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Genetic Variation at the *DNASE I* Locus in an Australian Cohort of SLE Patients

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Abstract

Objective: *DNASE I* serum concentrations and activity, as well as sequence mutations have been implicated in the pathology of systemic lupus erythematosus (SLE). This study was undertaken to assess the serum *DNASE I* concentrations and assess the *DNASE I* sequence variation in a cohort of SLE patients and controls.

Methods: *DNASE I* serum concentrations were assayed in 56 SLE patients and 33 age and sex matched controls. All SLE patients and controls were genotyped for exomic alleles at the *DNASE I* locus and for the variable number tandem repeat alleles present in intron 4 (VNTR - HumDN1).

Results: Skewed *DNASE I* protein concentration distributions were observed with the mean value for SLE patients being 44.2 U/mL compared to 56.4 U/mL in controls (NS). No sample tested negative for *DNASE I* protein. Only two of the previously reported six exomic alleles (*DNASE**1, *DNASE**2) were identified, together with four VNTR alleles (three-six repeats). Both loci manifested Hardy-Weinberg equilibrium. Linkage disequilibrium was observed between exomic alleles and the VNTR alleles, especially between *DNASE I** and 4 repeat VNTR (HumDN1) allele. No significant associations were observed between *DNASE I* concentrations and genotypes. Estimations of haplotype frequencies showed similar distributions for both SLE and the control cohorts, although it was noted that haplotypes containing *DNASE**2 had an elevated frequency of longer VNTR alleles than did *DNASE**1.

A meta-study of *DNASE I* exomic allelic frequencies showed similar frequencies to those obtained in other populations. For the VNTR locus, the longer alleles (five and six repeats) were more frequent, although there was no difference between SLE patients and controls.

Conclusion: This study does not support the hypothesis that specific two locus *DNASE I* genotypes predispose to SLE in the Western Australian cohort.

Keywords: Systemic lupus erythematosus; Serum *DNASE*; Autoimmune disease

Introduction

Systemic lupus erythematosus (SLE) is an important systemic autoimmune disease characterised by the presence of autoantibodies binding double stranded DNA (henceforth dsDNA ab) and decreased levels of serum complement components C3 and C4 [1]. It has been postulated that defective apoptotic clearance of immune complexes incorporating histone associated DNA predisposes to SLE [1]. *DNASE I*, is an endonuclease [2] believed to facilitate the removal of cellular debris thereby preventing aberrant inflammation [3-5]. There have been reports that *DNASE I* deficiency is a predisposing factor for SLE. Lower levels of *DNASE I* enzyme activity may be associated with lupus nephritis [6,7] and correlate with disease activity state and clinical parameters [8]. Yasuda and colleagues [2,9-12] and Iida et al. [13] have identified exomic polymorphisms of the *DNASE I* gene, some of which appear to be associated with susceptibility to SLE [14]. Furthermore, Koji and colleagues [15] have described an A-G transversion in exon two that results in defective synthesis of *DNASE I* in an SLE patient manifesting lower *DNASE I* activity. The *DNASE I* gene also contains a polymorphic 56bp variable number tandem repeat (VNTR-HumDN1) locus within intron four manifesting six alleles corresponding to molecular product sizes of 469, 525, 581, 637 and 693 for two, three, four, five and six repeats respectively [16-18]. Yasuda and colleagues [16] reported that the three repeats allele of VNTR (HumDN1) was in linkage disequilibrium with *DNASE I**1 allele whilst the VNTR alleles four and five were in linkage disequilibrium with *DNASE I**2.

In this study, genetic polymorphisms at both the *DNASE I* locus and at the VNTR (HumDN1) locus were typed in a Western Australian population of SLE patients and controls with known serum concentrations of *DNASE I* protein. The genetic diversity of *DNASE I* polymorphisms in these cohorts was compared with other studies and associations sought with disease and serological parameters indicative of SLE pathology.

Methods

Patients, controls and ethics

Fifty-six (56) treated systemic lupus erythematosus patients aged 47 ± 15 yrs. were recruited between March 2014 and December 2015 from Sir Charles Gairdner (SCGH) and Royal Perth Hospitals (RPH-Perth, Western Australia). The diagnoses were based upon the American College of Rheumatology (ACR) classification criteria. In addition to the SLE diagnosis, patients had co-morbidities including renal disease, liver pathologies as well as Sjögren and Raynaud's syndromes. SLE patients in this cohort were treated with various therapeutic regimens including steroids (low dose prednisolone (≤ 5 mg/day)), immunosuppressant therapies (azathioprine, cyclosporine, cyclophosphamide, methotrexate, mycophenolate, mofetil) and

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Received September 20, 2017; **Accepted** October 16, 2017; **Published** October 18, 2017

Citation: Margery-Muir AA, Wetherall JD, Groth DM (2018) Genetic Variation at the *DNASE I* Locus in an Australian Cohort of SLE Patients. Lupus Open Access 2: 130.

antimalarial therapies (hydroxychloroquine). Thirty-three age and sex-matched healthy control subjects (47 ± 15 yrs.) were recruited from the Perth metropolitan area. Human ethics approval: Curtin University, Sir Charles Gairdner Hospital and Royal Perth Hospital Human Ethics Committees approved the human studies (approval numbers HR 202/2013, HREC 2013-174). Written and informed consent was obtained from all study participants.

Sample collection

Peripheral blood from SLE patients was collected at one time point during the course of their disease monitoring and management into ethylenediaminetetraacetic acid (EDTA), heparin and serum tubes at PathWest collection centres. Serum samples were separated at PathWest laboratories within one hour of collection using centrifugation and frozen to between -70°C and -80°C. Samples from healthy human controls were also collected into EDTA, heparin and serum vacutainer tubes (Beckton Dickinson (BD), San Jose, USA) and processed in the same fashion at the Curtin University laboratory (Perth, WA).

Identification of phenotypes and genotypes at the *DNASE I* and intron 4 VNTR (HumDN1) loci

Stored DNA samples were used in a PCR reaction using MyFi™ DNA polymerase (Bioline, USA) to amplify exons (or part of) two, five, six, seven and eight using primers sets (from GeneWorks, Australia-table S4). The *DNASE I* gene was detected by PCR amplification using primers as indicated in Supplementary Table S4 in PCR microfuge tubes containing 1 µL of test DNA, together with 5 µL of 5x MyFi reaction buffer (Bioline, USA), 0.5 L of each forward and reverse primers 10 µM and 1 µL of MyFi DNA polymerase (Bioline, USA) as well as 17 µL of nuclease free water for a total volume of 25 µL per reaction. PCR assays were performed with cycle parameters set at 95°C for 1 min (1 cycle) and 35 cycles consisting of 95°C for 15 s followed by 61°C for 15 s (*DNASE I*) or 57°C for 15 s (VNTR-HumDN1).

PCR products were then sequenced (MACROGEN, Seoul, Korea) to identify exonic alleles of Human *DNASE I* as indicated in Table 1.

The number of VNTR (HumDN1) repeats within intron 4 of the Human *DNASE I* was determined using a PCR reaction with MyFi™ DNA polymerase (Bioline, USA) to amplify intron four spanned by exons four and five using the primers pair shown in Supplementary Table S4 (GeneWorks, Australia). The lengths of the amplicons were then estimated on a 4% agarose gel electrophoresis using a 1kb DNA ladder. As a control measure, five samples were sent for sequencing (details as above) and the results compared with the gels. Alleles manifested three, four, five and six repeats with corresponding molecular weights of 525, 581, 637 and 693bp respectively [19].

DNASE I enzyme concentrations

A sandwich ELISA kit (Creative Diagnostics, USA) was used to assay human *DNASE I* serum concentrations per manufacturer's instructions. Briefly, stored patient sera were incubated in microtiter wells coated with a *DNASE I* specific antibody. *DNASE I* present in the samples was immobilized and unbound products removed. Biotin labelled *DNASE I* specific antibody bound to immobilized *DNASE I* was detected with avidin conjugated Horseradish Peroxidase (HRP). Test sample concentrations were interpolated using a four-parameter logistic calibration curve fitted to the standard sera provided by the manufacturer (100, 50, 25, 12.5, 6.25, 3.12, 1.56 and 0 ng/mL).

Other serological parameters associated with SLE

We have previously reported the methods for assaying C1q, C3, and C4 concentrations in the serum of the SLE and control cohorts [20].

Data analysis

Nucleotide and peptide sequences were analysed using Geneious version 10 [21]. Linkage disequilibrium and population parameters were determined using "Genepop on the web" [22,23], LinkDos [24] and SNPStats [25]. Statistical significance was calculated using Mann-Whitney rank sum U-test with the GraphPad PRISM 7 application (GraphPad Software Inc., California, USA). P values of <0.05 were considered statistically significant.

Results

DNASE I protein concentrations in SLE patients and controls

DNASE I protein concentrations observed in serum samples of SLE patients and controls are shown in Figure 1. The distribution of concentrations in both cohorts was skewed; the mean concentration of *DNASE I* protein in SLE patients was lower than that of the controls (44.2 U/mL *vs.* 56.4 U/mL) although the difference was not significant. A preliminary investigation of associations between *DNASE I* serum concentrations and co-morbidities and drug therapies in the SLE patients was undertaken using heat maps is shown in Figures 2C and 2D respectively.

Allele and genotype frequencies at the *DNASE I* and intron 4 VNTR (HumDN1) loci

Allele frequencies at the *DNASE I* exomic and VNTR (HumDN1) loci are summarised in Table 2.

Alleles at both intragenic loci exhibited Hardy-Weinberg equilibrium and linkage disequilibrium. The frequencies of haplotypic combinations at both the *DNASE I* and VNTR (HumDN1) loci are shown in Figure 3. Haplotypes with VNTR alleles of 4, 5 and 6 repeats were more prevalent in both the SLE and control cohorts.

Ethnic variation in *DNASE I* polymorphisms

The allele frequencies reported in this study are in general agreement with the metastudy of ethnic allele frequency variation shown (see Table 3). Only the Ovambos' of African origin have higher frequencies of the *DNASE I**1 relative to the *DNASE I**2 allele. No apparent significant differences between *DNASE I* allele frequencies were observed for either the SLE patients or the healthy controls. A comparison of ethnic variation in the frequencies of the internal VNTR (HumDN1) locus within the different

DNASE I allele	Exon 2	Exon 5	Exon 6	Exon 7	Exon 8
*DNASE I**1	CAG-Gln	GTG-Val	CCC-Pro	CGC-Arg	CAA-Gln
*DNASE I**2	CAG-Gln	GTG-Val	CCC-Pro	CGC-Arg	CGA-Arg
*DNASE I**3	CAG-Gln	GTG-Val	GCC-Ala	CGC-Arg	CAA-Gln
*DNASE I**4	GAG-Glu	GTG-Val	CCC-Pro	CGC-Arg	CGA-Arg
*DNASE I**5	CAG-Gln	ATG-Met	CCC-Pro	CGC-Arg	CGA-Arg
*DNASE I**6	CAG-Gln	GTG-Val	CCC-Pro	TGC-Cys	CGA-Arg

Table 1: PCR products sequenced to identify exonic alleles of Human *DNASE I*.

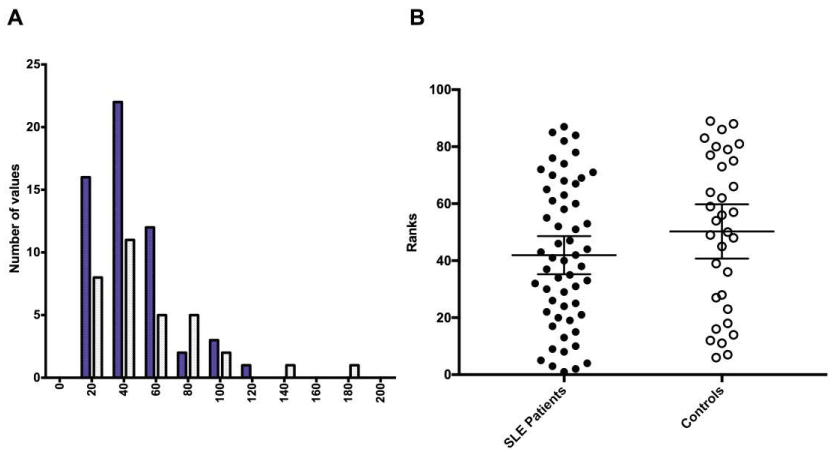

Figure 1: Serum *DNASE I* concentrations. A: purple bars-the frequency distribution (U/mL) in the SLE (mean=44.2 U/mL) and light grey bars-control (mean=56.4 U/mL) populations. B: Ranks of serum *DNASE I* concentrations in SLE and control cohorts showing means ± standard deviation-not significantly different.

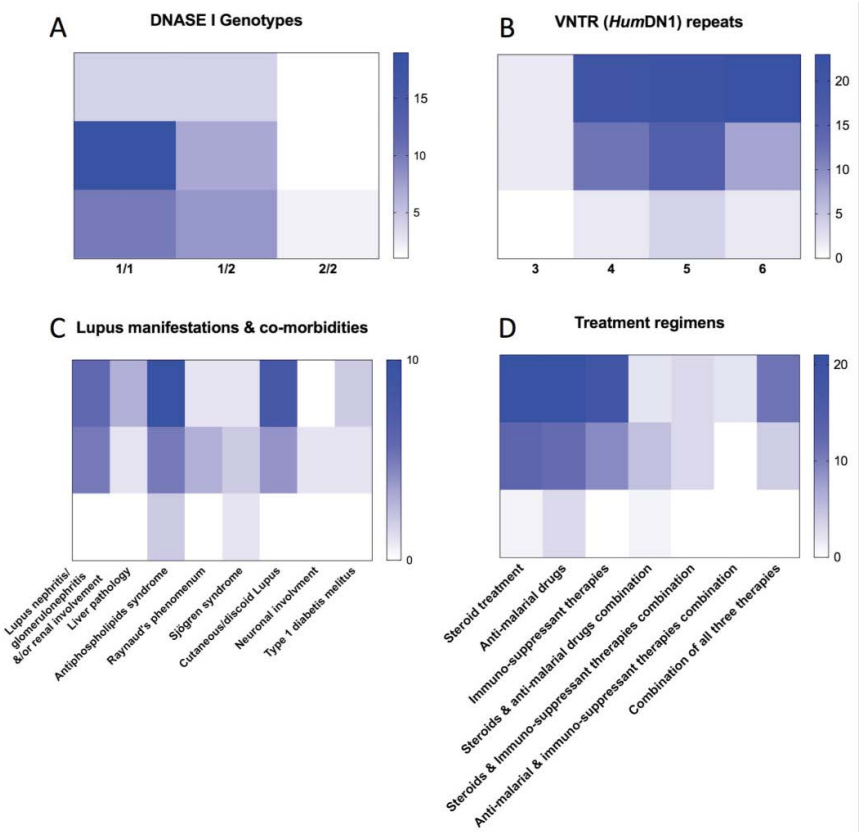

Figure 2: Heat maps showing associations between mean concentrations of serum *DNASE I* in SLE patients stratified in three groups defined as low concentration range, mid-concentration range and high concentration range with mean concentrations of *DNASE I*=29 U/mL top row, *DNASE I*=59 U/mL middle row, and *DNASE I*=103 U/mL bottom row respectively. Figures 2A and 2B: *DNASE I* concentrations versus *DNASE I* genotypes and VNTR (HumDN1) repeats genotypes respectively. Figures 2C and 2D: *DNASE I* concentrations in SLE patients versus co-morbidities and drug therapies respectively.

Population	*DNASE* */*1*	*DNASE* */*2*	VNTR (HumDN1) 3	VNTR (HumDN1) 4	VNTR (HumDN1) 5	VNTR (HumDN1) 6
SLE (n=56)	0.393 (n=44)	0.607 (n=68)	0.036 (n=4)	0.304 (n=34)	0.366 (n=41)	0.295 (n=33)
Control (n=33)	0.394 (n=26)	0.606 (n=40)	0.000	0.409 (n=27)	0.348 (n=23)	0.242 (n=16)

Table 2: Allele frequencies for the *DNASE I* locus and the intron 4 VNTR (HumDN1) locus in Australian SLE patients and healthy controls (numbers in brackets indicate the number of alleles in each group).

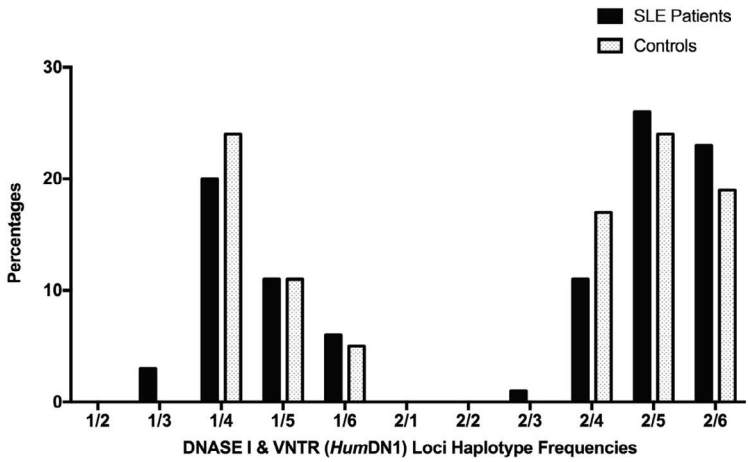

Figure 3: DNASE I (left number) & VNTR (HumDN1) (right number) Loci Haplotypes. The figure shows percentage haplotype frequencies in both loci for the SLE patients and control cohort.

Population (References)	Allele *DNASE I**1 Not diseased	Allele *DNASE I**2 Not diseased	Allele *DNASE I**1 SLE	Allele *DNASE I**2 SLE
Mixed Europeans (this study) Controls (n=25) (SLE n=24)	0.38	0.62	0.375	0.625
Mixed Asians (this study) Controls (n=5) SLE (n=11)	0.5	0.5	0.454	0.545
Mixed other (this study) Controls (n=3) SLE (n=21)	0.333	0.667	0.381	0.619
Germans [26,27]	0.294	0.705		
Germans [28]	0.265	0.728		
Spanish [29]	0.3003	0.6997	0.2246	0.7754
Japanese [26]	0.547	0.445		
Japanese [28]	0.595	0.405		
Koreans [28]	0.597	0.403		
Chinese (Han) [30]	0.536	0.464		
Chinese (Shenyang) [31]	0.567	0.433		
Chinese (Guangzhou) [31]	0.5417	0.4583		
Argentineans [14]	0.28	0.72	0.34	0.66
Turks [18]	0.221	0.779		
Ovambos [18]	0.872	0.128		
Ovambos [32]	0.872	0.128		
Ovambos [28]	0.889	0.111		
Indian Tamils [33]	0.52	0.49	0.495	0.505

Table 3: Frequency data for the *DNASE* alleles *1 and *2 are derived from published reports. The rare *DNASE I**3; *4; *5 and *6 alleles are not included due to insufficient published data. "Mixed other" control group contained individuals of Middle Eastern and Indian ethnicity whereas the "Mixed other SLE" group included individuals from African, South American and Australian Indigenous ethnicities which were grouped together as there were only a small number of individuals in each.

ethnic groups is shown in Table 4. The frequencies of the six alleles reported in this study were similar to those observed in other population with the exception that the long six repeat allele was more frequent in both the control and SLE populations reported herein. The genetic differentiation of the Ovambo and Japanese populations was evident with the three-repeat allele being more prevalent than in the other populations reported.

Are serum *DNASE I* concentrations dependent on genotype?

DNASE I serum concentration distributions are shown ranked by genotype in Figure 4.

There were no significant associations between *DNASE I* serum concentrations and either the *DNASE I* exomic genotypes or the repeat number variation of the VNTR (HumDN1) locus. The lowest concentration levels of serum *DNASE I* were associated with *DNASE I**2 genotype, and with the higher number of repeats at the VNTR (HumDN1) locus.

Associations between serological parameters and *DNASE I* and VNTR (HumDN1) alleles

A correlation matrix was generated for age, ethnicity, [C1q], [C3], [C4] and [*DNASE I*] versus VNTR (HumDN1) and *DNASE I* alleles (Supplementary Table S1). No significant correlations between the various parameters were observed except for serum concentrations of *DNASE I* and C1q complement protein (r=0.653; p=0.05). In the SLE cohort, the VNTR (HumDN1) repeat number was significantly correlated with [C3] and [C4] (0.304; p=0.03 and 0.279; p=0.04 respectively). However, specific alleles at both the VNTR (HumDN1) and *DNASE I* loci were significantly correlated in the SLE and control cohorts (r=0.760; p=0.0001; 0.569; p=0.0005 respectively) (Supplementary Tables S2 and S3).

In the absence of well-defined associations between specific *DNASE I* genotypes and SLE, heat maps were used to visualize trends in disease severity that may be related to *DNASE I* genotypes or serum concentrations in both the SLE patient and control cohorts. The heat map analyses (Figure 2) show qualitatively that SLE patients with higher numbers of VNTR (HumDN1) repeats (≥ 5) had lower serum concentrations of *DNASE I* protein and required more intensive drug regimens for management of their disease than persons with low numbers of repeat motifs.

Discussion

In this investigation only the two most frequent (ie. *DNASE I**1 and *DNASE I**2) of the six exonic alleles defined at the *DNASE I* locus were observed, although the typing system (shotgun sequencing) would have detected them if present. No variations in the nucleic acid sequence within exons two, five and seven were present in any of the individuals tested. The remaining four alleles (*DNASE I**3, *4, *5 and *6) are rare and there are few reports of their frequencies in the other populations [26]. The frequencies of the *DNASE I**1 and *2 in persons of European and Asian ethnicity were similar to previously published reports [26-

Population (References)	Allele 1 Not diseased	Allele 2 Not diseased	Allele 3 Not diseased	Allele 4 Not diseased	Allele 5 Not diseased	Allele 6 Not diseased	Allele 1 SLE	Allele 2 SLE	Allele 3 SLE	Allele 4 SLE	Allele 5 SLE	Allele 6 SLE
Mixed Europeans (this study) controls (n=50) SLE (n=48)	0	0	0	0.38	0.34	0.28	0	0	0	0.25	0.44	0.31
Mixed Asians (this study) controls (n=10) SLE (n=22)	0	0	0	0.6	0.4	0	0	0	0.05	0.41	0.36	0.18
Mixed other (this study) controls (n=6) SLE (n=42)	0	0	0	0.17	0.5	0.33	0	0	0.07	0.31	0.29	0.33
German [16]	0	0.01	0.26	0.34	0.39	0.01						
German [34]	0	0	0.39	0.33	0.28	0						
Japanese [16]	0	0.01	0.56	0.27	0.16	0						
Japanese [35]	0	0.03	0.58	0.21	0.18	0						
Chinese [35]	0	0.02	0.47	0.3	0.21	0						
Turks [35]	0	0	0.24	0.37	0.4	0						
Kuwaiti [17]	0	0.01	0.33	0.39	0.16	0.1	0	0.03	0.27	0.26	0.45	0
Kuwaiti [19]	0	0.02	0.31	0.37	0.18	0.12	0	0.05	0.28	0.26	0.42	0
Ovambo [35]	0	0.09	0.79	0.12	0.01	0						
Ghanaian [34]	0.01	0.08	0.77	0.09	0.05	0						
Iranian [36]	0	0	0.32	0.41	0.21	0.06	0	0	0.25	0.41	0.28	0.06

Table 4: Frequency data for the VNTR (humDN1) number of repeats are derived from referenced published reports. The rare *DNASE I**3; *4; *5 and *6 alleles are not included due to insufficient data. "Mixed other" control group contained individuals of Middle Eastern and Indian ethnicity whereas the "Mixed other SLE" group included individuals from African, South American and Australian Indigenous ethnicities which were grouped together as there were only a small number of individuals in each.

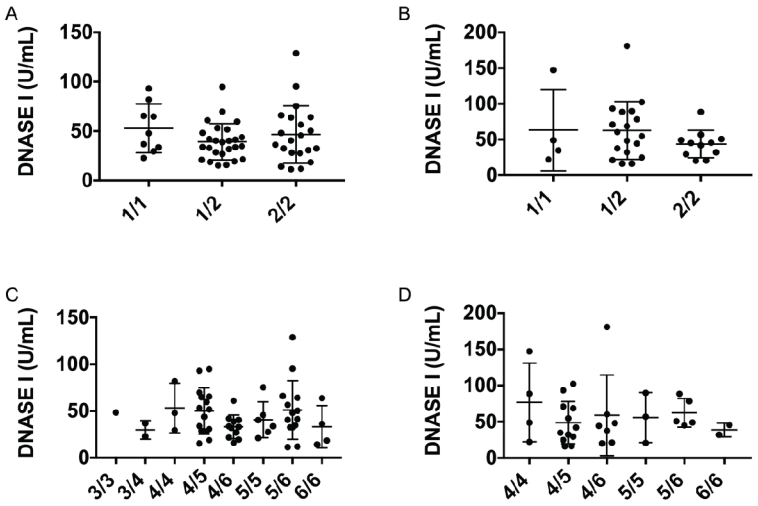

Figure 4: Shows the mean concentrations (U/mL ± SD) of the *DNASE I* protein in SLE patients (A & C) and controls (B & D) associated with *DNASE I* phenotype *DNASE I**1 or *DNASE I**2 (A and B) and with the VNTR (HumDN1) number of repeats (C and D).

28]. Four alleles were observed at the VNTR locus with the number of repeats varying from three to six. A greater frequency of the longest six repeat allele was observed in both cohorts in this study which may reflect improved typing techniques. Only in the Ovambo and Ghanian populations did allele frequencies at both loci differ markedly from the values reported in other populations. This is consistent with an ancestral version of the *DNASE I* gene being present in modern day African populations [18].

Allele frequencies at both the *DNASE I* and VNTR (HumDN1) loci manifested Hardy-Weinberg equilibrium despite the relatively small sample size of the populations typed. As would be expected, both intragenic loci exhibited linkage disequilibrium (LD) as has been previously reported [16,37].

Of the 36 possible genotypes comprising observed alleles at these two loci, 13 were observed in the SLE patients and 12 in the control group. These data demonstrate the presence of a restricted number of haplotypes in our populations. Al Fadhli and co-workers have shown that higher number of the VNTR (HumDN1) repeats are a risk factor for SLE and that the allele with five repeats was associated with a reduction of *DNASE I* enzyme activity [17]. It was not possible to measure the *DNASE I* enzyme activity in this study, however serum protein *DNASE I* concentrations were assayed in both populations. All persons tested exhibited *DNASE I* protein in their serum. Significant differences in serum *DNASE I* protein concentrations between SLE patients and the control cohort were not observed, although the mean concentration in the SLE cohort was slightly lower than in the control group. If *DNASE I* activity is proportional to the concentration of *DNASE I* protein it is unlikely that non-expressed mutant genes were present in the cohorts investigated.

Associations between the VNTR (HumDN1) locus and complement C3 and C4 serum concentrations were observed in the SLE cohort but

not in the control group. Hence, the number of VNTR (HumDN1) repeats in the SLE patients may influence clinical parameters. AlFadhli and co-workers have shown that higher number of the VNTR (HumDN1) repeats are a risk factor for SLE, and moreover, that the five-repeat allele was associated with a reduction of *DNASE I* enzyme activity [17].

The heat map analysis (Figure 2) showed qualitatively that SLE patients with higher numbers of VNTR (HumDN1) repeats (≥ 5) had lower serum concentrations of *DNASE I* protein than the controls and required more intensive drug regimens for management of their disease. However, quantitative analysis did not reveal any significant correlations between the serum concentrations of *DNASE I* protein and either phenotypes at the *DNASE I* and VNTR (HumDN1) loci or with disease severity (Figure 4). Other reports [17,36] in particular that of Al Fahdi [17] show that a high number of VNTR repeats are a risk factor for SLE and may cause decreased *DNASE I* activity. Positive correlations between *DNASE I* and C1q serum concentrations and the length of the VNTR allele with serum concentrations of complement components C3 and C4 were observed and noted. If low *DNASE I* activity and/or concentration impairs removal of apoptotic material thereby predisposing to SLE, it might be expected that VNTR alleles negatively correlate with C3, C4 and C1q serum concentrations in SLE patients. Hence this report does not support the hypothesis that specific two locus *DNASE I* genotypes predispose to SLE in the Western Australian cohort.

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