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ORIGINAL ARTICLE

Human telomerase reverse transcriptase in papillary thyroid cancer: gene expression, effects of silencing and regulation by BET inhibitors in thyroid cancer cells

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Abstract

Purpose Mutations in TERT promoter have been detected in the more aggressive papillary thyroid cancers (PTCs). To elucidate the role of TERT as an eligible molecular target in these tumors, the expression of hTERT was analyzed in a series of PTCs and the effects of both pharmacological and RNA-interference-induced *hTERT* silencing were investigated in two human PTC cell lines (K1 and BCPAP).

Methods The expression levels of *hTERT* mRNA and protein were evaluated by real-time PCR and western blot assays, respectively. Effects of *hTERT* silencing on PTC cell lines were analyzed by MTT, migration and western blot assays. Pharmacological inhibition of *hTERT* was performed using two bromodomain and extra-terminal (BET) inhibitors, JQ1 and I-BET762.

Results *hTERT* expression results increased in 20 out of 48 PTCs, including tumors either positive or negative for the presence of *hTERT* promoter and/or *BRAF* mutations. In K1 and BCPAP cells, *hTERT* silencing determined a reduction in cell viability (~50% for K1 and ~70%, for BCPAP, vs control) and migration properties that were associated with a decrease of AKT phosphorylation and β-Catenin expression. Moreover, *hTERT* mRNA levels were down-regulated by two BET inhibitors, JQ1 and I-BET762, which at the same dosage (0.5 and 5 μM) reduced the growth of these thyroid cancer cells.

Conclusions These findings demonstrate that *hTERT* may represent an excellent therapeutic target in subgroups of aggressive PTCs.

Keywords Papillary thyroid cancer · siRNA anti-*hTERT* · phospho-AKT · BET inhibitors

Introduction

The prevalence of differentiated thyroid carcinomas (DTCs) has increased rapidly in the last years [1]. In general, DTCs have a good prognosis which is mainly due to the efficacy of radioactive iodine-based treatments of recurrent and metastatic diseases following surgical intervention [2]. However, in those DTCs unable to concentrate radioiodine and with a worse prognosis, alternative therapeutic approaches must be taken [3, 4]. Elucidation of the almost complete scenario of genetic abnormalities present in thyroid cancer has opened the way for novel personalized therapeutic strategies directed against specific molecular targets in cancer cells. For this purpose, the use of protein multikinase inhibitors, a targeted therapy currently approved for use in patients with radioiodine-refractory

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thyroid cancer, is still limited by the presence of intolerable side effects and the frequent development of resistance [5, 6]. Thus, the search for novel molecular targets is an ongoing challenge.

Telomerase reverse transcriptase (TERT), the catalytic subunit of telomerase, has attracted particular attention since it is constitutively active in many cancer cells, contributing to their unlimited proliferation rate [7]. Its action is mainly attributed to its ability in telomere maintenance, but other mechanisms have also been described to explain its contribution to tumorigenesis [8]. The role of *TERT* in thyroid cancer is suggested by the discovery of somatic mutations in the human *TERT* (*hTERT*) promoter in the majority of poorly differentiated (PDTC) and anaplastic thyroid cancers (ATC) [9–11] and, to a lesser extent, also in papillary thyroid cancer (PTC), especially those with a particularly aggressive behavior [12–14]. Overexpression of *hTERT* has also been described in human thyroid tumors [15, 16].

Many studies have demonstrated the role of epigenetics, i.e., the modulation of acetylation/methylation residues on histone tails [7, 17], in the regulation of *hTERT* expression in normal and cancer cells, highlighting novel epigenetic-related proteins as useful targets also for the treatment of those tumors expressing this protein [18, 19]. In this context, bromodomain and extra-terminal (BET) inhibitors, which are able to hinder gene expression interfering with bromodomain-based acetylation reading, have shown anti-proliferative effects in thyroid cancer cells [20, 21]. It has been elucidated that BET inhibition modulated *hTERT* expression in glioblastoma [22, 23], but no data are currently available on its regulation in thyroid cancer. As an alternative strategy to inhibit TERT activity in cancer cells, the use of oligonucleotides for *hTERT* silencing has been successfully adopted to hinder the growth of several types of cancer cells [24–27], and the same approach has been demonstrated to be effective even in experimental models of ATC [16, 28, 29].

In this study, we first analyzed the expression levels of TERT transcript and protein in a series of PTC tissues. We then investigated the effects of small-interfering RNA-mediated silencing of *hTERT* on the viability and migration properties of two human PTC cell lines (K1 and BCPAP), exploring the molecular mechanism associated with this action. Moreover, we tested the ability of two BET inhibitors to modulate *hTERT* expression and the viability of these cell lines.

Material and methods

Collection of thyroid tissues

Forty-eight patients with PTC were enrolled at the "Sapienza" University Hospital of Rome (Italy). Samples of tumor or normal tissue were collected and immediately frozen after thyroidectomy. PTC histotypes were determined by histopathological examination and included 35 classical type (cl), 8 follicular variants (fv), and 5 uncommon variants (others) (i.e., 2 oxyphilic, 2 mixed fv and cl, 1 trabecular). Samples with a tumor cellularity more than 60% were selected, as well as controlateral normal thyroid tissues in the absence of signs of inflammation or other types of disease. For each tumor, the risk of recurrence was classified as low (LR, 27.5%) or high/intermediate (H/IR, 62.5%) in accordance with the 2015 ATA Guidelines for the Management of Adult Patients with Thyroid Nodules and Differentiated Thyroid Cancer [30]. *BRAF* and *hTERT* mutational status was determined by Sanger sequencing, as previously described [16, 31]. Table 1 shows comparison of clinico-pathological features among the groups with different mutations (Table 1A) or between groups with lower or higher TERT expression levels (Table 1B). The present study was approved by the Policlinico Umberto I ethical committee of Rome (Rif. 4798/21.12.17). Consent has been obtained from each patient or subject after full explanation of the purpose and nature of all procedures used.

Extraction of RNA and gene expression studies

Total RNA was isolated from tissues and thyroid cell lines using TRIzol reagent (Thermo Fisher Scientific Inc., Waltham, MA, USA), according to the manufacturer's protocol [32]. For human thyroid tissue samples, 1 µg of total RNA was reverse-transcribed with a High Capacity cDNA Reverse Transcription kit (Thermo Fisher Scientific) and *hTERT* expression was quantified by Real Time PCR in a 7900HT Fast Real Time PCR System (Thermo Fisher Scientific), as previously described [16]. Each sample was run in duplicate. Final results were determined by the $2^{-\Delta Ct}$ method using *β-actin* as an endogenous control.

For cell lines, 300 ng of RNA were reverse transcribed to cDNA using random primers and Superscript III reverse transcriptase (Thermo Fisher Scientific). qPCRs were performed using Platinum Quantitative PCR SuperMix (Thermo Fisher Scientific) with the QuantStudio3 Real Time PCR system (Thermo Fisher Scientific). Pre-designed TaqMan Assays (probe and primer sets) for *TERT*, *Fibronectin1* (*FN1*), and *β-actin* were purchased from Thermo Fisher Scientific. Data analyses were carried out using SDS 2.4 software (Thermo Fisher Scientific). Final results were determined by the comparative $2^{-\Delta\Delta Ct}$ method using *β-actin* as endogenous control and the silencing control or vehicle-treated control as calibrator. Experiments were repeated at least three times and each sample was run in triplicate.

Table 1 Comparison of clinico-pathological features among the groups with different mutations (A) or between groups with lower or higher *hTERT* expression levels (B)

Characteristic	Patients	(A) Mutational Status					(B) *TERT* mRNA Expression Levels		
		BRAF and *hTERT*	*hTERT*	*BRAF*	*hTERT* and *BRAF* wild type	*p*	Lower	Higher	*p*
	N = 48	*N* = 4	*N* = 2	*N* = 25	*N* = 17		*N* = 28	*N* = 20	
Sex									
Female	30	2	1	17	10	0.84[a]	16	14	0.36[a]
Male	18	2	1	8	7		12	6	
Median Age at diagnosis	44.8	61	58.5	42	37	0.04[b]	39.5	47	0.09[b]
years (range)	(19–71)	(45–65)	(52–65)	(22–71)	(19–60)		(19–71)	(22–65)	
Histologic Variant									
PTC-cl	35	3	0	23	9	<0.0001[a]	21	14	0.14[a]
PTC-fv	8	1	0	0	7		6	2	
PTC-other	5	0	2	2	1		1	4	
Median Tumor Size, mm	15.5	16	45	12	12	0.08[b]	11.5	13	0.67[b]
(range)	(3–45)	(9–38)		(5–35)	(3–27)		(3–35)	(5–45)	
Tumor Foci									
Unifocal	38	3	1	19	15	0.56[a]	21	17	0.40[a]
Multifocal	10	1	1	6	2		7	3	
Extrathyroidal extension									
No	25	1	0	10	14	0.016[a]	18	7	0.04[a]
Minor	23	3	2	15	3		10	13	
Lymph node metastases									
No	31	3	0	15	12	0.48[a]	16	15	0.20[a]
Yes	17	1	1	10	5		12	5	
ATA Risk									
Low	18	1	0	5	12	0.0054[a]	12	6	0.36[a]
Intermediate/ High	30	3	2	20	5		16	14	

[a]Chi-square test

[b]Mann–Whitney test with Dunnett's multiple comparisons test

Thyroid cancer cell lines, hTERT silencing, and treatment with BET inhibitors

Two human PTC cell lines, K1 and BCPAP, were used for in vitro experiments, both characterized by BRAFV600E mutations, as well as C228T and CC229, 228TT mutations of the *hTERT* promoter, respectively [10, 33, 34]. Cells were grown in DMEM or RPMI 1640 medium (Thermo Fisher Scientific) supplemented with 10% fetal bovine serum (FBS, Thermo Fisher Scientific), penicillin (100 IU/ml), streptomycin (100 mg/ml), and amphotericin B (2.5 mg/ml) (Sigma Aldrich, Milan, Italy). Short tandem repeat profiling was performed to confirm the identity of cell lines. Cells were transfected with two different *hTERT*-specific siRNA (si*hTERT*) using Lipofectamine RNAiMAX (Thermo Fisher Scientific), according to the manufacturer's instructions [16]. K1 and BCPAP cells were plated in 6-well plates (60×10^3/well or 130×10^3/well, respectively) with a medium containing 10% FBS and reached 60–80% confluence at the time of transfection. In all experiments, the control is indicated as siCtrl and represents cells transfected with Stealth RNAi Negative Control Duplexes (Thermo Fisher Scientific). BET inhibitors JQ1 and I-BET762 were purchased from Cayman Chemical (Cayman Chemical, Ann Arbor, MI, USA) and dissolved in dimethylsulfoxide (DMSO, Sigma Aldrich). K1 and BCPAP cells were treated with either BET inhibitors (0.5 or 5 μM) or vehicle (DMSO) for 48 h.

Protein extraction and western blot analysis

Total proteins were extracted as previously described [35]. Twenty micrograms of protein extracts were run on a 9% SDS-PAGE gel and transferred to PVDF membrane (VWR, Milan, Italy), blocked with PBS/Triton/milk (PBS 1×, Triton 0.1 and 5% non-fat dry milk) and incubated overnight with affinity-purified anti-TERT, anti-β-Catenin, anti-Fibronectin1, anti-AKT, anti-phospho-AKT, anti-ERK, anti-phospho-ERK, anti-tubulin, or anti-GAPDH antibodies (Supplementary Table 1). The membranes were incubated with different concentrations of horseradish peroxidase-conjugated anti-rabbit or anti-mouse antibodies (Transduction Laboratories, Lexington, KY, USA) in PBS/Triton/milk (Supplementary Table 1). Western blot detection

system ECL Plus (Perkin Elmer, Monza, Italy) was used to visualize the proteins.

Analysis of cell viability and migration

K1 and BCPAP cell viability after *hTERT* silencing or treatment with BET inhibitors was evaluated by MTT assay [36]. Cells were seeded in 96-well plates at a density of 5×10^3. After treatments, the solubilized product was quantified with a microplate spectrophotometer (xMark, Biorad, Milan, Italy) at a wavelength of 540 nm and a reference wavelength of 690 nm. Results are expressed as a percentage over control (siCtrl or Ctrl).

Transwell inserts with 8 µm pores were used for cell migration assay (Costar, Euroclone, Milan, Italy). After *hTERT* silencing, 60×10^3 cells were plated in the upper chamber of the inserts suspended in serum-free medium containing 1% BSA. As a chemiotactic agent, 600 µL of medium containing 10% FBS were added in the bottom wells. After 6 h of incubation, unmigrated cells were removed with cotton swabs from the upper surface of the filters which were fixed and stained with Diff-Quick Stain (Bio Map, Monza, Italy). Finally, cells were counted using a microscope provided with an eyepiece and equipped with a counting grid. Results from the count of five random fields are expressed as percentages over control (siCtrl).

Statistical analysis

Data were analyzed by Chi-square test, Mann–Whitney test, one-way ANOVA followed by the Tukey–Kramer or Dunnett's multiple comparisons test, as indicated. All results are expressed as mean ± standard deviation (SD) and *p*-values lower than 0.05 were considered statistically significant. All statistical analyses were performed using GraphPad Prism version 5.0 statistical software (GraphPad Software Inc., San Diego, CA, USA).

Results

Expression levels of hTERT in thyroid tumor tissues

First, we analyzed *hTERT* expression levels in 48 tissues originating from patients affected by sporadic PTC. The characteristics of the patients are summarized and compared with the biological features (mutational status and *hTERT* expression) in Table 1. A statistically significant association between mutational status and median age at diagnosis, histological variant type, extrathyroidal extension, and ATA risk of recurrence was found (Table 1A). Figure 1a shows the $2^{-\Delta Ct}$ values of *hTERT* mRNA expression in our cohort of PTCs, as well as details on mutational status and ATA risk of each case. Twenty tumor tissues presented higher *hTERT* transcript levels than those detected in normal tissues. They included 14 out of 30 classified as ATA high or intermediate risk and 6 out of 18 classified as ATA low risk. No significant association was found between *hTERT* expression levels and all clinico-pathological features but extrathyroidal extension ($p = 0.04$) (Table 1B). As shown in Fig. 1A and Table 2, six of the PTCs carrying an *hTERT* promoter mutation showed the highest levels of *hTERT* expression. Four of them also had a *BRAF* mutation (three with *BRAFV600E* and one with *BRAFV600_K601>E*) in addition to an *hTERT* promoter mutation. Two tissues with only *hTERT* promoter mutations showed high expression levels of *hTERT*, as well as 10 samples with only *BRAFV600E* mutations and 4 PTCs without genomic alterations. Subsequently, western blot analysis performed on the available protein extracts confirmed the presence of TERT protein in the tumors displaying high *hTERT* mRNA levels (Fig. 1B).

Effects of siRNA-mediated silencing of hTERT on PTC cells

In order to evaluate the effects of *hTERT* silencing on PTC cell lines, we transfected K1 and BCPAP cells with two different anti-*hTERT* siRNA. First, we observed a decrease in the levels of TERT protein expression with si*hTERT*-a rather than si*hTERT*-c, in accordance with our previous results in ATC cell lines [16] (Fig. 2A). Analysis of cell proliferation after *hTERT*-a silencing showed a significant reduction in cell viability in both cell lines (~50%, $p < 0.001$ for K1 and ~70%, $p < 0.001$ for BCPAP, vs siCtrl) (Fig. 2B). Next, we examined the effects on different signaling pathways. By western blot analysis, we observed a reduction in β-Catenin and phospho-AKT expression after *hTERT*-a silencing in both cell lines, while no change was found in the phosphorylation levels of ERK (Fig. 3).

In addition, *hTERT*-a silencing determined a significant reduction in the migration properties of both cell lines (~80%, $p < 0.001$ for K1 and ~85%, $p < 0.001$ for BCPAP, vs siCtrl) (Fig. 4a), associated with a significant decrease ($p < 0.001$ vs siCtrl) in both mRNA and protein levels of Fibronectin1 (FN1), a known molecular marker of epithelial–mesenchymal transition associated with increased migration ability of thyroid cancer cells [32] (Fig. 4b).

Effects of BET inhibitors on viability and hTERT expression in PTC cells

Since BET inhibition has clearly demonstrated a consistent antiproliferative effect in ATC cell lines [20, 21] and it has been previously shown that BRD4 is highly enriched on *hTERT* promoter [37], K1 and BCPAP cells were treated

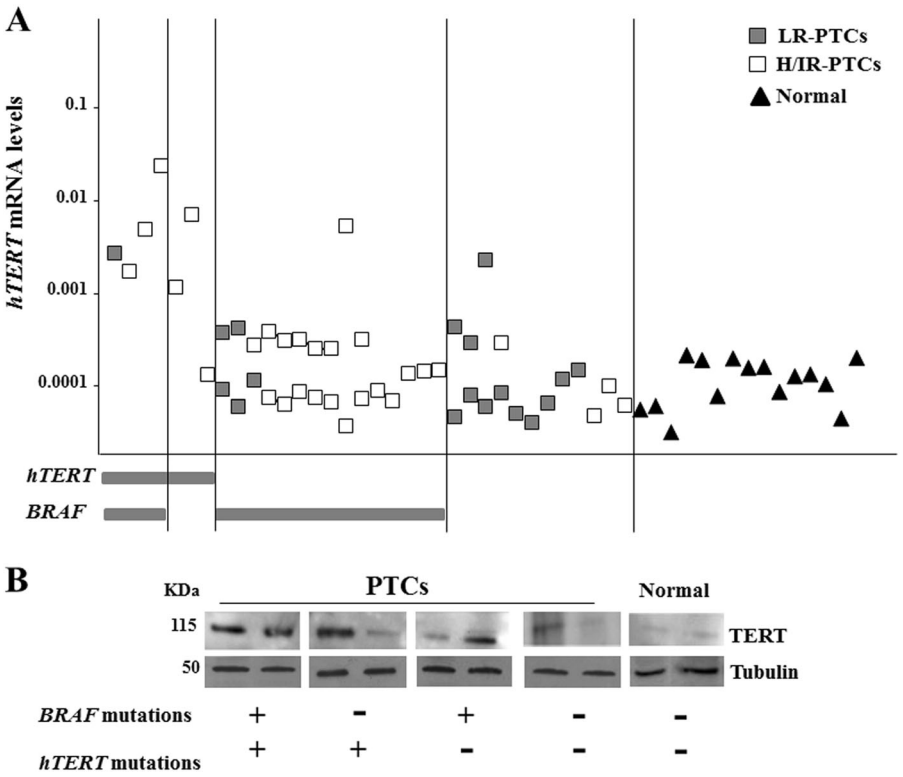

Fig. 1 Expression of *hTERT* mRNA and protein in PTCs. A *hTERT* mRNA levels in 48 thyroid tumor tissues from PTC patients are expressed as comparative $2^{-\Delta Ct}$ values using *β-actin* as endogenous control. White squares represent PTC with high/intermediate risk (H/IR); gray squares represent PTC with low risk (LR). Black triangles indicate values of 15 normal tissues. Gray lines below the *x* axis indicate samples harboring *hTERT* or *BRAF* mutations. B A band of approximately 115 kDa corresponding to hTERT protein was revealed by western blot analysis in 8 representative PTCs with high *hTERT* mRNA levels and 2 normal tissues. Tubulin was used as a loading control

Table 2 *hTERT* expression in PTCs with a different mutational status

hTERT expression	*BRAF*[a] and *hTERT*-mutations $N=4$	*hTERT*-mutations *BRAF*-wild type $N=2$	*BRAF* mutations *hTERT*-wild type $N=25$	*hTERT* and *BRAF*-wild type $N=17$	p[b]
Higher	4	2	10	4	0.013
Lower	0	0	15	13	

[a]Three with *BRAFV600E* and one with *BRAFV600_K601>E*

[b]Chi-square test

with two commercially available BET inhibitors (JQ1 and I-BET762). As shown in Fig. 5a, BET inhibitors at 0.5 and 5 µM significantly hindered the viability of both K1 and BCPAP cells ($p<0.001$ vs vehicle). Contextually, a reduction of *hTERT* mRNA levels was observed under the same experimental conditions (Fig. 5B).

Discussion

Limited therapeutic options are currently available for thyroid cancers which are refractory to radioiodine treatment [38]. Molecular analysis of the mutational landscape of these tumors has helped to identify novel therapeutic strategies and pharmacological agents able to target cancer-specific bullets [27, 39, 40]. Thus, two multikinase inhibitors (Lenvatinib and Sorafenib) are now approved for the treatment of these tumors, but their initial results, though promising, are limited by the presence of frequent intolerable side effects as well as the development of resistance in a percentage of patients [41]. In the search for alternative/additional targets, attention has focused on *hTERT*, whose gene promoter mutations are very frequent in PDTC and ATC (reviewed in [12]). This has led to the hypothesis that these genetic abnormalities may have a role in determining the aggressiveness of these rare tumors, especially in the presence of *BRAF* mutations [9, 11, 12, 42]. Mutations of *hTERT* promoter are much less frequent in PTCs, but a strong association between these mutations and the *BRAFV600E* mutation has been described in the more aggressive PTCs, and is also believed to contribute to the aggressive behavior of these cancers [43–45].

Recently, we have demonstrated that the overexpression of TERT may occur in ATCs even in the absence of the most common mutations in the promoter, and that the silencing of *hTERT* is effective in inhibiting growth and migration of ATC cells in vitro and in xenograft tumors in vivo [16, 29]. In the present study, investigation of a

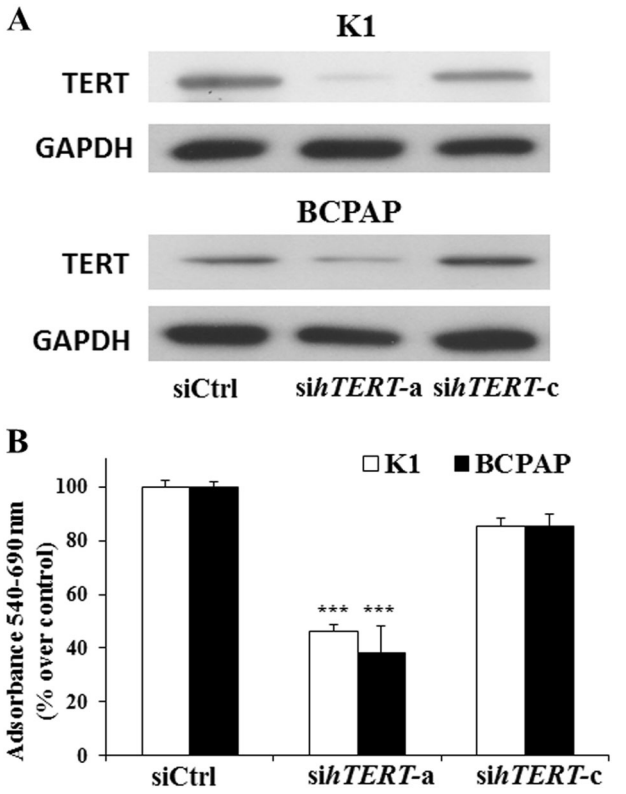

Fig. 2 Effects of *hTERT* silencing in K1 and BCPAP cells. A TERT protein expression after silencing of *hTERT* by two siRNAs. Western blot analysis is representative of three different experiments, as described in Material and methods. GAPDH was used as an internal control. B Cell viability evaluated by MTT assay 24 h after *hTERT* silencing with si*hTERT*-a and si*hTERT*-c, as described in Material and methods. siCtrl are cells transfected with Stealth RNAi Negative Control Duplexes. Results are mean ± SD of three independent experiments performed in eightplicate. Statistical analysis was performed using the Tukey–Kramer multiple comparisons test. ***$p < 0.001$ vs siCtrl

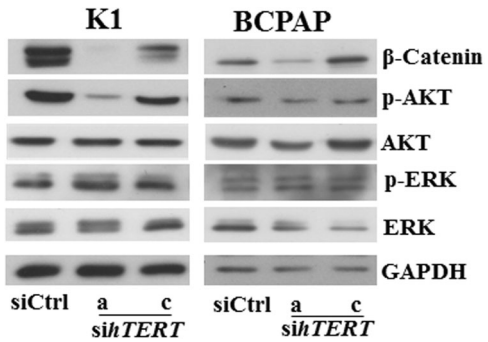

Fig. 3 Effects of *hTERT* silencing on β-Catenin, and AKT and ERK phosphorylation. Immunoblot analysis of β-Catenin, phosphorylated AKT (p-AKT) and AKT, phosphorylated ERK (p-ERK) and ERK, in K1 and BCPAP cells after *hTERT* silencing. Experiments were performed as described in the Material and methods section. Each result shown is representative of three different experiments. GAPDH was used as a loading control

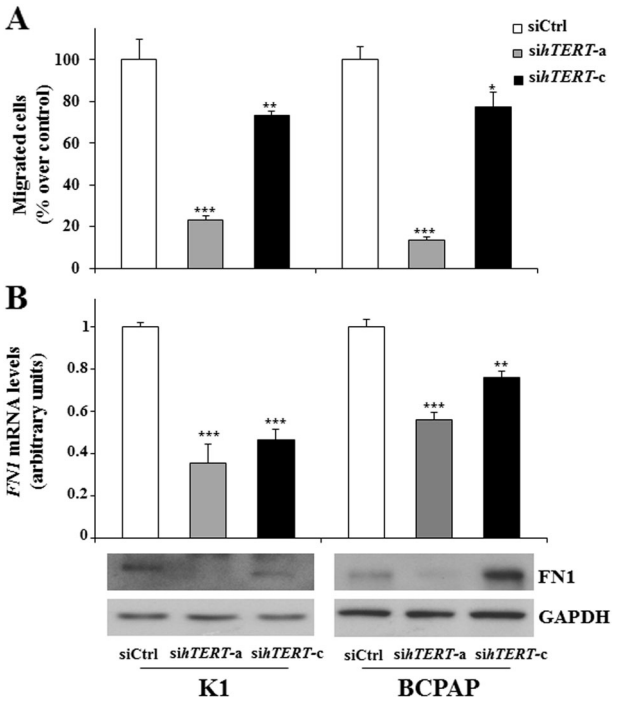

Fig. 4 Effects of *hTERT* silencing on migration property and Fibronectin1 expression in K1 and BCPAP cells. K1 and BCPAP cells treated with si*hTERT*-a, si*hTERT*-c, or siRNA sequence control (siCtrl) were prepared for migration, real-time PCR, and western blot assays as indicated in Material and methods. A For migration assays after 6 h, filters were stained, photographed at 10× magnification, and the cells counted. B mRNA levels of Fibronectin1 (FN1) were determined by $2^{-\Delta\Delta Ct}$ method using GAPDH as endogenous control and the siCtrl as calibrator. **c** Representative immunoblot of FN1 expression after silencing. GAPDH was used as loading control. Each experiment was performed in triplicate and values are expressed as mean ± SD. Statistical analysis was performed using the one-way ANOVA test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs siCtrl

preliminary series of PTCs demonstrated that the overexpression of *hTERT* occurs in a discrete percentage of PTCs (41.6%), especially in the most aggressive ones, and is not necessarily dependent on the presence of *hTERT* promoter mutations. Indeed, *hTERT* overexpression was found not only in tumor tissues carrying an *hTERT* promoter mutation, whether associated or not with a *BRAF* mutation, but also in PTCs carrying only the *BRAF* mutation, and in PTCs without genomic alterations, suggesting the validity of targeting TERT as an alternative therapeutic option even in PTCs. The potential validity of this approach was confirmed by the present finding. In fact, after silencing *hTERT* in PTC cells, we found a reduction in both the viability and migration properties of PTC cells, probably determined by an inhibition of the oncogenic WNT/β-Catenin and PI3K-AKT pathways which play a major role in the dysregulation of proliferation in these cell lines as well as the majority of PTCs [39, 46]. On the contrary, phospho-ERK levels were not influenced, consistent with

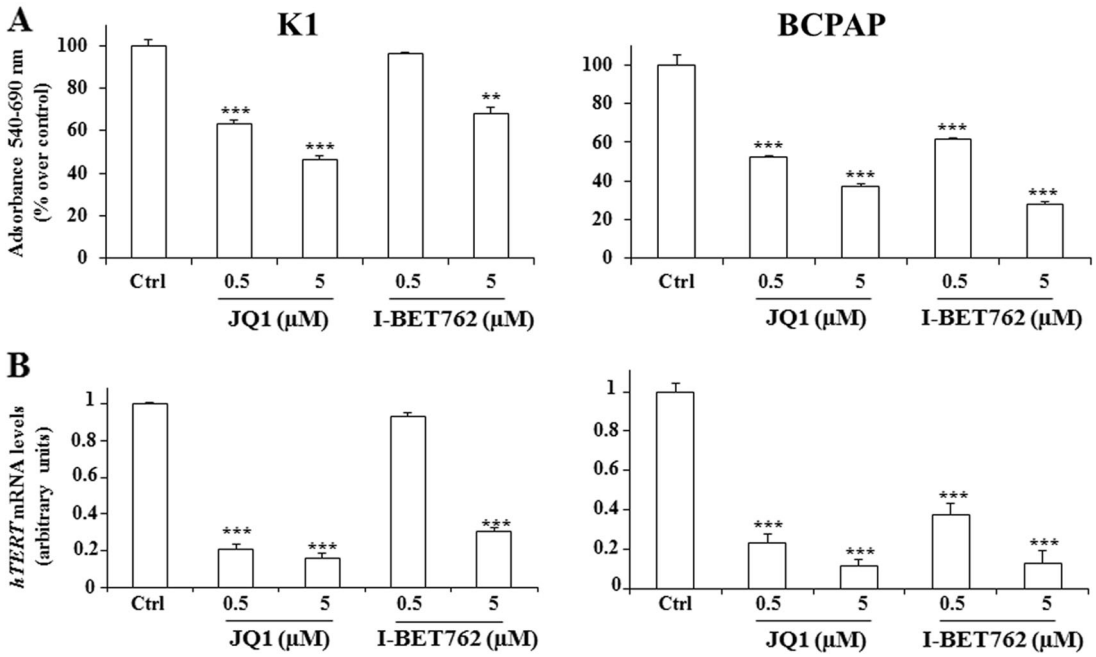

Fig. 5 Effects of BET inhibitors on cell viability and *hTERT* mRNA levels in PTC cell lines. K1 and BCPAP cells were treated with JQ1 or I-BET762 (0.5 or 5 μM). A Effects of BET inhibitors on cell viability was analyzed by MTT assays after 48 h of incubation. Results are expressed as a percentage over control (Ctrl) and were obtained from three independent experiments performed in eightplicate. **B** For qPCR assays, *hTERT* mRNA levels were evaluated after 24 h. Vehicle-treated control (Ctrl) cells were arbitrarily set at 1.0 and mRNA levels are indicated as relative expression values. All samples were run in quadruplicate. Results are shown as mean ± SD. $***p < 0.001$, $**p < 0.01$ vs Ctrl

the recent findings of Liu et al., who demonstrated a synergistic effect when combining *hTERT* and *BRAF* silencing in the same experimental model [47].

Another approach to reducing TERT expression and its tumorigenic-related potential is represented by pharmacological treatments meant to interfere with *hTERT* gene expression. Epigenetic therapies are nowadays receiving much attention in tumor management and novel insights have recently been made regarding *hTERT* epigenetic regulation [7, 17, 48]. Thus, in this study, we focused on pharmacologically-induced inhibition of *hTERT* expression by means of BET inhibitors. BET proteins have extensively been studied in cancer research and their inhibition has already been assessed as a potential antineoplastic tool in both solid tumors and leukemia [49, 50]. BET inhibitors target a well-known family of chromatin readers which interpret lysine acetylation on histone tails. Previously published results highlighted how the use of BET inhibitors (i.e., JQ1 and I-BET762) decreased ATC cell viability by interfering with diverse cellular pathways [20, 51]. Herein, we have demonstrated how two different BET inhibitors, able to decrease thyroid tumor cell growth, down-regulate *hTERT* mRNA levels in two PTC cell lines. These data shed light on both the epigenetic regulation of *hTERT* expression in thyroid cancer and on the possible use of these compounds in PTCs.

Finally, our findings suggest that targeting TERT may be taken into consideration in patients with aggressive PTC.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethics approval All procedures performed in the studies involving human participants were in accordance with the ethical standards of the institutional research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent Informed consent was obtained from all individual participants included in the study.

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