Lack of Telomerase Activity in Lung Carcinoids Is Dependent on Human Telomerase Reverse Transcriptase Transcription and Alternative Splicing and Is Associated with Long Telomeres

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Abstract

Purpose: Preliminary evidence indicates that telomerase activity is significantly less expressed in typical carcinoids than in large cell neuroendocrine carcinomas or in small cell lung cancers. Knowledge of the mechanisms by which telomerase is differentially regulated in neuroendocrine lung tumors is important for a better understanding of the pathogenesis of these malignancies.

Experimental Design: We investigated telomerase activity in 86 neuroendocrine lung tumors and correlated the enzyme activity with the expression of the enzyme subunits [human RNA component (hTR), human telomerase reverse transcriptase (hTERT), and alternatively spliced hTERT variants], with the telomere-associated protein human protection of telomere-1, and with the telomere length pattern.

Results: A significantly ($P = 0.0001$) lower frequency of telomerase-positive cases was found in typical carcinoids (14%) than in large cell neuroendocrine carcinomas (87%) and small cell lung cancers (92%). hTR was constitutively expressed in all carcinoids. Telomerase-negative carcinoids were characterized by the absence of any hTERT transcript, only displayed the β^- alternatively spliced variant, or concomitantly expressed the $\alpha^+\beta^+$ full-length message with different combinations of alternatively spliced variants. However, in these tumors, a more abundant level of alternatively spliced transcripts than that of the $\alpha^+\beta^+$ full-length transcript was generally found. No significant difference was observed in human protection of telomere-1 expression between telomerase-negative and telomerase-positive carcinoids. Telomeres were significantly ($P < 0.05$) longer in telomerase-negative carcinoids than in telomerase-positive carcinoids (median value, 9.15 versus 4.47 kb). However, alternative lengthening of telomeres, as shown by associated promyelocytic leukemia bodies, was not observed in these tumors.

Conclusions: Our results indicate that telomerase is repressed in most lung carcinoids and that hTERT transcription and alternative splicing play a role in such a negative regulation. Moreover, the absence of any telomerase maintenance mechanism may contribute to the favorable prognosis of this malignancy.

Neuroendocrine lung tumors, which account for 20% to 25% of all lung cancers, include different clinicopathologic entities, corresponding to typical and atypical carcinoids, large cell neuroendocrine carcinomas (LCNEC), and small cell lung cancers (SCLC; ref. 1). Although they show some common morphologic and functional features, these tumors are characterized by a variable degree of biological aggressiveness and a different clinical behavior. Specifically, typical carcinoids have an indolent clinical course and favorable prognosis (2), in contrast with the high aggressiveness and rapid evolution of SCLCs (3), whereas atypical carcinoids and LCNECs exhibit intermediate behaviors (4).

Based on specific patterns of genetic alterations (5–7) and gene expression profiles (8), it has been proposed that neuroendocrine lung tumors can be divided into low-grade (typical and atypical carcinoids) and high-grade (LCNECs and SCLCs) tumors and that the two groups may represent genetically distinct and unrelated entities rather than steps of a progression spectrum (9, 10). With regard to genes peculiarly expressed in specific neuroendocrine lung tumor types, we previously showed a differential expression of telomerase activity, as the enzyme was present in <10% of typical carcinoids but in ~90% of LCNECs and SCLCs (11). Moreover, when telomerase activity was correlated with the immunocytochemical expression of a panel of genes involved in cell proliferation and apoptosis pathways, we found that the absence of telomerase activity in carcinoids was associated with a lack of bcl-2, p53, and c-kit expression and was characterized by a low proliferation

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index consistent with the absence of cyclin-dependent kinase-4 expression and the presence of the cyclin-dependent kinase inhibitor Rb. Conversely, telomerase-positive LCNECs and SCLCs generally showed an immunophenotype consistent with multiple gene product alterations (including high expression of bcl-2, p53, and c-kit and loss of Rb) and were characterized by a high proliferation index (5).

Telomerase is a RNA-dependent DNA polymerase that stabilizes telomeres and allows cells to avoid the senescence checkpoint (12, 13). Several lines of evidence indicate that telomerase is involved in the attainment of immortality in cancer cells and may therefore contribute to tumorigenesis and neoplastic progression (14–16). Overall, telomerase activity has been found in a high percentage of human tumors (>85%), whereas only a limited fraction of cancers maintains telomeres through an alternative lengthening of telomere (ALT) recombination mechanism (17). The core enzyme consists of a RNA component (hTR) that provides the template for the *de novo* synthesis of telomeric DNA (18) and a catalytic subunit [human telomerase reverse transcriptase (hTERT)] with reverse transcriptase activity (19, 20). Telomerase expression and function are strictly controlled at multiple levels, including transcription and alternative splicing of the catalytic component hTERT (21–23), assembly of telomerase in a large complex holoenzyme mediated by hsp90 and hsp70 chaperones (24), modification of the hTERT phosphorylation pattern (25, 26), and interaction with telomere-associated proteins, including TRF1, TRF2, tankyrase, and human protection of telomere-1 (hPOT1; refs. 27–29). Specifically, hPOT1 is a recently discovered single-stranded telomeric DNA binding protein that has been shown to act as a telomerase-dependent positive regulator of telomere length (30).

Knowledge of the mechanisms by which telomerase is differentially regulated in the different human tumor types might be important for a better understanding of the pathogenesis of such malignancies. The present study was undertaken with the double aim: (*a*) to confirm in a large series of neuroendocrine lung tumors the differential expression of telomerase activity as a function of tumor type and (*b*) to gain insight into the molecular mechanisms responsible for the markedly lower frequency of telomerase activity observed in carcinoids than in LCNECs and SCLCs. The latter aim was achieved by correlating the presence/absence of enzyme activity with the expression of core enzyme subunits hTR and hTERT and the telomere-associated protein hPOT1, with the presence of alternatively spliced hTERT variants, and with the telomere length pattern in individual tumors.

Materials and Methods

Tumor material. A total of 86 neuroendocrine lung tumors were obtained from patients who underwent surgery at the Istituto Nazionale per lo Studio e la Cura dei Tumori (57 cases), the European Institute of Oncology (19 cases), and the Istituto Clinico Humanitas (10 cases). For tumor classification, we applied the Armed Forces Institute of Pathology criteria (31), which subdivide neuroendocrine lung carcinomas into typical carcinoids, atypical carcinoids, SCLCs, and LCNECs. Besides SCLC, recently renamed grade 3 neuroendocrine carcinoma, which is characterized by cytologic features of scanty cytoplasm, finely granular chromatin, absent or inconspicuous nucleoli, and frequent mitoses, all the three remaining entities showed neuroendocrine morphology. Typical carcinoid, recently renamed grade 1 neuroendocrine carcinomas (32), is a tumor with carcinoid morphology and <2 mitoses per 10 high-power fields lacking necrosis. Atypical carcinoid, recently renamed grade 2 neuroendocrine carcinomas, is a tumor with carcinoid morphology that can be distinguished from typical carcinoid by the higher degree of nuclear atypia, greater mitotic activity (≥5 mitotic figures per 10 high-power fields), and foci of spontaneous necrosis that are often dystrophically calcified. LCNEC, recently renamed grade 3 neuroendocrine carcinoma, is a tumor with a neuroendocrine morphology, >11 mitoses per 10 high-power fields, necrosis, and cytologic features of a non–small cell carcinoma coupled with positivity for one or more neuroendocrine markers by immunohistochemistry for neuroendocrine granules by electron microscopy. According to these criteria, our case series included 44 typical carcinoids, 15 LCNECs, and 27 SCLCs, whereas atypical carcinoids were not represented.

At the time of surgery and for molecular analyses, the tissue sample of each case was immediately flash frozen in liquid nitrogen and stored at −80°C until use. The representativity of the tumor tissue sampled for molecular analyses was confirmed on frozen sections successively stained with H&E. For each case, telomerase activity, hTR, hTERT, and hPOT1 expression, and telomere length were determined in three contiguous pieces of the same tumor lesion. In a subset of 10 cases, for which a large amount of frozen tumor material was available, we carried out all the molecular assays in the three contiguous tumor pieces, and results of the replicate assay were almost superimposable. The human SCLC cell line POGB (33) was used as a positive control throughout the study.

Telomerase activity detection assay. Cell extracts were obtained as described previously (34). Telomerase activity was measured by the PCR-based telomeric repeat amplification protocol (TRAP) with some modifications (16). Samples containing 0.6 and 6 μg protein were analyzed in the TRAP reaction for each tissue specimen by the TRAPeze kit (Intergen Co., Oxford, United Kingdom) according to the manufacturer's protocol. After extension of the substrate TS (5′-AATCCGTCGAGCAGAGTT-3′) oligonucleotide by telomerase, the telomerase products were amplified by PCR in the presence of 5′ ^{32}P-end-labeled TS primer for 28 cycles and resolved in 10% polyacrylamide gels. Each reaction product was amplified in the presence of a 36-bp internal TRAP assay standard. A TSR8 quantitation standard (which serves as a standard to estimate the amount of product extended by telomerase in a given extract) was included for each set of TRAP assays. Quantitative analysis was done as reported previously (35). Telomerase activity obtained with 1.0 μg protein extract from the POGB cell line was arbitrarily defined as 1.00 unit. The relative telomerase activity of each tissue sample represents the ratio between the arbitrary unit of the tested sample and that of POGB cells.

RNA extraction and reverse transcription-PCR analysis of hTR, human telomerase reverse transcriptase, and human protection of telomere-1. Total cellular RNA was extracted from frozen samples with the TRIzol reagent (Life Technologies, Gaithersburg, MD) according to the manufacturer's instructions. Total RNA (0.5 μg) from each sample was used for cDNA production using the Reverse Transcription-PCR (RT-PCR) Core kit (Applied Biosystems, Branchburg, NJ) with random hexamers. The cDNA samples were then amplified with the same kit. Amplification of hTR cDNA was obtained using TR-46S (5′-CTAACCCTAACTGAGAAGGGCGTAG-3′) and TR-148AS (5′-GAAGGCGGCAGGCCGAGGCTTTTCC-3′) oligonucleotides with an initial heating at 95°C for 3 minutes followed by 28 cycles at 95°C for 30 seconds, 65°C for 45 seconds, 72°C for 30 seconds, and 72°C for 5 minutes. Amplification of hPOT1 cDNA samples was obtained using POT1S (5′-TCAGTCTGTTAAACTTCATTGCCC-3′) and POT1AS (5′-TGCACCATCCTGAAAAATTATATCC-3′) oligonucleotides with an initial heating at 94°C for 30 seconds followed by 28 cycles at 94°C for 5 seconds, 60.4°C for 50 seconds, 72°C for 50 seconds, and 72°C for 5 minutes.

Amplification for *β-actin* as a constitutively expressed gene was done using the primers 774S/775AS (36) and ACTBs/ACTBa (37) for hTR and POT1, respectively. β-actin PCR amplification conditions

were identical to those described above, except that they were carried out for 20 cycles. Amplification of alternatively spliced hTERT cDNA was obtained using TERT-2164S (5′-GCCTGAGCTGTACTTTGTCAA-3′) and TERT-2620AS (5′-CGCAAACAGCTTGTTCTCCATGTC-3′) oligonucleotides with initial heating at 95°C for 3 minutes followed by 35 cycles at 95°C for 30 seconds, 62°C for 50 seconds, 72°C for 50 seconds, and 72°C for 5 minutes. The amplification was done in a mixture containing 0.3 μCi [α-^{32}P]dCTP (3000 Ci/mmol, Amersham Pharmacia Biotech, Milan, Italy). Primers for internal control were added at 72°C of cycle 15. All RT-PCR experiments were carried out in duplicate. Amplified products were electrophoresed on 1% agarose gel in 1× Tris-acetate EDTA buffer (hTR and hPOT1) and on 5% nondenaturing polyacrylamide gel in 1× Tris-borate EDTA buffer (hTERT). The agarose gel was photographed and the polyacrylamide gel was dried and autoradiographed.

All amplified products were analyzed by a ScanJet IIcx/T scanner (Hewlett Packard, Milan, Italy) and quantified by ImageQuant software (Molecular Dynamics, Sunnyvale, CA). To evaluate the relative levels of expression of the target genes, the value of the *β-actin* was used as the baseline gene expression in the samples, and the relative value was calculated for each of the target genes (densitometric value of the target gene/densitometric value of β-actin). The values were then used to compare expression across tested samples. The hTERT ratio (calculated by dividing the expression value of the full-length transcript signal by that of the alternatively spliced species) was also determined for each tissue sample.

DNA isolation and Southern blotting for measuring telomere length. Total DNA was isolated using DNAzol (Life Technologies Italia, Milan, Italy). For each sample, DNA (10 μg) was digested with 40 units *Hin*fI and then electrophoresed on 0.8% agarose gels. Following electrophoresis, gels were denatured, neutralized, transferred to a nylon membrane (Hybond-N, Amersham Pharmacia Biotech), and then cross-linked with UV light. The membrane was hybridized with a 5′ [γ-^{32}P]dATP-labeled telomeric oligonucleotide probe $(TTAGGG)_4$ as reported previously (36). Autoradiographs were scanned (ScanJet IIcx/T) and digitalized by ImageQuant, and the peak telomere restriction fragment length was calculated as reported previously, with minor modifications (38).

Assessment of alternative lengthening of telomere–associated promyelocytic leukemia bodies. Frozen tumor sections were dropped onto slides, fixed with methanol and acetone (1:1) for 10 minutes, and labeled with the primary rabbit anti–promyelocytic leukemia (PML) polyclonal antibody (Chemicon International, Temecula, CA). FITC-conjugated anti-rabbit secondary antibody (Sigma-Aldrich, Milan, Italy) was used to detect primary antibody. Fluorescence *in situ* hybridization analysis was carried out using the telomere PNA/Cy3 probe (Applied Biosystems) according to the manufacturer's instructions. Images were captured with a Nikon Eclipse E600 microscope using ACT-1 (Nikon, Tokyo, Japan) image analysis software and processed using Adobe Photoshop Image Reader 7.0 software.

Statistical analysis. Differences in frequency of tumors exhibiting telomerase activity by histologic type were assessed using the χ^2 test. The Wilcoxon rank-sum test was used to assess the statistical differences between telomere length in telomerase-positive and telomerase-negative carcinoids. All *P*s were two sided.

Results

Telomerase activity was measured in a series of 86 human neuroendocrine lung tumors, including 44 typical carcinoids, 15 LCNECs and 27 SCLCs, by the TRAP assay. The reliability of the assay was investigated by testing two protein concentrations (0.6 and 6 μg) for each sample, and results showed that in telomerase-positive tumors the extent of the TRAP signal was dependent on protein concentration (Fig. 1). In fact, in no case was a tumor that showed telomerase activity at the protein concentration of 0.6 μg negative at the highest protein concentration. Consequently, TRAP results obtained with 6 μg protein were considered as the best indicator of enzyme activity, and this concentration was used to compare telomerase activity across samples. Based on these data, a highly significant ($P = 0.0001$) difference in the frequency of telomerase-positive cases was observed among the three tumor types. Specifically, telomerase activity was observed in 6 of 44 (14%) carcinoids, 13 of 15 (87%) LCNECs, and 25 of 27 (92%) SCLCs. Examples of the TRAP assay for the different tumor types are illustrated in Fig. 1. To compare the extent of enzyme catalytic activity across samples, telomerase activity was expressed as a relative value with respect to the activity (arbitrarily defined as 1.00 unit) observed in the POGB human SCLC cell line, which was used as a positive control throughout the study. The extent of telomerase activity varied widely among TRAP-positive samples, ranging from 0.0059 to 0.93 (median value, 0.36 unit) in carcinoids, from 0.04 to 2.82 (median value, 0.30 unit) in LCNECs, and from 0.03 to 36.23 (median value, 0.53 unit) in SCLCs.

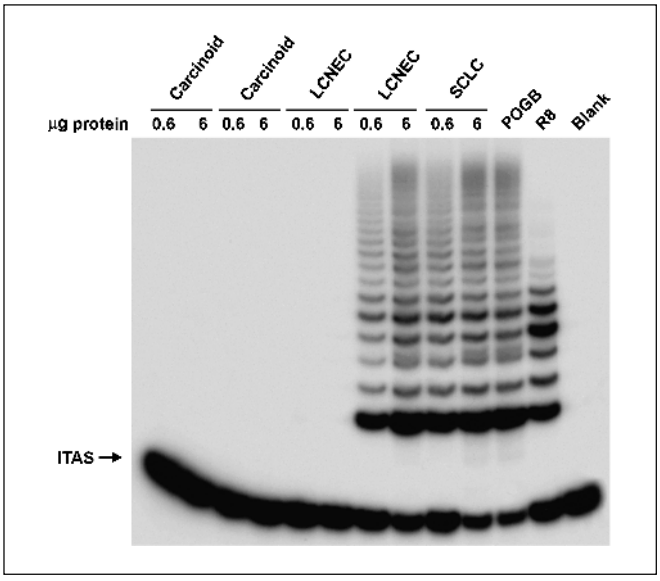

Fig. 1. A representative series of typical carcinoids, LCNECs, and SCLCs in which telomerase activity was detected by the TRAP assay using 0.6 and 6 μg protein. POGB, a positive control containing 1 μg protein of the telomerase-positive POGB human SCLC cell line; Blank, a negative control to which no cell extract was added; ITAS, internal amplification standard.

To investigate at a molecular level possible determinants responsible for the significantly lower frequency of telomerase activity observed in carcinoids than in LCNECs and SCLCs, we analyzed the expression of the components of telomerase core enzyme, hTR and hTERT, in a subset of 52 neuroendocrine lung tumors, including 24 carcinoids (19 TRAP negative and 5 TRAP positive), 12 LCNECs (4 TRAP negative and 8 TRAP positive), and 16 SCLCs (2 TRAP negative and 14 TRAP positive). Results of RT-PCR experiments showed that hTR was expressed in all clinical specimens, independently of telomerase activity, although the extent of hTR expression varied among the individual tumor specimens, ranging from 0.60 to 0.95 (median value, 0.79 unit) in carcinoids, from 0.63 to 0.81 (median value, 0.72 unit) in LCNECs, and from 0.72 to 1.08 (median value, 0.95 unit) in SCLCs.

Because it has been shown that alternative splicing of the hTERT is involved in the regulation of telomerase activity (21), we analyzed the expression of the different hTERT transcripts by RT-PCR using a specific primer set for the reverse transcriptase domain of the hTERT transcript, which allows the detection of four amplification products: the hTERT full-length transcript (457 bp), the α^- splicing variant that lacks 36 nucleotides from the 5′ end of exon 6 (which codes for the A domain belonging to the reverse transcriptase motifs of the enzyme, 421 bp), the β^- splicing variant (which is characterized by total deletion of exons 7 and 8, which in turn gives rise to a termination codon causing premature ending of translation, 275 bp), and the $\alpha^-\beta^-$ splicing variant (which displays the combination of both deletions, 239 bp; ref. 21). RT-PCR experiments were carried out on the same subset of 52 neuroendocrine lung tumors screened for hTR expression. Representative examples of the different hTERT slicing patterns observed in lung neuroendocrine tumors are shown in Fig. 2. Results showed that in the five carcinoids expressing telomerase activity the full-length transcript was always present: in one case it was coexpressed with the β^- variant but at a higher expression level than the deletion transcript (hTERT ratio, 1.21), whereas in four cases it was coexpressed with two (α^- and β^-) or all spliced variants at a comparable expression level (mean hTERT ratios, 1.02 and 0.85, respectively). With regard to telomerase-negative carcinoids, the full-length transcript was not detected in nine samples: seven samples were completely negative for hTERT expression and two samples showed only the β^- variant. In the remaining 10 cases, the full-length transcript was always present with different combinations of α^-, β^-, or $\alpha^-\beta^-$ deletion transcripts. Specifically, the abundance of the full-length transcript was comparable with that of the β^- deletion transcript in two cases (mean hTERT ratio, 1.065), whereas the remaining eight carcinoids generally showed a slightly lower expression of the full-length than that of the alternatively spliced variants, with mean hTERT ratios ranging from 0.455 to 0.687 for the different transcript combinations. Telomerase-positive LCNECs revealed expression of the full-length transcript alone (one case) or in association with different combinations of spliced variants α^-, β^-, or $\alpha^-\beta^-$ (seven cases). In all these cases, the level of expression of the full-length transcript was lower than that of the deletion variants, with mean hTERT ratios ranging from 0.17 to 0.413 for the different transcript combinations. All four telomerase-negative LCNECs showed only the β^- variant. With regard to the 14 telomerase-positive SCLCs, they all expressed the full-length transcript in association with the β^- transcript in 1 case, with the β^- and $\alpha^-\beta^-$ transcripts in 2 cases, and with all spliced variants in 11 cases. These tumors were characterized by a lower level of full-length transcript expression than that of the deletion variants, with the exception of one case in which the full-length transcript abundance was higher than that of β^- + $\alpha^-\beta^-$ spliced messages. Conversely, the two telomerase-negative SCLCs showed only the β^- variant.

As the next step of the study, we evaluated the expression of hPOT1, a telomere-associated protein that has been suggested to recruit telomerase to the ends of chromosomes and facilitate telomere elongation by the enzyme (39), in the same subset of 52 neuroendocrine lung tumors. Representative examples of hPOT1 expression patterns in the different tumor types are shown in Fig. 3. Results showed that hPOT1 was expressed in all tumor specimens, with expression levels ranging from 0.59 to 0.99 (median value, 0.77 unit) in carcinoids, from 0.86 to 1.13 (median value, 1.00 unit) in LCNECs, and from 1.00 to 1.45 (median value, 1.25 unit) in SCLCs. However, no significant difference in the level of hPOT1 mRNA was observed between telomerase-negative and telomerase-positive samples.

Finally, telomere length was determined by Southern blot hybridization in 30 neuroendocrine lung tumors, including 17 carcinoids (14 telomerase positive and 3 telomerase negative), 6 LCNECs, and 7 SCLCs (all telomerase positive). Representative examples of the different patterns of terminal restriction fragments are depicted in Fig. 4. Overall, the median telomere length in carcinoids (8.8 kb) was similar to that observed in

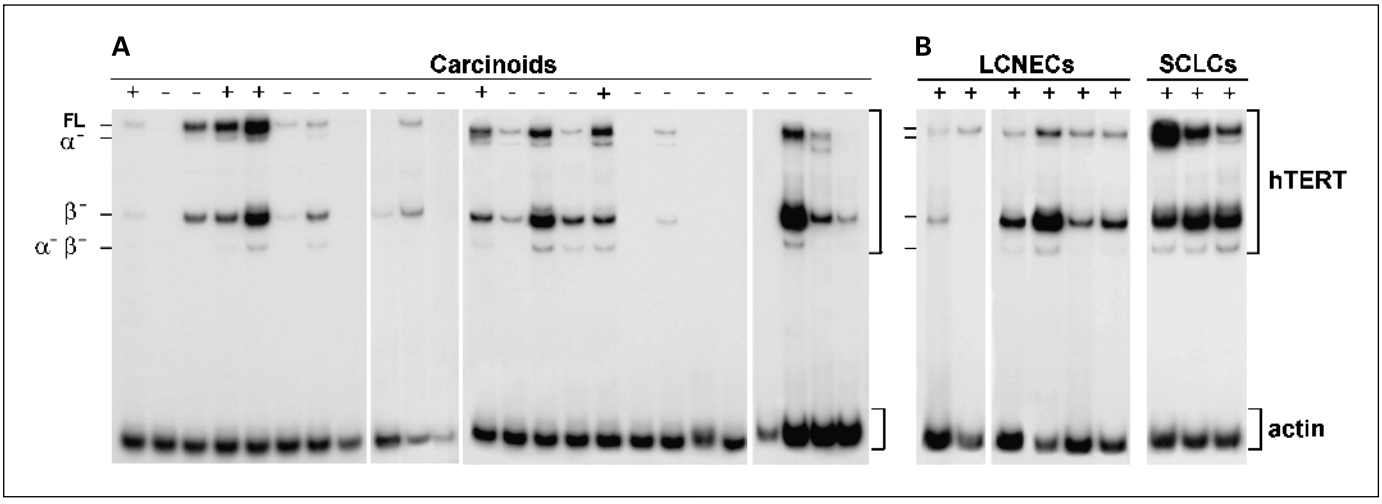

Fig. 2. Expression of hTERT mRNA transcripts (full-length and alternative splicing variants α^-, β^-, and $\alpha^-\beta^-$) as detected by RT-PCR in the overall series of typical carcinoids (*A*) and a representative series of LCNECs and SCLCs (*B*). Telomerase subunits were coamplified with β-actin as the internal standard. The POGB cell line was used as reference external control. The blank represents a negative control to which no RNA was added. The presence (+) or absence (−) of telomerase activity, as detected by the TRAP assay using 6 μg protein, is reported for each sample.

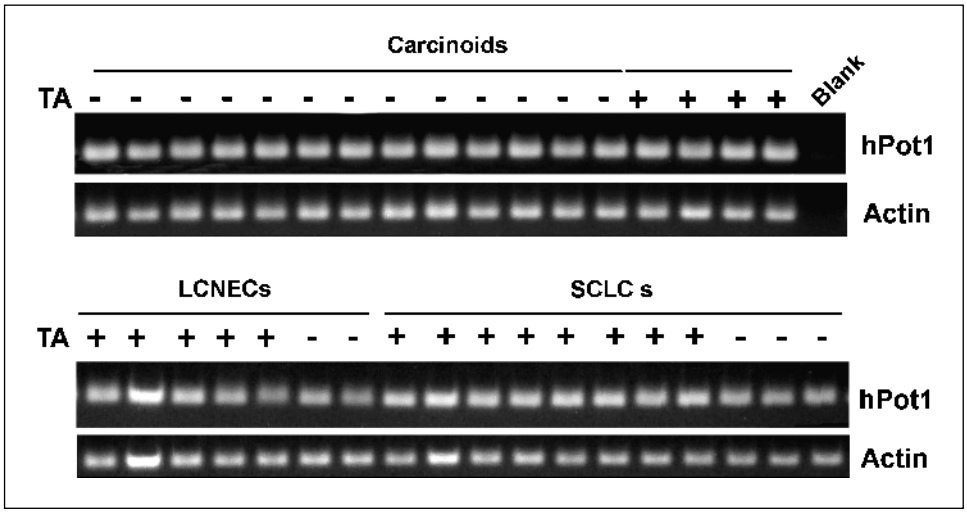

Fig. 3. hPOT1 mRNA expression as detected by RT-PCR in a representative series of typical carcinoids, LCNECs, and SCLCs. Actin was used as the external standard. The blank represents a negative control to which no RNA was added. The presence (+) or absence (−) of telomerase activity, as detected by theTRAP assay using 6 μg protein, is reported for each sample.

LCNECs and SCLCs (7.4 and 9.4 kb, respectively; Table 1). Among telomerase-positive tumors, carcinoids were characterized by a lower, although not statistically different, median terminal restriction fragment value (4.47 kb) than that observed in LCNECs and SCLCs. Moreover, a breakdown analysis within carcinoids as a function of the presence/absence of telomerase activity showed that telomerase-positive specimens were characterized by a significantly lower median terminal restriction fragment value than that observed in telomerase-negative samples (4.47 versus 9.15 kb; $P < 0.05$). To address the possibility that ALT mechanisms are operating in telomerase-negative carcinoids, we screened them for the expression of specific nuclear structures called ALT-associated PML bodies. These structures, which represent a novel form of nuclear PML bodies in which PML protein colocalizes with telomeric DNA, are peculiarly expressed in ALT tumor cells and absent in telomerase-positive tumor cells (40). However, results we obtained through a combined immunostaining/fluorescence *in situ* hybridization approach failed to show the presence of ALT-associated PML bodies in any carcinoid sample considered (data not shown).

Discussion

In the present study, we confirmed on a large series of neuroendocrine lung tumors previous evidence obtained by our group (11) and other groups (41) indicating a significantly lower frequency of telomerase-expressing cases in atypical carcinoids than in more aggressive tumor types, such as LCNECs and SCLCs. Such data would suggest that, according to the telomerase hypothesis (42), carcinoid tumor cells are mortal pre-M2 stage cells, whereas SCLC and LCNEC cells are immortal post-M2 stage cells. They corroborate and extend the previously reported evidence for the existence among neuroendocrine lung tumors of two genetically unrelated entities (SCLCs and LCNECs versus carcinoids) that have distinct phenotypic profiles.

To gain insight into the molecular mechanisms responsible for the repression of telomerase activity in most carcinoids, we assessed the expression of core enzyme components hTR and hTERT in individual tumors. hTR was expressed in all cases independently of telomerase activity, in accord with the results of previous studies indicating that the presence of hTR was not

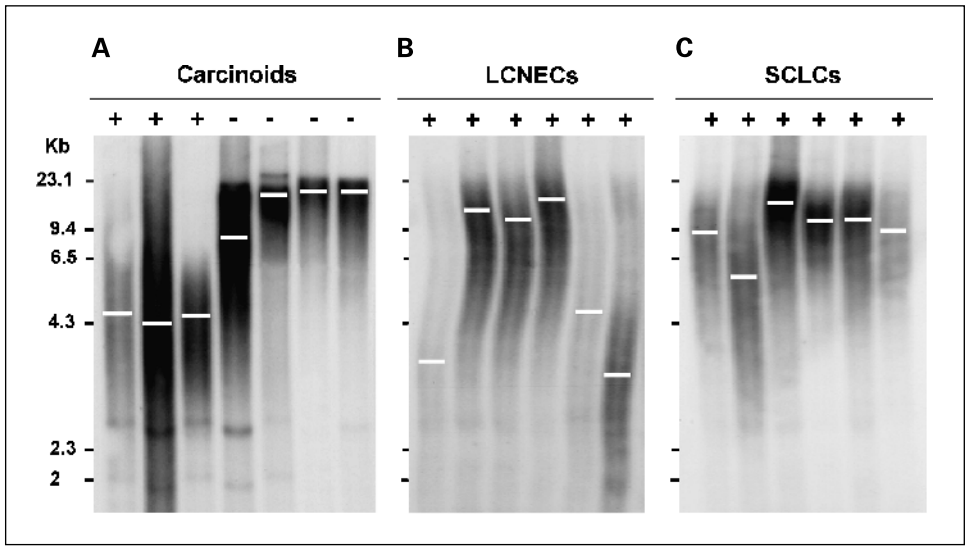

Fig. 4. Autoradiograms showing terminal restriction patterns in a representative series of carcinoids (*A*), LCNECs (*B*), and SCLCs (*C*) after hybridization of *Hin*fI-digested genomic DNA with a $(TTAGGG)_4$ probe.

Table 1. Telomere length of neuroendocrine lung tumors

	Terminal restriction fragment (kb)					
	Telomerase-positive cases			Telomerase-negative cases		
	No. cases	Median	Range	No. cases	Median	Range
Carcinoid	3	4.47*	4.35-4.6	14	9.15*	7-23
LCNEC	6	7.4	3.5-17	—	—	—
SCLC	7	9.4	5-14	—	—	—

*$P < 0.05$ (Wilcoxon rank-sum test). Median value of all carcinoids, 8.8.

correlated with telomerase activity in cellular models and clinical case series of different tumor types (43). Moreover, the extent of hTR expression was comparable in the different neuroendocrine lung tumor types, although a slightly higher median expression value was observed for SCLCs than for LCNECs and carcinoids. In this context, Sarvesvaran et al. (44) showed that lung carcinoids displayed a significantly lower frequency of hTR expression than SCLCs as detected by RNA *in situ* hybridization. A possible explanation for such discrepancy is that *in situ* hybridization is a less sensitive method than RT-PCR; therefore, low levels of hTR expression could be missed in some samples.

With regard to the telomerase catalytic subunit, several studies have shown a strong correlation between telomerase activity and hTERT mRNA expression in experimental and clinical models of different tumor types, suggesting that transcription of the *hTERT* gene is a major regulatory step in the process of telomerase reactivation (45, 46). Moreover, it has been proposed that besides gene transcription, alternative splicing of hTERT transcripts may be involved in the regulation of telomerase activity not only during human tissue development (21) but also in different human tumor types (22, 23, 34). Here, we assessed the presence of the hTERT full-length transcript and alternatively spliced variants in neuroendocrine lung cancers and correlated it with telomerase activity. hTERT splicing variants, including α^-, β^-, and $\alpha^-\beta^-$ deletion transcripts, were detected in most of the tumors. As alternative splicing removes reverse transcriptase motifs, it is unlikely that deletion transcripts code for functional proteins. In particular, the β^- transcript has been shown to be incapable of reconstituting an active telomerase enzyme *in vitro* (47). Moreover, the α^- splicing variant has been suggested recently to act as a dominant-negative inhibitor of telomerase activity (48). In this context, we showed recently that oligomer-mediated modulation of hTERT splicing pattern affects telomerase activity in prostate cancer cells. Specifically, by using a panel of 2′-*O*-methyl-RNA phosphorothioate oligonucleotides targeting the splicing site in the hTERT pre-mRNA, we were able to decrease to a variable extent the full-length hTERT transcript and to concomitantly increase the alternatively spliced transcripts. Moreover, the extent of telomerase activity in oligomer-treated samples was influenced by the relative abundance of alternatively spliced transcripts, and a statistically significant direct correlation was found between the catalytic activity of the enzyme and the ratio of the full-length transcript over the α^- + $\alpha^-\beta^-$ deletion transcripts (49).

Carcinoids expressing telomerase activity were characterized by the presence of the complete hTERT transcript together with one or more alternatively spliced variants. However, in these lesions, the full-length transcript was more abundant than or as abundant as alternatively spliced variants. Conversely, telomerase-negative carcinoids did not express any hTERT transcript or, alternatively, displayed the presence of an alternatively splice message alone or different combinations of full-length and deletion transcripts, with a generally higher level of alternatively spliced variants compared with that of the complete hTERT transcript. This finding would indicate for the first time the involvement of alternative splicing of hTERT in the regulation of telomerase activity in lung carcinoids. With regard to LCNECs and SCLCs, we found that the expression of the hTERT full-length transcript, alone or in association with one or more splice variants, was sufficient to account for telomerase activity in these tumors independently of its relative abundance with respect to that of deletion transcripts.

To obtain information on the possible role of telomere-associated proteins in controlling telomerase activity in carcinoids, we evaluated the expression of the telomere-associated protein hPOT1 (30) in neuroendocrine lung cancer specimens. This protein is thought to be a telomerase-dependent positive regulator of telomere length because of forced hPOT1 expression in a telomerase-positive human cell line lengthened telomeres, whereas hPOT1 was unable to lengthen telomeres in a telomerase-negative cell line (39). Moreover, antisense oligonucleotide-mediated hPOT1 down-regulation induced telomerase inhibition and telomere shortening (50), thereby suggesting that a function of hPOT1 is to facilitate telomere elongation by telomerase. However, the exact role of this protein in telomere dynamics is far from being completely understood. In fact, other authors (29) have proposed a possible role as a negative regulator of telomerase for hPOT1 via its interaction with the TRF1 complex. Results obtained in the present study failed to show appreciable difference in the abundance of hPOT1 mRNA between telomerase-negative and telomerase-positive carcinoids. The same was also true for LCNECs and SCLCs. Consequently, our data do not allow us to envisage a role of hPOT1 in the regulation of telomerase activity in neuroendocrine lung tumors.

The analysis of telomere length pattern in the different types of neuroendocrine lung tumors showed that terminal restriction fragments observed in carcinoids were comparable with those found in LCNECs and SCLCs. However, a breakdown analysis as a function of the presence/absence of telomerase activity showed that telomerase-negative carcinoids were characterized by significantly longer telomere restriction fragments than telomerase-positive carcinoids. It has been shown that some human tumors that do not express telomerase activity maintain the length of their telomeres by one or more mechanisms called ALT (51). Telomere dynamics in ALT cells are consistent with a recombination-based mechanism, and characteristics of ALT cells include unusually long and heterogeneous telomeres and nuclear structures called ALT-associated PML bodies, which besides the PML protein also contain telomeric DNA, telomere-specific binding proteins,

and proteins involved in DNA recombination and replication (40). To address the possibility that ALT mechanisms are operating in telomerase-negative carcinoids, we investigated the presence of ALT-associated PML bodies. However, based on the results obtained with a combined immunostaining/fluorescence *in situ* hybridization approach, we were unable to detect the presence of such structures in any tumor considered. Such data would indicate the absence of both the currently known telomere maintenance mechanisms, telomerase activity and ALT, in most carcinoids. They are also consistent with recent experimental data suggesting that the acquisition of a telomere maintenance mechanism is not strictly required for tumorigenesis in human fibroblasts (52) but may occur as a later event in tumor progression. Moreover, results from a clinical study aimed at correlating the expression of telomere maintenance mechanisms with clinical outcome of osteosarcoma patients showed that telomere-negative/ALT-negative tumors were clinically less aggressive and defined a subset of patients with increased overall survival (53).

Overall, results from our study indicate that telomerase is repressed in most lung carcinoids probably through mechanisms related to hTERT transcription and alternative splicing. They suggest that the absence of any telomerase maintenance mechanism may contribute to the favorable long-term prognosis of this malignancy (54), although the prevalence of early-stage tumors in our case series (80% presenting at stage I) and their relatively short median follow-up (5 years) did not allow us to appreciate differences in the clinical outcome between patients with telomerase-positive and telomerase-negative typical carcinoids.

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