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A diagnostic model based on gene biomarkers for Crohn's disease

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Abstract. Crohn's disease (CD) is a segmental chronic inflammatory bowel disease, which seriously affects the patient's quality of life. The etiology of CD is not yet clear, and there is still a lack of effective treatments. Therefore, in this study, we focus on developing a useful model for early diagnosis and targeted therapy of CD. The expression datasets of CD were collected to filter differentially expressed genes (DEGs) by overlapping "limma" package and "WGCNA" package. Then, functional enrichment analysis and protein-protein interaction (PPI) network analyses were performed. Hub genes were screened with "cytoHubba" plug-in and filtered with LASSO and stepwise regression analyses. The logistic regression model and nomogram were established based on the selected hub genes. The 45 DEGs were identified and the top 30 hub genes were chosen out for further study. Finally, 11 genes were selected to construct the logistic regression model and nomogram. The receiver operating characteristic (ROC) curve shows that the area under the curve (AUC) value was 0.960 in the training dataset and 0.760 in the validation dataset. A 11-gene diagnostic model was constructed with IL1B, CXCL10, CXCL2, LCN2, MMP12, CXCL9, NOS2, GBP5, FPR1, GBP4 and WARS, which may become potential biomarkers for early diagnosis and targeted therapy of CD.

Key words: Crohn's disease — Diagnosis — Logistic model — Nomogram — Biomarker

Abbreviations: AUC, area under the curve; BP, biological process; CC, cellular component; CD, Crohn's disease; DEGs, differentially expressed genes; GEO, Gene Expression Omnibus; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; MF, molecular function; PPI, protein-protein interaction; ROC, receiver operating characteristic.

Introduction

Crohn's disease (CD) is a chronic inflammatory bowel disease that affects the entire gastrointestinal tract, and its incidence is increasing worldwide. As shown in previous studies, the incidence and prevalence of CD varied greatly worldwide. The highest reported incidence of CD was founded in Oceania (29.3 *per* 100,000 in Australia) and North America (23.82 *per* 100,000 in Canada), and the highest reported prevalence was founded in Western Europe (322.0 *per* 100,000 in Germany) and North America (318.5 *per* 100,000 in Canada) (Ng et al. 2017). As for the pediatric-onset CD patients (first diagnosed at the age less than 19 years old), the reported highest incidences were 13.9 *per* 100,000 in North America and 12.3 *per* 100,000 in Europe (Sýkora et al. 2018). Moreover, an increasing incidence of CD has been observed worldwide. For example, the average incidence of CD increased from 7.16 to

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9.56 *per* 100,000 person-year (1998-2007) in the Tayside region, 8 to 13 *per* 100,000 person-year (2000-2020) in Finland, 5.1 to 15.6 *per* 100,000 person-year (1980-2017) in Denmark, and 4.48 to 9.8 *per* 100,000 person-year (2017-2020) in South Korea (Steed et al. 2010; Choe et al. 2022; Dorn-Rasmussen et al. 2023; Kontola et al. 2023). There was no significant difference in the incidence of CD between men and women.

CD is a progressive bowel inflammatory disease that leads to damage and disability of the gastrointestinal tract. The most common affected segments were terminal ileum and colon. The typical changes in the affected bowel were segmental, asymmetrical, and transmural, and nearly half of the patients finally develop complications such as strictures, fistulas, or abscesses (Torres et al. 2017). The typical clinical symptoms are abdominal pain, diarrhoea, rectal bleeding, fatigue and weight loss (Torres et al. 2017; Veauthier et al. 2018). CD patients cost a lot of medical care, and nearly 35% of them were hospitalized. Systemic steroids, immunomodulatory therapy, biological therapy and surgery are the mainly therapy for CD patients (Chaparro et al. 2021; Sáiz-Chumillas et al. 2022).

CD is a dysregulation of mucosal immune response and epithelial barrier function resulted from the interplay between genetic susceptibility, environment, and dysbiosis (Torres et al. 2017; Roda et al. 2020). However, the cause is still unclear. Combination therapy with early immunosuppression or biologicals can help patients have long-term remission and reduce complications (Torres et al. 2017). For example, microbiomarkers have been proposed to distinguish the CD pateints and act as a therapy (Pascal et al. 2017; Zhang et al. 2021). Traditional drug treatments for CD include antibiotics, nutritional treatment (Caio et al. 2021), corticosteroids and immunomodulators. At present, there are a variety of new therapies that have promoted the remission, such as anti-TNF (Adegbola et al. 2018) and JAK inhibitors (Rogler 2020).

Due to different disease development and drug treatment responsiveness, the impact of CD on the quality of life of patients varies greatly, which can range from interference to severe disability or death (Noor et al. 2020). Early diagnosis and personalized treatment can significantly improve the prognosis of patients, reduce the need for surgery. Therefore, screening of biomarkers is essential to achieve personalized treatment of CD patients.

In this research, we focused on the gene biomarkers of CD. The significantly differentially expressed genes between CD and control samples were identified. Then, the protein-protein interaction (PPI) network was constructed to pick up the hub genes. Moreover, logistic regression model and nomogram were established to filter the most significant genes associated with CD. The Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses were also performed to reveal the important molecular functions and pathways.

Materials and Methods

Data processing

The RNA expression datasets of CD were extracted from Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo/) (Clough et al. 2016). GSE75214 (75 CDs and 22 controls) and GSE179285 (168 CDs and 31 controls) were combined to establish the diagnostic model, and GSE48634 (34 CDs and 69 controls) was used as the test dataset. The batch effects between GSE75214 and GSE179285 were removed with "sva" package.

Screening of differentially expressed genes (DEGs)

The DEGs between CD samples and control samples were screened with two methods, including "limma" package (Ritchie et al. 2015) and "WGCNA" package (Langfelder et al. 2008). The final identified DEGs are the overlap of the differentially expressed genes screened by the "limma" package and the gene clusters that are significantly related to CD screened by the "WGCNA" package.

Function enrichment analysis

The enriched GO and KEGG terms were analyzed with "clusterProfiler" package (Yu et al. 2012) based on the screened DEGs. The significantly enriched terms were filtered with the $p < 0.05$.

Construction of protein-protein interaction (PPI) network

The PPI network was constructed by the Search Tool for the Retrieval of Interacting Genes (STRING, version 11.5, https://cn.string-db.org/) (Szklarczyk et al. 2021) based on screened DEGs, and was visualized by Cytoscape (version 3.8.2). The hub genes of the network were identified with "cytoHubba" plug-in based on "MCC" algorithm.

Establishment of a diagnostic model

The identified hub genes were used to establish the diagnostic model filtered with LASSO analysis and stepwise regression analysis. The logistic regression (Stoltzfus 2011) model was then constructed with the filtered genes using "glm" package. A nomogram consisting of these model genes was built. Then, the model was validated in the test dataset (GSE48634). The receiver operating characteristic (ROC) curve and calibration plot were used to appraise the

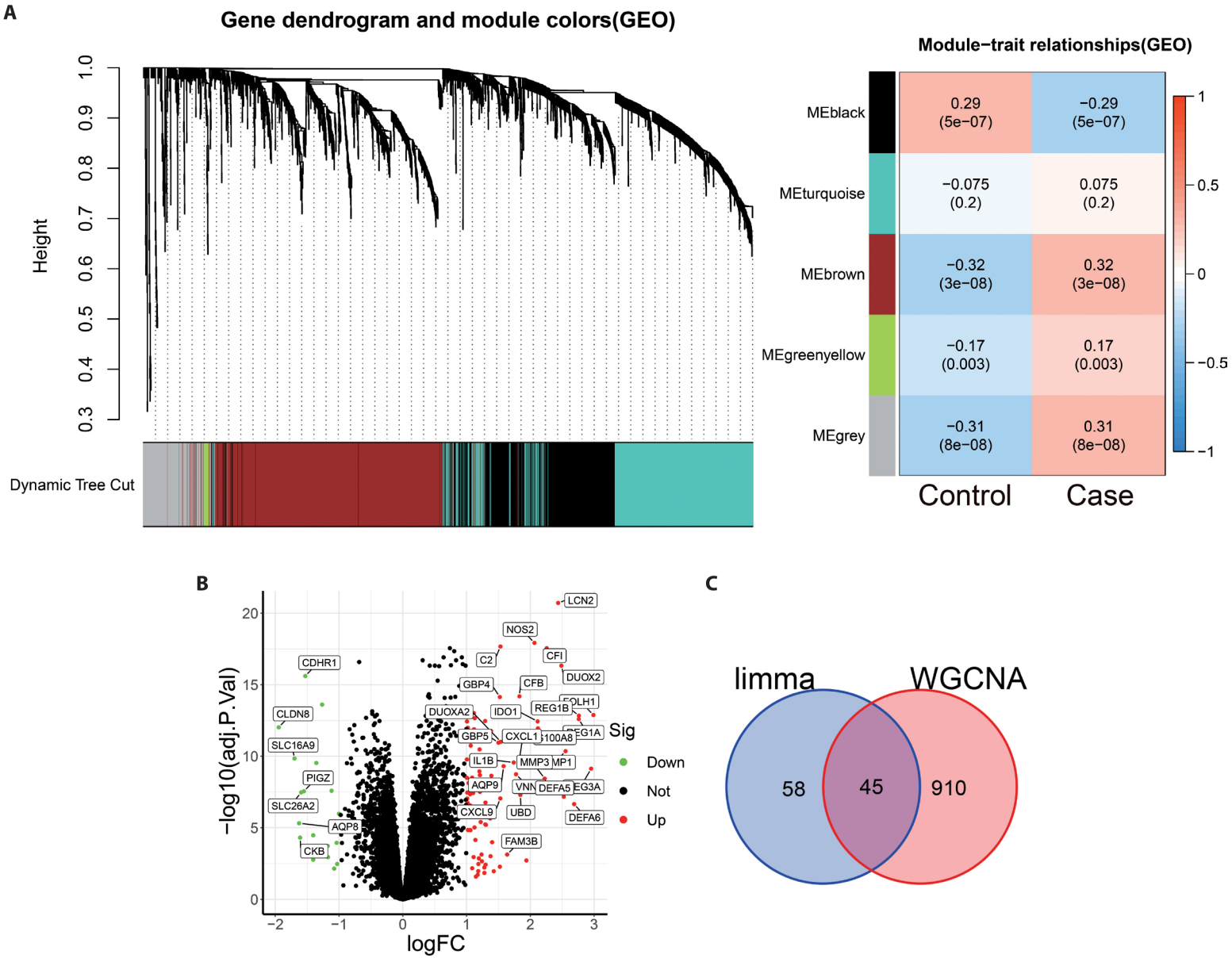

Figure 1. Identification of DEGs between CD and control samples. **A.** The gene modules of WGCNA analysis. **B.** The volcano plot of limma analysis. The green points stand for downregulated genes, and the red points stand for upregulated genes. **C.** Venn diagram shows the overlapping of limma and WGCNA results.

diagnostic ability. Ten-fold cross validation was applied to appraise the accuracy of this model.

Results

Screening of DEGs

By conducting the "WGCNA" package, the brown module was identified as the most significant gene cluster associated with CD, which contains 955 genes (Fig. 1A,B). In total, 103 DEGs were screened by "limma" package based on combined GEO datasets (Fig. 1C). Finally, 45 DEGs were picked by overlapping the results of above two methods (Fig. 1D).

Function enrichment analysis

The results of function enrichment analysis based on the 45 DEGs were shown in Figure 2A and B. The enriched GO terms consisted of three parts of results, including biological process (BP), cellular component (CC) and molecular function (MF). These genes were significantly enriched in response to lipopolysaccharide, response to molecule of bacterial origin and neutrophil chemotaxis at BP level, specific granule lumen, secretory granule lumen and cytoplasmic vesicle lumen at CC level, and CXCR chemokine receptor binding, chemokine activity and cytokine activity at MF level. The enriched KEGG pathways were IL-17 signaling pathway, TNF signaling pathway, NOD-like receptor signaling pathway, Cytokine-cytokine receptor interaction and Chemokine signaling pathway.

Construction of PPI network

The PPI network was constructed based on the 45 DEGs *via* STRING and Cytoscape. These genes were calculated with "MCC" algorithm and the top 30 genes were filtered as hub genes (Fig. 2C).

Establishment of a diagnostic model

The 30 hub genes were included in the establishment of the diagnostic model. To choose as few variables as possible, LASSO and stepwise regression analyses were successively conducted. By performing LASSO analysis, 18 hub genes were picked up (Fig. 3A,B). Then, a total of 11 genes were filtered by stepwise regression analyses, including IL1B, CXCL10, CXCL2, LCN2, MMP12, CXCL9, NOS2, GBP5, FPR1, GBP4 and WARS. The logistic regression model was established based on these 11 genes and visualized by nomogram (Table 1 and Fig. 3B). Moreover, this diagnostic model was appraised with ROC and calibration plot. GSE48634 was downloaded as the external validation dataset. The AUC values were 0.960 in the training dataset and 0.760 in the validation dataset (Fig. 3C), which confirmed the high stability of this model. The calibration plot also proved the diagnostic ability of this model (Fig. 3D).

To further certify the accuracy of this model, ten-fold cross validation was performed *via* "caret" package. The results revealed that the model accuracy was 0.898. All of the above results demonstrated that the 11-genes diagnostic model can predict the CD patients.

Discussion

CD is a segmental chronic inflammatory disease of the gastrointestinal tract, which seriously affects the patient's quality of life. The etiology of CD is not yet clear, and there is still a lack of effective treatments. Various studies have shown that early and personalized treatment can significantly improve the prognosis of patients and reduce complications such as surgery. Therefore, there is an urgent need to discover the biomarkers of CD in order to achieve the purpose of early diagnosis and targeted treatment.

In this study, two CD datasets containing a total of 243 CD samples and 53 control samples were combined to identify the DEGs. To remove the batch effects of the two datasets, the "sva" package was performed. Then, 45 DEGs were filtered with "limma" package and "WGCNA" package. Functional enrichment analysis confirmed that cellular response, lumen and chemokine and cytokine were the significantly enriched GO terms, and IL-17, TNF, NOD-like receptor, Cytokine and Chemokine signaling pathways were extremely enriched KEGG terms. Because CD is a chronic inflammatory disease, the production of inflammatory cytokines has been studied for a long time. Previous studies reported that TNF-α, IL-1β, and IL-6 significantly increased in the intestinal biopsies, which provided new ideas for CD treatment. NOD-like receptor is part of innate immune system that recognizes various ligands from microbial pathogens, host cells, and environmental sources, and activate inflammatory responses, such as inflammasome formation, and transcriptional regulation through NF-kB and MAPK pathways (Barbé et al. 2014). NOD-like receptors such as NLRP6 and NLRP3 have been shown to be involved in the regulation of CD (Ranson et al. 2018; Zhen et al.2019). In CD, innate lymphoid cells respond to stimuli by producing cytokines such as TNF-α, IL-17, IL-22 and INF-γ (Torres et al. 2017). Chemokines and inflammatory cytokines such as TNF-α and IL-1β can active immune cells (macrophages, lymphocytes, etc.), which can further produce chemokines and cytokines to promote inflammation. IL-17 is the main downstream cytokine of IL-12 and IL-23, whose targeting antibody – Ustekinumab – has been applied to the treat-

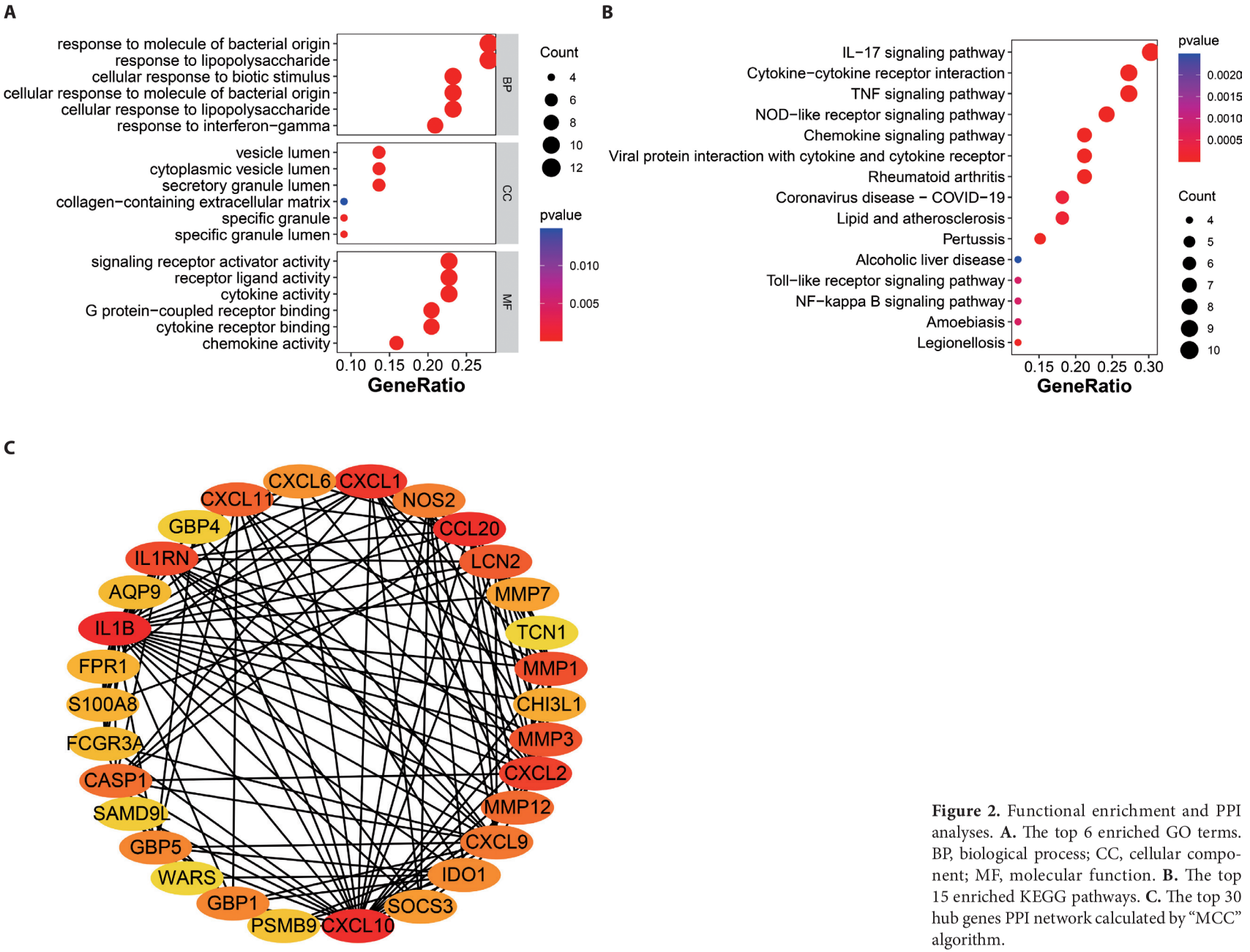

Figure 2. Functional enrichment and PPI analyses. **A.** The top 6 enriched GO terms. BP, biological process; CC, cellular component; MF, molecular function. **B.** The top 15 enriched KEGG pathways. **C.** The top 30 hub genes PPI network calculated by "MCC" algorithm.

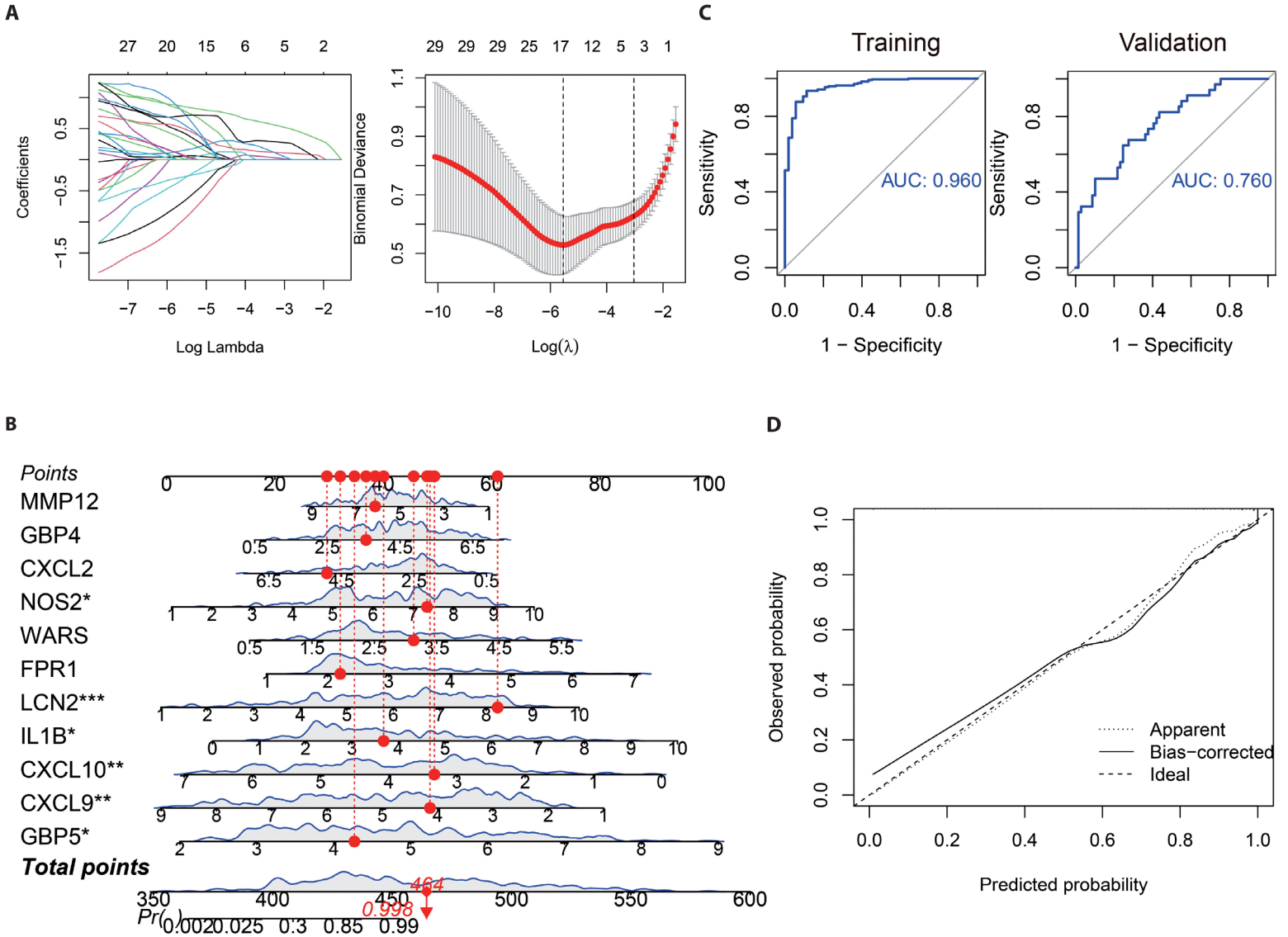

Figure 3. The construction and validation of diagnostic model. **A.** The coefficients of selected genes and lambda selection of LASSO regression analysis. **B.** The nomogram of the diagnostic model. **C.** ROC curves of the training and validation datasets. AUC, area under the curve. **D.** The calibration curve for nomogram.

ment of CD (Moschen et al. 2019). However, anti-IL-17 therapy cannot achieve remarkable clinical efficacy, and it can lead to new onset or worsening of CD (Yang et al. 2014; Hohenberger et al. 2018; Fauny et al. 2020). Unlike IL-17, anti-TNF therapy such as infliximab is the basis of CD treatment and has been used in clinical treatment for a long time (Pouillon et al. 2016; D'Haens et al. 2021). This part of results revealed the potential mechanisms of CD.

To develop a gene model for the diagnosis of CD, PPI network was constructed and the "MCC" algorithm was performed by "cytoHubba" plug-in to filter the top 30 hub genes. Then, LASSO and stepwise regression analyses were applied to screen the most relevant genes. Besides, these selected genes were involved in the construction of logistic regression model and nomogram. As the result revealed that IL1B, LCN2, NOS2, GBP5, FPR1, GBP4 and WARS were positively related to CD, but CXCL10, CXCL2, MMP12 and CXCL9 were negatively related to CD. Most of these genes were involved in the regulation of immune response. IL1B (interleukin 1β, IL-1β) is a type of cytokine secreted by macrophages and acts as a promotor of inflammation synergistically with other cytokines, such as TNF-α and IL-6 in CD. Besides, inhibition of IL-1β signaling relieves colitis (Lu et al. 2022). LCN2 (lipocalin 2), also known as neutrophil gelatinase-associated lipocalin (NGAL), usually acts as a biomarker of acute kidney injury. It also plays an important role in innate immunity by sequestering iron-containing siderophores to limit bacterial growth. The expression of IL1B and LCN2 were upregulated in CD samples confirmed by several studies (Thorsvik et al. 2018; da Silva et al. 2020; Mitsialis et al. 2020). CXCL (C-X-C motif chemokine ligand) is a series of chemokines that participate in immunoregulatory and inflammatory processes and expressed at the site of inflammation. CXCL2 may suppress the proliferation of hematopoietic progenitor cell. CXCL9 and CXCL10 are mainly involved in T lymphocyte recruitment and induce the IFN-γ secretion at the site of inflammation. Previous studies confirmed that CXCL9 and CXCL10 may be the potential biomarkers of CD and are related to the recurrence (Mello et al. 2021; Boucher et al. 2022; Walshe et al. 2022). NOS2 (nitric oxide synthase 2) can be induced by lipopolysaccharide and cytokines. When cells stimulated by pathogens, NOS2 helps macrophages against pathogens by utilizing oxidative stress of nitric oxide. GBP (guanylate binding protein), which located in the plasma surface of cell membrane, can bind to GDP or GTP and regulate the concentration of second messengers. GBP4 can be induced by interferon and hydrolyze GTP. GBP5 can regulate the NLRP3 inflammasome assembly and play important roles in inflammation. FPR1 (formyl peptide receptor 1) is a member of G protein-coupled receptor of phagocytic cells, and mediates the immunity and inflammation when host stimulated by microorganisms. WARS (tryptophanyl-tRNA synthetase) is induced by interferon and catalyzes the aminoacylation of tRNA with tryptophan. MMP12 (matrix metallopeptidase 12) is a member of matrix metalloproteinases and participates in the degradation of extracellular matrix. The remaining model genes have not yet been reported in CD research, and their relationship with CD needs further research and exploration in the future. It has been reported by some studies that IL1B, CXCL10, CXCL2 and NOS were regulated by PDE super family (cyclic nucleotide phosphodiesterase super family), which is a regulator of second messengers (cAMP and cGMP) (Reimund et al. 2001; Gustafsson et al. 2002; Lugnier 2006). PDE4 is a key member of PDE family that involved in the inflammation and acts on TNF-α and IL-1β (Tetsi et al. 2017). Increasing of PDE4 activity has been investigated in the mucosa of CD patients. Today, PDE4 inhibitors have been developed for the treatment of inflammation diseases and are considered as a hopeful treatment for CD patients. These results indicate that the model genes may be involved in other signaling pathways like PDE signaling to regulate the inflammation of CD. Besides, these signaling pathways may regulate inflammation synergistically with other pathways in CD. For example, previous study reported that TNF-α, IL-1β and MAPK signaling are involved in PDE4 signaling (Lugnier 2022). These model genes may be potential biomarkers of CD.

Some limitations of this study are as follows: 1. The biomarkers in this study were selected based on the expression levels of the genes, and did not include some key genes, such as PDE4, whose expression levels did not change significantly. 2. CD is an inflammatory disease that can affect the whole gastrointestinal tract. This study only involves intestinal specimens; more specimens from other parts of gastrointestinal tract should be included. 3. More expression datasets of CD should be involved to validate the diagnostic ability of this model. 4. Many of these model genes have little research on CD, and further experiments are needed to reveal them.

Conclusion

A 11-gene diagnostic model was constructed with IL1B, CXCL10, CXCL2, LCN2, MMP12, CXCL9, NOS2, GBP5, FPR1, GBP4 and WARS, which may become potential biomarkers for early diagnosis and targeted therapy of CD.

Data availability. In this study, the analyzed datasets were extracted from GEO database (https://www.ncbi.nlm.nih.gov/geo/).

Conflicts of interest. The authors declare that there is no conflict of interest regarding the publication of this article.

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Ethics approval. The data analyzed in this study is de-identified data extracted from GEO databases, and the ethics approval is not required.

Author contributions: All authors contributed to the study conception and design. SW collected data, conducted data analysis, and wrote the manuscript. JW contributed to design of the study. LZ participated in data and statistical analyses. All authors confirmed the final manuscript version.

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