Genetic Alterations in Iraqi Leukaemia Patients as Indicator for Polluted Environment

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ABSTRACT
Fifty blood samples of leukemia patients were analyzed to detect the genetic alterations associated with disease. The results showed a high significant DNA damage leukemia group. A significant (P< 0.05) increase in MN is observed in Acute Lymphoblastic Leukemia (ALL) patients (0.012 ± 0.00056, 0.014± 0.00049 MN/cell respectively) and decreased Nuclear Division Index (NDI) level (1.615 ± 0.0052, 1.597± 0.0039 respectively) when compared with control group (0.001 ± 0.00016; 1.845 ± 0.0091 respectively). DNA damage detected in Acute Lymphoblastic Leukemia (ALL) and Chronic Myeloid Leukemia (CML) patients with micronucleus (MN) is confirmed with statistically significantly higher COMET parameters (tail length =5.11±0.85, 5.66±0.91; % DNA mean in tail= 0.072 ± 0.041%, 0.082±0.039 and tail moment=0.00044 ± 6.87, 0.00052±5.99 respectively). The results indicated that the leukemia patients were exposed to a high genotoxicity which damaged the lymphocytes DNA.
INTRODUCTION

Environmental pollution in Iraq has been increased since the war with Iran in 1980. Since then the country has been moving from one war to another. These wars destroyed the Iraqi environment by distributing various carcinogens and mutagens that exist in the ammunitions used in these wars or from Iraqi chemical destroyed targets (Al-Azawi, 2006; Alaani, et al., 2011). The consequences and damages of these toxic hazards among Iraqi environments and peoples are varied according to the type of toxic hazard and took long time to appear. The extent of damage was detected to be vast and has affected ever aspect of the Iraqi environment (Habib et al., 2007; Al-Humadi, 2009; AL-Hashimi and Wang, 2013).

This ranges from the land destruction, water pollution as well as air pollution and finally depletion of the forests which is also a major environmental component (Buffler et al., 2005). Among the consequences of exposing to the toxic hazards, cancer was detected to be arisen among Iraqi population (Iraqi Cancer Board, 2012). The total number of malignant cases which were registered from 1995 to 1999 reached to more than 2500 cases but the number increased to 10,888 in the year 2000 (The Ministry of Health, 2004).

Leukemia was the fifth commonest cancer in 1998 responsible for 5.8% of all tumors (ranked seventh in 1989) (The Ministry of Health, 2004). The number and proportion of leukemia cases in the southern, middle and north governorates were increased since 1993. For example in Basra, leukemia constituted 8.5%, 9.1%, 8.4%, and 9.2% in 1993, 1995, 1997, and 1998 respectively compared to 5.4% in 1989, Maysan are 6.2%, 7.4%, 14.3% and 13% compared with 4.5% in 1989 (Iraq Cancer Board, 2000; Habib et al., 2007; WHO, 2008c), Mousel is 7.6% of all the types of cancer in 2010, while its 2.53% in 1980. It ranks eighth to ninth in term of all cancers during the period (1980-2000) and its rank third after breast and lung cancer during (2001-2010) and the average number of leukemia incidence cases diagnosed each year during (1980-2010) increased from (0.4) to (8) (AL-Hashimi, 2013) and Kurdistan, hematological malignancies are the most frequent cancer in males (21.13% of all cancer in males) and followed breast cancer in female population (18.8% of all cancer in female) (Othman et al., 2011).

However, overall, the proportion of leukemia cases was 5.8%, 6.5%, and 3.4% of all cancers in 1998, 1999, and 2000 respectively (Iraq Cancer Board, 2000). Currently, we are facing about 140,000 cases of cancer, with 7000 to 8000 new ones registered each year. More than 24% of diseases including cancers are estimated to be caused by toxic hazards (Lifang et al., 2012). Growing evidence suggests that these diseases arisen via genetic alterations of some genome regions. Leukemia is a cancer of the blood-forming system and has been defined as the uncontrolled proliferation of hematopoietic cells that have lost the capacity to differentiate normally to mature blood cells (Sawyers et al., 1991). It arises when an immature blood cell in the bone marrow (progenitor cell) develops uncontrollably and suppresses the production of healthy blood cells. The unregulated proliferating cells usually replace normal marrow, interfere with normal marrow function, may invade other organs and eventually cause death if untreated (Meng, 2001).

Leukemia is caused by several types of genetic alterations including structural alterations in the genetic material that resulting in mutations, activation of proto-oncogenes, tumor suppressor genes or chromosomal abnormalities (Karapetis et al., 2008; Case et al., 2008; Baldus et al., 2009; Lin et al., 2012).
The current study aimed to detect DNA damages associated with leukaemia as indicator for Iraqi polluted environment.

Materials and Methods Subjects
Requirements of research had been done for the samples under study in the molecular genetics laboratory at the Institute of Genetic Engineering and Biotechnology for Higher Studies / the University of Baghdad.
Five ml of blood has been collected by vein puncture from 35 acute lymphoblastic leukemia-ALL (2-70 yrs, 12 female +23 male), 15 chronic myeloid leukemia-CML (40-70 yrs,9 female +6 male) patients who were admitted to the Centre of Hematology / Yarmouk Hospital from February 2011 till September 2012. Thirty apparently healthy individuals (3-75 years, 14 female + 16 male) had been selected to be as control group. Patients and healthy were from Arab ethnic group.

Micronucleus Assay
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 resuspended 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, spread on clean slides and stained with Giemsa stain . 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 x 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 or 4 nuclei and the NDI is calculated by using the formula :
\[ NDI = \frac{(M1 + 2 \times M2 + 3 \times M3 + 4 \times M4)}{N} \]
where M1 to M4 represent the number of cells with one to four nuclei and N is the total number of stained cells scored.

Comet Assay
Comet assay was performed according to Bagdonas and Lazutka, 2007. Slides were stained with Ethedium bromid and examined with 40X objective of fluorescent...
microscope. 50 randomly selected cells are analysed per sample. Comet images were analysed using comet 5 image analysis software linked to a digital camera to quantify the DNA damage, three different parameters were evaluated: the tail length (TL), % DNA Mean in Tail and the tail moment (TM).

**Statistical Analysis**

The Statistical Analysis System- SAS (2010) was used for some analysis and students T-test at the comparative between means in this study.

**Results and Discussion Micronucleus**

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. Therefore seen as distinctly separate objects within the cytoplasm of the daughter cells (Counteryman and Heddle, 1976; Fenech and Morley, 1985). A significant (P< 0.05) increase in MN is observed in ALL and CML patients as compared with the control. The average of MN per cell (Mean ± SE) for ALL and CML patients are 0.012 ± 0.00056, 0.014 ± 0.00049 respectively MN / cell when compared with control group (0.001 ± 0.00016). The NDI is calculated via binucleated, trinucleated and quadrinucleated lymphocyte cell per 1000 lymphocytes. Table 1 shows that the average of NDI (Mean ± SE) is significantly (P< 0.05) decreased in ALL and CML patients (1.615 ± 0.0052, 1.597 ± 0.0039 respectively) when compared with the control (1.845 ± 0.0091) (Table 1) (Figure 1).

**Table (1): Mean ± SE of micronucleus(MN) /cell and nuclear division index(NDI) for the acute lymphocyte leukemia patients and control group.**

<table>
<thead>
<tr>
<th>Studied groups</th>
<th>No. of samples</th>
<th>Age (year) (Range ) Mean ± SE</th>
<th>Cells /MN (Mean ± SE)</th>
<th>NDI (Mean ± SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALL Patients</td>
<td>35</td>
<td>(7 -70 ) 18.8 ±4.12</td>
<td>0.012 ± 0.00056 A</td>
<td>1.615±0.0052 D</td>
</tr>
<tr>
<td>CML Patients</td>
<td>15</td>
<td>(40-70) 44.4 ± 6.23</td>
<td>0.014 ± 0.00049 A</td>
<td>1.597 ± 0.0039 D</td>
</tr>
<tr>
<td>Control</td>
<td>30</td>
<td>(3-75) 21.78 ±1.69</td>
<td>0.001±0. 00016 B</td>
<td>1.845 ±0.0091 C</td>
</tr>
</tbody>
</table>
These results showed that lymphocytes of leukemia patients are mitotically active with DNA damage which indicated the genotoxicity to the environment toxic agents. Such human lymphocytes genotoxicity was also reported by others who found that MN and NDI parameters are good biomarkers to estimate the toxic effect of biological, physical and chemical agents (Fenech, 1997; Iarmarcovai et al., 2008; Fenech and Bonassi, 2010; Milesovic-Djordievic et al., 2010). Titenko-Holland et al. (1997) studied micronucleus formation in human lymphocytes as a biomarker of genotoxicity both in vitro and in vivo. The findings show that the cytogenetic effects and response to chemotherapy on the MN frequencies in peripheral blood lymphocytes studied in vitro, using the micronucleus assay. Also, the NDI assay is performed for the same purpose according to the description of Eastmond and Turcker (1989b). The nuclear division index, a biomarker of cell proliferation in cultures, is considered a measure of general cytotoxicity. The relative frequencies of the cells may be used to define cell cycle progression of the lymphocyte after mitogenic stimulation, and it is affected by the exposure (Eastmond and Turcker, 1989b and Fenech, 2000). Higher spontaneous mean MN frequencies are observed in cancer patients suggesting a higher background level of genetic instability (Guler et al., 2005; El-Zein et al., 2006). Thus, significant increase of 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 is observed in a molecular epidemiology case control study (Bolognesi et al., 2002; Bolognesi et al., 2005). This result is in agreement with studies mentioned previously (Fenech et al., 2003; Bolognesi et al., 2005; Fenech, 2007). Decrease in the NDI shows increased cytotoxicity of chemotherapy. The NDI test is based on the fact that this marker estimates general toxicity (Eastmond and Turcker, 1989a; El-Zein et al., 2006). The explanations for this behaviour may come from recently published data showing that NDI is significantly lower in peripheral blood lymphocytes of patients with cancer after chemotherapy treatment (Ionescu et al., 2011). Assessment of DNA Damage Using the Comet Assay

The comet assay is a good technique to evaluate the genotoxic effects of various environmental hazard agents on human. The results of the comet assay in patients and healthy control group are summarized in the table 2. The mean tail length, % DNA tail mean and tail moment of ALL patients showed increased significant levels (P < 0.01) as compared with control (Figure 2). The mean of tail length (Mean ± SE) of ALL and CML patients and control group are 5.11±0.85; 5.66±0.91 and 2.95±0.44, respectively. The % DNA mean in tail (Mean ± SE) of ALL and CML patients and controls are 0.072±0.041%; 0.082±0.039 and 0.017±0.0055%, respectively. There is a significant increase in the % DNA Mean in tail of ALL and CML patients. DNA damage is statistically significant increasing in patients for tail moment (Mean ± SE) 0.00044±6.87; 0.00052±5.99 than in control subjects 0.000142±4.6. Most toxic hazards produce immediate toxicities. Kopjar et al., (2002) used the comet assay to evaluate the genotoxicity of different types of chemicals used as drugs towards human peripheral lymphocytes they found that high toxicity was detected among exposed cells as compared with control group. Such toxicity was also detected in ALL patients treated with these chemicals (Halder et al., 2002). High base line level of DNA damage may also point to an impaired
DNA-repair ability among cancer patients, the probability is that the neoplastic disease itself is associated with increased DNA damage or that these patients have a more fragile DNA than healthy individuals (Çeçener et al., 1998; Kopjar et al., 2006; Ursini et al., 2006).

Table (2): Comet assay results for patients and control.

<table>
<thead>
<tr>
<th>Studied groups</th>
<th>No. of samples</th>
<th>Comet parameters evaluation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Tail length (px) (Mean ± SE)</td>
</tr>
<tr>
<td>ALL Patients</td>
<td>35</td>
<td>5.11±0.85 B</td>
</tr>
<tr>
<td>CML Patients</td>
<td>15</td>
<td>5.66±0.91 B</td>
</tr>
<tr>
<td>Control</td>
<td>30</td>
<td>2.95 ±0.44 A</td>
</tr>
</tbody>
</table>

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

Figure (2) : comet images by fluorescent microscope (400X). (A) Undamaged DNA (B) Damaged DNA.

REFERENCES
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