Lung cancer is the leading cause of death in oncologic patients of western countries (1). Approximately 85% of lung cancer patients have a histologic diagnosis of non–small cell lung cancer (NSCLC). Due to the delay in clinical diagnosis, only a relatively small proportion of patients (20-25%) have a resectable disease at presentation and the 5-year estimated survival rates are disappointing (2-4). Therapeutic failure and the side effects of anticancer therapy remain very important issues for future research (5). Therefore, the study of tumor and patient genetic profiles, relative to drug-related genes, may offer new opportunities for tailoring treatments.

Telomeres are repetitive, noncoding DNA [TTAGGG] elements at the ends of chromosomes. Telomerase is a multimeric ribonucleoprotein complex consisting of at least a functional RNA (hTR) that contains the template region complementary to the telomeric sequence and a reverse transcriptase protein component (hTERT) that catalyzes the addition of telomeric repeats to the ends of chromosomes (6–9). In most human somatic cells, telomeres become shorter with each cell division due to incomplete lagging DNA strand synthesis and oxidative damage (10, 11). Telomere lengths are affected by their starting set points, the cellular activity of telomerase, the cell’s history of cell division, and environmental effects. Among the different molecular factors implicated in carcinogenesis, telomere dysfunction emerges as an early event associated with genetic instability. Genetic integrity is gradually lost as telomeres progressively become shorter with each cell replication cycle (12). This telomere shortening is a result of end-replication problems caused by DNA polymerase having difficulty in replicating the very ends of linear DNA. Telomere shortening may induce cells to undergo apoptosis or may induce chromosomal instability (10). In fact, telomere dysfunction (short telomeres) has been associated with the initiation and progression of human neoplasia (13).

Conclusions: Telomerase functional polymorphism in the hTERT gene may contribute as a prognostic factor in NSCLC patients. Our findings indicate that hTERT genetic variants, by modulating telomere length, may confer an advantage in chemotherapy response. The assessment of telomerase genetic variants could supplement prognosis of survival in the course of NSCLC and may be a promising molecular marker of treatment response in these patients.
Telomerase activity or hTERT expression or both are increased in cancers and both are prognostic factors in various cancer types (15–22). Furthermore, several studies using animal models and human NSCLC tissues have reported that TERT mRNA and TERT protein are over-expressed in lung cancer biopsies compared with normal lung tissues (23–25).

Although telomere shortening is inversely associated with age, telomere length has been found to vary considerably in human peripheral blood lymphocytes from individuals of the same age (26, 27). Matsubara and colleagues (28) screened the promoter region of hTERT for functional polymorphisms, and a frequent T to C transition was found 1,327 bp upstream the transcription starting site (−1327T/C). Individuals homozygous for the −1327T/C genotype showed lower telomerase activity and shorter telomere length in their peripheral leucocytes compared with the −1327T/T and −1327T/C genotypes.

The purpose of our study was to determine whether hTERT genetic variants are of prognostic and/or predictive value in NSCLC patients who have undergone a platinum-based doublet chemotherapy in combination with a third-generation cytotoxic compound. There are several studies regarding the hTERT −327T/C polymorphism. So far, this genetic variant has been associated with telomerase activity, telomere length, and coronary artery disease development (28–30). To the best of our knowledge this is the first study reporting a role of these telomerase genetic variants in cancer, specifically in NSCLC patients.

Materials and Methods

Study population

Starting in 1997, 226 consecutive Caucasian patients admitted to the Portuguese Institute of Oncology of Porto (IPO-Porto), Portugal, with cytologically or histologically confirmed NSCLC, were prospectively recruited to the study (median age, 63.5 years; mean age, 62.5 years; SD, 10.2). The recruited NSCLC patients were divided in two groups, according to tumor stage and treatment of the disease: stage I and II patients with surgical resection done at IPO-Porto, and stages III and IV patients treated with platinum-based chemotherapy between 1997 and 2009, which had follow-up data. The patients were evaluated according to the tumor-node-metastasis staging system, and the assessment of tumor response to chemotherapy was based on the Response Evaluation Criteria in Solid Tumors. The first-line chemotherapeutic protocol consisted of platin-based doublet chemotherapy in combination with a third-generation cytotoxic compound such as paclitaxel, gemcitabine, or docetaxel. The chemotherapy protocols were as follows: cisplatin (80 mg/m² on day 1) plus paclitaxel (175 mg/m² on day 1 every 3 weeks); cisplatin (100 mg/m² on day 1) plus gemcitabine (1,250 mg/m² on days 1 and 8 every 3 weeks); carboplatin [area under curve (AUC) 6 on day 1] plus paclitaxel (175 mg/m² on day 1 every 3 weeks); carboplatin (AUC 6 on day 1) plus gemcitabine (1,000 mg/m² on days 1 and 8 every 3 weeks).

The median follow-up time was 26 months (range, 1-135 months). Patients’ distribution according to the stage at the time of diagnosis was 29 patients (12.5%) presenting localized disease (stages I and II) and 203 (87.5%) with advanced disease (stages III and IV). Considering the patients’ gender, 49 (21.1%) were female and 183 (78.9%) were male individuals. Regarding smoking habits, 63 (27.2%) were nonsmokers and 169 (72.8%) were smokers or former smokers. For all patients, the histologic type distribution was as follows: 87 patients (37.5%) with epidermoid NSCLC, 112 (48.3%) with adenocarcinoma, and 33 (14.2%) with other NSCLC histologic types.

This study was conducted according to Helsinki Declaration principles. Antecubital peripheral venous blood sample was collected from each subject at the time of recruitment. DNA was extracted from peripheral blood samples using the QiAamp DNA Mini Kit (Qiagen), according to the manufacturer’s protocol.

hTERT −1327 T/C genotyping

The hTERT −1327 T/C polymorphism (rs 2735940) was analyzed by allelic discrimination with real-time PCR, through the 5′-nuclease assay (TaqMan) using the ABI Prism 7300HT Sequence Detection System (Applied Biosystems). Assay and PCR conditions were according to the included protocol except that PCR was run on 10 ng DNA in a 10 μL reaction volume. PCR plates were read and data were analyzed using Allelic Discrimination Program (SDS v2.1 software, Applied Biosystems).

Quality control procedures implemented for genotyping included double sampling in about 10% of the samples to assess reliability and the use of negative controls to prevent false positives. Two authors independently
reviewed the genotyping results, data entry, and statistical analyses.

**Statistical analysis**

Genotype proportions among groups were compared with Pearson $\chi^2$ test. Overall survival was the end point of this analysis and was calculated from the date of diagnosis to the patient’s date of death. Data were collected from patients’ medical records. The associations between hTERT polymorphism and survival were estimated by Cox regression analysis. Cox regression models were used to adjust for potential confounders with hTERT genotypes fitted as indicator variables. Analysis of data was done using the computer software SPSS for windows (version 13.0).

**Results**

Table 1 describes the genotype distributions of the hTERT −1327C/T functional polymorphism among NSCLC cases. The frequencies of CC, CT, and TT genotypes were 0.25, 0.43, and 0.32, respectively. Using the recessive model, we found the frequency of the CT and TT genotypes (T-carrier genotypes) to be 0.75. Observed versus expected genotype frequencies were calculated, and no deviation from Hardy-Weinberg equilibrium was observed ($P = 0.33$).

We found no statistically significant differences in genotype distributions according to the patients’ clinicopathologic characteristics, namely, histologic type (epidermoid and nonepidermoid cases; $P = 0.870$) and tumor stage (stages I/II and III/IV; $P = 0.837$). Moreover, the hTERT genotype frequencies did not differ significantly among NSCLC cases considering smoking habits (smoker/former smoker and nonsmoker cases; $P = 0.061$), gender (male and female; $P = 0.174$), and age (age <64 years and >64 years; $P = 0.246$).

Regarding survival analysis, Fig. 1 presents the Cox regression analysis of survival curves of patients with III and IV tumor stages considering hTERT genotypes. Using a multivariate Cox regression model, we found an increased overall survival time for T-carrier patients, when compared with CC genotype, with histologic type ($P = 0.523$), gender ($P = 0.691$), smoking status ($P = 0.707$), and age ($P = 0.459$) as covariates [hazard ratio (HR), 0.52; 95% confidence interval (95% CI), 0.35-0.77; $P = 0.001$; Table 2]. The median estimated cumulative survival in T-carrier patients was significantly higher at 26.5 months, compared with that of CC patients at 19.3 months (Fig. 1).

This difference was more evident when considering non-epidermoid tumor histologic type, with a statistically significant increase in overall survival of patients with T-carrier genotypes, compared with CC genotype, with an estimated median overall survival of 29.8 months for T-carrier patients and 19.3 months for CC patients (HR, 0.46; 95% CI, 0.27-0.78; $P = 0.004$), adjusted by gender ($P = 0.689$), smoking status ($P = 0.675$), and age ($P = 0.872$; Fig. 2).

Regarding epidermoid histologic type, we found no association between hTERT genotypes and overall survival (HR, 0.53; 95% CI, 0.27-1.05; $P = 0.068$). We found no

<table>
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<tr>
<th>hTERT genotypes, $n$ (%)</th>
<th>$P^*$</th>
<th>hTERT genotypes, $n$ (%)</th>
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* $\chi^2$ test.
Cisplatin selectively reduces telomerase activity in a specific cancers, it is potentially a universal anticancer drug target. Telomeres, by the enzyme telomerase (8, 33).

Some fusions that lack TTAGGG repeats at the fusion point. This may be due, for example, to oxidative stress. Total loss of telomeric sequences can become dysfunctional by several mechanisms, including one that can induce apoptosis (32).

Cellular activities by activating genetic programs of cell cycle arrest, differentiation, or senescence. Moreover, when telomeres become critically shortened and compromise genomic stability, chromosome ends activate DNA damage response pathways that can induce apoptosis (32). In cancer, these mechanisms are often inactivated, and telomeres can become dysfunctional by several mechanisms, such as loss or alterations of telomere-binding proteins involved in telomere maintenance, and DNA damage due, for example, to oxidative stress. Total loss of telomeric DNA can promote the formation of end-to-end chromosome fusions that lack TTAGGG repeats at the fusion point. Telomere loss may be compensated by the recombination-based ALT pathway or, as seen in the majority of human cancers, by the enzyme telomerase (8, 33).

Because of the general requirement for telomerase in cancers, it is potentially a universal anticancer drug target. Cisplatin selectively reduces telomerase activity in a specific manner in tumor cells so that telomerase inhibition might be a component of the efficacy of cisplatin in the treatment of cancer. Interestingly, cisplatin-adduct formation in the telomere unit may block the telomerase reaction, and DNA adducts of cisplatin may inhibit the conventional replication of the telomere repeats (34).

$hTERT$ mRNA expression seems to be most important for telomerase activity, but alternative splicing, posttranslational alterations, and hTERT localization in the cell contribute as well (35). Less is known about the impact of hereditary $hTERT$ gene variations. Matsubara et al. (28) screened the promoter region of $hTERT$ for functional polymorphisms in a Japanese healthy population, and a frequent T to C transition was found 1,327 bp upstream of the transcription starting site ($-1327T/C$). Individuals homozygous for the $-1327T/C$ genotype showed shorter telomere length in their peripheral leukocytes compared with the $-1327T/T$ and $-1327T/C$ genotypes.

In this study we report for the first time a role of $1327T/C$ $hTERT$ genetic variants in cancer prognosis and clinical outcome of NSCLC patients. So far, this genetic variant has been associated with telomerase activity, telomere length, and coronary artery disease development (28–30).

In this study, our results indicate an influence of the telomerase genetic variants in overall survival of NSCLC patients. Multivariate Cox regression analysis indicated an increased overall survival for T-carrier patients, when compared with CC genotype, after adjustment for tumor histologic type, stage, smoking status, age, and gender (HR, 0.52; 95% CI, 0.35−0.77; $P = 0.001$). The median estimated cumulative survival in T-carrier patients of 26.5 months was significantly higher compared with that of CC patients of 19.3 months.

This difference was more evident regarding nonepidermoid NSCLC histologic type, with the median estimated cumulative survival of 29.8 months in T-carrier patients being significantly higher compared with the 19.3 months of CC patients (HR, 0.46; 95% CI, 0.27−0.78; $P = 0.004$). Matsubara et al. (28) showed that the $-1327T/C$ polymorphism within the $hTERT$ promoter region has functional roles: the $-1327T$ sequence is associated with higher transcriptional activity, lack of age-dependent telomere shortening, longer telomere length, and telomerase activity. The relationship of the $-1327T/C$ polymorphism to telomere shortening, telomere length, and telomerase activity was found in normal peripheral leukocytes. Transcriptional regulation of $hTERT$ has a key role in telomerase activity and telomere shortening. Approximately 25% higher promoter activity in the $-1327T$ sequence was found compared with the $-1327C$-sequence, and the T allele was strongly associated with longer telomere length. Thus, the $hTERT$ T allele with higher $hTERT$ transcriptional activity is associated with more effective extension of the telomeric end during cell division. Another study found an overrepresentation of the $-1327T/C$ genotype in patients with coronary artery disease compared with controls, presenting shorter telomeres compared with other patients with alternative genotypes, indicating that...
a subgroup of patients is more prone to telomere shortening (29).

Tumors with excessive telomere alterations are therefore likely to possess the most extensive phenotypic variability and have the greatest probability of containing cells capable of invasion, extravasation, and metastasis, i.e., an aggressive tumor phenotype. Numerous groups have hypothesized that altered telomere length could predispose cells to gain the necessary properties to metastasize and cause recurrent disease, and thereby be a predictor of clinical outcome (36). Although not entirely consistent on the underlying mechanisms, several studies have indicated that telomere alterations are associated with parameters of clinical outcome in patients with lung cancer (37–39). A recent study (39) indicated a significant poor clinical outcome in NSCLC patients presenting telomere shortening, a finding that emerged as an independent prognostic marker in multivariate analysis. Therefore, considering that most NSCLCs display telomerase activity (36) and may be a promising molecular marker of treatment response in these patients. Furthermore, because telomerase has been associated with poor prognosis in NSCLC patients, and the hTERT T allele correlated with telomere elongation, this genetic variant may confer an advantage in treatment response in these patients. Therefore, because cisplatin mechanism of action involves aduct formation, the modulation of long telomeres through hTERT T variant may originate a more available and easier target for the cytotoxic activity of this compound, strengthening cisplatin activity and conferring an improved response of NSCLC patients presenting the long telomere variant.

Recently, telomerase has been intensively studied as a target for novel cancer gene therapy and therapeutics (43). Our findings that hTERT genetic variants, by modulating telomere length, may confer an advantage in chemotherapy response, according to different types of NSCLC, suggest that patients with long telomeres could have better responses to telomerase-based therapies. The assessment of telomerase genetic variants could supplement prognosis of survival in the course of NSCLC and may be a promising molecular marker of treatment response in these patients. Platinum and taxol compounds play a central role in cancer chemotherapy, and although treatment is limited by side effects, they continue to have widespread application. One of the main aims of clinical or hazard of 5.02 compared with tumors with greater telomere content (40). Moreover, studies in breast cancer have shown that telomere attrition is associated with parameters of increased risk and poor outcome, with low telomere content conferring an adjusted relative hazard of 4.43 (36, 41).

Because hTERT reactivation is a mechanism for cancer cells to avoid senescence (42) and the latter could be induced by chemotherapy, the predictive value of hTERT genetic variations for benefit to first-line chemotherapy needs evaluation. Because short telomeres have been associated with poor prognosis in NSCLC patients, and the hTERT T allele correlated with telomere elongation, this genetic variant may confer an advantage in treatment response in these patients. Furthermore, because telomerase mechanism of action involves aduct formation in the telomere unit, the modulation of long telomeres through hTERT T variant may originate a more available and easier target for the cytotoxic activity of this compound, strengthening cisplatin activity and conferring an improved response of NSCLC patients presenting the long telomere variant.

Recently, telomerase has been intensively studied as a target for novel cancer gene therapy and therapeutics (43). Our findings that hTERT genetic variants, by modulating telomere length, may confer an advantage in chemotherapy response, according to different types of NSCLC, suggest that patients with long telomeres could have better responses to telomerase-based therapies.

The assessment of telomerase genetic variants could supplement prognosis of survival in the course of NSCLC and may be a promising molecular marker of treatment response in these patients. Platinum and taxol compounds play a central role in cancer chemotherapy, and although treatment is limited by side effects, they continue to have widespread application. One of the main aims of clinical or
Translational research in cancer is the search for genetic factors that could foresee treatment outcomes, in biological activity and toxic effects. This genetic analysis might allow the selection of patients who would have the greatest benefit from chemotherapy. Furthermore, a better knowledge of the underlying molecular profile of the host and the tumor will facilitate screening for lung cancer susceptibility and tailoring of chemotherapy in individual patients, choosing those most likely to respond, adjusting doses more precisely in order to reduce less adverse effects, and establishing safety profiles based on individual genetic analyses.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References


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Clinical Cancer Research

Prognostic Significance of Telomerase Polymorphism in Non–Small Cell Lung Cancer

Raquel Catarino, António Araújo, Ana Coelho, et al.


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