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# TELOMERE REDUCTION IN SCLERODERMA PATIENTS: A POSSIBLE CAUSE FOR CHROMOSOMAL INSTABILITY

C. M. ARTLETT, C. M. BLACK,‡ D. C. BRIGGS,* C. O. STEVENS† and K. I. WELSH

*Tissue Typing, Oxford Transplant Centre, Churchill Hospital, Oxford, *Tissue Typing, Middlesex Hospital, London, †Department of Rheumatology, Middlesex Hospital, London and ‡Department of Rheumatology, Royal Free Hospital, London*

SUMMARY

We have hypothesized that the chromosomal instability observed in scleroderma patients and their family members may result from the loss of long stretches of the telomeric repeat which is found at the ends of all linear chromosomes. We examined the telomere lengths in scleroderma (SSc) patients ($n = 43$), their family members ($n = 182$) and in age-matched controls ($n = 96$) using restriction fragment length polymorphism (RFLP) and chemiluminescent labelled probes. The average loss of telomeric DNA in SSc patients and family members was found to be 3 kb when compared to the controls. This loss was not related to age or the duration of the disease. These results may reflect a genetic predisposition for chromosomal instability in these families, or exposure to a common environmental agent. A wide variety of common environmental agents are known to produce chromosomal aberrations; these include fungicides, pesticides, air pollutants and drugs. Scleroderma-like syndromes may be induced by some of these agents.

KEY WORDS: Chromosomal instability, Scleroderma, Telomere.

CHROMOSOMAL instability is a feature of scleroderma and was first reported in 1969 by Housset *et al.* [1]. It is an acquired form of chromosomal damage, thought to be caused by a clastogenic agent. Common chromosomal rearrangements seen are acentric fragments, dicentrics and ring chromosomes. Other abnormalities are derived from translocation or deletion of chromosome fragments, or from intrachromosomal rearrangements [2] and double minute chromosomes [3]. Chromosomal aberrations are not only a consistent feature of scleroderma (SSc) patients, but an increased rate of aberrations is seen in first-degree relatives and patients with Raynaud's phenomenon who later go on to develop SSc [4].

Telomeres, first identified by Muller in 1938 [5], are defined structurally as the terminal sequences of linear chromosomes. They function as the elements that give stability to, and ensure complete replication of, the chromosome end. Telomeres protect natural double-stranded DNA ends from degradation, fusion and recombination with internal chromosomal DNA [5, 6]. The reduction in telomere length, as a potential source for chromosomal damage and alteration through either end–end fusions, associations or deletions, has previously been analysed in other diseases [7, 8], but not in SSc. The evidence for telomere fusions in SSc is strong; multiple telomeric fusions have been reported, where the chromosomes have been seen to form a giant ring or chain. Fusions between sister chromatids in SSc patients have been shown to last for more than two cell generations [9]. It is known that the elderly have shorter telomeres and more chromosomal abnormalities pertaining to telomeric fusions [10, 11]. Tumours are also known to have shorter telomeres and telomeric fusions are also observed in high frequency in these cell lines [12, 13].

The single common structural feature of a telomere is its tandem array of simple repeat units. These units were found to be necessary for telomeric function [14]. The telomeric sequence differs across many species, but can be represented by the consensus sequence $5'\text{-}C_{1\text{-}8}T_{0\text{-}1}A_{0\text{-}4}\text{-}3'$ [15]. All telomeric DNA sequences have distinctly G- and C-rich strands. The G-rich strand is oriented 5′ to 3′ towards the molecular terminus of the chromosome. Oligonucleotide probes used to detect the telomeric smear also detect interstitial sites of telomeric repeats. These internal sites are inherited and can be analysed as a DNA fingerprint [12]. They are thought to be recombination 'scars', and the internal telomere-like stretches may be hot spots for chromosome breakage, fragility and recombination [16].

In this study, we have analysed the telomeric lengths and the interstitial fingerprint pattern from lymphocytes of 43 SSc patients [diffuse cutaneous systemic sclerosis (dcSSc), $n = 17$; limited cutaneous systemic sclerosis (lcSSc), $n = 26$], 182 family members and 96 controls. We have also analysed the fibroblasts from 'involved' and 'uninvolved' skin biopsies and lymphocytes in 27 patients. Any loss of telomeric DNA is directly measurable by telomere-specific probes and may reflect a feature of the abnormalities that are present in SSc chromosomes.

## MATERIALS AND METHODS

DNA was collected from blood lymphocytes using the salting-out method [17] and standardized by spectrophotometry such that all DNA concentrations

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Correspondence to: C. M. Artlett, Department of Rheumatology, Bluemle Lifesciences Building, Rm 522, Thomas Jefferson University, Philadelphia, PA 19107, USA.

were 1 $\mu$g/$\mu$l. From the same individual, fibroblasts were cultured and DNA extracted from abnormal and normal skin; and from blood lymphocytes ($n = 27$) of SSc patients. DNA was also extracted from 10 control fibroblast cell lines. The term 'abnormal' skin has been used for areas on the patient where the SSc phenotype is present in the fibroblasts and this is the typical skin thickening that is indicative of excess collagen deposition. Both biopsies for normal and abnormal skin were taken at the same time. The involved skin biopsy was taken from active lesions in both the lcSSc ($n = 11$) and dcSSc ($n = 16$) patients. The unaffected skin biopsy is taken from the area on the upper arm of the patient with lcSSc and from the back in the patient with dcSSc. Fibroblasts were obtained by punch biopsy performed in the Rheumatology Department at the Royal Free Hospital. Cell lines which grew exceptionally slowly, i.e. taking >3 weeks to reach confluence, were discarded from the study. There were six cell lines which were discarded, four were dcSSc and two were lcSSc. The reasoning for this is that very slow-growing cells are approaching senescence and will therefore have more chromosomal abnormalities [18, 19] than those cells which are not approaching senescence. The cells were cultured at 7%$CO_2$ in Dulbecco's modified Eagle's medium, supplemented with 15% fetal calf serum, 4500 ml/l D-glucose, 2 mM L-glutamine, 30 mg gentamycin, 0.6 mM sodium pyruvate, 600 U penicillin/streptomycin and 1500 U fungisone [20]. All cell lines were cultured to passage 6. Harvesting of the cells and the DNA extraction method have been described previously.

All the individuals studied were Caucasoid and all patients fulfilled the established criteria for SSc [21]. Scleroderma patients were divided into two groups: those with dcSSc in whom skin sclerosis was extensive and rapid, and where organ involvement was an early event ($n = 17$); and those with lcSSc in whom sclerodactyly, facial involvement, telangiectasia and calcinosis were prominent features, and where organ involvement may have been a late complication ($n = 26$). The average length of disease was 16.1 yr (S.D. = 8.3 yr). The SSc families were collected from across the UK and treatments differed from centre to centre, but no SSc patient was known to be taking any clastogenic drugs. Twenty-four spouses were amongst the family members.

Two micrograms of DNA were digested with 10 U *Msp*I and 10 U *Rsa*I [11]. The DNA was then size fractionated on a 0.8% agarose gel overnight at 50 V or until the bromophenol blue dye (0.25% bromophenol blue, 30% glycerol) had reached the end of the gel. The DNA was then capillary blotted onto Hybond N+ (Amersham) overnight. The hybridization and stringency wash temperatures for the telomere probe $(TTAGGG)_5$ were 37°C and 42°C, respectively. Probe labelling with digoxigenin and chemiluminescent detection methods were as follows: 100 pmol of the telomere probe were labelled with 1 mM digoxigenin-11-2',3'-dideoxy-uridin-5'-triphosphate (Dig-11-ddUTP) (Boehringer) with 25 U of terminal transferase (Boehringer). The probe was precipitated with 0.25 M ammonium acetate, 0.2 mM glycogen and 70% ethanol at −40°C overnight, and then centrifuged in a microfuge for 10 min. The supernatant was removed, and the pellet dried briefly and dissolved in 100 $\mu$l distilled water. The non-specific binding sites on the nylon were blocked with 0.1% casein and 0.05% lauroylsarcosine for 30 min at the hybridization temperature. This was then replaced with the hybridization solution that contained 100 pmol of the labelled probe and incubated at the hydridization temperature for 60 min. The filters were rinsed in 5 × SSPE/0.1% SDS, incubated in fresh hybridization solution at the stringency temperature for 15 min, then rinsed in 0.05 M Tris (pH 7.5)/0.02 M NaCl. The filters were re-blocked in 1% casein for 30 min, then 4 $\mu$l of anti-digoxigenin–alkaline phosphatase-conjugated Fab fragments (Boehringer) were added to the blocking solution and incubated at room temperature for 60 min with shaking. Unbound antibody was removed from the filter with four changes of 0.1 M Tris (pH 9.0)/0.01 M $MgCl_2$/0.005 M NaCl over 60 min. Diluted Luminogen PPD [4-meth-4-(3-phosphate phenyl) spiro-(1,2-dioxetane-3,2'-adamantane)] substrate (Boehringer) 1:100 in 0.1 M Tris (pH 9.0)/0.01 M $MgCl_2$/0.005 M NaCl was coated to the DNA side of the piece of nylon, wrapped in plastic and exposed to Kodak X-OMAT AR film for 30 min. The telomere restriction fragment length was determined by densitometric analysis by scanning of the autoradiograms. The end point of the telomeric smear was determined at that point where the smear was twice the background density. The midpoint of the smear was calculated from these data. To determine the reproducibility of this method, we analysed six patients on four specific occasions and found that the midpoints of their telomeric length were within 2–5% of each other.

## RESULTS

The telomeric lengths were measured, and the number and position of the interstitial repeat units were also noted. The consistency of DNA loaded was confirmed by the intensity of the band marked with the arrow in Fig. 1. This figure shows an autoradiograph of the telomeric smear and interstitial repeat fingerprint in SSc patients and family members from lymphocytic DNA. It was found that the average loss of the telomeric repeats in lymphocytes of patients and family members was ~3 kb when compared to the controls (Fig. 2). Our control measurements confirm these previously published [10, 11]. Figure 3 shows the mean lymphocytic telomeric length as a function of age and shows that although the telomeric lengths in the SSc families are shorter than in the controls, there is a gradual decrease in the telomeric length with age in these family members.

Analysis of the fibroblast cell lines revealed that there was little difference between the length of the telomere in the involved and uninvolved skin biopsies in the scleroderma patients, but the lymphocytes were found to have significantly shorter telomeres. It was observed

that the average telomeric length for lymphocytes was 5.45 kb (S.D. ± 0.82) of DNA, whereas the fibroblast length was 7.47 kb (S.D. ± 1.24) for the uninvolved skin and 7.1 kb (S.D. ± 0.87) for the involved skin. Control fibroblast telomeric length was ~8 kb (S.D. ± 1.36).

When we analysed individual family members, we observed a large range of telomeric lengths. We also noted that 11/15 spouses of the patients had significantly shorter telomeres than the controls (Fig. 4). None of the spouses were consanguineous in the SSc families, therefore their decreased telomeric length is intriguing. Most family members were found to have an average length within ±1 kb of the patient length (Table I).

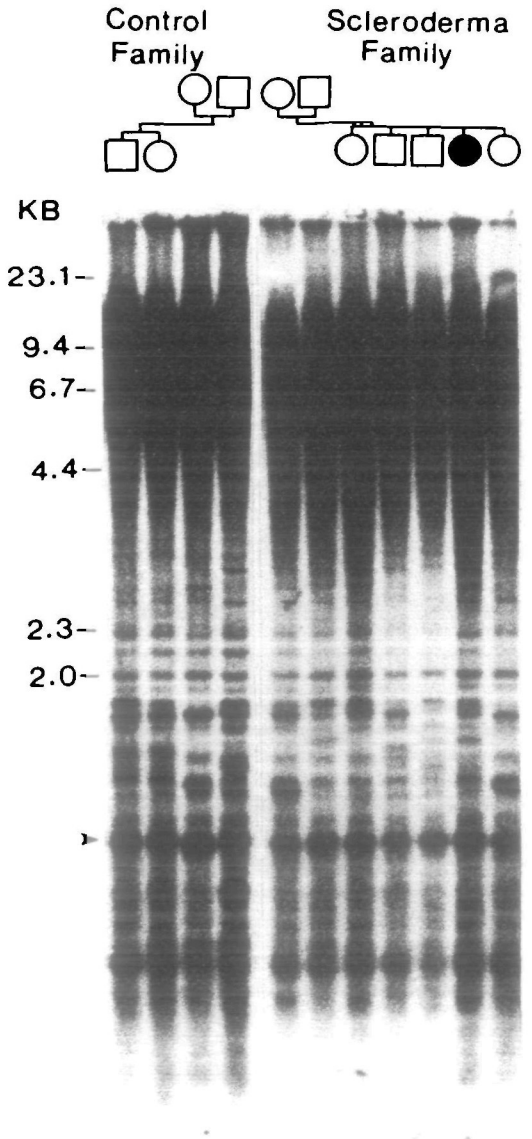


FIG. 1.—Autoradiograph of the telomeric smear and interstitial fingerprint. These two families were age matched by parental age and offspring, and exhibit difference in telomeric length. The offspring are age matched to within 5 yr.

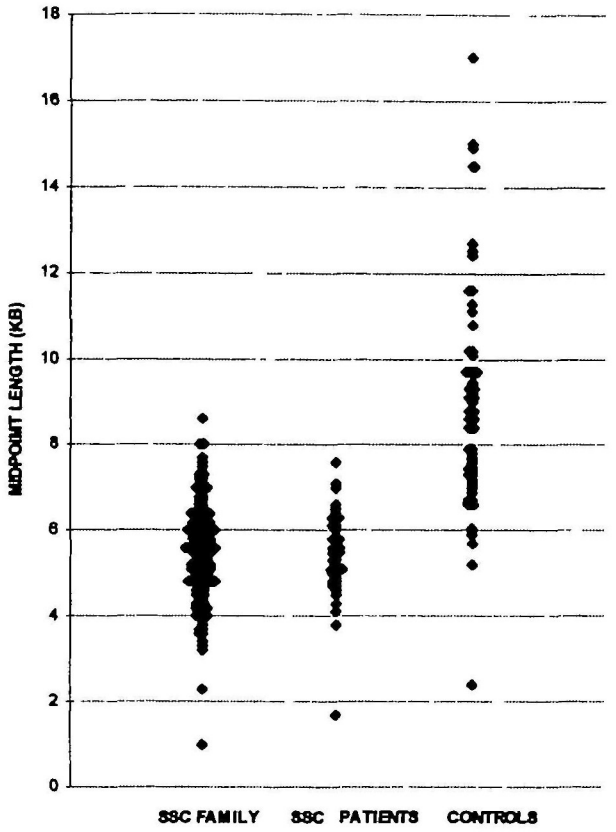


FIG. 2.—Telomeric midpoints in SSc patients, SSc families and controls. SSc family $n = 182$ (mean age 47.8; S.D. = ± 20.6), SSc patients $n = 43$ (mean age 50.4; S.D = ± 19.6), controls $n = 96$ (mean age 22.3; S.D. = 15.9).

The interstitial telomeric repeats were correlated to the family members, and the appearance of new bands not present in parents was noted. The numbers of new bands in the SSc patients were not found to be many, ~10%, but on average more interstitial telomeric bands were found in SSc family members than in the controls. These new bands, although not consistent in size, are most probably due to chromosomal rearrangements. The majority of the interstitial bands have a size which is <2.3 kb. Several bands appear to be present in all individuals, but ~20–25 bands depict the interstitial fingerprint pattern.

## DISCUSSION

Previous reports have shown that normal adults have ~8 kb of telomeric DNA at the end of their chromosomes [11, 12]. In our control group, we were able to confirm previous studies showing that average telomeric length decreased significantly with increasing

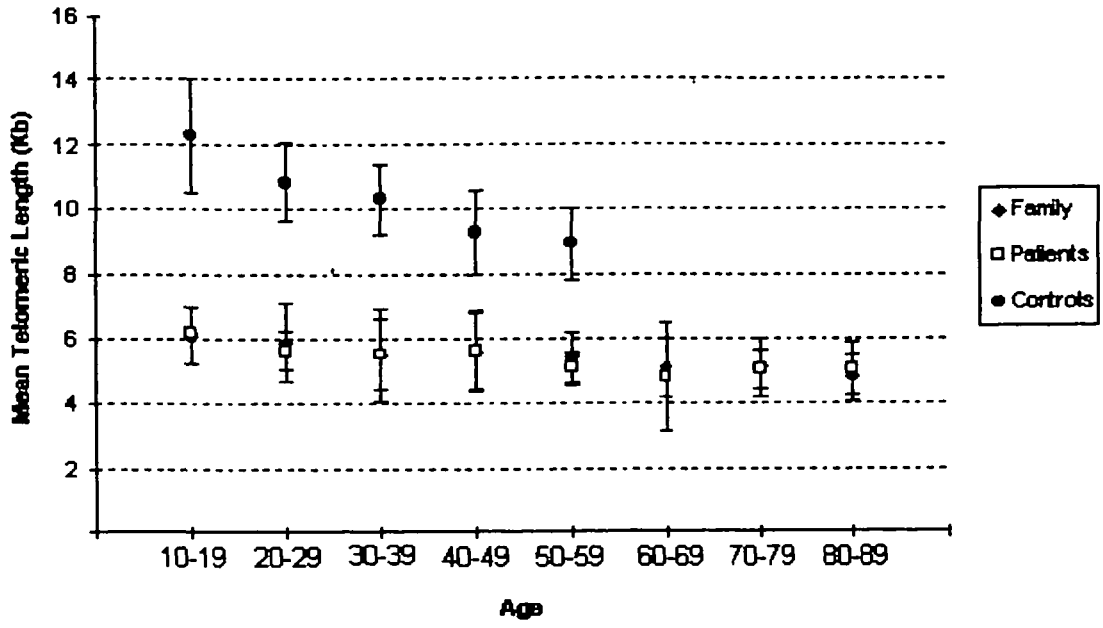


FIG. 3.—Relationship between telomeric length and age in SSc families and controls. Number of individuals in each age group and S.D. (in parentheses) are: 10–19: C = 10 (1.8), P = 1, F = 6 (0.9); 20–29: C = 9 (1.2), P = 3 (0.6), F = 41 (1.2); 30–39: C = 24 (1.1), P = 9 (1.1), F = 30 (1.4); 40–49: C = 14 (1.3), P = 8 (1.2), F = 25 (1.2); 50–59: C = 4 (1.1), P = 10 (0.5), F = 28 (0.8); 60–69: P = 6 (1.7), F = 24 (0.9); 70–79: P = 4 (0.6), F = 15 (0.9); 80–89: P = 2 (0.8), F = 12 (0.7).

age (Fig. 3). In contrast, telomeric length in the SSc families did not decrease significantly with age, but overall was shorter when compared to the controls.

We hypothesize that this shortened telomeric length could reflect exposure to a common environmental agent in these families and also to lymphocyte activation. The average telomeric length of the SSc fibroblasts was found to be slightly shorter than that of the control fibroblasts, but the major loss of the telomere repeat was observed in SSc lymphocytes. Antinuclear antibodies have been observed in 93% of the SSc patients in this study [22], but in only 24% of

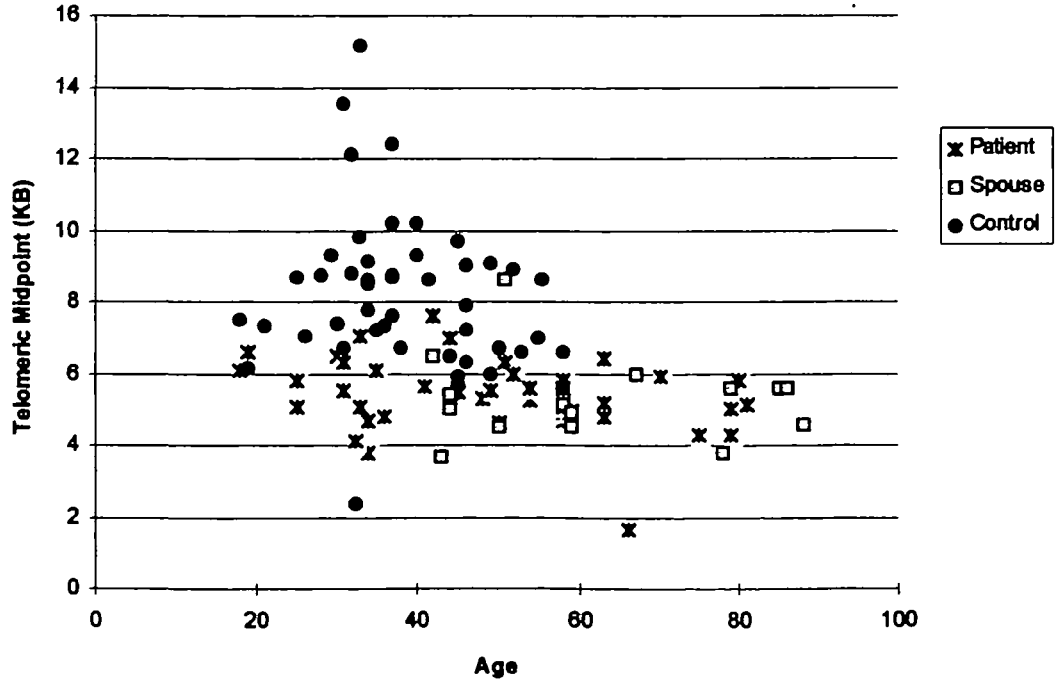


FIG. 4.—Relationship between age and telomere length in controls, SSc patients and spouses. Control ($n$ = 46), patient ($n$ = 43), spouse ($n$ = 15).

TABLE I
Mean telomeric length in SSc patients and family members

| Family code | Patient length (kb) | Mean family length (kb) |
|---|---|---|
| BE | 4.1 | 4.9 |
| LA | 4.7 | 4.8 |
| LJ | 5.6 | 5.9 |
| MA | 7.1 | 5.9 |
| N | 4.6 | 4.7 |
| SC | 6.1 | 6.2 |

spouses and 27% of relatives. We did not identify an association between the antibodies to topoisomerase I or the centromeric proteins and telomere shortening. Furthermore, many of the family members had shorter telomeres than the patients (Fig. 4) and this was seen at all ages. Where the patient telomeric length is low, the average telomeric length within these families is also low. These observations may also implicate a genetic predisposition for telomeric loss within these families. These results also reveal that the shortened telomere in SSc patients does not cause the disease, as some family members have shorter telomeres than patients.

Scleroderma patients and family members were observed to have an increase in the number of interstitial telomeric fragments of ~10%. This increase is not significant, and may reflect the overall genetic variation in DNA fingerprints in the population that results in sampling differences. This increase in fingerprint density may also be a result of chromosomal rearrangements. Certainly, the novel bands found in a small proportion of patients and family members would reflect this.

Telomeres have been found to shorten during the ageing of cultured fibroblasts [11], peripheral blood lymphocytes [23] and colon mucosa epithelia [12]. Although ageing in cells, tissues and organs occurs at many levels and is polygenic, several observations implicate a role for telomere shortening in the senescence of cells. There is an increased frequency of chromosomal abnormalities, especially telomeric associations or dicentrics in senescing fibroblasts [24]. Such chromosomal abnormalities are an indicator of terminal deletions. Telomeric loss may initiate cell cycle exit once a critical or threshold number of telomeric TTAGGG repeats is reached [25]. The loss of the telomere is thought to play an important role in the formation of cancer [24].

We do not see a general shift in the telomeric smear of the type seen in tumour cell lines [10], rather we observe a broadening of the smear. This implies that: (1) not all chromosomes are affected or, alternatively, (2) not all cells are affected.

It has been reported that there are markers of lymphocyte activation associated with SSc [25–27]. Further analysis is needed to determine whether it is these activated cells that have a higher rate of turnover and, therefore, shorter telomeres or if other cells are involved as well. Chromosome sorting and cell sorting will need to be performed to determine the importance of these terminal regions in lymphocyte activation and, therefore, possibly the aetiology of SSc. It is reasonable to assume that there is either increased lymphocytic cell turnover, or proliferation of another group of circulating blood cells causing the shortening of the telomere. Although SSc is not considered to be primarily a genetic disorder, genetic analysis of the MHC has revealed some HLA associations [28, 29]. Additionally, cell sorting will help to determine which of the cell populations, if any, are important in the progression of the disease. Chromosomal instability similar to that observed in SSc, such as strand breaks and dicentrics, has been identified in several other connective tissue diseases, including RA and systemic lupus erythematosus [4, 30, 31]. Further evaluation of these diseases will be important to establish whether shortened telomeres are specific to SSc, a feature of connective tissue diseases, or an indicator of lymphocyte activation and the immune response.

Exposure of cells to known clastogens, such as bleomycin [32], is known to induce SSc-like illness in susceptible people and analysis of telomeric lengths will give a greater understanding of the involvement of clastogens in this disease. We hypothesize that the clastogen which affects the chromosomes in the families is the first stimulus. In the patient, there is a second event or stimulus which pushes that individual on into SSc. We have recently found that SSc patients have molecular alteration at some VNTR sites and this type of instability may be due to the shorter telomere (in preparation).

For the first time at the DNA level, we have been able to demonstrate that SSc patients and family members have shorter telomeres, although as yet the overall implication of this feature for the disease is not well understood. The effect we have observed is not treatment related since it is also observed in family members. However, it may still be environmental agent induced. SSc is known to follow on from a variety of environmental agents, some clastogenic; agents to which family members may also be exposed.

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