Prognostic value and association of Lauren classification with VEGF and VEGFR-2 expression in gastric cancer

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Abstract. Gastric cancer (GC) is one of the most common malignant tumors in the world. As anti-angiogenic therapy shows efficacy in the treatment of GC, but only works in certain patients, the identification of potential beneficiaries are urgently required in order to apply appropriate treatments. The Lauren classification demonstrates numerous differences in etiology, epidemiology and pathology; however, the association between Lauren classification and pro-angiogenic factors remains unclear. The present study aimed to investigate the clinicopathological factors associated with Lauren classification and the prognostic significance of Lauren classification and vascular endothelial growth factor (VEGF) and VEGF receptor-2 (VEGFR-2) expression in GC. Paraffin-embedded GC tissues and clinical information of 255 patients with GC were collected. The clinicopathological factors associated with Lauren classification were evaluated by Logistic regression analysis. Kaplan-Meier survival and Cox regression analyses were used to examine the prognostic significance of Lauren classification and of VEGF and VEGFR-2 expression in patients with GC. The results demonstrated that there was no association between Lauren classification and VEGF and VEGFR-2 expression. Furthermore, results from survival analysis demonstrated that Lauren classification (P=0.001) and Tumor-Node-Metastasis stage (stage II, P=0.002; stage III, P<0.001) were independent prognostic factors in GC. Following subgroup analysis based on Tumor-Node-Metastasis stage, Lauren classification was demonstrated to be an independent prognostic factor in patients with stage III GC (P=0.010) but not in patients with stage I or II GC. Furthermore, VEGFR-2 overexpression was an independent predictor of survival in intestinal-type GC (P=0.040) but not in diffuse-type GC. Taken together, these results indicate that Lauren classification may serve as an independent prognostic factor for patients with GC. In addition, although the expression of VEGF and VEGFR-2 was not associated with Lauren classification, VEGFR-2 overexpression may be considered as an independent prognostic factor in intestinal-type GC.

Introduction

Gastric cancer (GC) is one of the most common malignant tumors in the world, notably in China, where it has the highest incidence compared with other countries (1). In China, GC is the second most frequent type of cancer and the second leading cause of cancer-associated mortality. In 2015, GC accounted for 679,100 new cases and 498,000 mortalities (2). Despite the development of comprehensive treatment approaches, such as anti-HER2 therapy, immunotherapy and anti-angiogenic therapy, the prognosis of GC remains poor and its current understanding remains limited.

Lauren classification has been widely accepted and used by pathologists and physicians since its introduction in 1965 (3). Lauren classification allows GC classification into three histological types, including intestinal-, diffuse- and mix-types, according to histopathological features of GC tissues (3). In intestinal-type GC, tumor cells exhibit adhesion and are arranged in tubular or glandular formations, whereas in diffuse-type GC, tumor cells infiltrate the stroma as single cells or small clusters due to lack of adhesion (3). Mix-type GC possesses all these characteristics (3). The proportion of men and elderly patients is higher in intestinal-type GC, whereas diffuse-type GC is more likely to happen in women and younger patients (4,5). Lauren classification presents differences in etiology, epidemiology and pathology, which means that certain tumor development pathways are characteristics of different Lauren classifications (4,6,7).
Angiogenesis, a complex process involving multiple growth factors and signaling pathways, such as vascular endothelial growth factor (VEGF), angiopoietin, fibroblast growth factor and platelet-derived growth factor, is recognized as one of the ‘hallmarks of cancer’ and serves crucial role in tumor growth and progression (8). VEGF and its receptor VEGF receptor 2 (VEGFR-2) are the most important pro-angiogenic factors (9). Whether the angiogenic phenotype differs between the intestinal-type and diffuse-type of GC is controversial. Previous studies reported that intestinal-type GC is more dependent on angiogenesis than diffuse-type (10,11); however, some studies reported opposite results and demonstrated that microvessel density was higher in diffuse-type GC compared with intestinal-type GC (12,13).

Considering the poor prognosis of GC, it is crucial to determine prognostic factors for identifying high-risk patients and provide them with the appropriate treatment. The present study aimed to identify factors associated with Lauren classification and clarify whether VEGF and VEGFR-2 expression is associated with Lauren classification. Furthermore, the present study aimed to analyze the prognostic value of Lauren classification in patients with GC, and to investigate the prognostic value of VEGF and VEGFR-2 expression in different Lauren classifications.

Materials and methods

Patients. The present study was approved by the Ethics Committee of Xinhua Hospital, and written informed consent was obtained from all patients prior to the study. Data from 255 patients with GC who underwent surgical gastrectomy between July 2009 and July 2014 at the Xinhua Hospital, School of Medicine, Shanghai Jiao Tong University (Shanghai, China) were retrieved. The inclusion criteria were as follows: i) Gastric cancer was histologically confirmed; ii) adequate paraffin-embedded tumor tissue samples were provided for further analyses; and iii) complete medical records with regular follow-up data were accessible (the final follow-up time was August 2018 and the survival times were recorded). The exclusion criteria were as follows: i) Patients who suffered recurrences or multiple cancers; ii) patients who underwent preoperative chemotherapy or radiotherapy; and iii) patients with incomplete clinical information. The present study included the patients who received postoperative chemotherapy. A total of 255 patients with GC were included in the present study. The mean age at diagnosis was 63.8 years (range, 27-88 years) and the male-to-female ratio was 1.8:1.0.

Immunohistochemistry. Tumor tissues were obtained during surgery and fixed in 10% neutral buffered formalin for 24 h at room temperature. Tissues were then dehydrated with 70% ethanol for 40 min, 95% ethanol for 40 min, 95% ethanol for 40 min, 100% ethanol for 40 min and then 100% ethanol for 40 min, all at room temperature. Tissues were incubated in xylene twice for 40 min at room temperature and then incubated in paraffin twice for 40 min at 60°C. The tumor tissues were then embedded in paraffin to create a formalin-fixed, paraffin-embedded block and were stored at room temperature for the subsequent analyses.

Immunohistochemical analysis of VEGF and VEGFR-2 expression were conducted on paraffin-embedded tissue samples. Each paraffin-embedded sample was cut into 5 μm slices and tissue slices were deparaffinized in xylene twice for 10 min at room temperature, rehydrated with 100% ethanol for 10 min, 100% ethanol for 10 min, 95% ethanol for 5 min and 75% ethanol for 5 min, all at room temperature, and placed in 3% H₂O₂ dissolved in methanol for 10 min at room temperature. Slices were then incubated with 10% normal goat serum (Beijing Solarbio Science & Technology Co., Ltd.) for 60 min at room temperature. Following overnight incubation at 4°C with primary antibody against VEGF (cat. no. ab1316; 1:100; Abcam) and VEGFR-2 (cat. no. ab2349; 1:100; Abcam), slices were incubated for 30 min at room temperature with the horseradish peroxidase-conjugated secondary antibody Envision TM Detection kit (cat. no. G500705; Sener Biotechnology) according to the manufacturer's instructions. Finally, all slices were incubated with diamino-benzidine (Beyotime Institute of Biotechnology) for 3-5 min at room temperature and counterstained with hematoxylin for 30 sec at room temperature.

The slides were examined under light microscope (Olympus Corporation) at x200 magnification and the assessment of VEGF and VEGFR-2 staining was performed by two blinded pathologists as previously described (14). For VEGF expression, the staining intensity was scored as follows: i) 0, no coloration; ii) 1, light brown; iii) 2, brown; and iv) 3, dark brown. The percentage of stained cells was scored as 1, 2, 3, 4 and 5, for 0-20, 21-40, 41-60, 61-80 and 81-100% of positively stained cells, respectively. The total score was defined as follows: staining intensity score x percentage of positively stained cells. Total scores of 0-5 and ≥6 were defined as VEGF (−) and VEGF (+), respectively. For VEGFR-2 expression, staining intensity was scored as follows: i) 0, no coloration; ii) 1, light brown; iii) 2, brown; and iv) 3, dark brown. The percentage of stained cells was scored as 0, 1, 2, 3 and 4 for 0-5, 6-25, 26-50, 51-75 and 76-100% of positively stained cells, respectively. Total scores of 0-1, 2-4 and 5-12 were defined as VEGFR-2 (−), VEGFR-2 (+) and VEGFR-2 (++ - ++++), respectively.

Lauren classification. Tumor tissue samples were examined by two experienced pathologists who were blinded to the patients' information and classified according to Lauren classification (3). The intestinal-type GC preserved the tubular or glandular appearance, whereas diffuse-type GC did not present tubular structures and comprised single or small clusters of cells. The mix-type GC was described as the combination of diffuse-type and intestinal-type.

Statistical analysis. SPSS 23.0 (IBM Corp.) and GraphPad Prism 7 (GraphPad Software, Inc.) software programs were used for all statistical analyses. A two-sided P<0.05 was considered to indicate a statistically significant difference. Factors associated with diffuse-type GC were assessed using logistic regression analysis to calculate an odds ratio (OR) with a corresponding 95% confidence interval (CI). Overall survival (OS) time was calculated from the first day of surgery to the final day of follow-up (August 2018) or mortality. Univariate and multivariate Cox regression analyses were performed to calculate the hazards ratio (HR) and 95% CI for identifying
factors associated with GC prognosis. All variables in the univariate analysis were entered into the multivariate analysis to determine independent prognostic factors. Survival curves were calculated using Kaplan-Meier curve analysis, and a log-rank test was used to compare survival times within subgroups.

## Results

### Patient characteristics.

The clinicopathological characteristics of the 255 patients with GC included in this study are presented in Table I. Almost half of the patients presented with stage I GC (47.8%), and the majority of patients had poor differentiation (65.9%), non-cardia location (92.9%) and tumor diameter ≤4 cm (70.6%). Among the 255 patients with GC, 45 (17.6%) cases presented VEGF (+), and 82 (32.2%) and 135 (52.9%) cases presented VEGFR (+) and VEGFR (++ - +++), respectively. Representative pictures of VEGF and VEGFR-2 expression are presented in Fig. 1. There were 159 (62.4%) patients with intestinal-type GC, 91 (35.7%) patients with diffuse-type GC and 5 (2.0%) patients with mix-type GC. Because only 2 patients presented with stage IV GC and 5 patients presented with mix-type GC, data from these patients were excluded. The data from 248 patients with GC were therefore used for further analysis.

### Factors associated with Lauren classification.

Univariate analysis demonstrated that stage III (P<0.001), poor differentiation (P<0.001), tumor diameter >4 cm (P=0.001), patients who received adjuvant chemotherapy (P<0.001) and VEGFR-2 (+) (P=0.048) were variables that were significantly associated with diffuse-type GC (Table II). Following multivariate analysis, poor differentiation (OR, 30.060; 95% CI, 8.651-104.453; P<0.001), non-cardia location (OR, 4.681; 95% CI, 1.025-21.376; P=0.046) and patients who received adjuvant chemotherapy (OR, 2.307; 95% CI, 1.066-4.993; P=0.034) remained significantly associated with diffuse-type GC. The expression of VEGF and VEGFR-2 were not associated with Lauren classification following multivariate analysis (Table II).

### Survival analysis for all patients.

After a median follow-up period of 6.31 years, 168 (67.7%) patients had survived and 80 (32.3%) patients had died. Following univariate analysis, TNM stage (stage II, P<0.001; stage III, P<0.001), differentiation (P=0.017), tumor diameter >4 cm (P=0.001), patients who received adjuvant chemotherapy (P<0.001) and VEGFR-2 (+) (P=0.048) were variables that were significantly associated with diffuse-type GC (Table II). Following multivariate regression analysis, only TNM stage (stage II HR, 3.492; 95% CI, 1.604-7.602; P=0.002; stage III HR, 6.208; 95% CI, 2.127-19.376; P=0.001) and Lauren classification (HR, 3.492; 95% CI, 1.604-7.602; P=0.002) were significantly associated with OS time (Table III). Following multivariate regression analysis using only TNM stage (stage II HR, 3.492; 95% CI, 1.604-7.602; P=0.002; stage III HR, 6.208; 95% CI, 2.127-19.376; P=0.001) and Lauren classification (HR, 3.492; 95% CI, 1.604-7.602; P=0.002) were significantly associated with OS time (Table III). The association between Lauren classification and TNM stage for OS was evaluated using subgroup analyses. Kaplan-Meier curve and log-rank test demonstrated that Lauren classification was significantly associated with OS in stage III subgroup (P=0.001); however, survival analysis in subgroups. The association between Lauren classification and TNM stage for OS was evaluated using subgroup analyses. Kaplan-Meier curve and log-rank test demonstrated that Lauren classification was significantly associated with OS in stage III subgroup (P=0.001); however, survival analysis in subgroups. The association between Lauren classification and TNM stage for OS was evaluated using subgroup analyses. Kaplan-Meier curve and log-rank test demonstrated that Lauren classification was significantly associated with OS in stage III subgroup (P=0.001); however.
this was not the case in stage I (P=0.372) or stage II (P=0.222) subgroups (Fig. 2). Furthermore, multivariate analysis demonstrated that Lauren classification was an independent prognostic factor in stage III subgroup (HR, 2.870; 95% CI, 1.293-6.371; P=0.010) (data not shown).

Based on the results of previous studies, VEGF/VEGFR-2 expression and Lauren classification are associated with clinical outcomes (15-17). Intestinal-type is more dependent on angiogenesis than diffuse-type (10), it is possible that the impact of VEGF and VEGFR-2 expression on clinical outcomes might differ between intestinal-type and diffuse-type GC. Therefore, we analyzed the impact of VEGF and VEGFR-2 expression on OS in different Lauren classifications. The results demonstrated a significant difference for VEGFR-2 expression only in the intestinal-type subgroup (P=0.001) (Fig. 3A). Subsequently, multivariate regression analysis in intestinal-type subgroup was performed, and demonstrated that VEGFR-2 (++ - ++++) may be considered as an independent prognostic factor for OS (HR, 4.903; 95% CI, 1.076-22.354; P=0.040) (data not shown).

Discussion

Lauren classification can divide GC into intestinal-, diffuse- and mix-types (18). Since mix-type GC possesses the characteristics of intestinal- and diffuse-types, only intestinal- and diffuse-types GC were included in the present study. Previous studies investigating the clinicopathological characteristics of GC according to Lauren classification reported distinct clinical characteristics between the intestinal- and diffuse-types GC (4,19-21). It has been demonstrated that there are more patients >65 years and more male patients in intestinal-type GC compared with diffuse-type GC, and that intestinal-type GC is associated with less aggressive features, including smaller tumor size, well-differentiated tumors, less tumor invasion depth and less lymphovascular invasion (19). Conversely, diffuse-type GC is characterized by more aggressive features, including advanced pathological T and N stages and advanced TNM stage (4,20).

In the present study, the proportion of diffuse-type GC was higher in patients with poor differentiation and non-cardia location, which was consistent with previous studies (4,21). Furthermore, patients who had received adjuvant chemotherapy mostly suffered from diffuse-type GC, which could be explained by the higher proportion of patients with poor differentiation histological grade in this subgroup.

A more aggressive behavior of diffuse-type GC may contribute to the poor prognosis of patients with diffuse-type GC. Qiu et al (4) and Chen et al (19) demonstrated that the Lauren classification was an independent prognostic factor for survival time, which was consistent with the results of the present study. However, a number of studies have demonstrated that the Lauren classification represents a significant prognostic factor for survival following the univariate analysis, but was not identified as an independent predictor following the multivariate analysis (22,23). This discrepancy may arise from different populations, limited sample size, various study design, among other things. Yamashita et al (22) suggested that diffuse-type advanced GC presenting with dismal prognosis was characterized by deeper invasion and emerging peritoneal cancer cell. The present study supports this suggestion, as it was also demonstrated that diffuse-type GC was a poor prognostic factor in stage III patients compared with stages I or II in the subgroup analysis.
Angiogenesis serves a crucial role in tumor cell survival and proliferation, and anti-angiogenic therapy has become a novel approach to treat cancer (24). Recently, numerous clinical studies on anti-angiogenic drugs have been performed in patients with GC (25-27). Ramucirumab, which is a human monoclonal antibody, can target the extracellular domain of VEGFR-2 and block the binding of VEGF, thereby preventing activation of the pro-angiogenic signaling pathway VEGF/VEGFR-2 (28). Furthermore, results from two randomized phase III trials (RAGARD and RAINBOW trials) allowed the US Food and Drug Administration (FDA) to approve ramucirumab monotherapy or combined with paclitaxel as

Table II. Factors associated with Lauren classification in patients with gastric cancer.

| Variables                  | Univariate analysis                  | Multivariate analysisa |
|---------------------------|--------------------------------------|------------------------|
|                           | OR (95% CI)  | P-value  | OR (95% CI)  | P-value  |
| Age, years                |                        |          |                        |          |
| ≤65                       | 1.00       |          | 1.00       |          |
| >65                       | 0.807 (0.474-1.375) | 0.430    | 0.941 (0.451-1.963) | 0.872    |
| Sex                       |                        |          |                        |          |
| Male                      | 1.00       |          | 1.00       |          |
| Female                    | 1.203 (0.702-2.062) | 0.502    | 1.125 (0.542-2.334) | 0.752    |
| TNM stage                 |                        |          |                        |          |
| I                         | 1.00       |          | 1.00       |          |
| II                        | 1.142 (0.553-2.358) | 0.720    | 0.637 (0.247-1.639) | 0.349    |
| III                       | 2.948 (1.622-5.358) | <0.001b  | 1.274 (0.531-3.058) | 0.587    |
| Differentiation           |                        |          |                        |          |
| Moderate/well             | 1.00       |          | 1.00       |          |
| Poor                      | 31.289 (9.495-103.108) | <0.001b  | 30.060 (8.651-104.453) | <0.001b  |
| Tumor location            |                        |          |                        |          |
| Cardia                    | 1.00       |          | 1.00       |          |
| Non-cardia                | 2.819 (0.788-10.909) | 0.111    | 4.681 (1.025-21.376) | 0.046c   |
| Tumor diameter, cm        |                        |          |                        |          |
| ≤4                        | 1.00       |          | 1.00       |          |
| >4                        | 2.525 (1.428-4.466) | 0.001b   | 1.646 (0.740-3.660) | 0.221    |
| Smoking history           |                        |          |                        |          |
| No                        | 1.00       |          | 1.00       |          |
| Yes                       | 1.038 (0.577-1.867) | 0.902    | 0.977 (0.413-2.31) | 0.958    |
| Drinking history          |                        |          |                        |          |
| No                        | 1.00       |          | 1.00       |          |
| Yes                       | 1.276 (0.580-2.809) | 0.545    | 2.639 (0.742-9.390) | 0.134    |
| Family history            |                        |          |                        |          |
| No                        | 1.00       |          | 1.00       |          |
| Yes                       | 0.519 (0.164-1.642) | 0.264    | 0.557 (0.127-2.431) | 0.436    |
| Chemotherapy              |                        |          |                        |          |
| No                        | 1.00       |          | 1.00       |          |
| Yes                       | 2.659 (1.558-4.537) | <0.001b  | 2.307 (1.066-4.993) | 0.034b   |
| VEGF expression           |                        |          |                        |          |
| (-)                       | 1.00       |          | 1.00       |          |
| (+)                       | 0.819 (0.408-1.647) | 0.576    | 0.619 (0.248-1.545) | 0.304    |
| VEGFR-2 expression        |                        |          |                        |          |
| (-)                       | 1.00       |          | 1.00       |          |
| (+)                       | 2.400 (1.009-5.707) | 0.048b   | 0.862 (0.278-2.669) | 0.796    |
| (++ - ++++)               | 1.808 (0.788-4.147) | 0.162    | 0.631 (0.208-1.909) | 0.415    |

aMultivariate analysis was carried out by adjusting all parameters listed in Table II. bP<0.05. CI, confidence interval; OR, odd ratio; TNM, Tumor-Node-Metastasis; VEGF, vascular endothelial growth factor; VEGFR-2, VEGF receptor 2.
second-line treatment for patients with GC (29,30). In addition, apatinib, which is a tyrosine kinase inhibitor that selectively inhibits VEGFR2, has been approved by the China FDA for patients with advanced GC (31). Furthermore, results from a
phase III trial demonstrated that apatinib treatment can significantly extend OS and progression-free survival (PFS) times in patients with GC who were refractory to at least two lines of chemotherapy (32). Bevacizumab is a recombinant humanized monoclonal antibody with high affinity for VEGF (33). A randomized, double-blind, phase III study demonstrated that bevacizumab combined with capecitabine-cisplatin as first-line treatment for GC can improve PFS but not OS in patients with

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Figure 2. Kaplan-Meier survival analysis of the prognostic value of Lauren classification in patients with different Tumor-Node-Metastasis stages. (A) OS rate of patients with stage I GC according to Lauren classification. (B) OS rate of patients with stage II GC according to Lauren classification. (C) OS rate of patients with stage III GC according to Lauren classification. GC, gastric cancer; OS, overall survival.

Figure 3. Kaplan-Meier survival analysis of the prognostic value of VEGF and VEGFR-2 expression in patients with GC according to Lauren classification. (A) OS rate of patients with intestinal-type GC according to VEGFR-2 expression. (B) OS rate of patients with diffuse-type GC according to VEGFR-2 expression. (C) OS rate of patients with intestinal-type GC according to VEGF expression. (D) OS rate of patients with diffuse-type GC according to VEGF expression. GC, gastric cancer; OS, overall survival; VEGF, vascular endothelial growth factor; VEGFR-2, VEGF receptor 2.
GC; however, following subgroup analysis, bevacizumab was reported to prolong OS in the pan-America group (34).

As not many effective biomarkers for anti-angiogenic targeted therapy have been identified, their efficacy may be underestimated. Clarifying the association between Lauren classification and VEGF and VEGFR-2 expression, and performing subgroup survival analysis for VEGF/VEGFR-2 expression in different Lauren classifications may help with the identification of high-risk patients and provide them with the appropriate treatment. It has been demonstrated that VEGF and VEGFR-2 are responsible for the formation of new blood vessels in intestinal-type GC (35). Similarly, Chen et al (36) indicated that VEGF expression in intestinal-type GC is significantly higher compared with in diffuse-type GC; however, other studies suggested that VEGF overexpression in intestinal-type GC is significantly associated with diffuse-type GC (37,38). The results from the present study demonstrated that VEGF and VEGFR-2 expression was not associated with Lauren classification, which was consistent with previous studies (39,40). In addition, VEGF and VEGFR-2 expression were not associated with OS in all patients with GC. However, the results following subgroup survival analysis suggested that VEGFR-2 overexpression may be considered as an independent prognostic factor in intestinal-type GC. Whether patients with intestinal-type GC and VEGFR-2 overexpression could benefit from anti-angiogenic targeted therapy requires further investigation.

The present study exhibited some limitations. Firstly, it was a retrospective study and was conducted by a single-institution. Secondly, the sample size was relatively small and only patients with GC treated by surgical gastrectomy were included. Thirdly, ~50% patients included in the study presented with stage I GC and the median OS was not reached. Large-scale and prospective multi-center studies are therefore required.

In conclusion, the results from the present study suggest that Lauren classification may be considered as an independent prognostic factor in patients with GC. Furthermore, Lauren classification exhibited prognostic significance for patients with stage III GC. The results also demonstrated that VEGF and VEGFR-2 expression was not associated with Lauren classification; however, results suggested that VEGFR-2 expression may be considered as an independent predictor of OS in patients with intestinal-type GC.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author upon reasonable request.

Authors’ contributions

MZ and LZ designed the study and helped to draft and revise the manuscript. XL collected the follow-up data, performed the statistical analysis and wrote the manuscript. XZ and YW collected the clinical data and performed immunohistochemistry. RW and LW analyzed immunohistochemistry data and classified the gastric cancer cases. All authors read and approved the final manuscript.

Ethics approval and consent to participate

This study was approved by the Ethics Committee of Xinhua Hospital Affiliated to Shanghai Jiaotong University School of Medicine, Shanghai, China (approval no. XHEC-D-2015-152). Written informed consent was obtained from all patients prior to the study.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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