



# A Novel Pathogenic Haplotype in *CDH23* Causing DFNB12: The Combined Effect of Two Individually Benign Variants

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**Background:** Aberrations in cadherin-related 23 (*CDH23*) account for a significant proportion of familial autosomal recessive non-syndromic hearing loss (DFNB12), a common subtype of hereditary hearing loss worldwide.

**Aims:** To elucidate the molecular basis and pathogenic mechanism of DFNB12 in an affected girl from a nine-member pedigree.

**Study Design:** Family-based genetic study with pedigree analysis.

**Methods:** Clinical whole-exome sequencing combined with pedigree analysis was used to identify disease-causing mutations. The potential functional consequences of these mutations were investigated using structural bioinformatic approaches, including homology modeling, molecular dynamics simulations, and other relevant tools.

**Results:** The proband carried compound heterozygous variants: a known pathogenic maternal variant (c.6049G > A) and a paternal haplotype comprising two linked variants (c.3262G > A and c.6911G > A), each individually classified as benign. Pedigree segregation analysis demonstrated that the paternal haplotype acts as a single pathogenic allele.

**Conclusion:** Two individually benign variants can combine to form a novel pathogenic haplotype (c.3262A–c.6911A). This mechanism may be under-recognized in routine variant interpretation pipelines. Our findings underscore the importance of evaluating the combined effects of linked benign variants to ensure accurate genetic counseling.

## INTRODUCTION

Non-syndromic hearing loss (NSHL) is one of the most frequently observed neurosensory impairments in newborns worldwide.<sup>1,2</sup> Although NSHL can result from a wide range of causes, hereditary factors account for 50–60% of reported cases. Approximately 80% of NSHL cases are classified as autosomal recessive NSHL (DFNB12), which is associated with severe to profound hearing impairment.<sup>3,4</sup> To date, 105 loci linked to DFNB12 have been identified, involving 67 disease-causing genes (<http://hereditaryhearingloss.org>).<sup>5-7</sup> The most prevalent genes associated with hereditary hearing loss include *GJB2*, *SLC26A4*, *MYO15A*, *OTOF*, and cadherin-related 23 (*CDH23*).<sup>8</sup>

Among these, *CDH23* plays an important role in DFNB12. The gene is located on the long arm of chromosome 10 (10q22.1, Gene ID 64072)

and encodes *CDH23*, a glycoprotein involved in Ca<sup>2+</sup>-dependent cell–cell adhesion.<sup>9,10</sup> *CDH23* is highly expressed in the stereocilia of inner ear sensory neurons and in retinal photoreceptor cells.<sup>11,12</sup> It is often located at the junction of the inner and outer segments of cilia and participates in the calyx-like process of pyramidal photoreceptors.<sup>13</sup> *CDH23* plays a crucial role in maintaining cell connections, mediating cell communication, and ensuring proper morphogenesis of hair bundles in inner ear neurosensory cells.<sup>12</sup> Disruption of hair cell stereocilia in animal models, mimicking *CDH23* variants, results in noise-induced hearing loss and age-related deafness.<sup>14</sup>

Given its importance in auditory perception, deleterious mutations in *CDH23* account for most hearing loss in DFNB12 patients. However, some pathogenic variants remain undiscovered.



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## MATERIAL AND METHODS

### Patients and subjects

The study included members of a single pedigree, comprising one affected patient and eight unaffected healthy relatives. The proband was a 4-year-old girl who was first diagnosed with bilateral congenital profound hearing loss in 2019 during her initial hospital visit. The study was approved by the Ethics Committee of the Chongqing Medical University (approval number: 20200631, date: 20.05.2020). Written informed consent was obtained from all participants and their legal guardians. Clinical examinations, including auditory steady-state response (ASSR) audiogram, distortion product otoacoustic emission (DPOAE), and auditory brainstem response (ABR), were performed following standard clinical procedures in a double-blind manner.

### Clinical whole-exome sequencing (cWES), Sanger sequencing, and pedigree analysis

Genomic DNA from the proband was sequenced using the BioelectronSeq4000 platform at Capitalbio Medlab (Beijing, China) according to a standard clinical whole-exome sequencing (cWES) workflow (Supplementary Figure 1). Potential pathogenic variants identified through high-throughput sequencing were validated by PCR-Sanger sequencing in the proband and her family members. The sequence-specific primers used were as follows:

- For *CDH23* c.3262G > A: F, 5'-GCAGCTGCTAACACCTGTCT-3'; R, 5'-TTCAGGGATGTCCTCAGGGA-3'
- For *CDH23* c.6049G > A: F, 5'-CAAGACATCTACGCCGTGGT-3'; R, 5'-GTCCAGCACTGAGAAGGGAG-3'
- For *CDH23* c.6911G > A: F, 5'-CAAGCTGACTGTCAACGTCT-3'; R, 5'-TGTAGTGACCAGCCCATTA-3'

### Bioinformatics analyses of *CDH23* variants

Bioinformatics analyses are described in detail in the Supplementary Materials and Methods. The physicochemical properties of the protein were characterized using multiple parameters. A linear model was then proposed to evaluate the clinical outcomes of combined variants based on the quantification of multidimensional structural characteristics, including secondary structure, flexibility, root-mean-square deviation (RMSD), isoelectric point (pI)/molecular weight, stability, surface charge distribution, hydrogen bonding, and hydrophobicity. A scoring formula quantifying the potential deleterious effects of combined mutations on molecular function was defined as follows:

$$Score = \sum_{j=1}^m \sum_{i=1}^n k_{ij} (a_{1,j}, a_{2,j}, \dots, a_{i-1,j}, a_{i+1,j}, \dots, a_{n,j}, \dots) a_{ij}$$

$$i = 1, 2, \dots, n; j = 1, 2, \dots, m$$

The variable  $a_{ij}$  represents the effect of mutation on structural parameter  $i$ , which can be assigned a numeric value. The variable  $k_{ij}$  denotes the weight of  $a_{ij}$ , which is independent of other mutations and structural parameters in an additive model but dependent on them in a cooperative model. The value  $m$  indicates

the number of amino acid residue substitutions, and  $n$  denotes the number of structural parameters included in the model. The clinical significance of combined mutations can be inferred by comparing their scores with those of known variants, which serve as internal references.

## RESULTS

### Clinical description of hearing characteristics and genetic analyses

Nine family members—four males and five females—spanning three generations were recruited for this study. The proband was first identified as unresponsive to sound at 10 months of age. ABR, ASSR, and DPOAE tests confirmed severe hearing loss in the proband, leading to a diagnosis of congenital bilateral profound deafness (Figures 1a–c). No family history of congenital hearing loss was reported in the pedigree (Figure 1d). Temporal bone computed tomography and magnetic resonance imaging were performed to exclude potential inner ear malformations.

Subsequently, standard cWES was conducted on lymphocyte DNA extracted from the proband's peripheral blood. Compound heterozygous variants were identified in *CDH23*, including a maternal c.6049G > A variant and a paternal haplotype comprising c.3262G > A in linkage with c.6911G > A. All three loci were genotyped via Sanger sequencing in all nine family members (Supplementary Figure 2). The potential deleterious effects of these *CDH23* variants were assessed using conventional prediction tools typically included in the cWES protocol (Supplementary Tables 1 and 2).

According to the predictions, the c.6049G > A variant was classified as pathogenic or likely pathogenic, consistent with previous clinical reports. In contrast, the c.3262G > A and c.6911G > A variants were of uncertain significance or likely benign, making it difficult to draw definitive conclusions. Notably, the clinical significance of these two variants has not been previously reported.

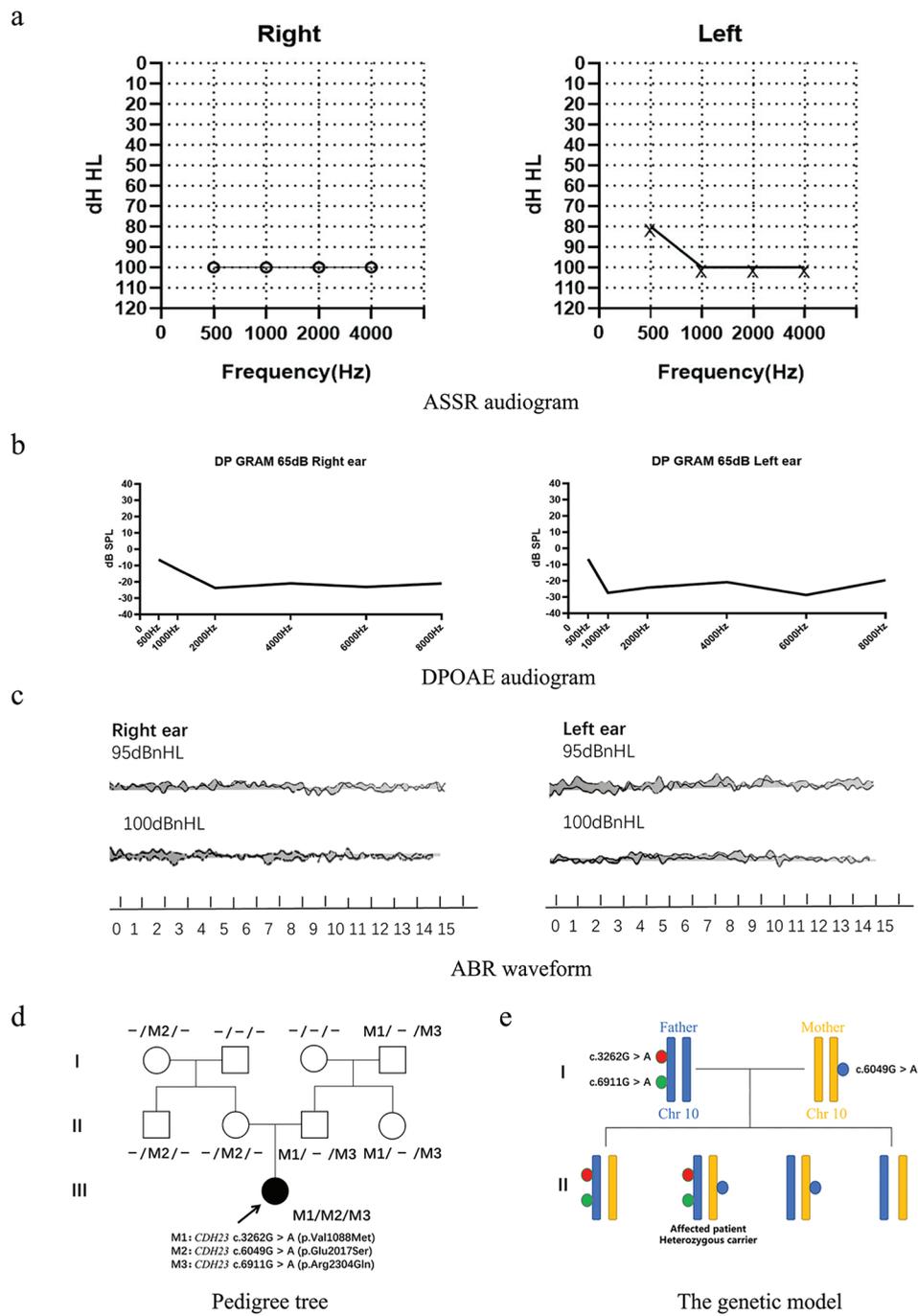
Analysis of genotype–phenotype correlations within the family revealed an intrafamilial segregation pattern consistent with autosomal recessive inheritance (Figure 1e). The affected individual's clinical phenotype aligned with *CDH23*-related prelingual deafness. Based on cWES and pedigree analysis, the compound heterozygous variants c.6049G > A (p.Glu2017Ser) and the paternal haplotype c.3262G > A (p.Val1088Met) in linkage with c.6911G > A (p.Arg2304Gln) in *CDH23* were confirmed as pathogenic.

### Compound heterozygous mutations in conserved regions of *CDH23*

The three confirmed pathogenic variants in the *CDH23* gene, initially identified by cWES (Figure 2a), include c.3262G > A (p.Val1088Met), c.6049G > A (p.Glu2017Ser), and c.6911G > A (p.Arg2304Gln). All three variants are located within conserved coding regions (Figure 2b). Sanger sequencing confirmed that the proband harbors compound heterozygous alleles of c.3262G > A, c.6049G > A, and c.6911G > A. The c.6049G > A variant was inherited from the mother, whereas the paternal haplotype carrying c.3262G > A in linkage with c.6911G > A was inherited from the father (Figures 1e and 2c).

The affected amino acid residues are highly conserved across species due to their location within essential domains. Sequence alignment of *Homo sapiens CDH23* with seven other species using constraint-

based multiple protein alignment tool revealed strong evolutionary conservation at these positions (Figure 2d).



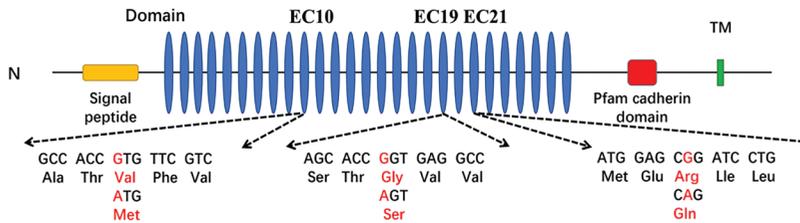
**FIG. 1.** The auditory steady-state response audiogram revealed (a) flat-type configuration with thresholds exceeding 90 dB nHL across 0.5, 1, 2, and 4 kHz, indicating profound hearing loss diagnosed as DFNB12. (b) Distortion product otoacoustic emission responses were absent at all tested frequencies (0.5–8 kHz), indicating outer hair cell dysfunction. (c) Auditory brainstem response of the patient to the click stimulus demonstrated no waveforms at 95 dB HL and 100 dB HL in both ears. (d) Pedigree of the family. Circles and squares denote females and males respectively, and the black arrow indicates the proband. (e) Analyses of the inherited pattern (also see Supplementary Figure 2 in the Supplementary Materials and Methods). ASSR, auditory steady-state response; ABR, auditory brainstem response; DPOAE, distortion product otoacoustic emission.

a

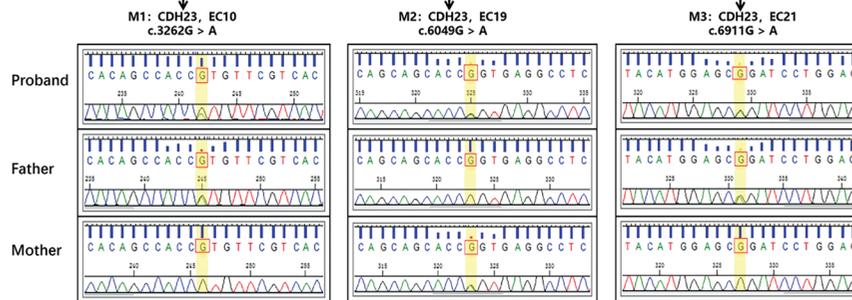
The filtered data of clinical Whole Exome Sequencing

Gene	Chr	GT	A0/DP	Trans	Exon	C.	p.	OMIM	Hert	Dis	CLINSIG
CDH23	chr10	het	104/177	NM_022124	exon27	c.3262G>A	p.Val1088Met	601386	Autosomal_recessive	AR	Uncertain significance
CDH23	chr10	het	100/184	NM_022124	exon44	c.6049G>A	p.Gly2017Ser	601386	Autosomal_recessive	AR	Likely pathogenic
CDH23	chr10	het	59 /145	NM_022124	exon48	c.6911G>A	p.Arg2304Gln	601386	Autosomal_recessive	AR	Uncertain significance

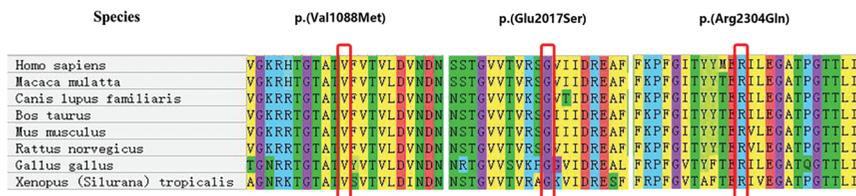
b



c



d



**FIG. 2.** Variations in the conserved motifs of *CDH23*. (a) The filtered result of cWES. (b) Schematic illustration of the primary structure of cadherin-related 23 encoded by *CDH23* and the extracellular domains comprising the three variations (directed by the dashed lines). (c) Sequencing chromatograms of the samples showing compound heterozygous variations in the conserved coding regions. Genomic DNAs were isolated from peripheral blood samples of the proband, and her parents. (d) Multiple alignment indicated that relevant substitutions of p.Val1088Met, p.Glu2017Ser and p.Arg2304Gln were located in highly conserved motifs across the species. The conserved amino acid residues are highlighted in red boxes. *EC*, extracellular; *CDH23*, cadherin-related 23.

### Bioinformatics analysis of *CDH23* mutations

To explore the potential molecular basis, a series of structural bioinformatics methods were applied. First, homology modeling of *CDH23* variants was performed using SWISS-MODEL, followed by structural optimization through molecular dynamics simulation. An array of structural analyses was then conducted to assess the structural implications of the mutants.

The secondary structures of *CDH23* extracellular (EC) domains were analyzed using SOPMA, which indicated that the alpha-helical content decreased in the EC19 and EC21 variants (Supplementary Table 3). According to PredyFlexy, the molecular flexibility of the EC19 variant was notably increased, whereas EC10 and EC21 showed minimal changes (Supplementary Figures 3a–c). Domain stability analysis revealed a significant decrease for the EC10 (p.Val1088Met) and EC19 (p.Glu2017Ser) variants (Supplementary Table 4). RMSD analysis showed a value of 0.254 Å for EC19, which was higher than 0.076 Å for EC10 and 0.059 Å for EC21 (Supplementary Figure 3d). These analyses provided insights into the possible structural and functional impacts of the variants.

The 3D structures of the native and mutant EC domains were examined using Swiss PDB Viewer. Compared with native structures, the mutant domains showed fluctuations that could influence molecular dynamics and function (Supplementary Figure 4). The pl of EC21 was lower than those of EC10 and EC19 (Supplementary Figure 4b). Substitution of native amino acids with mutant residues also altered charge distributions in EC10 and EC19 (Supplementary Figure 4c).

Changes in hydrogen bonding were observed in EC19 and EC21 mutants. In EC19, a hydrogen bond formed between Ser55 and Ile23 that was absent in the native domain, while in EC21, a bond appeared between Gln4 and Glu91 (Supplementary Figure 4d).

Hydrophobicity analysis using ProtScale indicated alterations in EC10 (Supplementary Figure 5).

Overall, the cumulative structural scores were consistent with the pedigree analysis (Table 1; Supplementary Figures 3–5; Supplementary Tables 3 and 4).

## DISCUSSION

Congenital deafness is one of the most prevalent sensory impairments in newborns, with an estimated incidence of 1–3 per 1,000 across populations, irrespective of sex or racial background.<sup>15,16</sup> The recurrence risk increases substantially when both parents carry the same pathogenic variants associated with hearing loss.<sup>17</sup> Among cases of non-syndromic hearing impairment (DFNB12), *CDH23* variants are the most frequently observed genetic lesions.<sup>18,19</sup>

The confirmed novel compound heterozygous variants of *CDH23*, c.6049G > A (p.Glu2017Ser) and c.3262G > A (p.Val1088Met) linked with c.6911G > A (p.Arg2304Gln), jointly contribute to autosomal recessive congenital hearing loss. While c.6049G > A (p.Glu2017Ser) alone is disease-causing, our study demonstrates that c.3262G > A (p.Val1088Met) in combination with c.6911G > A (p.Arg2304Gln) represents a novel pathogenic haplotype. These findings expand the known pathogenic mutation spectrum of *CDH23* and have clinical significance for genetic counseling in hereditary deafness.

In recent years, an increasing proportion of phenotypic variability may be attributed to combinations of variants at multiple loci rather than a single locus. For example, a linear cis combination of single nucleotide polymorphisms (SNPs) explained significantly more variance than the best individual SNP.<sup>20</sup> The additive effects of linked variants (haplotypes) on biomolecular functions warrant further investigation.

**TABLE 1.** Comprehensive Assessment of the Compound Heterozygous Mutations.

Variations	E10 V1088M	EC19 G2017S	E21 R2304Q	EC10-EC21 V1088M-R2304Q
Secondary structures	effect↓in α-helix	N/A	effect↓in α-helix	↓effect-effect↓in α-helix
Flexibility	N/A	↓effect	N/A	N/A-N/A
RMSD	N/A	↓effect	N/A	N/A-N/A
pl/Mw	N/A	N/A	effect↓	N/A-effect↓
Stability	↓effect	↓effect	N/A	↓effect-N/A
SCD	effect	effect	N/A	effect-N/A
H-bond	N/A	effect	effect	N/A-effect
Hydrophobicity	↓effect	N/A	N/A	↓effect-N/A
Score	4 (benign)	5 (pathogenic)	3 (benign)	6 (pathogenic)

Abbreviation of SCD represents surface charge distribution. To evaluate the potential synergistic effects of the combined variants, a cumulative structural impact scoring system was adopted based on the linear model. Given the variable  $k_{ij}$  is hard to accurately determine in a practical application milieu, a simplified model was then presented instead, in which  $k_{ij}$  was taken as a constant of “1”, and  $a_{ij}$  only took a value of “1” (effect) or “0” (without effect). In the case ( $j = 2$  mutations,  $i = 8$  parameters), score of c.6049 G > A (p.Glu2017Ser) (pathogenic) is 5, and those of c.3262 G > A (p.Val1088Met) (benign) and c.6911 G > A (p.Arg2304Gln) (benign) are 4 and 3, respectively. Interestingly, the score of combination of c.3262 G > A (p.Val1088Met) with c.6911 G > A (p.Arg2304Gln) was elevated to 6, which was beyond the score of the known pathogenic variation of c.6049 G > A (p.Glu2017Ser) (internal reference).

*CDH23* is a large protein consisting of 3,354 amino acid residues and plays a critical role in stereocilia organization and hair bundle formation. Although the biological effects of accumulated mutations on protein function remain unclear, our study is the first to reveal that substitutions of amino acid residues located far apart in the primary structure can result in *CDH23* dysfunction. Individual substitutions did not significantly impair the normal function of *CDH23*, suggesting a minimal impact on protein activity. However, the cooperative interplay between the two substitutions appears to amplify the effect on protein function. In theory, such additive or cooperative effects could substantially disrupt polypeptide folding, conformational stability, and physicochemical properties, leading to functional deficiencies. Although pedigree and structural bioinformatics analyses provide strong evidence for pathogenicity, the lack of experimental validation remains a limitation, warranting functional corroboration in future studies.

## CONCLUSION

Our pedigree-based study demonstrates that the combination of two benign variants can generate a novel pathogenic variant, a type of deleterious effect that has received little attention. Unlike *cis*-variants in *GJB2*, which often remain benign, this *CDH23* haplotype represents a rare case in which individually benign variants synergistically induce pathogenicity. These findings highlight the importance of evaluating complex variant interactions to ensure accurate genetic diagnosis and counseling. Structural bioinformatics can play a critical role in interpreting such complex coding-region mutations and providing mechanistic insight into their clinical significance. While our conclusions are grounded in robust genetic evidence from the pedigree, we propose that integrated structural bioinformatics modeling may offer a valuable strategy for assessing the clinical relevance of complex variants in cases where pedigree data are unavailable. This approach is also applicable for interpreting *de novo* mutations, which are frequently encountered in clinical genetics.

**Ethics Committee Approval:** The study was approved by the Ethics Committee of the Chongqing Medical University (approval number: 20200631, date: 20.05.2020). The procedures used in this study adhere to the tenets of the Declaration of Helsinki.

**Informed Consent:** Written informed consent was obtained from all participants and their legal guardians.

**Data Sharing Statement:** The datasets analyzed during the current study are available from the corresponding author upon reasonable request.

**Authorship Contributions:** Concept- J.Z.; Design- J.Z.; Supervision- J.Z.; Funding- J.Z.; Materials- Z.X.T., Y.T.Z.; Data Collection or Processing- Z.X.T., Z.X.W., Y.T.Z., Y.Y.H.; Analysis and/or Interpretation- Z.X.T., Z.X.W., X.S.; Literature Review- J.Z., X.S., Y.Y.H.; Writing- Z.X.T., J.Z.; Critical Review- J.Z.

**Conflict of Interest:** The authors declared that they have no conflict of interest.

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**Supplementary Table 1:**  
<https://balkanmedicaljournal.org/img/files/supp-table-1.xls>

**Supplementary Tables 2-4:**  
<https://balkanmedicaljournal.org/img/files/5-supplement-table-2-4-y.pdf>

### Supplementary Figures:

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### Supplementary Materials and Methods:

<https://balkanmedicaljournal.org/img/files/5-supplementary-Materials-and-Methods-y.pdf>

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