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Investigation of Aneusomy of Chromosome 21 in the Micronuclei of 13 Patients with Early Onset Alzheimer's Disease Using Fluorescence *in Situ* Hybridization: A Pilot Study

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Abstract

Background: Alzheimer's disease is one of the most common neurodegenerative and dementia disorders in people between the ages of 30 and 65. When symptoms appear in this age group, the disease is referred to as early-onset Alzheimer's disease (EOAD). Unfortunately, the symptoms are progressive and no current treatments are effective.

Methods: In this research, 13 patients, aged 37 to 65 years with symptoms of early-onset Alzheimer's disease, were studied. First, patient lymphocytes were isolated and cultured in RPMI 1640 medium using a special micronucleus (MN) culture method. Next, the lymphocytes were harvested and prepared on slides. The slides were then examined by fluorescent microscopy using a unique FISH protocol specific for MNs. The patients were divided into groups aged 30-39, 40-49, and 50-65.

Results: We found that 19.76% of the MNs from our EOAD patients originated in chromosome 21. Micronuclei originated in chromosome 21 in 21.20 and 16.52% of patients without and with family histories of Alzheimer's, respectively. This difference was not significant. Also, the percentage of micronuclei originating in chromosome 21 was not dependent on the patient age at the time of the study, or symptom onset age or duration.

Conclusions: This study shows that the rate of micronuclei with the origin of chromosome 21 is high in these patients. However, the micronucleus increased has no significant relationship with age and duration of disease or family history of it.

Keywords: Early-onset Alzheimer's disease (EOAD), Chromosomal instability, Fluorescence *in Situ* Hybridization (FISH), Neurodegenerative diseases, Micronucleus (MN).

Introduction

Alzheimer's disease (AD), in addition to affecting seniors, is one of the most common neurodegenerative and dementia disorders in people between the ages of 30 and 65 (1, 2). Clinical symptoms of AD include progressive memory loss and gradual devastation of other mental abilities (3). On average, the disorder lasts from three to nine years and eventually leads to death. Alzheimer's disease encompasses more than 50% of dementia cases, and more than 35 million people throughout the world are currently afflicted with this disorder (2). It was estimated in 2016 that about 5.2 million Americans had AD at that time (4). The number of people living with AD in Iran in not known precisely; however, estimates place the number around 300,000, and the Alzheimer's Association of Iran gives credence to this estimate. The data indicates that

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age is the most important risk factor for development of AD. A direct significant association exists between age and AD. Based on age, AD is categorized into early-onset AD (EOAD) and late-onset AD (LOAD). About 6% of AD cases are EOAD, mostly affecting people between the ages of 30 and 65, while LOAD, also known as the above-65 AD type, is far more common. Both AD types are commonly seen in families with histories of the disease. Sixty percent of EOAD cases have another affected family member, and autosomal dominant inheritance is found in 13% of familial cases. Monogenic AD has autosomal dominant inheritance; however, in most cases the disease is complex and influenced by both genetic and environmental factors. Although the probability of developing LOAD in first-degree relatives of an affected case is double that of the normal population, the inheritance of AD is barely compatible with the Mendelian pattern of inheritance (2, 5). Mutations in amyloid precursor protein (APP) (6), presenilin 1 (PS1), and presenilin 2 (PS2), have been implicated in the pathogenesis of EOAD (7). The loci of APP is localized on chromosome 21 in the region 21q11.2-q213 (8).

Although age is considered the most important risk factor for the development of AD, other factors including brain damage from head injuries, diabetes, hypertension, hypercholesterolemia, inactivity, and genetic factors are known to be involved (9, 10). Considering the role of inheritance in AD, chromosomal instability resulting from a microtubule deficiency, is critical to the development of the disease. Increased damage, such as micronucleus (MN) formation, a sign of improper or incomplete chromosome segregation and DNA cleavage, is seen in AD patients' lymphocytes and fibroblasts (11). The condition in which an organism is made up of cells that contain different numbers of chromosomes is called aneusomy (12). Various studies indicated that some neurodegenerative disorders, including AD, show high levels of chromosomal instability and DNA cleavage in somatic cells in affected individuals (13-18). The mean prevalence of cells with micronuclei in the normal populations is 0 to 0.9% and in EOAD cases was 21.1%, any increase in micronucleus count is a Represent of chromosomal instability (19, 20).

Some studies have shown a significant increase in MN in AD patient cells; however, the origin of these MNs has not yet been determined. According to the loci of APP on chromosome 21, therefore the aim of this study was to identify the origin of MNs using a fluorescence *in situ* hybridization (FISH) probe specific to chromosome 21.

Materials and methods

Subjects

This work is the continuation of an original work performed in the Genetics Research Centre of the University of Social Welfare and Rehabilitation Sciences (19). Twenty patients with EOAD aged 37 to 65 years were examined. Their mean age was 55.77 years. Of these, seven patients were omitted from the study because of problems including history of head injury, insufficient cell numbers in their cultured specimens, demise, or resource limitations.

All the patients voluntarily participated in this study; their next of kin completed a questionnaire that included questions regarding family history of AD, smoking habits, clinical histories, and other background diseases. Each subject in the study signed a consent form. A neurologist assessed the subjects based on their clinical symptoms and verified their AD. Additionally, all the patients were examined with the required laboratory and psychological tests to verify the clinical diagnosis. The inclusion criteria were as follows; willingness to participate in the study, AD onset age below 65, lack of chronic disease condition, and lack of prior participation in similar research. This study was validated by the Ethics Committee of the University of Social Welfare and Rehabilitation Sciences.

The subjects were separated into age groups of 30-39 (two subjects), 40-49 (one subject), and 50-65 (10 subjects). Of the 13 subjects, nine had family histories of AD while four did not, one was a smoker while 12 were not, and 11 were women while two were men.

Cell Culture

Ficoll-Paque was used to isolate lymphocytes from whole blood. The cells were cultured for 72 h in RPMI 1640 medium supplemented with 10% fetal calf serum (Life Technologies), 5 mg/ml phytohemagglutinin (PHA) (Inep-Zemun), and antibiotics (1% cocktail of penicillin/streptomycin, BioWhittaker). Cytochalasin-B was added to the lymphocytes after 44 h of culture. After 72 h of culture the cells were treated for 20 min with hypotonic solution and fixed in 3:1 methanol/acetic acid for 3 × 20 min. Fixed cells were dropped on clean chilled glass slides and air-dried at room temperature (21).

Slide Preparation for Fluorescence in Situ Hybridization (FISH)

Slides were incubated in a Coplin jar with 100 mL of 0.05% Rnase A solution in 2× SSC buffer for 60 min at 37 °C. The slides were washed twice with 100 mL of 2× SSC for 5 min at room temperature in a Coplin jar. They were then incubated in 100 mL of 0.005% pepsin prepared in 10 mM HCl for 10 min at 37 °C in a Coplin jar, followed by washing in 1× phosphate-buffered saline for 5 min at room temperature. Slides were dehydrated in a 50, 75, and 100% ethanol series in a Coplin jar for 5 min each at room temperature and then air-dried (22).

Hybridization

A 10 λ probe specific for the critical 21q22 region of chromosome 21 was placed onto the slide and covered with a coverslip. The probe and samples were denatured simultaneously by incubating the slides for 4 min at 90 °C on a heat block. Slides were then incubated overnight at 37 °C in a humidity chamber to allow probe-to-sample hybridization.

Post-hybridization Wash and Counterstaining

Slides were washed in 100 mL of 2× SSC at room temperature in a Coplin jar to remove the coverslip, followed by another wash in 1× PBS for 5 min at room temperature in a Coplin jar. Slides were dehydrated in a 50, 75, and 100% ethanol series in a Coplin jar for 5 min each at room temperature. Samples were then counterstained with two to three drops of 2 μg/mL DAPI in antifade mounting medium and covered with a coverslip. The slides were incubated for at least 1 h at 4 °C before analyzing.

Analysis

The slides were analyzed with a Zeiss fluorescence microscope with an appropriate filter combination. One thousand cytochalasin-B-blocked binucleated cells were scored per culture. The standard MN scoring criteria of 1/3 diameter, no overlap, and shape were used (23). Cytovision software was used to analyze the results.

Statistical analysis

All the data were analyzed using the latest version of SPSS package software and the differences were examined by ANOVA. The different groups were compared to determine whether age, family history, and disease onset age or duration affected the chromosome 21-related MN origin.

Results

The mean percent of micronuclei originating from chromosome 21 was determined for each age group. Most of the cells in all age groups had no micronuclei (Table 1). No significant differences were found between the age groups for the origin of micronuclei in chromosome 21 ($p < 0.05 < 0.05$).

The mean percentage of MN originating in chromosome 21 was 19.76%, and ranged from 2.2 to 46% (Table 2). No significant association was found between the percent of MN originating in chromosome 21 and disease duration or onset age.

The Mean, median, standard deviation, minimum, and maximum pertaining to the MN data and chromosome 21 aneuploidy were calculated for each EOAD patient sample. Most (80.23%) of the cells counted had micronuclei that showed no signal with the chromosome 21 probe, while 9.31 and 9.95% of the cells had micronuclei with one and two signals, respectively, for the chromosome 21 probe (Table 3).

No significant difference was found between the various age groups with respect to chromosome

21 of micronucleus origin.

In our study, in patients with family histories of AD, 21.2% of micronuclei originated in chromosome 21; in patients with no family histories of AD, 16.52% of micronuclei originated in chromosome 21. This difference was not significant.

The number of micronuclei originating in chromosome 21 was not significantly correlated with EOAD onset age or duration. In binary cells, no signal in the MN meant that the MN did not contain a chromosome 21 sequence complementary to our probe. One or two signals in the MN indicated that one or two whole or partial chromosome 21 sequences were present, respectively. Having a MN with two chromosomes 21 have resulted in monosomy in adjacent cells (Fig. 1).

Table 1. Micronuclei mean data for the EOAD 30-39, 40-49, and 50-65-year-old age groups.

	Age group	Number of subjects (n)	Mean
Micronuclei with no Signal	30-39	2	87.9
	40-49	1	61.3
	50-65	10	80.6
Micronuclei with One Signal	30-39	2	8
	40-49	1	6.9
	50-65	10	10.47
Micronuclei with Two Signals	30-39	2	4.1
	40-49	1	31.8
	50-65	10	8.93

Table 2. Patient data, including MN, origin after using FISH test with a probe specific to chromosome 21, age data and illness duration.

Alzheimer's Patients				
AD / Patient no	Percent of micronuclei originating in chromosome 21 (In%)	Disease Duration in years	Age of Onset	Number of Cells
1	9.2	3	37	1000
2	5.7	15	64	1000
3	2.2	10	64	1000
4	45	6	66	1000
5	5	4	54	1000
6	37.9	4	66	1000
7	46	10	64	1000
8	11.6	3	60	1000
9	38.7	3	46	1000
10	16	6	58	1000
11	12	6	58	1000
12	15	7	37	1000
13	12.6	6	51	1000

Table 3. Mean, median, standard deviation, minimum, and maximum of chromosome 21 MN calculated for EOAD patient cells.

		Mean	Median	Std. Deviation	Minimum	Maximum
Data of Micronucleus Chromosome 21	Micronuclei with No Signal	80.23	87.4	15.97	54	97.8
	Micronuclei with One Signal	9.81	8	7.18	1.2	27
	Micronuclei with Two Signals	9.95	4.8	10.7	1	32

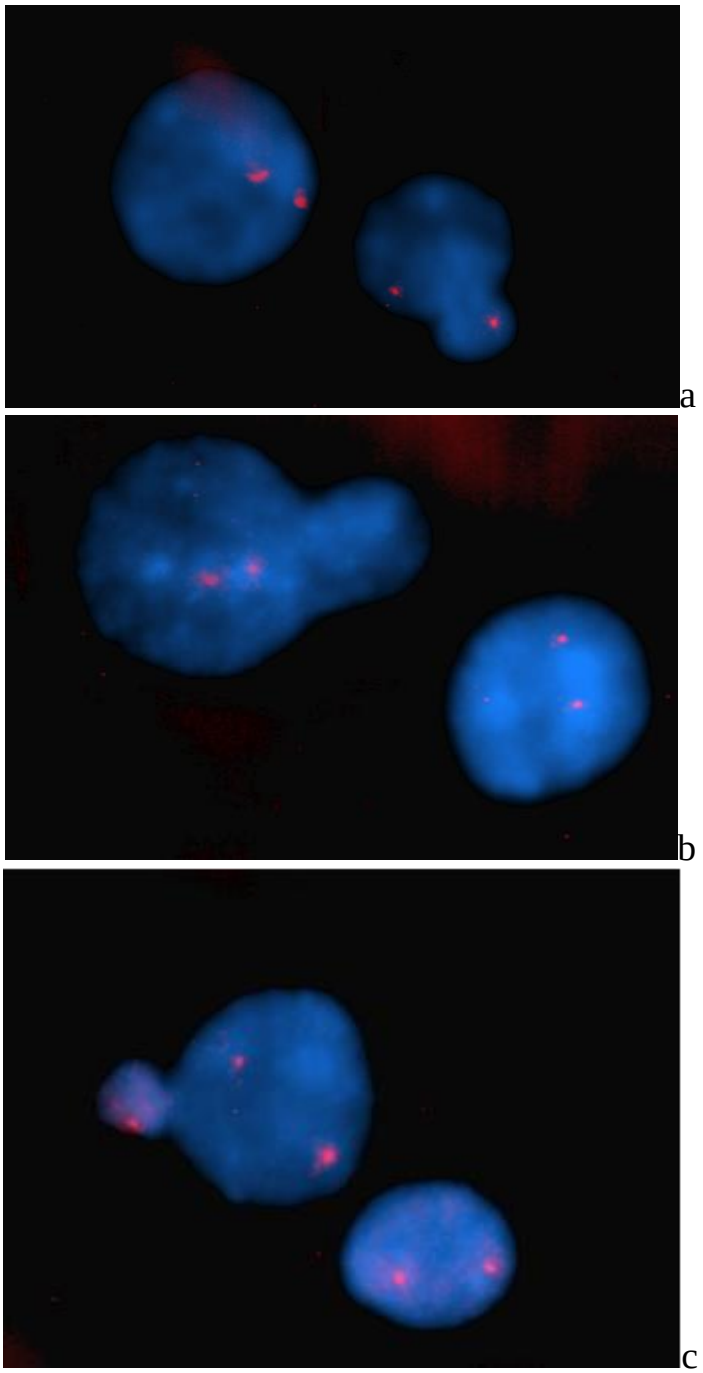

Fig. 1. (a) Two cells, one normal (left) and one with monosomy state (right), with a MN (arrow) showing a signal for a chromosome 21-specific probe, (b) two normal cells showing one with a MN (left) with no signal for the chromosome 21- specific probe, (c) two normal cells, one with a MN (arrow) showing a signal for the chromosome 21- specific probe.

Discussion

It is well known that alterations in the structure and/or function of several mitotic apparatus components, including tubulins, topoisomerase II, microtubule- and centromere-associated proteins, cause mistakes in chromosome distribution to daughter cells, leading to aneuploidy. It is therefore reasonable to predict that some of these components, which could be altered in AD patient neuronal cells (24), may also be altered in peripheral cells.

Biomarkers such as MNs, which can indicate improper chromosome segregation or DNA cleavage, can be utilized as tools to investigate chromosomal instabilities in neurodegenerative

diseases (17). Increased MN formation is a hallmark of neurodegenerative disorders and MNs are useful markers for studies on these disorders (25).

Currently, the best approaches to study these chromosomal instabilities are common cytogenetic and molecular cytogenetic techniques (26). Using these methods, individual cells can be investigated for chromosome number changes. Furthermore, FISH allows researchers leeway to assess and observe the level and percentage of hypo- and hyperdiploid mosaicism in specific cells.

This work is the continuation of an original study performed in the Genetics Research Centre (19). That study included 18 EOAD patients and 20 normal controls with no family histories of neurodegenerative diseases. In this study, we performed FISH in the micronuclei of 13 patients from that cohort. In that study, the mean of micronucleus frequency in the control group was about 8.3 percent.

In our study. 19.76% of micronuclei in the EOAD subjects originated in chromosome 21. Of these, 9.81% and 9.95% showed one and two signals, respectively. These results, showing more MNs and MNs with chromosome 21 origins in EOAD patients than in sporadic AD cases of demonstrate the different genetic mechanisms and genes that are involved in EOAD. In subjects in which chromosome 21 was not detected as the origin of micronuclei, other chromosomes may be involved.

We found no significant differences between the age groups and the origin of micronuclei in chromosome 21 ($p< 0.05$). Furthermore, we observed no significant association between the number of micronuclei originating in chromosome 21 with disease onset age or duration. These observations show that neither the disease onset age nor duration effect the micronuclei chromosomal origin.

Micronuclei frequency was previously shown to increase in lymphocytes and fibroblasts of AD patients (17, 18, 27-29). Recent findings illustrate that the MN level is significantly greater in AD patients' buccal cells than in those of normal individuals of the same age and gender. Moreover, the level of basal cells, which have highly condensed chromatin, is significantly less in AD patients than in normal individuals (30). It has also been shown in AD patient lymphocytes that aggregation of Aβ42 in reduced folic acid conditions induces DNA damage biomarker expression, including MN formation (31).

Another study reported a significantly greater level of MNs in AD and Parkinson's disease patients than in healthy controls. This study also showed that MNs of AD patients are chromosome-shaped with centromeres, while those of Parkinson's disease patients are fragmented in shape with no detectable centromere (28). In another study, 21.1% of AD patient cells had MNs, while only 8.3% of cells from normal controls had MNs. This difference was significant ($p< 001$) (19).

Another study reported that Down syndrome patients with trisomy 21 mosaicism can develop of AD in their third or fourth decade of life (32). Another study found a higher level of MNs in patients with sporadic or hereditary AD than in normal healthy subjects. This MN level increase was detectable in both spontaneous and induced alternatives (18). Increased MN formation in AD patients is an indicator of a deficiency in chromosome segregation during mitosis. Therefore, mutations responsible for AD probably function through causing a crack in the mitotic spindle leading to improper chromosome segregation.

Our study differs from those referenced above in that our samples came from EOAD patients, while in most studies, the samples came from LOAD patients. Another difference is that we used a probe specific to 21q22, a critical region of chromosome 21, while most other studies used a probe for the centromere region of chromosome 21, which overlaps with the centromere region of chromosome 13.

To extend our research work, it is necessary to increase the population size of the study to investigate genomic instabilities. By doing this, we can extrapolate our results to the greater population of EOAD patients. We can also use our method to identify individuals who are at risk of developing AD before the onset of the symptoms. Additionally, we propose using multicolor FISH to identify the origins of all

chromosomes involved in MN formation. In this way, we hope to better understand of the roles of other chromosomes in MN formation.

In conclusion, we showed, 1) in patients with EOAD, 19.76% of MNs originate from chromosome 21; 2) no significant difference was seen between age groups for MNs originating from chromosome 21; 3) the number of MNs that originate from chromosome 21 has no significant correlation with EOAD onset age or disease duration; and 4) the number of MNs that originate from chromosome 21 has no significant correlation with family history of AD.

To investigate genomic instabilities in populations, it will be necessary to increase the subject pools of the study. We also hope to use our method to identify at-risk individuals before the onset of AD symptoms. Additionally, we propose using multicolor FISH to precisely identify all the chromosomes involved in MN formation.

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