Sequence Analysis of Canine LINE-1 Elements and p53 Gene in Canine Transmissible Venereal Tumor

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Abstract

LINEs (long interspersed nuclear elements or long interspersed repeated DNA elements) contains two open reading frames (ORFs), ORF1 and ORF2. We analysed the ORF2 located in the 5' region to the first exon of oncogene c-myc in canine transmissible venereal tumor (TVT) cell. We also showed the transcription activation was induced by this TVT-LINE sequence using CAT assay. To identify the mutation of tumor suppressor gene, sequence analysis of p53 from TVT cell was performed. We identified the point mutation of 964 nucleotide (T→C) resulting in the change of amino acid (Phe→Ser) of p53 tumor suppressor protein.

Introduction

Mammalian genomic DNA contains several families of frequently repeated sequences and some have been shown to be mobile elements, such as SINE and LINE [9,21]. They are known to be mostly flanked by direct repeats of 7-20 base pairs (bp) that are believed to result in the duplication of target site. LINEs, long interspersed nuclear elements or long interspersed repeated DNA elements, are considered to be a nonviral retroposon found in all mammals. The LINE-1 elements, which are mostly truncated at the 5' end existed on the genomic DNA with 10,000 to 50,000 copies [5] and they encode two open reading frames(ORFs), ORF1 and ORF2. From the results of sequence analysis and purified components, the ORF2 of the LINE-1 encodes such biological functions as reverse transcriptase, endonuclease and possibly RNase H [8,15,17]. In contrast, the ORF1 has no activity showing a known enzymatic function [13]. The LINE-1 also can transpose near to oncogenes or other structural genes, and occasionally causes malignant diseases, such as transmissible venereal tumor (TVT) in dog [11,12] and human breast carcinoma [19].

The p53 tumor suppressor protein plays a central role in the maintenance of genomic integrity. Inactivation of the p53 tumor suppressor gene by point mutation and translocation events has been associated with a large number of human neoplasms [6]. Recently similar mutations within the canine cancer types including thyroid carcinoma [3], mammary tumors [22], osteosarcoma [10], circumanal gland adenoma [18] and lymphoma [23]. The vast clinical knowledge concerning the identification and treatment of canine cancer and the apparent similarity of p53 inactivation in the tumors of some cancer patients identifies canine p53 as a potential target for anti-cancer therapy in the dog [24].

The canine TVT is a naturally occurring neoplastic disease that affects the external genitalia of both sexes. It is transmitted during coitus. In addition to the natural mode of transmission, the TVT can be transferred from one dog to another by experimental transplantation of living tumor cells [2]. Canine TVT cells have truncated LINE-1 sequences from the 5' region to the first exon of oncogene c-myc [1, 11, 12]. However, little is known about that the genetic structure of the canine LINE-1 gene, the molecular relationship between the canine LINE-1 element and the LINE-1 element (TVT-LINE) inserted in rearranged c-myc (rc-myc) of TVT cell and the functional relationship between the TVT-LINE element and TVT disease.

To estimate the molecular structures of the canine LINE-1 element and the TVT-LINE integrated in TVT, we determined their DNA sequences. In addition, we have examined the transcription activation property of TVT-LINE element and investigated the point mutation of p53 gene in TVT cells. Here we report the complete ORF2 DNA sequences of the canine LINE-1 element and the TVT-LINE element isolated from the TVT lines in Korea and the results of experiments that examine the transcription activity property of TVT-LINE element using the CAT assay.
Materials and Methods

Samples and DNA Preparation
TVT tissues were removed surgically from the TVT dogs and were frozen and kept at -80°C until used. Genomic DNA was extracted as described by Okumura et al. [20]. The canine fibroma cell line A72 was cultured in Dulbecco’s modified Eagles medium (DMEM) supplemented with 10% fetal bovine serum and antibiotics. Baby hamster kidney (BHK) cell line was cultured in MEM medium contained 10% tryptose phosphate, 20mM HEPES and 2mM glutamine with 5% fetal bovine serum and antibiotics. The cell line A72 was used as a source to isolate total RNA and BHK-21 cell line was used as a target cell for CAT assay and production of the virus like particles of SFV.

Construction of plasmids
5’ truncated LINE-1 element of TVT (TVT-LINE) was subcloned into pBLCAT2 vector (PBL cat (F); TVT-LINE inserted into Xba I and Bam HI sites upstream of CAT gene, PBL cat (P); TVT-LINE inserted into Smal I and Kpn I sites of downstream of CAT gene, PBL cat (5D); 5’ deleted TVT-LINE which was digested with Hind III restriction enzyme was inserted into Hind III and Bam HI sites upstream of CAT gene, PBL cat (5D); 5’ deleted TVT-LINE which was amplified with primers T8 (5’-CGAGAAAGATC ATTTGAAAGAAGG-3’) and T9 (5’-CTACCACCCCTAGTTCGTTT-3) was inserted into Xba I and BamHI sites upstream of CAT gene (Fig. 1).

PCR, DNA manipulation and DNA sequencing
The PCR was performed with synthetic primers in 50μl reaction volume for 30 cycles. Long and Accurated (LA) PCR was performed in 50μl reaction volume for 10 sec at 98°C and for 15 min at 68°C for 30 cycles. The total RNAs were extracted from a canine fibroma cell line A72 and primary TVT tissue with TRIZOL reagent (GIBCO, USA), and mRNAs were purified with Oligotex-dT 30 (Takara, Japan). The cDNA templates were synthesized by reverse transcription (RT)-PCR from 5μl of mRNA using a first strand synthesis kit (Pharmacia, Sweden). The amplified PCR products and pBluescript SK(+) cloned DNA fragments were sequenced by the dyeoxy chain-termination method using a 373S sequencer (Perkin Elmer Applied Biosystems Division, USA) with a Taq Dye Deoxy Terminator Cycle Sequencing FS Kit. Synthetic DNA primers were used for the PCR, RT-PCR, LA-PCR and DNA sequencing. The DNA sequence data were analyzed by GENETYX-MAX software (Software Development Co. Ltd, Japan) for multiple sequence alignments and homology search.

Chloramphenicol acetyltransferase (CAT) assay
TVT-LINE cloned pBLCAT plasmids were transformed into BHK and A72 cell lines by electroporation using Bio-Rad Gene Pulser. 10μl of plasmid DNAs was used for each 1×106 BHK and A72 cells. After 48 hrs of transfection, the cells were harvested at 250×g for 10min 4°C. After 3 times washing with 5ml of pre-cooled PBS (4°C), the cell pellet was mixed with 1ml of lysis buffer (MOPS-buffered saline containing Triton X-100, pH 6.5) and stand for 30min at room temperature. The cell lysates were spun in a microcentrifuge at 15,000×g for 10min at 4°C. Cell extracts were used for ELISA test using CAT ELISA kit (Boehringer Mannheim GmbH, Germany) or stored at -70°C until used. Protein standard curve was made with Bio-Rad protein assay (Bio-Rad, CA). 100μl of cell extract protein was used per well of CAT ELISA plate. The absorbance of the samples at 450 nm using a THEROMO max microtiter plate (ELISA) reader.

Results

DNA sequence of Canine LINE-1 ORF2
To obtain the functional canine LINE-1 sequence, RT-PCR was performed with mRNA from cell line A72. The ORF2 was constructed from six overlapping PCR fragments. The six PCR products generated contiguous 4,251-bp cDNA sequences encoding 1,275 amino acids (hatched region of LINE-1 in Fig. 1). A comparison with the human ORF2 revealed that canine ORF2 sequences had a 63% homology with those of human ORF2 at the amino acid level.

DNA sequences of TVT-LINE in rc-myc of TVT
To estimate the relationship between the TVT-LINE and canine LINE-1, the TVT-LINE region was amplified with several primers and was determined for its sequences. Four TVT-specific primers (T1 to T4) reported by Ameriglio et al. [1] were used for the PCR but some non-specific DNA fragments were observed in normal dog samples. Therefore three genomic DNA primers (T6, T7 and T8) flanking the TVT-LINE elements were newly synthesized, and the TVT-LINE was amplified from three TVT samples. When the primer pairs of T6-T7 or T6-T8 were used in PCR, normal samples yielded 267bp or 158bp bands, which were different from those from the TVT samples, 1,645bp and 1,536bp, respectively. These results suggested that the primer pairs of T6-T7 or T6-T8 were specific primer pairs for diagnosis of TVT using PCR method. These PCR products were extracted from the gel, were determined for DNA sequences directly, and the DNA sequences of the TVT-LINE were aligned with those of the canine ORF2 region (Fig. 2). The DNA sequences of the inserts from three TVT lines were identical with the 1,378bp in size and were flanked by 10bp direct repeats (ATTCCTGGC). The sequence homology analysis with canine ORF2 suggested that the TVT-LINE contained a 416bp length homologous to the complementary strand of canine LINE-1 and was followed by a 5bp deletion; DNA sequences of the TVT-LINE showed a 98% and 63% homology to canine LINE-1 and human LINE-1 sequences, respectively.
Fig. 1. TVT-LINE element as a whole or truncated from was subcloned into pBLCAT2 vector.

CAAGGCTCTCATTCAAATGGAAGGAGAGATAAAGAGCTTCCAAGACAGGCAGGAACTGAAAGAATATGTGACCTCCAAA 80
CCAGCCTGCAAGAAATTTTAAGGGGGACTCTTAAAATTCCCCTTTAAGAAGAAGTTCAGTGGAACAGTCCACAAAAACA 160
MMTVLSYNLSTIVTLNVNLNDP21
AGGACTGAATAGATATCATGATGACACTAAACTCATATCTCTGAATGTGAACGGGCTTAATGACCCC240
IKRRRVSDWIKKQDPSCIICLQETHRFQ48
ATCAAAAGGCCAGGTCTTCACTGCCATATGGCTTCTGCTCTACAAAGAAGACTCTTTTAGACA320
KDTYSLLKIKGWRTRIYHSNPGPKKAGV74
GAAGGACACCTACAGCCTGAAAATTAAAAAGTTGGAGAACCATTACCATGGAATGTGCCCTAAAAAGGACAGGGTAG400
AILISDKLKTPTKTVRDEEGYHIILK101
CCATCCTATATCAGATACACTAAAAATTACCACAAAAAGCTGAGAGAAGAGAGGCCAGCTATTATCATACTAAAA480
GSIQEDLTILNYAPNVGAAYINQL128
GGATCTATCCAACAAGAGGACTTAACAATCCTCAATATATATGCCCCGAATGTGGGAGCTGCCAAATATAAATCAATT560
RSSLHPSKETRANLDQDMFTDIY181
GTTCTCTAAACAAACTCTCCTCAAAAAGAGAGGCCAGCTTTAATGATACACTGGAGAGGCCAGAATCTACTAC720
RTLHPNSTETYFFSSAHGTFSRIDHIL208
Fig. 2. NA sequences of the ORF2 canine LINE-1 and the TVT-LINE. The ORF2 of canine LINE-1 has one open reading frame from 178 to 4002 nucleotide. The DNA sequences of the TVT-LINE in TVT lines are compared with those of canine LINE-1. The underlined below the sequences shows the reverse complementary 416-bp sequences of TVT-LINE. The ten direct repeats found in the TVT-LINE are marked by a box.
Activation property test of TVT-LINE element in vitro

To investigate the activation property of the truncated LINE-1 element inserted in the upstream of the c-myc gene (TVT-LINE), we performed CAT assay in the BHK and A72 cell lines. Four plasmids were constructed with pBLCAT2 vector [TLPBL cat (F); TVT-LINE element was inserted upstream of the CAT gene, TLPBL cat (P); TVT-LINE element was inserted downstream of the CAT gene, TLPBL cat; 5’ D) and 3’ (D) truncated TVT-LINE element was inserted upstream of the CAT gene]. Each of these 10 DNA was transformed into 1×106 - 2×106 BHK and A72 cells at the electroporation condition of the 2500V/0.83KV. After 48 hours of transfection infected cells were harvested and then determined the CAT concentration of each infected cell lines (Fig. 3). TLPBL cat (F) contained BHK cell lines and A72 cell lines were shown 0.29 - 0.89 ng/ml and 0.21 -0.63 ng/ml of CAT enzyme activity, respectively. However, TLPBL cat (P) and TLPBL cat (D) showed similar CAT activity with control cell lines. These results suggested that TVT-LINE element in the upstream of c-myc oncogene could activate the c-myc gene in the TVT cell line.

Fig. 3. Activation property test of TVT-LINE elements in vitro using CAT assay. Control; pBLCAT2 plasmid, BHK(F) and A72(F); pBLCAT-TVT(F) plasmid, BHK(P) and A72 (P); pBLCAT-TVT(P) plasmid, BHK(D) and A72(D);pBLCAT-TVT(5D) and (3D).

Identification of point mutation of p53 gene in TVT

The p53 gene is frequently mutated in diverse human cancer [7, 14]. The isolation of canine p53 cDNA was carried out using a RT-PCR method and canine p53 gene specific primers (caFOR: 5’-ATGGAGGAGTCGAGTCA-3’, caREV: 5’-TTTT ATGGCGAGAGGTAGATTGC-3’) from the primary TVT tissues. This fragment was purified at the 0.7% agarose gel and cloned into TA cloning vector (Novagen, USA) and then nucleotide sequence was determined. These sequences were compared with normal canine p53 gene mRNA from Genbank (Fig. 4). We found a single point mutation at 964 nucleotide position from T → C. From the this point mutation amino acid was changed from Phe to Ser at codon 316.

To confirmed the point mutation at the TVT genomic DNA, we designed the primers (ca p53 931: 5’ - CGAAAA GAAGAAGCC ACTA-3’ and caREV) for the exon 9 and 10. The PCR products were purified at the 0.7% agarose gel and cloned into TA cloning vector and then sequenced. We confirmed the point mutation at the TVT genomic DNA samples. These results suggested that codon 316 might be the one of the causes of oncogenesis in the TVT.

Discussion

In this study, we determined the DNA sequences of the ORF2 for canine LINE-1 element and those of the LINE-1 integrated in c-myc gene of TVT. However, we failed to isolate the full sequence of ORF1 for canine LINE-1 element. At an initial investigation, we tried to isolate canine LINE-1 element from the cDNA clone reveled that clone 16 has nucleotide mutations or deletions, although the sequence identity between both clones was over 93%. These results indicated that even if genomic LINE-1 genes integrate with high copies in a canine genome and they would be transcripted by arbitrary promoters, the functional LINE-1 protein would not be produced as much. The functional ORF2 of LINE-1 was constructed from six RT-PCR products, but a long full-length ORF2 was not amplified in this study. This evidence suggests that the transcripts of LINE-1 have a small quantity of total RNA from canine cell line A72. In fact, we could not detect and distinct signals for LINE-1 gene by Northern blot analysis with total RNA from A72 (data not shown).

From molecular analysis of canine LINE-1 and TVT-LINE integrated in TVT lines, the TVT specific insert in front of c-myc gene appears to originate from the 3 region of canine LINE-1. Katzir et al.[11, 12] reported that the 1,314-bp LINE-1-like element flanked by 10-bp direct repeats integrate into the c-myc region of TVT, which is 64-bp smaller than the TVT-LINE of our study with different nucleotides. However, the 10-bp repeat sequence and the molecular basic structure, including the inversion and deletion, commonly occur in inserts of TVT lines from Israel, Japan and Korea. This result indicates that the insertion of a truncated LINE-1 element upstream of the c-myc gene may be the genetic event specific for the tumorigenesis of TVT disease.

An interesting question remains whether the TVT is caused by insertion of the truncated LINE-1 element upstream of the c-myc gene. Katzir et al.[12] pointed out the possibility that the inserted repetitive sequence has an effect on the transcriptional activity of the c-myc gene. To investigate this question, we performed CAT assay of TVT-LINE element.
in the upstream of CAT gene, CAT enzyme concentration was raised but the concentrations of CAT enzyme were not consistent as 0.284 - 0.89 ng/ml. This result suggests that the insertion of a truncated LINE-1 element upstream of c-myc gene can activate the c-myc gene but maybe the other factors are more needed.

Loss of p53 function is a common event in many types of human [6,14] and animal [24]. Although there is evidence that p53 functions through several mechanism, it is clear that the ability of p53 to act as a sequence specific transcriptional activator can play an important role in mediating both cell cycle arrest and apoptosis [3,18].

Mutant p53 can regulate the expression of the endogenous c-myc gene and is a potent activator of the c-myc promoter [6]. We showed here that mutation of p53 gene at codon 316 in the TVT tissues although it wasn't existed from exon 3 to 8 where were commonly occurred mutation in the human but it maybe the one of the etiological reasons of TVT.
References


