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FULL LENGTH ARTICLE

Transcriptomic analysis reveals the promotion of lymph node metastasis by *Helicobacter pylori* infection via upregulating chemokine (C-X-C motif) receptor 2 expression in gastric carcinoma

Lang Zha [a], Xiong Guo [a], Xiaolong Liang [a], Yuedong Chen [b], Deyong Gan [b], Wenwen Li [c], Ziwei Wang [a], Hongyu Zhang [a,*]

[a] *Department of Gastrointestinal Surgery, The First Affiliated Hospital of Chongqing Medical University, Chongqing 400010, China*
[b] *Department of Gastrointestinal Surgery, The Tongliang Hospital of the First Affiliated Hospital of Chongqing Medical University, Chongqing 400010, China*
[c] *Department of Pathology, Faculty of Basic Medicine, Chongqing Medical University, Chongqing 400016, China*

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Abstract Gastric carcinoma (GC) progression is mainly caused by local aggression and lymph node metastasis. However, some patients with early T-stage disease have lymph node metastasis, whereas some patients with late T-stage disease do not have lymph node metastasis, which indicates that invasion and metastasis are not always sequential in some GC patients. In the present study, the data of 101 GC cases were acquired from TCGA and divided into T-late-N-negative and T-early-N-positive groups according to pathological stages. A total of 338 genes were identified as differential genes between the T-late-N-negative and T-early-N-positive groups. GSEA showed that epithelial cell signaling in the *Helicobacter pylori* (*HP*) infection pathway was enriched in the T-early-N-positive group. MB staining indicated that the *HP* infection rate was 63% (39/62) in N-positive patients compared to 42% (16/38) in N-negative patients. To investigate the potential mechanism, we focused on the gene chemokine (C-X-C motif) receptor 2 (CXCR2), which was not only clustered in the gene set of epithelial cells signaling in the *HP* infection pathway but also significantly upregulated in T-early-N-positive GC by the analysis of the different genes based on the TCGA dataset. A meta-analysis showed that CXCR2 expression was positively correlated with N-stage but not with T-stage in

* Corresponding author.
E-mail address: hongyuzhang@gmail.com (H. Zhang).
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GC. This study indicated that invasion and metastasis could be independent processes driven by different molecular mechanisms in some GC patients. *HP* infection was a potential factor that promoted lymph node metastasis by upregulating CXCR2 expression.

Introduction

Gastric carcinoma (GC) is the fourth most frequent malignant neoplasm and the second leading cause of cancer death worldwide.[1] Death from GC is mainly caused by invasion and metastasis. Over the past decades, research on malignant neoplasms has primarily concentrated on differences between tumors and normal tissues, including GC. Many factors are involved in the progression of GC, such as *Helicobacter pylori* (*HP*) infection,[2] p53,[3] Her-2,[4] CDH1,[5] and c-erbB-2.[6] GC is a heterogeneous disease affected by multiple environmental and genetic factors.[7] Moreover, these confounding factors undergo intricate crosstalk, and distinct subtypes of GC can be shaped under different aspects. Therefore, the treatment of GC has not made significant progress in recent decades. In this scenario, targeting distinct mechanisms could be an attractive therapeutic strategy to overcome gastric cancer heterogeneity.

The clinical pathological stage is usually determined by histological grade, aggressive depth, and lymph node metastasis in malignancy. The invasion depth, measured by the *T* stage, is a fundamental property of carcinoma, and proliferation, invasion, and metastasis are considered sequential processes during tumor development.[8–10] However, early GC is defined as a gastric malignancy limited to the mucosa or submucosa, irrespective of lymph node metastasis.[11] Interestingly, some patients had deep local infiltration without lymph node metastasis (T-late-N-negative group), and some had the opposite, with shallow local infiltration accompanied by lymph node metastasis (T-early-N-positive group). This kind of GC patient is not rare. This would indicate that invasion and metastasis are not always sequential processes in some GC patients. It is possible that the T-late-N-negative GC and T-early-N-positive GC are different subtypes shaped by distinct mechanisms.

To investigate further, we interactively analyzed the differential gene expression in the T-late-N-negative GC and the T-early-N-positive GC based on the TCGA dataset to better disclose the distinct molecular mechanisms between invasion and metastasis in some GC patients. The results show that more lymph node metastasis is involved in *HP* infection in GC. CXCR2 overexpression induced by activation of the pathway was the potential molecular mechanism. Eradication of *HP* could be a novel strategy to prevent the recurrence and metastasis of GC.

Materials and methods

Data acquisition characteristics

The transcriptome data of gastric carcinoma and matched clinical information were obtained from the TCGA-STAD project (https://tcga-data.nci.nih.gov/tcga/). Patients with "0" gene expression values and incomplete clinical data were excluded. According to pathological characteristics, 101 GC cases were divided into two groups: local infiltration deep (T_3 and T_4) without lymph node metastasis (N_0), named the T-late-N-negative group, and local infiltration shallow (T_1 and T_2) but with lymph node metastasis (N_1, N_2, and N_3), named the T-early-N-positive group. The human GTF file was downloaded from Ensemble (http://asia.ensembl.org). The limma package of R software was utilized to screen differential genes between the T-late-N-negative GC and T-early-N-positive GC groups (FC $>$ 2, $P < 0.05$). This study was approved by the Ethics Committee of the First Affiliated Hospital of Chongqing Medical University.

Gene ontology (GO) analysis

GO analysis was conducted with Cytoscape (V3.6.0) software, and enrichment analysis maps were acquired from an online data analysis website (http://www.bioinformatics.com.cn/).

Gene set enrichment analysis (GSEA)

GSEA (http://software.broadinstitute.org/gsea/index.jsp) was utilized to analyze the biological functions of differentially expressed genes between T-late-N-negative GC and T-early-N-positive GC.[12,13] Annotated files c2.cp.kegg.v7.5.1.symbols.gmt were selected as the reference gene sets.[14] A false defection rate (FDR) *q* value $<$ 25%, NOM *P*-Val $<$0.05, and normalized enrichment score (NSE) $>$ 1 or $<$ −1 were selected to sort the pathways enriched in each phenotype.

Methylene blue (MB) staining in GC

Methylene blue (MB) staining was performed to detect *HP* infection in GC. An *HP* staining assay kit (methylene blue) was used for MB staining (SSS-reagent Co. Ltd., Shanghai, No. 52015). Tissue microarrays (TMAs) were purchased from Zhongkeguanghua Co. Ltd., Xian (D1060201), and the manufacturer's instructions were followed: the tissue microarray (TMA) was routinely dewaxed and hydrated and then stained with gastric *HP* staining solution for 10 min. After washing, the TMA was air dried before mounting with neutral resin.[15]

Meta-analysis assesses the relationship between CXCR2 expression and lymph node metastasis and local aggression

We comprehensively searched the PubMed, Google Scholar, Wily, Science Direct, Springer, and NIH databases on 2 May 2022 for chemokine (C-X-C motif) receptor 2 (CXCR2)-

related studies published before May 2022 according to the preferred Reporting Items for Systematic Review and Meta-Analysis (PRISMA) guidelines. The following keywords were used: CXCR2, chemokine (C-X-C motif) receptor 2, gastric cancer, and gastric carcinoma. The inclusion criteria for this study were as follows: (i) all patients in the studies were diagnosed with GC by pathological examination; and (ii) correlations between CXCR2 expression and clinicopathological characteristics. The exclusion criteria were as follows: (i) reviews, letters, and comments; (ii) laboratory research (including cell line studies and animal experiments); (iii) the language of the papers was not in English; and (iv) duplicated papers or data. Then, we extracted data from the included studies after the study quality evaluation. The following data were extracted from the full text: name of the first author, publication year, number of cases, number of patients with high CXCR2 expression and low expression, T-stage, and nodal status.

Statistical analyses

Data are expressed as the mean ± standard deviation. All statistical analyses were performed via the statistical programming language R for Windows (cran.r-project.org). The relationship between *HP* infection and lymph node metastasis was analyzed by the chi-square test. A two-tailed *P* value less than 0.05 was considered statistically significant. For the meta-analysis, HRs and corresponding 95% CIs were estimated by statistical analysis using Stata 16 software (Stata Corporation, College Station TX, USA). Correlations between CXCR2 expression and pathological characteristics (T stage and *N* stage) were assessed. A *Q* statistic *P* value < 0.01 or $I^2 > 50\%$ was considered significant heterogeneity. A random-effects model was applied as the heterogeneity was significant. Otherwise, a fixed Mantel–Haenszel effects model was used. Begg's funnel plots were used to assess publication bias. A *P* value greater than 0.5 was considered to indicate no publication bias.

Results

Screening differential genes between T-late-N-negative GC and T-early-N-positive GC

The raw and processed data of 101 GC cases were acquired from TCGA. The patients were divided into the T-late-N-negative group (late T-stage without lymph node metastasis, $n = 58$) and the T-early-N-positive group (early T-stage with lymph node metastasis, $n = 43$) according to pathological stages. A total of 338 genes were identified to be differentially expressed between the T-late-N-negative group and the T-early-N-positive group (logFC < -1 or logFC >1) (Fig. 1A). Ninety-nine of them were upregulated, and 239 were downregulated (Fig. 1B) (Raw data shown in supplement 1).

Molecular functions of the differentially expressed genes

The molecular functions of 99 downregulated genes (also representing overexpression in the T-early-N-positive group) were mainly clustered to RAGE receptor binding, peptide receptor activity, chemokine binding, and chemokine receptor activity (Fig. 2A), and 239 upregulated genes (also representing overexpression in the T-late-N-negative group) were mainly clustered to gated channel activity, passive transmembrane transporter activity, and fibroblast growth factor receptor binding (Fig. 2B).

GSEA identified the differential gene-related signaling pathways

To explore the enriched signaling pathways in the T-early-N-positive and T-late-N-negative groups, GSEA was performed based on the TCGA dataset. The enriched signaling pathways were selected based on NSE >1 or < -1, false defection rate (FDR) *q* value $< 25\%$, and NOM *P*-Val <0.05.

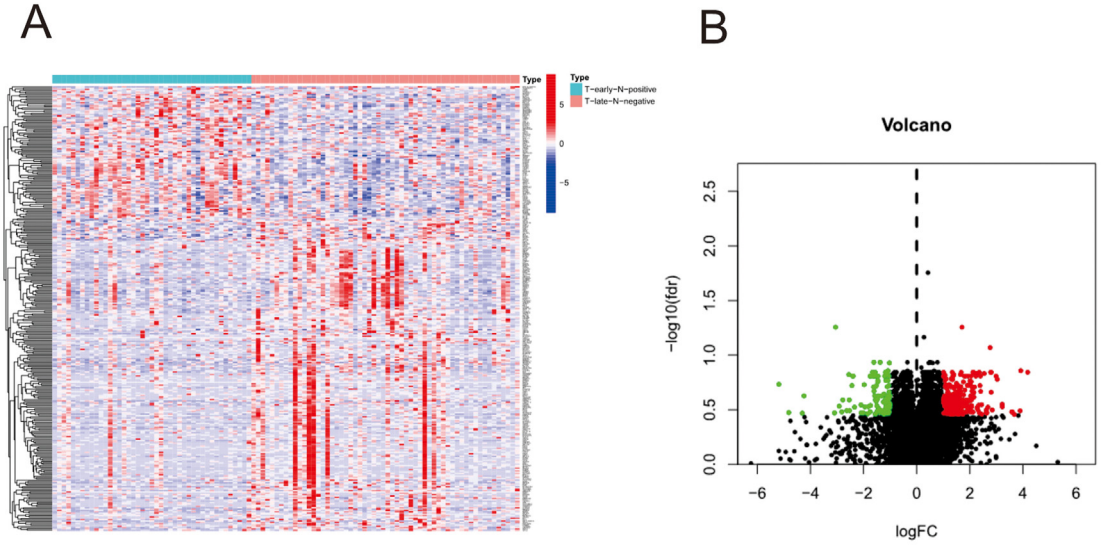

Figure 1 The differentially expressed genes between T-late-N-negative GC and T-early-N-positive GC. **(A)** Heatmap of the differentially expressed genes between the T-late-N-negative and T-early-N-positive groups. **(B)** Volcano map of the differentially expressed genes between the T-late-N-negative and T-early-N-positive groups.

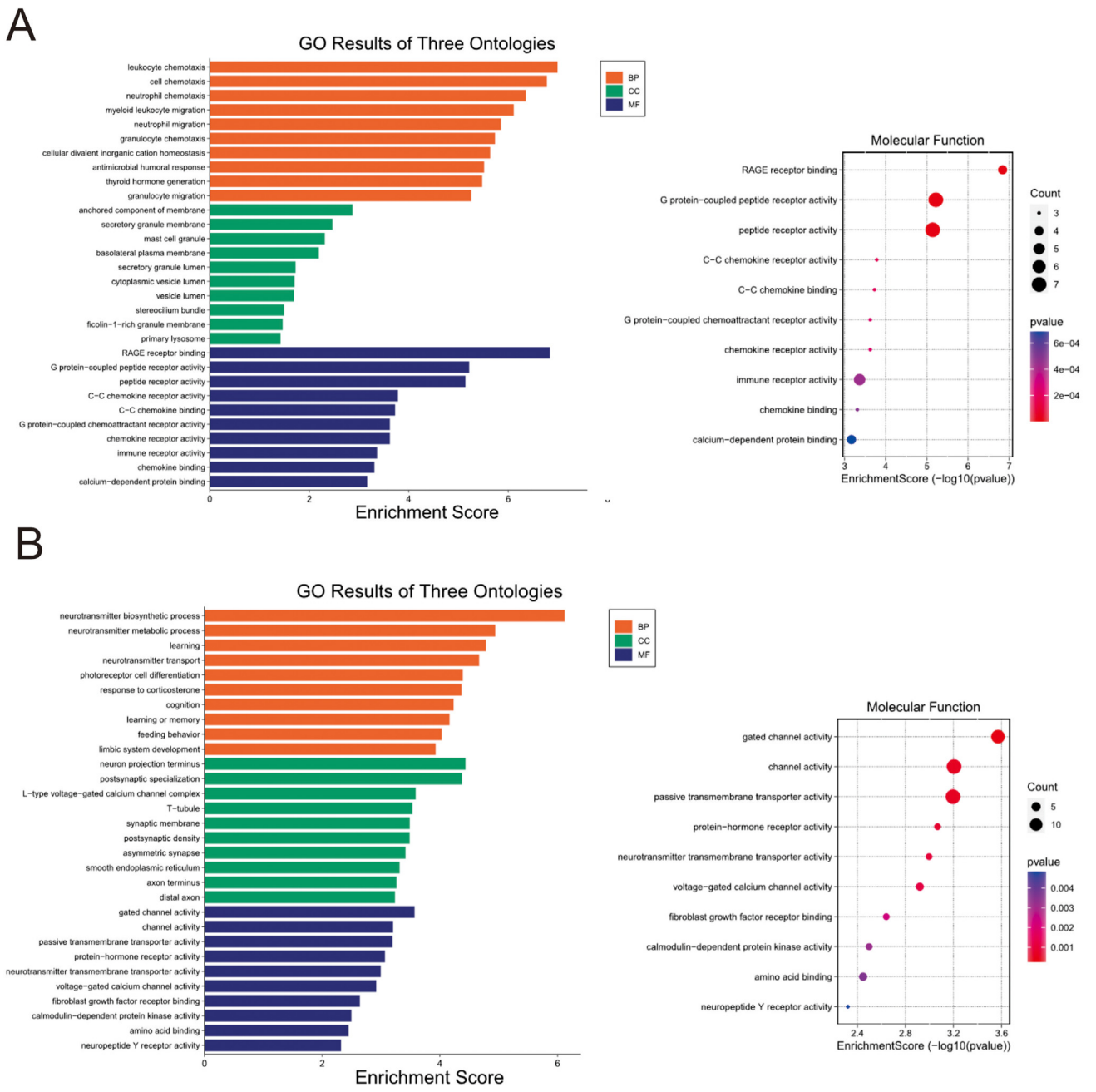

Figure 2 Molecular functions of the differentially expressed genes between T-late-N-negative GC and T-early-N-positive GC. **(A)** Molecular functions of the 99 downregulated genes. **(B)** Molecular functions of the 239 upregulated genes.

In total, 70/178 gene sets were downregulated in the T-late-N-negative group, and 108/178 gene sets were upregulated in the T-early-N-positive group. Twelve gene sets were significantly enriched at nominal NOM *P*-Val <5% in the T-late-N-negative group, and 38 gene sets were significantly enriched at nominal NOM *P*-Val <5% in the T-early-N-positive group. As shown in Figure 3A, DNA replication, mismatch repair, vascular smooth muscle contraction, *etc*. Were enriched in the T-late-N-negative group, and lysosome, fatty acid metabolism, galactose metabolism, *etc*. Were enriched in the T-early-N-positive group. Notably, epithelial cell signaling in the *HP* infection pathway was activated in the T-early-N-positive group (NES = −1.7704431, nominal *P* value < 0.00, FDR *q*-value = 0.017) (Fig. 3B). The genes related to KEGG epithelial cell signaling in *HP* infection are shown in the blue−pink o'gram (Fig. 3C). The results showed that *HP* infection could be associated with lymph node metastasis in GC.

HP infection is involved in lymph node metastasis in GC

To further verify that *HP* infection was involved in lymph node metastasis of GC, MB staining of TMAs was performed. Specimens on the TMAs were from 106 patients,

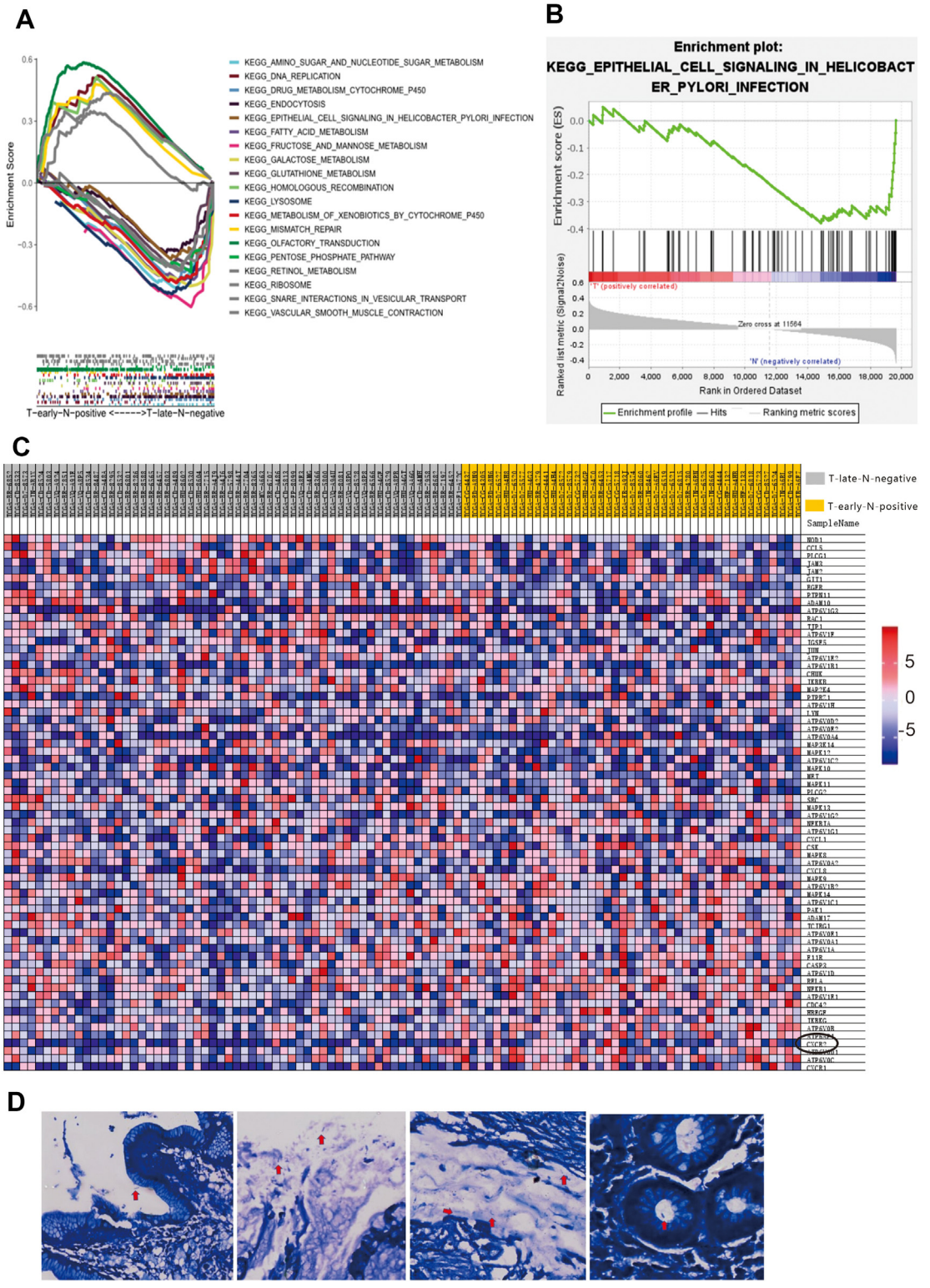

Figure 3 The relationship between *HP* infection and lymph node metastasis in GC. **(A)** The enriched signaling pathways of GSEA and the epithelial cell signaling in the *HP* infection pathway were markedly activated in the T-early-N-positive group. **(B)** Enrichment plot: KEGG epithelial cell signaling in the *HP* infection profile of the running ES scores & positions of gene set members on the rank-ordered list. **(C)** KEGG epithelial cell signaling in the *HP* infection blue–pink o'gram in the space of the analyzed gene set. **(D)** *HP* was stained as rods in the gastric pits (left) or mucus layer (right).

containing 100 GC cases, 3 cases from paracancerous tissues, and 3 cases from normal tissues. In this cohort, 2 cases were in pathological tumor node metastasis (pTNM) stage I, 56 cases were in pTNM stage II, 41 cases were in pTNM stage III, and 1 case was in pTNM stage IV. In 100 GC cases, 38 of them had no lymph node metastasis (N-negative), and 62 had lymph node metastasis (N-positive). As shown in Figure 3D, *HP* was stained as curved, spiral bacilli or rods in the epithelial surface, mucus layer, or gastric pits under the microscope. MB staining showed that the *HP* infection rate was 63% (39/62) in N-positive patients compared to 42% (16/38) in N-negative patients ($P < 0.05$) (Table S1).

The expression of CXCR2 was significantly correlated with *N* stage but not with *T* stage in GC

To explore the potential mechanism by which *HP* infection provokes lymph node metastasis in GC, we further analyzed data from GSEA based on the TCGA dataset. CXCR2 was not only clustered in the gene set of the epithelial cell signaling in the *HP* infection pathway but also significantly upregulated in T-early-N-positive GC by the analysis of the different genes based on the TCGA dataset (Fig. 3C). Therefore, the upregulation of CXCR2 may be a potential mechanism by which *HP* infection promotes lymph node metastasis. A total of 44 studies were obtained. According to the inclusion and exclusion criteria, six studies, including 1151 patients, were finally included for data extraction.[16–21] The detailed patient characteristics are shown in supplement 2. IHC staining was performed to detect CXCR2 expression in 6 studies. The correlations between CXCR2 expression and pathological characteristics (*T*-stage and *N*-stage) were assessed in 1151 GC patients from the 6 studies. CXCR2 expression was significantly associated with the *N* stage (*OR* = 0.488, 95%CI: 0.275–0.867, I^2 = 80%) (Fig. 4A) but not with *T* stage (*OR* = 0.528, 95%CI: 0.223–1.254, I^2 = 86.1%) (Fig. 4B). Begg's funnel plots showed that there was no publication bias in the meta-

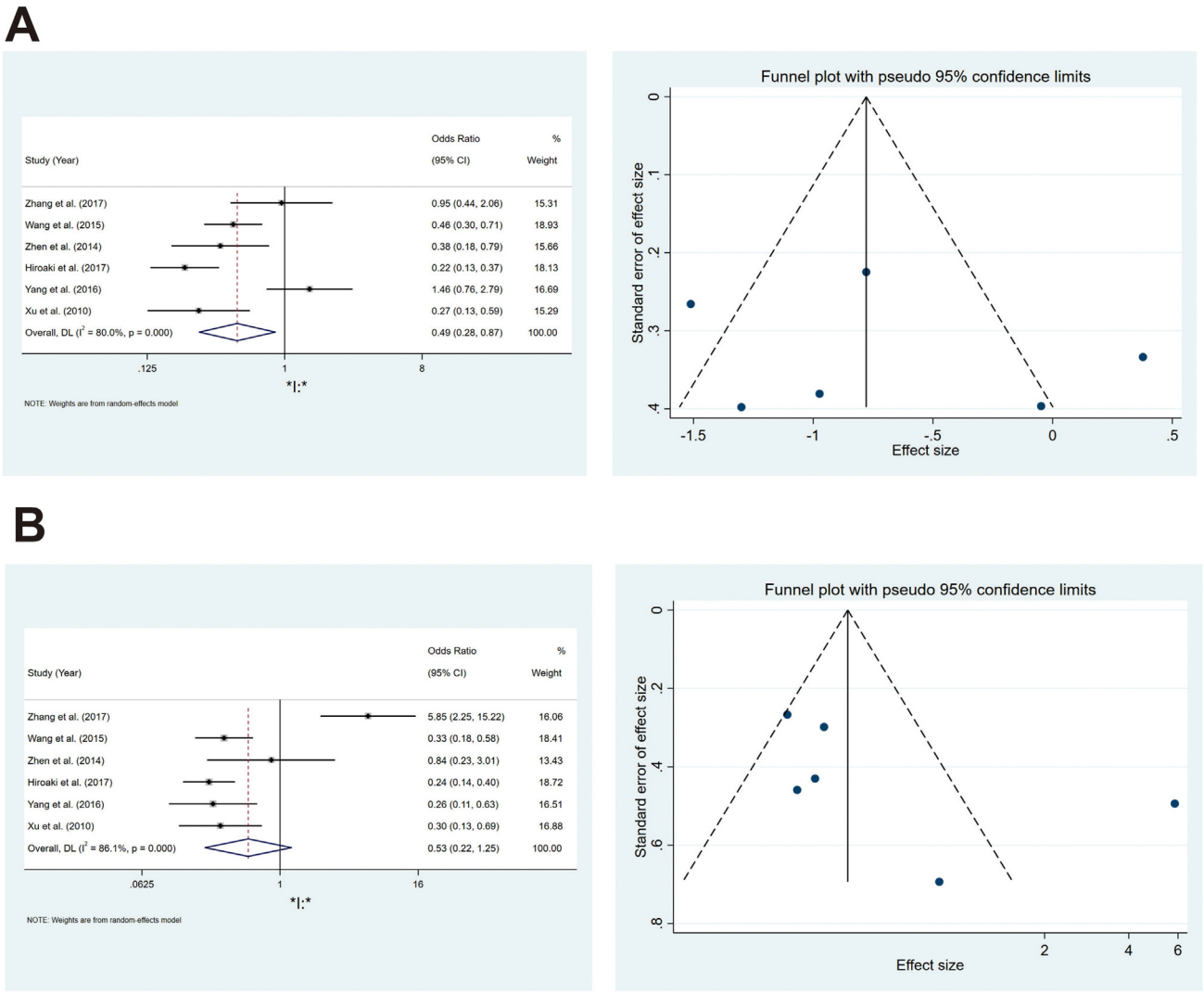

Figure 4 Meta-analysis of the correlations between CXCR2 expression and T stage or *N* stage in GC. **(A)** Forest plot of the association of CXCR2 expression with *N* stage and funnel plot of studies used in the analysis. **(B)** Forest plot of the elevated association of CXCR2 expression with *T* stage and funnel plot of studies used in the analysis.

analysis in terms of *N*-stage ($z = -0.19$, $P = 0.851$) (Fig. 4A) and *T*-stage ($z = 1.69$, $P = 0.091$) (Fig. 4B).

Discussion

GC is a malignant tumor with high mortality that threatens human health, and metastasis is the most common cause of death. Over the last few years, an increasing number of studies have shown that epithelial–mesenchymal transition (EMT) plays a crucial role in the progression and metastasis of cancer.[22,23] In the EMT process, proliferation, invasion, and metastasis are often coincident.[24] However, there is a clinical phenomenon in which some patients have deep invasion but no lymph node metastasis, and some have shallow invasion but have lymph node metastasis in GC. Our previous study also found that some GC cell lines obtained mobility and invasion abilities during EMT. Nevertheless, the proliferative capacity is reduced.[25] This would indicate that invasion and metastasis are not always sequential in some GC patients. In the present study, we interactively analyzed the differential gene expression in T-late-N-negative GC and T-early-N-positive GC based on the TCGA dataset and disclosed the distinct molecular mechanisms between invasion and metastasis in some GC patients. What caught our attention was that epithelial cell signaling in the *HP* infection pathway clustered in T-early-N-positive GC. Both *HP* infection and lymph node metastasis are risk factors for GC recurrence after radical surgery.[26] *HP* infection can activate neutrophils and mononuclear cells to produce proinflammatory cytokines, such as IL-1, IL-6, and IL-8,[27] and promote the formation of new lymphatic vessels or lymphangiogenesis.[21] Our study found that *HP* positivity more frequently exists in GC with lymph node metastasis.

To explore the potential mechanism by which *HP* infection promotes lymph node metastasis in GC, we analyzed the related genes from the epithelial cell signaling gene set in the *HP* infection pathway, which was clustered by GSEA. CXCR2 was not only clustered in the epithelial cell signaling gene set in the *HP* infection pathway but was also significantly upregulated in T-early-N-positive GC according to the analysis of the different genes based on the TCGA dataset. Therefore, we speculated that CXCR2 might have a crucial role in *HP* infection caused by lymph node metastasis. CXCR2 is located at 2q34-q35,[28] forming a gene cluster with CXC motif chemokine receptor 2 pseudogene 1 (CXCR2P1). Overexpression of CXCR2 has been observed in various neoplasms, including breast cancer,[29] laryngeal squamous cell carcinoma,[30] esophageal carcinoma,[31] colorectal cancer,[32] ovarian cancer,[33] lung cancer,[34] pituitary adenomas,[24] cholangiocellular carcinoma,[35] and pancreatic ductal adenocarcinoma.[36] Not unexpectedly, CXCR2 is also closely related to invasion and metastasis in GC. Unlike other studies, some reported that CXCR2 was only associated with N-stage but not with *T*-stage.[16,31] There are indications that CXCR2 could be a crucial factor in lymph node metastasis but not in local aggression in some GC cases. *HP*-infected patients expressed higher levels of CXCR2 than uninfected patients.[27] Then, we performed a meta-analysis of CXCR2 expression in GC, and the results showed that CXCR2 expression was markedly associated with the *N* stage but was not associated with the *T* stage. This finding indicated that CXCR2 could be a specific factor of lymph node metastasis in some GC cases. To our knowledge, lymph node metastasis of GC has two crucial steps. One is premetastatic niche formation; the other is immune evasion. First, CXCR2 overexpression occurs on lymphatic endothelial cells, which induces migration of these cells and tube formation, leading to new lymphatic vessels or lymphangiogenesis.[21] Furthermore, CXCR2 may promote GC immune evasion by changing immune inflammation. Knockdown of CXCR2 decreased PD-L1 expression and consequently improved the macrophage shift to the M1 phenotype.[37] CXCR2 plays an important role in lymph node metastasis by improving premetastatic niche formation and inducing immune evasion in GC.

In summary, the findings of this study suggested that distinct mechanisms in some cases of GC may dominate lymph node metastasis and local invasion. *HP* infection can promote lymph node metastasis by upregulating CXCR2 expression. *HP* can stimulate macrophages to produce APRIL, promoting lymphomagenesis and B-cell proliferation in gastric MALT lymphoma.[38] *HP* eradication therapy is an effective treatment for patients with gastric MALT lymphoma.[39,40] Lymph node metastasis and *HP* infection were risk factors for GC recurrence after radical surgery. Therefore, *HP* eradication after surgery could be an effective way to reduce the relapse of GC.

Author contributions

Lang Zha and Hongyu Zhang designed the experiments and wrote the paper. Xiong Guo and Xiaolong Liang analyzed the data. Yuedong Chen and Deyong Gan prepared the figures and tables. Wenwen Li performed the MB staining. Ziwei Wang and Hongyu Zhang reviewed and edited the manuscript.

Conflict of interests

The authors declare no conflict of interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.gendis.2022.10.027.

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