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ORIGINAL ARTICLE – CANCER RESEARCH

# Molecular subtyping of gastric cancer with respect to the growth pattern of lymph-node metastases

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## Abstract

**Purpose** Gastric cancer is the third leading cause of cancer-related death. Recently, innovative diagnostic and prognostic molecular subtypes have been proposed. We revealed that extranodal extension (ENE) of lymph-node metastases independently influences survival. Therefore, the aim of the present study was to evaluate novel molecular subtyping with regard to the growth pattern of lymph-node metastases.

**Methods** A total of 189 gastric carcinomas with lymph-node metastases were analyzed. The expression of p53, SOX2, SOX9, and the mismatch-repair gene products MLH1, PMS2, MSH2, and MSH6 were analyzed by immunohistochemistry. To determine the correlation with EBV infection, in situ hybridization for EBV-encoded small RNA (EBER) was applied.

**Results** ENE was present in 36% of patients. EBV-positive carcinoma was evident in 5.8%, and p53 aberrant (chromosomal instable) tumors in 22.2%, a gastric cancer with deficient mismatch-repair status in 9%, and MSS/p53neg/EBVneg tumors were seen in 63% of patients. There was no significant correlation between the presence or absence of ENE and the molecular subtypes. However, a significant association between molecular subgroups and the Lauren classification, the oncogene SOX2, and tumor grading was detected.

**Conclusion** The present findings suggest that alterations in gastric cancer leading to ENE are not associated with alterations underpinning the molecular subgroups. Nonetheless, molecular subtyping on the basis of IHC and ISH is feasible and might become clinical routine. Thus, further studies are needed to clarify the mechanisms of extranodal extension in gastric cancer.

**Keywords** Gastric cancer · Molecular subtyping · Extranodal extension · Immunohistochemistry · Prognosis

## Abbreviations

| | |
|---|---|
| 95% CI | 95% Confidence intervals |
| ACRG | Asian Cancer Research Group |
| CIN | Chromosomal instability |
| dMMR | Deficient mismatch repair |
| pMMR | Proficient mismatch repair |
| EBER | EBV-encoded small RNA |
| EBV | Epstein–Barr virus |
| ENE | Extranodal extension |
| FFPE | Formalin-fixed and paraffin-embedded |
| GC | Gastric cancer |
| ING | Intranodal growth |
| IHC | Immunohistochemistry |
| ISH | In situ hybridization |
| MSI | Microsatellite instability |
| TCGA | The Cancer Genome Atlas |
| TMA | Tissue microarray |
| WHO | World health organization |

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## Purpose

Gastric cancer (GC) remains still a major health problem and is the third leading cause of cancer-related death (Ferlay et al. 2015). In Western countries, GC is often diagnosed at an advanced stage with overall survival rates of 30–40% (Cunningham et al. 2006; Noh et al. 2014), whereas the incidence in East Asia is higher (Jemal et al. 2011). Treatment of GC patients involves multimodal treatment strategies with perioperative (radio-) chemotherapy and surgery. For decades, GC has been classified according to Lauren on the basis of microscopic appearance into diffuse, intestinal, and mixed type tumors (Lauren 1965). In addition, the World Health Organization (WHO) groups GC into morphologic subtypes [papillary, tubular, mucinous (colloid), and poorly cohesive carcinomas] (Hu et al. 2012). However, morphological-based classification systems imply that each stomach cancer has common pathogenic properties and responses similarly to treatment (Fakhri and Lim 2017).

In this respect, in the last decade, efforts have been made to improve these classification systems. Therefore, the underlying genetic and epigenetic alterations should be identified, enabling a tailor-made therapeutic approach. Thus, the Research Network of The Cancer Genome Atlas (TCGA) and the Asian Cancer Research Group (ACRG) addressed the heterogeneity of GC and introduced four subtypes of GC that have distinct molecular features (Cancer Genome Atlas Research 2014; Cristescu et al. 2015). Although this approach helps pave the way to a tailor-made treatment, the methodology used in these key reference studies is complex and expensive. Thus, studies have recently been published successfully validating the molecular subtypes by immunohistochemistry (IHC) and in situ hybridization (ISH) (Ahn et al. 2017; Birkman et al. 2018; Gonzalez et al. 2016; Setia et al. 2016). In the latter studies, the tumors were divided into molecular subgroups based on the presence of Epstein–Barr virus (EBV), p53 aberrations (as a surrogate marker for chromosomal instable subtype), and microsatellite instability (MSI). The genomically stable subtype was defined as EBV-negative, regular p53 signature, and microsatellite stable (MSS) (MSS/p53neg/EBVneg) (Cancer Genome Atlas Research 2014).

In addition, our working group demonstrated that extranodal extension (ENE) of lymph-node metastases, defined by the extension of neoplastic cells beyond the lymph-node capsule, independently influences overall survival in GC (ENE 16.21 months vs. ING 37.07 months) (Link et al. 2018), which has also been shown for colorectal cancer (Veronese et al. 2016), squamous cell carcinoma of the head and neck region, and of the vulva (Luchini et al. 2016). Moreover, we evaluated distinct stem cell phenotypes and revealed an association of SOX2 with the lymph-node growth pattern (Link et al. 2018).

Therefore, the aim of the present study was to evaluate and analyze the prognostic relevance of the recently introduced molecular subtypes in a well-characterized patient cohort of lymph-node-positive GC.

## Materials and methods

### Tissue selection

Formalin-fixed and paraffin-embedded (FFPE) samples of gastric cancers from 189 patients who underwent surgical tumor resection with standardized D2 lymph-node dissection at the Department of General, Visceral, and Transplant Surgery, Ludwig-Maximilians-University Munich between 2003 and 2014 were obtained from the archives of the Institute of Pathology. In the present manuscript, the 8th edition of the TNM Classification of Malignant Tumours was used. The corresponding clinicopathological data sets were retrieved from the databases of the Department of General, Visceral, and Transplant Surgery and the Institute of Pathology. Tumors were classified using the Lauren classification into intestinal, diffuse, and mixed type tumors (Lauren 1965). Cases were either categorized as ENE or intranodal growth (ING) if at least one metastatic lymph node showed ENE as previously described (Link et al. 2018).

### Immunohistochemistry and in situ hybridization

Immunohistochemical staining was performed using 5 µm serial tissue sections of representative FFPE tumor samples. A detailed description of the antibodies and immunohistochemical protocols used in this study are provided in Table 1. IHC for the mismatch-repair products MLH1, MSH2, MSH6, and PMS2 as well as for p53 were performed on a Ventana Benchmark XT autostainer (Ventana Medical Systems, OroValley, AZ) following the manufacturer's instructions.

For SOX9-specific IHC, first heat-induced epitope retrieval was performed by applying ProTaqs IV Antigen-Enhancer (Quartett, Germany) before adding a primary SOX9-specific antibody for 60 min at room temperature. Thereafter, a development step was introduced by adding a detection system and subsequently substrate-chromogen containing a DAB+ system (DAKO, Germany) according to the manufacturer's protocols.

For SOX2-specific IHC, heat-induced epitope retrieval was performed by applying Epitope Retrieval Solution (Novocastra, Germany) followed by adding a primary SOX2-specific antibody for 60 min at room temperature. The

**Table 1** Antibodies and subsequent detection systems used for this study

| Antibody | Company | Source | Clone | Dilution | Detection system |
|---|---|---|---|---|---|
| MLH1 | Leica Biosystems, Newcastle upon Tyne, UK | Mouse monoclonal | ES05 | 1:200 | Ventana OptiView DAB IHC Detection Kit |
| MSH2 | Cell Marque, Rocklin, CA | Mouse monoclonal | G219-1129 | Ready-to-use | Ventana OptiView DAB IHC Detection Kit |
| MSH6 | Ventana Medical Systems, Oro Valley, AZ | Mouse monoclonal | SP93 | Ready-to-use | Ventana OptiView DAB IHC Detection Kit |
| PMS2 | Ventana Medical Systems, Oro Valley, AZ | Rabbit monoclonal | EPR3947 | Ready-to-use | Ventana OptiView DAB IHC Detection Kit |
| SOX2 | Cell Signalling Technology, Danvers, MA | Rabbit monoclonal | D6D9 | 1:50 | AEC Solution, Invitrogen, Carlsbad, CA |
| SOX9 | Cell Signalling Technology, Danvers, MA | Rabbit monoclonal | Clone D8G8H | 1:100 | Vectastain ABC-Kit Elite Universal, Biozol, Germany |
| P53 | Thermo Fisher Scientific, Waltham, MA | Mouse monoclonal | D07 | 1:800 | Ventana OptiView DAB IHC Detection Kit |

development step was accomplished by adding the detection system (ImmPRESS Reagent Kit anti-rabbit Ig, Vector, Germany) and substrate-chromogen containing DAB+ system (DAKO, Germany). Finally, all slides were counterstained using hematoxylin (Vector Laboratories, Burlingame, CA). To control for nonspecific binding of antibodies, isotype controls were included (data not shown).

For EBV, the samples were pretreated for 28 min with ISH Protease and then incubated for an hour, followed by the application of a ready-to-use EBER (EBV-encoded small RNA) probe (Ventana/Roche) and signal detection with an ISH iVIEW Blue Detection Kit.

To exclude nonspecific reactions of antibodies and/or reagents, isotype and system controls were performed for all staining procedures.

### Scoring of immunohistochemistry and in situ hybridization

Loss of MLH1, PMS2, MSH2, and MSH6 expression (reflecting deficient mismatch-repair status/dMMR), a surrogate marker for MSI, was recorded when nuclear staining was absent in malignant cells by positive background staining including the stromal cells or normal epithelial cells. Cases with preserved nuclear MLH1, PMS2, MSH2, and MSH6 expression in tumor cells were classified as cases with proficient mismatch-repair status (pMMR) (Fig. 1a–f). A strong diffuse nuclear reaction for EBER was assessed as positive (Fig. 1g, h). The aberrant expression of p53 was defined as strong diffuse nuclear staining in > 90% of tumor cells or complete absence of p53 expression in all tumor cells (Fig. 1i, j). Nuclear staining of SOX9 and SOX2 was reported as both the percentage and intensity of stained nuclei throughout the tumor. Intensity values were categorized as follows: no staining—0, weak—1, moderate—2, and strong—3. The staining of SOX9 was evaluated as follows: when less than 30% of the tumor cells were stained and/or the intensity was missing (0) or weak (1), immunostaining was reported to be negative. When less than 10% of tumor cells were stained for SOX2 and/or the intensity was missing (0) or weak (1), immunostaining was reported to be negative (Fig. 1k–n).

Two blinded observers (FB and RT) independently evaluated all IHC markers. Discrepant cases were discussed with an experienced histopathologist (JN), and a consensus was reached.

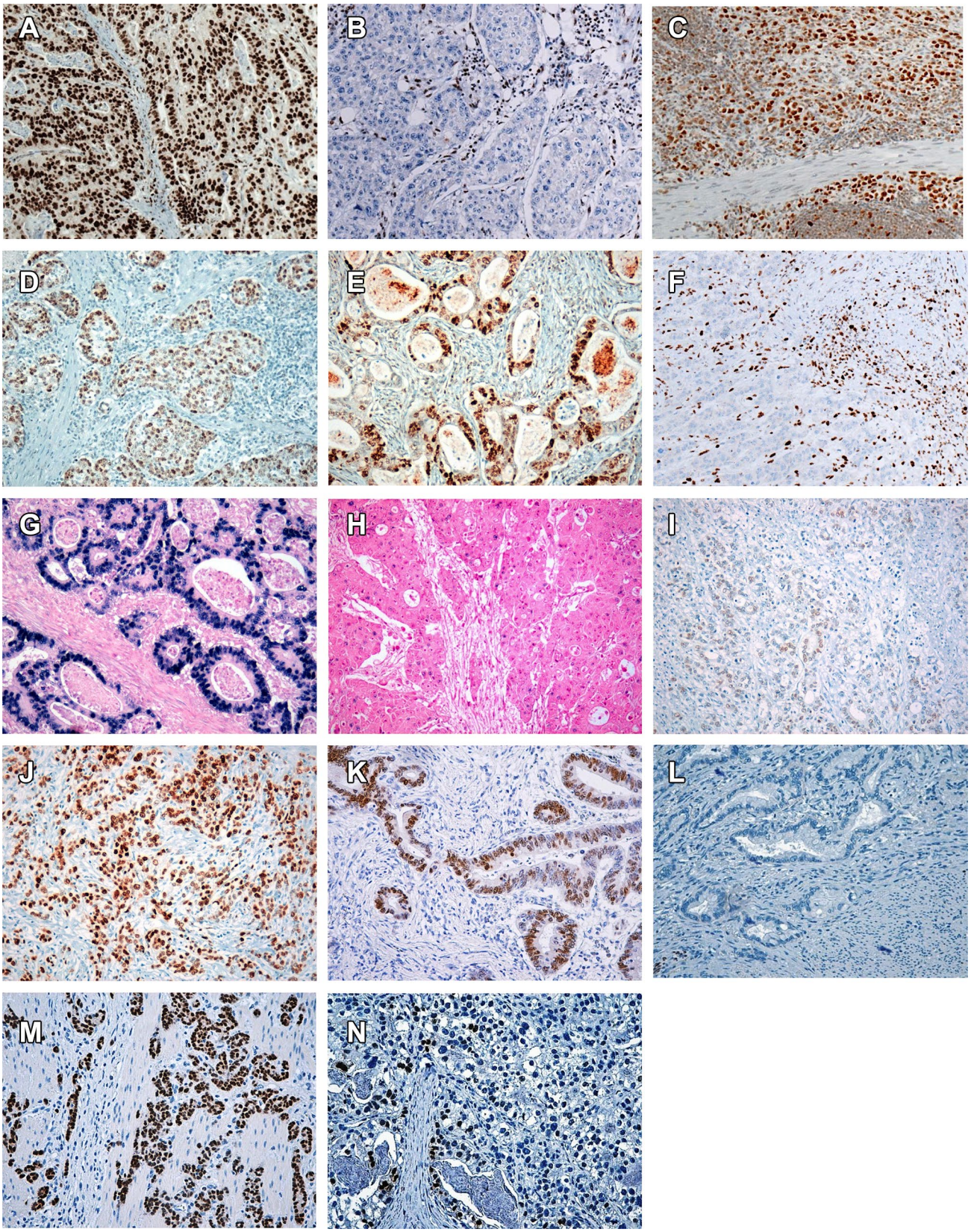
### Statistics

The significance of the correlations of the immunohistochemical analyses was tested using a two-sided Chi-square test (SPSS v. 25.0, IBM Inc., Armonk, NY). For all tests, a $p$ value less than 5% ($p < 0.05$) was considered statistically significant.

## Results

### Clinicopathological characteristics

In total, 189 patients were included, of whom all underwent resection of their primary tumor. Of these 189 patients, 36% ($n = 68$) had ENE, and 64% ($n = 121$) showed an ING pattern of the lymph-node metastases. The median age of the analyzed cohort was 67 years (range 21-91 years). A statistically significant correlation of the growth pattern of lymph-node metastases was observed with primary tumor stage (T) and lymphangiosis (L). Of the patients with GC leading to ENE, 61.2% ($n = 41/67$) had advanced primary tumors (pT3/4), whereas 39.7% ($n = 48/121$) of patients with ING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◂**Fig. 1** Immunohistochemical and in situ hybridization analysis in gastric cancer specimens (**a** hMLH1 positive; **b** hMLH1 negative; **c** hMSH2 positive; **d** hMSH6 positive; **e** hPMS2 positive; **f** hPMS2 negative: **g** EBER positive; **h** EBER negative; **i** regulated p53 expression pattern; **j** deregulated strong positive p53 expression pattern; **k** SOX2 positive; **l** SOX2 negative; **m** SOX9 positive; **n** SOX9 negative)

($p < 0.05$) had advanced primary tumors. Information about lymphangiosis was complete for the entire cohort and was detected significantly more often in primary tumors leading to ENE (82.3%) than in tumors with ING (67%) ($p < 0.05$).

Furthermore, in the analyzed cohort, there were 5.8% ($n = 11$) GC EBV-positive tumors, 9% ($n = 17$) dMMR status tumors, 22.2% ($n = 42$) p53 dysregulated primary tumors, and 63% ($n = 119$) MSS/p53neg/EBVneg tumors. The clinicopathological characteristics of the analyzed cohort are displayed in Table 2. Molecular subtyping showed no significant correlation with the growth pattern of the lymph-node metastases. However, the molecular subtyping correlated significantly with the Lauren classification, the tumor grading, and the oncogene SOX2 ($p < 0.05$).

Moreover, there were further observations unique to the analyzed cohort. EBV-positive tumors exhibited intestinal differentiation in 81.8% of cases and were associated with ING in 72.7% of cases. p53 aberrant primary tumors were more often seen in male patients (71%), commonly poorly differentiated (G3: 78.6%), and had a high probability of lymphangiosis (69%). ENE and ING were almost equally distributed in this group (ING: $n = 23$; 54.8% vs. ENE: $n = 19$; 45.2%). Tumors with dMMR status more often showed intestinal differentiation (82.4%), lymphangiosis (76.5%), and poor differentiation (G3: 88.2%); these tumors were predominantly negative for SOX2 (88.2%). MSS/p53neg/EBVneg primary tumors constituted the main subgroup (63%) and were associated with ING in 66.4% and with lymphangiosis in 74% of patients. These tumors were poorly differentiated in 86.6% of patients.

### Survival analyses

Our previous analyses revealed that ENE is an independent risk factor for overall survival (Link et al. 2018). In this respect, patients with ENE had a mean overall survival of 15.5 months [95% CI 10.4; 20.5 months] compared to 38.1 months [95% CI 30.5; 45.6 months] in patients with ING patterns ($p < 0.05$). The survival analysis with respect to the molecular subtypes provided no further distinctive feature ($p > 0.05$). Nonetheless, dMMR tumors had the best prognosis with a mean overall survival of 39.3 months [95% CI: 19.9; 58.7 months] followed by MSS/p53neg/EBVneg tumors with a mean overall survival of 32.2 months [95% CI 24.7; 39.7 months] and p53 aberrant tumors with a mean overall survival of 24.1 months [95% CI 15.2; 33.1 months]. The worst prognosis was observed for EBV-associated carcinomas with a mean overall survival of 18.6 months [95% CI: 6.1; 31 months] (Fig. 2).

## Discussion

Traditionally, GC is classified according to morphological growth patterns (Lauren 1965). Treatment follows the one-size-fits-all approach disregarding differences in cancer development and the underlying molecular alterations (Rokutan et al. 2018). The novel approach of molecular subtyping of stomach cancer in part addresses this unsolved issue. Furthermore, ENE has recently been demonstrated to be an independent risk factor for overall survival in GC (Alakus et al. 2010; Lee et al. 2014; Link et al. 2018). However, whether stomach cancer leading to ENE represents an independent subtype of GC remains ill-defined. Therefore, the aim of the present study was to analyze a well-characterized cohort of lymph-node-positive GCs according to novel molecular subtyping.

The findings of the present study suggest that there is no direct correlation between the novel diagnostic approach of molecular subtyping and the growth pattern of lymph-node metastases. Thus, ENE represents an independent risk factor not associated with the evaluated molecular classification. Since the lymph-node growth pattern can be determined only postoperatively, it would be of great value to detect a tool to estimate the probability of ENE preoperatively. Furthermore, the present findings suggest that the genetic and epigenetic alterations of tumors leading to ENE are different from those analyzed in the present manuscript.

The molecular subtyping of our cohort revealed that 5.8% of patients had an EBV-associated tumor, which is in accordance with previously published data (Cancer Genome Atlas Research 2014; Setia et al. 2016). The frequency of tumors with dMMR/MSI was 9% in our cohort, which is lower than that observed by Setia et al. (2016) (16%) and the ACRG (22.7%) (Cristescu et al. 2015), but consistent with the frequency observed by Ahn et al. (2017) (6.9%) and Birkman et al. (2018) (10.2%). This difference might in part be explained by the fact that lymph-node metastases are less frequently observed in dMMR/MSI tumors (Cristescu et al. 2015; Setia et al. 2016). The frequency of p53 aberrant tumors and MSS/p53neg/EBVneg tumors is comparable to that reported by Gonzalez et al. (Gonzalez et al. 2016), but diverges from the TCGA (Cancer Genome Atlas Research 2014). The TCGA used high-throughput technologies, which might be more accurate, and did not exclusively analyze lymph-node-positive patients. A direct comparison to the other IHC studies does not seem appropriate, since they investigated five groups (Ahn et al. 2017; Setia et al. 2016).

**Table 2** Clinicopathological characteristics of the analyzed cohort

| Variable | Total $n=189$ (100%) | EBV positive $n=11$ (5.8%) | p53 aberrant $n=42$ (22.2%) | dMMR/MSI $n=17$ (9%) | MSS/p53neg/EBVneg $n=119$ (63%) | $p$ value |
|---|---|---|---|---|---|---|
| Median age | | | | | | n.s. |
| < Median age | 93 (49.2%) | 7 (63.5%) | 21 (50%) | 6 (35.2%) | 59 (49.6%) | |
| > Median age | 96 50.8%) | 4 (36.5%) | 21 (50%) | 11 (64.8%) | 60 (50.4%) | |
| Gender | | | | | | n.s. |
| Female | 70 (37%) | 4 (36.5%) | 12 (28.6%) | 8 (47%) | 46 (38.7%) | |
| Male | 119 (63%) | 7 (63.5%) | 30 (71.4%) | 9 (53%) | 73 (61.3%) | |
| Lauren classification | | | | | | 0.012 |
| Intestinal | 102 (54%) | 9 (81.8%) | 27 (64.3%) | 14 (82.3%) | 52 (43.7%) | |
| Diffuse | 65 (34.4%) | 1 (9.1%) | 12 (28.6%) | 2 (11.8%) | 50 (42%) | |
| Mixed type | 22 (11.6%) | 1 (9.1%) | 3 (7.1%) | 1 (5.9%) | 17 (14.3%) | |
| Growth pattern of lymph nodes | | | | | | n.s. |
| ING | 121 (64%) | 8 (72.7%) | 23 (54.8%) | 11 (64.8%) | 79 (66.4%) | |
| ENE | 68 (36%) | 3 (27.3%) | 19 (45.2%) | 6 (35.2%) | 40 (33.6%) | |
| Tumor grade (WHO) | | | | | | 0.013 |
| G1 | 2 (1.1%) | 0 | 1 (2.4%) | 0 | 1 (0.8%) | |
| G2 | 28 (14.8%) | 6 (54.5%) | 7 (16.6%) | 2 (11.8%) | 13 (10.9%) | |
| G3 | 156 (82.5%) | 5 (45.5%) | 33 (78.6%) | 15 (88.2%) | 103 (86.6%) | |
| Gx | 3 (1.6%) | 0 | 1 (2.4%) | 0 | 2 (1.7%) | |
| T category | | | | | | n.s. |
| T1 | 2 (1.1%) | 0 | 1 (2.4%) | 0 | 1 (0.8%) | |
| T2 | 95 (50.3%) | 6 (54.5%) | 22 (52.4%) | 8 (47%) | 59 (49.6%) | |
| T3 | 57 (30.2%) | 2 (18.2%) | 13 (31%) | 7 (41.2%) | 35 (29.4%) | |
| T4 | 32 (16.9%) | 3 (27.3%) | 4 (9.5%) | 2 (11.8%) | 23 (19.4%) | |
| Tx | 2 (1.1%) | 0 | 2 (4.7%) | 0 | 1 (0.8%) | |
| N category | | | | | | n.s. |
| N0 | 0 (0%) | 0 | 0 | 0 | 0 | |
| N1 | 84 (44%) | 6 (54.5%) | 20 (47.6%) | 11 (64.8%) | 47 (39.5%) | |
| N2 | 55 (29%) | 3 (27.3%) | 12 (28.6%) | 3 (17.6%) | 37 (31.1%) | |
| N3 | 50 (27%) | 2 (18.2%) | 10 (23.8%) | 3 (17.6%) | 35 (29.4%) | |
| L category | | | | | | n.s. |
| L0 | 52 (27.5%) | 4 (36.5%) | 13 (31%) | 4 (23.5%) | 31 (26.1%) | |
| L1 | 137 (72.5%) | 7 (63.5%) | 29 (69%) | 13 (76.5%) | 88 (73.9%) | |
| SOX2 | | | | | | 0.018 |
| SOX2 neg. | 116 (61.4%) | 5 (45.5%) | 20 (47.6%) | 15 (88.2%) | 76 (63.9%) | |
| SOX2 pos. | 73 (38.6%) | 6 (54.5%) | 22 (52.4%) | 2 (11.8%) | 43 (36.1%) | |
| SOX9 | | | | | | n.s. |
| Negative | 1 (0.5%) | 0 | 0 | 0 | 1 (0.8%) | |
| Weak pos. | 12 (6.4%) | 2 (18.2%) | 3 (7.1%) | 1 (5.9%) | 6 (5.1%) | |
| Moderate pos. | 133 (70.3%) | 7 (63.5%) | 30 (71.4%) | 16 (94.1%) | 80 (67.2%) | |
| Strong pos. | 43 (22.8%) | 2 (18.2%) | 9 (21.5%) | 0 | 32 (26.9%) | |

*ENE* extranodal extension, *EBV* Epstein–Barr virus, *ING* intranodal growth, *L* lymphangiosis, *dMMR/MSI* microsatellite instability, *T* tumor stage

Moreover, we present unique results due to analyzing only lymph-node-positive patients. Previously, we demonstrated that ENE is associated with SOX9 but not with SOX2 (Link et al. 2018). The stem cell marker SOX2 seems to act as an oncogene, induce chemoresistance (Tian et al. 2012), and blocking of SOX2 was found to decrease the tumorigenicity of gastric cancer cell lines (Hutz et al. 2014; Matsuoka et al. 2012). In the present study, the expression of SOX2 was inversely correlated with dMMR/MSI tumors. Therefore, the suppressed SOX2 expression (11.8%) of dMMR/

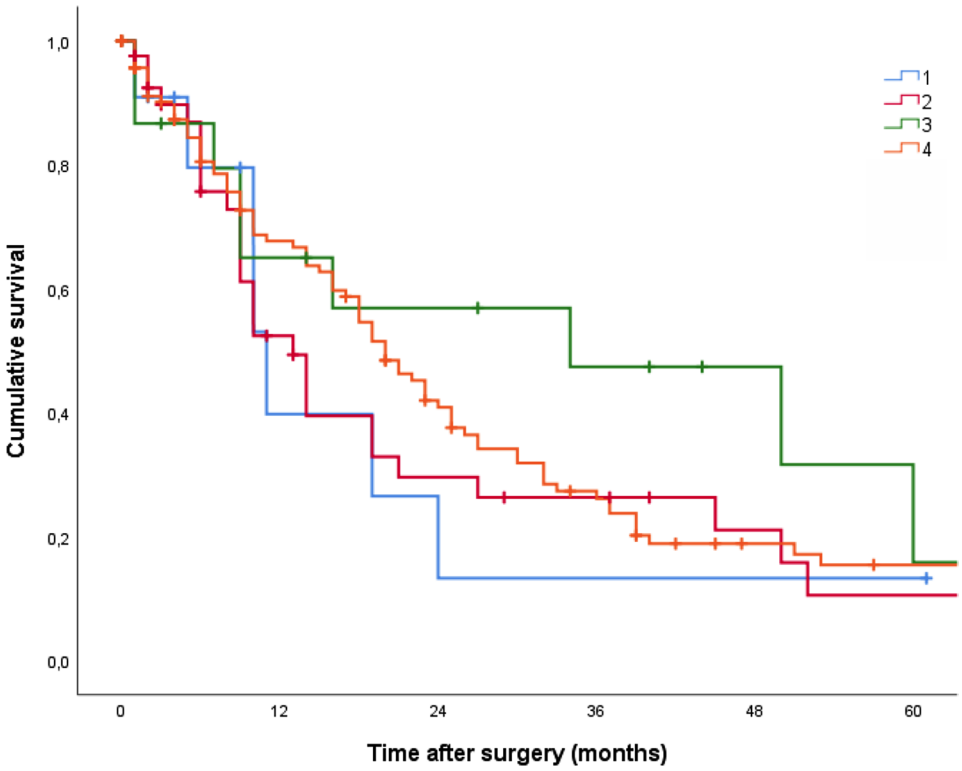


**Fig. 2** Mean calculated overall survival stratified by molecular subtyping. (1: EBV—Epstein–Barr virus; 2: p53 aberrant; 3: dMMR/MSI—microsatellite instability; 4: MSS/p53neg/EBVneg)

MSI tumors may in part contribute to the favorable outcome of this subgroup. In contrast, patients with aberrant p53 or EBV-associated tumors, who had the worst survival rates, and SOX2 positivity were 52.4% and 54.5%, respectively. In accordance with published data, we saw a higher rate of intestinal differentiated tumors in the EBV-positive or microsatellite-unstable tumor subtypes (Ahn et al. 2017). Surprisingly, aberrant p53, dMMR/MSI, and GS tumors are all associated with an increased G3 probability.

In addition, the Lauren classification showed a significant correlation with the molecular subtypes, which has also been seen in previously published studies and is controversial (Ahn et al. 2017; Birkman et al. 2018; Gonzalez et al. 2016; Setia et al. 2016). We detected an intestinal-type tumor in more than 80% of EBV-positive and microsatellite-unstable tumors, which implies that there are tumors of diffuse type with EBV positivity and dMMR/MSI status. These findings are in accordance with most published results (Ahn et al. 2017; Cancer Genome Atlas Research 2014; Cristescu et al. 2015; Kim et al. 2016), but are in contrast to those of Brinkman et al. (2018). Other working groups (Setia et al. 2016) have shown a strong correlation between genetically stable tumors and the intestinal type, which is not supported by our findings.

Although a carefully selected patient cohort was analyzed, patients with EBV-associated carcinomas had reduced survival rates. dMMR/MSI carcinomas have the best prognosis, which is consistent with previously published studies (Birkman et al. 2018; Cancer Genome Atlas Research 2014; Cristescu et al. 2015). In the present manuscript, molecular subtyping provided no significant prognostic relevance, although a trend was clear.

In the present manuscript, GC was classified according to the work published by the TCGA (Cancer Genome Atlas Research 2014), which was recently tested by IHC (Birkman et al. 2018). Subsequently, these results were validated in a larger cohort by the ACRG by gene expression analyses (Cristescu et al. 2015). The TCGA and ACRG published an innovative classification system using high-throughput technologies, which are expensive and complex. However, this sophisticated methodological approach might not find its way into the routine processing of surgical specimens within the coming years. However, it was recently demonstrated that molecular subtyping of GC is feasible with IHC and ISH (Ahn et al. 2017; Birkman et al. 2018; Gonzalez et al. 2016; Setia et al. 2016). These results were obtained utilizing tissue microarrays (TMAs) (Ahn et al. 2017; Birkman et al. 2018; Setia et al. 2016). Although the use of TMAs has been validated in various studies (Ahn et al. 2017; Bosch et al. 2019; Setia et al. 2016), their utilization might contain an element of uncertainty. Thus, in the present manuscript, only whole tumor block sections were used. Furthermore, patients were included irrespective of preoperative treatment, which is consistent with Birkman et al. (2018), who used the same diagnostic approach with IHC and ISH. However, this issue is in conflict with the TCGA and ACRG (Cancer Genome

Atlas Research 2014; Cristescu et al. 2015). Preoperative treatment might have influenced the results, but the frequency of preoperative treatment was evenly distributed throughout the analyzed cohort. Therefore, a significant impact seems unlikely. The analysis of only lymph-node-positive patients might represent a limitation to our study. However, the aim of the present study was to evaluate the correlation between ENE- and ING-inducing tumors and molecular subtypes. The exact molecular alterations in GC might not be represented by IHC and ISH. However, this methodological approach has been successfully proven recently (Ahn et al. 2017; Birkman et al. 2018; Gonzalez et al. 2016; Setia et al. 2016).

## Conclusions

The novel diagnostic approach of molecular subtyping is not associated with GC leading to ENE, but is associated with the Lauren classification and the oncogene SOX2. Nonetheless, molecular subtyping on the basis of IHC and ISH is feasible and might become clinically routine. The independent prognostic factor ENE still cannot be estimated preoperatively. Thus, the findings of the present study need to be validated in a larger cohort of GC patients.

### Compliance with ethical standards

**Conflict of interest** The authors wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

**Ethical approval** The study was carried out according to the recommendations of the local ethics committee of the Medical Faculty of the Ludwig-Maximilians-University Munich, Germany. This retrospective study was performed with irreversibly anonymized data sets and tissue specimens. Thus, ethical approval or written consent was not required. The study was performed according to the standards laid out in the declaration of Helsinki 1975. All researchers were blinded to the patient data during the experimental analysis.

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