

Germline Fumarate Hydratase Mutations and Evidence for a Founder Mutation Underlying Multiple Cutaneous and Uterine Leiomyomata

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Abbreviations: *FH/FH*, fumarate hydratase gene/protein; *FHD*, fumarate hydratase deficiency; *HLRCC*, hereditary leiomyomatosis and renal cell cancer; *MCL*, multiple cutaneous and uterine leiomyomata syndrome; *PCR*, polymerase chain reaction.

Abstract

Multiple cutaneous and uterine leiomyomata syndrome (MCL) is an autosomal dominant disease characterized by the presence of concurrent benign tumors of smooth muscle origin (leiomyoma) in the skin and uterus of affected females, and in the skin of affected males. MCL can also be associated with type II papillary renal cell cancer (HLRCC).

The genetic locus for MCL and HLRCC was recently mapped to chromosome 1q42.3-43 and subsequently, dominantly inherited mutations in the fumarate hydratase gene (*FH*) were identified. Importantly, analysis of the *FH* gene in tumors of MCL patients revealed a second mutation inactivating the wild-type allele in some tumors. Based on these findings, it has been suggested that *FH* may function as a tumor suppressor gene in MCL. Here, we report the analysis of the *FH* gene in a group of 11 MCL families, with the identification of 8 different mutations accounting for the disease in all families. One of the mutations, 905-1G>A, has been identified in four families of Iranian origin. The analysis of highly polymorphic markers in the vicinity of the *FH* gene showed a shared haplotype in these four families, suggesting that 905-1G>A represents a founder mutation. Collectively, identification of five novel and three recurrent mutations further supports the role of *FH* in the pathogenesis of MCL.

INTRODUCTION

Cutaneous leiomyomas are rare, benign tumors arising from the arrector pili muscle of the hair follicle. They can be found in association with uterine fibroids in multiple cutaneous and uterine leiomyomata syndrome (MCL; OMIM 150800), inherited as an autosomal dominant trait.^{1,2,3} Association of MCL with familial development of type II renal cell cancer has been characterized in a syndrome called hereditary leiomyomatosis and renal cell cancer (HLRCC; OMIM 605839).^{4,5}

Recently, the genetic locus for MCL and HLRCC was mapped to chromosome 1q42.3-43.^{3,6} Subsequently, germline mutations in the fumarate hydratase gene (*FH*) were found in MCL and HLRCC.⁷⁻¹⁰ Fumarate hydratase is an enzyme that functions as part of the Krebs cycle, responsible for cellular energy production and amino acid metabolism. *FH* has been predicted to act as a tumor suppressor gene, since loss of the wild-type allele has been found in cutaneous, uterine, and renal tumor biopsies of MCL patients. Moreover, the FH enzymatic activity is low or absent in tumors from individuals with MCL.⁷

While the tumorigenic mechanism of *FH* mutations remains elusive, evidence is clear that dominant mutations in succinate dehydrogenase, also an enzyme of the Krebs cycle, cause tumors of the carotid body and adrenal gland, paraganglioma and pheochromocytoma, respectively.¹¹ In addition, recessive mutations in the *FH* gene cause fumarate hydratase deficiency (FHD; OMIM 606812), characterized by neurological impairment, encephalopathy, and premature death in infants. Interestingly, MCL has been reported in the carrier mother of one infant with FHD.⁷ However, a recent

study has identified other carrier parents of FHD infants who are asymptomatic. Thus, it remains controversial whether all heterozygous *FH* mutations predispose to MCL.⁸

We previously reported five *FH* mutations in patients affected with MCL, including missense, nonsense, and frameshift mutations.⁹ Here, we have identified four novel and three recurrent mutations in seven MCL families, in addition to a recurrent novel splicing mutation, 905-1G>A, in four MCL families. Haplotype analysis of these four families using polymorphic markers surrounding the *FH* gene provides evidence for a founder effect.

MATERIALS AND METHODS

Human Subjects

We have identified eleven families (MCL-6-16) with dominantly inherited MCL, comprising 36 affected individuals and 96 unaffected family members, of which 18 affected and 13 unaffected individuals have been available for this study. Blood samples were collected following informed consent. Families were recruited from different areas of Israel (MCL-6-9), the United States (MCL-10-12, 14-16), and Spain (MCL-13). Of these, MCL 6-9 are Jewish families who report ancestors originating from Iran, MCL-11, 12, 13 and 14 are Caucasian families, MCL-10 originated from Ecuador, and MCL-15 and 16 originated from the Dominican Republic.

Mutation analysis

Genomic DNA was isolated from peripheral blood collected in EDTA-containing tubes using the PureGene DNA Isolation Kit (Gentra Systems, Minneapolis, MN). To screen for mutations in the human *FH* gene, all exons and splice junctions were PCR-

amplified from genomic DNA. PCR primers have been described previously.⁹ PCR products were sequenced in an ABI Prism 310 Automated Sequencer, using the ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems, Foster City, CA), following purification in Centriflex Gel Filtration Cartridges (Edge Biosystems, Gaithersburg, MD). Mutations were identified by visual inspection and comparison with control sequences generated from unrelated, unaffected individuals.

To confirm the mutations identified, direct or mismatched PCR followed by restriction enzyme digestion and/or PCR direct sequencing were used. DNA variants H92R and R190C were confirmed by digestion of the corresponding PCR-amplified products with *Bcl*II and *Mae*II, respectively. In the case of P149L, a forward primer (5'-GCTGGGTTTTGAGTAGTTAGTTGG-3') and a reverse mismatched primer (5'-GCAGCAGCAATGTGCATTGCTCTG-3') were used to introduce a *Dde*I restriction site. Mutation 905-1G>A was tested by PCR direct sequencing. Finally, controls for 1176del6 mutation were run on a 6% non-denaturing polyacrylamide gel and visualized by ethidium bromide staining. These mutations were tested in a mixed control population of 142-152 chromosomes.

Haplotype Analysis

Microsatellite markers covering the *FH* locus were selected from the Human Genome Working Draft at UCSC (www.genome.ucsc.edu). Eight polymorphic microsatellite markers, *DIS517*, *DIS2785*, *DIS304*, *DIS180*, *DIS204*, *DIS547*, *DIS1634*, and *DIS1609*, spanning an interval of 4.81 Mb (11.57 cM; Fig 3) surrounding

the *FH* gene on chromosome 1 were chosen. The PCR-amplified markers were electrophoresed in 6% non-denaturing polyacrylamide gels and visualized by ethidium bromide staining.

RESULTS

Clinical findings

The patients in the eleven families with MCL originated in five different countries, namely, four Jewish families from Israel (MCL-6-9), Ecuador (MCL-10), Spain (MCL-13), Dominican Republic (MCL-15, 16), and the United States (MCL-11, 12 & 14; Fig 1). These families comprised a total of 11 and 7 affected women and men, respectively, available for this study. Of 96 unaffected members in these families, 13 were available for this study. Skin leiomyomas were found in all of 18 affected individuals and none of the unaffected family members. The patients reported sensitivity of the lesions to cold temperature and touch. The typical skin lesions were firm, skin colored to red, and ranged from a few in number to approximately 100 in older individuals (Fig 2). The size of the lesions varied between 0.2 to 1.0 cm in diameter and clustered with time.

Of the eleven female patients, nine had coexisting skin and uterine leiomyoma and two of the female patients exhibited skin leiomyoma alone, without any uterine lesion. Five of the patients with affected uteri eventually underwent hysterectomy or myomectomy.

Mutation analysis of the *FH* gene

We have analyzed eleven families with dominantly inherited MCL for *FH* mutations. All of the families were found to carry heterozygous mutations in the *FH* gene (Table 1). Five of the mutations, H92R, P149L, R190C, 905-1G>A, and 1176del6 represent novel mutations, whereas Q4X, K187R, and R190H, on the other hand, have

been previously described.^{7,10} The five novel mutations have not previously been reported in patients with MCL or fumarate hydratase deficiency, nor have they been detected in 142-152 control chromosomes, arguing against them being common polymorphisms.

A G-to-A transition at the invariant G of the 3' acceptor site of intron 6 (905-1G>A) was identified in four families from the Middle East (MCL-6-9) (Table 1). The seven mutations in the coding region, on the other hand, are each unique to a single family. Nonsense mutation Q4X (amino acid residue 47 in the mitochondrial isoform), was found in family MCL-14. A six nucleotide in-frame deletion starting at nucleotide position 1176 that led to deletion of residues A350 and V351 (amino acid residues 393 and 394 in the mitochondrial isoform) was identified in family MCL-16. Five missense mutations were also identified: H92R in family MCL-15 (mitochondrial amino acid residue 135), P149L in family MCL-13 (mitochondrial amino acid residue 192), K187R in family MCL-11 (mitochondrial amino acid residue 230), R190C in family MCL-10 (mitochondrial amino acid residue 233), and R190H in family MCL-12 (mitochondrial amino acid residue 233). In families with more than one affected individual available for study, MCL-7, 9, 11, 13, cosegregation of the mutation with the disease phenotype was confirmed. Two unaffected carriers, III-3 and III-1, were identified in MCL-6 and MCL15, respectively (Fig 1).

Haplotype analysis

Closer examination of ancestry revealed that the four Jewish families sharing the 905-1G>A mutation reported ancestors originating from Iran, specifically Tehran (MCL-

6 and 7) and Shiraz (MCL-8 and 9) (Fig 3). From these four families, a total of eight affected and nine unaffected family members were available for the study. Genotyping of microsatellite markers *DIS517*, *DIS2785*, *DIS304*, *DIS180*, *DIS204*, *DIS547*, *DIS1634*, and *DIS1609* revealed that all affected individuals in these four families shared a common haplotype for markers *DIS304*, *DIS180*, *DIS204*, *DIS547*, and *DIS1634*. The shared haplotype covers an interval of 0.95 Mb (3.97 cM) surrounding the *FH* gene. Although for one of the families, MCL-8, we had access to only one family member, the genotypes for the microsatellite markers analyzed were compatible with the common haplotype. Given that the *FH* gene is positioned between *DIS204* and *DIS547*, the common haplotype highly suggests that mutation 905-1G>A represents a founder mutation. One unaffected individual, III-3 from MCL-6 (Fig 3), was shown to carry the disease haplotype. Sequence analysis confirmed that he is a carrier of the 905-1G>A splice site mutation.

DISCUSSION

Including our previous work,^{6,9} we have studied a group of 16 MCL families and have identified a total of 13 different mutations accounting for the disease in all families. Interestingly, previous studies have identified mutations in 60%, 76%, and 89% of the families studied.^{7,8,10} The observed variability in mutation detection rate may be attributed to the different mutation detection methods employed in each study and to mutations present in the non-coding region. Based on the number of MCL families in which no *FH* mutations have been detected, it still remains possible that the predisposition to MCL is genetically heterogeneous. Further studies will be necessary to discriminate between undetected mutations and locus heterogeneity.

Pooling the data from this study and our previous report,^{6,9} there were 38 affected female patients from 16 MCL pedigrees, including family members not available for mutation studies. Twenty-six affected females developed concurrent skin and uterine lesions (67%), eight of them developed uterine tumors only (20%), and five developed skin leiomyomas only (13%). It remains possible that patients with only uterine leiomyoma may have coexisting skin lesions that are difficult to detect. We have observed in this and our previous study,^{6,9} that patients from the same family may display intra-familial variability of the disease.

Predisposition to type II papillary renal cell carcinoma has been documented in a subset of MCL families.^{8,10,12} Although we have not encountered MCL probands with renal cell carcinoma, one family, MCL-12 (R190H) reported a sibling affected with MCL that developed renal cell cancer of unspecified histology. Renal screening of II-2 in MCL-15 revealed a renal cyst, with no suggestion for malignancy. One of the five MCL

families reported by Toro et al with renal cell cancer had a missense mutation in the same residue as MCL-12, R190L.¹⁰ The overall frequency of HLRCC is variable depending on the studies, ranging from 1-2% and 14.3%.^{8,10} In addition to type II papillary renal cell carcinoma, association between MCL and collecting duct carcinoma of the kidney has been recently reported.^{8,10,13}

One of the mutations identified in our patient collection is a novel splice site mutation, 905-1G>A, present in four Jewish families recruited in the Middle East (MCL-6-9). The fact that all four families originate from Iran, together with the presence of a common haplotype surrounding the *FH* gene, suggests that 905-1G>A is most likely a founder mutation. Four previously described *FH* mutations, N64T, K187R, R190H, and G354R, were also found in multiple families and analysis of three microsatellite markers within the *FH* gene showed a common haplotype shared by the carriers of each mutation.⁸ The missense mutation R190H found in this and two other studies^{8,10} deserves special mention. While two of R190H MCL families described by Alam et al⁸ originated from Spain and shared a common haplotype as described above, haplotype analysis of eleven families with the same mutation from North America showed no shared haplotype.¹⁰ In our study, the MCL proband with the R190H mutation is a Caucasian American. Collectively, the recurrence of R190H mutation in families of diverse ethnic origin and geographic distribution highlights the crucial function of this residue for the activity of the protein. According to the proposed structural model,⁸ R190, along with K187 and E312, are required to maintain a charge-charge interaction that is important to stabilize an active site loop. In this regard, it is interesting that two other missense mutations affect this residue, R190L¹⁰ and R190C (this study).

The three novel missense changes identified in this study, H92R, R190C, and P149L, have not been identified in a collection of 142-152 control chromosomes. Although they could still represent rare variants present in the general population, it is noteworthy that 13 out of 21 and 13 out of 20 *FH* mutations detected thus far were missense mutations.^{8,10} The five missense mutations reported here, H92R, K187R, P149L, R190H, and R190C, affect highly conserved residues among human, pig, rat, mouse, yeast, and *E. coli* FH (data not shown). As mentioned above, the proposed FH crystal structure suggests that the interaction between K187 and R190 residues, along with E312, would be crucial for the stability of the active site loops.⁸ According to the same model, P149 is positioned adjacent to an active site required for tetramerization of the FH protein. Mutations in these highly conserved residues would very likely disrupt the folding and tetramerization of the enzyme.

In addition to nonsense, splice site, and missense mutations, we have also identified a novel 6 bp deletion starting at nucleotide 1176, 1176del6, in MCL-16, resulting in an in-frame deletion of two amino acids, A350 and V351. Amino acid residues A350 and V351 are conserved in human, pig, rat, and mouse FH. The mutation V351L, affecting one of the residues involved in the deletion, was identified in one of our previously reported MCL families.⁹

We have identified two unaffected carriers of *FH* mutations. The first one, III-3 from MCL-6 family (Fig 1), is a 17-year-old male. Sequence analysis confirmed the haplotype results (Fig 3), with the identification of the splice site mutation 905-1G>A in III-3 and his mother, II-6, who reported onset of MCL symptoms at the age of 23. Exon-skipping is likely to be the mechanism in which the involved exon is spliced out of the

mRNA.¹⁴ Skipping of exon 7 would result in an in-frame deletion. Unfortunately, mRNA sample from this family was not available for further testing. The second unaffected carrier identified in our study is a 12-year-old child, III-1 from pedigree MCL-15, who carries the missense mutation H92R (Fig 1). The age of onset of MCL in her mother (II-2) was 30 years. Given the typical delayed onset of MCL in their affected parents, III-3 (MCL-6) and III-1 (MCL-15) are most likely predisposed to develop MCL later in life.

An important implication of identifying susceptibility genes for tumorigenesis such as MCL is the possible role they may play in the more common sporadic presentation of the same type of tumors. Families with MCL present with benign skin and uterine leiomyomas, the latter being a major public health issue for women. In addition, the aggressive papillary renal cell carcinoma and collecting duct carcinoma of the kidney can also be associated with MCL, as well as malignant leiomyosarcoma.^{5,8,10} So far, several groups have studied different types of malignancies, and have found *FH* mutations in a small number of tumors, including uterine and cutaneous leiomyomas, leiomyosarcomas and soft tissue sarcoma.^{12,15,16} Two other studies have excluded *FH* involvement in the pathogenesis of familial and sporadic prostate cancer.^{17,18} Recently, a novel alternatively-spliced transcript of the human alpha-methylacyl-CoA racemase, known to be elevated in prostate cancer, was found to have 88% identity with a 521-bp sequence spanning exons 7-10 of *FH*.¹⁹ In all, the role of *FH* mutations in sporadic tumorigenesis remains inconclusive.

The identification of the particular *FH* mutation in each MCL family will allow for close follow-up of those individuals at risk for the development of the aggressive

papillary renal cell cancer and collecting duct cancer of the kidney.^{4,5} The finding of skin leiomyoma in a patient should promote the examination for multiple leiomyomata in this patient as well as in all family members, together with the screening of the *FH* gene. Special attention should be paid for the early-onset of uterine fibroids in females and for the existence of kidney cancer in male and females within the family.

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Table 1. Germline *FH* Mutations in MCL Families

Family	Wildtype sequence	Mutant sequence	Amino-acid change ^a	Exon
MCL-14	CAA	TAA	Q4X	2
MCL-15	CAT	CGT	H92R	4
MCL-4*	AGC	ATC	S115I	4
MCL-2*	CAG	TAG	Q142X	4
MCL-13	CCC	CTC	P149L	5
MCL-11	AAG	AGG	K187R	5
MCL-10	CGT	TGT	R190C	5
MCL-12	CGT	CAT	R190H	5
MCL-6	905-1G	905-1G>A	-	Intron 6
MCL-7	905-1G	905-1G>A	-	Intron 6
MCL-8	905-1G	905-1G>A	-	Intron 6
MCL-9	905-1G	905-1G>A	-	Intron 6
MCL-1*	CAT	TAT	H275Y	7
MCL-5*	GAAAATGAACCA	GAAAACCA	1081del4 ^b	7
MCL-16	CATGTTGCTGTCACT	CATGTCACT	1176del6	8
MCL-3*	GTC	CTC	V351L	8

^a Amino-acid positions are derived from the cytosolic enzyme sequence (468 aa). The exon number corresponds to the entire gene; exon 1 encodes the 43 aa that form the mitochondrial signal peptide and the first amino acid of the cytosolic protein.

^b1081del4 mutation corresponds to a 4-bp deletion between amino acids 318 and 319.

* denote mutations reported in our previous study.⁹

LEGENDS FOR FIGURES

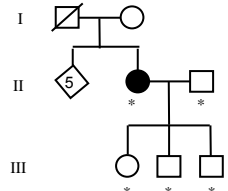
Fig 1. Pedigrees with MCL. Black, gray, and empty symbols indicate affected, unknown, and unaffected family members, respectively. *Asteriks* indicate individuals whose blood samples were available. No pedigree information was available for family MCL-10.

Fig 2. Clinical Pathology of Cutaneous Leiomyoma. Note the lesions on legs (MCL-13) (A), neck (MCL-9) (B), lower back (MCL-8) (C), and upper back (MCL-6) (D).

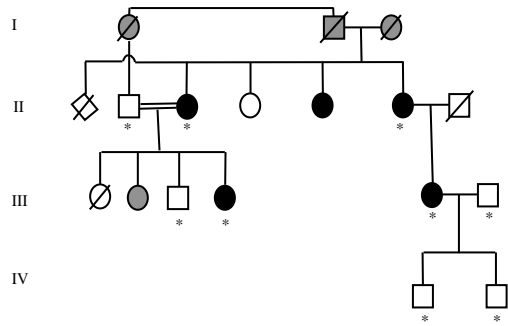
Clustered lesions typically present on upper trunk and back. Lesions clustering in the neck and leg as shown in (A) and (B) are uncommon.

Fig 3. Haplotype Analysis of MCL-6-9 Families. (A) Distances between polymorphic markers and the *FH* gene are shown in Mb and cM. (B) The genotypes shown for each individual correspond to markers *DIS517*, *DIS2785*, *DIS304* *DIS180*, *DIS204*, *DIS547*, *DIS1634*, and *DIS1609* (from centromere to telomere, top to bottom). +/- denotes the carrier of an *FH* mutation in MCL-6.

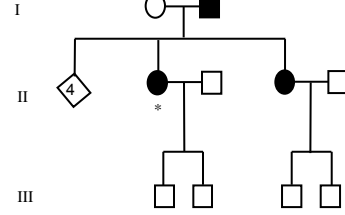
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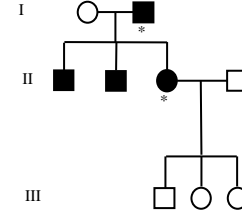
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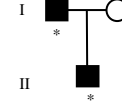
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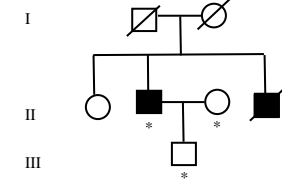
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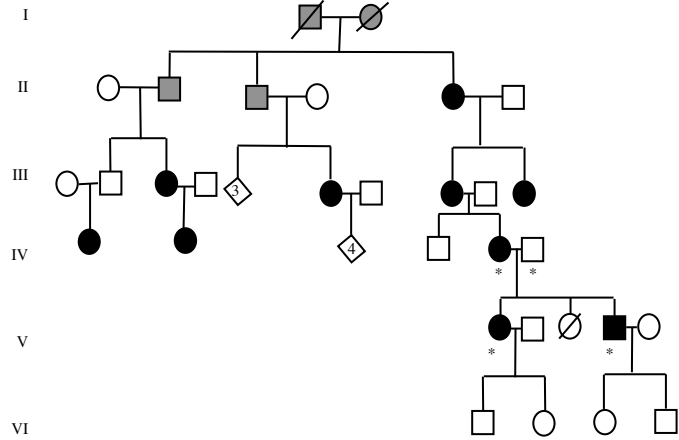
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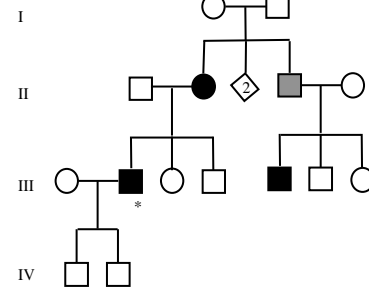
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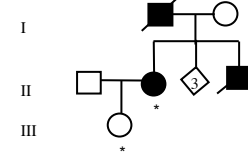
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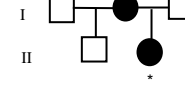
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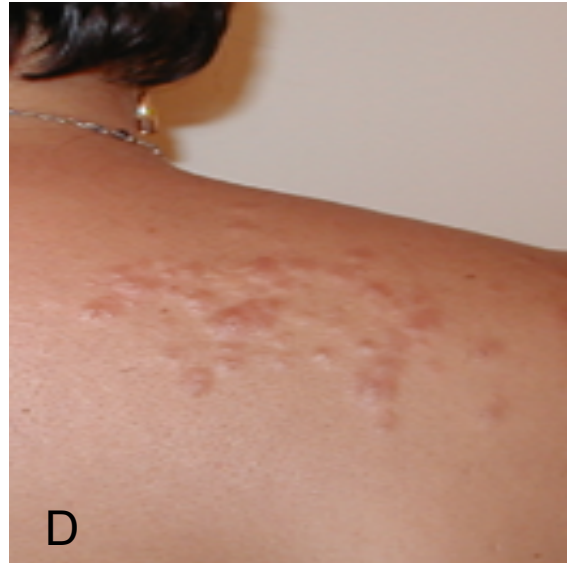
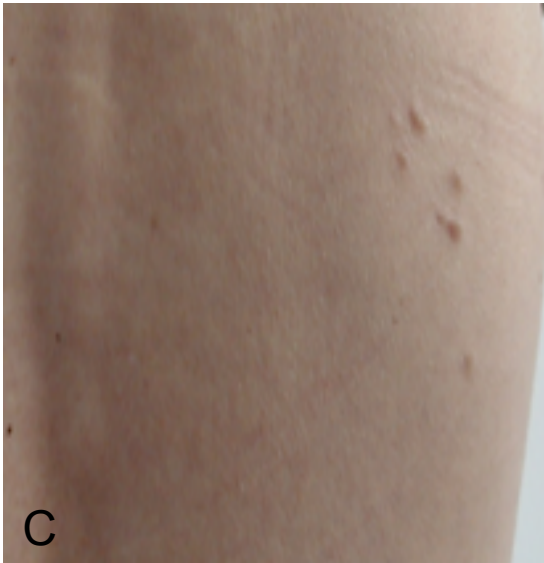
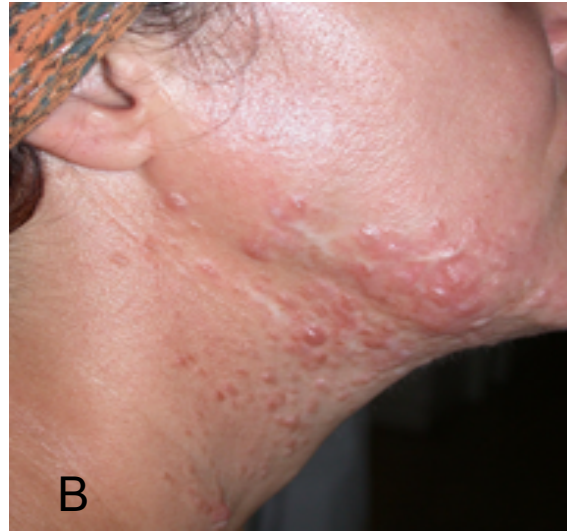
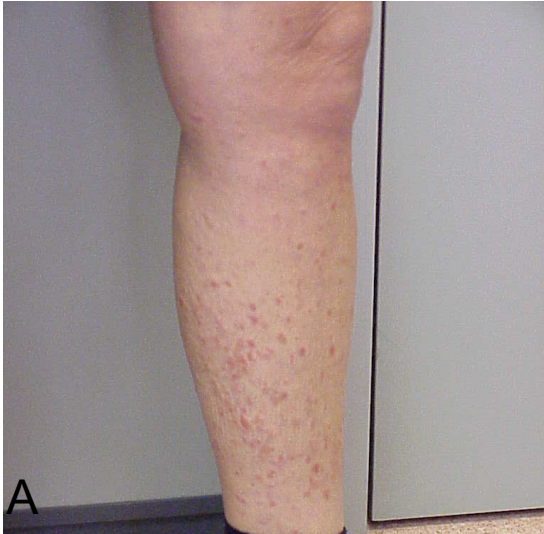


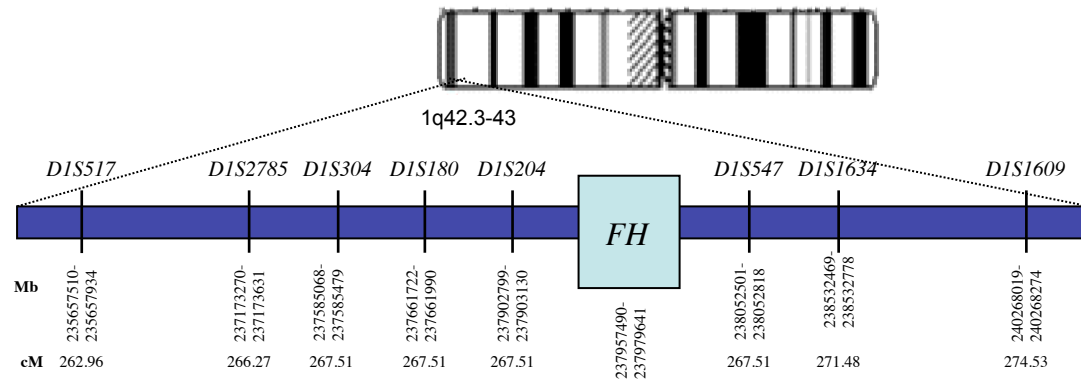
MCL-15



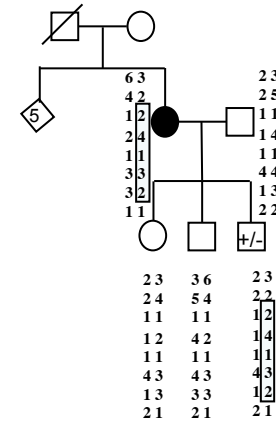
MCL-16



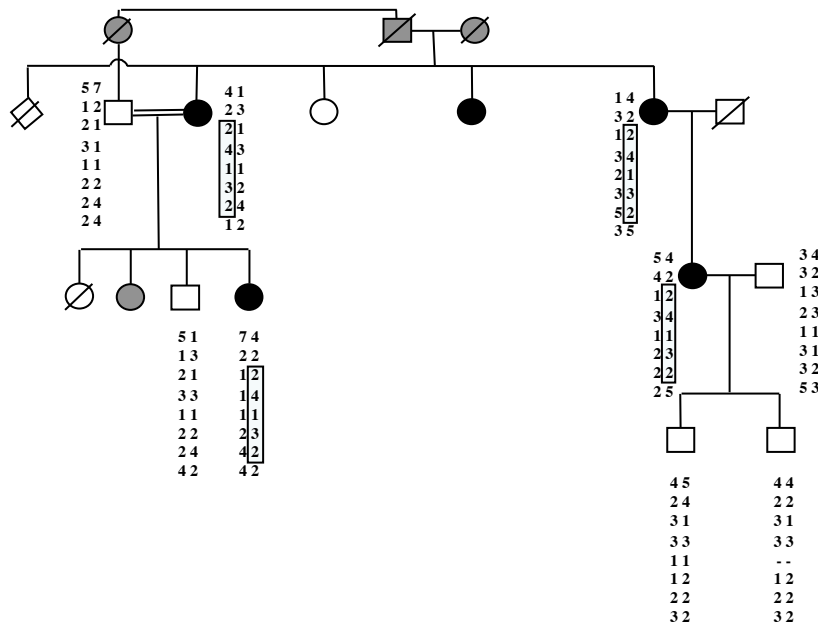




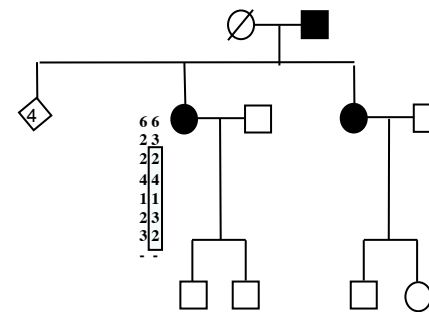
MCL-6



MCL-7



MCL-8



MCL-9

