Original Article

Screening and Identification of a Novel FHL2 Mutation by Whole Exome Sequencing in Twins With Familial Waldenström Macroglobulinemia

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BACKGROUND: Waldenström macroglobulinemia (WM) is a rare chronic B-cell lymphoma. Familial clustering of WM has been observed over the years. However, little is known about the contribution of inherited genetic variants to familial WM cases. **METHODS:** The authors performed whole exome sequencing (WES) of germline DNA samples from twins, one diagnosed with WM and the other diagnosed with immunoglobulin M monoclonal gammopathy of undetermined significance, and their healthy siblings. Bioinformatics analysis of public biological databases was used to identify the most relevant familial WM candidate from WES. Transcript expression and protein levels of the familial WM candidate were evaluated in the WM patient and 2 unaffected members of the kindred. **RESULTS:** Among the 10 shared candidate mutations in the twins, the authors identified a novel heterozygous germline mutation in four and a half LIM domains protein 2 (*FHL2*; c.G226A, p.V76M) as a familial WM–associated mutation. *FHL2* appeared to be connected with reported signaling pathways and disease-driving genes such as *IL6* and *HCK* in WM. In addition, the authors found reduced *FHL2* messenger RNA and protein expression in peripheral blood samples from the patient with WM in comparison with the healthy siblings. **CONCLUSIONS:** Taken together, these findings indicate that an *FHL2*^{g226a} mutation may play an important role in familial WM, and they provide new screening possibilities for familial cases. ***Cancer* 2021;127:2039-2048**. © *2021 American Cancer Society*.

LAY SUMMARY:

- Familial clustering in Waldenström macroglobulinemia (WM) has been observed over the years.
- The authors performed whole exome sequencing of germline DNA samples from twins, one diagnosed with WM and the other diagnosed with immunoglobulin M monoclonal gammopathy of undetermined significance, and their healthy siblings.
- Among the 10 shared candidate mutations in the twins, a novel heterozygous germline mutation in four and a half LIM domains protein 2 (*FHL2*; c.G226A, p.V76M) was identified as the most relevant familial WM candidate through bioinformatics analysis of a public database.
- Also, messenger RNA and protein expression of *FHL2* was significantly lower in peripheral blood mononuclear cells of the WM patient in comparison with the healthy siblings, and this suggested that the function of FHL2 was impaired when mutated.

KEYWORDS: familial Waldenström macroglobulinemia, four and a half LIM domains protein 2 (FHL2), genetic predisposition, germline mutation, twins.

INTRODUCTION

Waldenström macroglobulinemia (WM) is a subtype of non-Hodgkin lymphoma that is highly distinctive on the basis of its hypersecretion of monoclonal immunoglobulin M (IgM) and bone marrow infiltration with lymphoplasmacytic cells.[1] WM is a very rare disease with an incidence rate of 3 to 4 cases per million people per year.[2] However, immunoglobulin M monoclonal gammopathy of undetermined significance (IgM-MGUS) is more frequent in the population. Patients with IgM-MGUS can progress to WM at the rate of 1.5% to 2% per year.[3] Genetic factors are also important in determining the etiology of WM. Whole genome sequencing studies have shown that *MYD88* and *CXCR4* WHIM-like somatic mutations occur in more than 90% and 30% to 35% of WM patients, respectively.[4-6]

Familial clustering in WM has been previously documented. The earliest report of familial WM dates back to 1962 and describes 2 brothers with WM and their mother with asymptomatic IgM monoclonal gammopathy.[7] Since then,

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many researchers have shown familial aggregation of WM as well as other B-cell lymphoproliferative disorders, including non-Hodgkin lymphoma, multiple myeloma, chronic lymphocytic leukemia, monoclonal gammopathy of undetermined significance, acute lymphoblastic leukemia, and Hodgkin lymphoma, among first-degree relatives of patients with WM.[8-12] All these findings support the role of common susceptibility genes in the genetic predisposition to WM and other lymphoproliferative disorders.

Furthermore, enhanced ex vivo survival of peripheral blood B cells along with overexpression of the antiapoptotic BCL-2 protein has also been demonstrated among kindred of WM patients.[13] Roccaro et al[14] performed whole exome sequencing (WES) and identified *LAPTM5*^{c403t} and *HCLS1*^{g496a} in 3 WM members of an index family and in 8% of the extended familial cases. However, there is a need to further understand the importance of genetic factors in familial predisposition to WM. Germline-based genetic studies in patients with familial WM are very limited, and they may include important clues for deepening our understanding of the pathogenesis of WM. For this reason, in this study, we examined the phenotypes of 2 identical twins, one diagnosed with WM and the other with IgM-MGUS. WES of germline DNA obtained from these twins and their healthy siblings was also performed to further investigate these genetic disorders.

MATERIALS AND METHODS

Study Oversight

This study was approved by the medical ethics committee of Renji Hospital (School of Medicine, Shanghai Jiaotong University). This research was conducted according to the criteria set by the Declaration of Helsinki, and each subject signed an informed consent form before participating in the study.

Study Participants

We performed a family-based study by using WES for a pair of identical twins, one affected by WM and the other affected by IgM-MGUS, and 3 healthy siblings without any B-cell lymphoproliferative disorders, who were considered to be controls. On the basis of previous identification of familial aggregation of WM cases and clustering of B-cell lymphoproliferative disorders (WM, chronic lymphocytic leukemia, non-Hodgkin lymphoma, multiple myeloma, IgM-MGUS, and IgG/IgA-MGUS) among first-degree relatives of WM patients, we considered the twins to be a familial WM case.

Whole Exome Sequencing

Genomic DNA of the twins and their siblings was isolated from saliva cells. An adaptor-ligated library was prepared with the Paired-End Sample Prep Kit V1 (Illumina). Exome capture was performed with the SureSelect Human All Exon V6 (Agilent Technologies). Massively parallel sequencing was performed on a HiSeq 2500 (Illumina), which generated 150 base reads. Sequences were aligned for variant calling and annotation with the human genome reference sequence (hg38 build) with the BWA aligner.[15] Downstream processing was performed with VarScan2,[16] SAMtools,[17] and Picard Tools (http://picard.sourceforge.net/). VarScan2 was used to identify substitution and indel variant calls. Calls with a read coverage of ≤20× and a Phred-scaled single-nucleotide polymorphism quality of ≤20 were filtered out. All variants were annotated with SnpEff.[18]

Screening of Candidate Familial WM Variants

We first selected variants that occurred in twins but were absent in the 3 healthy siblings. High-confidence variants were chosen with a sequencing depth greater than 5 and a variant allele fraction greater than 0.2. Variants obtained with a minor allele frequency less than 0.01 in the general population according to the Exome Aggregation Consortium database (Broad Institute) were supposed to be novel or rare and thus potentially significant. Deleterious variants were predicted with the Mendelian Clinically Applicable Pathogenicity (M-CAP) score. Variants in the GeneHancer database were also taken into consideration. Variants located in noncoding regions were filtered out, and the remaining variants were defined as candidate familial WM variants.

Gene Expression Data From the Gene Expression Omnibus Database

GSE6691(https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE6691) was used to define genes that were differentially expressed between bone marrow–derived primary WM cells and normal B cells. Differential expression was analyzed with the Bioconductor package limma (http://www.bioconductor.org/) in the R statistical computing environment (https://www.r-project.org/).[19] We determined empirical Bayes-moderated *t* statistics to calculate *P* values for 2-class unpaired samples. Differentially expressed genes were identified according to the cutoff criteria of $P < .05$ and $|\log_2 FC| > 1.0$ (where FC is the fold change).

Prioritization of Candidate Variants

Protein-protein interaction (PPI) networks were predicted with the differentially expressed genes from GSE6691 and with the candidate familial WM genes from WES through the functional protein association networks (STRING database). The interacting candidate familial WM genes were selected. In combination with the M-CAP score and hemopathy or immune-related research, top likely familial WM mutations were prioritized.

Bioinformatic Prediction of Protein Structures

Secondary structures of four and a half LIM domains protein 2 (FHL2) were predicted by PSIPRED (http://bioinf.cs.ucl.ac.uk/psipred/). Three-dimensional FHL2 protein reconstruction was obtained from the Phyre2 server. DUET was used to assess the stability of protein.

Quantitative Reverse Transcriptase–Polymerase Chain Reaction

Total RNAs were extracted from peripheral blood mononuclear cells (PBMCs) with TRIzol reagent (Invitrogen, Carlsbad, California) and then reverse-transcribed with the Transcriptor First Strand cDNA Synthesis Kit (Roche, Switzerland). The resulting complementary DNAs (cDNAs) were used for quantitative polymerase chain reaction analysis with the SYBR-Green PCR Master Mix Kit (Qiagen, Valencia, California). The relative gene expression levels were normalized to those of GAPDH and calculated with the ΔCT method. Primers for quantitative reverse transcriptase–polymerase chain reaction are shown in Supporting Table 1.

Western Blot

PBMCs were lysed in a radioimmunoprecipitation assay buffer, and protein concentrations were determined with a bicinchoninic acid assay (Beyotime, Shanghai, China). Whole cell lysates were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis with 10% gels and then were transferred to nitrocellulose membranes. After blocking with 5% nonfat milk, the membranes were incubated with the primary antibody (FHL2 [ab12327; Abcam, United Kingdom] and β-actin [30101ES10; Yeasen, Shanghai, China]) and the horseradish peroxidase–conjugated secondary antibody (Beyotime, Shanghai, China) according to the manufacturer's directions. Bands were visualized with a chemiluminescent horseradish peroxidase substrate (Millipore, Massachusetts) and an electrogenerated chemiluminescence imaging system (Tanon, Shanghai, China).

Sanger Sequencing

The observed *FHL2* variant was validated by Sanger sequencing. Genomic DNA was extracted from saliva cells. Primers were designed from the intron regions to span the splice site and the exon/intron boundaries with 5′-tagged M13 sequences. After successful polymerase chain reaction amplification, reactions were enzymatically purified with the Illustra ExoStar Kit (GE Healthcare, Buckinghamshire, United Kingdom). New asymmetric polymerase chain reaction reactions were prepared according to the cycle sequencing method with the BigDye Terminator v1.1 Cycle Sequencing Kit (Life Technologies). The obtained polymerase chain reaction products were purified with Performa DTR (EdgeBio, Gaithersburg, Maryland) and analyzed via capillary electrophoresis in a 3130xl genetic analyzer (Life Technologies). Sequencing data analysis was performed with SeqScape V2.5 software (Life Technologies). Primers for Sanger sequencing are shown in Supporting Table 1.

To detect the base sequence of the WM patient's messenger RNA (mRNA), the resultant reverse transcriptase–polymerase chain reaction products of twin 1 and 2 healthy siblings were TA-cloned (Invitrogen), and individual colonies were picked and submitted for Sanger sequencing as before. Primers for mRNA Sanger sequencing are shown in Supporting Table 1.

Statistics

Raw sequencing metrics analyses were performed with the limma package in an R/Bioconductor computational environment. Differentially expressed genes were identified according to the cutoff criteria of $P < .05$ and $|\log_2 FC| > 1.0$. Data obtained from triplicate experiments were expressed as mean ± SD and were analyzed with Prism software. The significance test for the differences between the mean scores was determined with an independent-sample *t* test. *P* values less than .05 were considered to be statistically significant.

RESULTS

Patient Phenotypes

We investigated a family in which one identical twin was affected by WM and the other was affected by IgM-MGUS (Fig. 1). Clinical features are summarized in Supporting Table 2. Twin 1 was a 63-year-old male reporting persistent fatigue lasting more than 2 years. The hemoglobin level at the time of diagnosis was 96 g/L. Immunological findings showed an increased level of IgM (4690 mg/dL). Also, IgM-kappa M protein was detected by immunofixation electrophoresis. Moreover, the *MYD88*^{L265P}

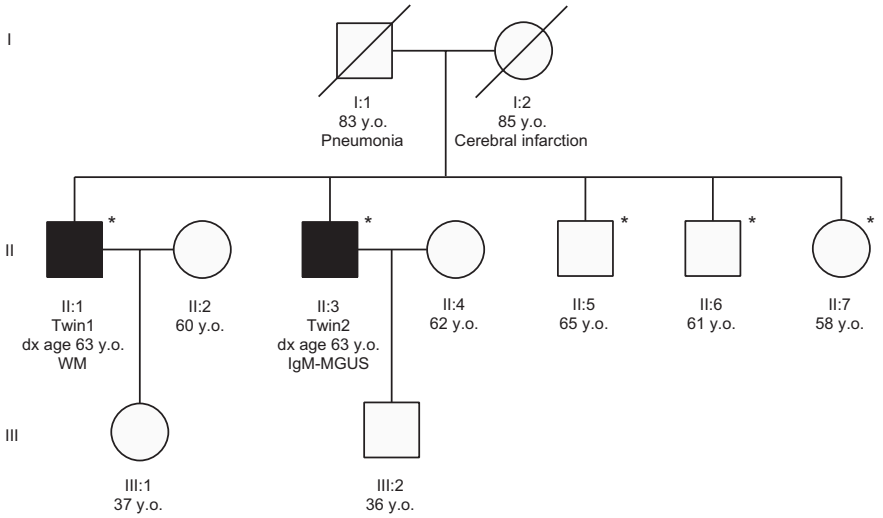

Figure 1. Family pedigree. Open symbols indicate healthy family members (II:2, II:4, II:5, II:6, II:7, III:1, and III:2), whereas black, filled symbols indicate individuals diagnosed with familial WM (II:1 [WM] and II:3 [IgM-MGUS]). Slashes indicate deceased family members. *Whole-exome-sequenced samples. dx indicates diagnosis; IgM-MGUS, immunoglobulin M monoclonal gammopathy of undetermined significance; WM, Waldenström macroglobulinemia; y.o., years old.

mutation was positive. Bone marrow morphology showed that the lymphoid system had proliferated abnormally, mainly with mature small lymphocytes. Plasmacytoid lymphocytes (2.5%), large lymphocytes, and occasionally prolymphocytes were observed. Bone marrow immunology demonstrated that $CD19^{+}$ cells expressed HLA-DR, CD19, CD20, CD22, FMC-7, Kappa, cKappa, and CD10 (partial). Furthermore, bone marrow biopsy displayed diffuse hyperplasia of lymphocytes, plasmacytoid lymphocytes, and plasma cells in the intertrabecular area of the bone. Twin 1 met the criteria for WM. He received rituximab, bortezomib, and dexamethasone therapy for 4 months. Reexamination of the blood showed that the hemoglobin level was up to 129 g/L and that the IgM level was down to 1920 mg/dL. IgM had decreased by 59%. Twin 2 presented with increased levels of IgM for more than 8 months. The highest level of IgM was 2960 mg/dL. He had no relevant clinical symptoms. IgM-lambda M protein was detected by immunofixation electrophoresis. The *MYD88*^{L265P} mutation was also positive. His bone marrow morphology and immunology were normal. He was diagnosed with IgM-MGUS and was followed up for observation.

Identification of a Novel FHL2^{g226a} *Mutation in Twins With Familial WM*

WES was performed on the twins and the 3 healthy siblings. The workflow for selecting candidate variants is described in Figure 2. This initial screen identified 10

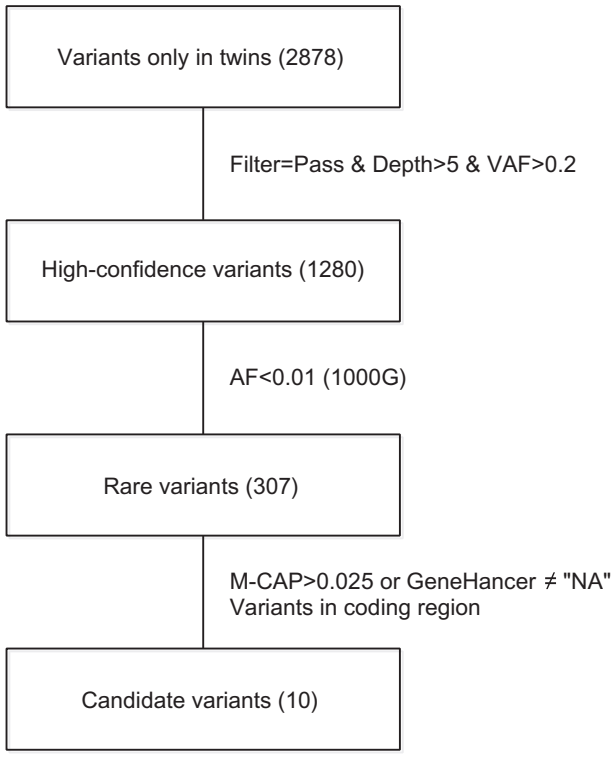

Figure 2. Workflow for selecting candidate Waldenström macroglobulinemia–associated variants. The number of variants identified in each step is shown. 1000G indicates 1000 Genomes; AF, allele fraction; M-CAP, Mendelian Clinically Applicable Pathogenicity; NA, not available; VAF, variant allele fraction.

rare, exonic, and nonsynonymous familial WM variants as candidates that also mapped to 10 genes (Table 1 and Supporting Table 3). All 10 germline variants were missense and heterozygous mutations. Among them, mutations of *FHL2* and *ZMAT5* were found to be novel on the basis of the Single Nucleotide Polymorphism Database. The variants were annotated and interpreted by implementation of M-CAP, as previously described,[20] and this allowed us to prioritize the deleteriousness of variants shared among twins. The M-CAP scores of *ZMAT5* and *DOCK2* mutations were less than 0.025, but these 2 mutations were already included in the GeneHancer database. Thus, we considered them to be candidate variants as well.

Next, we performed gene prioritization to select a familial WM–associated mutation. First, a publicly available gene expression data set (GSE6691)[21] was evaluated to define a WM cell mRNA signature. We compared the bone marrow–derived $CD19^+$ WM tumor cells obtained from newly diagnosed, untreated WM patients with B lymphocytes from peripheral blood obtained from healthy donors. We found that 111 genes were significantly different between WM patients and healthy donors ($P < .05$ and $|\log_2 FC| > 1.0$), and this confirmed the presence of a specific WM mRNA signature (Fig. 3A and Supporting Table 4). We identified 65 upregulated genes and 46 downregulated genes in WM patients. Furthermore, for these differentially expressed genes and candidate familial WM genes, a PPI networks analysis was performed (Fig. 3B and Supporting Table 5). A total of 294 protein pairs were obtained through PPI analysis, and their corresponding genes included 76 differentially expressed genes (48 upregulated and 28 downregulated) from GSE6691 and 4 candidate familial WM genes (*FHL2*, *MICALL1*, *CHST4*, and *DOCK2*). However, these 4 candidate genes were not differentially expressed in GSE6691. Among them, FHL2 was predicted to interact with IL-6 and HCK, which were upregulated in WM. Yang et al[22] showed that knockdown of *HCK* reduced survival and attenuated BTK, PI3K/AKT, and mitogen-activated protein kinase/extracellular signal-regulated kinase signaling in mutated *MYD88* WM and/or activated B cell–like diffuse large B-cell lymphoma cells. In myeloma cells, HCK is activated by IL-6 through the IL-6 coreceptor IL-6ST (GP130).[23] IL-6 is also a potent growth and survival factor in WM.[24] Previous studies have indicated that IL-6 from the tumor microenvironment promotes IgM secretion and cell growth via binding to the IL-6R on WM cells.[25-27] Recent results have shown that targeting IL-6R reduces IgM levels and tumor growth in WM.[28]

TABLE 1. Candidate Variants Likely Associated With Waldenström Macroglobulinemia in the Family

HUGO Symbol	Chr	POS	Reference Allele	Alternate Allele	dbSNP ID	Transcript ID	cDNA Change	Protein Change	M-CAP	GeneHancer
NEUROG1	5	135535455	C	A	rs752557883	NM_006161	c.G236T	p.R79L	0.529468143	NA
FHL2	2	105373664	C	T	NA	NM_001318894	c.G226A	p.V76M	0.221313566	Score = 1.30; name = GH02J105369
BRWD1	21	39258600	C	A	rs758709322	NM_018963	c.G1958T	p.S653I	0.085212325	NA
MICALL1	22	37940792	C	T	rs201526262	NM_033386	c.C2554T	p.R852C	0.066601116	NA
NR1H3	11	47261364	C	T	rs138301852	NM_001130102	c.C488T	p.P163L	0.048159321	NA
LARGE1	22	33283240	C	A	rs745392097	NM_133642	c.G1839T	p.L613F	0.038199314	NA
PCDHA5	5	140823261	C	T	rs781894789	NM_018908	c.C1486T	p.R496W	0.031213462	NA
CHST4	16	71537020	C	T	rs775213676	NM_001166395	c.C343T	p.P115S	0.030132263	NA
ZMAT5	22	29738365	C	A	NA	NM_001003692	c.G348T	p.K116N	0.012400343	Score = 0.38; name = GH22J029737
DOCK2	5	169803081	G	A	rs201511979	NM_004946	c.G2578A	p.V860I	0.005706219	Score = 1.38; name = GH05J169801

Abbreviations: cDNA, complementary DNA; Chr, chromosome; dbSNP, Single Nucleotide Polymorphism Database; HUGO, Human Genome Organisation; M-CAP, Mendelian Clinically Applicable Pathogenicity; NA, not available; POS, genomic position.
POSs (GRCh38) as well as cDNA and protein changes are listed. The deleteriousness of a variant was predicted with an M-CAP score greater than 0.025.

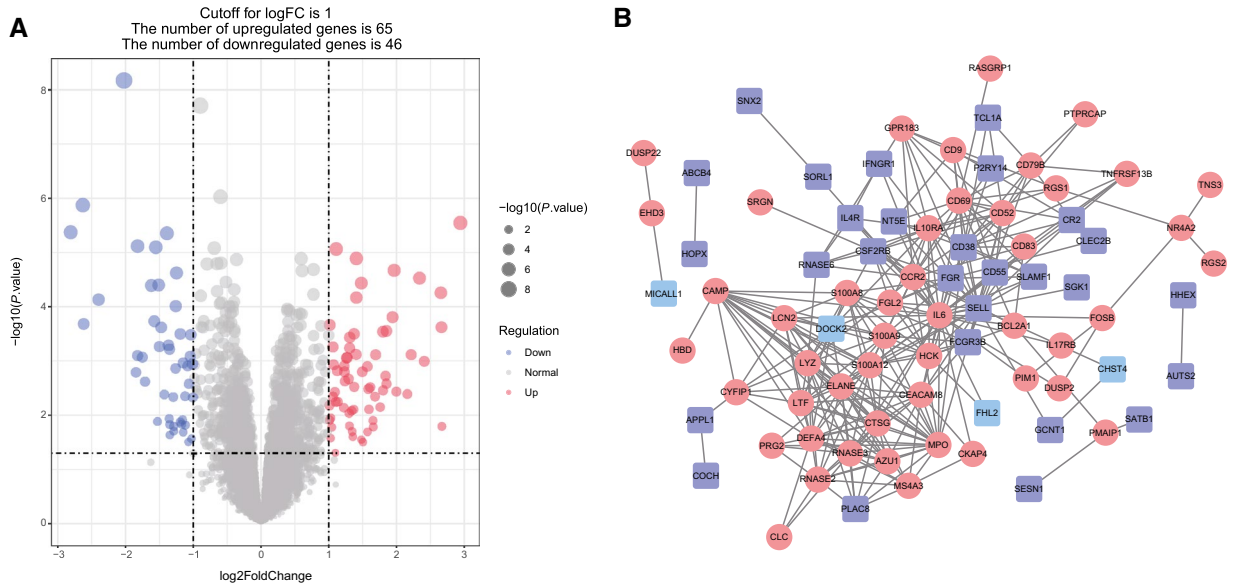

Figure 3. Identifying potential WM-associated variants. (A) Differential expression analysis of WM tumor cells and normal B lymphocytes using a public data set (GSE6691, https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE6691). The upregulated and downregulated genes are plotted in red and blue, respectively, according to the cutoff criteria of $P < .05$ and $|\log_2 FC| > 1.0$. Nonsignificant genes are shown in gray. (B) Protein-protein interaction networks for differentially expressed genes from GSE6691 and candidate familial WM genes from whole exome sequencing. The red, round nodes are upregulated genes from GSE6691, and the purple, square nodes are downregulated genes from GSE6691. The light blue, square nodes are candidate family WM genes that are not differentially expressed in GSE6691. FC indicates fold change; WM, Waldenström macroglobulinemia.

Genes containing deleterious variants that are significantly related to the WM mRNA expression signature and also related to hematological diseases or immunity may help us to identify the most promising familial WM–associated targets. FHL2 was reported to be unexpressed or underexpressed in lymphocytic leukemia, promyelocytic leukemia, and Burkitt lymphoma cell lines.[29] However, high FHL2 expression was observed in patients with acute lymphoblastic leukemia and complex karyotypes,[30] and enhanced expression of FHL2 could promote cell-cycle entry of myeloid progenitor cells and increase the frequency of apoptosis of bone marrow cells.[31] *FHL2* was suggested to be a potential tumor suppressor gene or oncogene according to this research. Therefore, we hypothesized that *FHL2* is strongly related to hematological diseases or immunity. By combining the results of the PPI networks analysis, we identified *FHL2* as the most relevant gene target among the 4 candidates.

Characteristics of the FHL2^{g226a} Mutation

Our Sanger sequencing confirmed the *FHL2*^{g226a} mutation in the family, which was present in the twins and absent in the 3 healthy siblings (Supporting Fig. 1). The twins' children whose immunoglobulin tested normal did not have the *FHL2*^{g226a} mutation either (Supporting Fig. 1). In addition, the variant allele fraction of the *FHL2*^{g226a} mutation was 0.3984 in twin 1 (WM) and 0.4872 in twin 2 (IgM-MGUS). The mutation is located on exon 3 of the *FHL2* gene (Fig. 4A). FHL2 is a 32-kDA protein consisting of helixes, strands, and coils. The mutation is predicted to be a missense mutation that causes an amino acid substitution from valine to methionine at the coil site (Fig. 4B,C). The stability change ($\Delta\Delta G$) of the *FHL2*^{g226a} mutation was –0.549 kcal/mol, which could result in destabilization.

Expression Levels of the FHL2^{g226a} Mutation in the Proband

The germline mutation *FHL2*^{g226a} was also present in the twins' peripheral blood. We performed quantitative reverse transcriptase–polymerase chain reaction and Western blotting to detect the expression of mutated FHL2 in PBMCs from twin 1 (diagnosed with WM) and the 2 unaffected siblings. Unfortunately, we were unable to obtain peripheral blood samples from twin 2. Results showed that the mRNA and protein expression of FHL2 in PBMCs of twin 1 was significantly lower in comparison with the healthy siblings (Fig. 5). This suggested that the function of FHL2 was affected and impaired by mutation. To detect whether the reduced

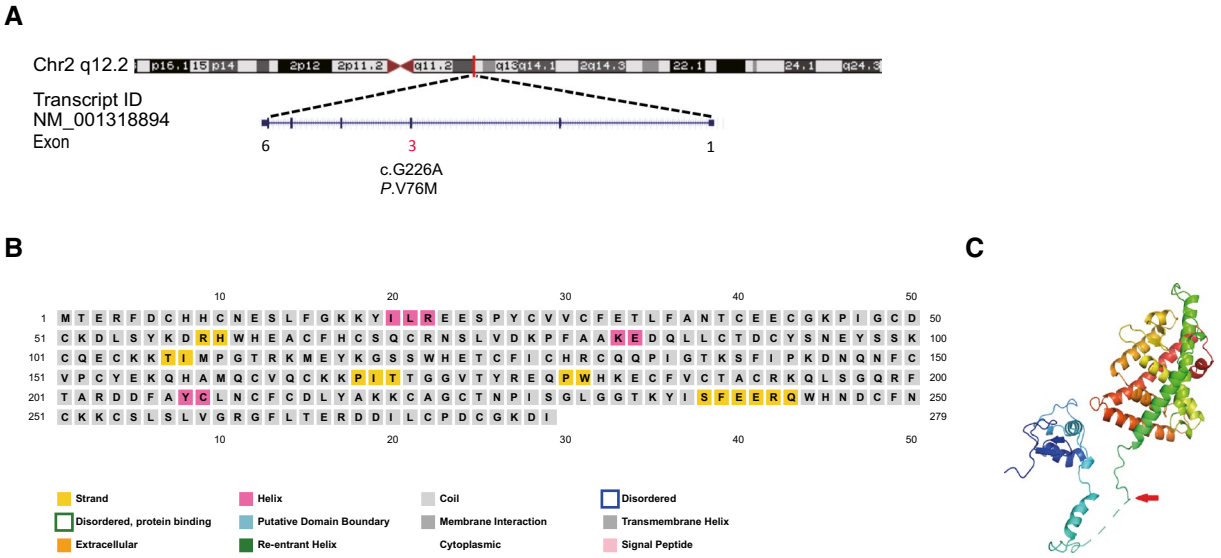

Figure 4. Characteristics of the *FHL2*^{g226a} mutation. (A) Genomic structure of *FHL2*^{g226a}. The location of the *FHL2* locus is shown by a red line; the centromere is indicated by red triangles. The gene structure is also shown (National Center for Biotechnology Information Reference Sequence Database No. NM_001318894). The exon is indicated by a blue box. The variant localizes in exon 3. (B) Secondary structures for FHL2 were predicted by PSIPRED (http://bioinf.cs.ucl.ac.uk/psipred/). Mutation positions and amino acid changes are indicated in red squares. (C) Three-dimensional reconstruction of the protein as predicted by Phyre2. NH_2-terminal and carboxyl-terminal domains are indicated in blue and red, respectively; V76M is indicated by a red arrow. Chr indicates chromosome; FHL2, four and a half LIM domains protein 2.

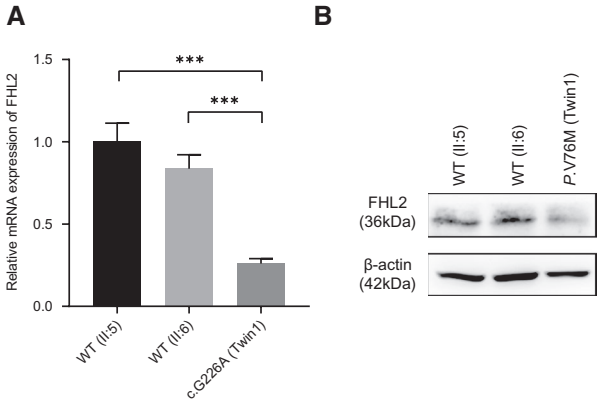

Figure 5. FHL2 expression levels in PBMCs of healthy siblings (II:5 and II:6) and twin 1 with Waldenström macroglobulinemia. (A) The relative mRNA expression of *FHL2* was assessed via quantitative reverse transcriptase–polymerase chain reaction in PBMCs. (B) The protein expression level of FHL2 was assessed via Western blotting in PBMCs with the indicated antibodies. β-Actin was used as an internal control. ***$P < .001$. FHL2 indicates four and a half LIM domains protein 2; mRNA, messenger RNA; PBMC, peripheral blood mononuclear cell; WT, wild type.

mRNA expression was caused by nonsense-mediated mRNA decay, RNA was extracted from PBMCs of twin 1 and 2 healthy siblings and was reverse-transcribed into cDNA for Sanger sequencing. The results showed that there was only a point mutation and that there was no nucleotide deletion in the mature mRNA (Supporting Fig. 2).

DISCUSSION

Previous reports as well as our own clinical experience prompted us to examine familial predisposition in WM. In this work, we have examined a family with twins, one diagnosed with WM and the other diagnosed with IgM-MGUS. IgM-MGUS is an early asymptomatic stage of WM. Twin 2 was found to have a progressive increase in IgM levels during the follow-up, and this meant that he was highly likely to progress to WM. The patients included in this research are very rare. They are monozygotic twins with highly similar genomic characteristics. If they are affected by the same type of disease, this may suggest the existence of a genetic driver and thus lead us to find the susceptibility genes. Here, the evaluation of cancer occurrence in a family with WM is significant for unraveling the molecular events that are driving the tumorigenesis.

Germline DNA from twins and 3 healthy siblings was exome-sequenced, and the following data analysis produced 10 rare and potentially damaging variants. All

of the 10 variants were present only in the twins and absent in their healthy siblings. Unfortunately, we were unable to obtain samples from other elder family members. In this specific case, the parents had already passed away, and it was difficult to trace the parents' siblings, who lived before WM was described and whose life expectancies were lower. For many patients, there may still be plenty of cases of familial WM because of the late age of onset and its indolent nature. Thus, we assume that the true familial susceptibility to WM actually may be higher than what we have observed in this study.

According to the screening criteria and data set analysis, the most promising candidate for familial WM is the *FHL2*^{g226a} mutation. However, a role for the other variants, especially mutations of *MICALL1*, *CHST4*, and *DOCK2*, or a combined effect of these mutations together cannot be ruled out. Notably, Hasan et al[32] showed that Wnt5a induces ROR1 to recruit and activate DOCK2, and this leads to activation of ERK1/2, which appears to be responsible for the capacity of Wnt5a to enhance chronic lymphocytic leukemia cell proliferation. Such Wnt5a-induced activation could be inhibited by cirmtuzumab but not by ibrutinib. This suggests that the ROR1/DOCK2-dependent pathway is related to ibrutinib resistance, and it could be assumed that DOCK2 also plays an important role in WM. However, the pathogenicity prediction of a *DOCK2* mutation was benign. Therefore, we did not consider this mutation to be a significant candidate in priority.

The *FHL2*^{g226a} mutation has not been reported before. It should be validated within other familial WM populations. The rarity of the *FHL2*^{g226a} mutation aligns with the estimates of WM prevalence in the overall population. WM represents a rare phenotype that probably requires more predisposing genetic factors than other common B-cell lymphoproliferative malignancies. This would be consistent with the findings of our study, which detected the *FHL2*^{g226a} mutation in the twins. On the other hand, the *FHL2*^{g226a} mutation might just be a benign variant. There is a high probability of finding the same mutation in a random pair of twins. Although the siblings and children did not have the *FHL2*^{g226a} mutation, this does not mean that they could not come down with B-cell lymphoproliferative disorders in the future.

FHL2 is a multifunctional adaptor protein that interacts with cell surface receptors, cytosolic adaptor and structural proteins, kinases, and nuclear transcription factors.[33,34] Its roles in cardiovascular diseases and carcinogenesis have been discussed comprehensively in recent years.[35-37] Different studies have unraveled the interconnections between FHL2 and a number of signaling cascades, including WNT, TNF-α, MAPK, TGF-β, androgen receptor, AP1, CREB/CREM, E4F1, and EpCAM.[38-47] Furthermore, WM cells have been reported to be associated with the WNT/β-catenin pathway and the ERK/MAPK pathway.[21,48] Thus, mutated FHL2 might contribute to WNT/β-catenin or ERK/MAPK modulation in WM cells. Also, PPI analysis has shown that FHL2 may interact with IL-6 and HCK, which previously have been confirmed to have a significant pathogenic effect in WM. Further studies to assess the precise signaling cascades of FHL2^{g226a}, IL-6, and HCK are, therefore, needed.

As mentioned earlier, *FHL2* may be an oncogene or a tumor suppressor gene in different tumors. However, the low expression of FHL2 in lymphocytic leukemia and Burkitt lymphoma[29] suggests that *FHL2* may play a tumor-suppressive role in lymphocytic tumors. In this study, we have found that the *FHL2*^{g226a} mutation would result in function loss of FHL2. Therefore, the decreased expression of FHL2 after mutation may be related to the occurrence and development of WM and IgM-MGUS. The reduced mRNA expression of *FHL2* suggested the possibility of nonsense-mediated mRNA decay. However, Sanger sequencing of mRNA ruled this out. One possible explanation is that the point mutation created a new initiation codon. Another possibility is that targeted binding of microRNA inhibits mRNA expression. The reasons for this deserve further study.

The identification of new predisposition genes may offer new and important insights into the molecular mechanisms of WM pathogenesis. It could also provide valuable tools for mutation screening and early intervention possibilities for carriers of mutations. The rareness of familial WM cases at our center prevents us from genetically validating the role of our variants. Further studies are needed to explore the relationship between the *FHL2*^{g226a} mutation and relevant signaling pathways, especially WNT and ERK cascades. The effect of mutation-induced FHL2 dysfunction on disease should be further investigated. Our findings suggest that the *FHL2*^{g226a} mutation may play an important role in WM susceptibility.

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CONFLICT OF INTEREST DISCLOSURES

The authors made no disclosures.

AUTHOR CONTRIBUTIONS

Yike Wan: Research, data analysis, and writing of the article. **Yuexin Cheng:** Data analysis and writing of the article. **Yabin Liu:** Data analysis. **Lijing Shen:** Data analysis. **Jian Hou:** Research study design and editing of the article.

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