A high and equal prevalence of the Q703K variant in *NLRP3* patients with autoinflammatory symptoms and ethnically matched controls

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ABSTRACT

Objective. *Cryopyrin associated periodic syndromes (CAPS) comprise a spectrum of autoinflammatory disorders of varying severity caused by mutations in the NLRP3 gene. The NLRP3-Q703K allele has been reported both as a functional polymorphism and as a low penetrance mutation.*

Methods. *To describe the clinical phenotype of subjects with the Q703K allele and to report the frequency of this allele among patients with autoinflammatory symptoms and healthy controls. To this end, a cohort of 10 ethnically-matched controls per each Q703K-carrying patient, was composed.*

Results. *Ninety patients suspected of harbouring a systemic autoinflammatory disease (SAID), exclusive of FMF, were referred to our centre for genotyping between 2012 and 2015. Fourteen of them (15.5%) were found to carry the Q703K allele, compared to 22 of 130 (16.9%) healthy, ethnically matched controls.*

Conclusion. *The similar carrier rate of the NLRP3-Q703K allele among patients with manifestations of a SAID and an ethnically matched control group suggest that this variant, does not determine the clinical phenotype. This reiterates the importance of testing a control group to avoid erroneously attributing a causative role to a gene polymorphism.*

Introduction

Cryopyrin-associated periodic syndromes (CAPS) comprise a continuum of rare autoinflammatory diseases, which can be subdivided into three main clinical subtypes. The most common is familial cold autoinflammatory syndrome (FCAS), manifested by cold induced fever and urticarial-like rash, many times accompanied by arthralgia and conjunctivitis. Muckle Wells syndrome is characterised by the same signs and symptoms in addition to frank arthritis, hearing loss and the development of AA amyloidosis in up to 30% of the patients. Neonatal onset multisystem inflammatory syndrome (NOMID) also known as chronic infantile neurologic and cutaneous and arthritis (CINCA) represents the severe end of the CAPS spectrum in which patients also develop mental retardation, meningitis, papilledema and vision loss (1). CAPS is inherited in an autosomal dominant mode with a high rate of *de novo* mutations in the *NLRP3* gene (2). Pathogenic, gain-of-function variants have been shown to be associated with caspase1 mediated upregulation of IL-1β secretion (3). Accordingly, treatment of CAPS with IL-1 antagonists has proven to be extremely effective (4, 5). More than 170 variants have been described thus far in *NLRP3*, 149 of which have been associated with the CAPS clinical phenotype and are considered pathogenic. Most of the mutations are found in exons in exons 3, 4 and 6 (fmf.igh.cnrs.fr/Infevers).

Over the years a number missense variants with unknown clinical significance have been described in this gene, including V198M, I313V and Q703K (6). The Q703K is the most controversial of these variants as it has been reported in several case series of patients with an autoinflammatory phenotype and yet was shown to be frequent in the general population (7). In this study

Table I. Ethnic origin and symptoms of patients with Q703K heterozygous *NLRP3* variation.

	Origin	Cold induced	Fever	Arthralgia	Myalgia	Abdominal pain	Conjunctivitis	Oral ulcers	Rash	Deafness	Fatigue
1	Ashkenazi	Yes	Yes	Yes	Yes	No	Yes	No	Yes	No	Yes
2	Ashkenazi	Yes	Yes	Yes	Yes	No	No	No	Yes	No	Yes
3	Ashkenazi	No	Yes	Yes	Yes	No	No	No	Yes	No	No
4	Iraq	No	Yes	No	Yes	Yes	No	No	Yes	No	Yes
5	Egypt/ Ashkenazi	No	No	No	Yes	Yes	No	No	No	No	No
6	Yemen	No	Yes	Yes	Yes	No	No	No	Yes	No	No
7	North African	No	No	Yes	No	No	No	No	Yes	Yes	Yes
8	Europe (non-Jew)	No	Yes	No	Yes	Yes	No	No	Yes	No	Yes
9	Arab -Muslim	No	Yes	No	Yes	No	No	No	Yes	No	Yes
10	Ashkenazi	No	Yes	No	No	No	No	No	No	No	Yes
11	Arab-Muslim	No	Yes	Yes	Yes	No	No	No	Yes	No	No
12	North- African/Buchara	No	Yes	No	Yes	Yes	No	No	No	No	No
13	Ashkenazi	No	Yes	Yes	Yes	No	No	Yes	Yes	No	Yes
14	Ashkenazi	No	Yes	Yes	No	No	No	No	Yes	No	Yes

we tested for Q703K in patients who had presented with diverse symptoms within the range of autoinflammatory diseases. Most were sent for analysis after the initial search for common mutations in the *MEFV* gene was negative, as part of second line screening for other relatively common autoinflammatory disorders in our region.

Herein we summarise our findings in 14 patients, exhibiting a wide range of autoinflammatory symptoms, who underwent molecular analysis and were found to be heterozygous for the Q703K variant and in an ethnically matched control group. We present clinical and population frequency evidence that suggest that Q703K is a polymorphism rather than as a disease causing mutation.

Methods

The study was approved by the Institutional Review Board and participants gave informed consent. Ninety patients were referred to our laboratory of autoinflammation and amyloidosis, between the years 2012-2015, on account of "autoinflammatory symptoms" including but not limited to recurrent febrile attacks, conjunctivitis, arthritis, rash and hearing impairment. The patients were tested with our autoinflammatory panel which includes screening for the common FMF mutations (*MEFV*), sequencing of exons 2, 3 and 4 of the TNF alpha receptor gene (*TNFRSF1A*), sequencing of exon 3 of *NLRP3*, and all exons of the mevalonate kinase gene (*MVK*). Clinical and demographic information about the Q703K positive patients was obtained from their referring physicians and medical records. For these Q703K positive patients, we created a control group by matching to each patient 10 controls of the same ethnic origin. Q703K was identified using a restriction assay. DNA amplification was performed with the primers: 5'- CTGAAATGGATTGAAGTGAAAGC-3' and 5'-TGAAGGAGTCTCAAACAGACAGTG-3', in a 25 μl reaction containing 50 ng of DNA, 10 ng of each primer, 12.5ul of RM (Thermo Scientific) and 9.5ul of water. After an initial denaturation of 2 min at 95°C, 30 cycles were performed (95°C for 30 sec, 59°C for 45 sec and 72°C for 40 sec), followed by a final extension of 10 min at 72°C. PCR amplified segments were cut with Hph1 restriction enzyme to yield 3 segments (483, 290 and 190 bp) in the heterozygotes as opposed to a single segment in the wild type. Statistical analysis was done using the Fisher exact test.

Results

Fourteen of the 90 patients (15.5%) screened were found to be heterozygous for Q703K. Their clinical presentation is summarised in Table I. Interestingly, only one patient fulfilled established criteria for CAPS (8) (patient no. 1), presenting with a cold induced rash, fever, arthralgia and conjunctivitis and showing a good response to treatment with IL-1 inhibitors. In total 12/14 of the patients presented with a rash and 11/14 with fever, which were the two most prominent complaints. One had sensorineural hearing loss. None of the 14 patients suffered from amyloidosis. We did not find any other exon 3 *NLRP3* sequence variations in these patients. Patient no. 8 was found to have two pathogenic variants in the *MVK* gene, patient no. 4 had one pathogenic variant in the *MEFV* gene, and patient no. 6 was found heterozygous for R92Q in the *TNFRSF1A* gene. Five of the patients were treated with IL-1 inhibitors, 3 with a good, one with a partial and one with no response. The ethnic distribution of the control group is presented in Table II. The frequency of Q703K in the patient cohort and control group was similar (15.5% vs. 16.9%, respectively, p=0.85). An especially high frequency of the Q703K variant was noted in the Ashkenazi controls.

Of the remaining 76 patients referred for genetic work-up in our centre, *NLRP3* mutations were detected in 5, two of whom had a family history consistent with CAPS. A *de novo* mutation was found in one patient and somatic mutations were identified in two additional patients, appearing in DNA extracted from blood samples but not in DNA from buccal smears.

Discussion

A low rate of detection of clearly pathogenic variants among patients with a clinical suspicion of CAPS alongside the observation that the Q703K variant

Table II. Genetic work-up, acute phase reactants and response to therapy in patients with Q703K heterozygous *NLRP3* variation.

	MEFV	TNFRSF1A	MVK	Inflammatory markers during attacks	Therapy and response*
1	Negative	Negative	ND	ESR 100 mm/Hg	Anti IL-1 - Good
2	Negative	Negative	ND	ESR 40 mm/Hg	NSAIDs- Good
3	Negative	Negative	ND	Plt 630K ESR 60 mm/Hg Ferritin 500	Anti TNF – Good (adalimumab)
4	Negative	Negative	ND	Normal	Anti IL-1-Partial
5	V726A	Negative	ND	Plt 720K CRP 215 (N<5)	Rituximab - Good
6	Negative	R92Q/G362	ND	ESR 36 mm/Hg	Anti IL-1 - Good
7	Negative	ND	ND	Plt 402K CRP 6.4 (N<5)	Anti TNF – Good (etanercept)
8	Negative	Negative	V250I	ESR 50 mm/Hg	Corticosteroids - Poor
9	Negative	Negative	ND	ESR 80 mm/Hg Ferritin 1000	Anti IL-1 - Poor Anti IL-6- Good
10	Negative	Negative	ND	CRP 345 (N<5)	Anti IL-1 - Good
11	Negative	Negative	Negative	CRP - Normal	NSAIDs- Partial
12	Negative	Negative	Negative	CRP - Normal	Colchicine- Partial
13	Negative	Negative	ND	CRP 2 (N<0.5)	Corticosteroids- Partial
14	Negative	Negative	ND	CRP 9 (N<0.5)	IVIG, Anti IL-6, Abatacept- Partial

ND: not done; ESR: erythrocyte sedimentation rate; CRP: C- reactive protein; NSAIDs: non-steroidal anti-inflammatory drugs; IL: interleukin; TNF: tumour necrosis factor; IVIg: intravenous immunoglobulins.
*Good response ≥75% decline in attack frequency, partial response 25-75% reduction in attack frequency, poor response ≤25% reduction in attack frequency.

Table III. Rate of *NLRP3* Q703K variation in ethnically matched controls*.

	Controls	Positive (%)
Ashkenazi	75	14 (18.6)
Arab	17	2 (11.7)
Yemen	10	2 (20.0)
Buchara	5	0 (0)
Iraq	9	0 (0)
Morocco	14	4 (28.6)
Total	130	22 (16.9)

*Fisher's exact probability test non-significant for each ethnicity *vs*. other ethnicity and *vs*. total.

is frequently found in these cohorts have prompted investigation into the role of the latter in disease pathogenicity.

The effect of Q703K on NLRP3 function and its clinical spectrum have been a matter of ongoing debate. Herein, we have found an unprecedented Q703K carrier rate of 16.9% in an ethnically matched cohort of 130 healthy individuals. Initially, Hoffman *et al*. found a 6% carrier rate of this variant in Caucasians (9) while a Swedish study reported a carrier rate of 13% in the local population (10).

Short case series have described the presence of this variant in patients with autoinflammatory disorders, the majority of which had some features consistent with CAPS, however only a few fully fulfilled established criteria for the disease and most did not respond to IL-1 inhibitors. This was also the case in our Q703K cohort in which the majority of patients suffered from fever, rash and arthralgia but only a fraction had cold induced symptoms and just one patient suffered from associated deafness.

Indeed, a 10-year multi-centre study performed in several Italian centres reiterates the mildness of the clinical features associated with Q703K carriage (11). Moreover, the authors note that a large proportion of patients carrying the Q703K variant eventually receive an alternative final diagnosis. Consistent with their findings, 2 of our patients were eventually diagnosed with other autoinflammatory diseases, mevalonate kinase deficiency in one and small vessel vasculitis in another, who also was heterozygous for the *MEFV* V726A mutation. One patient was found heterozygote for the R92Q variant in *TNFRSF1A*, however this variant is also usually considered a polymorphism without an associated specific clinical phenotype.

The lack of the most typical CAPS associated manifestations, especially hearing loss, the high percentage of patients who did not require anti IL-1 therapy and conversely, the relatively high rate of IL-1 treatment failure, lend further support to the notion that the Q703K variant is neutral in its clinical effect. Moreover, it is necessary to clarify that IL-1 responsiveness cannot serve to diagnose CAPS as this effect is nonspecific and may be present in non-specific autoinflammatory conditions. On a similar note, in one study, Q703K was seemingly overrepresented in patients with neurologic complaints; 13.8% of patients in a neuroimmunology department who had undergone extensive work up for headaches were found to harbour this variant which was also found to be associated with multiple sclerosis and responsiveness to anti IL-1 therapy in over half the cases (12). Taking into consideration the absence of controls in this study and the lack of specificity of anti IL-1 therapy, this observation should not be interpreted as implying an association to disease.

As for the *in vivo* effect of Q703K on inflammation, Blomgran *et al*. found non capase-1, IL-1β dependent delayed apoptosis in neutrophils from patients with this variant suggesting it may elicit a hyperinflammatory response (13) and a recent study by Kuemmerle-Deschner *at al*. found that transfected cell lines and whole blood from patients with low penetrance *NLRP3* variants, most of

them with Q703K (19 of 45 patients), showed an intermediate functional activation within NLRP3 inflammasome pathways of cell death, NF-kB activation, caspase-1 activity and cleavage and IL-1β release (14).

In contrast Reiber *et al*. failed to detect increased IL-1β secretion in individuals carrying low penetrance *NLRP3* mutations (including Q703K) (15). Summarising these studies one may conclude that the inflammatory effect exerted by Q703K is equivocal at best.

One of the key methods to differentiate a mutation from a benign polymorphism is to assess its frequency in a cohort of patients compared to controls. A true mutation should be overrepresented in the patient group, even if it is of low penetrance and needs interaction with other genes and/or environmental factors. If the contribution of the variant is strong enough, the over representation should hold even if the sample is heterogeneous, that is if some of the patients suffer from a completely different, unrelated disorder. In the case of Q703K, only two studies tested for such an association, and both had reported a low and almost equal frequency of this variant in patients with autoinflammatory features and controls, suggesting that Q703K is a benign polymorphism rather than a disease causing mutation (1, 11).

It seems that the majority of patients presenting with an ill-defined autoinflammatory phenotype cannot be clinically nor genetically classified into any one of the 3 studied autoinflammatory diseases. The quest to discover the genetic basis of the nebulous autoinflammatory phenotype has yet to achieve its goal. Meanwhile, these patients are treated on a clinical basis and some of them indeed respond to anti IL-1 treatment. In summary, our results support the notion that Q703K is a polymorphism rather than a disease associated mutation. In addition, we demonstrate the importance of properly choosing controls for such studies in order not to introduce bias.

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