Identification of Copy Number Abnormalities and Inactivating Mutations in Two Negative Regulators of Nuclear Factor-κB Signaling Pathways in Waldenström’s Macroglobulinemia


Abstract

Waldenström’s macroglobulinemia (WM) is a distinct clinicobiological entity defined as a B-cell neoplasm characterized by a lymphoplasmacytoid infiltrate in bone marrow (BM) and IgM paraprotein production. Cytogenetic analyses were historically limited by difficulty in obtaining tumor metaphases, and the genetic basis of the disease remains poorly defined. Here, we performed a comprehensive analysis in 42 WM patients by using a high-resolution, array-based comparative genomic hybridization approach to unravel the genetic mechanisms associated with WM pathogenesis. Overall, 83% of cases have chromosomal abnormalities, with a median of three abnormalities per patient. Gain of 6q was the second most common abnormality (17%), and its presence was always concomitant with 6q loss. A minimal deleted region, including MIRN15A and MIRN16-1, was delineated on 13q14 in 10% of patients. Of interest, we reported biallelic deletions and/or inactivating mutations with uniparental disomy in tumor necrosis factor (TNF) receptor–associated factor 3 (TRAF3), two negative regulators of the nuclear factor-κB (NF-κB) signaling pathway. Furthermore, we confirmed the association between TRAF3 inactivation and increased transcriptional activity of NF-κB target genes. Mutational activation of the NF-κB pathway, which is normally activated by ligand receptor interactions within the BM microenvironment, highlights its biological importance, and suggests a therapeutic role for inhibitors of NF-κB pathway activation in the treatment of WM. [Cancer Res 2009;69(8):3579–88]

Introduction

Waldenström’s macroglobulinemia (WM) is an incurable low-grade B-cell lymphoproliferative disorder characterized by bone marrow (BM) infiltration of a clonal population of small B lymphocytes, plasmacytoid lymphocytes, and plasma cells that secrete monoclonal IgM antibody (1). The etiology of the disease is still unknown. Although believed to be predominantly sporadic, familial cases suggest a possible predisposing genetic defect (2, 3).

The genetic basis of the disease remains poorly defined. Few recurrent chromosomal abnormalities have been reported in WM, reflecting the difficulty in obtaining tumor metaphases for karyotype studies. Deletion of 6q is the most common abnormality, identified in approximately half of the patients when analyzed by fluorescence in situ hybridization (FISH; ref. 4). To a lesser extent, trisomy of chromosome 4 (5) and 13q14 and 17p13 deletions have been described. Deletions on 13q14 and 17p13 are mainly associated with advance disease (6). On the other hand, no high-resolution whole-genome approaches have been used in the study of WM.

Nuclear factor-κB (NF-κB) comprises a family of transcription factors that regulate the transcription of hundreds of genes involved in inflammation, innate immunity, cell growth, and apoptosis (7). NF-κB transcription factors are homodimeric and heterodimeric complexes formed by five members of the Rel family: NF-κB1 (p50), NF-κB2 (p52), RELA (p65), RELB, and c-REL. Two signaling pathways are involved in the regulation of NF-κB complexes (canonical and noncanonical), which are respectively responsible for the activation of p50 and p52 from their inactive precursors p105 and p100 (8, 9). The activation of these pathways results in the translocation of p50/RELA and p52/RELB complexes into the nucleus and the subsequent transcriptional activation of target genes. Constitutive activation of the NF-κB pathways, either by inactivating mutations of negative regulators or up-regulation of positive regulators, has been linked to several tumor types (10, 11), but the molecular basis has remained largely unknown. Recently, we identified abnormalities affecting 11 regulator genes of the NFκB pathways in at least 17% of multiple myeloma (MM) patients and 41.3% of human myeloma cell lines (HMCL), resulting in the activation of NF-κB (12). Tumor necrosis factor (TNF) receptor–associated factor 3 (TRAF3), a negative regulator of the noncanonical NF-κB pathway, was the most commonly affected gene, with inactivating abnormalities identified in ~12% of patients (12). Additional inactivating abnormalities were identified in other negative NF-κB regulators (CYLD, cIAP1, cIAP2, and TRAF2), as well as gain-of-function mutations in positive regulators (MAP3K14/NIK, NF-κB1, NF-κB2, CD40, LTBR, and TNFRSF13B/TACI) of NF-κB signaling, albeit less frequently (12, 13). Based on these observations and previous studies in other B-cell neoplasias (14–16), we hypothesized that abnormalities in genes that regulate the NF-κB pathways might also be present in WM.
The aim of this study was to perform a comprehensive, high-resolution, array-based comparative genomic hybridization (aCGH) analysis to identify genomic abnormalities present in WM, especially focusing on the status of NF-κB pathway key regulators. We identified biallelic deletions and loss of heterozygosity with inactivating mutations in two negative regulators of the NF-κB signaling pathways, TRAF3 and TNFα-induced protein 3 (TNFAIP3), highlighting the role of these pathways in WM pathogenesis.

Materials and Methods

Patients. Fifty-seven WM patients were included in this study. Clinical and laboratory features of the patients are shown in Supplementary Table S1. BM samples and lymph node biopsies were collected after informed consent was obtained in accordance with the Declaration of Helsinki. The Mayo Clinic Institutional Review and Dana-Farber Cancer Institute review boards approved the study. For FISH experiments from BM samples, cytospin slides were prepared from ACK lysis buffer–treated BM aspirates to remove red blood cells. For aCGH and gene expression profiling (GEP), tumor cells were enriched with anti-CD19+ immunomagnetic beads (AutoMACS; Miltenyi-Biotec) and stored in TRIZOL reagent. RNA and DNA were extracted as recommended by the manufacturer with subsequent clean-up using the RNAeasy Mini kit (Qiagen) and standard phenol-chloroform extraction methods, respectively.

aCGH. High-resolution aCGH was performed in 42 patients with the human genome 244A microarray (Agilent Technologies). Additionally, to reach the maximum available resolution on 6q arm, a custom array was used in 11 patients with 6q monosomic deletions (Supplementary Table S2). Therefore, we designed a 4 × 44K array (Agilent Technologies), covering all the exons plus 700 bp of each adjacent intronic region with an average resolution of 200 bp from 6q15 to 6q telomere. The remaining intronic and intragenic regions were covered on an average resolution of 15 kb. Digestion, labeling, and hybridization steps were done according to the manufacturer’s protocols with some modifications. Briefly, 1.2 μg of tumor and reference DNAs were separately digested with bovine DNaseI (Ambion) for 12 min at room temperature. The normal human reference DNA is composed of a mixture of DNA derived from multiple female donors (Promega). In patients with low DNA yields, a linear whole-genome amplification procedure was incorporated before the DNA digestion step (GenomiPhi DNA amplification kit; GE Healthcare). Next, random primers and exo-Klenow fragment were used to differentially label tumor (Cy5) and reference (Cy3) genomic DNA samples (Agilent Technologies). Labeled genomic reactions were cleaned up with Microcon YM-30 columns (Millipore) and hybridized at 65°C for 40 h. Microarrays were scanned in a DNA Microarray Scanner (Agilent Technologies). Feature extraction was performed with Feature Extraction Software, version 9.5 (Agilent Technologies). Data were analyzed using the CGH Analytics 3.5.1 software (Agilent Technologies). The abnormalities were identified using a two-probe filter and aberration detection modules 1 and 2 algorithms (17) with thresholds of 7.5 and 5.5, respectively. Based on the study done in 50 healthy Caucasian males by de Smith and colleagues (18), we identified and eliminated copy number variation regions existing in at least 5% of individuals. Using this high-resolution genome-wide data (average resolution, 500 bp), only 5% of the genome was eliminated based on the analysis compared with up to 30% when other public databases were used. An in-house algorithm was developed to smooth the data.5 Unsupervised clustering analyses were done with Genespring 7 software (Agilent Technologies).

cIgM-FISH. FISH DNA probes to validate numerical and structural abnormalities were selected for BAC and fosmid clones using the University of California-Santa Cruz genome browser. The specificity of each probe at chromosome and gene level was confirmed by hybridization to normal metaphase preparations and gene-specific PCR, respectively. A list of probes used and chromosomal localization is provided in Supplementary Table S3.

The abnormalities were assessed at an average resolution of 200 bp from 6q15 to 6q telomere. The remaining intronic and intragenic regions were covered on an average resolution of 15 kb. Digestion, labeling, and hybridization steps were done according to the manufacturer’s protocols with some modifications. Briefly, 1.2 μg of tumor and reference DNAs were separately digested with bovine DNaseI (Ambion) for 12 min at room temperature. The normal human reference DNA is composed of a mixture of DNA derived from multiple female donors (Promega). In patients with low DNA yields, a linear whole-genome amplification procedure was incorporated before the DNA digestion step (GenomiPhi DNA amplification kit; GE Healthcare). Next, random primers and exo-Klenow fragment were used to differentially label tumor (Cy5) and reference (Cy3) genomic DNA samples (Agilent Technologies). Labeled genomic reactions were cleaned up with Microcon YM-30 columns (Millipore) and hybridized at 65°C for 40 h. Microarrays were scanned in a DNA Microarray Scanner (Agilent Technologies). Feature extraction was performed with Feature Extraction Software, version 9.5 (Agilent Technologies). Data were analyzed using the CGH Analytics 3.5.1 software (Agilent Technologies). The abnormalities were identified using a two-probe filter and aberration detection modules 1 and 2 algorithms (17) with thresholds of 7.5 and 5.5, respectively. Based on the study done in 50 healthy Caucasian males by de Smith and colleagues (18), we identified and eliminated copy number variation regions existing in at least 5% of individuals. Using this high-resolution genome-wide data (average resolution, 500 bp), only 5% of the genome was eliminated based on the analysis compared with up to 30% when other public databases were used. An in-house algorithm was developed to smooth the data.5 Unsupervised clustering analyses were done with Genespring 7 software (Agilent Technologies).

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Table 1. Delineation of MDRs and MARs based on recurrent abnormalities identified in >5% of patients

<table>
<thead>
<tr>
<th>Cytoband</th>
<th>Chromosome position (bp)</th>
<th>Loss (%)</th>
<th>Gain (%)</th>
<th>Size (Mb)</th>
<th>Expressed CIG</th>
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<tr>
<td>3q13.3-q28</td>
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</table>

NOTE: In regions smaller than 20 Mb were listed the cancer implicated genes expressed in at least 50% of WM patients. Cancer implicated genes include tumor suppressor genes and/or genes with inactivated mutations already described.

Abbreviation: CIG, cancer implicated genes.

*No CIG were found in 13q14, but microRNAs with proposed cancer implicated function were identified.
WM cells were identified by cytoplasmic anti-IgM staining concomitantly with the FISH technique (clgM-FISH) as previously described (19).

GEP. RNA isolation, purification, and microarray hybridization were performed in 22 patients using Affymetrix U133A arrays (Affymetrix), as previously reported (20). The complete data set has been deposited in the National Center for Biotechnology Information Gene Expression Omnibus (GEO) and is accessible through GEO series accession number GSE12668. In 11 of them, we also possess the aCGH data (see Supplementary Table S2).

Gene expression intensities were MAS5 transformed, and median normalized values were analyzed using GeneSpring 7.

A NF-κB index, a measure of NF-κB transcriptional activity, was calculated based on a gene expression index developed from HMCLs, as previously described (12). Briefly, we used an ANOVA test using the error estimate from a cross-gene error model with multiple testing correction to identify probe sets differentially expressed in HMCLs with identified NF-κB pathway mutations compared with those without identified abnormalities.

Figure 1. Overview of the copy number abnormalities identified in WM. Red blocks, copy number gains; blue blocks, copy number losses. A, penetrance plot summarizing the copy number imbalances per chromosome. The amplitude of each abnormality corresponds to its prevalence. B, hierarchical unsupervised clustering done from the genomic imbalances detected by aCGH per chromosome.
The presence of mutations in the NF-κB pathways was clearly associated with a higher level of NF-κB transcription activity in the HMCLs. The mean expression level of four-probe set corresponding to CD74, IL2RG, and TNFAIP3 (2) was used to calculate the index. The probes used were 209619_at (CD74), 204116_at (IL2RG), 202643_s_at, and 202644_s_at (TNFAIP3).

DNA sequencing. Genome sequencing was performed on the PAX5 (n = 16), PRDM1 (n = 9), TNFAIP3 (n = 24), and TRAF3 (n = 24) coding exons and adjacent intron-exon junctions. All the coding regions were amplified using 10 ng of genomic DNA in 25-μL reactions. The specific primers used in this study are listed in Supplementary Table S4. Capillary electrophoresis was performed on an ABI3730 sequencer (Applied Biosystems). DNA sequences were analyzed using Sequencher V4.5.

Immunofluorescence. NF-κB2 subcellular localization was determined by immunofluorescence staining. Cells were fixed in 4% paraformaldehyde, permeabilized in 0.5% Triton-100, and incubated with a 1:50 dilution of anti–NF-κB2 (Cell Signaling Technology) and subsequently stained with

![Figure 2. Chromosomal imbalances on chromosome 6. In the graphical output, negative values (left) indicate losses and positive values (right) indicate gains of tumor DNA. A, the simultaneous presence of 6p gain/6q loss was commonly identified. These chromosomal imbalances could involve either a region or the whole chromosomal arm. B, FISH validations of the above-described cases. Left, a sample with a whole 6p arm gain, including the centromere; center, a FISH pattern compatible with the presence of an isochromosome 6p; right, a 6p interstitial gain.](image-url)
1:100 Alexa-Fluor 647 (Invitrogen). Cytoplasmic staining with anti–Igκ/Igλ identified WM cells. Confocal imaging was performed on a Zeiss LSM510 microscope using a 63× objective.

Results

General overview of CGH. We performed aCGH in 42 WM patients. The complete data set is accessible through GEO series accession number GSE12668. Overall, 35 of 42 (83.3%) cases showed chromosomal abnormalities, with a median of three abnormalities per patient (range, 0–27). In total, 187 abnormalities were found, with 110 deletions and 77 gains. All the abnormalities identified are detailed in Supplementary Table S5. We identified 16 recurrent regions of copy number change found in >5% (identified in three or more patients): 10 deleted and 6 amplified regions. Minimal deleted regions (MDR) and minimal amplified regions (MAR) are detailed in Table 1.

A penetrance plot and a hierarchical clustering, including all the chromosomal abnormalities, are shown in Fig. 1A and B, respectively. The most frequent abnormality was the whole or partial deletion of the 6q arm in 17 of 42 patients (40.4%; Table 1; Fig. 1A) and the sole abnormality in 3 of 17 cases (Fig. 1B). Other recurrent deletions identified by aCGH include the deletion of...

Figure 3. TNFAIP3 abnormalities. A, delineation of four MDRs on 6q based on aCGH data (dashed lines). PRDM1 and TNFAIP3 were localized in MDR2 and MDR3, respectively. B, partial DNA sequences from a normal sample (top) and one with TNFAIP3 frame-shift deletion (red arrow; T155fsX215). The absence of the wild-type allele indicates the homozygous status of the mutation. The position of TNFAIP3 mutation at protein level is based on NP_006281.1, which represents the accepted full-length TNFAIP3 polypeptide. C, the TNFAIP3 transcript expression level was significantly lower in patients with one copy of the gene (1N) when compared with the group with two copies of TNFAIP3 (2N).
Figure 4. TRAF3 abnormalities. A, chromosome (left) and gene view (right) showing monoallelic and biallelic deletions on 14q32.32, including TRAF3 (red, inside the dashed box). B, clgM-FISH validations. A TRAF3 monoallelic deletion was identified in a third patient (MC1383) with no aCGH data. The NF-κBIA probe was used as a surrogate CEP14 probe. C, partial DNA sequences of both cases with TRAF3 monoallelic deletion (MC1341 and MC1383), confirming the presence of a mutation in the remaining allele (c.1800G>A and c.1209A>T leading to D483N and K286X substitutions, respectively). The positions of TRAF3 mutations at the cDNA and protein level are based on NM_145725.1 and NP_663777.1, which represent the accepted full-length TRAF3 transcript and polypeptide, respectively.
13q14 in 4 of 42 patients (10%; Table 1; Supplementary Fig. S1) and deletions of 7q22, 8p, 11q22-23, 11q23.2-24, and 17p11.2-p13.3 in three patients each (7.1%; Table 1).

Partial or whole gains on chromosome 18 and 6p arm were the most common gains (7 of 42 cases each, 16.6%), followed by chromosomes 4 (5 of 42, 11.9%), 3 (4 of 42, 9.5%), 8q (9.5%), and Xq27-q28 (9.5%; Table 1). Whole or partial gains on chromosomes 3 and 18 were concomitantly identified in three patients, none of them with 6q deletion. On the other hand, gains on chromosome 4 were identified irrespective of 6q deletion (Fig. 1B).

Gain of 6p was always concomitant with 6q loss (concomitant 6q loss/6p gain was found in seven patients, but no 6p gain/6q normal cases were identified; Fig. 1B); thus, 6p gain would be a cytogenetic consequence of 6q loss. We analyzed five patients with 6p gain/6q loss and identified three different cytogenetic abnormalities (Fig. 2A and B). By using a three-color FISH strategy, we observed two of five patients with three aqua (CEP6), three red (CCND3 probe at 6p21), and three green (6p telomeric probe) signals, indicating the gain of an extra 6p arm (Fig. 2A and B, left). Alternatively, two patients showed 2A/3R/3G signals, suggesting the presence of an isochromosome 6p (Fig. 2A and B, center). The remaining patient has a partial 6p gain, and our findings are compatible with the presence of an interstitial duplication (2A/2R/3G signals; Fig. 2A and B, right). These results highlight that more than one cytogenetic mechanism is involved in the origin of these abnormalities. By using a similar approach, the presence of an i(8q) was confirmed in two cases (Supplementary Fig. S2).

**Delineation of 6q MDRs and analysis of potential target genes.** In patients with 6q deletion, a single MDR was not identified. Instead, four MDRs (MDR-1 to MDR-4) were delineated, and they were present in at least 14 of 17 patients each (Table 1; Fig. 3A). The most common were MDR-2 (1.4 Mb) and MDR-3 (3.4 Mb), which were present in 16 patients each (Fig. 3A). These regions include AIM1, PRDM1 (MDR-2), and TNFAIP3 (MDR-3), three candidate tumor suppressor genes expressed in at least 50% of WM patients (Table 1). A special interest was put on PRDM1 and TNFAIP3, which have been previously reported as inactivated in other B-cell neoplasias (21–23).

![Figure 5](image-url). TRAF3 inactivation is associated with the activation of noncanonical NFκB pathway. A, NFκB index in WM. Our MM cohort is included for comparison. The patients with TRAF3 and cIAP1-cIAP2 inactivation are highlighted in red and blue, respectively. An association between patients with TRAF3 inactivation and high NFκB index is clearly observed in WM. The case with the second highest index value showed a very low TRAF3 expression (green dot; see Supplementary Table S6), suggesting its inactivation. However, no DNA or cDNA was available for aCGH or DNA sequencing. Bars, median values. B, immunofluorescence staining confirmed the association between TRAF3 inactivation and nuclear localization of NFκB, indicating p100 to p52 processing (patient MC1353). A patient with a close-to-median NFκB index and active TRAF3 was used as a negative control (MC1344).
Given the historical interest on 6q deletions in WM, we also used a custom aCGH microarray to reach the maximum available resolution in the region between 6q15 and 6q telomere. Analyzing 6q with the custom array on 11 patients (see Supplementary Table S2), we did not identify any biallelic deletion involving PRDM1, TNFAIP3, or other tumor suppressor genes.

Next, we sequenced the entire coding region of PRDM1 on nine patients with monoallelic deletion, and no inactivating mutations were found. On the other hand, we detected a frame-shift deletion (T15555X215) in 1 of 24 patients wherein TNFAIP3 was sequenced (Fig. 3B). DNA obtained from the whole genome amplification procedure was used in this sample, but the mutation was confirmed by independent PCR and DNA sequencing reactions done from genomic nonamplified DNA from the same patient. The mutation was found in one case with two copies of TNFAIP3, but the exclusive identification of the mutated allele by DNA sequencing suggests the existence of some uniparental disomy (UPD) mechanism involved in the loss of the wild-type allele (Fig. 3B). The truncated protein lacks the 575 COOH terminal amino acids, including the deubiquitinating region and multiple zinc finger regions. In the remaining patients with monoallelic TNFAIP3 deletions, no mutations affecting the other allele were identified. However, by comparing the median normalized GEP, a significantly lower expression level was observed in the group of patients with TNFAIP3 monoallelic deletion than patients with two copies of the gene (P = 0.0005; Fig. 3C).

Recurrent interstitial deletions on 13q14 containing MIRN15A and MIRN16-1. Biallelic deletions were rare events in this cohort, with only two abnormalities identified. One of the biallelic deletions comprises a 1.65-Mb region on 13q14. Small monoallelic deletions were also found on the same chromosome region in other three patients, thus delineating a 1.1-Mb MDR present in 4 of 42 cases (Table 1; Supplementary Fig. S1). MDR was similar to the described in chronic lymphocytic leukemia (CLL) and also includes the microRNA genes MIRN15A and MIRN16-1, which were already described as playing a key role in CLL pathogenesis (24). Besides the microRNAs, DLEU7 was localized inside the MDR.

TRAF3 inactivation is a recurrent event in WM, and its inactivation is associated with constitutive activation of the noncanonical NF-κB pathway. The remaining biallelic deletion was identified on 14q32.32. By aCGH, we found one biallelic deletion, including TRAF3, AMN, and CDC42BPB, in one patient (MC1353; Fig. 4A). Additionally, a focal monoallelic deletion was identified in a second patient in the same region (MC1341; Fig. 4A). These findings were validated by clgM-FISH, showing the presence of the abnormality in >90% of IgM-positive cells in both cases (Fig. 4B). In the sample with the monoallelic deletion, we identified a missense mutation (D483N) in the remaining allele (Fig. 4C). This substitution affects an amino acid highly conserved throughout vertebrate evolution, suggesting its functional relevance. Next, to determine the frequency of TRAF3 abnormalities in our entire WM cohort, we screened by clgM-FISH 15 additional patients without aCGH data. We found a TRAF3 monoallelic deletion in one additional patient (Fig. 4B), and again, the abnormality was found in the majority of the IgM-positive cells (88%). Moreover, we identified a nonsense mutation (K286X) on the remaining allele (Fig. 4C). This truncated form of TRAF3 lacks the MATH domain, which is critical for the interaction of TRAF3 with NIK and TNF receptors (25, 26). In summary, 3 of 57 (5.3%) patients studied have TRAF3 biallelic deletions or inactivating mutations and UPD. TRAF3 abnormalities were found in one of the three patients at diagnosis, suggesting that its presence is not a consequence of treatment.

We previously showed in MM that TRAF3 inactivation is correlated with an increased NF-κB transcriptional signature, measured as a NF-κB index (12). Analyzing our WM cohort, 4 of 22 WM patients clearly split as an outlier subgroup with the highest NF-κB indices (Fig. 5A). Two of these four patients do not produce active TRAF3 (MC1341 and MC1353), consistent with the association between TRAF3 inactivation and increased NF-κB transcriptional activity. Additionally, other remaining patients from the outlier group with the highest NF-κB indices expressed TRAF3 at the second lowest level (normalized expression value of 0.62 versus median normalized expression value of 1.41 in the entire cohort; see Supplementary Table S6), suggesting its potential inactivation. However, no DNA or RNA samples were available, and neither aCGH nor sequencing could be performed to confirm the TRAF3 status in this patient. Finally, to confirm that TRAF3 inactivation was associated with activation of the noncanonical NF-κB pathway, we examined the NF-κB2 subcellular localization. As predicted, nuclei of patients with TRAF3 inactivation contained NF-κB2 whereas NF-κB2 was cytoplasmic in patients without inactivation (Fig. 5B).

Abnormalities in other NF-κB regulators. Next, in an attempt to identify additional genetic abnormalities affecting NF-κB pathways, we screened the GEP and aCGH data for spiked expression and abnormalities of other regulator genes. By GEP, we did not identify differential expression of any of the main target genes involved in the regulation of NF-κB pathways. By aCGH, we identified large monoallelic deletions comprising clAP1/clAP2 (~35 Mb) and CYLD (~15 Mb) in one patient sample each (Supplementary Table S7). These two genes are negative regulators of the NF-κB signaling, and their inactivation was previously shown in MM patients and HMCls (12). Next, we screened our entire cohort of 57 patients by clgM-FISH, but no additional imbalances in clAP1/clAP2 or CYLD were identified. Finally, all patients were screened for NIK translocations/amplifications, but no abnormalities were identified. A summary of the observed abnormalities affecting genes involved in the NF-κB signaling pathways is shown in Supplementary Table S7.

Finally, we found a monoallelic deletion affecting exons 1 to 4 of PAX5, which was confirmed by PCR, and sequencing across the breakpoint (data not showed). This gene encodes a B-cell lineage-specific activator protein involved at the early stages of B-cell differentiation (27). PAX5 is involved in t(9;14)(p13;q32) translocations recurring in small lymphocytic lymphomas of the plasmacytoid subtype and in derived large-cell lymphomas (28). We analyzed our entire cohort by clgM-FISH and aCGH, but no additional imbalances affecting this gene were identified. We sequenced the entire coding and promoter region of PAX5 in 16 patients, but no mutations were identified.

Discussion

Although WM is considered a distinct clinicopathologic entity, the absence of morphologic, immunophenotypic, or chromosomal disease-specific markers makes its differential diagnosis from other B-cell neoplasias, such as MM, marginal zone lymphomas (MZL), and CLL, difficult. We present here the first high-resolution genomic study in WM, aiming to elucidate the genetic basis of the disease.
Overall, the WM karyotype is stable, even by using this high-resolution approach, with few recurrent abnormalities identified. Considering the amount and type of recurrent chromosomal abnormalities observed, it is clear that WM placed closer to MZL and CLL than MM, as was previously suggested by using gene expression signatures (20). Thus, WM shares with MZL the simultaneous presence of trisomies 3 and 18 (29). Furthermore, we found 7q and 11q deletions in our WM cohort, comprising the same chromosome regions previously identified in MZL and B-CLL (30, 31).

Chromosome 13 deletions are recurrent events in CLL and MM besides WM, but different chromosomal regions are affected by the deletion, ranging from monosomies in MM to small 13q14.3 interstitial deletion in CLL patients (24, 32). WM patients show the same type of the interstitial deletion pattern found in CLL patients, including \(MIRN15A\) and \(MIRN16-1\) in the MDR (24). It was proposed that these \(MIR\)s have tumor suppressor functions because their expression is inversely correlated to the expression of the anti-apoptotic BCL2 protein in CLL. Both \(MIR\)s negatively regulate BCL2 at a posttranscriptional level, and BCL2 repression induces apoptosis in a leukemic cell line model (33, 34). These findings, together with a recent report in SMZL (35), lead to the assumption that this abnormality is not restricted to B-CLL and its dysregulation is common to several indolent B-cell malignancies.

On the other hand, other recurrent chromosomal abnormalities, as whole or partial gains of chromosomes 4 and 8q arm, seem to be specific of WM, helping in distinguishing it from other indolent B-cell malignancies. Gain of 6p arm was always concomitant with 6q deletion, thus suggesting that its origin is secondary to 6q deletion. A similar mechanism seems to be operating in chromosome 8, as simultaneous 8p losses and 8q gains occur in a recurrent fashion, thus highlighting the presence of abnormalities involving both chromosomal arms as common events in WM.

At gene level, remarkable findings affecting genes involved in the regulation of the NF-\(\kappa\)B signaling pathways were identified. In fact, we found biallelic deletions or inactivating mutations and UPD affecting two negative regulators of NF-\(\kappa\)B pathways, \(TRAF3\) and \(TNFAIP3\).

\(TRAF3\) encodes a putative ubiquitin ligase, which plays a key role as a negative regulator of the noncanonical NF-\(\kappa\)B pathway (36). Loss of \(TRAF3\) results in the accumulation of the serine/threonine protein kinase NIK, leading to the constitutive activation of the noncanonical NF-\(\kappa\)B pathway (37). Recent studies have characterized \(TRAF3\) as a tumor suppressor gene (12, 38). In \(TRAF3\)/\(-/-\) HMCLs, the reintroduction of \(TRAF3\) was associated with an inhibition of NF-\(\kappa\)B2 processing (12). Furthermore, the conditional knockout of \(TRAF3\) in B cells is associated with extended B-cell survival and B-cell hyperplasia (38). Here, we identified biallelic \(TRAF3\) inactivation in at least 5.3% (3 of 57) WM samples. Our findings suggest that the constitutive activation of the noncanonical pathway, as a consequence of \(TRAF3\) inactivation, might be a recurrent event in WM pathogenesis. Considering that we analyzed only \(\sim 70\%\) of patients by aCGH and sequencing, we assume that the prevalence of \(TRAF3\) abnormalities might be even higher. These findings and our previous work showing \(TRAF3\) as the tumor suppressor gene more commonly inactivated in MM (12) confirm that \(TRAF3\) inactivation is not specific of MM, being a common event in B-cell malignancies.

In addition, an inactivating mutation was found in \(TNFAIP3\). \(TNFAIP3\) encodes A20, a TNF/CD40/Toll-like receptor–inducible zinc finger protein that acts as a negative regulator of the NF-\(\kappa\)B signaling pathway by negatively modulating the function of upstream I\(\kappa\)B kinase (IKK)–activating signaling proteins, such as TRAF6, RIP, and, possibly, IKK\(\gamma\) (39, 40). Its inactivation leads to constitutive activation of the inhibitor of IKK. \(TNFAIP3\) knockout mice develop severe inflammation and cachexia, failing to terminate TNF-induced NF-\(\kappa\)B responses (41). Recently, \(TNFAIP3\) polymorphisms have been associated with autoimmunedeneseas, such as rheumatoid arthritis and systemic lupus erythematosus (42–44). In addition, \(TNFAIP3\) biallelic deletions have been identified in diffuse large B-cell lymphomas and ocular adnexal marginal B-cell lymphomas; thus, \(TNFAIP3\) inactivation might be relevant in the pathogenesis of B-cell tumors (22, 23).

Here, we identified a homozygous inactivating mutation at \(TNFAIP3\) in 1 of 24 patients. Additionally, monoallelic deletions, including \(TNFAIP3\), were identified in 38% of patients. In this subset of patients, no mutations were identified in the remaining allele, but the \(TNFAIP3\) transcript expression levels were significantly lower than in the group of patients with two copies of the gene. These findings are suggestive of \(TNFAIP3\) haploinsufficiency in WM. Indeed, a recent report using \(TNFAIP3\)/\(-/-\) knockout mice have suggested that \(TNFAIP3\) haploinsufficiency is associated with increased expression of NF-\(\kappa\)B target genes (45). However, additional studies are needed to prove the effect of \(TNFAIP3\) monoallelic deletions in the activation of the NF-\(\kappa\)B target genes in WM patients.

To date, few recurrent cytogenetics abnormalities have been reported in WM, and the molecular consequences of these aberrations are largely unknown (4, 5). To our knowledge, this is the first study showing the inactivation of tumor suppressor genes in WM and identifying a potential target gene on the 6q arm. Overall, we showed \(TRAF3\) and \(TNFAIP3\) inactivation in 5.3% and 4.2%, respectively. In addition, 38% of patients have monoallelic \(TNFAIP3\) deletions. Moreover, these genes are negative regulators of NF-\(\kappa\)B signaling pathways, highlighting the importance of these pathways in sustaining growth in WM. Additional mutational screening of other regulators is ongoing to determine the extent of constitutive NF-\(\kappa\)B activation in this malignancy.

Finally, our findings might have therapeutic implications. Bortezomib, a first in class proteasome inhibitor, has shown antitumor activity in refractory and relapsed MM (46) and more recently in untreated and relapsed WM (47–49). The inhibition of the NF-\(\kappa\)B pathways is thought to be a principal mechanism of action of bortezomib. The identification of mutations affecting regulators of the NF-\(\kappa\)B pathways highlights their role in WM pathogenesis and identifies a subset of patients who might benefit from proteasome inhibitor–based treatments.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Acknowledgments**

Received 9/25/08; revised 1/18/09; accepted 1/7/09; published OnlineFirst 4/7/09.

**Grant support:** IWMF 2S grant (E. Braggio), MMRF Fellowship and Gene and Mary-Lou Kurtz Fellowship (J.J. Keats), grants R01-AG020686 and SPORE P50-CA110077-01 (D.J. Bergsagel), grant R01-CA83724-01, SPORE P50-CA110077-01 and P01-CA62242 from the National Cancer Institute, and the Donaldson Charitable Trust Fund (R. Fonseca), and the International Waldenström Macroglobulinemia Foundation. R. Fonseca is a clinical investigator of the Damon Runyon Cancer Research Fund.

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We thank Chris Gooden, Michael Bittner, Wee Joo Chng, Michael Sebag, and Sandra Montgomery for helpful assistance.
References

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