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BRIEF REPORT

Cell-free DNA in the plasma of patients with systemic sclerosis

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Abstract The aim of the present study was to evaluate the concentration of cell-free DNA (cf-DNA) in the plasma of patients with systemic sclerosis (SSc) and to examine the correlation of cf-DNA with clinical variables of the disease. The study population consisted of 122 SSc patients and 16 healthy controls. Epidemiological and clinical data were collected by direct assessment. The β-globin gene was used to determine the total amount of DNA in the plasma by real-time quantitative PCR analysis. cf-DNA was found in all patients (mean concentration 1,420.7 copies/ml) and controls (mean concentration 1,462.5), with no significant difference. In SSc patients, no correlation was found between cf-DNA and the type of organ involvement, but patients with active disease presented significantly higher cf-DNA concentrations than those with inactive disease ($p<0.05$). Our data suggest that cf-DNA could provide a useful biomarker for the assessment of disease activity in SSc patients.

Keywords Biomarkers · Cell-free DNA · Disease activity · Plasma cell-free DNA · Scleroderma · Systemic autoimmune diseases

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Introduction

Increased concentrations of cell-free DNA (cf-DNA) have been reported in the plasma and serum of patients with pre-eclampsia, cancer, pulmonary embolism or pregnancy, as well as some systemic autoimmune diseases [1–16]. Recently Zhong et al. reported increased levels of cf-DNA in rheumatoid arthritis patients (RA) compared with healthy controls [10] and suggested that cf-DNA could serve as a marker for RA.

We evaluated cf-DNA levels in the plasma of patients with systemic sclerosis (SSc) and studied their correlation with disease activity, clinical manifestations, and treatment in order to determine whether cf-DNA could be used as a biomarker in the monitoring of this complex disease.

Patients and methods

Patients

The study population consisted of 122 female SSc patients being monitored at the rheumatology units of the University of Naples and the University of Pisa. Patients were diagnosed with SSc based on the American College of Rheumatology classification criteria [17] and their disease was classified as limited or diffuse SSc according to LeRoy et al. [18]. Sixteen healthy subjects, all females, were selected as controls.

Epidemiological and clinical data were gathered by direct assessment and recorded on a specially prepared clinical chart. Organ involvement was evaluated by clinical examination and assessments including: pulmonary function tests to measure forced vital capacity (FVC) and diffusion capacity for carbon monoxide (DLCO), standard chest X-ray, high-resolution computed tomography (HRCT) of the lung, standard and 24-h electrocardiography, echocar-

diography, videocapillaroscopy, and barium esophagram. Kidney involvement was evaluated based on urinalysis, serum creatinine levels, and the glomerular filtration rate. Skin involvement was assessed by the Rodnan's skin score [19].

Disease activity was evaluated using the European Scleroderma Study Group Disease Activity Index. A value greater than 3 was taken to indicate active disease [20–22].

Disease duration was calculated based on the first appearance of Raynaud's phenomenon. Recent onset disease was arbitrarily defined as a disease duration of ≤3 years at the time of evaluation.

Real-time quantitative PCR analysis

Five milliliters of EDTA-anticoagulated blood was collected at the time of enrolment. Samples were centrifuged at 400×*g* for 15 min, and the plasma was carefully removed, transferred to cryovials and stored at −20°C until use. Genomic DNA was isolated (under a biosafety hood to avoid contamination) using a QIAamp Blood Kit (Qiagen GmbH, Hilden, Germany) and the blood and body fluid protocol. The extracted DNA was eluted to a final volume of 80 μl and stored at −20°C until use.

The β-globin gene was employed to determine the total amount of DNA in the plasma and the quality of the extracted genomic DNA. Real-time quantitative PCR (Rt-PCR) analysis was carried out using a PE Applied Biosystems 7700 Sequence Detector, and the amplification data was analyzed by Sequence Detection System software (Applied Biosystems, Foster City, CA, USA).

The localizations, sequences, and specificities of sequence-specific primer pairs used for the β-globin gene were determined as described by Lo et al. [16]. Each mix was prepared following an in-house procedure and used at a final volume of 50 μL. To quantify the specific β_2-globin, 10 μL of the extracted plasma DNA was amplified using an Rt-PCR assay and a cycling program of 95°C for 10 min (one cycle), 95°C for 15 s and 60°C for 1 min, for a total of 45 cycles, following the manufacturer's instructions (Applied Biosystems).

Each sample was analyzed in duplicate and standard curves were run simultaneously with each analysis. The plasma DNA concentration (copies per milliliter) was calculated by means of the equation: $C = (Q \times V_{DNA})/(V_{PCR} \times V_0)$ [where Q is the number of DNA copies determined by the sequence detector, V_{DNA} is the total DNA volume (80 μL), V_{PCR} is the volume of extracted DNA used for the PCR, and V_0 is the volume of plasma used to extract the genomic DNA (400 μL)].

Statistical analysis

The Mann–Whitney *U* test was used to correlate the cell-free plasma DNA levels with the epidemiological and clinical variables for the patients. Where appropriate, the χ^2 test was used for the categorical variables.

Results

Our patient cohort consisted of 122 female SSc patients; 99 had the limited form of the disease and 23 the diffuse form. The mean disease duration at the time of evaluation was 177±130 months (min. 6–max. 684, median 156). Using a cut-off point of 3 years, 13% of the patients had recent onset disease. The mean disease activity was 2±1.6 (min. 0–max. 8, median 1). Using a cut-off value of 3, a total of 28 patients (23%) had active disease and 94 (77%) had inactive disease. Of these, 12 (43%) had the diffuse and 16 (57%) the limited form of the disease.

The most frequent disease manifestations were Raynaud's phenomenon (120 patients, 98%), gastrointestinal involvement (102 patients, 84%), skin involvement (99 patients, 82%), and pulmonary involvement (90 patients, 74%). Joint or heart manifestations were observed in 35 (29%) and 32 (26%) patients, respectively; only one patient presented with kidney involvement.

At the time of the evaluation, 22 patients (18%) were being treated with a combination of low-dose corticosteroids and immunosuppressive (IS) drugs (15 cyclophosphamide, five azathioprine, two cyclosporin); ten (8%) with IS drugs alone (five cyclophosphamide, three azathioprine, two cyclosporin); 39 (32%) with low-dose corticosteroids alone; and 51 (42%) were not receiving any treatment.

cf-DNA was found in all patients, with a mean concentration of 1,420.7 copies/ml (min. 11–max. 18,975 copies/ml, median 566.5). No differences were observed between patients and healthy controls (mean concentration 1,462.5; range 450–2,700; median 1,125).

No correlation was observed between cf-DNA levels and the disease type (limited/diffuse) or organ involvement. No differences were observed between patients taking or not taking immunosuppressive drugs (mean values 1,391.5 vs. 1,431, respectively).

A significant difference was observed between patients with higher and lower disease activity, 1,910.4 versus 1,274.8 copies/ml ($p<0.05$).

Patients with a disease duration ≤3 years showed higher cf-DNA concentrations (2,251.5 copies/ml vs. 1,222.9 copies/ml), although the difference was not statistically significant.

Discussion

In this study, we evaluated cell-free DNA (cf-DNA) levels in plasma as a possible marker in scleroderma. Although no differences were observed between patients and healthy

controls, significantly higher concentrations of cf-DNA were observed in patients with active versus inactive disease. No correlations were observed between cf-DNA and the disease type (limited or diffuse), organ involvement, or drug treatment. These data suggest that cf-DNA might serve as marker of disease activity among SSc patients.

It is a well-established fact that plasma and serum contain cf-DNA, and higher concentrations of cf-DNA have been reported in a variety of conditions associated with increased cell turnover, cell damage, or apoptosis such as cancer, pulmonary embolism, trauma, pre-eclampsia, and some systemic autoimmune diseases [1–16, 23]; this could be related with an increased release related with cell death and or to an incomplete removal from the circulation [24, 25]

Higher cf-DNA levels have been found in patients with breast cancer, and this parameter appears to be associated with tumor size and diffuse disease [2–5]. Higher cf-DNA concentrations have also been seen in trauma patients who develop multiple organ damage [3]. Barada et al. found cf-DNA in patients with suspected pulmonary embolism, suggesting that it might be used to screen for pulmonary embolism [6].

Data has been published on the presence and concentration of cf-DNA in autoimmune diseases, in particular systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA) [10–12]. Increased concentrations of cf-DNA have been demonstrated in SLE and some studies have shown a correlation with disease activity. In a recent report, Zhong et al. evaluated 28 RA patients and observed a significant increase in cf-DNA concentrations in both serum and plasma [10].

Taken together, these data suggest that cf-DNA levels could serve as a biomarker for the assessment of the severity, extension, degree of activity, and the prognosis of different pathological conditions.

SSc is a systemic autoimmune disease characterized by a variable clinical picture and varying degrees of severity [22, 26]. Assessing disease activity can be difficult because of a dearth of laboratory markers and data on their use in the clinical setting. The identification of biomarkers linked to disease activity could greatly facilitate the follow-up of patients in clinical practice [26].

One limitation of our study resides in the relatively low number of patients with active disease, which makes impossible an analysis based on disease subset or on activity in different organs. However, the observed correlation with disease activity, perhaps reflecting (as in other pathological conditions studied) increased cell damage and cell turnover, may suggest that the cf-DNA may represent a marker of disease activity in SSc. These data may constitute the basis to conduct further studies on larger populations aimed at confirming the correlation of cf-DNA with disease activity, with specific organ involvement and possibly at studying changes in cf-DNA concentrations in the course of the disease.

Disclosures None

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