Molecular and Cellular Endocrinology 399 (2015) 288–295

Contents lists available at ScienceDirect

Molecular and Cellular Endocrinology

journal homepage: www.elsevier.com/locate/mce

Telomerase in differentiated thyroid cancer: Promoter mutations, expression and localization

Marina Muzza [a,b,1], Carla Colombo [a,b,1], Stefania Rossi [c], Delfina Tosi [d], Valentina Cirello [a,e], Michela Perrino [a,b], Simone De Leo [a,b], Elisa Magnani [f,g], Elisa Pignatti [f,g], Beatrice Vigo [a,b], Manuela Simoni [f,g], Gaetano Bulfamante [c,d], Leonardo Vicentini [h], Laura Fugazzola [a,e,*]

[a] Endocrine Unit, Fondazione IRCCS Ca' Granda, Milan, Italy
[b] Department of Clinical Sciences and Community Health, University of Milan, Italy
[c] Division of Pathology, San Paolo Hospital, Milan, Italy
[d] Department of Health Sciences, University of Milan, Italy
[e] Department of Pathophysiology and Transplantation, University of Milan, Italy
[f] Azienda USL of Modena, Italy
[g] Department of Biomedicine, Metabolism and Neural Sciences, University of Modena and Reggio Emilia, Italy
[h] Endocrine Surgery Unit, Fondazione IRCCS Ca'Granda, Milan, Italy

ARTICLE INFO

Article history:
Received 3 July 2014
Received in revised form 16 October 2014
Accepted 21 October 2014
Available online 27 October 2014

Keywords:
TERT
Outcome
BRAF
RAS
Genomics
Epidemiology

ABSTRACT

Telomerase-reverse-transcriptase (*TERT*) promoter mutations have been recently described in tumors. In the present large series, *TERT* mutations were found in 12% of papillary thyroid cancers (PTCs) and in 14% of follicular thyroid cancers (FTCs), and were found to significantly correlate with older age at diagnosis and poorer outcome. Interestingly, the prognostic value of TERT mutations resulted to be significantly stronger than that of $BRAF^{V600E}$. Moreover, the outcome was not different among tumors with isolated *TERT* mutation and those with coexistent mutations (*TERT/BRAF* in PTCs or *TERT/RAS* in FTCs). *TERT* rs2853669 polymorphism was found in 44.4% of tumors. At WB, TERT was significantly more expressed in tumors than in normal samples, being the highest levels of expression recorded in *TERT* mutated cases. At IHC, in tumors and in metastatic lymph-nodes TERT staining was significantly higher in the cytoplasm than in the nucleus, whereas in normal tissue the degree of staining did not differ in the two cellular compartments.

In conclusion, *TERT* mutations were shown to strongly correlate with a poorer outcome in differentiated thyroid tumors, and neither *BRAF* nor *RAS* mutation were found to confer an additional effect in the disease persistence. TERT protein was found to be more expressed in neoplastic than in normal tissues, and to display a different cellular localization, suggesting that it could contribute to thyroid cancer progression by mechanisms taking place in the cytoplasm.

1. Introduction

Telomerase is a ribonucleoprotein polymerase that maintains telomere repeat TTAGGG at the ends of chromosomes, and consists of a protein component with reverse transcriptase activity, TERT, and a RNA component which serves as a template (Harrington et al., 1997). Telomerase expression is normally repressed in postnatal somatic cells, resulting in the progressive shortening of telomeres which leads to growth arrest (replicative senescence). Most cancer cells constitutively express telomerase, thus allowing telomeres maintenance and unlimited cellular proliferation (Skvortzov et al., 2009). It has been shown that the genetic mechanisms responsible for telomerase reactivation in tumors could include engagement of *TERT* alternative splicing, *TERT* gene amplification, and mutations in the *TERT* promoter (Skvortzov et al., 2009). In particular, the mutations –124 C > T (C228T) and –146 C > T (C250T) in the *TERT* promoter have been shown to occur in several tumors (Killela et al., 2013; Liu et al., 2013). These mutations generate *de novo* consensus binding motifs for E-twenty-six (ETS) transcription factors and in cancer cell lines have been shown to increase the transcriptional activity of the TERT promoter by two- to six-folds (Huang et al., 2013).

These two *TERT* promoter mutations have been very recently reported in thyroid cancer cell lines and in thyroid tumors, either well differentiated (DTCs) or poorly differentiated and anaplastic (Landa et al., 2013; Liu et al., 2013, 2014; Melo et al., 2014; Vinagre et al.,

* Corresponding author. Endocrine Unit-Fondazione IRCCS Ca' Granda, Via F. Sforza, Milan 35-20122, Italy. Tel.: +39 02 55033498; fax: +39 02 50320605.
E-mail address: laura.fugazzola@unimi.it (L. Fugazzola).
[1] Equal contributors.

http://dx.doi.org/10.1016/j.mce.2014.10.019
0303-7207/

2013; Wang et al., 2014). In DTCs, an association between these *TERT* mutations ($TERT^{MUT}$) and older age at diagnosis, male gender and tumor size (Liu et al., 2014; Melo et al., 2014; Vinagre et al., 2013), and a correlation with a reduced progression free survival and overall survival have been shown (Liu et al., 2013; Melo et al., 2014). Discordant data are available about the coexistence of *TERT* and *BRAF* or *RAS* mutations. In particular, $TERT^{MUT}$ were found to be significantly more frequent in $BRAF^{V600E}$ than in $BRAF^{WT}$ papillary thyroid cancers (PTCs) (Liu et al., 2013; Melo et al., 2014) and in RAS^{MUT} than in RAS^{WT} follicular thyroid cancers (FTCs) (Wang et al., 2014), but these data were confirmed only for advanced thyroid tumors in another series (Landa et al., 2013). The clinical impact of the association is still controversial and indeed, the coexistence of *TERT* and *BRAF* mutations in PTCs was found to correlate with more aggressive features in a Chinese series (Liu et al., 2014). On the other hand, in a series from Portugal, no differences in the outcome were noted between $TERT^{MUT}/BRAF^{MUT}$ and $TERT^{MUT}/BRAF^{WT}$ cases, indicating that *BRAF* per se does not have an addictive impact in the disease persistence (Melo et al., 2014).

Very scarce, contradictory and not reliable data are available about TERT expression and cellular localization in the normal and neoplastic thyroid gland (Capezzone et al., 2009). Moreover, at the thyroid level no data exist about the possible modifying role of a common *TERT* polymorphism (rs2853669) located within a ETS binding site and shown in bladder cancer to be able to modify the effect of –124 C > T (C228T) and –146 C > T (C250T) mutations on survival and recurrence (Rachakonda et al., 2013).

In the present study, we performed a comprehensive evaluation of *TERT* promoter, $BRAF^{V600E}$ and N- and H-*RAS* mutations in a large series of sporadic and familial tumors and in metastatic lymph-nodes. The allelic frequency of rs2853669 SNP was also studied for the first time in thyroid cancer. Moreover, the expression and the localization of TERT was investigated by Western blot (WB) and immunohistochemistry (IHC).

2. Materials and methods

2.1. Patients tissue samples

A large series of 254 primary thyroid tumors were included in this study. In particular, the following histologic types were analyzed: 182 PTCs (143 conventional, 34 follicular, 2 oncocytic and 3 diffuse sclerosing variants), 58 FTCs (42 conventional and 16 Hurtle variants), and 14 MTC. In addition, 3 diffuse toxic goiters, 3 multinodular goiters and 8 lymph-nodal metastases were studied. Moreover, 10 familial PTCs (fPTC) cases were included and analyzed for *TERT* mutations at the germline level.

2.2. Histological classification and outcome definition

All specimens were reviewed by a senior pathologist (S.R.) to confirm the diagnosis. Tumors were classified and staged according to the 7th edition of the TNM staging (Compton et al., 2012). Criteria used to identify remission or persistent/recurrent disease were drawn on the bases of the European and American guidelines for the management of differentiated thyroid cancer (Cooper et al., 2009; Pacini et al., 2006) and have been previously reported in detail (Perrino et al., 2009).

2.3. Nucleic acids extraction

Tissues were collected during surgery, immediately frozen and stored at –80 °C. To ensure a pure tumor tissue isolation in tumors <1.5 cm, microdissection was performed. In larger tumors, the core of the neoplastic nodule was dissected macroscopically. DNA was extracted from tumor tissues or peripheral leukocytes by means of commercial kits (Puregene® Core Kit A, Qiagen, Germantown, MD, USA). All procedures performed for handling the tissues were approved by each Hospital Ethics Committee. An informed consent was obtained from all screened subjects.

2.4. Molecular analysis

The *TERT* proximal promoter was amplified from tissue and blood DNA using the primers TERTPforward: 5′-AGTGGATTCGCG GGCACAGA-3′ and TERTPreverse: 5′-GCAGCGCTGCCTGAAACTC-3′. Exon 15 of the *BRAF* gene was analyzed using specific intronic primers, as previously described (Fugazzola et al., 2004). Exon 2 of N-*RAS* was PCR amplified by means of newly designed specific intronic primers, forward: 5′-ACCTTGGCAATAGCATTGCAT-3′ and reverse: 5′-TAGTGTGGTAACCTCATTTCC-3′, whereas exon 2 of H-*RAS* was PCR amplified using previously described primers (Moura et al., 2011). PCR products were purified and directly sequenced (ABI 3130, Perkin Elmer Applied Biosystem, Foster City, CA).

2.5. Immunohistochemical studies

Paraffin blocks, including 19 PTCs, 5 FTCs and corresponding contralateral normal tissues, and 8 lymph-nodal metastases, were selected according to the following major criteria: (a) good morphology and (b) preservation of follicular structures (extensive fibrotic or hemorrhagic areas were excluded). From each paraffin block, 3 μm sections were obtained and tested for human TERT reactivity by means of a specific rabbit polyclonal antibody (Rockland Immunochemicals Inc. Gilbertsville, PA) at 1:2000 dilution, using an immunoperoxidase technique. The reaction was detected by Novolink Max polymer detection system (Novocastra Laboratories L.T.D., Leica Microsystem, Nussloch, Germany). In order to test the specificity and sensitivity of the antibody, control neoplastic tissues (gliomas) were also stained. The degree of positive staining for TERT was defined based on both the intensity of the staining and the percentage of stained cells. In particular, the intensity was defined as negative (0), low (1), medium (2) and high (3), whereas the percentage of stained cells were defined as none (0), 1–20% stained (1), 20–50% stained (2) and >50% stained (3).

2.6. Protein extraction and Western blotting

Briefly, thyroid tissue was homogenized in lysis buffer (Cell Signaling Technology, Inc., Danvers, MA), supplemented with protease inhibitors (Roche, Basel, CH) and immunoblotting was performed with a rabbit anti-human TERT antibody (Rockland Immunochemicals Inc. Gilbertsville, PA) at 1:1000 dilution, and with an anti-actin antibody (Novus Biologicals, Littleton, CO) or anti-GAPDH antibody (Ambion, Life Technologies, Foster City, CA). Chemiluminescence was detected using the Chemi-Doc-IT Imaging System (UVP, Upland, CA, USA). Band intensities were analyzed with the image analysis program NIH ImageJ and the TERT signal was normalized to actin signal.

2.7. Statistical analysis

Correlations between clinical features including outcome and genetic alterations were evaluated by means of Fisher's, $\chi 2$ test or *t*-test, as appropriate. Multivariate analysis of prognostic variables was carried out by stepwise logistic regression. Statistical significance was defined as $P < 0.05$. All tests were performed using the Version 13.2.2 of the MedCalc Software (B-8400 Ostend, Belgium).