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TERT mRNA expression as a novel prognostic marker in papillary thyroid carcinomas

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Abstract:	Background: TERT promoter mutations have been found in a subset of papillary thyroid carcinomas (PTCs) and are associated with tumor aggressiveness and worse prognosis. However, little is known about the

	status of TERT mRNA expression and its relation to TERT promoter mutations and clinicopathological features. Methods: We analyzed 159 PTC samples for TERT promoter mutations using direct DNA sequencing. TERT expression was measured using quantitative RT-PCR. To examine low allelic frequency of TERT promoter mutations with high sensitivity, we used droplet digital PCR (ddPCR). The relationship between the status of the TERT promoter mutation/expression and clinicopathological features including recurrence risk was statistically analyzed. Results: TERT promoter mutations were found in 20 cases (12.6%). However, TERT expression was observed not only in the mutation-positive tumors but also in 56 of 139 (40.3%) mutation-negative tumors. Among them, we detected low allelic frequency of TERT promoter mutations in three samples (5.4%) using ddPCR. We confirmed a significant association between TERT promoter mutations and aggressive clinicopathological features in this series. The risk of recurrence of TERT mutation-negative/expression-positive tumors was significantly higher than that of the mutation-negative/expression-negative tumors, suggesting that TERT expression even in absence of a mutation confers a negative influence on PTCs. Moreover, when we reclassified the mutation-negative cases into two groups based on the TERT expression levels: expression-negative/expression levels below the 80th percentile and expression levels above the 80th percentile because minimal expression may have a negligible clinical impact, a higher hazard ratio for recurrence was observed. Interestingly, TERT expression levels in the mutation-negative PTCs were inversely correlated with patient age and the presence of BRAF mutations. Conclusions: We confirm a strong correlation between the presence of TERT promoter mutations and aggressive clinicopathological features in this PTC series. In addition, there were PTCs showing high TERT mRNA expression even in the absence of TERT promoter mutations. These cases also showed a significantly higher recurrence rate. Since the TERT promoter mutations are observed only in elderly patients, TERT mRNA expression can be a useful prognostic marker especially in younger PTC patients.

TERT mRNA expression as a novel prognostic marker in papillary thyroid carcinomas

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Running title: *TERT* expression as a prognostic marker in PTC

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ABSTRACT

Background: *TERT* promoter mutations have been found in a subset of papillary thyroid carcinomas (PTCs) and are associated with tumor aggressiveness and worse prognosis. However, little is known about the status of *TERT* mRNA expression and its relation to *TERT* promoter mutations and clinicopathological features.

Methods: We analyzed 159 PTC samples for *TERT* promoter mutations using direct DNA sequencing. *TERT* expression was measured using quantitative RT-PCR. To examine low allelic frequency of *TERT* promoter mutations with high sensitivity, we used droplet digital PCR (ddPCR). The relationship between the status of the *TERT* promoter mutation/expression and clinicopathological features including recurrence risk was statistically analyzed.

Results: *TERT* promoter mutations were found in 20 cases (12.6%). However, *TERT* expression was observed not only in the mutation-positive tumors but also in 56 of 139 (40.3%) mutation-negative tumors. Among them, we detected low allelic frequency of *TERT* promoter mutations in three samples (5.4%) using ddPCR. We confirmed a significant association between *TERT* promoter mutations and aggressive clinicopathological features in this series. The risk of recurrence of *TERT* mutation-negative/expression-positive tumors was significantly higher than that of the mutation-negative/expression-negative tumors, suggesting that *TERT* expression even in absence of a mutation confers a negative influence on PTCs. Moreover, when we reclassified the mutation-negative cases into two groups based on the *TERT* expression levels: expression-negative/expression levels below the 80th percentile and expression levels above the 80th percentile because minimal expression may have a negligible clinical impact, a higher hazard ratio for recurrence was observed. Interestingly, *TERT* expression levels in the mutation-negative PTCs were inversely correlated with patient age and the presence of *BRAF* mutations.

Conclusions: We confirm a strong correlation between the presence of *TERT* promoter

mutations and aggressive clinicopathological features in this PTC series. In addition, there were PTCs showing high *TERT* mRNA expression even in the absence of *TERT* promoter mutations. These cases also showed a significantly higher recurrence rate. Since the *TERT* promoter mutations are observed only in elderly patients, *TERT* mRNA expression can be a useful prognostic marker especially in younger PTC patients.

INTRODUCTION

The incidence of papillary thyroid carcinoma (PTC) has been increasing worldwide (1). PTC has generally a favorable prognosis; however, approximately 10–15% of patients have recurrences either locally or/and at distant sites, some of which become refractory to treatment (2, 3). To distinguish between high-risk and low-risk cases, there have been many studies evaluating the value of molecular markers to predict PTC aggressiveness and prognosis.

The *BRAF*^{V600E} mutation is the most frequent genetic change in PTC. Its prevalence varies from 30 to 80% (4), probably depending on the population. Many studies have indicated an association between the presence of the *BRAF*^{V600E} mutation and aggressive clinicopathological features; however, its prognostic value, especially as an independent marker, is still debated. According to our results, the mutation rate in Japan is high (~80%), and we did not find any correlation between the presence of the *BRAF*^{V600E} mutation and aggressive clinicopathological features and worse prognosis in our series (5).

Recently, mutations in the promoter region of the telomerase reverse transcriptase (*TERT*) gene have been found in many types of cancers including thyroid carcinomas. There are two hot spots, called C250T (chr5: 1,295,250C>T) and C228T (chr5: 1,295,228C>T) (6, 7), and these are mutually exclusive. The average rate of the presence of these mutations in PTC has been reported to be around 10% (8), and there seems to be no major differences between populations. The mutations create a binding motif for the E26 transformation-specific (ETS) transcription factors and upregulate *TERT* mRNA expression, especially when the ETS family members are activated (e.g. by BRAF^{V600E}) (9-11). While the primary function of TERT is to maintain telomere length, there is increasing evidence regarding its telomerase-independent oncogenic functions through NF-κB, Wnt/β-catenin, and MYC pathways (12-14). In PTC, many studies have

demonstrated that the co-existence of the *BRAF*^{V600E} mutation and *TERT* promoter mutations is strongly associated with aggressive features and worse prognosis (5, 15-27). Moreover, *TERT* promoter mutations seem to be also associated with anaplastic transformation (28).

However, regarding the impact of *TERT* promoter mutations, two recent findings should be considered. First, Paulsson *et al.* have reported that there is a subset of cases showing *TERT* mRNA expression in the absence of *TERT* promoter mutations in follicular thyroid tumors (adenomas, follicular tumors with uncertain malignant potential, and carcinomas) (29). In that study, the authors have also demonstrated that tumors with positive *TERT* mRNA expression showed a shorter time to recurrence compared with *TERT* expression-negative carcinomas (29). However, many of the *TERT*-expressing tumors harbored *TERT* promoter mutations, and the prognostic value of *TERT* mRNA expression independent of the presence of *TERT* promoter mutations has not been analyzed. Muzza *et al.* also demonstrated TERT protein expression in PTCs without *TERT* promoter mutations (30). However, the number of analyzed cases was limited, and a correlation analysis between presence of TERT expression and clinicopathological findings was not presented (30). Secondly, according to The Cancer Genome Atlas (TCGA) data, *TERT* promoter mutations were not clonal in PTCs (31). The mutant allele frequency varied from 5% to 50% (average 23%) and there were cases with low allelic frequency that may not be detectable by regular Sanger sequencing (31).

In PTC, little is known about the relationship between *TERT* mRNA expression and clinicopathological features. Therefore, we measured *TERT* mRNA expression levels and allelic frequency of the *TERT* promoter mutations in PTCs in the present study, and analyzed their impact on clinicopathological features, especially as a prognostic marker.

MATERIALS AND METHODS

PTC samples and patient information

We collected 159 PTC samples operated between November 2001 and December 2017 at Nagasaki University Hospital (Nagasaki, Japan) and Kuma Hospital (Kobe, Japan). Clinicopathological data were collected from the patients' medical records. Patient age at operation ranged 14–81 years old (median age: 54 years old, 17.0% male). For staging, the AJCC/TNM staging system (8th edition) was used (32, 33). The histological subtypes were: 146 classic PTCs (25 were microcarcinomas), 10 follicular variant of PTCs (four were microcarcinomas), two diffuse sclerosing variant of PTCs, and one tall cell variant of PTC. The study protocol was approved by the institutional review boards of Nagasaki University and Kuma Hospital. Written informed consent was obtained from each patient. Fresh tumor tissue samples were obtained during surgical operations, snap-frozen in liquid nitrogen, and stored at -80°C. DNA and total RNA were extracted at the same time using ISOGEN reagent (Nippon Gene) according to the manufacturer's protocol.

Direct DNA sequencing

The mutational status of *BRAF* (around V600) and the promoter region of *TERT* were analyzed by direct DNA sequencing (the Sanger method) as described previously (5).

Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Total RNA was reverse transcribed using a High Capacity RNA-to-cDNA kit (Applied Biosystems). The following PCR reactions were done using SYBR Premix Ex *Taq*II (TaKaRa Bio) in a Thermal Cycler Dice real-time system (TaKaRa Bio). The cycle threshold (CT) value, which was determined using the second derivative method, was used to calculate relative expression. The *TERT* mRNA levels were normalized using *TATA-binding protein (TBP)* mRNA expression as a reference. Primer sequences are: *TERT* ex6–7 F, 5′-AGCCACGTCTCTACCTTGAC-3′ and *TERT* ex7–8 R, 5′-

CTCATTCAGGGAGGAGCTCT-3′; *TBP* ex2 F, 5'-CCTGCCACCTTACGCTCAG-3' and *TBP* ex3 R, 5'-TGGTGTTCTGAATAGGCTGTGG-3'.

Droplet digital PCR (ddPCR)

ddPCR was performed using ddPCR Supermix for Probes (Bio-Rad Laboratories) in a QX100 droplet generator (Bio-Rad Laboratories), a C1000 Touch thermal cycler (Bio-Rad Laboratories), and a QX100 droplet reader (Bio-Rad Laboratories). Probes used for the ddPCR were: *TERT* mut, 5′-/56-FAM/C+CC+C+T+TC+CGG/3IABkFQ/-3′, and *TERT* wt 228, 5′-/5HEX/C+CC+C+C+TC+CGG/3IABkFQ/-3′ (a base preceded by + is Locked Nucleic Acid). Primers were same as those used in direct DNA sequencing. It is possible to discriminate between C228T and C250T in the two-dimensional (2D) display using above two probes at the same time because the *TERT* mut probe can bind to both C228T and C250T, but the wt probe can bind to only C228. In the case of the C250T mutation, both FAM and HEX signals are detected, while only a FAM signal is detected when the mutation is C228T.

Recurrence as an endpoint

Disease recurrence was defined as a surgically removed and pathologically verified local lesion or regional metastasis/distant metastasis detected by ultrasound, scintigraphy, or other imaging not earlier than six months after initial treatment. The time to recurrence was calculated based on the date of reoperation or on the date of recurrence detection by medical imaging.

Statistical Analysis

Univariate Fisher's or Fisher-Freeman-Halton exact tests were used for categorical data. Pairwise statistical comparisons of proportions in more than two groups were performed with the COMPPROP macros in SAS (http://www2.sas.com/proceedings/sugi31/204-

31.pdf). Nonparametric Mann-Whitney or Kruskal-Wallis tests followed by Dwass, Steel, Critchlow-Fligner multiple comparison procedure for continuous data were used to compare characteristics in different PTC subgroups. To analyze recurrence-free survival (RFS), the Kaplan-Meier method and log-rank test were used. Factors affecting RFS were assessed in Cox proportional hazard models. To determine a threshold of the *TERT* expression level based on the concept that minimal expression compared with relatively higher expression by the *TERT* promoter mutations could have negligible effect on clinical behavior, we first calculated hazard ratios (HRs) in serial optimal Cox models for each cut-off percentile (mRNA expression-negative plus expression levels below each percentile cases *vs* expression levels above the percentile cases) in five percentile increment. We next ran Kaplan-Meier analyses using percentiles which demonstrated relatively higher HR. Then optimal threshold was selected based on the log-rank p-value. Multivariable logistic regression models were used to identify factors associated with extrathyroidal extension or pT category. Analyses with very small numbers of outcomes (< 5 per cell) or when quasi-complete separation was observed were conducted using Firth's approach to bias-reducing penalized maximum likelihood fit. Non-automatic model optimization was routinely performed using the Akaike information criteria. Stepwise variable selection was applied to the models amendable to automatic optimization. Once the most appropriate model was determined, the maximum likelihood estimates of the respective parameters and their Wald-type 95% confidence intervals were calculated. Statistical assessments were performed using the 3.71 release of SAS Studio for the 9.4M5 version of SAS (SAS Institute) or IBM SPSS Statistics Version 24 software (IBM). Graphs were drawn using GraphPad Prism 6 (GraphPad). All p-values were 2-sided and considered significant if $p < 0.05$.

RESULTS

Mutational status of the TERT promoter region and TERT expression

We first screened 159 PTC samples for mutations of the *TERT* promoter region (C228T and C250T) by direct DNA sequencing. *TERT* promoter mutations were found in 20 (12.6%) samples, all of which were C228T, and there was no C250T substitution in the current series. We next examined *TERT* mRNA expression by real-time qRT-PCR. *TERT* expression was confirmed in all of the *TERT* promoter mutation-positive samples. Interestingly, even among 139 mutation-negative samples, 56 (40.3%) showed *TERT* expression. We then explored the possibility that there are tumors with low allelic frequency of *TERT* promoter mutations that are not detectable by regular sequencing. To investigate the presence of the mutations with high sensitivity, we used ddPCR. First, the detection limit of the ddPCR for the two *TERT* promoter mutations was determined using serial dilutions of the PCR product of the *TERT* promoter region containing C228T or C250T in the PCR product of the wild-type promoter. In our hands, the detection limit of the mutant allele frequency was approximately 0.25% (Supplementary Fig. S1). We then analyzed all of the 56 *TERT*-expressing samples using ddPCR. We identified the mutation with low allelic frequency in three samples (5.4%), hereafter PTC A, B, and C (Fig. 1A). According to the 2D display, all harbored a C228T mutation. The allelic frequencies of the mutant were 17%, 10%, and 5% in PTC A, B, and C, respectively (Supplementary Table S1). Since tumor tissues consist not only of tumor cells but also of stromal, endothelial, and blood cells, the allelic frequency of the mutant in the tumor cells was corrected for tumor purity based on the ratio of the *TERT* promoter mutation to the $BRAF^{V600E}$ mutation because the $BRAF^{V600E}$ mutation is considered a clonal monoallelic mutation in all tumor cells according to the data obtained using next-generation sequencing and immunohistochemistry (34, 35). However, one should note that there is also some debate about the allelic frequency of the $BRAF^{V600E}$ mutation. Guerra *et al.* reported variable *BRAF* mutant allelic frequencies in PTCs using pyrosequencing (36). For the present study, we assumed that all tumor cells were $BRAF^{V600E}$ positive. After the

correction, the allelic frequencies of the *TERT* promoter mutation in tumor cells were as follows: PTC A, 14%; PTC B, 4%; and PTC C, 3%, which means that 28%, 8%, and 6% of the tumor cells had the *TERT* promoter mutation in PTC A, B, and C, respectively (Supplementary Table S1). We then retrospectively checked the chromatograms of the direct DNA sequencing results of these samples. There were discrete peaks of C228T (Fig. 1B), but it was impossible to confidently discriminate these signals from background signals. The results of direct DNA sequencing, expression analysis, and ddPCR are summarized in Fig. 2A–C. Taken together, *TERT* expression was observed in all of the tumors with the *TERT* promoter mutation as expected, even in the cases with low allelic frequency; however, *TERT* expression was also detected in 38% of mutation-negative cases, suggesting that there are other mechanisms to upregulate *TERT* expression.

Relationship between TERT mutational/expression status and clinicopathological features

We analyzed the relationship between the status of the *TERT* promoter mutation/expression and clinicopathological features. We classified the 159 cases into three groups: the *TERT* promoter mutation-negative/mRNA expression-negative group (mut-/exp-), the *TERT* promoter mutation-negative/mRNA expression-positive group (mut-/exp+), and the *TERT* promoter mutation-positive group (mut+/exp+). As shown in Table 1, tumors with the mutation (mut+/exp+) showed statistically significant differences in age, extrathyroidal extension, stage II/III/IV, compared with the other two groups (mut-/exp- and mut-/exp+) (1 *vs* 3 and 2 *vs* 3). These findings suggest that the *TERT* promoter mutation confers aggressive properties to PTCs.

RFS was evaluated using the Kaplan-Meier method and Cox proportional hazard models. In this analysis, we excluded four cases that had distant metastasis at the time of operation and additional 20 cases that were followed for less than six months. The survival curves

of the three groups separated, and there was a statistically significant trend (Fig. 3A; log-rank trend, p<0.001). The HRs for recurrence of the mut+/exp+ group relative to the mut-/exp- group and the mut-/exp+ group after adjustment for age, sex, tumor size, extrathyroidal extension, and lymph node metastasis were 20.47 (95% CI: 4.54 to 114.1, p<0.001) and 5.38 (95% CI: 1.14 to 30.32, p=0.046), respectively (Table 2, 1st and 2nd comparisons). In the optimal models, the HRs of the mut+/exp+ group relative to the mut-/exp- group and the mut-/exp+ group were 23.39 (95% CI: 4.49 to 121.85, p<0.001) and 6.24 (95% CI: 1.44 to 27.13, p=0.015), respectively (Table 2, 1st and 2nd comparisons).

In two-group analysis, based on the mutational status (mut-/exp- and mut-/exp+ *vs* mut+/exp+) and the expression status (mut-/exp- *vs* mut-/exp+ and mut+/exp+), the Kaplan-Meier curves and the HRs are shown in Supplementary Fig. S2A, B and Supplementary Table 2, respectively.

Regarding the grouping based on the expression status (regardless of the mutational status), we attempted to set a threshold based on the concept that minimal expression could have a negligible effect on the clinical behavior because many of the mut-/exp+ tumors showed very low expression compared with the mut+/exp+ group (Fig. 2C). First, we calculated HRs in serial optimal Cox models for each cut-off percentile (mRNA expression-negative plus expression levels below each percentile cases *vs* expression levels above the percentile cases) (Supplementary Fig. S3A). We then ran Kaplan-Meier analyses between the 50th and 70th percentiles showing high HRs (Supplementary Fig. S3B). This allowed us to identify the optimal threshold, the 65th percentile (Supplementary Fig. S3B). Based on this, the HR adjusted for age, sex, tumor size, extrathyroidal extension, and lymph node metastasis was 4.12 (95% CI: 1.55 to 10.72, p=0.005). In the optimal model, the HR was 4.44 (95% CI: 1.71 to 11.53, p=0.002) (Supplementary Table S2, 3rd comparison). Other results using different adjustments are

listed in Supplementary Table S2.

Relationship between TERT expression and clinicopathological features in the mutation-negative cases

Next, we focused on the *TERT* promoter mutation-negative cases. Interestingly, there were no statistical differences between the mut-/exp- group and the mut-/exp+ group in all clinicopathological parameters except mean recurrence time (Table 1, 1 *vs* 2). The Kaplan-Meier curve showed a significant difference (Fig. 3B, p=0.046), and Cox proportional hazard models also demonstrated statistical significance after adjustment for covariates (Table 2, 3rd comparison). The HR of the mut-/exp+ group relative to the mut-/exp- group adjusted for age, sex, tumor size, extrathyroidal extension, and lymph node metastasis was 4.25 (95% CI: 1.15 to 17.71, p =0.041) (Table 2, 3rd comparison). In the optimal model, the HR was 4.24 (95% CI: 1.13 to 15.90, p=0.032) (Table 2, 3rd comparison). Since the unadjusted HR was not significant (p=0.067), we analyzed the relationship between age and the amount of *TERT* expression. Surprisingly, *TERT* expression was inversely correlated with age (Fig. 2D, p=0.002), a finding that is opposite to the presence of *TERT* promoter mutations. These results indicate that *TERT* expression, even without presence of a *TERT* promoter mutation, has a negative influence on PTC prognosis.

Because many cases of the mut-/exp+ tumors showed very low *TERT* expression compared to the mut+/exp+ group (Fig. 2C), we also attempted to reclassify all mut- cases into two new groups based on the *TERT* expression level using the same method described above (Supplementary Fig. S3C). Kaplan-Meier analyses were run between the 75th and 85th percentiles, and the optimal threshold was determined to be the 80th percentile (Supplementary Fig. S3D). Using this threshold, the unadjusted HR was 5.72 (95% CI: 1.39 to 19.23, p=0.01) (Table 2, 4th comparison), which was higher than the HR

calculated based on the presence or absence of the expression (exp- *vs* exp+). In the optimal model, the HR was 4.34 (95% CI: 1.11 to 16.94, p=0.035) (Table 2, 4th comparison).

Using this grouping, there were significant differences in age (the expression levels above the 80th percentile group was younger), stage, recurrence, recurrence time, the prevalence of the *BRAF* mutation (Table 3). Interestingly, all cases with an expression level above the 80th percentile were stage I and displayed a significantly lower rate of the *BRAF* mutation (30.0% *vs* 69.9%) (Table 3). Even though tumors in which the expression levels were above the 80th percentile were present in patients with younger age, lower stage, and a lower *BRAF* mutation rate, their prognosis was worse. The Kaplan-Meier curve also demonstrated a significant difference (Fig. 3C, p=0.007).

DISSCUSSION

First, in the present study, we have successfully reconfirmed the strong correlation between the presence of *TERT* promoter mutations and aggressive clinicopathological features in this PTC series. Second, we have demonstrated that there is a subset of PTCs that express *TERT* mRNA even in the absence of the *TERT* promoter mutation (mut-/exp+). In this cases, *TERT* expression conferred a significant negative impact on PTC prognosis, which was, however, not as high as in the cases with a *TERT* promoter mutation. Since *TERT* promoter mutations are only observed in elderly patients, *TERT* expression may be a promising marker in younger patients.

Among *TERT* mut-/exp+ cases (determined by regular sequencing), there seems to be a small number of tumors with low allelic frequency of the *TERT* promoter mutation. Hence, in addition to a low allelic frequency of *TERT* promoter mutations, there are other

mechanisms that upregulate *TERT* expression in the majority of the *TERT* mut-/exp+ cases. In other types of cancers, it has been reported that amplification, structural variants, alternative splicing, and promoter methylation also upregulate *TERT* expression (37-44). Of note, the allelic frequency depends on tumor cell purity and sensitivity of a detection method. It is still unclear whether the low allelic frequency of the *TERT* promoter mutation has a significant influence on tumor aggressivity because the number of such cases in the current series was too small. Further studies are needed to fully understand the mechanisms of the *TERT* mRNA upregulation in PTCs.

In the current series, all of the *TERT* promoter mutation-positive cases were 55 years of age or older, and among the eight recurrent cases in this age group, seven had a *TERT* promoter mutation. Thus, the presence of a *TERT* promoter mutation is probably a good marker in elderly patients. In contrast, since there was no *TERT* promoter mutation-positive case in the patients younger than 55 years, this mutation is not useful in younger patients presenting with PTC. However, in these patients, *TERT* expression appears to have prognostic value.

In the *TERT* mut-/exp+ group, there were many tumors with a very low amount of *TERT* expression compared to the levels of *TERT* expression in the *TERT* promoter mutation-positive cases. Such a minimal expression may not have clinical significance; therefore, we attempted to seek the best threshold based on the *TERT* expression levels. In the present study, the highest HR was obtained when the mutation-negative cases were categorized into two groups: cases with expression levels above the 80th percentile and others. However, it is necessary to analyze a much larger number of cases to determine a proper threshold. In addition, the threshold may be influenced by the detection method. This is a limitation of the current study. Moreover, since an active surveillance management approach has been used for low-risk micro-PTCs in Japan, current cases

may not be fully representative of the whole PTC spectrum. It should rather be considered as a proof of principle that high *TERT* expression is associated with risk for recurrence.

The *TERT* expression levels above the 80th percentile cases were associated with larger tumor size, more frequent lymph node metastasis and extrathyroidal extension, and more T3 and T4 tumors compared to those in the other group, yet these differences were not statistically significant (Table 3), which could be due to the very small number of cases (only 10) in the group. For the same reason, it was difficult to compare these cases with the mutation-positive ones. Intriguingly, in the mutation-negative cases, *TERT* expression levels were inversely correlated with patient age and the frequency of the *BRAF* mutation. We have reported that tumor size is inversely correlated with the presence of the *BRAF* mutation in pediatric and adolescent PTCs (45). Taken together, tumors without the *BRAF* mutation may likely have higher *TERT* expression levels and more aggressive features in young PTC patients.

In summary, as reported by others, we confirm the association between presence of *TERT* promoter mutations and aggressive clinicopathological characteristics in PTCs. Moreover, high *TERT* expression levels were observed in PTCs even in *TERT* promoter mutation-negative tumors in patients of all ages, and *TERT expression* was associated with worse prognosis. Since *TERT* promoter mutations are only found in elderly patients, *TERT* expression can be also a useful marker, especially in younger patients with PTC.

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DISCLOSURE STATEMENT

The authors have nothing to disclose.

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673 **FIGURE LEGENDS**

674 **Figure 1.** Low allelic frequencies of the *TERT* promoter mutation in three samples, PTC
675 A, PTC B, and PTC C. (A) Droplet digital PCR results. Each dot represents a positive
676 droplet of a mutant allele or a wild-type allele. NTC: non-template control, Wt: wild-type
677 control, hetMut: both mutant and wild-type control (monoallelic). (B) Sanger sequencing
678 chromatograms of indicated samples. The hot spots of the *TERT* promoter mutation are
679 shown as arrowheads. All of PTC A, PTC B, and PTC C had the C228T mutation.

680

681 **Figure 2.** Summary of the *TERT* mutational and expression status in the current series.
682 (A) The flowchart of the *TERT* mutation/expression screening. (B) Pie chart of the results.
683 (C) Relative *TERT* expression level in each group classified using the above status. (D)
684 Correlation between the *TERT* expression level and patient age. The mutation-
685 negative/expression-positive cases are plotted. Solid line represents the linear regression
686 model with 95% confidence intervals indicated by dotted lines. Arrow heads indicate
687 cases with *TERT* mRNA expression higher than the 80th percentile shown in C.

688

689 **Figure 3.** Kaplan-Meier curves of recurrence-free survival. The vertical tick marks
690 correspond to censored data. (A) The recurrence-free survival curves of the indicated
691 groups. p-value of a log-rank trend test is shown. (B) and (C) Only mutation-negative
692 cases were analyzed. p-values of log-rank tests are shown.

693

694

Table 1. Association between *TERT* mutational/expression status and clinicopathological features

TERT mutational/expression status Parameter		Whole group Mean ± s.d. (range) or n (%)	mut-/exp- (1)	mut-/exp+ (2)	mut+/exp+ (3)	p-value (1 vs 2)	p-value (1 vs 3)	p-value (2 vs 3)
Number of cases		159	83	53	23			
Age ± sd (range)		52.0 ± 15.8 (14–81)	49.2 ± 15.7 (16–78)	50.3 ± 15.6 (14–76)	66.3 ± 7.0 (55–81)	ns	<0.001	<0.001
Sex F/M, ratio		132/27 (4.9:1)	68/15 (4.5:1)	47/6(7.8:1)	17/6 (2.8:1)	ns	ns	ns
Tumor size, mm		21.5 ± 13.0 (0.3–62)	21.2 ± 11.9 (0.3–60)	21.8 ± 15.5 (3–62)	21.9 ± 10.7 (6–45)	ns	ns	ns
pN		111 (69.8%)	59 (71.1%)	37 (69.8%)	15 (65.2%)	ns	ns	ns
M		4 (2.5%)	2 (2.4%)	1 (1.9%)	1 (4.3%)	ns	ns	ns
Extrathyroidal extension		82 (52.6%)	41 (51.3%)	23 (43.4%)	18 (78.3%)	ns	<0.05	<0.05
pT3 and 4		89 (56.0%)	46 (55.4%)	25 (47.2%)	18 (78.3%)	ns	ns	<0.05
Stage								
	I	88 (55.3%)	49 (59.0%)	37 (69.8%)	2 (8.7%)	ns	<0.05	<0.05
	II	61 (38.4%)	29 (34.9%)	14 (26.4%)	18 (78.3%)	ns	<0.05	<0.05
	III	7 (4.4%)	4 (4.8%)	1 (1.9%)	2 (8.7%)	ns	ns	ns
	IV	3 (1.9%)	1 (1.2%)	1 (1.9%)	1 (4.3%)	ns	ns	ns
Stage II, III, and IV		71 (44.7%)	34 (41.0%)	16 (30.2%)	21 (91.3%)	ns	<0.05	<0.05
Recurrence[a]		17 (11.0%)	4 (4.9%)	6 (11.5%)	7 (31.8%)	ns	<0.05	ns
Mean recurrence time [95% CI], months[a]		115.2 [108.6–121.8]	119.5 [114.1–124.8]	104.7 [92.8–116.7]	91.6 [68.3–114.9]	<0.05	<0.001	ns
Total thyroidectomy		89 (56.0%)	48 (57.8%)	26 (49.1%)	15 (65.2%)	ns	ns	ns
LN dissection		147 (92.5%)	76 (91.6%)	49 (92.5%)	22 (95.7%)	ns	ns	ns
BRAF mutation		111 (69.8%)	57 (68.7%)	33 (62.3%)	21 (91.3%)	ns	ns	<0.05

ns: not significant, p≥0.05

mut-/exp-: *TERT* promoter mutation-negative/mRNA expression-negative

mut-/exp+: *TERT* promoter mutation-negative/mRNA expression-positive

mut+/exp+: *TERT* promoter mutation-positive/mRNA expression-positive

[a]four cases with distant metastasis and 20 cases that were followed for less than six months were not included.

Table 2. Hazard ratios of disease recurrence

	HR	95%CI	p-value	HR	95%CI	p-value	HR	95%CI	p-value	HR	95%CI	p-value
mut-/exp-	1.00			1.00			1.00			1.00		
mut+/exp+	7.53	2.39–26.69	0.001	20.25	4.54–109.26	<0.001	20.47	4.54–114.1	<0.001	23.39	4.49–121.85	<0.001
adjustment:					age, sex			age, sex, size, Ex, N			optimal model (age)*	
mut-/exp+	1.00			1.00			1.00			1.00		
mut+/exp+	2.34	0.80–6.99	0.131	5.07	1.24–24.03	0.034	5.38	1.14–30.32	0.046	6.24	1.44–27.13	0.015
adjustment:					age, sex			age, sex, size, Ex, N			optimal model (age)*	
mut-/exp-	1.00			1.00			1.00			1.00		
mut-/exp+	3.27	0.98–11.87	0.067	4.65	1.31–18.51	0.026	4.25	1.15–17.71	0.041	4.24	1.13–15.90	0.032
adjustment:					age, sex			age, sex, size, Ex, N			optimal model (age)*	
mut-/exp- and exp below 80th percentile	1.00			1.00			1.00			1.00		
mut-/exp above 80th percentile	5.72	1.39–19.23	0.010	5.09	1.21–17.81	0.022	3.36	0.80–11.75	0.095	4.34	1.11–16.94	0.035
adjustment:					age, sex			age, sex, size, Ex, N			optimal model (age)*	

*In the optimal model, only “age” was used for adjustment.

mut-/exp-: *TERT* promoter mutation-negative/mRNA expression-negative

mut-/exp+: *TERT* promoter mutation-negative/mRNA expression-positive

mut+/exp+: *TERT* promoter mutation-positive/mRNA expression-positive

mut-/exp- and exp below 80th percentile: *TERT* promoter mutation-negative/mRNA expression-negative and mRNA expression levels below the 80th percentile of the mut-/exp+ cases

mut-/exp above 80th percentile: *TERT* mRNA expression levels above the 80th percentile of the mut-/exp+ cases

Table 3. Association between *TERT* expression and clinicopathological features in the *TERT* promoter mutation-negative cases

TERT expression		Whole group Mean ± s.d. (range) or n (%)	*TERT* exp- and exp below 80th percentile	*TERT* exp above 80th percentile	Univariate p-value
Number of cases		136	126	10	
Age		49.6 ± 15.6 (14–78)	50.5 ± 15.6 (14–78)	38.8 ± 12.6 (20–52)	<0.05
Sex F/M, ratio		115/21 (5.5:1)	106/20 (5.3:1)	9/1 (9.0:1)	ns
Tumor size, mm		21.4 ± 13.4 (0.3–62)	21.1 ± 13.1 (0.3–62)	24.7±16.9 (4–55)	ns
pN		96 (70.6%)	88 (69.8%)	8 (80.0%)	ns
M		3 (2.2%)	3 (2.4%)	0	ns
Extrathyroidal extension		64 (48.1%)	58 (47.2%)	6 (60.0%)	ns
pT3 and 4		71 (52.2%)	65 (51.6%)	6 (60.0%)	ns
Stage					
	I	86 (63.2%)	76 (60.3%)	10 (100.0%)	<0.05
	II	43 (31.6%)	43 (34.1%)	0	<0.05
	III	5 (3.7%)	5 (4.0%)	0	ns
	IV	2 (1.5%)	2 (1.6%)	0	ns
Stage II, III and IV		50 (36.8%)	50 (39.7%)	0	<0.05
Recurrence[a]		10 (7.5%)	7 (5.7%)	3 (30.0%)	<0.05
Mean recurrence time [95% CI], months[a]		115.2 [109.4–121.0]	117.6 [112.1–123.0]	86.3 [53.9–118.7]	<0.05
Total thyroidectomy		74 (54.4%)	67 (53.2%)	7 (70.0%)	ns
LN dissection		125 (91.9%)	116 (92.1%)	9 (90.0%)	ns
BRAF mutation		90 (66.2%)	87 (69.0%)	3 (30.0%)	<0.05

ns: not significant, p≥0.05

[a]three cases with distant metastasis and 20 cases that were followed for less than six months were not included.

TERT exp- and exp below 80th percentile: *TERT* mRNA expression-negative and mRNA expression levels below the 80th percentile

TERT exp- above 80th percentile: *TERT* mRNA expression levels above the 80th percentile

Figure 1

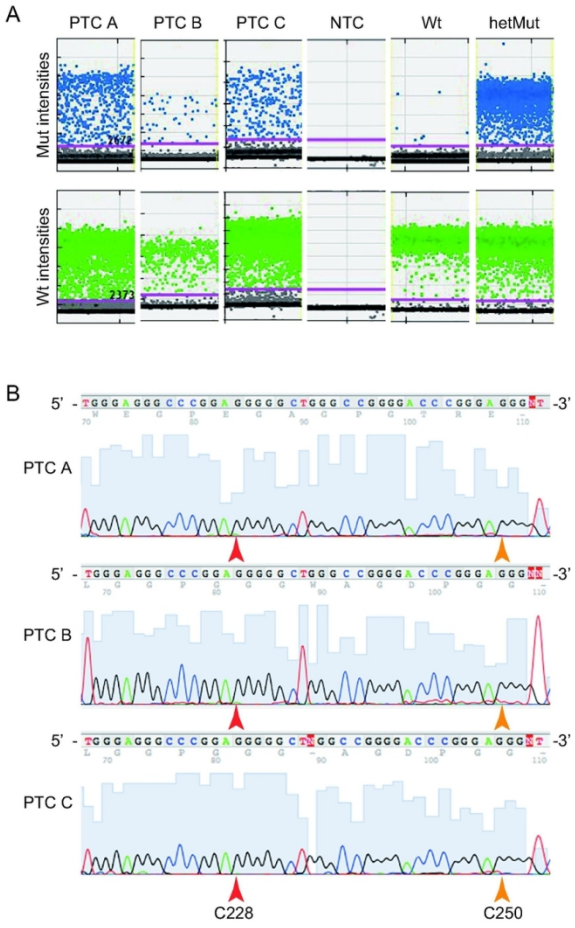

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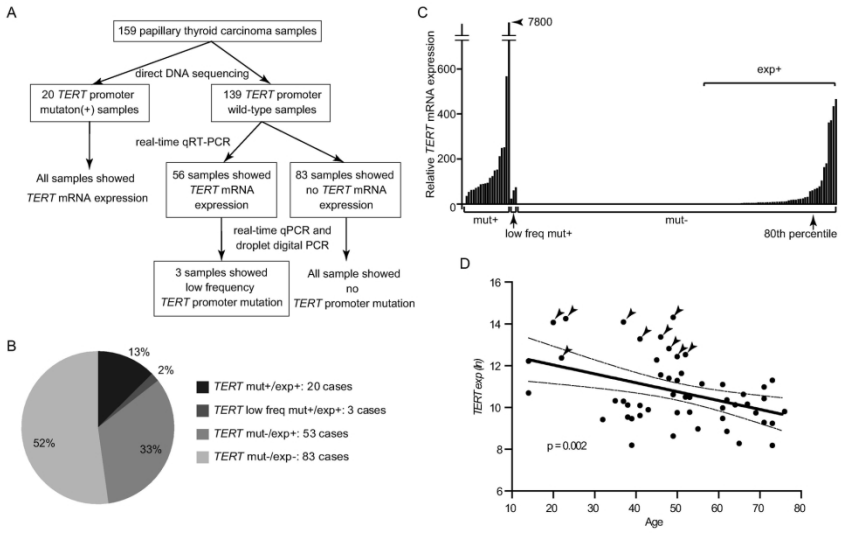
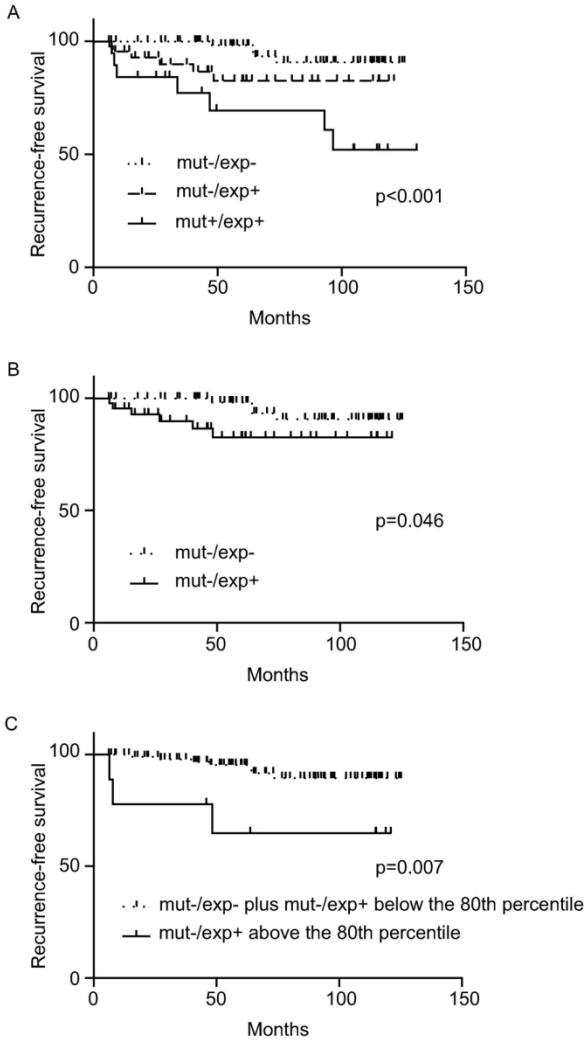
Figure 2



189x143mm (300 x 300 DPI)

Figure 3



126x178mm (300 x 300 DPI)

Supplementary Table 1. Allelic frequencies of the *TERT* promoter mutation in cancer cells.

	Concentration (copies/μl)[a]		*TERT* mutant frequency[b]	Concentration (copies/μl)[a]		*TERT*/*BRAF*	Allelic frequency in cancer cells
	TERT wt	*TERT* mut		*TERT* mut	*BRAF* mut		
PTC A	289	58.9	0.17	46	164	0.28	0.14
PTC B	46.2	4.9	0.1	5.4	67	0.08	0.04
PTC C	743	42.7	0.05	27.7	483	0.06	0.03

[a]average of the multiple experiments

[b]mutant frequency = mutant droplet copy number/(mutant droplet copy number + wild-type droplet copy number))

Supplementary Table 2. Hazard ratios of disease recurrence.

	HR	95%CI	p-value	HR	95%CI	p-value	HR	95%CI	p-value	HR	95%CI	p-value	HR	95%CI	p-value	HR	95%CI	p-value
mut-/exp-	1			1			1			1			1			1		
mut+/exp+	7.53	2.39–26.69	0.001	20.25	4.54–109.26	<0.001	19.44	4.36–103.95	<0.001	14.91	3.42–78.73	0.001	20.47	4.54–114.1	<0.001	23.39	4.49–121.85	<0.001
adjustment:					age, sex			age, sex, size			age, sex, size, Ex			age, sex, size, Ex, N			optimal model (age)*	
mut-/exp+	1			1			1			1			1			1		
mut+/exp+	2.34	0.80–6.99	0.131	5.07	1.24–24.03	0.034	4.75	1.17–22.23	0.042	3.12	0.77–14.97	0.14	5.38	1.14–30.32	0.046	6.24	1.44–27.13	0.015
adjustment:					age, sex			age, sex, size			age, sex, size, Ex			age, sex, size, Ex, N			optimal model (age)*	
mut-/exp-	1			1			1			1			1			1		
mut±/exp+	4.50	1.63–14.85	0.007	6.00	2.10–20.64	0.002	6.08	2.11–21.03	0.002	6.62	2.26–23.21	0.002	6.41	2.26–22.03	0.002	4.32	1.40–13.29	0.011
adjustment:					age, sex			age, sex, size			age, sex, size, Ex			age, sex, size, Ex, N			optimal model (Ex)*	
mut-/exp±	1			1			1			1			1			1		
mut+/exp+	4.68	1.75–11.90	0.002	11.14	2.95–49.55	0.001	10.55	2.79–46.26	0.001	8.01	2.12–35.59	0.004	13.80	3.39–66.67	0.001	11.83	2.84–49.37	0.001
adjustment:					age, sex			age, sex, size			age, sex, size, Ex			age, sex, size, Ex, N			optimal model (age)*	
mut±/exp- and exp below 65th percentile	1			1			1			1			1			1		
mut±/exp above 65th percentile	4.64	1.79–11.84	0.002	5.2	1.94–13.69	0.001	5.17	1.91–13.74	0.001	4.42	1.65–11.69	0.003	4.12	1.55–10.72	0.005	4.44	1.71–11.53	0.002
adjustment:					age, sex			age, sex, size			age, sex, size, Ex			age, sex, size, Ex, N			optimal model (age)*	
mut-/exp-	1			1			1			1			1			1		
mut-/exp+	3.27	0.98–11.87	0.067	4.65	1.31–18.51	0.026	4.83	1.36–19.34	0.024	5.21	1.46–21.14	0.019	4.25	1.15–17.71	0.041	4.24	1.13–15.9	0.032
adjustment:					age, sex			age, sex, size			age, sex, size, Ex			age, sex, size, Ex, N			optimal model (age)*	
mut-/exp- and exp below 80th percentile	1			1			1			1			1			1		
mut-/exp above 80th percentile	5.72	1.39–19.23	0.01	5.09	1.21–17.81	0.022	5.43	1.29–18.88	0.017	4.56	1.05–16.29	0.037	3.36	0.8–11.75	0.095	4.34	1.11–16.94	0.035
adjustment:					age, sex			age, sex, size			age, sex, size, Ex			age, sex, size, Ex, N			optimal model (age)*	

* In the optimal model, only age was used for adjustment.

mut±/exp- and exp below 65th percentile: *TERT* mRNA expression-negative and *TERT* mRNA expression levels below the 65th percentile, regardless of the mutational status

mut±/exp higher 35%: *TERT* mRNA expression levels above the 65th percentile, regardless of the mutational status

mut-/exp- and exp below 80th percentile: *TERT promoter mutation-negative*/mRNA expression-negative and mRNA expression levels below the 80th percentile of the mut-/exp+ cases

mut-/exp above 80th percentile: *TERT* mRNA expression levels above the 80th percentile of the mut-/exp+ cases

Supplementary Table 3. Association between *TERT* expression and clinicopathological features in all cases (including *TERT* promoter mutation-positive cases)

TERT mutational status		Whole group Mean ± s.d. (range) or n (%)	mut±/exp- and exp below 65th percentile	mut±/exp above 65th percentile	Univariate comparison
Number of cases		159	132	27	
Age		52.0 ± 15.8 (14–81)	51.2 ± 15.6 (14–78)	56.0 ± 16.4 (20–81)	ns
Sex F/M, ratio		132/27 (4.9:1)	112/20 (5.6:1)	20/7 (2.9:1)	ns
Tumor size, mm		21.5 ± 13.0 (0.3–62)	21.4 ± 13.0 (0.3–62)	21.8 ± 13.1 (4–55)	ns
pN		111 (69.8%)	92 (69.7%)	19 (70.4%)	ns
M		4 (2.5%)	3 (2.3%)	1 (3.7%)	ns
Extrathyroidal extension		82 (52.6%)	63 (48.8%)	19 (70.4%)	ns
pT3 and 4		89 (56.0%)	70 (53.0%)	19 (70.4%)	ns
Stage					
	I	88 (55.3%)	76 (57.6%)	12 (44.4%)	ns
	II	61 (38.4%)	48 (36.4%)	13 (48.1%)	ns
	III	7 (4.4%)	6 (4.5%)	1 (3.7%)	ns
	IV	3 (1.9%)	2 (1.5%)	1 (3.7%)	ns
Stage II, III and IV		71 (44.7%)	56 (42.4%)	15 (55.6%)	ns
Recurrence[a]		17 (11.0%)	9 (7.0%)	8 (30.8%)	<0.05
Mean recurrence time [95% CI], months[a]		115.2 [108.6–121.8]	116.1 [110.4–121.7]	91.6 [69.9–113.4]	<0.05
Total thyroidectomy		89 (56.0%)	72 (54.5%)	17 (63.0%)	ns
LN dissection		147 (92.5%)	122 (92.4%)	25 (92.6%)	ns
BRAF mutation		111 (69.8%)	93 (70.5%)	18 (66.7%)	ns

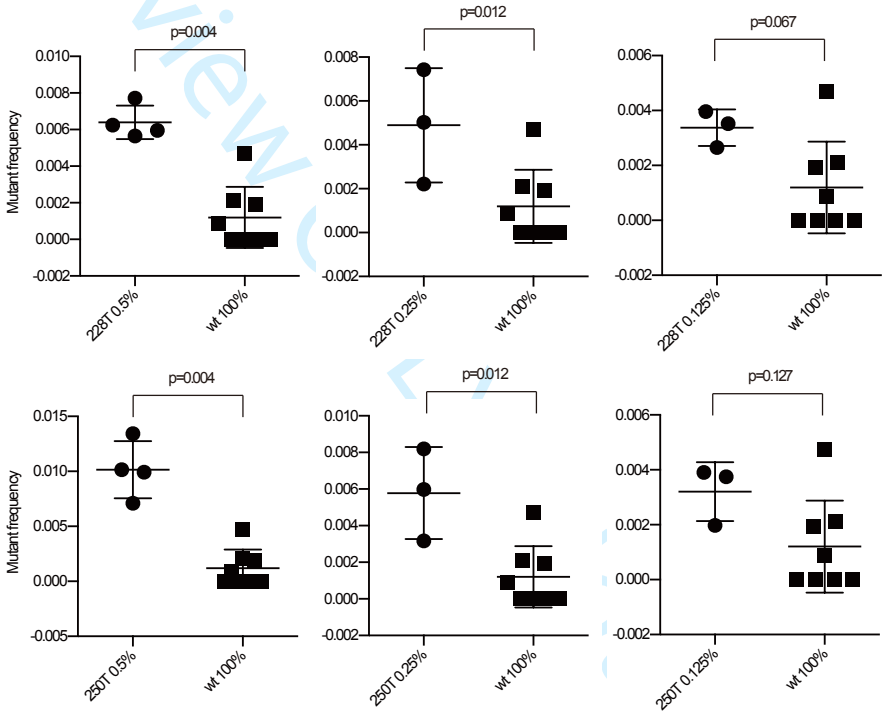
ns: not significant, p≥0.05

[a]four cases with distant metastases and 20 cases that were followed for less than six months were not included.

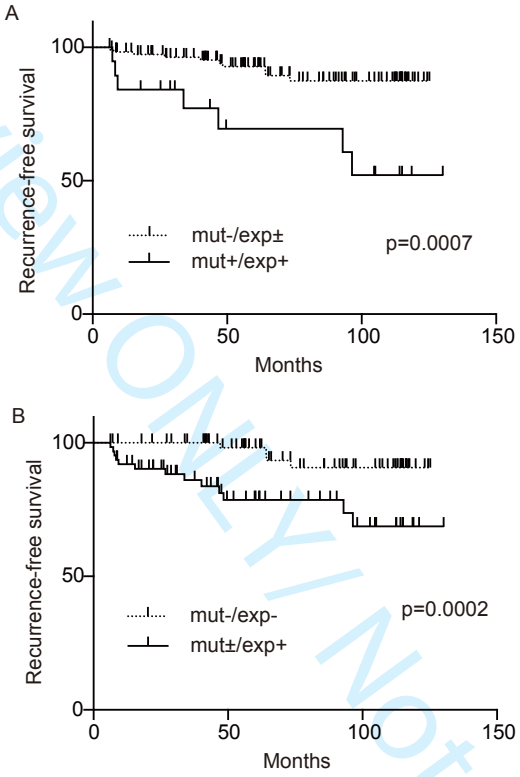
mut±/exp- and exp below 65th percentile: *TERT* mRNA expression-negative and *TERT* mRNA expression levels below the 65th percentile, regardless of the mutational status

mut±/exp above 65th percentile: *TERT* mRNA expression levels above the 65th percentile, regardless of the mutational status

Supplementary Figure S1

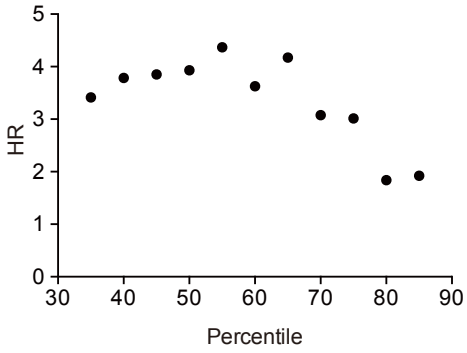


Supplementary Figure S2



Supplementary Figure S3

A



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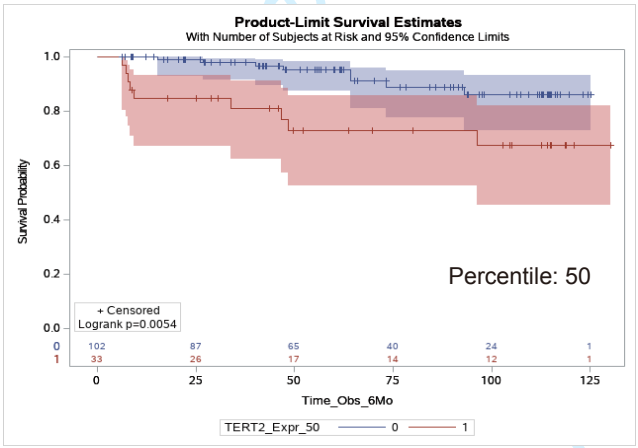
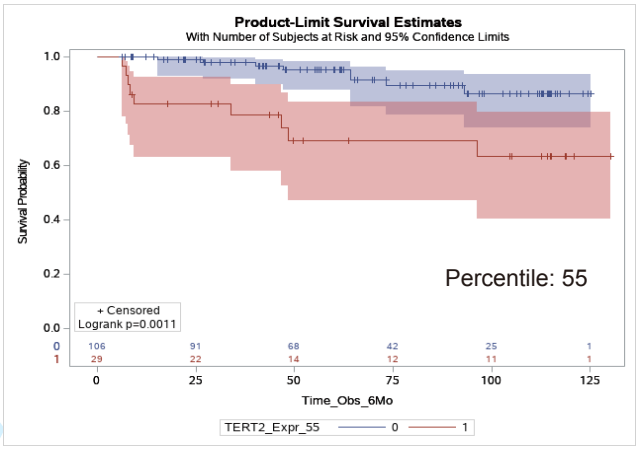
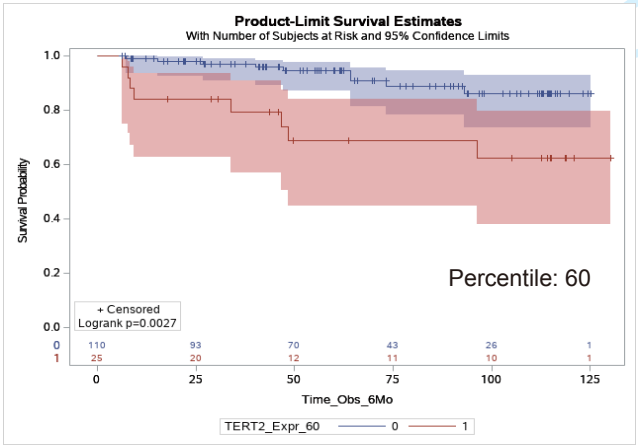
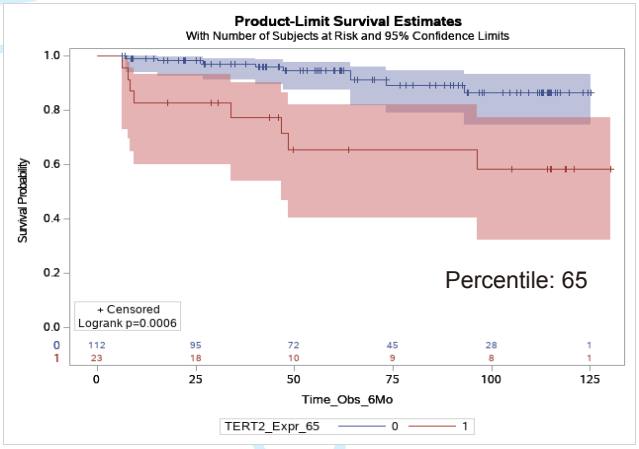
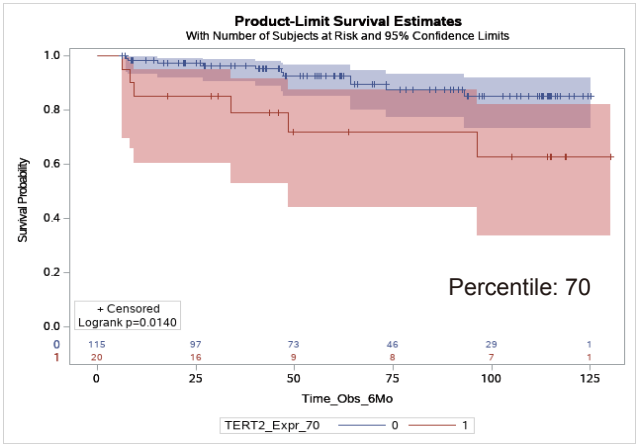




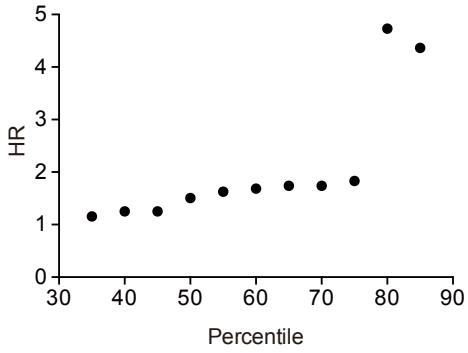






Supplementary Figure S3

C



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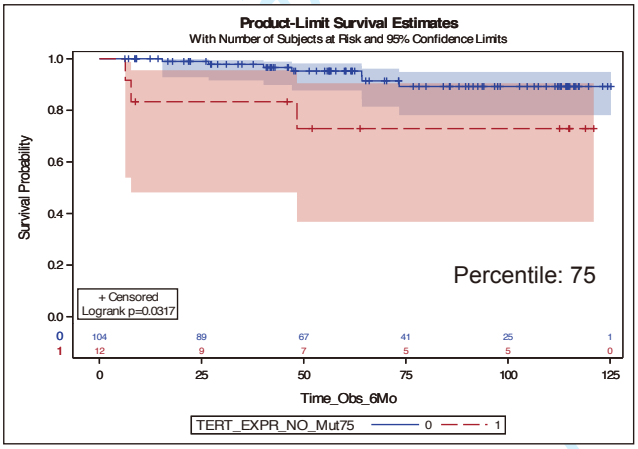

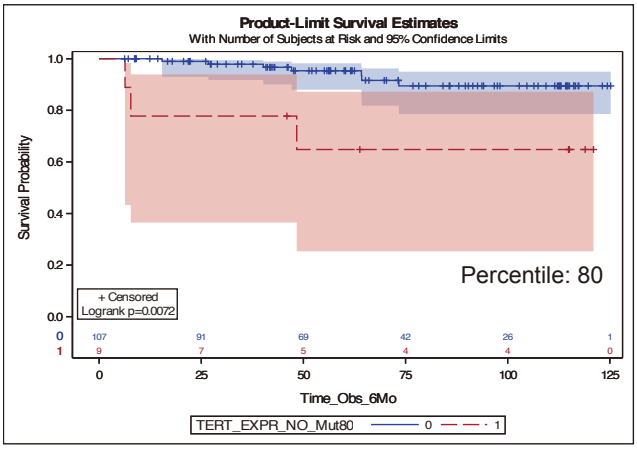

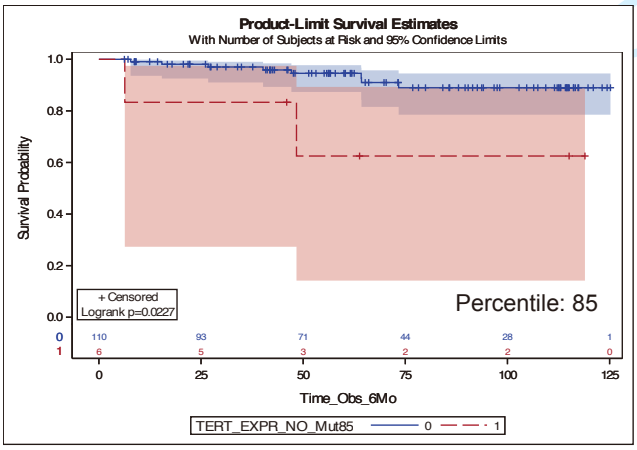

SUPPLEMENTARY FIGURE LEGENDS

Figure S1. Detection limit of ddPCR.

The detection limit of ddPCR was determined using serial dilutions of the PCR product of the *TERT* promoter mutation (C228T or C250T) in the wild-type PCR product. The *TERT* promoter region (163 bp) was amplified using DNA extracted from cell lines having only wild-type, C228T, and C250T and purified. We prepared a total 3,000 copies of the mixture of the mutant product (0%, 0.125%, 0.25%, and 0.5%) and the wild-type product and measured the mutant copy number using ddPCR. The results were compared with those of the 100% wild-type product, and the difference was analyzed by Mann-Whitney U test using the GraphPad Prism software. Significant difference was only observed between the 0.25% or more mutant allele samples (both C228T and C250T) and the wild-type samples.

Figure S2. Kaplan-Meier curves of the different grouping based on the *TERT* mutational/expression status.

The vertical tick marks correspond to censored data. p-values of log-rank tests are shown. (A) The current cases were divided based on the mutational status. (B) The current cases were divided based on the expression status.

Figure S3. Hazard ratios and Kaplan-Meier curves of each cut-off percentile based on the *TERT* mRNA expression level.

(A) Hazard ratios were calculated including mutation-positive and negative cases. (B) Kaplan-Meier curves of the indicted cut-off percentiles (expression-negative plus expression levels below the indicated percentile *vs* expression levels above the indicated percentile) (C) Hazard ratios were calculated using only mutation-negative cases. (D) Kaplan-Meier curves of the indicted cut-off percentiles (expression-negative plus expression levels below the indicated percentile *vs* expression levels above the indicated percentile)