Detection of SRS19-6MuLV in mouse dendritic cell sarcoma and its tumorigenesis

YANYAN LIU, XIAOCUI BIAN, BEI GU, HAILIANG FENG,
ZHENLI YANG, MIN ZHANG, WENJING ZHAO and YUQIN LIU

Department of Pathology, Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences
and Peking Union Medical College, Tsinghua University, Beijing 100005, P.R. China

Abstract. SRS19-6MuLV is a member of the MuLV family originally isolated from the Tianjin-Shanghai-Zunyi complex of murine leukemia. A notable characteristic of this virus is that it induces tumors of multiple hematopoietic lineages, including myeloid, erythroid, T-lymphoid and B-lymphoid. In a previous study, a sequence with high homology to SRS19-6MuLV in a murine dendritic cell sarcoma (DCS) was identified through cDNA expression screening with mAb 983D4. To investigate the relationship between SRS19-6MuLV and DCS, the existence of a specific SRS19-6MuLV DNA fragment in DCS cells, 15 murine tumor cells, 2 murine tumor tissues, 12 normal murine cells/tissues, 11 human tumor cell lines and SRSV/3T3 (NIH/3T3 cells infected with SRS cell supernatant) was detected by PCR. The specific fragment of SRS19-6MuLV was detected in DCS, mouse fore-gastric cancer cells, LII tumor tissue from which DCS is derived and SRSV/3T3. In addition, the integration sites of SRS19-6MuLV in the positive cells were examined by inverse PCR. Thus, 7 integration sites for SRS19-6MuLV were detected in DCS and 3 in SRSV/3T3. Analysis of sequences by BLAST revealed that some of the integration sites were associated with common fragile sites and some Ras-regulating miRNAs. Our results indicate that SRS19-6MuLV not only induced four types of leukemia, but also induced DCS. This virus does not infect human cells. Multiple integration of SRS19-6MuLV into chromosomes around fragile sites accounts for its carcinogenic effects.

Correspondence to: Dr Yuqin Liu, Department of Pathology, Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences and Peking Union Medical College, Tsinghua University, Beijing 100005, P.R. China
E-mail: liuyuqin@public.bta.net.cn

Introduction

SRS19-6MuLV, which was isolated from the Tianjin-Shanghai-Zunyi (TSZ) complex of murine leukemia in the 1960s, is a murine leukemia retrovirus. Unlike other murine leukemia viruses that cause individual types of leukemia, SRS19-6MuLV can cause at least four kinds of leukemia, including erythroid, myeloid, T-lymphoid and B-lymphoid (1,2). In a previous study, we identified a sequence with high homology to SRS19-6MuLV in a murine dendritic cell sarcoma (DCS) through cDNA expression screening with mAb 983D4 (3,4). The DCS cell line originated from a LII tumor tissue, as did the other leukemia cell lines in the TSZ complex. DCS is a sarcoma of dendritic cell (DC) origin and the direct origination of DCs from hematopoietic stem cells has been supported by expression chip analysis (5). Genomic analysis of DCS suggest the existence of a viral gene; secretion proteome studies also revealed the existence of a viral envelope-like protein (6). These results suggest a possible connection between SRS19-6MuLV and murine DCSs. Retroviruses that do not carry oncogenes usually induce tumors in susceptible host animals by proviral insertional mutagenesis, in which proto-oncogenes are activated via promoter or enhancer insertion as a consequence of integrating the viral DNA genome into the host cell chromosome (7).

The purpose of this study was to investigate the relationship between SRS19-6MuLV and DCS. Using PCR and inverse PCR (IPCR) (8-10), the existence of SRS19-6MuLV in different cells and tumors was explored and multiple integration sites were identified.

Materials and methods

Cell culture and animals. The mouse/human cell lines and mouse tumor tissues (Table I) used in this study were obtained from the Cell Resource Center, Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences and Peking Union Medical College. The cells were cultured according to the recommended protocol (http://www.cellresource.cn), and grown in 5% CO_2 at 37°C. The mice used in this study were SPF [Scxk (jing) 2009-0017] and were obtained from the Resource Center for Experimental Animal, National Institution for Food and Drug Control. The thawed cell suspensions of

LII and FC were injected subcutaneously into 615 male mice weighing 16-18 g, aged 4-6 weeks.

SRS19-6MuLV DNA determination by PCR. The genomic DNA of the cultured cells and tissues was isolated using the PureLink Genomic DNA kit (Invitrogen), following the manufacturer's instructions. A pair of primers (F, 5'-ACC TCCAGTCAAGCTACCCCAATAT-3' and R, 5'-TGGCTC ATACTCCAAAGGTT-3') that amplify a 1,401-bp fragment of SRS19-6MuLV end sequences (GenBank AF019230.1), including the SRS19-6MuLV' specific PRO-HV area (11), were designed with Primer Premier 5. For PCR reactions, a 50 μl reaction mixture was prepared by adding 5 μl of template genomic DNA, 25 μl of 2X EasyTaq PCR Supermix (TRANS), 2 μl each of primers (10 μM) and 16 μl H$_2$O. A total of 30 cycles of PCR were carried out, each consisting of 94°C for 30 sec, 58°C for 30 sec and 72°C for 80 sec. The 1,401-bp fragment of SRS19-6MuLV was amplified in genomic DNA from 15 samples of murine tumor cells, 2 samples of murine tumor tissue, 12 samples of murine normal cells/ tissues, 11 samples of human tumor cell lines and SRSV/3T3. The amplified fragments were sequenced (Sangon) and compared to SRS19-6MuLV gene sequences (GenBank AF019230.1) using DNAMAN.

Examination of SRS19-6MuLV particles by transmission electron microscopy (TEM). DCS and SRSV/3T3 cells were harvested during the logarithmic phase and 1x10^6 cells were aliquoted into 1.5-ml centrifuge tubes after washing twice in PBS. After washing, 1.4 ml of 2.5% glutaraldehyde was added gently to the centrifuge tubes, which were then stored at 4°C. Ultrathin sections were prepared and analyzed by TEM (JEOL JEM-1010, the core instrument facility, CAMS/PUMC).

Cloning of the SRS19-6MuLV integration sites by IPCR. Genomic DNA (1 μg) was digested for 1-6 h at 37°C with *Bam*HI (Takara), cutting the 3' end of SRS19-6MuLV at nucleotide position 7164 (Fig. 1). After digestion, the enzyme was heat-inactivated at 68°C for 10 min. Digested DNA was ligated using T4 DNA Ligase (Fermentas) for 10 min at 22°C at a DNA concentration of 2 ng/μl, and then heat-inactivated at 70°C for 5 min. Two pairs of nested primers (F1, 5'-GTTGCCCCAAAGACCTGAAACGACC-3' and R1, 5'-GTAGTTCCTGTTCCTATCCCTGCGG-3'; F2, 5'-CCTG AAACGACCTTGCGCCTCATTTGAA-3' and R2, 5'-AACA ATAGGGCCAATGTTAACGACACCG-3') were designed. For the first PCR reaction, 20 μl of reaction mixture was prepared by adding 8 μl of template cyclized genomic DNA, 10 μl 2X Premix LA *Taq* (Takara), and 1 μl each of F1 and R1 primers (10 μM). Then, 30 cycles of PCR were carried out (98°C for 10 sec, 68°C for 4 min). Using 1 μl of the first-round PCR product as a template, 30 cycles of second-round PCR were carried out with primers F2 and R2 (98°C for 10 sec, 68°C for 4 min). Purified PCR products were cloned into the pMD19-T vector (Takara) and sequenced.

Sequence analysis. The authenticity of the integration site sequence was verified by the following criteria: i) the sequence contained portions of both the SRS19-6MuLV

Table I. Cell lines and tumor tissues.

	Cell lines	Origination
1	DCS	Mouse dendritic cell sarcoma
2	MFC	Mouse fore-stomach cancer
3	YAC-1	Mouse lymphoma
4	P3/NSI/7-A	Mouse myeloma
5	MEL	Mouse erythroid leukemia
6	RAW264.7	Mouse macrophage leukemia
7	P19	Mouse testicular teratoma
8	LA795	Mouse lung adenocarcinoma
9	B16	Mouse melanoma
10	B16F1	Mouse melanoma
11	Ca761	Mouse breast carcinoma
12	P815	Mouse mastocytoma
13	CT26.WT	Mouse colon cancer
14	Hepa1-6	Mouse hepatic carcinoma
15	EL4	Mouse lymphoma
16	NIH/3T3	Mouse embryo fibroblast cells
17	3T3-L1	Mouse embryo fibroblast cells
18	WEHI-3	Mouse blood cell
19	FPC-P1	Mouse bone narrow cell
20	J774.A.1	Mouse monocyte-macrophage cells
21	NSC34	Mouse neuron-like cells
22	3T3-Swiss	Mouse embryo fibroblast cells
23	BALB/C3T3	Mouse embryo fibroblast cells
24	LII	KM mouse reticulum sarcoma
25	FC	615 mouse fore-stomach cancer
26	K562	Human erythromyeloid leukemia
27	THP-1	Human acute monocytic leukemia
28	Daudi	Human Burkkit's lymphoma
29	HL-60	Human acute promyelocytic leukemia
30	Calu-3	Human lung adenocarcinoma
31	MD-MB-435S	Human breast carcinoma
32	HGC-27	Human gastric cancer
33	HeLa	Human cervical cancer
34	HT-29	Human colorectal adenocarcinoma
35	HepG2	Human hepatic carcinoma
36	HCT116	Human colorectal adenocarcinoma
37	SRSV/3T3	Mouse NIH/3T3 cells infected by supernatant of SRS cells

3' end DNA sequence and the linked host DNA sequence; ii) the fragment between F2 and the integration site was identical to the corresponding region of the 3' end of SRS19-6MuLV, as well as the fragment between the putative *Bam*HI cutting site and R2 (Fig. 1); and iii) the host DNA region (containing ≥20 nucleotides) from the integration site sequence to the putative *Bam*HI cutting site showed ≥96% identity to the mouse genomic sequence, as shown by a BLAST homology search. Database analysis of the authenticated integration site sequences was performed by BLAST homology searches.
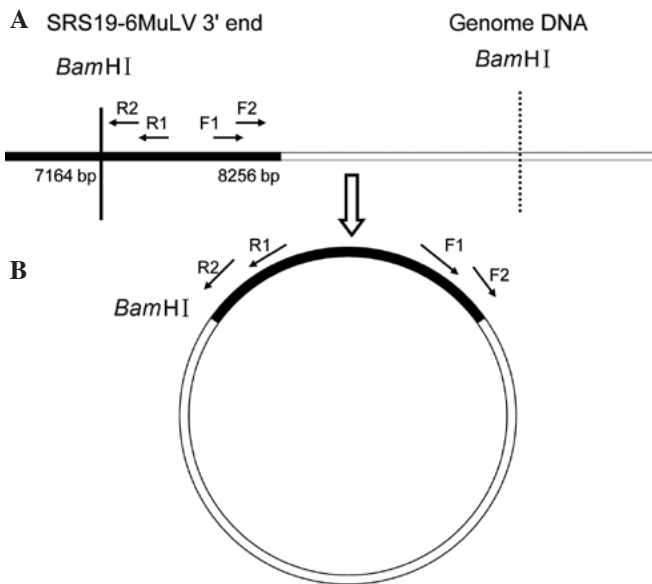
Figure 1. Schematic of inverse PCR (IPCR). (A) The shaded area denotes the region of the SRS19-6MuLV 3' end that has a *Bam*HI cutting site. The blank area denotes flanking genomic DNA that has a putative *Bam*HI cutting site. F1, R1 and F2, R2 denote two pairs of nested primers. (B) Circularization of this fragment puts the flanking genome DNA between the primers, and the flanking genome DNA can be amplified.

Results

Determination of SRS19-6MuLV DNA in 15 mouse tumor cells. To verify the possible connection between SRS19-6MuLV and murine DCS, the 1,401-bp fragment of SRS19-6MuLV DNA was amplified in DCS cells and 14 other mouse tumor cells. Analysis of PCR products by 1% gel electrophoresis (Fig. 2A) revealed a band in the DCS and mouse fore-gastric cancer (MFC) cell lines. The PCR products of DCS and MFC were sequenced and the sequences showed ≥98% identity to the 1,401 bp of the SRS19-6MuLV genome sequence.

Determination of SRS19-6MuLV DNA in 12 normal mouse cells or tissue samples. The 1,401-bp fragment of SRS19-6MuLV DNA was amplified in 12 normal mouse cells or tissues to verify the pathogenic specificity of SRS19-6MuLV. Analysis of PCR products by 1% gel electrophoresis (Fig. 2B) revealed that the normal cells/tissues were negative, indicating that the SRS19-6MuLV DNA fragment could only be detected in tumors.

Determination of SRS19-6MuLV DNA in 2 mouse tumor tissue samples and in SRSV/3T3 cells. Since SRS19-6MuLV DNA was detected in DCS and MFC cells, their parent tumor tissues, LII and FC, were examined. Analysis of PCR products by 1% gel electrophoresis (Fig. 2C) revealed that LII was positive, while FC was negative for SRS19-6MuLV. The SRSV/3T3 cells are NIH/3T3 cells infected with SRS cell supernatant (2); our PCR results were positive for SRS19-6MuLV (Fig. 2D). The PCR products of LII and SRSV/3T3 were sequenced and the sequences exhibited ≥98% identity to the 1,401-bp SRS19-6MuLV genome sequence. These results indicate that this virus infected MFC cells during the process of subculture
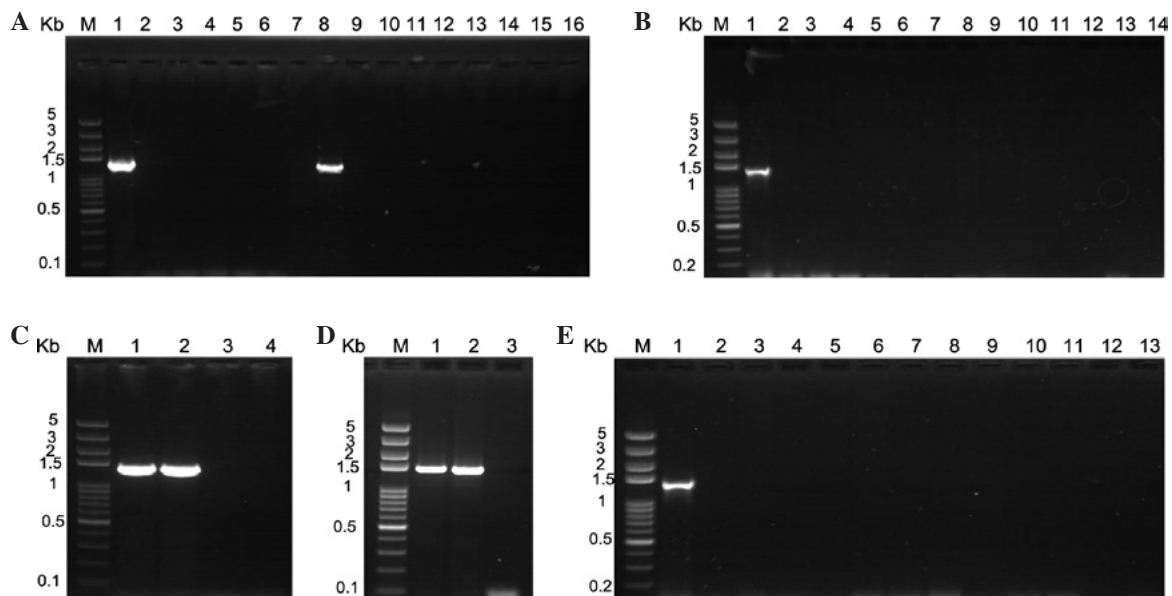

Figure 2. Amplification of SRS19-6MuLV DNA in 15 mouse tumor cells, 12 murine normal cells/tissues, 2 mouse tumor tissues, SRSV/3T3 cells and 11 human tumor cells. (A) Amplification of SRS19-6MuLV DNA in 15 mouse tumor cells. Lanes 1-15 are DCS, YAC-1, P3/NSI/7-A, MEL, RAW264.7, P19, LA795, MFC, B16, B16F1, Ca761, P815, CT26.WT, Hepa1-6 and EL4, respectively. Lane 16 is H₂O as the negative control. Lane M is 100 bp plus DNA ladder. (B) Amplification of SRS19-6MuLV DNA in 12 murine normal cells/tissues. The 12 murine normal cells/tissues (Lanes 2-13) are the spleen of 615 mouse, lung of 615 mouse, spleen of KM mouse, lung of KM mouse, NIH/3T3, 3T3-L1, WEHI-3, FDC-P1, J774.A.1, NSC34, 3T3-swiss and BalB/c3T3, respectively. Lane 1 is DCS as the positive control and Lane 14 is H₂O as the negative control. Lane M is 100 bp plus DNA ladder. (C) Amplification of SRS19-6MuLV DNA in 2 mouse tumor tissue samples. Lanes 2 and 3 are LII and FC, respectively. Lane 1 is DCS as the positive control and Lane 4 is H₂O as the negative control. Lane M is 100 bp plus DNA ladder. (D) Amplification of SRS19-6MuLV DNA in SRSV/3T3 cells. Lane 2 is SRSV/3T3. Lane 1 is DCS as positive control and Lane 3 is H₂O as negative control. Lane M is 100 bp plus DNA ladder. (E) Amplification of SRS19-6MuLV DNA in 11 human tumor cells. The 11 human tumor cells (Lanes 2-12) are K562, THP-1, Daudi, HL60, Calu-3, MD-MB-435S, HGC-27, Hela, HT-29, HepG2 and HCT116, respectively. Lane 1 is DCS as the positive control and Lane 13 is H₂O as the negative control. Lane M is 100 bp plus DNA ladder.
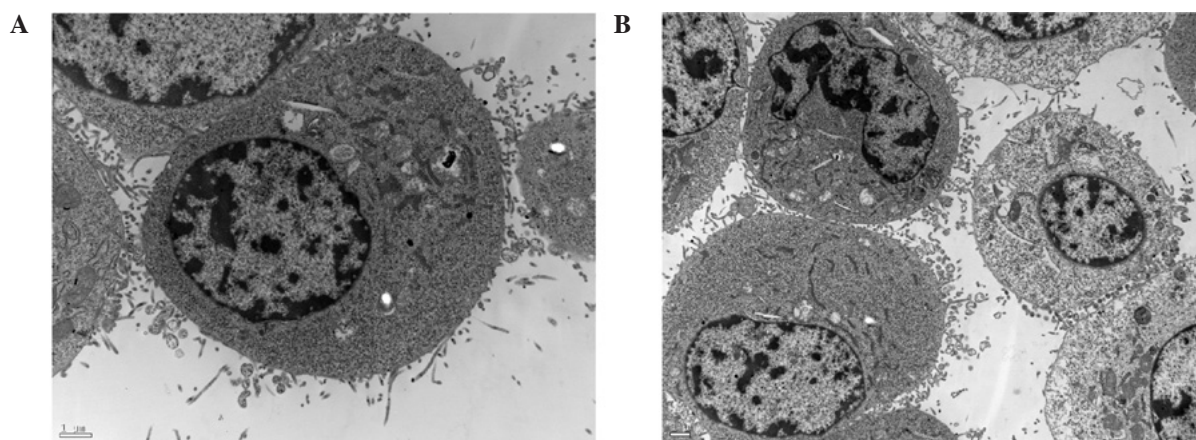
A **B**

Figure 3. Electron microscopic observations on the virus particles in DCS and SRSV/3T3 cells. No virus particles were observed in (A) DCS and (B) SRSV/3T3 cells. Original magnification, x10,000.
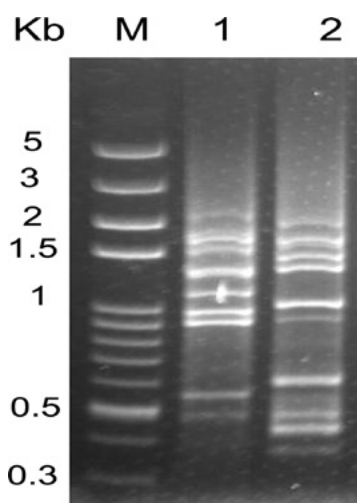

Figure 4. Amplification of SRS19-6MuLV integration sites. Lane 1 is DCS and Lane 2 is SRSV/3T3 cells. Lane M is 100 bp plus DNA ladder.

and the SRSV/3T3 cell genome contains an SRS19-6MuLV DNA fragment.

Determination of SRS19-6MuLV DNA in 11 human tumor cells. To check the carcinogenic species-specificity, the 1,401-bp fragment of SRS19-6MuLV DNA was amplified in 4 human blood system tumor cells (K562, THP-1, Daudi and HL-60) and 7 other human tumor cells. Analysis of PCR products by 1% gel electrophoresis (Fig. 2E) revealed that human tumor cells were negative, indicating that SRS19-6MuLV does not infect human cells.

Electron microscopic observations of the virus particles in DCS and SRSV/3T3 cells. No virus particles were observed in DCS or SRSV/3T3 cells by TEM (Fig. 3), indicating that the SRS19-6MuLV virus particles do not exist in these two cell lines.

Cloning SRS19-6MuLV integration sites in DCS and SRSV/3T3 cells. For further study of the role of SRS19-6MuLV in the tumorigenesis of mouse DCS, SRS19-6MuLV integration sites were amplified by IPCR in DCS and SRSV/3T3

cells. Analysis of the second-round PCR products by 1% gel electrophoresis revealed that there were multiple integration sites of SRS19-6MuLV in both DCS and SRSV/3T3 cells (Fig. 4). Subsequently, gel-purified PCR products were cloned into the pMD19-T vector. Clones (n=20) of DCS and SRSV/3T3 were determined by PCR using universal primers for the pMD19-T vector. Of the 20 amplified fragments, 14 were singled out and sequenced (Sangon).

Bioinformatics analysis of SRS19-6MuLV integration sites in mouse DCS and SRSV/3T3. The authenticated integration site sequences were singled out of those 14 clones and the same sequences were removed. Subsequently, 7 integration sites for SRS19-6MuLV were detected in DCS and 3 were detected in SRSV/3T3 (Table II). This result is consistent with the characteristics of the retrovirus. A common integration site for SRS19-6 MuLV in both DCS and SRSV/3T3 was identified in chromosome 6B3 (Table II), indicating that the integration of SRS19-6MuLV may have a preference for this site.

Many retroviruses cause cancer by activating proto-oncogenes or by inhibiting tumor suppressor genes and enhancer elements in the proviral LTR influence gene expression over hundreds of kilobase pairs (kbp), and can affect several genes simultaneously. The identity of the nearest genes within ±50 and 100 kbp, miRNA within ±100 kbp and associated common fragile sites of each SRS19-6MuLV integration site were examined (Table II). We did not identify a common proto-oncogene or tumor suppressor associated with the SRS19-6MuLV integration sites. However, after analysis for association with common fragile sites and miRNA, 2 integration sites in common with the fragile sites Fra6B3 and Fra12C1 were observed. In addition, 3 integration sites were near miRNAs, such as miRNA801, miRNA683 and 3 let7 miRNA family members (miRNAlet7d, miRNAlet7f1 and miRNAlet7a-1), which are important tumor-associated miRNAs (Table II). These factors may play important roles in the tumorigenesis of mouse DCS.

Discussion

SRS19-6MuLV is closely associated with a variety of mouse leukemias. In the 1960s, Professor Yang Gang established an

Table II. Characteristics of SRS19-6MuLV integration sites identified in murine DCS and SRSV/3T3 cells.

Cell no.	Site of integration		Gene(s) within indicated distance of integration site		miRNA within ±100 kbp of integration site	Common fragile site
	Cytoband	Nucleotide position	±50 kbp	±100 kbp		
DCS						
1[a]	6B3	56874802	Nt5c3, V1rc21, LOC100042317	Fkbp9, V1rc19 V1rc10, EG434016		Fra6B3
2	13A4	43380635	Gfod1	Sirt5		
7	13A5	48648456	Aw456871, A83005F24Rik	2fp169	miRNAlet7d, miRNAlet7f1, miRNAlet7a-1, miRNA683	
9	1H2.3	168899701	LOC622102, C30014k22Rik			
10	4D2.3	131908746	Snhg3, Rcc1, Trspap1, Phactr4, LOC100039864	9530096D07Rik, Gmeb1, Taf12	miRNA801	
13	12C1	55811004 or 55839900	Eapp, LOC673582, LOC100039735, LOC100039764	1110002B05Rik, Snx6		Fra12C1
14	3E1	65796002	LOC100044387, LOC630761, EG624866			
SRSV/3T3						
2[a]	6B3	56874802	Nt5C3, V1rc21, LOC100042317	Fkbp9, V1rc19 V1rc10, EG434016		Fra6B3
12	15F1	99546080	Gd1, Smarcd1, Accn2, Lass3, 2310016M24Rik, LOC100043481	Racgap1, Lima1, LOC10043473		
13	5F	123225463	Camkk2, P2rx4, Anapc5	Rnf34, Fbx100, P2rx7		

[a]The same integration site of SRS19-6MuLV in DCS and SRSV/3T3 cells.

implantable LII solid-type reticulum cell sarcoma through subcutaneous inoculation with a suspension of spleen tissue from KM mice with spontaneous leukemia. Thereafter, a series of cell lines and tumor strains were established on the basis of LII (2). The most significant outcome was the TSZ complex containing more than 12 types of cell lines and tumor strains, including SRS ascites-type reticulum cell sarcoma, from which SRS19-6MuLV was separated. What renders this leukemia virus unusual is its ability to induce four types of leukemia, including erythroid, myeloid, T-lymphoid and B-lymphoid (1). Currently, the focus of study on SRS19-6MuLV is to understand the molecular mechanism of its multiple leukemogenesis (12,13).

Our data support previous reports that SRS19-6MuLV induces DCS. The DCS cell line, originating from dendritic cells, was acquired by planting LII into 615 mice (5,14). A cDNA expression library of DCS with mAb 983D4 was screened and a sequence with high homology to SRS19-6MuLV was obtained, which indicates a relationship between SRS19-6MuLV and DCS. We hypothesized that SRS19-6MuLV exists in DCS and may play an important role in its carcinogenic ability. First, the existence of a 1,401-bp fragment of SRS19-6MuLV was identified containing the PRO-HV region in DCS and MFC cells, but not in 13 other tumor cell lines, thus excluding the possibility that the genomes of mouse normal cells/tissues contain SRS19-6MuLV DNA fragments. In accordance with our expectations, this specific fragment was detected in LII, the parent tumor tissue of DCS, but not detected in FC, the parent tumor of MFC. Since LII was derived from KM mouse spontaneous leukemia, SRS19-6MuLV may participate in the process of its tumorigenesis. It is most likely that this virus infected MFC cells during the process of subculture, and this virus is not the carcinogen. Our results expand the spectrum of tumorigenesis of SRS19-6MuLV to include not only erythroid, myeloid, T-lymphoid and B-lymphoid leukemias,

but also DC tumors. Another cell line, SRSV/3T3, was also observed, which originated from NIH/3T3 cells infected with the supernatant of SRS cells (2), containing this DNA fragment of SRS19-6MuLV. This virus-specific fragment was not detected in 11 human tumor cell lines, which means that this murine leukemia virus is not involved in human tumorigenesis.

No virus particles were observed in DCS and SRSV/3T3 cells by TEM, indicating that the SRS19-6MuLV did not replicate in these two cells. The following results prove that the virus had integrated into their genomes.

Altered expression of tumor suppressors or proto-oncogenes induced by retrovirus integration is one important mechanism of tumorigenesis (7). To further study the pathogenesis of SRS19-6MuLV, IPCR was used to search for the integration sites of SRS19-6MuLV in DCS and SRSV/3T3 cells. Although IPCR has some limitations, for example, in selecting appropriate restriction enzymes or the possibility of multiple DNA fragments cloning into the T vector, this method is superior for searching for integration sites accurately and precisely. In this study, 7 integration sites for SRS19-6MuLV were detected in DCS and 3 integration sites were detected in SRSV/3T3. It was found that SRS19-6MuLV had a common integration site in these two cells, located in chromosome 6B3, indicating that the integration of SRS19-6MuLV may have a preference for this site. The identity of the nearest genes within ±50 and 100 kbp, miRNA within ±100 kbp and associated common fragile sites of each SRS19-6MuLV integration site were examined. Although there were some tumor-associated genes around the integration sites, such as Nt5c3 (15,16), we did not find any important oncogenes or tumor suppressor genes. Additionally, 2 integration sites were located in the mouse common fragile sites, Fra6B3 and Fra12C1 (17). Previous studies have revealed that common fragile sites are extremely unstable in the process of carcinogenesis and are always destroyed and rearranged in many tumor cells (18,19). Researchers have noted that most miRNAs play significant roles in tumorigenesis and metastasis (20). miRNAlet7d, miRNAlet7f1 and miRNAlet7a-1, members of the let7 miRNA family, were found to be located 14-17 Kb downstream of the integrated SRS19-6MuLV. The let7 miRNA family is closely related to certain tumors through the regulating oncogene RAS, such as carcinomas of the lung, breast and ovary (21-23). We speculate that these factors may play important roles in SRS19-6MuLV-induced DCS.

In conclusion, SRS19-6MuLV DNA integrated into DCS genomic DNA and was involved in the tumorigenesis of DCS. This finding expands our knowledge regarding SRS19-6MuLV. Not only can it induce four types of leukemia, but it also causes DCS. Infection by this virus is species-specific and it is not present in human cell lines. By integration, SRS19-6MuLV may alter the expression of genes and miRNA near the integration sites.

Acknowledgements

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