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Exploratory study of MYD88 L265P, rare *NLRP3* variants and clonal hematopoiesis prevalence in patients with Schnitzler's Syndrome

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Abstract (250 words)

Objective: Assess the prevalence of the MYD88 L265P mutation and variants within *NLRP3*, also to evaluate the status of oligoclonal haematopoiesis in 30 patients with Schnitzler Syndrome (SchS).

Methods: 30 patients with SchS were recruited from 3 clinical centres. 6 patients with known acquired cryopyrin associated periodic syndrome (aCAPS) were included as controls. Allele-Specific Oligonucleotide PCR (ASO-PCR) for detection of the MYD88 L265P variant, Next-Generation Sequencing (NGS) of *NLRP3* and 28 genes associated with Myelodysplastic Syndrome (MDS) and Gene Scanning for X inactivation.

Results: Activating *NLRP3* mutations were not present within 11 SchS patients, who have not been sequenced for this gene previously. The MYD88 L265P variant was present in 9/30 SchS patients and somatic mutations associated with Clonal Hematopoiesis (CH) were identified in 1/30 SchS and 1/6 aCAPS patients. Evidence of non-random X inactivation was detected in one female with SchS and one female aCAPS patient.

Conclusion: A shared molecular mechanism accounting for the pathogenesis of inflammation in SchS remains elusive. CH is not associated with other somatic mutations found in SchS or aCAPS patients.

Introduction

Schnitzler Syndrome (SchS) is a rare, systemic autoinflammatory disease (SAID) with two major defining features - an IgM paraprotein and an urticarial-like rash – variously associated with recurrent fever, leucocytosis, elevated C-reactive protein and bone remodeling(1). SchS patients typically present in the 5th decade of life without any suggestion of inheritance. The clinical phenotype of SchS closely resembles that of the hereditary fever syndrome known as Cryopyrin Associated Periodic Syndrome (CAPS). Both SchS and CAPS are responsive to IL-1 blockade, further implying overlapping aetiology. CAPS is caused by gain-of-function mutations in *NLRP3* gene, a critical component of the NLRP3 inflammasome, which is a multimolecular complex involved in the processing and release of active IL-1β. Lately, cases of acquired late-onset CAPS (aCAPS) have been characterised, caused by somatic mutations in the *NLRP3* gene (2, 3). One study suggested that myeloid-restricted somatic mutations in *NLRP3* may contribute to the pathogenesis of SchS (4), although a larger study of 21 SchS patients failed to identify any somatic mutations in either *NLRP3* or 31 other genes associated with autoinflammatory disorders(5).

We also postulated that CH, a process associated with aging of the bone marrow and development of MDS, might be a contributory factor in patients with SchS and aCAPS. This consideration is substantiated by frequent findings of inflammatory components in MDS alongside inappropriate NLRP3 inflammasome activation (6). Research has shown MDS-

related somatic mutations in TET-2 and U2AF1, genes encoding for transcription and splicing factors respectively, stimulate the generation of reactive oxygen species (7). In turn, this triggers NLRP3 inflammasome formation, as evaluated by the presence of ASC specks and subsequently pyroptosis – a type of caspase-1 mediated cell death, habitually seen in patients with autoinflammatory conditions (8).

Furthermore, a shrinking pool of hematopoietic stem cells (HSCs) may provide favourable conditions to acquire additional somatic mutations in alternate genes with proinflammatory effects. A particular gene of interest is *MYD88,* coding for an adaptor protein involved in Toll-like Receptor (TLR) and IL-1R signalling pathways (9). Somatically acquired mutation resulting from a single nucleotide variation (SNV); T > C (3:38182641) in exon 5 of *MYD88* results in substitution of leucine to proline at position 265 (L265P), which is located in the Toll/IL-1 Receptor (TIR) domain of the protein. This gain of function (GOF) mutations leads to increased IRAK1 phosphorylation and in B cell derived malignancies promotes NF-κB and JAK–STAT3 signalling to enhance cell survival (9). Interestingly 20% of SchS patients develop a clinically overt lymphoproliferative disorder such as multiple myeloma, marginal zone lymphomas and Waldenström's Macroglobulinemia (WM) (10), in which the somatic MYD88 L265P mutation is considered an independent risk factor and has been reported in over 90% of cases of WM (11). Given its role in driving expression of pro-inflammatory genes downstream of TLRs and IL-R, the presence of the MYD88 L265P variant in myeloid cells could also result in excessive IL-1 release and development of symptoms typical of SchS.

Taking the aforementioned concepts into consideration, we hypothesised that SchS patients harbor the MYD88 L265P mutation, whilst demonstrating evidence of clonality, as a result of a shrinking pool of HSC's.

Patients and Methods

Patients

Thirty SchS patients and 6 CAPs patients were included in the study. Informed consent was provided by all participants in accordance with the Declaration of Helsinki.

NLRP3 variant exclusion from another 11 patients

Primers were designed to encompass the entirety of the *NLRP3* gene, with library preparations generated using the NEBNext DNA Library Prep Kit for Illumina (NEB, USA), according to the manufacturer's instructions. The individually barcoded samples were pooled together and subject to a 150bp paired-end sequencing run on MiSeq (Illumina, UK). Data analysis was carried out using the open access software Galaxy (usegalaxy.org) and Integrated Genomics Viewer (Broad Institute, USA).

ASO-PCR for *MYD88*

Allele Specific Oligonucleotide-PCR (ASO-PCR) was utilised to screen for the single nucleotide variant at chromosomal position 3: 38141150, using *MYD88* reference sequence NM_001172567.1. DNA was extracted from peripheral blood (PB) samples and carried out using two forward primers to discriminate between the wild-type allele (T) and the mutant allele (C), with a common reverse primer. Positive controls down to 1% of the mutant allele and negative controls were included.

MDS targeted panel sequencing

The Fluidigm custom made 48x48 access array was used for Illumina library preparation. Utilising the D3™ Assay Design Service (Fluidigm®, USA), amplicons were designed to encompass the hotspots of 28 genes frequently mutated in myeloid malignancies

(Supplementary table 1). DNA libraries were generated with an initial input of 50–100 ng/µl per sample, and created using the Targeted DNA Seq Library Reagent kit (Fluidigm®, USA). The resulting amplicons were subject to QC before and after ligation of the sequencing adaptor, using the Agilent 2100 Bioanalyser (Agilent, UK). The pooled library was run on the MiSeq (Illumina, UK), on a 150bp paired-end sequencing run. An in-house pipeline was employed to perform initial trimming, read alignment and variant calling.

X inactivation

Inactivation of the X chromosome (XCI) was assessed by determination of the androgen receptor (AR) locus methylation status using the HUMARA assay. PCR amplification of the polymorphic CAG repeat within the AR locus was performed using a 6-FAM labelled primer. Fragment analysis was carried out using the ABI 3100 genetic analyser (Applied Biosystems, UK). The XCI ratios were estimated as previously described (12), and reported as a percentage: <80% is considered random X inactivation, and percentages >80% were deemed as skewed.

Results

NLRP3 exclusion from another 11 patients

Deep sequencing of the *NLRP3* gene in 11 SchS patients (Patients 1-11; Table 1) did not identify any somatic variants.

ASO PCR

The notion that the activating somatically acquired MYD88 L265P variant could be present in SchS patients was first postulated by de Koning in 2014 (13), therefore we hypothesized that MYD88 L265P expressed in myeloid cells could explain the IL-1β-dependent character of SchS. Significantly, this variant was detected in PB of 9/30 SchS patients by ASO-PCR technique, which has detection sensitivity down to 1% of the mutant allele, and thus can detect mutations in circulating myeloid cells. This technique is highly utilised for the diagnostic assessment of WM and other B cell chronic lymphoproliferative disorders across laboratories worldwide (11, 14, 15).

MDS Panel

We postulated that OH could be a predisposing factor for developing further somatic mutations associated with inflammatory conditions. We sequenced PB derived DNA from 30 SchS and 6 aCAPS patients, who were included as disease controls, since these were known to harbour somatic mutations in *NLRP3.* We studied a panel of 28 genes associated with development of clonal haematopoiesis (CH) and MDS. Solely SchS patient 20 (Table 1) had a nonsense mutation in *STAG2* (c.559C>T p.Gln187), with a low variant allele fraction (VAF) of

0.081. In isolation, the latter may represent a CHIP (clonal haematopoiesis of indeterminate potential) mutation. Of the aCAPS cases however, one patient had three pathogenic variants. One mutation in *DNMT3-A* (c.2645G>A p.Arg882His), with two mutations in *TET-2* (c.4585C>T p.Gln1529; p.Gln1699*), all with a significant VAF (0.407, 0.388 and 0.402 respectively).

X inactivation

We used the HUMARA assay to identify X-allelic skewing in female patients, indicative of CH. Busque et al (16), first determined that CH is selectively enriched in women with *TET-2* mutation and one in seven subjects with CH and a *TET-2* mutation developed a haematological malignancy; corroborated by data demonstrating that allelic skewing was more pronounced in samples with somatic mutations (17), typical of OH. This agrees with our observations in aCAPS, as allelic skewing was found in the patient with 3 significant mutations. However, only one SchS patient showed non-random X inactivation, and in this patient the 28 gene panel did not identify any genetic variants. This suggests that in this patient the genetic change underlying the clonal process is not targeted by this panel. Unfortunately, skewing could not be investigated in the patient with the *STAG2* mutation due to the limitations of the HUMARA technique.

Discussion

The emergence of aCAPS demonstrated that low level somatic mutations in *NLRP3* can lead to development of acquired SAID, but this is not the case for SchS. Through sequencing the entirety of the *NLRP3* gene, we excluded the notion that *NLRP3* mutations are responsible for SchS pathology, strengthening our previous findings. Including the 21 patients we have reported previously (5), we have now excluded somatic *NLRP3* mutations in 32 SchS patients. Nevertheless, NLRP3 activation is still likely play a part in the aetiology of SchS, given previous data establishing elevated levels of ASC aggregates and IL-18 in SchS patients. Similar quantities were found in CAPS patients, not only but reinforcing the similar pathology between the two diseases but highly indicative of inflammasome activation (5).

In addition, we found that SchS patients have similar frequency of the somatic MYD88 L265P variant in PB as do patients with WM (18), a condition which is not associated with the inflammatory complications seen in SchS. Given that 20% of SchS patients develop WM, and more than 90% of the latter cohort harbour the MYD88 L265P mutation, we sought to seek evidence of this within our cohort. Demonstrating that a third of SchS patients bear this mutation could partly explain the clinical phenotype of systemic inflammation and neutrophilic urticarial dermatosis (NUD), with this novel finding in parallel with the frequency of those SchS patients who go onto develop B cell malignancy. Having not investigated bone marrow-derived DNA we cannot categorically exclude the presence of the MYD88 variant in mutation negative cases. Certainly, in cases where SchS is suspected, screening of this mutation could corroborate a diagnosis, using either ASO-PCR or Sanger sequencing (19). A de-novo germline GOF *MYD88* mutation (c.666T>G, p.Ser222Arg) has recently been reported as the cause of severe arthritis, intermittent fever and rash(20).

Although the rash in this case was different from typical NUD other symptoms are seen frequently in SchS and related SAID. It is possible that distinct clinical phenotypes associated with *MYD88* variants are in part dependent on a type of inheritance and cell-specific function of mutated protein. taking this into account it is therefore plausible to consider the occurrence of other variants in the *MYD88* gene in regards to SchS patients.

Our basis for investigating the mutational status of 28 genes associated with MDS was founded by several studies implicating the NLRP3 inflammasome responsible for directing CH and pyroptosis, specifically within MDS as compared to other bone-marrow malignancies(6). Thus, for SchS patients, harboring initiating mutations in MDS related genes would predispose to a generally pro-inflammatory environment, leading to the development of additional SAID-associated somatic mutations. However, our findings did not support this hypothesis. On average, 1.3 ±0.2 somatic mutations are acquired per hematopoietic stem cell (HSC) per decade and it has been demonstrated that age related hematopoietic clones are a common finding in individuals over the age of 55-65 years (21). Considering the average age in our patient cohorts was 55 years, and the fact that only 2 patients were found to have the initiating mutations and non-random X inactivation, it is likely these are age-related incidental findings, rather than specifically contributing to SchS or aCAPS pathogenesis.

In view of our results, it is possible that the biology of the SchS clonal cells has no substantial bearing on the development of inflammatory disease. However, it remains plausible to consider that an inherent pro-inflammatory property of the monoclonal protein, which is different in every patient with IgM MGUS, is more likely to be related to the aetiology of inflammation.

Study limitations

In an era where whole-exome/genome sequencing (WES/WGS) is considered as the gold standard - cost, timing and applicability to this disease cohort had to be considered. Screening a total of over 60 genes associated with auto inflammation and MDS over the past few years and given the apparent heterogeneity of SchS, it is plausible to consider that WES/WGS would detect various mutations, a majority of which are likely to be unrelated to SchS. Furthermore, SchS patients have very low levels of monotypic B cells in the marrow, and therefore may have a lower probability of circulating B cell clones. Thus, we cannot exclude the possibility that the MYD88 L265P 'mutation negative' patients do not harbor this mutation in their B cells.

Conclusions/Future directions

Our study has revealed the presence of the activating MYD88 L265P mutation in a third of SchS patients, the most common genetic factor found within SchS patients till date. Although this finding provides clues to the pathogenesis of this hetereogenetic disease, a unifying mechanism tying all SchS patients together remains to be discovered. In this context, research efforts should be geared towards characterising the link between the innate and adaptive immune networks.

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Authorship Contributions

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Savic had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design

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Disclosure of Conflicts of Interest

There is no other financial support or other benefits from commercial sources for the work reported on in this manuscript, or any other financial interests that any of the authors may have, which could create a potential conflict of interest or the appearance of a conflict of interest with regard to the work.

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Patient number	Sex	Age at symptom onset	Para-protein	IgMk para-protein levels (g/l)	Response to IL-1 inhibition	Bone marrow histology	Genes/Variants identified by MDS panel Prediction/VAF	*MYD88* L265P?	*NLRP3* mutational status	X inactivation result (if applicable)
1	male	62	IgMk	6	complete	No overt LPL		Yes		-
2	female	43.5	IgMk	17	good	No overt LPL		Yes		random
3	male	51.5	IgMk	8	good	No overt LPL		Yes		-
4	male	67	IgMk	8	complete	No overt LPL		Yes		-
5	female	54.5	IgMk	3	good	No overt LPL		No		random
6	female	72.5	IgMk	11	complete	No overt LPL		No		random
7	female	55.5	IgMk	5	complete	No overt LPL		No		random
8	female	53	IgMk	5	complete	No overt LPL		No		random
9	female	60	IgGk	N/A	good	Low-grade myeloma		No		non-random - skewed (80%)
10	female	72.5	IgMk	3	good	No overt LPL		No	Q703K*	-
11	male	62	IgMk	3	good	No overt LPL		Yes		-
12	male	36.8	IgMk	3	Partial	No overt LPL		No		-
13	female	37.9	IgMk	3	complete	No overt LPL		No		random
14	male	43.9	IgGλ	N/A	complete	15% BM plasma cell		No		-
15	female	44.8	IgMk	1	complete	No overt LPL		No		random
16	female	49.6	IgMk	4	complete	not done		No		random
17	male	49.9	IgMk	9	complete	No overt LPL		No		-
18	male	52.8	IgMλ (IF)	N/A	complete	No overt LPL		No		-
19	male	57.1	IgMk	7	complete	LPL		Yes	V198M*	-
20	male	58.1	IgMk	3	complete	LPL	*STAG-2* c.559C>T p.Gln187* Predicted: pathogenic VAF: 0.081	No		-
21	male	59.6	IgMλ	7	died before treatment	No overt LPL		No		-
22	male	61.7	IgMκ	5	complete	No overt LPL		No		-
23	female	60.7	IgMk	9	complete	low grade marginal zone lymphoma		No		random
24	female	68.4	IgMk	8	complete	No overt LPL		No		random
25	male	78.9	IgMk	7	complete	No overt LPL		Yes		-
26	female	39.7	IgMk	16	complete	No overt LPL		No		random
27	male	40.7	IgMk	4	complete	No overt LPL		No		-
28	male	61.2	IgMk	8	complete	No overt LPL		No		-
29	male	43.6	IgMk	6	complete	MGUS		Yes		-
30	female	59	IgMk	5	complete	Marginal Zone Lymphoma		Yes		random
CAPS - 1	male	48	N/A	N/A	complete	N/A		No	E567K	-
CAPS - 2	male	79	N/A	N/A	complete	N/A		No	G569V	-
CAPS - 3	female	67	N/A	N/A	complete	N/A	*DNMT3-A* c.2645G>A p.Arg882His Predicted: pathogenic VAF: 0.407 *TET-2* c.4585C>T p.Gln1529* Predicted: pathogenic VAF:0.388 *TET-2* p.Gln1699* Predicted: pathogenic VAF: 0.402	No	A352T	non-random – skewed (80%)
CAPS - 4	male	63	N/A	N/A	complete	N/A		No	Y563C	-
CAPS – 5	female	62	N/A	N/A	complete	N/A		No	Y563C	random
CAPS – 6	female		N/A	N/A	complete	N/A		No	Y536C	random

Table 1: Clinical and laboratory findings of 30 SchS patients and 6 CAPS patients LPL = lymphoproliferative lymphoma IF: immunofluorescence VUS: variant of unknown significance

*common non-pathogenic *NLRP3* variants unlikely to be disease causative and have shown to be present in healthy individuals