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ARAŞTIRMA RESEARCH ARTICLE

Incidence of micronucleus in primary immunodeficiency diseases: detection by micronucleus and FISH techniques

Primer immünyetmezlikli hastalarda mikronükleus sıklığının mikronükleus yöntemi ve FISH tekniği ile değerlendirilmesi

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ABSTRACT

Objective: Micronucleus (MN) assay is considered as a useful and fast screening test to determine MN frequency indicating the chromosomal damage and instability. The aim of this study was to determine MN formation by using MN assay and fluorescence in situ hybridization (FISH) technique in patients with primary immunodeficiency (PID).

Materials and Methods: This study included fifteen patients with PID (5 with ataxia telangiectasia, 5 with common variable immunodeficiency, 3 with severe combined immunodeficiency and 2 with agammaglobulinemia) and 15 healthy age-matched children.

Results: There was a significant increased MN frequency in cultured peripheral blood lymphocytes with Cyt-B in all patients ($p< 0.05$) when compared with the control group. When we compared the MN frequency among patients, the incidence of micronuclei (MNs) was found to be similar in cellular and humoral immunodeficiencies (IDs). Howe-

ÖZET

Giriş: Mikronükleus (MN) değerlendirmesi, kromozom hasarı ve instabilitesini tanımlamada kullanılan yararlı ve hızlı bir tarama testi olarak kabul edilmektedir. Bu çalışmanın amacı primer immünyetmezlikli hastalarda MN oluşumunu, MN yöntemi ve floresan in situ hibridizasyon (FISH) tekniğini kullanarak değerlendirmektir.

Gereç ve Yöntem: Çalışmaya 15 immünyetmezlikli hasta (5 ataksi telenjiektazi, 5 yaygın değişken immünyetmezlik, 3 ağır kombine immünyetmezlik ve 2 agammaglobulinemi) ile yaşları uygun 15 sağlıklı çocuk kontrol grubu olarak alındı.

Bulgular: Primer immünyetmezlikli hastaların sitoklasin-B ile kültüre edilen periferik kan lenfositlerindeki MN oluşumu, kontrol grubu ile karşılaştırıldığında önemli derecede yüksek bulundu ($p< 0.05$). Hücresel ve hümoral immünyetmezlikli hastaların MN oluşumları benzer olmasına rağmen, bir sentromerli MN oluşumu hücresel immünyetmezlikli hastalarda daha fazlaydı ($p< 0.05$).

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ver, the incidence of one-centromere-positive MNs was significantly higher in cellular IDs ($p< 0.05$).

Conclusion: In conclusion, the increased MN incidence and centromere positivity detected in PID diseases may be consistent with the chromosome instability, which contributes to the follow-up in these disorders.

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Key words: Micronucleus, immunodeficiency, chromosomal damage, chromosomal instability, FISH technique

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Sonuç: Sonuç olarak primer immünyetmezlikli hastalardaki artmış MN oluşumu ve sentromer pozitifliği, bu hastalardaki kromozomal instabilite ile uyumlu olabilir ve bu hastaların izlemine katkıda bulunabilir.

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Anahtar kelimeler: Mikronükleus, immünyetmezlik, kromozom hasarı, kromozomal instabilite, FISH tekniği

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INTRODUCTION

Primary immunodeficiency diseases (PID) are a group of disorders that increase susceptibility to infection, malignancy, and autoimmunity[1-3]. Specific advances within the past decade in understanding of molecular and genetic mechanisms of the PIDs have greatly improved the treatment options. Currently available treatment techniques such as bone marrow transplantation, immunoglobulin replacement, and gene therapy have contributed to the survival of these patients[3]. In the last few decades, the survival rate has improved substantially, consequently, the short-term and long-term effects of the disease and its treatment gained importance. Recently, chromosomal radiosensitivity and chromosome aberrations have been reported in especially cellular immunodeficiencies (IDs) due to the some alterations in genes of DNA repair and apoptosis[4-9]. In addition, enhanced radiosensitivity was suggested to be a marker of cancer-predisposing genes involved in the processing of DNA damage[9]. One of the biomarkers for determining chromosome alterations such as chromosomal loss and chromosomal damage in vitro is scoring micronucleus (MN) which plays a role in the formation of MN in dividing cell[9-11].

The MN assay is considered to be a useful screening test to determine MN formation which reflects chromosomal abnormalities, such as chromosome breaks and deletions, that originated in the last division of nucleated cells which are basically distinguishable of their chromosomal origin by using fluorescence in situ hybridization (FISH) with chromosome specific probes. The centromere negative MN is segment of the chromoseme p arm or q arm. The MN with one centromere includes a centromere of any chromosomes. The MN with centromere show the cells that have aneuploidy (monosomy or trisomy). The number of the centromeric MN reflect the higher instability in the genom[9-13]. The incidence of MN is determined by a technique in which the process of cytokinesis was blocked, by adding cytochalasin-B (Cyt-B) to the cell culture[9]. The cytokinesis-block MN assay have proved to be reliable, in vitro method for assessing the spontaneous and/or radiation-induced chromosomal damage and chromosomal instability in peripheral blood lymphocytes[9,14-17].

Previos studies evaluated chromosomal sensitivity and chromosomal aberrations in common variable immunodeficiency (CVID) and ataxia telangiectasia (AT)[5,6,8,15,16]. Van Bull (1995) showed a high chromosomal abnormalities induced by X-ray in SCID mouse. Very recently Gisselson et al. (2005) demonstrated the chromosome behaviour in interphase nuclei of patients with ICF Syndrome. According to our best knowledge, there was no report for MN fre-

quency and MN origine in other ID patients. The aim of present study was to determine the incidence of MN formation and the features of the MN by FISH technique as well as to assess whether it varies in different PIDs.

MATERIALS and METHODS

Fifteen PID patients and 15 healthy age-matched children were included in the study during three-year-period. All patients and the control group gave their informed consent before participating in the study. The control group was randomly selected from the population of the same area. The diagnosis of PID was done using the criteria described previously by the International Union of Immunodeficiency Societies (IUIS)[18]. The ages of the patients were between 6 months and 13 years. Clinical details of the patients are given in Table 1. The patients were divided into two groups as cellular and humoral IDs depending upon the predominantly affecting system to investigate the relation between the MNs formation and the classification. It was also investigated the relationship between incidence of the MNs formation and the diagnosis of the patients (Table 1). Blood samples were obtained from the antecubital vein in heparinized tubes and each blood sample was divided into two aliquots. One aliquot was directly processed for cytogenetic analysis, and the other for MN and FISH studies.

Metaphase Analysis

In both patient and controls, conventional cytogenetic analysis was done using the first aliquot of peripheral blood samples. Five metaphase spreads for each T-B-SCID group were analysed and at least 10 metaphase spreads were analysed for the other patients by using the standard peripheral blood protocol. Metaphase spreads were analysed with GTG-banding technique.

Micronucleus Assay

The other aliquot was also divided into two aliquots. One is processed for the conventional MN assay (direct preparation) where peripheral blood cells were treated with directly cold 0.05 M KCl and followed by centrifugation[19]. The cell pellet was fixed two times very gently with a cold mixture of methanol: acetic acid (3:1). The cell suspension was dropped onto clean slides and stained with Giemsa after aged overnight. The second aliquot was processed for the culture of peripheral blood sample with Cyt-B. Peripheral blood culture was carried out 37°C for 72 hours. The Cyt-B was added to Cyt-B culture assay at 44^{th} hours[20]. The slides were prepared as above mentioned protocol in the direct preparation. The slides were screened blind by two evaluators. There was no significant difference between evaluators. For each subject, 1000 nucleated cells were analysed for the presence of MN at a final 100x magnification. The MN described by Countryman et al.[14] and Vian et al.[17] was used as: diameter less than 1/3 of the main nucleus, non-refractility, not touching and the same colour as the nucleus or lighter.

FISH Analysis

FISH analysis was done only with the cultured peripheral blood sample with Cyt-B which showed the high frequency of MN. In order to remove the cytoplasma around the blocked-cell, the cell suspension was further treated gently with methanol: Acidic acid (3:2) fixative mixture once or twice. The slides were then prepared for FISH analysis.

The FISH study was performed by using commercial all chromosome-centromere-specific probes labelled with biotin (CAMBIO, Cambridge, UK). The probe in hybridization buffer was denaturated at 65°C for 10-15 min while the slide was denaturated in 70% formamide/2 x SSC for 2-4 min at 65°C, chilled with ice-cold 70% ethanol, and dehydrated with sequential washes in 90 and 100% ethanol and air-dried. The probe was dropped onto the denatured slides and placed by coverslips and then sealed with rubber cement. The hybridization was carried out in a water bath at 39-41°C for 16 h. The washes were done in 2 x SSC twice and 50% formamide/2 x SSC twice and 4 x SSC once, respectively, at 39-41°C for 5 min

Table 1. Characteristics of the patients with primary immunodeficiency and the controls, and micronucleus formation in peripheral blood lymphocytes

No	Age (year)	Gender	Diagnosis	Cellular/ Humoral	Disease type	Dead/ Alive	Micronucleus (%) Direct	Cyt-B (-)	Cyt-B (+)
1	0.5	F	T-B-SCID	C	SCID	D	1.2	0.5	9.3
2	1	M	T-B-SCID	C	SCID	D	1.2	0.2	2.2
3	0.5	F	T-B-SCID	C	SCID	D	1.0	0.3	7.1
4	8	M	AT	C	S	A	0.0	0.4	14.0
5	9	M	AT	C	S	A	0.3	0.5	2.0
6	7	M	AT	C	S	A	0.3	0.2	11.0
7	6	M	AT	C	S	A	0.2	0.3	9.0
8	3	F	AT	C	S	A	0.3	0.2	3.3
9	12	F	CVID	H	CVID	D	0.3	0.4	3.0
10	6	F	CVID	H	CVID	D	0.5	2.0	6.6
11	13	M	CVID	H	CVID	A	0.6	0.8	6.0
12	5	F	CVID	H	CVID	A	0.5	0.4	5.0
13	11	M	XLA	H	XLA	A	0.1	0.1	7.3
14	4	M	XLA	H	XLA	A	0.1	0.2	4.8
15	10	F	CVID	H	CVID	A	0.0	0.6	9.6
						Mean ± SD	0.40 ± 0.4	0.47 ± 0.46	6.68 ± 3.5*,**
Controls (n= 15)						Mean ± SD	0.42 ± 0.11	0.45 ± 0.2	0.88 ± 0.25

* Shows significant difference from the controls at $p< 0.05$.
** Shows significant difference from direct preparation and cultured without Cyt-B at $p< 0.05$.
M: Male, F: Female, SCID: Severe combined immunodeficiency, CVID: Common variable immunodeficiency, XLA: X-linked agammaglobulinemia, AT: Ataxia telangiectasia,
C: Cellular immunodeficiency, H: Humoral immunodeficiency, S: Well-defined immunodeficiency syndromes, D: Dead, A: Alive, Cyt-B: Cytochalasin-B, SD: Standart deviation.

each. The slides were treated avidin-fluoresceinisothiocyanate (FITC) at 37°C for 20 min, and then washed in 4 x SSC-tween-20 at 39-41°C for 5 min twice. The slides were then stained with a counterstain medium containing DAPI (4' 6-diamidino-2-phenyl-indole) and/or PI (propidium iodide). Slides were examined with an epifluorescence microscope (Nikon, Optiphot, Japan) for verifying of chromosomal origin of MN in the approximately same number of MN which were observed by Cyt-B MN assay.

Statistical Analysis

Statistical analysis was done by SPSS for Windows computer program. Parameters were compared using Mann-Whitney U and Chi-square tests. The logistic regression analysis was used for evaluating the prognostic value of independent factors on survival. $p< 0.05$ was considered statistically significant.

RESULTS

The clinical findings of the patients were summarized in Table 1. Conventional cytogenetic analysis of 15 patients with PID by tripsin G-banding were normal. Both direct (0.40 ± 0.4) and cultured peripheral blood lymphocytes without Cyt-B (0.47 ± 0.46) did not show an increase of MN incidence when compared with the control group (0.42 ± 0.11 and 0.45 ± 0.2) ($p> 0.05$) (Table 1). However, there was a significant incidence of MN in cultured peripheral blood lymphocytes with Cyt-B in the patients with PIDs (0.88 ± 0.25 versus 6.68 ± 3.5) ($p< 0.05$). In addition, there was significant increase of MN incidence in cultured peripheral blood with Cyt-B when compared with either direct preparation ($p< 0.05$) or with cultured peripheral blood without Cyt-B ($p< 0.05$). When we compared the MN frequency among patients, the incidence of micronuclei (MNs) was found to be similar in cellular and humoral immunodeficiencies (IDs).

In order to verify the chromosomal origin of MN in cultured peripheral blood sample with Cyt-B, which had high percentage of MN, FISH analysis with all chromosome centromere specific probe was used. The results showed that the incidence of centromere-positive MNs was higher in cellular IDs than in humoral IDs ($p< 0.05$). In addition, the incidence of one-centromere-positive MNs was also significantly higher in cellular IDs ($p< 0.05$). There was no relation between the incidence of centromere-positive MNs and the gender of the patients, and the diagnosis of the patients (Table 2).

Five of the patients died in four years (Table 2). Three of them were SCID patients who died because of pneumonitis while two were CVID patients one of whom had T-cell lymphoma and the other died because of pneumonitis. The number of the centromere positive MNs was higher in the dead patients than in the alive patients ($p< 0.05$). The number of the MNs with one-centromere was also higher in the dead patients than in the alive patient ($p< 0.05$). The effective factors on mortality was attributed to the age of the patients, the diagnosis of the patients and one-centromere-positive MN in evaluating the prognostic value of independent factors by logistic regression analysis.

DISCUSSION

Chromosomal abnormalities are known entities in PIDs such as CVID, AT, Nijmegen breakage syndrome (NBS) and immunodeficiency, centromeric instability and facial anomalies (ICF) syndrome[4-8,16]. In the previous studies, the MN assay has been used for investigation of radiation-induced chromosomal sensitivity, spontaneous chromosomal instability and chromosomal aberrations in CVID, AT, ICF syndrome[5,6,8,15,16,21,22]. However, there was no report about the use of this technique for evaluating spontaneous chromosome aberration and micronucleus incidence in the patients with SCID and agammaglobulinemia. In this study, the incidence of the MNs also increased in the patients with SCID and XLA as in the patients with CVID and AT.

The MN assay can be used to obtain a quantitative index of chromosome breakage and loss and/or instability because the formation of a MN is the end point of both chromosomal da-

Table 2. Dual-colour FISH results of the patients with primary immunodeficiency

No	Gender	Diagnosis	Cellular/ Humoral	Dead/ Alive	Total MN	MN-(C-)	%	MN-(C+)*	%	MN-(C)**	%	MN-(2C)	%	MN-(> 2C)	%
1	F	SCID	C	D	58	6	10.3	52	89.7	36	62.1	14	24.1	2	3.4
2	M	SCID	C	D	23	2	8.7	21	91.3	13	56.5	5	21.7	3	13.0
3	F	SCID	C	D	36	4	11.1	32	88.9	26	72.2	5	13.9	1	2.8
4	M	AT	C	A	51	33	64.7	18	35.3	8	15.7	6	11.8	4	7.8
5	M	AT	C	A	16	2	12.5	14	87.5	4	25.0	1	6.2	9	56.2
6	M	AT	C	A	27	6	22.2	21	77.8	12	44.4	5	18.5	4	14.8
7	M	AT	C	A	15	2	13.3	13	86.7	4	26.7	1	6.7	8	53.3
8	F	AT	C	A	19	1	5.2	18	94.8	4	21.0	4	21.0	10	5.3
9	F	CVID	C	D	29	7	24.1	22	75.9	6	20.7	5	17.2	11	37.9
10	F	CVID	C	D	48	5	10.4	43	89.6	38	79.2	3	6.25	2	4.2
The mean values for Cellular PID					**33.3**	**9.6**		**23.8**		**14.1**		**5.1**		**4.3**	
11	M	CVID	H	A	26	18	69.2	8	30.8	2	7.7	3	11.5	3	11.5
12	F	CVID	H	A	45	13	28.9	32	70.1	28	62.2	3	6.7	1	2.2
13	M	XLA	H	A	18	8	48.3	10	51.7	5	27.7	4	22.2	1	5.6
14	M	XLA	H	A	47	12	25.5	35	74.5	9	19.1	15	31.9	11	23.4
15	F	CVID	H	A	20	4	20.0	16	80.0	11	55.0	3	15.0	2	10.0
The mean values for Humoral PID					**30.6**	**7**		**23.6**		**13.4**		**5.1**		**5.1**	

* Statistically difference between cellular and humoral IDs ($p< 0.05$).
** Statistically difference between dead and alive patient ($p< 0.05$).
SCID: Severe combined immunodeficiency, CVID: Common variable immunodeficiency, XLA: X-linked agammaglobulinemia, AT: Ataxia telangiectasia, C: Cellular, H: Humoral, D: Dead, A: Alive, MN: Micronucleus, MN-(C-): MN centromere negative, MN-(C+): MN centromere positive, MN-(C): MN with one centromere, MN-(2C): MN with two centromere, MN-(>2C): MN with more than two centromere.

mage and segregation errors[15]. In the present study, the incidence of MN was significantly higher in the primary IDs than in the control group. However, there was no difference in the incidence of MN between cellular (SCID, AT) and humoral (CVID, XLA) IDs. These findings indicate the presence of chromosomal aberration and instability in humoral IDs as previously described in AT and ICF[15,16,21,22].

The present report is the first to use the MN assay and FISH technique to determine the incidence and the features of the MN in the patients with primary ID. Present FISH analysis showed that there were significant differences in the incidence of centromere positive MNs between cellular and humoral IDs. The MNs with one centromere were found to be higher in cellular IDs than in humoral IDs. This finding may indicate the higher chromosomal inastability in cellular IDs. These findings suggest that there is an association between low frequency of MN with one centromere in the patients with agammaglobulinemia. As for SCID patients, peripheral blood lymphocytes exhibited higher MN frequency with one centromere, indicating chromosome loss. There was also an association between high frequency of MN with one centromere and the chromosomal instability in SCID patients. There might be an association between low incidence of MN with one centromere and chromosomal stability in agammaglobulinemia.

The transformation of a normal cell to a malignancy can result from a variety of different factors that cause genomic instability. These transforming events may occur spontaneously by random mutations or gene rearrangements; alternatively they may be induced by chemical, physical or viral carcinogens. An association between radiosensitivity and predisposition to cancer was found in AT[8]. Genomic instability was also demonstrated in the patients with CVID by using radiosensitivity methods[5,6]. CVID is associated with risk of tumours and the most frequent types are similar to those found in AT such as lymphomas and leukaemias[4,6]. One of our CVID patients developed a T-cell lymphoma which supports the results of these previous studies. There might be a relation between genomic instability and development of T-cell lymphoma because chromosomal instability is a very common issue in PIDs. The increased MN incidence, indicating chromosomal instability, detected in PIDs may be consistent with the chromosome damage which contributes to the risk of cancer in these disorders.

In conclusion, our findings indicated that there was a high incidence of MN indicating chromosomal loss and damage in PIDs. The Cyt-B blocked MN assay, as well as radiosensitivity methods, may be a useful technique to show the chromosomal alterations and genomic instability. In the present study, the increased MN incidence and centromere positivity, especially MN with one centromere, detected in PID diseases may be consistent with the chromosome instability, which contributes to the follow-up in these disorders. Therefore, further studies in a large patient group for investigating the genomic instability in PIDs may help the understanding of the factors influencing the survival of these patients.

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