Review

The role and diagnostic value of cell-free DNA in systemic lupus erythematosus

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ABSTRACT

Cell-free DNA (cfDNA) represents a small fraction of total DNA pool that circulates freely in the blood both in normal and pathological conditions. Data indicate that cfDNA plays an important role in the pathogenesis of systemic lupus erythematosus (SLE) and hypomethylation may be crucial for its immunogenic properties. Although differences in quantification methodology hinder the comparison of results between the studies, it appears that levels of cfDNA are abnormally elevated in SLE patients and correlate with various antibody titers, but not with disease activity. Increased cfDNA concentration, however, may be associated with active lupus nephritis. Most of the studies confirmed apoptosis as the major cfDNA release mechanism in various conditions, but formation of neutrophil extracellular traps may significantly contribute to the cfDNA generation in SLE patients. In this review, we summarise current knowledge about the role and possible origin of cfDNA in SLE patients, and discuss why cfDNA testing for diagnostic and prognosis of SLE remains questionable.

Introduction

Most of the human DNA is located within a cell – in the nucleus and mitochondria. Cell-free DNA (cfDNA) represents a small fraction of total DNA pool that circulates in the bloodstream of healthy individuals. Its cellular origin and release mechanisms are still not fully understood. Interestingly, changes in cfDNA concentration have been associated with many disorders including various types of cancers (1-6), myocardial infarction (7-8), stroke (9), pregnancy pathologies (10-12), and autoimmune diseases (13-15). Circulating cfDNA appears to play a central role in the pathogenesis of systemic lupus erythematosus (SLE), therefore, it was extensively studied in lupus patients. SLE is a chronic autoimmune disease with a wide spectrum of clinical presentations. It can affect almost every organ of the body and its severity ranges from mild to potentially life-threatening. The course of disease is unpredictable, with periods of flares and remissions. Common clinical manifestations include mucocutaneous features, arthritis, Raynaud’s phenomenon, nephritis, vasculitis, gastrointestinal tract and nervous system involvement, lymphadenopathy, pleuritis, and pericarditis (16). Lupus pathogenesis is based on genetic and environmental factors, but precise pathomechanism still remains elusive (17). The disease may occur in genetically susceptible individuals and is initiated by certain environmental factors. Triggering stimuli, that may be relevant to the pathogenesis of SLE, include ultraviolet light, drugs, chemical factors, and possibly infectious agents (18-23). Lupus is characterised by several immunological abnormalities, both in the adaptive and innate immunity. Defective immunoregulation leads to the production of autoantibodies, lymphocytes hyperactivation, chronic inflammation, and immune complexes deposition, which causes tissue and organ damage (24).

Cell-free DNA levels

Ranges and mean cfDNA concentration, both in healthy and ill subjects, vary between the studies. Moreover, differences in sample processing and lack of standardisation of cfDNA extraction and quantification do not permit a direct comparison of the results (25). Most of the investigators confirmed that plasma cfDNA concentration in healthy individuals ranges from 0 ng/ml to about 89.8 ng/ml (26-28). Cell-free DNA levels in SLE patients were

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significantly increased comparing with healthy controls in all studies (29-38). In the analysis performed by Tug et al. in 59 SLE patients mean concentration of total cfDNA was 44.7±53.5 ng/ml, with concentration ranging from 2.05 ng/ml to 223.2 ng/ml (35). Cell-free DNA levels observed by Hendy et al., in comparable group of patients (n=52) were lower, with mean concentration of 16.31±2.58 ng/ml (36). Using different quantification method, Zhang et al. reported higher cfDNA ranges in both SLE patients (mean 236.66±40.09 ng/ml) and healthy controls (mean 187.96±40.55 ng/ml) (37). Therefore, in order to compare the results between the studies and to determine whether analysis of cfDNA levels might be useful for discriminating between healthy individuals and SLE patients, a standardisation of cfDNA measurements is necessary (Table 1).

**Release mechanisms**

Multiple sizing experiments revealed significant differences between cfDNA fragments length in SLE and in healthy controls. Suzuki et al. found that in healthy population plasma cfDNA mostly consists of 176 bp fragments, with no units larger than 2000 bp (40). Further sequencing studies confirmed the size distribution of plasma cfDNA molecules, showing the major peak around 166-169 bp, with the number of smaller peaks at intervals of approximately 10 bp (41-42). This periodicity was observed only in autosomal fragments of cfDNA, whereas mitochondrial cfDNA fraction exhibited no major peaks and a wider range of sizes (42). Circulating cfDNA in SLE patients was characterised by an abnormal pattern of cfDNA fragmentation. Gel electrophoresis studies revealed that plasma cfDNA from SLE patients consists of 150-200 bp, 400 bp, 600 bp, and 800 bp fragments (43). Additionally, Galeazzi et al. observed a high molecular weight DNA band of approximately 33kb length in all SLE samples, but not in healthy individuals and systemic sclerosis (SSc) patients (44). Sequencing studies revealed that cfDNA in SLE patients contained fewer 166-bp fragments and more DNA molecules of different length, particularly fragments shorter than 115 bp, comparing with healthy controls (45). Those changes were more pronounced in SLE patients classified as the active group, with SLEDAI over 6. Interestingly, the percentage of cfDNA fragments shorter than 115 bp increased comparing with healthy controls (45).

**Table 1. Comparison of methods used for cfDNA analysis in SLE.**

<table>
<thead>
<tr>
<th>Author, year</th>
<th>Patients</th>
<th>Concentration of cfDNA</th>
<th>Isolation method</th>
<th>Quantification method</th>
<th>Material</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raptis et al., 1980 (29)</td>
<td>n=7</td>
<td>293±57 ng/ml (inactive SLE) 3231±1122 ng/ml (active SLE)</td>
<td>Phenol-based extraction</td>
<td>DNA labelling by nick translation</td>
<td>Plasma</td>
</tr>
<tr>
<td>McCoubrey-Hoyer et al., 1984 (30)</td>
<td>n=31</td>
<td>66.90±83.68 ng/ml</td>
<td>Phenol-based extraction</td>
<td>Counterimmunoelectrophoresis and precipitin line comparison</td>
<td>Plasma</td>
</tr>
<tr>
<td>Chen et al., 2007 (31)</td>
<td>n=12</td>
<td>78.1 ng/ml</td>
<td>Silica-based extraction</td>
<td>PicoGreen fluorometric assay</td>
<td>Serum</td>
</tr>
<tr>
<td>Atamanuik et al., 2011 (33)</td>
<td>n=13</td>
<td>90 (64–103) pg/μl*</td>
<td>Not specified</td>
<td>Vistra Green fluorometric assay</td>
<td>Plasma</td>
</tr>
<tr>
<td>Bartoloni et al., 2011 (34)</td>
<td>n=44</td>
<td>35.0±9.0 ng/ml</td>
<td>Silica-based extraction</td>
<td>Real-time PCR (hTERT gene)</td>
<td>Plasma</td>
</tr>
<tr>
<td>Cepika et al., 2012 (32)</td>
<td>n=15</td>
<td>Not specified</td>
<td>Silica-based extraction</td>
<td>Real-time PCR (Alu-Ya5 gene)</td>
<td>Serum</td>
</tr>
<tr>
<td>Tug et al., 2014 (35)</td>
<td>n=59</td>
<td>28.9±42.8 ng/ml (L1PA2222)</td>
<td>Analysis of unpurified plasma</td>
<td>Real-time PCR (L1PA2222, L1PA290 genes)</td>
<td>Plasma</td>
</tr>
<tr>
<td>Zhang et al., 2014 (37)</td>
<td>n=54</td>
<td>236.66±40.09 ng/ml</td>
<td>Analysis of unpurified plasma</td>
<td>PicoGreen fluorometric assay</td>
<td>Plasma</td>
</tr>
<tr>
<td>Fragoulis et al., 2015 (38)</td>
<td>n=5</td>
<td>60.0 (9.8–88.0) ng/ml*</td>
<td>Silica-based extraction</td>
<td>Real-time PCR (β-globin)</td>
<td>Serum</td>
</tr>
<tr>
<td>Hendy et al., 2016 (36)</td>
<td>n=52</td>
<td>16.31±2.58 ng/ml</td>
<td>Silica-based extraction</td>
<td>Real-time PCR (GAPDH gene)</td>
<td>Plasma</td>
</tr>
</tbody>
</table>

Data are presented as mean ± SD, unless stated otherwise. *median (quartiles).

cfDNA: cell-free DNA; SLE: systemic lupus erythematosus; PCR: polymerase chain reaction; hTERT: human telomerase reverse transcriptase; GAPDH: glyceraldehyde 3-phosphate dehydrogenase.
lowing programmed cell death. However, the precise mechanisms by which excessive amounts of highly fragment-
ed cfDNA enter the circulation in SLE remain unclear. Accumulation of short cfDNA fragments might be caused by an abnormal DNA cleavage during cell death, since it was found that SLE pa-
tients show decreased ability to degrade chromatin released from both necrotic and viable cells (50). Moreover, based on the observation that short cfDNA fragments were enriched in the IgG-bound fraction, Chan et al. proposed a preferential binding of anti-DNA anti-
bodies to those fragments, which protect them from enzymatic degradation and clearance mechanisms (45). It is also possible that observed differences in cfDNA size distribution are caused by the increase of DNA secretion from living cells, possibly in the form of neu-
trophil extracellular traps (NETs).

**Neutrophil extracellular traps**

NETs formation is the unique antiimi-
crobial mechanism, in which neutro-
phils secrete netlike structures to trap and kill microbes (51). This process, termed NETosis, is distinct from apo-
tosis and necrosis and does not require DNA fragmentation (52). Neutrophil traps are composed of DNA, histones, and various antimicrobial proteins (53). In healthy individuals, NETs are secreted at the site of inflammatory re-
sponse and are subsequently degraded by endonuclease DNase I (54). Interest-
ingly, it was shown that the exces-
sive production and impaired clearance of NETs might be associated with in-
creased levels of cfDNA in SLE. Pa-
tients with SLE have decreased abil-
ity to degrade NETs, which is mainly caused by the presence of DNase I inhibitors and anti-NETs antibodies that protect neutrophil traps from en-
zymatic cleavage (54, 55). Interest-
ingly, NETs-degrading ability changes with the course of disease and could be partially restored by the addition of exogenous DNase I (55). Inefficient NETs degradation was also associated with SLEDAI, kidney involvement and pleuritis (54-56). NETs-degrading sta-
tus was also strongly correlated with anti-NETs and anti-dsDNA antibody
titters (54). Additionally, low density granulocytes (LDGs), an abnormal neutrophil subset with the enhanced ca-
pacity to form NETs, secrete excessive amounts of NETs in SLE patients (57). All NETs expose dsDNA, hence it was postulated that NETs formation could be an additional source of circulating cfDNA. Zhang et al. found that the percentage of LDGs was significantly higher in SLE patients compared with healthy controls, and plasma cfDNA levels correlated positively with LDG and neutrophil levels (37). Moreover, cfDNA concentration was higher in SLE patients with elevated LDG level. Those correlations were not observed in healthy controls. The authors postulat-
ed that in SLE patients about 41.2% of the cfDNA level elevation is caused by the excessive release of NETs by LDGs and neutrophils. It also appears that the contribution of NETosis to the plasma cfDNA generation in healthy subjects is negligible. Interestingly, despite the fact that DNase I activity in SLE pa-
tients was significantly lower compared with healthy individuals, no significant correlation was observed between cfDNA level and DNase I activity (37). The authors proposed that lack of correla-
tion might have been caused by the in-
terference of other factors that prevent-
ed DNase I from degrading NETs, for example anti-NETs antibodies (Fig. 1).

**Genomic distribution**

Circulating cfDNA in SLE exhibits sig-
nificant changes in genomic distribu-
tion comparing with healthy controls. High-throughput sequencing of serum cfDNA in healthy subjects revealed that 87% of sequences wereattrib-
able to known database sequences, of which 97% were genomic, and 3% were xenogeneic. There were no differ-
ence in genes, pseudogenes, transcribed regions, and protein-coding DNA se-
quences between cfDNA and genomic DNA, but circulating DNA tended to have higher proportion of Alu repeti-
tive elements and lower proportion of long interspersed nuclear elements L1 and L2 (41, 58-59). Representation of serum cfDNA sequences correlated with chromosome size, with the excep-
tion for chromosome 19 which showed only 81% of the expected expression (41). Interestingly, chromosome 19 has the highest gene density, amount of Alu elements, and GC content of all chromosomes (60). Further analysis did not reveal any significant correla-
tions between chromosomal represen-
tation in the serum and chromosome gene density or GC content (41). Dif-
ferently, plasma cfDNA profiles in SLE patients showed significant differences in genomic distribution (45). Multiple under- and over-representations called aberrant measured genomic represen-
tations (MGRs) were found in cfDNA pool of SLE patients, but not in healthy individuals. The median percentage of genome segments with aberrant MGRs was 8.1% in SLE patients with active disease (range 1.1–52%), and 6.5% in patients with inactive disease (range 0.5–32.1%). Interestingly, the extent of aberrant MGRs correlated with anti-
-dsDNA antibody level, but not with the disease activity measured by SLE-
DAI. Also, more IgG binding occurred at genomic locations with increased MGRs.

Data obtained from sequencing stud-
ies suggest that non-random degrada-
tion or clearance of cfDNA occurs both in healthy individuals and in SLE pa-
tients (42). Beck et al. postulated that cfDNA generated as a result of apop-
totic or necrotic unspecific nuclear DNA cleavage should be distributed uniformely in the entire genome. Only some highly histone protected regions would be overrepresented in cfDNA pool (41). Such a distribution was ob-
erved neither in healthy population nor in SLE patients. Moreover, uneven genomic representation was more pro-
nounced in SLE patients. This could be explained by the significant distur-
bances in the process of non-random DNA degradation. However, based on the observation that IgG antibody bind-
ing of plasma cfDNA may be related to the presence of aberrant MGRs, Chan et al. proposed a different hypothesis. Preferential binding of anti-DNA anti-
tibody to particular cfDNA sequences and formation of antibody-cfDNA im-
mune complexes could protect certain cfDNA molecules from enzymatic de-
gradation and clearance mechanisms.
Retention of antibody-bound cfDNA would increase the genomic representation in some regions, whereas regions with less preferential antibody binding would be underrepresented. These differences might have been detected as increased or decreased MGRs (45). Why preferential binding and retention of certain antibody-cfDNA complexes would be observed only in SLE patients remains unclear. Further studies are necessary to determine what is the significance of aberrant MGRs in cfDNA pool of SLE patients.

Methylation status and immunogenicity
Circulating cfDNA in patients with SLE may be immunogenic. It was found that plasma DNA obtained from patients with active SLE induces polyclonal and monoclonal production of anti-DNA antibodies after injection into experimental animals (61). Interestingly, hypomethylation may be crucial for cfDNA to exhibit its immunogenic properties. Administration of syngeneic apoptotic DNA triggered SLE-like disease with proteinuria and anti-DNA antibodies production in non-susceptible mice, but only when DNA was hypomethylated (62). The relevance of DNA demethylation in the pathogenesis of SLE was further confirmed in several studies (63-66). Both genomic DNA in T cells (67) and plasma cfDNA is hypomethylated in patients with SLE (45). Interestingly, the extent of cfDNA hypomethylation correlated with the anti-dsDNA antibody level and SLEDAI. Also, in patients with active disease cfDNA was more hypomethylated compared with both the inactive SLE group and with healthy individuals. Further analysis showed that especially cfDNA fragments of ≤115 bp showed decreased methylation density (45). These findings suggest that cfDNA hypomethylation may be associated with the pathogenesis of SLE. Based on the observation that plasma cfDNA exhibits immunogenic properties and its methylation status is associated with SLE activity, it seems probable that changes in cfDNA methylation influence the course of the disease, possibly by causing excessive anti-dsDNA production and lupus flares.

Clinical significance
SLE activity
The association between cfDNA level and SLE activity was confirmed only partially so far. Although it was shown that after therapy with cyclophosphamide, azathioprine, mycophenolate mofetil, or chloroquine cfDNA levels were significantly reduced (31, 36), investigators did not find any correlations between cfDNA levels and SLE Disease Activity Index (SLEDAI) (32-36). Despite no differences in cfDNA concentrations between SLE patients with certain clinical manifestations such as nephritis, arthralgia, myalgia, and cutaneous features, Tug et al. observed a significant correlation between the medical evaluation of SLE patients and cfDNA level fluctuations (35). Changes in disease activity from stagnation/remission to deterioration were especially represented by cfDNA levels. Additionally, Zhang et al. reported that cfDNA level in patients with lupus nephritis (LN) was significantly higher compared with patients without kidney involvement. Moreover, the level of cfDNA in patients with active LN was signifi-
Systemic lupus erythematosus is characterised by the presence of numerous autoantibodies directed against double stranded DNA (dsDNA), histones, and other nuclear components (39). Multiple investigators confirmed significant correlations between cfDNA levels and autoantibodies titers in SLE patients (Table II). Concentration of cfDNA correlated positively with anti-nucleosome (36) and anti-histone antibodies (33), and negatively with antinuclear (ANA) and anti-dsDNA antibody titers (35, 36). Additionally, patients negative for anti-dsDNA antibodies had higher cfDNA levels compared to those with positive anti-dsDNA-antibodies (35). To explain these results, the authors suggested that analysis of cfDNA may be hindered in patients with high autoantibody titers, possibly by the formation of antibody-DNA immune complexes that directly interfere with cfDNA detection or accelerate the clearance of antibody-bound DNA from the circulation.

**Inflammatory response**

Contradictory results were reported on the associations between cfDNA levels and complement proteins C3, C4, and C-reactive protein (CRP) levels. Tug et al. found a positive correlation between cfDNA concentration and levels of C3 and C4 in SLE patients and in healthy controls (35). Hendy et al. obtained an inverse correlation between cfDNA level and C3 level in SLE group (36), whereas Bartoloni et al. did not confirm any correlations between cfDNA and complement levels (34); Hendy et al. found a positive correlation between cfDNA level and CRP (36). In contrast, Tug et al. did not find any relation between cfDNA concentration and CRP levels in SLE patients (35). These conflicting results might have been caused by the differences in methodology and clinical diversity of patients’ populations. Levels of C3, C4, and CRP change over time, thereby results may vary depending on SLE activity at the point of examination. Nonetheless, findings suggest the possible involvement of cfDNA in the inflammatory response. Tug et al. postulated that cfDNA in SLE may act as an acute-phase reactant and the increased cfDNA concentration reflects inflammatory conditions (35). This notion seems to be further supported by the positive correlation between cfDNA and CRP levels observed by Hendy et al. in SLE patients (36).

**Summary**

Available data indicate that cfDNA is involved in the pathogenesis of SLE. Multiple disturbances in the immunoregulatory mechanisms lead to the excessive cfDNA release into the bloodstream. Apoptosis and NETosis are the two most significant mechanisms in this process. Abnormally elevated cfDNA level correlate with various autoantibodies titers and is associated with active lupus nephritis, which indicates the future prospects for cfDNA utility as a novel biomarker for SLE diagnostics. Unfortunately, considerable differences in sample processing and quantification methodology, as well as the heterogeneity of patients’ population, do not permit a direct comparison of the results between the studies. Therefore, standardisation of cfDNA measurement is necessary to accurately examine its potential applications in clinical practice. Also, further studies are necessary to truly understand the interplay between cfDNA and various autoantibodies in SLE, and to determine the role of cfDNA-antibody complexes in lupus nephritis development.

### Table II. Studies evaluating association between plasma cfDNA levels and disease activity parameters in SLE patients.

<table>
<thead>
<tr>
<th>Author, year</th>
<th>Patients</th>
<th>SLEDAI</th>
<th>ANA</th>
<th>Anti-dsDNA antibodies</th>
<th>Anti-nucleosome antibodies</th>
<th>Anti-histone antibodies</th>
<th>C3</th>
<th>C4</th>
<th>CRP</th>
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</thead>
<tbody>
<tr>
<td>Atamaniuk et al., 2011 (33)</td>
<td>n=13</td>
<td>4 (3-8)*</td>
<td>N</td>
<td>N</td>
<td></td>
<td>P</td>
<td>N</td>
<td>N</td>
<td></td>
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<tr>
<td>Bartoloni et al., 2011 (34)</td>
<td>n=44</td>
<td>2 (0-16)*</td>
<td>N</td>
<td></td>
<td></td>
<td></td>
<td>N</td>
<td>N</td>
<td></td>
</tr>
<tr>
<td>Tug et al., 2014 (35)</td>
<td>n=59</td>
<td>5.74±4.5</td>
<td>I</td>
<td>I</td>
<td></td>
<td>P</td>
<td>P</td>
<td></td>
<td>N</td>
</tr>
<tr>
<td>Hendy et al., 2016 (36)</td>
<td>n=52</td>
<td>7.31±5.26</td>
<td>I</td>
<td>I</td>
<td></td>
<td>P</td>
<td>I</td>
<td>N</td>
<td>P</td>
</tr>
</tbody>
</table>

Data are presented as mean ± SD, unless stated otherwise. *median (quartiles).
Cell-free DNA in SLE / A. Truszewska et al.

References


37. VAN DER VAART M, PRETORIUS PJ: Circulat-


