Telomere Abnormalities and Chromosome Fragility in Patients Affected by Familial Papillary Thyroid Cancer

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Introduction: Genomic instability has been proposed to play a role in cancer development and can occur through different mechanisms including telomere association and telomere loss. Studies carried out in our unit have demonstrated that familial papillary thyroid cancer (fPTC) patients display an imbalance, at the germinal level, in telomere-telomerase complex.

Aim: We aimed to verify whether familial fPTC patients show an increased spontaneous chromosome fragility.

Methods: To this purpose, we compared telomeric fusions and associations as well as other chromosomal fragility features by conventional and molecular cytogenetic analyses, in phytohemagglutinin stimulated T-lymphocytes from fPTC patients, unaffected family members, sporadic papillary thyroid cancer patients, and healthy subjects.

Results: We demonstrate that fPTC patients have a significant increase in spontaneous telomeric associations and telomeric fusions compared with healthy subjects and sporadic cases in the frame of an otherwise common spontaneous chromosome fragility pattern. A quantitative fluorescence *in situ* hybridization analysis demonstrates that familial cases display a significant decrease in the telomeric peptide nucleic acid-fluorescence *in situ* hybridization signal intensity in the metaphase chromosome. Moreover, three copies of the *hTERT* gene were found only in familial cases, although the result was not statistically significant.

Conclusions: These results contribute in defining familial thyroid cancer as a clinical entity characterized by an altered telomere stability, which may be associated with the predisposition to develop the familial form of thyroid cancer. **(*J Clin Endocrinol Metab* 97: E1327–E1331, 2012)**

Genomic instability has been proposed to play a pivotal role in cancer development by accelerating the accumulation of genetic changes at the basis of cancer evolution (1). It can occur through different mechanisms including an elevated rate of chromosome alterations (chromosome instability), which comprises telomere associations (2) and telomere loss (3). Telomeres are specialized nucleoprotein structures consisting of tandem arrays of double-stranded TTAGGG repeats ending by a 3′ single-strand overhang and capped by a specialized telomere protein complex named shelterin (4). The role of telomeres is to protect chromosome ends (5), and, in the absence of functional telomeres or when telomeres are critically short, cells undergo the formation of breakage-fusion bridge and other genomic changes such as chromosome rearrangements, chromosome arm gain and losses, chromosome fusions, and deletions or amplifications (6), responsible for a widespread karyotypic instability.

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Abbreviations: DTC, Differentiated thyroid cancer; FISH, fluorescence *in situ* hybridization; fPTC, familial papillary thyroid cancer; FTU, telomere fusions; HS, healthy subjects; PNA-FISH, peptide nucleic acid FISH; Q-FISH quantitative FISH; Q-PCR, quantitative PCR; sPTC, sporadic papillary thyroid cancer; TA, telomere associations; UFM, unaffected family members.

Differentiated thyroid cancer (DTC) may have a familial occurrence, with a prevalence of up to 10% (7). Most of the familial papillary thyroid cancer (fPTC) kindred have thyroid cancer as the only disease manifestation not associated with a distinct phenotype. So far, no candidate gene(s) has been discovered for this form of isolated familial DTC, and only in a minority of cases, different susceptibility genes have been identified at 1q21 (8, 9), 2q21 (10), 6q22 (9), 8q24 (11), 8p23.1 (12), and 19p13.2 (13).

A few studies have been reported on telomere-telomerase complex in fPTC patients (14–16), and dissimilar observations on telomere length of fPTC and sporadic papillary thyroid cancer (sPTC) patients have been described, opening a debate on the issue. To contribute observations from a different point of view, we aimed to verify, through a conventional/molecular cytogenetic approach, whether fPTC patients show a predisposition toward spontaneous chromosome fragility, telomere associations (TA), and telomere fusions (FTU). To this purpose, we analyzed these cytogenetic features in phytohemagglutinin-stimulated T lymphocytes from fPTC patients, unaffected family members (UFM), sPTC, and healthy subjects (HS).

Patients and Methods

Subjects

Ten milliliters of peripheral venous blood were obtained, at the time of the study, after signed informed consent according to the rules of the local ethic committee, from 13 fPTC patients [nine females, 11 classical papillary thyroid cancer, one follicular variant, and one with the tall cell variant] belonging to eight kindred (mean age 51.2 ± 11 yr, range 38–73 yr). Six (46.2%) had a parent-child relationship (four families), and seven (53.8%) had a sibling relationship (four families). Familial recurrence of the disease was defined as the presence of at least one first-degree relative with DTC. Moreover, we studied 11 sPTC patients (seven females, 10 classical papillary thyroid cancers, one follicular variant, mean age 56 ± 13 yr, range 35–74 yr), and six UFM belonging to three kindred (four females, mean age 34 ± 9 yr, range 29–52 yr) (Supplemental Table 1, published on The Endocrine Society's Journals Online web site at http://jcem.endojournals.org). As control, 10 HS (six females) without any clinical evidence of thyroid disease (by thyroid ultrasound) or past history of major illness or malignancies were enrolled (mean age 47 ± 12.5 yr, range 32–71 yr). Eleven of the 13 fPTC patients have been included in a previous study of our group (14). A history of radiation exposure has been excluded in all patients and controls.

Cell culturing

Phytohemagglutinin-M (GIBCO, Milan, Italy) stimulated lymphocytes, from 250 μl heparinized venous blood, were cultured in DMEM-F12 (1:1) (GIBCO Brl, Milano, Italy), supplemented with 16% fetal bovine serum (GIBCO), 100 μl/ml penicillin-streptomycin (GIBCO), and L-glutamine 200 mM (GIBCO). Cell cultures were incubated at 37 C (5% CO_2) for 72 h. One hour before harvesting, 0.5 μg/ml of Colcemid (Sigma-Aldrich, Milano, Italy) was added to cell cultures to obtain metaphase arrest. Cells collected by centrifugation were resuspended in a hypotonic KCl (0.075 M) solution for 10 min, fixed in acetic acid-methanol [1:3 (vol/vol)] and resuspended in 200 μl of fixative. Metaphase spreading was accomplished by dropping 10 μl of cell suspension onto cleaned slides. Air-dried preparations were kept at −20 C until use.

Conventional cytogenetic analysis, and Giemsa stain

Cytogenetic preparations were stained with 5% Giemsa (Sigma-Aldrich) in 0.06 M phosphate buffer for 5 min and then washed with water. The presence of telomeric associations, chromatid and chromosome breaks, gaps, acentric fragments, centric fissions, ring chromosomes, and double minutes were evaluated by analyzing 200 metaphases per subject under light microscopy (Olympus BX41 microscope; Olympus, Tokyo, Japan). Adjacent telomeric regions of proximal chromosomes touching each other were considered as TA. The digital images were recorded with the CytoVysion system (software 3.9; Applied Imaging, Inc., Santa Clara, CA). The mean chromosome abnormalities were given by the number of chromosome breakages per total number of cells.

Molecular cytogenetic analysis, telomere peptide nucleic acid fluorescence in situ *hybridization (PNA-FISH), and quantitative fluorescence* in situ *hybridization (Q-FISH)*

Telomere fluorescence *in situ* hybridization (FISH) assay was performed on lymphocyte metaphase chromosomes using a fluorescein-conjugated telomere PNA probe (Dako, Glostrup, Denmark), according to the manufacturer's instructions. Telomere hybridization signals were evaluated scoring a mean of 43 complete and evenly stained metaphases per individual by use of a digital image analysis system based on an epifluorescence Olympus BX41 microscope using blue (excitation 470–495 nm; emission 510–550 nm) and green filters (excitation 530–550 nm; emission 575 nm) and charge-coupled device camera (Cohu, San Diego, CA), interfaced with the CytoVysion System (Applied Imaging). The metaphases were scored for the presence of TA, FTU, and PNA-FISH signal. TA were defined as very close telomeres of adjacent chromosomes (touching each other or in proximity) showing FISH signals remaining separate. FTU were defined either as telomeres of adjoining chromosomes fused into a single FISH signal and with the counterstaining remaining continuous, either as two joint chromosomes with complete loss of telomeres and appearing as a long dicentric chromosome. For Q-FISH, telomere fluorescence signals were integrated from 20 metaphases and quantified by the ImageJ software, version 1.43u (National Institutes of Health, http://rsbweb.nih.gov/) according to the provided guidelines. The software measures fluorescence intensity of individual telomeres, expressed as the product of the telomere area and the average gray value within the selected telomere. The method provides excellent quantitation as the fluorescence intensity directly correlates to the length of the telomeres.

hTERT *gene copy number FISH analysis*

Double-target FISH was performed with a specific *hTERT* gene probe (BAC clone RP11-117B2; CHORI Oakland, CA) and a reference probe located at 5q31 (BAC clone RP11-461014; CHORI), directly labeled with Spectrum Orange and Spectrum Green fluorophore-conjugated deoxyuridine 5-triphosphate (Abbott Molecular/Vysis, Downers Grove, IL), respectively. Both probes were denatured 5 min at 75 C, and hybridization was carried on for 16 h at 37 C in Hybrite (Abbott Molecular/Vysis). After hybridization, slides were incubated three times for 5 min in 0.1× saline sodium citrate at 60 C and counterstained with 4′,6′-diamino-2-phenylindole. Hybridization signals were evaluated by scoring both metaphases (mean 32/case) and interphase nuclei (mean 248/case) under a UV light microscope with the above-described filters and a filter for 4′,6′-diamino-2-phenylindole (excitation 360–370 nm; emission 420 nm).

Statistical analysis

Comparisons between groups were performed using a two-tailed Student's t test. Values of $P < 0.05$ were considered significant. The *hTERT* gene copy number was evaluated by a χ^2 test.

Results

Spontaneous metaphase chromosome breaks by Giemsa stain

The mean frequency of spontaneous chromosomal abnormalities observed in 200 metaphases of each subject were compared. fPTC patients had a slightly higher, although not statistically significant, frequency of spontaneous chromosome abnormalities (mean 10.4%) when compared with HS (mean 8%), sPTC patients (mean 8.6%), and UFM (mean 9.5%) (Supplemental Table 2). The most frequent observed abnormalities were chromatid (Fig. 1A) and chromosome breaks together with gaps,

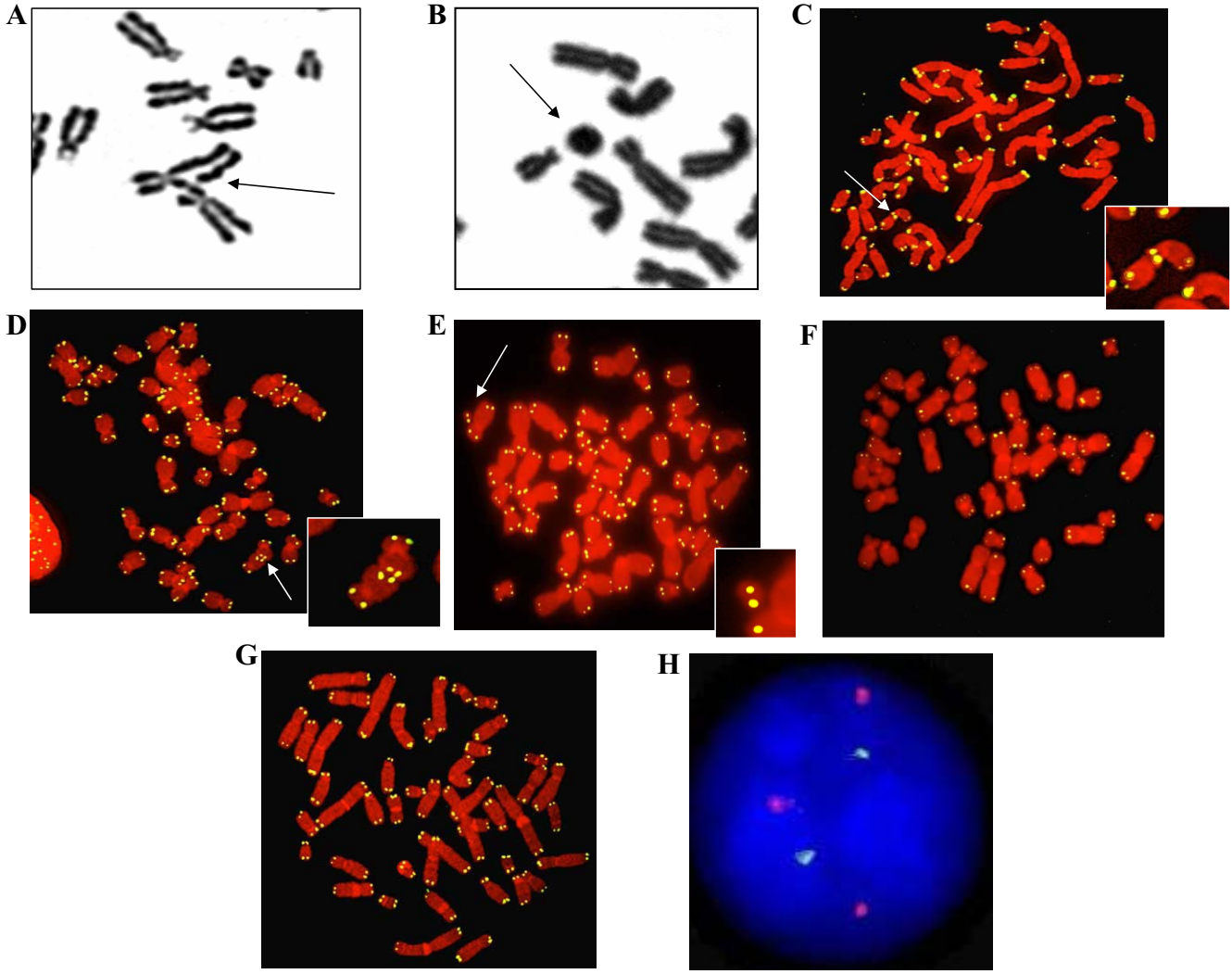

FIG. 1. Representative examples of spontaneous chromosome fragility (Giemsa stain) and telomere PNA-FISH in metaphase chromosomes from phytohemagglutinin-stimulated lymphocytes of fPTC patients: chromatid break (A), ring chromosome (B), telomeric fusion (C), telomeric association (D), telomeric fragment with two sister chromatid signals (E), telomere fluorescence intensity in a fPTC patient (F) and in a HS (G), three copies of h*TERT* gene (*red signals*) compared with the two copies (*green signals*) (H) of a reference gene located at 5q31.

TABLE 1. Spontaneous TA and FTU by telomere PNA-FISH

	Patients, n	Metaphases, n	FTU	Mean frequency (%)	TA	Mean frequency (%)
fPTC	13	620	10	1.6	19	3
sPTC	11	556	0	0	4	1
UFM	6	199	0	0	3	1.5
HS	10	500	0	0	5	1.2

acentric fragments, ring chromosomes (Fig. 1B), and centric fissions.

Spontaneous TA, FTU, and acentric fragments by telomere PNA-FISH

fPTC patients displayed a mean frequency of FTU significantly higher ($P < 0.001$) when compared with sPTC patients, HS, and UFM in which no FTU were present (Table 1). An acrocentric chromosome was implicated in the majority of FTU observed in fPTC subjects (Fig. 1C). Similarly, the mean frequency of chromosome association, calculated considering only the TA between nonacrocentric chromosomes, was significantly ($P < 0.05$) higher (mean 3%) compared with sPTC patients and HS but not UFM (Table 1). A representative example of TA is reported in Fig. 1D. Telomere associations were assessed also by Giemsa stain with similar results (not shown).

Acentric telomeric fragments were found in seven of 13 fPTC patients (53.8%) for a total of nine events ($P < 0.005$) compared with one of six UFM (17%), one of 11 sPTC (9%), and none of 10 HS. Among fPTC, four patients had only one event with telomeric fragment with one single signal, two patients had both one event with fragments with one single signal and one event with fragment with two sister signals, one patient had only one event with fragment with two sister signals. Fragments with two sister signals were observed only in fPTC patients (Fig. 1E and Supplemental Table 3).

Telomere length by Q-FISH

We analyzed 20 metaphases per subject of each group and compared the Q-FISH mean values (expressed as pixels). fPTC (Fig. 1F), UFM and sPTC displayed a reduction in telomere fluorescence (Q-FISH value: 146.5, 147, and 150.6, respectively) compared with HS (176.5) (Fig. 1G and Supplemental Table 4). The values of the three groups *vs.* HS were lower ($P = 0.00001$ for fPTC, $P = 0.0001$ for UFM, and $P = 0.000097$ for sPTC).

hTERT gene

hTERT copy number gain (at least one nucleus with three signals for the *hTERT* gene and two signals for the reference gene located on 5q31), was found only in fPTC patients (four of 13 cases, 30.8%) (Fig. 1H). The result was not statistically significant.

Discussion

Telomeres play a critical role in sheltering chromosomal ends, preventing chromosomes from erosion, end-to-end fusion, and other damaging events. Evidences that abnormally short telomeres play an important role in the early development of cancer and chromosome instability have been reported in several studies but almost exclusively at the somatic level (17). Recently we demonstrated abnormalities in the telomere-telomerase complex in the peripheral blood of fPTC (14). In the present study, we expanded our observations, demonstrating that the formation of telomeric fusions was an exclusive feature of fPTC patients, suggesting their nonrandom occurrence and the possibility to predispose to the formation of telomeric fusions in proliferating cells as a consequence of breakage/fusion/bridge cycles. Interestingly, fPTC patients have a frequency of spontaneous chromosome fragility slightly higher than HS and sPTC and show a preponderance of acentric fragments carrying telomeric sequences (positive Q-FISH signals), hence resulting from subtelomeric chromosomal breakage. No amplification (*i.e.* multiple gene copy number) of *hTERT* was observed in fPTC; however, one extra copy was scored in a minority of cells in four fPTC patients but not in any of the other groups. This figure, although statistically not significant, might indicate a propensity toward *hTERT* gene gains. This would be in keeping with our previous observation of *hTERT* gene amplification in fPTC compared with sPTC (not confirmed in a recent study from another group) (16), considering that FISH and quantitative PCR (Q-PCR) are two different detection methods, with unlike sensitivity toward genetic heterogeneity.

Q-FISH results on telomere length, emphasize a statistically significant difference between HS and all the other groups but not between fPTC and sPTC. This seems to be in contrast with our previous report by Q-PCR on nonstimulated interphase nuclei (14) but in agreement with a recent paper on the same subject (16). In this respect, however, it should be pointed out that Q-FISH experiments were carried out on phytohemagglutinin-stimulated T lymphocytes, whereas Q-PCR has been done on the whole white cell population, and thus, the two methods have different sensitivity.

Taken together, our results indicate that subtelomeric fragility, leading to acentric fragment formation, which

were highly preponderant in fPTC, is a feature associated with fPTC. It is intriguing to note that among fPTC, sPTC, and UFM, which do not differ significantly in their Q-FISH values but which do have Q-FISH values significantly lower than HS, only fPTC show an increased prevalence of telomeric fusions. Actually, the Q-FISH *P* values of UFM and sPTC *vs.* HS are approximately the same ($P = 1 \times 10^{-4}$), whereas the Q-FISH *P* values of fPTC is much more significant ($P = 1 \times 10^{-5}$) in comparison with HS. Moreover, considering that Q-FISH technology detects dark telomeres (*i.e.* no bright UV light signal is visible under a certain threshold of DNA repeats) but it is not able to quantify different number of telomeric repeats, it can be speculated that fPTC dark telomere might have a higher probability to be short enough to enable telomeric fusions than sPTC and UFM telomeres.

In addition, the finding of UFM telomere length very close to that of sPTC and constitutionally shorter than those of the general population, may suggest that UFM possibly need a lower series of environmental/(epi)genetic steps to achieve the critical telomere length associated with tumor development, thus showing a propensity to cancer development in the subsequent years than the general population.

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