

Tumor suppressor protein p53 and anti-p53 autoantibodies in pediatric rheumatological diseases

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The tumor suppressor protein p53 plays an important role in cell cycle regulation. One of the major features in rheumatic diseases is the abnormal proliferation of lymphocytes. p53 expression in peripheral blood mononuclear cells (by flowcytometry) and serum anti-p53 antibodies (by ELISA) were therefore measured in 18 children and adolescents with juvenile rheumatoid arthritis (JRA) and 17 with systemic lupus erythematosus (SLE) in comparison to 20 healthy controls, to determine their role. p53 expression in patients was insignificantly higher than that of controls ($2.28 \pm 2.71\%$ vs. $1.08 \pm 1.02\%$, respectively, $p > 0.05$) with 29.4% of the patients showing values above a cut-off level of 2.55% (95th percentile of controls). SLE patients with active disease had significantly higher p53 expression compared to controls and to patients with quiescent disease although no significant correlation with ESR or complement 3 was detected. Seropositivity to anti-p53 antibodies was observed in none of controls but in 22.8% of patients, all of whom, except one, had active disease. Seropositivity to anti-p53 antibodies was more prominent in lupus nephritis than in other presentations of SLE ($p < 0.05$). The mean p53 expression in seropositive patients was insignificantly higher than in seronegatives. p53 expression and seropositivity to anti-p53 were slightly higher in SLE than in JRA and were not significantly affected by the mode of therapy. Thus, the overexpression of p53 in some patients with active SLE and JRA might explain the abnormal proliferation of autoreactive lymphocytes that perpetuates the inflammatory response. The presence of anti-p53 antibodies might cause malfunctioning of p53 protein interfering with its regulatory functions.

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The cell cycle is controlled through several tightly-regulated checkpoints, among which the p53 tumour suppressor protein plays a special role (1). p53 is important in cell growth control, DNA repair, angiogenesis and apoptosis (2). Inactivation of this protein (e.g. by mutation) produces an abnormal protein with loss of regulation of cell cycle leading to uncontrolled cell proliferation and in some situations, neoplastic growth (3).

Rheumatological diseases are characterized by chronic inflammation with defective elimination and uncontrolled proliferation of autoreactive lymphocytes (4). Furthermore, synovial hyper-

plasia and the development of normal synovial fibroblasts into transformed cells are typical features of rheumatoid arthritis (RA). They may be mediated not only by up-regulation of proto-oncogenes, but also by genetic alteration of anti-proliferative genes such as p53 (5). Existing data concerning the role of p53 in pediatric rheumatological diseases are scanty and controversial.

The aim of this study was two-fold: first, to determine the expression of p53 in JRA and SLE, and second, to investigate a potential antibody response to p53 that might interfere with its regulatory functions.

Subjects and methods

Setting

This case control cross-sectional study took place in the Pediatric Allergy and Immunology Unit of Ain Shams University, Cairo, Egypt.

Subjects

The study comprised 35 children and adolescent patients: 17 with SLE and 18 with juvenile rheumatoid arthritis (JRA) satisfying the criteria of the American College of Rheumatology (6,7). Their ages ranged from 2.5 to 18 yr (mean $\pm$ SD: 12.6 ± 4.7 yr) with a male : female ratio of 1 : 3.2 (Table 1). All patients were on longterm anti-inflammatory therapy.

A control group of 7 males and 13 females with an age range of 5–16 yr (mean $\pm$ SD: 13.1 ± 3.8 yr) were also studied. Exclusion criteria for controls were the presence of personal or family history of rheumatological diseases, chronic illness, or weights or heights below the 5th or above the 95th percentiles for age (8).

Table 1. Some clinical and laboratory characteristics of patients in relation to anti-p53 seropositivity

	Total n	Anti-p53 positive n	Anti-p53 negative n	χ^2 (p)
Control group	20	0	20	
All patients	35	8	27	5.35 (0.02*) (vs. controls)
SLE	17	5	12	6.8 (0.009*) (vs. controls)
Active	12	5	7	2.95 (0.08)
Quiescent	5	0	5	
With nephritis	6	4	2	6.2 (0.01*)
Without nephritis	11	1	10	
C3 consumed	13	5	8	1.68 (0.19)
C3 normal	3	0	3	
Steroids only	12	3	9	0.38 (0.53)
Steroids + cytotoxics	5	2	3	
JRA	18	3	15	3.62 (0.057) (vs. controls)
Active	14	2	12	0.26 (0.6)
Quiescent	4	1	3	
Polyarticular	12	3	9	
– on MTX	7	1	6	1.03 (0.3)
– on NSAIDs	5	2	3	
Non-polyarticular	6	0	6	1.8 (0.18)
– oligoarticular	2	0	2	
–systemic onset	4	0	4	
On MTX	7	1	6	0.05 (0.8)
Not on MTX	11	2	9	

*significant ($p < 0.05$).

SLE: systemic lupus erythematosus; JRA: juvenile rheumatoid arthritis;
C3: Complement component 3; MTX: methotrexate; vs.: versus.

Clinical methods

Assessment of disease activity in SLE patients was carried out according to the criteria of Winfield *et al.* (9) with the aid of complete blood counts (CBC), erythrocyte sedimentation rate (ESR), urinalysis for proteinuria, creatinine clearance, serum complement 3 (C3) and anti-double stranded DNA (anti ds DNA). Accordingly, the disease was classified as quiescent in 5 SLE patients and as active in 12 patients (6 had nephritis, 4 had vasculitis, one had hemolytic anaemia, and one had arthritis).

Concerning JRA, disease activity was assessed clinically and by ESR (10). The disease was considered active in 14 patients and quiescent in 4 patients. Twelve patients had polyarticular JRA (3 were rheumatoid factor positive), 2 had oligoarticular JRA while 4 were of the systemic onset form.

Laboratory methods

These included CBC (Coulter Counter T660; Coulter Corp., Miami, FL, USA), ESR (Wester-gren method), urine protein and renal functions (Beckman Synchron Cx5; Beckman Instruments Inc., Fullerton, CA, USA), complement 3 (C3) by turbidimetry (Turbiquant C3; Behring Diagnostics GmbH, Marburg, Germany), antinuclear antibodies and anti ds DNA by indirect immunofluorescent microscopy (Sanofi Diagnostics-Pasteur, Chaska, MN, USA), and rheumatoid factor by latex agglutination (Avitex-RF; Omega diagnostics, Alloo, UK).

Measurement of intracellular total p53 expression in peripheral blood mononuclear cells was performed by flowcytometry using fluorescein isothiocyanate (FITC)-conjugated monoclonal mouse antihuman p53 oncoprotein that detects wild and mutant p53 (DAKO, Glostrup, Denmark). The procedure starts with isolation and wash of mononuclear cells from a fresh EDTA blood sample using ficoll hypaque density gradient centrifugation, cell fixation by 1% paraformaldehyde, incubation and wash, followed by permeabilization with cold 70% methanol, wash, and incubation with FITC-conjugated anti-p53. A negative control tube (mouse IgG1/FITC) was prepared for each test sample. Gating was set on mature lymphocytes. The number of cells was adjusted to $10\,000 \times 10^9/\text{ml}$. The percent fluorescence was determined on a flowcytometer (EPICS Profile II, Coulter Electronics, Miami, FL, USA).

Antibodies against p53 (in 1 : 20 diluted serum samples) were determined by sandwich-ELISA using extracts from human tumour cell native

p53 protein fixed by a monoclonal antibody onto the antigen-plate (ORGA-MED p53-antibody, Cat.-No: 04-0007; Dr med. Klopfer GmbH). A second microtiterplate coated with a control tumour cell extract without p53 was used to detect nonspecific binding of antigen-antibody complex. Absorbance was read at 450 nm using an ELISA reader. Values were first corrected for the reagent blank. The specificity of the signal was controlled by comparing the absorbance values between antigen and control plates. Samples giving higher values in the control wells were regarded as negative. Results were calculated and interpreted according to the manufacturer's instructions.

Statistical methods

Analysis was carried out using standard computer software, 'Statistica for Windows 95'. Results were displayed as mean and standard deviation as well as median and interquartile range for nonparametric data. Chi square and Mann-Whitney *U*-tests were used to compare different groups, and the Spearman's rank test to correlate quantitative data. A cut-off value for percent mononuclear p53 expression was taken at the 95th percentile of the control group above which a result was considered high.

Results

The ESR of all patients ranged from 5 to 100 mm/h (1st hour) with a mean of 32.63 ± 23.04 mm/h. Their C3 ranged from 22 to 121 with a mean of 56 ± 33.2 mg/dL. C3 was consumed (< 50 mg/dL) in 12/17 SLE patients.

Expression of p53 in peripheral blood mononuclear cells

p53 expression of all patients (15 with SLE and 9 with JRA) was insignificantly higher than that of controls ($n = 20$) [mean $\pm$ SD: $2.28 \pm 2.71\%$ vs. $1.08 \pm 1.02\%$, median (IQ range): 1% (0.15–3.1%) vs. 0.65% (0.1–2.0%), respectively, $z = 0.97$, $p > 0.05$]. Seven patients (29.2%), 6 with active SLE and one with active polyarticular JRA, had values above the cut-off level of 2.55%. p53 expression was significantly higher in active than in quiescent disease (Fig. 1). This applied to SLE rather than JRA. No significant correlation was noticed between p53 and either C3 or ESR. The expression of p53 in SLE, though higher, was insignificantly different from that in JRA [mean $\pm$ SD: $2.81 \pm 3.17\%$ vs. $1.32 \pm 1.27\%$, median: 2% vs. 1%, respectively ($z = 0.7$, $p > 0.05$)].

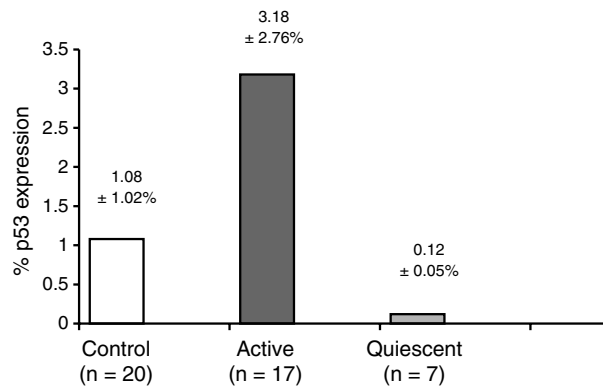


Fig. 1. Effect of disease activity on the mean % mononuclear p53 expression of all patients. Active vs. control: $z = 2.27$, $p < 0.05$. Active vs. quiescent: $z = 3.16$, $p < 0.01$.

Prevalence of anti-p53 seropositivity

Whilst antibodies against p53 were detected in 8/35 patients (22.8%), it was found in none of the controls ($p < 0.05$). Of the positive samples, 5 belonged to SLE and 3 to JRA ($p > 0.05$); all, but one, were exhibiting active disease. Seropositivity to p53 was significantly more prevalent in patients with lupus nephritis (66.6%) as compared to other clinical patterns of SLE (10%). (See Table 1).

Relationship of anti-p53 antibodies to p53 expression

Comparative analysis revealed that the mean p53 expression of seropositive patients ($3.8 \pm 4.16\%$) was insignificantly higher than that of seronegative ones ($1.65 \pm 1.69\%$) [$z = 0.86$, $p > 0.05$]. Combined anti-p53 positivity and p53 expression ($>$ cut-off level) occurred in 4 patients. These patients had active lupus nephritis.

Effect of different therapeutic modalities

The expression of p53, though higher in SLE patients receiving combination of steroids and cytotoxic drug, was insignificantly different from those receiving steroids only ($4.1 \pm 3.8\%$ vs. $1.3 \pm 1.2\%$, $z = 1.09$, $p > 0.05$). Similar findings were observed with NSAIDs as compared to MTX ($2.1 \pm 1.6\%$ vs. $0.9 \pm 1.1\%$, $z = 1.2$, $p > 0.05$). No significant differences were noticed in the anti-p53 seropositivity with different lines of therapy (Table 1).

Discussion

Assessment of p53 in patients with SLE and JRA revealed that overexpression of this protein is not

a constant finding in these diseases, as it was present in nearly 1/3 of all patients. It appears from the significantly higher mononuclear cell p53 expression in SLE patients with active disease as compared to controls and to patients with quiescent disease that overexpression of this protein is mainly related to disease status.

Wild type p53 has a short half-life and is rarely detected in normal non-neoplastic cells. Overexpression can be the result of overproduction of the wild type or a mutation of the p53 gene with production of a protein with a prolonged half-life permitting its detection (11). Another possible explanation is that a conformational change of p53 might be taking place upon cell activation (12). The last assumption offers a good explanation of the findings in this study.

Commercially available antibodies used in detection of p53, like the one used in this study, are directed against both the wild and the mutant types of p53, therefore it was difficult to ascertain whether a mutation of the p53 gene was behind the overexpression of the protein in patients with active disease. However, long standing inflammation and oxidative stress create a genotoxic environment that can lead to DNA damage and hence somatic mutations (13). Such mutations are expected to cause permanent overexpression of p53 in active as well as in quiescent disease. Since p53 is expressed by active lymphocytes and not by quiescent cells (2), its overexpression only during active disease would seem to be logical. Although little is known about the consequences of such mutations, it might be speculated that they impair the function of p53 and hence contribute to the chronic inflammatory process by extending the life span of aberrant immunocompetent cells (5).

Studies concerning p53 expression in patients with SLE are scarce. In a Mexican study, bone marrow cells of 8 SLE patients showed significant down-regulation of p53 gene (14). In another study, overexpression was noticed in lung tissues of collagen vascular disease patients with interstitial lung inflammation (15).

In the present study, mononuclear p53 expression in JRA patients was insignificantly different from controls with only one patient (in activity) surpassing the cut-off level. Overexpression of p53 has been reported in early and in longstanding RA synovium as well as in uninvolved joints (11,12) and dominant negative p53 mutations were also observed (16). A study from Germany failed to show a specific mutational pattern in JRA (5). These widely varying results among different studies might indicate heterogeneity among patients or might signify ethnic variations.

Antibodies against p53 were found in 22.8% of patients: 29.4% of SLE patients and 16.6% of JRA patients. In adult patients with SLE, the prevalence of anti-p53 antibodies was reported to be 32% (17), whereas in RA, the only available study reported the rare occurrence of these antibodies (in 2 out of 106 patients) (18). An autoimmune response to p53 has so far been described in some malignancies such as carcinomas and lymphomas and in chronic inflammatory bowel disease (19). Thus, in as much as the antibody response to p53 is not specific for rheumatic patients, it is by no means a constant finding among them. The question of whether these antibodies impair the function of p53 in cell cycle regulation is difficult to answer in view of the lack of significant relation between anti-p53 seropositivity and p53 expression. However, it seems possible that they do hinder p53 function causing enhancement of the autoimmune response, as most of the seropositive patients had severe and difficult to control disease.

Steroids, cyclophosphamide, methotrexate and NSAIDs were found to enhance p53 expression (20–22). The higher, albeit statistically insignificant, p53 expression in SLE patients receiving cytotoxic drugs might be drug-induced or might reflect the severe nature of the disease that prompted administration of these drugs. However, p53 expression among JRA patients receiving NSAIDs is probably a drug-related effect.

It is concluded that p53 is overexpressed in patients with active SLE and to a lesser extent in patients with active JRA. The relative contribution of overproduction of the wild type p53 protein and gene mutation remains to be elucidated. The abnormal p53 expression could disturb cell cycle regulation resulting in abnormal proliferation of autoreactive lymphocytes that perpetuate the inflammatory response. The presence of an autoantibody response to p53 protein in some patients with SLE and JRA might contribute to the malfunctioning of p53 protein interfering with its regulatory functions, however, further work is needed to decide their role. The answer to the question of whether the overexpression of p53 in some patients is predictive of future development of lymphoproliferative malignancies awaits long-term prospective studies.

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