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Concise Communication **In-Press Preview** **Oncology**

Replicative immortality is a hallmark of cancer governed by telomere maintenance. About 90% of human cancers maintain their telomeres by activating telomerase, driven by transcriptional upregulation of telomerase reverse transcriptase (*TERT*). Although *TERT* promoter mutations (TPMs) are a major cancer-associated genetic mechanism of *TERT* upregulation, many cancers exhibit *TERT* upregulation without TPMs. In this study, we described *TERT* Hypermethylated Oncological Region (THOR), a 433-bp genomic region encompassing 52 CpG sites located immediately upstream of the *TERT* core promoter, as a cancer-associated epigenetic mechanism of *TERT* upregulation. Unmethylated THOR repressed *TERT* promoter activity regardless of TPMs status, and hypermethylation of THOR counteracted this repressive function. THOR methylation analysis in 1,352 human tumors revealed frequent (>45%) cancer-associated DNA hypermethylation in 9 of 11 (82%) tumor types screened. Additionally, THOR hypermethylation — either independently or along with TPMs — accounted for how approximately 90% of human cancers can aberrantly activate telomerase. Thus, we propose THOR hypermethylation as a prevalent telomerase activating mechanism in cancer that can act independently or in conjunction with TPMs, further supporting the utility of THOR hypermethylation as a prognostic biomarker.

DNA hypermethylation within *TERT* promoter upregulates *TERT* expression in cancer

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Abstract

Replicative immortality is a hallmark of cancer governed by telomere maintenance. About 90% of human cancers maintain their telomeres by activating telomerase, driven by transcriptional upregulation of telomerase reverse transcriptase (*TERT*). Although *TERT* promoter mutations (TPMs) are a major cancer-associated genetic mechanism of *TERT* upregulation, many cancers exhibit *TERT* upregulation without TPMs. In this study, we described *TERT* Hypermethylated Oncological Region (THOR), a 433-bp genomic region encompassing 52 CpG sites located immediately upstream of the *TERT* core promoter, as a cancer-associated epigenetic mechanism of *TERT* upregulation. Unmethylated THOR repressed *TERT* promoter activity regardless of TPMs status, and hypermethylation of THOR counteracted this repressive function. THOR methylation analysis in 1,352 human tumors revealed frequent (>45%) cancer-associated DNA hypermethylation in 9 of 11 (82%) tumor types screened. Additionally, THOR hypermethylation – either independently or along with TPMs – accounted for how ~90% of human cancers can aberrantly activate telomerase. Thus, we propose THOR hypermethylation as a prevalent telomerase activating mechanism in cancer that can act independently or in conjunction with TPMs, further supporting the utility of THOR hypermethylation as a prognostic biomarker.

Introduction

Replicative immortality is an attribute of cancer cells governed by telomere maintenance (1). Telomeres are repetitive nucleoprotein structures that protect chromosomal ends and shorten after each replicative cycle, playing important roles in genome stability and cancer prevention (2, 3). To achieve replicative immortality, ~90% of human cancers reactivate telomerase – a holoenzyme responsible for elongating telomeres – through re-expression of the catalytic subunit telomerase reverse transcriptase (*TERT*) (4).

Previous studies have identified two prevalent cancer-associated *TERT* promoter mutations (TPMs) causing a cytidine to thymidine change at genomic loci chr5:1,295,228 (C228T) and 1,295,250 (C250T) as a genetic mechanism of *TERT* upregulation (5-9). However, TPMs do not prevent initial bulk telomere shortening at the time of malignant transformation and only act on cells with critically short telomeres to delay replicative senescence (10), implying that TPMs alone are insufficient for cancer cells to exhibit telomerase activity required for telomere length maintenance. Moreover, common tumor types, including breast, lung, prostate, colorectal, and hematological malignancies, rarely exhibit TPMs (11-16) yet display telomerase activity, supporting the idea that other undefined *TERT*-upregulating mechanisms must exist.

One mechanism not yet thoroughly investigated is the epigenetic regulation of *TERT*. Previous seminal studies have reported an association between *TERT* promoter hypermethylation and elevated *TERT* expression in cancer (17, 18), leading to our recent work which uncovered this association in a specific region within the *TERT* promoter termed Upstream-of-the Transcription-Start-Site (UTSS) (19). Several studies have reported frequent *TERT* promoter (UTSS) hypermethylation in various *TERT*-expressing cancer types (19-23), suggesting for an epigenetic

mechanism of telomerase activation in multiple cancers. However, the boundaries and functional impact of this region on *TERT* promoter activity have not been examined in detail.

In this study, we use conventional and next-generation sequencing (NGS) to uncover *TERT* Hypermethylated Oncological Region (THOR). Unmethylated THOR acts as a repressive element on *TERT* promoter activity, while methylation of THOR counteracts this repressive effect. Importantly, activating effects of TPMs on the *TERT* promoter is reduced when unmethylated THOR is present, implying that TPMs and THOR are two distinct *TERT* regulatory mechanisms. Finally, we find that THOR hypermethylation is a prevalent phenomenon in *TERT*-expressing tumor types regardless of their TPMs status.

Results and Discussion

Methylation landscape of the *TERT* promoter was examined up to ~650-bp of the transcription start site (TSS) with bisulfite-sequencing of DNA samples from normal cell lines/tissues (n=43) and *TERT*-expressing cancer cell lines (n=18). Using both pyrosequencing and targeted NGS technologies which produced highly consistent results (Supplemental Figure 1A–B), we defined THOR as a 433-bp genomic region within the *TERT* promoter (Chr5:1,295,321–1,295,753, GRCh37/hg19) that encompasses 52 CpG sites and is located upstream of common TPM sites (Figure 1A). Methylation of the CpG sites within THOR was significantly increased in cancer cell lines compared to normal samples with a mean methylation difference of 58% across THOR (P=1.49×10^{-9}; Figure 1A and Supplemental Figure 2). Unsupervised clustering based on CpG methylation status within THOR clearly separated every *TERT*-expressing cancer cell line from normal samples (Figure 1B).

Increased THOR methylation across all 52 CpG sites was validated and confirmed in tumors from various tissues (n=87; Figure 2A), and unsupervised clustering separated 82% (71/87) of these tumors from normal samples (Figure 2B). To examine the extent of THOR methylation in larger tumor cohorts, we tested and confirmed that 5 CpG sites (UTSS) (19) within THOR accurately represent average THOR methylation (Supplemental Figure 3). Using these CpG sites, we screened 1,352 tumor and 80 normal samples from various tissues to assess THOR methylation in cancer. The median THOR methylation of normal samples was 7.0% with no samples exceeding 18.3%, while most adult cancer types exhibited significantly higher median THOR methylation (P<0.05) with 91.4% of all tumors exceeding the median THOR methylation level of normal tissues. Of note, low THOR methylation observed in thyroid cancers may be associated with their known lower malignant potential and better prognosis compared to other tumor types. Other

tumors exhibiting low and heterogeneous methylation levels such as skin and bone cancers utilize other mechanisms for telomere maintenance, such as TPMs and Alternative Lengthening of Telomeres (ALT) pathway respectively (24). Furthermore, we compared patient-matched normal-tumor samples in a subset of lung (*n*=32) and prostate (*n*=67) cancers, in which 89.9% (89/99) of tumours exhibited higher THOR methylation compared to matched normal tissue by a median of 13.9% (Figure 2D), corroborating THOR methylation as a cancer-associated epigenetic event.

Prevalence of THOR methylation in cancer was assessed by dichotomizing tumor samples as hypomethylated or hypermethylated using a cut-off value of 16.1%, by adding 2 standard deviations to the mean methylation of normal samples as previously described (*see methods*) (19, 20). All cancer types screened exhibited high prevalence (>45%) of THOR hypermethylation except thyroid cancers (3%) and melanomas (30%; Figure 2E). Interestingly, tumors in which TPMs are rarely observed (i.e. prostate, breast, blood, and colon cancers) (11, 12, 15, 16) displayed higher prevalence of THOR hypermethylation compared to those in which TPMs are commonly found (i.e. skin, bladder, and brain cancers) (5, 8, 25) (Figure 2E), suggesting that THOR hypermethylation may be a crucial *TERT*-activating mechanism in tumors without TPMs. Together, these observations highlight a potential cancer-associated mechanism of telomerase activation through THOR hypermethylation and suggest the usefulness of THOR hypermethylation signature in differentiating normal and cancerous tissue.

To examine the role of THOR as a transcriptional regulatory element of *TERT,* we first performed 5' truncation analysis of the *TERT* promoter using reporter gene constructs spanning both the TPM sites and THOR. A continual increase in reporter gene expression was observed in a cancer cell line (LN229) as THOR was gradually truncated from the 5' end, with the pTERT-214 construct displaying peak promoter strength (Figure 3A). Further truncation of 82-bp (pTERT-

132) resulted in a ~2-fold reduction of reporter gene expression, indicating that the minimal *TERT* core promoter is a region up to 214-bp upstream of the *TERT* TSS, encompassing the TPM sites and proximal 75-bp of THOR. Importantly, addition of rTHOR to the *TERT* core promoter resulted in a significant decrease in reporter gene expression compared to the *TERT* core promoter alone in LN229 (Figure 3A) and multiple cancer cell lines from different tissues (Supplemental Figure 4). This region – which we defined as repressive THOR (rTHOR, Chr5:1,295,395–1,295,743) – is a functional region within THOR that represses *TERT* expression when unmethylated. We then tested the effect of rTHOR re-methylation using a unique reporter gene plasmid completely devoid of CpG sites (pCpGfree-promoter-Lucia, *Invivogen*). rTHOR was cloned upstream of the modified CpG-free *hEF*1 promoter (pCpG(+rTHOR)) and was efficiently methylated in vitro (pCpG(+rTHOR^{Meth}); Supplemental Figure 5). Hypermethylation of rTHOR resulted in a significant increase in reporter gene expression in LN229 and two additional cancer cell lines (HeLa and HT1080) (Figure 3B), implying that methylation of THOR counteracts the repressive effect of rTHOR. Finally, we treated brain cancer and glioma stem cell lines which harbor THOR hypermethylation and high *TERT* expression with the demethylation agent Decitabine, which resulted in reduced THOR methylation and *TERT* expression (Supplemental Figure 6). In contrast, *TERT* expression was not altered in normal embryonic stem cells lacking THOR hypermethylation (Supplemental Figure 6). These observations infer that cancer cells hypermethylate THOR to counteract the repressive effect of rTHOR and promote *TERT* upregulation.

High prevalence of THOR hypermethylation in tumor types that commonly harbor TPMs (Figure 2E) and the previous finding that TPMs alone are insufficient in preventing initial bulk telomere shortening at the time of malignant transformation (10) led us to investigate the relationship between THOR and TPMs. In contrast to the cancer-associated hypermethylation

observed within THOR, the average DNA methylation of the CpG sites at the TPM locus (Chr5:1,295,225 – 1,295,263, GRCh37/hg19) was ubiquitously low (<10%) in normal tissue (0.8%), tumor samples (4.4%) and *TERT*-expressing cancer cell lines (9.2%; Figure 1A and 2A). This suggests that the genomic loci encompassing either THOR or TPMs are distinct *TERT* regulatory regions. To functionally investigate the repressive effect of unmethylated rTHOR in the context of TPM, we compared reporter gene expression in four constructs: pTERT(+rTHOR) and pTERT(−rTHOR), which contain the *TERT* core promoter with and without rTHOR respectively, and in the presence and absence of C228T TPM (Figure 4A). As expected, the addition of rTHOR to the *TERT* core promoter decreased promoter strength by an average ~5-fold in all 3 cancer cell lines tested, while C228T mutation resulted in an average ~5-fold increase (Figure 4A). The addition of rTHOR to the C228T mutated *TERT* core promoter effectively counteracted the activating effect of this mutation and the activity was comparable to that of the wild-type *TERT* promoter without rTHOR. These findings indicate that THOR and TPMs are non-redundant but discrete regulatory mechanisms of *TERT* expression in cancer.

To explore the impact of these mechanisms on telomerase activation in cancer, we first examined whether they can co-exist in 10 *TERT*-expressing cancer cell lines. All cell lines exhibited THOR hypermethylation and, importantly, five showed co-existence of the two *TERT* activating mechanisms (Figure 4B). However, there was no significant difference observed in the level of *TERT* expression between cancer cell lines when TPMs were present or absent (Supplemental Figure 7). This suggests that in certain cancers where TPM is absent, other mechanisms such as THOR hypermethylation contribute to *TERT* expression. Second, we assessed the prevalence and co-existence of these mechanisms in cancer types in which TPMs are either frequently or rarely present (Figure 4C). In a cohort of cancers that commonly exhibit TPMs

(glioma/melanoma, *n*=396), we frequently (43%) observed TPMs and THOR hypermethylation together while 45% of the tumors exhibited only one of the mechanisms. Meanwhile, in a cohort of cancers that lack TPMs (prostate, lung, breast, and colon cancers, *n*=78), 90% of the tumors exhibited only THOR hypermethylation. Nonetheless, in both groups, the prevalence of THOR methylation together with TPMs reflected the overall frequency of telomerase-dependent tumors (~90%). Third, we screened a subset of gliomas – tumors that exhibit heterogeneous telomere maintenance mechanisms including THOR hypermethylation, TPMs, and ALT (*n*=21; Figure 4D). Interestingly, 93.3% (14/15) of *TERT*-expressing gliomas displayed THOR hypermethylation alone or with TPMs, while 83.3% (5/6) of gliomas that lacked *TERT* expression were missing either *TERT*-activating mechanism but were primarily dependent on ALT for telomere maintenance. These observations highlight the role of THOR hypermethylation as one of *TERT*-upregulatory mechanisms in cancer, either independently or in conjunction with TPMs.

Although we describe THOR hypermethylation as an additional *TERT*-upregulatory mechanism, understanding its biological mechanism needs further investigation. A recent study has associated allele-specific hypomethylation and active histone marks (H3K4me2/3) in cancer cell lines that harbor TPM (26). In this case, THOR hypermethylation may act as a regulatory mechanism strictly through transcription factor binding, enabling *TERT* expression even in the allele without active histone marks. *Cis*-acting transcriptional repressors such as WT1 and MZF-2 are known to bind the genomic region within THOR (27), but whether or not their binding is methylation-sensitive requires further investigation. Another interesting aspect of THOR hypermethylation is that it may regulate other genes in the proximity of *TERT* which ultimately affect *TERT* expression, such as *hTERT* antisense promoter associated (*hTAPAS*) non-coding RNA whose promoter overlaps with the *TERT* promoter (28). Lastly, to explore the co-existence and

interplay between THOR hypermethylation and TPMs in more detail, single-cell level analyses should be performed in the future.

In summary, this study defined THOR as a region of cancer-associated DNA hypermethylation, located adjacent to the *TERT* core promoter and common TPM sites. We demonstrated that unmethylated rTHOR is a repressive element of the *TERT* promoter and hypermethylation counteracts this effect, suggesting that cancer cells methylate THOR to upregulate *TERT* expression and activate telomerase. In addition, our study proposes two clinically relevant implications. First, unraveling the exact mechanism of THOR hypermethylation-driven *TERT* expression will uncover potential therapeutic targets for cancer treatment. Second, together with TPMs, the discovery of this *TERT*-upregulating mechanism in cancer accounts for how ~90% of human cancers activate telomerase. Our findings provide biological insight as to why tumors with THOR hypermethylation are associated with poorer clinical outcome (19, 20, 22, 23), further highlighting its value as a potential prognostic biomarker.

Materials and Methods

A complete description of the methods and statistical analysis is provided in the Supplementary Materials. Unsupervised clustering heatmap was created with modified version of Methylation plotter (29). NGS Data is available on GEO public Database [Accession#: GSE120511].

Study approval

All experiments were performed with appropriate approval by the research ethics board of the Hospital for Sick Children.

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Author contributions

P.C.B. and U.T. were responsible for study concept. D.L., P.C.B., and U.T. designed experiments; D.L. conducted all in-vitro experiments, methylation analyses, and statistical analyses; R.L. collected and conducted methylation analysis for prostate and bladder samples; M.G., C.Z., and T.L. conducted Decitabine-treatment experiments; M.K. and A.D. analyzed MiSeq data, supervised by T.J.P.; A.H. analyzed HiSeq data; N.M.N. and J.A. assisted with data interpretation; R.M., J.D., D.H., T.H., P.W., R.V., G.Z., J.K., S.D., M.T., C.H., J.W., A.F., R.J.H., M.M., K.W.,

H.Y., K.A., M.R.A., and P.B.D. provided patient samples; D.L., P.C.B., and U.T. co-wrote the manuscript.

Figures

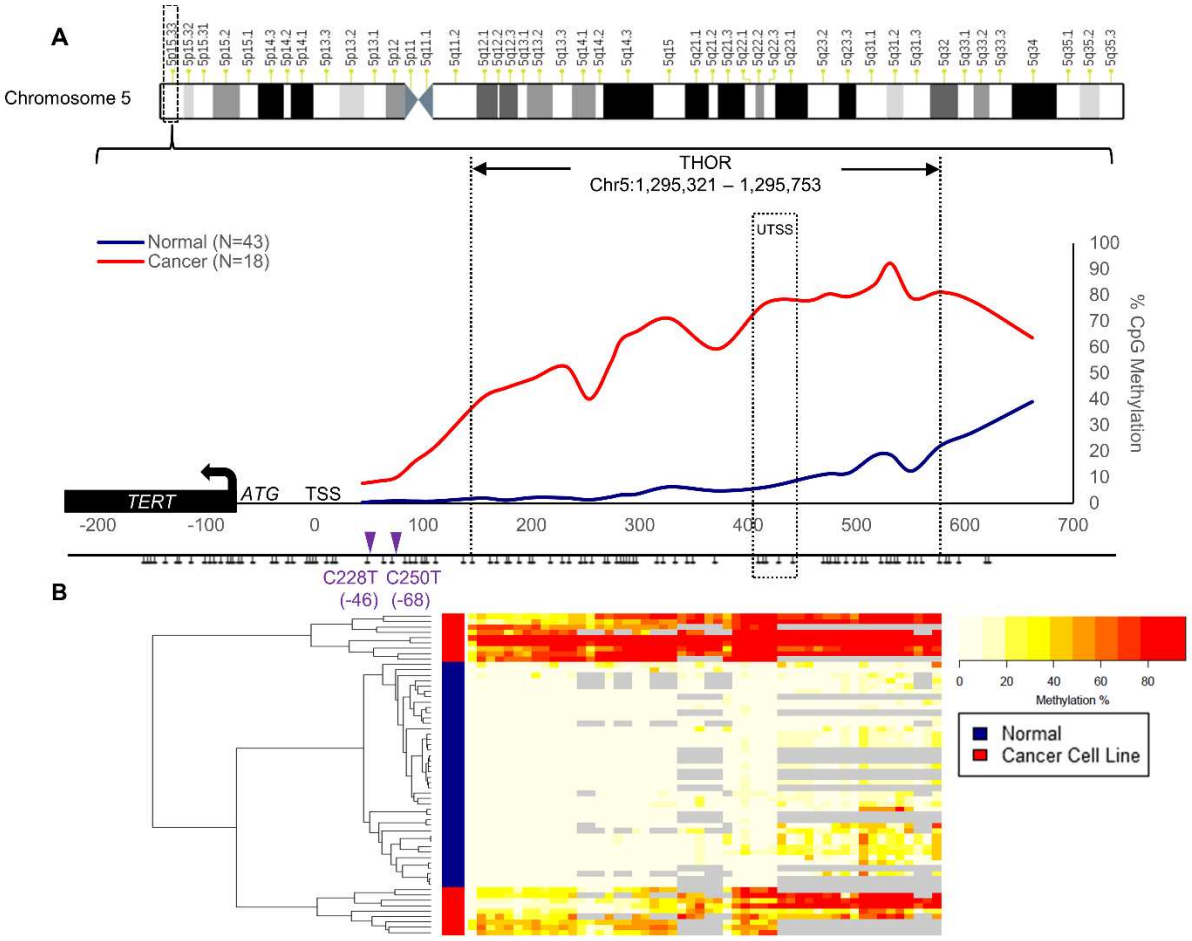
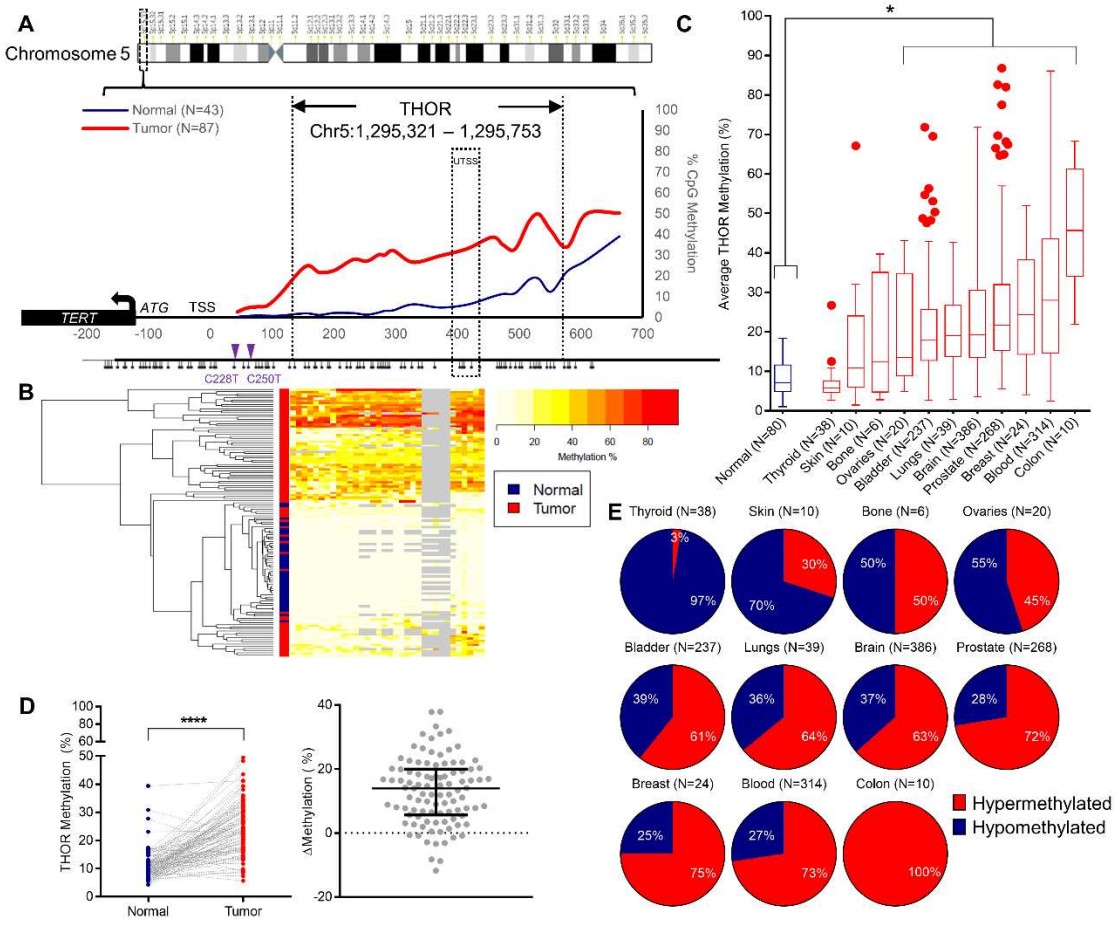


Figure 1. Defining THOR through DNA CpG methylation analysis of the *TERT* promoter. (**A**) Average CpG methylation of the *TERT* promoter in normal cell lines/tissues (*n*=43, blue) and *TERT*-expressing cancer cell lines (*n*=18, red). THOR (*TERT* Hypermethylated Oncological Region) is a 433-bp region (-140 to -572, relative to TSS) comprising of 52 CpG sites and located adjacently upstream of the common C228T and C250T TPMs (purple triangles). UTSS encompasses 5 CpG sites within THOR. ATG and TSS are start codon and transcription start site of the *TERT* promoter, respectively. Lollipops represent individual CpG sites. (**B**) Methylation heatmap generated from unsupervised clustering displays methylation percentage of each CpG site

within THOR for normal cell lines/tissues (*n*=43, blue) and *TERT*-expressing cancer cell lines (*n*=18, red). Grey color indicates unavailability of data.



409

410 **Figure 2. THOR hypermethylation is prevalent in human cancers.** (**A**) Average DNA CpG
411 methylation of the *TERT* promoter in normal cell lines/tissues (*n*=43, blue) and tumor samples
412 (*n*=87, red). (**B**) Methylation heatmap generated from unsupervised clustering displays
413 methylation percentage of each CpG site within THOR for normal cell lines/tissues (*n*=43) and
414 tumor samples (*n*=87). Grey color indicates unavailability of data. (**C**) Box-and-whisker plot
415 shows the median and distribution of the average THOR methylation levels in normal samples
416 (*n*=80, blue) and samples from various tumor tissue types (*n*=1,352, red; Sidak's multiple
417 comparisons test, **P*<0.05). (**D**) Difference in average THOR methylation level between each
418 paired normal-tumor samples (*n*=99, left plot) and distribution of differences in THOR methylation

(right plot, median&IQR; Paired *t*-test, ****P<0.0001). (**E**) Pie charts display the frequencies of THOR hypermethylation signature across various tumor types.

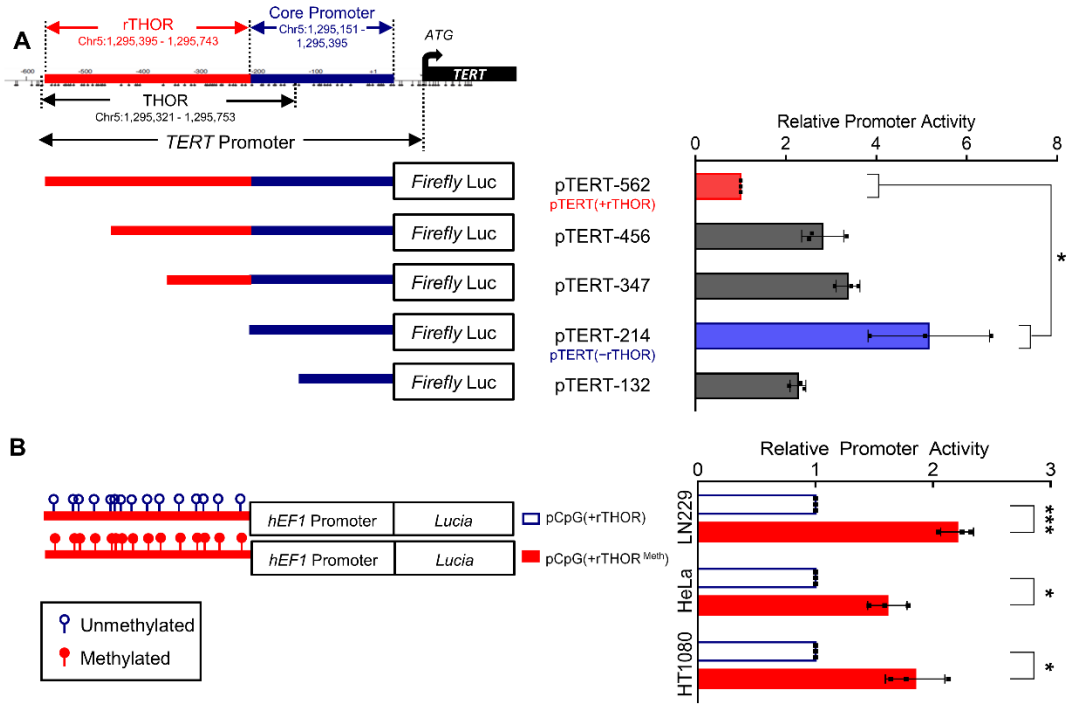


Figure 3. Hypermethylation counteracts the repressive effect of rTHOR on *TERT* promoter activity. For the data shown, each experiment was performed in triplicates. (**A**) Schematic representation of the *TERT* promoter is shown. Repressive THOR (rTHOR, red) is a transcriptional regulatory element within THOR, upstream of the *TERT* core promoter (blue). Normalized fold changes in *TERT* promoter activity are shown for the specified luciferase constructs transfected into glioblastoma cell line LN229. The numbers in the plasmid constructs indicate the distance (bp) from *TERT* TSS. (Unpaired *t*-test, **P*<0.05). **(B)** Normalized fold changes in *hEF1* promoter activity are shown for CpG-free constructs when rTHOR is unmethylated or methylated (in-vitro) in cancer cell lines LN229, HeLa, and HT1080 (Unpaired *t*-test, **P*<0.05).

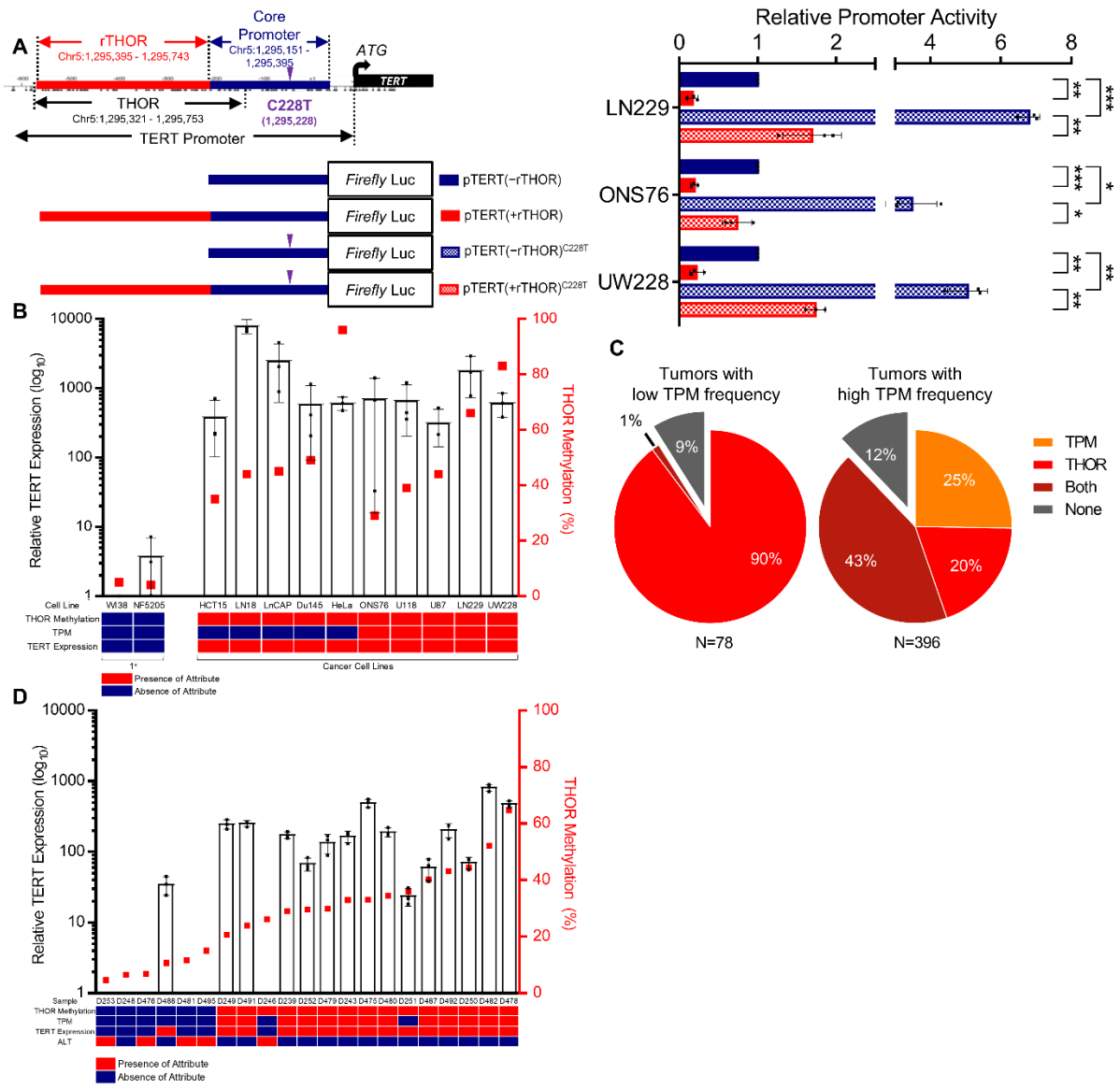


Figure 4. Co-existence and interplay of TPM and THOR hypermethylation in human cancers. For the data shown, each experiment was performed in triplicates. (**A**) Normalized fold changes in *TERT* promoter activity are shown for the specified luciferase constructs, with presence and absence of THOR and/or C228T TPM, in glioblastoma cell line LN229 and medulloblastoma cell lines ONS76 and UW228 (Unpaired *t*-test, **P*<0.05, ***P*<0.01, ****P*<0.001). (**B**) *TERT* expression (mean±SD, black bars/dots, left Y-axis) and average THOR methylation level (red dots, right Y-axis) are shown in human primary (1°) and cancer cell lines. *TERT* regulation-associated

characteristics for all cell lines are shown below the graph. (**C**) Pie charts display the frequencies of TPMs and THOR hypermethylation signature in TPM-common tumors (glioma/melanoma) and TPM-independent tumors (prostate/lung/colon/breast cancers). (**D**) *TERT* expression (mean±SD, black bars/dots, left Y-axis) and THOR methylation level (red dots, right Y-axis) are shown in a subset of adult gliomas (n=21). *TERT* regulation-associated characteristics for these samples are shown below the graph.