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Basic science

Reduced digestion of circulating genomic DNA in systemic sclerosis patients with the DNASE1L3 R206C variant

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Abstract

Objectives: Polymorphism in a coding region of deoxyribonuclease I-like III (*DNASE1L3*), causing amino acid substitution of Arg-206 to Cys (R206C), is a robustly replicated heritable risk factor for SSc and other autoimmune diseases. DNASE1L3 is secreted into the circulation, where it can digest genomic DNA (gDNA) in apoptosis-derived membrane vesicles (AdMVs). We sought to determine the impact of DNASE1L3 R206C on digestion of circulating gDNA in SSc patients and healthy controls (HCs).

Methods: The ability of DNASE1L3 to digest AdMV-associated gDNA was tested *in vitro*. The effect of R206C substitution on extracellular secretion of DNASE1L3 was determined using a transfected cell line and primary monocyte-derived dendritic cells from SSc patients. Plasma samples from SSc patients and HCs with DNASE1L3 R206C or R206 wild type were compared for their ability to digest AdMV-associated gDNA. The digestion status of endogenous gDNA in plasma samples from 123 SSc patients and 74 HCs was determined by measuring the proportion of relatively long to short gDNA fragments.

Results: The unique ability of DNASE1L3 to digest AdMV-associated gDNA was confirmed. Extracellular secretion of DNASE1L3 R206C was impaired. Plasma from individuals with DNASE1L3 R206C had reduced ability to digest AdMV-associated gDNA. The ratio of long: short gDNA fragments was increased in plasma from SSc patients with DNASE1L3 R206C, and this ratio correlated inversely with DNase activity.

Conclusion: Our results confirm that circulating gDNA is a physiological DNASE1L3 substrate and show that its digestion is reduced in SSc patients with the DNASE1L3 R206C variant.

Keywords: SSc, scleroderma, DNASE1L3

Rheumatology key messages

- The systemic sclerosis-associated *DNASE1L3* polymorphism resulting in R206C substitution impairs the extracellular secretion of DNASE1L3.
- Digestion of circulating genomic DNA is reduced in systemic sclerosis patients with DNASE1L3 R206C.

Introduction

Genetic association studies have revealed several SSc-susceptibility loci [1], including identification of a single-nucleotide polymorphism (SNP) in the coding region of deoxyribonuclease I-like III (*DNASE1L3*) resulting in amino acid substitution of Arg-206 to Cys (R206C) [2, 3]. DNASE1L3, an endonuclease expressed predominantly by dendritic cells and macrophages in the liver, spleen and gastrointestinal tract [4–6], has an essential immune-regulatory role. Loss-of-function *DNASE1L3* mutations were identified in familial SLE and hypocomplementaemic urticarial vasculitis syndrome (HUVS) [7, 8], and *Dnase1l3*-null mice spontaneously develop autoantibodies to double-stranded DNA (dsDNA) and protein antigens [5, 6]. The polymorphism encoding DNASE1L3 R206C was also recently identified as a heritable risk factor for RA and SLE [9, 10].

DNASE1L3 is secreted into the circulation and has a unique ability to digest genomic DNA (gDNA) in the lipid-rich environment of apoptosis-derived membrane vesicles (AdMVs) [5]. AdMVs (variably referred to as microparticles, apoptotic

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bodies or apoptotic cell-derived extracellular vesicles), are a subset of extracellular vesicles that are larger than exosomes, are abundant in the circulation as detected by flow cytometry, and contain nucleosomal DNA from apoptotic cells [5, 11–14]. Sisirak *et al.* found excess gDNA in circulating AdMVs (referred to in that paper as apoptotic cell microparticles) in plasma from *Dnase1l3*-null mice and familial SLE patients with rare *DNASE1L3* mutations, and evidence that this DNA can contribute to development of autoantibodies [5]. Regarding functional effects of DNASE1L3 R206C substitution, prior biochemical studies suggested that it reduces DNase activity [15, 16], while a recent study showed impaired extracellular secretion of the DNASE1L3 R206C variant [10].

Elucidation of DNASE1L3's physiological DNA substrate(s) and the effect of the R206C variant on their digestion is critical for understanding the mechanism by which this variant increases SSc susceptibility. In this study, we examined the effect of R206C substitution on DNASE1L3 extracellular secretion by primary cells from SSc patients. Then, we analysed plasma samples from SSc patients and matched healthy controls (HCs) to determine the impact of the DNASE1L3 R206C variant on digestion of circulating gDNA.

Methods

SSc and HC subjects

SSc patients and HCs had been recruited for the Scleroderma Family Registry and DNA Repository (Registry), a multicentre US and Canadian registry including plasma samples stored in the Division of Rheumatology at UTHealth Science Center at Houston (UTHSC-H) [17]. SSc patients met the 1980 preliminary ACR classification criteria, and therefore would also have met the 2013 ACR/EULAR classification criteria for SSc [18, 19]. Patients' demographics, disease duration (defined as time since the first non-Raynaud's symptom) and clinical features were recorded at the time of blood collection. Peripheral blood was collected in EDTA tubes and shipped overnight to the UTHSC-H laboratory, centrifuged at 1217 *g* for 10 min, and the supernatant stored in aliquots at −80°C. SSc patients and HCs whose plasma was analysed were White, not Hispanic or Latino/a, as determined by self-report. Additional SSc patients, who met the 2013 ACR/EULAR classification criteria, and HCs were recruited from our local clinic for peripheral blood mononuclear cells (PBMCs). HCs were not blood relatives of SSc patients and had no personal history of autoimmune disease. See the Supplementary Material, available at *Rheumatology* online, for details on genotyping. All participants provided written informed consent. This research was approved by the UTHSC-H Committee for the Protection of Human Subjects, protocol HSC-MS-02-046.

HUVS subjects

HUVS patients were recruited from the Division of Rheumatology at Bambino Gesù, Ospedale Pediatrico, Rome, Italy. Targeted sequencing of *DNASE1*, *DNASE2*, *DNASE1L3* and *TREX1* was performed using the Illumina (San Diego, CA, USA) NextSeq platform. All variants identified were confirmed by Sanger sequencing. Additional details can be found in Ranalli *et al.* [20]. Peripheral blood was collected in EDTA tubes, then centrifuged at 1160 *g* for 10 min. Plasma aliquots were stored at −80°C and shipped on dry ice to UTHSC-H. Recruitment of HUVS patients was approved by the Institutional Ethical Committee of Bambino Gesù Children's Hospital in Rome, protocol 1666_OPBG-2018.

Cloning and transfection

Plasmids containing the open reading frames (ORF) of human *DNASE1* and *DNASE1L3* were purchased from Genscript (Clones OHu18732, accession no. NM005223.3 and OHu20141, accession no. NM004944.3). Mutations encoding R206C or R206A were created via site-directed mutagenesis using reagents from New England Biolabs (no. E0554). *DNASE1* and *DNASE1L3* wild type (WT), R206C, and R206A were PCR subcloned into pcDNA3.1(+)IRES green fluorescent protein (GFP) vector [gift from Kathleen L. Collins [21]; Addgene (Watertown, MA, USA) plasmid no. 51406]. This vector contains the coding sequence for GFP separated from the inserted gene by an internal ribosomal entry site (IRES), and each gene has its own start and stop codons. GFP is not covalently tagged to the inserted gene, but its expression level serves as an indicator of transfection efficiency. Plasmids were propagated in DH5α *E. coli*, then purified [Qiagen (Germantown, MD, USA), no. 12143]. Each construct was confirmed by restriction digest and sequencing of the ORF. HEK293T cells were transfected via calcium phosphate precipitation. See the Supplementary Material, available at *Rheumatology* online, for details including harvesting the medium and cells, immunoblotting, and RT–quantitative PCR (RT-qPCR).

Primary monocyte-derived dendritic cells

PBMCs were isolated using Ficoll, followed by monocyte enrichment [StemCell Technologies (Vancouver, Canada), no. 19359]. Cells were differentiated *ex vivo* with GM-CSF and IL-4, then harvested for immunoblotting. See the Supplementary Material, available at *Rheumatology* online, for details.

Preparation of DNA or apoptosis-derived membrane vesicles from Jurkat T cells

An aliquot of Jurkat T cells was used for DNA isolation as described in the Supplementary Methods, available at *Rheumatology* online. This DNA was used for qPCR standard curves, and for a biochemical assay noted below. Another aliquot of Jurkat T cells was treated with 1 μM staurosporine [Abcam (Waltham, MA, USA), no. ab120056] for 24 h to induce apoptosis. After confirming >75% cell death via Trypan Blue staining, the cell culture was centrifuged at 400 *g* for 5 min to pellet the cells and large cell fragments. The supernatant containing AdMVs was aliquoted and stored at −80°C. For the experiments utilizing AdMVs, this supernatant was thawed and centrifuged at 16 000 *g* for 20–30 min to pellet AdMVs, then the pellet was resuspended for subsequent analyses. The presence of Jurkat T cell-derived AdMVs and their relative size were confirmed by flow cytometry by staining for CD3 [BioLegend (San Diego, CA, USA), no. 317343] and Annexin-V (Biolegend no. 640912), and using reference particles of 2.0-, 1.0- and 0.5 μm diameter [Thermo Fisher Scientific (Waltham, MA, USA), no. F13839]. See the Supplementary Material, available at *Rheumatology* online, for details.

DNase activity assay using media from transfected HEK293T cells

Medium from transfected HEK293T cells was incubated with either purified DNA or AdMVs from apoptotic Jurkat T cells in a reaction buffer containing calcium and magnesium. After this incubation, DNA was extracted [Wako Chemicals (Richmond, VA, USA), no. 296-60501], and quantified by qPCR. See the Supplementary Material, available at *Rheumatology* online, for details.

Flow Cytometry of AdMVs with DNA labelling

DNase reaction using medium from transfected HEK293T cells and AdMVs was performed as noted above, but with volumes scaled up 100-fold. AdMVs were then labelled with PicoGreen (Thermo Fisher Scientific, no. P11496) and for Annexin V (BioLegend, no. 640935) and analysed by flow cytometry. See the Supplementary Material, available at *Rheumatology* online, for details.

DNase activity assay within plasma samples

Plasma was thawed at room temperature, then 200 µl was centrifuged at 16 000 *g* for 30 min. The lipid layer on top of the supernatant was aspirated, then 100 µl of plasma supernatant was incubated at a ratio of 1:1 with a reaction buffer, for final concentrations of 50 mM Hepes pH 7.5, 4 mM $CaCl_2$ and 4 mM $MgCl_2$. This mixture was incubated at 37°C for 1 h to allow coagulation factors to form a clot, which had the appearance of a gel-like substance in the bottom of the tube. This clot material was manually removed using a pipet tip. The remaining soluble plasma–buffer mix was used for DNase reaction with Jurkat T cell-derived AdMVs as substrate. After incubation the DNA was extracted (Wako Chemicals, no. 296-60501), then quantified by qPCR against *Alu*. See the Supplementary Material, available at *Rheumatology* online, for details.

Measurement of endogenous genomic DNA in plasma

Plasma was thawed. After gentle mixing, an aliquot was diluted 1:500 in TE buffer (10 mM Tris pH 8.0, 1 mM EDTA), then 1 µl was used per 10 µl qPCR reaction; 1:500 dilution was chosen based on empirical testing of a plasma dilution series to determine the dilutions at which the detected DNA concentrations were proportionate to the extent of plasma dilution (interference with the qPCR was observed when plasma was not sufficiently diluted). Each sample was analysed with each of two primer pairs targeting 83-base pair and 244-base pair segments of *Alu*, respectively [10]. See the Supplementary Material, available at *Rheumatology* online, for details.

Statistical analyses

Graphs and statistics were performed using GraphPad (San Diego, CA, USA) Prism 9.3.1. Differences between groups were assessed by Student's *t*-test or one-way ANOVA for continuous data or by chi-square test for categorical data, as specified in the figure and table legends. Correlation was assessed by Pearson's *r*. All *P*-values are two-tailed, and <0.05 was considered significant.

Results

R206C substitution causes reduced extracellular secretion of DNASE1L3

After transfection of HEK293T cells, extracellular secretion of DNASE1L3 R206C, as determined by immunoblotting of the cell culture medium, was reduced compared with that of DNASE1L3 WT (Fig. 1A, lanes 3–4). Mutation of Arg-206 to Ala (R206A) rescued extracellular secretion of DNASE1L3 (Fig. 1A, lane 5), implying that the reduced extracellular secretion of the R206C variant is caused by the Cys residue, rather than the lack of an Arg residue *per se*, at this position. Intracellular expression of each DNASE1L3 variant, as determined by immunoblotting of cell lysates, was similar. Similar transfection efficiency was confirmed by immunoblotting of the cell lysates for GFP, which was expressed on the same plasmid separated by an IRES. RT-qPCR of cells transfected in parallel also confirmed similar transfection efficiency.

Consistent with recent observations [10, 22], monocytes isolated from human peripheral blood samples could be induced to express endogenous DNASE1L3 via their differentiation into monocyte-derived dendritic cells (moDCs) with GM-CSF and IL-4. Comparing moDCs from six individuals (three HCs and three SSc patients) without the DNASE1L3 R206C variant with those from three SSc patients homozygous for DNASE1L3 R206C, DNASE1L3 extracellular secretion was diminished in moDCs with DNASE1L3 R206C (Fig. 1B). Intracellular DNASE1L3 expression, as determined by immunoblotting of the cell lysates, was similar between *DNASE1L3* genotypes, suggesting that the mechanism causing reduced extracellular secretion is post-translational in nature. These results confirm the recent observations by Coke *et al.* who showed reduced extracellular secretion of the DNASE1L3 R206C variant by moDCs from a group of HCs [10].

Plasma from SSc patients and healthy controls with DNASE1L3 R206C has impaired ability to digest AdMV-associated genomic DNA

To determine the impact of the DNASE1L3 R206C variant on digestion of AdMV-associated gDNA within the circulation, we used a biochemical assay modified from that described by Sisirak *et al.* [5]. We first confirmed the unique ability of DNASE1L3 to digest gDNA associated with AdMVs, using recombinant DNase secreted by transfected HEK293T cells, with AdMVs from apoptotic Jurkat T cells as substrate. Flow cytometry confirmed the presence of AdMVs, detected between the 0.5- and 1.0-µm reference beads on forward scatter (Supplementary Fig. S1A, available at *Rheumatology* online) and staining positive both for CD3 and Annexin-V (Supplementary Fig. S1B and C, available at *Rheumatology* online). When purified DNA was used as substrate, both DNASE1 and DNASE1L3 digested gDNA, as demonstrated by qPCR against the short interspersed nuclear element *Alu* (Fig. 2A, left). However, when AdMVs were used as substrate, gDNA digestion was only seen with DNASE1L3 (Fig. 2A, right). This finding was confirmed by flow cytometry, using Annexin V to label the plasma membranes of AdMVs and PicoGreen to label DNA. A population of AdMV-associated DNA was reduced after the AdMVs were incubated with DNASE1L3, but not with DNASE1 (Fig. 2B).

To measure DNase activity within plasma samples against AdMV-associated gDNA, AdMVs were incubated in plasma

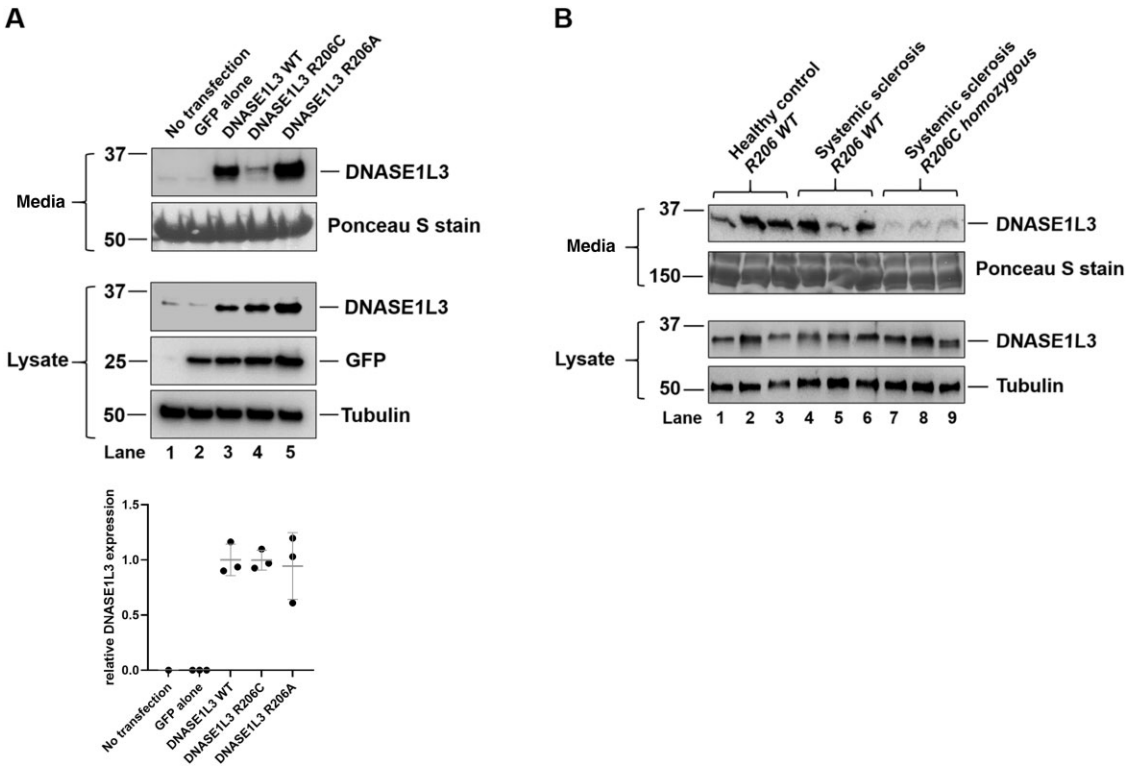

Figure 1. Reduced extracellular secretion of the DNASE1L3 R206C variant. (**A**) HEK293T cells were transfected as indicated. Two days later, the medium and cells were harvested for immunoblotting as indicated. Cells transfected in parallel were harvested for RT-qPCR, shown in the bottom panel. Triplicates were prepared for each condition except for no transfection. Red bars show the mean and S.D. Relative expression was determined based on comparison to DNASE1L3 WT (lane 3). (**B**) Monocytes were isolated from the indicated groups, then differentiated into moDCs for 8 days. Medium and cells were harvested for immunoblotting as indicated. DNASE1L3: deoxyribonuclease I-like III; GFP: green fluorescent protein; moDC: monocyte-derived dendritic cell; qPCR: quantitative PCR; WT: wild type

with a reaction buffer containing magnesium and calcium, after which the DNA was extracted and quantified by qPCR against *Alu* (Fig. 2C). Demographics and clinical features of the HCs ($n = 31$) and SSc patients ($n = 42$) whose plasma was assayed are shown in Table 1. Demographics were closely matched, except for the HC, R206C homozygous group, since there were only two HCs with this genotype with available plasma samples. SSc patients were also matched for disease duration, limited *vs* diffuse cutaneous involvement, and SSc-specific autoantibodies. The extent of digestion of AdMV-associated gDNA was significantly lower in plasma samples from those homozygous for DNASE1L3 R206C compared with those with DNASE1L3 R206 WT (mean DNA digested 4.7% in SSc patients homozygous for DNASE1L3 R206C *vs* 44.7% in SSc patients homozygous for R206 WT, $P < 0.0001$) (Fig. 2D). A partial defect in gDNA digestion was observed in plasma from those heterozygous for DNASE1L3 R206C, indicating a dose effect.

Incompletely digested genomic DNA in the circulation of SSc patients and healthy controls with the DNASE1L3 R206C variant

We initially hypothesized that SSc patients with the DNASE1L3 R206C variant have an excess of endogenous gDNA within the circulation. However, to our surprise, overall gDNA abundance in plasma from SSc patients with the DNASE1L3 R206C variant, as determined by qPCR against a relatively short fragment of *Alu* 83 base pairs in length, was not higher than that of patients without the R206C variant (Fig. 3D, left panel and additional data not shown).

Two recent studies examined the proportion of relatively long fragments of gDNA to shorter fragments as an indicator of the extent of gDNA digestion [10, 23]. Additionally, next-generation sequencing of gDNA extracted from the plasma of *Dnase1l3*-null mice showed an increased frequency of long, multinucleosomal gDNA fragments [24]. We therefore hypothesized that the downstream effect of DNASE1L3 deficiency is an increased proportion of incompletely digested gDNA fragments within the circulation, rather than an increase in overall gDNA abundance. To validate this hypothesis, we examined plasma samples from paediatric HUVS patients homozygous for a rare frameshift mutation of *DNASE1L3* (Ranalli *et al.* [20]). This mutation (c.290_291delCA) results in a premature stop codon and substantially reduced transcript levels of *DNASE1L3* [8]. Homozygosity therefore results in loss of function. After confirming primer pairs targeting relatively short (83 base pairs) or long (244 base pairs) fragments of *Alu* (Fig. 3A and B), we measured the abundance of each gDNA fragment size and determined the ratio of long: short gDNA fragments in plasma from HUVS patients with *DNASE1L3* mutation compared with HUVS patients without the mutation. Although plasma from patients with *DNASE1L3* mutation had lower overall abundance of each fragment size compared with patients without the mutation, they had a higher ratio of long: short gDNA fragments (mean ratio of 0.68 in patients with *DNASE1L3* mutation *vs* 0.09 in patients without *DNASE1L3* mutation) (Fig. 3C). This finding supports the

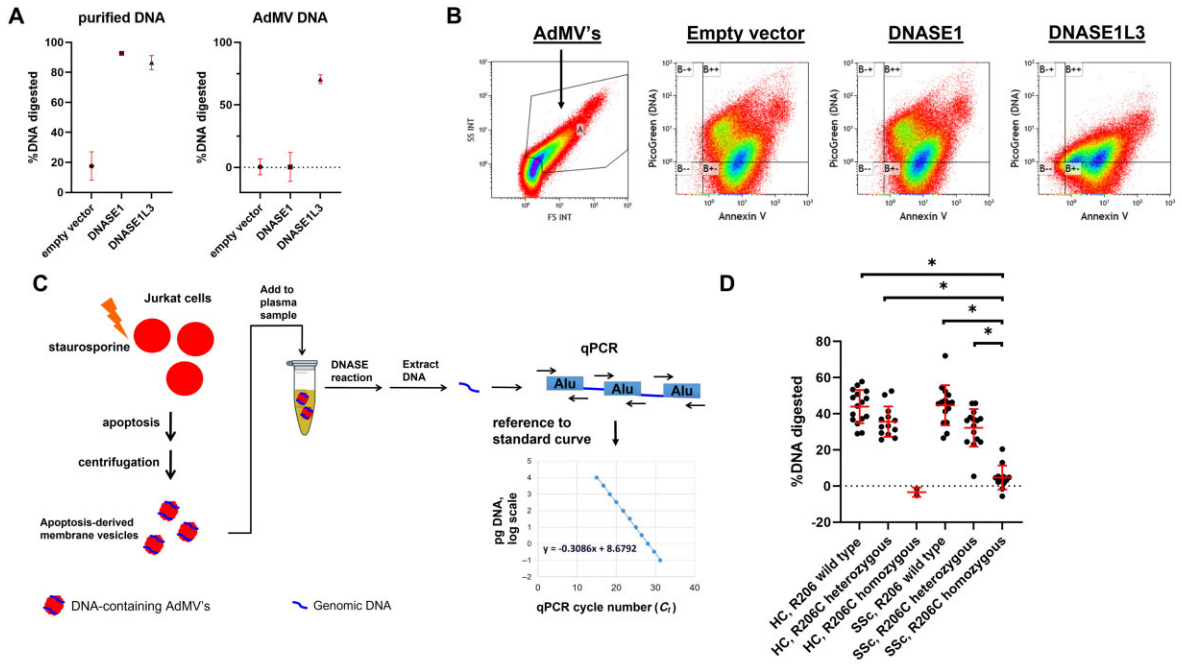

Figure 2. Reduced digestion of AdMV-associated gDNA by plasma from SSc patients and HCs with DNASE1L3 R206C. (**A**) Medium from HEK293T cells that had been transfected as indicated was incubated in a reaction buffer with either purified human DNA (left) or AdMVs from apoptotic Jurkat T cells (right). The DNA was then extracted and quantified by qPCR. Points and error bars represent the mean and S.D., respectively, of triplicates for each condition. (**B**) Same as (**A**), except that after the DNase reaction the AdMVs were labelled with PicoGreen and Annexin V and analysed by flow cytometry. The left panel shows forward (*x*-axis) and side (*y*-axis) scatter, which was used to set a gate for AdMVs. (**C**) Schematic representation of assay to determine plasma DNase activity against AdMV-associated gDNA. (**D**) gDNA digestion by groups as indicated. Each dot represents one subject. Lines and error bars in red represent the mean and S.D., respectively, for each group. $^{*}P < 0.05$. AdMV: apoptosis-derived membrane vesicles; DNASE1L3: deoxyribonuclease I-like III; gDNA: genomic DNA; HC: healthy control; qPCR: quantitative PCR

Table 1. Demographics and clinical features of subjects whose plasma was assayed for DNase activity

	Healthy control ($n = 31$)			Systemic sclerosis ($n = 42$)		
	R206 WT ($n = 16$)	R206C heterozygous ($n = 13$)	R206C homozygous ($n = 2$)	R206 WT ($n = 15$)	R206C heterozygous ($n = 15$)	R206C homozygous ($n = 12$)
Age, mean (S.D.), years	56.5 (9.1)	55.3 (11.8)	35.5 (13.4)	55.9 (7.5)	56.5 (7.1)	57.0 (7.4)
Female, *n* (%)	14 (87.5)	12 (92.3)	2 (100)	13 (86.7)	13 (86.7)	11 (91.2)
Disease duration, mean (S.D.), years				8.7 (6.9)	8.3 (5.1)	7.9 (7.1)
Diffuse skin involvement, *n* (%)				6 (40.0)	6 (40.0)	5 (41.7)
SSc-associated autoantibody, *n* (%)						
Centromere				5 (33.3)	4 (26.7)	5 (41.7)
Topoisomerase-I				3 (20.0)	3 (20.0)	2 (16.7)
RNA Pol III				4 (26.7)	5 (33.3)	3 (25.0)

R206C: Arg→Cys at amino acid 206 of DNASE1L3; WT: wild type (homozygous for the major allele encoding Arg-206).

premise that DNASE1L3 deficiency results in incomplete digestion, albeit not necessarily increased overall gDNA abundance, within the circulation.

We then applied this approach to determine the impact of DNASE1L3 R206C on the digestion status of circulating gDNA in SSc patients and HCs. Demographics and clinical features of the HCs ($n = 74$) and SSc patients ($n = 123$) whose plasma was assayed for endogenous gDNA are shown in Table 2, with matching for age, sex, disease duration, limited *vs* diffuse cutaneous involvement and SSc-associated autoantibodies. Matching was done to minimize potential effects of other variables such as disease severity, age or medication use on circulating DNA digestion across genotype groups so that any observed differences in DNA digestion were more likely attributable specifically to the presence or absence of the DNASE1L3 R206C variant. Accordingly, amongst those with available granular data on disease severity, there were no significant differences across genotype groups in modified Rodnan skin score (mRSS), forced vital capacity (FVC), presence of lung fibrosis on chest imaging, diffusion capacity of the lungs for carbon monoxide (DLCO) or estimated right ventricular systolic pressure (RVSP) by echocardiogram (Supplementary Tables S1–S5, available at *Rheumatology* online). Plasma from SSc patients homozygous for the

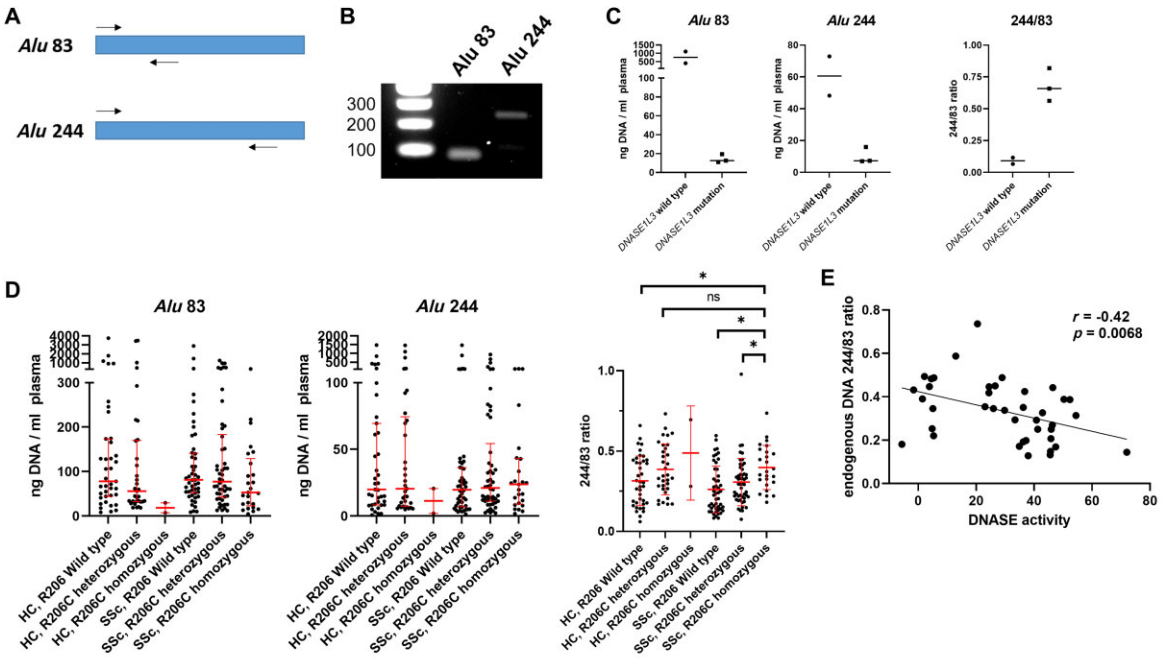

Figure 3. Increased ratio of incompletely digested gDNA in plasma from SSc patients with DNASE1L3 R206C. (**A**) Schematic representation of qPCR-based detection of short (*Alu* 83) and long (*Alu* 244) fragments of gDNA. (**B**) Agarose gel showing PCR products after performing PCR with a plasma sample using these primer pairs. (**C**) Endogenous gDNA abundance as determined using each primer pair with plasma samples from HUVS patients with *vs* without *DNASE1L3* frameshift mutation. Each dot represents one subject. Horizontal lines represent the median for each group. (**D**) Same as (**C**), but comparing HC and SSc groups by genotype as indicated. Each dot represents one subject. Red bars in the *Alu* 83 and *Alu* 244 figures indicate median and interquartile range. Red bars in the *Alu* 244/83 figure indicate mean and S.D. $^{*}P < 0.05$, ns: non-significant. (**E**) Correlation between *Alu* 244/83 ratio (from Fig. 3D) and plasma DNase activity against AdMV-associated DNA (from Fig. 2D) in SSc patients. AdMV: apoptosis-derived membrane vesicles; gDNA: genomic DNA; HC: healthy control; HUVS: hypocomplementaemic urticarial vasculitis syndrome; qPCR: quantitative PCR

Table 2. Demographics and clinical features of subjects whose plasma was assayed for endogenous gDNA

	Healthy control ($n = 74$)			Systemic sclerosis ($n = 123$)		
	R206 WT ($n = 39$)	R206C heterozygous ($n = 33$)	R206C homozygous ($n = 2$)	R206 WT ($n = 48$)	R206C heterozygous ($n = 50$)	R206C homozygous ($n = 25$)
Age, mean (S.D.), years	56.3 (11.1)	53.2 (11.7)	35.5 (13.4)	58.2 (9.3)	58.1 (10.4)	58.0 (9.5)
Female, *n* (%)	33 (84.6)	28 (84.8)	2 (100)	45 (93.8)	47 (94.0)	24 (96.0)
Disease duration, mean (S.D.), years				11.1 (7.0)	11.3 (7.9)	11.6 (7.0)
Diffuse skin involvement, *n* (%)				11 (22.9)	12 (24.0)	5 (20.0)
SSc-associated autoantibody, *n* (%)						
Centromere				28 (58.3)	32 (64.0)	17 (68.0)
Topoisomerase-I				5 (10.4)	5 (10.0)	2 (8.0)
RNA Pol III				6 (12.5)	8 (16.0)	2 (8.0)

R206C: Arg→Cys at amino acid 206 of DNASE1L3; WT: wild type (homozygous for the major allele encoding Arg-206).

DNASE1L3 R206C variant had a significantly higher ratio of long: short gDNA fragment sizes on average than those with R206 WT (mean ratio of 0.40 in SSc patients homozygous for DNASE1L3 R206C *vs* 0.26 in SSc patients homozygous for R206 WT) (Fig. 3D). Intermediate values were again observed in plasma from those heterozygous for R206C.

Amongst patients whose plasma was examined both for biochemical DNase activity against AdMV-associated gDNA (Fig. 2D) and for the endogenous long: short gDNA fragment ratio (Fig. 3D), there was significant inverse correlation between the two (Fig. 3E). This correlation confirms a relationship between circulating DNASE1L3 activity and the digestion status of endogenous circulating gDNA.

Discussion

In this study we confirmed the unique ability of DNASE1L3 to digest AdMV-associated gDNA [5] and impairment of DNASE1L3 extracellular secretion caused by R206C substitution [10]. We performed the largest study to date, and the first to include SSc patients, on the impact of *DNASE1L3* polymorphism on circulating DNA. Our results indicate that extracellular secretion of DNASE1L3 R206C is impaired, reducing the ability to digest AdMV-associated gDNA within the circulation. Consistently, physiological circulating gDNA is not as thoroughly digested in SSc patients with DNASE1L3 R206C compared with those with R206 WT.

In a prior study, qPCR-based quantification of circulating gDNA showed increased overall gDNA abundance in plasma from *Dnase1l3*-null mice [5]. In the same study, this trend was also observed in small groups of patients with rare *DNASE1L3* loss-of-function mutations or heterozygosity for DNASE1L3 R206C. However, conclusions regarding the impact of heritable *DNASE1L3* loss-of-function in humans were limited by small sample sizes. We did not find an increase in the overall abundance of circulating gDNA in SSc patients or HCs with DNASE1L3 R206C, despite reduced ability to digest AdMV-associated gDNA in a biochemical assay. Overall DNA abundance in the circulation is likely influenced by other factors besides *DNASE1L3* genotype, including rates of cell turnover, clearance of AdMVs and other DNA populations from the circulation via endocytosis within lymphoid organs, and the activity of DNASE1, which is able to digest populations of circulating DNA not associated with AdMVs, such as neutrophil extracellular trap DNA [25]. Further study is needed to define the impact of each of these variables on the overall abundance of circulating DNA. Nevertheless, we observed an increased ratio of long: short endogenous gDNA fragment sizes, indicative of incomplete gDNA digestion, in those with DNASE1L3 R206C. This finding is consistent with the observation that *Dnase1l3*-null mice had an increased frequency of long, multinucleosomal gDNA fragments in the circulation [24]. We also demonstrated a relationship between circulating DNASE1L3 activity and the extent of digestion of physiological circulating gDNA.

Although SSc patients and HCs in this study were matched for demographic and clinical variables, our data showed variation in the extent of gDNA digestion within genotype groups, implying that this *DNASE1L3* allele is not the sole determinant of circulating gDNA digestion. Identification of other determinants, which could include other *DNASE1L3* genotypic variants, transcriptional or post-transcriptional regulation of DNASE1L3 expression, DNASE1L3-regulatory modifications or binding proteins, and the extent of gDNA digestion intracellularly during apoptosis prior to its release into the circulation, will require further research. Because this study only included samples from White, not Hispanic or Latino/a subjects, interaction of the DNASE1L3 R206C variant with race and ethnicity could not be assessed. Medication use data are not available in the Registry, so potential effects of medication on circulating DNA digestion cannot be excluded. This concern is partially mitigated by the matching across genotype groups for clinical data including disease duration, year of blood draw, limited *vs* diffuse skin involvement, and SSc-specific autoantibodies, which are associated with distinct disease trajectories. This matching reduces the risk that there would have been differential medication usage across genotype groups, and therefore any potential medication effects on circulating DNA digestion would likely have affected each group similarly.

The downstream immunological consequences of DNASE1L3 R206C, specifically the mechanisms whereby this variant increases susceptibility to multiple autoimmune diseases (SSc, RA and SLE), are incompletely defined. Evidence from mouse models and paediatric SLE patients indicates that DNASE1L3 deficiency can lead to production of anti-dsDNA antibodies relevant to SLE [5, 6]. However, since anti-dsDNA antibodies are not a feature of SSc or RA, these disease associations suggest that DNASE1L3 deficiency can lead to other aberrations in immune function. Our results implicate circulating gDNA as a population of DNASE1L3-substrate DNA that is incompletely digested in those with the R206C variant, and thus further research on the downstream effects of this DNA pool is warranted.

Additional research is also needed to determine the prognostic significance of the DNASE1L3 R206C variant within SSc, for example its potential impact on skin disease severity and the risk of internal organ involvement. Within the Registry we did not find significant associations between the DNASE1L3 R206C variant and mRSS, lung fibrosis as determined by chest imaging, FVC, DLCO, or pulmonary hypertension as determined by right heart catheterization (data not shown). However, the Registry does not include complete data for these metrics and is a cross-sectional prevalent cohort, sub-optimal for analyses of these outcomes. Research in longitudinal cohorts of early SSc patients is needed.

In summary, we confirmed that circulating gDNA is a physiological DNASE1L3 substrate and demonstrated its incomplete digestion within the circulation of SSc patients with the DNASE1L3 R206C variant. These findings can guide research on the downstream mechanisms by which this variant increases susceptibility to SSc and other autoimmune rheumatic diseases.

Supplementary material

Supplementary material is available at *Rheumatology* online.

Data availability

The group-level data underlying this article are available in the article and in its online supplementary material. Anonymized, subject-level data will be shared on reasonable request to the corresponding author.

Contribution statement

B.S. and S.A. designed the study. B.S., X.G., Y.J.L., J.C., K.T.P., J.C. and D.E.L. performed or assisted with experiments. C.B. and I.C. provided plasma samples from HUVS patients. All authors helped with interpretation of the results and with manuscript preparation.

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