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Concise Communication**Oncology**

Relicative immortality is a hallmark of cancer cells governed by telomere maintenance. Approximately 90% of human cancers maintain their telomeres by activating telomerase, driven by the 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 describe the *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 TPM 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 that THOR hypermethylation is a prevalent telomerase-activating mechanism in cancer that can act independently of or in conjunction with TPMs, further supporting the utility of THOR hypermethylation as a prognostic biomarker.

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

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Relicative immortality is a hallmark of cancer cells governed by telomere maintenance. Approximately 90% of human cancers maintain their telomeres by activating telomerase, driven by the 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 describe the *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 TPM 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 that THOR hypermethylation is a prevalent telomerase-activating mechanism in cancer that can act independently of or in conjunction with TPMs, further supporting the utility of THOR hypermethylation as a prognostic biomarker.

Introduction

Relicative 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, approximately 90% of human cancers reactivate telomerase — a holoenzyme responsible for elongating telomeres — through reexpression of the catalytic subunit telomerase reverse transcriptase (*TERT*) (4).

Previous studies have identified 2 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 the 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 that has not yet been 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

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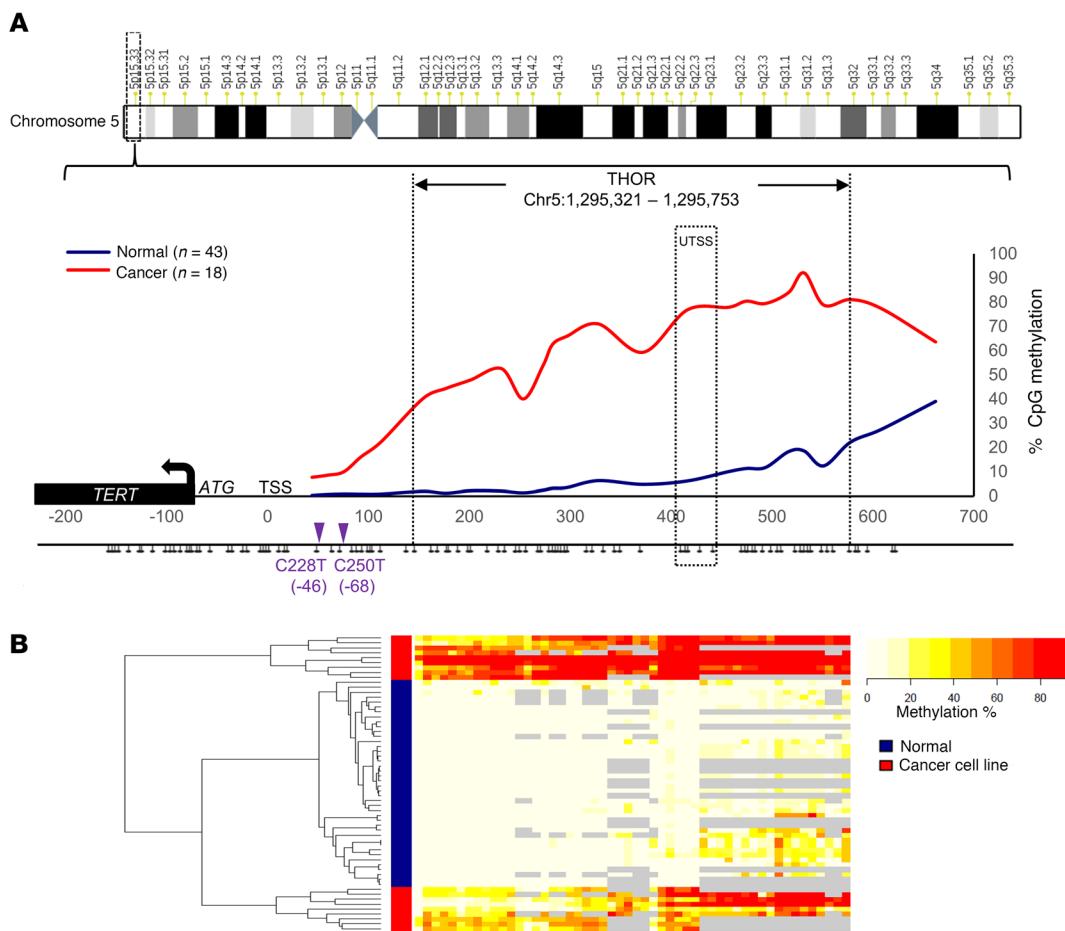


Figure 1. Defining THOR through DNA CpG methylation analysis of the *TERT* promoter. (A) Average CpG methylation of the *TERT* promoter in normal cell lines and tissues ($n = 43$, blue) and *TERT*-expressing cancer cell lines ($n = 18$, red). THOR is a 433-bp region (-140 to -572, relative to the TSS)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

transcription start site (UTSS) (19). Several studies have reported frequent *TERT* promoter (UTSS) hypermethylation in various *TERT*-expressing cancer types (19–23), suggesting 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 used 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 are reduced when unmethylated THOR is present, implying that TPMs and THOR are 2 distinct *TERT* regulatory mechanisms. Finally, we found that THOR hypermethylation is a prevalent phenomenon in *TERT*-expressing tumor types, regardless of their TPM status.

Results and Discussion

We examined the methylation landscape of the *TERT* promoter, which included approximately 650 bp upstream of the transcrip-

tion start site (TSS), with bisulfite-sequencing of DNA samples from normal cell lines and 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 1, A and B; supplemental material available online with this article; <https://doi.org/10.1172/JCI121303DS1>), 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 with normal samples, with a mean methylation difference of 58% across THOR ($P = 1.49 \times 10^{-9}$; Figure 1A and Supplemental Figure 2). Unsupervised clustering based on the 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

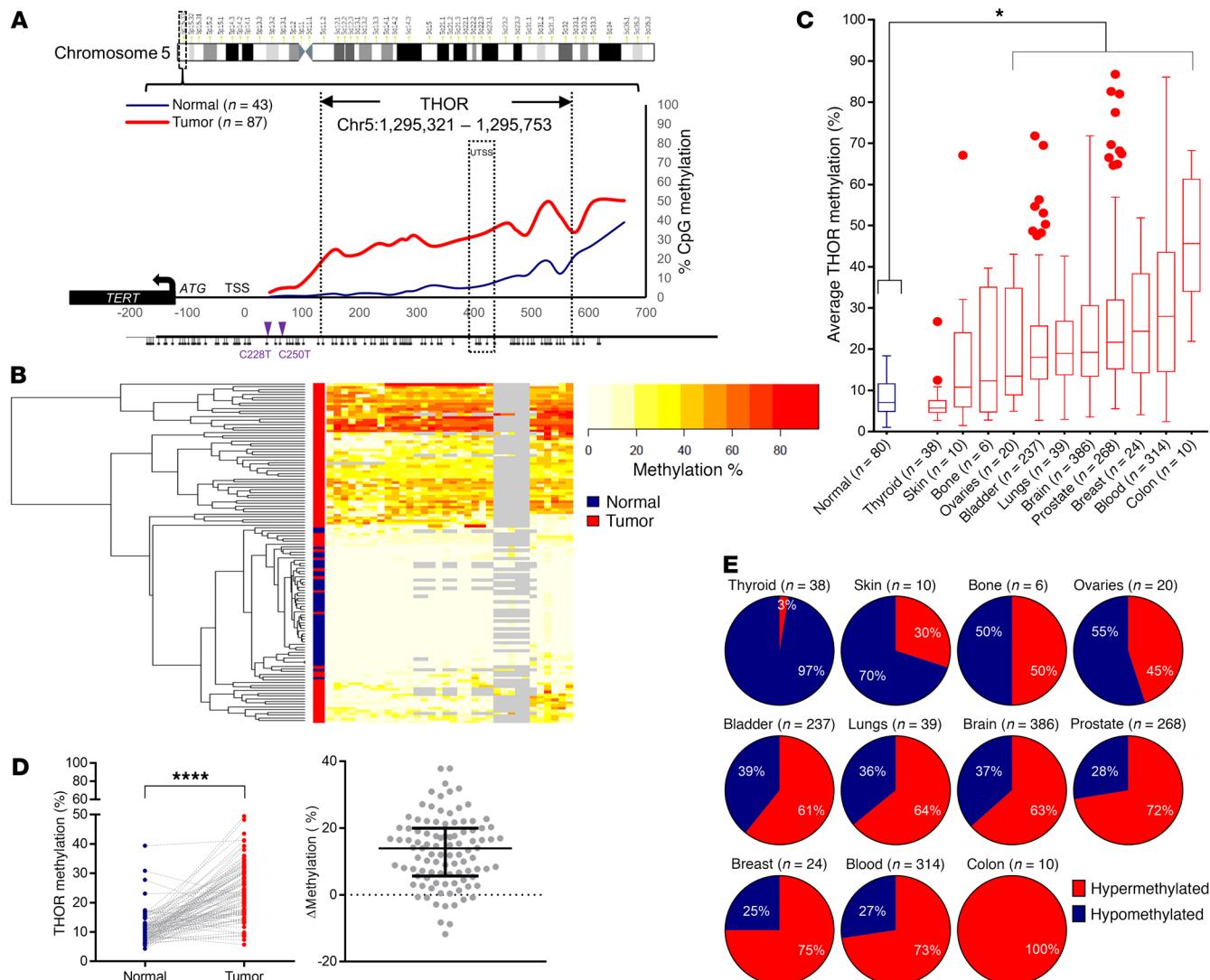


Figure 2. THOR hypermethylation is prevalent in human cancers. (A) Average DNA CpG methylation of the *TERT* promoter in normal cell lines and tissues (*n* = 43, blue) and tumor samples (*n* = 87, red). (B) Methylation heatmap generated from unsupervised clustering shows the methylation percentage of each CpG site within THOR for normal cell lines and tissues (*n* = 43) and tumor samples (*n* = 87). Gray color indicates unavailability of data. (C) Box-and-whisker plot shows the median and distribution of the average THOR methylation levels in normal control samples (*n* = 80, blue) and samples from various tumor tissue types (*n* = 1,352; red). *P < 0.05, by Sidak's multiple comparisons test. (D) Difference in average THOR methylation levels between each pair of normal tumor samples (*n* = 99; left plot) and distribution of differences in THOR methylation (right plot; median and IQR). ****P < 0.0001, by paired t test. (E) Pie charts display the frequencies of the THOR hypermethylation signature across various tumor types.

extent of THOR methylation in larger tumor cohorts, we tested and confirmed that 5 CpG sites (UTSS) (19) within THOR accurately represented the 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 (Figure 2C). 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, the low THOR methylation levels detected in thyroid cancers may be associated with their known lower malignant potential and better prognosis compared with other tumor types. Other tumor types such as skin and bone showing low and heterogeneous methylation

levels utilize other mechanisms for telomere maintenance, such as TPMs and the alternative lengthening of telomeres (ALT) pathway, respectively (24). Furthermore, we compared matched normal control and tumor samples in a subset of lung (*n* = 32) and prostate (*n* = 67) cancers, in which 89.9% (89/99) of the tumors had higher THOR methylation levels than matched normal tissue samples by a median of 13.9% (Figure 2D), corroborating THOR methylation as a cancer-associated epigenetic event.

We assessed the prevalence of THOR methylation in cancer by dichotomizing tumor samples as hypomethylated or hypermethylated using a cutoff value of 16.1%, adding 2 SDs to the mean methylation of normal samples as previously described (see Methods) (19, 20). All cancer types screened had a high prevalence (>45%) of THOR hypermethylation, with the exception of thyroid (3%)

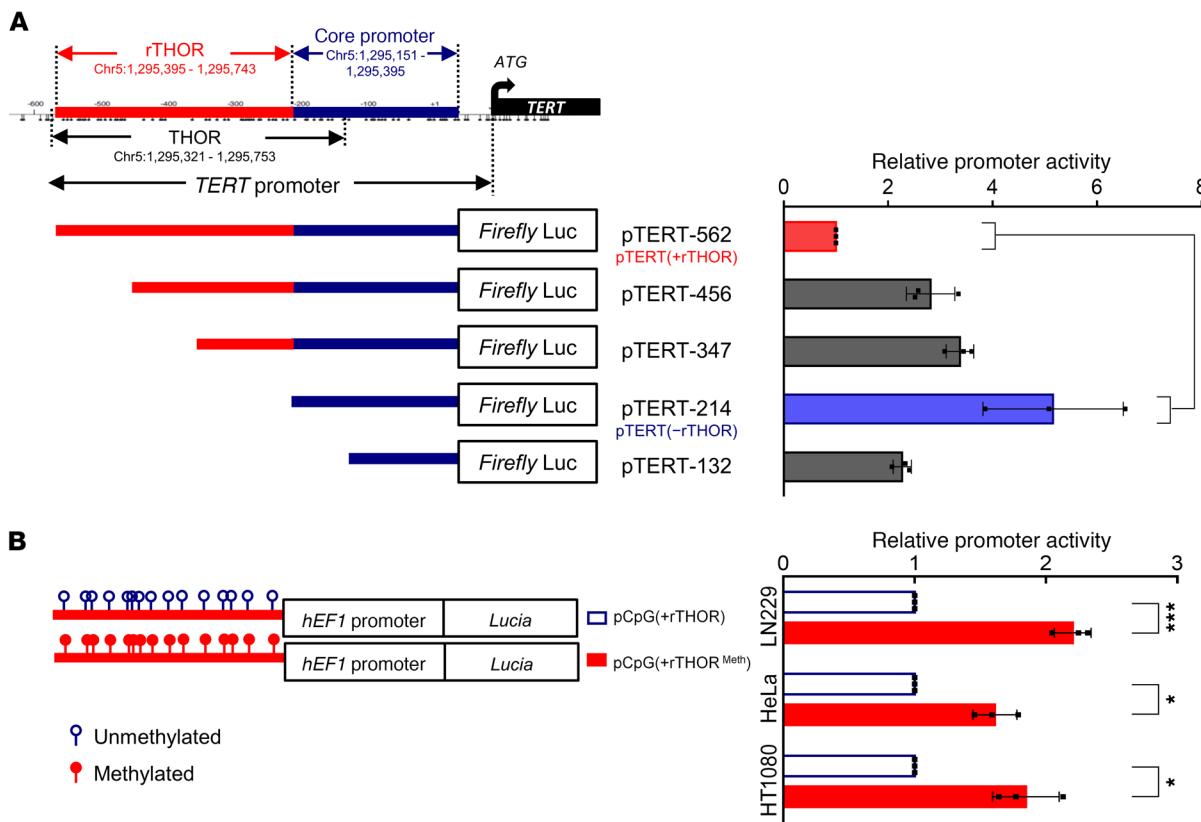


Figure 3. Hypermethylation counteracts the repressive effect of rTHOR on *TERT* promoter activity. For the data shown, each experiment was performed in triplicate. (A) Schematic representation of the *TERT* promoter. rTHOR (red) is a transcriptional regulatory element within THOR, located upstream of the *TERT* core promoter (blue). Normalized fold changes in *TERT* promoter activity are shown for the specified luciferase constructs transfected into the glioblastoma cell line LN229. The numbers in the plasmid constructs indicate the distance (bp) from the *TERT* TSS. *P < 0.05, by unpaired t test. (B) Normalized fold changes in *hEF1* promoter activity are shown for CpG-free constructs when rTHOR was unmethylated or methylated (in vitro) in the cancer cell lines LN229, HeLa, and HT1080. **P < 0.05 and ***P < 0.001, by unpaired t test.

and skin (30%) cancers (Figure 2E). Interestingly, tumors in which TPMs are rarely observed (i.e., prostate, breast, blood, and colon cancers) (11, 12, 15, 16) displayed a higher prevalence of THOR hypermethylation compared with 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 the 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. We observed a continual increase in reporter gene expression 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 an approximately 2-fold reduction of reporter gene expression, indicating that the minimal *TERT* core promoter is a region located up to 214 bp upstream of the *TERT* TSS, encompassing the TPM sites and the proximal 75 bp of THOR. Importantly, addition of repressive THOR (rTHOR) to the

TERT core promoter resulted in a significant decrease in reporter gene expression compared with expression of the *TERT* core promoter alone in LN229 cells (Figure 3A) and in multiple cancer cell lines from different tissues (Supplemental Figure 4). This region, which we defined as 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 remethylation using a unique reporter gene plasmid completely devoid of CpG sites (pCpG-free-promoter-Lucia; InvivoGen). rTHOR was cloned upstream of the modified CpG-free *hEF1* 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 cells and in 2 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, resulting in reduced THOR methylation and *TERT* expression (Supplemental Figure 6). In contrast, we found that *TERT* expression was not altered in normal embryonic stem cells lacking THOR hypermethylation (Supplemental Figure 6) after treatment with decitabine. These observations infer that can-

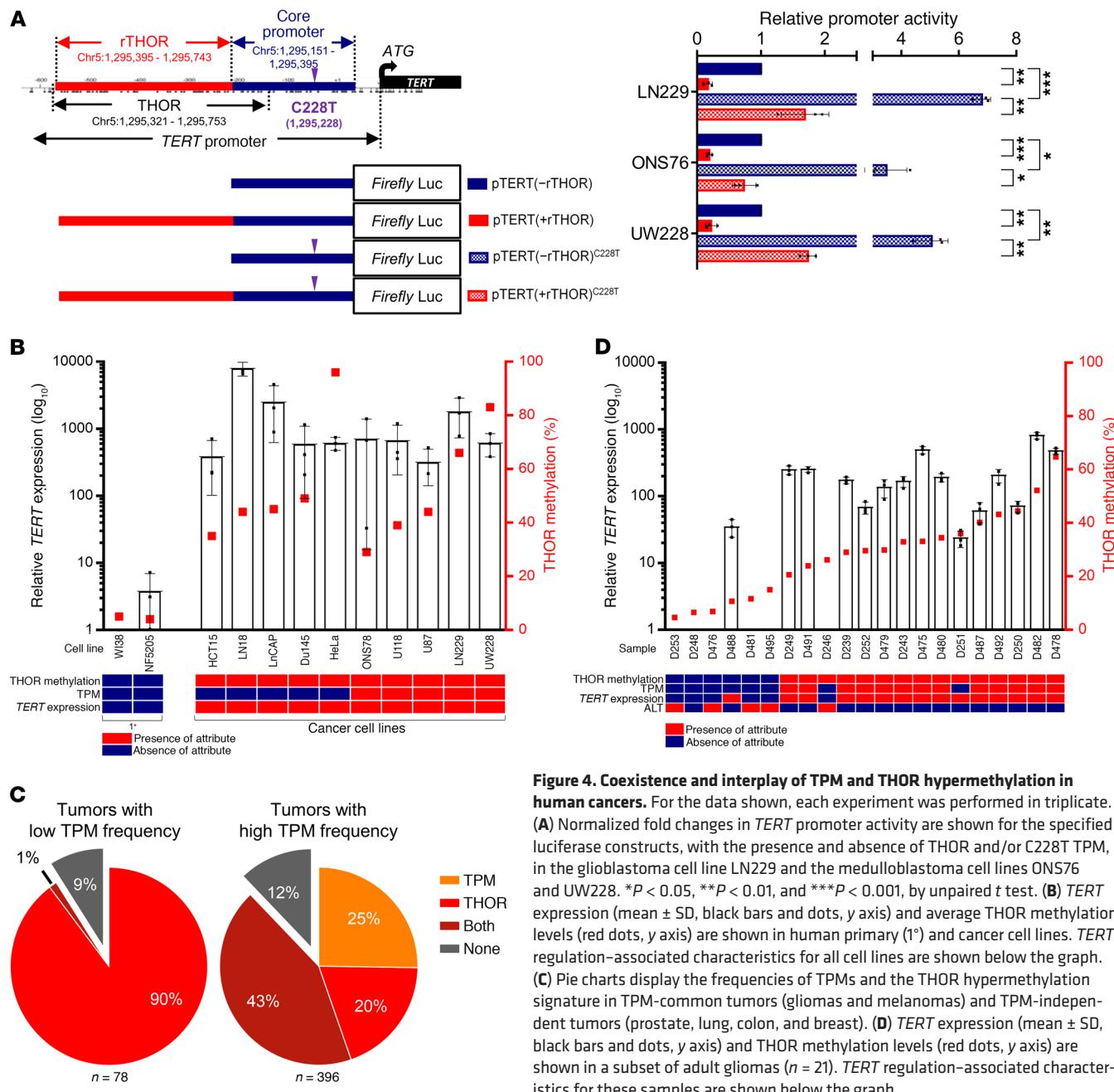


Figure 4. Coexistence and interplay of TPM and THOR hypermethylation in human cancers. For the data shown, each experiment was performed in triplicate. **(A)** Normalized fold changes in *TERT* promoter activity are shown for the specified luciferase constructs, with the presence and absence of THOR and/or C228T TPMs, in the glioblastoma cell line LN229 and the medulloblastoma cell lines ONS76 and UW228. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$, by unpaired t test. **(B)** *TERT* expression (mean \pm SD, black bars and dots, y axis) and average THOR methylation levels (red dots, y axis) are shown in human primary (1 $^{\circ}$) 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 the THOR hypermethylation signature in TPM-common tumors (gliomas and melanomas) and TPM-independent tumors (prostate, lung, colon, and breast). **(D)** *TERT* expression (mean \pm SD, black bars and dots, y axis) and THOR methylation levels (red dots, y axis) are shown in a subset of adult gliomas (n = 21). *TERT* regulation-associated characteristics for these samples are shown below the graph.

cer cells hypermethylate THOR to counteract the repressive effect of rTHOR and promote *TERT* upregulation.

A high prevalence of THOR hypermethylation in tumor types that commonly harbor TPMs (Figure 2E) and the previous finding that TPMs alone are insufficient at 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 Figure 2A). This suggests that the genomic loci encompassing either

THOR or TPMs are distinct *TERT* regulatory regions. To investigate the functional repressive effect of unmethylated rTHOR in the context of TPMs, we compared reporter gene expression in the constructs pTERT(+rTHOR) and pTERT(-rTHOR), which contained the *TERT* core promoter with and without rTHOR, respectively, and in the presence and absence of C228T TPMs (Figure 4A). As expected, the addition of rTHOR to the *TERT* core promoter decreased promoter strength by an average of approximately 5-fold in all 3 cancer cell lines tested, while the C228T mutation resulted in an average increase of approximately 5-fold (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 WT *TERT*

promoter without rTHOR. These findings indicate that THOR and TPMs are nonredundant 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 could coexist in 10 *TERT*-expressing cancer cell lines. All cell lines showed THOR hypermethylation and, importantly, 5 of the cell lines showed coexistence of the 2 *TERT*-activating mechanisms (Figure 4B). However, we observed no significant difference in the levels of *TERT* expression between the cancer cell lines when TPMs were present or absent (Supplemental Figure 7). This suggests that in certain cancers in which TPM is absent, other mechanisms such as THOR hypermethylation contribute to *TERT* expression. Second, we assessed the prevalence and coexistence of these mechanisms in cancer types in which TPMs are either frequently or rarely present (Figure 4C). In a cohort of cancers that commonly have TPMs (gliomas and melanomas, $n = 396$), we frequently (43%) observed TPMs and THOR hypermethylation together, while 45% of the tumors exhibited only 1 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, which are tumors that exhibit heterogeneous telomere maintenance mechanisms including THOR hypermethylation, TPMs, and ALT ($n = 21$) (Figure 4D). Interestingly, 93.3% (14 of 15) of the *TERT*-expressing gliomas had THOR hypermethylation alone or with TPMs, while 83.3% (5 of 6) of the gliomas that lacked *TERT* expression were missing either one of the *TERT*-activating mechanisms but were primarily dependent on ALT for telomere maintenance. These observations highlight the role of THOR hypermethylation as one of the *TERT*-upregulating mechanisms in cancer, either independently or in conjunction with TPMs.

Although we describe THOR hypermethylation as an additional *TERT*-upregulating mechanism, an understanding of its biological mechanism requires further investigation. A recent study has associated allele-specific hypomethylation and active histone marks (H3K4me2/3) in cancer cell lines that harbor TPMs (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 their binding is methylation sensitive requires further investigation. Another interesting aspect of THOR hypermethylation is that it may regulate other genes in the proximity to *TERT* that ultimately affect *TERT* expression. One example is the *hTERT* antisense promoter-associated (*hTAPAS*) noncoding RNA, whose promoter overlaps with the *TERT* promoter (28). Last, to explore the coexistence 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 that is 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 that hypermethylation counteracts this effect, suggesting that cancer cells methylate THOR to upregulate *TERT* expression and activate telomerase. In addition, our study proposes 2 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 approximately 90% of human cancers activate telomerase. Our findings provide biological insight into why tumors with THOR hypermethylation are associated with a poorer clinical outcome (19, 20, 22, 23), further highlighting its value as a potential prognostic biomarker.

Methods

A complete description of the methods and statistical analysis is provided in the Supplemental Materials. An unsupervised clustering heatmap was created with a modified version of Methylation plotter (29). NGS data are publicly available in the NCBI's Gene Expression Omnibus (GEO) database (GEO GSE120511).

Statistics. General statistical analyses were performed using a Fisher's exact test or a χ^2 test for categorical variables, and a 2-tailed *t* test (GraphPad Prism, version 6.0) for continuous variables. *P* values of less than 0.05 were considered statistically significant.

Study approval. All experiments were performed with the approval of the research ethics board of the Hospital for Sick Children (Toronto, Ontario, Canada).

Author contributions

PCB and UT were responsible for the study concept. DDL, PCB, and UT designed the experiments. DDL conducted all in vitro experiments, methylation analyses, and statistical analyses. RL collected data and conducted methylation analyses for prostate and bladder samples. MG, CHZ, and TL conducted the decitabine treatment experiments. MK and AD performed the MiSeq data analysis, which was supervised by TJP. AH and AJP analyzed the HiSeq data. NMN and JDA assisted with data interpretation. MR, RADM, JSD, DH, TH, PJW, RV, GZ, JK, SD, MDT, CEH, JDW, AF, RJH, MDM, KW, BD, HY, KA, MRA, and PBD provided patients' samples. DDL, PCB, and UT cowrote the manuscript.

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