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TERT promoter mutation and its synergistic interaction with *BRAF* and *RAS* mutations in thyroid cancer

갑상선암에서 *TERT* promoter 변이의 의의와 *BRAF*, *RAS* 변이와의 시너지 상호작용

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Abstract

TERT promoter mutation and its synergistic interaction with *BRAF* and *RAS* mutations in thyroid cancer

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Recent reports suggest that mutations in the promoter of the gene encoding telomerase reverse transcriptase (*TERT*) affect thyroid cancer outcomes. I aimed to investigate the clinical significance of *TERT* promoter mutation in thyroid cancer and its synergistic interaction with *BRAF* and *RAS* mutations. Furthermore, molecular mechanisms of the oncogene interaction by genomic analysis using next-generation sequencing database were explored. *TERT* promoter mutations were detected in 4.5% of all differentiated thyroid cancers and associated with poor prognosis. These mutations were more frequent in tumors also harboring either *BRAF* (4.8%) or *RAS* mutations (11.3%). The

prevalence of *TERT* promoter mutations was higher in high-risk patients: 9.1% and 12.9% in the ATA high-risk and advanced TNM stage groups, respectively. Among high-risk patients, the presence of *TERT* promoter mutations additively increased the risk of both recurrence and disease-specific mortality. The coexistence of *BRAF* and *TERT* promoter mutations had a synergistic effect on the clinicopathological characteristics and long-term prognosis of papillary thyroid cancer (PTC) and I firstly confirmed this by meta-analysis. From the analyses of RNA sequencing data and *in vitro* experiments, I could confirm that *TERT* mRNA expression was increased by adding the *BRAF* mutation to the *TERT* promoter mutation (fold change, 17.00; q-value = 1.36×10^{-13}). Furthermore, this increase was due to, at least in part, the upregulated expression of E-twenty-six (*ETS*), especially *ETV1*, *ETV4*, and *ETV5* by *BRAF* mutation. The coexisting mutations showed changes in the almost same intracellular signaling pathways as *BRAF* mutation alone, however, amplified the changes of the expression level of genes associated with altered pathways. Moreover, the inflammation and adhesion-related pathways were activated by adding *TERT* expression in *BRAF*-mutated PTCs. Notably, I firstly reported that the coexistence of *RAS* and *TERT* promoter mutations was associated with a higher rate of recurrence, suggesting that they had additive effects on the

prognosis, similarly to *BRAF* and *TERT* promoter mutations. As for the mechanism, I could confirm that this genetic duet significantly increased *TERT* expression (fold change, 5.58; *q*-value = 0.004) compared with the expression in tumors harboring *RAS* or *TERT* promoter mutation alone. Moreover, adding the *TERT* promoter mutation or expression to the *RAS* mutation, there were significant changes in transcriptional profile, which activated the aggressive intracellular pathways including MAPK pathways. In conclusion, genetic screening for *TERT* promoter mutations in high-risk patients with thyroid cancer might bolster the prediction of mortality and recurrence. In addition, molecular testing of *TERT* promoter mutation with *BRAF* or *RAS* mutation together may be useful in assisting with risk stratification in clinical settings. Furthermore, I can suggest that the mechanism of synergistic oncogene interaction between *TERT* and *BRAF* or *RAS* be explained by increased *TERT* expression, which may result from the *BRAF* or *RAS*-induced upregulation of several ETS transcription factors. Pathways related to aggressive behaviors of tumors are activated by the genetic duet; *BRAF* and *TERT* or *RAS* and *TERT*.

Keywords: *TERT*, *BRAF*, *RAS*, thyroid cancer, prognosis, transcriptome analysis

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Introduction

1. Increase in the prevalence of thyroid cancer and importance of predicting prognosis

In the past two decades, the incidence of thyroid cancer has increased dramatically worldwide: 15 fold in South Korea and more than double in the United States (Ahn et al., 2014; Howlader et al., 2016). The incidence of papillary thyroid cancer (PTC), the most common type of thyroid cancer, has increased drastically worldwide; however, majority of them show an excellent prognosis (Ahn et al., 2014; Davies and Welch, 2014; Howlader et al., 2016). Therefore, the recommended management of PTC has been changed to a less-extended therapy (Haugen et al., 2016). Follicular thyroid cancer (FTC) is the second most common type of thyroid malignancy following PTC, which accounts for 10%-32% of cases of differentiated thyroid cancer (DTC) (Hundahl et al., 2000; Lin et al., 1999). The increasing trend in the prevalence of thyroid cancer of the last decades was especially predominant for small PTC, which shows less aggressive features and favorable outcomes (Cho et al., 2013a; Ho et al., 2015). However, long-term trends in the characteristics and outcomes of FTC, distinct from other types of thyroid cancer, have not been reported.

Although the majority of thyroid cancer patients have excellent overall survival, 15%–20% experience either recurrence or distant metastasis with an associated overall 10-year survival rate of 40%–85% (Cho et al., 2014; Schlumberger, 1998). Therefore, the importance of precise risk stratification has been emphasized to compare treatment-associated benefits against adverse effects (Xing et al., 2013). Moreover, it is important to minimize overtreatment of patients who are likely to have a good prognosis, as well as to identify more accurately high-risk patients who would benefit from aggressive treatment and monitoring.

2. Prevalence of genetic alterations in thyroid cancer

Alterations of driver genes such as *BRAF* and *RAS* can cause DTC which can progress to poorly-differentiated or anaplastic thyroid cancer by additional hits (Gianoukakis et al., 2011). Recent advances of next generation sequencing started to provide important insights for our understanding about the molecular pathogenesis of thyroid cancer. In The Cancer Genome Atlas (TCGA) study of PTC (The Cancer Genome Atlas Data Portal), low density of somatic alterations was observed relative to other cancers, and the frequency of alterations was found as: *BRAF* 59.7%, *RAS* 13.0%, and gene fusions 12.4% of

PTCs. In addition to the PTC alone, I reported the genomic landscape of follicular thyroid carcinoma and adenoma, and subtypes of PTC, using the next generation sequencing (NGS) technique (Yoo et al., 2016). The frequency of mutation of each driver gene is different according to its histologic type. *BRAF* and *RAS* are the most frequently mutated genes; especially, *BRAF* in classical PTC, *RAS* in follicular-variant PTC (FVPTC) and FTC.

Several previous studies have presented temporal changes in the mutational frequencies associated with PTC. In the United States, the overall prevalence of *BRAF* mutations was stable, but increased from 50.0% to 76.9% in conventional PTC over the last four decades (Jung et al., 2014). Moreover, *RAS* mutations increased from 2.7% to 24.9% due to an increase in FVPTC. In Europe, the frequency of *BRAF* mutations increased gradually from 28.0% to 58.1% over the last 15 years (Romei et al., 2012). The incidence of the *RET/PTC* rearrangement, in contrast, decreased from 33.0% to 9.8% over the same period. In Korea, which is a *BRAF* mutation-prevalent country, *BRAF*-mutated PTCs increased from 62.2% to 73.7% over the last two decades (Hong et al., 2014a). However, no study has evaluated changes in the mutational frequencies of FTC over time.

In 2013, a novel mutation, telomerase reverse transcriptase (*TERT*)

promoter mutation started to be proposed as an important genetic alteration on the progression of thyroid cancer (Liu et al., 2013a). The prevalence of *TERT* mutations in PTC and FTC was reported to be 11% and 17%, respectively, and about 40% in poorly-differentiated and anaplastic thyroid cancer (Liu and Xing, 2016). However, the frequency of the mutations is strongly associated with geography, with clear differences reported between Asian and Western countries: for example, Korean patients exhibiting the highest rate of *BRAF*-associated thyroid cancers in the world. Given the geographic variability and temporal changes in the genetic alterations of thyroid cancer, the prevalence of *TERT* promoter mutation in Korea needs to be evaluated.

3. Telomerase reverse transcriptase (*TERT*) and the promoter mutations

TERT is a catalytic subunit of telomerase with the RNA component (TERC). *TERT* is undetectable in most somatic tissues, while normally present at low levels in cells that require high rates of self-renewal such as stem cells and germ cells (Kim et al., 1994). However, in human cancer cells, telomerase is often reactivated by upregulation of *TERT* transcription, which maintains telomere length and consequently does not enter the cellular replicative senescence

(Blasco, 2005). On the other hand, it has been described that *TERT* has the nontelomeric function which can regulate expression of various genes involved in cell proliferation and cellular signaling, and this noncanonical role may contribute to tumorogenesis and cancer progression (Li and Tergaonkar, 2014; Low and Tergaonkar, 2013).

The promoter region of *TERT* gene has two hot spots where are susceptible to point mutation: chr5, 1,295,228 C>T and 1,295,250 C>T, the positions 124 and 146 bp respectively upstream of the *TERT* transcription start site, and both mutations create a binding motif for the E-twenty six (ETS) family of transcription factors (Horn et al., 2013; Huang et al., 2013). After the first finding of mutations in the promoter of *TERT* gene in melanoma early in 2013 through whole-genome sequencing (Horn et al., 2013; Huang et al., 2013), the mutations were found also in other human cancers, such as bladder cancer and glioblastoma (Killela et al., 2013; Liu et al., 2013b) as well as thyroid cancer (Liu et al., 2013a).

TERT promoter mutation, recently described to be associated with aggressive clinicopathologic features and poor long-term prognosis in thyroid cancer, has received considerable attention as a novel prognostic molecular marker (Liu et al., 2013a). However, the prevalence of *TERT* promoter mutations is variable

across countries with results of 7.5%–25.5% (median 11.9%) for PTC (Gandolfi et al., 2015; Landa et al., 2013; Liu et al., 2014a; Liu et al., 2013a; Liu et al., 2014b; Muzza et al., 2015; Vinagre et al., 2013; Xing et al., 2014a; Xing et al., 2014c), and 13.8%–36.4% (median 17.1%) for FTC (Liu et al., 2014a; Liu et al., 2013a; Liu et al., 2014b; Melo et al., 2014; Muzza et al., 2015; Vinagre et al., 2013; Wang et al., 2014). In terms of cost-effectiveness, especially in areas with low prevalence, *TERT* promoter mutation assays are difficult to use as routine prognostic tests for all DTCs.

4. Effects of the coexistence of *BRAF* and *TERT* promoter mutations on clinical outcomes in thyroid cancer

Several molecular markers have been studied to identify potential prognostic markers, and an association between the $BRAF^{V600E}$ mutation and poor prognosis of PTC has been largely demonstrated (Kim et al., 2012; Tufano et al., 2012; Xing et al., 2005). However, owing to its high prevalence in PTC, clinical application of the $BRAF^{V600E}$ mutation has limitations, especially in the mutation-prevalent area of $BRAF^{V600E}$ (Ito et al., 2009; Kim et al., 2005; Song et al., 2015). Recently, *TERT* promoter mutation has been proposed as a strong prognostic biomarker (Liu et al., 2013a), and meta-analyses have demonstrated

an association between the *TERT* promoter mutation and aggressive clinicopathological characteristics (De-Tao et al., 2016; Liu and Xing, 2016). In 2014, Xing et al. firstly introduced and proved the synergistic role of $BRAF^{V600E}$ and *TERT* promoter mutations in the aggressive features and recurrence of PTC (Xing et al., 2014c), and PTC-related mortality (Xing et al., 2014b). Since then, the concept of the role of genetic duet on the prognosis has drawn great attention (Ngeow and Eng, 2014) and reflected later studies. Remarkably, although it was reported that the *TERT* promoter mutation of PTC without $BRAF^{V600E}$ or *RAS* mutations did not increase the risk of recurrence or mortality, the risk effect of the *TERT* mutation was observed when $BRAF^{V600E}$ or *RAS* mutations coexisted (Song et al., 2016b; Xing et al., 2014c). Moreover, the effect of this coexistence on clinicopathological characteristics is inconclusive because most studies did not provide data on both the *TERT* promoter mutation and $BRAF^{V600E}$.

5. Effects of the coexistence of *RAS* and *TERT* promoter mutations on clinical outcomes in thyroid cancer

The most frequent genetic alterations in FTC are *RAS* mutations (Nikiforova et al., 2003). The prognostic value of *RAS* mutations is still unclear, although some

evidence suggests that *RAS*-mutated FTCs may be at risk for a poor prognosis (Fukahori et al., 2012; Garcia-Rostan et al., 2003)) or distant metastasis (Jang et al., 2014; Manenti et al., 1994). On the other hand, recent studies have demonstrated that *TERT* promoter mutations, especially when they co-exist with *BRAF* mutations, are associated with a poor prognosis in PTC (Xing et al., 2014c). It is possible that, like the coexisting *TERT* promoter mutations and *BRAF* mutations in PTC, coexisting *TERT* promoter and *RAS* mutations may also play a cooperative role in tumor aggressiveness and poor clinical outcomes of thyroid cancer. However, there have been few reports about the prognostic effect of the coexistence of *TERT* promoter and *RAS* mutations, so that, biological and clinical significance of this association remains to be investigated.

6. Potential molecular mechanisms of synergistic oncogene interaction between *TERT* and *BRAF* or *RAS*

Transcription of *TERT* can be regulated by its promoter site, such as modulation of methylation status or various transcription factors recognizing their consensus sequence. Regarding the mechanism for the synergistic effect between *TERT* and $BRAF^{V600E}$ mutations, one potential mechanism has been

proposed that MAPK pathway activation by *BRAF*^{V600E} mutation may upregulate the ETS transcription factors, which leads to increased *TERT* expression by binding to the ETS binding site generated by the *TERT* promoter mutation (Xing et al., 2014c). One previous study (Vinagre et al., 2013) 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 3 samples of thyroid cancer harboring both mutations. Therefore, this potential mechanism of synergistic duet in thyroid cancer has not been fully proven in a large sample.

Several studies have reported 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). A recent study demonstrated that GABP which is one of the ETS transcription factors selectively binds to mutant *TERT* promoter using glioblastoma samples and cell lines of neuroblastoma, melanoma and hepatocellular carcinoma (Bell et al., 2015). Moreover, another recent study of melanoma showed that the ETS1 transcription factor, which was increased as a downstream target of activated MAPK pathway, could increase the transcriptional activity of *TERT* in melanoma cells harboring *TERT* promoter mutation (Vallarelli et al., 2016).

However, there have been no report about the direct link *TERT* expression and $BRAF^{V600E}$ or *RAS* mutation in thyroid cancer.

7. Hypothesis

I hypothesized that the prevalence of *TERT* promoter mutations in Korea would be different from other countries, because of the highest incidence of thyroid cancer in the world, the larger portion of small tumors, and the *BRAF*-prevalent area. Moreover, as a molecular marker for prognosis prediction, *TERT* promoter mutations would be related to poor clinical outcomes in thyroid cancer, especially when they coexist with *BRAF* or *RAS* mutations. The oncogene interaction between *TERT* and *BRAF* or *RAS* is probably due to increased ETS transcription factors, as previously known. Furthermore, there might be novel mechanisms in the transcriptomic changes by the genetic duets.

8. Aims of study

In Part I, I aimed to investigate the frequency of *TERT* promoter mutations in thyroid cancer patients in Korea, and examine the association of *TERT* promoter and other driver mutations - *BRAF* or *RAS*. Moreover, I evaluated whether *TERT* promoter mutation can be a molecular biomarker for the

prediction of long-term prognosis in addition to the conventional risk stratification system, therefore, defined patient subsets that might benefit from *TERT* promoter mutation tests for prognostication.

In Part II, I investigated the effects of *BRAF* and *TERT* promoter mutations on clinicopathological characteristics and prognosis of PTC patients in our institute, and performed a comprehensive meta-analysis to verify the synergistic effect of *BRAF* and *TERT* promoter mutation on clinical outcomes of PTC. Lastly, I explored the mechanism of the synergistic effect by transcriptome analysis using NGS database of TCGA and our institute.

In Part III, I evaluated changes of the prevalence of *RAS* and *TERT* promoter mutations in FTC over 15 years, as well as associations between these genetic alterations and clinicopathological outcomes. Furthermore, I aimed to identify the molecular mechanism of the synergism between *RAS* and *TERT* promoter mutations on high-risk clinical outcomes by genomic analysis using NGS database.

Chapter I. Prevalence and clinical significance of *TERT* promoter mutation in thyroid cancer

Materials and methods

Patients and tissue samples

Total of 551 patients (472 females and 79 males) with DTC, including 432 PTCs and 119 FTCs, were studied, who underwent thyroidectomy between 1993 and 2012 at the Seoul National University Hospital, Seoul, Korea. I included 308 patients with PTC from our previous study of *BRAF* mutations (Hong et al., 2014b), whose tumor DNA samples were available to analyze *TERT* promoter and *RAS* mutations. The prevalence of *BRAF* mutations in Korea (including our hospital) is the highest in the world, whereas that of *RAS* mutations is lower than in other countries (Song et al., 2015). Therefore, I additionally examined 124 patients with *BRAF*-wild-type PTC in order to investigate the effects of *TERT* promoter mutations in *BRAF*-wild-type as well as *RAS*-mutated tumors. Their median follow-up duration was 4.8 years (interquartile range, 3.4–10.6 years). The treatment protocol was same as in previous studies (Cho et al., 2013b; Choi et al., 2014). The high-risk group of American Thyroid Association (ATA) staging system was defined as the presence of any of the following: macroscopic tumor invasion, incomplete tumor resection, and distant metastasis. This study was conducted according to the guidelines of the Declaration of Helsinki. The research protocol was

approved by the Institutional Review Board Committee of the Seoul National University Hospital (No. H-1207-124-420). Informed consent was also obtained from all the subjects.

Mutational analyses

Standard polymerase chain reaction (PCR) was carried out for genetic sequencing to identify *BRAF*, *RAS*, and *TERT* promoter mutations. Briefly, a fragment of the *BRAF*, *RAS*, or *TERT* promoter was amplified by PCR from genomic DNA by using appropriate primers for *BRAF* codon 600, *N-RAS* codon 61, *H-RAS* codon 61, *K-RAS* codon 61, *N-RAS* codon 12/13, *H-RAS* codon 12/13, *K-RAS* codon 12/13, and for *TERT* (Table 1). The PCR analysis was conducted using the following amplification protocol: initial denaturation at 95℃ for 5 minutes, then 40 cycles of denaturation at 94℃ for 20 seconds, annealing at 56℃ for 20 seconds, elongation at 72℃ for 20 seconds, and a final extension at 72℃ for 10 minutes. The denatured PCR products were digested with restriction endonuclease TspRI (New England Biolabs, Beverly, MA, USA) and electrophoresed onto an agarose gel. The PCR product for *TERT* promoter was 191 bp, including the mutation sites C228T and C250T. Sequencing PCR was performed using the BigDye Terminator v3.1 Cycle Sequencing Ready

Reaction Kit (Applied Biosystems, Foster City, CA, USA) and ABI PRISM 3130xl Genetic Analyzer (Applied Biosystems). The mutation-positive samples by sequencing were confirmed using both forward and reverse primers.

Table 1. Nucleotide sequences of primers used for direct sequencing

Target	primers	Nucleotide sequence
BRAF V600E	Forward	GCTTGCTCTGATAGGAAAATGAG
	Reverse	GATACTCAGC AGCATCTCAGG
NRAS 12/13	Forward	TACTGTACATGTGGCTCGCC
	Reverse	CCGACAAGTGAGAGACAGGA
NRAS 61	Forward	CCAGATAGGCAGAAATGGGC
	Reverse	CCTTCGCCTGTCCTCATGT
HRAS 12/13	Forward	CAGTCCTTGCTGCCTGGC
	Reverse	CTCCCTGGTACCTCTCATGC
HRAS 61	Forward	GCATGAGAGGTACCAGGGAG
	Reverse	TGATGGCAAACACACACAGG
KRAS 12/13	Forward	AAGCGTCGATGGAGGATTT
	Reverse	TGTATCAAAGAATGGTCCTGCA
KRAS 61	Forward	CGTCATCTTTGGAGCAGGAA
	Reverse	ACTCCACTGCTCTAATCCCC
TERT	Forward	CCCTTCACCTTCCAGCTC
	Reverse	CAGCGCTGCCTGAAACTC

Statistical analyses

All statistical analyses were performed using SPSS version 20.0 (IBM Co, Armonk, NY, USA). Data are presented either as frequencies (%) or as mean ± standard deviation. Comparisons of categorical variables were performed using either the Pearson's χ^2 or Fisher's exact test (if the number was <5). Either the independent *t* or Wilcoxon-Mann-Whitney test was used for continuous variables. Survival curves were plotted using the Kaplan-Meier method with log-rank statistics. Cox proportional hazard regression was used to assess the risk of recurrence and disease-specific mortality. Statistical significance was defined as two-sided *P* values < .05.

Results

Prevalence of TERT promoter mutations

TERT promoter mutations were detected in 25 DTCs (4.5%). Mutations were detected in 18 of 432 PTCs (4.2%) and in 7 of 119 FTCs (5.8%). *BRAF* mutations were found in 58.1% of PTCs, while *RAS* mutations in 9.6% of DTCs, 3.2% of PTCs, and 32.8% of FTCs. Upon estimation of the actual frequency of *TERT* promoter mutations in PTC using the reported frequency of *BRAF* mutations in our country (Hong et al., 2014b) (72.7%, instead of 58.1% in this study), it showed a slight increase to 4.4%.

TERT promoter mutation frequencies were directly proportional to tumor size in PTCs (1.6%, 3.1%, 8.6%, and 28.6% of ≤ 1.0, 1.1–2.0, 2.1–4.0, and ≥ 4.1 cm, respectively [*P* for trend < 0.001]), and FTCs (0.0%, 3.4%, and 16.1% of ≤ 2.0, 2.1–4.0, and ≥ 4.1 cm, respectively [*P* for trend = .005]). *TERT* promoter mutations were more frequent in tumors harboring either *BRAF* (4.8%, 12 of 251; *P* = 0.257 vs. neither *BRAF* nor *RAS* mutations) or *RAS* (11.3%, 6 of 53; *P* = 0.006 vs. neither *BRAF* nor *RAS* mutations) mutations than in those harboring neither (2.8%, 7 of 247). However, this difference was not statistically significant for *BRAF* mutations because of the small number of *TERT*-mutated cases.

Of 551 DTC patients, 176 (31.9%) belonged to the ATA high-risk, while 139 (25.2%) belonged to the tumor-node-metastasis (TNM) stage III–IV groups (Table 1). Additionally, prevalence of the *TERT* promoter mutations was increased in the ATA high-risk (9.1% vs. 2.3% in low-risk or 2.5% in intermediate-risk; $P = 0.005$) and TNM stage III–IV (12.9% vs. 1.7% in TNM stage I–II, $P < 0.001$) groups.

Association of TERT promoter mutations with clinicopathologic characteristics

In the DTC patients harboring *TERT* promoter mutations, most clinicopathologic characteristics, such as older age, larger tumor size, more lymph node metastasis/distant metastasis, and higher rates of recurrent/persistent disease and disease-specific mortality, were more aggressive than in those with no mutations. Similar observations were made in the PTC patients (Table 2).

Table 2. Association of *TERT* promoter mutations with clinicopathologic outcomes

	PTC			DTC		
Variable	*TERT* (−)	*TERT* (+)	*P*[a]	*TERT* (−)	*TERT* (+)	*P*[a]
N	414 (95.8)	18 (4.2)		526 (95.5)	25 (4.5)	
C228T/C250T	-	15/3		-	21/4	
Sex, male	52 (12.6)	3 (16.7)	.490	73 (13.9)	6 (24.0)	.158
Age at diagnosis, years[b]	45.0 ± 13.2	56.8 ± 13.4	<.001	44.9 ± 13.4	56.3 ± 13.1	<.001
Tumor size, cm[c]	1.2 (0.8–1.9)	2.5 (1.3–4.1)	<.001	1.5 (0.8–2.5)	3.3 (2.0–4.5)	<.001
Extrathyroidal extension	247 (59.7)	14 (77.8)	.124	314 (59.7)	19 (76.0)	.103
Microscopic	134 (32.4)	3 (16.7)		174 (33.1)	7 (28.0)	
Gross	113 (27.3)	11 (61.1)		140 (26.6)	12 (48.0)	
Lymph node metastasis[d]	146 (37.5)	10 (55.6)	.124	147 (31.0)	11 (52.4)	.040
Distant metastasis	3 (0.7)	5 (27.8)	<.001	7 (1.3)	6 (24.0)	<.001
Disease status			.002			<.001
No evidence of disease	372 (89.9)	11 (61.1)		479 (91.1)	16 (64.0)	
Persistence	1 (0.2)	1 (5.6)		2 (0.4)	2 (8.0)	
Recurrence	41 (9.9)	6 (35.3)		45 (8.6)	7 (28.0)	
Disease-free survival, years[c]	4.4 (3.2–10.3)	3.2 (1.6–5.9)		4.6 (3.2–10.5)	4.2 (1.9–6.0)	
Death of disease	3 (0.7)	4 (22.2)	<.001	4 (0.8)	5 (20.0)	<.001
Disease-specific survival, years[c]	4.7 (3.7–10.6)	6.3 (3.2–10.2)		5.3 (3.8–10.9)	5.3 (3.2–10.3)	
ATA stage			<.001			.002
Low risk	127 (30.7)	2 (11.1)		170 (32.3)	4 (16.0)	
Intermediate risk	156 (37.7)	2 (11.1)		196 (37.3)	5 (20.0)	
High risk	131 (31.6)	14 (77.8)		160 (30.4)	16 (64.0)	
TNM stage						
I–II	318 (76.8)	5 (27.8)	<.001	405 (77.0)	7 (28.0)	<.001
III–IV	96 (23.2)	13 (72.2)		121 (23.0)	18 (72.0)	

Abbreviations: DTC, differentiated thyroid cancer; PTC, papillary thyroid cancer.

[a]*P* value for comparison between wild-type and mutant *TERT*. [b]Data presented as means ± standard deviations. [c]Data presented as medians (interquartile ranges).

[d]Missing cases: 56 of total DTC, 52 of *TERT* wild-type and 4 of *TERT* mutated DTC; 25 of total PTC, 25 of *TERT* wild-type and none of *TERT* mutated PTC.

Impact of TERT promoter mutations on recurrence and disease-specific mortality

For DTCs, the tumor recurrence rate was 8.6% (13.43/1,000 person-years [PY]) in patients with wild-type *TERT*, vs. 28.0% (59.55/1,000 PY) in those harboring its mutant counterpart. The presence of *TERT* promoter mutations was associated with significantly increased recurrence risk (log rank $P < 0.001$; Fig. 1A).

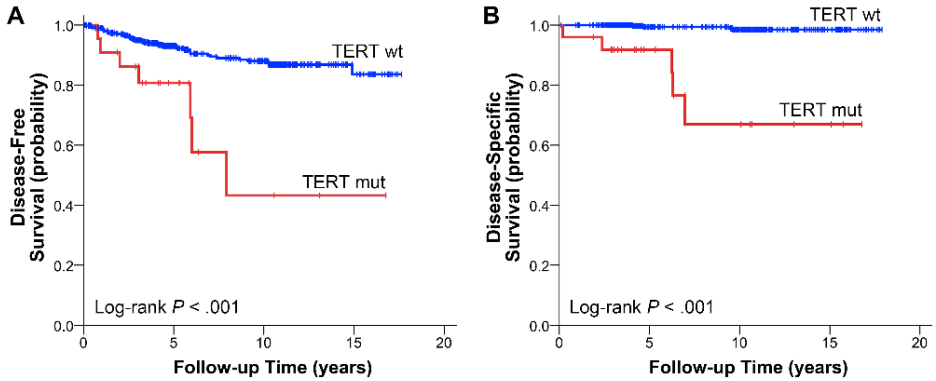


Figure 1. Effects of *TERT* promoter mutations on (A) disease-free and (B) disease-specific survival for patients with differentiated thyroid cancer. mut indicates mutant; *TERT*, telomerase reverse transcriptase; wt, wild type.

Cox regression analysis revealed that the hazard ratio (HR) of *TERT* promoter mutations for recurrence was 2.98 (95% confidence interval [CI], 1.20–7.39; $P = 0.019$) after adjustment for tumor size, extrathyroidal extension, lymph node metastasis, and mutational status of *BRAF* and *RAS* (Table 3).

Further, the disease-specific mortality rate was 0.8% (1.01/1,000 PY) in patients with wild-type *TERT*, vs. 20.0% (29.82/1,000 PY) in those with mutant *TERT*. *TERT* promoter mutations were related to increased disease-specific mortality (log rank $P < 0.001$; Fig. 1B). The HR was 21.14 (95% CI, 3.60–124.23; $P = 0.001$) after adjustment for age at diagnosis, sex, aggressive tumor behaviors, and mutational status of *BRAF* and *RAS* (Table 4).

Similar effects of *TERT* promoter mutations were observed when I analyzed them in all subjects with PTCs and with PTCs over 1 cm. However, for FTCs, the small number of events precluded the analysis (Tables 3 and 4).

Table 3. Hazard ratios of *TERT* promoter mutations for recurrence

	Recurrence/N (%)				Recurrences per 1,000 PY		Hazard Ratio (95% CI)			
Type of Cancer	Overall	*TERT* wt	*TERT* mut	*P*[a]	*TERT* wt	*TERT* mut	Unadjusted	*P*	Adjusted[b]	*P*
DTC	52/551 (9.4)	45/526 (8.6)	7/25 (28.0)	<.001	13.43	59.55	4.22 (1.90–9.38)	<.001	2.98 (1.20–7.39)	.019
PTC	47/432 (10.9)	41/414 (9.9)	6/18 (33.3)	<.001	16.04	76.66	4.60 (1.95–10.87)	.001	3.72 (1.43–9.65)	.007
PTC >1 cm	38/246 (15.4)	32/232 (13.8)	6/14 (42.9)	<.001	20.54	95.58	4.57 (1.89–11.04)	.001	7.03 (2.34–21.11)	.001
FTC	5/119 (4.2)	4/112 (3.6)	1/7 (14.3)	.135	5.03	25.46	4.57 (0.51–40.94)	.175	–	–

Abbreviations: PY, person-years; CI, confidence interval; wt, wild-type; mut, mutant; DTC, differentiated thyroid cancer; PTC, papillary thyroid cancer; FTC, follicular thyroid cancer.

[a]Log-rank *P* values.

[b]Adjusted for age at diagnosis, sex, tumor size, extrathyroidal extension, lymph node metastasis, and mutational status of *BRAF* and *RAS*.

Table 4. Hazard ratios of *TERT* promoter mutations for death from thyroid cancer

	Mortality/N (%)				Deaths per 1,000 PY		Hazard Ratio (95% CI)			
Type of Cancer	Overall	*TERT* wt	*TERT* mut	*P*[a]	*TERT* wt	*TERT* mut	Unadjusted	*P*	Adjusted[b]	*P*
DTC	9/551 (1.6)	4/526 (0.8)	5/25 (20.0)	<.001	1.01	29.82	30.43 (8.13–113.83)	<.001	21.14 (3.60–124.23)	.001
PTC	7/432 (1.6)	3/414 (0.7)	4/18 (22.2)	<.001	1.00	31.26	33.57 (7.46–151.09)	<.001	20.48 (2.95–142.08)	.002
PTC >1 cm	6/246 (2.4)	3/232 (1.3)	3/14 (21.4)	<.001	1.59	27.25	19.65 (3.91–98.88)	<.001	19.20 (2.56–144.15)	.004
FTC	2/119 (1.7)	1/112 (0.9)	1/7 (14.3)	.003	1.06	25.18	20.66 (1.26–337.80)	.034	–	–

Abbreviations: PY, person-years; CI, confidence interval; wt, wild-type; mut, mutant; DTC, differentiated thyroid cancer; PTC, papillary thyroid cancer; FTC, follicular thyroid cancer.

[a]Log-rank *P* values.

[b]Adjusted for age at diagnosis, sex, tumor size, extrathyroidal extension, lymph node metastasis, and mutational status of *BRAF* and *RAS*.

Additional prognostic effects of TERT promoter mutations on conventional risk assessment systems

I stratified all patients by using the risk assessment models, ATA and TNM staging systems, and then subdivided patients of the ATA high-risk group and the TNM stage III–IV into two groups based on the mutational status of the *TERT* promoter.

Among the ATA high-risk patients, those with *TERT*-mutated tumors showed 5.79 times higher recurrence risk than those carrying wild-type tumors, even after adjustment for age at diagnosis, sex, tumor size, and mutational status of *BRAF* and *RAS* (95% CI, 2.07−16.18; $P = 0.001$; Fig. 2A). Moreover, in the TNM stage III–IV group, the HR for recurrence was 3.60 after adjustment for age at diagnosis, sex, and mutational status of *BRAF* and *RAS* (95% CI 1.19–10.85; $P = 0.023$; Fig. 2B and Table 5). Stratified analysis for the HR among patients with either PTCs or with PTCs over 1 cm revealed that the presence of *TERT* promoter mutations additively increased the recurrence risk in high-risk patients by both models (Table 5).

Despite the study being limited by low number of deaths (9 of 551 [1.6%]; 2.19/1,000 PY), presence of *TERT* promoter mutations significantly increased disease-specific mortality in the ATA high-risk (adjusted HR, 16.16; 95% CI,

2.10–124.15; *P* = 0.007) and advanced-TNM stage (adjusted HR, 9.06; 95% CI 2.09–39.26; *P* = 0.003) patients (Figs. 2C and D; Table 6). Similar results were obtained when this analysis was performed in either patients with PTCs or those with PTCs over 1 cm (Table 6).

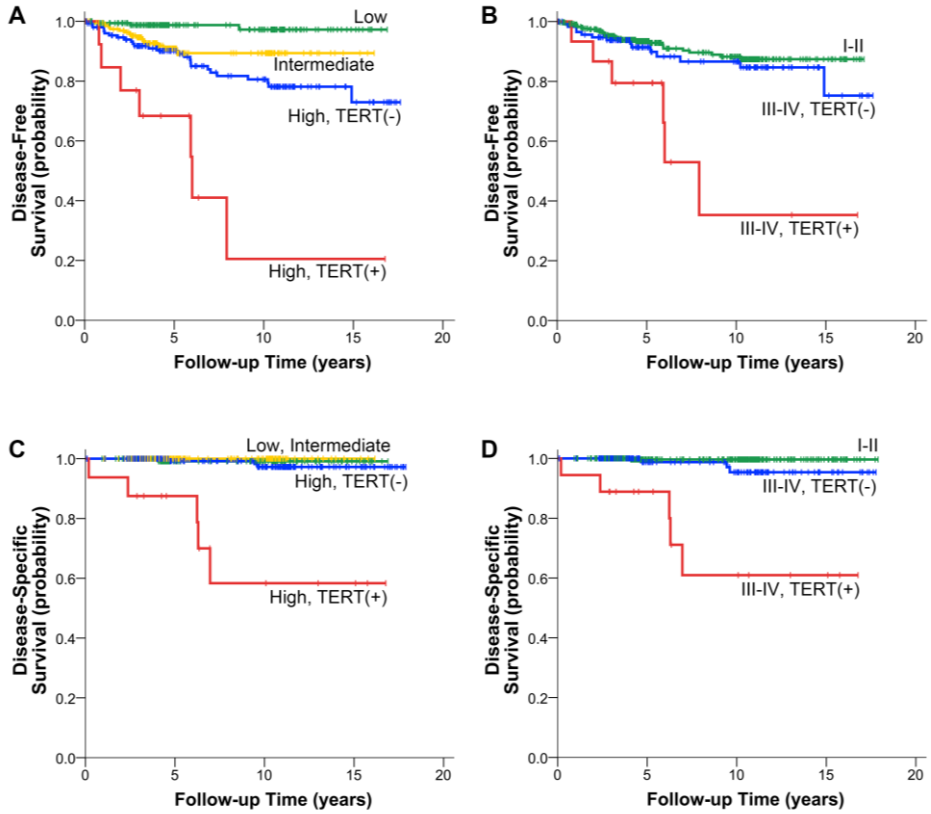


Figure 2. Additional prognostic effects of *TERT* promoter mutations on high-risk patients as defined by (A,C) American Thyroid Association and (B,D) TNM stages: (A,B) disease-free and (C,D) disease-specific survival. *TERT* indicates telomerase reverse transcriptase.

Table 5. Addition of *TERT* promoter mutations to high-risk patients defined by ATA or TNM stage for recurrence

		Unadjusted				Adjusted[a]			
Type of Cancer		HR (95% CI)	*P*	HR (95% CI)	*P*	HR (95% CI)	*P*	HR (95% CI)	*P*
DTC	ATA stage								
	Low	1.00	–	–	–	1.00	–	–	–
	Intermediate	5.46 (1.59–18.77)	.007	–	–	5.45 (1.57–18.98)	.008	–	–
	High, *TERT*(-)	8.69 (2.63–28.72)	<.001	1.00	–	8.71 (2.63–28.88)	<.001	1.00	–
	High, *TERT*(+)	37.91 (9.77–147.03)	<.001	4.53 (1.94–10.56)	<.001	48.91 (11.51–207.87)	<.001	5.79 (2.07–16.18)	.001
	TNM stage								
	I-II	1.00	–	–	–	1.00	–	–	–
	III-IV, *TERT*(-)	1.34 (0.71–2.50)	.368	1.00	–	2.15 (0.98–4.70)	.055	1.00	–
	III-IV, *TERT*(+)	5.20 (2.17–12.45)	<.001	3.82 (1.46–10.02)	.006	11.06 (3.74–32.70)	<.001	3.60 (1.19–10.85)	.023
PTC	ATA stage								
	Low	1.00	–	–	–	1.00	–	–	–
	Intermediate	5.14 (1.50–17.69)	.009	–	–	6.82 (1.78–26.11)	.005	–	–
	High, *TERT*(-)	6.40 (1.91–21.39)	.003	1.00	–	7.87 (2.21–28.06)	.001	1.00	–
	High, *TERT*(+)	26.92 (6.71–108.05)	<.001	4.50 (1.79–11.29)	.001	46.39 (10.14–212.24)	<.001	7.57 (2.51–22.87)	<.001
	TNM stage								
	I-II	1.00	–	–	–	1.00	–	–	–
	III-IV, *TERT*(-)	1.18 (0.60–2.30)	.633	1.00	–	2.14 (0.93–4.89)	.072	1.00	–
	III-IV, *TERT*(+)	5.39 (2.08–13.95)	.001	4.34 (1.51–12.49)	.007	15.05 (4.58–49.45)	<.001	4.00 (1.21–13.22)	.023
PTC >1 cm	ATA stage								
	Low	1.00	–	–	–	1.00	–	–	–
	Intermediate	4.07 (0.89–18.65)	.071	–	–	7.48 (1.26–44.23)	.027	–	–
	High, *TERT*(-)	4.41 (1.03–18.87)	.045	1.00	–	7.29 (1.37–38.66)	.020	1.00	–
	High, *TERT*(+)	17.67 (3.54–88.19)	<.001	4.17 (1.64–10.61)	.003	58.18 (8.97–377.26)	<.001	7.90 (2.56–24.45)	<.001
	TNM stage								
	I-II	1.00	–	–	–	1.00	–	–	–
	III-IV, *TERT*(-)	0.82 (0.38–1.78)	.618	1.00	–	1.91 (0.70–5.25)	.208	1.00	–
	III-IV, *TERT*(+)	3.82 (1.45–10.09)	.007	4.50 (1.48–13.71)	.008	14.04 (3.75–52.59)	<.001	4.69 (1.34–16.38)	.015

Abbreviations: DTC, differentiated thyroid cancer; PTC, papillary thyroid cancer; HR, hazard ratio; CI, confidence interval.

[a]Adjusted for age at diagnosis, sex, tumor size, and mutational status of *BRAF*/*RAS* in ATA stage; age at diagnosis, sex, and mutational status of *BRAF*/*RAS* in TNM stage.

Table 6. Addition of *TERT* promoter mutations to high-risk patients defined by ATA or TNM stage for thyroid cancer-specific death

Type of Cancer		Death, N (%)	Unadjusted		Adjusted[a]	
			HR (95% CI)	*P*	HR (95% CI)	*P*
DTC	ATA stage					
	Low	1/174 (0.6)	–	–	–	–
	Intermediate	0/201 (0.0)	–	–	–	–
	High, *TERT*(-)	3/160 (1.9)	1.00	–	1.00	–
	High, *TERT*(+)	5/16 (31.3)	23.33 (5.50–98.94)	<.001	16.16 (2.10–124.15)	.007
	TNM stage					
	I-II	1/412 (0.2)	–	–	–	–
	III-IV, *TERT*(-)	3/121 (2.5)	1.00	–	1.00	–
	III-IV, *TERT*(+)	5/18 (27.8)	13.20 (3.14–55.48)	<.001	9.06 (2.09–39.26)	.003
PTC	ATA stage					
	Low	1/129 (0.8)	–	–	–	–
	Intermediate	0/158 (0.0)	–	–	–	–
	High, *TERT*(-)	2/131 (1.5)	1.00	–	1.00	–
	High, *TERT*(+)	4/14 (28.6)	27.24 (4.90–151.39)	<.001	94.50 (2.03–4406.31)	.020
	TNM stage					
	I-II	1/323 (0.3)	–	–	–	–
	III-IV, *TERT*(-)	2/96 (2.1)	1.00	–	1.00	–
	III-IV, *TERT*(+)	4/13 (30.8)	18.10 (3.29–99.66)	.001	15.27 (2.60–89.80)	.003
PTC >1 cm	ATA stage					
	Low	1/48 (2.1)	–	–	–	–
	Intermediate	0/76 (0.0)	–	–	–	–
	High, *TERT*(-)	2/109 (1.8)	1.00	–	1.00	–
	High, *TERT*(+)	3/13 (23.1)	18.03 (2.97–109.55)	.002	88.64 (1.80–4376.90)	.024
	TNM stage					
	I-II	1/165 (0.6)	–	–	–	–
	III-IV, *TERT*(-)	2/69 (2.9)	1.00	–	1.00	–
	III-IV, *TERT*(+)	3/12 (25.0)	10.53 (1.75–63.46)	.010	17.75 (2.00–157.41)	.010

Abbreviations: DTC, differentiated thyroid cancer; PTC, papillary thyroid cancer; HR, hazard ratio; CI, confidence interval.

[a]Adjusted for age at diagnosis, sex, tumor size, and mutational status of *BRAF*/*RAS* in ATA stage; age at diagnosis, sex, and mutational status of *BRAF*/*RAS* in TNM stage.

Additionally, I performed the same analysis in high-risk patients by the age-metastasis-extent-size (AMES) and metastasis-age-completeness of resection-invasion-size (MACIS) scoring systems. AMES grouped 16.3% of DTC patients as high-risk, and among them, 18.9% were positive for *TERT* promoter mutations. Meanwhile, 20.1% of the subjects exhibited a MACIS score ≥6.0, and 15.3% were *TERT*-positive. Similarly, the AMES high-risk patients harboring *TERT* promoter mutations presented significantly higher recurrence (HR, 5.38; 95% CI, 1.97–14.71; $P = 0.001$) and disease-specific mortality (HR, 10.33; 95% CI, 2.44–43.85; $P = 0.002$) than those without the mutations. This observation was similar to that in the group with MACIS score ≥6.0 for recurrence (HR, 6.53; 95% CI, 2.34–18.27; $P < 0.001$) and mortality (HR, 8.68; 95% CI, 1.93–39.11; $P = 0.005$) (Figure 3).

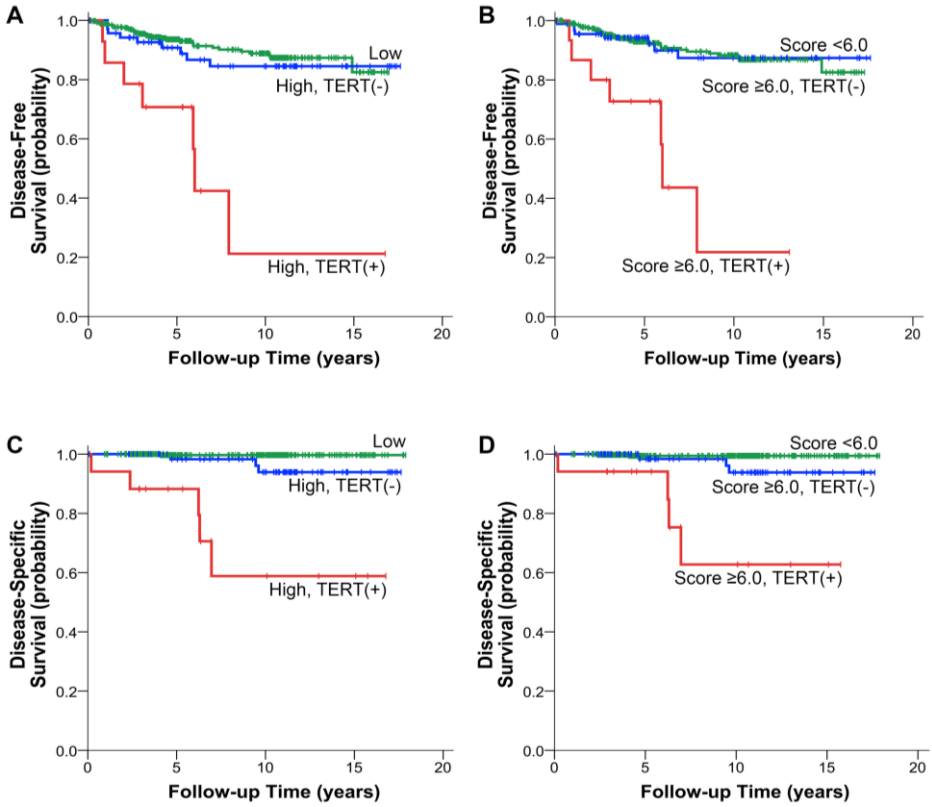


Figure 3. Additional prognostic effects of *TERT* promoter mutations on high-risk patients defined by AMES (A, C), and MACIS (B, D) scoring systems. Effects of *TERT* promoter mutations on disease-free (A, B) and disease-specific (C, D) survival.

Discussion

TERT promoter mutations were detected in 4.5% of all DTCs and associated with poor prognosis. These mutations were more frequent in tumors also harboring either *BRAF* (4.8%) or *RAS* mutations (11.3%). The prevalence of *TERT* promoter mutations was higher in high-risk patients: 9.1% and 12.9% in the ATA high-risk and advanced TNM stage groups, respectively. Among high-risk patients, the presence of *TERT* promoter mutations additively increased the risk of both recurrence and disease-specific mortality.

The adverse effects of *TERT* promoter mutations on clinicopathologic characteristics, recurrence, and mortality in this study were similar to those reported by previous studies (Gandolfi et al., 2015; Landa et al., 2013; Liu et al., 2014a; Liu et al., 2013a; Liu et al., 2014b; Muzza et al., 2015; Vinagre et al., 2013; Xing et al., 2014a; Xing et al., 2014c). The strong association between *TERT* promoter mutations and thyroid cancer-specific mortality indicates that these mutations are promising prognostic markers for DTC. However, because the incidence rates of thyroid cancer are gradually increasing, especially for small-sized tumors, it would be important to identify an optimal subset for *TERT* promoter mutation tests. Since the presence of either *BRAF* or *RAS* mutations could increase the risks associated with *TERT* promoter mutations,

routine tests of the latter in subjects harboring either *BRAF* or *RAS* mutations might provide additional prognostic information. However, the clinical usefulness of the *BRAF* mutational status has limitations in *BRAF*-prevalent areas. Therefore, I tried to adapt the *TERT* mutational status to staging systems for DTC to predict long-term outcomes. Although several staging systems have been proposed for better prediction of long-term prognosis of DTC (American Thyroid Association Guidelines Taskforce on Thyroid et al., 2009; Cady and Rossi, 1988; Edge and Compton, 2010; Hay et al., 1993), currently there is no single, best staging system for both recurrence and mortality. The ATA staging system (American Thyroid Association Guidelines Taskforce on Thyroid et al., 2009) was designed to assess the risk of recurrence in DTC while the TNM staging system (Edge and Compton, 2010) was developed to predict risk for death. However, I found that the limitation with respect to predictability of each staging system could be overcome by additional information on the *TERT* mutational status. Moreover, the frequencies of *TERT* promoter mutations were enriched in high-risk patients; the proportion of these patients among those with DTC is usually less than one third (Tuttle et al., 2010). Proportions of patients with DTC in the ATA high-risk and TNM stage III–IV groups in this study were 31.9% and 25.2%, respectively. Therefore, these high-risk subsets could benefit

from the prediction of recurrence and mortality by routine *TERT* promoter mutation tests. Furthermore, I confirmed additional increase in risks of recurrence and mortality using other risk scoring systems, AMES (Cady and Rossi, 1988) and MACIS (Hay et al., 1993). Further studies on the cost-effectiveness of the tests are required, considering the different prevalence of *TERT* promoter mutations and proportions of high-risk patients in each country.

The overall prevalence of *TERT* promoter mutations in the current study was lower than that reported in other countries (Liu et al., 2014a; Liu et al., 2014b; Melo et al., 2014; Xing et al., 2014a; Xing et al., 2014c). Since the frequency of *TERT* promoter mutations is strongly influenced by tumor size, the relatively large portion of small-size tumors in this study might be one of the reasons behind low frequency of the mutations. In this study, 56.9% of patients had PTCs 1 cm or less compared to 13.7% in a previous study with a 7.5% frequency of *TERT* promoter mutations in PTC (Melo et al., 2014). The second possible reason for the low rate of *TERT* promoter mutations is the geographic/ethnic difference. A recent study in European population reported *TERT* promoter mutations in 4.7% of microcarcinomas (de Biase et al., 2015). This is higher than the prevalence in our study, which was 1.6% of microcarcinomas and 3.1% of tumors 1.1–2.0 cm in size. There may be a

selection bias due to the addition of 124 *BRAF*-wild-type PTC cases, which can affect the results especially the mutational frequency. Therefore, I analyzed without the additional patients and the frequency of *BRAF* mutations was raised from 58.1% to 81.5% in PTC patients, as in previous reports (Hong et al., 2014b), but *TERT* promoter mutation rate was left unchanged as 4.2% (13/308) in PTC. However, there still remains the possibility of some confounding effects due to selection bias in this study.

In conclusion, the presence of *TERT* promoter mutations strengthened the prognostic predictions of conventional staging systems in DTC patients. Genetic screening of *TERT* promoter mutations in high-risk patients with DTC might bolster the prediction of mortality and recurrence.

Chapter II. *TERT* promoter and *BRAF* mutations in papillary thyroid cancer

II-1. Clinical significance of *TERT* and *BRAF* mutations in papillary thyroid cancer

Materials and methods

Patients and tissue samples

Total of 432 patients with PTC, who underwent thyroidectomy between 1993 and 2012 at the Seoul National University Hospital, were studied, and 308 patients with PTC from our previous study of $BRAF^{V600E}$ mutations were included (Hong et al., 2014b). The treatment protocol was same as in previous studies (Cho et al., 2013b; Choi et al., 2014). This study was conducted according to the guidelines of the Declaration of Helsinki. The research protocol was approved by the Institutional Review Board Committee of the Seoul National University Hospital (No. H-1207-124-420). Informed consent was also obtained from all the subjects.

Mutational analyses

Standard PCR was carried out for genetic sequencing to identify *BRAF* and *TERT* promoter mutations. The PCR protocol for amplifying *BRAF* exon 15 and *TERT* promoter used the following primers: 5'-

GCTTGCTCTGATAGGAAAATGAG-3' (forward) and 5'- GATACTCAGC AGCATCTCAGG-3' (reverse) for *BRAF*; 5'- CCCTTCACCTTCCAGCTC-3' (forward) and 5'- CAGCGCTGCCTGAAACTC-3' (reverse) for *TERT*. The PCR analysis was conducted using the following amplification protocol: initial denaturation at 95℃ for 5 minutes, then 40 cycles of denaturation at 94℃ for 20 seconds, annealing at 56℃ for 20 seconds, elongation at 72℃ for 20 seconds, and a final extension at 72℃ for 10 minutes. The denatured PCR products were digested with restriction endonuclease TspRI (New England Biolabs, Beverly, MA, USA) and electrophoresed onto an agarose gel. The digestion of the PCR products with TspRI yielded three major bands at 125, 87, and 12 base pairs (bp) for the wild-type allele. The T1799A mutation abolished the restriction site and resulted in a prominent 212 bp band from the mutant allele and residual bands from the normal allele. To confirm the reliability of the PCR-RFLP results, DNA from 13 PTC samples were chosen at random and sequenced. The sequencing was performed with a PTC-225 Peltier Thermal Cycler (MJ Research, Waltham, MA, USA) using an ABI PRISM BigDye Terminator Cycle Sequencing Kit and AmpliTaq DNA polymerase (Applied Biosystems, Foster City, CA, USA). All 13 sequencing results confirmed the $BRAF^{V600E}$ mutation statuses that were indicated by the PCR-RFLP method. The

PCR product for *TERT* promoter was 191 bp, including the mutation sites C228T and C250T. Following purification of the PCR products, direct DNA bidirectional sequencing was conducted with the BigDye Terminator v3.1 Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA, USA) and ABI PRISM 3130xl Genetic Analyzer (Applied Biosystems). The mutation-positive samples by sequencing were confirmed using both forward and reverse primers.

Statistical analyses

Comparisons of categorical variables were performed using either the Pearson's $\chi 2$ or Fisher's exact test (if the number was < 5), and the analysis of variance test was used for comparisons of continuous variables between three or more groups. A post-hoc Bonferroni test were used to determine which groups have statistically different characteristics. Survival curves were plotted using the Kaplan-Meier method with log-rank statistics. Cox proportional hazard regression was used to assess the risk of recurrence and disease-specific mortality. Statistical significance was defined as two-sided *P* values < 0.05.

Results

Association of coexistence of BRAF^{V600E} and TERT promoter mutations with clinicopathologic characteristics

The effects of coexistence of *BRAF^{V600E}* and *TERT* promoter mutations on clinicopathologic outcomes of 432 patients with PTC were investigated (Table 7). *BRAF^{V600E}* mutation alone was associated with larger tumor size, extrathyroidal extension, and high ATA risk. Coexistence of *BRAF^{V600E}* and *TERT* promoter mutations conferred additive effects with most aggressive characteristics and worse clinical outcomes. However, *TERT* promoter mutation alone failed to show a significant risk effect because of the number of subjects.

Table 7. Impact of *BRAF* and *TERT* promoter mutations, and their coexistence on clinicopathologic outcomes

Variable	No mutation	*BRAF* only	*TERT* only	*BRAF* + *TERT*
N	162	239	5	12
Sex, male	13 (8.0)	36 (15.1)[a]	0 (0.0)	3 (25.0)
Age at diagnosis, years[d]	44.5 ± 13.9	45.1 ± 12.6	50.4 ± 18.8	59.5 ± 11.1[a,b]
Tumor size, cm[e]	1.0 (0.7–1.5)	1.2 (0.8–2.0)[a]	1.0 (0.5–1.7)	3.0 (2.5–4.2)[a,b,c]
Extrathyroidal extension	82 (50.6)	162 (67.8)[a]	2 (40.0)	12 (100.0)[a,b,c]
Microscopic	53 (32.7)	78 (32.6)	0 (0.0)	3 (25.0)
Gross	29 (17.9)	84 (35.1)	2 (40.0)	9 (75.0)
Lymph node metastasis[f]	62 (40.8)	83 (37.1)	3 (60.0)	7 (58.3)
Distant metastasis	1 (0.6)	2 (0.8)	1 (20.0)	3 (25.0)[a,b]
Disease status				
NED	144 (88.9)	215 (90.0)	4 (80.0)	7 (58.3)[a,b]
Persistence	0 (0.0)	1 (0.4)	0 (0.0)	1 (8.3)
Recurrence	18 (11.1)	23 (9.7)	1 (20.0)	4 (36.4)[a,b]
DFS, years[e]	3.8 (2.7–4.7)	6.1 (4.1–10.7)[a]	3.0 (2.4–6.8)	4.5 (1.2–6.0)
Death of disease	1 (0.6)	2 (0.8)	0 (0.0)	4 (33.3)[a,b]
DSS, years[e]	4.0 (3.3–7.5)	9.6 (4.4–10.8)[a]	3.0 (2.4–10.3)	6.3 (4.4–13.1)[a]
ATA stage				
Low risk	57 (35.2)	60 (25.1)[a]	2 (40.0)	0 (0.0)[a,b]
Intermediate risk	66 (40.7)	87 (36.4)	1 (20.0)	1 (8.3)[a,b]
High risk	39 (24.1)	92 (38.5)	2 (40.0)	11 (91.7)[a,b]
TNM stage				
I–II	127 (78.4)	179 (74.9)	3 (60.0)	2 (16.7)[a,b]
III–IV	35 (21.6)	60 (25.1)	2 (40.0)	10 (83.3)[a,b]

Abbreviations: DTC, differentiated thyroid cancer; PTC, papillary thyroid cancer.
[a]Significantly different from No mutation group.
[b]Significantly different from *BRAF* only group.
[c]Significantly different from *TERT* only group.
[d]Data presented as means ± standard deviations.
[e]Data presented as medians (interquartile ranges).
[f]Missing cases: 25 of total PTCs (10 of no mutation and 15 of *BRAF* only)

Additional effects of coexisting mutations of BRAF^{V600E} with TERT promoter mutations on recurrence and disease-specific mortality

Next, I evaluated whether the risks of recurrence or mortality were influenced by the coexistence of *BRAF^{V600E}* mutation with *TERT* promoter mutations. The effects of *BRAF^{V600E}* mutation were analyzed separately in PTC patients. The presence of *BRAF^{V600E}* or *TERT* promoter mutation alone did not significantly alter the recurrence risk, and the mortality risk of each mutation could not be calculated because of the small number of deaths. Interestingly, their coexistence increased the risk of both recurrence and mortality (Fig. 4, Tables 8 and 9), and the HRs were significant even after adjustments for age at diagnosis and sex. However, the statistical significance disappeared after additional adjustments for tumor size, extrathyroidal extension, and lymph node metastasis.

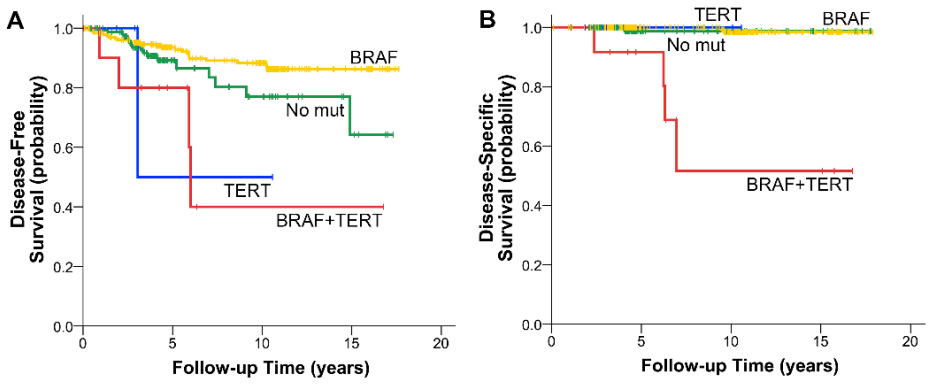


Figure 4. Effects of *TERT* promoter and *BRAF*^{V600E} mutations and their coexistence on (A) disease-free and (B) disease-specific survival for patients with papillary thyroid cancer. mut indicates mutant; *TERT*, telomerase reverse transcriptase.

Table 8. Hazard ratios of *TERT*, other driver mutations, or their coexistence for recurrence

	N (%)	Recurrences per 1,000 PY	Unadjusted HR (95% CI)	*P*	Adjusted HR[a] (95% CI)	*P*	Adjusted HR[b] (95% CI)	*P*
PTC								
No mutation	18/162 (11.1)	23.53	1.00	–	1.00	–	1.00	–
BRAF only	23/239 (9.6)	13.08	0.56 (0.30–1.05)	.073	0.58 (0.31–1.09)	.091	0.71 (0.36–1.39)	.314
TERT only	1/5 (20.0)	46.66	1.92 (0.26–14.43)	.525	2.21 (0.29–16.70)	.441	2.53 (0.33–19.74)	.375
BRAF + *TERT*	4/12 (33.3)	71.38	2.98(1.00–8.84)	.049	4.64 (1.42–15.18)	.011	2.30 (0.66–8.02)	.192

Abbreviations: PY, person-years; CI, confidence interval; PTC, papillary thyroid cancer.
[a]Adjusted for age at diagnosis and sex.
[b]Adjusted for age at diagnosis, sex, tumor size, extrathyroidal extension, and lymph node metastasis.

Table 9. Hazard ratios of *TERT*, other driver mutations, or their coexistence for mortality

	N (%)	Deaths per 1,000 PY	Unadjusted HR (95% CI)	*P*	Adjusted HR[a] (95% CI)	*P*	Adjusted HR[b] (95% CI)	*P*
PTC								
No mutation	1/162 (0.6)	1.07	1.00	–	1.00	–	1.00	–
BRAF only	2/239 (0.8)	0.99	0.68 (0.06–7.55)	.751	0.57 (0.51–6.44)	.651	0.83 (0.03–2.64)	.158
TERT only	0/5 (0.0)	0	–	–	–	–	–	–
BRAF + *TERT*	4/12 (33.3)	42.11	36.31 (4.01–328.92)	.001	15.13 (1.55–148.23)	.020	9.58 (0.42–219.74)	.157

Abbreviations: PY, person-years; CI, confidence interval; PTC, papillary thyroid cancer.
[a]Adjusted for age at diagnosis and sex.
[b]Adjusted for age at diagnosis, sex, tumor size, extrathyroidal extension, and lymph node metastasis.

II-2. Meta-analysis of synergistic effects of coexisting *TERT* and *BRAF* mutations on clinical outcomes

Materials and methods

Search strategy

I conducted a literature search from PubMed and Embase from inception to September 16, 2016. Two independent investigators (S.M. and Y.S.S.) selected articles with a combination of the following important terms: "*TERT*", "*TERT* promoter", "telomerase reverse transcriptase promoter", "telomerase reverse transcriptase", "mutation", "mutations", "thyroid", "neoplasms", "cancer", "carcinoma", and "tumor". The language of the literature was limited to English.

Study selection

All articles were electronically downloaded and screened for inclusion by a two-step method. First, titles and abstracts were evaluated according to predefined criteria. Articles were excluded if: 1) it did not report any clinical information of PTC subjects (e.g. gender, age, lymph node metastasis,

extrathyroidal extension, disease stage, recurrence or mortality); 2) there was no information of any *TERT* promoter or $BRAF^{V600E}$ mutations; 3) the study was published as a form of an abstract, an expert opinion, a letter, a conference article, or a review. I was conscientious of avoiding data from duplicate articles. If studies had multiple reports, the latest or most complete article was enrolled. Then, full texts of the selected, potentially relevant articles were reviewed independently by the two investigators based on the criteria listed above. Any disagreements were resolved by a third investigator (Y.J.P.).

Data extraction

The following variables were extracted by the two investigators independently based on the same rules: first author, publication year, country, number of patients by each *TERT* promoter and $BRAF^{V600E}$ mutation, number of males or females, mean age at diagnosis, the TNM stages, lymph node metastasis, extrathyroidal extension, distant metastasis, recurrence, and mortality. Disagreements were discussed with the third investigator.

Data analyses and statistical methods

I calculated the pooled odds ratios (ORs) with 95% confidence intervals (CIs)

using the Mantel-Haenszel method, and mean difference (MD) with 95% CIs using inverse variance, according to the Cohen method. The Higgins' I^2 statistic was used to test for heterogeneity. When $I^2 \leq 50\%$, the included studies were considered to have little heterogeneity, and a fixed-effects model was used. When $I^2 > 50\%$, the heterogeneity was defined and a random-effects model was used. Subgroup and sensitivity analyses were used to determine the cause of heterogeneity. The potential for publication bias was assessed using a funnel plot analysis. To examine the strength of the outcome, I conducted a sensitivity analysis to estimate the effects of the remaining studies without the larger one's effect. All statistical analyses were calculated by the statistical program R (R version 3.1.0, 2014, www.r-project.org).

Results

Characteristics of eligible studies for meta-analysis

Next, to confirm and quantify the additional effects of coexisting mutations of $BRAF^{V600E}$ and *TERT* promoter mutations on clinical outcomes, I performed meta-analysis. Literature search yielded 327 potentially relevant articles, of which 195 were screened for further review. 13 articles were ultimately selected for meta-analysis (Bullock et al., 2016; Gandolfi et al., 2015; Jin et al., 2016; Kim et al., 2016; Lee et al., 2016; Liu et al., 2016; Liu et al., 2014a; Liu et al., 2014b; Melo et al., 2014; Song et al., 2016b; Sun et al., 2016; The Cancer Genome Atlas Data Portal; Xing et al., 2014c) The detailed procedure of the study selection is summarized in Figure 5. In total, 4,347 patients with PTC were enrolled in this analysis. Overall, 283 (median 8.3%, ranges 2.8–21.6%) of these patients had coexisting $BRAF^{V600E}$ and *TERT* promoter mutations. Sample sizes of these studies ranged from 51 to 1051 patients. Because several variables contained in each article were different, I conducted the meta-analysis using the relevant variables for each study. The examined variables of the selected studies are summarized in Table 10.

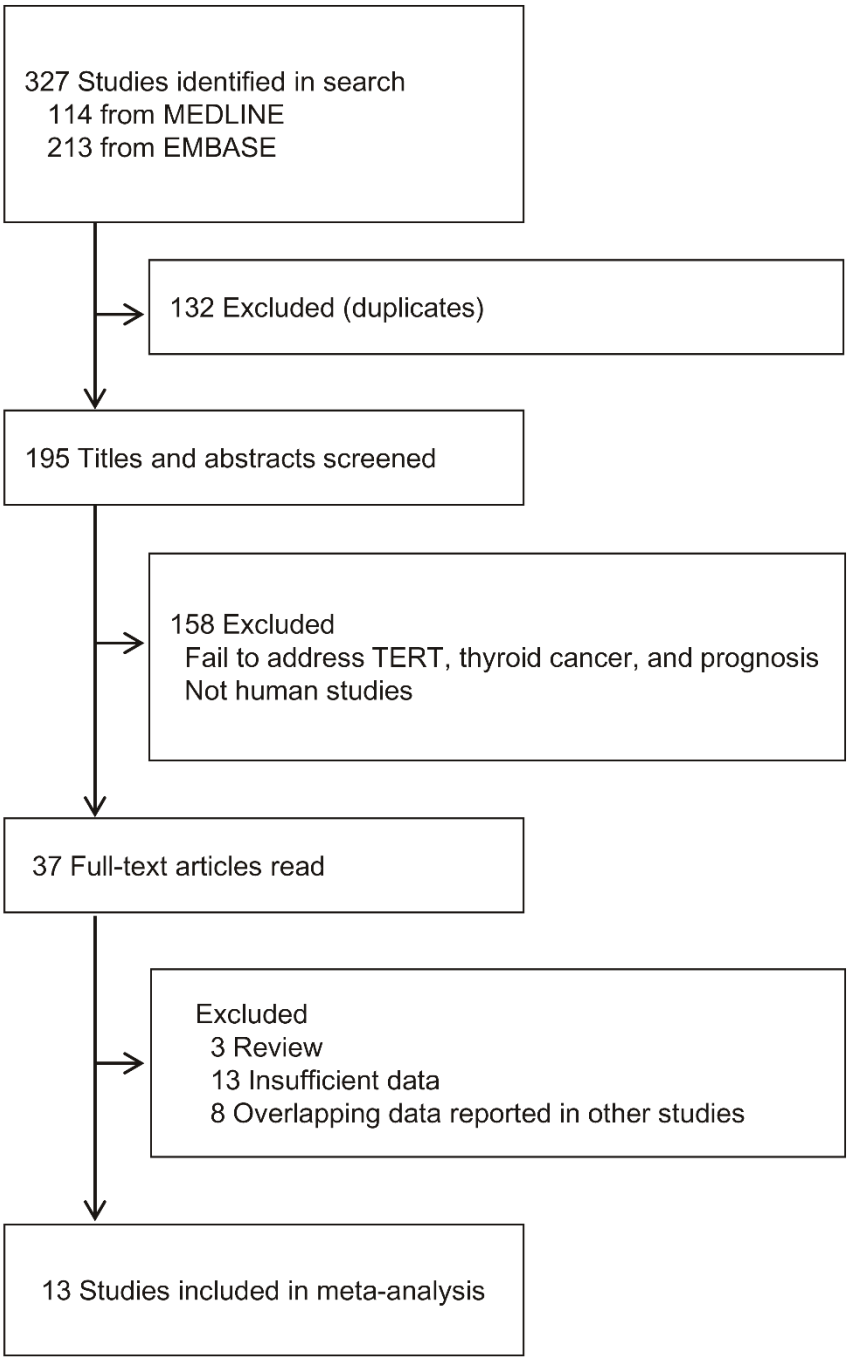


Figure 5. Representation of the search strategy.

Table 10. A summary of the 13 studies included in the meta-analysis

Study	Country	Cases	No. of *TERT* + *BRAF* (%)	Age	Gender	Lymph node metastasis	Extrathyroidal extension	TNM stage	Distant metastasis	Recurrence	Mortality
Liu T, 2014	Sweden	51	11 (21.6)	Y	Y	N	N	N	Y	N	N
Liu X 2014	China	367	36 (9.8)	Y	Y	Y	Y	Y	N	N	N
Xing M, 2014*	USA	507	35 (6.9)	Y	Y	Y	Y	Y	Y	Y	N
Melo M, 2014	Portugal, Spain	301	18 (6)	Y	Y	Y	Y	Y	Y	N	Y
Gandolfi G, 2015	Italy	121	12 (9.9)	Y	Y	Y	Y	Y	N	N	N
Song YS, 2016	South Korea	432	12 (2.8)	Y	Y	Y	Y	Y	Y	Y	Y
Bullock M, 2016	Australia	80	8 (10.0)	N	N	N	N	N	N	N	Y
Jin L, 2016	China	653	22 (3.4)	Y	Y	Y	Y	Y	N	N	N
Sun J, 2016	China	434	18 (4.1)	Y	Y	Y	N	Y	N	N	N
Lee S, 2016	Korea	207	27 (13.0)	Y	Y	Y	Y	Y	N	N	N
Liu R, 2016*	USA	1051	66 (6.3)	Y	Y	Y	Y	Y	N	N	Y
Kim TH, 2016	Korea	264	24 (9.1)	N	N	N	N	N	N	N	Y
TCGA, 2014	USA	386	29 (7.5)	Y	Y	Y	Y	Y	Y	Y	N

Abbreviations: Y, the study was evaluated; N, the study was not evaluated. *These studies used same database. Data of age, gender, lymph node metastasis, extrathyroidal extension, TNM stage and mortality was extracted from Liu R et al. Data of distant metastasis and recurrence was extracted from Xing M et al.

Associations of TERT promoter mutation with BRAF^{V600E} mutation

In Part I, *TERT* promoter mutations were more frequent in tumors harboring *BRAF* (4.8%, 12 of 251; P = 0.257 vs. neither *BRAF* nor *RAS* mutations) mutation than in those harboring neither (2.8%, 7 of 247). However, this difference was not statistically significant for *BRAF* mutations because of the small number of *TERT*-mutated cases. Then, I investigated the association between *TERT* promoter and *BRAF*^{V600E} mutation using a large number of pooled samples by meta-analysis. *TERT* promoter mutation was found in 11.4% of patients with *BRAF*^{V600E} (median, 12.9%; range 4.8–34.4%) vs. 6.3% of those without *BRAF*^{V600E} (median, 5.4%; range 0.9–14.3%) (OR, 2.46; 95% CI, 1.93–3.14; I^2, 0%; Fig. 6), which confirmed the significant association between two mutations.

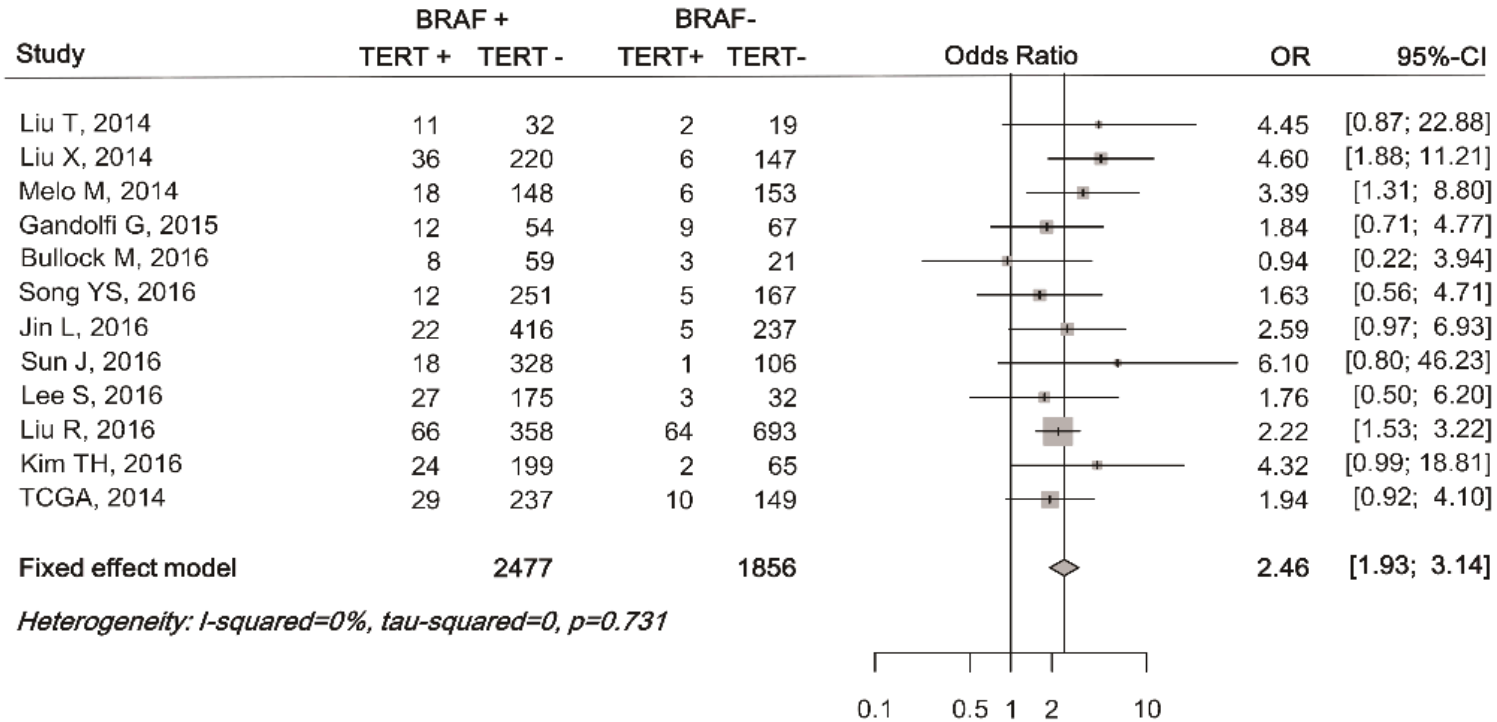
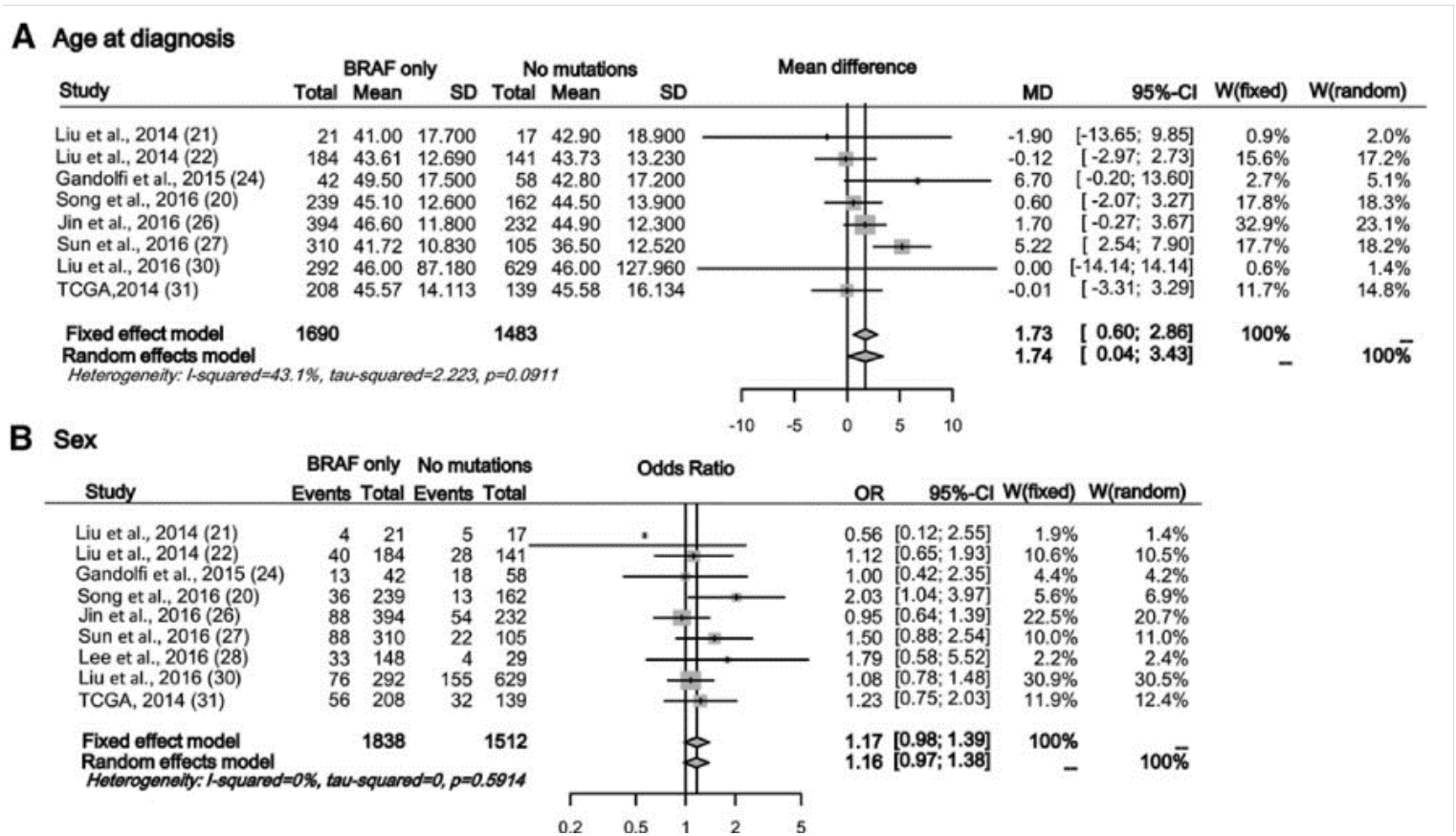


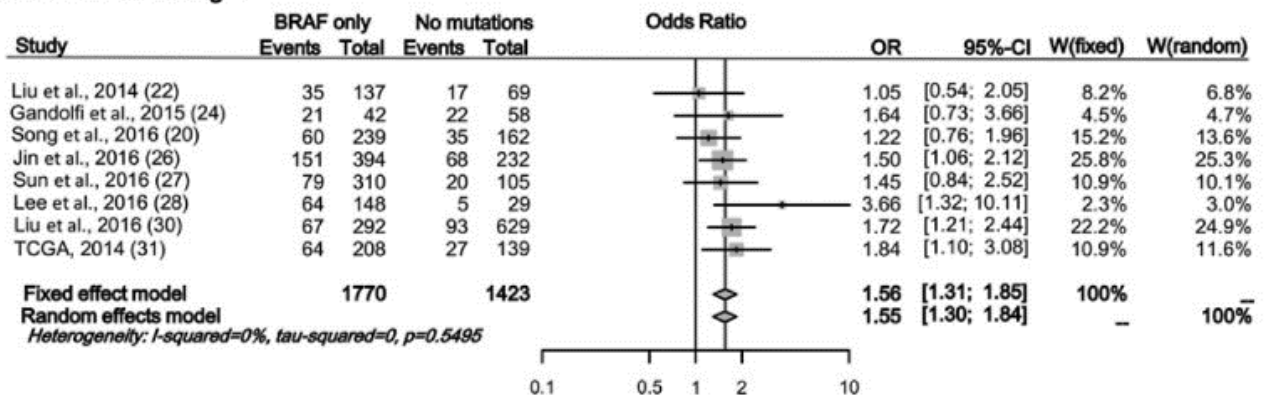
Figure 6. Forest plot showing the association between the *BRAF*^{V600E} and *TERT* promoter mutations. The forest plot displays the effect size and 95% CIs for each study and overall.

Effect of BRAF^{V600E} mutation alone over no mutation on clinicopathological characteristics

BRAF^{V600E} mutation alone was modestly associated with age at diagnosis (MD, 1.73; 95%CI, 0.6-2.86), advanced TNM stage (TNM stage III-IV; OR, 1.56; 95% CI, 1.31–1.85), extrathyroidal extension (OR, 2.45; 95% CI, 1.99–3.03), and lymph node metastasis (OR, 1.52; 95% CI, 1.12–2.05) as compared to no mutation (Fig. 7). However, there were no differences in gender, or distant metastasis between the two groups. No significant heterogeneity was found, except for lymph node metastasis. Sensitivity analysis was performed to identify heterogeneity for lymph node metastasis. When the study of Liu R *et al*. (Liu et al., 2016) was excluded, heterogeneity decreased to 48.1% (OR, 1.34; 95% CI, 1.11–1.62). Since analysis with the funnel plot was asymmetric, the trim-and-fill method to adjust for publication bias was conducted by adding one estimated missing studies (OR, 1.60; 95% CI, 1.17–2.20). The statistical significance remained after sensitivity analysis and adjustment of publication bias.


A Age at diagnosis
BRAF only
No mutations
Mean difference
Study Total Mean SD Total Mean SD MD 95%-CI W(fixed) W(random)
Liu et al., 2014 (21) 21 41.00 17.700 17 42.90 18.900 -1.90 [-13.65; 9.85] 0.9% 2.0%
Liu et al., 2014 (22) 184 43.61 12.690 141 43.73 13.230 -0.12 [-2.97; 2.73] 15.6% 17.2%
Gandolfi et al., 2015 (24) 42 49.50 17.500 58 42.80 17.200 6.70 [-0.20; 13.60] 2.7% 5.1%
Song et al., 2016 (20) 239 45.10 12.600 162 44.50 13.900 0.60 [-2.07; 3.27] 17.8% 18.3%
Jin et al., 2016 (26) 394 46.60 11.800 232 44.90 12.300 1.70 [-0.27; 3.67] 32.9% 23.1%
Sun et al., 2016 (27) 310 41.72 10.830 105 36.50 12.520 5.22 [2.54; 7.90] 17.7% 18.2%
Liu et al., 2016 (30) 292 46.00 87.180 629 46.00 127.960 0.00 [-14.14; 14.14] 0.6% 1.4%
TCGA,2014 (31) 208 45.57 14.113 139 45.58 16.134 -0.01 [-3.31; 3.29] 11.7% 14.8%
Fixed effect model 1690 1483 1.73 [0.60; 2.86] 100%
Random effects model 1.74 [0.04; 3.43] 100%
Heterogeneity: I-squared=43.1%, tau-squared=2.223, p=0.0911
-10 -5 0 5 10
B Sex
BRAF only
No mutations
Odds Ratio
Study Events Total Events Total OR 95%-CI W(fixed) W(random)
Liu et al., 2014 (21) 4 21 5 17 0.56 [0.12; 2.55] 1.9% 1.4%
Liu et al., 2014 (22) 40 184 28 141 1.12 [0.65; 1.93] 10.6% 10.5%
Gandolfi et al., 2015 (24) 13 42 18 58 1.00 [0.42; 2.35] 4.4% 4.2%
Song et al., 2016 (20) 36 239 13 162 2.03 [1.04; 3.97] 5.6% 6.9%
Jin et al., 2016 (26) 88 394 54 232 0.95 [0.64; 1.39] 22.5% 20.7%
Sun et al., 2016 (27) 88 310 22 105 1.50 [0.88; 2.54] 10.0% 11.0%
Lee et al., 2016 (28) 33 148 4 29 1.79 [0.58; 5.52] 2.2% 2.4%
Liu et al., 2016 (30) 76 292 155 629 1.08 [0.78; 1.48] 30.9% 30.5%
TCGA, 2014 (31) 56 208 32 139 1.23 [0.75; 2.03] 11.9% 12.4%
Fixed effect model 1838 1512 1.17 [0.98; 1.39] 100%
Random effects model 1.16 [0.97; 1.38] 100%
Heterogeneity: I-squared=0%, tau-squared=0, p=0.5914
0.2 0.5 1 2 5

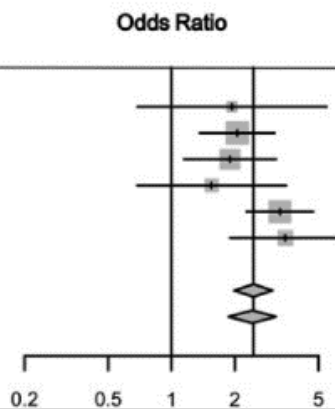
C Advanced TNM stage



Study	BRAF only Events	BRAF only Total	No mutations Events	No mutations Total	OR	95%-CI	W(fixed)	W(random)
Liu et al., 2014 (22)	35	137	17	69	1.05	[0.54; 2.05]	8.2%	6.8%
Gandolfi et al., 2015 (24)	21	42	22	58	1.64	[0.73; 3.66]	4.5%	4.7%
Song et al., 2016 (20)	60	239	35	162	1.22	[0.76; 1.96]	15.2%	13.6%
Jin et al., 2016 (26)	151	394	68	232	1.50	[1.06; 2.12]	25.8%	25.3%
Sun et al., 2016 (27)	79	310	20	105	1.45	[0.84; 2.52]	10.9%	10.1%
Lee et al., 2016 (28)	64	148	5	29	3.66	[1.32; 10.11]	2.3%	3.0%
Liu et al., 2016 (30)	67	292	93	629	1.72	[1.21; 2.44]	22.2%	24.9%
TCGA, 2014 (31)	64	208	27	139	1.84	[1.10; 3.08]	10.9%	11.6%
Fixed effect model		**1770**		**1423**	**1.56**	**[1.31; 1.85]**	**100%**	
Random effects model					**1.55**	**[1.30; 1.84]**		**100%**

Heterogeneity: I-squared=0%, tau-squared=0, p=0.5495

D Extrathyroidal extension



Study	BRAF only Events	BRAF only Total	No mutations Events	No mutations Total	OR	95%-CI	W(fixed)	W(random)
Liu et al., 2014 (22)	18	137	5	69	1.94	[0.69; 5.46]	5.1%	5.7%
Song et al., 2016 (20)	162	239	82	162	2.05	[1.36; 3.09]	27.8%	24.5%
Jin et al., 2016 (26)	68	394	23	232	1.90	[1.15; 3.14]	21.2%	18.7%
Lee et al., 2016 (28)	72	148	11	29	1.55	[0.69; 3.51]	8.3%	8.7%
Liu et al., 2016 (30)	79	292	64	629	3.27	[2.27; 4.72]	26.2%	28.1%
TCGA, 2014 (31)	66	193	16	123	3.48	[1.90; 6.36]	11.4%	14.3%
Fixed effect model		**1403**		**1244**	**2.45**	**[1.99; 3.03]**	**100%**	
Random effects model					**2.42**	**[1.87; 3.13]**		**100%**

Heterogeneity: I-squared=26.7%, tau-squared=0.0274, p=0.234

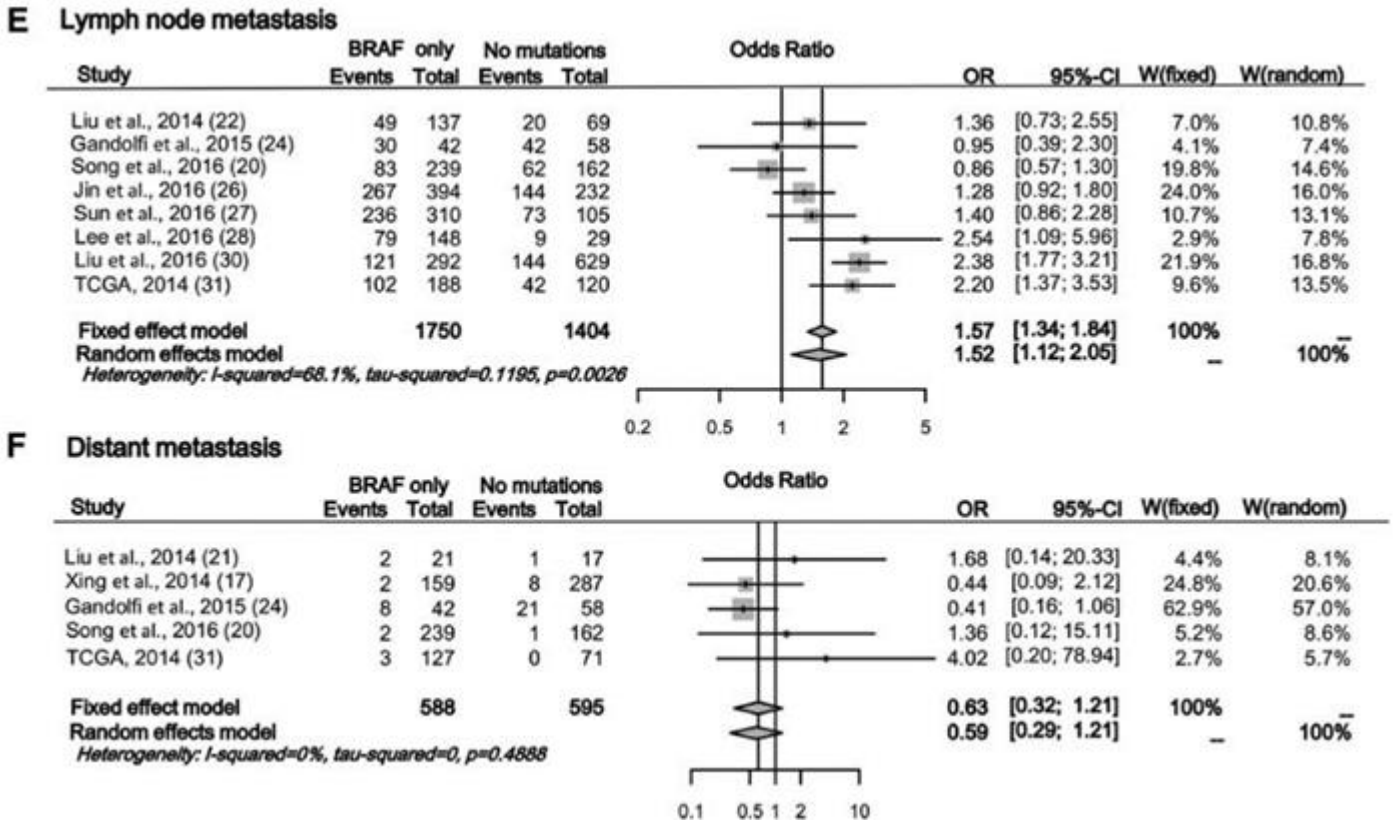
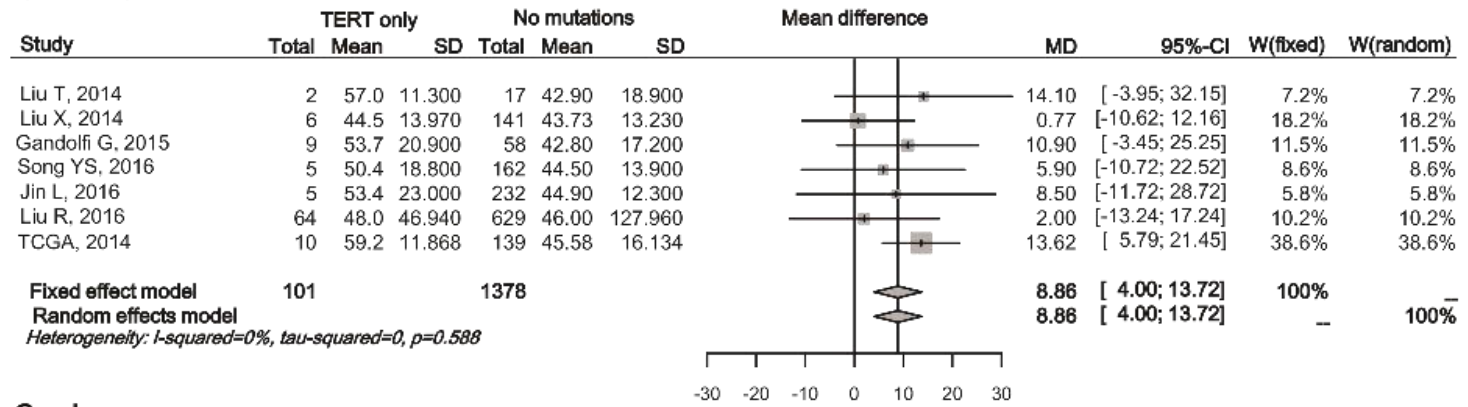


Figure 7. Forest plot showing the effects of *BRAF*^{V600E} mutation alone over no mutation on clinicopathological characteristics. The forest plot displays the effect size and 95% CIs for each study and overall.

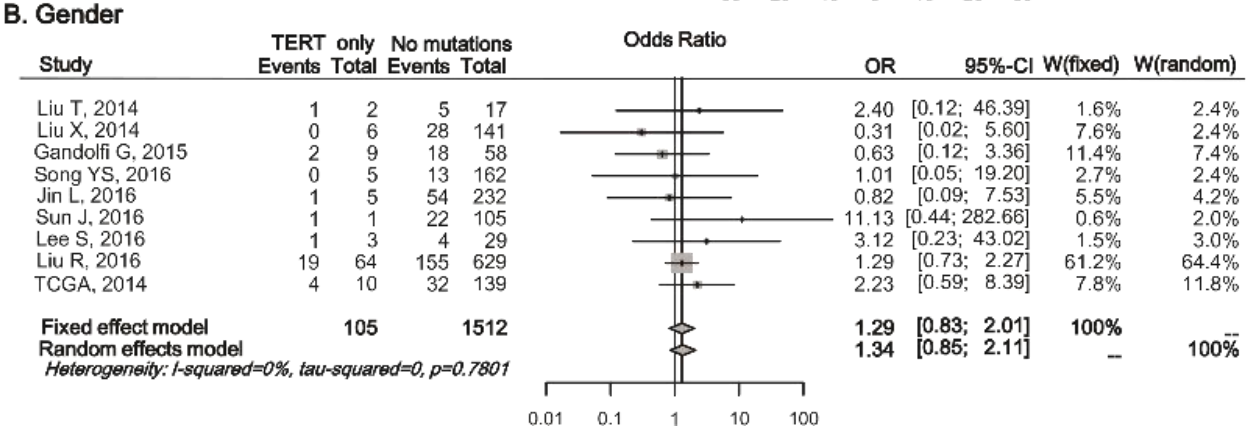
Effect of TERT promoter mutation over no mutation on clinicopathological characteristics

In comparison with the group negative for either mutation, *TERT* promoter mutation alone was not significantly associated with gender, advanced TNM stage, and extrathyroidal extension, while it was moderately associated with age at diagnosis (MD, 8.86; 95% CI, 4.00–13.72), lymph node metastasis (OR, 1.58; 95% CI, 1.02–2.46). In addition, a significantly higher risk for distant metastasis was found in patients with *TERT* promoter mutation alone than those without mutations (OR, 7.28; 95% CI 2.85–18.58; Fig. 8). No significant heterogeneity was found among these studies.

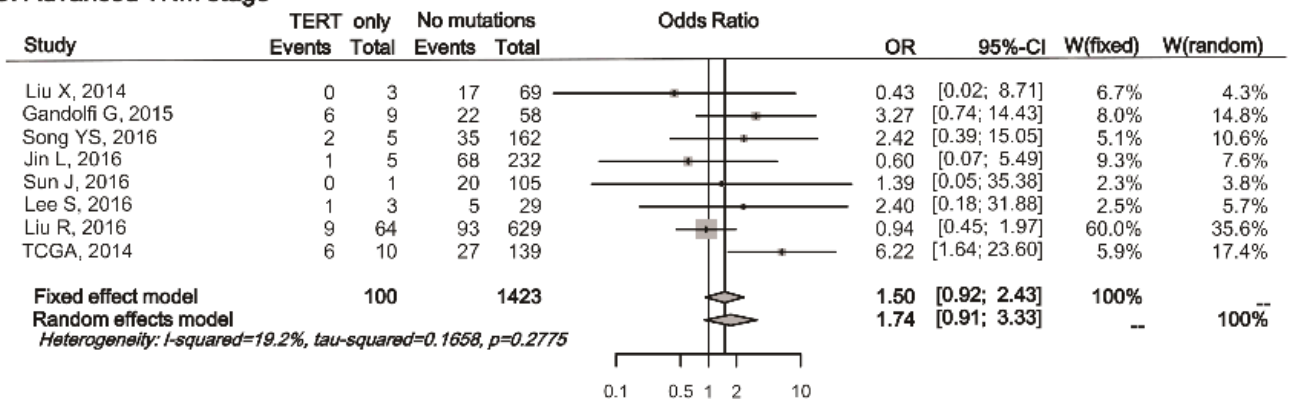
A. Age at diagnosis



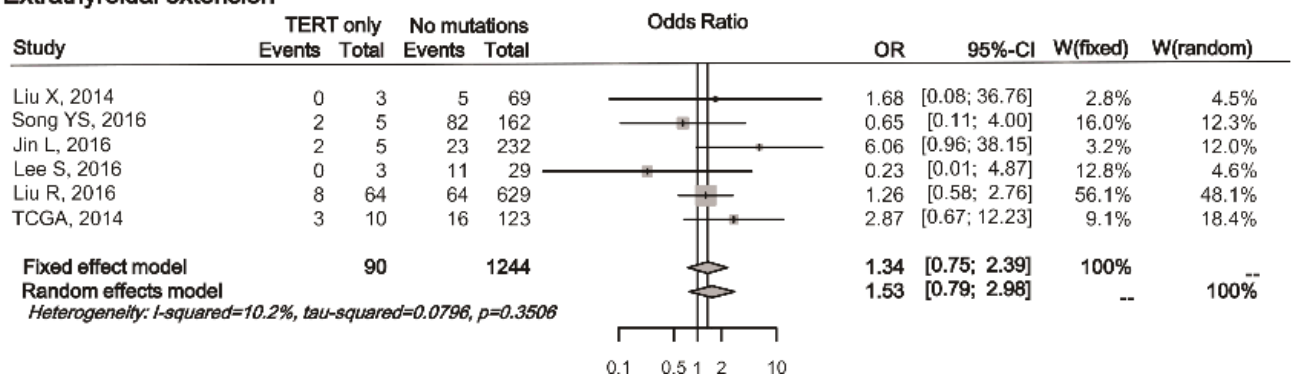
B. Gender



C. Advanced TNM stage



D. Extrathyroidal extension



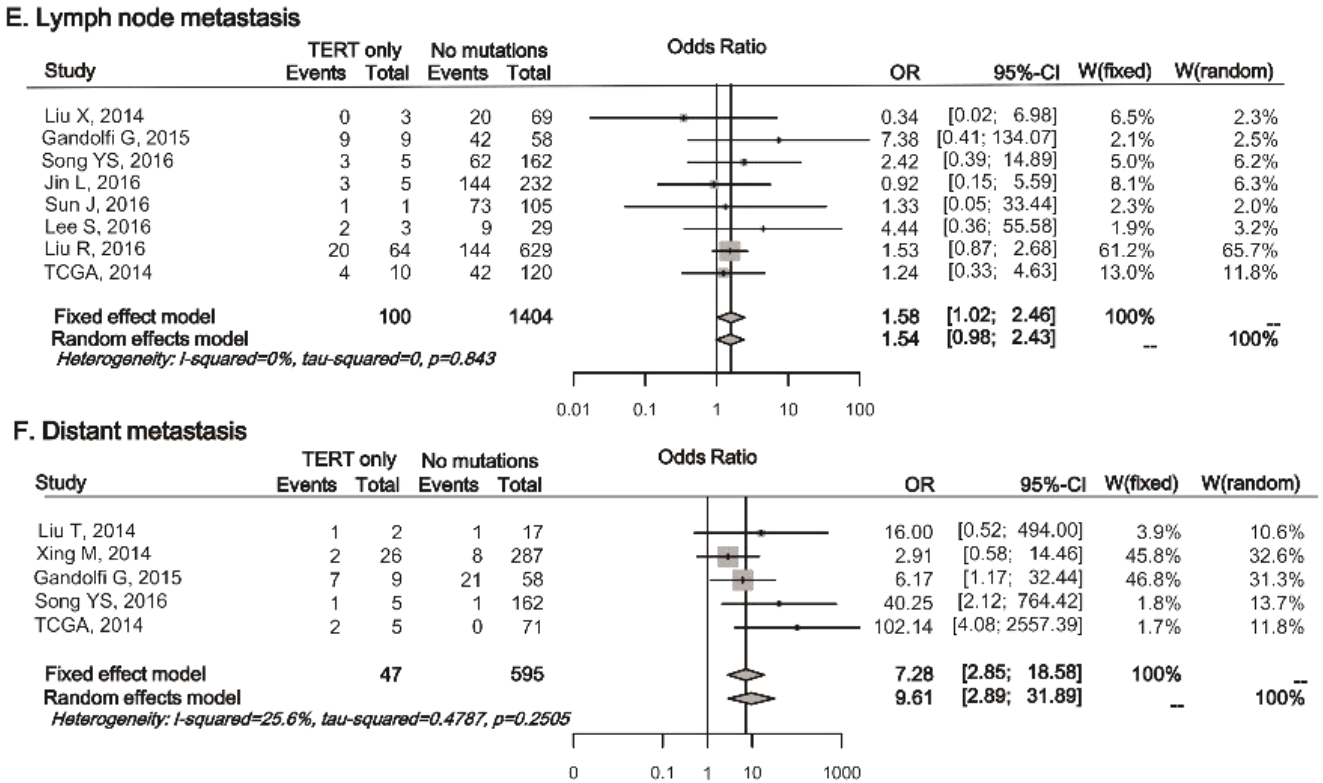
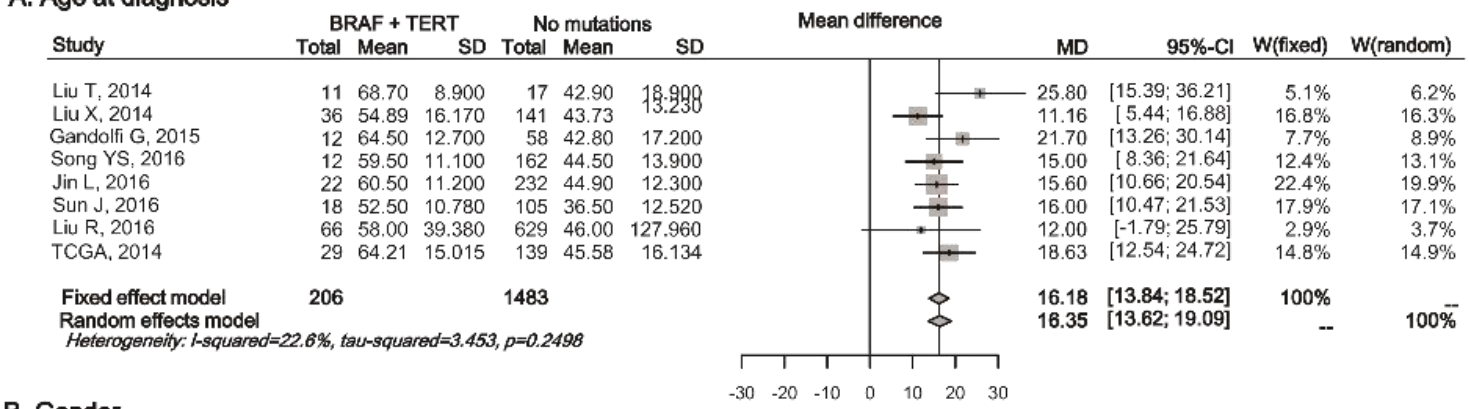


Figure 8. Forest plot showing the effects of *TERT* promoter mutation alone over no mutation on clinicopathological characteristics. The forest plot displays the effect size and 95% CIs for each study and overall.

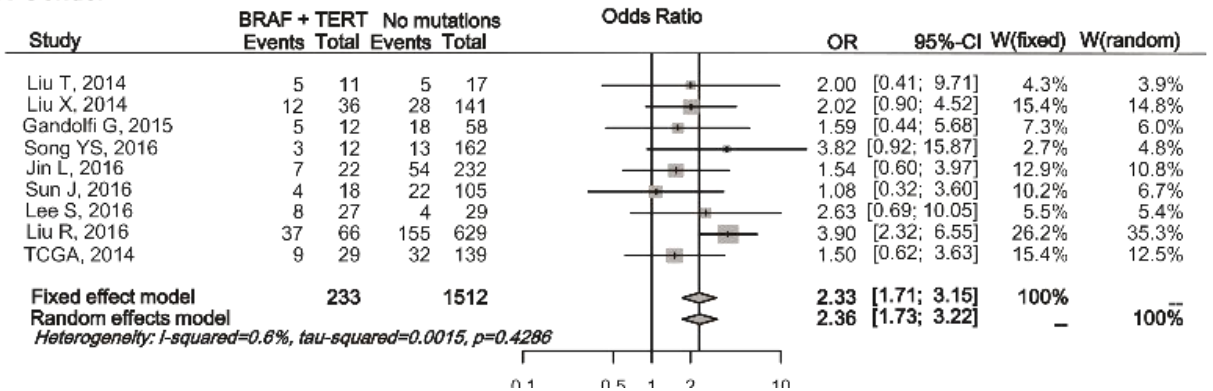
Effect of the coexistence of BRAF^{V600E} and TERT promoter mutations over no mutations on clinicopathological characteristics

The coexistence of *BRAF*^{V600E} and *TERT* promoter mutations was significantly associated with age at diagnosis (MD, 16.18; 95% CI, 13.84–18.52), gender (OR for male, 2.33; 95% CI, 1.71–3.15), advanced TNM stage (OR, 7.51; 95% CI, 5.38–10.48), extrathyroidal extension (OR, 8.14; 95% CI, 5.55–11.94), lymph node metastasis (OR, 2.94; 95% CI, 2.12–4.09), and distant metastasis (OR, 8.36; 95% CI, 4.13–16.95) as compared to no mutations (Fig. 9). No significant heterogeneity was found among these studies.

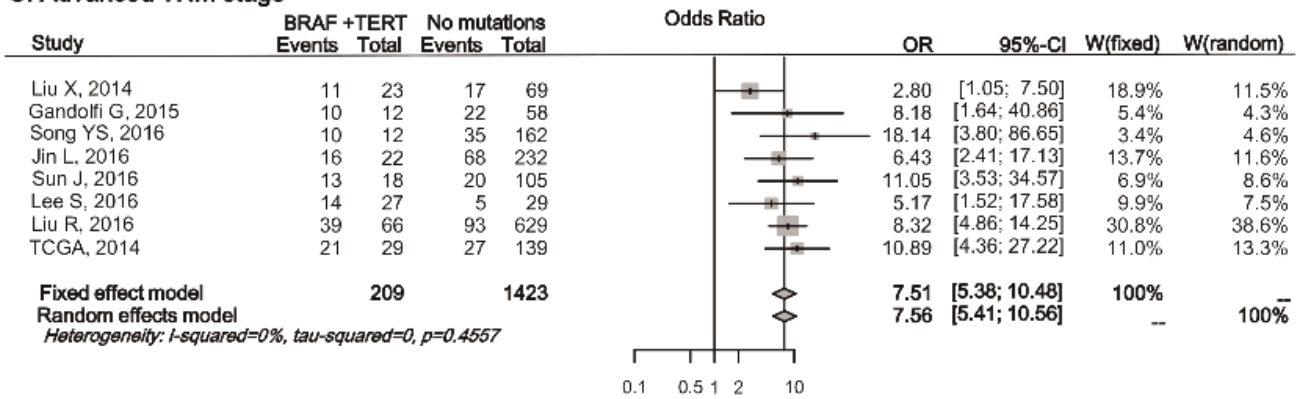
A. Age at diagnosis



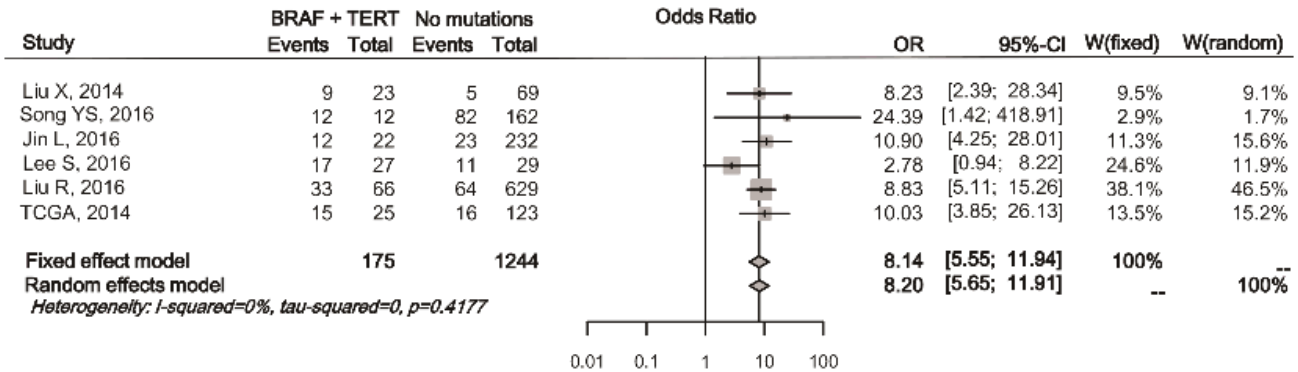
B. Gender



C. Advanced TNM stage



D. Extrathyroidal extension



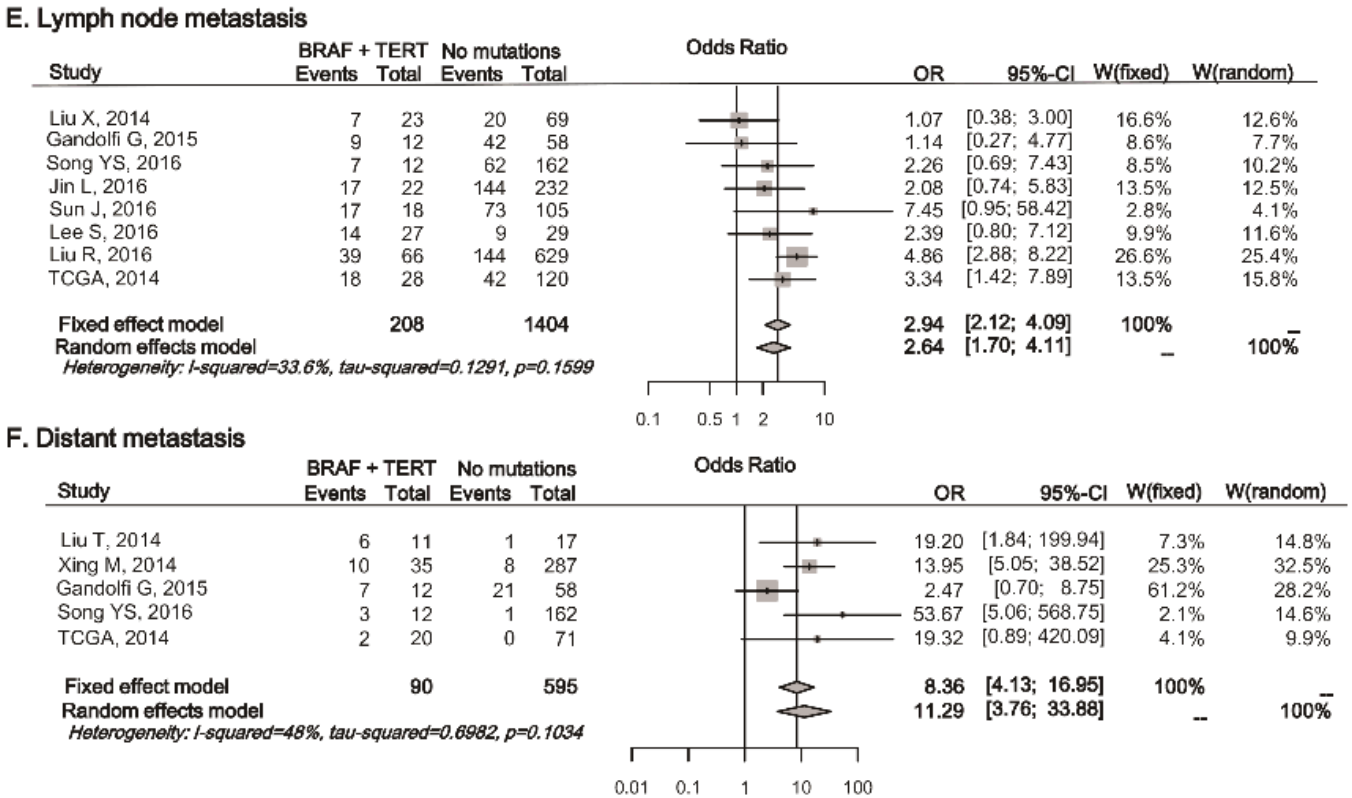
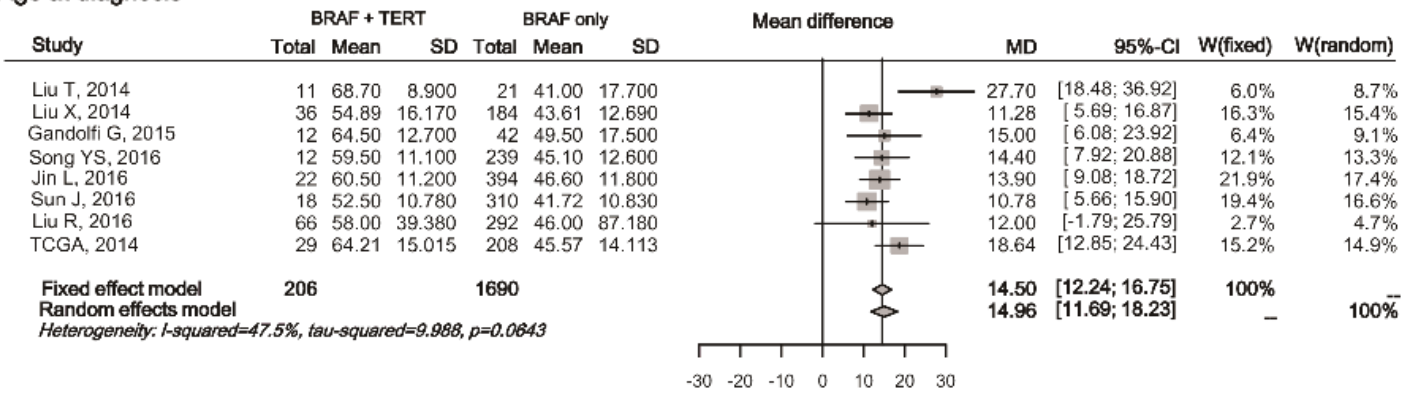


Figure 9. Forest plot showing the effects of the coexistence of *BRAF*^{V600E} and *TERT* promoter mutations over no mutations on clinicopathological characteristics. The forest plot displays the effect size and 95% CIs for each study and overall.

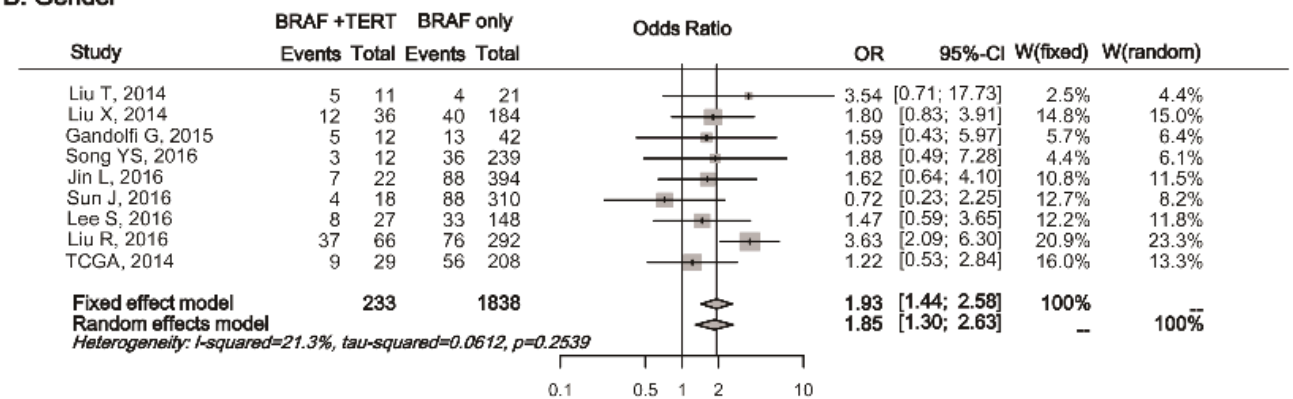
Effect of the coexistence of BRAF^{V600E} and TERT promoter mutations over BRAF^{V600E} mutation alone on clinicopathological characteristics

In comparison with *BRAF^{V600E}* mutation alone, the coexistence of *BRAF^{V600E}* and *TERT* promoter mutations was significantly associated with all variables including older age (MD, 14.50; 95% CI, 12.24–16.75), greater portion of male patients (OR, 1.93; 95% CI, 1.44–2.58), advanced TNM stage (OR, 4.19; 95% CI, 3.07–5.71), and higher risks of extrathyroidal extension (OR, 3.1; 95% CI, 2.2–4.37), lymph node metastasis (OR 1.59; 95% CI, 1.16–2.17), and distant metastasis (OR, 11.76; 95% CI, 5.63–24.58; Fig. 10). No significant heterogeneity was found among these studies.

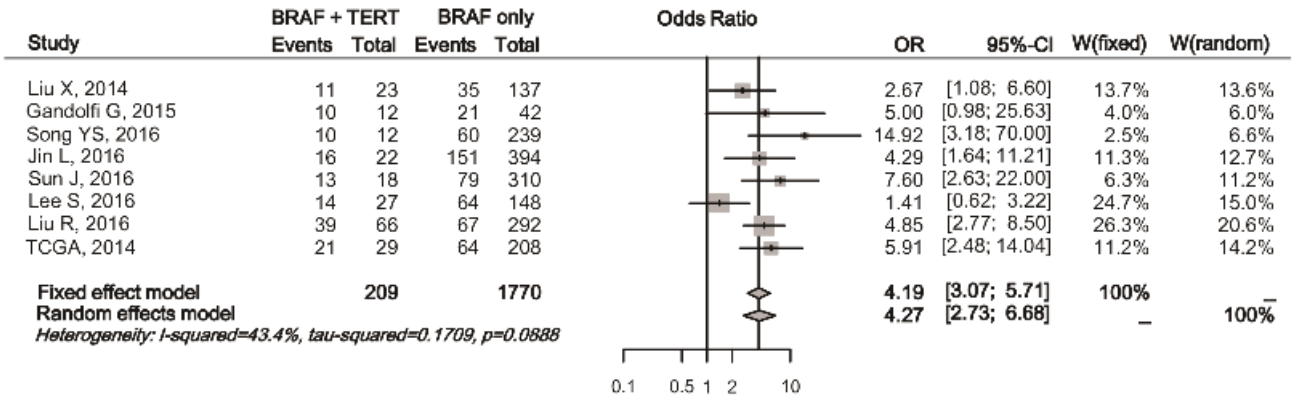
A. Age at diagnosis



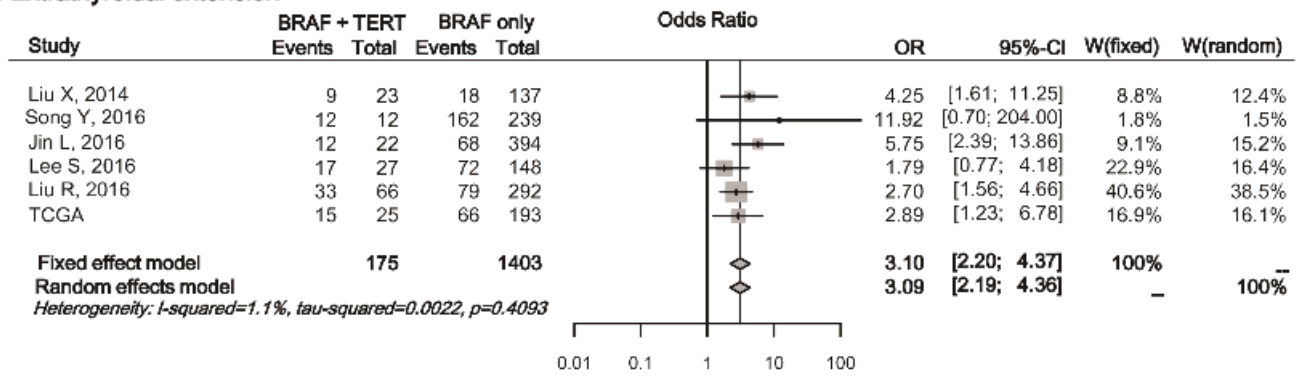
B. Gender



C. Advanced TNM stage



D. Extrathyroidal extension



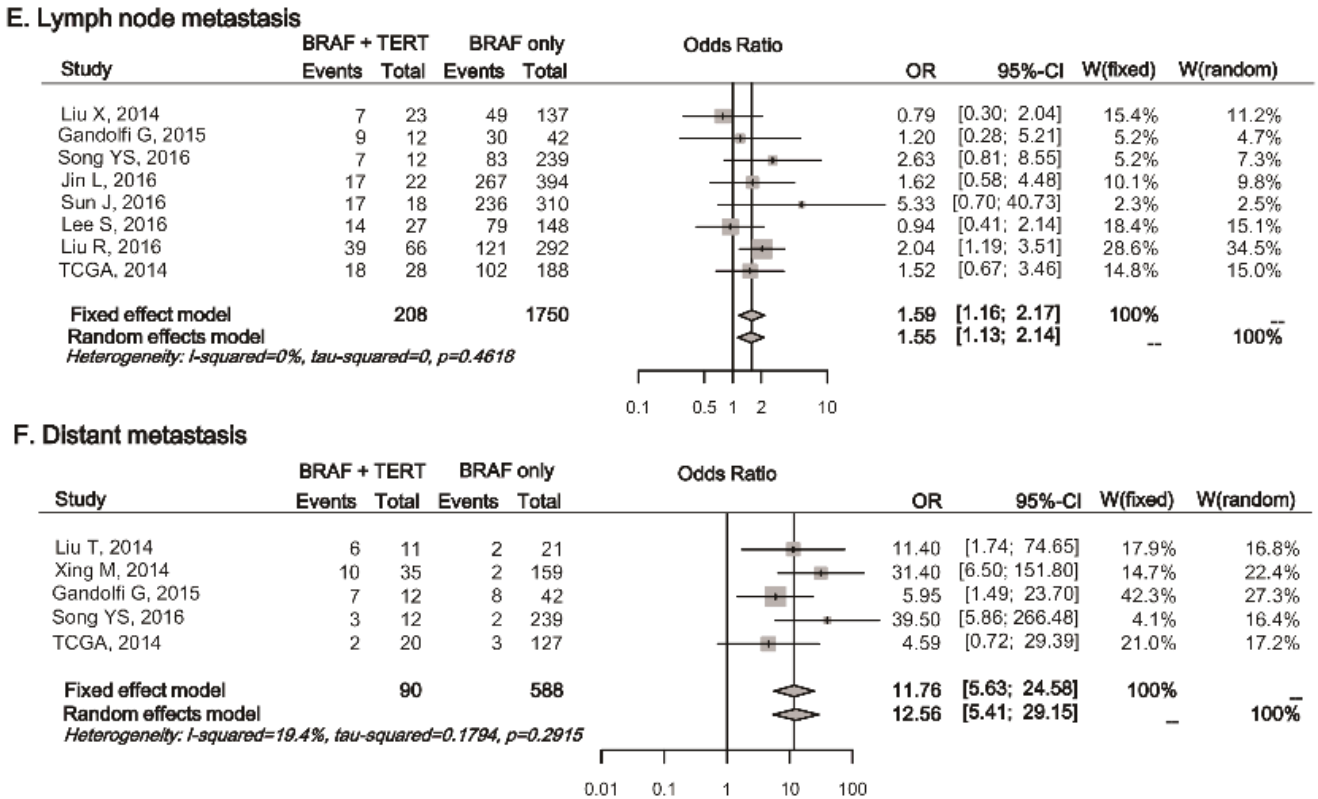
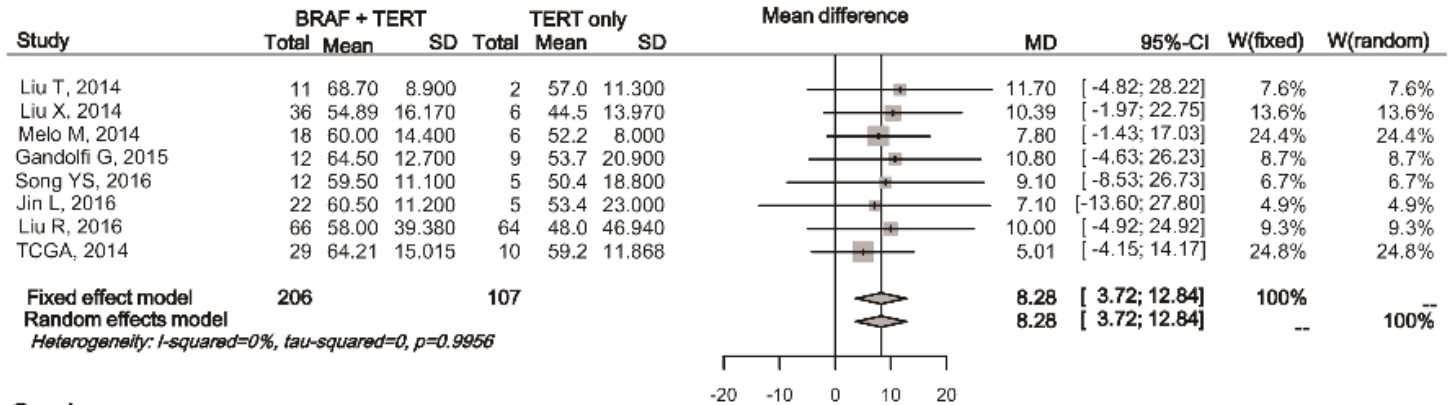


Figure 10. Effects of the coexistence of *BRAF*^{V600E} and *TERT* promoter mutations over *BRAF*^{V600E} mutation alone on clinicopathological characteristics. The forest plot displays the effect size and 95% CIs for each study and overall.

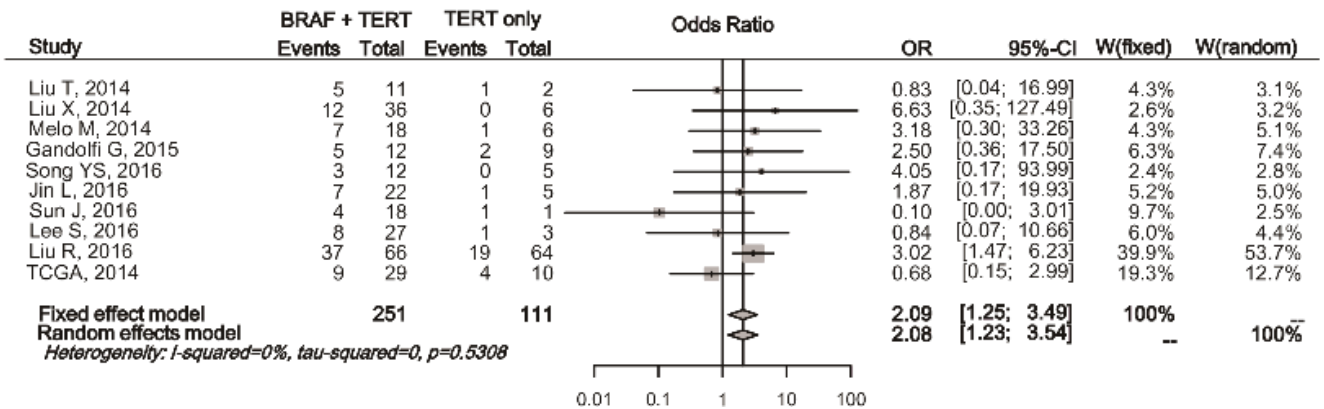
Effect of the coexistence of BRAF^{V600E} and TERT promoter mutations over TERT promoter mutation alone on clinicopathological characteristics

The coexistence of *BRAF^{V600E}* and *TERT* promoter mutations was significantly associated with age at diagnosis (MD, 8.28; 95% CI, 3.72–12.84), gender (OR for male, 2.09; 95% CI, 1.25–3.49), advanced TNM stage (OR, 4.66; 95% CI, 2.67–8.13), extrathyroidal extension (OR, 5.66; 95% CI, 3.02–10.6), and lymph node metastasis (OR, 2.03; 95% CI, 1.22–3.38) as compared to *TERT* promoter mutation alone. However, there was no significant difference in and distant metastasis between the two groups (Fig. 11). No significant heterogeneity was found among these studies.

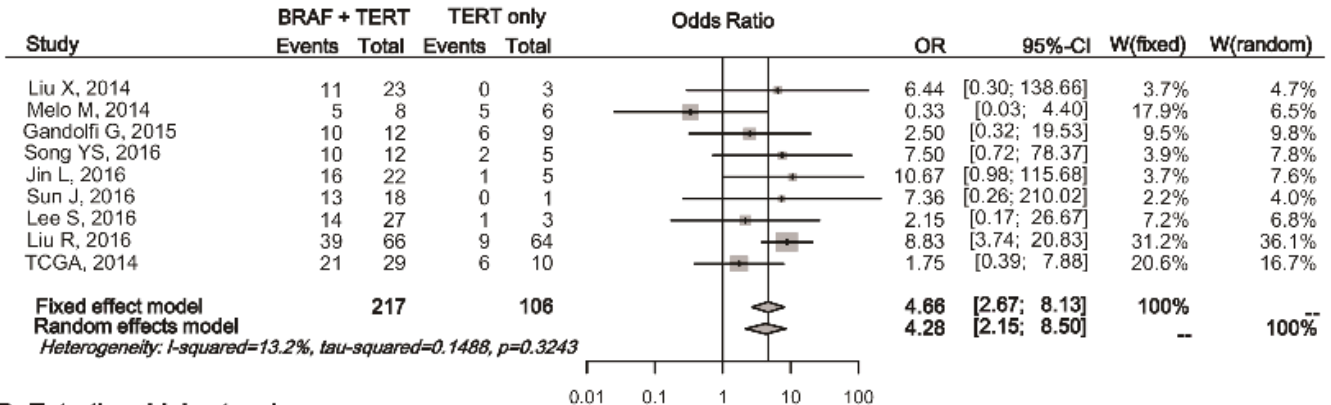
A. Age at diagnosis



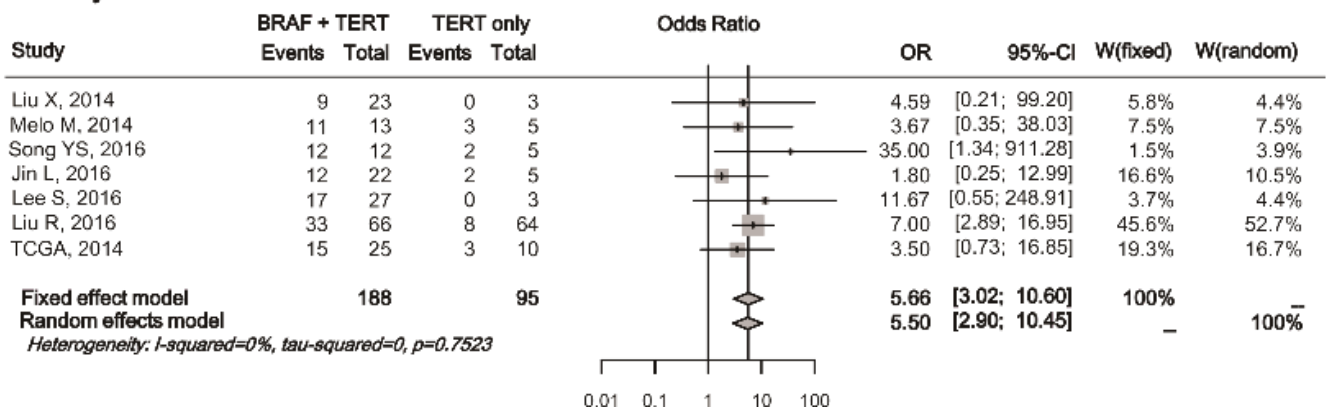
B. Gender



C. Advanced TNM stage



D. Extrathyroidal extension



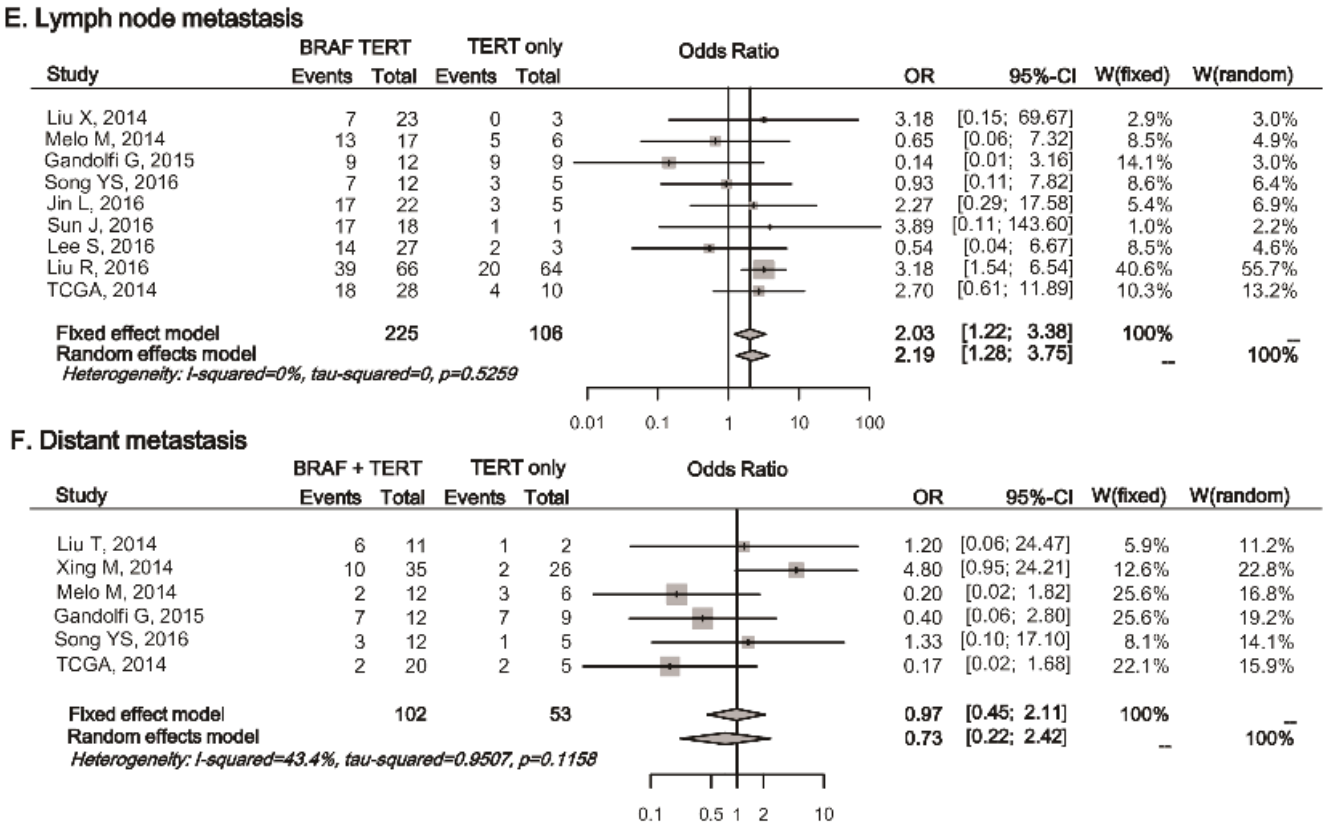


Figure 11. Effects of the coexistence of *BRAF*^{V600E} and *TERT* promoter mutations over *TERT* promoter mutation alone on clinicopathological characteristics. The forest plot displays the effect size and 95% CIs for each study and overall.

Risk effects of the long-term outcomes of BRAF^{V600E} mutation, TERT promoter mutation, or their coexistence

In comparison with the group negative for either mutation, the highest risk of recurrence of PTC was found in the coexistence of *BRAF^{V600E}* and *TERT* promoter mutations after adjustment for age and gender (the coexistence vs. no mutations, hazard ratio [HR], 6.60; 95% CI, 3.82–11.40; *BRAF^{V600E}* alone vs. no mutations, HR, 1.31; 95% CI, 0.49–3.46; *TERT* alone vs. no mutations, HR, 3.38; 95% CI, 0.85–13.35; Fig. 12). However, owing to limited data, I could not analyze the effects of the coexistence of two mutations over either mutation alone on recurrence.

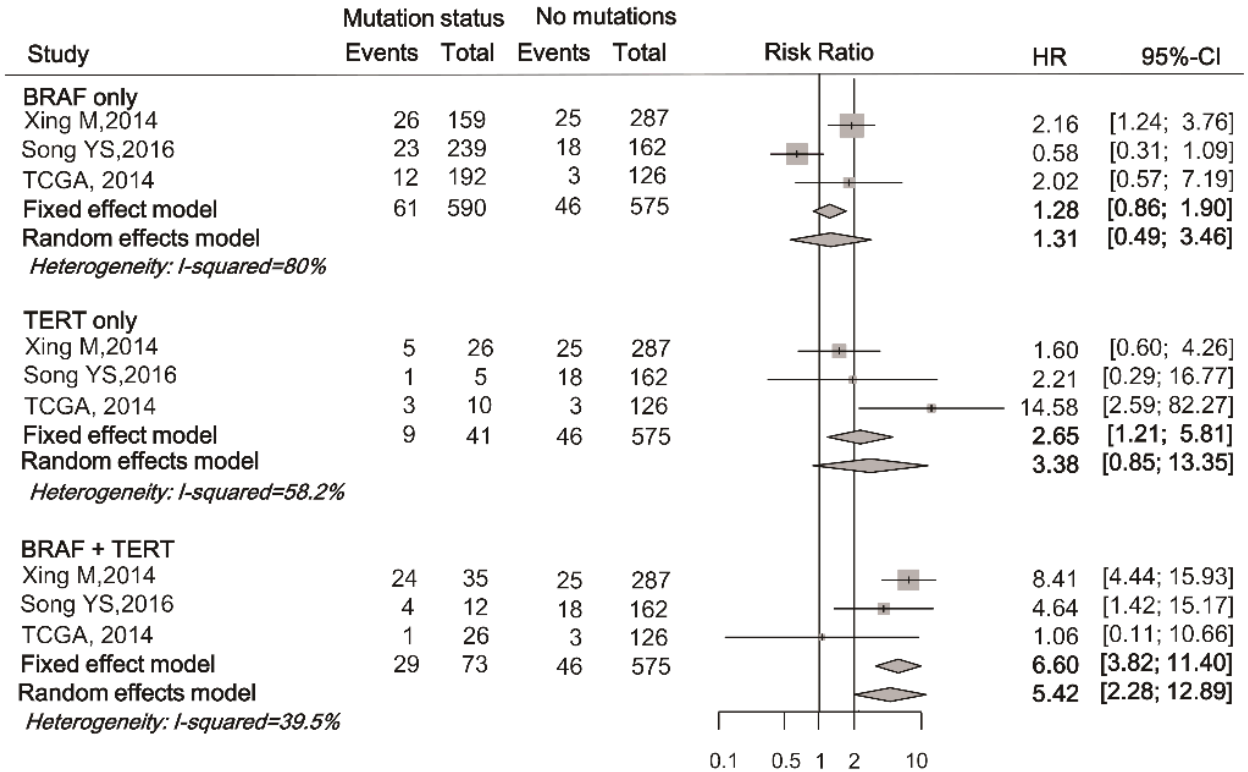


Figure 12. Forest plot showing individual and pooled hazard ratios of the recurrence of PTC in *BRAF*^{V600E} or *TERT* promoter mutation or their coexistence in comparison to no mutations with the adjustment for age at diagnosis and gender.

In the analyses for PTC-related mortality risk, TCGA data (The Cancer Genome Atlas Data Portal) was excluded because it has all-cause mortality, and two studies (Liu et al., 2013a; Song et al., 2016b) were included. The HR of *TERT* promoter mutation alone group could not be derived, since only one study (Liu et al., 2013a) had the events of death in that group. However, I could find that the coexistent $BRAF^{V600E}$ and *TERT* promoter mutations had a significantly higher PTC-related mortality than no mutations or $BRAF^{V600E}$ alone (the coexistence vs. no mutations, HR, 9.38; 95% CI, 2.81–31.29; $BRAF^{V600E}$ alone vs. no mutations, HR; 1.05; 95% CI, 0.29–3.76; the coexistence vs. $BRAF^{V600E}$ alone, HR; 20.07; 95% CI, 8.37–48.09, Fig. 13). Moreover, to confirm the synergistic effect of genetic duet on mortality, I further analyzed the ORs for mortality using five studies with PTC-related mortality rate (Bullock et al., 2016; Kim et al., 2016; Liu et al., 2016; Melo et al., 2014; Song et al., 2016b). The coexistence of the two mutations was more strongly associated with high-risk of mortality than either mutation alone, demonstrating a synergistic role of $BRAF^{V600E}$ and *TERT* promoter mutations (The coexistence vs. $BRAF^{V600E}$, OR, 17.46; 95% CI, 8.20–37.18; vs. *TERT*, OR, 2.81; 95% CI, 1.17–6.76, Fig. 14).

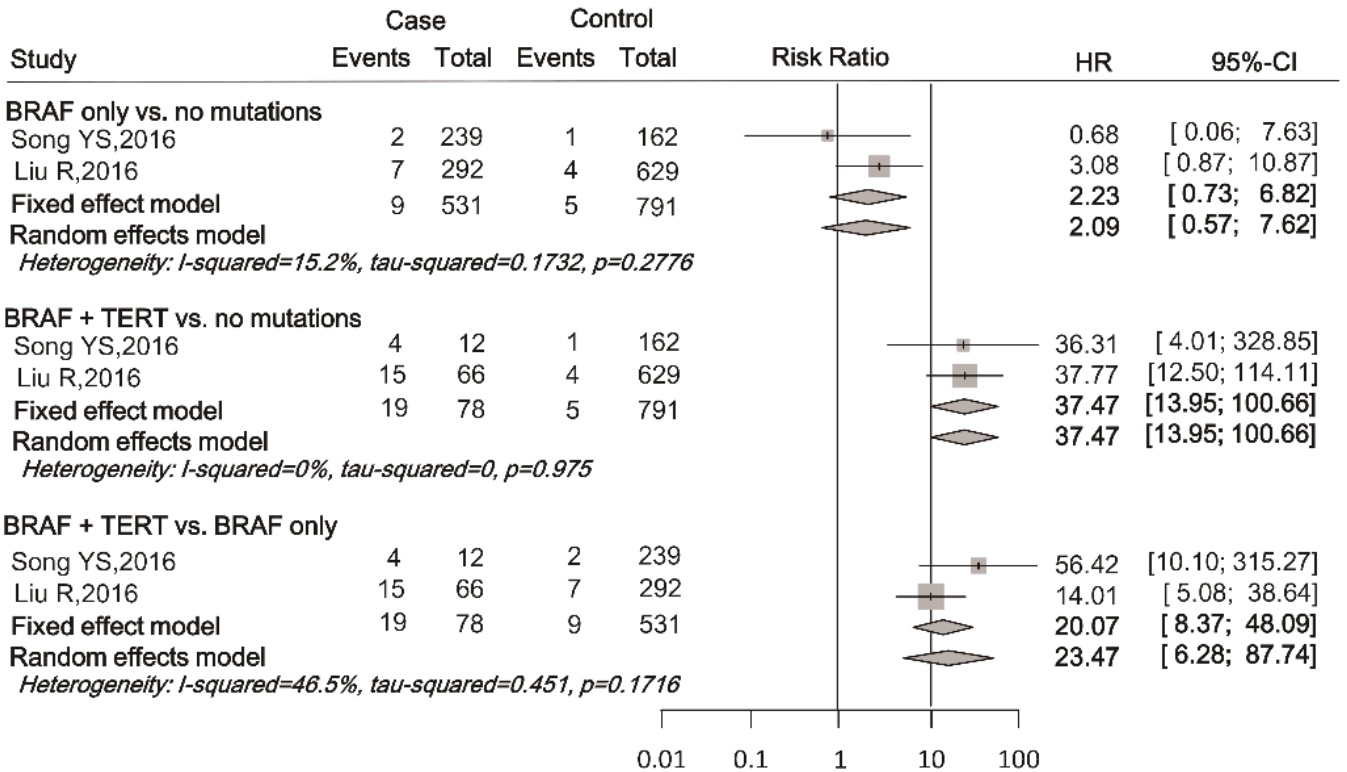


Figure 13. Forest plot showing individual and pooled hazard ratios of PTC-related mortality in *BRAF*^{V600E} mutation or the coexistence of *BRAF*^{V600E} and *TERT* promoter mutations.

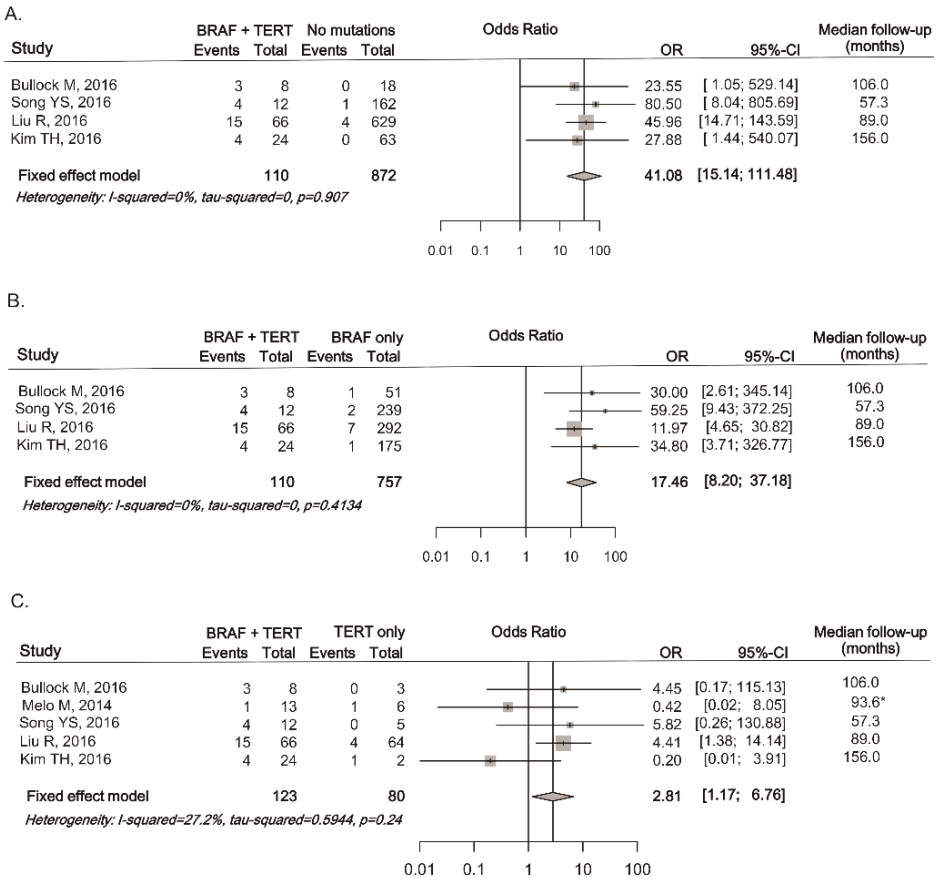


Figure 14. Forest plot showing the effects of the coexistence of *BRAF*^{V600E} and *TERT* promoter mutations over no mutations and either alone on PTC related mortality. The forest plot displays the effect size and 95% CIs for each study and overall. The coexistence vs. no mutations (A), the coexistence vs. *BRAF*^{V600E} mutation alone (B), the coexistence vs. *TERT* promoter mutation alone (C).

*Follow up duration was presented as mean

II-3. Molecular genetic mechanisms of synergistic interaction between *TERT* promoter and *BRAF* mutations

Materials and methods

Data acquisition and patient selection

From the genomic data on anonymized patients with PTC that are available from TCGA data portal (https://portal.gdc.cancer.gov/), I 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. DNA methylation analysis was performed by means of the Illumina Infinium HM450 array (Bibikova, et al. 2011), and methylation levels were quantified via β-values, which represent the proportion of methylation and range from 0 to 1. 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, 60 cases with other driver mutations and 57 cases with any driver fusions were then excluded (Table 11). 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, 2014). Finally, 270 patients with PTC were included in the present analysis, and I classified them by mutational status: 41 patients negative for the driver genetic alterations including *BRAF*^{V600E} and *TERT* promoter mutations (no mutation), 199 patients with the *BRAF*^{V600E} mutation only (*BRAF* only), 2 patients with a *TERT* promoter mutation only (*TERT* only), and 28 patients with coexistent *BRAF*^{V600E} and *TERT* promoter mutations (*BRAF*+*TERT*) (Fig. 15). For the comparison of differentially expressed gene (DEG), *TERT*-only group was excluded because of the small number of subjects in this group.

As a validation set of genomic analysis, 86 PTC samples having RNA sequencing data from our previous research (hereafter, SNU database; (Yoo et al., 2016)) were used in this study. In addition to the previous study, I performed Sanger sequencing for detection of *TERT* promoter mutation by a previously described method (Song et al., 2016a). After excluding 22 patients with other driver mutations and 8 with other driver fusions, 56 patients with PTC were included in the final analysis: 6 patients in the no-mutation group, 46 patients in the *BRAF*-only group, no patient in the *TERT*-only group, and 4 patients in the *BRAF*+*TERT* group (Fig. 15).

Table 11. List of driver mutations and fusions of excluded patients

List	N
Driver mutations	
BRAF, non-V600E	5
NRAS	31
HRAS	14
KRAS	3
EIF1AX	5
TSHR	1
CHEK2	1
Driver fusions	
AGK-BRAF	1
AKAP13-RET	1
ALK-MALAT1	1
ALK-STRN	1
AP3B1-BRAF	1
BRAF-CCNY	1
BRAF-ERC1	1
BRAF-FAM114A2	1
BRAF-MACF1	1
BRAF-MKRN1	1
BRAF-SND1	1
CCDC6-RET	15
ERC1-RET	2
ETV6-NTRK3	4
FGFR2-OFD1	1
FKBP15-RET	1
FLJ10661-THADA	1
GTF2IRD1-ALK	1
IGF2BP3-THADA	2
IRF2BP2-NTRK1	1
MET-TFG	1
NCOA4-RET	4
NTRK1-SQSTM1	1
NTRK1-SSBP2	1
NTRK1-TFG	1
PAX8-PPARG	4
RET-SPECC1L	1
RPS2P32-THADA	3
UACA-LTK	1
VCL-FGFR2	1

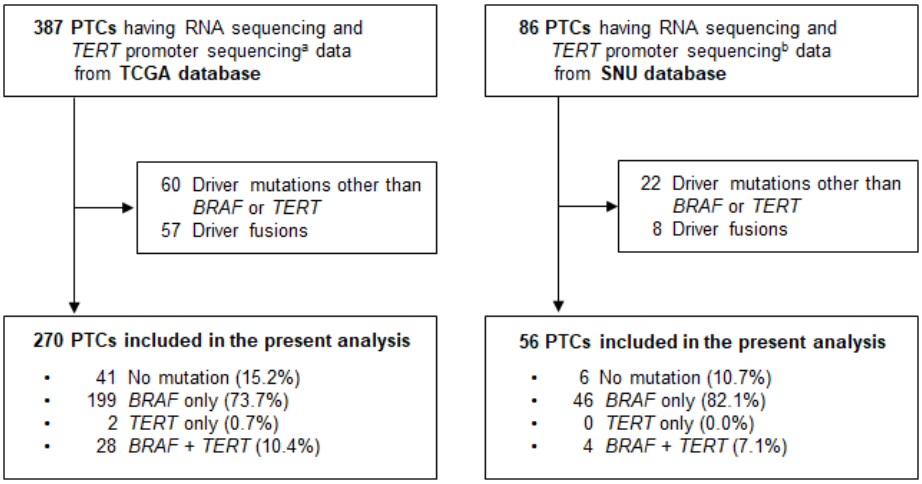


Figure 15. Flow chart and the mutational status of samples included in the study. [a]MiSeq or whole genome sequencing for *TERT* promoter region; [b]Sanger sequencing for *TERT* promoter region

Gene expression profiling and differentially expressed gene analysis

Using counted numbers of reads aligned to each gene, I normalized them via regularized log (rlog) transformation method of DESeq2 (Love et al., 2014). The DEGs were determined by DESeq2 to have *q*-value <0.05, $|\text{Log}_2$ (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. For heatmap display, 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, I used the Kyoto Encyclopedia of Genes and Genomes pathway database (Ogata et al., 1999). For comparison of mRNA expression in each group, normalized count data were subjected to the analyses.

Cell culture

Nthy-ori 3-1 (hereafter referred to as Nthy) is an immortalized thyroid follicular epithelial cell line derived from normal adult thyroid tissue, and Nthy cells expressing either wild-type *BRAF* (Nthy/WT) or mutant *BRAF* (Nthy/V600E) were established (Kim et al., 2017). BCPAP, a human PTC cell line harboring

both *BRAF*^{V600E} and *TERT* promoter mutations, were kindly provided by Dr. Minho Shong (Chungnam National University, Daejon, South Korea). All cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum and grown at 37 ℃ in a humidified 5% CO2 atmosphere.

RT-PCR analysis

The mRNA from the cultured Nthy/WT and Nthy/V600E cells was extracted by using TRIzol reagent (Invitrogen, Carlsbad, CA). First-strand cDNA was synthesized from 1 μg of total RNA using Superscript II reverse transcriptase (Invitrogen). Human *TERT*, *EHF*, *ELF3*, *ETV1*, *ETV4*, and *ETV5* gene expressions were quantified by PCR using a SYBR Green PCR master mix (Takara) and StepOne Plus real-time PCR system (Applied Biosystems, Foster City, CA). The primers used are listed in Table 12. The reaction was attempted to thermal cycling conditions as, 2 min at 95 ℃, 40 cycles of 15 s at 95 ℃, 1 min at 60 ℃.

BCPAP cells were treated with PLX4720 (10uM, Selleckchem, Houston, TX), a *BRAF* inhibitor. After 24 h from the treatment, the cells were harvested, and the mRNAs were extracted for the analysis of *TERT*, *EHF*, *ELF3*, *ETV1*, *ETV4*, and *ETV5* gene as described above.

Table 12. Sequences of PCR primers used to amplify each of the genes in RT–PCR

Gene		Primer sequence
TERT	Forward	5’-ATGCGACAGTTCGTGGCTCA-3’
	Reverse	5’-ATCCCCTGGCACTGGACGTA-3’
ETV1	Forward	5’-AGCTGAGATTTGCGAAGAGC-3’
	Reverse	5’-CTTCTGCAAGCCATGTTTCC-3’
ETV4	Forward	5’-CGCCTACGACTCAGATGTCA-3’
	Reverse	5’-CGCAGAGGTTTCTCATAGCC-3’
ETV5	Forward	5’-GACACAGATCTGGCTCACGA-3’
	Reverse	5’-GGCATGAAGCACCAGGTTAT-3’
ELF3	Forward	5’-CAACTATGGGGCCAAAAGAA-3’
	Reverse	5’-GAGTGGTCCGTGAGTTTGGT-3’
EHF	Forward	5’-AACCACCAGTCACCTTCCTG-3’
	Reverse	5’-GGGTTCTTGTCTGGGTTCAA-3’

Statistical analysis

To compare the clinicopathological characteristics of subjects according to mutational status, the Pearson's χ^2 or Fisher's exact test (if the number was <5) was used for categorical variables and analysis of variance for continuous variables. A post-hoc Bonferroni test were used to determine which groups have statistically different characteristics. I compared *TERT* promoter methylation according to mutational status using the Student's t test and investigated associations between the methylation and *TERT* expression with the Spearman's correlation test. The paired t-test was used to compare differences in the expression levels of each common DEG between *BRAF*-only and *BRAF+TERT* groups or between $BRAF^{V600E}$ mutation with *TERT* expression and without *TERT* expression groups. Statistical significance was defined as 2-sided *P* values <0.05. All statistical analyses were performed with SPSS 20.0 (IBM Corp., Armonk, NY, USA) or R v3.1.0 (www.r-project.org).

Results

Effects of coexistence of BRAF^{V600E} and TERT promoter mutations on poor clinicopathological outcomes of PTC in the dataset for genomic analysis

To identify the mechanism of interaction between *BRAF^{V600E}* and *TERT* promoter mutations in PTC, I performed the genomic analyses using public repository database of TCGA and our database as a validation set. The clinicopathological characteristics of subjects are presented in Table 13. 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, a higher proportion of extrathyroidal extension, especially for moderate or advanced invasion, lymph node metastasis, 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.

Table 13. Clinicopathological characteristics of subjects according to *BRAF*^{V600E} and *TERT* promoter mutational status

	TCGA				SNU			
	No mutation	*BRAF* only	*BRAF+TERT*	*P*	No mutation	*BRAF* only	*BRAF+TERT*	*P*
	41	199	28		6	46	4	
Male sex, n (%)	6/41 (14.6)	53 (26.6)	9/28 (32.1)	0.188	1 (16.7)	10 (21.7)	2 (50.0)	0.389
Age at diagnosis, years	52.7 ± 13.4	45.6 ± 14.1[b]	65.3 ± 14.0[b,c]	<0.001	52.8 ± 11.4	45.9 ± 12.1	52.5 ± 10.3	0.273
Histology, n (%)								
Conventional	16/37 (43.2)	155/190 (81.6)[b]	17/24 (70.8)[b]		2 (33.3)	40 (84.0)[b]	3 (75.0)	0.011
Follicular variant	20/37 (54.1)	11/190 (5.8)[b]	1/24 (4.2)[b]		4 (66.7)	6 (13.0)[b]	1 (25.0)	
Tall cell variant	0/37 (0.0)	21/190 (11.1)[b]	6/24 (25.0)[b,c]		-	-	-	
Other type	1/37 (2.7)	3/190 (1.6)	0/24 (0.0)		-	-	-	
Tumor size, cm[a]	3.1 (1.9–4.5)	2.5 (1.5–3.5)	3.0 (2.4 –4.5)[c]	0.007	2.0 (0.8-4.5)	1.0 (0.7-1.4)[b]	1.9 (1.2-2.1)[c]	0.014
Extrathyroidal extension, n (%)	3/34 (8.8)	65/185 (35.1)[b]	15/24 (62.5)[b,c]	<0.001	1 (16.7)	27 (58.7)	4 (100.0)[b]	0.028
Minimal	3/34 (8.8)	63/185 (34.1)[b]	8/24 (33.3)[b]		1 (16.7)	18 (39.1)	4 (100.0)[b]	
Moderate/advanced	0/34 (0.0)	2/185 (1.1)	7/24 (29.2)[b,c]		0 (0.0)	9 (19.6)	0 (0.0)	
Multifocalty, n (%)	21/40 (52.5)	89/196 (45.4)	12/28 (42.9)	0.666	0 (0.0)	12 (26.1)	3 (75.0)[b]	0.030
Lymph node metastasis, n (%)	6/33 (18.2)	99/179 (55.3)[b]	17/27 (63.0)[b]	<0.001	0 (0.0)	15 (32.6)	2 (50.0)	0.187
Distant metastasis, n (%)	0/17 (0.0)	3/121 (2.5)	2/19 (10.5)	0.198	0 (0.0)	1 (2.2)	0 (0.0)	1.000
ATA stage, n (%)				<0.001				0.073
Low risk	24/36 (66.7)	55/186 (29.6)[b]	5/24 (20.8)[b]		4 (66.7)	18 (39.1)	0 (0.0)	
Intermediate risk	12/36 (33.3)	125/186 (67.2)[b]	12/24 (50.0)		1 (16.7)	26 (56.5)	4 (100.0)	
High risk	0/36 (0.0)	6/186 (3.2)	7/24 (29.2)[b,c]		1 (16.7)	2 (4.3)	0 (0.0)	
TNM stage, n (%)				<0.001				0.309
I–II	31 (75.6)	134 (67.3)	7 (25.0)[b,c]		4 (66.7)	31 (67.4)	1 (25.0)	
III–IV	10 (24.4)	65 (32.7)	21 (75.0)[b,c]		2 (33.3)	15 (32.6)	3 (75.0)	
MACIS score	5.5 ± 1.1	5.2 ± 1.4	7.5 ± 1.8[b,c]	<0.001	5.6 ± 0.9	4.8 ± 1.1	5.7 ± 0.9	0.097

[a]Data presented as medians (interquartile ranges); [b]Significantly different from the no-mutation group; [c]Significantly different from the *BRAF*-only group

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

To identify the mechanism for the clinical aggressiveness of tumors harboring both *$BRAF^{V600E}$* and *TERT* promoter mutations, I examined the mRNA expression level of *TERT* using TCGA database (Fig. 16). Compared with the no-mutation group, *BRAF*-only or *TERT*-only group did not show a significant difference in *TERT* expression (fold change, 2.27 and 1.57, respectively; *q*-value = 0.108 and 0.866, respectively), whereas the *BRAF*+*TERT* group showed significantly higher *TERT* expression (fold change, 17.00; *q*-value = 1.36×10^{-13}; Fig. 16A). Nevertheless, it was unexpectedly found that the mRNA expression of *TERT* was detected in 25.1% of tumors in the *BRAF*-only group (50 of 199), and 14.6% of tumors in the no-mutation group (6 of 41; Fig. 16B). 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 (Table 14).

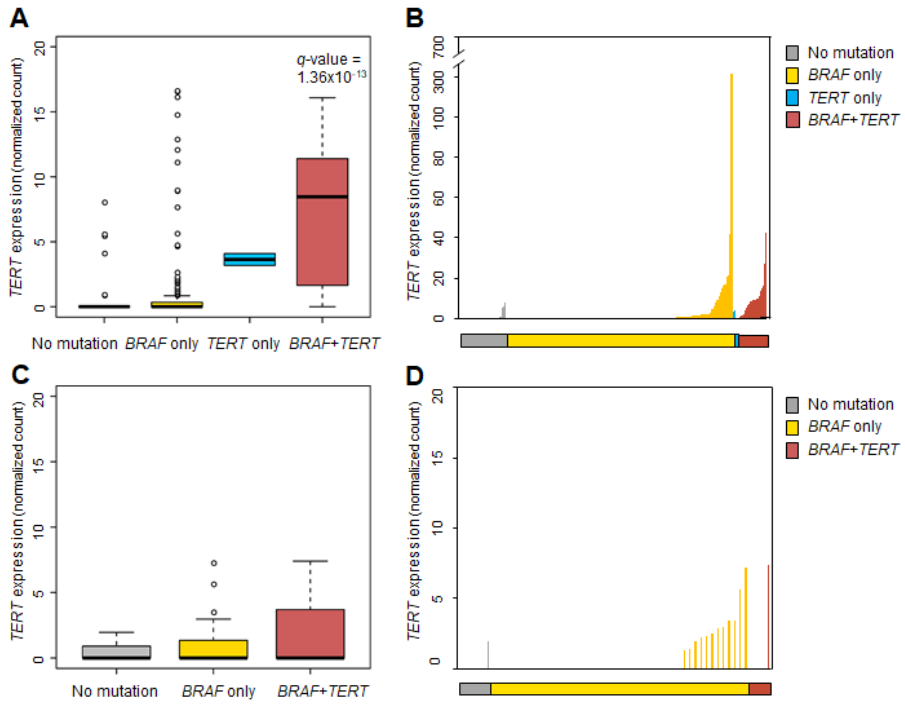


Figure 16. *TERT* mRNA expression according to mutational status. *TERT* mRNA expression levels from RNA sequencing data of TCGA (A, B) and SNU (C, D) database. (A, C) Median expression levels of *TERT* expression according to mutational status. (B, D) Each column represents an individual sample.

Table 14. Clinicopathological characteristics of subjects from TCGA database according to *TERT* expression status in *BRAF*-only group

Variable	*BRAF* only		P
	TERT expression (-)	*TERT* expression (+)	
N	149	50	
Follow-up duration, months[a]	22.5 (14.8–48.6)	27.3 (17.0–50.5)	0.292
Male sex, n (%)	37/149 (24.8)	16/50 (32.0)	0.321
Age at diagnosis, years	44.9 ± 13.3	47.7 ± 16.3	0.289
Histology, n (%)			0.115
Conventional	121/143 (84.6)	34/47 (72.3)	
Follicular variant	8/143 (5.6)	3/47 (6.4)	
Tall cell variant	13/143 (9.1)	8/47 (17.0)	
Other type	1/143 (0.7)	2/47 (4.3)	
Tumor size, cm[a]	2.2 (1.5–3.5)	2.8 (1.6–4.3)	0.144
Extrathyroidal extension, n (%)	51/140 (36.4)	14/45 (31.1)	0.516
Minimal	50/140 (35.7)	13/45 (28.9)	
Moderate/advanced	1/140 (0.7)	1/45 (2.2)	
Multifocalty, n (%)	67/146 (45.9)	22/50 (44.0)	0.817
Lymph node metastasis, n (%)	62/131 (47.3)[b]	37/48 (77.1)	<0.001
Distant metastasis, n (%)	2/94 (2.1)	1/27 (3.7)	0.535
Recurrence, n (%)	4/139 (2.9)	8/45 (17.8)	0.002
Disease-free survival, months[a]	22.6 (15.3–49.6)	25.8 (15.4–41.4)	0.784
Death, n (%)	1/148 (0.7)	3/50 (6.0)	0.050
Overall survival, months[a]	22.5 (14.8–48.6)	27.3 (17.0–50.5)	0.292
ATA stage, n (%)			0.567
Low risk	44/141 (31.2)	11/45 (24.4)[b]	
Intermediate risk	93/141 (66.0)	32/45 (71.1)[b]	
High risk	4/141 (2.8)	2/45 (4.4)	
TNM stage, n (%)			0.352
I–II	103/149 (69.1)	31/50 (62.0)	
III–IV	46/149 (30.9)	19/50 (38.0)	
MACIS score	5.5 ± 1.1	5.1 ± 1.3	0.144

[a]Data presented as medians (interquartile ranges)

These results were similar for PTC tumors of the SNU dataset, although statistical significance was absent due to the limited number of subjects (Fig. 16, C and D; Table 15).

Table 15. Clinicopathological characteristics of subjects from SNU database according to *TERT* expression status in *BRAF*-only group

Variable	*BRAF* only		P
	TERT expression (-)	*TERT* expression (+)	
N	34	12	
Male sex, n (%)	9 (26.5)	1 (8.3)	0.252
Age at diagnosis, years	44.0 ± 12.6	51.4 ± 8.6	0.066
Histology, n (%)			1.000
Conventional	29 (85.3)	11 (91.7)	
Follicular variant	5 (14.7)	1 (8.3)	
Tumor size, cm[a]	0.9 (0.7-1.5)	1.1 (0.6-1.2)	0.544
Extrathyroidal extension, n (%)	20 (58.8)	7 (58.3)	1.000
Minimal	12 (35.3)	6 (50.0)	
Moderate/advanced	8 (23.5)	1 (8.3)	
Multifocalty, n (%)	9 (26.5)	3 (25.0)	1.000
Lymph node metastasis, n (%)	11 (32.4)	4 (33.3)	1.000
Distant metastasis, n (%)	1 (2.9)	0 (0.0)	1.000
ATA stage, n (%)			1.000
Low risk	13 (38.2)	5 (41.7)	
Intermediate risk	19 (55.9)	7 (58.3)	
High risk	2 (5.9)	0 (0.0)	
TNM stage, n (%)			0.165
I–II	25 (73.5)	6 (50.0)	
III–IV	9 (26.5)	6 (50.0)	
MACIS score	4.7 ± 1.1	5.0 ± 0.9	0.462

[a]Data presented as medians (interquartile ranges)

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

Transcription of *TERT* can be regulated by its promoter site, such as modulation of methylation status or 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, first, the analysis of *TERT* promoter methylation was performed. I compared *TERT* promoter methylation status at 3 CpG sites available in TCGA database, which were located upstream of the transcription start site of *TERT* (chromosome 5: 1,295,737–1,295,988; cg26006951, cg17166338, and cg11625005). The expression of *TERT* mRNA correlated positively with two hypomethylated CpG sites of the *TERT* promoter (cg11625005 r = 0.412, $P < 0.001$; cg26006951 r = 0.164, $P = 0.006$; Fig. 17A). In the *BRAF+TERT* group, methylation levels of the CpG sites slightly increased, but the statistical significance was observed only for one CpG site of cg26006951 as compared with the no-mutation ($P = 0.001$) or *BRAF*-alone group ($P < 0.001$) even without hypermethylation (Fig. 17, B-D).

Next, I 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 (Fig. 17E; Table 16). 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.

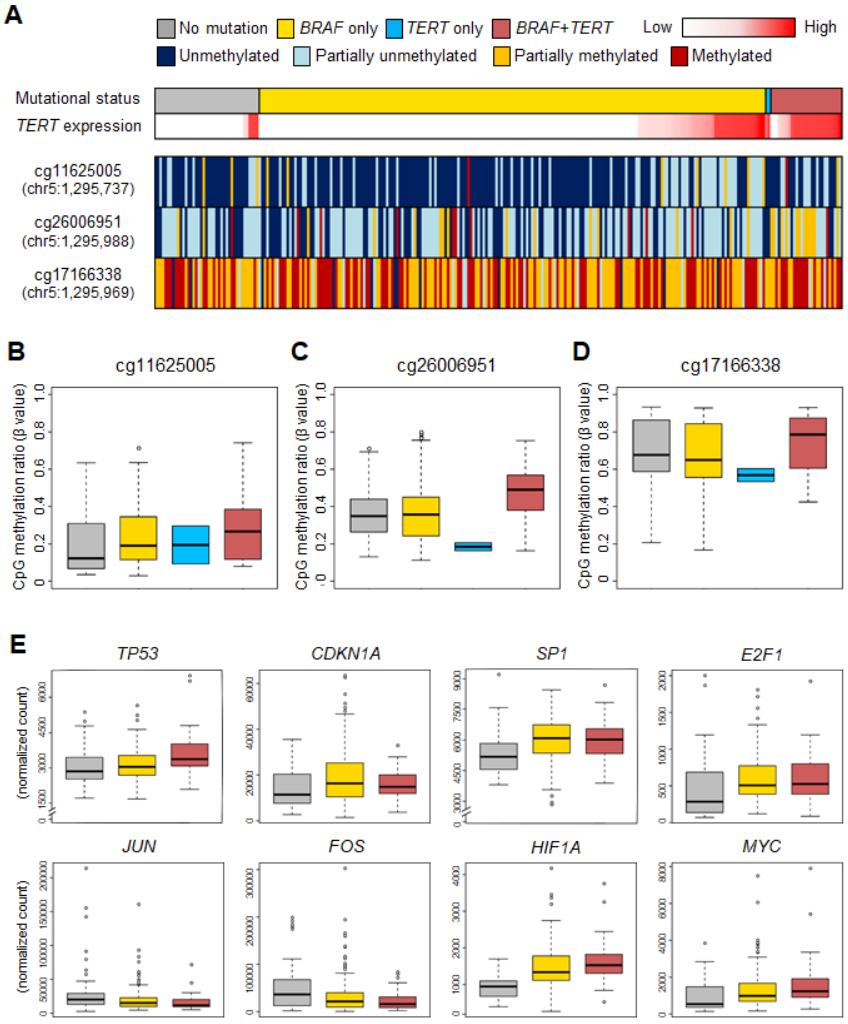


Figure 17. Molecular mechanisms of upregulated *TERT* expression. (A-D) Methylation status of *TERT* promoter according to the mutational status. The 3 CpG sites of *TERT* promoter were available in TCGA database and methylation levels were quantified using β-values ranging from 0 to 1. (A) The methylation status was defined as follows: >0.7, methylated (red); 0.5 to 0.7, partially methylated (orange); 0.3 to <0.5, partially unmethylated (cyan); and <0.3, unmethylated (blue). (B-D) Median methylation level of each CpG site according to mutational status. (E) Transcription factors containing recognition sequences at the *TERT* promoter site. Median expression level of each gene of the transcription factor according to mutational status, presented as normalized count value.

Table 16. Clinicopathological characteristics of subjects from SNU database according to *TERT* expression status in *BRAF*-only group

	No mutation vs. *BRAF* only			No mutation vs. *BRAF+TERT*		
	q-value	$\|Log_2$ (FC)$\|$	baseMean	*q*-value	$\|Log_2$ (FC)$\|$	baseMean
TCGA						
TP53	0.762	0.03	3155.22	0.037	0.25	3069.08
CDKN1A	0.011	0.42	18354.28	0.634	0.13	13576.41
SP1	8.71×10^{-4}	0.17	5969.68	0.012	0.20	5246.62
E2F1	0.021	0.36	582.24	0.203	0.40	488.47
JUN[*]	4.30×10^{-6}	-0.82	22575.60	5.81×10^{-4}	-1.04	25365.84
FOS[*]	0.004	-0.73	37776.08	0.003	-1.09	40265.23
HIF1A	3.12×10^{-12}	0.66	13795.42	8.87×10^{-10}	0.81	11195.14
MYC	0.034	0.37	1240.48	0.003	0.90	1247.56
SNU						
TP53	0.465	0.12	788.36	0.754	-0.10	781.70
CDKN1A	0.795	0.12	3743.58	0.879	-0.13	3655.45
SP1	0.003	0.44	2188.50	0.100	0.57	2194.86
E2F1	0.599	0.25	166.46	0.900	0.12	160.85
JUN[*]	0.063	-0.93	4986.88	0.003	-1.95	6843.07
FOS[*]	0.052	-1.22	7250.94	2.39×10^{-4}	-2.48	11780.48
HIF1A	2.58×10^{-4}	0.72	4987.89	0.007	0.89	4699.44
MYC	0.808	0.11	294.25	0.668	-0.36	270.68

Abbreviation: FC, fold change

[*] Differentially expressed gene, which is determined by DESeq2 to have *q*-value <0.05, $|Log_2$ (fold change)$| \geq 1$, and baseMean ≥ 100, of *BRAF*-only group compared to no-mutation group

Then, to evaluate the previously suggested mechanism, I determined whether the *BRAF*^{V600E} mutation upregulates *TERT* by increasing *ETS* expression because a *TERT* promoter mutation creates a binding motif for ETS transcription factors (Hoang et al., 2007; Horn 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 3 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. 18A and B; Fig. 19A; Table 17). When I analyzed 55 PTC samples of the SNU dataset, similar results were obtained: *EHF*, *ELF3*, *ETV1*, *ETV4*, and *ETV5* in the *BRAF+TERT* group and *EHF*, *ELF3*, *ETV7*, *ELF4*, *ETV1*, *ETV4*, and *ETV5* in the *BRAF*-only group were found to be upregulated DEGs (Fig. 18C; Fig. 19B; Table 18).

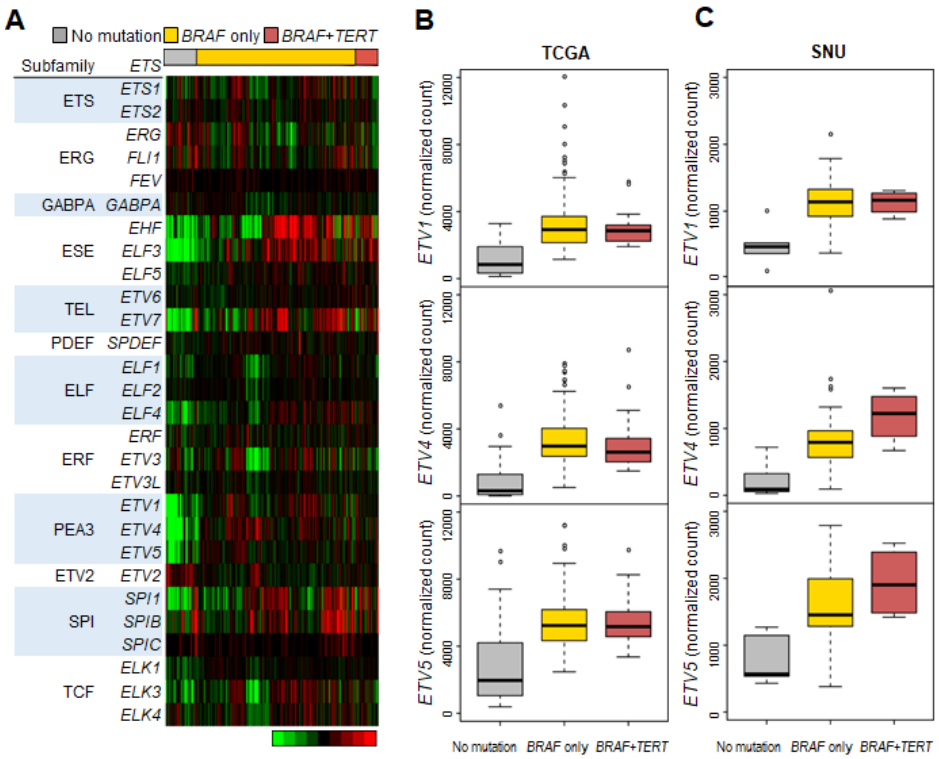


Figure 18. Upregulated expression of *ETV1*, *ETV4*, and *ETV5* by the *BRAF*^{V600E} mutation. (A) Heatmap showing discriminatory *ETS* genes according to mutational status of TCGA database. (B, C) Median expression level of *ETV1*, *ETV4*, and *ETV5* according to mutational status in TCGA (B) and SNU (C) database.

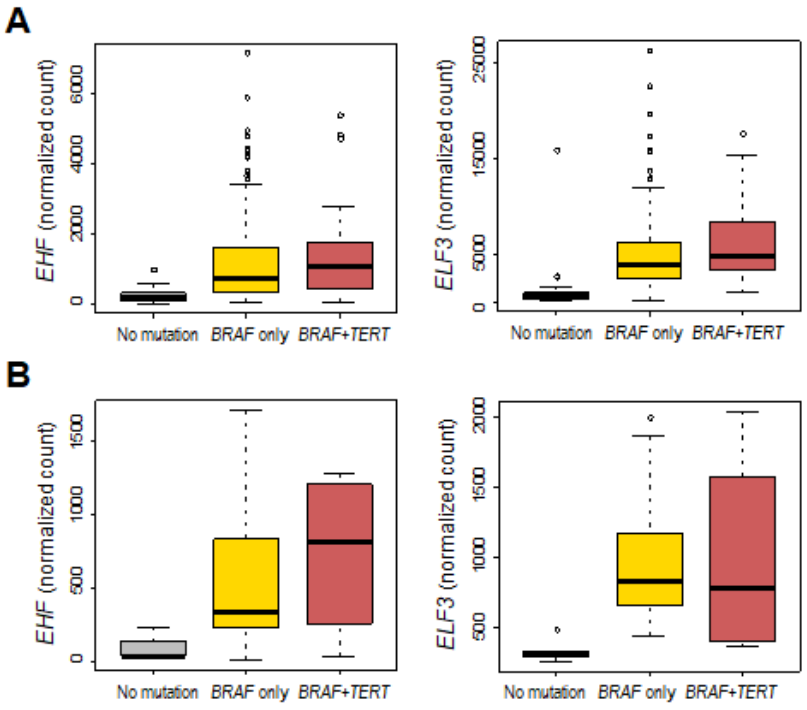


Figure 19. Changes in the *EHF* and *ELF3* expression by the *BRAF*^{V600E} mutation. (A, B) Median expression levels of *EHF* and *ELF3* according to mutational status in TCGA (A) and SNU (B) database.

Table 17. Changes in *ETS* gene expression in *BRAF*-only and *BRAF+TERT* groups compared to no-mutation group of TCGA databse

Subfamily	Gene	No mutation vs. *BRAF* only			No mutation vs. *BRAF+TERT*		
		q-value	$\|Log_2$ (FC)$\|$	baseMean	*q*-value	$\|Log_2$ (FC)$\|$	baseMean
ETS	*ETS1*	0.211	0.17	7958.02	0.940	-0.03	6546.49
	ETS2	0.858	0.02	4864.44	0.967	-0.01	4379.03
ERG	*ERG*	6.38×10^{-5}	-0.51	910.36	0.011	-0.56	957.01
	FLI1	0.004	-0.35	1055.51	0.078	-0.41	1052.94
	FEV	0.079	-0.88	0.49	0.579	-0.44	0.69
GABPA	*GABPA*	0.024	-0.15	2386.01	0.082	-0.18	2256.82
ESE	*EHF**,†	7.00×10^{-23}	2.35	1029.19	1.39×10^{-15}	2.68	708.14
	ELF3*,†	3.40×10^{-56}	2.53	4499.49	3.53×10^{-15}	2.38	3112.57
	ELF5	2.43×10^{-12}	1.87	6.49	8.23×10^{-9}	2.63	6.27
TEL	*ETV6*	0.246	0.07	2473.01	0.006	0.27	2357.05
	ETV7	5.26×10^{-4}	0.83	311.69	0.076	0.83	231.06
PDEF	*SPDEF*	8.62×10^{-5}	0.90	7.63	0.265	0.44	4.62
ELF	*ELF1*	0.002	0.26	5268.59	0.171	0.20	4400.89
	ELF2	0.578	0.04	1880.80	0.684	0.04	1704.58
	ELF4	2.10×10^{-12}	0.76	1028.87	1.01×10^{-5}	0.89	810.05
ERF	*ERF*	0.155	0.12	2894.91	0.151	0.20	2617.82
	ETV3	0.782	0.07	310.65	0.853	-0.07	267.23
	ETV3L	0.761	-0.09	5.70	0.871	-0.09	5.37
PEA3	*ETV1**,†	6.75×10^{-26}	1.44	2866.81	1.42×10^{-6}	1.27	1727.42
	ETV4*,†	2.31×10^{-27}	1.86	2934.37	9.69×10^{-6}	1.70	1673.91
	ETV5†	1.50×10^{-17}	0.91	4988.89	2.17×10^{-5}	1.02	3782.12
ETV2	*ETV2*	1.29×10^{-16}	-1.24	23.41	2.72×10^{-6}	-1.02	32.32
SPI	*SPI1*	0.033	0.43	1116.00	0.022	0.87	1077.01
	SPIB	0.246	0.50	75.23	0.599	-0.36	45.36
	SPIC	0.389	-0.57	1.300	0.068	-1.69	1.14
TCF	*ELK1*	3.29×10^{-21}	0.46	2182.44	1.41×10^{-8}	0.48	1769.47
	ELK3	1.38×10^{-9}	0.83	1933.12	0.000	0.73	1365.76
	ELK4	0.669	0.07	229.65	0.753	0.08	206.86

Abbreviation: FC, fold change

*,† Differentially expressed gene, which is determined by DESeq2 to have *q*-value <0.05, $|Log_2$ (fold change)$| \geq 1$, and baseMean ≥100, of **BRAF*-only group, and † *BRAF+TERT* group compared to no-mutation group

Table 18. Changes in *ETS* gene expression in *BRAF*-only and *BRAF+TERT* groups compared to no-mutation group of SNU database

Subfamily	Gene	No mutation vs. *BRAF* only			No mutation vs. *BRAF+TERT*		
		q-value	$\|Log_2$ (FC)$\|$	baseMean	*q*-value	$\|Log_2$ (FC)$\|$	baseMean
ETS	*ETS1*	0.454	0.21	2702.71	0.649	0.27	2821.66
	ETS2	0.011	-0.47	1508.03	0.348	-0.40	1981.75
ERG	*ERG*	0.007	-0.77	390.28	0.132	-0.77	564.26
	FLI1	0.511	-0.25	524.53	0.500	-0.44	597.70
	FEV	NA	0.11	0.17	NA	NA	0.00
GABPA	*GABPA*	0.929	-0.02	1083.57	0.743	0.12	1246.20
ESE	*EHF**,†	2.18×10^{-6}	2.41	491.61	0.004	2.45	372.34
	ELF3*,†	4.02×10^{-10}	1.62	851.61	0.008	1.58	622.61
	ELF5	0.017	1.60	3.90	0.020	2.02	3.50
TEL	*ETV6*	0.916	0.02	815.00	0.627	0.13	916.62
	ETV7*	6.95×10^{-4}	1.65	100.02	0.0956	1.06	50.40
PDEF	*SPDEF*	0.590	0.35	2.88	0.955	-0.07	2.50
ELF	*ELF1*	0.006	0.41	2076.72	0.0976	0.44	2015.68
	ELF2	0.359	0.13	930.07	0.501	0.20	1001.81
	ELF4*	5.86×10^{-7}	1.05	335.38	0.111	0.91	260.13
ERF	*ERF*	0.633	0.09	802.16	0.620	0.22	889.59
	ETV3	0.930	-0.02	564.32	0.787	0.08	642.10
	ETV3L	NA	0.19	0.24	NA	0.06	0.20
PEA3	*ETV1**,†	9.31×10^{-7}	1.20	1067.20	0.044	1.11	814.03
	ETV4*,†	1.03×10^{-6}	1.85	783.83	0.009	2.02	656.98
	ETV5*,†	2.35×10^{-5}	1.06	1499.65	0.003	1.30	1338.03
ETV2	*ETV2*	9.22×10^{-6}	-1.61	12.93	2.83×10^{-5}	-2.43	24.17
SPI	*SPI1*	0.175	0.68	441.19	0.677	0.35	341.35
	SPIB	0.049	1.54	41.96	0.577	0.65	14.04
	SPIC	0.760	0.31	0.83	NA	0.25	0.78
TCF	*ELK1*	1.94×10^{-4}	0.52	653.82	0.240	0.32	571.07
	ELK3	0.129	0.36	1955.78	0.277	0.48	1987.81
	ELK4	0.002	0.65	1239.97	0.007	0.98	1270.34

Abbreviation: FC, fold change

*,† Differentially expressed gene, which is determined by DESeq2 to have *q*-value <0.05, $|Log_2$ (fold change)$| \geq 1$, and baseMean ≥100, of **BRAF*-only group, and † *BRAF+TERT* group compared to no-mutation group

In vitro validation of changes in TERT and ETS expression by the BRAF^{V600E} mutation

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, I 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. 20A; Fig. 21A). 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. I next treated the *BRAF* inhibitor (PLX4720) to BCPAP PTC cells harboring both *BRAF^{V600E}* and *TERT* C228T mutations. The expression levels of genes *TERT*, *ETV1*, *ETV4*, and *ETV5* were significantly lowered by *BRAF* inhibition, while *ELF3* was upregulated (Fig. 20B; Fig. 21B).

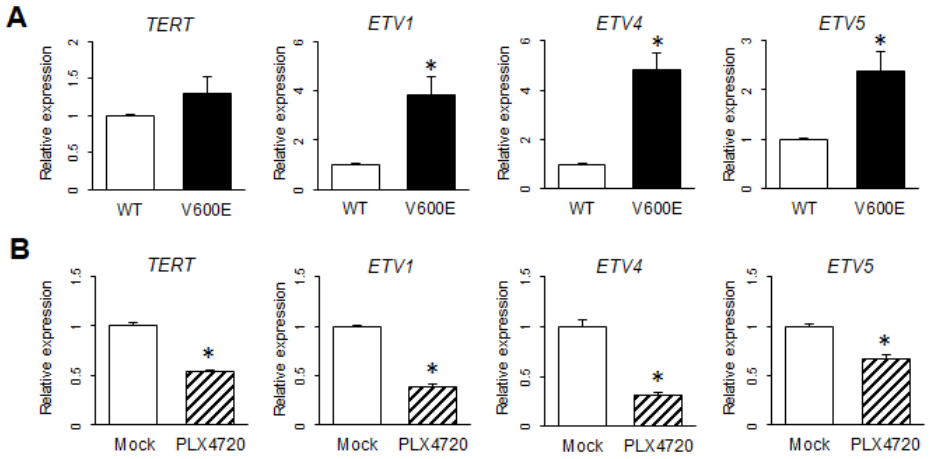


Figure 20. *In vitro* validation of changes in *TERT* and *ETS* expression by the $BRAF^{V600E}$ expression. (A) In human thyroid cell lines harboring the wild-type *BRAF* gene (Nthy/WT) and the mutant-type *BRAF* gene (Nthy/V600E), *TERT*, *ETV1*, *ETV4*, and *ETV5* expression was analyzed by real-time RT-PCR. **P* < 0.05 versus Nthy/WT. (B) BCPAP cells, a human PTC cell line harboring both $BRAF^{V600E}$ and *TERT* promoter mutations, were treated with PLX4720 (a *BRAF* inhibitor), and *TERT*, *ETV1*, *ETV4*, and *ETV5* expression was analyzed by real-time RT-PCR. **P* < 0.05 versus no treatment of PLX4720.

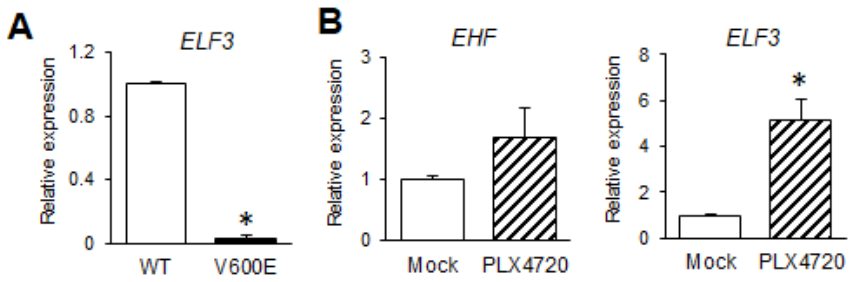


Figure 21. *In vitro* experiment of changes in *EHF* and *ELF3* expression by the $BRAF^{V600E}$ expression in (A) Nthy/WT and Nthy/V600E. *EHF* was not detected. **P* < 0.05 versus Nthy/WT. (B) BCPAP cells were treated with PLX4720 (a *BRAF* inhibitor). **P* < 0.05 versus no treatment of PLX4720. *EHF* and *ELF3* expression was analyzed by real-time RT-PCR.

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, I 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. 22A 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. 22C). Only 5 genes showed a statistically significant difference but with modest changes (fold changes, 2.0–2.7): *CILP*, *PODNL1*, *CREB3L1*, *WISP2*, and *CDKN2A* (Table 19). 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* (Fig. 23, A-C). Notably, the sets of DEGs of groups *BRAF*-only and *BRAF+TERT* strongly overlapped (Fig. 22D). Nevertheless, when I 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: 5.86×10^{-5} and 5.18×10^{-18}, respectively; Fig. 22E), 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. 22F; Table 20). Similar results were obtained in our database (Fig. 23, D-F).

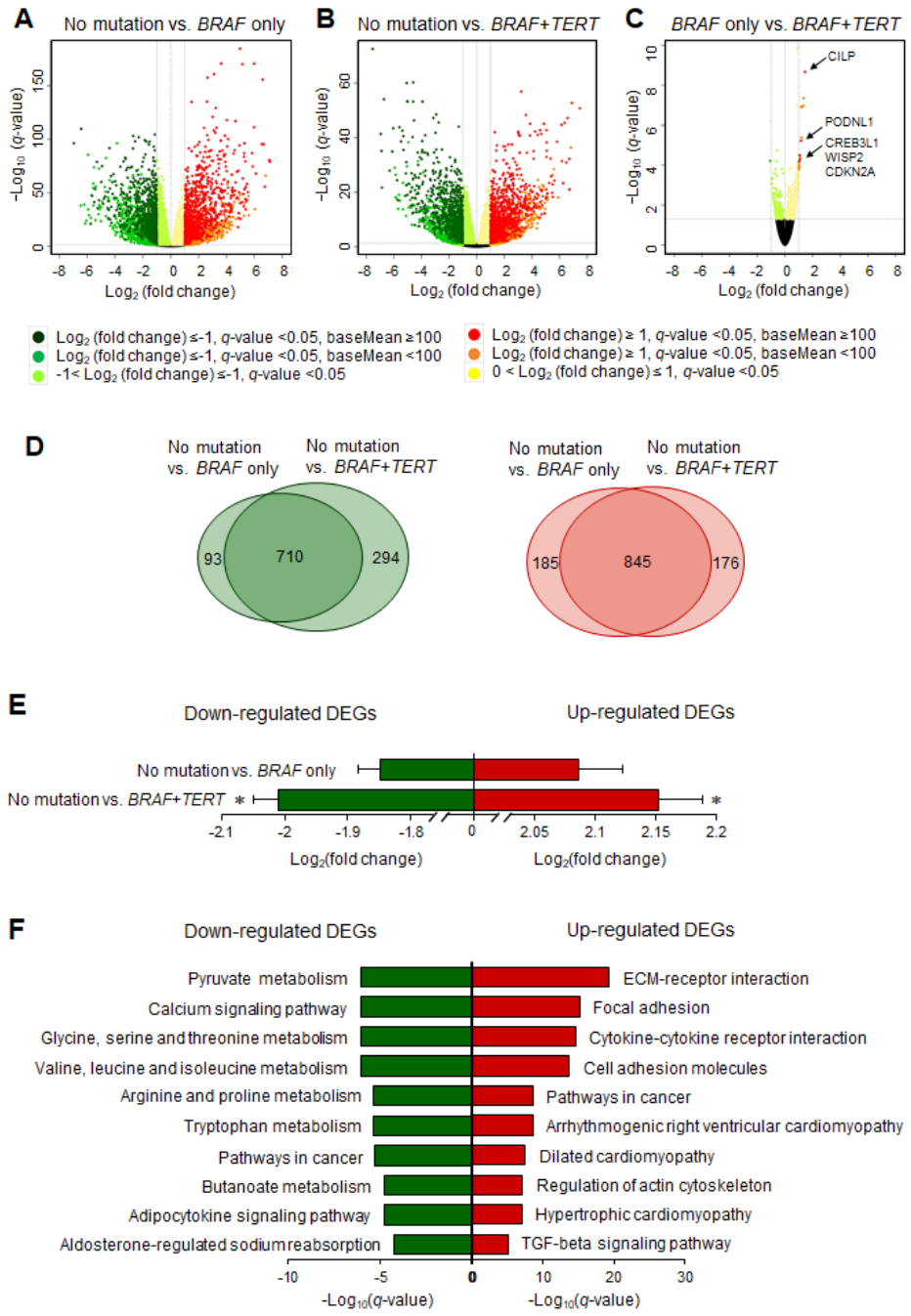


Figure 22. Transcriptional changes according to the mutational status of *BRAF*^{V600E} and *TERT* promoter. (A-C) Differentially expressed genes (DEGs) from RNA sequencing analyses of TCGA database were illustrated as the dark-

green dots (down-regulated) and the red dots (up-regulated) of volcano plots. (D) Venn diagram summarizing the overlap down-regulated (green) and up-regulated (red) DEGs between *BRAF*-only group and *BRAF+TERT* group compared with no-mutation group. (E) The degree of changes of the overlap DEGs. *Statistically significant. (F) The top 10 most significantly enriched molecular pathways of the overlap DEGs.

Table 19. Differentially expressed genes between *BRAF*-only group and *BRAF+TERT* group

DEG	*q*-value	$\lvert Log_2$ (fold change)$\rvert$	baseMean
CILP	$2.12x10^{-9}$	1.45	464.66
PODNL1	$6.03x10^{-6}$	1.16	221.70
CREB3L1	$3.18x10^{-5}$	1.05	455.88
WISP2	$4.91x10^{-5}$	1.09	246.81
CDKN2A	$6.42x10^{-5}$	1.04	319.57

Table 20. Common differentially expressed genes and enriched molecular pathways of *BRAF* mutation only and coexistence of *BRAF* and *TERT* promoter mutations

Pathways	*q*-value	Genes
Upregulated DEGs		
ECM-receptor interaction	4.76×10^{-20}	*ITGB7*, *ITGA9*, *ITGB8*, *ITGA2*, *ITGA3*, *ITGA11*, *TGB4*, *ITGB6*, *FN1*, *LAMA3*, *LAMB3*, *LAMC2*, *THBS1*, *COMP*, *THBS2*, *COL1A1*, *COL1A2*, *COL3A1*, *COL5A1*, *COL6A3*, *COL11A1*, *TNC*, *SDC4*, *SDC3*
Focal adhesion	7.66×10^{-16}	*ITGB7*, *ITGA9*, *ITGB8*, *ITGA2*, *ITGA3*, *ITGA11*, *ITGB4*, *ITGB6*, *FN1*, *LAMA3*, *LAMB3*, *LAMC2*, *THBS1*, *COMP*, *THBS2*, *COL1A1*, *COL1A2*, *COL3A1*, *COL5A*, *COL6A3*, *COL11A1*, *TNC*, *EGFR*, *PDGFRA*, *MET*, *ACTN1*, *CCND1*, *FLNA*, *RASGRF1*
Cytokine-cytokine receptor interaction	2.70×10^{-15}	*EGFR*, *PDGFRA*, *MET*, *TGFB2*, *IL6*, *TGFBR1*, *FAS*, *IL8*, *INHBA*, *INHBB*, *ACVR1*, *CXCL2*, *CCL13*, *CCR6*, *CXCL16*, *CCL17*, *CCL18*, *CCL20*, *CCL22*, *CXCL14*, *TNFRSF10A*, *IL1RAP*, *CLCF1*, *LIF*, *OSM*, *OSMR*, *NGFR*, *TNFRSF21*, *LTB*, *TNFRSF12A*, *TNFSF4*, *IL18R1*
Cell adhesion molecules	1.93×10^{-14}	*ITGB7*, *ITGA9*, *ITGB8*, *SDC4*, *SDC3*, *L1CAM*, *CD22*, *ICAM1*, *HLA-G*, *HLA-DQA2*, *PTPRF*, *PVRL1*, *CLDN1*, *CLDN16*, *CLDN4*, *CLDN10*, *CLDN2*, *CDH3*, *CDH4*, *VCAN*, *NRCAM*, *CD276*, *CNTNAP1*
Pathways in cancer	2.86×10^{-9}	*ITGA2*, *ITGA3*, *FN1*, *LAMA3*, *LAMB3*, *LAMC2*, *EGFR*, *PDGFRA*, *MET*, *CCND1*, *TGFB2*, *IL6*, *TGFBR2*, *IL6*, *TGFBR1*, *FAS*, *IL8*, *FGF2*, *CDKN2B*, *BMP4*, *RXRG*, *PTGS2*, *BID*, *CDKN2A*, *DAPK2*, *TGFA*, *CCNA1*, *RUNX1*, *WNT10A*
Arrhythmogenic right cardiomyopathy	2.86×10^{-9}	*ITGB7*, *ITGA9*, *ITGB8*, *ITGA2*, *ITGA3*, *ITGA11*, *ITGB4*, *ITGB6*, *ACTN1*, *CACNG4*, *CACNB1*, *DMD*, *DSC2*, *PKP2*
Dilated cardiomyopathy	3.31×10^{-8}	*ITGB7*, *ITGA9*, *ITGB8*, *ITGA2*, *ITGA3*, *ITGA11*, *ITGB4*, *ITGB6*, *TGFB2*, *CACNG4*, *CACNB1*, *DMD*, *ADCY8*, *ADCY7*
Regulation of actin cytoskeleton	6.47×10^{-8}	*ITGB7*, *ITGA9*, *ITGB8*, *ITGA2*, *ITGA3*, *ITGA11*, *ITGB4*, *ITGB6*, *FN1*, *EGFR*, *PDGFRA*, *ACTN1*, *FGF2*, *TIAM1*, *MYH10*, *WASF1*, *MSN*, *GSN*, *IQGAP3*, *TMSL3*
Hypertrophic cardiomyopathy	9.42×10^{-8}	*ITGB7*, *ITGA9*, *ITGB8*, *ITGA2*, *ITGA3*, *ITGA11*, *ITGB4*, *ITGB6*, *TGFB2*, *IL6*, *CACNG4*, *CACNB1*, *DMD*
TGF-beta signaling pathway	8.50×10^{-6}	*THBS1*, *COMP*, *THBS2*, *TGFB2*, *TGFBR1*, *INHBA*, *INHBB*, *ACVR1*, *CDKN2B*, *BMP4*, *LTBP1*

Downregulated DEGs		
Pyruvate metabolism	8.70×10^{-7}	*DLD*, *ALDH2*, *ALDH1B1*, *ACAT1*, *PDHA1*, *ACACB*, *ACSS1*, *ME1*, *LDHD*
Calcium signaling pathway	8.70×10^{-7}	*PRKCA*, *PRKACB*, *ADCY1*, *EDNRB*, *SLC25A4*, *SLC25A5*, *VDAC3*, *ITPR1*, *RYR2*, *AGTR1*, *AVPR1A*, *GRIN2C*, *PLCD4*, *PDE1B*, *GNAL*, *GNA14*
Glycine, serine and threonine metabolism	8.70×10^{-7}	*DLD*, *MAOB*, *GATM*, *CTH*, *GCAT*, *GLDC*, *DMGDH*, *PSPH*
Valine, leucine and isoleucine degradation	8.70×10^{-7}	*DLD*, *ALDH2*, *ALDH1B1*, *ACAT1*, *AOX1*, *ALDH6A1*, *ACADSB*, *BCAT2*, *ACAD8*
Arginine and proline metabolism	4.37×10^{-6}	*ALDH2*, *ALDH1B1*, *MAOB*, *GATM*, *GOT1*, *ALDH4A1*, *CKB*, *CKMT1B*, *CKMT2*
Tryptophan metabolism	4.37×10^{-6}	*ALDH2*, *ALDH1B1*, *ACAT1*, *MAOB*, *AOX1*, *OGDH*, *OGDHL*, *IDO1*
Pathways in cancer	5.65×10^{-6}	*PRKCA*, *AKT2*, *PIK3R3*, *TCF7L1*, *WNT4*, *WNT11*, *KIT*, *FZD8*, *EGF*, *RAC3*, *FGF7*, *FGF11*, *FGF12*, *FGF13*, *CYCS*, *BCL2*, *PGF*, *RARB*, *CEBPA*, *HSP90B1*
Butanoate metabolism	1.63×10^{-5}	*ALDH2*, *ALDH1B1*, *ACAT1*, *PDHA1*, *ACSM5*, *BDH1*, *ACSM3*
Adipocytokine signaling pathway	1.71×10^{-5}	*ACACB*, *AKT2*, *IRS1*, *PPARGC1A*, *PRKCQ*, *ACSL1*, *CD36*, *SLC2A4*, *ADIPOR2*
Aldosterone-regulated sodium reabsorption	5.89×10^{-5}	*PRKCA*, *PIK3R3*, *IRS1*, *ATP1B2*, *HSD11B2*, *SCNN1B*, *KCNJ1*

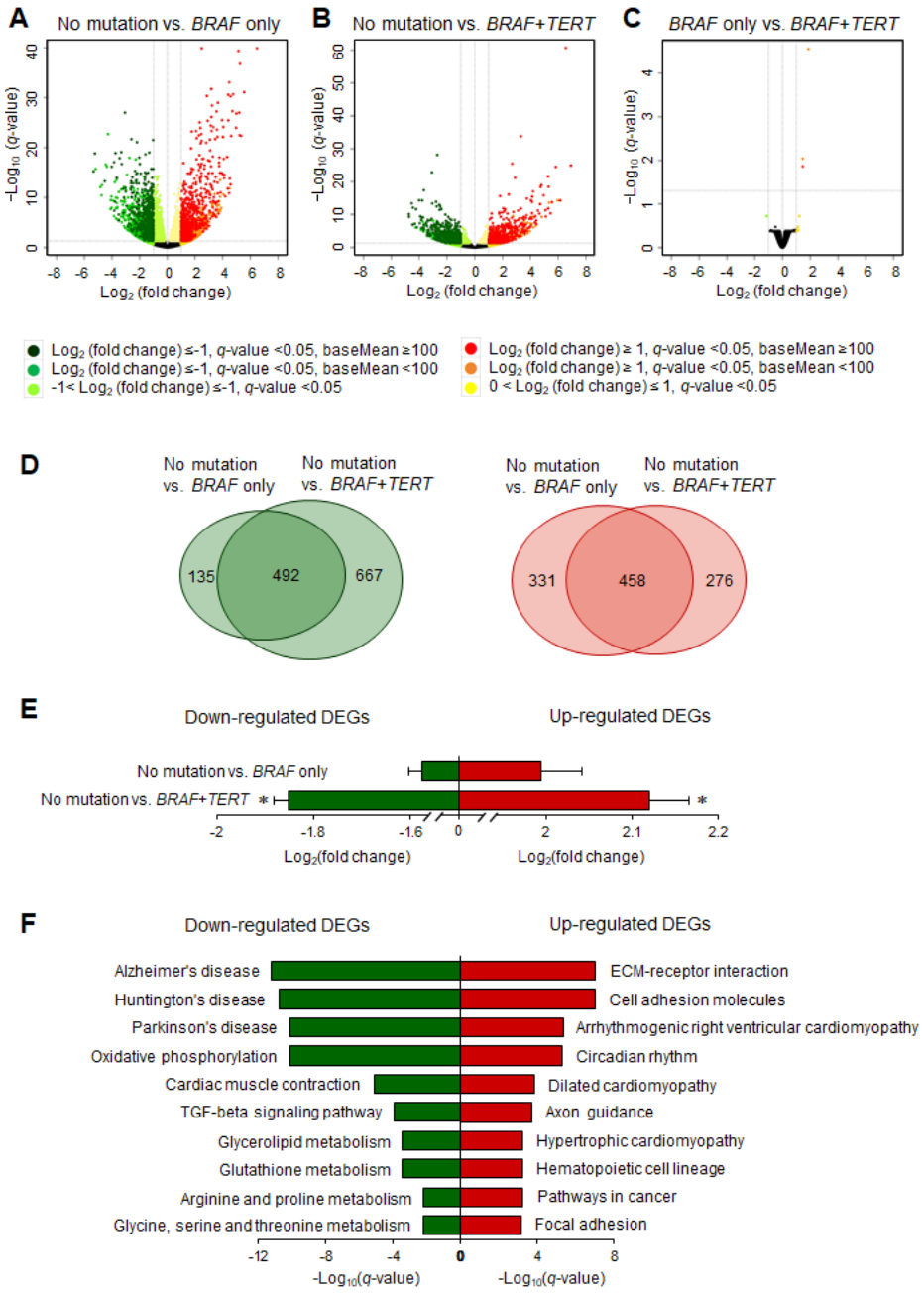


Figure 23. Transcriptional changes according to mutational status of the *BRAF*^{V600E} and *TERT* promoter in SNU dataset. (A-C) DEGs from RNA sequencing analyses were illustrated as volcano plots. (D) Venn diagram summarizing the overlap DEGs between *BRAF*-only group and *BRAF+TERT* group compared with no-mutation group. (E) The degree of changes of the overlap DEGs. *Statistically significant. (F) The top 10 most significantly enriched molecular pathways of the overlap DEGs.

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

As shown in Fig. 16, B 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. 22C Then, I reclassified the samples according to the *TERT* expression status rather than *TERT* promoter mutation (Fig. 24A). 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. 24, B 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 (Fig. 25, A and B). Moreover, when I 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 (Fig. 25C). 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. 24D). Most of these upregulated

genes belonged to pathways related to inflammatory cytokines or adhesion molecules (Fig. 24E; Table 21). The expression of representative genes including *CD28*, *CTLA4*, and *VCAM1* was significantly increased by *TERT* expression (Fig. 24F). I validated these results on SNU data (Fig. 26).

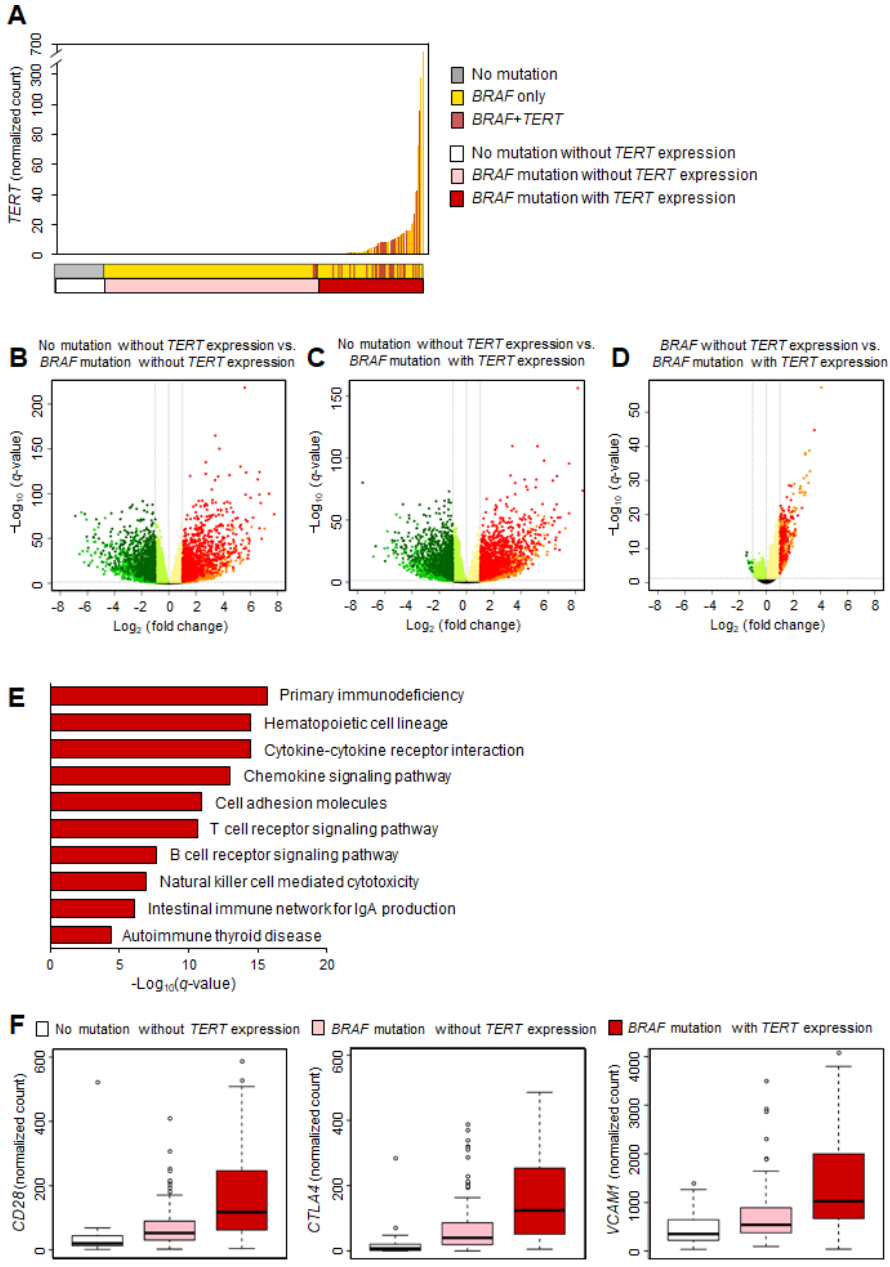


Figure 24. Transcriptional changes by *TERT* expression in addition to the *BRAF*^{V600E} mutation. (A) Each column represents the *TERT* mRNA expression level of an individual sample of TCGA database. (B-D) Differentially expressed genes (DEGs) from RNA sequencing analyses were illustrated as the dark-green dots (down-regulated) and the red dots (up-regulated) of volcano plots. (E) The top 10 most significantly enriched molecular pathways of the upregulated DEGs in tumors with *TERT* expression compared with those without *TERT* expression in *BRAF*-mutated PTCs.

Table 21. Differentially expressed genes and enriched molecular pathways between the groups of *BRAF* mutation with *TERT* expression and *BRAF* mutation without *TERT* expression

Pathways	*q*-value	Genes
Upregulated DEGs		
Primary Immunodeficiency	3.82×10^{-16}	*IL7R*, *CD8A*, *CD3D*, *CD3E*, *CD19*, *IL2RG*, *PTPRC*, *LCK*, *ZAP70*, *BTK*, *CD79A*
Hematopoietic cell lineage	7.12×10^{-15}	*IL7R*, *CD8A*, *CD3D*, *CD3E*, *CD19*, *CD2*, *CR2*, *CR1*, *CD38*, *CD5*, *CD7*, *MS4A1*, *CD37*
Cytokine-cytokine receptor interaction	7.61×10^{-15}	*IL7R*, *IL2RG*, *CXCR4*, *CCL5*, *CXCL10*, *CCL4*, *CXCL9*, *CXCL11*, *CXCL13*, *CXCR6*, *CCR7*, *CCL19*, *TNFRSF17*, *CSF2RB*, *IL12RB1*, *IL21R*, *LTB*, *CD27*
Chemokine signaling pathway	8.81×10^{-15}	*CXCR4*, *CCL5*, *CXCL10*, *CCL4*, *CXCL9*, *CXCL11*, *CXCL13*, *CXCR6*, *CCR7*, *CCL19*, *PIK3CG*, *ITK*, *PRKCB*, *WAS*, *DOCK2*, *RASGRP2*
Cell adhesion molecules	2.69×10^{-14}	*CD8A*, *PTPRC*, *CD2*, *CD28*, *CTLA4*, *PDCD1*, *ITGAL*, *HLA-DOA*, *HLA-DOB*, *HLA-DQA1*, *VCAM1*, *VCAN*, *SELL*, *CD6*
T cell receptor signaling pathway	1.30×10^{-12}	*CD8A*, *CD3D*, *CD3E*, *PTPRC*, *LCK*, *ZAP70*, *PIK3CG*, *ITK*, *CD28*, *CTLA4*, *PDCD1*, *CD247*
B cell receptor signaling pathway	8.85×10^{-10}	*CD19*, *BTK*, *CD79A*, *CR2*, *PIK3CG*, *PRKCB*, *DAPP1*, *CD72*, *CD79B*
Natural killer cell mediated cytotoxicity	9.07×10^{-9}	*LCK*, *ZAP70*, *PIK3CG*, *PRKCB*, *ITGAL*, *CD247*, *GZMB*, *KLRK1*, *SH2D1A*, *CD48*
Intestinal immune network for IgA product	2.72×10^{-8}	*CXCR4*, *TNFRSF17*, *CD28*, *HLA-DOA*, *HLA-DOB*, *HLA-DQA1*, *PIGR*
Autoimmune thyroid disease	1.84×10^{-6}	*CD28*, *CTLA4*, *HLA-DOA*, *HLA-DOB*, *HLA-DQA1*, *GZMB*

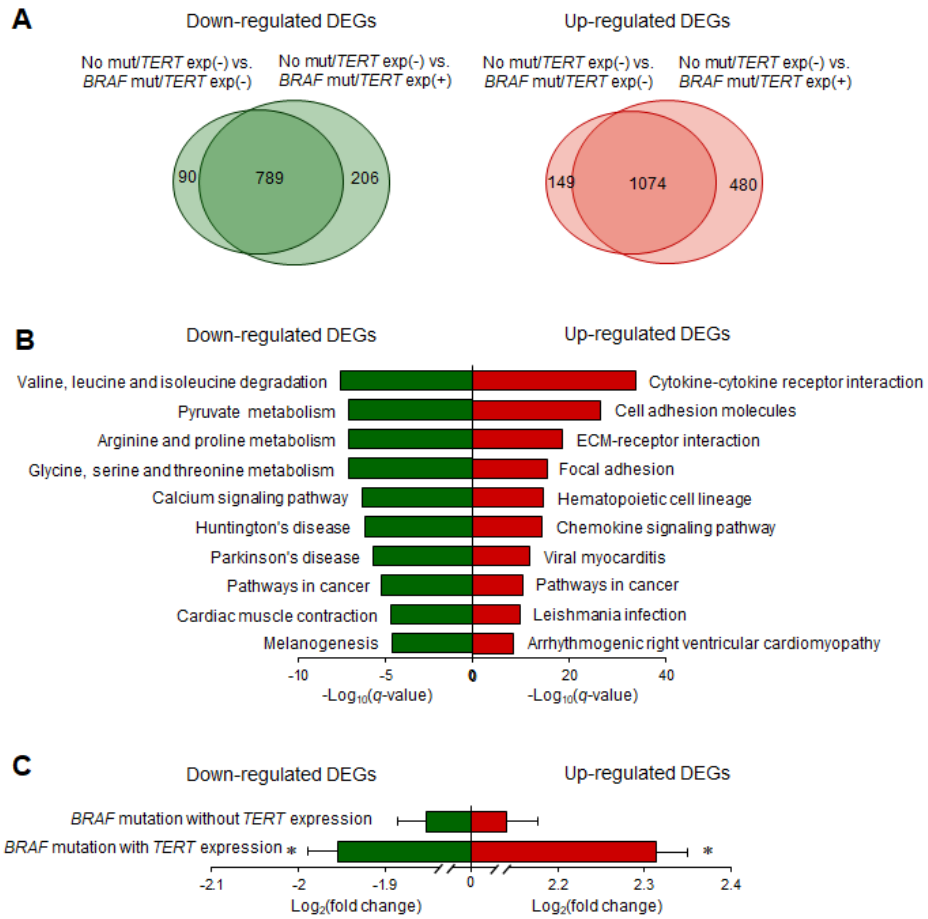


Figure 25. Common DEGs between groups "*BRAF*^{V600E} mutation without *TERT* expression" and "*BRAF*^{V600E} mutation with *TERT* expression" compared to the "no mutation without *TERT* expression" group. (A) Venn diagram summarizing the overlap down-regulated (green) and up-regulated (red) DEGs between groups "no mutation without *TERT* expression" [No mut/*TERT* exp(-)] vs. "*BRAF*^{V600E} mutation without *TERT* expression" [*BRAF* mut/*TERT* exp(-)] and vs. "*BRAF*^{V600E} mutation with *TERT* expression" [*BRAF* mut/*TERT* exp(+)]. (B) The top 10 most significantly enriched molecular pathways of the overlap DEGs. (C) The degree of changes in the gene expression of common DEGs, expressed as mean ± standard error of the mean. *Statistically significant.

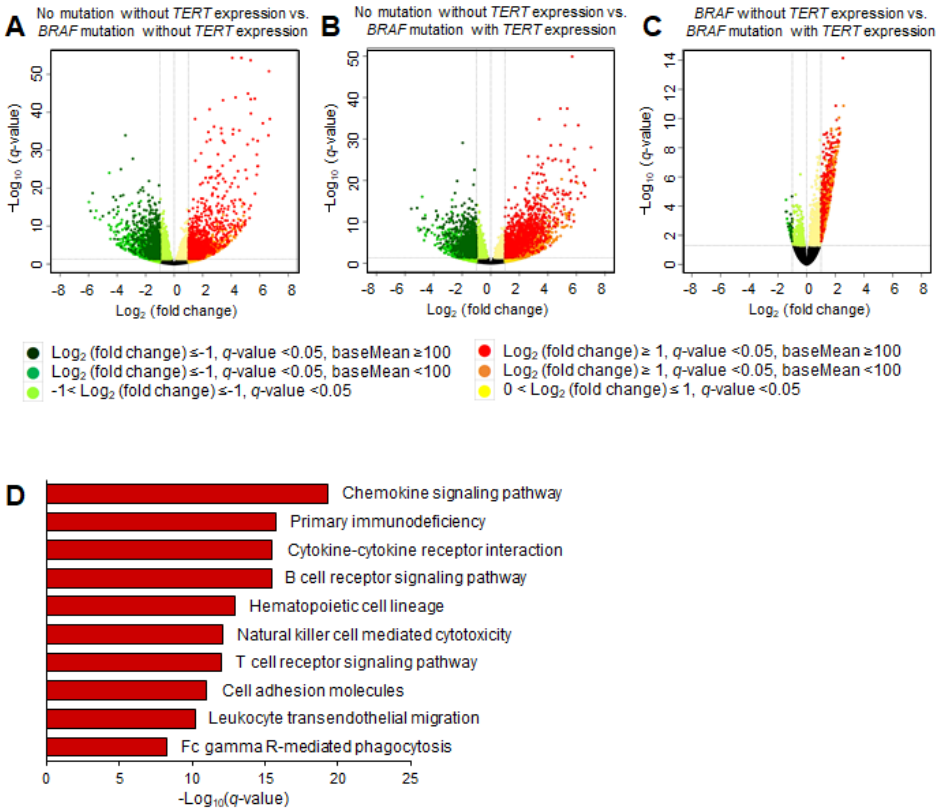


Figure 26. Transcriptional changes by *TERT* expression in addition to *BRAF*^{V600E} mutation of SNU dataset. (A-C) Differentially expressed genes (DEGs) from RNA sequencing analyses were illustrated as the dark-green dots (down-regulated) and the red dots (up-regulated) of volcano plots. (D) The top 10 most significantly enriched molecular pathways of the upregulated DEGs in tumors with *TERT* expression compared with those without *TERT* expression in *BRAF*-mutated PTCs.

Discussion

The coexistence of *TERT* promoter and *BRAF* mutations presented outcomes worse than each mutation alone as reported previously (Schlumberger, 1998; Tufano et al., 2012; Xing et al., 2013). Moreover, this study is the first meta-analysis investigating an association between the coexistence of $BRAF^{V600E}$ and *TERT* promoter mutations vs. each mutation alone and the associated prognostic factors. As for the mechanism, from the analyses of TCGA and SNU RNA sequencing data and *in vitro* experiments, I could confirm that *TERT* mRNA expression was increased by adding the $BRAF^{V600E}$ mutation to the *TERT* promoter mutation. Furthermore, this increase was due to, at least in part, the upregulated expression of *ETS*, especially *ETV1*, *ETV4*, and *ETV5* by $BRAF^{V600E}$ mutation. On the other hand, coexisting mutations showed changes in the almost same intracellular signaling pathways as $BRAF^{V600E}$ mutation alone, however, amplified the changes of the expression level of genes associated with altered pathways. Moreover, the inflammation and adhesion-related pathways were activated by adding *TERT* expression in *BRAF*-mutated PTCs. From these results, I could firstly provide an evidence for the possible molecular mechanism of the synergistic effects of $BRAF^{V600E}$ and *TERT* promoter mutation in thyroid cancer cells.

The synergistic effects of coexistence of *BRAF*^{V600E} and *TERT* promoter mutations on poor clinical outcomes have been controversial because some studies did not show similar synergistic effects (Gandolfi et al., 2015; Melo et al., 2014; Muzza et al., 2015). However, this pooled analysis clearly demonstrated that coexistence of *BRAF*^{V600E} and *TERT* promoter mutations were far more strongly associated with poor clinicopathological features and long-term outcomes than either *BRAF*^{V600E} or *TERT* promoter mutation alone in PTC. Especially considering the recent increase in studies that proposed the *TERT* promoter mutation as a significant prognostic molecular marker in PTC, the limited effect of the *TERT* promoter mutation alone was an unexpected finding. Most of these studies, however, presented the *TERT* promoter mutation without discriminating between *TERT* promoter mutation alone and the mutation with *BRAF*^{V600E}.

A positive association of *TERT* promoter mutations with *BRAF*^{V600E} has been reported in previous studies including a recently published meta-analysis (Liu and Xing, 2016). Likewise, in the present meta-analysis study, *TERT* promoter mutation was significantly more frequent in 11.4% of patients with *BRAF*^{V600E} vs. 6.3% of those without *BRAF*^{V600E}. Considering the existence of such a high association of *TERT* promoter mutation with *BRAF*^{V600E} and the

limited effect of *TERT* promoter mutation alone in this study, it is possible that the results of previous studies on the prognostic effects of *TERT* promoter mutation in PTC reflect, at least in some part, the effects of $BRAF^{V600E}$ mutation as well as true prognostic efficacy of the *TERT* promoter mutation. In addition, although data was limited, several studies also reported a positive relationship between *TERT* promoter and *RAS* mutations in thyroid cancer (Melo et al., 2014; Muzza et al., 2015; Song et al., 2016a). Therefore, reevaluation of the effect of each mutation alone is needed for risk stratification of thyroid cancer.

Recently, there have been several studies on the clinical meaning of the coexistence of *TERT* promoter and $BRAF^{V600E}$ or *RAS* mutations, reporting that the coexistence of the two mutations was associated with more aggressive clinical outcomes than either mutation alone (Song et al., 2015; Xing et al., 2014c). Xing *et al*. reported that the coexistence of two mutations was far more strongly associated with high-risk factors, recurrence of PTC, or disease-specific mortality than groups with no mutations, $BRAF^{V600E}$ mutation alone, or *TERT* promoter mutation alone. $BRAF^{V600E}$ and *TERT* promoter mutations alone each had a modest effect (Liu et al., 2016; Xing et al., 2014c). This meta-analysis fully confirmed the findings in their study.

The mechanism of their synergistic effects on prognosis can be explained

at a molecular level. I could confirm that this genetic duet significantly increased *TERT* expression compared with the expression in tumors harboring *BRAF* or *TERT* promoter mutation alone. One previous study (Vinagre et al., 2013) also showed the coexistence of $BRAF^{V600E}$ and *TERT* promoter mutations was associated with the highest levels of *TERT* mRNA expression, although they included only 3 samples of thyroid cancer harboring both mutations. However, I could confirm the expression of *TERT* was further upregulated by $BRAF^{V600E}$ mutation in PTCs with mutant *TERT* promoter in a relatively large number of samples and validated it using the PTC cell line harboring both mutations.

Moreover, among the 3 mechanisms known to control *TERT* promoter activity—*TERT* promoter methylation, other transcription factors, and ETS—ETS 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; Vinagre et al., 2013). Although binding the core recognition sequence 5′-GGA(A/T)-3′ is a common property of ETS transcription factors, a genomewide analysis of all

ETS family members has established some differences in DNA-binding preferences within this family. A recent study 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 (Bell et al., 2015). Moreover, another recent study on melanoma 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 (Vallarelli et al., 2016). Therefore, our results for the first time show a direct link between *TERT* expression and the $BRAF^{V600E}$ mutation in thyroid cancer: $BRAF^{V600E}$ upregulates *TERT* transcription by activating ETS factors, especially the PEA3 subfamily, in PTC harboring a mutant *TERT* promoter. Nevertheless, the present study uncovered transcriptional changes of those genes; therefore, further studies are needed to confirm the direct DNA–protein interaction and binding.

In methylation analysis, although I checked *TERT* promoter methylation status in only 3 CpG sites available from TCGA database, the degree of methylation was increased and, paradoxically, *TERT* mRNA expression was positively correlated with *TERT* promoter methylation. On the other hand,

when the association between *TERT* promoter methylation and *ETS* expression was examined, there was no significant association between them. These results suggest the possibility that epigenetic modification through *TERT* promoter CpG methylation may be an alternative pathway for *TERT* reactivation, but the methylation and *ETS* expression are independent mechanisms to regulate *TERT* expression. Moreover, this is consistent with the previous results that *TERT* mRNA expression was positively associated with *TERT* promoter methylation, and *TERT*-hypermethylated patients showed worse prognosis in the several studies of brain tumor (Castelo-Branco et al., 2013), melanoma (Fan et al., 2016) and gastric cancer (Wu et al., 2016), even though studies of *TERT* promoter methylation has been controversial. Therefore, the result in this study suggests the possibility that epigenetic modification through *TERT* promoter CpG methylation may be an alternative pathway for *TERT* reactivation. However, because I could check *TERT* promoter methylation status in only 3 CpG sites available from TCGA database and the correlation was not strong, it is not enough to take it as a main mechanism. Moreover, the expression of transcription factors other than ETS binding to *TERT* promoter was not significantly changed by $BRAF^{V600E}$ mutation.

The upregulation of *TERT* reactivates telomerase activity, and the activation

of telomerase plays both the canonical function maintaining telomere length and the noncanonical function modulating expression of genes affecting various molecular pathways including Myc, Wnt/β-catenin, phosphoinositide 3-kinase/Akt, Erk1/2, and NFκB signaling (Li and Tergaonkar, 2014; Low and Tergaonkar, 2013). Most of these molecular pathways are overlapped with those activated by $BRAF^{V600E}$ mutation, and the similarity of pathways activated by both mutations may result in an amplification of the pathways instead of activation of new pathways when they coexist. Commonly enriched molecular pathways were related to the signal pathways or ontologies representing invasion, adhesion, carcinogenesis of thyroid nodules, or cancer invasiveness (Nucera et al., 2011). On the other hand, *TERT* expression itself activated the inflammation and adhesion related-pathways in addition to the *BRAF* mutation, which is consistent with the non-canonical role of *TERT* such as activating the NFκB signaling pathway.

Some cases of *BRAF*-only group in which *TERT* expression was increased showed poorer clinicopathological outcomes than *BRAF*-mutated cases without *TERT* expression. There was a report of urothelial cancer that the *TERT* mRNA expression predict prognosis more accurately than *TERT* promoter mutation (30). Considering these results and the cost-effectiveness, *TERT* mRNA

expression test can be useful for the subgroup of high-risk patients with *BRAF* mutation alone, and I may target the molecular mechanism of increased *TERT* expression for them.

There were several limitations of the meta-analysis. First, the small number of studies and their small sample sizes led to insufficient statistical power, which affect the stability of the results. Second, because of the lack of data, I was unable to conduct a subgroup meta-analysis based on other factors, such as subtype of PTC. Therefore, considering these limitations, larger well-designed studies are necessary to prove the synergistic role of *TERT* promoter and $BRAF^{V600E}$ mutations in the diagnosis and prognosis of PTC patients.

In conclusion, the coexistence of $BRAF^{V600E}$ and *TERT* promoter mutations had a synergistic effect on the clinicopathological characteristics of PTC including advanced TNM stage, extrathyroidal invasion, lymph node metastasis, and distant metastasis. Importantly, according to the available evidence, coexistence of $BRAF^{V600E}$ and *TERT* promoter mutations were significantly associated with recurrence and PTC-related mortality as well. Therefore, molecular testing of $BRAF^{V600E}$ and *TERT* promoter mutations together may be useful in assisting with risk stratification of PTC in clinical settings. Moreover, the mechanism of synergistic effect between $BRAF^{V600E}$ and *TERT* promoter mutations on aggressiveness in PTC may be explained by

increased *TERT* expression, which may result from the *BRAF*-induced upregulation of several ETS transcription factors. Therefore, I can suggest that the $BRAF^{V600E}$ mutation upregulates 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.

Chapter III. *TERT* promoter and *RAS* mutations in follicular thyroid cancer

III-1. Clinical significance of *TERT* and *RAS* mutations in follicular thyroid cancer

Materials and methods

Patients and tissue samples

Among the FTC patients who underwent thyroidectomy at Seoul National University Hospital (Seoul, Korea) in 1997-2003 and 2009-2012, genetic analyses were performed of 134 patients (67 patients in each period) whose formalin-fixed paraffin-embedded (FFPE) DNA samples were available, including 119 patients from our previous report (Song et al., 2016b). For the mutational analysis, all tissue samples were reviewed by an expert pathologist who specializes in thyroid pathology, and the FTC tumor region of the samples was microdissected for subsequent DNA extraction. Pathological diagnoses were made according to the latest World Health Organization classifications for thyroid cancer (DeLellis et al.). This study was conducted according to the guidelines of the Declaration of Helsinki. The research protocol was approved by the institutional review board committee of the Seoul National University Hospital (H-1207-124-420).

Mutational analyses

All samples of the FFPE tumor block were digested with proteinase K (Sigma, St. Louis, MO, USA) for more than 24 hours at 56℃, and DNA was then isolated from the digested tissue using a Tissue SV Mini kit (General Biosystem Inc., Seoul, Korea). *NRAS* (codon 12/13 and codon 61), *HRAS* (codon 12/13 and codon 61), *KRAS* (codon 12/13 and codon 61), and *TERT* promoter (C228T and C250T) mutations were examined using PCR and amplified using appropriate primers (Table 22). PCR was performed using a BioMix kit (Bioline, Taunton, MA, USA). Purified PCR products obtained using a QIAquick Gel Extraction kit (Qiagen, Düsseldorf, Germany) were used for sequencing with a Big Dye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems, Foster City, CA, USA). The sequences were analyzed using ABI Prism 3730 Genetic Analyzer (Applied Biosystems). Each DNA sample was assayed at least twice in order to confirm its *RAS* and *TERT* promoter mutation status by sequencing with both forward and reverse primers. *RAS* mutation status was assessed in all 134 samples, but *TERT* promotor mutation status was only assessed in 120 samples because of PCR failure in 14 samples.

Table 22. Nucleotide sequences of primers used for direct sequencing

Target	primers	Nucleotide sequence
NRAS 12/13	Forward	TACTGTACATGTGGCTCGCC
	Reverse	CCGACAAGTGAGAGACAGGA
NRAS 61	Forward	CCAGATAGGCAGAAATGGGC
	Reverse	CCTTCGCCTGTCCTCATGT
HRAS 12/13	Forward	CAGTCCTTGCTGCCTGGC
	Reverse	CTCCCTGGTACCTCTCATGC
HRAS 61	Forward	GCATGAGAGGTACCAGGGAG
	Reverse	TGATGGCAAACACACACAGG
KRAS 12/13	Forward	AAGCGTCGATGGAGGATTT
	Reverse	TGTATCAAAGAATGGTCCTGCA
KRAS 61	Forward	CGTCATCTTTGGAGCAGGAA
	Reverse	ACTCCACTGCTCTAATCCCC
TERT	Forward	CCCTTCACCTTCCAGCTC
	Reverse	CAGCGCTGCCTGAAACTC

Statistical analyses

Data are presented as frequencies and percentages, as means and standard deviations, or as medians and interquartile range. Categorical variables were compared with the Pearson chi-square test or the Fisher exact test (if the number was <5). The independent *t* test or the Wilcoxon-Mann-Whitney test was used for continuous variables. In order to compare trends of the clinicopathological characteristics of FTC over three periods, the linear-by-linear association test was used for categorical variables and analysis of variance for continuous variables. The log-rank test was used to compare variables based on the time of events. Cox proportional hazards regression was used to assess the risk of recurrence. Statistical significance was defined as two-sided *P* values <0.05.

Results

Prevalence of RAS and TERT promoter mutations in FTC

The characteristics of the 134 patients who underwent genomic tests of *RAS* and *TERT* promoter mutations were shown in Table 23. Among them, 43 (32.1%) and 7 (5.8%) patients had tumors with *RAS* and *TERT* promoter mutations, respectively (Table 24). The prevalence of *RAS* mutations decreased from 40.3% to 23.9% (P = 0.042), and the *HRAS* codon 61 mutation mainly contributed to this decrease. The *NRAS* codon 61 mutation was most common in both periods. The codon 12/13 mutations of *NRAS*, *HRAS*, and *KRAS* were not found. *TERT* promoter mutations in each period were found in three (4.5%) and four (7.4%) cases, respectively (P = 0.699). The major type of *TERT* promoter mutation was *TERT* C228T in both periods, although *TERT* C250T was observed in one case in 2009-2012. The *PAX8/PPARγ* rearrangement was found in one (2.1%) case in 2009-2012.

Table 23. Clinicopathological characteristics of patients with follicular thyroid cancer

	Years		
	1997−2003	**2009−2012**	***P***
Cases	67	67	
Follow-up duration, years[a]	13.4 (5.6−14.5)	5.4 (4.3−6.5)	
Age at diagnosis	44.6 ± 14.3	45.0 ± 13.7	0.868
Male sex, n (%)	12 (17.9)	18 (26.9)	0.214
Body mass index, kg/m^2	24.3 ± 3.6	24.1 ± 3.8	0.788
Tumor size (cm)	3.6 ± 1.8	3.4 ± 1.8	0.467
Widely invasive FTC, n (%)	17 (25.4)	6 (9.0)	0.012
Vascular invasion, n (%)	16 (23.9)	11 (16.4)	0.282
Multiplicity, n (%)	10 (14.9)	6 (9.0)	0.287
Extrathyroidal extension, n (%)	15/56 (26.8)	6/66 (9.1)	0.010
Microscopic/gross	1/14 (1.8/25.0)	6/0 (9.1/0.0)	<0.001
Resection margin, n (%)	3 (4.5)	1 (1.5)	0.619
Lymph node metastasis, n (%)	3 (4.5)	0 (0.0)	0.244
Distant metastasis, n (%)	7 (10.4)	2 (3.0)	0.291[b]
At initial presentation	3 (4.5)	0 (0.0)	0.122
During follow-up	4 (6.0)	2 (3.0)	
5 years/10 years	5/5 (7.5/7.5)	0/NA (0.0/NA)	
Site of distant metastasis, n (%)			
Lung/bone/mediastinum/ brain/others	4/4/1/0/1 (57.1/57.1/ 14.3/0.0/14.3)	2/0/0/0/0 (100.0/0.0/ 0.0/0.0/0.0)	
Type of thyroidectomy, n (%)			0.225
Total thyroidectomy	24 (35.8)	25 (37.3)	
Subtotal thyroidectomy	6 (9.0)	1 (1.5)	
Lobectomy	19 (28.4)	17 (25.4)	
Lobectomy and completion	18 (26.9)	24 (35.8)	
RAI treatment, n (%)	27 (40.3)	38 (56.7)	0.057
Remnant ablation	21 (31.3)	36 (53.7)	
For distant metastasis	6 (9.0)	2 (3.0)	
Total dose of RAI, mCi[a]	153 (90−550)	60 (60−123)	<0.001
Prognosis			
No evidence of disease, n (%)	59 (88.1)	65 (97.0)	0.162[b]
Persistence, n (%)	4 (6.0)	1 (1.5)	0.365
Recurrence, n (%)			
Overall	4 (6.0)	1 (1.5)	0.596[b]
5 years/10 years	0/0 (0.0/0.0)	0/NA (0.0/NA)	
Disease-specific mortality, n (%)			
Overall	2 (3.0)	0 (0.0)	0.174[b]
5 years/10 years	2/2 (3.0/3.0)	0/NA (0.0/NA)	

[a] Values presented as median (interquartile range); [b] Log-rank *P* values
NA, not applicable; RAI, radioactive iodine; FTC, follicular thyroid cancer.

Table 24. Changes of mutational frequencies in follicular thyroid cancer between 1997-2003 and 2009-2012

	Years		
	1997−2003	**2009−2012**	***P***
Cases	67	67	
RAS mutation, n (%)	27 (40.3)	16 (23.9)	0.042
NRAS codon 61	19 (28.4)	14 (20.9)	
HRAS codon 61	8 (11.9)	1 (1.5)	
KRAS codon 61	0 (0.0)	1 (1.5)	
N/H/KRAS codon 12, 13	0 (0.0)	0 (0.0)	
TERT promoter mutation, n (%)	3/66 (4.5)	4/54 (7.4)	0.699
C228T	3/66 (4.5)	3/54 (5.6)	
C250T	0/66 (0.0)	1/54 (1.9)	

NA, not applicable.

Clinicopathological characteristics and outcomes of FTC according to mutational status

Comparing the clinicopathological features of FTC according to *RAS* mutational status (Table 25), distant metastasis occurred more frequently in *RAS*-mutated FTCs (log-rank $P = 0.030$), even though other features did not differ between *RAS* wild-type and *RAS*-mutated FTCs. *RAS* mutations were significantly associated with a lower frequency of no evidence of disease (log-rank $P = 0.011$) and a higher proportion of persistent disease ($P = 0.037$). However, no differences in recurrence or disease-specific mortality were found.

TERT promoter mutations were significantly associated with distant metastasis (log-rank $P = 0.001$), in particular to the lung (log-rank $P = 0.006$), and advanced TNM stage ($P = 0.045$; Table 26). Furthermore, the patients with a *TERT* promoter mutation tended to have a poor prognosis and were less likely to remain disease-free (log-rank $P = 0.001$). The percentage of patients with persistent disease was higher in the *TERT* promoter mutation group, although this was not statistically significant due to its low incidence. Moreover, the overall recurrence and disease-specific mortality rates in *TERT* promoter wild-type versus mutant samples were significantly different (2.7% vs. 28.6% and 0.9% vs. 14.3%, respectively) (log-rank *P*, for recurrence = 0.002; for disease-specific mortality = 0.007).

Table 25. Clinicopathological characteristics and outcomes of follicular thyroid cancer according to the mutational status of *RAS*

	RAS		
	Wild type	**Mutant**	***P***
Cases, n (%)	91 (67.9)	43 (32.1)	
Follow-up duration, years[a]	6.5 (4.5−13.3)	6.1 (4.5−13.4)	
RAS mutation type, n (%)			
N/H/KRAS	–	33/9/1 (24.6/6.7/0.7)	0.002
Age at diagnosis	45.9 ± 14.4	42.7 ± 13.0	0.223
Male sex, n (%)	21 (23.1)	9 (20.9)	0.781
Body mass index, kg/m^2	24.4 ± 3.6	23.9 ± 3.9	0.470
Tumor size (cm)	3.6 ± 2.0	3.2 ± 1.3	0.267
Widely invasive FTC, n (%)	16 (17.6)	7 (16.3)	0.852
Vascular invasion, n (%)	20 (22.0)	7 (16.3)	0.443
Multiplicity, n (%)	11 (12.1)	5 (11.6)	0.939
Extrathyroidal extension, n (%)	15 (17.9)	6 (15.8)	0.779
Microscopic/gross	6/9 (7.1/10.7)	1/5 (2.6/13.2)	
Resection margin, n (%)	2 (2.2)	2 (4.7)	0.593
Lymph node metastasis, n (%)	1 (1.1)	2 (4.7)	0.241
Distant metastasis, n (%)	3 (3.3)	6 (14.0)	0.030[b]
At initial presentation	0 (0.0)	3 (7.0)	0.031
During follow-up	3 (3.3)	3 (7.0)	
5 years/10 years	2/2 (2.2/2.2)	3/4 (7.0/9.3)	
Site of distant metastasis, n (%)			
Lung	3 (3.3)	3 (7.0)	0.385
Bone	1 (1.1)	3 (7.0)	0.097
Other sites	1 (1.1)	1 (2.6)	
TNM stage, n (%)			0.644
I–II/ III–IV	73/18 (80.2/19.8)	33/10 (76.7/23.3)	
No evidence of disease, n (%)	88 (96.7)	36 (83.7)	0.011[b]
Persistence, n (%)	1 (1.1)	4 (9.3)	0.037
Recurrence, n (%)			
Overall	2 (2.2)	3 (7.0)	0.195[b]
5 years/10 years	1/2 (1.1/2.2)	1/2 (2.3/4.7)	
Disease-specific mortality, n (%)			
Overall	1 (1.1)	1 (2.3)	0.572[b]
5 years/10 years	1/1 (1.1/1.1)	1/1 (2.3/2.3)	

[a] Values presented as median (interquartile range).
[b] Log-rank *P* values.

Table 26. Clinicopathological characteristics and outcomes of follicular thyroid cancer according to the mutational status of *TERT* promoter

	TERT		
	Wild type	**Mutant**	***P***
Cases, n (%)	113 (94.2)	7 (5.8)	
Follow-up duration, years[a]	6.7 (4.9−13.5)	6.4 (4.5−14.3)	
TERT mutation type, n (%)			
C228T/C250T	−	6/1 (5.0/0.8)	0.002
Age at diagnosis	44.6 ± 14.2	51.9 ± 15.7	0.191
Male sex, n (%)	24 (21.2)	2 (28.6)	0.644
Body mass index, kg/m^2	24.3 ± 3.8	22.6 ± 3.5	0.290
Tumor size (cm)	3.5 ± 1.8	4.8 ± 2.6	0.071
Widely invasive FTC, n (%)	22 (19.5)	1 (14.3)	1.000
Vascular invasion, n (%)	22 (19.5)	3 (42.9)	0.157
Multiplicity, n (%)	13 (11.5)	1 (14.3)	0.590
Extrathyroidal extension, n (%)	21 (20.8)	0 (0.0)	0.341
Microscopic/gross	7/14 (6.9/13.9)	0/0 (0.0/0.0)	
Resection margin, n (%)	4 (3.5)	0 (0.0)	1.000
Lymph node metastasis, n (%)	1 (0.9)	1 (14.3)	0.114
Distant metastasis, n (%)	6 (5.3)	3 (42.9)	0.001[b]
At initial presentation	2 (1.8)	1 (14.3)	0.166
During follow-up	4 (3.5)	2 (28.6)	
5 years/10 years	4/4 (3.5/3.5)	1/2 (14.3/28.6)	
Site of distant metastasis, n (%)			
Lung	4 (3.5)	2 (28.6)	0.039
Bone	3 (2.7)	1 (14.3)	0.216
Other sites	1 (0.9)	1 (14.3)	
TNM stage, n (%)			0.045
I−II/ III−IV	90/23 (79.6/20.4)	3/4 (42.9/57.1)	
No evidence of disease, n (%)	106 (93.8)	4 (57.1)	0.001[b]
Persistence, n (%)	4 (3.5)	1 (14.3)	0.263
Recurrence, n (%)			
Overall	3 (2.7)	2 (28.6)	0.002[b]
5 years/10 years	2/2 (1.8/1.8)	0/1 (0.0/14.3)	
Disease-specific mortality, n (%)			
Overall	1 (0.9)	1 (14.3)	0.007[b]
5 years/10 years	1/1 (0.9/0.9)	1/1 (14.3/14.3)	

[a] Values presented as median (interquartile range).
[b] Log-rank *P* values.

Association between the RAS and TERT promoter mutations and the effect of their coexistence on FTC recurrence

A significant association of *RAS* mutations with *TERT* promoter mutations was observed ($P = 0.045$; Table 27). *TERT* promoter mutations were found in 2.5% of *RAS* wild-type cases versus 12.2% of *RAS*-mutated cases, and conversely, *RAS* mutations were found in 31.9% of *TERT* wild-type cases versus 71.4% of *TERT*-mutated cases.

Table 27. Association between *RAS* and *TERT* promoter mutations in follicular thyroid cancer

***TERT* promoter mutations**				***RAS* mutations**				
***RAS*−**		***RAS*+**		***TERT*−**		***TERT*+**		
No	%	No	%	No	%	No	%	*P*
2/79	2.5	5/41	12.2	36/113	31.9	5/7	71.4	0.045

The patients with a *RAS* mutation, especially with coexistent *RAS* and *TERT* promoter mutations, showed higher rates of recurrence or persistence than those without any mutations (Table 28; Fig. 27A). Two patients had a *TERT* promoter mutation alone, and FTC recurrence was not observed in them. The coexistence of two mutations had the highest recurrence rate and increased the recurrence risk 6.27-fold compared to the absence of mutations, although statistical significance was lost after adjustment for age at diagnosis and sex. Moreover, I could confirm this synergistic effects on prognosis using data from 551 DTC patients of Part I. The presence of *BRAF*, *RAS*, or *TERT* promoter mutations alone did not significantly alter the recurrence risk, and the mortality risk of each mutation could not be calculated because of the small number of deaths. However, their coexistence increased the risk of both recurrence (HR of the coexistence of *BRAF* and *TERT*, 4.64; 95% CI, 1.42-15.18; HR of the coexistence of *RAS* and *TERT*, 5.36; 95% CI, 1.20-24.02; Table 29; Fig. 27B) and mortality (HR of the coexistence of *BRAF* and *TERT*, 15.13; 95% CI, 1.55-148.23; HR of the coexistence of *RAS* and *TERT*, 14.75; 95% CI, 1.30-167.00; Table 29; Fig. 27C), even after adjustments for the age at diagnosis and sex. However, the statistical significance disappeared after additional adjustments for tumor size, extrathyroidal extension, and lymph node metastasis except for mortality with coexisting *RAS* and *TERT* promoter mutations.

Table 28. Hazard ratios of *RAS* and *TERT* promoter mutations for recurrence of follicular thyroid cancer

Mutation status	Cases	Recurrence/ persistence, n (%)	Recurrence, n (%)	Unadjusted			Adjusted[a]		`
				HR	95% CI	*P*	HR	95% CI	*P*
No mutation	77	3 (3.9)	2 (2.6)	1			1		
RAS only	36	4 (11.1)	1 (2.8)	1.13	0.10−12.46	0.922	2.86	0.21−39.57	0.433
TERT only	2	0 (0.0)	0 (0.0)	–			–		
RAS + *TERT*	5	3 (60.0)	2 (40.0)	13.59	1.88−98.55	0.010	6.27	0.76−51.68	0.088

[a] Adjustment for age at diagnosis and sex; HR, hazard ratio.

Table 29. Hazard ratios *RAS* and *TERT* promoter mutations for recurrence and disease-specific death of differentiated thyroid cancer

	N (%)	Event per 1,000 PY	Unadjusted HR (95% CI)	*P*	Adjusted HR[a] (95% CI)	*P*	Adjusted HR[b] (95% CI)	*P*
Recurrence								
No mutation	21/240 (8.8)	15.94	1.00	–	1.00	–	1.00	–
RAS only	1/47 (2.1)	3.64	0.24 (0.03–1.79)	0.165	0.24 (0.03–1.77)	0.160	0.48 (0.06–3.77)	0.486
TERT only	1/7 (14.3)	32.34	1.86 (0.25–13.81)	0.546	2.03 (0.27–15.18)	0.489	2.59 (0.33–20.13)	0.364
RAS + *TERT*	2/6 (33.3)	65.39	4.16 (0.97–17.79)	0.054	5.36 (1.20–24.02)	0.028	3.09 (0.64–14.81)	0.159
Mortality								
No mutation	2/240 (0.8)	1.27	1.00	–	1.00	–	1.00	–
RAS only	0/47 (0.0)	0	–	–	–	–	–	–
TERT only	0/7 (0.0)	0	–	–	–	–	–	–
RAS + *TERT*	1/6 (16.7)	28.78	20.70 (1.87–228.53)	0.013	14.75 (1.30–167.00)	0.030	24.34 (1.51–392.20)	0.024

Abbreviations: PY, person-years; HR, hazard ratio; CI, confidence interval.

[a]Adjusted for age at diagnosis and sex.

[b]Adjusted for age at diagnosis, sex, tumor size, extrathyroidal extension, and lymph node metastasis.

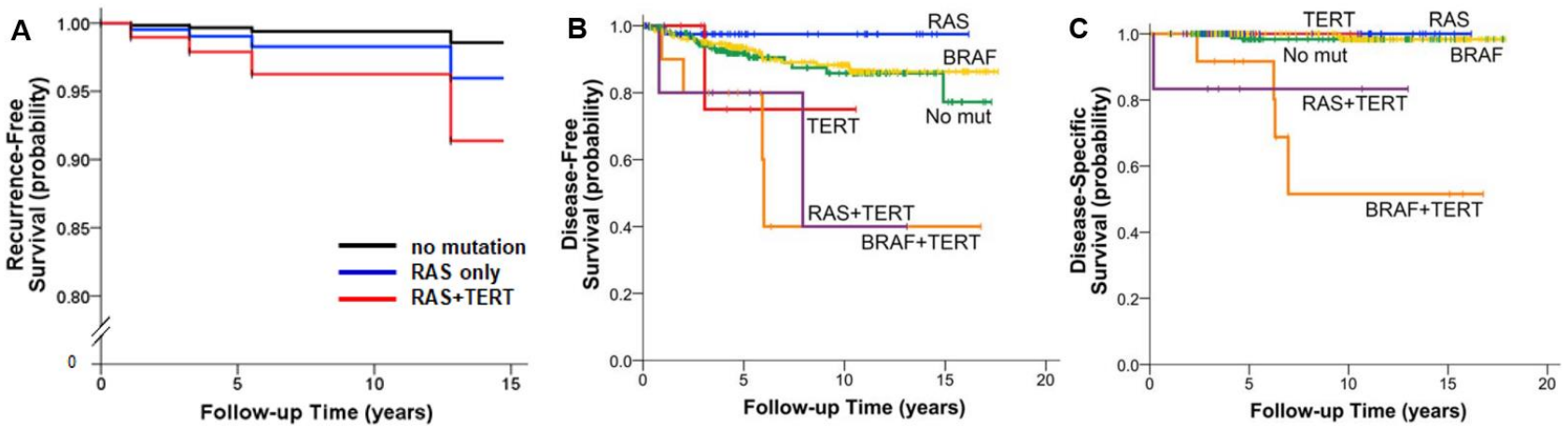


Figure 27. Effects of the coexistence of *RAS* and *TERT* promoter mutations on (A, B) disease-free and (C) disease-specific survival for patients with (A) follicular thyroid cancer and (B, C) differentiated thyroid cancer

III-2. Molecular genetic mechanisms of synergistic interaction between *TERT* promoter and *RAS* mutations

Materials and methods

Data acquisition and patient selection

Among the genomic data of anonymized patients with PTC were available from TCGA data portal (https://portal.gdc.cancer.gov/), I downloaded the data on clinical information, somatic mutations, mRNA expression, and DNA methylation in September 2016. The whole exome, and mRNA sequencing were performed on the Illumina HiSeq 2000 platform and DNA methylation analysis was performed using the Illumina Infinium HM450 array (Bibikova et al., 2011). For each CpG site, methylation levels were quantified using β-values, which represent the proportion of methylation and range from 0 to 1. A total of 387 samples have the *TERT* promoter sequencing results by either Illumina MiSeq or whole genome sequencing. To remove possible influences from driver mutations or fusions other than *RAS* or *TERT* promoter mutation, 239 with other driver mutations and 57 with any driver fusions were further excluded. The driver genetic alterations were defined as the significantly

mutated genes and driver fusions, which were definitions used in TCGA study (Cancer Genome Atlas Research, 2014). Finally, 91 patients with PTC were included in the present analysis, and I classified them according to the mutational status: 41 of negative for the driver genetic alterations including *RAS* and *TERT* promoter mutations (no mutation), 42 of *RAS* mutation only (*RAS* only), 2 of *TERT* promoter mutation only (*TERT* only), and 6 of the coexistence of *RAS* and *TERT* promoter mutations (*RAS*+*TERT*). For the DEG analysis, *TERT* only group was excluded because of small number of subjects in this group. As a validation set of genomic analysis, 33 FTC samples having RNA sequencing data from our previous research (SNU database; (Yoo et al., 2016)) were used in this study. In addition to the previous study, I performed Sanger sequencing for detection of *TERT* promoter mutation with previously described method (Song et al., 2016a). After excluding other driver mutations and other driver fusions, 24 patients with FTC were included: 3 of no mutation, 14 of *RAS* only, 0 of *TERT* only, and 7 of *RAS*+*TERT* group.

Gene expression profiling and differentially expressed gene analysis and statistical analysis

Same as the methods of in Part II-3.

Results

Effects of coexistence of RAS and TERT promoter mutations on poor clinicopathological outcomes in DTC

Next, to identify the mechanism of interaction between *RAS* and *TERT* promoter mutations, I performed the genomic analyses using public repository database of TCGA and our database as a validation set. The clinicopathological characteristics of subjects from TCGA are presented in Table 30. Among the subjects of TCGA database, in comparison with the no-mutation group, the characteristics of *RAS*-only group was not significantly different except for the younger age at diagnosis. However, *RAS+TERT* group was strongly associated with virtually all high-risk features such as higher proportion of male gender, older age at diagnosis, higher frequency of tall-cell variant PTC, larger tumor size, higher proportion of distant metastasis and recurrence and higher stage or sore of the prognosis prediction models, compared to no-mutation or *RAS*-only group.

Among the subjects from SNU database, similar to the TCGA database, *RAS+TERT* group showed high-risk clinicopathological characteristics, although there were some cases in which the number of subjects in each group did not prove statistical significance (Table 31).

Table 30. Clinicopathological characteristics of subjects of TCGA database according to *RAS* and *TERT* promoter mutational status

	No mutation	*RAS* only	*RAS+TERT*	*P*
	41	42	6	
Male sex, n (%)	6 (14.6)	9 (21.4)	4 (66.7)	0.027
Age at diagnosis, years	52.7 ± 13.4	42.3 ± 14.0[b]	61.5 ± 9.6[c]	0.001
Tumor size, cm[a]	3.1 (1.9–4.5)	2.3 (1.8–3.0)	4.4 (2.0 –6.7)[c]	0.058
Extrathyroidal extension, n (%)	3/34 (8.8)	4/37 (10.8)	2/6 (33.3)	0.270
Multifocalty, n (%)	21 (52.5)	18 (42.9)	3 (50.0)	0.753
Lymph node metastasis, n (%)	6/33 (18.2)	8/38 (21.1)[b]	2/6 (33.3)	0.752
Distant metastasis, n (%)	0/17 (0.0)	0/26 (0.0)	2/2 (100.0)[b,c]	0.001
Recurrence	1/38 (2.6)	2/39 (5.1)	3/6 (50.5)[b,c]	0.004
All-cause death	2 (4.9)	0 (0.0)	0 (0.0)	0.340
TNM stage, n (%)				0.003
I–II	31 (75.6)	35 (83.3)	1 (16.7)[b,c]	
III–IV	10 (24.4)	7 (16.7)	5 (83.3)[b,c]	

[a]Data presented as medians (interquartile ranges); [b]Significantly different from the no-mutation group; [c]Significantly different from the *RAS*-only group

Table 31. Clinicopathological characteristics of subjects of SNU database according to *RAS* and *TERT* promoter mutational status

	No mutation	*RAS* only	*RAS+TERT*	*P*
	3	12	9	
Male sex, n (%)	0 (0.0)	6 (50.0)	5 (55.6)	0.436
Age at diagnosis, years	35.0 ± 19.3	45.4 ± 11.4	61.5 ± 9.6[b,c]	0.006
Tumor size, cm[a]	4.4 (3.7–5.2)	2.9 (1.8–3.7)	3.1 (2.2 –3.8)	0.323
Widely invasive FTC, n (%)	0 (0.0)	0 (0.0)	6 (66.7)[c]	0.001
Extrathyroidal extension, n (%)	0 (0.0)	0 (0.0)	4 (44.4)[c]	0.024
Lymph node metastasis, n (%)	0 (0.0)	0 (0.0)	3 (33.3)	0.073
Distant metastasis, n (%)	0 (0.0)	0 (0.0)	7 (77.8)[b,c]	<0.001
TNM stage, n (%)				
I–II	3 (100.0)	11 (91.7)	3 (33.3)[c]	0.012
III–IV	0 (0.0)	1 (8.3)	6 (66.7)[c]	
MACIS	4.9 ± 0.5	4.7 ± 1.0	8.1 ± 2.5[b,c]	0.001

Upregulated TERT mRNA expression in coexistence of RAS and TERT promoter mutations

To identify the mechanism for the clinical aggressiveness of tumors harboring both *RAS* and *TERT* promoter mutations, I examined the mRNA expression level of *TERT* using TCGA database (Fig. 28). Compared with no-mutation group, *RAS* only or *TERT* only group did not show a significant difference in *TERT* expression (fold change, 1.25 and 1.57, respectively; *q*-value = 0.508 and 0.866, respectively), while, *RAS*+*TERT* group showed significantly higher *TERT* expression (fold change, 5.58; *q*-value = 0.004) (Fig. 28A). The mRNA expression of *TERT* was detected in 4.8% of tumors in *RAS*-only group (2 of 42), and 14.6% of tumors in no-mutation group (6 of 41) (Fig. 28B). The clinicopathological characteristics of the coexistence of *RAS* mutation with *TERT* expression showed more aggressive compared with the *RAS* mutation without *TERT* expression, but in the *RAS*-only group, the number of tumors harboring *TERT* expression was so small (n = 2) that it was impossible to compare those with and without *TERT* expression.

These results were similarly observed in FTC tumors of SNU (Fig. 28, C and D).

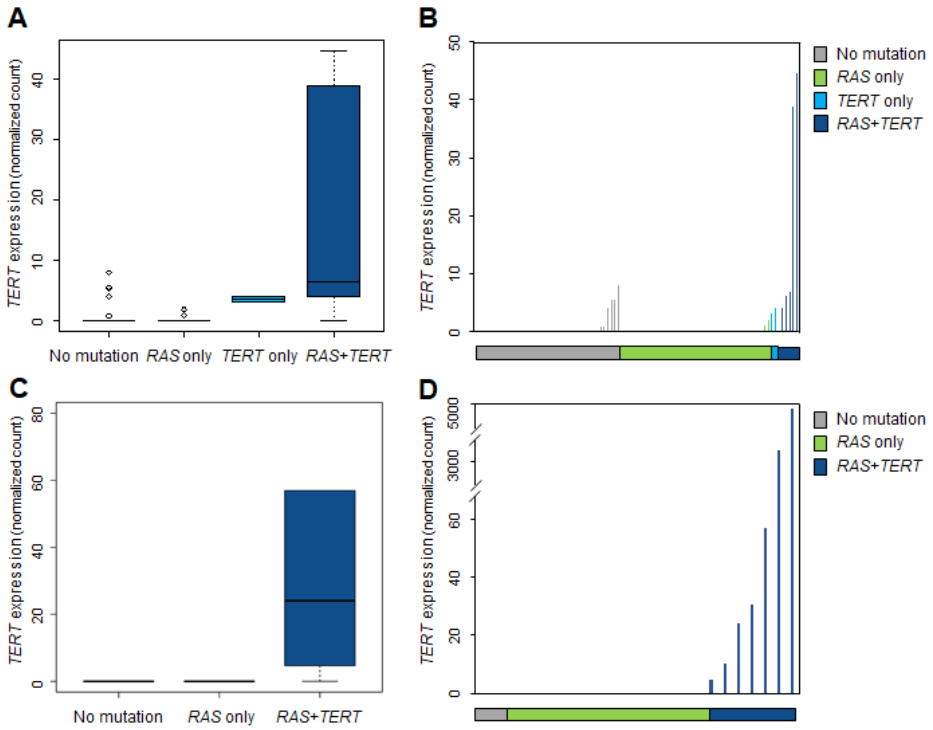


Figure 28. *TERT* mRNA expression according to mutational status. *TERT* mRNA expression levels from RNA sequencing data of TCGA (A, B) and SNU (C, D) database. (A, C) Median expression levels of *TERT* expression according to mutational status. (B, D) Each column represents an individual sample.

Molecular mechanisms of upregulated TERT expression by adding RAS mutation to TERT promoter mutations

Transcription of *TERT* can be regulated by its promoter site, such as modulation of methylation status or various transcription factors recognizing their consensus sequence (Akincilar et al., 2016). To elucidate the possible mechanism of upregulated *TERT* expression in the coexistence of *RAS* and *TERT* promoter mutations, firstly, the analysis of *TERT* promoter methylation was performed using methylation array data of TCGA study. I compared *TERT* promoter methylation status in three CpG sites available in TCGA database, which were located upstream of the transcription start site of *TERT* (chromosome 5: 1,295,737-1,295,988; cg26006951, cg17166338, and cg11625005). The expression of *TERT* mRNA was correlated positively with one hypomethylated CpG sites of *TERT* promoter (cg11625005 r = 0.421, P = 0.005) (Fig. 29A). In *RAS*+*TERT* group, methylation levels of the CpG sites slightly increased but the statistical significance was observed in one CpG site of cg11625005 compared with *RAS* alone group (q-value = 0.044), even though not to be hypermethylated (Fig. 29, B-D).

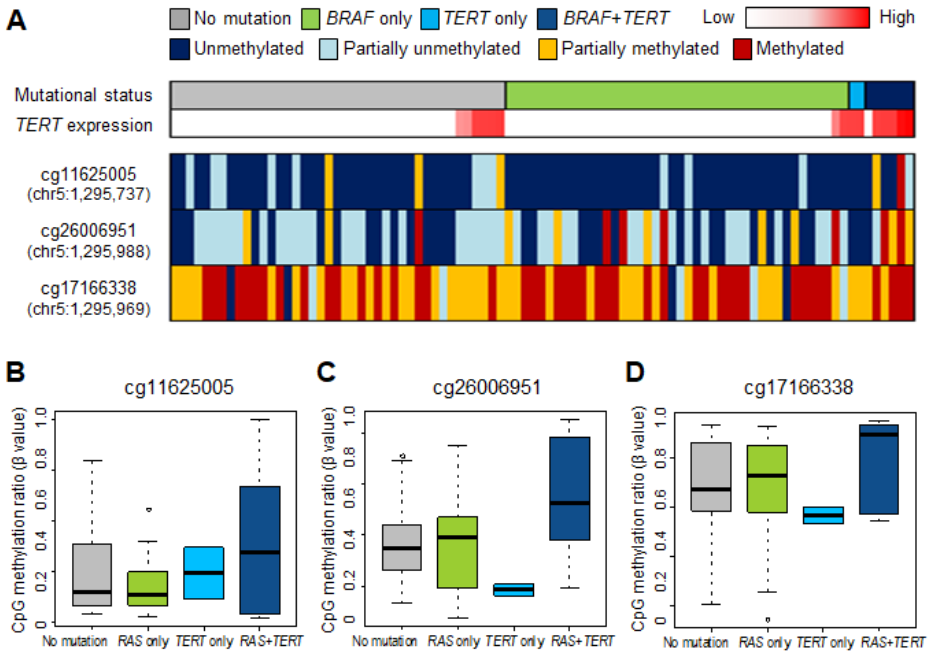


Figure 29. Molecular mechanisms of upregulated *TERT* expression. (A-D) Methylation status of *TERT* promoter according to mutational status. The 3 CpG sites of *TERT* promoter were available in TCGA database and methylation levels were quantified using β-values ranging from 0 to 1. (A) The methylation status was defined as follows: >0.7, methylated (red); 0.5 to 0.7, partially methylated (orange); 0.3 to <0.5, partially unmethylated (cyan); and <0.3, unmethylated (blue). (B-D) Median methylation levels of each CpG site according to mutational status.

Then, to evaluate the previously suggested mechanism, I next examined whether *RAS* mutation upregulates *TERT* expression by increasing *ETS* expression, since *TERT* promoter mutation creates a binding motif for ETS transcription factors (Horn et al., 2013; Huang et al., 2013). Among whole ETS family which consists of 28 genes and 12 subfamilies, 3 genes (*ETV1*, *ETV4*, and *ETV5*) of PEA3 subfamily were significantly upregulated, which was satisfied with the criteria of upregulated DEGs, in the *RAS*+*TERT* and *RAS*-only groups compared to the no-mutation group. (Fig. 30A and B). When I analyzed with FTC samples of SNU dataset, the similar results were found: *ETV1*, *ETV4*, and *ETV5* in the *RAS*+*TERT* and *RAS*-only groups were included in the upregulated DEGs (Fig. 30C).

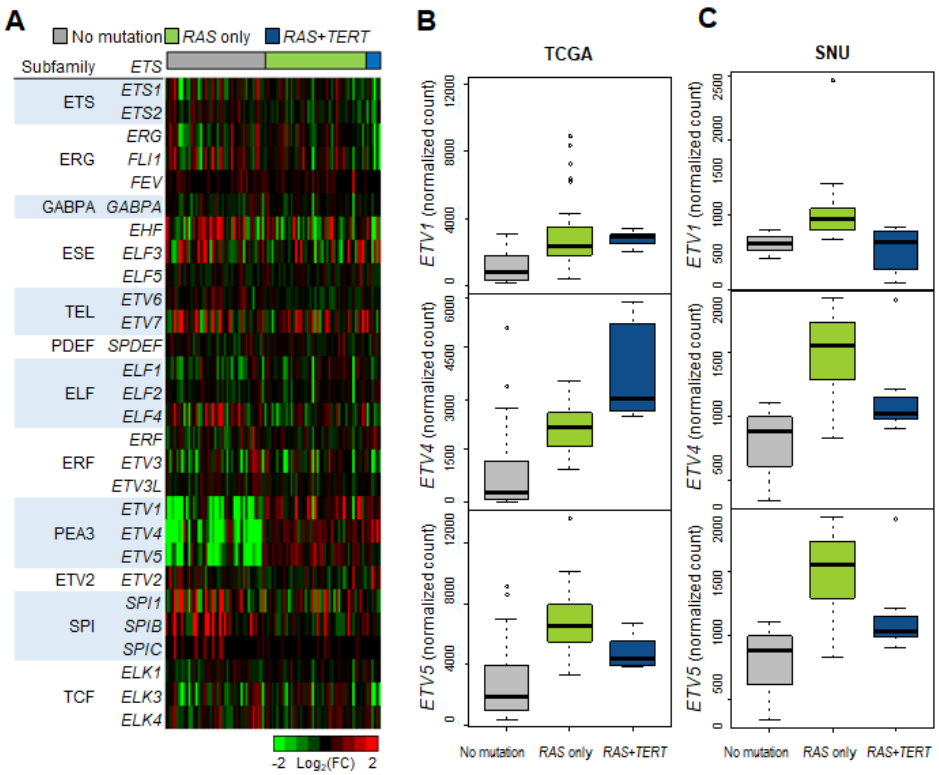


Figure 30. Upregulated expression of *ETV1*, *ETV4*, and *ETV5* by the *RAS* mutation. (A) Heatmap showing discriminatory *ETS* genes according to mutational status of TCGA database. (B, C) Median expression levels of *ETV1*, *ETV4*, and *ETV5* according to mutational status in TCGA (B) and SNU (C) database.

Changes in the intracellular signaling pathways by the coexistence of RAS and TERT promoter mutations

To investigate whether the increased *TERT* expression by the coexistence of *RAS* and *TERT* promoter mutations makes some changes in the intracellular signaling pathways, which might the aggressive tumor behavior, I next compared the transcriptional profiles according to the mutational status. The results were illustrated by volcano plots, in which up-regulated DEGs were denoted by red dots and down-regulated DEGs by dark-green dots. The *RAS*-only and *RAS*+*TERT* groups showed a significant change in transcriptional profiles demonstrating a number of DEGs compared to the no-mutation group (Fig. 31A and B). Moreover, there were a number of DEGs between *RAS*-only and *RAS*+*TERT* groups, while there were few DEGs between *BRAF*-only group and *BRAF*+*TERT* groups in the analysis of Part II (Fig. 31C). In the analysis with SNU dataset, the similar pattern of transcriptional changes was found, and there was no DEG between the *RAS*-only and the *RAS*+*TERT* groups (Fig. 31, D-F). I observed that the pathways related to the aggressive behaviors of cancer including MAPK signaling pathway were upregulated by adding the *TERT* promoter mutation to the *RAS* mutation (Fig. 32). And I could see the same results when I classified the subjects with *RAS* mutation by the *TERT*

expression status, because most of the patients with high expression were the *RAS+TERT* group (Fig. 33).

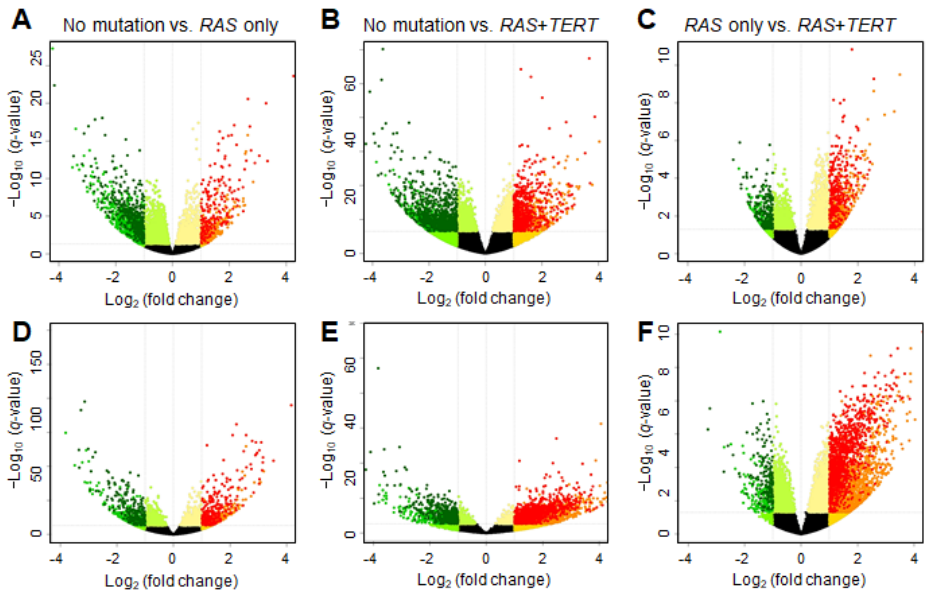


Figure 31. Transcriptional changes according to mutational status of the *RAS* and *TERT* promoter. Differentially expressed genes from RNA sequencing analyses of (A-C) TCGA and (D-E) SNU database were illustrated as the dark-green dots (down-regulated) and the red dots (up-regulated) of volcano plots.

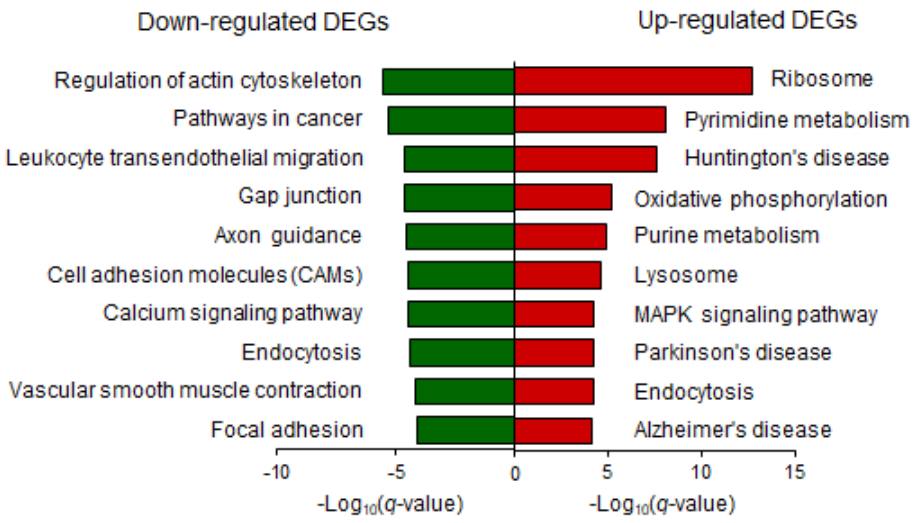


Figure 32. The top 10 most significantly enriched molecular pathways of the DEGs in tumors with *TERT* expression compared with those without *TERT* expression in *RAS*-mutated FTCs in SNU database.

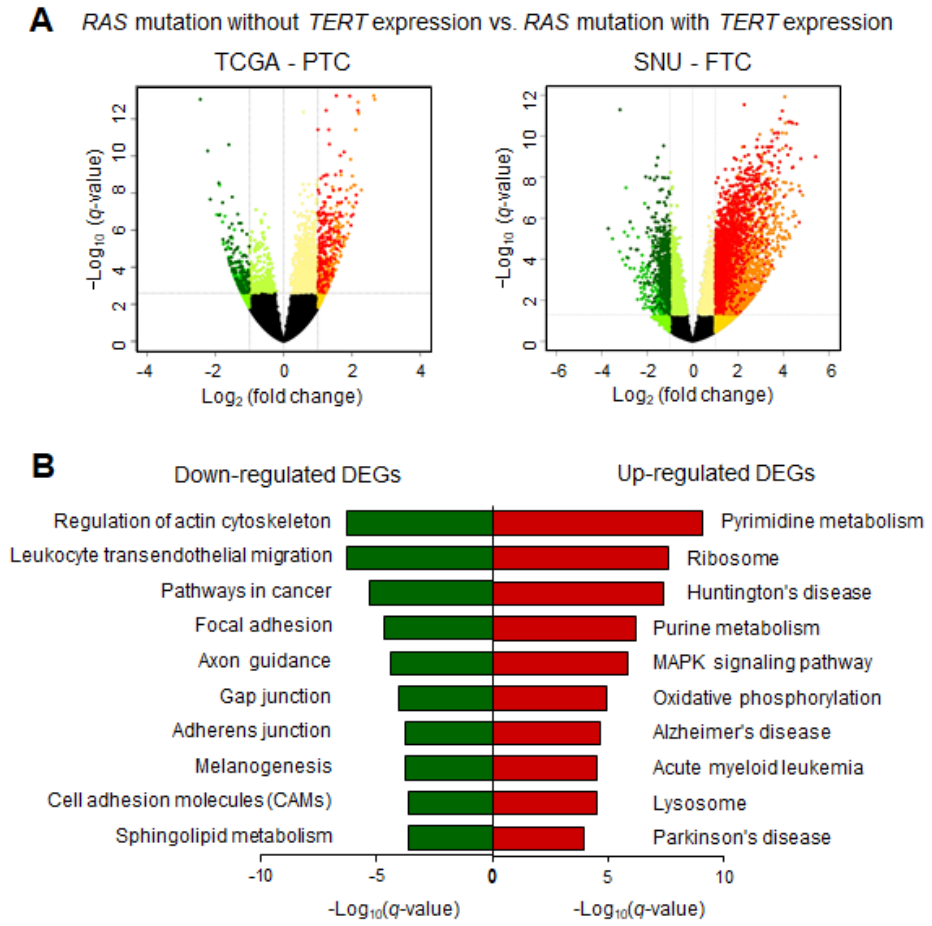


Figure 33. Transcriptional changes by *TERT* expression in addition to the *RAS* mutation. (A) Differentially expressed genes between *RAS* mutation without and with *TERT* expression were illustrated as the dark-green dots (down-regulated) and the red dots (up-regulated) of volcano plots. (E) The top 10 most significantly enriched molecular pathways of the DEGs in tumors with *TERT* expression compared with those without *TERT* expression in *RAS*-mutated FTCs in SNU database.

Discussion

The prevalence of *RAS* mutations decreased in recent years, while that of *TERT* promoter mutations did not change. *RAS* mutations were associated with distant metastasis, persistent disease, and frequent *TERT* promoter mutations. The coexistence of *RAS* and *TERT* promoter mutations was associated with a higher rate of recurrence, suggesting that they had additive effects on the prognosis, similarly to *BRAF* and *TERT* promoter mutations (Song et al., 2016b; Xing et al., 2014c). As for the mechanism, I could confirm that this genetic duet significantly increased *TERT* expression compared with the expression in tumors harboring *RAS* or *TERT* promoter mutation alone. Furthermore, this increase may be due to, at least in part, the upregulated expression of *ETS*, especially *ETV1*, *ETV4*, and *ETV5* by *RAS* mutation. Moreover, adding the *TERT* promoter mutation or expression to the *RAS* mutation, there were significant changes in transcriptional profile, which activated the aggressive intracellular pathways including MAPK pathways.

Recently, NGS has improved our understanding of the genetics and biology of thyroid cancer, including FTC and PTC (Cancer Genome Atlas Research, 2014; Jung et al., 2016; Yoo et al., 2016), and *RAS* point mutations are the most representative driver mutations in FTC. As the results of our study, *RAS*

mutations have been reported to be associated with distant metastasis (Jang et al., 2014; Manenti et al., 1994)and poor prognoses in FTC (Fukahori et al., 2012; Garcia-Rostan et al., 2003). This prognostic impact of *RAS* mutations in FTC seems to be in contrast to the favorable prognosis of PTC with *RAS* mutations, which is usually FVPTC without aggressive tumor behavior (Medici et al., 2015; Xing, 2016). Moreover, a similar frequency of *RAS* mutations was also observed in follicular adenoma (Sobrinho-Simoes et al., 2011; Yoo et al., 2016). Therefore, I suggest that the same *RAS* mutations might play different prognostic roles depending on the type of cancer.

Interestingly, as was found for *BRAF* mutations in PTC, *RAS* mutations were also associated with *TERT* promoter mutations in FTC, and their coexistence was associated with worse prognoses. *TERT* promoter mutations have not been detected in benign thyroid nodules, and have only uncommonly been found in cancer with a benign nature (Vinagre et al., 2013). In this study, patients with *TERT*-mutated FTC showed more frequent instances of distant metastasis, and higher recurrence and mortality. Although I could not demonstrate the effects of *TERT* promoter mutations alone, when they coexisted with a *RAS* mutation, the hazard ratio of recurrence was increased by more than 6 times (Table 5). Thus, *TERT* could be a useful prognostic marker

of malignancy, especially for *RAS*-mutated follicular neoplasm.

Regarding other oncogenes of FTC rather than *RAS* and *TERT*, Nikiforova et al. (Nikiforova et al., 2003) tested 12 cancer genes using targeted sequencing panel in 36 FTCs and identified that the second most common mutations after *RAS* (n = 12) were *TSHR* (n = 4) and *TP53* (n = 4) mutations in conventional and oncocytic FTCs, respectively. However, in the two recently published studies of FTC genomics in Korean using a NGS approach, including a study of our institute (Jung et al., 2016; Yoo et al., 2016), the prevalence of *TSHR* and *TP53* mutations were relatively very low: there was no FTC patient harboring *TSHR* mutation in both studies, and only one of 30 patients (3.3%) with *TP53* mutation who had a favorable prognosis in our study (Yoo et al., 2016). Moreover, according to the recent NGS studies on anaplastic or poorly-differentiated thyroid cancer (Kunstman et al., 2015; Landa et al., 2016), *TP53*, unlike *TERT*, might be related the aggressiveness of undifferentiated thyroid cancer rather than well-differentiated thyroid cancer.

The common mechanism of synergistic oncogene interaction between *TERT* and *BRAF* or *RAS* was explained by increased *TERT* expression, which may result from the *BRAF* or *RAS*-induced upregulation of several ETS transcription factors. Therapeutic targeting of transcription factors has been

considered to be quite a challenging work, however, new insights of the molecular mechanisms that control the ETS activity have opened a new chapter for the novel strategies to target this ETS transcription factor family (Sizemore et al., 2017). For example, in ETS fusion-positive prostate cancer and Ewing sarcoma, there have been attempts to target proteins stabilizing ETS or promote degradation, although they are still in phase I or II clinical trials or preclinical studies (Brenner et al., 2011; Choy et al., 2014). On the other hand, an indirect inhibition of ETS through inhibiting MAPK, KIT, or PDGFR has been reported to be effective, because ETS factors are the downstream effectors of them. Currently, PDGFR-mutated patients with gastrointestinal stromal tumor are being recruited for a phase III trial of PDGFR inhibitors (US National Library of Medicine). Targeting ETS may have a potential problem which might inhibit negative feedback in the MAPK pathway (Tetsu and McCormick, 2017), thus, double blocking of ETS and its upstream pathway such as MAPK can be a good option to overcome the limitation. Therefore, ETS-targeted therapy might be one of the promising therapeutic strategies especially for refractory thyroid cancer harboring the coexistence of *TERT* promoter mutation with *BRAF* or *RAS* mutation. However, further preclinical and clinical studies of ETS-targeted therapy in thyroid cancer are needed.

In conclusion, the prevalence of *RAS* mutations decreased. *RAS* mutations may be associated with poor clinical outcomes in FTC, especially in the coexistence with *TERT* promoter mutations. This synergistic effect of two mutations may be attributed to the activation of intracellular pathways related to the aggressive tumor behavior.

References

1. Ahn, H.S., Kim, H.J., and Welch, H.G. (2014). Korea's thyroid-cancer "epidemic"--screening and overdiagnosis. The New England journal of medicine *371*, 1765-1767.
2. Akincilar, S.C., Unal, B., and Tergaonkar, V. (2016). Reactivation of telomerase in cancer. Cell Mol Life Sci *73*, 1659-1670.
3. American Thyroid Association Guidelines Taskforce on Thyroid, N., Differentiated Thyroid, C., Cooper, D.S., Doherty, G.M., Haugen, B.R., Kloos, R.T., Lee, S.L., Mandel, S.J., Mazzaferri, E.L., McIver, B., *et al.* (2009). Revised American Thyroid Association management guidelines for patients with thyroid nodules and differentiated thyroid cancer. Thyroid *19*, 1167-1214.
4. Bell, R.J., Rube, H.T., Kreig, A., Mancini, A., Fouse, S.D., Nagarajan, R.P., Choi, S., Hong, C., He, D., Pekmezci, M., *et al.* (2015). Cancer. The transcription factor GABP selectively binds and activates the mutant TERT promoter in cancer. Science *348*, 1036-1039.
5. Bibikova, M., Barnes, B., Tsan, C., Ho, V., Klotzle, B., Le, J.M., Delano, D., Zhang, L., Schroth, G.P., Gunderson, K.L., *et al.* (2011). High density DNA methylation array with single CpG site resolution. Genomics *98*, 288-295.
6. Blasco, M.A. (2005). Telomeres and human disease: ageing, cancer and beyond. Nat Rev Genet *6*, 611-622.
7. Brenner, J.C., Ateeq, B., Li, Y., Yocum, A.K., Cao, Q., Asangani, I.A.,

Patel, S., Wang, X., Liang, H., Yu, J., *et al.* (2011). Mechanistic rationale for inhibition of poly(ADP-ribose) polymerase in ETS gene fusion-positive prostate cancer. Cancer Cell *19*, 664-678.

8. Bullock, M., Ren, Y., O'Neill, C., Gill, A., Aniss, A., Sywak, M., Sidhu, S., Delbridge, L., Learoyd, D., de Vathaire, F., *et al.* (2016). TERT promoter mutations are a major indicator of recurrence and death due to papillary thyroid carcinomas. Clin Endocrinol (Oxf) *85*, 283-290.
9. Cady, B., and Rossi, R. (1988). An expanded view of risk-group definition in differentiated thyroid carcinoma. Surgery *104*, 947-953.
10. Cancer Genome Atlas Research, N. (2014). Integrated genomic characterization of papillary thyroid carcinoma. Cell *159*, 676-690.
11. Castelo-Branco, P., Choufani, S., Mack, S., Gallagher, D., Zhang, C., Lipman, T., Zhukova, N., Walker, E.J., Martin, D., Merino, D., *et al.* (2013). Methylation of the TERT promoter and risk stratification of childhood brain tumours: an integrative genomic and molecular study. Lancet Oncol *14*, 534-542.
12. Cho, B.Y., Choi, H.S., Park, Y.J., Lim, J.A., Ahn, H.Y., Lee, E.K., Kim, K.W., Yi, K.H., Chung, J.K., Youn, Y.K., *et al.* (2013a). Changes in the clinicopathological characteristics and outcomes of thyroid cancer in Korea over the past four decades. Thyroid *23*, 797-804.
13. Cho, B.Y., Choi, H.S., Park, Y.J., Lim, J.A., Ahn, H.Y., Lee, E.K., Kim, K.W., Yi, K.H., Chung, J.K., Youn, Y.K., *et al.* (2013b). Changes in the clinicopathological characteristics and outcomes of thyroid cancer in Korea over the past four decades. Thyroid *23*, 797-804.

14. Cho, S.W., Choi, H.S., Yeom, G.J., Lim, J.A., Moon, J.H., Park do, J., Chung, J.K., Cho, B.Y., Yi, K.H., and Park, Y.J. (2014). Long-term prognosis of differentiated thyroid cancer with lung metastasis in Korea and its prognostic factors. Thyroid : official journal of the American Thyroid Association *24*, 277-286.

15. Choi, H., Lim, J.A., Ahn, H.Y., Cho, S.W., Lee, K.E., Kim, K.W., Yi, K.H., Sung, M.W., Youn, Y.K., Chung, J.K., *et al.* (2014). Secular trends in the prognostic factors for papillary thyroid cancer. European journal of endocrinology / European Federation of Endocrine Societies *171*, 667-675.

16. Choy, E., Butrynski, J.E., Harmon, D.C., Morgan, J.A., George, S., Wagner, A.J., D'Adamo, D., Cote, G.M., Flamand, Y., Benes, C.H., *et al.* (2014). Phase II study of olaparib in patients with refractory Ewing sarcoma following failure of standard chemotherapy. BMC Cancer *14*, 813.

17. Davies, L., and Welch, H.G. (2014). Current thyroid cancer trends in the United States. JAMA otolaryngology-- head & neck surgery *140*, 317-322.

18. De-Tao, Y., Kun, Y., Run-Qing, L., Xianghua, L., Jianhui, X., Mengyuan, L., Hongqiang, L., Yongfei, W., and Zhen, L. (2016). Clinicopathological significance of TERT promoter mutation in papillary thyroid carcinomas: a systematic review and meta-analysis. Clin Endocrinol (Oxf).

19. de Biase, D., Gandolfi, G., Ragazzi, M., Eszlinger, M., Sancisi, V.,

Gugnoni, M., Visani, M., Pession, A., Casadei, G., Durante, C., *et al.* (2015). TERT Promoter Mutations in Papillary Thyroid Microcarcinomas. Thyroid *25*, 1013-1019.

20. de Hoon, M.J., Imoto, S., Nolan, J., and Miyano, S. (2004). Open source clustering software. Bioinformatics *20*, 1453-1454.

21. DeLellis, R., Lloyd, R., Heitz, P., Eng, C., and eds. WHO classification of tumors, pathology and genetics—tumors of endocrine organs. IARC Press, Lyon 2004.

22. Edge, S.B., and Compton, C.C. (2010). The American Joint Committee on Cancer: the 7th edition of the AJCC cancer staging manual and the future of TNM. Annals of surgical oncology *17*, 1471-1474.

23. Fan, Y., Lee, S., Wu, G., Easton, J., Yergeau, D., Dummer, R., Vogel, P., Kirkwood, J.M., Barnhill, R.L., Pappo, A., *et al.* (2016). Telomerase Expression by Aberrant Methylation of the TERT Promoter in Melanoma Arising in Giant Congenital Nevi. J Invest Dermatol *136*, 339-342.

24. Fukahori, M., Yoshida, A., Hayashi, H., Yoshihara, M., Matsukuma, S., Sakuma, Y., Koizume, S., Okamoto, N., Kondo, T., Masuda, M., *et al.* (2012). The associations between RAS mutations and clinical characteristics in follicular thyroid tumors: new insights from a single center and a large patient cohort. Thyroid *22*, 683-689.

25. Gandolfi, G., Ragazzi, M., Frasoldati, A., Piana, S., Ciarrocchi, A., and Sancisi, V. (2015). TERT promoter mutations are associated with distant metastases in papillary thyroid carcinoma. European journal of

endocrinology / European Federation of Endocrine Societies *172*, 403-413.

26. Garcia-Rostan, G., Zhao, H., Camp, R.L., Pollan, M., Herrero, A., Pardo, J., Wu, R., Carcangiu, M.L., Costa, J., and Tallini, G. (2003). ras mutations are associated with aggressive tumor phenotypes and poor prognosis in thyroid cancer. J Clin Oncol *21*, 3226-3235.

27. Gianoukakis, A.G., Giannelli, S.M., Salameh, W.A., and McPhaul, L.W. (2011). Well differentiated follicular thyroid neoplasia: impact of molecular and technological advances on detection, monitoring and treatment. Molecular and cellular endocrinology *332*, 9-20.

28. Haugen, B.R., Alexander, E.K., Bible, K.C., Doherty, G.M., Mandel, S.J., Nikiforov, Y.E., Pacini, F., Randolph, G.W., Sawka, A.M., Schlumberger, M., *et al.* (2016). 2015 American Thyroid Association Management Guidelines for Adult Patients with Thyroid Nodules and Differentiated Thyroid Cancer: The American Thyroid Association Guidelines Task Force on Thyroid Nodules and Differentiated Thyroid Cancer. Thyroid *26*, 1-133.

29. Hay, I.D., Bergstralh, E.J., Goellner, J.R., Ebersold, J.R., and Grant, C.S. (1993). Predicting outcome in papillary thyroid carcinoma: development of a reliable prognostic scoring system in a cohort of 1779 patients surgically treated at one institution during 1940 through 1989. Surgery *114*, 1050-1057; discussion 1057-1058.

30. Ho, A.S., Davies, L., Nixon, I.J., Palmer, F.L., Wang, L.Y., Patel, S.G., Ganly, I., Wong, R.J., Tuttle, R.M., and Morris, L.G. (2015). Increasing

diagnosis of subclinical thyroid cancers leads to spurious improvements in survival rates. Cancer *121*, 1793-1799.

31. Hoang, J.K., Lee, W.K., Lee, M., Johnson, D., and Farrell, S. (2007). US Features of thyroid malignancy: pearls and pitfalls. Radiographics *27*, 847-860; discussion 861-845.

32. Hong, A.R., Lim, J.A., Kim, T.H., Choi, H.S., Yoo, W.S., Min, H.S., Won, J.K., Lee, K.E., Jung, K.C., Park, D.J., *et al.* (2014a). The Frequency and Clinical Implications of the BRAF(V600E) Mutation in Papillary Thyroid Cancer Patients in Korea Over the Past Two Decades. Endocrinol Metab (Seoul) *29*, 505-513.

33. Hong, A.R., Lim, J.A., Kim, T.H., Choi, H.S., Yoo, W.S., Min, H.S., Won, J.K., Lee, K.E., Jung, K.C., Park do, J., *et al.* (2014b). The Frequency and Clinical Implications of the BRAF(V600E) Mutation in Papillary Thyroid Cancer Patients in Korea Over the Past Two Decades. Endocrinol Metab (Seoul) *29*, 505-513.

34. Horn, S., Figl, A., Rachakonda, P.S., Fischer, C., Sucker, A., Gast, A., Kadel, S., Moll, I., Nagore, E., Hemminki, K., *et al.* (2013). TERT promoter mutations in familial and sporadic melanoma. Science *339*, 959-961.

35. Howlader, N., Noone, A., Krapcho, M., Miller, D., Bishop, K., Altekruse, S., Kosary, C., Yu, M., Ruhl, J., Tatalovich, Z., *et al.* (2016). SEER Cancer Statistics Review, 1975-2013. Bethesda, MD: National Cancer Institute.

36. Huang, F.W., Hodis, E., Xu, M.J., Kryukov, G.V., Chin, L., and

Garraway, L.A. (2013). Highly recurrent TERT promoter mutations in human melanoma. Science *339*, 957-959.

37. Hundahl, S.A., Cady, B., Cunningham, M.P., Mazzaferri, E., McKee, R.F., Rosai, J., Shah, J.P., Fremgen, A.M., Stewart, A.K., and Holzer, S. (2000). Initial results from a prospective cohort study of 5583 cases of thyroid carcinoma treated in the united states during 1996. U.S. and German Thyroid Cancer Study Group. An American College of Surgeons Commission on Cancer Patient Care Evaluation study. Cancer *89*, 202-217.

38. Ito, Y., Yoshida, H., Maruo, R., Morita, S., Takano, T., Hirokawa, M., Yabuta, T., Fukushima, M., Inoue, H., Tomoda, C., *et al.* (2009). BRAF mutation in papillary thyroid carcinoma in a Japanese population: its lack of correlation with high-risk clinicopathological features and disease-free survival of patients. Endocrine journal *56*, 89-97.

39. Jang, E.K., Song, D.E., Sim, S.Y., Kwon, H., Choi, Y.M., Jeon, M.J., Han, J.M., Kim, W.G., Kim, T.Y., Shong, Y.K., *et al.* (2014). NRAS codon 61 mutation is associated with distant metastasis in patients with follicular thyroid carcinoma. Thyroid *24*, 1275-1281.

40. Jin, L., Chen, E., Dong, S., Cai, Y., Zhang, X., Zhou, Y., Zeng, R., Yang, F., Pan, C., Liu, Y., *et al.* (2016). BRAF and TERT promoter mutations in the aggressiveness of papillary thyroid carcinoma: a study of 653 patients. Oncotarget *7*, 18346-18355.

41. Jung, C.K., Little, M.P., Lubin, J.H., Brenner, A.V., Wells, S.A., Jr., Sigurdson, A.J., and Nikiforov, Y.E. (2014). The increase in thyroid

cancer incidence during the last four decades is accompanied by a high frequency of BRAF mutations and a sharp increase in RAS mutations. The Journal of clinical endocrinology and metabolism *99*, E276-285.

42. Jung, S.H., Kim, M.S., Jung, C.K., Park, H.C., Kim, S.Y., Liu, J., Bae, J.S., Lee, S.H., Kim, T.M., Lee, S.H., *et al.* (2016). Mutational burdens and evolutionary ages of thyroid follicular adenoma are comparable to those of follicular carcinoma. Oncotarget *7*, 69638-69648.

43. Killela, P.J., Reitman, Z.J., Jiao, Y., Bettegowda, C., Agrawal, N., Diaz, L.A., Jr., Friedman, A.H., Friedman, H., Gallia, G.L., Giovanella, B.C., *et al.* (2013). TERT promoter mutations occur frequently in gliomas and a subset of tumors derived from cells with low rates of self-renewal. Proceedings of the National Academy of Sciences of the United States of America *110*, 6021-6026.

44. Kim, B.A., Jee, H.G., Yi, J.W., Kim, S.J., Chai, Y.J., Choi, J.Y., and Lee, K.E. (2017). Expression Profiling of a Human Thyroid Cell Line Stably Expressing the BRAFV600E Mutation. Cancer Genomics Proteomics *14*, 53-67.

45. Kim, N.W., Piatyszek, M.A., Prowse, K.R., Harley, C.B., West, M.D., Ho, P.L., Coviello, G.M., Wright, W.E., Weinrich, S.L., and Shay, J.W. (1994). Specific association of human telomerase activity with immortal cells and cancer. Science *266*, 2011-2015.

46. Kim, T.H., Kim, Y.E., Ahn, S., Kim, J.Y., Ki, C.S., Oh, Y.L., Kim, K., Yun, J.W., Park, W.Y., Choe, J.H., *et al.* (2016). TERT promoter mutations and long-term survival in patients with thyroid cancer.

Endocr Relat Cancer *23*, 813-823.

47. Kim, T.H., Park, Y.J., Lim, J.A., Ahn, H.Y., Lee, E.K., Lee, Y.J., Kim, K.W., Hahn, S.K., Youn, Y.K., Kim, K.H., *et al.* (2012). The association of the BRAF(V600E) mutation with prognostic factors and poor clinical outcome in papillary thyroid cancer: a meta-analysis. Cancer *118*, 1764-1773.

48. Kim, T.Y., Kim, W.B., Song, J.Y., Rhee, Y.S., Gong, G., Cho, Y.M., Kim, S.Y., Kim, S.C., Hong, S.J., and Shong, Y.K. (2005). The BRAF mutation is not associated with poor prognostic factors in Korean patients with conventional papillary thyroid microcarcinoma. Clin Endocrinol (Oxf) *63*, 588-593.

49. Kunstman, J.W., Juhlin, C.C., Goh, G., Brown, T.C., Stenman, A., Healy, J.M., Rubinstein, J.C., Choi, M., Kiss, N., Nelson-Williams, C., *et al.* (2015). Characterization of the mutational landscape of anaplastic thyroid cancer via whole-exome sequencing. Hum Mol Genet *24*, 2318-2329.

50. Landa, I., Ganly, I., Chan, T.A., Mitsutake, N., Matsuse, M., Ibrahimpasic, T., Ghossein, R.A., and Fagin, J.A. (2013). Frequent somatic TERT promoter mutations in thyroid cancer: higher prevalence in advanced forms of the disease. The Journal of clinical endocrinology and metabolism *98*, E1562-1566.

51. Landa, I., Ibrahimpasic, T., Boucai, L., Sinha, R., Knauf, J.A., Shah, R.H., Dogan, S., Ricarte-Filho, J.C., Krishnamoorthy, G.P., Xu, B., *et al.* (2016). Genomic and transcriptomic hallmarks of poorly

differentiated and anaplastic thyroid cancers. J Clin Invest *126*, 1052-1066.

52. Lee, S.E., Hwang, T.S., Choi, Y.L., Han, H.S., Kim, W.S., Jang, M.H., Kim, S.K., and Yang, J.H. (2016). Prognostic Significance of TERT Promoter Mutations in Papillary Thyroid Carcinomas in a BRAF(V600E) Mutation-Prevalent Population. Thyroid *26*, 901-910.

53. Li, Y., and Tergaonkar, V. (2014). Noncanonical functions of telomerase: implications in telomerase-targeted cancer therapies. Cancer Res *74*, 1639-1644.

54. Li, Y., Zhou, Q.L., Sun, W., Chandrasekharan, P., Cheng, H.S., Ying, Z., Lakshmanan, M., Raju, A., Tenen, D.G., Cheng, S.Y., *et al.* (2015). Non-canonical NF-kappaB signalling and ETS1/2 cooperatively drive C250T mutant TERT promoter activation. Nat Cell Biol *17*, 1327-1338.

55. Lin, J.D., Huang, M.J., Juang, J.H., Chao, T.C., Huang, B.Y., Chen, K.W., Chen, J.Y., Li, K.L., Chen, J.F., and Ho, Y.S. (1999). Factors related to the survival of papillary and follicular thyroid carcinoma patients with distant metastases. Thyroid *9*, 1227-1235.

56. Liu, R., Bishop, J., Zhu, G., Zhang, T., Ladenson, P.W., and Xing, M. (2016). Mortality Risk Stratification by Combining BRAF V600E and TERT Promoter Mutations in Papillary Thyroid Cancer: Genetic Duet of BRAF and TERT Promoter Mutations in Thyroid Cancer Mortality. JAMA Oncol.

57. Liu, R., and Xing, M. (2016). TERT promoter mutations in thyroid cancer. Endocr Relat Cancer *23*, R143-155.

58. Liu, T., Wang, N., Cao, J., Sofiadis, A., Dinets, A., Zedenius, J., Larsson, C., and Xu, D. (2014a). The age- and shorter telomere-dependent TERT promoter mutation in follicular thyroid cell-derived carcinomas. Oncogene *33*, 4978-4984.

59. Liu, X., Bishop, J., Shan, Y., Pai, S., Liu, D., Murugan, A.K., Sun, H., El-Naggar, A.K., and Xing, M. (2013a). Highly prevalent TERT promoter mutations in aggressive thyroid cancers. Endocrine-related cancer *20*, 603-610.

60. Liu, X., Qu, S., Liu, R., Sheng, C., Shi, X., Zhu, G., Murugan, A.K., Guan, H., Yu, H., Wang, Y., *et al.* (2014b). TERT promoter mutations and their association with BRAF V600E mutation and aggressive clinicopathological characteristics of thyroid cancer. The Journal of clinical endocrinology and metabolism *99*, E1130-1136.

61. Liu, X., Wu, G., Shan, Y., Hartmann, C., von Deimling, A., and Xing, M. (2013b). Highly prevalent TERT promoter mutations in bladder cancer and glioblastoma. Cell cycle *12*, 1637-1638.

62. Love, M.I., Huber, W., and Anders, S. (2014). Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol *15*, 550.

63. Low, K.C., and Tergaonkar, V. (2013). Telomerase: central regulator of all of the hallmarks of cancer. Trends Biochem Sci *38*, 426-434.

64. Manenti, G., Pilotti, S., Re, F.C., Della Porta, G., and Pierotti, M.A. (1994). Selective activation of ras oncogenes in follicular and undifferentiated thyroid carcinomas. Eur J Cancer *30A*, 987-993.

65. Medici, M., Kwong, N., Angell, T.E., Marqusee, E., Kim, M.I., Frates, M.C., Benson, C.B., Cibas, E.S., Barletta, J.A., Krane, J.F., *et al.* (2015). The variable phenotype and low-risk nature of RAS-positive thyroid nodules. BMC Med *13*, 184.

66. Melo, M., da Rocha, A.G., Vinagre, J., Batista, R., Peixoto, J., Tavares, C., Celestino, R., Almeida, A., Salgado, C., Eloy, C., *et al.* (2014). TERT promoter mutations are a major indicator of poor outcome in differentiated thyroid carcinomas. The Journal of clinical endocrinology and metabolism *99*, E754-765.

67. Muzza, M., Colombo, C., Rossi, S., Tosi, D., Cirello, V., Perrino, M., De Leo, S., Magnani, E., Pignatti, E., Vigo, B., *et al.* (2015). Telomerase in differentiated thyroid cancer: promoter mutations, expression and localization. Molecular and cellular endocrinology *399*, 288-295.

68. Ngeow, J., and Eng, C. (2014). TERT and BRAF in thyroid cancer: teaming up for trouble. J Clin Oncol *32*, 2683-2684.

69. Nikiforova, M.N., Lynch, R.A., Biddinger, P.W., Alexander, E.K., Dorn, G.W., 2nd, Tallini, G., Kroll, T.G., and Nikiforov, Y.E. (2003). RAS point mutations and PAX8-PPAR gamma rearrangement in thyroid tumors: evidence for distinct molecular pathways in thyroid follicular carcinoma. The Journal of clinical endocrinology and metabolism *88*, 2318-2326.

70. Nucera, C., Lawler, J., and Parangi, S. (2011). BRAF(V600E) and microenvironment in thyroid cancer: a functional link to drive cancer

progression. Cancer Res *71*, 2417-2422.

71. Ogata, H., Goto, S., Sato, K., Fujibuchi, W., Bono, H., and Kanehisa, M. (1999). KEGG: Kyoto Encyclopedia of Genes and Genomes. Nucleic Acids Res *27*, 29-34.

72. Ramlee, M.K., Wang, J., Toh, W.X., and Li, S. (2016). Transcription Regulation of the Human Telomerase Reverse Transcriptase (hTERT) Gene. Genes (Basel) *7*.

73. Romei, C., Fugazzola, L., Puxeddu, E., Frasca, F., Viola, D., Muzza, M., Moretti, S., Nicolosi, M.L., Giani, C., Cirello, V., *et al.* (2012). Modifications in the papillary thyroid cancer gene profile over the last 15 years. The Journal of clinical endocrinology and metabolism *97*, E1758-1765.

74. Schlumberger, M.J. (1998). Papillary and follicular thyroid carcinoma. The New England journal of medicine *338*, 297-306.

75. Sizemore, G.M., Pitarresi, J.R., Balakrishnan, S., and Ostrowski, M.C. (2017). The ETS family of oncogenic transcription factors in solid tumours. Nat Rev Cancer *17*, 337-351.

76. Sobrinho-Simoes, M., Eloy, C., Magalhaes, J., Lobo, C., and Amaro, T. (2011). Follicular thyroid carcinoma. Mod Pathol *24 Suppl 2*, S10-18.

77. Song, Y.S., Lim, J.A., Choi, H., Won, J.K., Moon, J.H., Cho, S.W., Lee, K.E., Park, Y.J., Yi, K.H., Park, D.J., *et al.* (2016a). Prognostic effects of TERT promoter mutations are enhanced by coexistence with BRAF or RAS mutations and strengthen the risk prediction by the ATA or TNM staging system in differentiated thyroid cancer patients. Cancer

122, 1370-1379.

78. Song, Y.S., Lim, J.A., Choi, H., Won, J.K., Moon, J.H., Cho, S.W., Lee, K.E., Park, Y.J., Yi, K.H., Park do, J., *et al.* (2016b). Prognostic effects of TERT promoter mutations are enhanced by coexistence with BRAF or RAS mutations and strengthen the risk prediction by the ATA or TNM staging system in differentiated thyroid cancer patients. Cancer *122*, 1370-1379.

79. Song, Y.S., Lim, J.A., and Park, Y.J. (2015). Mutation Profile of Well-Differentiated Thyroid Cancer in Asians. Endocrinol Metab (Seoul) *30*, 252-262.

80. Sun, J., Zhang, J., Lu, J., Gao, J., Ren, X., Teng, L., Duan, H., Lin, Y., Li, X., Zhang, B., *et al.* (2016). BRAF V600E and TERT Promoter Mutations in Papillary Thyroid Carcinoma in Chinese Patients. PloS one *11*, e0153319.

81. Tetsu, O., and McCormick, F. (2017). ETS-targeted therapy: can it substitute for MEK inhibitors? Clin Transl Med *6*, 16.

82. The Cancer Genome Atlas Data Portal Available at https://gdc-portal.nci.nih.gov. Accessed September 6, 2016.

83. Tufano, R.P., Teixeira, G.V., Bishop, J., Carson, K.A., and Xing, M. (2012). BRAF mutation in papillary thyroid cancer and its value in tailoring initial treatment: a systematic review and meta-analysis. Medicine *91*, 274-286.

84. Tuttle, R.M., Tala, H., Shah, J., Leboeuf, R., Ghossein, R., Gonen, M., Brokhin, M., Omry, G., Fagin, J.A., and Shaha, A. (2010). Estimating

risk of recurrence in differentiated thyroid cancer after total thyroidectomy and radioactive iodine remnant ablation: using response to therapy variables to modify the initial risk estimates predicted by the new American Thyroid Association staging system. Thyroid *20*, 1341-1349.

85. US National Library of Medicine ClinicalTrials.gov https://clinicaltrials.gov/ct2/show/NCT02847429 (2017).

86. Vallarelli, A.F., Rachakonda, P.S., Andre, J., Heidenreich, B., Riffaud, L., Bensussan, A., Kumar, R., and Dumaz, N. (2016). TERT promoter mutations in melanoma render TERT expression dependent on MAPK pathway activation. Oncotarget *7*, 53127-53136.

87. Vinagre, J., Almeida, A., Populo, H., Batista, R., Lyra, J., Pinto, V., Coelho, R., Celestino, R., Prazeres, H., Lima, L., *et al.* (2013). Frequency of TERT promoter mutations in human cancers. Nat Commun *4*, 2185.

88. Wang, N., Liu, T., Sofiadis, A., Juhlin, C.C., Zedenius, J., Hoog, A., Larsson, C., and Xu, D. (2014). TERT promoter mutation as an early genetic event activating telomerase in follicular thyroid adenoma (FTA) and atypical FTA. Cancer *120*, 2965-2979.

89. Wu, Y., Li, G., He, D., Yang, F., He, G., He, L., Zhang, H., Deng, Y., Fan, M., Shen, L., *et al.* (2016). Telomerase reverse transcriptase methylation predicts lymph node metastasis and prognosis in patients with gastric cancer. Onco Targets Ther *9*, 279-286.

90. Xing, M. (2016). Clinical utility of RAS mutations in thyroid cancer: a

blurred picture now emerging clearer. BMC Med *14*, 12.

91. Xing, M., Haugen, B.R., and Schlumberger, M. (2013). Progress in molecular-based management of differentiated thyroid cancer. Lancet *381*, 1058-1069.

92. Xing, M., Liu, R., and Bishop, J. (2014a). TERT promoter and BRAF mutations cooperatively promote papillary thyroid cancer-related mortality. Thyroid *24*, A-131.

93. Xing, M., Liu, R., and Bishop, J. (2014b). TERT promoter and BRAF mutations cooperatively promote papillary thyroid cancer-related mortality. Thyroid *24:A-131*.

94. Xing, M., Liu, R., Liu, X., Murugan, A.K., Zhu, G., Zeiger, M.A., Pai, S., and Bishop, J. (2014c). BRAF V600E and TERT promoter mutations cooperatively identify the most aggressive papillary thyroid cancer with highest recurrence. J Clin Oncol *32*, 2718-2726.

95. Xing, M., Westra, W.H., Tufano, R.P., Cohen, Y., Rosenbaum, E., Rhoden, K.J., Carson, K.A., Vasko, V., Larin, A., Tallini, G., *et al.* (2005). BRAF mutation predicts a poorer clinical prognosis for papillary thyroid cancer. The Journal of clinical endocrinology and metabolism *90*, 6373-6379.

96. Yoo, S.K., Lee, S., Kim, S.J., Jee, H.G., Kim, B.A., Cho, H., Song, Y.S., Cho, S.W., Won, J.K., Shin, J.Y., *et al.* (2016). Comprehensive Analysis of the Transcriptional and Mutational Landscape of Follicular and Papillary Thyroid Cancers. PLoS Genet *12*, e1006239.

Summary and conclusions

The prevalence of *TERT* promoter mutations was lower in Korea than in other countries. The mutations were more frequent in tumors harboring either *BRAF* or *RAS* mutations. *TERT* promoter mutations were associated with aggressive clinicopathologic characteristics and poor prognosis, which strengthened the prognostic predictions of the conventional staging systems. Genetic screening of *TERT* promoter mutations could aid predictions of mortality and recurrence in DTC patients, particularly in high-risk patients.

The coexistence of *BRAF* and *TERT* promoter mutations had a synergistic effect on the clinicopathological characteristics of PTC including advanced TNM stage, extrathyroidal invasion, lymph node metastasis, distant metastasis, recurrence and PTC-related mortality. Therefore, molecular testing of *BRAF* and *TERT* promoter mutations together may be useful in assisting with risk stratification of PTC in clinical settings. The synergistic effects of *BRAF* and *TERT* promoter mutations on poor clinical outcomes may be attributed to augmentation of pathways activated by *BRAF* and some immune and adhesion-related pathways.

The prevalence of *RAS* mutations in FTC decreased in recent years, while that of *BRAF* mutations in PTC increased, and that of *TERT* promoter

mutations did not change. The coexistence of *RAS* and *TERT* promoter mutations was associated with a higher rate of recurrence, suggesting that they had additive effects on the prognosis, similarly to *BRAF* and *TERT* promoter mutations. The synergistic effects of *RAS* and *TERT* promoter mutations on poor clinical outcomes may be attributed to activation of aggressive intracellular pathways including MAPK pathways.

The common mechanism of synergistic oncogene interaction between *TERT* and *BRAF* or *RAS* was explained by increased *TERT* expression, which may result from the *BRAF* or *RAS*-induced upregulation of several ETS transcription factors. The immune response and cell adhesion-related pathways were activated by *TERT* gene expression, consistent with the non-canonical role of *TERT*.

국문초록

갑상선암에서 *TERT* promoter 변이의 의의와 *BRAF*, *RAS* 변이와의 시너지 상호작용

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최근 갑상선암에서 telomerase reverse transcriptase (*TERT*) promoter 변이가 불량한 예후와 관련 있는 것으로 알려지면서 주목을 받고 있다. 따라서 *TERT* promoter 변이의 임상적 의의 및 갑상선암의 대표적인 암유발 유전자 변이인 *BRAF*와 *RAS* 변이와의 시너지 상호작용에 대해 알아보고자 하였다. 또한 차세대염기서열분석 데이터베이스를 이용하여 이 상호작용의 분자 유전학적 기전을 밝히고자 하였다. *TERT* promoter 변이는 전체 분화갑상선암의 4.5%에서 발견이 되었고 나쁜 예후와 연관이 있었다. 다른 변이 *BRAF* (4.8%) 또는 *RAS* (11.3%)와 함께 동반된 경우가 더 빈번하였고,

ATA 고위험군(9.1%)과 TNM 병기가 높은 (12.9%) 경우에 빈도가 높았다. 고위험군 환자에서 *TERT* promoter 변이가 있는 경우 재발 및 갑상선암으로 인한 사망의 위험이 추가적으로 더 증가하였다. 갑상선유두암에서 *BRAF*와 *TERT* promoter 변이가 함께 있는 경우 나쁜 임상병리학적 특성이나 장기적 예후에 시너지 효과를 보였고, 이 효과에 대해 처음으로 메타분석을 통해 증명하였다. RNA 서열 분석 및 in vitro 실험을 통해 *TERT* promoter 변이에 *BRAF* 변이가 더해지는 경우 *TERT* 유전자의 발현이 증가하는 것을 확인하였다. (fold change, 17.00; q−value = 1.36×10^{-13}) 그리고 *TERT* 발현의 증가는 *BRAF* 변이에 의한 *ETS* 발현의 증가, 특히 *ETV1*, *ETV4*, *ETV5*의 활성화가 부분적으로 기여할 것이다. *TERT*와 *BRAF* 변이가 공존하는 경우, *BRAF* 변이 단독인 경우와 거의 같은 세포 내 신호 경로의 변화를 보였지만 유전자 발현 변화의 정도는 보다 증폭되는 것을 확인할 수 있었다. 또한 *BRAF* 변이가 있는 갑상선유두암에서 *TERT* 유전자 발현이 있는 경우에는 면역 반응 또는 세포 접착 관련 경로가 활성화되는 것을 관찰하였다. *RAS*와 *TERT* promoter 변이가 공존하는 경우에도 *BRAF*와 마찬가지로 재발의 위험이 추가

적으로 증가하는 것을 확인하였다. 그 기전으로는 *TERT* 발현이 증가하였고 (fold change, 5.58; q−value = 0.004), *TERT* promoter 변이나 *TERT* 발현이 *RAS* 변이 동반되는 경우 MAPK 경로를 포함한 종양의 공격적 특성과 관련된 세포 내 신호 경로들이 활성화되었다. 결론적으로, *TERT* promoter 변이 검사는 갑상선암 고위험군 환자들을 대상으로 시행하는 것이 사망이나 재발을 예측하는데 도움이 되겠으며, *BRAF* 또는 *RAS* 변이 검사를 함께 시행하는 것이 더욱 정확히 위험도를 예측할 수 있을 것이다. *TERT*와 *BRAF* 또는 *RAS* 간의 시너지 상호작용은 *BRAF* 또는 *RAS*에 의한 ETS 전사 인사의 활성화로 인한 *TERT* 발현의 증가로 설명될 수 있겠다. 또한 *BRAF*와 *TERT*, *RAS*와 *TERT* 유전자 듀엣은 종양의 공격적인 특성과 관련된 세포 내 경로를 활성화 시킨다.

주요어: *TERT*, *BRAF*, *RAS*, 갑상선암, 예후, 유전자 발현 분석

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