Studies on the Genetic Characterization of Waldenström Macroglobulinemia

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CHAPTER 1

General introduction

In 1944 the Swedish physician Jan Gösta Waldenström (1906-1996) reported two patients, displaying symptoms of oronasal bleeding, lymphadenopathy, anemia, thrombocytopenia, increased erythrocyte sedimentation rate (ESR) and an abnormal serum protein of high molecular weight.[1,2] The absence of bone pain, lack of lytic bone lesions on radiographs and the presence of an excess of lymphoid cells in bone marrow, made him understand that this disease entity was different from multiple myeloma, in which lytic bone lesions are common and bone marrow is infiltrated by plasma cells. To this day, his original description of clinical characteristics and laboratory abnormalities still forms the basis of the diagnosis of a distinct clinicopathologic entity, which bears his name, Waldenström macroglobulinemia.

WALDENSTRÖM MACROGLOBULINEMIA

Waldenström macroglobulinemia (WM) is a B-cell malignancy characterized by a monoclonal lymphoplasmacytosis, producing a monoclonal immunoglobulin M (IgM) paraproteinemia. Clinical manifestations are related either to infiltration of the bone marrow and organs by the monoclonal B-cells (anemia, thrombocytopenia, granulocytopenia, organomegaly), or to the effects of the IgM paraproteinemia (hyperviscosity, peripheral neuropathy).[3] Lack of universally accepted diagnostic criteria makes it difficult to determine a precise incidence of this disorder. Nevertheless, it is believed that WM is 6 times less common than multiple myeloma, with an estimated incidence of 3.4 per million in males and 1.7 per million in females in the United States.[4,5] WM is more common in whites and familial clustering of cases has been reported, suggesting a role for genetic factors.[6]

Median age of patients with WM at diagnosis is 64 years. Compared to other hematological malignancies, the clinical course of WM is usually indolent. Generally the disease does not require immediate treatment. The median survival of patients with WM is 5 years and 10% of these survive beyond 15 years after diagnosis, indicating the variability in outcome for WM patients. Several retrospective studies have been undertaken to identify clinical parameters impacting on prognosis.[7-11] Most studies show age, hemoglobin value and β2-microglobulin levels as strong predictors of survival. In recent years, the detection of characteristic cytogenetic abnormalities of the malignant clone has added prognostic information with regards to several hematological malignancies. Currently, for WM there is no cytogenetic abnormality that shows association with outcome.

Asymptomatic WM is diagnosed in one third of patients and is often referred to as 'smoldering' WM. This group generally only needs expectant observation. Two thirds of WM patients need some sort of treatment during their course of the disease. Plas-

mapheresis is effective if symptoms are attributable to the hyperviscosity syndrome (oronasal bleedings, retinal hemorrhages and neurological abnormalities). Anemia is the most common indication for initiation of start systemic treatment. Several systemic therapies are available for first-line treatment: alkylating agents, nucleoside analogs and anti-CD20 monoclonal antibodies (rituximab). Often these regimens are combined with corticosteroids, which seem to be particularly useful in patients who develop auto-immune hemolytic anemia, mixed cryoglobulinemia or cold agglutinin disease, but have no additional effect on survival or response rate. Small numbers of patients have received autologous/allogeneic stem cell transplantation, but further evaluation of such therapies is needed and is currently only considered in refractory or relapsing disease. Sometimes painful splenomegaly or hypersplenism requires splenectomy.[12]

THE CLONAL B-CELL IN WALDENSTRÖM MACROGLOBULINEMIA

The pathologic designation for WM is lymphoplasmacytic lymphoma, as defined by the Revised European-American Lymphoma classification and WHO criteria.[13] This means that pleiomorphic B-lineage cells are found in WM bone marrow samples displaying a spectrum of morphologic features of small lymphocytes, plasmacytoid lymphocytes and plasma cells. A consensus panel at the Second International Workshop on WM in 2002 refined the clinicopathological diagnosis of WM as characterized by bone marrow infiltration by lymphoplasmacytic lymphoma, together with a monoclonal IgM protein of any concentration.[14]

Analysis of the immunoglobulin variable region (V) gene sequences and immunophenotyping has helped to determine the origin of the malignant B-cell clone in WM. V-genes encode antigen specificity and increase of affinity can be established by a mechanism called somatic hypermutation. This somatic hypermutation takes place in the follicular germinal centre of peripheral lymphoid tissue, normally after antigen exposure. The variable region of the immunoglobulin heavy chain (V_H) in WM shows that somatic hypermutation has taken place and that intraclonal heterogeneity is absent, which indicates that the WM B-cell is of (post)-germinal monoclonal origin[15,16]. After somatic hypermutation, a normal B-cell can either develop into a memory B-cell or undergo isotype class-switch recombination from IgM,IgD to IgG,IgA or IgE. WM B-cells do not undergo class-switch recombination. Recent reports reveal the potential of class-switch recombination in subsets of WM B-cells (in vitro and in vivo), which suggests an intact class-switch recombination machinery, but possible lack of appropriate stimuli[17,18].

Immunophenotyping has proven to be an essential part of diagnosis and classification of hematological malignancies. The immunophenotypic profile of WM cells include pan B-cell antigens CD19, CD20, CD22, CD79 together with surface IgM in virtually all cases.[13,19] Surface markers CD5, CD10 and CD23 are helpful to distinguish B-cell malignancies, chronic lymphocytic leukemia, follicular lymphoma and mantle-cell lymphoma. In WM CD5 and CD10 expression seem to be uncommon and CD23 expression was found in 35%-61% of cases, often showing co-expression with CD5 and CD10.[20] Expression of CD25, CD27, FMC7, BCL-2 and CD52 is seen regularly. CD103 is never expressed and CD138 expression, a plasma cell marker, is seen rarely.

Conventional cytogenetic analysis has revealed many disease specific chromosomal abnormalities in hematological malignancies and, especially in acute leukemia. These aberrations are of pathophysiologic, diagnostic and prognostic importance. Also, in several B-cell malignancies, such as multiple myeloma, chromosomal translocations at the immunoglobulin heavy chain locus (chromosome 14q32) are commonly seen.[21-23] Translocations result in the fusion of genes that as a result of the structural consequences may express abnormal function or as the result of their location under another promoter may become abnormally expressed. For instance, the chromosomal translocation involving 14q32 generally results in deregulation of oncogenes located at four partner chromosomes such as, MMSET and FGFR3 (both on 4p16), CCND1 (on 11q13), CCND3 (on 6p21) and c-Maf (on 16q23). Other frequently found cytogenetic abnormalities were deletions of chromosome 13 and 17p which involves the loss of genetic material.[24] Unfortunately, due to the low mitotic index of WM cells, informative karyotype studies have remained comparatively scarce.

In recent years new cytogenetic techniques have elucidated pathogenetic mechanisms, have been useful for characterizing biologically distinct subtypes and have contributed to the development of risk-stratified treatment in various hematological malignancies.

Fluorescence in situ hybridization (FISH) is a technique that can overcome the problems of karyotyping in non-dividing cells as it allows for screening of large numbers of interphase cells for chromosomal abnormalities.[25,26]

High throughput array-based techniques are rapidly becoming the mainstay in genomic and proteomic analysis of human cancers nowadays. Together with the sequencing of the entire human genome, these techniques are able to screen for variability and aberrations at distinct cellular levels such as protein, RNA, microRNA, single nucleotide polymorphisms, DNA and DNA methylation. In hematology, gene expression profiling using transcript microarrays has already provided interesting insights in the distinction of different leukemia and lymphoma subtypes.[27-29] Knowledge of prognosis and biology of these different subtypes have resulted in

the development of subtype-specific treatment regimens which eventually could evolve in patient tailored treatment. Gene expression profiling may also generate new ideas about specific oncogenic pathways and steers new drug development toward targeted molecular therapy.

AIMS AND OUTLINE OF THIS THESIS

The genetic basis of Waldenström macroglobulinemia entity is poorly understood and no genetic marker is available for this disease. Cytogenetic techniques have become available that allow for the analysis of genetic aberrations in non-dividing cells.

This thesis describes the characterization of the genetic profile of WM. In chapter 2, the prevalence and clinical consequences of two common cytogenetic aberrations is investigated for WM. These aberrations are known to be of clinical relevance in other related B-cell malignancies such as multiple myeloma, chronic lymphocytic leukemia and follicular lymphoma. Chapter 3 studies the presence of chromosomal translocations involving the immunoglobulin heavy chain locus in WM. Also the ploidy-status was assessed. In addition a new common aberration (deletion) for WM was detected. The prevalence of this new common aberration was subsequently investigated in IgM monoclonal gammopathy of undetermined significance (MGUS) (a precursor state to WM) (chapter 4). In chapter 4 the incidence of a minimal region of deletion in WM was also studied. The clinical impact of this deletion for WM patients is discussed in chapter 5. Finally, chapter 6 describes the comparison of WM with multiple myeloma, chronic lymphocytic leukemia and normal B-cells by gene expression profiling.

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CHAPTER 2

Deletions of 17p13.1 and 13q14 are uncommon in Waldenström macroglobulinemia clonal cells and mostly seen at the time of disease progression

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ABSTRACT

Waldenström macroglobulinemia (WM) is a plasma cell (PC) dyscrasia characterized by a monoclonal IgM paraproteinemia. Deletions of 17p13.1 and 13q14 are associated with tumor progression and worsened outcome in multiple myeloma (MM), and we thus investigated WM patients for their presence. Patients (*n*=40) were required to have a ≥ 1.5g/dL serum IgM paraproteinemia and a monoclonal lymphoplasmacytic infiltrate. We used interphase fluorescence *in situ* hybridization (FISH) with probes that localized to 17p13.1 (LSI *p53*/CEP 17) and 13q14 (D13S319 and LSI 13 *Rb*). Of 40 successfully studied patients for 17p13.1 (*p53*) deletions, 6 were abnormal, consistent with hemizygous deletion (15%). Of 37 cases successfully studied for the 13q14 deletions, 6 were also abnormal with one pair of signals deleted (16%). Patients with deletions were more likely to be later in the course of the disease. No obvious clinical associations were noted with the exception that patients with 17p13.1 (*p53*) deletions had a higher percent involvement of clonal cells in the BM. Deletions of these two regions are uncommon in WM, being more common in the late stages of the disease, thus unlikely playing a role in primary disease pathogenesis.

INTRODUCTION

Waldenström macroglobulinemia (WM) is an indolent B-cell malignancy, characterized by lymphoplasmacytic clonal cell involvement of the bone marrow (BM) and an associated IgM paraproteinemia [1,2]. While WM has clinical and pathologic similarities to multiple myeloma (MM), there is little understanding of its disease pathogenesis. The median survival of patients averages 5 years [1,2]. Ages of 65 or older, albumin less than 4.0 g/dL, presence of cytopenias or cryoglobulinemia, weight loss and hepatomegaly have all been proposed as prognostic factors [3,4]. There is no known correlation between variables specific to the clonal cells and prognosis. It would be desirable to identify patients at risk of early progression and thus in need of closer surveillance or treatment.

Genomic deletions of the *p53* tumor-suppressor-locus (17p13.1) have been observed in about 10% of patients with MM as detected by interphase fluorescence *in situ* hybridization (FISH), and are thought to negatively influence prognosis [5,6]. Similarly, deletions at 13q14 are detected in about 50% of patients with MM when studied by interphase FISH and appear to be associated with a shortened survival [7-10]. Interphase FISH deletions of 13q14 commonly indicate large deletions of the q-arm or monosomy [9,11], and no area of minimal deletion has been identified in MM [12]. There is no proven pathogenetic role for either deletion in the PC dyscrasias, but both are present in the large majority of clonal cells when detected, suggesting clonal selection [6-9,11]. Deletions of these two sites in MM are suspected to be associated with progression of the disease as they increase in prevalence with advancing stages of the disease [6-9,13]. Because deletions of 13q14 and 17p13.1 have been shown to be of prognostic significance in MM, and no systematic study of them in WM has been performed, we wanted to assess patients for their prevalence.

PATIENTS AND METHODS

Patients and bone marrow samples

We studied a total of 40 patients, who fulfilled the clinical and pathological diagnosis of WM [1,2]. Patients were required to have an IgM monoclonal protein of at least 1.5 g/dL, by serum protein electrophoresis and/or nephelometry, and a clonal lymphoplasmacytic infiltration comprising at least 20% of the BM cellularity. Patients were also eligible if they had a monoclonal IgM greater than 3 g/dL and lower percentage BM involvement as could be seen in previously treated patients.

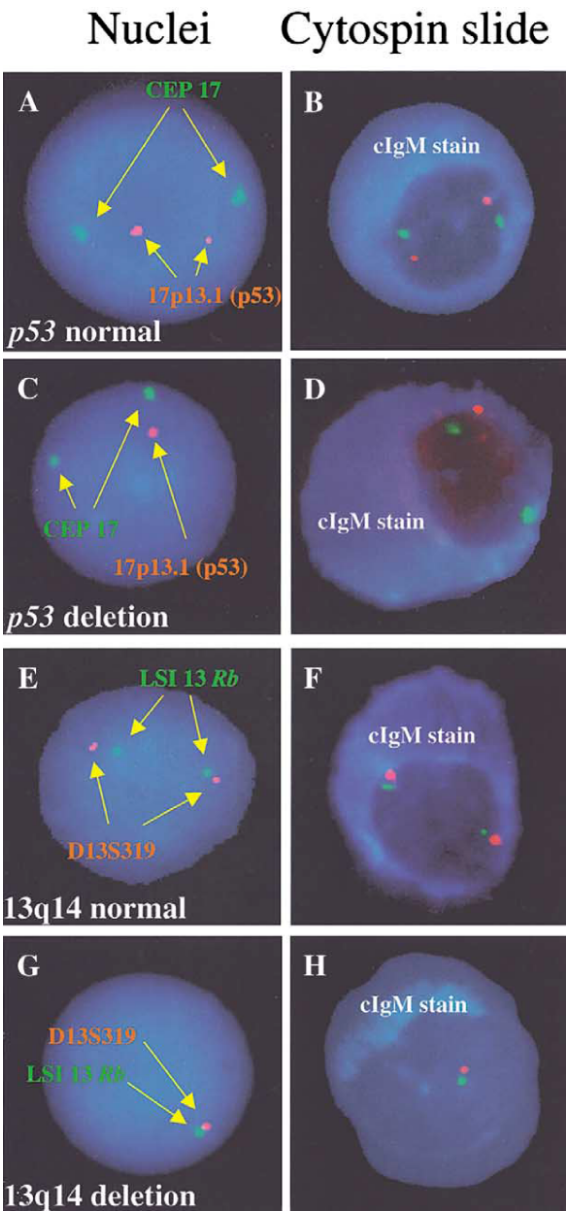

Figure 1. The normal and abnormal patterns of hybridization for both the 13q14 and 17p13.1 (*p53*) probes. Each one of the images depicts both nuclei as observed in the drop slides (left) and a cell with the immunofluorescent detection of the cytoplasmic IgM (cIg-FISH) (right). (A) and (B) show the normal pattern of hybridization with the 17p13.1 (*p53*) probe (red) and CEP 17 (green) probes. (C) and (D) show the abnormal pattern observed in the cells with hemizygous deletion of 17p13.1 (*p53*) but with two remaining CEP 17 signals indicative of disomy. (E) and (F) show clonal cells with the normal pattern consisting of two pairs of hybridization signals for LSI 13 (*Rb*) (green) and D13S319 (red). (G) and (H) show samples with hemizygous deletion of the 13q14 locus and displaying only one pair of signals. (Images captured with the Leica DMR-XA microscope and the Leica Q-FISH imaging software, ×100 magnification.)

The study was conducted under IRB approval, and samples were obtained from all cases at the time of routine clinical procurement. In 24 cases, fresh BM aspirates were enriched for mononuclear cells by the Ficoll method and 15 of these samples were further enriched by concurrent positive selection with CD138 and CD19 positive magnetic beads (Miltenyi Biotec). Cytospin slides were made to preserve the cytoplasm of cells and thus their morphology. In an additional 16 patients stored BM samples, previously cultured for metaphase analysis and arrested in mitosis with Colcemid, were available for study. A single drop of BM mononuclear cell suspension was dropped on a microscope slide for these specimens.

Cytoplasmic staining

In the 24 cases where cytospin slides were available we also performed cytoplasmic immunoglobulin (cIg) staining using an AMCA conjugated anti-IgM antibody (Vector Laboratories Inc.) in a variation of our previously published technique[14]. Cytospin slides were fixed in 95% ethanol for 5 minutes and incubated with an anti-IgM antibody in the dark for 20 minutes. The slides were washed and incubated with an AMCA-labeled anti-goat antibody. Slides were washed in 1X PBD and incubated in 2% paraformaldehyde for 5 minutes. Each cytospin slide was subjected to an enzyme treatment of either proteinase K (10 µg/ml) for 15 minutes, or a 0.1% pepsin solution at 37°C for 2 minutes. The slides were then passed through an ethanol series (70%, 80% and 100%).

Interphase FISH

We used the combination of a 17p13.1 probe labeled in Spectrum Red, (LSI *p53*, Vysis Inc) probe simultaneously hybridized with a chromosome 17 alpha-satellite-DNA centromere probe (labeled in Spectrum Green, CEP17, Vysis Inc.). To test for 13q14 deletions we used the commercially available probes LSI 13 (*Rb*) (Spectrum Green) and D13S319 (Spectrum Red, Vysis Inc.) (Figure 1). The normal pattern for cells was that of two pairs of signals (2R2G). Loss of one or more signals was indicative of deletion (2R1G, 1R2G, or 1R1G).

These probes have been extensively tested and produce discrete signals at the expected hybridization sites in normal metaphases, and a pair of discrete signals in interphase FISH cells. The upper limit of normal (ULN) value was calculated using the mean plus three standard deviations of 1000 normal cells. A patient was said to have a deletion if the percentage of cells with one signal exceeded the mean plus three standard deviations of normal. The ULN was estimated at 6% but to further improve in our specificity we arbitrarily decided to consider a sample as abnormal only if the cells with only one signal exceeded 10%. This is in agreement with our

experience with other assays currently used, as for similar (two color, centromere and locus specific probes) interphase FISH, the normal range cut off at 95% confidence interval for deletion is 8.5% (S. Jalal, personal communication).

We intended to score at least 100 clonal cells of each case, additionally identified by morphologic features and cytoplasmic anti-IgM staining in selected (n=24) cases. In all cases, but two, we could score 100 cells (50 cells in one patient for the 13q deletion assay and 70 cells in one patient for the 17p13.1 deletion assay). The estimated percentage of clonal cells with deletions in the cytospin samples were the actual number of cells with deletion, because the cells are scored only if they were cIg-positive. The estimated percentage of clonal cells for the drop slides was calculated by dividing the percent of total cells with only one signal by the percentage of BM involvement by the clonal cells.

Images were captured using a Leica epi-fluorescence microscope with a fluoroisothiocyanate, Texas red, and a DAPI filter and the Leica Q-FISH software (Leica, Wetzlar, Germany). Images were processed using the Adobe Photoshop software (Adobe Systems Inc., Seattle, WA) and printed using a color laser printer (Tektronix Phaser 840, Wilsonville, OR, USA).

Statistical analysis

The χ^2 test was used to compare patients and nominal variables. The Wilcoxon non-parametric test was used to compare continuous variables. The Wilcoxon and log-rank test were used to evaluate the difference in survival between patients with and without the abnormalities. Multiple comparisons were done with the most important prognostic and biologic variables. The following variables were studied both as continuous variables and as dichotomous variables (in parenthesis, and using the previously published prognostic models in WM); BM involvement, age (≥ 65 versus others), hemoglobin (≥ 12g/dL versus others), albumin (≥ 4g/dL versus others), creatinine, white blood cell count (≥ 4,000 X 10^6/L versus others), platelet count (≥ 150,000 X 10^6/L versus others), serum calcium, M-spike (≥ 2.5g/dL versus others), nephelometric serum IgM level (≥ 2.5g/dL versus others), β_2-microglobulin (≥ 2.7 mg/dL versus others), serum viscosity, labeling index, gender, and previous treatment. The methods of Kaplan and Meier were used to perform a survival analysis.

RESULTS

A total of 40 patients were studied and their clinical features are shown in Table 1. The median follow-up time since diagnosis was 52 months (range 2-209 months). At last follow-up 32 patients were alive with disease and 8 patients had died. The samples were obtained at diagnosis in 11 patients and later in the course of the disease in 29 patients.

Deletion of 17p13.1

We studied 40 patients for deletions of 17p13.1 (*p53*) and were successful in 24 cytospin cases and in 16 cases with cell suspensions. A total of 6 cases showed an abnormal pattern indicative of hemizygous deletion (15%). (Table 2) In these 6 cases the pattern was that of an interstitial deletion with loss of one 17p13.1 (*p53*) signal and two remaining CEP17 probe-signals. The estimated median-percentage of clonal cells with only one signal was 67% (range 29-95%). Only one of these 6 patients had the 17p13.1 (*p53*) deletion detected at the time of diagnosis and in the remaining 5 patients the samples were obtained later in the course of the disease.

Deletion of 13q14

We successfully studied 37 patients for 13q14 deletions (22 with cytospin slides and 15 with cell suspension dropped slides). A total of 6 cases were found to have a

Table 1. Clinical and laboratory features of patients studied

Features	Median	(Range)
Patient age	63	(45-82)
patients aged ≥65	40%	
Male (%)	24	(60%)
Female (%)	16	(40%)
Hemoglobin (g/dL)	9.6	(5.2-14.8)
patients with hemoglobin <10 g/dL	42.5%	
Serum monoclonal spike (g/dL)	3.14	(0.67-6.9)
Nephelometric IgM (mg/dL)	4678	(1530-14260)
Percentage clonal cells in the BM	40	(10-95)
β_2-microglobulin (mg/dL)	3.11	(0.15-6.89)
Viscosity (cpm)	2.6	(1.2-11)
Patients with known prior exposure to alkylator treatment (%)	21	(53)
Patients with leukopenia (%)[1]	11	(28)
Patients with thrombocytopenia (%)[1]	12	(30)
Patients with hepatomegaly (%)	1	(3)
Patients with splenomegaly (%)	5	(13)

[1] Leukopenia was defined as a white blood cell count of less than $4,000\times10^6$/L and thrombocytopenia was defined as a platelet count of less than $150,000\times10^6$/L.

Table 2. Percentage of clonal cells with deletions for 17p13.1 (p53) and 13q14

Patient	Sample	% BM involvement	17p13.1 (*p53*)		13q14		% estimated clonal cells with deletion
			status	% abnormal cells	status	% abnormal cells	
17p13.1 deletions							
1	Cytospin	95	deleted	95	unsuccessful		95
2	Cytospin	90	deleted	29	unsuccessful		29
3	Drop slide	40	deleted	38	normal	1	95
4	Drop slide	70	deleted	47	normal	3	67
5	Drop slide	90	deleted	40	normal	0	44
17p13.1 and 13q14 deletion							
6	Drop slide	70	deleted	46	deleted	33	66/47 [a]
13q14 deletions							
7	Cytospin	30	normal	2	deleted	98	98
8	Cytospin	80	normal	4	deleted	96	96
9	Cytospin	20	normal	2	deleted	53	53
10	Cytospin	30	normal	3	deleted	25	25
11	Drop slide	55	normal	2	deleted	55	100

[a] values for 17p13.1 and 13q14 deleted cells, respectively.

pattern indicative of hemizygous deletion (16%) (Table 2). The estimated median-percentage of clonal cells with only one signal was 75% (range 25-100%). In all cases there was concomitant loss of one pair of signals suggesting large deletions at 13q14 or monosomy. Only one of 6 patients had the 13q14 deletions detected close to the time of diagnosis (4 months from the time of original diagnosis) and in the remaining 5 patients the samples were obtained later in the course of the disease.

There was no association between having deletion of 13q14 and 17p13.1 (*p53*) as only one patient had deletions of both sites. To ensure that we were scoring the correct number of signals for both probes we also used six chromosome enumeration probes to asses ploidy status and found that in all cases the clonal cells were mostly diploid.

Clinical correlations

The only significant association was that patients with 17p13.1 (*p53*) deletion had a significantly higher percentage of BM involvement by the clonal process (mean 38% versus 75%, Wilcoxon p=0.002). Deletions of 17p13.1 (*p53*) appeared to be more common among women (deleted in 5/16 women versus 1/24 of men, Fisher's exact test p=0.029). Patients with deletions of 17p13.1 (*p53*) were more likely to have received prior therapy than others (6/6 versus 15/29, Fisher's exact test p=0.06). Patients with 13q14 deletions were more likely to have a platelet count of less than 150,000 $X10^6$/L than others (Fishers exact p=0.052). There was a trend for patients

with 13q14 towards a higher serum IgM level and a higher β_2-microglobulin (*p*= NS). Survival since the time of diagnosis was not clearly different when patients were stratified according to the presence or absence of deletions at either 13q14 or 17p13.1 (*p53*). Patients with deletions of 17p13.1 (*p53*) were more likely to be dead at the time of last follow-up as compared to others (4/6 versus 5/34, Fisher's exact *p*=0.0163). However no conclusive comments can be made with regards to these observations because of the heterogeneous nature of the patients and management strategies, the small number of events, and different time of detection of the chromosomal abnormalities (at diagnosis versus later).

DISCUSSION

In this study we have shown that deletions of 17p13.1 (*p53*) and 13q14 are uncommon in the clonal cells of patients with WM. The low frequency of these deletions is in agreement with what would be expected for an indolent clonal B-cell disorder such as WM. Furthermore, it should be noted that both deletions were almost exclusively seen in the advanced stages of the disease and not at the time of diagnosis. This particular study cannot be used to examine the influence of deletions at 13q14 and 17p13.1 (*p53*) on survival because of the aforementioned reasons. The study of a much larger number of patients is needed to evaluate the possible prognostic significance of deletions at these two sites. However with such a low prevalence of the abnormality in the newly diagnosed patients these studies are likely to be noncontributory.

The deletion prevalence of 17p13.1 (*p53*) (~15%) is similar to what we and others have observed in the clonal cells of MM patients (*R. Fonseca, manuscript in preparation*) [15], and also increase in frequency with disease progression. The incidence of 13q14 deletions is less than that commonly reported in B-cell chronic lymphocytic leukemia [16-25] (CLL) and MM [7-10]. As with 17p13 deletions, those of 13q14 are also uncommon at the time of diagnosis. These facts attest to a different biologic nature of WM as compared to MM or B-cell CLL.

The two chromosomal abnormalities are unlikely to play a role in the etiology of the disease and it is currently unknown whether loss of DNA at these regions contributes in a mechanistic fashion to progression. Since these deletions usually afflicted the majority of the clonal cells, as is seen in MM [9,11], they appear to be evolutionary selected. This would imply that these abnormalities are seen in a minority of cells at the time of diagnosis or are acquired with progression and ultimately the percentage

of abnormal cells progressively increases with clonal growth. It is also possible that deletions of these two sites are merely reflective of ongoing genomic instability[26], or that they were present since the clone origin and persist through its evolution, although this seems less likely given their higher prevalence in the more advanced cases. The sensitivity of our assays and the limited number of patients studied do not allow us to reach firm conclusions. We propose that deletions at 13q14 or 17p13.1 (*p53*) do not contribute to the early stage pathogenesis.

The underlying pathogenetic mechanisms responsible for WM are unknown. We have recently shown that patients with WM harbor no 14q32 translocations including the t(9;14)(p13;q32) as has been reported in the non-secretory lymphoplasmacytic lymphomas. The few reported series would suggest that aneuploidy is not common although few studies have addressed this issue in detail. The search for the underlying molecular lesion responsible for disease pathogenesis must continue.

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CHAPTER 3

Waldenström macroglobulinemia neoplastic cells lack immunoglobuline heavy chain translocations but have frequent 6q deletions

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ABSTRACT

Lymphoplasmacytic lymphoma (LPL) is characterized by t(9;14)(p13;q32) in 50% of patients who lack paraproteinemia. Waldenström macroglobulinemia (WM), which has an immunoglobulin M (IgM) paraproteinemia, is classified as an LPL. Rare reports have suggested that WM sometimes is associated with 14q23 translocations, deletions of 6q, and t(11;18)(q21;q21). We tested for these abnormalities in the clonal cells of WM patients.

We selected patients with clinicopathologic diagnosis of WM (all had IgM levels greater than 1.5g/dL). Southern blot assay was used to detect legitimate and illegitimate IgH switch rearrangements. In addition to conventional cytogenetic (CC) and multicolor metaphase fluorescence *in-situ* hybridization (M-FISH) analyses, we used interphase FISH to screen for t(9;14)(p13;q32) and other IgH translocations, t(11;18)(q21;q21), and 6q21 deletions. Genomic stability was also assessed using chromosome enumeration probes for chromosomes 7, 9, 11, 12, 15 and 17 in 15 patients.

There was no evidence of either legitimate or illegitimate IgH rearrangements by the Southern blot assay (n=12). CC (n=37), M-FISH analyses (n=5), and interphase FISH (n=42) failed to identify IgH or t(11;18) translocations. Although tumor cells from most patients were diploid for the chromosomes studied, deletions of 6q21 were observed in 42% of patients.

In contrast to LPL tumors that are not associated with a paraproteinemia and have frequent t(9;14)(p13;q32) translocations, IgH translocations are not found in WM, a form of LPL tumor distinguished by an IgM paraproteinemia. However, WM tumor cells, which appear to be diploid or near diploid, often have deletions of 6q21.

INTRODUCTION

Waldenström macroglobulinemia (WM) is a B-cell lymphoproliferative disorder characterized by an immunoglobulin M (IgM) paraproteinemia and the accumulation of clonal lymphoplasmacytic cells in the bone marrow (BM)[1,2]. With advancing disease, patients may develop organomegaly, anemia and hyperviscosity. A serum monoclonal IgM that exceeds a concentration of 1.5 g/dl is characteristic of WM, and is useful in differentiating WM from other neoplasms with plasmacytoid differentiation[3]. The diagnosis is based on the presence of the characteristic accompanying clinical features and the pathologic findings, most commonly from the bone marrow (Table 1). Because of its morphologic and immunophenotypic features, the pathologic designation for WM has been lymphoplasmacytic lymphoma (LPL), as proposed by the revised European-American lymphoma classification [3]. Because LPL is not always associated with paraproteinemia, it is possible that LPL without paraproteinemia and WM (LPL with IgM paraproteinemia) are distinct pathological entities.

It has been reported that the t(9;14)(p13;q32) translocation occur in at least 50% of patients with LPL [4]. The t(9;14)(p13;q32) results in up-regulation of *PAX-5* [5,6], implicating this gene as a putative oncogene in the pathogenesis of LPL [7-9]. Other *PAX* genes are known to be involved in diverse human neoplasia [10]. *PAX-5* encodes for the transcription factor, B-cell specific activation protein (BSAP), which is a 50-kD protein critical for B-cell development. BSAP up-regulation appears to cause an increase in B-cell proliferation [11] and is characteristically absent in both normal and malignant plasma cells [12-15]. BSAP/*PAX-5* is known to down-regulate the Iε promoter and the IgH 3'α enhancer, with consequent down-regulation of IgH transcription [16-19]. In the original series of LPL tumors with the t(9;14)(p13;q32) translocation, none of

Table 1. Clinical and laboratory features of patients

Characteristic		All Patients	
		***n*=74**	**%**
Gender	male	46	63
	female	28	37
Light chain type	κ	55	74
	λ	18	24
	biclonal	1	2
β_2-microglobulin > 2.7 (mg/L)		24 of 39	62
Albumin < 3.5 (n = 46) (g/dL)		23 of 46	50
Labeling index 1 * (%)		1	2
Hemoglobin < 11 (g/dL)		47 of 73	64
BM clonal involvement (%)		50 (median)	5-95 (range)

* Includes plasma cells and lymphocyte clonal cells (defined by light chain restriction).

the patients had paraproteinemia, a finding that is consistent with the possibility that overexpression of *PAX-5* prevents the expression of high levels of immunoglobulin that result in paraproteinemia. If this hypothesis is correct, one would expect that the t(9;14)(p13;q32) translocation would not be present in WM, a form of LPL with an IgM paraproteinemia.

The underlying genetic abnormalities responsible for WM have not been identified. From a limited number of conventional cytogenetic (CC) studies, no recurrent cytogenetic abnormalities have been detected in tumor cells from patients with WM. However, there are sporadic reports of WM tumors with IgH or t(11;18)(q21;q21) translocations or 6q abnormalities, each of which occurs in other kinds of B-cell tumors. Thus, we decided to test a cohort of patients with well-defined WM to address 3 issues. First, despite the expression of IgM in WM, does legitimate or illegitimate IgH switch recombination occur, and perhaps mediate IgH translocations, as previously shown in multiple myeloma and other B cell tumors?[20] Second, using a highly sensitive and specific interphase fluorescence *in-situ* hybridization (FISH) assay, what is the incidence of the t(9;14)(p13;q32) or other IgH translocations in WM? Third, through a combination of CC, multicolor metaphase fluorescence *in-situ* hybridization (M-FISH), and interphase FISH assays, what is the incidence of recurrent numeric or structural karyotype abnormalities – including the t(11;18)(q21;q21) translocation or abnormalities of 6q21 – in WM?

PATIENTS AND METHODS

Patients and bone marrow samples

We identified patients who fulfilled the clinical and pathological diagnosis of WM [1,2] (Table 1). The study was conducted under institutional review board approval. Each patient was required to have an IgM paraproteinemia of 1.5 g/dL or greater and clonal lymphoplasmacytic infiltration comprising at least 20% of the mononuclear cells in the bone marrow (BM), but BM involvement could be lower (10% to 19%) if the patient's monoclonal protein exceeded 3 g/dL. The cohort consisted of 74 patients who fulfilled these criteria (65% had prior chemotherapy exposure). Samples available for study included BM aspirate samples obtained from 33 cases at the time of routine clinical procurement (all studies with cIg-FISH), Fixed cell pellets in Carnoy fixative and cultured for the purpose of clinical karyotype analysis from 17 patients, or both types of samples from 24 patients (cultured and fixed cell pellets and uncultured bone marrow aspirate).

In 19 patients, bone marrow research aspirates were further enriched by simultaneous CD138+ and CD19+ magnetic bead selection (Miltenyi Biotec, Auburn, CA). Owen et al[21,22] have found that clonal cells in WM express CD19, CD138 or both. Because of the pleomorphic nature of the clonal process in WM, we felt that with the combination of CD138 and the B cell marker CD19, we could enrich for the clonal cells of patients. When we only had stored BM samples previously cultured for metaphase analysis, it was not possible to perform magnetic bead selection or concurrent immunofluorescent staining of the cytoplasmic immunoglobulins.

This study was conducted under the approval of the Mayo Clinic institutional review board, and patients gave informed consent for the sample collection. The study was conducted in accordance with the Declaration of Helsinki for research with human subjects.

Southern blot

The Southern blot analysis was performed according to our previously published technique [20]. In brief, we used a J_H probe to detect the germ line and clonal bands to ensure the presence of clonal DNA. We subsequently performed sequential hybridization with probes flanking the 5' and the 3'ends of the Sμ switch region. The presence of fully concordant 5'Sμ and 3'Sμ bands indicates the absence of IgH switch recombination. Alternatively, discordant 5'Sμ and 3'Sμ bands that do not hybridize with other IgH switch probes strongly suggest illegitimate IgH switch recombination caused by a chromosomal translocation[20]. A total of 2.5-10 μg of total BM DNA (these samples were not magnetic micro-bead enriched) was loaded into each well after complete digestion with restriction enzyme *Hin*dIII. (Figure 1)

Standard cytogenetic analysis

Conventional karyotypes performed for clinical evaluation were available for 37 patients. BM specimens were processed by both direct and short-term culture techniques, as described previously [23]. At least 20 banded metaphases were analyzed for each patient, with representative karyotypes prepared from at least 2 metaphases from each clone. The karyotype was described according to the International System for Human Cytogenetics Nomenclature (ISCN, 1995).

Multicolor-FISH assay

M-FISH was performed as previously described.[24,25] Slides with informative karyotypes in 5 cases were processed using the Spectra Vysion (Vysis, Downer's Grove, IL) reagent. Probes for M-FISH were placed on the hybridization site, protected with a coverslip, sealed with rubber cement, and placed in the HYBrite (Vysis)

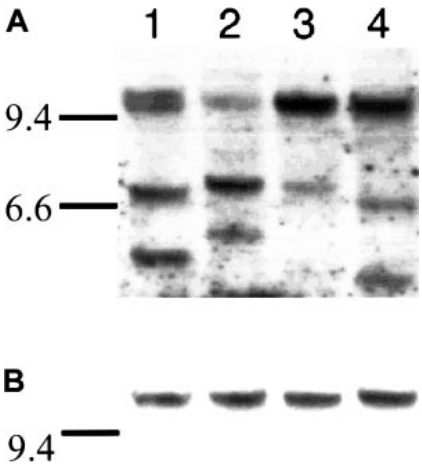

Figure 1. Southern blot assay used to detect legitimate and illegitimate rearrangements at the IgH locus. Southern blot of 4 WM samples that were fractionated on an agarose gel, blotted, and hybridized sequentially with a JH probe (A), and a 5′ Sμ probe (B). Horizontal bars at the left of each exposure correspond to λ HindIII markers, with the 9.4-kb marker in each lane but the 6.6-kb marker included for panel A. Panel A shows the genomic DNA probes with a JH oligonucleotide probe, and the corresponding clonal bands (variable size) and germline fragments (approximately 10 kb). Panel B shows the constant size of the 5′ Sμ probe indicating lack of legitimate or illegitimate rearrangements. The total amount of genomic DNA loaded was 2.5 μg per lane.

for co-denaturation. Slides were viewed with a Zeiss (Thornwood, NY) microscope powered by a 100-W mercury bulb. Filter sets for capturing M-FISH images and viewing metaphases were from Vysis.

Interphase FISH

We used 2 different probe strategies to detect IgH rearrangements by interphase FISH. We first used a break-apart strategy (segregation), using a cosmid probe (V_H) (labeled red) specific for the IgH variable region and a BAC clone (C_H) specific for the IgH constant (labeled green) [26]. Under separate experiments and to detect the t(9;14)(p13;q32) translocation, we used a fusion strategy (colocalization), using a BAC clone (clone 112gO2; Incyte Genomics, Palo Alto, CA) specific for the *PAX-5* gene at 9p13 (labeled red) (Figure 2) together with the V_H/C_H probes (both labeled in green). [26] To validate these probes, we tested them in the cell line KIS-1, which harbors the t(9;14)(p13;q32) and was kindly provided by Dr Hitoshi Ohno (Kyoto University, Japan) and in a patient sample with t(9;14)(p13;q32) kindly provided by Dr Herve Avet-Loiseau (Nantes, France). In both cases our probes showed 2 fusion signals of the IgH probe and the *PAX-5* BAC clone, on both derivative chromosomes in each case, indicating its usefulness for detection of this translocation.

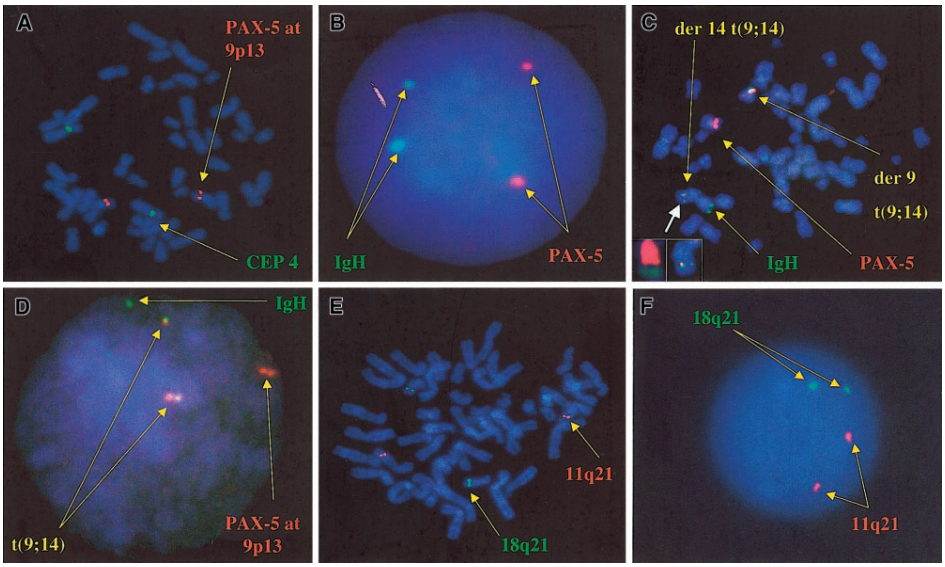

Figure 2. Correct localization of the probes used for the detection of the t(9;14)(p13;q32) and the t(11;18)(q21;q21). (A) Localization of the *PAX-5*-specific probe to chromosome 9 at band p13 (red). The green signal is that of chromosome 4 centromeric probe. (B) Normal pattern of hybridization for the IgH/*PAX-5* probes, consistent with 2 pairs of discrete green and red signal, respectively, without fusion signals. (C) Metaphase FISH assay in a patient with known t(9;14)(p13;q32) serving as the positive control. Metaphase shows 2 fusion signals indicating a balanced reciprocal translocation and the germline configuration in for the IgH probes (green) and the 9p13 *PAX-5* probe (red). Smaller boxes in the lower left corner depict an enlarged view with the der(14)t(9;14) chromosome, showing the fusion signal indicative of the translocation (right small box) and the same chromosome after stripping of the probes and hybridizing with whole chromosome paints (WCPs). Left small box: red indicates WCP 14, and green in WCP 9). (D) Interphase FISH results on the same patient and clearly show 2 distinct fusion signals indicative of the balanced translocation. (E) Correct localization of the probes utilized for the detection of the t(11;18)(q21;q21). (F) Normal interphase FISH pattern for these same 2 probes, again consistent with 2 pairs of discrete green and red signal, respectively, without resulting fusion signals

To detect t(11;18)(q21;q21) we used a set of probes, kindly provided by Dr David James (Mayo Clinic), that span both breakpoints in all reported t(11;18)(q21;q21) translocations and that result in a double fusion, as validated by E. Remstein et al [27] (Figure 2). To screen for possible aneuploidy, we used commercially available centromere enumeration probes (CEPs) for chromosomes 7, 9, 11, 12, 15 and 17 (Vysis). To screen for 6q21 deletions we used the clone RPCI 91C23 (obtained form Oakland Childrens Hospital, CA) with simultaneous hybridization for CEP 6. This same cohort of patients has also been studied for deletions at 13q14 and 17p13 by interphase FISH and the results are compared to those obtained from this study [28].

Noncommercial probes were directly labeled using standard nick translation with SpectrumRed or SpectrumGreen (Vysis). Slides and probes were co-denatured for 7 minutes at 80°C, placed in a humidified chamber and allowed to hybridize in a

37°C oven overnight. Slides were then washed and an anti-fade mounting medium (Vectashield H-1000) was applied to each and coverslipped. For slides not assessed for the cytoplasmic immunoglobulin stain, DAPI (Vector Laboratories, Burlington, CA) was added to the medium.

Cytoplasmic staining

In all patients (n = 33) from which cytospin slides were available, we performed cytoplasmic staining of the IgM cytoplasmic protein using an AMCA (7-amino-3-acetic acid)-conjugated anti-IgM antibody (Vector Laboratories), in a variation of our previously published technique [29]. If we could not use the cIg-FISH technique, or if the original cytospin slides were depleted, we used slides made from the cultured and fixed cells. Because of these limitations these slides were scored on unselected cells without regard to cellular morphology.

Scoring statistics

Using a Leica epifluorescence microscope with a fluorescein isothiocyanate, Texas red, and a DAPI filter, we attempted to score at least 100 clonal cells from each patient. In the cytospin slides, only cells identified by morphologic features or cytoplasmic anti-IgM staining were scored. Fusion of probe signals (colocalization) was defined, as 2 probe signals making contact (Figure 2). Break-apart of probe signals (segregation) was defined by a distance of more than 3 signal widths between 2 differently labeled probe signals. A sample was said to have an abnormal pattern if the percentage of cells exceeded the mean percentage background level plus three standard deviations found for normal cells. The mean percentage background was determined in normal and abnormal samples, and at least 1000 cells were scored for each set of probes.

RESULTS

Southern blot analysis

To detect IgH rearrangements we conducted a pilot study with the Southern blot analysis, but we did not find evidence of either legitimate or illegitimate IgH switch recombination fragments. In all cases the presence of clonal cell DNA was confirmed by the finding of clonal rearrangements by hybridization with a J_H probe, but there was complete concordance of the 5'Sμ and the 3'Sμ bands in sequential hybridizations (n = 12). (Figure 1)

Conventional cytogenetics and M-FISH

We reviewed the karyotype results of 45 patients using conventional cytogenetic analysis performed for routine clinical purposes. Six patients were excluded because chromosomal analysis was performed at the time of diagnosis of secondary myelodysplasia or leukemia. Abnormal findings on BM examination and resultant abnormal karyotypes were consistent with the diagnoses. Of the remaining 39 patients, 35 had sufficient number of metaphases to be evaluable (Table 2). Of these 35 patients, 22 (63%) patients were found to have only normal metaphases, possibly originating from the normal myeloid elements in the BM, including 2 patients whose only abnormality was the loss of chromosome Y. Abnormal metaphases were obtained in 13 (37%) patients, 5 of whom were not previously treated. Structural and numerical chromosomal abnormalities were encountered in the abnormal karyotypes, but usually we observed single abnormalities in an otherwise normal karyotype (Table 2). For the 13 patients with abnormal karyotype, 4 (31%) had recurrent abnormalities of chromosome 13, with del(13q14) in 3 and monosomy 13 in one; 2 (15%) had del(6)(q13q21)(q13q25); 1 (8%) had an addition at chromosome 6q27(add(6)(q27)): 2 (15%) had abnormalities of chromosome 17q25 and 1 (8%) had chromosome 17 monosomy; 2 had del(5q)(13q35) (one with and one without prior therapy); and 4 had diverse abnormalities in chromosome 8 (t(Y;8)(q11;p23), i(8)(q10), der(8;9)

Table 2. Karyotypic abnormalities in patients with informative karyotypes

Patient	Karyotype	Additional information provided by M-FISH [number of metaphases analyzed]
Patients not previously treated		
1	~4n with fragmentation[5]/46,XX [20]	48,XX,der(9),der(17) [8]
2	47,X,t(Y;8)(q11.2;p23),del(5)(q13q31), del(6)(q13q21),der(19) t(19;?)(q13.1;?),+2mar [10]/ 46,XY [10]/10=	Not done
3	46,XX,del(6)(q13q25)[1]/46,XX [29]	No further information [4]
4	46,XY,del(13)(q14q22)[2]/46,XY [18]	49X,-X+der(3),+4,der(9),+18,+19 [2]
5	47,X,-Y,add(3)(q21),add(4)(q21), add(6)(q27),add(7)(q11.2),i(8)(q10),+10,-13,-13,-15, add(17)(q25),+add(18)(q23),+19,-20,+3mar[cp3]/46,XY [17]	No further information [4]
Previously treated patients		
6	47-48,XY,der(16)t(16;?)(q24;?),der(17) t(17;?)(q25;?),+1-2mar [5]/46,XY [14]	Not done
7	45,X,-X,der(8;9)(q10;q10),+9,-17,-19,+2mar[1]/ 45,idem,t(7;18)(p15;q21)[1]/46,XX [28]	Not done
8	45,XY,-7[7]/46,XY [23]	45,XY,-7 [3]
9	46,XX,-12,+r[8]/46,XX [10]	Not done
10	46,XX,del(13)(q14q22)[3]/46,XX [17]	Not done
11	46,XX,del(13)(q12q14) [3]/46,XX [5]	Not done
12	46,XY,+add(1)(p12),-15[1]/46,XY [29]	Not done
13	47,XY,+8,?del(5)(q13q35),del(6)(p21.3?p23),tdic(13;?) [20]	Not done

(q10;q10) and -8). As a follow-up to conventional cytogenetics in 5 of 13 patients with an abnormal karyotype, M-FISH studies identified additional karyotypic abnormalities in 2 patients but provided no additional information in 3 patients (Table 2).

Interphase FISH

Of the 31 patients studied, 30 had no evidence of IgH translocations by the V_H/C_H strategy, and one patient had 37% of cells harboring the abnormality. In addition, in none of these 31 patients could we find fusion signals indicative of t(9;14)(p13;q32) (Figure 2). When we extended our study to include 18 additional patients in whom we had CC cell suspension to study, we found that none of them had evidence of IgH translocations by the V_H/C_H break-apart strategy (n = 17) or the t(9;14)(p13;q32) translocation by the fusion strategy (n = 18). One patient had 3 or more signals from the *PAX-5* BAC clone in 69% of the cells but no fusions (17% with 3 signals, and 52% with 4 signals). This same patient had evidence of trisomy for chromosome 9 in the metaphase analysis. None of the 24 patients studied had evidence of the t(11;18) (q21;q21) as detected by FISH.

To screen for possible aneuploidy, tumor cells from 15 patients were studied by interphase FISH using CEP probes for chromosomes 7, 9, 11, 12, 15 and 17. With the exception of one patient, all chromosomes were normal; one patient had only one signal for chromosome 9, indicative of monosomy in 26% of the clonal cells. Although some patients showed abnormalities in the karyotype consistent with aneuploidy (Table 2), interphase FISH showed that this is the exception, and patients with karyotype abnormalities likely represent aggressive variants of the disease. There is probably an inherent bias in the patient population that is subjected to scrutiny of karyotype analysis (*i.e.,* worsening disease). In addition it is highly likely, as in multiple myeloma, that the ability to obtain informative metaphases is closely related to the tumor burden and proliferative activity[30].

Deletions of 6q

We also tested 24 patients (in whom we had metaphase culture slides but could not perform the cIg-FISH assay) for evidence of 6q21 deletions and found that 10 (42%) had abnormalities in more than 25% of the cells. In addition 5 (21%) more patients had between 10% and 25% abnormal cells. Because in many of these latter patients the percentage of clonal involvement of the bone marrow was between 10% and 20%, it is conceivable that they could also have had deletions at this site. Therefore between 42% and 63% of patients had deletions of 6q21.

Correlation between interphase FISH and karyotype analysis

Three patients had chromosome abnormality detected by metaphase analysis; we also performed specific interphase FISH testing. In one case of a patient –13 was confirmed by FISH, and in one patient with the deletion of 17p this was confirmed by FISH. In yet in another patient, del(6)(q13q25) was detected by our FISH probe.

DISCUSSION

In this study we have found that clonal cells from patients with clinically defined WM do not have the t(9;14)(p13;q32) translocation, as previously found in 50% of patients with LPL but without paraproteinemia. Thus, WM, a type of LPL with IgM paraproteinemia, and LPL with no paraproteinemia differ not only in phenotype but also in the presence of the t(9;14)(p13;q32) translocation. This result is consistent with the hypothesis that the predicted biologic features of B-cell clones with *PAX-5* up-regulation are incompatible with the phenotype of WM [17-19]. Notably, BSAP is absent in plasma cells, and overexpression of *PAX-5*/BSAP abrogates the production of the J peptide. [13] This protein is an integral component of the IgM pentamers, which give rise to the hyperviscosity of patients with WM. [31] In addition, *PAX-5*/BSAP negatively regulates the 3'α enhancer, with likely consequent inhibition of transcription at the IgH locus, [17-19,32,33] a result inconsistent with the IgM paraproteinemia observed in these patients. Finally, the inhibition of *PAX-5* transcription with antisense oligonucleotide down-regulates immunoglobulin class switching [11], a result consistent with our failure to identify evidence for legitimate or illegitimate IgH isotype switch recombination in the clonal cells of IgM-producing WM.

Our finding that IgH translocations are not present in WM is consistent with the previously reported karyotypic abnormalities in these patients. In a series of 45 WM cases reported by Louviaux et al, 12 patients had abnormal metaphases, but no abnormalities of chromosome 14 were noted [34]. In our review of the cytogenetic database at the Mayo Clinic of patients we report here, which we report here, we were unable to find any patient with 14q32 abnormalities. Unlike multiple myeloma [20,35,36] and low-grade lymphomas, [37,38] 14q32 translocations appear not to be initiation events for disease pathogenesis in WM. Scattered reports in the literature describe patients with WM and 14q32 translocations, including t(8;14)(q24;q32). [39-42] Some of these reports are confounded either by the lack of consistent clinical features associated with a diagnosis of WM or by the samples originating from pleural effusions and, thus, of unknown relation to the original clone. [39-42]

There are rare reports of WM patients in whom the tumor cells had a t(11;18)(q21;q21) translocation, [43,44] an abnormality also seen in approximately 20% of extranodal marginal zone lymphomas. [45] Because we have been unable to detect this translocation in the tumor cells of 24 WM patients, it must be, at best, rare.

Despite an apparent paucity of structural and numeric karyotypic abnormalities in tumor cells from our cohort of WM patients, we did identify a high prevalence of deletions of the long arm of chromosome 6. This is consistent with what has been previously reported by conventional cytogenetic studies in selected patients with WM [46,47] and other B-cell neoplasias in which abnormalities in this region are common. [48-50] This is also consistent with findings from our karyotype analysis that 3 patients had 6q deletions. Further work is under way to characterize the area of minimal deletion, and the search for putative genes involved in disease pathogenesis. We suspect that the inactivation of a tumor suppressor gene at this locus, as is seen in other B-cell neoplasias, is likely to be of importance for clone immortalization.

Our data suggest less genomic instability in WM than in multiple myeloma, as determined by metaphase analyses for structural and numeric abnormalities and interphase FISH for numeric abnormalities. Moreover, we have recently reported that, unlike multiple myeloma and B-cell chronic lymphocytic leukemia (B-CLL), deletions of 13q14 are rarely observed at the time of diagnosis [28]. As are deletions of 17p13.1 (see below), 13q14 deletions are seen mostly in advanced stages and in a small fraction (approximately 15%) of patients with WM at the time of diagnosis. [28] Therefore, we can conclude that WM is clearly different from myeloma in that it lacks IgH translocations, has only infrequent deletions of 13q14, and has limited numeric and structural karyotypic abnormalities. WM is also different from B-CLL in the lower incidence of 13q14 deletions (seen in approximately 50% of patients with B-CLL) and the low prevalence of trisomy 12 (seen in approximately 15% of patients with B-CLL).[51] WM is also different from the extranodal marginal zone lymphomas in that we could not detect any case with the t(11;18)(q21;q21). The lack of IgH translocations also differentiates WM from many kinds of B lymphomas (follicular, mantle cell, diffuse large cell).[52] We thus conclude that the biologic nature of WM is different from multiple myeloma and most lymphomas, but appears more similar to post-germinal center B-CLL (Table 3).

The normal counterpart of the malignant cell in WM might be a postgerminal center IgM memory B cell that has undergone somatic hypermutation but fails to undergo isotype class switching. Our Southern blot results, failing to identify legitimate or illegitimate IgH switch recombination rearrangements indicative of isotype class

Table 3. Genetic comparison between WM, myeloma and B-CLL

Feature	WM	Myeloma	B-CLL
Clinical course	Usually indolent	Aggressive	Usually indolent
Genomic stability (ploidy)	Diploid	Aneuploid	Diploid
Structural abnormalities (other than 6q)	Infrequent	Frequent, multiple	Infrequent
IgH translocations	Rare	75%	Rare
Chromosome 13 abnormalities at diagnosis	Rare[28]	~50%	~50%
Deletions of 17p13	Rare at diagnosis[28]	Rare (10%-15%)	Rare (5%)
Proliferation by the labeling index	Low/absent	Active	Not done
Somatic hypermutation	Yes	Yes	Variable

switching, and the presence of the IgM paraproteinemia are consistent with this. In addition, individual WM tumor cells show heterogeneous morphological features consistent with variable differentiation from a B cell to a plasma cell but failure to fully differentiate into plasma cells. We speculate that the presumed genetic event(s) responsible for generating an immortalized clone of tumor cells in WM are directly or indirectly responsible for the failure of isotype class switching and lack of differentiation to plasma cells. With the exception of frequent deletions of the long arm of chromosome 6, our knowledge of specific primary or secondary genetic events in WM remains elusive.

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CHAPTER 4

6q deletion discriminates Waldenström macroglobulinemia from IgM monoclonal gammopathy of undetermined significance

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ABSTRACT

IgM monoclonal gammopathy of undetermined significance (IgM MGUS) and Waldenström macroglobulinemia (WM) are sometimes clinically difficult to distinguish. In our previous study, deletion of the long arm of chromosome 6 (6q) was found in about half of WM patients. To further clarify the area of minimal deletion at 6q (6q-) and to address the issue of whether 6q- occurs in IgM MGUS, 12 IgM MGUS and 38 WM patients were studied by fluorescence *in situ* hybridization (FISH) using probes targeting different chromosomal segments of 6q. No 6q deletions were found in IgM MGUS samples. Of 38 successfully studied WM patients, 21 (55%) showed a deletion of 6q. The area of minimal deletion was between 6q23 and 6q24.3, but the deletion usually encompassed a large fragment of the 6q arm. These results indicate that 6q- can distinguish WM from IgM MGUS and is likely to be a secondary event.

INTRODUCTION

Waldenström macroglobulinemia (WM) is a B-cell lymphoproliferative disorder characterized by the accumulation of monoclonal cells in bone marrow and peripheral lymphoid tissues and is associated with an IgM serum monoclonal protein [1]. The pathology designation for WM is lymphoplasmacytic lymphoma (LPL), as proposed by the World Health Organization Classification of Tumours. We have reported that the most common genomic aberration in WM clonal cells is loss of the long arm of chromosome 6 (6q)[2,3]. As of yet, however, no minimal area of deletion and no unique gene expression consequences of these deletions have been identified. It is important to delineate the minimal area of deletion, which could harbor possible tumor suppressor genes (TSG). To do so we conducted a fluorescence in situ hybridization (FISH) study using a set of probes representative of different regions of 6q.

Likewise, it is currently unclear whether 6q- represents an early immortalizing genetic event or whether it is associated with disease progression. The study of 6q in IgM monoclonal gammopathy of undetermined significance (IgM MGUS) can help elucidate this question. IgM MGUS is a precursor state to lymphoproliferative disorders, particularly WM. In a population based study we were able to show that patients with IgM MGUS have a 46-fold higher risk of developing Waldenström macroglobulinemia than the general population [4]. In a Mayo Clinic series of 242 patients with IgM MGUS, 40 (17%) developed malignant lymphoproliferative disorders of which 22/40 (55%) developed WM[5]. During long-term follow up, the risk of progression of MGUS of IgM type to lymphoma or related disorders averaged 1.5% per year [6]. Little is known about the biology of IgM MGUS and the factors associated with its malignant progression to WM.

Finally, the similarity of clinical presentation and laboratory parameters between IgM MGUS and Waldenström macroglobulinemia makes the discrimination of these two diseases difficult and the diagnostic criteria frequently overlap. An international consensus panel concluded that the level of the monoclonal protein alone could not be used as the sole discriminant between IgM MGUS and WM [7]. Furthermore, cases with classic lymphoplasmacytic morphology in the bone marrow, even with low concentration of a serum monoclonal protein, have been classified as WM. It is evident that better markers are needed to make this diagnostic differentiation.

In the present FISH study we determine a more accurate prevalence of 6q- in WM by concurrently using cytoplasmic staining of the clonal cells. We also delineate the area of minimal deletion in WM, by using a set of 10 FISH probes located at 6q. Finally, we assess the prevalence of 6q- in IgM MGUS, an assumed precursor state of WM.

PATIENTS AND METHODS

Patients

We studied 38 WM patients and 12 IgM MGUS who fulfilled the clinical diagnosis of WM or IgM MGUS [7,8] (Table 1). The study was conducted under the approval of Mayo Clinic Institutional Review Board and in accordance with the Declaration of Helsinki for research with human subjects. For this particular study, and to attain greater stringency in our diagnostic criteria, each WM patient was required to have a serum IgM paraprotein level of 1,500 mg/dL or greater and clonal lymphoplasmacytic infiltration comprising at least 20% of the mononuclear cells in the bone marrow (BM) biopsy. The BM involvement at diagnosis could be 10-19% if the patient's monoclonal protein level exceeded 3,000 mg/dL. Patients were classified as having IgM MGUS according to standard criteria, namely 10% or fewer monotypic plasma cells (PCs) or lymphoplasmacytic cells in the BM biopsy and an IgM monoclonal serum protein concentration of 3,000 mg/dL or less, and no evidence of MM or WM. In seven IgM MGUS patients the BM sample was taken at the time of diagnosis.

Samples and FISH probes used

In the cases studied, BM aspirates were enriched for mononuclear cells using the Ficoll method and cytospin slides were made. For the FISH assays, we used our previously published techniques [2]. Briefly, we used FISH combined with cytoplasmic immunoglobulin M (cIgM) staining with an AMCA (7-amino-4-methylcoumarin-3-acetic acid)-conjugated anti-IgM antibody (cIgM-FISH). We also used cytomorphology for the detection of the clonal cells on cytospin slides. A total of 10 probes were

Table 1. Clinical characteristics IgM MGUS and WM patients.

	IgM MGUS (n = 12)		WM					
			6q present (n = 17)		6q deleted (n = 21)		WM Total (n = 38)	
Sex								
Male	6	(50%)	9	(53%)	14	(67%)	23	(61%)
Female	6	(50%)	8	(47%)	7	(33%)	15	(39%)
Light chain type (%)								
κ	10/12	(83%)	11/17	(65%)	14/20	(70%)	25/37	(68%)
λ	1/12	(8%)	6/17	(35%)	6/20	(30%)	12/37	(32%)
biclonal	2/12	(17%)	0/17	(0%)	3/20	(14%)	3/37	(8%)
B2-microglobulin > 3.5 mg/dL	0/9	(0%)	3/16	(19%)	6/17	(35%)	9/33	(27%)
Albumin < 3.5 g/dL	1/12	(8%)	7/17	(41%)	6/20	(30%)	13/37	(35%)
Hemoglobin < 11 g/dL	0/12	(0%)	4/16	(25%)	12/21	(57%)	16/37	(43%)
Serum IgM > 3,000 mg/dL	0/12	(0%)	11/15	(73%)	15/20	(75%)	26/35	(74%)
Serum IgM spike > 3.0 g/dL	0/9	(0%)	7/16	(44%)	7/20	(35%)	14/36	(39%)
Median % PC BM involvement (range)	5 (0-10)		20 (10-90)		50 (10-90)		30 (10-90)	

used, including 2 commercially available probes (CEP 6 and Telomere 6q, Vysis Inc., Downers Grove, IL USA) and eight BAC or PAC clones (Invitrogen, Carlsbad, CA USA) developed in our laboratory. Clones were selected based on a specific gene encompassed in the insert and/or localization on the 6q arm and validated by PCR and normal metaphase slides. Genes of interest included *BLIMP1* (RPCI-1 134E15, RPCI-1 101M23), *MYB* (RPCI-1 32B1), *BTF/BCLAF1* (CTD 2600I3), *SHPRH* (RPCI-11 164A17), *LATS1* (RPCI-11 52G20) and *FOP/FGFR1OP* (RPCI-1 167A14). Finally, two probes localizing to 6q13 (RPCI-11 553A21) and 6q15 (RPCI-11 113K07) were applied. In IgM MGUS samples, only the probes encompassing genes *BLIMP1*, *MYB* and *SHPRH* were applied simultaneously with the CEP 6 probe.

Statistics and scoring criteria

The signal pattern of each sample was identified under an epifluorescence microscope with fluorescein isothiocyanate, Texas red, and a DAPI filter. We attempted to score at least 100 clonal cells from each patient, but a sample was considered evaluable if at least 20 cells were scored. In the cytospin slides, only cells identified by morphologic features as lymphoplasmacytic were scored. Based on our previous FISH studies[2,9], an abnormal pattern is determined if the percentage of cells exceeded the mean percentage background level plus 3 standard deviations found for normal cells. Therefore a cut-off of 10% or greater was used to indicate a patient sample with evidence of deletion for a probe studied. This cut-off was not critical because, in most cases, the deletions involved the majority of the clonal cells.

RESULTS

MGUS Patients

A total of 12 IgM MGUS patients were selected for this study (six women, six men; median age at diagnosis, 64 years). Two cases showed a pattern that was indicative of a biclonal (IgMκ/ IgGκ) B-cell involvement by protein electrophoresis and immunofixation, but IgM was the predominant protein. Five patients had IgM MGUS associated peripheral neuropathy, a common characteristic of IgM paraproteinemia[10]. One patient had received chlorambucil and prednisone as treatment for a painful peripheral neuropathy. Two patients had been successfully treated with plasmapheresis for their peripheral neuropathy, but no hyperviscosity symptoms were observed. One patient had received prednisone and azathioprine for ulcerative colitis treatment. The BM samples showed PCs in all but three cases (range; 0-10% involvement). Three BM samples contained a mixture of small lymphocytes and plasmacytoid cell

comprising 5, 9 and 10% of the BM, respectively. None of the IgM MGUS patients showed progression to WM or other malignant stage during follow up.

Waldenström macroglobulinemia patients

Thirty-eight WM were included (15 women, 23 men; median age at diagnosis, 67 years). Three patients had histological proof of amyloid deposition. Seventeen patients had received no treatment for WM prior to their BM sample. The other patients had received prior treatment with alkylating agents, purine analogs, anti-CD20 antibodies and/or corticosteroids. Cytogenetic results of this cohort of WM patients were successful in 14 cases (data not shown). Seven cases (50%) showed an abnormal karyotype but no 6q deletion was detected. Clinical parameters of IgM MGUS and WM patients are summarized in Table 1.

cIgM-FISH

In the 12 IgM MGUS cases studied, no abnormal pattern was detected by using two probes, encompassing *BLIMP1* and *MYB*, simultaneously with CEP 6. In nine IgM MGUS cases we were also able to apply the probe covering gene *SHPRH* together with CEP 6. No abnormal pattern was scored. Based on these results we decided not to apply the other probes in IgM MGUS. In five cases previously performed FISH studies did not detect evidence of chromosome 14 translocation using the 'break-apart' strategy (data not shown). As expected, 6q deletions were detected in 21 WM cases (55%) using any of our probes on 6q (Figure 1). One WM case had a monosomy for chromosome 6 (case 21, Figure 1).

Area of minimal deletion in WM

To define the area of minimal deletion at chromosome 6q, 29 WM cases were successfully studied by using all ten probes. In nine WM cases probe 'coverage' was not complete, either due to hybridization problems or lack of slides. In the 21 WM cases showing deletion of 6q, three had an interstitial deletion (cases 2, 3 and 13, Figure 1).

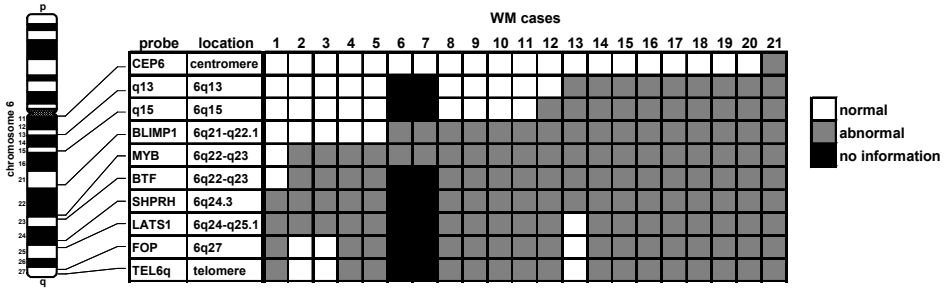

Figure 1. FISH results in WM cases with 6q deletions.

The remainder of these cases showed deletions of large segments, including the telomeric probe. The probe encompassing *SHPRH* (at 6q24) was the most frequently deleted of the ten probes. Because FISH cannot precisely define the outside margins of the deletion, we conclude that the likely boundaries of the "area of minimal deletion" are 6q23 and 6q24.3.

DISCUSSION

Although WM and IgM MGUS are considered closely related diseases, we demonstrate in this study that 6q deletions are uncommon in IgM MGUS. Recent data showed that 6q deletion is uncommon in nodal LPL, which suggests that WM has a different cytogenetic and biologic profile compared to nodal LPL [11]. We speculate that 6q- could serve as a specific genetic marker for WM.

Our data show that 6q deletions assessed by cIgM-FISH are the most common chromosomal abnormalities in WM, with a prevalence of 55%. Strikingly, although many WM cases display loss of almost the complete 6q arm as detected by cIgM-FISH, conventional cytogenetics was not able to detect this abnormality in any of the cases. Therefore we suggest that the cIgM-FISH assay is a sensitive test to detect 6q- in WM.

Deletions of 6q have been described in other hematological malignancies and solid tumors. In non-Hodgkin lymphomas (NHL) three distinct regions have been defined and associated with disease grade/sub-type: 6q21-23 in high grade NHL; 6q25-27 in intermediate grade NHL, and 6q23 in low grade NHL. In this study we delineate the area of minimal deletion between 6q23 and 6q24.3. Several candidate tumor suppressor genes have been suggested for this area. The FISH probe SHPRH covering the *SRPRH* gene locus at 6q24, was deleted most frequently. The protein product (SHPRH) of this gene contains five functional domains (SNF2, histone linker, PHD, RING and helicase). It is speculated that SHPRH plays a role in chromatin-mediated transcription and therefore is a candidate tumor suppressor gene. However, functional studies are required to elucidate its exact biological role[12].

In WM the 6q deletion is probably a progression event most commonly observed in WM, but not in the pre-malignant phase of the disease. Our preliminary clinical observations suggest a correlation between 6q deletions and more aggressive clinical features. Other authors have associated deletions of 6q23 with a low-grade subset NHL, with small lymphocytic and plasmacytic morphology[13].

The cell of origin in IgM MGUS is unknown. For IgG and IgA MGUS it has been well established that BM clonal cells harbor similar recurrent aberrations, compared to the malignant stages (multiple myeloma and related disorders). Therefore, BM is

most likely the location of the main clonal population. IgM MGUS will progress to a malignant stage in 17% of the cases. In the progression of IgM MGUS to lymphoma, it is likely that a lymph node harbors the cell of origin. In the progression of IgM MGUS to WM, primary amyloidosis and chronic lymphocytic leukemia, the BM is likely the residing place of the clonal cell. The unknown progression path hampers IgM MGUS studies based on BM tissue. Ideally, a study using paired samples from patients having progressed from IgM MGUS to WM would be more informative.

In conclusion, the prevalence of 6q- is 55% in WM and therefore the most common aberration. We have also shown that deletions at this region are uncommon in IgM MGUS. The probe for 6q24 could be used to distinguish WM from IgM MGUS, but more study is necessary to prove its utility as a clinical marker. Finally, we delineated an area of minimal deletion (6q23-24.3) in WM and speculate that this is a secondary event involving a tumor suppressor gene at this locus which results in a more aggressive subset of the disease.

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CHAPTER 5

6q deletion in Waldenström macroglobulinemia is associated with features of adverse prognosis

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ABSTRACT

Fluorescence in situ hybridisation (FISH) is an effective technique for the cytogenetic analysis of Waldenström macroglobulinemia (WM) but the potential impact of molecular cytogenetics on disease evolution and as a prognostic marker is still unknown. Deletion of the long arm of chromosome 6 (6q-) is the most frequent cytogenetic abnormality in WM. This study analysed the prevalence of this aberration in 102 WM patients, and we have correlated it with disease characteristics. The incidence of 6q21 deletion was 7% by conventional cytogenetics and 34% when analysed by FISH (54% when cytoplasmic immunoglobulin M-FISH was used). Patients with deletion of 6q displayed features of adverse prognosis such as higher levels of β2M and monoclonal paraprotein and a greater tendency to display anaemia and hypoalbuminemia. Interestingly, there was a correlation between the presence of 6q deletion and the International Staging System prognostic index (incidence of 6q- among patients stratified in stages 1, 2 and 3 was 24%, 42% and 67% respectively). Those patients diagnosed with smouldering WM who displayed the abnormality showed a trend to an earlier requirement of treatment. Finally the survival analysis did not show differences between the two groups of patients, probably due to the short follow up of our series.

INTRODUCTION

Waldenström macroglobulinemia (WM) is a B-cell clonal disorder that affects elderly patients. The rarity of this disorder, as well as its location at a crossroads between chronic lymphoproliferative disorders, low grade lymphomas and monoclonal gammopathies, creates frequent controversies surrounding the WM pathogenesis. Immunophenotypic[1,2], cytogenetic and molecular [3-5] studies have been carried out to gain insight into the characteristics of the malignant clone. Cytogenetic studies in WM have been hampered by the low mitotic index of the tumour cells. Thus, only five meaningful series of patients have reported on the karyotypic findings of WM, and included altogether 165 patients[6-8]. The frequency of cytogenetic abnormalities in these series was quite low (35%, range: 17%-37%) and the most recurrent change was 6q deletion with an occurrence of 6%-16%. Other less frequent abnormalities were the loss of 13q mostly seen in cases with advanced disease[9], and trisomy of chromosome 5 and chromosome 8 but observed in less than 10% of patients.

To overcome the low yield of karyotype in low proliferative diseases alternative techniques such as fluorescence in situ hybridization (FISH) have been used for the study of the disease[9-11]. Deletions of the long arm of chromosome 6 are reported with a frequency of up to 63% [8] while some other aberrations, typical of related B-cell neoplasms such as loss of *RB1* or immunoglobulin heavy chain (IgH) locus rearrangements, have been only rarely detected in WM patients (9%-16% [9,11] and 3-14% [8,11] respectively). Because of the small number of patients studied, a comprehensive analysis in a large series of patients is warranted in order to better define the prevalence of chromosomal changes present in WM.

Although the clinical course is usually indolent, the disease may evolve in two patterns: around one third of the patients are asymptomatic at the time of diagnosis and do not require treatment but only observation. This group is also often referred to as 'smouldering' WM. The rest are symptomatic due to lymphoid infiltration (with consequent bone marrow insufficiency) and/or to the presence of immunoglobulin M (IgM) paraprotein (and the complications arising from it). Among this second group of patients, some display features of aggressive disease and have a shorter survival. While there are some prognostic models devised to estimate the prognosis of WM patients, these models are mostly based on host features (e.g. age, cytopenias, albumin, organomegaly),[12-17] and not in intrinsic features of the clonal cells. Dimopoulos *et al* (2004) recently demonstrated that the International Staging System (ISS) for multiple myeloma (MM), by using albumin and β2-microglobulin (β2M) levels, could also be applied to WM. The different pathways for clinical behavior have stimulated investigation of new prognostic factors, particularly genetic ones that can better predict disease outcome. So far no studies have been carried out

that explore the potential impact of cytogenetics on WM disease evolution, despite genetic changes dictating clinical heterogeneity among many other hematological neoplasms, such as chronic lymphocytic leukemia (CLL) [18-21]and MM[22-26].

In the present study we have analysed the prevalence of 6q deletion in a relatively large number of patients with WM and we have correlated the results with the clinical and biological features of these patients.

PATIENTS AND METHODS

Patients and samples

Bone marrow samples from 102 patients diagnosed with WM from two different institutions were included in the study. Sixty-two of them had been studied at Mayo Clinic (USA) while the other 40 were from Salamanca (Spain). Diagnostic criteria used were those proposed at the consensus panel of the Second International Workshop on Waldenström's Macroglobulinemia[1]: presence of IgM paraprotein in the serum and infiltration in the bone marrow trephine biopsy by lymphoplasmacytic lymphoma as defined by the presence of an infiltrate of lymphocytes, lymphoplasmocytes and plasma cells. Immunohistochemistry was also performed in some of the cases, showing in all of them light chain restriction, presence of pan-B antigens such as CD79a and CD20 and expression of CD38 in the plasma cell population. Other B cell lymphoprolipherative disorders were excluded by the lack of expression of CD5, CD10 and CD23. In those cases in which a trephine biopsy was not available a lymphoplasmacytic involvement of at least 20% in bone marrow aspirate was required. The clinical features of this cohort of patients are summarized in Table 1. Thirty patients (29%) were diagnosed with smouldering WM. This diagnosis required the absence of any symptom attributed to the tumour burden or to the paraprotein and at least 12 months without treatment after diagnosis. Most of the patients were studied at diagnosis, while 35% of them had received treatment previously to the cytogenetic analysis. The median follow-up of the patients included in the analysis was 36 months.

Patients were stratified according to the ISS in three categories: Stage I: albumin ≥ 35 g/L and β2M < 35 mg/L; stage II: albumin < 35 g/L and β2M < 35 mg/L or β2M 35-55 mg/l and stage III: β2M > 55 mg/l.

Conventional cytogenetics

Bone marrow cells were cultured in RPMI medium supplemented with 15% fetal calf serum, penicillin and L-glutamine. All samples were cultured for 72 h with TPA (12-O-tetradecanoylphorbol 13-acetate) as previously described. At least 20

Table 1. Clinical and laboratory characteristics of the 102 patients included in the study.

Parameter		% of patients
Age (years)*	68 (44-94)	
Sex	Male	65%
	Female	35%
Light chain	Kappa	76%
	Lambda	24%
Previous treatment	Yes	35%
	No	65%
WM	Symptomatic	71%
	Asymptomatic	29%
IgM (mg/l)	> 20	79%
	< 20	21%
BM lymphoid infiltration (%)	> 40	36%
	< 40	64%
Hemoglobin (g/l)	> 110	50%
	< 110	50%
Albumin (g/l)	> 40	32%
	< 40	68%
β2M (mg/l)	> 40	29%
	< 40	71%
CRP (mg/l)	> 35	25%
	< 35	75%
ESR (mm/h)	> 100	56%
	< 100	44%
ISS	1	45%
	2	41%
	3	14%

* Continuous variable: results given as median (range)

metaphases were necessary to consider a case evaluable for analysis. Chromosomes were identified by G-banding and karyotypes were described according to the International System for Human Cytogenetics Nomenclature (ISCN, 1995)[27]. Conventional karyotype studies were available in 55 patients (27 from Mayo Clinic and 28 from Spain).

Fluorescence in situ hybridization

In 98 WM patients, deletions of 6q were assessed by either simple interphase FISH performed on cell nuclei (n = 63, Salamanca and Mayo Clinic) or cytoplasmic IgM (cIgM)-enhanced FISH (n = 39, Mayo Clinic) according to our previously published techniques[28,29]. The principal aim of the present study was to analyze the prognostic implications of deletion of 6q in WM patients, so, although a number of different probes were used, we only strived to ascertain in a qualitative manner whether deletions of chromosome 6 were present or not. In 29 of these patients, 6q21 deletion

was screened using a probe derived from clone RP11 91C23 (obtained from Oakland Children's Hospital, Oakland, CA) with simultaneous hybridization of a centromeric probe for chromosome 6 CEP 6 (Vysis, Downers Grove IL)[8]. In the other 73 patients, two probes, derived from clones RP1-134E15 and RP1-32B1 (Oakland Children's Hospital, Oakland, CA) that include genes *BLIMP-1* and *MYB*, were hybridized simultaneously with CEP 6.

A minimum of 100 cells were analysed in all patient samples using Vysis scoring criteria. The cut-off point for the identification of alteration was set at ≥10% cells with abnormal signal

Statistical analysis

The Chi-square and the Mann-Whitney *U*-tests were used to estimate the statistical significance of differences between groups. A *P*-value <0.05 was used to define statistical significance. Survival analysis was performed using Kaplan-Meier method and the statistical significance inter-groups was evaluated with the log-rank test. The statistical analyses were carried out using the Statistical Package for the Social Scienses (SPSS) software-11.0 version (SPSS Inc. Chicago, IL, USA).

RESULTS

Conventional cytogenetics

Abnormal metaphases were present in 22 (40%) of the 55 patients that had metaphase cytogenetics information available, probably selecting a bias of the ordering physicians, secondary to perceived clinical need. Four cases presented with 6q deletion (7%). Abnormalities in chromosome 14 were observed in five patients [30], with +14 in four cases and add(14)(q32) in the remaining case. Other recurrent abnormalities were del(11)(q21), an additional chromosome 5 and monosomy of chromosomes 8 and 18. Three patients displayed a hyperdiploid karyotype.

FISH

Of the 102 patients included in the study, deletion in the 6q21 region was present in 40 of them [30]. Obviously, due to the different sensitivity of both techniques, the percentage of cases with 6q deletion was higher in patients analyzed by cIgM-FISH (54%) than in cases studied by conventional FISH (30%). Given our desire to only ascertain in a qualitative fashion for the presence of 6q deletions, and to increase our sample size for the clinical correlations we decide to include all patients tested, whether studied by cIgM-FISH or not. Only in four of these 39 patients with 6q

deletion detected by FISH was the abnormality also detected by conventional cytogenetics.

In 73 patients, two different probes localised at 6q21 were investigated: one containing *BLIMP-1* and one containing *MYB*. In 30 out of the 31 patients with deletion who were analysed with these two probes, the *MYB* signal was lost. By contrast, the signal for *BLIMP-1* which represents the most centromeric region of 6q21, was deleted in only 26 cases. CEP 6 was lost in only one of the patients analysed, indicating that the existence of monosomy in these cases is uncommon.

Correlation between molecular cytogenetic changes and disease characteristics

Upon comparing the FISH results with the clinical characteristics of patients, we observed that cases with 6q deletion displayed features which, in previous studies, have been associated with worse prognosis in WM (Table 2). Patients with 6q- presented significantly with higher levels of β2M (β2M ≥ 40 mg/L: 52% vs. 14%, P=0.001), anaemia (Hb < 11o g/L in 66% vs. 40%, P=0.01) and hypoalbuminemia (albumin < 4o g/L in 90% vs. 54% of cases respectively P=0.001). The amount of paraprotein has also been described in some reports as having prognostic influence in WM, and, in the present series, IgM levels were higher than 20 g/l in 90% of patients with loss of 6q as compared with 72% of patients without the deletion (P=0.03). In contrast, age showed no difference between both groups of patients (69±8 vs. 66±11, P=0.26). Patients with the cytogenetic aberration also displayed more frequently elevated rates of acute phase reactants, such as erythrocyte sedimentation rate [31] and

Table 2. Clinical and biological features of WM patients with and without deletion of 6q.

	6q- (n=40)	*Non 6q- (n=62)*	*p*
β2M > 40 mg/l	52%	14%	0.001
Albumin < 40 g/l	90%	54%	0.001
CRP > 35 mg/l	47%	12%	0.006
Hb < 110 g/l	66%	40%	0.01
IgM > 20 g/l	90%	72%	0.03
ESR > 100 mm/h	73%	46%	0.04
ISS 1	29%	55%	
ISS 2	46%	37%	0.05
ISS 3	25%	8%	
Requirement of treatment	87%	67%	0.02
Age (years)	69 ± 8	66 ± 11	0.26
WM symptomatic	75%	68%	0.28
Sex male	70%	62%	0.28
BM infiltration >40%	40%	33%	0.381
Previously treated	41%	32%	0.27
Time from diagnosis to test (months)*	35.6 (53.2)	29.4 (44.7)	0.61

* Continuous variable: results given as mean (standard deviation)

C-reactive protein (CRP). There was no difference in the prevalence of 6q deletion between patients with symptomatic and indolent WM (42% vs 33%, *P*=0.28).

We also analysed if there was any correlation between the deletion of 6q and the ISS prognostic index. And we observed that, with differences close to the limit of significance, the frequency of 6q- increased with the stage of the ISS. In this sense, only 24% of patients in stage 1 displayed the abnormality as compared to 42% and 67% in patients included in stage 2 and 3 respectively (*P*=0.05).

No differences were observed in the frequency of 6q deletion among those cases analysed at the moment of diagnosis and those who had been previously treated (35% vs. 44% respectively, *P*=0.27). Similarly, 6q- was not correlated with the time spent from diagnosis to the cytogenetic analysis, as it was 35.6 months for patients with the deletion as compared with 29.4 months for those without the abnormality (*P*=0.61).

Prognostic impact of molecular cytogenetic changes in WM

Regarding the prognosis, patients that had deletion of 6q frequently required treatment; 87% of patients with 6q- required treatment while only 67% of patients without the abnormality were treated during the evolution of their disease (*P*=0.02). We further investigated the importance of loss of 6q in the timing of treatment among those cases diagnosed with smouldering disease who, for this reason, were not treated at the moment of diagnosis. And in this subset of patients, those with 6q deletion displayed a shorter treatment free survival (median of 55.2 months), when compared to not reached after a follow-up of 100 months for patients without the deletion (*P*=0.03) (Figure 1).

The survival analysis did not reveal a clear association with 6q deletion in the global subset of patients. When only those patients with symptomatic WM were analysed, the survival curve of patients displaying the abnormality was slightly worse than that of patients without the abnormality, although differences were not statistically significant (Figure 2).

DISCUSSION

Information on cytogenetic abnormalities in WM is scarce due to the low proliferative index of the tumour cell population and the rarity of the disease. As a consequence, studies that seek to correlate the cytogenetic profile and clinical outcome of WM patients are, by their nature, complex and may require the use of aggregate data from multiple institutions.

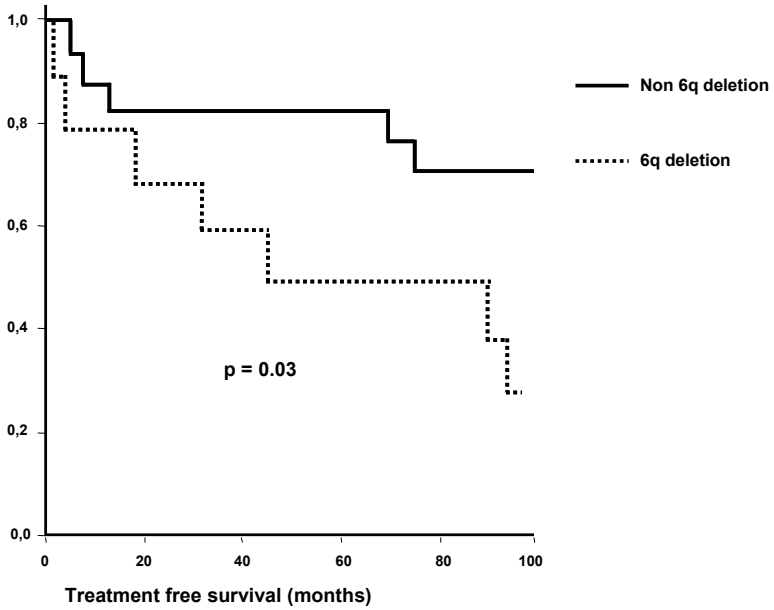

Figure 1. Treatment free survival for patients with smouldering WM. Patients with 6q deletion (n=10) required treatment sooner than those without 6q deletion (n=18).

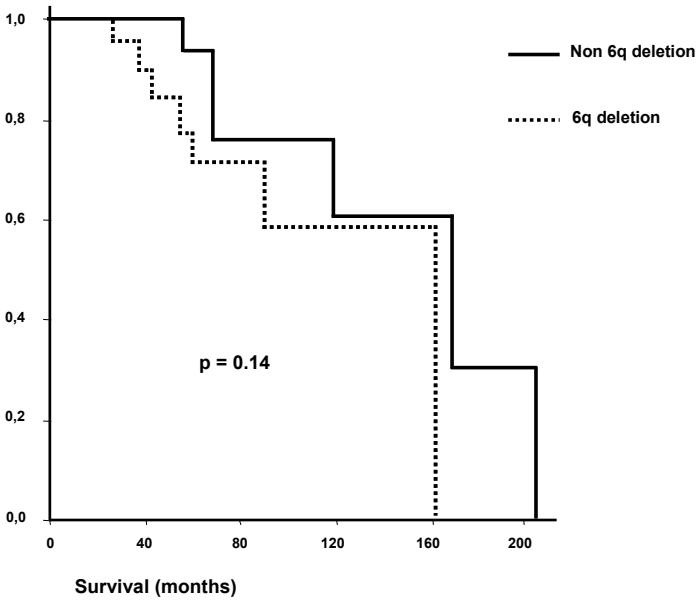

Figure 2. Overall survival for patients with symptomatic WM. Patients with 6q deletion (n=30) did not displayed an statistically significant poorer survival than those without 6q deletion (n=39).

By necessity, the present study combined the patients of two institutions. All patients were analyzed by FISH. In one-third of cases, tumour population was detected and scored by use of additional cIgM staining (cIgM FISH). Although the sensitivities of the two techniques may differed slightly, we believe that the overall sensitivity is sufficient on a population scale to assess the role of 6q status in WM.

In the present series, by performing conventional cytogenetics (CC) and FISH analysis targeting 6q region, up to 55% of WM patients displayed cytogenetic aberrations. Previous reports have shown that 6q- is the most common abnormality, either by conventional cytogenetics[6-8] or FISH[8,32], up to the point that it has become a WM-associated marker[6,7,33]. The prevalence of 6q deletion by means of FISH was 39% in our series, although we could find some differences among those patients analysed by cIgM-FISH and those analysed with conventional FISH, because of the different sensitivity of both techniques. In this sense, 54% of patients analysed by cIgM-FISH displayed the abnormality when compared with only 30% among those patients analysed by interphase FISH. Nevertheless, even when employing the less sensitive of these techniques, 6q deletion was the most frequent abnormality of all those tested in WM. Although this abnormality is also observed in other B-cell neoplasms, it is usually present at a much lower frequency[34-36]. In addition, a detailed mapping study has shown that 6q deletions are large deletions and clonally selected, and that is present in the majority of clonal plasma cells (R. Fonseca, personal communication). Because of its recurrent nature it has been suspected that the chromosome 6q region harbours a tumour suppressor gene of pathogenetic significance for WM[8,36]. The precise timing of deletions as a mechanism of loss of heterozygosity is unknown (i.e. at disease initiation *versus* as a progression event). Limited information suggests the latter, given that 6q deletions appear to be uncommon in IgM monoclonal gammopathy of undetermined significance (MGUS), a precursor state of WM. Our study showed no difference in the frequency of 6q deletion at diagnosis compared to previously treated patients. The time spent from diagnosis to performance of FISH analysis was similar in both groups.

When we stratified WM patients according to 6q deletion, we observed that, in line with our previous observations in a smaller group, 6q- patients were more likely to display laboratory features associated with adverse prognosis[14-17], such as high levels of β2M and paraproteinemia, or hypoalbuminemia. The ISS, a recently proposed prognostic index for WM, showed a trend (P=0.05) to display more frequently 6q- in those cases with advanced stages of the disease. Other differentiating markers were some acute phase reactants, like CRP or ESR, which displayed higher levels in cases with the deletion. The similar incidence of 6q deletion in asymptomatic and symptomatic WM, despite its tendency to display adverse prognosis, is intriguing. Nevertheless a parallel situation has been observed for 13q deletion and t(4;14) in

MM; while these cytogenetic abnormalities have been associated with worse outcome in MM[26,37], their incidence is similar in both MGUS and symptomatic MM[38].

This correlation of the presence of 6q deletion with clinical and biological parameters of advance disease was also confirmed by the fact that these patients also had a higher requirement of chemotherapy treatment. In fact, the great majority of patients (87%) with loss of 6q were treated during the evolution of the disease, while one-third of the patients without loss of 6q never required treatment in our series (P=0.02). This data, combined with the shorter time to receive treatment for patients diagnosed with indolent WM who have 6q deletion, suggest that patients with the deletion who are asymptomatic or with mild symptoms at diagnosis should be carefully followed because most of them will progress to a clear symptomatic form and treatment should be started as soon as possible.

In our opinion, the lack of impact of 6q deletion in the survival analysis can be explained by the short follow-up of the patients included in the study. The indolent nature of WM and the long survival these patients are well known, and makes it necessary to follow the patients for a long time to be able to show differences in survival. In our relatively large study, although some of the patients have a very long follow-up (longer than 10 years), half of them were diagnosed in the last 3 years (median follow-up 36 months), and therefore, a longer follow-up of these groups of patients could reveal greater differences in their outcome.

In summary, our results show that cytogenetic abnormalities in WM may contribute to a greater understanding of the disease heterogeneity and could help to identify patients in different prognostic groups.

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CHAPTER 6

Gene-expression profiling of Waldenström macroglobulinemia reveals a phenotype more similar to chronic lymphocytic leukemia than multiple myeloma

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ABSTRACT

Waldenström macroglobulinemia (WM) is a B-cell malignancy characterized by the ability of the B-cell clone to differentiate into plasma cells. Although the clinical syndrome and the pathological characteristics are well defined, little is known about its biology and controversy still exists regarding its cell of origin. In this gene-expression study, we compared the transcription profiles of WM with those of other malignant B cells including (chronic lymphocytic leukemia [CLL] and multiple myeloma [MM]) as well as normal cells (peripheral blood B-cells and bone marrow plasma cells). We found that WM has a homogenous gene-expression regardless of 6q deletion status and clusters with CLL and normal B-cells on unsupervised clustering with very similar expression profiles. Only a small gene set has expression profiles unique to WM compared to CLL and MM. The most significantly up-regulated gene is *IL6* and the most significantly associated pathway for this set of genes is MAPK signaling. Thus, IL6 and its downstream signaling may be of biologic importance in WM. Further elucidation of the role of IL-6 in WM is warranted as this may offer a potential therapeutic avenue.

INTRODUCTION

Waldenström macroglobulinemia (WM) is a clinico-pathologically distinct B-cell malignancy characterized by intramedullary monoclonal expansion of predominantly small B-lymphocytes with variable plasmacytoid differentiation in the bone marrow (BM), associated with serum IgM paraprotein. Histologically, this represents bone marrow involvement by lymphoplasmacytic lymphoma [1].

Although the clinical disease spectrum has been well established, little is known about its biology. The cell of origin is thought to be an unusual memory B-cell that has undergone somatic hypermutation in the absence of antigenic selection with failure in the class switch recombination [2,3]. Genetically, unlike many other B-cell malignancies, translocations involving the IgH locus on chromosome 14q32 are rare in WM [4,5]. Karyotypic analysis is hampered by a low yield of abnormal metaphases. The most common genetic abnormality is deletion of the long arm of chromosome 6 [5,6]. However, this cytogenetic abnormality is not unique to WM and its biological and clinical significance in WM has yet to be elucidated.

In this study, we undertook a gene-expression profiling (GEP) study of WM using $CD138^+$ and $CD19^+$ selected malignant bone marrow cells, and compared their transcription program with related B-cell malignancies chronic lymphocytic leukemia (CLL) and multiple myeloma (MM) as well as normal B-cell and plasma cell (PC) counterparts. The selection of cells using both CD138 and CD19 should encompass the full spectrum of malignant cells, so that we can define a molecular signature for WM, its relation to other normal and malignant B-cell counterparts, and identify biologically relevant and important pathways, new disease markers and potential therapeutic targets in an unbiased fashion. We found that the transcription profile of WM is very similar to CLL and normal B-cells, and distinct from MM and normal PCs. Only a small gene set is unique to WM. Pathway analysis of genes unique to WM provide further insights into the biology of this disease.

MATERIALS AND METHODS

Samples

Twenty-three WM, 8 CLL, 101 MM, 24 SMM, 22 monoclonal gammopathy of unknown significance (MGUS; including 1 IgM MGUS), 15 normal PC and 7 normal B cells were included in this study. Blood and BM samples were procured after informed consent was obtained in accordance with the Declaration of Helsinki. The study was approved by the Institutional Review Board of the Mayo Clinic. The samples were enriched for cell population of interest using immunomagnetic beads

(AutoMACS, Miltenyi-Biotec, Auburn, CA). For WM and the case of IgM MGUS, $CD19^+$ and $CD138^+$ (concurrent but not sequential) selected BM cells were used. For MM, SMM, MGUS and normal PCs, $CD138^+$ selected BM cells were used. For CLL and normal B cells, $CD19^+$ selected peripheral blood cells were used. The CLL, WM and PC neoplasm samples were consecutive samples with quantitatively and qualitatively adequate RNA for gene-expression study. The common cytogenetic abnormalities and categories were present (6q deletion for WM, IgH translocations, 13 deletion and hyperdiploidy for MM, and mutated and unmutated IgVH for CLL) in these patients at frequencies similar to published data (data not shown).

Gene-expression profiling

RNA was extracted from the enriched cells. Gene-expression analysis was performed using the Affymetrix U133A chip for WM, MM, SMM, MGUS and normal PCs and the U133A and U133B chips for CLL and normal B cells (Affymetrix, Santa Clara, CA). RNA isolation, purification and microarray hybridization have been previously reported [7]. Gene-expression intensity values were log transformed, normalized to the median and analyzed using GeneSpring 7 (Agilent Technologies, Palo Alto, CA).

Gene-expression analysis

For all analysis, the universal gene list was all genes on the U133A chip minus immunoglobulin genes (total 22104 genes). To define the relation between WM, CLL, PC neoplasm, normal PCs, and B-cells, we first generated a list of genes whose expression varied significantly across individual samples by Welch ANOVA using variance computed by applying the Cross-Gene Error Model (CGEM) based on Deviation from 1 available within GeneSpring. This overcomes the lack of replicates and variance associated with the individual samples and is similar in principle to variance filtering. The generated gene list was then used for unsupervised clustering of the CLL, WM, PC neoplasm, normal PCs and B-cell samples using a hierarchical agglomerative algorithm. To detect possible heterogeneity and identify subtypes within WM, all genes were used for unsupervised clustering. The Pearson correlation coefficient and centroid linkage were used as similarity and linkage methods, respectively.

To identify genes with unique gene-expression profiles for WM, CLL and MM, we first, identify genes with significantly different expression between WM, MM and CLL by ANOVA with Benjamini and Hochberg multiple testing corrections. Genes whose *P* value is less than .05 and that pass a false discovery rate (FDR) of 5% were selected for further filtering by fold change (2-fold or higher different expression between WM and others).A similar sequence was applied to MM and CLL. Finally, a Venn diagram incorporating the 3 filtered gene sets (1 each for WM, MM and CLL) was used to identify genes unique to each disease. The specificity of the WM

signature was further validated by leave-one-out cross-validation (LOOCV) using 2 algorithms: the *k*-nearest neighbor (KNN) and the support vector machines (SVMs). In the KNN method, a sample is classified based on a majority vote of the classes of the *k* neighbor that are closest to it in terms of Euclidean distance. In the SVM method, a sample is classified based on its position relative to an optimal linear decision boundary constructed on a transformed feature space of the microarray data. For both methods, the predictor genes are those with unique profiles in WM compared to CLL or MM (significant by ANOVA after multiple testing corrections and greater than 2-fold difference in expression) as outlined. For the KNN method, optimal settings are number of neighbors of 15 and *P* value ratio of 0.1. For SVM, radial kernal function was used.

Inspection of some of these disease-specific gene lists revealed genes that could represent contaminating cell populations acquired during selection; pre-B cells (expressing *NPTT* [tdt], *MME* [CD10], *VPREB1*), and monocyte/macrophages (expressing *CD36, CD163, CD14, APOE*). Subsequently, we generated lists of genes whose expression may be the results of these contaminating cell populations by finding genes whose expression profiles were correlated with tdt (for Pre-B cells) and CD36 (for monocytes) using a Pearson's correlation coefficient of 0.7 as the cutoff value.

Because the initial unsupervised clustering of samples may be affected by the presence of these contaminating cells, we extracted previously published CLL signature (unique to CLL when compared to different B cell types and B cell malignancies although not including WM or MM) [8]. Unique MM signature has not been previously defined. As a compromise we extracted genes differentially expressed between MM and normal PCs previously published [9] as a surrogate for a MM signature. Interestingly, the 2 gene sets do not overlap. Using both gene signatures, we clustered WM with CLL and MM samples to confirm the similarity between WM and CLL but not MM

Gene ontology and pathway / network analysis was performed using a web-based software, MetaCore (GeneGo Inc, St Joseph, MI). The software contains an interactive, manually annotated database derived from literature publications on proteins and small molecules that allows for representation of biological functionality and integration of functional, molecular, or clinical information. Several algorithms to enable both the construction and analysis of gene networks were integrated as previously described [10]. The output *P* values reflect scoring, prioritization and statistical significance of networks according to the relevance of input data.

RESULTS

Gene-expression profile of WM is related to CLL and normal B-Cells

On unsupervised clustering, WM clustered together with normal B cells and CLL samples (first branching of the dendogram, Figure 1a). Furthermore, inspection of the heatmap suggested very similar profiles between these conditions. Of interest, the one case of IgM MGUS in the dataset clustered with WM instead of MGUS or normal PCs. WM samples were split into 2 groups with CLL and IgM MGUS clustered in between. The separation of WM into 2 clusters had no relation to 6q deletion status as both clusters had similar number of patients with 6q deletion (Supplementary Figure 1)[11]. The expression profile of the smaller WM cluster (which

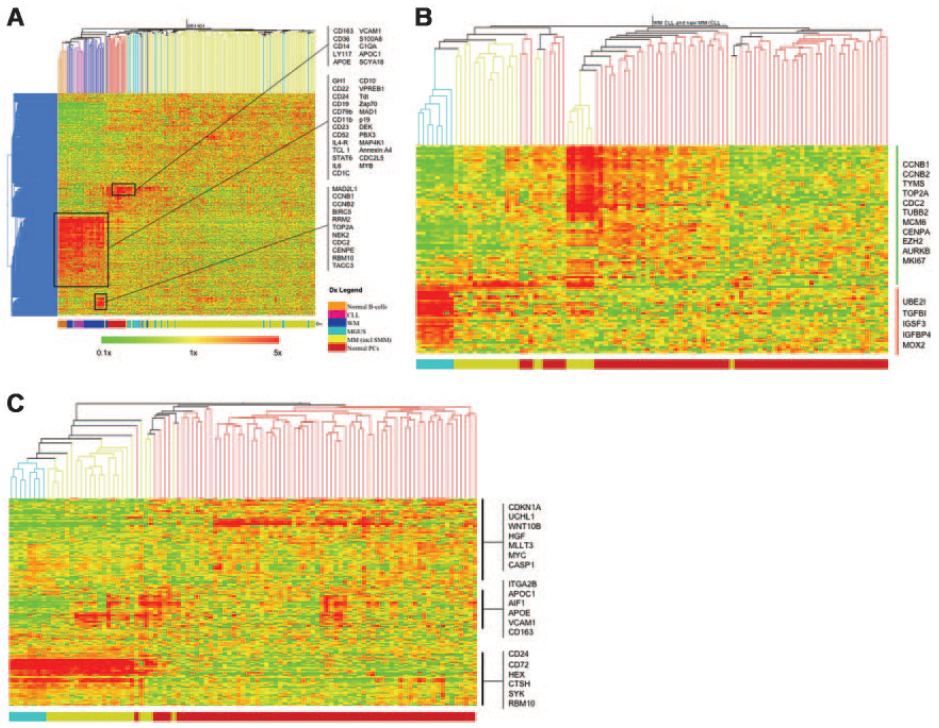

Figure 1. Gene-expression profile of WM is closer to CLL than MM. (a) Using 2162 gene probes that are variably expressed across the WM, CLL and MM samples, unsupervised clustering of these samples together with normal B-cells, PCs, MGUS and SMM was performed. Selected genes that clustered together and were overexpressed in different sample clusters are highlighted. Restricting our analysis to only WM, CLL and MM samples, unsupervised clustering was performed using published b) CLL and c) MM signatures. WM samples clustered predominantly with CLL samples and also exhibited closer approximation of gene-expression profile using both signatures to CLL. Some genes of interests are highlighted. In both heatmap, the colored bar at the bottom indicates the tumor type: blue = CLL, yellow = WM and red = MM. The scale of the gene-expression data is similar to figure 1a.

clustered with IgM MGUS, WM cluster 1) was closer to normal B-cells than the larger WM cluster (WM cluster 2; Figure 1a). In addition, 2 WM samples clustered next to normal PCs and 1 sample clustered with MM and MGUS (WM cluster 3). In general, the percentage of plasma cells in the bead purified cells used for gene-expression profile increased from WM cluster 1 to WM cluster 3, suggesting the clustering of WM into these groups may reflect a difference in degree of plasmacytic differentiation of the tumor cells (Table 1).

Normal PCs and most of the MGUS samples clustered together. A significant number of genes overexpressed by these samples are genes expressed in monocytes/macrophages (*CD163, CD36, CD14, APOE, VCAM1*). These genes were also overexpressed in some WM cases (Figure 1a). This probably represents genes expressed by contaminating monocytes/macrophages resulting from immunomagnetic bead selection of BM cells. This could be due to non-specific binding of beads to Fc receptors present on the surface of monocytes/macrophages or phagocytosis of these beads by the monocytes/macrophages. This was most apparent when the percentage of cells

Table 1. Percentage of Plasma Cells and Bone Marrow involvement by tumor in WM samples and their correlation with contamination signature and clustering.

Samples	PCs in purified samples (%)	BM involvement (%)	Contamination Signature	Clustering
WM8	25	20-30	Nil	WM 1
WM15	16	50-60	Nil	WM 1
WM22	NA	60-70	Nil	WM 1
WM25	8	80	Nil	WM 1
WM2	45	10	Monocytes	WM 1
WM10	26	80-90	Nil	WM 1
WM24	70	10-20	Nil	WM 2
WM17	38	80	Nil	WM 2
WM3	18	30	Monocytes	WM 2
WM12	26	20	Nil	WM 2
WM5	90	60-70	Nil	WM 2
WM19	NA	NA	Nil	WM 2
WM20	NA	NA	Monocytes	WM 2
WM11	28	30	Monocytes	WM 2
WM4	36	20-25	Pre-B	WM 2
WM18	NA	NA	Pre-B	WM 2
WM7	NA	30	Pre-B	WM 2
WM13	23	20	Pre-B	WM 2
WM16	14	20-30	Pre-B	WM 2
WM9	22	10	Pre-B	WM 2
WM23	95	30	Monocytes	WM 3
WM26	94	10	Monocytes	WM 3
WM14	49	<5	Monocytes	WM 3

Abbreviation: NA = Not available

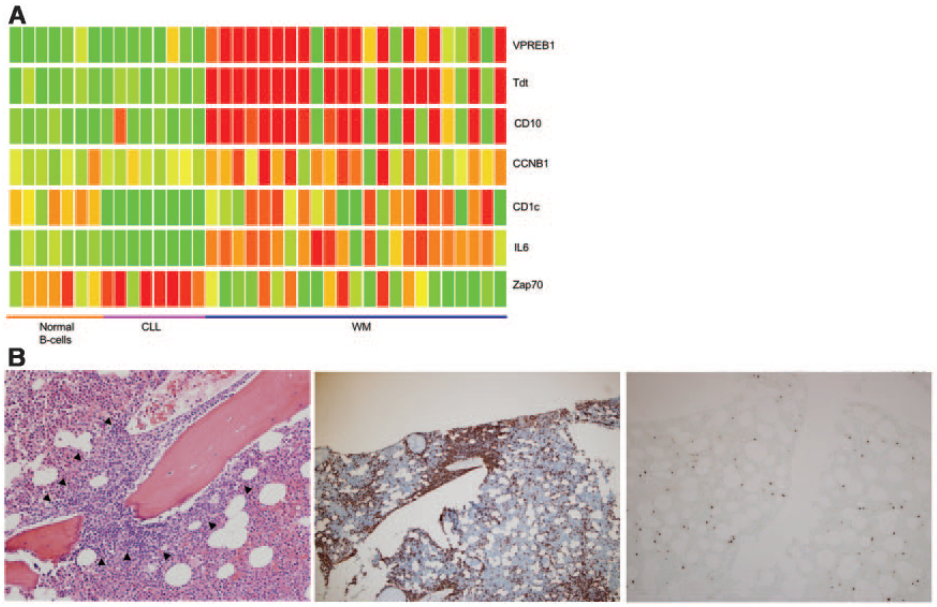

Figure 2. Contributions of genes from contaminating cell populations. (a) Closer inspection of some of the genes within the gene cluster overexpressed in normal B-cell, CLL and WM revealed some interesting differences. In particular, the concurrent overexpression of *NPTT* (tdt), *MME* (CD10), and *VPREB1* in the WM samples suggested likely contamination with pre-B cells. In addition, *ZAP70* and *CCNB1* (as a representative gene from the proliferation cluster overexpressed in a subset of WM patients) overexpression is tightly associated with samples overexpressing *NPTT* (tdt), *MME* (CD10), and *VPREB1*. (b) To verify that tdt expression was originating from contaminating pre-B cells, immunostaining for tdt was performed on bone marrow biopsies from WM patients with and without the pre-B cell contamination signature. This figure is representative for samples with the contamination signature. Malignant cells (CD20 positive) formed intra-medullary clusters (marked by arrows; top panel H+E staining; middle panel CD20 staining). In contrast, nuclear tdt staining was seen in scattered interstitial cells that represented pre-B cells (right panel). In samples without the contamination signature, no tdt positive cells were seen. All images were at 40x magnification. The microscope used is a Zeiss Axioskop (Carl Zeiss Microimaging Inc, Thornwood, NY). Images were captured by the Olympus DP70 CCD camera using the DP controller image capture software (Olympus, Center Valley, PA).

selected for was low in the bone marrow (in this case $CD138^+$ PCs in normal and MGUS BM or WM samples with fewer malignant cells). This problem is less apparent if the tissue source of selection is peripheral blood (normal B cells and CLL samples) because monocytes are very minor populations of peripheral-blood cells. As corroborating evidence, the WM cases with this monocyte contamination signature had the lowest degree of BM tumor involvement (Table 1). In view of the possible impact of these contaminating cells and their gene-expression on interpretation of results, we created list of genes that are highly correlated with these pre-B cell and monocytes/macrophage genes, so that they can be subtracted from subsequent analysis of genes with expression profiles unique to the individual tumor types. There were also cases of MGUS that clustered with MM samples (Figure 1a). This is consistent with the well-known similarity in global gene-expression between MGUS and MM [9].

The 2-dimensional clustering also revealed different clusters of genes that were overexpressed in the different tumor/tissue types. The expression profiles of normal B cells, WM and CLL samples were very similar (Figure 1a). These samples overexpressed a cluster of genes that included B-cell markers such as *CD22*, *CD19, CD79b*, and *CD11b*, potential therapeutic targets such as *CD52*, prognostic markers (*ZAP70*), a set of genes normally expressed in pre-B cells (*MME* [CD10], *VPREB1, NPTT*[tdt]) and genes of potential biological relevance (*IL6*) (For a full list of genes in this cluster refer to supplementary material)[11]. The appropriate expression of B-cell markers provided some internal validation to the gene-expression data. We decided to look closer at the expression of *IL6*, *ZAP70*, *MME, VPREB1* and *NPTT* in CLL and WM (Figure 2a). *IL6* expression was high (>2-fold higher than CLL and MM) in most WM compared to CLL and normal B-cells. *ZAP70* expression was high in 7 of the 8 CLL samples and a subset of WM patients. *MME, VPREB1* and *NPTT* were overexpressed in the same WM samples and most likely represent contaminating bone marrow pre-B cells resulting from the CD19 selection process. Interestingly, the expression of *ZAP70* in WM was highest in the samples with the presumed pre-B cell contamination. In view of previous studies showing *ZAP70* expression in normal B-cells [12,13] and lack of *ZAP70* expression in WM [14], the *ZAP70* expression in the subset of WM was most likely because of the contaminating pre-B cells.

A subset of WM appeared to overexpressed genes involved in cell cycle and proliferation (*MAD2L1, CCNB1, CCNB2, TOP2A, NEK2, CENPE, CDC2*). Interestingly these corresponded to the WM samples with the highest expression of *ZAP70* but they did not constitute all the samples with the contaminating 'pre-B cell signature' (Figure 1a and 2a). As ZAP-70 is overexpressed in "activated" B cells [12], it is possible that these samples are contaminated with more activated and proliferating pre-B cells as compared to other WM samples.

To validate that these genes were expressed by contaminating cells, staining for tdt by immunohistochemistry was performed by an expert hemato-pathologist for 5 samples with the contamination signature and 5 without and showed that tdt was positive only in the non-malignant pre-B cells (hematogones) in the 5 samples with the contamination signature and tdt staining was absent in the 5 samples without the contamination signature. A representative example is shown in figure 2b. This strongly suggested that overexpression of tdt and the strongly correlated set of proliferation genes including *ZAP-70* was due to the presence of contaminating pre-B cells.

Because our unsupervised clustering may be affected by these contaminating cells, we sought to verify the observation that the expression profile of WM was similar to CLL but different from MM by using published CLL and MM signature (see "Material and methods"). It was clear that using either signature, WM was clustered together

with CLL and not MM. Furthermore, the expression profile of genes constituting the 2 signatures was more similar between WM and CLL than MM (Figure 1b-c).

WM has a homogeneous gene-expression profile.

Initial attempts to identify genes with variable expression across the 23 WM samples using ANOVA yielded a very small list of genes (<50). Therefore, we performed unsupervised clustering of WM samples using all genes. This analysis suggested a relatively homogeneous expression profile amongst the WM samples except for 4 samples which underexpressed a subset of genes (Supplementary Figure 1)[11]. On closer inspection, these cases had the highest PC percentage and low BM involvement, and the under-expression of these genes were also seen in normal PCs and MGUS but not in CLL or B cells (data not shown). These 4 cases were not IgM myeloma as the patients had no bone disease and were negative for t(11;14) [15], instead, they had lymphadenopathy or splenomegaly and in 1 case also a 6q deletion by in situ hybridization (FISH), which were all hallmarks of WM. There were no obvious difference in the expression profiles between samples with the 6q and those without the deletion because they clustered together on unsupervised clustering and no genes were differentially expressed between 6q-deleted and non-deleted cases.

Analysis of expression of cluster of differentiation (CD) markers, and genes involve in cell cycle regulation

In an attempt to identify possible diagnostic markers, we next analyzed the expression of cluster of differentiation (CD) markers among the samples. The expression of CD markers in WM was similar to CLL and B-cells and different from MM and normal PCs (Supplementary Figure 2a)[11]. The expression of B-cell markers such as *CD19, CD20, CD22, CD23* and the common leukocyte antigen (*CD45*) in normal B-cells, CLL and WM but not MM; expression of *CD5* in CLL but not normal B-cells, WM, MM or normal PCs; expression of PC markers such as *CD38* and *CD138* in MM and normal PCs but not in normal B-cells, CLL or WM provided internal validation of the gene-expression results as these were known immunophenotypic markers for the respective cell types (Supplementary Figure 2b)[11]. The low but higher expression of *CD45* and *CD19* in normal PCs compared to MM and expression of *CD56* in MM but not normal PCs are consistent with the difference in the expression of these markers between normal and malignant plasma cells [16,17]. In terms of potential new disease markers, *CD1C* was highly expressed in almost all cases of WM but was not expressed in CLL or MM; however strong expression was also seen in normal B-cells (Figure 2a and supplementary Figure 2b)[11]. *CD200* was strongly expressed in CLL but not in WM or MM and expression was weaker in normal B-cells. Of therapeutic interest, CD52 was expressed in CLL and WM but not MM whereas *CD117* (c-kit) was expressed in MM but not WM or CLL. *CD20* appears to be expressed strongly in

normal B cells, CLL, WM with weak expression in MM (supplementary Figure 2b)[11]. Consistent with published data [18], the expression of CD20 was found predominantly in patients with t(11;14).

Because deregulation of the cell cycle is common in B-cell malignancies and these deregulated pathways are potential therapeutic targets [19], we investigated and compared the gene-expression of genes involved in these processes between CLL, WM and MM (supplementary Figure 2)[11]. The expression of cell-cycle genes was very similar between CLL and normal B-cells, whereas there were more differences between MM and normal PCs. WM seemed to have an intermediate pattern of expression for these genes. In terms of the D-type cyclins, CLL and WM only expressed *CCND3* whereas MM expressed all 3 cyclin D genes. Of note, the CDKI genes were predominantly up-regulated in the malignant conditions compared to their normal cellular counterpart. In MM, *p14*, *p15*, *p18* and *p21* were up-regulated and only *p27* and *p57* were down-regulated compared to normal PCs. In WM, p15, p18, and p57 were up-regulated compared to normal B-cells (Supplementary Figure 3)[11]. This suggests that on the whole, CDKIs are up-regulated in these B-cell malignancies and we speculate that these are secondary responses to check proliferation of tumor cells. The expression of *CCNB1* and *CDC2* is usually an indication of proliferation. These were not expressed in CLL consistent with its known low proliferative index. The expression of these genes was significantly higher in WM and correlated with expression of *DNTT*, probably representing pre-B-cell contamination (Figure 2a).

Genes with distinct expression profile in WM, CLL and MM are involved in different pathways relevant to their biology

Seventy-three genes had a distinct expression profile in WM compared to CLL and MM (Figure 3 and Table 2). The specificity of this WM signature was further tested by LOOCV. Using both KNN and SVM, all the WM were correctly predicted. Of all the non-WM cases, only 1 case was not predicted by KNN (did not pass the *P* value ratio) and wrongly predicted by SVM. Forty-eight of these were up-regulated and 25 down-regulated in WM. Interestingly, the most significantly up-regulated gene in WM is *IL6*. The aforementioned *CD1C* was also among the top 10 most significantly up-regulated genes.

A total of 396 genes had a distinct expression profile in CLL compared to WM and MM; 174 of these genes were up-regulated and 222 down-regulated in CLL. As expected, up-regulated genes included *BCL2* and *ZAP70*. Among the down-regulated genes were cell-cycle-related genes (*CDC2, CDC20, NEK2*), and several CDKI (*CDKN1C, CDKN1A, CDKN2C*).

A total of 1247 genes had a distinct expression pattern in MM compared to CLL and WM; 577 of these were up-regulated and 670 down-regulated in MM. Many of

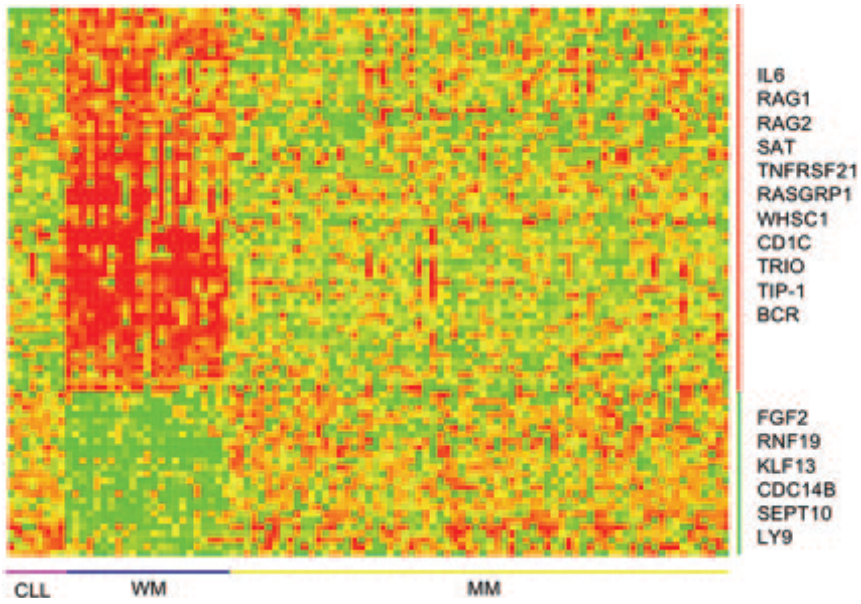

Figure 3. Gene-expression signature unique to WM. Seventy-three genes, 48 up-regulated and 25 down-regulated constitute a gene-expression signature unique to WM. Here the samples are ordered according to diagnosis and genes according to fold difference in expression between WM and CLL and MM. Some interesting genes are highlighted. For a more complete list see supplementary material. The scale of the gene-expression data is similar to figure 1a.

the up-regulated genes are involved in signaling pathways known to be important in MM such as WNT signaling (*DKK1, FRZB, WNT10B, WNT5A, FZD6, WNT6*), *IL6R, IGF1R, MET* (HGF receptor) and *HGF*. Among the down-regulated genes were several known to be important in early B cell receptor signaling and differentiation that tend to be down-regulated with terminal differentiation to PCs (*PAX5, CD19, VAV*). The top 10 up- and down-regulated genes and other selected genes of interest in WM, CLL and MM are appended in table 2, 3 and 4, respectively (for the full gene lists, please refer to supplementary material)[11].

When these disease specific genes were analyzed for relevant processes and pathways using Metacore, interesting differences were obtained. The gene ontology (GO) processes most associated with the genes unique to MM were involved in signal transduction and intracellular signaling, in particular cell-surface receptor linked signaling whereas the most relevant pathways includes AKT, IGF-1R and WNT signaling as well as prostacyclin synthesis, angiopoietin signaling, and integrin mediated cell adhesion. For CLL, the relevant processes were immune response, apoptosis and cell cycle regulation and the most relevant pathways were involved in apoptosis regulation (data not shown). Due to the small list of genes unique to WM, no significant pathways related to the set of genes were detected. However, the most

Table 2. Genes with unique gene-expression profile in WM (The top 10 up- and down-regulated genes plus other genes of interest are shown).

Probe	P-Value	Gene Names	Fold Change Rel to MM	Fold Change Rel to CLL	Descriptions
Up-regulated in WM compared to CLL and MM					
205207_at	4.31E-10	IL6	4.39	6.41	interleukin 6 (interferon, beta 2)
204081_at	2.49E-08	NRGN	3.23	5.38	neurogranin (protein kinase C substrate, RC3)
201310_s_at	2.51E-07	P311	4.9	6.29	P311 protein
209626_s_at	1.02E-06	OSBPL3	3.63	2.77	oxysterol binding protein-like 3
205987_at	1.06E-05	CD1C	24.4	17.99	CD1C antigen, c polypeptide
210640_s_at	1.80E-05	GPR30	9.75	8.93	G protein-coupled receptor 30
205240_at	2.82E-05	HSU54999	2.28	3.66	LGN protein
211829_s_at	4.80E-05	GPR30	6.08	8.93	G protein-coupled receptor 30
202497_x_at	5.01E-05	SLC2A3	7.55	4.39	solute carrier family 2 (facilitated glucose transporter), member 3
215464_s_at	5.75E-05	TIP-1	4.1	4.85	Tax interaction protein 1
209053_s_at	0.0035779	WHSC1	3.12	2.94	Wolf-Hirschhorn syndrome candidate 1
Down-regulated in WM compared to CLL and MM					
222154_s_at	3.70E-08	DKFZP564A2416	6.33	6.64	DKFZP564A2416 protein
219878_s_at	2.57E-07	KLF13	5.99	6.04	Kruppel-like factor 13
221163_s_at	1.66E-06	WBSCR14	3.39	4.58	
216869_at	2.01E-06	PDE1C	2.42	3.21	PDE1C3 splice variant; 3',5' cyclic nucleotide phosphodiesterase
218182_s_at	6.47E-06	CLDN1	2.05	3.55	claudin 1
219630_at	6.83E-06	DD96	2.16	3.22	epithelial protein up-regulated in carcinoma, membrane associated protein 17
206736_x_at	1.12E-05	CHRNA4	3.09	3.75	cholinergic receptor, nicotinic, alpha polypeptide 4
206994_at	1.26E-04	CST4	3.24	2.97	cystatin S
215967_s_at	1.33E-04	LY9	3.27	2.93	lymphocyte antigen 9
207553_at	1.48E-04	OPRK1	3.88	3.09	opioid receptor, kappa 1

Table 3. Genes with unique expression profile in CLL (The top 10 up- and down-regulated genes plus other genes of interest are shown).

Probe	P-Value	Gene Names	Fold Change Rel to MM	Fold Change Rel to WM	Description
Up-regulated in CLL compared to WM and MM					
218704_at	1.80E-27	FLJ20315	3.34	3.15	hypothetical protein FLJ20315
221010_s_at	8.59E-21	SIRT5	3.44	2.86	sirtuin silent mating type information regulation 2 homolog 5
203072_at	3.00E-18	MYO1E	7.97	7.77	myosin IE
204155_s_at	6.06E-16	KIAA0999	3.51	2.83	ESTs
204446_s_at	1.74E-15	ALOX5	9.13	8.97	arachidonate 5-lipoxygenase
208858_s_at	3.47E-15	KIAA0747	4.95	4.93	KIAA0747 protein
214366_s_at	7.54E-15	ALOX5	7.66	4.36	arachidonate 5-lipoxygenase
208269_s_at	1.82E-14	ADAM28	38.87	5.47	a disintegrin and metalloproteinase domain 28
205389_s_at	4.34E-14	ANK1	10.23	4.74	ankyrin 1, erythrocytic
209670_at	2.15E-12	TRAC	13.03	7.38	T-cell receptor alpha-chain (VDJC); Human T-cell receptor active alpha-chain mRNA from JM cell line, complete cds.
203685_at	1.38E-09	BCL2	3.96	3.01	B-cell CLL/lymphoma 2
214032_at	1.46E-04	ZAP70	11.79	6.71	zeta-chain (TCR) associated protein kinase (70 kD)
Down-regulated in CLL compared to WM and MM					
222315_at	8.41E-24	ESTs	28.09	40	ESTs
202768_at	2.64E-22	FOSB	82.6	107.76	FBJ murine osteosarcoma viral oncogene homolog B
202708_s_at	2.37E-19	H2BFQ	4.88	3.07	H2B histone family, member Q
201830_s_at	8.07E-16	NET1	19.4	23.7	neuroepithelial cell transforming gene 1
201694_s_at	4.47E-14	EGR1	8.62	6.41	early growth response 1
205780_at	7.42E-14	BIK	9.61	16.8	BCL2-interacting killer (apoptosis-inducing)
202733_at	1.64E-12	P4HA2	3.32	3.1	procollagen-proline, 2-oxoglutarate 4-dioxygenase (proline 4-hydroxylase), alpha polypeptide II
211143_x_at	1.71E-12	NR4A1	3.95	3.19	nuclear receptor subfamily 4, group A, member 1
202095_s_at	2.42E-12	BIRC5	4.78	6.41	baculoviral IAP repeat-containing 5 (survivin)
209911_x_at	1.13E-11	H2BFB	4.88	2.94	H2B histone family, member B
204493_at	1.19E-07	BID	9.26	6.21	BH3 interacting domain death agonist
213182_x_at	3.86E-07	CDKN1C	4.33	6.94	cyclin-dependent kinase inhibitor 1C (p57, Kip2)
200670_at	1.30E-05	XBP1	13.73	7.81	X-box binding protein 1
203362_s_at	2.68E-05	MAD2L1	3.34	3.53	MAD2 mitotic arrest deficient-like 1 (yeast)
206665_s_at	3.01E-05	BCL2L1	8.2	4.67	BCL2-like 1
209642_at	5.75E-05	BUB1	3.05	3.1	BUB1 budding uninhibited by benzimidazoles 1 homolog (yeast)
202284_s_at	7.99E-05	CDKN1A	15.27	9	cyclin-dependent kinase inhibitor 1A (p21, Cip1)
202870_s_at	2.07E-04	CDC20	4.44	7.58	CDC20 cell division cycle 20 homolog (S. cerevisiae)
210559_s_at	2.37E-04	CDC2	3.36	4.08	Homo sapiens mRNA for CDC2 delta T, complete cds.
211792_s_at	0.001266	CDKN2C	3.25	3.05	cyclin-dependent kinase inhibitor 2C (p18, inhibits CDK4)
204641_at	0.005091	NEK2	3.51	5.6	NIMA (never in mitosis gene a)-related kinase 2

relevant GO process is in activation of MAPK activity. Of note, the MAPK cascade is one of signaling pathways activated by IL-6 [20].

Table 4. Genes with unique expression profile in MM (The top 10 up- and down-regulated genes plus other genes of interest are shown).

Probe	P-Value	Gene Name	Fold Change Rel to WM	Fold Change Rel to CLL	Description
Up-regulated in MM compared to CLL and WM					
212253_x_at	3.74E-28	BPAG1	3.52	5.62	bullous pemphigoid antigen 1 (230/240kD)
201064_s_at	4.27E-24	PABPC4	2.36	4.22	poly(A) binding protein, cytoplasmic 4 (inducible form)
209968_s_at	2.62E-23	NCAM1	2.3	4.1	neural cell adhesion molecule 1
204271_s_at	1.23E-20	EDNRB	17.6	12.5	endothelin receptor type B
205309_at	3.40E-17	ASML3B	3.41	4.67	acid sphingomyelinase-like phosphodiesterase
204602_at	5.92E-17	DKK1	9.9	7.41	dickkopf homolog 1 (Xenopus laevis)
202973_x_at	8.46E-17	KIAA0914	5.56	6.53	KIAA0914 gene product
203697_at	2.07E-16	Frizzled-related protein	13.61	17.6	FRZB; frizzled protein homolog
202170_s_at	4.42E-16	AASDHPPT	2.11	2.58	aminoadipate-semialdehyde dehydrogenase-phosphopantetheinyl transferase
215059_at	1.27E-15	Homo sapiens mRNA; cDNA DKFZp564G112 (from clone DKFZp564G112)	6.76	10.76	Homo sapiens mRNA; cDNA DKFZp564G112 (from clone DKFZp564G112)
210755_at	2.15E-12	HGF	4.18	3.36	hepatocyte growth factor (hepapoietin A; scatter factor)
209347_s_at	1.15E-06	MAF	2.43	2.78	v-maf musculoaponeurotic fibrosarcoma oncogene homolog (avian)
205071_x_at	2.94E-10	XRCC4	5.26	6.58	X-ray repair complementing defective repair in Chinese hamster cells 4
206213_at	5.20E-06	WNT10B	2.72	4.72	wingless-type MMTV integration site family, member 10B
213693_s_at	1.02E-05	MUC1	2.22	2.61	mucin 1, transmembrane
213425_at	4.20E-05	WNT5A	3.33	4.55	wingless-type MMTV integration site family, member 5A
203510_at	5.69E-05	MET	4.27	4.9	met proto-oncogene (hepatocyte growth factor receptor)
212097_at	7.15E-05	CAV1	17.45	18.7	caveolin 1, caveolae protein, 22kD
205945_at	5.72E-04	IL6R	2.48	3.41	interleukin 6 receptor
203987_at	7.81E-04	FZD6	2.87	4.97	frizzled homolog 6 (Drosophila)
221609_s_at	9.71E-04	WNT6	2.26	2.73	wingless-type MMTV integration site family, member 6
203628_at	0.018357	IGF1R	2.33	2.85	insulin-like growth factor 1 receptor
Down-regulated in MM compared to CLL and WM					
209269_s_at	3.99E-38	SYK	30.17	26.91	Spleen tyrosine kinase
34210_at	1.84E-34	CD52	94.71	180.4	CAMPATH-1 (HUMAN);, mRNA sequence.
215537_x_at	3.06E-29	DDAH2	5.04	7.25	dimethylarginine dimethylaminohydrolase 2
204661_at	6.88E-28	CD52	62.91	115.2	CDW52 antigen (CAMPATH-1 antigen)
201721_s_at	2.21E-26	LAPTM5	9.51	16.39	Lysosomal-associated multispanning membrane protein-5
203037_s_at	1.96E-25	KIAA0429	10.66		KIAA0429 gene product
216237_s_at	3.75E-23	MCM5	5.2	8.1	MCM5 minichromosome maintenance deficient 5, cell division cycle 46 (S. cerevisiae)
201954_at	5.40E-23	ARPC1B	2.08	3.51	actin related protein 2/3 complex, subunit 1B (41 kD)
41220_at	3.86E-22	MSF	3.8	7.59	MLL septin-like fusion
200934_at	8.18E-22	DEK	21.98	28.82	DEK oncogene (DNA binding)
221969_at	1.51E-15	PAX5	6.58	12.04	ESTs, Weakly similar to S57447 HPBRII-7 protein [H.sapiens]
206398_s_at	8.54E-15	CD19	23.57	45.66	CD19 antigen
205536_at	1.39E-10	VAV2	2.76	4.82	vav 2 oncogene
218806_s_at	1.42E-06	VAV3	2.86	5.21	vav 3 oncogene
202414_at	2.53E-06	ERCC5	2.03	3.01	excision repair cross-complementing rodent repair deficiency, complementation group 5 (xeroderma pigmentosum,)
204054_at	2.93E-05	PTEN	2.05	2.64	phosphatase and tensin homolog
212888_at	3.40E-05	DICER1	2.32	2.88	Homo sapiens clone 23938 mRNA sequence
209193_at	5.19E-05	PIM1	3.18	3.06	pim-1 oncogene
201700_at	2.22E-04	CCND3	3.07	3.78	cyclin D3
205647_at	6.49E-04	RAD52	2.45	4.22	RAD52 homolog (S. cerevisiae)
216299_s_at	0.001976	XRCC3	3.1	3.17	X-ray repair complementing defective repair in Chinese hamster cells 3

DISCUSSION

To our knowledge, this is the first gene-expression profiling study in WM and its contrast to other B-cell malignancies. In this study, we attempt to define a molecular signature of WM in the context of normal cellular counterparts (B-cells and PCs) and malignant cells in related B-cell malignancies (CLL and MM).

WM has a homogeneous transcription profile, clusters with CLL and normal B-cells on unsupervised clustering and clearly has a similar expression profile to CLL. In contrast, the expression profile of WM is very different from MM and normal PCs. Previous studies have shown that CLL, regardless of immunoglobulin heavy chain (IgH) mutation status, has a homogeneous expression profile [8,21] very similar to peripheral blood resting B-cells [11,21]. In another study comparing CLL to different tonsillar B-cell populations, the expression profile of CLL is most closely related to memory B-cells,[8] which also constitutes a significant population of the peripheral-blood B-cell pool [22]. The similarity between the expression profile of WM and CLL is perhaps not surprising given the known biological and clinical characteristics of these diseases. Both are indolent tumors characterized by low proliferation. Unlike

other B-cell malignancies, chromosomal translocations involving the IgH locus are relatively uncommon in both CLL and WM [23,24]. These IgH translocations are thought to occur either during immunoglobulin VDJ recombination in maturing B-cells or during Ig somatic hypermutation and isotype switching in mature B cells within the germinal center (GC) [25]. They are therefore common in B-cell lymphomas which are usually derived from GC B-cells [26]. This observation is consistent with the notion that both CLL and WM are derived from memory B-cells where these mechanisms have been inactivated. Despite these similarities, differences such as differential expression of immunophenotypic markers and demographics in IgVH mutation status (almost all WM are IgV mutated [3,27] compared to 50-70% of CLLs [28,29]) suggest that they may be derived from different memory B-cell populations [22].

It is possible that the expression profile of WM is closer to normal B-cells and CLL than MM and normal PCs due to the additional $CD19^+$ selection (normal B-cells and CLL are $CD19^+$ selected) as compared to only $CD138^+$ selection in MM and normal PCs. There are several arguments against this; (1) the bulk of $CD19^+$ cells should represent the cell of interest (malignant WM and CLL cells and normal B-cells); (2) the tissue source for CD19 selection is different (BM for WM and peripheral blood for normal B-cells and CLL) and hence the predominant non-malignant B-cell population selected should be different (pre-B cells for WM and mature B-cells for CLL); (3) WM but not CLL samples are additionally CD138 selected. Furthermore, our strategy of using both CD138 and CD19 to select for malignant WM cells has the added advantage of allowing the analysis of the entire malignant population. Therefore, the respective expression signature should, in general, reflect tumor phenotype rather than contaminating cells or other by-products resulting from CD19 selection. The similarity between WM and CLL and not MM is further confirmed using independently derived CLL and MM signatures.

Detailed analysis of gene-expression for CD markers and genes involve in cell-cycle regulation also show similarities between WM and CLL and normal B-cells but not MM and normal PCs. In total our analysis suggests that WM is defined by a B-cell-like signature and in terms of gene transcription signature is closer to CLL than MM. The clustering of the IgM MGUS case together with WM and the similar expression of genes with expression profile unique to WM (IL6 and CD1c; data not shown) suggest a shared phenotype between IgM MGUS and WM. This is consistent with current notion that a subset of IgM MGUS represents the precursor state of WM [30].

Defining genes with unique expression in a disease may provide insight into its biology. This would be particularly useful in WM where little is known about its biology. Despite the purity of greater than 90% in most samples after immunomagnetic bead-positive selection, presence of contaminating normal cells may still contribute to the overall gene-expression profile. In our dataset, we noted signatures of con-

taminating monocyte/macrophages and pre-B-cells, particularly in the samples with lower number of target cells (eg, MGUS, normal samples and WM with lower BM involvement). To ensure that all the genes with unique expression profiles in each of the malignant conditions are relevant to the malignant cells, we subtracted genes that constitute the signature of contaminating cell types. Only a small set of genes have a unique expression profile in WM. Among the up-regulated genes, *IL6* is the most significant. It has been demonstrated that IL-6 levels are elevated in WM [31,32] and that IL-6 is required for plasmacytic differentiation of the clonal B-cells in WM [33]. However, remarkably little is published regarding the potential role of IL-6 in WM biology given its prominent role as a growth and survival factor in MM [34,35]. Our data regarding *IL6* expression in WM is validated by 2 abstract presentations that used gene-expression approaches. In the first study, GEP identified deregulated elements in the IL-6 signaling cascade [36] whereas in the second study utilizing similar analysis approach as ours they found that *IL6* expression is higher in WM B-cells than normal B-cells [37]. Its potential relevance to WM biology is highlighted by the fact that the GO process most relevant to the WM unique genes in our study is activation of MAPK, which is involved in IL-6 signaling [20]. Furthermore, as our gene-expression analysis is performed on highly purified malignant cells and not total bone marrow cells (ie, including stromal cells), the differential expression of IL-6 in WM cells is more supportive of an autocrine source for IL-6 in WM compared to MM where the source of IL6 is predominantly from the stromal cells. The functional and biological importance of IL6 in WM should be investigated as this may represent an important therapeutic avenue.

As for CLL and MM, the differentially expressed genes suggest that apoptosis regulation and receptor mediated signal transduction are critical processes central to CLL and MM biology, respectively. These results are consistent with the known biochemical and signaling pathways found in the malignant B cells from MM and CLL patients. It is well known that MM growth and survival is mediated through various cytokines secreted by stromal cells in the bone marrow milieu upon interaction with MM cells [34,35]. Similarly, the fundamental role of anti-apoptosis mechanisms is well established in CLL [38]. More importantly, this comparative analysis shows that different pathways are deregulated in these tumors.

In conclusion, our GEP study suggests that WM samples have a homogenous expression profile very similar to CLL and normal peripheral blood B-cells. A small set of genes is distinctly expressed in WM, with *IL6* the most significantly up-regulated gene. These genes are most significantly associated with MAPK signaling. This in turn suggests that IL-6 and its signaling network may be of biological significance in WM.

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CHAPTER 7

Discussion and future directions

This thesis deals with the genetics and cytogenetics of Waldenström macroglobulinemia (WM). Knowledge of the genetic profile may increase our understanding of the disease etiology and pathogenesis. In addition, the identification of cytogenetic aberrations or aberrant gene expression can be used as disease specific and/or prognostic markers and ultimately also as a focus for the development of targeted therapeutics.

In the studies described in this thesis we found that WM displays a distinct genetic pattern, which is different from that of multiple myeloma (MM) but more similar to that of chronic lymphocytic leukemia (CLL). In this chapter we shall summarize the genetic findings in WM and discuss their implications for clinical diagnosis.

IGH TRANSLOCATION

According to the WHO classification of tumours, WM and lymphoplasmacytic lymphoma (LPL) share the same pathologic designation.[1] The translocation t(9;14)(p13;q32) involving the *PAX-5* and IgH genes has been detected in 50% of LPL patients and therefore are often assumed also to be present in WM.[2,3] The translocation results in the up-regulation of *PAX-5*, a gene that encodes a transcription factor, B-cell specific activation protein (BSAP).[4] BSAP is a protein that is critical for B-cell development, but it is not expressed in normal and malignant plasma cells.[5,6] The original publication had reported the t(9;14) in 4 cases of LPL and none of these patients had presented with paraproteinemia. These observations are consistent with the potential effect of *PAX-5* overexpression to down-regulate IgH transcription.[7,8] Hence, t(9;14)(p13;q32) would not be expected to be present in WM, a form of LPL *with* paraproteinemia.

In the fluorescence in situ hybridization (FISH) studies of chapter 3, t(9;14) could not be demonstrated in 31 cases of WM. Thus, unlike MM, translocations involving the IgH locus (at 14q32) appear uncommon in WM. Our finding is in agreement with other previously reported karyotype studies in patients with WM, where chromosome 14 abnormalities were not detected.[9] Also, in a FISH study by Chang et al IgH translocation were described in only 3 out of 22 WM cases, while no common partner chromosome (4p14, 8q24, 11q13 or 16q32) could be detected.[10] In a third larger FISH study among 69 WM cases IgH translocations were identified in two cases only.[11] Of note, the infrequent presence of IgH translocations in WM could probably be useful in diagnostic dilemmas in particular patients. Avet-Loiseau et al showed that the presence of t(11;14) discriminates IgM MM from WM.[12]

Finally, a study by Cook et al reassessed the prevalence of t(9;14) in 12 nodal LPLs. Contrary to the original report by Offit et al, no t(9;14) was found and all LPL cases were negative for IgH translocations.[13]

Altogether, these studies show that IgH translocations are quite uncommon in LPL/WM as they are seen in a minority subset of cases. Especially t(9;14) should not be considered a LPL/WM specific cytogenetic characteristic, as defined by the WHO classification of tumours. These cytogenetic data also suggest that WM differs from MM, in which IgH translocations are commonly detected and may represent early events in the pathogenesis.

DELETION 6q

Based on karyotype studies, deletion of the long arm of chromosome 6 (6q) was identified as the most common chromosomal abnormality in WM (chapter 3).[9] Using the more sensitive interphase FISH technique, hemizygous deletions 6q were detected in 50% of cases. A recent FISH study by Chang et al used probes located at 6q21 and 6q25 and detected the presence of 6q deletions (either 6q21 or 6q25) in 13 out of 34 WM cases (38%).[14] Our collaborative study with the Spanish group from Salamanca involves a total of 102 WM cases. In the latter series, described in chapter 5, the same prevalence of deletion 6q21 was found (39%). In this study, due to different sensitivities of techniques, the percentages of cases with 6q deletions was higher in patients analyzed by cytoplasmic IgM enhanced FISH (54%) than in cases studies by conventional FISH (30%). The highest prevalence of 6q deletions (55%) was found in our study when we used multiple FISH probes along the whole 6q arm in 38 WM cases (chapter 4). We also observed that many WM cases displayed loss of almost the complete 6q arm. Nevertheless, a minimal region of deletion was delineated between 6q23 and 6q24.3 and the FISH probe covering the *SRPRH* gene was deleted most frequently. A pathogenetic role of the latter gene and several other candidate tumor suppressor genes such as *BLIMP-1* and *MYB*, have been suggested for this area but additional structural and functional studies will be needed to elucidate the biological basis of the 6q deletion in WM.

Due to the absence of 6q deletions in IgM MGUS, a precursor stage of particularly WM, we speculate that this deletion is a secondary event and maybe associated with disease progression. The prognostic significance for 6q deletion in WM was investigated in the cohort of 102 WM patients (chapter 5). Cases of WM with a 6q deletion displayed features of adverse prognosis (higher levels of β2-microglobulin and M-protein, anemia and hypoalbuminemia) and showed a trend to have more advanced stages of disease according to the International Staging System (ISS).[15]

Also, the treatment-free survival was negatively influenced by the presence of the 6q deletion in smoldering WM patients. Survival analysis in our limited series did not reveal a negative impact of the presence of a 6q deletion. However the lack of an apparent effect of 6q- deletions on survival is not too surprising as this is most likely explained by the limited statistical power and the short follow-up time of this indolent disease. Contrary to our results, Chang et al had not found evidence for a correlation between 6q deletion and clinical or biological parameters. In their study of 34 cases, a significant difference in overall survival was neither observed between the 6q deletion and non-deletion subgroups. For the time being it remains unclear if the 6q deletion can be used as prognostic marker for WM outcome. The 6q deletion is the most frequent chromosomal structural aberration in WM. The high prevalence of 6q deletion and its unique presentation in WM, compared to nodal LPL[16] or IgM MGUS, suggest a differential cytogenetic profile associated with this abnormality.

OTHER STRUCTURAL CHROMOSOMAL ABERRATIONS

Abnormalities, like deletions 13q and 17p are commonly found in MM and are generally recognized as adverse prognostic abnormalities. We showed that compared to MM, deletions 13q and 17p are less common in WM at time of diagnosis, but they may be observed in 15% at the time of disease progression (chapter 2). Chang et al found deletions of 13q or 17p in 9% of 22 WM.[10] Nevertheless, due to low sample number, this study was unable to address the question of the prognostic significance of these deletions.

Sporadic WM case studies have reported the translocation t(11;18)(q21;q21).[17] This translocation is associated with extranodal marginal zone lymphoma. In our series of 24 WM cases this translocation was not detected by FISH.

Aneuploidy is common in MM and based on the type of numerical chromosomal abnormalities, four categories can be identified: hypodiploid, pseudodiploid, hyperdiploid and near-tetraploid. IgH translocations and deletion 13 are closely related with the the non-hyperdiploid MM categories. Based on these cytogenetic data two prognostically different groups can be detected: non-hyperdiploid MM with IgH translocation (unfavorable) and hyperdiploid MM without IgH translocations (favorable). It is postulated that these two groups represent different pathogenic pathways of MM.[18,19] To assess the ploidy status in WM we performed multicolor (M)-FISH in 5 cases and interphase FISH using centromere enumeration probes (CEPs) in 15 cases (chapter 3). Although some patients featured abnormalities in the karyotype consistent with aneuploidy, interphase FISH showed that this is the exception in WM. The absence of aneuploidy (hyperdipliody) together with the infrequent pres-

ence of IgH translocations, suggest that WM does not share either of the proposed pathogenic pathways of MM.

COMPARATIVE GENE EXPRESSION PROFILING

In the gene expression profiling (GEP) study of WM in chapter 6 we compared $CD19^+$ and $CD138^+$ WM bone marrow cells with other B-cell malignancies (CLL and MM) and B-cells and plasma cells as their normal cellular counterparts. The 23 WM cases display a homogeneous single expression pattern. Importantly, 6q deleted and non-deleted WM cases do not form distinct clusters, when we use all genes on the Affymetrix U133A chip. This suggests that loss of genes located at 6q does not lead to profound transcriptional changes. Several reasons can be given, why 6q deleted and non-deleted do not form distinct clusters in our GEP study. First, this could be explained by the small samples size of WM cases. Second, it is easier to detect a gene dosage effect, when there is gain of chromosome because the dynamic range for increase in gene expression is much higher compared to deletion or under-expression. Third, the microarray platform we used only represented a part of the human genes. Consequently, it is possible that genes critical in WM pathogenesis are missed in this GEP study. Finally, we compared 6q deleted and non-deleted WM, using all genes on the U133A chip. A supervised analysis, using only the 6q genes, could reveal specific expression differences between the 6q deleted and non-deleted WM.

The WM expression pattern resembles that of CLL and normal B-cells. The gene expression pattern of WM is different from MM and normal plasma cells.

Only a small set of genes was found to be specific for WM. Among the upregulated genes, *IL6* was the most significant. IL-6 levels are known to be high in WM and this cytokine is required for plasmacytic differentiation in WM B-cells.[20,21] Moreover, IL-6 is among the most important proliferation and survival factors in MM.[22,23] The potential pathogenic role for IL-6 in WM was also suggested by Guttierrez et al.[24] In their gene expression profiling study they compared clonal WM B-cells with clonal WM plasma cells. They observed a different gene expression pattern between the WM B-cell population and the WM plasma cell population. IL-6 was elevated in the B-cell subset of WM clonal cells. This cytokine is currently considered as possible therapeutic target and could explain the elevated levels of C-reactive protein in WM patients.

In summary, we conclude that WM shows a gene expression profile more similar to that of CLL than that of MM and that further study is warranted to elucidate the potential pathogenic role of IL-6 production in WM clonal cells.

FUTURE PERSPECTIVES

Together with the sequencing of the entire human genome, microarray techniques appear useful for understanding tumorigenesis at distinct cellular levels, such as protein, RNA, micro-RNA, single nucleotide polymorphisms (SNPs), DNA and DNA methylation.

A combined strategy using different microarray platforms in the same tumor samples could screen for recurrent or sporadic aberrations and at the same time could give more insights of its consequences. For example, recurrent small biallelic deletions could identify regions that potentially harbor tumor suppressor genes. If those identified genes belong to a known common pathway, a working hypothesis can be formulated about its role in tumorigenesis. Subsequent analysis of GEP data could reveal dysregulated expression of those target genes involved in this pathway. Additional mutation analyses can establish an overall rate of altered target genes. To validate the role of possible tumor suppressor genes, reintroduction of the wild-type gene into a tumor cell line model can be used to test the hypothesis. It is this combined strategy that was used in a recent MM paper that identified an array of mutations that result in activation of the noncanonical NF-κB pathway.[25] These observations provided valuable insight into the significant contribution of the non-canonical NF-κB pathway in the pathogenesis of MM, which was also highlighted by the clinical success of bortezomib, a postulated NF-κB inhibitor.

A pathway-centric approach was also suggested by Wood et al. as a new paradigm of cancer research.[26] Genome-wide sequencing of gene transcripts in breast and colorectal cancers identified a few gene mutations at high frequency and a much larger number of genes mutated at low frequency. It appears that those sporadic mutated genes exert tumorigenesis through known cellular signaling pathways. The authors stated that through array-based techniques future cancer research will easily identify genetic alterations, but will be challenged to comprehend its precise role of these alterations in cellular pathways and tumorigenesis.

Using the same approach for WM, will benefit the understanding of molecular pathways involved in the pathogenesis of WM. These array-based techniques may also evolve into practical clinical tools and therefore guide (WM) patient management in the near future.

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CHAPTER 8

Summary / Samenvatting

Summary

Waldenström macroglobulinemia (WM) is a rare incurable disease, characterized by an expansion in the bone marrow of a subpopulation of white blood cells, the B-lymphocytes. These cells produce an abnormal excessive amount of antibody protein, immunoglobulin M (IgM).

High blood concentration of this monoclonal gammopathy or IgM M-protein, eventually leads to thickening of the blood, resulting in hampered blood flow causing the hyperviscosity syndrome. Important symptoms of this syndrome are fatigue, visual problems, a tendency to bleed easily, headache, dizziness, deafness, kidney failure and heart failure. Also, bone marrow expansion of B-cells compromises normal blood cell production, causing anemia, shortage of normal white blood cells, resulting in a higher infection risk, and lowered platelet counts, necessary for blood clotting.

In some patients, the IgM protein interacts with nerve tissue, resulting in tingling of hands and feet, loss of sensation and muscle weakness.

Because of the relative indolent course of the disease WM, treatment is usually not immediately required at the time of diagnosis. WM patients can be without treatment for many years. Nevertheless, some patients have progressive disease and need instant chemotherapy. The cause of this heterogeneity in clinical behavior is unclear.

More understanding of the cause of WM could give more insight. Many acquired chromosomal abnormalities have been found in other malignant blood diseases and sometimes gave insight into biological processes that contribute to the development of that disease. Knowledge of these processes can provide potential targets for novel drug development.

In addition, in some cases certain chromosomal abnormalities can predict the clinical course of the disease. Specific chromosomal abnormalities are characteristic for a particular disease and therefore can be helpful in making the correct diagnosis and subsequent treatment choices.

This thesis contains studies that give more insight into the presence of chromosomal abnormalities in WM compared to related disorders such as monoclonal gammopathy of undetermined significance (MGUS) and malignant blood diseases like chronic lymphocytic leukemia and multiple myeloma.

Besides the search for chromosomal abnormalities in WM, we used the microarray technique, to look more detailed at gene expression level patterns in WM compared to the above mentioned related diseases.

Chapter 1 is a general introduction, which describes the clinical characteristics of Waldenström macroglobulinemia and the necessity of more knowledge of chromosomal abnormalities.

Chapter 2 contains the results of cytogenetics analysis on bone marrow tissue of WM patients by using the fluorescent in situ hybridization (FISH) technique. We looked specific for the presence of loss of chromosome 13 (13q) and the short arm of chromosome 17 (17p). These abnormalities are known to be associated with an aggressive course of multiple myeloma. Our results showed that both chromosomal abnormalities are uncommon in WM. Nevertheless, when present in WM, then they are associated with a worse prognosis in WM.

In **chapter 3** we present FISH data of 31 WM patients indicating that translocations between chromosome 14 and other chromosomes, specifically chromosome 9, are rare. Also numerical abnormalities of complete chromosomes were uncommon. In contrast, both translocations involving chromosome 14 and numerical chromosome changes are frequently found in multiple myeloma. Our findings in WM suggest that this disease might differ from multiple myeloma on the genetic level.

Finally, in this chapter we present data of the most frequently found chromosomal abnormality in WM; the deletion of the long arm of chromosome 6 (6q). Initially, this 6q abnormality was found in 50% in our 24 WM patients.

Chapter 4 focuses on the 6q deletion in WM and a pre-malignant state that often progresses to WM, IgM MGUS. No 6q deletion was found in bone marrow tissue of 12 IgM MGUS cases. This could mean that this abnormality is acquired later in the development of WM. This deletion also seems to be limited to a specific region of chromosome 6, although most of the time the WM patient has lost one whole 6q arm. By using FISH we screened the whole 6q arm for deletions. The minimal region of deletion is between chromosome band 6q23 and 6q24.3. Knowing the minimal deleted region could be helpful identifying important genes within this deleted region. Consequently, deletion of these genes can influence their function, potentially contributing to the development of WM.

Combining FISH data of WM patients from Spain with our cases, we were able to determine the presence prevalence of 6q deletion in a large WM group. In **chapter 5** we show that the frequency is at least 34% using the FISH technique in 102 WM cases. Although not statistically significant, there seems to be a tendency towards a worse prognosis when a patient has the 6q deletion. Confirmation is needed studying larger groups of WM patients, with a longer follow-up time.

In **chapter 6** we compare mircoarray data of patient material of chronic lymphocytic leukemia, multiple myeloma and WM. The microarray technique generates data on the the expression level of thousands of genes simultaneously, making it possible to assemble specific gene expression profiles. Irrespective of the presence of the 6q deletion, our study shows that the gene expression profile of WM is more similar to chronic lymphocytic leukemia than to multiple myeloma. Only a small amount of the investigated genes distinguishes WM from chronic lymphocytic leukemia and multiple myeloma. One of the genes that was more up-regulated in WM compared to the other diseases, was the gene that codes for interleukin 6 (IL-6). Previous studies have demonstrated that IL-6 is important for B-cell development in WM. Also, IL-6 is an important factor for growth of multiple myeloma tumor cells. More study is needed to know more about the role of IL-6 in the biology of WM clonal B-cells.

Chapter 7 contains a general discussion about our findings in WM compared to data of other research groups. We expect that new (cyto)genetic techniques will give more insight into the contribution of specific genetic abnormalities to important biological pathways in tumor cells in general and in WM B-cells in particular.

Samenvatting

Waldenström macroglobulinemia (WM) is een zeldzame ongeneeslijke kwaadaardige ziekte, die gekenmerkt wordt door een ongebreidelde deling in het beenmerg van een subpopulatie van witte bloedcellen, de B-lymfocyten. Deze cellen maken een abnormale hoeveelheid afweereiwit, immuunglobuline M (IgM).

Deze zogenaamde monoklonale gammopathie of M-proteïne van het type IgM leidt uiteindelijk bij hoge bloedconcentraties tot stroperigheid van het bloed en doorstroombelemmering, resulterende in het hyperviscositeit syndroom. De belangrijkste klachten van dit syndroom zijn vermoeidheid, stoornissen van het zien, verhoogde bloedingsneiging, hoofdpijn, duizeligheid, doofheid, verslechterende nierfunctie en hartproblemen. Tevens zorgt de woekering van de B-lymfocyten in het beenmerg voor verdringing van de aanmaak van andere gezonde bloedcellen, leidend tot bloedarmoede, verminderde hoeveelheid afweercellen, resulterend in een verhoogde kans op infecties, en verlaagde bloedplaatjes, nodig voor de stolling.

Tenslotte tast in sommige gevallen het abnormale eiwit IgM de zenuwen aan, zich uitend in tintelingen aan handen en voeten, gevoelsstoornissen en krachtsverlies.

Door het relatief milde beloop van de ziekte is vaak bij het stellen van de diagnose WM behandeling niet meteen noodzakelijk. Bij afwezigheid van klachten kan soms jaren gewacht worden met behandeling. Echter, er zijn ook patienten waarbij de ziekte sneller verloopt, waardoor behandeling middels chemotherapie noodzakelijk wordt. Wat de onderliggende oorzaak van dit verschil in ziektebeloop is onduidelijk.

Meer begrip van de oorzaak van WM zou meer inzicht kunnen geven. Verworven afwijkingen van chromosomen, dragers van DNA, zijn in andere kwaadaardige bloedziekten gevonden en blijken soms duidelijkheid te geven over processen die bijdragen aan het ontstaan van de ziekte. Kennis over deze processen biedt aangrijpingspunten voor de ontwikkeling van nieuwe werkzame geneesmiddelen. Ook is bekend dat chromosoom afwijkingen een voorspellende waarde kunnen hebben voor het beloop van de ziekte. Soms zijn kenmerkende chromosoom afwijkingen gerelateerd aan een specifieke bloedziekte, zodat onderzoek naar deze afwijking behulpzaam kan zijn bij het stellen van de diagnose en het maken van een passende behandelkeuzes.

In dit proefschrift worden studies beschreven die meer inzicht geven over de aanwezigheid van chromosomale afwijkingen in WM in vergelijking tot de gerelateerde aandoening IgM monoclonale gammopathie “of undetermined significance” (MGUS), en de kwaadaardige bloedziekten, chronische lymfatische leukemie en multipel myeloom. Naast cytogenetisch onderzoek naar chromosoom afwijkingen is met behulp van een moleculair biologische techniek, microarray, meer gedetaileerd gekeken naar kenmerkende verschillen tussen bovengenoemde gerelateerde ziekten.

Hoofdstuk 1 is een algemene introductie waarin de kenmerken van Waldenström macroglobulinemie worden beschreven en de noodzaak voor meer kennis over chromosomale afwijkingen.

Hoofdstuk 2 beschrijft de resultaten van cytogenetisch onderzoek op beenmerg materiaal van WM patienten met behulp van de techniek fluorescence in situ hybridization (FISH). Er is specifiek gekeken naar verlies van een deel van chromosoom 13 (13q) en verlies van de korte arm van chromosoom 17 (17p). Het is bekend dat deze afwijkingen ook in multipel myeloom voorkomen en geassocieerd zijn met een aggressiever ziektebeloop. In WM bleek deze afwijkingen zelden voor te komen. Wel bleken ze geassocieerd te zijn met slechter prognose van WM.

In **hoofdstuk 3** wordt aangetoond dat met behulp van FISH technieken translocaties tussen chromosoom 14 en andere chromosomen zeldzaam zijn in WM. In het bijzonder translocatie tussen chromosoom 9 en 14, welke geassocieerd zou zijn met WM, werd in onze studie van 31 patienten niet gevonden. Ook numerieke afwijkingen van hele chromosomen waren zeldzaam. Zowel translocaties waarbij chromosoom 14 betrokken is, als numerieke chromosomale afwijkingen zijn veel voorkomende afwijkingen in multipel myeloom. Onze bevindingen in WM wijzen erop dat deze ziekte ook op genetisch niveau verschilt van multipel myeloom.

Tenslotte wordt in dit hoofdstuk de bevinding van de meest voorkomende chromosomale afwijking in WM gepresenteerd; de deletie van de lange arm van chromosoom 6 (6q). In deze studie wordt deletie 6q in 50% van de 24 WM patienten gevonden.

Hoofdstuk 4 focust zich op de 6q chromosoom afwijking in WM en de premaligne aandoening IgM MGUS, die zich vaak uiteindelijk ontwikkeld in WM. In 12 IgM MGUS patienten kon in het beenmerg geen 6q deletie worden aangetoond. Dit kan betekenen dat deze afwijking pas later in de ontwikkeling van WM plaatsvindt. Ook lijkt de deletie inWM zich te beperken tot een bepaald gebied op de 6q arm, alhoewel meestal de gehele 6q ontbreekt. Met behulp van FISH werd op verschillende lokaties op chromosoom 6 gescreend voor deleties. De minimale grote van de deletie lijkt zich te concentreren tussen chromosoom band 6q23 en 6q24.3. De kennis van de minimale grootte van de deletie kan behulpzaam zijn bij het vinden

van eventuele belangrijke genen gelegen binnen deze deletie, die door deze deletie minder functioneel worden en daardoor een bijdrage aan de ontwikkeling van WM kunnen leveren.

Samenvoeging van een WM patientengroep uit Spanje met onze patienten, biedt de mogelijk te komen tot een beter inzicht in het voorkomen van de deletie 6q. In **hoofdstuk 5** wordt de analyse van deze groep van 102 WM patienten beschreven, waaruit blijkt dat het percentage van 6q deletie minimaal 34% is met behulp van de FISH techniek. Alhoewel niet statistisch significant, lijkt de aanwezigheid van de 6q deletie in WM patienten, geassocieerd te zijn met een slechtere prognose. Het zal noodzakelijk zijn een grotere groep patienten langdurig te analyseren om aan te tonen of de 6q deletie in WM daadwerkelijk betekenis heeft voor het beloop van de ziekte.

Hoofdstuk 6 beschrijft de resultaten van een vergelijkend onderzoek tussen patienten materiaal van chronische lymfatische leukemie, multipel myeloom en WM, waarbij gebruik wordt gemaakt van microarrays, waarmee simultaan het expressieniveau van duizenden genen kan worden bepaald. Het totale expressieprofiel van WM in onze studie vertoont meer overeenkomsten met chronische lymfatische leukemie dan met multipel myeloom. Dit resultaat is onafhankelijk van de aan of afwezigheid van de deletie 6q. Van de onderzochte genen, is slechts een klein aantal genen dat WM onderscheidt van chronische lymfatische leukemie en multipel myeloom. Van deze genen, was in WM het expressieniveau van het gen voor interleukine 6 (IL-6) verhoogd in vergelijking tot de andere ziekten. Eerdere studies toonden aan dat het boodchappereiwit IL-6 een belangrijke rol speelt in de uitrijping van de B-cellen in WM. Ook in multipel myeloom is IL-6 een belangrijke factor voor groei en overleving van de tumorcel. Verdere studies moeten uitwijzen welke rol IL-6 speelt in de biologie van klonale B-cellen in WM.

Tenslotte, betreft **hoofdstuk 7** een algemene discussie, waarin onze bevindingen worden vergeleken met resultaten van andere studies in WM. Ook wordt een toekomst perspectief geschetst waarbij met nieuwe (cyto)genetische technieken, meer inzicht zal worden verkregen in de cumulatieve bijdrage van specifieke genetische afwijkingen aan belangrijke biologische processen in tumorcellen in het algemeen en mogelijk WM B-cellen in het bijzonder.

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Curriculum vitae

De schrijver van dit proefschrift werd geboren op 5 juni 1974 te 's-Gravenhage. Hij behaalde in 1992 het eindexamen Gymnasium B aan het Erasmiaans Gymnasium te Rotterdam. Hierna werd begonnen met de studie Geneeskunde aan de Katholieke Universiteit te Leuven, België. Vervolgens werd in 1993 deze studie voortgezet aan de Rijksuniversiteit Leiden, leidend tot het doctoraalexamen in 1997. Een wetenschappelijke stage werd gelopen op de afdeling hemato-pathologie, Leids Universitair Medisch Centrum (begeleiders: prof. dr. Ph.M. Kluin en dr. S.A. Riemersma), waar het eerste contact werd gelegd met dr. R. Fonseca, die daar gelijktijdig werkzaam was. Na in 2000 aan de Rijksuniversiteit Leiden zijn artsexamen te hebben behaald, verbleef hij gedurende anderhalf jaar als research fellow in het laboratorium van dr. Fonseca, Mayo Clinic, Rochester Minnesota, VS. Hier werd een begin gemaakt met het onderzoek dat uiteindelijk heeft geleid tot de resultaten beschreven in dit proefschrift. In mei 2002 werd begonnen met de opleiding tot internist in het Erasmus Medisch Centrum te Rotterdam (opleiders: prof. dr. H.A.P. Pols en dr. J.L.C.M. van Saase), hierna vervolgd in het Havenziekenhuis te Rotterdam (opleider: dr. P.J. Wismans). Deze opleiding werd in 2005 gedurende één jaar onderbroken om opnieuw te werken in het laboratorium van dr. Fonseca, Mayo Clinic, Scottsdale Arizona, VS. Op 1 mei 2008 vond de registratie plaats als internist. Sinds 1 september 2007 is hij begonnen met het aandachtsgebied hematologie (opleider: prof. dr. B. Löwenberg) en werkt momenteel in het Erasmus Medisch Centrum, locatie Daniel den Hoed.

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