Author’s response to reviews

Title: Testicular sex cord-stromal tumor in a boy with 2q37 deletion syndrome

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Author’s response to reviews: see over
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Dr. Tim Sands
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BMC-series Journals
BioMed Central
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MS: 1368424977102729

Dear Dr. Sands,

Thank you very much for your kind management of our manuscript, “Testicular sex-cord stromal tumor in a boy with 2q37 deletion syndrome” by Sakai et al. (MS 1368424977102729). We have addressed all the reviewers’ questions and carefully inspected the revised manuscript not to miss remaining errors. Please find the document for details of our point-by-point response attached to this letter.

We hope that the revised manuscript would fit well with the acceptable format of your journal.

Thank you for your consideration and review of our manuscript.

Yours sincerely,

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Point-by-Point Response to Reviewers

We appreciate very much extensive reviews and kind words from the reviewers. We herein report that we have successfully corrected ambiguous descriptions in our original manuscript in light of their helpful comments. Below is a point-by-point response to reviewers' comments. Please note that the reviewers’ comments are quoted in bold letters for ease of reference.

Referee 1’s comments:
- Major Compulsory Revisions

1. **The manuscript does not discuss the strong evidence that haploinsufficiency for HDAC4 – including frameshift mutations within the gene itself – cause brachydactyly and some other features of the 2q37 deletion syndrome (Williams et al, 2010, Am J Hum Genet 87: 219-228).** Other CNVs may still modify the phenotype, but a clear role for this gene has been established.

   This comment prompted us to realize that our original manuscript had a core defect in discussing the genetic causes of brachydactyly and mental retardation phenotypes in 2q37 deletion syndrome. To clarify that we appreciate previous studies demonstrating the implication of HDAC4 in phenotypic presentation, we inserted the following description at the second paragraph of Discussion:

   "The human HDAC4 gene encodes a chromatin remodeling factor, histone deacetylase 4, which cooperatively regulates gene expressions with other transcription factors in the physiological process of development and differentiation of various tissues [19, 20]. Haploinsufficiency of HDAC4 has been implicated as a responsible gene for the phenotypes of brachydactyly and developmental delay since the gene locus (chr2:240016312-240220334) was mapped to the deleted regions in individuals with del2q37.3 syndrome, who presented these phenotypes [21]. Moreover, Williams et al. clearly demonstrated that frame-shift mutations of HDAC4 itself caused brachydactyly mental retardation phenotypes [21]."

2. **Multiple reports have noted that brachydactyly and obesity may evolve with age, so the absence of these features in a two-year-old is not so surprising.**

   We thank the reviewer's professional advice for making a concrete discussion. We appended the following description to the end of above discussion:

   "The deleted region in this study encompassed the HDAC locus, whereas he did not show the brachydactyly at two years of age. Given that brachydactyly and obesity is typically absent in early childhood of those with del2q37 syndrome, the present case may develop such phenotypes later in his life. It is thus likely that HDAC4 works as an essential regulator of gene expressions both in embryonic and postnatal development."

3. **The unique feature of this case is the testicular sex-cord stromal tumor, but there is little discussion about this. 2q37 deletions have been associated with malignancies, especially Wilms tumor, and mutations have been found in the gene DIS3L2 (Astuti et al, 2012, Nat Genet. 44:277-84). It would be important to indicate the position of DIS3L2 in Figure 3 and discuss whether or not the malignancy in this child is related to this locus or not.**

   As pointed by the referee, we had overlooked one of the most important studies in this field. We
thus mapped the \textit{DIS3L2} locus (chr2:232,826,293-233,208,678) in new Fig 2C and Fig 3. Accordingly, we added the following descriptions to the revised manuscript.

- **Result** (p6, lines 8-11): "We confirmed that one of Wilms tumor-associated genes, \textit{DIS3L2} (chr2:232,826,293-233,208,678), was located outside of the proximal breakpoint of our case (chr2: 234,275,216-234,264,038) (Fig 3C)."

- **Discussion** (P7, line 3-9): "Drake et al. [10] studied a series of sporadic Wilms tumors and found evidence of a tumor suppressor role for a 360-kb critical region at 2q37 encompassing the DIS3 mitotic control homolog (S. cerevisiae)-like 2 (DIS3L2) locus. More recently, the germline mutations within the DIS3L2 gene were identified to cause Perlman syndrome, a congenital overgrowth syndrome that is predisposed to Wilms tumor [2]. In this report, we verified that this case had a heterozygous deletion of HDAC4, but not DIS3L2 (Fig 2C and 3). DIS3L2 was therefore unlikely to cause the testicular tumor in this case, although it cannot be completely excluded that the DIS3L2 gene expression was deregulated in the affected tissue."

- **Figure Legends** (P12): The legend to Fig 2 (B-D) was updated, "Only the genes that were selected for qPCR and \textit{DIS3L2} are annotated for simplicity."

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**- Minor Essential Revisions**

1. **Breakpoint coordinates should include the build of the human genome to which they refer, since they may change in future builds.**

   We thank the kind advice and insert the following note in the Materials and Methods: "The coordinates of CNV breakpoints were defined according to the UCSC genome assembly, GRCh37/hg19 (http://genome.ucsc.edu/)."

**Referee 2's comments:**

**Minor**

1) **Remove "the" from "affected genes at the chromosome 2q37" in the Abstract**

   We thank Referee #2's helpful advice. Yes, we have removed the article.

2) **Reference 2 : Leroy et al was published in 2013, not 2012.**

   This is our mistake. We corrected it to 2013.

**Major**

1) **What other indications, apart from this case, are there that the 2q37 deletion confers a risk for "tumorigenic conditions"? There would appear to be no other cases/reports of testicular sex-cord stromal tumors in 2q37 deletion patients and, as pointed out, this is a novel complication and thus the possibility that it is coincidental should be discussed. How common is sex-cord stromal tumor in this population? This case should be entered into DECIPHER so that if/when other testicular sex-cord stromal tumors occur in patients with any of these CNVs, a cause and effect could be established. Has this case been entered into DECIPHER? If not, will it be?**

   The referee's questions raised a crucial discussion over the complex phenotypes of this case and others with 2q37 deletion syndrome. As discussed above, we had not rationalized sufficiently in the
original text why we thought 2q37 deletion might heighten the risk for testicular tumor (Please see our response to the item 3 by the Referee #1).

On the other hand, there is no evidence for association of sex cord-stromal tumor with specific chromosomal abnormality. Thus, we agree to the referee's critical viewpoint - we cannot exclude the possibility that the complication may be irrelevant with the 2q37 deletion or accompanying CNVs because this is only one case of 2q37 deletion syndrome with a complication of testicular tumor, as far as we know. For this reason, however, we strongly hoped to share the unique features of the present case with worldwide readers through reporting the boy to an open-access journal, BMC Med Genomics.

With regard to DECIPHER, we are planning to enter the profiles to the database while we wish to prioritize our efforts primarily on publication of our case. Collectively, we added the following sentences at the end of the new discussion (Referee #1, No.3) to provide a fair perspective to the readers:

"On the other hand, one could argue that testicular sex cord-stromal tumor is coincidental just in this case unless other cases of 2q37 deletion syndrome with similar complications are reported in the future. For better accessing the clinical findings of this case to worldwide physicians and genetists, we are currently submitting the content of this report to the open resource, DECIPHER (http://decipher.sanger.ac.uk/)."

2) There are over 115 cases in the literature (Leroy, 2013) but the authors discuss only 39 previous cases from five studies; why?

The reason is simple - because the breakpoint data are not always available for all patients with 2q37 deletion syndrome. We thus collected FISH and CGH data showing breakpoints at 2q37 from previously reported 39 patients and mapped them on a graphic overview using UCSC (hg19). To clarify this reason, we added the following sentence at the beginning of the second paragraph of result:

"Among those with del2q37 syndrome thus far reported, we successfully identified the breakpoints at 2q37 for 39 individuals using FISH or CGH data in the literature [1, 2, 4-8]."

3) Why do the authors propose haploinsufficiency of HDAC4, rather than any other genes within the deletion, as the relevant candidate gene contributing to testicular sex-cord stromal tumors?

While we inspected the past literature more carefully, we found that reduced expression of HDAC4 is rather associated with regression than proliferation of cancer cells in most studies. Knowing that it is better not to confuse readers, we decided to eliminate the description that links HDAC4 haploinsufficiency to the onset of sex cord-stromal tumor. While little evidence has been documented for contributions of other genes affected by 2q37 to tumorigenesis, we are unable to exclude this possibility. We therefore supplemented our discussion with the following sentences:

"Notably, elevated expression of HDAC4 is known to promote tumor formations, whereas its chemical inhibitors and siRNA-mediated knockdown of HDAC4 are associated with regressions of cell growth [27]. Therefore, it is unlikely that haploinsufficiency of HDAC4 contributed per se to the testicular tumor formation in this case, whereas there is little evidence that other genes within the deleted region are associated with carcinogenesis. Concerning various unknown mechanisms underlying the unique phenotypes of this case, further genome-wide analyses to identify the unique genetic backgrounds in this case must be considered for future studies."

4) 2q37 deletions are associated with intellectual deficiency (Leroy, 2013) so why suggest the 1p36
duplication contributed to intelligence disability? Is the deficiency in this case unusually profound compared to other 2q37 deletions?

This is another arguable point. We tried to understand the biological effects of co-existing CNVs on the developmental phenotypes although we failed to mention that the case did not show distinct phenotypes in intellectual disability from other cases with 2q37 deletion. We were particularly interested in the duplication of 1p36.33–p36.32 because we were aware that it was a critical region for autism and intellectual disability when deleted (Pinto D, Nature 2010; Kaminsky EB, Genet Med 2011; Girirajan S, N Engl J Med 2012; Battaglia A, Eur J Paediatr Neurol 2013). Among them, Pinto et al. presented the cases with duplication of 1p36.32 in a large cohort and described the CNV as one of potentially pathogenic CNVs for intellectual disability and autism.

Considering the CNVs at 16p11.2, one of the most recurrent hotspots of autism, both segmental deletion and duplication in this region are known to cause neuro-psychiatric disorders. While precise mechanisms for neuro-developmental phenotypes with increased gene dosage remain to be elusive, it is not rare that duplication types of CNVs are associated with intellectual disability and other cognitive disorders. We therefore inserted the sentence at the end of the first paragraph of discussion:

"While the present case did not show profound phenotype of intellectual disability compared to other cases in the literature, we focused on duplication of 1p36.33–p36.32, since the the CNVs in this region was described to confer the risk for neurodevelopmental disorders, such as autism and intellectual disability [20-22]."

5) The literature shows incomplete penetrance of haploinsufficiency of HDAC4 and CAPN10 resulting in BDMR and obesity, presumably due to variation in genetic background, mutations in the remaining (recessive) allele, epigenetic effects, multigenic inheritance and stochastic factors. Copy number variation (CNV), which is a part of the genetic background, is far from unusual (especially duplications), but is more obvious and easy to detect when present. Thus the significance of the co-occurrence of two duplications in this case is far from clear or unexpected.

We admit that there will be long-lasting arguments over the concomitant CNVs in their pathogenic effects for unusual complications and in modifying the syndromic phenotypes. We also understand that we cannot conclude anything for functional significance of other CNVs from clinical profiles of a single case. Nonetheless, we believe that this is our mission as physicians to report new cases with rare complications for accumulating genetic evidence towards unraveling the molecular basis of syndromic disorders. We added the following sentence to the last part of discussion:

"Also, since genome-wide data in this case are limited to the conventional CGH analysis, we must continue our efforts to identify the parental origin of accompanying CNVs, co-existence of single-nucleotide variations and their associated epigenetic as well as signaling effects [7] through genome-wide scans in future studies."

6) Please comment on the possible relationship, if any, between the inv (1) (p36.1p36.3) in the father and 1p36.33-p36.32 duplication in the patient. Was CGH performed on the parents? If not, why not? If so what were the results?

The CNV, duplication of 1p36.33–p36.32, was possibly converted from a balanced inversion, inv (1) (p36.1p36.3), which occurred in his father. However, we could not perform further CGH analyses
for the parents because informed consents were not successfully obtained from the parents.

7) **The human genome reference version needs to be included, presumably hg19.**

Yes, we use UCSC hg19. As also pointed by the referee #1, we inserted the description at Materials and Methods.

**Discretionary**

1) **Presumably because the authors used an Agilent oligonucleotide array they were not able to determine the parental origins of the CNVs detected? SNP genotyping of parents and child could be performed to determine parental origins of the CNVs detected.**

We do agree with the referee's point. As described above, however, we were not allowed to determine the parental origin of CNVs because the parents did not agree with our further genetic analyses.

2) **Please comment on the possible relationship, if any, between the inv (1) (p36.1p36.3) in the father and 1p36.33-p36.32 duplication in the patient. Was CGH performed on the parents? If not, why not? If so what were the results?**

This is a repeated question of Major 6). Although we do understand the value, we were unable to conduct CGH studies for the parents since they did not agree with our further analyses.

**Others (from the authors):**

- We corrected "sex-cord stromal tumor " in Title, Abstract and Text to use "sex cord-stromal tumor" for precise terminology.
- In Abstract, we corrected the description of deletion coordinate (chr2:242,674,807-234,275,216) to (chr2:234,275,216-242,674,807).
- Along with the upgraded discussion, the order of citations were reorganized and the following citations were added:
  [27] Marks P, Rifkind RA, Richon VM, Breslow R, Miller T, Kelly WK: Histone deacetylases and

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