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Candida Albicans Alcohol Dehydrogenase 1 Gene in Oral Dysplasia and Oral Squamous Cell Carcinoma

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This study was designed to examine the prevalence of Candida albicans alcohol dehydrogenase 1 (CaADH1) gene in oral dysplasia as well as oral squamous cell carcinoma (OSCC) with and without lymph node metastasis (LNM) using reverse transcription polymerase chain reaction (RT-PCR) in order to determine its role in initiation, propagation and metastasis of oral dysplasia and carcinoma. Formalin-fixed paraffin-embedded specimens were grouped into four groups: 7 control, 16 oral dysplasia, 16 OSCC without LNM and 15 OSCC with LNM. All specimens were examined by periodic acid Schiff (PAS) stain to detect Candida hyphae, while CaADH1 gene was detected using RT-PCR. Candida hyphae were detected by PAS stain in one specimen of oral dysplasia group, 5 OSCC without LNM and 5 OSCC with LNM. CaADH1 gene was detected in 29 specimens (one case of severe dysplasia, 16 OSCC without LNM and 12 OSCC with LNM), with a highly statistically significant between the groups. CaADH1 gene is associated with OSCC with and without metastasis whereas in oral dysplasia it could not be estimated. Further studies with larger sample size are needed to confirm the role of CaADH1 in oral dysplasia and OSCC.

Key words: Candida albicans, alcohol dehydrogenase 1, oral dysplasia, oral carcinoma.

Introduction

Oral dysplasia and oral squamous cell carcinoma (OSCC) have various risk factors as tobacco smoking/chewing, alcohol intake, Candida albicans and human papilloma virus infection [1].

The association of oral leukoplakia with Candida was first reported by Jepsen and Winther [2] and since then various hypotheses related to the role of Candida in oral dysplasia and cancers were widely discussed [3]. Low socioeconomic status, poor oral health and nutritional deficiency all are associated with the risk of oral cancer and Candida infection [1, 4].

Alcohol dehydrogenase is a family of enzymes responsible for the production of acetaldehyde i.e. involved in acetaldehyde metabolism which is linked to carcinogenesis. Candida albicans is one of the agents responsible for the conversion of alcohol into acetaldehyde intra- orally through Candida albicans alcohol dehydrogenase 1 (CaADH1) mRNA gene [5, 6].

Studies that evaluated the potential role of Candida and CaADH1 mRNA in initiation and progression of oral dysplasia and OSCC as well as the metastasis of OSCC are relatively rare. Therefore, the current study was conducted to examine the prevalence of CaADH1 mRNA in oral dysplasia as well as OSCC with and without lymph node me-
tastasis (LNM) in order to determine its role in initiation, propagation and metastasis of oral squamous cell carcinoma.

Material and methods

Specimens collection

Being a retrospective study using archival blocks, this work was approved by the Ethical committee of Faculty of Dentistry, Cairo University.

Fifty four archival formalin embedded (FFPE) blocks were collected between 2011 and 2015 from the Oral and Maxillofacial Pathology Department, Faculty of Dentistry; General Pathology Department, Faculty of Medicine and Surgical Pathology Department, National Cancer Institute, Cairo University and they were grouped as follows: 7 control specimens of normal gingiva taken after gingivectomy for esthetic reasons (Group A), 16 specimens of oral dysplasia (Group B), 16 specimens of OSCC without LNM (Group C) and 15 specimens of OSCC with regional LNM (Group D).

The clinical information (age, gender and site) were obtained retrospectively from clinical records supplied with specimens and presented in Table I. Specimens documented as white lesions were only included in this study.

Hematoxylin and eosin stained sections were prepared from each FFPE block and immediately placed in 1.5 ml microcentrifuge tubes saved in 4°C refrigerator. After that sections were placed in Schiff reagent then washed in water. Finally, sections were counterstained in Mayer’s hematoxylin. A positive control slide (purchased from American Master-Tech Scientific Laboratory Supplies, USA) underwent the PAS stain procedure to ascertain the validity of PAS stain kit and accuracy of the technique.

Specimens processing for RNA extraction

Specimens were deparaffinized in xylene and washed with 100% ethanol and incubated in 240 µl PKD buffer. Ten µl proteinase was added to the previous mixture and mixed gently by pipetting up and down, then incubated in heating block at 56°C for 15 minutes, then at 80°C for 15 minutes to digest Candida cell walls. Tubes were incubated on ice for 3 minutes, then centrifuged for 15 minutes at 12,500 xg. The supernatants were then transferred to a new microcentrifuge tube without distortion of the pellet.

RNeasy FFPE kit (Qiagen, USA) was used to extract the RNA from the specimens following the manufacturer’s instructions. The extracted RNA was treated with DNase I solution to eliminate the residual of DNA in the RNA.

cDNA synthesis and RT-PCR

The reverse-transcription master mix was prepared using quantiscpt reverse transcriptase, quantiscript RT buffer, RT primer mix (Qiagen, USA). Template RNA (14 µl) was added to each tube containing reverse-transcription master mix, mixed and then stored on ice. The mixture was incubated for 15 min at 42°C in the thermal cycler (Biometra, USA). Then the mixture was incubated in thermal cycler for 3 min at 95°C to inactivate reverse transcriptase. Twelve and half µl of Top Taq was added to 3 µl CaADH1/RTPCR F primer sequence (5’–3’), (CCTCAGATGTTCTACGTCC, (10 Pmol), 3 µl CaADH1/RT primer sequence (5’–3’), (AAGATTCGTTGACGATC, (10 Pmol), 1.5 µl cDNA, then the mixture was vortexed in a total volume of 2.5 µl. Samples were put in thermal cycler for 95°C for 5 minutes (for separation of the strands), then 35 cycles of 94°C for 30 seconds, 51°C for 30 seconds (for annealing), 72°C for 60 second (for extension), then samples were put in 72°C for 10 minutes for long extension. Five positive control sections of 5 µm thickness each were scratched from the positive control slides and underwent the previous technique in comparison to the marker (100 bp) and study groups and had successfully ascertained the technique.

Statistical analysis

Data were statistically described in terms of frequencies (number of cases) and percentages. Comparison between the study groups was done using χ² test. Exact test was used instead when the expected frequency is less than 5. Within group comparisons were done using McNemar test.
Results

PAS stain

*Candida* hyphae were detected in FFPE specimens stained magenta by PAS staining, whereas other negative tissue appeared blue. *Candida* was detected in the superficial layers and penetrated deeper into the epithelium and connective tissue. Only 11 specimens (20.3% of all studied specimens) were positive by PAS stain among the whole studied specimens.

One severe oral dysplasia (Figs. 1, 2). Five OSCC without LNM and five OSCC with LNM. *Candida* hyphae were recognized between the invading malignant epithelial cells located deep in the connective tissue in a well and poorly differentiated OSCC specimens (Figs. 3, 4). All control specimens (group A) were negative. The Chi-square test revealed that the difference was not statistically significant within the different studied groups. Moreover, pairwise comparisons using Fisher Exact test did not show any statistically significant difference. Summary of results for all specimens is shown in Table II.

RT-PCR

*Candida albicans* ADH1 mRNA gene was detected in 29 specimens (53.7% of whole studied specimens). The same positive severe dysplasia specimen stained by PAS, sixteen OSCC without LNM and twelve OSCC with LNM, whereas all control specimens were negative for the *CaADH1* mRNA gene (Figs. 5-7).

The χ² test revealed that the difference in the expression of *CaADH1* mRNA in different groups was

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Table I. Clinical data for all specimens of the patients

| Code | Age (years) | Gender | Site         |
|------|-------------|--------|--------------|
| A1   | 70          | M      | Gingiva      |
| A2   | 41          | F      | Gingiva      |
| A3   | 49          | M      | Gingiva      |
| A4   | 47          | M      | Gingiva      |
| A5   | 35          | M      | Gingiva      |
| A6   | 30          | F      | Gingiva      |
| A7   | 39          | F      | Gingiva      |
| B1   | 49          | M      | Hard palate  |
| B2   | 37          | M      | Tongue       |
| C1   | 55          | F      | Cheek        |
| B8   | 50          | M      | Hard palate  |
| B9   | 36          | F      | Hard palate  |
| B10  | 36          | M      | Floor of the mouth |
| B11  | 62          | M      | Tongue       |
| B12  | 53          | F      | Lip          |
| B13  | 51          | F      | Tongue       |
| B14  | 19          | M      | Lip          |
| B15  | 56          | M      | Tongue       |
| B16  | 88          | F      | Cheek        |
| C1   | 55          | F      | Gingiva      |
| C2   | 54          | F      | Gingiva      |
| C3   | 56          | M      | Soft palate  |
| C4   | 61          | M      | Tongue       |
| C5   | 58          | F      | Hard palate  |
| C6   | 57          | M      | Tongue       |
| C7   | 40          | M      | Tongue       |
| C8   | 47          | F      | Tongue       |
| C9   | 50          | M      | Cheek        |
| C10  | 45          | F      | Tongue       |
| C11  | 34          | F      | Lip          |
| C12  | 63          | M      | Tongue       |
| C13  | 69          | M      | Lip          |
| C14  | 36          | F      | Tongue       |
| C15  | 60          | M      | Hard palate  |
| C16  | 70          | M      | Soft palate  |
| D1   | 65          | M      | Tongue       |
| D2   | 64          | M      | Cheek        |
| D3   | 45          | F      | Tongue       |
| D4   | 70          | M      | Lip          |

Table I. Cont.

| Code | Age (years) | Gender | Site         |
|------|-------------|--------|--------------|
| D5   | 65          | M      | Tongue       |
| D6   | 52          | M      | Cheek        |
| D7   | 50          | F      | Cheek        |
| D8   | 45          | F      | Tongue       |
| D9   | 57          | M      | Gingiva      |
| D10  | 53          | M      | Lip          |
| D11  | 70          | F      | Cheek        |
| D12  | 43          | M      | Cheek        |
| D13  | 50          | F      | Cheek        |
| D14  | 45          | F      | Tongue       |
| D15  | 49          | M      | Hard palate  |

A – specimen of control group; B – specimen of oral dysplasia group; C – specimen of oral squamous cell carcinoma without lymph node metastasis group; D – specimen of oral squamous cell carcinoma with lymph node metastasis group; M – male; F – female
The significance of difference in PAS stain and RT-PCR positivity according to age, gender, site and histopathological grading for all groups revealed a non-significant difference.

Discussion

Persistent mucosal colonization with bacteria, viruses and yeasts have been postulated to induce cancer by triggering cell proliferation, inhibiting apoptosis, interfering with cellular signaling pathways and up-regulating tumour promoters [7].

An association between fungal infection especially Candida albicans and oral cancer has long been discussed in the literature however the exact mechanisms by which Candida albicans share in OSCC developing still require much research. It may promote cancer development, progression and metastasis through its ability to produce carcinogens (e.g. nitrosamines, acetaldehyde) [8, 9, 10, 11].
Many techniques have been used to detect Candida colonies. Kumar et al. [12] used various laboratory tests such as culture, germ tube test, carbohydrate fermentation test, Papanicolaou (PAP) and Calcofluor-White (CFW) staining as cytopathological techniques, in addition to tissue sections were stained by PAS and CFW staining. In addition, Bakri et al. [5] used PAS stain, immunohistochemistry and RT-PCR to detect Candida albicans and CaADH1 mRNA gene.

In the present study, investigation of the Candida and CaADH1 mRNA gene prevalence was performed using PAS stain and RT-PCR, respectively. Although using PAS stain to detect Candida in a FFPE samples is less expensive and easier in comparison to the other mentioned techniques, it could not differentiate Candida albicans from other yeast species and it can give false positive and negative results [13]. Detection of Candida albicans specific genes as CaADH1 mRNA by RT-PCR is more expensive and complicated but it is more sensitive, in addition to its ability to detect the exact Candida species and the exact carcinogenic gene [5].

Our cases were chosen based on many studies that suggested the role of Candida albicans in oral dysplasia and OSCC. Therefore, the studied specimens were divided into oral dysplasia and OSCC which was further subdivided into OSCC without and with LNM in order to focus on the effect of Candida albicans infection in the metastatic potential based on the finding of Rodríguez-Cuesta et al. [14] who reported that Candida albicans enhance melanoma metastasis into the liver both at early and late stages of the metastatic process. The effect of Candida albicans on promoting metastasis seems to be based on an inflammatory process, the role of Candida albicans in tumor adhesion and metastasis has been linked to pro-inflammatory cytokines as TNF-α and IL-18. Metastasis is also mediated by an increase in adhesion-molecule expression [14, 15].

Despite the fact that Candida albicans is considered as a commensal organism in normal oral cavity and many studies reported that the oral carriage of Candida infection could be ranged between 30–45% in the general healthy (non-symptomatic) population [16], all the control specimens included in this study were negative by PAS stain and RT-PCR, this finding was in line with Bakri et al. [5].

The present work revealed Candida hyphae infection by PAS stain in only one case of the oral dysplasia group. This is nearly similar to the results obtained by Bakri et al. [5] who reported only two positive specimens from ten oral dysplastic leukoplakia specimens examined by PAS stain and along with Wu et al. [17] in their retrospective cohort study which investigated the clinicopathologic features of Candida infection in biopsies from patients with premalignant oral leukoplakia and reported that the prevalence
of PAS positive candidal infection in 396 biopsies was 15.9%. This low prevalence of Candida in oral dysplasia implies that it does not play a role in carcinogenesis initiation. Kumar et al. [12] detected Candida hyphae in 15 of the 45 PAS stained oral dysplastic specimens (33%). This value is remarkably higher than that recorded by the current study and may be attributed to subjectivity of the technique, where examiners may differ in their interpretation of the histological findings related to Candida hyphae leading to false positive results.

Results of the present study showed Candida infection in 31% of the 16 OSCC without LNM specimens and in 33% of the 15 OSCC with LNM specimens examined by PAS stain. This is nearly similar to results obtained by Jahanshahi and Shirani [13] who detected the Candida hyphae by PAS stain in only 25 of 100 OSCC specimens (25%), however, they detected Candida infection in 76 of 100 OSCC (76%) of the same studied cases by fluorescence staining. The prevalence of PAS positivity in OSCC in the current study is higher than that reported by Sanketh et al. [11] who detected Candida in only 10 out of 100 OSCC examined with CFW stain, in contrast to Kumar et al. [12] who detected Candida by PAS stain in 19 of 45 oral cancer specimens (42%).

This controversy in results of PAS stain for FFPE specimens points out to the significance of the plan of section cuts in detecting Candida by PAS staining and emphasizes the importance of serial sectioning to properly examine oral specimens for Candida hyphae. Another considerable problem is the observer’s uncertainty about the positive or negative Candida presentation because of the morphological properties of the Candida which make it appear as rod-shaped bacteria in pink to reddish color or as collagen fibers aggregated to form short bundles. Moreover, in the epithelium itself, the existing keratin has high similarity to Candida hyphae. Therefore, the PAS staining is not a very reliable procedure to study and determine the presence or absence of fungi, because it involves many false positive and false negative results. Moreover, the experience of the examiner highly affects the reliability and repeatability of PAS findings [5, 13].

By using RT-PCR to detected CaADH1 mRNA gene expression, only one oral dysplasia specimen was positive, a finding almost identical to that found by Bakri et al. [5] who detected two positive specimens out of the 10 oral dysplastic leukoplakia. In coincidence, the same oral dysplastic specimen that was positive for Candida hyphae by PAS was also positive for CaADH1 mRNA. This is identical to the results obtained by Bakri et al. [5] who reported that the same two dysplastic specimens that were positive by PAS were also positive by immunocytochemistry and RT-PCR. This similarity in results between the PAS staining and RT-PCR suggests that the Candida species present in the specimens was Candida albicans.

In the current study, the low prevalence of CaADH1 mRNA detected by RT-PCR and Candida hyphae and spores detected by PAS stain in oral dysplasia specimens can be explained by the favorable patient’s overall status that does not increase its predisposition to Candida infection.

To the extent of our knowledge this is the first study of CaADH1 mRNA gene prevalence in OSCC without and with LNM. In the current work, CaADH1 mRNA gene expression was observed in 16 specimens of OSCC without LNM and 12 specimens of OSCC with LNM. This finding was quite surprising since the patients having OSCC routinely receive antifungal treatment for Candida before the excisional biopsies. The high prevalence of CaADH1 mRNA detected in OSCC, may be explained by notion of Fox and Nobile [18] that Candida albicans biofilms are resistant to standard antifungal treatments. This drug resistance can be attributed to the fact that biofilms provide physical protection against drug penetration. Moreover, the biofilm cells themselves are intrinsically resistant to treatment [18].

Our results showed high CaADH1 mRNA gene prevalence in the specimens of OSCC with LNM. This finding is in line with Rodríguez-Cuesta et al. [14] who reported that Candida albicans significantly

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Table II. Pairwise comparison between groups

| COMPARED GROUPS                      | PAS      | RT-PCR   |
|--------------------------------------|----------|----------|
| Control vs. oral dysplasia           | 1\(^{ns}\) | 1\(^{ns}\) |
| Control vs. OSCC without LNM         | 0.272\(^{m}\) | < 0.0001* |
| Control vs. OSCC with LNM            | 0.155\(^{m}\) | < 0.0001* |
| Oral dysplasia vs. OSCC without LNM  | 0.172\(^{m}\) | < 0.0001* |
| Oral dysplasia vs. OSCC with LNM     | 0.083\(^{m}\) | < 0.0001* |
| OSCC without LNM vs. OSCC with LNM   | 1\(^{ns}\) | 0.101\(^{m}\) |

PAS – periodic acid Schiff; RT-PCR – reverse transcription polymerase chain reaction; OSCC – oral squamous cell carcinoma; LNM – lymph node metastasis; NS – non-significant.

* statistically significant at confidence level 95%.
increased metastasis of melanoma to liver. However, statistical analysis revealed that the difference in the prevalence of Candida infection was not significantly different when comparing OSCC with and without LNM.

However, the discrepancy in results between PAS and RT-PCR in OSCC without and with LNM may be explained by the possibility that the species of Candida present in the samples detected by PAS were not Candida albicans which is detected specifically by RT-PCR. Moreover, Candida albicans do not always have the carcinogenic CaADH1 mRNA gene. In addition, the plan of sections may affect the detection of the yeast, as stated before [5].

The non-significant difference of PAS stain and RT-PCR positivity according to age, gender, site and histopathological grading for all groups is attributed to the small sample size. And because of inadequate medical history reporting, one of the limitations of this study is that it can not be confirmed whether the candidal infection observed in the studied specimens is related to a previous procedure such as incisional biopsy or therapeutic interventions as chemotherapy. Therefore, it can not be concluded whether Candida arose spontaneously or as a consequence of a previous intervention. Consequently, the primary or secondary role of Candida albicans could not be determined at all times.

In many developing countries, patients may not come to attention to cancer diagnosis at all, either because of fear or the inability of poor people to access hospital services. Data may be even more unreliable because follow-up, even of treated cases, is impossible in many developing countries and there is limited international standardization with regard to the categories for cause of death recorded in the death certification [19]. For that correlation with smoking habits, alcohol consumption, oral hygiene, immunocompromised state, disability and follow-up status were missed in reports.

In conclusion, CaADH 1 gene was associated with oral squamous cell carcinoma with and without metastasis whereas in oral dysplasia it could not be estimated. A role of it could not be detected in the initiation of oral dysplasia. However, it may be involved in progression and metastasis of oral squamous cell carcinoma. Further studies with larger sample size with clinical parameters are needed to confirm its role in pathogenesis of oral dysplasia and cancer.

The authors declare no conflict of interest.

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