Lack of Telomerase Gene Expression in Alternative Lengthening of Telomere Cells Is Associated with Chromatin Remodeling of the \textit{hTR} and \textit{hTERT} Gene Promoters

Stuart P. Atkinson, Stacey F. Hoare, Rosalind M. Glasspool and W. Nicol Keith

Centre for Oncology and Applied Pharmacology, University of Glasgow, Cancer Research UK Beatson Laboratories, Bearsden, Glasgow, United Kingdom

Abstract
The presence of active telomere maintenance mechanisms in immortal cells allows the bypass of senescence by maintaining telomere length. In most immortal cell lines and tumors, telomere maintenance is attributable to telomerase reactivation. However, a number of immortal cell lines and tumors can achieve telomere maintenance in the absence of detectable telomerase activity by the alternative lengthening of telomere (ALT) mechanism. Epigenetic mechanisms have been implicated in the regulation of telomerase expression. We show that specific modifications within the chromatin environment of the \textit{hTR} and \textit{hTERT} promoters correlate with expression of \textit{hTR} and \textit{hTERT} in ALT, normal and telomerase-positive tumor cell lines. Lack of expression of \textit{hTR} and \textit{hTERT} in ALT cell lines is associated with histone H3 and H4 hypoacetylation and methylation of Lys\textsuperscript{9} histone H3. Conversely, \textit{hTR} and \textit{hTERT} expression in telomerase-positive cell lines is associated with hyperacetylation of H3 and H4 and methylation of Lys\textsuperscript{9} H3. Methylation of Lys\textsuperscript{360} H4 was not linked to gene expression but instead was specific to the \textit{hTERT} promoters of ALT cells. This may provide an insight into the differences between ALT and telomerase-positive cells as well as a novel marker for the ALT phenotype. Treatment of normal and ALT cells with 5-azadeoxycytidine in combination with Trichostatin A caused chromatin remodeling of both promoters and reactivation of \textit{hTR} and \textit{hTERT} expression in ALT and normal cell lines. This data establishes a definite link between the chromatin environment of the telomerase gene promoters and transcriptional activity. (Cancer Res 2005; 65(17): 7585-90)

Introduction
In most cancer cell lines and tumors, telomere maintenance is achieved by the reactivation of telomerase (1). However, some cancer cell lines and tumors can maintain their telomeres in the absence of telomerase by the alternative lengthening of telomere (ALT) mechanism (2), but it is unclear why some cells use the ALT pathway whereas others cells reactivate telomerase. ALT lines represent an interesting model for telomerase gene regulation as some express \textit{hTR} but not \textit{hTERT} and others lack expression of both components. \textit{hTR} and \textit{hTERT} gene expression is regulated on many levels during development and tumorigenesis and evidence suggests that epigenetic mechanisms may also be involved. We have previously shown that CpG methylation at the promoter of \textit{hTR} in ALT cell lines is associated with gene repression (3), whereas other groups have linked CpG methylation with transcriptional status of the \textit{hTERT} promoter, although the correlation is not clear (4, 5). The influence of DNA methylation of gene expression suggests that histone modification may also regulate telomerase gene expression and previous studies have provided links between transcription factor binding and chromatin remodeling leading to changes in \textit{hTERT} gene expression (6), although these studies have analyzed a limited number of modifications in the \textit{hTERT} promoter alone. The histone code hypothesis (7) proposes that it is through the combination of several histone modifications that gene expression can be altered. Thus, to investigate how the chromatin environment might influence telomerase gene expression and how this may influence the mechanism of telomere maintenance in different cell types, we studied histone acetylation (histones H3 and H4 and Lys\textsuperscript{9} histone H3) and histone methylation (Lys\textsuperscript{3} and Lys\textsuperscript{9} H3 and Lys\textsuperscript{360} H4) at the \textit{hTR} and \textit{hTERT} promoters in tumor, normal, and ALT cell lines.

Materials and Methods
\textbf{Cell lines.} Cell lines used were C33a (cervical carcinoma); A2780 (ovarian adenocarcinoma); 5637 (bladder carcinoma); WI38 (normal fetal lung fibroblast); and SUSM-1, SKLU, GM847, KMST-6, and WI38-SV40 (all ALT).

\textbf{Chromatin immunoprecipitation assays.} Cells were used when at 70% to 80% confluence and chromatin immunoprecipitation (ChIP) assays were done following the instructions recommended by the kit supplier (Upstate Biotechnology, Dundee, United Kingdom). Sonication was optimized to give chromatin fragments of around 500 bp to 1 kb in length (8 × 10-second pulses at 5 μm with 20-second rest between each pulse on ice using an MSE Soniprep150 sonifier). Resultant DNA from each immunoprecipitate was purified using the QIAquick PCR Purification Kit (Qiagen, West Sussex, United Kingdom). Also included in each experiment was a no antibody control immunoprecipitate to detect any background, which if present was subtracted from each immunoprecipitate within that experiment.

\textbf{Antibodies.} Antibodies used are the following: TriMeK4 H3, TriMeK9 H3, and TriMeK9 H3 (all Abcam, Cambridge, United Kingdom); DiMeK4 H3, DiMeK20 H4, TriMeK20 H4, AChH3, AChH4, and AcrK9 H3 (all Upstate Biotechnology).

\textbf{Quantitative PCR.} Products from the ChIP assay were quantified by quantitative PCR on an Opticon2 DNA Engine (MJ Research, Inc., Waltham, MA) using primers to \textit{hTR} and \textit{hTERT} core promoter sequences and the SYBR Green Q-PCR Buffer (Finnzymes, Espoo, Finland). \textit{hTR} primers, 29SF 5’-CCGCCCGAGAGGGTGAC-3’ and 5ALTR 5’-AAGTCAGCGAGAAAAA-CAGC and \textit{hTERT} primers, TERTSF 5’-TCCCTTACAGTCCGAGATT-3’ and TERTSR 5’-AGCGAGAGAGGTCGAATCG-3’.
Expression analysis. hTR expression was analyzed by quantitative PCR using the following primers: TRC3F, 5'-CTAACCCTAAGTGAGAAGGGCGTA-3' and TRC3R, 5'-GGCGAACGCGCACGCTGACATT-3' and adjusted to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression (primers GAPDH0.45F 5'-ACCACGTCCATGGCCATCAC-3' and GAPDH0.45R 5'-CCACCACCTGTGCTGTA-3'). hTERT expression was analyzed by methods outlined in Keith et al. (8) using primers which detect all splice variants (primers HT2026F 5'-GCTGAGCTGTACTTTGCAA-3' and HT2482R 5'-GCTGAGCTGTACTTTGCAA-3'). hTERT expression is displayed as total amounts of all four main splice variants (wild type, α-deletion, β-deletion, and αβ-deletion).

Trichostatin A and 5-azadeoxycytidine treatment. Cells were treated twice for 24 hours with 5-azadeoxycytidine (5-azadC, Sigma, Dorset, United Kingdom) to a final concentration of 2.5 μmol/L and treated for 16 hours with Trichostatin A (TSA, Upstate Biotechnology) at a final concentration of 350 nmol/L.

Results

Profile of repressive histone lysine methylation states at the hTR promoter. To understand the relationship between expression levels of hTR and chromatin remodeling at the promoter, we first examined hTR gene expression levels from each cell line. In Fig. 1A, we see that hTR is expressed in GM847, 5637, A2780, SKLU, C33a, and WI38, whereas expression levels were negligible in SUSM-1, KMST-6, and WI38-SV40.

To examine the relationship between expression and histone modifications, ChIP assays using antibodies against specific histone modifications were used to generate a profile of the chromatin environment surrounding the hTR promoter in each cell line. Methylation of Lys3 histone H3 (MeK9 H3) facilitates the formation of heterochromatin (9, 10), and elevated levels of...
MeK9 H3 at promoter sequences are associated with repression of gene expression (11, 12). Figure 1B shows that MeK9 H3 is elevated in cells with low hTR expression (WI38, SUSM-1, KMST-6, and WI38-SV40) when compared with cells expressing relatively high levels of hTR (GM847, 5637, A2780, SKLU, and C33a), which were nearly devoid of MeK9 H3, showing that increased levels of MeK9 H3 at the hTR promoter are associated with repression of hTR gene expression.

Methylation of Lys 20 histone H4 (MeK20 H4) is a mark of constitutive and facultative heterochromatin (13), but Fig. 1C shows that there is no clear link between MeK20 H4 and hTR expression. In this study, MeK20 H4 was only elevated in the ALT cell lines (GM847, SKLU, SUSM-1, KMST-6, and WI38-SV40), which may indicate a novel role for this modification.

Profile of transcriptionally permissive histone lysine modifications at the hTR promoter. Acetylated histones H3 and H4 (AcH3 and AcH4) are associated with euchromatin (14), and acetylation of promoter proximal histones is associated with gene expression (15, 16). Figure 1D shows that high levels of AcH3 and AcH4 are linked to elevated levels of hTR gene expression, with GM847, 5637, A2780, SKLU, C33a, and WI38 exhibiting higher levels of AcH3 and AcH4 than SUSM-1, KMST-6, and WI38-SV40. Higher levels of AcK9 H3 (Fig. 1E) are also observed in cell lines with higher expression.

Methylation of Lys 4 histone H3 (MeK4 H3) is also associated with active gene transcription (17) and elevated levels have been observed at active gene promoters (18). Figure 1F indicates that high levels of MeK4 H3 are associated with elevated hTR gene expression.
expression with GM847, 5637, A2780, SKLU, C33a, and WI38 exhibiting higher levels of MeK4 H3 than SUSM-1, KMST-6, and WI38-SV40. This data shows that increased levels of MeK4 H3, AcH3 and H4, and Ack9 H3 at the hTERT promoter sequence correlate with increased levels of hTERT gene expression.

Overall, the data shows that distinct patterns of modifications present at the hTERT promoter sequences are associated with gene expression (histone hyperacetylation, MeK4 H3, and lack of MeK9 H3) and gene repression (histone hypoacetylation, MeK9 H3, and lack of MeK4 H3). Interestingly, MeK20 H4 seems to mark for the ALT phenotype rather than being linked to gene repression.

**Profile of repressive histone lysine methylation at the hTERT promoter.** As for hTR, we first established hTERT gene expression levels from each cell line. As Fig. 2A shows, of the cell lines studied, only the tumor cell lines (C33a, A2780, and 5637) have appreciable levels of hTERT gene expression. Apart from the GM847 cell line, which expresses very low amounts of hTERT, the ALT and normal cell lines showed no hTERT expression. The profile of the chromatin environment surrounding the hTERT promoter was then investigated.

MeK9 H3 is associated with gene repression and consistent with this the levels of MeK9 H3 were lowest in the cell lines expressing high levels of hTERT (C33a, A2780, and 5637) compared with the normal and ALT cell lines (Fig. 2B).

As with the hTR promoter, MeK20 H4 is observed at higher levels at the hTERT promoter in the ALT cell lines in which only GM847 has any hTERT expression. In contrast normal fibroblasts (WI38) have levels of MeK20 H4 that are comparable with those found in hTERT-expressing tumor lines suggesting that either different mechanisms of repression exist in ALT and normal cells or that MeK20 H4 has a role other than in gene repression (Fig. 2C).

**Profile of transcriptionally permissive histone lysine modifications at the hTERT promoter.** Acetylation of promoter histones H3 and H4 are generally considered to allow the gene to be permissive for transcription. Figure 2D and E show that the two tumor cell lines expressing the highest levels of hTERT (C33a and A2780) have the highest levels of AcH3, AcH4, and Ack9 H3. However, 5637, which express a relatively high level of hTERT, has acetylation levels comparable with that of the nonexpressing cell lines. The ALT and normal cell lines that do not express hTERT (except for very low amounts in GM847) show low acetylation of AcH3, AcH4, and Ack9 H3.

Although MeK4 H3 is associated with active gene expression all the cell lines exhibited some degree of MeK4 H3 whether they express hTERT or not. However, apart from the KMST-6 cell line, MeK4 H3 is higher in the hTERT-expressing tumor cell lines (C33a, A2780, and 5637) than in the hTERT-nonexpressing normal and ALT cell lines (Fig. 2F).

Thus, as with hTR, the data shows that distinct patterns of modifications present at the hTERT promoter sequences are associated with gene expression (histone hyperacetylation, MeK4 H3, and lack of MeK9 H3) and gene repression (histone hypoacetylation and MeK9 H3). Interestingly, MeK20 H4 again seems to mark for the ALT phenotype rather than being linked to gene repression.

**Reactivation of telomerase gene expression by chromatin remodeling.** As active hTR and hTERT promoters show similar patterns of histone modifications (high levels of AcH3, AcH4, Ack9 H3, and MeK4 H3), we asked whether expression of repressed telomerase genes in telomerase-negative cells could be induced by promoting such modifications. TSA, a histone deacetylase inhibitor and 5-aza-dC, a DNA methylation inhibitor were used in combination to induce chromatin remodeling.

Treatment of KMST-6, WI38, and WI38-SV40 with TSA and 5-aza-dC in combination resulted in increases in hTERT gene expression (Fig. 3A) associated with increases in acetylation (Fig. 3B and C), apart from AcH3 level in KMST-6 which does not change upon treatment. Increases in MeK4 H3 were also associated with increases in expression in KMST-6 and WI38, although they decreased in WI38-SV40 (Fig. 3D).

hTERT expression was reactivated in KMST-6, WI38, and WI38-SV40 following treatment with TSA and 5-aza-dC in combination.

![Figure 3](image-url)
repression and activation of both hTR and hTERT in normal, ALT, and cancer cell lines, and forced chromatin remodeling of the hTR and hTERT promoter sequences can induce the reexpression of hTR and hTERT. Our results are consistent with a recent study by Kumakaru et al. (19) on hTERT; furthermore, the proposal that the chromatin-mediated regulation of expression is of importance for both hTR and hTERT. We have also shown that the normal cell line W38 has a promoter chromatin profile which shows neither euchromatic nor heterochromatic hallmarks compared with the normal and ALT cell lines. It may be in this cell line that the lack of permissive modifications may be enough to repress hTERT transcription without needing the added effect of the repressive modifications. This may also be the hallmark of a short-term repression of hTERT expression, whereas in the ALT high levels of repressive methylation marks are present perhaps pointing to a more long-term repressive effect. This study has also uncovered a novel association of methylated Lys20 histone H4 with the ALT phenotype and may represent a new marker for the ALT phenotype alongside telomere length analysis and ALT-associated PML body presence. The role of Lys20 histone H4 methylation is not clear. It is associated with heterochromatin but as in this study is not necessarily associated with regulation of gene expression and it has recently been implicated in DNA damage response pathways (20). Further investigation of its association with the ALT phenotype may help to shed new light on the role of this modification.

This study also suggests that modulation of the chromatin environment of the telomerase gene promoters could influence which telomere maintenance mechanism is used. Telomerase reactivation may occur by chromatin remodeling allowing a more permissive state of transcription to occur at the hTR and hTERT promoters. Conversely, the ALT phenotype may arise due to the tight repression of the hTR and/or hTERT promoter, caused by chromatin remodeling of the promoter sequences. Chromatin remodeling events that cause increased levels of repressive modifications to concentrate at promoter sequences may “force” a cell to use the ALT telomere maintenance mechanism rather than that of telomerase activity, due to the inability of the cell to reexpress either hTR or hTERT. Alternatively, the promoter may accumulate heterochromatic changes because there is no need for telomerase in these cells once ALT is activated.

Further studies identifying the proteins involved in modulating the chromatin environment of the hTR and hTERT promoters may allow identification of new regulatory pathways, provide therapeutic drug targets, and allow further understanding of the processes controlling telomerase gene expression in normal development and tumorigenesis. Specific targeting by chromatin remodeling enzymes to the hTR and hTERT promoters to allow a level of transcriptional control is also an exciting prospect.

Overall our results suggest that the modulation of the chromatin environment has an important role in regulating telomerase gene expression and may be a significant factor in the acquisition of the ALT phenotype by some tumor cells.

Discussion

This study emphasizes the importance of the chromatin environment in the regulation of telomerase gene expression. Common chromatin patterns at promoter sequences are associated with

Figure 4. A, hTERT expression analysis before and after treatment with TSA and 5-azadC. ChIP analysis of the hTERT promoter was completed for (B) AcH3 and AcH4, (C) AcK9 H3, and (D) Di-MeK4 and Tri-MeK4 H3. Each immunoprecipitate was quantified in triplicate by quantitative PCR analysis and related back to an input sample in each experiment to normalize the data.

This data shows that for hTR and hTERT, an increase in the levels of histone modification linked to gene expression at the promoter can cause reexpression or increases in expression.

Acknowledgments

Received 5/18/2005; revised 6/13/2005; accepted 6/22/2005.

Grant support: Cancer Research UK, European Community grant LSHC-CT-2004-502943 and Glasgow University.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
References

Lack of Telomerase Gene Expression in Alternative Lengthening of Telomere Cells Is Associated with Chromatin Remodeling of the hTR and hTERT Gene Promoters


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/65/17/7585

Cited articles
This article cites 19 articles, 7 of which you can access for free at:
http://cancerres.aacrjournals.org/content/65/17/7585.full#ref-list-1

Citing articles
This article has been cited by 12 HighWire-hosted articles. Access the articles at:
http://cancerres.aacrjournals.org/content/65/17/7585.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, use this link http://cancerres.aacrjournals.org/content/65/17/7585.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.