PEER REVIEW HISTORY

BMJ Open publishes all reviews undertaken for accepted manuscripts. Reviewers are asked to complete a checklist review form (**http://bmjopen.bmj.com/site/about/resources/checklist.pdf**) and are provided with free text boxes to elaborate on their assessment. These free text comments are reproduced below.

ARTICLE DETAILS

TITLE (PROVISIONAL)	Non-invasive prenatal diagnosis using foetal DNA in maternal plasma: A preliminary study for identification of paternally-inherited alleles using single nucleotide polymorphisms
AUTHORS	Chen, Jang Jih; Tan, Mary Anne Jin Ai; Chua, Kek Heng; Tan, Peng Chiong; George, Elizabeth

VERSION 1 - REVIEW

REVIEWER	Thessalia Papasavva The Cyprus Inst of Neurology and Genetics Cyprus
REVIEW RETURNED	02-Apr-2015

GENERAL COMMENTS	The authors present a study in which they describe non invasive prenatal diagnosis for identification of paternally inherited alleles using single nucleotide polymorphisms. The basic approach is good however, there are several flaws/problems regarding methodology and results as described below: Major: -Haplotyping of parental DNA (Page 8): This is not the adequate strategy for haplotyping. This is rather genotyping. You cannot phase the allele as wild-type or mutant by simply genotyping. The methodolody described here does not show or prove that the SNPs are in cis or inherited in cis. In order to associate and phase alleles you need to analyze more members of the family or the CVS with the final dagnosis in order to be able to infer haplotypes. Another approach to do this is single molecule haplotyping. Therefore, there is no way to know the inherited mutation based on the results derived from the mentioned strategy. -Foetal DNA enrichment (page 7): the method used (extraction of DNA fragments from a gel) has several drawbacks such as contamination and loss of fragment and it has not been adopted as a safe method. -the SNPs reported there are not the internationally accepted SNP names. You schould consider finding their "rs" numbers. They can be found in databases such as the db SNP database of the NCBI. -The bibliography used in this stude is somewhat outdated. Consider adding more recent and updated references. -There is a mix up at various locations. Texts that belong to Results section appear in Discussion. Please adjust. -Discussion just reports the results without any justification or

	discussion. -Not clear whether parents share the same mutation -The text needs language editing.

REVIEWER	Prof. Wolfgang Holzgreve University Hospital Bonn Germany
REVIEW RETURNED	07-Apr-2015

GENERAL COMMENTS	This is an important and well written manuscript, and I have only two criticisms which could be addressed by the authors easily: 1. When chorionic villus sampling as a standard method of prenatal diagnosis is addressed in the text (page 4, second paragraph) "limb reductions" are mentioned as compliction, although these findings have only beeen reported in the 80ies when CVS started, but hey are not of concern with a proper sampling technique. 2. In the results section (page 10) the sentence: " Comparison of these results carried out earlier using CV DNA showed concordance" is not clear, and it should be stated whether the CVS took place before or after the non-invasive testing. This should also be mentioned clearly in the abstract.

VERSION 1 – AUTHOR RESPONSE

Reviewer Name Thessalia Papasavva
Institution and Country The Cyprus Inst of Neurology and Genetics
Cyprus
Please state any competing interests or state 'None declared': Non declared

Please leave your comments for the authors below
The authors present a study in which they describe non invasive prenatal diagnosis for identification of paternally inherited alleles using single nucleotide polymorphisms. The basic approach is good however, there are several flaws/problems regarding methodology and results as described below:

Major:
-Haplotyping of parental DNA (Page 8): This is not the adequate strategy for haplotyping. This is rather genotyping. You cannot phase the allele as wild-type or mutant by simply genotyping. The methodolody described here does not show or prove that the SNPs are in cis or inherited in cis. In order to associate and phase alleles you need to analyze more members of the family or the CVS with the final dagnosis in order to be able to infer haplotypes. Another approach to do this is single molecule haplotyping. Therefore, there is no way to know the inherited mutation based on the results derived from the mentioned strategy.

Answer

Haplotyping of parental DNA is carried out by amplification of either allele (mutant or wild-type allele).

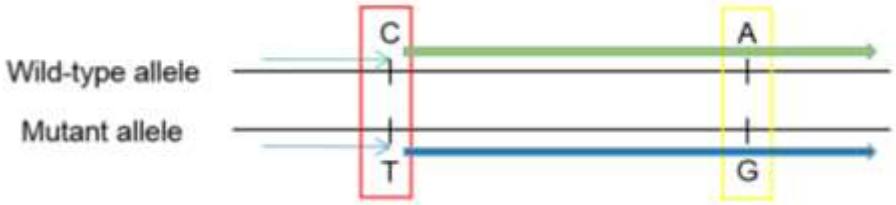

Figure above shows an example of DNA of a heterozygous patient (carrier of IVS2-654 C>T). The genotype of the patient has been determine earlier. Box in red refers to the location of point mutation, C i s the wild-type allele and T is the mutant allele. Specific primer was designed to bind at either the allele containing A (green primer) or C (blue primer). The green and blue block arrow refers to the amplification product generated from the amplification using their respective primer (ARMS).

The sequence of the amplification product generated from each primer was determined and aligned using clustal x to detect any difference between the two sequences. For instance, box in yellow shows nucleotide polymorphism. Adenine is located in cis with the wild-type allele (cytosine) while the guanine is located in cis with the mutant allele (thymine).

However, we have amended haplotyping to genotyping in the manuscript.

-Foetal DNA enrichment (page 7): the method used (extraction of DNA fragments from a gel) has several drawbacks such as contamination and loss of fragment and it has not been adopted as a safe method.

We agree this method has some risk of contamination. However, the contamination in this study is minimal or none as the gel tank, comb gel excision blade are cleaned with DNA-away spray and expose to UV for 30 minutes. TBE buffer and gel are prepared fresh. Only one sample will be process at any one time. We agree that there may be some loss of DNA fragment, however, the degree of loss is expected to be proportional between maternal and foetal DNA. The result in our study showed that gel excision to reduce the background maternal DNA does improve the resolution of base call for the foetal DNA (page 9- Discussion-foetal enrichment).

-the SNPs reported there are not the internationally accepted SNP names. You schould consider finding their "rs" numbers. They can be found in databases such as the db SNP database of the NCBI.
The revised names are as follows:

IVS2-81	**rs 7946748**
-469	**rs 10742584**
-521	**rs 200771769**
-528	**rs 74234654**
-541	**rs 10768684**
-551	**rs 35755129**

-The bibliography used in this stude is somewhat outdated. Consider adding more recent and updated references.
We have added three citations as below:

Chua YA, Abdullah WZ, Yusof Z, Gan SH. A New Nested Allele-Specific Multiplex Polymerase Chain Reaction Method for Haplotyping of VKORC1 Gene to Predict Warfarin Sensitivity. BioMed Research International. 2014;2014:6.

Gayden T, Regueiro M, Martinez L, Cadenas AM, Herrera RJ. Human Y-chromosome haplotyping by allele-specific polymerase chain reaction. Electrophoresis. 2008;29(11):2419-23.

Pettersson M, Bylund M, Alderborn A. Molecular haplotype determination using allele-specific PCR and pyrosequencing technology. Genomics. 2003;82(3):390-6.

-There is a mix up at various locations. Texts that belong to Results section appear in Discussion. Please adjust.
Proper adjustments will be made if the authors are allowed to revise the manuscript and resend for reviewing.

-Discussion just reports the results without any justification or discussion.
Thank you for the comment, revision will be carried out in the discussion to include research carried out by other investigators and justifications

-Not clear whether parents share the same mutation
Yes, they share the same mutation. In methodology, Parents 1, 2 and 3 are carrier for CD41/42 etc. Parents mean father and mother.

-The text needs language editing.
This will be attended to in the revision

Reviewer Name Prof. Wolfgang Holzgreve
Institution and Country University Hospital Bonn
Germany
Please state any competing interests or state 'None declared': None declared

Please leave your comments for the authors below
This is an important and well written manuscript, and I have only two criticisms which could be addressed by the authors easily:
1. When chorionic villus sampling as a standard method of prenatal diagnosis is addressed in the text (page 4, second paragraph) "limb reductions" are mentioned as compliction, although these findings have only beeen reported in the 80ies when CVS started, but hey are not of concern with a proper sampling technique.

Thank you for the correction. We agree that "limb reductions" were complications in the earlier times when CV sampling was done. We will revise accordingly.

2. In the results section (page 10) the sentence: " Comparison of these results carried out earlier using CV DNA showed concordance" is not clear, and it should be stated whether the CVS took place before or after the non-invasive testing. This should also be mentioned clearly in the abstract.

Genotypes of the parents were carried out prior to prenatal diagnosis. CVS sampling was carried out before the non-invasive protocols were studied, This will be stated more clearly in the revised manuscript.

VERSION 2 – REVIEW

REVIEWER	Thessalia Papasavva The Cyprus Institute of Neurology and Gebet
REVIEW RETURNED	09-Jun-2015

GENERAL COMMENTS	The authors present a study in which they descibe non invasive prenatal diagnosis for identification of paternally inherited alleles using single nucleotide polymorphisms. The basic approach is good, with the advantage that it is not a complicated method, straight forward and cost effective, with no need of sophisticated and expensive technology. However, a disadvantage of this approach is that on the short amplified fragment to ensure the fetal DNA which should be less than 300bp with an optimal of 145bp, the number of SNPs that will give sufficient information is limited. Therefore, accuaracy is limited to 1 or 2 SNPs. Neverthe less, the study design and approach is adequate.