Original article:

Histological assessment of stromal maturity as a prognostic factor in surgically treated gastric adenocarcinoma

Short title: Stromal maturity and prognosis in GC

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Abstract

Background: Histological assessment of stromal maturity is a potential prognostic factor in colorectal cancer, but its applicability in gastric adenocarcinoma is completely unknown. The aim of this study was to evaluate the feasibility and prognostic significance of assessing stromal maturity in gastric adenocarcinoma.

Methods: This study was conducted retrospectively in a cohort of 583 gastric adenocarcinoma patients treated surgically in Oulu University Hospital, Finland, between the years 1983-2016. The original diagnostic slides were used for assessment of stromal maturity. Patients were divided into mature stroma and immature stroma groups, and stromal maturity was analysed in relation to five-year and overall survival. Primary outcome of the study was five-year survival, and secondary outcome was overall survival.

Results: The Kappa-coefficient for interobserver agreement was 0.609. Patients with immature stroma had worse five-year survival compared to patients with mature stroma (adjusted HR 1.32, 95% CI 1.06-1.64). Stromal maturity was significantly associated with five-year survival in intestinal type subgroup (adjusted HR 1.63, 95% CI 1.20-2.21), but not in diffuse type subgroup (adjusted HR 1.21, 95% CI 0.87-1.70).

Conclusions: Stromal maturity is an independent prognostic factor in gastric adenocarcinoma, and it can be analysed with moderate reproducibility.

Keywords: Gastric cancer, Prognosis, Stroma, Collagen

Introduction

Despite falling incidence during the last decades, gastric cancer remains as the fifth most common cancer worldwide.[1] The treatment of gastric cancer is challenging and shadowed by poor prognosis.[2] Tumour-node-metastasis (TNM) classification is used estimate the prognosis of gastric cancer patients. However, patients with tumours of similar TNM stage can have very different outcomes.[3]

Stromal tissue and stromal fibroblasts participate in tumour growth.[4] In colorectal cancer, classifying tumours based on the type of desmoplastic stroma has prognostic significance[5-7] Less mature desmoplasia with myxoid stroma or thick, eosinophilic collagen fibres is associated to worse prognosis, compared to a desmoplastic reaction with mature, thin collagen fibers.[5, 6] The analysis can be performed on routine haematoxylin-eosin (HE) slides.[5, 6]

The prognostic relevance of stromal maturity based on analysing HE-stained slides is unknown in gastric cancer. Previously, a Chinese immunohistochemistry study utilizing second harmonic generation imaging has shown that gastric cancers containing thick, immature collagen fibres have poor prognosis.[8] The aim of present study was to evaluate the feasibility of stromal maturity assessment based on HE-stained slides and its prognostic value in gastric cancer.

Materials and methods

Study design

This study was a single-institution retrospective cohort study in Oulu University Hospital during 1983-2016.[9, 10] Briefly, 601 patients underwent gastrectomy for gastric adenocarcinoma with diagnostic HE-stained slides available for 583 of the patients. The Oulu University Hospital Ethics Committee approved the study (15.2.2016 §51) and the Finnish National Authority for Medicolegal Affairs (VALVIRA) waived the need to obtain informed consent from the study patients.

Data collection

Identification of the patients was done by electronically searching Oulu University Hospital pathology and administrative records. Patient records, operation charts and pathology reports were used for data retrieval. The immutable national personal numbers assigned to each Finnish resident were used to combine the 100% complete follow-up data from the Causes of Death Registry at the Statistics Finland to the study database. Follow-up data was available until the end of 2016.

After retrieval and review of the original, prospectively collected haematoxylin-eosin diagnostic glass slides used for clinical decision-making, multiple HE-stained sections from each patient were viewed with light microscope. A representative section with deepest invasion was used for further analysis. Sections were digitized using Aperio AT2 (Leica Biosystems, Wetzlar, Germany).

Exposure (Stromal maturity)

Stromal maturity was analysed from scanned HE-stained slides using Aperio ImageScope independently by two researchers (N.K. and M.E.) blinded to the clinical and outcome data.

Maturity of tumour-associated stroma was analysed from the intratumoral stroma of the invasive tumour and the desmoplastic reaction in front of the invasive edge of the tumour. The studies on colorectal cancer have focused on the stroma in the desmoplastic area in front of the invasive edge of the tumor.[5, 6] In our material there were a lot of tumours without assessable desmoplastic reaction, especially T4-tumors growing to serosal adipose tissue. Assessing stroma from the whole tumour area was therefore considered to be more accurate. Immature stroma was defined as presence of thick, hypocellular collagen bundles with eosinophilic hyalinization previously described as keloid-like collagen.[7] The area with most immature stroma was considered decisive. If there were no keloid-like collagen bundles or if they were present only in under 5% of the intratumoral stromal area and desmoplastic stromal area in front of tumour combined, the stroma was considered mature. Previous studies in colorectal cancer have used a three-tiered categorization classifying tumours with myxoid stroma as immature, and tumours that do not contain myxoid stroma but contain keloid-like collagen as intermediate.[5, 6] In our material there were only a handful of tumours that contained myxoid stroma for a three-tiered categorization, and cases with myxoid stroma or keloid-like collagen were both classified as immature. The assessment of stromal maturity was solely based on presence or absence of keloid-like collagen or myxoid stroma, and no other properties of stroma, like fibroblast composition and stromal inflammation were considered.

The slides on which the researchers disagreed were reassessed together and consensus was reached. The few controversial cases were reassessed with an expert gastrointestinal pathologist (V-M.P).

Outcomes

Primary outcome of the study was 5-year survival, defined as death for any cause during the time between date of surgery and death of patient during 5 years or the end of 5-year follow up.

Secondary outcome of the study was overall survival, defined as death for any cause during the time between date of surgery and death of patient or the end of follow up.

Statistical analysis

The study was conducted according to an *a priori* analysis plan. Interobserver agreement was assessed by calculating Cohen's kappa. Categorical variables were compared using χ^2-test, while T-test was used for continuous variables. Survival curves were compared with Kaplan-Meier method and log rank test. Cox regression provided hazard ratios (HR) with 95% confidence intervals (CI). Where indicated, Cox regression was adjusted for potential confounding variables: 1) year of surgery (<2000 or ≥2000), 2) age at diagnosis (continuous variable), 3) sex (male or female), 4) administration of perioperative chemotherapy (yes or no), 5) tumour stage (stage I-II or stage III-IV), 6) Laurén classification (intestinal, diffuse or mixed) and 7) radical resection (R_0 or $R_{1/2}$). Subgroup analyses were performed in Laurén intestinal, and diffuse type gastric adenocarcinomas separately, adjusted for other confounders listed above. For subgroup analysis of the intestinal type subgroup, an additional confounder for histological grade (I-II, or III) was used. A post-hoc survival

analysis in tumour stage II patients was done after obtaining the primary results. In the post-hoc

analysis, the confounders specified above were used. All analyses were done with IBM SPSS

Statistics 24.0 (IBM corp., Armonk, NY).

Results

Patients

There were 583 surgically treated gastric adenocarcinoma patients in the study. Median age was 69 years and 353 (60.4%) of the patients were male and 231 (39.6%) female. Perioperative chemotherapy was given to 22 (3.8%) patients. Microscopically confirmed R_0 resection was achieved for 437 (75.0%) patients, while 146 (25.0%) had $R_{1/2}$ resection. These patients with unradical resection included some patients with palliative intent, including 34 (5.8%) patients that had distant metastases at the time of surgery. Median follow-up time was 26 months (range 0-396 months) and it was complete for all patients.

Assessment of stromal maturity

The Cohen's kappa value for interobserver agreement for the first analysis was 0.609. Reassessment was needed for 108 (18.5%) of the slides. The main reason for reassessment was large number of borderline tumours, for which it turned out to be difficult to define precisely if the collagen bundles fulfilled the criteria of being considered as keloid-like. Assessment was especially challenging when the stromal collagen fibres were thick but not clearly arranged in bundles, in which case the stroma was considered mature in the reassessment. The stroma of cases without clear desmoplastic reaction was classified as mature, as keloid-like collagen was only seen in cases with desmoplastic reaction. Examples of mature and immature stroma are shown in Figure 1.

Of the patients 360 had mature stroma, while 223 patients had immature stroma. Immature stroma was associated with younger age at diagnosis, higher tumour stage, diffuse type histology and unradical resection (Table 1).

Primary outcome: 5-year survival

During 5-year period after surgery 387 (66.4%) of the 583 patients died. The patients with immature stroma had significantly worse 5-year survival (21.1%) than the patients with mature stroma (37.3%, log rank test p<0.001, Figure 2). The immature stroma group had significantly worse five-year survival compared to the mature stroma group in both univariate analysis (HR 1.56, 95% CI 1.28-1.91, Table 2) and multivariate analysis (HR 1.32, 95% CI 1.06-1.64, Table 2).

In a subgroup analysis of the patient group with intestinal histological type, the patients with immature stroma had significantly worse 5-year survival (18.4%) compared to patients with mature stroma (34.6%, log rank test p=0.001, Figure 2). In the univariate analysis 5-year survival was significantly worse in the immature stroma group compared to the mature stroma group (HR 1.63, 95% CI 1.20-2.21, Table 2). In the multivariate analysis, the immature stroma group also had significantly worse 5-year survival compared to the mature stroma group (HR 1.41, 95% CI 1.04-1.93).

In the subgroup of patients with diffuse type histology, the immature stroma group had significantly worse 5-year survival (21.6%) compared to the mature stroma group (40.6%, log rank test p=0.004, Figure 2). The difference in 5-year survival between the immature stroma and the mature stroma groups was significant also in univariate analysis (HR 1.54, 95% CI 1.14-2.07, Table 2). In

multivariate analysis the difference in 5-year survival was not significant (HR 1.21, 95% CI 0.87-1.70).

Secondary outcome: overall survival

The patients with immature stroma had significantly worse survival in univariate analysis compared to the patients with mature stroma (HR 1.53, 95% CI 1.27-1.84, Table 2). The difference in overall survival between the immature and mature stroma groups was significant also in multivariate analysis (HR 1.35, 95% CI 1.11-1.65).

In the intestinal type histology subgroup, the patients with immature stroma had significantly worse overall survival compared to the patients with mature stroma (HR 1.45, 95% CI 1.09-1.91, Table 2). The difference between the overall survival of the groups was not significant in multivariate analysis (HR 1.25, 95% CI 0.94-1.68.) In the diffuse type subgroup, the patients with immature stroma had significantly worse overall survival compared to patients with mature stroma in univariate (HR 1.66, 95% CI 1.26-2.19, Table 2), and multivariate analysis (HR 1.36, 95% CI 1.02-1.82).

Post-hoc analysis of outcomes in stage II patients.

Due to the strong association between stromal maturity and tumour stage, as well as strong confounding by tumour characteristics indicated by large changes in the HRs in the primary analyses, a post-hoc analysis in stage II patients (n=221) was done to examine the value of stromal maturity in early stage gastric cancer. The post-hoc analysis showed that the immature stroma group

(n=97) had worse five-year survival compared to the mature stroma group (n=124) (univariate HR 1.49, 95% CI 1.09-2.05). The difference in five-year survival was similar in multivariate analysis (HR 1.47 95% CI 1.04-2.08). The Kaplan-Meier curve is shown in Supplementary Figure 1.

Discussion

This is the first study describing the association between stromal maturation and prognosis in gastric adenocarcinoma, showing that the assessment of stromal maturity in gastric adenocarcinoma using HE-stained slides is feasible. Stromal maturity is an independent prognostic factor in gastric adenocarcinoma.

The present study has some strengths and limitations: The study was conducted in a large cohort size with good statistical power in the main analysis. No patients were lost to follow-up as Statistics Finland has 100% complete coverage of mortality data.[11] The treatment of gastric cancer has changed during the study period, but the year of surgery was taken into account in the multivariate analyses. The small number of patients treated with neoadjuvant therapy limits the applicability of the results to this patient group, as neoadjuvant therapy is known to cause stromal changes.[12]

A total of seven studies have studied the prognostic significance of stromal maturity in colorectal cancer, using a three-tier categorization.[5-7, 13-16] The HRs in multivariate for mortality or recurrence have been between 2.0 (95% CI 1.4-2.9)[14] and 5.4 (95% CI 2.9-10.4)[16] comparing immature to mature stroma, while the corresponding multivariate HRs for mortality or recurrence in intermediate stroma group compared to mature stroma group have been more modest, between 1.3 (95% CI 0.7–2.3)[6] and 2.9 (95% CI of 1.6–5.4) in colorectal cancer.[16]

The prognostic value of stromal maturity in gastric cancer seems to be more limited than in colorectal cancer based on the results of this study, as two-tiered categorization of stroma resulted in a HR of 1.32 with 95% CI of 1.06-1.64 for five-year survival in immature stroma compared to mature stroma group. Its prognostic value seems to be also smaller than collagen width analysed

with second generation harmonic imaging, for which a Chinese study suggested a HR of 2.67 with 95% CI of 1.76-4.06 for overall survival in wide collagen group compared to thin collagen group in two independent cohorts.[8] However, this study was lacking in quality of follow-up, which was not based on registries, it did not adjust for all relevant confounders and selected only patients with R0 resection in the analysis. In the present study, stromal maturity was associated with five-year survival in the intestinal type subgroup in multivariate analysis but not with overall survival, while in the diffuse type subgroup stromal maturity was associated with overall survival but not with five-year survival. Due to the moderately low HRs, the subgroup analyses might suffer from low statistical power making strong conclusions about prognostic significance of stromal maturity in different histological types of gastric cancer difficult.

Stromal maturity was also strongly associated with higher TNM stage, poor differentiation in intestinal type and $R_{1/2}$ resections. That might indicate that immature stroma is associated with more aggressive behaviour of the tumour, and when these associations are taken into account in the multivariate analyses, assessment of stromal maturity brings less additional prognostic value compared to colorectal cancer. Due to these issues, a further examination of the association between stromal maturity and survival was conducted in a post-hoc analysis of stage II patients. These results show that stromal maturation may have prognostic value in early stage gastric cancer. Due to the post-protocol nature of these analyses, the prognostic value of stromal maturation in stage II gastric cancer needs to be validated in an independent large cohort.

The mechanisms contributing to worse outcomes of patients with immature stroma are incompletely known. It is well known that fibroblasts have an important role in tumour development.[4, 17]

Fibroblasts of innate stromal tissue restrict tumour growth, but cancer-associated fibroblasts (CAFs) of desmoplastic tumour stroma support tumour progression.[4, 18] CAFs can secrete signalling molecules that directly improve tumour growth and they can secrete proteases that degrade extracellular matrix, which might improve invasion potential of tumour cells.[17] CAFs also can promote the tumour cells to undergo epithelial-mesenchymal transition, which in turn increases invasive and metastatic capabilities of tumour cells.[19] Tumours with immature stroma might have more CAFs or a more growth-supporting CAF-composition and a growth-supporting tumour microenvironment.

The biological differences between mature and immature stromal reactions are currently unknown. A strong desmoplastic reaction is especially common phenomenon in pancreatic cancer.[20] The way desmoplastic collagen fibres align has been associated with prognosis in pancreatic cancer.[21] In gastric cancer, width of desmoplastic collagen fibres has previously been associated with prognosis.[8] In colorectal cancer, changes in structure of the extracellular matrix in the desmoplastic stroma has been suggested to be a potential marker for early carcinoma.[22] Changes in collagen composition and structure of extracellular matrix might explain differences between mature and immature stroma in gastric cancer.

The results of this study merit further research. Stromal maturity might be a clinically applicable prognostic factor in gastric cancer, even though only moderate interobserver agreement was reached in this study, which might limit its usefulness. Obviously, classification of stromal response in this tumour type would benefit from better criteria, preferably based on those biological mechanisms of the response mediating the prognostic effect. Additional large, preferably nationwide retrospective

studies, and prospective studies are needed to better understand prognostic value of stromal maturity in gastric cancer. Studies in neoadjuvant treated patients and early stage gastric cancer are warranted.

In conclusion, the analysis of stromal maturity in gastric cancer is feasible with moderate reproducibility, and stromal maturity is an independent prognostic factor in gastric adenocarcinoma.

Acknowledgements

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Table 1. Associations between stromal maturity and clinicopathological variables in 583 surgically resected patients with gastric adenocarcinoma.

	Mature stroma (n=360)	Immature stroma (n=223)	P-value
Year of surgery			0.092
≥2000	148 (41.1%)	105 (47.1%)	
<2000	212 (58.9%)	118 (52.9%)	
Mean age at diagnosis	68.6	64.0	**<0.001**
Sex			
Male	227 (63.1%)	125 (56.1%)	0.056
Female	133 (36.9%)	98 (43.9%)	
Neoadjuvant chemotherapy			0.30
Yes	11 (3.1%)	11 (4.9%)	
No	349 (96.9%)	212 (95.1%)	
Tumour stage			**<0.001**
1 or 2	245 (68.1%)	114 (51.1%)	
3 or 4	115 (31.9%)	109 (48.9%)	
Laurén class			**<0.001**
Intestinal	216 (60.0%)	77 (34.5%)	
Diffuse	132 (36.7%)	138 (61.9%)	
Mixed	12 (3.3%)	8 (3.6%)	
Histological grade in intestinal type			**<0.001**
I or II	148 (68.5%)	35 (45.5%)	
III	68 (31.5%)	42 (54.5%)	
Radicality of resection			**<0.001**
R0	290 (80.6%)	147 (65.9%)	
R1 or R2	70 (19.4%)	76 (34.1%)	

Table 2. Univariable and multivariable analysis of stromal maturity's effect on prognosis in 583 patients with gastric adenocarcinoma.

	Number of patients	Mature stroma HR (95% CI)	Immature stroma HR (95% CI)
5-year survival			
All patients (Crude)	583	1.00 (Reference)	1.56 (1.28-1.91)
All patients (Adjusted)[a]	583	1.00 (Reference)	1.32 (1.06-1.64)
Subgroup analysis			
Intestinal type (Crude)	293	1.00 (Reference)	1.63 (1.20-2.21)
Intestinal type (Adjusted)[b]	293	1.00 (Reference)	1.41 (1.04-1.93)
Diffuse type (Crude)	270	1.00 (Reference)	1.54 (1.14-2.07)
Diffuse type (Adjusted)[c]	270	1.00 (Reference)	1.21 (0.87-1.70)
Overall survival			
All patients (Crude)	583	1.00 (Reference)	1.53 (1.27-1.84)
All patients (Adjusted)[a]	583	1.00 (Reference)	1.35 (1.11-1.65)
Subgroup analysis			
Intestinal type (Crude)	293	1.00 (Reference)	1.45 (1.09-1.91)
Intestinal type (Adjusted)[b]	293	1.00 (Reference)	1.25 (0.94-1.68)
Diffuse type (Crude)	270	1.00 (Reference)	1.66 (1.26-2.19)
Diffuse type (Adjusted)[c]	270	1.00 (Reference)	1.36 (1.02-1.82)

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

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

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

Figure legends

Figure 1. Examples of immature stroma in intestinal type gastric adenocarcinoma (A), immature stroma in diffuse type gastric adenocarcinoma (B), mature stroma in intestinal type gastric adenocarcinoma (C) and mature stroma in diffuse type gastric adenocarcinoma (D) at 200x total magnification
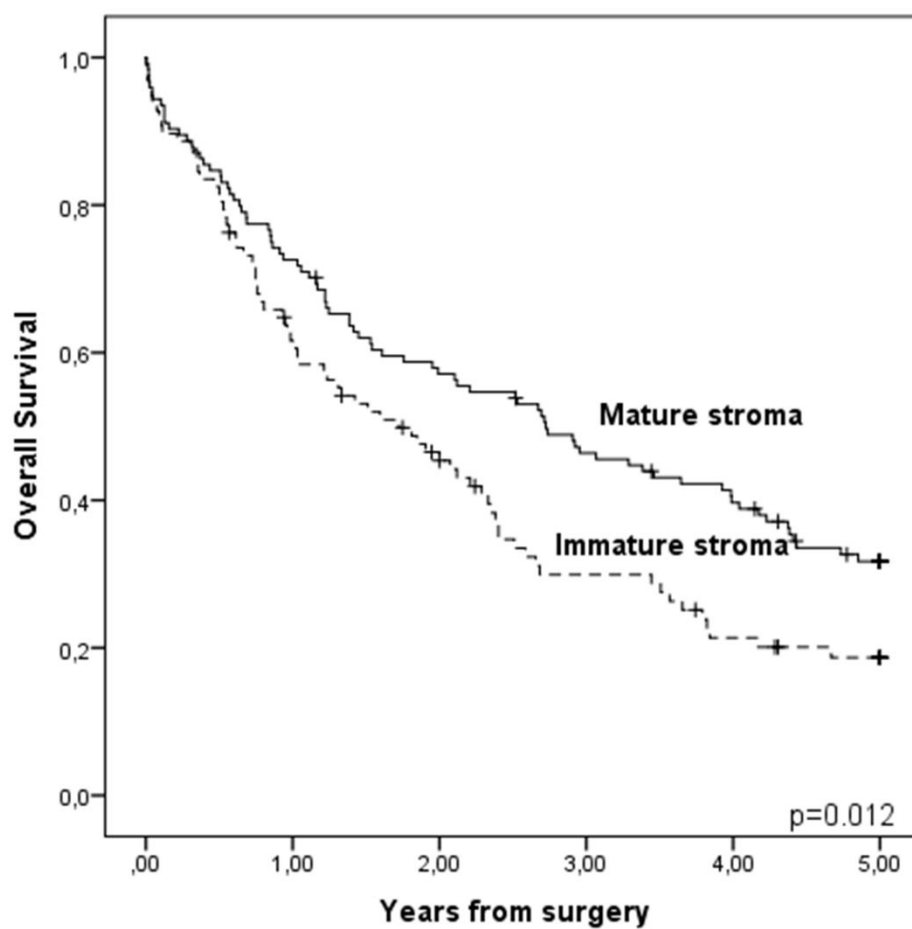
Figure 2. The Kaplan-Meier figures of five-year overall survival of patients with gastric adenocarcinoma (A), five-year overall survival of patients with intestinal type gastric adenocarcinoma (B) and five-year overall survival of patients with diffuse type gastric adenocarcinoma (C) stratified by stromal maturity.

Figures
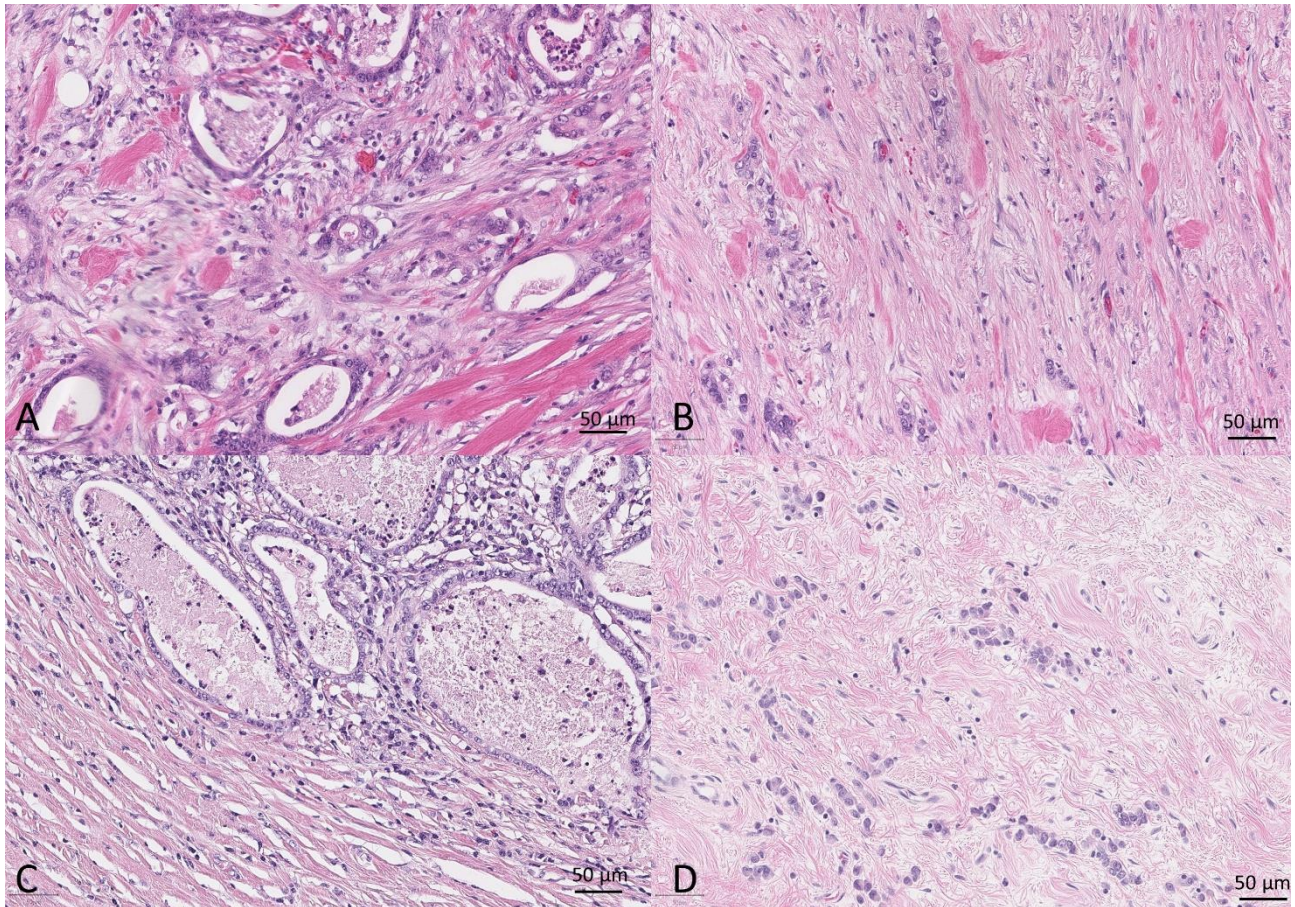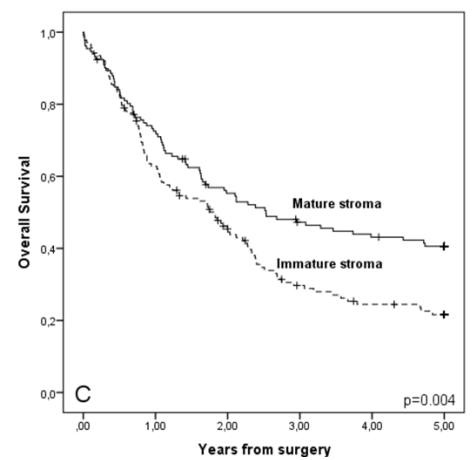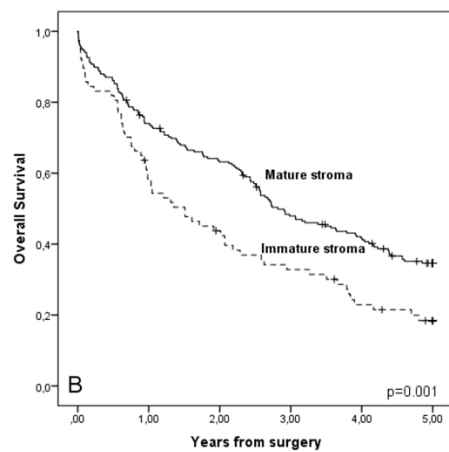
Supplementary figures

Supplementary figure 1. The Kaplan-Meier figure of five-year overall survival of patients with stage II gastric adenocarcinoma stratified by stromal maturity