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Gene Copy-Number Variations (CNVs) of Complement *C4* and *C4A* Deficiency in Genetic Risk and Pathogenesis of Juvenile Dermatomyositis

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Abstract

Objective—Complement-mediated vasculopathy of muscle and skin are clinical features of juvenile dermatomyositis (JDM). We assess gene copy-number variations (CNVs) for complement C4 and its isotypes, C4A and C4B, in genetic risks and pathogenesis of JDM.

Methods—The study population included 105 JDM patients and 500 healthy European Americans. Gene copy-numbers (GCNs) for total *C4, C4A, C4B* and *HLA-DRB1* genotypes were determined by Southern blots and PCRs. Processed activation product C4d bound to erythrocytes (E-C4d) was measured by flow cytometry. Global gene-expression microarrays were performed in 19 JDM and 7 controls using PAXgene-blood RNA. Differential expression levels for selected genes were validated by qPCR.

Results—Significantly lower GCNs and differences in distribution of GCN groups for total *C4* and *C4A* were observed between JDM and controls. Lower GCN of *C4A* in JDM remained among *HLA DR3*-positive subjects (p=0.015). Homozygous or heterozygous *C4A*-deficiency was present in 40.0% of JDM compared to 18.2% of controls [odds ratio (OR)=3.00 (1.87–4.79), $p=8.2x10^{-6}$]. JDM had higher levels of E-C4d than controls (p=0.004). In JDM, *C4A*-deficient subjects had higher levels of E-C4d (p=0.0003) and higher frequency of elevated levels of multiple serum muscle enzymes at diagnosis (p=0.004). Microarray profiling of blood RNA revealed upregulation

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CHS, AP, KEL, LGR, LP and CYY conceived and designed the study. AP, RAA, LGR, FWM and CHS recruited JDM patients and investigated patients' clinical presentations. KJ, YLW, KEL and CYY recruited healthy controls. KEL, YLW, BZ, EL, AA, DN, TPO and CYY performed experiments. KEL, RAA, CHS, PW, LP, LGR and CYY performed data interpretation and analyses. KEL, CHS, LGR and CYY wrote the manuscript. All authors (except DN who is deceased) revised and approved the manuscript as written.

of type I Interferon-stimulated genes and lower abundance of transcripts for T-cell and chemokine function genes in JDM, but this was *less* prominent among *C4A*-deficient or *DR3*-positive patients.

Conclusions—Complement *C4A*-deficiency appears to be an important factor for the genetic risk and pathogenesis of JDM, particularly in patients with a *DR3*-positive background.

Keywords

erythrocyte-bound C4d (E-C4d); elevated serum muscle enzyme levels; *HLA-DRB1*0301*; *HLA-DRB1*1501*; gene expression profiles

INTRODUCTION

Juvenile dermatomyositis (JDM) is a rare, autoimmune, multi-system inflammatory disease affecting primarily muscle and skin in children. Characteristic clinical features and diagnostic criteria include proximal muscle weakness and inflammation, increased levels of serum muscle enzymes, distinct skin rashes such as Gottron's papules or heliotrope rash, and pathological changes on muscle biopsy or magnetic resonance imaging (MRI) [1–7].

The HLA class II gene *DRB1* allele **0301* (also known as *DR3*) has been identified as a major immunogenetic risk factor for JDM and was reaffirmed as the predominant risk locus of juvenile and adult dermatomyositis in a genome-wide association study [3–5 8]. HLA class I and class II genes are engaged in antigen presentation and processing. The class III genes are heterogeneous in structures and function, and include genes encoding for components of the complement system C4, C2 and factor B, and for cytokines such as tumor necrosis factor-α (TNF-α), and α and β lymphotoxins (Figure 1) [9]. Previous studies revealed that class II genes *DRB1* allele **0301* and *DQA1* allele **0501*, class III gene *TNFA*–308A allele, and class I gene variants *B*08* and *A*01* are in strong linkage disequilibrium among human subjects of European descent and this is described as the ancestral haplotype AH8.1 [10–12]. Also present in AH8.1 is a single *C4B* gene but the *absence* of a *C4A* gene [10 13 14]. Remarkably, there are extensive inter-individual gene CNVs and gene-size dichotomy for complement *C4*. Briefly, two to eight copies of *C4* genes can be present in a diploid genome [15 16]. Segmental duplications for complement *C4* genes occur as RCCX modules, which always include the *RP* (*STK19*) gene upstream of *C4*, and the downstream genes *CYP21* and *TNX* (RCCX). Each *C4* gene can be a long gene of 20.6-kb or a short gene of 14.2-kb [17 18]. Each *C4* gene either encodes for an acidic C4A or a basic C4B protein, with only four amino acid changes (**PC**PV**LD** 1101–1106 for C4A and **LS**PV**IH** for C4B), but these result in substantial differences in chemical reactivity for peptide and carbohydrate antigens [19–22].

Complement-mediated destruction of perivascular endothelium and perifascicular ischemia of muscle fibers in biopsies from dermatomyositis patients have been demonstrated by multiple investigators [23–28]. Circulating immune complexes, immunoglobulins IgG and IgM, complement C3 and C5b-9 membrane attack complex were shown in dermatomyositis muscle and skin biopsies. However, the initiation for complement activation and the potential role of complement in the breakdown of immune tolerance in JDM remain unclear.

Continuous CNVs with 1 to 4 copies of *C4* genes on a haplotype with different combinations of *C4A* and *C4B* genes in human populations have only been established since 1999 [16 29 30]. Many earlier epidemiologic studies of complement *C4A* and *C4B* in rheumatic diseases, including JDM, were based on an incomplete or inaccurate model with two-locus (*C4A*-*C4B*) on a haplotype for data interpretation, and thus conclusions drawn became uncertain [31–34]. Here we perform a fresh and meticulous investigation of *C4* genetic diversity and examine their effects on the risk and pathogenesis of JDM, with further considerations to the presence and absence of *HLA-DRB1* risk and protective alleles.

PATIENTS AND METHODS

Study populations

IRB approval was obtained from Nationwide Children's Hospital (NCH) and the National Institutes of Health (NIDDK/NIAMS, NIH). One hundred five JDM patients were enrolled, of which 45 were from NCH and 60 were from the NIH. Each patient met the diagnostic criteria for JDM according to the Bohan and Peter criteria [1 2]. Typical characteristic MRI abnormalities of muscle were applied in place of biopsy as a modification of the Bohan and Peter criteria for the NCH cohort [35]. The mean age (±SD) at recruitment was 10.8±7.6 years old, and at disease diagnosis was 7.4±4.2 years old. The self-reported racial distribution was 90.5% Caucasian, 6.7% African American, and 2.9% Hispanic. Complete demographics and disease characteristics are displayed in Table 1. Ten non-Caucasian JDM patients were *not* included in the genetic analysis. Race-matched healthy control subjects included 500 European Americans residing in Midwest-America.

Determination of total *C4, C4A* and *C4B* genotypes and phenotypes

Previously, we described protocols for genotyping and phenotyping of complement C4 by Southern blot analyses and immunofixation, respectively [36–38]. In cases of limited DNA quantities or ambiguous results, quantitative real-time PCR experiments for GCN of total *C4*, *C4A*, and *C4B* were performed as described [15]. All *C4*-CNV calls were validated rigorously by independent technology, or multiple amplicons in qPCR, and matched genotype and phenotype interpretations (Figures S1–S3, Tables S1 and S2).

Flow cytometric detection of erythrocyte-bound complement activation fragments

Erythrocytes from whole blood were used for antibody staining and flow cytometry. Mouse monoclonal antibodies specific for human C4d, for human C3d, or the isotype-matched control MOPC21 (Quidel, San Diego, CA) were used [39 40]. PE-conjugated goat anti-mouse IgG $F(ab')_2$ (Jackson ImmunoResearch, West Grove, PA) was used as a secondary antibody. FlowJo software (Tree Star Inc., Version 7.6) was used to electronically gate erythrocytes based on forward and sideward scatter properties. Among the gated cells, E-C4d or E-C3d was reported as median fluorescence intensity (MFI), which was calculated using C4d-specific (or C3d-specific) MFI minus the MOPC-isotype control MFI.

HLA-DRB1 Typing

Genotyping of *HLA-DRB1* alleles for JDM and all control samples were determined using genomic DNA for sequence-specific primer PCR [41]. *HLA-DRB1* frequency was calculated by the number of allele-positive subjects divided by the total number of subjects.

Gene expression profiling

RNA was extracted from whole blood using PAXgene collection tubes (PreAnalytiX, Becton, Dickinson and Company). Microarray analysis was performed by the Biomedical Genomics Core facility at the NCH. RNA samples passing quality control were labelled with Agilent's One-Color microarray-based gene expression analysis labeling protocol and hybridized to the SurePrint G3 Human v2 GE 8x60K Microarray (AMADID 039494). Images were analyzed with Feature Extraction 10.9 (Agilent Technologies). Median foreground intensities were obtained for each spot and imported into the mathematical software package R. After pre-processing, the data were quantile normalized using the LIMMA package [42]. Statistical analysis was performed via Significance Analysis of Microarray (SAM) implemented using the Bioconductor Siggenes package to identify differentially expressed genes between JDM and control groups [43]. Changes in expression ≥1.5 fold and a 15% false discovery rate as estimated by SAM were considered significantly different. Selected genes were validated by SYBR-Green qPCR using PAXgene RNA.

Statistical analysis

Statistical analyses were performed using Prism6 (GraphPad Software Inc., San Diego, CA) and JMP Genomics 6.0 (SAS Institute Inc., Cary, NC) software. Descriptive statistics are displayed as mean ± standard deviation (SD) for normally distributed data, and simple comparisons were made using Student's t-test for continuous data, or by chi-square analysis for categorical data. Odds Ratios (OR) with 95% confidence intervals (CI) are reported. For non-normally distributed data, median with interquartile range (IQR) is reported, and Mann-Whitney test was used for comparisons. For all analyses, $p \leq 0.05$ was considered to be significant.

RESULTS

Gene CNVs of total *C4*, *C4A*, and *C4B* in JDM and race-matched healthy controls

Total C4 genes—In healthy controls (N=500), the variation of *C4* GCN showed a normal distribution pattern, ranging from 2–7 total copies. In Caucasian JDM (N=95), total *C4* genes ranged from 2–5 copies, with a shift of distribution to the lower copy-number compared to controls [χ^2=20.7; degree of freedom (df)=4; p=0.0004, χ^2 analysis) (Figure 1). JDM had a lower mean GCN than did controls (JDM: 3.49±0.71; controls: 3.83±0.69; $p=1.8\times10^{-5}$, t-test; Table 2). Low copy-number of total *C4* (*C4T*, GCN≤3) was present in 50.5% of JDM and 29.2% of controls [OR=2.48 (1.59–3.87); $p=7.5\times10^{-5}$].

Copy-numbers of C4A and C4B genes—The reduction in copy-number of total *C4* in JDM can be the result of a decrease in *C4A*, *C4B*, or both. We observed a significant shift to lower GCN of *C4A* in JDM patients (p=0.0004). The presence of homozygous or heterozygous deficiency of *C4A* genes (GCN=0 or 1) had a frequency of 40.0% in JDM,

compared to 18.2% in controls [OR=3.00 (1.87–4.79); p=8.2x10^{-6}]. The overall mean GCN of *C4A* observed in JDM was 1.79±0.86 compared to 2.09±0.75 in controls (p=0.0004).

As for *C4B*, no significant differences in the distribution of *C4B*-GCN or *C4B*-deficiency were observed between JDM and controls. Therefore, the basis for decreased GCN of total *C4* in Caucasian JDM patients was attributable to lower GCN of *C4A*.

HLA-DRB1 alleles, *C4A*-GCN and *C4A*-deficiency on disease risks of JDM

The frequency of *HLA-DRB1*0301* alleles (*DR3*) was 46.3% in Caucasian JDM patients (N=95), compared with 25.8% in a race-matched healthy population (N=500). *HLA-DR3* was associated with JDM with an OR=2.48 (1.58–3.89) and a p-value of 9.5x10^{-5}. The concurrence of *C4A*-deficiency and *DR3* in a subject was present in 36.8% of JDM and 15.4% of controls, with an odds ratio of 3.20 (1.98–5.19) and a p-value of 4.8x10^{-6}. By contrast, the frequency of HLA-*DRB1*1501* (*DR2*) was 11.6% in Caucasian JDM compared to 27.8% in healthy controls. *DRB1*1501* was a *protective* factor against JDM with OR=0.34 (0.18–0.66) and p=0.0004 (Figure 1E; Table 2A).

Multiple logistic regression analyses were performed to investigate if *C4A*-deficiency, the presence of *DR3* and the presence of *DRB1*1501* could serve as independent risk factors for JDM, conditional upon presence of other factor(s) in five different combinations of regression models (Table 2B). In models when *DR3+* and *C4A*-deficiency were put together as individual factors (model *a* or *b*), the relevance of *DR3+* as an independent parameter became insignificant. The presence of *DRB1*1501* plus *C4A*-deficiency, or *DR3+*, or *C4A*-deficiency with *DR3+* all yielded statistical significance to account for JDM genetic risks. The last model yielded the best fit: *C4A*-deficiency plus *DR3+* was a strong risk factor with odds ratio of 2.96 (1.84–4.80) and *DRB1*1501* was protective factor with odds ratio of 0.34 (0.21–0.55).

To further evaluate the relative roles of *C4A*-deficiency and *DRB1*0301* on disease risk of JDM, we performed subgroup analyses (Table 2C). Among the *DR3+* subjects, JDM patients had a significantly lower mean-GCN of *C4A* than controls (JDM: 1.18±0.54; controls: 1.47±0.72; p=0.015). Similarly among the *DR3+* subjects, *C4A*-deficiency had a greater prevalence in JDM (79.6%) than controls (59.7%) [OR=2.63 (1.17–5.92), p=0.014]. Among *DR3*-negative subjects, however, there were no apparent differences in mean-GCN of *C4A* or the prevalence of *C4A*-deficiency between JDM and controls, suggesting the heightened risk of lower *C4A*-GCN or *C4A*-deficiency on JDM required a *DR3+* background.

Reciprocal subgroup analyses to compare the prevalence of *DR3+* between JDM and controls among *C4A*-deficient subjects (GCN≤1), or among *C4A*-proficient subjects (GCN≥2) revealed slight but insignificant increases in the frequency of *DR3* in JDM (Table 2C).

Levels of erythrocyte-bound C4d (E-C4d) or C3d (E-C3d) in JDM and controls

Cell-bound complement levels were determined in 40 Columbus JDM patients and 206 healthy subjects of European ancestry by flow cytometry. Comparing between JDM and

healthy controls, significant elevation of E-C4d levels (p=0.004, Mann Whitney test, Figure 2A) but not E-C3d levels (Figure 2B) was observed in JDM. The mean fluorescent intensities (MFI) for E-C3d levels were substantially lower than those of E-C4d levels, which is consistent with the presence of complement regulation mechanisms on self cell surfaces.

We investigated if there was a correlation of E-C4d levels with *C4A or C4B* gene dosages in JDM. The *C4A*-deficiency group (GCN≤1; *N*=15) had a median MFI of 1426 (IQR: 601–1744), which was significantly *higher* than that of the *C4A*-proficient group (GCN≥2; N=25; median MFI=454 (234–718); p=0.0003, Mann-Whitney test). On the other hand, the *C4B*-deficiency group (GCN≤1; N=11) had a median E-C4d MFI of 308 (226–505), which was significantly *lower* than that of the *C4B*-proficient group (GCN≥2, N=29; median MFI=775 (495–1458); p=0.003, Mann-Whitney test). Thus, C4A and C4B appeared to play opposite roles on the deposition of cell-bound E-C4d: high GCN of *C4A* dampened activation, while high GCN of *C4B* amplified activation.

JDM patients with elevated levels of multiple serum muscle enzymes had low GCN of *C4A*

JDM patients exhibited elevated levels of a variety of serum muscle enzymes. We performed intragroup comparisons to investigate if *C4A*-deficiency was related to elevated muscle enzyme levels at the time of disease diagnosis. Indeed, patients with *C4A*-deficiency had higher prevalence of abnormal serum muscle enzymes such as creatine kinase (C4A-deficient: 86.1%, C4A proficient: 58.2%; p=0.0034) and aldolase (*C4A*-deficient: 94.1%, *C4A*-proficient: 78.4%; p=0.038) and elevations of multiple serum muscle enzymes (*C4A*-deficient: 97.1%; *C4A*- proficient: 74.5%; p=0.0025) than *C4A*-proficient patients. The prevalence of elevated levels of serum aspartate aminotransferase and lactate dehydrogenase was not associated with *C4A*-deficiency (Table 3).

Differential gene expression profiling of JDM and controls

Global gene expression profiling was performed using PAXgene blood RNA from 19 consecutive JDM patients and 7 controls (Figure 3A and Tables S3 and S4). Expression profiles revealed differential expression of transcripts in JDM from 56 genes that were significantly different using SAM criteria. Differentially expressed genes included 24 upregulated and 32 downregulated genes. Of the upregulated genes, the most remarkable are nine type I interferon (IFN-I) response genes and three genes related to B-cell functions. Of the downregulated genes, the most distinct were genes related to T-cell functions, chemokines and chemokine receptors. Six JDM patients exhibited the most polarized upregulation of IFN-I genes and downregulation of genes for chemokine/chemokine receptor and T-cell functions (Figure 3A). Of interest, five of these six JDM patients were *C4A*-proficient (GCN≥2), and did *not* carry the *HLA-DR3* allele.

To validate gene expression changes from microarray, we performed SYBR-Green qPCR using cDNA from PAXgene-blood RNA for *IFI44*, *IFI17*, *CXCR6*, and *CCR5* transcripts. Results revealed in JDM upregulation of *IFI44* (2.7-fold; p=0.028) and *IFI17* (3.0-fold; p=0.0054), and downregulation of *CXCR6* (1.9 fold; p=0.031) and *CCR5* (1.5 fold;

p=0.048) (Figure 3B). The housekeeping gene *GAPDH* was used as a normalization standard [44].

DISCUSSION

A great challenge for studying complex diseases associated with the *HLA* region, including JDM, is to determine which gene(s) or polymorphic variants contribute to disease development under the background of strong allelic associations or linkage disequilibrium. This is the first study to decipher the gene CNVs for complement *C4* and its isotypes *C4A* and *C4B* in JDM with *definitive* techniques, and to dissect the relative roles of HLA-*DRB1*0301* (*DR3*) and *C4A*-deficiency on JDM disease risk in subjects of European ancestry.

The carriage of *DRB1*0301* was present in 46.3% of our JDM patients compared to 25.8% of race-matched healthy controls. *DR3* is a medium effect-size risk factor for JDM (OR=2.48). Homozygous and heterozygous deficiency of complement *C4A* had a frequency of 40.0% in Caucasian JDM and 18.2% in healthy controls (OR=3.00). The co-existence of *HLA-DR3* and *C4A*-deficiency confers higher risk than either individual risk factors, and such concurrence in a diploid genome was present in 36.8% of JDM and 15.4% of controls with an OR=3.20. The independent roles of *DRB1* variants and *C4A*-deficiency in JDM were further validated by multiple logistic regression analyses. Moreover, among *DR3*-positive subjects, lower mean GCN of *C4A* or higher prevalence of *C4A*-deficiency persisted in JDM versus controls. An interpretation to this phenomenon is that *DR3*-positivity contributes to a permissive background and *C4A*-deficiency significantly elevated the vulnerability to an autoimmune disease including JDM. While HLA-DRB1 and complement C4 each is engaged in specific immunologic functions such as antigen presentation to effector T-cells, and complement-mediated cytolysis and immunoclearance, they are both involved in the recognition of self and non-self, and are key players for the process of archiving memory and tolerance in the immune system.

Destruction of perifascicular capillaries by complement and subsequent ischemia of muscle fibers in dermatomyositis have been demonstrated by multiple investigators over the past three decades [23–28]. Activation of complement can be initiated via the classical pathway that is triggered by immune complexes formed between myositis-associated or myositis-specific autoantibodies and self-antigens abundant in muscles and skin. Physiologically, low GCN or low production of C4A protein systemically may dampen immune complex clearance and therefore promote autoimmunity. Compared with controls, we observed a moderate but significant increase in the deposition of C4d on red-blood cells among JDM patients, which reiterates involvement of complement activation in the pathogenesis of JDM. Almost all JDM patients with two or more elevated muscle enzymes at disease diagnosis had a *C4A*-deficiency but normal mean GCN of *C4B*. In other words, immune-mediated tissue injuries in JDM might have been resulted through the activation of C4B. Consistent with this phenomenon, we observed increased deposition of processed complement activation product E-C4d in JDM patients than in controls. Interestingly, the levels of E-C4d were directly proportional to the GCN of *C4B*, and inversely proportional to the GCN of *C4A*. Physiologically, activated C4B protein is highly reactive and over-activation could lead to

complement-mediated injuries. In addition to its role in immunoclearance and protection against autoimmunity, the presence of activated C4A may attenuate the activity of C4B and minimize its potential deleterious effect.

Among the JDM patients, 63.2% were *not* associated with *C4A*-deficiency on a *DR3*+ background, and the underlying genetic risk factors in this group of patients (*C4A-proficient* and *DR3-negative*) are yet to be identified. An emerging feature in JDM is the upregulation of type I interferon-stimulated gene expression in many patients [45–47]. Our microarray studies of peripheral blood samples revealed marked increase in transcripts in JDM for many IFN-I stimulated genes and B-cell specific genes, but diminished transcript levels of many genes related to chemokines and T-cell functions. Such differential levels of transcripts reflect both different gene expression levels and also compositions of leukocytes in the peripheral blood samples. The polarized upregulation of IFN-stimulated genes and B-cell function genes, and downregulation of chemokine receptor and T cell function genes were more marked among *C4A*-proficient or *DR3*-negative patients, implying the presence of additional or alternative mechanisms leading to the pathogenesis of JDM.

In conclusion, we report the novel finding of low GCN of complement *C4* and *C4A*-deficiency associated with JDM. JDM patients with *C4A*-deficiency were more likely to have elevated levels of multiple serum muscle enzymes at diagnosis and high levels of E-C4d. Further in-depth studies through *HLA-DRB1* and *C4A* genotypes, cell-bound C4d levels and differentially expressed genes including those engaged in muscle-cell functions and signaling, and characterization of clinical phenotypes [48] may help understanding the pathogenic mechanisms, enable patient stratification and facilitate genotype and gene expression guided therapies of JDM.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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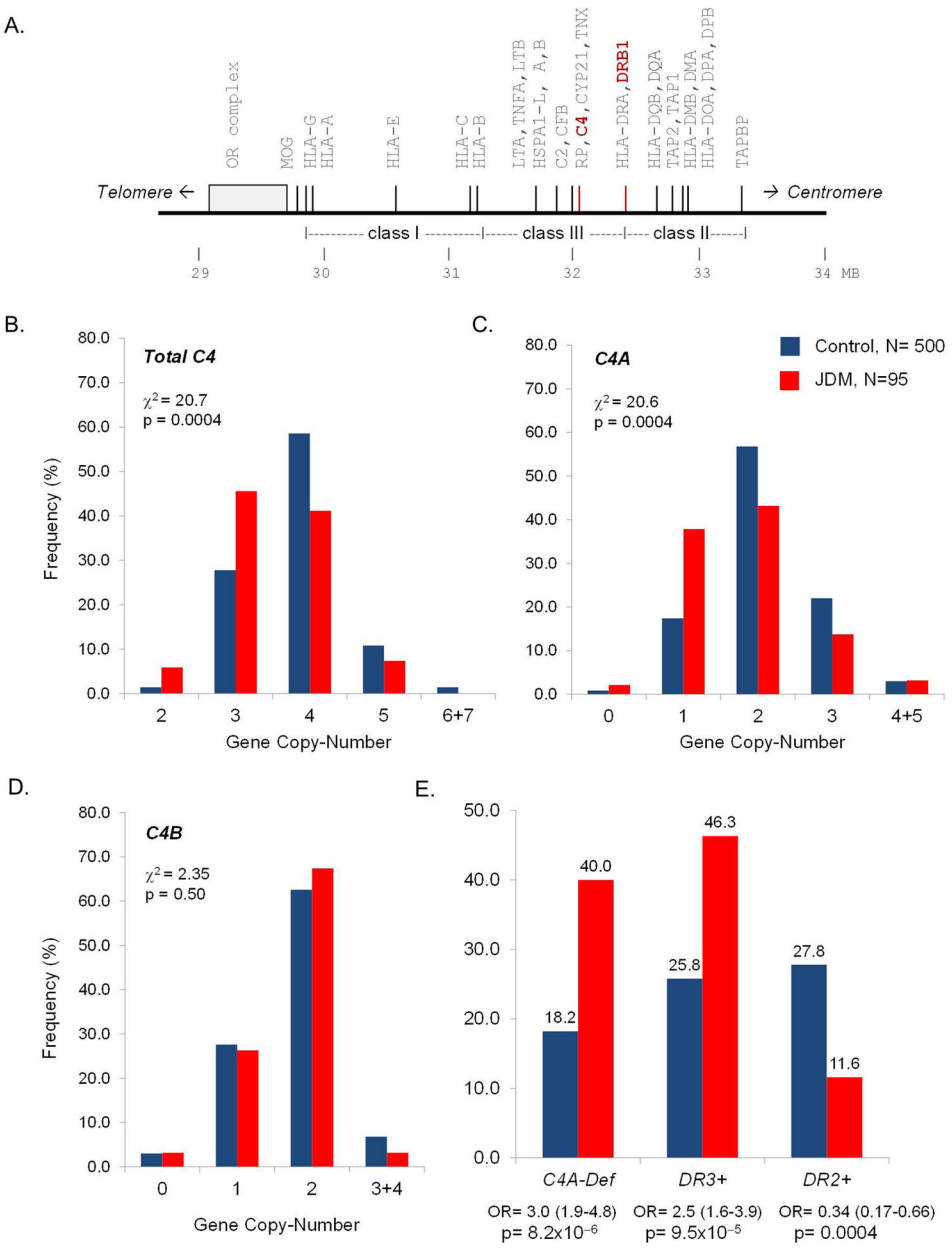

Figure 1.

Variations of *C4* haplotypes and gene copy-numbers (GCNs) of total *C4*, *C4A* and *C4B* in JDM subjects and race-matched healthy controls. **A**. A simplified map of the human major histocompatibility complex (MHC) showing genes of immunologic functions; *RP-C4-CYP21-TNX* (RCCX) and *HLA-DRB* are characterized by inter-individual copy-number variations. **B, C and D.** Gene copy-number variations of complement *C4, C4A and C4B* in JDM and controls. The p-values and chi-square values are indicated for each analysis. **E.** A summary of genetic risk factors in the MHC for JDM.

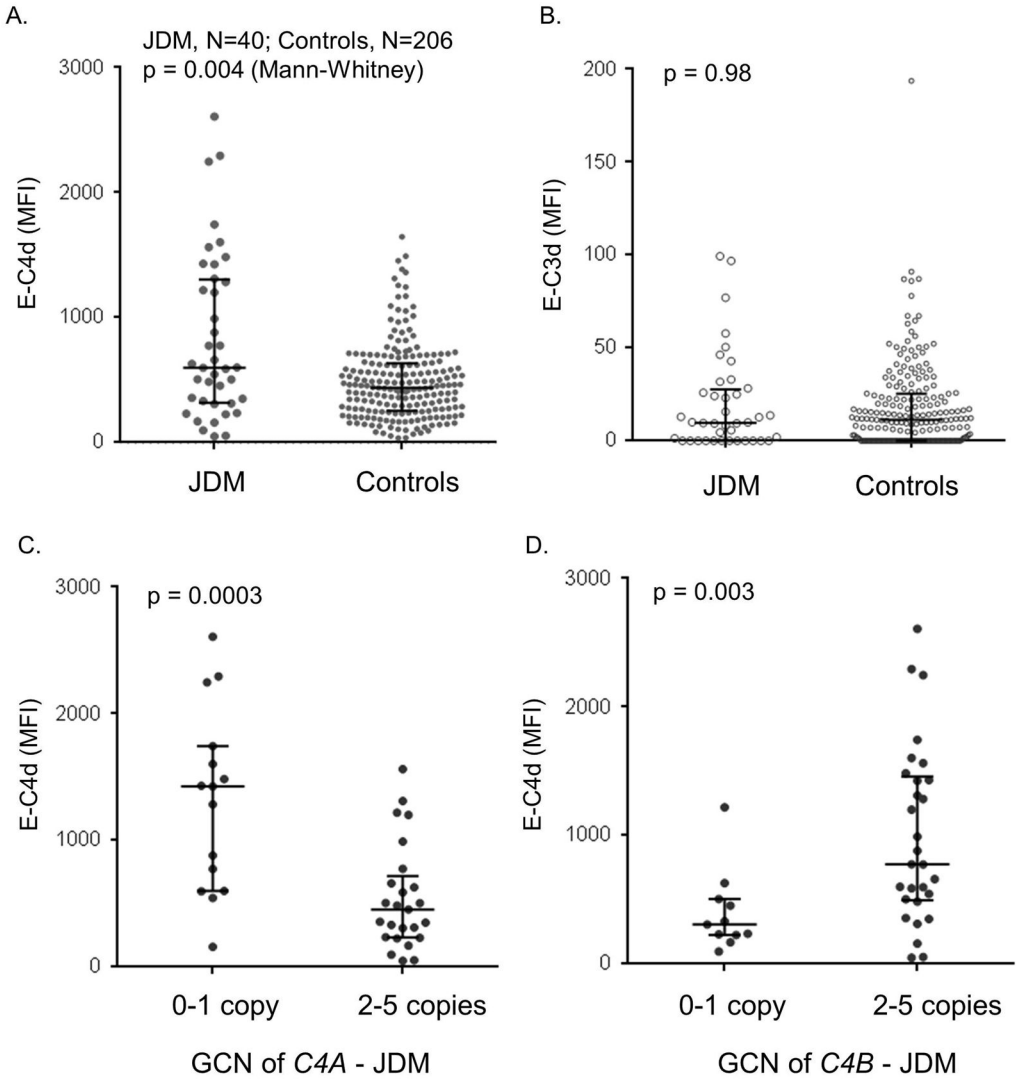

Figure 2.

Erythrocyte-bound E-C4d and E-C3d in JDM patients and controls. A and **B**. A comparison of E-C4d and E-C3d, respectively, in JDM and controls. **C**. A comparison of E-C4d in C4A-deficient and C4A-proficient JDM patients. **D**. A comparison of E-C4d in C4B-deficient and C4B-proficient JDM patients. The median for each group is indicated by a horizontal bar, while the shorter bars represent interquartile ranges; the p-value for Mann Whitney test is indicated.

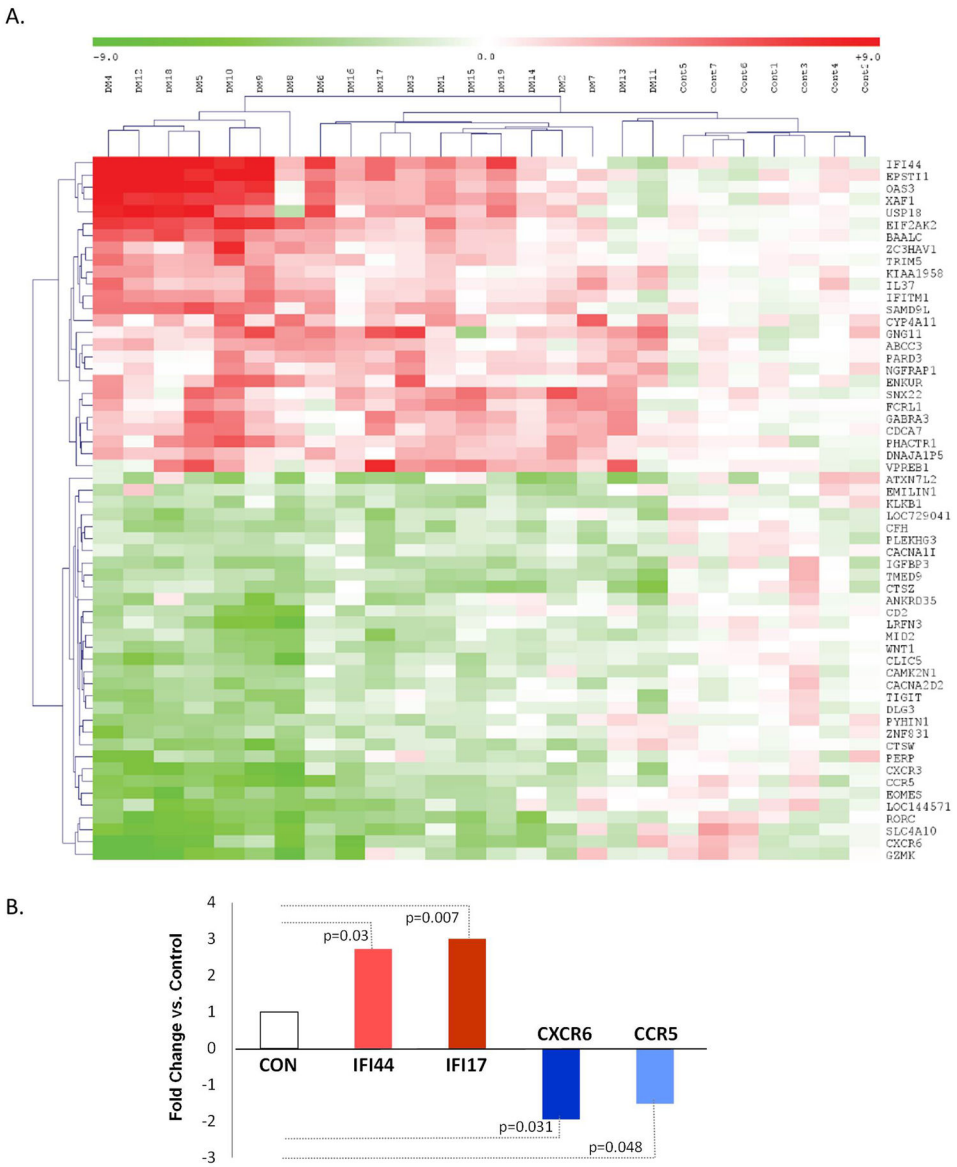

Figure 3.

Gene expression profiling of PAXgene blood RNA in JDM and healthy controls. **A**. Hierarchical clustering analysis of PAXgene blood RNA gene expression microarray data in 19 JDM and 7 healthy subjects; red and green represent upregulated and downregulated genes, respectively; vertical columns represent the data for each subject, and rows indicate different genes (see Table S3 for details). **B**. Four genes from microarray results were selected for SYBR-green qPCR analysis using cDNA from PAXgene blood RNA to validate the upregulation and downregulation in JDM of the chosen genes. The white column shows normalized value to 1 for controls, while the fold-change in JDM for each respective gene is indicated by colored columns; p-values for Mann-Whitney tests are indicated. Number of subjects (N) used for SYBR-Green qPCR assays are: *IFI44* - 28 JDM and 14 controls; *IFI17*

- 17 JDM and 15 controls; *CXCR6* - 24 JDM and 19 controls; and *CCR5* - 24 JDM and 19 controls.

Table 1

Demographic Features and Clinical Characteristics of JDM Patients *

Features	N (%)
Age at recruitment: mean ± SD, yrs. old	10.8 ± 7.6
Age at diagnosis: mean ± SD, yrs. old	7.4 ± 4.2
Sex: female / male	67 (63.8%) / 38 (36.2%)
Race/Ethnicity: Caucasian / African / Hispanic	95 (90.5%) / 7 (6.7%) / 3 (2.9%)
Calcinosis	15 / 97 (15.5%)
Ulcerations	17 / 97 (17.5%)
Lipodystrophy	8 / 96 (8.3%)
Disease course †	
Monocyclic	16 / 66 (24.2%)
Polycyclic	12 / 66 (18.2%)
Chronic continuous	38 / 66 (57.6%)
Positive ANA	62 / 82 (75.6%)

* Values given indicate number of subjects (percentage) for which data was available.

† Only patients who have been followed ≥2 years were categorized by disease courses.

Table 2

HLA-DRB1 Alleles and *C4* Gene CNVs in JDM and Controls

A. Analysis of single genetic risk factors in JDM				
	JDM (N=95)	**Control (N=500)**	**p-value**	**OR (95% CI)**
a. Distribution, N (%)				
C4T GCN ≤ 3	48 (50.5%)	146 (29.2%)	$7.5x10^{-5}$	2.48 (1.59–3.87)
C4A deficiency, GCN ≤1	38 (40.0%)	91 (18.2%)	$8.2x10^{-6}$	3.00 (1.87–4.79)
C4B deficiency, GCN ≤1	28 (29.5%)	153 (30.6%)	0.83†	
*HLA DRB1*1501*	11 (11.6%)	139 (27.8%)	0.0004	0.34 (0.18–0.66)
*HLA DRB1*0301 (DR3+)*	44 (46.3%)	129 (25.8%)	$9.5x10^{-5}$	2.48 (1.58–3.89)
C4A-deficiency with *DR3+*	35 (36.8%)	77 (15.4%)	$4.8x10^{-6}$	3.20 (1.98–5.19)
b. Mean GCN ± SD				
Total *C4* genes	3.49 ± 0.71	3.83 ± 0.69	$1.8x10^{-5}$	
C4A genes	1.79 ± 0.86	2.09 ± 0.75	0.0004	
C4B genes	1.71 ± 0.58	1.73 ± 0.63	0.68†	

B. Multiple logistic regression models for genetic factors of JDM				
Models and parameters	**R^2**	**χ^2**	**p-value**	**OR (95% CI)**
*a. C4A-deficiency, DRB1*1501 and DR3+*	0.058	30.2	$1.2x10^{-6}$	
*DRB1*1501*		8.95	0.0028	0.39 (0.19–0.74)
C4A-deficiency		6.21	0.013	2.27 (1.19–4.40)
DR3+		0.70	0.40†	1.31 (0.69–2.44)
b. C4A-deficiency and DR3+	0.041	21.3	$2.4x10^{-5}$	
C4A-deficiency		6.05	0.014	2.26 (1.18–4.39)
DR3+		1.39	0.24†	1.47 (0.77–2.74)
*c. C4A deficiency and DRB1*1501*	0.057	29.5	$3.9x10^{-7}$	
*DRB1*1501*		9.64	0.0045	0.38 (0.19–0.71)
C4A-deficiency		16.8	$2.7x10^{-5}$	2.76 (1.71–4.43)
*d. DR3+ and DRB1*1501*	0.046	24.0	$6.1x10^{-6}$	
*DRB1*1501*		8.80	0.003	0.40 (0.19–0.74)
DR3+		11.3	0.0008	2.21 (1.39–3.49)
*e. C4A-deficiency with DR3+ and DRB1*1501*	0.059	30.6	$2.2x10^{-7}$	
C4A-deficiency with *DR3+*		17.9	$2.4x10^{-5}$	2.96 (1.81–4.80)
*DRB1*1501*		9.7	0.0018	0.34 (0.21–0.55)

C. Subgroup analyses of *C4A*-GCN, *C4A*-deficiency and HLA-*DR3* in JDM				
	JDM	**Control**	**p-value**	**OR (95% CI)**
Mean *C4A* GCN, *DR3+*	1.18±0.54	1.47±0.72	0.015	
Mean *C4A* GCN, *DR3-*	2.31±0.73	2.31±0.63	0.97†	

C. Subgroup analyses of *C4A*-GCN, *C4A*-deficiency and HLA-*DR3* in JDM				
	JDM	**Control**	**p-value**	**OR (95% CI)**
C4A-deficient, *DR3*+, N (%)	35 (79.6)	77 (59.7)	0.014	2.63 (1.17–5.92)
C4A-proficient, DR3+, N *(%)*	9 (20.5)	52 (40.3)		
C4A-deficient, *DR3*−, N (%)	3 (5.9)	14 (3.8)	0.50†	
C4A-proficient, *DR3*−, N (%)	48 (94.1)	357 (96.2)		
DR3+, *C4A*-deficient, N (%)	35 (92.1)	77 (84.6)	0.23†	
DR3−, C4A-deficient, N (%)	3 (7.9)	14 (15.4)		
DR3+, *C4A*-proficient, N (%)	9 (15.8)	52 (12.7)	0.53†	
DR3−, C4A-proficient, N (%)	48 (84.2)	357 (87.3)		

Abbreviations: C4T, total copies of *C4* genes; CI, confidence interval; GCN, gene copy-number; N, number; OR, odds ratio. *C4A*-deficient: *C4A* GCN =0 or 1; *C4A*-proficient: *C4A* GCN ≥2

† not statistically significant; categorical data were compared by χ^2 analyses; continuous data were compared by t-tests.

Table 3

Elevation of serum muscle enzymes at disease diagnosis in JDM patients with and without *C4A*-deficiency

Muscle Enzyme	**N (%) with elevation of muscle enzyme levels**		
	***C4A-de*ficient**	***C4A-pro*ficient**	**p-value***
Aldolase	32 (94.1)	40 (78.4)	0.038
Aspartate aminotransferase	27 (81.8)	36 (67.9)	NS
Creatine kinase	31 (86.1)	32 (58.2)	0.0034
Lactate dehydrogenase	24 (82.8)	31 (77.5)	NS
≥ 2 Muscle enzymes	33 (97.1)	38 (74.5)	0.0025

*by χ^2 analysis;

NS, not significant