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Methods

Micronucleus evaluation in mitogen-stimulated lymphocytes of patients with acromegaly

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ABSTRACT

Acromegaly is a syndrome characterized by a sustained elevation of circulating growth hormone and insulin-like growth factor–1 (IGF-1). Insulin-like growth factor–1 is a potent mitogen and has a role in the transformation of normal cells to malignant cells. This study aims to evaluate the spontaneous micronucleus (MN) frequency by using the cytokinesis-block MN assay to determine genetic damage in the lymphocytes of patients with acromegaly. The study was carried out in 20 patients who had active acromegaly and in 20 age- and sex-matched healthy controls. The MN values were measured in binucleated cells obtained from mitogen-stimulated lymphocytes of patients and control subjects. The distribution of binucleated cells with 1, 2, 3, or more MNs was also measured. We found significantly higher MN frequency values in the lymphocytes of acromegalic patients than in those of the control subjects (2.23 ± 0.68 vs 1.03 ± 0.54, $P = .001$). The MN frequency increased with increasing IGF-1 levels of acromegalic patients ($P = .036$, $R = 0.47$). We observed that the number of binucleated cells with 2 MNs was higher for the majority of patients with acromegaly than for control subjects. Furthermore, the receiver operating characteristic curve (area under the curve = 0.914, $P < .0001$) was calculated to assess the discriminative power of the MN frequency. Our results indicate that increased MN frequency in the lymphocytes of patients with acromegaly may reflect genomic instability and this increased MN frequency may be associated with elevated levels of circulating growth hormone and IGF-1.

1. Introduction

Acromegaly, a rare endocrine disease, is associated with increased morbidity and premature mortality, mainly due to cardiovascular, respiratory, rheumatologic, and metabolic diseases [1-3]. In addition, patients with acromegaly may have a higher risk of developing cancer [4,5]. Acromegaly is a syndrome caused by long-term exposure to elevated levels of growth hormone (GH) and peripheral insulin-like growth factor–1 (IGF-1). The GH hypersecretion is mainly caused by a somatotroph cell adenoma (more than 95% of cases). Rarely, this syndrome may be associated with hypothalamic or an ectopic GH-releasing hormone–producing tumor [2,4,6]. Although the relationships among GH, IGF-1, and cancer are documented in the literature [5,7], there is no clinical evidence that acromegalic patients have a higher "generic" cancer risk

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than the healthy population [7-9]. However, a number of studies have reported that acromegalic patients have increased risk of developing variety of cancers [8,10,11].

Micronucleus (MN) frequency is a biomarker of chromosomal damage, genome instability, and cancer risk [12,13]. The cytokinesis-blocked micronucleus (CBMN) assay is the most extensively used method for measuring MNs in human peripheral blood lymphocytes. Micronucleus is formed through different processes, such as chromosome breakage or whole chromosome loss, which lag behind in anaphase during cell division [12,14].

To the best of our knowledge, there are no data in the literature on MN formation in the lymphocytes of patients with acromegaly. The present study is the first report concerning MN frequency in patients with acromegaly. Our objective, therefore, was to determine the spontaneous MN frequency in the phytohemagglutinin (PHA)-stimulated lymphocytes of patients with acromegaly using CBMN assay.

2. Materials and methods

2.1. Patients and controls

The study was performed in 20 patients diagnosed with active acromegaly who were admitted to the Department of Endocrinology and Metabolism at Erciyes University's Medical Faculty from December 2008 to December 2009. Twenty age- and sex-matched healthy controls were included in the study.

The diagnosis of acromegaly was confirmed by GH response to oral glucose tolerance test and IGF-1 levels. After 75-g oral glucose load [15,16], nadir GH levels were higher than 1 μ/L in all patients. Serum IGF-1 levels were also higher than the age- and sex-related reference ranges in acromegalic patients [17].

Seventeen of the 20 patients included in the study had been newly diagnosed and had not received any medical or surgical therapy for acromegaly. Three patients had previously been operated on using the transsphenoidal approach and had been diagnosed with pituitary adenoma secreting GH, but had not received any medical therapy; however, they all had active disease states, with increased serum IGF-1 levels according to the normal sex and age reference levels and a failure to suppress GH levels after 75-g glucose loading. The oral glucose tolerance test was not performed on 4 of the 20 patients because they had diabetes (4 patients' ID: 3, 9, 13, and 20). Fifteen of the patients had macroadenoma, 3 had microadenoma, and 2 had partial empty sella. There was deficiency of corticotrophin, thyrotrophin, and gonadotrophin axis in 2 and of thyrotrophin in only 1 of the 20 patients. These 3 patients were given appropriate replacement therapies before taking the sample.

The control group was selected from healthy subjects matched for age, sex, and smoking habits; and none of them was known to be receiving any drugs for medical or other reasons. A questionnaire designed to collect information on medical history and drug and smoking habits was completed for each patient and control subject.

Blood samples were obtained from patients for the measurement of GH, IGF-1, follicle-stimulating hormone, luteinizing hormone, total testosterone, free testosterone, free thyroxine, free triiodothyronine, prolactin, estradiol, adrenocorticotropic hormone, thyroid-stimulating hormone, and cortisol. All serum samples were collected in the early morning after an 8-hour fasting period. Neither pituitary deficiencies nor excessive hormone secretion except for GH/IGF-1 was detected in any of the patients.

The local ethics committee approved the study protocol, and all patients gave written informed consent. The study was conducted in accordance with the Declaration of Helsinki and local laws, depending on which afforded greater protection to the patients.

2.2. Determinations of IGF-1 and GH levels

Serum concentrations of IGF-1 and GH were measured by sensitive and specific immunoradiometric assay (Active Non-Extraction IGF-1 IRMA DSL-2800 and Active Growth Hormone IRMA DSL-1900; Diagnostics System Laboratories, Webster, TX). For IGF-1, the intraassay and interassay coefficients of variation were 4.9% and 5.1%, respectively. For GH, the intraassay and interassay coefficients of variations were 4.1% and 8.7%, respectively. The use of these kits is one of the procedures recommended for IGF-1 measurements, and they have high sensitivity [18].

2.3. Whole-blood cultures of human lymphocytes

Antecubital heparinized 3-mL blood samples were taken after informed consent had been obtained from all patients and control subjects. Approximately 0.4 mL of heparinized whole-blood samples was cultured for 72 hours at 37°C in 5 mL of peripheral blood karyotyping medium that was supplemented with 1.5% PHA-M to stimulate T-lymphocytes (all sourced from Biological Industries, Kibbutz Beit Haemek, Israel). To determine intraindividual differences, duplicate cultures were prepared for each patient and control at the specified time.

2.4. CBMN assay

Forty-four hours after the initiation of cultures, cells were blocked from entering cytokinesis by the addition of cytochalasin-B to each culture tube (Sigma-Aldrich, St Louis, MO; final concentration, 3 μg/mL) [19]. The cultures were stopped at 72 hours after initiation, treated with hypotonic solution (0.1 mol/L KCl) for 4 minutes, and fixed using 2 changes of methanol–acetic acid (3:1) [20]. The fixed cells were spread onto glass slides and stained with 5% Giemsa (Merck) in Sorensen buffer for 10 minutes.

To determine the intraindividual differences, the different slides of the 2 parallel cultures for each patient and control subject were prepared and evaluated. All slides were scored blindly using a Nikon Alphaphot-2 light optical microscope. A minimum of 1000 binucleate cells (BNCs) with 2 macronuclei wells surrounded by cytoplasm were scored from each patient and control subject, and the frequency of binucleate cells with MN (MNBNCs) was determined. Published criteria for MN determination were followed [21,22].

Table 1 – Patients' profile at study entry and the total number of BNCs scored, total number of MNs, MN frequency, and distribution of MNBNCs in PHA-stimulated lymphocytes

ID	Sex	Age (y)	Neurosurgery (by transsphenoidal approach)	Pituitary adenoma diameter (mm)	IGF-1 level (mU/L)	z-SDS IGF-I	Nadir GH after 75-g glucose loading (μIU/mL)	z-SDS GH	No. of BNCs with				Total no. of MNs[a]	Total no. of BNCs scored	MN frequency (%)
									1 MN	2 MNs	3 MNs	4 MNs			
1	M	43	–	9	994	–0.26	10.62	0.482	11	3	1	–	20	1069	1.87
2	M	57	–	14	1971	2.16	18.03	2.036	28	1	1	–	33	1021	3.23
3	F	55	–	22	316	–1.94	–	–	18	–	–	–	18	1015	1.77
4	F	49	–	13	1526	1.06	4.90	–0.717	11	3	1	2	28	1085	2.58
5	M	35	–	14	1184	0.21	15.5	1.505	25	–	–	–	25	1048	2.39
6	M	41	+	11	1305	0.51	4.19	–0.865	14	2	1	–	21	1003	2.09
7	F	27	–	31.67	1175	0.19	16.99	1.818	19	1	–	–	21	1062	1.98
8	M	56	–	6	1369	0.67	4.44	–0.813	26	3	2	–	38	1004	3.78
9	M	47	+	Empty sella	1131	0.08	–	–	24	1	2	–	32	1074	2.98
10	F	51	+	Empty sella	1082	–0.04	2.49	–1.222	18	4	–	–	26	1016	2.56
11	M	44	–	12	1039	–0.15	11.04	0.570	20	5	1	–	33	1004	3.29
12	M	61	–	12	1127	0.07	11.93	0.757	12	–	–	–	15	1029	1.46
13	F	66	–	7.49	355	–1.85	–	–	14	–	1	1	19	1045	1.82
14	M	27	–	27	706	–0.98	3.35	–1.042	15	–	–	–	14	1010	1.39
15	M	24	–	41	1019	–0.19	9.59	0.266	16	3	–	–	21	1015	2.07
16	F	32	–	100	933	–0.41	8.00	–0.067	18	2	–	–	24	1028	2.33
17	F	27	–	13	1750	1.61	10.43	0.442	9	2	1	–	25	1045	2.39
18	M	34	–	15	1521	1.04	3.66	–0.977	15	3	–	–	15	1010	1.49
19	F	47	–	11	680	–1.04	1.71	–1.386	11	–	2	–	21	1140	1.84
20	M	30	–	11	1305	5.51	–	–	11	1	–	–	13	1023	1.27

z-SDS indicates standard deviation score.

[a] (1 MN × 1) + (2 MNs × 2) + (3 MNs × 3) + (4 MNs × 4).

2.5. Statistical analysis

Statistical comparisons of the frequency of MNBNCs, age, and IGF levels between the patients with acromegaly and the control subjects were performed using the nonparametric Mann-Whitney *U* test for 2 independent samples. Spearman ρ correlation analysis was used to assess the relationships among age, IGF-1 level, nadir GH level, pituitary adenoma diameters, duration of acromegaly, and MN frequency.

A receiver operating characteristic (ROC) curve analysis was performed to assess the potential of MN frequencies to discriminate between cases and controls. The ROC curve analysis was used to assess predictive accuracy. The area under the ROC curve serves as one means for evaluating the discriminatory power of diagnostic and predictive test systems. The ROC curve describes this ability by giving the sensitivity and specificity for the entire range of measurements. Sensitivity and specificity were calculated at all possible cutoff points to find the optimal cutoff value.

3. Results

The characteristics of the patients are given in Table 1. The mean age of the patients was 42.65 ± 12.69 (range, 24-66) years. Eight of the patients were female, and 12 were male. Three of the patients were smokers, and 17 were nonsmokers. Results from the statistical analysis of age, IGF-1 levels, and MN frequency (frequency of MNBNCs) in the PHA-stimulated lymphocytes of 20 patients with acromegaly and 20 healthy controls are given in Table 2.

When compared with the control subjects, the MN frequency in patients with acromegaly was found to be significantly higher than (2.23 ± 0.68 vs 1.03 ± 0.54, $P = .001$, Table 2); and it was observed that the MN frequency of lymphocytes from acromegalic patients increased with IGF-1 ($P = .036$, $R = 0.47$, Fig. 1). There was no statistically significant correlation between MN frequency and the nadir GH level after oral glucose load or pituitary adenoma diameters of patients and duration of acromegaly ($P = .09$, $R = 0.46$; $P = .61$ $R = 0.15$; $P = .49$, $R = 0.161$; respectively).

Table 2 – Biochemical and MN frequency (percentage) findings of patients and controls

Groups	Age (y) (mean ± SD)	IGF-1 level (mU/L) (mean ± SD)	MN frequency (%) (mean ± SD)
Acromegalic patients (n = 20)	42.65 ± 12.69	1099.60 ± 403.28	2.23 ± 0.68
Controls (n = 20)	39.95 ± 10.49	262.00 ± 105.12	1.03 ± 0.54
P value	.524	.001[a]	.001[a]
Z value	0.638	5.058	4.477

[a] Patients with acromegaly exhibit statistically higher BNCs with MN and IGF-1 levels than controls according to the 2-tailed nonparametric Mann-Whitney *U* test for the comparison of the means of independent variables.

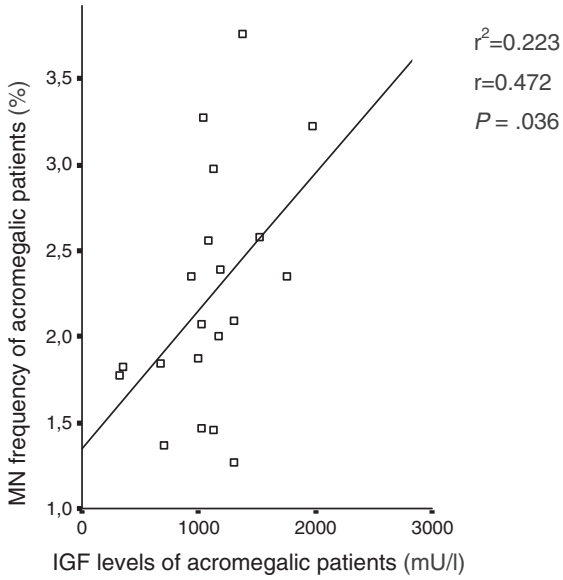

Fig. 1 – Correlation between the IGF-1 levels and MN frequencies of acromegalic patients.

The MN number in patients varied between 13 and 38 per approximately 1000 BNCs (Table 1). However, the MN number in control subjects ranged from 3 to 20 per approximately 1000 BNCs (Table 3). We found that the MN frequency ranged from 0.29% to 1.89% in control subjects and from 1.27% to 3.78% in acromegalic patients. In addition, the MN frequency ratio (MN mean of patients to MN mean of

Table 3 – The total number of BNCs scored, total number of MNs, MN frequency, and distribution of MNBNCs in PHA-stimulated lymphocytes from control subjects

ID code	No. of BNCs with		Total no. of MNs[a]	Total no. of BNCs scored	MN frequency (%)
	1 MN	2 MNs			
1	3	–	3	1042	0.29
2	5	–	5	1011	0.49
3	8	–	8	1009	0.79
4	16	2	20	1061	1.89
5	12	–	12	1069	1.12
6	15	2	19	1017	1.87
7	6	2	8	1036	0.77
8	10	–	10	1009	0.99
9	19	–	19	1024	1.86
10	8	–	8	1013	0.79
11	10	–	10	1017	0.98
12	5	–	5	1074	0.47
13	7	2	11	1065	1.03
14	16	–	16	1010	1.58
15	9	–	9	1024	0.88
16	18	–	18	1021	1.76
17	6	–	6	1006	0.60
18	3	–	3	1011	0.30
19	16	–	16	1018	1.57
20	6	–	6	1021	0.59

[a] (1 MN × 1) + (2 MNs × 2).

controls) between patients with acromegaly and control subjects was computed according to Iarmarcovai et al [13] and was determined to be 2.17.

The total number of BNCs scored, the total number of MNs, the MN frequency, and the distribution of MNBNCs in PHA-stimulated blood cells from the patients and control subjects are shown in Tables 1 and 3, respectively. We observed the presence of BNCs with 2 MNs in the stimulated lymphocytes of almost all the acromegalic patients (except IDs 3, 5,12,13,14, and 19). Ten patients were found to have BNCs with 3 MNs, and 2 patients were found to have BNCs with 4 MNs. However, cells with 2 or more MNBNCs were not observed in most control subjects (except IDs 4, 6, 7, and 13).

No significant relationship was found between MN frequency and either age or sex in either of the groups ($P = .30$, $R = 0.21$; $P = .62$, $R = 0.12$; respectively).

The ROC curves showed that using the cutoff value for MN frequency of patients and controls was identified with a sensitivity of 100%, a specificity of 70%, a positive predictive value of 76.9%, and a negative predictive value of 100% (area under curve, 0.914; 95% confidence interval, 0.781-0.978).

4. Discussion

High levels of IGF-1 in the circulation have been reported to enhance cell proliferation [9]. An increase in the rate of cell proliferation may lead to impaired fidelity of control or repair of genomic integrity during the cell cycle due to time limitations [23]. Micronuclei are small, extranuclear bodies consisting of an acentric fragment or a whole chromosome fragment that lags behind in anaphase during cell division [19]. Using the CBMN assay, MN is scored in binucleated cells that have completed nuclear division [14,19]. In the present study, we found significantly higher MN frequency in the lymphocytes of patients with acromegaly than control subjects. Elevated IGF-1 levels may increase the number of DNA replication errors and induce aneuploidy due to stimulation of cell proliferation. Therefore, the stimulation of cell proliferation resulting from high levels of IGF-1 in the circulation may be responsible for increased MN frequency in patients with acromegaly.

In this study, we determined a moderate positive correlation between IGF-1 levels of patients with acromegaly and the MN frequency of their mitogen-stimulated lymphocytes. However, in patients with normalized IGF-1 levels, we do not know whether the MN frequency returned to normal values. Therefore, a long-term follow-up of the patients is necessary to reevaluate the effects of treatment.

It has been reported that patients with acromegaly have an increased risk of developing cancers, particularly colorectal, prostate, and thyroid cancers [1,3]. Correa et al [11] suggest that men with acromegaly should be carefully screened for prostate cancer. However, the relationship between patients with acromegaly and other cancers, including breast, prostate, and hematological cancers, has been a point of debate [7-10]. It has been reported that the increased incidence of cancer in acromegalic patients has several possible biological explanations, the most probable one being related to the effects of elevated levels of circulating GH and IGF-1 [5,8,10]. Genetic damage in lymphocytes reflects similar damage in cells undergoing carcinogenesis. For this reason, both chromosome and genome alterations relevant to cancer in peripheral blood lymphocytes have been extensively investigated using CBMN assay [13,14]. The presence of an association between the frequency of MN in lymphocytes and cancer risk has been supported [24,25]. The structural chromosomal aberrations and aneuploidy may be the cause of this association. It has also been reported that MN frequency in the general population is predictive of increased cancer risk, suggesting that increased MN formation is associated with the early stages of carcinogenesis [24].

Because MN has been associated with chromosomal instability and cancer risk, the goal of the present study was to contribute to the controversial issue concerning whether or not acromegalic individuals have an increased risk of cancer. We propose that elevated MN frequency may predict an increased risk of malignancy in acromegalic patients. Furthermore, carcinogenesis is a multistep and/or multifactorial process. Unfortunately, there are limited data regarding malignant or premalignant lesions in study populations. Further studies investigating associated malignancies in patients with acromegaly may be useful for demonstrating the clinical relevance of the present findings.

On the other hand, the number of MNs in a single binucleated cell normally ranges from 0 to 3 in the lymphocytes of healthy individuals [22]. An important observation in our study was that the majority of patients with acromegaly showed a significant elevation in the frequency of BNCs, with 2 or 3 MNs in stimulated peripheral lymphocytes, whereas healthy subjects did not show the presence of BNCs with 2 MNs (except IDs 4, 6, 7, and 13) or 3 MNs. The increase of MN numbers in single binucleated cells in acromegalic patients may be due to elevated genome alterations or the impairment of the genomic repair of cells.

Age and sex have been shown to influence MN frequency in the cultured peripheral blood lymphocytes of humans [14,21]. However, in the present study, no significant relationship was found between MN frequency and either age or sex in either of the groups.

In a recent meta-analysis by Iarmarcovai et al [13], it was reported that MN frequency (26 studies; years 2002-2006) in 1000 binucleated cells ranged from 2.0 to 36.0 (mean 14.8) in control subjects and from 2.2 to 60.0 (mean 20.3) in untreated cancer patients. Furthermore, in another meta-analysis by the same authors [13], it was reported that the MN frequency ratio between patients with cancer and control subjects ranged between 0.45 and 2.39 (mean, 1.45). Previously, it was reported that the MN frequency ranged from 0.99 to 3.21 (mean, 1.87%) in control subjects and from 1.40 to 5.85 (mean, 3.41%) in untreated patients with leukemia [20]. We found that the MN frequency in 1000 binucleated cells ranged from 2.8 to 18.8 (mean, 1.02%) in the control subjects and from 1.27 to 3.78 (mean, 2.23%) in acromegalic patients. We also found that the MN frequency ratio between patients with cancer and control subjects had a mean of 2.17. Micronucleus frequency may be related to other clinical factors such as nutrition (folate and riboflavin concentration) or ageing (Alzheimer disease etc). In addition, MN frequency is affected by factors such as lifestyle, smoking, tea and coffee intake, and exposure to occupational or environmental toxicants and genetic factors [12,25,26].

Therefore, there may be natural differences between the average MN frequencies in most of the studies performed. [20,27-30]. Our patients and control subjects were free from conditions affecting MN frequency, such as malnutrition and Alzheimer disease; smoking and tea and coffee intake levels were similar between the patients and control subjects.

An ROC analysis has been performed for the present study. The area under the ROC curve was 0.914 (95% CI, 0.781%-0.978%), indicating a high ability to discriminate between patients and controls. However, MN frequency was not found to be a new diagnostic test for acromegaly; but our results still indicate that MN frequency can be predictive for acromegalic patients.

It is reported that there is normally a delay of 7 to 10 years between onset of the first symptoms and the diagnosis of acromegaly [31]. In acromegaly, because of the slow nature of the phenotypical changes and the existence of a long period between the emergence of changes and the establishment of diagnosis, objective assessment regarding the length of the illness is hardly possible. However, based on the estimated duration provided by the patients in connection with the illness, the relation between MN and the length of illness was assessed; but no relationship was found. Although the findings of the present study are important, the clinical relevance needs to be further evaluated.

Few studies have shown a significant increase in DNA damage in the peripheral blood lymphocytes of endocrine-related disease and hormone-dependent cancers [32,33]. In this study, it was found that there was increased MN frequency in acromegalic patients. Our data indicated that increased MN frequency in patients with acromegaly may reflect genomic instability. We think that the MN frequency enhancement in our patients might have been caused by increased cell proliferation rate resulting from elevated IGF-1 levels. However, further studies are needed to support the findings of the present study.

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