

Analysis of Novel Metastasis-associated Gene *TI-227*

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***TI-227* is a cancer metastasis-associated gene isolated from the B16-F10 mouse melanoma subline that preferentially metastasizes to the lung following intravenous injection. *TI-227* is more highly expressed in the B16-F10 and colon26-nl17 sublines, which preferentially metastasize to the lung following intravenous injection, than in the B16-BL6 and colon26-nl22 sublines, which preferentially metastasize to the lung following subcutaneous injection. Since *TI-227* could not be detected in normal tissues, there is a possibility that it is expressed specifically in tumors that preferentially metastasize to the lung following intravenous inoculation. Although the 5' end of *TI-227* was analyzed, its total sequence contained no obvious open reading frame. However comparison with the isolated human counterpart, *TI-227H* gene, revealed a highly homologous region near the 3' end, which was expected to contain a very short open reading frame.**

Key words: Metastasis-associated gene — Differential screening — B16 melanoma — *TI-227*

Blood-borne tumor metastasis comprises sequential steps: growth at a primary site, vascularization, invasion into blood vessels, attachment to capillary endothelial cells, invasion and growth in target organs.^{1,2} Several metastatic cell lines have been established and studies with those cells have revealed the existence of genes expressed in association with metastatic potential and behavior. Various methods, including differential screening,³ have been developed to clarify the underlying molecular mechanisms involved in cancer metastasis. Several metastasis-associated genes were found by comparing the expression levels in low- and high-metastatic cell lines, and such studies have revealed metastasis as a consequence of molecular events.⁴⁻⁷ We have previously used B16 mouse melanoma sublines with different metastatic behaviors.⁸⁻¹⁰ B16-F10 cells were established by 10 successive selections for lung metastasis following intravenous injection,⁸ while B16-BL6 cells were established from B16-F10 cells that penetrated the mouse bladder membrane.¹⁰ Although B16-F10 and B16-BL6 cells are both malignant and highly metastatic, B16-BL6 cells implanted into the subcutis are capable of spontaneously metastasizing to the lung, whereas B16-F10 cells colonize the lung only after direct inocula-

tion into the blood vessels. By comparing the genes expressed in B16-F10 cells and B16-BL6 cells, we isolated four that were highly expressed in B16-F10 but lower in B16-BL6 cells: *TI-225* (polyubiquitin), *TI-229* (pyruvate kinase), *TI-241* (LRF-1 homologue), and *TI-227* (novel gene).¹¹ Six other isolated genes (triosephosphate isomerase, 10-formyltetrahydrofolate dehydrogenase, tyrosinase related protein 2, cytochrome *c* oxidase, ATP synthetase α subunit, ribosomal protein), however, showed higher expression in B16-BL6 than in B16-F10 cells, while the other isolated genes showed no differences between the two lines.¹¹ In this study we further analyzed the characteristics of the *TI-227* gene.

MATERIALS AND METHODS

Cell lines and DNA library The sublines of colon26-nl17 and nl22 cells were cultured in DMEM containing 10% FBS, 100 μ g/ml of kanamycin, and 2 mM glutamine. All cells were maintained in a humidified atmosphere of 5% CO₂ at 37°C. The human fibroblast cDNA library was purchased from CLONETECH (Palo Alto, CA). The genomic library of the mouse was kindly provided by Dr. Tetsuo Noda, Japanese Foundation for Cancer Research.

Construction of the DNA libraries Poly(A)⁺ RNA was prepared from 8 \times 10⁷ exponentially growing cells using a Fast-Track mRNA isolation kit (Stratagene, La Jolla, CA), according to the instruction manual. Randomly primed and oligo(dT)-primed cDNA libraries of B16-F10 cells were prepared from 5 μ g of each poly(A)⁺ RNA using a Uni-ZAP XR2 kit (Stratagene) and recloned into Uni-ZAP XR vectors, according to the supplied instructions. The titer of the library was approximately 5 \times 10⁵ PFU before amplification, and the average length of the cDNA was 2 kbp.

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Abbreviations used are: DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; PFU, plaque-forming unit; AMPPD, disodium 3-(4-methoxyspiro{1,2-dioxethane-3,2'-tricyclo-[3.3.1.1^{3,7}]decan}-4-yl)phenyl phosphate; SDS, sodium dodecyl sulfate; SSC, sodium citrate-sodium chloride-buffer; SSPE, sodium phosphate-sodium chloride-EDTA buffer; nNOS, neural nitric oxide synthase.

Library screening Recombinant clones were plated at a density of 13000 plaques/90-mm plate and transferred to Hybond N(+) nylon filters (Amersham, Buckinghamshire, UK), according to the manufacturer's instructions, except that baking was done at 120°C for 20 min. Probes were prepared using the nucleotide labeling mixture containing digoxigenin-labeled dUTP (Boehringer-Mannheim, Mannheim, Germany). Duplicate filters were hybridized with 26 ng/ml of digoxigenin-labeled cDNA probes in hybridization buffer (50% formamide, 5× SSC, 0.05 M sodium phosphate (pH 7.0), 7% SDS, 0.005% yeast RNA, 0.1% lauroylsarcosine, 2% blocking reagent at 39°C for 16 h. Hybridized filters were washed twice in 2× SSC-0.1% SDS at room temperature for 5 min and twice in 0.5× SSC-0.1% SDS at 65°C for 15 min. Filters were hybridized with anti-alkaline phosphatase antibody and AMPPD according to the digoxigenin detection kit instruction (Boehringer-Mannheim), then exposed to X-ray films at room temperature for 1–60 min.

Northern blot analysis Poly(A)⁺ RNA (0.5 μg) was fractionated by electrophoresis on 1% agarose gel plates containing formaldehyde and transferred to nylon membranes (Amersham). Multi-tissue northern blot membrane containing 2 μg of mRNA isolated from various mouse organs was purchased from CLONETECH. The cDNA probe was labeled using a multi-primer DNA labeling kit (Amersham) according to the manufacturer's instruction. Membranes were hybridized with ³²P-labeled probes in a solution containing 50% formamide, 4× SSPE, 1% SDS, 0.5% Irish cream (R and A Bailey and Co., Dublin, Ireland), and 125 μg/ml of salmon testis DNA at 42°C for 24 h, washed in 2× SSC-0.1% SDS at 42°C four times and autoradiographed. The membranes were dehybridized at 100°C for 5–60 min and rehybridized with other cDNA probes.

PCR Template DNA (10 pmol) and primer DNA (10 pmol) were mixed to the total volume of 39 μl, then 3 μl of dNTP mix and 5 μl of 10× buffer along with 1 μl of TaKaRa Taq (TaKaRa, Kyoto) were added. The conditions of the PCR was 95°C for 1 min, 58°C for 2 min, 72°C for 3 min, for a total of 35 cycles. PCR products were then separated by electrophoresis on 2% agarose gel plates containing ethidium bromide and the plates were photographed.

Sequencing of cDNA clones Plasmid DNAs of the isolated clones were prepared by the alkaline-lysis method and purified with QIAGEN-tip 20 (QIAGEN, Hilden, Germany), according to the instruction manual. Sequencing of cDNA clones was performed using double-stranded templates and plasmid-specific primers in an automatic DNA sequencer (Applied Biosystems Model 373A, Ver. 2.0, Foster City, CA), according to the instruction manual of the Taq sequencing kit of Applied Biosystems. A homology search was performed using Genbank and EMBL data

bases. The possibility of translation was calculated using MacMolly software (Soft Gene GmbH, Berlin, Germany). The coding probability was calculated by using Staden's algorithm.¹²⁾

RESULTS

DNA sequencing The first isolated clone of *TI-227* at the time of differential screening was only 1.5 kbp, while the predicted length from the northern blot was 3.5 kbp. From the random primer library we reisolated two clones that were both about 3.5 kbp. Though we tried the primer extension method with the primer near the 5' end, no clones longer than the known 5' end were obtained (data not shown). The 3.5 kbp sequence (Genbank Accession No. D50523), determined from those clones, was compared with other known cDNA sequences using the Genbank/EMBL database, but no homologous genes were detected. Possible frames are shown in Fig. 1a, but there is no obvious open reading frame, and the longest is only about 200 bp. The calculated coding possibility is shown in Fig. 1b. The human homologue gene *TI-227H* (Genbank Accession No. D50525) was isolated from the human fibroblast cDNA library with the *TI-227* probe. The comparison between *TI-227* and *TI-227H* revealed a

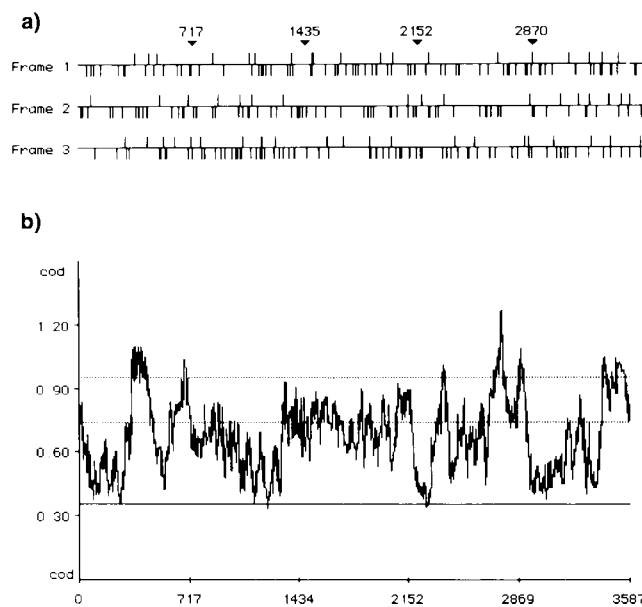


Fig. 1. (a) The possible open reading frames of *TI-227*. The upward bar represents the ATG codon; the downward bar represents stop codons. (b) The possibility of the translation of *TI-227*. The vertical axis represents the possibility of translation; the longitudinal axis represents base numbers from the 5' end. The stricter cut-off value and the weaker cut-off value are shown by the two dotted lines.

short but highly conserved region near the 3' end (Fig. 2). We then isolated two clones (G-10,19) whose determined sequences were identical from the mouse genomic library with the probe of the 5' end fragment of the isolated clone (Fig. 3a). TATA box sequences were not included. We made a set of primers and performed PCR between the 5'-3' primer (A, B or C) and the 3'-5' primer (D) (Fig. 3b). Both A and B primers gave bands of the expected length with the genomic DNA library as a template. Only the C primer gave the band of the expected length with the iso-

lated *TI-227* gene as a template. These were control studies and the results were as expected, but when we used the whole cDNA library as a template, B and C primers gave the band, but A primer did not. The exon-intron junction consensus sequence (C or T rich region/N/C or T/AG/A or G) was not included within the region. From these results, the real 5' end was expected to be located between the A and B primers.

Northern blot analysis Colon26-nl17 and nl22 cells are mouse colon cancer sublines that preferentially metastas-

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3424 GCCAGTACAG AGGGAAATGG AGCCAAAAGA GGAAGAGACT TGAGTTCTGA TGATCACATT 3483
      ||||| ||| || || | ||||| ||| | ||||| ||| | ||| ||| | ||
3841 GCCAGTGCAG GGGAAAGCAG AGCCAAAATA TGGAGAGACT TGAATCCTGA TGACAGCGTT 3900

3484 TATGCCCTG
      | |||||
3901 TGTGCCCTG
    
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Fig. 2. Comparison between *TI-227* (top) and *TI-227H* (bottom) near the 3' end.

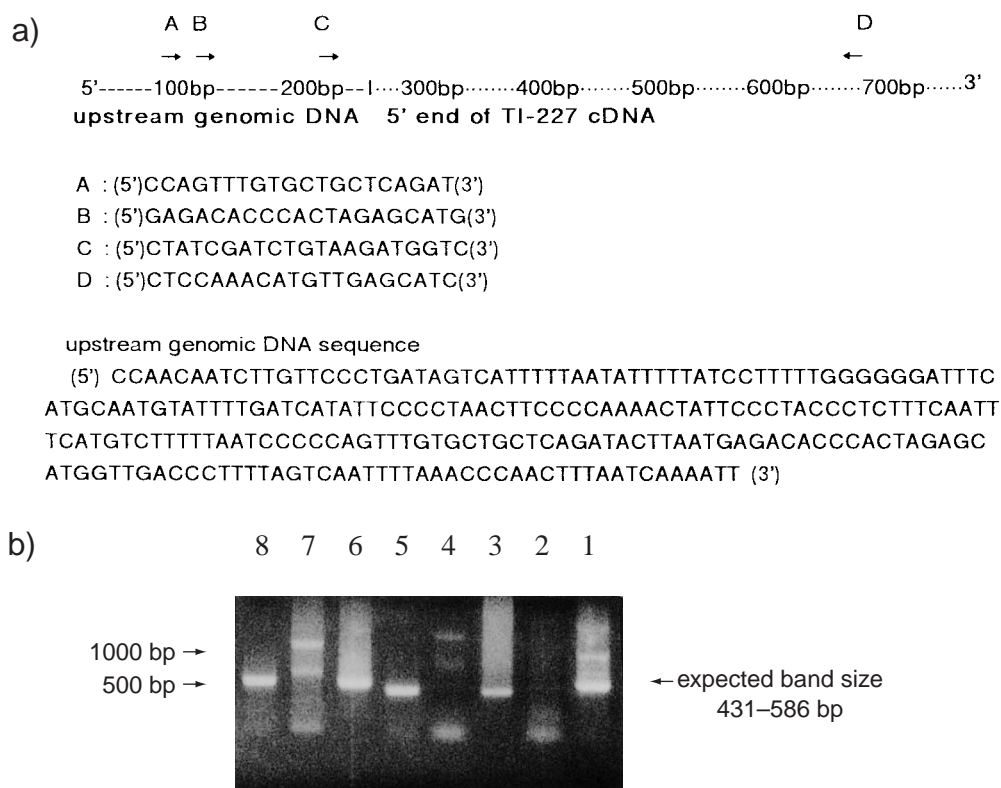


Fig. 3. (a) The sequence analysis of the 5' upstream region of *TI-227* and the primer set of *TI-227*. A and B are primers in the 5'-3' direction located in the 5' upstream region. C and D are primers located in the *TI-227* cDNA directed 5'-3' and 3'-5', respectively. (b) The result of PCR using A, B, C and D primers on the template of a genomic DNA, *TI-227* cDNA and a whole cDNA library. The arrow shows the band of expected length. Templates used: 1, 6, G-10; 2, 3, 7, *TI-227*; 4, 5, 8, cDNA. Primers used: 1, A, D; 2, A, D; 3, C, D; 4, A, D; 5, C, D; 6, B, D; 7, B, D; 8, B, D.

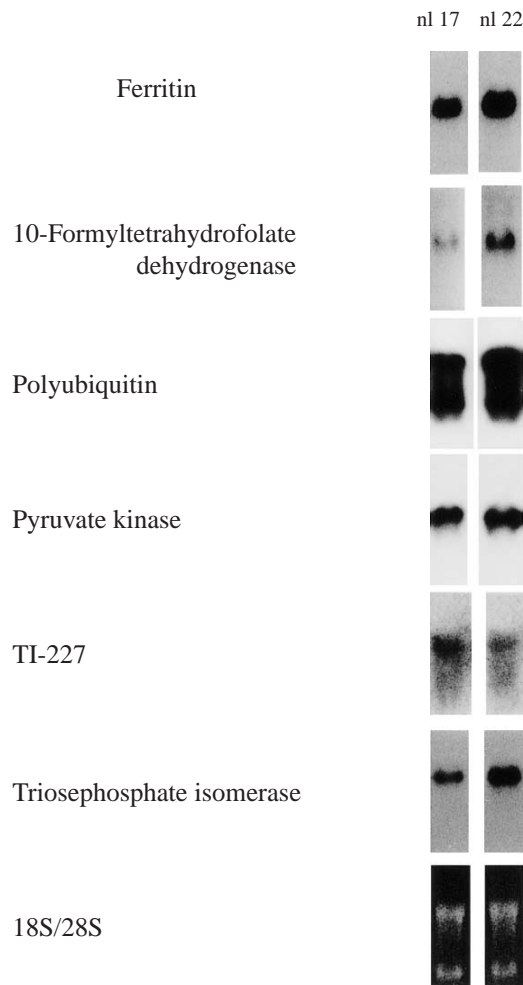


Fig. 4. Northern blot analysis of *TI-227*, expressed in colon26-nl17 and nl22 cells. Samples of poly(A)⁺ RNA (0.5 μ g) were electrophoresed in 1% formaldehyde-agarose gels and transferred to nylon membranes. The membranes were hybridized with the cDNA probes.

size to the lung following intravenous and subcutaneous injection, respectively (established by Tsuruo *et al.*). Also mouse B16 melanoma F10 and BL6 preferentially metastasize to the lung after intravenous and subcutaneous injection, respectively. The relationship of colon26-nl17 and nl22 sublines is thought to be similar to that of B16 melanoma F10 and BL6, so we performed northern blot analysis using *TI-227* cDNA and compared the mRNA expression levels in colon26-nl17 and nl22 with other cDNAs that were expressed more in B16-F10 cells. *TI-227* was the only clone that was expressed more in the colon26-nl17 subline (Fig. 4). To determine the function of *TI-227*, we attempted to detect mRNA of *TI-227* in nor-

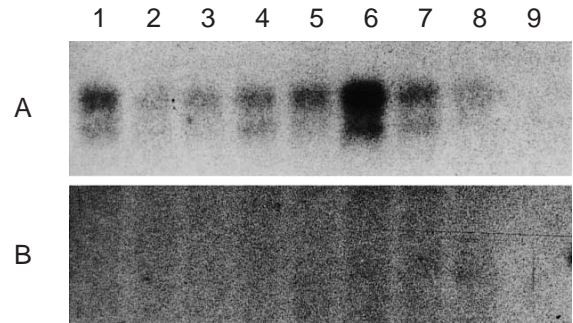


Fig. 5. Northern blot analysis of 10-formyltetrahydrofolate dehydrogenase (A) and *TI-227* (B) in normal tissues. Samples of poly(A)⁺ RNA (2.0 μ g) were transferred to nylon membranes. 1, heart; 2, brain; 3, spleen; 4, lung; 5, liver; 6, skeletal muscle; 7, kidney; 8, testis; 9, none (control).

mal tissues (Fig. 5) but could not. The control gene (10-formyltetrahydrofolate dehydrogenase) showed higher expression levels in the skeletal and heart muscles.

DISCUSSION

Colon26 adenocarcinoma nl17 subclone preferentially metastasized to the lung after intravenous inoculation, but not after subcutaneous inoculation. The nl22 subclone, however, preferentially metastasized to the lung after subcutaneous inoculation, but not after intravenous inoculation. The relationship of nl17 and nl22 is thought to be similar to the relationship between B16 melanoma F10 and BL6, respectively. Since *TI-227* was more highly expressed in colon26 adenocarcinoma nl17 than in nl22, it was expected to correlate with tumor metastasis following intravenous inoculation. Although the control gene, 10-formyltetrahydrofolate dehydrogenase, was expressed in actively metabolizing organs such as muscle, the expression of *TI-227* was not detected by northern blot analysis in normal tissues. There is a possibility that *TI-227* functions under the special circumstances of blood-borne metastasis.

Sequence analysis of *TI-227* revealed the insert to be 3751 bp with no obvious open reading frame or signal for translation identification. We have not been able to extend the sequence of mRNA further than the formally determined 5' end by primer extension (data not shown). The human homologue of *TI-227* isolated from the human fibroblast cDNA library was about 3.9 kbp long and showed 88.7% homology with *TI-227* at its 3' end, while the overall homology was 74.8%. If the open reading frame exists, it is expected to be located around that short region, but the real open reading frame has not been determined yet. Neuronal expression of nNOS protein was reported to be suppressed by an antisense RNA transcribed

from *NOS* pseudogene.¹³⁾ This suggests that transcribed pseudogenes are not without purpose and are a potential new class of regulatory gene. Even if *TI-227* is not translated, there remains a possibility that it might control the expression of other blood-borne metastasis-related genes, as an mRNA. Since *TI-227* is expressed more highly in B16-F10 (colon26-nl17) than in B16-BL6 (colon26-nl22), *TI-227* might be related to events after the attachment to capillary endothelial cells in the lungs. Although we examined the expression of *TI-227* in other tumor cell lines, we could not detect its expression by northern blot analysis.

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From the analysis of the 5' upstream region, this gene was not expected to be under TATA regulation, but the details are still unknown. We are now studying this gene further in association with other genes expressed during blood-borne metastasis.

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