Author's response to reviews

Title: A mutation in H/ACA box of telomerase RNA component gene (TERC) in a young patient with myelodysplastic syndrome

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Author's response to reviews: see over
Dear Dr. Sands; 

Re: MS: 5004894711128530  
A mutation in the H/ACA box of telomerase RNA component gene (TERC) in a young patient with myelodysplastic syndrome

Telomeres are repeated sequences located at chromosome ends of eukaryotes, protecting DNA from end joining and degradation. Telomeres become shorter in each cell cycle, but telomerase, a ribonucleoprotein complex, alleviates this attrition. The telomere RNA component (TERC) is an essential element of telomerase, serving as a template for telomere elongation. Mutations in the TERC gene cause accelerated telomere attrition, leading to diseases including bone marrow failure syndromes.

Through revising our manuscript, we found that the A377G mutation of TERC was reported in an aplastic anemia (AA) patient (Vulliamy et al, PLoS One, 2011;6(9):e24383).

Although the finding of the A377G mutation in the AA patient was shown, no analysis (even telomere length measurement) was performed in the report, making it uncertain whether the mutation was responsible for causing the disease.

Our work clearly shows the close association of the A377G mutation and the disease using functional assays and a familial analysis. Moreover, analyses of the pedigree demonstrated interesting observations including recovery of telomere length in a descendant carrying the mutation. Surprisingly, the telomere length of the patient’s sperm was much longer than that of the patient’s white blood cells. Disease anticipation has been reported with TERC mutations, and one affected individual had azospermia (Vulliamy et al, Nat Genet. 2004 May;36(5):447-9., Goldman et al, Proc Natl Acad Sci U S A. 2005 Nov 22;102(47):17119-24.). Our case suggests that severity of telomere shortening during spermatogenesis might be different depending on mutation sites of TERC. For this extended analyses, we added two new authors as indicated in the manuscript.

Although the title of the manuscript has been changed, we believe that our findings are of great importance in the field of telomere biology.

All the authors contributed to the work described in the paper and all take responsibility for it. Moreover, none of the work described in the paper has been published elsewhere.

We have addressed all the comments by the two reviewers as indicated on the attached pages.

We hope our explanations and revisions satisfactory and that the revised version of our paper is now suitable for publication in BMC Medical Genetics.
We thank you for considering our paper for publication in BMC Medical Genetics, and look forward to hearing from you at your earliest convenience.

Yours sincerely,

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We are grateful to Dr. Wong for the critical comments and useful suggestions that have helped us to improve our paper. As indicated in the responses that follow, we have taken all these comments and suggestions into account in the revised version of our paper.

**Reviewer 1**
**Major Compulsory Revision**

1) Given the importance of the H-box in binding with H/ACA proteins, and the steady-state accumulation of TERC, it is surprising that the patient’s father, who inherited the same TERC-377G mutation, did not exhibit any notable clinical symptoms. The authors should comment on this, and its implications for the patient’s siblings.

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The authors should comment on this:
Previous studies have demonstrated that TERC mutations are inherited, but penetrance can vary, even within pedigrees although the mechanism is still not understood: two references were sited below as examples.

1. In our previous work, we identified C116T and C204G TERC mutations in two families, respectively. Affected family members showed short-for-age telomere lengths, but not all of them exhibited clinical manifestations [1].
2. Vulliamy T et al. investigated the telomere lengths and disease status of individuals from eight families with autosomal dominant inheritance of dyskeratosis congenital [2]. Affected individuals in these eight families displayed heterozygous TERC mutations although individual families had mutations in different sites of the TERC gene. Some members carrying TERC mutations from five families remained asymptomatic well into adulthood (even at age 60 or 61).

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Its implications for the patient’s siblings:
As described in “Medical history” in the Results section, two younger siblings were healthy. The patient’s son had normal telomere length, but he was one year old at that time. Close follow-up would be necessary. Interestingly, telomeres were much longer in the patient’s sperm compared to his leukocytes. Disease anticipation has been reported with TERC mutations, and one affected individual had azospermia [2, 3]. Our case suggests that severity of telomere shortening during spermatogenesis might be different depending on mutation sites of TERC.

We added these points in the Discussion section.
2) Figure 1 depicts the secondary structure of TERC, and the H-box location. The authors’ rendition (using sticks and balls) is not entirely accurate. This can easily be corrected using available RNA-drawing software. While TERC structure can be obtained from the literature, it would be helpful if an up-to-date accurate version be included in the manuscript.

We revised Figure 1, according to the reviewer’s advices and suggestions.

3) a) In the methods section, the authors describe generating T7-promoter-driven WT and mutant TERC, subcloned into the pUC57 plasmids. T7 is a bacteriophage-derived promoter, and pUC57 lacks any mammalian promoter sequence. The method, as it is written, is not predicted to mediate recombinant TERC expression in VA13 cells. To clarify, the authors should state whether the TERC gene (with its endogenous promoter) was cloned, or if VA13 cells were cotransfected with a T7 polymerase expression vector.

In this study, pUC57-wtTERC and pUC57-mutTERC plasmids were constructed by applying the TERC-gene cassette design (a HindIII site and the T7 promoter sequence at a 5′ end; an engineered FspI site and a BamHI site at a 3′ end) described by Autexier et al.[4]. The authors confirmed using the in-vitro transcription system that the RNA sequence made from their wtTERC plasmid contained only the TERC sequence.

These wtTERC and mutTERC gene cassettes with the T7 promoter, but without the endogenous TERC promoter, were synthesized and subcloned into a pUC57 vector (pUC119-derived plasmid vector pUC57 and used as a subcloning vector by the company) by GenScript (Piscataway, NJ, USA), instead of pUC119 used in the previous work.

We did not cotransfect each of these plasmids with a T7 polymerase expression vector. However, it is evident from previous studies and our current data (please see below) that the plasmid constructs expressed wtTERC and mutTERC.

1. It has been demonstrated that in the absence of T7 RNA polymerase, the human growth hormone or luciferase was expressed using two different vector (pUC18 and pGEM) systems under control of the T7 promoter by subcloning each encoding gene downstream of the T7 promoter [5]. The authors compared expression levels from pUC18-T7, pGEM-T7, and pRSV (carrying the viral LTR/enhancer promoter) plasmids using the two reporter genes (the growth hormone and luciferase genes). The ratios of expression between the three plasmid types are essentially the same for both genes tested. The expression level obtained with the pGEM-T7 or pUC18-T7 was 90% or 30% of that detected with pRSV, respectively. By further experiments, it was revealed that the mammalian RNA polymerase II was responsible for transcription for
the T7 promoter. Therefore, the authors have concluded that the T7 promoter can function as a polymerase II promoter in mammalian cells.

2. In our current work, telomerase activity was indeed detected by cotransfection of pUC57-wtTERC or pUC57-mutTERC with pcDNA3-Flag-TERT into VA-13 cells (telomerase-deficient cells), in which the pUC57 vector was used as a negative control. The results clearly showed that wtTERC or mutTERC was expressed from pUC57-wtTERC or pUC57-mutTERC plasmid, respectively.

3. Although data were not shown in our submitted manuscript, we performed the following experiments.

- Quantitative PCR assay was carried out with adequate primers and probes in order to examine wtTERC or mutTERC expression from pUC57-wtTERC or pUC57-mutTERC plasmid, respectively, confirming the expression of these RNAs.
- Further, RNA FISH was performed using proper probes and antibodies to address the expression and localization of wtTERC or mutTERC in subcellular compartments, resulting in the detection of wtTERC or mutTERC in the nuclei.

*RNA FISH results were added to this revised manuscript as Figure 5, following the comment of Reviewer 2.

b) Cells extracts were assayed for telomerase activity two days after the transient transfection of two recombinant telomerase subunits (TERT; WT versus mutant TERC) in VA13 cells. Relative telomerase activities, measured in WT-TERC-transfected cells versus mutant TERC-transfected cells, were used as a functional indicator of the mutation’s effect on telomerase activity. Transient transfection efficiency could be highly variable between different treatments. How would the authors control for this discrepancy?

1. As the reviewer pointed out, transient transfection efficiency could be highly variable. It is ideal if we could use the TERT-expressing VA13 cells, instead of cotransfection of TERT and TERC plasmids into the cells, but we could not obtain the cells. In published studies [3, 6], telomerase activity was measured by cotransfection of TERT and TERC plasmids using the TRAP assay. Therefore, we employed cotransfection experiments in this work.

2. We repeated transient transfection experiments twice in a duplicated manner (biological and technical replicates) using the VA13 cells. Indeed, we obtained variable fluorescence values generated with the TRAPEZE® XL kit products between two experiments when measured by a fluorescence plate reader. However, in the two experiments, telomerase activity in the VA 13 cells cotransfected with mutTERC and Flag-TERT plasmids was lower (50-70%) than that in the cells cotransfected with wtTERC and Flag-TERT plasmids, in which the empty pUC57 vector was used as a negative control. Thus, as mutTERC showed decreased
telomerase activity compared with wtTERC in the two experiments, we concluded that the A377G TERC mutation was responsible for telomere shortening.

c) How sure are the authors that the mutant TERC affects normal telomerase activity by 50%? The over-expression of the mutant TERC through transient transfection in VA13 cells may alleviate some of the pre-RNP assembly defects, leading to measurement of higher telomerase activity, compared to constitutively low TERC expression levels. The authors should include this caveat in their comments.

According to the previous report [5] cited elsewhere, the pUC plasmid carrying the T7 promoter displayed lesser expression of the two reporter genes. This was the one reason why we used the pUC57 with the T7 promoter for this study as we did not want too much expression of TERC although we did not actually compare the expression level of TERC using other vectors in this work.

We corrected the manuscript accordingly as we agree that we could not accurately quantify the reduction rate of telomerase activity of mutTERC in our assay.

**Minor Essential Revisions:**

1) page 3, “recruit TERC into a unique structure called cajal body in the nucleolus”. This statement is not accurate. Cajal bodies are unique nuclear bodies that are structurally distinct from the nucleolus, which is an organelle.

   The statement was corrected accordingly.

2) page 7, TERC accumulation and RNA stability refer to the same observation in this context; the use of both terms in the same sentence is redundant.

   The sentence was revised accordingly.
We are grateful to Dr. Beattie for the critical comments and useful suggestions that have helped us improve our paper. As indicated in the responses, we have taken all these comments and suggestions into account in the revised version of our manuscript.

**Reviewer 2**

**Compulsory Revisions:**

_The work that the authors have done is very nice and is the beginning of a very interesting study, however there are a number of additional experiments that would greatly enhance this study. Since the authors have access to patient samples it would be nice to see telomerase activity from the actual blood samples that the telomere length analysis was done._

_In addition, since this region of the molecule is known to affect the stability and accumulation of hTR and well as interaction with the protein dyskerin, it would be nice to include these studies as well (ideally in the patient samples)._  
_Since it is also postulated that this region is not necessary for telomerase activity per se and that mutations in the H/ACA box still retain the ability to bind to hTERT, inclusion of these experiments would also be interesting._

We thank the reviewer for the positive assessment of our work and its implications.

1) Unfortunately, fresh peripheral blood sample of the patient is not available, as the patient died of Epstein-Barr virus-associated B-cell lymphoma in 2012.

2) We performed RNA FISH experiments in which pUC57-wtTERC or pUC57-mutTERC was cotransfected with pcDNA3-Flag-TERT into VA13 cells, and cells were subjected to RNA FISH 48 hours post transfection. RNA FISH data (Figure 5) and the discussion were added to the revised manuscript.

* mutTERC was accumulated in Cajal bodies in a similar manner to wtTERC. However, we were unable to clearly observe difference of accumulation levels between wtTERC and mutTERC in our RNA FISH system which was not quantitative. Therefore, we cannot exclude the possibility that A377G mutation caused TERC instability, whereby the decreased telomerase activity was observed in the TRAP assay.

* Although dyskerin appeared to colocalize with mutTERC in our RNA FISH assay, it is still unclear whether dyskerin was actually interacted with mutTERC and whether the assembly of mutTERC and H/ACA proteins was functionally intact.

* We tried to examine whether mutTERC still retained the ability to bind to hTERT. After staining with anti-TERT antibody, hTERT was present diffusely throughout the nucleus. Therefore, we could not draw a conclusion from our data.
• Acumulating evidence has suggested that TERC localization pattern is dependent on ectopically expressed or endogenous TERC, cell types, cell cycles, time course, experimental methods, and so on. Therefore, to clarify the mechanism of impaired telomerase activity due to A377G TERC mutation, different lines of experiments will be required.

Minor Revisions:

On minor comment in the abstract, I don’t believe the authors can conclude from these studies that the mutation in TERC was responsible for the bone marrow failure….It correlates with and is consistent with, but the data does not address cause of the disease.

Etiology of MDS is still unknown, but genetic instability is hypothesized to be a major cause of the disease. Telomere shortening was reported to contribute to chromosomal instability, causing leukemia [7]. Our patient showed a number of clones with critically short telomere length by STELA. Besides, primary MDS is quite rare in young (<40) population: less than 3% in Sweden [8] and 0.8% in the United States [9]. Based on these observations, it is strongly suggested that TERC mutation contributed to the pathogenesis of MDS in our patient. However, as the reviewer pointed out, it is difficult to determine what caused MDS in our patient and we don’t think the TERC mutation solely caused the disease. Thus, we have revised the abstract accordingly.


