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Original Article (Pages: 7193-7200)

A Missense Mutation of G257A at Exon 3 in PEX7 CDS Was Responsible for the Incidence of Rhizomelic Chondrodysplasia Punctata Type 1

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Abstract

Background

Rhizomelic chondrodysplasia punctata (RCDP) type 1 is among of the rare autosomal recessive peroxisome biogenesis disorders caused by mutations in the *PEX7* gene. RCDP patients with the classic form of RCDP1 do not live more than 10- year.

Materials and Methods

In the present study, a two-year-old girl with skeletal abnormalities and dysmorphic facial appearance is reported to be suffered from RCDP. The patient's parents were second cousins and healthy and there was no similar case in the parents' family. *PEX7* gene was sequenced in the patient and her parents.

Results

A homozygous mutation, G257A, was identified *PEX7* in the genome of patient while the parents were compound heterozygous.

Conclusion

Taken together, clinical presentation and peroxisome profile of the patient suggested a missense mutation led to formation of a pathogenic *PEX7*, responsible for incidence of RCDP.

Key Words: Fibroblast, Peroxisome biogenesis disorder, *PEX7*, PTS2, RCDP.

*Please cite this article as: Alamatsaz M, Ghaedi K, Hashemi M, Shafeghati Y, Faghihi M, Nasr-Esfahani MH. A Missense Mutation of G257A at Exon 3 in PEX7 CDS Was Responsible for the Incidence of Rhizomelic Chondrodysplasia Punctata Type 1. Int J Pediatr 2018; 6(2): 7193-7200. DOI: **10.22038/ijp.2017.26197.2240**

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Received date: Nov.11, 2017; Accepted date: Dec.22, 2017

1- INTRODUCTION

Peroxisome biogenesis disorders (PBDs) belong to autosomal recessive inherited disorders which are genetically heterogeneous types with severe defects in peroxisome assembly and activity. PBDs are divided into two groups of Zellweger spectrum including Zellweger syndrome (ZS) and rhizomelic chondrodysplasia punctata (RCDP).

Rhizomelic chondrodysplasia punctata (RCDP) type 1 (OMIM, #215100) is an autosomal recessive disorder induced by a mutation in the *PEX7* gene (1). RCDP patients suffer from congenital cataracts, growth and mental retardation, symmetrical shortening of the thigh and arm (rhizomelia), epiphyseal cartilage (chondrodysplasia punctate) (2, 3).

Unfortunately, the prognosis of this disease is poor, hence results in early childhood death (3). Rhizomelic chondrodysplasia punctata type 1(RCDP1) patients compare to patients with Zellweger syndrome, have a higher level of phytanic acid and show a more severe defect in plasmologenesis. In these patients, peroxisomes lack of four enzymes, dihydroxyacetone phosphate acyltransferase (DHPAT), phytanic acid oxidase, thiolase and alkyl dihydroxyacetone phosphate synthase (ADAPS) as they could not enter to peroxisomes (4).

In fibroblast cells derived from RCDP 1 patients, the morphology of peroxisomes is normal, and the amount and content of the enzyme catalase and PMP70 are not altered compared with normal peroxisomes (5). However, peroxisomal localization of perxisomal targeting signals type 2 (PTS2) containing proteins are abrogated. The pathogenic responsible gene is *PEX7* which encodes a cytosolic receptor for peroxisomal sorting of PTS2 containing proteins. There are several hotspots which have been identified to be vulnerable to mutations in *PEX7* gene (6).

Here, we report a homozygous missense mutation, responsible for the onset of this RCDP type 1 case which suffered from a missense mutation of G257A at exon 3 in *PEX7* CDS.

2- MATERIALS AND METHODS

2-1. Ethical issue

This study was approved by the ethical committee of Royan Institute while the informed consent of the patient's parents was obtained by our colleagues in Sarem Hospital, Tehran, Iran. Also, there was no intervention done on patient to declare in this study.

2-2. Patient

The patient was a two-year-old girl, born to first-degree consanguineous couple who showed the typical clinical characteristic of RCDP type 1, including the shortening of the upper extremities (rhizomelia), bilateral congenital cataract, stippled epiphyses (chondrodysplasia punctata), joint stiffness, microcephaly, depressed nasal root, and high arched eyebrows with synophrys (**Figure.1**).

Patient's parents were second cousins and healthy and there was no similar case in the family of the parents. Approximately 5 mL of blood sample was taken from the patient and her parents for further analyses.

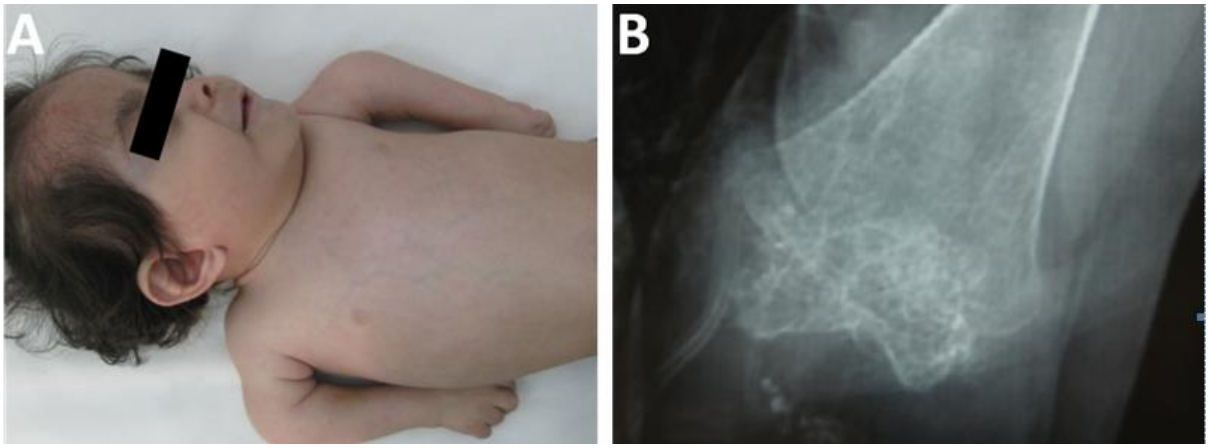

Fig.1: (A) RCDP1 patient, with severe main clinical manifestations related to RCDP type 1with a very short hand humerus. (B) X-ray radiograph of relatively short femora indicates a stippled appearance and a thickened shaft (chondrodysplasia punctata).

2-3. Whole-exome sequencing

Genomic DNA from the patient's blood was extracted by DNeasy Blood and Tissue Kit (Qiagen, Germany) and was send to whole-exome sequencing. Whole-exome sequence was performed with a next generation sequencing was performed to sequence close to 100 million reads on Illumina sequencer (Illumina, USA). Test platform examined >95% of the targeted regions with a sensitivity of above 99%. Bioinformatics analysis of the sequencing results was performed using international databases and standard bioinformatics software.

2-4. Skin cell culture

Skin biopsies of pro-band was carried out according to standard method and sample was transferred into in Dulbecco's modified Eagle's medium (DMEM; supplemented with 15% volume/volume (v/v) fetal bovine serum (FBS), and 1% (v/v) penicillin and streptomycin (all purchased from Thermo Fisher Scientific, UK). Fibroblasts were incubated at 37 ℃ in 5% CO_2 under a humid condition.

2-5. Mutation analysis

Polymerase chain reaction (PCR) and sequencing were used for the identification of the *PEX7* gene mutation at genomic level in patient and her parents. As described genomic DNA was extracted from the blood samples and amplification of the exon 3 was performed with a primer pair as: 5'-TTTTTTGTTGTGTAGCTGCC-3' (sense) and 5'-CGTTTAAACTTGTCCCCTGA-3' (anti sense). PCR products were directly purified and send for Sanger sequencing using the sense primer (Pishgam Company, Tehran, Iran). Meanwhile, total RNA from the cultured patient fibroblasts was extracted by RNeasy Mini Kit (Qiagen, Germany) followed by a cDNA synthesis step using Oligo (dT) 18 Primer according to the manufacturer's protocol (Thermo Fisher Scientific, UK). At the next step, patient derived *PEX7* CDS was amplified by PCR using primer pair 5′-AATGCTAGCGTCTGCCTGGTCTCTCT AAC-3′ (forward) and 5′-ACCCTCGAGCAACATCCTCTGTTTC TGAC-3′ (reverse) that amplified one 1100-bp fragment (7). Then, PCR product was sub-cloned into pTZ vector (Thermo Fisher Scientific, UK) and bi-directionally sequenced to get rid of undesired mutation during amplification. Finally *Nhe*I- *Xho*I fragment of pTZ vector containing the full length of patient derived *PEX7* coding sequence (CDS) was transferred to the

same sited in pDB2 as already described (7). In this study we also used pDB2 vector with the normal *PEX7* CDS.

2-6. Immunofluorescence microscopy

Normal and mutated *PEX7* CDS (N*PEX7* and M*PEX7*, respectively) were transfected into the patients derived fibroblast cells using Lipofectamine (LTX) (Invitrogen, USA) according to the manufacturer's instruction. To ensure of complementation by the N*PEX7*, fibroblast cells including patient derives fibroblasts and human normal fibroblasts (7) were stained against rabbit polyclonal anti human thiolase antibody. Briefly cells were fixed for 30 min with 4% paraformaldehyde in phosphate-buffered saline (PBS). Then, cells were washed and permeabilized one hour with 0.4% (v/v) Triton X-100 in PBS at 37 °C.

Both blocking and incubation with primary antibody (polyclonal rabbit anti-thiolase, 1:100 dilution; Abcam, UK) was performed simultaneously for 1 h at 37 °C. At next step, slides were washed several times with PBS and exposed to compatible secondary antibody (Fluorescein isothiocyanate [FITC] conjugated anti-rabbit Immunoglobulin G, 1:50 dilution, Sigma, USA) for 1.5 h at 37 °C. Finally, stained cells were analyzed with a fluorescent microscope (Olympus, Tokyo, Japan) and images were acquired with an Olympus DP70 camera (Olympus, Tokyo, Japan).

2-7. Western blot analysis

Normal and patient's fibroblast cells were lysed with TRI reagent (Sigma, USA) as previously described (7). Approximately, 30µg of extracted proteins was subjected to Sodium Dodecyl Sulfate PolyAcrylamide Gel Electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membrane. After blocking with 10% skim milk powder (w/v) and washing in Tris-buffered saline with Tween-20 (TBST), proteins were detected by subsequent incubation of the membrane with primary rabbit anti-thiolase antibody (1:5000 dilution; Abcam, UK) for 1.5 h. After several washings with TBST, membrane was incubated with goat anti-rabbit IgG (1:16000, Santa Cruz, USA) conjugated to horseradish peroxidase (HRP) in 2% skim milk at room temperature for 45 minutes with constant shaking. The same condition was used for mouse anti-β-tubulin antibody (1:2500; Sigma, USA) and goat anti-mouse IgG (1:5000, Dako, Japan) conjugated to horseradish peroxidase (HRP), as a loading control. Subsequently, membrane was washed with TBST and each protein band was visualized by an Amersham ECL Advance Western Blotting Detection Kit (GE Healthcare, USA) as described (7).

3- RESULTS

3-1. Mutational analysis of PEX7 in patient and her parents

Whole-exome sequencing of DNA revealed a homozygous missense mutation (G to A transition at nucleotide 257 in CDS of *PEX7*) resulting in an amino acid substitution C86Y in the first WD motif of Pex7p. As expected, patient's parents showed G>A mutation as heterozygous by direct sequencing of genomic DNA (**Figure.2**).

3-2. Dysfunction of patient derived PEX7

To confirm the obtained results of sequencing, and determine of patient derived Pex7 protein disability in import of PTS2 containing proteins into peroxisome, subcellular localization of peroxisomal 3-ketoacyl-CoA thiolase was assessed by immunostaining of cultured fibroblast cells transfected with either *MPEX7* cDNA or *NPEX7* cDNA. As indicated, normal fibroblast cells showed a punctuate fluorescence pattern due to localization of

thiolase in peroxisome (**Figure.3A**). Interestingly *MPEX7* CDS was not able to restore peroxisomal localization of thiolase as it showed an evenly distributed (cytosolic) fluorescence pattern (**Figure.3B**) despite of *NPEX7* CDS which its transfection resulted in complementation of thiolase sorting to peroxisomes (**Figure.3C**). Cytosolic fraction of thiolase is a type of unprocessed protein detectable as 44-kDa band in immunoblot of lysate cells. However, mature type of thiolase (41 kDa) translocates into the peroxisomes. This difference in molecular weight between the unprocessed precursor and mature protein could be readily detected by a mobility shift on immunoblots. Therefore, immunoblot analysis of peroxisomal thiolase was performed on lysate cells of normal and patient's fibroblasts. Data showed a mature type of thiolase in lysates of normal fibroblast cells while unprocessed 44 kDa precursor form of thiolase was found in lysate of patient's fibroblasts (**Figure.4**), which is in agreement with the defective peroxisome import of this protein in patient's fibroblast cells.

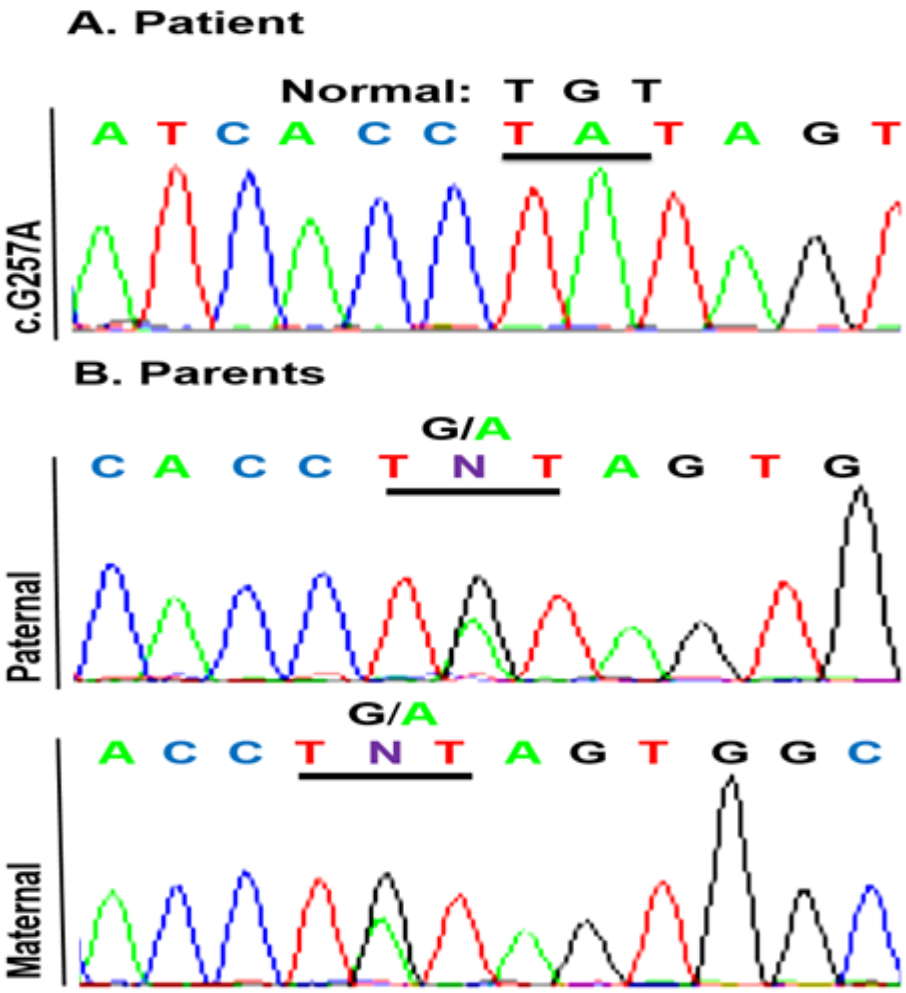

Fig.2: Genomic sequence analysis of a part of exon 3 in *PEX7* containing C86Y mutation from patient (A) and (B) her parents. The point mutation (G to A transition) was detected in patient, homozygously, and in parents, heterozygously.

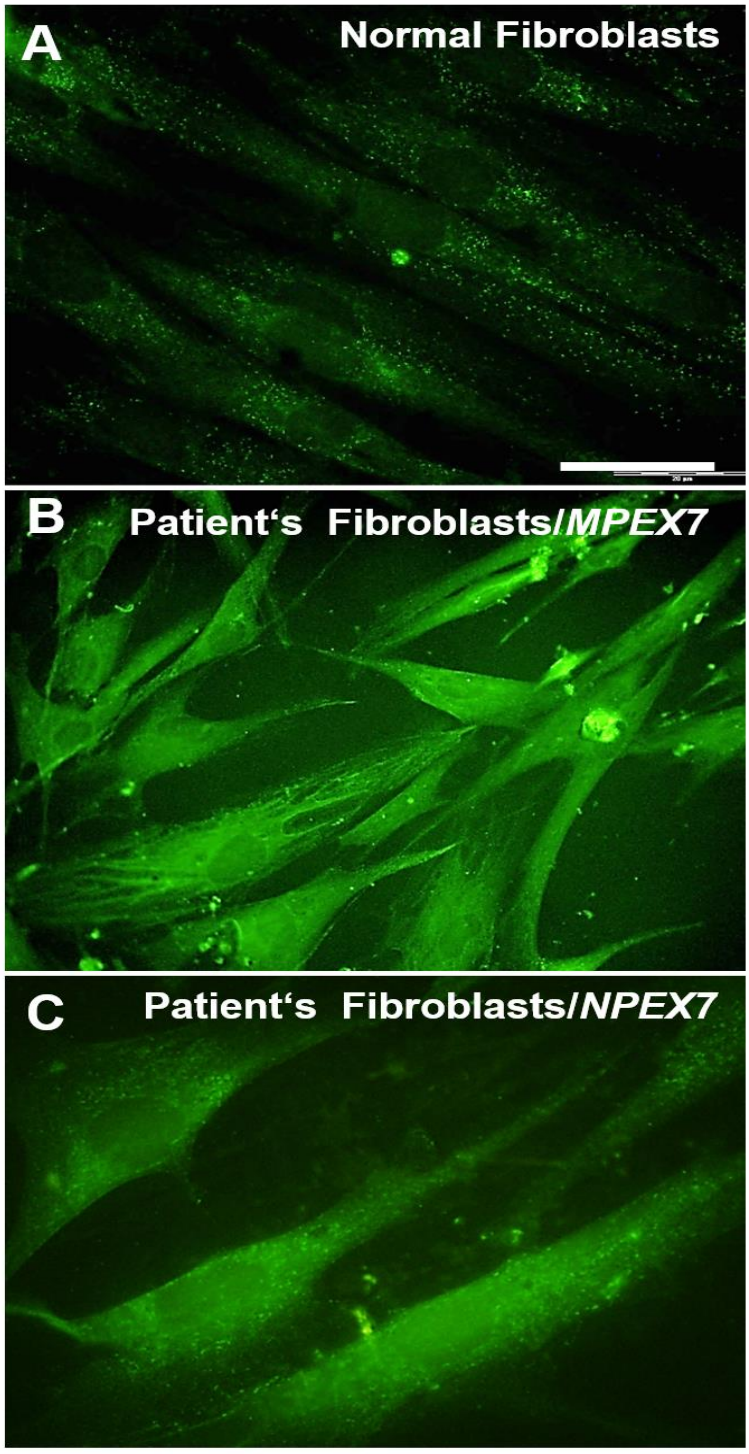

Fig.3: Immunofluorescent staining of thiolase in fibroblasts. Peroxisomal localization of peroxisomal 3-ketoacyl-CoA thiolase in normal fibroblast cells (A) compared to cytosolic pattern (B) of this enzyme in patient's fibroblast cells transfected with patient derived *PEX7* CDS (*MPEX7*) and restored punctuate pattern of thiolase (C) in patient's fibroblasts transfected with normal human *PEX7* CDS (*NPEX7*). Bar is equal to 50 µm.

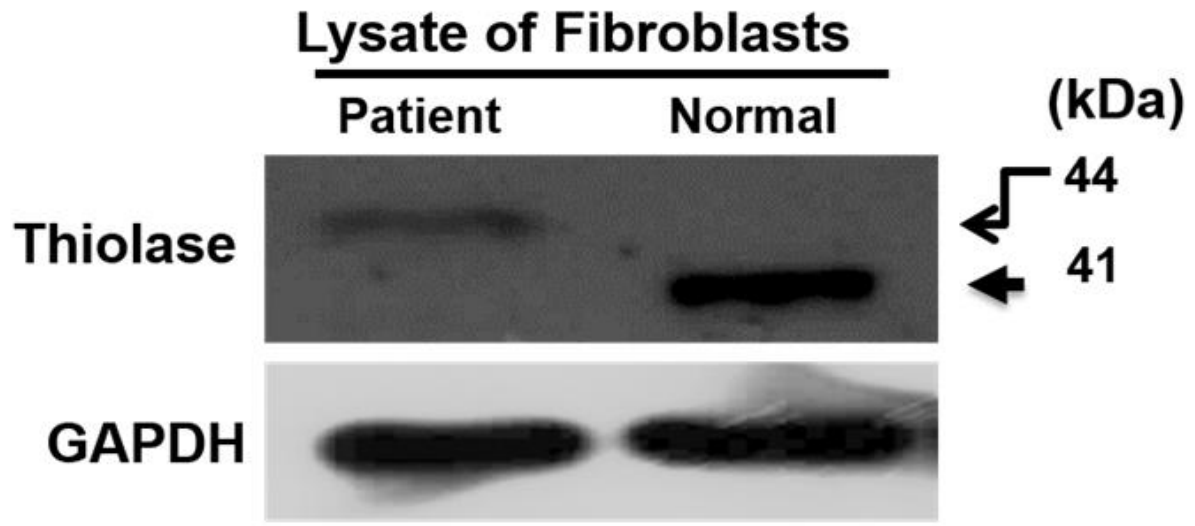

Fig.4: Western blotting analysis with an antibody against peroxisomal 3-ketoacyl-CoA thiolase. A mature form of thiolase (41 kDa) was detected in lysates of normal fibroblast cells, while an unprocessed precursor form of thiolase (44 kDa) was detected in lysate of patient's fibroblasts. The input of each lane was normalized with GAPDH antibody as the loading control marker.

4- DISCUSSION

RCDP1 is the most common phenotype of RCDP which is due to mutations in *PEX7* gene. Previous studies have shown a large mutational spectrum in the *PEX7* gene (8, 9). Braverman et al. have performed a wide mutation analysis of *PEX7* on a crowd of 60 RCDP pro-bands and identified a spectrum of genetic abnormalities (9). In a different study performed by Motely et al., multiple abnormalities that involved deletion, insertion, and missense, nonsense, and splice-site mutations were reported in *PEX7* structure (8). One of the missense mutations detected in that study was the substitution of G to A (at CDS nucleotide position 257) which caused an amino acid change (C86Y), similar to our reported publication (10). Interestingly, Motely et al. have reported this mutation, as a heterozygote mutation in genomic DNA analysis, in only one Dutch patient with an allele frequency of 0.7. We already indicated this mutation as a homozygous deterioration in both *PEX7* alleles of two Iranian patients (10).

5- CONCLUSION

In the present study we identified one patient with RCDP1 who was homozygous for the C86Y mutation responsible for Pex7p dysfunction. Although, the precise mechanism of the C86Y mutation in the loss of Pex7p functionality is still unclear, we suggest that C86Y mutation causes the targeting loss of PTS2 containing proteins to peroxisome including peroxisomal 3-ketoacyl-CoA thiolase. It also is possible that this mutation causes inconsistency of Pex7p. Considering the low incidence rate of RCDP in population, the presence of this mutation in this RCDP patient could be reflecting the occurrence of high frequency rate of this mutation in Iranian population which needs more attention. Therefore, we suggest studying more RCDP cases to confirm such hypothesis.

6- ABBREVIATIONS

ADAPS: Alkyl dihydroxyacetone phosphate synthase,
DHPAT: dihydroxyacetone phosphate acyltransferase,

DMEM: Dulbecco's modified Eagle's medium,
FBS: fetal bovine serum; HRP, horseradish peroxidase,
PBDs: Peroxisome biogenesis disorders; PBS, phosphate-buffered saline,
PTS2: perxisomal targeting signal type 2,
PVDF: polyvinylidene difluoride,
RCDP: rhizomelic chondrodysplasia punctate,
TBST: Tris-buffered saline with Tween-20, and
ZS: Zellweger syndrome.

7- AUTHORS CONTRIBUTIONS

Marziyeh Alamatsaz*:* Conception and design of the work, acquisition of data, analysis and interpretation of data, and drafted sections of manuscript.

Kamran Ghaedi*:* Contributed to design of work, analysis and interpretation of data, and finalized the manuscript.

Motahare-Sadat Hashemi*:* acquisition of data and data analysis.

Yousef Shafeghati*:* Acquisition of data and drafted sections of manuscript and finalized the manuscript.

Mohammad Faghihi*:* analysis and interpretation of data, Acquisition of data and drafted sections of manuscript and finalized the manuscript.

Mohammad Hossein Nasr-Esfahani*:* Contributed to design of work, analysis and interpretation of data, and finalized the manuscript.

8- CONFLICT OF INTEREST: None.

9- ACKNOWLEDGMENTS

All authors support submission of manuscript to this journal. There was no financial support to declare.

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