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ORIGINAL PAPER

The 752delG26 mutation in the *RFXANK* gene associated with major histocompatibility complex class II deficiency: evidence for a founder effect in the Moroccan population

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Abstract Major histocompatibility complex class II plays a key role in the immune response, by presenting processed antigens to CD4+ lymphocytes. Major histocompatibility complex class II expression is controlled at the transcriptional level by at least four *trans*-acting genes: *CIITA, RFXANK*, *RFX5* and *RFXAP*. Defects in these regulatory genes cause MHC class II immunodeficiency, which is frequent in North Africa. The aim of this study was to describe the immunological and molecular characteristics of ten unrelated Moroccan patients with MHC class II deficiency. Immunological examinations revealed a lack of expression of MHC class II molecules at the surface of peripheral blood mononuclear cells, low CD4+ T lymphocyte counts and variable serum immunoglobulin (IgG, IgM and IgA) levels. In addition, no MHC class II (HLA DR) expression was observed on lymphoblasts. The molecular analysis identified the same homozygous 752delG26 mutation in the *RFXANK* genes of all patients. This finding confirms the association between the high frequency of the combined immunodeficiency and the defect in MHC class II expression and provides strong evidence for a founder effect of the 752delG26 mutation in the North African population. These findings should facilitate the establishment of molecular diagnosis and improve genetic counselling for affected Moroccan families.

Keywords MHC class II immunodeficiency · *RFXANK* gene · Molecular diagnosis · Founder effect · Morocco

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Introduction

Major histocompatibility complex (MHC) class II molecules are transmembrane proteins playing a key role in the development and control of the immune system [6]. The genes encoding these proteins are tightly regulated and form a multigene family. Defective MHC class II expression causes MHC class II deficiency [8] due to defects in the transacting factors essential for the transcription of MHC class II genes. There are four genetic complementation groups (A, B, C and D), reflecting the existence of four major MHC class II transcription factors [4, 12, 14, 18]. Genes encoding all these factors have been identified. The *MHC2TA* gene (MIM 600005), from complementation group A, encodes the Class II transactivator protein, the master control factor determining cell-type specificity and controlling the induction of MHC II expression [20, 30,

31]. The *RFXANK* (MIM 603200) [19, 21], *RFX5* (MIM601863) [24, 29] and *RFXAP* (MIM 601861) [7, 32] genes belong to complementation groups B, C and D, respectively. These three proteins associate to form the RFX complex, which binds to the X-box *cis*-acting sequence present in MHC II promoters [25]. RFX binding is necessary, but not sufficient for MHC II gene transcription. The expression of a fourth factor, CIITA (Class II transactivator), is also required for transcription [30]. Patients with no membrane expression of MHC class II antigens by antigen-presenting cells cannot mount antigen-specific immune responses and thus present early-onset combined immunodeficiency. They are prone to severe and recurrent bacterial, protozoan and viral infections of the respiratory and intestinal tracts, leading to malabsorption, growth arrest and progressive organ failure. The average life expectancy of these patients is 4 years [13].

MHC II deficiency is inherited as an autosomal recessive trait. A high degree of consanguinity has been observed in affected families, regardless of the complementation group affected. The first reported patient with RFX-ANK deficiency was of North African origin and North African populations remain the most frequently affected [17, 26]. However, MHC II deficiency has been reported in various ethnic groups. Since the first report of combined immunodeficiency due to a lack of expression of MHC class II molecules and the description of four complementation groups, the genes of complementation group B have been found to be the most frequently affected. Most of the patients in this group were of North African origin, and most had the same mutation in the *RFXANK* gene (752delG26) [34]. This gene encodes a transcription factor of 260 amino acids. It consists of ten exons spanning approximately 10 kb and located on chromosome 19p12. No molecular analysis of Moroccan patients has ever been carried out in Morocco. We investigated the possibility of a founder effect in this population, with a view to providing prenatal diagnosis. This approach would also pave the way for haematopoietic stem cell transplantation (HSCT), which is currently not available to our population. We therefore characterised the immunological and genetic features of ten Moroccan patients with MHC class II deficiency.

Materials and methods

Patients

We enrolled ten unrelated Moroccan patients (7 girls and 3 boys) with MHC class II deficiency originating from different regions. The median age was 6.3 years (range, 0.6–12 years). Parents were consanguineous in eight of the ten patients. The medical history of patients was marked by several episodes of diarrhoea and recurrent respiratory tract infections. We were able to include both of the parents for seven of the families included in this study. Informed consent for participation in this study was obtained from the parents.

Immunological analysis and cell culture

Serum immunoglobulin concentration was determined by radial immunodiffusion in all patients. HLA DR expression on PHA blasts was assessed on peripheral blood leukocytes (PBL) cultured in 96-well plates (10^6 cells/ml), at 37°C, under an atmosphere containing 5% CO_2. These cells were cultured in RPMI 1640 supplemented with 10% inactivated human serum AB, L-glutamine (100 mM), penicillin (100 units/ml) and streptomycin (100 µg/ml). PHA (10 µg/ml) was added, and the cells were incubated for a further 3 days. We then assessed expression of the CD25 and HLA DR activation markers on blasts, with a FACScan flow cytometer (Becton Dickinson).

Classical procedures were used for immunofluorescence assays on the FACScan flow cytometer. Cells were stained with monoclonal antibodies directed against CD3, CD4, CD8, CD19 and HLA class II molecules, labelled with either fluorescein (FITC) or phycoerythrin (PE). We analysed about 5,000 cells by flow cytometry.

Molecular analysis

We extracted genomic DNA from whole blood with the Qiagen DNAeasy kit (Qiagen, Hilden, Germany). We screened for the 752delG26 mutation (deletion of 26 bp) in all patients, by amplifying the intron5/exon6 region of the *RFXANK* gene by PCR, in a total volume of 50 µl containing 100 ng template DNA and 10 pmol of each primer: F-GGTTCTCTAGATTGGCAGCACTGGGGATAG and R-GCTACGAATTCCAGCAGACA CAGCCAAAAC, as previously described [34]. The PCR conditions were as follows: 94°C for 5 min, followed by 35 cycles of 94°C for 30 s, 60°C for 30 s and 72°C for 45 s and a final DNA extension at 72°C for 5 min. PCR products were separated by electrophoresis in a 2% agarose gel. The primer pair used generates a PCR product of 244 bp. A 218 bp PCR product is generated if the 752delG26 deletion is present. The presence of this deletion was also confirmed by automatic sequencing with the ABI BigDye Terminator v 3.1 standard kit and an ABI 3130 Genetic Analyzer.

Results

Clinical examination showed that all patients had severe chronic diarrhoea and recurrent respiratory tract infections. However, eight patients had also mucocutaneous candidi-

asis, and six patients had alveolar/interstitial pneumonia. The median age at onset of the first clinical signs was 7 months. All patients were vaccinated at birth with bacillus Calmette–Guérin (BCG), and none developed BCGosis. All patients received monthly treatment with intravenous immunoglobulin (IVIG) (0.5 g/kg) for a mean of 2 years and 3 months. The diagnosis of MHC II immunodeficiency was confirmed by the defective expression of MHC class II antigens on peripheral mononuclear blood cells (PBMCs), with an absence of HLA DR surface expression on resting lymphocytes (Fig. 1B).

Immunological investigations (Table 1) showed that all patients had a small proportion of CD4+ T lymphocytes (10 to 19%) with normal levels of CD19+ B lymphocytes (9% from 36%). Flow cytometry showed CD8+ T lymphocyte levels to be normal in four patients, high in four patients and low in two patients.

Serum immunoglobulin concentrations were heterogeneous, with different levels of IgG, IgM and IgA in different patients. Studies of PHA-activated lymphocytes showed that the CD25 activation marker was normally expressed, whereas there was no HLA DR expression on these PHA lymphoblasts (Table 2; Fig. 1C and D).

We analysed the size of the PCR products encompassing exon 6 for each patient and the members of his or her family. The 752delG26 mutation, consisting of a deletion of 26 bp, was detected in all patients in the homozygous state (a single 218 bp band was observed in the agarose gel). All the parents tested presented this mutation in the heterozygous state, with two bands, 244 bp and 218 bp in size, detected on agarose gels (Fig. 2). The 752delG26 deletion spans the 3′ splice site of exon 5, extending 1 bp into exon 6. The presence of this deletion therefore leads to loss of the splice acceptor site of exon 6. The 752delG26 mutation results in a frame shift and a premature stop codon. Consanguinity was strongly implicated in primary MHC class II deficiency in Morocco, eight out of ten patients were born to consanguineous parents.

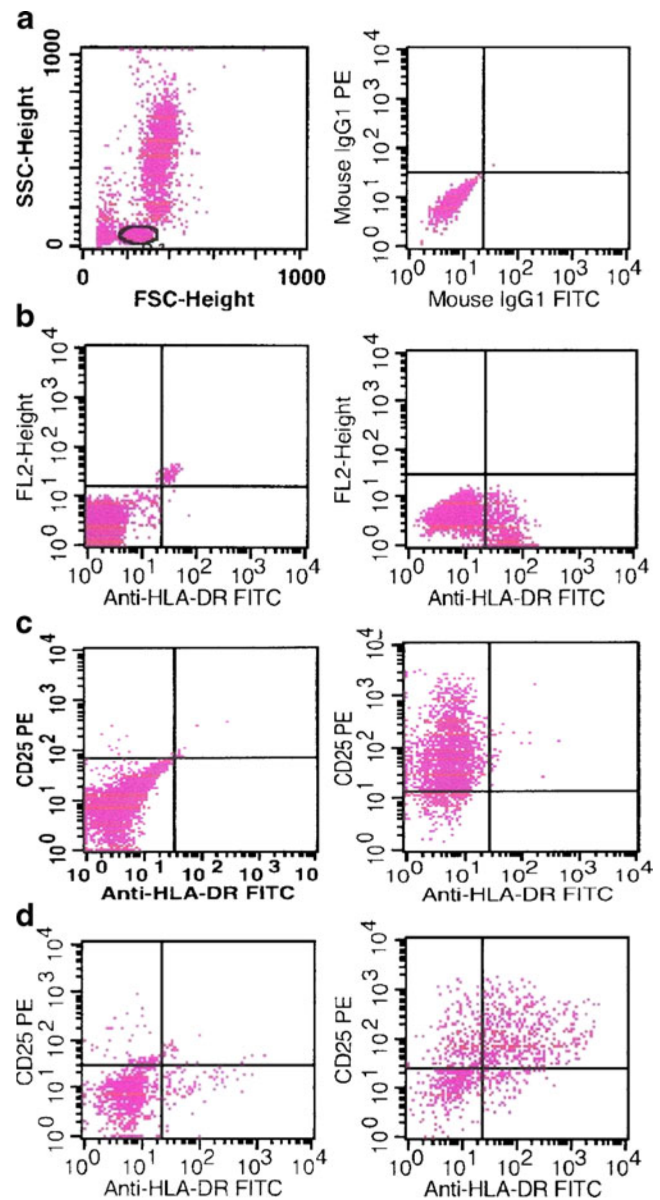

Fig. 1 Histograms generated by FACScan flow cytometry. **A**: Isotypic control; **B**: On the left, a patient with MHC II deficiency; on the right, a subject with normal MHC II expression; **C**: on the left, a patient before PHA stimulation (no expression of CD25 and HLA DR); on the right, a patient after PHA stimulation (expression of activation molecule CD25, but not of HLA DR); and **D**: control showing HLA DR expression before PHA stimulation (*on the left*) and the expression of both HLADR and CD25 after PHA stimulation (*on the right*)

Discussion

Combined immunodeficiency associated with the defective expression of MHC class II, is a rare hereditary disease with autosomal recessive transmission [9, 11, 34]. This disorder is genetically heterogeneous but is considered to be associated with a single phenotype. Four different complementation groups (A, B, C and D) have been identified, corresponding to mutations in four different MHC II regulatory genes: *MHC2TA* for group A, *RFXANK* for group B, *RFX5* for group C and *RFXAP* for group D [4, 12, 18].

This disease seems to be particularly common in North Africa, where the frequency of consanguineous marriages is high [1, 3, 5, 15]. The first clinical manifestations usually appear early in life (mean of 4 months), generally between the ages of 3 and 8 months [10, 13]. Age at onset of the first clinical signs was slightly later in our series than in other series.

The clinical findings for our ten patients are conclusive. Similar signs or severe infection and death at an early age were reported for one or several of the siblings of the

Table 1 Immunological features of Moroccan patients with MHC II deficiency

	Age	Serum Ig concentration (g/l)			Immunophenotyping of leukocytes				
		IgG*	IgM*	IgA*	CD3+*	CD4+*	CD8+*	CD19+*	DR
1	6M	5.2 (2.4–8.8)	0.36 (0.2–1)	0.09 (0.1–0.5)	39% (51–77)	19% (35–56)	20% (12–23)	26% (11–41)	NE
2	6M	3.92 (2.4–8.8)	0.34 (0.2–1)	0.28 (0.1–0.5)	72% (51–77)	12% (35–56)	57% (12–23)	22% (11–41)	NE
3	2Y3M	2.5 (3.7–15.8)	0.32 (0.5–2.2)	0.42 (0.3–1.3)	50% (56–77)	16% (28–47)	34% (16–30)	17% (14–33)	NE
4	2Y6M	5.08 (4.9–16.1)	0.37 (0.5–2)	0.42 (0.4–2)	70% (56–77)	10% (28–47)	50% (16–30)	22% (14–33)	NE
5	3Y4M	ND	ND	ND	24% (56–77)	12% (28–47)	07% (16–30)	36% (14–33)	NE
6	1Y6M	ND	ND	ND	22% (53–75)	14% (32–51)	07% (14–30)	32% (16–35)	NE
7	6M	2.5 (2.4–8.8)	0.32 (0.2–1)	0.42 (0.1–0.5)	84% (51–77)	12% (35–56)	65% (12–23)	09% (11–41)	NE
8	8M	2.91 (3–10.9)	0.48 (0.6–2.1)	0.42 (0.2–0.7)	40% (49–76)	27% (31–56)	11% (12–24)	32% (14–37)	NE
9	12Y	8,55 (5.4–16.1)	0.59 (0.5–1.8)	0.35 (0.7–2.5)	83% (56–84)	10% (31–52)	61% (18–35)	10% (6–23)	NE
10	6M	2.11 (4.9–16.1)	0.32 (0.5–2)	0.35 (0.4–2)	45% (51–77)	15% (35–56)	23% (12–23)	31% (11–41)	NE

Ig Immunoglobulin, *ND* Not determined, *Y* Years, *M* Months

* Normal values for age are given between brackets [28, 33]

NE: Not expressed

patients. The infectious symptoms were refractory chronic diarrhoea, mucocutaneous candidiasis and recurrent respiratory tract infections leading to a failure to thrive and rapidly leading to diagnosis of the immune defect. Opportunistic infections caused by bacteria, *Pneumocystis jirovici*, *Candida* and viruses were also generally found. The clinical signs of our patients were similar to those reported in other studies of patients with MHC class II deficiency [2, 13, 27].

All the patients in this group had the same immunological phenotype, with an absence of HLA DR on the surface of resting lymphocytes and low CD4+ T lymphocyte counts. Our findings for humoral immunity (defective co-operation between T and B cells) are consistent with previous reports, with no hyperIgM cases identified [2].

Genetic analysis showed that all our patients had the same mutation, the 752delG26 mutation in the *RFXANK* gene. These results are similar to those previously reported for the North African population [34]. Several studies have also shown that about half the patients have mutations affecting complementation group B and that 73% of the corresponding kindreds are of North African descent (Morocco, Algeria and Tunisia). The 752delG26 mutation in the *RFXANK* gene is present in 92% of affected North African kindreds, and the most frequent molecular defect identified in cases of MHC class II deficiency is *RFXANK* mutation [16, 22, 34]. However, other mutations in the *RFXANK* gene have been characterised in patients of different ethnic origins (French/Spanish, Italian and Turkish) [19, 21, 34, 35]. These mutations are all clustered in exons 5 to 9, in a region encoding the ankyrin repeats, which are important for the function of the protein [23]. It has been suggested that the most frequent mutation, 752delG26, originated in an ancestor from the Maghreb (North Africa) [34]. This deletion leads to the formation of an mRNA lacking exons 5 and 6. The excision of these exons results in a frame shift and the introduction of a premature stop codon in exon 9. Our molecular analysis of the *RFXANK* gene

Table 2 Marker expression after lymphocyte activation without (–) and with PHA (+) in the control and patient number 5

Markers	Control		Patient	
	–PHA	+PHA	–PHA	+PHA
DR	20%	72%	NE	0.5%
CD25	9%	67%	2%	64%
CD2	87%	82%	29%	58%
CD5	9%	22%	18%	44%
CD19	82%	85%	35%	73%

NE Not expressed

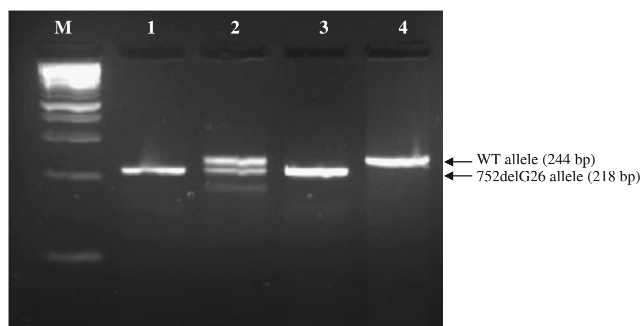

Fig. 2 Photograph of a gel showing the 752delG26 deletion of the *RFXANK* gene. M: Size markers. 1 and 3: Patients homozygous for the mutant allele with the 26 bp deletion. 2: Subject heterozygous for this deletion. 4: Control subject without the deletion

showed that all ten patients were homozygous for the 752delG26 mutation. These results further confirm previous reports of a high frequency of this mutation in patients from Morocco and the Maghreb [19, 34]. Eight of the patients died at various ages, from 2 to 12 years, and the main causes of death are primarily due to severe infections, and none of them received a bone marrow transplantation. However, two patients are still alive, and one of the patients has undergone bone marrow transplantation.

These results demonstrate that the 752delG26 deletion, in the *RFXANK* gene, is the most common mutation in Moroccan patients with MHC II deficiency, consistent with a possible founder effect of this mutation, as previously suggested by Wiszniewski et al. [34]. The existence of such a founder effect should facilitate molecular diagnosis and improve genetic counselling for the Moroccan population. Further surveys warrant the study of a cohort of patients in order to determine more precisely the role of genetics, epigenetic and environmental factors that influence the phenotype of patients with MHC II deficiency, in Morocco.

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