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Gly111Ser mutation in CD8A gene causing CD8 immunodeficiency is found in Spanish Gypsies

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Abstract

We describe the second case of CD8 immunodeficiency. It confirms the pathogenic effect of p.Gly111Ser, leading to complete deficit of CD8+ lymphocytes, although the clinical manifestations may vary in severity. Similarly to the first case reported, our patient is also from Spanish Gypsy origin and homozygous for the p.Gly111Ser mutation in CD8α chain. The patient has suffered repeated respiratory infections from childhood but with conservation of her pulmonary parenchyma, on the contrary to the first patient, who died because of his respiratory injury. We developed an AluI–PCR-RFLP assay to screen a total of 1127 unrelated control individuals: 734 subjects of Gypsy ancestry from different sub-isolates and geographic locations in Europe, and 393 of Spanish (non-Gypsy) ethnicity. The results indicate that p.Gly111Ser is confined to the Spanish Gypsy population, where it occurs at a carrier rate of 0.4%. Analysis of microsatellite markers flanking the CD8A mutated gene revealed a shared polymorphic haplotype suggesting a common founder for p.Gly111Ser mutation that causes CD8 deficiency in the Spanish Gypsy population. CD8 immunodeficiency should be given diagnostic consideration in Spanish Gypsies with recurrent infections. Our findings may also have implications for these patients in terms of specific recommendations in vaccination and healthy habits and for genetic counseling of affected families.
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1. Introduction

CD8+ T lymphocytes are defensive cells that recognize antigens presented by MHC class I molecules at the cellular membrane. After recognition by the T cell receptor (TCR), CD8+ lymphocytes activate to become cytotoxic T lymphocytes (CTL), able to lyse the presenting cell. By this mechanism, CTLs recognize and eliminate tumour, infected or allogeneic cells (Henkart, 1999). CD8 is a homo (αα) or heterodimer (αβ) coreceptor for the TCR (Zamoyska, 1994). Its expression characterizes T lymphocytes with cytotoxic capabilities and is necessary for the positive selection of the CD8+ cellular lineage in the thymus (Nakayama et al., 1994).

Human CD8 immunodeficiency (Notarangelo et al., 2006) was defined in 2001 (MIM # 608957) by the finding of a unique case in a 25-year-old Spanish Gypsy male from a consanguineous family (Family 1) (De la Calle-Martín et al., 2001). The disease was characterized by a history of recurrent

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bacterial and viral respiratory infections from childhood, presence of disseminated bronchiectasias and severe ventilatory disturbances. After excluding other immunodeficiencies, lymphocyte phenotyping revealed a total absence of CD8+ cells. Additionally two asymptomatic younger sisters also lacked CD8+ cells. Analysis of the genes encoding CD8 α and β chains revealed a G to A mutation in *CD8A* gene that changed glycine to serine at position 111 of the CD8α subunit (GenBank accession no. AY039664). The familial segregation supported a defect of autosomal recessive inheritance.

Based on: (a) the clinical symptoms of the patient, (b) the presence of high titers of antibodies against CMV, herpes zoster, herpes simplex and rubella that demonstrate antiviral immunocompetence, and (c) the absence of disease in the CD8 negative sisters, CD8 immunodeficiency was considered non-severe and compatible with life (De la Calle-Martín et al., 2001; Regueiro and Español, 2007).

Here we report the second case of CD8 immunodeficiency due to the presence of homozygous p.Gly111Ser mutation in CD8α chain, in a 16-year-old girl with repeated respiratory infections. The affected subject (Family 2) is also of Spanish Gypsy descent although unrelated with the family previously reported by De la Calle-Martín et al. (2001). This fact prompted us to analyse the presence of the mutation in Spanish Gypsy and non-Gypsy individuals and in several European Gypsy populations.

2. Materials and methods

2.1. Clinical data of the CD8 deficient patients

The proband is a 16-year-old Spanish Gypsy girl that was referred to our department due to repeated respiratory infections since childhood and failure to thrive. She was clinically evaluated and her immune status (immunoglobulin levels, lymphophenotype and lymphocytic proliferation) was determined in the laboratory. Her medical history was recovered by review of medical records. The parents were interviewed to obtain familial pedigree and history of the disease. The clinical history of family 1 was also revised.

2.2. Sample populations and families

The sample groups studied included a total of 734 unrelated subjects representing different European Gypsy populations: 242 from Spain; 394 from Bulgaria, representing 8 sub-isolates; and 98 from Romania, Hungaria and the Czech Republic. The analysis also included 393 unrelated Spanish (non-Gypsy) controls. Three familial pedigrees were analysed: (1) family 1, with 7 members including a CD8 deficient proband (5), (2) family 2, with 7 members, including the second CD8 negative patient and (3) family 3, with 12 members, including 4 with a carrier status for p.Gly111Ser mutation. Informed consent was obtained from all sample donors and the studies were approved by the corresponding ethical committees.

2.3. Analysis of the mutation

Total RNA and genomic DNA extraction was performed from peripheral blood lymphocytes by standard procedures. For the genetic analysis of family 2, the exons 1 and 2 of *CD8A* gene and surrounding genomic sequences (wild type *CD8A* gene sequence M27161) were amplified using polymerase chain reaction (PCR). Primer pairs used were 5′GAGCTTCGAGCCAAGCAGCG3′ (E1-2F) and 5′CAGGTGCGCTAAGAGGCTTG3′ (E1-2R). The remaining full length cDNA of CD8A was amplified with primers 5′GTCATGGCCTTACCAGTGAC3′ (CD8MF) and 5′GTAATGTAGTGGCTGTTGCAC3′ (CD8MR). Amplification conditions were 95 °C for 5 min, followed by 38 cycles of 95 °C for 15 s, 61.5 °C for 30 s and 72 °C for 80 s, with a final extension of 72 °C for 10 min. PCR products were purified using the QIAquick Gel Extraction kit (Qiagen, Hilden, Germany) and sequenced in an automatic 3100 Avant DNA sequencer (Applied Biosystems, Foster City, CA).

The population samples and healthy relatives of patients were screened for the *CD8A* c.331G>A mutation by genomic DNA amplification of exons 1 and 2 and flanking intronic sequences with primers E1-2F and E1-2R (above described). The PCR product (636bp) was digested with AluI restriction enzyme and run in 10% acrylamide gels. AluI digestion rendered two fragments of 386 and 250 bp in a wild type individual, while a new restriction site was generated by the c.331G>A mutation, and then three restriction fragments of 250, 212 and 174 bp were observed (Fig. 1).

2.4. Microsatellite genotyping and haplotype analysis

Subjects of the three families carrying the c.331G>A mutation were genotyped for microsatellite markers spanning the genetic interval containing the *CD8A* and *CD8B* genes on chromosome 2p32 (D2S2232, D2S388, D2S417, STSCD8A, STSCD8B, D2S2216, D2S2181). The novel markers STSCD8A and STSCD8B are intragenic to *CD8A* and *CD8B*, respectively, and were developed by searching for tandem repeats of CA dinucleotide in sequence contig NT_022184 (NCBI database, Homo sapiens genome view, build 36.1) and by designing flanking primers (CD8A-forward: 5′-TTAGATGCTACCACAGGGATGC-3′; CD8A-reverse: 5′-CAGCTCAAGTGACTGCCTCATC-3′; CD8B-forward: 5′-TAAACTTTTAGTGAGATCAAATGTGG-3′; CD8B-reverse: TAAAATTTTCCAAAATATAAATGAGATTA-3′). Conditions for PCR amplification of microsatellite markers used in this study were as follows: 1 cycle of denaturation at 94 °C for 5 min; 35 cycles of denaturation at 94 °C for 40 s, annealing at 56 °C for 40 s; and a final extension step of 72 °C for 10 min. The order of markers was established by integrating genetic and physical maps (NCBI, http://www.ncbi.nlm.nih.gov). For comparative purposes, we provide allele sizes for individual 134702 available from CEPH (Dib et al., 1996), which was used as a standard in genotyping assays. Allele sizes in the CEPH:134702 individual for the novel STSCD8A and STSCD8B markers, as well as for the D2S417 and D2S2181 markers were determined by DNA sequencing of the corresponding amplimers.

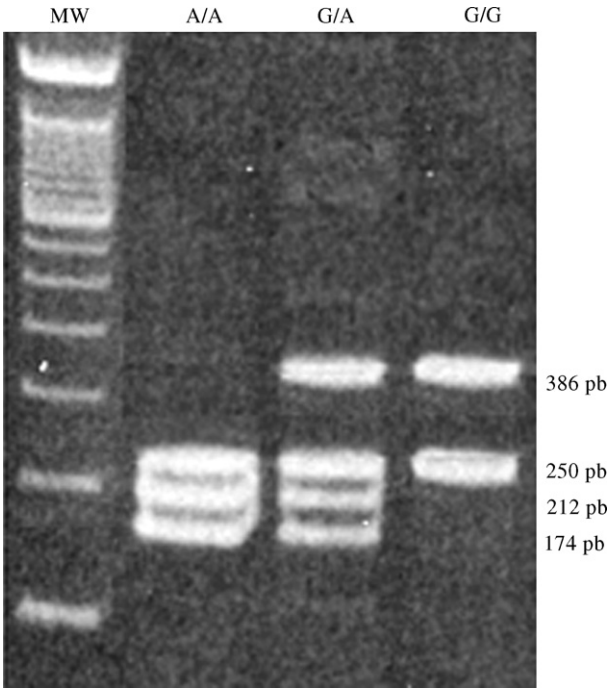

Fig. 1. Migration pattern following AluI digestion: screening for the CD8A c.331G>A mutation. Genomic DNA amplification of exons 1 and 2 (636 bp) was digested with AluI restriction enzyme and run in 10% acrylamide gels. In a wild type individual (G/G) the digestion rendered two fragments of 386 and 250 bp. The described c.331G>A mutation in the homozygous individual creates an AluI restriction site and three fragments of 250, 212 and 174 bp were observed (A/A). The heterozygous pattern is also shown (G/A).

3. Results

3.1. Clinical and laboratory findings

Proband in family 2 is a 16-year-old girl, first of three siblings (subject III:1, Fig. 2b). She suffered two bronchiolitis episodes at 1 and 2 months of age, the second of which was positive for RSV and required hospitalization. From years 1 to 4 she suffered several episodes of pneumonia and otitis, together with repeated lower respiratory tract infections. On one occasion, serology positive for parainfluenza 3 virus was detected. At year 4 she was re-admitted to hospital and diagnosed with asthma, ENT infections and pulmonar LIL atelectasis (this had been observed since age 2). She was correctly vaccinated, alergical test were negative, and immunoglobulin serum levels were normal. Infections resolved with antibiotic treatment. At 7 years of age the patient was referred to endocrinology with failure to thrive. In the last year she had suffered two episodes of pneumonia together with several respiratory infections with high temperature and productive cough. At present she is under psychological evaluation to exclude a possible nutritional disturbance.

The current immunological laboratory tests revealed normal levels of serum IgG, IgA, IgM and IgG subclasses, and normal lymphocyte proliferation and subpopulations except for an absolute absence of the CD8+ T lymphocyte subset. Double negative T cells (TCRαβ+CD4−CD8−) were increased (16% versus 2% in normal controls). Serology was positive for CMV, EBV and rubella, and negative for HIV, A, B and C hepatitis, sifilis and toxoplasma.

Since, 2001, proband in family 1 (subject II:1, Fig. 2a) suffered repeated respiratory infections due to continuous contamination of the extensive bronchiectasias (pseudomonas aeruginosa was detected in sputum on several occasions). By age 33 his ventilatory function had deteriorated to the point of being wheelchair-bound and completely dependent on oxygen therapy. Before being able to undergo lung transplantation the patient deceased. Interestingly, both of his CD8 negative sisters are healthy at present day.

3.2. Mutation screening and haplotype analysis

Once excluded other immunological abnormalities, immunophenotype data and absence of surface CD8 expression in patient III:1 from the Spanish Gypsy family 2, together with parents consanguinity, were suggestive of the hereditary recessive form of CD8 deficiency. Therefore, we designed primers to amplify and sequence the six exons of *CD8A* gene and surrounding genomic sequences. This analysis revealed a G-to-A transition at nucleotide 331 in exon 2 (c.331G>A) of *CD8A* (Fig. 2b). This mutation is predicted to cause the substitution of a glycine by a serine at codon 111 (p.Gly111Ser) of the CD8α protein. The glycine residue is placed in the immunoglobulin domain of the CD8α chain and is conserved in all reported species through evolution. The mutation creates an additional restriction site for the enzyme AluI at exon 2. We took advantage of this finding to develop a screening test specific for the mutation (see Section 2). This test allowed us to verify that the mutation was in homozygous state in the patient III:1 and, as expected, in heterozygous state in both parents (obligate carriers). In the patient's healthy sibs the mutation was only detected in heterozygous state in her brother (III:2) and was absent in her sister (III:3) (Fig. 2b).

Interestingly, the mutation had been previously reported as causative of CD8 deficiency in another Gypsy family (family 1, Fig. 2a) (De la Calle-Martín et al., 2001). This novel case confirms its pathogenic condition and suggests it might be prevalent among Gypsies. By applying the screening test we investigated the frequency of the mutation in sample groups of healthy Gypsy and non-Gypsy populations (see Section 2). We found the c.331G>A mutation in heterozygous state in one of the 242 Spanish Gypsy subjects analysed, indicating he was just a coincidental carrier (family 3, Fig. 2c). The mutation was not found in non-Gypsy population or Gypsy groups out of Spain. The three Gypsy families carrying the mutation do not share geographic origin in Spain. Family 1 lived in Barcelona and was in fact of Portuguese origin, family 2 lives in Madrid and family 3 in Sevilla. Genealogic data from these families, as far as available, revealed no relationship between them, but their common ethnicity suggested the possibility of a common founder for the mutation. We investigated the evolutionary origins of the c.331G>A by studying haplotypes associated with this mutation. Seven microsatellite markers spanning the *CD8A* region were selected for this study. Their relative order and

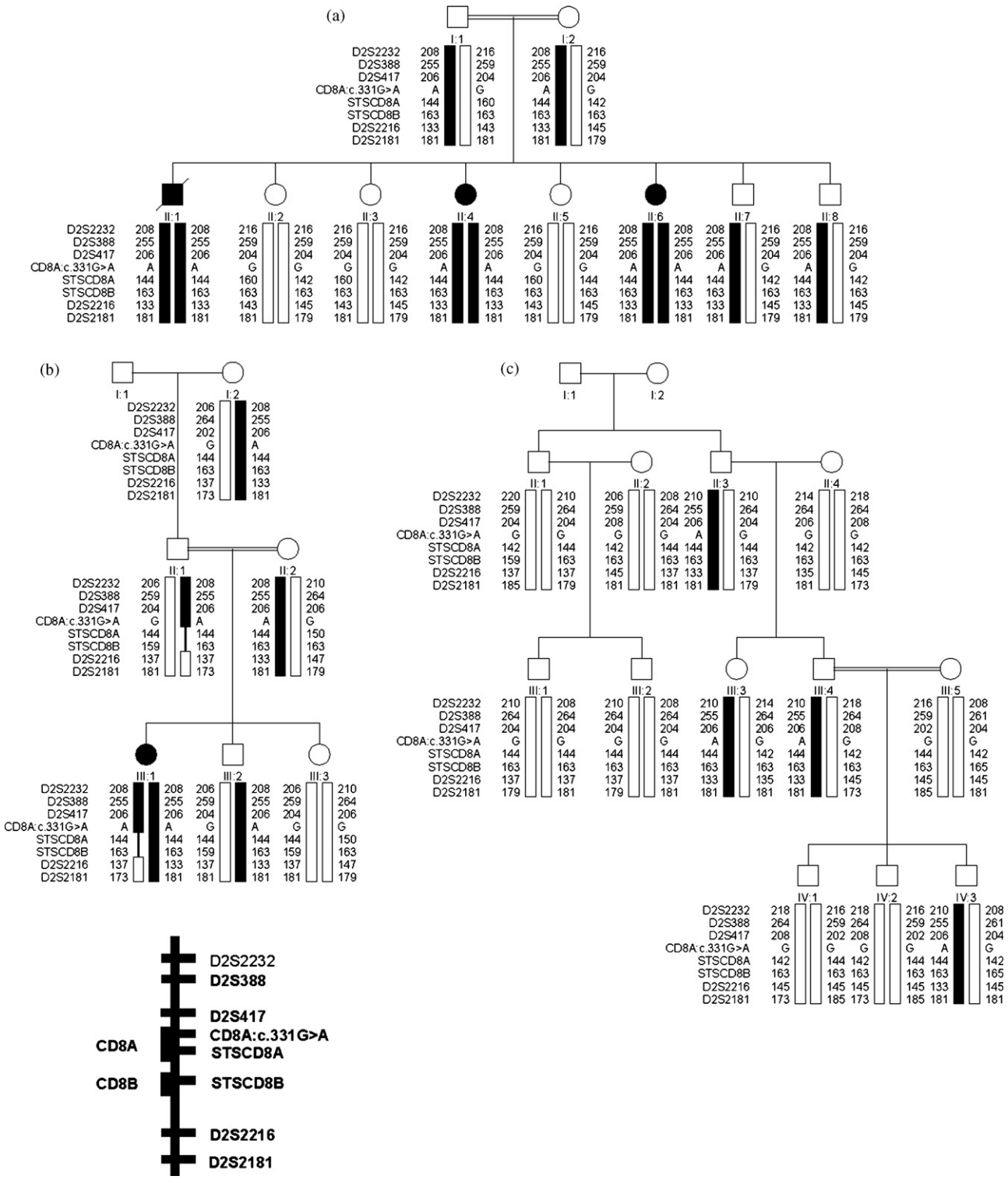

Fig. 2. Pedigrees and haplotype analysis of families 1 (a), 2 (b) and 3 (c) based on the indicated polymorphic markers spanning the *CD8A* region on chromosome 2p32 (inset, shared core haplotype in bold face). Affected clinical status and disease haplotype is depicted in black. The novel found mutation is denoted as CD8A:c.331G>A. Homozygous haplotype segregation was observed in patient II:1 and II:4 from family 1 and patient III:1 from family 2. The order of markers was established by integrating genetic and physical maps.

physical distances were as follows: D2S2232–60 kb–D2S388–936 kb–D2S417–23 kb–*CD8A*:c.331G>A–3 kb–STSCD8A–40 kb–STSCD8B–1464 kb–D2S2216–81 kb–D2S2181 (Fig. 2). The results of this analysis showed that the three families shared a core haplotype associated with the mutation composed of alleles 255, 206, 143, 163, 133 and 181 of markers D2S388, D2S417, STSCD8A, STSCD8B, D2S2216 and D2S2181, respectively, where the D2S417, STSCD8A and STSCD8B are

Table 1
Haplotypes of gypsy origin associated with the c.331G>A mutation in the *CD8A* gene

Marker	Family 1 and family 2	Family 3	Genotype for CEPH individual 134702	Heterozygosity (%)	Allele frequency in gypsy controls, $N = 58$
D2S2232	208	210	210/218	83	208: 14%, 210: 20%
D2S388	255	255	260/264	69	255: 9%
D2S417	206	206	204/204	72	206: 21%
STSCD8A	144	144	144/160	73	144: 36%
STSCD8B	163	163	163/163	28	163: 84%
D2S2216	133	133	135/145	79	133: 4%
D2S2181	181	181	181/181	70	181: 46%

Relative order and physical distances are as follows: D2S2232-60 kb–D2S388–936 kb–D2S417–23 kb–*CD8A*:c.331G>A–3 kb–STSCD8A–40 kb–STSCD8B–1464 kb–D2S2216–81 kb–D2S2181. The marker heterozygosity and allele frequencies were calculated from a sample group of 58 Spanish Gypsies. To allow other laboratories to compare their data with those reported in this work, we provide allele sizes for individual 134702, available from CEPH (Dib et al., 1996).

in close proximity to the mutation (Table 1, Fig. 2). These data indicate the possibility of a common founder allele bearing the c.331G>A mutation causing CD8 deficiency in the population of Spanish Gypsies.

4. Discussion

We have characterized the second case of CD8 deficiency by identifying the p.Gly111Ser mutation of CD8α chain in homozygous state in a Spanish Gypsy simplex case. This mutation had been previously identified in another CD8 deficient Gypsy family in Spain. The study of this second case confirms that the p.Gly111Ser change causes a complete absence of CD8+ lymphocytes and high numbers of double negative (TCRαβ+CD4−CD8−) lymphocytes in peripheral blood. Both affected individuals from families 1 and 2 showed an increased susceptibility to respiratory infections with repeated episodes from childhood, but had positive antiviral serologies that demonstrate previous contact with a diversity of virus and immunological competence to defeat them. The predominant immunophenotype found in the α/β double negative T cells was suggestive of effector cytotoxic cells (De la Calle-Martín et al., 2001). However, patient in family 1 suffered an extensive destruction of the pulmonary parenchyma with bronchiectasias that continuously re-infected with bacteria and severe ventilatory disturbances culminating in the patient's death, while patient in family 2 shows (at present) conservation of the lung tissue (with only a small area of atelectasis in the left inferior lobe) and its functional properties. Data recorded in CD8 deficient families 1 and 2 show that the homozygous status for the p.Gly111Ser mutation translates into a variety of phenotypes, ranging from severe pulmonary disease and death in family 1 proband to absence of symptoms in his sisters, along with a more intermediate disease form exhibited by proband in family 2. Two mechanisms are usually invoked to explain the variability of phenotypes in human immunodeficiencies: (a) the effect of the particular constellation of polymorphisms in other genes with immune function in each individual, and (b) the effect of environmental factors. Proband in family 1 was a heavy chronic smoker (more than 20 cigarettes per day from age 14), which could explain, at least in part, the more aggressive form of the disease he suffered.

The AluI–PCR-RFLP test developed here allowed a rapid analysis of relatives in families 1 and 2 and of sample populations of unrelated individuals. The familial studies confirmed that the p.Gly111Ser mutation is inherited in autosomal recessive form. In the screening of populations we detected another individual (subject IV:3, Fig. 2c) also of Spanish Gypsy origin, that carried the p.Gly111Ser mutation in heterozygous state, while the mutation was not found in non-Gypsies or different Gypsy subpopulations from other European countries. This observation, together with the fact that the p.Gly111Ser mutation is associated with the same CD8-region haplotype in the three families (Fig. 2), suggests that a founder effect for this mutation is responsible for the CD8 deficiency in the subpopulation of Spanish Gypsies.

It is currently accepted that after 100 generations, the average region identical by descent flanking a disease gene will be about 0.7 cM. The disease core haplotype shared by the three families, as far as investigated, spans around 2.5 cM of genetic distance between markers D2S388 and D2S2181. Consequently, the age of the common ancestor of the p.Gly111Ser mutation could be estimated to be around 30 generations. A simple arithmetic calculation, assuming that one generation is 20–25 years, would trace back the origin of the mutation around six to eight centuries ago, after the arrival of the Romani people in Europe in the 11th century in successive migration from the Indian subcontinent. The founder effect observed could suggest that the p.Gly111Ser mutation arose after the entry of Gypsy population in the Iberian Peninsula (dated in the early 15th century) (Fraser, 1992; Singhal, 1992; Liegeois, 1994). The p.Gly111Ser carrier rate (0.4%) is lower than other frequencies reported for single gene disorders in Gypsies, which argues for a relatively young origin of the mutation (Kalaydjieva et al., 2001). However, it cannot be excluded an earlier origin for the mutation occurred in an ancestral genetic isolate that finally gave rise to the Spanish Gypsy subpopulation. Altogether, our data are consistent with the present view of the European Gypsy community like a group of genetic sub-isolates which resulted from secondary founder effects occurred 6–24 generations ago (Gresham et al., 2001; Kalaydjieva et al., 2005), the p.Gly111Ser mutation being probably one of them.

In summary, data until present support that CD8 deficiency is a single gene Mendelian disease of autosomical recessive inheri-

tance and phenotypical variability, prevalent in Spanish Gypsies and caused by the founder mutation p.Gly111Ser in CD8α chain. However, it cannot be excluded that other genetical abnormalities in CD8 protein chains causing the disease may appear in the future. A complete and definitive picture of the clinical spectrum for the disease awaits the study of new cases.

CD8 immunodeficiency should be suspected in young Spanish Gypsy patients suffering repeated and/or severe respiratory infections and a lymphophenotype should be ordered to confirm or discard the absence of CD8+ T cells. Specific recommendations in vaccination and healthy habits (specially avoiding smoking) may be proposed to the affected individuals. The molecular diagnosis should be initially addressed in search of the c.331G>A mutation by means of the PCR-RFLP screening test developed here, which would permit the rapid and inexpensive detection of the mutation in the affected families, genetic counseling and the prevention of this Mendelian disorder.

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