Determination of MYD88 and CXCR4 mutation for clinical detection and their significance in Waldenström macroglobulinemia

Running Title: MYD88 and CXCR4 Mutations in Waldenström Macroglobulinemia

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Competing Interests

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Abstract

Purpose: This study aims to explore the incidence and clinical features of MYD88 and CXCR4 mutations in patients with Waldenström macroglobulinemia (WM) and determine the optimal method for routine clinical practice. Additionally, we seek to evaluate the prognostic significance of these features across various therapeutic backgrounds [cytotoxic group, the Rituximab/Bortezomib-based group, and the Bruton's tyrosine kinase inhibitor (BTKi) group]. **Experimental Design:** 385 symptomatic WM patients were analyzed for MYD88 and CXCR4 mutations using Sanger sequencing, next-generation sequencing (NGS), allele-specific quantitative polymerase chain reaction (AS-PCR), and/or droplet digital PCR (ddPCR). **Results:** The overall MYD88 mutation rate was 87.8%, relatively lower than that in Western cohort. Both AS-PCR and ddPCR demonstrated high sensitivity in unsorted samples, detecting 98.5% and 97.7% of mutations, respectively, including those with low tumor burdens. The total CXCR4 mutation rate was 30.9%, with NGS exhibiting the highest sensitivity of 78.0%. CXCR4 mutation was significantly linked to shorter OS only within the BTKi treatment group. The multivariate analysis indicated that MYD88 and CXCR4 mutations were not independent prognostic factors in the non-BTKi group when considering IPSSWM clinical staging. However, in the BTKi treatment group, these mutations emerged as independent adverse prognostic factors, overshadowing the prognostic significance of IPSSWM classification (MYD88: HR=0.229, *P*=0.030; CXCR4: HR=3.349, *P*=0.012). **Conclusions:** Testing for MYD88 mutations using AS-PCR or ddPCR in unsorted samples is viable for routine clinical practice. Under BTKi treatment, MYD88 and CXCR4 mutations hold greater prognostic importance than IPSSWM staging in WM.

Translational relevance

The detection of MYD88 and CXCR4 mutations is crucial for WM, yet there is a lack of standardized and unified methods for their detection. This comprehensive study includes a large cohort to investigate the prevalence, clinical manifestations, and prognostic significance of MYD88 and CXCR4 mutations in Chinese patients with WM. We employed various methods to simultaneously test for MYD88 and CXCR4 mutations in the same sample, directly comparing the sensitivity of different approaches. Additionally, we explored the prognostic value of MYD88 and CXCR4 mutations against diverse treatment backgrounds, including non BTKi therapy and BTKi therapy. Furthermore, the research integrates these mutations with the traditional IPSSWM scoring system, assessing their independent prognostic value across various treatment protocols. It was discovered that under treatment with BTKi, MYD88 and CXCR4 mutations carry greater prognostic impact than the IPSSWM staging system in WM.

Introduction

Lymphoplasmacytic lymphoma (LPL) is characterized by the monoclonal proliferation of small B lymphocytes, plasmacytoid lymphocytes, and plasma cells. Waldenström macroglobulinemia (WM), a subtype of LPL, is marked by bone marrow involvement and the presence of an immunoglobulin M (IgM) monoclonal protein(1). Recent research advancements in WM have underscored the importance of two key somatic mutations: MYD88 and CXCR4(2, 3). MYD88 mutation ($MYD88^{MT}$) present in approximately 93%-97% of WM cases(2), is crucial for both diagnosis and prognosis. Mutations in the CXCR4 gene, the second most common somatic mutation, are found in about 30%-40% of WM patients(3). Identifying the status of these mutations is essential for evaluating the efficacy of Bruton's tyrosine kinase inhibitor (BTKi) treatment(4-6). Despite these advancements, a standardized method for the detection of these mutations remains to be established.

Initial studies on the incidence of MYD88 mutations utilized AS-PCR on CD19-selected samples to optimize sensitivity(7). AS-PCR is an economical and practical assay that offers higher sensitivity compared to Sanger sequencing. Subsequent studies highlighted ddPCR as a superior technique that provides improved sensitivity, precision and reproducibility over AS-PCR(8, 9). NGS also delivers dependable results on CD19-selected samples, with the added benefit of detecting both non-L265P MYD88 mutations and other genetic alterations such as those in CXCR4 and TP53(2, 3). However, the necessity of CD19 sorting for all specimens in current clinical practice remains debated. On one hand, sorting can be time-consuming and costly. An additional challenge is that for patients with lower tumor infiltration, at least 5ml of bone marrow fluid needs to be collected in order to isolate a sufficient quantity of CD19+ cells for MYD88 detection. Moreover, our routine clinical assays, including immunotyping, Fluorescence In Situ Hybridization (FISH) and target sequencing, demand a considerable volume of bone marrow sample in addition to MYD88 testing. On the other hand, some WM patients encountered challenges such as hyperviscosity syndrome or dry tap during bone marrow aspiration, further complicating the collection of adequate bone marrow fluid volume. This scenario could elevate the likelihood of experimental failure. Additionally, recent studies have highlighted the potential for false negatives that may arise from selecting CD19+ cells, in samples from treated WM patients who have undergone extensive B cell-depletion treatments(10, 11). Acknowledging the impracticalities of CD19 enrichment in routine clinical settings, our study seeks to assess the sensitivity and specificity of direct MYD88 mutation detection on un-sorted clinical samples using various methods. Our goal is to identify a testing approach that is not only more convenient, cost-effective, and efficient but also

has a higher success rate. Ultimately, we aim to establish a method that can be seamlessly integrated into standard clinical testing protocols, addressing both the practical and technical challenges currently faced.

Our research not only evaluates the effectiveness of various techniques in identifying MYD88 and CXCR4 mutations but also aims to elucidate the mutation frequency of WM within the Chinese population. Previous research suggests that WM has a lower age-adjusted incidence rate in East Asians compared to Western populations(12, 13). The MYD88 mutation rate in China is reported to be relatively low, ranging from 60% to 90%(14-17). The occurrence of CXCR4 mutations among Chinese WM population remains ambiguous, possibly due to the reliance on less standardized and accurate methods like Sanger sequencing in past studies. To address this, we have meticulously confirmed each MYD88-negative case using at least three distinct methodologies and re-examined some negative samples after enhancing them with CD19 cell enrichment. This rigorous approach aims to test the hypothesis that the mutation rate of MYD88 among the Chinese cohort is indeed lower than observed in Western populations. Furthermore, our study compiles and analyses data on the clinical characteristics, genetic variances, and survival rates of MYD88-negative WM patients in China, offering a detailed insight into this specific patient group.

This comprehensive study encompasses a large cohort to delve into the incidence, clinical manifestations, and prognostic implications of MYD88 and CXCR4 mutations in Chinese WM patients. It offers a direct comparison of the efficiency across various detection methods and explore whether the prognostic significance of these mutations remains consistent across different therapeutic approaches, including non BTKi therapy and BTKi therapy.

Materials and Methods

2.1 Patients and samples collection

In our study, 418 patients diagnosed with WM according to the Second International Workshop on WM criteria(18) were admitted from June 2013 to June 2023 at the Institute of Hematology & Blood Diseases Hospital. Of these, 33 patients did not require treatment during the follow-up period and were consequently excluded from the study. Thus, a total of 385 symptomatic WM patients were included in the final analysis. All participants in our study underwent comprehensive bone marrow evaluations, including immunophenotyping and biopsy. These examinations revealed infiltration of the bone marrow by monoclonal B cells and plasma cells in all patients. Comprehensive clinical and biological data were collected for each patient at time of diagnosis. Comprehensive clinical and biological data were collected at

diagnosis. Of these, 336 patients were available for analysis of treatment response and outcome. They were categorized into three groups based on their first-line therapy: Cytotoxic therapy group, Rituximab/bortezomib-based therapy group and burton's tyrosine kinase inhibitor (BTKi) therapy group. More details on these treatment regimens were in Supplementary Table 1.

Tumor cell samples were prospectively collected from unsorted specimens prior to the initiation of any therapeutic interventions. The collection encompassed bone marrow (BM, in 356 cases), peripheral blood (PB, in 22 cases), and lymph nodes (LN, in 7 cases). The processes for sample collection, storage, and nucleic acid extraction are detailed in a previous publication(14). Due to the potential significant underestimation of tumor burden by the multiparameter flow cytometry (FCM) method, we utilized immunohistochemistry (IHC) from biopsies to estimate the tumor load. Since precise quantification is not feasible with IHC, we have categorized the tumor burden into four groups: 0.5-10%, 10-30%, 30-50%, and greater than 50%.

To be classified as MYD88 wild-type ($MYD88^{WT}$), each sample underwent at least three different detection methods, including Sanger sequencing, NGS, ddPCR, and AS-PCR. Given the absence of a universally acknowledged 'gold standard' for MYD88 L265P mutation detection, we considered a positive result from any detection method as sufficient evidence to classify a patient as MYD88 mutation-positive ($MYD88^{MT}$). Given that the tumor burdens in most $MYD88^{WT}$ patients were relatively high, we supposed that CD19 sorting might deplete the sample material significantly while offering minimal improvement to the detection results. As such, we opted not to re-test all MYD88-negative samples post-sorting. Instead, we strategically selected and re-tested eight patients who were not only MYD88-negative but also had a notably low tumor infiltration rate of less than 5%. This selective approach allowed us to maximize the use of our limited sample material and enhance the reliability and specificity of our detection methods for MYD88 mutations.

All patient samples were obtained after written informed consent, in accordance with the Declaration of Helsinki and approved by the Institute of Hematology and Blood Disease Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College Ethics Committee.

2.2 Efficacy and outcomes

Assessment of the treatment response in patients with measurable WM was based on the latest response criteria consensus from 11th International Workshop on Waldenström macroglobulinemia and NCCN guidelines (Version 2.2022)(19, 20). Overall survival (OS) was calculated from diagnosis to either the date

of death or the date of the last follow-up examination. Progression free survival (PFS) was calculated from diagnosis to either the date of first relapse, death or the date of the last follow-up examination. The response to BTKi was evaluated at a minimum of three months in BTKi therapy. The overall response rate (ORR) encompassed the rates of minor response (MR), partial response (PR), very good partial response (VGPR), and complete response (CR). The deep response rate was defined as the combined rate of CR and VGPR.

2.3 Detection of the MYD88 and CXCR4 mutation by Sanger sequencing, AS-PCR, ddPCR, and NGS

MYD88 mutations were identified by Sanger sequencing, AS-PCR, ddPCR, and NGS. CXCR4 mutations were identified by Sanger sequencing, AS-PCR, and NGS. The details of the methods were seen in Supplementary Martials.

2.4 Statistical analysis

The association between two categorical variables was assessed using Pearson's chi-square test and Fisher's exact test. The comparison of continuous variables among two groups of patients was evaluated using Student's t-test. Survival curves were constructed using the Kaplan-Meier method, and differences were estimated through the log-rank test. Multivariate Cox regression analysis was used to assess the independent prognostic impact. Sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) were calculated. The concordance between different assays was investigated using the kappa (κ) test, with agreement deemed almost perfect between 0.8 and 1. A *P*-value less than 0.05 was considered statistically significant. All the statistical analyses were performed using IBM SPSS (RRID:SCR_002865); GraphPad Prism (RRID:SCR_002798) and/or R package version 3.5.1.

Data Availability Statement

Data is available from the corresponding author on reasonable request. The original data (bam files) have been deposited in the Genome Sequence Archive of the National Genomics Data Center from China and are accessible under BioProject ID PRJCA028747 (accessible at https://ngdc.cncb.ac.cn/bioproject/browse/PRJCA028747) and Accession ID HRA008237 (accessible at https://bigd.big.ac.cn/gsa-human/browse/HRA008237).

Results

1. Mutation rate and test performance of different methods for MYD88 and CXCR4

The cohort comprised 385 WM patients with a median age of 62 years old, ranging from 20 to 86 years old. Clonal immunoglobulin M protein was detected all patients. The median serum immunoglobulin M level was 3430 mg/dL at diagnosis.

All the patients underwent assessment for MYD88 mutation. We performed AS-PCR in 322 patients, NGS in 246 patients, Sanger sequencing in 233 patients and ddPCR in 214 patients. To enhance the sensitivity of MYD88 mutations detection, we simultaneously performed at least three testing methods in each $MYD88^{WT}$ samples. We defined a sample as $MYD88^{WT}$ only if all the assays yielded negative results. If any one of the testing methods produced a positive result, the sample was defined as $MYD88^{MT}$ sample. In total, MYD88 mutation was observed in 338 (87.8%) of the 385 patients. The patients' clinical characteristics and treatment choices are detailed in Supplementary Table 2. The mutation detection rates were 87.6% (312/356) in BM samples and 90.9% (20/22) in PB samples. Only two mutations of those mutations were situated at a non-L265P site identified by NGS. Examining the mutation rate revealed by each method, Sanger sequencing detected a rate of 61.4%, NGS of 74.4%, AS-PCR of 84.2%, and ddPCR of 79.0%, respectively (Supplementary Table 3). The detection rate of MYD88 using ddPCR was slightly lower than that of AS-PCR, but the difference was not statistically significant. This difference does not imply that ddPCR is less sensitive than AS-PCR; there was selection bias in the samples tested by the two methodologies. Therefore, we separately analyzed 196 patients who underwent MYD88 testing with both ddPCR and AS-PCR. The positive detection rates for ddPCR and AS-PCR were 78.6% (154/196) and 77.6% (152/196), respectively, showing similar detection rates for both methods.

We evaluated the test performance of the four methods in detecting MYD88 mutations (Supplementary Table 3). AS-PCR and dd-PCR exhibited the highest sensitivity (98.5%, 95% CI, 96.1-99.5 and 97.7%, 95% CI, 93.8-99.3) and NPV (91.7%, 95% CI, 79.1-97.3, and 91.1%, 95% CI, 77.9-97.1). AS-PCR and dd-PCR also demonstrated the lowest false-negative rate (1.5% and 2.3%). However, Sanger sequencing and NGS failed to detect MYD88 mutation effectively in patients with low tumor load, and exhibited a high false-negative rate in patients with tumor burden less than 10% (55.6% and 31.3%, respectively, Figure 1). Whereas, there was no significant difference in the MYD88 mutation rate detected by AS-PCR and ddPCR among patients with varying tumor infiltration fraction (P=0.149 and 0.316, respectively, Supplementary Figure 1). The eight MYD88-negative samples mentioned in the Methods section were retested using ddPCR after CD19 magnetic bead sorting, and the results remained negative. Subsequently, we analyzed

the concordance among the four methods, the highest concordance was found between ddPCR and AS-PCR (Kappa 0.911) and the lowest concordance was found between Sanger sequencing and ddPCR (Kappa 0.200, Supplementary Table 4).

Due to the significant limitations of AS-PCR and Sanger sequencing in detecting CXCR4 mutations, especially since AS-PCR cannot detect non-hotspot or frameshift mutations, we did not include patients who were tested for CXCR4 using only one method (either AS-PCR or Sanger sequencing). As a result, our study included a total of 362 patients conducting CXCR4 mutation testing. All included patients were either tested with both AS-PCR and Sanger sequencing (n=273), both AS-PCR and NGS (n=202), or solely with NGS (n=44), as illustrated in Supplementary Table 5. The observed overall mutation rate was 30.9%. Examining each method individually, NGS demonstrated the highest detection rate of CXCR4 mutation among the three methods, with a mutation positive detection rate reaching up to 26.0%, following by AS-PCR with a mutation rate of 18.2%, and Sanger sequencing of 12.8%. Due to the limitation of AS-PCR in detecting only point mutations and the low sensitivity of Sanger sequencing, we adopted a joint testing approach by combining both methods. A sample was defined as positive if either method detected a mutation and negative if both methods were negative. This combined approach increased the positive detection rate to 26.7%. Among the methods, NGS exhibited the highest sensitivity at 78.0% (95% CI, 67.3-86.1), followed by AS-PCR at 58.6% (95% CI, 48.2-68.3) and Sanger sequencing at 41.2% (95% CI, 30.8-52.4, Supplementary Table 6). The AS-PCR and Sanger sequencing combined approach reached a sensitivity of 85.9%, showcasing its feasibility as an alternative to NGS. Focusing solely on the two hotspot S338X mutations detectable by AS-PCR, we observed that AS-PCR outperformed NGS in sensitivity for these specific mutations, registering at 93.4% compared to NGS's 62.5%. Nonetheless, AS-PCR's capability was confined to identifying hotspot point mutations. The mutation detection results for CXCR4 via the three methods lacked uniformity, with concordance among the methods measuring below 0.5 (Supplementary Table 7). Additionally, Supplementary Table 5 presents the detail deletion results for MYD88 and CXCR4 across the entire cohort using different methods.

2. The clinical and genetic characteristics of MYD88 mutated and wild-type patients

Out of the 385 WM patients examined, 47 (12.2%) were categorized as $MYD88^{WT}$ patients. $MYD88^{WT}$ patients exhibited significantly lower proportion of males (59.6% vs. 74.0%, *P*=0.039) and lymphadenopathy (25.0% vs. 43.4%, *P*=0.035) and higher proportion of hepatomegaly (33.3% vs. 19.3%,

P=0.037) and splenomegaly (64.3% vs. 47.6%, *P*=0.043). Additionally, $MYD88^{WT}$ patients showed a notably higher proportion of elevated lactic dehydrogenase (LDH) (28.3% vs. 11.8%, *P*=0.003, Table 1).

Subsequently, we identified the differences in IGHV mutation status and VDJ rearrangements between $MYD88^{MT}$ and $MYD88^{WT}$ patients. Productive IGHV-D-J rearrangements were obtained and analyzed in 260 WM patients. The majority of WM cases (94.6%) were found to carry mutated IGHV genes as determined by a IGHV germline homology cutoff of 98%. The proportion of mutated IGHV was comparable between $MYD88^{MT}$ and $MYD88^{WT}$ patients (95.9% vs. 85.2%, *P*=0.055). However, there was a notable difference in the distribution of the IGHV mutation percentage between the two groups (Figure 2A). We observed that IGHV3-30, IGHD3-22 and IGHJ3 were significantly over-represented, and IGHV3-23 and IGHJ4 significantly under-represented in the $MYD88^{WT}$ group compared to the mutated group (*P*<0.05, Figure 2B-D). Concurrently, CDR3 length was significantly longer in $MYD88^{WT}$ patients (median 19 vs. 14, *P*=0.006, Figure 2E). The detailed IGHV-D-J repertoire of the two groups were shown in Supplementary Figure 2.

The cytogenetic features were assessed through FISH and G-banded metaphase karyotypes. No significant difference in cytogenetic features was observed between $MYD88^{MT}$ and $MYD88^{WT}$ patients. Complex karyotype was significantly more common detected in $MYD88^{WT}$ patients than $MYD88^{MT}$ patients (18.4% vs. 7.3%, *P*=0.047, Table 1).

3. The clinical and genetic characteristics of CXCR4 mutated and wild-type patients

Given the individual sensitivities of AS-PCR and Sanger sequencing are relatively low, we employed a combination of AS-PCR and Sanger sequencing as a unified detection strategy. We tested a total of 362 cases for CXCR4 mutations, identifying 114 mutations in 112 cases, representing a mutation rate of 30.9%. The majority of CXCR4 mutations were truncated mutations (112 out of 114, 98.2%), located in the regulatory C-terminal domain that extends spanning amino acids 313 to 345 (Supplementary Figure 3A). Nonsense and frameshift mutations accounted for 64.0% and 33.3% respectively (Supplementary Figure 3B). The most prevalent alteration was the amino acid change S338X as nonsense mutation at the nucleotide position 1013, comprising 64 of the 114 mutations (56.1%). Regarding the nucleotide changes contributing to this alteration, C > G was observed in 65.6% of cases, and C > A in 34.3%. The second most commonly affected site was at amino acid 341 and 318, accounting for 6.1% and 5.3% of the mutations respectively

(Supplementary Figure 3C). This analysis highlights the diversity and prevalence of CXCR4 mutations in the examined patient population.

Patients with CXCR4 mutations demonstrated distinctive clinical characteristics compared to wild-type patients (Table 1). Notably, the mutated group exhibited a significantly higher proportion of patients older than 65 years (42.9% vs. 31.6%, P=0.038), and higher proportion of anemia (83.9% vs. 73.6%, P=0.035) and thrombocytopenia (37.4% vs. 19.6%, P<0.001). Additionally, these patients displayed an elevated tumor burden in the bone marrow, as evidenced by a median of 12.2% versus 6.8% determined by FCM (P<0.001). The CXCR4-mutated group had a significantly higher proportion of patients with serum IgM levels exceeding 40g/L (46.4% vs. 34.8%, P=0.035) and serum β2-MG more than 3 mg/L (74.5% vs. 61.3%, P=0.019). Consequently, there was a notably lower percentage of patients in CXCR4 mutated group classified as International Prognostic Scoring System (IPSSWM) of low-risk (18.4% vs. 31.2%, P=0.016). In contrast, patients with wild-type CXCR4 were more prone to develop lymphadenopathy (45.7% vs. 25.6%, P=0.001).

IGHV mutation status and gene repertoire were also analyzed between CXCR4 wild-type and mutated groups. We observed that IGHD3-22 were significantly over-represented and IGHD3-10 were significantly low-represented in the CXCR4 wild-type group compared to the mutated group (P=0.012, P=0.008). Besides, we observed no significant difference in the distribution of IGHV hypermutation rate, IGHVJ gene repertoire, and CDR3 length between the CXCR4 wild-type and mutated groups (Supplementary Figure 4-5).

We observed a significantly higher proportion of patients exhibiting abnormal karyotypes in the CXCR4 mutation group compared to the wild-type group (39.4% vs. 21.1%, P=0.001, Table 1). Specifically, the mutation group displayed a significantly elevated incidence of abnormal karyotypes with -Y (6.5% vs. 1.0%, P=0.018) and del 7 (5.4% vs. 0%, P=0.004) in comparison to the CXCR4 wild-type group. This comprehensive analysis elucidates the varied clinical and genetic profiles between patients with CXCR4 mutations and those with wild-type CXCR4.

4. Impact of MYD88/CXCR4 mutations on treatment outcomes

We initiated our analysis by examining the response rate and survival outcomes of patients based on their MYD88 and CXCR4 mutation status across different therapeutic approaches. Among the 336 patients who received systemic treatment, 172 patients (51.1%) received Rituximab/Bortezomib-based therapy and 104 patients (31.0%) received BTKi-based regimens (Supplementary Table 1). There was no obvious difference

in the option of treatment regimens among patients with different MYD88 and CXCR4 mutation status (Table 1). With a median follow-up of 33.3 months, the median PFS for the entire cohort was 50.0 months (95% CI: 38.3–61.7) and the median OS was not reached. The 5-year overall survival rate was 80.1% (Supplementary Figure 6A-B).

Overall, there were no significant differences observed in either PFS or OS between $MYD88^{WT}$ and $MYD88^{MT}$ groups (*P*=0.112 and 0.451, respectively) (Figure 3A-B). Then, we assessed the prognostic value of MYD88 mutation across various treatment regimens (Figure 3C-H). Across the non-BTKi therapy groups, patients with $MYD88^{WT}$ exhibited similar ORR compared to those with MYD88 mutations in first-line therapy (Supplementary Table 8-9). Notably, $MYD88^{WT}$ patients exhibited significantly lower ORR compared to those with MYD88 mutations under BTKi-based therapy (44.4% vs. 83.8%, *P*=0.019, Supplementary Table 10). Interestingly, patients treated with non BTKi therapy displayed worse PFS and OS in the $MYD88^{WT}$ group compared to the mutated group (median PFS: 34.3 months vs. 60.7 months, *P*=0.005; median OS: 62.9 months vs. not reached, *P*=0.011; Figure 3E-F). Moreover, the $MYD88^{WT}$ group exhibited inferior PFS as compared to the mutated group under BTKi-based therapy (median 17.8 months vs. 34.0 months, *P*=0.014), but no difference in OS was observed (Figure 3G-H).

Notably, CXCR4 mutated group manifested significantly worse PFS and OS compared to the wild-type group (median PFS: 38.5 months vs. 61.6 months, *P*=0.032; median OS: 103.8 months vs. not reached, *P*=0.048) (Figure 4A-B). We then conducted a detailed analysis on response rates and survival outcomes based on CXCR4 mutation status across different therapeutic options in first-line therapy. We did not observe significant difference in treatment response between CXCR4 wild-type and mutated patients across varied treatment strategies (Supplementary Table 8-10). CXCR4 wild-type and mutated patients exhibited analogous PFS and OS within the cytotoxic therapy group (Figure 4C-D). However, under Rituximab/Bortezomib-based therapy, the CXCR4 mutation group demonstrated diminished PFS compared to the wild-type group (median 40.5 months vs. 58.4 months, *P*=0.039) (Figure 4E-F). Likewise, under BTKi-based regimens, the CXCR4 mutated group exhibited significantly inferior survival outcomes compared to the wild-type group (Figure 4G-H).

We further explored the outcomes of patients with different CXCR4 mutation types, amino acid sites, and cancer cell fraction (CCF) (Supplementary Figure 7). Our results indicated that neither types of mutation nor amino acid sites of CXCR4 mutation significantly influenced outcomes. Although a trend was observable suggesting that patients with higher CXCR4 clonality (CCF > 50%) experienced inferior PFS and OS

outcomes compared to those with lower CXCR4 clonality (CCF < 50%), the impact of varying CCF did not reach statistical significance (PFS: *P*=0.741; OS: *P*=0.521; Supplementary Figure 7E-F). This is likely due to the small number of patients with subclonal CXCR4 mutations. A larger sample size is warranted for more conclusive validation.

To sum up, both MYD88 mutation and CXCR4 mutation had a prognostic impact on PFS in patients undergoing BTKi-based therapy, but only CXCR4 mutation exerted significant impact on OS. When MYD88 and CXCR4 mutations were combined in the survival curve, we did not observe any prognostic significance of MYD88 and CXCR4 mutation in patients treated with cytotoxic therapy (Supplementary Figure 8A-B). CXCR4 mutation status emerged as an essential factor distinguishing outcomes in patients with Rituximab/Bortezomib-based therapy, while $MYD88^{WT}$ patients and those with both MYD88 and CXCR4 mutations exhibited comparably poor prognoses (Supplementary Figure 8C-D). Notably, in the era of BTKi treatment, both MYD88 and CXCR4 mutations were significant determinants of patients' outcomes. If we categorized the patients into three groups, those with MYD88 mutations but without CXCR4 mutations had the most favorable prognosis no matter in PFS or OS (Supplementary Figure 8E-F). It's worth noting that conducting multiple hypothesis testing could increase the risk of statistical errors. Consequently, we also applied the Bonferroni correction to the *P*-values obtained from our survival analysis. This adjustment revealed that the presence of MYD88 mutations continued to show prognostic relevance for PFS in the Rituximab/Bortezomib-based group. However, OS ceased to maintain statistical significance after the application of multi-hypothesis correction. Furthermore, following the Bonferroni adjustment, the prognostic value of CXCR4 mutations for OS no longer held statistical significance across any of the subgroups. This may be attributed to the wide array of treatment options available to WM patients following the failure of first-line therapies, including the opportunity to participate in diverse clinical trials. The advent of new therapeutic agents has markedly enhanced the survival prospects for these patients. Further validation of our findings will require more uniform treatment cohorts or clinical trial cohorts.

5. Prognostic prediction under different treatment backgrounds

IPSSWM system was developed and validated in cohorts treated with chemotherapy or immunotherapy (Supplementary Figure 9A). However, we found IPSSWM staging system could not stratify the survival in patients treated with BTKi therapy (*P*=0.498, Supplementary Figure 9B). To integrate genetic mutations with clinical prognostic index, we incorporated the IPSS staging along with MYD88 and CXCR4 mutations for multivariate analysis of OS. The results elucidated that, across the entire cohort, CXCR4 mutation served

as an independent adverse prognostic factor irrespective of IPSSWM staging, while MYD88 mutation did not exhibit significance in the multivariate analysis (Table 2). Subsequently, we segregated patients into BTKi treatment group and non-BTKi treatment group. We found that in the BTKi treatment group, IPSSWM classification did not hold prognostic significance in univariate and multivariate analysis (Supplementary Figure 9B; Table 2). MYD88 and CXCR4 mutation serving as an independent adverse prognostic factor for OS (MYD88: HR=0.229, 95%CI 0.061-0.865, *P*=0.030; CXCR4: HR=3.349, 95%CI 1.302-8.612, *P*=0.012). In contrast, in the non-BTKi treatment group, IPSSWM was an independent adverse prognostic factor (HR=1.596, *P*=0.003). Upon considering IPSSWM classification, both CXCR4 and MYD88 mutations relinquished their prognostic significance in the non-BTKi treatment group (Table 2). Hence, prognostic factors varied under different treatment backgrounds. In the era of BTKi-based therapy, the detection of gene mutations has assumed an increasingly pivotal role in prognosis prediction.

Discussion

MYD88 and CXCR4 mutations have been identified as highly recurrent somatic mutations in WM by whole genome sequencing(2, 3). The direct comparison of the efficacy of different detection methods and the mutation rates in Chinese WM patients remain unclear. Moreover, the clinical implications of MYD88 and CXCR4 mutations in the eras of immunochemotherapy and targeted therapy warrant further exploration. This study represents the largest integrative studies on the incidence, clinical characteristics, and prognostic significance of MYD88 and CXCR4 mutations. Additionally, it conducts subgroup analyses of survival data across different treatment regimens, shedding light on the real-world clinical significance of detecting MYD88 and CXCR4 mutations.

Previous research has highlighted the high sensitivity of CD19-selected bone marrow with AS-PCR for detecting MYD88 mutations(7). However, the practicality of sorting B-cells for this testing is limited in routine clinical settings. To ensure the accuracy of MYD88 testing on unsorted samples, we employed a three-pronged approach. First, for patients identified as MYD88 negative, confirmation was sought using at least three distinct tests, with a consensus of negativity required across all tests. Second, we stratified patients based on tumor cell proportions, enabling us to compare mutation detection rates across different tumor loads and evaluate the influence of tumor cell purity on detection rates. Third, we performed CD19 magnetic bead sorting on eight $MYD88^{WT}$ samples characterized by low tumor loads. After enriching for tumor cells, we retested for MYD88 mutations using ddPCR to confirm their negative status. Through these comprehensive detection methods, we determined that the MYD88 mutation rate in Chinese WM patients

was 87.8% (338/385), which is notably lower than the 93.5% observed in a European WM cohort using comparable methods on unsorted samples (chi-square test *P*=0.003)(21). The distinction between East and West in WM extends beyond MYD88 mutation frequencies. Research has reported that age-standardized incidence rates in Asians are lower than those reported in the United States and Europe(12). Retrospective studies indicate that Asian populations present with WM at a younger age, exhibit a higher prevalence of anemia, and contain a larger proportion of IPSSWM high-risk patients(22). Furthermore, our previous research has identified differences in the usage of IGHV segments between Chinese and Western WM patients, with a higher usage of V4 segments(14). These differences may be attributed to distinct microbiological and genetic backgrounds between Eastern and Western populations, possibly exposing them to different antigenic stimuli spectra. Further research is needed to elucidate these underlying mechanisms. However, the study does have certain limitations. Although we have used multiple methods to minimize the impact of unsorted samples on detection accuracy, the most direct and reliable approach would be to sort all samples and retest MYD88 using multiple methods. Comparing these results with those from unsorted samples would directly demonstrate whether sorting affects the detection results and further validate the conclusion that the MYD88 mutation detection rate is lower in China. On the other hand, to prove this rigorously, additional studies on other Chinese cohorts using the same methodology are needed.

Our study found that ddPCR and AS-PCR are highly sensitive for detecting MYD88 mutations in unsorted samples, outperforming NGS and Sanger sequencing(23). This supports the use of ddPCR and AS-PCR for routine clinical testing. However, it's important to note that 92.4% of our samples were from bone marrow, and peripheral blood was tested only in cases with high white blood cell counts. Our study didn't include samples with extremely low tumor loads (<0.5%), so our findings don't apply to such cases. While AS-PCR is cost-effective, it's semi-quantitative and requires standard reference curves and strict standardization, which may limit its use. The variation in results between studies could be due to the small patient cohort and differing experimental conditions(8). Therefore, ddPCR, on the other hand, offers a more stable and quantitative approach for MYD88 mutation detection. Despite their lower sensitivity for MYD88 mutations, NGS and Sanger sequencing can detect non-L265P MYD88 mutations and CXCR4 mutations, which is a significant advantage. Even though the detection rate of non-L265P MYD88 mutations in our study was lower than in some other studies(3), this capability should not be underestimated.

CXCR4 mutations are essentially unique to WM and can influence disease presentation and therapeutic responses, particularly to classic BTKi treatment(5). Unlike the MYD88 mutation, individuals can have

various CXCR4 mutations, with differences in type, location, and clonality. Prior research indicated that the subtype of CXCR4 mutation (nonsense or frameshift) influences treatment response and survival outcomes. Additionally, the allelic burden of CXCR4 has been found to impact the response to Ibrutinib and PFS. A retrospective analysis showed that a clonality of ≥25% was linked to lower response rates and worse PFS than clonality <25%(24). In contrast, our study did not find any prognostic significance in the type or clonality of CXCR4 mutations. Considering the limited sensitivity of Sanger sequencing and the restrictive capability of AS-PCR in detecting only hotspot mutations, our results endorse NGS as the premier method for identifying CXCR4 mutations. The integrative strategy of combining Sanger sequencing with AS-PCR could offer a viable alternative for CXCR4 mutation detection in scenarios where NGS is not accessible.

Patients with $MYD88^{WT}$ genotype are rare due to the low prevalence of this genotype of WM. Given that patients with wild-type MYD88 have a lower response rate to BTKi, in our clinical strategy selection, we tend to opt immunotherapy or combination immunotherapy treatments for patients with wild-type MYD88, such as the BR regimen, and less frequently use BTKi monotherapy. Following the adaptation of treatment strategies based on MYD88 mutation status, we observed that the overall survival rates of patients with the wild-type MYD88 closely aligned with those harboring MYD88 mutations (Figure 3A-B). A comparison of the clinical characteristics between patients with wild-type and mutant MYD88 revealed that $MYD88^{WT}$ patients had higher LDH levels and a higher proportion of complex karyotypes, which may suggest a slightly poorer response to non-BTKi therapy compared to $MYD88^{MT}$ patients. However, the limited number of patients with $MYD88^{WT}$, further reduced after stratification into treatment groups, along with variability in follow-up duration and the generally short follow-up periods across these groups, necessitates a cautious interpretation of the survival curves. To definitively determine the prognostic value of MYD88 mutations across various treatment modalities, larger-scale prospective studies with extended follow-up periods are essential for a more thorough elucidation.

Moreover, patients with CXCR4 mutations typically have higher IgM levels and lower platelet counts, which are also adverse indicators. We found patients with CXCR4 mutations had significant worse outcome than wild-type patients, especially in Rituximab/Bortezomib and BTKi treated group. While many studies have explored how MYD88 and CXCR4 mutations affect response to BTKi treatment and survival prognosis in patients with WM, few have combined these mutations with clinical prognostic factors in a multivariate analysis. This leads to the question of whether MYD88 and CXCR4 mutations are independent prognostic factors separate from these clinical features. To investigate, we included MYD88 mutations,

CXCR4 mutations, and IPSSWM staging in our analysis. We found that IPSSWM and CXCR4 mutations were independent adverse prognostic factors for survival (Table 2). Adding CXCR4 mutation testing to IPSSWM stratification improves prognosis prediction. However, MYD88 mutations didn't show prognostic significance in our multivariate model. Considering the known lesser response of patients with wild-type MYD88 and mutated CXCR4 to BTKi therapy, we divided patients into two groups: those undergoing BTKi therapy and those receiving traditional non-BTKi (cytotoxic drugs and Rituximab/Bortezomib) treatment. We discovered that in the non-BTKi group, the CXCR4 mutation didn't significantly impact survival after adjusting for IPSSWM in the analysis. This suggests that in patients with non-BTKi therapy, the prognostic value of MYD88 and CXCR4 mutations is not crucial, and the need for additional mutation testing beyond IPSSWM stratification is debatable. However, in the BTKi treatment era, the effects of MYD88 and CXCR4 mutations on survival are independent of clinical IPSSWM stratification. Therefore, testing for these mutations is crucial for determining prognosis in patients treated with BTKi therapy.

Our study concludes that both AS-PCR and ddPCR are highly effective for detecting MYD88 mutations in unsorted samples, providing high sensitivity suitable for routine clinical use. For CXCR4 mutations, NGS showed the highest sensitivity. In the context of non-BTKi treatments, neither MYD88 nor CXCR4 mutations emerged as independent prognostic factors when considering the IPSSWM clinical staging. However, in the era of BTKi treatment, the prognostic relevance of both MYD88 and CXCR4 mutations becomes significant, independent of the IPSSWM score. This indicates that testing for these mutations is crucial for predicting survival outcomes in patients receiving BTKi therapy. Our findings highlight the importance of understanding how disease risk factors can vary depending on the treatment context.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author Contributions

SHY conceptualized the study design. YTY, YY, WJX and JW analyzed the data, performed statistical analyses, and wrote the manuscript. YTY, WX, YY, YJJ, YSH, YXL, TYW, RL, HS, HXW, QW, WL, GA, WWS, YX, WYH, ZY, and DHZ acquired the data and managed the patients. MH, ZJX, JXW, LGQ and SHY revised the manuscript critically and approved the final version.

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Figure legends

Figure 1. The false negative rates of MYD88 testing using Sanger sequencing **(A)** and next-generation sequencing (NGS) **(B)** in patients with different tumor burdens. Tumor burden is estimated by immunohistochemistry of bone marrow biopsies. **P*<0.05; ***P*<0.01.

Figure 2. The IGHV-D-J gene repertoire of patients with mutated and wild-type MYD88. (**A**) Distribution of patients with different identity to germline IGHV gene; (**B**) Distribution of major VH family gene segments; (**C**) Distribution of major DH family gene segments; (**D**) Distribution of major JH family gene segments; **(E)** Distribution of CDR3 length. **P*<0.05.

Figure 3. The survival outcomes in patients with mutated and wild-type MYD88 according to the option of different first-line therapies. The progression free survival and overall survival in the whole cohort (**A-B**); in patients with cytotoxic therapy (**C-D**); in patients with Rituximab/Bortezomib-based therapy (**E-F**); and in patients with BTKi-based therapy (**G-H**).

Figure 4. The survival outcomes in patients with mutated and wild-type CXCR4 according to the option of different first-line therapies. The progression free survival and overall survival in the whole cohort (**A-B**); in patients with cytotoxic therapy (**C-D**); in patients with Rituximab/Bortezomib-based therapy (**E-F**); and in patients with BTKi therapy (**G-H**).

Table 1. The clinical and genetic characteristics of MYD88 mutated and wild-type patients

Characteristic	$MYD88^{WT}$ (N=47)	$MYD88^{MT}$ (N=338)	*P*	$CXCR4^{WT}$ (N=250)	$CXCR4^{MT}$ (N=112)	*P*
Age, ≥65 years, n (%)	16 (34.0)	121 (35.8)	0.814	79 (31.6)	48 (42.9)	0.038
Gender, male, n (%)	28 (59.6)	250 (74.0)	0.039	181 (72.4)	79 (70.5)	0.716
Laboratory parameters, n (%)						
Serum IgM>40 g/L,	16 (34.0)	130 (38.5)	0.559	87 (34.8)	52 (46.4)	0.035
HGB ≤11 g/dL	40 (83.1)	257 (76.0)	0.165	184 (73.6)	94 (83.9)	0.031
PLT≤100x10^9/L	14 (29.8)	92 (27.2)	0.712	49 (19.6)	52 (37.4)	0.000
Serum albumin<35g/L	22 (47.8)	166 (51.9)	0.607	127 (52.9)	47 (44.8)	0.163
Serum β2-MG>3 mg/L	33 (78.6)	205 (70.9)	0.304	164 (74.5)	57 (61.3)	0.019
LDH≥250U/L	13 (28.3)	37 (11.8)	0.003	37 (15.6)	11 (10.7)	0.230
Malignant cells fraction in BM detected by FCM, median (range)	11.3 (0.50-71.5)	9.4 (0.51-90.2)	0.886	6.8 (0.50-84.5)	12.2 (0.51-85.3)	0.000
Clinical manifestations, n (%)						
Lymphadenopathy	9 (25.0)	112 (43.4)	0.035	86 (45.7)	23 (25.6)	0.001
Splenomegaly	27 (64.3)	141 (47.6)	0.043	108 (49.5)	51 (50.0)	0.939
Hepatomegaly	14 (33.3)	57 (19.3)	0.037	48 (22.3)	20 (19.4)	0.554
IPSS score, n (%)						
Low risk	9 (20.9)	86 (27.4)	0.369	72 (31.2)	19 (18.4)	0.016
Intermediate risk	17 (39.5)	106 (33.8)	0.455	76 (32.9)	38 (36.9)	0.477
High risk	17 (39.5)	122 (38.9)	0.932	83 (35.9)	46 (44.7)	0.130
FISH examination, n (%)						
Del (11q22-23)	0	2 (1.0)	1.000	2 (1.4)	0	0.853
Del (13q14)	0	5 (2.8)	0.991	2 (1.6)	2 (3.4)	0.783
Trisomy 12	0	3 (3.0)	1.000	1 (1.3)	2 (6.1)	0.458
Del (17p13)	0	16 (5.4)	0.318	12 (5.7)	2 (2.0)	0.241
Del (6q23)	1 (9.1)	19 (32.8)	0.221	15 (32.6)	5 (21.7)	0.348
G-banded metaphase karyotypes, n (%)						
Normal karyotype	30 (78.9)	209 (73.1)	0.415	165 (78.9)	57 (60.6)	0.001
Complex karyotype	7 (18.4)	21 (7.3)	0.047	18 (8.6)	7 (7.5)	0.761
Treatment regimen, n (%)						
Cytotoxic therapy	7 (17.5)	53 (17.9)	0.950	40 (18.3)	15 (15.0)	0.300
Rituximab/Bortezomib-based therapy	24 (60.0)	148 (50.0)	0.235	112 (51.4)	49 (49.0)	0.694
BTKi-based therapy	9 (22.5)	95 (32.1)	0.218	66 (30.3)	36 (36.0)	0.310

HGB, hemoglobin; PLT, platelet; β2-MG, β2 microglobulin; IgM, immunoglobulin M; BM, bone marrow; FCM, flow cytometry; LDH, lactate dehydrogenase; IPSS, international prognostic scoring system for WM; FISH, fluorescence in situ immunohybridization; BTKi, Bruton's tyrosine kinase inhibitor

Table 2. Univariate and multivariate Cox regression analysis for overall survival

The whole cohort (N=336)				
Variable	Univariate analysis		Multivariate analysis	
	HR (95%CI)	*P*	HR (95%CI)	*P*
IPSSWM	1.531 (1.112-2.107)	0.008	1.425 (1.006-2.018)	0.046
MYD88 mutation	0.788 (0.358-1.734)	0.554	0.481 (0.291-1.254)	0.176
CXCR4 mutation	1.795 (1.038-3.104)	0.036	1.830 (1.023-3.271)	0.042
BTKi-based therapy cohort (N=104)				
Variable	Univariate analysis		Multivariate analysis	
	HR (95%CI)	*P*	HR (95%CI)	*P*
IPSSWM	1.388 (0.780-2.468)	0.265	1.219 (0.686-2.164)	0.500
MYD88 mutation	0.312 (0.087-1.117)	0.073	0.229 (0.061-0.865)	0.030
CXCR4 mutation	3.099 (1.228-7.825)	0.017	3.349 (1.302-8.612)	0.012
Non-BTKi therapy cohort (N=232)				
Variable	Univariate analysis		Multivariate analysis	
	HR (95%CI)	*P*	HR (95%CI)	*P*
IPSSWM	1.596 (1.090-2.337)	0.016	1.596 (1.039-2.450)	0.003
MYD88 mutation	1.256 (0.491-3.213)	0.634	0.859 (0.321-2.229)	0.762
CXCR4 mutation	1.440 (0.689-3.014)	0.333	1.268 (0.578-2.450)	0.553

IPSSWM, international prognostic scoring system for WM

Figure 1

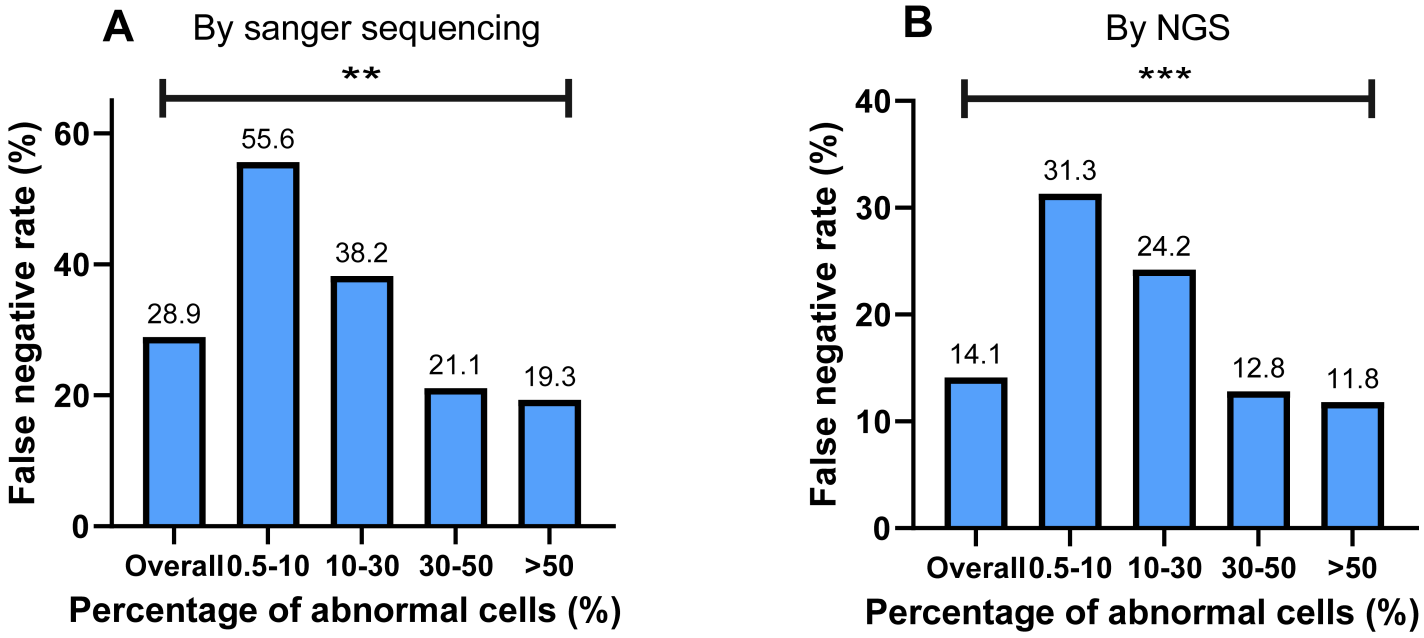

Figure 2

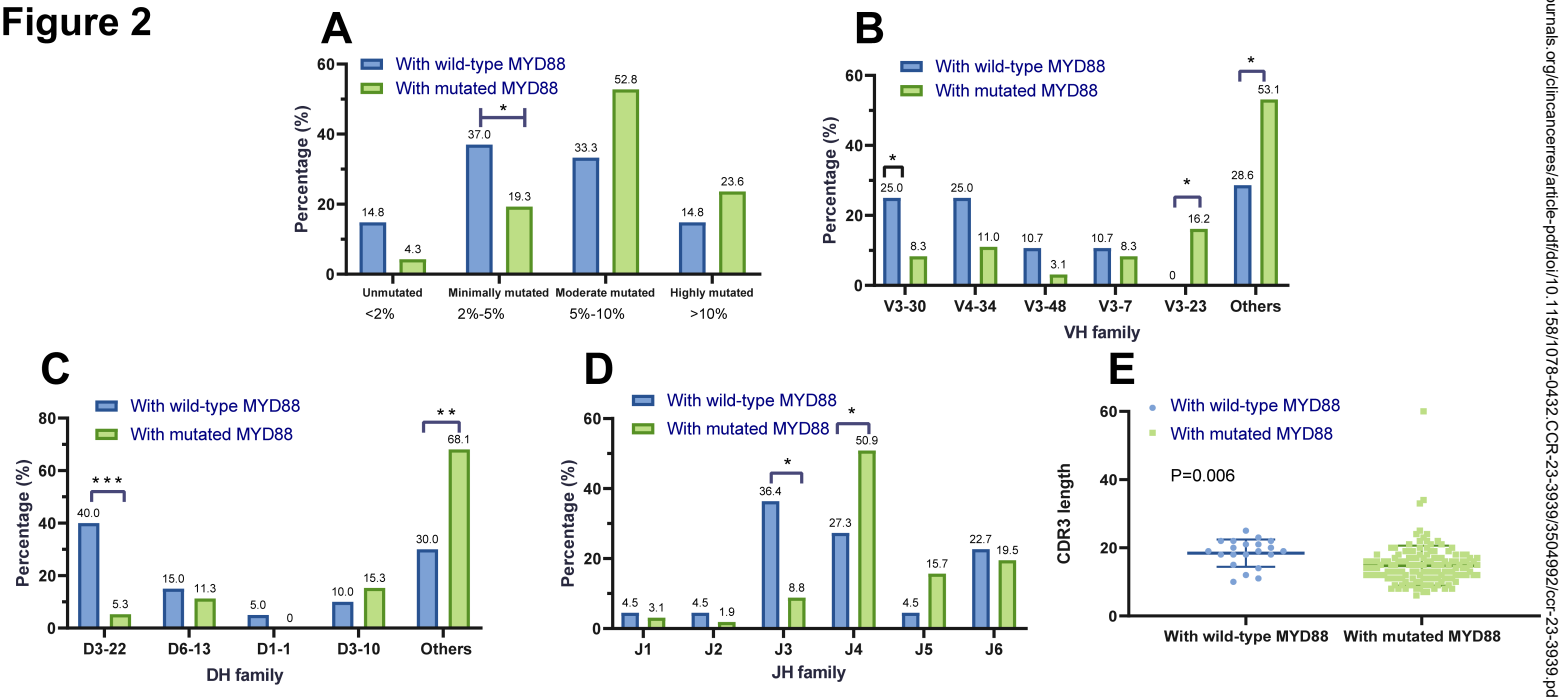

Figure 3

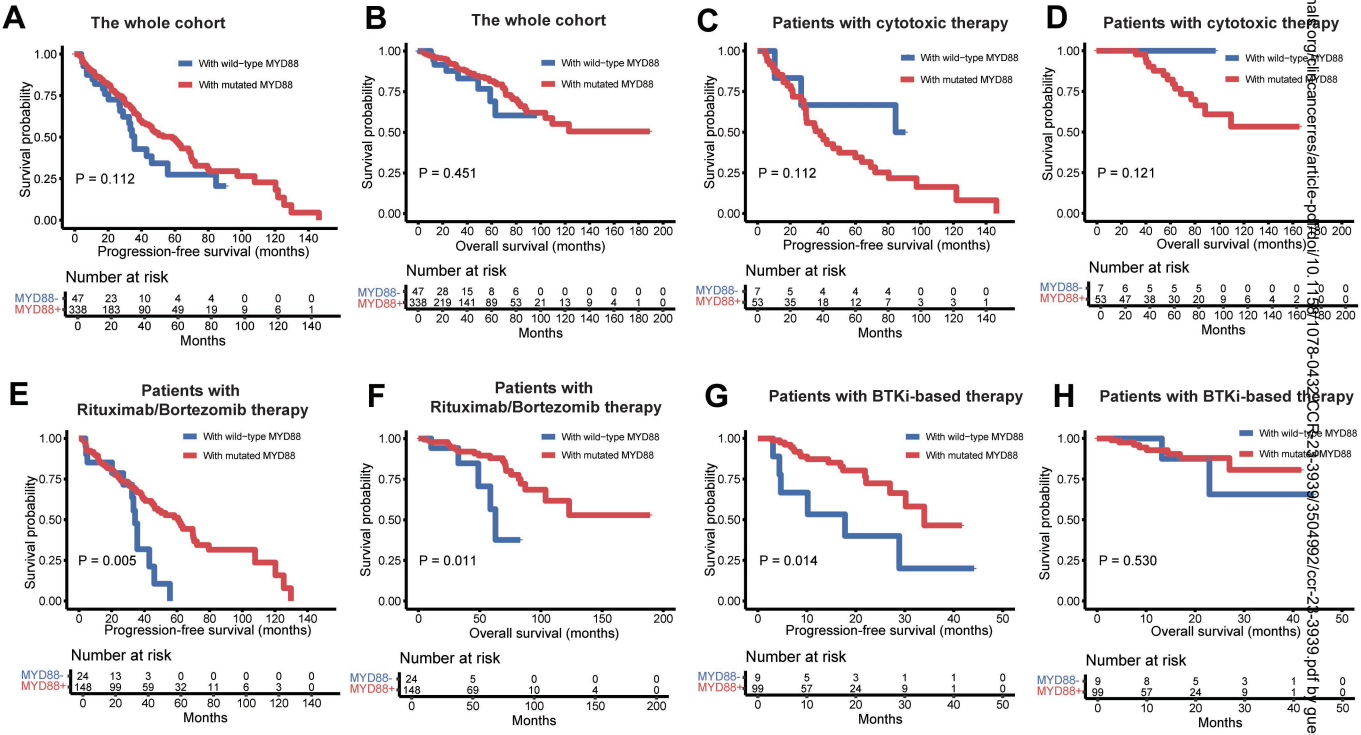

Figure 4