Proteomic Analysis of Waldenstrom Macroglobulinemia

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Abstract

To better understand the molecular changes that occur in Waldenstrom macroglobulinemia (WM), we employed antibody-based protein microarrays to compare patterns of protein expression between untreated WM and normal bone marrow controls. Protein expression was defined as a >2-fold or 1.3-fold change in at least 67% of the tumor samples. Proteins up-regulated by >2-fold included Ras family proteins, such as Rab-4 and p62DOK, and Rho family proteins, such as CDC42GAP and ROKα. Other proteins up-regulated by >1.3-fold included cyclin-dependent kinases, apoptosis regulators, and histone deacetylases (HDAC). We then compared the samples of patients with symptomatic and asymptomatic WM and showed similar protein expression signatures, indicating that the dysregulation of signaling pathways occurs early in the disease course. Three proteins were different by >2-fold in symptomatic versus asymptomatic, including the heat shock protein HSP90. Elevated protein expression was confirmed by immunohistochemistry and immunoblotting. Functional significance was validated by the induction of apoptosis and inhibition of proliferation using specific HDAC and HSP90 inhibitors. This study, therefore, identifies, for the first time, multiple novel proteins that are dysregulated in WM, which both enhance our understanding of disease pathogenesis and represent targets of novel therapeutics. [Cancer Res 2007;67(8):3777–84]

Introduction

Waldenstrom macroglobulinemia (WM) is a low-grade lymphoproliferative disorder characterized by the presence of a lymphocytic, plasma cell and lymphoplasmacytic infiltrate in the bone marrow and the presence of a serum monoclonal protein immunoglobulin M (IgM; refs. 1–3). To date, WM remains incurable, with a median survival of 5 years (4–6). The current therapeutic modalities available include alkylator agents, purine nucleoside analogues, and rituximab. The main risk factor for the development of WM is preexisting IgM-monoclonal gammopathy of undetermined significance (MGUS; refs. 7, 8).

Comprehensive investigation of the underlying molecular alterations in WM has been made possible with the introduction of gene expression profiling. A recent study of gene expression profiling of 23 cases of WM compared with cases of multiple myeloma and chronic lymphocytic leukemia (CLL) revealed that WM had a homogenous expression profile on unsupervised clustering analysis and had a similar expression profile to CLL (9). A small set of genes had a unique expression profile in WM, which included the mitogen-activated protein kinase (MAPK) pathway and interleukin-6 (IL-6). However, gene expression profiling determines the level of mRNA in the samples and controls, and changes in mRNA levels do not always translate into changes at the protein level (10).

The recent development of protein array techniques allows for a comprehensive analysis of molecular changes at a functional protein level. Conventional methods of proteins quantification, such as immunoblotting, ELISA, and two-dimensional gel electropheresis are not amenable to high-throughput applications. The antibody-based protein microarray allows the screening of multiple samples simultaneously and generation of signature profiles (11–14). In this study, we defined the differential expression of proteins in tumor samples from patients with WM versus normal bone marrow lymphocytes and plasma cells and then validated in functional assays their biological importance as potential targets of novel therapeutics in WM.

Materials and Methods

Patient samples. This study was approved by the Mayo Foundation and the Dana-Farber Cancer Center Institutional Review Boards and conducted in accordance with the Declaration of Helsinki. All patients gave written informed consent for their tissue to be used for research and for review of their clinical records. For the protein array analysis, 10 frozen bone marrow samples were obtained from patients with newly diagnosed WM between August 1999 and February 2003. Another four samples were obtained from patients with symptomatic WM, between September 2005 and April 2006, for confirmation studies by immunoblotting. The diagnosis of WM was based on the characteristic tumor cell morphology and immunophenotype, as well as the demonstration of an IgM monoclonal protein by immunoelectropheresis and immunofixation. Cells were obtained from bone marrow aspirates using immunomagnetic bead selection (Miltenyi Biotech, Auburn, CA) for concomitant isolation of $CD19^+$ and $CD138^+$ cells as previously described (9). This technique led to the selection of malignant cells with more than 85% homogeneity as confirmed by κ and λ immunostaining of purified cells.

Normal $CD19^+$ lymphocytes and $CD138^+$ plasma cells were isolated in a similar fashion from four bone marrows that were pooled and used as nondiseased controls for all microarray experiments to provide a constant reference point for arrays. These cells were obtained from bone marrows because they represent the normal counterpart of the WM cells in the bone marrow microenvironment context. Another three normal controls were

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used for confirmation studies with immunoblotting and immunohistochemistry. A total of 11 separate experiments with antibody-based protein microarrays were done. All samples were compared with a pooled control sample of $CD19^+$ and $CD138^+$ cells from four normal bone marrow controls. One experiment was done with a normal control extract versus itself to detect any nonspecific binding to the antibodies imprinted on the microarray slides and to provide a normalized baseline ratio for comparison of all WM samples to control.

To determine differences between symptomatic and asymptomatic WM, we selected 10 cases of WM, 5 with symptomatic WM and 5 asymptomatic. The asymptomatic WM/MGUS samples were selected by the presence of an IgM monoclonal protein with an M-spike <1.0 g/dL, and the presence of lymphoplasmacytic cells of <10% in the bone marrow and the absence of symptoms related to WM. In addition, follow-up of these patients showed no progression to symptomatic WM to the date these studies were done.

Protein microarray procedure. The Ab Microarray (BD Clontech, Palo Alto, CA) detects a wide variety of proteins (both cytosolic and membrane bound) representing a broad range of biological functions, including signal transduction, cell-cycle regulation, gene transcription, and apoptosis. The microarray contains 512 highly specific and sensitive monoclonal antibodies (mAb) against human polypeptides. Because each antibody is printed twice on each microarray slide for internal control of each sample, each slide contains 1,024 antibodies.[5]

After CD19 and CD138 enrichment, cells ($1–6 \times 10^6$) were sedimented. Total protein was extracted by a single freeze-thaw cycle of the cell pellet in liquid nitrogen followed by homogenization in the manufacturer's extraction/labeling buffer. After measurement of the total protein concentration by the bicinchoninic acid method (Pierce, Rockford, IL, USA), each sample was diluted to a final total protein concentration of 1.1 mg/mL. For each sample and control, 90 μL of total protein was labeled with 10 μL of labeling dyes, either Cy3 or Cy5 (Amersham Biosciences, Piscataway, NJ). The dual-fluorescence detection method is designed so that inherent variations in dye labeling do not affect the outcome of the experiment. After 90 min of incubation and 30 min of blocking, the unbound dye was removed using PD-desalting columns (Amersham Biosciences). Protein concentration was again determined using the bicinchoninic acid method with subtraction of the dye's contribution to the overall absorbance at 562 nm. The average number of dye molecules covalently coupled to each protein was measured as per the user manual and usually ranged from two to four molecules of Cy3 or Cy5 per molecule of protein.

Two slides, each imprinted with 512 mAbs, were provided for reverse color labeling to allow normalization of the samples. The arrays are printed on standard-size (75 × 25 × 1-mm) glass slides with 512 antibodies printed in duplicate on each slide. Protein samples from normal B cells labeled with Cy5 were mixed with protein samples from WM B cells labeled with Cy3 protein and added to slide 1. For slide 2, the protein samples from normal B cells were labeled with Cy3, and the proteins from WM B cells labeled with Cy5. Labeling the proteins from both the normal and malignant B cells with Cy3 and Cy5 allows the microarray to detects differences in specific protein abundance between the WM sample and the control sample with each experiment. Total protein (20 μg) was added to each slide and incubated at room temperature for 30 min before a series of washes. The slides were dried and scanned according to the instructions of the supplier using the Axon GenePix 4000B scanner set to 635 nm (Cy5 channel), PMT 670 V, power 33%, and 532 nm (Cy3 channel), PMT 550 V, Power 33% to produce a text file with signal intensities. Two ratios were generated from the spot images, WM-Cy5/normal-Cy3 (slide 1) and normal-Cy5/MCL-Cy3 (slide 2), for each protein target.

Data analysis and clustering. The mean of the ratios of Cy5/Cy3 of both slides were analyzed using Clontech Excel software developed specifically for each microarray lot by the manufacturers. The two ratios were used to calculate an internally normalized ratio (INR), or ratio of ratios, for each spot on the array. This calculation normalizes for differences due to labeling efficiency and antibody-antigen binding affinity, greatly enhancing the precision and accuracy of the assay. The replicate values within each slide were then averaged, and an INR was calculated where INR = $\sqrt{\text{ratio 1/ratio 2}}$ and ratios 1 and 2 correspond to slides 1 and 2. ratio 1 = normal-Cy5 relative fluorescent units/WM-Cy3 relative fluorescent units, and ratio 2 = WM-Cy5 relative fluorescent units/normal-Cy3 relative fluorescent units. The average INR was calculated for each antibody; and duplicate INR values that varied by >30% were discarded. The Genespring software was used for analysis of all 10 experiments and normalized to the control versus control experiment. An unsupervised clustering analysis was done; and changes that were 1.3- or 2-fold or higher in at least 6 of 10 (67%) WM samples compared with the control were identified. These differences were statistically significant at $P = 0.05$.

Cell lines and reagents. The WM cell lines (BCWM.1 and WSU-WM) and IgM-secreting low-grade lymphoma cell lines (MEK1, RL) were used. The BCWM.1 is a recently described WM cell line that has developed from a patient with untreated IgMκ WM (15). The cells express the typical lymphoplasmacytic phenotype (15). WM-WSU was a kind gift from Dr. AlKhatib (Wayne State University, Detroit, MI). MEK1 was a kind gift from Dr. Neil Kay (Mayo Clinic Rochester, MN). In addition, we used a multiple myeloma cell line, MM.1S, a kind gift from T. Hideshima (Dana Farber Cancer Institute, Boston, MA) to determine the expression of proteins in plasma cells. All cell lines were cultured in RPMI 1640 containing 10% fetal bovine serum (Sigma Chemical, St. Louis, MO), 2 μmol/L L-glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin (Life Technologies, Grand Island, NY). The histone deacetylase (HDAC) inhibitor trichostatin A (Upstate, New York, NY) and HSP90 inhibitor 17-AAG (A.G. Scientific Inc., San Diego, CA) were used to determine the functional effect of inhibition of these proteins on survival of WM cell lines.

Immunohistochemistry. Paraffin-embedded bone marrow biopsies available from the same patients with the same dates of sample acquisition were analyzed by immunohistochemistry using monoclonal antibodies to human proteins: cyclin-dependent kinase 2 (CDK2; LabVision Neomarker), Rab4, HDAC3, RCC-1, fatty acid synthase (FAS), and HSP90 (BD PharMingen, San Diego, CA). Anti-CD20 antibodies (Ventana Stainer, Ventana Medical System, Tucson, AZ) were used to localize the areas of lymphocyte aggregates. Another four normal controls were used to confirm the expression level of proteins in the control samples. Images were visualized under a Leica DM IL microscope (Wetzlar, Germany) using an objective lens of 40/0.6 × 2 (magnification, 80×) equipped with a camera Leica dfc300 FX and software Leica IM50 version 6.2.1.

Immunoblotting. WM and IgM-secreting lymphoma cell lines, along with primary $CD19^+$ or $CD138^+$ cells or concomitant $CD19^+/CD138^+$ cells from patients with WM ($n = 10$) and normal donors ($n = 5$), were harvested and lysed. To determine that the changes in protein expression were not due to different ratios of plasma cells and lymphocytes in the samples, we did immunoblotting using primary WM cells ($n = 3$) sorted separately for CD19 and for CD138 and compared them to normal controls ($n = 2$) that were selected in a similar fashion. Similarly, we confirmed the expression of these proteins using another three samples of WM cells selected for concomitant $CD19^+/CD138^+$. Immunoblotting was done using lysis buffer (Cell Signaling Technology, Beverly, MA) reconstituted with 5 mmol/L NaF, 2 mmol/L Na_3VO_4, 1 mmol/L phenylmethylsulfonyl fluoride, 5 μg/mL leupeptine, and 5 μg/mL aprotinin. Whole-cell lysates were subjected to SDS-PAGE and transferred to polyvinylidene diflouride membrane (Bio-Rad Laboratories, Hercules, CA). The antibodies used for immunoblotting included Rab4, p62DOK, ROK, CDC42, and HSP90 (Cell Signaling Technology). Anti-actin antibody (Santa Cruz Biotechnology, Santa Cruz, CA) was used for loading control.

Apoptosis assay. Apoptosis was detected by using annexin V/propidium iodide (PI) staining. In brief, cells (1×10^6) from 24- and 48-h cultures with trichostatin or 17-AAG were washed with ice-cold PBS and resuspended in binding buffer [10 mmol/L HEPES (pH, 7.4), 140 mmol/L NaCl, 2.5 mmol/L $CaCl_2$]. WM cells were incubated with annexin V-FITC (5 μL/mL; Caltag Laboratories, Burlington, CA) for 15 min at 4°C. Analysis of the data was

[5] The complete list of the arrayed antibodies, including Swiss-Prot ID numbers of the target antigens, is available at http://bioinfo2.clontech.com/abinfo/ab-list-action.do.

done using flow cytometry (Beckman Coulter Inc., Fullerton, CA) as previously described (18). Similarly, WM cells from two patients were cultured with serial concentrations of trichostatin or 17-AAG for 48 h and analyzed for apoptosis using annexin/PI staining.

Results

Baseline patient characteristics. The median age of the 10 patients of whom samples were used for protein microarray was 69.4 years (range, 55.8–78). There were six men and four women in this cohort. At the time of sample collection, all patients had no previous therapy. The median M-spike for the patients with asymptomatic WM/MGUS was 0.7 mg/dL (range, 0–1.3) and 3.3 mg/dL (range, 1.6–5.9) for those with symptomatic WM ($P = 0.009$). The percent bone marrow involvement with lymphoplasmacytic malignant cells was 3% (range, <5–5%) for patients with asymptomatic WM/MGUS and 30% (range, 10–60) for patients with symptomatic WM.

Protein expression. Unsupervised clustering of the WM samples showed a homogenous pattern of expression in all the samples (Fig. 1*A*). We analyzed polypeptides that were up- or down-regulated by >1.3-fold or >2-fold as compared with normal control. The >1.3-fold expression cutoff was recommended by the manufacturer as a significant value for up-regulated proteins. We also used a cutoff of >2-fold because it has been the standard cutoff used in the analysis of cDNA array data. The expression level of each of the polypeptides dysregulated by >1.3-fold is shown in the unsupervised clustering analysis in Fig. 1*A*, and the fold change in the level of each of these proteins in all the samples as compared with the control is shown in Fig. 1*B*. Using the >2-fold cutoff, the microarrays identified six dysregulated polypeptides in at least 60% samples of WM (Table 1 and Fig. 1*C*). All polypeptides were overexpressed in the WM cells as compared with control cells. These polypeptides were signal transduction regulators, such as Ras-related proteins, including Rab4 and p62DOK; Rho-related proteins, including CDC42GAP, and ROKα; and other proteins, such as SNX-1, Roaz, and FAS. Using the >1.3 cutoff, 105 polypeptides were up-regulated, and 74 were down-regulated in at least 60% samples of WM (Tables 2 and 3). These included polypeptides involved in cell cycle regulation, such as CDK2 and RCC-1, histone deacetylases such as HDAC3, and modulators of apoptosis, such as the proteins in the phosphoinositide-3-kinase pathway and proteosome/ubiquitin pathway.

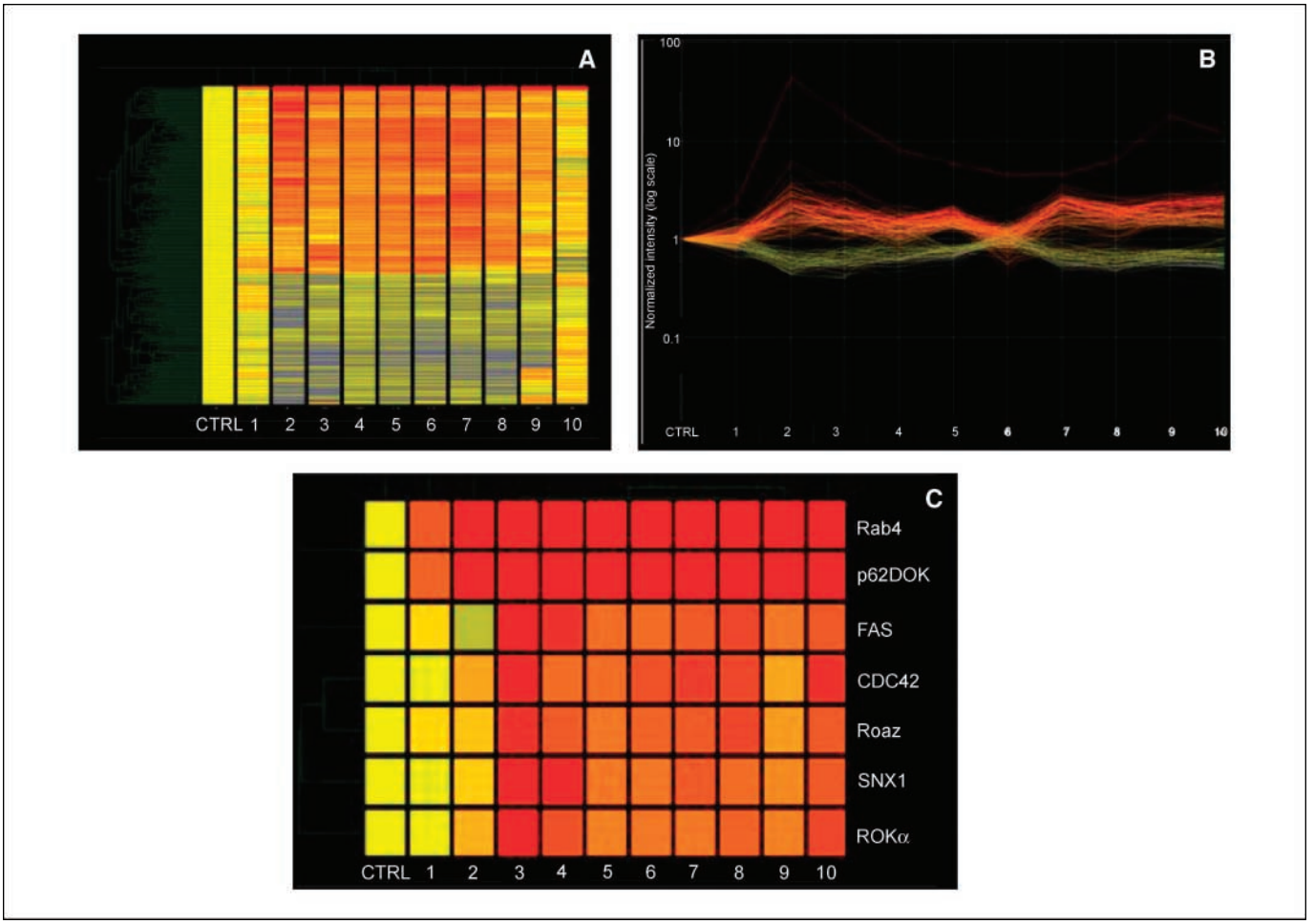

Figure 1. *A,* heat map of unsupervised clustering analysis of polypeptides up-regulated by 1.3-fold in samples of 10 patients with WM as compared with normal control (*CTRL*). All samples were normalized to control versus control samples (*first sample, left*). *Yellow,* protein level similar to the control; *red/orange*, overexpressed; *blue/green*, underexpressed. *B,* expression levels of polypeptides up-regulated by 1.3-fold or more in samples of patients with WM. Rab4 and p62DOK were up-regulated by 8-fold as compared with the normal control. *C,* heat map of polypeptides up-regulated by 2-fold or more in the 10 samples as compared with control. These included seven proteins, including Rab4, p62DOK, FAS, CDC42, Roaz, SNX1, and ROKα.

Table 1. Proteins up-regulated by 2-fold or more in WM samples compared with control

Protein	Function
Ras-related protein (Rab4)	Small GTPase in the Ras family that regulates recycling of proteins from the early endosomes to the cell surface. Overexpression of Rab4 causes a redistribution of receptors on plasma membrane versus endocytic compartments.
RasGAP-associated docking protein $p62^{DOK}$ (P62DOK)	p62dok is a major substrate for many tyrosine kinases, including c-kit, v-abl, vFPS, epidermal growth factor, and platelet-derived growth factor. Upon phosphorylation by kinases, p62dok forms a complex with Ras GTPase-activating protein.
FAS	Fatty acid synthesis. Up-regulated in most human carcinomas. False positive in WM.
CDC42GAP	Along with other members of the GTP binding proteins (Rho and Rac), CDC42GAP is implicated in regulating a variety of cellular functions, including actin cytoskeleton organization, cell growth control and development, transcriptional activation, membrane trafficking, and cell transformation.
Sorting nexin 1 (SNX1)	Involved in intracellular trafficking. This endosomal protein regulates the cell surface expression of epidermal growth factor receptor. This protein also has a role in sorting protease-activated receptor-1 from early endosomes to lysosomes.
Rat O/E-1–associated zinc finger protein (Roaz)	Alters the expression of target genes during cell lineage determination and differentiation.
ROKα	Downstream of Ras/Raf-1. Binds RhoA, B, and C. Involved in reorganization of the cytoskeleton.

We then determined whether there was a difference in protein expression in patients with asymptomatic disease/MGUS as compared with those with symptomatic WM who required therapy. As shown in Fig. 2, unsupervised clustering showed no difference in protein expression between samples of patients with symptomatic versus asymptomatic disease. However, there were three proteins identified as up-regulated in symptomatic WM as compared with asymptomatic WM//MGUS by >2-fold expression level. These included the heat shock protein HSP90, the Ras family protein CDC25C, and the chemotaxis protein p43/EMAPII.

Validation of the results. To assess reproducibility, a protein array was done of protein from normal $CD19^+$ and $CD138^+$ cells against the protein from the same cells, and no difference was found between the two samples, indicating equal labeling of the proteins by the two fluorescent dyes (data not shown). To validate the results of the protein microarray, immunohistochemistry on paraffin-embedded tissue from the same biopsies used for the protein array analysis was done. Figure 4 shows the expressions of Rab4, HDAC3, and RCC1 that were overexpressed in symptomatic WM samples as compared with control. In addition, to further confirm the overexpresssion of HSP90 in symptomatic WM, we determined the level of HSP90 on samples of symptomatic WM as compared with control and showed that HSP90 is overexpressed in these samples consistent with the protein array data (Fig. 3). Although some of the predictions of the arrays were confirmed, immunohistochemistry also showed that the antibody arrays had false-positive results. FAS was identified as an overexpressed protein in the WM samples compared with the normal controls. On validation with immunohistochemistry, we determined that FAS identifies only adipocytes present in the bone marrow, which may have been present in a higher quantity in the WM samples as compared with the control, leading to a false-positive result. To

Table 2. Proteins up-regulated by >1.3-fold (n = 105) in WM as compared with control

Signaling pathways	Proteins
Apoptosis/proliferation	14-3-3e; caspase-7/Mch3, iNOS/type II, FADD/Mort-1, Smac/DIABLO, caspase-8/FLICE, PCNA, perforin, Inhibitor 2, PTP1B, PI-4 kinase b, Janus-activated kinase 1, Stat 3, HSF4, PKC I
Cell cycle	Cdk2, cdk1, FBP, Rb2, c-Myc, HDAC3, E2F-2, GCIP, RCC1.
Protein kinases	FYB/SLAP-130, STI1, NM23-H1 (nucleoside Di-P kinase), NEK3, Clk1 (Sty), guanylate kinase, CaM kinase kinase, MKP2, TNIK, Rac1
Adhesion, invasion, and cell motility	Integrin b3 (CD61), MDC9 (ADAM), JAM-1, Mena, Maspin, LAR, annexin II, p62 lck ligand, pp120 src substrate, CLA-1 (CD36), RPTPb, nexilin, contactin, tensin
Transcription-related proteins	CBP, DP-1 Ku70, Max, p54nrb, TLS, Pax-5
Others	AP-180 (AP-3) adenovirus 5E1A, GFAP, SIII p15, IGFBP-3, M33, DBP2, SV40 large T antigen, La Protein, Brm, Neurogenin3, Rch-1, annexin IV, CD3z, ECA39, SRP54, ZFP-37, PECI, DCC, Lamp-1, AKAP 79, cGB-PDE, IL-12 (p70), GAGE, SH2-B, FEN-1, rSec8, PYK2, Striatin, L1, GRIP, Rag-1, PDI, DLP1, JIP-1, GIT1, G3BP, Rad 50, RanBP3

Table 3. Proteins down-regulated by >1.3-fold in WM compared with control (n = 74)

Signaling pathways	Proteins
Survival and cell cycle	JNKK1, NPAT, hRAD9, RalA, GOK/Stim1, PP2A Catalytic a, PKC b, PKC a, PKCλ, IKKg/3/NEMO, NF-κB, IKKa/1, IkBe, PARP, Btf, p55Cdc
Adhesion, cell motility	Integrin a3 (CD49c/VLA-3 a), Fibronectin, Phospholipase Cγ 1, p140mDia, MCP-3, Melusin
Transcription- and translation-related proteins	eIF-4g, p300, NTF2, TFII-I/BAP-135
Other proteins	Cyclooxygenase-2/PGHS, CDC27, heme oxygenase 1, acetylcholine receptor (b), IL-3, IL-13, Mint1, Gelsolin, cathepsin L, VHL, Attractin, PMCA2, annexin VI, ORC5, Tim23, CTBP1, chromogranin A, caveolin 1, LEDGF syntaxin 11, TRF2, ZAP70 kinase nNOS, WT1 (Wilm's tumor protein), Mint3, MyoD, L-Caldesmon, K+ Channel a, XIN, COMT, SATB1, CAF-1 p150, SRPK2, FMS (CD115), annexin XI, plakophilin 2a, Mxi-1, AMACR, Rab24, amphiphysin, MUPP1, KSR-1, Psme3/PA28-g, CNTFRa, PMF-1, TFIIB, SIP1, MONA

further confirm that the level of expression in normal controls was reproducible in multiple samples, we analyzed three different normal bone marrow samples and showed that the levels of Rab4, HDAC3, RCC1, and HSP90 were similar in all samples (data not shown).

In addition, to further validate our results, we did immunoblotting on 10 samples of newly diagnosed symptomatic WM and 5 different controls that were not included in the protein array analysis. As shown in Fig. 4*A*, p62DOK, Rab4, and HSP90 were overexpressed in WM samples (n = 3) compared with normal control cells. Similarly, the WM cells lines and IgM-secreting lymphoma cells lines (BCWM1, RL, and WM-WSU) had a high expression of all three proteins. In this experiment, we used $CD19^+$ cells and $CD138^+$ cells selected separately to detect a true differential expression of these proteins between malignant cells and normal control and exclude changes that may occur due to the ratio of lymphocytes and plasma cells in each sample. As shown in Fig. 4*A* and *B*, both the $CD19^+$ cells and the $CD138^+$ cells of the same samples expressed a higher protein concentration of HSP90, p62DOK, and Rab4 compared with control. We then confirmed those changes in a different set of WM samples (n = 4) and control normal BM $CD19^+$ cells (n = 2) as shown in Fig. 4*C*. Finally, we used a third group of primary WM samples (n = 3) that were selected for concomitant $CD19^+/CD138^+$ cells and compared with normal controls selected in a similar fashion. As shown in Fig. 4*D*, the expression of HSP90, p62DOK, and Rab4 was higher in the WM samples compared with normal control. These data confirm the overexpression of p62DOK, Rab4, and HSP90 in 10 symptomatic WM samples that were not used in the protein array analysis.

The HDAC inhibitor trichostatin and HSP90 inhibitor 17-AAG showed inhibition of proliferation and induction of apoptosis in WM cell lines and patient samples. To confirm the functional significance of protein elevation, we used the HDAC inhibitor trichostatin and HSP90 inhibitor 17-AAG. BCWM1 and WM-WSU cells were cultured for 24 and 48 h in the presence of trichostatin (1–100 ng/mL) or 17-AAG (300–900 nmol/L). As shown in Fig. 5, trichostatin inhibited WM cell survival at 24 and 48 h in a dose-dependent fashion. Similarly, 17-AAG (900 nmol/L) induced apoptosis at 24 and 48 h (Fig. 5*B*). Finally, we confirmed the apoptotic effects of 17-AAG and trichostatin on primary WM cells obtained from patients and showed significant induction of apoptosis at 48 h in response to both 17-AAG and trichostatin.

Discussion

There is an urgent need to elucidate the molecular pathways that mediate proliferation and resistance to apoptosis in WM to provide targets for novel therapies. Transcriptional profiling in WM has identified some pathways that are up-regulated in WM (9). Proteomic analysis represents a technique that yields more information at the functional protein level. The antibody array technology represents a high-throughput new technology to identify novel proteins and rapidly screen multiple samples yielding molecular signatures and profiles. However, this technique has its limitations with false-positive and negative results (11, 13, 16–20), and confirmation studies with conventional techniques are therefore essential.

In this study, we identified for the first time novel proteins that are dysregulated in WM compared with normal controls. Using >2 cutoff, the microarrays identified seven up-regulated polypeptides involved in signaling pathways regulating Ras-related proteins, including Rab4 and p62DOK; Rho-related proteins, including

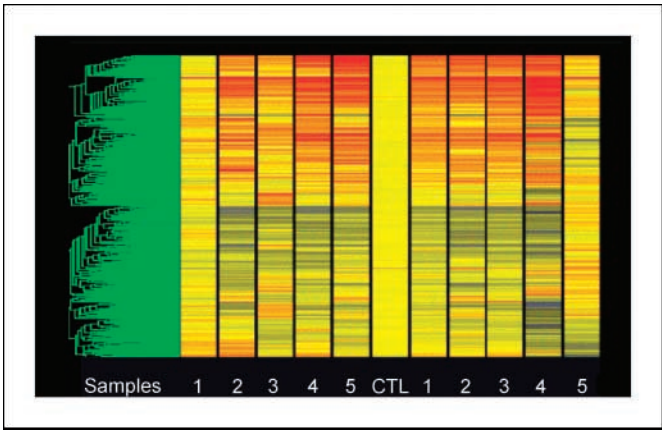

Figure 2. Heat map of unsupervised clustering analysis of samples of patients with symptomatic WM (n = 5, *left*), control normal samples, and asymptomatic WM/MGUS (n = 5, *right*). All samples were normalized to control versus control samples (center of the clustering analysis). The clustering analysis showed similar expression patterns in the symptomatic and asymptomatic samples. Three proteins were differentially expressed in the symptomatic compared with the asymptomatic samples by >2-fold expression difference. These included HSP90, CDC25C, and p43/EMAPII.

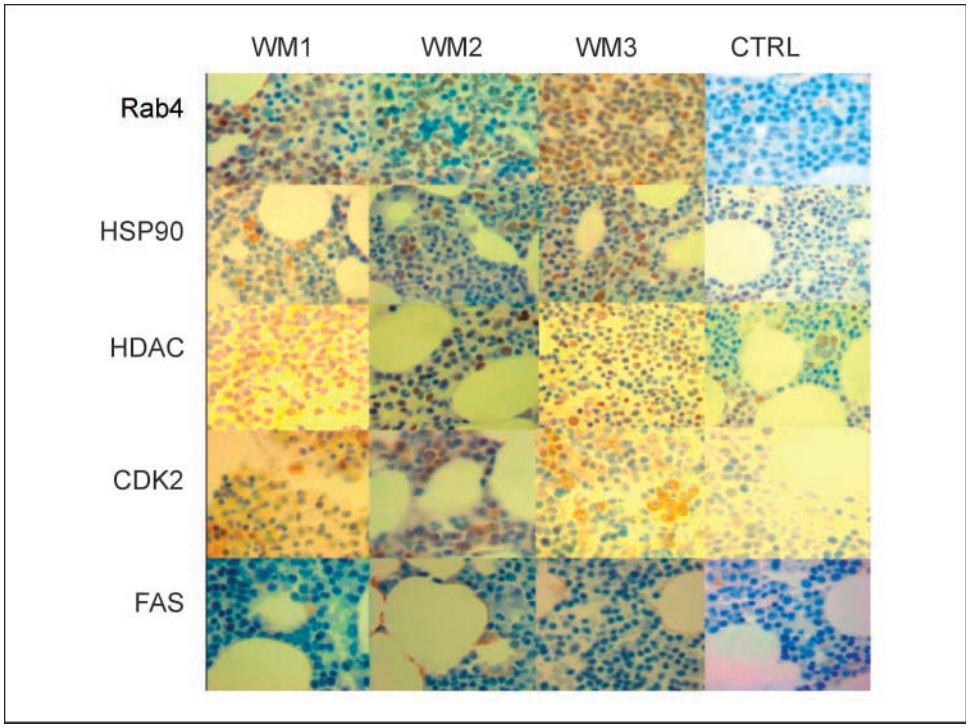

Figure 3. Immunohistochemistry for HDAC3, HSP90, CDK2, Rab4, and FAS antibodies in three samples of patients with symptomatic WM compared with the expression levels of normal bone marrow control. The expression of Rab4, HDAC3, CDK2, and HSP90 was elevated in WM as compared with control. FAS expression was only present in the fat globules. The presence of lymphocytes was confirmed by CD20 staining (data not shown). Confirmation with three other normal controls was done (data not shown). Images were visualized under a Leica DM IL microscope using an objective lens of 40/0.6 × 2 (magnification, 80×) equipped with a camera Leica dfc300 FX and software Leica IM50 version 6.2.1.

CDC42GAP, and ROKα; and other proteins, such as SNX-1, Roaz, and FAS. We then confirmed the expression of these polypeptides using immunohistochemistry and immunoblotting and delineated some of the biological networks that these proteins signal through. Although the number of samples analyzed in this study was small, we confirmed the results using another 10 WM samples using immunoblotting. Therefore, protein array techniques can be used as an exploratory tool, and other traditional techniques, such as immunoblotting or immunohistochemistry, can be used for confirmation of the identified proteins in a larger number of samples.

Some of the polypeptides identified in this analysis might contribute to the pathogenesis of WM, including those in the Ras and Rho families of kinases. Ras proteins included Rab4 and p62DOK. Oncogenic *Ras* expression occurs in up to 40% of multiple myeloma cases and correlates with aggressive disease (21, 22). This study, therefore, identifies a role of Ras signaling pathway in WM. Rab4 is a Ras-like small GTPase that coordinates protein transport

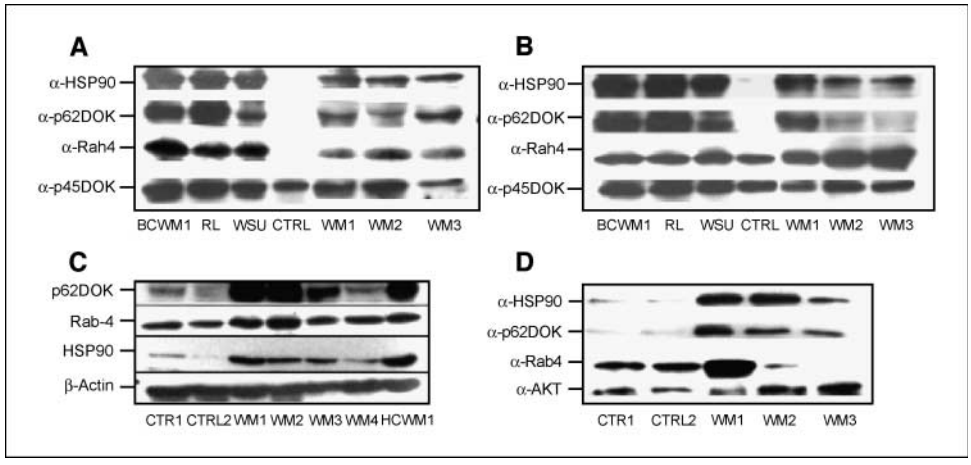

Figure 4. *A,* immunoblotting for Rab4, p62DOK, and HSP90 in $CD19^+$ symptomatic WM samples ($n = 3$) and $CD19^+$ control cells ($n = 2$) and WM and IgM-secreting cell lines (BCWM.1, RL, and WM-WSU). Expression of these proteins was higher in the WM samples and in the cell line as compared with the control samples. p45DOK was used as loading control. *B,* immunoblotting for Rab4, p62DOK, and HSP90 in the same WM cell used in (*A*), but using the $CD138^+$ fraction of the cells and control cells. Expression of these proteins was higher in the WM samples compared with the control samples. p45DOK was used as loading control. *C,* immunoblotting for Rab4, p62DOK, and HSP90 in another group of symptomatic WM patient samples ($n = 4$) and $CD19^+$ control cells ($n = 2$) and BWM.1 cell line. Expression of these proteins was higher in the WM samples compared with the control samples, confirming the results obtained in (*A*). Actin was used as loading control. *D,* immunoblotting for Rab4, p62DOK, and HSP90 in another set of WM samples ($n = 3$) and control samples ($n = 2$) that were selected using concomitant $CD19^+/CD138^+$ selection. Expression of these proteins was higher in the WM samples compared with the control samples. AKT was used as loading control.

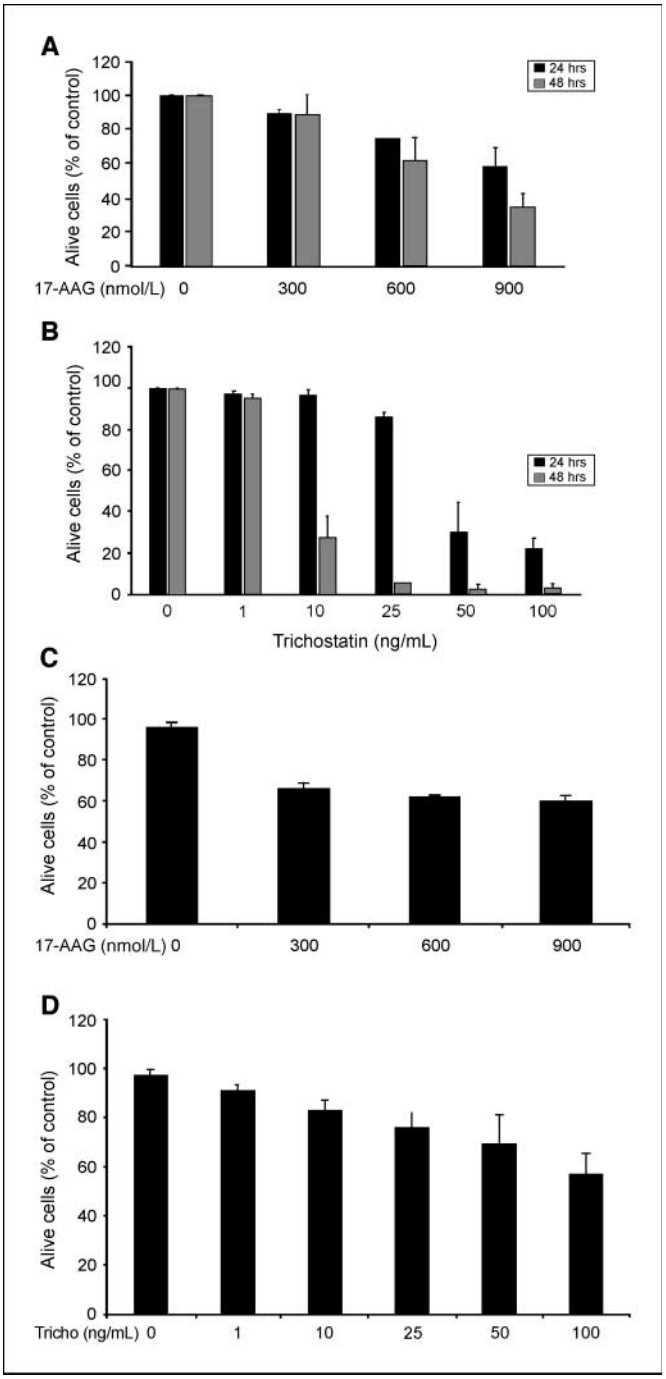

Figure 5. *A,* apoptosis assay using annexin V/PI staining showed induction of apoptosis with 17-AAG at 24 and 48 h incubation. *B,* apoptosis assay using annexin V/PI staining showed induction of apoptosis with trichostatin at 24 and 48 h incubation. *C,* apoptosis assay using annexin V/PI staining showed induction of apoptosis with 17-AAG at 48 h incubation in patient samples. *D,* apoptosis assay using annexin V/PI staining showed induction of apoptosis with trichostatin at 48 h incubation in patient samples.

from the endosome to the plasma membrane (23). It is associated with prolonged activation of MAP kinase in some malignancies (24). P62DOK or RasGAP-associated docking protein was originally defined as a tyrosine-phosphorylated 62-kDa protein that coimmunoprecipitated with p21Ras GTPase-activating protein (RasGAP; ref. 25). RasGAP is an essential component of Ras-activated signaling pathways (26, 27). RasGAP down-regulates Ras activity and plays a role in cell growth and differentiation (26, 27).

Similarly, proteins in the Rho pathway were up-regulated in WM as compared with normal controls. The GTPase RhoA has been implicated in various cellular activities, including the formation of stress fibers, motility, and cytokinesis (28, 29). Cdc42 belongs to the Rho family of small GTP binding proteins along with Rac and Rho (30). It is involved in regulating a variety of cellular functions, including actin cytoskeleton organization, cell growth control and development, transcriptional activation, membrane trafficking, and cell transformation (30). ROKα is a p150 serine/threonine kinase binding RhoA only in its active GTP-bound state, promoting the formation of stress fibers and focal adhesion complexes (31).

Other polypeptides that were up-regulated by 1.3-fold include HDAC3. Histone acetyltransferases can stimulate gene transcription by acetylating histones, facilitating an open chromatin state (32). Alteration in the chromatin structure allows access of transcription factors to the promoter regions and results in the activation of gene transcription (32). HDACs play a critical role on the pathogenesis of B cell malignancies, such as in large B cell lymphoma and multiple myeloma (32). In addition, we showed that the HDAC inhibitor trichostatin inhibited growth and survival of primary WM cells and WM cells lines, confirming that HDACs are important regulators of survival in WM.

We further showed that the molecular changes occurred early in the disease in cases with asymptomatic WM/MGUS analogous to results in patients with multiple myeloma where the molecular abnormalities identified in MGUS are similar to those identified in symptomatic multiple myeloma (33). HSP90 was up-regulated in symptomatic WM as compared with asymptomatic WM/MGUS, indicating that this protein is up-regulated with the progression of disease. HSP90 has been implicated in the pathogenesis and resistance of many malignancies including multiple myeloma, another plasma cell dyscrasia (34). We further confirmed the functional significance of this protein in the survival of WM cells by demonstrating that the HSP90 inhibitor 17-AAG induced significant apoptosis and inhibition of growth in WM cell lines and primary patient samples.

Previous studies of gene expression profiling in 23 patients diagnosed with WM identified a homogenous expression profile of WM cells that was similar to that of CLL. The most significantly up-regulated gene was *IL-6*, and the most significantly associated pathway for this set of genes was MAPK signaling. Although changes in mRNA levels do not always translate into changes at the protein level, we have identified multiple members of the Ras/MAPK pathway up-regulated in this protein array analysis reflecting consistency between gene and protein expression profiling.

In summary, our studies have identified for the first time novel proteins that are differentially dysregulated in WM, which both enhances our understanding of disease pathogenesis and represent targets for novel specific inhibitors.

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