

4-28-2017

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Recommended Citation

Machado, M. A., D'Agulham, A. C., Chaiben, C. L., Castro, A. S., Bonfim, C. M., & de Lima, A. A. Exfoliative Cytology in the Oral Mucosa of Patients with Fanconi Anaemia: A Morphometric Approach. J Dent Indones. 2017;24(1): 13-18

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ORIGINAL ARTICLE

Exfoliative Cytology in the Oral Mucosa of Patients with Fanconi Anaemia: A Morphometric Approach

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ABSTRACT

Patients with Fanconi anaemia (FA) are prone to develop squamous cell carcinomas at an early age. Exfoliative cytology can be used to detect changes to the health of the oral mucosa. **Objective:** The aim of this study was to evaluate morphometrical and morphological changes using exfoliative cytology and to analyse and quantify the proliferative activity using silver nucleolar organiser regions (AgNOR) in epithelial cells of the tongue in FA patients, after haematopoietic stem cell transplantation (HSCT). **Methods:** Oral smears were collected from the tongues of 20 FA children and adolescents after haematopoietic stem cell transplantation (FA) and 20 healthy children (C) using exfoliative cytology. The smears were stained using the Papanicolaou technique and silver impregnation. The cells were morphologically analysed and the nuclear area (NA), the cytoplasmic area (CA), and the nucleus-to-cytoplasm area ratio (NA/CA) were calculated. **Results:** Mean values for the FA and C groups were: NA (71.85 and 55.21µm²; $p < 0.01$); CA (2127.48 and 1441.61µm²; $p < 0.01$); NA/CA (0.03 and 0.04; $p < 0.01$), respectively. A significant increase in the NA and CA for the FA group ($p < 0.01$) was seen, and an alteration in the NA/CA ratio. No morphological differences were found between the groups. Class I smears were predominant in both groups. No differences were found between the groups for the mean values of AgNORs per nucleus. **Conclusion:** This study suggests that morphological changes occurred in the oral epithelium cells of children and adolescents with Fanconi anaemia when subjected to HSCT.

Keywords: carcinoma, cytology, Fanconi anaemia, hematopoietic stem cell transplantation, Nucleolus Organizer Region

INTRODUCTION

Fanconi anaemia (FA) is an autosomal recessive disease with congenital anomalies, progressive bone marrow failure, aplastic anaemia, pancytopenia and leukaemia susceptibility.¹⁻³ The prevalence of the disease is approximately 1:350,000 births, with an equal distribution in both genders.^{1,4}

Haematopoietic stem cell transplantation (HSCT) is the only curative treatment for bone marrow failure in FA. These patients are susceptible, with a markedly increased risk, to squamous cell carcinomas (SCC) of the head and neck and genitourinary tract.⁵ Survivors of allogeneic HSCT who presented with

oral complications of graft-vs-host disease have a significantly increased risk for developing cancer. Incidence of secondary solid tumours is between 2-6% at 10 years and 6-13% at 15 years.^{6,7} The most common secondary solid malignancies are SCC of the skin and mouth representing for one third of all secondary solid tumours.⁶⁻⁹ Studies using exfoliative cytology or liquid based exfoliative cytology have demonstrated that the nuclear and cytoplasmic areas of the epithelial cells in the oral mucosa may be modified due to systemic conditions, such as anaemia, kidney transplant, and human immunodeficiency virus.⁹⁻¹²

Silver stainable nucleolar organiser regions (AgNORs) are markers of cell proliferation that are able to evaluate cellular changes and the biological behaviour of lesions

indicating potential malignancy.^{13,14} Early diagnosis of SCCs in transplanted FA subjects and follow up of lesions with malignant potential is essential for a good prognosis, decreased mortality, and morbidity.^{1,15}

Patients with Fanconi anaemia are more prone to develop premalignant and malignant epithelial lesions mainly after HSCT. In this way, exfoliative cytology is a useful and non-invasive tool that is important to evaluate cell changes even in areas considered healthy. The aim of this study was to evaluate quantitative changes in oral epithelial cells and analyse proliferative activity using exfoliative cytology in children and adolescents with FA that were submitted to HSCT.

METHODS

The committee of Ethics in Research approved the experimental protocol of the present study at the Universidade Federal do Paraná, Curitiba, Brazil. The patients and/or their parents or guardians were informed about the objective and other aspects of the research and signed the terms of agreement.

Twenty patients with the diepoxybutane (DEB) analysis by chromosome-breakage assay with confirmed diagnosis of Fanconi anaemia (FA) were recruited from the Bone Marrow Transplantation Unit of Hospital de Clínicas and 20 healthy patients (control group – C) were recruited from the School of Dentistry, both from Universidade Federal do Paraná, Curitiba, Brazil. Their name, age, relevant medical history, and drugs in use were recorded for the FA group. These patients underwent haematopoietic stem cell transplantation within the past one to five years.

Both groups aged from 7 to 18 years old, paired regarding sex and age were considered for the study. Oral examinations were performed in both groups and no clinically detectable premalignant or malignant oral lesions were observed. Patients who utilised mouthwashes or those with orthodontic braces were excluded from the sample, in order to minimise possible effects of such conditions upon the epithelial cell morphology. In the control group, children with any systemic diseases were also excluded from the sample.

Exfoliated cells from the clinically normal tongue were obtained by oral liquid-based exfoliative cytology. The squamous epithelial cells were collected using Universal Collection Medium kit of DNA-Citoliq System™ (Digene, São Paulo, Brazil) (Figure 1).

Cytological smears were prepared and stained using the Papanicolaou stain. Cytomorphometric and cytological analysis was done as previously described.⁹ Each slide was assessed using light microscopy and fifty cell images were captured. Nuclear area (NA)



Figure 1. Collection of smears from the tongue using cytobrush.

and cytoplasmic area (CA) were measured using the AnalySIS image system™ (Olympus Soft Imaging Solutions GmbH, Münster, Germany) for Windows XP™ 2008 (Figure 2 and 3). The NA/CA ratio was calculated.

Cytological analysis was assessed using light microscopy. The identification of basic inflammatory, dysplasia, or malignant alterations in all smears cells was done according to Papanicolaou classification; Class I: absence of abnormal or atypical cells; Class II: normal cells with inflammatory changes, no evidence of malignancy; Class III: suggestive, but not conclusive for malignancy; Class IV: cytology strongly suggestive of malignancy; Class V: cytology conclusive for malignancy.^{16,17}

The type of predominant cell (cellularity) in each smear was analysed also. The quantitative analysis of the AgNOR was carried out through light microscopy by binocular Olympus BX41™ (Olympus, Tokyo, Japan), adapted with a WH 10×-H/22 ocular and PLAN 100×/0.25. Prior to the analysis, the slide identification was covered to avoid bias. The parameter utilised for the AgNOR counting was determined as well-defined blackened points in the interior of the nucleoli in accordance with the criteria established by Crocker et al. (1989).¹⁸ The same observer carried out the process of counting the AgNOR. The number of dark points coloured by silver was evaluated in 100 cell nuclei for each smear. Nuclei with the absence of AgNOR were not counted. The nucleoli coloured by silver similar to a ring were counted as if they were one. A value of one AgNOR was assigned during analysis when the assemblage of blackened points could not be viewed as AgNOR.^{18,19}

Statistical tests were performed with SPSS for Windows 13.0 (SPSS Inc., Chicago, IL, USA). Significant statistical differences between groups were examined using Student's *t*-test (NA, CA, NA/CA, and AgNORs), $p < 0.05$.

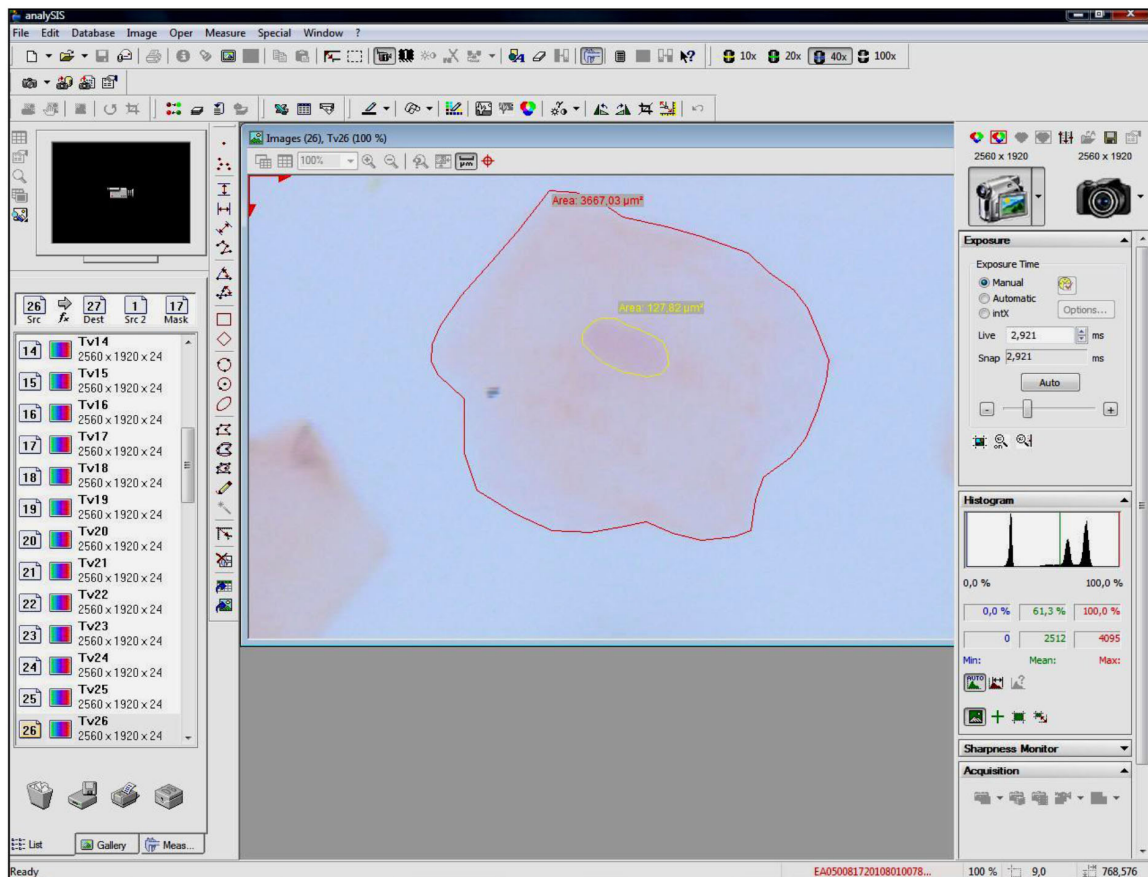


Figure 2. Layout image analysis software (Analysis) showing the delimitation of the nuclear and cytoplasmic area and their values.

RESULTS

The characteristics of the FA and control groups are described in Table 1. The mean time of HSCT for the FA group was 37.05 months (± 28.8), ranging 8-86 months; 11 subjects (55%) were submitted to HSCT from a non-related donor and 9(45%) had allogeneic transplantation.

There was a predominance of enucleated cells in the superficial layer in both groups. The enucleated (95%) and nucleated cells (5%) of the superficial layer showed the same distribution between groups. No statistically significant differences between groups were observed ($p=1.00$). There were no statistically significant differences between groups in morphological analysis of oral smear cells. According to the Papanicolaou classification there was a predominance of class I smears (cells with normal morphology, absence of atypical or abnormal) in 55% of the sample in the FA and C groups. Class II smears (cells with normal morphology and inflammatory changes) were also observed in 45% of the sample in both groups. There were no smears from class III (cells with the presence of dysplastic changes and some criteria of malignancy, but with minor alterations), IV (smears with cell

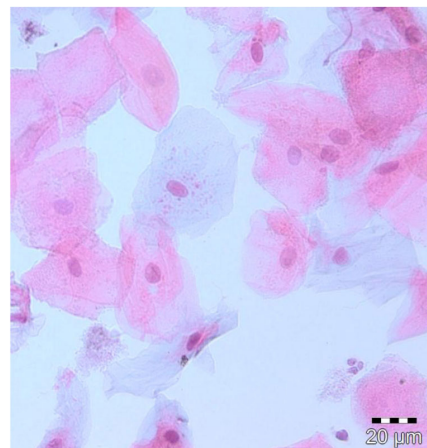


Figure 3. Epithelial cells exhibiting nuclear and cytoplasmic enlargement in oral smear of Fanconi anaemia patient ($\times 400$).

alterations, strongly suspected malignancy, and a number of abnormal cells) and V (smears with cell alterations consistent with the presence of malignancy) in the FA and C groups. A total of 2000 epithelial cells were assessed. The values for the NA, CA, and NA/CA ratio are illustrated in Table 2.

Table 1. Characteristics in the FA and C groups

Groups		FA	Control
Age (Mean \pm SD)		11.2 \pm 3.4	11.2 \pm 3.4
Gender	Male	4.0 (44.46)	5.0 (55.56)
	Female	6.0 (54.55)	5.0 (45.45)
Race	White	3.5 (35)	4.0 (40)
	Non-white	6.5 (65)	6.0 (60)

Table 2. Mean and standard deviation of NA, CA, NA/CA in the FA (20) and C (20) groups.

Groups	FA	C	p value
Variables	Mean \pm SD	Mean \pm SD	
NA (μm^2)	71.85 \pm 15.05	55.21 \pm 8.63	< 0.01*
CA (μm^2)	2127.48 \pm 441.20	1441.61 \pm 247.23	< 0.01*
NA/CA	0.03 \pm 0.006	0.04 \pm 0.005	< 0.01*

Table 3. Mean and standard deviation of number of AgNOR dots per nucleus and nuclear area in FA and C groups.

Groups	N	Mean \pm SD	p value
FA	20	3.05 \pm 0.44	0.28
C	20	2.93 \pm 0.20	

Student's t-test, *Statistics difference ($p < 0.05$); FA: Fanconi Anemia, C: Control

Student's t-test, *Statistics difference ($p < 0.05$); NA: Nuclear area, CA: Cytoplasmic area, NA/CA: Nucleus-to-cytoplasmic area ratio, FA: Fanconi Anaemia, C: Control

The normality test of Shapiro-Wilk and homogeneity of variance Levene's test revealed that data showed a normal distribution and homogeneous variances for NA and CA between groups ($p > 0.05$). Student's t-test revealed a significant increase in NA and CA for the FA group (Figure 3) compared to the C group ($p < 0.01$). There was a statistically significant difference in the mean values of NA/CA (Student's t-test; $p < 0.01$) between groups. No significant statistical differences in quantitative analysis of the AgNOR were observed between groups using Student's t-test ($p = 0.28$) (Table 3).

DISCUSSION

FA is a rare disorder resulting from autosomal or rarely X-linked recessive inheritance of mutations in

at least 13 genes.²⁰ Manifestations of FA appear in the first decade of life and include various malformations such as short stature, abnormality of the thumbs, microcephaly, café au lait hypopigmented spots, renal malformation, and growth retardation.²¹

Patients with FA that underwent haematopoietic cell transplantation have an increased susceptibility to the development of squamous cell carcinomas (SCC) of the head and neck compared to FA patients.²² The tongue is the prevalent site for SCC development in the oral cavity.²⁰ Thus intraoral examinations are relevant for detecting dysplasia. This justifies the choice of the tongue as the collection area in this study.

Oral exfoliative cytology appears as a useful tool that may increase the sensitivity in early diagnosis and management of potentially malignant lesions.^{15,20,23} In the present study there was a significant increase in NA and CA and a decrease in the NA/CA ratio of epithelial cells for the FA group when compared to the C group. The increase in the NA and CA could be associated with chronic anaemia caused by medullar aplasia. Oral keratinocytes proliferate and differentiate along the epithelial layers until they exfoliate. Thus keratinocytes of the spinous layer have a nuclear increase and as they pass into the granular layer to the surface layer, the nucleus and cytoplasm naturally decreases. However, papillar atrophy of the tongue of some children and adolescents in this study was clinically observed. In epithelial atrophy, the thickness of the epithelial layers is reduced. Furthermore, keratinocytes do not exhibit a normal maturation process and are exfoliated before the decrease of the nucleus and the cytoplasm occurs.

These results can be a reflex of the epithelial stratification, which tends to increase the size of keratinocytes as they become more superficial. However, this hypothesis only could be proved by histopathological examination. The morphological alterations in epithelial cells of non-transplanted FA patients could be related to the immunosuppression associated with persistent bone marrow failure and genetic instability. This may predispose these patients to cellular changes in carcinogenesis.²² However it is not possible to confirm this hypothesis, because the group of patients with only bone marrow aplasia was not evaluated in this study.

Smeters et al. related that most head and neck squamous cell carcinoma develop in large precancerous fields of genetically altered mucosal epithelium, which can present as visible lesions, but the majority are macroscopically not detectable.²⁴ AgNORs are a valuable parameter of cell kinetics that are associated with the rapidity of cell duplication, which may characterise dysplasia.^{12,14,19} The quantitative analysis of AgNOR allows us to evaluate the level of cellular proliferation and follow up the lesions with a susceptibility of malignancy. However, this could not

be confirmed because nucleolar quantitative changes were not observed in this study.

The exfoliative cytology rarely removes cells from the deeper layers of the epithelium, especially when it is rigid.²⁵ Moreover, the dorsal surface of the tongue is lined by keratinising stratified squamous epithelium, consequently it was expected that enucleated and nucleated superficial cells would be more prevalent in smears obtained from this region.²⁶

Morphologically, all smears of both the FA and C groups were classified as class I and II, as per the Papanicolaou system for cytology. In the original Papanicolaou classification system, class I is defined as the absence of atypical or abnormal cells. Class II is defined as the presence of inflammatory cells. This result was expected because all smears were obtained from the region of a clinically normal tongue.

Hypothetical models describing the possible role for the FA pathway in cancer susceptibility has been discussed, but until now remains unclear.²⁷ We cannot predict when FA patients will develop neoplastic diseases. For the management of these patients we recommend oral examinations and exfoliative cytology. Exfoliative cytology is an inexpensive and non-invasive technique that can be used as a tool for oral cancer prevention in patients with FA.²⁸⁻³⁰

These examinations are important tools of oral screenings and they may decrease the need for surgical approaches and can improve survival. Further investigation should be performed to elucidate the mechanisms involved in oral mucosal changes in children and adolescents with FA, who were submitted to hematopoietic stem cell transplantation, especially those related to the early development of squamous cell carcinoma.

CONCLUSION

This study suggests that morphological changes occurred in the oral epithelium cells in children and adolescents with Fanconi anaemia, who were submitted to hematopoietic stem cell transplantation, but not in cell proliferation.

ACKNOWLEDGMENTS

We would like to thank all patients who kindly collaborated with this research, as well as the Bone Marrow Transplantation Unit of Clínicas Hospital, Federal University of Paraná, Curitiba, Brazil.

CONFLICT OF INTEREST

There are no conflicts of interest or any financial or personal relationships with other people or organisations

that could inappropriately bias the conduct and findings this study.

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(Received August 20, 2016; Accepted March 6, 2017)