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Correlation between ploidy status using flow cytometry and nucleolar organizer regions in benign and malignant epithelial odontogenic tumors

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ABSTRACT

Objective: Differentiation between the aggressive benign odontogenic tumors and their malignant counterparts is controversial and difficult. While flow cytometry (FCM) allowed DNA analysis in neoplasia, argyrophilic organizer regions (AgNORs) number and/or size in a nucleus are correlated with the ribosomal gene activity and therefore with cellular proliferation. The aim of this research was to study the diagnostic accuracy of FCM and AgNORs staining in differentiating between benign and malignant epithelial odontogenic tumors and to correlate between these two interventions.
Design: Sixteen benign cases [8 cases of ameloblastoma (AB) and 8 cases of keratocystic odontogenic tumor (KCOT)] and 13 malignant epithelial odontogenic tumors [8 cases of ameloblastic carcinoma (ABC) and 5 cases of clear cell odontogenic carcinoma(CCOC)] were included in the current study. For FCM analysis, a single cell suspension from Formalin fixed paraffin-embedded (FFPE) tumors was prepared according to a modified method described by Hedley (1989) and AgNORs staining were performed in accordance to the Ploton protocol (1986). Analysis of AgNORs was performed using both quantitative and qualitative methods.
Results: The work revealed that all the examined tumors were diploid, except for 40% of CCOC cases. The S-phase fraction (SPF) value, AgNORs count and AgNORs area/cell showed statistically significant difference on comparing benign and malignant groups. A weak positive correlation was observed between SPF and AgNORs count.
Conclusion: The SPF value was considered to be more sensitive and specific in differentiation between aggressive benign and malignant epithelial odontogenic tumors in comparison to AgNORs counting.

1. Introduction

Odontogenic tumors are proliferations of tooth-producing tissues with a wide range of biologic potentials and behaviors. They are classified according to their histogenesis into: epithelial, ecto-mesenchymal and mixed tumors (Marx & Stern, 2013). Although benign odontogenic tumors are typically asymptomatic, some of them run in aggressive course causing jaw expansion, movement of teeth, root resorption, and bone loss. Among the aggressive and highly recurrent benign epithelial odontogenic tumors, AB and KCOT are the most common (Diana, Maya, Sekar, Murali, & Ramesh, 2014).

However, the aggressive behavior of these tumors and their pathogeneses are not fully understood. To date, a limited number of investigations were accomplished on odontogenic tumors to study their invasive potentials and proliferative activity. Among these investigations, FCM has allowed DNA analysis which would be very beneficial for better understanding and definite differentiation between these tumors and their malignant counterparts (Tabll & Ismail, 2011). However, SPF cutoff value in these tumors was not calculated. Moreover, AgNORs staining has represented a simple, reliable and most cost-effective technique for assessment of cellular proliferation. It has represented a cell kinetics parameter and has been used for prognostic purposes (Jain, Uma, Soundarya, Sangeetha, & Smitha, 2012). Correlation between AgNORs staining and SPF value

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Accordingly, our goals were to compare between common aggressive benign and malignant epithelial odontogenic tumors using FCM DNA analysis and AgNORs staining with calculating the SPF cutoff value in epithelial odontogenic tumors; which is variable among the different tumors. Our work also aimed to investigate the role of morphometric analysis of AgNORs staining; area and roundness in differentiating these tumors. Moreover, the sensitivity and specificity of these techniques as malignant diagnostic tests were examined. Furthermore, correlation between ploidy status, SPF value and AgNOR count of the examined lesions was performed.

2. Material & methods

2.1. Specimens

The archives of the Oral & Maxillofacial Pathology Departments, Faculty of Oral and Dental Medicine (FODM), Cairo University and Alexandria University were reviewed, to search for AB, KCOT and identification of the most common malignant epithelial odontogenic tumors during the period from 2000 to 2015. The review revealed that ABC and CCOC were the most common. The sample size was estimated after consultation a statistician at the evidence based dentistry center at FODM. All cases of AB, KCOT, ABC and CCOC in the previously mentioned period with sufficient tissue amount for FCM were included. In total, 16 benign cases (8 cases of AB and 8 cases of KCOT) and 13 malignant epithelial odontogenic tumors (8 cases of ABC and 5 cases of CCOC) were included in the current study.

2.2. H&E staining

The collected cases were serially cut into 5 μm thick sections and stained routinely with H&E stain for re-evaluation of the diagnosis. The cases were grouped into 4 groups: AB group, KCOT group, CCOC group and ABC group.

2.3. Flow cytometric analysis

All steps took place in the Tissue Culture and Cytogentic Unit, Pathology Department, National Cancer Institute, Cairo university. DNA cycle test kit (BD Cycle test Plus DNA Reagent Kit, catalogue no. #340242) was used. The single cell suspension from FFPE tumors were prepared according to a modified method described by Hedley (1989): 3–5 sections (20 μm thick) were cut from each block. Sections were deparaffinized, rehydrated and centrifuged for 10 min (1500 RPM). Each section was cut into tiny pieces to facilitate digestion by 5 ml pepsin 0.125/25 ml pH 1.5 at 37 °C in water bath for 90 min with intermittent tapping every 5 min followed by centrifugation. The disaggregated tissues were filtered through 50 μm pore size nylon mesh in order to remove debris and cell clumps.

Then, trypsin inhibitor and ribonuclease A were added to the sample to stop the action of pepsin and digest RNA. After washing using PBS, nuclear staining for DNA content was done using 50 μg/ml propidium iodide at room temperature for 10 min in the dark. Then the stained samples were acquired by FCM device (Becton & Dickinson FACScan, USA). Normal human peripheral blood lymphocytes were used to identify the normal diploid peak that served as reference peak for subsequent analysis. From each sample, at least 10,000 cells were acquired by flow cytometer at a rate of 100–200 cells/s. Interpretation of DNA histograms were done according to Baretton et al. (1995), Das et al. (2005).

2.4. Assessment of flow cytometric analysis

While histograms showing a single G0/G1 peak were defined as diploid, the tumors that exhibit two distinct G0/G1 peaks (second peak is >10% away from diploid one) were classified as aneuploid. SPF was obtained as the percentage or proportion of cells preparing for mitosis by their active doubling of DNA. SPF value above 10% was considered high (El-Deftar, El Gerzawi, Abdel-Azim, & Tohamy, 2012). The analysis was repeated to assess the reliability.

2.5. AgNOR staining

All steps took place in the Pathology laboratory, Pathology Department, National Cancer Institute, Cairo university. FFPE sections were stained using silver nitrate puriss. p.a., ACS reagent Ph. Eur. (catalogue no. #31630), purchased from SIGMA-ALDRICH, USA in accordance to the Ploton protocol (Ploton et al., 1986): The sections were deparaffinized in xylene and dehydrated in graded alcohol series. The working solution was prepared as follows: (50% Silver nitrate solution, gelatin Solution; Gelatin 2 g, Formic acid 1 ml, Distilled water 100 ml) and the two solutions A & B were mixed immediately before use in the ratio of 2:1, respectively. The slides were then incubated in freshly prepared working solution for 45 min at room temperature. Finally, slides were rinsed in distilled water for one minute, dehydrated, cleared and mounted in Di-*n*-butyl Phthalate in Xylene (DPX).

2.6. Assessment of AgNORS staining

Analysis of AgNORs were performed using two parameters:

2.6.1. A. Quantitative method

Counting of each slide was performed manually using conventional light microscope (Leica) × 1000 magnification with oil immersion as recommended by Crocker, Boldy, and Egan (1989). Only well-defined and sharply stained intra-and extra-nucleolar AgNOR dots were included in the counting and morphometric analysis. AgNORs within the nuclei at the border of the microscopic field or superimposed nuclei were excluded. The mAgNOR dots/cell in 100 cells was calculated for each case.

2.6.2. B. Qualitative method

Using image analyzer (Leica Qwin 500 Switzerland), the area and roundness of each AgNOR were measured in 100 cell, then the computer system calculated the mean value of each parameter. The total AgNORs area/cell for each lesion was estimated by the following equation: mAgNORs count/cell × mean area of each AgNOR. Regular AgNORs were given a score 1, any deviation from 1 denoted more irregularity of the AgNOR.

2.7. Statistical analysis

For evaluation of statistical significance among studied lesions, analysis of variance (ANOVA) test was used. Unpaired Student's *t* test was used to compare between benign and malignant tumors as two groups. P-values ≤ 0.05 were considered statistically significant. Mean value of AgNORs count and FCM were correlated using Pearson's correlation coefficient. The sensitivity and specificity of both tests in diagnosis of benign and malignant epithelial odontogenic tumors were estimated.