Short Telomeres, Telomerase Reverse Transcriptase Gene Amplification, and Increased Telomerase Activity in the Blood of Familial Papillary Thyroid Cancer Patients

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Background: Differentiated papillary thyroid cancer is mostly sporadic, but the recurrence of the familial form has been reported. Short or dysfunctional telomeres have been associated with familial benign diseases and familial breast cancer.

Objective: The aim of our work was to study the telomere-telomerase complex in the peripheral blood of patients with familial papillary thyroid cancer (FPTC), including the measurement of relative telomere length (RTL), telomerase reverse transcriptase (hTERT) gene amplification, hTERT mRNA expression, telomerase protein activity, and search of hTERT or telomerase RNA component gene mutations.

Patients: Cumulating a series of patients seen at the University of Siena and a series at the University of Rome, the experiments were conducted in 47 FPTC patients, 75 sporadic papillary thyroid cancer (PTC) patients, 20 patients with nodular goiter, 19 healthy subjects, and 20 unaffected siblings of FPTC patients.

Results: RTL, measured by quantitative PCR, was significantly ($P < 0.0001$) shorter in the blood of FPTC patients, compared with sporadic PTCs, healthy subjects, nodular goiter subjects, and unaffected siblings. Also by fluorescence *in situ* hybridization analysis, the results confirmed shorter telomere lengths in FPTC patients ($P = 0.01$). hTERT gene amplification was significantly ($P < 0.0001$) higher in FPTC patients, compared with the other groups, and in particular, it was significantly ($P = 0.03$) greater in offspring with respect to parents. hTERT mRNA expression, as well as telomerase activity, was significantly higher ($P = 0.0003$ and $P < 0.0001$, respectively) in FPTC patients, compared with sporadic PTCs. RTL, measured in cancer tissues, was shorter ($P < 0.0001$) in FPTC patients, compared with sporadic PTCs. No mutations of the telomerase RNA component and hTERT genes were found.

Conclusion: Our study demonstrates that patients with FPTC display an imbalance of the telomere-telomerase complex in the peripheral blood, characterized by short telomeres, hTERT gene amplification, and expression. These features may be implicated in the inherited predisposition to develop FPTC. (***J Clin Endocrinol Metab* 93: 3950–3957, 2008)**

Differentiated thyroid cancer (DTC), although mostly sporadic, may have a familial occurrence, with a prevalence of up to 10% (1, 2). The risk of developing the same tumor in first-degree relatives of subjects with DTC is significantly higher (between 3.2 and 8.6) than in the general population (3, 4). Several rare hereditary syndromes caused by germline mutations

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Abbreviations: DIG, Digoxigenin; DHPLC, denaturing HPLC; DTC, differentiated thyroid cancer; FISH, fluorescence *in situ* hybridization; FPTC, familial papillary thyroid cancer; hTERT, telomerase reverse transcriptase; PTC, papillary thyroid cancer; Q-PCR, quantitative PCR; RTL, relative telomere length; S, single copy gene; T, telomere; TERC, telomerase RNA component; Tg, thyroglobulin.

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of known tumor suppressor genes are associated with the occurrence of DTC, mainly of the papillary histotype, such as familial adenomatous polyposis, Cowden syndrome, Werner syndrome, and Carney complex (5–8). However, most of the patients have thyroid cancer as the only disease manifestation and are not associated with a distinct phenotype. So far, no candidate gene(s) has been discovered for this form of familial DTC, and only in a minority of cases, a locus of susceptibility has been identified: the locus TCO on 19p13.2 (9), the locus PRN1 on 1q21 (10), and the locus NMTC1 on 2q21 (11).

Genetic integrity is partly maintained by the architecture of telomeres, and it is gradually lost as telomeres progressively shorten with each cell replication due to incomplete lagging DNA strand synthesis and oxidative damage. Telomerase is a reverse transcriptase enzyme that counteracts telomere shortening by adding telomeric repeats to the G-rich strand. It is composed of a telomerase RNA component (TERC) that serves as a template for the addition of repeats and a protein component, telomerase reverse transcriptase (hTERT) (12). In humans, telomerase activity is abundant in germ cells, adult stem cells, and activated immune cells, whereas it is absent or low in adult differentiated cells and resting immune cells. In the absence of telomerase or when the activity of the enzyme is low, compared with the replicative erosion, apoptosis is triggered (12). Interestingly, patients who have inherited or acquired genetic defects in telomere maintenance seem to have an increased risk of developing familial benign diseases such as dyskeratosis congenital syndrome (13) and malignant diseases such as head, neck, lung, breast, and renal cancers (14). Some studies have shown that relative telomere length segregates in families (15, 16) and that the decrease in telomere length may play a role in age-related genetic instability (17).

Based on these observations, the present work was aimed to search for abnormalities of the telomerase complex in the peripheral blood of familial DTC, possibly implicated in the susceptibility to develop the disease.

Patients and Methods

Two series of patients were studied, one from the University of Siena (original series) and one from the University of Rome (confirmatory series).

Siena series (original series)

Of 300 patients affected by DTC, followed up in the Section of Endocrinology of the University of Siena (Italy) from 1978 to 2007 (230 females and 70 males; ratio female to male: 3:1, range 8–84 yr), 34 patients (11.3%), belonging to 16 kindred, had the familial recurrence of the disease, defined as the presence of at least one first-degree relative with DTC, possibly configuring the features of familial disease. All of them were of the papillary histotype and will be referred to as familial papillary thyroid cancer (FPTC).

Of the 34 FPTC patients, 27 (79.4%) had a parent-child relationship (13 families) and seven (20.6%) had a sibling relationship (three families). In two families there was both a parent-child relation and a sibling relation. Age at diagnosis of FPTC group was 48 ± 18.5 yr (range 14–77 yr). Age at diagnosis of probands was 54.3 ± 9.3 yr (range 25–77 yr) and that of affected familial members was 42.9 ± 15.7 yr (range 15–73 yr). Two FPTC patients presented other cancers: one had breast cancer diagnosed at age 64 yr and one had lymphoma at age 6 yr, 1 and 8 yr before the diagnosis of papillary thyroid cancer (PTC), respectively. No thyroid cancer-associated syndrome was present clinically in our patients.

Blood was available for genetic analysis in 29 FPTC patients (20 females). The five additional patients were not analyzed because two patients belonging to the first generation were deceased and three were not available. These patients were compared with 50 sporadic PTC patients (36 females) matched for age (mean age at diagnosis 49.5 ± 18.4 yr, range 18–87 yr), 20 patients with nodular goiter (one male and 19 females, mean age 59.4 ± 13.6 yr, range 36–80 yr), and 19 healthy subjects (five males and 14 females, mean age: 38.5 ± 17.8 yr, range 24–74 yr). In addition, we analyzed 10 out of 17 unaffected family members of FPTC patients (five males and five females, mean age 28.2 ± 11.5 yr, range 14–50 yr) belonging to six kindred, in which unaffected family members were present. Initial treatment in all patients consisted of total thyroidectomy and radioiodine ablation.

According to standard criteria (18, 19), patients with undetectable stimulated serum thyroglobulin (Tg) levels (<1.0 ng/ml), negative Tg antibody, and no evidence of disease (at clinical examination, neck ultrasound, negative diagnostic 131-I whole body scan, and/or other imaging techniques) were defined as free of disease, whereas patients with detectable basal or stimulated serum Tg and/or evidence of disease were classified as having persistent/recurrent disease. The FPTC group included 20 patients in complete remission (69%) and nine with persistent disease (31%). The group of sporadic PTCs included 42 (84%) patients in complete remission and eight with persistent disease (16%).

Rome series (confirmatory series)

We performed a validation series evaluating the telomere-telomerase complex in the blood of 18 FPTC patients (eight females), 10 unaffected family members, and 25 sporadic PTC patients followed up in the Section of Endocrinology, University La Sapienza, Rome, Italy, from 1984 to 2007. Mean age at diagnosis of FPTC was 45.5 ± 15.2 yr (range 26–74 yr), and all of them had PTC. Ten (55.5%) had a parent-child relationship (five families) and eight (44.5%) had a sibling relationship (four families). Age at diagnosis of probands was 41.1 ± 14.9 yr (range 23–65 yr) and age of diagnosis of affected familial members was 42.3 ± 14.1 yr (range 28–74 yr). No thyroid cancer-associated syndrome was present clinically in these patients. Fifteen patients (83.3%) were in complete remission and three (16.7%) had persistent disease. They were compared with 25 age/sex-matched sporadic PTC patients (four males and 21 females, mean age at diagnosis 56 ± 20.2 yr, range 21–80 yr). The group of sporadic PTCs included 18 patients in complete remission (72%) and seven with persistent disease (28%). In addition, we analyzed 10 unaffected family members of FPTC patients (five males and five females, mean age 41.5 ± 25.4 yr, range 10–83 yr). In this series the analysis was limited to the measurement of relative telomere length (RTL) and hTERT gene amplification.

DNA extraction

Blood samples were obtained at different time after surgery, ranging from 6 to 396 months, after informed consent obtained in accordance with local ethical committee guidelines. Genomic DNA was extracted using salting out procedures. For each sample DNA concentration was assessed by spectrophotometry and stock solutions of 200 ng per 50 μl were prepared.

DNA was also extracted from paraffin-embedded tissue available in 10 FPTC patients and 30 sporadic PTCs, using the DNeasy tissue kit (QIAGEN, Milan, Italy). Approximately 25 mg of tissue were incubated overnight at 56 C in lysis buffer in the presence of proteinase K (20 μl). Samples were then incubated for 15 min at 95 C to allow paraffin melting. After centrifugation for 3 min at 14,000 rpm, DNA was extracted following kit instructions. For each sample DNA concentration was assessed by spectrophotometry and stock solutions of 200 ng per 50 μl were prepared.

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Measurements of RTL

This analysis was performed using two different techniques: 1) quantitative PCR (Q-PCR), and 2) telomere fluorescence *in situ* hybridization (FISH).

The Q-PCR assay was performed as previously described (20) on 30 ng/μl of genomic DNA. Telomere length quantification involved determining the relative ratio of telomere (T) repeat copy number to a single copy gene (S) copy number (T/S ratio) in experimental samples using standard curves. This ratio is proportional to the average telomere length. The 36B4, encoding acidic ribosomal phosphoprotein P0, has been used as the single copy gene. Primers have been as follows and were used at 300 nM final concentration in a reaction mix containing 12.5 μl Syber Green PCR master mix for a final volume of 25 μl: telomere sense, 5′-GGTTTTTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGT-3′; telomere antisense, 5′-TCCCCGACTATCCCTATCCCTATCCCTATCCCTATCCCTA-3′; 36B4 sense, 5′-CCCATTCTATCACAACGGTACAA-3′, 36B4 antisense, 5′-CAGCAAGTGGGAAGGTGTAATCC-3′.

A standard curve was included in each run. The thermal cycling profile for the telomere amplification was: 95 C for 10 min followed by 30 cycles of 95 C for 15 sec and 54 C for 1 min, and for the 36B4, amplification was 95 C for 10 min followed by 40 cycles of 95 C for 15 sec and 60 C for 1 min. To exclude the presence of nonspecific binding between Syber Green and primers, a melting curve was added at the end of all PCR amplification reactions. The melting profile was as follows: 3 min at 95 C followed 15 sec at 95 C (40 repetitions), 30 sec at 60 C, 30 sec at 55 C, and 10 sec at 55 C. Because the T/S ratio will reflect RTL only if the number of copies of S per cell that are PCR amplified is the same in all samples studied, we determined by Q-PCR, as described by Cawthon (20), the relative ratio of 36B4 gene copies to β-globin gene copies using the following primers: β-globin forward, 3′-GCTTCTGACACAACTGTGTTCACTAGC-5′ and β-globin reverse, 3′-CACCAACTTCATCCACGTTCACC-5′ (400 nM each). The relative ratio 36B4 to β-globin obtained indicated that equal copy numbers of the 36B4 gene per cell were amplified in all DNA samples (not shown).

FISH was done with the Dako telomere/fluorescein isothiocyanate, kit PNA (Dako, Milan, Italy) on white cells isolated from fresh blood. Briefly, cells were fixed on slides with 3.7% pharaformaldeide for 2 min at room temperature and then washed with Tris-buffered saline twice for 5 min. Slides were then incubated for 10 min with the pretreatment solution and washed with Tris-buffered saline twice for 5 min. Slides were placed in 70% ethanol for 2 min, 85% ethanol for 2 min, and ethanol 100% for 2 min and then air dried (dehydratation). Ten microliters of a telomere PNA-probe fluorescein isothiocyanate (provided by the kit) were applied to each sample and incubated for 5 min at 85 C followed by 30 min at room temperature. Slides were washed with rinse solution, incubated for 5 min at 65 C in wash solution, and dehydratation steps with ethanol repeated. Nuclei were counterstained with 10 μl of mounting solution containing 4′,6′-diamino-2-phenylindole. Nuclear telomeric spots and fluorescence were read at ×40 magnification using a DMRB microscope (Leica, Heidelberg, Germany). Images were captured with DC350 FX camera (Leica) and fluorescence analyzed with FW4000 software (Leica). Spots were scored as 0 (no staining) or 1, 2, or 3 (when fluorescent spots were distributed in $<$10% of the cell, between 60 and 70%, or in 100% of the cell, respectively) (21). The final score was the mean obtained from the lecture of five fields for patient, each field containing at least five cells. Fluorescence intensity was scored as 0 (absent), 1 (low), 2 (medium), or 3 (high). The resulting score was the mean obtained from the lecture of three fields for patient, each field containing at least five cells.

hTERT studies

hTERT gene amplification

To investigate hTERT gene amplification, Q-PCR was performed using two different approaches: Syber Green and TaqMan.

For Syber Green analysis, 30 ng/μl of genomic DNA were amplified using a final concentration of 300 nM of the following primers: primer sense, 5′-TGACACCTCACCTCACCCAC-3′, primer antisense, 5′-CACTGTCTTCCGCAAGTT-3′ in a reaction mix containing 12.5 μl Syber Green PCR master mix (Bio-Rad, Milan, Italy) for a final volume of 25 μl. The DNA quantity standards were represented by serial dilutions of reference DNA sample (a mixture of several DNA isolated from cancer cells; Celbio, Milan Italy) to produce five final concentrations (200, 40, 8, 1.6, 0.32 ng/μl). In each run, we included a standard curve and a negative control. The thermal cycling profile was 95 C for 10 min followed by 30 cycles of 95 C for 15 sec and 60 C for 30 sec. To exclude the presence of nonspecific binding between Syber Green and primers, a melting curve was added at the end of all PCR amplification reactions. The melting profile was as follows: 3 min at 95 C, followed 15 sec at 95C (40 repetitions), 30 sec at 60 C, 30 sec at 55 C, and 10 sec at 55 C.

For TaqMan analysis, a specific fluorescence probe (FAM) 5′-GGTTACACACGTGGT-3′ (150 nM) was added to a PCR mix containing 12.5 μl master mix (Bio-Rad) and 300 nM of primers to a final volume of 25 μl (not shown).

hTERT mRNA expression

RNA was extracted from fresh blood samples of 19 FPTC patients and 40 sporadic PTCs of the Siena series who volunteered the blood for this experiment, using QIAamp RNA blood mini kit (QIAGEN). One microgram for each sample was retrotranscribed into complementary cDNA using an iScript cDNA synthesis kit (Bio-Rad) and 30 ng/μl used for Q-RT-PCR. To search for hTERT mRNA expression, 12.5 μl Syber Green PCR master mix (Bio-Rad) were added to 900 nM of each primers (primer forward, 3′-ACGGCGACATGGAGAACAA-5′, primer reverse, 3′-CACTGTCTTCCGCAAGTTCAC-5′) in a final volume of 25 μl. The RNA quantity standards were represented by serial dilutions of reference RNA sample (Celbio) to produce five final concentrations (200, 40, 8, 1.6, 0.32 ng/μl). The thermal cycling profile was 95 C for 10 min followed by 35 cycles of 95 C for 15 sec and 60 C for 1 min. A melting curve was introduced at the end of each amplification to exclude the presence of nonspecific binding between Syber Green and primers.

hTERT protein activity

Telomerase activity was measured following the protocol described in the TELOTAGGG telomerase PCR ELISA SAIC kit (Roche, Milan, Italy). White cells were isolated from fresh blood samples using PolymorphPrep (Axis-Shield, Rome, Italy), and protein concentrations were determined with Bradford method (Sigma, Milan, Italy). To exclude that the observed activity was due to a different protein content, we normalized protein concentration to add 50 μg of total protein in each well. Telomeric repeats were added to a biotin-labeled primer by telomerase, followed by the amplification of the elongation product by PCR. Aliquots of the PCR product were denatured, bound to a streptavidin-coated 96-well plate, and hybridized to a digoxigenin (DIG)-labeled telomeric repeat-specific probe. An antibody to DIG, conjugated to peroxidase, was subsequently bound to DIG and visualized by a colorimetric reaction due to enzyme's ability to metabolize tetramethyl benzidine. Sample absorbance was measured at 450 nm (reference wavelength 690 nm) using an ELISA plate reader within 30 min after the addition of the stop reagent. Internal positive and negative controls provided by the kit were used.

Search for TERC and hTERT mutations

We search for the presence of TERC and hTERT mutations in the blood of 29 FPTC and 50 sporadic PTC patients. To determine the presence of mutations in TERC gene, 25 μl/samples of AmpliTaq Gold PCR master mix (Applied Biosystems, Warrington, UK) were added to 300 nM of each primer in each PCR tubes containing 200 ng of DNA in a final volume of 50 μl. Primers were designed to amplify both the promoter and the transcription regions of TERC gene. PCR products were visualized with ethidium bromide in a 2% agarose gel and subsequently analyzed with the denaturing HPLC (DHPLC) technique to confirm the presence/absence of mutations. DHPLC results were validated by direct sequencing (in both directions) of each sample.

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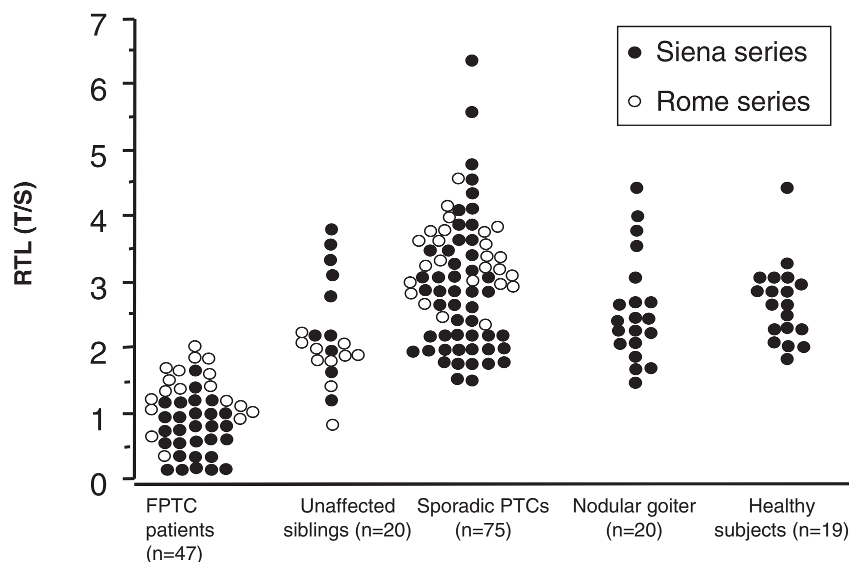

FIG. 1. RTL measured by Q-PCR and expressed as ratio T/S. FPTC patients have shorter telomeres, compared with all other groups ($P < 0.0001$ by one way ANOVA with Fisher's correction).

To analyze for mutations in hTERT gene, we amplified the 16 exons of the gene and its 3′ untranslated region with the use of specific primers (300 nM each). PCR was conducted in a final volume of 50 μl in the presence of 25 μl/samples of AmpliTaq Gold PCR master mix (Applied Biosystems). After visualization in agarose gel (2%), amplicons were analyzed with DHPLC and then sequenced in both directions.

Primer sequences, conditions, and temperature for PCR and DHPLC are reported in the supplementary table, published as supplemental data on The Endocrine Society's Journals Online Web site at http://jcem.endojournals.org.

Statistical analysis

Statistical analysis was performed with StatView for Windows, version 5.00.1 (SAS Institute, Cary, NC). One-way ANOVA with Fisher's correction was used to determine the difference in telomere length as a continuous variable. To calculate differences in hTERT gene or hTERT activity among different groups, the one-way ANOVA test with Dunn's multiple comparison was used. For analysis between first and second generation of FPTC patients, the paired *t* test was used. The staining scores were compared between sporadic PTCs and FPTC patients using Mann-Whitney *U* test.

Results

RTL

As shown in Fig. 1, RTL (measured by Q-PCR and expressed as T/S ratio) was significantly ($P < 0.0001$) shorter in FPTC patients of both Siena and Rome series (without difference between the two series) (mean ± SD 1.1 ± 0.03, range 0.18–1.8), compared with sporadic PTCs (mean ± SD 3 ± 0.97, range 1.5–6.7), healthy subjects (mean ± SD 2.69 ± 0.66, range 1.9–4.5), nodular goiter (mean ± SD 2.6 ± 0.8, range 1.87–4.5), and unaffected siblings (mean ± SD 2.16 ± 0.5, range 1.2–4).

To rule out that this result was bias by the presence of circulating tumor cells, we correlated the RTL with the clinical status of FPTC and sporadic PTC patients at the moment of the study. No significant difference was found between 35 FPTC disease-free patients (mean ± SD RTL: 1.17 ± 0.44) and 12 FPTC with persistent disease (mean ± SD RTL: 0.86 ± 0.31) ($P = 0.2$), between 60 disease-free sporadic PTC patients (mean ± SD RTL: 2.93 ± 1.04) and 15 sporadic PTCs with persistent disease (mean ± SD. RTL: 3.03 ± 0.86) ($P = 0.6$), indicating that our results were not biased by the presence or absence of disease (Table 1).

RTL was also measured by FISH analysis on white cells isolated from fresh blood. Results reproduced those obtained with Q-PCR technique. In terms of number of fluorescent spots, FPTC patients displayed significantly ($P = 0.0006$) fewer fluorescent spots (mean score ± SD 1.4 ± 0.7), compared with sporadic PTCs (mean score ± SD: 2.2 ± 0.8) (Fig. 2A). In addition, fluorescence intensity was higher in sporadic PTCs (mean score ± SD: 1.9 ± 0.6), compared with FPTC patients (mean score ± SD: 1.1 ± 0.6) ($P = 0.01$, Fig. 2B). A representative example of FISH reaction obtained in FPTC patients and sporadic PTCs is shown in Fig. 2C.

hTERT gene

We next studied the hTERT gene and found that it was significantly ($P < 0.0001$) amplified in FPTC patients (mean ± SD: 155 ± 27 ng/μl), compared with sporadic PTCs (mean ± SD 37.8 ± 15 ng/μl), healthy subjects (mean ± SD 11.2 ± 8 ng/μl), nodular goiter (mean ± SD 39.6 ± 12 ng/μl), and unaffected siblings (mean ± SD 52.2 ± 29 ng/μl) (Fig. 3, *lower panel*). In addition, it was significantly ($P = 0.03$) more represented in offspring (mean ± SD 187 ± 26 ng/μl) with respect to parents

TABLE 1. RTL and hTERT amplification according to the clinical status of FPTC and sporadic PTCs at the time of the study

	RTL (T/S)		hTERT gene amplification (ng/μl)	
	Mean ± SD	P	Mean ± SD	P
FPTC				
Disease free (n = 35)	1.17 ± 0.44	0.2	163.03 ± 52.95	0.1
Persistent disease (n = 12)	0.86 ± 0.31		189.04 ± 61.64	
Sporadic PTCs				
Disease free (n = 60)	2.93 ± 1.04	0.6	32.03 ± 43.79	0.7
Persistent disease (n = 15)	3.03 ± 0.86		36.03 ± 55.19	

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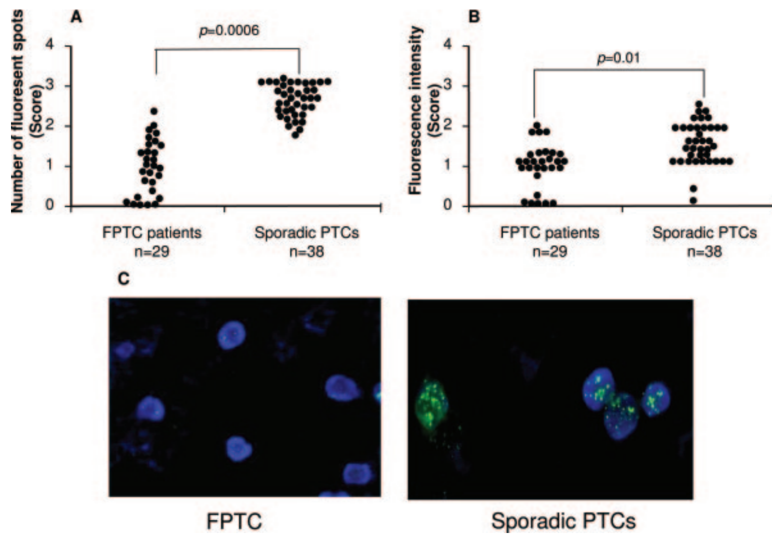

FIG. 2. RTL measured by FISH analysis in white blood cells of 38 sporadic PTCs and 29 FPTC patients of the Siena series. A, *Number of fluorescent spots* represented as arbitrary score ($P = 0.0006$ by Mann-Whitney *U* test). B, *Fluorescence intensity* represented as arbitrary score ($P = 0.01$ by Mann-Whitney *U* test). C, Representative example of FISH reaction in white blood cells of one FPTC patient and one sporadic PTC patient. Magnification, ×40.

(mean ± SD 137 ± 30 ng/μl) (Fig. 3, *upper panel*). To confirm these data, we performed TaqMan analysis and we obtained similar results. hTERT gene was significantly amplified ($P < 0.0001$) in FPTC patients (mean ± SD 184.7 ± 35 ng/μl), compared with other groups (sporadic PTCs, mean ± SD 49.6 ± 26 ng/μl; unaffected siblings, mean ± SD 37.6 ± 21 ng/μl; nodular goiter, mean ± SD 33.1 ± 13 ng/μl; healthy subjects, mean ± SD 7.6 ± 16 ng/μl) (data not shown).

No significant difference in hTERT gene amplification was observed between 35 FPTC disease-free patients (mean ± SD 163.03 ± 52.9 ng/μl) and 12 FPTC with persistent disease (mean ± SD 189.04 ± 61.64 ng/μl) ($P = 0.1$) between either 60 sporadic PTC disease-free patients (mean ± SD 32.03 ± 43.7 ng/μl) and 15 sporadic PTCs with persistent disease (mean ± SD 36.03 ± 55.1 ng/μl) ($P = 0.7$), ruling out the bias of circulating tumor cells (Table 1).

To ascertain that hTERT gene amplification was transcribed into mRNA, we measured mRNA levels by Q-RT-PCR in available patients of the Siena series. As shown in Fig. 4A, hTERT mRNA was significantly ($P = 0.0003$) increased in FPTC patients (mean ± SD 186.23 ± 37), compared with sporadic PTCs (mean ± SD 58.9 ± 15).

To verify that hTERT mRNA amplification found in FPTC patients was translated into a functional protein, we measured the activity of telomerase using the TELO-TAGGG telomerase PCR ELISA kit. The protein activity was higher in FPTCs than unaffected siblings ($P < 0.0001$) and sporadic PTCs ($P < 0.0001$) (Fig. 4B). In FPTC patients there was no difference between the first and second generation or patients with sibling relationship ($P = 0.4$).

Consistent with the presence of a functional protein, we did not find mutations of both the TERC and hTERT genes by DHPLC analysis of the PCR products performed in 29 FPTC and 50 sporadic PTC patients (data not shown).

Correlations between hTERT gene amplification, mRNA expression, telomere length, and protein activity

RTL was inversely correlated ($R^2 = -0.75$; $P < 0.0001$) with hTERT gene amplification in FPTC (Fig. 5A) but not the other groups. Moreover, hTERT gene amplification correlated with hTERT mRNA expression ($R^2 = 0.6$; $P < 0.001$) (Fig. 5B). A positive correlation was also found between hTERT mRNA and hTERT protein activity (Fig. 5C).

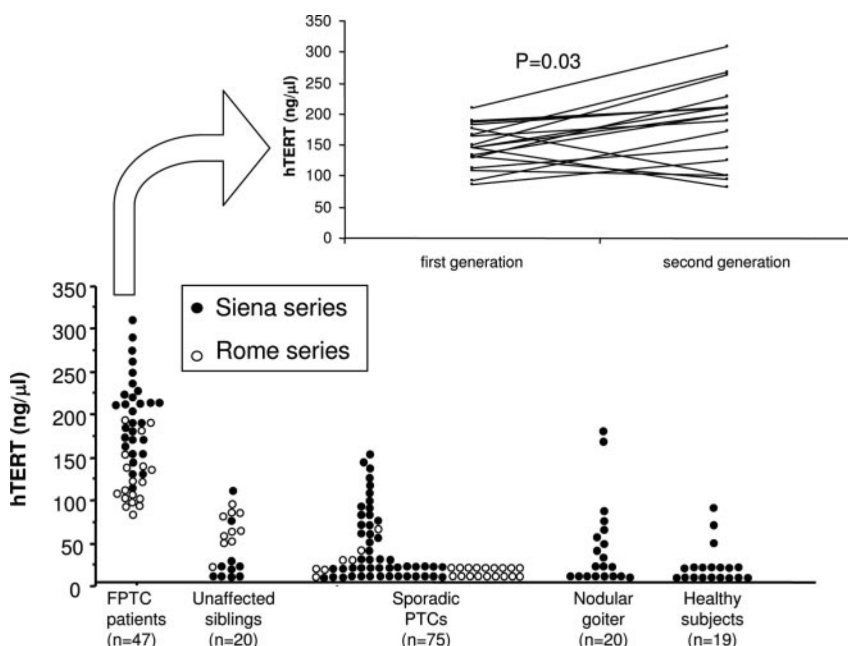

FIG. 3. hTERT gene amplification in FPTC patients and control groups (*lower panel*) ($P < 0.0001$, by one way ANOVA with Dunn's multiple comparison). hTERT gene amplification in the first and second generation (*upper panel*) ($P = 0.03$ by paired *t* test).

RTL in tissue samples of FPTC patients

RTL was also measured in 10 available cancer tissues of FPTC patients and 30 sporadic PTCs. As in peripheral blood, the RTL was also significantly ($P < 0.0001$) shorter in the tissues of FPTC patients (mean ± SD 0.96 ± 0.068, range 0.86–1.23), compared with sporadic PTCs (mean ± SD 1.8 ± 0.3, range 1.6–2.1) (data not shown).

Discussion

Differentiated thyroid cancer, although mostly sporadic, may have a familial occurrence, and some evidences suggest that

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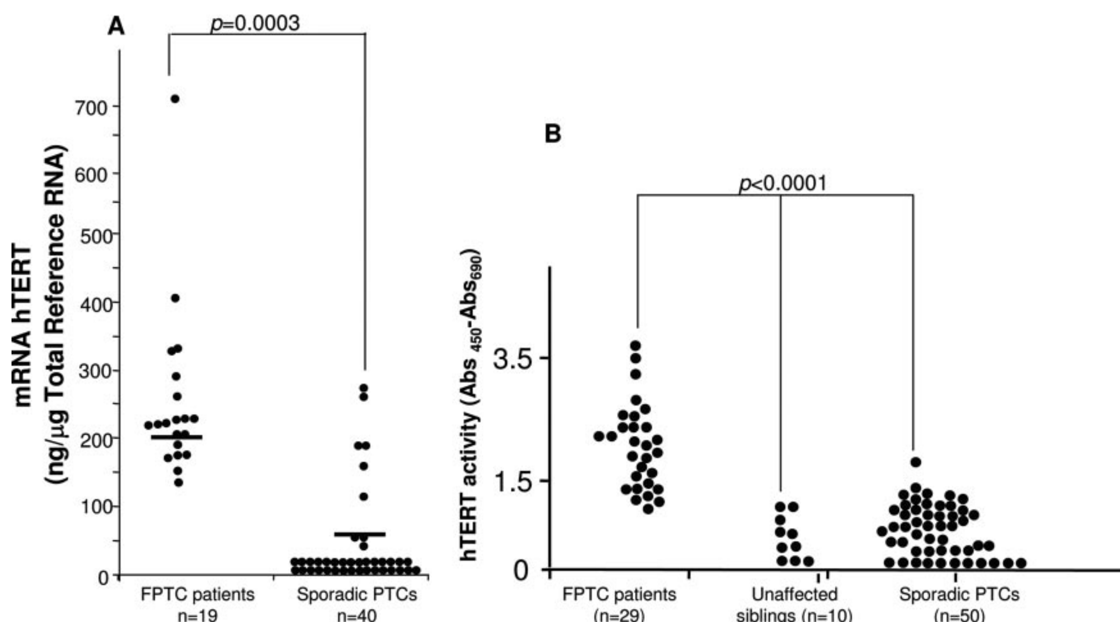

FIG. 4. A, hTERT mRNA levels in white blood cells of 19 FPTC patients and 40 sporadic PTCs of the Siena series ($P = 0.0003$, by one way ANOVA with Dunn's multiple comparison). B, Telomerase activity in white blood cells of FPTC patients, unaffected siblings, and sporadic PTCs of the Siena series ($P < 0.0001$ by one way ANOVA with Fisher's correction).

FPTC may have a genetic predisposition. Some of the cases have been associated with rare syndromes caused by germline mutations of known tumor suppressor genes (familial adenomatous polyposis, Cowden syndrome, Werner syndrome and Carney complex) (5–8). In addition, by linkage analyses of a few pedigrees, three different chromosomal regions have been shown to harbor a susceptibility gene, including 1q21, 19p13, and 2q21 coding for distinct phenotypes: familial PTCs enriched in papillary renal neoplasia (10), thyroid carcinoma with oxyphilia (9), and familial nonmedullary thyroid carcinoma type 1 (11). An autosomal dominant inheritance pattern with reduced penetrance appears likely in these pedigrees (22).

In this report we analyzed FPTC patients in which clinical evidence has excluded the presence of the above-mentioned hereditary syndromes. We studied the telomere-telomerase complex based on previous report of high telomerase activity and short telomeres in cancer development (14, 23–27). In sporadic thyroid carcinoma, telomerase activity is detectable in nearly 50% of thyroid cancer tissues, and some authors proposed that the detection of telomerase activity may be helpful in differentiating between benign and malignant thyroid tumors (28–30). The presence of short telomeres is reported in the blood of patients with sporadic head and neck, bladder, lung or renal cancers (14), and familial breast cancer (31), strengthening the hypothesis that telomere dysfunction may represent a risk factor for cancer development. Similarly, other studies described the presence of short telomeres in tissue samples of different cancers (32, 33). The association of RTL and hTERT activity in the blood is less investigated. *In vitro* (34) and *in vivo* studies (35) reported high telomerase activity associated with the initiation and progression of human neoplasia, but unfortunately, the large majority of these studies were conducted on tumor tissues.

The present study is probably the first report of short telomere length, hTERT gene amplification, and increased telomerase expression in peripheral white blood cells of patients with a familial cancer. FPTC patients display shorter telomeres, increased amplification in hTERT gene copy number, and higher telomerase activity, compared with sporadic PTCs. The hTERT gene amplification positively correlates with hTERT mRNA expression, which is translated into a functional protein. We observed that the relative telomere length found in FPTC patients of the second generation was similar (and sometimes even shorter) to that of the parents and was significantly shorter, compared with unaffected siblings. This result might be explained by the evidence that average RTL is partly transmitted throughout generations (15, 16) and an X-linked pattern of inheritance (36), and a paternal transmission has been proposed (37). Our patients of the first generation may also have inherited short telomere from their parents (that we could not study) or might have acquired the abnormality after mutations/alterations of specific control mechanisms, such as shelterin complex or transcription factors. Whatever the mechanism(s), our results suggest that patients born with short telomeres might reach earlier in life the threshold telomere length sufficient to trigger cancer development and/or progression. This is consistent with our data that patients of the second generation were always diagnosed with thyroid cancer at an earlier age, compared with their affected relative in the first generation (submitted for publication, Capezzone M, Marchisotta S, Cantara S, Busonero G, Brilli L, Pazaitou-Panayiotou K, Carli AF, Caruso G, Toti P, Capitani S, Pammolli A, Pacini F). An intriguing finding of our study is the association of short telomeres and elevated telomerase activity. Although we have no definitive explanation, this finding might be not surprising: short telomeres and high telomerase activity have been reported, at the tissue level, in several human cancers (23–25, 27) including thyroid cancer

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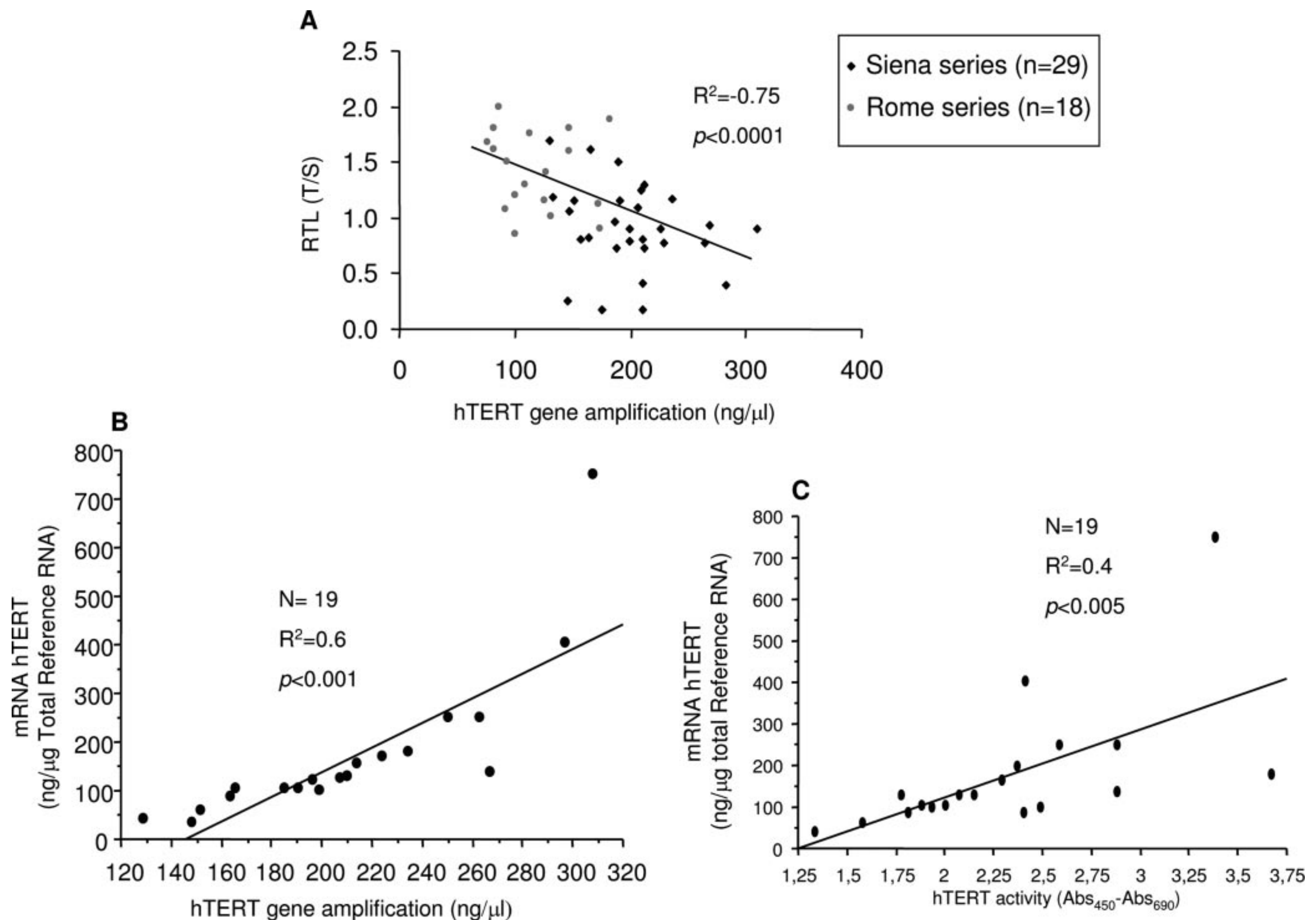

FIG. 5. A, Correlation between RTL and hTERT gene amplification in 29 FPTC patients of the Siena series and 18 patients of the Rome series. B, Correlation between hTERT mRNA and hTERT gene amplification in 19 FPTC patients of the Siena series. C, Correlation between hTERT activity and hTERT mRNA in 19 FPTC patients of the Siena series.

(26, 38). In particular, Matthews *et al.* (38) studying a series of apparently sporadic differentiated thyroid cancer found that, according to the relation between telomere length and telomerase activity, three different patterns might be identified: 1) telomerase-positive lesions, consistent with the conventional model of telomere erosion followed by telomerase reactivation, 2) telomerase-negative tumors, which maintain telomere length by a mechanism independent of telomerase, and 3) telomerase-negative tumors, which are still undergoing telomere erosion and may therefore be composed of mortal cancer cells. If we are allowed to extrapolate these models to our study, we might speculate that FPTCs fall into the first category, whereas sporadic PTCs might fall into one of the other two.

In agreement with other authors (39, 40), we can speculate that the high telomerase activity found in our FPTC patients represents a mechanism of telomere stabilization that precludes DNA-damaged cells from apoptosis and contributes to their genomic instability and immortalization, or might represent an ineffective tentative of telomere repair. Both hypotheses may be supported by the inverse correlation found between RTL and hTERT gene amplification. Alternatively, exaggerated hTERT activity may be an expression of an increase in hTERT gene copy number (or gene amplification), which *per se* represents a genetic abnormality associated with genomic instability, as recently demonstrated (41).

From the clinical point of view, our results suggest that RTL and hTERT activity might play a role in the predisposition to familial papillary thyroid cancer.

Acknowledgments

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