A pilot study: a possible implication of Candida as an etiologically endogenous pathogen for oral lichen planus

Hong He (✉ honghehh@zju.edu.cn)

Zhejiang University <https://orcid.org/0000-0003-1299-0868>

Mengyun Zhang

Zhejiang University School of Medicine Affiliated Stomatology Hospital

Xinyu Xia

Zhejiang University School of Medicine Affiliated Stomatology Hospital

Haiping Yang

Zhejiang University School of Medicine Affiliated Stomatology Hospital

Qiao Peng

Zhejiang University School of Medicine Affiliated Stomatology Hospital

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Abstract

Objectives—This study aimed to investigate the prevalence and genotypic profiles of *Candida albicans* from patients with oral lichen planus (OLP). **Materials and**

Methods—Genotypic profiles of *Candida albicans* strains from OLP patients and healthy controls were analyzed. Random amplified polymorphic DNA and internal transcribed spacer of ribosome DNA polymerase chain reaction were used to sequence the DNA of these strains, and then their genetic similarity was measured using BLAST, UIV Band, and Vector NTI Suite Sequence Analyses Software.

Results—The prevalence of *C. albicans* strains detected from erosive-OLP, non-erosive OLP, and normal individuals was 18.87%, 18.75%, and 7.92%, respectively. Four different genotypes were revealed by the two methods. To be specific, type I was found only in the healthy subjects; type II a and II b were found in non-erosive OLP, and type III was identified in erosive OLP. Intragroup similarity coefficients S_{AB} were 100%, and inter-groups similarity coefficients S_{AB} were less than 30%.

Conclusions—The genotypic evidence of *C. albicans* in OLP might inferred an endogenous infection and some etiologic sense contributing to professional recognition on the development and progression of OLP for more suitable diagnose and treatment.

Background

Oral lichen planus (OLP) is a common chronic inflammatory oral mucosal disease affecting 1–2% of the general population [1–3]. OLP is considered as a potentially malignant disorder with a unbenign transformation prevalence of 0–1% according to the World Health Organization [4, 5]. The etiology of OLP remains not largely known and far from being uncovered an common recognition, evidence demonstrated that microbial infection, psychological disorders, allergies, and immunodeficiency were closely associated with the pathogenesis of OLP [6–8].

Considering the oral microorganisms, *Candida* species were the commensal fungus in the mucosal flora of healthy individuals while were reported as an important existence in OLP [9–11]. Among the *Candida*, *Candida albicans* (*C. albicans*) was the predominant species in the OLP patients [12–14]. Arora et al. investigated the prevalence and phenotypic variation of *Candida* species in OLP cases, and found that *C. albicans* constituted the majority of the five identified *Candida* species [13]. Thus, *C. albicans* from OLP has gained increasing attention in recent years in clinical and laboratory researches.

Initially, the researches were focused on the phenotypic presence of *C. albicans* in OLP, but they failed to answer the following basic questions due to the low resolution achieved with phenotypic analysis: whether the pathogen was a fungus; whether *C. albicans* was an initial pathogen or a symbiont; whether it facilitated the infection in OLP, or vice versa; and whether *C. albicans* was a probable antigen for OLP. It is worth noting that a close correlation might exist between genotypic profiles and virulence attributes [15]. Moreover, genotyping can provide information about DNA and has stronger resolution than

phenotyping to distinguish variants among individuals or stages [15, 16]. Therefore, the relationship between genotypes of *C. albicans* and OLP dose merit to be further investigated.

In this study, genotyping analyses using BLAST, UIV Band, and Vector NTI Suite Sequence Analysis soft wares were conducted to investigate the relationship between OLP andgenotypes of *C. albicans*. Combination of random amplified polymorphic DNA (RAPD) and internal transcribed spacer (ITS) [17–19] of ribosome DNA polymerase chain reaction (PCR)was used to intergroup genotype *C. albicans* isolates from healthy individuals and OLP patients. The results showed that the phenotypes and intragroup genotype of *C. albicans* were homologous but the genotypes of *C. albicans* were heterogeneous among erosive(E)-OLP, non-erosive (NE)-OLP and healthy individuals. Both methods revealed three discrepant subtypes of *C. albicans* in clinical different stages of OLP, and one different subtype in healthy controls. Thus, it can be speculated that endogenous infection rather than exogenous infection of *C. albicans* might be the vital factor for OLP etiology.

Methods

This study was approved by the local research ethics committee. All patients provided written informed consent. The protocols were reviewed and approved by the local institutional review boards (IRB). The IRB numbers for our medical ethic files were No. 2015 (17) of the stomatology hospital and 2010 (137) of the 2nd hospital affiliated to Medical School Zhejiang University. Training session was brought forward to all participants to minimize the undesirable discrepancy.

For our power calculation, we assumed the standard deviation in groups of patients and control. With $\alpha = 0.05$, a power of 99 %, a total of 250 subjects (aged between 16 and 86 years), including 149 patients with OLP and 101 age-sex-matched healthy volunteers ($P > 0.05$), were recruited at the Affiliated Hospitals of Zhejiang University from February 2010 to March 2016 (Table 1 and Fig. 1). The patients were diagnosed with OLP based on clinical and histopathological criteria, as well as the criteria of Chinese Stomatological Association of Oral Medicine. The 101 healthy individuals had no diseases of oral mucosa. The exclusion criteria were as follows: patients were those with full or partial dentures, those taking broad-spectrum antibiotics, antifungals, glucocorticoids, or immunosuppressive agents for a long period of time or recently (within 3 months), and those with systemic diseases, including diabetes, thyroid hypofunction, and immune deficiency.

Table 1. Clinical features and positive culture rate of *Candida albicans* strains of the subjects

Groups	Gender		Age (Mean)	Positive cases	Positive rate
	M	F			
NE-OLP□n=96□	47	49	48.22 ± 16.43	18	18.75 %*
E-OLP□n=53□	27	26	51.37 ± 15.78	10	18.87%*
Control□n=101□	51	50	49.69 ± 17.19	8	7.92%

Isolates

All the samples were swabbed from subjects' oral mucosal membrane. The clinical isolates were randomly labeled, and all information about the isolates was blinded to the researcher who conducted phenotyping and genotyping analyses. All isolates were first analyzed using conventional microbiological identification methods [20, 21], and those with the morphological characteristics of *C. albicans* were analyzed using germ tube test and the API 20C AUX identification kit (Analytical Profile Index; BioMérieux S. A., France).

DNA Extraction

DNA was extracted using the Biospin Fungus Genomic DNA Extraction Kit (Bioflux, China) following the manufacturer's protocol. DNA concentration was examined using the Ultraviolet Spectrophotometer (Pharmacia Biotech, USA, 07450).

RAPD fingerprinting and S_{AB} Analysis

RAPD was conducted as described [22]. Two primers (C1: 5'-ACGGTACACT-3'; C2: 5'-GTTCCGCCC-3') were used in this study. After amplification, the products were electrophoresed in 2% of agarose gel. After staining with ethidium bromide (Kaiser Germany), the PCR products were qualified using the UV Band system. Information about the location and relative molecular mass of the main amplified bands was extracted for calculating the genetic similarity coefficient S_{AB} between any two bands. Strains were categorized into several families that share gene homology at a criterion of $S_{AB} = 0.8$.

ITS Sequence Determination

ITS analysis was conducted as described in a previous study (White et al.). PCR amplification was performed with primers (5'-GGAAGTAAAAGTCGTAACAAGG-3'; 5'-GCTGCGTTCTTCATCGATGC-3') that were designed based on the conserved regions of ITS 1 and ITS 2 rRNA genes. The DNA fragment amplified included the intervening 5.8S gene and the ITS 1 and ITS 2 noncoding regions. Primers were synthesized by the Eppley Molecular Biology Core Laboratory (NE, USA). The amplified PCR products were purified and sequenced by TaKaRa Bio-Engineering Co., Ltd. (Dalian, China). BLAST was used to compare the DNA sequence with standard ITS sequences of *C. albicans* in the GenBank at National Center for Biotechnology Information to determine the taxonomy of the isolates. Intra-species sequence similarity and variation for isolates were determined using the Vector NTI Suite software (www.liax.cn) and visually confirmed using pairwise nucleotide alignments. Referenced isolates were also aligned. The similarities of the sequences were determined with the expectation frequency minimized to 0.0001.

Statistical Analysis

A P value less than 0.05 was deemed as the standard significance level. All calculations were performed by χ^2 test using SPSS statistical software (SPSS 25.0; SPSS Inc., Chicago, IL, USA).

Results

Positive Rate of *C. albicans* culture—Prevalence of *C. albicans* in OLP

The results showed that *C. albicans* were identified from 16 of 96 NE-OLP (16.67%), 10 of 53 E-OLP (18.87%), and 8 of 101 healthy controls (7.92%) (Table 1). The prevalence of *C. albicans* in NE-OLP and E-OLP was significantly higher than that in the healthy symbiotic group (χ^2 test, $P < 0.05$), but no significant difference was found between NE-OLP and E-OLP (χ^2 test, $P > 0.05$) (Table 1). Using germ tube test and the API 20C AUX identification kit, eight isolates of *C. albicans* from NE-OLP, two from E-OLP, and two from the healthy controls were identified.

RAPD and UIV Band Analysis

Electrophoresis analysis of RAPD products showed that the healthy controls had only one band, both E-OLP isolates had multiple bands, and all the eight NE-OLP isolates had two bands (Fig. 2). S_{AB} analysis further showed that the NE-OLP formed a major clade, and the E-OLP formed another separate clade. The health control was basal to the NE-OLP and E-OLP clades.

Then, these RAPD genomes were analyzed by UIV Band. The results showed that genetic homology coefficient S_{AB} was less than 30% between the control, NE-OLP, and E-OLP groups, while it was 100% in a single subgroup. Additionally, the NE-group could be categorized into two subgroups, namely two genotypes, one including strains numbered 3–7 and 11 and the other including strains numbered 9, 10 and 12 (Fig. 3).

ITS Sequences

The DNA fragment with expected size (250bp) was successfully amplified from all isolates (Fig. 4). BLAST analyses of the sequences referring to NCBI databases clearly showed that all isolates belonged to the *C. albicans* species. Then, these ITS sequences were genotyped using Vector NTI Suite software, and the sequence analysis showed that the base pair genotype results of ITS regions of the strains were consistent with the results of the RAPD analysis (Fig. 5). Specifically, the type I was found from normal strains, type IIa and IIb were detected from NE-OLP cases, and type III was identified from E-OLP cases.

Discussion

The relationship between *C. albicans* and the etiology of OLP has long been of interest for many researchers, but it is still indistinct and remains to be elucidated. This study found that the prevalence of *C. albicans* in OLP was significantly higher than healthy individuals. Moreover, *C. albicans* isolates from both NE-OLP and E-OLP were genetically distinguishable from those in control group. These suggested that OLP might be a predisposing condition to candidal infection and that certain genotypes of *C. albicans* isolates were involved in the progression of OLP.

The prevalence of *C. albicans* in OLP patients generally differed from that in healthy individuals. It was reported that a positive *Candida* culture was more prevalent among OLP patients (48.9%) than among control subjects (26.7%) [14]. In the present study, we also identified a definitely higher positive cultivation frequency of *C. albicans* in the NE-OLP group and E-OLP group than in the healthy individuals. This discrepancy might cause the dysfunction of lymphocytes in OLP. As Simark-Mattsson et al. revealed, the proliferation and cytokines production of peripheral blood mononuclear cells from OLP were significantly reduced following the stimulation of *C. albicans*, reflecting a potential immune regulatory mechanism of OLP modulating by *C. albicans* [10]. The high prevalence of specific *C. albicans* isolates in OLP may indicate that they could have strong adaptability to the microenvironment provided by the dynamic interaction between OLP progression and the fungal strains [23]. However, the result differed from that observed by Artico et al., who found that the positive prevalence of colonization by *Candida* spp. was higher in the healthy subjects than in OLP [12]. The possible explanation of the opposite results may lie on the difference of the sample size, the experimental methods, and the site of sample collection.

Here, the RAPD and ITS sequence were both chosen, because previous other studies failed to further classify *C. albicans* among the groups and offered no better precision than just telling that they were all *C. albicans*. Unexpectedly, the results of both classifying identification showed a great coherence of consistency. And as we know, phenotyping was vulnerable to be influenced by environment leading to poor repeatability, weak identification, and instability [24]. In contrast, genotyping improved the precision of the classification of *C. albicans* from species to subtypes [25]. The fact that *C. albicans* does not differ in morphologic phenotype between symbiotic and pathogenic situations implies the importance of genotyping.

Although other technologies as multilocus sequence typing and microsatellite typing were thought as more efficient and reliable, possible scholar even viewed RAPD and ITS as obsolete, we got worthwhile facts. As result, RAPD products showed by the electrophoresis analysis that the healthy controls had only one band, both E-OLP isolates had multiple bands, and all the eight NE-OLP isolates had two bands, which was further confirmed by ITS sequence. Moreover, the genetic homology coefficient S_{AB} was less than 30% between the control, NE-OLP, and E-OLP groups, while it was 100% in subgroup. Although scholars who obtained genotyping results usually deduced that *C. albicans* infection in OLP was exogenous [26], authors here think that if *C. albicans* in OLP were exogenous, they should be disordered or similar in erosive or non-erosive OLP instead of the ordered genotypes. The exogenous colonization of

C. albicans should manifest some indefiniteness and randomness, but no study so far has found a common natural *C. albicans* symbionts in OLP. Thus, we did not support this conception. On the contrary, we proposed that it was the endogenous genotypic changes of *C. albicans* that led to the development in OLP under special oral environmental and ecological conditions. This might be because the specific microenvironment fostered a mutation of *C. albicans* from symbionts to further specific pathogenic genotypes to induce T-cell-mediated immune response. Microenvironmental elements in oral cavity include PH and temperature and salivary or gum fluids ingredients et al, were identified to induce key pathogen's virulence gene mutation and immune reaction, or new possible balance [27]. Meanwhile, it was the mutation that drives the *C. albicans* to evolve and better adapt to the current environment [28], and then formed the type of dominant bacteria, which, eventually, affected the severity of the disease upon its interaction with the host. In this study, the results of both RAPD and ITS sequence showed that both intraspecies homology and inter-species variations existed in *C. albicans* among the three clinical groups. The correlation between ITS sequence mutation of *C. albicans* and clinical progression of OLP has been reflected in the changes from type I for normal strains, to type IIa, type IIb for NE-OLP cases, and to type III for E-OLP strains. This is expected to be used not only as an indicator for evaluating the severity of OLP, but also to provide a therapeutic basis for individualized treatment.

However, other study reported that it was OLP that drew *C. albicans*' collection in lesions [16]. We hold the reverse viewpoint that *Candida* is probably the initial pathogen and probably is the antigen for OLP (Fig. 6), with the facts that it is easy to cure OLP by addressing oral hygiene and dental health problems which are so frequently involving fungi. If not so, solving the oral hygiene, dental and oral health, consuming antifungal drugs would never work. From the clinical aspect, the curative effect might be more obvious to cure E-OLP by controlling erosion to non-erosion with corticosteroids, and then let the situation left to address dental and hygiene problem. The reason for many other scholars to experience many prolonged healing and recurrences of OLP is that they ignore the patients' poor oral situation facts or habits. In fact, all through the author's practice experience, addressing oral hygiene and dental problems (which according to our common knowledge could mean numerous fungi and *Candida*) is a most effective and even key way to cure OLP within the motherland as a developing country—largely better and more correct also more accurate than other therapies including possibly blind and sole adoption of immune drugs or herb products and et al.

Accordingly, a strong positive relationship has been identified between the infection of yeast in oral cavity and the degree of epithelial dysplasia or OSCC [3, 29]. Gainza-Cirauqui et al. suggested that *C. albicans* isolated from potentially carcinogenic oral diseases could produce mutagenic amounts of acetaldehyde, which was involved in abnormal epithelial proliferation of mucosa [30]. This further induced events including disorder and strange cellular keratin to deterioration of abnormal proliferation, and abnormal nuclear divisions in epithelial plaque, developing to cancerous [31]. Therefore, clinicians should specifically note any possible source for the presence of *Candida* and any unbenign dental-to-mucosa friction injury in patients with OLP epithelial dysplasia or carcinoma.

Our results with the clinical *C. albicans* strains confirmed that the ITS sequences of *C. albicans* were obviously differential among E-OLP, NE-OLP and healthy individuals, which suggested that endogenous *C. albicans* in oral environment may play roles in the etiology and pathogenesis of OLP. Although the sample of clinical *C. albicans* strains selected in this study was small, and the mutual effect between OLP lesion and *C. albicans* gene mutation was dynamic and complex, our report's reason and facts are strong. Larger sample sizes or an OLP model would also be optimistic to verify our deductions and sense in the future.

Conclusions

An optimistic verification of present results would be important to confirm the possible etiology and some more correct, precise and effective therapeutic strategies for OLP. The etiology of OLP might lie in the endogenous infection of *C. albicans* and its gene mutation in specific and dynamic microenvironment of the patient's oral cavity, which is crucial in the fields of immunology and the reasonable intervention for premalignant progression in oral health.

Abbreviations

API: Analytical Profile Index

BLAST: Basic Local Alignment Search Tool

C. albicans: *Candida albicans*

DNA: DeoxyriboNucleic Acid

E:- erosive-

IRB: institutional review boards

ITS: internal transcribed spacer

NE:- non-erosive-

OLP: oral lichen planus

OSCC: oral squamous cell carcinoma

PCR: polymerase chain reaction

RAPD: random amplified polymorphic DNA

Declarations

Ethics approval and consent to participate

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent was obtained from all individual participants included in the study.

Consent to publish

Consent for publication has been obtained from the patient of figure1.

Availability of data and materials

All relevant data are within the present paper.

Competing interests

The authors declare that they have no conflict of interest in this work.

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Authors' Contributions

HH, YHP, ZMY and XXY drafted the manuscript. HH made critical revisions to include important intellectual content in the manuscript. YHP and PQ conducted the experiments, formatting and preparing the manuscript. All authors read and approved the final version.

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Figures

Figure 1

OLP Lesions in left buccal and tongue

Figure 2

Electrophoresis pattern of the RAPD results of *C. albicans* from the normal group (2a: 1-7 electrophoresis lanes), E-OLP group (2b: 1-2 electrophoresis lanes), and NE-OLP group (2c: 3-11 electrophoresis lanes). M, Marker; C, control. (Please be advised, the numbers of strains were not the numbers of cases).

Fig. 3

Figure 3

Genotyping tree of gene homology coefficient SAB of *C. albicans* by UIV Band analysis. horizontal axis = SAB; Vertical axis = genotypes of RAPD products of isolated strains. 1-2 = E-OLP strains no. 2, 8, SAB = 100%; 14, 16-19 = NE-OLP strains no. 3-7 and 11, SAB = 100%; 15, 20, 21 = NE-OLP strains no. 9, 10, and 12, SAB = 100%; 6 = No. 1 strain from the control group. (Please be advised, the numbers of strains were not the numbers of cases).

Figure 4

Electrophoresis patterns of the ITS PCR results (all 250 bp). 4a: N1 strain was from the control group, and E2 and E8 strains were from the E-OLP group. 4b: 3-7 and 9-12 strains were from the NE-OLP group. (Please be advised, the numbers of strains were not the numbers of cases).

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N-E3_(117) CCCAGCC-----TGCCGCCAGAG-GTCTAAACTTACAAACAAATTTTAT
N-E4_(120) CCCAGCC-----TGCCGCCAGAG-GTCTAAACTTACAAACAAATTTTAT
N-E5_(118) CCCAGCC-----TGCCGCCAGAG-GTCTAAACTTACAAACAAATTTTAT
N-E11_(118) CCCAGCC-----TGCCGCCAGAG-GTCTAAACTTACAAACAAATTTTAT
N-E6_(130) CCCACCG----CCAAAGCAAGTTTGTTCAAAGAAAAACACATGTGGTG-
N-E9_(131) CCCACCG----CCAAAGCAAGTTTGTTCAAAGAAAAACACATGTGGTG-
N-E10_(130) CCCACCG----CCAAAGCAAGTTTGTTCAAAGAAAAACACATGTGGTG-
N-E12_(130) CCCACCG----CCAAAGCAAGTTTGTTCAAAGAAAAACACATGTGGTG-
Consensus_(151) CCCAGCG      T   GCCAGAG GTTTAAAC AAAACAAATTT TTAT
                201                250

N1_(126)    CAACTTGTCAACCCAGATTATTACT-AATAGTCAAAACTTTCAACAACGG
E-2_(192)    TTTTACAGTTAGTCAAATTTTGAATTAATCTTCAAAACTTTCAACAACGG
E8_(192)    TTTTACAGTTAGTCAAATTTTGAATTAATCTTCAAAACTTTCAACAACGG
N-E3_(161)    CAACTTGTCAACCCAGATTATTACT-AATAGTCAAAACTTTCAACAACGG
N-E4_(164)    CAACTTGTCAACCCAGATTATTACT-AATAGTCAAAACTTTCAACAACGG
N-E5_(162)    CAACTTGTCAACCCAGATTATTACT-AATAGTCAAAACTTTCAACAACGG
N-E11_(162)    CAACTTGTCAACCCAGATTATTACT-AATAGTCAAAACTTTCAACAACGG
N-E6_(175)    ----CAATTAAGCAAATCAGTAATGATCCTTCGCGAGGTTCACTACGG
N-E9_(176)    ----CAATTAAGCAAATCAGTAATGATCCTTCGCGAGGTTCACTACGG
N-E10_(175)    ----CAATTAAGCAAATCAGTAATGATCCTTCGCGAGGTTCACTACGG
N-E12_(175)    ----CAATTAAGCAAATCAGTAATGATCCTTCGCGAGGTTCACTACGG
Consensus_(201) CA TTAA CAAATTATTAAT AATCTTCAAAACTTTCAACAACGG
                251                286

N1_(175)    ATCTCTTGGTTCTCGCATCGATGAAGAACGCAGC--
E-2_(242)    ATCTCTTGGTTCTCGCATCGATGAAGAACGCAGCAA
E8_(242)    ATCTCTTGGTTCTCGCATCGATGAAGAACGCAGCAA
N-E3_(210)    ATCTCTTGGTTCTCGCATCGATGAAGAACGCAGCAA
N-E4_(213)    ATCTCTTGGTTCTCGCATCGATGAAGAACGCAGCCA
N-E5_(211)    ATCTCTTGGTTCTCGCATCGATGAAGAACGCAGCCA
N-E11_(212)    ATCTCTTGGTTCTCGCATCGATGAAGAACGCAGCCA
N-E6_(220)    AAACCTTGTTCAGACTTTTACTTCCAA-----
N-E9_(221)    AAACCTTGTTCAGACTTTTACTTCCAA-----
N-E10_(220)    AAACCTTGTTCAGACTTTTACTTCCCA-----
N-E12_(220)    AAACCTTGTTCAGACTTTTACTTCCCA-----
Consensus_(251) ATCTCTTGGTTCTCGCATCGATGAAGAACGCAGC A

```

Figure 5

Sequences analysis results using Vector NTI Suite Type I: 5'-TCGACTGC...AAGAACGCAGC-3' was named for no. 1 strain from the control group of 101 subjects; Type Ia: 5'-GGAAGTAA...GAACGCAGCCA-3' was named for strains no. 3-5, 7 and 11 from the NE-OLP group of 96 cases; Type Ib: 5'-TGGCTGCG...TTTACTTCCCA-3' was named for strains no. 6, 9, 10 and 12 from the NE-OLP group of 96 cases; Type III: 5'-TGGAAGTA...GAACGCAGCAA-3' was named for strains no. 2 and 8 from the E-OLP group of 53 cases. (Please be advised, the numbers of strains were not the numbers of cases).

Figure 6

The authors view Candida pathogen/antigen as a vital part of etiology for OLP this figure's copyright belongs to the American Journal of Translational Research the authors transferred to.