Oncology and Translational Medicine
DOI 10.1007/s10330-021-0509-9
December 2021, Vol. 7, No. 6, P269–P274

ORIGINAL ARTICLE

Correlation analysis of breast fibroadenoma and the intestinal flora based on 16S rRNA sequencing*

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Abstract

Objective To analyze the characteristics of the intestinal microflora in patients with breast fibroadenoma using 16S ribosomal RNA (rRNA) high-throughput sequencing.

Methods Fecal samples from 20 patients with breast fibroadenoma and 36 healthy subjects were randomly collected and analyzed using high-throughput sequencing technology for 16S rRNA V4 region sequencing, and the alpha diversity (Chao index, Shannon index) was calculated using Mothur (v.1.39.5) software. Beta diversity was analyzed using QIIME (v1.80). SPSS software (version 23.0) and the t-test of two independent samples were used to analyze differences in the abundance of bacteria between the two groups.

Results Compared with that in the healthy control group, the α diversity of the intestinal microflora in breast fibroadenoma patients increased, but the difference was not statistically significant ($P > 0.05$). At the phylum level, significant differences were observed between the two groups. The abundance of Firmicutes was higher in the breast fibroadenoma group ($P < 0.05$), whereas the abundance of Synergistetes was higher in the healthy control group ($P < 0.005$). A total of five bacterial genera showed significant differences between the two groups: the breast fibroadenoma group showed higher levels of Bautia ($P < 0.005$), Coprococcus ($P < 0.005$), Roseburia ($P < 0.05$), and Ruminococcus ($P < 0.005$), whereas Sutterella was more abundant in the healthy control group than in the breast fibroadenoma group ($P < 0.05$).

Conclusion The diversity and abundance of the intestinal flora in patients with breast fibroadenoma are significantly different from those in healthy subjects, suggesting that an imbalance in the intestinal flora is correlated with the occurrence of breast fibroadenoma.

Received: 15 July 2021
Revised: 31 August 2021
Accepted: 21 October 2021

Key words: intestinal flora; estrogen; breast fibroadenoma; 16S ribosomal RNA; high-throughput sequencing

Breast fibroadenoma is the most common benign breast tumor in women. It is primarily composed of proliferative breast fibrous tissue and ducts, and its occurrence may be related to an abnormal quality or quantity of estrogen receptors contained in fibroblasts; however, the precise etiology remains unclear [1].

The intestinal tract is the largest digestive organ of the human body, which contains a large number of bacteria and has a genome approximately 100 times that of humans [2].The human intestinal flora contains genes that encode thousands of microbial enzymes and metabolites [3–4]. The intestinal flora is closely related to the estrogen metabolism in the body. The intestinal microbes contain genes related to estrogen metabolism and encode β-glucuronidase. When the content of this enzyme in the intestine increases, the glucuronidase-estrogen conjugate is decomposed; estrogen returns to the free state and is re-absorbed into the blood through the hepatointestinal circulation, thus leading to an increase in endogenous estrogen levels [5].Therefore, changes in estrogen levels caused by the imbalance of intestinal flora may be an important factor in the occurrence of breast

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* Supported by a grant from the Qingdao Pharmaceutical Research Guidance Plan 2019 (No. 2019-WJZD140).
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fibroadenoma.

A number of studies have confirmed that the intestinal flora can affect the occurrence and development of breast cancer through estrogen metabolism, immune regulation, and generation of short-chain fatty acids (SCFAs) [5–8]; however, a correlation between breast fibroadenoma and intestinal microbes has not been reported. This study involved the collection of stool samples from 20 female patients with breast fibroadenoma and 36 healthy adult women. Through 16S rRNA sequencing of the V4 area and variance analysis, the intestinal flora diversity and composition of the samples were evaluated. These results provide a new theoretical basis for the diagnosis and prevention of breast fibroadenoma.

Materials and methods

Case selection

Patients admitted to the Breast Surgery Department of Qingdao Central Hospital and diagnosed with breast fibroadenoma via postoperative paraffin section pathology in the Pathological Diagnostic Center and healthy adult females without any breast-related diseases, as confirmed by the Physical Examination Center, were selected. All subjects had a normal body mass index (BMI) and had not used antibiotics, probiotics, antacids, gastrointestinal motility agents, or other drugs that could affect the intestinal flora in the 6 months before enrollment. The subjects did not have hypertension, coronary heart disease, diabetes, cirrhosis, malignant tumors, or other primary diseases. A total of 56 female subjects were included in this study, including 20 patients with breast fibroadenoma and 36 healthy adult females. All subjects signed an informed consent form and volunteered to participate in the study.

Specimen collection

Fresh fecal samples (no less than 10 g) from the 56 subjects were collected with sterile cotton swabs, placed in a sterile container, and immediately stored in a refrigerator at –80°C for low-temperature preservation. All of the above procedures were performed under sterile conditions.

Amplifier sequencing

The collected fecal samples were cryopreserved and sent to Qingdao BGI Institute for gene sequencing. The process was as follows: (1) Genomic DNA extraction: The cetyltrimethylammonium bromide (CTAB) or sodium dodecyl sulfate (SDS) method was used to extract genomic DNA from the samples, and agarose gel electrophoresis was used to detect the purity and concentration of the DNA. An appropriate amount of the samples was placed in a centrifuge tube, and the samples were diluted to 1 ng/μL with sterile water. (2) PCR amplification: Diluted genomic DNA was used as a template. According to the selection of the sequencing region, specific primers with Barcode were used; the 16S V4 primer was 515F-806R. The Phusion® High-Fidelity PCR Master Mix and GC Buffer from New England Biolabs were used. PCR was performed using high-efficiency and high-fidelity enzymes to ensure amplification efficiency and accuracy. The PCR was conducted on the Bio-Rad T100 gradient PCR instrument. PCR products were detected by electrophoresis on a 2% agarose gel. (3) Mixing and purification of PCR products: The PCR products were mixed and purified according to the concentration of the PCR products, and the PCR products were mixed at the same concentration. After thorough mixing, the PCR products were purified by electrophoresis with a 1× TAE concentration of 2% agarose, and the target bands were recovered by gelling, using the Thermo Scientific Genejet Gel Recovery Kit. (4) Library construction and computer sequencing: The Illumina TruSeq DNA PCR-Free Library Preparation Kit was used to construct the library. After Qubit quantification and library testing, NovaSeq 6000 was used for computer sequencing of the qualified library.

Bacterial community information analysis

The software Mothur v.1.39.5 was used to remove all the redundant tags, and the software USEARCH (v7.0.1090) was used to cluster the spliced tags into operational taxonomic units (OTUs). After the OTU representative sequence was obtained, species annotation was carried out by comparing the OTU representative sequence with the Greengenes database using RDP Classifier (V2.2) software, and the confidence threshold was set to 0.8. Alpha diversity was calculated using Mothur (v.1.39.5) software, and beta diversity was analyzed using QIIME (v1.80).

Statistical analysis

SPSS software (version 23.0) was used for data analysis, and the *t*-test of two independent samples was used to analyze differences in the abundance of bacteria between the two groups. Statistical significance was set at $P < 0.05$.

Results

Sequencing data, sample out, and diversity analysis

A total of 5004,192 high-quality sequences were obtained from 56 samples in the two groups, with an average sequence length of approximately 252 bp, and a total of 3911 OTUs were generated. The sequencing coverage of all samples reached 99.9%. The dilution curve reflects whether the sequencing quantity of the sample was sufficient. If the curve flattens or reaches the

plateau stage, the sequencing depth can be considered to have covered all the species in the sample. The contrary means that the species diversity in the sample is high and there are more species that have not been detected by sequencing. As shown in Fig. 1, the Chao index dilution curve gradually flattened with the increase in sequencing data, as did the Shannon index dilution curve. Therefore, it can be concluded that the sequencing depth covered all species.

The α diversity index was calculated based on the species and abundance of OTUs.

The Chao index reflects the richness of the community in the sample; the higher the index, the richer the species. The Shannon index reflects the diversity of the community; the larger the Shannon index, the greater the diversity of the community, as shown in Fig. 2a and 2b. There was no significant difference in the abundance of intestinal microbial species ($P > 0.05$), but there was an increasing trend of α diversity in the breast fibroadenoma group. In addition, principal component analysis (PCA) was conducted based on the OTU level (Fig. 2c). The samples from the breast fibroadenoma group (shown in green in Fig. 2c) and the healthy control group (shown in blue in Fig. 2c) were compared and analyzed. The results showed that the bacterial flora compositions of the two groups were different.

Analysis of flora structure and relative abundance

In this study, the structure and composition of the intestinal flora of the breast fibroadenoma group and the healthy adult female group were analyzed at the phylum and genus levels, respectively.

(1) Relative abundance analysis of the intestinal flora at the phylum level

At the phylum level, the top 13 strains were selected

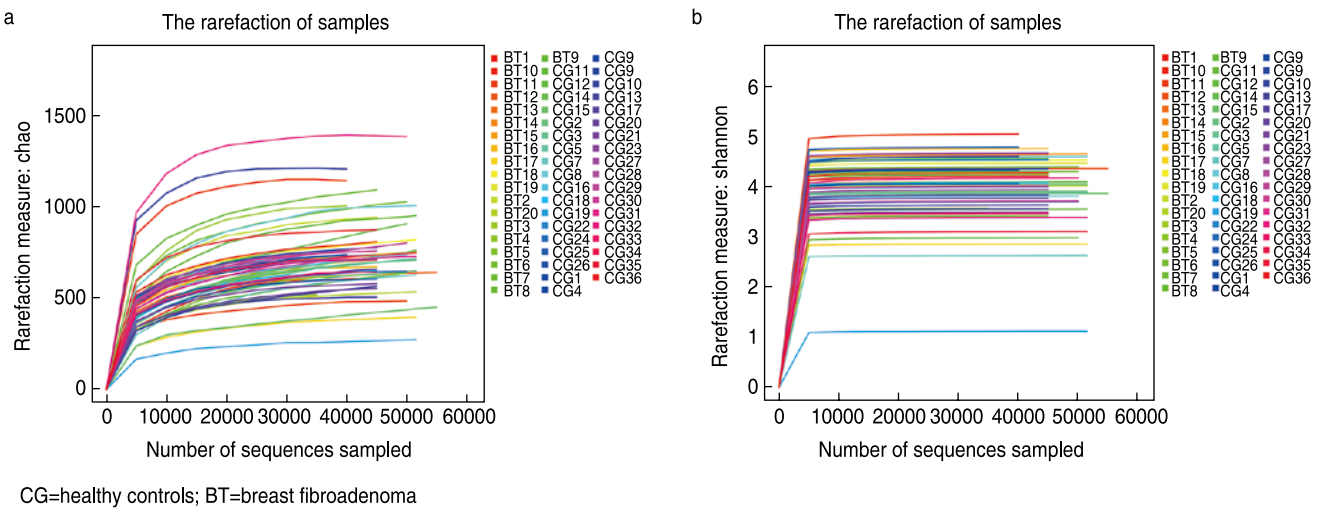


Fig. 1 Chao index and Shannon index dilution curve

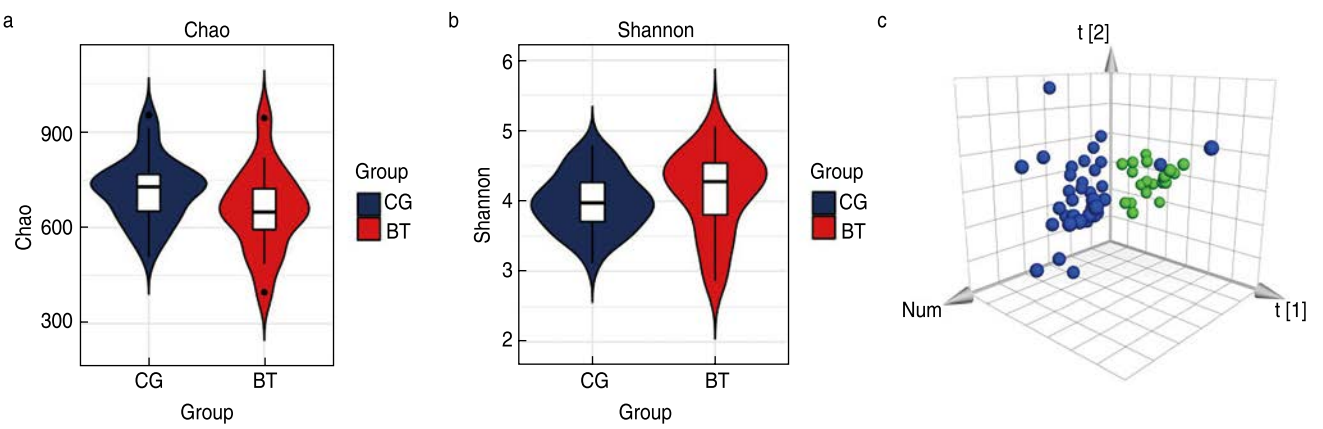


Fig. 2 α-diversity analysis and principal component analysis (PCA)

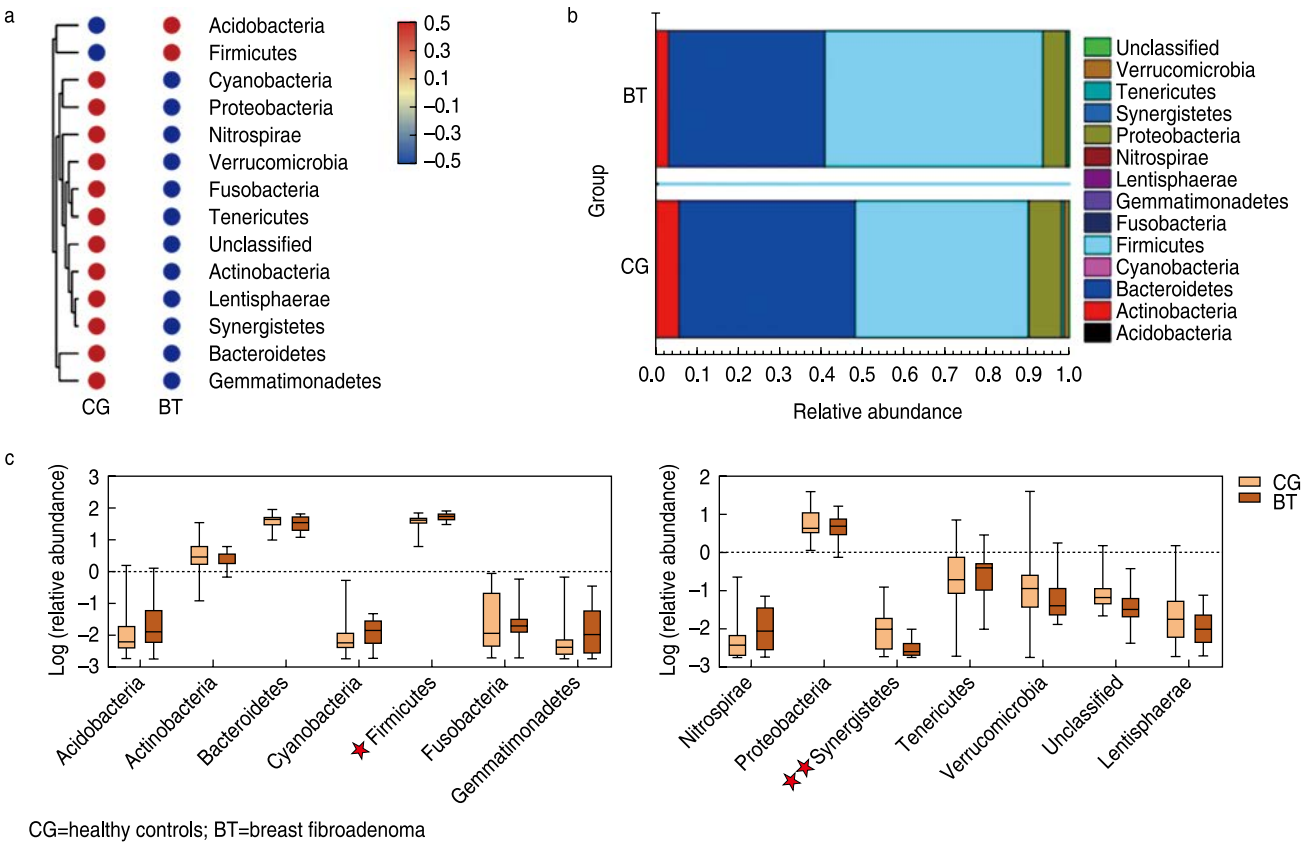
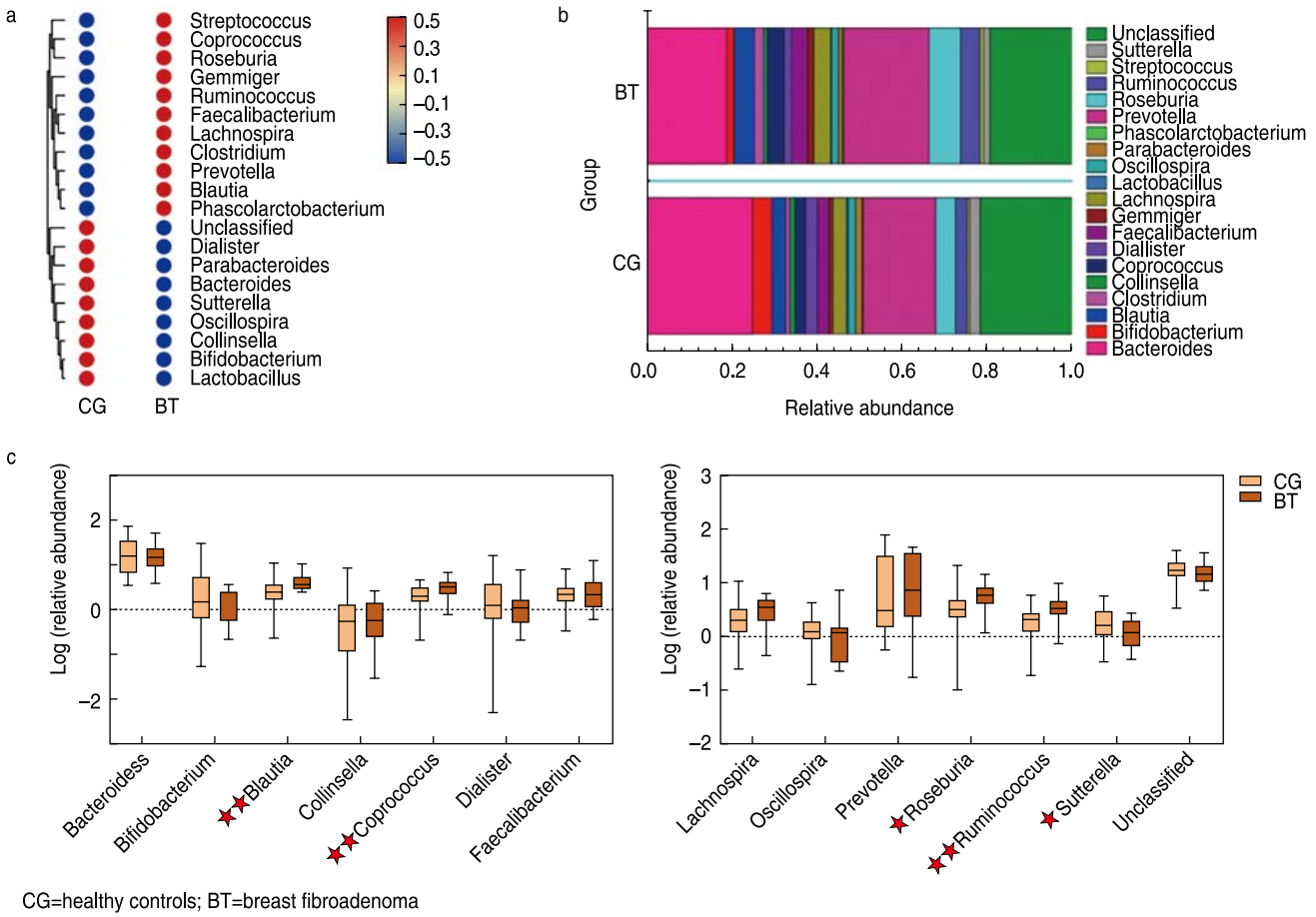


Fig. 3 Analysis of relative abundance of the intestinal flora at the phylum level between the healthy control group and the breast fibroadenoma group

for relative abundance analysis, as shown in Fig. 3a. The dominant bacterial phyla in the healthy control group were Cyanobacteria, Proteobacteria, Nitrospirae, Verrucomicrobia, and Fusobacteria, Tenericutes, Actinobacteria, Lentisphaerae, Synergistetes, Bacteroidetes and Gemmatimonadetes, whereas the dominant phyla in the breast fibroadenoma group were Acidobacteria and Firmicutes. As shown in Figure 3B, at the phylum level, the dominant species in both groups were Firmicutes, Bacteroidetes, Verrucomicrobia, and Actinobacteria. However, there were significant differences in species composition between the healthy control group and the breast fibroadenoma group. Based on the abundance data of the two groups, the *t*-test of two independent samples was used to analyze the species with different phylum levels in the intestinal flora of the breast fibroadenoma group and the healthy adult female group. As shown in Fig. 3c, there were significant differences in the two categories between the two groups. The abundance of Firmicutes was higher in the breast fibroadenoma group than in the healthy control group ($P < 0.05$). Synergistetes were more abundant in the healthy control group than in the breast fibroadenoma group ($P < 0.005$). According to these results, the two abovementioned phyla may be correlated with the occurrence of breast fibroadenoma; however, further analysis is necessary.

(2) Relative abundance analysis of the intestinal flora at the genus level

The top 19 bacterial genera were selected for further analysis of the relative abundance. As shown in Fig. 4a, the dominant genera in the healthy control group were Dialister, Parabacteroides, Bacteroides, Sutterella, Oscillospira, Collinsella, Bifidobacterium, and Lactobacillus. The dominant species in the breast fibroadenoma group were *Streptococcus*, *Coprococcus*, *Roseburia*, *Gemmiger*, *Ruminococcus*, *Facecalibacterium*, *Lachnospira*, *Clostridium*, *Prevotella*, *Blautia*, and *Phascolarctobacterium*. As shown in Fig. 4b, the dominant species in both groups were Bacteroides, Prevotella, and Roseburia. Based on the abundance data of the two groups, the t-test of two independent samples was used to analyze the species with a different intestinal flora at the genus level between the breast fibroadenoma group and the healthy adult female group. As shown in Fig. 4c, a total of five bacterial genera showed significant differences between the two groups of samples. The breast fibroadenoma group and the healthy control group



CG=healthy controls; BT=breast fibroadenoma

Fig. 4 Analysis of relative abundance of the intestinal flora at the genus level between the healthy control group and the breast fibroadenoma group

had comparable levels of Bautia ($P < 0.005$), Coprococcus ($P < 0.005$), Roseburia ($P < 0.05$) and Ruminococcus ($P < 0.005$). The abundance of Sutterella in the healthy control group was higher than that in the fibroadenoma group ($P < 0.05$).

Discussion

Breast fibroadenoma is the most common breast fibroepithelial tumor in women. These tumors are hormone dependent; they increase in size due to factors such as estrogen, progesterone, prolactin, and pregnancy and decrease after menopause [9, 10]. Currently, there are no effective preventive measures for breast fibroma, and the treatment primarily consists of surgical resection, which is associated with a risk of recurrence [11, 12]. Studies have shown that breast fibroadenoma is an independent risk factor for breast cancer, and the risk of breast cancer 20 years later in patients with breast fibroadenoma is twice that in healthy women [13]. Studies have confirmed that the intestinal flora plays an important role in the occurrence and progression of breast cancer [6–8]. However, it is still unclear whether there is a correlation between breast fibroadenoma and the intestinal flora. Therefore, this study used 16S rRNA high-throughput sequencing to evaluate the intestinal flora of patients with breast fibroadenoma. The results of this study showed that compared with that of the healthy control group, the intestinal microflora of the patients with breast fibroadenoma showed an increased α diversity, indicating an imbalance of the intestinal microflora in patients with breast fibroadenoma. The dominant phyla in the breast fibroadenoma group and the healthy control group were Firmicutes, Bacteroidetes, Verrucomicrobia, and Actinobacteria. However, the abundance of Firmicutes was higher in the breast fibroadenoma group than in the healthy control group, and the abundance of Synergistetes was higher in the healthy control group than in the breast fibroadenoma group. A total of five bacterial genera showed significant differences between the two groups. Compared with that in the healthy control group, the abundance of Blautia, Coprococcus, Roseburia and Ruminococcus in the breast fibroadenoma group was higher. The abundance of

Sutterella was higher in the healthy control group than in the fibroadenoma group. Chan *et al*. [14] pointed out that Firmicutes, Proteobacteria, and Bacteroidetes have β-glucuronidase activity. Exogenous estrogen levels are closely related to β-glucuronidase [15]. In this study, we found that the abundance of Firmicutes was higher in the breast fibroadenoma group than in the healthy control group, and the difference was statistically significant ($P < 0.05$). Therefore, the significant differences in the abundance of Firmicutes suggest that the imbalance of the intestinal flora may influence the development of breast fibroadenoma by affecting estrogen metabolism, which is consistent with the previous hypothesis. Patients with breast fibroadenoma have a relatively high Prevotella content; Prevotella can induce intestinal mucosal inflammation [16]. Therefore, patients with breast fibroadenoma may show intestinal mucosal injury. In addition, the content of SCFA-producing bacteria, such as Streptococcus, Coprococcus, Ruminococcus, Lachnospira, and Clostridium, in breast fibroadenoma patients was relatively high. SCFAs, primarily acetate, propionate, and butyrate, are bacterial fermentation products derived from soluble dietary fiber in the colon. A growing body of evidence suggests that SCFAs play a key role in maintaining the intestinal barrier by stabilizing specific transcription factors, promoting the composition of tight junctions and the secretion of mucins [17]. SCFAs also regulate the differentiation of T cells into effector cells or regulatory T cells and are considered potential predictors of immunotherapeutic responses in some cancers [18]. Therefore, the results of this study suggest that the intestinal flora of patients with breast fibroadenoma may be associated with an immune response. In conclusion, patients with breast fibroadenoma show an imbalance of the intestinal flora.

This study had the following limitations: a small sample size, analysis by 16S rRNA level observational studies, lack of validation using large samples and animal experiments. Nevertheless, when combined with relevant clinical indicators, the findings of this study might provide important theoretical guidance for the prevention, diagnosis, and treatment of breast fibroadenoma.

Conflicts of interest

The authors indicated no potential conflicts of interest.

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DOI 10.1007/s10330-021-0509-9
Cite this article as: Wang BD, Liu X, Bian YH, *et al*. Correlation analysis of breast fibroadenoma and the intestinal flora based on 16S rRNA sequencing. Oncol Transl Med, 2021, 7: 269–274.