# Patients with Systemic Sclerosis Present Increased DNA Damage Differentially Associated with DNA Repair Gene Polymorphisms

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***ABSTRACT.*** ***Objective.*** Patients with systemic sclerosis (SSc) exhibit increased toxicity when exposed to genotoxic agents. In our study, we evaluated DNA damage and polymorphic sites in 2 DNA repair genes (*XRCC1* Arg399Gln and *XRCC4* Ile401Thr) in patients with SSc.

***Methods.*** A total of 177 patients were studied for DNA repair gene polymorphisms. Fifty-six of them were also evaluated for DNA damage in peripheral blood cells using the comet assay.

***Results.*** Compared to controls, the patients as a whole or stratified into major clinical variants (limited or diffuse skin involvement), irrespective of the underlying treatment schedule, exhibited increased DNA damage. *XRCC1* (rs: 25487) and *XRCC4* (rs: 28360135) allele and genotype frequencies observed in patients with SSc were not significantly different from those observed in controls; however, the *XRCC1* Arg399Gln allele was associated with increased DNA damage only in healthy controls and the *XRCC4* Ile401Thr allele was associated with increased DNA damage in both patients and controls. Further, the *XRCC1* Arg399Gln allele was associated with the presence of antinuclear antibody and anticentromere antibody. No association was observed between these DNA repair gene polymorphic sites and clinical features of patients with SSc.

***Conclusion.*** These results corroborate the presence of genomic instability in SSc peripheral blood cells, as evaluated by increased DNA damage, and show that polymorphic sites of the *XRCC1* and *XRCC4* DNA repair genes may differentially influence DNA damage and the development of autoantibodies. (J Rheumatol First Release Feb 1 2014; doi:10.3899/jrheum.130376)

*Key Indexing Terms:*
SYSTEMIC SCLEROSIS | COMET ASSAY | DNA REPAIR
DNA DAMAGE | *XRCC1* AND *XRCC4*

Systemic sclerosis (SSc) is an autoimmune connective tissue disorder primarily characterized by inflammation, fibrosis, and degenerative changes in blood vessels of skin, joints, skeletal muscles, and internal organs[1,2]. Vasculopathy is considered one of the first events in SSc pathogenesis, and widespread vasculopathy may evolve to progressive interstitial and perivascular fibrosis[3]. These features are reported to be mediated by proinflammatory

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cytokines and angiogenesis factors, by the loss of cell redox control[4], and by the oxidative stress that may target DNA, leading to major DNA damage[5].

Compared to the general population, increased risk of certain cancers, particularly hematologic cancers, has been observed in autoimmune diseases, such as SSc and systemic lupus erythematosus (SLE)[6]. DNA damage[7] and deficiencies of the DNA repair system[8,9] have been implicated in the development of autoimmune disorders and cancer. Polymorphic sites in DNA repair genes, such as uracil-DNA glycosylase, have been associated with autoantibodies in patients with rheumatoid arthritis (RA)[8].

Several lines of evidence indicate that patients with SSc may present genomic instability, including (1) loss of chromosomes[10]; (2) high rates of lymphocyte chromosomal abnormalities, particularly in patients exhibiting anticentromere antibodies (ACA)[11,12,13]; (3) presence of cytogenetic micronuclei, which are chromatin bodies resulting from loss of whole chromosomes or chromosome fragments without a centromere[14], both in patients with SSc and in those presenting Raynaud phenomenon (RP)[15]; and (4) increased radiation toxicity, which has been reported in patients with SSc submitted to autologous stem cell transplantation[16].

Several DNA repair mechanisms controlling DNA damage and genomic instability have been described, based on a series of proteins displaying several functions depending on the type of DNA damage, i.e., single strand break (SSB), double strand break (DSB) or damaged bases [base excision repair (BER)]. A multiprotein complex is involved in SSB repair, including the radiographic repair complementing defective repair in Chinese hamster cells 1 (XRCC1) protein that interacts with DNA ligase III, DNA polymerase-β, and poly (ADP-ribose) polymerase[17,18]; SSB may be caused by endogenous factors, such as direct attack by reactive oxygen species during normal metabolism, or by exogenous factors, such as ionizing radiation. Additionally, SSB may be indirectly recruited as a consequence of BER[19].

Two polymorphisms in the *XRCC1* gene are more commonly studied, the first leading to a C to T substitution at codon 194 in exon 6 (C194T), and the second to a G to A substitution at codon 399 in exon 10 (G399A), causing the substitution of arginine (Arg) with tryptophan (Trp; Arg194Trp) in the first case, and of Arg with glutamine (Gln; Arg399Gln) in the second case. These polymorphic residues, located in conserved sites of the protein, may alter the efficiency of the BER mechanism, increasing the chance of permanent DNA damage[20,21]. The Arg399Gln polymorphic site has been associated with the development of cancer at distinct locations, including head and neck[22], colorectal[23], stomach[24], esophagus[25], and breast and lung cancer[26,27].

Nonhomologous end joining (NHEJ) is the most frequent DSB repair mechanism; however, it is less accurate than homologous recombinational repair, frequently resulting in nucleotide loss at the breaking site. In addition, NHEJ is responsible for rejoining the ends of immunoglobulin V(D)J segments during the V(D)J recombination processes[28]. The radiographic repair complementing defective repair in the Chinese hamster cells 4 (*XRCC4*) gene produces a protein that combines with DNA ligase IV, permitting the joining of DNA extremities during NHEJ repair[28]. Polymorphic sites in the *XRCC4* gene, including the nonconservative Ile401Thr polymorphism, have been reported[29]; however, the functional consequence of this genetic variation is unknown. On the other hand, some mutations in the *XRCC4* gene have been associated with increased DNA toxicity induced by radiation, with errors in V(D)J recombination and with chromosomal changes[30].

Considering that patients with SSc present evidence of genomic instability and considering that there are few studies regarding DNA damage and DNA repair genes in the disease, we evaluated peripheral blood leukocyte DNA damage, polymorphic sites in DNA repair genes involved in SSB (*XRCC1*) and DSB (*XRCC4*), and associated these polymorphic sites with the DNA damage in a series of patients with SSc.

## MATERIALS AND METHODS

*Subjects*. The study was conducted on 177 patients (156 women) with a mean age of 48.6 ± 6.65 years followed at the Unit of Rheumatology of the State University of Campinas, São Paulo, Brazil. This large group of patients was evaluated for DNA repair gene polymorphisms. DNA damage was evaluated in a subgroup of these patients (49 women and 7 men) with a mean age of 45.42 ± 5.55 years. Seven of those patients were not taking drugs and 49 were taking azathioprine, D-penicillamine, methotrexate (orally), and cyclophosphamide (by monthly intravenous pulses) in different combinations. All patients fulfilled the American College of Rheumatology classification criteria for SSc[31] and were divided into diffuse and limited SSc subtypes according to LeRoy, *et al*[32].

The magnitude and severity of skin thickening were evaluated by the modified Rodnan skin score that analyzes 17 anatomical sites, graded from 0 (normal skin) to 3 (intense skin thickening). The following criteria were used to define specific tissue/organ involvement: articular (inflammatory polyarthralgia or arthritis); vascular (ischemic ulcers of fingertips or extensive cutaneous necrosis, amputation, or both); esophageal (dysphagia with radiological evidence of distal esophageal hypomotility); intestines (altered intestinal habit associated with radiological evidence of motility disturbances in the small or large bowel); interstitial lung disease [dyspnea associated with evidence of interstitial lung involvement by chest radiograph or high-resolution computed tomography, and restrictive defect of lung function tests (forced vital capacity < 70%)]; pulmonary hypertension (pulmonary artery systolic pressure > 40 mmHg at Doppler echocardiogram, alone or associated with interstitial lung disease); heart (congestive heart failure or symptomatic pericarditis or symptomatic arrhythmia); and kidneys (scleroderma renal crisis, characterized by rapidly progressive renal insufficiency associated with malignant arterial hypertension). RP, calcinosis, and telangiectasis were also evaluated. Other laboratory investigations included complete blood cell count, erythrocyte sedimentation rate, and autoantibodies [antinuclear antibody (ANA), ACA, and anti-Scl70]. The clinical and laboratory features of patients with SSc are summarized in Table 1.

The control group consisted of 191 healthy individuals (95 women) with a mean age of 31.1 years, presenting no previous history of autoimmune disorders. DNA damage was evaluated in a subgroup of these

*Table 1*. Demographic, clinical, and laboratory features of patients with SSc (systemic sclerosis).

| Features | Limited SSc, n = 109 (%) | Diffuse SSc, n = 68 (%) | Total, n = 177 (%) |
|---|---|---|---|
| Men | 9 (8) | 12 (18) | 21 (12) |
| Women | 100 (92) | 56 (82) | 156 (88) |
| Median age, yrs | 48.3 | 49.6 | |
| White | 80 (62) | 44 (65) | 124 (70) |
| African-Brazilian | 29 (38) | 24 (35) | 53 (30) |
| Clinical followup (mos) | 96 | 70 | — |
| Raynaud phenomenon | 101 (94) | 62 (91) | 163 (92) |
| Vascular skin ulcers | 28 (26) | 20 (29) | 48 (27) |
| Telangiectasia | 44 (40) | 17 (25) | 61 (35) |
| Calcinosis | 23 (21) | 7 (10) | 30 (17) |
| Esophageal abnormalities | 90 (83) | 53 (78) | 143 (81) |
| Intestinal abnormalities | 4 (2) | 2 (3) | 6 (3) |
| Interstitial lung disease | 39 (36) | 24 (35) | 63 (36) |
| Pulmonary vascular disease | 19 (17) | 22 (32) | 41 (23) |
| Cardiac abnormalities | 8 (7) | 7 (10) | 15 (9) |
| Scleroderma renal crisis | 0 | 5 (7) | 5 (3) |
| Antinuclear antibody | 92 (84) | 59 (87) | 151 (85) |
| Anticentromere antibody | 17 (16) | 7 (10) | 24 (14) |
| Anti-Scl 70 antibody | 15 (14) | 16 (24) | 31 (18) |

individuals (48 women and 9 men) with a mean age of 40 ± 5.36 years. The study protocol was approved by the local ethics committee (protocol #9581/2002) and all subjects gave written informed consent to participate.

*Measurement of peripheral blood cell DNA damage*. The alkaline version of the comet assay was used according to Miller, *et al*[33]. Briefly, 10 µl of peripheral blood leukocytes isolated after red cell lysis were added to 120 µl of 0.5% low melting point agarose (Sigma) at 37°C, layered on slides precoated with 1.5% regular agarose, covered with a coverslip, and left to stand for 10 min at 4°C. Coverslips were carefully removed and the slides immersed in a lysis solution [2.5 M NaCl; 100 mM EDTA; 10 mM Tris (pH 10); 1% N-lauroylsarcosine sodium; 1% Triton X-100, and 10% dimethylsulfoxide)] overnight. Slides were then washed in phosphate buffered saline for 5 min, immersed in freshly prepared alkaline buffer (10 N NaOH, 200 mM EDTA, pH 13), and randomly distributed in a horizontal electrophoresis chamber. After a 20-min DNA unwinding period, electrophoresis was performed at 25 V and 300 mA (1 V/cm) for an additional period of 20 min. After electrophoresis, slides were rinsed 3 times with neutralization buffer (0.4 M Tris; pH 7.5), fixed with absolute ethanol, stained with 50 µl of 20 µg/ml ethidium bromide (Sigma), and scored under a fluorescence microscope at 400× magnification. Fifty cells per subject were randomly gathered using a fluorescence microscope coupled to a camera, and images were analyzed using the CometScore software (TriTek Corp, 2003, download at www.autocomet.com). DNA damage was evaluated according to ethidium bromide fluorescence intensity as scored with the fluorescence microscope. When no damage is observed in the nucleoid (DNA plus nuclear matrix), a concentrated round luminescence is observed, whereas when DNA damage is observed, a comet-shaped tail is seen. According to the DNA damage, the tail was scored from 0 (no DNA damage) to 5 (maximum DNA damage) in a total of 100 cells. Then, the variable that measures damaged DNA is the total tail intensity divided by the total number of cells (% DNA in the tail). Figure 1 illustrates nucleoids exhibiting scores of DNA damage ranging from 0 to 5.

*Polymorphism analyses*. Five milliliters of whole blood were collected into sterile EDTA-Vacutainer tubes (Becton-Dickinson). DNA was isolated using a salting out procedure, and single nucleotide polymorphic sites in the *XRCC1* (rs: 25487) and *XRCC4* (rs: 28360135) genes were evaluated using these primers[34]: (1) *XRCC1* Arg399Gln (exon 10), forward 5'-CCC CAA GTA CAG CCA GGT C-3' and reverse 5'-TGC CCC GCT CCT CTG AGT AG-3'; and (2) *XRCC4* Ile401Thr (exon 4): forward 5'-CTC AGA AGA AAT TGT GTA TGC T-3' and reverse 5'-ACC ACA AGC AAA CTG TGT ACA C-3'.

DNA amplification was performed in a total reaction volume of 20 µl, containing 20 mM Tris-HCl, pH 8.0, 50 mM KCl, 1.5 mM MgCl2, 0.5 µM of each primer, 0.2 mM dNTP, 100 ng of genomic DNA, and 1 U of Taq DNA polymerase (Invitrogen). Amplification conditions included an initial 5-min denaturation step at 94°C, followed by 35 cycles at 94°C for 30 s, 58°C for 1 min (*XRCC1*) or 52°C for 30s (*XRCC4*) and 72°C for 10 min. The variant alleles were identified by digestion with restriction endonuclease enzymes. For *XRCC1* Arg399Gln, the 242 bp amplicon was digested with MspI (New England Biolabs). The wild-type allele Arg was identified by the presence of 2 bands of 148 and 94 bp, and the mutant allele Gln by the uncut 242 bp product. The 276 bp amplicon of *XRCC4* Ile401Thr was digested with BsrDI (New England Biolabs) at 65°C overnight. The uncut 276 bp fragment identified the wild-type allele, while the presence of 169 and 107 bp characterized the mutant allele Thr.

*Statistical analysis*. The comparisons of DNA damage between patients and controls were performed using the nonparametric Mann-Whitney test (2 groups) or Kruskal-Wallis test (3 groups), followed by the Dunn posttest.

Allele and genotype frequencies were estimated by direct counting and compared between patients and controls using the Fisher's exact test, and the OR and 95% CI were also estimated. The adherence of observed genotype frequencies to the proportion theory of Hardy-Weinberg equilibrium was tested by the Fisher's exact test.

The statistical analyses were performed using SigmaStat and GraphPad InStat computer software programs, with the level of significance set at $p \leq 0.05$.

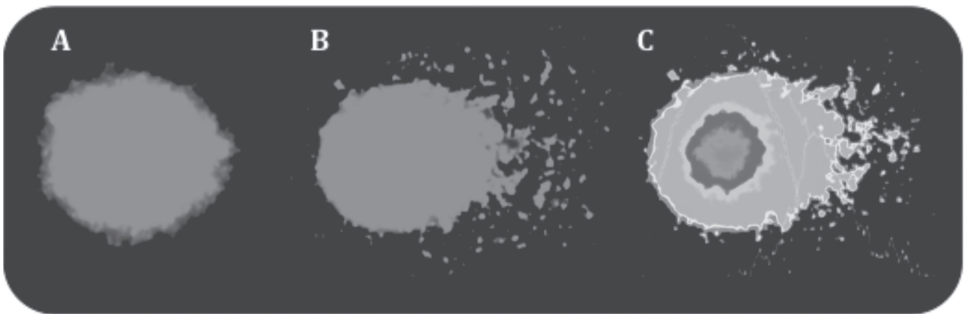


*Figure 1*. Typical comet assay showing a cell exhibiting no DNA damage (A = score 0) and a cell with moderate DNA damage (B = score 3). The cell exhibiting moderate damage (as shown in B) was submitted to fluorescence analysis (as shown in C), and the magnitude of DNA damage was measured as the percentage of tailed DNA.

## RESULTS

*Basal DNA damage*. Compared to healthy individuals, patients with SSc exhibited increased DNA damage (% DNA in the tail: 3.50 for controls and 14.30 for patients, $p < 0.001$). When patients were stratified according to major clinical variant (diffuse or limited skin involvement), the DNA damage observed in both variants was significantly increased compared to control ($p < 0.001$ for each comparison); however, no significant difference was detected when the diffuse form was compared to the limited form. The SSc group was also stratified according to treatment. Seven patients were not taking drugs and 49 were taking azathioprine, D-penicillamine, methotrexate, and cyclophosphamide in different combinations. No significant difference in the magnitude of DNA damage was observed between treated and untreated patients (% of DNA in the tail = 27.81 and 27.64, respectively); however, both groups presented increased DNA damage compared to control ($p < 0.001$, for each comparison). All these results are shown in Figure 2. No significant associations were observed regarding the influence of other clinical and laboratory manifestations (shown in Table 1) on DNA damage (data not shown).

*Genotype analysis*. The *XRCC1* and *XRCC4* genotype and allele frequency distributions observed in patients and controls are shown in Table 2. No significant differences were observed regarding the allele and genotype frequency of the 2 polymorphic sites when patients considered as a whole or stratified according to skin involvement were compared to healthy individuals. We also stratified patients according to their major clinical or laboratory features, and patients exhibiting the *XRCC1* Arg399Gln allele (at single and double dose) showed increased frequency of ANA compared to patients with the *XRCC1* Arg399Arg allele ($p = 0.01$), with an OR 3.5 (95% CI 1.35–7.57). In addition, patients exhibiting the *XRCC1* Arg399Gln allele (at single or double dose) showed increased frequency of ACA compared to patients presenting the *XRCC1* Arg399Arg allele ($p = 0.001$, OR 3.22, 95% CI 1.34–6.33; data not shown). Considering *XRCC4* Ile401Thr polymorphisms, no significant differences were observed according to patient major clinical or laboratory features.

*DNA damage according to the polymorphic sites of DNA repair genes*. To evaluate whether the DNA repair gene polymorphisms contributed to DNA damage, we determined associations between the magnitude of DNA damage and *XRCC1* and *XRCC4* gene polymorphic sites from 3 points of view, i.e., for healthy individuals and patients considered as individual groups, and comparing patients and controls (Table 3).

Considering only the group of healthy subjects, the *XRCC1* Arg399Gln allele was associated with a higher level of DNA damage compared to the *XRCC1* Arg399Arg allele ($p < 0.001$). In addition, individuals exhibiting the *XRCC4* Ile401Thr allele presented higher DNA damage than individuals exhibiting the Ile401Ile allele ($p < 0.001$).

Considering only patients with SSc, no significant differences were detected in DNA damage regarding the *XRCC1* alleles. On the other hand, patients presenting the *XRCC4* Ile401Thr allele exhibited increased DNA damage compared with patients presenting the Ile401Ile allele ($p < 0.001$).

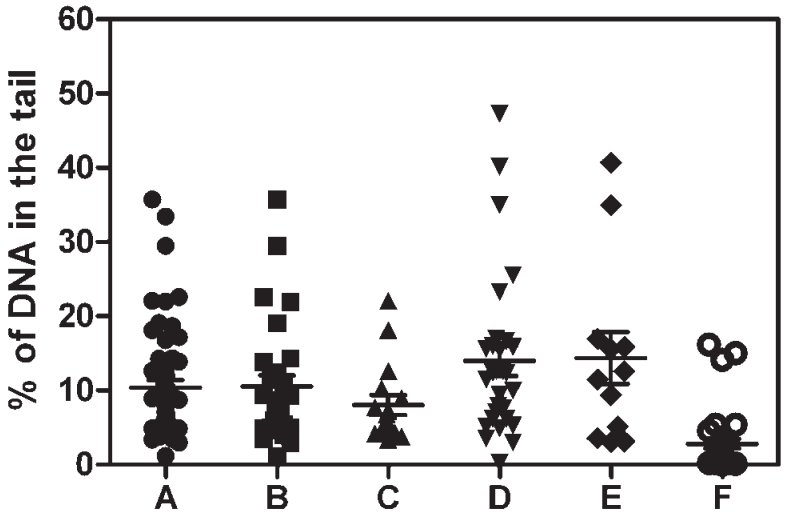


*Figure 2*. DNA damage as evaluated by the comet assay (% of DNA in the tail) in patients with systemic sclerosis (SSc; A: whole group, median 11.3), stratified according to the clinical variant (B: limited SSc, median 11.5, and C: diffuse SSc, median 9.85), and according to treatment (D: without treatment, median 15.64, and E: treated with combination of different drugs, median 16.11), and in healthy individuals (F: controls, median 3.5). All comparisons between patients and controls were significant ($p < 0.001$).

*Table 2*. Frequency of the DNA repair (*XRCC1* and *XRCC4*) gene polymorphic sites observed in patients with systemic sclerosis (SSc) considered as a whole and stratified according to the clinical variant (limited or diffuse) and in healthy individuals. No significant differences were observed. The probabilities of adherence to Hardy-Weinberg equilibrium (HWE) for patients and controls are also shown.

| *XRCC1* Arg→Gln codon 399 | | Patients with SSc, n = 173 (%) | Limited SSc, n = 104 (%) | Diffuse SSc, n = 69 (%) | Controls, n = 176 (%) | p* |
|---|---|---|---|---|---|---|
| Genotypes | Arg/Arg | 91 (52.60) | 55 (52.88) | 36 (52.18) | 85 (48.30) | 0.87 |
| | Arg/Gln | 55 (31.79) | 31 (29.81) | 24 (34.78) | 55 (31.25) | 0.75 |
| | Gln/Gln | 27 (15.61) | 18 (17.31) | 9 (13.04) | 36 (24.45) | 0.66 |
| Allele frequency | Arg | 237 (68.5) | 141 (67.79) | 96 (69.57) | 225 (63.92) | 0.9 |
| | Gln | 109 (31.5) | 67 (32.21) | 42 (30.43) | 127 (36.08) | 0.52 |
| *XRCC4* Ile→Thr codon 401 | | n = 163 (%) | n = 101 (%) | n = 62 (%) | n = 144 (%) | |
| Genotypes | Ile/Ile | 154 (82.9) | 95 (94.06) | 59 (95.16) | 137 (95.14) | 0.69 |
| | Ile/Thr | 9 (17.33) | 6 (5.94) | 3 (4.84) | 6 (4.17) | 0.85 |
| | Thr/Thr | 0 (0) | 0 (0) | 0 (0) | 1 (0.69) | 0.55 |
| Allele frequency | Ile | 317 (97.24) | 196 (97.03) | 121 (97.58) | 280 (97.22) | 0.35 |
| | Thr | 9 (2.76) | 6 (2.97) | 3 (2.42) | 8 (2.78) | 0.65 |
| HWE | | p < 0.05 | p < 0.05 | p < 0.05 | p < 0.05 | |

* Fisher's exact test.

*Table 3*. Associations between the DNA repair *XRCC1* and *XRCC4* polymorphic sites and DNA damage as evaluated by the comet assay in patients with SSc (systemic sclerosis) and controls. *XRCC1* Arg/Gln codon 399 polymorphism: only the *XRCC1*Arg399Gln allele was associated with increased DNA damage in healthy individuals. *XRCC4* Ile/Thr codon 401 polymorphism: SSc and healthy control individuals presenting the *XRCC4* Ile401Thr allele exhibited increased DNA damage. Patients, irrespective of the DNA repair allele, exhibited increased DNA damage when compared to controls.

| Polymorphism | Genotypes, n = 56 | Patients % of DNA (median) | p | Genotypes, n = 38 | Controls % of DNA (median) | p | Patients × Controls |
|---|---|---|---|---|---|---|---|
| *XRCC1* Arg→Gln codon 399 | Arg399Arg, n = 25 | 30.32 | p > 0.05 | Arg399Arg, n = 32 | 1.71 | p < 0.001 | p < 0.001 |
| | Arg399Gln, n = 17 | 24.93 | | Arg399Gln, n = 6 | 11.20 | | p < 0.001 |
| | Gln399Gln, n = 14 | 29.98 | | Gln399Gln, not found | | | |
| *XRCC4* Ile→Thr codon 401 | Ile401Ile, n = 46 | 20.56 | p < 0.001 | Ile401Ile, n = 36 | 2.83 | p < 0.001 | p < 0.001 |
| | Ile401Thr, n = 7 | 84.54 | | Ile401Thr, n = 2 | 15.52 | | p < 0.001 |
| | Thr401Thr, not found | | | Thr401Thr, not found | | | |

When patients with SSc were compared to healthy controls, irrespective of the DNA repair allele, patients exhibited increased DNA damage ($p < 0.001$ for each comparison).

## DISCUSSION

Many studies have identified the presence of spontaneous and induced chromosomal changes in patients with SSc[12]. One of the most important findings is the observation of an increased frequency of micronuclei in SSc peripheral blood lymphomononuclear cells. The micronuclei represent losses of blocks of DNA or even whole chromosomes[14], which may cause loss of regions that encode proteins important for adequate cell functioning[11,12,15,34,35,36].

There are many lines of evidence showing that the cells of patients with autoimmune diseases are more sensitive to genotoxic stress compared to healthy individuals. Lymphocytes from patients with autoimmune diseases, including juvenile RA, SLE, and SSc present elevated levels of DNA damage when subjected to gamma radiation, a potent

inductor of reactive oxygen species and DNA breaks. It has been reported that cells of patients with SLE, when subjected to ionizing radiation, are less efficient in repairing DNA damage, indicating an increased sensitivity to ionizing radiation[37,38,39].

Pre-transplant conditioning with gamma irradiation produces high toxicity to the cells of patients with autoimmune diseases; however, toxicity appears to be increased for patients with SSc compared to patients with other autoimmune diseases[16]. As a corollary, patients with breast cancer who undergo treatment with local irradiation and chemotherapy have a higher predisposition to develop skin manifestations similar to SSc[40].

In our study, we showed that the level of DNA damage, as measured by a single-cell alkaline assay, was from 2-fold to 9-fold higher in patients with SSc compared to healthy individuals, irrespective of disease variant and of drug treatment with azathioprine, methotrexate, and D-penicillamine. In addition, because cyclophosphamide, a drug currently used for the treatment of organ involvement in patients with SSc, has a well-known effect on DNA damage[41,42,43], we further studied 2 small groups of patients, 1 consisting of individuals using no drugs (2 patients) and the other consisting of patients treated with cyclophosphamide for more than 3 months (5 patients), and no difference in the magnitude of DNA damage was observed. Taken together, we can speculate that these findings indicate that the increased DNA damage observed in SSc cells could be caused by the disease itself rather than by drug therapy. Although the influence of DNA damage on SSc pathogenesis has not been completely elucidated, the results reported in our study, together with literature findings reporting that genomic instability may appear in patients with SSc even preceding clinical manifestations[15], indicate that DNA damage may contribute to SSc complications. Indeed, SSc has been associated with cancer, particularly breast cancer, in patients with SSc submitted to treatment with gamma irradiation and chemotherapy[40]. In the study population of 947 patients with SSc, there were 168 deaths in the period 1991–2010; among those deaths, 13.8% of the 58 non-SSc-related deaths were associated with cancer[44].

Besides SSc complications, DNA damage may also contribute to SSc pathogenesis, because DNA damage and genomic instability in different chromosomal regions have been associated with increased production of reactive oxygen species in chronic inflammatory and degenerative processes, and also in autoimmune disorders[45]. Increased reactive oxygen species production has been reported in patients with SSc compared to healthy subjects[46]. In addition, reactive oxygen species may also contribute to SSc pathogenesis, because increased reactive oxygen species are associated with silica inhalation, which may lead to chronic inflammatory processes and may also induce SSc-like manifestations[47]. Moreover, the redox balance, which is fundamental to protect the cells against oxidative stress[48], is not efficient in patients with SSc. Therefore, it is possible that oxidative stress may contribute to higher levels of DNA damage in patients with SSc.

Regarding DNA repair genes, it is interesting to note that individuals exhibiting defects in the DNA repair machinery genes, such as Bloom syndrome and Fanconi anemia, may present skin manifestations similar to SSc[49,50,51], indicating that defects in DNA repair mechanisms may contribute to SSc pathogenesis. Therefore, it is possible that the higher levels of DNA damage observed in SSc in our study may be due to a less efficient DNA repair capacity. To investigate this issue, we evaluated 2 polymorphic sites in important DNA repair genes responsible for SSB (*XRCC1*) and DSB (*XRCC4*) and associated these polymorphic sites with the magnitude of DNA damage.

Considering that polymorphic sites in DNA repair genes may be involved in DNA repair efficiency, we reported that the frequencies of the polymorphic sites in the *XRCC1* and *XRCC4* genes observed for patients with SSc considered as a whole or stratified into clinical variants were closely similar to those observed for healthy individuals. However, when we associated DNA damage with DNA repair gene polymorphic sites, the findings were interesting. Compared to the *XRCC1* Arg399Arg allele, the *XRCC1* Gln399Gln allele has been associated with increased DNA damage in healthy individuals[52,53,54], as we also observed. In addition, the *XRCC1* Arg399Gln allele has been associated with increased genomic instability and increased susceptibility to various cancers, such as head and neck[22], colorectal[23], stomach[24], esophagus[25], breast, and lung cancer[26,27]. Although we did not observe significant differences regarding *XRCC1* gene polymorphism, patients with SSc who exhibited *XRCC1* Arg399Gln, *XRCC1* Gln399Gln, or both alleles presented increased DNA damage compared to healthy individuals, suggesting that the increased DNA damage observed in patients may not depend exclusively on this polymorphic site.

After stratifying patients according to laboratory features, we showed that the presence of the *XRCC1* Arg399Gln allele was associated with the presence of ANA and ACA. Although DNA *per se* is not immunogenic, deficient DNA repair mechanisms may provide more nuclear and cytoplasm antigens, facilitating the production of autoantibodies[55]. The presence of genotoxic stimuli is critical for DNA immunogenicity, because DNA damage caused by exposure to superoxide anion may render DNA more immunogenic in patients with SLE[56]. In addition, guanine residues may be modified by reactive oxygen species in DNA and RNA, inducing circulating autoantibodies in SSc and patients with SLE[57]. Previous studies have associated the presence of allele *XRCC1* Arg399Gln with deficient DNA repair owing to accumulation of DNA

damage[58]. Therefore, it is possible that, under conditions of high levels of DNA damage, such as that observed in the case of chronic inflammation or exposure to environmental agents or the drugs used for treatment, individuals with the *XRCC1* Arg399Gln allele may repair DNA less efficiently, leading to abnormal DNA fragmentation, which in turn may lead to the production of anti-DNA antibodies[9].

Regarding the *XCCR4* gene, it is interesting to observe that patients and controls carrying the mutant Ile401Thr allele showed a significantly increased DNA damage compared to subjects with the wild type Ile allele or the Ile/Ile genotype. There are few functional studies regarding the *XCRR4* Ile401Thr polymorphism, one of them reporting that it is not associated with the genesis of the somatic mutations observed at birth[34]. In addition, the evaluation of polymorphic sites of DNA repair genes in patients with SLE shows that the *XRCC4* Ile401Thr polymorphism does not seem to be associated with an impaired DNA repair, although an additive effect with *XRCC1* Arg399Gln has been suggested[9]. This is the first study, to our knowledge, reporting a functional consequence of the *XRCC4* Ile401Thr polymorphism in patients with SSc. Because there are few therapeutic options to treat patients with SSc, particularly using cytotoxic drugs, and because autologous stem cell transplantation has been occasionally used in severe SSc cases (requiring conditioning pretreatment with high doses of cytotoxic drugs or total body irradiation[16]), a careful screening for aggravating factors should be performed, and the *XRCC4* Thr mutant allele may be a potential marker, deserving further investigation in a larger series of patients.

Peripheral blood cells of patients with SSc exhibited increased DNA damage irrespective of the clinical variant and type of treatment used. Although the frequencies of polymorphic sites in DNA repair *XCCR1* and *XCCR4* genes were closely similar for patients and healthy individuals, the presence of polymorphic sites associated with less efficient DNA repair in patients with SSc was associated with increased DNA damage. The identification of genetic factors associated with increased DNA damage in patients with SSc may be useful to unveil pathogenic features, disease complications, and morbidity in response to new treatments dependent on radiation therapy.

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