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Image cytometry analysis of normal buccal mucosa smears: influence of smoking and sex-related differences

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To get more information about the influence of smoking on chromatin pattern of normal buccal mucosa cells and to assess sex-related differences in nuclear features, quantitative analysis of normal buccal mucosa smears was performed. In this study, buccal smears were collected from 78 healthy subjects. Image cytometry analysis was performed on Feulgen-thionin stained smears. Probability distributions of 78 nuclear features were calculated for both, cell-by-cell and slide-by-slide classifications. Seven nuclear features showed discriminative ability between smokers and non-smokers; most of them were nuclear texture features. Statistical analysis of nuclear features in groups of females and males showed that only two nuclear features were different. It is concluded that smoking should be considered in image cytometry analysis of lesions in oral cavity.

Key words: mouth mucosa; image cytometry; smoking; cell nucleus; sex factors

Introduction

During the last decades, semi-automated and, lately, fully automated high-resolution image cytometry has been used in research work.[1,2] Using image cytometer, different morphometric, densitometric and texture features of cell nuclei can be measured and analysed. In a number of studies on tissue sections and cell samples from different precancerous and cancerous lesions, the diagnostic value and prognostic ability of quantitative methods have been already tested.[1-7,10,13] It seems that malignancy associated changes (MAC), the term that was introduced by Nieburgs *et at.*[14] and denotes subtle changes in chromatin organisation in the nuclei of normal-appearing cells in patients with malignant diseases, can be objectively assessed by image cytometry.[2,3,7,8] However, in the field of quantitative cytometry there are still several, yet unsolved methodological problems. It is supposed that, besides pathological processes themselves,[6,9,10,12] many factors from the environment may affect nuclear features. Hence, the buccal mucosa cells may be affected by age, sex, smoking, consumption of alcohol and infection.[3,4,16,17]

In the present study, we used high resolu-

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tion image cytometry to analyse the influence of smoking on nuclear features of normal squamous cells of the buccal mucosa as well as potential sex-dependent differences in nuclear features.

Materials and methods

The cell samples were taken from 78 healthy subjects. The study group consisted of 52 females and 26 males without any clinical evidence of cancer. Among them, there were 22 smokers and 56 non-smokers. Buccal mucosa cells were collected with a wooden spatula. Liquid transport medium was used to resuspend and transport cell samples. Membrane filtration method and filter imprint technique were used for smear preparation. Two smears were prepared parallelly, one for light-microscopy examination and another for image cytometry analysis. The slides for light-microscopy examination were Papanicolaou stained. All buccal mucosa smears were cytopathologically diagnosed as normal. The smears for image cytometry analysis were fixed in Delaunay fixative and air-dried. After fixation, the slides were postfixed in Böhm-Sprenger fixative and stoichiometrically stained for DNA by modified Feulgen-thionin method.

Image acquisition and analysis were performed by Cyto-Savant™ high resolution image cytometer (Oncometrics Technol. Corp., Vancouver, Canada).[15] The image analysis system consists of a MicroImager 1400 digital camera with a light transducer and a charge-coupled device (CCD), which is made up of 1320 x 1035 individual sensor elements. The size of each sensor element is 6,8 μm x 6,8 μm. The digital camera is mounted on a Nikon light microscope with 20 x PlanApo objective lens with numerical aperture 0,75. This assures an effective pixel size, a square of 0,34 μm x 0,34 μm (~ 0,1 m^2). The photometric resolution is 256 gray levels.

The slides were scanned automatically with Acquire program. Only intermediate cell nuclei of the buccal mucosa were selected for further analysis. The nuclei that were either overlapped or degenerated or out of focus, were manually separated and were not included in the statistical analysis. For each nuclear image, 78 nuclear features were calculated by the image analysis system. The statistical analysis was performed separately for smokers/non-smokers group and for females/males group. The probability distribution for each nuclear feature was calculated for 22 smokers (n=11665 cells) and for 56 non-smokers (n=24505 cells). Like for the smokers/non-smokers group, the probability distributions for each nuclear feature were also calculated for 52 females (n=25387 cells) and 26 males (n=10783 cells).

Results

Out of 78 analysed nuclear features, one morphometric and 6 nuclear texture features were discriminative between smokers and non-smokers: area, lowDNAarea, medDNAarea, low_av_dst, lowDNAamount, lowDNAcomp and range¯extreme (for description of nuclear features see Appendix 1). In cell-by-cell analysis, discriminative ability of those nuclear features ranged from 56,8 % to 58,6 %, whereas in slide-by-slide analysis, discriminative ability was between 55,8 % and 67,1 %. Nuclear features with best discriminative ability between smokers and non-smokers on the basis of cell-by-cell and slide-by-slide classifications are listed in Table 1.

The results of statistical analysis for sex-dependent groups (females and males) showed that only two nuclear features were different: range_extreme and fractal1_area (Appendix 1). In cell-by-cell analysis, discriminative ability ranged from 55 % to 56,8 % whereas in slide-by-slide analysis, it was between 60 % and 64,3 %. Nuclear features

Table 1. Nuclear features with best discriminative ability between smokers and non-smokers on the basis of cell-by-cell and slide-by-slide classifications

	Cell-by-cell classification (%)	Slide-by-slide classification (%)
Area	57,8	67,1
LowDNAarea	58,4	65,7
MedDNAarea	58,6	64,3
Low_av_dst	58,2	67,1
LowDNAamount	58,3	65,7
LowDNAcomp	58,3	65,7
Range_extreme	56,8	55,7

with best discriminative ability between females and males on the basis of cell-by-cell and slide-by-slide classifications and are presented in Table 2.

Discussion

In previous light microscopy and image cytometry studies about the effect of age and smoking on normal cells in head and neck region as well as the sex-related differences, discrepant results were obtained. In 1973, Pappelis *et al.*[17] reported that a diversified nuclear area might be an indicator of the maturation status of buccal mucosa epithelium rather than age dependent changes. On the other hand, Cowpe *et al.*[4] believed that size variation of buccal mucosa nuclei is significantly age dependent.

Burger *et al.*[3] and Reith *et al.*[13] studied the effect of smoking on buccal and nasal mucosa cells by image cytometer. In Burger's study, 17 smokers (n=1297 cells) and 46 non-smokers (n=3822 cells) were included. The linear discriminative analysis of nuclear features showed a 57,5 % correct cell classification, but differences between the specimens were not significant. Reith *et al.*[13] studied the effect of smoking on normal nasal mucosa cells of nickel workers. In this study, 9 smokers without dysplasia (n=608 cells) and 6 non-smokers (n=536 cells) were included. Although the number of analysed cells was small, they found significant differences in nuclear features between smokers and non-smokers. The results showed that 73,2 % of the cells of smokers and non-smokers could be correctly classified. In terms of specimen classification only one of non-smokers was incorrectly classified.

The results of our study, in which much larger number of cells was analysed than in previous studies, indicate that smoking can cause subtle changes of chromatin pattern. Our study group consisted of 22 smokers (n=11665 cells) and 56 non-smokers (n=24505 cells). Nuclear features showed discriminative ability between smokers and non-smokers in both, cell-by-cell and slide-by-slide analyses. In none of these studies, smoking habits, such as smoking period and number of cigarettes per day, were considered as factors which could also have an affect on nuclear chromatin pattern.

In addition, we investigated the sex-related differences in nuclear features of normal buccal mucosa cells. Two nuclear texture features showed 60 % and 64,3 % discriminative ability in slide classification. The statistical analysis was performed on the group of 52 females (n=25387 cells) and 26 males (n=10783 cells). Burger *et al.*[3] found four nuclear texture features that showed significant discriminative ability between sexes. According to them, sex dependent differences might be due to hormonal status or

Table 2. Nuclear features with best discriminative ability between females and males on the basis of cell-by-cell and slide-by-slide classifications

	Cell-by-cell classification (%)	Slide-by-slide classification (%)
Range_extreme	56,8	60
Fractal1_area	55	64,3

might be caused by an expression of X-chromosome (Barr body). Their study was performed on the group of 38 females (n=3338 cells) and 25 males (n=1781 cells). The overall correct cell classification was 62,8 % with significant specimen classification of 71,4 %. In contrast to the studies above, Cowpe *et al.*[4] studied nuclear area of buccal smears of 55 females and 50 males and did not find any differences. The relationship between nuclear and cell size of buccal mucosa smears and the stage of the menstrual cycle was not detected. In this study the smears were stained by Papanicolaou method. In our investigation, we analysed smears that were stoichiometrically stained for DNA by Feulgen-thionin method. Therefore, the discrepancy in results could be due to different staining procedures.

In conclusion, our pilot study suggests that smoking should be considered in image cytometry analysis of lesions in oral cavity. However, to get more reliable information about the influence of smoking on buccal cells as well as the sex-related differences, larger number of buccal mucosa smears should be analysed. Furthermore, for more reliable assessment of chromatin changes, also smoking habits, consumption of alcohol, medical history of healthy subjects and maturation of buccal mucosa epithelium should be considered.

Appendix 1

Nuclear feature named *area* is a morphological nuclear feature that represents the nuclear area. Nuclear features *lowDNAarea, medDNAarea, low_av_dst, lowDNAamount* and *lowDNAcomp* are discrete nuclear texture features that are based on segmentation of nuclei into discrete regions of high, medium and low chromatin condensation. Nuclear feature *range_extreme* is a local extreme nuclear texture feature that is calculated as the difference between the highest local maximum and the lowest local minimum of smoothed image. Nuclear feature *fractal1_area* is a fractal nuclear texture feature that represents measurements of the area of a three-dimensional surface, created by the nuclear optical density function.[11]

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