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*Original Article*

# Functional characterization of mutations in the promoter proximal region of the telomerase hTERC gene identified in patients with hematological disorders

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**Abstract:** Telomerase RNA gene (hTERC) mutations have been identified in a subset of patients with bone-marrow failure syndromes (BMFS). While most of the mutations were found in the coding region of hTERC, some rare disease-associated mutations as well as polymorphic sequence changes were found in the promoter proximal region of the gene, including the -99C/G sequence change that was thought to modulate hTERC gene expression by disrupting Sp1 transcriptional factor binding [1]. We and other researchers recently identified, in addition to the -99C/G mutation, several other sequence variations (-240delCT, -714+C insertion, and -771A/G) in the hTERC promoter in other cohorts of patients with blood disorders. Using a convenient telomerase reconstitution assay coupled with the hTERC-promoter driven luciferase reporter assay, we characterized each of the hTERC's promoter sequence variants and found that these rare sequence changes did not negatively affect telomerase gene expression or function. We therefore conclude that all known mutations in the promoter proximal region of the hTERC gene to date do not necessarily contribute to the pathogenesis of hematological disorders by directly affecting telomerase transcriptional activity and/or its enzymatic function.

**Keywords:** Telomeres, telomerase, bone-marrow failure syndromes, gene promoter analysis

## Introduction

Human telomeres at chromosome ends typically consist of more than 1,000 base pairs (bps) of simple repetitive DNA sequences and associated proteins [2]. In somatic cells, these DNA repeats are gradually lost with cellular replication and aging, owing to the inability of DNA polymerase to fully replicate the 3' end of the chromosomes. Telomere attrition eventually leads to critically short telomeres, which can induce cellular senescence and/or apoptosis [2]. In the germ line and stem cell compartments, however, telomere lengths are maintained by a specialized enzyme called telomerase. Telomerase is a ribonucleoprotein (RNP) complex with two main components: an RNA-dependent DNA polymerase (hTERT) and an integral hTERC RNA, which provides a template to synthesize DNA repeats onto chromosome ends [2]. It has been shown that telomeres are abnormally shortened in a subset of patients with bone-marrow failure syndromes (BMFS) as a result of pathogenic mutations in telomerase gene components that lead to an impairment in the proliferative capacity of hematopoietic stem cells [3-5]. Furthermore, an association has been established between the degree of telomere shortening and that of disease severity and the age of onset [3, 5].

Our laboratory and those of other researchers have recently identified numerous sequence changes, including disease-associated mutations and inconsequential polymorphic sequence variations, in both the hTERT and hTERC genes and shown that most of the patient mutations negatively affected telomerase enzymatic function in vitro in a haploinsufficiency manner (reviewed in [6]). While most of the mutations were found in the coding region of the genes, rare sequence changes were also noted in the regulatory regions, including but not limited to the promoter proximal region of the hTERC gene (**Figure 1**) [1, 4, 7-10]. These include the C to G change at position -99 in the promoter (-99C/G) identified in an 18 yr old man with paroxysmal nocturnal hemoglobinuria (PNH) [1] and in a 1

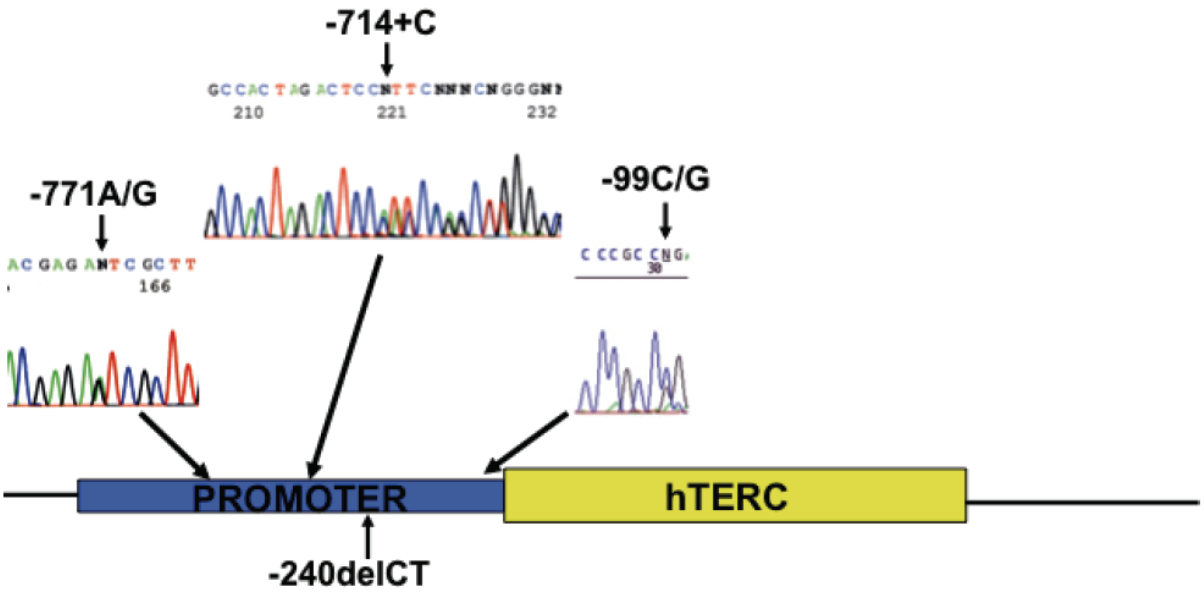


**Figure 1.** Schematic depiction of the hTERC gene with naturally occurring sequence variations that occur in its promoter sequence. Known sequence changes in the hTERC promoter proximal region include -99C/G, -240delCT, -714+C, and -771A/G. Electrophereograms showing the heterozygous nature of some of these sequence variations are also shown.

yr old child with juvenile myelomonocytic leukemia [10], a 2 base pair deletion at position -240 (-240delCT) in a 4 yr old child with myelodysplastic syndrome (MDS) [10], a cytosine insertion at position -714 (-714+C) and A to G change at position -771 (-771A/G) [7] of the hTERC promoter. Both the -714+C and -771A/G sequence changes were found at about the same frequency in MDS patients and healthy controls, and therefore were considered polymorphic sequence variations (SNPs) of the gene [7]. To the best of our knowledge, only one study so far has attempted to characterize one such hTERC promoter-associated mutation (-99C/G) and has implicated its role in the pathogenesis of PNH by modulating hTERC expression and telomerase enzymatic function [1]. Here, we have re-examined the -99C/G mutation as well as characterized for the first time all other known sequence variations to date and showed that none of the sequence variants negatively affects telomerase gene expression and function.

## Materials and methods

*Generation of point mutations*

The pLXSP-hTR plasmid (a kind gift of Dr. D. Broccoli) contains 2,442 bps of the hTERC sequence cloned into the pLXSP plasmid at the Sal I site. The hTERC promoter proximal sequence (1,457 bps) was subcloned via PCR amplification from the pLXSP-hTR plasmid into the pGL3-Basic vector at the Sac II and Blg II sites. The authenticity of the cloned hTERC promoter sequence was sequence verified. hTERC promoter mutants were created in both the pLXSP-hTR plasmid that carries 1,457 bps hTERC promoter proximal sequence (-1 to -1457) upstream of the hTERC coding region, and the pGL3-hTR plasmid that carries the same 1,457 bps of hTERC promoter proximal sequence upstream of the firefly luciferase gene, using the QuickChange site-directed mutagenesis kit (Strategene, CA, USA). All mutations were confirmed by direct DNA sequencing, and their quantity and quality routinely checked by spectrophotometric analysis and agarose gel electrophoresis.

*In vivo reconstitution of telomerase activity*

Wild-type or mutant pLXSN-hTR plasmids (2 μg) were transfected into VA13+hTERT cells (at approximately 70% confluency) in 6-well polystyrene dishes using SuperFect transfection reagent (Qiagen, Hilden, Germany) according to

the manufacturer's instructions. To monitor transfection efficiency, a plasmid (peGFP-N1) (Strategene, CA, USA) expressing green fluorescent protein was transfected in a parallel transfection reaction. The eGFP protein expression level was examined under fluorescence microscope. Approximately 48 hours after transfection, cells were scraped from the dish in the presence of 1 mL cold phosphate-buffered saline. Cellular extracts were then prepared in 1X CHAPS lysis buffer as suggested by the manufacturer (Chemicon International, CA, USA). Telomerase activity of the cellular extract from $2x10^4$ cells was assayed using the TRAPeze Telomerase Detection Kit following the manufacturer's directions (Chemicon International, CA, USA), except that PCR was performed as follows: 95˚C for 2 min; 25 cycles of 94˚C for 10 s, 50˚C for 30 s, 72˚C for 30 s; and 72˚C for 5 min. Products were analyzed on a 12% native polyacrylamide gel and examined by phosphor imaging (Molecular Dynamics, GE Healthcare Bio-Sciences Corp., NJ, USA).

*Luciferase activity of hTERC promoter variants*

Human kidney 293T epithelial cells were seeded at a density of $4\times10^5$ cells per well in 12-well plates 24 hours prior to transfection. The cells were transfected with 700 ng of the pGL3-hTR plasmid(s) and 100 ng of the pRL-CMV plasmid as an internal control of transfection efficiency, using Lipofectamine2000 transfection reagent (Invitrogen) according to the manufacturer's instructions. Cells were harvested 48 hours after transfection and lysed in 200μl reporter lysis buffer (Promega). Luciferase activity was measured using the Luciferase Assay System (Promega). Luciferase activity (arbitrary units) was divided by the internal control in the same sample to normalize for transfection efficiency and expressed as relative luciferase activity. Transfection data represents at least three independent experiments, each performed in triplicate.

## Results and discussion

We and other researchers have recently identified several sequence variations in BMFS patients or healthy controls in the proximal promoter region of the hTERC gene (**Figure 1**). These include the -99C/G mutations identified in a PNH patient [1] and a young child with juvenile myelomonocytic leukemia [10], the -240delCT mutation in another child with MDS [10], and the -714+C and -771A/G sequence changes that were found at about the same frequency in MDS patients and healthy controls [7]. Since some of these sequence changes were identified in patients with shortened telomeres, we tested the hypothesis that some of them would have an adverse effect on hTERC transcription. To do this, plasmids carrying 1,457 bps of promoter sequence upstream of the hTERC gene were transfected into VA13+hTERT cells, a human lung-derived cell line that lacks endogenous hTERC expression but has been engineered to express stably the hTERT protein. These cells cannot ordinarily produce functional telomerase, but can assemble the active enzymatic complex when transfected transiently with a vector that expresses a functional hTERC copy. When cell lysates containing either the wild type or mutated hTERC sequence were collected to perform the telomere repeats amplification (TRAP) assay, which is used to assess the ability of the reconstituted telomerase complex to add telomeric DNA repeats onto a synthetic DNA primer in vitro, we did not observe any significant defects in telomerase enzymatic activity (**Figure 2**). We therefore reason that these promoter-associated sequence variations did not affect hTERC gene expression. In order to validate this, we created plasmid constructs carrying the same 1,457 bps of hTERC promoter sequence upstream of a firefly luciferase reporter gene and used them to assess the possible effects of the hTERC-promoter sequence changes in transcribing the luciferase reporter gene. As shown in **Figure 3**, none of the sequence changes caused any significant effects in luciferase reporter activity.

It is noteworthy that the -99C/G mutation has previously been shown to affect the consensus sequence of one of four Sp1 binding sites in the core *hTERC* promoter [1]. In that study, gel shift (EMSA) assay was used to show that the -99C/G mutation could disrupt the binding of the Sp1 transcriptional factor to this site (named Sp1.2). In the minimal hTERC promoter context of 100 bps, the authors also showed that the -99C/G mutation produced an increase in luciferase reporter activity, suggesting to them that this site could act as a repressor of hTERC transcription [1]. Based on these findings, the authors of this study speculated that mutation of Sp1.2 sequence of the hTERC promoter proximal re-

gion altered telomerase gene expression and function (though they did not directly test this) and might contribute to the pathogenesis of PNH [1]. The authors did acknowledge however that the effect that they observed with the –99C/G mutation might be dependent on the relatively short hTERC promoter sequence used in their study [1]. It is unfortunate that the biological activities of the -99C/G mutation or of other known hTERC promoter-associated variants could not be assessed directly in cells collected in patients due to the fact that these were retrospective studies and no other biological samples besides genomic DNAs were available for further analysis. Regardless, our in vitro telomerase reconstitution assays show that none of the hTERC promoter mutations known to date play a role in modulating either telomerase transcription or its enzymatic function.

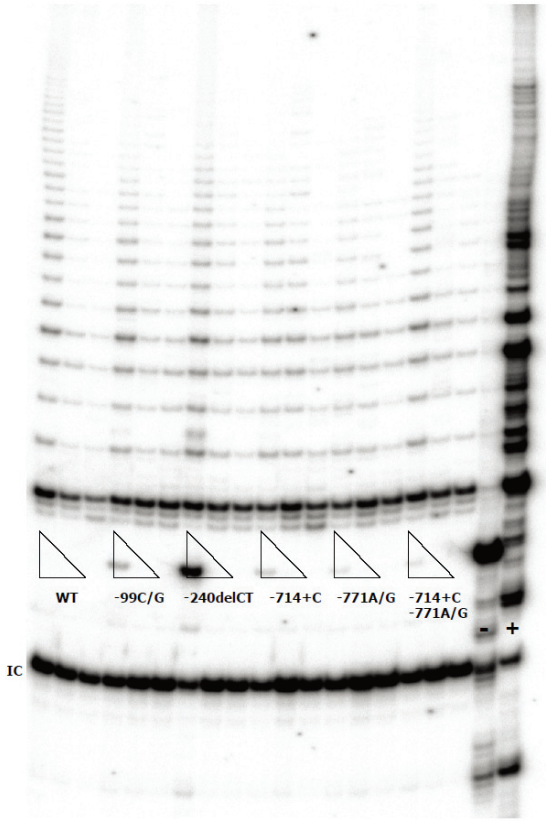


**Figure 2.** Naturally occurring hTERC promoter variants show no significant decrease in telomerase enzymatic activity. Telomerase enzymatic activities are determined in VA13+hTERT cells for the naturally occurring hTERC promoter mutations. A representative TRAP gel showing the relative telomerase enzymatic activities obtained from the promoter sequence changes is shown. Serial ten-fold dilutions of the transfected cell lysates (indicated by triangles) were assayed for each sample to ensure linearity of the assay. A negative control (-) is composed of an inactivated telomerase by denaturing cell lysate containing a wild-type telomerase at 95°C for 5 minutes prior to assaying. Positive control PCR products (+) amplified from a control TSR8 DNA template supplied in the TRAP kit. -771A/G + -714+C contains a combination of the two mutations in the hTERC promoter sequence. "IC" indicates PCR products amplified from an unrelated DNA template, which is included in each reaction as an internal control for PCR amplification efficiency.

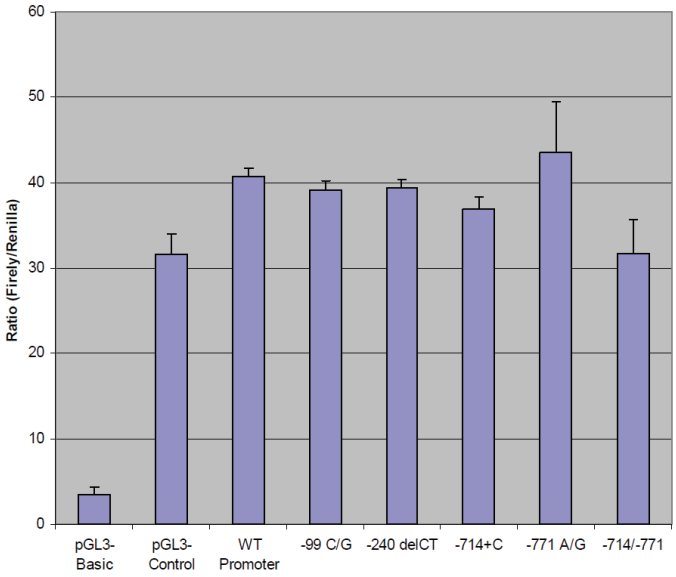


**Figure 3.** Naturally occurring hTERC promoter variants show no significant decrease in hTERC transcriptional activity. For each transfection of plasmids (pGL3-WT or different promoter-associated mutations) containing the hTERC-promoter driven firefly luciferase reporter gene, the luciferase activity was normalized to the Renilla luciferase activity expressed from the co-transfected pRL-CMV expression vector. The means from three independent experiments are shown for each construct; *bars*, SD. pGL3-Basic is a promoter-less construct and used as a negative control. The positive control pGL3-Control construct consists of the firefly luciferase gene driven by the SV40 viral promoter.

However, we cannot formally rule out the possibility that these sequence changes may play some role (s) in a cellular context. Thus, it remains to be learned why these patients carry abnormally shortened telomeres when these sequence changes do not appear to decrease telomerase promoter activity or its enzymatic function.

Recently, the spectrum of diseases presenting with shortened telomeres and carrying mutations in the key telomerase components, hTERT and hTERC, has expanded to include those not only affecting the hematopoietic system, but other highly-proliferative tissues, such as lungs and liver. These telomere-associated diseases include, but are not limited to, idiopathic pulmonary fibrosis (IPF), Cri du Chat syndrome, liver and heart fibroses and liver cirrhosis [11-14]. As more telomerase mutations are identified in a wide spectrum of human diseases, it is important to clarify which sequence changes might be disease-associated and which are not. Our data reported in this study suggest that all known mutations in the promoter proximal region of the telomerase hTERC gene thus far do not necessarily participate in disease pathogenesis by directly modulating telomerase gene expression or function. More research, therefore, is needed in order to fully understand the mechanisms by which certain telomerase mutations may lead to the onset of these diseases. This knowledge may someday lead to novel therapies to better treat or prevent these debilitating and deadly disorders.

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