An *in vivo* cytogenetic analysis of human oral squamous cell carcinoma

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Abstract

Background: Oral cancer ranks in the top three of all cancers in India, which accounts for over 30% of all cancers reported in the country. The micronucleus test (MNT) is one of the most widely applied short term tests used in genetic toxicology to evaluate the mutagenicity and carcinogenicity. **Aims:** The present study aims at an *in vivo* cytogenetic analysis of human oral squamous cell carcinoma and to assess the applicability of MNT in diagnosing early detection of oral carcinoma. **Materials and Methods:** Exfoliated scrape smears were collected from the clinically diagnosed 136 patients suffering from oral precancerous and cancerous lesions. The wet fixed smears were stained by adopting Papanicolaou's staining protocol and counter-stained with Giemsa's solution. **Results:** The frequency of micronucleated cells has been observed to be in increasing order with the increase of the age-groups and from control to precancerous to cancerous cases significantly in both sexes. **Conclusion:** Micronucleus formation in the oral mucosa could be a biomarker of genetic damage and also a potential onco-indicator in the long run of oral carcinogenesis. Therefore, MNT can be applied for the early detection of oral carcinoma in the human being.

Key words: Exfoliated scrape smear, micronucleated cell, micronucleus test, precancerous and cancerous epithelium, nuclear entropy, carcinogenicity and pathogenicity

Introduction

Oral cancer in biomedical term is known as oral squamous cell carcinoma (OSCC) ranks in the top three of all cancers in India, which accounts for over 30% of all cancers reported in the country.[1,2] Since, the oral cavity is more accessible to complete examination; it could be used in early detection of precancerous and cancerous lesions. However, either due to ignorance or inaccessibility of medical care, the disease gets detected in the later stages. As a result, the survival index continues to be small (50%), as compared to the progress in diagnosis and treatment of other malignant tumors.[3] No doubt, cytopathology followed by biopsy (histopathology) have become a routine procedure for the detection of various stages of cancer. Micronucleus test has been accepted as an *in vivo* cytogenetic analysis and a primary tool for the early detection of OSCC in human population.[4-6]

The MNT is one of the most widely applied short term tests used in genetic toxicology and is an important test implemented by the regulatory authorities of different countries to evaluate mutagenicity of, and sensitivity to, xenobiotics.[7] Although, the scope of this test is continuously broadening by incorporating new technologies for the detection of the different genetic alterations, the MNT is often used to predict the carcinogenic potential of various compounds.[8,9] In the present study, the frequency of micronucleated cells (MNCs) of human oral exfoliated scrape smears of normal, precancerous and cancerous epithelia have been scored, statistically analyzed and interpreted to assess the applicability of MNT in diagnosing early detection of oral carcinoma.

Materials and Methods

The patients

In this case-control study, a total of 136 cases, of which 82 (60.3%) males and 54 (39.7%) females were taken into account. All these were referral patients from different hospitals of Odisha and were registered for the 1st time treatment at the Out Patient Department of Acharya Harihar Regional Cancer Center, Cuttack, Odisha, during May 2007 to May 2009. The referral patients have undergone neither chemotherapy nor radiotherapy earlier. Prior to the collection of samples, case-history of the patients relating to their age, sex, food habits, oral hygiene, and addiction to the tobacco and alcohol were asked and recorded on the prepared questionnaire for detailed analysis.

The recorded age of the patients ranges from 30 to 87 years. Therefore, the collected samples were grouped into three broad age groups, such as 30-49, 50-69 and 70-89 years. The relative proportion of the patients was found to be more (51.5%) in the age group of 50-69 years than the other two. Considering the food habits of the patients, 92.7% were non-vegetarian who prefer to take meat, fish and eggs while 7.3% were vegetarian. In non-vegetarian group, the percentage of males was more (96.3%) than the females (87.1%). Along with the alcohol, the non-vegetarians prefer to take either meat or fish or eggs 3-4 times within a week. On the contrary, the percentage of females is more (12.9%) than the males (3.7%) under vegetarian category. Basing on the degree of pathogenicity, 32 (23.5%) males and 23 (16.9%) females were precancerous cases and 50 (36.8%) males and 31 (22.8%) females were cancerous ones. Considering the nature of addiction, out of 136 cancer-affected individuals, 126 (92.7%) were addicted to either different forms of tobacco or alcohol or both for more than 15 years and the rest 10 (7.3%) were non-addicted.

Age group, oral site and sex-matched parallel set of 136 samples were also collected from the non-addicted and non-cancerous healthy individuals from different regions of Odisha as normal or control group. In other words, out of 136 normal individuals, none of them was addicted to any habit in control group.

Collection of samples

Exfoliated scrape smears were collected from the clinically diagnosed 136 patients suffering from oral precancerous and cancerous lesions. Smearing was done on the pre-cleaned-coded microslides and the smeared slides were immediately fixed

Access this article online
Quick Response Code:
Website: www.sajc.org
DOI: 10.4103/2278-330X.173178

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How to cite this article: Mohanta A, Mohanty PK, Parida G. An *in vivo* cytogenetic analysis of human oral squamous cell carcinoma. South Asian J Cancer 2015;4:123-6.

in aceto-alcohol (1:3) fixative. Two slides were smeared and prepared from each affected sites of the patient.

Staining protocol and scoring of micronuclei

The wet fixed smears were stained by adopting Papanicolaou's staining protocol and counter-stained with Giemsa's solution. One thousand cells were screened and the micronucleated cells were scored from each stained sample following standard criteria.[10] Although more than one micronuclei were observed in oral squamous cells, the scored elements were the MNCs and not the number of micronuclei.[11]

Statistical analysis

Findings were statistically analyzed, interpreted and correlated with the age group, sex and degree of pathogenicity. Test of proportion (*Z*-test) was followed and the critical ratio (*Z*-value) was calculated for the test of significance.

Results

The MNCs in the exfoliated scrape smears of buccal mucosa were found to contain either a single micronucleus or more number of micronuclei within a cell [Figures 1 and 2]. The scored number of MNCs in control, precancerous and cancerous groups was tabulated and their frequencies were calculated [Table 1]. A total of 7 (0.021%) MNCs from 34 samples, 9 (0.026%) from 33 and 6 (0.068%) MNCs from 15 samples of male individuals were scored in the control group in the age group of 30-49, 50-69 and 70-89 years, respectively. The mean percentage of MNCs was calculated to be 0.026 in males. In the case of females, the number and percentage of MNCs were scored to be 2 (0.018%) from11, 7 (0.019%) from 36 and 2 (0.028%) from 7 individuals in 30-49, 50-69 and 70-89 year of age groups, respectively. The mean percentage of MNCs was recorded to be 0.020 in females.

In precancerous group, the number (percentage) of MNCs were recorded to be 8 (0.60%) from 8 males and 26 (0.52%) from 5 females, in 30-49 year age group. In the age group of 50–69 years, the scored MNCs were 139 (0.77%) in18 males and 85 (0.60%) in14 females. In 70-89 years of age group, 54 (0.90%) MNCs from 6 males and 30 (0.75%) MNCs from 4 females were scored. Thus, the mean percentages of MNCs were estimated to be 0.753 in males and 0.613 in females, respectively. The critical ratios in the precancerous group were calculated to be 14.91 in males and 11.442 in females which were observed to be more than the *Z*-values of normal distribution at 1% level of significance.

Scoring the samples from cancerous group, the number (percentage) of MNCs were reported to be 425 (1.70%) from 25, 336 (2.10%) from 16 and 232 (2.57%) from 9 males in 30-49, 50-69 and 70-89 years of age groups, respectively. The average percentage of MNCs was 1.986 in males. In case females, the recorded numbers (percentage) of MNCs were 79 (1.316%) from 6, 463 (2.104%) from 22and 87 (2.90%) from 3 patients in the respective age group of 30-49, 50-69 and 70-89 years. The average frequency was calculated to be 2.029 in females. The *Z*-values were found to be 31.285 in males and 25.016 in females. In both precancerous and cancerous groups, the *Z*-values in males and females were observed to be significantly ($P<0.001$) higher than the normal observed value.

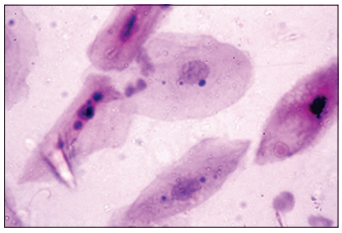

Figure 1: Bi-, tri- and tetramicronucleated condition of oral squamous cells (Giemsa's stain, ×400)

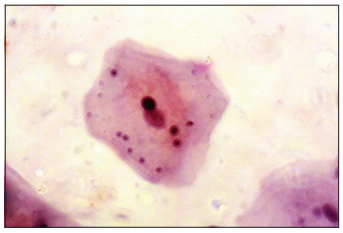

Figure 2: Anisonucleated octamicronucleated condition of an oral squamous cell (Papanicolaou's stain, ×400)

Discussion

World Health Organization has defined oral precancerous lesion as a morphologically altered tissue that is more likely to be transformed into cancer than its normal counterpart. Since, the formation of micronuclei in eukaryotic cells is an end point of chromosomal damage or segregation errors, the presence of micronuclei reflects a genotoxic or carcinogenic exposure.[12] The direct correlation between the micronuclei formation and genomic damage make the micronucleus assay an efficient alteration to the metaphase analysis. Reports of earlier workers have shown that analysis of micronuclei in oral mucosal cells is a sensitive method for monitoring genetic damage in human population.[13,14]

Working with high risk for oral cancer, Ghosh and Parida have reported that the frequency of MNCs is higher in males (7.37%) than the females (5.90%). They have also reported that the frequency of MNCs gradually increases from lower to higher age groups in both sexes.[15] After analyzing the frequency of MNCs in exfoliated buccal smears, Casartelli *et al.* have opined

Table 1: Age group and sex-wise frequency of MNCs in control and cancer affected groups

Group	Age group in years	Number of samples screened		Number of MNCs scored		Percentage of MNCs		Mean percentage of MNCs		Critical ratio (Z value)*	
		Male	Female	Male	Female	Male	Female	Male	Female	Male	Female
Control	30-49	33	11	07	02	0.021	0.018	0.026	0.020	-	-
	50-69	34	36	09	07	0.026	0.019				
	70-89	15	07	06	02	0.068	0.028				
Total	30-89	82	54	22	11	0.226	0.020	0.026	0.020	-	-
Pre-cancerous	30-49	08	05	48	26	0.60	0.52	0.753	0.613	14.910*	11.442*
	50-69	18	14	139	85	0.77	0.60				
	70-89	06	04	54	30	0.90	0.75				
Cancerous	30-49	25	06	425	79	1.70	1.316	1.986	2.029	31.285*	25.016*
	50-69	16	22	336	463	2.109	2.104				
	70-89	09	03	232	87	2.57	2.90				
Total cases	30-89	82	54	1234	770	1.504	1.425	1.504	1.425	34.472*	27.353*

*Significant at 1% level ($P<0.01$) of confidence, where Z=2.576. MNCs=Micronucleated cells

the gradual increase in MNC counts from normal mucosa to precancerous lesions to cancerous lesions.[16] Halder *et al.* have investigated and reported a comparative analysis of exfoliated oral mucosal cells with micronuclei frequency in normal, precancerous and malignant epithelium of 100 persons, in toto. They have observed that the average frequency of MNCs in the oral mucosal cells of control group was 0.35% (males-0.39% and females-0.32%). In the subjects of precancerous lesion, frequency of MNCs was 0.63% (males-0.55% and females-0.75%). The frequency of MNCs in the cancer patients (pre-operative) was 1.26% (males-1.66% and females-1.16%) and in cancer patients (post-operative) was 0.44% (males-0.51% and females-0.38%).[5] In the present study, although post-operative cases were not included, but it has been observed that the frequencies of MNCs were in increasing order from lower to higher age-groups and from normal to precancerous to cancerous epithelium significantly. In the same age group of all these three groups, the frequencies of MNCs were also observed to be in an elevated state. Thus, the present finding not only corroborates but also supports the results of earlier workers.[5,13,15,16]

Some of the earlier studies have shown no sex-related differences in their MNT results.[17,18] However, some authors have described sex as an important variable in the MNT with males generally being more sensitive than females to the induction of micronuclei.[19] In this study, it has also been found that the mean percentage of MNCs differ from a male to female in control, precancerous and cancerous groups and thus, corroborates with the earlier findings.[5,19] However, the mean percentage of MNCs in cancerous group was found to be less in males (1.986) than females (2.029) which contradicts the earlier outcomes.[15,18]

A genetic progression model for OSCC to explain the field cancerization theory has been proposed, by which an entire epithelial surface is primed for neoplastic changes following prolonged carcinogen exposure, leading to focal areas that progress at different rates towards invasive cancer. Microsatellite analysis in head-and-neck squamous cell cancer for allelic loss at 10 major chromosome loci demonstrated that the spectrum of chromosomal deletions progressively increases at each histopathological step from benign hyperplasia to dysplasia to carcinoma *in situ* to invasive cancer.[20] The most common gains in betel quid and/or tobacco chewing associated with oral cancers are on chromosomes 8p, 9p, 9q, 11q, 17q and 20q and the most frequent losses are in chromosome arms 3p (genes FHIT and RARB), 4q, 5q, 9q and 18q.[21,22]

In this study, the patients are reported to be habituated with chewing and smoking of tobacco and drinking of alcohol for more than 15 years. Over many years of such continued habit, cellular entropy has increased in the oral epithelia of the patients. Due to genotoxicity of tobacco and alcohol, cellular entropy increases and cells with abnormal chromosome complements are continuously formed. Mohanta *et al*. have recently reported that longer the duration of abuse of tobacco and alcohol, greater was the number of MNCs in oral epithelia. It has also been observed that chewing and smoking of tobacco and drinking of alcohol enhance the rate of formation of micronuclei along with other cytological atypias followed by OSCC. In their study, they have also reported that appearance of higher percentage of MNCs in non-addicted oral cancer patients than the single addicted (chewers, smokers and alcoholics) groups, has proved the MNC to be an onco-indicator and MNT to be the simplest tool for the test of genotoxicity as well as for the early detection of cancer of the oral cavity.[23]

Considering the frequency of MNCs in oral mucosa of normal, precancerous and cancerous groups and their appearance for many years, these seem to represent an early detectable marker of epithelial carcinogenic progression and micronucleus assay in human buccal cells is considered as a tool for biomonitoring DNA damage.[7,24] Jadhav *et al.* have reported that micronucleus could be a candidate to serve as a biomarker for the prediction of the grade of OSCC.[25]

Conclusion

Considering the above *in vivo* cytogenetic analysis of human OSCC, it is observed that increased frequency of MNCs from normal to precancerous to cancerous cases with respect to increased age groups in both sexes is a fact. Hence, as the second law of thermodynamics is concerned, the entropy of buccal mucosa increases with the onset of micronuclei formation due to the carcinogenic effect of tobacco and alcohol. Longer the period of addiction, greater is the nuclear entropy and greater is the genetic damage followed by genesis of more number of MNCs. Thus, formation of single micronucleus to more number of micronuclei within a cell as well as appearance of single MNC to more number of MNCs in the buccal mucosa indicates MNC as a bio-marker of genetic damage and also a potential onco-indicator in the long run of oral carcinogenesis. Therefore, MNT can be applied for the early detection of OSCC in the human being.

Acknowledgments

Authors are thankful to the Head, P.G. Department of Zoology, Utkal University, Vani Vihar, Bhubaneshwar, Odisha for providing laboratory and library facilities; to the Director and Head, Department of Oncopathology, Acharya Haihar Regional Cancer Center (AHRCC), Cuttack, Odisha for permitting us to collect samples from the oral cancer patients and also for providing library and laboratory facilities. One of us (AM) is grateful to the University Grants Commission (UGC), New Delhi, India for awarding UGC Research Fellowship to undertake this project.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

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