

ORIGINAL ARTICLE

Waldenström's macroglobulinemia harbors a unique proteome where Ku70 is severely underexpressed as compared with other B-lymphoproliferative disorders

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Waldenström's macroglobulinemia (WM) is a clonal B-cell lymphoproliferative disorder (LPD) of post-germinal center nature. Despite the fact that the precise molecular pathway(s) leading to WM remain(s) to be elucidated, a hallmark of the disease is the absence of the immunoglobulin heavy chain class switch recombination. Using two-dimensional gel electrophoresis, we compared proteomic profiles of WM cells with that of other LPDs. We were able to demonstrate that WM constitutes a unique proteomic entity as compared with chronic lymphocytic leukemia and marginal zone lymphoma. Statistical comparisons of protein expression levels revealed that a few proteins are distinctly expressed in WM in comparison with other LPDs. In particular we observed a major downregulation of the double strand repair protein Ku70 (*XRCC6*); confirmed at both the protein and RNA levels in an independent cohort of patients. Hence, we define a distinctive proteomic profile for WM where the downregulation of Ku70—a component of the non homologous end-joining pathway—might be relevant in disease pathophysiology.

Blood Cancer Journal (2012) 2, e88; doi:10.1038/bcj.2012.35; published online 7 September 2012

Keywords: Waldenström macroglobulinemia; proteomics; 2D-electrophoresis; *XRCC6*

INTRODUCTION

Waldenström's macroglobulinemia (WM)—alternatively designated as lymphoplasmacytic lymphoma—is an uncommon B-cell lymphoproliferative disorder (LPD) characterized primarily by a lymphoplasmacytic infiltration of the bone marrow and by an immunoglobulin M monoclonal gammopathy.^{1,2}

Although the precise molecular path(s) leading to WM remain largely unknown, there is clear evidence for the post-germinal center nature of the WM clonal cells, as the *IGH* (immunoglobulin heavy chain gene) is almost always subjected to somatic hypermutation,^{3,4} although without the presence of any intraclonal variation.^{5,6} Moreover and in contrast to immunoglobulin M multiple myeloma (MM) and chronic lymphocytic leukemia (CLL), post-switch clonotypic immunoglobulins are undetectable in WM B cells, suggesting therefore that absence of isotype switching;^{5,6} although the latter could be achieved *in vitro* in response to appropriate stimuli for example, CD40-ligand and IL-4.⁷ Immunoglobulin class switching requires a functional activation-induced cytidine deaminase⁸ and uses the robust non homologous end-joining (NHEJ) pathway.⁹ The Ku (Ku70/Ku80) heterodimer is a key factor in this pathway, acting as a scaffold for the recruitment of NHEJ core or such processing factors as the DNA-dependent protein kinase catalytic subunit (DNA-PKcs) and the *XRCC4*/ligase IV complex.^{10,11}

To progress in the understanding of molecular pathway(s) underlying the advent of the disease, gene-expression profiling of WM cells has been previously performed; revealing a homogeneous expression profile, more similar to that of CLL than that of MM.¹² A small set of genes was thereafter identified to be distinctly expressed in WM. They include interleukin-6 (IL6) and genes of the mitogen-activated protein kinase pathway. Upregulation of IL6 in WM was confirmed by an independent study.¹³ Aiming to compare WM cells with B-cell morphology and those with plasma cell morphology, this work concluded that B cells and plasma cells from WM patients exhibit distinct patterns of gene expression as compared with B cells and plasma cells from patients with CLL and MM.¹³

Few proteomic studies have been performed in WM. These include a proteomic analysis of signaling pathways performed in WM and MM samples, before and after treatment with a proteasome inhibitor.¹⁴ Clustering analysis allowed to identify proteins that were expressed by either of these disorders but not both, indicating differences in cellular responses to proteasome inhibition.¹⁴ Hatjiharissi *et al.*¹⁵ on the other hand compared—using an antibody-based protein microarray method—the patterns of protein expression between untreated WM cells and normal bone marrow controls. These analyses identified upregulation of proteins of the Ras and Rho family, as well as of

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Received 28 March 2012; revised 16 July 2012; accepted 7 August 2012

cyclin-dependent kinases, apoptosis regulators and histone deacetylases.¹⁵ Moreover, high expression of the heat shock protein HSP90 was observed in cells from symptomatic WM patients.¹⁵

Here, we aimed to perform a comprehensive proteomic analysis of WM versus closely linked diseases with likely overlapping pathophysiologies. Similarly to our recent work in CLL¹⁶ and using a two-dimensional gel electrophoresis (2D-E) approach,¹⁷ we compared WM with marginal zone lymphoma (MZL) and CLL. Three notable lessons were learnt in this study: (1) in contrast to observations using gene-expression profiling and limited proteomic analyses where WM is quasi indistinguishable from CLL, MM or normal B cells, we demonstrate a—statistically significant—distinct proteomic content for WM versus other LPDs; (2) we establish the existence for WM, of 17 majorly differentially expressed proteins in the same comparative analyses; (3) among the latter, we identify Ku70, part of the Ku heterodimer, and directly involved in class switch recombination, as one of the most differentially underexpressed proteins in WM cells as compared with other B-cell LPDs.

PATIENTS AND METHODS

Patients and B-cell selection

Twenty six untreated patients with LPD (10 WM, 4 MZL, 10 CLL and 2 mantle cell lymphoma; MCL), were enrolled in this study. Peripheral blood and/or bone marrow were obtained for each patient after informed consent in accordance with the Helsinki Declaration. Clinical and biological features of all patients are provided in Table 1. Cells corresponding to 3 WM, 3 MZL and 4 CLL samples were used for 2D-E analysis, whereas those from, respectively, 9 (4 WM, 3 MZL and 2 CLL) and 14 (5 WM, 2 MZL, 5 CLL and 2 MCL) untreated patients were used for western blot and quantitative RT-PCR experiments. B cells—for all LPDs with the exception

of WM and MZL used in 2D-E—were purified by negative selection using the Rosettesep B-cell enrichment cocktail (StemCell Technologies, Vancouver, Canada) followed by density gradient centrifugation on Ficoll-Paque PLUS (GE Healthcare, Saclay, France). B cells from whole blood or bone marrow of WM and MZL patients analyzed in 2D-E were sorted by flow cytometry (FACSARIA II, Becton Dickinson, Le Pont-De-Claix, France) using anti-CD19 monoclonal antibody (CD19-PerCP Cy5.5, BD Biosciences, Le Pont de Claix, France), kappa or lambda monotypy (kappa-FITC/lambda-PE, Dako) and the immunophenotypic characteristics of the malignant clone (CD79b for the MZL2 patient) (CD79b-APC, BD Biosciences). The purity of samples isolated by rosette and flow cytometry sorting cells was >95%; often >97%.

2D-ELECTROPHORESIS (2D-E)

Ten samples were used in 2D-E analysis. For each cell suspension, total proteins were extracted in iso-electric focusing-specific lysis buffer containing 7 M urea, 2 M thiourea, 1% CHAPS, 10% isopropanol, 10% isobutanol, 0.5% Triton X100, 0.5% SB3-10, 30 mM Tris, 65 mM DTT, 0.5% IPG buffer 3–10 and 30 mM spermine. After centrifugation at 16 000 g for 30 min at 4 °C, proteins were precipitated with the Perfect-Focus Kit from G-Biosciences (Maryland, Heights, MO, USA) and resuspended in a buffer containing 7 M urea, 2 M thiourea, 1% CHAPS, 10% isopropanol, 10% isobutanol, 0.5% Triton X100, 0.5% SB3-10 and 30 mM Tris. The total protein concentration of each sample was established using the Bradford assay (Protein Assay, Bio-rad, Ivry sur Seine, France) with bovine serum albumin as standard. All protein extracts (50 µg per sample) were labeled using fluorescent Cyanine (Cy) dyes, as per the manufacturer's instructions for minimal labeling (GE Healthcare). Cy3 and Cy5 were alternatively used to label protein extracts according to the dye switch method. For each gel, two labeled protein extracts—expected to

Table 1. Patients' characteristics

Samples	Origin	Experiments	Patient's age	Patient's sex	Diagnosis	Cytogenetics
WM1_BM	Bone marrow	2D-E and qPCR	79	M	WM (IgM kappa)	Trisomy 4
WM1_B	Blood	2D-E				
WM2_B	Blood	2D-E	66	M	WM (IgM kappa)	Undetermined
WM3_BM	Bone marrow	WB	72	M	WM (IgM kappa)	Undetermined
WM4_B	Blood	WB	58	M	WM (IgM kappa)	Undetermined
WM5_BM	Bone marrow	WB	59	F	WM (IgM kappa)	Normal
WM6_BM	Bone marrow	WB	59	M	WM (IgM kappa)	Undetermined
WM7_BM	Bone marrow	qPCR	63	F	WM (IgM lambda)	Undetermined
WM8_BM	Bone marrow	qPCR	64	M	WM (IgM kappa)	Undetermined
WM9_BM	Bone marrow	qPCR	51	M	WM (IgM lambda)	Normal
WM10_BM	Bone marrow	qPCR	32	M	WM (IgM kappa)	Undetermined
MZL1_B	Blood	2D-E	61	F	MZL (IgM kappa)	Undetermined
MZL2_B	Blood	2D-E	57	F	MZL (IgM lambda)	Trisomy 18 and 3
MZL2_BM	Bone marrow	2D-E and WB				
MZL3_BM	Bone marrow	WB and qPCR	59	F	MZL (IgM kappa)	Undetermined
MZL4_B	Blood	WB and qPCR	64	F	MZL (IgM kappa)	Normal
CLL1	Blood	2D-E	89	M	CLL (IGH unmutated)	Deletion 11q
CLL1'	Blood	2D-E				
CLL2	Blood	2D-E	67	F	CLL (IGH unmutated)	Deletions 13q and 17p
CLL3	Blood	2D-E	64	M	CLL (IGH mutated)	Normal
CLL4	Blood	WB	77	M	CLL (IGH undetermined)	Deletion 13q
CLL5	Blood	WB	78	M	CLL (IGH mutated)	Trisomy 12
CLL6	Blood	qPCR	54	M	CLL (IGH unmutated)	Deletions 16q and 17p
CLL7	Blood	qPCR	52	M	CLL (IGH mutated)	Normal
CLL8	Blood	qPCR	78	M	CLL (IGH undetermined)	Undetermined
CLL9	Blood	qPCR	79	F	CLL (IGH undetermined)	Deletion 13q
CLL10	Blood	qPCR	72	M	CLL (IGH undetermined)	Undetermined
MCL1_BM	Bone marrow	qPCR	65	M	MCL	t(11;14)
MCL2_B	Blood	qPCR	57	M	MCL	t(11;14)

Abbreviations: CLL, chronic lymphocytic leukemia; IGH, immunoglobulin heavy chain gene; IgM, immunoglobulin M; MCL, mantle cell lymphoma; MZL, marginal zone lymphoma; qPCR, quantitative polymerase chain reaction; WB: western-blot; WM, Waldenström's macroglobulinemia; 2D-E, two-dimensional electrophoresis.

co-migrate—were mixed to a strip's rehydration buffer containing 7 M urea, 2 M thiourea, 1% CHAPS, 10% isopropanol, 10% isobutanol, 0.5% Triton X100, 0.5% SB3-10, 40 mM DTT and 0.5% IPG buffer 4–7 for a total volume of 460 μ l. Rehydration of a 24 cm Immobiline pH 4–7 DryStrip (GE Healthcare) was achieved in the dark during 16 h. Iso-electric focusing was then performed at 20 °C for a total of 85 000 Vh using the Ettan II IPGphor system (GE Healthcare). After migration, the strips were equilibrated in SDS containing buffer (reduction and alkylation) before being loaded onto SDS polyacrylamide gels for separation according to molecular weight using an Ettan DALT Six Electrophoresis System (GE Healthcare). After migration, 2D-E gels were scanned using an Ettan DIGE Imager (GE Healthcare) according to the manufacturer's instructions. Image analysis and statistical calculations were performed using the Progenesis SameSpots software (NonLinear Dynamics, Newcastle, UK) and the 'Multiple stains per gel without internal standards' comparison method. All sample gel images were first aligned. Spots were then automatically detected and filtered to eliminate non-protein spots. Statistical analyses (analysis of variance and principal component analyses) were performed on normalized spots data. For multigroup analysis of variance test, a *q*-value (a FDR corrected *P*-value) of <0.05 was considered significant. The threshold chosen to appreciate variations was a 2.5-fold change when comparing WM versus CLL groups.

Protein identification

Analytical gels were stained with SYPRO Ruby (Invitrogen Corporation, Carlsbad, CA, USA) and used for robotized spot picking (EXQuest spot cutter, Bio-Rad, Marnes-la-Coquette, France). Gel plugs were washed twice with 25 mM ammonium bicarbonate in 50% acetonitrile then dehydrated in 100% acetonitrile, before being subjected to overnight in-gel trypsin digestion. Briefly, each spot was incubated in a solution containing 50 mM ammonium bicarbonate, 10% acetonitrile and 20 μ g/ml trypsin (G-Biosciences) on ice during 1 h, then overnight at 37 °C. The supernatants were collected and gel plugs were incubated in 0.1% TFA 50% acetonitrile in an ultrasonic bath during 10 min to extract residual peptides. The new supernatants were pooled with the former. Peptides were dried completely in a vacuum centrifuge then resuspended in 0.1% TFA 50% acetonitrile, to be analyzed by mass spectrometry (MS or MS/MS). Peptides were spotted onto a MALDI plate with matrix solution (50% acetonitrile, 0.1% TFA, HCCA at saturation) diluted four-fold in 50% acetonitrile, 0.1% TFA and analyzed by MS using an Autoflex MALDI-TOF (Bruker Daltonics, Bremen, Germany). This instrument was operated in positive ion mode and externally calibrated in the peptide mass range of 700–3200 m/z. MS/MS analysis was performed using the Ultimate 3000 nanoLC system (Dionex, Voisin le Bretonneux, France) coupled to an Esquire HCTultra nESI-IT-MS (Bruker Daltonics). A search for protein identity was carried out with MASCOT (<http://www.matrixscience.com>). Confident matches were defined by the MASCOT score and statistical significance (*P*<0.05), the number of matching peptides and the percentage of total amino acid sequences covered by matching peptides.

Western-blot analysis

Cell lysates were prepared by incubating purified B-cell pellets on ice for 30 min in 200 μ l of a lysis buffer containing 150 mM NaCl, 50 mM Tris pH8, 1% NP40, 10% glycerol, 0.5 mM EDTA and protease inhibitor Complete 1X (Roche Diagnostics, Mannheim, Germany). The samples were then centrifuged at 4 °C for 30 min at 16 000 *g*. Samples containing 50 μ g of protein were mixed with Laemmli buffer 1X and boiled for 5 min. Protein extracts were separated on 10% SDS-PAGE, then transferred to nitrocellulose membranes (Hybond-C Extra, Amersham Biosciences, Buckinghamshire, UK). Nonspecific binding sites were blocked for 1 h in 5% milk in T-TBS

(50 mM Tris, 150 mM NaCl, 0.5% Tween-20). The membranes were then probed with anti-Ku (p70) (clone N3H10, Kamiya Biomedical, Seattle, WA, USA) during 3 h at room temperature. After thorough washing in T-TBS, incubation was carried out for 1 h in the presence of horseradish peroxidase-conjugated anti-mouse IgG (Bio-Rad Laboratories, Hercules, CA, USA). All immunoblots were revealed by enhanced chemiluminescence using the Super Signal West Pico Substrate (Pierce Biotechnology, Inc., Rockford, IL, USA). Data were acquired by scanning-densitometry using a Chemi-Start (Vilber-Lourmat, Marne-La-Vallée, France).

Quantitative real-time RT-PCR

A quantitative real-time RT-PCR amplification of *XRCC6* (encoding Ku70) was performed as a second validation test. This was achieved for 14 subjects, respectively, 5 WM and 9 others B LPD including MZL (*n*=2), MCL (*n*=2) and CLL (*n*=5). Briefly, total RNA was extracted using the RNeasy mini kit (Qiagen, Courtaboeuf, France) and treated with DNase (Qiagen). cDNA was synthesized using Improm II reverse transcriptase (Promega, Lyon, France) and random hexadeoxynucleotide primers (Promega). The relative gene expression of *XRCC6* was determined by concomitant amplification of *GUSB* (beta-D glucuronidase) as a reference gene using a LightCycler 480 (Roche). Assays were performed in duplicate using 5 μ l of cDNA, 1X Taqman Universal Master Mix (Applied Biosystems, Warrington, UK) and 1X TaqMan Gene Expression Assays (Applied Biosystems, Foster City, CA, USA) for *XRCC6* and *GUSB* in a total volume of 25 μ l. LightCycler 480 Software (Roche) was used to calculate the relative gene expression of *XRCC6* ($2^{-\Delta\Delta CT}$ method).

RESULTS

Here we present a first comprehensive 2D-E analysis of WM versus other LPDs.

WM is a unique proteomic entity

Quantitative proteomic analysis by 2D-E was performed for 10 protein extracts obtained from B cells isolated from peripheral blood and/or bone marrow of 2 WM (3 samples), 2 MZL (3 samples) and 3 CLL (4 samples) patients. The inclusion of blood and bone marrow cells from the same patients (WM1 and MZL2) was aimed to foresee the extent to which cell origin/developmental stage might have any influence in proteomic profile in a same individual.

Multivariate analysis of protein expression was first performed using a principal component analysis. Used as an explorative tool to investigate the clustering of all WM, MZL and CLL samples, principal component analysis analysis demonstrated that WM clustered distinctly from MZL and CLL (Figure 1). Hence, the protein content of WM cells is significantly different from those of MZL or CLL. Of note, one WM and one MZL patient segregated quite differently in principal component analysis, although without overlapping with other LPDs, and the 'stray' WM patient still was nearest to the other two (Figure 1).

Several proteins are differentially expressed in WM as compared with MZL and CLL

Among the 1051 polypeptide spots analyzed within different samples, 356 showed a differential expression. Most differentially expressed polypeptide spots (with a 2.5-fold change) between WM and CLL samples, and between WM and MZL samples, as identified by mass spectrometry, are presented in Table 2. The first highlight from this analysis is that the detected differentially expressed proteins are not—for the vast majority—among the candidate genes/proteins, which would have expected to be critical in WM—that is, in direct connection with leukomogenesis,

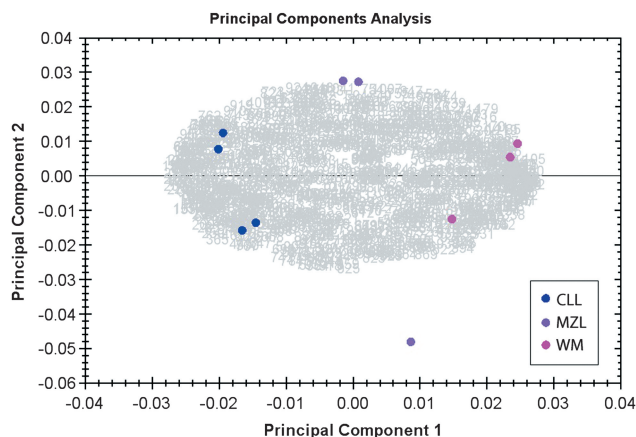


Figure 1. Principal component analysis (PCA) distinguishes WM, MZL and CLL cells. PCA as a 'score plot' of a spot map of the 10 protein extracts (3 WM, 3 MZL and 4 CLL) color-coded according to the legend, projected onto the first two principal components. The PC1 and PC2 axes segregate WM samples (pink spots) from both MZL (purple spots) and CLL (blue spots) groups.

immune-related loci and so on—but are rather confined to ubiquitously expressed structural/housekeeping proteins for example, cytoskeleton, metabolism. Remarkably the vast majority of these proteins show a similar expression pattern that is, up/downregulation in WM vs CLL or MZL hinting again to the fact that WM harbors a distinct proteome entity versus these two other closely considered LPDs. A few notable distinctly expressed proteins are the following: phosphoglucomutase-2 and annexin A6, which were strongly overexpressed, especially when comparing WM and CLL; conversely, peroxiredoxin and Ku70 (X-ray repair cross-complementing protein 6), showed the strongest downregulation in WM compared with CLL. The same trend, although less pronounced, was observed between WM and MZL. Several components of the cytoskeleton were also differentially expressed in WM cells, namely moesin, gelsolin and lamin-B1. Ku70 proteomic profile was examined more thoroughly: 2D and 3D representations together with statistical expression data are shown in Figure 2. No variation of the proteomic content between blood and bone marrow cells, neither for WM nor MZL was noted for this protein.

Lower expression of Ku70 (XRCC6) protein and gene expression in WM cells by comparison with other LPDs

Ku70 was—for obvious reasons of its direct implication in immunoglobulin class switching—the candidate protein retained for validation on a larger cohort of patients. First, western blot experiments performed on 9 untreated patients (whose characteristics are summarized in Table 1) clearly confirmed a much lower expression that is, quasi absence of Ku70 in WM as compared with MZL and CLL (Figure 3). Second, in order to investigate whether the quantitative defect of Ku70 protein was related to a lack of *XRCC6* RNA transcript or to a post-translational process, gene expression of *XRCC6* was assessed using quantitative RT-PCR (using *GUSB* as reference gene) in 14 patients with WM or other lymphoid malignancies. *XRCC6* expression was found to be significantly lower in WM cells as compared with other LPDs including MZL, CLL and MCL (Figure 4); thus suggesting that the observed protein downregulation was due to a transcriptional shut down.

DISCUSSION

WM, a rare, distinct, B LPD, is defined by lymphoplasmacytic infiltration of the bone marrow and a monoclonal immunoglobulin

M paraproteinemia, respectively, ascertained by bone marrow biopsy and serum protein immunoelectrophoresis/immunofixation. However, before performing the latter tests in order to reach diagnostic certitude, there is/are at present no unique non invasive diagnostic biomarker(s) available helping to guide the clinician in an often diversity of nonspecific early symptoms for example, fatigue, anemia, thrombocytopenia, hepatosplenomegaly and lymphadenopathy.

Array technology, especially transcriptome analysis, has been widely used in clonal hematological malignancies.^{18,19} WM has not escaped this trend, as mentioned earlier. Proteome analysis—more cumbersome presently—has been less widely used and in case of WM has been performed in only two reports with self imposed limitations/biases. Here, we present a first comprehensive 2D-E analysis of WM, as compared with other LPDs. This study yielded more significant differences for 17 proteins (Table 2). Besides, the possibly critical downregulation of Ku70, a few other modifications of housekeeping/structural proteins were observed. These include molecules involved in glucose metabolism, redox balance, cell communication, protein metabolism, regulation of translation or nucleic acid binding. Phosphoglucomutase-2 appears to be selectively overexpressed in WM compared with CLL and MZL.^{20,21} Inversely, glutaredoxin-3 (GLRX3), lactoylglutathione lyase (GLO1) and peroxiredoxin-6 (PRDX6) appeared to be underexpressed in WM cells as compared with CLL and MZL cells.²² GLO1 belongs to the glyoxalase complex, which catalyzes the conversion of methylglyoxal to D-lactate.²³ Peroxiredoxins are a ubiquitous family of antioxidant enzymes that functions as tumor suppressor to certain haematopoietic cancers in mice.²⁴ PRDX6, the most underexpressed protein in WM in our study, is a bifunctional enzyme having both peroxidase and phospholipase A2 activities, with roles in oxidative stress-induced and TNF-induced apoptosis.²⁵

Cytoskeleton proteins also appeared to be differentially expressed in WM. Gelsolin is an actin-binding protein that is a key regulator of actin filament assembly and disassembly.^{26,27} Gelsolin has been reported increased in vincristine (an anti-microtubule agent)-resistant acute leukemia, whilst moesin was decreased in the same cells.²⁸ Moesin, which expression was decreased in WM in our study, is a member of the ERM protein family, which appears to function as cross-linker between plasma membranes and actin-based cytoskeletons for cell-cell recognition, signaling and cell movement phenomena. Finally, we observed that several isoforms of annexin A6, a calcium-dependent membrane-binding protein, were upregulated in WM cells.²⁹

Again, perhaps the most notable alteration observed in our study was the silencing of Ku70 in WM cells. This was further confirmed by western blot and mRNA analysis in a second cohort of patients, strengthening the consistency of this anomaly. The Ku (Ku70/Ku86) heterodimer was first discovered as an auto-antigen in patients with autoimmune disorders.³⁰ The genes encoding these proteins are located on chromosomes 22q13 and 2q33-35.³¹ Ku is the DNA-targeting subunit of a DNA-dependent protein kinase (DNA-PK), which is a serine/threonine kinase consisting of a 465-kDa catalytic subunit (DNA-PKcs) and the heterodimeric Ku regulatory complex. The latter binds to DNA double-stranded ends and other discontinuities in the DNA³² and recruits the DNA-PKcs of the complex.³³ Classical NHEJ involves the DNA-PK complex, essential for lymphoid development, especially VDJ recombination and Ig switching. Indeed Ku70^{-/-} mice lack B-cell maturation and the absence of Ku70 confers hypersensitivity to ionizing radiation and deficiency in DNA double-stranded breaks repair,³⁴ translating in an extreme radiosensitivity and specific VDJ recombination defects,³⁵⁻³⁷ as well as high levels of chromosomal aberrations.^{38,39} In man, DNA-PK activity and increased expression of both Ku70 and Ku86 have been shown to correlate with resistance to therapeutic molecules notably in

Table 2. Most differentially expressed proteins between WM, CLL and MZL cells

Spot no. ^a	FC ^b WM vs CLL	FC ^b WM vs MZL	Access number ^c	Protein name ^d	Gene ^e	q-value ANOVA ^f	MASCOT score ^g	Sequence cover % ^g	No. of peptides matched ^g	Theoretical pI/MW ^h	Observed pI/MW ^h	Function ⁱ
685	14	3.3	Q96G03	Phosphoglucosyltransferase-2	PGM2	0.001	235	10%	5	6.28/68283	6.35/79175	Catalytic activity, metabolism, energy pathways
653	14	3	P08133	Annexin A6	ANXA6	0.0006	722	36%	23	5.42/75873	5.57/76803	Calcium ion binding, cell communication, signal transduction
769	5.8	1.6	P61978	Heterogeneous nuclear ribonucleoprotein K	HNRNPK	0.001	100	41%	15	5.39/50976	5.24/71014	Ribonucleoprotein, regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolism
476	5.2	1.2	P06396	Gelsolin	GSN	0.006	94	22%	12	5.9/80641	5.82/91105	Structural constituent of cytoskeleton, cell growth and/or maintenance
709	4.8	1.7	P20700	Lamin-B1	LMNB1	0.009	152	32%	21	5.11/66408	5.3/75379	Structural molecule activity, cell growth and/or maintenance
478	4.4	1.6	P06396	Gelsolin	GSN	0.01	450	26%	16	5.9/80641	5.74/91105	Structural constituent of cytoskeleton, cell growth and/or maintenance
811	3.9	2	P31150	Rab GDP dissociation inhibitor alpha	GDI1	0.006	68	27%	8	5.0/50583	5.05/68326	GTPase activator activity, cell communication, signal transduction
1017	3.4	3.4	Q8TBC4	NEDD8-activating enzyme E1 catalytic subunit	UBA3	0.01	75	21%	8	5.3/51852	5.38/55484	Ubiquitin-specific protease activity, protein metabolism
678	3.3	2.8	P08133	Annexin A6	ANXA6	0.004	722	36%	24	5.42/75873	5.57/76803	Calcium ion binding, cell communication, signal transduction
482	3.3	-1	P06396	Gelsolin	GSN	0.02	138	26%	15	5.9/80641	5.91/90785	Structural constituent of cytoskeleton, cell growth and/or maintenance
928	2.5	1.5	Q9CZ79	Ubiquitin activating enzyme E1 domain containing 1	UBA5	0.006	149	19%	5	4.79/45291	4.89/59448	Protein binding, protein metabolism
798	-2.5	-1.6	P14866	Heterogeneous nuclear ribonucleoprotein L	HNRNPL	0.006	268	12%	5	8.46/64133	6.83/70435	RNA binding, regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolism
1673	-2.6	1.6	Q04760	Lactylglutathione lyase	GLO1	0.005	66	26%	7	5.12/20778	5.01/22387	Lyase activity, metabolism, energy pathways
1291	-2.9	2.6	O76003	Glutaredoxin-3	GLRX3	0.0002	251	22%	6	5.31/37432	5.53/41031	Molecular function unknown, biological process unknown
1465	-3	-2.3	Q8WXX5	DnaJ homolog subfamily C member 9	DNAJC9	0.02	460	43%	13	5.58/29910	5.85/31685	Chaperone activity, protein metabolism
1046	-3.1	1.1	P13489	Ribonuclease inhibitor	RNH1	0.002	322	15%	6	4.71/49973	4.71/53948	Translation regulator activity, regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolism
463	-3.5	-1.6	P13639	Elongation factor 2	EEF2	0.003	571	18%	14	6.41/95338	6.75/96931	Translation regulator activity, protein metabolism, translation
462	-4	-2.1	P13639	Elongation factor 2	EEF2	0.01	99	3%	2	6.41/95338	6.83/96704	Translation regulator activity, protein metabolism, translation
651	-4.5	-2.5	P26038	Moesin	MSN	0.03	720	23%	13	6.09/67820	6.41/82098	Structural constituent of cytoskeleton, cell growth and/or maintenance
713	-5.6	-3.4	P12956	X-ray repair cross-complementing protein 6	XRCC6	0.04	114	7%	5	6.23/69843	6.47/77798	DNA binding, regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolism
712	-7.4	-3.3	P12956	X-ray repair cross-complementing protein 6	XRCC6	0.04	225	41%	23	6.23/69843	6.57/77344	DNA binding, regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolism
1	-12	-4.4	P30041	Peroxiredoxin-6	PRDX6	0.02	438	46%	10	6.0/25035	6.44/25730	Peroxidase activity, metabolism, energy pathways

Abbreviations: ANOVA, analysis of variance; CLL, chronic lymphocytic leukemia; FC, fold change; MW, molecular weight; MZL, marginal zone lymphoma; WM, Waldenström's macroglobulinemia. ^aSpot numbers refer to ID spots. ^bFC was calculated using Progenesis SameSpots software; negative FC signifying that polypeptide spot is underexpressed in WM samples as compared with CLL or MZL cells, and positive FC signifying that spot is overexpressed in WM cells. ^cAccession number in the UniProt database (<http://uniprot.org>). ^dFull protein name recommended by the UniProt consortium. ^eGene names according to HUGO Gene Nomenclature. ^fq-value from multigroup ANOVA was calculated using Progenesis SameSpots software. ^gMASCOT score indicates the confidence of protein identification using the Mascot search engine (<http://www.matrixscience.com>), depending on amino acid sequence coverage (in %) and on number of matched peptides used for identification in the Swiss-Prot database (<http://expasy.org/sprot/>). ^hTheoretical pI and molecular weight obtained from the Mascot database. Observed molecular weight and pI calculated by DeCyder version 6.5 software, according to location in the gel. ⁱProtein function and biological process were assigned in accordance with the Human Protein Reference Database (<http://hprd.org>).

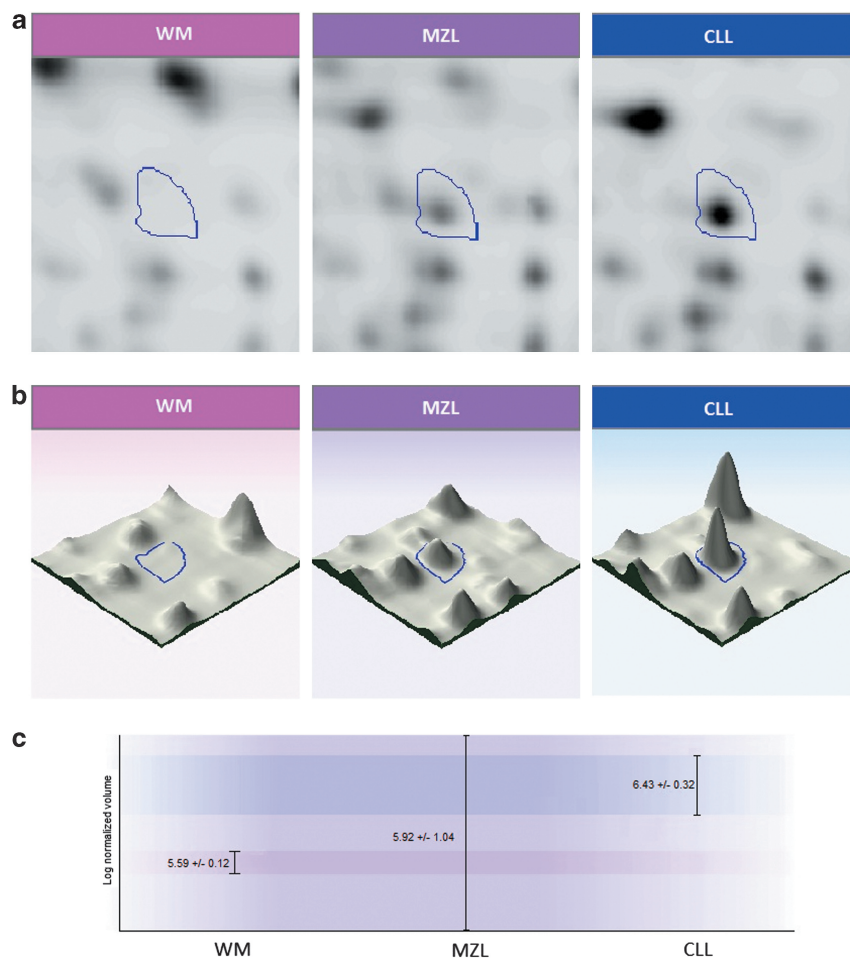


Figure 2. 2D-E analysis of Ku70 protein expression. Spots were analyzed with Progenesis SameSpots (NonLinear Dynamics). (a) Representative focus of the Ku70 protein (spot no. 712) on WM, MZL and CLL samples 2D-gel images. (b) 3D-representation of the Ku70 volume ratios difference between WM, MZL and CLL samples. (c) Statistical analysis of spot no. 712: significant decrease of Ku70 expression in WM cells as compared with CLL cells ($*P < 0.05$).

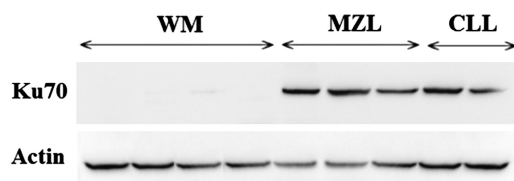


Figure 3. Western-blot validation of the Ku70 2D-E profile. Western blot results of Ku70 expression in samples from WM, MZL and CLL cells. Actin was used as loading control.

the context of CLL.⁴⁰ Minor defects in the NHEJ pathway moreover have been shown to confer predisposition to leukemia.⁴¹ Most LPDs arise via transformation of post-germinal center B cells with chromosomal mutations.⁴² Immunodeficiencies with both abnormal DNA repair⁴³ and genetic polymorphisms of NHEJ components^{44,45} have been associated to an increased susceptibility to the development of lymphoid malignancies, suggesting that an aberrant NHEJ pathway could lead to lymphomagenesis. However, when investigated, no mutation or deletion of Ku86 has been observed in CLL or acute lymphoblastic leukemia cells.⁴⁶ Acute lymphoblastic leukemia cells were found to express high levels of DNA-PKcs, Ku86 and Ku70 protein, whereas CLL cells displayed a lower expression of DNA-PKcs and

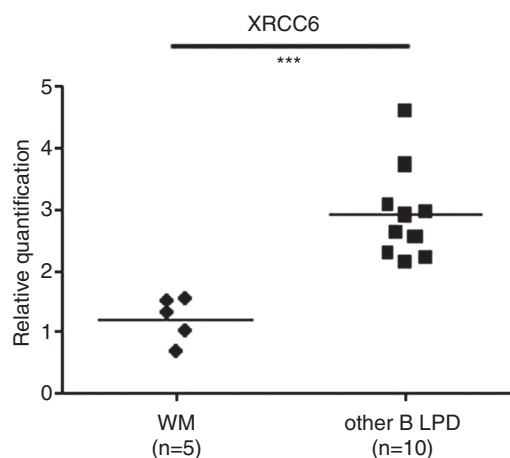


Figure 4. Quantitative real-time PCR validation for *XRCC6*. Relative gene-expression quantification for *XRCC6* (Ku70) and *GUSB* (beta-D glucuronidase) as reference gene, in WM and other B LPD (MZL, MCL and CLL) samples ($**P < 0.001$ by Student's *t*-test).

Ku86 but not Ku70.⁴⁷ By quantitative RT-PCR in several types of LPD (not including WM), a lower expression of Ku70-encoding *XRCC6* gene was observed compared with reactive lymph nodes.⁴⁸

Lower expression of Ku70 transcripts by quantitative RT-PCR could be the result of at least two phenomena: (a) genetic (point mutation etc.) or epigenetic (promoter methylation, microRNA etc.) mechanisms; recently described as key regulators in WM biology;⁴⁹ (b) as part of a larger transcriptional downregulation that is, expression of BLIMP1—a transcriptional repressor — involved in terminal differentiation of B cells to plasma cells has been shown to shut down immunoglobulin class switching through inhibition of activation-induced cytidine deaminase, Ku70, Ku86, DNA-PKcs and STAT6.⁵⁰

In conclusion, this is a comprehensive proteomic analysis of the WM cells in comparison with that of two other B LPDs. This study shows that WM harbors a unique proteome with regards to CLL and MZL cells. A rather limited set of proteins were found to be differentially expressed with no 'usual suspects' being part of this list where the majority of proteins were rather housekeeping/ structural proteins. The confirmation of the downregulation of Ku70—part of the Ku heterodimer, a critical factor in class switch recombination (lacking in WM)—and its mechanisms need to be further investigated in model systems.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

We would like to thank Izo Delic (CEA, Fontenay-aux-roses, France) and Ali Dalloul (EA RHEM, Nancy, France) for helpful advice and Manuel Chapelle (Plateforme Protéomique/ Spectrométrie de masse, Institut Jacques Monod, Paris, France) for allowing us to use the EXQuest spot cutter. AP was supported by a fellowship from the Fondation pour la Recherche Médicale. This work was supported by the Ligue régionale Alsace contre le Cancer, the Association pour la Recherche contre le Cancer (ARC), Fondation pour la Recherche Médicale (FRM) and Agence Nationale pour la Recherche (ANR) and the Laboratoire d'Excellence TRANSPLANTE; a member of the Strasbourg University 'Initiative in Excellence' (IDEX).

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