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Interaction of *BRAF*-induced ETS factors with mutant *TERT* promoter in papillary thyroid cancer

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Abstract

Synergistic effects of *BRAF*^{V600E} and *TERT* promoter mutations on the poor clinical outcomes in papillary thyroid cancer (PTC) have been demonstrated. The potential mechanism of this phenomenon has been proposed: MAPK pathway activation by the *BRAF*^{V600E} mutation may upregulate E-twenty six (ETS) transcription factors, increasing *TERT* expression by binding to the ETS-binding site generated by the *TERT* promoter mutation; however, it has not yet been fully proven. This article provides transcriptomic insights into the interaction between *BRAF*^{V600E} and *TERT* promoter mutations mediated by ETS factors in PTC. RNA sequencing data on 266 PTCs from The Cancer Genome Atlas and 65 PTCs from our institute were analyzed for gene expression changes and related molecular pathways, and the results of transcriptomic analyses were validated by *in vitro* experiments. *TERT* mRNA expression was increased by the coexistence of *BRAF*^{V600E} and *TERT* promoter mutations (fold change, 16.17; *q*-value = 7.35×10^{-12} vs no mutation). In the ETS family of transcription factors, *ETV1*, *ETV4* and *ETV5* were upregulated by the *BRAF*^{V600E}/MAPK pathway activation. These *BRAF*^{V600E}-induced ETS factors selectively bound to the mutant *TERT* promoter. The molecular pathways activated by *BRAF*^{V600E} were further augmented by adding the *TERT* promoter mutation, and the pathways related to immune responses or adhesion molecules were upregulated by *TERT* expression. The mechanism of the synergistic effect between *BRAF*^{V600E} and *TERT* promoter mutations on cancer invasiveness and progression in PTC may be explained by increased *TERT* expression, which may result from the *BRAF*-induced upregulation of several ETS transcription factors.

Key Words

- ▶ *TERT* promoter mutation
- ▶ *BRAF*^{V600E} mutation
- ▶ thyroid cancer
- ▶ prognosis
- ▶ mechanism

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Introduction

Telomerase reverse transcriptase (*TERT*) has a canonical role maintaining telomere length and the nontelomeric function, which can regulate the expression of various

genes involved in cell proliferation and cellular signaling, and both canonical and noncanonical roles may contribute to tumorigenesis and cancer progression

(Low & Tergaonkar 2013, Li & Tergaonkar 2014). The promoter region of the *TERT* gene has two hot spots susceptible to a point mutation: chr5, 1,295,228 C>T and 1,295,250 C>T, and both mutations create a binding motif for the ETS family of transcription factors (Horn *et al.* 2013, Huang *et al.* 2013).

TERT promoter mutation in thyroid cancer was first reported in 2013 (Liu *et al.* 2013) and has been shown to be associated with not only aggressive clinicopathological features but also poor prognosis (Liu *et al.* 2016). In particular, *TERT* promoter mutation is associated with the *BRAF*^{V600E} mutation (Liu *et al.* 2013), which is the most common alteration and is also related to aggressiveness of papillary thyroid cancer (PTC) (Xing 2010, Song *et al.* 2015). When they coexist, this genetic duet exerts a synergistic negative effect on clinical outcomes (Xing *et al.* 2014, Song *et al.* 2016, Liu *et al.* 2017, Moon *et al.* 2017). The potential mechanism behind the synergism between the two mutations has been proposed: MAPK pathway activation by *BRAF*^{V600E} may upregulate ETS transcription factors, which increase *TERT* expression by binding to the ETS-binding site generated by the *TERT* promoter mutation (Xing *et al.* 2014). Nonetheless, this mechanism of the synergistic duet in thyroid cancer has not been fully proven.

Recent advances in next-generation sequencing (NGS) started to provide important insights for our understanding of the molecular pathogenesis of thyroid cancers (Cancer Genome Atlas Research Network 2014, Landa *et al.* 2016, Yoo *et al.* 2016). Therefore, we investigated the molecular mechanism of the synergistic effects of *BRAF*^{V600E} and *TERT* promoter mutations in PTC by analyzing the transcriptomic changes according to the mutational status using the NGS database of The Cancer Genome Atlas (TCGA) and validated the findings with our data (Yoo *et al.* 2016).

Materials and methods

Data acquisition and selection of patients

From the genomic data on anonymized patients with PTC that are available from TCGA data portal (<https://portal.gdc.cancer.gov/>), we downloaded the data on clinical information, somatic mutations, mRNA expression and DNA methylation in September 2016. Whole exome sequencing and mRNA sequencing were performed on the Illumina HiSeq 2000 platform. A total of 387 samples had the *TERT* promoter sequencing results from either Illumina MiSeq or whole genome sequencing. To remove

possible influences from driver mutations or fusions other than *BRAF*^{V600E} or *TERT* promoter mutation, 64 cases with other driver mutations and 57 cases with any driver fusions were then excluded (Supplementary Table 1, see section on [supplementary data](#) given at the end of this article). The driver genetic alterations were defined as the significantly mutated genes and driver fusions, according to definitions in a TCGA study (Cancer Genome Atlas Research Network 2014). Finally, 266 patients with PTC were included in the present analysis, and we classified them by mutational status: 41 patients negative for the driver genetic alterations including *BRAF*^{V600E} and *TERT* promoter mutations (no mutation), 196 patients with the *BRAF*^{V600E} mutation only (*BRAF*-only), 2 patients with a *TERT* promoter mutation only (*TERT* only) and 27 patients with coexistent *BRAF*^{V600E} and *TERT* promoter mutations (*BRAF*+*TERT*) (Supplementary Fig. 1). For the comparison of differentially expressed genes (DEGs), the *TERT*-only group was excluded because of the small number of subjects in this group.

As a validation set of genomic analysis, among 125 PTC samples having RNA sequencing data from our previous research (hereafter, SNU database; Yoo *et al.* 2016), 24 patients with other driver mutations and 22 with other driver fusions were excluded. In addition to the previous study, we performed Sanger sequencing for the detection of *TERT* promoter mutation by a previously described method (Song *et al.* 2016). A total of 65 tumor DNA samples available in quantities enough to analyze *TERT* promoter mutations were included in the final analysis: 9 patients in the no-mutation group, 51 patients in the *BRAF*-only group, 1 patient in the *TERT*-only group and four patients in the *BRAF*+*TERT* group (Supplementary Fig. 1). This study's protocol was approved by the Institutional Review Board of Seoul National University Hospital, in accordance with the Declaration of Helsinki (approval ID: H-1108-041-372). Written informed consent was obtained from each subject.

Gene expression profiling and DEG analysis

Using counted numbers of reads aligned to each gene, we normalized them by the regularized log (rlog) transformation method of DESeq2 (Love *et al.* 2014). The DEGs were determined by DESeq2 to have *q*-value <0.05, |Log₂(fold change)| ≥ 1, and baseMean ≥ 100 and were illustrated using volcano plots which show the magnitude and the statistical significance of differential translation for each gene. The calculated *P* values were adjusted to *q*-values for multiple testing using

the Benjamini–Hochberg correction. To construct a heatmap, the centered *rlog* values were applied to the K-means clustering algorithm using cluster 3.0 (de Hoon *et al.* 2004). To identify molecular pathways that were significantly enriched in DEGs, we searched the Kyoto Encyclopedia of Genes and Genomes pathway database (Ogata *et al.* 1999). For comparison of mRNA expression among groups, normalized count data were subjected to the analyses.

Cell culture

Nthy-ori 3-1 is an immortalized thyroid follicular epithelial cell line derived from normal adult thyroid tissue, and Nthy-ori 3-1 cell clones expressing either wild-type *BRAF* (Nthy/WT) or mutant *BRAF* (Nthy/V600E) were established (Kim *et al.* 2017). BCPAP and MDA-T32, the human PTC cell lines harboring both *BRAF*^{V600E} and *TERT* promoter mutations, were kindly provided by Dr Minhong Shong (Chungnam National University, Daejeon, Korea) and purchased from the American Type Culture Collection, respectively. KTC-2, FRO, SW1736 and 8505C are the human anaplastic thyroid cancer cell lines harboring *BRAF*^{V600E} and *TERT* promoter mutations. The former two cell lines were kind gifts from Dr June-Key Chung (Seoul National University College of Medicine, Seoul, Korea), and SW1736 and 8505C cells were kindly provided by Dr Yoon Woo Koh (Yonsei University College of Medicine, Seoul, Korea) and Dr Seong Jin Lee (Hallym University College of Medicine, Chuncheon, Korea), respectively. KTC-2 cells were cultured in the RPMI 1640 medium supplemented with 5% of fetal bovine serum and all other cells were cultured in the RPMI 1640 medium supplemented with 10% of fetal bovine serum, and grown at 37°C in a humidified atmosphere containing 5% of CO₂.

RT-PCR analysis

The mRNA from the cultured Nthy/WT and Nthy/V600E cells was extracted by means of the TRIzol Reagent (Invitrogen). First-strand cDNA was synthesized from 1 µg of total RNA with the Superscript II Reverse Transcriptase Kit (Invitrogen). Human *TERT*, *EHF*, *ELF3*, *ETV1*, *ETV4* and *ETV5* gene expression levels were quantified by PCR with the SYBR Green PCR master mix (Takara) on a StepOne Plus real-time PCR system (Applied Biosystems). The primers are listed in Supplementary Table 2. The reaction was conducted under the thermal cycling conditions

as follows: 2 min at 95°C, and then 40 cycles of 15 s at 95°C and 1 minute at 60°C.

BCPAP cells were treated with PLX4720 (1 µM, Selleckchem), a *BRAF* inhibitor, or PD98059 (10 µM, Cell Signaling Technology), an ERK inhibitor. After 24 h of treatment, the cells were harvested, and mRNAs were extracted for the analysis of expression levels as described above.

Western blot analysis

BCPAP cells were treated with PLX4720 (0.1, 0.5, 1 and 10 µM), a *BRAF* inhibitor, and the cell lysates were harvested at 24 h. Proteins (15 µg) were separated by 8% SDS-PAGE and transferred to nitrocellulose membranes. Membranes were incubated with primary antibodies overnight at 4°C. The primary antibodies were pERK (1:1000, #4370), ERK (1:2000, #9102) from Cell Signaling Technology, and β-ACTIN (A5441) from Sigma-Aldrich. The secondary antibodies were anti-rabbit and anti-mouse. Bound primary antibodies were detected by incubation of the membranes with a secondary horseradish peroxidase-conjugated IgG antibody and visualized using Amersham Imager 600 (GE Healthcare).

Chromatin immunoprecipitation assay

The chromatin immunoprecipitation (ChIP) assay was performed as described previously (Jeong *et al.* 2011) using the EpiQuik Tissue Chromatin Immunoprecipitation kit (P-2002, EpiGentek, NY, USA). Briefly, Nthy-ori 3-1 (3 × 10⁶ cells), KTC-2 (3 × 10⁶ cells), BCPAP (3 × 10⁶ cells) and MDA-T32 (3 × 10⁶ cells) cells were cross-linked with 1% formaldehyde and blocked with 1.25 M glycine solution. The cells were resuspended in CP3 lysis buffer containing a protease-inhibitor cocktail, and sonicated until the cross-linked chromatin was sheared to an average DNA fragment length of 200–300 bp. Chromatin was immunoprecipitated with control IgG (#2729; Cell Signaling technology), anti-ETV1 (sc-55581; Santa Cruz Biotechnology), anti-ETV4 (ARP32263_P050, Aviva Systems Biology, CA, USA) and anti-ETV5 (13011-1-AP, Proteintech, IL, USA), purified, and then analyzed by qPCR using a CFX384 Touch Real-Time PCR Detection System (Bio-Rad Laboratories, Inc.). KTC-2, BCPAP and MDA-T32 cells were treated with 1 µM of PLX4720 for 24 h. The primers were validated by analysis of template titration and dissociation curves. The primer sequences are listed in Supplementary Table 2.

Statistical analysis

To compare the clinicopathological characteristics of subjects according to mutational status, Pearson's χ^2 or Fisher's exact test (if the number was <5) was carried out for categorical variables and analysis of variance for continuous variables. The Mann–Whitney *U* test was used to determine significance of difference between two groups in the assays of RT-PCR, western blotting and ChIP. The paired *t* test was performed to compare differences in the expression levels of each common DEG between groups *BRAF*-only and *BRAF*+*TERT* or between groups *BRAF*^{V600E} mutation with *TERT* expression and without *TERT* expression. For multiple comparisons, a Bonferroni correction method was applied. Statistical significance was defined as two-sided *P* values <0.05. All statistical analyses were performed in R v3.4.2 (www.r-project.org) or SPSS 23.0 (IBM Corp.).

Results

Effects of the coexistence of *BRAF*^{V600E} and *TERT* promoter mutations on poor clinicopathological outcomes in PTC

The clinicopathological characteristics of subjects are presented in Table 1. Among the subjects from TCGA database, in comparison with the no-mutation group, the *BRAF*-only group was significantly associated with younger age at diagnosis and a higher proportion of conventional and tall-cell variant PTC, extrathyroidal extension and lymph node metastasis. Furthermore, the *BRAF*+*TERT* group was strongly associated with virtually all high-risk features such as older age at diagnosis, higher frequency of tall-cell variant PTC, larger tumor size, higher proportion of extrathyroidal extension, especially for moderate or advanced invasion, lymph node metastasis, mortality and higher stage or score of the prognosis prediction models, as compared to the no-mutation or *BRAF*-only group.

Among the subjects from the SNU database, as in TCGA database, the *BRAF*+*TERT* group showed high-risk clinicopathological characteristics, although there were some cases in which the small number of subjects in each group did not support statistical significance.

Upregulated *TERT* mRNA expression in the coexistence of *BRAF*^{V600E} and *TERT* promoter mutations

To identify the mechanism behind the clinical aggressiveness of tumors harboring both *BRAF*^{V600E} and *TERT* promoter mutations, we examined the mRNA

expression level of *TERT* (Fig. 1). Compared with the no-mutation group, *BRAF*-only or *TERT*-only group did not show a significant difference in *TERT* expression (fold change, 2.30 and 1.54, respectively; *q*-value=0.095 and 0.886, respectively), whereas the *BRAF*+*TERT* group showed significantly higher *TERT* expression (fold change, 16.17; *q*-value=7.35 × 10^{−12}; Fig. 1A). Nevertheless, it was unexpectedly found that the mRNA expression of *TERT* was detected in 25.5% of tumors in the *BRAF*-only group (50 of 196), and 14.6% of tumors in the no-mutation group (6 of 41; Fig. 1B). Similar to cases of the mutational coexistence, some clinicopathological characteristics, such as lymph node metastasis, recurrence and mortality, of the group 'coexistence of *BRAF*^{V600E} mutation with *TERT* expression' showed greater aggressiveness as compared to the *BRAF*^{V600E} mutation without *TERT* expression (Supplementary Table 3).

These results were similar for PTC tumors of the SNU dataset, although statistical significance was absent due to the limited number of subjects (Fig. 1C, D and Supplementary Table 4).

Molecular mechanisms of upregulated *TERT* expression by the coexistence of *BRAF*^{V600E} and *TERT* promoter mutations

Transcription of *TERT* can be regulated various transcription factors recognizing their consensus sequence (Akincilar *et al.* 2016). To elucidate the possible mechanism of upregulated *TERT* expression for the combination of *BRAF*^{V600E} and *TERT* promoter mutations, we studied the expression levels of representative transcriptional factors known to contain recognition sequences in the *TERT* promoter region (Akincilar *et al.* 2016, Ramlee *et al.* 2016): p53, p21, SP1, E2F, AP1, HIF1 and c-myc. There was no transcription factor gene whose expression was significantly changed (to satisfy the criteria of a DEG) by the presence of the *BRAF*^{V600E} mutation, except for AP1 (Supplementary Fig. 2 and Supplementary Table 5). AP1, which is a transcription factor composed of the c-Jun and c-Fos subunits, is known to regulate *TERT* expression, either activating or repressing *TERT* transcription (Ramlee *et al.* 2016), and the expression of *JUN* and *FOS* was low in the *BRAF*+*TERT* group of both TCGA and SNU database; however, the *BRAF*-only group did not show significant changes.

Then, to evaluate the previously suggested mechanism, we determined whether the *BRAF*^{V600E} mutation upregulates *TERT* by increasing *ETS* expression because a *TERT* promoter mutation creates a binding motif for *ETS*

Table 1 Clinicopathological characteristics of subjects according to *BRAF*^{F600E} and *TERT* promoter mutational status.

	TCGA				SNU			
	No mutation	<i>BRAF</i> -only	<i>BRAF</i> + <i>TERT</i>	<i>P</i>	No mutation	<i>BRAF</i> -only	<i>BRAF</i> + <i>TERT</i>	<i>P</i>
Male sex, <i>n</i> (%)	41 6 (14.6)	196 52 (26.5)	27 9 (33.3)	0.170	9 2 (22.2)	51 11 (21.6)	4 2 (50.0)	0.385
Age at diagnosis, years	52.7 ± 13.4	45.5 ± 14.1 ^b	65.0 ± 14.2 ^{b,c}	<0.001	48.0 ± 13.2	46.5 ± 11.8	52.5 ± 10.3	0.604
Histology, <i>n</i> (%)								
Conventional	16/37 (43.2)	152/187 (81.3) ^b	16/23 (69.6) ^b		4 (44.4)	42 (82.4) ^b	3 (75.0)	0.031
Follicular variant	20/37 (54.1)	11/187 (5.9) ^b	1/23 (4.3) ^b		5 (55.6)	9 (17.6) ^b	1 (25.0)	
Tall-cell variant	0/37 (0.0)	21/187 (11.2) ^b	6/23 (26.1) ^{b,c}		–	–	–	
Other type	1/37 (2.7)	3/187 (1.6)	0/23 (0.0)		–	–	–	
Tumor size, cm ^a	3.1 (1.9–4.5)	2.4 (1.5–3.5) ^b	3.0 (2.4–4.4) ^c	0.011	2.2 (0.8–4.9)	1.1 (0.7–1.4) ^b	1.9 (1.2–2.1) ^c	0.006
Extrathyroidal extension, <i>n</i> (%)	3/34 (8.8)	65/182 (35.7) ^b	14/23 (60.9) ^{b,c}	<0.001	1 (11.1)	29 (56.9) ^b	4 (100.0) ^b	0.004
Minimal	3/34 (8.8)	63/182 (34.6) ^b	8/23 (34.8) ^b		1 (11.1)	20 (39.2)	4 (100.0) ^b	
Moderate/advanced	0/34 (0.0)	2/182 (1.1)	6/23 (26.1) ^{b,c}		0 (0.0)	9 (17.6)	0 (0.0)	
Multifocality, <i>n</i> (%)	21/40 (52.5)	88/193 (45.6)	12/27 (44.4)	0.709	0 (0.0)	14 (27.5)	3 (75.0) ^b	0.017
Lymph node metastasis, <i>n</i> (%)	6/33 (18.2)	96/176 (54.5) ^b	17/26 (65.4) ^b	<0.001	0 (0.0)	16 (31.4)	2 (50.0)	0.058
Distant metastasis, <i>n</i> (%)	0/17 (0.0)	3/119 (2.5)	2/18 (11.1)	0.116	0 (0.0)	1 (2.0)	0 (0.0)	1.000
Recurrence, <i>n</i> (%)	1/38 (2.6)	12/182 (6.6)	1/24 (4.2)	0.596	0 (0.0)	1 (2.0)	0 (0.0)	1.000
Disease-free survival, months ^a	18.1 (10.4–41.1)	24.0 (15.3–48.1)	17.0 (10.2–49.7)	0.140	58.6 (52.4–76.2)	68.2 (47.1–91.5)	75.5 (38.2–90.2)	0.859
Death, <i>n</i> (%)	2/41 (4.9)	4/196 (2.0)	5/27 (18.5)	<0.001	0 (0.0)	1 (2.0)	0 (0.0)	1.000
Overall survival, months ^a	17.8 (8.7–38.0)	24.6 (15.0–48.1)	17.8 (10.1–50.9)	0.076	58.6 (52.4–76.2)	68.5 (49.8–91.5)	75.5 (38.2–90.2)	0.817
ATA stage, <i>n</i> (%)				<0.001				0.021
Low risk	24/36 (66.7)	55/183 (30.1) ^b	5/23 (21.7) ^b		7 (77.8)	21 (41.2) ^b	0 (0.0) ^b	
Intermediate risk	12/36 (33.3)	122/183 (66.7) ^b	12/23 (52.2)		1 (11.1)	28 (54.9) ^b	4 (100.0) ^b	
High risk	0/36 (0.0)	6/183 (3.3)	6/23 (26.1) ^{b,c}		1 (11.1)	2 (3.9) ^b	0 (0.0) ^b	
TNM stage, <i>n</i> (%)				<0.001				0.191
I–II	31 (75.6)	133 (67.9)	7 (25.9) ^{b,c}		7 (77.8)	36 (70.6)	1 (25.0)	
III–IV	10 (24.4)	63 (32.1)	20 (74.1) ^{b,c}		2 (22.2)	15 (29.4)	3 (75.0)	
MACIS score	5.5 ± 1.1	5.2 ± 1.4	7.4 ± 1.8 ^{b,c}	<0.001	5.2 ± 1.0	4.8 ± 1.0	5.7 ± 0.9	0.204

^aData are presented as medians (interquartile ranges); ^bsignificantly different from the no-mutation group; ^csignificantly different from the *BRAF*-only group.

Figure 1

TERT mRNA expression according to mutational status. *TERT* mRNA expression levels from RNA sequencing data from TCGA (A and B) and SNU (C and D) database. (A and C) Median expression levels of *TERT* according to mutational status. (B and D) Each column represents an individual sample.

transcription factors (Horn *et al.* 2013, Huang *et al.* 2013). In the whole ETS family, which consists of 28 genes (and 12 subfamilies), 2 genes (*EHF* and *ELF3*) of the ESE subfamily and three genes (*ETV1*, *ETV4* and *ETV5*) of the PEA3 subfamily were significantly upregulated (in accordance with the criteria of upregulated DEGs) in the *BRAF*+*TERT* group compared to the no-mutation group. *EHF*, *ELF3*, *ETV1* and *ETV4* were similarly upregulated in the *BRAF*-only group compared with the no-mutation group (Fig. 2A, B, Supplementary Fig. 3A and Supplementary Table 6). When we analyzed PTC samples of the SNU dataset, similar results were obtained: *EHF*, *ETV4* and *ETV5* in the *BRAF*+*TERT* group and *EHF*, *ETV7*, *ETV1*, *ETV4* and *ETV5* in the *BRAF*-only group were found to be upregulated DEGs (Fig. 2C, Supplementary Fig. 3B and Supplementary Table 7).

***In vitro* validation of changes in *TERT* and *ETS* expression by the *BRAF*^{V600E} mutation and ETS binding to the mutant *TERT* promoter**

Among the possible mechanisms to regulate *TERT* expression, the *ETS* expression is consistently elevated in the presence of *BRAF*^{V600E} mutation, which can be deduced a major mechanism of the effect of coexistent *BRAF*^{V600E} and *TERT* promoter mutations.

To confirm the changes of *TERT* and *ETS* gene expression under the influence of the *BRAF*^{V600E} mutation observed in the transcriptome data, we conducted *in vitro* experiments on overexpression or inhibition of *BRAF*. The expression of *TERT* in Nthy/V600E cells failed to show a statistically significant difference from Nthy/WT cells, although this expression was slightly higher in Nthy/V600E cells than in Nthy/WT cells (Fig. 3A and Supplementary Fig. 3C). Nonetheless, *ETV1*, *ETV4* and *ETV5* were significantly upregulated as expected, whereas *ELF3* was downregulated in Nthy/V600E cells in contrast to the tissue expression pattern. The expressional changes of *EHF* could not be evaluated because its expression was undetectable in both Nthy/WT and Nthy/V600E cells. We next treated the *BRAF* inhibitor (PLX4720) or ERK inhibitor (PD98059) to thyroid cancer cell lines harboring both *BRAF*^{V600E} and *TERT* promoter mutations. The expression levels of genes *TERT*, *ETV1*, *ETV4* and *ETV5* were significantly lowered by *BRAF* and ERK inhibition, while *ELF3* was upregulated (Fig. 3B and Supplementary Fig. 3D). In addition, the phosphorylation of ERK was suppressed by *BRAF* inhibition in cells harboring both *BRAF*^{V600E} and *TERT* promoter mutations (Fig. 3C).

Next, to demonstrate direct binding of *ETV1*, *ETV4* and *ETV5* to the *TERT* promoter in a mutation-dependent manner, ChIP assays were performed in the Nthy-ori 3-1

Figure 2

Upregulated expression of *ETV1*, *ETV4*, and *ETV5* by the *BRAF*^{V600E} mutation. (A) Heatmap of mRNA expression of *ETS* according to mutational status in the samples from TCGA database. (B and C) Median expression levels of *ETV1*, *ETV4* and *ETV5* according to mutational status in TCGA (B) and SNU (C) database.

cells (wild-type for *BRAF*^{V600E} and *TERT* promoter) and KTC-2, BCPAP and MDA-T32 cells (harboring both *BRAF*^{V600E} and *TERT* promoter mutations). We demonstrated a significant enrichment of *ETV1*, *ETV4* and *ETV5* binding at the *TERT* promoter region of KTC-2 cells, compared to Nthy-ori 3-1 cells (Fig. 3D). Moreover, we confirmed that the increased enrichment of these factors at the mutant *TERT* promoter was reduced by the *BRAF* inhibitor, except for *ETV1* in BCPAP and MDA-T32 cells (Fig. 3D).

Changes in the intracellular signaling pathways by the coexistence of *BRAF*^{V600E} and *TERT* promoter mutations

To investigate whether the increased *TERT* expression by the coexistence of *BRAF*^{V600E} and *TERT* promoter mutations causes some changes in the intracellular signaling pathways, which might reflect aggressive tumor behavior, we next compared the transcriptional profiles by mutational status. The results were illustrated in volcano plots, in which upregulated DEGs are denoted by red dots and downregulated DEGs by dark-green dots. Groups *BRAF*-only and *BRAF* + *TERT* showed a significant change in transcriptional profiles revealing a number of DEGs as compared to the no-mutation group (Fig. 4A and B). On the other hand, there were few DEGs between groups *BRAF*-only and *BRAF* + *TERT*, indicating that there was little difference in the transcriptional profile between the two groups (Fig. 4C). Only 12 genes showed

a statistically significant difference but with modest changes (fold changes, 2.0–2.7): *CILP*, *SFTPA1*, *SFTPA2*, *PODNL1*, *TSPAN11*, *CREB3L1*, *EGLN3*, *WISP2*, *GAS1*, *SLC38A3*, *MFAP5* and *TFF3* (Supplementary Table 8). In the analysis of the SNU dataset, a similar pattern of transcriptional changes was observed, and there were no DEGs between groups *BRAF*-only and *BRAF* + *TERT* (Supplementary Fig. 4A, B and C). Notably, the sets of DEGs of groups *BRAF*-only and *BRAF* + *TERT* strongly overlapped (Fig. 4D). Nevertheless, when we compared the expression levels of common DEGs between groups no-mutation vs *BRAF*-only and no-mutation vs *BRAF* + *TERT*, the expression of upregulated DEGs was increased further and that of downregulated DEGs was decreased further in the *BRAF* + *TERT* group in comparison with the *BRAF*-only group (*P* values according to the paired *t* test: 3.88×10^{-5} and 1.62×10^{-11} , respectively; Fig. 4E), indicating that the degree of changes in the gene expression of *BRAF*-mutated PTCs was amplified by the addition of *TERT* promoter mutation. The results of functional enrichment analysis showed that the upregulated genes were related to the pathways including the extracellular matrix receptor interaction, focal adhesion, cytokine-cytokine receptor interaction and cell adhesion molecules, whereas downregulated genes were associated with metabolism-related pathways (Fig. 4F and Supplementary Table 9). Similar results were obtained in our database (Supplementary Fig. 4D, E and F).

Figure 3

In vitro validation of changes in *TERT* and *ETS* expression by the *BRAF*^{V600E} mutation and direct binding of ETS factors to the mutant *TERT* promoter. (A) In human thyroid cell lines expressing either wild-type *BRAF* (Nthy/WT) or mutant *BRAF* (Nthy/V600E), *TERT*, *ETV1*, *ETV4* and *ETV5* gene expression levels were quantified by RT-PCR. **P* < 0.05 vs. Nthy/WT. (B) BCPAP, KTC-2, SW1736, FRO, 8505C, the human thyroid cancer cell lines harboring both *BRAF*^{V600E} and *TERT* promoter mutations, were treated with PLX4720 (a *BRAF* inhibitor) or PD98059 (an ERK inhibitor), and *TERT*, *ETV1*, *ETV4* and *ETV5* gene expression levels were quantified by RT-PCR. **P* < 0.05 vs mock. (C) BCPAP cells were treated with PLX4720 (0.1, 0.5, 1 and 10 μ M) and proteins were harvested from total cell lysates 24 h after treatment. Western blot analysis was performed with anti-pERK and anti-ERK. **P* < 0.05 vs mock. (D) Chromatin immunoprecipitation (ChIP) assay for ETV1, ETV4 and ETV5 occupancy at *TERT* promoter in Nthy-ori 3-1 cells without *BRAF*^{V600E} and *TERT* promoter mutations and in KTC-2, BCPAP and MDA-T32 cells with both mutations. KTC-2, BCPAP and MDA-T32 cells were treated with PLX4720. The fold enrichment was the fold increase for the signal from each antibody-enriched chromatin relative to a control IgG. *Cyclophilin* (*Cyclo*) was used as negative control. All data are expressed as mean $\pm$ s.d. **P* < 0.05 vs Nthy-ori 3-1, #*P* < 0.05 vs mock.

Changes in the intracellular signaling pathways by *TERT* expression in PTCs harboring the *BRAF*^{V600E} mutation

As shown in Fig. 1B and D, in some tumors in the *BRAF*-only group, comparable expression of the *TERT* gene was observed relative to the *BRAF*+*TERT* group. This might be one of the reasons for the absence of DEGs between groups *BRAF*-only and *BRAF*+*TERT* (Fig. 4C). Then, we reclassified the samples according to the *TERT* expression status rather than *TERT* promoter mutation (Fig. 5A). The PTCs harboring the *BRAF*^{V600E} mutation without or with *TERT* expression showed a significant change in transcriptional profiles compared to those without either *BRAF*^{V600E} mutation or *TERT* expression (Fig. 5B and C). The common pathways of DEGs between the *BRAF* mutation groups with and without *TERT* expression

were almost identical to those between *BRAF*-only and *BRAF*+*TERT* mutation groups (Supplementary Fig. 5A and B). Moreover, when we compared the expression levels of common DEGs between the two groups, as with the *TERT* promoter mutation, the degree of changes in the gene expression of *BRAF*-mutated PTCs was amplified by the addition of *TERT* expression (Supplementary Fig. 5C). On the other hand, in contrast to the *TERT* promoter mutation, a number of genes were activated by the presence of *TERT* expression in the *BRAF*-mutated tumors (Fig. 5D). Most of these upregulated genes belonged to pathways related to inflammatory cytokines or adhesion molecules (Fig. 5E and Supplementary Table 10). The expression of representative genes including *CD28*, *CTLA4* and *VCAM1* was significantly increased by *TERT* expression (Fig. 5F). We validated these results on SNU data (Supplementary Fig. 6).

Figure 4

Transcriptional changes according to mutational status of the *BRAF*^{V600E} and *TERT* promoter. (A, B and C) Volcano plots for DEGs according to mutational status of samples from TCGA database: no-mutation vs *BRAF*-only (A), no-mutation vs *BRAF*+*TERT* (B), and *BRAF*-only vs *BRAF*+*TERT* (C). (D) Venn diagram for the overlapping downregulated (green) and upregulated (red) DEGs between groups no-mutation vs *BRAF*-only and no-mutation vs *BRAF*+*TERT*. (E) The degree of changes in the gene expression of common DEGs, expressed as mean $\pm$ standard error of the mean. (F) The top 10 most significantly enriched molecular pathways of common DEGs.

Figure 5

Transcriptional changes by *TERT* expression in addition to the *BRAF*^{V600E} mutation. (A) Each column represents the *TERT* mRNA expression level of an individual sample of TCGA database. (B, C and D) Volcano plots for DEGs according to *BRAF*^{V600E} mutation and *TERT* mRNA expression: no mutation without *TERT* expression vs. *BRAF*^{V600E} mutation without *TERT* expression (B), no mutation without *TERT* expression vs. *BRAF*^{V600E} mutation with *TERT* expression (C), and *BRAF*^{V600E} mutation without *TERT* expression vs. *BRAF*^{V600E} mutation with *TERT* expression (D). (E) The top 10 most significantly enriched molecular pathways of upregulated DEGs in *BRAF*-mutated PTCs with *TERT* expression compared to those without *TERT* expression. (F) Median expression levels of genes *CD28*, *CTLA4* and *VCAM1*, which were upregulated DEGs in *BRAF*-mutated PTCs with *TERT* expression compared to those without *TERT* expression.

Discussion

The clinicopathological outcomes of *BRAF*-mutated PTCs clearly became much more aggressive when they had a coexisting *TERT* promoter mutation. From the analyses of TCGA and SNU RNA sequencing data and *in vitro* experiments, we could confirm that *TERT* mRNA expression was increased by addition of the *BRAF*^{V600E} mutation to the *TERT* promoter mutation. Furthermore, this increase was at least in part due to the upregulated expression of *ETS*, especially *ETV1*, *ETV4* and *ETV5* by the *BRAF*^{V600E} mutation. On the other hand, coexisting mutations showed changes in almost the same intracellular signaling pathways as a *BRAF*^{V600E} mutation alone but amplified the changes of the expression level of genes associated with altered pathways. Moreover,

the inflammation and adhesion-related pathways were activated by addition of *TERT* expression in *BRAF*-mutated PTCs. From these results, we could obtain evidence for the possible molecular mechanism behind the synergistic effects of *BRAF*^{V600E} and *TERT* promoter mutation in thyroid cancer cells.

As for the mechanism, we confirmed that this genetic duet significantly increased *TERT* expression as compared with the expression in tumors harboring *BRAF*^{V600E} or *TERT* promoter mutation alone. One previous study (Vinagre *et al.* 2013) also showed that the coexistence of *BRAF*^{V600E} and *TERT* promoter mutations was associated with the highest levels of *TERT* mRNA expression although they included only three samples of thyroid cancer harboring both mutations. On the other hand, we confirmed that the expression of *TERT* was further upregulated by the

BRAF^{V600E} mutation in PTC tissues with a mutant *TERT* promoter on a relatively large number of samples and validated this finding using the PTC cell lines harboring both mutations. Moreover, the similarity of pathways activated by *BRAF*^{V600E} and both mutations may result in amplification of the pathways instead of activation of new pathways when they coexist. Commonly activated molecular pathways were found to be related to the signaling pathways or ontologies representing invasion, adhesion, carcinogenesis of thyroid nodules or cancer invasiveness (Nucera *et al.* 2011).

Moreover, among transcription factors which can control *TERT* promoter activity, the *ETS* expression showed the most significant difference, confirming the possibility that it plays an important role in the synergism between *BRAF* and *TERT*. Several research groups have reported that the major *ETS* transcription factors can actively bind to the mutated *TERT* promoter region because their binding motif was created by the mutation (Bell *et al.* 2015, Li *et al.* 2015). Although binding the core recognition sequence 5'-GGA(A/T)-3' is a common property of *ETS* transcription factors, a genome-wide analysis of all *ETS* family members has established some differences in DNA-binding preferences within this family. A previous study (Bell *et al.* 2015) revealed that the GABP transcription factor selectively binds to a mutant *TERT* promoter in glioblastoma samples and cell lines of neuroblastoma, melanoma and hepatocellular carcinoma. Moreover, Liu *et al.* (2018) recently reported the important findings that *BRAF*^{V600E}/MAPK pathway increased GABPB expression and GABPA/B complex via FOS, and this complex selectively binds and upregulates mutant *TERT* promoter in human cancer, including thyroid cancer. Another recent study on melanoma (Vallarelli *et al.* 2016) showed that the *ETS1* transcription factor, which was upregulated as a downstream target of the activated MAPK pathway, increases the transcriptional activity of *TERT* in melanoma cells harboring the *TERT* promoter mutation. However, in our study, *GABP* and *ETS1* expression was not significantly affected by *BRAF*^{V600E}, but we firstly showed *ETV1*, *ETV4* and *ETV5* were upregulated by the *BRAF*^{V600E}/MAPK pathway activation in thyroid cancer. We demonstrated that these *BRAF*^{V600E}-induced *ETS* factors bound directly to the *TERT* promoter in a mutation-dependent manner, using ChIP assays. Furthermore, we confirmed the expression of *ETV1*, *ETV4* and *ETV5* was increased by

BRAF overexpression and decreased by *BRAF* or ERK inhibition. The binding of these *ETS* factors to the mutant *TERT* promoter was also decreased by *BRAF* inhibition, although there was no difference in *ETV1* in several cell lines. However, one of the limitations of our study is that we did not show that each *ETS* factor directly induced *TERT* activation after binding to the mutant *TERT* promoter.

Some cases in the *BRAF*-only group exhibited *TERT* expression and this was associated with poorer clinicopathological outcomes. One of the potential mechanisms of *TERT* reactivation in this *BRAF*-only group is the *TERT* fusion gene which has been reported to markedly increase the expression of *TERT* in liposarcoma (Stransky *et al.* 2014) and kidney cancer (Karlsson *et al.* 2015). In addition, the methylation of *TERT* promoter also might be one of the mechanisms for regulating *TERT* expression. When we performed the *TERT* promoter methylation analysis using the DNA methylation array data from TCGA, there was a significant positive correlation between cg11625005, one of the CpG sites of the *TERT* promoter, and *TERT* mRNA expression levels in the *BRAF*-only group ($r=0.427$, $P<0.001$). The presence of *TERT* expression in the *BRAF*-mutated tumors was associated with activation of inflammation and adhesion-related pathways, in agreement with one of the noncanonical roles of *TERT* such as activating the NF- κ B signaling pathway (Low & Tergaonkar 2013, Li & Tergaonkar 2014). There was a report about the clinical importance of *TERT* expression in urothelial cancer, namely, that the *TERT* mRNA expression predicts clinical outcomes more accurately than does the *TERT* promoter mutation (Borah *et al.* 2015).

In conclusion, the mechanism of synergistic effects between *BRAF*^{V600E} and *TERT* promoter mutations on aggressiveness of PTC may be explained by increased *TERT* expression, which may result from the *BRAF*-induced upregulation of several *ETS* transcription factors, especially *ETV1*, *ETV4* and *ETV5*. Therefore, we can suggest that the *BRAF*^{V600E} mutation upregulates the *ETS* transcription factors, and then, the *TERT* promoter mutation may turn on the switch of catastrophe by creating the *ETS*-binding sites and increasing *TERT* expression. Furthermore, the molecular pathways activated by the *BRAF*^{V600E} mutation are further augmented by the *TERT* promoter mutation or *TERT* expression, which can synergistically enhance the effects on cancer invasiveness and progression.

Supplementary data

This is linked to the online version of the paper at <https://doi.org/10.1530/ERC-17-0562>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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