Immunogenetics of complement in mixed cryoglobulinaemia

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ABSTRACT

Objective. *A low C4 level is one of the hallmarks of mixed cryoglobulinaemia (MC). However, several reports suggest that other factors may be involved in C4 depletion. The C4 gene is located in a multiallelic CNV locus in the human MHC region. We studied the C4 gene copy number (GCN) and both C4A and C4B isotypes, as well as the presence of the hypofunctional C4A6 allotype (rs41315824) and C4A0 allotype (rs367709216) in 41 MC patients, 16 SLE patients and 78 healthy controls.*

Methods. *GCN of the C4 gene were evaluated by real time PCR. C4A6 allotype (p.Arg458Trp) and ins 2-bp mutation in exon 29 were screened by primer extension. Correlation with clinical signs of the disease (cutaneous ulcers, peripheral neuropathy, GN, purpura, hepatitis) have been performed by cluster analysis, (K-means algorithm).*

Results. *C4 GCN analysis showed that fewer MC patients had more than 2 copies of the C4A gene as well as a lower C4A gene-copy index (1.90 ± 0.54 vs. 2.21 ± 0.78) as compared to healthy controls. SNP rs41315824 analysis showed a significant increase in the frequency of the p.Arg458Trp (C4A6) variant in cryoglobulinaemic patients. Lastly, cluster analysis allowed us to identify two separate clusters of patients. The cluster that included patients with three or less C4 gene copies was found to have a greater prevalence of the most severe complications such as glomerulonephritis, neuropathy and severe cutaneous ulcers.*

Conclusion. *These data suggest there may be a relationship between polymorphisms of the C4 gene and clinical presentation.*

Introduction

Mixed cryoglobulinaemia (MC) is a multisystem disorder strongly associated with HCV infection. Circulating mixed cryoglobulins, low C4 levels and orthostatic skin purpura are the hallmarks of MC, and it is commonly accepted, that the severe hypocomplementaemia observed is mostly due to early complement component consumption. Several observations (1) suggest that C4 depletion does not depend solely on immunocomplex activation or HCV infection, but that other factors, such as C4 gene polymorphisms, could be involved. C4 gene is highly polymorphic, it is located in a multiallelic copy number variation (CNV) locus called RCCX module (6p21.1) (2-4). Each C4 gene can code for either an acid C4A protein or a basic C4B protein which, at nucleotide level, show 99% sequence identity. Moreover, more than 40 allotypes have been described for both C4A and C4B genes due to the presence of SNPs (5); among these, C4A6 allotype (6) and the null allele C4AQ0, due to a di-nucleotide insertion in exon 29, could be involved in C4 deficiency.

In the present study, we investigated the copy number of total C4 genes and either C4A and C4B isotypes and C4A6 and C4AQ0 allotypes in patients with HCV-associated MC.

Patients and methods

Patients

The study included 41 unrelated italian patients (26 females and 15 males, age 45 - 83 years) affected by MC and 16 patients affected by SLE as a diseased control group. Every patient with MC was carefully evaluated by two clinicians with specific expertise (DR,SS) (7). Patients fullfilled the criteria recently published by Quartuccio *et al.* (8). MC was diagnosed on the basis of criteria described elsewhere (7).

An analysis was also performed on 78 gender- and age-matched healthy subjects recruited from among the healthy blood donor population.

Gene copy number analysis
CNVs of the C4 gene were evaluated by quantitative real time polymerase chain reaction (qPCR LightCycler 480 II (Roche Diagnostics) (9). Amplification data were analysed using the 2-ΔΔCT method.

Genotyping for C4 mutations
C4A6 (p.Arg458Trp, rs41315824) and C4AQ0 allotype (ins 2-bp mutation in exon 29 rs367709216), were screened using Multiplex fluorescent primer extension (SNaPshot Multiplex Ready Reaction Mix Applied Biosystems).

Statistical analysis
Chi-square and Fisher's exact test analyses were performed to determine the differences in total C4, C4A, and C4B gene CNV among groups. Cluster analysis was performed using the K-means algorithm to group patients with similar profiles together (10). Analyses were performed using SPSS 19.0 software (Chicago, Illinois, USA).

Results

The distribution and frequencies of C4 GCN for our three groups *i.e*. MC patients, systemic lupus erythematosus and controls (CTRL) are presented in Figure 1 and Table I.
As compared to the healthy control group, fewer patients in the MC group had 2 or more copies of the C4A gene (MC 9.75% *vs*. CTRL 29.49% p=0.01). The gene-copy index, mean copy number of a specific gene that manifests interindividual gene CNV in a selected population (6) of C4A in healthy controls, was 2.21±0.78, while in patients with MC was 1.90±0.54 (p=0.02).

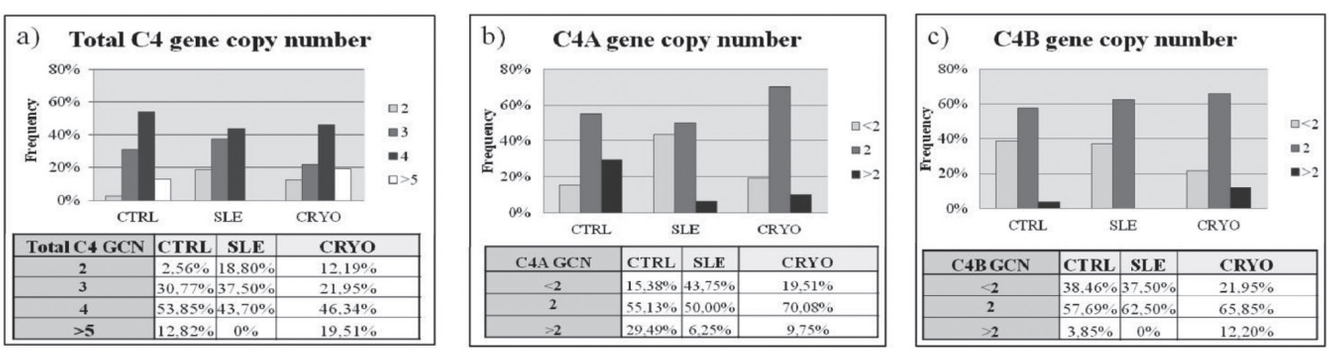
C4A and C4B allotype analysis
The p.Arg458Trp variant was detected in 6 of 42 patients (14.3%) with MC, and in 1 of 16 SLE (6.25%) patients, though only in the C4A gene. No C4A6 allotype was detected in the control group. All six cryoglobulinaemic patients showed four copies of C4 (two copies of C4A and two copies of C4B) at CNV analysis, whereas the SLE patient had only two copies of total C4 (one of C4A and one of C4B). None had a 2 bp insertion in exon 29.

C4 serum levels
C4 levels were measured in cryoglobulinaemic patients at disease onset. Values ranging between 0.15 and 0.4 g/l were considered normal.
By grouping cryoglobulinaemic patients by total C4 gene copy number (considering the presence of C4A6 allotype), we found that patients with two copies of C4 showed median values of serum C4 levels of 0.04g/l (range 0–0.1 g/l) at disease onset; patients with three copies of C4 showed median values of serum C4 levels of 0.03g/l (range 0.01–0.14 g/l), patients with four or more copies of C4 showed median values of serum C4 levels of 0.09g/l (range 0.00–0.24 g/l).

Cluster analysis of C4 GCN and C4A6 allotype
Considering the low haemolytic activity that characterises the C4 protein carrying p.Arg458Trp variant (C4A6 allotype), we considered patients with tryptophan in position 458 as if they had fewer C4 gene copies than what was actually found by quantitative PCR.
Therefore, we performed cluster analysis assuming that patients carrying the p.Arg458Trp variant a defective C4 gene.
Cluster analysis showed two clusters, and the one including patients with 3 or less than 3 C4 gene copies showed the highest prevalence of the most severe complications of cryoglobulinaemia such as glomerulonephritis, neuropathy and severe cutaneous ulcers. Of particular interest, this subset was made up solely of female patients. In the second cluster patients with more than three C4 gene copies showed hepatitis and longer disease duration.

Discussion

MC is a multisystemic disorder strongly associated to HCV infection that shows severe C4 hypocomplementaemia. Complement C4 fraction depletion is a sensitive and important finding in patients affected by cryoglobulinaemic vasculitis that is found in 70–90% of patients. Nevertheless, C4 fraction depletion does not correlate with the level of cryoglobulins, it may persist after apparent clearance of the HCV virus, and it correlates poorly with clinical signs (1). These observations suggest that mechanisms other than complement cascade activation are involved in MC. It has been shown that C4 may be involved in HCV infection, as both HCV core and NS5A proteins cause a decrease in C4 production by inhibiting transcription of C4 mRNA (11, 12). Moreover, greater C4 activity in HCV infections was also associated with better response to standard HCV treatment (13, 14). The presence of low C4 GCNs has been implicated in disease susceptibility, low copy numbers of C4A have



Total C4 GCN	CTRL	SLE	CRYO
2	2,56%	18,80%	12,19%
3	30,77%	37,50%	21,95%
4	53,85%	43,70%	46,34%
>5	12,82%	0%	19,51%

C4A GCN	CTRL	SLE	CRYO
<2	15,38%	43,75%	19,51%
2	55,13%	50,00%	70,08%
>2	29,49%	6,25%	9,75%

C4B GCN	CTRL	SLE	CRYO
<2	38,46%	37,50%	21,95%
2	57,69%	62,50%	65,85%
>2	3,85%	0%	12,20%

Fig. 1. C4 gene copy number distribution in cryoglobulinaemic patients and in SLE and control group.

Table I. C4 Gene-copy index of cryoglobulinaemic patients (CRYO), systemic lupus erythematosus (SLE) and control (CTRL) group. CRYO (p.Arg458Trp): assuming that patients with tryptophan in position 458, as if they had fewer C4 gene copies than what was actually found by quantitative PCR.

		Gene copy index ± SD		
Disease	n°	C4 tot	C4A	C4B
CTRL	78	3.87 ± 0.96	2.21 ± 0.78	1.67 ± 0.60
SLE	16	3.25 ± 0.77	1.63 ± 0.62	1.63 ± 0.50
CRYO	41	3.73 ± 0.92	1.90 ± 0.54	1.83 ± 0.74
CRYO (p.Arg458Trp)	41	3.54 ± 1.00	1.70 ± 0.72	
p-value (*t*-test) two-tailed				
SLE *vs*. CTRL		0.017	0.006	0.795
CRYO *vs*. CTRL		0.442	0.0283	0.196
CRYO (p.Arg458Trp) *vs*. CTRL		0.776	0.0009	0.196
SLE *vs*. CRYO		0.069	0.128	0.312
SLE *vs*. CRYO (p.Arg458Trp)		0.307	0.690	0.312

been shown to be associated with SLE and a low copy number of C4B has been related to morbidity and mortality in patients with cardiovascular disease, especially among smokers.

Because of these inter-relationships between C4 serum levels, HCV infection and mixed cryoglobulinaemia (15), we hypothesised that C4 CNV might be related to the onset of MC in HCV positive patients and/or to the onset of the most severe clinical manifestations such as glomerulonephritis, neuropathy and skin lesions.

Our findings showed a significant increase in the frequency of tryptophan in MC patients (p.Arg458Trp) when compared to both normal samples and SLE patients. C4A6 allotype shows low haemolytic activity and a reduced ability to bind C5 within the C5 convertase complex. The mutant protein behaves like its wild-type counterpart with respect to covalent binding to C1 bearing targets, but with substantially less C5 cleavage. Therefore, it is likely that patients with the C4A6 allotype bear fewer C4A gene copies than one might presume based on the results of real time PCR due to the presence of a functionally defective copy. The high frequency of p.Arg458Trp variant in MC patients is intriguing. Few data are available on the frequency distribution of this variant in immune-mediated diseases or on the biological consequences of the presence of C4A6 allotype in immune complex removal and antiviral defense mechanisms. No correlation has been found between the presence of C4A6 allotype in either the homozygous or heterozygous state and clinical signs.

C4 serum levels are related to C4 gene copy number: Patients with fewer copies have lower levels of the protein. Nevertheless, the rate of C4 depletion in MC may be influenced by several environmental factors which could explain the high variability of serum levels in patient groups.

Cluster analysis allowed us to identify two separate clusters of patients, each showing a distinct range of C4 copies associated with patterns of demographic characteristics and clinical manifestations. One cluster, which included patients with three or less than three C4 gene copies, showed a greater prevalence of patients suffering from glomerulonephritis, neuropathy and severe cutaneous ulcers. A second cluster, which included patients with more than three C4 gene copies was found to have the highest prevalence of hepatitis and longer disease duration.

These data suggest a definite relationship between polymorphisms of the C4 gene and clinical presentation. Abnormal C4 gene dosage and/or function could alter or limit the complement-mediated clearance of immune complexes and modulate both the release of downstream complement components and tissue inflammation.

This is an explorative monocentric study focused on a cohort of severe cases of cryoglobulinaemic patients, nevertheless, this is the largest observational database-driven study to attempt to identify correlations and cluster profiles based on demographic characteristics, C4 patterns and clinical manifestations in cryoglobulinaemic patients. These findings suggest that cryoglobulinaemic vasculitis may be viewed as a disease with a dual spectrum of manifestations. High *vs*. low number of C4 copies may influence disease expression, however replicate results in a larger subset of patients are needed, including less severe cases of MC without renal involvement, HCV patients with or without circulating cryoglobulins, and no syndromic symptoms and signs.

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