The role of chitotriosidase duplication gene polymorphism in the susceptibility to sarcoidosis

Vloga duplikacijskega polimorfizma v genu za hitotriozidazo pri sarkoidozi

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Abstract

Background: Sarcoidosis is a systemic granulomatous disease that most commonly affects respiratory system, and may present with an acute onset or follow a chronic course. Sarcoidosis has an established genetic component, supported by evidence from familial, population and linkage genetic studies. Several attempts at discerning the exact genetic background of sarcoidosis have produced mostly inconclusive results. Recently, the roles of fungal infection and exposure to fungal antigens have been implicated in sarcoidosis pathogenesis. A mammalian form of chitinolytic enzyme – chitotriosidase has been discovered, and it has been hypothesised to play a role in defense against chitin-containing pathogens and innate immunity. A 24 base pair duplication polymorphism in the chitotriosidase gene (CHIT1) has been described, causing abnormal mRNA splicing patterns, leading to enzymatically inactive chitotriosidase.

Methods: An association study has been performed, comparing frequencies of duplication polymorphism in groups of 159 Slovenian patients with sarcoidosis and 272 healthy controls. Genotyping was performed by polymerase chain reaction (PCR), followed by size discrimination of PCR products with agarose gel electrophoresis. Statistical analyses have been performed using Pearson's Chi-Square test.

Results: Our results did not show significant association of 24bp duplication polymorphism in CHIT1 gene with the susceptibility to sarcoidosis.

Conclusions: In our study, we were unable to demonstrate a significant effect of duplication polymorphic variation in the CHIT1 gene on the susceptibility to sarcoidosis. However, other polymorphisms in CHIT1 gene should be investigated and additional studies performed in other genetically distinct populations.

Izvleček

Izhodišča: Sarkoidoza je sistemska granulomatozna bolezen, ki najpogosteje prizadene dihala. Lahko poteka v akutni ali kronični obliki. Dokazi za genetsko ozadje sarkoidoze izvirajo iz več vrst študij, ki so pokazale povečano pogostost bolezni v družinah bolnikov, razlike v prevalenci, poteku in resnosti bolezni med predstavniki različnih rasnih skupin in povečano sopojavljanje bolezni pri enojajčnih dvojčkih. Prevladuje mnenje, da se sarkoidoza razvije kot posledica medsebojnega delovanja številnih okoljskih dejavnikov na dedno obremenjenega posameznika. Številni poskusi iskanja genov, ki sodelujejo pri genetsko pogojenem ozadju sarkoidoze, nam niso dali enovitih rezultatov. Do danes tako še ne poznamo povsem, kateri geni vplivajo na dedno nagnjenost k sarkoidozi.

V zadnjem času se kot možni patogenetski mehanizem za nastanek sarkoidoze uveljavlja teorija, da gre za okužbo z glivami in stik z antigeni celične stene gliv. V podporo tej teoriji so nedavno ugotovili povišane ravni encima hitotriozidaze v serumu in bronhoalveolnem izpirku (BAL) bolnikov. Encim hitotriozidaza razgrajuje hitin in mu pripisujejo pomen pri obrambi pred boleznimi, ki jih povzročajo organizmi, ki vsebujejo hitin. V genu, ki nosi zapis za hitotriozidazo (CHIT1), je opisan polimorfizem – 24 baznih parov dolga duplikacija, ki povzroči moteno izrezovanje intronov iz prepisov mRNA, kar vodi v izgubo 29 aminokislin iz končnega encima ter okvari njegovo funkcijo. To duplikacijo so že povezali z dovzetnostjo za okužbe s filarijami, z malarijo in bakteriemijo pri bolnikih z akutno mieloično levkemijo. Pogostost homozigotov za to duplikacijo, ki pomeni popolno odsotnost hitinolitične funkcije hitotriozidaze, v populaciji belcev ocenjujejo na približno 5 %.

Metode: Opravili smo asociacijsko študijo, v kateri smo primerjali pogostost duplikacije med

skupinama 159 slovenskih bolnikov s sarkoidozo in 272 zdravih krvodajalcev. Genotipizacijo smo opravili s polimerazno verižno reakcijo (PCR), tej pa je sledilo ločevanje različno velikih produktov reakcije na agaroznem gelu. Statistične analize smo opravili s pomočjo Pearsonovega testa hi-kvadrat.

Rezultati: Rezultati naše študije niso pokazali statistično značilne povezanosti med prisotnostjo duplikacije v genu CHIT1 in pojavnostjo sarkoidoze pri preiskovancih.

Zaključki: V naši študiji nismo uspeli dokazati povezave med duplikacijskim polimorfizmom v genu CHIT1 in pojavnostjo sarkoidoze. Pred izključitvijo vloge omenjenega polimorfizma moramo preiskati obstoj morebitne povezave tudi pri predstavnikih drugih rasnih in etničnih skupin. Izpostaviti je potrebno, da obstajajo v genu CHIT1 tudi drugi polimorfizmi, ki bi lahko vplivali na pojavnost sarkoidoze, kar prav tako zahteva nadaljnje preiskave pred izključitvijo njegove vloge pri nastanku sarkoidoze.

Introduction

Sarcoidosis is a multisystemic granulomatous disorder, most commonly affecting respiratory system in the forms of bilateral hilar lymphadenopathy and lung parenchymal involvement. Other organ systems are also commonly involved–most notably the eye, skin, cardiac muscle and the nervous system. Sarcoidosis may present as an acute form, which is commonly characterised by self-limiting course, or as a slowly progressing chronic form that can result in relentless fibrosis of the lung and organ systems involved. It has been estimated that 10–20 % of patients with sarcoidosis suffer from permanent sequelae in pulmonary or extrapulmonary systems, and 1–5 % mortality rates due to respiratory insufficiency, central nervous system and cardiac involvement have been associated with sarcoidosis.[1,2]

The aetiology of sarcoidosis is unknown at present.[1] However, various studies suggest that sarcoidosis occurs as a result of the action of several inciting environmental factors on a genetically susceptible individual.[2] Several lines of evidence support the genetic background of sarcoidosis – familial aggregation of cases with sarcoidosis, differences in sarcoidosis prevalence among racial groups and increased disease state concordance in identical twins.[3] Due to the complex nature of sarcoidosis genetic background, there have been several difficulties in the process of studying specific genetic factors conferring susceptibility to sarcoidosis and the results were mostly inconsistent across different genetic studies.[3] Therefore, the need to further characterize its genetic background remains in place.

Recently, it has been hypothesised that exposure to fungal infection and fungal antigens may play a significant role in sarcoidosis pathogenesis.[4] In accordance with this hypothesis, it has been noted that levels of chitotriosidase, a mammalian form of chitinolytic enzyme, are significantly increased in sarcoidosis.[5-7] Chitotriosidase has been implicated in the defence against chitin-containing pathogens, such as fungi, nematodes and insects.[8,9] The dysfunction of this pathway may therefore alter natural defence against fungal infection, cause persistence of fungal infection and prolonged contact of an individual with triggering fungal antigens, consequently affecting susceptibility to sarcoidosis.

The gene coding for human chitotriosidase (CHIT1) is located on chromosome 1q31-q32, and several genetic polymorphisms have been found in this region.[10] A 24 basepair duplication in exon 10 of CHIT1 gene has been described.[11] The duplication introduces a cryptic splice site into CHIT1 gene coding region, causing in-frame deletion of 87 nucleotides and subsequent loss of 29 amino acid residues from chitotriosidase enzyme, causing functional inactivation of its chitinolytic activity.[11] This deficiency is inherited in recessive manner and it is estimated that roughly 5 % of Caucasians are homozygous for this duplication.[9] It has been shown that CHIT1 duplication polymorphism is associated with susceptibility to filarial infection, malaria and Gram-negative bacteraemia in children with acute myeloid leukaemia.[12-14]

We therefore aimed to investigate whether CHIT1 duplication polymorphism plays a role in the genetic susceptibility to sarcoidosis.

Patients and Methods

Subjects

Subjects diagnosed with sarcoidosis were recruited from an ongoing sarcoidosis registry started in year 2000 at the University Medical Centre Ljubljana, Department of Pulmology and Allergic diseases. Diagnosis of sarcoidosis was based on the clinical picture, radiographic presentation, bronchoalveolar lavage (BAL) and biopsy specimens from the lung, skin or lymph nodes after other granulomatous diseases had been excluded. A hundred and fifty-nine patients were included in the study. There were 67 males (42.1 %) and 92 females (57.9 %), their mean age being 42 at the time of diagnosis with a standard deviation of 13.3 years. The patients were followed up from 3 to10 years after diagnosis confirmation. The clinical presentation at diagnosis is presented in Table 1, taking into consideration the standard classification system.[1]

The control groups consisted of 272 age and sex matched healthy blood donors, 112 males (41.2 %) and 150 females (58.8 %), their mean age being 42.5 years with a standard deviation of 11 years. All patient and control subjects were Slovenian blood donors, not related to each other.

All subjects participated in the study after they had given their full informed consent. Study was approved by the National Ethical Committee.

Genotyping

After isolating DNA from blood leukocytes by standard protocols, genotyping of 24bp CHIT1 duplication was performed as described previously, by polymerase chain reaction with primers aligned around the duplication region, resulting in 24 base pairs longer amplicon length where duplication was present. The final reaction mix consisted of: 1× PCR buffer, 0.2mM dNTP, 2.0 mM MgCl2, 500 nM concentration of each primer, 0.2 units of Tfi polymerase (Invitrogen, Carlsbad, CA, USA), bidestilated H2O and 500ng of DNA. Primer sequences were:

Table 1: Patients with sarcoidosis; clinical presentation.

Clinical characteristics	No. of patients
Pulmonary lymph nodes	143
Lung interstitium	131
Radiologic stage:	
Stage I	26
Stage II	101
Stage III	26
Stage IV	2
Different types of skin involvement	47
Löfgren's syndrome	28
Arthralgias	27
Extrapulmonary node involvement	16
Salivary glands involvement	6
Involvement of parenchymal organs (liver,spleen,kidney or heart)	26
Different types of neural involvement	14

CHIT1-F: 5'AGCTATCTGAAGCAGAAG3'; CHIT1-R: 5'GGAGAAGCCGGCAAAGTC 3'.

The protocol of PCR amplification was as follows: initial denaturation at 94°C for 3 min, then 35 cycles of denaturation at 95°C for 30 sec, annealing at 56°C for 30 sec and elongation at 72°C for 30 sec, and final extension at 72°C for 5 min.

Differences of amplicon length were inspected after performing electrophoresis on 3 % agarose gel stained with SYBRSafe® DNA Gel stain (Invitrogen, Carlsbad, CA, USA).

Statistical analyses

The significance of association was analyzed using the Pearson's Chi-Square test (χ^2). Odds ratios (OR) and their respective 95 % confidence intervals (CI) were calculated to compare allelic and genotype distribution in patients and control subjects. Statistical analyses were computed using R statistical package, available at *http://www.r-project.org*. χ^2 goodness-of-fit tests for deviation from Hardy-Weinberg equilibrium were calculated. Tests of statistical power were performed using *gap* (Genetic analysis package) for R available from *http://cran.r-project.org/web/packages/gap/*. Associations were regarded as significant when they reached the p value of equal to or less than 0.05.

Results

Comparison of allele and genotype distributions at 24bp CHIT1 duplication polymorphism in the groups of sarcoidosis cases and controls revealed no significant association of particular allele or genotype with sarcoidosis. Results of genotyping at 24bp CHIT1 polymorphism are presented in Table 2.

Calculation of statistical power revealed that in our group of 159 cases and 272 controls, considering frequency of risk allele 24.5 % and frequency of sarcoidosis in general population estimated at 0.2 %, we reached a power of 80 % to detect relative genotype risk of approximately 4.5 under a recessive genetic model. Genotype frequencies in our study sample did not differ significantly from the frequencies predicted by calculation of Hardy-Weinberg equilibrium in our group of cases (p=0.70) and controls (p=0.28).

Subgroup stratification by disease characteristics revealed no association with particular disease features of our group of patients.

Discussion and Conclusions

In our study we have failed to demonstrate the association of 24bp duplication in the CHIT1 gene with susceptibility to sarcoidosis. Additionally, no association of variants at this polymorphism with sarcoid-

Table 2: Allele and genotype frequencies at 24bp duplication polymorphism in sarcoidosis cases and controls.

Allele/Genotype		Sarcoidosis n (%)	Controls n (%)	OR (95 % CI)	p
Allele	N*	246 (77.4)	420 (77.2)	1.01 (0.73 to 1.40)	1.00
	dup**	72 (22.6)	124 (22.8)		
	Total	318 (100.0)	544 (100.0)		
Genotype	N/N	96 (60.4)	159 (58.5)	dup/dup vs N/N + N/dup 0.70 (0.29 to 1.73)	0.44
	N/dup	54 (34.0)	102 (37.5)		
	dup/dup	9 (5.7)	11 (4.0)		
	Total	159 (100.0)	272 (100.0)		

** N signifies presence of normal allele at CHIT1 polymorphic site*
*** dup signifies presence of duplication at CHIT1 polymorphic site*

osis course, staging, Löfgren syndrome and particular organ system involvement could be demonstrated.

Fungal infection has been hypothesised to play a significant role in sarcoidosis pathogenesis. In this view, fungal cell wall antigens could act as a trigger for an overactive immune response in a genetically susceptible host.[4] Epidemiological studies have shown that increased occurrence of sarcoidosis is associated with employment in environments with mold exposure as well as living in environment with high humidity–also indirectly implying increased mold exposure.[1] Further evidence has been contributed by clinical studies, showing that the addition of antifungal treatment to corticosteroid regimen significantly benefited sarcoidosis patients, with improvements in symptoms, diffusion capacity and X-ray stages in comparison to patients treated exclusively with corticosteroids.[15]

Additional line of evidence to the hypothesis of fungal antigen trigger came from studies reporting elevated levels of chitotriosidase in sarcoidosis.[5,7] Chitotriosidase is a mammalian form of chitinolytic enzyme, involved in the defence against pathogens containing chitin, such as fungi, nematodes and insects.[8] Chitotriosidase has been used as a marker of disease activity in patients with Gaucher I disease, but the activity of this enzyme also increases in the serum of patients with other lysosomal storage diseases, acute ischemic stroke, thalassemia and infections, such as acute Plasmodium falciparum malaria and visceral leishmaniasis.[9]

It is not clear what causes increased levels of chitotriosidase in sarcoidosis. It is thought to be a nonspecific marker of macrophage activation and a marker of chronically activated tissue macrophages.[8] Interestingly though, it was shown to be a relatively specific marker of sarcoidosis activity, in comparison to other granulomatous and interstitial lung disease, where chitotriosidase activity is predominantly unchanged.[16,17] It remains to be ascertained whether an increase in chitotriosidase levels in sarcoidosis is attributable to compensatory rise in its excretion due to putative exposure to environmental agents or this phenomenon is a consequence of increased macrophage burden encountered in sarcoidosis.

Our results show that 5.7 % and 4 % of sarcoidosis cases and control subjects, respectively, have a genetically inherited deficiency of chitotriosidase enzyme. However difference in the proportion of subjects homozygous for mutant allele did not reach statistical significance when comparing case and control groups. The proportion of homozygous carriers in our study group is comparable to results from similar studies on healthy individuals from Dutch, Spanish, Sicilian and Ashkenazi Jewish populations.[9] Interestingly, African populations from Burkina Faso and Benin were characterised by greatly reduced frequency of chitotriosidase deficient individuals, which could, in part, be reflected by different course of sarcoidosis observed in this population.[12] In contrast, Asian population, characterised by a lower frequency of sarcoidosis, has a significantly hi frequency of 24bp mutant allele.[18,19]

The relatively large proportion of individuals with inherited defect of chitotriosidase may lead us to believe that this enzyme is redundant in humans. However, functional studies and previously reported positive genetic associations of 24bp CHIT1 duplication with susceptibility to various human diseases, speak against this argument.[12-14] It is possible that chitotriosidase function is redundant in the sense that when its activity is perturbed, other functionally related enzymes substitute for its function. Recently, one such enzyme – acidic chitinase, has been isolated and described.[20,21] Therefore, complete characterization of genes in this pathway is necessary in further studies on this topic. It is necessary to note that 24bp duplication investigated in this study is not the only polymorphism in the CHIT1 gene region. Other significant polymorphisms in CHIT1 region have been described, notably Gly102Ser and Ala442Gly polymorphisms and several others that could also affect chitotriosidase function and sarcoidosis susceptibility.[22]

In our study, we were unable to demonstrate a significant effect of duplication polymorphic variation in the CHIT1 gene on the

susceptibility to sarcoidosis. However, other polymorphisms in CHIT1 gene should be investigated and additional studies performed in other genetically distinct populations.

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