Author's response to reviews

Title: Absence of mutations in NR2E1 and SNX3 in MMEP (microcephaly, microphthalmia, ectrodactyly, and prognathism) and related phenotypes

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Author's response to reviews: see over
Re: Absence of mutations in \textit{NR2E1} and \textit{SNX3} in five patients with MMEP (microcephaly, microphthalmia, ectrodactyly, and prognathism) and related phenotypes

I am pleased to re-submit this manuscript for publication as a Research Article in \textit{BMC Medical Genetics}.

We thank the reviewers for their well-considered comments of our work. We had no difficulty incorporating their important suggestions in the revised manuscript and the process has no doubt strengthened the paper. For specific details on how we have responded to their comments, please see below.

Yours sincerely,

Elizabeth M. Simpson, Ph.D.
Reviewer One: Uta Franke

Major Compulsory Revisions

1. The authors report that they have not found any “coding mutations” in NR2E1, presumably meaning mutations that change an amino acid. However, they should report all sequence changes that they observed. It is well known that synonymous nucleotide substitutions that do not change the amino acid code can cause disease by activating cryptic splice sites (e.g. Berg et al, Hum. Mut. 1:24-34 (1992) or by altering splice enhancer binding sites. The effect of such mutations can only be studied at the RNA level. With brain tissue not easily available from patients, transcript analysis can be done by introducing the mutation into in vitro splicing systems (Liu et al, Nature Genet. 16:328-329 (1997).

Done. We have clarified the issue raised by the reviewer. Our study of NR2E1 in MMEP and related phenotypes did not detect any coding changes, including synonymous substitutions. We have clarified this point as follows:

Page 2 (Abstract): We did not detect any synonymous nor nonsynonymous coding mutations of NR2E1 or SNX3
Page 9 (Results and Discussion): We did not detect any synonymous nor nonsynonymous coding mutations.

2. The authors do report a single sequence change in the NR2E1 3’ UTR g.21502T>C that they call a “candidate regulatory mutation”, even though the same nucleotide change was present in the unaffected mother. They report that the same sequence change was seen in the previous study (reference 9) in a patient with microcephaly, but not in a population control sample. But the previous study reports that the g.21502T>C change was also present in the unaffected father of the microcephaly patient, a fact that the authors omitted from the present manuscript. Therefore, it is most likely that g.21502T>C is an innocuous sequence variant and not a “regulatory mutation”. Of note, the SNP database currently lists 62 sequence variants in NR2E1.

We agree. We have clarified this point as follows:

Page 9 (Results and Discussion): Interestingly, we previously reported the g.21502T>C candidate regulatory mutation (in addition to two other mutations) in a patient with microcephaly and the unaffected father but not in 344 control chromosomes nor in 188 ethnically-diverse chromosomes.

We agree with the reviewer that g.21502T>C could represent an innocuous sequence change. We have expanded our interpretation of the role, if any, of g.21502T>C by stating the following:

Page 10 (Results and Discussion): One possibility is that the g.21502 T>C is an innocuous substitution that does not contribute to disease but rather represents a rare variant in the general population.

3. The hypothesis that NR2E1 could be involved in MMEP is weak because the gene is brain-specific, and the authors have previously excluded mutations in this gene in a large series of patients with microcephaly and other brain malformations. MMEP is distinguished from these cases by the presence of ectrodactyly, a malformation of the
feet. In the absence of any evidence that NR2E1 is expressed during limb development, this is a poor candidate gene. The authors argue for NR2E1 as a candidate because it is the closest gene to SNX3, located about 22 kb downstream. However, on the other side of SNX3 there is another positional candidate, LACE1, located approximately 33 kb upstream of SNX3. This gene encodes a protein with possible ATPase function that is highly conserved in fly, yeast and bacteria. As LACE1 is more widely expressed in tissues compared to NR2E1, it would be a reasonable candidate to study in these five patients.

We take the reviewer’s point. Sequencing LACE1 in these patients will be a logical undertaking for a subsequent paper. Our selection of candidate genes for this study is driven by the 6q21 positional breakpoint data that implicates NR2E1 and SNX3 in MMEP and related phenotypes. We did not mean to propose that NR2E1 alone causes MMEP. We have clarified this point as follows:

Page 5 (Background): We propose that a gene(s) near the 6q21 translocation may underlie MMEP and related phenotypes in some patients. Here, we test the hypothesis that patients with MMEP or a related phenotype may harbor mutations in NR2E1 and/or SNX3.

Minor Essential Revisions
4. The paragraph on transcription factor binding site analysis is redundant because it is part of the previous manuscript (ref. 9) in which this candidate g.21502T>C mutation was already included.

We agree. We have omitted the methods section on “Transcription Factor Binding Site Analyses” on Page 6. We now reference the previous work on page 9.

5. The translocation designated as “6q21;7q31.2” should be presented in the standard nomenclature which is t(6;7)(q21;q31.2) in the text. In the reference section, the incorrect nomenclature needs to be left because this is what the authors of the original paper called it.

We agree. The translocation designation has been changed throughout the text to t(6;7)(q21;q31.2) to reflect the standard nomenclature as pointed out by the reviewer.

Discretionary Revisions (which the author can choose to ignore)
6. The authors could also consider sequencing other genes not located at 6q21, such as PAX3 known to be involved in cortical phenotypes. Their best bet, however, would be to focus on genes that are expressed during development of brain and limbs.

We take the reviewer’s point. Sequencing of other genes involved in cortical development, such as PAX3, will be a logical undertaking for a subsequent paper.
Reviewer Two: Annick Raas-rothchild

General
I would suggest to see that the manuscript has been shortened and modified before publication.

1. Is the question posed by the authors new and well defined? The question is new in part and defined

2. Are the methods appropriate and well described, and are sufficient details provided to replicate the work? Yes

3. N/A.

4. Does the manuscript adhere to the relevant standards for reporting and data deposition? Yes

5. Are the discussion and conclusions well balanced and adequately supported by the data? Not completely.

6. Do the title and abstract accurately convey what has been found? No I would add the number of patients studied

We agree. The title has been changed as follows:

Absence of mutations in NR2E1 and SNX3 in five patients with MMEP (microcephaly, microphthalmia, ectrodactyly, and prognathism) and related phenotypes.

7. Is the writing acceptable? Manuscript should be shortened.

Kumar et al reported the study of SNX3 and NR2E genes in 4 patients with MMEP syndrome and one patient with a translocation including the breakpoint 6q21. One patient with related MMEP harbored the g.21502T>C change previously reported in a patient with Microcephaly who also had additional NR2E sequence mutations. The authors already showed in a former paper [genes brain behavior 2006] that NR2E1 coding mutations do not contribute to cortical and behavioral abnormalities in patients with Microcephaly.

I would suggest writing that the patient with ulnar aplasia was included in the work only because of the translocation breakpoint locus.

We agree. We have revised the text as follows:

Page 7 (Methods): Patient 5 was included in the study on the basis of having a de novo chromosome translocation involving the 6q21 region t(6;7)(q21;q31.2).

The manuscript should be shortened. The background first paragraph could be synthesized for example. In this work SNX3 was sequenced in 4/5 patients although no mutation had been found in one previous MMEP patient. This should be stated clearly.
Information such as "...33.5 kb sequence data" [page 7] ; "we confirmed this variant by sequencing both strands etc..." are not needed.

Done. We thank the reviewer for the suggested wording. The first paragraph of the abstract background as been shortened, as suggested.

The g.21502T>C change was reported in the paper by Kumar et al, as a candidate regulatory mutation and not a mutation as it is stated in page 7, in addition to two more mutations which is different from the present report. I would suggest adding this. I would suggest adding to the discussion that the unaffected father in the paper by kumar [2006] was a g.21502T>C carrier.

We agree. The text has been revised as follows:

Page 9 (Results and Discussion): We genotyped g.21502T>C in the unaffected parents and identified the candidate regulatory mutation in the mother but not the father.

Interestingly, we previously reported the g.21502T>C candidate regulatory mutation (in addition to two other mutations) in a patient with microcephaly and the unaffected father but not in 344 control chromosomes nor in 188 ethnically-diverse chromosomes.

Did the author sequenced pax 6 in the studied patients? It would be interesting to add the information.

PAX6 has not been studied in the patients as we focused on positional candidates in this paper. Sequencing PAX6, along with other brain and eye functional candidates, represents feasible undertakings for subsequent papers.
Reviewer Three: Maria Giuseppina Miano

Major points:
1. In Methods, the authors should include a brief clinical description of each examined patient. Data reported in Table 1, in my opinion, are not enough to explain the choice of the patients to undergo the screening analysis. Based on these considerations, the authors should be adding the criteria used in this selection.

We agree. We have now included a brief clinical description of each examined patient. Table 1 has been deleted from the paper since all the information is presented in Methods as follows:

Approval for this study was obtained from The University of British Columbia and Child & Family Research Institute. The research followed Canada’s Tri-Council Statement on ‘Ethical Conduct for Research Involving Humans’. Approval was also obtained through the Institutional Review Board of Self Regional Healthcare (Greenwood, SC). Patients were ascertained and examined from five centers: The University of the Witwaterstrand, Johannesburg; The University of Cape Town, South Africa; Cedars-Sinai Medical Center, Los Angeles; Universita Cattolica, Rome; and The University of California, San Francisco. Patients were referred to the Greenwood Genetic Center for molecular research studies on split-hand/foot malformation. Controls were ascertained from the Greenwood Genetic Center.

All cases were sporadic and born to non-consanguineous parents. Patient 1 is the original patient with MMEP and t(6;13)(q21;q12) described previously by Viljoen and Smart [12]. This is a 44-year-old Caucasian female with severe mental retardation, congenital microphthalmia causing complete blindness, central cleft lip and palate, ectrodactyly with absence of toes 2-4 on both feet, finger-like thumbs, and a broad, prominent jaw.

Patients 2-4 had normal blood chromosome analyses and were included in this study on the basis of having clinical features that significantly overlapped those of the MMEP phenotype. Patient 2 was felt to have possible MMEP versus an unusual variant of the ectrodactyly-ectodermal dysplasia-clefting (EEC) syndrome and has not been described previously. She is a Hispanic female with congenital microcephaly, bilateral microphthalmia, colobomas of the right iris and retina, left chorioretinal coloboma, left lacrimal duct stenosis, unilateral cleft lip and palate, dysplastic ears, soft tissue syndactyly of fingers 3-4 on the right hand, ectrodactyly of the left foot with a hallux and two digital rays, unilateral mixed versus sensorineural hearing loss requiring a hearing aid, sparse scalp hair and eyebrows, and narrow, deep-set nails. She had a prolonged hospitalization after birth and required gastrostomy tube placement. She had no evidence of a TP73L (previously TP63 and P63) mutation, which is known to be involved in split hand/foot malformation [13].

Patients 3 and 4 were felt to have an unusual variant of the EEC syndrome. A brief summary of their clinical findings was reported previously [13]. Patient 3 is a 10-year-old Caucasian female with microcephaly, bilateral iris colobomas, microphthalmia with significant vision impairment, bilateral ectrodactyly of the hands and feet, unilateral cleft lip, and patchy alopecia of the scalp hair. She required gastrostomy feedings until age 9 due to ongoing problems with feeding, failure to thrive, and severe gastroesophageal reflux. A gastric emptying study revealed delayed emptying with a non-functioning section of the stomach and reverse peristalsis, which resolved with gastric Botox
therapy. She has experienced significant dental problems due to ectodermal dysplasia. She attends a regular school program with visual assistance. Patient 4 is an African-American female seen at 3 months of age with congenital microcephaly, congenitally sealed eyelids with small to absent globes, ectrodactyly of the hands and feet, a notch in the upper lip resembling a mild midline cleft, absent scalp hair, underdeveloped eyelashes and eyebrows, underdeveloped nails, minor differences in ear shape, unilateral hearing loss, pelvic kidney, anteriorly placed anus, and tethered spinal cord. Both patients had no evidence of a TP73L mutation [13].

Patient 5 was included in the study on the basis of having a de novo chromosome translocation involving the 6q21 region (t(6;7)(q21;q31.2)) and congenital ulnar ray aplasia. His findings were described previously by Gurrieri et al. [11]. He is a Caucasian male evaluated in the newborn period with congenitally bowed radii, absent ulnae, absence of fingers 3-5 on the right hand, syndactyly of fingers 2-3 and absence of fingers 4-5 on the left hand, and a cyst of the septum pellucidum. He did not have microcephaly, ocular abnormalities, or other features of the MMEP phenotype. We cannot exclude the possibility that breakage at 7q31 may disrupt a gene(s) involved in MMEP.

2. In the case of the patient carrying the translocation 6q21;7q31.2, it is unclear why the authors exclude the involvement of an alteration on the chromosome 7. This point should be clarified.

We agree. We have clarified this point as follows:

Page 7, description of patient 5. We cannot exclude the possibility that breakage at 7q31 may disrupt a gene(s) involved in MMEP.

3. Can the authors exclude the presence of other rearrangements? The authors should verify it by karyotype analysis.

Yes, we can exclude the presence of other rearrangements based on karyotype analyses that have been performed for each patient.

4. The authors should add a picture describing the physical map of the SNX3 locus, underlying the distances between it and NR2E1 gene and also the translocation breakpoint.

We agree. A physical map of the SNX3 locus is now included as Figure 1.

Note to Editor: We have removed Table 1 (Clinical data), which has been replaced by text. Therefore, the number of display items has not changed.

5. The finding of g.21502T>C in the MMEP patient and in the patient with microcephaly is suggestive of a relationship with brain disease. However, to support this hypothesis, the authors should examine its effect on mRNA stability.

We take the reviewer’s point. Elucidating the effects of g.2150T>C on mRNA stability is a logical next step for future studies but is beyond the scope of this paper.
6. In Conclusion Section, the authors should be discussing the heterogeneity of the disease because in my opinion this is a crucial point.

We agree. Phenotypic heterogeneity of the patients has been discussed at length in the Methods section. We have also discussed genetic heterogeneity in Conclusions, as requested by the reviewer:

Page 10 (Conclusions). MMEP and related phenotypes represent a spectrum of heterogeneous conditions for which multiple loci may be involved, including NR2E1 and SNX3 on Chromosome 6q21-22 [10, 11]. The present study does not support involvement of NR2E1 or SNX3 coding mutations in MMEP or related phenotypes. However, we cannot exclude the possibility that regulatory NR2E1 or SNX3 mutations, such as g.21502T>C of NR2E1, may underlie abnormal human cortical development in some families. In addition, we cannot exclude the possibility that deletions at NR2E1 or SNX3 may underlie MMEP, given that sequencing is unable to distinguish between homozygosity across loci versus large deletions. The lack of obvious mutations in NR2E1 and SNX3 contribute to the genetic complexity underlying this heterogeneous syndrome. Follow-up studies of other positional candidates such as LACE1, would be a next logical undertaking.
Reviewer Four: Diane W Cox

The expression pattern of the two selected genes, either from experimental data or from databases, should be included.

We agree. The text now includes a description of the known expression patterns for these genes as follows:

Page 4 (Background, paragraph 1). Sorting nexin 3 (SNX3) is ubiquitously expressed and belongs to the sorting nexin family, which are involved in intracellular protein trafficking [1].

Page 4 (Background, paragraph 2). NR2E1 is also a strong functional candidate, given that mice deleted for Nrt2e1 present with a complex MMEP-related phenotype that includes forebrain hypoplasia, eye abnormalities, and cognitive impairment [4-6], which is consistent with the brain and eye expression pattern of this gene [7, 8]

Deletions of one allele of the two genes have not been excluded. This is probably not apparent from heterozygous SNPs appearing within the sequence, as only one is noted (NR2E1). If deletions have not been excluded, that should be stated in the text. The statement that the present study 'does not support involvement of the two genes' is too strong, considering the possibility of deletion that has not been excluded.

We agree. We clarify this point as follows:

Page 10 (Conclusions): In addition, we cannot exclude the possibility that deletions at NR2E1 or SNX3 may underlie MMEP, given that sequencing is unable to distinguish between homozygosity across loci versus large deletions.