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Gene Copy-Number Variations (CNVs) and Protein Levels of Complement C4A and C4B as Novel Biomarkers for Partial Disease Remissions in New-Onset Type 1 Diabetes Patients

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Abstract

Objective—To determine the roles of complement *C4A* and *C4B* gene CNVs and their plasma protein concentrations in residual insulin secretion and loss of pancreatic beta-cell function in new-onset type 1 diabetes patients.

Methods—We studied 34 patients of European ancestry with new-onset type 1 diabetes, aged between 3 and 17 years (10.7 ± 3.45), at Nationwide Children's Hospital in Columbus, Ohio. Gene copy-number and size variations of complement *C4A* and *C4B* were determined by genomic Southern blot analyses. *C4A* and *C4B* protein phenotypes were elucidated by immunofixation and radial immunodiffusion. Two-digit *HLA-DRB1* genotypes were determined by sequence-specific PCR. At 1 month and 9-month post diagnosis, stimulated C-peptide levels were measured after a standardized mixed-meal tolerance test.

Results—The diploid gene copy-numbers of *C4A* varied from 0 to 4, and those of *C4B* from 0 to 3. Patients with higher copy-number of *C4A* or higher *C4A* plasma protein concentrations at diagnosis had higher C-peptide levels at 1 month post diagnosis ($p=0.008$; $p=0.008$). When controlled by the Z-score of body-mass index, *C4A* copy-numbers, *C4A* protein concentrations, the age of disease-onset, the number of *HLA-DR3* but not *DR4* alleles were significant parameters in determining C-peptide levels. At 9-month post diagnosis, 42.3% of patients remained in partial remission, and these patients were characterized by lower total *C4B* copy-numbers or lower *C4B* protein concentrations ($p=0.02$, $p=0.0004$).

Conclusions—*C4A* appears to associate with the protection of residual beta-cell function in new-onset type 1 diabetes; *C4B* is correlated with the end of disease remission at 9-month post diagnosis.

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Keywords

Type 1 Diabetes; Partial disease remission; C-Peptide; Complement C4; CNV; HLA-DRB1

Introduction

Type 1 diabetes (T1D) is an autoimmune disease characterized by the destruction of β -cells in the pancreas, leading to inability to synthesize endogenous insulin and poor glycemic controls. Multiple genetic and environmental factors have been implicated in the etiology of T1D (1, 2). The strongest genetic factor associated with human T1D lies in the major histocompatibility complex (MHC), also known as the HLA, on the short arm of chromosome 6. In particular, specific polymorphic variants of HLA class II genes are thought to contribute to over 50% of the genetic risk for T1D (2, 3), with the highest risk conferred by haplotypes with *DRB1*0301-DQB1*0201-DQA1*0501* (also known as *HLA-DR3* haplotype), *DRB1*0405-DQB1*0302-DQA1*0301*, or *DRB1*0401-DQB1*0302-DQA1*0301* (*HLA-DR4* haplotypes) (4, 5). While T1D is considered an organ-specific disease that mainly involves T-cell mediated autoimmunity, B-cells and other immune effector proteins may also be involved in disease pathogenesis. Autoantibodies against antigens of pancreatic islet cells are detectable years before the disease onset or development of hyperglycemia (6).

At disease presentation, most T1D patients have already lost 80–90% of their original beta-cell mass (7, 8). After diagnosis and medical treatment, many patients experience a partial remission period or “honeymoon” characterized by residual endogenous insulin secretion, requiring less frequent or lower doses of exogenous insulin injections, which may last between months and years after disease onset. T1D patients who produce more endogenous insulin for a longer duration have better prognosis: a lower total daily insulin requirement, better glucose control, fewer episodes of severe hypoglycemia and relatively less nephropathy and retinopathy (9–13). While variants of HLA class II genes are important genetic factors in T1D disease onset or susceptibility, they do *not* appear to play a major role in modulating the partial disease remission. Understanding the etiologic factors that differentiate preservation of beta-cell function and development of full-blown T1D disease may yield new insights into pathogenesis and effective intervention and therapies.

Complement *C4* gene maps to the class III region of human MHC, which is 544 kb telomeric from *HLA-DRB1* (14). Recent advance in the molecular genetic studies of the *C4* locus lead to the establishment of a novel concept in human genetics, which features frequent, multi-allelic copy-number variations (CNV) of *C4* genes. One to four copies of *C4* gene in an HLA *haplotype*, or two to eight copies of total *C4* genes in a *diploid* genome, are present among different individuals (15–18). The CNV of *C4* genes is a result of segmental duplications involving four contiguous genes encoding for serine/threonine kinase RP, complement C4, steroid 21-hydroxylase CYP21, and tenascin TNX, a phenomenon termed as the RCCX modular duplications (15, 19). *C4*-CNV is coupled with complex secondary variations in (a) the physical size of *C4* genes with a long form (L) of 20.6 kb and a short form (S) of 14.2 kb, and (b) DNA sequences leading to two classes of polymorphic proteins,

the acidic C4A and the basic C4B with a large range of plasma protein concentrations (17, 20, 21). Dysregulation of complement activation can lead to complement-mediated cellular destruction or tissue injuries and thus affect autoimmunity. Although extremely rare in prevalence, human subjects with *complete* deficiencies of both C4A and C4B are almost always afflicted with systemic lupus erythematosus (SLE) or a lupus-like syndrome (22, 23). Furthermore, low gene copy-number of *C4A* is a risk factor for SLE disease susceptibility, while high copy-number of *C4A* is a protective factor against SLE (17). Thus, complement C4, and C4A in particular, is important in the protection against *systemic* autoimmunity (23). However, the roles of *C4A* and *C4B* multi-allelic CNVs in organ-specific autoimmunity such as T1D have not been investigated vigorously.

Here we determine whether complement C4 is associated with changes in pancreatic β -cell function at T1D disease onset and during the partial remission period. Pancreatic beta-cell function was examined through measurements of serum C-peptide levels before and after mixed-meal stimulation. Plasma C4 protein levels, C4A and C4B protein polymorphisms, gene CNVs of *C4A* and *C4B*, plus HLA-*DRB1* genotypes were determined. Our data reveals a novel phenomenon with significant associations between *C4A* gene copy-numbers or C4A protein concentrations and C-peptide levels at 1-month post T1D diagnosis. By contrast, patients who remained in partial disease remission at 9-month post T1D onset are characterized by low copy-number of *C4B* genes and low C4B protein levels.

Methods

Study population

We recruited 35 pediatric subjects of European ancestry who were recently diagnosed with T1D at Nationwide Children's Hospital (NCH; Columbus, OH) from 2009–2010. These new onset T1D patients aged between 3 and 17 years old, among them were 28 males and 6 females. The prevalence of T1D in males and females is roughly similar (24). The higher participation of male patients in this serial clinical study reflected voluntary participations in this study, and young boys were more willing and amendable to several venipunctures during the mixed meal tolerance tests. The diagnosis of T1D was based on a fasting blood sugar >126 mg/dL (7 mmol/L) or a random blood sugar >200 mg/dL (11.1 mmol/L) with symptoms of hyperglycemia, including polydipsia and polyuria. Autoimmune diabetes was confirmed by the presence of at least one of the diabetes associated autoantibodies, including GAD65, ICA512 and insulin autoantibody. All diagnosed patients used insulin therapy with multiple daily insulin injections of rapid-acting insulin and a single daily injection of long-acting insulin, or insulin pump therapy. Informed consent and assent (for subjects 8–17 years of age) were obtained in accordance with Institutional Review Board approved protocols for human subject research.

Mixed-meal tolerance test (MMTT)

MMTT was performed in the study subjects 1-month and 9-month post diabetes diagnosis in the NCH Clinical Research Services Center. Subjects were fasted overnight, and the MMTT was performed in the next morning. The usual morning dose of rapid acting insulin was withheld, though subjects still continued basal insulin. Baseline blood samples were

obtained to determine fasting glucose and C-peptide levels (25–27). The subjects ingested 6ml/kg (maximum of 360 ml) of the mixed-meal, Boost (8 fluid ounces with 240 kcal; 33g carbohydrate, 15g protein, and 6g fat) in a period less than 10 minutes. Ninety minutes after ingestion, a blood sample was obtained for determination of stimulated C-peptide and glucose levels. Glucose levels were determined in the clinical laboratory at NCH. Plasma C-peptide levels were measured by Esoterix Laboratories (Calabassas Hills, CA).

Determination of T1D-associated autoantibodies

Serum samples were processed and kept frozen at -86°C until the evaluation of the diabetes-associated autoantibodies against GAD-65, ICA-512 and insulin were performed (Esoterix Laboratories, Calabassas Hills, CA). Briefly, the measurements of anti-GAD-65 and anti-ICA-512 were performed by immunoprecipitation using radioactive recombinant proteins for GAD-65 and ICA-512, respectively, and protein-A as a carrier for immune complexes. The sensitivity of assays and intra-assay precision were 0.5 units and $>97\%$, respectively, for anti-GAD-65; and 0.75 units and $>90\%$, respectively, for anti-ICA512. As provided by Esoterix, Inc., for anti-GAD assays, the intra-assay variance for quadruplicate assays of same samples were between 4.8 and 10%. The inter-assay variance among 246 assays was between 12–15%. For the assays of ICA-512, the intra-assay variance (quadruplicates) was between 1.3 and 2.8%. The inter-assay variance among 153 assays was between 6 and 7%. For the measurement of total insulin antibodies (all Ig classes), insulin complexed with antibodies were dissociated from immune complexes, and free insulin in serum were removed by binding to acidified-charcoal and centrifugation. Serum autoantibodies against insulin were then measured through interaction with radioactive insulin and precipitation with polyethylene glycol (total binding). Nonspecific binding (NSB) was determined by adding unlabelled insulin to replicate samples to compete with radiolabeled insulin for binding to insulin autoantibodies. Specific binding counts were calculated by subtracting NSB from total counts and converted to μU of insulin based on specific activity of labeled insulin used in the assay, and data were shown as total insulin antibody binding capacity in μU of insulin/ml of serum tested (28). The intra-assay and inter-assay variations for insulin autoantibodies (20 assays) were between 2.5 and 6.5%, and between 8.2 and 11%, respectively.

HLA-DRB1 genotyping

Two-digit genotyping of HLA-*DRB1* alleles was determined using genomic DNA for sequence-specific primer (SSP) PCR. Using customized oligos for PCR, the *DRB1* alleles were determined with 20 independent reactions for each sample, followed by agarose gel electrophoresis (29). Specific patterns of PCR products after electrophoresis were interpreted based on the presence or absence of PCR products.

Complement C4 genotyping and phenotyping

Detailed procedures for genotyping and phenotyping of complement C4 have been described (18, 30). Briefly, genomic DNA samples were isolated from peripheral blood leukocytes by Genra Kits (Valencia, CA). CNVs and genotypes of complement *C4* and *RCCX* modules were determined by genomic Southern blot analyses (Fig. 1A). *TaqI* restriction fragment

length polymorphisms (RFLP) were determined using specific probes corresponding to 3' region of *RP* and 5' region of *C4*, *CYP21*, and *TNX* (*RCCX*). Results of *TaqI* RFLP yielded information on the *C4* CNV, *C4* gene size and CNVs of other three constituent genes in *RCCX* modules. The relative copy-numbers of *C4A* and *C4B* genes were determined by *PshAI-PvuII* RFLP, using a *C4d*-specific probe for hybridization (Fig. 1B).

To determine the polymorphism and relative levels of *C4A* and *C4B* proteins, EDTA-plasma samples were digested with neuraminidase and carboxyl peptidase B to eliminate irregularities in glycosylations, and at the carboxyl termini of the beta and alpha chains, and resolved through their gross difference in electric charges by high voltage agarose gel electrophoresis (Fig. 1C). The *C4* proteins were immunofixed with goat antisera against human *C4*, washed and stained. The *C4A* and *C4B* protein allotypes were scanned and their relative intensities quantified by ImageQuant Software. The *C4A* and *C4B* protein concentrations were calculated from their total *C4*. Plasma protein levels of *C4* and *C3* were determined by single radial immunodiffusion using EDTA-plasma and commercial RID-kits (The Binding Site, UK).

Statistical analyses

All statistical analyses were performed using JMP software (SAS Institute, Cary). Linear regressions or bivariate fits were used to evaluate the correlation between two parameters such as between *C4* protein levels and C-peptide levels. Student's t-test was used to compare means of two groups. Logistic regression models were built to evaluate independent contribution of multiple parameters for C-peptides at 1-month or 9-month, or for the presence and absence of disease remission at 9-month post T1D diagnosis.

Results

Patient demographics and summary statistics

Of the 35 subjects enrolled in the study, one subject was negative for all three autoantibodies (GAD65, ICA-512 and insulin autoantibody) and was therefore excluded for data analyses. The mean age of T1D disease onset was 10.66 ± 3.45 years (mean \pm SD). The average body mass index (BMI) at disease onset was 18.35 ± 3.60 (Tables 1 and 2). Thirty-two subjects completed the MMTT at 1-month post diabetes diagnosis, and twenty-six subjects completed the MMTT at both 1-month and 9-month post diabetes diagnosis. Six patients had a first-degree relative with T1D (frequency: 0.176), and two patients had a family history of other autoimmune diseases.

Complement C3 and C4 protein levels

Total *C4* protein level in each patient at diagnosis was measured three times and averaged. *C4* protein ranged from <30 to 505 mg/L with a mean of 245 mg/L. *C4A* protein levels ranged from 0 (*C4A* deficiency) to 248 mg/L with a mean of 131 mg/L. *C4B* protein levels ranged from 0 (*C4B* deficiency) to 303 mg/L with a mean of 115 mg/L. Total *C3* protein levels ranged from 485 to 2,681 mg/L with a mean of 1,326 mg/L (Tables 1 and 2).

Gene CNVs of total *C4*, *C4A*, *C4B*, long *C4*, short *C4* and *RCCX* modules

The total *C4* CNV in a diploid genome ranged from 2 to 6 copies among the subjects (Figure 1, Table 1). The median *C4* CNV was 4 and had a frequency of 0.38. *C4A* CNV ranged from 0 to 4 and *C4B* ranged from 0 to 3 copies. Approximately one-third of T1D subjects had 0 or 1 copies of *C4A*. Two subjects were found to have homozygous deficiency (0 copy) of *C4A*. Most subjects have 2 copies of *C4B* (frequency=0.50); one subject was homozygously deficient of *C4B*. Overall, 65% of the *C4* genes were in the long form, and 35% were in the short form.

RCCX and *HLA-DRB1*

RCCX haplotypes with monomodal-L had a frequency of 0.07, while monomodal-S had a frequency of 0.19. Bimodal-LL was the most common haplotype with a frequency of 0.35, followed by bimodal-LS with a frequency of 0.28. Trimodal haplotypes of LLS and LSS were far less common in our patients with a frequency of 0.03 and 0.07 respectively.

The two most common *HLA-DRB1* genotypes among our subjects were *DRB1*04* (also known as *DR4*) and *DRB1*03(17)* (or *DR3*) with a frequency of 0.32 and 0.22, respectively. Five subjects were heterozygous for *DRB1*03/*04* (frequency: 0.15). Only one patient had a *DRB1*15* (or *DR2*) haplotype. Detailed results for patient demographics, CNVs of total *C4*, *C4A*, *C4B* and *RCCX* modules, C3 and C4 protein levels, and *HLA-DRB1* are shown in Tables 1 and 2.

C-peptide levels

Stimulated C-peptide levels at 1-month post diabetes diagnosis ranged from 0.5 to 3.9 ng/mL with a mean (\pm SD) of 2.1 ± 1.0 ng/mL. Nine-month stimulated C-peptide levels ranged from 0.0 to 3.8 ng/mL with a mean of 1.6 ± 1.0 ng/mL. (SI conversion: C-peptide nmol/L = $0.333 \times$ C-peptide ng/mL)

Complement *C4A* gene CNV and *C4A* plasma protein concentrations strongly correlate with stimulated C-peptide levels at 1-month post T1D diagnosis

To investigate if variations of *C4* genes and proteins are associated with the outcome for the loss of beta-cell function in the new onset T1D patients, we determined the relationship between stimulated C-peptide levels at 1-month post diagnosis and the *C4* gene copy-numbers. Total *C4* CNV was significantly correlated with 1-month C-peptide levels ($R^2=0.13$; $p=0.04$), such that subjects with higher *C4* CNV had higher C-peptide levels (Fig. 2A). The correlation with C-peptide was even greater for *C4A* CNV, which had an R^2 of 0.21 and a p-value of 0.008 (Fig. 2B). By contrast, there was no apparent relationship between *C4B* CNV and 1-month C-peptide values (Fig. 2C).

The correlation of *C4* CNVs with stimulated C-peptide levels was further manifested in the positive correlation between protein levels of plasma C4 and C-peptides. Total C4 protein concentrations were marginally correlated with C-peptide levels ($R^2=0.11$; $p=0.058$) at 1-month post T1D diagnosis (Fig. 2D). When the C4 proteins were separated into *C4A* and *C4B*, it became clear that *C4A* protein at disease diagnosis was significantly correlated with

1-month C-peptide levels ($R^2=0.21$; $p=0.008$) (Fig. 2E). Similar to the case for the CNV of *C4B* genes, there was no clear relationship between 1-month C-peptide levels and C4B protein at diagnosis (Fig. 2F).

There were no significant relationships between diabetes-associated autoantibodies, anti-GAD-65, anti-ICA-512 or insulin autoantibody with C-peptide levels at 1 month or 9 months post diagnosis.

C4B CNV was inversely correlated with C-peptide levels at 9-month post T1D diagnosis

At 9-month post diabetes diagnosis, C-peptide levels were no longer significantly correlated with total *C4* CNV or *C4A* CNV. On the contrary, a *negative* correlation between 9-month C-peptide levels and *C4B* gene CNV emerged ($R^2=0.15$; $p=0.048$). Higher *C4B* copy-number correlated with *lower* C-peptide levels. *C4A* and *C4B* protein levels at diagnosis also did not correlate with 9-month C-peptide levels.

C4A, BMI or BMI z-score are independent parameters for stimulated C-peptide levels at 1-month

C4A CNV ($R^2=0.215$, $p=0.0075$), *C4A* protein concentration ($R^2=0.209$; $p=0.0085$), dosage of *HLA-DR3* allele ($R^2=0.138$; $p=0.036$), BMI ($R^2=0.310$; $p=0.0009$) or BMI Z-score ($R^2=0.184$; $p=0.014$) were *each* significantly correlated with 1-month (stimulated) C-peptide levels (Table 3). We further investigated if the correlation of *C4A* with stimulated C-peptide was independent of other parameters such as age of disease onset, BMI, and Z-score of BMI by performing multiple logistic regression analyses. BMI and *C4A* gene CNVs co-existed as positive and independent parameters for variations of C-peptides (overall $R^2=0.40$, $p=0.0005$; BMI, $p=0.005$; *C4A* CNV, $p=0.04$). The same phenomenon held for BMI and *C4A* protein levels at diagnosis (overall $R^2=0.423$, $p=0.0003$; BMI, $p=0.003$; *C4A* conc., $p=0.024$). Similar but smaller effects on C-peptide levels were observed for BMI Z-score plus *C4A* CNV or *C4A* protein concentrations. Age of onset, however, could not coexist in multiple logistic regression models with BMI, and *C4A* CNV or *C4A* protein concentration as an independent parameter of C-peptide levels.

Partial disease remission at 9-month post diabetes diagnosis had significantly lower copy-number of C4B genes, lower plasma C4B proteins, and the presence of HLA-DR3/DR4 heterozygotes

Twenty-six subjects were followed through 9 months. All had been previously studied at 1-month. Mean hemoglobin (Hgb) A1c for all subjects at 9-month was 7.5%. These subjects were divided into two groups based on insulin dose adjusted hemoglobin A1c (IDAA1c): $HbA1c(\%) + 4 \times \text{insulin dose (units/kg/day)}$. A patient with an IDAA1c < 9 is defined as being in partial remission (31). Eleven of 26 subjects (42.3%) at 9-month remained in partial remission. Of the subjects who *had ended* the partial remission period, they had higher *C4B* CNV (mean copy-number \pm SD: 1.88 ± 0.7 vs 1.27 ± 0.62 ; $p=0.02$), higher *C4B* protein concentrations at diagnosis (mean \pm SD: 156.2 ± 66.2 mg/L vs 70.0 ± 44 mg/L; $p=0.0004$; t-test) (Fig. 3C, 3F), and higher *C4B* protein concentration at 9-month post-diagnosis (148.5 ± 79.3 mg/L vs 90.3 ± 56.7 mg/L; $p=0.02$; t-test). While there was no relationship between *C4A* CNV or *C4A* protein concentration and subjects with a defined honeymoon

period (Fig. 3B, 3E), the patient group who *had ended* partial remission at 9-month had significantly higher total C4 protein concentrations at diagnosis (310 ± 112 mg/L vs. 207 ± 56 mg/L; $p=0.005$; t-test) (Fig. 3D) and marginal difference in total C4 gene copy-number ($p=0.056$) (Fig. 3A).

The dosages of *HLA-DR3* alleles ($p=0.26$, χ^2 analysis) or *HLA-DR4* alleles ($p=0.90$, χ^2 analysis) did not associate with partial disease remission at 9 months. On the other hand, T1D subjects who had *HLA DR3/DR4* heterozygotes all remained in partial disease remission at 9 months ($p=0.017$, χ^2 analysis).

To determine if *DR3/DR4* heterozygotes and *C4B* CNV or C4B protein concentrations are independent risk factors for partial disease remission at 9 months, multiple logistic regression analyses were performed (Table 3, panel B). Lower *C4B* CNV ($\chi^2=6.88$, $p=0.0087$) and the presence of *DR3/DR4* heterozygotes ($\chi^2=6.85$, $p=0.0089$) independently contributed to disease remissions at 9-month (model p -value=0.0018; $R^2=0.355$). In an alternative model, *DR3/DR4* heterozygotes ($\chi^2=4.09$, $p=0.043$) and lower C4B protein concentration ($\chi^2=11.0$, $p=0.0009$) were both significant and independent contributing factor for partial disease remission (model p -value=0.0002, $R^2=0.471$).

As a negative control, no apparent correlations were observed between plasma C3 concentration and stimulated C-peptide levels at 1 or 9-month post diabetes diagnosis ($p=0.51$; $p=0.36$), or partial disease remission ($p=0.21$).

Discussion

T1D has been extensively characterized as a T-cell mediated autoimmune disease but the roles for other immune effector proteins including those of the complement system in disease pathogenesis and progression are unclear. We determined the gene CNVs and their corresponding plasma protein levels for the two isotypes of complement C4, C4A and C4B in new-onset T1D patients. We showed that T1D subjects with higher gene copy-numbers of *C4A*, or with higher plasma C4A protein concentrations had higher levels of stimulated C-peptide at 1-month post diabetes diagnosis. On the other hand, T1D patients with higher *C4B* gene copy-number or higher C4B protein concentrations were *less* likely to retain the capability to secrete endogenous insulin or C-peptide at 9-month post diagnosis and therefore ended partial disease remission. The gene CNVs of *C4A* and *C4B* and their protein concentrations at T1D disease onset can be novel biomarkers relevant for the prediction and intervention of T1D disease remission.

From a physiologic standpoint, activated C4A proteins bind effectively to antigens with *amino* groups such as proteins or immune complexes. Through covalent binding to target molecules and serving as a ligand to complement receptor CR1 on erythrocytes, activated C4, particularly those from C4A, is engaged in the clearance of immune complexes and waste materials in the circulation (32, 33). Activated C4B proteins bind vehemently to antigens with *hydroxyl* groups such as carbohydrates or glycoproteins (34, 35), and is highly efficient in the propagation of complement activation pathways that cumulate to the formation of the membrane attack complex. Such differential chemical reactivity between

C4A and C4B offers an explanation for the “Yin-Yang” phenomenon of C4A and C4B in the pathogenesis and progression of the new-onset T1D.

At least two sets of parameters appeared to be confounding factors. First, there is a strong and inverse correlation between the number of *HLA-DR3* alleles and the copy-number of *C4A* genes ($R^2=0.388$, $p=0.00084$), or *C4A* protein concentration ($R^2=0.248$, $p=0.0027$). Second, serum C4 levels among healthy subjects modestly correlated with BMI (20), probably because adipose tissues also synthesize complement proteins.

To resolve the confounding factors in determining the residual beta-cell activities 1-month post disease onset, we performed multiple logistic regression analyses. *C4A* gene CNV or dosage of *HLA-DR3* alleles each can co-exist with BMI Z-score as significant and independent parameters on determining the stimulated C-peptide levels in regression models. When controlled by BMI Z-scores, the strengths of association with C-peptide for *C4A* CNV (t-ratio=2.68, $p=0.012$) and *C4A* protein concentration (t-ratio=2.58, $p=0.015$) appeared to be slightly stronger than that for *HLA-DR3* allele dosage (t-ratio=2.35, $p=0.026$).

At 9-month post diagnosis, BMI Z-scores, *HLA-DR3* alleles, *C4A* CNV or *C4A* protein concentrations at diagnosis were no longer significant determining factors for stimulated C-peptides. Instead, the copy-number of *C4B* genes ($p=0.048$) and the age of disease onset ($p=0.022$) become significant determining factors. Among those subjects who had ended partial remission, they had significantly higher copy number of *C4B* genes and higher *C4B* protein concentration levels. Under hyperglycemic conditions, high *C4B* gene copy-number and/or high levels of activated *C4B* protein could potentiate complement-mediated tissue-injuries during an inflammatory response and aggravate pancreatic beta-cell destructions, leading to shorter honeymoon period in new-onset T1D. An alternative explanation would be that hyperglycemia associated with the end of remissions might have altered C4 secretion. Against the latter are earlier findings that C4 levels were not significantly altered in poorly controlled diabetes patients without infection (36), or among patients with intensive insulin therapy during coronary bypass surgery (37).

Previous studies have identified several risk factors for a non-existence or shortened honeymoon period including younger age of disease onset, severity of disease at presentation (DKA), female gender, and higher HbA1c at T1D onset (11, 12, 38–42). One study investigated the association of *HLA-DRB1* alleles *DR3* and *DR4* with stimulated C-peptides after disease onset and found no significant correlation (11). Our data revealed that *HLA-DR4* has no role on stimulated C-peptide levels during the honeymoon period. Therefore, lumping *HLA-DR3* and *DR4* alleles together in an analysis would have masked the effect of *DR3*. In our study population, 83.3% of T1D subjects who had an *HLA-DR3* allele also had a specific RCCX haplotype characterized by the presence of a short *C4B* gene (coding for C4B1 protein) and the absence of *C4A*. This haplotype is similar or probably identical to the described “extended” or “ancient” haplotype with HLA A1 B8 DR3 and complotype SC01 (which stands for complement factor B-slow, C2C, C4AQ0 and C4B1) (43, 44).

This study enrolled a limited number of new-onset T1D patients in a serial clinical study to identify risk factors of *large effect-size* (i.e., prominent risk factors) associated with residual C-peptide levels at one month after disease onsets, and the existence of partial remissions 9 months after diagnosis. Results of this study favorably justify an in-depth prospective study with greater number of new-onset patients for quarterly mixed meal tolerance tests, assays of complement *C4A* and *C4B* gene CNVs, and serial variations of *C4A* and *C4B* protein levels over a two-year period. Such extended study will allow us to identify medium and low effect-size risk factors associated with T1D pathogenesis, disease remission and progression. If the Yin-Yang phenomenon for *C4A* and *C4B* in T1D “honeymoon period” is replicated in larger and more extensive studies, then enhancing the expression of *C4A* and reducing the levels of functional *C4B* would be a reasonable goal to intervene the disease progression and to achieve prolonged disease remission of T1D. Future investigations of the remission period following diagnosis with reagents that modulate *C4A* and *C4B* biosynthesis or turnover can lead to new exciting discoveries for treatment and therapies of T1D.

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Abbreviations

(Hgb)	Hemoglobin
(MMTT)	mixed meal tolerance test
(T1D)	Type 1 Diabetes

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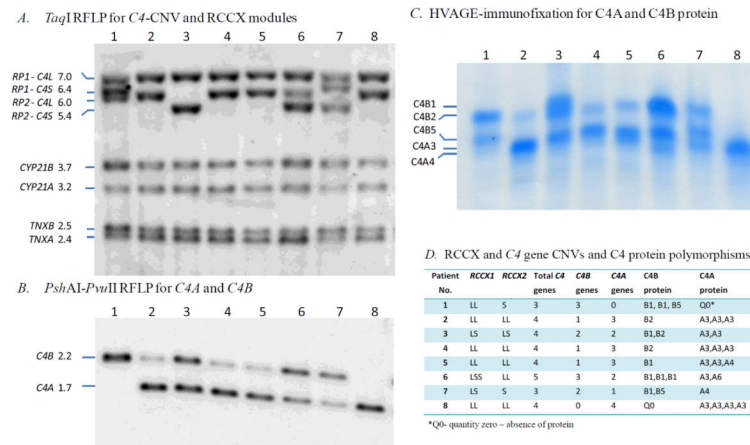


Fig. 1. Genotypic and phenotypic characterizations of complement C4 in the MHC class III region. **A.** Genomic *TaqI* RFLP to determine *RP-C4-CYP21-TNX* (RCCX) modules and copy-number variations of long and short *C4* genes. **B.** *PshAI-PvuII* RFLP to elucidate the relative gene copy-numbers of *C4A* and *C4B*. **C.** High voltage agarose gel electrophoresis (HVAGE) and immunofixation of plasma protein to demonstrate polymorphisms of native C4A and C4B allotypes based on gross differences in electric charges. **D.** Interpretations of C4 genotypes and phenotypes in eight T1D patients shown in panels A to C.

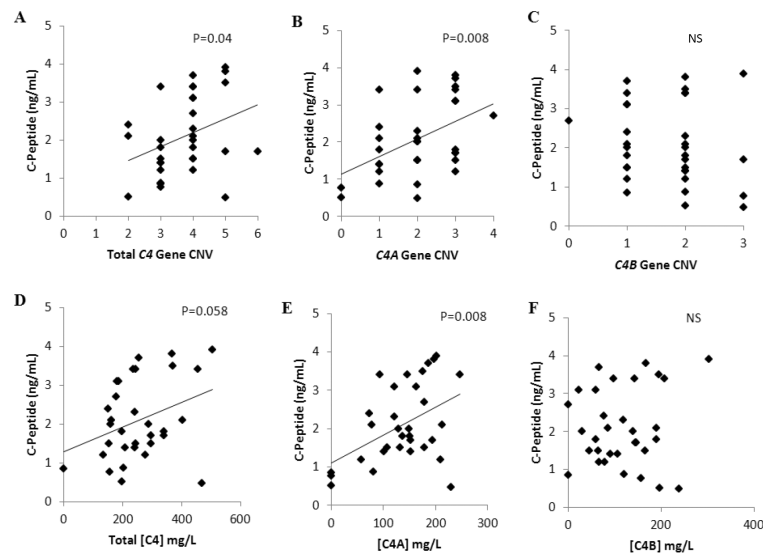


Fig. 2. Correlation of total *C4* gene CNV (A), *C4A* gene CNV (B), *C4B* gene CNV (C), total C4 protein (D), C4A protein (E) and C4B protein (F) concentrations with stimulated C-peptide levels at 1-month after disease onset. Note that higher total *C4* CNV or *C4A* gene is positively correlated with C-Peptide levels ($p=0.04$, $p=0.008$ respectively), and higher total C4 protein concentration or C4A protein concentration is positively correlated with C-peptide levels ($p=0.058$, $p=0.008$ respectively).

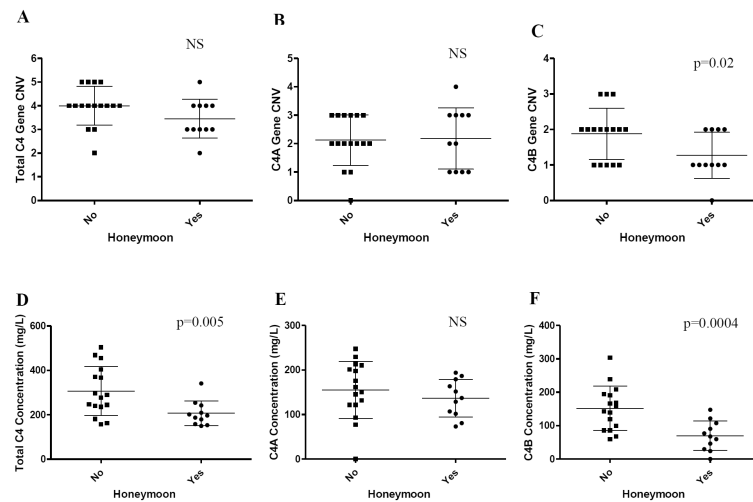


Fig. 3. Comparison of *C4* gene CNV (A), *C4A* gene CNV (B), *C4B* CNV (C), total C4 protein (D), C4A protein (E), and C4B protein (F) concentrations in subjects with and without disease partial remission (honeymoon) at 9-month post disease onset. Lower total C4 protein concentration, *C4B* CNV or C4B protein concentration are correlated with the presence of partial disease remission ($p=0.005$, $p=0.02$, $p=0.0004$, respectively).

TABLE 1

Demographics, complement and HLA-DRB1 in new onset T1D

Patient	Sex	Age (yrs)	BMI (kg/m ²)	[C3] (mg/L)	[C4] (mg/L)	[C4]-9 mon. (mg/L)	Total C4 CNV	C4A CNV	C4B CNV	RCCX H1	RCCX H2	DRB1 H1	DRB1 H2
1	M	9	15.7	1532	158	188	3	0	3	LL	S	17(G)*	13.1
2	F	11	16.3	1137	187	138	4	3	1	LL	LL	17(G)	4
3	M	10	15.6	1592	456	406	4	2	2	LS	LS	1	4
4	M	12	23.0	1414	254	236	4	3	1	LL	LL	4	13
6	M	9	23.9	2681	504	556	5	2	3	LL	LLS	7	9
7	M	13	17.2	1157	242	434	3	1	2	LS	S	17(G)*	4
8	M	11	17.3	1288	179	362	4	4	0	LL	LL	1	4
9	M	4	14.4	2244	202	266	3	1	2	LL	S	17(G)*	4
10	M	9	15.5	1222	209	271	3	1	2	LL	S	17(G)*	11
11	M	10	17.5	1855	341	n.d.	3	1	2	LL	S	17(G)*	4
12	M	14	15.4	849	181	128	4	3	1	LL	LS	1	4
13	M	14	17.0	1355	241	195	4	2	2	LS	LS	7	8
14	M	13	12.5	849	273	n.d.	3	1	2	LL	S	17(G)*	4
15	M	10	21.8	1493	371	484	5	3	2	LS	LSS	12	13.1
16	F	12	17.0	1031	163	102	2	1	1	L	S	1	17(G)
17	M	14	15.5	1493	469	222	5	2	3	LS	LSS	1	4
18	M	9	17.3	1306	278	261	4	3	1	LL	LL	4	4
19	M	12	16.3	1183	289	276	4	2	2	LS	LS	7	8
20	M	16	29.2	732	245	279	4	3	1	LL	LS	4	7
21	M	8	23.9	1984	298	399	4	2	2	LS	LS	17(G)	17(G)
22	M	4	19.4	1006	297	397	6	3	3	LSS	LLS	7	13.1
23	M	10	25.4	1306	335	n.d.	3	1	2	LL	S	4	4
24	M	16	17.6	1006	197	237	4	3	1	LL	LL	4	12
25	F	11	21.3	1123	236	196	3	1	2	LS	S	4	15
26	F	8	17.1	2441	404	321	4	2	2	LS	LS	4	4
27	M	11	17.5	485	158	246	3	2	1	LS	L	13.1	1
28	M	6	19.7	1006	153	323	3	2	1	LL	L	4	7

Patient	Sex	Age (yrs)	BMI (kg/m ²)	[C3] (mg/L)	[C4] (mg/L)	[C4]-9 mon. (mg/L)	Total C4 CNV	C4A CNV	C4B CNV	RCCX H1	RCCX H2	DRBI H1	DRBI H2
30	M	12	15.4	1386	341	340	5	3	2	LSS	LL	1	7
31	M	7	15.2	912	198	n.d.	2	0	2	S	S	17(3)*	17(3)*
32	M	13	19.8	1386	150	175	2	1	1	L	S	17(3)*	17(3)
33	M	17	20.7	1761	244	n.d.	4	3	1	LL	LS	13.1	12
34	F	3	14.9	727	<30	n.d.	3	2	1	LS	L	13.1	4
35	M	16	17.0	735	135	n.d.	3	1	2	LL	S	17(3)*	14.1

* an asterisk signifies an extended haplotype with *HLA-DR3* and C4A deficiency; [C4], C4 protein concentration; [C3], C3 protein concentration; H1, haplotype 1; H2, haplotype 2; n.d., not determined.

TABLE 2

Summary statistics of demographic and laboratory data for new onset T1D patients

	at diagnosis or 1-month	at 9 months
	<i>mean ± s. d.</i>	<i>mean ± s. d.</i>
Age (yrs)	10.73 ± 3.45	
Body Weight (kg)	43.21 ± 15.92	
Height (m)	1.51 ± 0.22	
Body mass index	18.35 ± 3.6	
Z-score, BMI	-0.04 ± 3.6	
anti-GAD-65 (U/ml)	10.78 ± 27.98	
anti-ICA512 (U/ml)	11.39 ± 14.36	
anti-insulin (U/ml)	16.84 ± 30.78	
Total C4-CNV	3.65 ± 0.95	
C4A-CNV	1.97 ± 0.10	
C4B-CNY	1.71 ± 0.72	
	<i>frequency</i>	
HLA-DR3 (0/1/2)	0.647/ 0.265/ 0.088	
HLA-DR4 (0/1/2)	0.441/ 0.471/ 0.088	
DR3/DR4 (0/1)	0.853/ 0.157	
	<i>mean ± s. d.</i>	<i>mean ± s. d.</i>
Hb-A1c (%)	11.37 ± 2.43	7.45 ± 1.42
Fasting glucose (mg/dL)	108.7 ± 38.6	151.9 ± 73.8
Stimulated glucose (mg/dL)	189.7 ± 54.3	277.1 ± 101.9
Fasting C-peptide (ng/ml)	0.774 ± 0.484	0.695 ± 0.418
Stimulated C-peptide (ng/ml)	2.10 ± 1.02	1.63 ± 1.01
Total C4 cone. (mg/L)	245.1 ± 113.0	288.0 ± 110.7
C4A cone. (mg/L)	130.5 ± 66.8	161.6 ± 74.7
C4B cone. (mg/L)	114.7 ± 71.2	121.9 ± 77.4
C3 cone., mg/L	1325.5 ± 490.5	1752.9 ± 562.6

Table 3

Effects of individual contributing factors and multiple logistic regression models for risk factors associated with C-peptide levels at 1-month (A), and the end of partial remission at 9-month (B) post T1D diagnosis.

A. Stimulated C-peptides at 1-month post diagnosis			
	R ²	t-ratio	P
<i>Individual parameters</i>			
C4A CNV	0.215	2.87	0.0075
C4A cone.	0.209	2.82	0.0085
HLA-DR3 *	0.138	-2.20	0.036
BMI	0.310	3.67	0.0009
Z-BMI	0.184	2.61	0.014
age of onset	0.06	1.43	0.162
<i>Regression Model 1: C4A CNV and BMI</i>			
model	0.405	9.85*	0.0005
C4A CNV		2.15	0.040
BMI		3.04	0.005
<i>Regression Model 2: C4A protein cone, and BMI</i>			
model	0.423	10.6 [¶]	0.0003
C4A cone.		2.39	0.024
BMI		3.28	0.0027
<i>Regression Model 3: HLA-DR3 and BMI</i>			
model	0.405	9.87 [¶]	0.0005
HLA-DR3		-2.16	0.040
BMI		3.61	0.0012
B. End of partial disease remission at 9-month			
	R ²	χ ²	P
<i>Individual parameters</i>			
C4B CNV	0.162	5.74	0.0166
C4B cone.	0.355	12.6	0.0004
DR3/DR4 *	0.161	5.71	0.0169
<i>Regression Model 4: C4B-CNV and DR3/DR4</i>			
model	0.355	12.6	0.0018
C4B-CHV		6.88	0.0087
DR3/DR4 *		6.85	0.0089
<i>Regression Model 4: C4B cone. and DR3/DR4</i>			
model	0.471	16.8	0.0002
C4B cone.		11.0	0.0009
DR3/DR4 *		4.09	0.043

* inverse association

[¶]F-ratio value is presented.