Identification of gut microbiome and transcriptome changes in ulcerative colitis and pouchitis

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\textbf{ABSTRACT}

\textbf{Background:} Pouchitis is the common postoperative complication of ulcerative colitis (UC) and is also considered as inflammatory bowel disease. The aim was to investigate the microbiological and transcriptional differences between the two illnesses.

\textbf{Methods:} Eighty-five participants were enrolled (37 UC, 15 healthy UC pouches, 15 pouchitis and 18 healthy volunteers) and stool samples were collected. Microbial populations were analyzed by pyrosequencing of 16S ribosomal DNA. Furthermore, transcriptome data of 119 UC and 28 pouch patients were obtained from two data sets for bioinformatics analysis.

\textbf{Results:} The results of gut microbiota community analysis showed that with aggravation of UC, intestinal microorganisms were characterized by a gradual decreased in diversity and numbers of butyrate-producing bacteria and \textit{Bacteroides}. Besides, in addition to the decrease of probiotics, the proliferation of \textit{Escherichia–Shigella} and \textit{Ruminococcus gnavus} was observed in pouchitis which is related to multiple infection pathways. The function enrichment of differential expression genes and hub genes, as well as the immunological condition was shown to be distinct using transcriptome bioinformatics analysis between UC and pouchitis. A stronger immune response occurs in UC and may be associated with high expression of tumor necrosis factor and interleukin, while multiple hub genes such as \textit{CDK1} in pouchitis are associated with cell cycle regulation.

\textbf{Conclusions:} The characteristics of gut microbiota disturbance and transcriptome alteration in UC and pouchitis are different. Our findings suggested that pouchitis may have a unique pathogenesis which was separated from UC.

\textbf{Abbreviations:} UC: ulcerative colitis; IPAA: ileal pouch-anal anastomosis; FAP: familial adenomatous polyposis; PDAI: pouchitis disease activity index; OTUs: operational taxonomic units; RDP: Ribosome Database Project; PCoA: principal co-ordinates analysis; PCA: principal component analysis; LEfSe: Linear discriminant analysis Effect Size; KEGG: Kyoto Encyclopedia of Genes and Genomes pathway; GO: Gene Ontology; DEGs: differentially expressed genes; BP: biological process; CC: cellular component; MF: molecular function; PPI: protein–protein interaction; ssGSEA: Single-sample Gene Set Enrichment Analysis

\textbf{Background}

Ulcerative colitis (UC) is an autoimmune disease that occurs in the colon and rectum, and most patients with active UC achieve remission through standardized medical treatment [1]. Surgical intervention is a better choice when medical treatment fails or when serious complications occur. Total colorectal resection with ileal pouch-anal anastomosis (IPAA) is the standard surgical treatment for UC [2,3]. However, approximately 50% of patients with pouch develop inflammation within 5 year [4], and the incidence is much higher than those with familial adenomatous polyposis (FAP) who undergo the same surgery [5,6].

Inherited early transcriptomic changes are associated with the UC-pouch, including those characteristic of enhanced immune and inflammatory responses, as well as remodeling of the extracellular matrix [7–9]. Furthermore, similar to UC, fever, abdominal pain, diarrhea and hematochezia are common clinical symptoms in patients with pouchitis [4]. Therefore, one hypotheses propose that pouchitis represents the recurrence of UC in the normal small intestine [10].

However, although genetic predisposition does not directly account for pouchitis, it appears to serve a prerequisite for environmental susceptibility and potential microbial instability [9]. In contrast to predominant use of antibiotics to treat pouchitis, the use of antibiotics to treat UC is
controversial, even though meta-analysis has shown it helps improve clinical remission rates of patients with UC [11,12]. Gut microbiota may hold the key to triggering the inflammatory response [13]. Therefore, we hypothesized that the pathogenesis of UC and pouchitis is different, which would be elucidated by identifying the altered characteristics of the microbiome and transcriptome.

**Methods**

**Study subjects**

We recruited patients with UC or UC-pouch who were admitted to the General Hospital of Tianjin Medical University. Sex- and age-matched healthy volunteers served as the control group. All UC patients had a definite diagnosis, and they were divided into remission, mild, moderate, severe UC subgroup according to modified Mayo scores (Supplementary Table 1) [14]. All UC-pouch patients underwent ileostomy closure after at least 1 year, and inclusion criteria for patient with a normal pouch function were no inflammation and pouch dysfunction symptoms after IPAA, and their pouchitis disease activity index (PDAI < 7, Supplementary Table 2) [15], while pouchitis was defined as ≥2 episodes of pouchitis diagnosed according to clinical symptoms within 2 years and PDAI score ≥7.

All participants were not treated with antimicrobials for at least 4 weeks before analysis of their gut microbiota. Then, we collected fecal samples into sterile test tubes, which were stored at −80°C. The Ethics Committee of Tianjin Medical University General Hospital approved this study (number: IRB2021-WZ-199), and all subjects granted their written informed consent before the study commenced.

**DNA acquisition and 16S ribosomal DNA pyrosequencing**

According to the OMEGA Stool DNA Kit instructions (D4015, Omega, Inc., Norwalk, CT), genomic DNA extraction from Stool samples was performed. The concentration and purity of DNA samples were determined by NanoDrop2000 spectrophotometer and unqualified DNA samples will be discarded. Primers for the 16S ribosomal DNA V3–V4 regions of the bacterial genome were designed in the experiment. The primer sequence of 336F was 5’-GTACTCTACGGGAGGCAGCA-3’, and the primer sequence of 806R was 5’-GATGACTACHVGG GTWTCTAAAT-3’. A PCR system was established to specifically amplify the V3–V4 region of the 16S ribosomal DNA of the sample genome. Qualified agarose gels were sent to LC-Bio Technology (Hangzhou, China) for Miseq library construction then performed sequencing on Illumina Miseq PE300 platform.

**Microbial analysis**

Sequences comprising <200 bp, ambiguous bases, and with mismatches with primer sequences or barcode labels were excluded from the analysis [16]. Qualified reads were isolated using sample-specific barcode sequences and analyzed and pruned using Illumina pipeline version 2.6 (San Diego, CA). QLIME software was used to analyze the data. Sequences with 97% identity were grouped into operational taxonomic units (OTUs) [17], sparse curves were generated, and richness and diversity indexes were calculated [18]. Sequences were classified according to the tool provided by the Ribosome Database Project (RDP) [19].

We used R software to evaluate the similarities between different samples, and cluster analyses principal co-ordinates analysis (PCoA, based on unweighted UniFrac distance algorithm) were performed according to the OTU information. The distance between evolving microbial communities of each sample was calculated using the TAYC coefficient and is represented as an insignificant cluster tree between two groups. The arithmetic mean method was used to describe the difference (Δ = 1 – similarity) between multiple samples [20]. Tree files in Newick format were generated. To compare the community members and community structures of different samples, the top 50 community heat maps were generated using Mothur. The linear discriminant analysis Effect Size (LEfSe) method was used to analyze different species among the different groups of subjects (default LDA = 3) [21]. We used PICRUSt and PICRUSt2 to predict functions and Kyoto Encyclopedia of Genes and Genomes pathway (KEGG) based on different gut microbiota [22,23].

**Bioinformatics analysis**

We obtained transcriptome expression data of 119 UC and 28 pouch patients from Gene Expression Omnibus (ID: GSE107499, GSE50788, https://www.ncbi.nlm.nih.gov/geo). We used the ‘Limma’ R package to identify the differentially expressed genes (DEGs), protein–protein interaction network (PPI network, https://string-db.org/cgi/input.pl) and Cytoscape was used to visualize the associations of DEGs and obtain hub genes. In addition, we evaluated the immune status of UC and pouchitis by Single-sample Gene Set Enrichment Analysis (ssGSEA).

**Statistical analysis**

SPSS 22.0 (SPSS Inc., Chicago, IL) and R 4.0.1 software (R Foundation for Statistical Computing, Vienna, Austria) was used for statistical analysis. Uparse software was used to cluster OTUs with 97% similarity, and representative OUT sequences were compared and annotated with the corresponding species according to the SILVA128 16S reference database. The confidence threshold was defined as 80%, ANOVA was used to evaluate the differences in bacterial community structures, LEfSe was used to identify different species, and Tukey’s test was used to evaluate differences in the Shannon index. PICRUSt predicted the functional composition of metagenomes, and p<.05 (corrected using false discovery rates) indicated a significant difference.
Results

We included 37 patients with UC (51% females; average age, 41.6 ± 13 years), 30 patients with UC-pouch (50% females, average age 43.3 ± 11 years) and 18 healthy individuals (50% females; average age, 38.3 ± 13 years). According to their PDAI, 15 patients were included in the pouchitis group.

Gut microorganism analysis of ulcerative colitis

Clinical demographics of the UC and control groups are shown in Table 1. We summarized the changes of gut microbiota within different UC activity levels. The abundances of Firmicutes decreased in patients with moderate and severe activity UC, while that of Proteobacteria increased in patients with severe active UC (Figure 1(a)). The compositions of the gut microbiota of patients with UC patients were altered at the genus level (Figure 1(b)). We used Shannon’s α-diversity index to show that microbial diversities of the remission and mild groups, as well as that of the control group, were similar. In contrast, the diversities of the moderate and severe groups were significantly decreased (4.79 and 4.39 vs. 5.04, \( p = .012, p = .002 \), respectively) (Figure 1(c)). The results of principal coordinate analysis showed similar results, in that the microbial communities of the moderate and severe groups were significantly different compared with those of the control group (\( p < .001 \) and \( p < .001 \), respectively) (Figure 1(d)).

When we used LEfSe analysis to further search for different species among the subgroups, we found that Mogibacterium species increased in patients with mildly and moderately active UC, while Lachnoclostridium and Peptostreptococcus were increased in patients with severe, active UC (Figure 1(e,f)). Furthermore, Bacteroides, Roseburia and Blautia gradually decreased with the development of inflammation.

Gut microorganism analysis of pouchitis

Certain baseline characteristics of patients with pouch are shown in Table 2. At the level of the Bacteriophyta, compared with the normal pouch, Bacteroidetes decreased while...
Proteobacteria significantly increased in the pouchitis group (Figure 2(a)). The pouchitis group had a lower Shannon \( \alpha \)-diversity level (4.09 vs. 5.07, respectively, \( p = .049 \)) (Figure 2(b)) and a significantly different community structure compared with the normal pouch group (\( p < .001 \)) (Figure 2(c)). The relative abundances of Escherichia–Shigella and Bacteroides were respectively over-represented and significantly lower compared with controls (Figure 2(d,e)). Furthermore, the results of LEfSe analysis were similar in that Escherichia–Shigella increased while Bacteroides and Faecalibacterium decreased in the pouchitis group (Figure 2(f)).

**Functional analysis of gut microbiota in ulcerative colitis and pouchitis**

We used the PICRUSt2 algorithm to analyze the combined data for the moderate and severe UC subgroups (MSUC).

This analysis revealed 77 and 132 functional differences in patients with MSUC and those with pouchitis, respectively. For example, the Bifidobacterium shunt, inosine-5'-phosphate biosynthesis, and heterolactic fermentation were enhanced in patients with UC, while the L-alanine and demethylmenaquinol pathways were increased in those with pouchitis. Furthermore, the functional prediction indicates overproduction of enterobactin in patients with pouchitis (Figure 3(a,b)). Further Spearman’s correlation analysis of different microorganisms based on PICRUSt showed that Escherichia–Shigella, as well as other bacteria such as Ruminococcus gnavus, may be associated with the production of enterobactin (Figure 3(c)).

According to KEGG analysis, the most enrichment of pathways associated with the immune system and infectious diseases varied among the bacteria included in the heat map (Figure 3(d)). These results show that these pathogens, mainly Escherichia–Shigella, were involved in diverse infection pathways.

**Transcriptome changes in ulcerative colitis and pouchitis**

To more fully compare the differences between UC and pouchitis, we obtained the gut transcriptome data from 119 UC, 15 normal pouch and 13 pouchitis patients from two GEO cohorts. We identified 388 down-regulated genes and 865 up-regulated genes in UC and 432 down-regulated genes and 208 up-regulated genes in pouchitis compared to the normal mucosa (Supplementary Table 3). Principal component analysis (PCA) showed there were differences in transcriptome expression between inflammatory and non-inflammatory mucosa (Figure 4(a,d)). Volcano plot showed the DEGs that the screening condition was logFC > 1, \( p < .05 \) (Figure 4(b,e)). The heat map displayed 40 genes that differ mostly in UC and pouchitis (Figure 4(c,f)).

![Figure 2](image_url)

**Figure 2.** Changes of gut microbiota in pouchitis. (a) Bacteroidetes decreased and Proteobacteria increased in pouchitis, (b) microbial \( \alpha \)-diversity decreased in pouchitis, (c) different microbial structures between non-pouchitis and pouchitis and (d-f) a large increase of Escherichia–Shigella in pouchitis.
We further conducted functional enrichment analysis on DEGs. Functional enrichment followed a similar pattern as more genes in UC were up-regulated and more genes in pouchitis were down-regulated (Figure 5). Annotation for GO and KEGG pathways are presented in Tables 3 and 4. We found that the major enrichment pathways in UC are associated with the release of inflammatory factors, including leukocyte migration, leukocyte cell–cell adhesion, cell chemotaxis and IL-17 signaling pathway. Nevertheless, multiple
metabolic pathways are down-regulated in pouchitis, including fatty acid metabolic process, carboxylic acid biosynthetic process and biosynthesis of amino acids.

Identification the hub genes in UC and pouchitis

We analyzed the interaction between DEGs in UC and pouchitis based on PPI network and Cytoscape (Figure 6(a,d)), and the MOCDE plug was used for modular analysis of DEGs (Figure 6(b,e)). Finally, we identified the top 10 hub genes in UC and pouchitis by Degree algorithm (Figure 6(c,f)). We discovered that the hub genes of active UC and pouchitis are very different. The overexpression of TNF, interleukin and other inflammatory genes was up-regulated in active UC while the hub genes in pouchitis such as CDK1 and CCNA2 are involved in regulating the cell cycle (Supplementary Figure 1).

Evaluation of immune infiltrating cells in UC and pouchitis

We used ssGSEA algorithm to score 16 immune cells and 13 immune functions in UC and pouchitis compared to healthy mucosa (Figure 7). We found that all immune cells except natural killer cells were enriched in the inflamed mucosa with UC which suggested a really quite different immune signature from pouchitis (Figure 7(a,b)), and the immune strength of UC is stronger than that of pouchitis (Figure 7(c,d)).

Discussion

The incidence rate of inflammatory bowel disease is increasing year by year. It has brought great challenges to social and public health. The pathogenesis of inflammatory bowel disease is complex, which involves susceptibility of the host, gut microbiota imbalance, environmental factors and immune overactivation [24], and multiomics technology (such as microbiomics, metabonomics and transcriptomics) provided some promising way to reveal the pathogenesis landscape of IBD. However, some specific clinical phenomena need further explanation. For example, pouch inflammation is often found in the patients with UC-pouch, but the treatment strategies between UC and pouchitis are greatly
different. Clinical remission is achieved using metronidazole/ciprofloxacin for patients with acute pouchitis (whether or not they are relapse), while 5-aminosalicylic acid and biological agents should be considered in chronic antibiotic-refractory pouchitis which share more autoimmune features [4,25]. These effects of treatment indicate similarities in microbial pathogenesis between specific types of pouchitis and UC, but most cases of pouchitis differ from UC. Therefore, this study systematically analyzed the characteristics of microbiome and transcriptome changes in UC and pouchitis and proposed that pouchitis may have a unique pathogenesis which was separated from UC.

Table 3. Annotation for GO and KEGG analysis results in UC.

| Ontology ID | Description                          | GeneRatio |
|------------|--------------------------------------|-----------|
| BP GO:0005000 | Leukocyte migration                  | 104/1139  |
| BP GO:0007159 | Leukocyte cell–cell adhesion         | 80/1139   |
| BP GO:00060326 | Cell chemotaxis                      | 75/1139   |
| BP GO:00030595 | Leukocyte chemotaxis                 | 61/1139   |
| BP GO:00022409 | Positive regulation of cell–cell adhesion | 65/1139 |
| CC GO:0009897 | External side of plasma membrane     | 81/1187   |
| CC GO:0062023 | Collagen-containing extracellular matrix | 63/1187 |
| CC GO:0045177 | Apical part of cell                  | 54/1187   |
| CC GO:0030667 | Secretory granule membrane           | 44/1187   |
| CC GO:0016324 | Apical plasma membrane               | 45/1187   |
| MF GO:0005125 | Cytokine activity                    | 48/1117   |
| MF GO:0005539 | Glycosaminoglycan binding            | 47/1117   |
| MF GO:0042379 | Chemokine receptor binding           | 22/1117   |
| MF GO:0008009 | Chemokine activity                   | 19/1117   |
| MF GO:0048018 | Receptor ligand activity             | 69/1117   |
| KEGG hsa04060 | Cytokine–cytokine receptor interaction | 67/613   |
| KEGG hsa05323 | Rheumatoid arthritis                 | 34/613    |
| KEGG hsa04640 | Hematopoietic cell lineage           | 33/613    |
| KEGG hsa04061 | Viral protein interaction with cytokine and cytokine receptor | 33/613 |
| KEGG hsa04514 | Cell adhesion molecules              | 40/613    |
| KEGG hsa04657 | IL-17 signaling pathway              | 28/613    |
| KEGG hsa05150 | Staphylococcus aureus infection      | 27/613    |
| KEGG hsa04380 | Osteoclast differentiation           | 31/613    |
| KEGG hsa05146 | Amoebiasis                           | 27/613    |
| KEGG hsa05144 | Malaria                              | 18/613    |

GO: Gene Ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes pathway; BP: biological process; CC: cellular component; MF: molecular function.

Table 4. Annotation for GO and KEGG analysis results in pouchitis.

| Ontology ID | Description                          | GeneRatio |
|------------|--------------------------------------|-----------|
| BP GO:0006631 | Fatty acid metabolic process         | 49/449    |
| BP GO:0046394 | Carboxylic acid biosynthetic process | 52/449    |
| BP GO:0016053 | Organic acid biosynthetic process    | 52/449    |
| BP GO:0044282 | Small molecule catabolic process     | 48/449    |
| BP GO:0044242 | Cellular lipid catabolic process     | 34/449    |
| CC GO:0016324 | Apical plasma membrane               | 37/457    |
| CC GO:0045177 | Apical part of cell                  | 40/457    |
| CC GO:0042627 | Chylomicron                          | 7/457     |
| CC GO:0024361 | Very-low-density lipoprotein particle | 8/457  |
| CC GO:0034385 | Triglyceride-rich plasma lipoprotein particle | 8/457 |
| MF GO:0046906 | Tetrapyrrole binding                 | 19/448    |
| MF GO:0008238 | Exopeptidase activity                | 16/448    |
| MF GO:0016616 | Oxidoreductase activity, acting on the CH-OH group of donors, NAD or NADP as acceptor | 16/448 |
| MF GO:0020037 | Heme binding                         | 17/448    |
| MF GO:0016614 | Oxidoreductase activity, acting on CH-OH group of donors | 16/448 |
| KEGG hsa04975 | Fat digestion and absorption         | 13/281    |
| KEGG hsa03320 | PPAR signaling pathway               | 15/281    |
| KEGG hsa00590 | Arachidonic acid metabolism          | 13/281    |
| KEGG hsa00480 | Glutathione metabolism               | 11/281    |
| KEGG hsa00982 | Drug metabolism – cytochrome P450    | 12/281    |
| KEGG hsa04597 | Cholesterol metabolism               | 10/281    |
| KEGG hsa03330 | Arginine and proline metabolism      | 10/281    |
| KEGG hsa01230 | Biosynthesis of amino acids          | 12/281    |

GO: Gene Ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes pathway; BP: biological process; CC: cellular component; MF: molecular function.

Over the past decade, changes in gut microorganisms have been found in many human diseases, and some therapies based on regulating intestinal microorganisms have been highly expected. A recent study reported that the difference of microbiome in autism spectrum disorder may be due to the preference of patients’ diet, rather than the cause of the disease [26], therefore, it is important to understand whether microbial changes are the cause or the result. Intestinal inflammation may be triggered by the following two pathways. The classic approach is that pathogenic microorganisms and their metabolites are recognized by pattern recognition receptors, and then activate the innate

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immune system, activating NF-κB and inflammasome, which stimulates the production of chemokines and proinflammatory cytokines [27], or the instability of the composition of gut microbiota can lead to overactivity of the immune system by immunoglobulin [28]. We showed here that the diversity of the gut microbiota decreased in patients with UC or pouchitis and that UC and pouchitis were associated with different microbial structures compared with the control group, which were consistent with previous study [29]. When it comes to active UC, though evidence indicates that Lachnoclostridium contributes to produce sulfide that inhibits butyrate utilization [30], Peptostreptococcus triggers a proinflammatory response based on activating nuclear factor kappa-B (NF-κB) activation [31]. However, it is unlikely that a single infection could trigger the IBD [24], and the increased abundance of these bacteria suggests that their role in promoting the occurrence of UC is limited (Figure 1(b)).

In contrast, KEGG analysis showed that many pathogens enriched in pouchitis were related to numerous pathways associated with infectious diseases (such as vibrio cholerae infection, hepatitis, influenza A and pathogenic Escherichia coli infection). That means infectious bacteria play an important role in the occurrence of pouchitis. What is more, we found that, enterobactin, a siderophore secreted by many pathogens, will confer a survival advantage upon E. coli in the inflammatory bowel [32], was associated with many pathogens enriched in pouchitis. Therefore, we concluded that the proliferation of these pathogenic bacteria could be the direct cause of UC-pouchitis, which likely explains why the use of antibiotic monotherapy can achieve clinical remission in most patients with pouchitis.

Beneficial bacteria are important for intestinal barrier function. Butyrate is an energy source required for gut epithelial cells to maintain the function of gut barrier [33–35]. Bacteroides produces vitamin K and has anti-inflammatory potential [36,37]. The depletion of these bacteria weakens the gut barrier and lead to more severe inflammation [38]. These reductions in beneficial bacteria have been observed in both UC and pouchitis, and we suggested that there are more likely to be a result of gut inflammation. It should be emphasized that dysbacteriosis in IBD may reflect the response of microbial community to gut inflammation [39]. In fact, inflammation increases the oxidation state of the gut cavity; however, probiotics are often anaerobic and difficult to survive in this oxygenated environment [40]. Therefore, according to the results of microbiome, we proposed that the quantity of infectious bacteria is closely related to the occurrence of pouchitis while dysbacteriosis might not be...
the driver event but might exacerbate later in the progression of UC and contribute to the chronicity disease [39].

To more fully evaluate the pathogenesis of UC and pouchitis, we analyzed the transcriptome alteration characteristics based on bioinformatics. Our results confirmed that there were significant differences in the characteristics of transcriptome changes between UC and pouchitis. In active UC, more genes related to inflammatory factors are overexpressed while more genes showed down-regulation in pouchitis. Fyn and LCK belong to the Src family of protein kinases, Fyn may influence epicardial disorders by regulating B cell differentiation during the early stages of B cell development [41] and LCK plays a vital role in various cellular processes such as leukocyte cell adhesion and proliferation [42]. TNF, IL6 and IL1B also are key genes more widely reported in UC. CYP3A4 has a wide range of substrates and is involved in the metabolism and transportation of roughly half of all currently prescribed medicines, and was observed a decrease in Crohn’s disease [43]. Biological process analysis in pouchitis showed hub genes are mainly involved in regulating the cell cycle (Supplementary Figure 1). In addition, the immune response is more strong in active UC than pouchitis. The analysis of transcriptome data supports our previous hypothesis, conceivably, microbial disorder could not be sufficient to trigger strong immunity in UC, because the dysbiosis is more severe in pouchitis, but the immune response is less severe.

In summary, we characterized transcriptome and microbiome changes in UC and pouchitis. We promote that the proliferation of infectious bacteria is closely related to the occurrence of pouchitis while the pathogenesis of UC should fully consider gene expression level. Our results provide a new perspective on the pathogenesis of IBD.

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Consent for publication
Not applicable.

Ethics approval and consent to participate
Our study was approved by the Ethics Committee of Tianjin Medical University General Hospital. All participants provided a written informed consent for participating in this study.
consent upon enrolment. The methods were carried out in accordance with the relevant guidelines.

Authors contributions
Li-Sheng Yang and Di Huang recruited patients for this study. Xin Gao and An-Qi He performed the bioinformatics analysis. Xin Gao and Li-Sheng Yang drafted the manuscript and all authors reviewed it. Xin Gao and Kai-Yu Li approved final version. Xin Gao and Tong Liu performed the statistical analysis.

Disclosure statement
The authors of this manuscript report no conflict of interest.

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Data availability statement
The data and code that support the findings of this study are available from the corresponding author upon reasonable request.

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