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The Micronucleus Test in Urothelial Cells and Uterine Smears of Cervix Cancer Patients: A Comparison

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ABSTRACT Cytogenetic damage was assessed using the (MN) test in urothelial cells and cervix smears of local cervix cancer patients since the genetic end-point screening for micronuclei provides a measure of both, chromosome breakage and loss. The MN data were grouped for stage-types of cancer, age-groups, age-at-marriage, parity levels and socio-economic status. A comparison of the results obtained from both the tissues has revealed that the percent frequency of MNd cells was elevated in urothelial cells except when the variable for age-groups of the patients was compared. If validated, the MN assay in urothelial cells may prove useful for screening programmes for cervix cancer as besides scoring effectively for cytogenetic damage, it utilizes a non-invasive process of sample collection.

INTRODUCTION

Cervix cancer is a serious problem behesting Indian women as estimates have recorded that of all cancers among females, almost 24 % were of the uterine cervix (Chander et al. 1999). It had also been predicted (Murthy and Mathew 1999) that in the absence of any control programme, the incidence of cervical cancer would rise to 140,000 by the end of the year 2000. This is rather unfortunate since its early detection makes it treatable if not curable. There has been observed reduction in morbidity and mortality from this cancer among the screened persons (Hodge et al. 1998). In fact, the goal of cervix cancer screening is to detect it in its earlier stages and so the symptoms should alert women to the need for a medical examination.

Among the worldwide efforts being carried out for the determination of the effects of environmental, genetic and life-style factors on the genomic stability in human populations, the Micronucleus test (MNT) is one of the techniques adopted by numerous laboratories (Fenech et al. 1999). Micronuclei provide a measure of both, chromosome breakage and chromosome loss. Chromatid or chromosome fragments and entire 'aberrant' chromosomes which do not get included in the newly formed daughter cells, form micronuclei. The MN assay is more rapid and simpler than chromosomal analysis. In the present investigation the MNT in exfoliated cells of urine and in uterine smears has been employed for scoring cytogenetic damage in cervix cancer patients. The bladder exfoliated cells provide a non-invasive method of sample collection and comprise cells not directly involved in uterine cancer. The smears can be requested during the examination of the cervix which is the target site for this cancer.

MATERIALS AND METHODS

Subjects: Samples were obtained from cervix cancer patients visiting various hospitals of Amritsar, viz. Guru Ram Das Medical College and Hospital, Guru Teg Bahadur Hospital and Karam Singh ward of Radiotherapy. The patients (generally housewives belonging mostly to low-socio-economic status families) were visiting these hospitals for some gynaecological complaints like, intermenstrual bleeding, post-coital bleeding, leuchorrea, prolongation of the menstrual period, etc. Of these, 25 tested positive with the Pap smear and so comprised the subject group. Those testing negative (n=25) comprised the control group since periodic gynaecological examination by normal, healthy women is generally not favoured.

Each individual was questioned as to her age (being also her age-at-detection), age-at-marriage, age at first pregnancy, reasons for consultation, economic status, diet, smoking and alcohol drinking patterns, any disease incidence, environmental or medical exposure, etc. The data were kept as individual records on pre-designed questionnaires. The nature of the study was explained to the individuals and those who signed a consent form willingly were requested to provide urine and cervix smear samples. The protocol for the micronucleus test, as used by Chakrabarti and Dutta (1988) with some

alterations, was followed for analyzing the genetic damage in both the tissues. Data obtained for percent frequencies of MNd cells were subjected to Student's t-test for statistical analysis.

The MN Test in Urothelial Cells: The sources of urothelial cells are the renal tubular, bladder and squamous epithelia. Urine sampling is a non-invasive process. Mid-stream samples (~5ml) were requested in aspectic vials and were transported to the laboratory on ice,. They were processed within 3-4 hrs of sample collection. The procedure, briefly, entailed sample wash thrice in phosphate buffered saline (PBS) with alternate centrifugations at 1200 rpm for 10 min. From the pellet, smear preparations were made on pre-cleaned glass slides. Upto 2-3 slides per case were made and these were allowed to air dry. The cells were fixed in methanol for 20 min and then were stained in May-Grunwald's stain (0.25%) for 5 min, counter-stained with Giemsa (1%) for 3 min and mounted in DPX. The slides were coded and scored blind. Depending on the cell population available, a total of 200-500 cells were initially scored under the low power (40x) of a binocular microscope, while the presence of micronucleated (MNd) cells was confirmed under oil immersion (100x) and independently by another observer. The results were expressed as percent frequency of MNd cells.

The MN Test in Uterine Smears: Cancer of the cervix develops from cells that cover the surface of the cervix. It takes 3weeks to >20 yrs for the cells to become truly malignant. The epithelial cells from the cervix can be sampled during a routine gynaecological examination. The smears were obtained by the attending gynaecologist. A sterilized wooden spatula was rotated in a 360º fashion and the scrapings were collected in PBS. The samples were brought to the laboratory on ice and were processed within 2-4 hrs. The protocol for the MN test, as given above, was adhered to. Depending upon the cell population available, 600-1000 cells per individual were scored for the presence of micronuclei under 40x with MNd cells confirmed at 100x.

RESULTS AND DISCUSSION

In the present study an attempt has been made to compare the percent frequency of MNd cells in different tissues of just-diagnosed cervix cancer patients in order to observe whether marked differences in damage exist. The MN data observed in both the tissues were statistically significant from their respective controls (results communicated). In this paper only the comparative study is being presented. Data for the MNd cells have been grouped with respect to the stage-types of cancer, age of patients and some of the recognized risk factors in cervix cancer etiology (illiteracy, low socio-economic status, early menarche, early marriage, multiparity, first child birth at an early age, poor genital hygiene and genital infections; Dutta et al. 1990; Thompson 1992). Data were not compared within those sub-groups lacking MNd cells and/or with less number of subjects. The total number of cells scored in both the tissues varied depending on the samples provided by the subjects. While ~5ml of urine was on an average provided by the subjects, the cervix smears were obtained by the gynaecologist during the cervix exmination. Therefore scorable cells per individual varied from 600-1000 in smears and 200-500 in urine samples.

The patients were in different cervix cancer stages (Table 1) and the general trend observed was the higher percent frequency of MNd cells in urothelial cells as compared to in cervix smears. This is interesting since more cells were scored in smear samples and it is also the site for development of cancer. The data from stage I were comparable in both the tissues while significantly elevated frequencies of MNd cells were recorded in urothelial cells at stages II and III, in the sum total as also in control data.

The age-range of the patients varied from 21-70 yrs.; this was also their age-at-detection (Table 2). Significant damage was observed only in patients who were 21-30 and 51-60 years of age. The number of MNd cells were higher in their smears though it was reverse for data in total patient and control groups.

The trend for percent frequencies of MNd cells at different parity levels (Table 3) exhibited non-significant difference at the low parity level but statistical significance at the higher levels with more damage in urothelial cells, both among patients and controls.

For the factor age-at-marriage, the 11-15 years' age-range depicted significance for MN data , again elevated in urothelial cells (Table 4). The middle socio-economic status (SES) group too had more significant damage in urothelial cells of patients while non-significant results were

Table 1: Frequency of MNd cells in patients at different stages of cervix cancer

Patient group	*MNd cells/total urine cells scored*	*Mean[a]±SE (% frequencey MNd cells)*	*MNd cells/total cervis cells scored*	*Mean[a]±SE (% frequency MNd cells)*	*t-test*	*d.f.*	*p-value*
Stages of Cervix Cancer							
(Ia+Ib)(8)	6/2217	0.229** ±0.075	17/6780	0.228** ±0.046	0.1137	14	NS
(IIa+IIb)(8)	6/2769	0.242** ±0.091	30/6212	0.426** ±0.080	3.54	14	<0.01
(IIIa+IIIb)(9)	15/2566	0.580** ±0.075	24/6663	0.319** ±0.064	5.18	16	<0.001
Total (25)	27/7552	0.359** ±0.058	71/19655	0.324** ±0.024	2.98	48	<0.01
Control group (25)[b]	4/7143	0.058 ±0.065	7/18446	0.031 ±0.023	2.35	47	<0.02

** -Highly significant compared to respective total control groups, p≤0.05 and p≤0.01 (Student's t-test)
a – calculated as an average of individual frequencies of micronucleated cells in that group.
b-n=24 for urine samples as one sample did not yield cells.

Table 2: Frequency of MNd cells in different age groups of cervix cancer patients and control individuals

Age/Age-at-detection (yrs)	*No of MNd cells/total urine cells scored*	*Mean[a]±S.E. (% frequency MNd cells)*	*No. of MNd cells/total cervix cells scored*	*Mean[a]±S.E. (% frequency MNd cells)*	*t-test*	*d.f.*	*p-value*
Patients Group							
21-30(3)	3/909	0.298**±0.129	16/3010	0.507** ± 0.044	3.35	4	<0.05
31-40(7)	6/2002	0.314**±0.111	17/5725	0.313** ± 0.037	0.27	12	NS
41-50(7)	6/2204	0.239**±0.088	15/5068	0.210** ± 0.053	1.67	12	NS
51-60(6)	9/1935	0.508**±0.115	21/4552	0.433** ± 0.028	2.31	10	<0.05
65(1)	1/232	0.431**	0/800	-			
75(1)	2/270	0.740**	2/500	0.200** ± 0.140			
Total(25)	27/7552	0.359**±0.058	71/19655	0.324** ± 0.024	2.98	48	<0.01
Controls Group							
21-30(10)[b]	1/2538	0.049 ±0.140	2/7010	0.022 ± 0.021	1.16	17	NS
31-40(7)	2/2152	0.092 ±0.104	3/5325	0.049 ± 0.022	1.95	12	NS
41-50(4)	0/1225	-	1/2426	0.033 ± 0.028			
51-60(2)	0/612	-	1/1945	0.050 ± 0.035			
61-70(2)	1/616	0.161	0/1740	-			
Total(25)[b]	4/7143	0.058 ±0.066	7/18446	0.031 ± 0.023	2.35	47	<0.02

** - Highly significant compared to respective total control groups, p≤0.05 and p≤0.01 (Student's t-test)
a – calculated as an average of individual frequencies of micronucleated cells in that group.
b-n=9 and n=24, respectively for urine samples as one sample did not yield cells.

observed for both categories in controls (Table 5). It has to be recalled that these results were statistically different from their respective control values while here only a comparison with respect to MN frequencies in different tissues is being made.

Citations in the literature include chromosomal damage in peripheral blood lymphocytes (Sreekantiah et al. 1988; Murty et al. 1988), in carcinoma *in situ* (Sreekantiah et al,1987; Atkin et al.1990; Mark et al. 1999), in different cell lines (Gebhart et al.1984 ; Han et al. 1991), besides observing sites of tumour suppressor genes (Mitra et al.1994), DNA

Table 3: Frequency of MNd cells and number of pregnancies in cervix cancer patients and control individuals

Groups	*MNd cells/ total urine cells scored*	*Mean[a]±SE (% frequency MNd cells)*	*MNd cells/ total cervix cells scored*	*Mean[a]±SE (% frequency MNd cells)*	*t-test*	*d.f.*	*p-value*
Patient Group							
1-3 (7)	8/2339	0.359**±0.097	19/5642	0.347** ± 0.046	1.02	12	NS
4-6 (11)	13/3274	0.406**±0.099	21/7818	0.231** ± 0.051	3.76	20	<0.01
7-9 (7)	6/2039	0.286**±0.051	31/6195	0.447** ± 0.091	3.85	12	<0.001
Total (25)	27/7552	0.359**±0.058	71/19655	0.324** ± 0.024	2.98	48	<0.01
Control Group							
1-3 (13)[b]	3/3594	0.091 ±0.092	3/9247	0.025 ± 0.013	2.76	23	<0.02
4-6 (11)	1/3336	0.029 ±0.092	4/8245	0.042 ± 0.022	1.21	20	NS
7-9 (1)	0/213	-	0/954	-			
Total (25)[b]	4/7143	0.058 ±0.066	07/18446	0.031 ± 0.023	2.35	47	<0.02

** Highly significant compared to respective total control groups, $p \leq 0.05$ and $p \leq 0.01$ (Student's t-test)
a – calculated as an average of individual frequencies of micronucleated cells in that group.
b–n=12 and n=24, respectively for urine samples as one sample did not yield cells.

Table 4: Frequency of MNd cells and age-at-marriage of cervix cancer patients and control individuals

Age-at-marriage (yrs)	*MNd cells/ total urine cells scored*	*Mean[a]±SE (% frequency MNd cells)*	*MNd cells/ total cervix cells scored*	*Mean[a]±SE (% frequency MNd cells)*	*t-test*	*d.f.*	*p-value*
Patient Group							
11 – 15 (3)	6/912	0.659**±0.105	10/2330	0.406** ± 0.064	4.09	4	<0.02
16 – 20 (20)	20/6037	0.335**±0.061	56/15315	0.319** ± 0.048	1.62	38	NS
21 (1)	0/274	-	4/1020	0.392**			
26 (1)	1/329	0.303**	1/990	0.131**			
Total (25)	27/7552	0.359** 0.058	71/19655	0.324** ± 0.024	2.98	48	<0.01
Control Group							
11 – 15 (2)	1/530	0.223 ±0.223	0/1950	-			
16 – 20 (18)[b]	3/5232	0.056 ±0.029	6/13088	0.039 ± 0.016	0.762	33	NS
21 (5)	0/1381	-	1/3408	0.180 ± 0.016			
Total (25)[b]	4/7143	0.058 ±0.066	7/18446	0.031 ± 0.023	2.35	47	<0.02

** Highly significant compared to respective total control groups, $p \leq 0.05$ and $p \leq 0.01$ (Student's t-test)
a – calculated as an average of individual frequencies of micronucleated cells in that group.
b–n=17 and n=24, respectively for urine samples as one sample did not yield cells.

damage in lymphocytes and epithelial cells of the cervix (Jaiswal et al. 1994; Udumudi et al. 1998; Desai et al.1998) as well as MN in uterine smears (Chakrabarti and Datta1988) of cervix cancer patients.

None of these nor other studies have used urothelial cells for investigating cytogenetic damage in this cancer. The single gel electrophoresis assay performed with peripheral blood lymphocytes and cervical epithelial cells has shown greater damage in the latter which was further step-wise elevated with the cancer stage (Jaiswal et al. 1994;Udumudi et al.1998; Desai et al. 1998). In the results of the present study rather, damage in urothelial cells was increased despite the lesser number of scorable cells. Although the damage in cervix smears was also significantly more than in controls yet it was generally lesser than in urothelial cells except when analysed for the age variable. These are surprising results since urothelial cells do not constitute cells of the cervix but comprise cells from the renal tubular, squamous and bladder epithelia (Soods 1994). Further, in the control individuals the background rate is higher than in cervix smears, which could possibly be due to

Table 5: Frequency of MNd cells and socio-economic status of cervix cancer patients and control individuals

Socio-economic Status	*MNd cells/ total urine cells scored*	*Mean[a]±SE (% frequency MNd cells)*	*MNd cells/total cervix cells scored*	*Mean[a]±SE (% frequency MNd cells)*	*t-test*	*d.f.*	*p-value*
Patient Group							
Low (19)	20/5668	0.361** ±0.071	60/14755	0.363** ±0.045	0.532	36	NS
Middle(6)	7/1884	0.356** ±0.066	11/4900	0.199** ±0.069	4.150	10	<0.01
Total (25)	27/7552	0.359** ±0.058	71/19655	0.324** ±0.024	2.980	48	<0.01
Control Group							
Low (11)[b]	2/2873	0.076 ±0.110	3/7084	0.032 ±0.021	1.870	19	NS
Middle (14)	2/4270	0.046 ±0.079	4/11382	0.031 ±0.014	1.520	26	NS
Total (25)[b]	4/7143	0.058 ±0.066	7/18446	0.031 ±0.023	2.350	47	<0.02

** Highly significant compared to respective total control groups, $p \leq 0.05$ and $p \leq 0.01$ (Student's t-test)
a – calculated as an average of individual frequencies of micronucleated cells in that group.
b–n=10 and n=24, respectively for urine samples as one sample did not yield cells.

the gynaecological complications in them. This requires investigations on the MN frequencies in urine cells of an age-, sex- and socio-economic-matched healthy control group from this region though 0.073±0.099 percent frequency of MNd cells has been observed in healthy women of Amritsar in another study (Gandhi et al. unpublished results). Other studies have recorded varying, yet low, MN frequency in urothelial cells of healthy controls (0.73± 0.22 in males was observed by Cid et al. 1990; 0.1% in females by Reali et al. 1987; 0.47±0.31 by Lehucher-Michel et al. 1996).

It may further be stated that on calculating the specificity and efficiency of these assays, the MN test in cervix smears scored better (Gandhi et al, unpublished results) which makes it a more reliable index of damage besides it being the target site for this cancer. However, emergent views from this study indicate that the MN test in urothelial cells , after validation, may prove beneficial to perform in screening programmes of cervix cancer given the non-invasive method of sample collection, easier availability of urine and the rapidity of the assay.

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