Identification of the Cylindromatosis Tumor-Suppressor Gene Responsible for Multiple Familial Trichoepithelioma

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Multiple familial trichoepithelioma (MFT) is an autosomal dominant skin disease characterized by the presence of many small benign tumors with pilar differentiation predominantly on the face. The first locus has been previously mapped to chromosome 9p21, but no gene for MFT has been identified to date. To identify the disease gene in a large Chinese family, we initially performed linkage analysis with microsatellite markers from 9p21, but failed to confirm the linkage to this region. Previous publications showed MFT and familial cylindromatosis (FC) can occur within one family and in a single person. Therefore, we speculated that the cylindromatosis gene (CYLDI gene) responsible for FC may be related to the pathogenesis of MFT. In view of that, we genotyped all available individuals using 11 microsatellite markers spanning the CYLDI gene region at 16q12–q13. We identified the linkage of MFT to this region. Mutation analysis in the CYLDI gene detected a frameshift mutation, designated as c.2355–2358delCAGA. The study firstly identified the cylindromatosis gene responsible for MFT and showed that different mutations of the CYLDI gene can give rise to distinct clinical and histological expression such as FC and MFT.

Key words: CYLD1 gene/heterogeneity/linkage analysis/multiple familial trichoepithelioma/mutation
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Trichoepithelioma is a benign cutaneous tumor that originates from hair follicles. It occurs as a sporadic non-familial and a multiple-familial type. The multiple familial trichoepithelioma (MFT) (OMIM number 601606) is a relatively common genetic dermatosis characterized by the presence of many skin-colored small tumors located predominantly not only in the nasolabial folds but also on the nose, forehead, upper lip, and occasionally on the scalp, neck, and upper trunk, which is inherited in an autosomal dominant pattern. Because the histological features are dermal aggregates of basaloid cells with connection to or differentiation toward hair follicles, this disorder has been thought to represent a benign hamartoma of the pilosebaceous apparatus.

Harada *et al* (1996) have carried out linkage analysis in three American MFT families and mapped the first locus for MFT to a 4 cM region at 9p21 involved in the tumorigenesis. Nevertheless, to date, no other research groups have replicated the finding on chromosome 9p21. The sporadic trichoepithelioma occurs more commonly than MFT and is not inherited, and loss of heterozygosity studies demonstrated deletions at 9q22.3 similar to that of sporadic basal cell carcinoma (BCC) (Matt *et al*, 2000).

Familial cylindromatosis (FC) (OMIM number 132700) is also a dominant predisposition to multiple neoplasms of the skin appendages. The locus for FC on chromosome 16q12–q13 was demonstrated (Biggs *et al*, 1995; Takahashi *et al*, 2000) and several groups were able to show loss of heterozygosity at 16q in sporadic cylindromas as well as FC (Biggs *et al*, 1995; Verhoef *et al*, 1998; Thomson *et al*, 1999; Leonard *et al*, 2001). Twenty germline mutations and six somatic mutations have been detected in the cylindromatosis gene (CYLD1 gene) (Bignell *et al*, 2000; Poblete Gutierrez *et al*, 2002). MFT and FC can occur within one family and in a single person (Gerretsen *et al*, 1995), which suggests that the two types of dermatosis may be caused by dysfunction of the same gene. It may be that there are two forms of MFT: one that occurs in isolation is determined by a gene in the region at 9p mapped by Harada *et al* (1996), and another that co-occurs with FC is determined by a gene on chromosome 16.

The gene for MFT is unknown to date. To identify the disease gene, we herein studied a three-generation Chinese family with MFT. Linkage analysis using markers on chromosome 9p21 excluded the linkage to this region in this family, which suggested that MFT was a genetically heterogeneous disorder. Then we focused our investigation on 16q12–q13 and identified the locus for this family at this region. By mutation analysis, we firstly found a frameshift mutation of c.2355–2358delCAGA in the CYLD1 gene, which was responsible for MFT.

Results

Clinical findings The proband (III-6) is a 9-y-old girl who developed numerous tumors on her face from 5 y old. Most of the lesions were dome-shaped, skin-colored, firm

Abbreviations: BCC, basal cell carcinoma; CYLD1 gene, cylindromatosis gene; FC, familial cylindromatosis; MFT, Multiple familial trichoepithelioma

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papules between 5 and 8 mm in size. Other affected individuals also developed the characteristic facial trichoepitheliomas. The lesions increase steadily both in number and size along with age. Subjective symptoms were limited to occasional itching and psycho-social problems due to disfigurement. The clinical features of this family were listed in Table I. The mean age of onset was 12 y varying from 5 to 16 y. The lesions increased in number and size till approximately 25 y of age, and then gradually regressed along with age.

Skin specimen obtained from the nasolabial region of the proband revealed the typical histopathological characteristics of trichoepithelioma (Fig 1).

Two-point linkage analysis We initially analyzed seven microsatellite markers from the previously mapped region at 9p21 and observed pairwise LOD scores of less than -2 for each marker. As a result, the linkage to this region was excluded for this family. To identify the locus for MFT in this family, we analyzed the same set of genomic DNAs with three microsatellite markers at 16q12–q13 (D16S3112, D16S308, D16S3116). The presence of linkage to chromosome 16q was suggested by a two-point LOD score of 2.41 at D16S308 ($\theta = 0.00$). For fine mapping of this gene, a further eight microsatellite markers from this region were tested. Two-point LOD scores between the relevant markers and the disease locus are shown in Table II. The maximum two-point LOD score was 3.31 ($\theta = 0.00$) for marker D16S3044.

Haplotype analysis Haplotype was constructed with Cyrillic version 2.02 software to define the borders of the critical region (Fig 2). The crossover in normal individual III-8 places the MFT locus distal to D16S3116. The distal border of MFT is defined to D16S415 by a crossover event in individual II-7. These results indicate that the gene responsible for MFT lies in the 16 cM interval between D16S3116 and D16S415.

Identification of a causative mutation in the CYLD1 gene The CYLD1 gene falls within the identified locus at 16q12–q13 for MFT. By direct sequencing, we detected a four-basepair deletion at nucleotide position 5–8 of exon 18 in the CYLD1 gene leading to a frameshift mutation (Fig 3) in genomic DNAs of all seven patients, designated c.2355–2358delCAGA corresponding to the cDNA sequence of the gene beginning with A of the ATG translation start site (Antonarakis *et al*, 1998). We did not find this frameshift mutation in 12 normal individuals of this MFT family and 200 unrelated, population-matched control individuals.

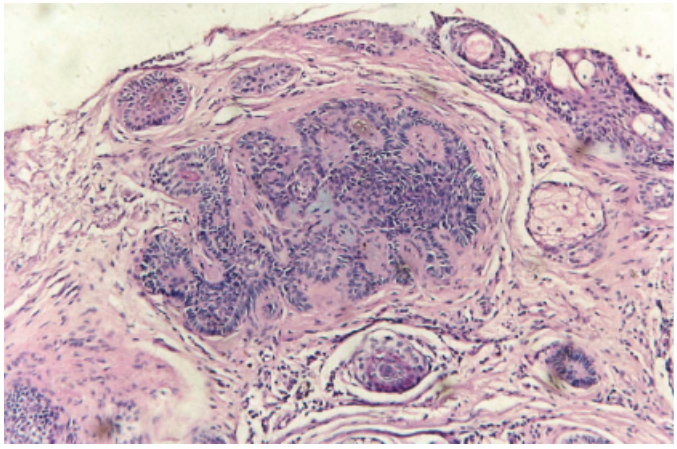

Figure 1
Histology of trichoepithelioma excised from the face of the proband (hematoxylin and eosin staining). Well-demarcated nodules consist of small basaloid cells that are arranged in a palisade-like pattern; multiple horn cysts with a fully keratinized center surrounded by basaloid cells lie free in the fibrous stroma.

Discussion

In this study, we identified a frameshift mutation in the CYLD1 gene responsible for MFT in a Chinese family. For the first time we were able to present evidence that MFT and FC were allelic disorders caused by different mutations in the same gene CYLD1 and demonstrated that MFT was a genetically heterogeneous disorder on the molecular level.

Differentiation of MFT from FC is important because they are associated with the same gene. Clinically, FC begins to appear in the second or third decades predominantly in

Table I. Review of clinical findings in affected members of the family with MFT

ID no.	Sex	Age at examination	Location	Number	Size (mm)	Age of onset (y)
I–2	F	68 y	A+B+C+D	++++	5–20	14
II–2	M	—	A+B+C	++++	5–15	16
II–5	M	—	A+B	+++	5–20	11
II–7	M	35 y	A +C	++	5–15	15
II–10	F	29 y	A+B+C+D	++++	5–20	13
III–1	F	17 y	A+B+C	+++	5–8	14
III–2	F	16 y	A+B+C	++	5–10	10
III–6	F	9 y	A+B+C+D	++++	5–8	5
III–9	F	15 y	A+B+C	++	5–10	10

Note: ID no, individual number in the pedigree; —, the individual died; A, nasolabial folds; B, nose; C, forehead; D, upper lip; +, 1–5; ++, 5–10; +++, 10–20; ++++, >20.

Table II. Two-point LOD scores between the MFT locus and markers on chromosome 9p and 16q

Markers	Location (cM)	LOD score at θ = 0.00	0.10	0.20	0.30	0.40	Z_{max}	θ_{max}
D9S157	31.8	−13.55	−3.15	−1.56	−0.74	−0.26	−0.26	0.40
D9S162	33.3	−4.11	−0.81	−0.31	−0.09	0.00	0.00	0.40
D9S1870	36.5	−14.43	−3.14	−1.56	−0.74	−0.26	−0.26	0.40
D9S171	42	−6.69	−1.51	−0.71	−0.31	−0.09	−0.09	0.40
D9S1833	45	−13.93	−2.89	−1.36	−0.59	−0.18	−0.18	0.40
D9S169	48.2	−10.82	−2.21	−0.97	−0.38	−0.09	−0.09	0.40
D9S161	50.3	−6.89	−1.06	−0.37	−0.08	−0.03	−0.03	0.40
D16S3076	44.2	−1.20	0.58	0.62	0.51	0.30	0.62	0.20
D16S3068	46.6	2.41	1.97	1.50	0.98	0.44	2.41	0.00
D16S3116	49.6	0.31	1.81	1.55	1.09	0.53	1.81	0.10
D16S3093	50.8	2.61	2.19	1.71	1.15	0.54	2.61	0.00
D16S409	56.2	2.10	1.78	1.43	1.02	0.55	2.10	0.00
D16S3044	56.7	3.31	2.76	2.15	1.46	0.70	3.31	0.00
D16S308	61.2	2.41	1.97	1.50	0.98	0.44	2.41	0.00
D16S416	63.2	0.68	0.60	0.49	0.35	0.19	0.68	0.00
D16S415	65.6	−0.77	0.90	0.84	0.63	0.34	0.90	0.10
D16S3137	66.9	2.10	1.78	1.43	1.02	0.55	2.10	0.00
D16S3112	72.1	0.01	1.55	1.35	0.97	0.51	1.55	0.10

Note: LOD scores were calculated under an autosomal dominant mode of inheritance, a penetrance of 99.9% at variant recombination fractions. Genetic coordinates in centimorgans according to The Genethon Human Genetic Linkage Map (http://www.genethon.fr/genethon.en.html) are in the column "Location".

hairy areas of the body, accumulating in number and increasing slowly in size throughout adult life (van Balkom and Hennekam, 1994). The tumors sometimes lead to the formation of a confluent mass that may ulcerate or become infected. Malignant change with distant metastasis does occur (Gerretsen *et al*, 1993; Sybert, 1997), but is unusual. For MFT, it occurs in childhood or adolescence. The lesions characteristically grow for a limited time and stabilize in size within a few years. Some of the lesions show spontaneous remission over the years and rare malignant changes occur. Histologically, numerous isolated islands of epithelial cells that are surrounded and separated from each other by PAS positive staining material suggest the features of FC, whereas primitive hair structures, cornified cysts, a palisade pattern, and stromal fibrosis favor a diagnosis of MFT. The clinical and histopathological findings in our patients are fully consistent with MFT. MFT can usually be differentiated from BCC because of its characteristic clinical presentation. Histopathologic criteria for differentiation between BCC and MFT are well estabished (Johnson *et al*, 1993). In addition, the positive stain of CD_{34} in trichoepithelioma is a useful distinguishing feature (Kirchmann *et al*, 1994). Genetically, the gene for hereditary BCC has been assigned to chromosome 9q22.3–q31 and mutations of the PTCH gene are detected in hereditary BCC (Bale *et al*, 1994).

The CYLD1 gene is composed of 20 exons, of which the first three are untranslated, and extends over approximately 56 kb genomic DNA. The full-length cDNA was predicted to encode a protein of 956 amino acids. In 2000, Bignell and colleagues reported the first mutations in the CYLD1 gene causing FC. All the mutations are located in the 3′ two-thirds of the CYLD1 coding sequence. Loss of heterozygosity of the wild-type allele of CYLD1 gene suggested that it is a tumor-suppressor gene that follows the classic two-hit model (Knudson, 1971). Recently, Poblete Gutierrez and colleagues detected a frameshift mutation in the CYLD1 gene, designated 2253delG, and were able to show that a single mutation can induce the co-occurrence of cylindromas and trichoepitheliomas. The mutation study in our isolated MFT family was further able to demonstrate that MFT is allelic to FC. The location and type of each mutation detected in the CYLD1 gene to date are depicted in Fig 4, which did not reveal any clear phenotype–genotype correlation. Therefore, the data mentioned above strongly demonstrate that the CYLD1 gene characterizes pleiotropy that mutations in the same gene can induce several different phenotypes, and that different genes such as CYLD1 and a hitherto unknown gene on 9p21 can respectively lead to the same disorder MFT.

The CYLD1 protein is believed to be a cytoskeletal protein containing four recognizable groups of sequence motifs: three CAP-GLY domains, one proline-rich repeat, two UCH (ubiquitin carboxy-terminal hydrolases) catalytic domains, and four Cys-X-X-Cys pairs. The function of each part has been demonstrated in previous data (Bignell *et al*, 2000). It contains three conserved domains, and the

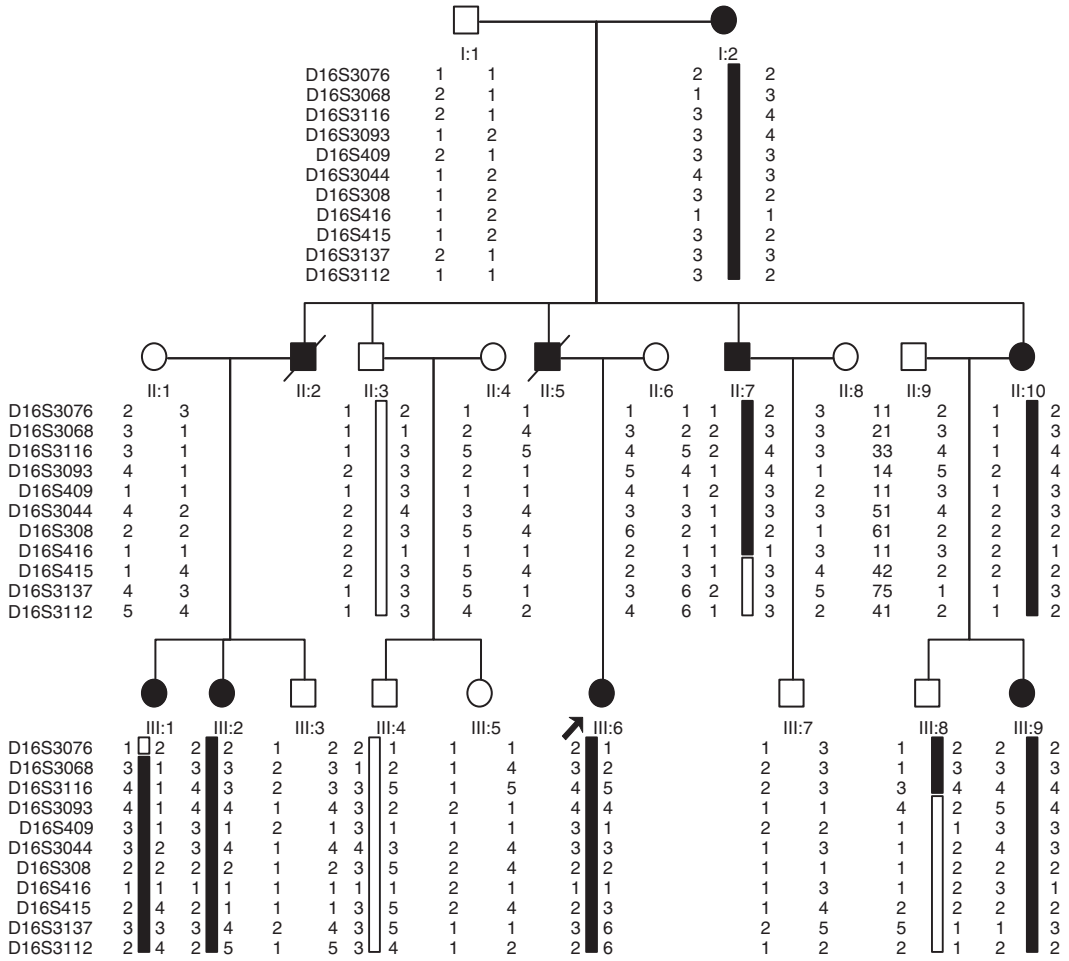

Figure 2
Pedigree and haplotype analysis of the MFT family. The arrow indicates the proband; affected and unaffected individuals are respectively represented by black and open symbols; disease-gene-bearing chromosomes are indicated with boxes; and blackened bars represent disease-carrying haplotypes.

frameshift mutation identified in this study locates in the UCH conserved domain. UCH catalyzes the hydrolysis of ubiquitin, resulting in deubiquitination and hence reduced degradation of target proteins by the proteasome (D'Andrea *et al*, 1998). Therefore, inactivation of the CYLD1 protein may contribute to oncogenesis by enhancing the degradation of proteins that suppress cell proliferation or promote apoptosis. This mutation occurs at the amino acid codon 785–786 of the CYLD1 protein and leads to the formation of a premature termination codon 132 nucleotides downstream of the deletion site. Prediction of the potential transmembrane segments of the mutant protein consists of six transmembrane segments, indicating loss of the seventh segment of the normal CYLD1 protein. It is possible that the presence of 44 aberrant amino acids plays a role in the pathogenesis of MFT, or the seventh segment of the normal CYLD1 protein is critical in keeping from the tumorigenesis.

The CYLD1 gene may play a critical role in governing cell fate decisions. Mutational changes in the CYLD1 gene could affect the normal regulation of apocrine or eccrine stem cell populations, which then give rise to the growth of distinct tumor types because the physiologic function of the encoded protein is disabled (Poblete Gutierrez *et al*, 2002). In a further study, we will perform functional studies of this frameshift mutation and further demonstrate the molecular mechanism of MFT.

Materials and Methods

Subjects A three-generation family with MFT consisting of seven affected and 12 unaffected individuals was identified through proband from Anhui province in China (Fig 2). The diagnosis of proband (Fig 5) was confirmed by typical clinical findings and histopathological examinations obtained from three tumor specimens. All family members received careful examinations by experienced clinical dermatologists. The medical history of the family members revealed neither cylindromas nor consistent involvement of other systems. Clinical and histological characteristics supported the diagnosis of MFT. After informed consent was obtained, blood samples were collected from available family members and 200 unrelated, unaffected control individuals. Genomic DNAs were extracted from peripheral blood using the Wizard Genomic DNA purification kit (Promega, Madison, WI). We make the statement of institutional approvals and the statement of adherence to Declaration of Helsinki Guidelines.

Genotyping We initially analyzed this family using polymorphic microsatellite markers from chromosome 9p21. Linkage analysis excluded the linkage to this region in this family. We then genotyped this family with microsatellite markers spanning the

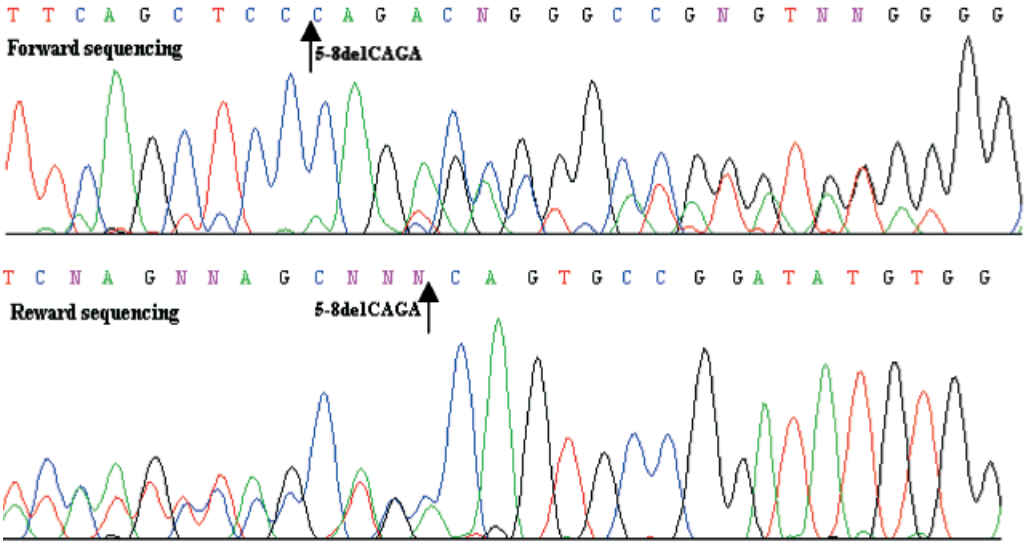

Figure 3
Mutation analysis of exon 18 of the CYLD1 gene in the proband. Bidirectional automated sequence analysis of exon 18 of the CYLD1 gene indicated a four-basepair deletion at the nucleotide position 5–8 in exon 18 of the CYLD1 gene, designated as c.2355–2358delCAGA.

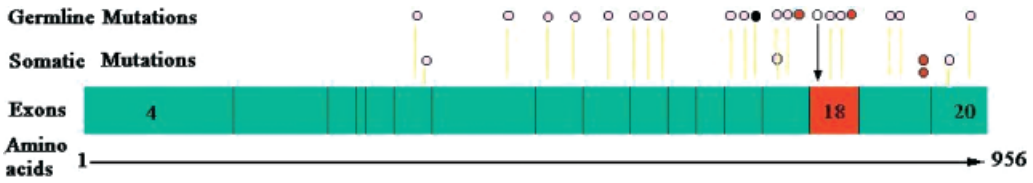

Figure 4
All mutations in the CYLD1 gene. The mutation detected in this study is represented by a closed circle, and its location is indicated by a black arrow pointing to exon 18. Each circle represents a specific mutation whose location is indicated by a yellow vertical line. Notes: ○, truncating mutation responsible for MFT detected by us; ■, exon 18 of the CYLD1 gene; ○, truncating mutations responsible for FC; ●, splicing mutations responsible for FC; ●, truncating mutations detected in an FC family co-occurring with MFT; |, splice site.

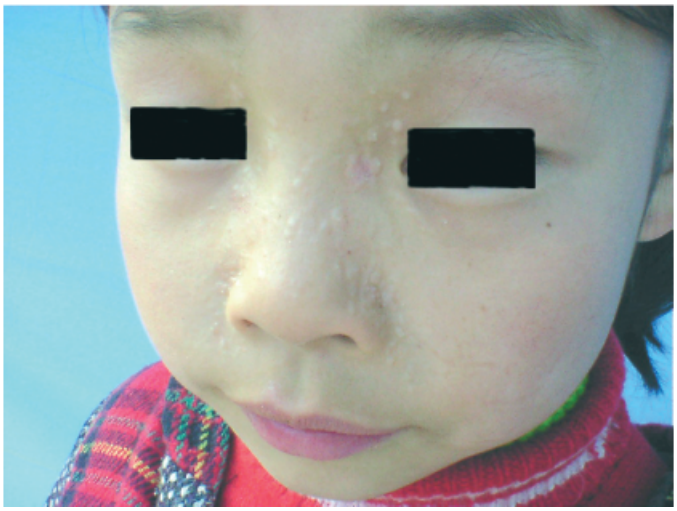

Figure 5
Clinical findings of the proband. Numerous small trichoepitheliomas in the nasolabial folds.

locus for FC at 16q12–q13. Genethon linkage map (Dib *et al*, 1996) was used to establish marker locations. The markers and their location are shown in Table II. For the linkage study, genotypes were obtained by polymerase chain reaction (PCR) with microsatellite markers of chromosomes 9p21 and 16q12–q13. The reactions were performed with a touchdown program in a 5 μL volume containing 10 mM Tris–HCl (PH 8.3), 50 mM KCl, 0.1 mg per mL gelatin, 3.0 mM $MgCl_2$, 0.2 mM of each dNTP, 0.04 μM of each primer, 10 ng genomic DNA, and 0.2 U AmpliTaq Gold (Applied Biosystems, Foster City, CA). The PCR conditions were as follows: taq activation at 94°C for 12 min, followed by 40 cycles, each having denaturation at 94°C for 30 s, annealing at 56°C for 60 s and extension at 72°C for 90 s, except that in the first 15 cycles the annealing temperature decreased from 63°C to 56°C by 0.5°C per cycle, and the final extension was 72°C for 10 min. The PCR products were separated on a MegaBACE-1000 DNA sequencer (Amersham BioScience, Piscataway, NJ). The size of the allele was determined on the basis of MegaBACE ET400-R Size Standard. Following the electrophoresis, data were collected and analyzed with Intrument Control software version 2.1 and Genetic Profiler software version 1.1. Genotypes of each locus were further reviewed and scored independently by two observers and retyped in case of discrepancy.

Linkage and haplotype analysis Two-point linkage analysis was performed by Linkage programs version 5.10 (Lathrop and Lalouel, 1984). Autosomal dominant inheritance with 99.9% penetrance was assumed. The affected allele frequency was taken as 0.00001 (Harada *et al*, 1996). Marker allele frequencies were evaluated to be uniformly distributed. The recombination frequency was assumed to be equal for both sexes. Haplotype was constructed with Cyrillic version 2.02 software (Sobel and Lange, 1996).

Mutation detection of the CYLD1 gene The localization of the CYLD1 gene within the critical region that we mapped on chromosome 16q12–q13 encouraged us to search for mutations in the CYLD1 gene. Amplification of all coding exons of the CYLD1 gene and the adjacent splice sites was carried out using PCR primers designed by us shown in Table III.

Table III. Primers used for screening of the CYLD1 gene sequence from genomic DNA

Exon	Exon (bp)	Coding	Forward primer (5′–3′)	Reverse primer (5′–3′)
4a	627	Y	CTTGAATGCCCCACAGAAC	GTCTTCAGTCTTAATTTTCAGTCA
4b			CTGAAGCGTACAACTCCAGGAA	AAGAATGCAGCGTTACAGACAAA
4c			ATTACAGAAGACTCTTGGGAAAAT	TTCAGCCTGTTTAAAAACAGAAA
5a	303	Y	TCCCCAGGACCTGCGTAAT	TTTCAGCATCAGATACAACTTCTT
5b			CAAGTAAATTGGGCCTAAAACT	GTGATGAAGATTGTGGCGTGTT
6	106	Y	GTAACCAAACACCACCTG	CCTAATGTATTCTTTCTTTCTTTT
7	9	Y		
8	99	Y	GTCTACATACATGTTCAGAAGTAA	TTTTTCCCCCATAGTATTATC
9	117	Y	ACAAAGAGCGGTCTACTTATTCTG	TTATTGGTTATGTAAACAGTTATT
10a	380	Y	GCATGGCCAAGGGTGGACTC	GGTCAGGTGGGCAGTTGGATAGA
10b			TCTGCAGTGATAGCTTTTCTGACA	CAGTCTCACCAAGATGCCCAATAC
11	166	Y	AAGCTTAATGAGCGTTGGCAAGAA	GAAAGGGTATACTAGAAACAGGTC
12	142	Y	GAGGCAGATGGCAGTTTCTTTCAC	AAGCCTACTTTCCACTTA
13	123	Y	CATAAGAACTAATTTTCACCCATCT	TTGTTATGTTATTTGTTCTCCAGA
14	92	Y	CACATTTCAGCTAATACTTTGACA	ATCGGGCCACTGCACACTCC
15	67	Y	CCCCAGCCTATTAAGAGCCCTAAA	TTAATTGAAATAATATTGGAGAAT
16	133	Y	ACAGACTTTCACATAAAATACCAC	TAATCACTAGGAGAACTTTCCAGT
17	109	Y	ATACTGAATTGCCTTGGGAAATAC	TAAGGCAGAACTGTTTTGTTTGAC
18	119	Y	AAAAACTTTCCAAACGAGCATTGT	TTTTTGAAGCCATAACATTGTCCT
19	227	Y	CTCAGAAAATATCACTCAGAACAGG	CATCTTGATCAGGCTGGTCTTGAA
20a	2294	Y	TCCCGATGACCCCAGTTATTTGT	TTGGCCCCAGAATCCTTTCAGAAAC
20b		Y	TCTTATTAAAACTTCAGGGACACA	ACCCCATGCCCAGAAGTAGGA
20c		N	TTCTTCCTCAAGGCACCGTTAGC	ATTCGAGCTGGCAGTTCTGTTCAC
20d		N	GCATTTGGGGACAGAATCACTCA	AAGCCTCTTTTAGTCCATTGA
20e		N	CTCTCCCAGGAAATTGTAATAAT	TTTTTCTGTGTTTCTGCAATAGTG
20f		N	CATCTATGGTCCTGGTCTACAACA	AATATTTTATGGATGGGGCAAAGT
20g		N	AAGATGAGTTTTTGTACCTTACTG	GAAATGTTGCAGCAGCTTCATCC
20h		N	TATTACAGGGTGATTGGACTTCTA	TACAAACCCTGCAATTACTTTTTA

Notes: (a) Exons 1–3 are untranslated. The primers were designed with Primer3.0 to amplify all 17 coding exons and adjacent splice sites; (b) to facilitate sequencing, large exons (4, 5, 10, 20) were subdivided into several overlapping PCR fragments.

PCR was carried out in a 25 μL total volume, containing 20 ng genomic DNA, 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 3.0 mM $MgCl_2$, 0.01% gelatine, 0.2 mM dNTPs, 10 pmol of each primer, and 0.75 U HotStar®Taq (QIAgene, Germany). The PCR conditions were: HotStar®Taq activation at 95°C for 15 min, followed by 40 cycles, each having denaturation at 94°C for 40 s, annealing at 58°C for 40 s and extension at 72°C for 40 s, except that in the first 10 cycles the annealing temperature decreased from 63°C to 58°C by 0.5°C per cycle, and the final extension was 72°C for 10 min. PCR products were directly sequenced on an ABI 377 DNA sequencer (Perkin-Elmer).

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Appendix: Electronic Database Information

Accession numbers and URLs for data in this paper are as follows:

- Accession numbers: OMIM: MFT, *601606; FC, #132700; *CYLD1*, *605018; Genebank: *CYLD1*, AJ250014; cDNA of *CYLD1*: NM_015247.
- Genethon: http://www.genethon.fr/genethon.en.html;
- GenBank: http://www.ncbi.nlm.nih.gov/web/search/index.html;
- Online Mendelian Inheritance in Man (OMIM): http://www.ncbi.nlm.nih.bov/OMIM;
- Expert Protein Analysis System (ExPASy): http://www.expasy.ch.