PEER REVIEW HISTORY

ARTICLE DETAILS

TITLE (PROVISIONAL)	SPECTRUM OF NOVEL MUTATIONS FOUND IN WAARDENBURG SYNDROME TYPE 1 AND TYPE 2: IMPLICATIONS FOR MOLECULAR GENETIC DIAGNOSTICS
AUTHORS	Wildhardt, Gabriele; Zirn, Birgit; Graul-Neumann, Luitgard; Wechtenbruch, Juliane; Suckfüll, Markus; Buske, Annegret; Bohring, Axel; Kubisch, Christian; Vogt, Stefanie; Strobl-Wildemann, Gertrud; Greally, Marie; Bartsch, Oliver; Steinberger, Daniela

VERSION 1 - REVIEW

REVIEWER	Yong Feng, Prof and Director of Department of Otolaryngology, Xiangya Hospital, Central South University, Changsha, Hunan, China. There are no competing interests.
REVIEW RETURNED	27-Sep-2012

THE STUDY	-The clinical imformation is too brief . For example, it's unclear the clinical phenotype of the affected family members; How many male and famale patients in this study and how many patients had unilateral or bilateral hearing loss respectively? -There are few references in recent 5 years. In addition, the mutation, p.Arg217Ile, is not a novel mutation which is reported previously(Chen H,Jiang L,Xie Z,et al.Novel mutations of PAX3,MITF,and SOX10 genes in Chinese patients with type Ⅰ or type Ⅱ Waardenburg syndrome. Biochemical and Biophysical Research Communications.2010, 18;397(1):70-74). - There is also an incomplete sentence on page 5, line 9.
RESULTS & CONCLUSIONS	The MS has a good originality and relatively high novelty. But Much of the discussion and information contained therein has been published previously. It might be useful to capture the quintessential information in a short report.

REVIEWER	Dr. Ignacio del Castillo, PhD Principal Investigator Unidad de Genetica Molecular Hospital Universitario Ramon y Cajal, IRYCIS CIBERER Madrid SPAIN
REVIEW RETURNED	19-Oct-2012

THE STUDY	1) The criteria of inclusion of patients in this study should be specified in more detail, and the number of cases with a clinical diagnosis of WS1 and WS2 should be reported as well. Taking into account Table 1, how could you include cases 6, 10 and 18 (n.a. in

	all columns describing their phenotypes) in this study? 2) Mutations in MITF are not a major cause of WS2 (Introduction, paragraph 3, line 1), since it is estimated that they only account for 15% of the cases (see references 1 and 18). The contribution of SNAI2 is controversial (see comment on this point in reference 18), but almost irrelevant anyway. Also, the involvement of SOX10 in WS2 is not mentioned (see Bondurand et al., Am J Hum Genet 2007; 81:1169). It is estimated that SOX10 mutations are found in 15% of WS2 cases. The Introduction section should include these data. 3) More information is needed about the MLPA assay that was used. Were you using the MRC Holland kit? If so, it should be mentioned; if not, the method should be described in more detail.
RESULTS & CONCLUSIONS	1) Mutations in MITF only account for 15% of the WS2 cases. The contribution of SNAI2 is controversial, but almost irrelevant anyway. It is estimated that SOX10 mutations are found in 15% of WS2 cases. Given this background, it is surprising that the authors have found MITF mutations in all their WS2 cases. If really so, this point should be discussed in detail in the Discussion section. But, are only elucidated cases being reported? 2) Some data are just provided without any indication of supporting evidence (see also comment 3). Results, paragraph 1, last sentence. How did you prove that the two mutations were in the same chromosome? By segregation analysis? Please specify. 3) No data are presented that really support the de novo origin of the mutations that are reported in the manuscript. Was paternity investigated? I would not ask for a formal paternity test, but at least 6-7 polymorphic microsatellite markers from different chromosomes should be examined.
REPORTING & ETHICS	In the Materials and Methods section, Patients, nothing is said about Informed Consents and/or approval of the study by any Ethics Committee.
GENERAL COMMENTS	This manuscript reports on 16 novel mutations in the PAX3 and MITF genes in cases of Waardenburg syndrome, which is a significant contribution to the spectra of mutations in these genes. Major comments 1) In the Materials and Methods section, Patients, nothing is said about Informed Consents and/or approval of the study by any Ethics Committee. 2) The criteria of inclusion of patients in this study should be specified in more detail, and the number of cases with a clinical diagnosis of WS1 and WS2 should be reported as well. Taking into account Table 1, how could you include cases 6, 10 and 18 (n.a. in all columns describing their phenotypes) in this study? 3) Mutations in MITF are not a major cause of WS2 (Introduction, paragraph 3, line 1), since it is estimated that they only account for 15% of the cases (see references 1 and 18). The contribution of SNAI2 is controversial (see comment on this point in reference 18), but almost irrelevant anyway. Also, the involvement of SOX10 in WS2 is not mentioned (see Bondurand et al., Am J Hum Genet 2007; 81:1169). It is estimated that SOX10 mutations are found in 15% of WS2 cases. The Introduction section should include these data.

	Given this background, it is surprising that the authors have found MITF mutations in all their WS2 cases. If really so, this point should be discussed in detail in the Discussion section. But, are only elucidated cases being reported? This issue is related to major comment 2. 4) Some data are just provided without any indication of supporting evidence (see also major comment 5). Results, paragraph 1, last sentence. How did you prove that the two mutations were in the same chromosome? By segregation analysis? Please specify. 5) No data are presented that really support the de novo origin of the mutations that are reported in the manuscript. Was paternity investigated? I would not ask for a formal paternity test, but at least 6-7 polymorphic microsatellite markers from different chromosomes should be examined. Minor comments 1) Abstract, Objectives, Line 3 (and also in Page 2, Article Focus). Craniofacial dysmorphism is found only in WS1 and WS3, and so it is not a general feature of WS. 2) Abstract, Results. Last sentence is a comment, not a result, and it should be deleted or rephrased. 3) Abstract, Conclusion. The abbreviation "TS" should be avoided. 4) Introduction, paragraph 1, last line. Complete the last sentence "in addition to WS2", since WS4 does not include the craniofacial or limb malformations that are characteristic of WS1 or WS3. 5) More information is needed about the MLPA assay that was used. Were you using the MRC Holland kit? If so, it should be mentioned; if not, the method should be described in more detail. 6) Page 6, paragraph 3, line 4. Was the parent affected or not? 7) Page 6, paragraph 3, lines 6-7. Figures 1 and 2 do not summarize all previously published mutations, just their locations. 8) Nomenclature of mutations should be checked with the freely available Mutalyzer software. The names of the p.Arg37fs and p.Ser197fs mutations are incomplete. 9) The severity of the hearing impairment should be reported according to the standard rules (mild-moderate-severe-profound). What does (+) mean in Table 1, case 1? 10) Page 8, line 4. "All of these presented hearing" ?? 11) Page 8, last line. The sentence should be completed: "loss of protein function leading to haploinsufficiency seems to be the disease-causing mechanism for WS1". The mechanism is haploinsufficiency, not loss of function (one allele remains functional). 12) Some typos: caucasian (Caucasian; abstract, design and patients, line 1); fotographs (photographs; abstract, design and patients, line 5); german (German; abstract, setting, line 1); larger deletions (large deletions; page 2, key messages, and page 8, last paragraph, line 4).

VERSION 1 – AUTHOR RESPONSE

Reviewer 1:
1) Every single clinical feature of the affected family members is described in table 1. We listed expression of inclusion criteria for the molecular analyses such as dystopia canthorum, hetero-chromia iridis, and hearing loss. Additional clinical information such as uni- or bilateral hearing loss or other symptoms are presented in the respective rows of the table. We included in this table another column which contains information concerning the gender of the patient.
2) We included the reference (Chen H et al.) and added the distinct mutation in table 1 as suggested.
3) We changed the sentence on page 5, line 9 into:
Products were separated electrophoretically and the signals captured by a CCD camera. To evaluate quantity and size of the fragments the software Sequence Pilot (JSI Medical Systems, Kippenheim, Germany) was used.

Reviewer 2:
Major comments
1) We added to the manuscript the sentence: Clinical information and specimens were obtained with informed consent in accordance with German law for genetic diagnostics.
2) Molecular analyses was done for patients if a clinical suspicion of Waardenburg syndrome occured. Inclusion criterion for the description of patients in this manuscript was the detection of a mutation in the genes investigated for the respective study population. We did not evaluate the ratio of cases with molecular findings in the analyzed genes versus the total number of cases with clinical suspicion that has given the indication for the molecular analyses.
We added the clinical designation and therefor indication for the analyses for the cases 6 and 18. For case 10 details concerning phenotypic expression of the leading criteria and additional clinical symptoms are included in the table.
3) The latter situation is the basis of the study design that we have focused in our manuscript: As described above, with PAX3 and MITF analyses elucidated cases were described. As mentioned before, with our study we had not the intention to evaluate the ratio of cases with molecular findings in all genes ever described to be associated with any form of WS versus the total number of cases with clinical suspicion that has given the indication for the molecular analyses.

We included the reference that describes SOX 10 mutations as a cause for WS2 in the introduction of our manuscript.
4) The indication is now given in the table. We have also added the information concerning the segregation analyses in the section “results” of the manuscript.
5) The description de novo was used in terms of consideration of provided information concerning kinship.

Minor comments
1) We changed this sentence according to these suggestions in: … is clinically characterized by congenital hearing loss, pigmentation anomalies and depending on the subtype of the disease, presence or absence of craniofacial dysmorphism.
2) Intention of the last sentence was to describe a conclusion of our genotype-/phenotype analysis. We have indicated this and the nature of this information now as following:
Furthermore, our genotype-/phenotype analyses indicate that WS without dystopia (WS2) and TS correspond to a clinical spectrum that is influenced by MITF mutation type and position.
3) We replaced TS by Tietz syndrome.
4) We completed the sentence as suggested by the reviewer. “WS4 or Waardenburg-Shah syndrome has features of Hirschsprung disease in addition to WS2.”
5) We added the following information:
For this purpose MRC-Holland® SALSA MLPA P186 was used.
6) We added the information in this sentence: …shown to be inherited from one parent that presented

signs and symptoms of WS), whereas…
7) Figure 1 and 2 summarize the positions of all mutations described so far. The focus was to depict the location of all these gene variations.
8) For the description of the mutations the mutation nomenclature and recommendations of the Human Genome Variation Society HGVS were used: http://www.hgvs.org/mutnomen/recs-prot.html#fs
9) The symbols to indicate these standard rules are now explained in the legend of the table. In addition the explanation of the (+) is added here.
10) This sentence is completed now: “All of these presented hearing loss.”
11) Since loss of protein function and haploinsufficiency have not the same meaning we realized the suggestion of the reviewer as following: Therefore, loss of protein function respectively haploinsufficiency seems to be the disease causing mechanism for WS1.
12) All typos were corrected.

VERSION 2 – REVIEW

REVIEWER	Professor Feng Yong, Department of Otolaryngology, Xiangya Hospital, Central South University, Changsha, Hunan, China. I declare no conflicts of interest.
REVIEW RETURNED	20-Dec-2012

- The reviewer completed the checklist but made no further comments.

REVIEWER	Ignacio del Castillo Principal Investigator Unidad de Genetica Molecular Hospital Ramon y Cajal Madrid Spain No competing interests
REVIEW RETURNED	10-Dec-2012

GENERAL COMMENTS	The authors have complied with some of the requests in my previous referee's report, but some important comments have not been addressed, as follows. 1) In this class of study, it is not acceptable to present only cases who were positive in the molecular screening. The authors are not presenting a case, but a large cohort. The proportion of patients with a clinical diagnosis of WS who were confirmed (and those who were not) at the molecular level is meaningful. It allows comparison between cohorts of different studies, it allows to check the accuracy of the clinical criteria of inclusion, and it indicates how many cases remain unelucidated (who are candidate subjects for investigating the hypothetical implication of novel genes in the syndrome). I do not understand the bizarre reticence of the authors to present the whole data, since for sure they know how many cases they screened at the molecular level. Accordingly, it is also not acceptable to indicate "clinical designation" as a criterion for including cases 6 and 18. This "clinical designation" is for sure based on clinical findings that should be reported in Table 1. 2) In the legend to Table 1, for hypothetically de novo mutations, it should be added that "no paternity testing was performed". I

	recommend that "de novo?" should replace "de novo" for patients 8, 13 and 19 in Table 1. 3) Page 7, lines 2-3. I must insist on changing the sentence to say "The positions of these mutations and all previously published mutations are summarized...." 4) The authors have used the "short description" format of HGVS rules. I strongly recommend to use the "long description" format, which is much more informative, and can be checked by using the Mutalyzer software. Note that this also allows to detect incorrect names. For example, c.111dupC in PAX3 results in p.Val38Argfs*76. In its short form, it would be p.Val38fs (instead of p.Arg37fs). 5) Page 8, lines 5-6. "Loss of protein function respectively haploinsufficiency seems to be the disease causing mechanism for WS1". Please rephrase this sentence, it can be hardly understood. 6) Table 1. Reporting two sequence variants in the same allele should follow the HGVS rules: [c.28T>A; c.33+6del7] (patient 11).

VERSION 2 – AUTHOR RESPONSE

1) Reviewer:
In this class of study, it is not acceptable to present only cases who were positive in the molecular screening. The authors are not presenting a case, but a large cohort. The proportion of patients with a clinical diagnosis of WS who were confirmed (and those who were not) at the molecular level is meaningful. It allows comparison between cohorts of different studies, it allows to check the accuracy of the clinical criteria of inclusion, and it indicates how many cases remain unelucidated (who are candidate subjects for investigating the hypothetical implication of novel genes in the syndrome). I do not understand the bizarre reticence of the authors to present the whole data, since for sure they know how many cases they screened at the molecular level.

Authors:
We apologize, if the presentation of our observations can be understood as a resistance against other, of course highly plausible and meaningful forms of data presentation as the reviewer suggested.
Our data were generated in a clinical context in combination with a request for molecular genetic diagnostics. We have collected the clinical data of this cohort of patients that are tested to be positive for a mutation in the genes investigated over a time period of 5 years. In this period, we performed the molecular diagnostics for more than 120 patients referred from more than 60 clinicians from all over the world. The reviewer is perfectly right, that it allows checking the accuracy of the clinical criteria of inclusion, if the cohort of mutation positive and the cohort of mutation negative can be compared. However, it would have been beyond our resources, to obtain for all mutation negative patients clinical data of satisfying quality. Thus, we have concentrated on the spectrum of mutations compared with the clinical expression of symptoms. This led us at least to the observation that previously not found or described mutations that we have identified are associated with the clinical phenotype of Waardenburg syndrome and that the frequency of certain mutation types (deletions/duplications) could have consequences for rational procedures for molecular genetic diagnostics procedures. Furthermore we presented, that one previously decribed position in MITF-gene which was shown to be mutated in a patient with Tietz syndrome can be also affected in patients with WS. Therefore, both WS2 and TS Tietz syndrome most probably correspond to a common clinical spectrum that is influenced by mutation type and position.

We think that this is worth to communicate it for the clinical and diagnostic community. Although we could not compare with the mutation negative cohort, which would led to other meaningful results without a doubt, our findings based on the concentrated view on the mutation positive cohort seem to us a relevant result that was hard enough to obtain for this relative rare disorder.

2) Reviewer:
Accordingly, it is also not acceptable to indicate "clinical designation" as a criterion for including cases 6 and 18. This "clinical designation" is for sure based on clinical findings that should be reported in Table 1.

Authors:
We added more clinical description for the cases 6 and 18 in the table.

3) Reviewer:
Page 7, lines 2-3. I must insist on changing the sentence to say "The positions of these mutations and all previously published mutations are summarized...."

Authors:
We have changed that as suggested in the manuscript.

4) Reviewer:
The authors have used the "short description" format of HGVS rules. I strongly recommend to use the "long description" format, which is much more informative, and can be checked by using the Mutalyzer software. Note that this also allows to detect incorrect names. For example, c.111dupC in PAX3 results in p.Val38Argfs*76. In its short form, it would be p.Val38fs (instead of p.Arg37fs).

Authors:
We have changed that as suggested in the manuscript.

5) Reviewer:
Page 8, lines 5-6. "Loss of protein function respectively haploinsufficiency seems to be the disease causing mechanism for WS1". Please rephrase this sentence, it can be hardly understood.

Authors:
We have changed that as suggested in the first review.

6) Reviewer:
Table 1. Reporting two sequence variants in the same allele should follow the HGVS rules: [c.28T>A; c.33+6del7] (patient 11).

Authors:
We have changed that as suggested in the manuscript.

VERSION 3 - REVIEW

REVIEWER	Dr. Ignacio del Castillo, PhD Principal Investigator Unidad de Genetica Molecular Hospital Universitario Ramon y Cajal Madrid

	Spain
REVIEW RETURNED	06-Feb-2013

GENERAL COMMENTS	The authors have modified the manuscript according to the criticisms in my previous reports. I have only a couple of minor comments: 1) I recommend to include a sentence in "Patients" explaining what the authors have replied to this referee about the composition of the cohort under study, i.e. that the 19 reported patients are those positive for mutation identification among over 120 cases with a clinical temptative diagnosis of Waardenburg syndrome, referred to their laboratory by many different clinicians. 2) Abstract, Results paragraph. "16" patients are mentioned here, but the right figure is 15. 3) Table 1. The "n.a." abbreviation should be explained.

VERSION 3 – AUTHOR RESPONSE

Reviewer:
1) I recommend to include a sentence in "Patients" explaining what the authors have replied to this referee about the composition of the cohort under study, i.e. that the 19 reported patients are those positive for mutation identification among over 120 cases with a clinical temptative diagnosis of Waardenburg syndrome, referred to their laboratory by many different clinicians.
Authors:
We have added that as suggested in the manuscript.

Reviewer:
2) Abstract, Results paragraph. "16" patients are mentioned here, but the right figure is 15.
Authors:
We have changed that as suggested in the manuscript.

Reviewer:
3) Table 1. The "n.a." abbreviation should be explained.
Authors:
We have added that as suggested in the manuscript.