Author’s response to reviews

Title: Polymorphisms in the glucocerebrosidase gene and pseudogene urge caution in clinical analysis of Gaucher disease allele c.1448T>C (L444P)

Authors:

Justin T Brown (jbrown@asuragen.com)
Cora Lahey (clahey@asuragen.com)
Walairat Laosinchai-Wolf (wlaosinchaiwolf@asuragen.com)
Andrew G Hadd (ahadd@asuragen.com)

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Justin T Brown
Asuragen
2150 Woodward St
Austin TX 78744 USA
512-651-0191 x 6176
jbrown@asuragen.com

Editors, BMC Medical Genetics

Dear editors:

Please accept the revised manuscript "Polymorphisms in the glucocerebrosidase gene and pseudogene urge caution in clinical analysis of Gaucher disease allele c.1448T>C (L444P)" for final editorial review and potential publication in BMC Medical Genetics. We greatly appreciate this opportunity.

Each reviewer returned a very insightful list of comments. We have addressed each in the detailed list below. For example, we have added clarification on disease mutation and information on assay advantages in response to reviewer Bembi. A common request from Bembi and Sidransky involved mutation nomenclature; therefore, nomenclature has been updated throughout the manuscript. We were pleased that reviewers Bembi and Guerrini recognized the contribution of this work and did not require any major compulsory revisions prior to publication. Importantly, we have responded to the discrepant opinions of reviewer Sidransky. Overall, numerous positive changes have been made in response to all three reviews. All changes and responses below have been read and agreed to by all authors.

This improved manuscript highlights the limitations of using publicly-available sequence databases for multiplex assay design. As multiple, unique genomic DNA sequence information is not always readily available, the sequence data contributed here should aid in the future design of genetic assays in this region of the GBA gene. Furthermore, whereas the available sequence information suggested discrimination capabilities with a shorter product, we identified a single-nucleotide polymorphism in the pseudogene that required the use of a larger product for effective gene/pseudogene discrimination. We successfully demonstrated that larger amplified products can be directly hybridized to bead-bound capture probes in a liquid bead array. We are happy to have this opportunity to contribute this work to the scientific literature.

Thank you for your time and efforts.

Sincerely,
Justin T Brown PhD

Response to Reviewer Bruno Bembi

1. The reviewer asked why L444P alone was chosen for the screening of Gaucher disease in light of the fact that the more prevalent mutation N370S exists in the Ashkenazi population. In the Results and
Discussion sections we have clarified that three additional mutations (including N370S) were in fact chosen for screening. The focus of the paper remains the technical challenges associated with allele L444P.

Results:
"This assay includes wild-type and mutant genetic determinations for Gaucher disease allele L444P."
now reads
"This assay includes wild-type and mutant genetic determinations for Gaucher disease alleles c.1226A>G (N370S), 84GG, IVS2+1G>A, and c.1448T>C (L444P)."

Discussion:
"...we developed a set of reagents that allows genetic determinations for Gaucher allele L444P. In our preliminary design strategy, the parallel primer matched both gene and pseudogene.

now reads
"...we developed a set of reagents that allows genetic determinations for Gaucher disease alleles c.1226A>G (N370S), 84GG, IVS2+1G>A, and c.1448T>C (L444P). In our preliminary design strategy, the c.1448T>C-targeting parallel primer matched both gene and pseudogene."

2. The reviewer would appreciate a review of the advantages and disadvantages of the test presented here. Whereas a thorough review of such issues would be outside the scope of the manuscript, this reviewer’s comment affirms the desire for such discussion in the target audience. To this end, we illustrate some areas that already exist in the text and others that have been added in order to address this curiosity without weakening the focus of the work.

Methods:
"This software package allows review of selected alleles and/or diseases within the panel."
now reads
"This software package can address ethical and policy considerations by revealing only those alleles and/or diseases which have been requested by the physician or genetic counselor. This software package also allows configuration of analysis parameters, stores test data in a database, queries the database with or without sample identifiers, and produces sample and batch reports."

Discussion:
"The benefit of consolidating multiple-disease testing into a single procedure includes reduced training, labor costs, and turnaround time.[6]"
now reads
"The benefits of consolidating multiple-disease testing into a single procedure such as the test presented in this work include reduced training, labor costs, and turnaround time.[6]"

Discussion:
new sentence
"These reagents use multiplex PCR and bead array hybridization to facilitate an assay that detects 23 mutations per sample in fewer than 5 hours. Only a single transfer step is required with no intervening purification steps. Allele stringency is maintained without post-hybridization wash steps."

Response to Reviewer Renzo Guerrini
1. In a minor comment, the reviewer expressed the opinion that there is some redundancy between the initial parts of the Background and Discussion sections. While it is true that multiplexing is discussed in both sections, we feel both (a) that it is necessary in both sections to give the reader the proper background to understand the Results and then to explore the ramifications in the Discussion and (b) that the treatment of this central theme is different between the two sections. This comment is unfortunately somewhat discrepant with reviewer Bembi who requested additional—not less—information in these sections.

2. The reviewer requests that in the Background section of the Abstract that we specify the 8 diseases in the Ashkenazi population included in the multiplex assay. We have done so.

Abstract:
"We have developed a multiplexed genetic assay for eight diseases prevalent in the Ashkenazi population that includes an allelic determination for GBA allele L444P (1448C)."
now reads
"We have developed a multiplexed genetic assay for eight diseases prevalent in the Ashkenazi population:
Tay-Sachs, Gaucher type I, Niemann-Pick types A and B, mucolipidosis type IV, familial dysautonomia, Canavan, Bloom syndrome, and Fanconi anemia type C. This assay includes an allelic determination for GBA allele c.1448T>C (L444P).

3. The reviewer requested that the nomenclature for the mutations be changed. Gaucher disease nomenclature has been notoriously divergent between institutions for many years. We believe this particular request illustrates a difference between academic and clinical nomenclature for these alleles. However, this request was also made by another reviewer. Therefore, this has been changed in the title and throughout the manuscript. Where appropriate, the amino acid designation L444P has been included parenthetically for clarification for those who are not yet using the c.1448T>C nomenclature.

Response to Reviewer Ellen Sidransky

Major

1. The reviewer states correctly that "There are well-established databases detailing the sequences for GBA and pseudoGBA available." However, it is clear in the work that our assay system targets genomic DNA and that the majority of databases to which the reviewer refers offer sequence based on RNA-derived cDNA sequences. An example statement follows.

   Background:
   "In the case of GBA, there are relatively few publicly available unique genomic DNA sequences.[1, 15, 16] Sequences derived from cDNA are more abundant[17] but are not so useful as a design aid for assays targeting genomic DNA."

2. The reviewer identifies the lack of assay coverage for gene:pseudogene recombinant mutants. As mentioned in the Background section, "Such design challenges in clinical molecular analysis of GBA are well established.[5]" The scope of this work does not include all design challenges in Gaucher disease testing, nor does it purport to do so. Rather, we highlight the limitations of using publicly-available sequence databases for multiplex assay design. Whereas the available sequence information suggested discrimination capabilities with a shorter amplification product as preferred by modern multiplexed assay systems, we identified a single-nucleotide polymorphism in the pseudogene that required the use of a larger product for effective gene/pseudogene discrimination.

3. The reviewer requested that the nomenclature for the mutations be changed. Gaucher disease nomenclature has been notoriously divergent between institutions for many years. We believe this particular request illustrates a difference between academic and clinical nomenclature for these alleles. However, this request was also made by another reviewer. Therefore, this has been changed in the title and throughout the manuscript. Where appropriate, the amino acid designation L444P has been included parenthetically for clarification for those who are not yet using the c.1448T>C nomenclature.

4. The reviewer is concerned about anonymization of samples. The manuscript and cover letter were previously updated per editorial review for clarification of sample de-identification, approval and consent. In this study, samples were randomly selected from residual patient materials by a laboratory technologist that was not part of the research team. No identifying information was provided with the samples and no coding was performed. Neither the investigators nor the provider could link the samples with the donors. This study does not meet the definition of human subject research. The federal wide assurance number for Asuragen is FWA00009951. In order to further clarify this critical ethical issue, we have updated the following statements with either additional information or more precise wording.

Abstract:
"...evaluations were conducted using anonymized patient DNA samples..." now reads
"...evaluations were conducted using de-identified patient DNA samples..."

Methods:
"Samples were randomly selected and provided without coding or identifying information to members of the research team." now reads
"Samples were randomly selected and provided without coding or identifying information to members of the research team. Investigators were not provided with previously determined genotypes until after testing was completed. In addition, the investigators did not have access to the laboratory information systems of the facilities."
Methods:
"These anonymized patient DNA samples..."
now reads
"These de-identified patient DNA samples..."

Results:
"An anonymized set of four discrepant...
now reads
"A de-identified set of four discrepant..."

5. The reviewer quotes a portion of the following sentence from the Background section: "This report details our identification of common pseudoGBA and GBA polymorphisms not reported in the literature." Through citation and text, we recognize that there are prior reports of a portion of these polymorphisms. This was previously noted in the text of the manuscript (see example below). The reviewer led us to an article by Martinez-Arias et al. Due to a discrepancy between nucleotide numbering systems, we had previously overlooked that one of the nucleotide changes had been observed in this previous work, but in only one of ten populations tested: Maya. We regret this small oversight. However, ours is the first report of the change in the Ashkenazi population—a group that (unlike the Maya) carry Gaucher disease with the high frequency of 1:17. The included clarification is below.

Discussion (original text example):
"As expected based on previous reports,[1, 15-17] all sequenced genes had T and all pseudogenes had C at this site."

Discussion:
"Although all patients' genes exhibited C at nucleotide 7159, two patients' pseudogenes were heterozygous for C, rendering this an unreliable universal primer position for primer mispair-based differentiation."
now reads
"Although all patients' genes exhibited C at nucleotide 7159, two patients' pseudogenes were heterozygous for C, rendering this an unreliable universal primer position for primer mispair-based differentiation. This nucleotide change—observed here in samples submitted for Ashkenazi carrier screening—has previously been reported in the Maya population.[37]"

6. The reviewer states an opinion that one of our hypotheses is very reasonable and even obvious. Hypotheses are the cornerstone of the scientific method and generally refer to an interpretation of a practical situation or condition taken as the ground for action. Hypotheses thus require action (experimentation) to bear out their assertions. This particular hypothesis is tied to the theme of the work as it pertains to the limitations of using publicly-available sequence databases for multiplex assay design. Whereas the available sequence information suggested discrimination capabilities with a shorter amplification product preferred by modern multiplexed assay systems, we identified a single-nucleotide polymorphism in the pseudogene that required the use of a larger product for effective gene/pseudogene discrimination.

Minor

1. The reviewer alerted us to the fact that residual enzymatic activity with this mutation is actually quite variable. We have updated the sentence to which the reviewer referred.

Background
"When found in the functional gene, the L444P mutation can cause disease[19] and has been demonstrated to reduce enzymatic activity by 77%.[20]"
now reads
"When found in the functional gene, the c.1448T>C mutation can cause disease[19] and has been demonstrated in one system to reduce enzymatic activity by 77%.[20]"

2. The reviewer requests that we clarify genomic versus cDNA sequence. This was addressed in major item #3 above. The reviewer further requests reference sequence information. This has been addressed in minor item #5 below.

3. The author requests a reference for fluorescent microspheres on page 11. We have now cited the origina peer-reviewed description of the technology used.
...immobilized on fluorescently addressed microspheres.

4. The reviewer points out a difference in terminology concerning reviews and analyses of literature data. We have changed this term to match that of the reviewer.

Discussion:
"We expected to detect L444P at a frequency similar to that based on meta-analysis of the literature..."
now reads
"We expected to detect c.1448T>C at a frequency similar to that based on review of the literature..."

Tables:
"Table 3 - Meta-analysis of carrier frequencies of Gaucher allele L444P"
now reads
"Table 3 - Review of carrier frequencies of Gaucher allele L444P"

5. The reviewer would like the reference sequence to appear in the text. We have added such a reference in the first place in which a nucleotide number is used per section (once in the Results and once in the Discussion).

Results:
"However, the downstream primer spanned nucleotide 7368 with its 3' terminus at nucleotide 7354 (see supplemental file)."
now reads
"However, the downstream primer spanned nucleotide 7368 with its 3' terminus at nucleotide 7354 (reference sequence [GenBank:J03059.1]; see additional file: Multiple sequence alignment of GBA and GBA)."

Discussion:
"As seen in Table 5, nucleotide 6844 may be a candidate...
now reads
"As seen in Table 5, nucleotide 6844 (reference sequence [GenBank:J03059.1]) may be a candidate..."

6. The reviewer offers that these divergent nucleotides may have previously been published. This thought was not offered by the other two reviewers. Our own review of Gaucher disease literature certainly reveals prior reporting of a small portion of these nucleotides. This was previously noted in the text of the manuscript. The reviewer led us to an article by Martinez-Arias et al. See discussion and changes made based on this minor comment in major item #5 above.