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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], and **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, 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

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 bidirectional 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.

Keywords

Mantle cell lymphoma; Small lymphocytic lymphoma; Suppression subtractive hybridization; Differential gene expression; Human lymphoma

*Corresponding author: Dr. Cindy Malone, California State University Northridge, 18111 Nordhoff St., Northridge, CA 91330, USA. Tel: (818) 677-6145; fax: (818) 677-2034. cmalone@csun.edu (C.S. Malone).

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 4 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 M. et al., 2008). This mouse model also clinically showed organ involvement similar to the human disease (Wang M. 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 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 (5–10μm) were placed into 5mL RNA STAT-60 (Tel-Test, Friendswood, TX) for total RNA extraction according to the manufacturer’s instructions.

Suppression subtractive hybridization

cDNA was synthesized from 0.3μg 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). Bidirectional 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 0.5M NaOH, 1M NaCl and neutralized for 5 min in 0.5M Tris pH8.0, 0.5M 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) *Lemna 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 (0.5M $NaPO_4$ pH7.0, 1mM EDTA, 7% SDS, 1% BSA) at 62°C overnight. Membranes were washed three times with 0.1% SDS, 0.1X SSC at 62°C for 15m. 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 protein databases and the BLAST algorithm. Clones were assigned to functional categories based on GeneCard information.

“Virtual” Northern blot analysis

cDNA was synthesized from 0.2–0.7μg total tumor RNA from three FL, one MZL, one SLL, and five MCL patient samples using the SMART PCR cDNA Synthesis Kit (Clontech) essentially as described (Patrone et al., 2003). After PCR amplification, cDNA was

precipitated, washed, and resuspended in TNE (10mM Tris pH8.0, 10mM NaCl, 0.1mM EDTA). cDNA concentrations were determined using the PicoGreen dsDNA Quantitation Reagent (Molecular Probes, Eugene, OR). 0.5μg cDNA was fractionated in 1% agarose gels in 1X TBE running buffer (equal lane loading was confirmed by ethidium bromide staining), denatured in 0.5M NaOH, 1.5M NaCl at RT for 30m, neutralized in 0.5M Tris pH7.0, 1.5M NaCl at RT for 30m, transferred to Nytran nylon membranes (Schleicher & Schuell, Keene, NH) using the TurboBlotter Rapid Downward Transfer System (Schleicher & Schuell) overnight in 10X SSC, and baked at 80°C for 30m.

cDNA fragments to be used as probes were prepared by PCR amplification of SSH-generated clones using Advantage cDNA Polymerase Mix and Nested Primers 1 and 2 under the following conditions: 94°C for 1m; 25 cycles of 94°C for 15s, 68°C for 4m; 68°C for 8m. Overnight digestion with Rsa I (Promega, Madison, WI) was performed to remove SSH linkers and reactions were then concentrated using the StrataPrep PCR Purification Kit (Stratagene, La Jolla, CA). PCR products were subsequently fractionated in 1% agarose gels, followed by agarose gel purification using Ultrafree-MC Centrifugal Filter Units (Millipore, Bedford, MA). DNA concentrations were determined by UV spectroscopy. Random-primed [biotinylated]CTP-labeled probes were generated using the SpotLight Random Primer Labeling Kit (Clontech).

Probe hybridizations were performed individually using the SpotLight Chemiluminescent Hybridization & Detection Kit (Clontech). Hybridization signals were detected by chemiluminescence (Clontech). Membranes were stripped by incubating twice with 50mL 0.4M NaOH, 0.1% SDS at 60°C for 15m.

Results

To identify genes that are differentially expressed between MCL and SLL, we performed bi-directional SSH between an MCL patient sample and an SLL patient sample. RNA for SSH was extracted from tumor samples visually selected for a high proportion of tumor vs. non-tumorous or non-viable cells (Fig. 1). Approximately 1600 clones from the MCL-SLL direction of the subtraction and approximately 900 clones from the SLL-MCL direction of the subtraction were analyzed. To reduce the number of false positives identified by SSH prior to sequencing analysis, we confirmed differential expression of clones through hybridization with cDNA starting material and subtracted material. For this purpose, dot-blot analyses of the SSH clones were performed using 96-well format. PCR-amplified cDNA was stamped onto membranes in duplicate and identical membranes were then hybridized with SLL cDNA, MCL cDNA, SLL-MCL subtracted cDNA, or MCL-SLL subtracted cDNA probes (data not shown). Clones showing differential expression in the appropriate direction of the subtraction were analyzed further.

Our previous work using SSH showed that a large number of identified clones are differentially expressed between two lymphocyte-derived tumor samples but are not likely to be involved in the tumorigenic process (Patrone et al., 2003; Teitell et al., 1999). In order to minimize the analyses of these “common” genes in our study, additional dot-blot analyses were performed. PCR-amplified cDNA was stamped onto membranes in duplicate and the

membranes were hybridized with the probe cocktail containing a variety of the “common” gene fragments not likely to be involved in the tumorigenic process (data not shown). SSH clones on the membranes showing detectable expression using this “common” gene cocktail were excluded from further analyses.

Clones showing differential expression and no “common” gene expression by dot-blot analyses were subsequently sequenced, analyzed using GenBank, and categorized according to function using GeneCard. A large number of genes involved in many different cellular processes were identified in both directions of the subtraction. Seventy-three different genes were identified in the MCL-SLL direction (Table 1), and 131 different genes were identified in the SLL-MCL direction (Table 2). Since many genes were identified more than once, the identification frequency of each gene is included (Tables 1 and 2). The percentage of genes in each of the designated functional groups is shown in Figures 2 and 3, where genes falling into multiple functional groups are included in each of these groups. It is interesting to note that while genes involved in immunity and signal transduction, for example, are similar in percentage, the aggressive, higher-rate of replication MCL has a significantly higher percentage of differential cell cycle-related and DNA damage and repair-related genes than were found differential in SLL (Figures 2 and 3). Interestingly, the signature gene for MCL, cyclin D1 (*CCND1*), was not detected as differentially expressed between the MCL and SLL samples used. This is not surprising as the defining genetic alterations in MCL, a t(11;14)(q13;q32) translocation, does not massively overproduce transcripts encoding *CCND1* (Gladkikh et al., 2010), and the depth of our sequencing analysis may not have been deep enough to statistically detect this difference. More likely, *CCND1* has sequence homology with other cyclin encoding genes, such as cyclin D2 (*CCND2*) and D3 (*CCND3*) (NCBI Genbank accession codes NM_001759.3 and NM_001760.3, with 73% and 71% identity respectively), and the SSH procedures could have artifactually eliminated this differentially expressed gene. Gratifyingly, another signature gene that distinguishes histologically indistinguishable SLL from MCL, *CD23* (official name *FCER2*), was identified in SLL-specific genes and absent from the MCL-specific genes, further validating our SSH.

“Virtual” Northern blotting was performed on a subset of MCL-SLL genes in order to confirm differential expression between the tumor samples analyzed by SSH and to expand the analysis to include a larger panel of lymphoma samples. Since conventional Northern blotting could not be used due to limited primary sample RNAs, “Virtual” Northern blotting was performed in which total RNA was converted into cDNA and subsequently analyzed as a representation of the tumor RNA (Teitell et al., 1999). All genes analyzed by “Virtual” Northern blot were differentially expressed between the SLL and MCL cases used in SSH (compare lanes 5 and 10 in all panels, Fig. 4), indicating successful performance of our SSH and dot-blot analyses. In addition, many of these genes showed broad differential expression across the larger panel of samples, including *RBBP8*, *CCNB1*, *CCNB2*, *DCK*, *MCM7*, *RAD51AP1*, *NCOA4*, *SHOC2*, *SKAP2*, and *ZWINT*. In contrast to the pattern of broad differential expression seen for many of these genes, *CDC2*, *GPNMB*, and *GPR34* were only differentially expressed between the SSH tumor samples and showed variable expression across the larger panel of samples. The data from these “Virtual” Northern blots confirmed

the differential expression of all genes analyzed and identified a subset of genes with preferential expression in typically aggressive versus indolent lymphoma.

Discussion

In order to identify genes that distinguish MCL and SLL, bi-directional SSH was performed and approximately 2500 clones were screened for differential expression using a variety of techniques. The combination of dot-blot analyses using SSH-generated probes and a "common" gene cocktail probe resulted in the successful removal of false positive and non-tumorigenic clones from the cDNA pool. After the completion of these screening techniques, 133 clones from the MCL-SLL direction and 179 clones from the SLL-MCL direction were sequenced and analyzed for identity using GenBank. Non-redundant gene fragments were identified for 73 different genes in the MCL-SLL direction and 131 genes were identified in the SLL-MCL direction. The clustering of genes into functional group categories highlights the similarities, but more importantly the differences between typically aggressive lymphoma versus a typically indolent lymphoma gene profile. "Virtual" Northern blot analyses were performed on a subset of MCL-SLL genes and differential expression between the tumor samples analyzed by SSH was confirmed for all genes tested. In addition, these "Virtual" Northern blot analyses expanded the study to include additional indolent FL and MZL patient samples and additional aggressive MCL patient samples, identifying genes with preferential expression in aggressive lymphoma. These genes include *RBBP8, CCNB1, CCNB2, DCK, MCM7, RAD51AP1, NCOA4, SHOC2, SKAP2*, and *ZWINT*. Many of these genes and several others have been identified in recent microarray analyses of MCL, SLL, CLL, FL, and other B cell lymphomas, both compared to each other and to normal tonsillar B cells. Interestingly, most of the differentially expressed genes identified here have not been found in other analyses, highlighting the unique ability of SSH to identify potentially rare or low expression genes (Gadgil et al., 2002).

When the differential genes identified in the MCL versus SLL and the SLL versus MCL SSH were categorized and displayed as a percentage of all differential genes found in the SSH, several interesting similarities and glaring differences were revealed (See Figures 2 and 3). Functional categories with expected similar percentages of represented genes include those in immunity, signal transduction, translation, and those categorized as proteins with no currently designated function. The cancer-related gene frequency between the two SSH results were very similar with the MCL versus SLL SSH having 6% (as listed in Figure 2 as metastasis and oncogenesis genes), and the SLL versus MCL SSH having 4% of the total genes fall into this category. It is notable to mention that the genes from the MCL versus SLL SSH functioned specifically in metastasis and oncogenesis, while the SLL versus MCL SSH genes were more loosely associated with cancer in general using GeneCard analysis. The categories that stand out as most different between MCL versus SLL SSH profiles are the cell cycle associated genes and the DNA damage and repair associated genes. None of the known genes found in the SLL versus MCL SSH were associated with DNA damage and repair, and only 4% of genes were associated with the cell cycle, as strongly contrasted with to 12% and 20% for MCL versus SLL SSH, respectively. These results are concordant with MCL being an aggressive, highly proliferative lymphoma (Jares and Campo, 2008). Although a pre-GC MCL may show differences in gene expression compared with a post-

GC SLL based on derivation from distinct stages of B cell lineage development, it is nevertheless striking that the cell cycle and DNA repair gene categories represent the most differentially expressed genes. Both pre-GC mantle zone cells and post-GC marginal zone cells are not thought to be rapidly cycling or undergoing active DNA damage, as compared with the highly proliferative and DNA-damage induced intervening GC B cell stage in development (Klein and Dalla-Favera, 2008). Therefore, we favor the differential regulation of these gene regulatory packages as most likely arising from the pathology of rapid cell cycling with arrest and DNA damage known to occur in MCL versus SLL, rather than reflecting distinct cells/stages of origin. Finally, both SSH directions resulted in genes with unknown function and genes that have yet to be described, but there were significantly more (17% vs. 5%) unknown genes found in the SLL versus MCL SSH directions for unclear reasons.

The subset of MCL-SLL genes analyzed by “Virtual” Northern blot was chosen for further analysis based on unknown biology or biology with a potential relevance to aggressive disease. CDC2 (cell division cycle 2, promoting G1 to S and G2 to M transitions) and CDC28A are cyclin-dependent kinases (Draetta and Beach, 1988; Draetta et al., 1988a; Draetta et al., 1988b; Lee M. and Nurse, 1988; Lee M. G. et al., 1988; Lee M. G. and Nurse, 1987) that interact with cyclin B1 (Pines and Hunter, 1989; Pines and Hunter, 1992) and cyclin B2 (Jackman et al., 1995) to drive entry into mitosis, and these complexes in turn interact with cyclin A and cyclin E to promote the G1/S transition (Aleem et al., 2005; Kaldis and Aleem, 2005). Additionally, CDC2 appears to be the molecular target for G2 cell cycle arrest that occurs in response to DNA damage (Stark and Taylor, 2006). CDC2 also functions in meiotic cell cycle control and causes arrested mammalian oocytes to reenter the cell cycle and complete meiosis (Han and Conti, 2006). RBBP8 (retinoblastoma binding protein 8), also called RIM and CTIP, is a transcriptional co-repressor that interacts with Rb (Fusco et al., 1998; Meloni et al., 1999), BRCA1 (Li S. et al., 1999; Wong et al., 1998; Yu et al., 1998), and IKAROS (Koipally and Georgopoulos, 2002). RBBP8 is required for double strand break repair by homologous recombination in S phase and G2 (Huertas and Jackson, 2009; Limbo et al., 2007). Specifically, RBBP8 must interact with BRCA1 to drive double strand break repair by homologous recombination (Yun and Hiom, 2009) which may be required to repair replication-related DNA damage in rapidly cycling MCL cells. DCK (deoxycytidine kinase) is found at high levels in normal mononuclear leukocytes and may be found at lower levels in non-lymphoid tissues with high levels of DCK found in corresponding tumors of these tissues (Eriksson et al., 1994). The physiologic function of DCK is to phosphorylate several deoxyribonucleosides, dA, dC, and dG (Arner and Eriksson, 1995; Hazra et al., 2009) and also to activate several antileukemic nucleoside analogues (Smal et al., 2007). Decreased expression of DCK is associated with resistance to anticancer chemotherapeutic agents (Chottiner et al., 1991; Kobayashi et al., 1994; Song J. H. et al., 2009). GPNMB (glycoprotein non-metastatic melanoma protein B) is a transmembrane glycoprotein that has increased expression in low-metastatic melanoma cell lines (Weterman et al., 1995), primary dendritic cells (Ahn et al., 2002), glioma cells (Kuan et al., 2006), melanoma cells (Tse et al., 2006), macrophage-related tissues (Ripoll et al., 2007), aggressive bone metastatic breast cancer cells (Rose et al., 2007), and in melanocytes and melanoblasts (Loftus et al., 2009; Tomihari et al., 2009). GPNMB has a variety of

ascribed functions including suppression of macrophage inflammatory responses (Ripoll et al., 2007), the adhesion of melanocytes to keratinocytes (Tomihari et al., 2009), and increases breast cancer metastasis to bone in a mouse model (Rose et al., 2007; Rose and Siegel, 2007). GPR34 (G protein-coupled receptor 34), a P2Y-like receptor, is a ubiquitously-expressed (Schoneberg et al., 1999b), but predominately in the brain (Bedard et al., 2007; Marchese et al., 1999b), and is a G protein-coupled receptor of unknown function (Marchese et al., 1999a; Schoneberg et al., 1999a). MCM7 (minichromosome maintenance complex component 7), is a component of the DNA replication machinery (Fujita et al., 1996a; Fujita et al., 1996b) and has increased expression in a variety of tumor tissues including neuroblastoma, prostate, cervical, endometrial, oral squamous cell, and hypopharyngeal carcinomas (Cromer et al., 2004; Feng et al., 2008; Hiraiwa et al., 1997; Li S. S. et al., 2005; Ren et al., 2006). In a mouse model, MCM7 played a role in the initial formation of tumor and more importantly in their progression to malignant tumors (Honeycutt et al., 2006). RAD51AP1 (RAD51 associated protein 1), also called PIR51, is a protein that interacts with RAD51, DNA, and RNA (Kovalenko et al., 1997; Mizuta et al., 1997). RAD51AP1 plays a role in DNA repair by preventing chromosomal breaks (Henson et al., 2006) through the enhancement of RAD51 recombinase (Wiese et al., 2007). RAD51AP1 has increased expression in primary hepatocellular carcinoma (Song H. et al., 2004) and is implicated in both the development and progression of intrahepatic cholangiocarcinoma (Obama et al., 2008). NCOA4 (nuclear receptor coactivator 4) interacts with the androgen receptor (Gao et al., 1999) and is translocated to the RET oncogene in thyroid carcinoma (Peng et al., 2008). NCOA4 appears to promote cell growth and invasion of prostate cancer cells (Peng et al., 2008). SHOC2 (soc-2 suppressor of clear homolog) is a positive regulator of Ras-mediated signal transduction (Li W. et al., 2000; Sieburth et al., 1998) and specifically activates the MAPK pathway (Rodriguez-Viciana et al., 2006) to drive cell proliferation. SKAP2 (src kinase associated phosphoprotein 2) functions as an adaptor protein for the src family of kinases (Kouroku et al., 1998; Liu et al., 1998; Marie-Cardine et al., 1998) and is implicated in myeloid differentiation and growth arrest (Curtis et al., 2000). ZWINT (ZW10 interactor) interacts with the kinetochore during mitosis and is required for proper chromosome segregation (Famulski et al., 2008; Kops et al., 2005; Lin et al., 2006; Starr et al., 2000; Wang H. et al., 2004) and is essential for mitotic spindle checkpoint control.

Several genes identified in the MCL-SLL SSH have also been identified in microarray studies (See Table 1). For example, *TOP2A* (topoisomerase II alpha) has been identified by microarray analyses as a component of an MCL proliferation signature (Rosenwald et al., 2003) and an MCL gene cluster (Thieblemont et al., 2004), by protein microarray analysis as a protein with upregulated expression in MCL compared to normal tonsillar B cells (Ghobrial et al., 2005), and by immunohistochemistry as a prognostic factor in MCL (Schrader et al., 2004). *TOP2A* was identified in additional microarray studies as a gene with downregulated expression in CLL compared to normal tonsillar B cells (Aalto et al., 2001) and compared to diffuse large B cell lymphoma (DLBCL), Burkitt lymphoma (BL), and FL (Klein et al., 2001). *GPNMB* was identified as a gene with significantly upregulated expression in MCL compared to normal B cell populations (Ek et al., 2002). *CDC2* was identified as a component of an MCL proliferation signature (Rosenwald et al., 2003) and as

a gene with downregulated expression in SLL compared to reactive lymph node (Schmechel et al., 2004). *BUB1*, *CDC2*, *CCNB1*, *CCNB2*, and *ZWINT* were all identified as genes downregulated in CLL compared to DLBCL, BL, and FL (Klein et al., 2001). *YWHAQ* (tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide) was identified as a protein with upregulated expression in MCL compared to normal tonsillar B cells (Ghobrial et al., 2005). *IFI44L* (interferon-induced protein 44-like) was identified as a gene with upregulated expression in MCL compared to FL (Schmechel et al., 2004). Interestingly, both *CDC2* and *IFI44L* did not show up in the specific MCL-SLL microarray, even though they were found in these other array comparisons (Schmechel et al., 2004), highlighting the ability of SSH to identify differentially expressed genes that standard microarray technologies miss. *ENPP2* (Ectonucleotide pyrophosphatase/ phosphodiesterase 2) and *GLUL* (glutamate-ammonia ligase) were identified as genes with upregulated expression in MCL compared to naïve B cells (Ek et al., 2002; Rizzatti et al., 2005).

Several genes identified in the SLL-MCL SSH direction (SLL-specific genes) have also been identified in microarray studies (See Table 2). Most importantly, *FCER2* (Fc fragment of IgE, low affinity II, receptor for CD23), also called *CD23*, is a major diagnostic marker for the classification of SLL and was identified as differentially expressed in SLL and absent from gene identified in the MCL-specific gene pool. *RHOH* (Ras homolog gene family member H) and *GPR183* (G protein-coupled receptor 183) were identified as genes with down-regulated expression in MCL compared to normal tonsillar B cells (Zhu et al., 2001). *NFE2L2* (nuclear factor erythroid-derived 2-like 2) was identified as a gene with downregulated expression in MCL compared to FL (Schmechel et al., 2004). *PAX5* (paired box 5), also called *BSAP*, was identified as upregulated in CLL compared to tonsillar B cells (Aalto et al., 2001). The identification of many of the same genes in this study in SLL and in similar microarray studies of indolent lymphoma supports the use of SSH as a technique for successfully identifying differentially expressed genes.

Interestingly, many genes were identified having preferential expression in MCL versus SLL that have not been identified elsewhere that may play a role in the development and progression of MCL and other aggressive malignancies. These genes may not have met the criteria for microarray analysis, but were picked up by SSH due to its ability to detect rare transcripts, even those with modest changes in gene expression (Gadgil et al., 2002). SSH also picks up differentially expressed sequences that have no matches in the database other than to BAC clones or chromosome open reading frames (ORFs) (Gadgil et al., 2002). These rare transcripts, modestly expressed genes, and unknown genes should not be overlooked when describing genes associated with certain cell types such as aggressive lymphomas, as any of these may contribute to the aggressive phenotype of these diseases (Gadgil et al., 2002) or provide potentially useful biomarkers in future studies. The identification of all these gene types in our study validate the use and utility of SSH especially for rare mRNA detection.

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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

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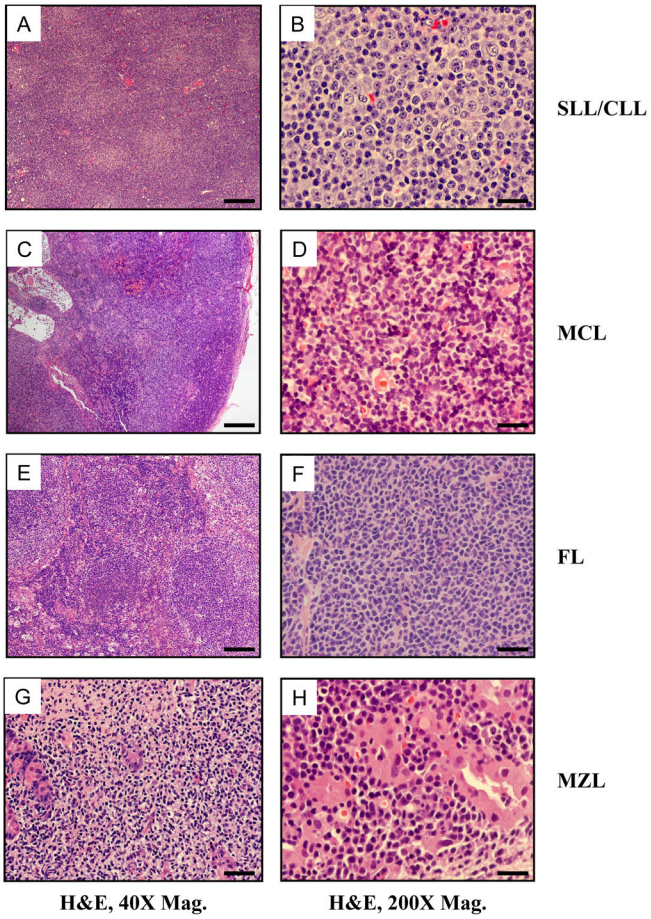

Figure 1. Representative histologic tissue sections of lymphomas used in this study

Histological 5 μm-thick tissue sections of typically indolent small lymphocytic lymphoma [SLL/CLL; Hematoxylin and eosin, 40× mag (A) and 200X mag (B)], follicular lymphoma [FL; Hematoxylin and eosin, 40× mag (C) and 200X mag (D)], marginal zone lymphoma [MZL; Hematoxylin and eosin, 40× mag (E) and 200X mag (F)], and typically aggressive mantle cell lymphoma [MCL; Hematoxylin and eosin, 40× mag (G) and 200X mag (H)] patient samples are shown. Note the similar appearance and tumor cell size of MCL and SLL samples as well as the nodular appearance of FL. Panels A, C, E, G scale bar = 40 μm and B, D, F, H scale bar = 200 μm. All images courtesy of Jonathon W. Said.

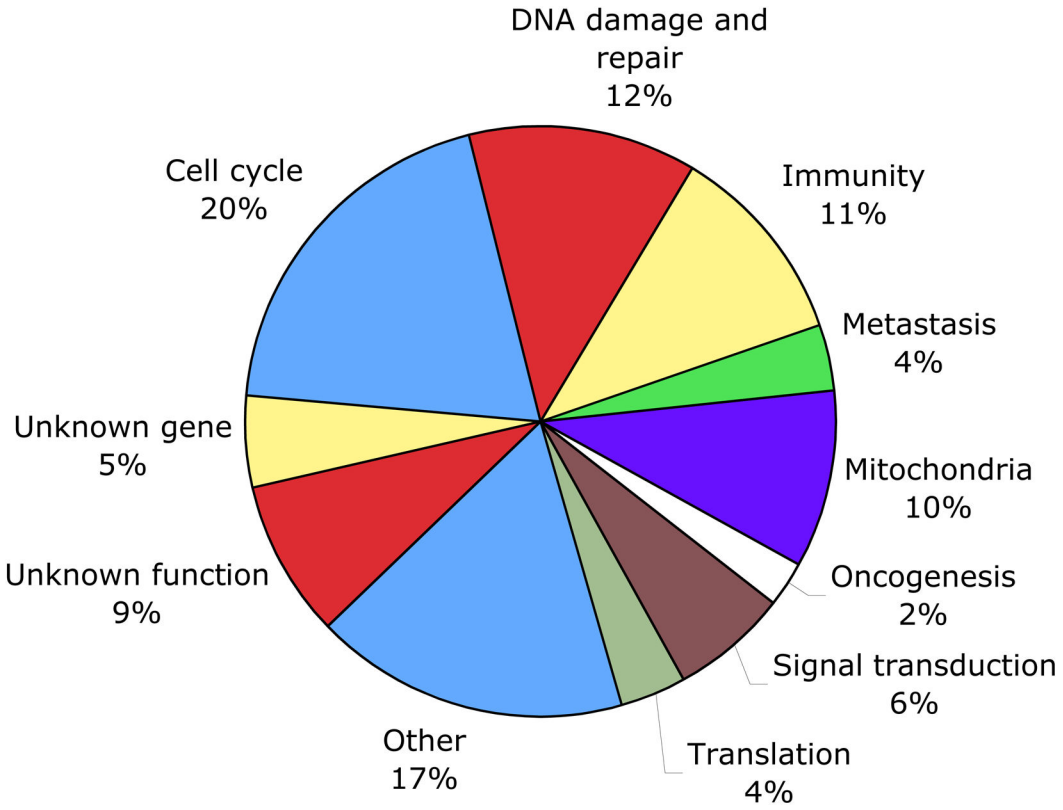

Figure 2. Differentially expressed genes identified by SSH in the MCL versus SLL direction

Graphical analysis of functional group characterizations from Table 1, where genes are included in all categories in which they meet specific functional criteria according to GeneCard. Functional groups are graphed alphabetically and are by percentage as follows: Other 17%, Cell Cycle 20%, DNA damage and repair 12%, Immunity 11%, Montochondria 10%, Unknown function 9%, Signal transduction 6%, Unknown gene 5%, Metastasis 4%, Translation 4%, and Oncogenesis 2%.

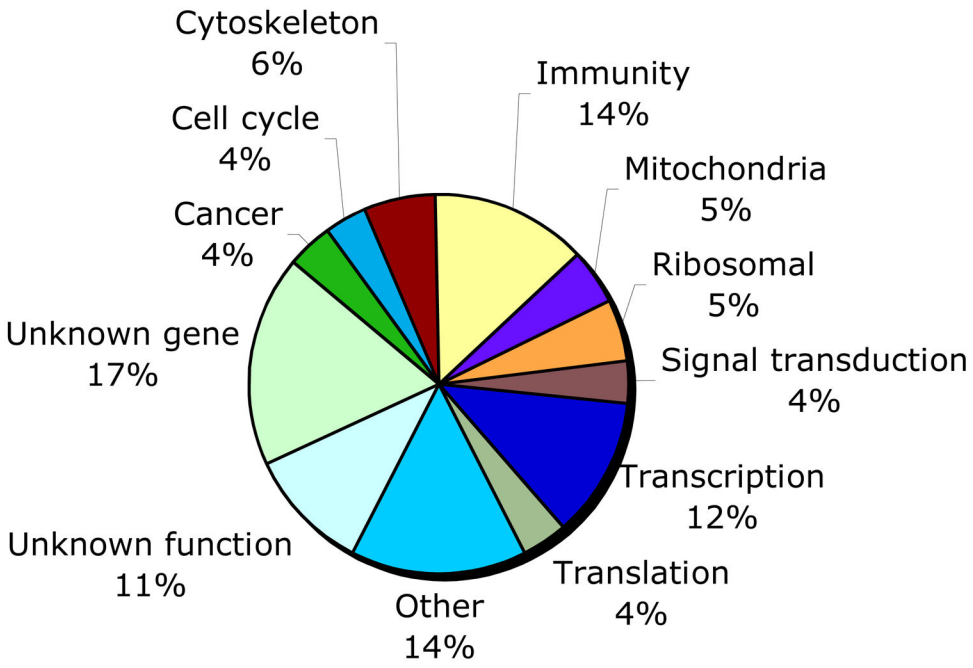

Figure 3. Differentially expressed genes identified by SSH in the SLL versus MCL direction

Graphical analysis of functional group characterizations from Table 2, where genes are included in all categories in which they meet specific functional criteria according to GeneCard. Functional groups are graphed alphabetically and are by percentage as follows: Unknown gene 17%, Immunity 14%, Other 14%, Transcription 12%, Unknown function 11%, Cytoskeleton 6%, Mitochondria 5%, Ribosomal 5%, Cancer 4%, Cell cycle 4%, Signal transduction 4%, and Translation 4%.

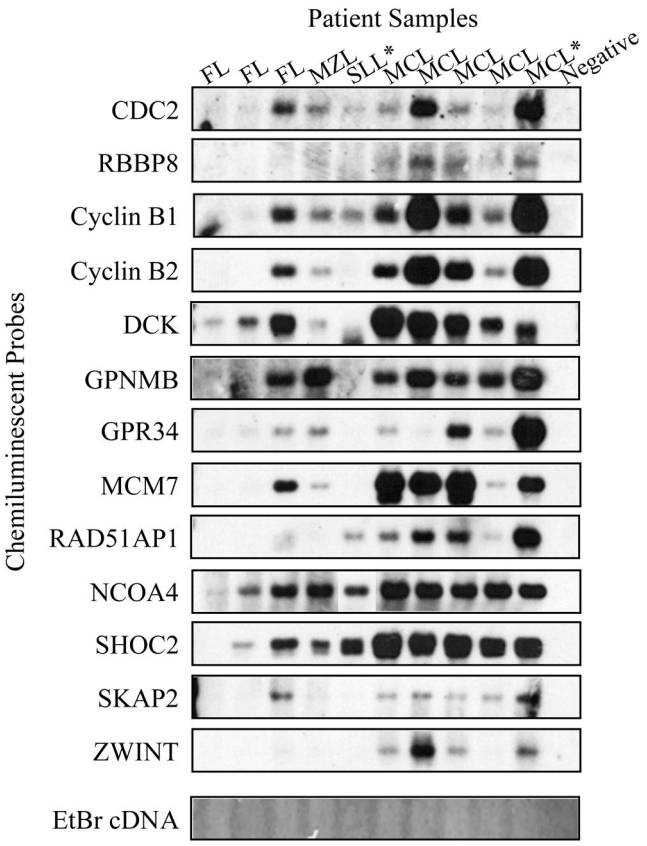

Figure 4. "Virtual" Northern analysis of a subset of genes identified in the MCL versus SLL SSH direction

Total RNA isolated from 10 patient tumor samples was subjected to RT-PCR using the SMART PCR cDNA Synthesis Kit (Clontech). 0.5μg cDNA was separated on 1% agarose gels, transferred to nylon membranes, and hybridized with the biotin-labeled probes indicated to the left of each individual blot. Hybridization signals were detected by chemiluminescence. The specific tumor samples used in the suppression subtractive hybridization (SSH) are indicated with asterisks. FL: Follicular lymphoma. MZL: Marginal zone lymphoma. SLL: Small lymphocytic lymphoma. MCL: Mantle cell lymphoma. Negative: H_2O. Bottom panel: cDNA was stained with EtBr prior to transfer to show equivalent cDNA loading. One representative EtBr-stained gel of the cDNA is shown to illustrate equivalent loading.

TABLE 1

Differentially expressed genes identified by SSH in MCL vs. SLL

Function	Genes Identified by SSH	Accession #	Hits	Confirmed	References
Cell Cycle	**ANLN**: anillin, actin binding protein (also Scraps, scra)	NM_018685	1		
	BUB1: budding uninhibited by benzimidazoles 1 homolog (yeast) (also BUB1A, BUB1L, hBUB1)	NM_004336	1	MA	(Klein et al., 2001)
	CCNB1: cyclin B1	NM_031966	6	MA	(Klein et al., 2001)
	CCNB2: cyclin B2 (also HsT17299)	NM_004701	3	MA	(Klein et al., 2001)
	CDC2: cell division cycle 2, G1 to S and G2 to M (also CDK1, CDC28A)	NM_001786	1	VN, MA	This study and (Klein et al., 2001; Rosenwald et al., 2003; Schmechel et al., 2004)
	FANCI: Fanconi anemia, complementation group I	NM_018193	1		
	*__HMGB1__: high-mobility group box 1 (also HMG1, HMG3, SBP-1)	NM_002128	3		
	*__HMGN2__: high-mobility group nucleosomal binding domain 2 (also HMG17)	NM_005517	1		
	*__MCM7__: minichromosome maintenance complex component 7 (also CDABP0042, CDC47, MCM2, P1.1-MCM3, P1CDC47, P85MCM, PNAS-146)	NM_005916	2	VN	This study
	NAP1L1: nucleosome assembly protein 1-like 1 (also NAP1, NAP1L, NRP)	NM_139207	1		
	NCAPG2: non-SMC condensin II complex, subunit G2 (also CAP-G2, LUZP5, MTB, hCAP-G2)	NM_017760	2		
	NUSAP1: nucleolar and spindle associated protein 1 (also ANKT, BM037, LNP, PRO0310p1, Q0310, SAPL)	NM_016359	4		
	*__POLE3__: polymerase (DNA directed), epsilon 3 (p17 subunit) (also CHARAC17, CHRAC17, YBL1, p17)	NM_017443	2		
	*__RBBP8__: retinoblastoma binding protein 8 (also RIM; CTIP)	NM_002894	5	VN	This study
	*__TOP2A__: topoisomerase (DNA) II alpha 170kDa (also TOP2, TP2A)	NM_001067	4	MA	(Aalto et al., 2001; Ghobrial et al., 2005; Klein et al., 2001; Rosenwald et al., 2003; Schrader et al., 2004; Thieblemont et al., 2004)
	ZWINT: ZW10 interactor (also HZwint-1, KNTC2AP, ZWINT1)	NM_007057	1	VN, MA	This study and (Ghobrial et al., 2005; Klein et al., 2001)
DNA Damage and Repair	*__FANCI__: Fanconi anemia, complementation group I	NM_018193	1		
	*__HMGB1__: high-mobility group box 1 (also HMG1, HMG3, SBP-1)	NM_002128	3		

Function	Genes Identified by SSH	Accession #	Hits	Confirmed	References
	*__HMGN2__: high-mobility group nucleosomal binding domain 2 (also HMG17)	NM_005517	1		
	*__MCM7__: minichromosome maintenance complex component 7 (also CDABP0042, CDC47, MCM2, P1.1-MCM3, P1CDC47, P85MCM, PNAS-146)	NM_005916	2	VN	
	__PMS2__: PMS2 postmeiotic segregation increased 2 (S. cerevisiae) (also HNPCC4, PMS2CL, PMSL2)	NM_000535	1		
	__RAD51AP1__: RAD51 associated protein 1 (also PIR51) (also PIR51)	NM_006479	1	VN	
	*__RBBP8__: retinoblastoma binding protein 8 (also RIM; CTIP)	NM_002894	5	VN	
	__SF3B3__: splicing factor 3b subunit 3 130kDa (also RSE1, SAP130, SF3b130, STAF130)	NM_012426	1		
	*__TOP2A__: topoisomerase (DNA) II alpha 170kDa (also TOP2, TP2A)	NM_001067	4	MA	(Aalto et al., 2001; Ghobrial et al., 2005; Klein et al., 2001; Rosenwald et al., 2003; Schrader et al., 2004; Thieblemont et al., 2004)
Immunity	__AICDA__: activation-induced cytidine deaminase (also AID, ARP2, CDA2, HIGM2)	NM_020661	2		
	__CLEC2D__: C-type lectin domain family 2, member D (also CLAX, LLT1, OCIL)	NM_013269	2		
	__CXCL10__: chemokine C-X-C motif ligand 10 (also C7, IFI10, INP10, IP-10, SCYB10, crg-2, gIP-10, mob-1)	NM_001565	1		
	__IFI44L__: interferon-induced protein 44-like (also C1orf29)	NM_006820	4	MA	(Schmechel et al., 2004)
	__IGJ__: immunoglobulin J polypeptide, linker protein for immunoglobulin alpha and mu polypeptides	NM_144646	1		
	__IGKC__: immunoglobulin kappa locus	AB022653	14		
	__OAS2__: 2′-5′-oligoadenylate synthetase 2	NM_002535	6		
	__RBPJ__: recombination signal binding protein for Ig kappa J region (also CBF1, IGKJRB, IGKJRB1, KBF2, RBP-J, RBPJK, RBPSUH, SUH, csl)	NM_203284	1		
	__TNFSF8__: tumor necrosis factor (ligand) superfamily, member 8 (also CD153, CD30L, CD30LG)	NM_001244	1		
Metastasis	__ENPP2__: ectonucleotide pyrophosphatase/phosphodiesterase 2 (also ATX, ATX-X, AUTOTAXIN, LysoPLD, NPP2, PD-IALPHA, PDNP2)	NM_006209	2	MA	(Ek et al., 2002; Rizzatti et al., 2005)
	__GPNMB__: glycoprotein (transmembrane) nmb (also HGFIN, NMB, osteoactivin)	NM_002510	1	VN, MA	This study and (Ek et al., 2002)
	__NCOA4__: nuclear receptor coactivator 4 (also ARA70, ELE1, PTC3, RFG)	NM_005437	1	VN	This study
Mitochondria	__ACAA2__: acetyl-coenzyme A acyltransferase 2 (also DSAEC)	NM_006111	1		
	__ATP5A1__: ATP synthase, H+ transporting, mitochondrial F1 complex, alpha subunit 1, cardiac muscle (also ATP5A, ATP5AL2, ATPM, MOM2, OMR, ORM, hATP1)	NM_004046	2		
	__COX15__: COX15 homolog, cytochrome c oxidase assembly protein (yeast)	NM_004376	1		

Function	Genes Identified by SSH	Accession #	Hits	Confirmed	References
	MDH1: malate dehydrogenase 1 NAD (soluble) (also MDH-s, MDHA, MOR2)	NM_005917	1		
	SLC25A19: solute carrier family 25 (mitochondrial thiamine pyrophosphate carrier), member 19 (also DNC, MUP1, TPC)	NM_021734	1		
	SLC25A46: solute carrier family 25, member 46	NM_138773	1		
	SLC25A5: solute carrier family 25 (mitochondrial carrier; adenine nucleotide translocator), member 5 (also 2F1, AAC2, ANT2, T2, T3)	NM_001152	1		
Oncogenesis	**AFF3**: AF4/FMR2 family, member 3 (also LAF4, MLLT2-like)	NM_002285	1		
	*__POLE3__: polymerase (DNA directed), epsilon 3 (p17 subunit) (also CHARAC17, CHRAC17, YBL1, p17)	NM_017443	2		
Signal Transduction	**SHOC2**: soc-2 suppressor of clear homolog (C. elegans) (also SOC-2, SOC2, SUR-8, SUR8)	NM_007373	1	VN	This study
	RICTOR: RPTOR independent companion of MTOR, complex 2 (also AVO3)	NM_152756	1		
	SKAP2: src kinase associated phosphoprotein 2 (also PRAP, RA70, SAPS, SCAP2, SKAP-HOM, SKAP55R)	NM_003930	1	VN	This study
	TBL1XR1: transducin b-like 1X-linked receptor 1 (also C21, DC42, IRA1, TBLR1)	NM_024665	3		
	YWHAQ: tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide (also KCIP-1, 14-3-3)	NM_003406	1	MA	(Ghobrial et al., 2005)
Translation	**EIF1AY**: eukaryotic translation initiation factor 1A,Y-linked	NM_004681	1		
	HNRNPC: heterogeneous nuclear ribonucleoprotein C (C1/C2) (also C1, C2, HNRNP, HNRPC, SNRPC)	NM_004500	2		
	NARS: asparaginyl-tRNA synthetase (also ASNRS, NARS1)	NM_004539	1		
Other	**ACOX3**: acyl-Coenzyme A oxidase 3, pristanoyl	NM_003501	1		
	CYP51A1: cytochrome P450 family 51 subfamily A polypeptide 1 (also CP51, CYP51, CYPL1, LDM, P450-14DM, P450L1)	NM_000786	1		
	DCK: deoxycytidine kinase	NM_000788	1	VN	This study
	ETNK1: ethanolamine kinase 1 (also EKI, EKI1, Nbla10396)	NM_018638	1		
	GART: phosphoribosylglycinamide formyltransferase (also AIRS, GARS, GARTF, PAIS, PGFT, PRGS	NM_000819	1		
	GLUL: glutamate-ammonia ligase (glutamine synthetase) (also GLNS, GS, PIG43, PIG59)	NM_002065	2	MA	(Rizzatti et al., 2005)
	GPR34: G protein-coupled receptor 34 (P2Y-like receptor)	NM_005300	2	VN	This study
	H2AFV: H2A histone family, member V (also H2AV)	NM_138635	1		
	LYZ: lysozyme (renal amyloidosis) (also LZM)	NM_000239	3		
	PSME1: proteasome (prosome, macropain) activator subunit 1 (also PA28A, PA28alpha, REGalpha)	NM_006263	2		
	SNRPD1: small nuclear ribonucleoprotein D1 polypeptide (also HsT2456, SMD1, SNRPD, Sm-D1)	NM_006938	1		
	TFRC: transferrin receptor (p90, CD71) (also CD71, TFR, TFR1, TRFR)	NM_003234	1		

Function	Genes Identified by SSH	Accession #	Hits	Confirmed	References
	USP47: ubiquitin specific protease 47 (also TRFP)	NM_017944	1		
	VPS4B: vacuolar protein sorting 4 homolog B (S. cerevisiae) (also MIG1, SKD1, SKD1B, VPS4-2)	NM_004869	1		
Unknown Function	**CBWD2**: COBW domain containing 2	NM_172003	1		
	FAM111A: family with sequence similarity 111, member A	NM_022074	1		
	NPM1P7: nucleophosmin 1 (nucleolar phosphoprotein B23, numatrin) pseudogene 7 (also NG7-6; NPMP7; NPM1P7)	L15320	2		
	PDLIM1: PDZ and LIM domain 1 (also CLIM1, CLP36, CLP-36, hCLIM1)	NM_020992	1		
	PMS2L13: postmeiotic segregation increased 2-like 13	AB017004	1		
	PTER: phosphotriesterase related (also RPR-1)	NM_030664	1		
	TMED5: transmembrane emp24 protein transport domain containing 5 (also CGI-100)	NM_016040	1		
Unknown Gene	BAC clone 12 RP11-290L1	AC011611	1		
	BAC clone RP11-191J2 from 4	AC096952	1		
	BAC clone RP11-77403 from 4	AC104825	1		
	mitochondrion, complete genome	NC_001807	1		
	Seq from clone RP3-384F21 on chr1q24	AL022171	1		

* genes found in more than one functional group

VN = virtual Northern

MA = microarray

TABLE 2

Differentially expressed genes identified by SSH in SLL versus MCL

Function	Genes Identified by SSH	Accession #	Hits	Confirmed	References
Cancer-related	**HECA**: headcase homolog (Drosophila) (also HDC, HDCL, HHDC)	NM_016217	1		
	SRPK2: SFRS protein kinase 2 (also SFRSK2)	NM_182692	1		
	TMBIM6: transmembrane BAX inhibitor motif containing 6 (also BAXI1, BI-1, TEGT)	NM_001098576	1		
	TNFAIP8: tumor necrosis factor, alpha-induced protein 8 (also GG2-1, MDC-3.13, SCC-S2, SCCS2)	NM_001077654	1		
	UVRAG: UV radiation resistance associated gene (also DHTX, p63)	NM_003369	1		
Cell Cycle	**CCND2**: cyclin D2	NM_001759	1		
	CDC16: cell division cycle 16 homolog (S. cerevisiae) (also APC6)	NM_003903	1		
	MAD2L1BP: MAD2L1 binding protein (also CMT2)	NM_014628	1		
	MDM4: Mdm4 p53 binding protein homolog (mouse) (also HDMX, MDMX, MRP1)	NM_002393	1		
	USP8: ubiquitin specific peptidase 8 (also HumORF8, UBPY)	NM_005154	1		
Cytoskeleton	**ACTR6**: ARP6 actin-related protein 6 homolog (yeast) (also ARP6, CDA12, hARP6, hARPX)	NM_022496	1		
	APPBP2: amyloid beta precursor protein (cytoplasmic tail) binding protein 2 (also PAT1)	NM_006380	1		
	CCT6A: chaperonin containing TCP1, subunit 6A (zeta 1) (also CCT-zeta, CCT-zeta-1, CCT6, Cctz, HTR3, MoDP-2, TCP-1-zeta, TCP20, TCPZ, TTCP20)	NM_001762	1		
	DMD: dystrophin (also BMD, CMD3B, DXS142, DXS164, DXS206, DXS230, DXS239, DXS268, DXS269, DXS270, DXS272)	NM_004023	3		
	FLJ42562: PREDICTED: Homo sapiens similar to echinoderm microtubule associated protein like 5, transcript variant 2	XM_001726921	1		
	MYL12A: myosin, light chain 12A, regulatory, non-sarcomeric (also MLCB, MRLC3, MYL2B)	NM_006471	1		
	TMSB4Y: thymosin beta 4, Y-linked (also TB4Y)	NM_021109	3		
	TPT1: tumor protein, translationally-controlled 1(also HRF, TCTP, p02)	BC003352	1		
Immunity	**ADAM28**:ADAM metallopeptidase domain 28 (also ADAM23, MDC-Lm, MDC-Ls, MDCL, eMDCII)	NM_014265	1		
	ADAM28: ADAM metallopeptidase domain 28 (also ADAM23, MDC-Lm, MDC-Ls, MDCL, eMDCII)	NM_014265	2		
	ADAMDEC1: ADAM-like, decysin (also decysin, disintegrin protease	NM_014479	1		
	BTLA: B and T lymphocyte associated (also BTLA1, CD272)	NM_001085357	1		
	CASP1: caspase 1, apoptosis-related cysteine peptidase (interleukin-1 beta convertase) (also ICE, IL1BC, P45)	NM_001223	1		
	CD200: CD200 molecule (also MOX1, MOX2, MRC, OX-2)	NM_001004196	2		
	CLLU1: chronic lymphocytic leukemia up-regulated 1	NM_001025232	1		

Function	Genes Identified by SSH	Accession #	Hits	Confirmed	References
	CR2: complement component (3d/Epstein Barr virus) receptor 2 (also C3DR, CD21, SLEB9)	NM_001006658	1		
	CRTAM: cytotoxic and regulatory T cell molecule	NM_019604	2		
	FCER2: Fc fragment of IgE, low affinity II, receptor for (CD23) (also CD23, CD23A, CLEC4J, FCE2, IGEBF)	NM_002002	1		
	GPR18: G protein-coupled receptor 18	NM_001098200	1		
	GPR183: G protein-coupled receptor 183 (also EBI2)	NM_004951	1	MA	(Aalto et al., 2001)
	*__HSPD1__: heat shock 60kDa protein 1 (chaperonin) (also CPN60, GROEL, HLD4, HSP60, HSP65, HuCHA60, SPG13)	NM_002156	1		
	IBTK: inhibitor of Bruton agammaglobulinemia tyrosine kinase (also BTKI)	NM_015525	1		
	*__IKZF3__: IKAROS family zinc finger 3 (Aiolos) (also AIO, AIOLOS, ZNFN1A3)	NM_183232	2		
	*__TCF4__: transcription factor 4 (also E2-2, ITF2, PTHS, SEF2, SEF2-1, SEF2-1A, SEF2-1B, bHLHb19)	NM_001083962	1		
	*__TLE1__: transducin-like enhancer of split 1 (E(sp1) homolog, Drosophila) (also ESG, ESG1, GRG1)	NM_005077	1		
	*__ZBTB20__: zinc finger and BTB domain containing 20 (also DPZF, HOF, ODA-8S, ZNF288)	NM_015642	1		
Mitochondria	**COX11**: COX11 homolog, cytochrome c oxidase assembly protein (yeast) (also COX11P)	NM_004375	1		
	DNAJC19: DnaJ (Hsp40) homolog, subfamily C, member 19 (also TIM14, TIMM14)	NM_145261	1		
	*__HSPD1__: heat shock 60kDa protein 1 (chaperonin) (also CPN60, GROEL, HLD4, HSP60, HSP65, HuCHA60, SPG13)	NM_002156	1		
	MRPL42: mitochondrial ribosomal protein L42 (also HSPC204, MRP-L31, MRPL31, MRPS32, PTD007, RPML31)	NM_014050	1		
	MRPL45: mitochondrial ribosomal protein L45	NM_032351	1		
	MRPS6: mitochondrial ribosomal protein S6 (also C21orf101, MRP-S6, RPMS6, S6mt)	NM_032476	1		
Ribosomal	**NCL**: nucleolin (also C23)	NM_005381	1		
	RPL26: ribosomal protein L26	NM_000987	1		
	RPS18: ribosomal protein S18 (also DADB-159G18.1, D6S218E, HKE3, KE-3, KE3)	NM_022551	1		
	RPS20: ribosomal protein S20	NM_001023	1		
	RPS27: ribosomal protein S27 (also MPS-1, MPS1)	XM_001484	1		
	RPS27L: ribosomal protein S27-like	NM_015920	1		
	RPS3A: ribosomal protein S3A (also FTE1, MFT)	NM_001006	2		
Signal Transduction	**APLP2**: amyloid beta (A4) precursor-like protein 2 (also APPH, APPL2, CDEBP)	NM_001642	1		
	MAP4K5: mitogen-activated protein kinase kinase kinase kinase 5 (also GCKR, KHS, KHS1, MAPKKKK5)	NM_006575	1		

Function	Genes Identified by SSH	Accession #	Hits	Confirmed	References
	PTP4A2: protein tyrosine phosphatase type IVA, member 2 (also HH13, HH7-2, HU-PP-1, OV-1, PRL-2, PRL2, PTP4A, PTPCAAX2, ptp-IV1a, ptp-IV1b)	NM_003479	1		
	RAP1A: RAP1A, member of RAS oncogene family (also KREV-1, KREV1, RAP1, SMGP21)	NM_002884	1		
	RPS6KA3: ribosomal protein S6 kinase, 90kDa, polypeptide 3 (also RP11-393H10.3, CLS, HU-3, ISPK-1, MAPKAPK1B, MRX19, RSK, RSK2, S6K-alpha3, p90-RSK2, pp90RSK2)	NM_004586	1		
Transcription	**ATRX**: alpha thalassemia/mental retardation syndrome X-linked (RAD54 homolog, S. cerevisiae) (also ATR2, DXHXS6677E, HP1-BP38, Hp1bp2, Hp1bp38, MRXS3, RAD54L, Rad54, XH2, Xnp, ZNF-HX)	NM_000489	1		
	FOXP1: forkhead box P1 (also QRF1, hFKH1B)	NM_032682	1		
	FOXR1: forkhead box R1 (also DLNB13, FOXN5)	NM_181721	1		
	*__IKZF3__: IKAROS family zinc finger 3 (Aiolos) (also AIO, AIOLOS, ZNFN1A3)	NM_183232	2		
	MEF2C: myocyte enhancer factor 2C (also MADS box transcription enhancer factor 2)	NM_002397	1		
	NCOA3: nuclear receptor coactivator 3 (also RP5-1049G16.3, ACTR, AIB-1, AIB1, CAGH16, CTG26, KAT13B, RAC3, SRC-3, SRC3, TNRC14, TNRC16, TRAM-1, bHLHe42, pCIP)	NM_181659	1		
	NOP58: NOP58 ribonucleoprotein homolog (yeast) (also HSPC120, NOP5, NOP5/NOP58)	NM_015934	1		
	PAX5: paired box 5 (also BSAP)	NM_016734	1	MA	(Aalto et al., 2001)
	PDCD6IP: programmed cell death 6-interacting protein (also AIP1, Alix, DRIP4, HP95)	NM_013374	1		
	POLR3B: polymerase (RNA) III (DNA directed) polypeptide B (also C128, RPC2)	NM_018082	1		
	*__TCF4__: transcription factor 4 (also E2-2, ITF2, PTHS, SEF2, SEF2-1, SEF2-1A, SEF2-1B, bHLHb19)	NM_001083962	1		
	TCF12: transcription factor 12 (also HEB, HTF4, HsT17266, bHLHb20)	NM_003205	1		
	*__TLE1__: transducin-like enhancer of split 1 (E(sp1) homolog, Drosophila) (also ESG, ESG1, GRG1)	NM_005077	1		
	TNFAIP1: tumor necrosis factor, alpha-induced protein 1 (endothelial) (also B12, B61, EDP1)	NM_021137	1		
	YTHDC1: YTH domain containing 1 (also YT521, YT521-B)	NM_001031732	1		
	*__ZBTB20__: zinc finger and BTB domain containing 20 (also DPZF, HOF, ODA-8S, ZNF288)	NM_015642	1		
Translation	**DENR**: density-regulated protein (also DRP, DRP1, SMAP-3)	NM_003677	1		
	EEF1A1: eukaryotic translation elongation factor 1 alpha 1 (also CCS-3, CCS3, EEF-1, EEF1A, EF-Tu, EF1A, GRAF-1EF, LENG7, PTI1, eEF1A-1)	XM_004398	9		
	EIF1: eukaryotic translation initiation factor 1 (also A121, EIF-1, EIF1A, ISO1, SUI1)	NM_005801	1		
	EIF4B: eukaryotic translation Initiation factor 4B (also EIF-4B, PRO1843)	NM_001417	1		
	MTIF2: mitochondrial translational initiation factor 2 (also IF-2mt)	NM_002453	1		
Other	**ALDH3A2**: aldehyde dehydrogenase 3 family, member A2 (also ALDH10, FALDH, SLS)	XM_008516	1		

Function	Genes Identified by SSH	Accession #	Hits	Confirmed	References
	AP1S2: adaptor-related protein complex 1, sigma 2 subunit (also DC22, MGC:1902, MRX59, SIGMA1B)	XM_351139	1		
	ATP1B3: ATPase, Na+/K+ transporting, beta 3 polypeptide (also ATPB-3, CD298)	NM_001679	2		
	collagenase, stromelysin, metalloelastase	U78045	1		
	EVI2A: ecotropic viral integration site 2A (also EVDA, EVI2)	NM_014210	1		
	LUM: lumican (also LDC, SLRR2D)	NM_002345	1		
	METTL8: methyltransferase like 8 (also TIP)	NM_024770	1		
	NFE2L2: nuclear factor (erythroid-derived 2)-like 2 (also NRF2)	NM_006164	1	MA	(Schmechel et al., 2004)
	PALM2-AKAP2: PALM2-AKAP2 readthrough transcript (also AKAP2)	NM_147150	1		
	PIKFYVE: phosphoinositide kinase, FYVE finger containing (also CFD, FAB1, PIP5K, PIP5K3)	NM_015040	1		
	PNPLA8: patatin-like phospholipase domain containing 8 (also IPLA2(GAMMA), IPLA2-2, IPLA2G)	NM_015723	1		
	PSMA6: proteasome (prosome, macropain) subunit, alpha type, 6 (also IOTA, PROS27, p27K)	NM_002791	1		
	RHOH: Ras homolog gene family member H (also ARHH, TTF)	NM_004310	1	MA	(Zhu et al., 2001)
	RNGTT: RNA guanylyltransferase & 5′-phosphatase (also CAP1A, HCE, HCE1, hCAP)	XM_004349	1		
	SERPINE2: serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 2 (also GDN, PI7, PN1, PNI)	NM_006216	2		
	SNX18: sorting nexin 18 (also SH3PX2, SH3PXD3B, SNAG1)	NM_052870	1		
	SUPT16H: suppressor of Ty 16 homolog (S. cerevisiae) (also CDC68, FACTP140, SPT16/CDC68)	AF152961	1		
	TMED5: transmembrane emp24 protein transport domain containing 5 (also CGI-100)	NM_016040	1		
	TMEM66: transmembrane protein 66 (also FOAP-7, HSPC035, XTP3)	NM_016127	1		
	UBE2G1: ubiquitin-conjugating enzyme E2G 1 (UBC7 homolog, yeast) (also E217K, UBC7, UBE2G)	NM_182682	1		
	UTS2: urotensin 2 (also PRO1068, U-II, UCN2, UII)	NM_021995	1		
Unknown Function	**GTSF1**: gametocyte specific factor 1 (also FAM112B)	NM_144594	1		
	ITM2A: integral membrane protein 2A (also BRICD2A, E25A)	NM_004867	1		
	L1TD1: LINE-1 type transposase domain containing 1 (also ECAT11)	NM_019079	1		
	MBNL1: muscleblind-like (Drosophila) (also EXP, EXP35, EXP40, EXP42, KIAA0428, MBNL)	NM_021038	2		
	MIRHG2: microRNA host gene 2 (non-protein coding) (also BIC, MIR155HG, NCRNA00172)	NR_001458	1		
	MLLT10: myeloid/lymphoid or mixed-lineage leukemia (trithorax homolog, Drosophila); translocated to 10 (also AF10)	NM_001009569	1		
	RBM25: rNA binding motif protein 25 (also RED120, RNPC7, S164, fSAP94)	NM_021239	1		

Function	Genes Identified by SSH	Accession #	Hits	Confirmed	References
	SH3BGRL: SH3 domain binding glutamic acid-rich protein like (also SH3BGR) acid-rich protein like (also SH3BGR)	NM_003022	1		
	SIPA1L1: signal-induced proliferation-associated 1 like 1 (also E6TP1)	NM_015556	1		
	SSBP2: single-stranded DNA binding protein 2 (also HSPC116)	NM_012446	1		
	STARD7: StAR-related lipid transfer (START) domain containing 7 (also GTT1)	NM_020151	1		
	TMEM167A: transmembrane protein 167 (also TMEM167)	NM_174909	1		
	TOP1P1: topoisomerase (DNA) I pseudogene 1	XM_012935	1		
	UBL3: ubiquitin-like 3 (also HCG-1, PNSC1)	NM_007106	1		
Unknown Gene	BAC clone R-85K15 of library RPCI-11 on chr14	AL049776	1		
	BAC clone RP11-218F10 from 4	AC093788	1		
	BAC clone RP11-438F11 from 4	AC093841	1		
	BAC clone RP11-517F1 from 2	AC104698	1		
	BAC clone RP11-632F7 from 4	AC131953	1		
	BAC clone RP11-709L9 from 4	AC023150	1		
	BAC RP11-436A20 3	AC008009	1		
	C11orf59: chromosome 11 open reading frame 59	NM_017907	1		
	C12orf35: chromosome 12 open reading frame 35	NM_018169	1		
	C1orf162: chromosome 1 open reading frame 162	NM_174896	2		
	C1orf43: chromosome 1 open reading frame 43 (also NICE-3, NS5ATP4, S863-3)	NM_015449	1		
	C6orf62: chromosome 6 open reading frame 62	NM_030939	1		
	Chr16 clone CTA-345G4	AC130454	1		
	Chr5 clone CTC-547D20	AC026701	1		
	Chr5 clone CTD-3136I2	AC122700	1		
	Chr9 clone hRPK.538_E_7	AC007172	1		
	Clone IMAGE:5297467	BC043003	1		
	Hypothetical protein RP5-1022P6 on chr20	NM_019593	1		
	mitochondrion, complete genome	NC_001807	5		
	Seq from clone RP11-248J23 on chr10	AL513355	1		
	Seq from clone RP11-393H10 on chrX	AL732366	1		
	Seq from clone RP11-50K12 on chr9	AL354937	8		

Function	Genes Identified by SSH	Accession #	Hits	Confirmed	References
	Seq from clone RP11-551G24	AL512637	1		
	Seq from clone RP4-747L4 on chr1q23-24	AL009051	1		

* genes found in more than one functional group

VN = virtual Northern

MA = microarray