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pTERT C250T mutation: A potential biomarker of poor prognosis in metastatic melanoma

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ABSTRACT

Melanoma is the most aggressive form of skin cancer and the leading cause of death from cutaneous tumors. Several studies have associated alterations in the *TERT* promoter region (*pTERT*) with gene overexpression, aggressiveness and poor prognosis of the disease. The aim of this study was to clarify the role of *pTERT* molecular status in paired samples of primary melanoma and metastasis using tissue and plasma to establish a correlation with disease progression and survival.

A total of 88 FFPE tissue samples from 53 patients with advanced melanoma were analyzed. Of these, 35 had paired samples. We also examined cfDNA samples from plasma of 25 patients. We detected a good correlation between primary tumors and metastases in *pTERT* mutation and methylation status. We were also able to identify *pTERT* mutations in plasma samples that correlated with mutational status in tissue samples. Interestingly, the C250T mutation was associated with worse survival and higher *TERT* mRNA expression, compared to the other most common mutation: C228T. In addition, hyper-methylation of the promoter region seems to be related to the progression of *pTERT* wild type (WT) patients. These results suggest that *TERT* gene alterations plays an important role during tumor progression, with the detection of the C250T mutation in tissue and plasma as a potential biomarker of poor prognosis in patients with advanced melanoma.

Abbreviations: FFPE, fixed and formalin paraffin embedded; cfDNA, circulating free DNA; WT, wild-type; *pTERT*, *TERT* promoter; ddPCR, droplet digital PCR; MS-PCR, Methylation specific PCR; RT-qPCR, Reverse transcription quantitative PCR.

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1. Introduction

The discovery of targetable oncogenic driver mutations (*BRAF* 40–60% and *NRAS* 15–20%) in most melanomas, which are consistent during tumor progression, has led to the emergence of specific effective inhibitors with good responses in clinical practice [1–3]. However, a high percentage of these patients eventually develop resistance and a proportion of patients lack known driver mutations, making the identification of new biomarkers and therapeutic targets a priority for researchers.

Between 2013 and 2014, several reports discovered two common recurrent mutations in the promoter region of the *TERT* (telomerase reverse transcriptase) gene, detectable at a high frequency in primary melanomas and cell lines derived from metastatic melanomas [4–7]. Both C > T mutations, C228T and C250T, corresponding to the positions −124 and −146 from the ATG start site, respectively, contain a consensus binding site for E-twenty-six (ETS) transcription factors, which generates a 2–4–fold increase in *TERT* transcriptional activity. In addition, several authors have associated these mutations with aggressive tumor progression and poor survival [5–13]. Recent studies have also shown the presence of *pTERT* mutations in plasma samples from total peripheral blood in patients with metastatic melanoma using highly sensitive methods (digital PCR, ddPCR) [14–16].

In addition to *pTERT* mutations, other genetic and epigenetic alterations appear to be involved in *TERT* overexpression during tumor development, including promoter hypermethylation, chromosomal rearrangements, amplifications and transcriptional fusions [17,18]. Unlike most genes, *TERT* expression is upregulated when a specific region of the promoter is hypermethylated, avoiding binding of the repressor CCCTC-binding factor (CTFC) [19]. Hypermethylation occurs in the *TERT* hypermethylated oncological region (THOR) located immediately upstream of the *TERT* core promoter. Several studies have shown that THOR is hypermethylated in different tumor types and is associated with poor survival [17,20,21]. However, conflicting results have been published on this topic; therefore, further studies are required to clarify the effect of *TERT* regulation on tumor progression, especially in metastatic melanoma [22,23].

The goal of this study was to analyze the effect of *pTERT* mutation and/or hypermethylation in *TERT* gene expression in paired samples of melanoma patients (primary tumors, metastases and plasma) to define the role of *TERT* in the progression and outcome of patients in advanced stages of disease.

2. Materials and methods

The detailed protocols are described in Supplemental Materials and Methods (Additional file S1).

2.1. Patients and tumor samples

We studied 88 FFPE tumors from 53 patients with advanced melanoma (stages III and IV, according to American Joint Committee

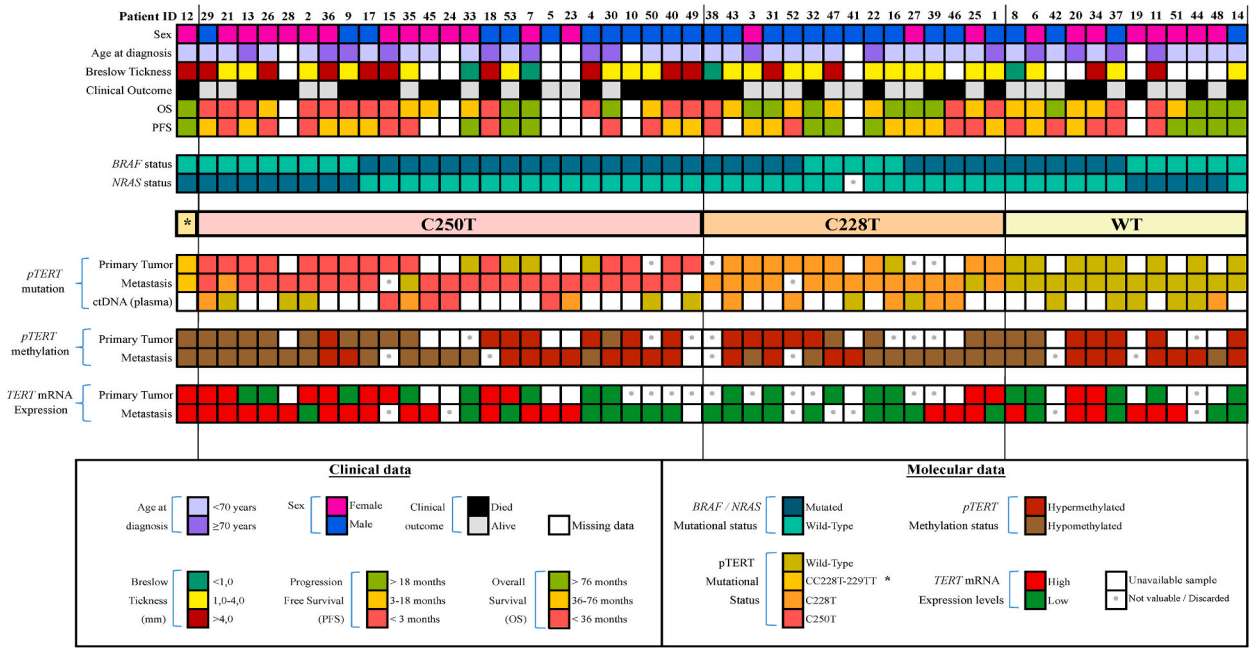

Fig. 1. Collection of the clinical, histopathological and molecular data of 53 melanoma patients included in the study. All data are grouped according to the mutational status of *pTERT* (mutated in C250T, C228T or C228-229 TT; or wild-type). Each patient is represented in a column, which includes the results obtained in their paired FFPE samples of primary melanoma and metastasis; and, if appropriate, their liquid biopsy (plasma).

on Cancer, AJCC [24]), obtained before treatment. Representative tumoral regions were selected by a pathologist's review of the H&E-stained slides. After review, we studied 35 patients with paired samples available of primary melanoma and metastases showing good quality for molecular analyses. These patients had several metastases in different anatomical locations but only one metastases per patient was tested. Plasma samples from the patients treated in 12 de Octubre Hospital (25 of 53 patients) were collected after recurrence and before systemic treatment. In addition, 13 benign samples (7 normal skin and 6 benign melanocytic nevus) were included as control in our melanoma sample cohort. The samples were collected from the Biobank of Hospital 12 de Octubre. The clinical, histological, and molecular data of the patients are detailed in Fig. 1 and Table S1.

2.2. Pyro-sequencing

Pyrosequencing of DNA tissue samples was used to detect *TERT* $^{-124[C > T]}$ (C228T) and *TERT* $^{-146[C > T]}$ (C250T) mutations. Amplifications were carried out using the PyroMark PCR Kit (Qiagen, Hilden, Germany) and pyrosequencing was performed using a PyroMark Q24 system (Qiagen) following the manufacturer's protocol (Table S2). A negative control (*pTERT* wild-type [WT]), two positive controls (C228T and C250T), and a non-template control (NTC) were used for each run. Discordant results between paired tissue samples (primary tumor and metastases) were confirmed by Sanger sequencing under the same conditions as those used in the pyrosequencing analysis.

2.3. Droplet digital PCR (ddPCR)

ddPCR in cfDNA from plasma samples was used to detect *TERT* $^{-124[C > T]}$ (C228T) and *TERT* $^{-146[C > T]}$ (C250T) mutations using a QX200 droplet reader (Biorad, Hercules, California) following the manufacturer's protocol. A negative control (*pTERT* WT), two positive controls (C228T and C250T), and a NTC were used for each run. This technique was also used to confirm the discordant results between FFPE tissue and plasma samples.

2.4. Quantitative PCR

Quantitative PCR from DNA tissue samples was used to detect *BRAF* and *NRAS* mutations by using the automated Idylla System technology (Biocartis, Mechelen, Belgium) following the manufacturer's protocol. Only one sample per patient (primary tumor or metastasis) was analyzed, as these are driver mutations that are consistent during melanoma progression and only change after inhibitor treatment [3].

2.5. Methylation specific PCR (MS-PCR)

DNA tissue samples treated with EpiTect Bisulfite Kit (Qiagen) was amplified by MS-PCR to evaluate the methylation status of four CpG islands which accurately represent the mean methylation of the region described as THOR (hypermethylated TERT oncology region) [20] (Fig. S1). MS-PCR was carried out according to the protocol described by Xin Y et al. [25] (Table S3). Non-bisulfite-treated DNA, methylated DNA (Merck, Darmstadt, Germany), unmethylated DNA (Qiagen) and NTC were used in each run as controls. PCR products were analyzed in a 3% agarose gel (Fig. S2) and the intensity of the amplified bands for methylation and non-methylation primers was measured by densitometry using the ImageJ program (version 1.53e for Windows, NIH, Bethesda, Maryland). Double standardization was performed with the values obtained. First an internal normalization between the methylation and non-methylation bands belonging to each sample, and then an external normalization with the mean of the normal skin samples; thus obtaining a numerical value for *pTERT* methylation status. From these values, the samples were distributed in two groups: hypermethylated and hypomethylated, using the median of normalized *pTERT* methylation levels of melanoma samples as a cut-off point.

2.6. Reverse transcription quantitative PCR (RT-qPCR)

RT-qPCR of mRNA from FFPE samples was performed to evaluate *TERT* expression levels in a relative quantitative PCR using a 7500 Fast Real-Time PCR System (Thermo Fisher Scientific, Waltham, Massachusetts) following the manufacturer's protocol. *GAPDH* was used as an endogenous control. Because *TERT* mRNA expression levels were undetectable in tissue samples of normal skin and benign melanocyte nevus, normalization of melanoma expression was carried out only with the expression of endogenous *GAPDH* using the ΔCT method. From the obtained values, the samples were distributed in two groups: high and low expression, using the median of the normalized *TERT* mRNA expression levels of the melanoma samples as a cut-off point.

2.7. Statistical and survival analysis

Comparative statistical analyses were performed using Chi-square (χ^2), ANOVA and Student's t-test. The effect size was estimated using Odds ratio (OR) and 95% confidence interval (CI). Survival studies were performed using Kaplan-Meier survival curves and Cox regression analyses. GraphPad Prism, GP (version 8.0.1 for Windows, San Diego, California), SPSS Statistics (version 21.0.0 for Windows, IBM, New York, USA) and R statistical analysis software (version 4.0.3 for MacOS, Vienna, Austria) were used to perform these analyses. Statistical significance was set at P value < 0.05.

3. Results

3.1. pTERT mutational status

We detected *pTERT* mutation in 69% (61/88) of the tumor samples, being C250T the most frequent mutation (Table 1). When comparing paired samples, *pTERT* mutational status was preserved in 77% (27/35) of patients between primary tumors and their metastases (χ^2 OR 16.0, 95% CI 2.56–100.08, p = 0.001). Altogether, eight of 35 patients had a discordant *pTERT* mutational status, of which 62.5% (5/8) acquired the mutation, 25% (2/8) lost it and 12.5% (1/8) change it in the metastatic tumor (Fig. 1, Table 2A).

We studied *pTERT* mutational status in plasma samples available in order to correlate the results obtained in tissue samples (Table 1). We were able to detect *pTERT* mutations in 52% (13/25) of plasma samples. In addition, *pTERT* mutational status was preserved in 56% (14/25) of paired melanoma samples between tissue (primary or metastatic) and plasma samples (χ^2 OR 2.67, 95% CI 1.09–6.52, p = 0.038) (Fig. 1, Table 2B).

3.2. BRAF and NRAS mutations based on pTERT mutational status

BRAF^{V600E} and *NRAS* mutation was detected in 62% (33/53) and 26% (14/53) of the patients, respectively (Table S1). After excluding patients with discordant results for the *pTERT* mutation (8/53) or non-valuable for *BRAF/NRAS* (1/53), we observed the coexistence of *BRAF* and *pTERT* mutations in 48% (21/44) of melanoma patients, of which 57% (12/21) showed C250T and 43% (9/21) C228T mutations. *NRAS* and *pTERT* mutations coexisted in 18% (8/44) of patients, 88% (7/8) of them corresponding to C250T mutation (Fig. 1, Table 3). Therefore, we observed that there was a statistically significant association between *BRAF* or *NRAS* mutational status, and the position of *pTERT* mutation (C250T or C228T), being the C250T mutation the most frequent in patients with *NRAS* mutation (χ^2 OR 2.0, 95% CI 1.34–2.98, p = 0.017).

3.3. pTERT methylation status

To clarify the effect of other molecular alterations on *TERT* regulation, we analyzed *pTERT* methylation levels in our patient cohort and control samples (Fig. 2 Fig.S2). Normal skin and melanocytic nevi showed lower *pTERT* methylation levels compared to melanoma samples (Fig. 2A). After excluding those samples who were discarded or had a non-valuable result (8/88), we used the median value of methylation levels detected in our cohort as a cut-off point to defined two methylation status in melanoma: hypermethylated (48%, 38/80) and hypomethylated (52%, 42/80) (Fig. 2B).

Interestingly, we observed high levels of methylation in 68% (15/22) of *pTERT* WT melanomas compared to 40% (23/58) of *pTERT* mutated samples. Therefore, melanoma tumors without *pTERT* mutations were more likely to have *pTERT* hypermethylation (χ^2 OR 3.2, 95% CI 1.15–9.09, p = 0.023) (Fig. 2C). However, we did not detect differences in *pTERT* methylation status associated with *BRAF* or *NRAS* mutation status (data not shown).

In addition, eighty-one percent of patients with a valuable result (25/31) maintained methylation levels between primary and metastases paired samples (χ^2 OR 17.33, 95% CI 2.92–103.02, p = 0.001). Thirty-nine percent (12/31) of the cases showed *pTERT* hypermethylation, whereas 42% (13/31) were hypomethylated in both lesions, with the remaining 19% (6/31) of discordant cases (Fig. 1).

3.4. TERT mRNA expression levels

After excluding those samples that were discarded or had a non-valuable result (12/88), the median of standardized expression levels in melanoma samples was used as a cut-off point to define two expression groups: high (49%, 37/76) and low (51%, 39/76) mRNA expression. Control samples lacked *TERT* mRNA expression (Fig. 3A). Fifty percent (26/52) of *TERT* mutated samples and 46% (11/24) of *TERT* WT samples showed high mRNA expression levels (no statistically significant difference was observed) (Fig. 3B). However, when analyzing each type of mutation independently, we observed that *pTERT* C250T mutation was associated with higher

Table 1
TERT promoter mutation frequencies in melanoma (primary, metastasis and plasma) and control samples analyzed by pyro-sequencing.

pTERT	Melanoma samples			Control samples	
	Primary[1] (n = 38)	Metastasis[1] (n = 50)	Plasma[2] (n = 25)	Normal skin (n = 7)	Nevus (n = 6)
WT	13 (34%)	14 (28%)	12 (48%)	7 (100%)	6 (100%)
Mutated	25 (66%)	36 (72%)	13 (52%)	–	–
C250T	15 (60%)	21 (58%)	4 (31%)	–	–
C228T	9 (36%)	14 (39%)	9 (69%)	–	–
CC228–229TT	1 (4%)	1 (3%)	–	–	–

[1] Sanger sequencing and droplet digital PCR (ddPCR) were used to confirm discordant results between paired samples from the same patient (primary vs metastasis; tissue vs plasma).
[2] *pTERT* mutational status in plasma samples was analyzed by ddPCR.
pTERT = *TERT* promoter.

Table 2
TERT promoter mutational status in paired samples from melanoma patients. **A.** Paired tissue samples of primary tumors and their metastases from 35 melanoma patients analyzed by pyro-sequencing and confirmed by Sanger sequencing. **B.** Paired samples of tissue tumors (primary or metastases) and plasma from 25 melanoma patients analyzed by droplet digital PCR (ddPCR).

A *pTERT* mutational status	Metastatic tumors			
	Wild Type (n = 10)	C250T (n = 15)	C228T (n = 9)	CC228-229 TT (n = 1)
Primary tumors				
Wild Type (n = 13)	**8 (23%)**	4 (11%)	1 (3%)	0 (0%)
C250T (n = 13)	1 (3%)	**11 (31%)**	1 (3%)	0 (0%)
C228T (n = 8)	1 (3%)	0 (0%)	**7 (20%)**	0 (0%)
CC228-229TT (n = 1)	0 (0%)	0 (0%)	0 (0%)	**1 (3%)**

B *pTERT* mutational status [1]	Plasma samples		
	Wild Type (n = 11)	C250T (n = 4)	C228T (n = 9)
Tissue samples [2]			
Wild Type (n = 6)	**5 (21%)**	0 (%)	1 (4%)
C250T (n = 11)	4 (17%)	**4 (17%)**	3 (13%)
C228T (n = 7)	2 (8%)	0 (%)	**5 (21%)**

[1] One patient (1/25) had to be excluded from the cross table because two different types of pTERT mutation were observed in their paired samples of primary and metastasis (C228T and C250T).
[2] pTE*RT* mutational status in tissue samples was analyzed by pyro-sequencing. Droplet digital PCR (ddPCR) were used to confirm discordant results between paired samples from the same patient (tissue vs plasma).
pTERT = *TERT* promoter.

Table 3
Coexistence of *pTERT* mutations and *BRAF/NRAS* driver mutations in tissue samples from 44 melanoma patients, after excluding patients with discordant results for *pTERT* mutation (8/53) or non-valuable for *BRAF/NRAS* (1/53).

Melanoma patients (n = 44)	*BRAF/NRAS* mutational status [2]		
	Wild Type (n = 4)	*BRAF* mutated (n = 27)	*NRAS* mutated (n = 13)
***pTERT* mutational status** [1]			
Wild Type (n = 12)	1 (2%)	6 (14%)	5 (11%)
C250T (n = 19)	0 (0%)	**12 (27%)**	**7 (16%)**
C228T (n = 12)	3 (7%)	**9 (21%)**	**0 (0%)**
CC228-229TT (n = 1)	0 (0%)	**0 (0%)**	**1 (2%)**

[1] pTERT mutational status was analyzed by pyro-sequencing. Sanger sequencing were used to confirm discordant results between paired samples from the same patient (primary vs metastasis).
[2] BRAF/*NRAS* mutational status was analyzed by Quantitative PCR (qPCR).
pTERT = *TERT* promoter.

levels of *TERT* mRNA expression in 59% (19/32) of cases, compared with 28% (5/18) of samples with high *TERT* mRNA expression and C228T mutation (χ^2 OR 3.8, 95% CI 1.09–13.26, p = 0.032; and *t*-test *P*-value = 0.041) (Fig. 3C).

In addition, we observed a trend toward to maintain *TERT* mRNA expression levels in patients with paired primary and metastatic melanoma samples, so that 65% (19/29) showed consistent levels (χ^2 OR 4.3, 95% CI 0.84–21.76, p = 0.071). We identified 31% (9/29) of high levels, 34.5% (10/29) of low *TERT* mRNA levels and 34.5% (10/29) with discordant results in paired samples (Fig. 1).

Although no significant association was detected between *pTERT* methylation and *TERT* mRNA expression levels (data not shown), we observed that 46% (21/46) and 73% (16/22) of melanomas with *BRAF* and *NRAS* mutations, respectively, showed high mRNA expression levels of *TERT*. However, none of the eight *BRAF/NRAS* WT samples showed high mRNA expression of *TERT*. Therefore, the presence of *NRAS* mutations is associated with higher *TERT* mRNA expression levels in melanoma (χ^2 OR 4.2, 95% CI 1.41–12.41, p = 0.007; and ANOVA *P*-value = 0.0007) (Fig. 4). Subsequently, a bidirectional analysis revealed that there was no interaction between the *NRAS* mutational status and the mutation type present in *pTERT* (C250T or C228T), so that both factors have an independent effect on *TERT* mRNA expression (two-way ANOVA P Interaction-value = 0.971).

3.5. Clinical and survival analyses

For clinical and survival analyses, patients previously mentioned who showed discordant results for *pTERT* mutation, methylation, and/or mRNA expression were not included. Those for which overall survival (OS) and progression-free survival (PFS) data were not available were also not included (Fig. 1, Table S1). Univariate Cox regression analyses of clinical variables in these series showed that age at diagnosis (<70 or ≥ 70 years) was significantly associated with OS in melanoma patients, whereas Breslow thickness (low, medium or high) was related to PFS (Table 4). We also observed that Breslow thicknesses greater than 1.0 mm appeared to be more frequent in those patients with *pTERT* mutations in their primary tumor (χ^2 OR 1.4, 95% CI 0.96–1.98, p = 0.006). While all patients (3/3) with a low Breslow index (<1.0 mm) lacked *pTERT* mutations, 74% (14/19) and 79% (11/14) of patients with medium (1.0–4.0

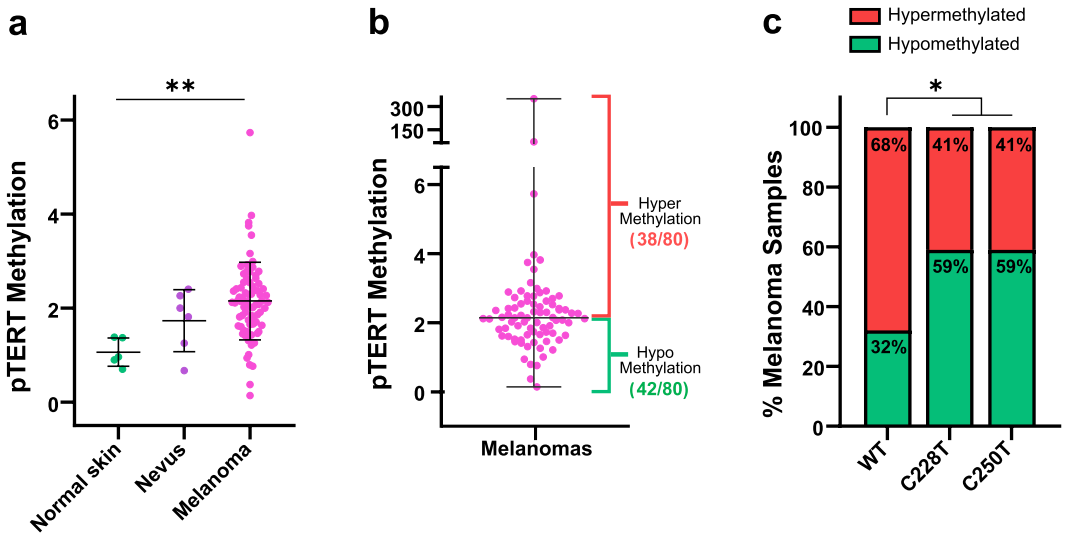

Fig. 2. ***pTERT*** methylation status correlate with *pTERT* mutational status. **A.** *pTERT* methylation normalized values with mean±standard deviation (SD) in normal skin (n = 5), nevus (n = 6) and melanoma samples (n = 78). *pTERT* methylation levels were obtained by performing a methylation-specific PCR (MS-PCR) and a subsequent double normalization, internal and external, using normal skin samples as the baseline. Control samples showed lower levels of *pTERT* methylation compared to melanoma samples. **B.** Distribution of hyper (48%, 38/80) and hypo (52%, 42/80) methylated samples using the median of the normalized methylation levels in our cohort as cutoff. **C.** Positive correlation between *pTERT* methylation and mutational status in melanoma samples from a Chi-square (χ^2) test (OR 3.2, 95% CI 1.15–9.09, p = 0.023). *pTERT* mutational status: mutated C250T (n = 34), mutated C228T (n = 22) and wild-type (n = 22). P value style GP: *P < 0.050, **P < 0.010, ***P < 0.001, and ****P < 0.0001.

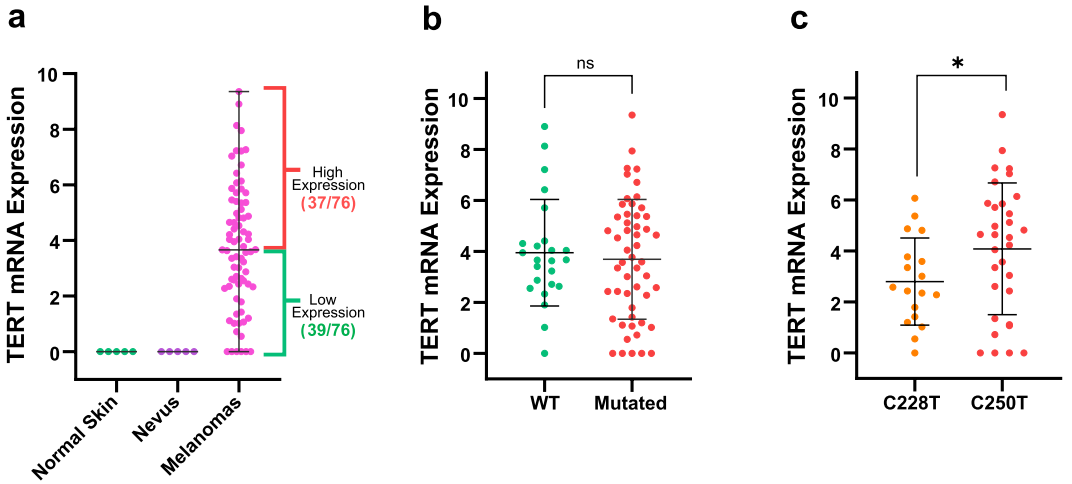

Fig. 3. ***pTERT*** **C250T/C228T mutations correlate with different levels of *TERT* mRNA expression. A.** Normalized values of *TERT* mRNA expression in normal skin (n = 5), nevus (n = 5) and melanoma samples (n = 76), detected from reverse transcription quantitative PCR (RT-qPCR). Control samples lack *TERT mRNA* expression. Distribution of high (49%, 37/76) and low (51%, 39/76) mRNA expression according to the median of the normalized expression levels in melanoma samples. **B.** Means±standard deviation (SD) comparison of *TERT* mRNA expression levels between melanoma tumors with *pTERT* wild-type (n = 24) and *pTERT* mutated (n = 52). Unpaired *t*-test no significant (ns) with *P*-value = 0.627. **C.** Means ±SD comparison of *TERT* mRNA expression levels between melanoma tumors with *pTERT* mutation C228T (n = 18) or C250T (n = 32). Unpaired *t*-test statistically significant with P value = 0.041; and Chi-square (χ^2) test OR 3.8, 95% CI 1.09–13.26, P value = 0.032. P value style GP: *P < 0.050, **P < 0.010, ***P < 0.001, and ****P < 0.0001.

mm) and high (>4.0 mm) Breslow index, respectively, showed the *pTERT* mutations, with the C250T being the most frequent mutation in thicker tumors (57%, 8/14) (Table S1, Fig. 1).

Subsequent univariate Cox regression analyses of the molecular variables revealed that *pTERT* mutational status (WT, C228T, or C250T) and *TERT* mRNA expression level (low or high) were significant predictors of clinical outcome and tumor progression. The effect on survival of C250T mutation and C228T mutation were analyzed separately, being the C250T mutation the only one

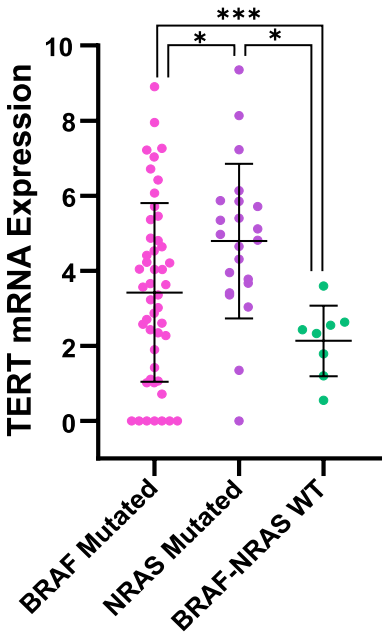

Fig. 4. Association between *NRAS/BRAF* status and *TERT* mRNA expression levels. Means±standard deviation (SD) comparison of *TERT* mRNA expression levels between melanoma tumors with *BRAF* mutated (n = 48), *NRAS* mutated (n = 22), or wild-type (WT) for both genes (n = 8). Variance analysis by ANOVA one way unpaired test with *P*-value = 0.0007. Multiple comparisons test: *NRAS* mutated vs *BRAF* mutated (*P*-value = 0.046); *BRAF* mutated vs WT for both genes (*P*-value = 0.031), and *NRAS* mutated vs WT for both genes (*P*-value = 0.0001). P value style GP: *P < 0.050, **P < 0.010, ***P < 0.001, and ****P < 0.0001.

Table 4
Univariate Cox Regression Analyses in 53 melanoma patients. Ten variables (3 clinical and 7 molecular) were studied separately for each analysis (PFS and OS).

Variable	HR	95% CI	P value*	P adjust [1]
Overall Survival (OS)				
Clinical data [2]				
Age at diagnosis	2.492	1.043–5.953	**0.040***	0.133
Sex	0.721	0.314–1.656	0.440	0.550
Breslow Tickness	1.648	0.779–3.486	0.191	0.347
Molecular data [3]				
BRAF mutational status	1.354	0.572–3.204	0.491	0.578
NRAS mutational status	0.884	0.322–2.424	0.810	0.853
pTERT mutational status	2.825	1.417–5.631	**0.003***	**0.030***
pTERT C250T mutation	5.854	2.134–16.056	**0,001***	**0.020***
pTERT C228T mutation	0.625	0.252–1.554	0.312	0.446
pTERT methylation status	0.771	0.300–1.983	0.589	0.853
TERT mRNA expression levels	2.744	1.004–7.504	**0.049***	0.122
Progression Free Survival (PFS)				
Clinical data [2]				
Age at diagnosis	1.611	0.647–4.015	0.306	0.471
Sex	0.421	0.159–1.115	0.082	0.182
Breslow Tickness	2.347	1.011–5.449	**0.047***	0.134
Molecular data [3]				
BRAF mutational status	2.016	0.793–5.124	0.141	0.282
NRAS mutational status	0.517	0.172–1.556	0.241	0.402
pTERT mutational status	3.387	1.529–7.505	**0.003***	**0.020***
pTERT C250T mutation	5.106	1.663–15.677	**0.004***	**0.020***
pTERT C228T mutation	1.040	0.388–2.784	0.938	0.938
pTERT methylation status	0.641	0.222–1.852	0.412	0.549
TERT mRNA expression levels	3.594	1.064–12.139	**0.039***	0.156

*Values of P < 0.05 were considered statistically significant.
[1] The p values were adjusted by Benjamini-Hochberg method (BH).
[2] Patients that did not have clinical and survival data available were not included.
[3] Patients who showed discordant results for *pTERT* mutation, methylation and/or mRNA expression were excluded.
HR= Hazard ratio; CI = Confidence Interval; *pTERT* = *TERT* promoter.

significantly associated with PFS and OS (Table 4). Accordingly, the Kaplan-Meier survival curves indicated that patients with the C250T mutation showed a worse OS (average of 36 months) and PFS (average of 5 months) than those with the C228T mutation (average OS 106 months, and PFS 23 months) or *pTERT* WT (average OS 223 months, and PFS 55 months) (OS log-rank test *P*-value = 0,001; PFS log-rank *P*-value = 0.004) (Fig. 5A and B). Furthermore, patients with high *TERT* expression levels showed a significantly worse OS (average of 51 months) and PFS (average of 8 months) than patients with low mRNA expression levels (OS, average of 88 months; PFS, 20 months) (log-rank *P*-value = 0,041; and *P*-value = 0.028) (Fig. 5C and D). However, after adjusting the p values by the Benjamini & Hochberg multiple comparison method, we observed that only *pTERT* mutational status was significantly associated with OS and PFS, where the C250T mutation showed the greatest effect on progression and outcome in advanced melanoma patients.

4. Discussion

We identified *pTERT* mutation in 66% and 72% of primary and metastatic melanoma samples, respectively, being C250T the most frequently mutation detected. Previous studies reported similar frequencies in metastatic and cell line samples. However, mutation frequencies were considerably lower in primary melanoma samples compared to those in our study [4–6]. This increase could support the association of *pTERT* mutation status with a more aggressive disease, since patients in our cohort are in higher stages of the disease (III and IV, according to AJCC) [6,7,9,10,12].

Additionally, we observed that *pTERT* mutation and methylation status were preserved in 77% and 81% paired tissues samples of primary melanomas and their corresponding metastatic, respectively. These results provide strong evidence that p*TERT* alterations occur early during the clonal expansion of tumor cells and are important for metastatic progression [5,26,27]. However, in contrast to previous studies in paired samples [28–30], we observed that more than half of the discordant cases for *pTERT* mutation (5 of 8 cases; 62%) were mutated in the metastatic lesion and not in the primary tumor. Accordingly, we observed that in discordant cases for mRNA expression, 70% (7/10) showed high levels in metastasis compared to primary lesion. These data support the hypothesis that *pTERT*

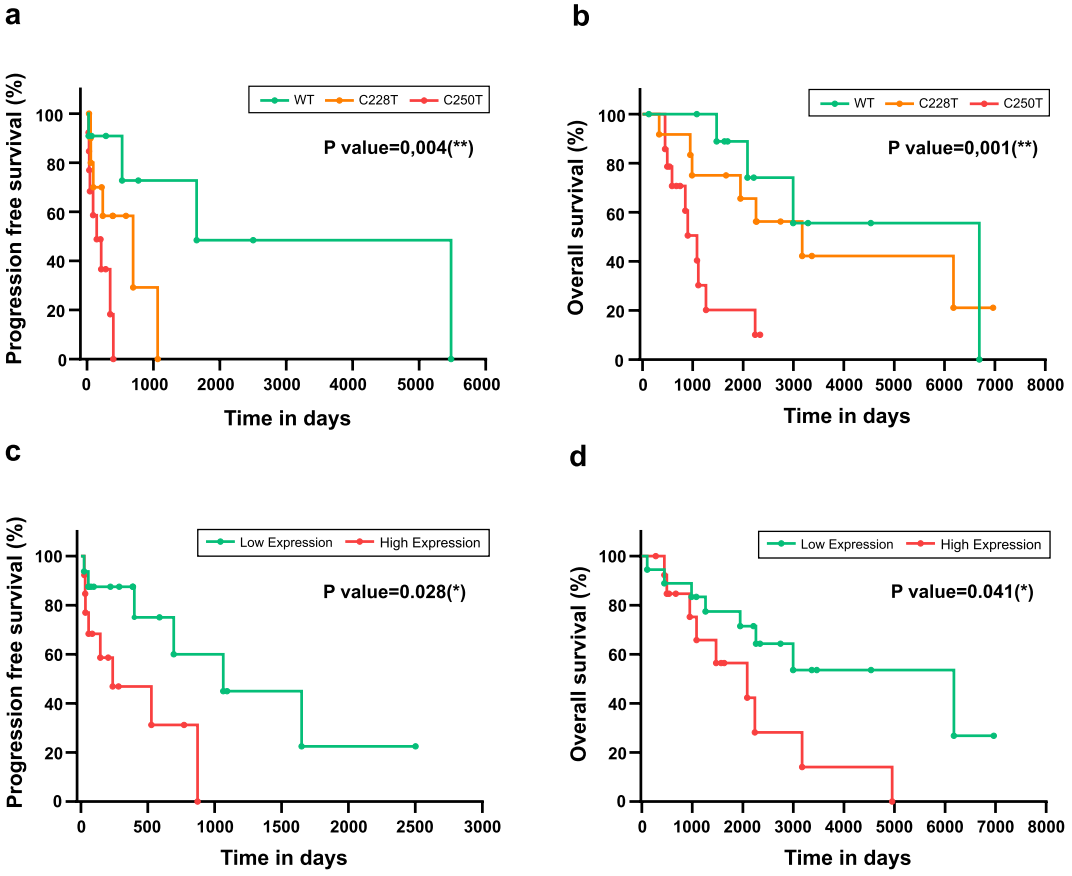

Fig. 5. Effect of *TERT* alterations in melanoma patients' survival. A. Kaplan-Meier overall survival (OS) analysis between melanoma patients with *pTERT* mutation C250T (n = 14), C228T (n = 12) and wild-type (n = 11). Log-Rank test *P*-value = 0.001. **B.** Kaplan-Meier progression free survival (PFS) analysis between melanoma patients with *pTERT* mutation C250T (n = 13), C228T (n = 11) and wild-type (n = 11). Log-Rank test *P*-value = 0.004. **C.** Kaplan-Meier OS analysis between melanoma patients with high *TERT* mRNA expression levels (n = 14) and low levels (n = 18). Log rank test P value = 0.041. **D.** Kaplan-Meier PFS analysis between melanoma patients with high *TERT* mRNA expression levels (n = 13) and low levels (n = 16). Log rank test *P*-value = 0.028. P value style GP: *P < 0.050, **P < 0.010, ***P < 0.001, and ****P < 0.0001.

mutations and gene overexpression are related to more aggressive disease behavior and may play a relevant role in the metastatic progression of stage III and stage IV lesions [6,7,9,12]. The failure to detect *pTERT* mutations in metastatic samples of discordant paired patients (2/8; 25%) may be due to the fact that these patients had more metastases in other anatomical locations, which were not tested for *pTERT* mutations. Another possibility is that metastatic tissue did not originate from the primary tumor we tested but from another unknown or occult primary tumor.

Similarly, we detected *pTERT* mutations in plasma samples that correlated with mutational status in tissue samples with similar proportions to those detected in previous studies (50% to 70%) [14,15]. This suggests that *pTERT* mutations could represent a valuable biomarker for monitoring of melanoma patients [14–16]. The inability to detect *pTERT* mutations in the plasma of discordant cases (7/11), can be explained by the variable tumor shedding into the bloodstream. While the presence of discordant *pTERT* mutations between plasma and tissue (4/11) can be explained by tumor heterogeneity in tissue samples [31,32]. Even so, considering that plasma detection rates of *pTERT* mutations are lower than those of other driver genes such as *BRAF* (>80%) [33–35], larger cohort studies would be needed to confirm its usefulness as a potential biomarker in melanoma. In addition, it would be useful to also analyze the driver mutations (*BRAF* and *NRAS*) in plasma samples to confirm that cfDNA of the samples is representative of the tumor. However, we obtained a limited quantity of cfDNA to perform these analyses.

We observed that *TERT* mRNA expression levels were present in melanoma samples and absent in control samples (normal skin and nevus), as previously described [36]. In contrast to previous reports, we did not find differences between the transcriptional expression levels shown by samples with *pTERT* mutated and those observed in samples with *pTERT* WT. Unlike ours, most of these previous reports were carried out with melanoma cell lines and not with patients [4,5,37], while others were performed only with young patients [11,21]. In our cohort, we observed high *TERT* mRNA expression levels in tumors with *pTERT* WT, which could be explained by the presence of other alterations such as hypermethylation, chromosomal rearrangements, amplifications or transcriptional fusions [17,18]. These alterations may not be activated by environmental factors in cell lines or not manifest in young patients, such as *pTERT* methylation which has been shown to increase with age [25]. Interestingly, if we stratified patients according to the type of *pTERT* mutation, we found a significant association between the C250T mutation and higher *TERT* mRNA expression levels compared to the C228T mutation; unlike previous studies in which they observed otherwise [38,39]. In 2015, Li et al. demonstrated that *pTERT* C250T mutation, unlike C228T, requires non-canonical NF-κB signaling in cooperation with ETS factors to efficiently activate *TERT* transcription in glioblastoma multiforme tumors (GMB) [40]. Knowing that *TERT* expression depends on a complex interlocking of genetic and epigenetic events, future functional studies should be carried out to explain the different role of C250T and C228T mutations in *TERT* transcriptional activation in melanoma.

Recent studies have reported the presence *pTERT* hypermethylation in *TERT*-expressing cancers demonstrating that it functions as an epigenetic mechanism of telomerase activation [20,21,41,42]. In our study, we detected higher *pTERT* methylation levels in melanoma samples than in control samples (normal skin and nevus), as previously described [17,22]. Additionally, *pTERT* WT samples showed higher methylation levels than the mutated samples. However, in contrast to previous studies [17], this finding was not statistically associated with higher mRNA expression levels, probably because the low proportion of WT samples in our cohort. Larger series of melanoma would be necessary to study the effect that hypermethylation can have on *TERT* overexpression independently of *pTERT* mutations. In addition, other studies suggest that although THOR hypermethylation may be a prevalent telomerase-activating mechanism in cancer, other epigenetic mechanisms, such as chromatin accessibility are necessary for *TERT* mRNA expression [22,43].

When we assessed the associations between *TERT* status with clinic-pathological features, we confirmed that the presence of *pTERT* mutations correlate with a higher Breslow index and worse prognosis, as indicated by previous studies [6,7,10,13,36]. In particular, we observed that C250T mutation was more frequent in melanomas with high Breslow thickness (>4 mm) and was also associated with worse progression and poor survival compared to C228T mutation and *pTERT* WT melanomas. In addition, we found a dismal prognosis in melanoma patients with high *TERT* expression levels. In 2013, Horn et al. found that the most common *pTERT* mutations present in melanoma, C228T and C250T, were mutually exclusive, concluding that both were functionally redundant [4]. However, recent studies have observed differences in their effect on patient survival and transcriptional activity. In contrast to our results, Andrés-Lencina et al. and Chang et al. observed that the C228T mutation was associated with adverse prognostic markers in melanoma and poorer survival, compared with patients with C250T mutation [39,44]. However, both studies have been carried out with a large majority of patients in early stages of the disease (stages I and II), while our cohort consists of melanoma patients in advanced stages (III and IV). This is consistent with the results obtained by Del Bianco et al. who analyzed samples of metastatic melanoma patients with mutated *BRAF*, in which they associated the *pTERT* C250T mutation with poorer prognosis and survival [45]. In the same line, Blateau et al. observed that the presence of the *pTERT* C228T mutation was a marker of good prognosis in patients *BRAF* mutated with markers of poor prognosis (presence of brain metastases and increased LDH) [46]. Therefore, we hypothesize that C250T and C228T *pTERT* mutations have different effects on disease progression and outcome. In particular, our results confirm that the presence of C250T mutation could be a significant predictor of poor survival and worse clinical outcome in patients with advanced melanoma (III and IV).

Finally, we observed the coexistence of mutations in *pTERT* and *BRAF* or *NRAS* in higher proportions than those reported in previous studies [6,9,10,47], probably due to melanoma advanced stages in our cohort. The coexistence of these mutations has been associated with increased tumor growth and worse prognosis of the disease [9,10,39,47,48]. In our cohort, we observed a significant association between the *BRAF* or *NRAS* mutation status and the position of the *pTERT* mutation (C250T or C228T). Additionally, we found that *NRAS* mutations have an independent effect on *TERT* mRNA expression. Accordingly, Manrique-Silva et al. observed in their patient cohort a pronounced effect of *NRAS* mutations on survival in combination with *pTERT* mutations [48]. Therefore, there appears to be a synergy between *BRAF/NRAS* and *TERT* in patients with melanoma, which could be explained by the activation of the *TERT* gene promoted by *MAPK* signaling [48,49]. However, further studies in larger series of melanoma samples are necessary to

clarify whether the coexistence of *BRAF* or *NRAS* and *pTERT* mutations affects melanoma progression and influences therapeutic strategies, as reported in previous studies [45,46,50–52].

Among the limitations of our study were the small size of our melanoma patients' cohort, as well as the inability to have plasma samples from all patients in these series. In addition, some patients came from other centers, so not all clinical data were available for statistical and survival analyses, therefore selection bias cannot be ruled out and generalizability is not guaranteed, so more prospective and larger studies are needed in the future.

In conclusion, our findings confirm the wide prevalence of *pTERT* mutations in metastatic melanoma and strongly suggest that the C250T mutation is more aggressive than C228T showing higher levels of *TERT* mRNA expression and worse outcomes in advanced stages of the disease. Future functional studies should be carried out to explain the role of the C250T mutation and to confirm its potential biological differences from C228T. Hyper-methylation of the promoter region seems to play a role in the progression of *pTERT* WT, but no definitive conclusion can be drawn from our limited series of cases. In spite of this, we found a strong positive correlation between paired samples of melanoma patients in *pTERT* mutational status and methylation levels, which supports that *TERT* alterations play an important role during tumor progression, having a prognostic value. In particular, detection of C250T *pTERT* mutation in tissue or plasma could be used as a potential biomarker of poor outcome and prognosis in patients with advanced melanoma. Further research in larger series of melanoma patients would be necessary to confirm these findings.

Declarations

Author contribution statement

Leyla Blanco García: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Yolanda Ruano: Conceived and designed the experiments; Wrote the paper.

Raquel Blanco Martínez-Illescas: Analyzed and interpreted the data.

Rocío Cubo and Paula Jiménez Sánchez: Performed the experiments.

Erica Riveiro Falkenbach: Conceived and designed the experiments.

Pablo Ortiz Romero: Contributed reagents, materials, analysis tools or data.

María Concepción Garrido, José Luis Rodríguez Peralto and Víctor Javier Sánchez-Arévalo Lobo: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Data availability statement

Data will be made available on request.

Ethics approval and consent to participate

This study was approved by the Research with Medicines Ethics Committee (CEIm by its acronym in Spanish) of 12 de Octubre Hospital, and all the patients provided written informed consent for inclusion in the study.

Consent for publication

Not applicable.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e18953.

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