + II	25 (67.6%)	24 (68.6%)	137 (56.1%)	108 (57.8%)	294/503	0.348
III + IV	12 (32.4%)	11 (31.4%)	107 (43.9%)	79 (42.2%)	209/503	
Histological grade in intestinal type						
I	14 (48.3%)	17 (60.7%)	87 (63.5%)	42 (60.9%)	160/263	0.505
II + III	15 (51.7%)	11 (39.3%)	50 (36.5%)	27 (39.1%)	103/263	
Laurén classification						
Intestinal	29 (78.4%)	28 (80.0%)	137 (56.1%)	69 (36.9%)	263/503	**<0.001**
Diffuse	6 (16.2%)	7 (20.0%)	101 (41.4%)	108 (57.8%)	222/503	
Mixed/not classified	2 (5.4%)	0 (0.0%)	6 (2.5%)	10 (5.3%)	18/503	
Perioperative chemotherapy						
Yes	0 (0.0%)	0 (0.0%)	13 (5.3%)	8 (4.3%)	21/503	0.247
No	37 (100.0%)	35 (100.0%)	231 (94.7%)	179 (95.7%)	482/503	
Radicality of resection						
R0	33 (89.2%)	32 (91.4%)	175 (71.7%)	132 (70.6%)	372/503	**0.008**
R1 or R2	4 (10.8%)	3 (8.6%)	69 (28.3%)	55 (29.4%)	131/503	

Statistically significant values are shown in bold type. Other* = mixed, not classified; aberr: aberrant; EBV = Epstein–Barr virus; MMR = mismatch repair; MSI = microsatellite-instability; MSS = microsatellite-stable; neg = negative; pos = positive; wt = wild-type.

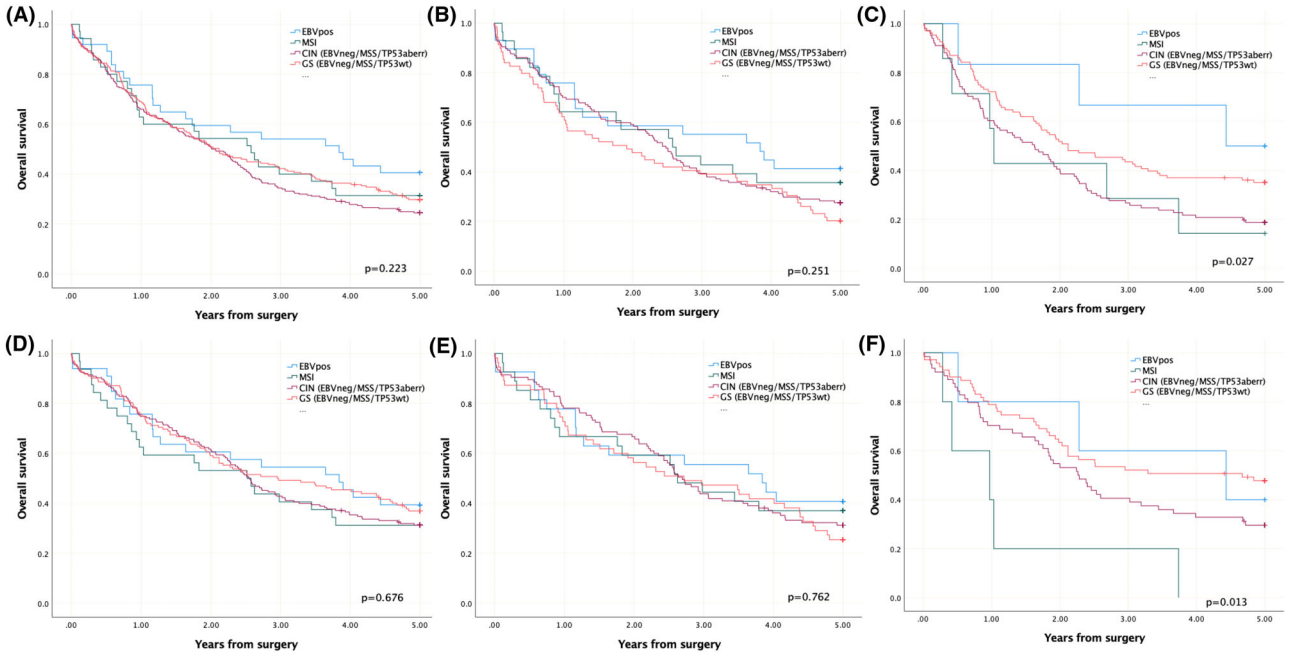

Figure 3. The Kaplan–Meier figures presenting 5-year survival stratified by molecular subgroups in gastric adenocarcinoma. **A,** All patients, **B,** intestinal-type histology subgroup, **C,** diffuse-type histology subgroup, **D,** all patients with radically resected tumours, **E,** intestinal-type tumours with radical resection and **F,** diffuse-type tumours with radical resection.

but not as accurate as genetic testing, for determination of MSI,[21] further complicated by the use of different probes and antibodies, tissue preparation and evaluation criteria.[12] Contrary to most previous studies having used only one marker, usually MLH1, to define MSI subtype, all four MSI markers were examined for the present study. The reliability of TP53 status evaluation using IHC has been criticised, as TP53 cannot identify all CIN tumours.[9,15,22] Some misclassification may occur, as some tumours may harbour alterations not evaluated by the markers used. Lastly, due to the classification method, some tumours possessed more than one feature: none were EBV-positive/MSI/TP53 aberrant, four were EBV-positive/TP53 aberrant and eight were MSI/TP53 aberrant. However, a sensitivity analysis excluding these patients showed results similar to the main analysis.

Previous molecular profiling studies based on IHC and ISH suggest that the EBV-positive subtype is associated with younger age, male sex, intestinal histology and better prognosis, while MSI and TP53 aberrant tumours are more common in females and older patients. Patients with MSI tumours also have better prognosis than patients with MSS tumours.[11–17] However, only some of the previous studies have included comparisons between all the four subtypes, and others have considered each variable as either positive or negative (e.g. MSI versus MSS and EBV+ versus EBV−), which makes comparisons between studies more difficult. Some studies have included E-cadherin as a classification marker. However, the current results are in line with previous studies, as well as TCGA and ACRG studies. We observed a proportion of 7.4% EBV-positive tumours (versus 3.3–10.4% in previous studies) and 7.0% MSI tumours (versus 4.8–20.9% in previous studies). In our study, EBV-positive tumours were all MSS and mainly TP53 wt, as EBV positivity and MSI status were mutually exclusive.

The 5-year survival was notably worse among patients with diffuse MSI tumours (14.3%) compared to patients with intestinal MSI tumours (35.7%). Also, in the subgroup of R0-resected diffuse tumours, the 5-year survival of patients with MSI tumours was 0%. Among patients with intestinal-type tumours, those with an MSI subtype had a slightly but not significantly better prognosis. However, only one previous study has included a subgroup analysis by histological subtype, but in that study none of the diffuse tumours had an MSI phenotype.[11] Although the sample size of this study was relatively large, the statistical power in the subgroup analysis was low. It has been suggested that MSI tumours are easily detected by the immune system, leading to tumour-suppressive immune responses.[20] In diffuse gastric cancer, however, these antitumoural

Table 3. Univariable and multivariable analysis of EBV, MSI, CIN and GS status and 5-year survival in 503 patients with gastric adenocarcinoma

	No. of patients	EBV-pos HR (95% CI)	MSI HR (95% CI)	CIN (EBV-neg, MSS, TP53 aberr) HR (95% CI)	GS (EBV-neg, MSS, TP53 wt) HR (95% CI)
5-year survival					
All patients (crude)	503	0.75 (0.47–1.17)	0.97 (0.63–1.50)	1.14 (0.91–1.43)	1.00 (Ref)
All patients (adjusted)*	503	0.77 (0.48–1.23)	1.07 (0.68–1.67)	1.11 (0.88–1.41)	1.00 (Ref)
R0 resected patients (crude)	372	0.95 (0.58–1.54)	1.23 (0.77–1.97)	1.14 (0.86–1.50)	1.00 (Ref)
R0 resected patients (adjusted)*§	372	0.83 (0.50–1.37)	1.32 (0.81–2.16)	1.12 (0.84–1.49)	1.00 (Ref)
Subgroup analysis					
Intestinal type (crude)	263	0.60 (0.35–1.04)	0.71 (0.42–1.21)	0.84 (0.60–1.16)	1.00 (Ref)
Intestinal type (adjusted)†	263	**0.57 (0.33–0.99)**	0.84 (0.49–1.45)	0.76 (0.54–1.07)	1.00 (Ref)
R0 resected patients intestinal type (crude)	214	0.74 (0.42–1.32)	0.89 (0.48–1.48)	0.89 (0.61–1.31)	1.00 (Ref)
R0 resected patients intestinal type (adjusted)†§	214	0.65 (0.36–1.19)	0.96 (0.54–1.71)	0.92 (0.63–1.36)	1.00 (Ref)
Diffuse type (crude)	222	0.59 (0.19–1.88)	1.63 (0.71–3.75)	**1.53 (1.11–2.11)**	1.00 (Ref)
Diffuse type (adjusted)‡	222	0.54 (0.17–1.75)	1.13 (0.48–2.65)	**1.57 (1.14–2.18)**	1.00 (Ref)
R0 resected patients diffuse type (crude)	145	1.06 (0.33–3.45)	**3.92 (1.53–10.05)**	**1.55 (1.00–2.40)**	1.00 (Ref)
R0 resected patients diffuse type (adjusted)‡§	145	0.75 (0.22–2.49)	**3.26 (1.20–8.82)**	1.43 (0.90–2.28)	1.00 (Ref)

Statistically significant values are shown in bold type. CIN = chromosomal instability; aberr = aberrant; EBV = Epstein–Barr virus; GS = genomically stable; MSI = microsatellite-instability; MSS = microsatellite-stable; neg = negative; pos = positive; Ref = Reference; R0 = radically resected; wt = wild-type.

*Adjusted for year of diagnosis, age, sex, tumour stage, Laurén classification, perioperative chemotherapy and radical resection.

†Adjusted for year of diagnosis, age, sex, tumour stage, tumour grade, perioperative chemotherapy and radical resection.

‡Adjusted for year of diagnosis, age, sex, tumour stage, perioperative chemotherapy and radical resection.

§Not adjusted for radical resection.

mechanisms may not be sufficient. Therefore, larger studies based on the histological subtype of the tumours are required.

The majority of CIN tumours have mutations in the TP53 tumour suppressor gene. For example, 71% of the CIN tumours had a TP53 mutation in the TGCA study.[3] In the present study, the detection of TP53 immunohistochemistry was used to determine CIN subtype. CIN tumours were associated with intestinal subtype and lymph node metastasis. Although the GS subtype was associated with diffuse-type histology and negative lymph node status, the CIN subtype in diffuse adenocarcinomas was associated with poorer prognosis compared to GS subtype.

The current analyses suggest that intestinal- and diffuse-type tumours both exhibit all four molecular subtypes, and that there may be differential prognostic effects between molecular subtypes in intestinal- and diffuse-type GC. A previous study has suggested several major molecular differences between intestinal- and diffuse-type cancers,[23] some of which could explain these differential prognostic effects. Compared to previous similar studies, the classification in this study was based on Laurén histological subtypes[24] but, due to relatively low power in some subgroups, larger studies are required in the future.

Conclusion

The molecular subtypes of gastric cancer based on ISH and IHC are associated with histological type and other clinicopathological variables in gastric cancer,

but not with prognosis. As the prognostic effects of molecular subtypes in intestinal and diffuse cancers may differ, combining histological and molecular information is recommended for future studies.

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Conflicts of interest

The authors have no potential conflicts of interest to declare.

Data availability statement

Data are available upon reasonable request from the corresponding author.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Summary of ISH and IHC data for 503 gastric cancer patients.

Table S2. Relationship between EBV, MMR and TP53 status.