RESEARCH ARTICLE

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The Micronuclei Scoring as a Biomarker for Early Detection of Genotoxic Effect of Cigarette Smoking

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Abstract

Objective: The main aim of this study is to evaluate the micronuclei scoring as a biomarker for early detection and screening of genotoxic effect of cigarette smoking in the peripheral blood T- lymphocytes. **Methods:** A total number of eligible 148 individuals have participated in the study; 78 Current smokers and 70 never smokers. Cytokinesis-block micronucleus assay was performed for all the participants in the peripheral blood T-lymphocytes. Assessment of the smoking status of the participants was conducted through the detailed smoking history, Fagerström test for nicotine dependence (FTND) scoring, and determination of the urinary cotinine creatinine ratio (CCR). **Result:** A significantly higher frequency of micronuclei in the binucleated T-lymphocytes(BMNi) was identified in the smokers group as compared to the nonsmokers; OR=4.9, 95% CI=1.9-12.5), *P-value*=0.006. Both of the pack years and the smoking duration of the smokers could significantly predict the BMNi scoring; *P-value*=0.001, 0.002 respectively. **Conclusion:** Our results indicate the association between BMNi and cigarette smoking, suggesting that BMNi Scoring can be a useful biomarker for early detection and screening of the genotoxic effect of cigarette smoking as a primary preventive measure for various smoking induced cancers.

Keywords: Cigarette smoking- micronuclei- lymphocytes- genotoxicity

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Introduction

Cancer is ranked as the second leading cause of death globally, and is the responsible cause of an estimated 9.6 million deaths in 2018. Globally, about one in six deaths is due to cancer and approximately 70% of cancer causing deaths occur in low and middle-income countries (WHO, 2019). Tobacco use is considered as the most important risk factor for cancer and is responsible for approximately 22% of cancer deaths (GBD, 2016). Cigarette smoke contains over 4000 chemical carcinogens, with 200 known carcinogens that show toxicity (Paradeep et al., 2014). Nicotine consumption by cigarette smokers expresses significant genotoxic effects in human cells, including upper aerodigestive tract, as well as peripheral lymphocytes (Kleinsasser et al., 2005). The early biomarkers are divided into three groups: The first is to define the exposure to carcinogenic agents, the second is to show the biological effects on the target tissue and the third is to detect the individual susceptibility (Jois et al., 2010). Scoring of micronuclei (MNi) is considered a DNA damage biomarker which expected in the future to be predictive of increased genotoxicity and can be utilized as a biomarker to distinguish different preneoplastic conditions much prior than the appearances of clinical symptoms especially in the high risk populations (Shashikala et al., 2015). It is assumed that nicotine-induced DNA damage to be as a consequence of oxidative stress (Ginzkey et al., 2012). The micronuclei (MNi), originate either from acentric chromosome fragments or a whole chromosome lagging behind in anaphase and are left outside the daughter nuclei. They can originate from chromosome breakage due to unrepaired or misrepaired DNA lesions or chromosome malsegregation due to mitotic malfunctioning (Migliore et al., 2011).

Scoring of the MNi can be performed relatively easily and on different cell types such as Lymphocytes, fibroblasts and exfoliated epithelial cells (Alexandrescu et al., 2006). MNi scoring in the exfoliated oral buccal epithelial cells is noninvasive but can be interfered by the bacteria that are commonly found in the mouth (Kashyap et al., 2012).

The main aim of this work is to evaluate the micronuclei (MNi) scoring in peripheral blood T- lymphocytes as a biomarker for early detection and screening of genotoxic effect of cigarette smoking. It has been supposed that this procedure may be a solid future strategy for the prediction and primary prevention of human cancer dangers among the tobacco users. To the best of our knowledge, this is the first Egyptian study that addresses this issue.

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Materials and Methods

Study design and participants

This is a descriptive cross sectional study, has been undertaken at the National Research Centre, Egypt. A total number of eligible 148 individuals have participated in the study; 78 Current smokers are included as cases, in addition to 70 age and sex matched non smokers group were included as controls. The control group was cancer-free and apparently healthy individuals. All participants are clinically assessed, and a well structured questionnaire form was fulfilled through personal interviews. The questionnaire included their detailed smoking history, alcohol consumption, special habits, medical history, with emphasis on diabetes mellitus,occupational history and family history of cancers. All participants were males and were non exposed to DNA-damaging cytostatic drugs or ionizing radiation within one year before collection of the blood samples. All diabetics and those having occupational histories of exposure to chemicals such as organic solvents or paints were excluded from this study. All individuals with medically compromising conditions were excluded also from the study.

Smokers in this study were defined as, individuals who had smoked more than 100 cigarettes during their lifetimes. Cumulative lifetime smoking was measured by pack-years smoked, which was calculated individually for each smoker as described in other studies (Nersesyan et al., 2011; Metwally et al., 2016). A Written informed consent has been obtained from each subject before the sampling. The score of Fagerström test for nicotine dependence (FTND) was calculated individually for each smoker as described in Previous studies (Fagerström et al., 1978; Saad-Hussein et al., 2017).The body Mass index was calculated as described in WHO (2006) and Hegazy et al., (2016). Prior to the study, an ethical clearance has obtained from the ethical committee of the national research centre, in accordance with Helsinki's Declaration,world medical association (2013).

Laboratory analysis

1- Quantitative determination of uinary cotinine concentrations: the morning urine specimens were collected in plastic Falcon's tubes and the samples centrifuged at the speed of 2,000-3,000 rpm for 20-min. The supernatant was removed.The concentration of cotinine in the urine was determined by Enzyme Linked Immuno-sorbent assay (ELISA), a colorimeteric method, using a commercial cotinine Elisa kits from Sigma-Aldrish Co.LLC.,USA,.according to the manufacturer's instructions(Sigma-Aldrish, 2019).Urinary cotinine concentration then expressed as Pg/ml.

2- Urinary creatinine concentration was determined using calorimetric, Alkaline picrate method (Jaffe) as described by Bartles (1971). Calorimetric assay kit (Cayman, USA) was used. The concentration was expressed as mg/dl.

3- Correction of Cotinine concentration for Creatinine Excretion was performed as recommended by Benowitz (1983).Urinary Cotinine Creatinine Ratio(CCR) then was calculated and expressed as Pg/mg.Cr.

Cytokinesis-block micronucleus assay (CBMN)

The cytokinesis-block MN assay was performed using the protocol of Fenech (2007). Venous blood sample (3-3.5 ml) was taken from each patient and healthy volunteer under aseptic conditions into a sterile heparin-coated vacutainer. For each individual 2 culture tubes were set up under laminar air flow to avoid any contamination with the following constitutes; each 100 ml bottle of RPMI 1640 was added to 25 ml Foetal bovine serum, 4 ml L-glutamine, 1.5 ml penicillin/streptomycin solution and 5 ml of phytohemagglutinine. They were mixed well and distributed in sterile falcon's flat tipped 5ml tubes, and then 0.4 ml blood was added for each culture tube. Cytokinesis-block micronucleus assay (CBMN) in peripheral blood lymphocytes was done to detect chromosomal malsegregation. After initiation of culture at 37°C by 44 hours, blocking of cytokinesis with cytochalasin-B (Cytochalasin B from Drechslera dematioidea, Sigma – Aldrich (now Merck)) have been done in which scoring is selectively directed to binucleated cells (BN), as these are cells that can present MNi (Fenech et al., 2003), then the culture was completed for another 24-28 hours before harvest and Giemsa staining. Five hundred BN cells were studied and multiplied by two for each case for scoring of MNi. BN cells with MNi were photographed using a computer supported image analyzer (computer-assisted camera system; Applied Imaging, San Jose, California, USA). Scoring of MNi in BN cells prevents confusing effects caused by altered cell division kinetics (Fenech et al., 2011).

Statistical analysis

The collected data and the Laboratory results have been statistically analyzed using IBM SPSS version 20.0 software (Statistical Package for Social Science). Quantitative data were expressed as mean values ± standard deviation (SD) . Ranges and frequency distributions were estimated for qualitative variables. Normally distributed data were compared for 2 groups using Student's t test or using non parametric Mann-Whitney U test and Mood's median test. The significance of differences between proportions was tested by Chi square test (χ^2). Differences were considered significant with p value <0.05 (two-tailed). A logistic regression analysis was used to test the association between the smoking and BMNi and presented as odds ratios (OR) with confidence interval (95 % CI). Multiple linear regression analysis was used to determine the relationship between the smoking and BMNi scoring.

Results

Smoking Status characteristics

The mean age of smoking onset in the smokers group was 15.7±4.2 years old with mean total years of smoking duration; 21.5±9.3 years and mean pack years; 27.3±17.5. Fagerstrom Nicotine dependence Score ranged from 1-10 with mean 6.4±2.5 and median 7. The estimated Cotinine Creatinine Ratio (CCR) was significantly higher among the smokers group; 30 ±7 pg/mg.cr as compared to nonsmokers group; 10±6 pg/mg.cr, *P-value*=0.0001

Table 1. A Summary for the Characteristics of the Study Participants

Variable	Current smokers (n=78)	Nonsmokers (n=70)	p-value
	Mean±SD	Mean±SD	
Age (yrs.) /(Mean±SD)	43.5±11.3	40.9±9.9	0.4
Body Mass index (kg/m^2)	29.3±5.3	30±5.1	0.6
Age of smoking onset (years)	15.7±4.2	--	
Number of cigarettes/day	24.7±10.3	--	
Years of smoking	21.5±9.3	--	
Pack years (PY)	27.3±17.5	--	
Fagerström Nicotine dependence Score	6.4±2.5	--	
Median (total Range)	7 (1-10)		
IQR	4 (4-8)		
Cotinine Creatinine Rtio (CCR) (pg/mg cr.)	30±7	10±6	<0.0001*
Micronuclei Scoring (BMNi) (Mean Rank)	64.9	50.7	0.02*
Zero	7 (9.0)	23 (33.0)	0.01*
BMNi score ≤4	46 (59.0)	26 (37.0)	
BMNi score >4	25(32.0)	21 (30.0)	

*Student t-test, Mann-whitney U test, $\chi 2$ test, two sided P-value<0.05

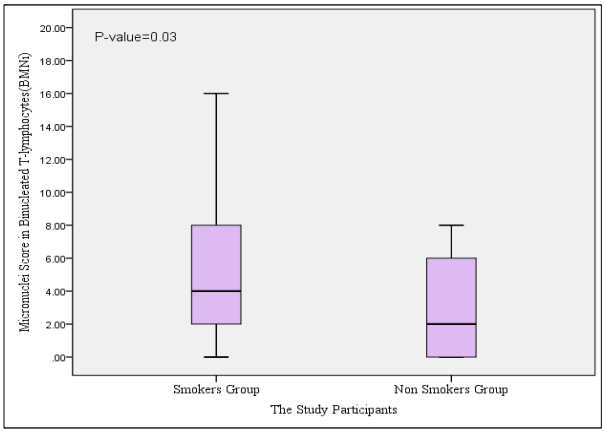

Figure 1. The Frequency of Micronuclei in Binucleated T-Lymphocytes of Smokers and Nonsmokers

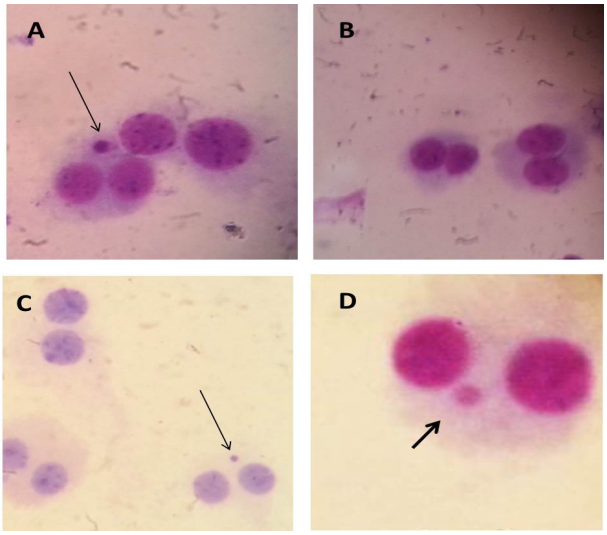

Figure.2. A Photomicrograph Representing the Images of the Detected Micronucleated, Binucleated Peripheral Blood T- Lymphocyte Cells (A, C, D), and the Detected Normal Binucleated T-Lymphocyte Cells (B). Arrow pointed to the micronucleus(MNi).

(Table 1).

Micronuclei Score in binucleated cells(BMNi)

As demonstrated in Figure 1, the median micronuclei score in binucleated cells(BMNi) among the smokers was 4 with a total range (0-16 MNi) versus 2 in the non smokers with a total range (0-8 MNi). Moreover, The frequencies of micronuclei (BMNi), as well as their median score in the smokers group, were significantly comparable to the non smokers group, *P-value*<0.05. The mean rank of micronuclei score was significantly higher in the smokers than non smokers; 64.9 vs.50.7, *P-value*=0.02. The MNi in the binucleated cells were

Table 2. A Linear Regression Analysis Model to Predict the Relationship of Cigarette Smoking with Micronuclei Score in the Binucleated T-Lymphocytes

Variable	Unstandardized Coefficients		Standardized Coefficients	p-value	95% CI
	B	SE	Beta		-0.5
Age at smoking onset (yr.)	-0.17	0.1	-0.1	0.2	- 0.4- 0.1
Pack years	0.1	0.03	0.3	0.001*	0.04-0.17
Smoking duration (yr.)	0.2	0.06	0.3	0.002*	0.07-0.3
Nicotine Dependence Score	0.4	0.2	0.1	0.1	-0.03-0.8
CCR (pg/mg.cr.)	0.4	2.4	0.02	0.8	-4.4-5.3

CCR, Cotinine Creatinine Ratio; *, High Statistically significant

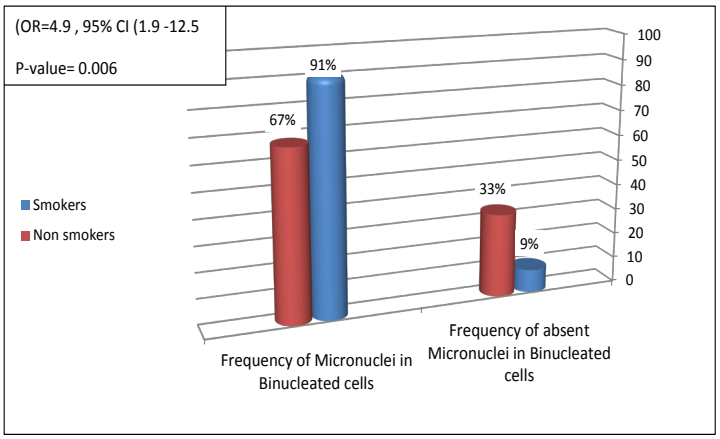

Figure 3.The Association between Micronuclei in the Binucleated T-lymphocytes and Smoking Status of the Participants.

absent in 9% (n=7) of the smokers versus 33% (n=23) of the non smokers,in addition; BMNi score was ≤4 in 59% (n=46) of the smokers versus 37% (n=26) of the nonsmokers. Moreover, BMNi score was> 4 in 32% (n=25) of the smokers versus 30% (n=21) of the nonsmokers (Table1).

In Figure 2, we can notice that micronuclei in the binucleated cells (BMNi) have specific morphological criteria; they are identical to but smaller in size than the main nuclei, round or oval in shape, they are not connected to the main nuclei and having the same staining intensity as the main nuclei.

Association of Micronuclei in binucleated cells with smoking status of the participants

As shown in Figure 3, a significantly higher frequency of BMNi was identified in the smokers group as compared to the nonsmokers ;OR=4.9, 95% CI=1.9-12.5, *P-value*=0.006. A multiple linear regression analysis revealed that, both of the pack years and the smoking duration of the smokers could significantly predict the BMNi scoring; *P-value*=0.001, 0.002 respectively. On the other hand, the age of smoking onset of the smokers as well as their nicotine dependence score, and urinary cotinine creatinine ratio (CCR) as well, didn't add significantly to the regression model; *P-value*>0.05 (Table 2).

Discussion

It is widely known that cigarette smoking is the most important risk factor for lung cancer, which is considered as one of the most malignant tumors with the highest incidence and mortality among others (Malyankar et al., 2004). However, the mechanism of carcinogenesis by cigarette smoking is still not fully understood.

It is well known that cigarette smoke contains large amounts of reactive oxygen species (ROS) and ROS-induced oxidative DNA damage has been reported to play a major role in lung cancer (Chen et al., 2015). Tobacco-specific nitrosamines have been reported to be potent clastogenic and mutagenic agents that are thought to be responsible for the induction of chromatid/ chromosomal aberrations resulting in the production of micronuclei (MNi) (Kumar et al., 2000).

The present study evaluated the micronuclei (MNi) scoring in the peripheral blood T-lymphocytes, as a useful biomarker for early detection and screening of the cigarette smoking induced genotoxic effect. We found that the Fagerström nicotine dependence score among the studied smokers varied from 1 to 10 with mean value 6.4±2.5, median=7, IQR(4-8) which denote that the nicotine dependence score of 50% of the studied smokers was moderately to highly dependent (FTND ≥7). Whilst, the other 50% was moderately low dependent (FTND≤7). Cotinine is a major metabolite of nicotine found in the urine of smokers and could be used to validate smoking status of the individuals(Shirtcliff et al.,2003). Thus, the urinary cotinine creatinine ratio was significantly higher among the smokers group; 30±7 pg/mg. cr., as compared to the non smokers group; 10±6 pg/mg.cr., *P-value*<0.0001 (Table 1).

Micronuclei may be detected in normal healthy individuals due to their exposure to environmental pollutants such as drugs, chemicals, food, and free radical injuries (Samantha et al., 2010). This study showed a significant higher median score of BMNi in the smokers group as compared to the non smokers,*P-value*=0.03 (Figure 1). Moreover, the mean rank of micronuclei score was significantly higher in the smokers when compared to the non smokers. In addition; it was observed that 33% of the nonsmokers hadn't any BMNi versus 7% of the smokers whilst, 59% of the smokers had BMNi score≤4 versus 37% of the nonsmokers. Furthermore, there were 32% of the smokers had BMNi score≥4 versus 30% of the nonsmokers (*P-value*=0.01), (Table 1).

It was an interesting finding in this result, that there was an association between the smoking status of the participants and the micronuclei (MNi), where the smokers were about fivefold more prone to have micronuclei in their peripheral blood T-lymphocytes than the non smokers; OR=4.9, *P-value*= 0.006 (Figure 3).

Our study evaluated the linear relationship of cigarette smoking and the BNMi scoring to predict the effect of cigarette smoking on BNMi scoring. It has been significantly predicted that every increase in the pack years of the smokers by one pack year, will be associated with an increase by 0.1 score of BMNi;

P-value=0.001. Further, every one year increase in the duration of smoking will be associated with an increase by 0.2 score of BMNi; *P-value*=0.002. On the other hand, the age of smoking onset of the smokers, their nicotine dependence score and urinary cotinine creatinine ratio (CCR) as well, didn't show any effect on the BMNi scoring; *P-value*>0.05, which may be explained partly by the variations in the individuals genetic susceptibility and the genetic variations in the nicotine metabolism of the participants (Table 2).

These findings indicate that the BMNi as biomarker of DNA damage was significantly higher among the smokers which is in agreement with Kleinsasser et al., (2005); who emphasized that, the cigarette smoking expresses significant genotoxic effects in human cells including the peripheral lymphocytes and hence increasing the risk of cancer. Larramendy et al., (1991), has been strongly suggested that smoking increases the rate of micronuclei formation in both CD8 and B lymphocytes. Our study approved that cigarette smoking increases the micronuclei also in the peripheral blood T-lymphocytes.

In conclusion, our study emphasizes the association between smoking as an environmental pollutant and the binucleated micronuclei (BMNi) in the peripheral blood T-lymphocytes, as an early marker of DNA damage,suggesting that BMNi can be a useful biomarker for early detection and screening of genotoxic effect of cigarette smoking.The study highlights the importance of smoking cessation, as a modifiable environmental risk factor of various types of cancers, in prevention of the disease incidence or prevention of the disease aggravation in the patients. Avoidance of exposure to smoking may decrease the potential occurrence of the cancer in the community. Further work is recommended on verification of MN assay, such as simultaneous work on DNA adduct for future research.

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