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Candidate genes contributing to the aggressive phenotype of mantle cell lymphoma

Sarah E. Henson[a], Travis Morford[b], Mary-Pat Stein[b], Randolph Wall[a,c,d], Cindy S. Malone[b,*]

[a] Department of Microbiology, Immunology, and Molecular Genetics, David Geffen School of Medicine at the University of California Los Angeles, Los Angeles, CA 90095, USA
[b] Department of Biology, California State University Northridge, 18111 Nordhoff St., Northridge, CA 91330, USA
[c] Molecular Biology Institute, David Geffen School of Medicine at the University of California Los Angeles, Los Angeles, CA 90095, USA
[d] Jonsson Comprehensive Cancer Center, David Geffen School of Medicine at the University of California Los Angeles, Los Angeles, CA 90095, USA

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ABSTRACT

Mantle cell lymphoma and small lymphocytic lymphoma are lymphocyte cancers that have similar morphologies and a common age of onset. Mantle cell lymphoma is generally an aggressive B cell lymphoma with a short median survival time, whereas small lymphocytic lymphoma is typically an indolent B cell lymphoma with a prolonged median survival time. Using primary tumor samples in bi-directional suppression subtractive hybridization, we identified genes with differential expression in an aggressive mantle cell lymphoma versus an indolent small lymphocytic lymphoma. "Virtual" Northern blot analyses of multiple lymphoma samples confirmed that a set of genes was preferentially expressed in aggressive mantle cell lymphoma compared to indolent small lymphocytic lymphoma. These analyses identified mantle cell lymphoma-specific genes that may be involved in the aggressive behavior of mantle cell lymphoma and possibly other aggressive human lymphomas. Interestingly, most of these differentially expressed genes have not been identified using other techniques, highlighting the unique ability of suppression subtractive hybridization to identify potentially rare or low expression genes.

Introduction

Mantle cell lymphoma (MCL) is a B cell lymphoma derived from naïve, pre-germinal center B cells of the primary lymphoid follicles or the mantle zone of secondary lymphoid follicles (Campo et al., 1999; Perez-Galan et al., 2010). MCL is typically characterized by an aggressive disease course that is unresponsive to conventional therapies, with a short median survival time of only three to four years (Campo et al., 1999; Decaudin, 2002; Hartmann et al., 2009; Leonard et al., 2001). The range of survival time can be as little as a few months to more than ten years (Hartmann et al., 2009). This rapid disease onset and fatal course is replicated in two recent MCL mouse models described below.

MCL is commonly characterized by a t(11;14) chromosomal translocation that juxtaposes the *BCL-1* gene and the *IgH* gene enhancer and results in over expression of *BCL-1* encoded Cyclin D1 proteins (Bertoni et al., 2004; Campo et al., 1999; Weisenburger et al., 1996). *Cyclin D1* transgenic mice however, do not develop lymphoid tumors (Bodrug et al., 1994; Lovec et al., 1994), suggesting that additional genetic alterations are necessary for disease development. New mouse models of MCL have been developed by crossing IL-14α and *c-MYC* transgenic mice resulting in double transgenic mice that developed aggressive monoclonal tumors and resulted in death by lymphoma by four months of age (Ford et al., 2007). In these mouse models, biomarkers and organ involvement are similar to that seen in human MCL (Ford et al., 2007). A second mouse model was generated using SCID-hu immunodeficient mice as recipients of human patient MCL samples (Wang et al., 2008). This mouse model also clinically showed organ involvement similar to the human disease (Wang et al., 2008).

Small lymphocytic lymphoma (SLL), the tissue counterpart of chronic lymphocytic leukemia (CLL), is a B cell lymphoma that is probably derived from either pre- or post-germinal center (GC) B cells (Klein et al., 2001; Rosenwald et al., 2001). Pre-GC SLL lacks the genetic refinements observed in generating high-affinity antibodies during the GC reaction and can have a more aggressive clinical

Abbreviations: BL, Burkitt lymphoma; CLL, chronic lymphocytic leukemia; DLBCL, diffuse large B cell lymphoma; EtBr, ethidium bromide; FL, follicular lymphoma; GC, germinal center; MCL, mantle cell lymphoma; MZL, marginal zone lymphoma; ORFs, open reading frames; SLL, small lymphocytic lymphoma; SSH, suppression subtractive hybridization; VN, virtual Northern.

* Corresponding author.
E-mail address: cmalone@csun.edu (C.S. Malone).

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course than does post-GC SLL, which shows no genetic evidence of antibody gene alterations from passing through the GC (Klein and Dalla-Favera, 2008; Perez-Galan et al., 2010). Unlike MCL and many other classes of lymphoma, pre- or post-GC CLL and SLL are not characterized by a common oncogenic translocation (Guipaud et al., 2003; Swerdlow et al., 1995). Despite a poor response to conventional therapies, post-GC CLL/SLL is most often characterized by a prolonged indolent period with a median survival time between seven and ten years (Binet et al., 1981; Dighiero et al., 1981).

In this study, we identified differentially expressed genes that distinguish MCL from SLL. Bi-directional suppression subtractive hybridization (SSH) was performed between an aggressive MCL patient sample and an indolent SLL patient sample. Briefly, SSH is similar to mRNA differential display and subtractive cDNA hybridization techniques, comparing cDNA from a "tester" population of cells to mRNA or cDNA from a "driver" population (Diatchenko et al., 1996). The advantage to SSH over many other methods is that non-target DNA amplification is specifically reduced, while amplification of the target population of differentially expressed cDNAs between the two samples is enhanced. Herein, several screening strategies were employed to reduce the number of false positive and non-tumorigenic clones from the cDNA pool prior to sequence identification and SSH identified a large number of differentially expressed genes in both MCL minus SLL (MCL-SLL) and SLL minus MCL (SLL-MCL) directions of the subtraction.

Differential expression of a subset of the "MCL-specific" genes (MCL-SLL subtraction) was further confirmed using "Virtual" Northern blot analyses with a larger panel of primary samples representing both aggressive and indolent human lymphomas. The "Virtual" Northern blot was utilized because the tissue samples were too small to use in a traditional Northern blot. The "Virtual" Northern used RT-PCR linear range generated cDNA of all expressed RNAs from each of the small tissue samples available (Teitell et al., 1999). Thus, this study identified two sets of genes that are differentially expressed between MCL and SLL and a subset of genes that is differentially expressed between a larger panel of aggressive and indolent lymphoma samples. The diverse collection of "MCL-specific" genes potentially contribute to the aggressive behavior of MCL and provide candidate genes for MCL biomarkers and targets for therapeutics that combat aggressive human lymphomas.

Materials and methods

Patient samples

Five indolent lymphoma samples were used in this study. Three follicular lymphoma (FL), one marginal zone lymphoma (MZL), and one small lymphocytic lymphoma (SLL) patient samples were kindly provided by Jonathan W. Said (UCLA, Los Angeles, CA). Five aggressive lymphoma samples were used in this study. Two MCL patient samples were kindly provided by Jonathan W. Said (UCLA, Los Angeles, CA) and three MCL patient samples were kindly provided by Thomas M. Grogan (University of Arizona Cancer Center, Tucson, AZ). Samples were examined histologically and tissue blocks were trimmed to exclude areas of necrosis or surrounding non-lymphoid tissues. Microtome sections (5–10 μm) were placed into 5 mL RNA STAT-60 (Tel-Test, Friendswood, TX) for total RNA extraction according to the manufacturer's instructions.

Suppression subtractive hybridization

cDNA was synthesized from 0.3 μg total tumor RNA from one MCL patient sample and one SLL patient sample using the SMART PCR cDNA Synthesis Kit (Clontech, Palo Alto, CA) with 16 rounds of amplification. The resulting cDNA PCR products were each purified using the Qiaquick PCR purification kit (Qiagen, Valencia, CA). SSH to generate subtracted cDNA libraries from 1.3 μg of pooled cDNA was performed essentially as described (Diatchenko et al., 1996) with reagents and procedures provided in the PCR-Select cDNA Subtraction kit (Clontech). Bi-directional suppression subtractive hybridization (SSH) was performed using MCL cDNA as tester and SLL cDNA as driver, and SLL cDNA as tester and MCL cDNA as driver. After the two hybridization steps (1st for 8 h and 2nd for 22 h), differential PCR products were generated by sequential amplifications (primary for 27 rounds and secondary for 12 rounds). The resulting differential cDNA populations were subcloned into the TOPO TA cloning vector pCR2.1 (Invitrogen, Carlsbad, CA) and transformed into DH5α *Escherichia coli*. White colonies containing gene inserts were selected by isopropyl-B-D-thiogalactoside/5-bromo-4-chloro-3-indolyl-B-galactoside screening and seeded into 96-well microtiter plates for growth with antibiotic selection. Approximately 1600 clones generated in the MCL-SLL direction and 900 clones generated in the SLL-MCL direction were randomly selected and grown in 96-well plates for further analysis. These subtracted populations were used as probes for miniarray screening, as described below.

Miniarray analysis

Samples of bacterial culture lysates in 96-well plates were stamped with the Multi-Blot Replicator replicating tool (V&P Scientific, San Diego, CA) into fresh 96-well Thermowell plates (Corning Costar, Lowell, MA) for PCR amplification of cDNA inserts. cDNA fragments were amplified by PCR using Advantage cDNA Polymerase Mix (Clontech) and Nested Primers 1 and 2 from the PCR-Select cDNA Subtraction kit. PCR was performed for 30 cycles (94 °C for 30 s, 68 °C for 3 min), and the average size of insert fragments was 1 kilobase as determined by ethidium bromide (EtBr) stained 1% agarose gels (data not shown). These PCR products were identically stamped onto quadruplicate MagnaCharge nylon membranes (Osmonics, Minnetonka, MN) using the Multi-Blot Replicator. The first position on each membrane was stamped with an 850-bp *Pst*I fragment of the plant *Lemna gibba* RuBPCase gene. The membranes were denatured for 10 min in 0.5 M NaOH, 1 M NaCl and neutralized for 5 min in 0.5 M Tris pH8.0, 0.5 M NaCl, followed by UV crosslinking in a Spectrolinker (Specktronics, Westbury, NY). Miniarray analysis was performed as described (Patrone et al., 2003) using the following random-primed [α-^{33}P]ATP (NEN, Boston, MA)-labeled probes generated using the Prime-It II Random Primer Labeling Kit (Stratagene, La Jolla, CA): (1) SLL cDNA; (2) MCL cDNA; (3) SLL-MCL subtracted cDNA population; (4) MCL-SLL subtracted cDNA population; (5) "common" gene cocktail; (6) *L. gibba* RuBPCase. Probes were spiked with 0.3 ng of the 850-bp RuBPCase gene fragment before radiolabeling to allow semi-quantitative comparisons of hybridization intensities between membranes. Hybridizations were performed in aqueous hybridization buffer (0.5 M $NaPO_4$ pH7.0, 1 mM EDTA, 7% SDS, 1% BSA) at 62 °C overnight. Membranes were washed three times with 0.1% SDS, 0.1× SSC at 62 °C for 15 min. Hybridization signals were determined visually by autoradiography and quantitatively with a PhosphorImager (GE Healthcare, Piscataway, NJ) by using the program IMAGEQUANT (GE Healthcare).

Sequencing and GenBank analysis

Sequencing of cDNA fragments was performed using cycle sequencing (Laragen, Los Angeles, CA) with T7 and M13Rev primers. Sequences were identified using GenBank's nucleotide and pro-