Mutation of the TERT promoter, switch to active chromatin, and monoallelic TERT expression in multiple cancers

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Somatic mutations in the promoter of the gene for telomerase reverse transcriptase (TERT) are the most common noncoding mutations in cancer. They are thought to activate telomerase, contributing to proliferative immortality, but the molecular events driving TERT activation are largely unknown. We observed in multiple cancer cell lines that mutant TERT promoters exhibit the H3K4me2/3 mark of active chromatin and recruit the GABPA/B1 transcription factor, while the wild-type allele retains the H3K27me3 mark of epigenetic silencing; only the mutant promoters are transcriptionally active. These results suggest how a single-base-pair mutation can cause a dramatic epigenetic switch and monoallelic expression.

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The telomerase ribonucleoprotein (RNP) complex maintains telomeric DNA in normal stem cells as well as in most cancer cells. This telomere maintenance is necessary to perpetuate indefinite cellular proliferation. Most human cells express the telomerase RNA subunit hTR, while normal somatic cells other than stem cells do not express telomerase reverse transcriptase (TERT), the catalytic subunit of telomerase. In such somatic cells, TERT gene expression is epigenetically silenced at the transcriptional level (Atkinson et al. 2005; Liu et al. 2007; Zhu et al. 2010).

The failure to discover recurrent mutations or gene rearrangements that activate TERT expression was incongruous with its fundamental role in cancer. This situation changed when two cancer-specific somatic mutations in the TERT promoter were identified (Horn et al. 2013; Huang et al. 2013). These TERT promoter mutations occur more commonly than any other observed mutation in a number of cancers, including melanomas, glioblastomas [GBMs], hepatocellular carcinomas (HCCs), and urothelial carcinomas (UCs) (Killela et al. 2013; Kinde et al. 2013; Vinagre et al. 2013). The mutations are uniformly C>T transitions, predominantly located either −124 base pairs [bp] or −146 bp upstream of the TERT translational start site [ATG]. Data based on reporter constructs suggest that TERT promoters with these mutations are about two-fold more effective at driving expression than wild-type promoters [Horn et al. 2013; Huang et al. 2013; Rachakonda et al. 2013]. The mutations are associated with functional increases in TERT protein, telomerase activity, and telomere length [Borah et al. 2015] and decreased survival for cancer patients [Rachakonda et al. 2013; Griewank et al. 2014; Borah et al. 2015]. Genome editing of the TERT promoter at −124 suggests that the mutations are causative for increased TERT expression in normal bladder stem cells and bladder cancer cells (Li et al. 2015; Xi et al. 2015) and are capable of preventing TERT silencing upon differentiation of stem cells (Chiba et al. 2015).

Both the −124 and −146 promoter mutations create consensus binding sites for E-twenty-six [ETS] family transcription factors, of which there are 27 members, many of which are predicted to bind to the same sequence [Hollenhorst et al. 2011]. A recent study implicated GABPA as a relevant ETS factor [Bell et al. 2015]. However, the pathway by which an epigenetically silenced TERT gene is activated by the promoter mutation remains largely unknown. In the current study, we addressed this question and observed monoallelic expression of TERT from the promoter bearing the −124 mutation. Thus, TERT provides a model system for investigating central questions about how subtle genetic mutations can drive major epigenetic alterations.

Results and Discussion

The chromatin state of wild-type and mutant TERT promoters is different

As TERT promoter mutations are almost always heterozygous [Fig. 1A], we explored the possibility that the allele bearing a promoter mutation is selectively active. Initially, we chose to test this hypothesis in HCC-derived cell lines and, in preparation, used PCR and Sanger sequencing as well as next-generation sequencing to identify lines that are heterozygous for TERT promoter mutations at −124 bp from the ATC [66 bp from the transcriptional start site [TSS]] [Fig. 1A; Supplemental Table S1]. The cell lines spanned a very large range of TERT expression.

[Keywords: TERT; chromatin; monoallelic; noncoding mutations; promoter; telomerase]

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and telomerase activity, with no clear difference between lines with and without promoter mutations (Supplemental Tables S1, S2; Supplemental Fig. S1A–C). The lack of a genotype–phenotype correlation differs from results obtained in UC cell lines (Borah et al. 2015), but the number of cell lines studied here may be insufficient to reveal an association, if there is one, in HCC.

We selected HepG2, SNU-423, and SNU-475 as lines with promoter mutations and detectable telomerase and performed chromatin immunoprecipitation (ChIP) with antibodies to RNA polymerase II (Pol II) [e.g., Fig. 1B]. Sequencing of PCR products obtained from these ChIP experiments indicated that Pol II had a strong preference for occupying the mutant TERT promoter in all three lines (Fig. 1C,D). We confirmed that these results were not due to a bias introduced by PCR (Supplemental Fig. S2).

Pol II recruitment to DNA frequently correlates with an open chromatin state that can be identified by post-translational modifications on nucleosomal histone proteins. Dimethylation or trimethylation of the first lysine of histones H3 (H3K4me2/3) is established by the Trithorax group of proteins and is associated with gene activation (Schuettengruber et al. 2011). Consistent with the results from the Pol II ChIP, sequencing revealed a strong preference for the H3K4me2/3 mark on the mutant compared with the wild-type allele (Fig. 1C,D).

The Polycomb-repressive complex PRC2 is thought to be solely responsible for deposition of H3K27me3, a hallmark of facultative heterochromatin containing repressed genes (Steffen and Ringrose 2014). We found high levels of H3K27me3 at the TERT promoter in telomerase-negative human primary cells compared with the HCC line SNU-475 (Supplemental Fig. S3A,B), consistent with a previous report (Atkinson et al. 2005) that TERT is epigenetically silenced in telomerase-negative somatic cells. We then examined the allele specificity of the H3K27me3 mark in HCC lines with TERT promoter mutations by PCR and sequence analysis of DNA immunopurified with an antibody against H3K27me3. We found a substantial depletion of the H3K27me3 mark on the promoter mutant allele relative to the wild-type allele [Fig. 1C,D]. We obtained similar results for two UC-derived lines and one GBM-derived line (Supplemental Fig. S4). Thus, the TERT allele with a promoter mutation has an active chromatin mark, while the wild-type allele in the same cell bears an epigenetic hallmark of inactive chromatin.

Transcriptional up-regulation of GABPB1 and allele-specific binding of GABPA in HCC

The TERT promoter mutation creates a consensus binding site for an ETS family transcription factor, leading to the prediction that the TERT promoter mutation drives telomerase in response to the recruitment of such a factor. RNA sequencing [RNA-seq] analysis was performed on a panel of nine HCC lines, five of which were heterozygous for the TERT promoter mutation (Supplemental Tables S1, S2). Many of the 27 ETS factors were expressed among the cell lines [Supplemental Table S3], but only GABPB1 was expressed at significantly higher levels in lines with promoter mutations compared with those without (P = 0.005) [Fig. 2A,B; Supplemental Table S3].

GABPB1 contains a transcriptional activation domain and forms a heterodimer with the ETS factor GABPA (LaMarco et al. 1991; Gugneja et al. 1995). The latter protein performs the sequence-specific DNA-binding function and was also expressed in all HCC lines tested (Supplemental Table S3). Using ChIP, we found that GABPA bound to the TERT promoter in both HCC and UC lines [Fig. 2C]. This binding was specific for the mutant promoter in the two heterozygous HCC lines that we tested [Fig. 2D]. In support of allele-specific binding, ENCODE ChIP-seq [ChIP combined with deep sequencing] data for two mutant cell lines show GABPA bound at the TERT promoter, while two wild-type cell lines did not (Supplemental Fig. S5). Using siRNA knockdown, we found that GABPA contributed to TERT transcription (Supplemental Fig. S6). While our work was in preparation, Bell et al. [2015] reported that GABPA exhibited an allele bias in binding to the TERT promoter in several different cancers. Our results extend this conclusion to new HCC cell lines and indicate an associated up-regulation of GABPB1, but not GABPA or GABPB2, in the mutant promoter HCC cells. The selective up-regulation of the transactivating subunit GABPB1 provides some insight into how these cells specifically enhance expression of GABPA-bound genes such as TERT despite the apparent redundancy for similar consensus binding sequences among the 27 ETS transcription factors.

Many of the ETS transcription factors are predicted to bind to the same sequence [Hollenhorst et al. 2011]. The
ETS factor ELF1 was recently implicated in melanoma progression; evaluation of promoter mutations in the cancer-associated gene succinate dehydrogenase (SDHD) suggested that they disrupt ELF1 binding (Weinhold et al. 2014). When we compared the proposed ELF1-binding site in SDHD with the TERT promoter sequence, we found that these promoters share a similar bipartite consensus ETS site (Supplemental Fig. S7). This sequence similarity led us to test whether ELF1 occupied the TERT promoter. ChIP showed ELF1 bound at the TERT promoter in HCC and UC cell lines, but, in contrast to GABPA, ELF1 did not demonstrate a preference for the TERT promoter sequence, we precipitated the transcribed allele from UMUC3, while the RNA Pol II ChIP DNA isolated by either RNA Pol II ChIP of UMUC3 cells or H3K27me3 ChIP of T24. Because the RNA Pol II ChIP sequencing of a region that encompasses both the TERT promoter mutation and the SNP in exon 2. We used template DNA isolated by either RNA Pol II ChIP of UMUC3 cells or H3K27me3 ChIP of T24. Because the RNA Pol II ChIP precipitated the transcribed allele from UMUC3, while the H3K27me3 ChIP isolated the nontranscribed allele from T24, we conclude that the allele harboring the TERT promoter mutation also contains the variant observed by RT–PCR (Fig. 3B,D). We expected that cancer cells with no TERT promoter mutation would show expression of both alleles of TERT. Indeed, TERT cDNA from the colon cancer cell line HCT-116, which does not bear any known mutation for TERT mRNA expression in these cancer cells. To test this, we used two tools to identify from which allele transcripts are derived. First, using UC cells that were heterozygous for promoter mutations, we searched their exome sequence data for naturally occurring heterozygous single-nucleotide polymorphisms (SNPs) in the TERT gene body (Supplemental Table S6). UMUC3 and T24 cells both bear a SNP in one copy of TERT in exon 2 (rs2736098). PCR and sequencing of genomic DNA demonstrated that these cells are indeed heterozygous for rs2736098, while sequencing of the RT–PCR products indicated that, in each case, the cDNA exhibited a single variant of the SNP (Fig. 3A), although the expressed variant differed for the two lines. As controls, we detected no amplification in samples where reverse transcriptase was omitted, indicating that the products were indeed derived from cDNA (Supplemental Fig. S12). In addition, we were able to detect biallelic expression of heterozygous SNPs in the transcripts of STAG1 and RNase H [in UMUC3 cells] and p53 and NBN [in SNU-475 cells], indicating faithful amplification of these variant cDNA templates (Supplemental Fig. S13).

Second, to assess whether the TERT transcripts from T24 and UMUC3 were derived from the allele bearing the TERT promoter mutation, we performed PCR and sequencing of genomic DNA based on RT–PCR analysis of a naturally occurring SNP in exon 2 (Fig. 3C). We expected that cancer cells with no TERT promoter mutation would show expression of both allelic forms of TERT mRNA. Instead, TERT cDNA from the colon cancer cell line HCT-116, which does not bear any known mutation in the TERT promoter, exhibited an allelic ratio similar to that observed for the naturally occurring SNP in exon 2. We used template DNA isolated by either RNA Pol II ChIP of UMUC3 cells or H3K27me3 ChIP of T24. Because the RNA Pol II ChIP precipitated the transcribed allele from UMUC3, while the H3K27me3 ChIP isolated the nontranscribed allele from T24, we conclude that the allele harboring the TERT promoter mutation also contains the variant observed by RT–PCR (Fig. 3B,D).
cate that the mutation results in binding of GABPA in
predisposed to activation by the mutation. Our data indi-
cell lines is consistent with the idea that these cells were
The higher expression of GABPB1 in the promoter mutant
dimer (Fig. 3D) by virtue of the
and abundant transcription factor GABPA/B1 hetero-
sites for the common
promoter state in precancerous cells versus cancer cells with
unknown. Chromosomal coordinates are for hg38. (for review, see Li and Clevers
model, where tumors arise from stem cells with active telomerase, and the TERT promoter mutation
causes that allele to remain selectively active while the
the other allele becomes repressed. In short, the tumor cell
lines show the result of an epigenetic switch, but future
experiments will be required to determine whether the
mutant allele was switched on or the wild-type allele
was switched off.

Other important questions remain to be answered. The
prevalence of these mutations, together with the critical
function of TERT in telomere maintenance, suggests
that they may function as gatekeepers to cancer develop-
ment. If these mutations convert TERT from a repressed
state to an expressed state, what is the temporal order of
events? In the scenario that the mutation is the initiating
event, as suggested by the genome-editing studies in UCs
(Li et al. 2015), recruitment of sequence-specific pioneering
factors to the mutated site could constitute the
second event. What are these pioneering factors? These
recruitment events may lead to the methylation of
H3K4 on the mutant allele, which in turn drives H3 acety-
lation (Crump et al. 2011). Histone modifications such as
these promote the transition of Pol II from an initiating
form to an elongating form (Stasevich et al. 2014), result-
ing in gene expression. That many mutations concomi-
tantly form allele-specific transcription factor-binding
sites and associate with epigenetic changes (Kilpinen
et al. 2013; McVicker et al. 2013) suggests that genetic
changes can indeed drive epigenetic changes.

Because many ETS factors are reported to bind to similar
consensus sequences, do ETS factors other than GABPA/
B1 also bind and activate mutant TERT promoters? Con-
versely, given the ubiquitous nature of the ETS factors,
do other family members discriminate among their target
genes using a different bipartite sequence? For example,
the bipartite sequence identified by Bell et al. (2015) differs
significantly from the proposed binding site in the SDHD
promoter (Supplemental Fig. S7). Identifying the mecha-
isms controlling GABPA/B1 activity and expression likely
will be important to understand TERT expression in
these cells. Finally, TERT promoter mutations distinguish
cancer cells from normal telomerase-expressing cells.
Thus, from a translational point of view, full understand-
ing of the mechanistic differences in the transcription of
TERT among these cell types may provide a therapeutic
approach or a biomarker for stratifying tumors.

Materials and methods

Cell lines
HCC lines and UMUC3 and T24 were obtained from American Type Cul-
ture Collection. UMUC3 and T24 were grown as described (Guin et al.

Figure 3. Monoallelic expression of TERT in tumor-derived cell
lines with mutations in the TERT promoter. (A) PCR and Sanger se-
quencing show a heterozygous coding SNP (rs2736098) in exon 2 of
TERT in two UC lines [genomic DNA], but only one sequence is ex-
pressed [cDNA] [see the model in D]. (B) Linking the TERT promoter
mutation with the exon 2 SNP. PCR and sequencing were performed
for a region spanning the promoter mutation and rs2736098; PCR
template DNA was derived from allele-specific ChIP for RNA Pol II
or H3K27me3 (e.g., Fig. 1B). (C) No allelic bias in expression of
TERT in HCT-116 colon carcinoma cell line with no known TERT
regulatory mutations. The ratio of the two sequences is the same in
genomic DNA and cDNA; the cause of the different peak heights is
unknown. Chromosomal coordinates are for hg38. (D) Model of the
TERT promoter state in precancerous cells versus cancer cells with
TERT promoter mutations. The SNP rs2736098 is found on different
alleles in T24 and UMUC3, as shown by the allele-specific ChIP in B.
RNA extraction and cDNA preparation

Following RNA extraction with Trizol (Life Technologies), reverse transcription was performed by treating with 10 µg of RNA with 5 U of RQ1 DNase (Promega) according to the manufacturer’s protocol, followed byphenol extraction (pH 7), chloroform extraction, and then ethanol precipitation. The cDNA was generated from 2 µg of RNA synthesized using random hexamers, oligo(dT)$_{30}$, and SuperScript III (Life Technologies). Following treatment with RNase H (New England Biolabs), quantitative PCR was performed with iQ SYBR Green PCR mix (Bio-Rad) using a Roche LightCycler 480 with the program 10 min at 98°C, 30 sec at 95°C; 30 sec at 60°C, 30 sec at 72°C; 5 min at 72°C, followed by quantification using the Roche LightCycler 480 software. Melt curve analyses were examined to ensure the uniformity of relevant PCR amplicons.

Primers for rs2736098 in UMUC3 and T24 were forward (5′-CTGTTGTTCTTGTGTTGGTGTC-3′) and reverse (5′-CCTTGTGCGCCCTGAGAGATAG-3′). Primers for assessing HCT-116 TERT promoter were forward for both cDNA and genomic DNA (5′-GCCAGGTGTA CGGCTTCTGCT-3′), reverse for genomic DNA (5′-CTCCCTCAACCTCG GCTCTCT-3′), and reverse for cDNA (5′-CAGGATCTCCTCCACCGACA-3′). The heterozygous SNP in HCT-116 was first identified using the COS-MIC cell line browser (Forbes et al. 2014).

ChiP

ChiP was performed as previously described (Schwartz et al. 2012; Davidovich et al. 2013) with the exceptions noted in the Supplemental Material. For immunoprecipitation, 5–25 µg of solubilized chromatin was used with 2 µg of α-RNA Pol II antibody (EMD Millipore, catalog no. 05-623), α-H3 (Abcam, ab-1791), α-H3K4me2/3 (Abcam, ab-6000), α-H3K27me3 (EMD Millipore, 07–449), or 4 µg of α-GABPA (Santa Cruz Biotechnology, H-180 sc-22810) and incubated overnight at 4°C. All replicates reported in this study represent independent biological samples.

PCR and sequence analysis of the TERT promoter, TERT expression, and telomerase activity

Quantitative PCR for the TERT promoter was performed on a Roche LightCycler 480 using iQ SYBR Green PCR mix (Bio-Rad) with primers for the TERT promoter forward (5′-GTCCTGCCCCTTCACCTTCT-3′) and reverse (5′-AGCGCTGCCTGAAACTCG-3′). For immunoprecipitation, 5 µg of α-GABPA (Santa Cruz Biotechnology, H-180 sc-22810) and incubated overnight at 4°C. All replicates reported in this study represent independent biological samples.

RNA-seq expression analysis

Total RNA was isolated from cell lines using Qiagen reagents and following the recommended protocol. RNA-seq libraries were constructed using TrueSeq RNA kit from Illumina according to the manufacturer’s protocol. The major steps in the protocol were: [1] depletion of RNA with the use of probes complementary to rRNA sequences, [2] generation of cDNA, and [3] generation of next-generation sequencing libraries. The libraries were sequenced on a HiSeq 2500 (Illumina). The sequencing data were matched to the human reference genome version hg19 using the CASAVA pipeline (Illumina) with the ELAND algorithm set for RNA analysis. The expression profiles were compiled on a Genome Studio RNA expression module (Illumina) using reads that passed the chastity quality filter by Illumina. The data are reads per kilobase per million mapped reads. Data for each gene are normalized for the length of the transcripts.

Competing interest statement

T.R.C. is on the board of directors of Merck.

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2014 except as noted below and were authenticated by the University of Colorado Cancer Center Protein Production Shared Resource using an Applied Biosystems Profiler Plus kit, which analyzed nine STR loci (Life Technologies, 4303326). After authentication, cells were frozen within 1–2 wk. Viols of cells were resuscitated 2 mo prior to being used in experiments in this study. All cells were cultured in DMEM (WVR Scientific) with GlutaminePlus (Atlanta Biologiicals), 10% FBS (Seradigm), penicillin/streptomycin (GIBCO), glutamax (GIBCO), and sodium pyruvate (GIBCO), except HepG2 cells were cultured in EMEM (American Type Culture Collection) without sodium pyruvate.
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