Expression of regulatory genes for lymphoplasmacytic cell differentiation in Waldenstrom Macroglobulinemia

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Summary

Waldenstrom Macroglobulinemia (WM) is a B-cell malignancy characterized by excess bone marrow (BM) lymphoplasmacytic cells (LPC). The accumulation of LPC in WM may represent a failure of B-cells to properly differentiate into plasma cells. The present study investigated transcriptional expression of genes involved in late B-cell differentiation, including *PRDM1*, *PAX5*, *XBP1* transcripts and *ERN1,* in BM B-cells from 31 patients with WM and six healthy donors. Real time reverse transcription polymerase chain reaction (RT-PCR) determined that approximately 80% of the patients had high *XBP1* spliced mRNA expression, 80% of whom had high mRNA *ERN1α* expression. *XBP1*, *PRDM1* and *PAX5* mRNA was present in all patients studied. Using relative quantitative RT-PCR we isolated two groups with low and high expression of *XBP1*, *XBP1* spliced and *ERN1α*. Sequence analysis showed germline polymorphisms in all genes studied. These data depict for the first time a heterogeneous expression pattern of the genes involved in late differentiation process of plasma cells in patients with WM and propose a role of *XBP1-ERN1α* in WM pathogenesis.

Keywords: Waldenstrom Macroglobulinemia, *XBP1*, *ERN1*.

The terminal differentiation of B cells into immunoglobulin-secreting plasma cells is orchestrated by several major transcription factors including PRDM1 (BLIMP1), XBP1 and PAX5. These transcription factors are involved in cell cycle arrest, induction of immunoglobulin secretion and inhibition of germinal centre function. PRDM1 is predominantly expressed at the plasma cell stage where it represses directly PAX5 and indirectly through PAX5 the expression of XBP1 (Shapiro-Shelef & Calame, 2005). Paired box protein 5 (*PAX5*) is a gene essential for B cell development, and continuous expression of *PAX5* is required throughout the B cell lineage to maintain the functional identity of B cells. During B cell activation, *PAX5* is downregulated by *PRDM1* before terminal differentiation into antibody-secreting plasma cells, and enforced expression of *PAX5* prevents plasmacytic development (Johnson *et al*, 2005; Hagman & Lukin, 2006). *XBP1* is required for transcription of IgM chains and for optimization of antibody secretion (Iwakoshi *et al*, 2003; Gunn *et al*, 2004). *XBP1* mRNA undergoes splicing (*XBP1s*) by the gene product of *ERN1α* (IRE1) via site-specific cleavage leading to a more potent and stable transcription factor than the unspliced form (Yoshida *et al*, 2001; Lee *et al*, 2002).

Waldenstrom Macroglobulinemia (WM) is an IgM secreting B-cell neoplasm that is encompassed within the pathological diagnosis of lymphoplasmacytic lymphoma as defined by the World Health Organization and the Revised

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European–American Lymphoma classification system (Owen *et al*, 2003). An excess of mature lymphocytes, lymphoplasmacytic cells and mature plasma cells is present in the bone marrow of patients with WM, and represents a single B-cell clonal process capable (at least in part) of differentiation (Bartl *et al*, 1983). The most common genetic abnormality described in WM is the heterozygous loss of 6q21–23 in 30–50% of WM patients by fluorescence *in situ* hybridization (FISH) analysis (Schop *et al*, 2006; Treon *et al*, 2006), a site which encompasses *PRDM1*. We therefore investigated the expression of genes involved in late stage B-cell differentiation, including *PAX5*, *PRDM1*, *XBP1* and IREα in lymphoplasmacytic cells taken from the bone marrow (BM) of patients with WM.

Patients and methods

Patients and cells

Thirty-one patients with the consensus panel definition of WM were included in this study, which was approved by the Institutional Review Board at Dana-Farber Cancer Institute. The median age of patients was 62 (range 41–85) years and the male/female ratio was 1·6. All WM patients were previously untreated. Table I summarizes the characteristics of the patients. Bone marrow aspirates from healthy donors were used in comparison (AllCells LLC, Berkeley, CA, USA).

BM $CD19^+$ and $CD138^+$ cells were obtained after density-gradient centrifugation using single layer extraction [Ficoll-Paque Plus (Pharmacia Biotech, Piscataway, NJ, USA)]. $CD19^+$ selection was performed using CD19 beads obtained from Milteny Biotec (Auburn, CA, USA) according to manufacturer's instructions. The purity of isolated B-cells by this technique was over 90% as confirmed by flow cytometric analysis with a monoclonal antibody reactive to human CD20-phycoerythrin (PE) (BD-Bioscience, San Jose, CA, USA). BM $CD138^+$ cells were isolated using CD138 beads (Milteny Biotec) from the negative fraction of the $CD19^+$ selected mononuclear cells.

In addition to primary cells, we also included several IgM-secreting cell lines in these studies: Namalwa, RL and Ramos which were obtained from the American Type Culture Collection (Manassas, VA, USA), MEC1 from DMSZ (Braunschweig, Germany), the WM-WSU (al-Katib *et al*, 1993) and BCWM.1 (Santos *et al*, 2005) WM cell lines. The multiple myeloma cell line, MM.1S was a kind gift from Dr Steven Rosen (Northwestern University, Chicago, IL, USA). All cells were cultured in RPMI1640 supplemented with 10% fetal bovine serum, 50 units/ml of penicillin, 50 μg/ml streptomycin and 2 mmol/l L-Glutamine.

Table I. Characteristics of the patients with Waldenstrom macroglobulinemia included in the study.

	WM n = 31	
Characteristic	Median	Range*
Haemoglobin (g/l)	126	93–159
IgM (g/l)	23·6	10–63
β2m (mg/l)	2·9	1·5–13
Albumin (g/l)	38	30–45
Serum viscosity (cp)	1·95	1·1–5·4

*Range: minimum–maximum.

Semi-quantitative reverse transcription polymerase chain reaction (RT-PCR) analysis

Total RNA was extracted using RNeasy Mini kit according the manufacturer's instructions (Qiagen, Valencia, CA, USA). PCR was performed using the PTC-200 DNA Engine thermal cycler (MJ Research Inc., Waltham, MA, USA). Thirty-five cycles of PCR amplification were performed for *XBP1s*, *ERN1α* and *PAX5* and 31 cycles for *PRDM1* and *XBP1*. Thirty cycles were used for *ACTB* amplification in order to keep the reaction in a linear range. Primers used were as follow: *XBP1*: sense, 5′-CGCTGAGGAGGAAACTGAA-3′ and antisense, 5′-GGG-AGGCTGGTAAGGAACT-3′; *PRDM1*: sense, 5′-TCGGGT-CGTTTACCCCATC-3′ and antisense, 5′-CACAGCGCTCAGG CCATTA-3′. *PAX5* sense, 5′-CCGACTCCTCGGACCAGC-3′ and antisense, 5′-GCCGAATCCGTGCTCACC -3′. *ERN1α*: sense, 5-TCCAGAGATGCTGAGCGAAGACTG-3 and antisense, 5-TCTGCAGGCAGCTCCCGGTAGT-3. Human *XBP1s* primers were previously published (Yoshida *et al*, 2001).

Quantitative RT-PCR analysis (qRT-PCR)

XBP1 transcript expression was also determined using real time quantitative RT-PCR using TaqMan® fluorescence methodology and expression of *XBP1s* was studied using SYBR green technique, following manufacturer protocols (Applied BioSystems, Foster City, CA, USA). The primers used for *XBP1s* in these studies were previously published (Back *et al*, 2005). Human 18S rRNA was used as the endogenous control gene. Taqman's primer-probe set for these studies were designed by Applied Biosystems (Assays on Demand Gene Expression Product, PN4331182). PCR reactions were performed in the 7500 Real Time PCR System (Applied BioSystems). All reactions including controls were performed in triplicate. The relative level of target gene was normalized to the endogenous reference gene (18S rRNA) and determined using the ΔC_T method.

Gene sequence analysis

Gene sequence analysis was performed for *PRDM1*, *PAX5*, *XBP1* and *ERN1α* using the ABI PRISM 3100 fluorescent-based automated capillary electrophoresis system. Genomic DNA was isolated from BM-derived $CD19^+$ cells and cheek cells from 31 WM patients, along with 100 samples North

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American human variation panel and 10 samples Ashkenazi panel (Coriell Institute for Medical Research, Camden, NJ, USA) consisting of 56 (51%) male and 54 (49%) female samples, as well as 54 healthy donor samples provided by the Dana-Farber Molecular Diagnostics Core Facility. Genomic DNA was extracted using the Gentra PureGene kit (Qiagen, Valencia, CA, USA). Exons with flanking intron regions were amplified and sequenced using ABI dye-primer chemistry-based reagents.

Data analysis

All results of gene mRNA expression were normalized as a ratio over actin expression unless otherwise specified. Comparisons were performed using the Mann–Whitney *U* test and correlations were tested through the Pearson and Spearman correlation coefficient according to the variable tested. P value < 0·05 was considered statistically significant.

Results

Gene expression studies in CD19+ selected BM cells

We first investigated the mRNA expression of *PRDM1*, *PAX5*, *XBP1*, *XBP1s* spliced and *ERN1α* genes in $CD19^+$ selected BM cells from WM patients compared to healthy donors using semi-quantitative RT-PCR analysis (Fig 1). *PRDM1*, *PAX5*, *XBP1* transcripts were present in all patients studied as for the six healthy donors. In contrast, approximately 80% of the patients had *XBP1s* mRNA expression, 80% of whom had *ERN1α* mRNA expression, compared to healthy donors.

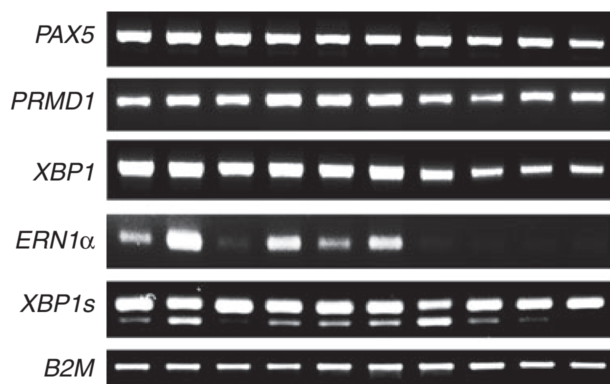

Fig 1. Semi-quantitative RT-PCR transcripts expression of *PAX5*, *PRDM1*, *XBP1*, *ERN1α* and *XBP1-s* genes was perform on bone marrow (BM) $CD19^+$ selected cells from 7 representative patients with Waldenstrom macroglobulinemia (from the initial cohort of 31 patients tested, corresponds to lanes 1–7) and 3 BM $CD19^+$ selected cells from different healthy donors (corresponds to lanes 8–10). WM, Waldenstrom macroglobulinemia; NBM, normal bone marrow.

Assessment of XBP1 transcripts and ERN1α gene expression in WM using quantitative RT-PCR (qPCR)

Given the degree of variability for *XBP1s* and *ERN1α* expression among WM patients, we next utilized qPCR to quantify *XBP1s* and *ERN1α* gene expression in $CD19^+$ BM cells isolated from WM patients (n = 16) and healthy donors (n = 4) (Fig 2A). The mean expression level of *XBP1* transcript levels among WM patients was significantly higher when compared to healthy donors (P = 0·048). Although not significant, the mean expression level of *XBP1s* was also higher than healthy donors.

We then sought to clarify whether the level of expression of these transcripts could distinguish certain subsets of patients with WM. Using a cutoff that corresponds to the mean gene expression in healthy donors for each gene, we were able to identify two groups of patients with *XBP1s* expression with 11/16 (61%) and 5/16 (38%) patients demonstrating high and low *XBP1s* expression respectively; and with *ERN1α* expression

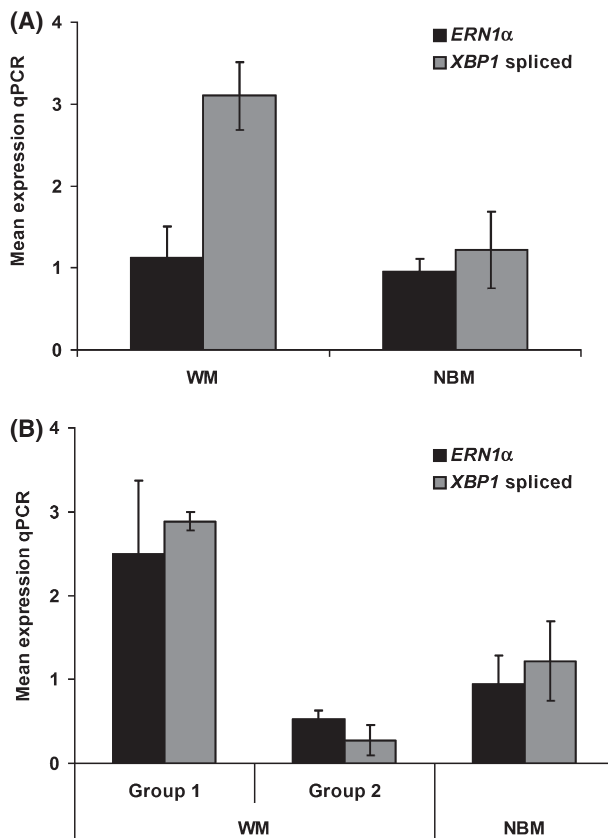

Fig 2. Mean expression of *ERN1α* and *XBP1-s* transcripts expression studied by quantitative RT-PCR. (A) BM $CD19^+$ selected cells from 16 patients with the consensus panel definition of Waldenstrom macroglobulinemia compared to four BM $CD19^+$ selected cells from healthy donors. (B) BM $CD19^+$ selected cells from the two groups of WM patients with high and low *XBP1 s* and *ERN1α* transcripts expression. The cut-off to separate the two groups was obtained from the mean of the corresponding transcript expression of four BM $CD19^+$ selected cells from healthy donors.

with 11/16 (68%) and 5/16 (31%) patients demonstrating high and low *ERN1α* expression respectively (Fig 2B). Overall, 6/16 (37%) of the patients presented with a discrepancy between *ERN1α* and *XBP1-s* transcripts expression. There was no relationship with markers of tumor burden or prognosis and *XBP1*, *XBP1s* and *ERN1α* gene expression using qPCR analysis.

Genetic sequencing

Lastly, as part of these efforts, we sequenced the exons including flanking intronic sequences for *ERN1α* and *XBP1*. Genomic DNA, collected from $CD19^+$ selected BM cells along with buccal cells with active WM disease, was sequenced for *ERN1α* and *XBP1*. As a control, genomic DNA from 164 normal donor controls was utilized as controls for these sequencing studies. Four variants were observed within the first exon of *XBP1* located on chromosome 22q12.1 for 31 patients with WM, which were then sequenced. Two of these variants were untranslated (G/t, Chr 27526434 and rs2269575) while rs5762809 and a novel silent variant (C/t, Chr 27526306) were present in the translated region of the exon. All of these variants occurred in the normal controls at greater frequency than those found in patients though only reached significance for rs5762809 ($P = 0{\cdot}044$). Finally, we sequenced *ERN1α* in 31 patients with WM, and identified several novel variants, all of which were all present in the germline of patients in WM patients. The incidence of these variants ranged from 2% to 8% and did not significantly differ from the normal donor population (Table II). No relationship was found between presence of a variant with markers of tumor burden or prognosis, as we did not find any significant correlation with the expression *XBP1* and *ERN1α* transcripts.

Table II. *XBP1* and *ERN1* sequences analysis.

SNP ID	Location	Position	Genomic change	AA change	Allelic frequency (%)
XBP1					
NA	Exon 1	27526534	G/t	–	0·052
rs2269575	Exon 1	27526526	C/t	–	0·069
rs5762809	Exon 1	27526494	G/a	Ala/Thr	0·121
NA	Exon 1	27526306	C/t	Pro/Pro	0·052
ERN1					
rs196952	Intron 2	59512032	G/a	–	0·672
rs196951	Intron 2	59512010	A/g	–	0·734
NA	Intron 3	59510874	A/g	–	0·078
NA	Intron 4	59510749	T/c	–	0·016
NA	Intron 4	59510736	C/t	–	0·078
NA	Exon 5	59506310	G/c	Val/Val	0·047
rs196940	Intron 6	59499460	T/c	–	0·719
rs196939	Intron 6	59499404	T/c	–	0·742
rs16947425	Intron 7	59498076	G/t	–	0·145
rs187826	Exon 10	59495187	T/c	Ile/Ile	1
NA	Exon 11	59491662	C/t	Leu/Leu	0·031
NA	Exon 14	59485892	G/a	Gly/Gly	0·031
rs10689374	Intron 16	59484121	AGG Insert	–	1
NA	Intron 18	59479143	A/g	–	0·032
NA	Exon 19	59479010	G/a	Ala/Ala	0·016
rs196923	Exon 19	59479004	T/c	His/His	1
NA	Intron 19	59478924	A/c	–	0·016
rs196912	Exon 22	59475212	A/g	Thr/Thr	0·703

SNP ID, Single nucleotide polymorphism identity number; NA, not applicable (previously undocumented); AA, amino acid; T, thymidine; G, guanine; C, cytidine; A, adenosine; Val, valine; Ile, isoleucine; Leu, leucine; His, histidine; Thr, threonine; Pro, proline; Gly, glycine; Ser, serine; Asp, Aspartic acid; Asn, Asparagine.

Discussion

As part of these studies, we performed detailed gene expression and sequencing analysis for several key factors with a role in lymphoplasmacytic cell differentiation: *PRDM1, PAX5, XBP1* and *ERN1α, PRDM1, PAX5* and *XBP1* transcripts were present in all WM patients samples studied. Normal *PRDM1* and *PAX5* expression is consistent with our sequence analysis that did not demonstrate any novel mutants (data not shown). The normal expression of *PRDM1* denotes with report in B-cell diffuse large cell lymphoma where absence of PRDM1 protein expression has been recently reported (Pasqualucci *et al*, 2006). A previous study found *PAX5* overexpression in patients with lymphoplasmacytic expression on the basis of the chromosomal translocation t(9;14) (Poppe *et al*, 2005). However, in a subsequent series of WM patients this abnormality could not be replicated (Merzianu *et al*, 2006).

Interestingly, we identified variations for *ERN1α* and *XBP1s* transcripts expression in patients with WM compared to healthy donors, the majority of patients having higher *ERN1α* and *XBP1s* transcripts expression than healthy donors. Although we did not identify any mutations in either *XBP1* or *ERN1α* to account for these events, we did observe a high incidence of germline single nucleotide polymorphisms in *ERN1α* that may modulate *XBP1* expression. Further studies are necessary to delineate what changes if any may occur in protein expression of *ERN1α* and *XBP1* as a consequence of such germline variants. The overexpression observed in some patients could be the consequence of the presence of lymphoplasmacytic and plasma cells in the tumoral clone (San Miguel *et al*, 2003). Lastly, the silencing of growth regulatory genes in cancer by hypermethylation has been described, which may explain the heterogeneous pattern of transcript expression observed in the *ERN1α–XBP1* pathway (Rivenbark *et al*, 2006). Other mechanisms, such as microRNAs, can be responsible for mRNA degradation or translational inhibition (Hagan & Croce, 2007).

In summary, we have identified a heterogeneous pattern of loss in key regulatory genes involved in lymphoplasmacytic cell expression, such as *ERN1α* and *XBP1s*. Further studies are required to better identify possible mechanisms for these losses in gene transcripts and determine whether it might contribute to WM pathogenesis.

Disclosure

The authors declare no competing financial interests.

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Author contributions

XL, EF, RC, IMG, SPT: designed research, analyzed data, wrote the paper; XL, ZH, VB, SH, SV: sequence analysis; LX, DDS, EH, ASM, SA, AMR, AWH, CJP, RJM: performed research.

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