Evaluation of Micronucleus and Nuclear Division Index in the Lymphocytes of some Iraqi Patients with Acute Lymphocyte Leukemia

تقييم النوى الصغيرة ومعامل الانقسام النووي في الخلايا اللمفاوية لدم بعض المرضى العراقيين المصابين بسرطان الدم اللمفاوي الحاد

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Abstract

The aim of our study was to determine micronucleus MN frequency and nuclear division index in peripheral blood lymphocytes of patients diagnosed with Acute Lymphoblastic Leukemia ALL, who had undergone chemotherapy. Patients were treated with nine of drugs, which included Vincristine , Methotrexate , Cytosar-U, L-asparaginase , Etoposide, , dexamethasone (Decadron) , Indoxan , Steroids. The study was carried out on fifty Iraqi patients (34 Male, 16 Female), aged 2-70 years with Acute Lymphoblastic Leukemia ALL. These samples included 20 pretreatment aged 7-70 years, 15 under treatment aged 2-57years and 15 relapsed aged 9-40 years, compared with a sample consisted of 50 apparently healthy normal individuals collected randomly from population living in Baghdad aged 3-75 years. Results of the of MN in the human lymphocyte observed a significant increase $p<0.05$ in the males and females of human peripheral blood lymphocytes of patients with Acute Lymphoblastic Leukemia, before and after the chemotherapy as compared with the control. While, a significant decrease $P> 0.05$ in nucleic division index NDI was observed in the human peripheral blood lymphocytes of Acute Lymphoblastic Leukemia Patients (males and females), before and after the chemotherapy as compared with the control. In addition, the results of MN and NDI were compared in the genders of the groups studied. In conclusion, the results indicated that there is a possibility of using the changes in the mean of MN frequency and NDI as biomarkers for the assessment of DNA damage in the human peripheral blood lymphocytes of patients with Acute Lymphoblastic Leukemia ALL before and after the chemotherapy treatment and the increase frequencies of MN in ALL patients indicate the effect of antileukemic agents in inducing somatic genetic damage.

المستخلص

هدفت الدراسة الحالية إلى تحديد تردد النوى الصغيرة و معامل الانقسام النووي في الخلايا اللمفاوية لدم المرضى العراقيين المصابين بسرطان الدم اللمفاوي الحاد قبل وبعد العلاج الكيميائي ، اذ استخدم ت 9 أنواع من الأدوية الكيميائية. أجريت الدراسة على 50 مريضا (34 ذكر و16 أنثى)، بعمر 2-70سنة. وشملت 20 مريضا قبل المعالجة الكيميائية بعمر 7 – 70سنة ، 15 مريضا بعد المعالجة20 مريضا قبل المعالجة بعمر 7-70 سنة ، 15 مريضا بعد المعالجة الكيميائية بعمر 2- 57 سنة و15 مريضا بعد المعالجة الكيميائية في فترة الانتكاسه بعمر 9 – 40 سنة ، إضافة إلى مجموعة السيطرة والتي شملت 50 فرد بعمر 3- 75 سنة من بغداد. أظهرت النتائج وجود زيادة معنوية $p<0.05$ في معدل تردد النوى الصغيرة MN في الخلايا اللمفاوية ثنائية النواة لذكور وإناث مرضى ابيضاض الدم اللمفاوي الحاد قبل وبعد المعالجة الكيميائية مقارنة مع مجموعة السيطرة . بينما لوحظ انخفاض معنوي $p<0.05$

Key words: Micronuclei, Human Lymphocytes, Chemotherapy, Acute Lymphocyte Leukemia

في معامل الانقسام النووي NDI في الذكور والإناث لمرضى ابيضاض الدم الحاد قبل وبعد المعالجة الكيميائية مقارنة مع مجموعة السيطرة . كما أظهرت النتائج زياده معنويه في MN في الذكور والاناث للمرضى المصابين بمرض سرطان الدم اللمفاوي الحاد مقارنة مع مجموعة السيطره . نستدل من نتائج الدراسة إلى امكانية استخدام التغيرات في معدل النوى الصغيرة و معامل الانقسام النووي في الخلايا اللمفاوية لمرضى ابيضاض الدم الحاد بعد وقبل المعالجة الكيميائية كمؤشراً بايولوجياً يمكن استعماله في في تقدير الضرر في جزيئة الدنا لمرضى ابيضاض الدم الحاد وان الزيادة في تردد النوى الصغيرة دليل على ان العوامل المضادة للسرطان تسبب أضرار وراثية جسدية .

Introduction

In 1902, Theodor Boveri introduced a hypothesis mechanistically linking chromosomal abnormalities to the pathogenesis of cancer. Cancer is a genomic disease associated with accumulation of genetic damage [1].

Over the last decades, biomarker-based approaches have been applied in the exposure assessment of genotoxic agents and the increases of these biomarkers are considered early events, associated with disease-related changes. Assuming that the mechanisms for the induction of chromosomal damage are similar in different tissues, the extent of chromosomal damage evaluated in lymphocytes and other surrogate tissues is likely to reflect the level of damage in cancer-prone tissues and in turn cancer risk [2].

Micronuclei are formed from chromosomal fragments or lagging chromosomes at an anaphase due to mitotic spindle damage which are not included in the nuclei of the daughter cells Figure (1). They are therefore seen as distinctly separate objects within the cytoplasm of the daughter cells [3,4].

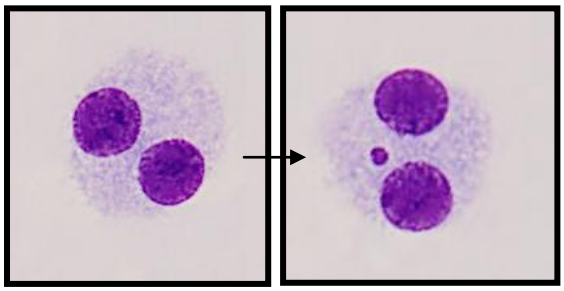
There is an increasing interest in the assessment of biological markers that detect the damage produced after cancer chemotherapy. As the target cells in determination of the correlation between exposure of cancer patients to antineoplastic drugs and/or radiation and alterations of DNA repair efficiency, peripheral blood lymphocytes are frequently used [5]. Therefore, we decided to investigate the effect of the mentioned drug on the frequency of micronuclei MN in peripheral blood lymphocytes of patients as a consequence of intra-tumoral application *in vivo*.

Since 1985, cytokinesis- block micronucleus test CBMN in human peripheral blood lymphocytes has been accepted by many laboratories as an optimal method for evaluation of genotoxic effect [6,7]. It is known that micronuclei `increase consequence of chromosomal damages in dividing cells [4], and that there is a positive correlation between these two biological endpoints [7]. Chemotherapy can result in a significant increase in MN frequencies in lymphocytes patients with acute lymphocytic leukemia in comparison to the levels before treatment and those in healthy controls [8,9].

The nuclear division index NDI is a marker of cell proliferation in cultures which is considered a measure of general cytotoxicity, the relative frequencies of the cells may be used to define cell cycles progression of the lymphocyte after mitogenic stimulation and how this has been affected by the exposure [10,11]. The hypothesis behind it, is that cells with greater chromosomal damage will either die before cell division or may be less likely to enter this phase [12,13]. The lowest NDI value is 1.0, which occurs if all of the viable cells have failed to divide during the cytokinesis-block period and so, all will be mononucleotide. If all viable cells complete one division there will be all binucleated, the

NDI value is 2.0. An NDI value can be greater than 2.0 if certain viable cells have completed more than one nuclear division during the cytokinesis-block phase and therefore contain more than two nuclei [14]

The aim of our study was to determine MN frequency and nuclear division index in peripheral blood lymphocytes of some Iraqi patients diagnosed with Acute Lymphoblastic Leukemia, before and after chemotherapy.



-A- -B-

Fig. (1): (A) Binucleated lymphocyte cell without micronuclei
(B) With 1 micronuclei (1000X).

Materials and Methods

Patients Studies

The study was carried out on fifty Iraqi patients 34 Male, 16 Female, age 2-70 years with Acute Lymphoblastic Leukemia. The clinically diagnosis of the patients has been made at a consultant medical staff in the following medical centers: The National Center of Hematology/ Al Mustansyria University; Central Child Hospital and Baghdad Teaching Hospital. These samples included 15 under treatment age 2-57 years, 15 relapsed age 9-40 years and 20 pretreatment age7-70 years, compared with a sample consisted of 50 apparently healthy individuals collected randomly from population living in Baghdad age 3-75 years.

Antineoplastic Drugs

In the course of the study, All patients were treated with antineoplastic drugs according to nine different chemotherapeutic protocols, mainly as Vincristine, methotrexate, Cytosar-U, L-Asparaginase, Etoposide, dexamethasone (Decadron), Indoxan and steroids.

Blood sampling

Five ml of human peripheral blood from all patients and control subjects were collected by venipuncture into heparinzed tubes during the period February 2011 till June 2012. The blood samples were placed in a cool - box under aseptic conditions and transfer to the laboratory.

Culture technique

The MN was performed as described by [4]. Under sterile conditions, 0.5ml of whole heparinized blood were cultured into tissue culture tube containing 4.5 ml RPMI- 1640 (sigma) with 20% fetal calf serum (sigma) and 0.2 mg/ml PHA-M (sigma). Cultures were incubated at 37°C for 72 hours, at 44 h of incubation cytochalasin B (cyto B, Sigma) in a final concentration of 4.5 µg/ml of the culture medium was added. Then, the incubation was completed and the cultures were harvested at 72 h of incubation.

Treatment with hypotonic solution and fixation

Cultures were centrifuged at 1500rpm for 10 min., the supernatant was carefully removed, and the cells were re-suspended in 10 ml of hypotonic solution 0.075 M KCl and incubated for 20 min at 37°C. Then, suspension was centrifuged at 1500 rpm for 10 min and the supernatant was discarded to harvest the cells. After removal of the supernatant, the pellet was fixed with freshly prepared 3:1 methanol/glacial acetic acid and centrifuged as described before. This procedure was repeated 4 times. All supernatant was removed and the pellet suspended in a few drops of freshly prepared fixative solution, dropped on clean slides and stained with Giemsa stain that was prepared according to [15,16]. The slides were stained for 2-3 min.

Scoring of micronuclei

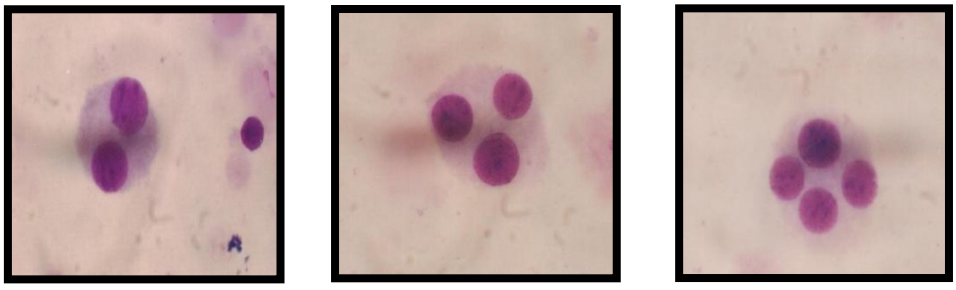
The analyses of MN were carried out on 1000 cytochalasin blocked binucleated lymphoblasts (CB cells) using 400 x magnification for surveying the slides while 1000 × magnification was used to confirm the presence or absence of MN in the cells.

Nuclear division index (NDI)

When scoring CB lymphocyte preparations one observes cells with 1, 2, 3, etc, main nuclei. One thousand stained cells are scored to determine the frequency of cells with (1,2,3,4) nuclei and the NDI is calculated to define cell cycles progression of the lymphocyte after mitogenic stimulation by using the formula [11]:

NDI = (M1 + 2 x M2 + 3 x M3 + 4 x M4)/N

Where M1 to M4 represent the number of cells with one to four nuclei and N is the total number of stained cells scored Figure(2).



A **B** **C**

Fig.(2): Cytokinesis blocked human lymphocyte cell, (A): Binucleated lymphocyte cell, (B): Trinucleated lymphocyte cell and (C): Qudrinucleated lymphocyte cell (1000X).

Data Analysis and Statistics

The data of this study were compiled into the computerized data file and frequency, distribution and statistical description Mean, SE was divided using SPSS statistical software. We used statistical analysis of variance ANOVA test and least significantly difference LDS test by probability of less than 0.05 ($p<0.05$) according to Duncan [17]. A program based on the U-test was employed to test the data for MN distribution.

Results and Discussions

CBMN test had been applied to investigate mutagenic effect of chemotherapy on the MN frequency in peripheral blood lymphocytes of 50 ALL Iraqi patients 34 Male, 16 Female, age 2-70 years before and after the chemotherapy. The result of MN/cell in peripheral

blood lymphocytes of patients diagnosed with Acute Lymphoblastic Leukemia (ALL), before and after the completed therapy and control group are shown in table1. A significant P< 0.05 increase in MN was observed in ALL patients (under treatment, relapse and pretreatment) as compared with the control. However, significant P< 0.05 variation in MN was observed in ALL patients under treatment, relapse and pretreatment. The average of MN per cell (Mean ± SE) for under treated, relapsed and pretreated ALL patients were 0.048 ± 0.007, 0.063 ± 0.0066, 0.009 ± 0.00056 MN/cell, respectively when compared with the control (0.001 ± 0.00016). The average of MN frequency was increased in under treated, and relapsed ALL patients when it was compared to the average of MN in the ALL patients before the therapy(pretreated) with the statistical significance of P< 0.05Table (1) .

Micronuclei in mitotically active cells arise from structural chromosomal aberrations or disturbed function of mitotic spindle. That is why some authors consider that the follow up of MN frequencies in peripheral blood lymphocytes in the samples of human individuals could be a very effective test to estimate the effects of biological, physical and chemical agents [17]. The MN assay is generally used to determine the *in vivo* genotoxicity of carcinogens in normal cells like human lymphocytes [18.19]. Titenko-Holland were studied micronucleus formation in human lymphocytes as a biomarker of genotoxicity both in vitro and in vivo [20]. Here we present our findings on the cytogenetic effects of chemotherapy on the MN frequencies in peripheral blood lymphocytes studied in vitro, using the micronucleus assay. Also, in the table 1 shown that the average of NDI (Mean ± SE) for undertreated, relapsed and pretreated ALL patients were 1.216 ± 0.0048, 1.440 ± 0.0009, 1.701 ± 0.0052, respectively when compared with the control (1.845 ± 0.0091). A significant (P> 0.05) decrease in NDI was observed in ALL patients under treated and relapsed ALL patients in comparison with pretreated ALL patients and control Table (1).

Table (1): Mean ± SE of MN /Cell and NDI for the acute lymphocyte leukemia patients and control group

Studies groups	No. of samples	Age (year) (Range) Mean ± SE	MN / Cells (Mean ± SE)	NDI (Mean ± SE)
All Patients after chemotherapy treatment (Under treatment)	15	(2-57) 20.3 ± 3.99	0.048 ± 0.007 A	1.216 ± 0.0048 a
All Patients after chemotherapy treatment Relapse)	15	(9 – 40) 18.4 ± 2.49	0.063 ± 0.0066 B	1.440±0.0009 B
All Patients before hemotherapy treatment (Pretreatment)	20	(7 -70) 20.8 ± 3.35	0.009± 0.00056 C	1.701 ± 0.0052 c d
Control(Baghdad)	50	(3- 75) 21.78 ± 1.69	0.001±0. 00016 D	1.845 ±0.0091 d

•Similar latter in a column (for comparison between studies groups) mean there is no significant difference p < 0.05, according to Duncan test.

The nuclear division index as biomarker of cell proliferation in cultures which is considered a measure of general cytotoxicity, the relative frequencies of the cells may be used to define cell cycles progression of the lymphocyte after mitogenic stimulation and how this has been affected by the exposure [10,11] .

Table (2) shown that the comparison between gender and MN frequency and NDI (Mean ± SE) in the acute lymphocyte leukemia patients and control group. The separation on genders shows that MN mean was significant P< 0.05 increase in the males of relapsed ALL patients as compared with females in this group and controls. Also, the MN showed no significant difference P> 0.05 between males and females in other groups of ALL patients, while, the separation on genders shows that MN mean is more significant p < 0.05 in females than in males of control group. Regarding NDI , statistical analysis showed that there was no significant difference P> 0.05 between males and females in each groups of ALL patients and control groups table(2) .

Table (2): Comparison between gender and MN frequency and NDI (Mean ± SE) in males and females for the acute lymphocyte leukemia patients and control group.

Studies groups	No. of samples	Age (year)	Gender	MN /Cell (Mean ± SE)	NDI (Mean ± SE)
ALL Patients after chemo-therapy treatment (Under treatment)	11	4-57	♂	0.047±0.0086	1.218 ± 0.0058
	4	2-53	♀	0.048 ± 0. 0060	1.211 ± 0.0091
ALL Patients after chemotherapy treatment (Relapse)	12	2-40	♂	0.062 ± 0.0075 *	1.431 ± 0.0096
	3	9-22	♀	0.044 ± 0.0097	1.468 ± 0.0153
ALL Patients before chemotherapy treatment (Pretreatment)	11	8-70	♂	0.0089± 0.00072	1.704 ±0.0070
	9	7-51	♀	0.0094±0. 0009	1.704 ± 0.0092
Control (Baghdad)	34	6-66	♂	0.0014± 0.0015	1.849 ±0.0099
	16	4 -51	♀	± 0.00033* 0.0020	1..849 ±0.0193

***Significant differences between genders in studies groups and control using t-test.**

When the control and the all groups were compared, the induced micronuclei show an increase in both genders; this effect is more pronounced in females. The findings of the present study are in agreement with those reported previously by [21, 22 .23] who have showed high micronucleus frequency in peripheral blood lymphocytes of untreated cancer patients irrespective of gender, smoking and cancer sites .Higher spontaneous mean MN frequencies were also observed in cancer male and females patients suggesting a higher background level of genetic instability [24,25]. Significant increased chromosomal damage in patients with all cancer types with a doubled MN frequency when compared with healthy controls, subjects at increased risk and cancer patients was observed in a molecular epidemiology case control study [26,27].

Table (3) shows that the distribution of the micronuclei among of cytokinesis lymphocytes block (CB) cells are over dispersed in the acute lymphocyte leukemia patients compared to control group using the *U* value (Test quality :an approximate to a unite normal deviate),

CB cells lymphocytes having one or more micronuclei . They were rendered evident according to the fenech [28] criteria.

Table (3): Distribution of micronuclei (Mean ± SE) in the lymphocytes of the acute lymphocyte leukemia patients and control group

Studies groups	MN Distribution(Mean ± SE)				No. of BN cells with MN (Mean ± SE)	*I*	*U*
	0MN	1MN	2MN	≥3MN			
ALL Patients after chemotherapy treatment (under-treatment)	953.13 ± 6.54	45.27 ± 5.89	0.87 ± 0.292	0.27 ± 0.145	46.4 ± 0.0070	1.19	3.21
ALL Patients after chemotherapy treatment (Relapse)	935.4 ± 5.00	62.87 ± 4.67	1.4 ± 0.24	0.33 ± 0.191	64.6 ± 0.0066	0.98	1.39
ALL Patients before chemotherapy treatment (Pretreatment)	991.5 ± 0.394	8.05 ± 0.293	0.35 ± 0.132	0.1 ± 0.069	8.5 ± 0.0 0056	1.23	2.20
Control (Baghdad)	998.52 ± 0.102	1.36 ± 0.101	0.12 ± 0.046	0.00 ± 0.00	1.48 ± 0.00016	1.85	14.06

***U*, test quality approximate to a unit normal deviate, I, dispersion index**

The Frequencies of MN and NDI in peripheral blood lymphocytes of males of patients with acute lymphocyte leukemia before and after chemotherapy treatment and control group are shown in Table (4). The average of MN frequency was significant P< 0.05increase in the males of ALL patients (under treatment, relapse and pretreatment) as compared with the males of control group. Also, the average of NDI was significant P> 0.05 decrease in the males of under treated, and relapsed ALL patients when compression to average of NDI in the ALL patients before the chemotherapy (pretreated) and control group, but the difference in the NDI average between ALL patients before the chemotherapy treatment (pretreated) and control group was not significant P> 0.05.

Table (4): MN Frequency and NDI (Mean ± SE) in the males for the acute lymphocyte leukemia patients and control group.

Studies groups	No. of samples	Age (year)	MN /Cell (Mean ± SE)	NDI (Mean ± SE)
ALL Patients after chemotherapy treatment (Under treatment)	11	4 -57	0.047±0.0086 A	1.218 ± 0.0058 a
ALL Patients after chemotherapy treatment (Relapse)	12	2-40	0.062 ± 0.0075 B	1.431 ± 0.0096 b
ALL Patients before chemotherapy treatment (Pretreatment)	11	8-70	0.0084 ± 0.00072 C	1.704 ± 0.0070 cd
Control (Baghdad)	34	6-66	0.0014 ± 0.0015 D	1.849 ±0.0099 d

•Similar latter in a column (for comparison between studies groups) mean there is no significant difference p < 0.05, according to Duncan test.

Table (4) shown that the average of MN frequency was significant higher P< 0.05 in the females of under treated, relapsed and pretreated ALL patients as compared with the females of control group. Also, the MN showed no significant difference P> 0.05 in females of under treated ALL patients compared with the females of relapsed ALL patients. Micronuclei frequency increases as the age increases naturally; there are well known ageing events which take place in the genetic material, too. As we could notice in our study, in males the process is going differently than in females, where the MN frequency is even more intense, the events taking place in direct connection with the exposure duration to the harmful environment.

Antileukemic agent produced a significant genetic damage, which was proved by the increased incidence of chromosomal aberration and MN formation in human as well as in animal model [29]. [31] Were reported the significantly higher MN frequencies in patients with ALL after the treatment with antileukemic agent such as vincristine, methotrexate, daunomycine, prednisone and asparaginase [30].

Table (5): MN Frequency and NDI (Mean ± SE) in the females for the acute lymphocyte leukemia patients and control group.

Studies groups	No. of samples	Age (year)	MN /Cell (Mean ± SE)	NDI (Mean ± SE)
ALL Patients after chemotherapy treatment (Under treatment)	4	2-53	0.048 ± 0. 0060 A	1.211 ± 0.0091 a
ALL Patients after chemotherapy treatment (Relapse)	3	9-22	0.044 ± 0.0097 AB	1.468 ± 0.0153a b
ALL Patients before chemotherapy treatment (Pretreatment)	9	7-51	0.0094± 0. 0009 C	1.704 ± 0.0092 c d
Control (Baghdad)	16	4-51	0.0020 ± 0.00033 D	1.849 ±0.0193 d

•Similar latter in a column (for comparison between studies groups) mean there is no significant difference (p < 0.05), according to Duncan test.

The average of NDI are shown in table (5), was significant P> 0.05 decrease in the females of under treated, relapsed and pretreated ALL patients as compared with the average of NDI in the females of control group, but NDI showed no different significant P> 0.05 in females of under treated ALL patients compared with the females of relapsed ALL patients table (5). This result is in agreement with studies mentioned previously [14,28]. Decrease in the NDI shows increased cytotoxicity of chemotherapy, the tested of NDI based on the fact that this marker estimates general toxicity [30,31,32]. The proportion of binucleated cells may be used as a biomarker of the lymphocytes mitogen response, immune functions and cytostatic effects of various studied agents [33].The explanations for this behavior may come from recently published data showing that NDI is significantly lower NDI in peripheral blood lymphocytes of patients with cancer before and after chemotherapy treatment.

Conclusions

These data clearly suggested the validity of the methodology in pointing out the role played by antileukemic agents in inducing somatic genetic damage. The results indicated that there is a possibility of using the changes in the mean of MN frequency and NDI as biomarkers for the assessment of DNA damage in the human peripheral blood lymphocytes of patients with Acute Lymphoblastic Leukemia.

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