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Clinicopathological characteristics of gastric adenocarcinoma with enteroblastic differentiation and gastric adenocarcinoma with enteroblastic marker expression

--Manuscript Draft--

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Abstract:	Gastric adenocarcinoma (GA) with enteroblastic differentiation (GAED) is an aggressive carcinoma histologically characterized by a glycogen-rich clear cytoplasm and fetal gut-like structures. GAED shows the expression of at least one of the following enteroblastic markers (EMs): glypican-3 (GPC3), spalt-like transcription factor 4 (SALL4), and α-fetoprotein (AFP). Despite the absence of clear cytoplasm, we often encounter GA with EMs expression (GA with EM); however, the clinicopathological characteristics of GA with EM remain unclear. Immunohistochemical (IHC) expression of three EMs (AFP, GPC3, and SALL4) was examined on tissue microarray. According to the status of the clear cytoplasm of tumor cells, GAs showing IHC expression of EMs were classified as either GAED or GA with EM, and this analysis categorized 688 GAs into 94 GAEDs (13.7%), 58 GAs with EM (8.4%), and 536 conventional GAs (CGAs). Both GAED and GA with EM showed frequent lymphovascular invasion, lymph node metastasis, and liver metastasis compared to CGA. However, a higher frequency of venous invasion, but not of lymphatic invasion, was noted for GAED in

	comparison to CGA. GAED and GA with EM showed similar overall survival. GAED had significantly poorer prognosis than CGA; however, not for GA with EM. Furthermore, GA showing EM expression had a worse prognosis than CGA. Interestingly, GA showing EM-positive group was more aggressive than CGA group as they had frequent venous invasion and liver metastasis despite its smaller tumor size. GAED and GA with EM can be clinically classified as aggressive tumors but pathologically they seem to be slightly different.
Response to Reviewers:	July, 15h, 2023 Abbas Agaimy, M.D. Editor-in-Chief Virchows Archiv Dear Dr. Abbas Agaimy and reviewers, Thank you for inviting us to submit a revised manuscript entitled, “Clinicopathological characteristics of gastric adenocarcinoma with enteroblastic differentiation and gastric adenocarcinoma with enteroblastic marker expression” (Manuscript ID: VIAR-D-23-00242) to Virchows Archiv. We also appreciate the time and effort you and each of the reviewers have dedicated to providing insightful feedback on ways to strengthen our paper. Thus, it is with great pleasure that we resubmit our article for further consideration. We have incorporated changes that reflect the detailed suggestions you have graciously provided. We also hope that our edits and the responses we provide below satisfactorily address all the issues and concerns you and the reviewers have noted. To facilitate your review of our revisions, the following is a point-by-point response to the questions and comments delivered in your letter. The changes made during the revision are highlighted by yellow. Reviewers' comments: Reviewer #1: Maybe it would be useful for readers to comment the vague definition of the category of AFP-producing gastric carcinomas including hepatoid carcinoma, YST-like gastric carcinoma, GAED and well differentiated tubular/papillary adenocarcinoma (sic!) published in the current WHO classification of neoplasms of digestive system. RE) Thank you very much for the reviewer’s comments. According to the comments, we added comments regarding the current vague definition of the category of AFP-producing gastric carcinomas in the Introduction section. Reviewer #2: The authors investigated the significance of enteroblastic differentiation, defined as the expression of enteroblastic markers with or without the high grade clear cell histology, in gastric cancer. The abstract is informative, however, a language correction of the second half would be welcome, e.g., for lines 41-42 I would suggest "However, a higher frequency of venous invasion, but not of lymphatic invasion, was noted for GAED in comparison to CGA." if that is what the authors meant and the word count allows; RE) Thank you very much for the reviewer’s suggestion. According the suggestion, we changed this paragraph as your advice. lines 42-48 could also use rephrasing for a better clarity, but I will leave it to the authors' invention. RE) Thank you very much for the reviewer’s suggestion. We asked the English native speaker to rephrase these last parts of the abstract. Introduction is well-written and informative, adequately summarizing the data on enteroblastic differentiation in digestive tract adenocarcinomas. RE) Thank you very much for the positive comments. We really appreciate. The methodological approach is adequate and clearly described. In terms of the statistical analysis, the addition of Cox proportional hazard analysis for survival data would provide a more meaningful estimation of the magnitude of prognostic differences; for the non-significant comparisons a sensitivity analysis would be valuable (i.e., what is the minimal difference that would be called significant given the number of cases and events in the comparison). RE) Thank you very much for the reviewer’s comments. We employed Cox proportional hazard analysis for survival data. Sensitivity analysis was also performed

for parameters showing marginal significance (such as p=0.06) using several different statistical methods. However, these analyses did not change the results.

For the result section, please add the number or percentage of cases positive for all three markers (e.g., in line 137/138).
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RE) Thank you very much for the positive comment to our manuscript.

Again, thank you for giving us the opportunity to strengthen our manuscript with your valuable comments and queries. We have worked hard to incorporate your feedback and hope that these revisions persuade you to accept our submission.

Sincerely,

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July, 15h, 2023

Abbas Agaimy, M.D.
Editor-in-Chief
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Clinicopathological characteristics of gastric adenocarcinoma with enteroblastic differentiation and gastric adenocarcinoma with enteroblastic marker expression

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Keywords: Gastric adenocarcinoma with enteroblastic differentiation; clear cytoplasm; enteroblastic marker positive gastric adenocarcinoma; lymphatic invasion; venous invasion; aggressive behavior

Conflicts of Interest and Source of Funding

The authors declare that there are no competing interests. This study was supported by Grants-in-Aid from the Japan Society for the Promotion of Science (JSPS) KAKENHI (Grant Numbers: #21K06931 to T.Y., #20K07415 to T.S.).

Abstract (250 words).

Gastric adenocarcinoma (GA) with enteroblastic differentiation (GAED) is an aggressive carcinoma histologically characterized by a glycogen-rich clear cytoplasm and fetal gut-like structures. GAED shows the expression of at least one of the following enteroblastic markers (EMs): glypican-3 (GPC3), spalt-like transcription factor 4 (SALL4), and α-fetoprotein (AFP). Despite the absence of clear cytoplasm, we often encounter GA with EMs expression (GA with EM); however, the clinicopathological characteristics of GA with EM remain unclear. Immunohistochemical (IHC) expression of three EMs (AFP, GPC3, and SALL4) was examined on tissue microarray. According to the status of the clear cytoplasm of tumor cells, GAs showing IHC expression of EMs were classified as either GAED or GA with EM, and this analysis categorized 688 GAs into 94 GAEDs (13.7%), 58 GAs with EM (8.4%), and 536 conventional GAs (CGAs). Both GAED and GA with EM showed frequent lymphovascular invasion, lymph node metastasis, and liver metastasis compared to CGA. However, a higher frequency of venous invasion, but not of lymphatic invasion, was noted for GAED in comparison to CGA. GAED and GA with EM showed similar overall survival. GAED had significantly poorer prognosis than CGA; however, not for GA with EM. Furthermore, GA showing EM expression had a worse prognosis than CGA. Interestingly, GA showing EM-positive group was more aggressive than CGA group as they had frequent venous invasion and liver metastasis despite its smaller tumor size. GAED and GA with EM can be clinically

classified as aggressive tumors but pathologically they seem to be slightly different.

Introduction

Gastric adenocarcinoma (GA) with enteroblastic differentiation (GAED) is a special type of gastric carcinoma with a glycogen-rich clear cytoplasm and fetal gut-like structures [1-4]. α-fetoprotein (AFP)-producing gastric carcinomas including hepatoid carcinoma, yolk-sac tumor-like carcinoma, GAED and well differentiated tubular/papillary adenocarcinoma are described in the current WHO classification of tumours of digestive system as hepatoid adenocarcinoma and related entities [5], however, these tumors are vaguely defined, because these tumors closely overlap each other. The type previously referred to as AFP-producing gastric cancer is also considered a subtype of GAED [6-10]. Glypican-3 (GPC3), spalt-like transcription factor 4 (SALL4), and AFP are known as biomarkers of GAED [11-13]. The incidence of AFP-producing gastric carcinoma is within the range of 1.3–15.1% worldwide [7, 14-17]. The frequency of GAED is reported to be approximately 2.2–10.9%, however, this largely depends on the corresponding diagnostic criteria [10, 17].

GAED is histologically defined as that showing positive staining for at least one of the enteroblastic markers (EMs), AFP, GPC3, and SALL4, and tumor cells with a clear cytoplasm are indispensable [18]. Almost all cases have a coexisting conventional GA (CGA) [18-22]. In addition, GAED is a high-grade cancer that exhibits an aggressive behavior with higher rates of lymphatic and venous invasion, lymph node metastasis, and liver metastasis than CGA [18, 20]. We previously demonstrated that copy number variation (CNV) from next generation

sequencing (NGS) analysis exhibited a high frequency of *ERBB2* amplification, which introduces the possibility of using trastuzumab as an effective therapy, as in CGA [10]. Additionally, we reported that GAED has a higher rate of *TP53* mutations than CGA, that LOH and methylation are considered inactivation mechanisms of *TP53*, and that reduced SMAD4 expression and methylation of *SMAD4* may contribute to the acquisition of the aggressive phenotype of GAED [23, 24].

On the other hand, we also noticed that a substantial proportion of GAs showed positive staining for enteroblastic markers, despite the absence of histologically recognizable clear cytoplasm. However, the clinicopathological characteristics of this GA subset remain unclear. We have reported that the status of clear cell differentiation within tumors did not affect any clinicopathological or molecular pathological differences in colorectal carcinoma with the expression of enteroblastic markers [25]. Based on this finding, we propose that colorectal adenocarcinoma with enteroblastic marker expression could be classified together regardless of clear cell differentiation as colorectal adenocarcinoma with enteroblastic differentiation (CAED), which is a colorectal counterpart of GEAD [25]. In addition, CAED has similar clinicopathological and molecular pathological characteristics to GAED, including aggressive behavior, high rates of lymphovascular invasion and liver metastasis, and high frequency of *TP53* mutation [25]. Therefore, we speculated that the same phenomenon could also be observed in GA.

Materials and methods

Case selection and preparation of tissue microarray (TMA)

Out of 1341 cases of GA operated in our hospital from 2008–20, 688 cases of advanced GA were included, excluding early gastric carcinoma and Barrett's adenocarcinoma. A total of 688 GA samples from 686 patients (one patient with synchronous double GAs and one patient with non-synchronous double GAs) were analyzed using tissue microarray (TMA) or whole sections. TMA was prepared from 688 GA cases, as previously described [25]. In addition, three patients diagnosed with GAED between 2012–13, whose samples were not included on TMA, were also included in this series [10]. These three cases were included in our previous study and contained a clear cytoplasm [10]. This study was reviewed and approved by the Juntendo University School of Medicine Institutional Review Board (E21-0345).

Immunohistochemistry (IHC) and histological evaluation

Immunohistochemistry (IHC) was first performed on TMA with antibodies against AFP, GPC3, and SALL4. Any staining pattern, regardless of the staining intensity and staining area, was considered positive. In cases showing an expression of at least one of three enteroblastic markers, we re-evaluated the whole section for the presence of clear cytoplasm and growth patterns [1, 10, 23, 24]. Furthermore, in cases where clear/pale cytoplasm was observed within tumor cells on TMA, the cases were re-evaluated on whole sections for IHC. The enteroblastic marker was considered positive when at least 10% of the positive area was

confirmed. In our previous studies, growth patterns in GAED were classified into two types. The "solid type," in which sheet-like growth patterns are seen even in small areas, and the "tubulo-papillary type," in which only tubular to papillary growth patterns are seen [10, 23, 24]. In this study, we classified GAED into two groups: "solid-type" and "non-solid type." "Non-solid type" mainly comprised of "tubulo-papillary type" in our previous study and small amounts of cases showing "mucinous, por2 (scirrhous), and signet-ring cell carcinoma." Histologically, cases were classified either as "GAED" showing clear cytoplasm of tumor cells and expression of at least one enteroblastic marker or "enteroblastic marker positive gastric adenocarcinoma without clear cytoplasm (here defined as GA with EM)," which does not have clear cytoplasm but shows expression of enteroblastic markers. The remaining cases were classified as "CGA'. The histopathological diagnoses were reviewed by two pathologists (T.S. and D.A.) and validated by a gastrointestinal pathologist (T.Y.) at our hospital.

Clinicopathological features

Clinicopathological features, such as age, sex, tumor location, tumor size, macroscopic type, invasion depth, TNM stage, lymphatic invasion, venous invasion, lymph node metastasis, liver metastasis, growth pattern, presence or absence of clear cytoplasm, and IHC staining for AFP/GPC3/SALL4, were examined. TNM stage was based on the 8th edition of the American Joint Committee on Cancer (AJCC)/ Union for International Cancer Control (UICC) pathological tumor-node-metastasis (pTNM) staging system for gastric cancer [26].

Survival and statistical analyses

Categorical and continuous variables were analyzed using the Fisher's exact test, χ2 test, and t-test, respectively. For survival analysis, we performed Kaplan– Meier survival analysis and log-rank tests. Statistical analyses were performed using the statistical software 'EZR'(Easy R)(Saitama Medical Center, Jichi Medical University, Saitama, Japan) [27], which is a graphical user interface for R (R Foundation for Statistical Computing, Vienna, Austria). Cox proportional hazard model was also employed for survival analysis. $P < 0.05$ were considered statistically significant.

Results

Clinicopathological findings

Supplementary Table 1 shows the clinicopathological characteristics of the 688 GA patients enrolled in this study.

The 688 cases of advanced GA were classified into 94 GAEDs (13.7%), 58 GAs with EM (8.4%), and 536 CGAs. All GAED cases were accompanied by a conventional adenocarcinoma area as the minor component. Supplementary Table 2 summarizes the histological and IHC findings. Histologically, a clear cytoplasm was observed in 116 of 688 cases (16.9%) including 94 GAEDs and 22 CGAs. Positive IHC staining for AFP, GPC3, and SALL4 was observed in 5.5%, 14.1%, and 16.9% of 688 cases, respectively. Cases positive for all three markers were 3.9% and 22.1% showed positive staining for at least one of the enteroblastic markers. AFP, GPC3, and SALL4 expression was detected in 30.9%, 69.1%, and 80.9% of 94 GAED cases, respectively. Among 58 GA with EM cases, AFP, GPC3, and SALL4 expressions were observed in 15.5%, 55.2%, and 69.0% of cases, respectively. Figure 1 shows typical histological and IHC findings in GAED and IHC findings in GA with EM.

Table 1 summarizes the comparison of 94 GAEDs and 58 GAs with EM. Comparisons between GAED and GA with EM showed that a mean age ($p = 0.189$), sex ($p = 0.832$), tumor location ($p = 0.056$), tumor size ($p = 0.483$), TNM stage ($p = 0.126$), and lymph node metastasis rate ($p = 0.067$) were not statistically different. However, there were statistically significant

differences in macroscopic type ($p < 0.05$), invasion depth ($p < 0.05$), lymphatic invasion rate ($p < 0.05$), venous invasion rate ($p < 0.05$), and growth patterns ($p < 0.05$). Particularly, the liver metastasis rate ($p < 0.01$) was significantly higher in GAED. The lymphatic invasion was more frequent in GA patients with EM. As for IHC findings, there were no statistical differences in the GPC3 ($p = 0.086$) and SALL4 positivity ($p = 0.117$); however, the positive rate of AFP ($p < 0.05$) was significantly higher in GAED than in GA with EM.

Table 2 summarizes the comparison between the 94 GAEDs and the 536 CGAs. Comparisons between GAED and CGA showed that TNM stage ($p = 0.053$), lymphatic invasion rate ($p = 0.191$), and lymph node metastasis rate ($p=0.092$) were not statistically different. Meanwhile, there were statistically significant differences in terms of sex ($p < 0.05$), tumor location ($p < 0.05$), and tumor size ($p < 0.05$). Especially for a mean age ($p < 0.01$), the macroscopic type ($p < 0.01$), invasion depth ($p < 0.01$), venous invasion rate ($p < 0.01$), liver metastasis rate ($p < 0.01$) were significantly higher in GAED.

Table 3 summarizes the comparison of 58 GAs with EM and 536 CGAs. Comparisons between GA with EM and CGA showed that only tumor location ($p<0.05$) and venous invasion rate ($p < 0.05$) were statistically different. Enteroblastic differentiation was frequently observed in the upper gastric area, while venous invasion was more frequently observed in GA with EM.

Regardless of the presence of a clear cytoplasm, the patients were further divided into two groups: enteroblastic marker-positive gastric adenocarcinoma and negative gastric

adenocarcinoma (CGA). Under this classification, there were statistically significant differences in mean age ($p < 0.01$), sex ($p = <0.01$), tumor location ($p < 0.05$), macroscopic type ($p < 0.01$), invasion depth ($p < 0.01$), venous invasion rate ($p < 0.01$), and liver metastasis rate ($p < 0.01$). There were no statistically significant differences in tumor size ($p = 0.057$), TNM stage ($p = 0.119$), lymphatic invasion rate ($p = 0.844$), and lymph node metastasis rate ($p = 0.489$). Interestingly, the tumor size tended to be smaller in the EM-positive group than in the EM-negative group; however, venous invasion and liver metastasis rates were significantly higher in the EM-positive group (Table 4).

We also noticed that CGA (EM-negative GA) sometimes had a clear cytoplasm; thus, CGAs were further divided into two groups according to the presence or absence of clear cytoplasm (CC-positive GA and CC-negative GA). This comparison revealed that venous invasion was more frequently observed in CC-positive CGA and that CC-positive CGA preferentially occurred in elderly and male patients (Supplementary Table 3).

Prognosis in GAED and GA with EM

The five-year overall survival rates for GAED, GA with EM, and CGA were 46.6%, 47.9%, and 58.2%, respectively. GAED and GA with EM showed similar OS trends in overall survival ($p = 0.78$, Figure 2A). GAED had a significantly poorer prognosis than CGA ($p = 0.035$, Figure 2A; Hazard ratio: 1.428 (1.024-1.990), P=0.036); however, there was no statistically significant survival difference between GA with EM and CGA ($p = 0.157$, Figure 2A; Hazard

ratio: 1.337 (0.893-2.003), P=0.158). Furthermore, GA showing EM expression, regardless of the presence of a clear cytoplasm (GAED and GA with EM), showed a worse overall survival rate than CGA (p = 0.018, Figure 2B; Hazard ratio: 1.391 (1.057-1.832), P=0.019). In the CGA group, the status of clear cytoplasm within the tumor cells did not affect the overall survival rate (data not shown).

Survival analyses for each factor were also performed for GAED and GA with EM. In GAED, survival analysis revealed that lymphatic invasion (p < 0.01, Figure 3A), liver metastasis (p < 0.01, Figure 3B), growth pattern (solid type, p < 0.01, Figure 3C), and expression of GPC3 (p < 0.05, Figure 3D) were significantly associated with a poor overall survival. In addition, lymph node metastasis (p = 0.053) was associated with a poor overall survival. In GA with EM, the survival analysis revealed that lymph node metastasis (p < 0.01, Figure 4A) and liver metastasis (p < 0.01, Figure 4B) were significantly associated with a poor overall survival. Moreover, lymphatic invasion (p = 0.071) and expression of AFP (p = 0.053) tended to be associated with a poor overall survival.

Discussion

The pathological concept of GAED has been gradually established since the identification of GPC3 and SALL4 as enteroblastic markers in addition to AFP together, with an increasing number of studies on AFP-producing gastric carcinoma [1, 11, 12, 18]. We have previously reported a few GAED studies describing its clinicopathological characteristics; however, we could not enroll consecutive GAED cases in these studies since we employed a keyword search for GAED such as AFP and clear cytoplasm in the electronic pathological record [10]. Therefore, we identified GAED and GA with EM using TMA-based screening in this study. IHC revealed that the frequencies of enteroblastic markers in all GAs were 5.5% for AFP, 14.1% for GPC3, and 16.9% for SALL4, which was in line with the previously reported ratios [4, 11, 12, 28, 29]. A total of 152 out of 688 (22.1%) GA cases in this series showed the expression of at least one of the three enteroblastic markers, thereby providing evidence that a significant proportion of GA showed enteroblastic differentiation by IHC. This study demonstrated that both GAED and GA with EM tended to show higher frequencies of venous invasion and liver metastasis than CGA, even in their smaller size. These findings suggested that the expression of enteroblastic markers itself is associated with aggressive behavior [11, 12, 14, 30].

Regarding prognosis, the five-year overall survival rates of patients with GAED and those with GA with EM were 46.6% and 47.9%, respectively. Long term survival analysis in GAED

and survival analysis of GA with EM as an independent subgroup have not been reported so far, and these were almost similar to or slightly better than those reported in AFP-producing gastric carcinoma and hepatoid adenocarcinoma [14, 15, 31, 32]. Regarding the significance of clear cytoplasm within GA, GAED had a considerably worse prognosis than CGA, and GA with EM had a worse prognosis than CGA, although the difference was not statistically significant. Furthermore, the presence of clear cytoplasm was associated with frequent venous invasion, but not with the overall survival rate in the CGA group. These findings suggest that the presence of a clear cytoplasm partly contributes to the acquisition of more aggressive behavior in GA, regardless of enteroblastic differentiation.

GAED is defined as the expression of at least one of the enteroblastic markers, wherein a clear cytoplasm is histologically confirmed [18]. However, the presence of clear cytoplasm is not required in CAED, which is a colorectal counterpart of GAED, since the clinicopathological and molecular pathological differences cannot be identified according to the status of clear cytoplasm within the tumor [25]. This finding suggested that CAED could be classified only by enteroblastic marker expression. In GAED, from the viewpoint of overall survival rates, similar trends were confirmed between GAED and GA with EM; however, patients with GAED seemed to show inferior survival rates during the first 3 years compared to GA with EM. Furthermore, patients with GAED show adverse outcomes even at an early stage [20]. In addition, several clinicopathological differences were observed between the two groups.

Positive rates for three EM markers, venous invasion, liver metastasis, and solid growth pattern, were more frequently detected in GAED than in GA with EM, while lymphatic invasion was more prevalent in GA with EM. Thus, although both tumors are high-grade, further comprehensive analysis is required to draw a conclusion on whether GAED and GA with EM could be grouped together under a single pathological category as GAED.

In a clinical setting, it has been demonstrated that the expression of oncofetal proteins is associated with a poor prognosis in various cancers [30]. Therefore, it is highly recommended to perform IHC for EM in addition to identifying specific histological features, such as solid patterns and tumor cells with clear cytoplasm during routine pathological diagnosis. Trastuzumab and Trastuzumab deruxtecan are approved as molecular target therapies for GA, for GA with HER2 overexpression; nivolumab has been approved as well [33-37]. Although promising therapeutic targets have not been identified in these types of tumors, we have previously reported that a subset of GAED shows amplification of *ERBB2* at a similar frequency in CGA [10]. Further molecular pathological analysis might provide new therapeutic targets for these subsets of GA, in addition to the understanding of tumorigenesis including the acquisition of aggressive phenotypes.

This study has several limitations. First, since we excluded cases of Stage IA and part of Stage IB during TMA preparation, we only enrolled patients with advanced GA. This sample selection might have affected the prognostic analysis, because the aggressive behavior of

tumors becomes clearer, especially in early-stage tumors. In fact, the 3-year overall survival rate in GAED in this series is almost similar to that in our previous study, which included approximately 30% of Stage I GAED [10], and GAED showed an aggressive clinical course even in the early stages [20]. GA with EM might have revealed an adverse overall survival rate compared to CGA if the early-stage GAs were also included. Second, we overlooked possible cases of GAED and GA with EM by TMA-based IHC screening, although extensive histological examinations were performed to check for the presence of clear cytoplasm using all available slides in cases where at least one of the enteroblastic markers was positively stained on TMA cores. In contrast, there were 22 cases with a clear cytoplasm within the CGA group which were negative for EM by whole-section IHC (Supplementary Table 3). Patients with CGA and a clear cytoplasm also showed few clinicopathological characteristics, such as frequent venous invasion, which were similar to that of the EM-positive group. Likewise, although we performed EM IHC by whole section in case clear cytoplasm was confirmed on TMA slide, few cases of CGA with a clear cytoplasm may be classified as GAED through a more extensive search.

In conclusion, in addition to the previously defined GAED, we found that a considerable portion of GA showed an expression of enteroblastic markers by IHC but did not have a clear cytoplasm, despite extensive histological examinations. We define this subset of GA as GA with EM, which also showed worse prognosis than CGA with GAED. Both GAED and GA with

EM demonstrated similar clinicopathological features, such as a high frequency of liver metastasis and lymphovascular invasion, although venous invasion was more prevalent in GAED and vice versa for lymphatic invasion.

DATA AVAILABILITY

The data used during the current study are available from the corresponding author on reasonable request.

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Figure legends

Figure 1: Histological and immunohistochemical findings in GAED and GA with EM.

Boundary area between gastric adenocarcinoma with enteroblastic differentiation (GAED), which consists of columnar cells with clear cytoplasm with enteroblastic differentiation, and a conventional adenocarcinoma component (A). High-power view of the blue-framed GAED

component in A (B). High-power view of the red-framed conventional adenocarcinoma component in A (C). Immunohistochemical (IHC) staining for α-fetoprotein (AFP) (D), Glypican-3 (GPC3) (E), and palt-like transcription factor 4 (SALL4) (F). Gastric adenocarcinoma with enteroblastic marker expression showing both solid and nonsolid growth patterns (G). Immunohistochemical (IHC) staining for alpha-fetoprotein (AFP) (H), Glypican-3 (GPC3) with a high-power-view image of a partially positive area (inset at lower right) (I) and Spalt-like transcription factor 4 (SALL4) (J).

Figure 2: Comparison of Overall survival in GAED, GA with EM, and CGA.

(A) All three curves (GAED, GA with EM and CGA) were put in one panel, and each pairwise comparison was performed. GAED and GA with EM show similar trends in overall survival rates (p=0.78), however, patients with GAED show rather worse prognosis than GA with EM especially during first 3 years. GAED has a significantly poorer overall survival than CGA (p=0.035), but survival difference between GA with EM and CGA loses statistical significance (p=0.157). (B) GAs showing expression of EM regardless of the presence of clear cytoplasm (GAED and GA with EM) have worse overall survival than CGAs (p=0.018).

Figure 3: Comparison of overall survival by each factor in GAED.

(A) lymphatic invasion (p<0.01), (B) liver metastasis (p<0.01), (C) growth pattern (Solid type,

p<0.01) and (D) expression of GPC3 (p<0.05) were significantly associated with poor overall survival.

Figure 4: Comparison of overall survival by each factor in GA with EM.

(A) lymph nodes metastasis (p<0.01) and (B) liver metastasis (p<0.01) were significantly associated with poor overall survival.

Supplementary Table 1

Clinicopathological characteristics of study patients (n=688)

Abbreviations: SD, Standard deviation; U, Upper third; M, Middle third; L, Lower third; MP, Muscularis mucosae; SS, Subserosa; SE, Tumor penetration of serosa; SI, Tumor invasion of adjacent structures; TNM, Tumor-node-metastasis.

Supplementary Table 2

Histological and immunohistochemical findings in 688 GAs, including GAED and GA with EM

Abbreviations: GA, gastric adenocarcinoma; GAED, gastric adenocarcinoma with enteroblastic differentiation; EM, enteroblastic marker; CC, clear cytoplasm; AFP, α-fetoprotein; GPC3, Glypican-3; SALL4, Spalt-like transcription factor 4.

Table 1

Clinicopathological findings (GAED vs GA with EM)

Abbreviations: GAED, gastric adenocarcinoma with enteroblastic differentiation; GA, gastric adenocarcinoma; EM, enteroblastic marker; SD, Standard deviation; U, Upper third; M, Middle third; L, Lower third; MP, Muscularis mucosae; SS, Subserosa; SE, Tumor penetration of serosa; SI, Tumor invasion of adjacent structures; TNM, Tumor-node-metastasis; AFP, α-fetoprotein; GPC3, Glypican-3; SALL4, Spalt-like transcription factor 4.

Table 2

Clinicopathological findings (GAED vs CGA)

Abbreviations: CGA, conventional gastric adenocarcinoma

Table 3

Clinicopathological findings (GA with EM vs CGA)

Table 4

Clinicopathological findings according to EM expression

Supplementary Table 3

Clinicopathological findings in CGA according to clear cytoplasm

Abbreviations: CC, clear cytoplasm

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Author contributions statement

D Abe, T.S., T.H., H.U., A.N., and T.Y. planned this project and D Abe, T.S., Y.A., N.Y., and T.Y. contributed to the diagnosis of GAED and GA with EM cases. D Abe and T.S. performed the main parts of the experiments and D Abe, T.S., T.H., H.U., S.M., T.F. and A.N. collected and analyzed the clinical data. D Abe and T.S. wrote the majority of the manuscript. All authors approved the final version of the manuscript.

Clinicopathological characteristics of gastric adenocarcinoma with enteroblastic differentiation and gastric adenocarcinoma with enteroblastic marker expression

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Conflicts of Interest and Source of Funding

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Abstract (250 words).

Gastric adenocarcinoma (GA) with enteroblastic differentiation (GAED) is an aggressive carcinoma histologically characterized by a glycogen-rich clear cytoplasm and fetal gut-like structures. GAED shows the expression of at least one of the following enteroblastic markers (EMs): glypican-3 (GPC3), spalt-like transcription factor 4 (SALL4), and α-fetoprotein (AFP). Despite the absence of clear cytoplasm, we often encounter GA with EMs expression (GA with EM); however, the clinicopathological characteristics of GA with EM remain unclear. Immunohistochemical (IHC) expression of three EMs (AFP, GPC3, and SALL4) was examined on tissue microarray. According to the status of the clear cytoplasm of tumor cells, GAs showing IHC expression of EMs were classified as either GAED or GA with EM, and this analysis categorized 688 GAs into 94 GAEDs (13.7%), 58 GAs with EM (8.4%), and 536 conventional GAs (CGAs). Both GAED and GA with EM showed frequent lymphovascular invasion, lymph node metastasis, and liver metastasis compared to CGA. However, a higher frequency of venous invasion, but not of lymphatic invasion, was noted for GAED in comparison to CGA. GAED and GA with EM showed similar overall survival. GAED had significantly poorer prognosis than CGA; however, not for GA with EM. Furthermore, GA showing EM expression had a worse prognosis than CGA. Interestingly, GA showing EM-positive group was more aggressive than CGA group as they had frequent venous invasion and liver metastasis despite its smaller tumor size. GAED and GA with EM can be clinically

classified as aggressive tumors but pathologically they seem to be slightly different.

Introduction

Gastric adenocarcinoma (GA) with enteroblastic differentiation (GAED) is a special type of gastric carcinoma with a glycogen-rich clear cytoplasm and fetal gut-like structures [1-4]. α-fetoprotein (AFP)-producing gastric carcinomas including hepatoid carcinoma, yolk-sac tumor-like carcinoma, GAED and well differentiated tubular/papillary adenocarcinoma are described in the current WHO classification of tumours of digestive system as hepatoid adenocarcinoma and related entities [5], however, these tumors are vaguely defined, because these tumors closely overlap each other. The type previously referred to as AFP-producing gastric cancer is also considered a subtype of GAED [6-10]. Glypican-3 (GPC3), spalt-like transcription factor 4 (SALL4), and AFP are known as biomarkers of GAED [11-13]. The incidence of AFP-producing gastric carcinoma is within the range of 1.3–15.1% worldwide [7, 14-17]. The frequency of GAED is reported to be approximately 2.2–10.9%, however, this largely depends on the corresponding diagnostic criteria [10, 17].

GAED is histologically defined as that showing positive staining for at least one of the enteroblastic markers (EMs), AFP, GPC3, and SALL4, and tumor cells with a clear cytoplasm are indispensable [18]. Almost all cases have a coexisting conventional GA (CGA) [18-22]. In addition, GAED is a high-grade cancer that exhibits an aggressive behavior with higher rates of lymphatic and venous invasion, lymph node metastasis, and liver metastasis than CGA [18, 20]. We previously demonstrated that copy number variation (CNV) from next generation

sequencing (NGS) analysis exhibited a high frequency of *ERBB2* amplification, which introduces the possibility of using trastuzumab as an effective therapy, as in CGA [10]. Additionally, we reported that GAED has a higher rate of *TP53* mutations than CGA, that LOH and methylation are considered inactivation mechanisms of *TP53*, and that reduced SMAD4 expression and methylation of *SMAD4* may contribute to the acquisition of the aggressive phenotype of GAED [23, 24].

On the other hand, we also noticed that a substantial proportion of GAs showed positive staining for enteroblastic markers, despite the absence of histologically recognizable clear cytoplasm. However, the clinicopathological characteristics of this GA subset remain unclear. We have reported that the status of clear cell differentiation within tumors did not affect any clinicopathological or molecular pathological differences in colorectal carcinoma with the expression of enteroblastic markers [25]. Based on this finding, we propose that colorectal adenocarcinoma with enteroblastic marker expression could be classified together regardless of clear cell differentiation as colorectal adenocarcinoma with enteroblastic differentiation (CAED), which is a colorectal counterpart of GEAD [25]. In addition, CAED has similar clinicopathological and molecular pathological characteristics to GAED, including aggressive behavior, high rates of lymphovascular invasion and liver metastasis, and high frequency of *TP53* mutation [25]. Therefore, we speculated that the same phenomenon could also be observed in GA.

Materials and methods

Case selection and preparation of tissue microarray (TMA)

Out of 1341 cases of GA operated in our hospital from 2008–20, 688 cases of advanced GA were included, excluding early gastric carcinoma and Barrett's adenocarcinoma. A total of 688 GA samples from 686 patients (one patient with synchronous double GAs and one patient with non-synchronous double GAs) were analyzed using tissue microarray (TMA) or whole sections. TMA was prepared from 688 GA cases, as previously described [25]. In addition, three patients diagnosed with GAED between 2012–13, whose samples were not included on TMA, were also included in this series [10]. These three cases were included in our previous study and contained a clear cytoplasm [10]. This study was reviewed and approved by the Juntendo University School of Medicine Institutional Review Board (E21-0345).

Immunohistochemistry (IHC) and histological evaluation

Immunohistochemistry (IHC) was first performed on TMA with antibodies against AFP, GPC3, and SALL4. Any staining pattern, regardless of the staining intensity and staining area, was considered positive. In cases showing an expression of at least one of three enteroblastic markers, we re-evaluated the whole section for the presence of clear cytoplasm and growth patterns [1, 10, 23, 24]. Furthermore, in cases where clear/pale cytoplasm was observed within tumor cells on TMA, the cases were re-evaluated on whole sections for IHC. The enteroblastic marker was considered positive when at least 10% of the positive area was

confirmed. In our previous studies, growth patterns in GAED were classified into two types. The "solid type," in which sheet-like growth patterns are seen even in small areas, and the "tubulo-papillary type," in which only tubular to papillary growth patterns are seen [10, 23, 24]. In this study, we classified GAED into two groups: "solid-type" and "non-solid type." "Non-solid type" mainly comprised of "tubulo-papillary type" in our previous study and small amounts of cases showing "mucinous, por2 (scirrhous), and signet-ring cell carcinoma." Histologically, cases were classified either as "GAED" showing clear cytoplasm of tumor cells and expression of at least one enteroblastic marker or "enteroblastic marker positive gastric adenocarcinoma without clear cytoplasm (here defined as GA with EM)," which does not have clear cytoplasm but shows expression of enteroblastic markers. The remaining cases were classified as "CGA'. The histopathological diagnoses were reviewed by two pathologists (T.S. and D.A.) and validated by a gastrointestinal pathologist (T.Y.) at our hospital.

Clinicopathological features

Clinicopathological features, such as age, sex, tumor location, tumor size, macroscopic type, invasion depth, TNM stage, lymphatic invasion, venous invasion, lymph node metastasis, liver metastasis, growth pattern, presence or absence of clear cytoplasm, and IHC staining for AFP/GPC3/SALL4, were examined. TNM stage was based on the 8th edition of the American Joint Committee on Cancer (AJCC)/ Union for International Cancer Control (UICC) pathological tumor-node-metastasis (pTNM) staging system for gastric cancer [26].

Survival and statistical analyses

Categorical and continuous variables were analyzed using the Fisher's exact test, χ2 test, and t-test, respectively. For survival analysis, we performed Kaplan– Meier survival analysis and log-rank tests. Statistical analyses were performed using the statistical software 'EZR'(Easy R)(Saitama Medical Center, Jichi Medical University, Saitama, Japan) [27], which is a graphical user interface for R (R Foundation for Statistical Computing, Vienna, Austria). Cox proportional hazard model was also employed for survival analysis. $P < 0.05$ were considered statistically significant.

Results

Clinicopathological findings

Supplementary Table 1 shows the clinicopathological characteristics of the 688 GA patients enrolled in this study.

The 688 cases of advanced GA were classified into 94 GAEDs (13.7%), 58 GAs with EM (8.4%), and 536 CGAs. All GAED cases were accompanied by a conventional adenocarcinoma area as the minor component. Supplementary Table 2 summarizes the histological and IHC findings. Histologically, a clear cytoplasm was observed in 116 of 688 cases (16.9%) including 94 GAEDs and 22 CGAs. Positive IHC staining for AFP, GPC3, and SALL4 was observed in 5.5%, 14.1%, and 16.9% of 688 cases, respectively. Cases positive for all three markers were 3.9% and 22.1% showed positive staining for at least one of the enteroblastic markers. AFP, GPC3, and SALL4 expression was detected in 30.9%, 69.1%, and 80.9% of 94 GAED cases, respectively. Among 58 GA with EM cases, AFP, GPC3, and SALL4 expressions were observed in 15.5%, 55.2%, and 69.0% of cases, respectively. Figure 1 shows typical histological and IHC findings in GAED and IHC findings in GA with EM.

Table 1 summarizes the comparison of 94 GAEDs and 58 GAs with EM. Comparisons between GAED and GA with EM showed that a mean age ($p = 0.189$), sex ($p = 0.832$), tumor location ($p = 0.056$), tumor size ($p = 0.483$), TNM stage ($p = 0.126$), and lymph node metastasis rate ($p = 0.067$) were not statistically different. However, there were statistically significant

differences in macroscopic type ($p < 0.05$), invasion depth ($p < 0.05$), lymphatic invasion rate ($p < 0.05$), venous invasion rate ($p < 0.05$), and growth patterns ($p < 0.05$). Particularly, the liver metastasis rate ($p < 0.01$) was significantly higher in GAED. The lymphatic invasion was more frequent in GA patients with EM. As for IHC findings, there were no statistical differences in the GPC3 ($p = 0.086$) and SALL4 positivity ($p = 0.117$); however, the positive rate of AFP ($p < 0.05$) was significantly higher in GAED than in GA with EM.

Table 2 summarizes the comparison between the 94 GAEDs and the 536 CGAs. Comparisons between GAED and CGA showed that TNM stage ($p = 0.053$), lymphatic invasion rate ($p = 0.191$), and lymph node metastasis rate ($p=0.092$) were not statistically different. Meanwhile, there were statistically significant differences in terms of sex ($p < 0.05$), tumor location ($p < 0.05$), and tumor size ($p < 0.05$). Especially for a mean age ($p < 0.01$), the macroscopic type ($p < 0.01$), invasion depth ($p < 0.01$), venous invasion rate ($p < 0.01$), liver metastasis rate ($p < 0.01$) were significantly higher in GAED.

Table 3 summarizes the comparison of 58 GAs with EM and 536 CGAs. Comparisons between GA with EM and CGA showed that only tumor location ($p<0.05$) and venous invasion rate ($p < 0.05$) were statistically different. Enteroblastic differentiation was frequently observed in the upper gastric area, while venous invasion was more frequently observed in GA with EM.

Regardless of the presence of a clear cytoplasm, the patients were further divided into two groups: enteroblastic marker-positive gastric adenocarcinoma and negative gastric

adenocarcinoma (CGA). Under this classification, there were statistically significant differences in mean age ($p < 0.01$), sex ($p = <0.01$), tumor location ($p < 0.05$), macroscopic type ($p < 0.01$), invasion depth ($p < 0.01$), venous invasion rate ($p < 0.01$), and liver metastasis rate ($p < 0.01$). There were no statistically significant differences in tumor size ($p = 0.057$), TNM stage ($p = 0.119$), lymphatic invasion rate ($p = 0.844$), and lymph node metastasis rate ($p = 0.489$). Interestingly, the tumor size tended to be smaller in the EM-positive group than in the EM-negative group; however, venous invasion and liver metastasis rates were significantly higher in the EM-positive group (Table 4).

We also noticed that CGA (EM-negative GA) sometimes had a clear cytoplasm; thus, CGAs were further divided into two groups according to the presence or absence of clear cytoplasm (CC-positive GA and CC-negative GA). This comparison revealed that venous invasion was more frequently observed in CC-positive CGA and that CC-positive CGA preferentially occurred in elderly and male patients (Supplementary Table 3).

Prognosis in GAED and GA with EM

The five-year overall survival rates for GAED, GA with EM, and CGA were 46.6%, 47.9%, and 58.2%, respectively. GAED and GA with EM showed similar OS trends in overall survival ($p = 0.78$, Figure 2A). GAED had a significantly poorer prognosis than CGA ($p = 0.035$, Figure 2A; Hazard ratio: 1.428 (1.024-1.990), P=0.036); however, there was no statistically significant survival difference between GA with EM and CGA ($p = 0.157$, Figure 2A; Hazard

ratio: 1.337 (0.893-2.003), P=0.158). Furthermore, GA showing EM expression, regardless of the presence of a clear cytoplasm (GAED and GA with EM), showed a worse overall survival rate than CGA (p = 0.018, Figure 2B; Hazard ratio: 1.391 (1.057-1.832), P=0.019). In the CGA group, the status of clear cytoplasm within the tumor cells did not affect the overall survival rate (data not shown).

Survival analyses for each factor were also performed for GAED and GA with EM. In GAED, survival analysis revealed that lymphatic invasion (p < 0.01, Figure 3A), liver metastasis (p < 0.01, Figure 3B), growth pattern (solid type, p < 0.01, Figure 3C), and expression of GPC3 (p < 0.05, Figure 3D) were significantly associated with a poor overall survival. In addition, lymph node metastasis (p = 0.053) was associated with a poor overall survival. In GA with EM, the survival analysis revealed that lymph node metastasis (p < 0.01, Figure 4A) and liver metastasis (p < 0.01, Figure 4B) were significantly associated with a poor overall survival. Moreover, lymphatic invasion (p = 0.071) and expression of AFP (p = 0.053) tended to be associated with a poor overall survival.

Discussion

The pathological concept of GAED has been gradually established since the identification of GPC3 and SALL4 as enteroblastic markers in addition to AFP together, with an increasing number of studies on AFP-producing gastric carcinoma [1, 11, 12, 18]. We have previously reported a few GAED studies describing its clinicopathological characteristics; however, we could not enroll consecutive GAED cases in these studies since we employed a keyword search for GAED such as AFP and clear cytoplasm in the electronic pathological record [10]. Therefore, we identified GAED and GA with EM using TMA-based screening in this study. IHC revealed that the frequencies of enteroblastic markers in all GAs were 5.5% for AFP, 14.1% for GPC3, and 16.9% for SALL4, which was in line with the previously reported ratios [4, 11, 12, 28, 29]. A total of 152 out of 688 (22.1%) GA cases in this series showed the expression of at least one of the three enteroblastic markers, thereby providing evidence that a significant proportion of GA showed enteroblastic differentiation by IHC. This study demonstrated that both GAED and GA with EM tended to show higher frequencies of venous invasion and liver metastasis than CGA, even in their smaller size. These findings suggested that the expression of enteroblastic markers itself is associated with aggressive behavior [11, 12, 14, 30].

Regarding prognosis, the five-year overall survival rates of patients with GAED and those with GA with EM were 46.6% and 47.9%, respectively. Long term survival analysis in GAED

and survival analysis of GA with EM as an independent subgroup have not been reported so far, and these were almost similar to or slightly better than those reported in AFP-producing gastric carcinoma and hepatoid adenocarcinoma [14, 15, 31, 32]. Regarding the significance of clear cytoplasm within GA, GAED had a considerably worse prognosis than CGA, and GA with EM had a worse prognosis than CGA, although the difference was not statistically significant. Furthermore, the presence of clear cytoplasm was associated with frequent venous invasion, but not with the overall survival rate in the CGA group. These findings suggest that the presence of a clear cytoplasm partly contributes to the acquisition of more aggressive behavior in GA, regardless of enteroblastic differentiation.

GAED is defined as the expression of at least one of the enteroblastic markers, wherein a clear cytoplasm is histologically confirmed [18]. However, the presence of clear cytoplasm is not required in CAED, which is a colorectal counterpart of GAED, since the clinicopathological and molecular pathological differences cannot be identified according to the status of clear cytoplasm within the tumor [25]. This finding suggested that CAED could be classified only by enteroblastic marker expression. In GAED, from the viewpoint of overall survival rates, similar trends were confirmed between GAED and GA with EM; however, patients with GAED seemed to show inferior survival rates during the first 3 years compared to GA with EM. Furthermore, patients with GAED show adverse outcomes even at an early stage [20]. In addition, several clinicopathological differences were observed between the two groups.

Positive rates for three EM markers, venous invasion, liver metastasis, and solid growth pattern, were more frequently detected in GAED than in GA with EM, while lymphatic invasion was more prevalent in GA with EM. Thus, although both tumors are high-grade, further comprehensive analysis is required to draw a conclusion on whether GAED and GA with EM could be grouped together under a single pathological category as GAED.

In a clinical setting, it has been demonstrated that the expression of oncofetal proteins is associated with a poor prognosis in various cancers [30]. Therefore, it is highly recommended to perform IHC for EM in addition to identifying specific histological features, such as solid patterns and tumor cells with clear cytoplasm during routine pathological diagnosis. Trastuzumab and Trastuzumab deruxtecan are approved as molecular target therapies for GA, for GA with HER2 overexpression; nivolumab has been approved as well [33-37]. Although promising therapeutic targets have not been identified in these types of tumors, we have previously reported that a subset of GAED shows amplification of *ERBB2* at a similar frequency in CGA [10]. Further molecular pathological analysis might provide new therapeutic targets for these subsets of GA, in addition to the understanding of tumorigenesis including the acquisition of aggressive phenotypes.

This study has several limitations. First, since we excluded cases of Stage IA and part of Stage IB during TMA preparation, we only enrolled patients with advanced GA. This sample selection might have affected the prognostic analysis, because the aggressive behavior of

tumors becomes clearer, especially in early-stage tumors. In fact, the 3-year overall survival rate in GAED in this series is almost similar to that in our previous study, which included approximately 30% of Stage I GAED [10], and GAED showed an aggressive clinical course even in the early stages [20]. GA with EM might have revealed an adverse overall survival rate compared to CGA if the early-stage GAs were also included. Second, we overlooked possible cases of GAED and GA with EM by TMA-based IHC screening, although extensive histological examinations were performed to check for the presence of clear cytoplasm using all available slides in cases where at least one of the enteroblastic markers was positively stained on TMA cores. In contrast, there were 22 cases with a clear cytoplasm within the CGA group which were negative for EM by whole-section IHC (Supplementary Table 3). Patients with CGA and a clear cytoplasm also showed few clinicopathological characteristics, such as frequent venous invasion, which were similar to that of the EM-positive group. Likewise, although we performed EM IHC by whole section in case clear cytoplasm was confirmed on TMA slide, few cases of CGA with a clear cytoplasm may be classified as GAED through a more extensive search.

In conclusion, in addition to the previously defined GAED, we found that a considerable portion of GA showed an expression of enteroblastic markers by IHC but did not have a clear cytoplasm, despite extensive histological examinations. We define this subset of GA as GA with EM, which also showed worse prognosis than CGA with GAED. Both GAED and GA with

EM demonstrated similar clinicopathological features, such as a high frequency of liver metastasis and lymphovascular invasion, although venous invasion was more prevalent in GAED and vice versa for lymphatic invasion.

DATA AVAILABILITY

The data used during the current study are available from the corresponding author on reasonable request.

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Figure legends

Figure 1: Histological and immunohistochemical findings in GAED and GA with EM.

Boundary area between gastric adenocarcinoma with enteroblastic differentiation (GAED), which consists of columnar cells with clear cytoplasm with enteroblastic differentiation, and a conventional adenocarcinoma component (A). High-power view of the blue-framed GAED

component in A (B). High-power view of the red-framed conventional adenocarcinoma component in A (C). Immunohistochemical (IHC) staining for α-fetoprotein (AFP) (D), Glypican-3 (GPC3) (E), and palt-like transcription factor 4 (SALL4) (F). Gastric adenocarcinoma with enteroblastic marker expression showing both solid and nonsolid growth patterns (G). Immunohistochemical (IHC) staining for alpha-fetoprotein (AFP) (H), Glypican-3 (GPC3) with a high-power-view image of a partially positive area (inset at lower right) (I) and Spalt-like transcription factor 4 (SALL4) (J).

Figure 2: Comparison of Overall survival in GAED, GA with EM, and CGA.

(A) All three curves (GAED, GA with EM and CGA) were put in one panel, and each pairwise comparison was performed. GAED and GA with EM show similar trends in overall survival rates (p=0.78), however, patients with GAED show rather worse prognosis than GA with EM especially during first 3 years. GAED has a significantly poorer overall survival than CGA (p=0.035), but survival difference between GA with EM and CGA loses statistical significance (p=0.157). (B) GAs showing expression of EM regardless of the presence of clear cytoplasm (GAED and GA with EM) have worse overall survival than CGAs (p=0.018).

Figure 3: Comparison of overall survival by each factor in GAED.

(A) lymphatic invasion (p<0.01), (B) liver metastasis (p<0.01), (C) growth pattern (Solid type,

p<0.01) and (D) expression of GPC3 (p<0.05) were significantly associated with poor overall survival.

Figure 4: Comparison of overall survival by each factor in GA with EM.

(A) lymph nodes metastasis (p<0.01) and (B) liver metastasis (p<0.01) were significantly associated with poor overall survival.

Supplementary Table 1

Clinicopathological characteristics of study patients (n=688)

Abbreviations: SD, Standard deviation; U, Upper third; M, Middle third; L, Lower third; MP, Muscularis mucosae; SS, Subserosa; SE, Tumor penetration of serosa; SI, Tumor invasion of adjacent structures; TNM, Tumor-node-metastasis.

Supplementary Table 2

Histological and immunohistochemical findings in 688 GAs, including GAED and GA with EM

Abbreviations: GA, gastric adenocarcinoma; GAED, gastric adenocarcinoma with enteroblastic differentiation; EM, enteroblastic marker; CC, clear cytoplasm; AFP, α-fetoprotein; GPC3, Glypican-3; SALL4, Spalt-like transcription factor 4.

Table 1

Clinicopathological findings (GAED vs GA with EM)

Abbreviations: GAED, gastric adenocarcinoma with enteroblastic differentiation; GA, gastric adenocarcinoma; EM, enteroblastic marker; SD, Standard deviation; U, Upper third; M, Middle third; L, Lower third; MP, Muscularis mucosae; SS, Subserosa; SE, Tumor penetration of serosa; SI, Tumor invasion of adjacent structures; TNM, Tumor-node-metastasis; AFP, α-fetoprotein; GPC3, Glypican-3; SALL4, Spalt-like transcription factor 4.

Table 2

Clinicopathological findings (GAED vs CGA)

Abbreviations: CGA, conventional gastric adenocarcinoma

Table 3

Clinicopathological findings (GA with EM vs CGA)

Table 4

Clinicopathological findings according to EM expression

Supplementary Table 3

Clinicopathological findings in CGA according to clear cytoplasm

Abbreviations: CC, clear cytoplasm

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Author contributions statement

D Abe, T.S., T.H., H.U., A.N., and T.Y. planned this project and D Abe, T.S., Y.A., N.Y., and T.Y. contributed to the diagnosis of GAED and GA with EM cases. D Abe and T.S. performed the main parts of the experiments and D Abe, T.S., T.H., H.U., S.M., T.F. and A.N. collected and analyzed the clinical data. D Abe and T.S. wrote the majority of the manuscript. All authors approved the final version of the manuscript.

Figure 1

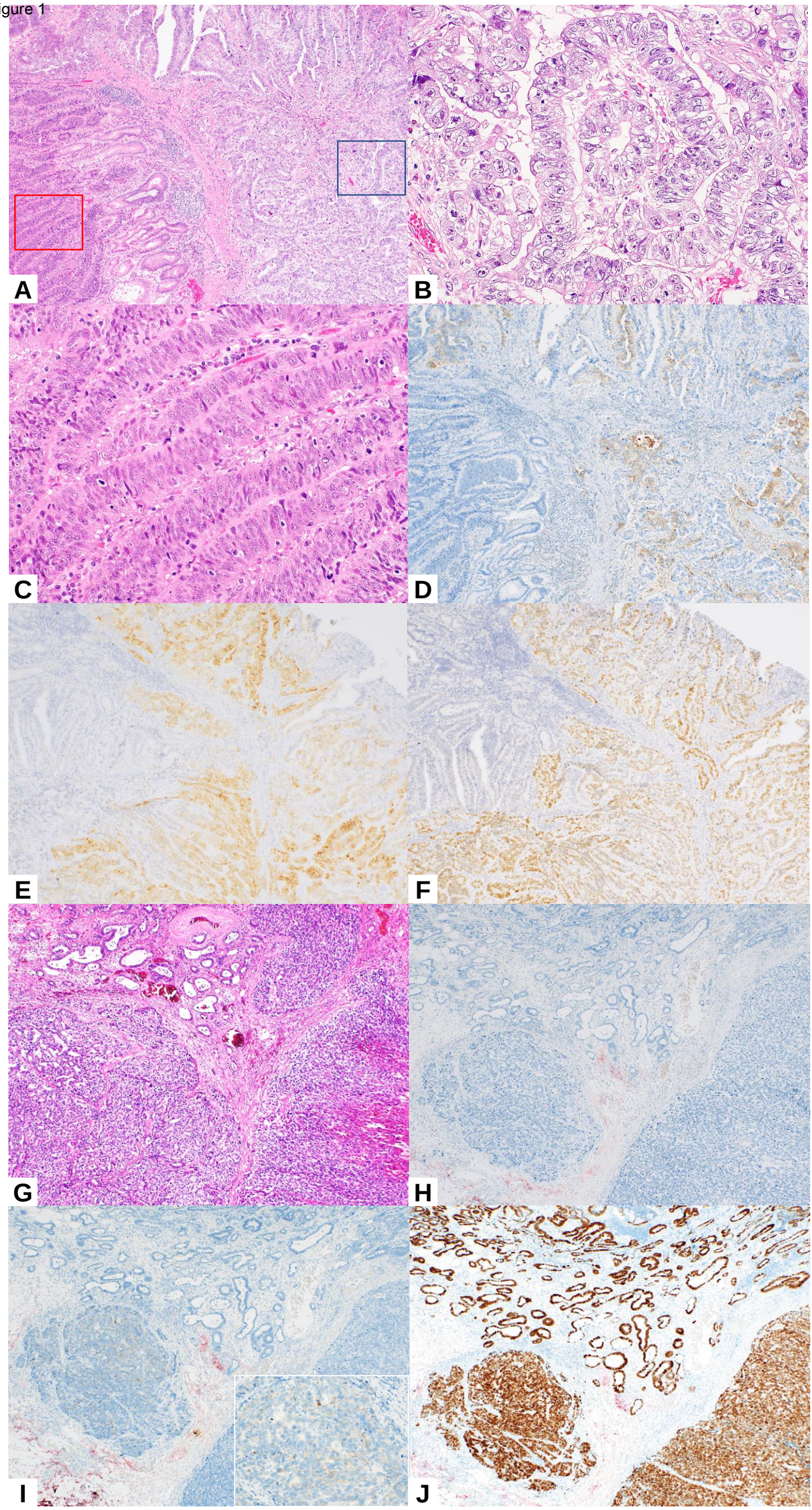


Figure 2

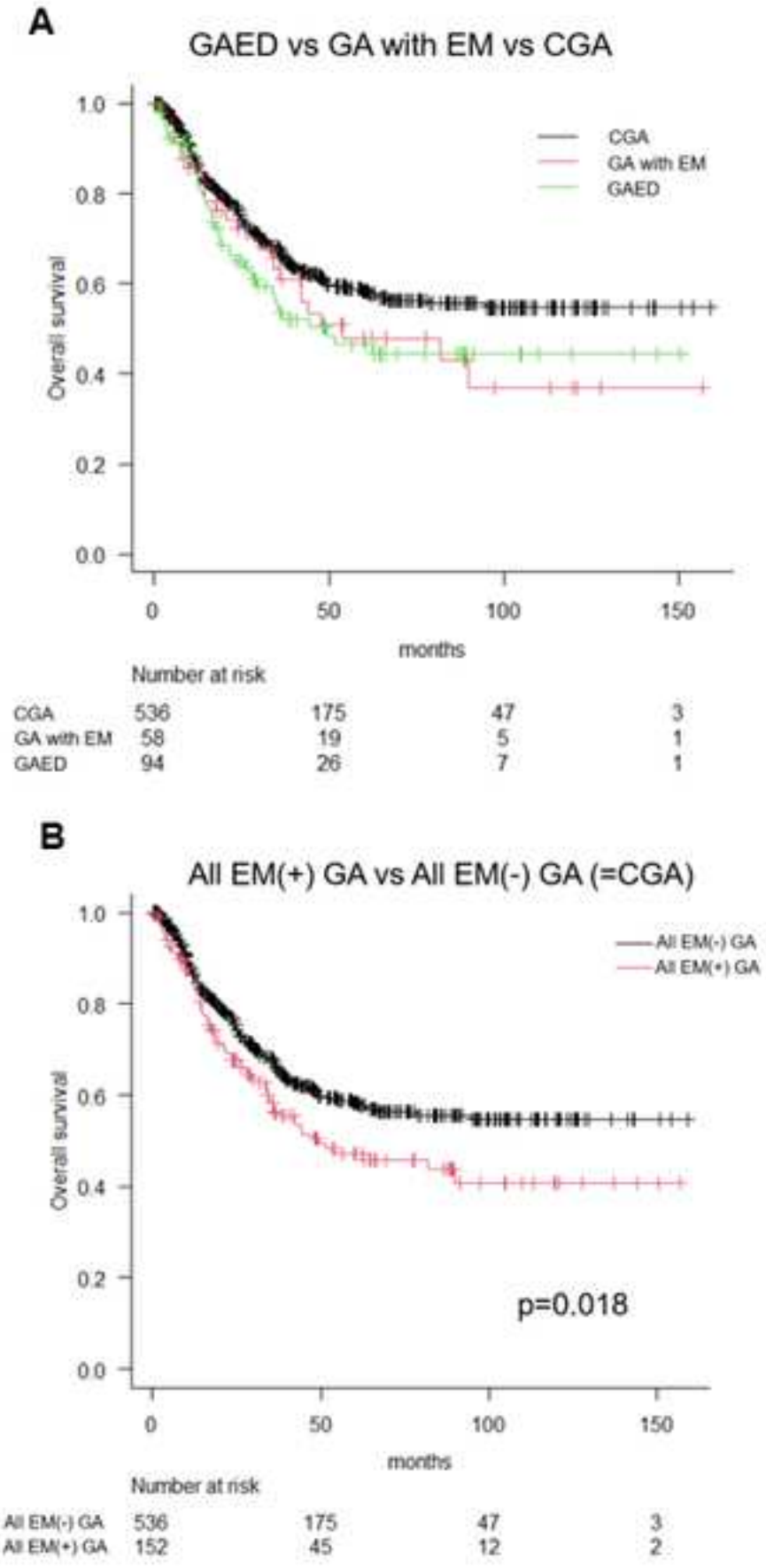
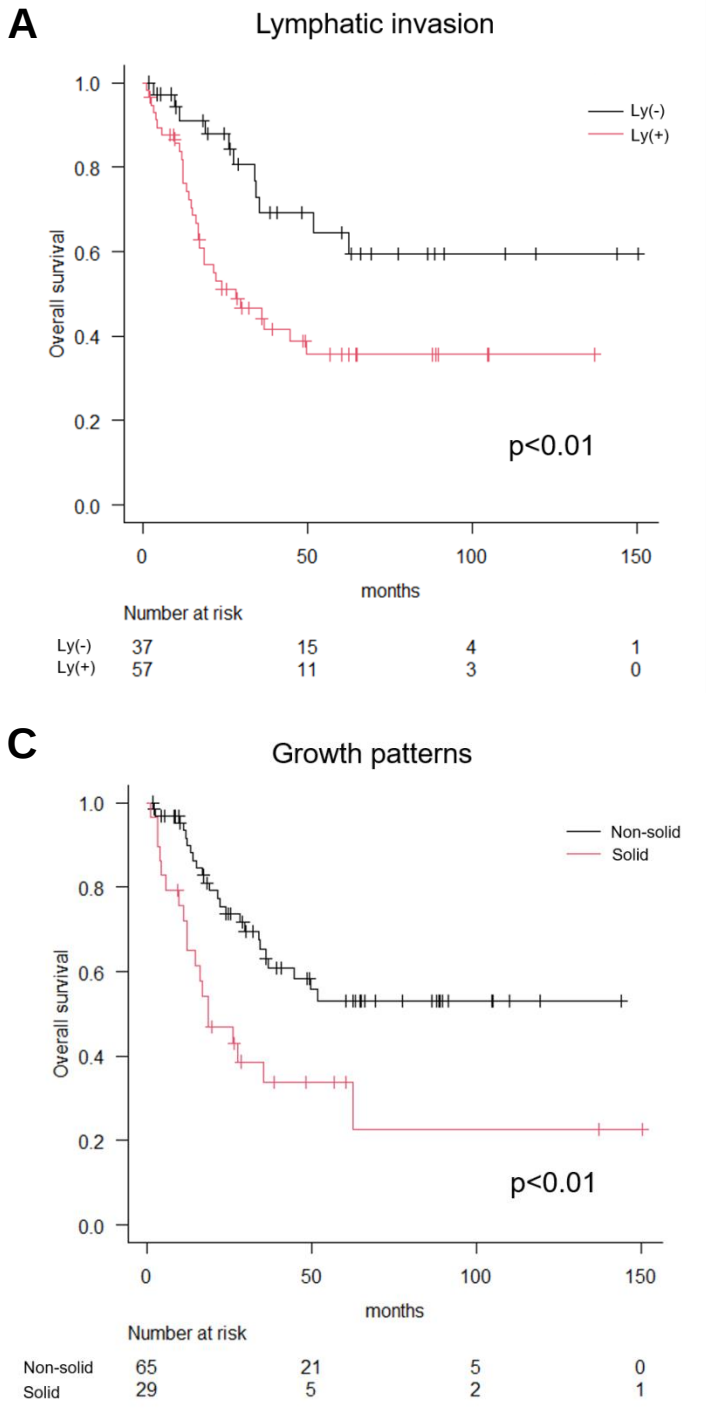


Figure 3


A
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Number at risk
Ly(-) 37 15 4 1
Ly(+) 57 11 3 0
B
Liver metastasis
Overall survival
(-)
(+)
p<0.01
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Number at risk
(-) 55 21 7 1
(+) 39 5 0 0
C
Growth patterns
Overall survival
Non-solid
Solid
p<0.01
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Number at risk
Non-solid 65 21 5 0
Solid 29 5 2 1
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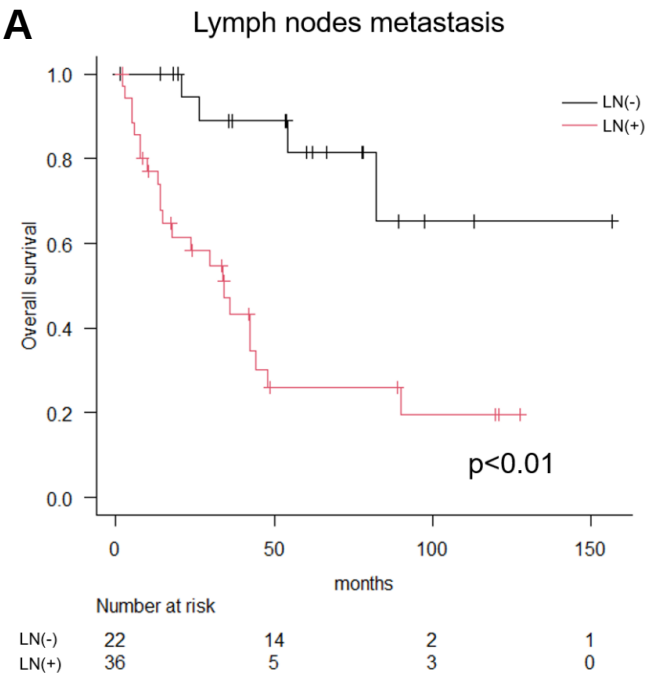
Figure 4


A
Lymph nodes metastasis
Overall survival
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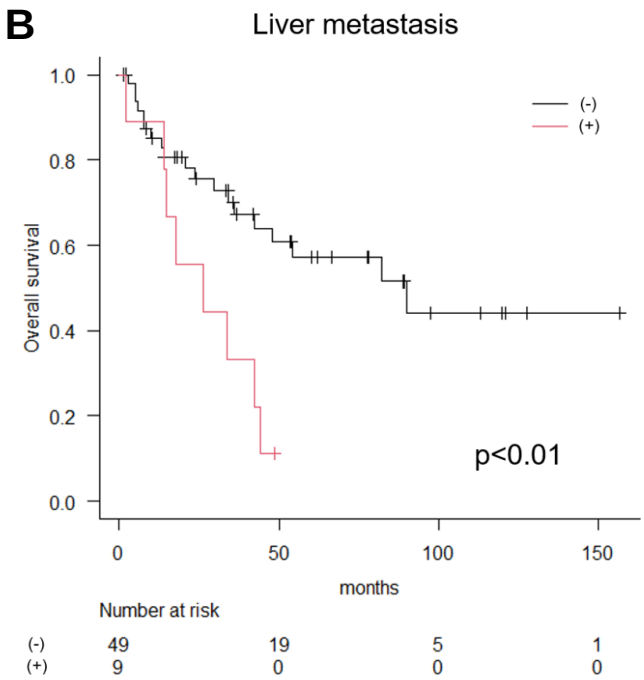
B
Liver metastasis
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Number at risk
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Table 1. Clinicopathological findings (GAED vs GA with EM)

	GAED n=94 (13.7%)	GA with EM n=58 (8.4%)	p value
Age (years) (mean±SD)	72.5±10.6	70.2±9.9	0.189
Sex (male/female)	76/18	48/10	0.832
Tumor location (U/M/L)	34/21/39	28/17/13	0.056
Tumor size (mm) (mean±SD)	60.9±32.3	65.3±40.6	0.483
Macroscopic type (Borrmann1/2/3/4/5)	10/46/24/2/12	2/21/17/6/12	<0.05
Invasion depth (MP/SS/SE/SI)	17/57/17/3	7/27/23/1	<0.05
TNM stage (I/II/III/IV)	9/37/38/10	5/22/19/12	0.373
TNM stage (IB/IIA/IIB/IIIA/IIIB/IIIC/IV)	9/15/22/17/18/3/10	5/14/8/12/4/3/12	0.126
Lymphatic invasion (+) n (%)	57 (60.6%)	45 (77.6%)	<0.05
Venous invasion (+) n (%)	63 (67.0%)	29 (50.0%)	<0.05
Lymph node metastasis (+) n (%)	72 (76.6%)	36 (62.1%)	0.067
Liver metastasis (+) n (%)	39 (41.5%)	9 (15.5%)	<0.01
Growth patterns (Solid/Non-solid type)	29 (30.9%)/65 (69.1%)	8 (13.8%)/50 (86.2%)	<0.05
Immunohistochemical findings			
AFP (+)	29 (30.9%)	9 (15.5%)	<0.05
GPC3 (+)	65 (69.1%)	32 (55.2%)	0.086
SALL4 (+)	76 (80.9%)	40 (69.0%)	0.117

Abbreviations: GAED, gastric adenocarcinoma with enteroblastic differentiation; GA, gastric adenocarcinoma; EM, enteroblastic marker; SD, Standard deviation; U, Upper third; M, Middle third; L, Lower third; MP, Muscularis mucosae; SS, Subserosa; SE, Tumor penetration of serosa; SI, Tumor invasion of adjacent structure; TNM, Tumor-node-metastasis; AFP, α-fetoprotein; GPC3, Glypican-3; SALL4, Spalt-like transcription factor 4

Table 2. Clinicopathological findings (GAED vs CGA)

	GAED n=94 (13.7%)	CGA n=536 (77.9%)	p value
Age (years) (mean±SD)	72.5±10.6	68.5±11.7	<0.01
Sex (male/female)	76/18	379/157	<0.05
Tumor location (U/M/L)	34/21/39	163/189/184	<0.05
Tumor size (mm) (mean±SD)	60.9±32.3	69.1±41.7	<0.05
Macroscopic type (Borrmann1/2/3/4/5)	10/46/24/2/12	34/128/168/93/113	<0.01
Invasion depth (MP/SS/SE/SI)	17/57/17/3	114/181/206/35	<0.01
TNM stage (I/II/III/IV)	9/37/38/10	65/176/211/83	0.433
TNM stage (IB/IIA/IIB/IIIA/IIIB/IIIC/IV)	9/15/22/17/18/3/10	65/92/84/82/69/60/83	0.053
Lymphatic invasion (+) n (%)	57 (60.6%)	364 (67.9%)	0.191
Venous invasion (+) n (%)	63 (67.0%)	188 (35.1%)	<0.01
Lymph node metastasis (+) n (%)	72 (76.6%)	362 (67.5%)	0.092
Liver metastasis (+) n (%)	39 (41.5%)	43 (8.0%)	<0.01

Abbreviations: GAED, gastric adenocarcinoma with enteroblastic differentiation; CGA, conventional gastric adenocarcinoma; SD, Standard deviation; U, Upper third; M, Middle third; L, Lower third; MP, Muscularis mucosae; SS, Subserosa; SE, Tumor penetration of serosa; SI, Tumor invasion of adjacent structure; TNM, Tumor-node-metastasis

Table 3. Clinicopathological findings (GA with EM vs CGA)

	GA with EM n=58 (8.4%)	CGA n=536 (77.9%)	p value
Age (years) (mean±SD)	70.2±9.9	68.5±11.7	0.228
Sex (male/female)	48/10	379/157	0.064
Tumor location (U/M/L)	28/17/13	163/189/184	<0.05
Tumor size (mm) (mean±SD)	65.3±40.6	69.1±41.7	0.503
Macroscopic type (Borrmann1/2/3/4/5)	2/21/17/6/12	34/128/168/93/113	0.288
Invasion depth (MP/SS/SE/SI)	7/27/23/1	114/181/206/35	0.093
TNM stage (I/II/III/IV)	5/22/19/12	65/176/211/83	0.489
TNM stage (IB/IIA/IIB/IIIA/IIIB/IIIC/IV)	5/14/8/12/4/3/12	65/92/84/82/69/60/83	0.299
Lymphatic invasion (+) n (%)	45 (77.6%)	364 (67.9%)	0.178
Venous invasion (+) n (%)	29 (50.0%)	188 (35.1%)	<0.05
Lymph node metastasis (+) n (%)	36 (62.1%)	362 (67.5%)	0.38
Liver metastasis (+) n (%)	9 (15.5%)	43 (8.0%)	0.081

Abbreviations: GA, gastric adenocarcinoma; EM, enteroblastic marker; CGA, conventional gastric adenocarcinoma; SD, Standard deviation; U, Upper third; M, Middle third; L, Lower third; MP, Muscularis mucosae; SS, Subserosa; SE, Tumor penetration of serosa; SI, Tumor invasion of adjacent structure; TNM, Tumor-node-metastasis

Table 4. Clinicopathological findings according to the EM expression

	All EM(+) GA n=152 (22.1%)	All EM(-) GA (=CGA) n=536 (77.9%)	p value
Age (years) (mean±SD)	71.6±10.4	68.5±11.7	<0.01
Sex (male/female)	124/28	379/157	<0.01
Tumor location (U/M/L)	62/38/52	163/189/184	<0.05
Tumor size (mm) (mean±SD)	62.6±35.6	69.1±41.7	0.057
Macroscopic type (Borrmann1/2/3/4/5)	12/67/41/8/24	34/128/168/93/113	<0.01
Invasion depth (MP/SS/SE/SI)	24/84/40/4	114/181/206/35	<0.01
TNM stage (I/II/III/IV)	14/59/57/22	65/176/211/83	0.513
TNM stage (IB/IIA/IIB/IIIA/IIIB/IIIC/IV)	14/29/30/29/22/6/22	65/92/84/82/69/60/83	0.119
Lymphatic invasion (+) n (%)	102 (67.1%)	364 (67.9%)	0.844
Venous invasion (+) n (%)	92 (60.5%)	188 (35.1%)	<0.01
Lymph node metastasis (+) n (%)	108 (71.1%)	362 (67.5%)	0.489
Liver metastasis (+) n (%)	48 (31.6%)	43 (8.0%)	<0.01

Abbreviations: GA, gastric adenocarcinoma; EM, enteroblastic marker; CGA, conventional gastric adenocarcinoma; SD, Standard deviation; U, Upper third; M, Middle third; L, Lower third; MP, Muscularis mucosae; SS, Subserosa; SE, Tumor penetration of serosa; SI, Tumor invasion of adjacent structure; TNM, Tumor-node-metastasis