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 twofold 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
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 HCC lines (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 to 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 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.
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 with and without TERT promoter mutations. (C) Quantitative ChIP for GABPA occupancy at the TERT promoter in HCC (HepG2, SNU-475, and SNU-423) and UC (T24 and UMUC3) cell lines with promoter mutations. Error bars indicate ±SEM. n = 1−5; P > 0.03 from a one-tailed paired t-test between each ChIP and its IgG control. (D) ChIP followed by PCR and Sanger sequencing of the TERT promoter in two HCC lines (SNU-423 and SNU-475).

Figure 2. ETS transcription factor expression and binding of GABPA to the TERT promoter in HCC and UC cell lines. (A) The 10 most highly expressed ETS transcription factors in the tested HCC lines using RNA-seq. P-values were derived from a two-tailed t-test assuming heteroscedasticity. Expression levels are ±SEM. (B) GABPA and GABPB1 expression levels from RNA-seq analysis of HCC lines with and without TERT promoter mutations. (C) Quantitative ChIP for GABPA occupancy at the TERT promoter in HCC (HepG2, SNU-475, and SNU-423) and UC (T24 and UMUC3) cell lines with promoter mutations. Error bars indicate ±SEM. n = 1−5; P > 0.03 from a one-tailed paired t-test between each ChIP and its IgG control. (D) ChIP followed by PCR and Sanger sequencing of the TERT promoter in two HCC lines (SNU-423 and SNU-475).

TERT is expressed monoallelically from mutant promoters

Reporter genes driven by TERT promoters with a mutation at −124 show modestly higher expression than wild-type promoters [Horn et al. 2013; Huang et al. 2013; Rachakonda et al. 2013]. However, the allele-specific phenotypes that we observed at the endogenous TERT promoter (Figs. 1C, 2D) suggest that such comparisons of exogenous plasmids may underestimate the contribution of the promoter mutation. If the mutation acts as a switch and is a key component of activating TERT expression, then the mutated allele should be largely or solely responsible 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 are 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 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 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 in the TERT promoter, exhibited an allelic ratio similar to genomic DNA based on RT–PCR analysis of a naturally occurring SNP in exon 2 [Fig. 3C].

With few exceptions, such as imprinted genes and X-chromosome inactivation, monoallelic expression as dramatic as that observed here for TERT is highly unusual. Pedigree analysis of inherited disease-associated TERT
mutations (Armanios et al. 2005) does not support maternal or paternal imprinting. For nonimprinted genes, the allelic bias in gene expression is typically on the order of twofold (Yan et al. 2002; Li and Clevers 2010).

Model for an epigenetic switch

In normal somatic cells other than stem cells, TERT expression is repressed, and telomerase activity is not detectable (Kim et al. 1994; Wright et al. 1996; Meyerson et al. 1997; Atkinson et al. 2005). Whether cancers are derived from normal stem cells (with active telomerase) or transiently proliferating cells (with inactive telomerase) is currently unknown (for review, see Li and Clevers 2010). Our data are consistent with a model in which cancer cells bearing one of the recurrent TERT promoter mutations are derived from cells (perhaps transiently proliferating ones) in which TERT is normally silenced. Such cells gain a de novo binding site for the common and abundant transcription factor GABPB1/B1 heterodimer (Fig. 3D) by virtue of the TERT promoter mutation. The higher expression of GABPB1 in the promoter mutant cell lines is consistent with the idea that these cells were predisposed to activation by the mutation. Our data indicate that the mutation results in binding of GABPA in HCC cells, leading to recruitment of Pol II either concomitantly with or subsequent to an epigenetic shift from a repressed to an active chromatin state. At the same time, the remaining wild-type allele in the same cell remains silent, residing in inactive chromatin.

Introduction of TERT promoter mutations by genome editing in human embryonic stem cells indicates that the mutations have the capacity to prevent the programmed silencing that TERT normally undergoes upon terminal differentiation (Chiba et al. 2015) and suggests another mechanism by which such mutations may contribute to oncogenesis. Our data are equally consistent with such a model, where tumors arise from stem cells with active telomerase, and the TERT promoter mutation causes that allele to remain selectively active while 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 development. 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 secondary 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 acetylation (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), resulting in gene expression. That many mutations concomitantly 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? Conversely, 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 mechanisms 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 understanding 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 Culture Collection. UMUC3 and T24 were grown as described (Guin et al.
TERT promoter mutations and monoallelic expression

RNA extraction and cDNA preparation

Following RNA extraction with Trizol (Life Technologies), reverse transcription was performed by treating 10 µg of RNA with 5 U of RQ1 DNase (Promega) according to the manufacturer’s protocol, followed by phenol extraction (pH 7), chloroform extraction, and then ethanol precipitation. The cDNA was generated from 2 µg of RNA synthesized using random hexamers, oligoDT30, 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, and 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.

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′-CTTCTGTTGCCTGTC‘-3′) and reverse (5′-CCTGTTGCGCCCTGAGGAGTAG-3′). Primers for assessing HCT-116 TERT SNP (COSM3136609) were forward for both cDNA and genomic DNA (5′-CAGGTTGTA CGGCTTCGGT-3′), reverse for genomic DNA (5′-CTCCCTACCTGGCCTC‘-3′), and reverse for cDNA (5′-CAGGATCCTCTCCAGCAGA-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, 2–5 µg of solubilized chromatin was used with 2 µg of a 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-GAP45 (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 rRNA 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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