Cellular and molecular biological studies of a retroviral induced lymphoma transmitted via breast milk in a mouse model

Hussein Saeed Bagalb
Medical University of Ohio

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# Health Science Campus

## FINAL APPROVAL OF THESIS
Master of Science in Biomedical Sciences

Cellular and Molecular Biological Studies of a Retroviral Induced Lymphoma, Transmitted via Breast Milk in a Mouse Model

Submitted by:
Hussein Bagalb

In partial fulfillment of the requirements for the degree of Master of Science in Biomedical Sciences

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<th>Examination Committee</th>
<th>Signature/Date</th>
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<tr>
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Senior Associate Dean
College of Graduate Studies
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Date of Defense: September 15, 2008
Cellular and Molecular Biological Studies of a Retroviral Induced Lymphoma, Transmitted via Breast Milk in a Mouse Model

By

HUSSEIN SAEED BAGALB

Master of Science in Biomedical Science

Departments of Medicine and Physiology/Pharmacology

College of Medicine
University of Toledo
2008
Dedication

In the name of God, the Compassionate and Merciful

I dedicate this thesis to my family, especially…

to my father and my dear mother for instilling the importance of hard work and higher education;

to my lovely wife for her patience and understanding;

to my wonderful son abdullah and to my expecting baby.

May this work serve as an inspiration to my children as they strive to reach their goals.
I would like to thank my major advisor, Dr. Joana Chakraborty for her advice and guidance, I would also like to thank my committee members, Dr. Joan Duggan and Dr. Sonia Najjar for taking the time to review this work and for their instruction. Also, I would like to thank the in charge of Dr. Chakraborty laboratory, Henry Oknta, for all help and support.
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INTRODUCTION

The HIV pandemic remains the most serious of infectious disease challenges to public health. In 2007, worldwide an estimated 15.4 million total number of women are living with Human Immunodeficiency virus (HIV). An average of 420,000 children are newly infected with HIV annually (WHO/UNAIDS 2007). Mother to child transmission (MTCT) of HIV is a major route of HIV acquisition in children. Although treatment of the infected pregnant mothers with anti-retroviral therapy (ART) has limited MTCT of HIV-1 in developed countries (Connor et al., 1994), in Africa and other developing countries it still remains a significant source of infection. Due to practical and ethical constraints involving human subjects, the mechanism of perinatal transmission of HIV-1 is not yet fully understood. Therefore, a suitable animal model may provide an alternative solution. Our lab has successfully developed a murine model for mother-to-pups transmission of MoMuLV-ts-1 retrovirus. It has been demonstrated that the transmission of ts-1 can occur in utero, intrapartum and/or postpartum. Postpartum transmission through breast milk can produce an immunodeficiency state in mice and development of lymphoma in some pups, mimicking HIV infection in humans (Clark et al., 2001; Duggan et al., 2004; Duggan et al., 2006).

(Chakraborty et al., 2008) have found that ts-1 provirus integration leads to an over expression of four genes associated with lymphoma in BALB/c mice. This
model will allow us to demonstrate ts-1 retrovirus transmission via breast milk and the molecular mechanism of the lymphoma development through natural transmission of a retrovirus to the offspring.

**Objective/Hypothesis:**

The following hypotheses have been tested during the current investigation:

Alteration in the expression of specific genes in the ts1 murine model after provirus integration may contribute to lymphomagensis and may be associated with distinct types of lymphoma.

**Specific Aims**

- **Specific aim 1:**
  Grow the virus, setup the experiment, infect the animals, perform PCR assay to detect infectivity and to obtain lymphomas.

- **Specific aim 2:**
  Identify the phenotypic of lymphoma in ts1 murine model.

- **Specific aim 3:**
  Analysis of the gene expression near to the ts1 provirus integration site and distinguish the variation relative to route of transmission.
Tumor induction by retroviruses

Cancer is the result of a series of genetic alterations each providing a selective advantage to the cell at a specific stage of tumorigenesis. The first discovery of retroviruses associated with cancer was reported in early 20th century when an avian erythroblastosis virus (AEV) was isolated from spontaneous erythro-leukemia in a chicken (Ellerman and Bang, 1908). And shortly after, Peyton Rous demonstrated that chicken sarcomas were infectious and can induce tumor when transmitted into healthy birds (Rous, 1910). This was followed by many other researcher who were able to isolate retroviruses from rodent, cats, cow and other animals. In human, the human T-lymphotropic virus type 1 (HTLV-1) was the first retrovirus isolated in 1980 (Poiesz et al., 1980), and has been shown to induce adult T-cell leukemia (ATL)(Barmak et al., 2003).

Retroviruses are classified into seven genera as shown in Table I. Oncogenic retroviruses (retroviruses that induce tumorigenesis) belong to one of the first five groups of these genera (Goff, 2007):
Table I: Retrovirus genera

<table>
<thead>
<tr>
<th>Genus</th>
<th>Example</th>
<th>Virion Morphology</th>
<th>Genome</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Alpharetrovirus</td>
<td>Rous sarcoma virus (RSV)</td>
<td>central, spherical core (C type)</td>
<td>simple</td>
</tr>
<tr>
<td>2. Betaretrovirus</td>
<td>Mouse mammary tumor virus (MMTV)</td>
<td>eccentric, spherical core (B, D-type)</td>
<td>simple</td>
</tr>
<tr>
<td>3. Gammaretrovirus</td>
<td>Moloney murine leukemia virus (Mo-MLV)</td>
<td>central, spherical core (C type)</td>
<td>simple</td>
</tr>
<tr>
<td>4. Deltaretrovirus</td>
<td>Human T-cell leukemia virus (HTLV)</td>
<td>central, spherical core (C type)</td>
<td>complex</td>
</tr>
<tr>
<td>5. Epsilonretrovirus</td>
<td>Walleye dermal sarcoma virus</td>
<td>central, spherical core (C type)</td>
<td>simple</td>
</tr>
<tr>
<td>6. Lentivirus</td>
<td>human immunodeficiency virus (HIV)</td>
<td>Cone-shaped core</td>
<td>complex</td>
</tr>
<tr>
<td>7. Spumavirus</td>
<td>human foamy virus</td>
<td>Immature</td>
<td>complex</td>
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The general mechanism by which oncogenic retroviruses induce tumor can be divided into two classes: acute and slow transforming viruses. Acute transforming retroviruses induce polyclonal tumours within 2 to 3 weeks after infection of the host. These retroviruses induce tumors through acquisition and expression of cellular proto-oncogenes that have been captured by the virus (Figure1) (Uren et al., 2005). An example is v-Abl in the Abelson Murine leukaemia Virus (Shore et al., 2002).
In contrast, slow transforming retroviruses induce mono- or oligo-clonal tumours with a longer latency of several months. These types of retroviruses do not carry viral oncogenes and can cause tumors by activating cellular proto-oncogenes close to the proviral DNA integration site on the host genome (Figure 2).
Elements in the proviral genome that regulate the viral transcript also act in *cis* on cellular gene transcripts. Depending on whether the provirus integrates into or in the vicinity of genes, these elements can enhance or disrupt normal transcription and thus induce oncogenic mutations. This class of retroviruses have been found to induce tumors in many animals. i.e. type of virus in birds (ALV and REV) and murine leukemia virus (MLV) in mice (Uren et al., 2005). In addition to these two general transformation classes, a small number of retroviruses induce tumors by expression of their own oncogenic protein. For example, human T-cell leukemia virus types 1 and 2 (HTLV-1 and HTLV-2, respectively) induce adult T-cell immortalization and leukemia in human by expression of viral Tax protein. Tax has no cellular homologue and it works in trans to disrupt cellular checkpoints and destabilize genome integrity (Jeang and Gatignol, 1994). leading to transformations that directly cause human cancer (Jeang, 2005).

In AIDS-related lymphoma, patient studies indicated that oncogene activation by insertional mutagenesis might be another mechanism by which HIV-1 can induce cancer (Herndier et al., 1992; Shiramizu et al., 1994). The release of the complete mouse genome sequence and the availability of reliable methods for isolation of proviral flanks has introduced the retroviral insertion mutagenesis screen in mice as a powerful procedure to identify genes contributing to tumorigenesis. Many oncogenes identified in these screens have given a valuable basis for better understanding the development of human cancer (Uren et al., 2005).
The moloney murine leukemia virus, because it utilizes a slow transformation mechanism to induce leukemia or lymphoma in mice, is one of the retroviruses that provide an excellent model to identify and study the oncogenes involved in retrovirus-induced tumorigenesis.

**Moloney Murine Leukemia Virus (Mo-MuLV)**

Mo-MuLV was first discovered by Moloney in 1960. Mo-MuLV is a slow transforming retroviruses which induces T-cell lymphoma within 3 to 4 months when inoculated in mice (Fan, 1997). The mechanism by which Mo-MuLVs lead to cell transformation is by activation of cellular proto-oncogenes or inactivation of tumor suppressor genes. This is a result of the proviral DNA integration site on the host genome (Uren et al., 2005). The Mo-MuLV retrovirus consists of single positive strand of RNA coated with an envelope. It belongs to the Gamma-retrovirus genus of the Retroviridae family as seen in Table 1. Mo-MuLV is classified as simple retroviruses which carries only three genes (\textit{gag}, \textit{pol}, and \textit{env}). Gag genes are group specific antigens which encode viral capsid protein matrix (MA), capsid (CA) and nucleocapsid (NC). The pol or polymerase genes encode viral protease enzymes (PR) essential for the cleavage of gag proteins during maturation, reverse transcriptase (RT) and integrase (IN), the viral enzyme responsible for provirus integration into the host genome, and env genes for envelop which encode the surface protein (SU) and the transmembrane protein (TM) (Coffin et al., 1997).
Assembly and structure of Mo-MuLV

The assembly site of Mo-MuLV occurs at the plasma membrane of the infected cells. Assembly starts when the Gag, Gag-Pol-precursors and Env protein are synthesized. They accumulate together to form of progeny virions. After budding from the cells, the released virion particles have a spherical shape and are about 100-120 nm in diameter. They are immature and they undergo maturation steps which are essential for viruses infectivity. On maturation, the precursor proteins in the immature particles are cleaved by viral protease mediated processing of Gag-and Gag-Pol-precursors. The Gag protein, is both necessary and sufficient to mediate the assembly and release of immature virus particles. Most gag proteins are processed to generate the matrix protein (MA), the capsid protein (CA) and the nucleocapsid protein (NC). The MA proteins are closely associated with the lipid of the envelope as peripheral membrane proteins. The CA forms the virion core, and the NC is associated with the viral RNA genome. At the same time that Gag-precursors are cleaved, the Pro and Pol region of the Gag-Pro-Pol precursor are also cleaved. The Pol is processed to form the viral protease enzymes (PR), reverse transcriptase (RT) and integrase (IN). Cleavage of Gag precursors also yields a P12-protein which is necessary for late stages of assembly and release of virus (Goff, 2007). P12 may also play a role during reverse transcription and integration of viral DNA into the host genome (Yuan et al., 2002). The proteolytic cleavage of the env-protein occurs during its transport to the assembly site by the host protease. The cleavage forms two subunits: the
surface part (SU) and transmembrane part (TM). The SU is the outer envelope of glycoprotein that is responsible for major receptor binding to recognize the tropism- cells. The TM subunit, anchored in the virion envelope and holds the SU subunit, and also plays a major role in membrane fusion. The length of each monomer in the RNA viral genome is about 7-12 kb in size, which is maintained in the middle of the particles (Figure 3) (Goff, 2007).

Figure 3: Mo-MuLV Structure
The Mo-MuLV Life Cycle

The life cycle initiates with the specific binding of SU while is the viral glycoprotein envelope, to specific surface receptors on the appropriate target cell. The receptor used by ecotropic Mo-MuLV (viruses that only infect mouse cells) is the cationic amino acids transporter mCAT-1 (Albritton et al., 1989). The receptor for amphotropic Mo-MuLV (viruses that infect both mouse and non mouse cells) is a type III sodium-dependent phosphate transporter called PiT2 (Feldman et al., 2004). After binding occurs the fusion of the viral envelop with the cellular plasma membrane allows the entry of the retrovirus into the host cell. In Mo-MuLV, fusion mechanism is controlled by the isomerization of a disulphide bond that binds the the SU- and TM-subunit together. Receptor binding induces rearrangement. Protein destabilization of the inter-subunit disulfide within the motif in SU leads to the release of SU from TM, which facilitates fusion (Wallin et al., 2004). Fusion will release the viral core into the infected cell that contains two single stranded identical genomic RNA positive strands, reverse transcriptase (RT), structural proteins, viral integrase (IN) and specific tRNAs. After entry, the genomic RNA is reverse transcribed into DNA (-) strands by the RT protein utilizing a tRNA that anneals to the viral RNA genome. tRNA anneals at a complementary sequence known as primer binding site (PBS) downstream of the 5’ long terminal repeat (LTR) as shown in Figure 4a. Synthesis of minus strand DNA occurs toward the 5’ end of the RNA to generate U5 and a repeated sequence of the R region which is present on the LTR at both the 5’ and 3’ of the
viral RNA. The product of this step is known as minus strand strong stop DNA (Haseltine et al., 1976). The RNA complementary to the newly synthesized DNA is degraded by the ribonuclease H (RNase H) activity of RT (Figure 4b). Next, the resulting minus-strand DNA performs translocation or jumps to the 3’ end of the viral RNA and anneal to the homologous R region (Figure 4c). Thereafter, reverse transcription of the 3’ part of the viral RNA begins and continues to elongate the minus strand strong stop DNA to form long minus strand DNA. RNase H activity subsequently degrades the minus RNA strand (Figure 4d). Mo-MuLVs, as well as other retroviruses, contain a polypurine tract (PPT) that is resistant to the activity of RNase H. This sequence is located upstream from the 3’ LTR RNA that cannot be degraded. The non-degraded RNA serves as primer for the synthesis of the plus stranded DNA (Figure 4 e & f). Then, a circular intermediate is formed until elongation of the minus strand DNA is completed (Figure 4g), this results in the formation of a double-stranded linear viral DNA containing a protein-DNA complex called the preintegration complex (PIC) (Goff, 2007) (Bowerman et al., 1989). Next, PIC enters the nucleus and is required to wait until the mitosis stage of cell division for nuclear translocation (Lewis and Emerman, 1994). After nuclear translocation, IN mediates the insertion of linear DNA into the host chromosomal DNA to form a provirus (Goff, 2007).
Figure 4: Steps of Reverse Transcription of Mo-MuLV RNA genome

A retrovirus-specific cellular tRNA binds to the primer binding site region (PBS) of the plus sense RNA genome, after binding a minus strand DNA synthesis start and proceeds to the 5' end of viral genome RNA using the viral reverse transcriptase (RT) (a). Following RNase H degradation of r and u5 regions of the complementary RNA (b). The minus ssDNA transfer and anneal to the complementary 3' end of genomic RNA and reverse transcription of the 3' part of the viral genome start (c), accompanied by RNase H digestion of the genomic U3 and R region, reverse transcription continues through to the PBS site yielding the -ssDNA(d). After completing of the –ssDNA most viral RNA is removed by RNase H except the the ppt (polypurine tract) persists and primes plus strand DNA synthesis until part of the tRNA is copied (e) the tRNA primer is removed (f). The annealing of PBS region of the second strand hybridizes with the PBS region of the first strand will allow extension on both Plus and minus strand DNA using each other as a template(g).
The proviral DNA structure has two LTRs sequences located at both ends (Figure 5) with each LTR divided into U3, R and U5 regions. The U3 region contains the promoter and enhancer elements required for initiation of provirus transcription. Transcription starts at the U3-R boundary, and RNA cleavage/polyadenylation occurs at the R-U5. The integrated provirus serves as a template for the production of viral RNA and proteins (Fan, 1997). The provirus is transcribed by cellular RNA polymerase II into: i) a spliced mRNA for translation of the env-protein, ii) a genomic RNA, and iii) a mRNA for translation of the Gag-precursor and Gag-Pol-precursor which are assembled into viral cores of the immature virion that bud through the plasma membrane of infected cells (Goff, 2007).

Figure 5: The provirus contains two long terminal repeats (LTRs). LTR can subdivided into three reagions: U3, R and U5 contains the enhancer and promoter sequences. The R domains encode the 5’ capping sequences and the polyA (pA) signal. Gag, Pol and env encode the viral components required for assembly of viral particles. The splice donor (SD) and splice acceptor (SA) necessary for the formation of the subgenomic mRNA that encodes different viral proteins. (Fan, 1997)
Mechanisms of Retroviral Insertional Mutagenesis

The integrated provirus mediated alteration of a cellular gene which may contribute to tumorigenesis can be divided into two groups: activation of proto-oncogenes and inactivation of tumor suppressor genes. The ability of an integrated provirus to alter a cellular gene transcription depends on the proviral elements that are involved in the transcriptional regulation of the viral DNA present in the LTRs of the provirus at each end (Mikkers and Berns, 2003).

Activation of cellular gene expression by proviral insertions can be achieved by several mechanisms. Example, the promoter insertion, when the provirus integrates in the promoter region 5’of a target gene in the same transcriptional orientation, that will separate the gene from its cellular promoter and it may activate the gene through the viral promoters presents in the 5’ or 3’LTR (Mikkers and Berns, 2003). This has first seen in the avian leukosis virus (ALV) provirus where integrates adjacent to c-myc gene and can increase expression that leads to lymphoid leukosis (Hayward et al., 1981).

When the 5’ LTR promoter is used to initiate transcription of the downstream cellular gene it usually results in the formation of chimeric transcripts containing both viral and cellular sequences (Voronova et al., 1987). Gene activation by promoter insertion at 3’ LTR is frequently associated with deletion of the 5’ LTR suggesting that removal of the 5’ LTR promotes 3’ LTR driven transcription of the cellular gene (Westaway et al., 1984). The viral enhancer of integrated provirus is
another example of a mechanism by which provirus insertion activates cellular genes. This is probably the major mechanism of gene activation by retroviral insertional mutagenesis. The activity of enhancer insertion can act over large distances. Enhancer insertion involves integration of a provirus either at the 5’ end of a gene in the reverse transcriptional orientation or at the 3’ in the same transcriptional orientation (Cuypers et al., 1984; Nusse et al., 1984; Selten et al., 1985; Uren et al., 2005; West et al., 2005).

Also viral integration can occur within the transcription unit of the cellular gene (intragenic integration) and may disrupts coding domains of the protein sequence that lead to inactivated or mutated expression of target genes. For instance, the tumor suppressor genes P53 and Nf1 were inactivated by viral integration. In some cases intragenic insertion may also result in expression of truncated protein; for example, proviral insertion in c-Myb results in expression of a truncated protein (Ben David et al., 1988; Buchberg et al., 1990; Mukhopadhyaya and Wolff, 1992)
**HIV as a retrovirus**

**Classification of HIV**

The human immunodeficiency virus (HIV) is an enveloped RNA virus belonging to the Lentivirus subfamily of Retroviruses, and is a sexually transmitted pathogenic retrovirus. It can be divided into two types. HIV-1 is the predominant strain of the current pandemic, and it begins showing symptoms within 5 years of infection. HIV-2 is localized mainly in West Africa, and the clinical manifestations of HIV-2 are similar to those of HIV-1 but progression is typically slower than HIV-1. In 1983-84 HIV was identified as the causative agent of the acquired immunodeficiency syndrome (AIDS). HIV-1 is classified into three sub-groups based on the sequences of *gag* and *env* genes: group O (outliers), group M (majority), and group N (non-M/O). Within group M there are 10 clades, classified form A through J. HIV subtyping may allow tracking of the epidemic to a certain extent. Clade B is the most common in North America and Western Europe, B and F is prevalent in Brazil, E is prevalent in South Eastern Asia, and A, C, D and E is localized to the developing world. The HIV targets the host immune system, making it a very difficult pathogen for the human body to fight. In addition to making the host highly susceptible to secondary infections, rapid mutation rates within the viral genome makes vaccine and drug development difficult (Lythgo, 2004).
**Viral Structure**

The HIV virion contains a genome composed of two copies of positive single-stranded RNA. As a retrovirus, HIV codes for reverse transcriptase, and is thus able to produce DNA from its native RNA. The mature virion is composed of a central core surrounded by a spherical lipid envelope acquired by budding from the surface of an infected cell. The core contains reverse transcriptase, integrase and protease in association with two strands of RNA. HIV has a long genome with at least 9 genes: two regulatory genes called tat and rev, four accessory genes called vif, vpu, vpr, nef, and three structural genes. The structural genes are:

I. gag (inner core polypeptides): p17, p24, p7, p9

II. pol (viral enzymes): enzymes – reverse transcriptase, protease, integrase

III. env (envelop proteins) – gp 120, gp 41

Figure 6: Schematic diagram of HIV-1 Structure
**Viral Entry**

In order for HIV to infect a cell, HIV must be able to transfer its genome to the targeted cells across both the viral and cellular membranes. Under the right conditions, specific integral membrane proteins found on the surface undergo conformational changes that lead the viral and cellular membranes to fuse with one another, providing a portal of entry (Hernandez et al., 1996). The entry process can be divided into three components: attachment of the virus to the target cell surface after recognition and binding by a specific receptor on the target cell surface; binding event which induces the viral fusion protein to undergo conformational changes; and the membrane fusion reaction itself. Viral tropism is restrictive to presence or absence of the receptor type on the target cell surface (Doms and Trono, 2000).

**First contact**

The Env protein in HIV-1 is responsible for mediating viral attachment and membrane fusion. Transcribed as a single polypeptide precursor that forms trimers, env-precursor is subsequently cleaved by a cellular protease to generate two noncovalently associated subunits, gp120 and gp41. The gp120 binds virus to the cell surface, whereas the membrane-spanning gp41 subunit is largely responsible for membrane fusion (Wyatt and Sodroski, 1998). The primary receptor for HIV-1 is CD4, which is found on the cell surface of two cells which play a major role in Immune system (Th cells and macrophages). Therefore, infection of these cells leads to immune dysfunction (Doms and Trono, 2000).
Coreceptor binding

Binding of gp120 to CD4 alone can not fuse the membrane, it also need a second receptor (co-receptor). After CD4/ gp120 binding conformational changes within gp120 occur which enable it to bind to the co-receptor. This binding event leads to membrane fusion. The second receptor is a chemokine receptor, either CXCR4 or CCR5 depending on the HIV strain (Lythgo, 2004).

Fusion

After co-receptor triggering, the hydrophobic amino-terminal fusion peptide gp41 undergoes conformational changes, which lead it to be exposed. It interacts with the membrane of the target cell through the formation of a triple-stranded coiled-coil, effectively bridging the two membranes. The coiled-coil structure has one amino-terminal leucine/ isoleucine heptad repeat domain from each env subunit (Figure 7). It contains hydrophobic grooves into which the carboxy terminal heptad repeat regions of each gp41 subunit attach, forming a six-helix bundle (Chan et al., 1997).
Figure 7: Schematic drawing of attachment process of HIV to the target cell

“Model for HIV-1 entry. Binding of CD4 to gp120 results in exposure of a conserved coreceptor (CoR) binding site in gp120, perhaps by movement of the V3 and V1/2 loops. Coreceptor binding causes the fusion peptide of gp41 to be exposed and inserted into the membrane of the target cell in a triple-stranded coiled-coil. Formation of a helical hairpin structure in which gp41 folds back on itself is coincident with membrane fusion. The bottom portion of the figure displays gp41 alone. Addition of the T20 peptide blocks membrane fusion by preventing the formation of the hairpin structure” (Doms and Trono, 2000).
**Life Cycle**

After the virus envelope fuses with the target cell membrane, the viral capsid gets into the cell. Immediately after entry, viral reverse transcriptase (RT) transcribes the viral genome into cDNA (Figure 8), using a cellular tRNA molecule as a primer (Moore and Chaisson, 1999). The only (+) sense RNA virus whose RNA genome does not serve as mRNA is HIV. The RTase’s nuclease activity degrades the viral RNA template, and the cDNA travels to the nucleus. Nuclear entry is mediated by Vpr and Vif accessory proteins, and also by nuclear localization signals within the Vpr and p17 matrix sequences. Viral integrase proteins insert the viral genome into the host’s chromosomal DNA (Garcia and Gaynor, 1994). The HIV RNA genome contains 9 open reading frames and is about 9 kb in length. After integration, cellular factors mediate transcription of viral transcription factors located on the pol or env genes. A protein called Tat increases transcription rate (Cladera et al., 2001). Upon transcription of the env gene, the Rev (a regulatory protein) binds a specific region on both spliced RNA (used for structural components) and un-spliced RNA (used for genome packaging) and mediates env’s export from the nucleus (Lythgo, 2004). Viral structural components are located on the gag gene, and are initially transcribed and translated in the cytoplasm into a pr55 pre-protein, which is cleaved upon maturation of the virus. A gag-pol precursor, also translated in the cytoplasm, yields the viral protease, integrase, and reverse transcriptase. Both the gag and gag pol genes contain 2 different reading frames which are differentially expressed,
favoring transcription of structural products over enzymatic ones (Jacks et al., 1988). The mRNA of the *env* gene, which codes for viral surface glycoproteins, is translated in the ER to yield a gp160 pre-protein. This precursor travels to the cell membrane where it remains in a non-covalent trimer. During virion formation, the gp160 polyprotein is cleaved into gp120 and gp41, and also yields 3 regulatory proteins. *Vpr* is involved in the transport of cDNA into the nucleus, *Vpu* is involved in virion assembly, and *Vif* is important in maintaining infection efficiency (Emerman and Malim, 1998).

Figure 8: The life cycle of HIV and the mechanism of infection.

AIDS-defining illnesses

The most frequent diseases that are associated with patients who have a weakened immune system referred to AIDS-defining illnesses, and generally fall under four categories, including opportunistic infections, diseases affecting the central and peripheral nervous systems, malignancies and wasting syndrome. One of the most common malignancies associated with human immunodeficiency virus infection is non-Hodgkin’s lymphoma (NHL).

HIV-1 Associated Non-Hodgkin’s Lymphomas (NHL)

Prevalence

The incidence rate of non-Hodgkin's lymphomas (NHL) among HIV patients significantly increased between 1980-1985. The U.S. Centers for Disease Control and Prevention's (CDC) considered NHL as an additional manifestation of AIDS in 1985 (Harnly et al., 1988). Prior to the use of effective antiretroviral therapy, the percentage of HIV-1-associated lymphomas was 3 to 4% of all AIDS-defining illnesses reported to the CDC (Moore et al., 1991). In 1992, review study suggest that HIV-1-associated lymphomas in USA were estimated between 8-27% of approximately 36,000 newly diagnosed cases of lymphoma (Gail et al., 1991). Without effective antiretroviral therapy, it is estimated that 5-10% of all HIV-infected individuals will have lymphoma as either an initial or subsequent AIDS-defining condition (Hamilton-Dutoit et al., 1991). The risk of NHL among HIV patients has increased nearly 200 fold and accounts for a greater percentage
of AIDS defining illness in the US and Europe since the advent of HAART therapy (Wood and Harrington, 2005). Therefore, HIV-1-associated lymphomas represent a significant clinical entity within the spectrum of HIV-1-associated illnesses.

Clinical and histological classification

The immunophenotype of the malignant cells in approximately 95% of HIV-1-associated lymphomas are considered to be of B-cell origin. In a review study done in 1991, approximately 80% from total 2,500 cases of HIV-1-associated lymphomas developed systemic lymphomas and 20% arose in the central nervous system (CNS) (Baumgartner et al., 1990; Beral et al., 1991).

HIV-1-associated lymphomas can be categorized into two groups; Lymphomas can occur in presence or absence of HIV. The vast majority of these lymphomas are high grade B-cell lymphoma (Figure 9), Lymphomas occur specifically in HIV positive patients and include two rare entities: primary effusion lymphoma and plasmablastic lymphoma of the oral cavity (Carbone and Gloghini, 2005).
Biological research on AIDS-NHL highlights two factors that can contribute to the development of NHL heterogeneous features. First, the level of the host’s immune deficiency may select the type of AIDS-related lymphoma that will develop in a patient. Second, the different clinicopathologic variants of AIDS-NHL derive from distinct B-cell subsets and may be associated with different pathogenetic pathways (Carbone, 2002).

**Diffuse Large B-Cell Lymphomas (DLBCL)**

The major type of NHL seen in AIDS patients are DLBCL. It is more common and more aggressive in AIDS patients compared to immune-competent individuals. DLBCL could be further divided into the centroblastic and immunoblastic...
subtype. The risk for AIDS- centroblastic and immunoblastic lymphoma increases substantially as immune function decreases, and affected patients display a more severe immunodeficiency compared with AIDS-Burkitt Lymphoma (BL) patients. The molecular pathogenesis of systemic AIDS-centroblastic and immunoblastic lymphoma is complex and more heterogeneous than that of AIDS-BL (Table II) (Carbone and Gloghini, 2005). Previous studies of gene expression indicated that AIDS-DLPCL is associated with over expression of the proto-oncogene T-cell leukemia 1 \((TCL-1)\) (Teitell et al., 1999).

Table II: Immunological status and genetic lesions in AIDS-NHL

<table>
<thead>
<tr>
<th>Histology</th>
<th>Host’s immunodeficiency</th>
<th>EBV (%)</th>
<th>HHV8 (%)</th>
<th>c-MYC (%)</th>
<th>BCL6 rearrangement (%)</th>
<th>P53 (%)</th>
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<td></td>
<td></td>
<td>Infection</td>
<td>LMP1 status</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AIDS-BL</td>
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<td>30</td>
<td>–</td>
<td>100</td>
<td>–</td>
<td>60</td>
</tr>
<tr>
<td>AIDS-CB</td>
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<td>40</td>
<td>–</td>
<td>–</td>
<td>20</td>
<td>Rare</td>
</tr>
<tr>
<td>AIDS-IBL</td>
<td>Marked</td>
<td>90</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>Rare</td>
</tr>
<tr>
<td>AIDS PCNSL</td>
<td>Marked</td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>AIDS-CB</td>
<td>Marked</td>
<td>100</td>
<td>–</td>
<td>–</td>
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<td>nd</td>
</tr>
<tr>
<td>AIDS-IBL</td>
<td>Marked</td>
<td>100</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>nd</td>
</tr>
<tr>
<td>AIDS PEL</td>
<td>Marked</td>
<td>90</td>
<td>–</td>
<td>100</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

AIDS-BL, AIDS-related Burkitt’s lymphoma; AIDS-CB, AIDS-related centroblastic lymphoma; AIDS-IBL, AIDS-related immunoblastic lymphoma; AIDS-PCNSL, AIDS-related primary central nervous lymphoma; AIDS-PEL, AIDS-related primary effusion lymphoma. -, absent infection or expression; in case of positive infection or expression, the percentage of positive cases is indicated. (Carbone and Gloghini, 2005)
**Molecular Features**

The pathologic heterogeneity of AIDS-NHL is correlated with the heterogeneity of the molecular pathway associated with these lymphomas. The molecular pathway associated with AIDS-BL involve activation of \( c\)-MYC and disruption of \( p53 \), and less frequent infection by Epstein-Barr virus (EBV) with failure to express the viral transforming antigens of the latent membrane protein 1 (LMP-1). EBV infection occurs in 40% of AIDS-CB cases and in 90% of AIDS-IBL cases. Rearrangements of \( BCL-6 \) are detected in 20% of AIDS-CB cases (Table II). In the presence of EBV infection, BCL-6 expressing AIDS-CB fails to express LMP1 antigen. In contrast, Expression of LMP1 is found in AIDS-IBL with absent BCL-6 expression. Consistently, the molecular pathways of viral infection and lesions of cancer-related genes associated with AIDS-NHL vary substantially in different clinicopathologic categories of the disease. The biological heterogeneity of AIDS NHL is highlighted by their histogenetic differences, because AIDS NHL is related to distinct B cell subsets (i.e., germinal center [GC] or post-GC B cells). The B-cell within the GC display the BCL-6+ and CD138/syndecan-1(syn-1) phenotype, whereas B-cell that have exited the GC and have undergone further maturation toward the plasma cell stage exhibit the BCL-6−/syn-1+ phenotype. The phenotypic pattern of AIDS-BL and systemic AIDS-CB closely reflects B cells residing in the GC. Conversely, the phenotype of AIDS-IBL, is either systemic or localized primarily to the central nervous system, and AIDS-PEL reflects post-GC B cells in all cases (Figure10) (Carbone, 2002).
Figure 10: A model for the histogenesis of AIDS-NHL and its linking with the molecular pathways.

“The model is derived from the expression profile of BCL-6 and CD138/syn-1 throughout physiologic B cell maturation. B cells within the GC display the BCL-6+/syn-1− phenotype, whereas B cells that have exited the GC and have undergone further maturation toward the plasma cell stage exhibit the BCL-6−/syn-1+ phenotype” (Carbone, 2002)
**Mother to Child Transmission of HIV**

Globally the number of children (less than 15 years old) living with Human immunodeficiency virus-1 (HIV-1) infection reached 2.5 million, with approximately 420,000 new infections among children reported in 2007((UNAIDS) and (WHO), 2007). It has been estimated that most HIV-1 infections cases in children results from transmission of HIV-1 from mother-to-child (MTCT), which can occur in utero (during pregnancy), intrapartum (labor/delivery) or postpartum through breast milk (Newell, 1998). The prevalence of mother-to-child transmission of HIV is about 25-40% overall and studies indicated that 60-70% of theses HIV infections in infants occur through intrapartum routes and in utero route accounts for 20-30% (Thorne and Newell, 2003). The additional risk of acquiring HIV infection through breast-feeding is approximately 14%(Dunn and Newell, 1992), and some studies indicate that avoidance of breast-feeding can decrease the risk of mother-to-child transmission of HIV by 44% (Nduati et al., 2000). Exclusive breast-feeding for limited time has been found to be less associated with HIV transmission and it improves child survival more than other forms of infant feeding (Coovadia et al., 2007).

**Breastfeeding**

Human breast milk provides an ideal source of nutrition to the infant compared with commercially prepared formula. In addition to the most valuable nutrition that breastfeeding provides, human milk also provide protection against a
number of infectious diseases by specific immunological components (antibodies, T cells, B cells, accessory cells) and non-specific factors (e.g. complement, lactoferrin, properdin, glycoconjugates), and other factors thought to be inhibitory to the gp 120-CD4 binding process, but this does not appear to prevent transmission through breast feeding (Newell, 1998; Nicoll et al., 1995; Rassin DK et al., 2001). In 1992 the World Health Organization, recommended women with HIV who lived in areas where the main cause of infant mortality is not infectious disease, and the rate is low, to use a safe alternative to breast feeding. However, where the opposite is the case and mortality is caused mainly by infectious disease or malnutrition, women should be advised to breast feed even if they have HIV (Newell, 1998). Studies reported that higher MTCT of HIV-1 has been associated with mixed feeding (breastfeeding and formula-feeding) than either alone, possibly because the gastrointestinal mucosa is being inflamed by contaminated formula, thus compromising mucosal barriers to HIV entry (Coutsoudis et al., 1999; Tess et al., 1998). HIV-1 has been isolated in both the cellular and non-cellular parts of breast milk colostrum (Thiry et al., 1985). It is unknown whether infection occurs through cell-free HIV-1 in breastmilk or through HIV-infected cells. Cell-free viruses can directly enter into the bloodstream via mucosal breaches, or could penetrate the mucosal lining of the gastrointestinal tract of infants thus infecting cells. If HIV infection occurs through cell-associated virus, colostral milk may be more infectious because of its high cellular content, although the other components of colostrum may have a protective effect (Tess BT
et al., 1997). The immature gastrointestinal tract of the newborn may facilitate transmission (Datta et al., 1994).

Risk factors

Maternal viral load has been found to be the most important risk factor correlated with transmission (Fang et al., 1995; Mock et al., 1999). Weak immune function status of the mother as determined by low CD4+ counts, especially those with vitamin A deficiency is associated with higher rates of transmission (Nduati et al., 1995), unprotected sexual intercourse and injection of drugs during pregnancy may also increase the risk of mother-to-child HIV-1 transmission (Bulterys et al., 1997). The mother and child Human Leukocyte Antigen (HLA) concordance and maternal HLA homozygosity play a role in increasing risk of the vertical transmission of HIV-1 (mackelprang et al., 2008). Previous study data suggest that low concentration of alpa-defensins in the breast milk is associated with high rate of the transmission (Kuhn et al., 2005). Zidovudine reduces the risk of maternal-infant HIV transmission by approximately two thirds when it is given to pregnant women during pregnancy and to the newborn for six weeks (Connor et al., 1994). Mother-to-child transmission remains an important route of HIV transmission to children especially in the developing countries. Since ts1, a temperature-sensitive mutant of Moloney Murine Leukemia Virus TB (MoMuLV-TB), has similar properties of infection and transmission in mice as HIV has in humans, it is useful for studying retroviral mother-to-child transmission.
Mo-MuLV-ts1 model

ts-1 was first isolated by propagating MoMuLV in a thymus bone marrow cell line (TB) taken from CFW/D mice. ts-1 virus has a defect in the intracellular processing of the envelope precursor polyprotein (Pr80env) at the restrictive temperature (Wong and McCarter, 1973; Wong et al., 1983). Moloney murine leukemia virus (MoMuLV)-TB is a murine type C retrovirus and can induce T-cell lymphomas in susceptible strains of mice after a long incubation period (Yuen and Szurek, 1989). Newborn BALB/c mice infected with ts1 virus have neurodegenerative disease resulting in hind limb paralysis and immunologic disease characterized by severe thymic atrophy associated with immunodeficiency due to destruction of T-lymphocytes, and generalized body wasting (Wong et al., 1989; Wong et al., 1983). Infectivity of ts-1 is significantly related to its temperature (Wong et al., 1983), and can replicate optimally at permissive temperature 34 Cº (Yuen et al., 1985). That may explained why the ts-1 can produce hind limb paralysis in newborn mice and not the adult, regarding to the lower body temperature of the newborn mice (~34 Cº) than adult (~38.4 Cº) (Crispen, 1978). ts-1 is unique among other murine retrovirus in that it can cause degenerative diseases in mice similar to HIV in humans, by affecting both the central nervous system (CNS) and immune system. Infected T-lymphocytes have impaired function (Saha and Wong, 1991; Wong et al., 1992). Murine ts-1 model has been extensively used as small-animal model for retrovirus induced
neurodegenerative disease (Wong, 1990). Oxidative stress has been suggested as a major mechanism for ts1 induced neurodegeneration and T-cell loss in infected newborn mice (Jiang et al., 2006). The U3 region of ts-l controls the pathogenicity and targets cell type. Exchanging the U3 region of ts-l with the Cas-Br-E will change the primary site of viral replication from the thymus and spleen to the spleen alone (Wong et al., 1991).

Transfer of humoral immunity to ts-1 can be passed from mother to baby via breast milk and can provide protection from neurodegenerative and immunologic disease in neonatal mice (Saha et al., 1994). (Chakraborty et al., 2003) have developed a murine model to study the ts-1 retroviruses induced perinatal transmission. Infected mother can transmit the ts-1 virus vertically to offspring. Later on, results indicate that mother-to-offspring transmission via breast milk can occur at about 100% incidence (Duggan et al., 2006), and can cause lymphomas when pups suckle from ts-1 infected mothers, The pattern of proviral ts-1 integration sites observed in these lymphoma tissues correlated with up-regulation of mRNA expression of candidate genes that may contribute to lymphomagenesis (Chakraborty et al., 2008).

In previous investigations, the availability of the mouse genome sequence allowed for the identification of proviral integration sites following ts1 infection. Using these integrations sites, potential genes were found that could be involved in murine cancer. Since ts-1 infection in mice mimics HIV infection in humans, these findings can be used to better understand HIV-induced tumorigenesis in
humans. This study is designed to continue using this model to further investigate lymphoma development in mice infected with ts1 through breast milk transmission.
Materials and Methods

Virus Culture and Assay

To grow and obtain the appropriate concentration of virus particles, we used TB cells (thymus-bone marrow) as shown in figure 11, for viral culture and 15F cells for viral assay. The ts1 viral stock, TB cells and 15F cells were kindly supplied by Dr. P.K.Y Wong at the M.D. Anderson Cancer Center, Smithville, Texas.

Procedure of growing ts-1 in TB cells

1- Thawed 1 ml vial of cells till pellet moved. By using sterile pipette, the cells were transferred to a 75 cm² flask (vented cap), containing 10 ml of complete DMEM.

2- Gently rocked the flask side to side until the media spread all over the bottom equally, and incubated overnight at 37°C with a 5% CO₂ atmosphere.

3- Next day, the old media was replaced with a 15 ml complete DMEM, and incubated at 37°C for 2-3 days (until the cells are 70-80% confluent).

4- To split the cells, the flask was gently shaken to get rid of the loosely attached cells off the bottom of the flask. Then, the medium was poured off and
immediately the cells were rinsed with 5 ml saline citrate solution.

5-The saline citrate solution was poured off and 5 ml of 2.5% trypsin in saline citrate solution was added and incubated 37°C for 5-10 min. The cells were checked for ~ 90% detached from the flask.

6- 5 ml complete DMEM was added to neutralize the trypsin reaction. Then the solution was pipetted up and down gently to avoid any cell clusters.

7- Then 10 ml of cell suspension is transferred into a 50 ml tube, and centrifuged for 5 min at 1000 rpm.

8- The supernatant was carefully poured off and the pellet was re-suspended in 5 ml complete DMEM. The solution was pipetted up and down several times.

9- The cells were counted and diluted to 1:5 (desired amount of cells between 30 – 300 cells per square on hemocytometer ), 100 µl cell suspension diluted in 400 µl completed DMEM, mixed well and sufficient drops (<20 µl) were added to the hemocytometer and the four corner were counted for cell number. Total numbers from all corners were added together and the average was calculated. The average then was multiplied by five (diluted factor), and the result was multiplied by $10^4$ (factor of the hemocytometer). The result indicates the
number of cells per milliliter.

Example: \(83 + 67 + 72 + 89 = 311/4 = 77.8\)

\[77.8 \times 5 = 388.7 \Rightarrow 388.7 \times 10^4\]

\[3.89 \times 10^6 \text{ cells/ml}\]

10-To grow a new cell generation, \(1 \times 10^6 \text{ cells/ml}\) was needed. To calculate the volume of cell suspension to be added to a new 75 cm\(^2\) flask containing 15 ml completed DMEM; the number of cells needed was divided by the number of cells per ml obtained as above.

Example: \[\frac{1.0 \times 10^6}{3.89 \times 10^6 \text{ (from above example)}} = 257 \mu l/\text{flask}\]

So, in this example we need 257\(\mu l\) of cell suspension added to 15 ml complete DMEM per flask.

11-The flask was incubated at 37°C for 2-3 days (until the cells are 70-80% confluent).

At this point, the cells were either split for further growth (seeding), split for freezing, or split for viral culture (TB cells only).

12-For viral culture, we only use TB cells. First, a concentration of \(1 \times 10^6\) TB cells /ml are seeded in 75 cm\(^2\) flask containing 10 ml polybrene DMEM incubated at 37°C overnight.
The next day, old media was poured off and TB cells were infected with ts1 virus in 3-5 ml of polybrene media at a multiple of infection (MOI) of 1.0.

\[ \text{MOI} = \frac{\text{No# of virus per ml}}{\text{No# of cells}} \]

Therefore, if cells were 1 x 10^6 cells/ml the viral titer should be 1 x 10^6 FFU/ml. The virus can be diluted with polybrene media to obtain the appropriate titer if needed, and then, added to the 3-5 ml of polybrene media.

After the virus suspension is added to the flask, it was incubated at 34°C for 40 min.

After 40 min, the inoculum was poured off and 10 ml complete DMEM was added to the flask. The flask was then incubated at 34°C for approximately 3 day (until the cells are 70-80% confluent).

At that point culture medium was harvested. The flask was gently rocked side to side and the medium was transferred to a 50 ml falcon tube.

The medium was filtered through a 0.45 μM filter to avoid cellular debris into another 50 ml falcon tube.
18-A 1 ml of filtered medium was liquated into screw-capped tubes cryotubes for storage in -80°C. Because gradual freezing is required, the tubes are stored in -4°C overnight then transfer to -20°C and finally to -80°C.

The viral titer of the batch will be determined from the next step (modified 15F cells assay).

Figure11: Thymus-bone marrow (TB) cells
15F Cells for ts-1 Viral Assay

1- A 75 cm² flask containing 70-80% confluent 15F cells was gently shaken to get rid of the loosely attached cells off the flask. Next, the medium was poured off and immediately the cells rinsed with 5 ml saline citrate solution.

2- The saline citrate solution was poured off and 5 ml of 2.5% trypsin in saline citrate solution was added and incubated in 37°C for 5-10 min. The cells were checked for ~ 90% detached from the flask.

3- 5 ml complete DMEM was added to neutralize the trypsin reaction. Then the solution was pipetted up and down gently to avoid any cell clusters.

4- The 10 ml cell suspension was transferred through a 40 µ strainer into a 50 ml tube, and centrifuged for 5 min at 1000 rpm.

5- The supernatant was carefully poured off and the pellet was re-suspended in 5 ml complete DMEM. The solution was pipetted up and down several times.

6- Next, the cells were counted. The cells are diluted to 1:5 (desired amount of cells between 30 – 300 cells per square on hemocytometer ), and 100 µl cell suspension diluted in 400 µl complete DMEM. After mixing well, sufficient drops (<20 µl) were added to the hemocytometer and the four corners were
counted for the number of cells. Total numbers from all corners were added
together and the average was calculated.

The average was multiplied by five (diluted factor), and the result was
multiplied by $10^4$ (factor of the hemocytometer). Results indicate the number of
cells per milliliter.

Example: $83+67+72+89 = 311/4=77.8$

$$77.8 \times 5 = 388.7 \Rightarrow 388.7 \times 10^4$$

$$3.89 \times 10^6 \text{ cells/ml}$$

For the 15F assay, the amount of cells needed per plate (60 mm culture plate) was
$9.6 \times 10^4$ cells/plate equal to $(1 \times 10^6 \text{ cells/ml})$. The appropriate number of cells
was calculated,

i) \[
\frac{\# \text{ of cells needed}}{\# \text{ of cells obtained}} = \text{Vol. of cells suspension need/plate}
\]

ii) To calculate the entire volume of cell suspension needed for the assay,

the vol. of cells suspension need/plate is multiplied by total number of plates
needed (each dilution from $10^{-1}$ to $10^{-7}$ in duplicate included polybrene control).

iii) The entire volume of cells suspension added into 4 ml of polybrene medium

for each plate.

Example: $9.6 \times 10^4 \text{ cells/plate} / 3.89 \times 10^6 \text{ cells/ml} = 0.025 \text{ ml}$

$$0.025 \text{ ml} \times 16 \text{ plates} = 0.4 \text{ ml of cells suspension}$$

$$4 \text{ ml} \times 16 \text{ plates} = 64 \text{ ml polybrene medium}$$
Final answer: we need to add 0.4 ml cells suspension to 64 ml polybrene medium.

In addition, we labeled two more plates as control plates to make sure that there is no viral contamination. 4 ml completed DMEM was added for each plate plus the appropriate volume of cell suspension needed.

\[
0.025\text{ ml x 2 plates} = 0.05\text{ ml of cells suspension} \\
4\text{ ml x 2 plates} = 8\text{ ml complete DMEM}
\]

10-All plates were labeled for the virus dilution factor, control, and date.

11- 4 ml of total cell suspension was added to each 60 mm Petri dish according to labeled plates, and incubated at 34°C or 37°C overnight.

12-Next day, plates were checked for even distribution of cells under the microscope.

13-A 24 well titer plate was labeled for the dilution factors and was used for serial dilutions. To perform the dilution steps, first we added 1.8 ml of polybrene medium to all labeled wells, and then we added 0.2 ml from virus stock (after thawed) to the first well and mixed it well by pipetting up and down. Next, 0.2 ml from first well was taken and transferred to the next well, mixed well, and so on. 0.2 ml from the last well was removed and discarded.
<table>
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<th>Dilution</th>
<th>$10^{-1}$</th>
<th>$10^{-2}$</th>
<th>$10^{-3}$</th>
<th>$10^{-4}$</th>
<th>$10^{-5}$</th>
<th>$10^{-6}$</th>
<th>$10^{-7}$</th>
</tr>
</thead>
<tbody>
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<td>Polybrene medium</td>
<td>1.8 ml</td>
<td>1.8 ml</td>
<td>1.8 ml</td>
<td>1.8 ml</td>
<td>1.8 ml</td>
<td>1.8 ml</td>
<td>1.8 ml</td>
</tr>
<tr>
<td>Virus stock</td>
<td>0.2 ml</td>
<td>0.2 ml</td>
<td>0.2 ml</td>
<td>0.2 ml</td>
<td>0.2 ml</td>
<td>0.2 ml</td>
<td>Discarded</td>
</tr>
</tbody>
</table>

14-After pouring off the old media from each plates (not controls plates), 0.5 ml of dilutions were pipetted into the corresponding plates (starting from highest to lowest, $10^{-7}$ to $10^{-1}$). For polybrene control plates, old media was replaced with 0.5 ml of polybrene media alone. No need to change the media of DMEM control plates.

15-All plates were placed into a 34°C incubator for 40 min. After 40 min the medium was aspirated off and 4 ml of completed DMEM was added to all plates including polybrene and completed DMEM control plates.

16-Plates were incubated at 34°C for 3 days, after 3 days the medium was changed with fresh complete DMEM.

17- Five to six days after infection, the foci in each plates were counted using an inverted microscope (Figure 12). The viral titer was calculated as follows:
Virus titer (infection units per ml) = \frac{\text{Number of foci}}{\text{Virus dilution}} \times 2

Determine titer from plate with more foci for less statistically error.

For example: 200 foci per plate / 10^{-4} \text{ plate titer} \times 2 = 400 \times 10^4

Final answer: 4.0 \times 10^6 \text{ IU/ml or Focus Forming Unit (ffu)/ml}

Figure 12: Shows the formation of foci due to ts-1 virus infected 15f cells at 34°C.
Mice and Experimental Protocol

Eighteen timed pregnant BALB/c female mice were purchased from Charles River Co., Wilmington, Maine, USA. All experiments were performed under Institutional Animal Care and Use Committee protocol and under direct supervision of the trained personnel of the Department of Laboratory Animal Medicine at the College of Medicine, Health Science Campus at the University of Toledo, OH. Two to three days after arrival, the eighteen females produced 68 pups in total. These 68 pups were divided into experimental and control groups. 72 h after birth, 54 (33 female and 21 male) of these 68 pups were injected intraperitoneally (ip) with 0.1 ml of $4.0 \times 10^6$ ffu/ml of ts-1 virus and designated as infected (experimental) group. The other 14 control (11 female and 3 males) pups were injected with 0.1 ml DMEM only (control) group. All pups were allowed to reach adulthood. 11 more males were purchased from Charles River laboratories for mating purpose. All females were allowed to mate with control males. The 21 experimental males were euthanized. Thirty three experimental females produced 99 pups and 11 control females produced 47 pups. As soon as the pups were born (6-12 hr), they were allowed to suckle from either control or ts1 infected mothers. These 146 pups were divided into 5 groups. Please see the flow diagram (Figure 13). Twenty-four experimental pups (Group #1) were allowed to suckle from their infected biological mother. Group # 2, twenty-nine experimental pups suckled from ts-1 infected surrogate mothers. Group #4, forty-six experimental pups suckled from surrogate control mothers. Thirty-eight control pups (group #3)
suckled from surrogate ts-1-infected mothers. And 9 control pups (group #5) suckled from their non infected biological mother (Figure 13). After weaning, all mothers were euthanized by lethal injection of sodium pentobarbital intraperitoneally in the abdominal region.

Figure 13: Flow chart shows Pups divided to five groups and the transmission mode of MoMuLV-ts-1-retroviruses.
**Tissue collection**

The offspring were observed for 6 months for development of clinical symptoms and evidence of symptomatic infection. Animal’s weight and general observation were recorded on biweekly basis. At the end of this period, the pups were euthanized and tissues from their adrenals, thymus, spleens and lymph nodes were collected. Portions of each tissue sample from all animals were fixed in 4% formalin for histology slide preparation. The remainders of the tissue samples were stored at -80°C for DNA extraction for PCR analyses. All tissues were placed in pre-labeled tubes. All procedures were performed under strict aseptic conditions, including change of gloves, operation pads, wiping, washing, and sterilization of instruments to ensure that cross-contamination did not occur between animals.

**DNA Extraction**

DNA extraction of all tissues was done using 200 µl of PBND buffer and 1 µl of proteinase K per 10-30 mg piece of tissue. Each piece of tissue was placed in 1.5 ml eppendorf tube containing the PBND and proteinase K, and were incubated overnight at 55°C. Next day, samples were spun down for 1 min at 14,000 rpm and the supernatant was transferred to clean new sterile tube. This then was stored at -20°C, and later was used for PCR analysis.
Polymerase Chain Reaction (PCR)

PCR analysis was performed following the same guidelines for all tissues using Perkin Elmer GeneAmp® PCR Reagent Kit with AmpliTaq® DNA Polymerase. The only variable item was the amount of DNA, which was determined based on intensity of bands. Reactions were carried out in 0.2 ml microcentrifuge tubes. Each PCR reactions tube contained 25 µl of Redmix (Gene Choice, Fredrick, MD), 0.1 µM of each primer, variable amounts of DNA, and deionized water to a total volume of 50 µl. Two sets of primers were used. The first set of primer was for ts1 env gene, and amplified a 264-bp region of the ts1 viral envelope gene. The forward primer was 5’AACCCGCGAGGCCCCCTAATCCCCT3’ and the reverse primer was 5’CCCGGGGGAGAAGAAAACGGGGATTGA3’. The second one was an internal control primer (IMR015 and IMR016), which amplified a portion of a T-cell receptor murine gene of 200 bp (http://jax.org). The forward primer for this was 5’CAAATGTTGCTTGTCTGGTG3’, and the reverse primer was 5’GTCAGTCGAGTGCACAGTTT3’. Negative controls were identical except that no DNA was added to the reaction mixture. Positive control was from BALB/c mouse splenic DNA with known ts1 infection. The thermocycler setting was as follows: 94°C for 2 min, 94°C for 30 sec, then 52.3°C for 1 min, and 72°C for 30 sec repeated 29 times and then 5 min at 72°C. PCR bands were obtained by using agarose gel electrophoresis using Amresco 3:1 HRS agarose to cast 1% gels in 1X TBE. DNA reference markers were obtained by using 100-bp DNA Ladder from GibcoBRL®.
Inverse-PCR (I-PCR) and DNA Cloning

Spleens and lymph nodes from infected mice that developed lymphoma and from infected mice that did not develop lymphoma were examined for viral integration sites. High molecular weight genomic DNA was extracted from the tissues using Qiagen Genomic DNA Purification kit (Qiagen, Valencia, CA.), digested with BamH1, purified and ligated using T4 ligase for I-PCR (Li et al., 1999). Primary PCR was performed using a set of inverse primers [forward 1AF: CAG ACA CAG ATA AGT TGC TGG CCA G (211-235) and reverse 1AR: AAG ACG CTT GGA GAT TTG GTT AGA G (1872-1896)] designed from MoMuLV ts1 sequence at the 5’end products of BamH1 digest. Numbers in the parenthesis indicate positions of bases on MoMuLV ts1 sequence (Mouse Genome Database). The solution consisted of 20 pmol each for the inverse primers, 2 μl DNA template, 500 μM dNTP’s, 1.3 U of Expand Long Template Polymerase and 5 μl Expand Buffer System 3 (Roche Diagnostics Corp., Indianapolis, IN) and the volume brought to 50 μl with distilled water. Thermal cycling conditions were as follows: 94°C for 2 min followed by 10 cycles of 94°C for 10 sec, 55°C for 30 sec and 68°C for 10 min; 25 cycles of 94°C for 10 sec, 55°C for 30 sec and 68°C for 10 min (auto-extension 20 sec per cycle) followed by a final extension at 68°C for 10 min. The secondary PCR reaction was performed using primary PCR product as template and using the same parameters as in primary PCR except with nested (secondary) primers in place of primary primers [forward 2AF: AGA CCA CGA TTC GGA TGC AAA CAG (61-84) and reverse 2AR: GAG AGA TGA GCA
AGC TAT TGG CCA C (2035-2059)]. PCR products were run on a gel, bands were cut out, purified and, cloned using TOPO TA cloning kit (Invitrogen, Carlsbad, CA) following the manufacturer’s instructions. E-coli was chosen as the cloning vector following routine procedure. Colonies with viral inserts were identified and analyzed. PCR products cloned ranged from 800 b to 2 kb, which was expected to contain approximately 500 to 1800 bases of host genomic sequence. Colonies were selected and purified with Qiagen Miniprep kit (Qiagen, Valencia, CA) and sequenced at MWG Biotech (MWG-Biotech Inc., High Point, NC).

Figure 14: Summary of the inverse PCR process.
**Quantitative reverse transcriptase-PCR (qRT-PCR)**

RNA later®-ICE from Ambion®

Stabilization of RNA in the animal tissues:

1- Pipetted 250 μl of RNAlater-ICE® in a centrifuge labeled tube. (RNA later solution must be 10X of sample tissue weight). Then tubes were placed in dry ice.

2- Took 25 mg of tissue sample (spleen) from infected mice that developed lymphoma (group 1 and 3-a), infected mice that did not develop lymphoma (group 3-b), and uninfected control mice (group 5). Tissues were immediately placed in the labeled tubes containing 250 μl of RNA according to the sample number.

3- The tubes were tightly capped and mixed well until all tissue samples were completely submerged. Tubes were stored overnight at -20°C.

RNA Extraction (using RNeasy Mini Kit):

RNeasy® Mini Kit (Qiagen, Valencia, CA) for purification of total RNA from animal tissues. Store at room temperature.

1- β-Mercaptoethanol (β-ME) was added to Buffer RLT (supplied with a kit):

   10 μl (β-ME) per 1 ml Buffer RLT.

2- Buffer RPE is supplied as a concentrate, and it was diluted with 4X volume of ethanol (96-100%) before used.
**Procedure**

1-RNAs free tubes were labeled according to the tissue samples in RNAlater stabilized tubes from the previous step. Tubes were placed on ice.

2-Tissues in RNAlater-ICE tubes were picked up from -20°C (one tube at a time) and the entire tissue was removed from the reagent using sterile forceps and placed directly in the appropriate RNAs free tube.

3-300 μl of (β-ME) Buffer RTL was added for disruption and homogenization using a motor and pestle, then another 300 μl of (β-ME) Buffer RTL was added and gently redistributed to get full homogenization.

4-Homogenized sample was stored at -20°C, and same procedure was repeated for the other sample.

5-After all samples were homogenized, sample tubes were removed from the freezer and spun down for 3 min at 13,200 rpm and the supernatant was carefully transferred to a new labeled microcentrifuge tube.

6-600 μl of 70% ethanol was added to the supernatant, and mixed well by pipetting up and down.

7-Up to 700 μl of the mixture from each sample was transferred to an RNeasy spin column placed in a 2 ml collection tube (supplied with a kit) then spun in the centrifuge for 15 sec. at 11,000 rpm. Then, the flow-through was discarded. Step was repeated for the remainder of each mixture.

8-To wash the spin column membrane, 700 μl Buffer RW1 was added to the RNeasy spin column, and spun in the centrifuge for 15 sec. at 11,000 rpm.
Then the flow-through was discarded with the collection tube.

9- The RNeasy spin column was carefully placed in a new 2 ml collection tube.
   500 μl Buffer RPE was added to the RNeasy spin column, then spun in
   the centrifuge for 15 sec. at 11,000 rpm. The flow-through was discarded.

10- Step 9 was repeated with 2 min centrifuge to ensure that the spin column
    membrane was washed properly.

11- The RNeasy spin column was transferred to a new labeled microcentrifuge
    tube.

12- To elute the RNA, 30 μl RNase-free water was added directly to the RNeasy
    spin column, and spun for 1 min at 11,000 rpm.

13- The spin column was discarded, and the collected RNAs were stored at -20°C.

**Quantitation of RNA**

The concentration of RNA for all samples was determined by measuring the
absorbance at 260 nm (A260) in a spectrophotometer.

An absorbance of 1 unit at 260 nm corresponds to 40 μl of RNA per ml.

\[ A_{260} = 1 \implies 40 \, \mu l/ml \]

1- The RNA sample was diluted to 1:50 in RNase-free water.

2- 50 μl of the diluted sample was transferred to RNase-free cuvette. A cuvette was
    placed in the spectrophotometer, and the concentration result was obtained.

3- Water was used to zero the spectrophotometer before sample reading.
The concentration of each sample was used to calculate the required volume the total RNA for Gel Electrophoresis to checked the quality and integrity of RNA and to convert total RNA to cDNA.

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Volume amount of RNA (μl) equivalents to</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gel Electrophoresis</td>
<td>2 μg RNA</td>
</tr>
<tr>
<td>cDNA</td>
<td>5 μg RNA</td>
</tr>
</tbody>
</table>

Example: the concentration = 0.714 μg/μl

For Gel Electrophoresis: 2 μg (RNA concentration needed) / 0.714 μg/μl = 2.8 μl of RNA required

For cDNA: 5 μg (RNA concentration needed) / 0.714 μg/μl = 7.0 μl of RNA required

**Formaldehyde Agarose Gel for RNA Electrophoresis**

All material and solution required for FA gel (1.2% agarose) were prepared in advanced as following:

**10x FA gel buffer**
200 mM 3-[N-Morpholino]propanesulfonic acid (MOPS) (free acid)
50 mM sodium acetate
10 mM EDTA
pH to 7.0 with NaOH

**1x FA gel running buffer**
100 ml 10x FA gel buffer
20 ml 37% (12.3 M) formaldehyde
880 ml RNase-free water
10 μl of a 10 mg/ml ethidium bromide (1 μl/100 μl buffer)
5x RNA loading buffer
16 µl saturated aqueous bromophenol blue solution*
80 µl 500 mM EDTA, pH 8.0
720 µl 37% (12.3 M) formaldehyde
2 ml 100% glycerol
3.084 ml formamide
4 ml 10 x FA gel buffer
RNase-free water added to bring the total volume to 10 ml.
Stability: Approximately 3 months at 4°C

(1.2%) FA gel preparation
1.8 gm agarose 3:1
15 ml 10x FA gel buffer
135 ml RNase free-water

The mixture was heated in the microwave to melt the agarose, and then cooled to 65°C in water bath for (10-15 min). then the following was added:
2.7 ml 37% (12.3 M) formaldehyde
1 µl 10 mg/ml ethidium bromide.

Then were mixed thoroughly and poured onto gel support. Prior to running the gel, gel was equilibrated in 1x FA gel running buffer.

RNA sample preparation for FA gel electrophoresis

The equal volume of 2 µl RNA of each sample was added to 5 µl of 5X loading buffer and RNase free-water was added to bring the total volume to 25 µl. Total volume 20 µl of RNA ladder was prepared (3 µl RNA ladder + 17 µl H2O + 5 µl of 5X loading buffer). All samples including ladder were mixed thoroughly and incubated at 65°C for 4 min in thermal block.

Load the sample and Gel running

25 µl of each Samples and 20 µl of RNA ladder was loaded in the appropriate wells in the gel, and to avoid crossover one well was left empty.

Gel ran at 70 mV for approximately 30 min.
Intact total RNA run on a denaturing gel will have sharp 28S and 18S rRNA bands.

(qRT-PCR) Procedure

Prepare the cDNA

Master-mixed for RNA to cDNA Reverse Transcriptase was prepared as follows:

4.0 µl  5X first strand buffer (Invitrogen)
1.0 µl  100 mM DTT (Invitrogen)
0.4 µl  dNTP stock (25 mM of each dNTP)
1.0 µl  RNaseOUT (40 U/ µl, Invitrogen)
1.0 µl  SuperScript™ III Reverse Transcriptase (200U/ µl, Invitrogen)

7.4 µl  Total volume for each sample (ingredients multiplied by the number of samples needed with an extra 2 reaction)

Procedure:

1-Volume amount equivalent to 5 µg of RNA of each sample were added to a labeled sterile PCR tube. Each sample ran in duplicate.

2-1.0 µl of RandomHexamers (0.4 µg/ µl QIAGEN) added.

3-DEPC (treated H2O) added to bring the total volume to 12.6 µl.

5-Samples were heated at 65°C for 15 min. Then placed in ice to cool for 1 min.

Followed by a quick spin.

6-7.4 µl Master-mixed added to each sample.

7- All samples were incubated a 50°C for 1 hr.

8-Duplicate samples were combined and diluted 1:5, (i.e. 160 µl of dH2O added to 40 µl of reaction mixture), called the cDNA stock solution.

9- cDNA stock aliquated in to 8 tubes of 25 µl each. Tubes were properly labeled.
Check the quality of cDNA samples

Real-time PCR was performed with mouse GAPDH a “house keeping” gene primer pairs. All RT-PCR was performed as described by using iCycler thermal cycling instrument and MyiQ Single color real-time PCR. Protocol as follows:

1-5 µl stock cDNA diluted with 196 µl dH2O, to make 1:150 dilution of cDNA were prepared for all samples. Tubes were labeled properly and stored at -20°C.

2-20 µl of 100 pmol/µl of mouse GAPDH primer stock solution (forward and reverse primer each from stock solution) diluted with 760 µl dH2O to make 2.5 µM working solution and store at 20°C.

3-Master mix was prepared as follows:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
<th>Multiplicator</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molecular grade Sterile dH2O, Cellgro®</td>
<td>6.5 µl</td>
<td>X number of sample</td>
</tr>
<tr>
<td>Primer pair mix (2.5 µM, gc-955, gc-956)</td>
<td>1.0 µl</td>
<td>X number of sample</td>
</tr>
<tr>
<td>iQ™ SYBR green supermix (Bio-Rad)</td>
<td>12.5 µl</td>
<td>X number of sample</td>
</tr>
</tbody>
</table>

5-5 µl of 1:150 dilutions cDNA was added (in duplicate for each sample) to the optically clear plate (ABI plate). Then 20 µl master mix was added.

6-Three wells of negative control were included (contain primer plus dH2O instead of cDNA)
7- Plates were loaded and run on the iCycler using 2step-MeltBioRadSJL.tme

   PCR program as follows:

   Cycle 1: (1X) at 95.0ºC for 03:00

   Cycle 2: (40X)
      Step 1: at 95.0ºC for 00:30
      Step 2: at 60.0ºC for 00:30

   Cycle 3: (1X) at 95.0ºC for 1 min

   Cycle 4: (1X) at 55.0ºC for 1 min

   Cycle 5: (80X) at 55.0ºC for 10 sec.

   Increase set point temperature after cycle 2 by 0.5ºC

Ct values (threshold cycle) for GAPDH were obtained (average Ct value 18-21).

qRT-PCR procedure for a gene of interest:

The qRT-PCR technique was used to examine differential expression of selected candidate genes. Gene-specific primers were designed using Primer Express software (version 1.5; Applied Biosystems ABI, Foster City, CA) to amplify 75-100 bp PCR products. Expression of GAPDH, was used to normalize the data.

1-cDNA samples were diluted to appropriate dilutions. The Ct value result must be within range (18 -21). i.e., if 1:50 dilution of sample was tested and the Ct value wasn’t in the accepted range, 1:10 dilution will be tested.

2-RT-PCR was performed on three replicates per sample included “No-template” controls to ensure amplification specificity.

3- Master mix with a primer pairs of selected candidate gene-specific 3' and 5'
primer was prepared as follows:

Molecular grade Sterile dH₂O, Cellgro®  6.5 µl  X  number of sample
Primer pair mix of specific gene (2.5 µM)  1.0 µl  X  number of sample
iQ™ SYBR green supermix (Bio-Rad)  12.5 µl  X  number of sample

4-5 µl of 1:50 dilutions cDNA of each sample was added to the optically clear plate (ABI plate). Then 20 µl master mix was added.

5-RT-PCR performed as above.

Melt-curve analysis was performed immediately after amplification to confirm amplification of a single PCR product. An RNA expression levels were calculated for each gene using comparative dCt method and then normalized for house keeping gene, GAPDH expression levels. Gene expressions are presented as relative values and expressed as the “fold change”.
**Histology**

Neutral buffered formalin fixed spleen tissue samples were embedded in paraffin. 4 micron thick sections were cut and stained with hematoxyline and eosine (H & E) stained. Some sections were prepared for immunohistochemistry staining. This was done by the histology laboratory at The University of Toledo Medical Center.

**Immunohistochemistry**

Reagents and material:

1-0.01 M Phosphate Buffered Saline (PBS), pH 7.4. SIGMA®, (St. Louis, MO)

One pack dry powder dissolved in 1 L dH₂O.

2-3% Hydrogen Peroxide purchased from SIGMA®, (St. Louis, MO)

Store at 4°C

3-Ethyl alcohol purchased from Pharmco™ (Brookfield,CT)

4-Sigma DAB (Daiminobenzidine tetrahydrochloride) Tablet. (store at -20°C)

Dissolved prior to use as follows:

5 ml dH₂O
1 DAB tablet
1 Hydrogen peroxide/Buffer Tablet

5-Citrate Buffer- 10mM pH 6.0 (store at 4°C)

30.7gm Citric Acid, Anhydrous C₆H₈O₇ FisherBiotech (Fair Lawn, NJ)
16.0 L dH₂O.

pH adjusted by adding 10N NaOH.

8-Xylene was purchased from Chempure™. (Houston, TX).
9-Kits Used:

Vectastain ABC-Peroxidase Universal IgG, were purchased from Vector Laboratories, Inc. (Burlingame, CA)

10- Primary antibody:

<table>
<thead>
<tr>
<th>Antibody Name</th>
<th>Description</th>
<th>Manufacture</th>
<th>Address</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD3</td>
<td>Rat monoclonal anti-human recognizing CD3, T-cell associated antigen</td>
<td>AbD Serotec</td>
<td>Oxford,UK</td>
</tr>
<tr>
<td>CD79a</td>
<td>Mouse monoclonal IgG1 antibody recognizing CD79a, B cell associated antigen</td>
<td>Affinity BioReagents™</td>
<td>Golden,CO</td>
</tr>
</tbody>
</table>

Procedure

1-Deparaffinization

The slides were incubated at 60°C for 1 hr. and immediately placed in:

- Xylene; (2 X) for 5 min.
- 100% ethanol; (2 X) for 3 min.
- 90% ethanol; (2 X) for 3 min.
- PBS; (2 X) for 2 min.

2-Antigen retriever was performed to unmask the antigens on paraffin embedded tissue sections by placing slides in 10 mM citric buffer (pH 6.0) at 90°C in a 2100 Retriever (PickCell Laboratories, Amsterdam. Netherlands) for 20min.

3-Slides washed in PBS for 10 min.

4-Slides treated in 3% Hydrogen Peroxide for 5 min to inactivate any endogenous peroxidase activity.

5-Slides washed in PBS (3X) for 3 min.

6- The tissue sections were incubated in blocking serum for 10 min.
5 ml PBS, and 1 drop horse serum (supplies in Vector kit).

After 10 min slides were shaken off to remove the blocking serum.

8- The tissue sections were incubated with the primary antibodies at 1:100 dilution for 30 minutes at room temperature.

9- Slides washed in PBS (3X) for 3 min.

10- The sections were then stained with the appropriate secondary antibody with avidin-biotinylated horseradish peroxidase complex (ABC) for 30 minutes at room temperature.

5 ml PBS, 2 drops horse serum, 2 drop biotinlyated antibody (supplies in Vector kit).

11-Slides washed in PBS (3X) for 3 min.

13- The slides were stained with DAP solution to visualize immunostaining.

14-Slides were rinsed with dH₂O.

15-Slides were dehydrated, mounted in permount and covered with coverslips.
**Statistical analysis**

All statistical analysis was performed by using Statistical Package for the Social Sciences (SPSS) software. The Mann-Whitney test (non-parametric test) used to compare the difference of the gene expression between control group and each individual group of the three experimental groups including: ts1 infected pups with lymphoma development after they suckled from their biological infected mother (group 1), control pups with lymphoma development after they suckled from surrogate infected mother (group 3-a), and control pups with no lymphoma after they suckled from surrogate infected mother (group 3-b). A $p$ value less than 0.05 was used to indicate a statistically significant difference.
RESULTS

Almost 100% of pups from the first three groups (Group 1, 2 & 3) tested positive for ts-1 when they suckled from infected surrogate or biological mother. 78% Pups from infected mother (Group 4) tested positive when they suckled from uninfected surrogate mother. None of 9 control pups (Group 5) tested positive for ts-1 when they were allowed to suckled from surrogate control mother. Infectivity was determined by PCR assay (Figure 15).

Figure 15: PCR band of DNA samples from 5 groups tested for the presence of ts-1

Lymphoma Development in offspring

Lymphoma was diagnosed and scored based on the weight and size data of the spleen and visual estimation of the lymph nodes (mesenteric) and thymus (Figure 17, 18). Thirteen out of 91 infected pups developed lymphoma. The 13 pups were from three different experimental groups. Five of 24 pups of infected mothers developed
lymphoma when they suckled from their ts-1 infected biological mothers (Group 1). Two of 29 pups from infected mothers that suckled from ts-1 infected surrogate mothers developed lymphoma (Group 2). Six of 38 control pups from uninfected mothers developed lymphoma when they were allowed to suckle from infected surrogate mothers (Group 3). None of the 46 pups from infected mothers developed lymphoma when they suckled from uninfected surrogate mothers (Group 4). None of nine control pups had lymphoma (Group 5)(Figure 16).

Figure 16: five groups of animals show route of transmission, rate of infection and development of lymphoma

Exp.- experimental ts-1 infected
NC- No change ( biological mother)
Cont.- non infected animal
Figure 17: Picture of normal spleen (A) & lymphoma spleen (B)

Spleen of ts-1 infected mouse with lymphoma, note the severe splenomegaly (B) in comparison to the normal size spleen (A) of control mouse.

Figure 18: Histogram shows the spleen mean size of all groups
Histology and Immunohistochemistry

Spleen sections were used for this study. Hematoxylin and eosin (H&E) stained slides of 13 tumors samples induced by ts-1 showed massive or partial destruction of the normal architecture with some infiltration of large cells and multinucleated giant cells (Figure 19D, G & J). In the control group, spleen section showed intact splenic architecture (Figure 19A). Immunohistochemical staining showed light staining for T-cell marker (CD3) as shown in Figure 19E & H, with no staining for B-cell marker (CD79a) (Figure 19F & I) in seven tumor samples from both group 1 and group 2. Indicate that these tumors are a T cell lymphoma. These pups of infected ts-1 mothers were allowed to suckle either from infected ts-1 biological or surrogate mother. On the other hand tumor samples collected from pups of control mother who allowed to suckled from surrogate ts-1 infected mother (group 3-A) showed apparent strong staining for both T-cell markers (CD3) (Figure 19K) and B-cell markers (CD79a) (Figure 19L). These results indicate that Lymphomas produced in this group originate from both B and T cells. Results were compared with control sections from control group (group 5) (Figure 19B & C). See results of each animal in (Table III).
Figure 19: Immunohistochemistry Slides: Stain with H&E stain, anti CD3 (T-cell marker) and Anti CD79a (B-cell marker)

<table>
<thead>
<tr>
<th>Group</th>
<th>H&amp;E</th>
<th>CD3 (T cell marker)</th>
<th>CD79a (B cell marker)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group # 5</td>
<td>A</td>
<td>B</td>
<td>C</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group # 1</td>
<td>D</td>
<td>E</td>
<td>F</td>
</tr>
<tr>
<td>(Exp./NC)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group # 2</td>
<td>G</td>
<td>H</td>
<td>I</td>
</tr>
<tr>
<td>(Exp./Exp)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group # 3</td>
<td>J</td>
<td>K</td>
<td>L</td>
</tr>
<tr>
<td>(Con./Exp)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Paraffin-embedded tissue sections stained with hematoxylin and eosin [A, D, G, J] and by the avidin-biotin complex immunohistochemical method for CD3 T-cell marker [B, E, H, K], and for CD79a B-cell marker [C, F, I, L].
Table III: immunohistochemistry results from all three lymphoma groups and control animals.

<table>
<thead>
<tr>
<th>Group</th>
<th>Animal #</th>
<th>TISSUE ID</th>
<th>SEX</th>
<th>H&amp;E Comment</th>
<th>CD3 (T-cell) stain cells (stain intensity)</th>
<th>CD79a (B-cell) stain cells (stain intensity)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group#1 (Exp./NC)</td>
<td>27</td>
<td>07-070</td>
<td>F</td>
<td>observed a lot destruction of the architecture</td>
<td>few (+2)</td>
<td>no stain</td>
</tr>
<tr>
<td></td>
<td>29</td>
<td>07-071</td>
<td>F</td>
<td>infiltration large cells with a lot destruction</td>
<td>few (+1)</td>
<td>no stain</td>
</tr>
<tr>
<td></td>
<td>44</td>
<td>07-072</td>
<td>F</td>
<td>some area preserve intact structure with partial destruction</td>
<td>few (+1)</td>
<td>no stain</td>
</tr>
<tr>
<td></td>
<td>45</td>
<td>07-073</td>
<td>F</td>
<td>some area preserve intact structure with partial destruction</td>
<td>few (&lt;+1)</td>
<td>no stain</td>
</tr>
<tr>
<td></td>
<td>49</td>
<td>07-074</td>
<td>F</td>
<td>strong destruction of the architecture</td>
<td>few (+1)</td>
<td>no stain</td>
</tr>
<tr>
<td>Group#2 (Exp./Exp.)</td>
<td>18</td>
<td>06-490</td>
<td>F</td>
<td>some area preserve intact structure with partial destruction</td>
<td>few (+1)</td>
<td>no stain</td>
</tr>
<tr>
<td></td>
<td>19</td>
<td>06-491</td>
<td>F</td>
<td>some area preserve intact structure with partial destruction</td>
<td>few (+1)</td>
<td>no stain</td>
</tr>
<tr>
<td></td>
<td>53</td>
<td>06-492</td>
<td>F</td>
<td>massive destruction of the architecture with preserve in some area</td>
<td>many (+3)</td>
<td>few (+1)</td>
</tr>
<tr>
<td></td>
<td>56</td>
<td>06-493</td>
<td>M</td>
<td>massive destruction with a lot of giant cells and multiple nuclei</td>
<td>many (+3)</td>
<td>many (+4)</td>
</tr>
<tr>
<td></td>
<td>58</td>
<td>06-494</td>
<td>M</td>
<td>massive destruction with a lot large cells invasion</td>
<td>many (+3)</td>
<td>many (+4)</td>
</tr>
<tr>
<td></td>
<td>59</td>
<td>06-495</td>
<td>M</td>
<td>intact structure with some infiltration large cells</td>
<td>many (+3)</td>
<td>many (+4)</td>
</tr>
<tr>
<td></td>
<td>62</td>
<td>06-496</td>
<td>F</td>
<td>massive destruction of the architecture with preserve in some area</td>
<td>many (+3)</td>
<td>many (+4)</td>
</tr>
<tr>
<td></td>
<td>78</td>
<td>06-497</td>
<td>M</td>
<td>massive destruction of the architecture with preserve in some area</td>
<td>many (+3)</td>
<td>many (+4)</td>
</tr>
<tr>
<td>Controls Gr.5 (Normal)</td>
<td>137</td>
<td>07-374</td>
<td>M</td>
<td>intact structure with some cells scatter in the periphery</td>
<td>few (+2)</td>
<td>few (+2)</td>
</tr>
<tr>
<td></td>
<td>123</td>
<td>07-371</td>
<td>F</td>
<td>intact structure</td>
<td>few (+2)</td>
<td>few (+1)</td>
</tr>
<tr>
<td></td>
<td>127</td>
<td>07-370</td>
<td>M</td>
<td>intact structure</td>
<td>few (+2)</td>
<td>no stain</td>
</tr>
<tr>
<td></td>
<td>130</td>
<td>07-500</td>
<td>F</td>
<td>intact structure</td>
<td>few (+3)</td>
<td>few (+1)</td>
</tr>
<tr>
<td></td>
<td>135</td>
<td>07-346</td>
<td>M</td>
<td>intact structure</td>
<td>few (+3)</td>
<td>few (+2)</td>
</tr>
</tbody>
</table>

Graded from +1 to +4 used for mild positive to intense positive staining

Proviral Integration sites by Inverse-PCR

Spleen and lymph node tissues were used for this study in determining viral integration sites. Tissues from infected pups with lymphoma and pups infected with no lymphoma were used for identification of viral integration sites. Of 720 colonies screened, 368 were white colonies indicating the insertion of viral genome into the murine genome these were further analysed. Of these 368 colonies 209 proviral genomic insertion sites were found. Fifty-two were intragenic and 157 were intergenic sites with over 90 unique intra and intergenic combined viral integration sites identified in mouse genome. The maximum
number of insertion sites (> 10) were found in chromosomes 1, 2, 5, 7, 10, 11, 12 and 13, with the most on chromosomes 10, 12 and 13 with 26, 32 and 45 insertions, respectively. Interestingly, of all 45 inserts in chromosome 13, 39 were at the same location from one animal and none of the animals showed viral inserts in 18 or Y chromosomes. These data was reported in a previous study (Chakraborty et al., 2008).

The mRNA expression of candidate genes

Twenty-seven genes were selected for mRNA analysis by examining the effect of viral integration on their expression level based on 1) their proximity to specific genes (within 100kb); 2) listing in Retroviral Tagged Cancer Gene Database (http://RTCGD.ncifcrf.gov) for genes associated with cancer in relation to retroviral insertions; and , 3) their association with cancer development. Spleen tissues from 7 control animals (group 5), 4 with infection and lymphoma (Group 1), 6 with infection and lymphoma (group 3-a) were used in this studies. The change in mRNA expression has been depicted in Table IV & V. The expression values of 27 genes examined included 11 candidate genes with intragenic viral integration site (VIS) (Table IV), and 16 candidate genes with intergenic VIS (Table V). When compared to the control group, group 1, mRNA expression of Tacc3 and Ahi1 genes showed statistically significant up-regulation with an average 11.3 and 3.6 fold increases respectively. Also, in the same group, Ccnd1, Bcl11a and Irf2bp2 mRNA expression showed statistically significant
down-regulation of with approximately half-fold decrease compared to the control group. In group 3-a, the mRNA expression of Tacc3, Ahi, Gfi and Ncor2 genes showed significant up-regulation (P value less than 0.05) with an average of 12.6, 7.7, 3.2 and 2.4-fold increases in mRNA expression respectively, while Vamp8 showed significant downregulation compared to control group. In addition to these three groups, we extended group 3 spleenic tissues analysis to 4 mice who were infected with no clinical symptoms lymphoma (group 3-b) to test whether these animals have the same patterns of differential gene expression levels as the other mice from the same group who developed lymphoma. No similarities were found except for two genes, Ahi1 and Gfi1, which showed non significant upregulation with an average 2-fold increase. These animals may have developed lymphoma if the end time point of the experiment were set longer. Figure 20 and Table VI summarize results for all gene expression data and statistically significant fold change of all tested groups.
Table IV: mRNA expression analysis of 11 genes with intragenic Viral Integration Site (VIS) and 16 genes with intergenic (VIS).

<table>
<thead>
<tr>
<th>Candidate genes</th>
<th>Ahi1</th>
<th>Elk3</th>
<th>Ncor2</th>
<th>Evi5</th>
<th>Serinc3</th>
<th>Tgfa</th>
<th>Ppp1r16b</th>
<th>Rgs3</th>
<th>Cit</th>
<th>Il17ra</th>
<th>Mid1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (not Infected)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control 1</td>
<td>1.87</td>
<td>2.48</td>
<td>3.02</td>
<td>0.84</td>
<td>0.81</td>
<td>0.95</td>
<td>2.96</td>
<td>2.37</td>
<td>1.09</td>
<td>1.06</td>
<td>0.77</td>
</tr>
<tr>
<td>Control 2</td>
<td>0.28</td>
<td>0.20</td>
<td>0.09</td>
<td>0.53</td>
<td>0.62</td>
<td>0.73</td>
<td>0.19</td>
<td>0.12</td>
<td>0.90</td>
<td>0.66</td>
<td>1.05</td>
</tr>
<tr>
<td>Control 3</td>
<td>2.24</td>
<td>0.79</td>
<td>1.34</td>
<td>0.67</td>
<td>0.61</td>
<td>0.80</td>
<td>1.26</td>
<td>1.56</td>
<td>0.61</td>
<td>1.37</td>
<td>0.95</td>
</tr>
<tr>
<td>Control 4</td>
<td>0.22</td>
<td>0.09</td>
<td>0.14</td>
<td>2.45</td>
<td>1.22</td>
<td>1.04</td>
<td>0.07</td>
<td>0.17</td>
<td>0.75</td>
<td>0.67</td>
<td>1.11</td>
</tr>
<tr>
<td>Control 5</td>
<td>0.98</td>
<td>0.88</td>
<td>0.78</td>
<td>1.03</td>
<td>1.56</td>
<td>0.82</td>
<td>1.32</td>
<td>1.36</td>
<td>1.25</td>
<td>1.23</td>
<td>1.22</td>
</tr>
<tr>
<td>Control 6</td>
<td>0.62</td>
<td>1.53</td>
<td>0.86</td>
<td>1.08</td>
<td>0.82</td>
<td>1.93</td>
<td>0.65</td>
<td>0.49</td>
<td>1.72</td>
<td>1.16</td>
<td>1.09</td>
</tr>
<tr>
<td>Control 7</td>
<td>0.80</td>
<td>1.02</td>
<td>0.76</td>
<td>0.40</td>
<td>0.79</td>
<td>0.74</td>
<td>0.55</td>
<td>0.54</td>
<td>0.68</td>
<td>0.85</td>
<td>0.82</td>
</tr>
</tbody>
</table>

Average fold expression: 1.00 1.00 1.00 1.00 1.00 1.00 1.00 1.00 1.00 1.00 1.00
SEM: 0.32 0.33 0.40 0.28 0.13 0.17 0.40 0.34 0.16 0.11 0.07

SEM: Standard error of the mean.

The table shows the alteration in gene expression by mRNA quantitation by using comparative ddCT method. The effect of viral integration sites (VIS) in three groups of experimental animals were studied in comparison to the control animals. Shaded boxes show statistically significant level change of the genes compared with control group.

The table shows the alteration in gene expression by mRNA quantitation by using comparative ddCT method. The effect of viral integration sites (VIS) in three groups of experimental animals were studied in comparison to the control animals.

SEM: Standard error of the mean.

Shaded boxes show statistically significant level change of the genes compared with control group.
Table V: mRNA expression analysis of 16 genes with intergenic Viral Integration Site (VIS).

<table>
<thead>
<tr>
<th>Candidate genes</th>
<th>Tacc3</th>
<th>Aurka</th>
<th>Gfi1</th>
<th>Evi1</th>
<th>Vamp8</th>
<th>Dusp22</th>
<th>Irfl4</th>
<th>Ccnd1</th>
<th>Prdm16</th>
<th>Bcl11a</th>
<th>Zfp42</th>
<th>Irfl2bp2</th>
<th>Il5</th>
<th>Cldn5</th>
<th>Myc</th>
<th>Irfl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (not Infected)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 20: Histogram showing the mRNA expression level of 27 genes for all groups.

Summarized from table IV and V, using comparative ddCt method, p value < 5 indicate the significant increase or decrease in gene expression of each individual experimental groups included animals with infection and lymphoma (Gr.1: Exp./ No Change), animals with infection and lymphoma (Gr.3: Cont./Exp.), and animal with infection and no lymphoma (Gr.3: Cont./Exp.), in compared to the control animal.
DISCUSSION

Transmission of retrovirus via breast milk is important, since this is a major source of HIV post-partum transmission from mother to infants in humans (Kourtis et al., 2003). Our previous studies have shown that MoMuLV-ts1 is a suitable animal retroviral model to study HIV perinatal transmission and pathogenesis. Transmission of ts-1 via breast milk can transmit infection to newborn BALB/c mice and lead to develop of lymphomas. In this study, we used immunohistochemical analysis in an attempt to identify the possible phenotypic heterogeneity of lymphoma cells developed in this model. MoMuLV typically induces T-cell lymphoma in 100% of the infected mice with a mean latency period of 3–4 months (Fan, 1997). Immunohistochemistry showed little cells staining for T-cell with no staining for B-cell lymphoma in seven tumor samples from both group 1 and 2, when pups of infected ts-1 mother were allowed to suckle either from infected ts-1 biological or surrogate mother. In contrast, six tumor samples collected from non infected ts-1 pups allowed to suckle from surrogate ts-1 infected mother (group 3-a) showed more interesting results with apparent strong staining for both T-cell and B-cell lymphoma (Table III and Figure 19). To our knowledge, this is the first demonstration that ts-1 can induce B-cell lymphoma. We suggested that the immune system background of pups may influence the status and type of lymphoma. Transfer of humoral immunity to ts-1 can be passed from infected mother to baby via breast and can provide some level of protection from neurodegenerative and immune diseases in neonatal mice (Saha et al., 1994). Since the majority of immunophenotypic malignant cells of HIV-1-associated with lymphomas
are considered to be of B-cell origin (Knowles, 1997), this MoMuLV-ts1 model may be of considerable benefit in the study of the induction of B-cell lymphoma in AIDS patients. However, further efforts should be directed towards elucidating the mechanism of ts-1 virus inducing B-cell lymphoma. During the current investigation, we attempted to answer the question of “how these lymphomas develop”. Therefore we used viral integration site (VIS) analysis to identify potential genes involved in lymphomas. It is known that retroviral insertional site analysis is a powerful method to isolate genes involved in cancer (Akagi et al., 2004). Several important genes have been identified using this method. The possibility exists that the provirus may integrate within or near a proto-oncogene and can cause inappropriate expression or it may also insert within a tumor suppressor gene causing inactivation, and this may lead to tumorigenesis (Glud et al., 2005; Uren et al., 2005). We have identified 27 candidate genes, 11 with intragenic viral integration site and 16 with intergenic viral integration site. Our results demonstrate patterns of differential gene expression levels distinct for each group compared to the control group, and correlate with lymphoma phenotypes produced in these groups. Our comparative results of gene expression reveal some interesting observation in relative to phenotype of lymphomas produced in both groups. i.e. tumor samples from pups (group 1) display T-cell lymphoma associated with up-regulation of two genes: Tacc3 and Ahi-1 (table IV,V). In the other group (group 3-a), six pups showed strong positive reaction for B-cell lymphoma in addition to T-cell lymphoma. The cells of these lymphomas expressed significantly higher levels of mRNA for four genes: Gfi1, Ahi1, Tacc3, and Ncor2. We noticed both Ahi1 and Tacc3 genes were found to be generally up-regulated in
both groups 1 and 3-a. These observations suggest that these two genes may be involved in T-cell lymphoma development, and might be in a different pathway than the other two genes, \textit{Gfi1} and \textit{Ncor2}. Since \textit{Gfi1} and \textit{Ncor2} were only found to be overexpressed in group 3-a lymphoma tissue, they might contribute to B-cell lymphoma development in these mice.

The alteration in mRNA expression levels compared to the control group observed for these genes may be due to the ts-1 provirus and may contribute, in part, to the lymphoma development observed in these mice. Also we noticed that expression levels tested in the four ts-1 infected no-lymphoma mice (Group 3-b) show an average 2-fold increase in two genes, \textit{Tace3} and \textit{Gfi1}, compared to the control group. It would have been interesting to see if these mice might have developed lymphoma if the end time point of the experiment were set longer.

In this study, out of the 27 candidate genes we have identified, \textit{Ahi1} (Abelson helper integration site-1) mRNA expression showed an average 3.6 and 7.7-fold increase in group 1 and group 3-a, respectively, compared to the control group. \textit{Ahi1} was discovered as a gene commonly activated by proviral insertional mutagenesis in v-able or myc-induced murine leukemias and lymphomas (Jiang et al., 2002). The oncogenic role of \textit{Ahi1} has been demonstrated in a cutaneous T-cell lymphoma cell line derived from a patient with Sezary syndrome (SS) (Ringrose et al., 2006). Also \textit{Ahi1} expression significantly increases in chronic myeloid leukemia (CML) (Jiang et al., 2004). Our model has a good potential for testing the role of \textit{Ahi-1} in potential cancer pathway involved in T-cell lymphoma development.
Nuclear corepressor 2 (Ncor2) genes is a transcriptional coregulatory protein showed significant increase in mRNA activity with an average 2.5-fold increase associated with lymphoma development in mice (group 3-a). Ncor2 previously called Silencing Mediator for Retinoid and Thyroid hormone receptor (SMRT), it is involved in regulating many transcriptional activities that are essential for cellular growth and differentiation (Jiang et al., 2001). Overexpression of Ncor 2 was commonly associated with prostate cancer cells (Khanim et al., 2004). Ncor2 protein showed 1.3-fold increase in 66% of patients with mantle-cell lymphoma (MCL), a unique subtype of B-cell non-Hodgkin lymphoma (Ghobrial et al., 2005). Our model might provide important insight into the molecular mechanisms of Ncor2 gene in the B-cell lymphoproliferative disorders.

All tumor samples from both groups, group 1 and group 3-a, showed increases in mRNA expression of transforming acidic coiled-coil protein 3 (Tacc3) gene compared to control group. Among the four upregulated genes, Tacc3 has the highest mRNA expression levels with an average of 11.3 and 12.6-fold increases in group 1 and group 3, respectively. Tacc3 are associated with centrosome and microtubule-associated proteins that are essential for mitotic spindle formation (Gergely et al., 2000). Tacc3 was identified as a novel prognostic marker in nonsmall cell lung cancer (Jung et al., 2006). Dysregulation of Tacc3 proteins have also been related to ovarian cancer (Lauffart et al., 2005). (Schneider et al., 2007) has demonstrated the important role of Tacc3 in spindle assembly and cellular survival, thus introducing Tacc3 as a potential therapeutic target in cancer cells. Deficiency of Tacc3 leads to P53 mediated apoptosis (Piekorz et al., 2002).
Therefore, overexpression of Tacc3 as observed in our mice may be causing inhibition of apoptosis, thus leading to lymphoma.

The growth factor independent 1 (Gfi1) gene observed with significant overexpression in tumor display biphenotypic (B-/T-cell) lymphomas (group 3-a), Gfi1 is a transcriptional repressor essential for function and development of different hematopoietic cells (Marteijn et al., 2007), also was found to be highly expressed during T-cell development and in early B-cell subpopulation (Yucel et al., 2004). Gfi1 was identified as a common integration site of Moloney murine leukemia virus in rat T-cell lymphoma cell lines (Gilks et al., 1993). Gfi1 is a proto-oncogene, strongly cooperated in the process of the T-cell lymphomagenesis with Myc and Pim-1 (Zornig et al., 1996). Alteration in Gfi1 gene expression in our mouse model might provide important insights into development and induction of B- and T-lympho-proliferative disorder in human.

Although in this investigation, we focused on the upregulated genes, there were also genes that were significantly downregulated in each group (Table IV & V). Based on the literature research that we have done, it is not yet clear how reduction of these genes may be involved in lymphoma development. Further investigation will be necessary to understand the role that these downregulated genes play in lymphogenesis. In table VI, summary of all results including mRNA analysis and immunohistochemistry.
Currently, we are using Western blot assay to determine whether the protein level of these candidate genes can correlate with their mRNA expression level. Also, we have started a new experiment with a new set of mice for confirming our data and it will allow us to expand our investigations, including histological analysis of the subclasses of lymphoma, study the mechanism of ts-1 virus inducing B-cell lymphoma and compare these results with lymphomas in AIDS patients.
CONCLUSIONS

We conclude that transmission of MoMuLV-ts1 via breast milk mediated integration activates the expression of four cellular genes including \textit{Ahi1, Ncor2, Tacc3, and Gfi1} and that this event may contribute to lymphomagenesis.

Also the immune system status of pups appears to play a major role in determining the phenotype of the lymphoma cells.
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The Journal of Infectious Diseases 197:1156–1161


APPENDICES

Appendix A:

Abstract title:-
Immunohistochemical and mRNA expression analysis of lymphoma associated with breast milk transmission of a retrovirus in a mouse model;

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Presented at the 47th Annual Meeting of American Society of Cell Biology, Dec-2007

Objective: To determine the type of lymphoma caused by postpartum transmission of a retrovirus, MoMuLV-ts1 via breast milk of infected mothers.

Methods: Two groups of 53 BALB/c pups from infected mothers were allowed to suckle either from their infected biological or surrogate mothers. The third group of 38 control pups suckled from their surrogate infected mothers. The fourth group of 46 pups of infected mothers, suckled from control surrogate mothers. Spleen, thymus and lymph node tissues were used for immunohistochemistry and qRT-PCR. Antibodies to CD3 and CD79a were used to identify the T-cell and B-cell lymphomas respectively. mRNA expression levels for each of 29 genes were determined using a standard curve method, normalized for GAPDH gene expression.

Results: Almost 100% of the first three groups of pups were infected when allowed to suckle from the infected mothers while 78% of pups of the fourth group were infected when they suckled from the control mothers and none developed lymphoma. Thirteen pups from the other 3 groups developed lymphoma. Two pups of infected mothers
suckled from infected surrogate mothers and 5 pups suckled from their biological infected mothers, developed T-cell lymphoma. The third group of six pups showed the most interesting results with more B-cell lymphoma and higher expression levels of mRNA for three genes including Tacc3, Aurka, and Ahi1, where the viral genome integration has occurred.

**Conclusion:** In this study, the types of lymphoma correlate with the source of the retroviral infection. In our model, the control pups exposed to breast milk of infected mothers may develop the more aggressive type B-cell lymphoma, with integration of viral genome into the site of cancer related genes, such as Tacc3, Aurka and Ahi1.

This work was supported by American Cancer Society, Ohio Division and F.M. Douglas Foundation, of St.Vincent Medical Center.
Appendix B:


**Title:** Retroviral gene insertion in breast milk mediated lymphomagenesis

**Running Title:** Breast milk transmitted lymphomagenesis

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Retroviral gene insertion in breast milk mediated lymphomagenesis

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Abstract:

We have demonstrated breast milk transmitted MoMuLV-ts1 retrovirus infection and subsequent lymphoma development in offspring of uninfected mothers suckled by infected surrogate mothers. Additionally, we have shown that the lymphoma development occurs as a result of viral gene integration into host genome. A total of 146 pups from Balb/C mice were divided into 5 groups; one control and 4 experimental. All offspring suckled from surrogate infected or control mothers, except one group of infected pups left with their biological mothers. Thirteen of 91 infected pups developed lymphoma. Inverse-PCR, DNA cloning, quantitative real time PCR (qRT-PCR) were used to study the virus integration sites (VIS) and alterations in gene expression. VIS were randomly distributed throughout the genome. Majority of insertion sites were found in chromosomes 10, 12 and 13. A total of 209 proviral genomic insertion sites were located with 52 intragenic and 157 intergenic sites. We have identified 29 target
genes. Four genes including Tacc3, Aurka, Gfi1 and Ahi1 showed the maximum upregulation of mRNA expression. These four genes can be considered as candidate genes based on their association with cancer. Upregulation of these genes may be involved in this type of lymphoma development. This model provides an important opportunity to gain insight into the relationship of viral gene insertion into host genome and development of lymphoma via natural transmission route such as breast milk.

**Key words:** Retrovirus, Moloney Murine Leukemia Virus, Breast milk, Mother-to-pup viral transmission, lymphoma, viral insertion sites, gene expression.

**Introduction:**

During the current investigation, we have used the Molony Murine Leukemia Virus - temperature sensitive mutant (MoMuLV-ts1) to induce lymphoma, by maternally transmitted retrovirus infection via breast milk in mice. Our laboratory is the first to show the development of lymphoma among the offspring of ts1 infected mothers. Lymphoma induction by integration of the Molony Murine Leukemia Virus (MoMuLV) genome into the mouse genome has been demonstrated by a number of investigators (Hwang et al., 2002; Johnson et al., 2005; Joosten et al., 2002; Kim et al., 2003; Lund et al., 2002; Mikkers et al., 2002; Sorensen et al., 1996; Suzuki et al., 2002). Induction of lymphoma by MoMuLV is a multistep process driven by both viral and non-viral mechanisms (Fan et al., 1997; Hartley et al., 1997). Although provirus integration is random in virus infected non-tumor cells, it shows regional specificity in these lymphomas, suggesting that insertional mutagenesis may play an important role in tumor induction and progression. MoMuLV, unlike transforming viruses, lacks oncogenes and employs mechanisms such as promoter insertion and enhancer
activation to induce tumors. MoMuLV infection activates Rel/NFκB transcription factor, (Pak & Faller, 1996) which may participate in induction of lymphoma. Several other viruses, such as human immunodeficiency virus 1 (HIV-1) and cytomegalovirus, activate NF-KB (Bachelerie et al., 1991; Cherrington & Mocarski, 1989). Existing literature shows that lymphomas associated with MoMuLV are clonal tumors and appear four to six months following virus inoculation. The proviral integration form of the retrovirus is generally located near an appropriate cellular proto-oncogene in tumor cells. Activation of the proto-oncogenes causes pathological changes in specific cell types leading to clonal expansion of the infected cell producing cancer (Kim et al. 2003). Targeted genes have been shown to play an important role in oncogenesis (Neel et al., 1981; Nusse & Varmus, 1982; Rosenberg & Jolicoeur, 1997). PCR and RT-PCR based methods were used by Paun et al. (2005) and by Shin et al. (2004) for LP-BM5 and ecotropic MuLV in C57 BL/6J, AKR and C58 mice respectively. Positions of provirus/host DNA junctions were identified by PCR-based techniques in murine tumors using SL3-3 retrovirus in T-cell lymphomas, (Rasmussen et al., 2005). According to Kim et al. (2003), candidate cancer genes in mouse T-cell lymphomas induced by SL3-3 retrovirus include transcription factors, such as Fos, Gfi1, Lef1, Myb, Myc, Runx3 and Sox3, all three D cyclins and Ras signaling pathway factors, including Rras 2/TC 21, Rasgrp 1 and Cmkbr7/CC R7. Rras 2 was the most frequent target with increased expression observed in insertions as far as 57 kb from the transcribed region. This study justified the importance of genome-based analysis of retroviral integration for identification of new cancer genes and their potential role in human cancer. Suzuki et al. (2002) identified 884 retroviral integration sites (RIS) in B-cell lymphomas in their
mouse model using retroviral tagging for gene discovery by inverse PCR (I-PCR), DNA sequencing and bioinformatics tool provided by Mouse Genome Project. These authors located 36 common retroviral integration sites (CIS) that are known or predicted to be genes involved in human cancer or their homologs. This approach has been extremely powerful for identification of a large number of genes for different diseases (Suzuki et al., 2002). Shin et al., (2004) located 62 common integration sites including 31 previously identified sites. Seven new sites were identified in marginal zone lymphoma (MZL) of NFS.V+ mice. They found differential patterns of gene expression and disease progression for Gfi1, Sox4, Brca2, Snf1lk, Nfkb1, Pou2af1, Prdm1, Stat6 and Blnk by using microarrays and qRT-PCR. Hansen et al. (2000) and Li et al. (1999) identified target genes and several candidate cancer genes. They demonstrated that genome-based analysis of retroviral insertion sites for identification of specific cancer candidate genes is a highly powerful technique. Identification of large numbers of candidate cancer genes are possible by analysis of retroviral common integration sites in tumors based on the mouse genome sequence (Kim et al., 2003; J. Li et al., 1999; Suzuki et al., 2002).

MoMULV-ts1 is an excellent model for studying the molecular mechanism of lymphoma development through natural transmission of a retrovirus via breast milk to the offspring of infected mothers, and their progression through clinical stages. Typically 100% of mice infected with wildtype MoMuLV develop lymphoma (Fan. 1997). In our model, about 13-15% of offspring develop lymphoma thus proving an excellent control system with pups of same litter with or without lymphoma but 100% rate of infection. Using this model, we have identified 29 target genes by inverse PCR (I-PCR)
for viral integration that could be candidate genes involved in lymphoma development. This model is interesting since no clear evidence is available to date on breast milk transmitted virus induced lymphomagenesis. Although Epstein-Barr virus (EBV) (Fan et al., 2005) has been implicated in lymphoma development in HIV infected patients, no conclusive evidence of the carcinogenicity of this virus has been presented thus far.

Non Hodgkins lymphoma is an AIDS defining illness. However, there is no direct established relationship between HIV infection and development of lymphoma. Although several investigators have reported about HIV-1 integration into host cells causing T-cell lymphoma, further studies are needed to understand the effect of HIV on lymphoma development. For example, HIV integration was reported within fur gene at upstream of c-fes/fps protooncogene causing T-cell lymphoma (Shiramizu et al, 1994). Additionally, integration of HIV-1 in T-lymphocytes has been shown to cause malignant transformation leading to lymphoma (Herndier et al, 1992). In other studies, tax gene has been shown to immortalize T-lymphocytes and cause T-cell lymphoma by human T-cell leukemia virus (HTLV-1) (Jeang et al. 2004 and Blattner, 1999). It must be noted that HIV-1 infection causes considerable increase in cancer burden, specifically Kaposi’s sarcoma. Therefore, a retroviral model for cancer induction needs careful investigation and our model will help provide information regarding the relationship between retroviral infection and lymphomagenesis.
Materials and Methods:

**Virus culture and assay:** The MoMuLV-ts1 viral stock, TB cells (Thymus-Bone marrow) for viral culture and 15F cells for viral assay were kindly provided by Dr. P.K.Y. Wong at the University of Texas M.D. Anderson Cancer Center, Smithville, Texas. Briefly, the ts1 virus was grown in TB cells. TB cells were cultured in Dulbecco Modified Eagle Medium (DMEM) with 6% fetal calf serum, 4% newborn calf serum and 1% penicillin/streptomycin at 37°C until 70-80% confluency. TB cells were then treated with Polybrene in DMEM containing 3% heat inactivated newborn calf serum and 1% penicillin/streptomycin. 1×10^6 focus forming units (ffu)/ml of ts1 virus were added to TB cells. The cell suspension with the virus was incubated at 34°C for 40 minutes. After that, the cells were cultured in fresh DMEM at 34°C for 3 days or until 70-80% confluency. Culture media containing viral particles was removed and filtered through a 0.45 μm filter and stored in 1 ml aliquots at -80°C (Chakraborty et al., 2003).

The 15F cells were grown for viral assay by the same method described above for the TB cells. 9.6×10^6 cells/ml were plated onto 60 mm culture plates in Polybrene. Serial dilutions of 0.2 ml viral stock to be assayed were added to the 24 well plates. Next, 0.5 ml of corresponding viral dilutions were transferred to respective 60 mm plates and incubated at 34°C for 40 minutes. Medium was aspirated and the cells were incubated in fresh DMEM for 3 days at 34°C. Fresh DMEM was added after 3 days and cells were again incubated for 2-3 more days at 34°C and ffu on the plates were counted. A final viral titer of 4.0 × 10^6 ffu/ml was obtained for the viral stock. (Chakraborty et al., 2003).
All experiments involving mice were carried out according to an approved IACUC protocol and under the direct supervision of trained personnel of the Department of Laboratory Animal Medicine at the College of Medicine, Health Science Campus, University of Toledo, OH.

**Lymphoma Induction in the offspring of ts1 infected mothers:** Eighteen timed pregnant BALB/c female mice were purchased from Charles River Co., (Wilmington, Maine). Two to three days after arrival, the 18 females delivered 68 pups at our animal facility. Seventy-two hours after birth, 54 (33 females and 21 males of these 68 pups were injected intraperitoneally (ip) with 0.1 ml of 4.0 x 10^6 ffu/ml of ts1 virus and designated as infected (experimental) group. The other 14 (11 females and 3 males) pups were injected with 0.1 ml DMEM only, producing an uninfected (control) group. All pups were allowed to reach adulthood. Eleven extra males were purchased from Charles River Laboratories for mating purpose. All females were allowed to mate with control males. The experimental males were euthanized. Thirty-three experimental females produced 99 pups and 11 control females produced 47 pups. Within 6 to 12 hours after birth, offspring were allowed to suckle from either control or ts1 infected mothers. These 146 pups were divided into 5 groups (see Table 1). Group 1 had 9 pups from control mothers, which suckled from control surrogate mothers. Forty-six group 2 experimental pups suckled from surrogate control mothers. Twenty-nine group 3 experimental pups suckled from ts-1 infected surrogate mothers. Thirty-eight group 4 control pups suckled from ts1 infected surrogate mothers. Twenty-four group 5 experimental pups suckled from their infected biological mothers. PCR were performed
using tissue samples from all experimental and control pups to identify ts1 viral genome to determine the infection. Thirteen of 91 pups from groups 3, 4 and 5 developed lymphoma. The lymphoma were scored by using the weight data of the spleen and visual estimation of the lymph nodes (mesenteric) and thymus (photographs published in our previous paper, Duggan et. al. 2004). An increase of 5 to 25 fold increase of the weight of the spleens were recorded as splenomegaly and increase in the size of the lymph nodes and thymus in combination with the increase in spleen weight was considered as the indication of lymphoma. Tissues from the spleens and lymph nodes were collected from all animals for analyses of viral genome integration sites. However for the current study only, tissues from spleens were used. Tissues from 5 control animals, 4 with infection but no lymphoma and 6 with infection and lymphoma were used for I-PCR and mRNA expression studies.

**Inverse-PCR (I-PCR) and DNA Cloning:** Spleens and lymph nodes from infected mice that developed lymphoma and from infected mice that did not develop lymphoma were examined for viral integration sites. High molecular weight genomic DNA was extracted from the tissues using Qiagen Genomic DNA Purification kit (Qiagen, Valencia, CA.), digested with BamH1, purified and ligated using T4 ligase for I-PCR (J. Li et al., 1999). Primary PCR was performed using a set of inverse primers [forward 1AF: CAG ACA CAG ATA AGT TGC TGG CCA G (211-235) and reverse 1AR: AAG ACG CTT GGA GAT TTG GTT AGA G (1872-1896) designed from MoMuLV ts1 sequence at the 5’end products of BamH1 digest. Numbers in the parenthesis indicate positions of bases on MoMuLV ts1 sequence (Mouse Genome Database). The solution consisted of 20 pmol each for the inverse primers, 2 μl DNA template, 500 μM dNTP’s,
1.3 U of Expand Long Template Polymerase and 5 μl Expand Buffer System 3 (Roche Diagnostics Corp., Indianapolis, IN) and the volume brought to 50 μl with distilled water. Thermal cycling conditions were as follows: 94°C for 2 min followed by 10 cycles of 94°C for 10 sec, 55°C for 30 sec and 68°C for 10 min; 25 cycles of 94°C for 10 sec, 55°C for 30 sec and 68°C for 10 min (auto-extension 20 sec per cycle) followed by a final extension at 68°C for 10 min. The secondary PCR reaction was performed using primary PCR product as template and using the same parameters as in primary PCR except with nested (secondary) primers in place of primary primers [forward 2AF: AGA CCA CGA TTC GGA TGC AAA CAG (61-84) and reverse 2AR: GAG AGA TGA GCA AGC TAT TGG CCA C (2035-2059)]. PCR products were run on a gel, bands were cut out, purified and, cloned using TOPO TA cloning kit (Invitrogen, Calsbad, CA) following the manufacturer’s instructions. E-coli was chosen as cloning vector following routine procedure. Colonies with viral inserts were identified and analyzed (see Table 2). PCR products cloned ranged from 800 b to 2 kb, which was expected to contain approximately 500 to 1800 bases of host genomic sequence. Colonies were selected and purified with Qiagen Miniprep kit (Qiagen, Valencia, CA) and sequenced at MWG Biotech (MWG-Biotech Inc., High Point, NC).

**Quantitative Reverse Transcriptase-PCR (qRT-PCR):** The quantitative real-time RT-PCR technique was used to examine differential expression of selected candidate genes. First-strand cDNAs was reverse transcribed from total RNA (5 μg/reaction) using Oligo(dT)20 as primers and SuperScript III (Invitrogen). Gene-specific primers were designed using Primer Express software (version 1.5; Applied Biosystems ABI, Foster City, CA) to amplify 75-100 bp PCR products. Expression of GAPDH, a
“house keeping” gene was used to normalize the data. qRT-PCR was performed essentially as described by Lee et al. (2005) using the iCycler thermal cycling instrument and MyiQ Single-Color Real-Time PCR Detection System software (Bio-Rad, Hercules, CA). Specifically, real-time PCR was performed on three replicates per sample using IQ SYBR Green Supermix kit (BioRad, Hercules, CA) on 5 µl of template cDNA of varying dilutions with 1 µl of gene-specific 3' and 5' primer mixtures (2.5 µM solution) optimized for a given primer pair per 25 µl reaction. The reaction mixture contained 12.5 µl of 2x master mix (100mM KCl, 40 mM Tris-HCl, pH 8.4, 6 mM MgCl₂, 0.4 mM dNTPs, 50 units/ml iTaq DNA polymerase, 20 nM SYBR Green (BioRad, Hercules, CA), 12.5 µl of cDNA at appropriate dilutions plus primer mixtures and distilled H₂O, run on the following thermocycler programs: 95°C for 3 min followed by 40 cycles of 95°C for 30 sec, 60°C for 30 sec. 0.5°C increments starting at 55°C for 80 cycles. Melt-curve analysis was performed immediately after amplification to confirm amplification of a single PCR product. "No-template" controls were included to ensure amplification specificity. RNA expression levels were calculated for each gene using comparative ddCt method and then normalized for house keeping gene, GAPDH expression levels. Gene expressions are presented as relative values and expressed as the “fold change”.

All statistical analysis was performed by using Statistical Package for the Social Sciences (SPSS) software. Student’s t-test was used to test the equivalence of mean gene expression between control and ts1 infected no lymphoma (N LYM) or ts1 infected with lymphoma (LYM) development. A p value of <0.05 was used to indicate a statistically significant difference.
Results:

**Lymphoma Development in Offspring of Uninfected and Infected Mothers via Breast Milk of Infected Biological and Surrogate Mothers:**

Nine control pups born to control mothers (DMEM injected) were suckled from surrogate control mothers, none of which had ts1 infection or lymphoma (Group 1). None of the 46 pups from infected mothers developed lymphoma when suckled from uninfected surrogate mothers (Group 2), although 78% of these pups tested positive for ts1. Thirteen pups which developed lymphoma were from three different experimental conditions: Two of 29 pups from infected mothers that suckled from infected surrogate mothers developed lymphoma (Group 3). Six of 38 control pups from uninfected mothers developed lymphoma when they were allowed to suckle from infected surrogate mothers (Group 4). Five of 24 pups from infected mothers developed lymphoma when they suckled from their ts1 infected biological mothers (Group 5) (Table 1).

**Identification of Proviral Genome Integration Sites by Inverse-PCR (I-PCR):**

Spleen and lymph node tissues were used for this study in determining viral integration sites. Tissues from uninfected pups suckled from uninfected control mothers were used to obtain baseline values for fold change in gene expression levels but not for determining viral integration sites. Tissues from infected pups with lymphoma (LYM) and pups with no lymphoma (N LYM) were used for identification of viral integration sites. Of 720 colonies screened, 368 were white colonies indicating the insertion of viral genome into the murine genome and applied towards further analysis. Of these 368
colonies 209 proviral genomic insertion sites were found (Table 2). Fifty-two were intragenic and 157 were intergenic sites with over 90 unique intra and intergenic combined viral integration sites identified in mouse genome (Table 3). The maximum number of insertion sites (≥ 10) were found in chromosomes 1, 2, 5, 7, 10, 11, 12 and 13, with the most on chromosomes 10, 12 and 13 with 26, 32 and 45 insertions, respectively (Fig 1). Interestingly, of all 45 inserts in chromosome 13, 39 were at the same location from one animal, i.e. LYM6 (Fig 1). No inserts were found in chromosome 18 or the Y chromosome (Fig 1).

**The mRNA Expression of Candidate Genes in Spleens of Control Mice and Mice with Lymphoma:** Twenty-nine genes were selected for mRNA analysis by examining the effect of viral integration on their expression level based on 1) their proximity to specific genes (within 100kb); 2) listing in Retroviral Tagged Cancer Gene Database (http://RTCGD.ncifcrf.gov) for genes associated with cancer in relation to retroviral insertions; 3) their association with cancer development. The change in mRNA expression has been depicted in Table 4a and 4b and summarized in Fig. 2. The expression values of 29 genes examined included 11 candidate genes with intragenic VIS (viral integration site) (Table 4a) and 18 candidate genes with intergenic VIS (Table 4b).

mRNA expression levels for genes interrupted by VIS (intragenic) or adjacent to VIS (intergenic) was examined in mice. The fold change of mRNA expression level compared to control group ranged from 0.10 for *Ifng* to 19.48 for *Gfi1* (Table 4b – clear boxes). The mRNA expression of *Tacc3* and *Aurka* genes with intergenic VIS were found to undergo the most upregulation with averages of 9.2 and 2-fold increases in
mRNA expression respectively compared to controls. *Gfi1* showed the highest levels of mRNA expression with a 19.48-fold increase in LYM1. Among other animals of this group (LYM), an average of 7.1-fold upregulation was noted compared to the control group. *Gfi1* may be a marker of clinical significance because when the value of LYM1 (19.48), an outlier, was removed the standard deviation changed from borderline significance at p<0.06 to 0.002 (significant). *Ahi1* with intragenic VIS also showed significant increases in mRNA expression levels with an average of 3.8-fold upregulation compared to the control mice. The mRNA expression showed a 3.4-fold upregulation in LYM1, a 4.5-fold increase in LYM5 and a 7.8-fold increases in LYM6. All samples from the lymphoma group for *Tacc3* showed a range of 2.7-fold to 17.4-fold increases in expression when compared to control group. LYM5 showed 17.4-fold increase and LYM6 showed 16.1-fold increase. Similarly, a 2.59-fold upregulation of mRNA for *Aurka* in LYM5 was observed. Other members of the same lymphoma group showed increases of *Aurka* ranging from 1.08 to 2.2-fold compared to the control group. Table 5 shows the detail descriptions of these 29 genes, the insertion sites, mouse and human chromosome location and gene functions.

**Discussion:**

Although extensive investigations have been carried out on development of lymphoma using Moloney Murine Leukemia Virus (MoMuLV), so far no study is available on MoMuLV-ts1 (temperature sensitive) virus causing lymphoma among offspring of ts1 infected females. Specifically, available literature does not address viral transmission causing lymphoma via breast milk from biological or surrogate infected mothers to the
offspring. In our previous investigation we have reported that breast milk is a major source of ts1 infection among offspring of BALB/c mice (Duggan et al., 2004; Duggan et al., 2006). Transmission of retrovirus via breast milk is important, since transmission of HIV through breast milk is a major source of infection for mother-to-child transmission (MTCT) in humans (De Cock et al., 2000). Although Naarding et al. (2005) reported the beneficial effect of Lewis X (Le\(^x\)), a sugar epitope in human breast milk and proposed that binding of Le\(^x\) to DC-SIGN may reduce the HIV transmission to the infants breast fed by infected mothers, there is no doubt that breastfeeding is the major route of HIV transmission to babies of mothers with HIV infection (Coutsoudis et al., 2004). More work is needed regarding how specific molecules, such as Le\(^x\) in breast milk can be used to deter the entry of pathogenic microorganisms into the cells. Many studies have shown that once a virus such as EBV enters the cell and the viral genes insert into the host DNA, the host genome becomes altered leading to malignancies (Fan et al., 2005; J. Li et al., 1999; Liao et al., 1995; Scheijen et al., 1997; Shin et al., 2004). Many of these genes produce proteins which are responsible for causing various types of cancer. In this report, we have shown that the insertion of ts1 viral genome into the infected pups is associated with lymphoma. MoMuLv typically induces T-cell lymphoma in 100% of the infected mice with a mean latency period of 3-4 months (Fan, 1997). The phenotypes of the tumor cells are T-cells with CD4\(^+\)/CD8\(^-\);CD4\(^+\)/CD8\(^-\);CD4\(^+\)/CD8\(^+\) or CD4\(^+\)/CD8\(^-\). According to Fan (1997), the role of activated proto-oncogenes for tumorogenesis is a multistep process, similar to humans and important insight can be obtained from the MoMuLV studies.
We have observed distribution of VIS throughout the entire mouse genome with no preferred sites except one on chromosome 13 with 39 hits in LYM6 mouse. This suggests that viral integration into the mouse genome is random, similar to that reported for the HIV integration into the human genome (Lewinski et al., 2006). Only two VIS out of nearly 90 were identified in both groups, i.e. mice with lymphoma and mice without lymphoma, suggesting that these two groups may be distinct and that some VIS are not relevant in the development of lymphoma while other VIS are involved in lymphoma development. This suggests lymphoma development is a random event, developing only when certain sites were targeted for viral genome integration. This fits well with the hypothesis that viral integration will lead to lymphoma development only when it occurs in areas near genes related to lymphoma development. Several genes may be especially good candidates for lymphoma development. Expression patterns on Tacc3 and Gfi1 genes were similar in mice with lymphoma revealing upregulation for these genes, though to different degrees. For example, the expression level of Gfi1 in LYM1 is an exaggerated response in expression rather than low or no response to the stimuli showing the importance of this gene in this model. These genes might be in the same pathway leading to lymphoma development in these mice and thus have the same molecular mechanisms for lymphoma development. The same patterns of change in expression for these two genes were also observed in one mouse without lymphoma, NLYM1 suggesting that this mouse might have developed lymphoma if allowed to live longer. The mRNA expression fold increases for gene Irf4 in NLYM1 and NLYM4 and Gfi1 in NLYM1 and NLYM3 in the infected but no lymphoma group compared to the control group may indicate the onset of lymphoma development in these 3 mice before
overt clinical manifestations. Although it is possible that we have missed VIS in these mice with this assay, their similar expression patterns compared to that of mice where the VIS were found, suggests that these genes may be involved in some way in the molecular mechanism for lymphoma development.

The change in mRNA expression levels compared to the control group observed for these genes may be due to the VIS and may contribute, in part, to the lymphoma development observed in these mice. This argument is strengthened by the fact that other mice developing lymphoma showed similar patterns of change of mRNA expression of these genes compared to the controls. All other genes tested for mRNA expression levels both with intergenic and intragenic VIS showed unremarkable levels of expression compared to controls at this time. Expression levels tested in the ts1 infected no lymphoma (N LYM) group were also unremarkable in most part except for gene \textit{Irf4} with intergenic VIS. \textit{Irf4} mRNA gene expression showed 1.5 and 1.7-fold increases in N LYM1 and N LYM4, respectively. \textit{Gfi1} mRNA expression was also upregulated in the infected but no lymphoma (N LYM) group by 3.2-fold and 2.8-fold for N LYM1 and N LYM3 respectively, compared to the control group. It would be interesting to see if this mouse might have developed lymphoma if the end time point was set longer. Other genes such as \textit{Irf4} and \textit{Ccnd1} showed upregulation in only one of the mice with lymphoma. The molecular mechanisms for lymphoma development in these mice may be different compared to the other groups that showed similar patterns of change in their expression. Alternatively a given gene product might be upstream of the pathway showing no change in its expression. Other genes located downstream in the same pathway changed by other VIS might show changes in their expression.
Of the 29 target genes we have identified during this study, transforming acidic-coiled coil protein 3 (Tacc3) gene showed an over 9 fold increase in mRNA expression compared to controls. Overexpression of Tacc3 was related to progression of non-small cell lung cancer (NSCLC) in humans (Jung et al., 2006). Tacc3 proteins have also been shown to contribute to breast, prostate, multiple myeloma and ovarian cancer (Dhanasekaran et al., 2001; Lauffart et al., 2005; Still, Hamilton et al., 1999; Still, Vince et al., 1999). Jung et al. (2006) recognized that both absence or overexpression of Tacc3 may contribute to cancer production. These investigators proposed that Tacc3 may be important for cancer progression rather than the cause for cancer production. In our study overexpression of Tacc3 may be contributing to progression of lymphoma. Note that in the no lymphoma (NLYMP) group, no significant increase in Tacc3 mRNA expression was observed, suggesting that its function was for cancer progression and not production. In this group, the lymphoma had not yet been produced, therefore no progression is underway. All three types of Tacc proteins, i.e. Tacc1, Tacc2 and Tacc3 are components of the centrosomal spindle involved in microtubule stabilization and hematopoetic development. Tacc3 interact with nuclear transcription factors (Piekorz et al., 2002; Still et al., 2004). Deficiency of Tacc3 leads to P53 mediated apoptosis. Therefore, overexpression of Tacc3 as it has been observed in our mice may be causing inhibition of apoptosis thus leading to lymphoma.

The other gene with higher than a 2 fold increase in mRNA activity is Aurora kinase A (Aurka). Overexpression of Aurka mRNA has been associated with a number of cancers including head and neck squamous cell carcinoma, esophageal squamous cell carcinoma, glioma, pancreatic cancer, hepatocellular carcinoma and bladder cancer.
Abnormal centrosome and spindle formation due to increased Aurka mRNA expression has been reported to promote tumor progression (Reiter et al., 2006) and Aurka inhibitors have been proposed for cancer treatment (Andrews, 2005). Upregulation of Aurka in breast milk mediated lymphoma in our mouse model has a good potential for testing the potential of Aurka inhibitors, which may be applied for therapeutic purposes.

Abelson helper integration site-1 (Ahi1) gene encodes a family of proteins involved in leukemogenesis (Jiang et al., 2004). Although Ahi1 mRNA expression has been proposed to contribute to the development of human leukemia (Jiang et al., 2004), possibly a different gene is involved in the process of lymphogenesis (Kim et al., 2003). The intragenic location of our VIS in Ahi1 makes it quite interesting to understand the interactions of nearby genes at this location for lymphoma. Since the function of Ahi1 protein has not yet been well established, it seems logical to speculate that this protein may be involved in the signal transduction pathway. Our model provides the opportunity to study it further regarding whether an increase in Ahi1 mRNA expression is involved in the development of lymphoma or its progression.

Although retroviral integration into the host genome of a mouse model has been very informative regarding how genomic alteration causes diseases, the process is not the same as observed in human retroviral integration (Lewinski et al., 2006). For example, HIV’s most preferred site of integration is in the active transcription units while murine leukemia virus (MLV) integration occurs near the transcription start sites and CpG islands. HIV can integrate at any time of the cell cycle, while MLV can integrate
only after mitosis (Lewinski et al., 2006). Using a MLV/HIV hybrid, Lewinski et al. (2006) showed distinct favored viral integration in HIV versus MLV. They concluded that viral integrase (IN) is the factor which determines the site of choice of integration. Therefore, further work is needed to understand the similarities and differences between the genomic involvements of murine versus HIV causing malignancy. Our MoMuLV-ts1 model provides an important system for this study of the viral gene integration and its relationship to lymphoma development, caused by natural transmission via breast milk.

Acknowledgement:

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References:


alters the pattern of insertional activation and identifies new common insertion sites. 


function evolution of the transforming acidic coiled coil genes revealed by analysis of

acidic coiled coil-containing gene family, TACC3, maps in 4p16, close to
translocation breakpoints in multiple myeloma, and is upregulated in various cancer
cell lines. *Genomics, 58*(2), 165-170.

(2002). New genes involved in cancer identified by retroviral tagging. *Nat Genet,
32*(1), 166-174.

(2005). The clinical significance of Aurora-A/STK15/BTAK expression in human

Overexpression of Aurora-A contributes to malignant development of human
Table 1: Five groups of animals showing route of transmission, rates of infection and development of lymphoma

<table>
<thead>
<tr>
<th>Group #</th>
<th>Offspring</th>
<th>Surrogate Mother</th>
<th>Sample Size</th>
<th>PCR Analysis</th>
<th>Lymphoma</th>
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<td></td>
<td></td>
<td></td>
<td></td>
<td># Positive ts1</td>
<td>%</td>
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<td>Ctrl</td>
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<td>Gr 3 (n=29)</td>
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<td>ts1</td>
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<td>29</td>
<td>100.0</td>
</tr>
<tr>
<td>Gr 4 (n=38)</td>
<td>Ctrl</td>
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<td>37</td>
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<td>Gr 5 (n=24)</td>
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<td>biological mother</td>
<td>24</td>
<td>24</td>
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</table>

Ctrl - Control (no infection)
ts1 - Infected (positive for MoMuLV-ts1)

Table 2: Summary of the analysis of colonies with virus insertion sites (VIS) from pups with infection but no lymphoma (NLYM) and pups with infection and lymphoma (LYM).

<table>
<thead>
<tr>
<th></th>
<th>Number of colonies analyzed</th>
<th>Number of colonies with inserts</th>
<th>Number of colonies with specific VIS sites</th>
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<tr>
<td>N LYM 1</td>
<td>61</td>
<td>23</td>
<td>4</td>
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<tr>
<td>N LYM 2</td>
<td>50</td>
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<tr>
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<td>12</td>
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<td>LYM 1</td>
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<td>LYM 4</td>
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<td>LYM 5</td>
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<td>LYM 6</td>
<td>111</td>
<td>43</td>
<td>39</td>
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<tr>
<td>Total</td>
<td>720</td>
<td>368</td>
<td>209</td>
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Table 3: Showing the intragenic and intergenic viral integration sites and their chromosome locations.

<table>
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<tr>
<th>Chromosome</th>
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Total no. of hits 52
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## Table 4a and 4b: Gene Expression (Comparative ddCT Method)

### Table 4a: Intragenic Viral Integration Sites (VIS)

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| **ts1 Infected Lymphoma** |      |       |      |      |         |      |          |      |     |        |      |
| LYM1             | 0.88 | 1.77  | 3.38 | 0.65 | 1.71    | 0.55 | 1.25     | 0.97 | 1.06| 1.14   | 0.40 |
| LYM2             | 1.55 | 0.71  | 1.25 | 0.39 | 1.92    | 0.91 | 0.37     | 0.13 | 0.69| 0.82   | 0.36 |
| LYM3             | 0.98 | 1.65  | 2.04 | 1.96 | 0.23    | 0.31 | 3.23     | 39.43| 0.39| 0.60   | 0.48 |
| LYM4             | 1.48 | 0.92  | 3.81 | 1.70 | 0.62    | 2.31 | 0.64     | 0.50 | 1.20| 0.70   | 0.95 |
| LYM5             | 0.79 | 1.11  | 4.50 | 3.06 | 6.64    | 1.44 | 0.67     | 0.95 | 1.79| 0.94   | 1.83 |
| LYM6             | 0.92 | 3.77  | 7.83 | 1.15 | 2.77    | 0.44 | 1.23     | 1.21 | 0.58| 1.08   | 0.86 |
| **Av. Fold Expr.** | 1.10 | 1.66  | 3.80 | 1.49 | 2.32    | 0.99 | 1.23     | 7.20 | 0.95| 0.88   | 0.81 |
| ± SEM           | 0.15 | 0.50  | 1.03 | 0.44 | 1.03    | 0.34 | 0.46     | 7.06 | 0.23| 0.09   | 0.25 |
| **p value < 0.05** | 0.70 | 0.26  | 0.03 | 0.39 | 0.23    | 0.99 | 0.69     | 0.38 | 0.91| 0.42   | 0.50 |

SEM: Standard error of the mean. Shaded boxes show the genes and the level of significance.
### Table 4b: Intergenic Viral Integration Sites

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| ts1 Infected No Lymphoma | | | | | | | | | | | | | | | | | | |
| N LYM1 | 1.97 | 1.46 | 2.72 | 0.50 | 1.22 | 3.21 | 0.74 | 1.19 | 1.75 | 1.23 | 1.91 | 1.08 | 1.79 | 1.66 | 0.28 | 0.29 | 0.81 | 1.40 |
| N LYM2 | 1.20 | 1.16 | 1.29 | 1.50 | 1.21 | 1.22 | 1.53 | 1.12 | 1.10 | 1.43 | 1.01 | 0.90 | 1.19 | 1.24 | 0.69 | 0.51 | 0.65 | 0.68 |
| N LYM3 | 0.95 | 1.16 | 0.69 | 1.13 | 0.82 | 2.76 | 1.33 | 1.09 | 0.72 | 1.40 | 1.23 | 0.84 | 0.54 | 0.78 | 0.53 | 0.37 | 0.49 | 0.59 |
| N LYM4 | 1.88 | 1.71 | 2.66 | 0.79 | 0.93 | 1.18 | 0.90 | 0.96 | 0.96 | 1.39 | 1.36 | 0.65 | 0.85 | 1.09 | 0.36 | 0.54 | 0.45 | 1.06 |
| **Av. Fold Expr.** | 1.50 | 1.37 | 1.84 | 0.98 | 1.05 | 2.09 | 1.13 | 1.09 | 1.13 | 1.36 | 1.38 | 0.87 | 1.09 | 1.19 | 0.47 | 0.43 | 0.60 | 0.93 |
| ± SEM | 0.29 | 0.15 | 0.58 | 0.25 | 0.12 | 0.60 | 0.21 | 0.06 | 0.25 | 0.05 | 0.22 | 0.10 | 0.31 | 0.21 | 0.11 | 0.07 | 0.10 | 0.22 |
| p value < 0.05 | 0.14 | 0.07 | 0.22 | 0.95 | 0.80 | 0.13 | 0.71 | 0.39 | 0.69 | 0.29 | 0.28 | 0.41 | 0.68 | 0.46 | 0.01 | 0.00 | 0.29 | 0.89 |

| ts1 Infected Lymphoma | | | | | | | | | | | | | | | | | | |
| LYM1 | 0.71 | 0.56 | 6.29 | 0.78 | 2.21 | | 0.58 | 1.25 | 0.49 | 0.68 | 0.13 | 1.12 | 0.59 | 0.72 | 0.18 | 1.11 | 0.49 | 0.70 |
| LYM2 | 1.83 | 1.03 | 8.71 | 5.00 | 1.30 | 5.96 | 1.18 | 0.97 | 1.08 | 0.52 | 0.48 | 1.82 | 0.44 | 0.47 | 0.84 | 1.28 | 3.00 | 0.81 |
| LYM3 | 1.16 | 1.03 | 2.66 | 0.75 | 1.08 | 6.52 | 0.93 | 0.96 | 0.65 | 0.94 | 0.11 | 0.68 | 1.39 | 0.97 | 0.27 | 0.63 | 0.13 | 0.18 |
| LYM4 | 1.28 | 1.04 | 3.71 | 1.36 | 1.86 | 3.04 | 1.33 | 1.01 | 0.53 | 1.12 | 0.15 | 0.90 | 1.01 | 1.52 | 0.43 | 0.49 | 0.29 | 0.13 |
| LYM5 | 1.20 | 1.26 | 17.42 | 1.11 | 2.59 | 4.15 | 1.10 | 1.81 | 1.02 | 0.07 | 1.14 | 1.76 | 0.85 | 1.14 | 0.72 | 0.67 | 0.95 | 1.15 |
| LYM6 | 1.12 | 2.68 | 16.14 | 1.23 | 1.95 | 3.87 | 0.52 | 0.66 | 0.38 | 0.32 | 0.10 | 1.14 | 0.58 | 0.61 | 0.88 | 0.60 | 0.51 | 0.40 |
| **Av. Fold Expr.** | 1.22 | 1.27 | 9.16 | 1.71 | 1.83 | 7.17 | 0.94 | 1.11 | 0.69 | 0.61 | 0.35 | 1.24 | 0.81 | 0.91 | 0.55 | 0.80 | 0.90 | 0.56 |
| ± SEM | 0.16 | 0.33 | 2.81 | 0.73 | 0.25 | 2.76 | 0.15 | 0.17 | 0.13 | 0.17 | 0.18 | 0.20 | 0.16 | 0.17 | 0.13 | 0.14 | 0.48 | 0.18 |
| p value < 0.05 | 0.27 | 0.44 | 0.02 | 0.35 | 0.01 | 0.06 | 0.84 | 0.57 | 0.28 | 0.28 | 0.08 | 0.32 | 0.39 | 0.69 | 0.02 | 0.21 | 0.85 | 0.38 |

SEM: Standard error of the mean. Shaded boxes show the genes and the level of significance. Clear boxes show the highest (Gfi1) and the lowest (Irfng) levels of fold changes in mRNA expression.
<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Gene Name</th>
<th>Mouse Chr no.</th>
<th>No. Hits</th>
<th>Human Homolog</th>
<th>Intragenic / Intergenic VIS</th>
<th>Function</th>
<th>Accession</th>
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<tr>
<td>Elk3</td>
<td>ETS oncogene family</td>
<td>10C-D1</td>
<td>9</td>
<td>12q23</td>
<td>Intragenic</td>
<td>Repressor of heme oxygenase 1 (HO-1) gene transcription involved in cell migration</td>
<td>NM_013508.1</td>
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<td>Dusp22 and Irf4</td>
<td>Dual specificity phosphatase 22 and Interferon regulatory factor 4</td>
<td>13A3</td>
<td>39</td>
<td>6p25</td>
<td>Intergenic</td>
<td>Dusp22 is a positive regulator of the JNK pathway IRF4 is central in protecting CD4(+) cells against proapoptotic stimuli</td>
<td>NM_0010379; (Dusp22) and NM_13674.1 (Irf4)</td>
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<tr>
<td>Ncor2</td>
<td>Nuclear receptor co-repressor 2</td>
<td>5F</td>
<td>3</td>
<td>12q24</td>
<td>Intragenic</td>
<td>NCoR/SMRT-interacting domain transforms AML1-ETO into a potent leukemogenic protein</td>
<td>NM_011424.1</td>
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<td>Ahi1</td>
<td>Abelson helper integration site</td>
<td>10 A3</td>
<td>1</td>
<td>6q23.3</td>
<td>Intragenic</td>
<td>Ahi-1/AHI-1 express in primitive hematopoietic cells</td>
<td>BC055400</td>
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<tr>
<td>Evi5 and Gfi1</td>
<td>Ecotropic viral integration site 5 and growth factor independent 1</td>
<td>5F</td>
<td>1</td>
<td>1p22</td>
<td>Intragenic</td>
<td>Ev5 is a common site for retroviral integration into T-cell lymphomas Gfi1b was found to be up-regulated in early stages of B-cell and in a subset of early T-cell development</td>
<td>NM_007964.1; (Evi5) and NM_010278.1 (Gfi1)</td>
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<td>Ccnd1</td>
<td>Cyclin D1</td>
<td>7F5</td>
<td>8</td>
<td>11q13</td>
<td>Intergenic</td>
<td>Cyclin D1 up-regulation in intestinal neoplasia is important for tumor progression rather than initiation. contribute to oncogene in malignancy</td>
<td>NM_007631.1</td>
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<tr>
<td>Tde1 (Serinc3)</td>
<td>Tumor differentially expressed protein 1 (serine incorporator 3)</td>
<td>2H3</td>
<td>3</td>
<td>20q13</td>
<td>Intragenic</td>
<td>Inhibits apoptosis and stimulates tumorigenesis</td>
<td>NM_012032.2</td>
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<tr>
<td>Aurka</td>
<td>Aurora kinase A</td>
<td>2H3</td>
<td>4</td>
<td>20q13</td>
<td>Intergenic</td>
<td>Overexpression promotes cell proliferation and inhibits apoptosis</td>
<td>NM_011497.2</td>
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<tr>
<td>Tgfa</td>
<td>Transforming growth factor alpha</td>
<td>6D1</td>
<td>1</td>
<td>2p13</td>
<td>Intragenic</td>
<td>Tumor-associated endothelial cells. TGF-alpha inhibits apoptosis in mouse blastocysts</td>
<td>NM_031199.1</td>
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<td>Gene Name</td>
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<td>Human Homolog</td>
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<tr>
<td>Tacc3</td>
<td>Transforming acidic coiled-coil containing protein 3</td>
<td>5B3</td>
<td>11</td>
<td>4p16</td>
<td>Intergenic</td>
<td>Role in hematopoietic stem cell function and interface with p53-regulated apoptosis. Stabilize microtubules and contribute to cancer</td>
<td>NM_11524.2</td>
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<td>Prdm16</td>
<td>PR domain containing 16</td>
<td>4E2</td>
<td>1</td>
<td>1p36</td>
<td>Intergenic</td>
<td>Aberrant expression associated with hypomethylation in adult T-cell leukemia cells</td>
<td>NM_027504.2</td>
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<tr>
<td>Bcl11a</td>
<td>B-cell CLL/lymphoma 11A (zinc finger protein)</td>
<td>11A3</td>
<td>2</td>
<td>2p16</td>
<td>Intergenic</td>
<td>Bcl11a is essential for postnatal development and normal lymphopoiesis.</td>
<td>NM_016707.1</td>
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<tr>
<td>Ppp1r16B</td>
<td>Protein phosphatase 1, regulatory (inhibitor) subunit 16B</td>
<td>2H1</td>
<td>1</td>
<td>20q11.23</td>
<td>Intragenic</td>
<td>mRNA is located in cell bodies and dendrites of neurons in four distinct regions of the brain</td>
<td>NM_153089.2</td>
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<tr>
<td>Rgs3</td>
<td>Regulator of G-protein signaling 3</td>
<td>4B3</td>
<td>1</td>
<td>9q32</td>
<td>Intragenic</td>
<td>Induces heat shock response element (HSE)-dependent gene transcription</td>
<td>NM_019492.1</td>
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<tr>
<td>Cit</td>
<td>Citron kinase</td>
<td>5F</td>
<td>1</td>
<td>12q24</td>
<td>Intragenic</td>
<td>Functions in the control of G(2)/M transition in the hepatocyte cell cycle</td>
<td>NM_007708.2</td>
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<tr>
<td>Il17ra</td>
<td>Interleukin 17 receptor A</td>
<td>6 F1</td>
<td>1</td>
<td>22q11</td>
<td>Intragenic</td>
<td>May belong to a novel growth-receptor like molecule with capability to support cellular mitogenesis</td>
<td>NM_008359.1</td>
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<tr>
<td>Mid1</td>
<td>midline 1</td>
<td>X F5</td>
<td>1</td>
<td>Xp22</td>
<td>Intragenic</td>
<td>X-linked polydactyly (Xpl) and Patchy-fur (Paf) mutant mice</td>
<td>NM_010797.1</td>
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<td>Evi1</td>
<td>Ecotropic viral integration site 1</td>
<td>3 A3</td>
<td>1</td>
<td>3q24-q28</td>
<td>Intergenic</td>
<td>Promotes cell proliferation. Evi1 directly binds to GATA-2 promoter as an enhancer</td>
<td>NM_007963.1</td>
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<tr>
<td>Gene Symbol</td>
<td>Gene Name</td>
<td>Mouse Chr no.</td>
<td>No. Hits</td>
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<td>Zfp42</td>
<td>Zinc finger protein 42</td>
<td>8 A4</td>
<td>2</td>
<td>4q35.2</td>
<td>Intergenic</td>
<td>Embryonic stem cell marker and pluripotent stem cell marker</td>
<td>XM_284454.5</td>
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<tr>
<td>Irf2bp2</td>
<td>Interferon regulatory factor 2 binding protein 2</td>
<td>8 E2</td>
<td>1</td>
<td>1q42.3</td>
<td>Intergenic</td>
<td>No gene reference into function</td>
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<td>Ilng</td>
<td>Interferon gamma</td>
<td>10 D2</td>
<td>1</td>
<td>12q14</td>
<td>Intergenic</td>
<td>Participates in death of dopaminergic neurons; protection against infection</td>
<td>NM_008337.1</td>
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<tr>
<td>Il5 and Irf1</td>
<td>Interleukin 5 / interferon regulatory factor 1</td>
<td>11 A5/B1 - 11 B1.3</td>
<td>1</td>
<td>5q31.1</td>
<td>Intergenic</td>
<td>Eosinophils on the development and mammary gland, uterus and ovary function; Mice lacking Irf1 develop CD30+ lymphoproliferative disease</td>
<td>NM_010558.1 (Il5) and NM_008390.1 (Irf1)</td>
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<tr>
<td>Cldn5</td>
<td>Claudin 5</td>
<td>16 A3</td>
<td>2</td>
<td>22q11.21</td>
<td>Intergenic</td>
<td>Claudin-5 is specifically altered in utrophin/dystrophin-deficient (double knockout, dko hearts)</td>
<td>NM_013805.2 (Cldn5)</td>
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<tr>
<td>Vamp8</td>
<td>Vesicle-associated membrane protein 8</td>
<td>6 C1</td>
<td>1</td>
<td>2p12-p11.2</td>
<td>Intergenic</td>
<td>VAMP-8 is required for release from dense core granules, alpha granules, and lysosomes. Regulate exocytosis of pancreatic acinar cells</td>
<td>NM_016794.2</td>
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<tr>
<td>Myc</td>
<td>Myelocytomatosis oncogene</td>
<td>15 D2-D3</td>
<td>2</td>
<td>8q24.21</td>
<td>Intergenic</td>
<td>Required for development of B but not pre-B cell lymphomas from cmyc overexpressing tumor progenitors. Myc is required for a normal hypertrophic response</td>
<td>NM_010849.4</td>
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<tr>
<td>Cd274</td>
<td>CD 274 antigen; programmed cell death 1 ligand 1</td>
<td>19 C2</td>
<td>1</td>
<td>9p24</td>
<td>Intergenic</td>
<td>Also known as B7-H1; PD-L1; Pdcd111; Pdcd11lg1. Interaction crucially controls the effector differentiation of autoreactive T cells to maintain self-tolerance</td>
<td>NM_021893.2</td>
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Figure 1

MoMuLV-ts1 Retroviral Insertion Sites on Mouse Genome

Mouse Chromosome Lenght (Mb)

Mouse Chromosome Number

Chromosome

Viral integration sites (ts1 + No Lymphoma)

Viral integration sites (ts1 + Lymphoma)

Total # Hits on each chromosomes
Gene Expression (Comparative ddCt Method)

-3.00 2.00 7.00 12.00

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Fold Expression</th>
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<td>Elk3</td>
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<td>Dusp22</td>
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<td>Ncor2</td>
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<td>Ahi1</td>
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<td>Irf4</td>
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<td>Evl5</td>
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<td>Tacc3</td>
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<td>Ccnd1</td>
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<td>Serinc3/Tde1</td>
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<td>Aurka</td>
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<td>Tgfα</td>
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<td>Gfi1</td>
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<td>Ppp1r16β</td>
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<td>Cd274</td>
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- Infected No Lymphoma
- Infected Lymphoma

Figure 2
Table 1: Five groups of offspring from DMEM injected control or MoMuLV-ts1 infected mothers, left to suckle from the control or infected surrogate mothers, except group 5 which suckled from their biological mothers. Gr 1 (control) had no infection or lymphoma. Gr 2 had 78% infection (column 5) but no lymphoma (column 8). Gr 3, 4 and 5 had almost 100% infection rate and 6.9%, 15.8% and 20.8%, respectively, developed lymphoma (bold numbers). A total of 13 offspring (column 8) of 126 infected (column 4) developed lymphoma. Control: ctrl; MoMuLV-ts1 infected: ts1.

Table 2: Table summarizing the number of colonies analyzed from tissues of individual animals and number of colonies with viral genome inserts.

Table 3: Table showing intragenic and intergenic viral integration sites with number of insertion site(s) in individual chromosomes. For intergenic insertion sites, the location of upstream and downstream genes have been included in column 1 and column 2.

Tables 4a and 4b: Tables showing the increase in gene expression by mRNA quantitation by using comparative ddC_T method. The effects of viral integration sites (VIS) in two groups of experimental animals were studied in comparison to the control animals. These two experimental groups included animals with infection but no lymphoma (NLYM) and animals with infection and lymphoma.
Boxed numbers show the original animals in which the mRNA expressions were measured.

Table 4a: This Table shows the effect of intragenic VIS. Significant increase of mRNA expression of one gene, i.e., Ahi1 was observed in animals with lymphoma (LYM). None of the other genes showed significant alteration.

Table 4b: Table showing the effect of intergenic VIS on mRNA expression of several genes, including Tacc3, Aurka and Vamp8. Tacc3 and Aurka show significant upregulation, while Vamp8 showed significant down regulation. Tacc3 and Aurka were affected in LYM group only, while Vamp8 was down regulated in both LYM and NLYM groups.

Table 5: Summarizes the 29 genes studied including their symbols, gene name, VIS location on mouse chromosomes, no. of hits on mouse chromosomes (column 4), human homolog, relative position of VIS to gene, gene function and their accession numbers.

Figure 1: This is a graphical representation, showing the MoMuLV-ts1 insertion sites on mouse chromosomes. This is a representative summary of the insertion sites and does not include all insertions in each chromosome. All animals showed the ts1 viral genome insertion sites (VIS) on their chromosomes. Maximum number of insertions were found on chromosomes 10 (26), 12 (32) and
13 (45). Moderate number of insertions were found on chromosomes 1 (10), 2 (13), 5 (18), 7 (13), 11 (11). Chromosomes 18 and Y did not have any VIS in any of the animals. One mouse (LYM6) had 39 hits on chromosome 13.

Figure 2: Graphical representation of the mRNA expression of 29 genes, summarized from Table 3A and 3B, using comparable ddC_T method. Ahi1, Tacc3 and Aurka showed significant increase in mRNA expression.
Appendix C:

Abstract Title:-
Determination Of Phenotype And mRNA Expression In a Retrovirus Induced Murine Lymphoma Model.
Hussein Bagalb1, Henry Okonta1, Joan M. Duggan, MD, FACP2, Joana Chakraborty, PhD1, Department of Physiology & Pharmacology1, Department of Medicine2, University of Toledo, Toledo, OH 43614

Presented at the joint meeting of ICAAC and IDSA, Oct-2008
46th Annual Meeting of the Infectious Diseases Society of America.

Background: Approximately 95% of HIV-1-associated lymphomas are considered to be of B-cell origin. The vast majority of these tumors are high grade B-cell lymphoma. The objective of the current study is to determine: 1) the phenotype of lymphoma associated with temperature sensitive Moloney Murine Leukemia Virus (Mo-MuLV-ts1) and 2) the alteration in mRNA expression of these lymphomas.

Methods: A total of 117 BALB/c pups were divided into 4 groups: 24 (group #1) from infected mothers were allowed to suckle from their infected biological mother; 38 control pups (group #2) from non-infected mother suckled from surrogate infected mothers; 46 pups of infected mothers (group #3), suckled from control surrogate mothers and 9 control pups (group #4) suckled from their non infected biological mother. Splenic tissues were used for immunohistochemistry and qRT-PCR. Antibodies to CD3 and CD79a were used to identify the T-and B-cell lymphomas respectively. mRNA expression levels for each of 28 genes were determined using a standard curve method, normalized for GAPDH gene expression.
**Results:** Group #1 and #2 pups that suckled from infected mother were almost 100% infected with 16% and 21% incidence rates for lymphoma. Group #3 pups were infected (78%) when they suckled from the control mothers, but none developed lymphoma. A total of 11 pups developed lymphoma of which 5 suckled from their infected biological mothers and developed T-cell lymphoma. Six pups from group #2, developed both T-and B-cell lymphoma. These lymphomas expressed significantly higher levels of mRNA for two genes (*Gfi1* and *Ncor2*), Two other genes (*Ahi1* and *Tacc3*) were found to be generally upregulated in all lymphomas.

**Conclusion:** We have concluded that the route of transmission for ts1 virus may 1) determine the type and progression of the lymphoma. 2) play a role in controlling the integration site and possible alteration of the nearby genes. This work was supported by American Cancer Society, Ohio Division and F.M. Douglas Foundation, of St.Vincent Medical Center.
ABSTRACT

The objective of this study is to determine the phenotype of lymphoma associated with temperature sensitive Moloney Murine Leukemia Virus (Mo-MuLV-ts1) retrovirus and the alteration in specific genes of mRNA expression in these lymphomas. MoMuLV-ts1 infection in BALB/c mice mimics HIV infection in humans. In previous work, we have demonstrated breast milk transmitted Mo-MuLV-ts1 infection and subsequent lymphoma development in offspring. In this experiment, a total of 146 pups from BALB/c mice were divided into 5 groups; one control and 4 experimental. Splenic tissues were used for immunohistochemistry, Inverse-PCR and quantitative real-time PCR (RT-PCR). Through studying viral integration sites, twenty-seven genes were identified as candidate genes in lymphoma development. mRNA expression levels for each of 27 genes were determined using a standard curve method, normalized for GAPDH gene expression. A total of 13 pups developed lymphoma of which 7 suckled either from their infected biological or surrogate mothers and developed T-cell lymphoma (Group #1 and #2). Six pups from control mother that suckled from infected surrogate mother (Group #3) developed T-cell lymphoma and B-cell lymphoma. The cells of these lymphomas expressed significantly higher levels of mRNA for two genes (Gfi1 and Ncor2). Two other genes (Ahi1 and Tacc3) were found to be generally significantly upregulated in all lymphomas. Our results indicated that ts1 integration activates the expression of four cellular genes including Ahi1, Ncor2, Tacc3, and Gfi1 that may contribute to lymphomagenesis.