Nuclear Protein Contents in Peripheral Blood Mononuclear Cells of Trisomy 21 Infants

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Objective: The trisomy 21 (Ts21) or Down’s syndrome (DS) phenotype is assumed to occur primarily by the expression/overexpression of some genes encoded by the extra chromosome 21. It has recently been shown by AgNOR staining that babies with Ts21 have more AgNOR area (more NOR proteins) and more RNA content in their peripheral blood mononuclear cells (PBMCs) than those of controls. The aim of this study was to test whether or not the nuclear proteins content of PBMCs from trisomy 21 babies/infants is higher than that of the controls.

Method: For this purpose, flow cytometric measurement of the stained PBMC nuclei was used. Nuclei from PBMCs was isolated and stained with propidium iodide and fluorescein isothiocyanate (PI/FITC) for DNA and protein estimation, respectively.

Results: Mean nuclear protein content of Ts21's (N = 30, mean age = 3.46 ± 3.05 years old) PBMCs was found statistically higher than that of the controls (N = 33, mean age = 3.79 ± 1.93 years old) (P = 0.005, nonparametric Mann–Whitney U test for two independent variables). This means that the average nuclear protein content of PBMC from Ts21 infants is higher than that of the controls. Furthermore, there is a moderate negative correlation between the ages of the studied DS patients and the protein content in the nuclei of their PBMCs (Linear regression analysis: P = 0.002, r = −0.55). This correlation is not found with controls (P = 0.186, r = −0.24).

Conclusion: We have concluded that average protein content of PBMCs' nuclei from DS infants is higher than that of the controls, decreasing significantly with age.

Key terms: Down’s syndrome; trisomy 21; nuclear protein contents; PBMC

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Trisomy 21 (Ts21) or Down's syndrome (DS) is by far the most common and best known of the chromosomal disorders. The DS phenotype is assumed to be due to expression/overexpression of some genes encoded by the extra chromosome 21 (1–7). Nevertheless, extensive phenotypic heterogeneity is observed in Ts21 patients (4,8).

Most cases of DS are caused by nondisjunction, which is the failure of homologous chromosomes to separate in meiosis. It is also known that nondisjunction of chromosome 21 is mostly seen in oogenesis than in spermatogenesis, and so the abnormal gamete in DS is usually the egg.

Cytogenetic and molecular studies of individuals with partial duplications of chromosome 21 and features of DS have sought a “critical region” on this chromosome, duplication of which is postulated to be both necessary and sufficient to produce the DS phenotype (9). This has been narrowed down to 21q22.1–q22.3, a region named as the DS Critical Region (DSCR) (10–14). This segment contains about 33 conserved genes (15) and...
has been associated with DS features including craniofacial abnormalities, short stature, joint hyperlaxity, hypotonia, and mental retardation (11,14,16). This DSCR has also been proposed to (be necessary but not sufficient for brain phenotypes of trisomic mice) contain a gene or genes responsible for mental retardation of the DS phenotype, which is characterized by deficits in speech, language, verbal short-term memory, spatial learning, and recall and long-term memory (14). Different studies have shown that DSCR1 gene product belongs to a family of proteins that binds and inhibits calcineurin, a serine-threonine phosphatase (17,18). Other genes identified in this region include DSCR2–6 (13,19–21), DSCR8 (22), and DSCR9–10 (23). The gene dosage effect hypothesis is criticized to be not sufficient to fully explain the DS phenotypes (20).

Our previous investigations have shown that peripheral blood mononuclear cells (PBMCs) of Ts21 infants (mostly neonates) contain more RNA than that of controls. This in vivo (24) and in vitro RNA levels (25) decline, however, with the ages of the patients, to become finally lesser than that of controls as early as in the age of 15–20s.

The presence of the extra chromosome 21 constitutes an inherent protein increase in trisomy 21 cell's nucleus, because these cells contain an extra set of Hsa21 (human 21 chromosome as Homo sapiens 21) histones and nonhistone proteins. Furthermore, silver (AgNOR) staining shows that DS infants' lymphocytes contain more nucleolus organizer regions (NORs) proteins in vivo (26) and in vitro (when stimulated with mitogen) (27–30) than those of controls. Transcription factors genes are encoded by Hsa21 (6) and they are essentially nuclear proteins. Present report shows that average nuclear protein content of PBMC measured by flow cytometry (FC) from DS infants is statistically higher than that of controls. The average nuclear protein levels of PBMC from DS patients decrease, however, with the age of the patients similar as seen in the case of RNA levels (24,25) in PBMC of these patients.

MATERIALS AND METHODS

Patients and Controls

PBMC of 30 patients with DS and 33 healthy controls were studied. Patients with DS were selected from Education and Rehabilitation Center and Pediatric Clinic of Medical Faculty, Erciyes University, Turkey. These patients had no associated anomalies. Healthy controls had normal diploid complement of chromosomes and clinically healthy individuals. The age range was 0–11 for both DS patients and control individuals. The ages of babies less than 6 months were considered as 0 year old and those aged between 6 and 17 months were considered as 1 year old. The other approximations have been made in the same way. DS patients consisted of 20 males and 10 females. Controls consisted of 23 males and 10 females. Mean ages were 3.46 ± 3.05 and 3.79 ± 1.93 years, respectively, for DS patients and controls. Statistical data show that the mean ages of DS and control group are not statistically different: Z = 0.728, P = 0.4616 (Mann–Whitney U tests for two independent variables, with two-tailed statistics). The mean age of cases and controls appears to belong to the same population according to age to compare the data obtained from each group.

The local ethics committee approved the study protocol. The study was conducted in accordance with the declaration of Helsinki and local laws depending on whichever afforded greater protection to the patients.

Isolation of the PBMCs' Nuclei

Nuclear isolation procedure is conducted according to Ref. 31 with a few modifications: 2 ml of heparinized blood was layered on 3 ml of ficoll-hypaque (sigma) and centrifuged for 25 min at 1,300 rpm at room temperature. PBMCs at the interface were aspirated with a Pasteur pipette and washed twice in PBS [5 g FA-Bacto Buffer (Difco, San Jose, CA; cat no: 223142) in 500 ml of distilled water] by centrifugation for 10 min at 900 rpm at room temperature and 500 μl of nuclear isolation buffer [NIB: 0.661 g Tris-HCl (Sigma, St. Louis, MO; cat no: T-3253), 0.097 g Tris-base (Sigma, cat no: T-1503), 0.29 g NaCl (Merck & Co, NJ, USA; cat no: 6400), 0.04 g EDTA-tetra Na (Sigma, cat no: ED455), 0.50 ml NP-40 (Sigma, cat no: 127087-87-0) QS to 100 ml, pH = 7.53] is added on the cells. The number of nuclei was adjusted to a concentration of 1 × 10⁸ nuclei/ml by diluting with NIB.

Nuclear Staining

Nuclear staining was conducted according to Ref. 31 with a few modifications: 1 ml of nuclear suspension is transferred to FC tube and mixed with 900 μl of NaCl-bicarbonate buffer (10.18 g NaCl + 0.25 g NaHCO₃ QS to 100 ml) plus 100 μl of FITC solution [6 mg fluorescein isothiocyanate (Sigma) F-7250 dissolved in 2 ml of bicarbonate buffer and diluted 1,000 folds with NaCl-bicarbonate buffer] and 1 ml of PI solution (6 mg of propidium iodide dissolved in 6 ml of NaCl-bicarbonate buffer and diluted 14.3 folds with NaCl-bicarbonate buffer). This mixture is maintained at 4°C for 30 min before FC measurement.

Flow Cytometry

For analysis of protein and DNA content of PBMC nuclei, Bectman Coulter Epics XL-MCL flow cytometer was used and 10,000 events were collected for each sample. Care was taken to measure both patients' and controls' samples in the same session. Each nucleus has generated fluorescence light scatter signal as it passed through the focus of a 488-nm argon-ion laser beam. The green fluorescence emission (F₁₅₂ mean-X; protein) and the red fluorescence emission (F₂₀₀ mean-X, DNA) for each cell were separated by optical filters and recorded in separate photomultipliers, and the integrated values of cells were stored in computer. The position of each...
marker including its minimum and maximum values was kept constant during the progress of the study for both patients and controls.

DNA staining and its FC measurement is used as a control of reliability of the measurement, because in vivo DNA value is almost constant in PBMC across different individuals. Furthermore, our experiments have shown that protein measurement of PBMC of the same individual in the presence of PI staining was found to be more reliable than protein measurement by FITC staining alone. In most applications, protein stains are not used alone, but are combined with DNA fluorochromes (32).

### Statistical Analysis

Differences between DS patients and controls in $F_{525}$ mean-X values were tested using nonparametric Mann–Whitney $U$ test for two independent variables. Significance was set at $P < 0.05$ with two-tailed probability (Table 1). Linear regression analysis was used to assess the relationship between the age and nuclear protein contents as $F_{525}$ mean-X values.

### RESULTS

#### Experiments to Ensure the Specificity/Reliability of Protein Measurement in PBMCs’ Nuclei by Flow Cytometry

Pollack et al. (33), using FITC/PI to quantitate simultaneously nuclear protein and DNA have been able to delineate nuclear protein and DNA increase during phytohemagglutinin stimulation of human peripheral blood lymphocytes. The same increases have been observed during the growth of HeLa cells (34). To ensure the specificity of protein staining, PBMC nuclei from three different healthy adults (average age ~35 years old) were double stained with FITC/PI as described in the Material and Methods section, and their nuclear protein/DNA levels have been evaluated by FC at zero time and after 48 h of culture. The results were as following: Protein ($F_{525}$ mean-X) and DNA ($F_{620}$ mean-X) at zero time were found equal to 166.00 ± 3.57 and 141.43 ± 5.52, respectively. After 48 h of phytohemagglutinin stimulation in Peripheral Blood Karyotyping Medium (cat no: 01-198-1B, Biological Industries, Haemek, Israel), their relative protein and DNA values were 257.50 ± 5.57 and 204.33 ± 7.16, respectively. This experiment has led us to conclude that our method used to estimate and to compare protein and DNA level in PBMC nuclei of DS patients and control individuals by FC measurement after FITC/PI double staining is sufficiently specific and reliable.

#### Comparison of Nuclear Protein Content in PBMCs from DS Patients and Healthy Controls

Table 1 resumes the statistics of protein and DNA contents in PBMC nuclei of 30 DS and 33 healthy controls. The average protein content of PBMC nuclei of DS patients is statistically different and higher from that of the controls, whereas the means of DNA content of the same cells’ nuclei are not different between DS and control individuals. Statistical data show that the mean age of DS and control group are not statistically different. The two groups appeared to belong to the same age population when the data obtained from each group

### Table 1

<table>
<thead>
<tr>
<th>Groups</th>
<th>Number</th>
<th>Nuclear protein$^a$ content, $F_{525}$ mean X (mean ± SD)</th>
<th>Nuclear DNA$^b$ content, $F_{620}$ mean X (mean ± SD)</th>
<th>Age$^c$ (year) (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DS patients</td>
<td>30</td>
<td>181.75 ± 13.46</td>
<td>142.30 ± 5.07</td>
<td>3.46 ± 3.05</td>
</tr>
<tr>
<td>Controls</td>
<td>33</td>
<td>172.71 ± 8.96</td>
<td>142.76 ± 5.57</td>
<td>3.79 ± 1.93</td>
</tr>
</tbody>
</table>

DS cases ($N = 30$) contain statistically higher nuclear protein level ($F_{525}$ mean-X) than that of the controls ($N = 33$) (Two-tailed nonparametric Mann–Whitney $U$ test for the comparison of the means of the two independent variables). The table shows that our FC measurement of nuclear DNA content after PI staining is not sensitive to the extra chromosome 21 DNA (~33.5 million bp) of DS patients, which constitutes only a ~0.5% of whole diploid human genome (~6.4 billion bp).

$^aZ = 2.787; P = 0.005.$

$^bZ = 0.158; P = 0.874.$

$^cZ = 0.728; P = 0.616.$
were compared. Furthermore, the graphic in Figure 1 shows that the average protein content of the PBMC nuclei from infant patients with DS decreases with the increasing age of these infants. This decrease with age is statistically significant (Linear regression analysis: $P = 0.002, P < 0.05$) and demonstrates a moderate negative relationship ($r = -0.236$) between the age and protein content ($35$) in PBMC nuclei of DS infants. Healthy controls do not show a relation between the age and protein content (35) in PBMC nuclei of DS infants. Healthy controls show more protein level in their PBMC nuclei than that of some DS patients. The apparent slope of the line is due largely to the nuclear protein level of an 11 year old infant, and if her data is not evaluated, the line will be quasi horizontal.

No significant relationship was found between the two sexes regarding their PBMC nuclear protein content for both DS patients and healthy controls ($P > 0.05$).

**DISCUSSION**

A study concerning the comparison of nuclear protein level between DS patients and healthy individual cells has not been found in the literature. The starting point of this study was the observation that DS infants’ interphase lymphocytes contain more NORs area (26,27,29,30) and their metaphase lymphocytes contain more AgNOR($+$) chromosomes (28,29) than that of controls. In other words, DS cells have shown more NORs proteins in their interphase nuclei and metaphase cells than those of controls. It might be interesting and worthwhile to compare nuclear protein content between DS patients and healthy individuals in the nuclei of a given tissue. For this, PBMCs have been used. The challenge (8) and highly variable phenotype (4,8) of DS patients appear also in this area. The age-dependent decrease in protein content in PBMC nuclei of DS patients is similar to the age-dependent decrease in RNA content in PBMC of DS patients (24,25).

The major Ag-NOR proteins associated with the ribosomal genes were found as the largest RNA polymerase I subunit, the 135-kD NOR protein, the UBF transcription factor, and a 50-kD protein (36). During interphase, the major Ag-NOR proteins in human cells correspond to nucleolin, protein B23 (nucleophosmin) and 42-, 40-, and 29-kD polypeptides (36). Nucleolin and protein B23 are related to the cell population doubling time in human (37) and rat (38) cancer cells. Protein B23 loss results in severe dysregulation of the developmental and growth-related events (39). A few number of nucleolar proteins were characterized and/or denominated. For example, about 50 nonribosomal proteins are associated with the early nucleolar pre-60S ribosomes (40).

It is plausible to admit that the average nuclear protein enhancement observed in PBMC nuclei of DS infants is due essentially to the nucleolar protein enhancement in these cells’ nuclei including ribosomal, nonribosomal and NORs proteins. The direct/indirect effects of high protein levels in PBMC nuclei of DS infants to their nuclear architecture and health are unknown. With the present data and knowledge, except for the observation that the total nuclear protein and total cellular RNA levels (24,25) with ages are well-adjusting, indicating an inherent rRNA/protein parallel-ism, we do not know which special type of RNA/protein plays a crucial role in the higher protein levels in PBMC nuclei of DS infants.

Although the mean value of protein contents in PBMC is higher in infants with DS, these subjects suffer nonetheless from immune deficiencies. In fact, RNA and protein excess in the cells of DS patients at the early stage of their development compared to controls appears unnecessary/useless, even proposed to be harmful because of the wasted energy in producing unnecessary proteins and RNA transcripts (28). Furthermore, ribosomal subunits after synthesis are exported to the cytoplasm in energy-dependent fashion (41).

PBMC are a mixture of monocytes and lymphocytes and it is worthwhile to compare them separately in their
protein and RNA content between DS patients and controls in function of age.

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LITERATURE CITED

Cytometry Part B: Clinical Cytometry