Telomerase Activity in Germ Cell Cancers and Mature Teratomas

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Background: **An inverse relationship has been reported between the presence of telomerase enzymatic activity and the induction of differentiation in human tumor cell lines. Male germ cell tumors represent an attractive clinical model to assess this relationship further because high telomerase activity is present in normal germ cell progenitors and in embryonal carcinomas that can differentiate into mature teratomas. To investigate how telomerase activity and the differentiation state of germ cell tumors are related, telomerase activities and telomere lengths were measured in benign testicular tissues, germ cell cancers, and mature or immature teratomas.** ***Methods:*** **By use of a modified telomeric repeat amplification protocol (TRAP) assay, telomerase activity was measured in four specimens of benign testicular tissue, in 27 germ cell cancers, in seven mature teratomas, and in one immature teratoma. Telomere lengths were measured in all specimens by restriction digestion of genomic DNA and Southern blot hybridization analysis. Associations between telomerase activity and tissue histopathology were assessed with two-sided Fisher's exact tests.** ***Results:*** **Telomerase activity was detected in all examined germ cell cancers and in the benign testicular tissue specimens. In marked contrast, telomerase activity was not detected in any mature teratoma (*P*<.0001). Very long telomeres were detected in some mature teratomas, consistent with telomerase repression as a late event in teratoma formation. The immature teratoma, with malignant transformation, had high telomerase activity.** ***Conclusion:*** **Telomerase is active in germ cell cancers and repressed in mature teratomas. The absence of telomerase activity may contribute to the limited proliferative capacity of mature teratomas. These findings support the existence of an inverse relationship between telomerase activity and the differentiation state of clinical germ cell tumors. [J Natl Cancer Inst 1999;91:1321–6]**

Telomerase is an enzyme that prevents critical telomere shortening during cell division, allowing cells to bypass replicative senescence *(1–5)*. Escape from senescence is required for tumorigenesis. Telomerase activation is a frequent finding in malignancy *(6)*. An inverse relationship is reported between telomerase activity and induced differentiation of tumor cell lines, including germ cell tumor lines *(7–9)*. Following treatment with differentiation-inducing agents, telomerase activity is repressed in differentiation-sensitive but not differentiation-resistant human germ cell tumor cell lines *(8)*. Repression of telomerase activity may play a role in regulating the differentiation state of clinical tumors.

Germ cell tumors are unique in their capacity to undergo extensive differentiation, known as teratoma formation *(10)*. Unlike undifferentiated embryonal carcinomas from which teratomas derive, mature teratomas have limited proliferative capacity, although malignant transformation can occur *(11)*. This biologic feature provides an opportunity to investigate in the clinical setting how telomerase activity and tumor cell differentiation state relate. Studies investigating telomerase activity in male germ cell tumors are also relevant because these tumors derive from germ cell progenitors that constitutively express telomerase to regulate their

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telomere lengths *(2,3,6,12).* These findings are of added interest because late-generation telomerase RNA null mice exhibit defective spermatogenesis with increased apoptosis and decreased proliferation rates observed in the testes *(13).* This suggests that regulation of telomerase activity and perhaps telomere lengths are required for the maturation of normal or transformed germ cells.

Male germ cell tumors are broadly classified as seminomas and nonseminomas. These tumors exhibit diverse histopathology, often with extensive somatic differentiation *(14).* This study was undertaken to contrast telomerase activities and telomere lengths in male germ cell cancers (seminomas, nonseminomas, and mixed germ cell tumors) with those in mature or immature teratomas to understand the relationship between the telomerase activity and the differentiation state of clinical germ cell tumors. A modified telomeric repeat amplification protocol (TRAP) assay *(6,15,16)* was used to quantify telomerase activities.

Materials and Methods

Tissue Bank

Forty-one tumor specimens were obtained from 35 patients having germ cell tumors, who underwent potentially curative or diagnostic surgical resections. Benign testicular tissues were obtained either from patients who underwent orchiectomy for prostate cancer or from patients who underwent orchiectomy for germ cell cancer and had adjacent benign testicular tissue available for examination. Use of these found tissue specimens was approved by the Institutional Review Board. Within 10 minutes of surgical resection, the specimens obtained were snap-frozen in liquid nitrogen. Histopathologic analyses confirmed that extensive lymphocytic infiltrates and germinal centers or contaminating normal tissues were not present.

Germ cell tumors often exhibit histopathologic heterogeneity. To confirm the histopathologic diagnosis of the specimens used for telomerase and telomere measurements, a portion of each frozen tissue specimen was also sent for histopathologic analyses. All tissue specimens were reviewed by a single reference pathologist (V. E. Reuter) to confirm the histopathology present and to exclude concurrent pathologic processes. For those specimens used for TRAP assays, terminal restriction fragment (TRF) length and alkaline phosphatase measurements were immediately adjacent to those processed for histopathologic diagnoses. This permitted statistical correlations to be made between telomerase activities, telomere lengths, and the histopathology of the examined germ cell tumors. A portion of the same specimen was available for protein extraction and for isolation of genomic DNA used to assess TRF lengths.

Protein Extraction

Frozen tissue specimens (50–100 mg) were homogenized in 100–200 μL of ice-cold CHAPS (3-[{3-cholamidopropyl}-dimethylammonio]-1-propane-sulfonate) lysis buffer by use of disposable pestles, incubated on ice for 30 minutes, and centrifuged at 12 000*g* for 30 minutes at 4 °C. The supernatant was immediately collected, and the protein concentration was measured by use of the BioRad protein assay kit (Bio-Rad Laboratories, Richmond, CA). Protein aliquots were stored at –80 °C as 1 μg/μL stocks, as previously described *(6,8).* The isolated protein extracts were independently analyzed for alkaline phosphatase activities, as previously reported *(16).* This analysis was used to confirm that protein extracts used for TRAP assays were of sufficient integrity to measure another enzymatic activity susceptible to degradation in clinical tissues. If alkaline phosphatase activities were not detected in isolated protein extracts, then those extracts were not used for subsequent TRAP analyses.

TRAP Assay

The telomerase TRAP assay was performed by use of a modified polymerase chain reaction (PCR)-based method, previously established *(15,16).* Two micrograms of desired protein extracts was assayed in reactions containing 50 μL of the TRAP reaction mixture. For each assay, a negative control and 0.1 amol of the quantitation standard oligonucleotide R8 were included. The results were quantitated as previously reported *(16).* Briefly, this assay incorporates an internal PCR control of a 36 base-pair (bp) product (designated TSNT), running 14 bp below the smallest size-fractionated, TRAP-derived species. The amount of telomerase activity (total product generated [TPG]) for each reaction was calculated by use of the formula:

$$\mathrm{TPG} = \frac{(T - B)/(CT)}{(\mathrm{R8} - B)/(\mathrm{CR8})} \times 100.$$

T = radioactive counts from telomerase bands from the protein extracts, B = radioactive counts from a negative control (background), R8 = radioactive counts from R8 (0.1 amol), CT = radioactive counts from the internal control TSNT (0.01 amol) of the protein extract, and CR8 = radioactive counts from TSNT (0.01 amol) of the R8 (0.1 amol). One unit of TPG was defined as 0.001 amol or 600 molecules of telomerase substrate (TS) primer *(15)* extended by at least three telomeric repeats by the telomerase activity present in the examined extract and corresponds approximately to the activity present within a single immortal cell. Linearity of the assay was confirmed over at least three logs of the target protein concentrations [*(16);* data not shown]. All of the protein extracts were analyzed in at least two independent TRAP assays. The average telomerase activity (TPG) was calculated for every analyzed specimen. Presence of a potential telomerase inhibitor in telomerase-negative specimens was assayed by TRAP experiments by use of a protein extract from a mature teratoma mixed with protein extract from a neuroblastoma cell line known to have telomerase activity without a telomerase inhibitor.

TRF Length Measurements

TRF length measurements were performed by use of 10 μg of genomic DNA digested with the restriction endonucleases *Msp*I and *Rsa*I (Boehringer Mannheim GmbH, Mannheim, Germany) and electrophoresis on a 0.5% agarose gel. Hybridizations with telomere-specific $(TTAGGG)_3$ radiolabeled oligonucleotide probe were performed. Mean TRF lengths were calculated, as previously reported *(16–18).*

Statistical Analyses

When several assays were performed on the same tissue specimen or several tissue specimens from the same patient were analyzed, the average results from these assays were used for statistical analyses. Mean and standard deviation (SD) values for TPG and TRF length measurements were assessed for each histopathologic subtype. The association between telomerase activity in mature teratoma compared with that of germ cell cancers was examined by Fisher's exact test. Fisher's exact test was calculated by use of BMDP version 1.1 for Windows. All P values are two-sided. A two-sided Pearson's correlation test was used to analyze the correlation between telomerase activities and telomere lengths. Differences between mean telomerase activity and telomere lengths by histopathologic subtype were analyzed by the one-way ANOVA (analysis of variance) test.

Results

This study examined telomerase activity present in four benign testicular tissues without histopathologic evidence of germ cell tumors, 27 germ cell cancers, seven mature teratomas, and one immature teratoma. The germ cell tumors were derived from 35 patients. The clinical characteristics, histopathologies, and pretreatment markers are shown in Table 1. Telomere lengths were measured by TRF Southern blot hybridization analysis *(16–18).* Tissue specimens were obtained from testicular primary tumors in 31 cases and from retroperitoneal or pelvic sites in four. The median age of the patients was 27 years (range, 18–45 years). Twenty-six of the 35 patients had metastases at diagnosis. Seven seminoma cases were analyzed. The nonseminomatous germ cell tumors were embryonal carcinoma (n = 14), mature teratoma (n = 7), mixed germ cell tumors (n = 5), immature teratoma (n = 1), or a yolk sac tumor (n = 1).

Telomerase activity was readily detected in the examined benign testicular tissues without histopathologic evidence of germ cell tumors. In these tissues, the average telomerase activity was 489 TPG (range, 248.5–846 TPG). In the examined germ cell cancers, telomerase activity was detected in each case in which the integrity of the protein tissue extracts was confirmed by results of alkaline phosphatase assays (Fig. 1; data not shown). For the

Table 1. Patient characteristics

Characteristic	No.
Patients	35
Male/female	35/0
Median age in y (range): 27 (18–45)	
Primary site	
Testis	31
Retroperitoneal/pelvis	4
Histopathology	
Seminoma	7
Embryonal carcinoma	14
Yolk sac tumor	1
Mixed tumors	5
Mature teratoma	7
Immature teratoma	1
No. of metastatic sites	
0	9
1	19
2	6
3	1
Elevated pretreatment markers*	
AFP only	8
β-HCG only	2
AFP and β-HCG	7
Neither	18

*AFP = alpha-fetoprotein and β-HCG = β-human chorionic gonadotropin.

examined germ cell tumors, the average telomerase activity was 222.08 ± 287.08 SD and the TPG range was 0–1007. These telomerase activities exceeded those previously found by use of this TRAP assay in other examined human malignancies *(16–18).* These high telomerase activities may reflect the finding that nontransformed human fetal, newborn, and adult testes constitutively express high telomerase activities *(6,12)* and the corresponding malignancies may have high basal telomerase levels. The distribution of telomerase activities as related to germ cell tumor histopathology is shown in Fig. 1. Table 2 compares telomerase activity and telomere length as a function of histopathology for the examined germ cell tumors. There is no statistically significant difference observed among germ cell tumor types in mean telomerase activities (P = .13) by one-way ANOVA statistical analysis.

Analysis of telomerase activity in mature teratomas indicated that all examined mature teratomas had no detectable telomerase activity. An inhibitory factor was not detected in these examined protein extracts (data not shown). This lack of telomerase activity was statistically significantly associated with mature teratomas versus the examined germ cell cancers (Fisher's exact test; P<.0001).

The absence of telomerase activity in teratomas was in marked contrast to the high levels detected in the examined germ cell cancers. Notably, a single immature teratoma having malignant transformation had high telomerase activity (data not shown). This provides independent confirmation of a link between telomerase activity present in teratomas and the malignant potential of these tumors. Very long telomeres were detected in some mature teratomas (*see* Fig. 2), indicating that telomerase repression can represent a late event in teratoma formation. To confirm that the telomerase activity measured in seminomas was not due to an extensive lymphoid infiltrate that may include germinal centers the reference pathologist (V. E. Reuter) reviewed the histopathology of these cases. These dissected tumors were not found to have extensive lymphoid infiltrates or germinal centers (data not shown).

Telomere lengths as assessed by TRF analyses were examined in benign testicular tissues and germ cell tumors. The average mean TRF was 15 ± 5.92 kilobases (kb), SD (range, 6.17–34.5 kb), as shown in Fig. 2 and Table 2. Telomere length did not correlate with telomerase activity (two-sided Pearson's correlation; P = .96). The distribution of TRF lengths by histopathology is displayed in Fig. 2 and Table 2. Mean TRF lengths were longer in mature teratomas (TRF 19.09 ± 9.41 kb, SD; range, 6.17–34.5 kb). The mean TRF lengths for seminomas were shorter than those for the mature teratomas (TRF 10.71 ± 1.6 kb, SD; range, 8.73–13.3 kb), but these telomere lengths were not statistically significantly different as assessed by germ cell tumor type (P = .069; one-way ANOVA test). However, long telomeres (mean TRF, >20 kb) were detected in seven germ cell tumors, four of which were mature teratomas, two of which were embryonal carcinomas, and one of which was a mixed germ cell tumor containing immature teratoma. Telomerase activities were not detected in these four mature teratomas. The mean telomerase activity was high (523.1 TPG) in the other three examined germ cell tumors. Telomerase

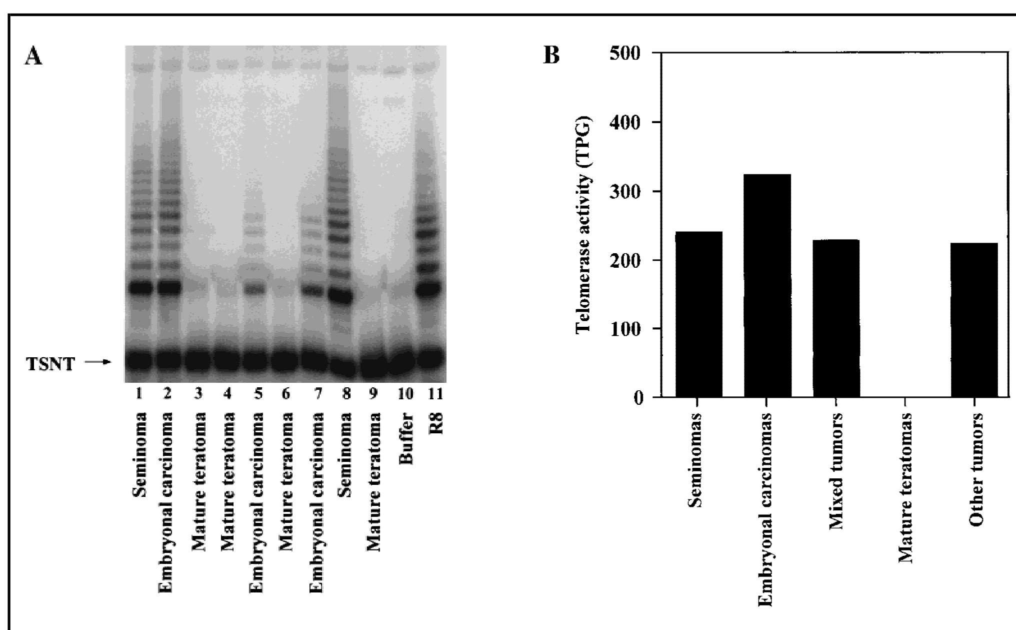

Fig. 1. Measurement of telomerase activity in germ cell tumor specimens. **A**) Representative polymerase chain reaction (PCR)-based telomeric repeat amplification protocol (TRAP) assays. Two micrograms of protein extracts was used in the assays displayed in **lanes 1–9,** a negative control (buffer without protein extract, **lane 10**) and a quantitation standard (R8, **lane 11**) were used for each of these TRAP assays. High telomerase activities were frequent in the germ cell cancer specimens, as indicated in **lanes 1, 2, 5, 7,** and **8,** while telomerase activity was not detected in the examined mature teratomas, as shown in **lanes 3, 4, 6,** and **9. B**) The telomerase activities (total product generated) from all of the examined germ cell tumors are shown in relation to the histopathologic subsets. The term "other tumors" refers to one immature teratoma and one yolk sac tumor. This study reveals that high telomerase activity present in germ cell cancers is not detected in mature teratomas. The standard deviations for each germ cell tumor subset are shown in Table 2.

Table 2. Telomerase activity and telomere length measurements in germ cell tumors in relation to histopathology*

Tumor type	No. of samples	Mean telomerase activity,† range	Mean telomere length,‡ range
Overall	35	222.08 (0–1007) [SD ± 287.08]	15 (6.17–34.5) [SD ± 5.92]
Seminoma	7	239.18 (70.6–437) [SD ± 174.66]	10.71 (8.73–13.3) [SD ± 1.60]
Embryonal carcinoma	14	322.35 (6.2–1007) [SD ± 365.93]	15.33 (8.47–28.63) [SD ± 4.96]
Mixed tumors	5	227.53 (2.18–703) [SD ± 299.28]	15.1 (11.65–20.15) [SD ± 3.09]
Yolk sac tumor	1	77	9
Mature teratoma	7	0 [SD ± 0]	19.09 (6.17–34.5) [SD ± 9.41]
Immature teratoma	1	371	17

*SD = standard deviation.

†Telomerase activity was assayed by the described telomeric repeat amplification protocol assay. Results were quantitated as the total product generated.

‡Telomere lengths were measured by terminal restriction fragment DNA analyses (kilobase) by Southern blot hybridization by use of a telomeric-specific probe.

activity is regulated during spermatogenesis and is undetectable in mature spermatozoa *(12,19)*. These observed differences in telomere lengths within germ cell tumors may reflect distinct stages of spermatogenesis from which these tumors derive.

Discussion

Prior work revealed that induced differentiation of maturation-sensitive but not of maturation-resistant human tumor cell lines represses telomerase activity *(7,8)*. This study extends this *in vitro* work by demonstrating that a similar relationship exists for clinical germ cell tumors. While telomerase activity was uniformly detected in germ cell cancers, telomerase activation was undetectable in the examined mature teratomas. This finding indicates that repression of telomerase activity accompanies maturation of these clinical tumors. A single immature teratoma with malignant transformation had telomerase activity. This observation provides additional evidence for a tight association between the differentiation state of germ cell tumors and telomerase activity. The high telomerase activity measured in the examined seminomas was not likely due to contaminating lymphoid cells with telomerase activity because these tumors did not have extensive lymphoid infiltrates or germinal centers. A study of human colon cancers *(18)* that examined inflammatory bowel lesions with extensive lymphoid infiltrates is reported. A minority of colitis cases had telomerase activity measured but at much lower levels than in colon cancers. This finding argues against contaminating lymphoid cells being responsible for the telomerase activity found in seminomas.

Some human tumors have no detectable telomerase activity *(20)*. Several mechanisms may contribute to this finding. Telomerase reactivation may not be required when tumor precursor cells have long telomere lengths and transformation results from few mutations. This is suggested by the absence of telomerase activity found in some retinoblastomas *(21)*. The absence of telomerase activity in some advanced stage neuroblastomas *(22)* may reflect tumor cell maturation. It is this mechanism for telomerase repression that is proposed as active in mature teratomas. It is notable that long telomeres were detected in some mature teratomas, indicating that telomerase repression is not an early step in teratoma formation.

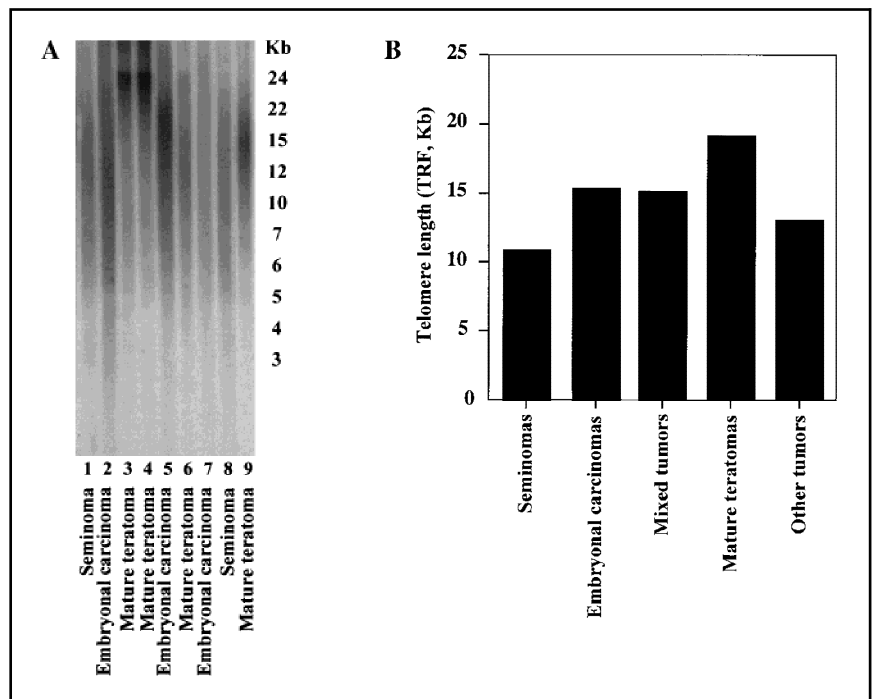

Fig. 2. Telomere length measurements of human germ cell tumors. **A**) Representative telomeric restriction fragment (TRF) length analyses of genomic DNA of human germ cell tumors are shown in **lanes 1–9.** Mean TRF lengths for germ cell tumors were longer (average TRF, 15 kilobases [kb]) than those previously reported for other adult human tumors *(16,17)*. Telomere lengths did not correlate with telomerase activity ($P = .96$; Pearson's correlation test). In the displayed mature teratomas, TRF lengths were quite long in two (>20 kb) but not in the other examined teratomas (**lanes 6** and **9**). **B**) Mean TRF lengths for all of the examined germ cell tumors are displayed in relation to histopathologic subsets. The term "other tumors" refers to one immature teratoma and one yolk sac tumor. The mean telomere lengths were somewhat longer in mature teratomas than in other germ cell tumors, but the mean telomere lengths assessed by germ cell tumor type were not significantly different ($P = .069$). Standard deviations for each germ cell tumor subset are shown in Table 2.

TRF lengths greater than or equal to 20 kb were reported in sperm and fetal tissues *(2,3)*. Three germ cell tumors in this study were found to have long telomeres and high telomerase activity. In other examined germ cell tumors, short TRF lengths (defined as TRF <10 kb) were measured with detectable telomerase activity, as shown in Table 2. However, the mean TRF lengths in the different germ cell tumor types were compared with a one-way ANOVA analysis and were not found to demonstrate a statistically significant difference ($P = .069$).

Telomerase repression might result from tumor cells exiting the cell cycle. An association is previously reported between telomerase activity and proliferation. Telomerase is repressed when tumor cells achieve quiescence *(23–26)*. In quiescent hematopoietic progenitors, basal telomerase activity is low but is rapidly induced when cells enter the cell cycle after exposure to hematopoietic growth factors *(25)*. Telomerase activity is inducible in some cultured somatic cells *(26)*. A relationship between telomerase activity and proliferation is reported. In breast cancer, telomerase activity is associated with S-phase fraction in lymph node-positive cancers *(27)*. In primary lung cancers, expression of the proliferation marker Ki-67 was associated with telomerase activity *(16)*. In germ cell tumors, expression of Ki-67 and proliferating cell nuclear antigen were detected in almost all of the examined germ cell tumors, including mature teratomas *(28)*.

Telomerase activity is regulated in spermatogenesis and in early embryogenesis *(12,19)*. Telomerase activity is not detected in mature spermatozoa and unfertilized eggs but is present in blastocysts and many fetal tissues. This developmental regulation of telomerase may account for detection of different telomerase activities or TRF lengths in the subsets of germ cell tumors present in Table 2. Male germ cell tumors are among the most sensitive to chemotherapy, and advanced stage germ cell tumors are often cured with cisplatin-based chemotherapy *(14)*. Perhaps chemotherapy treatments will affect telomerase activities in germ cell tumors. It is not yet known whether chemotherapy treatments alter telomerase activities in teratomas.

Alternative mechanisms may compensate for the end-replication problem and eliminate the need for telomerase activation in tumors. An alternative mechanism for the lengthening of telomeres was found in immortalized cell lines and in subsets of tumor-derived lines *(29)*. Some mature teratomas in this study had long telomeres. This is reminiscent of the long telomeres detected when alternative mechanisms for telomere lengthening are present *(29)*. However, telomere lengths similar to or shorter than those found in the benign testicular tissues (TRF length 14.3 kb; range, 12.52–14.4 kb) were observed in other teratomas. Therefore, an alternative mechanism is not likely to provide a consistent explanation for telomere lengthening in these mature teratomas. Other reasons for the absence of telomerase activity in teratomas may exist. Although an inhibitor of telomerase activity was not found, telomerase activation may precede repression after telomere lengthening in teratomas. Telomerase activity in teratomas may occur at levels below detection by this TRAP assay. While these or alternative telomere lengthening mechanisms are not formally excluded, the absence of telomerase activity in mature teratomas is hypothesized to result from signaling of tumor cell differentiation.

In summary, this study reports that telomerase activity was detected in all of the examined germ cell cancer specimens. In marked contrast, telomerase activity was not detected in the examined mature teratomas. No telomerase inhibitory activity was detected in any of these teratomas, and the integrity of the protein extracts used in this study was confirmed. These findings indicate that an inverse relationship exists between telomerase activity and the differentiation state of germ cell tumors. Notably, an immature teratoma exhibiting malignant transformation had telomerase activity detected. Thus, telomerase activation is commonly found in malignant germ cell tumors without extensive evidence of maturation. Absence of telomerase activity is a consistent feature of mature teratomas. Since long telomeres were detected in some mature teratomas, telomerase repression can be a late event in teratoma formation. Taken together, this study offers evidence for a direct link between telomerase activation and differentiation state of clinical germ cell tumors. Future work will determine whether this absence of telomerase activity is a marker or cause of teratoma formation.

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