Chitotriosidase deficiency in the Cypriot population: identification of a novel deletion in the CHIT1 gene

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Abstract

Objectives: The purpose of this study was to determine the normal range of chitotriosidase activity in the Cypriot population and the frequency of the 24bp duplication polymorphism. Furthermore, we compared the allele frequency of this polymorphism in two locations with different malaria endemicity in the past.

Design and methods: Plasma chitotriosidase activity was measured using a fluorogenic substrate. The 24bp polymorphism was detected using PCR analysis of exon 10 of the CHIT1 gene. Additional mutations were detected using direct sequencing.

Results: The normal range of chitotriosidase activity was found to be 9.5-44.0 nmol/ml/hr. Among 114 normal individuals genotyped for the 24bp duplication, 7% were found to be homozygous, 36% heterozygous and 57% wild type (allele frequency 0.25). The allele frequency of this polymorphism in individuals originating from two locations with different malaria endemicity in the past was not significantly different. A novel deletion mutation in the CHIT1 gene was identified associated with loss of chitotriosidase activity. This new deletion eliminates 29 nucleotides from exon 9 resulting in the generation of a premature stop codon, probably leading to the production of an aberrant protein molecule.

Conclusions: The normal range of chitotriosidase activity and the allele frequency of the 24bp duplication polymorphism in the Cypriot population were found to be similar to those of other Mediterranean populations. No evidence for an association between the presence of the 24bp duplication polymorphism and susceptibility to malaria was found. A novel deletion in exon 9 of the CHIT1 gene was identified (allele frequency 0.01).

Keywords: chitotriosidase, 24bp duplication, deficiency, malaria, Cyprus, CHIT1
Introduction

Human chitotriosidase (EC 3.2.1.14) is an endoglycosidase with the ability to hydrolyze chitin, a long chain N-acetyl glucosamine biopolymer which functions as a structural component of the coating of fungi, insects, nematodes and shellfish. The enzyme is synthesized by activated macrophages and is considered to be a component of the innate immune system, especially involved in defense against chitin containing pathogens [1-3]. It was first identified in patients with Gaucher disease, a lysosomal storage disorder characterized by the accumulation of lipid laden macrophages as a result of glucocerebrosidase (acid β-glucosidase) deficiency [2]. The enzyme was purified from the spleen of a Gaucher patient and characterized after cloning of its cDNA from a human macrophage cDNA library [4, 5]. Because of its high values in the plasma of Gaucher patients, chitotriosidase is used as a marker of disease progression and for monitoring response to treatment [2]. Moderately elevated levels of chitotriosidase are also observed in other lysosomal storage disorders (such as GM1 gangliosidosis, Krabbe disease, Niemann-Pick disease, mucopolysaccharidoses I and IVA) [6-8] as well as in patients with atherosclerosis and coronary artery disease [9-11], β-thalassaemia [12-14], malaria [13, 15] and other disorders involving activated macrophages.

Chitotriosidase is encoded by the CHIT1 gene, located on chromosome 1 at position q31-32 [16]. The gene consists of 12 exons and spans about 20 kb of genomic DNA. A polymorphism in exon 10 of the CHIT1 gene caused by a 24bp duplication (c.1049_1072dup24) and leading to enzyme deficiency is frequently encountered among different populations. The presence of this polymorphism results in the activation of a cryptic 3’ splice site leading to an in-frame deletion of 87 nucleotides and the production of an abnormal mRNA. The resulting protein lacks 29 amino acid residues (Val 344-Gln 372) and is catalytically inactive. The 24bp duplication polymorphism has been identified in high frequencies among different populations, both European and Asiatic [17-23].

Whereas the 24bp duplication polymorphism is quite common in populations of European and Asian ancestry, it is almost absent in African populations [18, 19]. This observation led to the suggestion that the presence of the mutant chitotriosidase allele might confer increased susceptibility to malaria and other infectious diseases. This notion was initially supported by a reported higher prevalence of the 24bp duplication polymorphism among Indian patients with filariasis (27% homozygotes) compared to the healthy population screened (10% homozygotes) [20]. It was therefore postulated that the absence of the 24bp duplication polymorphism in populations of African origin probably resulted from a selective advantage for the wild type allele. Other studies, however, did not support the aforementioned hypothesis. As shown for patients with filariasis in Papua New Guinea, there was no correlation between the presence of the mutant allele and the progression or the severity of the disease [24]. Moreover, data reported by two studies for the Sardinian population where contradictory with respect to the frequency of the mutant allele in areas of high and low malaria endemicity respectively. Piras et al. [21] reported similar frequencies for the wild type and mutant allele as those reported by Malaguarnera et al. [18] but their results regarding the association of the presence of the mutant allele with malarial infection significantly differed.

Several other mutations in the CHIT1 gene resulting in absent or reduced enzyme activity have been described. The p.Gly102Ser missense mutation (exon 4 of the CHIT1 gene) is frequently encountered (allele frequency 0.2-0.3) in all the populations screened (European,
Asian, African). The results reported regarding the impact of this polymorphism on chitotriosidase activity are controversial since some groups support that the polymorphism does not significantly affect the enzyme activity [25], whereas other groups suggest that the presence of the p.Gly102Ser polymorphism confers a 4-5 fold reduction in enzyme activity [19].

The p.Gly354Arg polymorphism (exon 10) is either encountered on its own or in combination with two more changes downstream (complex E/I 10 allele). In both cases a complete absence of chitotriosidase activity is observed [25]. The p.Ala442Gly polymorphism (exon 12) is frequently encountered among different populations but has not been associated with reduced enzyme activity, whereas the p.Ala442Val polymorphism, which is mostly encountered among individuals of African origin, has been associated with significantly reduced chitotriosidase activity [25].

The presence of polymorphisms that decrease chitotriosidase activity impedes the use of this biomarker for the diagnosis and monitoring of Gaucher disease. It is therefore important, for the correct interpretation of chitotriosidase levels, to establish the type and frequency of these polymorphisms in every population. In the present study we established the normal range of plasma chitotriosidase activity and the frequency of the 24bp duplication polymorphism in the Cypriot population. We also showed the presence of the p.Gly102Ser polymorphism and identified a new mutation in exon 9. Furthermore, we compared the frequency of the 24bp polymorphism in two locations of the island with low and high malaria endemicity in the past.
Materials and Methods

Subjects and samples
For the determination of the normal range of chitotriosidase activity and the frequency of the 24bp duplication in our population a total of 114 apparently healthy unrelated individuals were recruited. The number and geographic origin of samples were selected such that all regions of the island are correctly represented in the cohort. The study was approved by the Cyprus National Bioethics Committee and informed consent was obtained from all participating volunteers.

For the investigation of a potential correlation between the 24bp duplication polymorphism and malaria endemicity, a different set of DNA samples (195 in total) obtained from the DNA bank of the Cyprus Institute of Neurology and Genetics were used. The samples were randomly selected. One hundred samples came from individuals originating from a region at sea level where malaria endemicity was high, and 95 samples belonged to individuals from a village at an altitude of 880m where malaria was nearly absent.

Measurement of Chitotriosidase activity
EDTA plasma was used for the determination of chitotriosidase activity using an artificial substrate as previously described [2]. Briefly, 5μl of undiluted plasma was incubated at 37°C for 1 hour with 100μl of a solution containing 22 μmol/L of the artificial substrate 4-methylumbelliferyl-β-D-N,N’,N”triacetyl chitotrioside (Sigma M5639) in citrate-phosphate buffer, pH 5.2. The reaction was terminated by adding 1ml of 0.25 mol/L glycine-NaOH buffer, pH 10.4. The release of 4-methyl-umbelliferone was measured using a JASCO PTL-3965 fluorometer at an excitation and emission wavelength of 365 nm and 450 nm respectively. Chitotriosidase activity was calculated as nanomoles of substrate hydrolyzed per ml plasma per hour.

DNA Isolation
DNA was isolated from blood samples using the Qiagen Gentra Puregene Blood Kit (catalogue number 158467) according to the manufacturer’s instructions.

24bp duplication polymorphism detection by PCR
Genotyping for the 24bp duplication polymorphism was performed using previously described primers (5’-GAAGAGGTAGCCAGGCTCTGG-3’) and (5’-CTGCCGTAGCGTCTGGATGAG-3’) which enabled the detection of both the normal (75bp) and the mutant (99bp) allele [21]. PCR reactions were carried out in 50μl volumes containing 1 x PCR buffer (Qiagen), 0.5 mM dNTP’s, 20 pmol of each primer, 0.25 units of Taq polymerase (Qiagen) and 200ng of template DNA. DNA was initially denatured at 94°C for 4 min and then subjected to 36 cycles of 60 sec denaturation at 94°C, 30 sec annealing at 55°C and 60 sec of extension at 72°C. Amplified fragments were separated by electrophoresis in 3% agarose gel (NewSieve) and visualized by ethidium bromide staining [Fig.2].

Detection of additional CHIT1 mutations
DNA samples were subjected to sequencing in an attempt to identify additional mutations or polymorphisms present. Exons 4, 10 and 12 of the CHIT1 gene were initially sequenced and evaluated for the presence of known mutations associated with low enzyme activity. In those subjects, in whom none of the known mutations were detected, sequencing of the remaining exons of the CHIT1 gene was performed in order to identify any unknown mutations. For the screening of known mutations, previously described sets of primers were used [25], whereas
for the remaining exons primers were designed using the online primer design software Primer3 (http://frodo.wi.mit.edu/primer3/). All primer sets used for this study are shown in supplementary Table 1.

**Identification of the breakpoints of the new CHIT1 deletion in exon 9**

For the determination of the breakpoints of the new *CHIT1* deletion in exon 9, DNA from the subject was amplified by PCR using primers: 5’-GCCGCTCGAGCAATCTCTAGTCTCCTGGTG-3’ and: 5’-CTAGTCTAGACCCTTGAGGTCCTGAACTGT-3’ which carry an *XhoI* and *XbaI* restriction site respectively. The amplified fragments were cloned in the vector pBluescript (pBS-KSII) digested with the same enzymes. Bacterial colonies harboring either the cloned wild type or the mutant allele were identified by PCR on the basis of the difference in the amplicon size using the above primers. PCR products obtained from three independent bacterial clones were subjected to bidirectional sequencing.

**Screening for the new CHIT1 deletion in exon 9**

All 114 samples of our cohort were screened for the 29bp deletion in exon 9 on the basis of the presence of a shorter product after PCR amplification followed by agarose gel electrophoresis.

**Statistical Analysis**

The raw data obtained in this study were statistically processed using the SPSS (v.17) statistical software package. Descriptive statistics (mean, mode, variance, histograms, etc.) and inferential statistics (confidence interval, normality test, etc.) were obtained. The normal range and mean values of chitotriosidase activity were calculated after the exclusion of extreme values. The Kolmogorov-Smirnov statistic test was used to check the hypothesis that the data was normally distributed. The χ² test was used to check whether the population is within the Hardy-Weinberg equilibrium.
Results and Discussion

The present study was conducted on a cohort of 114 individuals originating from all major geographic areas of Cyprus. We initially sought to determine the normal range of chitotriosidase activity for the Cypriot population by an enzymatic assay performed in plasma. Following the exclusion of outliers, the normal range of chitotriosidase activity was determined to be 9.5-44.0 nmol/ml/hr (mean 26.77). We also determined the range, mean, standard deviation and median values for each genotype group, with respect to the 24bp duplication (wt/wt, wt/mut, mut/mut). There was an overlap between the values obtained for the wild type and heterozygous individuals [Fig. 1].

Among the 114 individuals genotyped for the 24bp duplication polymorphism, 8 (7.02%) were homozygous, 41 (35.96%) were heterozygous, whereas 65 (57.02%) were wild type. Based on these results, the mutant allele frequency is 0.25 (57/228 alleles), which is similar to that of other populations of European and Mediterranean ancestry (Table 1). The $\chi^2$ test revealed that the observed distribution of genotypes for the Cypriot population did not significantly vary from the Hardy-Weinberg predictions.

Table 1. Allele frequencies of the CHIT1 24bp duplication polymorphism associated with chitotriosidase deficiency in different ethnic groups.

| POPULATION ORIGIN      | No. | % wt/wt | % wt/mut | % mut/mut | wt allele frequency | mut allele frequency | Ref. |
|------------------------|-----|---------|----------|-----------|--------------------|--------------------|------|
| African                |     |         |          |           |                    |                    |      |
| Benin                  | 100 | 100     | 0        | 0         | 1                  | 0                  | 18   |
| Burkina Faso           | 100 | 98      | 2        | 0         | 0.98               | 0.02               | 18   |
| Mediterranean          |     |         |          |           |                    |                    |      |
| Corsica                | 194 | 74.8    | 24.2     | 1         | 0.869              | 0.131              | 21   |
| Sardinia               | 340 | 68.6    | 27.9     | 3.5       | 0.825              | 0.175              | 21   |
| Turkey                 | 95  | 66.3    | 29.5     | 4.2       | 0.811              | 0.189              | 21   |
| Portugal               | 295 | 60      | 37.3     | 2.7       | 0.786              | 0.214              | 21   |
| Spain                  | 103 | 61.2    | 32       | 6.8       | 0.772              | 0.228              | 21   |
| Israel                 | 68  | 60.3    | 33.8     | 5.9       | 0.772              | 0.228              | 21   |
| Cyprus                 | 114 | 57      | 36       | 7         | 0.75               | 0.25               | PS   |
| Sicily                 | 100 | 51.01   | 44.54    | 5.45      | 0.73               | 0.27               | 18   |
| European               |     |         |          |           |                    |                    |      |
| Basque Country         | 60  | 76.7    | 23.3     | 0         | 0.883              | 0.117              | 21   |
| Continental Italy      | 99  | 63.7    | 34.3     | 2         | 0.808              | 0.192              | 21   |
| Netherlands            | 171 | 58.5    | 35.1     | 6.4       | 0.761              | 0.239              | 21   |
| Continental France     | 128 | 59.4    | 31.3     | 9.3       | 0.75               | 0.25               | 21   |
| Asian                  |     |         |          |           |                    |                    |      |
| Papua New Guinea       | 906 | N/A     | N/A      | N/A       | 0.88               | 0.12               | 21   |
| South India            | 67  | N/A     | N/A      | N/A       | 0.56               | 0.44               | 21   |
| Korea                  | 231 | 20.3    | 47.2     | 32.5      | 0.439              | 0.561              | 23   |
| Taiwan                 | 82  | N/A     | N/A      | N/A       | 0.42               | 0.58               | 21   |

N/A: Not Available
PS: Present Study
In an attempt to investigate any association between the presence of the 24bp duplication polymorphism and malaria incidence, the frequency of the polymorphism was also determined in samples obtained from two altimetrically different locations in Cyprus, characterized by different malarial endemicity in the past. Until its eradication after the Second World War, malaria was endemic in coastal regions in Cyprus but virtually absent from mountainous regions above 900m [26]. The frequency of the mutant allele in the area at sea level was found to be 0.305 while the frequency in the mountainous area was 0.384 (Table 2). The difference between the two frequencies is not statistically significant ($\chi^2=2.73$, df=2, p=0.255). This implies that the hypothesis that low frequency of the mutant allele correlates to high incidence of malaria [18] does not appear to apply, at least for Cyprus. Our results are rather in agreement with previously published studies showing no significant association between the presence of the 24bp duplication polymorphism and increased susceptibility to malaria [21, 27]. Furthermore, the eradication of malaria is relatively recent, little more than two generations ago, which is too limited a period of time to cause allele differences [21].

Table 2 Allele frequencies of the CHIT1 24bp duplication polymorphism in the Cypriot population: comparison of two altimetrically different locations with different malaria endemicity in the past.

| GENOTYPE  | All areas n=114 | High altitude (low malaria endemicity) n=95 | Sea level (high malaria endemicity) n=100 |
|-----------|-----------------|------------------------------------------|------------------------------------------|
| wt/wt     | 57% (65/114)    | 42% (40/95)                               | 50% (50/100)                             |
| wt/mut    | 35% (40/114)    | 39% (37/95)                               | 39% (39/100)                             |
| mut/mut   | 7% (8/114)      | 19% (18/95)                               | 11% (11/100)                             |
| wt allele frequency | 0.75  | 0.616  | 0.695  |
| mut allele frequency | 0.25  | 0.384  | 0.305  |

As expected, all subjects homozygous for the 24bp duplication polymorphism had no detectable chitotriosidase activity. However, 8 individuals in our cohort which were genotyped as heterozygous carriers of the 24bp duplication polymorphism had plasma chitotriosidase activity below 10 nmol/ml/hr, while one individual heterozygous for the 24bp duplication polymorphism had virtually zero activity (Table 3). We therefore sought to investigate the possibility that these individuals carry a second mutation in the CHIT1 gene, associated with reduced enzyme activity. Towards this end, DNA from these 9 individuals was subjected to direct sequencing of CHIT1 exons. These subjects were initially sequenced for the presence of previously described polymorphisms (p.Gly102Ser in exon 4, p.Gly354Arg in exon 10 and p.Ala442Val in exon 12), known to be associated with low enzyme activity. When screened for the p.Gly102Ser polymorphism in exon 4, five out of the nine individuals were found to be heterozygous carriers of this polymorphism (Table 3). This result suggests that the p.Gly102Ser polymorphism is probably frequently encountered in the Cypriot population as in other European populations [19, 24, 28]. Screening of more DNA samples is required to establish the frequency of this mutant allele in the Cypriot population.
Table 3. Screening for a second mutation in heterozygotes for the 24bp duplication polymorphism with chitotriosidase activity less than 10nmol/ml/hr

| No. | Subject | Chitotriosidase activity (nmol/ml/hr) | Allele 1 | Allele 2 |
|-----|---------|-------------------------------------|---------|---------|
| 1   | CH 83   | 1                                   | 24bp duplication | New 29 bp deletion |
| 2   | CH 52   | 5                                   | 24bp duplication | g.4579G>A (p.Gly102Ser) |
| 3   | CH 53   | 5                                   | 24bp duplication | g.4579G>A (p.Gly102Ser) |
| 4   | CH 99   | 6                                   | 24bp duplication | g.4579G>A (p.Gly102Ser) |
| 5   | CH 107  | 6                                   | 24bp duplication | g.4579G>A (p.Gly102Ser) |
| 6   | CH 81   | 8                                   | 24bp duplication | ? |
| 7   | CH 46   | 9                                   | 24bp duplication | g.4579G>A (p.Gly102Ser) |
| 8   | CH 96   | 9                                   | 24bp duplication | ? |
| 9   | CH 30   | 10                                  | 24bp duplication | ? |

The remaining four subjects were tested for the p.Gly354Arg and the p.Ala442Val polymorphisms in exons 10 and 12 respectively. However, all four individuals were negative for these polymorphisms. We therefore proceeded to sequence the remaining exons of the CHIT1 gene in order to identify any new mutations or polymorphisms. In one of these individuals (subject 83) a new mutation, a 29bp deletion in exon 9, was detected (Table 3). This individual was a heterozygous carrier of the 24bp duplication polymorphism but exhibited near zero (0.65nmol/ml/hr) chitotriosidase activity. The mutation was initially detected when the PCR product of exon 9 of this individual was subjected to agarose gel electrophoresis and a second band, smaller than the normal chitotriosidase band, was evident, suggestive of the presence of a deletion mutation in one of the two alleles [Fig. 3A]. The electropherogram obtained from the sequencing of this PCR product from both ends was indeed indicative of a deletion mutation of 29 nucleotides. The exact breakpoints of the new deletion could not be determined due to the presence of a GG dinucleotide flanking the deleted DNA region. According to HGVS nomenclature, this new deletion is described as c.(965_993)del (ENST00000367229) and results in the removal of 10 amino acid residues (322-331) from the protein, followed by a frame shift and the generation of a premature stop codon 3 amino acids thereafter [Fig. 3B].

Evaluation of the parents of this subject for the presence of the new mutation revealed that the mother is heterozygous whereas the father is heterozygous for the 24bp duplication polymorphism. Screening the remaining 113 samples of our cohort for the new deletion revealed two additional carriers, giving a frequency of 0.01 for this mutant allele in the Cypriot population. The fact that the herein described deletion in exon 9 of the CHIT1 gene has not been reported in other populations may suggest that it could be a founder mutation which is unique for Cyprus.

This study has provided data for the plasma chitotriosidase levels in the Cypriot population and the polymorphisms in the CHIT1 gene associated with reduced enzyme activity. These data will be useful for the interpretation of chitotriosidase levels in Cypriot Gaucher patients. Our data are also important for completing the picture of the frequency of the 24bp duplication polymorphism in Mediterranean populations and for providing further evidence against an association between this polymorphism and malaria.
Figure 1: Chitotriosidase activity levels for the three genotype groups of the 24bp duplication in the Cypriot population.

Figure 2: Detection of the 24bp duplication polymorphism in the CHIT1 gene by PCR of genomic DNA. Lane 1, homozygous (wt/wt), lanes 2&3, heterozygous (wt/mut), lane 4, homozygous mutant (mut/mut), lane 5, DNA ladder (Fermentas, Fast ruler™, Low range).
Figure 3:

A. PCR amplification of exon 9 of the CHIT1 gene. In samples 5 and 6 a second band (261bp) is apparent in addition to the wild type product (290bp) suggestive of the presence of a deletion mutation in heterozygosity. In lanes 2, 3, 4 and 7 only the wild type product is present. Lane 1: DNA ladder (New England Biolabs, 100bp DNA ladder).

B. Part of the nucleotide and amino acid sequence of exon 9. The boxed region represents the sequence which is removed due to the deletion mutation. The deletion results in a frame shift and a premature stop codon indicated by an asterisk. A GG dinucleotide flanking the deleted DNA region prevents the accurate determination of the exact deletion breakpoints.
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