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Cancer Diagnostics

Real-Time RT-PCR Quantification of Human Telomerase Reverse Transcriptase Splice Variants in Tumor Cell Lines and Non–Small Cell Lung Cancer

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Background: **We developed and validated a real-time reverse transcription (RT)–PCR for the quantification of 4 individual human telomerase reverse transcriptase (*TERT*) splice variants ($\alpha+\beta+$, $\alpha-\beta+$, $\alpha+\beta-$, $\alpha-\beta-$) in tumor cell lines and non–small cell lung cancer (NSCLC).**

Methods: **We used in silico designed primers and a common TaqMan probe for highly specific amplification of each *TERT* splice variant, PCR transcript–specific DNA external standards as calibrators, and the MCF-7 cell line for the development and validation of the method. We then quantified *TERT* splice variants in 6 tumor cell lines and telomerase activity and *TERT* splice variant expression in cancerous and paired noncancerous tissue samples from 28 NSCLC patients.**

Results: **In most tumor cell lines, we observed little variation in the proportion of *TERT* splice variants. The $\alpha+\beta-$ splice variant showed the highest expression and $\alpha-\beta+$ and $\alpha-\beta-$ the lowest. Quantification of the 4 *TERT* splice variants in NSCLC and surrounding nonneoplastic tissues showed the highest expression percentage for the $\alpha+\beta-$ variant in both NSCLC and adjacent nonneoplastic tissue samples, followed by $\alpha+\beta+$, with the $\alpha-\beta+$ and $\alpha-\beta-$ splice variants having the lowest expression. In the NSCLC tumors, the $\alpha+\beta+$ variant had higher expression than other splice variants, and its expression correlated with telomerase activity, overall survival, and disease-free survival.**

Conclusions: **Real-time RT-PCR quantification is a specific, sensitive, and rapid method that can elucidate the biological role of *TERT* splice variants in tumor development and progression. Our results suggest that the expression of the *TERT* $\alpha+\beta+$ splice variant may be an independent negative prognostic factor for NSCLC patients.**

Telomerase is a cellular ribonucleoprotein reverse transcriptase, and telomerase RNA segments act as templates for the synthesis of telomeric DNA onto chromosomal ends *(1, 2)*. Human telomerase reverse transcriptase (*TERT*)[4,5] contains the enzyme's catalytic subunit *(1–3)*, and the expression of the functional hTERT protein is a prerequisite for acquisition of telomerase activity *(4)*. Telomerase has a direct role in early oncogenic transformation *(3, 5–7)*, and through its activation, cancer cells stabilize their telomere size and sustain their unlimited growth *(7–9)*. Moreover, the in vitro malignant transformation of normal human cells was achieved by reconstitution of telomerase activity by induction of *TERT* gene expression in combination with other oncogenes *(5, 10)*. More than 85% of most human tumors express telomerase activity *(11)*, and essentially all major types of cancer have been screened for the presence of *TERT* mRNA in a variety of clinical specimens *(12)*.

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[4] Nonstandard abbreviations: TERT, telomerase reverse transcriptase; NSCLC, non–small cell lung cancer; RT, reverse transcription; RTA, relative telomerase activity; DFI, disease-free interval.
[5] Human genes: *TERT*, telomerase reverse transcriptase (*Homo sapiens*); *HPRT*, hypoxanthine phosphoribosyltransferase.

Splice variants that are found predominantly in tumors have clear diagnostic value and may provide potential drug targets *(13–15)*. *TERT* has been shown to contain at least 6 alternative splicing sites *(16–20)*; however, only the full-length *TERT* transcript is associated with telomerase activity *(16–18)*. The α splice site causes a 36-bp deletion within the conserved reverse transcriptase motif A *(16)* and was found to be a dominant negative inhibitor of telomerase activity *(21)*. The β splice site causes a 182-bp deletion leading to a nonsense mutation truncating the protein before the conserved reverse transcriptase motifs, resulting in a nonactive TERT protein and catalytically inactive telomerase *(16–18)*. Alternative splicing of *TERT* may be a novel mechanism of telomerase regulation *(22)*, and *TERT* mRNA splicing patterns have been studied in immortal human cells *(23)* and benign and malignant breast tumors *(24)* in an attempt to elucidate their biological role. The quantitative relationship between functionally active telomerase and the major telomerase components *TERT* and hTR in acute leukemia cells was recently studied *(25)*. The expression of the α-negative splice variant can inhibit telomerase activity *(26)* in telomerase-positive cells and causes telomere shortening and eventually cell death. The function and biological role of *TERT* splice variants is still unknown, however *(2, 18–26)*.

Expression of *TERT* was found to be an independent negative prognostic factor for non–small cell lung cancer (NSCLC) patients *(27–30)*. Telomerase activity and *TERT* mRNA expression were detected at a very high percentage in lung carcinoma and are considered poor prognostic indicators in NSCLC. Telomerase activity was not found to correlate with clinicopathologic variables, however, and *TERT* was not associated with prognosis and did not correlate with any clinical variables *(31)*. Determination of telomerase activity by in situ hybridization, immunohistochemistry, and TRAP assay has been reported to be useful for evaluating the diagnosis and prognosis of lung carcinomas *(32)*. Very recently, alternative splicing of *TERT* was found to be involved in the control of telomerase activity in lung cancer *(33, 34)*.

We previously developed a quantitative luminometric hybridization assay for telomerase activity *(35)* and for *TERT* β-plus transcript *(36, 37)*. We developed a quantitative real-time reverse transcription (RT)-PCR method for 4 individual *TERT* splice variants to clarify their quantitative relationship in tumor cell lines and in NSCLC tissues and their surrounding noncancerous tissues. We also investigated the correlation of *TERT* splice variant expression and telomerase activity with the clinical outcome of NSCLC patients.

Materials and Methods

CELL LINES AND CULTURE

We used the human mammary carcinoma cell line MCF-7 for the development and validation of the assay and studied the expression of *TERT* splice variants in 6 human cancer cell lines, MCF-7, MDA-MB-231, COLO205, K562, T47D, and HeLa (http://www.atcc.org/common/catalog).

PATIENTS AND TISSUE SAMPLES

Lung carcinomas and adjacent nonneoplastic tissues were obtained from 28 NSCLC patients. All patients gave their informed consent to participate in the study, which was approved by the ethics and scientific committees of our institution. The tumor types and stages were determined according to the WHO classification. At the time of surgery, all tissue samples were immediately flash frozen in liquid nitrogen and stored at −80 °C. All samples were analyzed histologically to access the account of tumor component (at least 70% of tumor cells) and the quality of material (i.e., absence of necrosis).

TOTAL RNA ISOLATION AND CDNA SYNTHESIS

Total cellular RNA was isolated with the Qiagen RNeasy Mini Reagent Set (Qiagen) for lung tissue samples and the Trizol LS reagent (Invitrogen) for cell lines, according to the manufacturers' instructions. All preparation and handling steps of RNA took place in a laminar flow hood under RNase-free conditions. The isolated RNA was dissolved in RNA storage buffer (Ambion) and stored at −70 °C until used. RNA concentration was determined in the NanoDrop® ND-1000 spectrophotometer (NanoDrop Technologies). We used 1 μg of total RNA to perform reverse transcription of RNA with the SuperScript III Kit (Invitrogen) in a total volume of 20 μL, according to the manufacturer's instructions. RNA integrity of all cDNA preparations was tested by real-time PCR amplification of the *HPRT* (hypoxanthine phosphoribosyltransferase) gene (LightCycler-h-HPRT, Roche).

QUANTITATIVE REAL-TIME RT-PCR

Primer and probe design. Four highly specific primers and the TaqMan probe were in silico designed in our laboratory (see Table 1 in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol53/issue1). The principle of the developed real-time RT-PCR assay is shown in Fig. 1. Segments 1–5 do not represent the exons of the *TERT* gene; for simplicity, segment 2 represents the α-deletion site, and segment 4 represents the β-deletion site. Primer TE 1/2 was designed to amplify both *TERT* α+ splice variants (α+β+, α+β−), and TER3 was designed to amplify both α− splice variants (α−β+, α−β−). Primer KAT4b was designed to amplify β+ splice variants (α+β+, α−β+), and TER2 was designed to amplify β− splice variants (α+β−, α−β−). Primer combinations for each splice variant were as follows: TE 1/2–KAT4b, α+β+; TE 1/2–TER2, α+β−; TER3–KAT4b, α−β+; and TER3–TER2, α−β−. All primers were designed to have similar melting temperatures, so that all PCRs could be performed in the same run. Quantification of *TERT* splice variant mRNA was performed in the LightCycler (Roche).

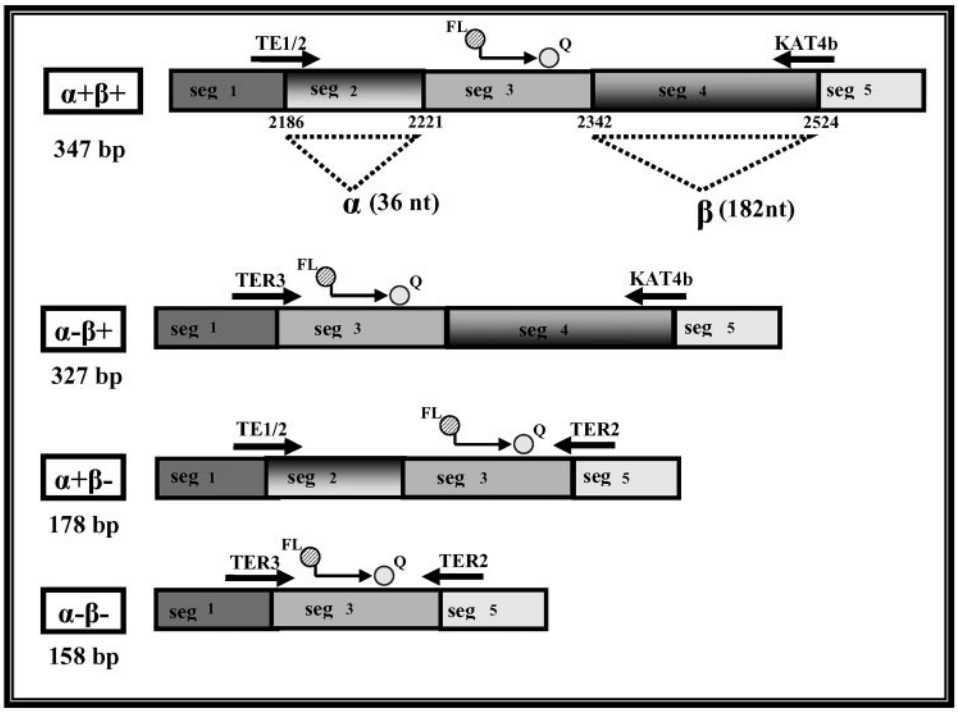

Fig. 1. Principle of the proposed real-time RT-PCR assay for the quantification of 4 *TERT* splice variants.

Abbreviations: seg, segment; FL, 6-carboxyfluorescein (FAM); Q, 5-carboxytetramethye rhodamine.

Real-time PCR conditions. Real-time-PCR was performed in a total volume of 10 μL per reaction. We placed 1 μL of cDNA into a 9-μL reaction mixture that contained 0.1 μL of Taq DNA polymerase (5 U/μL, Platinum DNA Polymerase; Invitrogen), 1 μL of the supplied 10× PCR buffer, 0.5 μL of $MgCl_2$ (50 mmol/L), 0.2 μL of dNTPs (10 mmol/L; Fermentas), 0.15 μL of bovine serum albumin (10 g/L; Serva), 0.5 μL of the appropriate sense primer for each splice variant, 0.5 μL of the corresponding antisense primer (3 μmol/L), and 1 μL of the TaqMan probe (3 μmol/L); finally, DEPC–H_2O was added to a final volume of 10 μL. The cycling protocol was identical for all splice variants and consisted of an initial 5-min denaturation step at 95 °C, followed by 50 cycles of denaturation at 95 °C for 10 s, annealing at 65 °C for 20 s, and extension at 72 °C for 20 s.

PREPARATION OF EXTERNAL STANDARDS FOR EACH INDIVIDUAL *TERT* SPLICE VARIANT

We prepared 4 external standards specific for the 4 *TERT* splice variants, as previously described *(38)*. cDNA was synthesized from total RNA extracted from 10^5 MCF-7 cells and served as a template for the amplification of each *TERT* splice variant by real-time PCR with the above-described specific primers. RT-PCR products were run on a 3% agarose gel, and splice variant–specific bands (see Fig. 1 in the online Data Supplement) were excised, the amplicons were purified (UltraClean™, GelSpin™ DNA Purification Kit; MO BIO) and quantified with the PicoGreen DNA Quantification Kit (Molecular Probes). Serial dilutions of these stock amplicon solutions, 1×10^1 to 1×10^4 copies/μL, were kept in 10-μL aliquots in DNase/RNase-free water at −20 °C until use and served as external standards throughout the study. For each target, a calibration curve was created by plotting the concentration of each external standard expressed as copy numbers per microliter vs the value of the corresponding crossing point (C_p).

TELOMERE REPEAT AMPLIFICATION PROTOCOL (TRAP) ASSAY FOR TELOMERASE ACTIVITY

Telomerase activity in NSCLC tissues samples was assessed with the Telo TAGGG Telomerase PCR-ELISA^{PLUS} Kit (Roche), according to the manufacturer's instructions *(8)*.

STATISTICAL ANALYSIS

For each *TERT* splice variant, we used the McNemar and Fisher exact tests (Statmost; DataMost Corp) to compare mRNA expression and telomerase activity. Correlations among *TERT* α+β+ mRNA quantitative levels, telomerase activity, and clinicopathologic features were assessed by the Mann–Whitney and Kruskal–Wallis tests. Survival times were calculated from the date of surgery to the occurrence of cancer-related events. Survival curves were estimated by the Kaplan–Meier method, and *P* values <0.05 were considered statistically significant (SPSS).

Results

QUANTITATIVE REAL-TIME RT-PCR FOR *TERT* SPLICE VARIANTS

Analytical characteristics. We performed extensive optimization of the primers and probe concentrations, $MgCl_2$

concentration, and reaction temperatures and times (data not shown).

To determine the analytical sensitivity and linearity of the proposed real-time RT-PCR assay, splice variant-specific *TERT* external standards were used. For each splice variant, a calibration curve was generated by serial dilutions of 1×10^1 to 1×10^4 copies of the target of interest per reaction. All calibration curves showed linearity over the entire quantification range (R^2, 0.9929–0.9997) (see Fig. 2 in the online Data Supplement).

To determine within-run precision of the assay, we tested 3 different concentrations of each *TERT* splice variant in the same run in 4 parallel determinations, and for between-run precision, we assayed 3 different concentrations of each *TERT* splice variant in 7 different experiments (all performed in a 1-month period) (Table 1).

Specificity. When cDNA, derived from 10^5 MCF-7 cells, was amplified after 50 cycles by real-time PCR, agarose gel electrophoresis of the PCR products revealed only one specific product of the desired length for each splice variant (see Fig. 1 in the online Data Supplement).

The specificity of our assay was further evaluated by running real-time RT-PCR experiments with the specific primers for each splice variant in samples containing purified PCR amplicons corresponding to the other individual *TERT* splice variants. Each *TERT* splice variant was amplified only by its corresponding specific primer pair, and the cross-reactions between them were negligible. In this way, each *TERT* splice variant was specifically quantified even in the presence of all the rest, a crucial technique to avoid false-positive quantitative results (see Table 2 in the online Data Supplement).

Sensitivity. The analytical detection limit of the method, defined as 3.3 times the SD of the crossing point for the less concentrated external standard for each splice variant divided by the mean slope of the corresponding calibration curve, was found to be to 3×10^0 copies/reaction for $\alpha+\beta+$, 7×10^0 copies/reaction for $\alpha-\beta+$, 2×10^0 copies/reaction for $\alpha+\beta-$, and 2×10^0 copies/reaction for $\alpha-\beta-$. The analytical limit of quantification, defined as 3 times the detection limit, was 9×10^0 copies/reaction for $\alpha+\beta+$, 2×10^1 copies/reaction for $\alpha-\beta+$, 6×10^0 copies/reaction for $\alpha+\beta-$, and 6×10^0 copies/reaction for $\alpha-\beta-$.

Table 1. Precision study of the real-time RT-PCR assay for *TERT* splice variants.

		Crossing point (SD)	
***TERT* splice variant**	**Copies/μL**	**Within-run ($n = 4$)**	**Between-run ($n = 7$)**
$\alpha+\beta+$	9×10^1	34.8 (1.1)	33.9 (0.8)
	9×10^2	30.9 (0.3)	30.5 (0.7)
	9×10^3	27.5 (0.2)	27.1 (0.4)
$\alpha+\beta-$	2×10^1	39.5 (1.4)	39.2 (1.1)
	2×10^2	35.8 (0.3)	35.6 (0.8)
	2×10^3	31.7 (0.2)	31.4 (0.6)
$\alpha-\beta+$	5×10^1	37.7 (1.3)	36.7 (0.7)
	5×10^2	33.3 (0.3)	33.2 (0.4)
	5×10^3	29.1 (0.2)	29.6 (0.2)
$\alpha-\beta-$	2×10^1	40.5 (1.0)	39.3 (1.2)
	2×10^2	35.9 (0.4)	35.1 (0.5)
	2×10^3	31.8 (0.04)	31.3 (0.6)

QUANTIFICATION OF *TERT* SPLICE VARIANTS IN TUMOR CELL LINES

In all cell lines except COLO-205, the most abundantly expressed splice variant was $\alpha+\beta-$ (51.0%–74.7%), followed by the full active transcript $\alpha+\beta+$ (25.3%–43.9%). The $\alpha+\beta+$ splice variant, which encodes the catalytically active *TERT* protein, was the most abundant splice variant in COLO-205 cells. The less abundant splice variants $\alpha-\beta+$ and $\alpha-\beta-$ were not detected at all in T47D cells, whereas the $\alpha-\beta+$ splice variant was also not detected in MDA-MB-231 and HeLa tumor cell lines. Both of them were detected at very low percentages in the remaining cell lines (see Fig. 3 in the online Data Supplement).

QUANTIFICATION OF *TERT* SPLICE VARIANTS IN NSCLC PAIRED TISSUE SAMPLES

Quantification of the 4 *TERT* splice variants was performed in 28 pairs of NSCLC tissues and their adjacent nonneoplastic tissues. Both $\alpha+\beta-$ and $\alpha+\beta+$ splice variants were the most abundant transcripts in NSCLC tissue samples (23 of 28; 82.1%), and the $\alpha-\beta+$ and $\alpha-\beta-$ splice variants had the lowest expressions [8 of 28 (28.6%) and 15 of 28 (53.6%), respectively]. In adjacent nonneoplastic tissue, the $\alpha-\beta+$ and $\alpha-\beta-$ splice variants were not expressed at all, the $\alpha+\beta+$ splice variant was detected in 2 of 28 (7.1%), and the $\alpha+\beta-$ was detected in 8 of 28 (28.6%), respectively. In NSCLC tissues, the $\alpha+\beta+$ had the highest expression of all (median, 119 copies/μg of total RNA; range, 0–1366), followed by $\alpha+\beta-$ (median, 75 copies/μg of total RNA; range, 0–351) (Fig. 2A). The $\alpha-\beta+$ and $\alpha-\beta-$ splice variants were found in very small concentrations ($\alpha-\beta+$ median, 3 copies/μg of total RNA, range, 0–20; $\alpha-\beta-$ median, 17 copies/μg total RNA, range, 0–393). *TERT* expression was significantly higher in tumor tissues than in nonneoplastic tissues ($\alpha+\beta+$, $P = 0.000$; $\alpha+\beta-$, $P = 0.000$; $\alpha-\beta+$, $P = 0.000$; $\alpha-\beta-$, $P = 0.001$; calculated with the Wilcoxon matched pairs signed-rank test).

In a bar chart showing the proportion of the alternatively splicing variants for each tumor specimen (Fig. 2B), remarkable heterogeneity is seen between NSCLC tumor tissues with respect to *TERT* splicing variant profiles. The $\alpha+\beta-$ splice variant was the most abundant of all, making up 100% of the *TERT* transcripts in 2 adenocarcinoma samples, whereas in most other tumors, it made up the majority of all *TERT* splice variants. The $\alpha+\beta+$ full active *TERT* splice variant represents the majority (>50%)

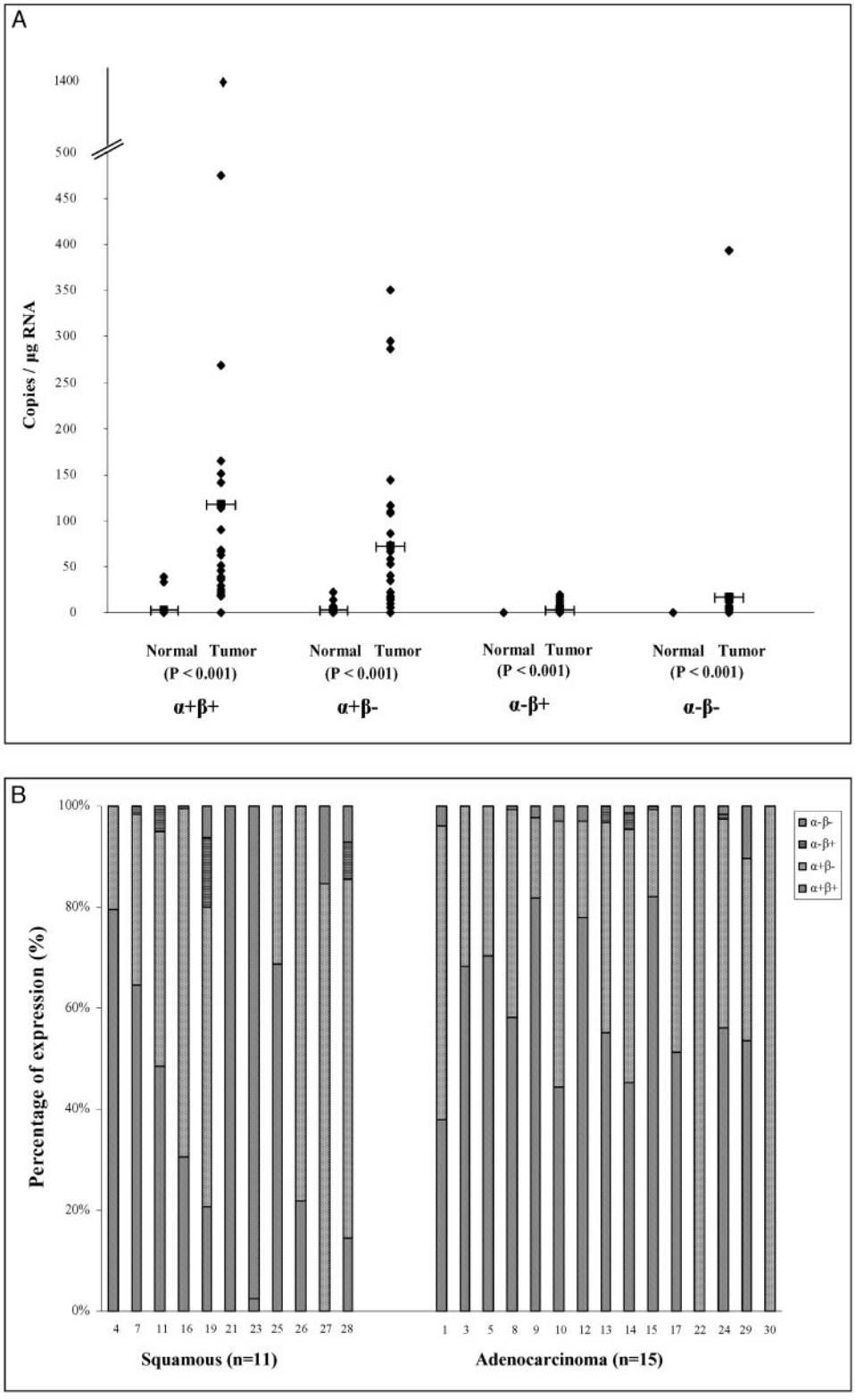

Fig. 2. Quantification of *TERT* splice variants in NSCLC and their adjacent nonneoplastic tissue samples.

(*A*), absolute quantification (copies per microgram of total RNA). (*B*), expression (%) of individual *TERT* splice variants in NSCLC tissues.

of *TERT* splice variants in many tumors, and the $\alpha-\beta-$ and $\alpha-\beta+$ splice variants were found in very low percentages. It is remarkable to note that the $\alpha-\beta-$ transcript was detected at a very high percentage in 1 squamous carcinoma sample.

CLINICAL RELEVANCE OF THE DETECTION OF *TERT* $\alpha+\beta+$ SPLICE VARIANT AND TELOMERASE ACTIVITY IN PATIENTS WITH NSCLC

Our analysis of the concordance between telomerase activity and *TERT* splice variant expression in 28 paired

NSCLC tumor and adjacent noncancerous tissues (Table 2) indicated that relative telomerase activity (RTA) did not correlate ($P > 0.05$) with any clinicopathologic feature of the patients in the population studied. We observed similar lack of correlation for *TERT* $\alpha+\beta+$ mRNA expression ($P > 0.05$) as quantified by the developed real-time RT-PCR methodology (Table 3).

Table 2. Relationship between telomerase activity and *TERT* splice variant mRNA in NSCLC ($n = 28$).

TERT splice variant		Telomerase activity, %		
		Positive	Negative	P^a
$\alpha+\beta+$	Positive	16 (57.2)	7 (25)	0.343
	Negative	1 (3.6)	4 (14.3)	NS[b]
$\alpha+\beta-$	Positive	14 (50.0)	9 (32.1)	0.149
	Negative	3 (10.7)	2 (7.1)	NS
$\alpha-\beta+$	Positive	6 (21.4)	2 (7.1)	0.026
	Negative	11 (39.3)	9 (32.1)	
$\alpha-\beta-$	Positive	10 (35.7)	5 (17.8)	0.773
	Negative	7 (25.0)	6 (21.4)	NS
All	Positive	17 (60.7)	9 (33.3)	0.0076
	Negative	0 (0)	2 (7.1)	

[a] McNemar and Fisher exact tests.
[b] NS, not significant.

Relapse. The disease-free survival curves in NSCLC patients demonstrated significantly shorter survival in patients with *TERT* $\alpha+\beta+$ mRNA-positive tumors than in patients with *TERT* $\alpha+\beta+$ mRNA-negative tumors. During the follow-up period, 16 of 28 patients (57.1%) developed metastases; 15 of these patients (93.8%) were *TERT* $\alpha+\beta+$ mRNA-positive and had significantly higher scores than the 6 patients who were also *TERT* $\alpha+\beta+$ mRNA-positive and had not relapsed (Table 3). The Kaplan–Meier estimates of the cumulative disease-free interval (DFI) for the *TERT* $\alpha+\beta+$ mRNA-positive and negative groups were significantly different (log rank test, $P = 0.0166$) in favor of patients who were *TERT* $\alpha+\beta+$ mRNA-negative (Fig. 3A). When all cases in which at least 1 splice variant was detected were considered *TERT* positive, however, there was not a significant difference (log rank test, $P = 0.6321$) in the cumulative DFI for the *TERT* mRNA-positive and negative groups (data not

Table 3. *TERT* $\alpha+\beta+$ mRNA expression and telomerase activity in relation to clinicopathologic features and clinical outcome of NSCLC patients.

Variable		*TERT* $\alpha+\beta+$ mRNA expression				Telomerase activity			
	n	Positive	%	Score, mean (SD) [a]	*P* value	Positive	%	RTA, mean (SD)	*P* value
Age, years									
≥60	13	11	84.6	73.4 (127)	NS[b,c]	8	61.5	0.56 (0.59)	NS[c]
<60	15	12	80.0	157 (342)		9	60.0	1.08 (1.15)	
Histologic type									
Adenocarcinoma	16	13	81.3	173 (341)	NS[c]	11	68.8	0.96 (0.89)	NS[c]
Squamous cell carcinoma	12	10	83.3	46 (51)		6	50.0	0.68 (1.04)	
Lymph node metastasis (N)									
N0	9	6	66.7	182 (447)	NS[d]	5	55.6	0.67 (0.76)	NS[d]
N1	13	12	92.3	73 (76)		7	53.9	1.01 (1.24)	
N2	6	5	83.3	123 (178)		5	83.3	0.74 (0.41)	
Stage									
I/II	22	18	81.8	117 (287)	NS[c]	12	54.6	0.87 (1.06)	NS[c]
III/IV	6	5	83.3	123 (178)		5	83.3	0.74 (0.41)	
Grade									
GII	14	10	71.4	47 (52)	NS[c]	8	57.1	0.94 (1.16)	NS[c]
GIII	14	13	92.9	191 (362)		9	64.3	0.75 (0.73)	
Depth of tumor invasion (T)									
T1	5	5	100	157 (184)	NS[c]	3	60.0	1.11 (1.46)	NS[c]
$T2/T_3$	23	18	78.3	110 (281)		14	60.9	0.79 (0.84)	
Relapse									
Yes	16	15	93.8	167 (327)	0.003[c]	11	62.5	1.14 (1.09)	NS[c]
No	12	8	66.7	55 (133)		6	50.0	0.45 (0.56)	
Overall survival									
Dead	12	12	100	209 (371)	0.003[c]	10	83.3	1.28 (0.97)	0.023[c]
Alive	14	10	71.4	57 (122)		5	35.7	0.47 (0.87)	

[a] TERT $\alpha+\beta+$ copies per μg of RNA.
[b] NS, not significant.
[c] Mann–Whitney test.
[d] Kruskal–Wallis test.

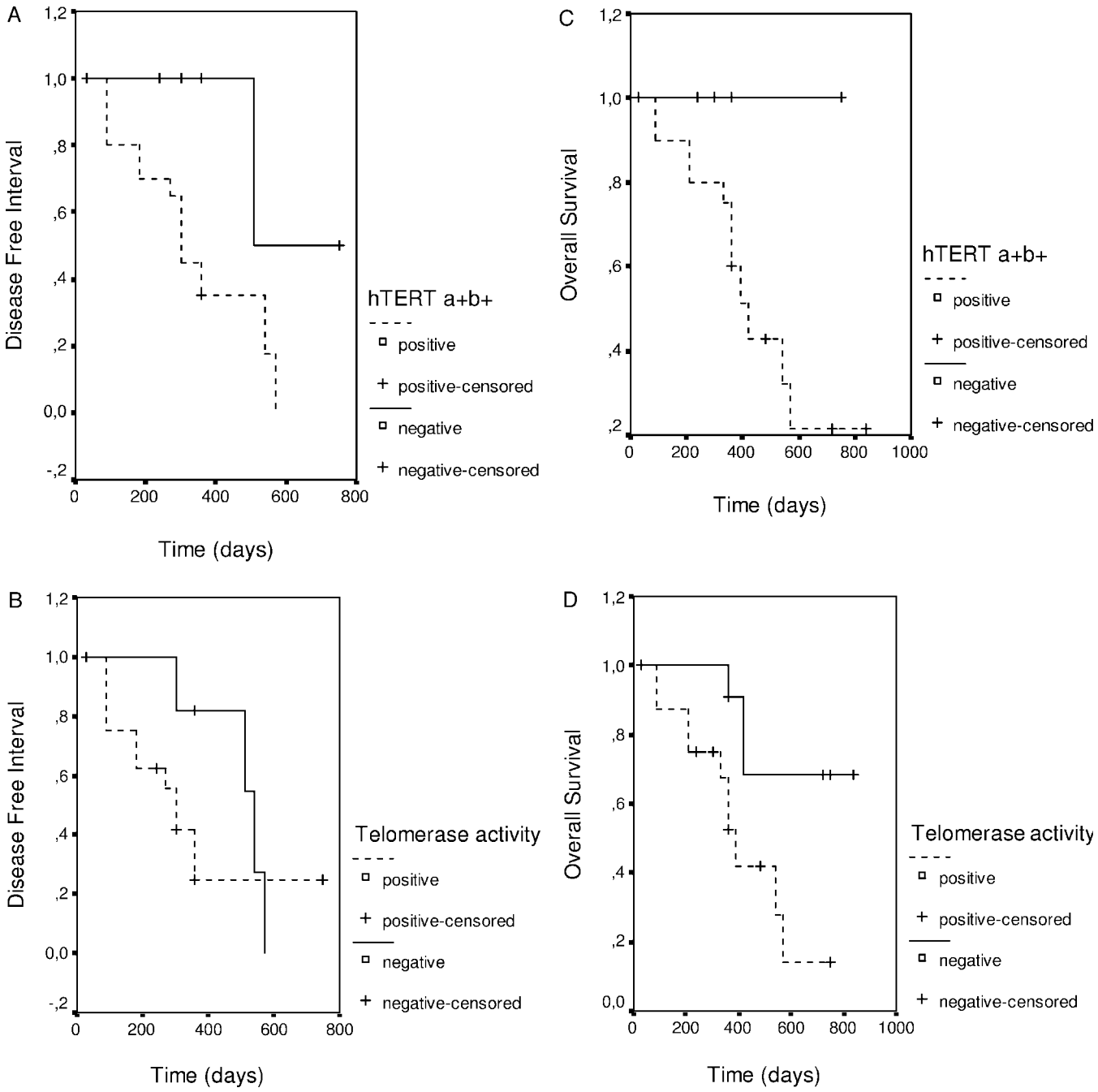

Fig. 3. Kaplan–Meier estimates of DFI and overall survival (OS).

(*A*), Kaplan–Meier estimates of DFI for the NSCLC patients with or without *TERT* $\alpha+\beta+$ mRNA expression ($P = 0.0166$). (*B*), Kaplan–Meier estimates of DFI for the NSCLC patients with or without telomerase activity ($P = 0.0499$). (*C*), Kaplan–Meier estimates of OS for the NSCLC patients with or without *TERT* $\alpha+\beta+$ mRNA expression ($P = 0.0296$). (*D*), Kaplan–Meier estimates of OS for the NSCLC patients with or without telomerase activity ($P = 0.0202$).

shown). Eleven of the 16 (68.8%) patients that relapsed were positive for telomerase activity, and there was no significant difference in RTA levels between those patients from those who were telomerase positive but did not show any relapse ($P > 0.05$; Table 3). The DFI curves estimated by the Kaplan–Meier method demonstrated that patients with positive telomerase activity had poorer prognoses (log rank test, $P = 0.0499$) than those with negative telomerase activity (Fig. 3B).

Overall survival. During the follow-up period, 14 of 28 (50%) patients died; however, 12 of 28 (42.8%) died from lung cancer. All patients who died were positive for *TERT* $\alpha+\beta+$ mRNA, with a score approximately 4 times higher

than those who were alive and positive for *TERT* $\alpha+\beta+$ mRNA (Table 3). The Kaplan–Meier estimates of the overall survival rates for the *TERT* $\alpha+\beta+$ positive and negative groups were significantly different (log rank test, $P = 0.0296$) in favor of patients who were *TERT* $\alpha+\beta+$ mRNA negative (Fig. 3C). Furthermore, 10 of 12 (83.3%) patients who died were positive for telomerase activity, with an RTA score ~3 times higher than those who were alive and positive (Table 3). Kaplan–Meier analysis showed a significant difference (log rank test, $P = 0.02$) in overall survival rates between patients with positive and negative telomerase activity in favor of those patients who were negative (Fig. 3D).

Discussion

We describe the development and analytical validation of a real-time RT-PCR assay for the absolute quantification of *TERT* splice variants $\alpha+\beta+$, $\alpha-\beta+$, $\alpha+\beta-$ and $\alpha-\beta-$, a method for quantifying the expression of each individual *TERT* splice variant in 6 tumor cell lines. All the cell lines studied expressed the *TERT* full-length message, together with different combinations of alternatively spliced variants, with surprisingly little variation in the proportion of alternatively spliced forms of *TERT* except in COLO-205. Our finding of a relatively high percentage for the $\alpha+\beta+$ splice variant (~50%) in all of these cell lines strikingly differs from the lower percentage (~4%) reported in a previous study *(23)*. The differing results may be attributable to our use of quantitative real-time RT-PCR for each splice variant, in the same cDNA and under the same experimental conditions, whereas in the previous study, a semiquantitative method was used *(23)*. As previously suggested *(23)*, however, our study also shows surprisingly little variation in the proportion of alternatively splicing forms of *TERT* among the different cell lines.

The expression of all *TERT* splice variants in NSCLC was significantly higher than in adjacent nonneoplastic tissues. The most abundant transcripts were $\alpha+\beta-$ and $\alpha+\beta+$, and the least abundant were $\alpha-\beta+$ and $\alpha-\beta-$. The most impressive difference between squamous and adenocarcinomatous samples was the very low expression of only one splice variant ($\alpha+\beta-$) in 2 of 15 (13.3%) adenocarcinomas, one of which was positive and the other negative for telomerase activity. The fact that there is a significant heterogeneity in the expression of *TERT* splice variants in NSCLC raises many questions about the possible role of the alternate transcripts of *TERT* in the regulation of telomerase and in malignant transformation.

Although in most cases, results of *TERT* $\alpha+\beta+$ expression and telomerase activity did not differ significantly (concordance, 20 of 28; 71.4%; $P = 0.343$), discordant results occurred in 8 cases. A possible explanation for the 7 cases negative for telomerase activity, in which *TERT* $\alpha+\beta+$ expression was detected could be the lower detection limit for the *TERT* assay and the coexpression of other *TERT* splice variants in these samples. There were 2 tumor samples with relatively low telomerase activity and very high *TERT* $\alpha+\beta+$ expression. In both of these samples, we detected a relatively high percentage of the $\alpha-\beta+$ splice variant, the α-dominant negative, which has been found to be a negative inhibitor of telomerase activity (*21*, *26*). In addition, the procedure for active telomerase isolation needed for *TERT* expression analysis has many more steps, and thus possibly lower recovery, than total RNA isolation. On the other hand, we found only 1 sample positive for telomerase activity and negative for *TERT* $\alpha+\beta+$, possibly because of PCR artifacts. According to our results, by using primers that amplify a region in the *TERT* mRNA that is common for all splice variants, we overestimated the positivity of our samples ($P = 0.0076$), because a high expression of the $\alpha-\beta+$ splice variant is a negative inhibitor of telomerase activity.

The detection of *TERT* $\alpha+\beta+$ splice variant had prognostic implications for NSCLC patients because it was associated with decreased DFI ($P = 0.003$). During the follow-up period, 12 patients died from disease relapse. Although statistical analysis suggests that there is an association between the quantification of *TERT* $\alpha+\beta+$ mRNA and the hazard of death ($P = 0.003$), the quite small number of patients make us cautious about the interpretation of this finding.

In conclusion, we have developed a highly specific, sensitive, and rapid real-time RT-PCR method for quantitative determination of individual *TERT* splice variants. We correlated individual *TERT* splice variant expression with telomerase activity and found that the use of primers that amplify a region in the *TERT* mRNA that is common to all splice variants leads to overestimation of sample positivity. Quantification of *TERT* $\alpha+\beta+$ mRNA is a significant prognostic indicator associated with shortened DFI and overall survival in NSCLC patients. Future studies are needed to further evaluate and confirm our findings in a larger number of patients. Through our results and those of many others, many questions have arisen about the possible role of the alternate transcripts of *TERT* in the regulation of telomerase and in malignant transformation.

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