

## Original Article

# The role of the A→C395 IFNGR1 mutation in determining susceptibility to intracellular infection in Malta

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## Abstract

**Background:** The first human mycobacterial susceptibility gene was identified amongst four children on the island of Malta in 1995. All affected children were homozygous for a nonsense mutation at position 395 of the interferon gamma receptor 1 (IFNGR1) gene, and all but one died of overwhelming mycobacterial infection.

The population of Malta has high rates of infection with intracellular pathogens; leishmania, brucellosis and tuberculosis are all endemic, while leprosy, which was previously endemic, has only recently been eradicated. We hypothesised that heterozygous carriers of the IFNGR1 gene mutation, while resistant to infection with poorly pathogenic organisms, may have increased susceptibility to infection with more virulent pathogens.

**Methodology and Result:** Screening patients with a past history of intracellular infection and healthy newborns for the presence of the IFNGR1 A→C395 mutation, using sequence specific primer PCR, did not identify any carriers of the mutation.

**Conclusion:** These results suggest that the IFNGR1 mutation is unlikely to be of public health significance on Malta.

## Introduction

The identification of the first human mycobacterial susceptibility gene in 1995 provided a major breakthrough in our understanding of the mechanism of mycobacterial infection.<sup>1,2</sup> In particular, it highlighted the critical role of the interleukin-12/interferon gamma (IL-12/IFN $\gamma$ ) pathway in the control of intracellular infection.<sup>3</sup> The study of patients with genetic mutations that selectively increase susceptibility to infection with mycobacteria, and, less commonly, salmonella has dramatically increased our understanding of the mechanism of immunity to mycobacteria. Parental consanguinity and familial forms are frequently observed leading to the description of the syndrome Mendelian Susceptibility to Mycobacterial Disease (MSMD - MIM 209950). The causal mutations of MSMD have been found in five different susceptibility genes: Interferon gamma receptor (IFN $\gamma$  R1) and IFN $\gamma$  R2, encoding the two chains of the receptor for IFN $\gamma$ ; IL-12B, encoding the p40 subunit of IL-12; IL12R $\beta$ 1, encoding the  $\beta$ 1 chain of the receptor for IL-12; and the signal transducer and activator of transcription-1 (STAT1), encoding an essential transducer of IFN $\gamma$ -mediated signaling (Figure 1). The inheritance is most often autosomal recessive, but X-linked recessive has been described in one family<sup>4</sup> and autosomal dominant inheritance has been reported in several other families.<sup>5,6</sup> In addition the defects can be partial or complete, leading to at least ten different disorders. Complete defects cause more severe disease than partial defects and there is a spectrum of severity such that children with complete IFN $\gamma$ -R deficiencies present with the most profound manifestation of the syndrome.

Four children, all of whom came from a small fishing town on the south east coast of Malta, presented with disseminated infection from poorly pathogenic mycobacteria. In addition, one child also developed a prolonged episode of salmonellosis. Three of the four children were known to be related: two were brothers, whose parents were second cousins and who were the fourth cousins of a third child. The fourth child was not knowingly

## Keywords

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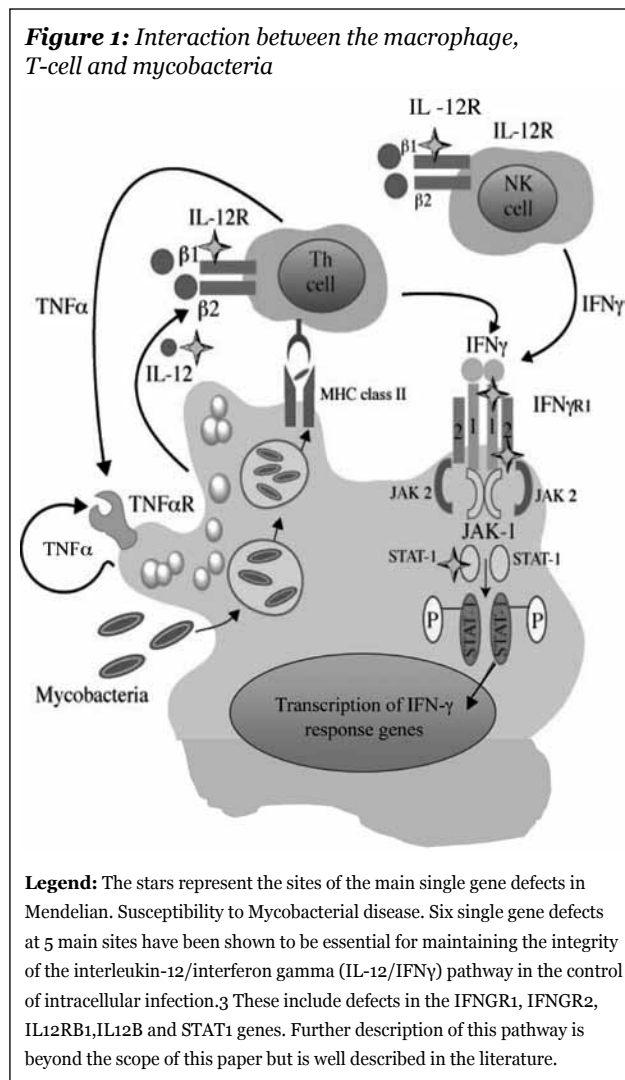
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related to the others but it was likely that all the children had inherited the same recessive mutation from a common ancestor. A mutation in the interferon gamma receptor 1 (IFNGR1) gene was identified and the children were found to be homozygous for the defect,<sup>3</sup> a nonsense mutation at nucleotide 395 of the IFNGR1 gene (A>C 395). The resultant stop codon produced a truncated protein lacking the transmembrane and cytoplasmic domain, with complete abrogation of receptor expression on cell surfaces and a functional defect in the up-regulation of tumour necrosis alpha (TNF-alpha) by macrophages in response to IFN $\gamma$ .<sup>7</sup>

Since this initial description, no further cases of IFNGR1 deficiency have been identified in Malta. However, the frequency of carriers of the mutation in the Maltese population remains unknown. While the heterozygous parents and siblings of the affected Maltese children remained healthy, functional studies showed them to have defective in vitro production of TNF $\alpha$  in response to lipopolysaccharide (LPS) and IFN $\gamma$ , together with defective T-cell proliferative responses and defective IFN $\gamma$  production in response to mycobacterial antigens.<sup>8,9</sup> This raised the intriguing question of whether heterozygous carriers of the defect, while resistant to poorly pathogenic mycobacteria, would have an increased susceptibility to infection with more virulent intracellular organisms.



The population of Malta, in common with other countries bordering the Mediterranean littoral, has relatively high rates of infection with intracellular organisms. Leishmaniasis, brucellosis and tuberculosis (TB) are all endemic to the island while leprosy, which was previously endemic, has only recently been eradicated. Both visceral (VL) and cutaneous leishmaniasis (CL) occur in Malta and are caused by *Leishmania infantum*, a member of the *L. donovani* complex. Since 1946, notification of any human cases of either CL or VL has been compulsory with a total of 1246 VL and 36 CL cases notified by 1999 (Department of Health, Government of Malta). Since then, the annual incidence has declined steadily, with only 1.07 cases (range 0-2.06) /100,000 population since 1995-2007.<sup>10</sup>

Leprosy was found in Malta for centuries and, at its peak in the late nineteenth century, a leprosarium was built which still exists. The first Maltese leprosy eradication programme began in June 1972 and, at the outset, a total of 261 cases were recorded, two thirds of whom had lepromatous leprosy (LL).<sup>11</sup> LL is characterised by multibacillary disease, a high number of skin lesions, chronicity and long-term complications,<sup>12</sup> consistent with an inability to mount an effective type 1 cytokine response and low IFN $\gamma$  production.

Brucellosis, a chronic granulomatous disease that may have an insidious or acute onset, is endemic in Mediterranean countries and only *Brucella melitensis* (*B. melitensis*) biovar 1 has been isolated on the Maltese islands.<sup>13</sup> Infection is acquired primarily by ingestion of contaminated raw milk or unpasteurised cheese. Human brucellosis cases number about 1 to 20 per year in Malta, with the highest number occurring in the 25 to 34 year age group.<sup>13</sup> While eradication of brucellosis from the Maltese islands is a foreseeable goal, this has not been achieved to-date.

The number of TB notifications in Malta over the period 1990-2008 was small, averaging 5.53 (1.5-12.3) cases per 100,000 per year (Personal communication, Dr David Pace, Paediatric Infectious Diseases Clinic, Mater Dei Hospital, Malta).

Given the high prevalence of intracellular infections in Malta, and the discovery of a cluster of families carrying a defect in the IFNGR1 gene, we postulated that patients from the island with a past history of infection with brucella, leishmania, leprosy and TB could have a higher rate of carriage of the IFNGR1 gene A>C 395 mutation compared with healthy controls. We therefore screened for the frequency of this mutation in healthy Maltese newborns and in a cohort of patients with a past history of intracellular infection using sequence specific primer PCR (SSP-PCR).<sup>14-16</sup>

## Materials and methods

In the absence of any data on the expected prevalence of the IFNGR1 gene (A>C 395) mutation in the Maltese population, we assumed a prevalence of the abnormal allele in the control population of 0.5%, and of 5% in patients with a history of intracellular disease. A total sample size of 414 controls and 138 patients was therefore required for a study with a 95% CI at 80% power.

Approval to complete the study was obtained from the ethics committees of St Luke's Hospital, Gwardamangia, and St Mary's Hospital, London.

Patients with a past history of brucellosis, leishmaniasis, leprosy or TB were identified from the Malta public health records available up to 1998 when the study was conducted. These individuals were contacted by post and invited to donate a sample of blood for DNA extraction when written informed consent was also obtained. Information regarding the disease type (extra-pulmonary TB *vs* pulmonary TB, lepromatous *vs* tuberculoid leprosy and VL *vs* CL) was obtained from public health records. No retrospective review of patient notes was carried out. Patients were not screened for HIV infection but at the time of completing this study the HIV seroprevalence rate was <0.1% amongst adults. Cord blood samples taken for routine neonatal screening from healthy newborn infants were obtained from the Malta Biobank at the University of Malta. DNA was extracted from whole blood by salting out, re-suspended in TE buffer and stored at -20°C prior to use.<sup>17</sup>

A rapid and simple, one-step genotyping method was used to test for the presence of the A>C 395 mutation in the IFNGR1 gene, using sequence specific primers (PCR-SSP) capable of distinguishing between the wild type and mutant sequence.<sup>14-16</sup> Primers were obtained from Cruachem (Glasgow) and designed by Dr Sarah Marshall, Tissue Typing Laboratory, Regional Transplant Centre, Churchill Hospital, Oxford, UK.

Primer sequences were as follows:

- common (sense) primer 5'-CAGAATGGATTGATGCCTGC-3'
- mutant (anti-sense) primer 5'-GGCATAACAGCAAATCTCTCTG-3'
- wild-type (anti-sense) primer 5'-GGCATAACAGCAAATCTCTCT-3'

Two internal control primers amplifying a human growth hormone sequence were also included in each reaction to confirm successful PCR amplification.

- control (sense) primer 1: 5' GCCTTCCCAACCATCCCTT 3'
- control (anti-sense) primer 2: 5' TCACGGGATTCTGTGTGTTTC 3'

PCR mix, cycling conditions and resolution of products were standardised, as previously described.<sup>14</sup> DNA from a known homozygous and heterozygous carrier of the mutation were amplified together with all patient samples to check for accuracy and consistency of the genotyping by SSP-PCR. Validation of the procedure was completed by sequencing five of the control DNA samples, together with DNA from a known homozygote and heterozygote for the IFNGR1 mutation, to confirm that genotyping by PCR-SSP was correct.

## Results

DNA was obtained from a total of 167 patients with a past history of intracellular infection and from 450 anonymised neonatal samples obtained from cord blood samples taken for the purposes of routine newborn screening. The type of intracellular infection and method used to confirm the diagnosis are summarised in Table 1.

**Table 1:** Cases of intracellular infection in study population

Intracellular infection	Disease type	Number	Diagnostic criteria
Brucellosis	NA	60	Serology
Leishmaniasis	Visceral	27	Bone marrow aspirate
Leprosy	Lepromatous	44	Clinical +/- slit skin smear
Tuberculosis	PTB	32	21 (66%) culture proven
	EPTB	4	1 (25%) culture proven

**Legend:** Details of past history of patients recruited to the study categorised by intracellular infection, together with disease type and method used to confirm diagnosis. (NA: not applicable, PTB: pulmonary TB, EPTB: extra-pulmonary TB).

Screening of DNA from all cases and controls using sequence specific primers (PCR-SSP) capable of distinguishing between the wild type and mutant sequence<sup>14-16</sup> revealed no carriers for the IFNGR1 A>C 395 mutation in both cases and controls.

## Discussion

Newport *et al* in their original description of the IFNGR1 gene defect in 1996,<sup>1</sup> detailed the clinical and functional phenotype of the affected children and their heterozygous, first degree relatives. To date, Although this particular mutation has not been reported in other populations outside Malta, since the initial report a large body of literature has reported numerous other mutations in both the IFNGR1 gene and other genes involved in Mendelian Susceptibility to Mycobacterial Disease (MSMD). The clinical features of a large worldwide cohort of patients with recessive and dominant IFNGR1 mutation are well described by Dorman in 2004,<sup>18</sup> together with the range of infecting pathogens. There is little doubt that, although rare, these conditions result in significant susceptibility to serious infections that may have severe clinical repercussions. Hence, this study addressed the question of whether the IFNGR1 gene mutation, described in a small group of consanguineous families from the island of Malta, was prevalent amongst patients from within the same population with a past history of intracellular infection. We hypothesised that an increased rate of carriage of the mutation would be found amongst patients with a history of intracellular infection. However, amongst the 167 patients screened, no heterozygotes were identified. Screening 450 healthy newborns for carriage of the mutation also failed to identify any heterozygotes. Although our hypothesis is not disproved, this finding suggests that the IFNGR1 A>C 395 mutation is very unlikely to be a significant contributor to intracellular infection in this island population and, therefore, has little or no public health significance.

One limitation of our study is that the numbers of both healthy controls and patients screened was small. However, the study was adequately powered and suggests that the mutation, if present in the population, is at a prevalence of less than 0.5%. Furthermore, we only looked for the presence of one mutation within the IFNGR1 gene and it is possible that mutations in other regions of the gene may account for the high rates of susceptibility to intracellular infection. To-date however, the range of pathogens associated with IFNGR1 mutations is small,<sup>18</sup> and these patients appear to be resistant to a wide range of ubiquitous bacteria, fungi, and parasites. Affected patients with IFNGR1 deficiency are likely to present with mycobacterial infections, especially those of low virulence, and occasionally with infections caused by non-typhoid salmonella, *H. capsulatum*, *L. monocytogenes*, human herpesvirus 8 and cytomegalovirus.

Finally, we did not screen for other mutations in the IL-12/IFN $\gamma$  pathway. For example, both IL12RB1 and IL12p40 deficient children and adults tend to present with infections caused by weakly virulent salmonella or mycobacteria that are usually associated with a favourable outcome.<sup>19</sup> Some individuals may be completely asymptomatic whilst three patients with IL12RB1 deficiency have developed clinical TB<sup>20</sup> raising the real possibility that defects in this gene may account for susceptibility to TB in the general population and possibly infection with other intracellular pathogens. Further screening of the population for defects in these genes is, therefore, warranted.

Although the island has a universal BCG vaccination programme administered at the age of 12 years, brucellosis, leishmaniasis and TB are all still prevalent and it is likely that any affected homozygotes would present with infection caused by poorly virulent mycobacteria.<sup>18</sup> However, to-date, no further homozygous cases for the IFNGR1 mutation have been described in Malta. Thus, the original finding of the mutation amongst three families, all of whom originated from one town on the island of Malta, is likely to have been an isolated occurrence restricted to a small group of consanguineous families and does not pose a significant public health problem.

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