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Clinical metagenomic analysis of bacterial communities in breast abscesses of granulomatous mastitis

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SUMMARY

Background: Granulomatous mastitis (GM) is a chronic inflammatory breast lesion. Its etiology remains incompletely defined. Although mounting evidence suggests the involvement of Corynebacterium in GM, there has been no systematic study of GM bacteriology using -omics technology.
Methods: The bacterial diversity and relative abundances in breast abscesses from 19 women with GM were investigated using 16S rDNA metagenomic sequencing and Sanger sequencing. A quantitative PCR (qPCR) assay was also developed to identify *Corynebacterium kroppenstedtii*.
Results: A bioinformatic analysis revealed that Corynebacterium was present in the 19 GM patients, with abundances ranging from 1.1% to 58.9%. Of note, Corynebacterium was the most abundant taxon in seven patients (more than a third of the subjects). The predominance of *Corynebacterium kroppenstedtii* infection (11 of 19 patients, 57.9%) was confirmed with Sanger sequencing and the qPCR assay.
Conclusions: This study profiled the microbiota of patients with GM and indicated an important role for Corynebacterium, and in particular *C. kroppenstedtii*, in the pathogenesis of this disease.

1. Introduction

Granulomatous mastitis (GM) is a rare chronic inflammatory lesion of the mammary gland that was first truly established as an individual disease by Kessler in 1972.[1–3] It is also known as 'granulomatous lobular mastitis', and usually affects women of reproductive age. This entity is characterized by the presence of an ill-defined mass, galactorrhea, abscess, sinuses, and/or cutaneous ulceration.[4] In clinical practice, its differential diagnosis poses a problem because it resembles other forms of non-lactating mastitis, such as periductal mastitis and breast tuberculosis. At present, the definitive diagnosis relies on histopathology.

There have been several theories concerning the initial causes of GM, including inflammatory reactions, autoimmunity or hypersensitivity, local trauma, breast-feeding, oral contraceptives, and microbial infection.[5–8] However, it remains a subject for debate, because there is insufficient evidence for a definitive conclusion to be drawn. As its etiology is ambiguous, no effective therapeutic algorithm or clinical management strategy for this condition has yet been established. Corticosteroids, surgical excision, and antibiotics have been reported as the first line of treatment.[9–11] However, the various protocols or differing combinations of these strategies often have variable consequences, including recurrence and unnecessary radical mastectomies.[12,13] In recent decades, a growing literature has suggested that Corynebacterium infection is closely related to the pathogenesis of GM. Bacterial culture, PCR, and 16S rDNA sequencing have been used in most of these studies. However, there has been no systematic study of GM bacteriology, especially with an -omics approach. Therefore, the microflora involved in GM is still incompletely characterized.

In China, the incidence of chronic non-lactating mastitis, especially GM, has gradually increased, and it is even more

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common than lactating mastitis. The identification of specific pathogens should contribute to the diagnosis and treatment of these diseases.

In the present study, the microbiota in breast pus was examined using metagenomic sequencing and a bioinformatic analysis. Corynebacterium was found in all 19 patients investigated, with abundances ranging from 1.1% to 58.9%. In seven patients, the most abundant taxon was Corynebacterium. More importantly, PCR sequencing confirmed the predominance of *Corynebacterium kroppenstedtii* infection in these subjects (11 of 19 patients, 57.9%). A specific qPCR assay was developed to quantitatively assess *C. kroppenstedtii*. This could be used to guide targeted therapy in the clinical context. These findings shed more light on the role of Corynebacterium in the development of GM and may facilitate a precise treatment for this disease entity.

2. Methods

2.1. Patients and specimens

This study was approved by the Institutional Ethics Board of Guangdong Women and Children Hospital; written informed consent was obtained from all of the participants. In total, 19 women histologically diagnosed with GM were recruited into the study. The patients ranged in age from 25 to 36 years, with a mean age of 30.5 years. The clinical symptoms included a breast lump, abscess, sinus, and/or cutaneous ulceration. Three age-matched women with acute lactating mastitis were also included in the study as controls. None of the patients in this series had received antimicrobial treatment. Specimens from breast core biopsies or surgical excision were sent for histological examination. The breast pus was aspirated with a sterile syringe and divided into two samples, one for metagenomic analysis and the other for bacterial culture.

2.2. Pathological evaluation

Hematoxylin–eosin (HE) staining was performed and sections of each sample were carefully reviewed by two independent pathologists. GM is principally characterized by the presence of non-necrotizing granulomas, confined to the breast lobules. In detail, the histological features of GM include signs of chronic granulomatous inflammation with clusters of multinucleate giant cells, leukocytes, epithelioid cells, and macrophages, as well as abscess.

2.3. Metagenomic analysis

Metagenomic sequencing has been described elsewhere.[14–16] In brief, it includes DNA extraction, library preparation, sequencing, and data processing. DNA was isolated with a TIANamp Genomic DNA Kit (catalog number DP304-02; TIANGEN Biotech (Beijing) Co., Ltd., Beijing), according to the manufacturer's instructions. The fusion primer set, which incorporated adapter sequences, indexing barcodes, and PCR primers (515F: GTGCCAGCMGCCGCGGTAA; 806R: GGACTACHVGGGTWTCTAAT), was used to amplify the V4 region of the 16S rRNA gene.[17] The PCR products were then checked for size and specificity by agarose gel electrophoresis. The amplicons were pooled in equimolar amounts and sequenced with an Ion PGM Hi-Q 400 Sequencing Kit. The sequences obtained were filtered to remove low-quality and polyclonal sequences, and the adapter sequences were trimmed. The filtered data for each sample (>50 000 sequenced reads) were used to construct operational taxonomic units. The resulting clusters of sequences were used for accurate taxonomic assignment to the genus level and the number of sequences in each genus was calculated using the 16S rDNA analysis pipeline.

Table 1
Primer sets and probe used for sequencing and qPCR

Primer/probe	Sequences (5′–3′)	Size of amplicons (bp)
16S-F	AGCGAACAGGATTAGATACCCT	560
16S-R	CGATTACTAGCGACTCCGACT	
C. K-F	AGAACCTTACCTGGGCTTGA	133
C. K-R	CGCTCGTTGCGGGACTTA	
C. K-Probe	FAM-ACTGGATGCGGCCAGA-MGB	

qPCR, quantitative PCR.

2.4. Sanger sequencing and quantitative PCR

For Sanger sequencing, a PCR assay was used to amplify the 16S rRNA gene in the 19 GM samples. The mixture contained PCR mix buffer, forward primer, reverse primer, and the genomic DNA template in a volume of 40 μl. Hot start PCR was initiated by incubation for 3 min at 95 °C, and ended with a 5-min extension at 72 °C, with 40 cycles of denaturation at 95 °C for 15 s, annealing at 60 °C for 25 s, and extension at 72 °C for 50 s. The PCR products were sent for sequencing. The relevant sequences and the expected sizes of the amplicons are shown in Table 1.

A qPCR assay was developed to specifically detect the species *C. kroppenstedtii*, which consisted of genomic DNA, Takara PCR Master Mix buffer (TAKARA Biotechnology (Dalian) Co., Ltd., Dalian), forward primer, reverse primer, and probe, in a volume of 40 μl. The PCR cycling conditions were as follows: 1 cycle at 50 °C for 2 min, followed by one cycle at 95 °C for 10 min and 40 cycles at 92 °C for 15 s, 58 °C for 20 s, and 65 °C for 45 s. The quantification cycle (Cq) values were measured at a fixed fluorescence threshold. Samples with Cq > 40 were considered negative. All analyses were performed on an ABI 7500 Real-Time PCR System (Applied Biosystems).

2.5. Bacterial culture

Breast pus was aspirated with a sterile syringe and cultured on 5% sheep blood agar after the plate was streaked. The inoculated plates were incubated at 35 °C in 5% CO_2 for 18–24 h. Isolates retrievable from stock cultures were identified by their colony morphology, Gram staining, biochemical tests, and the RapID CB Plus System (Remel Inc., USA), as recommended by the manufacturer.[18]

3. Results

3.1. Bacterial profiles in breast pus

The taxonomic assignment of the sequences determined classified them into diverse genera. The top 10 genera were Bacteroides, Blautia, Corynebacterium, Dialister, Escherichia–Shigella, incertae sedis, Lactobacillus, Prevotella, Staphylococcus, and Streptococcus. For convenience, other cultured and uncultured bacteria were combined into 'others'. As controls, the DNA was extracted from three pus samples obtained from patients with acute lactating mastitis associated with known Staphylococcus and Streptococcus infections, confirmed by bacterial culture. These DNA samples were used in the metagenomic analysis, together with the DNA of the 19 specimens from patients with GM. As expected, the genera *Staphylococcus* (89.1%, 96.6%) and *Streptococcus* (97.2%) clearly predominated in the three control samples (SA1, SA2, and SR1) (Fig. 1). These results indicate that the system is suitable for the analysis of additional unknown samples. In general, the bacterial profiles of the GM subjects differed clearly from those of the controls. Samples GM8 and GM16 had relatively high abundances of