[ABSTRACT WM3.1]

PROTEOMIC ANALYSIS IN WALDENSTROM MACROGLOBULINEMIA

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To better understand the molecular changes that occur in Waldenstrom's Macroglobulinemia (WM), we employed antibody-based protein microarrays to compare patterns of protein expression between untreated WM and normal bone marrow controls. The Antibody 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 against human polypeptides. 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 Mspike less than 1.0 gm/dL, and the presence of lymphoplasmacytic cells of less than 10% in the bone marrow and the absence of symptoms related to WM. In addition, follow up of these patients demonstrated no progression to symptomatic WM to the date these studies were performed. After CD19+ and CD138+ selection from the samples and another 3 bone marrow normal control samples, protein extraction was performed and the cell extracts were labeled with Cy3 and Cy5 dyes as per the manufacturer's protocol. 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. In addition, to confirm the expression patterns obtained in the protein microarray, we used WM and IgM secreting lymphoma cell lines along with primary CD19+ or CD138+ cells or concomitant CD19+/CD138+ cells from another group of patients with WM (N=10) and normal donors (N=5). Unsupervised clustering of the WM samples demonstrated a homogenous pattern of expression in all the samples. We analyzed polypeptides that were upor down-regulated by > 1.3 fold or >2 fold as compared to normal control. Using the >2 fold cutoff, the microarrays identified 6 dysregulated polypeptides in at least 60% samples of WM. All polypeptides were overexpressed in the WM cells as compared to 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 upregulated and 74 downregulated in at least 60% samples of WM. 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 PI3K pathway and proteosome/ubiquitin pathway. We then determined whether there was a difference in protein expression in patients with asymptomatic disease/MGUS as compared to those with symptomatic WM who required therapy. Unsupervised clustering showed no difference in protein expression between samples of patients with symptomatic versus asymptomatic disease. However, there were 3 proteins identified as upregulated in symptomatic WM as compared to 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. To validate the results of the protein microarray, immunohistochemistry on paraffin embedded tissue from the same biopsies used for the protein array analysis was performed and immunoblotting from another 10 samples of newly diagnosed symptomatic WM and 5 different controls that were not

included in the protein array analysis was performed. The proteins p62DOK, Rab4 and HSP90 were overexpressed in WM samples (N=3) compared to 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 3 proteins. To confirm the functional significance of protein elevation, we used the HDAC inhibitor Trichostatin and HSP90 inhibitor 17-AAG. Trichostatin and 17-AAG inhibited WM cells survival and induced apoptosis at 24 and 48h in a dose- dependent fashion. There is an urgent need to elucidate the molecular pathways that mediate proliferation and resistance to apoptosis in WM in order to provide targets for novel therapies. Transcriptional profiling in WM has identified some pathways that are upregulated in WM.1 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. 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 included Rab4 and p62DOK. Oncogenic Ras expression occurs in up to 40% of multiple myeloma cases and correlates with aggressive disease.2,3 This study, therefore, identifies a role of Ras signaling pathway in WM. Rab4 is a Ras-like small GTPase that coordinates protein transport from the endosome to the plasma membrane. It is associated with prolonged activation of MAPKinase in some malignancies. P62DOK or RasGAP- associated docking protein was originally defined as a tyrosine-phosphorylated 62-kDa protein that coimmunoprecipitated with p21Ras GTPase-activating protein (RasGAP). RasGAP is an essential component of Ras-activated signaling pathways.4,5 RasGAP down-regulates Ras activity and plays a role in cell growth and differentiation. 4,5 Similarly, proteins in the Rho pathway were upregulated in WM as compared to normal controls. The GTPase RhoA has been implicated in various cellular activities, including the formation of stress fibers, motility, and cytokinesis.6,7 Cdc42 belongs to the Rho family of small GTP binding proteins along with Rac, and Rho. 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.8 ROKα is a p150 serine/threonine kinase binding RhoA only in its active GTP-bound state promoting formation of stress fibers and focal adhesion complexes. Other polypeptides that were upregulated by 1.3 fold include HDAC3. Histone acetyltransferases (HATs) can stimulate gene transcription by acetylating histones, facilitating an open chromatin state. Alteration in the chromatin structure allows access of transcription factors to the promoter regions and results in activation of gene transcription. Histone deacetylases (HDACs) play a critical role on the pathogenesis of Bcell malignancies such as in large B-cell lymphoma and multiple myeloma. In addition, we demonstrated 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 demonstrated that the molecular changes occured 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.9 HSP90 was upregulated in symptomatic WM as compared to asymptomatic WM/MGUS indicating that this protein is upregulated with progression of disease. HSP90 has been implicated in the pathogenesis and resistance of many malignancies including multiple myeloma, another plasma cell dyscrasia.10 We further confirmed the functional signifcance of this protein in 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 upregulated 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.

References

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