Abnormal microRNA-16 Locus with Synteny to Human 13q14 Linked to CLL in NZB Mice*

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Running Title: Linkage Analysis of Murine Model of Human B-CLL

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*Abbreviations: SSLP, short sequence length polymorphisms; B-CLL, B- cell chronic lymphocytic leukemia; SLE, systemic lupus erythematosus; NZB, New Zealand Black mouse; LPD, lymphoproliferative disorder or disease; AIHA, autoimmune hemolytic anemia; PALS, periarteriolar lymphoid sheath; GC, germinal center; EMH, extramedullary hematopoiesis; MZL, marginal zone lymphoma; GN, glomerulonephritis; MCV, mean corpuscular volume; F1BC, F1 backcross; miRNA, microRNA

†NZB mir-16-1 sequence variant has been deposited in NCBI data base #EF042973
ABSTRACT

NZB mice, with autoimmune and B lymphoproliferative disease (B-LPD), are a model for human chronic lymphocytic leukemia (CLL). A genome-wide linkage scan of the NZB loci associated with lymphoma was conducted in F1 backcrosses of NZB and a control strain, DBA/2. Of 202 mice phenotyped for the presence or absence of LPD, surface marker expression, DNA content and microsatellite polymorphisms, 74 had disease. The CD5+, IgM+, B220 dim, hyperdiploid LPD was linked to three loci on chromosomes 14, 18 and 19 which are distinct from previously identified autoimmunity-associated loci. The region of synteny with mouse D14mit160 is the human 13q14 region, associated with human CLL, containing microRNAs, mir15a/16-1. DNA sequencing of multiple NZB tissues identified a point mutation in the 3’flanking sequence of the identical micro RNA, mir16-1 and this mutation was not present in other strains, including the nearest neighbor, NZW. Levels of miR-16 were decreased in NZB lymphoid tissue. Exogenous miR-16 delivered to an NZB malignant B-1 cell line resulted in cell cycle alterations and increased apoptosis. Linkage of the mir15a/16-1 complex and the development of B-LPD in this spontaneous mouse model suggest that the altered expression of the miR15a/16-1 is the molecular lesion in CLL.
INTRODUCTION

B-cell chronic lymphocytic leukemia (CLL) is the most common hematological malignancy in the Western world \(^1\); however, the molecular etiology and pathogenesis of CLL remains unknown but antigen exposure may play a role \(^2\). Normal B cell development and differentiation as well as CLL leukemogenesis are thought to be a multi-step process. Thus, CLL may represent an accumulation of several genetic alterations. CLL shows the highest incidence of familial leukemia and is believed to be polygenetic \(^3\). Although the cell of origin remains unknown, it is thought by some to arise in a CD5+ B cell splenic marginal zone sub-population whose immunoglobulin genes encode for polyreactive IgM autoantibodies \(^4\), \(^5\). Molecular cytogenetic abnormalities reported associated with CLL include 13q14 deletions \(^6\), \(^7\). A minimally deleted region (MDR) has been described and several genes in this region have been sequenced but no mutations in protein-coding regions have been identified \(^8\). However, mutations in miR-16 have been described \(^9\) as well as reduction in the levels of expression in the majority of CLL patients\(^{10}\). The NZB mouse has been studied extensively as a model to investigate autoimmune diseases such as systemic lupus erythematosus (SLE) \(^{11}\), \(^{12}\), as well as a model for the B cell lymphoproliferative disorder, CLL \(^{13}\). In both the NZB and human CLL, the disease is late appearing and similar to a subset of CLL patients, NZB mice develop autoimmune hemolytic anemia (AIHA). As NZB mice age, they develop a monoclonal lymphoproliferative expansion characterized by increased numbers of CD5+ B220 dull B cells which are hyperdiploid \(^{14}\). In addition, the NZB malignant CD5+ B clones frequently have increased IL-10 production and development of CLL-like clones is associated with elevated IL-10 in crosses of NZB with DBA/2 \(^{15}\). In contrast, the DBA/2 strain has very few CD5+ B cells and does not
demonstrate an age-related expansion of these cells. In the present report we used crosses of these two strains to determine the genetic loci linked to the development of LPD.

Microsatellite or simple sequence length polymorphisms (SSLP) are widely used in mouse genetics because they are numerous, highly polymorphic and widely dispersed throughout the genome and these SSLP's have been used to create dense linkage maps for at least 12 inbred strains of mice. The commercial availability of primers and the PCR-based identification of SSLP chromosome markers make it feasible to systematically search the entire mouse genome for linkage. In the present study, we performed a genome-wide scan to identify loci for murine lymphoproliferative disease (LPD) and additional direct DNA sequencing analysis was conducted on a locus on mouse chromosome 14 with synteny to human 13q14.

MATERIALS AND METHODS

Mouse colony. A mouse breeding colony was established within the FDA facility at the NIH campus and all protocols were approved by the Animal Use Committee. New Zealand Black (NZB) and DBA/2 (DBA) male and female mice were purchased from the Jackson Laboratories (Bar Harbor, ME). These mice and their progeny were housed under uniform conditions in the disease free animal rooms at the FDA (Bethesda, MD, USA). Male and female NZB and DBA/2 individuals were mated to produce a group of F1 individuals (NZB x DBA/2). F1 individuals were then backcrossed to NZB individuals to produce the population of [(NZB x DBA/2) F1 x NZB]. Animals were aged in single sex groups of ten. A total of 202 F1BC (read F1 back cross) animals were phenotyped of which 74 were positive microscopically for the presence of B cell LPD within the spleen. Additional subsequent crosses between NZB and DBA/2 were performed involving 230 additional study animals.

Phenotyping of Mice.
Autopsy. At the time of these studies, the mice were between 21-27 months of age. Animals were bled under light anesthesia [Metofane® (methoxyflurane, Pitman-Moore, Mundelein, IL, USA)] followed by cervical dislocation, peritoneal lavage and autopsy. The animals' total weight, sex, state of health and spleen weight were noted. Sections were obtained from the sternum, heart, lungs, salivary glands, liver, spleen, both kidneys, lymph nodes, and intestines for histological evaluation.

Histopathology. Tissue samples for microscopic examination were formalin fixed, paraffin embedded, sectioned and stained with H and E (EPL, Vienna, VA, USA). Spleens were systematically reviewed for overall size, pattern of the periarteriolar lymphoid sheath (PALS), number and size of germinal centers (GC), degree of extramedullary hematopoiesis (EMH), and whether GCs were inactive or reactive. LPD was classified as previously reported 17-19. Histology was performed using a Zeiss, model Axiophot microscope (Carl Zeiss, Inc., Thornwood, NY, USA) using a 5x objective with a 0.16 numerical aperture. The appearance of the bridging of two germinal centers by marginal zone cells was defined as minimal LPD or early spleen marginal zone lymphoma. Moderate involvement was infiltration of the red pulp by these marginal zone cells. Marked involvement was complete replacement of normal splenic architecture. A 40x objective (0.5-1.0 numerical aperture) was employed for nuclear morphology analysis. Photomicrographs were produced with a Cool Snap model HQ camera (Princeton Instruments, Trenton, NJ, USA) and the software for acquisition was IPLab (BD Biosciences, Rockville, MD, USA).

Blood. Blood was obtained from mice anesthetized with Metofane® via brachial artery exsanguination or cardiac puncture. This blood was used for a complete blood count (CBC), reticulocyte count, blood film and serum immunoglobulin levels (Analytics, Gaithersburg,
MD, USA). These results were compared to normal mouse values and cut-off values were determined (Table 1).

Flow Cytometric Analysis. Single cell spleen and peritoneal suspensions were obtained. Spleen suspensions were further treated with ammonium chloride lysis. The cells were analyzed for both surface markers and DNA content (cell cycle). For surface markers, cells (1 x 10^6) were stained with FITC conjugated goat anti-IgM, anti-CD5 conjugated with phycoerythrin (PE), anti-B220 conjugated with PE-Cy5 (TriChrome®) and isotype controls were all obtained from same manufacturer (Caltag, Burlingame, CA, USA). Unstained cells, isotype controls and singly and doubly stained cells in conjunction with AutoComp software were used to set compensation. A FACScan flow cytometer (BD Bioscience, San Jose, CA, USA) was used to collect 2 x 10^4 events. Lysis II and CellQuest software (BD Bioscience) and Mod Fit (Verity Software House, Topsham, ME, USA) were used for data analysis and display. For cell cycle analysis, cell suspensions (1 x 10^6 cells) were stained with the FITC anti-IgM reagent, fixed in cold 70% ETOH and stored overnight at 4°C. At the time of analysis, the ethanol fixed cells were incubated with propidium iodide (PI, 50 ug/ml) and ribonuclease (1 mg/ml) for 30 min at 37°C and analyzed immediately on a FACScan flow cytometer using CELLFIT software (BD Bioscience) for both the acquisition and analysis of 100,000 events. Chicken erythrocyte and calf thymus nuclei were used as linearity controls. A DNA index was derived by the software.

DNA Extraction for Genotyping. DNA was extracted from non-pathologic tissues, usually from the liver. DNA was purified by the standard phenol/chloroform method followed by the QIAamp tissue kit (Qiagen, Inc., Chatsworth, CA, USA). The samples were re-suspended in TE (10mM Tris-Cl pH 7.6, 1mM EDTA) at a volume close to 1 µg/µl.
**Primer pair selection.** Microsatellite oligonucleotide primer pairs were purchased from Research Genetics (Huntsville, AL, USA). SSLPs were typed by determining the size of the PCR products obtained with individual primer sets. The identification of a polymorphic locus in the two inbred strains (NZB and DBA/2) was first determined. Of 450 primers analyzed, 75 were deemed informative with detectable size difference in PCR product size between the two parental strains. Representative informative primers included: D1Mit68, D1Mit303, D1Mit49, D1Mit33, D2Mit15, D2Mit21, D3Mit60, D4Mit95, D4Mit259, D4 Mit 9, D4Mit37, D4Mit232, D5Mit294, D5Mit81, D5Mit8, D5Mit101, D6MIT84, D6Mit123, D6Mit188, D6Mit10, D6Mit25, D6Mit15, D7Mds5, D7Mit39, D8Mit4, D8Mit6, D8Mit137, D9Mit205, D9Mit45, D9Mit182, D9Mit52, D11Mit229, D11Mit29, D11Mit99, D11Mit50, D12Mit56, D12Mit35, D12Mit51, D13Mit221, D13Mit202, D13Mit110, D13Mit130, D13Mit78, D14Mit98, D14Mit129, D14Mit5, D14Mit160, D15Mit85, D16Mit131, D16Mit118, D17Mit176, D17Mit93, D18Mit61, D18Mit62, D18Mit4, D19Mit35, D19Mit6.

**PCR Conditions.** PCR reactions were performed using reagents and methods from Invitrogen (Carlsbad, CA, USA) with Taq enzyme from Boeringer-Mannheim (Roche Pharmaceuticals,). Thirty-five cycles were used of 2 min. 94°C, 2 minutes at 56 to 62°C, and 30 sec. at 72°C. Reactions were 20 µl volumes covered with oil in a microtiter plate. A hot start of 2 min. at 98°C was followed by the addition of enzyme at 85°C. The PCR products were resolved on a 3% Metaphor (Cambrex Corp formerlyFMC Inc., Rockland, ME, USA) agarose gel stained with ethidium bromide. For the 75 loci chosen for this study, the longest distance between a marker and an autosomal locus was 15.8 cM. The X chromosome was not evaluated.

**Linkage analysis.** For all linkage analysis studies using the informative primer pairs, three DNA controls were used: NZB parental, DBA/2 parental and F1 (NZB xDBA/2) DNA. The F1 would
show the presence of both parental bands whereas the F1BC could show either both bands or only the NZB band and the F2 could be homozygous for either parent or heterozygous. The samples were scored for heterozygosity versus homozygosity and the results were analyzed for expected distribution using the chi-square analysis. In additional studies, only three loci were reexamined: D14Mit160, D18Mit4 and D19Mit6.

*Sequencing and designing primers for mir 15a/16-1.* Using the miRBase Sequence Database (http://microrna.sanger.ac.uk/sequences/) and the nomenclature established \(^{20,21}\), the stem-loop and mature sequences, as well as the base coordinates were obtained for each of the following: hsa-miR 16-1, hsa-miR 15a (both human), mmu-miR 16-1, and mmu-miR 15a (both mouse). Using the NCBI BLAST 2 Sequences (www.ncbi.nlm.nih.gov), regions of homology were found among the miR 15a/16-1 regions between *Homo sapiens* chr 13 and *Mus musculus* chr 14. For amplification of mouse chromosome 14 region containing mmu-miR-15a and miR-16-1 (determined using the Ensemble Genome Browser www.ensembl.org), the following primers were produced by the NJMS Molecular Resource Facility, so as to create a 502 base pair amplicon: 5’cctggtatgcagtggtaaggc 3’ (forward primer), 5’ctattgaggtgctaggag 3’ (reverse primer).

*DNA extraction, PCR, and DNA sequencing.* Sources of DNA were obtained from the spleen, liver, kidney, bone marrow or lymph node from NZB/NJ, NZW, DBA/2J, C57Bl/6J, NOD/SCID/DR1, and SJL strains obtained from the Jackson Labs. DNA was extracted using the Qiagen DNeasy Tissue Kit, and amplified via PCR on the GeneAmp PCR system 9600 (Applied Biosystems, Foster City, CA, USA)) at an annealing temperature of 55°C for 35 cycles. The PCR products were purified using the Qiagen QIAquick PCR Purification Kit and sequenced using four-color fluorescent sequencing reactions on the Applied Biosystems model 3130xl sequencer.
Using NCBI’s BLAST 2, the DNA sequences obtained were compared to reference sequences. The sequence obtained in NZB mice has been reported to GenBank #EF042973.

*Functional Analysis of miR-16 RNA.* Tissue and cells were obtained and placed in TRizol (Invitrogen) and RNA extracted. Realtime PCR was used to quantitate miR-16 in tissues using the TaqMan microRNA Reverse Transcription Kit (Applied Biosystems) and the RT 391miR-16 5x RT Primer. The RT reaction was run on the GeneAmp PCR System 9600 for 1 cycle at 16°C for 30min, 42°C for 30min, and 85°C for 5min. Following the stem-loop RT reaction, real-time was performed using the TaqMan 2x Universal PCR Master Mix (Applied Biosystems) and TaqMan MicroRNA Assay TM 391 miR-16 20x Real Time primers (Applied Biosystems), and run on the Applied Biosystems 7500 Real Time PCR System at 95°C for 10min, 95°C for 15sec and 60°C for 60sec for 40 cycles. Change in gene expression was reported as change in the delta CT compared to the GAPDH control (TaqMan Rodent GAPDH Control; Applied Biosystems) and relative quantification study was performed on the RT products according to the manufacturer’s protocol. Northern analysis was also performed and the level of expression reported as normalized fmol (normalized to the median of tRNA signals).

To study the effects of exogenous miR-16, a total of 1-4 million LNC cells were transfected with a microRNA mimic using the using the Amaxa Nucleofector II. Experimental groups included untreated, electroporated, and transfected (miR-16 or control mimic) cells. Cells were transfected with 3ug of miRDIAN Mimic mmu-miR-16 (Dharmacon-C-310112-02) or 3ug of miRDIAN Mimic Negative Control #1(Dharmacon-CN-001000-01) according to the manufacturer’s protocol using program G-16, and solution T. Cells were plated at 0.5 x 10^6 per ml and incubated at 37°C for 24 hours. Following incubation, flow cytometry was used to detect cell cycle changes and apoptosis by staining the DNA with propidium iodide (Calbiochem).
PI staining, 1x10^6 cells were stained with hypotonic PI (0.05mg/ml PI, 0.1% Triton X-100). FACS data was acquired on Becton Dickinson FACS Calibur. Acquisition was done using CELLQUEST software (Becton Dickinson), and analysis was performed using ModFit LT software (Varioity House Inc).

RESULTS

Phenotype of F1Backcrosses. Based on the results of the phenotypic analysis of 202 mice examined the backcross mice were divided on the basis of the presence or absence of LPD (non-LPD and LPD) (Fig 1, Table 1). Enlarged spleens were present in both LPD and non-LPD mice, however greatly enlarged spleens are more frequently seen in LPD mice (55% versus 24%, see Table 1). Based on RBC, MCV, and percent reticulocytes there was no significant difference in the presence of autoimmune hemolytic anemia in LPD versus non-LPD mice. However, leukocytosis was more frequent in the LPD mice (17% versus 3%, see Table 1), and lymphocytosis was confirmed by review of the blood film. Peritoneal cell count and serum IgM mean levels are nearly identical and do not suggest a disease related difference. Table 2 suggests that there is an increase in aneuploidy in the LPD F1BC animals.

Histopathology. In all, 37% (74/202) of the mice demonstrated LPD. Ninety-four percent (94%)(67/71) of the splenic murine lymphomas were classified as marginal zone lymphomas (MZL). Various stages of marginal zone involvement could be seen (Fig 2): early marginal zone hyperplasia (Fig 2A-2B); the confluence of two or more germinal centers; massive infiltration of the red pulp (Fig 2B/C); and the entire replacement of the spleen (Fig 2D). Higher power views of tumor involved areas showed cellular details (Fig 2E-2H). The marginal zone cells were characterized as monocytoid lymphocytes with clumped chromatin and a slight nuclear
indentation (Fig 2E/F). The nuclear pattern became more vesicular in some mice (Fig 2G/H). This cellular spectrum suggests heterogeneity and marginal zone transformation. Other lymphocytic neoplasms (less than 5%) identified in mice with LPD included were lymphoplasmocytic lymphoma (2 cases) centroblastic lymphoma (one case) and small lymphocytic lymphoma (one case).

**Flow Cytometric Analysis.** Flow cytometric data from 12 representative F1BC mice with LPD as defined by histopathology are presented in Figure 3. Hyperdiploidy was detected in both spleen and peritoneal cavity cells (Fig 3 A/B), but not all mice with LPD had detectable aneuploidy. Further, the hyperdiploid cells were IgM positive B cells (Fig 3 C/D) confirming that the LPD was due to a B cell expansion. In the spleens shown in this figure, 75% of the mice (9/12) had hyperdiploid B cell clones, whereas in the peritoneal cells 50% of the mice (6/12) had no detectable hyperdiploid cells. Thus hyperdiploid cells may be present in the spleen yet undetectable in the matched peritoneum (See mouse 4 and 12).

The amount of hyperdiploid B cell clones varied among the individual mice. For instance in mouse #12 there is evidence of a small splenic hyperdiploid clone in the G2M peak. In contrast, mouse #4 clearly demonstrates a dominant hyperdiploid B clone in the spleen which is lacking in the peritoneal cells (Fig. 3 B and D). Three color analysis of surface marker (Fig 3E/F) expression indicates that the aneuploid population is within the IgM+, CD5 dull+, B220 dim+ population.

**Genomic Screening.** In order to establish linkage between disease phenotype and genetic loci, informative polymorphic microsatellite regions were identified. To establish a linkage map, 450 SSLP were screened. Only 75 primers were informative with a minimum coverage of 15.8 cM.
Two hundred and two F1BC animals were phenotyped. Thirty-seven percent (74/202) were found to have LPD upon histopathologic evaluation of their spleens. Of these, 67 of 74 were subjected to a genome-wide linkage analysis. Liver DNA from LPD positive animals was evaluated for these 75 informative SSLP loci. Of the 75 informative loci evaluated in diseased F1BC mice, deviation from expected frequencies were observed at three distinct loci. The p-values as determined by chi-square analysis were all 0.02 or less. These loci are located on mouse chromosomes 14, 18 and 19 (D14Mit160, D18Mit4 and D19Mit6) with deviations toward homozygosity for NZB.

*Candidate Gene Linkage:* To further establish linkage to candidate loci, a region of mouse chromosome 14, approximately 11Mb centromeric to the D14Mit160 location, was sequenced in both the NZB and the DBA/2 strain (Fig 4). There was a point mutation in the NZB in the flanking region 3’ to the stem loop structure of the pre-mir16-1. This mutation was not found in the DBA2 or other mouse strains (tissues/cell lines from C57Bl/6, SJLJ, NZW, Balb/c, NOD/SCID data not shown). This mutation was observed in multiple tissue sources of DNA derived from the NZB as well as DNA from two in vitro cell lines established from NZB, the LNC (an NZB-derived malignant B cell line) and 3C2 (an NZB derived CD8+ CTL line) (Fig 4 and data not shown). In both the NZB sequence and that reported for human CLL, the mutation is in a nearly identical location in the 3’flanking region of mir-16-1 (Fig 4C).

*miR-16 Function Analysis in NZB:* The level of expression of miR-16 was investigated by real-time PCR and Northern analysis of RNA from several tissue sources derived from NZB and compared to the expression in the control C57Bl/6 or DBA/2 strains (Table 3 and Fig 5). NZB
tissue sources of RNA showed a decrease in miR-16 in spleen; however, the NZB kidney was not decreased in miR-16 expression compared to the control strain expression (Table 3). In addition, the NZB derived malignant B cell line, LNC, had an even greater decreased expression of miR-16 compared to C57Bl6 spleen (Fig 5). To study the effects of reconstitution of miR-16, electroporation was used to deliver either miR-16 or control mimic into the NZB line, LNC. Cell cycle and subG0/G1 accumulation were used to monitor the induction of apoptosis by miR-16 (Fig 6). There was an increase in apoptosis following treatment with miR-16 and a decrease in cells in the S-phase. A non-NZB derived B cell line, NJ11107, did not demonstrate any effects following introduction of exogenous miR-16 (data not shown). As a control, both cell lines were transfected with pMax-EGFP vector and similar transfection efficiency was found (approximately 50%).

DISCUSSION

In the present report, genetic loci linked to the development of B cell malignancy was determined by using the NZB, a strain that develops a highly penetrant phenotype of B cell lymphoma and leukemia (http://cancermodels.nci.nih.gov/mmhcc). We have described the phenotypic characterization and genomic analysis of NZB LPD in crosses of NZB and DBA/2 mouse strains. The F1BC mice were derived from F1 crosses of NZB x DBA/2 and the F1 were backcrossed to the NZB parent. Aged F1BC mice were characterized for anemia, serum IgM globulin levels, spleen histopathology, spleen cell DNA content and surface marker expression and a genomic scan was conducted using microsatellite polymorphism typing. Splenic histopathology revealed LPD in 36.6% while splenic aneuploidy was found to be present in 43.2% in the F1BC colony. The majority of the F1BC mice that developed histopathological evidence of LPD were classified as marginal zone lymphomas expressing surface IgM, elevated
levels of CD5 and dim B220. This B cell malignancy is linked to the NZB allele at D14Mit160, D18Mit4 and D19Mit6. In addition, sequence analysis of the mir-16-1 loci located near D14Mit160, demonstrated a point mutation in NZB. This nucleotide change was not present in all other mouse strains analyzed, including the nearest neighbor strain, NZW. Since this nucleotide is non-coding and located in the 3’flanking region of mir-16-1, we examined expression of miR-16 in NZB tissues. Using RT-PCR and Northern analysis, there is reduced expression of miR-16 in NZB spleen and in an in vitro NZB B cell line. Add back experiments involving the miR-16 resulted in increased apoptosis and decreased cell growth. The finding of NZB MZL linkage to D14Mit160 is a new finding and the fact that its human analogue is 13q14 further strengthens the rationale for using the NZB as a model for human CLL.

The presence of LPD and aneuploidy occurred in approximately one third of the F1BC and was not unexpected. We were surprised with the finding of MZL. Based on LPD in the NZB as a model for human CD5+ CLL, one would have expected the spleen to show evidence of a small lymphocytic lymphoma. Although, hyperdiploidy has been previously described in the NZB spleen, human CLL does not typically show evidence of aneuploidy as determined by the flow cytometric analysis of propidium iodide (PI) stained cells (Marti, unpublished observation). However, chromosomal abnormalities are well known in CLL. It would now seem that the MZL histopathology, aneuploidy and surface IgM, CD5 and B200 expression are linked to a region near D14Mit160 (D14Mit160 is approximately 11Mb from mir-16-1).

As noted above, the 13q14 site is frequently involved in human CLL and 13q14 deletions also occur in other closely related human LPD such as mantle cell lymphoma and multiple myeloma. The process of leukemogenesis or lymphomagenesis is a multi-step process as evidenced by the fact that not all tumors in the F1BC were MZL but were rather other B cell
malignancies. In addition, even if individual mice were homozygous for all three loci, we did not always find histopathological evidence of MZL. Conversely mice could be homozygous for two of the loci and still have malignant disease. Therefore there must exist additional genetic loci controlling the expression of LPD in this murine model. In our familial CLL studies we find a variety of other lymphoid and hematological malignancies \(^3\). Of greater interest, we find a significant increase of 13q14 deletions in familial CLL \(^8\) and this thus makes the NZB a model not only for sporadic CLL but for familial CLL. This highlights the fact that additional factors influence the type of B cell malignancy that develops.

There are a number of candidate genes on chromosomes 14, 18 and 19 located near the site of SSLP identified linkage. However, because of the synteny of the mouse chromosome 14 to the human 13q14, this loci was further studied. The significance of the other two loci on mouse chromosomes 18 and 19 remains to be determined. In the present study, identification of loci linked to the development of LPD in the NZB mouse model has been consistent with suggested tumor suppressor loci in human CLL. In particular, the linkage of lymphoproliferative disease with the mouse chromosome 14 (human 13q14) is not unexpected, since up to 50% of cases of CLL have a loss of human 13q14.3 \(^{26}\). Two genes in this region have been shown to be highly conserved between mouse and humans, DLEU2 and RFP2 (also called LEU5) \(^{27}\). Despite the linkage to this particular region of DNA in CLL, no somatic mutations in this region have been detected in CLL patients \(^{28}\) and none were found in the NZB strain of mouse (data not shown). In addition to the presence of DLEU2 and RFP2 in the human 13q14 and the region of synteny in the mouse chromosome 14, there are two microRNAs genes found in the intronic region of DLEU2 in both murine and human, mir-15a and mir-16-1.
miRNAs have been found to play a role in oncogenesis \(^{29}\) and have differential expression in tissues\(^{30}\). Alterations in microRNAs in human B-CLL consist of both deletions and mutations\(^{31}\). Specifically the most frequently deleted genomic region contains the mir-15a and mir-16-1 genes. Recent SNP array studies also found that mir15a/16-1 genes were the targets of the recurrent 13q14 deletions and strengthen the conclusion that these two microRNAs are critical genes for CLL pathogenesis \(^{32}\). Mutations in this region have been reported (reviewed in \(^{31}\)). In the present report, we describe a mutation in NZB in the immediate 3’flanking region of mir-16-1. Analysis of levels of mature miR-16 indicated decreased expression in NZB spleen as well as in the NZB derived malignant B cell line, LNC. The flanking region, containing the mutation, may be important for proper tertiary structure of the stem-loop in the pre-mir \(^{33}\). This is consistent with the finding reported by others of decreased miR-16 in some CLL patients\(^{31}\). One possible outcome of decreased miR-16 is a failure to properly reduce target gene expression. One of the target genes for miR-16 (based on presence of complementary sequences to miR-16 in the target gene 3’UTR) is bcl-2 \(^{34}\). Due to the presence of the mutation, decreased miR-16 may result in increased bcl-2 expression. In both the murine model of CLL and some CLL patients with the disease, increased bcl-2 may lead to a failure to undergo apoptosis which may play a role in disease. To test this possibility, the NZB cell line, LNC, with the mir-16-1 point mutation, was treated with exogenous miR-16 and following treatment, induction of apoptosis was observed. This was not found in other non-NZB B cell lines. This suggests that miR-16 is important in the induction of apoptosis in the murine NZB CLL line. Of note, the mature miR-16 is also encoded by a second transcription unit, mir-15b-mir-16-2 located on chromosome 3\(^{35}\). It remains unclear if this transcription unit might partially compensate for the loss of expression of the mir-16-1 gene.
The genetic linkage of autoimmune disease in the NZB has been studied extensively as a model for SLE \(^{36,37}\), while the loci linked to leukemia and lymphoma have not been determined. Based on our findings, the loci linked to LPD in NZB mice were not linked to previously identified loci linked to autoimmunity. In our present study, we found linkage to chromosomes 14, 18 and 19 in NZB. In other mouse CLL studies, transgenic mice derived by using the human \(TCL1\) sequence (mouse homolog on chromosome 12), have been produced which develop a variety of B cell neoplasias including CLL, follicular lymphomas, Burkitt’s lymphoma and diffuse large cell lymphomas; however, no linkage was found to this gene locus in the present study \(^{38,39}\). It is of interest that a locus involving CD5 B cells in NZB has also been mapped to chromosome 4 but this was not linked to LPD in the present study \(^{40}\) while a locus on chromosome 13 has been found to be linked to marginal zone hyperplasia in NZB mice \(^{41}\).

The results from this present study describe three loci in NZB mice linked to the development of B cell lymphoproliferative disease. The significance of two of these loci located on mouse chromosomes 18 and 19 remains to be determined. The third locus was found to be the site of a point mutation in a microRNA in NZB mice. Alterations in miRNA miR-16 levels may be an important factor in the development of both human CLL and the NZB mouse model since overexpression of miR-16 in an NZB cell line induces apoptosis. The NZB mouse, because it spontaneously develops B lymphoproliferative disease in an environment characterized by anti-self reactivity, can shed insight not only into disease mechanisms but also in the development of therapy targeted at the loci linked to the development of CLL.
References


TABLE 1. Association of NZB phenotype and LPD.

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<th>Phenotype</th>
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<th>Above Cut-off</th>
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<tr>
<td></td>
<td>n</td>
<td>Non-LPD</td>
</tr>
<tr>
<td><strong>Spleen</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mg &gt; 500 mg</td>
<td>74</td>
<td>932±937</td>
</tr>
<tr>
<td>Cells x 10^9/ml</td>
<td>&gt;20,000 Cells x 10^9/ml</td>
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<tr>
<td>WBC</td>
<td>64</td>
<td>17.1±34.0</td>
</tr>
<tr>
<td>RBC</td>
<td>64</td>
<td>5.8±1.8</td>
</tr>
<tr>
<td>MVC</td>
<td>64</td>
<td>53.5±12.2</td>
</tr>
<tr>
<td>IgM</td>
<td>71</td>
<td>577±233</td>
</tr>
<tr>
<td>Percent RBC</td>
<td>70</td>
<td>17.2±24.5</td>
</tr>
<tr>
<td>Reticulocyte</td>
<td>70</td>
<td>17.2±24.5</td>
</tr>
<tr>
<td>Cell Type</td>
<td>Total Mice</td>
<td>Non-LPD Mice</td>
</tr>
<tr>
<td>------------</td>
<td>------------</td>
<td>--------------</td>
</tr>
<tr>
<td><strong>Spleen</strong></td>
<td></td>
<td></td>
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<tr>
<td>Diploid</td>
<td>135</td>
<td>93</td>
</tr>
<tr>
<td>Aneuploid</td>
<td>67</td>
<td>35</td>
</tr>
<tr>
<td><strong>Peritoneum</strong></td>
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<td></td>
</tr>
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<td>85</td>
<td>50</td>
</tr>
<tr>
<td>Aneuploid</td>
<td>90</td>
<td>59</td>
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**TABLE 3**  MicroRNA miR-16 Expression

A. Realtime PCR

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<tr>
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<th>NZB ΔCt</th>
<th>Control ΔCt</th>
<th>NZB ratio RQ#</th>
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<tr>
<td>Spleen</td>
<td>0.97±0.79</td>
<td>-0.08±0.21</td>
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<td>Kidney</td>
<td>-0.08±0.01</td>
<td>0.21±2.08</td>
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B. Northern

<table>
<thead>
<tr>
<th></th>
<th>NZB (fmol)</th>
<th>Control (fmol)</th>
<th>NZB ratio*</th>
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</thead>
<tbody>
<tr>
<td>Spleen</td>
<td>2.23±1.19</td>
<td>4.36±0.25</td>
<td>0.51</td>
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<tr>
<td>Kidney</td>
<td>3.8±2.26</td>
<td>2.88±0.58</td>
<td>1.32</td>
</tr>
</tbody>
</table>

*Real Time PCR analysis for miR-16. Expression of miR-16 in mouse tissue was detected via Real Time PCR. The expression of miR-16 was compared to GAPDH control and the delta (Δ) CT determined (average ± standard error). The relative quantification (RQ) was determined using the ΔΔCT method with a primer efficiency of 90% as reported by the manufacturer and compares the expression in NZB versus control strain, DBA/2 (mice were 5 weeks of age, n = 4).

*Northern Analysis of miR-16. Expression of miR-16 in mouse tissue was detected via Northern blot analysis and data are reported as normalized fmol (normalized to the median of tRNA signals). The results are from two experiments with the control strain C57Bl/6. The NZB expression is the ratio of the normalized miR-16 (fmol) in NZB divided by the normalized miR-16 (fmol) in C57Bl/6.
Figure Legends

Figure 1. Phenotypic Characterization of F1 backcross of NZB x DBA/2. The mice were divided into LPD (right panel) and Non-LPD (left panel) based on splenic histopathology. The above values were ranked ordered in each group based on splenic weight. Data is presented for serum IgM level (IgM), peritoneal cell count, White blood count (WBC), RBC (red blood cell count), MCV (mean corpuscular volume) and percentage of reticulocytes.

Figure 2. Splenic Marginal Zone Lymphoma in the F1BC NZB mice. The left column (A-D) contains low power (5.5x objective) (bar is 5 cm) photomicrographs of selected mice spleens showing the various stages of MZL seen: early marginal zone hyperpasia and the confluence of two or more germinal centers (A and B); moderate and massive infiltration of the red pulp (C and D). The right column (E-H) shows selected areas of involvement at a higher power (43x objective) (bar is 100 microns) for cellular detail.

Figure 3. Flow Cytometric Surface and Cell Cycle Analysis. Twelve representative mice with LPD were examined and their analysis presented in order in panels A-F. The mice are numbered from 1-12 and data from the same animal are shown for both spleen and peritoneal lavage cells (A-D). Panels A and B are the single parameter DNA content histograms for spleen and peritoneal cells. Panels C and D are two parameter IgM versus DNA content (FL2-A versus FL1-H) for spleen and peritoneal cells. Panels E and F show the expression of IgM versus CD5 (FL1-H versus FL2-H) and IgM versus B220 (FL1 versus FL3-H) respectively for spleen cells. Examples of splenic hyperdiploidy can be seen in panel A, mice 2, 4, 8, and 9. Further resolution of these aberrant subpopulations can be better appreciated in the corresponding Panel C.
smaller aneuploid populations seen in mice 11 and 12 can be better appreciated in the two color analysis shown in panel C.

**Figure 4.** Point mutation in 3’ DNA adjacent to pre-mir-16-1 region in NZB. (a) Nucleotide sequence comparison of the region of mouse chromosome (chr) 14 and human chr 13 on which miRNA mir-16-1 is located. The top sequence is the database reference sequence (antisense strand) in *Homo sapiens* for hsa-mir-16-1 with sense strand base coordinates (NCBI36)13:49521099-49521187. The second sequence is the antisense of the database reference sequence in *Mus musculus* for mmu-mir-16-1 with the sense strand base coordinates (NCBIM36)14:60585981-60586067. The sequence homology between the mir-16-1 region (including the 3’ flanking region) of *Mus musculus* (chr 14) and of *Homo sapiens* (chr 13) is shown (vertical arrows indicate the end of the pre-mir-16-1 in humans versus mouse). The third and fourth rows are sequence comparisons of splenic DNA from DBA/2J and NZB/BINJ mice. The sequences are identical to the reference sequence, except for a T→A point mutation (on the antisense strand; A→T point mutation at base 60585990 on the sense strand of chr 14) in the NZB/BINJ. In addition, DNA extracted from DBA/2J (5wks) liver, NZW (5wks) spleen, Balb/C B cell lymphoma cell line (CH27), C57BL/6 (4mos) kidney, SJL/J B cell lymphoma line (NJ117), and NOD SCID (7mos) liver showed no point mutation, whereas DNA from NZB/BINJ spleen, liver (14mos), kidney (15mos), T cell lymphoma line (3C2), and malignant B1 cell line (LNC) all had the same point mutation (data not shown). The precursor stem-loop structure sequences (pre-miRNA) are based on established nomenclature. In the mouse samples (pre-mmu-mir) for both pre-mir-15a (Accession # MI0000564) and pre-mir-16-1(MI0000565) and mature sequences for both mir-15a (MIMAT0000526) and 16-1
(MIMAT0000527) were also compared and no other mutation was found in these regions (data not shown). The shaded boxed region is the mature miR-16 and the unshaded boxed region is the 3’flanking region which is further compared in the lower figure. (b) Sequence comparison between human and mouse 3’adjacent to pre-mir-16-1. The point mutations in NZB/BINJ splenic DNA and in the reported DNA of patients with CLL are indicated. The NZB/BINJ splenic DNA shows an A→T mutation at the 60585984 base on chromosome 14, which is 6 bases from the end of the pre-mmu-mir-16-1 sequence. CLL DNA has a reported G→A mutation at the 49521103 base on chr 13, 7 bases from the pre-hsa-mir-16-1 sequence.

Figure 5. Northern Analysis. The expression of miR-16 was analyzed by Northern blots with RNA from four different tissues obtained from C57Bl/6 or NZB mice and the LNC cell line, a malignant B-1 cell line derived from NZB. The expression of mature miR16 (top gel, lower band) was quantitated and normalized to the expression of tRNA (middle gel). The gel was visualized following staining with ethidium bromide (lower gel). The upper arrow (top gel) indicates the precursor form of miR-16.

Figure 6. Functional Analysis of miR-16. The NZB malignant B cell line, LNC, was transfected with either the miR-16 or control mimics. The cells were harvested 24h post transfection and the cell cycle stages determined by flow cytometric techniques. The columns represent the mean percent change in the NZB B cell line treated with miR16-1 versus control mimic treatment (n=4).
Figure 1
Figure 2
Figure 3
Figure 4

a

Homo sapiens
Mus musculus
DBA/2J
NZB/BINJ

miR-16-1

13:49521110

14:60585990

3’ DNA adjacent to pre-mir 16-1

Chr 14: 60585984
Chr 13: 49521103

DBA/2J
NZB/BINJ (+6 mutation)
Homo sapiens chr 13
CLL DNA (+7 mutation)
Figure 5
Figure 6
Abnormal microRNA-16 locus with synteny to human 13q14 linked to CLL in NZB mice