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Early View

Research letter

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Exome sequencing and pathogenicity-network analysis of 5 French families implicate mTOR signalling and autophagy in familial sarcoidosis

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To the Editor,

Sarcoidosis is a complex disease characterised by the presence of epithelioid non-caseating granulomatous inflammation affecting multiple organs and whose aetiology has been related to microbial, environmental and genetic factors [1, 2]. However, no single antigenic trigger has been identified, although associations with *Propionibacterium acnes* and mycobacteria pathogen-associated molecular pattern (PAMP), deposition of serum amyloid A (SAA), and exposure to inorganic particles and insecticides have been suggested [3–5]. Occurrence of familial cases suggests that genetic variation contributes to disease pathogenesis with a heritability of about 39% [6]. Despite intensive genome-wide association studies (GWAS), no single nucleotide polymorphism (SNP) is as yet able to explain the “missing heritability” in the disease, especially for non-resolving, non-Lofgren’s syndrome (non-LS) sarcoidosis [5–7].

To overcome the issue of missing heritability in multifactorial diseases, different strategies to unravel the genetic complexity have been proposed. These include 1) a select focus on sequencing high susceptibility individuals with young age-at-onset, or with positive family history [8], 2) analysing a cohort of stringent, well-phenotyped families with accessible sibships, 3) pooling of rare variants for

analysis, and 4) elucidating the gene regulatory network of identified disease-genes by functional pathway gene analyses [7, 9]. Here, we incorporated the above-mentioned strategic guidelines and genotyped by whole exome sequencing (WES) a cohort of five clinically-screened families with non-LS sarcoidosis. Clinical diagnosis criteria used were according to ATS/ERS/WASOG guidelines [10] and diagnosis of sarcoidosis assessed in all cases based on histological confirmation of either lymph nodes and/or bronchial biopsies. Families F1 to F5 were collected through the national SARCFAM observational project, whose primary objective was to establish a clinical and genetic database on sporadic and familial sarcoidosis [8]. The 5 families, presenting an autosomal dominant pattern of inheritance, included a total of 14 affected individuals together with 8 family-matched non-affected first degree relatives (**Figure 1a**), which allowed a genotype comparison between affected cases and healthy controls within each family. Such a genomic subtraction between cases and internal controls of the same family would allow genetic triggers associated with the inherited predisposition to sarcoidosis to be highlighted; and assuming or considering an autosomal mode of transmission, select for highly penetrant variants/genes.

Next, we sought to pool susceptibility variants prioritized from a series of functional prediction software programs to accentuate the cumulative deleterious effects of variants found only in probands but not in internal healthy controls. Thus, variants subtracted between affected family members and healthy family-internal controls were selected when they met one of the following criteria: minor allele frequency (MAF) lower than 0.05 (5%), and a pathogenic score either by SIFT (< 0.05), POLYPHENv2 (> 0.400), Mutation Taster and Alamut Visual Interactive Biosoftware©. The variants per family were then pooled together from all 5 families, which resulted in a total of 227 disease susceptibility variants in 192 genes, including 223 (88.9%) missense variants, 2 (0.7%) splicing sites, 9 (3.6%) in-frame deletion / insertion, 1 (0.3%) % start-stop, and 8 (3.2%) nonsense variants.

To control for variant classification and annotation bias, we used Variant Effect Predictor to determine the functional effect of the 227 identified susceptibility variants on transcripts curated in Ensembl's database (http://www.ensembl.org/) mapped 20,000 bp upstream and downstream in the Ensembl human GRCh38 Assembly database. We then took all the HUGO gene identifiers encoding the affected transcripts and mapped them to pathway gene sets curated in the WikiPathway 2016 (https://www.wikipathways.org/) database. This resulted in enrichment of transcripts in various pathways, with TOR signaling (*DDIT4*, *MLST8*, *DDIT4L*, *MTOR*) identified as the top enriched pathway (**Figure 1b**). To further increase the robustness of our enrichment analysis, we performed an additional gene set over-representation pathway analysis using ConsensusPathDB, a meta-database that integrates different types of functional interactions from multiple data resources [11]. From this analysis, autophagy was found to be the top pathway enriched amongst the transcripts affected by

the selected variants (**Figure 1b**). This was followed by Phenyl ethylamine degradation, and Target Of Rapamycin (TOR) Signaling, the top enriched pathway when mapped to WikiPathway database alone.

Despite the differences in the study design approach, our results partially overlap with the observations from 6 German families with sarcoidosis with no internal familial controls and with our previous study [12, 13]. These include rare variants/genes that are implicated in calcium metabolism (*ANXA5*, *CDH23*, *DMXL2*, *TRPV2*), and those involved in cell growth, survival and migration (such as *PTPRD*, *FAT* atypical cadherins and *KIF*), highlighting the putative role of such processes in sarcoidosis.

In this study, pooling of *in silico* selected susceptibility variants derived from genomic subtraction of proband cases from matched internal controls in 5 French families (with at least two first degree relatives) additionally led to the identification of genes enriched in the regulation of mTOR signaling pathway and autophagy. Previously, Linke et al. described a mouse model where the deletion of the upstream mTORC1-inhibitor *TSC2* in macrophages led to spontaneous development of granulomas; and also showed that sarcoid granulomas from progressive-disease patients have active mTORC1 signaling [14]. Recently, mTORC1 signaling involvement was also identified in RNA-seq gene set enrichment data from a cutaneous sarcoidosis patients [15]. Moreover, successful treatment of a sarcoidosis patient with the mTOR inhibitor rapamycin was reported previously [16]. Here, we implicate a role of mTOR signalling in familial cases of sarcoidosis. As mTORC1 is a negative regulator of autophagy, it is particularly interesting that patients in predisposed families share mutations in genes involved in autophagy and intracellular vesicular transport, such as *ATG9B* and *SEC31B*, suggesting that cumulative defects in autophagy regulation may play a key role in sarcoidosis pathogenesis. In a previous study, we showed that some of the genes associated with mTOR regulation were also direct regulators of Rac1's functional hub, an important Rho GTPase in actin remodelling and cellular mobility and inhibiting the mTOR complex [17].

One limitation of this study is that healthy relatives in families might develop sarcoidosis later in life, even if onset of the disease in familial cases is usually early. Secondly, we are aware that we have only investigated 5 families in this study. Future studies should perform large-scale deep sequencing, and correlate the genotypes to disease phenotypes [18] using a similar approach as we propose here, and identify molecular targets useful for the therapeutic management of sarcoidosis patients. While we acknowledge the descriptive nature of this study, this work represents the first time to our knowledge that a whole exome sequencing study included internal familial controls in the study cohort, focused only on a subtype of patients (non-LF sarcoidosis), pooled low frequency and rare variants identified as deleterious from multiple software packages, and derived gene pathways *de*

novo enriched from the cohort's identified variants, without comparison to known sarcoidosis candidate genes. These are study design strategies, which we put forward for future next generation sequencing studies investigating familial sarcoidosis to consider.

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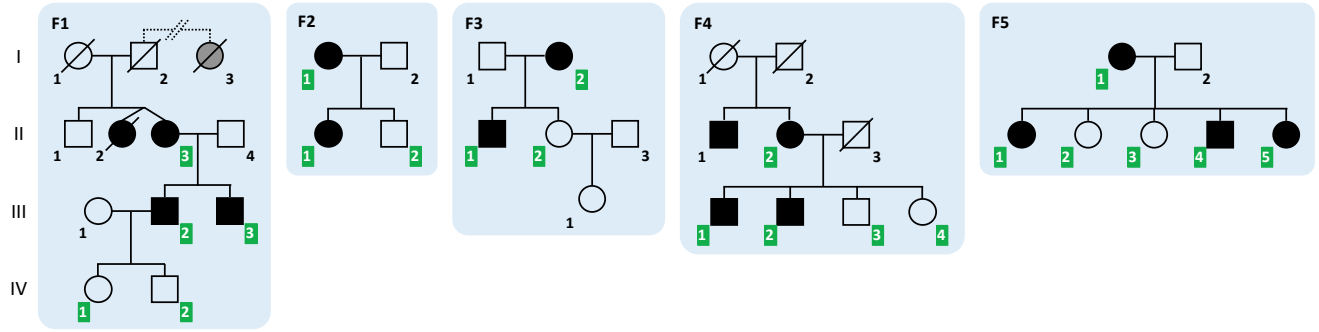
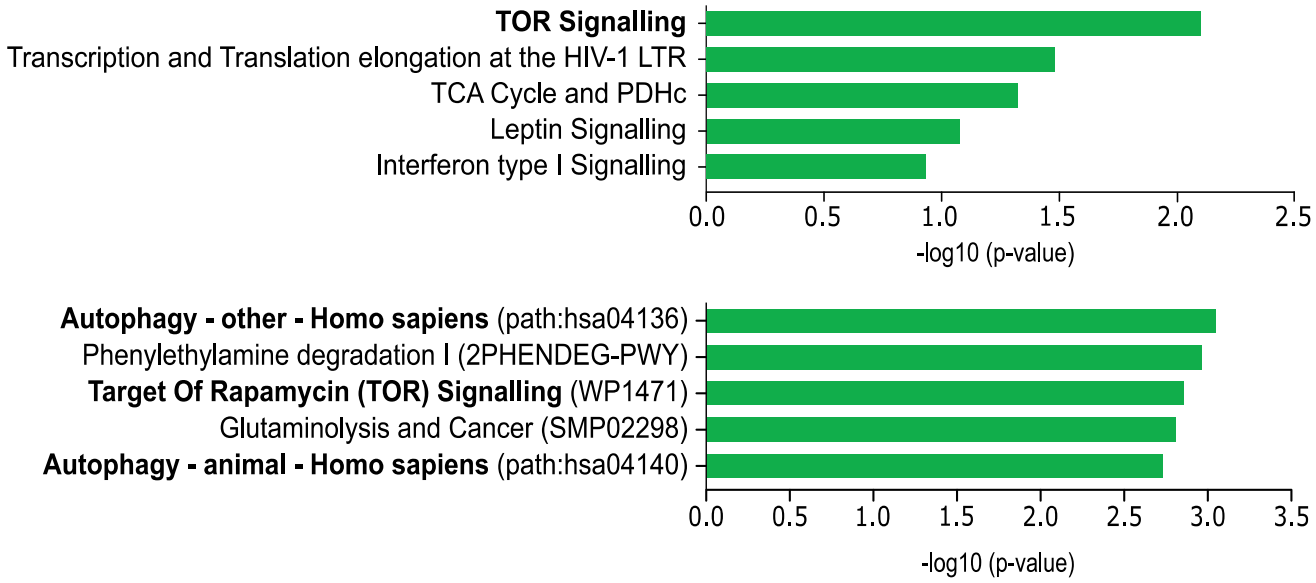
Figure legend

a) Families (F1to F5), with a total of 14 affected (filled symbols) and 8 non-affected (clear symbols) individuals were analysed. The labelled green squares indicate the analysed individuals. All protocols involving study participants outlined in this manuscript have been approved by the French CCP LYON SUD EST-2 Ethical Committee (REF IRB 00009118). All participants provided written consent and gave permission to use their DNA for research purposes. b) Exome sequencing and variant calling were performed as described previously [12]. Two independent NGS sequencing rounds were performed on the whole series (F1 to F5) and only the variants identified recurrently were kept. Gene identifiers from the selected 227 variants derived by the Ensembl Variant Effect Predictor were then used for gene set over-representation pathway analyses. Gene identifiers were mapped to the top 5 ranked pathway gene sets found in WikiPathways 2016, and ConsensusPathDB.

a)


F1
F2
F3
F4
F5
I
II
III
IV

b)


TOR Signalling
Transcription and Translation elongation at the HIV-1 LTR
TCA Cycle and PDHc
Leptin Signalling
Interferon type I Signalling
0.0
0.5
1.0
1.5
2.0
2.5
-log10 (p-value)
Autophagy - other - Homo sapiens (path:hsa04136)
Phenylethylamine degradation I (2PHENDEG-PWY)
Target Of Rapamycin (TOR) Signalling (WP1471)
Glutaminolysis and Cancer (SMP02298)
Autophagy - animal - Homo sapiens (path:hsa04140)
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
-log10 (p-value)