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From Cell Biology to Tissue Engineering

Expression of cyclooxygenase-2 has no impact on survival in adenocarcinoma of the esophagogastric junction but is associated with favourable clinicopathologic features

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Summary. Background. COX-2 expression induces carcinogenesis and is thought to be an adverse prognostic factor in gastric carcinomas while the prognostic value of DNA mismatch repair (MMR) is still controversial. Concerning adenocarcinomas of the esophagogastric junction, no comprehensive data regarding either factors are available as of yet.

Objective. We assessed expression of COX-2, MLH1 and MSH2 in adenocarcinoma of the esophagogastric junction in relation to patients' survival and various clinicopathologic features.

Design. Immunohistochemical studies (using antibodies against COX-2, MLH1 and MSH2) were performed in a study population of 228 tumours. Follow-up data was available for all patients with a mean follow-up time of 42.8 months.

Results. 78 (34.2%) tumours were COX-2 negative, 148 (64.9%) showed COX-2 positivity. Assessment of COX-2 expression and clinicopathologic features revealed an inverse correlation with depth of tumour invasion and number of metastatic lymph nodes (p=0,021 and p=0,004, respectively). No correlation with other features could be demonstrated. 62 cases (27.2%) showed loss of DNA repair enzymes MLH1 and/or MSH2. MMR differed significantly between COX-2 positive and negative cases (p=0,028). Kaplan-Meier survival analyses revealed no impact on patients' survival for COX-2 expression or MMR status (p=0.837 and p=0.972, respectively).

Conclusions. Expression of COX-2 in adenocarcinomas of the esophagogastric junction seems to have no prognostic effect or impact on patients' survival but is associated with favourable clinico-pathologic factors. MMR deficiency was more frequent in COX-2 negative tumours, but MMR status had no impact on survival and patients' outcome whatsoever.

Key words: Cyclooxygenase-2, COX-2, Adenocarcinoma of the esophagogastric junction, DNA mismatch repair

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Introduction

Recently, cyclooxygenase-2 (COX-2) - which converts arachidonic acid into prostaglandins and acts proinflammatory (Brown and DuBois, 2005) - has been shown to induce carcinogenesis (including angiogenesis, metastasis and immunosuppression) as well as tumour progression through induction of prostaglandin biosynthesis (Harris, 2007). Overexpression may open up additional therapeutic and possibly preventive options for patients through application of selective COX-2 inhibitors (Bertagnolli et al., 2006; Flossmann et al., 2007). COX-2 overexpression has been shown to be a frequent event in tumours of the lower gastrointestinal tract (especially colorectal cancer) and seems to be of prognostic significance (Zhang and Sun, 2002;

Soumaoro et al., 2004; Brown and DuBois, 2005). Overexpression has also been demonstrated in carcinomas of the upper gastrointestinal tract (Thiel et al., 2011). However, the prognostic value in gastric carcinomas is still controversial (Wang et al., 2015). Meta-analyses suggest COX-2 to be an adverse prognostic factor but convincing evidence is missing (Song et al., 2014). Concerning adenocarcinomas of the esophagogastric junction (AEG), no comprehensive studies addressing this topic have been reported.

Inactivation of DNA mismatch repair genes which causes genomic instabilities in different forms of cancer (i.e. colorectal carcinomas (Heinimann, 2013)) has recently been extensively discussed in relation to gastric carcinomas but meta-analyses show that the prognostic value of microsatellite instability is still open to debate (Choi et al., 2014), while other studies demonstrated a correlation with favourable clinicopathologic features such as lower tumour stage, fewer metastatic lymph nodes and intestinal type (Lee et al., 2002; Beghelli et al., 2006; Corso et al., 2009; Marrelli et al., 2016). Data regarding MMR deficiency in carcinomas of the esophagogastric junction are scarce and restricted to few studies with small numbers of patients (Yanagi et al., 2000; Theuer et al., 2002).

Currently, expression of COX-2 in adenocarcinomas of the upper gastrointestinal tract, especially in relation to patients' survival has not been comprehensively examined. In our study, we aimed to assess the effect of COX-2 expression on overall and event-free survival, taking into account tumour stage as well as other clinicopathologic characteristics like MMR status, TNM stage and nodal status.

Materials and methods

Case selection

228 tumour samples (formalin-fixed, paraffin-embedded tissue (FFPE) containing adenocarcinomas of

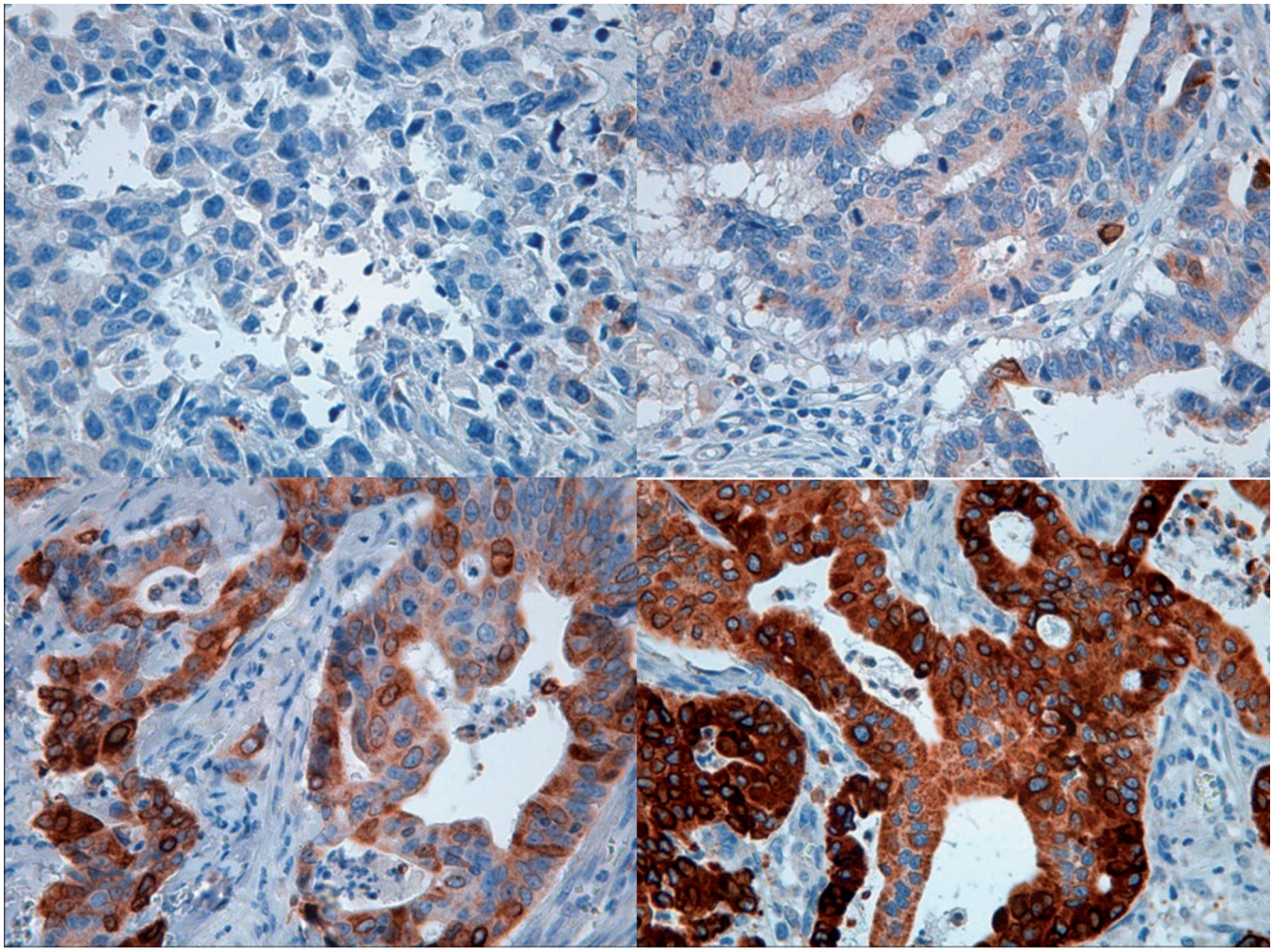

Fig. 1. Immunohistochemical expression of COX-2 in AEG tumors. Upper left: no staining (score 0); upper right: weak staining (score 1+); lower left: moderate staining (score 2+); lower right: strong staining (score 3+).

the esophagogastric junction, defined as showing their epicentre within 2 cm either side of the junction) were collected as part of standard clinical care at the University Hospital Schleswig-Holstein, Campus Luebeck during 1992-2014. The study was approved by the local Ethical Committee of the University of Luebeck. Follow-up data was available for all patients, mean follow-up period was 42.8 months (range 0-227 months).

Tissue microarray construction

Tissue microarrays (TMAs) were constructed using the manual QuickRay® kit (Unitma, Seoul, Korea) in accordance with the manufacturer's instructions. One representative core per tumour sample measuring 2 mm was dissected and mounted onto a tissue microarray. Appropriate controls were included in each slide (kidney and tonsil tissue).

Immunohistochemistry

Immunohistochemical studies were performed on FFPE sections of the TMA according to a standard, three-step immunoperoxidase technique using the automated Menarini Bond Max System (Menarini Diagnostics, Germany). The following antibodies were used: COX-2 (Dako, Glostrup, Denmark, dilution 1:100), MLH1 (Cell Marque, clone G168-728, dilution 1:50) and MSH2 (Cell Marque, Rocklin, California, USA, clone G219-1129, dilution 1:100). All immuno-histochemical stains were evaluated by two pathologists (JK, KR).

Table 1. Clinico-pathologic characteristics of adenocarcinoma of the esophagogastric junction according to COX-2 expression.

Characteristic	All cases*	COX-2 positive*	COX-2 negative*	p-value
Total n	228	148 (64.9)	78 (34.2)	
Gender				
Male	192 (84.2)	125 (84.5)	66 (84.6)	0.976
Female	36 (15.8)	23 (15.5)	12 (15.4)	
Mean age ± SD	61.82 ± 10.59			
Neoadjuvant therapy				
Yes	89 (39.0)	55 (37.2)	34 (43.6)	0.437
No	139 (61.0)	93 (62.8)	44 (56.4)	
pT				
(low) pT0	5 (2.2)	4 (2.7)	1 (1.3)	0.021
pT1a	6 (2.6)	4 (2.7)	0 (0)	
pT1b	31 (13.5)	23 (15.5)	7 (8.9)	
pT2	42 (18.4)	28 (18.9)	14 (17.9)	
(high) pT3	120 (52.6)	78 (52.7)	42 (53.8)	
pT4a	17 (7.5)	8 (5.4)	9 (11.5)	
pT4b	7 (3.1)	2 (1.4)	5 (6.4)	
pN				
pN0	88 (38.6)	66 (44.6)	20 (25.6)	0.004
pN1	39 (17.1)	24 (16.2)	13 (16.7)	
pN2	49 (21.5)	31 (20.9)	18 (23.1)	
pN3	52 (22.8)	27 (18.2)	25 (32.1)	
Grade				
G1	2 (0.9)	1 (0.7)	0 (0)	0.148
G2	51 (22.4)	36 (24.3)	14 (17.9)	
G3	102 (43.8)	67 (45.3)	35 (44.9)	
No grading	73 (32.4)	44 (29.7)	29 (37.2)	
Distant metastases				
Present	50 (21.9)	30 (20.3)	20 (25.6)	0.699
Absent	92 (40.4)	63 (42.6)	27 (34.6)	
Unknown	86 (37.7)	55 (37.2)	31 (39.7)	
WHO classification				
Tubular	168 (73.6)	112 (75.7)	54 (69.2)	0.488
Poorly cohesive	26 (11.4)	16 (10.8)	10 (12.8)	
Mucinous	10 (4.4)	6 (4.1)	4 (5.1)	
Papillary	2 (0.9)	1 (0.7)	1 (1.3)	
Undifferentiated	6 (2.6)	3 (2.0)	3 (3.8)	
Other	16 (7.0)	10 (6.7)	6 (7.7)	
MSI				
MMRp	163 (71.5)	114 (77.0)	48 (61.5)	0.028
MMRd	62 (27.2)	34 (23.0)	28 (35.9)	
Not evaluable	3 (1.3)		2 (2.6)	

*Numbers in parentheses show the proportion of tumours with a specific clinical or immunohistochemical feature in a given COX-2 subtype. p-values with statistical significance are typed in bold letters.

Immunohistochemistry for COX-2

COX-2 expression was evaluated as staining intensity in tumour cells and scored as either absent (0), weak (1+), moderate (2+) or strong (3+). Examples of COX-2 staining are shown in Fig. 1. Surrounding as well as tumour-infiltrating inflammatory cells served as positive controls (Ogino et al., 2006). In accordance with previous studies, moderate or strong COX-2 expression was determined as being COX-2 positive, while in cases of weak or absent staining tumours were classified as COX-2 negative (Ogino et al., 2008). All cases were evaluated as a consensus score by two pathologists (JK and KR) who were blinded to clinical outcomes and survival times.

MMR status

MMR status was assessed on Tissue Microarrays using two immunohistochemical markers (mismatch repair proteins MLH1 and MSH2). MMR deficiency (MMRd) was defined as loss of expression of one or both markers while MMR proficiency (MMRp) was defined as preserved expression of both markers.

Statistical analysis

Statistical analysis was conducted using SPSS Statistics version 22. Survival differences and outcome as well as overall survival were analysed with Kaplan-Meier estimates, including Log rank-test, while correlation of COX-2 expression with different clinicopathologic features was determined with χ^2- and Fisher-exact-test as well as Pearson's bivariate correlation. A p-value <0.05 was considered statistically significant.

Results

COX-2 expression in adenocarcinomas of the esophagogastric junction

Of 228 analysed cases, 226 were evaluable for COX-2 expression. 78 cases were COX-2 negative (34.2%), 148 showed moderate to strong COX-2 expression, thus being evaluated as COX-2 positive (64.9%). 2 cases showed not enough tumour cells for reliable determination (0.9%). Assessment of COX-2 expression and various clinicopathologic features (as

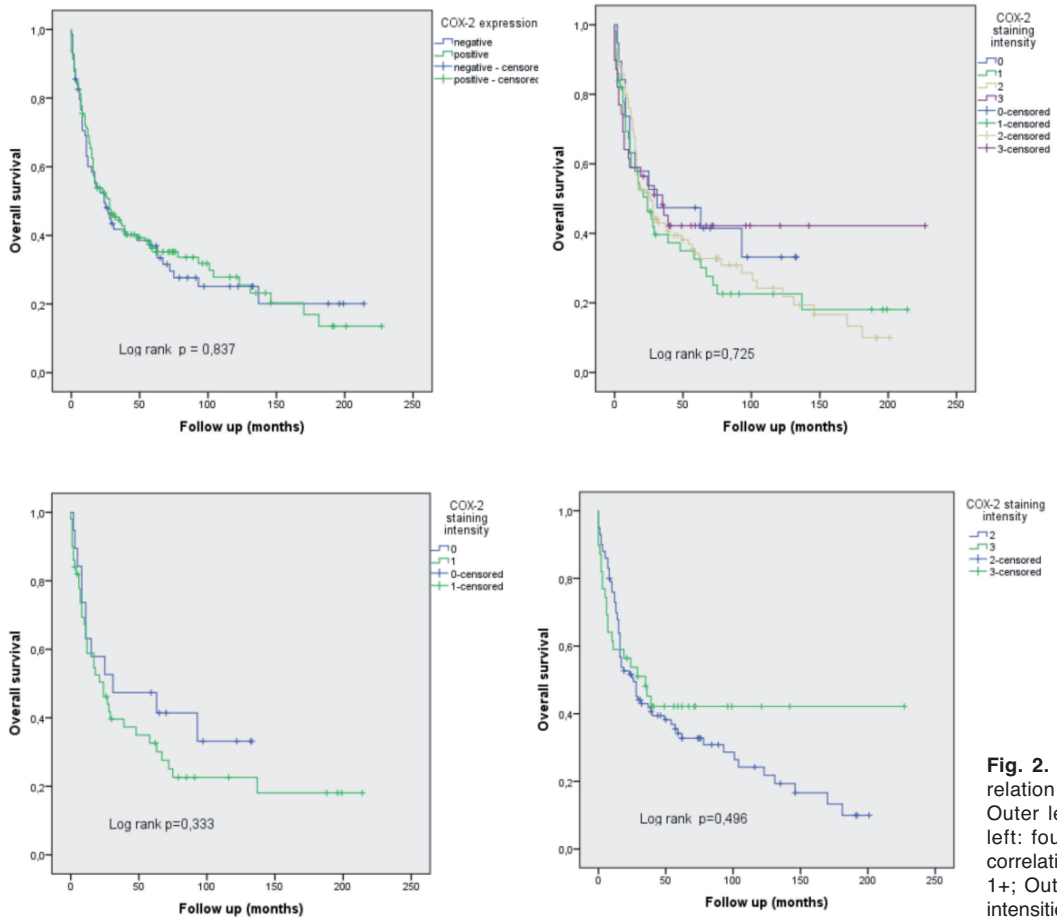

Fig. 2. Kaplan-Meier survival curve in relation to tumoral COX-2 expression. Outer left: Two-tier system; 2nd from left: four-tier system; 2nd from right: correlation of staining intensities 0 and 1+; Outer right: correlation of staining intensities 2+ and 3+.

shown in Table 1) revealed an inverse correlation between COX-2 expression and tumour stage (depth of invasion) as well as number of metastatic lymph nodes (p=0.021 and p=0.004, respectively). No correlation between COX-2 expression and histologic tumour type, tumour grade or presence of distant metastases could be demonstrated.

Applying Kaplan-Meier survival curves revealed that COX-2 overexpression did not influence patients' survival significantly (p=0.837), applying either a two-tier or four-tier system, although the graphic curves hint at a survival difference between 50-150 months follow-up. Survival times did not differ on a large scale between both groups; survival in COX-2 positive cases averaged 70.66 months, in COX-2 negative cases 67.54 months (Table 2). Further analysis showed that overall survival (OS) did not differ significantly in COX-2 positive cases when staining intensities 2+ and 3+ were compared directly (p=0.496). Paralleling of staining intensities 0 and 1+ likewise revealed no significant differences in overall survival (p=0.333). Appropriate Kaplan-Meier curves are depicted in Fig. 2.

MMR status in adenocarcinomas of the esophagogastric junction

A total of 62 cases (27.2%) showed loss of repair enzymes MLH1 and/or MSH2 (MMRd tumours) while the remaining 163 (71.5%) retained expression of both markers (MMRp tumours). 3 cases were not evaluable for analysis. MMR expression differed significantly between COX-2 positive and negative cases: MMRd could be demonstrated more often in COX-2 negative tumours (35.9% of cases vs. 23%) while COX-2 positive cases showed MMRp in a higher percentage (77% of cases vs. 61.5%; p=0.028) as shown in Table 1.

Applying Kaplan-Meier survival curves revealed that MMRd did not influence patients' survival significantly (p=0.972) as shown in Fig. 3. Survival times did not differ considerably between MMRd-and MMRp-groups; survival in MMRd tumours averaged 69.97 months, in MMRp tumours 64.99 months (Table 2).

Discussion

Incidence of adenocarcinomas of the esophagogastric junction has increased in incidence over the past years, in contrast to the decline which has been noticed with regard to carcinomas of the distal stomach (Bollschweiler et al., 2001). Our aim was to comprehensively examine adenocarcinomas of the esophagogastric junction with regard to outcome differences in relation to COX-2 expression and MMR status and correlation with various clinicopathologic parameters.

Cyclooxygenase-2 is a known promoter of carcinogenesis, angiogenesis, metastasis and tumour progression and overexpression has been shown to be of significance in colorectal carcinomas, with advanced stages and higher cancer-specific mortality, thus acting as a predictor of poor prognosis (Ogino et al., 2008). Data concerning carcinomas of the upper gastrointestinal tract are more scarce, some studies suggesting that overexpression in gastric carcinomas likewise leads to poorer survival but the results are still controversial (Thiel et al., 2011; Song et al., 2014). COX-2 overexpression might open up new therapeutic possibilities. In fact, it has been shown that application of selective COX-inhibitors is able to prevent colorectal adenomas from recurring as well as decreasing the risk for colorectal cancer (Arber et al., 2006; Chan et al., 2007). For gastric carcinomas, similar effects (i.e. prevention of gastric cancer and induction of apoptosis) have been shown in cell lines and mice (Futagami et al., 2008; Sun et al., 2008). In our study, we were able to demonstrate that the majority of carcinomas of the esophagogastric junction (approximately 2/3) show moderate to strong immunohistochemical COX-2 expression, making further therapeutic options potentially accessible to these patients. However, it remains to be studied whether patients benefit from the application of selective COX-2 inhibitors or if possible adverse side effects (cardiovascular, renal) might limit its use (Bhosale et al., 2015).

The percentage of COX-2 positive tumours in the literature varies, but several studies could demonstrate

Table 2. COX-2 expression and MMR status in relation to survival among patients.

	Total (n)	average survival$^{\Phi}$	standard deviation	95% CI*	p-value
COX-2 neg.	78	67.54	10.61	46.74-88.34	0.837
COX-2 pos.	148	70.66	8.02	54.94-86.37	
MMRp	163	64.99	10.56	44.3-85.68	0.972
MMRd	62	69.82	7.86	54.57-85.38	

*CI: confidence interval; $^{\Phi}$average survival time given in months.

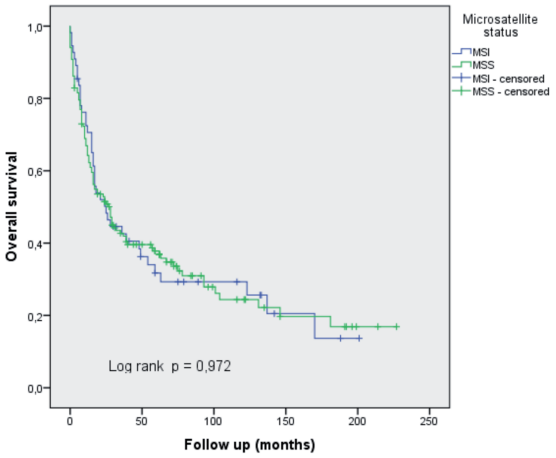

Fig. 3. Kaplan-Meier survival curve in relation to MMR status in tumors.

COX-2 expression in colorectal carcinoma and gastric carcinoma in approximately 70% of cases (Yamamoto et al., 1999; Zhang and Sun, 2002; Soumaoro et al., 2004). These findings are closely in line with our results, which show COX-2 overexpression in 64.9% of cases (Table 1). Other studies analysing breast cancer, non-small cell lung cancer, bladder cancer and various other entities also showed COX-2 overexpression in high proportions (>50%), establishing COX-2 expression as a common finding in many epithelial tumours, not being an exclusive feature of gastrointestinal carcinomas and hinting at a role in early carcinogenesis (Tabriz et al., 2013; Misron et al., 2015; Shimizu et al., 2015).

In our study COX-2 expression had no impact on patients' overall survival (OS) in AEG tumours, using either a two-tier or four-tier system (Fig. 2). OS was similar in COX-2 positive tumours (70.66±8.02 months) and COX-2 negative tumours (67.54±10.61 months). This is in contrast to previously published data regarding colorectal carcinoma where high expression marked advanced tumour stages and a poor prognosis (Ogino et al., 2008). To what extent these findings prove to be valid and reproducible remains to be clarified in further studies.

Regardless of OS, COX-2 expression was associated with favorable clinicopathologic features such as fewer lymph node metastases and lower pT-stage (Table 1). Why these parameters show no impact on overall survival in our study remains unclear, but it seems possible that other factors such as histologic tumor type, presence or absence of distant metastases and MMR status antagonize and outshine its effect.

In accordance with previous studies regarding gastric carcinomas we also demonstrated that COX-2 expression is less frequently associated with MMRd, suggesting that different underlying mechanisms (potentially in key regulating genes) might lead to different pathways of carcinogenesis in COX-2 positive and -negative tumors (Yamamoto et al., 1999).

MMRd itself was shown in 27.2% of all cases, regardless of COX-2 expression. This is in line with data concerning gastric carcinomas where microsatellite instability was demonstrated in 15-30% of cases (Velho et al., 2014). Again, no differences in OS were observed between both groups (MMRd and MMRp) as shown in Fig. 3 (p=0.972). Previously published data on gastric carcinomas reported improved survival in patients with MMRd compared to MMRp tumors (Velho et al., 2014) as well as association with favorable clinicopathologic factors (such as intestinal type and occurrence in the distal stomach), independent of disease stage (Beghelli et al., 2006).

As a limitation of our study, it should be mentioned that patients were recruited over a long period of time (1992-2014) and the study population therefore naturally contains differences in therapeutic and surgical procedures as well as chemotherapy protocols which might account for certain variations in survival times.

In conclusion, our data suggests that COX-2 expression and MMR status do not have substantial influence on patients' survival. However, COX-2 expression seems to be associated with favorable clinicopathologic features. In addition, MMRd was shown more frequently in COX-2 negative cases, which underlines previously published data, according to which COX-2 indicates poor prognosis and microsatellite instability indicates a favorable outcome.

Author contributions. JK and KR performed morphological and immunohistochemical studies, analyzed the data and wrote the manuscript. TH contributed cases and performed morphological studies. EP and UW contributed cases and clinical data and revised the manuscript. CT designed the research, performed morphological studies and revised the manuscript. All authors read and approved the final version of the manuscript.

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