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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, and 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. The 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.

Introduction

During the current investigation, we have used the Moloney 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 Moloney 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/NF-kB transcription factor, (Pak and 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 and 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 and Varmus, 1982; Rosenberg and 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

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Table 1
Five groups of animals showing route of transmission, rates of infection and development of lymphoma

Group #	Offspring		Surrogate mother	Sample size	PCR analysis				Lymphoma	
					Positive ts1		Negative ts1		#	%
					#	%	#	%		
Gr 1 (n=9)	Ctrl	→	Ctrl	9	0	0.0	9	100.0	0	0.0
Gr 2 (n=46)	ts1	→	Ctrl	46	36	78.3	10	21.7	0	0.0
Gr 3 (n=29)	ts1	→	ts1	29	29	100.0	0	0.0	**2**	**6.9**
Gr 4 (n=38)	Ctrl	→	ts1	38	37	97.4	1	2.6	**6**	**15.8**
Gr 5 (n=24)	ts1	→	Biological mother	24	24	100.0	0	0.0	**5**	**20.8**

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

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 is possible by analysis of retroviral common integration sites in tumors based on the mouse genome sequence (Kim et al., 2003; 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 developed lymphoma, thus providing an excellent control system. The pups of the same litter had a 100% infection rate, but with or without the lymphoma. 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 that 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* proto-oncogene 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) (Jeng et al., 2004 and Blattner, 1999). It must be noted that HIV-1 infection causes considerable increase in cancer burden through multiple mechanisms. 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.

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

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)

	Number of colonies analyzed	Number of colonies with inserts	Number of colonies with specific VIS sites
N LYM 1	61	23	4
N LYM 2	50	20	13
N LYM 3	50	38	20
N LYM 4	50	28	12
LYM 1	108	75	46
LYM 2	110	61	23
LYM 3	80	26	14
LYM 4	50	19	7
LYM 5	50	35	31
LYM 6	111	43	39
Total	720	368	209

Table 3
The intragenic (a) and intergenic (b) viral integration sites and their chromosome locations

Chromosome	# Intragenic hits	RIS name	Gene ID upstream	Gene ID downstream
1	2	5430435G22Rik	Ctse	Slc26a9
1	1	Traf3ip3	A130010J15Rik	Hsd11b1
2	3	Tde1/Serinc3	5830472M02Rik	Pkig
2	1	Ppp1r16b	Actr5	2310007D09Rik
4	1	Gpr51	A630005A	Samd6
4	1	170012312Rik	LOC666845	LOC383991
4	1	Rgs3	4933430I17Rik	Zfp618
5	1	Evi5	Gfi1	Rpl5
5	1	Snx8	Nudt1	Eif3s9
5	3	Ncor2	3110032G18Rik	Scarb1
5	1	Cit	Ccdc64	Prkab1
6	1	Tgfa	Add2	2010309E21Rik
6	1	Vamp5	AF119384	Vamp8
6	1	Il17ra	Jarid1a	Cecr6
7	1	A930008G19Rik	23100007H09Rik	2010208k18Rik
7	1	LOC665427	6430701C03Rik	Clcn4-2
9	1	2310001H13Rik	Ulk4	Cck
9	1	Trak1	Ctnnb1	Cck
9	1	Ube1l	Traip	D330022AD01Rik
10	1	Ahi1	Pde7b	Hbs1l
10	1	Al317395	BC021785	Slc16a10
10	9	Elk3	Pctk2	Lta4h
10	1	B4galnt1	4632413K17Rik	C78409
11	1	Jmjd3	2600017H02Rik	2900022L05Rik
11	2	Rnf43	Gm739	Supt4h1
12	1	Cpsf2	Mjd	Slc24a4
13	3	Aytl2	Mrpl36	Slc6a3
15	1	1110014D18Rik	E430025E21Rik	Trib1
16	2	Lpp	LOC433009	5430420C16RiK
17	1	Trml2	Trml4	B430306N03Rik
19	1	Exoc6	Hhex	LOC546726
19	2	Gprk5	Prdx3	BC029127
19	1	2810048G17Rik	Pdcd11	LOC546729
X	1	Mid1	G53001100Rik	LOC667853
Total no. of hits	52			

Chromosome	# Intergenic hits	RIS name	Gene ID upstream	Gene ID downstream
1	2	N/A	LOC624702	LOC624755
1	1	N/A	Xpr1	Acbd6
1	2	N/A	St8sia4	Gm1833
1	1	N/A	1700027A15Rik	Tns1
1	1	N/A	Pthr2	Crygf
1	1	N/A	Dtymk	Ing5
2	4	N/A	Aurka	Cstf1
2	1	N/A	Stxbp1	9130404D14Rik
2	1	N/A	Cd93	Nxt1
3	1	N/A	Evi1	Mds1
4	1	N/A	Prdm16	Arpm2
5	11	N/A	Tacc3	Fgfr3
5	1	N/A	BC003324	6330548G22Rik
5	2	N/A	Actb	Fscn1
6	1	N/A	Vamp5	Vamp8
7	8	N/A	Ccnd1	Tpcn2
7	1	N/A	Josd2	2310044H10Rik
7	1	N/A	Furin	Blm
8	2	N/A	LOC244558	LOC824855
8	2	N/A	Zfp42	Adam26b
8	1	N/A	Irf8	Foxf1a
8	1	N/A	Irf2bp2	A630001012Rik
9	1	N/A	D630044F24Rik	LOC628947
9	1	N/A	Ap1m2	Slc44a2
9	1	N/A	Uaca	Tle3
9	1	N/A	Rab11a	LOC665584
10	1	N/A	Ifng	Dyrk2
11	2	N/A	Bcl11a	LOC628476
11	2	N/A	Canx	LOC622343
11	1	N/A	Il5	Irf1
11	1	N/A	Prpsap1	Sphk1
11	1	N/A	Sfrs2	2600014M03Rik
12	35	N/A	LOC674120	LOC67121
12	1	N/A	Trib2	Lpin1
12	1	N/A	LOC627607	6430527G18Rik
12	2	N/A	Dus4l	Gpr22
13	1	N/A	Elov12	LOC268650
13	39	N/A	Dusp22	Irf4
13	1	N/A	Fbp1	2010111l01Rik
13	1	N/A	Ell2	Glrx
14	1	N/A	Olfr750/GA	Ang1
14	1	N/A	Extl3	Fzd3
14	1	N/A	Slc25a30	Tpt1
15	2	N/A	LOC432956/ A1bg	Myc
16	2	N/A	Cldn5	LOC622795
16	4	N/A	Sept5	Cldn5
17	1	N/A	Foxp4	1700067P10Rik
19	1	N/A	5033414D02Rik	Cd274
19	1	N/A	2700046G09Rik	Minpp1
X	1	N/A	Hcfc1	Irak1
X	1	N/A	Sat1	Acot9
X	1	N/A	Apln	Xpnpep2
Total no. of hits	157			

For intergenic insertion sites, the locations of upstream and downstream genes have been included in column 1 and column 2 R1s: Retrovirus insertion sites.

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 and none of the animals showed viral inserts in 18 or Y chromosomes (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 100 kb); 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) were 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 4a — clear boxes). The mRNA expression of *Tacc3* and *Aurka* genes with intergenic VIS was 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

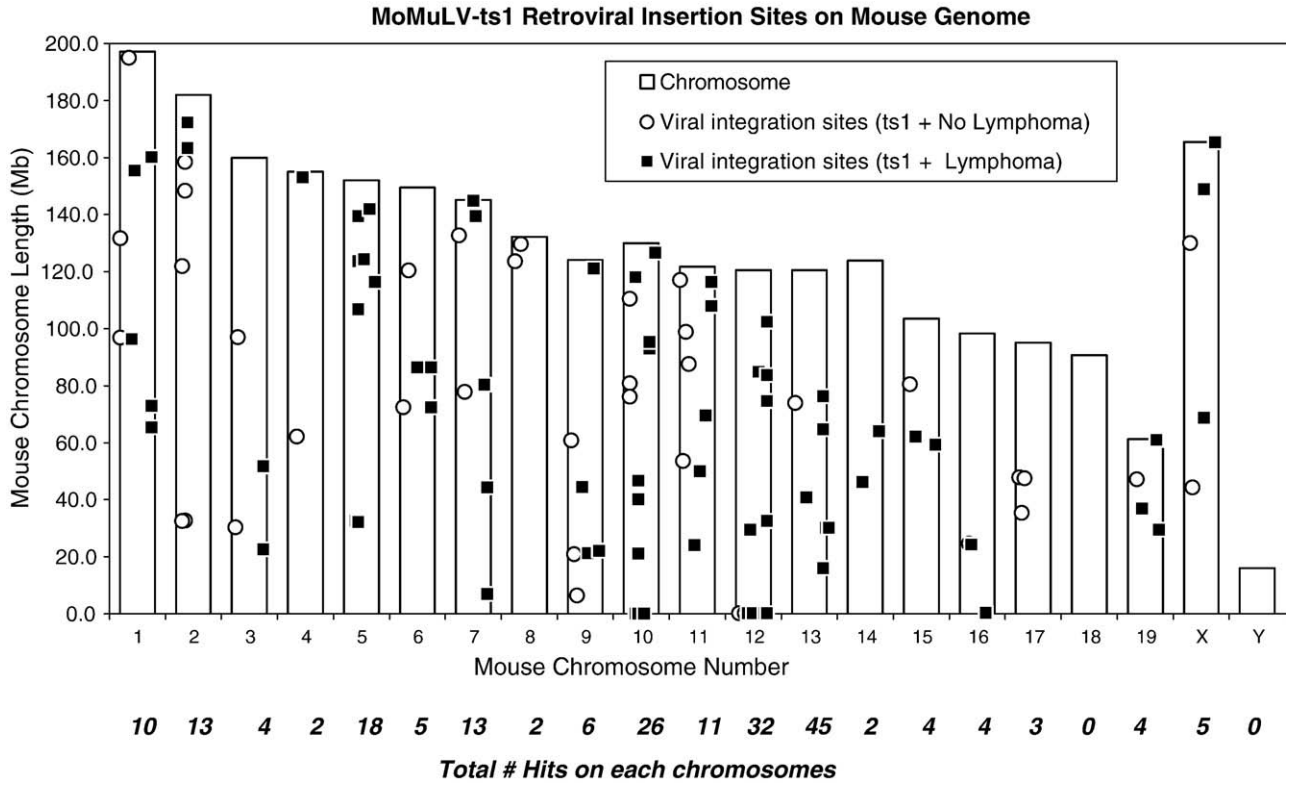

Fig. 1. This is a graphical representation, showing the MoMuLV-ts1 insertion sites on mouse chromosomes. This is a representative summary of the insertion sites and does not include all insertions in each chromosome. All animals showed the ts1 viral genome insertion sites (VIS) on their chromosomes. 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), and 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.

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 tumorigenesis 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 that 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

Table 4a
Intragenic Viral Integration Sites (VIS)

Candidate genes	*Elk3*	*Ncor2*	*Ahi1*	*Evi5*	*Serinc3*	*Tgfa*	*Ppp1r16b*	*Rgs3*	*Cit*	*Il17ra*	*Mid1*
Control (not infected)											
Control 1	0.99	0.68	0.85	0.92	1.63	0.94	1.18	1.29	1.49	1.31	1.26
Control 2	1.72	0.75	0.54	0.96	0.86	2.20	0.58	0.47	2.05	1.24	1.12
Control 3	1.15	0.66	0.69	0.36	0.82	0.85	0.49	0.89	0.81	0.92	0.84
Control 4	0.52	0.80	0.64	0.42	0.27	0.47	0.40	0.79	0.35	0.74	1.26
Control 5	0.62	2.12	2.30	2.34	1.42	0.54	2.33	1.56	0.29	0.80	0.53
Av. fold expr.	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
± SEM	0.24	0.31	0.37	0.40	0.27	0.35	0.40	0.21	0.38	0.13	0.16
ts1 infected no lymphoma											
N LYM1	0.69	0.64	1.68	1.17	0.47	0.44	1.90	1.30	0.52	1.30	0.40
N LYM2	0.69	0.53	0.92	0.55	0.91	1.55	0.36	0.41	0.88	0.77	0.35
N LYM3	0.35	0.33	0.62	0.16	0.54	1.23	0.40	0.52	0.67	0.48	0.91
N LYM4	1.18	0.97	1.36	1.54	0.91	0.57	0.72	0.73	0.40	0.38	0.72
Av. fold expr.	0.73	0.62	1.15	0.86	0.71	0.95	0.85	0.74	0.62	0.73	0.59
± SEM	0.20	0.15	0.27	0.36	0.14	0.31	0.41	0.23	0.12	0.24	0.15
p value < 0.05	0.35	0.27	0.74	0.77	0.32	0.90	0.77	0.38	0.33	0.31	0.07
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

The table shows 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 (LYM).
SEM: Standard error of the mean. Shaded boxes show the genes with significant change in expression levels.
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.

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, N LYM1 suggesting that this mouse might have developed lymphoma if allowed to live longer. The mRNA expression fold increases for gene *Irf4* in N LYM1 and N LYM4 and *Gfi1* in N LYM1 and N LYM3 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, suggest 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 *Irf4* with intergenic VIS. *Irf4* mRNA gene expression showed 1.5 and 1.7-fold increases in N LYM1 and N LYM4, respectively. *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 *Irf4* and *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 et al., 1999a,b). Jung et al. (2006) recognized that both absence and 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,

Table 4b
Intergenic Viral Integration Sites

Candidate genes	*Dusp 22*	*Irf4*	*Tacc3*	*Ccnd 1*	*Aurka*	*Gfi1*	*Prdm16*	*Bcl11a*	*Evi1*	*Zfp 42*	*Ifng*	*Irf2bp 2*	*Il5*	*Cldn5*	*Vamp8*	*Myc*	*Irf1*	*Cd274*
Control (not infected)																		
Control 1	0.93	0.91	0.85	1.01	0.96	0.98	1.12	0.96	0.52	0.91	0.69	1.07	1.00	1.32	1.03	1.08	1.07	0.95
Control 2	1.09	1.39	0.64	1.83	1.52	1.20	1.89	1.32	1.86	2.09	0.57	1.10	1.30	1.46	1.33	1.23	0.57	0.39
Control 3	0.60	0.71	0.82	0.83	0.76	1.10	0.99	0.83	0.74	0.87	0.74	0.88	0.85	0.69	1.02	0.83	0.72	0.58
Control 4	1.24	0.96	0.37	0.84	0.96	0.95	0.65	0.96	0.97	0.84	1.00	0.61	0.98	0.74	0.75	0.93	0.41	0.43
Control 5	1.14	1.04	2.32	0.49	0.80	0.77	0.35	0.94	0.91	0.30	2.01	1.33	0.70	0.79	0.87	0.93	2.22	2.66
Av. fold expr.	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.97	1.00	1.00	1.00	1.00	1.00
± SEM	0.13	0.12	0.38	0.25	0.15	0.08	0.29	0.09	0.26	0.33	0.29	0.13	0.11	0.18	0.11	0.08	0.36	0.48
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	19.48	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

The table shows 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 (LYM).
SEM: Standard error of the mean. Shaded boxes show the genes with significant change in expression levels. Clear boxes show the highest (Gfi1) and the lowest (Ifng) levels of fold changes in mRNA expression.
This table shows 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 downregulation. *Tacc3* and *Aurka* were affected in LYM group only, while *Vamp8* was downregulated in both LYM and NLYM groups.

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 hematopoietic development. *Tacc3* interacts 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 (Jeng et al., 2004; Klein et al., 2004; Li et al., 2003; Reiter et al., 2006; Sen et al., 2002; Tanaka et al., 2005; Tong et al., 2004). 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.

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.

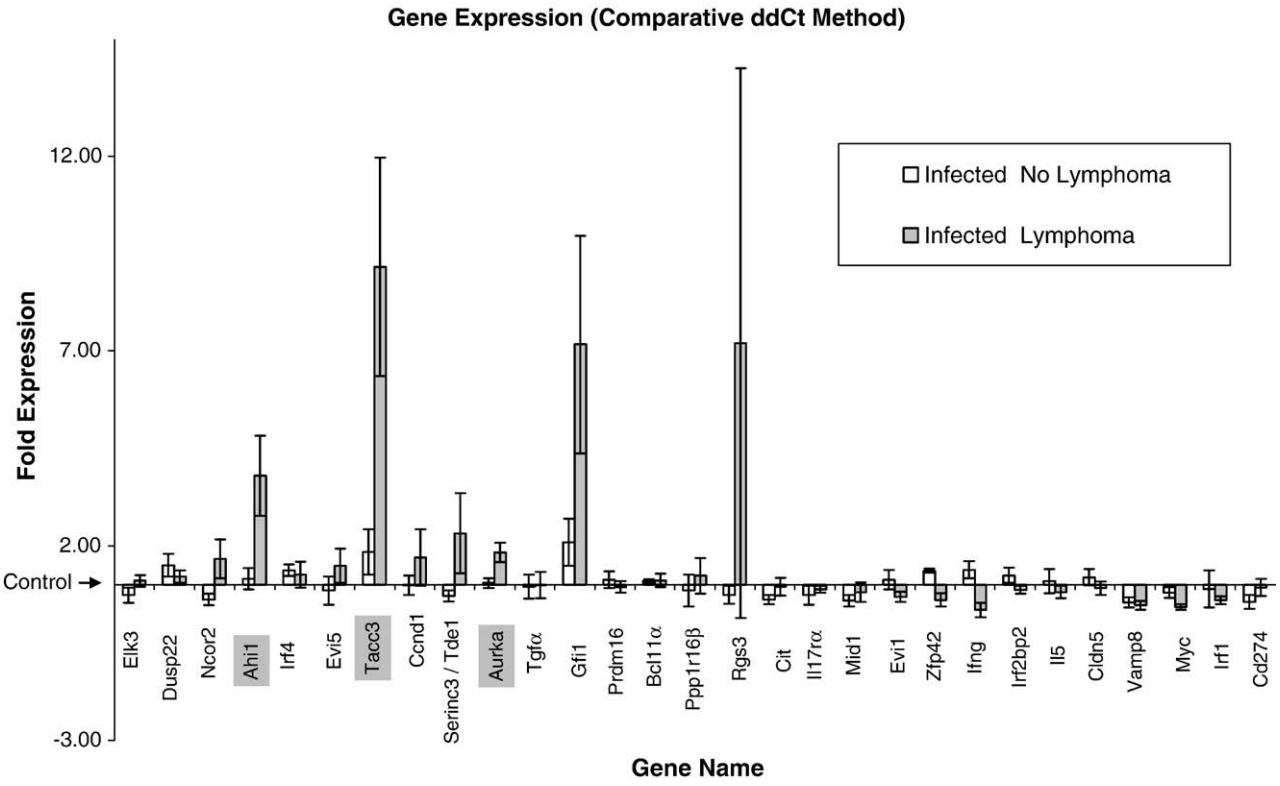

Fig. 2. Graphical representation of the mRNA expression of 29 genes, summarized from Table 3, using comparable ddC_T method. Ahi1, Tacc3 and Aurka showed significant increase in mRNA expression.

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 was added to TB cells. The cell suspension with the virus was incubated at 34 °C for 40 min. 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 min. 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 4.0×10^6 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 h 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 was 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 of the weight of the spleens was 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

Table 5
Summary of twenty-nine genes analyzed and their functions

Gene symbol	Gene name	Mouse chr no.	No. hits	Human homolog	Intragenic/ intergenic VIS	Function	Accession
Elk3	*ETS oncogene family*	10C-D1	9	12q23	Intragenic	Repressor of *heme oxygenase 1* (*HO-1*) gene transcription involved in cell migration	NM_013508.1
Dusp22 and *Irf4*	*Dual specificity phosphatase 22* and *Interferon regulatory factor 4*	13A3	39	6p25	Intergenic	*Dusp22* is a positive regulator of the *JNK* pathway. *IRF4* is central in protecting CD4(+) cells against proapoptotic stimuli	NM_0010379 (*Dusp22*) and NM_013674.1 (*Irf4*)
Ncor2	*Nuclear receptor co-repressor 2*	5F	3	12q24	Intragenic	*NCoR/SMRT*-interacting domain transforms *AML1-ETO* into a potent leukemogenic protein	NM_011424.1
Ahi1	*Abelson helper integration site*	10 A3	1	6q23.3	Intragenic	*Ahi-1/AHI-1* express in primitive hematopoietic cells	BC055400
Evi5 and *Gfi1*	*Ecotropic viral integration site 5* and *growth factor independent 1*	5F	1	1p22	Intragenic	*Evi5* is a common site for retroviral integration into T-cell lymphomas. *Gfi1b* was found to be upregulated in early stages of B-cell and in a subset of early T-cell development	NM_007964.1 (*Evi5*) and NM_010278.1 (*Gfi1*)
Ccnd1	*Cyclin D1*	7F5	8	11q13	Intergenic	*Cyclin D1* upregulation in intestinal neoplasia is important for tumor progression rather than initiation. Contribute to oncogene in malignancy	NM_007631.1
Tde1 (*Serinc3*)	*Tumor differentially expressed protein 1* (*serine incorporator 3*)	2H3	3	20q13	Intragenic	Inhibits apoptosis and stimulates tumorigenesis	NM_012032.2
Aurka	*Aurora kinase A*	2H3	4	20q13	Intergenic	Overexpression promotes cell proliferation and inhibits apoptosis	NM_011497.2
Tgfa	*Transforming growth factor alpha*	6D1	1	2p13	Intragenic	Tumor-associated endothelial cells. *TGF-alpha* inhibits apoptosis in mouse blastocysts	NM_031199.1
Tacc3	*Transforming acidic coiled-coil containing protein 3*	5B3	11	4p16	Intergenic	Role in hematopoietic stem cell function and interface with *p53*-regulated apoptosis. Stabilizes microtubules and contributes to cancer	NM_11524.2
Prdm16	*PR domain containing 16*	4E2	1	1p36	Intergenic	Aberrant expression associated with hypomethylation in adult T-cell leukemia cells	NM_027504.2
Bcl11a	*B-cell CLL/lymphoma 11A* (*zinc finger Protein*)	11A3	2	2p16	Intergenic	*Bcl11a* is essential for postnatal development and normal lymphopoiesis.	NM_016707.1
Ppp1r16B	*Protein phosphatase 1, Regulatory (inhibitor) subunit 16B*	2H1	1	20q11.23	Intragenic	mRNA is located in cell bodies and dendrites of neurons in four distinct regions of the brain	NM_153089.2
Rgs3	*Regulator of G-protein signaling 3*	4B3	1	9q32	Intragenic	Induces heat shock response element (HSE)-dependent gene transcription	NM_019492.1
Cit	*Citron kinase*	5F	1	12q24	Intragenic	Functions in the control of G(2)/M transition in the hepatocyte cell cycle	NM_007708.2
Il17ra	*Interleukin 17 receptor A*	6 F1	1	22q11	Intragenic	May belong to a novel growth-receptor like molecule with capability to support cellular mitogenesis	NM_008359.1
Mid1	*Midline 1*	X F5	1	Xp22	Intragenic	X-linked polydactyly (Xpl) and Patchy-fur (Paf) mutant mice	NM_010797.1
Evi1	*Ecotropic viral integration site 1*	3 A3	1	3q24-q28	Intergenic	Promotes cell proliferation. *Evi1* directly binds to GATA-2 promoter as an enhancer	NM_007963.1
Zfp42	*Zinc finger protein 42*	8 A4	2	4q35.2	Intergenic	Embryonic stem cell marker and pluripotent stem cell marker	XM_284454.5
Irf2bp2	*Interferon egulatory factor 2 binding protein 2*	8 E2	1	1q42.3	Intergenic	No gene reference into function	XM_284454.5
Ifng	*Interferon gamma*	10 D2	1	12q14	Intergenic	Participates in death of dopaminergic neurons; protection against infection	NM_008337.1
Il5 and *Irf1*	*Interleukin 5*/*interferon regulatory factor 1*	11 A5/ B1-11 B1.3	1	5q31.1	Intergenic	Eosinophils on the development and mammary gland, uterus and ovary function. Mice lacking *Irf1* develop CD30+ lymphoproliferative disease	NM_010558.1 (*Il5*) and NM_008390.1 (*Irf1*)
Cldn5	*Claudin-5*	16 A3	2	22q11.21	Intergenic	Claudin-5 is specifically altered in utrophin/dystrophin-deficient (double knockout), dko hearts	NM_013805.2 (*Cldn5*)
Vamp8	*Vesicle-associated membrane protein 8*	6 C1	1	2p12-p11.2	Intergenic	*VAMP-8* is required for release from dense core granules, alpha granules, and lysosomes. Regulates exocytosis of pancreatic acinar cells	NM_016794.2
Myc	*Myelocytomatosis oncogene*	15 D2-D3	2	8q24.21	Intergenic	Required for development of B but not pre-B cell lymphomas from cmyc overexpressing tumor progenitors. *Myc* is required for a normal hypertrophic response	NM_010849.4
Cd274	*CD 274 antigen*; *programmed cell death 1 ligand 1*	19 C2	1	9p24	Intergenic	Also known as *B7-H1*; *PD-L1*; *Pdcd1l1*; *Pdcd1lg1*. Interaction crucially controls the effector differentiation of autoreactive T cells to maintain self-tolerance	NM_021893.2

The table 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.

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 dNTPs, 1.3 U of Expand Long Template Polymerase and 5 μl Expand Buffer System 3 (Roche Diagnostics Corp., Indianapolis, IN) and the volume was 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 s, 55 °C for 30 s and 68 °C for 10 min; 25 cycles of 94 °C for 10 s, 55 °C for 30 s 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. 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 were 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 (BioRad, 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 2× master mix (100 mM KCl, 40 mM Tris–HCl, pH 8.4, 6 mM $MgCl_2$, 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 H_2O, run on the following thermocycler programs: 95 °C for 3 min followed by 40 cycles of 95 °C for 30 s, 60 °C for 30 s 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 using 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.

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