ARTICLE

Pattern of somatic mutations in patients with Waldenström macroglobulinemia or IgM monoclonal gammopathy of undetermined significance

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ABSTRACT

We analyzed *MYD88* and CXCR4 mutation status of 260 patients with Waldenström macroglobulinemia or IgM monoclonal gammopathy of undetermined significance using allele-specific real time quantitative polymerase chain reaction and Sanger sequencing, respectively. A subgroup of 119 patients was further studied with next-generation sequencing of 11 target genes (*MYD88*, *CXCR4*, *ARID1A*, *KMT2D*, *NOTCH2*, *TP53*, *PRDM1*, *CD79B*, *TRAF3*, *MYBBP1A*, and *TNFAIP3*). *MYD88* (L265P) was found at diagnosis in 91% of patients with Waldenström macroglobulinemia and in 60% of patients with IgM monoclonal gammopathy of undetermined significance using allele-specific polymerase chain reaction analysis. *MYD88* mutations other than the classical L265P (V217F, S219C and M232T) were found in four cases by next-generation sequencing. Waldenström macroglobulinemia patients with wild-type *MYD88* had a distinct clinical phenotype characterized by less bone marrow infiltration (P=0.01) and more frequent extramedullary involvement (P=0.001) compared to patients with mutated *MYD88*. Patients with wild-type *MYD88* did not show additional mutations in the other target genes. *CXCR4* mutations were found by Sanger sequencing in 22% of patients with Waldenström macroglobulinemia. With next-generation sequencing, a *CXCR4* mutation was detected in 23% of patients with Waldenström macroglobulinemia and 9% of those with IgM monoclonal gammopathy of undetermined significance. Asymptomatic Waldenström macroglobulinemia patients harboring a *CXCR4* mutation had a shorter treatment-free survival (51 months) than that of patients with wild-type *CXCR4* (median not reached) (P=0.007). Analysis of variant allele frequencies indicated that *CXCR4* mutations were present in the dominant clone in the majority of cases. Recurrent somatic mutations of *KMT2D* were found in 24% of patients with Waldenström macroglobulinemia and 5% of patients with IgM monoclonal gammopathy of undetermined significance and were primarily subclonal.

Introduction

Waldenström macroglobulinemia (WM) is a rare lymphoproliferative disorder characterized by the presence of a serum IgM paraprotein associated with infiltration of the bone marrow by lymphoplasmacytic lymphoma.[1] Familial aggregation of WM and related B-cell disorders strongly supports a role for genetic factors in the pathogenesis of the disease.[2-4]

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Some years ago, using whole genome sequencing Treon *et al.* identified *MYD88* (L265P) as the most common somatic mutation in WM.[5] Signaling studies showed that the mutant protein encoded by *MYD88* triggers tumor growth through the activation of nuclear factor kappa light-chain enhancer of activated B cells (NF-κB) via two independent pathways, namely IRAK1-4 and BTK.[6] The frequent recurrence of one single variant (i.e. L265P) in WM/lymphoplasmacytic lymphoma prompted the design of allele-specific polymerase chain reaction (PCR)-based strategies for the detection of the *MYD88* mutation.[7-8] Using this approach, the *MYD88* (L265P) mutation is detectable in more than 90% of WM patients, while it is rarely expressed in other indolent mature B-cell tumors, such as splenic marginal zone lymphoma or chronic lymphocytic leukemia.[7-10]

The diagnosis of WM may be preceded by a history of IgM monoclonal gammopathy of undetermined significance (IgM MGUS). Long-term follow-up studies show that patients with IgM MGUS have a risk of progression to WM or to other B-cell lymphoproliferative disorders of approximately 1.5-2% per year.[11] Using allele-specific PCR, 50-80% of patients with IgM MGUS were found to harbor the *MYD88* (L265P) mutation, suggesting that *MYD88* mutation is an early genetic event in the development of WM.[7-10,12] In a previous study, we demonstrated that IgM MGUS patients harboring the *MYD88* (L265P) mutation have a significantly higher risk of progression to WM or other lymphoproliferative disorder as compared with patients with the wild-type gene, regardless of the size of the serum IgM monoclonal protein.[13]

The second most common mutations in WM are nonsense or frameshift mutations in the carboxyl-terminal cytoplasmic tail of the *CXCR4* gene. *CXCR4* is overexpressed by cancer cells in many hematopoietic and solid cancers, but WM is the first human cancer in which somatic *CXCR4* mutations have been reported.[14] These mutations are similar to germline mutations typical of the WHIM syndrome, an inherited autosomal dominant disorder characterized by warts, hypogammaglobulinemia, infections and myelokathexis.[15] In WM, somatic *CXCR4* mutations result in impaired internalization of the CXCR4 receptor, leading to constitutive activation of the CXCR4 pathway, AKT and ERK activation and eventually WM cell survival.[16-17] Using Sanger sequencing, *CXCR4* mutations are detected in approximately 30% of WM patients, almost all of whom also harbor the *MYD88* (L265P) mutation. *CXCR4* mutations are usually subclonal, supporting the notion that they are acquired after the *MYD88* (L265P) mutation in the development of WM.[18] From a clinical standpoint, the presence of *CXCR4* mutations has been associated with a more aggressive clinical presentation, including higher levels of IgM serum monoclonal protein, a higher incidence of hyperviscosity syndrome and more extensive bone marrow infiltration,[19-21] as well as with clinical resistance to Ibrutinib.[16-17,22]

While there is an increasing body of data about *MYD88* and *CXCR4* mutations, little is known about the remaining genomic landscape of WM. Using whole genome sequencing, somatic mutations of *ARID1A* have been found in 17% of patients, while *CD79B*, *KMT2D* (formerly known as *MLL2*), *MYBBP1A* and *TP53* mutations have been described in less than 10% of cases each.

The aim of this study was to analyze the pattern of mutations of 11 genes in a cohort of well-annotated WM or IgM MGUS patients, and to evaluate correlations between somatic mutations and disease phenotype or patients' outcome. The following genes were studied: *MYD88*, *CXCR4*, *ARID1A*, *KMT2D*, *NOTCH2*, *TP53*, *PRDM1*, *CD79B*, *TRAF3*, *MYBBP1A*, and *TNFAIP3*.

In this study we demonstrated that *MYD88* wild-type patients have a distinct clinical phenotype and do not show additional somatic mutations in the other target genes; furthermore, we demonstrated that *CXCR4* mutations in WM patients are associated with an earlier need for treatment. Finally we report for the first time that subclonal *KMT2D* mutations are highly recurrent in WM patients.

Methods

These investigations were approved by the local Ethics Committee. The procedures followed were in accordance with the Helsinki Declaration of 1975, as revised in 2000, and samples were obtained after subjects had provided informed consent.

Patients

The study population included 130 patients with WM and 130 patients with IgM MGUS, in whom *MYD88* and *CXCR4* mutation status was evaluated with allele-specific real-time quantitative PCR (RT-qPCR) and Sanger sequencing, respectively. The methods are described in the *Online Supplements*. A subgroup of 119 patients (62 with WM and 57 with IgM MGUS) was further studied using next-generation sequencing (NGS) of 11 target genes, selected on the basis of data available in the literature. The patients' characteristics are reported in *Online Supplementary Table S1*. The diagnoses of WM and IgM MGUS were made according to the consensus recommendations from the Second International Workshop on WM.[1]

Sample collection and cell separation

Sample collection and cell separation are described in the *Online Supplement*.

Mutation analysis of target genes using next-generation sequencing

Mutation analysis was performed on paired tumor/germline gDNA in order to identify relevant somatic mutations of selected genes. A TruSeq Custom Amplicon panel (TSCA; Illumina, San Diego, CA, USA) targeting complete coding exons and their adjacent splice junctions from the 11 genes was designed using Illumina Design Studio software. The TSCA panel consisted of 249 amplicons, 425 bp in length, for a total of 69 kb targeted DNA. Dual-barcoded TSCA libraries were created from 250 ng of high quality DNA according to the manufacturer's protocol. Libraries were multiplexed and underwent 2x250-bp paired-end sequencing on a MiSeq sequencing system using the MiSeq Reagent Kit version 3 (Illumina). The resulting average depth of coverage for the 249 amplicons was 1009x.

Sequence reads were initially aligned to the human genome (GRCh37/hg19) using the Burrows-Wheeler[23] aligner. The Genome Analysis Toolkit[24] (*www.broadinstitute.org/gatk/*) was later used to clean up reads and make alignment data more reliable for the variant calling (GATK data cleanup best practice): single nucleotide variants and small insertions-deletions were identified by Mutect[25] and UnifiedGenotyper, respectively. Functionally annotated variants were filtered according to the following criteria: synonymous variants and variants located outside protein coding regions were filtered; polymorphisms described in dbSNP (ver-

sion 138) and the 1000 Genomes Project with a population frequency higher than 1% and 0.14%, respectively, were removed; variants with coverage <30X and less than ten supporting reads and variants with an allelic fraction lower than 1% were filtered; the remaining variants, evaluated as candidate somatic mutations, were manually reviewed and tagged as oncogenic using different criteria based on information retrieved from the literature, sequence conservation and in *silico* prediction of effect.

Statistics

Quantitative variables are summarized in terms of median and range. Categorical variables are described by absolute and relative frequencies. The association between two categorical variables was estimated by the Fisher exact test. Quantitative variables were compared between two groups of patients using the Wilcoxon rank-sum test. The correlation between two quantitative variables was tested using the Spearman rho correlation.

Overall survival was defined as the time from diagnosis to death from any cause or last follow-up (for censored patients) Treatment-free survival was measured (only in WM patients with mutational status evaluated at diagnosis) from the date of diagnosis to the date of first-line treatment or the last follow-up (for censored patients). Overall survival and treatment-free survival were estimated using the Kaplan-Meier product limit method and survival curves were compared by the log-rank test. The effect of variables on overall survival and treatment-free survival was evaluated using the proportional hazard Cox model. The agreement between NGS and PCR results and between methods was tested by the Cohen kappa coefficient. *P*-values less than 0.05 were considered statistically significant. All analyses were carried out using Stata 12.1 software (2011).

Results

MYD88 mutation status and allele burden as determined by polymerase chain reaction

Using allele-specific RT-qPCR, the *MYD88* (L265P) mutation was found in 78 of 130 (60%) of patients with IgM MGUS and in 112 of 130 (86%) of those with WM. In WM, 96/106 untreated patients (91%) and 16/24 (67%) of previously treated patients were found to harbor the *MYD88* (L265P) mutation. Among 24 previously treated WM patients, seven had wild-type *MYD88*. Two of them had progressive disease with a bone marrow infiltration of 50% and 70%. The other five *MYD88* wild-type patients were in very good partial response and did not have detectable bone marrow infiltration at the time of analysis. Among the patients with an *MYD88* mutation, the median allele burden was 1.05 (range, 0.1-18.4) in those with IgM MGUS and 15.4 (range, 0.1-96) in those with WM (P<0.001). Patients with symptomatic WM had a higher *MYD88* allele burden as compared with asymptomatic ones (28.5 *versus* 13; P=0.05) (Figure 1). However, there were overlapping values without a clear cut-off between WM and IgM MGUS patients. The prevalence of *MYD88* (L265P) mutation was not significantly higher in CD19$^+$-selected bone marrow samples than in unselected samples either in IgM MGUS patients (62% *versus* 58%; P= 0.722) or in WM patients (87% *versus* 85%; P=0.802).

Correlations of *MYD88* mutation status and *MYD88* allele burden with disease phenotype

Compared with WM patients harboring the *MYD88* (L265P) mutation, patients with wild-type *MYD88* had significantly less bone marrow infiltration (median 18% *versus* 35%; P=0.01) and higher β_2-microglobulin levels (median 3109 μg/L *versus* 2480 μg/L; P=0.03), borderline lower IgM levels (median 536 mg/dL *versus* 1170 mg/dL; P=0.07) and platelet counts (median 217 x 10^9/L *versus* 260 x 10^9/L; P=0.06), and more frequent extramedullary involvement (60% *versus* 12% of patients; P=0.001). No significant difference was found for age, sex or the other clinical characteristics. Among WM patients with the *MYD88* (L265P) mutation, the median *MYD88* allele burden was significantly higher in patients with hemoglobin levels <10 g/dL (P=0.01), β_2-microglobulin levels >3000 μg/L (P<0.001), serum albumin levels <3.5 g/dL (P=0.01), detectable Bence-Jones proteinuria (P=0.04), abnormal free-light chain ratio (P=0.046) or bone marrow infiltration >30% (P=0.01) (Figure 2). No significant correlation was found between *MYD88* allele burden and age, sex, platelet count, IgM levels, or presence of extramedullary disease.

CXCR4 mutations in patients with IgM monoclonal gammopathy of undetermined significance and Waldenström macroglobulinemia

Thirty-four of 260 patients (13%) had a *CXCR4* mutation. Nonsense and frameshift mutations accounted for 16 (47%) and 18 cases (53%), respectively, C1013G being the most common variant (14/34, 41% of all *CXCR4* mutations). The frequency of *CXCR4* mutations was 5/130 (4%) in IgM MGUS patients and 29/130 (22%) in WM patients (P<0.001). The prevalence of *CXCR4* mutations was not statistically different between CD19$^+$-selected and unsorted bone marrow samples (7% *versus* 2% in IgM MGUS patients; P=0.188 and 25% *versus* 19% in WM patients; P=0.519). In WM patients, the presence of a *CXCR4* mutation was associated with a significantly higher degree of bone marrow infiltration (50% *versus* 30%; P=0.04) and higher *MYD88* allele burden (24% *versus* 9%; P=0.01) (*Online Supplementary Table S2*). Taking into account that the threshold for mutant allele detection by Sanger sequencing is approximately 20%, we analyzed the prevalence of *CXCR4* mutations according to this cut-off and found that the rate of *CXCR4* mutations was 27% in patients with 20% or more bone marrow infiltration

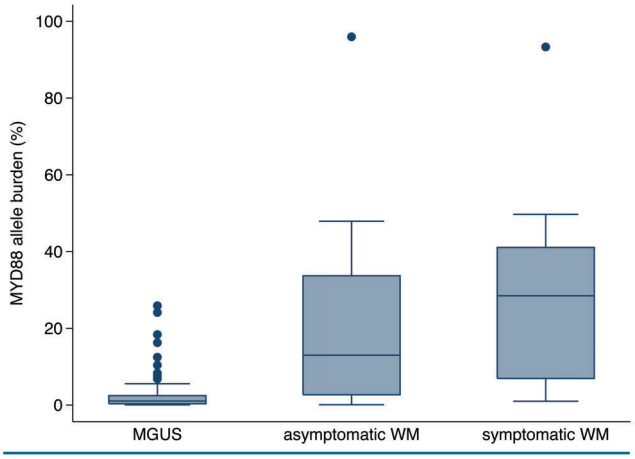

Figure 1. *MYD88* allele burden in patients with IgM monoclonal gammopathy of undetermined significance, asymptomatic and symptomatic Waldenström macroglobulinemia.

and 13% in patients with less than 20% bone marrow infiltration (*P*=0.179).

Prognostic impact of *CXCR4* and *MYD88* mutations

The median follow-up of the whole series was 43 months (38.9 months for IgM MGUS patients and 50.4 months for WM patients). During the follow-up, three of 130 IgM MGUS patients (2%) and 26 of 81 asymptomatic WM patients (32%) progressed to symptomatic WM requiring treatment. Twenty-five of 260 patients (10%) have died.

The median treatment-free survival was significantly shorter in asymptomatic WM patients harboring a *CXCR4* mutation at diagnosis (median 51 months) than in those with wild type *CXCR4* (median not reached) (*P*=0.007) (Figure 3). In multivariate analysis, *CXCR4* mutation and size of serum protein were independent prognostic factors for progression from asymptomatic to symptomatic WM requiring therapy (*Online Supplementary Table S3*). *MYD88* mutational status did not influence time to first treatment (*P*=0.19). Treatment-free survival was significantly shorter in asymptomatic WM patients harboring both *MYD88* and *CXCR4* mutations than in *MYD88*-mutated/*CXCR4*-wild type patients (*P*=0.019), confirming that *CXCR4* but not *MYD88* mutations were associated with an earlier need for treatment (Figure 4). Overall survival was not affected by either *MYD88* or *CXCR4* mutational status (*data not shown*).

Somatic mutations identified by next-generation sequencing

Overall, 88 of 119 patients studied with NGS (74%) had one or more somatic mutations in the 11 target genes. The median number of mutations was significantly higher in WM patients than in IgM MGUS patients (median 2 *versus* 1; *P*<0.001) and in those previously treated than in untreated ones (median 2 *versus* 1; *P*<0.001). *MYD88* mutations were found in 80/119 patients (67%), with a median variant allele frequency of 34.2% (range, 3.0-93.3%). The prevalence of *MYD88* mutations determined by NGS was 85% in WM and 47% in IgM MGUS patients (*P*<0.001) (Figure 5). *MYD88* mutations other than the classical L265P (n=76) were found in four patients and were V217F (n=2), S219C (n=1) and M232T (n=1). Fourteen patients who resulted *MYD88* (L265P) wild-type by NGS were found to have the mutation by allele-specific RT-qPCR (K coefficient of concordance between NGS and PCR, 65%; *P*<0.001). Twelve of them had a diagnosis of IgM MGUS and two had WM. Of these latter two patients, one was untreated with predominant extramedullary disease and minimal bone marrow infiltration, the other was a previously treated patient who had achieved a very good partial response and did not have detectable bone marrow infiltration at the time of mutation status analysis.

Using NGS a *CXCR4* mutation was found in 19 patients (16%) with a median variant allele frequency of 22.5% (range, 4.2-49.8%). The K coefficient of concordance between NGS and Sanger sequencing was 89.8% (*P*<0.001). The prevalence of *CXCR4* mutations was 23% in WM and 9% in IgM MGUS patients (*P*=0.047) (Figure 5). Of the two patients with a *CXCR4* mutation who were *MYD88*-wild type according to NGS, one was found to harbor the *MYD88* (L265P) mutation with a low allele burden (0.34%) using allele-specific RT-qPCR. The comparison of variant allele frequencies of *CXCR4* and *MYD88* mutations in individual patients indicated that *CXCR4* mutations were present in the dominant clone in the majority of cases (Figure 6).

NGS allowed the identification of somatic mutations in *KMT2D* (16% of patients), *TP53* (8%), *NOTCH2* (7%), *PRDM1* (4%), *ARID1A* (3%), *CD79B* (3%), and *TRAF3*

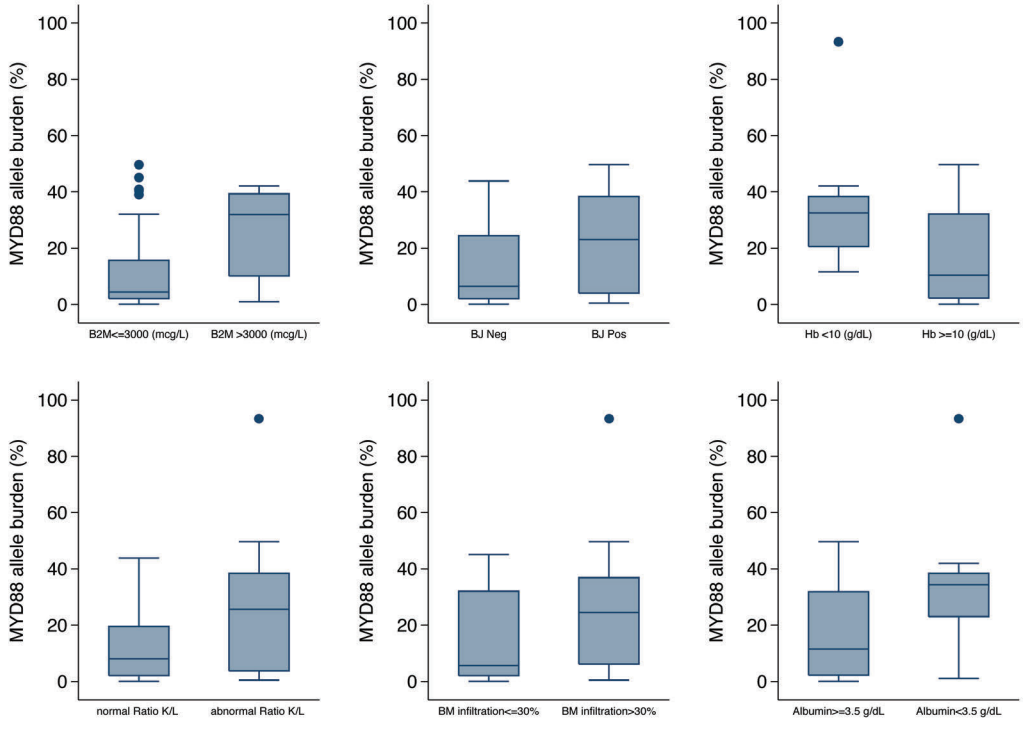

Figure 2. Correlation of *MYD88* allele burden with disease phenotype in patients with Waldenström macroglobulinemia.

(1%). No mutations were found in *MYBBP1A* or *TNFAIP3*. Overall, the prevalence of these mutations was significantly lower in patients with either wild-type *MYD88* or wild-type *CXCR4* than in those with *MYD88* and/or *CXCR4* mutations (16% *versus* 41%; *P*=0.007) (Figure 5). The prevalence of *KMT2D* mutations was significantly higher in WM patients than in patients with IgM MGUS (24% *versus* 5%; *P*=0.002) while the prevalence of mutations in other genes was not statistically different according to diagnosis (*P*>0.3 for all comparisons). The analysis of variant allele frequency in patients harboring either a *MYD88* or a *KMT2D* mutation showed that *KMT2D* mutations were primarily subclonal (Figure 7).

TP53 mutations were found in three of 57 patients with IgM MGUS (5%) and six of 62 patients with WM (10%). The variant allele frequency of *TP53* mutations was below 3.5% in IgM MGUS patients and untreated WM patients. In the four previously treated WM patients, the variant allele frequencies were 2.9%, 10.1%, 11.2% and 57.8%.

We did not find a statistically significant correlation of *KMT2D*, *TP53*, *NOTCH2*, *PRDM1*, *ARID1A*, *CD79B*, or *TRAF3* mutations with overall survival or time to first treatment.

Discussion

This study was conducted on two well-balanced subgroups of patients with an established diagnosis of IgM MGUS or WM. The study includes the largest series published so far of IgM MGUS patients screened for somatic mutations of genes with potential relevance to the pathogenesis of WM. Since progression from IgM MGUS to WM is likely a multi-step process, in which multiple genetic hits are required for progression from a pre-benign condition to an overt neoplastic disease, the analysis of mutation pattern in IgM MGUS patients is useful to understand whether a given mutation represents an early

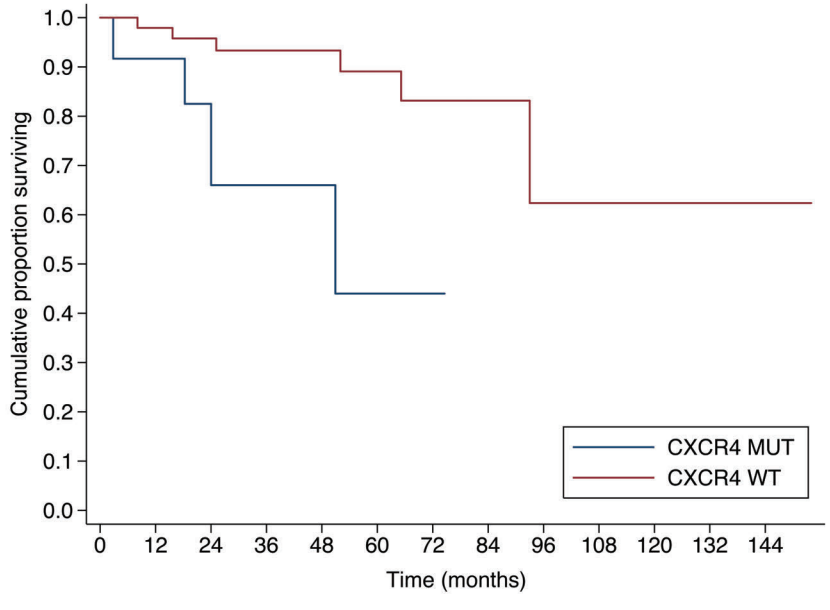

Figure 3. Treatment-free survival of asymptomatic patients with Waldenström macroglobulinemia according to *CXCR4* mutation status.

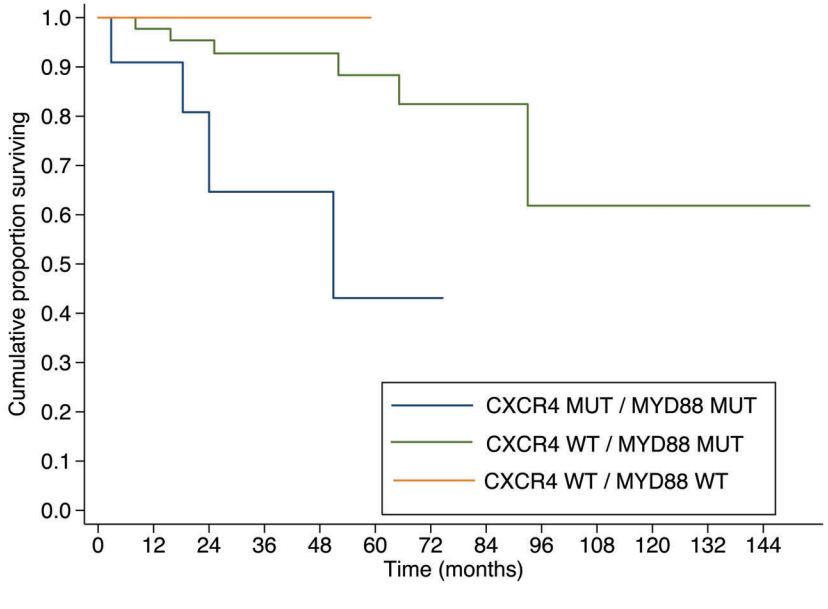

Figure 4. Treatment-free survival of asymptomatic patients with Waldenström macroglobulinemia according to *MYD88* and *CXCR4* mutational status.

or late event during the oncogenic process starting from MGUS and ending with WM. In agreement with this model, the high prevalence of *MYD88* mutations in IgM MGUS patients (60%) confirms that this mutation is an early event, while the low prevalence of *CXCR4* and *KMT2D* mutations in IgM MGUS (<10%) suggests that these mutations usually occur later.

As expected, the *MYD88* (L265P) mutation was confirmed as the most common somatic mutation in WM and IgM MGUS, in agreement with results reported by other groups.[5,7-10,12-14] Among the patients with WM, the rate of *MYD88* (L265P) mutation was significantly lower in previously treated patients than in untreated ones. The majority of WM patients who resulted *MYD88* wild-type

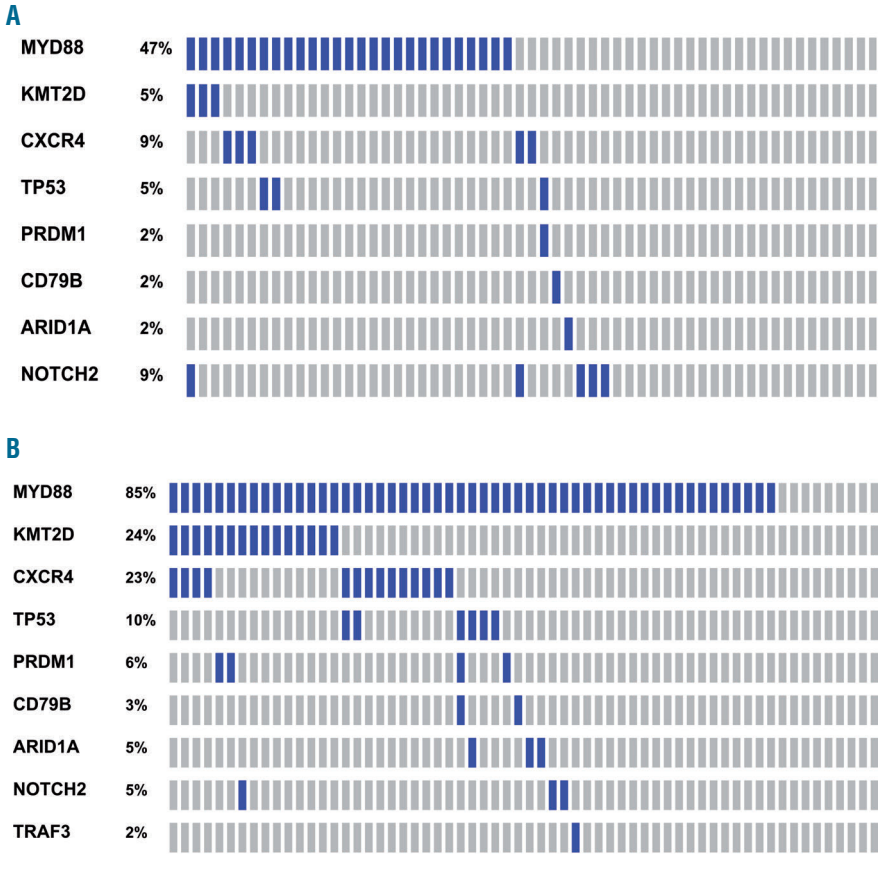

Figure 5. Pattern of mutations observed in patients with (A) IgM monoclonal gammopathy of undetermined significance or (B) Waldenström macroglobulinemia.

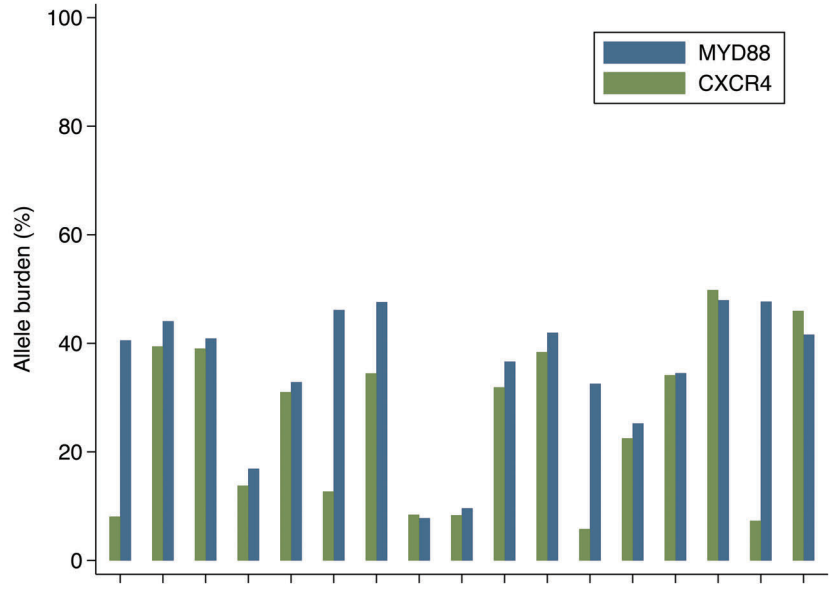

Figure 6. Variant allele frequency of *MYD88* and *CXCR4* mutations as determined by next-generation sequencing.

after therapy had been successfully treated with immunochemotherapy. The lower yield of clonal B cells following treatment with B-cell depleting agents may at least in part explain this finding, even though a baseline sample was not available to confirm this hypothesis.

Samples studied with RT-qPCR for *MYD88* (L265P) included either CD19$^+$-selected or unselected bone marrow mononuclear cells. The rate of *MYD88* (L265P) mutation was not significantly higher in sorted samples than in unsorted ones, either in IgM MGUS or in WM. This result was not surprising in WM patients, in line with a recent publication showing a high level of concordance in the rate of *MYD88* (L265P) mutation between selected and unselected bone marrow cells,[26] while it was less expected in the context of IgM MGUS. In CD19$^+$-selected samples, we did not assess CD19$^+$ clonality with light chain restriction, which would probably have increased the accuracy in detecting the *MYD88* (L265P) mutation, especially among patients with IgM MGUS. In fact, using this approach the Salamanca group found the *MYD88* (L265P) mutation in 87% of IgM MGUS patients, which is a higher rate than in our study as well as in other published series.[9] Finally, we did not specifically evaluate the plasma cell compartment, in which the presence of the *MYD88* (L265P) mutation has been previously reported.[5,26] These observations indicate that the source of DNA may affect the rate of *MYD88* mutations, and underline the need to define the best strategy for testing these mutations, balancing the gain in accuracy with time and costs required for CD19$^+$ sorting and light chain restriction assessment.

NGS allowed the identification of mutations other than the classical L265P variant in four patients. Among these, the M232T variant has been recently described in a WM patient,[27] whereas the V217F and S219C had been previously reported in diffuse large B-cell lymphoma, activated B-cell subtype.[28] Overall, there was a strong concordance between NGS and RT-qPCR findings, and discordant cases were mainly represented by IgM MGUS patients in whom the L265P mutation was found only with RT-qPCR. This finding confirms the greater sensitivity of RT-qPCR over NGS, which becomes evident when the B-cell clone is small.

WM patients with wild-type *MYD88* showed distinct clinical features, including lower IgM levels, less bone marrow infiltration and more frequent extramedullary disease as compared with patients harboring the *MYD88* mutation. *MYD88* wild-type patients were almost invariably *CXCR4* wild-type and did not have additional mutations in the other genes studied with NGS. The existence of a small subgroup of WM patients not harboring the *MYD88* (L265P) mutation has been increasingly recognized in recent studies. These patients seem to have lower response rates to the BTK inhibitor ibrutinib and a poorer outcome as compared with *MYD88*-mutated patients.[19,22] Furthermore, *MYD88* wild-type cases show lower expression of genes related to B-cell differentiation and a lower rate of *IGH* somatic hypermutation, suggesting that they constitute a distinct entity with respect to classical WM, possibly deriving from a B cell in an earlier stage of differentiation.[9,29]

Allele-specific RT-qPCR allowed the estimation of *MYD88* allele burden. Although WM patients usually have higher allele burdens as compared with IgM MGUS patients, we could not identify a threshold that reliably distinguished these two conditions.

In WM patients, the *MYD88* allele burden was found to be a good surrogate marker of clinical disease burden. Although clinical variables associated with a higher *MYD88* allele burden include some of the prognostic factors of the WM International Prognostic Scoring System (hemoglobin, albumin, β_2-microglobulin levels), a higher *MYD88* allele burden was not associated with worse survival.

In this study we analyzed *CXCR4* mutation status by means of Sanger sequencing and NGS. The threshold for mutant allele detection by Sanger sequencing is approximately 20% and the rate of *CXCR4* mutations was twofold higher in patients with a bone marrow infiltration of 20% or more than in patients with a bone marrow infiltration of less than 20%. Given the greater sensitivity of

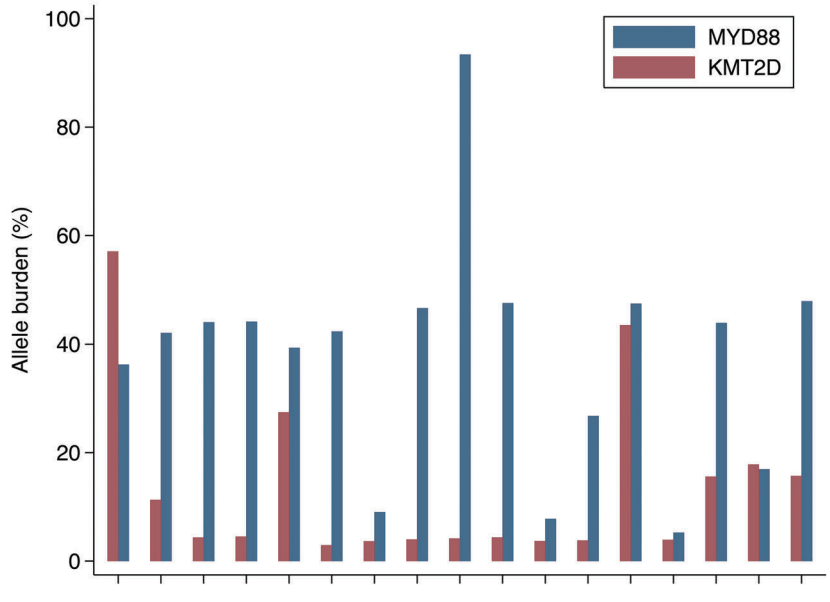

Figure 7. Variant allele frequency of *MYD88* and *KMT2D* mutations as determined by next-generation sequencing.

NGS, we expected to find a significantly higher rate of mutations with this technique than with Sanger sequencing, especially in patients with IgM MGUS in whom the B-cell clone is usually under the detection limits of Sanger sequencing. Indeed, less than 10% of IgM MGUS patients were found to harbor a *CXCR4* mutation by NGS, suggesting that *CXCR4* mutations are later events than the *MYD88* (L265P) mutation in the pathogenesis of WM/lymphoplasmacytic lymphoma. A *CXCR4* mutation was found in approximately a quarter of WM patients, in agreement with results reported by Poulain *et al.* in a series of WM patients studied with Sanger sequencing and NGS.[20] Combining Sanger technology with allele-specific PCR for the most common variant S338X, *CXCR4* mutations were found in up to 43% of WM patients, suggesting that differences among studies depend mainly on the sensitivity of the method used as well as the selection of patients.[18]

In a study published by Xu *et al.*, *CXCR4* mutations were shown by cancer cell fraction analysis to be primarily subclonal.[18] Although in a targeted sequencing approach with DNA it is often difficult to determine unequivocally whether a mutation is clonal or subclonal, because of problems with determining the tumor cell content in the biopsy, lack of knowledge about aneuploidy of chromosomes with mutations, and potential variation in the efficiency of amplifying the wild-type and mutated alleles, our data seem to indicate that *CXCR4* mutations pertain to the dominant *MYD88*-mutated clone in the majority of cases.

Of interest, *CXCR4* mutations were associated with an earlier need for treatment in patients with WM. In a study of 48 asymptomatic WM patients with a long follow-up, the cumulative probability of progression to symptomatic WM or other lymphoproliferative disorders was 59% at 5 years and 68% at 10 years. The main risk factors for progression were the extent of bone marrow infiltration by lymphoplasmacytic cells, the size of monoclonal protein and hemoglobin levels.[30] In our study we performed a multivariate analysis including *CXCR4* mutation status and the above reported clinical risk factors for progression. *CXCR4* mutation status and the size of serum monoclonal protein were independent risk factors for progression from asymptomatic to symptomatic WM requiring therapy. Based on these findings, a model including molecular and clinical variables may refine prognostication of asymptomatic WM patients and potentially lead to the design of risk-adapted follow-up strategies.

In the subgroup of patients studied with NGS we analyzed the occurrence of somatic mutations in other genes with potential relevance in WM based on evidence from the literature. One of the main strengths of this study is that we could validate the somatic origin of the mutations as a matched control, represented by bone marrow CD19$^-$ mononuclear cells was available in all cases. We found that subclonal mutations of *KMT2D* are highly recurrent in WM patients and may also be present in IgM MGUS patients. *KMT2D* encodes a histone methyltransferase that targets the Lys-4 position of histone H3. Somatically acquired *KMT2D* mutations have been reported in about 90% of follicular lymphomas, 30% of diffuse large B-cell lymphomas, 15% of splenic marginal zone lymphomas, as well as in 10-15% of mantle cell lymphomas.[31-35] Germline *KMT2D* mutations have been reported in 60-70% of patients with Kabuki syndrome, an autosomal dominant disorder characterized by craniofacial, intellectual, and cardiac abnormalities as well as by recurrent infections. In these patients, *KMT2D* mutations are associated with dysregulation of terminal B-cell differentiation, leading to humoral immune deficiency and autoimmune complications.[36] Abnormally low serum levels of IgA, IgG, or both have been observed in more than 50% of WM patients before treatment and remain low after treatment also in patients achieving a complete remission.[37] Whether there is a relationship between *KMT2D* mutations and hypogammaglobulinemia or autoimmune phenomena commonly observed in WM patients deserves further investigations.

Using NGS we found *TP53* mutations in 10% of WM patients and in 5% of IgM MGUS patients; however, so far these mutations have not been seen to have a prognostic impact, probably because of the short follow-up of patients studied with NGS. Interestingly, Poulain *et al.* recently reported a similar prevalence of *TP53* mutations in WM (11%) and showed that *TP53* alterations were associated with shorter overall survival, independently of WM International Prognostic Scoring System score.[38]

In conclusion, this study adds more knowledge about the clinical and prognostic implications of *MYD88* and *CXCR4* mutations and reveals the presence of further somatic mutations potentially relevant to the pathogenesis of WM. Longitudinal studies with sequential evaluations during the course of the disease are needed to understand the clonal evolution underlying progression from IgM MGUS to WM and clonal dynamics under treatment.

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