Circulating *hTERT* DNA in Early Breast Cancer

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Abstract. *Background: The objective of the study was to quantify the human telomerase reverse transcriptase (hTERT) gene in the circulating DNA of patients with primary breast cancer (BC) and to test its correlation with clinical parameters of the disease. Patients and Methods: One hundred and twenty-one BC patients, 30 patients with fibroadenoma (NBC) and 50 healthy women were enrolled. Results: The level of hTERT in the plasma was significantly different in BC, NBC and controls ($p<0.01$), showing a sensitivity of 50% and specificity of 90% in the ability to detect malignancy. The circulating hTERT DNA was significantly different in the estrogen receptor $(ER)^+$/progesterone receptor $(PgR)^+$ compared to the ER^-/PgR^- patients ($p=0.03$). Higher hTERT levels were associated with higher human epidermal growth factor receptor (HER)-2/Neu expression: score 0-1 vs. score 2+ ($p=0.01$) and vs. score 3+ ($p=0.02$). Finally, hTERT was significantly inversely correlated with the carbohydrate antigen (CA) 15.3 serum level ($p=0.001$). Conclusion: Circulating hTERT DNA has a better diagnostic value than CA 15.3 in early breast cancer disease and could be a possible candidate as a tumor marker in patients with infiltrating ductal carcinoma positive to steroid hormonal receptor and with amplification of HER-2/Neu.*

Breast cancer (BC) is a leading cause of malignancy in women and 35-40% of patients with operable breast cancer develop metastases after primary therapy. At the time of surgery, the clinical prediction of relapse is still based on the determination of prognostic parameters in the primary tumor or locoregional lymphonodes (1, 2). Various serum tumor markers have been evaluated regarding their utility for the detection and monitoring of BC patients (3). However, current screening methods fail to completely predict recurrency. Thus, a non-invasive test for early detection of the disease and for monitoring disease progression has been a goal for many researchers (4).

The finding that tumors can release DNA into the circulation has opened new areas in translational cancer research. Increased concentrations of cell-free DNA in plasma or serum have been found in patients with different types of cancer, but not in healthy individuals (5, 6). Accordingly, it is possible to detect tumor-specific DNA alterations such as oncogenes and tumor suppressor gene mutations, microsatellite and epigenetic alterations, and chromosomal translocations, identical to those found in the primary DNA in the plasma of patients carrying various types of cancer (7, 8). In particular, the level of the human telomerase reverse transcriptase (*hTERT*) gene in plasma has been suggested as a marker of tumor presence/aggressiveness at least for liver cancer (9).

hTERT is a single-copy gene, detectable with good correlation in serum and plasma (10). Telomerase is not expressed in most somatic tissues, but is widely expressed in cancer cells (11, 12). Telomerase expression is thought to be necessary for cells to divide continuously beyond replicative senescence and may, therefore, be a critical step in cellular immortality and carcinogenesis (13). The role of telomerase activation in human cancer development has been extensively studied in recent years. Telomerase reactivation appears to constitute a relatively early event in invasive breast carcinogenesis, in fact it increases in preinvasive lesions such as ductal carcinoma *in situ* (DCIs) (14, 15). Recent studies have correlated the expression of *hTERT* with the aggressiveness of the tumour and with an accelerated progression in diverse types of neoplasias (16, 17). The objective of this study was to quantify the *hTERT* gene in the plasma of patients with primary breast cancer and to test its correlation with the clinical parameters of disease: age, histology, stage, nodal status, hormonal receptors, proliferative activity (mitotic index: MIB-1), human epidermal growth factor receptor (HER)-2/Neu and carbohydrate antigen (CA) 15-3.

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Key Words: hTERT, circulating DNA, breast cancer, real-time PCR, tumor marker.

Patients and Methods

Patients. One hundred and twenty-one BC patients (median age 59 years: range 37-92) and 30 patients with benign breast disease, fibroadenoma (NBC: non-breast cancer) median age 45 years (range: 23-65) were enrolled in this study at the Istituto Tumori Giovanni Paolo II of Bari. Fifty women volunteers (median age 45: range 25-70 years) who came to the laboratory and were considered healthy by clinical and laboratory examination were included as healthy controls. The clinical diagnosis of the 121 patients was confirmed by histological examination. Among them, 97 had ductal infiltrating BC (IDC), 15 infiltrating intraductal and 10 infiltrating lobular BC. Peripheral blood samples (5 ml) were collected from each participant using a vacutainer system with lithium-heparin. Samples were collected before any invasive procedures or therapy. The plasma was immediately separated from the cellular fraction by centrifugation at 2,500 rpm for 10 min at 4˚C, and frozen at –80˚C.

Extraction of DNA from plasma and quantification of hTERT. The DNA was extracted from 200 μl of plasma using commercial kits based on affinity columns (QIAamp Blood Mini Kit; Qiagen, Hilden, Germany) following the manufacturer's recommendations. The quantification of *hTERT* was performed using a RT-PCR (real-time polymerase chain reaction), based method. The primers and the probe were designed by Applied Biosystems (Foster City, CA, USA) to specifically amplify the gene of interest, *hTERT* (The Quantifiler® Human DNA Quantification Kit). The Quantifiler kit contained all the necessary reagents for the amplification, detection and quantification of a human-specific DNA target (*hTERT*, location 5p15.33, amplicon length 62 bases pairs): Quantifiler® Human Primer Mix, Quantifiler Human DNA Standard, Quantifiler PCR Reaction Mix (AmpliTaq Gold® DNA Polymerase, deoxyribonucleotide triphosphate (dNTPs) with deoxyuridine triphosphate (dUTP), and optimized buffer components).

The RT-PCR reaction was carried out in 96-well plates with a total volume of 25 μl/well containing the following reagents: 12.5 μl of PCR Master Mix, 10.5 μl of Primer Mix and 2 μl of purified DNA from each sample. The reaction was carried out using the GeneAmp 7000 Sequence Detection System (Applied Biosystems) under the following conditions: the mixture of samples and reactants for the PCR reaction was pre-heated to 50˚C for 2 min and then 95˚C for 15 s and 60˚C for 1 min. This was followed by 40 cycles of 95˚C for 15 s and 60˚C for 1 min. A standard curve with Quantifiler Human DNA Standard ranging from 50 ng/μl to 0.023 ng/μl (50.0 ng/μl,16.7 ng/μl, 5.56 ng/μl, 1.85 ng/μl, 0.62 ng/μl, 0.21 ng/μl, 0.068 ng/μl, 0.023 ng/μl) was used for every plate. Every patient sample and DNA standard was carried out in duplicate and only the curves with a coefficient of correlation between 0.999 and 0.995 and a slope between 3.25 and 3.35 were accepted.

Detection of CA 15.3. CA 15.3 serum levels were determined using IMMULITE 2000 BR-MA (Medical System S.p.A., Genova, Italy), a two-step sequential chemiluminescent immunometric assay that using an anti-CA 15.3 murine monoclonal antibody, with 0.2 U/ml as the lower limit of sensitivity; 99% of the sera donated by the healthy women contained less than 40 U/ml of CA 15.3 in agreement with reported normal values (18).

Statistical analyses. The data were analysed by unpaired *t*-test, Mann Whitney *U*-test, Pearson correlation by *SPSS* software for window 9.0.1. The numerical data were expressed as mean±standard deviation (SD). A p-value ≤0.05 was considered statistically significant.

Results

The level of *hTERT* in the plasma was significantly different between the BC (0.98±0.94 ng/μl), NBC (0.55±0.25 ng/μl) and controls (0.03±0.09 ng/μl) ($p<0.001$, Mann-Whitney *U*-test) showing a sensitivity of 50% and specificity of 90% in the ability to detect malignancy (Figure 1). Table I lists the correlation between the plasma level of circulating *hTERT* DNA and the clinical characteristics of the patients: no statistically significant associations were found with age, menopausal status, stage, grading, nodal status or MIB-1.

Higher levels of circulating *hTERT* DNA were found in the patients with ductal infiltrating carcinoma with respect to the patients with other types of BC (1.04±0.97 ng/μl *vs*. 0.66±0.37 ng/μl; $p=0.05$). Higher levels of circulating *hTERT* DNA were also found in the 38 patients who had menarche at age >14 years (1.13±1.00 ng/μl) than in the 68 patients who had menarche at age <14 years (0.75±0.70 ng/μl) $p=0.02$.

A significant correlation was found between circulating *hTERT* DNA and steroid hormone receptor status (in the 115 analysed patients). Circulating *hTERT* DNA was significantly higher in the estrogen receptor $(ER)^+$/progesterone receptor $(PgR)^+$ patients (1.00±0.84 ng/μl) compared to the ER^-/PgR^- patients (0.48±0.42 ng/μl) ($p=0.03$).

HER-2/Neu amplification was analysed in 52 patients, in 14 of whom the protein was not expressed (score 0-1), 11 were border line (score 2+) and 27 were positive (3+). Interestingly, higher *hTERT* levels were associated with higher HER-2/Neu expression: 0-1 (0.70±0.43 ng/μl) *vs*. 2+ (1.18±0.67 ng/μl), $p=0.02$, and *vs*. 3+ (1.24±0.42 ng/μl), $p=0.01$.

Finally, the circulating *hTERT* DNA was significantly inversely correlated with CA 15.3 serum level ($p=0.001$) (Table I). When the circulating *hTERT* DNA levels and serum CA 15.3 concentrations were compared as continuous variables, a significant inverse correlation between the two markers was found (Pearson correlation=–0.2, $p=0.006$) (Figure 2). For further analysis, the patients were divided into four groups on the basis of the cut-off values of the two markers. For *hTERT,* the cut-off value was the median value of 1 ng/μl and for CA 15.3, 40 U/ml was the cut-off. The first group (N=31) consisted of patients who had an increase of *hTERT* only; the second group (N=68) did not have an increase of either tumor marker; the third group (N=22) had increased serum CA 15.3 only; and the fourth group (N=0) had both increased circulating DNA and serum CA 15.3. These groups were modelled on nodal status, grading, receptor status and *HER-2/Neu* amplification. Interestingly, no patients belonged in the fourth group: high level of *hTERT* DNA and high level of CA 15.3.

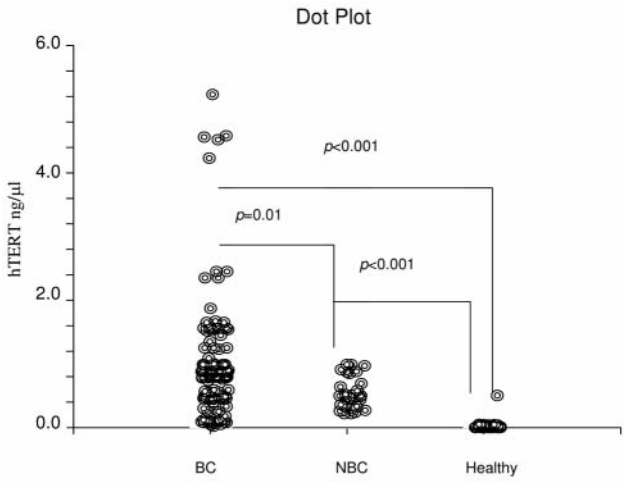

Figure 1. *Mean value of circulating hTERT DNA in BC patients (breast cancer), NBC patients (non-breast cancer) and in healthy women.*

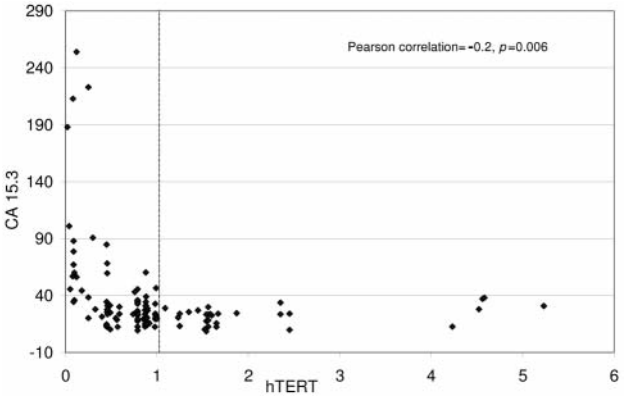

Figure 2. *Scatter plot showing correlation between circulating hTERT DNA and serum CA 15.3.*

Table I. *Association between patient characteristics and circulating hTERT DNA.*

Patient characteristics	N (%)	*hTERT* DNA (mean±SD) ng/µl	*p*-Value
Age (years)			
≥50	93 (77)	0.92±0.91	n.s.[a]
<50	28 (23)	1.08±0.85	
Menopausal status			
Premenopausal	51 (42)	0.97±0.84	n.s.[a]
Postmenopausal	70 (58)	0.94±0.94	
Age at menarche			
≥14 years	38 (36)	1.13±1.00	0.02[a]
<14 years	68 (64)	0.75±0.70	
Histology			
IDC	96 (79)	1.04±0.97	0.05[a]
Other	25 (22)	0.66±0.37	
Grading			
1	22 (18)	0.01±0.57	n.s.[b]
2	48 (40)	1.04±0.84	
3	51 (42)	1.02±1.02	
Stage			
I	56 (46)	0.93±0.74	n.s.[b]
II	41 (34)	1.14±1.12	
III	24 (20)	0.94±0.98	
Nodal status			
Negative	46 (38)	0.80±0.56	n.s.[a]
Positive	75 (62)	1.08±0.88	
Steroid hormonal receptor status			
ER+/PgR+	67 (57)	1.00±0.84	
ER–/PgR–	25 (23)	0.48±0.42	0.03[b]
ER+/PgR–	22 (18)	1.11±0.98	n.s.
ER–/PgR+	1 (2)	0.67±0.16	n.s.
MIB-1			
<20	40 (35)	0.81±0.46	n.s.[a]
≥20	73 (65)	1.11±1.10	
HER-2/Neu			
0-1	14 (27)	0.70±0.43	
2+	11 (21)	1.18±0.67	0.02[b]
3+	27 (52)	1.24±1.01	0.01[b]
CA 15.3			0.001[a]
<40 U/ml	98 (81)	1.11±0.87	
≥40 U/ml	23 (19)	0.48±0.64	

n.s.: Not significant, a: *p*-values were calculated with unpaired *t*-test, b: *p*-values were calculated with Mann-Whitney *U*-test. IDC: infiltrating ductal carcinoma, ER: estrogen receptor, PgR: progesterone receptor. HER-2/neu: human epidermal growth factor receptor, CA 15.3: carbohydrate antigen, MIB-1: proliferative activity.

Discussion

The present study was the first that examine circulating *hTERT* DNA with respect to the clinicopathological characteristics in breast cancer. Furthermore only a limited number of investigations have evaluated the relationship between human telomerase expression or activity and clinico-pathological parameters (19). In the current study, the mean level of circulating *hTERT* seemed to be a predictor of positive malignancy predictor with a specificity of 90% . In fact, it was significantly more elevated in the BC patients than in those with benign breast disease and it was even less in the healthy donors, confirming the observations of other authors that circulating DNA varies among individual cancer patients and that it can also be measured in healthy individuals (20, 21). Furthermore, telomerase activation was shown in premalignant lesions, such as cervical and prostate intraepithelial neoplasia, and even in some benign lesions, suggesting its early role in carcinogenesis (22, 23). However, Boddy *et al.,* while reporting significantly lower levels of circulating DNA in healthy individuals compared to prostate cancer patients, also stated that men with benign hyperplasia presented higher circulating DNA than prostate cancer patients (24). It is possible that genomic instability caused by telomere

dysfunction occurs in the early stages of carcinogenesis, when telomerase has not yet been activated. During subsequent progression, the telomeres may undergo further progressive shortening, generating rampant chromosomal instability, which threatens the survival of these cells. Therefore, telomerase activation necessarily occurs at this stage to stabilize the genome and confer unlimited proliferative capacity upon the emerging and evolving cancer cell (25, 26).

In this study, a direct correlation between circulating *hTERT* DNA and steroid hormone receptor status and older age at menarche was shown. Evidence has indicated that telomerase activity is critically regulated by steroid hormones in their target tissues (27). In particular, the human endometrium expresses telomerase activity despite its somatic origin, and this activity is tightly regulated in a menstrual phase-dependent manner, suggesting a control mechanism of telomerase by sex steroids (28). Hombach-Klonish *et al*. demonstrated that estrogen activates telomerase *via* the direct interaction of ligand-activated ER with the *hTERT* promoter (29). The present data demonstrated that *hTERT* could be a direct target of estrogen pathways confirming the existence of hormone-dependent mechanisms of telomerase activity control. The identification of *hTERT* as a target of estrogens is a novel finding which advances the understanding of telomerase regulation in hormone-dependent cells, potentially implicating hormones in senescence and malignant conversion.

To the best of our knowledge, this was also the first study to investigate the relationship between the plasma expression of circulating *hTERT* DNA and the serum level of CA 15.3 in BC patients before surgery, and to analyze their association with the clinicopathological characteristics. Currently, CA 15.3 is the most widely used serum marker for BC, it is recommended in the evaluation of response to therapy and for monitoring the course of BC, but there is no evidence of the efficacy of screening with this marker in BC (30). In fact, CA 15.3, is elevated in only 3% of patients with localised cancer while it is elevated in up to 70% of patients with metastatic disease (31). In the present study, high CA 15.3 levels were found in only 19% of the patients with no significant difference between the patients with and without nodal metastasis (11% *vs*. 23% , respectively). The CA 15.3 assay measures the protein product of the mucin 1 gene (*MUC-1*). This protein (episialin, epithelial membrane antigen, CA 15.3 antigen) is a highly *O*-glycosilated mucin-like transmembrane glycoprotein encoded by the *MUC-1* gene on chromosome 1q21 (32). In most normal glandular epithelium, MUC-1 is expressed at the apical surface. Entire membrane MUC-1 expression is more often seen in mucinous carcinoma than in ductal carcinomas of no special type (33). Several studies have described cell adhesion inhibition as well as an increased metastatic and invasive potential of tumor cells associated with the overexpression of MUC-1 (34). For that reason, apical expression in breast carcinoma, indicates normal routing of MUC-1 molecules and, as a consequence, relatively intact glandular differentiation (35). Thus, the present study indicated that circulating *hTERT* DNA has a better diagnostic value than CA 15.3 in early BC disease and *hTERT* could be a possible candidate as a tumor marker in patients with a histology of infiltrating ductal carcinoma positive to steroid hormonal receptor and with amplification of *HER-2/Neu*.

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