PEER REVIEW HISTORY

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ARTICLE DETAILS

TITLE (PROVISIONAL)	Mutation screening of the TPO gene in a cohort of 192 Chinese patients with congenital hypothyroidism
AUTHORS	Fu, Chunyun; Xie, Bobo; Zhang, Shujie; Wang, Jin; Luo, Shiyu; Zheng, Haiyang; Su, Jiasun; Hu, Xuyun; Chen, Rongyu; Fan, Xin; Luo, Jingsi; Gu, Xuefan; Chen, Shaoke

VERSION 1 - REVIEW

REVIEWER	Hakan Cangül Istanbul Medipol University, Turkey
REVIEW RETURNED	14-Dec-2015

GENERAL COMMENTS	Accept in its current form

REVIEWER	H. Bikker Academic Medical Center, the Netherlands
REVIEW RETURNED	19-Jan-2016

GENERAL COMMENTS	Fu and co-authors present the results of the TPO gene analysis in a cohort of 192 Chinese patients with CH. There are some key points that I believe require adjustments to the manuscript. 1. The application of targeted NGS analysis is useful, but the choice of genes needs some adjustments. NKX2.5 is not associated with CH (see Engelen et al.,PLoS ONE 7(12): e52685) and DUOXA2 and FOXE1 are not included. This should at least be addressed. 2. Data about the quality of the NGS analysis are missing, e.g. horizontal and vertical coverage, minimal coverage/ subsequent sanger sequencing of low coverage fragments, sensitivity of the test. Were there also data about deletions and duplications in the tested genes? This could be detected in the comparison of the NGS data of all patients. If that is not possible MLPA should be considered. These data are important. 3. The mutations in DUOX2 (R681L and L1343F) are frequent snp's in the East Asian population; 0.4 and 0.6% in the Exome Aggregation Consortium (ExAC) database (http://exac.broadinstitute.org), the pathogenicity of these mutations is therefore (very) questionable.

	4. There is an inconsistency between fig.1 and the text. In the text pat.1 is called compound heterozygous for the DUOX2 variants R681L and L1343F, in fig.1 L1343F is not detected in the mother and seems therefore de novo? This is should be addressed in the text. 5. Patient 2 was not detected in a newborn screening? Since treatment started late, a severe fenotype is expected and probably not the result of multiple mutations (in DUOX2). The suggestion that multiple mutations in patient 1 account for a more severe phenotype is also unnecessary, because the absence of functional TPO protein, caused by a homozygous nonsense mutation explains the inability of the thyroid gland to produce thyroid hormone 6. The fact that in patient 2 three TPO mutations seem to be present is intriguing. In theory, the presence of two pathogenic mutations on one allele is not very likely. In theory, it is also possible that the mother is heterozygous for a deletion of at least the exon in which T561 is located. MLPA or other ways of del/dup analysis will be necessary to test this. Alternative, a snp underneath one of the PCR primers could also result in conflicting results between sequence analysis/ NGS analysis of the father and son. 7. In silico analysis never confirms a causative nature, these analyses are only suggestive for pathogenicity, co-segregation in a large family, or functional (in vitro) tests are necessary to prove pathogenicity. 8. ref10 is from 2010; see HGMD (professional) for recent data (about 100 published mutations in the TPO gene). 9. In line 51 p 3; c.1497G>A does not lead to p.R648Q. 10. The NM nrs should be included. 11. According to http://www.hgvs.org/mutnomen/ p.C808AfsX24 should be p.C808Afs*24. 12. Fig.3 T561 is conserved up till Tetraodon, so it is possible to add more species. 13. Fig.4 typo “tructural”, should be “structural”. Some arrows in the figure would be helpful for clarity.

REVIEWER	Héctor M. Targovnik Instituto de Inmunología, Genética and Metabolismo INIGEM CONICET-UBA
REVIEW RETURNED	16-Feb-2016

GENERAL COMMENTS	It is a scientifically acceptable paper from two cases of congenital hypothyroidism associated to TPO and DUOX mutations in Chinese population. Next generation sequencing analysis showed that patient 1 was homozygous for c.2422delT TPO mutation combined with compound heterozygous mutations in DUOX2 gene,

	p.R683L/p.L1343F, whereas patient 2 carrier a triallelic TPO mutatios, p.R648Q/p.T561M/p.T561M. p.T561M is a novel mutation and c.2422delT, p.R648Q, p.R683L/p.L1343F are previously published mutations. bioinformatics studies are indicated for the mutation, but not functional studies were performed. The conclusion is supported by the data presented. The only weakness is the lack of functional studies. There are only a minor comment. 1) It should be indicated in the table 1 the reference values for thyroid parameters.

VERSION 1 – AUTHOR RESPONSE

Responses to the reviewer's comments:
Reviewer 1
Reviewer 2:
1. Response to comment: (The application of targeted NGS analysis is useful, but the choice of genes needs some adjustments. NKX2.5 is not associated with CH (see Engelen et al.,PLoS ONE 7(12): e52685) and DUOXA2 and FOXE1 are not included. This should at least be addressed.)
Response: Our CH capture panel included FOXE1 gene but not DUOXA2, and according to the Reviewer's suggestion, we have revised this part as shown in the first paragraph of the Mutation detection and interpretation part:"CH capture panel as Illumina Truseq Custom Amplicon v1.5 kit was designed and included 10 known CH associated genes (TPO, TSHR, PAX8, NKX2.1, FOXE1, DUOX2, TG, SLC5A5, SLC26A4 and IYD) with complete coding regions and flanking intronic regions, and DUOXA2 gene is not included".
2. Response to comment: (Data about the quality of the NGS analysis are missing, e.g. horizontal and vertical coverage, minimal coverage/ subsequent sanger sequencing of low coverage fragments, sensitivity of the test. Were there also data about deletions and duplications in the tested genes? This could be detected in the comparison of the NGS data of all patients. If that is not possible MLPA should be considered. These data are important.)
Response: as shown in the first paragraph of Results part: "PCR-based targeted enrichment, Illumina Truseq Custom Amplicon, of 10 known CH genes followed by 500 cycles paired-end sequencing on an Illumina MiSeq was performed, resulting in a total of 4.77 million reads. The average coverage is more than 95%, all TPO exons' depth were successfully covered with >40X. In addition, no deletion or duplication of exon in TPO was detected by manual read depth inspection."
3. Response to comment: (The mutations in DUOX2 (R683L and L1343F) are frequent snp's in the East Asian population; 0.4 and 0.6% in the Exome Aggregation Consortium (ExAC) database (http://exac.broadinstitute.org), the pathogenicity of these mutations is therefore (very) questionable.)
Response: DUOX2 defects are commonly inherited in an autosomal recessive fashion, and the worldwide incidence of CH is about 1/2000. In some region the incidence of CH can be high to 1/575 (Rastogi MV, et al. Orphanet J Rare Dis, 2010). The two variants (R683L and L1343F) have been previously reported and considered as probable pathologic mutations, and they can also be detected in heterozygous carriers who are euthyroid.
4. Response to comment: (There is an inconsistency between fig.1 and the text. In the text pat.1 is called compound heterozygous for the DUOX2 variants R681L and L1343F, in fig.1 L1343F is not detected in the mother and seems therefore de novo? This is should be addressed in the text.)
Response: We have made correction according to the Reviewer's comments, as shown in the first paragraph of Results part: " patient 1 was homozygous for c.2422delT TPO mutation combined with double heterozygous DUOX2 mutations (p.R683L/p.L1343F)"
5. Response to comment: (Patient 2 was not detected in a newborn screening? Since treatment started late, a severe phenotype is expected and probably not the result of multiple mutations (in DUOX2). The suggestion that multiple mutations in patient 1 account for a more severe phenotype is also unnecessary, because the absence of functional TPO protein, caused by a homozygous

nonsense mutation explains the inability of the thyroid gland to produce thyroid hormone)
Response: We agreed your comment, and there is probably some truth to both views. Our study demonstrated that three even more variations in one or more CH associated gene can be found in one patient with more severe disease, thereby indicating that they may combinedly affect the phenotype of the individual. Recently a study (Satoh M, et al. J Pediatr Endocrinol Metab. 2015) also supported our suggestion.
6. Response to comment: (The fact that in patient 2 three TPO mutations seem to be present is intriguing. In theory, the presence of two pathogenic mutations on one allele is not very likely. In theory, it is also possible that the mother is heterozygous for a deletion of at least the exon in which T561 is located. MLPA or other ways of del/dup analysis will be necessary to test this. a snp underneath one of the PCR primers could also result in conflicting results between sequence analysis/ NGS analysis of the father and son.)
Response: We agreed your comment that the presence of two pathogenic mutations on one allele is not very likely. We try our best to recall the patient' s mother. Unfortunately, she refused to provide a DNA sample. Several SNP databases covering the broad range of population-specific SNP information have been utilized to prevent mismatching caused by unexpected SNPs in the designed primers, in particular the 3' end.
7. Response to comment: (In silico analysis never confirms a causative nature, these analyses are only suggestive for pathogenicity, co-segregation in a large family, or functional (in vitro) tests are necessary to prove pathogenicity.)
Response: We are sorry that there was no facility to conduct functional tests in our current settings. We try our best to recall the relatives of the patients for a co-segregation study. Unfortunately, most of them refused to provide DNA sample.
8. Response to comment: (ref10 is from 2010; see HGMD (professional) for recent data (about 100 published mutations in the TPO gene)
Response: We have made correction according to the Reviewer's comments, as shown in the second paragraph of Introduction part: " Up to now, about 100 mutations were reported and recorded in the HGMD (www.hgmd.cf.ac.uk/ac/index.php)"
9. Response to comment: (In line 51 p 3; c.1497G>A does not lead to p.R648Q.)
Response: We are sorry for this mistake. We have made correction according to the Reviewer's comments, as shown in the third paragraph of Abstract part: "..... c.1943C>T/p.R648Q in TPO".
10. Response to comment: (The NM nrs should be included.)
Response: We have added the NM nrs according to the Reviewer's suggestion, as shown in the second paragraph of Introduction part: " TPO (NM_000547)"
11. Response to comment: (According to http://www.hgvs.org/mutnomen/ p.C808AfsX24 should be p.C808Afs*24.)
Response: We have re-written this part according to the Reviewer's suggestion.
12. Response to comment: (Fig.3 T561 is conserved up till Tetraodon, so it is possible to add more species.)
Response: We have revised this part according to the Reviewer's suggestion, as in Fig.3.
13. Response to comment: (13.Fig.4 typo "tructural", should be "structural". Some arrows in the figure would be helpful for clarity.)
Response: We are sorry for this mistake. We have revised this part according to the Reviewer's suggestion, as in Fig.4.
Reviewer 3:
1. Response to comment: (It should be indicated in the table 1 the reference values for thyroid parameters.)
Response: Considering the Reviewer's suggestion, we have added the reference values as shown in the table 1.

VERSION 2 – REVIEW

REVIEWER	H.Bikker AMC, the Netherlands
REVIEW RETURNED	23-Mar-2016

GENERAL COMMENTS	The manuscript is now more clear and better readable.