

Mutation detection of *CYP21A2* gene in nonclassical congenital adrenal hyperplasia patients with premature pubarche

Mahsa Kolahdouz, Mahin Hashemipour¹, Hossein Khanahmad, Bahareh Rabbani², Mansoor Salehi, Ali Rabbani³, Arman Ansari¹, Mona Mobalegh Naseri

Department of Genetics and Molecular Biology, School of Medicine, ¹Department of Pediatric Endocrinology, Isfahan Endocrine and Metabolism Research Center, Isfahan University of Medical Sciences, Isfahan, ²Department of Biochemistry and Genetic Group, Faculty of Medical Sciences, Qazvin University of Medical Sciences, Qazvin, ³Growth and Development Research Center, Tehran University of Medical Sciences, Tehran, Iran

Abstract

Background: Congenital adrenal hyperplasia (CAH) due to mutations in the gene encoding 21-hydroxylase is one of common disease with an autosomal recessive form. In this study, our aim is to detect the prevalence of eight common mutations in nonclassical congenital adrenal hyperplasia (NCAH).

Materials and Methods: A total of 30 patients with clinical and laboratory evidence of NCAH was selected. Gene-specific polymerase chain reaction (PCR) without contamination of pseudogene was carried out, and PCR product of this step was used to amplification-refractory mutation system PCR on eight common mutations in *CYP21A2* gene.

Results: Two heterozygote patients for I2G mutation and six heterozygote patients for Q318X mutation is reported in our study. These mutations associated with the classic form of CAH, and heterozygotes presented with NC symptom, including premature pubarche and hirsutism.

Conclusion: There are some data about the association of the mutation with the clinical form of CAH including classic (salt-wasting and simple virilizing) and NC form. I2G and Q318X mutations were reported in classic form in homozygote state, but the heterozygote form associated with NC form. CAH diagnosis with NC symptom and with measurement of 17-hydroxyprogesterone as NCAH is not a trusted assessment and require to molecular analysis for accurate diagnosis.

Key Words: 21-hydroxylase deficiency, amplification-refractory mutation system polymerase chain reaction, *CYP21A2* gene, nonclassic congenital adrenal hyperplasia

Address for correspondence:

Dr. Hossein Khanahmad, Department of Genetics and Molecular Biology, School of Medicine, Isfahan University of Medical Sciences, Isfahan, Iran.

E-mail: Hossein_khanahmad@yahoo.com

Received: 14.02.2015, Accepted: 14.6.2015

INTRODUCTION

Congenital adrenal hyperplasia (CAH) is one of the most common inborn endocrine disorders^[1] with the autosomal recessive pattern because of 21-hydroxylase

deficiency (21-OHD).^[2] 21-OHD included about 95% of CAH cases and were the most common cause of ambiguous genitalia in girls.^[3] About 5–8% cases are due to 11- β hydroxylase deficiency.^[4] 21-hydroxylase

Access this article online	
Quick Response Code:	Website: www.advbiores.net
	DOI: 10.4103/2277-9175.178794

This is an open access article distributed under the terms of the Creative Commons Attribution-NonCommercial-ShareAlike 3.0 License, which allows others to remix, tweak, and build upon the work non-commercially, as long as the author is credited and the new creations are licensed under the identical terms.

For reprints contact: reprints@medknow.com

How to cite this article: Kolahdouz M, Hashemipour M, Khanahmad H, Rabbani B, Salehi M, Rabbani A, *et al.* Mutation detection of *CYP21A2* gene in nonclassical congenital adrenal hyperplasia patients with premature pubarche. *Adv Biomed Res* 2016;5:33.

is an enzyme of cytochrome P450, encoded by the *CYP21A2* gene.^[5] The *CYP21A2* is located on the short arm of chromosome 6 (6p21.3) adjacent to its inactive pseudogene (*CYP21A1P*) within the HLA region.^[6] The *CYP21A2* and *CYP21A1P* have 98% homology in exons and 96% in introns.^[3] Each gene and pseudogene contain 10 exons with 3.1 kb in length.^[7]

The pseudogene is situated about 30 kb upstream of *CYP21A2*. In humans, these two genes are located immediately next to the 3' end of the *C4A* and *C4B* genes encoding the fourth component of serum complement.^[5] Due to a high degree of sequence homology between gene and pseudogene, unequal crossover, and gene conversion events usually occur in *CYP21A2* gene and, therefore, generate a mutation in the gene.^[1]

Enzyme 21-hydroxylase catalyze the conversion of 17-hydroxyprogesterone (17-OHP) to 11-deoxycorticosterone. Therefore deficiency of 21-hydroxylase decreases the rate of this conversion and leading to androgen excess and also disruption the balance of cortisol and aldosterone.^[5] Disruption of cortisol synthesis induces adrenocorticotropic hormone (ACTH) secretion by the hypophysis.^[8] Two main clinical forms of the disease include: (1) Classic CAH which is clinically categorized in two groups, the simple virilizing (SV) and salt-wasting (SW) and (2) nonclassic (NC) form.^[9] The frequency of classic form is about 1 of 10,000 live births. Approximately, 30% of classic form show SV features and about 70% show additional SW. SW form, a fatal inability to preserve dietary sodium, has shown a severe defect in both cortisol and aldosterone synthesis.^[10] Ambiguous external genitalia in female newborn is seen in the SW form. Immediate diagnosis and treatment with mineralocorticoid and salt supplementation after birth result in reduced mortality and morbidity.^[11] All patients with classical or NC disease can be treated with glucocorticoids.^[12] Impairment of cortisol synthesis is recognized in SV, whereas aldosterone synthesis is in normal range. The masculinization of external genitalia represents in the female. In the NC or late-onset form, a delicate defect in cortisol synthesis can typically solely be detected throughout stimulation with corticotropin.^[13] Female NC 21-OHD patients do not exhibit genital ambiguity at birth and may reveal some signs of androgen excess in late childhood or early adulthood.^[14] The most common symptoms are premature pubarche in children, severe acne, hirsutism and decreased fertility.^[15] Precocious pseudopuberty can happen in both sexes, but it is comparatively rare. The prevalence for the NC form is 1:53 for Hispanics, 1:27 for Ashkenazi Jews, 1:333 for Italians and 1:1000 for other Caucasians.^[16]

The molecular genetic basis of CAH has been entirely investigated, and different mutations were classified based on 21-hydroxylase activity to predict the related phenotype of the affected individuals.^[17]

Approximately, 65–70% of *CYP21A2* mutations are deleterious and due to microconversion derived from pseudogene *CYP21A1P*^[18] including eight common mutations (p.P30L, c. 293-13A/C >G in intron 2 splice site, 8-bp deletion in exon 3, p.I172L, exon 6 cluster [p.I236N, p.V237E, p.M239K], p.V281L, p.Q318X, p.R356W).^[19] About 25–30% are caused by unequal meiotic crossovers (or deletions).^[18] Point mutations in compound state indicates SW form, although a single mutation has mild effect.^[20]

Across mutations detection in previous studies, p.Q318X and p.R356W had 0% enzyme activity, c. 293-13A/C >G with minimal residual activity and p.I172L with 2–11% enzyme activity were associated with classic form of disease.^[21]

A large number of methods have been expanded which can detect mutations in *CYP21A2*,^[5] such as restriction fragment length polymorphism,^[22] amplification-refractory mutation system (ARMS) polymerase chain reaction (PCR),^[23] Allele specific oligonucleotide^[24] and other methods. The biggest problem in *CYP21A2* molecular screening is very highly homology between gene and pseudogene causes to contamination of PCR products with pseudogene.

In this study, we report the results of molecular screening on eight most common mutations in *CYP21A2* gene in 30 NC CAH patients in Isfahan province.

MATERIALS AND METHODS

Patients and sample preparation

Thirty children with NC symptom of CAH from Isfahan province participated in our study. The patients were cumulated based on 17-OHP level before and after ACTH stimulation test and premature pubarche from Isfahan endocrine center. Informed consent was obtained from their parents. The patient's age ranged from 3 to 12 years. Basal 17-OHP >10 ng/ml was seen in 60% cases with premature pubarche and hirsutism. Value more than 20 ng/ml was reported only in one case. In other cases, 17-OHP was <10 ng/ml but with NC symptom.

The blood sample was obtained from them. DNA was extracted from peripheral blood samples by GeNet Bio DNA extraction kit (Korea) according to its protocol. Agarose gel electrophoresis and spectrophotometry were used for assessment of DNA quality and quantity.

Positive controls were received from Tehran children's medical center.

Mutation analysis

Because of high homology between gene and pseudogene, at first a primary couple PCR were performed for specific amplification of the *CYP21A2* gene and discriminate gene from pseudogene [Figure 1]. Primers which used for this step did not amplify *CYP21A1P*. The primers are shown in Table 1. The products of this step were used as a DNA template for ARMS PCR.

ARMS PCR was performed for the detection of eight common mutations in *CYP21A2* gene. Three PCR reaction for splice mutation in intron 2 are required because there are two wild-types and one mutant alleles. Allele-specific PCRs with specific primers were done [Table 2].

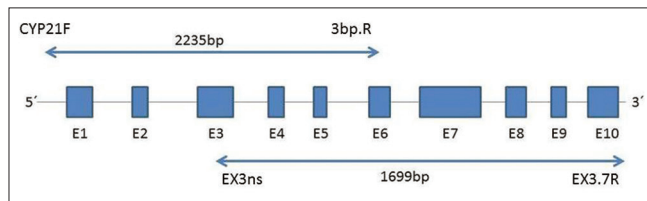


Figure 1: Typical polymerase chain reaction method for amplification of *CYP21* in two overlapping fragments with 2.2 and 1.7 kb

Table 1: The two couples primers for discrimination of gene from pseudogene

Primer	Sequence (5'→3')
CYP21F	F: ATTCCCAATTCTATTTTTTA
3bp.R	R: CTCAGCTGCATCTCCACGA
Ex3ns	F: CGGACCTGTCCTTGGGAGACTAC
EX3.7_R	R: CCAGCCTCCACCACATTTTGAC

Table 2: The primers used for allele specific PCR

Mutation	Forward primer (5'→3')	References	Reverse primer (5'→3')
P30L	5'-CCAGAGCCTCCACCTCCC-3' 5'-TCCAGAGCCTCCACCTCCT-3'		5'-GGAGCCTTTTGCTTGTC-3'
I2G	5'-TTCCACCCTCCAGCCCCAA-3' 5'-TTCCACCCTCCAGCCCCAC-3' 5'-TTCCACCCTCCAGCCCCAG-3'	[25] [25] [25]	5'-CCCTCCACTGGCCTGCCACG-3'
8bp-deletion	5'-CGGACCTGTCCTTGGGAGACTAC-3' 5'-ACTACCCGGACCTGTCCTTGTC-3'	[25] [25]	5'-CCAGCCTCCACCACATTTTGAC-3'
I172N	TTCTCTCCTCACCTGCAGCATCAT TTCTCTCCTCACCTGCAGCATCAA	[25] [25]	CCCTCCACTGGCCTGCCACG
EX6 cluster	TCACATCGTGGAGATGCAGCT AGGGACCACAACGAGGAGAA	[25]	GGAGCCTTTTGCTTGTC
V281L	ACAGCTCCTGGAAGGGCAGC ACAGCTCCTGGAAGGGCACT		CCAGCCTCCACCACATTTTGAC
Q318X	TTCGTGGTCTAGCTCCTCTG TTCGTGGTCTAGCTCCTCTA	[25] [25]	GAGGGATCACATCGTGGAGAT
R356W	CTAAGAGCACAACGGGCCG CTAAGAGCACAACGGGCCA	[25] [25]	TAAGAACTACCCGGACCTGTCC

PCR: Polymerase chain reaction

Direct sequencing

To confirm the results of allele-specific PCR, some heterozygote samples have been sequenced. The PCR products were purified and sequenced with SeqF: CCACCTCAGCCTCAAGTGT and Ex3ns: CGGACCTGTCCTTGGGAGACTAC primers.

RESULTS

DNA samples of 30 patients in Isfahan were analyzed for the existence of eight common mutations in *CYP21A2* gene. Approximately, 83% of cases were females and consanguinity was seen in 23% of patients. Based on clinical manifestation and biochemical tests of patients, all of them were NC form of CAH.

Results indicated heterozygosity in two patients for I2G mutation with clitoromegaly and premature pubarche manifestation and heterozygosity in some patients for Q318X mutation. Another mutation not found in our patients [Table 3].

This study suggested carriers with heterozygosity for I2G and Q318X mutations may represent as NC form.

DISCUSSION

This study was directed to detect eight common mutations in 30 patients from Isfahan province, Iran, with NC CAH by ARMS-PCR method.

The molecular diagnosis of the 21-OHD has a large pitfall due to the existence of a pseudogene. Almost all common mutations of *CYP21A2* are present in *CYP21A1P*. Contamination of gene with pseudogene causes false heterozygosity in all normal samples.

Table 3: The frequency of mutations in *CYP21* in our study

Mutation	Exon/intron	Nucleotide variation	Frequency (%)
I2G	Intron2	656A/C>G	3.3
Q318X	Exon8	1996C>T	15

Hence, discrimination of gene from pseudogene is a critical point in molecular detection of 21-OHD. To achieve reliable results, *CYP21A2* coding sequence has been divided to two overlapping fragments and amplified by two specific couple primers that amplify an only *CYP21A2* gene, and there was not any contamination with pseudogene. Some mutations have been identified using ARMS PCR on two overlapping amplified fragments to screen eight common mutations.

In according to other studies in Iran, which Q318X is most frequent,^[25,26] in our study also Q318X mutations is the most common in Isfahan, in contrast to one another study in Iran by Ramazani *et al.*^[23] and western European cohorts.^[27]

In the Anglo-Saxons a large deletion is prevalent; an R356W mutation is prevalent in the Croatians; a V281 L mutation is prevalent in Ashkenazi Jews; an IVS2 mutation is prevalent in the Middle-Eastern population and Iranians; and a Q318X mutation is prevalent in East Indians.^[28]

In 2014, Sharaf *et al.*^[19] analyzed I2G mutation by allele-specific PCR and reported 76% and 17.2% cases with heterozygous and homozygous mutation respectively. Another study in Macedonia population report 41.5% of patient with I2G mutation by PCR/ACRS method.^[22] This splice site mutation in intron 2 identify as one of the common mutation in Malaysian patients.^[29] This splice site mutation by activating another cryptic site in splicing activity change the premature mRNA splicing and, therefore, switch the reading frame.^[30]

In the Northeast Brazil and Tunisian, Q318X mutation is the most frequent mutation.^[1,31]

Six other mutations not found in the population of our study. It may require to a large sample size with all form of disease including SW and SV in addition to NC form of the disease. Moreover, it may be due to the prevalence of other common point mutations or deletion in Isfahan.

Various methods for *CYP21A2* point mutation detection was performed in several studies. The biggest problem in this survey is due to pseudogene, and it is important to prevent of pseudogene contamination

in the PCR-based method. In our study, according to previous studies^[7,32] gene amplified in two parts separating gene from pseudogene and then ARMS PCR for detection of four-point mutations had been used.

ARMS PCR is a rapid method for mutation detection. This method reduce time and cost rather than other methods which used in other study. However, this method is very sensitive and various condition including annealing temperature and primer density can eventuate to false negative or positive result. In our study, two patients with heterozygosity for I2G mutation were detected as homozygote with ARMS PCR, whereas direct sequencing was presented in heterozygote form.

Regarding to high incidence of CAH in Iran and Isfahan province, this highlights the importance of screening of CAH. Q318X and I2G mutations are seen more than other mutations in Iran population, therefore screening for these mutations can be the first step in newborn screening. If any patients are negative for these mutations, molecular diagnosis of other common mutations will assess.

Newborn screening (by molecular method) can predict the phenotypes type and reduce mortality due to SW form of the disease. Moreover, prenatal diagnosis can reduce ambiguous genitalia and genital virilization in the female fetus in SV form and premature puberche in NC form.

Although measurement of 17-OH progesterone with high level can determine form of CAH but cannot recognize SW from SV phenotype^[33] and also cannot detect carriers in the population. Moreover 17-OHP increases in stresses and can cause to false positive result. Therefore, molecular tests for accurate diagnosis of CAH and type of disease are required. The results of molecular diagnosis of CAH can be used in genetic counseling.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

REFERENCES

- Campos VC, Pereira RM, Torres N, Castro Md, Aguiar-Oliveira MH. High frequency of Q318X mutation in patients with congenital adrenal hyperplasia due to 21-hydroxylase deficiency in northeast Brazil. *Arq Bras Endocrinol Metabol* 2009;53:40-6.
- Stikkelbroeck NM, Hoefsloot LH, de Wijs IJ, Otten BJ, Hermus AR, Sistermans EA. *CYP21* gene mutation analysis in 198 patients with

- 21-hydroxylase deficiency in The Netherlands: Six novel mutations and a specific cluster of four mutations. *J Clin Endocrinol Metab* 2003;88:3852-9.
3. New MI, Wilson RC. Steroid disorders in children: Congenital adrenal hyperplasia and apparent mineralocorticoid excess. *Proc Natl Acad Sci U S A* 1999;96:12790-7.
 4. Witchel SF, Smith R, Suda-Hartman M. Identification of CYP21 mutations, one novel, by single strand conformational polymorphism (SSCP) analysis. *Mutations in brief no 218*. Online. *Hum Mutat* 1999;13:172.
 5. Cantürk C, Baade U, Salazar R, Storm N, Pörtner R, Höppner W. Sequence analysis of CYP21A1P in a German population to aid in the molecular biological diagnosis of congenital adrenal hyperplasia. *Clin Chem* 2011;57:511-7.
 6. Vrzalová Z, Hrubá Z, St'ahlová Hrabincová E, Pouchlá S, Votava F, Koloušková S, *et al.* Identification of CYP21A2 mutant alleles in Czech patients with 21-hydroxylase deficiency. *Int J Mol Med* 2010;26:595-603.
 7. Concolino P, Mello E, Minucci A, Giardina E, Zuppi C, Toscano V, *et al.* A new CYP21A1P/CYP21A2 chimeric gene identified in an Italian woman suffering from classical congenital adrenal hyperplasia form. *BMC Med Genet* 2009;10:72.
 8. Siegel SF, Hoffman EP, Trucco M. Molecular diagnosis of 21-hydroxylase deficiency: Detection of four mutations on a single gel. *Biochem Med Metab Biol* 1994;51:66-73.
 9. Rabbani B. Homozygous complete deletion of CYP21A2 causes a simple virilizing phenotype in an Azeri child. *Asian Biomed* 2011;5:889.
 10. Lako M, Ramsden S, Campbell RD, Strachan T. Mutation screening in British 21-hydroxylase deficiency families and development of novel microsatellite based approaches to prenatal diagnosis. *J Med Genet* 1999;36:119-24.
 11. Therrell BL. Newborn screening for congenital adrenal hyperplasia. *Endocrinol Metab Clin North Am* 2001;30:15-30.
 12. Speiser PW, White PC. Congenital adrenal hyperplasia. *N Engl J Med* 2003;349:776-88.
 13. Speiser PW, Dupont J, Zhu D, Serrat J, Buegeleisen M, Tusie-Luna MT, *et al.* Disease expression and molecular genotype in congenital adrenal hyperplasia due to 21-hydroxylase deficiency. *J Clin Invest* 1992;90:584-95.
 14. Bas F, Kayserili H, Darendeliler F, Uyguner O, Günöz H, Yüksel Apak M, *et al.* CYP21A2 gene mutations in congenital adrenal hyperplasia: Genotype-phenotype correlation in Turkish children. *J Clin Res Pediatr Endocrinol* 2009;1:116-28.
 15. Krone N, Roscher AA, Schwarz HP, Braun A. Comprehensive analytical strategy for mutation screening in 21-hydroxylase deficiency. *Clin Chem* 1998;44:2075-82.
 16. Speiser PW, Dupont B, Rubinstein P, Piazza A, Kastelan A, New MI. High frequency of nonclassical steroid 21-hydroxylase deficiency. *Am J Hum Genet* 1985;37:650-67.
 17. Wilson RC, Mercado AB, Cheng KC, New MI. Steroid 21-hydroxylase deficiency: Genotype may not predict phenotype. *J Clin Endocrinol Metab* 1995;80:2322-9.
 18. Xu Z, Chen W, Merke DP, McDonnell NB. Comprehensive mutation analysis of the CYP21A2 gene: An efficient multistep approach to the molecular diagnosis of congenital adrenal hyperplasia. *J Mol Diagn* 2013;15:745-53.
 19. Sharaf S, Hafez M, ElAbd D, Ismail A, Thabet G, Elsharkawy M. High frequency of splice site mutation in 21-hydroxylase deficiency children. *J Endocrinol Invest* 2014;38.5:505-511
 20. Haider S, Islam B, D'Atri V, Sgobba M, Poojari C, Sun L, *et al.* Structure-phenotype correlations of human CYP21A2 mutations in congenital adrenal hyperplasia. *Proc Natl Acad Sci U S A* 2013;110:2605-10.
 21. Krone N, Braun A, Roscher AA, Knorr D, Schwarz HP. Predicting phenotype in steroid 21-hydroxylase deficiency? Comprehensive genotyping in 155 unrelated, well defined patients from southern Germany. *J Clin Endocrinol Metab* 2000;85:1059-65.
 22. Anastasovska V, Kocova M. Intron 2 splice mutation at CYP21 gene in patients with congenital adrenal hyperplasia in the republic of Macedonia. *Balkan Journal of Medical Genetics* 2010;13:27-33.
 23. Ramazani A, Kahrizi K, Razaghiyar M, Mahdieh N, Koppens P. The frequency of eight common point mutations in CYP21 gene in Iranian patients with congenital adrenal hyperplasia. *Iran Biomed J* 2008;12:49-53.
 24. Lobato MN, Ordóñez-Sánchez ML, Tusié-Luna MT, Meseguer A. Mutation analysis in patients with congenital adrenal hyperplasia in the Spanish population: Identification of putative novel steroid 21-hydroxylase deficiency alleles associated with the classic form of the disease. *Hum Hered* 1999;49:169-75.
 25. Vakili R, Baradaran-Heravi A, Barid-Fatehi B, Gholamin M, Ghaemi N, Abbaszadegan MR. Molecular analysis of the CYP21 gene and prenatal diagnosis in families with 21-hydroxylase deficiency in northeastern Iran. *Horm Res Paediatr* 2005;63:119-24.
 26. Rabbani B, Mahdieh N, Ashtiani MT, Larjani B, Akbari MT, New M, *et al.* Mutation analysis of the CYP21A2 gene in the Iranian population. *Genet Test Mol Biomarkers* 2012;16:82-90.
 27. White PC, Speiser PW. Congenital adrenal hyperplasia due to 21-hydroxylase deficiency. *Endocr Rev* 2000;21:245-91.
 28. Wilson RC, Nimkarn S, Domic M, Obeid J, Azar MR, Najmabadi H, *et al.* Ethnic-specific distribution of mutations in 716 patients with congenital adrenal hyperplasia owing to 21-hydroxylase deficiency. *Mol Genet Metab* 2007;90:414-21.
 29. Menabò S, Balsamo A, Baldazzi L, Barbaro M, Nicoletti A, Conti V, *et al.* A sequence variation in 3'UTR of CYP21A2 gene correlates with a mild form of congenital adrenal hyperplasia. *J Endocrinol Invest* 2012;35:298-305.
 30. Witchel SF, Bhamidipati DK, Hoffman EP, Cohen JB. Phenotypic heterogeneity associated with the splicing mutation in congenital adrenal hyperplasia due to 21-hydroxylase deficiency. *J Clin Endocrinol Metab* 1996;81:4081-8.
 31. Kharrat M, Tardy V, M'Rad R, Maazoul F, Jemaa LB, Refai M, *et al.* Molecular genetic analysis of Tunisian patients with a classic form of 21-hydroxylase deficiency: Identification of four novel mutations and high prevalence of Q318X mutation. *J Clin Endocrinol Metab* 2004;89:368-74.
 32. Pinterova L, Garami M, Pribilincova Z, Behulova R, Mezenska R, Lukacova M, *et al.* PCR based diagnosis of 21-hydroxylase gene defects in Slovak patients with congenital adrenal hyperplasia. *Endocr Regul* 2000;34:65-72.
 33. New MI, Abraham M, Gonzalez B, Domic M, Razzaghy-Azar M, Chitayat D, *et al.* Genotype-phenotype correlation in 1,507 families with congenital adrenal hyperplasia owing to 21-hydroxylase deficiency. *Proc Natl Acad Sci U S A* 2013;110:2611-6.

To,
The Editor

Covering Letter

Submission of Manuscript for publication

Dear Sir,

We intend to publish an article entitled

in your journal.

On behalf of all the contributors I will act and guarantor and will correspond with the journal from this point onward.

Prior presentation of the data reported in this manuscript:

Organisation

Place

Date

We have done sufficient work in the field to justify authorship for this manuscript.

We hereby transfer, assign, or otherwise convey all copyright ownership, including any and all rights incidental thereto, exclusively to the journal, in the event that such work is published by the journal.

Thank you,

Yours' sincerely,

Name of corresponding contributor

Signature

Title of the manuscript:

Title Page

Type of manuscript:

Running title:

Contributors:

	First name	Middle name initial	Last name	Highest academic degree	Names of departments and institutions (including city and state)	Email addresses
1						
2						
3						
4						
5						
6						

Corresponding Author:

Name:

Address:

Phone numbers:

Facsimile numbers:

E-mail address:

Total number of pages:

Total number of tables:

Total number of figures:

Total number of supplementary files:

Word counts: For abstract:

For the text:

Acknowledgement:

Conflict of interest:

Financial Support:

Contribution details (to be ticked marked as applicable):

Contributors' form

	Contributor 1	Contributor 2	Contributor 3	Contributor 4	Contributor 5	Contributor 6
Concepts						
Design						
Definition of intellectual content						
Literature search						
Clinical studies						
Experimental studies						
Data acquisition						
Data analysis						
Statistical analysis						
Manuscript preparation						
Manuscript editing						
Manuscript review						
Guarantor						