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Identity of rearranged LINE/c-*MYC* junction sequences specific for the canine transmissible venereal tumor

(transposable elements/oncogene/polymerase chain reaction)

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ABSTRACT **The canine transmissible venereal tumor is a naturally occurring neoplastic disease that affects the external genitalia of both sexes and is transmitted during coitus. Cytogenetic and immunologic studies demonstrated that tumors from different parts of the world are very similar, suggesting that they are transferred from one animal to another by the transplantation of viable cells. We found that the c-*MYC* oncogene was rearranged in this tumor by the insertion of a transposable genetic element sequence (known as LINE, long interspersed element) 5′ to the first exon. The amplification of a DNA segment located in the junction of the LINE genome and c-*MYC* upstream sequences enabled the testing of the similarity of transmissible venereal tumor samples collected independently in different parts of the world. Oligonucleotide primers flanking the LINE/c-*MYC* junction were used to amplify a 340-base-pair segment and nested primers amplified a 280-base-pair segment. A fifth oligonucleotide used as a probe contained the actual junction sequence. All of the tumors analyzed revealed the existence of the specific bands, which were absent in normal canine DNA samples. The amplified segments obtained from all of the tumors analyzed were identical in size and nucleotide sequence, suggesting transmission of the original rearranged cell itself, as opposed to independent events of LINE insertion in a "hot spot."**

The canine transmissible venereal tumor (TVT) is a naturally occurring neoplastic disease that affects the external genitalia of both sexes and is transmitted during coitus (1, 2). Experimental transmission of TVT was described by Novinsky in 1876 (3). The precise cell type of the tumor is not yet known (1). This tumor can be transplanted by intact viable cells across the major histocompatibility complex barriers among dogs and other canines (1, 2). Cytogenetic and immunologic studies demonstrated that tumors from different parts of the world are very similar, suggesting that they are transferred from one animal to another by the transplantation of viable cells (4, 5). We found that the c-*MYC* oncogene was rearranged in this tumor by the insertion of a transposable genetic element sequence (known as LINE, long interspersed element) 5′ to the first exon (6). Fig. 1 illustrates schematically the structure of the rearranged c-*MYC* oncogene as determined by restriction mapping (6). Analysis of several TVT samples from various parts of the world by Southern blotting revealed that in all tumors the insertion of the same LINE repetitive element occurred in the vicinity of the c-*MYC* gene (7).

Amplification of a DNA segment located in the junction of the LINE genome and c-*MYC* upstream sequences enabled the testing of the similarity of TVT samples collected independently in different parts of the world.

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MATERIALS AND METHODS

Sample Collection. Seven TVT samples were collected independently in the United States, at the Universities of Connecticut and Illinois, and in Israel, at Ben-Gurion University, Beer-Sheva, and at the Veterinary Institute, Beit-Dagan. A sample of the spleen from a TVT-carrying dog was also analyzed. Tissues were frozen at −70°C. Normal canine peripheral blood cells were collected during routine tests performed at a veterinary clinic.

DNA Isolation. High molecular weight DNA was extracted from the tumor tissues and peripheral blood cells using the nucleic acid extractor (Applied Biosystems, model 340A) according to the manufacturer's instructions.

Primers. The following oligonucleotides were synthesized by the DNA synthesizer (Applied Biosystems, model 381A) according to the manufacturer's instructions. Numbers refer to their position in the sequence published in ref. 6.

T1 5′-ATGCACCAAGATTTTCTTCACTGC-3′ (positions 58–81)

T2 5′-GAGTCGACTGCAGCTACGAATGAAT-GATTGGCCAGAT-3′ (positions 100–123 with a 13-base-long tail for cloning at the 5′ end)

T3 5′-GAGTCGACTGCAGCTTCTTGCAAGATA-CATCCA-3′ (positions 350–331 with a 13-base-long tail for cloning at the 5′ end)

T4 5′-ATCCTAGAGAAGAACACAGGCAACAC-3′ (positions 400–375)

T5 5′-ATCCTTTAACATTCTCTGGCTGTCTTCGAC-3′ (positions 211–230)

Amplification Reactions. PCR was performed using the DNA thermal cycler (Perkin–Elmer/Cetus) (8, 9). A 100-μl reaction mixture containing 10–100 ng of genomic DNA in 50 mM KCl, 10 mM Tris·HCl (pH 8.3), 1.5 mM $MgCl_2$, 0.001% gelatin, 200 μM (each) deoxyribonucleotide triphosphates (dNTP: dATP, dCTP, dGTP, dTTP), 25 pmol of each of two primers (T1 plus T4 or T2 plus T3), and 1.3 units of Taq DNA polymerase (Cetus) was subjected to 25–45 cycles of amplification. Cycles were as follows: 30 sec at 94°C, 30 sec at 60°C, and 1.5 min at 72°C. The last cycle was terminated with an elongation step of 10 min at 72°C. The use of nested primers involved using the external pair (T1 plus T4) for the

Abbreviation: TVT, transmissible venereal tumor.

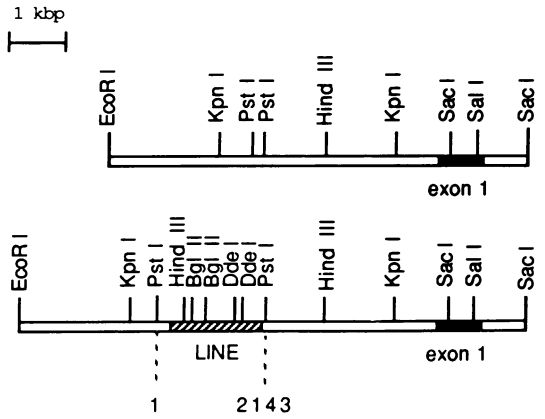

FIG. 1. Physical map of the first exon and 5′ flanking sequences of the normal canine c-*MYC* gene and of the rearranged c-*MYC* gene present in the canine TVT. The upper line represents the map of the canine c-*MYC*. The lower line shows the rearranged c-*MYC*. Numbers refer to the *Pst* I restriction sites according to ref. 6. kbp, Kilobase pairs.

first 15 cycles, then removing 50 μl of the reaction mixture into a fresh tube containing the internal set of primers (T2 plus T3), with no additional DNA, and subjecting this new reaction mixture to 25–40 more amplification cycles.

PCR was also performed using end-labeled oligonucleotide primers followed by separation on acrylamide gel (9).

Southern Blot Analysis. PCR products were separated by electrophoresis in 1.5% agarose and visualized under UV after ethidium bromide staining. DNA was transferred to nylon filters by Southern blotting. The nylon-bound DNA was hybridized with an end-labeled oligonucleotide probe (oligonucleotide T5) (9).

End-labeling of oligonucleotide probes and primers was carried out for 60 min at 37°C with T4 polynucleotide kinase according to the manufacturer's instructions (New England Biolabs).

Direct Sequencing of PCR Products. Direct sequencing of single-stranded amplified DNA was performed as described (9) with minimal modifications. One hundred nanograms of genomic DNA was amplified for 30 cycles by PCR as described above, using the set of external primers (T1 plus T4). Ten microliters of the amplification products was subjected to a second round of amplification, in which one of the oligonucleotide primers was omitted. The sample was then extracted with chloroform and the DNA was alcohol precipitated from 2.5 M ammonium acetate. The pellet was dissolved in 5 μl of H_2O immediately before use in the DNA sequencing reaction. The appropriate internal ^{32}P-labeled primer (T2 or T3) was used for sequencing. Five microliters of amplified single-stranded DNA was mixed with 3 μl of ^{32}P-labeled primer (≈25 pmol) and 2 μl of 5× buffer. Samples were annealed for 10 min at 60°C and cooled slowly to room temperature. The Sanger dideoxy termination sequencing reaction was performed using the Sequenase kit of United States Biochemical.

RESULTS

Amplification of a Tumor-Specific Segment. The DNAs of seven different TVT samples from various geographical locations and of seven normal peripheral blood cell samples were tested by PCR amplification using two sets of nested oligonucleotide primers. The amplified sequences were hybridized with an oligonucleotide probe corresponding to the LINE/c-*MYC* junction sequences (Fig. 2). Amplified LINE/c-*MYC* sequences were demonstrated in all of the analyzed TVT samples, whereas all of the normal control DNA samples were negative. DNA extracted from the spleen of a TVT-carrying dog gave a positive signal (Fig. 2, lane S1), whereas the same sample, when tested by Southern blotting, revealed only the nonrearranged pattern (6). This suggests the existence of a minute population of malignant cells in this macroscopically noninvolved spleen. Additionally, DNA samples from peripheral blood cells of two tumor-bearing dogs were negative when tested by the PCR (data not shown).

The same samples were also tested by the PCR using ^{32}P end-labeled primers (Fig. 3). Again, with this more rapid and sensitive method, all TVT samples gave a positive signal, whereas all normal controls were negative. The amplified segment was about 290 bp long, as was predicted from the sequence of the rearranged gene. Comparison between all of the amplified radioactive sequences by acrylamide gel electrophoresis revealed that the analyzed LINE/c-*MYC* junction segments, present in all of the analyzed TVT samples, are exactly of the same length.

Nucleotide Sequence of the Amplified LINE/c-*MYC* Junction Segments. The identity of the rearranged LINE/c-*MYC* sequences present in all of the analyzed TVT samples was tested by direct sequencing of amplified single-stranded DNA of the junction sequences. Both strands were independently sequenced, using the sense and the antisense internal primers (oligonucleotide T2 or T3). Fig. 4 depicts the LINE/c-*MYC* junction sequence detected in the DNA samples of all seven TVTs analyzed. All of the samples contained identical sequences. Comparison of the results obtained with our previously published sequence (6) revealed complete identity, except for a discrepancy of eight nucleotides clustered in a short segment located 3′ to the junction point (positions 237–247 in Fig. 4), where eight deoxycytosylate residues were replaced by a single deoxyguanosylate and seven thymidylate residues. The original tumor studied (6) revealed a sequence identical to that obtained in this study. We went back to the cloned, rearranged c-*MYC* (λ clone, ref. 6). The sequencing of the breakpoint region disclosed a sequence identical to that found in all of the tumors in the present study. We therefore assume that the present sequence is the correct one, and the discrepancy is due either to a sequencing error in the original study or to artifacts introduced during the subcloning procedure.

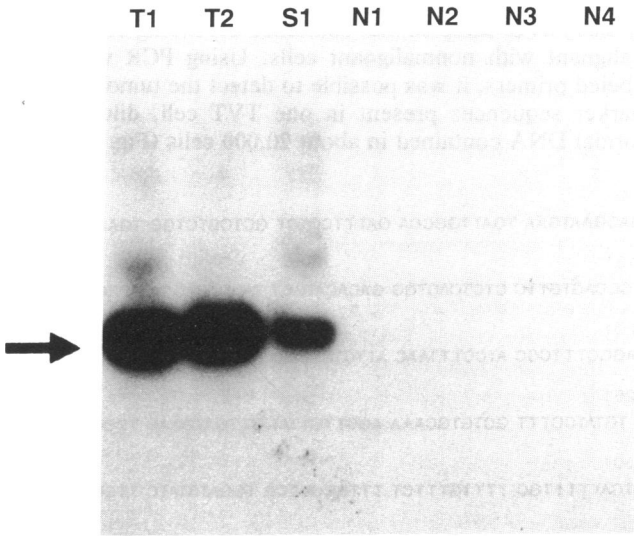

FIG. 2. Southern blotting of PCR products of two TVT samples (T1 and T2), of four normal controls (N1–N4), and of a spleen sample from a TVT-carrying dog (S1). The junction sequences were amplified using primers T1 and T4 for the first 15 rounds of amplification and reamplified using nested primers T2 and T3 for another 30 cycles. Oligonucleotide T5 was used as a radioactive probe. The arrow indicates amplified LINE/c-*MYC* sequences.

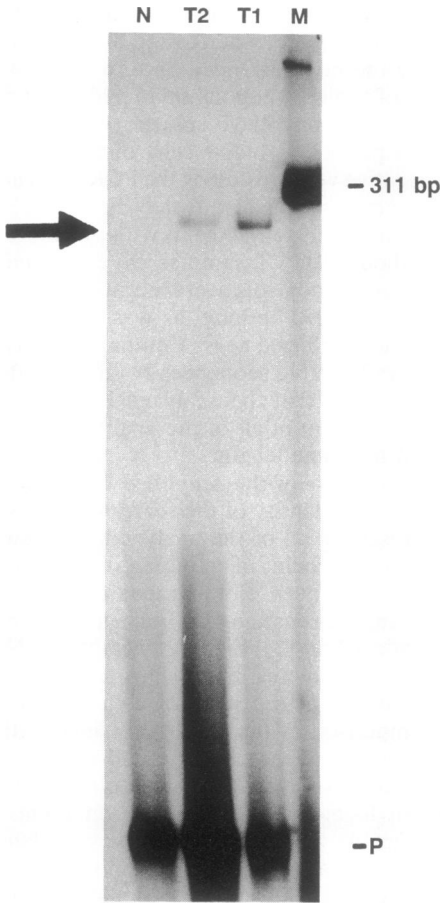

FIG. 3. Polyacrylamide gel electrophoresis of the products of an amplification reaction of two TVT samples (T1 and T2) and of a normal control (N) using radioactively labeled primers. The PCR reaction was carried out as described in the text, with end-labeling of the internal primers T2 and T3. The arrow indicates amplified LINE/c-*MYC* sequences. M, labeled size marker; P, primer.

Detection of Minute Cell Populations Carrying the TVT Marker. The sensitivity of detection of TVT cells was tested by analysis of samples that contained ascending dilutions of malignant with nonmalignant cells. Using PCR with end-labeled primers, it was possible to detect the tumor-specific marker sequences present in one TVT cell, diluted with normal DNA contained in about 20,000 cells (Fig. 5).

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101
TACGAATGAA TGATTGGCCA GATTTCGTCT GCTCGTCTGC TGAAGAGCTT
151
CCCAGTGTTC CTCTCACTGG GACACATGGT TAGCACAGGA AATACTGGTG
201                              ↓      C   C CCCC CC
AGGCTTTCCC ATCCTTTAAC ATTCTCTGGC TGTCTTTGAG TTTTGTTGAC
251
TGTATCCTTT GCTGTGCAAA AGCTTCTTAT CTTGATGAAG TCCCAATAGT
301
TCATTTTTGC TTTTGTTTCT TTTGCCTTCG TGGATGTATC TTGCAAGAAG
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FIG. 4. Nucleotide sequence of the LINE/c-*MYC* junction sequences detected in the seven different TVT samples analyzed and sequenced directly by the PCR. The arrow indicates the junction point between the c-*MYC* and the inserted LINE sequence. The oligonucleotides used for sequencing are underlined by a dashed line. The segment between positions 237 and 247 contains the previously published sequence in the upper line. Numbers refer to positions in the sequence published in ref. 6.

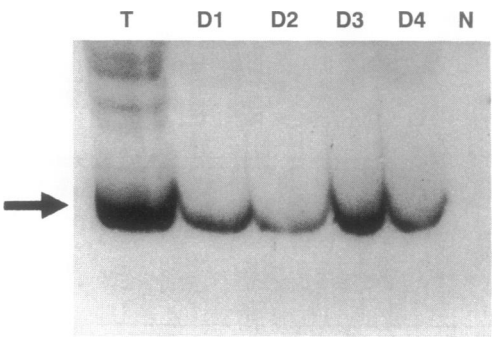

FIG. 5. Polyacrylamide gel electrophoresis of radioactively labeled PCR products of a TVT sample (T), of decreasing quantities of DNA from a TVT sample diluted with increasing amounts of normal canine DNA (D1–D4), and of a normal canine control (N). The arrow indicates amplified LINE/c-*MYC* sequences.

DISCUSSION

The uniqueness of the TVT model lies in the fact that this is the only proven example of a naturally occurring tumor that is transmitted by cell transplantation (1, 2). We found that the c-*MYC* oncogene was rearranged in this tumor due to the insertion upstream to the gene of a 1.5-kbp repetitive DNA element that belongs to the mammalian LINE family (6). Southern blot analysis showed that TVTs from individual dogs from various regions contain a similar rearranged gene, suggesting a common cellular origin for TVT in various dogs (7). The possibility that the rearranged c-*MYC* is a polymorphic allele in the animals examined so far was ruled out by the absence of this rearrangement in the peripheral blood cells of affected subjects.

The insertion of transposable genetic elements into preferred genome loci is well documented in prokaryotes, and similar "regional specificity" exists also in eukaryotic systems (10). Some oncogene loci may be preferred integration sites for certain repetitive movable genetic elements (11). For example, two independent events of integration of an endogenous retroviral-like intracisternal A-particle genome into the c-*MOS* oncogene were documented in different murine plasmacytomas (12–14). Therefore, one may argue that the rearranged c-*MYC* genes, demonstrated in different TVT samples, could result from independent events of LINE integration into the c-*MYC* in a preferred "hot spot" and are not identical. In all studied examples of regional specificity, the integration of the transposable elements occurred in a limited area but not at the same nucleotide. Therefore, the precise analysis of the integration points may unravel whether all TVTs carry the same rearranged gene or are just similar genes resulting from integration in a preferred site. We found in this study that the amplified LINE/c-*MYC* junction sequences from all of the analyzed tumors were exactly of the same length. All of the amplified sequences hybridized under stringent conditions to an oligonucleotide probe complementary to the nucleotides on both sides of the junction point. Moreover, an identical LINE/c-*MYC* junction sequence was demonstrated in all of the analyzed tumors. Therefore, the integration of the same LINE genome was found exactly at the same nucleotide of the c-*MYC* in all TVTs, indicating that the rearranged genes are not only similar but are, in fact, identical. This finding strongly supports the hypothesis that all canine TVTs originated from one tumor cell.

The ability to detect minute populations of malignant cells is of great clinical importance. The PCR technique enables the detection of minimal residual disease in cases in which the malignant cells are characterized by a specific genetic aberration that is not present in the host nonmalignant cells. The best-studied examples of the detection of malignant cells by

PCR are chronic myeloid leukemia and follicular lymphoma, in which the presence of chromosomal translocations 9;22 and 14;18, respectively, enables the amplification of tumor-specific sequences (15, 16). We describe here the application of PCR for the detection of minute cell populations by the amplification of tumor-specific sequences that are the result of a unique integration of a transposable genetic element. Approximately one-third of the mammalian genome is composed of repetitive sequences (10^3–10^5 copies). This includes endogenous retrovirus-like elements as well as short (SINE) and long (LINE) interspersed DNA elements (17–20). Some of these sequences can function as mobile genetic elements and transpose from one place to another in the mammalian genome (17–20). Several examples of integration of transposable elements in the vicinity of oncogenes and other cellular genes in tumors have been described (6, 7, 11–14, 21–26). Such tumor-specific somatic mutations can serve for the detection of minimal residual disease by PCR.

The canine TVT is a very unique model. This tumor can be transplanted by intact viable cells across the major histocompatibility complex barriers among dogs and other canines. After several months of progressive growth, the tumor usually regresses in adult dogs but metastasizes in puppies and immunosuppressed dogs (1, 2). In the present work, we describe the detection of decreasing numbers of malignant cells in the presence of a large excess of normal cells. In addition, the presence of an amplified rearranged segment in the Southern blot-negative spleen of a tumor-bearing animal illustrates possible uses of the method. The ability to follow minute TVT cell populations may serve for the study of basic phenomena, such as tumor transplantation, progression, and regression, in this model. Several reports documented rare events of accidental transplantation of malignant cells across major histocompatibility complex barriers in human beings (27–29). The study of the TVT model may, therefore, be relevant to human oncology as well.

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