

Plasma cell-free DNA levels in children with Henoch Schönlein purpura

[Henoch-Schönlein purpuralı çocuklarda plazma serbest DNA düzeyleri]

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ABSTRACT

Objective: Circulating free nucleic acids are found at low levels in healthy subjects and in some diseases. The source is reported to be apoptosis of lymphocytes and other nucleated cells. The aim of this study is to investigate cfDNA levels in children with Henoch Schönlein purpura (HSP) during acute and remission phases.

Methods: We investigated cfDNA levels in 21 children with HSP in acute phase and 21 in remission and in 25 healthy controls using real-time quantitative PCR.

Results: We found significantly increased cfDNA levels in acute HSP patients (median: 6632.5 pg/ml) compared to those in remission (4540.8 pg/ml) and healthy controls (4650 pg/ml) ($p<0.05$).

Conclusion: Significantly increased cfDNA levels in acute phase of HSP might be explained by increased apoptosis. Measuring cfDNA levels may have a potential role for monitoring HSP activity. For this finding, determination of complex interactions at cellular level and prognostic significance need further research.

Key Words: Apoptosis, cell-free DNA, Henoch-Schönlein purpura, children Plasma.

Conflict of Interest: The authors declare no conflict of interest.

ÖZET

Amaç: Sağlıklı bireylerde ve bazı hastalık durumlarında dolaşımda serbest nükleik asitler bulunurlar. Bu nükleik asitlerin kaynağının lenfosit ve diğer çekirdekli hücrelerin apoptozisi olduğu bildirilmektedir. Bu çalışmada Henoch Schönlein purpuralı (HSP) çocuklarda akut hastalık ve remisyon fazlarında plazma serbest nükleik asit (cfDNA) düzeylerinin araştırılması amaçlanmıştır.

Metod: Gerçek-zamanlı kantitatif PCR yöntemi kullanarak 21 akut, 21 remisyonunda olan HSP li çocuk ve 25 sağlıklı kontrolde plazma cfDNA düzeylerini araştırdık.

Bulgular: Akut HSP hastalarında cfDNA düzeylerini (medyan: 6632.5 pg/ml) remisyonundaki (4540.8 pg/ml) ve sağlıklı kontrollerdeki (4650 pg/ml) düzeylere göre istatistiksel olarak anlamlı düzeyde yüksek bulduk ($p<0.05$).

Sonuç: Akut HSP'li çocuklarda cfDNA düzeylerinin anlamlı düzeyde yüksek bulunması bu hastalardaki artmış apoptotik aktivite ile açıklanabilir. Hastalık aktivitesinin izleminde cfDNA ölçümünün potansiyel bir rolü olabilir. Bu bulgunun hücresel düzeydeki karmaşık etkileşimlerinin ve prognostik öneminin belirlenmesi için ileri çalışmalara gereksinim vardır.

Anahtar Kelimeler: Apoptozis, serbest nükleik asitler, Henoch-Schönlein Purpura, çocuklar.

Çıkar Çatışması: Yazarların çıkar çatışması yoktur.

Introduction

Small amounts of cfDNA are normally present in the plasma of healthy subjects. The origin is reported to be the apoptosis of lymphocytes and other nucleated cells or an active physiological secretion by lymphocytes [1-3]. Elevated levels of cell-free nucleic acids have been reported in various clinical conditions such as cancer, stroke, trauma, myocardial infarction, autoimmune disorders, pregnancy complications and peritoneal dialysis. This fact has stimulated researchers to investigate the possibility to use circulating nucleic acids as a potential marker for early detection or follow-up of some diseases [2,4-10]. Henoch-Schönlein purpura (HSP) is the most common vasculitic disease of childhood with potentially serious systemic involvement. Recent reports suggest an intriguing relationship between HSP and apoptosis [11-13]. In this study we aimed to investigate the cfDNA levels in children with HSP during acute and remission phases in order to analyze its relation with disease activity.

Materials and Methods

Patients

Twenty-one children (Male/Female: 15/6) with acute HSP and 21 in remission (Male/Female: 14/7) (median age 12: range 4-18) and 25 age and sex matched healthy children (M/F: 8/17) (median age: 10; range 6-16) were enrolled in the study. American College of Rheumatology criteria were used for the diagnosis of HSP [11]. Other inflammatory conditions like severe infection or diseases affecting liver function were accepted as the exclusion criteria. As for the remission group; children who had had HSP at least six months before and who had no systemic finding at the moment of blood sampling were included in the study. The present study was conducted under written informed consent of the parents and approved by the local Ethics Committee.

Blood sampling, measurements

Blood samples were taken in the morning at first presentation in acute phase patients and after an overnight fast in healthy controls and remission phase patients. They were stored at -70 °C until analyzed. We used the protocol sug-

gested by Chiu RWK et al. [14] Peripheral blood from each participant was collected in EDTA tubes and they were centrifuged at 1600g for 10 min at 4 °C, followed by further centrifugation of plasma in new tubes at 16 000 g at 4 °C. The plasma samples then were stored at -80 °C until analysis. DNA was extracted from 600 µL of plasma according to the protocol of the High Pure viral Nucleic acid kit (Roche Diagnostics GmbH, Penzberg, Germany), and eluted in 100 µL of H₂O. We then subjected 10 µL of the DNA to real time quantitative PCR for the β-globin gene as described in the protocol of LightCycler Control Kit DNA using LightCycler FastStart DNA Master HybProbe on LightCycler version 1.5 to amplify a 110 bp fragment from the human beta-globin gene with specific primers. Amplification of target DNA was monitored using Hybridization Probes that hybridize to an internal sequence of the amplified fragment. Results were expressed as “pg DNA /ml of plasma”.

Statistical methods

Median values of the measurements were used for statistical analysis. Kruskal-Wallis test was used for three-group comparison. Statistical differences between the two groups were analyzed with Wilcoxon and Mann-Whitney U-tests. P values less than 0.05 were considered significant.

Results

Demographic and laboratory characteristics of the groups are given in Table 1. All the patients had purpura. The clinical presentations of the patients were of mild to moderate severity with no central nervous system involvement. No renal involvement finding was present in acute phase group. But in remission group 7 patients (33%) had minimal urinary findings in their first presentation such as microscopic hematuria and/or non-nephrotic proteinuria and they were resolved completely at the time of blood sampling. Therefore in none of them kidney biopsy was indicated. Gastrointestinal and musculoskeletal system involvement were relatively more frequent ranging from 38 to 52% (Table 1) The median cfDNA levels were significantly increased in acute HSP patients (6632.5 pg/ml) compared to remission (4540.8 pg/ml) and control (4650.0 pg/ml) groups (p<0.05) (Figure 1).

Table 1. Demographic and laboratory characteristics of the groups

Findings	Acute phase (Group 1)	Remission (Group 2)	Control (Group 3)
Age (year)	6.2±2.8	8.0±3.0	7.5±2.9
Male/Female (n)	15/6	14/7	8/17
Median cfDNA levels (pg/ml)*	6632.5	4540.8	4650.0
Renal involvement	-	7 (33%)**	-
Gastrointestinal involvement	8/21 (38%)	11 (52%)**	-
Musculoskeletal involvement	11/21(52%)	8 (38%)**	-

*p<0.05, **At first presentation

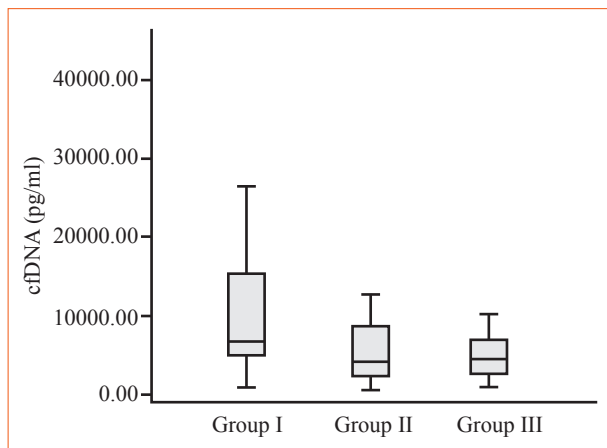


Figure 1. cfDNA levels in study groups.

Discussion

The idea to use cfDNA in the diagnosis or follow-up of certain diseases as a quick and relatively less invasive tool has been getting more popular in laboratory medicine especially in the fields of oncology, cardiology, obstetrics and internal medicine. However there is still a long way to go for widespread routine clinical application. [2,4-10] The exact mechanism of cfDNA presence in circulation is not clear. Release from apoptotic or necrotic cells are reported to be among the main sources [1,2]. In this study, for the first time we have investigated the cfDNA levels in children with HSP and demonstrated significantly increased cfDNA levels in acutely ill children compared to those in remission and healthy controls.

Although we did not confirm the significantly increased apoptosis by commonly known markers like caspases, we think that elevated cfDNA levels in acute HSP patients is of apoptotic origin. This thought is based on the fact that particular size of DNA segments that is 110 base pairs fragments, measured in our study is within the range of apoptotic origin of 80 to 200 base pairs as reported in previous studies [15,16]. In addition reports on the role of apoptosis in various clinical forms of HSP also agree with this hypothesis. In the study of Özaltın et al. [12] the authors reported increased apoptosis of peripheral blood neutrophils and lymphocytes in the acute phase of children with HSP compared to resolution phase and they suggested that this might have had a contributory role in the early control of the inflammatory response and repair in this self-limited vasculitis. In the immunohistochemical study of Bek et al. [13] investigating the renal expression of proapoptotic and antiapoptotic proteins “bcl-2” and “bax”, the authors reported predominance of antiapoptotic factors in children with severe HSP nephritis with unfavorable prognosis, a result seemingly in accordance with the suggestion of Özaltın et al. [12] addressing the apoptotic control of early inflammation. The evidence for apoptosis-vasculitis association is not limited only for HSP but also for other vasculitides or autoimmune dis-

eases [10,17-19]. For example Holdenrieder et al. [10] reported elevated levels of apoptotic products, caspases and nucleosomes in patients with SLE, Wegener’s Granulomatosis and Microscopic polyangitis. The nucleosomes measured in their study are the basic elements of chromatin formed by a core protein consisting of an octamer of histones and about 146 bp of ds-DNA that is wrapped around it [20], and the nucleosomes are among the forms of circulating DNA along with shed cells, apoptotic bodies, other nucleoproteins and free DNA [21].

However there are some limitations of our research. Inability to make experiments immediately on fresh samples might have affected the measurement of actual levels of cfDNA with very short half-life. But since we have control groups, this preanalytic confounding factor, if any, might have had operated for all groups equally. Thus we believe that this did not lead to a significant risk of sampling bias. Secondly, it is not possible to compare our results with the other studies since there is no similar study done either in children or in adults. In addition, the patient group in our study is of mild to moderate clinical severity with no serious systemic involvement. Therefore it is not possible to make any comparison or correlation analysis between the different clinical presentations of HSP and cfDNA levels. Especially renal or central nervous system involvements are of greater importance in terms of morbidity and prognosis in children with HSP. So, a study measuring and comparing the cfDNA levels in more severe forms of HSP such as severe renal and/or central nervous system involvement with long term prognosis might reveal very informative results. Correlation studies between disease activity/severity and cfDNA levels might be designed similar to the previous examples of such studies in the current literature. But some controversy does exist in these studies. While some of them reported correlation of cfDNA levels with disease activity or relationship with unfavorable outcome in situations like systemic sclerosis, experimental pulmonary thromboembolism, severe sepsis or intensive care unit patients; such a study done in SLE patients revealed not very promising results [22-26]. Atamaniuk et al. [26] in their research measuring the levels of circulating DNA as a potential tool to assess and to predict disease activity in patients with SLE indicated that cfDNA plasma levels in patients with SLE were significantly increased. The authors stated that especially apoptosis and necrosis would lead to an increase in measurable free plasma DNA, however they found no correlation between cfDNA levels and SLE activity. They attributed this result to cfDNA peaks being rather short-lived events and therefore they reported that their findings excluded measuring free plasma DNA as a marker to assess disease activity in patients with SLE. But we think that aforementioned controversy in such studies may still warrant designing such a study in children with HSP.

In conclusion this is a preliminary study investigating the cfDNA levels in children with HSP. Significantly

increased cfDNA levels in acute HSP might indirectly indicate increased apoptosis in the acute phase of this common vasculitis of children. However, since our study is not directly measuring the apoptotic activity, the comments about the role of apoptosis are completely based on indirect evidence from the literature and should be regarded with scientific scepticism. Due to cross-sectional and observational design of the study, it is not possible to withdraw a certain conclusion regarding the clinical significance of this finding. Prospective studies in HSP patients with more severe systemic involvement are needed to search the impact on or relevance with long term prognosis. Our results might serve as a base-line finding for future studies investigating the clinical significance of this elevation for more severe forms of the disease.

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Conflict of Interest

There are no conflicts of interest among the authors.

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