

# Homozygous mutations in *LPIN2* are responsible for the syndrome of chronic recurrent multifocal osteomyelitis and congenital dyserythropoietic anaemia (Majeed syndrome)

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**Background:** Majeed syndrome is an autosomal recessive, autoinflammatory disorder characterised by chronic recurrent multifocal osteomyelitis and congenital dyserythropoietic anaemia. The objectives of this study were to map, identify, and characterise the Majeed syndrome causal gene and to speculate on its function and role in skin and bone inflammation.

**Methods:** Six individuals with Majeed syndrome from two unrelated families were identified for this study. Homozygosity mapping and parametric linkage analysis were employed for the localisation of the gene responsible for Majeed syndrome. Direct sequencing was utilised for the identification of mutations within the genes contained in the region of linkage. Expression studies and in silico characterisation of the identified causal gene and its protein were carried out.

**Results:** The phenotype of Majeed syndrome includes inflammation of the bone and skin, recurrent fevers, and dyserythropoietic anaemia. The clinical picture of the six affected individuals is briefly reviewed. The gene was mapped to a 5.5 cM interval (1.8 Mb) on chromosome 18p. Examination of genes in this interval led to the identification of homozygous mutations in *LPIN2* in affected individuals from the two families. *LPIN2* was found to be expressed in almost all tissues. The function of *LPIN2* and its role in inflammation remains unknown.

**Conclusions:** We conclude that homozygous mutations in *LPIN2* result in Majeed syndrome. Understanding the aberrant immune response in this condition will shed light on the aetiology of other inflammatory disorders of multifactorial aetiology including isolated chronic recurrent multifocal osteomyelitis, Sweet syndrome, and psoriasis.

Multifactorial disorders, such as psoriasis and inflammatory bowel disease, result from complex interactions between a number of predisposing factors, including genotypes at one or more loci and a variety of environmental exposures that trigger, accelerate, or exacerbate the disease process. There are several approaches to the identification of aetiologic genetic factors in a complex disease, such as genome-wide linkage and association studies or targeted examination of a specific gene or region. In the latter approach, the specific gene that is targeted in a study is chosen due to its physiologic role, its presence in a chromosomal break point, or its causal role in a Mendelian disorder sharing a similar phenotype. The exploration of the role of a rare Mendelian disorder gene in a more common disorder that shares a phenotype with the Mendelian disorder has proven successful on multiple occasions, such as in *WFS1* and deafness,<sup>1</sup> *IRF6* and cleft lip and/or palate,<sup>2</sup> and rare variations in *ABCA1*, *APOA*, and *LCAT* and low plasma levels of HDL cholesterol.<sup>3</sup>

Chronic recurrent multifocal osteomyelitis (CRMO; MIM 259680) was clinically described in 1970, but it became recognised as a separate clinical entity in 1972.<sup>4–5</sup> CRMO is an autoinflammatory disorder, that is, it is characterised by seemingly unprovoked inflammation in the absence of autoimmunity or infectious causes. It is frequently associated with a cutaneous inflammatory condition such as pustulosis palmoplantaris (PPP),<sup>6–8</sup> Sweet syndrome,<sup>9–11</sup> and psoriasis.<sup>12–14</sup> Although CRMO usually occurs sporadically, several observations point to a genetic component to its aetiology. There are at least three reports of affected siblings with normal

parents, suggesting autosomal recessive inheritance.<sup>8 15 16</sup> A published article reports one monozygotic twin pair concordant for CRMO and a second monozygotic twin pair with CRMO diagnosed in one twin and PPP in the other.<sup>17</sup> In the same article, the authors report a child with CRMO whose father has developed chronic non-infectious osteomyelitis of the sternum as an adult, suggesting autosomal dominant inheritance with variable expression. Also, it has been recently shown through an association study that there is a susceptibility locus for isolated CRMO on chromosome 18q21.3–22.<sup>17</sup> Furthermore, there is a similar murine disorder (chronic multifocal osteomyelitis), resulting from a recessive mutation in a gene mapped to a region on mouse chromosome 18, which is homologous to the human region containing the CRMO susceptibility locus.<sup>18 19</sup>

The high prevalence of consanguinity among Jordanians makes it possible to identify inbred families with rare recessive disorders that are large enough to independently establish linkage.<sup>20–22</sup> In these families, individuals with rare recessive traits are likely to have inherited both copies of the mutated gene from a common ancestor and are thus identical by descent (homozygous) not only at the trait locus but also at neighbouring marker loci.

The syndrome of CRMO and congenital dyserythropoietic anaemia (CDA), or Majeed syndrome, is a rare autosomal recessive disorder first described in 1989.<sup>23</sup> It is composed of three prominent features: CRMO, CDA, and an inflammatory

**Abbreviations:** CDA, congenital dyserythropoietic anaemia; CRMO, chronic recurrent multifocal osteomyelitis; PPP, pustulosis palmoplantaris

dermatosis.<sup>23–25</sup> Majeed syndrome has been described only in two families, although two reported sporadic cases have a similar phenotype.<sup>26, 27</sup> The CRMO starts in infancy and continues relentlessly leading to retarded growth or joint contractures or both.<sup>23–25</sup> The CDA is variable in severity and characterised by microcytosis, both peripherally and in the bone marrow, which is not typical of any of the well characterised and classified forms of CDA.<sup>23–25</sup> The neutrophilic dermatosis, Sweet syndrome, is the common reported skin inflammation.<sup>23</sup> Our group has previously reported six individuals with Majeed syndrome from three sibships from two unrelated families.<sup>23–25</sup> The gene responsible for this complex syndrome was mapped to a 5.5 cM interval on the short arm of chromosome 18. We then identified that homozygous mutations in *LPIN2* are responsible for Majeed syndrome.

## METHODS

### Subjects

We identified a family from Jordan with four affected individuals. A detailed family history was obtained and a four generation pedigree was constructed (fig 1). The family members who agreed to participate in this study are four affected individuals and seven of their relatives (family A). A second family with two affected individuals in one sibship and first cousin parents was identified, but only the affected individuals participated in the study (family B). Informed consent, approved by a local (Jordanian) institutional review board, was provided by the participants or their legal guardian. We obtained 1064 controls from the HGDP-CEPH Human Genome Diversity Cell Line Panel,<sup>28</sup> an additional 86 unrelated CEPH controls, and 367 anonymous Jordanian control DNA samples.

### Genotyping

DNA was extracted from whole blood by standard procedures. A genome-wide scan for linkage was performed with the ABI PRISM linkage mapping set of fluorescent micro-satellite DNA markers, version 2 (PE Biosystems, Foster City, CA) using affected individuals from family A. To test the regions that showed homozygosity in the screen, typing of all family members with several markers within that region was performed. To refine the region of interest, an additional set of 17 micro-satellite markers was chosen from Marshfield genetic map.<sup>29</sup> Amplification of these markers was performed by PCR under standard conditions.<sup>30</sup> The amplified products were analysed on 6% denaturing polyacrylamide gel (8 M urea) and stained with silver nitrate according to standard protocols.<sup>31</sup>

### Statistical analysis

LOD score analysis was performed using MLINK of the LINKAGE 5.1 computer program package.<sup>32</sup> Multipoint linkage analysis was performed using SIMWALK2.<sup>33, 34</sup> The analysis was carried out assuming autosomal recessive inheritance, full penetrance, and no phenocopies. An assumed disease allele frequency of 0.001 was used in the initial analysis, but was varied from 0.01 to 0.001 to examine the strength of linkage. The allele frequencies for all the markers were assumed to be equal, since the true allele frequency for this population is unknown. We also varied the marker allele frequencies for measurement of the strength of linkage. A standard LOD score of 3 or higher was used for significance. The Marshfield genetic map was used as the reference map for linkage.<sup>29</sup>

### Exclusion of candidate genes and mutation detection

The region of linkage contains 11 known and predicted genes as obtained from the genome databases ([http://](http://www.ncbi.nlm.nih.gov)

<http://genome.ucsc.edu>; <http://www.ensembl.org>). We prioritised the genes in the region according to their likelihood for producing the phenotype of Majeed syndrome, as follows: *TGIF*, *HEC (KNTC2)*, *EMILIN2*, *LPIN2*, *METTL4*. The remaining six genes were tissue specific (muscle or neuronal tissue) or the predicted function could not be logically related to the phenotype. The genomic sequences of the candidate genes were obtained from the genome databases and the primer pairs were designed to amplify the exons and the splice sites of the genes using the Primer3 program ([http://frodo.wi.mit.edu/cgi-bin/primer3/primer3\\_www.cgi](http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi)). The PCR amplification products were run on an agarose gel, and the bands containing the amplified products were recovered and extracted from the agarose by column purification. The amplified products were sequenced using an automated ABI sequencer with dye terminator chemistry. Sequencing was performed for an obligate carrier and an affected individual from family A, an affected individual from family B, and a normal unrelated control. Once a mutation was identified in family A, the same exon was sequenced in all family members to see if it segregated with both the phenotype and haplotype. We then screened the control populations by both SSCP analysis and sequencing of suspicious bands. For family B, we sequenced the exon that carried the mutation in the other affected individual and developed an SSCP assay to screen the control populations for that mutation. The sequences of the primers used for the amplification of the exons and for the SSCP analysis are included in the supplementary material which is available at <http://jmedgenet.com/supplemental>.

### Structure of *LPIN2* and its transcript

The structure of *LPIN2* was obtained from the public genomic databases (D87436; NM\_014646). Five human overlapping cDNA clones covering the entire cDNA of *LPIN2* were obtained (Research Genetics, Invitrogen, Carlsbad, CA) and sequenced for confirmation of the cDNA sequence. These clones were used as positive controls in the expression studies.

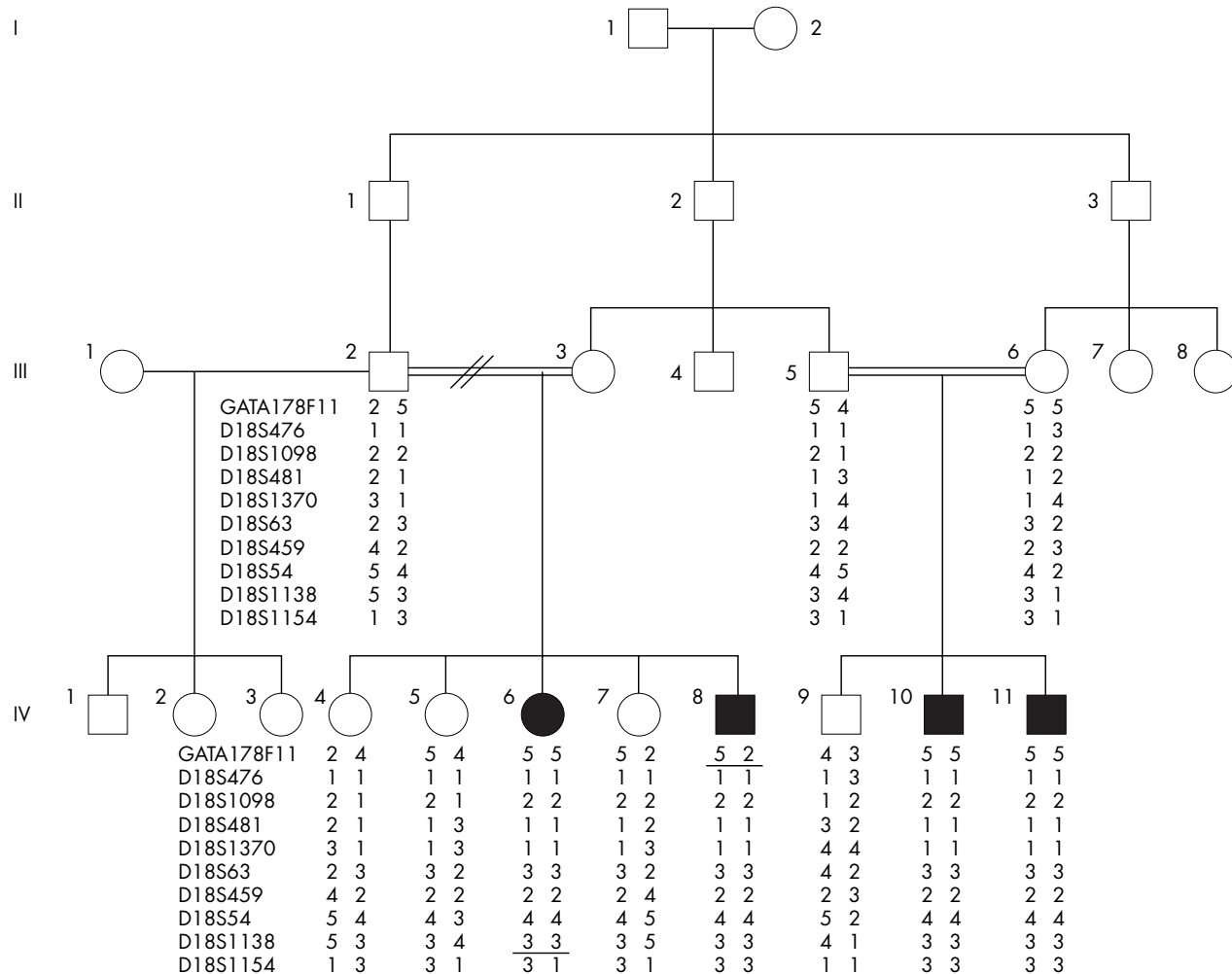
### Expression analysis

Pre-made northern blots containing 2 µg of poly(A)+RNA per lane from multiple human tissues were obtained commercially (Clontech Laboratories, Palo Alto, CA). Primers were designed to amplify a 348 bp stretch of mRNA or cDNA from exons 18, 19, and 20. The primers were used to generate a probe by amplification of one of the obtained clones and the probe was labelled radioactively by random priming (Amersham Pharmacia Biotech, Piscataway, NJ). The probe was hybridised overnight in hybridisation buffer at 42°C. The blot was washed three times with 2×SSC:0.1% SDS for 15 min each wash. The blot was then exposed to Kodak X-Omat film at –80°C. The first strand cDNA was obtained commercially (Invitrogen) and the same primers were used in a standard PCR reaction.

## RESULTS

### Clinical picture

Our group has identified two Jordanian families with Majeed syndrome. Family A had four affected individuals from two sibships (IV-6, IV-8, IV-10, IV-11; fig 1) and family B had two affected siblings (B-1, B-2). All six affected individuals from both families had: (i) CRMO (classic radiographs, multifocal isotope uptake on bone scan, prolonged course, and no response to i.v. antibiotic treatment) that was clinically manifest as recurrent bone and joint pain, (ii) CDA (anaemia with dyserythropoiesis, hypochromia, and microcytosis evident on both blood smear and bone marrow aspirate examination), (iii) recurrent fevers (occurring every 2–4 weeks,



**Figure 1** Pedigree of family A showing the haplotypes for 10 microsatellite markers within the region of linkage for all family members and showing the two cross over events.

lasting 3–4 days/episode), and (iv) growth failure. The phenotype was more severe in family B, presenting with higher fevers, more extensive osteomyelitis, and severe transfusion dependent anaemia (table 1).<sup>24</sup>

Cutaneous inflammation is also seen in Majeed syndrome. In family A, Sweet syndrome was definitively diagnosed in two affected individuals (IV-10 and IV-11), while two other affected individuals had a history of rash that was consistent with Sweet syndrome (IV-6 and IV-8). The two affected individual from family B did not have Sweet syndrome, but

one of them (B-1) had a history of cutaneous pustulosis. One obligate carrier (III-5) had severe psoriasis and III-3 was reported to have psoriasis as well but was not examined. One affected individual from family B (B-1), evaluated at the age of 21 years, had contractures of upper and lower extremity joints, no sexual development, and maxillary bone hyperplasia. The other affected individual from family B (B-2) was ascertained at the age of 2.5 years and had not yet developed contractures or any skin manifestation. The clinical picture of the six affected individuals is summarised in table 1.

**Table 1** Summary of the clinical features of the six affected individuals

Clinical feature	A-IV-6	A-IV-8	A-IV-10	A-IV-11	B-1	B-2
CRMO						
Bone and joint pain	+	+	+	+	+	+
Fever	Mild	Mild	Mild	Mild	High	High
Radioisotope scan	+	+	+	+	+	+
CDA						
Microcytosis	+	+	+	+	+	+
Blood transfusion	No	No	No	No	Frequent	Frequent
Hb electrophoresis	Normal	Normal	Normal	Normal	Normal	Normal
Skin						
Sweet syndrome	Yes	Yes	By report	Yes	No	No
Other					Pustulosis	
Relative with psoriasis	Yes	Yes	Yes	Yes	NA	NA

## Linkage analysis

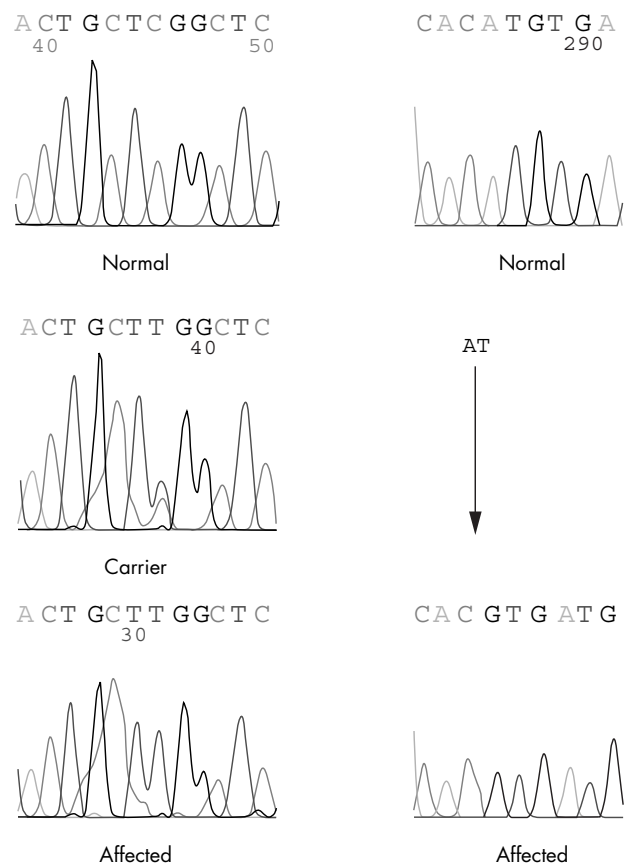
The genome-wide scan showed four regions of homozygosity and all but one were excluded by genotyping all family members for markers within the region of homozygosity. The region of interest, on the short arm of chromosome 18, was refined using 17 microsatellite markers. A maximum two-point LOD score of 3.48 was obtained for marker D18S1138 at a recombination fraction ( $\theta$ ) = 0 with recombination events at markers GATA178F11 and D18S1154, both marking the region of linkage (table 2). Multipoint linkage analysis produced a maximum LOD score of 4.4 (results not shown). The haplotypes for 10 different markers in the region of linkage were constructed for all the participating family members (fig 1). The minimal region of homozygosity in the affected individuals was 5.5 cM between the microsatellite markers GATA178F11 from the telomeric end and D18S1154 from the centromeric end.<sup>29</sup> The two affected individuals from family B were homozygous for the 10 markers but for different alleles and so there was no common haplotype (results not shown).

## Exclusion of candidate genes

The markers that define the Majeed syndrome critical region span about 5.5 cM (genetic distance), which is about 1.8 Mb (physical distance) and contains 11 known and predicted genes. *LPIN2*, which was identified due to a lipin domain it shares with *LPIN1*, was the fourth on our list of candidate genes. No mutations were identified by sequence analysis in the genes *TGIF*, *KNTC2*, and *EMILIN2*, but two mutations were found in *LPIN2*.

## Mutation detection

A missense mutation c.2201C→T (S734L) was identified, which segregates with the phenotype in family A (fig 2). The mutation replaces a polar serine with a non-polar leucine and was not found in 1064 controls obtained from the HGDP-CEPH Human Genome Diversity Cell Line Panel or in 86 unrelated CEPH controls (a total of 1150 individuals and 2300 chromosomes). However, this mutation has a frequency of 0.005 (four heterozygotes in 734 chromosomes) in 367 unrelated ethnically matched (Jordanian) controls. The serine at 734 is conserved in human and mouse lipin proteins, and is also conserved in chimpanzee, mouse, rat, chicken, frog, blowfish, mosquito, honey bee, fruit fly, and the nematode *Caenorhabditis elegans* (fig 3). In family B, we identified a frame shift mutation, c.540-541delAT, which changes the amino acid in position 180 from threonine to be followed by a stop codon in position 181 (fig 2). This mutation was not identified in any individual from the three groups of controls (a total 1517 individuals and 3034 chromosomes).



**Figure 2** (A) The sequence of exon 17 of *LPIN2* in a normal control, a carrier, and in an affected individual from family A showing the c.2201C→T (S734L) mutation. (B) The sequence of exon 4 in a normal control and in an affected individual from family B showing the c.540-541delAT frame shift mutation. The arrow is pointing to the homozygous deletion in the affected individual.

## Structure of *LPIN2* and its transcript

*LPIN2* (lipin 2), previously called *KIAA0249*,<sup>35</sup> consists of 20 exons spanning about 95 kb and producing a 6228 bp mRNA transcript. The coding sequence is 2691 bp producing an 896 amino acid protein. The translation initiation codon is in exon 2 and the stop codon is at the beginning of exon 20. All of exon 1 and most of exon 20 are untranslated, representing the 5' and 3' untranslated regions, respectively. The lipin 2 protein potentially localises to the nucleus as it has a putative nuclear localisation signal. Lipin proteins constitute a novel family of nuclear proteins containing at least three members

**Table 2** Linkage analysis results and LOD scores

Marker	Name	cM	Base pairs starts	Base pairs ends	Recombination fraction ( $\theta$ )						
					0.0	0.01	0.05	0.10	0.20	0.30	0.40
	GATA178F11	2.84	2113098	2113470	∞	1.29	1.74	1.72	1.37	0.90	0.42
D18S476	AFM299YF1	2.84	2167796	2168139	1.26	1.23	1.10	0.94	0.64	0.36	0.14
D18S1098	AFMA175YE9	5.58	2859597	2859941	1.68	1.64	1.5	1.32	0.96	0.60	0.26
D18S481	AFM321XC9	6.94	3056133	3056372	3.28	3.21	2.92	2.56	1.82	1.10	0.46
D18S1370	ATA45G06	6.94	3147651	3147789	3.07	3.00	2.73	2.38	1.67	0.99	0.39
D18S63	AFM205TD6	8.3	3428520	3428862	2.72	2.66	2.41	2.09	1.46	0.86	0.34
D18S459	AFM238YD8	8.3	3524819	3525147	1.95	1.91	1.74	1.53	1.08	0.65	0.28
D18S54	AFM080XD7	8.3	3649285	3649553	3.41	3.33	3.04	2.67	1.91	1.16	0.49
D18S1138	AFMB360YH1	8.3	3662022	3662357	3.48	3.41	3.12	2.74	1.98	1.22	0.54
D18S1154	AFMA056YE1	8.3	3882012	3882371	∞	0.88	1.33	1.31	0.97	0.54	0.17

<i>H sapiens</i>	HSINENGYKFLYCS	SARAIGMADMTRGYLHW
<i>P troglodytes</i>	HSINENGYKFLYCS	SARAIGMADMTRGYLHW
<i>M musculus</i>	HSINENGYKFLYCS	SARAIGMADMTRGYLHW
<i>R norvegicus</i>	HSINENGYKFLYCS	SARAIGMADMTRGYLHW
<i>G gallus</i>	HSINENGYKFLYCS	SARAIGMADMTRGYLHW
<i>X laevis</i>	HTIHENGYKFLYCS	SARAIGMADMTRGYLHW
<i>T nigroviridis</i>	HSVHENDYKFLYCS	SARAIGMADMTRGYLHW
<i>A gambiae</i>	IEENGYKMLYLS	SARAIGQAKTTRGYLQ
<i>A mellifera</i>	IKNNGYKLLYLS	SARAIGQAKVTREYL
<i>D melanogaster</i>	IEQNGYKLLYLS	SARAIGQSRLTREYL
<i>C elegans</i>	IKNNGYKMVYLS	SRAIGQSHTTKQYL

**Figure 3** The serine of the S734L mutation is conserved across species. The figure shows the conservation across chimpanzee, mouse, Norway rat, red jungle fowl, African clawed frog, blowfish, mosquito, honey bee, fruit fly, and the nematode *C elegans*.

in mammalian species and paralogs in distantly related organisms from human to yeast.<sup>36</sup>

### Expression analysis

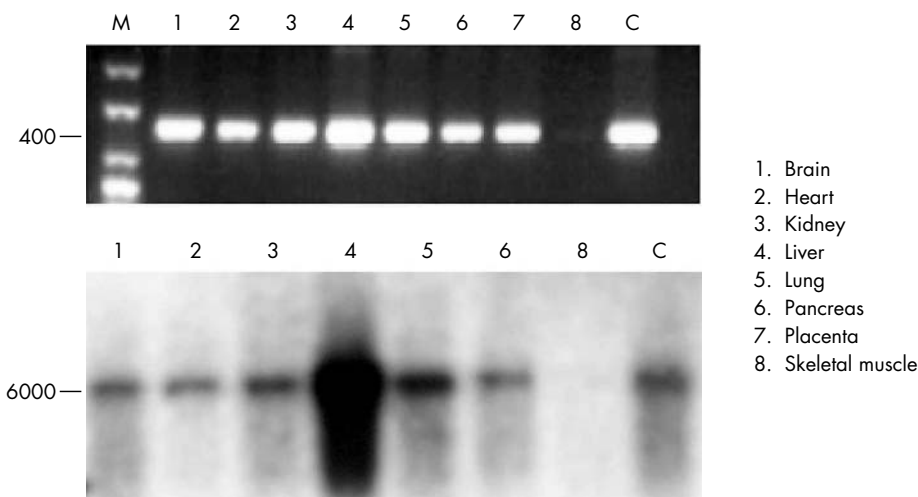
Global expression of *LPIN2* was determined by probing a multiple human tissue northern blot that contains a variety of adult tissues. This experiment determined that *LPIN2* is expressed in liver, lung, kidney, placenta, spleen, thymus, lymph node, prostate, testes, small intestine, and colon. It is not expressed in skeletal muscle or in colon that is stripped from its mucosa. There is only one identifiable transcript with a size of about 6 kb. First strand cDNA from multiple human tissues were obtained and expression was examined using primers designed to amplify a 348 bp stretch of cDNA. The expression pattern was similar to the results of the northern blot analysis (fig 4).

### DISCUSSION

Majeed syndrome is composed of three prominent clinical components. The first is CRMO which, unlike isolated CRMO, has an earlier age at onset, more frequent episodes, shorter and less frequent remissions, and is probably life long. The second is CDA, which is characterised by microcytosis both peripherally and in the bone marrow. It can be variable in severity ranging from a mild, unnoticeable anaemia to a blood transfusion dependent form. The third is an inflammatory dermatosis, which can be Sweet syndrome or

pustulosis. From clinical observation, it seems that the skin inflammation may manifest in carriers, although the number is too small to clearly state this fact. We propose that Majeed syndrome is an autoinflammatory disorder and falls into the category of periodic fever syndromes.

Majeed syndrome is an autosomal recessive disorder, since male and female siblings are affected and the parents are normal and consanguineous in the two described families. We identified that homozygous mutations in *LPIN2* are responsible for the phenotype in the affected individuals in both families. One mutation, c.540-541delAT, is a frame shift mutation that produces a premature stop codon producing a truncated protein that is 180 amino acids long. Since we do not have DNA samples from other family members and could not test for segregation of the mutation within the family, we elected to screen a large control population. We did not identify the mutation in 1150 CEPH controls or in 367 ethnically matched controls. The second mutation, S734L, is a missense mutation that replaces a highly conserved serine with a leucine and segregates with the phenotype in the family. It was not found in a panel of 1150 CEPH controls of different ethnicities. The mutant allele frequency in the ethnically matched controls was 0.005. It is possible that this mutation is more prevalent in the Jordanian population due to a founder effect. The mutation frequency in the Jordanian population gives a calculated prevalence of 1 in ~35 000 according to the Hardy-Weinberg equilibrium equation



**Figure 4** Agarose gel showing the results of PCR amplification of multiple tissue first strand cDNA by *LPIN2* cDNA primers (top panel), and the results of the northern blot analysis from similar tissues. C, clone; M, molecular weight marker. The molecular weight marker for the northern blot  $\beta$ -actin control is not shown.

( $q = 1/185$  and  $q^2 = 1/34\ 225$ ). Although this figure is higher than the estimated prevalence based on identified cases, we believe that this condition is underdiagnosed due to a lack of awareness of this clinical entity. Based on this data, we conclude that mutations in *LPIN2* cause Majeed syndrome.

The clinical description indicates the increased severity of the phenotype in affected individuals from family B as compared to affected individuals from family A. We speculate that this variability is related to the mutation identified in each family. In family A, the amino acid change probably leads to altered structure and function of the lipin 2 protein. In family B, the truncating mutation most likely leads to complete loss of function, since the protein is only 180 amino acids long.

Little is known about the function of *LPIN2*. It shares a lipin domain with the human ortholog of murine *lpin1* which plays a role in fat metabolism.<sup>36</sup> We placed *LPIN2* on our candidate gene list due to considerations regarding its structure and predicted function. Studies of the fission yeast (*Schizosaccharomyces pombe*) Ned1 gene, which belongs to an evolutionary conserved gene family which includes the mammalian *LPIN* genes, revealed that mutations have a high incidence of chromosome missegregation, aberrantly shaped nuclei, overdeveloped endoplasmic reticulum-like membranes, and increased sensitivity to microtubule destabilising agents.<sup>37</sup> This description is reminiscent of the dyserythropoiesis seen in Majeed syndrome. In addition, the role of human lipin 2 in fat metabolism is not clear, since *LPIN1* mutations have not been reported in patients with lipodystrophy.<sup>38</sup> Based on the presence of dyserythropoiesis in this syndrome, we propose that *LPIN2* also plays a role in mitosis and its absence or disturbed function leads to abnormal mitosis, especially in rapidly dividing cells such as the RBC lineage in the bone marrow.

We hypothesise that lipin 2 plays a role in the regulation of the innate immune system, and the defect in the protein would lead to increased production of proinflammatory signals. One way that this could occur would be if lipin 2 plays a role in promoting apoptosis in polymorphonuclear leukocytes and its normal function is to curtail the inflammatory milieu. Mutations in *LPIN2* will lead to a disturbed function and persistence of inflammation. The assumption would be that there is tissue specific redundancy in the role played by lipin 2 and its related pathway in the down regulation of inflammation, with a lower level of redundancy in skin and bone. We also hypothesise that variations in *LPIN2* may play an aetiologic role in psoriasis and related inflammatory skin disorders as it falls within a chromosomal region identified as a psoriasis susceptibility locus.<sup>39</sup> This hypothesis is further supported by the fact that at least two heterozygotes of *LPIN2* mutations and all homozygotes from Majeed syndrome families have psoriasis or another inflammatory dermatosis.

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## ELECTRONIC-DATABASE INFORMATION



Genome databases mentioned in this paper are to be found at <http://www.ncbi.nlm.nih.gov>, <http://genome.ucsc.edu>, and <http://www.ensembl.org>. The Primer3 program can be found at [http://frodo.wi.mit.edu/cgi-bin/primer3/primer3\\_www.cgi](http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi), while primer sequences are available from <http://jmedgenet.com/supplemental>.

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