Original Article

Detection of Candida Species in Non-Habit-Associated Oral Squamous Cell Carcinoma Using Periodic Acid–Schiff and Calcofluor White Staining: A Case-Control Study

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ABSTRACT

Background: Oral squamous cell carcinoma (OSCC) is the most common oral malignancy and may occur in individuals without established risk habits. Candida species have been implicated as possible contributors to oral carcinogenesis; however, conflicting evidence and variability in diagnostic methods have resulted in uncertainty regarding their true role.

Purpose: The present study aimed to detect the presence of candida species in non-habit-associated OSCC and to comparatively evaluate the diagnostic efficacy of periodic acid–Schiff (PAS) and calcofluor white staining methods.

Materials and Method: In this prospective case–control study, included 50 cases of non-habit-associated, histopathologically confirmed OSCC and 50 control samples. Tissue sections were subjected to PAS staining and calcofluor white fluorescent staining for the detection of candida species. Fluorescent evaluation was performed using a Radical RXLr5 fluorescence microscope. Data were analyzed using descriptive statistics and Fisher's exact test, with a significance level set at p Value < 0.05.

Results: PAS staining did not demonstrate the presence of candida in any OSCC or control samples. Calcofluor white staining detected candida in 2 (4%) OSCC cases, while all control samples were negative. The difference in candida detection between OSCC and control groups did not reach statistical significance (p> 0.05).

Conclusion: The findings indicate a low prevalence of intratissue candida in non-habit-associated OSCC. Calcofluor white staining demonstrated greater sensitivity than PAS in detecting sparse fungal elements, suggesting its potential role as an adjunctive diagnostic tool. However, the limited detection observed supports the view that candida is more likely a secondary colonizer or co-factor rather than a primary etiological agent in OSCC.

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Introduction

Oral squamous cell carcinoma (OSCC) constitutes over 90% of all oral malignancies and remains a major contributor to cancer-related morbidity and mortality. According to the International Agency for Research on Cancer (2020), cancers of the oral epithelium rank 16th in both incidence and mortality worldwide [1-2]. OSCC demonstrates marked geographical variation, with particularly high prevalence in South-East Asian countries, where it is among the most commonly diagnosed cancers. Traditionally, OSCC has shown a male predominance and is regarded as a multifactorial disease, with established risk factors including tobacco use, betel quid chewing, areca nut consumption, alcohol intake and additional lifestyle factors [3]. Nevertheless, despite the strong association with these habits, there is a growing

incidence of non-habit associated OSCC. Epidemiological studies indicate that approximately 4-6% of OSCC cases occur in individuals without any history of tobacco or alcohol use [4]. Of particular concern is the high proportion of younger patients present without identifiable oral habits, highlighting the potential involvement of alternative etiological factors in OSCC development [5].

Candida species are commensal organisms of the oral cavity with the potential to cause opportunistic infection under favorable conditions [6]. Several authors have proposed that candida may act as a precursor to dysplastic changes, with the severity of epithelial dysplasia showing a positive correlation with the degree of candidal invasion [7-8]. This hypothesis is supported by evidence suggesting increased mitotic activity and the production of carcinogenic nitrosamines by certain candida strains, which may directly contribute to oral carcinogenesis [9]. A systematic review reported candidal association in 6.8% to 100% of oral leukoplakias, with malignant transformation observed, thereby renewing interest in candida as a potential causative factor in OSCC [10]. Conversely, conflicting evidence exists, as demonstrated by a three-year prospective study in which only one out of 28 patients with oral leukoplakia progressed to OSCC despite the absence of prior candidal infection in the majority of cases [11]. Furthermore, findings in which *candida albicans* is not detected within lesional tissue pose significant challenges in establishing its precise role in the initiation of OSCC. This ongoing controversy is compounded by variability in diagnostic methodologies used to detect candida. Conventional approaches such as direct microscopy, culture techniques, and histochemical stains including periodic acid-Schiff (PAS) and Gomori methenamine silver (GMS) are widely employed but may differ in sensitivity and diagnostic yield. More recently, fluorescent staining techniques such as calcofluor white (CFW) staining have been used for the detection of fungal elements in tissue sections and smears [7]. However, despite these advances, there is no clear consensus regarding the most reliable method for detecting candida in OSCC, contributing to persistent uncertainty surrounding its true pathogenic significance.

In view of the conflicting evidence regarding the role of candida in OSCC and the lack of consensus on optimal detection methods, the present study aimed to detect the presence of candida species in non-habit-associated OSCC and to comparatively evaluate the diagnostic efficacy of PAS and CFW staining methods.

Materials and Method

Study design and setting

This prospective case–control study was conducted in the Department of Oral Pathology and Maxillofacial Pathology at Dr. R. Ahmed Dental College & Hospital.

Selection and description of participants

Study groups

A total of 100 participants were included in the study, comprising 50 clinically and histopathologically confirmed cases of OSCC and 50 control samples. The control group consisted of individuals with clinically normal oral mucosa who reported to the department for dental procedures unrelated to inflammatory or neoplastic conditions.

Inclusion criteria

The inclusion criteria for the study group were newly diagnosed, untreated cases of OSCC with no history of tobacco use, betel quid chewing, areca nut consumption, alcohol intake, or other oral habits. Only cases with adequate tissue samples suitable for histopathological and staining procedures were included. Control subjects included individuals without oral habits, systemic illness, or clinical evidence of oral mucosal disease.

Exclusion criteria

Participants with a history of antifungal therapy, prior radiotherapy or chemotherapy, recurrent OSCC, immunocompromised status, systemic diseases predisposing to fungal infection, or inadequate tissue samples were excluded from the study.

Histopathological confirmation

Incisional biopsy specimens from all suspected OSCC cases were obtained and processed routinely. Diagnosis was confirmed on hematoxylin and eosin (H&E) stained sections by two experienced oral pathologists prior to inclusion in the study.

Tissue processing

Biopsy specimens were fixed in 10% neutral buffered formalin, routinely processed, and embedded in paraffin. Sections of 4-5µm thickness were obtained using a rotary microtome and mounted on clean glass slides for staining procedures.

Staining procedures

PAS staining

Sections designated for PAS staining were deparaffinized in xylene and rehydrated through graded alcohols to distilled water. The slides were treated with periodic acid followed by Schiff reagent according to standard protocol. After washing, the sections were counterstained, dehydrated, cleared, and mounted. PAS positivity was defined by the presence of magenta-colored fungal hyphae or pseudohyphae within the epithelial or connective tissue components.

Calcofluor white staining

For CFW staining, deparaffinized and rehydrated sections were treated with CFW stain solution (Calcofluor White Stain, product no. 18909; Sigma-Aldrich, Merck, Germany). The stained sections were examined using a fluorescence microscope (Radical RXLr5, Radical Scientific Equipments Pvt. Ltd., India) under appropriate excitation and emission wavelengths. Fungal elements appeared as bright fluorescent structures against a dark background and were recorded as positive when hyphal or pseudohyphal forms were observed.

Evaluation criteria

All stained sections were independently evaluated for the presence or absence of candidal elements by two observers who were blinded to the study groups. In cases of disagreement, a consensus was reached following joint evaluation.

Statistical analysis

Data were entered and analyzed using Statistical Package for the Social Sciences (SPSS) software version 27.0 (IBM Corp., Armonk, NY, USA). Frequencies and percentages were calculated. Fisher's exact test was applied to compare candida detection between OSCC and control groups and between PAS and CFW staining methods. A p Value of <0.05 was considered statistically significant.

Ethical approval

The Institutional Ethics Committee of Dr. R. Ahmed Dental College & Hospital approved the study. Written informed consent was obtained from all participants prior to biopsy and sample collection.

Results

A total of 100 participants were included in the study, comprising 50 cases of non-habit-associated OSCC and 50 control samples (Table 1).

PAS staining results

PAS staining did not demonstrate the presence of candidal hyphae or pseudohyphae in any of the OSCC or control tissue sections (Figure 1). All 50 OSCC cases (100%) and 50 control samples (100%) were PAS-negative for candida species (Table 2).

Table 1: Demographic Characteristics of the study population

Variable	OSCC (n=50)	Control (n=50)
Age (years), mean±SD	55.1±10.25	44.02±9.8
Gender		
Male, n (%)	12 (24)	16 (32)
Female, n (%)	38 (76)	34 (68)

OSCC: Oral squamous cell carcinoma

Calcofluor white staining results

Calcofluor white fluorescent staining revealed the presence of candidal hyphae in 2 (4%) of the OSCC cases (Figure 2). The remaining 48 (96%) OSCC samples did not show fungal elements (Figure 3). None of the control tissue samples demonstrated candida positivity on CFW staining (Table 3).

Comparative analysis

Candida detection using CFW staining was observed exclusively in OSCC tissues, whereas all control samples were negative. Fisher's exact test did not demonstrate a statistically significant difference in candida detection between OSCC and control groups (p Value > 0.05).

A comparison between PAS and CFW staining in OSCC cases showed that PAS failed to detect candida elements in all samples, while CFW staining identified candida in 2 cases. This difference did not reach statistical significance (p> 0.05) (Table 4).

The prevalence of candida positivity in OSCC cases

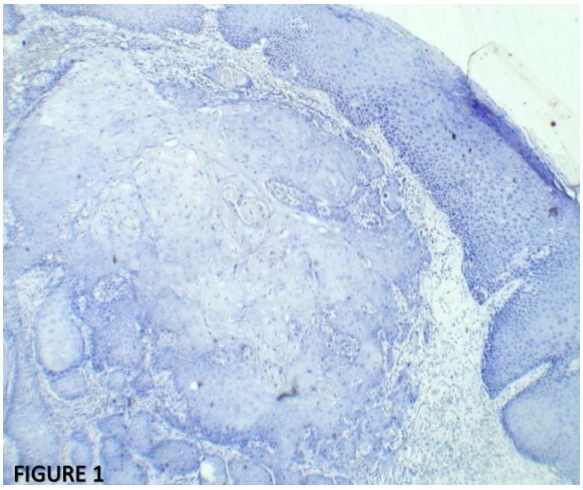

Figure 1: Photomicrograph of an oral squamous cell carcinoma tissue section stained with periodic acid–Schiff (PAS) showing absence of candidal hyphae or pseudohyphae within the epithelium. Magnification: 10×

Table 2: Detection of Candida species using PAS staining

Group	PAS Positive n (%)	PAS Negative n (%)
OSCC (n= 50)	0 (0)	50 (100)
Control(n=50)	0 (0)	50 (100)

PAS: Periodic acid–Schiff; OSCC: Oral squamous cell carcinoma

detected by CFW staining was 4% (95% confidence interval: 0.5–13.7).

Discussion

The present study evaluated the presence of candida species in non-habit-associated OSCC using PAS and CFW staining techniques. The findings demonstrated a low prevalence of candidal detection in OSCC tissues, with candida identified in 4% of cases using CFW staining, while all samples were negative on PAS staining. None of the control tissues showed evidence of candidal colonization with either staining method. Several studies have explored the association between candida species and oral epithelial dysplasia, potentially malignant disorders, and OSCC, reporting widely variable findings [5, 7, 9]. McCullough *et al.* [12] demonstrated that oral yeast carriage was significantly higher in patients with oral epithelial dysplasia and OSCC compared to individuals without dysplastic or neoplastic lesions, suggesting a possible interaction between oral yeast carriage and epithelial dysplasia, although the precise role of candida in disease progression remained unclear. Their findings emphasized colonization rather than tissue invasion, which may partly explain differences observed across studies.

Studies employing microbiological culture techniques have frequently reported higher rates of candidal carriage in malignant and pre-malignant conditions. Gonz-

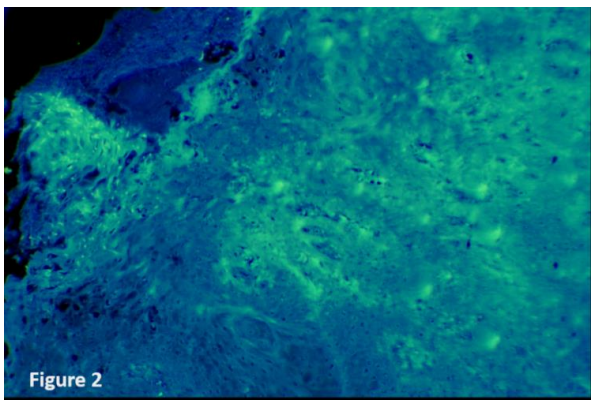

Figure 2: Fluorescence photomicrograph of an oral squamous cell carcinoma tissue section stained with calcofluor white demonstrating bright fluorescent fungal hyphae. Magnification: 10×

Table 3: Detection of Candida species using calcofluor white staining

Group	CFW Positive n (%)	CFW Negative n (%)
OSCC (n = 50)	2 (4)	48 (96)
Control (n = 50)	0 (0)	50 (100)

CFW: Calcofluor white; OSCC: Oral squamous cell carcinoma

ález-Gravina *et al.* [13], in a pediatric oncological population, observed a high prevalence of oral candidiasis, with *Candida albicans* being the predominant species, followed by non-albicans species. However, their study primarily reflected opportunistic infection in immunocompromised patients rather than a direct etiological association with oral carcinogenesis. Similarly, Anila *et al.* [14] and Saigal *et al.* [15] reported increased candidal carriage in malignant and potentially malignant oral lesions compared to healthy controls, though they concluded that the observed levels were within physiological limits and may not represent a direct causative factor in malignant transformation.

Cytological and histopathological studies have produced mixed results. Dany *et al.* [16] reported increased candidal presence in lesions with moderate dysplasia compared to mild or non-dysplastic lesions using PAS staining, suggesting a possible association between fungal colonization and increasing dysplastic severity. In contrast, the present study failed to demonstrate candidal presence using PAS staining in both OSCC and control tissues, highlighting the limited sensitivity of conventional histochemical stains when fungal burden is minimal or localized.

More recent investigations have continued to report elevated candidal carriage in OSCC patients using salivary and culture-based techniques. Sanjaya *et al.* [17], Csaba Berkovits *et al.* [18], and Yadav *et al.* [19] doc

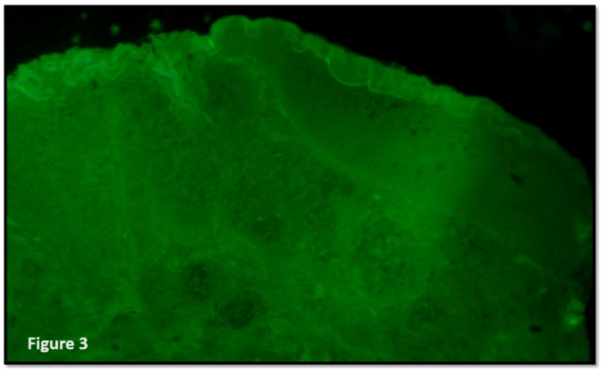

Figure 3: Fluorescence photomicrograph of an oral squamous cell carcinoma tissue section stained with calcofluor white showing absence of fungal elements. Magnification: 10×

Table 4: Comparison of PAS and calcofluor white staining in OSCC cases

Staining method	Positive n (%)	Negative n (%)
PAS	0 (0)	50 (100)
Calcofluor white	2 (4)	48 (96)

PAS: Periodic acid–Schiff; CFW: Calcofluor white

umented significantly higher isolation rates of candida, particularly *candida albicans*, in OSCC patients compared to controls. These studies primarily assessed surface colonization- through saliva or oral rinse samples, which may not accurately reflect true tissue invasion. This distinction between surface colonization and intratissue presence is critical when interpreting the pathogenic relevance of candida in OSCC.

Methodological variation appears to be a key factor contributing to conflicting results in the literature. Gallè *et al.* [20] emphasized that both microbiological and histological techniques have advantages and limitations and suggested that they should be considered complementary. In this context, the present study focused exclusively on tissue-based detection to evaluate true fungal invasion rather than surface carriage.

Overall, while numerous studies have reported increased candidal carriage in OSCC and related lesions, particularly through culture-based and salivary analyses, evidence of consistent intratissue candidal invasion remains limited. The low prevalence observed in the present study, especially in non-habit-associated OSCC, supports the view that candida may act as a secondary colonizer or co-factor rather than a primary etiological agent in oral carcinogenesis.

Histopathological examination remains a reliable approach for the detection of fungal organisms within tissue sections, as the demonstration of fungal elements provides direct evidence of tissue invasion rather than mere surface colonization. PAS staining is one of the most commonly employed histochemical techniques in routine pathology for the identification of fungi due to its ability to highlight chitin and other polysaccharide components of the fungal cell wall. Several studies have reported high sensitivity of PAS staining in detecting candida species in cytological and histological specimens, supporting its widespread use as a screening method [21].

Despite these advantages, PAS staining has inherent limitations, particularly in cases where fungal burden is minimal or sparsely distributed within tissue sections. In the present study, PAS staining failed to detect candidal elements in all OSCC and control samples. This finding suggests that PAS may lack sufficient sensitivity to identify low-level or focal fungal presence in malignant tissues, especially when candida is not extensively invasive.

Calcofluor white staining, a fluorescent brightener that binds to β-linked polysaccharides such as chitin and cellulose in fungal cell walls, has been increasingly utilized for rapid and sensitive detection of fungal organisms. Upon excitation with ultraviolet or violet light, CFW produces intense fluorescence, enhancing contrast and facilitating visualization of fungal structures even when present in low quantities. Earlier work by Lynch and Gibson [22-23] demonstrated strong concordance between CFW and Grocott's methenamine silver staining, highlighting the advantages of CFW in terms of speed, sensitivity, simplicity, and cost-effectiveness.

Multiple studies have reported higher detection rates of candida in OSCC using CFW staining compared to conventional histochemical methods. Rashmi Santosh Kumar [8], Jahanshahi and Shirani [24], and Sanketh *et al.* [25] observed increased candidal detection in OSCC tissues using CFW, though statistical significance and etiological implications varied across studies. Importantly, some authors emphasized that the mere presence of candida does not necessarily establish a causal role in carcinogenesis, suggesting that fungal colonization may represent a secondary phenomenon in malignant tissues.

Recent comparative diagnostic studies further support the methodological superiority of CFW in terms of sensitivity. Chinnasamy *et al.* [5] reported minimal agreement between PAS and CFW staining, with CFW demonstrating higher sensitivity but lower specificity and moderate concordance. These findings align with the observations of the present study, where CFW staining identified candida in a small subset of OSCC cases that were negative on PAS staining. This selective positivity highlights the ability of fluorescent techniques to detect sparse fungal elements that may be overlooked by conventional histochemical stains [5].

Taken together, the findings suggest that while PAS remains a well-established and widely accepted staining method, its diagnostic yield may be limited in detecting low-level fungal invasion in OSCC tissues. Calcofluor white staining appears to offer enhanced sensitivity and

may serve as a valuable adjunctive tool in histopathological evaluation. However, variability in specificity and detection rates across studies indicates that CFW results should be interpreted with caution and in conjunction with clinical and histopathological findings.

Although candidal presence was detected in only a minority of OSCC cases, the selective localization of fungal elements within malignant tissues raises the possibility that candida may act as a co-factor rather than a primary etiological agent in oral carcinogenesis. It remains unclear whether candidal colonization precedes malignant transformation or represents a secondary phenomenon facilitated by altered epithelial integrity and local immunosuppression within tumor tissues.

The present study specifically focused on non-habit-associated OSCC cases to minimize confounding effects of tobacco and alcohol exposure. The low prevalence of candidal detection in this subset suggests that candida alone is unlikely to be a dominant causative factor in the development of OSCC in the absence of established risk habits. However, its potential contributory role in select cases cannot be entirely ruled out.

Certain limitations of the present study should be acknowledged. The sample size was relatively limited, which may have reduced the statistical power to detect significant associations. Only histopathological staining techniques were employed for fungal detection, and species-level identification was not performed. Additionally, molecular diagnostic methods, which may offer higher sensitivity, were not included. Future studies incorporating larger sample sizes and advanced molecular techniques may provide further insights into the role of candida in OSCC.

Conclusion

The present study demonstrated a low prevalence of intratissue candida detection in non-habit-associated OSCC. Calcofluor white staining identified candida in a small subset of OSCC cases, whereas periodic acid–Schiff staining failed to detect fungal elements in both OSCC and control tissues. These findings suggest that while candida may be present selectively in malignant oral tissues, its role as a primary etiological factor in OSCC remains uncertain.

The superior sensitivity of CFW staining highlights its usefulness as an adjunctive diagnostic tool for detecting sparse fungal elements within tissue sections, particularly in cases where conventional histochemical stains may be insufficient. However, the low detection rate observed in the present study indicates that candidal presence is more likely to represent secondary colonization or a co-factor rather than a dominant causative agent in non-habit-associated OSCC.

Further studies incorporating larger sample sizes, species-level identification, and advanced molecular diagnostic techniques are warranted to clarify the biological significance of candida in oral carcinogenesis and to establish standardized, reliable methods for its detection in malignant oral lesions.

Author Contributions

Nikita Kashyap: Conceptualization, sample collection, methodology, data acquisition, statistical analysis, manuscript drafting. Jay Gopal Ray: Study supervision, critical review, interpretation of results, manuscript editing.

Ethical Approval

The study was approved by the Institutional Ethics Committee of Dr. R. Ahmed Dental College & Hospital, Kolkata, West Bengal, India. All procedures were performed in accordance with the ethical standards of the Declaration of Helsinki.

Informed Consent

Written informed consent was obtained from all participants prior to sample collection.

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Data Availability

The data supporting the findings of this study are available from the corresponding author upon reasonable request.

Conflict of Interest

The authors declare no conflict of interest.

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