Author's response to reviews

Title: A novel c.-22T>C mutation in GALK1 promoter is associated with elevated galactokinase phenotype

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Author's response to reviews: see over
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Dear Senior Editor, Scott Edmunds:

We deeply appreciate your review and comments. Our manuscript (1549084170227142) entitled “A novel c.-22T>C mutation in GALK1 promoter is associated with elevated galactokinase phenotype” was revised to incorporate the comments and suggestions raised by reviewers.

I hope the revised manuscript will better meet the requirements of the ‘BMC MG’ for publication.

Thank you for your judicious editorial effort and time.

Sincerely yours,

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<Response to the reviews on the submitted manuscript>

The following is a detailed listing of specific responses to points raised by reviewers. On the revised manuscript, we have marked changes in blue made in response to the comments.

Reviewer 1

Major Compulsory Revisions:
1. This paper would be considerably strengthened if the identity of the transcription factor regulating the expression of the gene was known. In addition, the data relating to the mobility-shift competition is not at all convincing. As it stands, the paper is limited by these facts. The assertion that the c-22C DNA-protein complex was completed more efficiently with cold c-22C rather than c-22T (Fig. 4) is dubious. This effect, if it exists, is modest and would need to be quantified before any conclusions could be drawn. The authors may also wish to try and identify, for example using specific (and commercially available) antibodies to, say, Sp1 or Egr1, the nature of the protein DNA complex.

We agree with the reviewer’s point that the manuscript would be strengthened if we were able to identify the transcription factor. We performed supershift assay using HEN1, Sp1 and Egr1 antibodies and could not detect supershifted bands. We have included the picture of supershift assay with HEN1 antibody in Fig 4A which was previously indicated as ‘data not shown’. We consider that determining the identity of the transcription factor would be beyond the scope of this manuscript. As the reviewer suggested, we have quantified the intensities of the shifted bands in the competition assay and indicated in the Fig 4B.

Minor essential revisions:
1. I am not sure why, in Figure 3, the data labelled ‘AAT’ in both panels have no error bars. I understand that the data has been normalised relative to the activity of the wild-type construct, but there still should be an error associated with this calculation.

For each experiment, the value of luciferase for ‘AAT ‘(changed to wt in the
revised manuscript) was set to 100 and the values for other constructs were normalized accordingly. In this analysis, therefore, we do not have error bars on the data labeled ‘AAT’.

2. The lanes in Figure 4 are referred to in the text as being numbered, but no numbers are on the figure itself.
*We have included the lane numbers in Fig 4.*

Reviewer 2

**Major Compulsory Revisions**

1. It would be better to refer to “GALK” as “GALK1” to be consistent with the gene name and to distinguish the protein from GALK2 (N-acetylgalactosamine kinase)
*We have changed “GALK” to “GALK1”.*

*We meant GALK1 activity and have corrected it.*

3. P4: Line 1: Galactose is converted to glucose 1-phosphate by those enzymes
NOT glucose.
*We have corrected it.*

4. P4: Galactosemia is not screened for “throughout the world”. I think it would be very surprising if the majority of newborns worldwide are screened.
*We have modified the sentence into the following: “Newborn screening tests for galactosemia have been performed in Korea as well as in many developed countries.”.*

5. P5: “...the absence of TATA box and CCAAT box”; this requires a citation to published literature.
*We have included the reference.*

6. P12/P13: Give units for GALK activities
*We have included the units*
7. Discussion: This is somewhat repetitive of results and the length could be cut; the overall conclusions seem essentially sound
We have rewritten the first paragraph of Discussion to remove repetition of results.

8. Figure 1B: It is hard to tell the difference between the two experimental lanes, partly due to smearing. This gel should be repeated or the differences highlighted on the figure. Some kind of explanation of the expected changes should be given in the legend.
We repeated experiments and changed the Figure 1. We have explained the expected changes in the Figure legends.

9. Figure 3: It would be useful to see any significant differences marked on the figure.
We performed the paired t-tests to see the significant differences in luciferase activities between wild type and transfected cell lines. We marked and described the statistical data in Figure 3.

10. Figure 4: The gel shift is very clear and unambiguous. However, the competition experiment is less clear cut. The difference between 22C and 22T does not seem to be that great. How many times was this repeated? Was any attempt made to quantify the bands and compare (perhaps statistically)?
The competition assay was performed at least three times and we obtained similar results. As the reviewer suggested, we have quantified the intensities of the shifted bands in the competition assay and included the result as a graph in Fig 4.

Minor Essential Revisions
11. P8: “NEB” not “NEB lab”; the “B” stands for “Biolabs”
As the reviewer indicated, we have changed the word “NEB lab” to “New England Biolabs”

12. P9: Give concentrations of protease inhibitors
No concentration is available for this commercially available protease mixtures. Instead, we have indicated the concentration suggested by the manufacturer as 1X.
13. P11: “with statistical significance set at {a p value of} 0.05” (suggested added text in {})
   As the reviewer suggested, we have included “a p value of”.

14. P13: nucleotide change(s)
We have corrected the typographic error.

15. P17: “...according to {a} study conducted on galactose metabolism...”
We have corrected the typographic error.

16. P18: “...such as {the} ovary...”
We have corrected the typographic error.

Discretionary Revisions
17. P5: 1-year-old probably better as single words not hyphenated
   We have changed “1-year-old” to “one year old”.

18. P7 (and elsewhere) use of the tilde (“~”) to designate nucleotide ranges
    looks odd and may be better replaced with “to”.
   As the reviewer suggested, we have changed “~” to “to”.

Reviewer 3

1) In Abstract, EMSA needs full spelling.
   As the reviewer suggested, we have changed “EMSA” to “electrophoretic mobility shift assay”.

2) In Methods, Authors should describe the method (for example, colorimetric or Paigen method) and the measurement materials for the galactosemia in newborn mass screening in Korea. The values of the measurement materials should be described in 63 positive patients, especially c.-22TC positive patients, to easily understand the situation of the patients.
   In Methods, we have indicated the type of detection methods (colorimetric assay) and the measurement material (on dried blood spot) for the galactosemia in newborn screening. And we described the mean and range of the total galactose level in 63 positive newborns.
3) In Table I, 65 subjects are written, but it is 63 subjects in the text. We have corrected the typographic error and change 65 to 63 in Table 1.

4) In Figure 3, authors should rewrite c.22T instead of AAT, c.177G instead of ACT, c.27C instead of ACT, c.22C instead of AAC. We have renamed the constructs AAT, GAT, ACT, and AAC to wt, c.-179G, c.-27C, and c.-22C, respectively.

5) In Figure 4, please show the lane number (1-10). We have included the lane numbers in Fig 4.

Reviewer 4

MAJOR COMPULSORY REVISIONS

It would be useful to specify the incidence of GALK elevated or reduced activity individuals. As far as we know, ‘the exact incidence of GALK elevated or reduced activity individuals’ has not been reported. But the incidence of GALK1 deficiency is estimated to be 1:1,000,000. We have included the following sentence in Background. “The exact incidence of GALK1 deficiency is unknown, but is probably 1:1,000,000 [1, 4] and the incidence with elevated GALK1 activity has not been reported.”

OMIM has to be included the first time GALK gene or galactosemia is introduced. We have indicated OMIM number for galactosemia (MIM 230400, 230350 and 230200)

Do authors calculate the frequency of observed and expected genotype? We have calculated the frequencies of expected genotypes from Hardy-Weinberg equation. There were no differences in genotypic frequencies between observed and expected genotypes. We have included the following sentence in Result. “Genotype distributions of the c.-179A>G, c.-27A>C and c.-22T>C variations did not deviate from those expected for Hardy-Weinberg proportion.”

Why the authors don’t perform any real time RT-PCR to evaluated the
quantity of GALK1 transcripts for each variants?
We agree with the reviewer’s point that the manuscript would be strengthened if we were able to perform the quantitation of GALK1 mRNA using RT-PCR. But unfortunately, it needed a fresh whole blood to get the mRNA, and it was very hard to request a re-sampling from the neonatal patients just for the purpose of study. Instead, we performed the promoter analysis.

MINOR ESSENTIAL REVISIONS
Abstract, methods: pag 2 line 4 ..activity and the molecular GALK gene variations
As the reviewer suggested, we have changed the sentence.

What is EMSA assay? This has to be clearly written within the abstract
As the reviewer suggested, we have changed “EMSA” to “electrophoretic mobility shift assay”.

last line:.nulceotide variation within GALK1 promoter
As the reviewer suggested, we have changed “nucleotide alteration in the GALK1 promoter” to “nucleotide variation within GALK1 promoter” in Abstract.

Reviewer 5

Major Issue:
1)The author should show the newborn screening data used to give a "positive newborn screen for galactosemia" using dried blood on filter paper and how increased galactokinase was determined for the 63 infants studied here. And we have described the mean and range of the total galactose level on dried blood spots in 63 positive newborns. GALK1 assay was performed by radiometric assay using erythrocytes derived from heparinized whole blood.

2) One infant was homozygous for the c.-22C "mutation". Fig 1B should show the homozygous effect on the RFLP analysis when both alleles have a gain of a Bsg1 cutsite.
We repeated experiments with the new samples including homozygotes for c.-22C and changed the Figure 1.

3)There are several important issues to clarify when postulating a positive
response element created by a single nucleotide change at c.-22 when this gene has a housekeeping promoter region and this nucleotide is 5'to the transcriptional start site. The authors should include at least a sequence analysis of potential positive response elements generated by the c.-22 T to C transition.

In the last two sentences in Results, we explained that we analyzed the nucleotide sequence for potential transcription factor that might differentially interact. We identified HEN1 as a potential candidate but we could not observe a supershift using HEN1 antibody. We have included this result in Fig 4A which was previously indicated as ‘data not shown’.

MINOR:
1) Clearly state that erythrocytes were used for GALK enzyme and Gal-1-P assays.
We have added “in erythrocytes” to clarify that the GALK enzyme and GAL-1-P assays were done with erythrocytes.

Also it would be of interest to discuss why the T/T (typographic error, it should be C/C) homozygote had less increase than four heterozygotes with the T/C genotypes. Perhaps the "gain of function" was complete with only one mutant allele/
Actually, a C/C homozygote had less increase in GALK1 values than three heterozygotes (only two in normalized values). It would be hard to conclude that “gain of function” was complete with only one mutant allele because the GALK1 value of CC genotype was higher than most of TC genotype.

2) Was there an increase in GALK mRNA? I see no Northern blots or quantitative RT-PCR data.
Unfortunately, we could not perform the quantitation of GALK1 mRNA because it needed a fresh whole blood to get the mRNA. It was very hard to request a re-sampling from the neonatal patients just for the purpose of study.

3) The gel shift assays should be discussed more fully. Fig.4 demonstrates that there is a shift when either the 22T or 22C probe is used with nuclear extracts and that either cold 22T or 22C competes for the presumed nuclear protein binding. This finding reduces the importance of the specificity of the c.-22 C to T transition in affecting increased transcription of GALK by an as yet to be identified DNA response element or a novel nuclear protein. Please
discuss these issues more fully in the Discussion and reduce reiteration of your Results section in the Discussion.

We have included the quantitation of the competition assay in Fig 4. We have rewritten the Discussion to reduce reiteration of Results section.

We hope that these revisions are satisfactory, and we look forward to hearing from you.