

In vitro chromosomal radiosensitivity in patients with common variable immunodeficiency

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Abstract

Common variable immunodeficiency (CVID) is one of the predominant antibody deficiency disorders, some evidence of which indicates that chromosome instability is present in these patients. An increased risk of cancer in patients with CVID has been documented. This study was undertaken to highlight radiation sensitivity in CVID patients and to clarify the genetic basis of this defect in these cases. Stimulated lymphocytes of the studied subjects were exposed to low-dose gamma-rays in the G2 phase or the G0 phase of the cell cycle and chromosomal aberrations were scored. Lymphocytes of healthy individuals, ataxia telangiectasia (AT) cases and a group of acute lymphoblastic leukemia (ALL) patients were investigated in the same way as controls. By two methods of analysis (one-way ANOVA and unpaired t-test), the CVID cases were significantly more radiosensitive than healthy controls based on the results of the G2 and the G0 assays. First-degree relatives of CVID patients were radiosensitive by the micronucleus assay which showed a significant difference as compared with normal controls ($p = 0.001$). In conclusion, this study may support that chromosomal radiosensitivity in CVID patients is a marker of genetic predisposition to the disease. The results might be a clue to describe the increased risk of cancer in CVID patients.

Key words: primary immunodeficiency, common variable immunodeficiency, chromosome radiosensitivity, acute lymphoblastic leukemia, ataxia telangiectasia.

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Introduction

Common variable immunodeficiency (CVID) constitutes the largest group of primary hypogammaglobulinemias [1, 2], manifested by low levels of serum immunoglobulins (Igs) and a wide variety of clinical signs and symptoms such as chronic and recurrent infections (mainly occurring in the respiratory and gastrointestinal tracts), autoimmune diseases and granulomatous lesions [3-7]. CVID patients also are at increased risk of different types of lymphoid malignancies [2, 5]. The malignancy phenotype in

CVID patients may associate with a state of immune deregulation characterized by various functional abnormalities of both B cells and T cells [8, 9]. Furthermore, impaired cellular immune response such as a decreased number and proportion of different lymphocyte populations, non-responsiveness of lymphocytes to mitogens and antigens, altered levels of cytokine production, and deficient expression of cell-surface molecules may be present in CVID patients [10]. The higher incidence of cancer in CVID cases has also

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been explained by genomic instability, manifested by an increased level of chromosomal damage after suitable mutagenic stress *in vitro* [11, 12].

Acute lymphoblastic leukemia (ALL), as a cancer disorder, is known by clonal proliferation, decreased apoptosis and accumulation of immature lymphoid cells, which is arrested at various differentiation stages within the bone marrow and lymphoid tissues [13, 14]. These patients usually have high white blood cell counts and may present with organomegaly, particularly mediastinal lymph nodes enlargement and central nervous system involvement [15]. Since chromosomal defects and molecular abnormalities in ALL patients have been identified [16], we considered ALL patients as a control group to compare radiation-induced chromosomal damage in CVID patients with ALL cases.

This study was carried out to explore radiation sensitivity in CVID patients and their first-degree relatives. This might elucidate the genetic basis of this primary immunodeficiency in these cases.

Material and methods

Study subjects

The study population consisted of 30 CVID patients registered in the Children's Medical Center Hospital affiliated by the Tehran University of Medical Sciences, which serves as a referral center for both adult and pediatric patients with primary immunodeficiency diseases in Iran [17, 18]. The study was performed between January 2007 and October 2011. Diagnosis of CVID was based on the criteria of the European Society for Immunodeficiency (ESID) and the Pan-American Group for Immunodeficiency (PAGID) in patients older than 4 years including decreases in serum IgG, IgA, and/or IgM levels by 2 or more standard deviations from the mean and absence of other well-defined antibody deficiencies [19, 20]. These patients were selected from all available CVID patients according to inclusion criteria including receiving regular intravenous immunoglobulin (IVIG) monthly and no history of smoking and alcohol exposure. Thirty age- and sex- matched healthy individuals served as negative controls, 24 first-degree relatives of CVID patients and 20 ALL patients were also recruited in this study. Moreover, samples obtained from six confirmed ataxia telangiectasia cases by mutation analysis were used as positive controls. Radiosensitivity is a major hallmark of the AT patients [21]. The process of this study was approved by the ethical committee of the Tehran University of Medical Sciences and all patients or their parents or legal guardians were asked to fill an informed consent form.

Cytogenetic methods

Experimental protocol for the G_2 assay: G_2 chromosomal radiosensitivity assay was performed essentially as described by Scott *et al.* [22] with a minor modification.

Prior to culturing, heparinized blood samples from all participants were kept within 4 hours at ambient temperature. For each blood sample, two tissue culture flasks were set up: one for *in vitro* γ -irradiation, the other served as control (un-irradiated) for analysis of the spontaneous chromosomal aberrations. To each flask, 0.5 ml of the blood was added in 4.5 RPMI-1640 culture medium supplemented with 10% fetal calf serum, 1% L-glutamine, 100 U/ml penicillin and 100 μ g/ml streptomycin. Phytohemagglutinin or PHA (Life Technologies GmbH, Frankfurt, Germany) at a final concentration of 1 μ g/ml was used to induce lymphocyte proliferation. The flasks were incubated in a humidified air atmosphere at 37°C with 5% CO_2 for 4 days. Four hours before harvesting, the cultures were exposed to gamma irradiation (Theratron 780e, MDS, Canada; ^{60}Co , 70cGy/min) with a dose of 100 cGy at ambient temperature. After 2-hour incubation, colcemid (Gibco, final concentration 0.15 μ g/ml in the medium) was added to arrest the cells at metaphase. Material of each flask was transferred to a centrifuge tube, and then centrifuged at 1200 RPM for 10 minutes to harvest lymphocytes. The supernatant was then removed and cells were treated with 5 ml of 0.075 M KCl for 15 minutes. After further centrifugation, the KCl was removed and the cell suspensions were fixed with fresh fixative (methanol/glacial acetic acid; 3/1) and this process was performed two more times. The cells in suspension were dropped on to clean coded slides. The slides were dried in air and stained with 2% Giemsa (in phosphate buffer saline, pH 7.0) for 5 minutes. Duplicate slides were made for each sample. For structural chromosome aberration study, 100 well-spread metaphases of both irradiated and non-irradiated samples were scanned and scored for aberrations such as chromatid breaks, gaps and exchanges, and chromosome breaks and fragments (Fig. 1). Two genetic experts scored each slide to eliminate scorer bias.

Experimental protocol for G_0 -micronucleus assay

Full details are given elsewhere [23], briefly, two tissue culture flasks were prepared and set based on a previous explained method of G_2 assay. One of the flasks of each donor was exposed to gamma rays uniformly, total dose of 300 cGy (Theratron 780e, MDS, Canada; ^{60}Co , 70cGy/min) at ambient temperature. Lymphocytes were stimulated to proliferate with PHA (final concentration of 1 μ g/ml). The flasks were incubated at 37°C (with 5% CO_2). Forty four hours later, cytochalasin B (Sigma) was added with a final concentration of 6 μ g/ml. After further incubation, cells were harvested at 92 hours post-stimulation by hypotonic shock with 0.075 M KCl, followed by fixation, three times, in methanol/acetic acid (3:1) solution. Slides were stained in 2% Giemsa for 5 min. Duplicate slides were made for each sample. For analysis, 500 binucleate cells (BNCs) per slide were scored for the number of micronucleus (MN) formation based on the international

standard protocol [24]. In total, 1000 binucleate cells were scored per sample. The number of BNCs containing two or three MN was also recorded.

Statistical analysis

The data were analyzed using SPSS statistical software (version 17.0). For each participant, frequency and percentage of chromatid or chromosome type aberrations in 100 metaphases were calculated as well as micronuclei per thousand binucleate lymphocytes. For each sample, the spontaneous yield of each aberration obtained from the non-irradiated flask was subtracted from the yield in irradiated cells to get the net radio-induced chromosomal aberration yield. The total number of chromatid gaps and breaks per 100 metaphases was also determined to give a ‘G2 score’. Different groups were compared using one-way ANOVA and unpaired *t*-test. Correlation between the G₂ and the G₀-micronucleus assays was analyzed by Pearson correlation coefficient test.

Results

Thirty patients (21 males, 9 females) with mean age of 17.4 ±10.7 years were evaluated. The mean age at onset and the mean age at diagnosis were 6.4 ±5.2 and 11.7 ±9.3 years, respectively (Table 1). The mean concentration of Igs (IgG: 175.8 ±165.3; IgA: 14.5 ±21.4; IgM: 20.6 ±16.5; all in µg/dl) and CD marker percentages (CD3:69.6 ±17.5; CD4:30.8 ±12.7; CD8:38.6 ±11.9; CD19:13.39 ±4.3; all in % of lymphocytes) were recorded. The parents of twenty two patients (73.3%) had consanguineous marriages. The mean G2 score for CVID patients (81.2 ±19.6) was significantly higher than that for the healthy control group (60.7 ±18.8, *p* = 0.001) (Table 2). The mean frequency of micronucleus yield per 1000 binucleate cells in CVID patients (71.4 ±24.6) was also significantly higher than that observed in healthy controls (17.3 ±5.9, *p* = 0.001) (Table 2). There was no significant difference in G2 score between first-degree relatives of CVID patients and healthy controls (*p* = 0.83), however there was a significant difference in micronucleus yield between CVID relatives and healthy

controls (45.9 ±19.6 vs. 17.3 ±5.9, *p* = 0.001) (Table 2). The number of CVID patients with parental consanguinity was higher than the number of those with non-consanguineous parents (22 vs. 8), though, there was no significant difference between CVID patients with parental consanguinity versus cases from non-consanguineous parents in either G2- or G0- type aberrations (Mean G2 Score: 81.4 ±22.2 vs. 80.6 ±10.1; Mean micronuclei yield: 71.7 ±21.7 vs. 70.5 ±32.9). ALL patients showed more sensitivity to radiation by G2 score or micronucleus formation comparing to healthy controls (*p* = 0.02, *p* = 0.001 respectively) (Table 2). There was no significant variation in G2 score or micronucleus yield between CVID patients and ALL cases. The correlation between G2 score and micronucleus yield in either CVID patients or in the healthy control group was not significant (*r* = 0.28, *p* = 0.12, *r* = 0.27, *p* = 0.14, respectively, Fig. 2). More information about distribution and frequency of chromosomal aberrations for CVID patients and other groups are presented in Table 2. Further analysis fails to show any correlation between radiosensitivity and early or late onset of the disease or any type of clinical phenotyping.

Discussion

There are several primary immunodeficiency disorders particularly ataxia telangiectasia and ligase IV deficiency, which are susceptible to conventional doses of radiation therapy. Moreover, other genetic diseases including Nijmegen breakage syndrome, Mre11 deficiency, and Fanconi’s anemia have been predictable for clinical radiosensitivity [25]. The higher incidence of chromosomal alterations after radiation has been demonstrated in the results of DNA repair deficiencies. Indeed, individuals with DNA repair deficiency may underlie the elevated cancer prevalence [26]. In this study we highlight chromosomal radiosensitivity in CVID patients with a greater sample size. We also recruit first-degree relatives of the patients to perceive their sensitivity in exposure to radiation compared with their affected child. In this investigation, radiation sensitivity was measured with both the G₂ and the G₀ – micronucleus assays to ensure presence or absence of radiosensitivity in the studied

Table 1. Characteristics of common variable immunodeficiency patients, their first-degree relatives and two different control groups of patients

| Group | Number | Sex (M/F) | Mean age (SD) | Number of metaphases examined | Mean percentage of aberrant metaphase (SD) |
|------------------|--------|-----------|---------------|-------------------------------|--|
| Healthy controls | 30 | 21/9 | 17.5 (9.3) | 3000 | 15.6 (13.9) |
| CVID patients | 30 | 21/9 | 17.4 (10.7) | 3000 | 20.4 (16.0) |
| CVID relatives | 24 | 11/13 | 42.0 (14.6) | 2400 | 17.0 (14.29) |
| ALL patients | 20 | 18/2 | 15.3 (2.7) | 2000 | 29.2 (23.2) |
| AT patients | 6 | 2/4 | 14.2 (7.2) | 600 | 22.2 (18.2) |

CVID – common variable immunodeficiency, ALL – acute lymphocytic leukemia, AT – ataxia telangiectasia, SD – standard deviation

Table 2. Details of G₂ and G₀ chromosomal aberration frequencies in irradiated peripheral blood lymphocytes of common variable immunodeficiency patients comparing with 4 different control groups

| Parameter | Healthy controls (n = 30) | CVID (n = 30) | CVID-relatives (n = 24) | ALL (n = 20) | AT (n = 6) | p-value ^a |
|--|------------------------------|--|----------------------------|-------------------------------|-----------------|----------------------|
| Chromatid breaks | | | | | | |
| Mean (SD) | 23.5 (9.5) | 33.3 (10.5) | 23.7 (9.4) | 33.50 (16.1) | 50.0 (12.2) | 0.001 |
| p-value ^b | – | Healthy (0.001) Relatives (0.001) AT (0.002) | Healthy (0.931) | Healthy (0.020) AT (0.031) | Healthy (0.002) | – |
| Chromatid gaps | | | | | | |
| Mean (SD) | 37.20(11.5) | 47.8 (10.0) | 38.1 (13.4) | 46.8 (16.8) | 70.1 (14.1) | 0.001 |
| p -value ^b | – | Healthy (0.001) Relatives (0.003) AT (0.001) | Healthy (0.783) | Healthy (0.033) AT (0.005) | Healthy (0.001) | – |
| G2 score (chromatid breaks + chromatid gaps) | | | | | | |
| Mean (SD) | 60.7 (18.8) | 81.2 (19.6) | 61.8 (21.6) | 80.3 (32.6) | 120.2 (21.7) | 0.001 |
| p-value ^b | – | Healthy (0.001) Relatives (0.001) AT (0.001) | Healthy (0.83) | Healthy (0.022) AT (0.01) | Healthy (0.001) | – |
| Chromosome breaks | | | | | | |
| Mean (SD) | 18.3 (10.7) | 22.5 (9.9) | 19.2 (8.4) | 27.0 (12.0) | 33.0 (7.4) | 0.002 |
| p-value ^b | – | Healthy (0.118) Relatives (0.181) AT (0.020) | Healthy (0.710) | Healthy (0.010) AT (0.262) | Healthy (0.002) | – |
| Chromosome gaps | | | | | | |
| Mean (SD) | 16.6 (7.2) | 21.4 (7.3) | 19.8 (7.7) | 23.7 (15.1) | 19.3 (7.5) | 0.104 |
| Fragmentations | | | | | | |
| Mean (SD) | 9.5 (4.1) | 11.7 (3.2) | 10.6 (5.1) | 12.4 (7.1) | 15.5 (3.1) | 0.037 |
| p-value ^b | – | Healthy (0.026) Relatives (0.324) AT (0.013) | Healthy (0.382) | Healthy (0.109) AT (0.151) | Healthy (0.002) | – |
| Exchange | | | | | | |
| Median (IQR) ^c | 1 (3-0) | 4 (5.2-3) | 2 (4.7-1) | 4 (9.7-3) | 5 (9-3.7) | – |
| p-value ^b | – | – | – | – | – | – |
| Micronucleus | | | | | | |
| Mean (SD) | 17.3 (5.9) | 71.4 (24.6) | 45.9 (19.6) | 73.9 (21.1) | 96.5 (13.7) | 0.001 |
| p-value ^b | – | Healthy (0.001) Relatives (0.001) AT (0.022) | Healthy (0.001) | Healthy (0.001) AT (0.022) | Healthy (0.001) | – |

ALL – acute lymphocytic leukemia, AT – ataxia telangiectasia, CVID – common variable immunodeficiency, SD – standard deviation

^a – ANOVA test, ^b – t-test; ^c – interquartile range (Q₃-Q₁)

individuals as the involved low penetrance genes in radio-sensitivity in G₀ and G₂ stages of cell cycles are different.

In this study, the data showed that the mean G2 score and the mean frequency of micronucleus yield were significantly higher in CVID patients than those in healthy controls. There are few reports which indicate the evidence of radiosensitivity of DNA in CVID cases after exposure to

radiation [27-29] or chemical agents [12] and its involvement with malignancy progress [13, 30, 31]. In our previous study we explained the dose-dependent chromosomal instability in CVID cases [27], however this property is not exhibited in other primary immunodeficiency syndromes [25]. Although, Vorechovsky *et al.* indicated the enhancement of chromosome damage in CVID cases, but they have

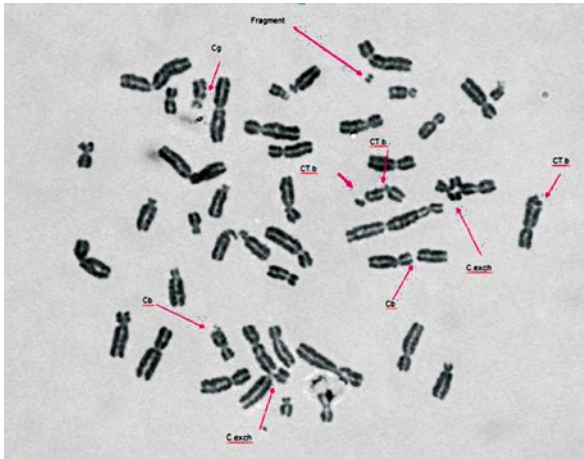


Fig. 1. Metaphase spread in peripheral blood lymphocytes after low dose rate γ -radiation exposure from a common variable immunodeficiency patient with a significant number of chromatid breaks, chromosome breaks and chromosome exchange noted. CT.b – chromatid break; Cb – chromosome break; Cg – chromosome gap; C.exch – chromosome exchange

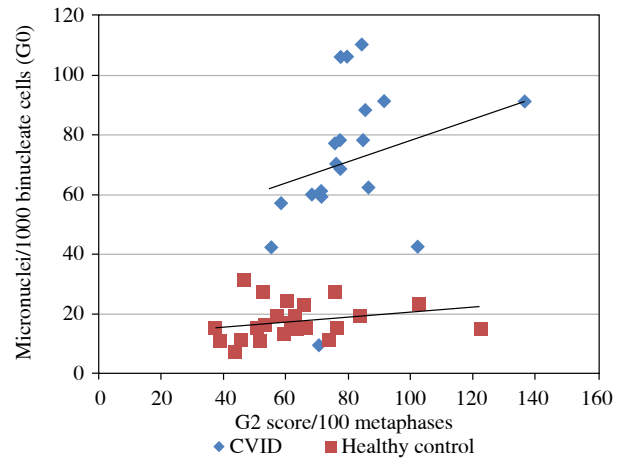


Fig. 2. Correlation between G2 score (chromatid breaks + chromatid gaps) and micronucleus yield in peripheral blood lymphocytes of common variable immunodeficiency (CVID) patients or of a healthy control group after low dose rate exposure of γ -irradiation. A poor correlation was found between the G2 and the G0 assays in both CVID patients and healthy control groups ($r = 0.28$, $p = 0.12$ and $r = 0.27$, $p = 0.14$, respectively)

considered total aberrations per cell as an indicator of radiosensitivity and did not study each aberration separately [28]. However, in the present study, we evaluated both G₂ chromosomal aberrations and G₀-micronucleus formation to demonstrate radiosensitivity in CVID patients. This indicates that radiation in CVID patients potentially leads to elevation of chromosomal aberrations. Patients with CVID suffer from a number of infections, and therefore undergo frequent medical imaging that exposes them to radiation. Since these patients might be sensitive to radiation, they should be protected from unnecessary medical techniques that incorporate radiation.

Results of this study showed that first-degree relatives of CVID patients were radiosensitive with the micronucleus assay which showed a significant difference as compared with normal controls. As the genes involved in the processing of radiation induced DNA damage in G₀ and G₂ phases of the cell cycle are different [32, 33], therefore our results might point to the defects in genes involved in the processing of radiation-induced DNA damage of G₀ phase in first-degree relatives of CVID patients. It is also reported that in breast cancer patients, chromosomal radiosensitivity is most evident using the micronucleus assay after exposure of lymphocytes with low dose rate radiation [32, 34].

The impact of these findings could be greatly improved by identifying the underlying genetic cause of the radiation sensitivity in CVID patients and potentially their first-degree relatives. Presumably not all CVID patients are radiation sensitive, since not all cases are due to disruption of DNA repair machinery, few cases might be due to receptor or downstream signaling dysfunction. It would also stand

to reason that not all family members carried risk variants for CVID. In surveys conducted in previous studies, we did not find a report on evaluation of radiosensitivity in first-degree relatives of CVID patients to compare our results with that. Altogether these findings support the theory of the autosomal recessive pattern of inheritance in selected cases with sensitivity to radiation [30]. Furthermore, we tried to compare the results of CVID patients and ALL cases to investigate chromosomal sensitivity of CVID patients compared with ALL as a malignancy originated from lymphocytes. We observed a higher frequency of G₂ chromatid type aberrations and micronucleus yield in CVID as well as in ALL patients. It is reported that the frequency of chromosomal aberrations in ALL cases were statistically higher than that of normal controls, as stated that 65% of the ALL patients were sensitive to γ -irradiation [35, 36]. Similar data between two groups indicate that high radiosensitivity in CVID may lead to later malignancies as our patients are mainly pediatric. An increased risk of cancer with an estimated incidence of 11-13%, particularly during the 5th and 6th decade of life in CVID patients has been reported [37]. It has also been demonstrated that the frequency of chromatid breaks and gaps is higher in individuals with genetic conditions predisposing to cancer [38].

Conclusions

Our data showed that the same percentage of patients with ALL and CVID were sensitive to radiation, meaning that maximum care should be taken during their diagnosis or treatments with unnecessary medical techniques that in-

corporate radiation. Furthermore, a homogenous group of patients with radiosensitivity may candidate for the same genetic defect involved in the upstream of class switching process. Further detailed investigations in CVID patients to characterize genome mutations or sites of double strand breaks are needed.

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The authors declare no conflicts of interest.

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