Molecular Cloning of the Feline Telomerase Reverse Transcriptase (TERT) Gene and Its Expression in Cell Lines and Normal Tissues

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ABSTRACT. Telomerase is a kind of reverse transcriptase which synthesizes and elongates telomeres. Telomerase activity is detected in many naturally occurring tumors and its expression appears to play an important role in the immortalization of tumor cells. In this study, cDNA encoding the feline telomerase reverse transcriptase (TERT) gene was cloned partially from a feline lymphoma cell line. The clone obtained in this study was 237 bp long including a reverse transcriptase motif 2, and was shown to have amino acid sequence similarity of 81.0% and 58.2% with human and mouse TERT cDNAs, respectively. TERT mRNA expression was detected in telomerase-positive cells (FL74, FT-1, 3201, FKNp, FONp, and FYMp), and was not detected in telomerase-negative cells (normal fibroblasts and CRFK). TERT mRNA was detected in various normal tissues including the spleen, pancreas, stomach, cerebrum, testis, bone marrow, lymph node and thymus, and relatively high-level expression was observed in the small bowel and large bowel. No expression of TERT mRNA was detected in the liver, adrenal gland, urinary bladder and lung. The TERT cDNA clone and the results obtained in this study will be useful for further investigation of feline tumors.

KEY WORDS: cloning, expression, feline, telomerase, TERT.

Telomeres are specific structures at the end of linear chromosomes that protect chromosomes from DNA degradation, end-to-end fusion, and rearrangement [3]. Telomeres become progressively shorter with repeated cell divisions. The decrease in telomere length results in the inability of cells to divide, and triggers cell senescence [1, 14, 30]. However, tumor cells can continue to divide because of telomere-maintenance mechanisms (e.g., telomerase) that add new telomeric sequences to the end of chromosomal DNA [21].

The telomerase consists of an essential RNA template (telomerase RNA component: TERC) and protein components including a kind of reverse transcriptase (telomerase reverse transcriptase: TERT) [9, 19, 21, 23]. Telomerase binds to the 3' end of the single-stranded region of chromosomal DNA. Then, the sequence repeats are added to the end of the DNA using TERC as a template [9, 19, 21, 23]. In humans, TERC is present in all cells, whereas the expression of TERT gene is confined to cells that have telomerase activity [19, 23]. The ectopic expression of the TERT gene enables telomerase-negative cells to have telomerase activity [4], indicating that TERT is the major determinant of telomerase activity.

Telomerase activity is detected in many naturally occurring tumors in humans [16, 26], dogs [28, 29] and cats [6]. On the other hand, most of the somatic cells in adults have undetectable or low telomerase activity, although gonadal cells and lymphocytes have distinct telomerase activity. Therefore, its activity can be thought of as a tumor marker. Furthermore, the results of some studies [10, 24, 27] indicate that telomerase inhibitors may have potential as new anticancer drugs.

In the present study, we report on the partial molecular cloning of the feline TERT gene and its expression in various types of cultured feline cells to compare the expression of the TERT gene with telomerase activity. Furthermore, we analyzed the expression of the TERT gene in various normal feline tissues, to provide fundamental information for prospective studies on feline tumors.

MATERIALS AND METHODS

Cell culture: In this study, we used normal fibroblasts, an epithelial-like feline kidney cell line (CRFK) [8]), three lymphoma cell lines (FL74, FT-1 [20], and 3201 [25]), and three mammary adenocarcinoma cell lines (FKNp, FONp, and FYMp). Primary fibroblasts originated from a skin biopsy specimen of a normal male cat. FKNp was established from a 12-year-old female Persian cat with mammary adenocarcinoma (clinical stage II). FONp was established from a 12-year-old female Siamese cat with mammary adenocarcinoma (clinical stage III). FYMp was established from a 15-year-old female Japanese domestic cat with mammary adenocarcinoma (clinical stage III). All mammary adenocarcinoma cells have been passaged for more than three years in our laboratory, and the major morphology of these cells did not change in this period. The normal fibroblasts and the CRFK cells were cultured in Dulbecco’s modified Eagle’s medium containing 10% heat-inactivated (56°C, 30 min) fetal bovine serum, penicillin (100 U/ml), and streptomycin sulfate (100 µg/ml) at 37°C in a humidified atmosphere of 5% CO2. The other cells (FL74, FT-1,
3201, FKNP, FONp, and FYMp) were cultured in RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum, penicillin (100 U/ml), and streptomycin sulfate (100 μg/ml) at 37°C in a humidified atmosphere of 5% CO₂. The cells were washed twice with phosphate-buffered saline (PBS), and divided into two samples, one for telomerase activity assay and the other for TERT messenger RNA (mRNA) detection, to be carried out with identical samples. These samples were rapidly frozen in liquid nitrogen, and stored at –80°C until analyzed.

**Tissue samples**: Specimens of normal tissues were obtained from a healthy male cat. All the tissue specimens were washed with saline (0.9% NaCl) solution immediately after collection, rapidly frozen in liquid nitrogen, and stored at –80°C until analyzed.

**Cloning and sequence analysis of feline TERT complementary DNA (cDNA)**: mRNA was extracted from the 3201 cells using affinity binding to oligo(dT) cellulose (Fast Track mRNA Isolation kit, Invitrogen, Carlsbad, CA). A cDNA sample was synthesized with a commercially available kit (cDNA Synthesis kit, Amersham Biosciences, Piscataway, NJ). Oligonucleotide primers to amplify a central region of feline TERT cDNA were designed based on the sequence of human TERT cDNA [23] (GenBank/EMBL/DDBJ accession number AF015950): forward primer, 5'-GACTCCGTTCATCCCCAAG-3' (nucleotides (nt) 1859–1878 in human TERT cDNA) and reverse primer, 5'-CATCCACCTTGACAAAGTAC-3' (nt 2137–2116 in human TERT cDNA). Using this primer pair, feline TERT cDNA was amplified from 3201 cDNA with Taq polymerase (Takara, Kyoto, Japan). Polymerase chain reaction (PCR) amplification was performed with denaturation at 94°C for 1 min, annealing at 50°C for 1 min and polymerization at 72°C for 1.5 min. The DNA fragment generated by the PCR was cloned into a plasmid vector (pCR2.1 vector, Invitrogen), and the obtained clone was sequenced by the dideoxy chain termination method (ABI Prism Big Dye Terminator, Invitrogen), and the obtained clone was sequenced by the PCR was cloned into a plasmid vector (pCR2.1 vector, Invitrogen), and the obtained clone was sequenced by the dideoxy chain termination method (ABI Prism Big Dye Primer Cycle Sequencing Ready Reaction Kit, Applied Biosystems, Foster City, CA).

**Determination of telomerase activity**: Telomerase activity was measured by the telomeric repeat amplification protocol (TRAP) assay using a commercially available kit (TRAPeze telomerase detection kit, Intergen, Purchase, NY) as described previously [29], with slight modifications. Briefly, frozen cell samples were homogenized in lysis buffer containing 10 mM Tris-HCl [pH 7.5], 1 mM MgCl₂, 1 mM ethylene glycol bis(β-aminoethyl ether) N,N,N',N'-tetraacetic acid (EGTA), 0.1 mM benzamidine, 5 mM β-mercaptoethanol, 0.5% 3-(3-cholamidopropyl) dimethylammonio)-1-propane sulfonate. The protein concentration in the cell extract was measured by the Lowry method (Bio-Rad DC protein assay kit, Bio-Rad Laboratories, Hercules, CA). Two microliters of the cell extract (equivalent to 50 ng) was incubated in a reaction mixture (TRAPeze telomerase detection kit, Intergen) consisting of 1 x reaction buffer (20 mM Tris-HCl [pH 8.3], 1.5 mM MgCl₂, 63 mM KCl, 0.05% Tween 20, 1 mM EGTA), deoxynucleoside triphosphate mix (dNTPs), TS primer (5'-AAATCCGTCGAGCAGAGTT-3'), TRAP primer mix containing a reverse primer and an internal control primer, and 2 U of Taq polymerase (AmpliTaq, Applied Biosystems) at 37°C for 30 min; this mixture was then subjected to 30 cycles of PCR consisting of denaturation at 94°C for 30 sec and annealing and polymerization at 59°C for 30 sec. As a control, the TRAP assay was performed using TSR8 control template (TS primer + 5 telomeric repeats; 5'-AAATCCGTCGAGCAGAGTTAG(GGTTAG)7-3') instead of the tissue extract to assess the PCR amplification step. The PCR products were separated by electrophoresis in a 12.5% non-denaturing polyacrylamide gel, and stained with SYBR Gold (Molecular Probes, Eugene, OR) for visualization.

**RNA sample preparation for detection of TERT mRNA**: Total RNA was extracted by a silica-gel membrane method (RNeasy Mini Kit, Qiagen, Hilden, Germany). To avoid contamination of genomic DNA, the RNA samples were treated with deoxyribonuclease I (Invitrogen). The integrity of RNA was checked by denaturing agarose gel electrophoresis (1.2% agarose gel in 1 x MOPS buffer (20 mM 3-[N-morpholino]propanesulfonic acid, 5 mM sodium acetate, 1 mM ethylenediamine-N,N,N',N'-tetraacetic acid (EDTA), pH was adjusted to 7.0 with NaOH) and 0.246 M formaldehyde) and SYBR Gold staining.

**Detection of transcriptions by reverse transcription (RT) and PCR**: In this study, an equal amount of RNA sample (500 ng each) was used for RT-PCR analysis to compare the expression level of TERT mRNA. Single-strand cDNA was synthesized from 500 ng total RNA in a reaction primed with oligo(dT) using a commercially available kit according to the manufacturer's manual (GeneAmp RNA PCR kit, Applied Biosystems). To amplify feline TERT cDNA, a primer pair was prepared: forward primer, 5'-AGCCAGTAGCAGAGGAGTTAG-3' (nt 45–64 in feline TERT cDNA) and reverse primer, 5'-TGGGTCCTGAGCCCGACAC-3' (nt 209–228 in feline TERT cDNA). PCR was performed using a commercially available Taq polymerase (AmpliTaq Gold, Applied Biosystems). After denaturation at 95°C for 2 min, 28 cycles of the reaction (95°C for 1 min [denaturation] and 60°C for 1 min [annealing and polymerization]) were performed, followed by a final extension procedure at 72°C for 7 min. The PCR products were electrophoresed through a 4% agarose gel, and stained with SYBR Gold for visualization. The gel images were captured with a CCD camera (XC-75 video camera module, SONY, Tokyo, Japan), and the densitometric intensities of the RT-PCR products were analyzed with an image analysis program (NIH Image Version 1.62, U.S. National Institute of Health). The expression level of TERT mRNA was estimated by comparison of the band intensity with that of FL74 (5, 50, 500 ng). RT-PCR signals below this range were tentatively designated as 'not detected'.

**RESULTS**

The feline TERT cDNA clone obtained in this study was...
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237 bp long, encoding 79 amino acid residues (GenBank/EMBL/DDBJ accession number AB094676). The deduced amino acid sequence encoded by the clone was shown to have 81.0% and 58.2% amino acid sequence similarities with human and mouse [11] (GenBank/EMBL/DDBJ accession number AF051911) TERT cDNAs, respectively. A BLAST search [2] reveals that the probabilities of these similarities occurring by chance are $1 \times 10^{-31}$ and $1 \times 10^{-21}$, respectively. This clone contained a reverse transcriptase motif 2 (amino acids 630–649 in human TERT [2]).

A TRAP assay was performed to evaluate the telomerase activity in feline cultured cells. Telomerase activity was detected in all of the tumor cell lines (FL74, FT-1, 3201, FKNp, FONp, and FYMp). However, no telomerase activity was detected in the normal fibroblasts or CRFK cells (Fig. 1), even with the use of an increased amount of protein up to 200 ng (data not shown).

Next, RT-PCR analysis was performed to examine the expression of the TERT gene in feline cultured cells. As shown in Fig. 1, TERT mRNA was detected in all the tumor cell lines (FL74, FT-1, 3201, FKNp, FONp, and FYMp), but not in the normal fibroblasts or CRFK cells. TERT mRNA in the normal fibroblasts and CRFK cells was not detected even when the PCR cycles were increased up to 35 (data not shown).

TERT mRNA was detected in various normal tissues including the spleen, pancreas, stomach, cerebrum, testis, bone marrow, lymph node and thymus, and relatively high-level expression was observed in the small bowel and large bowel. No expression of TERT mRNA was detected by RT-PCR in the liver, adrenal gland, urinary bladder and lung (Fig. 2).

DISCUSSION

In this study, feline TERT cDNA was partially cloned and its expression was assessed in normal fibroblasts, an in vitro immortalized cell line (CRFK), and six tumor cell lines (FL74, FT-1, 3201, FKNp, FONp, and FYMp), to compare the expression of the TERT gene with telomerase activity. TERT mRNA expression was detected in telomerase-positive cells (FL74, FT-1, 3201, FKNp, FONp, and FYMp),
and was not detected in telomerase-negative cells (fibroblasts and CRFK). Although we tested a small number of cells, this result is consistent with that in human cell lines [19, 23]. Therefore, TERT expression is the primary determinant regulating the telomerase activity in feline cells. Telomerase activity has been measured by a PCR-based telomerase activity assay, called the TRAP assay. In this method, the telomerase activity is measured in vitro by two steps. In the first step of the reaction, telomerase in a cell or tissue extract adds telomeric repeats onto the oligonucleotide primers. In the second step, the extended products are amplified by PCR. This method is sensitive, but some extracts from cells or tissues contain inhibitors of Taq polymerase [15]. In such a case, the extended products in the first step should be extracted with phenol/chloroform/isoamyl alcohol and ethanol precipitation before the second step. This extraction is complicated and may be a cause of false positive or negative results. Therefore, to assess the telomerase expression in cells or tissues, it is preferable to detect TERT mRNA by RT-PCR. Furthermore, TERT mRNA detection by in situ hybridization or TERT protein detection by immunohistochemistry may be suitable to detect the localization of telomerase expression.

Interestingly, the CRFK cells, an in vitro immortalized cell line that can divide infinitely [8], have no telomerase activity and TERT mRNA expression. This cell line may have a telomerase-independent mechanism for maintaining telomere length, as shown in human cell lines immortalized in vitro and tumor-derived cell lines [5]. For example, telomere recombination in yeast is considered to be a mechanism for maintaining the telomere length [18, 22]. Also, in human cells, the extreme ends of chromosomes are tucked into the double-strand repeat forming a loop [12]. Therefore, it is conceivable that embedded single-strand DNA can be elongated by conventional DNA polymerase, using the double-strand repeat as a template.

TERT mRNA was detected in various normal tissues including the spleen, pancreas, stomach, cerebrum, testis, bone marrow, lymph node and thymus, and relatively high-level expression was observed in the small bowel and large bowel. The expression pattern of TERT mRNA in normal feline tissues is consistent with that in humans [17]. Cells in the crypts are morphologically immature and divide rapidly (once per two days). There are lymphocytes in gut-associated lymphoid tissues including aggregated (Peyer’s patches and lymphoid nodules) and non-aggregated (luminal, intraepithelial and lamina propria lymphocytes) tissues [13]. These cells may be the origin of TERT mRNA in the normal feline small bowel and large bowel. To assess the TERT expression at the single-cell level in normal feline tissues, in situ hybridization or immunohistochemical staining should be used.

In conclusion, the feline TERT gene was partially cloned, and its expression in normal feline tissues was detected by RT-PCR. Although further research is required, these data will provide fundamental knowledge of telomerase expression in normal feline tissues. Furthermore, quantitative measurement of the TERT mRNA expression and in situ detection of TERT will provide useful information to establish a diagnostic system for feline tumors.

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REFERENCES


