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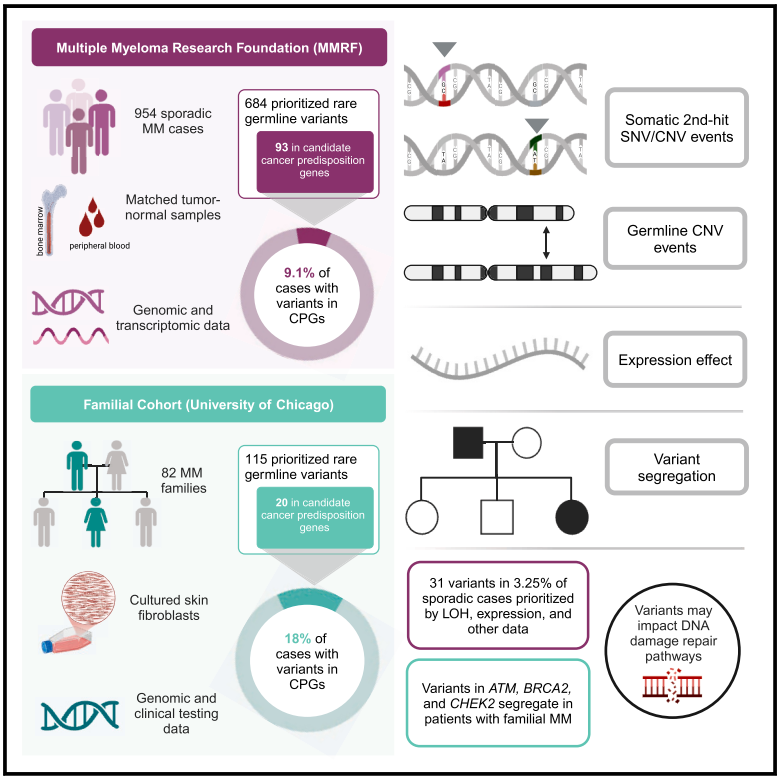
Germline predisposition in multiple myeloma

Graphical abstract



Highlights

- Putative rare risk variants were found in 9% of sporadic and 18% of familial MM cases
- LOH, expression, and other data identified 31 variants in 3.25% of sporadic MM cases
- Candidate variants were detected in genes related to cancer risk and MM
- Disruption of DNA damage repair pathways may play a role in MM susceptibility

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In brief

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Germline predisposition in multiple myeloma

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SUMMARY

We present a study of rare germline predisposition variants in 954 unrelated individuals with multiple myeloma (MM) and 82 MM families. Using a candidate gene approach, we identified such variants across all age groups in 9.1% of sporadic and 18% of familial cases. Implicated genes included genes suggested in other MM risk studies as potential risk genes (*DIS3*, *EP300*, *KDM1A*, and *USP45*); genes involved in predisposition to other cancers (*ATM, BRCA1/2, CHEK2, PMS2, POT1, PRF1,* and *TP53*); and *BRIP1, EP300,* and *FANCM* in individuals of African ancestry. Variants were characterized using loss of heterozygosity (LOH), biallelic events, and gene expression analyses, revealing 31 variants in 3.25% of sporadic cases for which pathogenicity was supported by multiple lines of evidence. Our results suggest that the disruption of DNA damage repair pathways may play a role in MM susceptibility. These results will inform improved surveillance in high-risk groups and potential therapeutic strategies.

INTRODUCTION

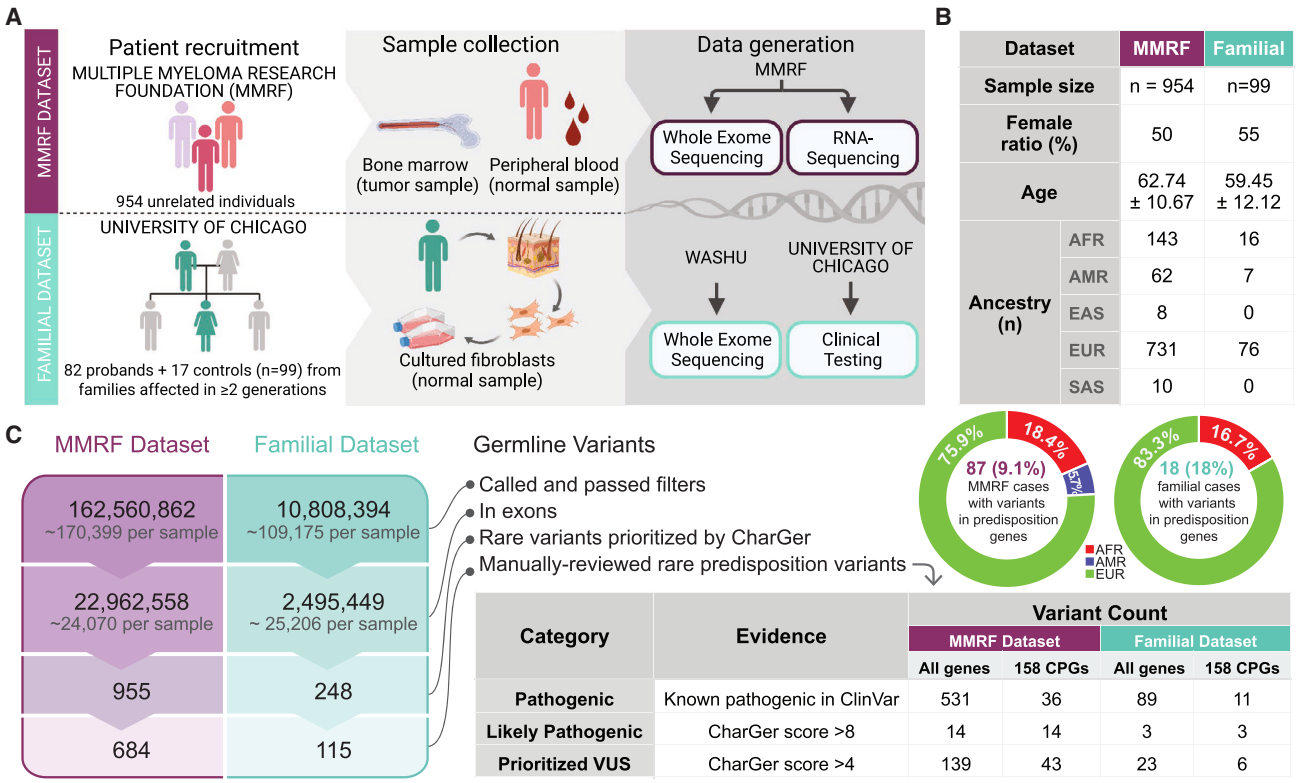
Multiple myeloma (MM) is an incurable malignancy, characterized by the clonal proliferation of malignant plasma cells in the bone marrow, monoclonal immunoglobulins in urine and serum, and organ dysfunction.[1,2] It is often preceded by a premalignant condition called monoclonal gammopathy of undetermined significance (MGUS), which progresses to asymptomatic smoldering MM (SMM), and ultimately, MM.[3–6]

Risk factors associated with MM include age, male gender, race/ethnicity, family history, and history of MGUS.[7–9] Individuals of African ancestry have a 2– to 3-fold higher risk of developing MGUS and MM and develop MM at earlier ages than others.[9–15] Additionally, first-degree relatives of MGUS/MM individuals have a 2– to 4-fold increased disease risk,[16–19] suggesting a germline contribution to MGUS and MM.

Our knowledge of inherited MM susceptibility has been informed mostly by genome-wide association studies (GWASs), which have identified 35 common risk single nucleotide polymorphisms (SNPs) at independent loci.[20–28] However, common variants rarely give a complete picture of disease inheritance.[29–34] The 35 risk loci explain less than 20% of MM heritability, suggesting that other genetic factors, such as rare germline events, play significant roles.[32–41] Prior studies have investigated rare germline predisposition variants (GPVs) in MM, identifying a few candidate predisposition genes, such as *DIS3*,[42,43] *CDKN2A*,[42,44,45] and *KDM1A*,[42,46] as well as genes with suggestive associations with MM, including *ARID1A*,[42,47] *EP300*,[42,48] *KIF18A*,[49] and *USP45*.[42,47] Although promising, these studies only involved populations of European ancestry not identifying variants relevant to other groups, and some may have been underpowered for the identification of moderately penetrant variants due to relatively small sample sizes. Moreover, they do not offer a comprehensive analysis of variant pathogenicity. Advancing beyond these limitations calls for larger cohorts, inclusion of additional ancestries, and more comprehensive detection and interpretation of rare germline variants.

Whole exome (WES) and/or whole genome sequencing (WGS) data from normal and tumor tissues, and from healthy controls, support the detection of both common and rare GPVs and genes through the analysis of both germline and somatic data. In recent years, >100 cancer predisposition genes (CPGs) (*i.e.,* genes in which rare pathogenic germline variants confer an increased cancer risk) have been reported, broadening our understanding of cancer predisposition.[50,51] Moreover, studies of CPGs involving multiple cancers (*i.e.,* pan-cancer) suggest that predisposing factors may be shared across cancer types.[51,52]

Recently, our group has published a pan-cancer study (>10K cases) that revealed new insights on GPVs across 33 cancer



Dataset		MMRF	Familial
Sample size		n = 954	n=99
Female ratio (%)		50	55
Age		62.74 ± 10.67	59.45 ± 12.12
Ancestry (n)	AFR	143	16
	AMR	62	7
	EAS	8	0
	EUR	731	76
	SAS	10	0

Category	Evidence	Variant Count			
		MMRF Dataset		Familial Dataset	
		All genes	158 CPGs	All genes	158 CPGs
Pathogenic	Known pathogenic in ClinVar	531	36	89	11
Likely Pathogenic	CharGer score >8	14	14	3	3
Prioritized VUS	CharGer score >4	139	43	23	6

Figure 1. Discovery of germline predisposition variants in 954 MM cases from the MMRF cohort and 82 families

(A) Recruitment of research subjects and sample collection details for both the Multiple Myeloma Research Foundation (MMRF) and University of Chicago familial datasets.

(B) Characteristics of the 954 MMRF and 99 familial samples, including sample size, female ratio, age distribution, and genetic ancestry for each cohort. Age is depicted as the average age +/− one standard deviation (MEAN +/− SD). Female ratio is the percentage (%) of female individuals in each cohort. Genetic ancestry is estimated from WES data by training a random forest classifier on variants detected in each cohort overlapping with the 1000 Genomes dataset, classifying samples into African (AFR); Ad Mixed American (AMR); East Asian (EAS); European (EUR); or South Asian (SAS). Accuracy on the test set was >99% for both datasets (see Figure S1).

(C) Summary of germline variant calling and CharGer results for both datasets, showing the percentage of affected cases by ancestry group. Variants passing manual review are used in downstream analyses.

types, and informed guidelines for the detection and classification of rare germline variants.[51] This study, however, did not include MM. Here we present progress in characterizing the landscape of rare germline variants underlying MM predisposition by leveraging these approaches and boosting analyses with the integration of two cohorts: 1) 954 cases of sporadic MM from the Multiple Myeloma Research Foundation (MMRF) CoMMpass (relating Clinical outcomes in MM to the personal assessment of genetic profile) Study; and 2) 82 families with a personal/family history of MM, other hematopoietic malignancies, and/or young-onset solid tumors (Figures 1A and 1B). Using a candidate gene approach, we focus on several candidate CPGs and other genes related to MM in different contexts. We hypothesize that the missing heritability of MM is largely explained by rare pathogenic GPVs in known and novel MM predisposition genes and CPGs. Revealing the full spectrum of GPVs in MM, their functional impacts, the interactions between them, the genetic background of individuals, and somatic mutations, move us closer to the personalized treatment of this disease.

RESULTS

Germline variant detection and quality control

We analyzed germline WES from 954 MMRF samples and 82 families (n = 99; Figures 1A and 1B) which passed quality control criteria, reaching ≥10X coverage across 158 candidate CPGs curated from the literature reported to carry rare, pathogenic variants associated with different cancer types, including genes with suggestive associations with MM (STAR Methods; Figure S1A; Table S1 - tab ST1A), as well as ≥20X coverage over target regions (Figure S1B). WES data were also used to predict genetic ancestry using a random forest classifier, achieving ~99% accuracy for both datasets (STAR Methods; Figure S1C; Table S1 - tabs ST1E and ST1F).

Samples passing quality control criteria were used to detect germline variants using GermlineWrapper (STAR Methods), which implements three tools: VarScan2,[53] GATK,[54] and Pindel.[55] Variants were filtered and annotated (STAR Methods), resulting in a total of 22,962,558 and 2,495,449 exonic calls for the MMRF and familial cohorts, respectively (Figure 1C). As expected, we

observe a higher number of variants for individuals of African (AFR) ancestry compared to others, with an average of 28,138 and 29,319 variants in the MMRF and familial datasets, respectively, compared to 23,300 and 24,338 respective variants for individuals of European (EUR) ancestry (Figure S1D). Variant quality control measures confirmed high data quality, with >99% concordance with dbSNP (release 151; Figure S1E), and an average transition-transversion (TiTv) ratio of 2.84 for both cohorts.

Discovery of candidate predisposition variants, genes, and DNA damage repair pathways in multiple myeloma susceptibility

We classified germline variants into pathogenic (P), likely pathogenic (LP), or prioritized variants of uncertain significance (PVUS) using CharGer,[56] which prioritizes variants based on guidelines by the American College of Medical Genetics and Genomics - Association for Molecular Pathology (ACMG-AMP)[57] (STAR Methods). CharGer prioritized 955 and 248 rare variants (allele frequency [AF] $\leq$0.05% in the 1000 Genomes Project or gnomAD r2.1.1) in the respective MMRF and familial cohorts (Figure 1C). From these, 684 passed manual review in both normal and tumor samples in MMRF, from which 93 were found in the 158 candidate CPGs listed in Table S1 (tab ST1A) (36 P, 14 LP, 43 PVUS) in 9.1% of the cohort (n = 87), with P/LP variants affecting 5.1% of cases (n = 51; Table S2 – tabs ST2A and ST2B). We also detected a subset of 31 variants for which pathogenicity was supported by multiple lines of evidence in 3.25% of the MMRF cohort (n = 31; Tables S2 and S8; also see Figure 5). For the families, 115 rare variants passed manual review, with 20 in CPGs (11 P, 3 LP, 6 PVUS) affecting 18% of the cohort (n = 18), with P/LP variants in 13.1% of cases (n = 13; Table S2 – tabs ST2A and ST2E). Of the 87 affected MMRF cases, the majority were of European ancestry, with 75.9% EUR, 18.4% of African ancestry (AFR), and 5.7% of Admixed American (AMR) ancestry (Table S2 – tab ST2B; Figure 1C). Moreover, affected individuals had an average age of 62.83 years (±10.45) and 52% were females. Of the 18 affected familial cases, 83.3% were EUR and 16.7% AFR, with an average age of 59.16 years (±8.62) and 66% were females (Table S2 – tab ST2E). For transparency of results, the full list of 684 variants detected in the MMRF cohort and 115 variants detected in the familial cohort are provided in Table S2 (tabs ST2D and ST2G, respectively). Later in discussion, we comment on findings pertaining to variants in the list of 158 candidate CPGs and other MM-related genes.

Among affected genes in our list of 158 candidate CPGs (listed in Table S1), we observe *DIS3*[43] and *KDM1A*[46] previously reported to harbor MM-associated GPVs, as well as genes with suggestive associations with the disease, including *EP300*[48] and *USP45*[47] (Figure 2; Table S2 – tabs ST2B and ST2E). Additionally, we found variants in genes beyond this list, linked to some of the 35 common, low-risk variants associated with MM[20–25,27,28] (Table S2 – tabs ST2C and ST2F; genes listed in Table S1 – tab ST1D). For example, a P frameshift variant in *POT1* (p.Q358fs) in a 48 year old (y/o) male with a family history of breast cancer (BRCA) and lymphoma (mother) in the familial dataset. This tumor suppressor gene (TSG) plays a role in telomere protection and chromosomal stability,[58,59] is somatically mutated in MM,[60] associated with melanoma predisposition,[61] and commonly mutated in chronic lymphocytic leukemia.[62–64] We also detected a P stop-gain mutation in *DNAH11* (p.R1445*) in a 62 years/o male with unknown history of cancer in the MMRF (Table S2 – tab ST2C). Both *POT1* and *DNAH11* contain common low-risk variants, rs58618031 and rs4487645,[20,25] respectively, but were not implicated as the main functional candidates for the loci. Further studies are needed to determine their involvement in MM risk. We also observed germline variants in other genes reported as somatically mutated in MM (*FGFR3, KDM5C, SAMDH1, TGDS, TRAF3IP1*)[60,65–67], and genes from a recent germline study (*AMPD3, ANO10, DARS2, DSP, FLNC, MYH7, PROM1, SLC6A19*, *WFS1*)[68] (Table S2 – tabs ST2C and ST2F).

Interestingly, we detected candidate GPVs in genes predisposing to other cancers, including a P *BRCA1* missense variant (p.M1796R) within MMRF, and truncating and missense variants affecting *BRCA2* in both cohorts (Table S2). Besides, well-known in ovarian cancer (OV) and BRCA predisposition,[51,52] and as Fanconi anemia genes,[69] germline variants in these genes are associated with a risk of developing therapy-related myeloid neoplasms (t-MN) and other hematopoietic malignancies,[70–74] including MM. In particular, *BRCA2* has been reported as a candidate MM predisposition gene in a family study.[75] Moreover, a recent study investigating *BRCA1/2* deleterious germline variants in patients with hematopoietic malignancies without previous diagnosis of other solid tumors and who had not undergone any kind of chemotherapy or radiotherapy showed a high prevalence of MM in *BRCA2* variant carriers.[76] Other known CPGs carrying candidate GPVs for MM include *ERCC2, FANCA, PMS2,* and *PRF1*.

Mutation burden analysis using the total frequency test (TFT)[51,77] (STAR Methods) against the Genome Aggregation Database (gnomAD) non-cancer cohort (n = 118,479), used as controls, revealed significant enrichment of P/LP events (FDR$\leq$ 0.05) in *BRCA2* (FDR = 0.023), *CHEK2* (FDR = 0.045), and *KDM1A* (FDR = 0.045), and suggestive enrichment (FDR $\leq$ 0.15) in *ATM* (FDR = 0.067), *POT1* (FDR = 0.069), *PMS2* (FDR = 0.081), *TP53* (FDR = 0.116), and *PRF1* (FDR = 0.116) in the familial cohort (Figure 2C; Table S3). These findings confirm previously reported gene-MM associations, such as *BRCA2*[76,78] and *KDM1A*,[46] and point to the involvement of genes that have only been reported as somatically mutated in MM, but also involved in susceptibility to other cancers, such as *ATM, CHEK2, POT1,* and *TP53.* Finally, they suggest novel gene-MM associations, such as *ATM, PMS2,* and *PRF1.*

To find shared predisposition factors between MM and other cancers, and potential MM specific associations, we also tested the burden of P/LP events in our MM cohorts using The Cancer Genome Atlas (TCGA) dataset[51] for comparison, which includes >10K adult cancer cases across 33 cancer types, excluding MM (STAR Methods). Several genes reported in somatic MM studies were potentially associated with MM risk in the families, such as *ATM* (FDR = 0.1) and *CHEK2* (FDR = 0.09) (Figure S2A; Table S3). In the TCGA cohort, *ATM* is associated with stomach (STAD), prostate (PRAD), pancreatic (PDAC), lung adenocarcinoma (LUAD), and BRCA.[51] *BRCA2* is associated with MM in our familial cohort (FDR = 4.92E-02), and with BRCA, OV, and PDAC in TCGA,[51] suggesting shared predisposition factors

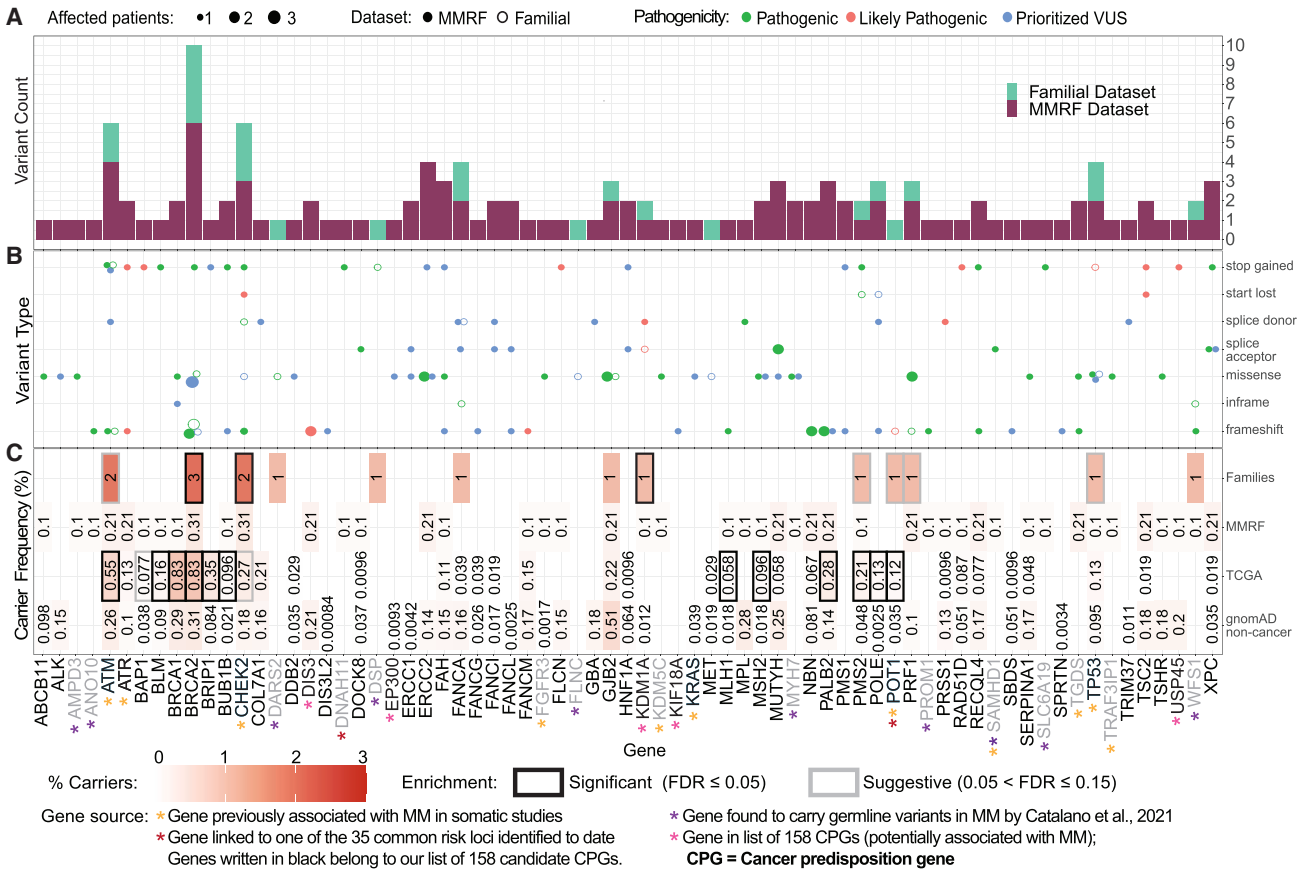


Figure 2. Distribution of rare germline predisposition variants across genes

(A) Sum of unique P, LP, and PVUS per gene in each dataset, represented by stacked bars.

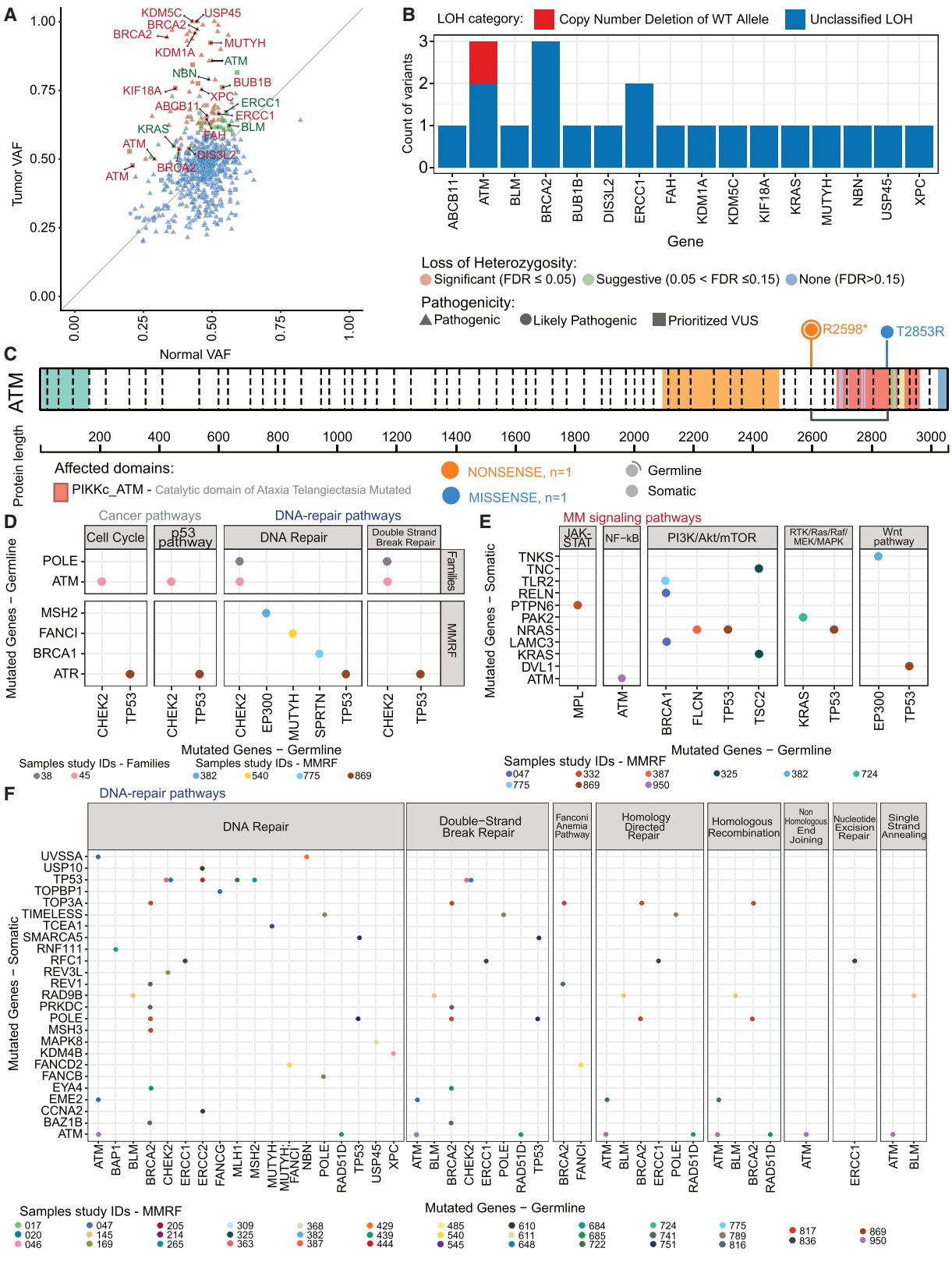
(B) Number of cases (represented by dot size) affected by P/LP/PVUS across genes in each dataset.

(C) Burden test results for MMRF and Familial datasets against the gnomAD non-cancer cohort. Results from our TCGA germline study by Huang et al.[51] were included for a pan-cancer level comparison. The numbers in each box indicate the percentage (%) of carriers (carrier frequency) of P/LP variants of each gene per cohort. The black outline indicates significant (FDR≤0.05) enrichment for P/LP variants of that gene; the gray outline indicates suggestive (FDR≤0.15) enrichment. Only variants in the 158 candidate CPGs and other MM-related genes are represented. See Table S2.

between MM and other cancers. Moreover, we observe suggestive specific associations of *TSC2* (FDR = 0.14) and *XPC* (FDR = 0.1) with MM when comparing the MMRF dataset to TCGA. *TSC2* is a TSG within the PI3K/Akt/mTOR pathway, which is commonly activated in MM, playing an important role in MM cell survival and growth.[79] Truncations of this gene lead to increased mTOR signaling and phosphorylation of its downstream effectors, resulting in cell growth and tumor development.[80–83] Loss-of-function of XPC impairs its ability to recognize DNA defects together with RAD23, CENT2, and UV-DDB, disrupting the NER pathway in MM.[84,85]

To account for the potential impact of genetic ancestry in our results, we also performed a burden test including only individuals of European ancestry from the MMRF and familial cohorts against the gnomAD non-cancer Non-Finnish subset (n = 51,377; STAR Methods). We can observe that our results do not differ significantly when controlling for this confounding variable (Figure S2B; Table S3). In fact, *BRCA2* remains significantly enriched for P events in the Familial EUR dataset when compared to the gnomAD non cancer Non-Finnish cohort (FDR = 8.18E-03; Figure S2B). *CHEK2* appears with suggestive enrichment in the families compared to gnomAD non-cancer (Figure S2A) and significantly enriched in that cohort after controlling for ancestry (FDR = 4.01E-02; Figure S2B). The results across the TCGA cancer types are also consistent.

Further, we find overlapping events between our MM cohorts and TCGA, suggesting a pleiotropic effect of these variants and genes. In total, 16 and 5 variants detected in TCGA were called in the MMRF and familial datasets, respectively (Table S3). We observe a P frameshift in *BRCA2* (p.V220fs) associated with OV and BRCA in TCGA. This variant was found in a 61 years/o female in MMRF with no family history of cancer, supporting a role for this gene in MM risk, consistent with previous studies.[76] Another example is a *CHEK2* frameshift (p.L465fs) associated with PDAC, lymphoid neoplasm diffuse large B-cell lymphoma (DLBC), and testicular germ cell tumors (TGCT) in TCGA. This variant was found in a 66 year/o female in the familial cohort with a family history of MM (mother) and



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prostate cancer (brother), supporting the role of *CHEK2* in MM susceptibility.

Finally, functional enrichment analyses of KEGG and Reactome pathways for affected genes belonging to our list of 158 CPGs (genes listed in Table S1 – tab ST1A) and other MM related genes (genes listed in Table S1 – tabs ST1B and ST1C; STAR Methods) revealed significant enrichment (FDR $\leq$0.05) of DNA damage repair pathways, such as Homologous Recombination (HR), which play a role in MM and other cancers[84,86–95] (Figures S3 and S3E; Table S4). The presence of P/LP events in *ATM, BRCA1,* and *BRCA2* may indicate the potential disruption of the HR pathway, likely not in the form of Homologous Recombination Deficiency (HRD) as it is observed in solid tumors, given that the presence of HRD in MM has been refuted and a topic of major discussion and controversy in the literature.[91–93,95,96] In the occurrence of the disruption of the HR pathway, Non-Homologous End-Joining (NHEJ) tends to be activated,[97] exemplified here by the significant enrichment of NHEJ in the MMRF (Figure S3A). Other pathways enriched in MMRF include Mismatch Repair (MMR) and Nucleotide Excision Repair (NER; Figures S3A and S3B). In the families, we see the enrichment of the Base Excision Repair (BER) and oncogenic signaling pathways,[98] such as cell cycle and p53 signaling. Additionally, although not significant, we observe P, LP, and PVUS events affecting genes in key signaling pathways in MM, such as JAK/STAT, NF-κB, RTK/Ras/Raf/MEK/MAPK, PI3K/Akt/mTOR, and Wnt signaling[79] (Figure S3C), and other important oncogenic signaling pathways, such as Notch, and TGF-beta (Figure S3D).

Analyses of two-hit events strengthen the characterization of germline predisposition variants

We examined loss-of-heterozygosity (LOH) events using allele fractions from matched tumor-normal data from MMRF to identify variants positively selected in the tumor in the context of the two-hit hypothesis.[52,99,100] We also investigated *cis* biallelic events to identify cases with both germline P/LP variants and missense or truncating somatic mutations in the same gene.

We identified 16 germline variants in CPGs and other MM-related genes undergoing significant (FDR $\leq$0.05) LOH in tumors (6 P, 2 LP, 8 PVUS) (Figure 3A; Table S5). Of these, 12 are in TSGs and 4 are in genes not classified as TSG or oncogene. Additionally, we observe 5 variants showing suggestive (FDR $\leq$0.15; tumor VAF >0.6 and normal VAF <0.6) LOH in tumors (3 P, 2 PVUS), of which 4 are in TSGs and one in an oncogene.

Three variants with significant LOH affect genes previously suggested to be associated with MM risk by single studies[46,47,49]: an LP splice variant in *KDM1A* (c.517 + 1G>A, FDR = 1.48E-13); a PVUS frameshift in *KIF18A* (p.P648fs, FDR = 1.36E-11); and an LP stop-gain in *USP45* (p.C38*, FDR = 4.39E-04). *KDM1A* and *KIF18A* are part of the PI3K/Akt/mTOR and cell cycle pathways, respectively, while *USP45* is involved in NER (Figures S3C–S3E). Additionally, these events affect cases with no family history of cancer, suggesting a contributing role in these patients' disease. Further studies and stronger evidence, however, are needed to confirm whether there is a true association of these genes with MM risk.

Two truncating *BRCA2* P variants (p.W993* and p.V220fs) showed strong LOH in these tumors (FDR = 5.39E-06 and FDR = 4.17E-09, respectively). Although these variants have P evidence from multiple submitters in ClinVar linked to OV and BRCA, both affect women with an unknown history of other cancers, suggesting their involvement in MM (Table S5). We also detect a *BRCA2* missense PVUS (p.T544I) with significant LOH (FDR = 1.82E-02) in a 69 year/o male with a family history of breast, lung, and colorectal cancer, and MM/MGUS (maternal grandmother), indicating the potential segregation of this variant with disease and common susceptibility between MM and the other cancers. Together, these results further underscore a role for *BRCA2* in MM susceptibility, consistent with previous studies.[75,76] Variants in CPGs not yet associated with MM susceptibility also showed significant LOH, including p.E297G in *ABCB11* (FDR = 1.52E-02), p.F581fs in *BUB1B* (FDR = 3.13E-04), p.R167W in *ERCC1* (FDR = 1.16E-02), and p.R415* in *XPC* (FDR = 1.32E-07; Table S5).

Two loss-of-function variants in *ATM* showed significant LOH. This gene is somatically mutated in MM,[60,65,101] but is not yet associated with MM risk. One is a stop-gain PVUS (p.E649*, FDR = 1.21E-05) in a 59 year/o female with an unknown family history of cancer. The other is a P frameshift (p.T237fs, FDR = 6.35E-03) in a 67 year/o male with a family history of BRCA. Interestingly, we observe a P stop-gain mutation (p.R2598*) in *ATM* undergoing suggestive LOH (tumor VAF = 0.86, normal VAF = 0.5) in a 45 year/o female with no family history of cancer. Analysis of tumor copy number variation (CNV) using GISTIC2 (STAR Methods) indicates that this LOH event may be due to the deletion of the wild-type allele (Figure 3B). Moreover, analyses of *cis* biallelic events indicated that this variant is coupled with a somatic missense (p.T2853R) that disrupts the catalytic domain of ATM disrupting its DNA repair functionality (Figure 3C). The presence of copy number deletion of *ATM* and this downstream somatic event supports the two-hit abruption of the gene and suggests a causal role for p.R2598*. Further experiments would be needed to investigate these events.

Figure 3. LOH and biallelic events in the MMRF dataset

(A) Comparison of variant allele frequencies (VAFs) in tumor and normal samples reveals events undergoing LOH in the tumor. Dots represent variants; diagonal line indicates equal tumor and normal VAFs (*i.e.,* neutral selection); green represents suggestive LOH (FDR$\leq$0.15); red represents significant LOH (FDR$\leq$0.05); blue represents events not statistically significant.

(B) Number of variants showing different types of LOH classified based on somatic copy number changes; only significant LOH events were classified. We highlight the LOH of an *ATM* P stop-gain variant (p.R2598*) due to copy number deletion of the wild-type allele (shown in red). Data are represented as the total number of variants.

(C) Lolliplot representing a candidate biallelic event of the same *ATM* variant coupled with a somatic event (p.T2853R) in a 45 year/o, female MM patient with no family history of cancer. See Table S8.

(D–F) Trans germline-germline (D) and germline-somatic events (E and F). Germline-germline events were also evaluated for the families. Dots represent a *trans* event, colored by sample. See Table S5.

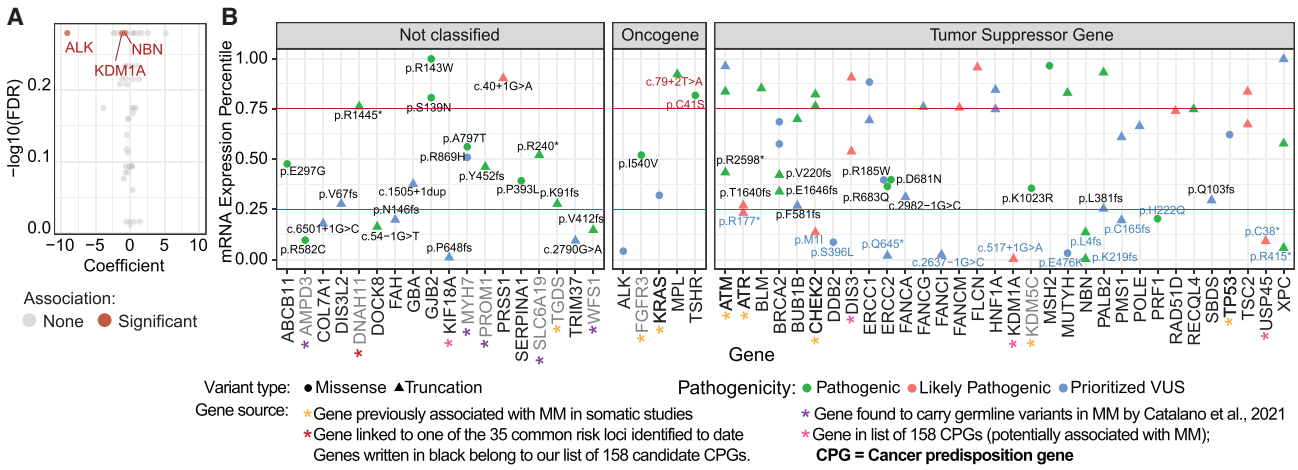


Figure 4. Effect of candidate predisposition variants on gene expression in the MMRF

(A) Genes significantly associated (FDR ≤ 0.05, linear regression) with higher or lower expression in carriers of P/LP/PVUS. Significance is represented as -log10(FDR) (y axis), and the estimated change in gene expression level is given as log2 fold change (coefficient on the x axis). Dots represent genes.

(B) Gene expression distribution in carriers of P/LP/PVUS. Dots indicate gene expression percentile in the carrier relative to other cases, depicted in the y axis. Variants in oncogenes associated with >50% expression are labeled, and those associated with >75% expression are written in red. Variants in TSGs associated with <50% expression are labeled, and those associated <25% expression are written in blue. Variants in genes not classified as tumor suppressor genes or oncogenes are also labeled.

Expanding beyond the classical two-hit hypothesis, we also investigated what we refer to as *trans* events, *i.e.,* genes in the same biological pathway affected by P/LP/PVUS germline variants (germline-germline), or by both P/LP/PVUS germline variants and missense or truncating somatic events (germline-somatic) (STAR Methods; Table S6). In the MMRF, we observe a 41 year/o woman with a missense and a stop-gain germline mutations in *TP53* and *ATR*, respectively (p.C141S and p.R177*) (Figure 3D). Both genes are part of the cell cycle and p53 signaling pathways, which are disrupted in cancer.[98] The same case also carries *trans* germline-somatic events affecting key MM signaling pathways, including PI3K/Akt/mTOR, RTK/Ras/Raf/MEK/MAPK (*TP53* germline p.C141S and *NRAS* somatic p.Q61R), and the Wnt signaling pathway (*TP53* germline p.C141S and *DVL1* somatic p.S190R) (Figure 3E). The latter pathway is involved in MM cell differentiation, proliferation, apoptosis, and migration.[79,102–105] *DVL1* has an important role in Wnt signaling,[106–108] promoting LRP6 phosphorylation and activating Wnt transcriptional activity.[109,110] Mutations in this gene are associated with aberrant Wnt signaling in several cancers, including MM.[105,107,111–114] We also found a 62 year/o male in the familial cohort with both a splice and stop-gain P variants in *ATM* and *CHEK2,* respectively (c.444 + 1G>A and p.Q2641*) (Figure 3D). These genes are involved in the cell cycle, p53 signaling, and double-strand break repair pathways.

Other examples of *trans* germline-somatic events detected in the MMRF affect other MM signaling pathways, such as JAK-STAT and NF-κB (Figure 3E), and oncogenic signaling pathways, including cell cycle, p53 signaling, NOTCH, and NRF2 (Figure S4). We also observe many germline-somatic events in genes within DNA damage repair pathways, including Fanconi anemia, HR, NHEJ, NER, and single strand annealing pathways (SSA), supporting the notion that DNA repair pathways may play a role in MM (Figure 3F). For example, we found a 61 year/o female with a P frameshift in *BRCA2* (p.V220fs) coupled with splice and missense mutations in *POLE* and *TOP3A*, respectively (c.2320-2A>C and p.Y8N), which are part of the HR pathway.

Gene expression changes in variant carriers in the Multiple Myeloma Research Foundation dataset

We calculated the percentile of gene expression for carriers of P/LP/PVUS events relative to non-carriers in the MMRF and performed a differential expression analysis to identify altered genes in carriers (STAR Methods). Our results show that the TSGs *KDM1A* and *NBN* are associated with lower expression in P/LP variant carriers (FDR = 3.63E-03 and FDR = 7.54E-03, respectively; Figure 4A; Table S7).

We observe truncating variants in TSGs and variants in oncogenes associated with lower and higher expression percentiles, respectively (Figures 4B and S5). Notably, truncations in *KDM1A* and *USP45*, which showed significant LOH, were associated with the bottom 25th expression percentile. These results are consistent with the two-hit hypothesis and previous reports of *KDM1A* and *USP45* having a TSG role in MM.[46,47]

We see 2 P frameshift variants in *BRCA2* associated with relatively lower expression values (<50%), one of which is undergoing LOH in the tumor (p.V220fs). *CHEK2* and *ATR* variant carriers showed expression values in the bottom 25th percentile (~13% and 24%, respectively). Both variants affect females with a family history of BRCA. Carriers of variants in other known predisposition TSGs were also associated with the bottom 25th expression percentile, such as *DDB2* (8.6%), *ERCC2* (1.8%), *FANCI* (2.4%), *MUTYH* (2.2%), *NBN* (13.5%), and *XPC* (5.8%).

Carriers of variants in the known predisposition oncogenes *MPL* (splice variant c.79 + 2T>A) and *TSHR* (missense variant p.C41S) were associated with the top 25th expression percentile

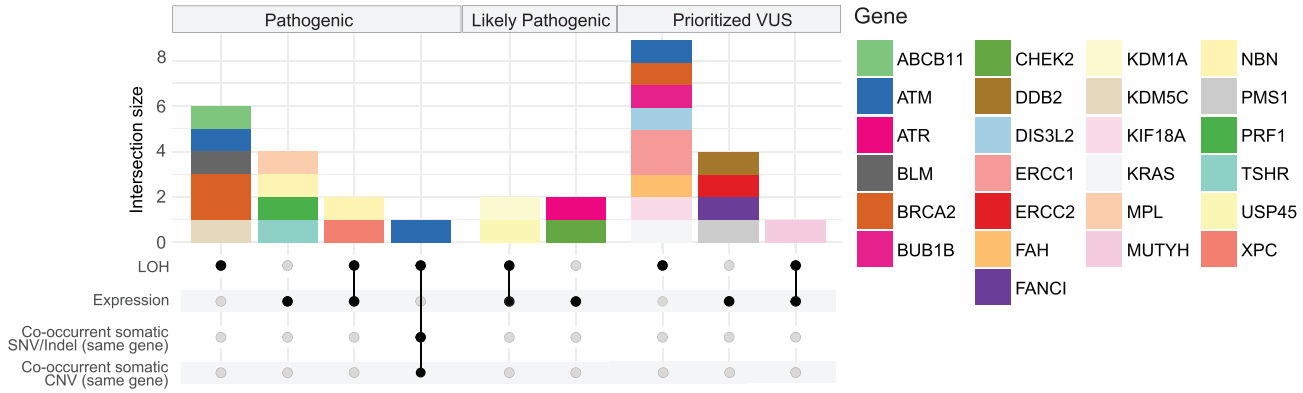


Figure 5. Germline predisposition variants supported by multiple lines of evidence in the MMRF cohort

UpSet plot showing variants prioritized by CharGer that present additional evidence of pathogenicity through analyses of LOH, expression association, and co-occurrence with somatic mutations and copy-number variants.

(92% and 82%, respectively). The MPL thrombopoietin receptor activates JAK-STAT signaling to drive MM and other hematological malignancies.[79,115] Deleterious *MPL* variants act as autosomal recessive variants, conferring risk for congenital amegakaryocytic thrombocytopenia.[116] However, some *MPL* variants, including the Ashkenazi Jewish founder splice donor variant (c.79 + 2T>A)[117] seen here linked to MM, have also been detected as heterozygous alleles associated with hereditary thrombocythemia[118] and predisposition to BRCA, uterine corpus endometrial carcinoma, sarcoma, and pheochromocytoma and paraganglioma in TCGA.[51] *TSHR* is part of the cAMP signaling pathway, which is commonly activated in cancer. Although variants in this gene have not been associated with MM, high expression of *TSHR* is consistent with reports from patients with MM under treatment with immunomodulatory drugs (IMiDs), such as thalidomide, which can result in thyroid disorders, including hypothyroidism, characterized by high levels of thyroid-stimulating hormone and *TSHR*.[119,120]

Multiple lines of evidence confirm the pathogenicity of putative predisposition single nucleotide variants and indels in the Multiple Myeloma Research Foundation cohort

We sought multiple lines of evidence to corroborate the pathogenicity of candidate GPVs in the MMRF, for which access to matched tumor-normal data allowed for the analyses of LOH, expression effect, and co-occurrence with somatic SNVs, indels, or copy-number variants within the same gene (STAR Methods). Considering variants with >1 additional piece of evidence of pathogenicity, we observed 13/49 high confidence P variants and 4/14 high confidence LP variants from the original 107 P/LP/PVUS events in the 158 candidate CPGs and other MM-related genes (Figure 5; Table S2 – tab ST2A; Table S8). We also nominated 14 GPVs from the initial list of 44 PVUS. Together, these 31 nominated P/LP/PVUS variants affect 3.25% of the MMRF cohort.

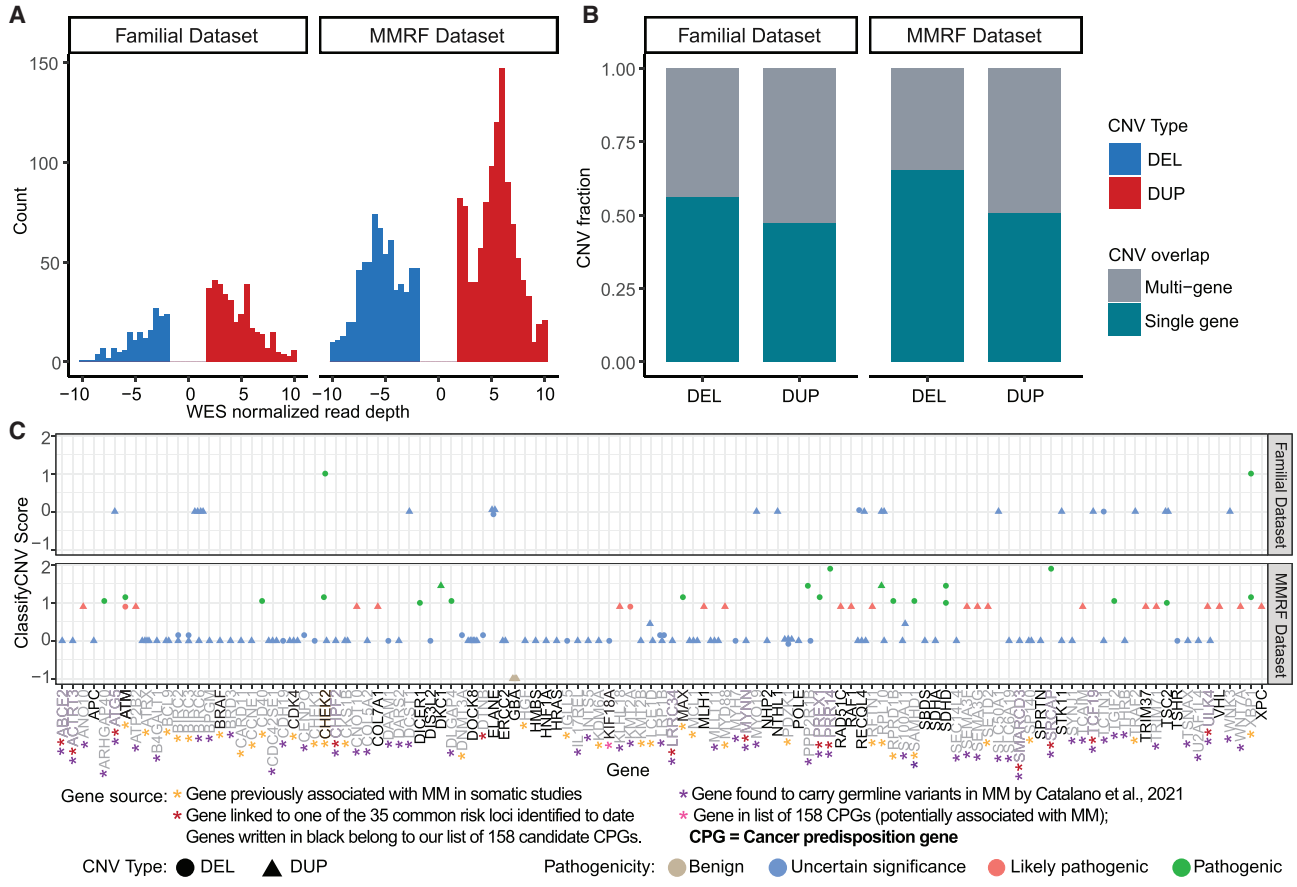
Among our high confidence P events, we see the *BRCA2* variants p.W993* and p.V220fs, which undergo significant LOH. We also identified the missense p.T544I in *BRCA2* amongst our nominated PVUSs, providing further support for this gene in MM risk. Our results also include *NBN*, *KDM1A, CHEK2, USP45,* and *ATR* truncations whose carriers all presented the bottom 25% expression compared to other cases, and a P *TSHR* missense variant associated with the top 25% expression.

Analyses of rare germline copy number variants identify pathogenic and likely pathogenic events in multiple myeloma

We searched for rare germline copy number variants (gCNVs) in WES data from both MMRF and familial cohorts using the exome Hidden Markov Model (XHMM).[121,122] Variants were assessed for pathogenicity according to the 2019 ACMG guidelines[123] using ClassifyCNV[124] (STAR Methods).

We observed a total of 1,727 rare gCNVs (AF <0.6% considering 50% overlaps) in the MMRF (Figure 6A; Table S9), with an average of 0.7 overlapping deletions and 1.1 overlapping duplications per case. Of these, 57% affect single genes, while 43% overlap multiple genes (Figure 6B). We observed 109 gCNVs affecting genes in our list of 158 candidate CPGs and other MM related genes (Figure 6C), from which 12 were classified as P and 7 as LP. We detected a P deletion of *PKD1* involving a partial deletion of *TSC2* in a 67 year/o male of EUR ancestry. This case also shows the bottom quantile expression of *TSC2* (22.9%; Figures 6D and 6E). Additionally, we observed a P deletion of *SDHD* in a 75 year/o male of AFR ancestry that shows a lower expression of this gene (33.6%). *SDHD* is a TSG involved in predisposition to paragangliomas and adrenal/extra-adrenal pheochromocytomas.[51,125–129] It has also been proposed that the succinate dehydrogenase (SDH) complex plays a role in the development of hematological malignancies,[130] while *SDHD* deletions are associated with mitochondrial dysfunction, affecting survival and maintenance of hematopoietic stem cells, and myeloid and B-lymphoid progenitors.[131]

Among gCNVs of uncertain significance in the MMRF, we detected a few affecting known CPGs and suggested MM predisposition genes, such as a partial deletion of *KIF18A* (exons 1–6) in a 68 year/o female of EUR ancestry. This case also shows the bottom expression of *KIF18A* (26.6%). We also pinpoint a



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Dataset	Affected genes	CNV Type	CNV value	Expression quantile	Pathogenicity
	CHEK2				
Familial	XBP1	DEL	-4.17	Validated via qPCR	P
Familial	ELANE	DUP	3.31	Validated via qPCR	VUS
Familial	ELANE	DEL	-2.16	Validated via qPCR	VUS
Familial	ELANE	DUP	2.53	Validated via qPCR	VUS
MMRF	DIS3L2	DEL	-5.06	0.107	VUS
MMRF	TSC2	DEL	-1.9	0.229	P
MMRF	KIF18A	DEL	-6.95	0.266	VUS
MMRF	CHEK1	DEL	-1.81	0.306	VUS
MMRF	SDHD	DEL	-2.41	0.336	P
MMRF	DKC1	DUP	3.05	0.758	P
MMRF	ERCC2	DUP	4.53	0.799	VUS
MMRF	CHEK2	DUP	5.74	0.813	VUS
MMRF	SDHA	DUP	5.06	0.838	VUS
MMRF	DOCK8	DUP	5.03	0.842	VUS
MMRF	RECQL4	DUP	2.12	0.853	VUS
MMRF	DOCK8	DUP	6.32	0.945	VUS
MMRF	MLH1	DUP	2.4	0.954	LP
MMRF	ERCC2	DUP	6.08	0.957	VUS
MMRF	TRIM37	DUP	1.98	0.957	LP
MMRF	RAD51C	DUP	1.98	0.970	LP
MMRF	SPRTN	DUP	2.19	0.976	VUS
MMRF	HRAS	DUP	5.06	0.981	VUS

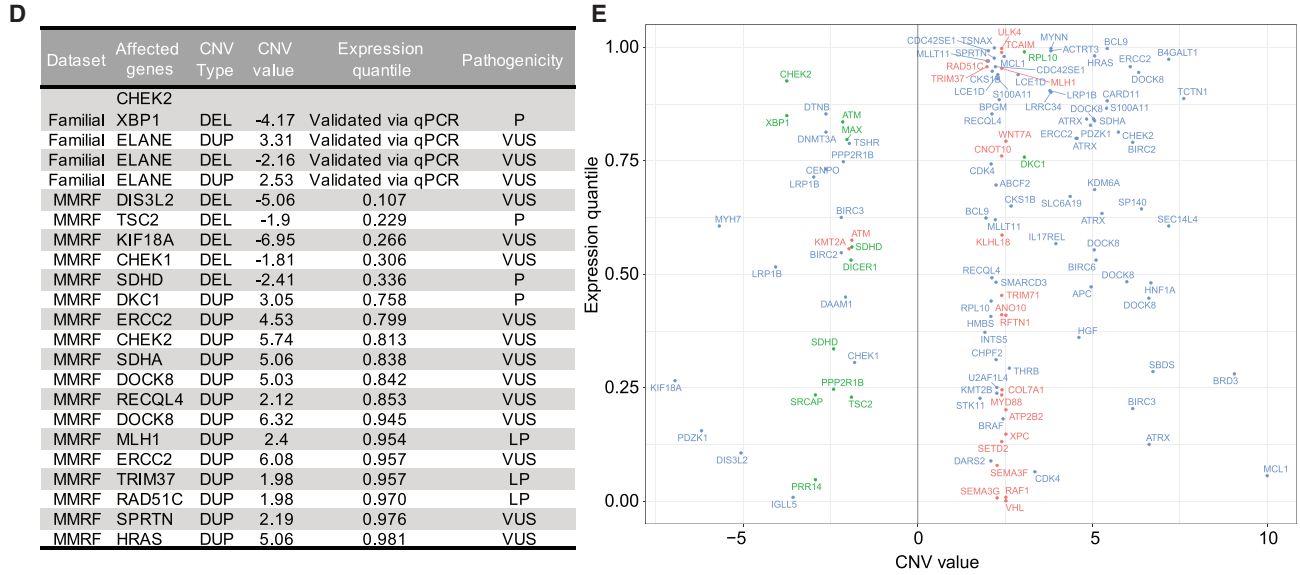


Figure 6. Rare germline copy number variants (gCNVs) in MM

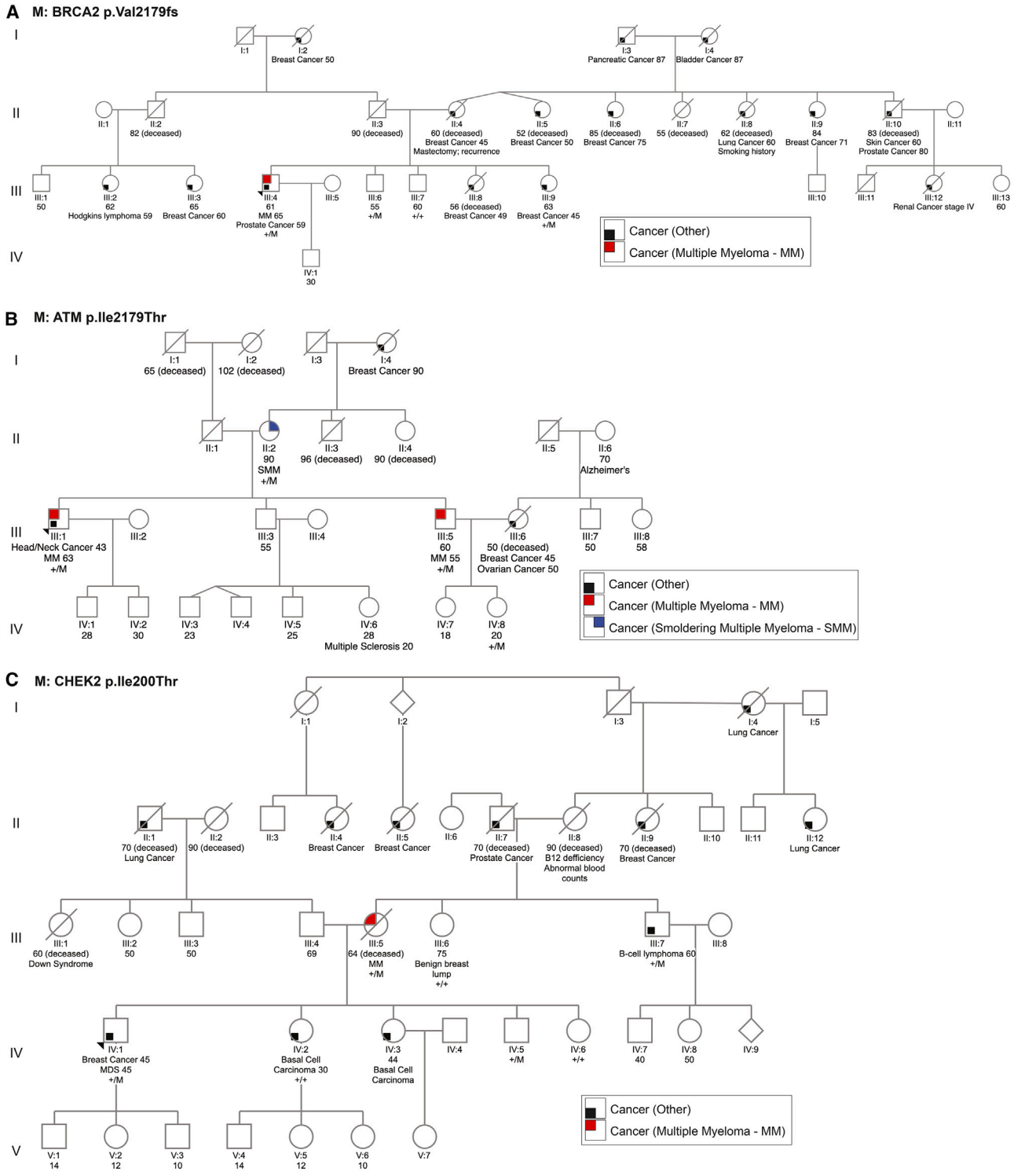
(A) Rare gCNVs (AF <0.6% considering 50% overlaps) detected from WES using XHMM. CNV value is represented by the normalized read depth of the genomic region (x axis).

(B) Percentage of gCNVs affecting single vs. multiple genes.

(C) Distribution and pathogenicity of rare gCNVs across 158 CPGs and other MM-related genes.

(D) gCNVs along with its CNV value and expression percentile. Only gCNVs validated by qPCR in the families are shown. For the MMRF, only gCNVs for which the expected transcriptional effect was observed are shown.

(E) Expression quantile associated with each gCNV in MMRF. Dots represent gCNVs, colored by pathogenicity. See Table S9.



M = mutated allele;
+ = reference allele;
MDS = Myelodysplastic syndrome.

(legend on next page)

deletion encompassing the entirety of *CHEK1* in a 75 year/o male of AFR ancestry, which exhibits lower expression of this gene (30.6%). Although we were able to validate the transcriptional effect of some gCNVs in the MMRF, not all events corresponded to the expected gene expression changes requiring further investigation, including P *CHEK2* and *ATM* deletions not associated with bottom quantile expression.

In the families, we identified 500 rare gCNVs, from which 22 affected the 158 candidate CPGs and other MM related genes (Figure 6; Table S9). We detected 3 P and 3 LP gCNVs, including a P deletion of the entire *CHEK2* gene in a 70 year/o male of EUR ancestry whose maternal uncle and cousin were also diagnosed with MM. This individual was not shown to carry other P/LP variants in any genes of interest, suggesting a causal role for the *CHEK2* deletion in this patient's disease. This gCNV was validated via qPCR. We also observed a partial deletion of *CHEK2* (exons 3–4) in a 66 year/o female of EUR ancestry with a family history of leukemia (son) and BRCA (sister). Although this gCNV was not identified by XHMM due to its limited ability to detect single exon/short CNVs, it was detected through qPCR during clinical testing. This case also carries an LP splice acceptor variant in *KDM1A* (c.1623-1G>A), which may also be involved in this patient's MM susceptibility. Therefore, further investigation is needed for a causality role for either variant. Finally, we detect two duplications and one deletion of uncertain significance involving the *ELANE* gene in patients with no other P/LP variants detected. Although validated via qPCR, their role in these patients is unknown, and further investigation is needed. *ELANE* is known to promote angiogenesis, tumor development, and metastasis.[132] Autosomal dominant mutations in this gene are associated with hematological disorders, such as severe congenital neutropenia (CN),[133–138] and patients with *ELANE* related CN have been shown to have a greater risk of developing MDS-acute leukemia.[139–142] However, its role in MM is unclear.

Segregation analyses reveal segregating *BRCA2, ATM,* and *CHEK2* variants in patients with familial multiple myeloma

We searched for variants segregating with MM in families for which we had sequencing information for >1 family member (Figure 7; Table S10). We detected a heterozygous P frameshift in *BRCA2* (p.V2179fs) in the proband, a male diagnosed with prostate cancer at age 59 and MM at 65 (Figure 7A). The variant is also present in the proband's brother, a healthy 55 years/o male, and in the sister diagnosed with BRCA at 45. It is unknown whether this event is in other family members across different generations, as those were not tested. This variant has been associated with hereditary cancer syndromes, breast and colorectal cancer.[143–145] Although we see several cancer types in different pedigree members, we do not observe breast or colorectal cancer in the proband, suggesting a role for this variant in this individual's disease. Further testing of additional family members would be necessary to validate these results.

Interestingly, we see a heterozygous rare missense variant (p.I2179T, gnomAD MAF = 4.061E-06) in *ATM* across three generations in an MM family (Figure 7B). This variant did not reach a high enough CharGer score to be classified as P or LP, and has multiple VUS reports in ClinVar. However, it is detected in the proband, a male diagnosed with head and neck cancer at age 43 and MM at 63; in the proband's mother, diagnosed with MM at age 90; and in the brother, diagnosed with MM at 55. It is also in the proband's niece, a healthy 20 years/o at the time of testing. The presence of this variant across three generations and the absence of other cancer types in almost all tested family members, except for the proband, indicates a possible causal role for this variant in this family's MM and offers further support for *ATM* as an MM predisposition gene.

Within MM families, we identified variants seen more commonly in populations that were detected by both WES and clinical testing in this study. One was a heterozygous *CHEK2* missense (p.I200T, gnomAD MAF = 0.004256) (Figure 7C) detected in the proband, a female diagnosed with myelodysplastic syndrome at age 44 and BRCA at 45. This event is also in the proband's mother, diagnosed with MM at age 64, in the uncle, diagnosed with B-cell lymphoma, and in the brother, a healthy individual at the time of testing. This variant impairs the activation of CHEK2 by DNA damage and its ability to bind and phosphorylate its downstream targets, leading to uncontrolled cell growth and proliferation.[146] It is associated with various cancers,[147–153] including lymphoid and myeloid malignancies,[154–161] and is a risk factor for clonal hematopoiesis.[162] Here, we observe several cancers in different members of this pedigree. Although further analysis involving additional relatives would be needed to validate these findings, this *CHEK2* variant is present in all family members with hematological malignancies, suggesting a potential role in MM risk.

Clinical association analyses suggest that putative predisposing variants influence multiple myeloma risk across age groups

We did not find any associations between age at onset and predisposition genes, which may reflect insufficient statistical power. Additionally, we do not observe any overall age differences between carriers and non-carriers of P/LP/PVUS events for either cohort (MMRF Wilcoxon p-value = 0.474; families Wilcoxon p-value = 0.986; Figures S6A–S6C). The median age of diagnosis for carriers and non-carriers of rare germline variants in predisposition genes for the MMRF was 64 and 63, respectively, and 63 and 61 for the familial cohort.

A similar power limitation applies to the identification of variants or genes associated with ancestry. However, given the diverse nature of our cohort, we still explored the distribution of variants across different groups (Figure S6D). We observe a missense PVUS (p.N1126S) in *EP300* in a 62 year/o male of AFR ancestry within MMRF with no family history of cancer. *EP300* is involved in cellular proliferation, cell cycle regulation,

Figure 7. Segregating variants in patients with familial MM

(A) Pedigree of familial MM kindred carrying *BRCA2* p.Val2179fs mutation.

(B) Pedigree of MM kindred carrying *ATM* p.Ile2179Thr mutation across 3 generations.

(C) Pedigree of familial MM kindred carrying *CHEK2* p.Ile200Thr mutation across 2 generations. See Table S10.

DNA damage repair, apoptosis, is known to carry rare MM-associated GPVs,[48] and is somatically mutated in MM.[60] Variants in this gene were not observed in other ancestry groups. Other genes exclusively mutated in the AFR group include *BRIP1, FANCM,* and *KIF18A.* Although underpowered to make any conclusions about ancestry-specific genes or variants, these results may point to candidates for the higher risk of MM in individuals of AFR ancestry.

We also investigated variants in the MMRF affecting individuals with a known family history of cancer (Figure S6E). We observe family history of breast and gynecological cancer for carriers of *BRCA2* variants, as expected. However, we also observe the history of MM/MGUS (maternal grandmother) in a carrier of *a BRCA2* variant (p.T544I) supporting the involvement of this gene in MM. We also see a family history of BRCA and leukemia in carriers of *CHEK2* variants, and BRCA in a carrier of an *ATM* variant, supporting the idea of shared predisposition factors across cancer types.

DISCUSSION

We present a large study of rare GPVs in MM, analyzing 954 unrelated individuals from the MMRF and 82 families (Figure 1). We identified 93 rare P/LP/PVUS variants in 9.1% of the MMRF cohort, with P/LP variants affecting 5.1% of cases, and 115 rare P/LP/PVUS variants in 18% of the familial cohort, with P/LP variants affecting 13.1% of cases. We also detected 31 P/LP/PVUS variants for which pathogenicity was supported by multiple lines of evidence (LOH, expression effect, co-occurrence with a somatic event) in 3.25% of the MMRF cohort (Figure 5; Table S2 – tab ST2A; Table S8).

This study provides additional evidence for previously suggested gene-MM associations, such as *DIS3*,[43] *EP300*,[48] *KDM1A*,[46] and *USP45*,[47] and genes linked to the 35 common, low-risk loci associated with this disease, such as *POT1* and *DNAH11*[20,25] (Figure 2). We also found putative GPVs in genes reported in somatic MM studies (*FGFR3, KDM5C, SAMDH1, TGDS,* and *TRAF3IP1*),[60,65–67] and in a germline study by Catalano et al.[68] (*AMPD3, ANO10, DARS2, DSP, FLNC, MYH7, PROM1, SLC6A19*, and *WFS1*). Interestingly, we identified genes known to be involved in predisposition to other cancers (*ATM, BRCA1*, *BRCA2*, *CHEK2, PMS2, POT1, PRF1,* and *TP53*) with *ATM, PMS2,* and *PRF1* being potential novel gene-MM associations. *CHEK2* was also reported to carry copy number deletions in the familial cohort, supporting its role in MM risk (Figure 6). Furthermore, we detected suggestive specific associations of *TSC2* and *XPC* with MM when comparing the MMRF dataset to TCGA,[51] and a potential pleiotropic effect of variants and genes among different cancer types (Figure S2).

The detection of putative P/LP/PVUS germline variants in genes such as *ATM, BLM, BRCA1, BRCA2, CHEK2,* and *RAD51D,* among others, suggests that the disruption of DNA damage repair pathways, including HR, MMR, NHEJ, NER, and BER, may play a role in MM risk. This aligns with previous findings that DNA damage repair pathways can influence genomic changes in MM, and disruption of these mechanisms - such as through deleterious mutations in key genes - may offer an explanation for the genomic instability seen in MM.[84–95,163–166] Given that germline variants are present in every cell of the human body, pathogenic germline variants in key DNA damage repair genes may indicate a global disruption of such repair mechanisms. In particular, the presence of deleterious germline variants in *ATM, BRCA1/2,* although well known in predisposition to solid tumors, such as breast and ovarian cancer, has also been associated with the risk of developing myeloid neoplasms hematopoietic malignancies in patients who have been exposed to DNA-damaging agents, such as chemotherapy or radiotherapy, *i.e.,* therapy-related myeloid neoplasms (t-MN).[70–74] Recently, deleterious germline variants in *BRCA1/2* have been shown in patients with hematopoietic malignancies without a prior diagnosis of other cancers or exposure to chemotherapy or radiotherapy, with a high prevalence of MM in *BRCA2* variant carriers.[76] Inactivation of *ATM* has also been shown to contribute to genomic instability in MM and other hematological malignancies, as this gene promotes DNA repair and activates checkpoints to suppress abnormal Ig and T cell receptor (TCR) rearrangements.[91,166–168] Therefore, further studies are needed to investigate how germline variants in key DNA repair genes interact with known genomic driver events in the pathogenesis of MM.

This study has provided the basis for a number of interesting findings related to MM susceptibility. Overall, our results suggest that rare GPVs influence MM risk at all age groups, challenging the common belief that GPVs always result in malignancies diagnosed at earlier ages than typically seen.[169] Our results also may suggest the involvement of genes *EP300*, *BRIP1, and FANCM* in MM susceptibility in individuals of AFR ancestry. We also observe a family history of breast, gynecological, and lung cancer, and MM/MGUS in a *BRCA2* variant carrier in the MMRF, further supporting the role of this gene in MM risk. Nonetheless, improved collection and curation of clinical and family history data would be pivotal for further investigation of these predisposition variants.

Regarding our familial dataset, we also acknowledge the limitations imposed by the lack of sequencing data from relatives of all 82 probands. However, despite these constraints, segregation analyses revealed three pedigrees showing the respective segregation of variants in *BRCA1, ATM,* and *CHEK2* with MM and other tumor formation (Figure 7), further underscoring the role of these genes in MM susceptibility.

In conclusion, this study allowed for the discovery of putative germline variants and genes underlying MM risk, which we hope will inform the development of better prevention and early detection strategies for this disease, especially in high-risk groups. Careful attention to the collection of personal and family history of malignancy in individuals with MM will be key in elucidating the role of GPVs in MM. Further validation of the functional impact of the genetic changes presented here is needed to better understand their prognostic and therapeutic implications in MM and to provide a higher level of precision in the personalized treatment of this disease.

Limitations of the study

This article should be interpreted considering its limitations. First, we recognize that our ability to detect variants and genes associated with specific clinical features, such as age and ancestry,

may be limited. While our dataset, which includes both the MMRF CoMMpass Study cohort and a familial cohort, is among the largest available for multiple myeloma (MM), we remain underpowered to detect these associations due to sample size. Additionally, the absence of sequencing data for relatives of all 82 probands in the familial cohort further limits our analyses, especially our ability to perform further segregation analyses of candidate predisposition variants detected in those probands. Consequently, future studies with larger, more statistically powered cohorts are needed. In addition, a more detailed and systematic collection of clinical and family history information will be essential for deeper investigation of predisposition variants proposed in this study.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by Dr. Li Ding (lding@wustl.edu).

Materials availability

This study did not generate new unique reagents.

Data and code availability

- Data was provided by The Multiple Myeloma Research Foundation (MMRF) CoMMpass (Relating Clinical Outcomes in MM to Personal Assessment of Genetic Profile) Study (NCT01454297). dbGaP Study Accession: phs000748. Data types analyzed in this study were RNA-seq, whole exome sequencing, and clinical information. The MMRF CoMMpass study can be accessed at https://www.ncbi.nlm.nih.gov/projects/gap/cgi-bin/study.cgi?study_id=phs000748.v7.p4. De-identified raw whole-exome sequencing data and germline variant calls for the familial dataset that support the findings of this study, as well as original MMRF case identifiers linked to study IDs used in this study are available on Synapse (Synapse ID: syn26844264; https://www.synapse.org/#!Synapse:syn26844264) to approved researchers upon reasonable request as of date of publication. Access can be requested by contacting Dr. Godley (L.A.G). These accession numbers are also listed in the key resources table.
- Code for tools and pipelines developed by our group used in this study, including CharGer, GermlineWrapper, and TinDaisy CWL, are publicly available in our group's GitHub repository: https://github.com/ding-lab/. Their specific URL's and DOIs are available in the key resources table as of the date of publication.
- Any additional information and codes required to reanalyze the data reported in this article are available from the lead contact upon reasonable request.

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AUTHOR CONTRIBUTIONS

L.D. and L.A.G. led the project design. J.J., M.P., A.K., L.M., A.S., B.D., and A.J.J. provided family-based data. F.M.R. generated data, led data analysis, wrote the article, and generated the figures and tables. E.S. performed ancestry predictions. F.M.R., B.D., A.J.J., M.C.W., M.F., J.F.D., R.V., L.A.G., and L.D. edited the article.

DECLARATION OF INTERESTS

The authors declare no competing interests.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Critical commercial assays		
KAPA Hyper Prep Kit	KAPA Biosystems	Cat # 7962363001
xGen Exome Research Panel v1.0	IDT Technologies	https://www.idtdna.com
Deposited data		
Whole Exome Sequencing, RNA Sequencing, and clinical data for individuals from the MMRF	The Multiple Myeloma Research Foundation (MMRF) CoMMpass (Relating Clinical Outcomes in MM to Personal Assessment of Genetic Profile) Study (NCT01454297)	dbGaP Study Accession: phs000748; https://www.ncbi.nlm.nih.gov/projects/gap/cgi-bin/study.cgi?study_id=phs000748.v7.p4
Whole Exome Sequencing data for 82 families from the University of Chicago	This study	Protected data available to approved researchers; Synapse ID: syn26844264; https://www.synapse.org/#!Synapse:syn26844264 Please contact L.A.G. or F.M.R. to request access.
Germline variant callset for the Familial cohort	This study	Protected data available to approved researchers; Synapse ID: syn26844264; https://www.synapse.org/#!Synapse:syn26844264
Oligonucleotides		
Primer: *ELANE* gene exon 4 - qPCR primer Forward: CCTGGGAGCCCATAACCTCT Reverse: AAGTTTACGGGGTCGTAGCC Product size: 93 bps Covered region (GRCh38): chr19:853,286–853,378 (exon 4)	This study	N/A
Primer: *RECQL4* gene intron 9–10 - qPCR primer Forward: ACTGCTGCTTGTCCCCTAAC Reverse: TTTGACCTGCTGCCAAGACT Product size: 85 bps Covered region (GRCh38): chr8:144,515,001–144,515,085 (intron 9–10)	This study	N/A
* Validation of *CHEK2* gCNVs were performed by Ambry Genetics; primer sequences are not provided	Ambry Genetics	N/A
Software and algorithms		
Ancestry prediction	Li Ding Lab	https://github.com/ding-lab/ancestry
bam-readcount v0.8	McDonnell Genome Institute	https://github.com/genome/bam-readcount
BWA v0.7.17-r1188	Li, 2013[170]	http://bio-bwa.sourceforge.net/
CharGer v0.5.4	Scott et al., 2019[56]	https://github.com/ding-lab/CharGer/tree/v0.5.4
ClassifyCNV v1.1.1	Gurbich and Ilinsky, 2020[124]	https://github.com/Genotek/ClassifyCNV
clusterProfiler v3.18.1	Yu et al., 2012[171]	https://bioconductor.org/packages/release/bioc/html/clusterProfiler.html
CNVkit v0.9.6	Talevich et al., 2016[172]	https://github.com/etal/cnvkit
Ensembl Variant Effect Predictor (VEP) v95	McLaren et al., 2016[173]	https://github.com/Ensembl/ensembl-vep
FastQC v0.11.8	Andrews, 2010[174]	https://www.bioinformatics.babraham.ac.uk/projects/fastqc/
featureCounts Rsubread v1.6.4	Liao et al., 2014b[175]	http://subread.sourceforge.net/
GATK DepthOfCoverage v3.8-0	McKenna et al., 2010[54]	https://github.com/broadinstitute/gatk
GATK HaplotypeCaller v4.0.0.0	McKenna et al., 2010[54]	https://github.com/broadinstitute/gatk
GATK VariantEval v3.8-0	McKenna et al., 2010[54]	https://github.com/broadinstitute/gatk

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
GermlineWrapper pipeline v1.1	Li Ding Lab	https://github.com/ding-lab/germlinewrapper
GenePattern GISTIC 2.0.22 module	Reich et al., 2016; Mermel et al., 2011[176,177]	https://www.genepattern.org/modules/docs/GISTIC_2.0
Integrative Genomics Viewer (IGV) v2.8.2	Robinson et al., 2011[178]	https://software.broadinstitute.org/software/igv/
Mosdepth v0.2.4	Pedersen and Quinlan, 2018[179]	https://github.com/brentp/mosdepth
Mutect v1.7.7	Cibulskis et al., 2013[180]	https://github.com/broadinstitute/mutect
Picard Toolkit v2.22.4–0	Broad Institute of MIT and Harvard[181]	https://github.com/broadinstitute/picard
Pindel v0.2.5	Ye et al., 2009	https://github.com/genome/pindel
Python v2.7 and v3.7	Python Software Foundation	https://www.python.org/
R v4.0.3	R Development Core Team	https://www.R-project.org
ReactomePA v1.34.0	Yu and He, 2016[182]	https://bioconductor.org/packages/release/bioc/html/ReactomePA.html
SNPRelate v1.24.0	Zheng et al., 2012[183]	https://www.bioconductor.org/packages/release/bioc/html/SNPRelate.html
STAR v2.5.0a	Dobin et al., 2013[194]	https://github.com/alexdobin/STAR
Strelka v2.9.2	Kim et al., 2018[184]	https://github.com/Illumina/strelka
TinDaisy CWL pipeline	Li Ding Lab	https://github.com/ding-lab/TinDaisy
Trimmomatic v0.38-1	Bolger et al., 2014[185]	https://github.com/usadellab/Trimmomatic/tree/V0.38
VarScan v2.3.8	Koboldt et al., 2012[53]	https://dkoboldt.github.io/varscan/
XHMM	Fromer and Purcell, 2014[121]	https://zzz.bwh.harvard.edu/xhmm/

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Multiple Myeloma Research Foundation (MMRF) dataset

We obtained Whole-Exome Sequencing (WES) data from 965 tumor-normal sample pairs for individual cases (n = 965; 479 males, 482 females, 4 with gender not reported; mean age 62.66 ± 10.71) generated by The Multiple Myeloma Research Foundation (MMRF) CoMMpass (Relating Clinical Outcomes in MM to Personal Assessment of Genetic Profile) Study (NCT01454297). Of 965 cases, 605 were self-reported Caucasian individuals. Other demographic information, such as ethnicity, was not available. RNA-Sequencing data for 797 of those tumors corresponding to individual patients were also obtained from MMRF, as well as clinical information. dbGaP Study Accession is phs000748. After quality control measures, 954 tumor-normal sample pairs were used in this study (n = 954; 475 males, 475 females, 4 with gender not reported; mean age 62.74 ± 10.67; 599 self-reported Caucasian individuals). See "germline variant calling and filtering" methods section for more details on sample quality control strategies. Ancestry was estimated based on whole-exome sequencing data for more accuracy (as detailed in the "ancestry prediction" methods section). Final ancestry predictions are provided in Table S1 – tab ST1E and Figure 1B.

Recruitment of research subjects for the familial dataset

All patients diagnosed with monoclonal gammopathy of undetermined significance (MGUS), multiple myeloma (MM), smoldering myeloma (SMM), plasma cell leukemia, plasmacytoma, and amyloidosis, who underwent bone marrow biopsy at The University of Chicago were queried for their personal and family history of cancer. Clinical germline testing was offered if: (i) the individual (also called proband) had been diagnosed with a second cancer, excluding non-melanoma skin cancer; or (ii) the individual was diagnosed with MM < 50 years old[46]; or (iii) the family history included a solid tumor diagnosed in an individual <50 years old within two generations of the proband. All of the research subjects included in this study chose to undergo clinical germline testing by the Genetic Services Laboratory at The University of Chicago as described[186] and consented to research participation under an IRB-approved protocol, 11–0014, for which Dr. Godley (L.A.G) served as Principal Investigator. Clinical testing was performed on DNA derived from cultured skin fibroblasts as previously described.[186] After anonymization, samples were shared with Washington University in Saint Louis for whole-exome sequencing. All of the variants identified in clinical testing were identified by the analysis at Washington University in Saint Louis. Samples overlapping the MMRF dataset, identified via identity-by-descent (IBD) analyses, were excluded from this dataset (n = 5 probands), leaving 82 probands and 17 relatives (n = 99; mean age 59.45 ± 12.12). See "identity-by-descent (IBD) analyses" methods section for more details. Of the 99 cases, 43 were males, 55 females, and 1 did not have gender information reported. Also, 65 were self-reported Caucasians, 16 African-Americans, 2 Hispanic/Latinos, and 16 did not self-report race information. Ethnicity information was not available. Ancestry was estimated based on whole-exome sequencing data for

more accuracy (as detailed in the "ancestry prediction" methods section). Final ancestry predictions are provided in Table S1 – tab ST1F and Figure 1B.

Skin fibroblast culture

Skin fibroblast cultures for samples from the familial dataset were generated as previously described.[186] Fibroblast cultures were initiated from 3mm skin punches. Using a sterile technique, subcutaneous fat was removed, and the remaining dermal and epidermal layers were manually minced with a scalpel in type I collagenase/α-MEM. The specimen was incubated overnight in collagenase at 37°C with 5% CO_2. To further dissociate the cells, the tissue was aspirated through a 20-gauge needle and cultured in AmnioMax medium (Thermo Fisher Scientific, Waltham, MA) in a T25 vented flask. DNA extraction was performed using Qiagen isolation. All of the cells used in this study were periodically tested free of mycoplasma contamination by MycoFluor Mycoplasma Detection Kit (Thermo Fisher).

METHOD DETAILS

DNA isolation, library preparation and whole-exome sequencing (WES)

WES data for the MMRF dataset was provided by the MMRF itself, as described. Familial samples from skin fibroblast cultures were sequenced at the McDonnell Genome Institute (MGI) at Washington University in Saint Louis. Genomic DNA (150-250ng) was fragmented on the Covaris LE220 instrument targeting 250bp inserts. Automated dual indexed libraries were constructed with the KAPA Hyper Prep Kit (KAPA Biosystems, Cat # 7962363001) on the SciClone NGS instrument platform (PerkinElmer). Samples were amplified post ligation (9 cycles) and then size selected with AMPure XP beads (1x) to tighten the final distribution size of each sample. Libraries were then pooled (1 pool of 8, 1 pool of 9) at an equimolar ratio yielding ~4-4.5μg per library pool prior to the hybrid capture. Library pools were hybridized with the xGen Exome Research Panel v1.0 reagent (IDT Technologies) that spans a 39 Mb target region (19,396 genes) of the human genome. The concentration of the library capture pools were accurately determined through qPCR utilizing the KAPA library Quantification Kit according to the manufacturer's protocol (KAPA Biosystems/Roche) to produce cluster counts appropriate for the Illumina NovaSeq6000 instrument. Library pools were run over 0.068 of a NovaSeq6000 S4 flow cell using the XP workflow and a 151 × 10 × 10x151 sequencing recipe in accordance with manufacturer's protocol.

Sequence alignment

Exome sequencing data for the MMRF were obtained as BAM files, already aligned to the hs37d5 human reference genome. Paired-end exome sequencing data from the familial cohort samples were first trimmed for adapter sequences and low quality reads using Trimmomatic[185] (v0.38-1 using its paired-end mode with default parameters, except where -threads 2, -phred33, ILLUMINACLIP:TruSeq3-PE.fa:2:30:10:8:TRUE, LEADING:3, TRAILING:3, SLIDINGWINDOW:4:15, MINLEN:36). After trimming, samples were aligned to the GRCh38 human reference genome using the BWA-MEM algorithm from the Burrows-Wheeler Alignment Tool[170] (BWA, v0.7.17-r1188 with default parameters, except where -t 8, -M). Duplicates were removed using Picard (MarkDuplicates; v2.22.24-0 with default parameters; https://github.com/broadinstitute/picard).

Somatic variant calling for the MMRF dataset

Somatic variants were detected from WES data in our MMRF dataset, for which we had paired tumor-normal samples. We used our in-house TinDaisy CWL pipeline (https://github.com/ding-lab/TinDaisy), which implements four standard tools: Strelka2,[184] Mutect[180] (v1.7.7), VarScan[53] (v2.3.8), and Pindel[55] (v0.2.5). The hs37d5 reference genome was used, as implemented for BAM generation by the MMRF CoMMpass Study.

We retained exonic SNVs called by at least two callers among Strelka, VarScan, and Mutect, and indels called by at least two callers among Strelka, VarScan, and Pindel. To generate high confidence variant calls, we implemented cutoffs of at least 14 total reads in the tumor and at least 8 in the normal and filtered variants by a minimal variant allele frequency (VAF) of 0.05 in tumors and a maximal VAF of 0.02 in normal samples. We further excluded indels longer than 100bp and required all variants to have an allele frequency of at least 0.005 based on the highest allele frequency observed between 1000 Genomes,[187] NHLBI-ESP,[188] or gnomAD,[189] as extracted from Ensembl Variant Effect Predictor (VEP)[173] (v95) annotation (MAX_AF field). For the remaining candidate exonic somatic variants, we also filtered low quality variants by bam-readcount (default parameters except where -q 10 -b 20) (https://github.com/genome/bam-readcount) and excluded calls present in dbSnP (v151) but not in COSMIC (v88). Finally, sequential SNVs on the same haplotype were combined into multi-nucleotide variants (MNVs) within TinDaisy by using our in-house tool DNPFilter (https://github.com/ding-lab/dnp_filter). Finally, somatic variants detected for the MMRF were lifted over to GRCh38 coordinates using Picard's LiftoverVcf tool (v2.22.4-0; https://github.com/broadinstitute/picard).

Germline variant calling and filtering

WES data from normal samples for both datasets were first assessed for quality using FastQC (v0.11.8 with default parameters).[174] Coverage within target regions was calculated using Mosdepth[179] (v0.2.4 with default parameters, except where -Q 20). Coverage ranged from 19.8X to 283X for the MMRF dataset and 45X to 196X for the familial dataset (Figure S1B). In total, 954 and 99 samples from MMRF and familial datasets, respectively, passed quality control criteria and had >20X average coverage (mapping quality ≥20) across target regions.

Germline variants for samples passing quality control criteria were identified using the GermlineWrapper pipeline (https://github.com/ding-lab/germlinewrapper), which integrates multiple tools: germline SNVs were identified using VarScan[53] (v2.3.8 with default parameters, except where –min-var-freq 0.08,–p value 0.10,–min-coverage 3,–strand-filter 1, -min-avg-qual 15, -min-reads2 2, -min-freq-for-hom 0.75) operating on a mpileup stream from SAMtools (v1.2 with default parameters, except where -q 1 -Q 13) and GATK[54] (v4.0.0.0, using its Haplotype Caller in single-sample mode excluding duplicate and unmapped reads and retaining calls with a minimum quality of 10). Germline indels were identified using VarScan[53] (version and parameters as above) and GATK[54] (version and parameters as above) in single-sample mode. We also applied Pindel[55] (v0.2.5b9 with default parameters, except where -m 6, -w 1, and excluded centromere regions [genome.ucsc.edu]) for indel prediction. For analyses of MMRF samples, we used the hs37d5 reference genome, as used for BAM generation by the MMRF CoMMpass Study, and specified an insertion size of 500 whenever this information was not provided in the BAM header. For familial samples, the GRCh38 reference genome was used. Single nucleotide variants (SNVs) were based on the union of raw GATK and VarScan calls. We required that indels were called by Pindel or at least two out of the three callers (GATK, VarScan, Pindel). Cutoffs of minimal 10X coverage and 20% VAF were used in the final step to report the high-quality germline variants. For consistency across datasets, germline variants detected for the MMRF were lifted over to GRCh38 coordinates using Picard's LiftoverVcf tool (v2.22.4-0) (https://github.com/broadinstitute/picard).

Variants called by GermlineWrapper were required to have an Allelic Depth (AD) $\geq$ 5 for the alternative allele. Additionally, we filtered out any indels longer than 100bp. A total of 162,560,862 and 10,808,394 variants passed these filters for the MMRF and familial datasets, respectively (Figure 1C). Variants were also filtered based on coding regions of full-length transcripts obtained from Ensembl release 95 plus the additional two base pairs flanking each exon that cover splice donor/acceptor sites, resulting in a total of 22,962,558 and 2,495,449 exonic variants for the MMRF and familial datasets, respectively (Figures S1D and 1C).

Finally, variants passing filters were assessed for quality by calculating concordance with dbSnP (release 151) and average transition-transversion (TiTv) ratio using GATK's[54] VariantEval tool (v3.8-0 with default parameters). For the MMRF dataset, we achieved 98.21% concordance with dbSnP, while for the familial dataset we achieved 99.17% concordance (Figure S1E). For both datasets, our germline exomes displayed high quality, with an average TiTv ratio of 2.84.

Somatic copy number variant (CNV) calling from the MMRF dataset

Somatic copy number variants were called from WES data following procedures as described in the Genomic Data Commons (GDC) copy number variation pipeline documentation (https://docs.gdc.cancer.gov/Data/Bioinformatics_Pipelines/CNV_Pipeline/). We first used CNVkit[172] (v0.9.6) to perform a circular binary segmentation (CBS)[190] analysis on MMRF's WES data. Then, copy number segments obtained from CNVkit were used as inputs for GISTIC2[176] to obtain focal-level CNV values. GISTIC2 was implemented using the GISTIC2 module in GenePattern,[177] with parameters as described by GDC. A 0.3 threshold was used to classify focal CNV values into the following categories: loss (−1), for genes with focal CNV values < −0.3; gain (+1) for genes with focal CNV values >0.3; and neutral (0), for genes with focal CNV values between or equal to −0.3 and 0.3.

Germline copy number variant (gCNV) calling

We used the exome Hidden Markov Model (XHMM) as previously described[122] on our WES normal data from 954 to 99 MMRF and familial samples, respectively, in order to detect rare germline copy number variations (gCNVs). We used GATK's DepthOfCoverage tool (v3.8.0; mapping quality $\geq$20) to estimate base-resolution coverage for Ensembl coding exon intervals obtained from UCSC Table Browser. Those exon targets with GC content >90% or <10%, repeat-marked bases >25%, length <10bp or >10kbp, or mean depth <10 were excluded and the resulting target-by-sample depth matrix was mean centered by target dimension. Following, a principal component analysis (PCA) was performed and the components with variance >70% of the mean variances of all components were removed. The resulting depth matrix was normalized to sample-level *Z* score while removing targets with high variance (standard deviation >50). Finally, the Viterbi Hidden Markov model (HMM) was applied for gCNV discovery using XHMM's default parameters and quality metrics for each CNV were obtained by the forward-backward HMM algorithm, as described.[121] Final calls obtained for the MMRF cohort were lifted over to GRCh38 coordinates using the UCSC's lifOver web application for consistency across the cohorts (https://genome.ucsc.edu/cgi-bin/hgLiftOver).

Germline CNV confirmation for familial samples

Quantitative polymerase chain reaction (qPCR) analysis was performed to detect predicted copy number variants that had not been tested in a clinical laboratory. Patients' genomic DNA samples from skin or saliva were evaluated using the Power SYBR Green PCR Master Mix. Primers were designed to cover the predicted regions of deletion or amplification and tested for optimal annealing temperatures on normal human placental DNA. Primer sequences are available in the key resources table. Twenty ng of each patient sample were run in triplicate with 1 μL of each primer at 10 μM, 10 μL of the SYBR Green Master Mix, and distilled water to a total final volume of 20 μL. Placental gDNA was used as a positive control and a sample lacking DNA was used as a negative control. PMP22 served as the house-keeping gene for diploid reference and DNA samples were merged to make 5 serial dilutions (1:4) to serve as standards for calculating the amplification efficiency for each primer set. PCR amplification was performed for 40 cycles using the Applied Biosystems 7500 Real-Time PCR Software. After PCR amplification, the melt curves of each reaction were checked to ensure specificity of the primers, as indicated by amplification of a single major peak. The cycle threshold values (CT) of the serial dilutions were plotted against the log values of their known concentrations, with the slope of this plot being used to calculate the

amplification efficiency: 2(-1/slope). The mean CT value for each triplicate was used to obtain the ΔCT between the experimental sample with primers covering genes of interest and the housekeeping gene *PMP22*. ΔΔCT was then calculated by subtracting the ΔCT value of the positive control reaction from each experimental ΔCT mean. To determine the relative expression level between the positive control and the experimental samples, the amplification efficiency raised to the power of -ΔΔCT was calculated. The presence of a copy number variant was declared if the relative expression was about 0.5 for deletions and 2 for duplications.

Ancestry prediction

We identified likely ancestry for each individual in our datasets by using a reference panel of genotypes and clustering based on principal components. First, we selected a set of 107,853 coding SNPs with minor allele frequency (MAF) > 0.02 from the 1000 Genomes Project[187] (http://ftp.1000genomes.ebi.ac.uk/vol1/ftp/release/20130502/) and measured their depth and allele counts in each sample using the tool bam-readcount (v0.8 with default parameters, https://github.com/genome/bam-readcount). We then genotyped each sample as follows: 0/0 if reference allele count $\geq$8 and alternative allele count <4; 0/1 if reference allele count $\geq$4 and alternative allele count $\geq$4; 1/1 if reference allele count <4 and alternative allele count $\geq$8; and./. (missing) otherwise. Further, we filtered out markers with missingness >5%, after which >72k and >65K markers remained for analysis for the MMRF (965 samples) and familial (104 samples) datasets, respectively. Note that ancestry predictions were performed before some samples were excluded during QC steps. Therefore, sample sizes depicted here may be larger than the final set. We performed principal component analysis (PCA) for each group of markers on the 1000 Genomes Project data to identify the top 20 principal components and projected our cohorts onto the 20-dimensional space representing the 1000 Genomes data. We then trained a random forest classifier with the 1000 Genomes dataset using the 20 principal components we identified, splitting the 1000 Genomes datasets 80/20 for training and validation, respectively. Our classifier achieved ~99% accuracy on the validation dataset using models trained with the markers for both MMRF and familial datasets (Figure S1C). The fitted classifiers were then used to classify samples into African (AFR), Ad Mixed American (AMR), East Asian (EAS), European (EUR), or South Asian (SAS). Final ancestry predictions are provided in Table S1 – tabs ST1E and ST1F.

Identity-by-descent (IBD) analyses

Analysis of identity-by-descent (IBD) was performed to confirm relationships between individuals in our pedigrees, find potential overlaps between the two cohorts. We used the R/Bioconductor package SNPRelate[183] (v1.24.0) to perform relatedness analysis using identity-by-descent (IBD) measures. A variant call format (VCF) file of the two cohorts combined was converted to GDS file using the SNPRelate. Linkage disequilibrium (LD) pruning using an LD threshold of 0.2, MAF cut-off of 0.05 and allowing a missing rate of 0.3 was performed. IBD estimation was done by implementing the maximum likelihood estimation (MLE) method.[191,192] Samples overlapping between the two cohorts (n = 5 probands) were excluded from the familial cohort.

QUANTIFICATION AND STATISTICAL ANALYSIS

Gene list curation for pathogenic variant classification

For this specific study, we extended the list of 152 cancer predisposition genes (CPGs) previously compiled by Huang et al.[51] to a total of 158 genes, by adding 6 genes which have been either shown to contribute to MM susceptibility in previous studies of rare germline variants in MM, such as *DIS3*[43] and *KDM1A*,[46] or in which potential risk rare variants had been suggested but not validated in individual MM studies, including *ARID1A*[47], *EP300*[48], *KIF18A*[49], and *USP45*,[47] as well as *CDKN2A*,[44,45] which was already included in the list of 152 CPGs. Although the latter are not completely recognized as MM risk genes by the scientific community yet, we have included them in our analysis in order to better understand and further investigate their role in MM. Results in these genes were carefully revised. This extended gene list was used as input for our tool CharGer (described below) using the *–inheritanceGeneList* parameter. The source and reference for each gene are provided in Table S1.

Pathogenicity assessment of germline variants

Germline variants called with GermlineWrapper were annotated with the Ensembl Variant Effect Predictor (VEP)[173] (v95 with default parameters, except where –everything) and their pathogenicity was determined with our automatic pipeline CharGer[56] (v0.5.4 with default CharGer scores, https://github.com/ding-lab/CharGer/tree/v0.5.4), which annotates and prioritizes variants based on published guidelines by the American College of Medical Genetics and Genomics - Association for Molecular Pathology (ACMG-AMP).[57] CharGer retrieves information from the ClinVar (release as of 08/15/2019 parsed using codes from MacArthur lab ClinVar, https://github.com/macarthur-lab/clinvar) and gnomAD (r2.1.1)[189] databases, as well as computational tools, including SIFT (v5.2.2)[193] and PolyPhen (v2.2.2)[194]; , to inform the implementation of 12 pathogenic and 4 benign evidence levels for the classification of germline variants. The detailed implementation and score of each evidence level, as well as parameters used are as previously described.[51]

We further selected rare variants with $\leq$0.05% allele frequency (AF) in gnomAD (r2.1.1) or 1000 Genomes.[187] We also performed read count analysis using bam-readcount (https://github.com/genome/bam-readcount; v0.8 with parameters -q 10, -b 15) to evaluate the number of reference and alternative alleles for each variant. Both normal and tumor samples were used for the MMRF samples, while only normal samples were provided for the familial dataset. We required variants to have at least 5 counts of the alternative

allele and a variant allele frequency (VAF) of at least 20%. Variants remaining after these filters were manually reviewed with the Integrative Genomics Viewer (IGV) software (v2.8.2).[178] A total of 684 and 145 variants were retained after manual review (Figure 1C). We considered variants to be pathogenic (P) if they were known pathogenic variants in ClinVar; likely pathogenic (LP) if CharGer score >8; and prioritized variant of uncertain significance (PVUS) if CharGer score >4. A list of all variants passing manual review and their information are displayed in Table S2. MMRF case IDs have been replaced with study IDs to ensure the privacy of the study participants. Original MMRF case identifiers linked to study IDs used here are available on Synapse (Synapse ID: syn26844264; https://www.synapse.org/#!Synapse:syn26844264). Data can be provided upon reasonable request.

Germline CNVs detected through XHMM were assessed for pathogenicity using ClassifyCNV (v1.1.1),[124] which applies the 2019 ACMG guidelines for the classification of germline duplications and deletions.[123]

Burden testing of rare pathogenic and likely pathogenic germline variants

We performed burden testing of pathogenic and likely pathogenic variants detected in the MMRF and familial datasets using the Total Frequency Test (TFT)[77] as previously described,[51] which implements a one-sided Fisher test to detect genes with potentially increased burden of pathogenic variants in our datasets in comparison with controls. For this study, we collapsed pathogenic and likely pathogenic germline variants to the gene level and then implemented the total allele counts of pathogenic and likely pathogenic variants detected in the gnomAD (r2.1.1) non-cancer cohort (n = 118,479) using the same CharGer pipeline as controls. In order to account for the potential impact of genetic ancestry in our results, we also performed a burden test including only individuals of European ancestry from the MMRF and familial cohorts against the gnomAD non-cancer Non-Finnish subset (n = 51,377) to remove the effect of this potentially confounding variable.

We also tested burdens of pathogenic and likely pathogenic variants in MM in comparison with other cancer types previously analyzed using The Cancer Genome Atlas (TCGA) cohort from a previous study by our group.[51] Here, we tested burden for each cancer type and each gene using all other cancer types as controls, subtracting out the cohorts with suggestive enrichment for the specific gene in the gnomAD analyses. As described above, this test was also performed in the EUR subset of MMRF, familial, and TCGA cohorts against the gnomAD non-cancer Non-Finnish subset (n = 51,377). We used the standard Benjamini-Hochberg procedure to adjust the resulting p-values to FDR. We define significant events if FDR $\leq$0.05, and suggestive events if FDR $\leq$0.15. The results for both the analyses using the complete cohorts and EUR cohorts only are provided.

Functional enrichment analysis

Our list of genes affected by P/LP/PVUS germline variants in both datasets was submitted through functional enrichment analysis for Gene Ontology (GO) terms and KEGG pathways using the R package clusterProfiler[171] (v3.18.1). We also performed enrichment analysis for REACTOME pathways using the R package ReactomePA[182] (v1.34.0). We used an FDR value of $\leq$0.05 to consider a GO term, KEGG, or REACTOME pathway significantly enriched.

LOH and biallelic events

Analysis of loss-of-heterozygosity (LOH) events can help identify germline variants that are positively selected in the tumor by comparing the VAF in the tumor to that in the normal. Here, we first estimated read counts for each variant in both normal and tumor samples for our MMRF cases using bam-readcount (https://github.com/genome/bam-readcount) (v0.8 with parameters -q 10, -b 15). Then, LOH events were identified by implementing a Fisher's exact test between tumor and normal samples in order to identify any variants for which VAF in the tumor was significantly greater than in the normal for any of the germline variants identified. The resulting p-values were adjusted to FDR using the Benjamini-Hochberg procedure. We considered a LOH to be significant if FDR $\leq$0.05 and suggestive if FDR $\leq$0.15. Moreover, we also considered events with tumor VAF >0.6 and normal VAF <0.6 as additional suggestive LOH events. Further, we classified significant and suggestive LOH events based on GISTIC CNV results as (1) copy number deletion of the wild-type allele, or (2) copy number amplification of the alternative allele, following the same procedures as previously described.[51]

We also performed an analysis of *cis* biallelic events, where we searched for cases carrying both germline pathogenic or likely pathogenic variants and missense or truncating somatic mutations, as well as somatic CNV events in the same gene. Additionally, we investigated potential *trans* events, i.e., genes in the same biological pathway affected by germline variants (germline-germline) or genes in the same pathway affected by both germline and missense or truncating somatic events (germline-somatic). The familial dataset was also investigated for *trans* germline-germline events. Genes were annotated with KEGG and Reactome pathways using the R packages clusterProfiler[171] (v3.18.1) and ReactomePA[182] (v1.34.0), respectively. The lolliplots are constructed and modified from the PCGP protein paint (https://pecan.stjude.cloud/proteinpaint).

Selection of candidate predisposing variants via multiple lines of evidence

We sought multiple lines of evidence to corroborate the pathogenicity of candidate GPVs in the MMRF, for which access to matched tumor-normal data allowed us to perform analyses of LOH, expression effect, and co-occurrence with somatic events in the same gene (SNVs, indels, or copy-number variants). We used an integrative approach to categorize variants into 3 classes: 1) High confidence P: variants classified as P during manual review that show at least one additional piece of evidence of pathogenicity (strong LOH, expression effect, or co-occurring with a somatic event); 2) High confidence LP: same as 1) but for variants initially classified as

LP; and 3) Nominated PVUS: same as 1) but for variants initially classified as PVUS. For expression effect, we considered variants in TSGs associated with the bottom 25% expression, and variants in oncogenes associated with the top 25%. For LOH, we considered variants having both suggestive and significant LOH.

RNA quantification and analysis

Raw RNA-Seq data were obtained from the MMRF CoMMpass Study, as described. Reads were assessed for quality with FastQC[174] (v0.11.8 with default parameters) and mapped to a modified version of the GRCh37 human reference genome based on the 1000 Genomes phase 2 reference assembly sequence, as used in the MMRF CoMMpass Study (http://ftp.1000genomes.ebi.ac.uk/vol1/ftp/technical/reference/phase2_reference_assembly_sequence/hs37d5.fa.gz). Alignment was performed using STAR (v2.5.0a).[195]

We obtained gene-level read counts, Fragments Per Kilobase of transcript per Million mapped reads (FPKM), and FPKM Upper Quartile (FPKM-UQ) values following GDC's mRNA analysis pipeline (https://docs.gdc.cancer.gov/Data/Bioinformatics_Pipelines/Expression_mRNA_Pipeline/). Read counts were obtained using the featureCounts function of the Rsubread package (v1.6.4)[175] that allowed counting the number of reads assigned to genes. These read counts were then converted to FPKM and FPKM-UQ using the formula described in GDC's pipeline documentation.

We calculated the expression percentile of each gene in the MMRF cohort using the empirical cumulative distribution function (ecdf) in R. Following, we implemented a linear regression model to compare the expression percentile differences between carriers and non-carriers of P/LP variants. Resulting *p*-values were adjusted using the standard Benjamini-Hochberg procedure.

Segregation analysis in familial dataset

For the probands whose additional family members were available, we performed segregation analysis using either exome sequencing data or targeted Sanger sequencing. Co-segregating variants shared by exomes are provided in Table S10.

Analysis of age at onset differences between carriers and non-carriers

We implemented a linear regression model to evaluate potential associations between germline variant carriers of predisposition genes and age at onset. We tested genes harboring 3 or more P and LP variants. Resulting *p*-values were adjusted with the Benjamini-Hochberg procedure. We also implemented a Wilcoxon Rank-Sum test to determine overall age differences between all carriers of P/LP/PVUS variants and non carriers for both datasets.