Original Research

GJB4 and *GJC3* variants in non-syndromic hearing impairment in Ghana

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Impact statement

Although connexins are known to be the major genetic factors associated with HI, only a few studies have investigated *GJB4* and *GJC3* variants among hearing-impaired patients. This study is the first to report *GJB4* and *GJC3* variants from an African HI cohort. We have demonstrated that *GJB4* and *GJC3* genes may not contribute significantly to HI in Ghana, hence these genes should not be considered for routine clinical screening in Ghana. However, it is important to study a larger population to determine the association of *GJB4* and *GJC3* variants with HI.

Abstract

The contribution of *GJB4* and *GJC3* gene variants to hearing impairment in Africa has not yet been studied. Here, we investigated the contribution of these genes to autosomal recessive non-syndromic hearing impairment in Ghanaian children. Hearing-impaired children from 141 simplex and 59 multiplex families were enrolled from 11 schools for the deaf in Ghana. The coding regions of *GJB4* and *GJC3* were amplified, sequenced, and analyzed for the study participants previously found to be negative for *GJB2* and *GJB6* variants. Seven *GJB4* and one *GJC3* variants were identified. One out of the seven *GJB4* variants was classified as likely pathogenic, while the others were either benign or synonymous. The likely pathogenic variant (p.Asn119Thr/rs190460237) was predicted to be likely associated with hearing impairment. We modeled the wild-type and mutant proteins of this variant (p.Asn119Thr) to evaluate the effect of the mutation on protein structure and ligand-binding properties. The mutant and not the wild type had the potential to bind N-Ethyl-5′-Carboxamido Adenosine (DB03719) which was due to a slight structural change that was observed. No clinically relevant variant was identified in the *GJC3* gene. We report for the first time a likely pathogenic *GJB4* variant that may be associated with non-syndromic hearing impairment in Ghana; the finding will add to the body of evidence of the contribution of *GJB4* to hearing impairment cases around the world.

Keywords: *GJB4*, *GJC3*, protein modeling, hearing impairment, *in silico*, virtual screening

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Introduction

Hearing impairment (HI), a disabling congenital disease, is globally known as one of the major age-standardized disabilities of life.[1,2] According to a World Health Organization (WHO) report in 2019, about 466 million people are estimated to be living with HI.[3] A higher prevalence is recorded in sub-Saharan Africa (about 6 out of 1000 live births) compared to the developed countries (about 1 out of 1000 live births).[4] Reports from different populations have shown that about 50% of congenital HI cases are of genetic origin[4,5] and about 80% of the genetic cases are non-syndromic.[6,7] The majority of all non-syndromic HI cases (nearly 80%) are inherited in the autosomal recessive fashion.[8,9] HI is genetically highly heterogeneous with over 140 genes identified to date[10] but the contribution of gene variants to HI has not been equally investigated across global populations, with limited studies from Africa. Hence, there is a great scarcity in the representation of known pathogenic genetic variants of African ancestry. As a result, a recent study of pathogenic and likely pathogenic (PLP) autosomal recessive non-syndromic hearing impairment (ARNSHI) variants

(selected from the ClinVar and Deafness Variation Databases with their frequencies from gnomAD database) estimated the prevalence of HI due to PLP as 5.2 per 100,000 individuals for Africans/African Americans, compared to a higher prevalence of 96.9 per 100,000 individuals for Ashkenazi Jews.[11] The knowledge deficit is likely hindering progress in understanding the mechanism of HI and ultimately affecting the development of therapeutic strategies, genetic diagnoses, prognosis and genetic counselling.[11]

Connexin genes are the most frequently reported known HI genes associated with HI cases, particularly in populations of European and Asian ancestries.[12–14] Connexins are a family of gap junction proteins expressed in almost all human tissues and are involved in intercellular communication,[15,16] and mutations in connexin genes have been implicated in about 28 genetic diseases,[17] with deafness and skin diseases as the most frequently associated condition.[16,18] Variations in the gene *GJB2* are most frequently associated with non-syndromic hearing impairment (NSHI).[18,19] Similar to *GJB2*, *GJB4* and *GJC3* gene variants are associated with skin disorders;[17] however, they are seldom associated with ARNSHI. Associations have been established previously between NSHI and *GJB4* in Iran[20,21] and Taiwan,[22] and between NSHI and *GJC3* in Taiwan[22] and India.[23] However, multiple evidence from independent populations is needed for the clinical validity of hearing impairment gene-disease pairs.[10] Earlier studies investigating *GJB4* mutations among hearing-impaired patients found missense variants such as p.R227W (c.679C>T), p.C169W (c.507C>T), and p.R151S (c.451C>A),[20–22] though the molecular mechanisms of the cause of deafness with respect to these variants were not well elucidated. However, it was suggested that these that these variants may be pathogenic since they were identified among patients and not control participants.[20–22] Interestingly, ClinVar and the Rat Genome Database contain *GJB4* variants associated with autosomal non-syndromic deafness,[24,25] further supporting the pathogenicity of the gene. Moreover, *GJB4* protein was found to be expressed in the cochlea of rats.[26] Similar to *GJB4*, some *GJC3* variants (e.g. p.I90A/c.569T>A and c.781 + 62 G > A) were reported only among hearing-impaired individuals without any extensive molecular study on their pathogenicity.[22,23] There is therefore the need to interrogate *GJB4* and *GJC3* variants from other populations across the world and to study the molecular mechanisms of pathogenicity of these gene variants.

To date, only *GJB2* and *GJB6* contributions to NSHI have been systematically investigated in Ghana[27–30] and other parts of Africa.[31,32] There is no study from Africa on the role of *GJB4* and *GJC3* variants in HI. In this study, we investigated the contribution of *GJB4* and *GJC3* to NSHI in Ghana. We report for the first-time, variants in *GJB4* and *GJC3* genes in a Ghanaian HI cohort, and we have used *in silico* protein modeling approaches to explore the possible molecular mechanisms through which a likely pathogenic variant found in *GJB4* could cause deafness.

Materials and methods

Ethics consideration

The set of ethical principles of the Declaration of Helsinki were adhered to in this study. Ethical approvals were sought and obtained from two ethics review boards: the Noguchi Memorial Institute for Medical Research Institutional Review Board (NMIMR-IRB CPN 006/16–17) and the University of Cape Town's Faculty of Health Sciences' Human Research Ethics Committee (HREC 104/2018). Prior to patient enrolment, the study was explained to each study participant in their native language and informed consent was confirmed by signature.

Study participants

The participants in this study were grouped into three categories: (1) isolated/non-familial simplex cases ($n = 141$) living with severe to profound HI with putative genetic cause of deafness; (2) multiplex/familial cases consisting of 59 individuals, each one selected from 59 families who had at least two affected family members with HI (Figure 1 and Figure S1); and (3) control participants ($n = 47$) randomly selected from a general Ghanaian population, with no personal and family history of HI. The medical records of the hearing-impaired students were evaluated to identify families with congenital HI. Both families and isolated cases were compatible with autosomal recessive inheritance, and each hearing-impaired participant was carefully examined and interviewed with a structured questionnaire to eliminate syndromic and environmental causes of HI as described previously.[27] All the study participants including the controls had been previously screened and were found to be negative for *GJB2* and *GJB6* gene variants (Figure 1).[27,30]

Genetic analyses

DNA extraction. At the West African Centre for Cell Biology of Infectious Pathogens (WACCBIP), University of Ghana, Accra, Ghana, DNA were extracted from the blood samples collected from each participant using QIAamp DNA Blood Maxi Kit ® (Qiagen, USA).

Polymerase chain reaction and Sanger sequencing. The molecular analyses were conducted at the Division of Human Genetics, University of Cape Town. Previously published primers[20] specific for *GJB4* exon 2 (F1B4: 5′-TCAATCGCACCAGCATTAAG-3′ and R1B4: 5′-GGGGG ACCTGTTGATCTTATC-3′) and *GJC3* exon 1 (F1C3: 5′-GCTCCCTCTGAAGGACAGTG-3′ and R1C3: 5′-GGGAG GAGATCATCAGGACA-3′) and *GJC3* exon 2 (F2C3: 5′-TGGGTACGCACTGTGAAAAA-3′ and R2C3: 5′-AGC TCCTCCTTGGACAGGAT-3′) were used to amplify the coding regions of *GJB4* and *GJC3*. The PCR amplicons were Sanger sequenced as described by Bosch *et al.* in 2014 using ABI 3130XL Genetic Analyzer® (Applied Biosystems, Foster City, CA).

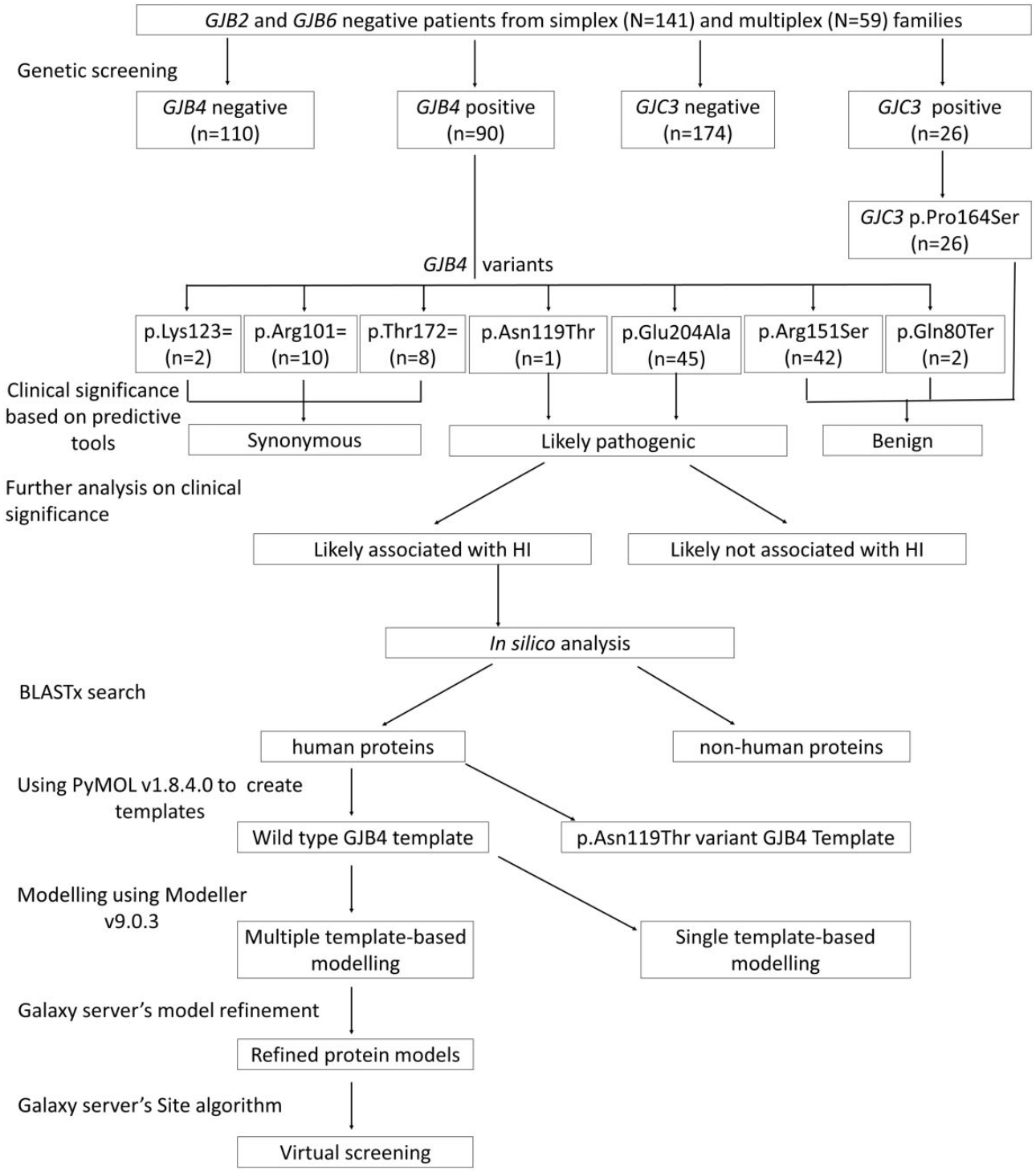

Figure 1. Flow chart of genetic screening of patients with *GJC3* and *GJB4* variants, and *in silico* analysis of *GJB4* c.356A>C (p.Asn119Thr) variant.

Data analysis

The Sanger sequence data were cleaned and analyzed using FinchTV chromatogram viewer and Unipro UGENE Integrated Bioinformatics Tools.[33,34] Odd ratios were calculated to examine how strongly the identified variables are associated with the HI phenotype. We used Fisher's exact test to determine if there is an association between the number of alleles obtained for each variant in different populations. *P*-values less than 0.05 were considered significant. We used the following online databases, genome browser, and predictive programs to predict the clinical significance of the identified gene variants: VarSome,[35] ClinVar,[25] Align GVGD (Align Grantham Variation/Grantham Deviation),[36,37] FATHMM (Functional Analysis Through Hidden Markov Models),[38–40] MutationAssessor,[41,42] MutationTaster,[43] MutPred2 (Mutation Prediction 2), PROVEAN (Protein Variation Effect Analyzer),[44–46] PolyPhen-2

(Polymorphism Phenotyping V-2),[47] SIFT (Sorting Intolerant From Tolerant),[48–51] EIGEN,[52] MPV (pathogenicity of missense variants), PrimateAI[53] and InterVar[54] (Table S1).

In silico analysis of c.356A>C (p.Asn119Thr) variant

The "ab1" file (obtained from the ABI 3130XL Genetic Analyzer®) of the sample with the *GJB4* c.356A>C (p.Asn119Thr) variant was trimmed and edited using the SnapGene Viewer v5.0.6 (https://www.snapgene.com/). The resulting sequence was then saved as a FASTA file which was used to perform a BLASTx search in the non-redundant protein data bank (nrPDB) accessed via the NCBI BLAST web interface. Six hits were obtained from the BLASTx search, of which four were human proteins which were retrieved as .pdb files. Only the "A" chain of the PDB hits showed homology with GJB4 protein; hence, they were the only chains considered for further analysis. The "A" chains were retrieved as PDB files using the "indicate chain" command of PyMOL v1.8.4.0.[55] For each of the four templates (the retrieved "A" chains), the wild type and mutant proteins of the *GJB4* c.356A>C (p.Asn119Thr) variant were modeled.

Modeler v9.0.3[56] was used to perform a template-based (TM) modeling of both the wild type and mutant proteins of the *GJB4* c.356A>C (p.Asn119Thr) variant using two strategies; (i) single template-based modeling and (ii) multiple-template-based modeling (Figure 1). All the scripts used for the modeling were obtained from the Modeler web tutorial and changes were made where necessary.

Single template-based modeling. To identify the best template, the four templates were compared against each other using multiple sequence alignment and phylogenetic tree reconstruction. Pairwise alignment of the best template was performed with both the wild type and mutant proteins of the *GJB4* c.356A>C (p.Asn119Thr) variant from which 50 models were built. The best model was selected based on the lowest DOPE (Discrete Optimized Protein Energy) score.[57]

Multiple template-based modeling. A multiple sequence alignment (MSA) was performed for all the four templates followed by pairwise sequence alignment with the wild type and mutant proteins of the *GJB4* c.356A>C (p.Asn119Thr) variant. Similar to the single template-based modeling, 50 models each of the wild type and mutant proteins were built and the top 10 models were selected based on the lowest DOPE score.[57] The best model was selected from the top 10 models based on the Ramachandran plot evaluation (RAMPAGE) and Z-score (ProsA-Web) (Figure 2).

Model refinement. The best models were run through the Galaxy server's[58,59] refinement (Refine2) pipeline, which iteratively optimizes the initial structure using global and local operators as loop modeling and hybridization. The top-ranked model based on Galaxy energy, in combination with other parameters, was selected for virtual screening of possible ligands.

Virtual screening. To assess the possible effect of the *GJB4* c.356A>C (Asn119Thr) variant on the binding property of the protein, virtual screening for ligands was performed using the Galaxy server's Site algorithm. The algorithm predicts binding by comparing the distance between an amino acid residue and a ligand atom with the sum of their van de Waals radii + 0.5angstrom. Binding site residues are considered as those with a smaller difference in distance.

Results

Molecular analysis of *GJB4* and *GJC3*

To identify *GJB4* and *GJC3* variants that may be associated with HI in Ghana, we investigated hearing-impaired patients identified to be negative for *GJB2* and *GJB6* gene variants, from both multiplex ($n = 59/127$ affected individuals from 59 unrelated families) and simplex ($n = 141$) unrelated families segregating ARNSHI (Figures 1 and S1). These patients were found to have severe to profound congenital HI and their clinical and demographic data were previously reported.[27] The *GJB4* and *GJC3* gene variants were identified in the hearing-impaired patients and were further examined among the control individuals not affected by HI (Table 1).

The clinical significance and pathogenicity of the identified variants were predicted using 2 online databases and 12 predictive bioinformatic tools (Table S1). The sensitivity, accuracy, and specificity of these predictive tools vary based on the algorithms used.[60] It was, therefore, important to use a combination of predictive tools.[61]

Variants in *GJC3*

The molecular, clinical, and pathogenic evaluation of the variants identified in heterozygous state a *GJC3* variant predicted as benign (p.Pro164Ser). Two familial cases were found to be homozygous for the same mutation (Table 1).

Variants in *GJB4*

Three *GJB4* synonymous variants (p.Lys123=, p.Arg101=, and p.Thr172=) were identified in all three groups of samples. Of the three synonymous variants, p.Lys123= was classified as benign and p.Thr172= as a variant of uncertain significance. The *GJB4* sequence analysis also identified one nonsense and two non-synonymous variants classified as benign (p.Gln80Ter, p.Arg151Ser, and p.Glu204Ala). An additional variant (p.Asn119Thr) was classified as likely pathogenic (Table 1). Although some predictive tools suggested that the *GJB4* p.Glu204Ala variant was likely pathogenic, this was not supported by clinical gene variants-disease correlations with HI since the homozygous form of the variant was identified in both affected hearing-impaired ($n = 25$) and unaffected hearing control individuals ($n = 3$) with an odds ratio of 0.81. In addition,

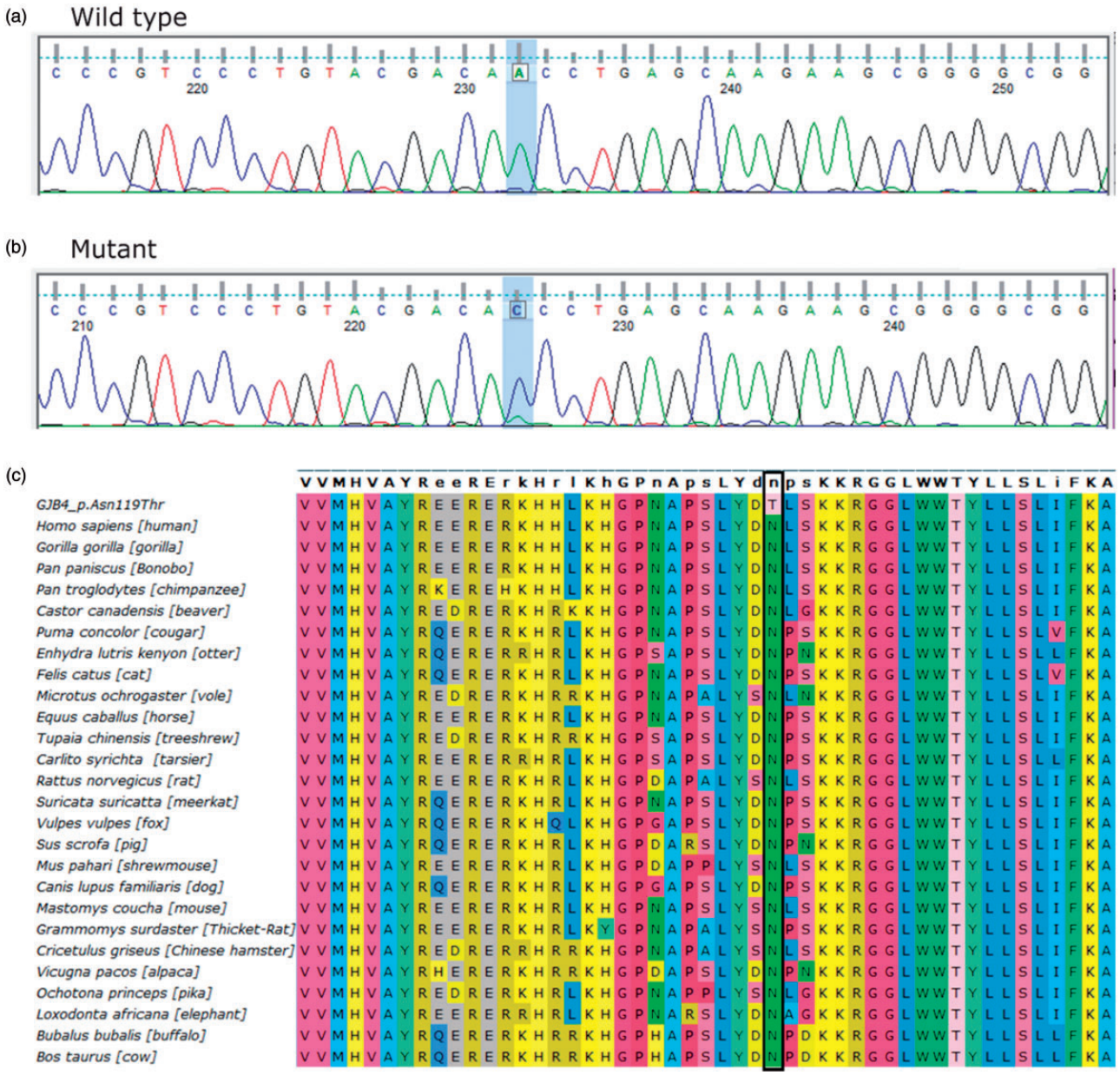

Figure 2. Chromatograms and multiple sequence alignment of *GJB4* p.Asn119Thr variant. Chromatogram of Sanger sequence of (a) wild type and (b) mutant of *GJB4* c.356A>C (p.Asn119Thr) variant. The position of the nucleotide change is highlighted in blue (c) Multiple sequence alignment of GJB4 protein in different species. Position 119 for the c.356A>C (p.Asn119Thr) variant is boxed.

the minor allele frequency of *GJB4* p.Glu204Ala within the global and African populations exceeds the threshold of 0.05, suggesting that it is not disease-causing (Table 2).

The *GJB4* p.Asn119Thr variant predicted as likely pathogenic was identified in only one hearing-impaired individual from a simplex family (Figure 2(a) and (b)). The sample from the participant in whom this variant was found was independently sequenced three times, with each run from a new PCR product. Furthermore, the *GJB4* p.Asn119Thr variant had less than 0.01 allele frequency in the global and African populations, indicating that it is a rare variant (Table 2). Since the *GJB4* p.Asn119Thr variant was predicted to be likely pathogenic, we examined it further using protein modeling approaches (Figures 3 to 5).

Evolutional evaluation of amino acid at position 119 of GJB4 protein

Since the molecular analysis suggested *GJB4* c.356A>C (p.Asn119Thr) as a likely pathogenic variant, a multiple sequence alignment was performed with *GJB4* protein sequences from different species to investigate the evolutional conservation of the amino acid residue at position 119 of the protein (Figure 2). Asparagine (Asn) at position 119 was conserved among all the different species investigated suggesting that the residue is important for the protein's function. It is worth mentioning that some of the amino acid residues around the asparagine 119 were not conserved among some of the species studied.

Table 1. *GJC3* and *GJB4* variants found in hearing-impaired patients and control subjects from Ghana.

Gene	Variants	Protein change	Clinical significance	Geno-types	Number of participants, *n* (%) Multiplex family *N* = 59	Simplex family *N* = 141	Total affected (*N* = 200)	Controls *N* = 47	Odds ratio	*P* value
GJC3	c.490C>T (rs73405465)	p.Pro164Ser	Benign	GG	50 (84.75%)	124 (87.94%)	174 (87.00%)	41 (87.23%)		
				GA	7 (11.86%)	17 (12.06%)	24 (12.00%)	6 (12.77%)	0.94	0.45
				AA	2 (3.39%)	0	2 (1.00%)	0	–	–
GJB4	c.611A>C (rs3738346)	p.Glu204Ala	Benign	AA	49 (83.05%)	106 (75.18%)	155 (77.50%)	38 (80.85%)		
				AC	4(6.78%)	16 (11.35)	20 (10.00%)	6 (12.77%)	0.81	0.34
				CC	6(10.17%)	19 (13.47%)	25 (12.50%)	3 (6.38%)	2.04	0.13
GJB4	c.451C>A (rs78499418)	p.Arg151Ser	Benign	CC	47 (79.66%)	111 (78.72%)	158 (79.00%)	40 (85.11%)		
				CA	8 (13.56%)	18 (12.77%)	26 (13.00%)	3 (6.38%)	2.19	0.11
				AA	4 (6.78%)	12 (8.51)	16 (8.00%)	4 (8.51%)	1.01	0.49
GJB4	c.516T>C (rs111693060)	p.Thr172=	Variant of uncertain significance	TT	56 (94.92%)	136 (96.45%)	192 (96.00%)	46 (97.87%)		
				TC	0	3 (2.13%)	3 (1.50%)	1 (2.13%)	0.72	0.39
				CC	3 (5.08)	2 (1.42%)	5 (2.50%)	0	–	–
GJB4	c.369G>A (rs142843509)	p.Lys123=	Benign	GG	59 (100%)	139 (98.58%)	198 (99.00%)	46 (97.87%)		
				GA	0	2 (1.42)	2 (1.00%)	1 (2.13%)	0.46	0.26
				AA	0	0	0	0	–	–
GJB4	c.356A>C (rs190460237)	p.Asn119Thr	Likely pathogenic	AA	59 (100%)	140 (99.29%)	199 (99.50%)	47 (100.00%)		
				AC	0	0	0	0	–	–
				CC	0	1 (0.71%)	1 (0.50%)	0	–	–
GJB4	c.303C>G (rs138184343)	p.Arg101=	Synonymous	CC	55 (93.22%)	135 (95.74%)	190 (95.00%)	46 (97.87%)		
				CG	1 (1.69%)	4 (2.84%)	5 (2.50%)	1 (2.13%)	1.21	0.43
				GG	3 (5.09%)	2 (1.42)	5 (2.50%)	0	–	–
GJB4	c.238C>T (rs114429815)	p.Gln80Ter	Benign	CC	59 (100%)	139 (98.58%)	198 (99.00)	45 (95.74%)		
				CT	0	1 (0.71%)	1 (0.50%)	2 (4.26%)	0.11	0.039
				TT	0	1 (0.71%)	1 (0.50%)	0	–	–

Modeling of wild type and mutant (c.356A>C (p. Asn119Thr)) GJB4 protein

We examined the possible molecular effect of the change in the conserved amino acid at position 119 of the protein by modeling and comparing the wild type and *GJB4* c.356A>C (p.Asn119Thr) mutant proteins. Good quality models with DOPE scores of $\sim -26{,}500$ were obtained from the modeling experiment from which the best models were selected. Multiple-template modeling performed better than the single-template modeling (Figure 1(e)). Both models were evaluated and found to be within the range of expected values for X-ray crystallography-determined and nuclear magnetic resonance (NMR)-determined proteins. Z-scores of -4.56 and -4.28 were obtained for wild type and mutant (c.356A>C (p.Asn119Thr)) *GJB4* proteins (Figure 3(a) and (b)), respectively. In addition, more than 98% of the residues were observed to fall within favorable and allowed regions on the Ramachandran plot with highly favorable ProsA Z-scores for both models (Figure 3(c) and (d)).

The Galaxy refinement of the wild type and mutant *GJB4* proteins produced 10 models, from which we selected the best-refined (Figure 4(a) and (b)). The model labeled "MODEL 1" appeared to be the overall best for the wild type, while the model "MODEL 7" appeared as the best-refined for the mutant (Figure 4(a) and (b)). Figure 3(a) and (b) shows the quality improvement of the selected refined models compared to the unrefined models.

The *GJB4* c.356A>C (p.Asn119Thr) mutation slightly modifies the protein structure, which we can observe when the mutant protein is compared with the wild type protein. On the wild type protein, asparagine at position 119 forms part of a random coil, however the same position in the mutant model harboring a threonine residue forms a helix (Figure 4). There was, generally, a high degree of conservation of the extracellular E1 and E2 loops, as expected. Refinement further saw the modeling of two short helixes in the C-terminus, in regions of random coil expected for gap junction proteins (Figure 5).

Virtual screening

Connexins are characterized by four transmembrane helices that form the transmembrane pore and extracellular domains, which form two loops (E1 and E2) that help in cell–cell recognition and docking. These loops are mostly involved in protein–protein interactions, while residues on the alpha-helix transmembrane domains are involved in the process of small molecule shuttling. To the best of our knowledge, the *GJB4* c.356A>C (p.Asn119Thr) mutation (rs190460237) has not been previously reported, hence we modeled the 3D structures of *GJB4* wild type and mutant proteins which revealed subtle but fundamental differences that may have significant implications on the protein function. To assess the possible effect of these differences, we performed virtual screening for ligands using the Galaxy server's Site algorithm. The virtual screening predicted four ligands and their corresponding binding sites for the wild type *GJB4* (1KS, SNT, A8T, and SG8) and five ligands for the mutant *GJB4* c.356A>C (p.Asn119Thr) (NEC, 1KS, SNT, A8T, and SG8) proteins. Although none of the ligands interacts with the position 119 residues of both the wild type and the mutant models, it appears that the residue

Table 2. Differential allele frequencies of *GJB4* and *GJC3* variants in the global population.

			Our data			Allele frequency (Ensembl)								
Gene	Variants	rs number	Allele	Cases	Control	*P*-value (cases vs. control)	Global	*P*-value (our cases vs. global)	Africa	*P*-value (our cases vs. Africa)	America	*P*-value (our cases vs. America)	East Asia	*P*-value (our cases vs. East Asia)	Europe	*P*-value (our cases vs. Europe)
GJC3	c.490C>T (p.Pro164Ser)	rs73405465	G	0.93	0.94	1.0000	0.98	0.0001	0.94	0.6441	0.99	0.0001	1.00	0.0001	1.00	0.0001
			A	0.07	0.06		0.02		0.06		0.01		0.00		0.00	
GJB4	c.611A>C (p.Glu204Ala)	rs3738346	A	0.83	0.87	0.3548	0.89	0.0001	0.75	0.0023	0.89	0.0022	0.88	0.0075	0.99	0.0001
			C	0.17	0.13		0.11		0.25		0.11		0.12		0.01	
GJB4	c.451C>A (p.Arg151Ser)	rs78499418	C	0.86	0.88	0.6197	0.96	0.0001	0.90	0.0134	0.92	0.0010	0.97	0.0001	1.00	0.0001
			A	0.14	0.12		0.04		0.10		0.08		0.03		0.00	
GJB4	c.516T>C (p.Thr172=)	rs111693060	T	0.97	0.99	0.4863	0.99	0.0001	0.98	0.1931	1.00	0.0001	1.00	0.0001	1.00	0.0001
			C	0.03	0.01		0.01		0.02		0.00		0.00		0.00	
GJB4	c.369G>A (p.Lys123=)	rs142843509	G	0.99	0.99	0.4699	1.00	0.0296	1.00	0.0532	1.00	0.1340	1.00	0.0806	1.00	0.0808
			A	0.01	0.01		<0.01		0.00		<0.01		0.00		0.00	
GJB4	c.356A>C (p.Asn119Thr)	rs190460237	A	0.99	1.00	1.0000	1.00	0.0156	1.00	0.1366	1.00	0.1335	1.00	0.0806	1.00	0.0808
			C	0.01	0.00		<0.01		<0.01		0.00		0.00		0.00	
	c.303C>G (p.Arg101=)	rs138184343	C	0.96	0.99	0.3282	1.00	0.0001	0.98	0.0302	1.00	0.0001	1.00	0.0001	1.00	0.0001
			G	0.04	0.01		<0.01		0.02		0.00		0.00		0.00	
GJB4	c.238C>T (p.Gln80Ter)	rs114429815	C	0.99	0.98	0.2425	0.99	0.7962	0.96	0.0013	1.00	0.6744	1.00	0.0228	1.00	0.0229
			T	0.01	0.02		0.01		0.04		<0.01		0.00		0.00	

change caused a perturbation in the protein structure that is significant enough to alter ligand binding (Figure 5).

Discussion

Mutations in connexin genes have been implicated in about 28 genetic diseases, with HI and skin disorders as the predominant cases.[17] Although the *GJC3* gene has been associated with NSHI with specific pathological alterations in the cochlea,[62,63] there are limited studies globally and especially from Africa. Unlike other epidermal disease-associated connexins, the role of *GJB4* variants in NSHI is not well elucidated.[64] To the best of our knowledge, this is the first report on *GJB4* and *GJC3* variants in African hearing-impaired patients and will add to the current knowledge, as well as help refine gene-disease pairs and clinical validity curation.

Mouse models created with alterations in the *GJC3* gene indicated that about 50% of homozygous *GJC3* null mice had delayed maturation of hearing thresholds, high-frequency hearing loss, and were vulnerable to noise-induced hearing loss.[65] An earlier study, however, did not describe any significant difference between the phenotypes (including auditory brainstem response) of the *GJC3* deficient and the wildtype control adult mice.[66] The authors stated that the gene might be functionally associated with other connexins such as connexin 32 and connexin 47 which suggested that it may not be independently associated with the HI phenotype. Our study identified p.Pro164Ser (c.490C>T/rs73405465) variant in the *GJC3* gene of both hearing-impaired and hearing individuals in Ghana with a 0.94 odds ratio. The missense *GJC3*-p.Pro164Ser variant had a minor allele frequency of 0.064 in the African population[67] which is greater than the 0.050 thresholds for calling uncommon variants. Considering the odds ratio, minor allele frequency, and occurrence of the variant in control hearing participants, the *GJC3*-p.Pro164Ser variant may not be associated with HI. The *GJC3* p.Pro164Ser variant had no record/phenotypic data in ClinVar[25] and Ensembl[67] and was labeled as benign, non-pathogenic, neutral, or polymorphism by the majority of predictive tools used (Table S1) as well as on the VarSome database,[35] further supporting its non-pathogenicity.

The expression pattern and contribution of *GJB4* to HI remain unclear. A *GJB4* deficient mouse model generated by replacing the coding region of *GJB4* with a lacZ gene did not show any auditory abnormality when assessed by brain stem evoked potentials.[68] Interestingly, these mice did not show any skin abnormality, which made it difficult to interpret the role of *GJB4* in humans; however, there have been some studies that investigated and detected *GJB4* gene variants in deaf individuals.[20–22] In a rat study, *GJB4* was found to be expressed in rat cochlea, suggesting its role in the hearing process. The present study identified synonymous *GJB4* variants (p.Lys123=, p.Arg101=, and p.Thr172=) in both affected and control samples of which p.Lys123= and p.Thr172= were classified as benign and variant of uncertain significance, respectively. But these three variants had no effect on the resultant protein; hence, they may not be responsible for HI pathogenesis. We also identified *GJB4*

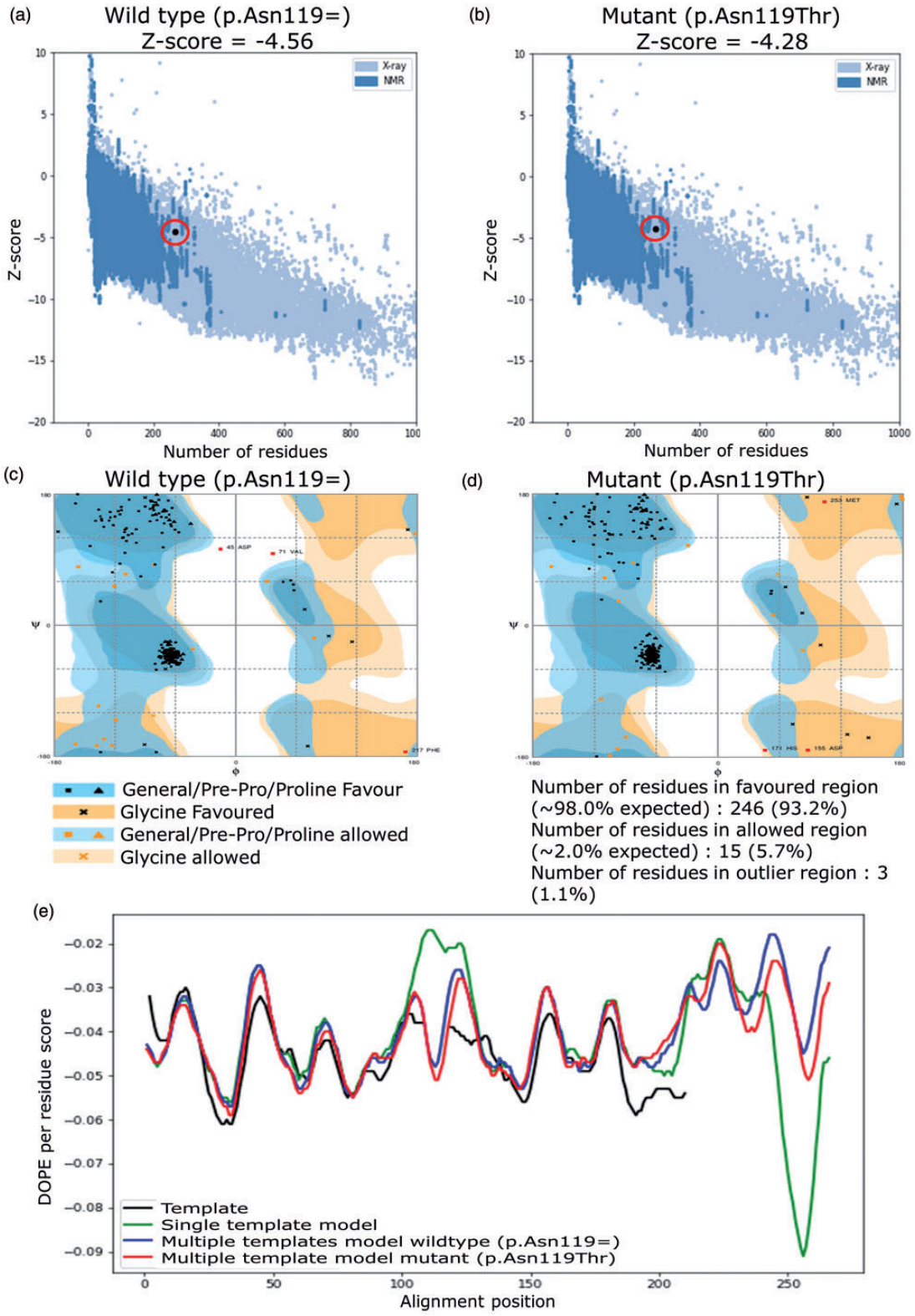

Figure 3. Evaluation and validation of GJB4 protein models: ProsA web evaluation of (a) wildtype (p.Asn119=) and (b) mutant (p.Asn119Thr) proteins. Ramachandran plot of (c) wildtype (p.Asn119=) and (d) mutant (p.Asn119Thr) proteins. (e) Discrete optimized protein energy (DOPE) profile for wildtype (p.Asn119=) and mutant (p.Asn119Thr) proteins.

p.Arg151Ser and p.Gln80Ter variants previously predicted to be benign. There was no published data on the *GJB4* p.Gln80Ter variant in hearing HI patients. Similar to our study results, *GJB4* p.Arg151Ser was found in both HI patients and controls in Iran[20] suggesting that it may not be associated with the HI phenotype. The variant was associated with skin disorders and found in patients without hearing loss[69,70] hence confirming the above observation.

(a) Wild type (p.Asn119=)

Model	RMSD	MolProbity	Clash score	Poor rotamers	Rama favored	GALAXY energy
Initial	0.000	3.158	76.6	2.5	91.7	-340.29
MODEL 1	3.385	1.258	1.7	0.0	95.1	-6421.90
MODEL 2	3.532	1.334	1.9	0.0	94.3	-6421.75
MODEL 3	3.263	1.190	0.6	0.0	92.0	-6420.64
MODEL 4	3.431	1.269	1.5	0.0	94.3	-6397.94
MODEL 5	4.018	1.473	2.5	0.0	93.2	-6389.18
MODEL 6	3.749	1.419	1.9	0.0	92.4	-6387.26
MODEL 7	3.496	1.268	1.2	0.0	93.6	-6378.46
MODEL 8	4.236	1.356	1.7	0.4	93.2	-6358.87
MODEL 9	3.332	1.306	1.5	0.0	93.6	-6353.99
MODEL 10	3.970	1.504	2.5	0.4	92.4	-6347.12

(b) Mutant (p.Asn119Thr)

Model	RMSD	MolProbity	Clash score	Poor rotamers	Rama favored	GALAXY energy
Initial	0.000	3.116	79.8	2.1	91.7	-419.16
MODEL 1	6.385	1.250	1.2	0.0	93.9	-6487.91
MODEL 2	6.519	1.322	1.7	0.0	93.9	-6478.79
MODEL 3	6.183	1.209	1.0	0.0	93.9	-6428.75
MODEL 4	6.535	1.401	1.7	0.0	92.0	-6421.41
MODEL 5	6.532	1.274	1.0	0.0	92.4	-6405.01
MODEL 6	10.315	1.459	1.9	0.4	91.3	-6402.54
MODEL 7	2.715	1.122	0.8	0.0	94.7	-6397.00
MODEL 8	6.880	1.405	1.5	0.4	90.9	-6396.68
MODEL 9	3.194	1.091	0.6	0.0	94.3	-6387.53
MODEL 10	8.916	1.339	1.5	0.0	92.8	-6375.11

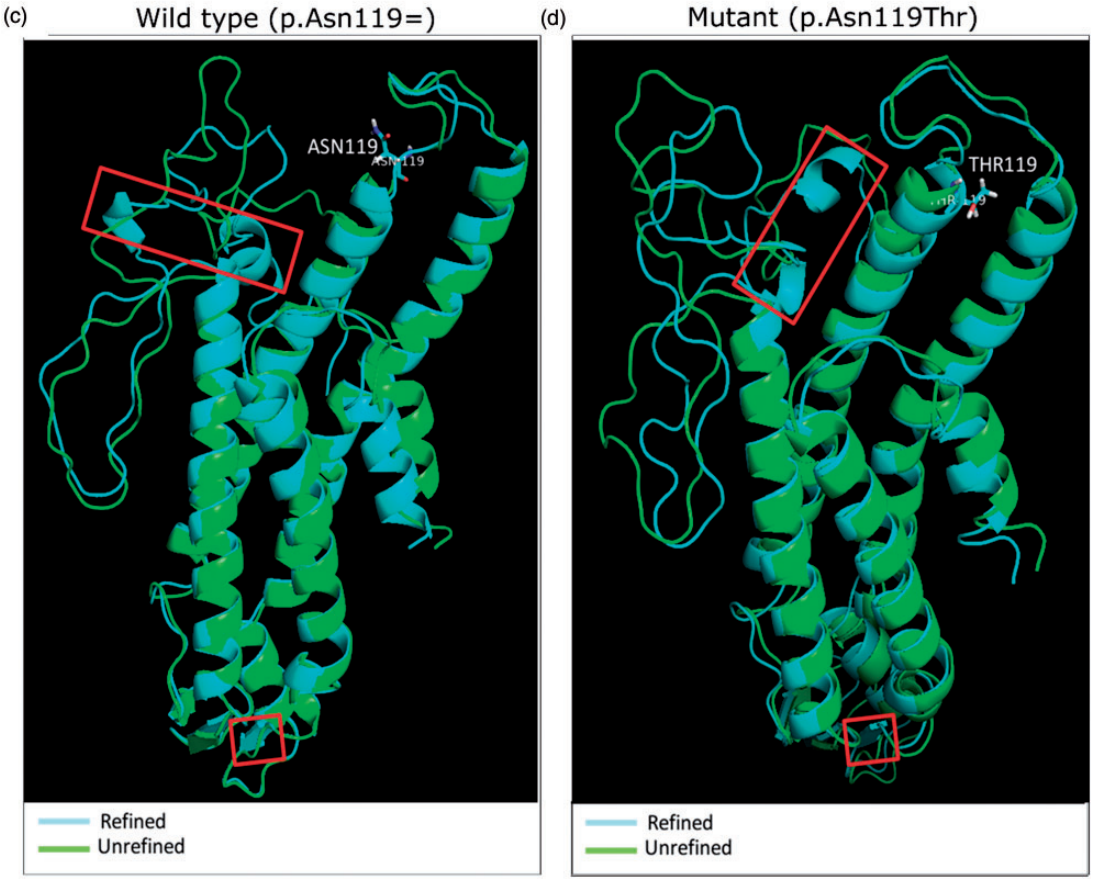

Figure 4. Refinement of *GJB4* protein models. Galaxy refinement of (a) wild type and (b) mutant c.356A>C (p.Asn119Thr) *GJB4* protein models. The best-ranked models are highlighted with red rectangles. Refined and unrefined models of (c) wild type and (d) mutant *GJB4* c.356A>C (p.Asn119Thr) *GJB4* protein models.

Similar to our findings, a Spanish study also identified the *GJB4*- p.Glu204Ala in hearing-impaired patients.[64] We found the variant in both control and affected samples which are consistent with findings from Iran[20]; our findings suggest that there is no likely association between the *GJB4* p.Glu204Ala variant and HI. The p.Asn119Thr variant may be of clinical significance since it was reported as "likely pathogenic," according to InterVar and the majority of the predictive tools (Table S1). *GJB4* p.Asn119Thr was predicted to be a variant of uncertain significance by VarSome.[35] According to the automated clinical interpretation of genetic variants by ACMG/AMP 2015 guideline,[54] the variant was found to fall within the categories of PM1, PM2, PP3, and BP1. This implies that the variant is located within a mutational hot spot or a well-established functional domain without benign variation (PM1), and absent from controls in the ESP, 1000Genomes, and ExAC databases with extremely low frequency if recessive (PM2) with multiple lines of computational evidence supporting a deleterious effect of the gene product (PP3).[54] A supporting evidence for benign status of a missense variant in a gene which when truncated are known to cause disease (BP1).[54] When the variant was analyzed for "Pathogenic variants Enriched Regions (PER) for genes and gene families" in

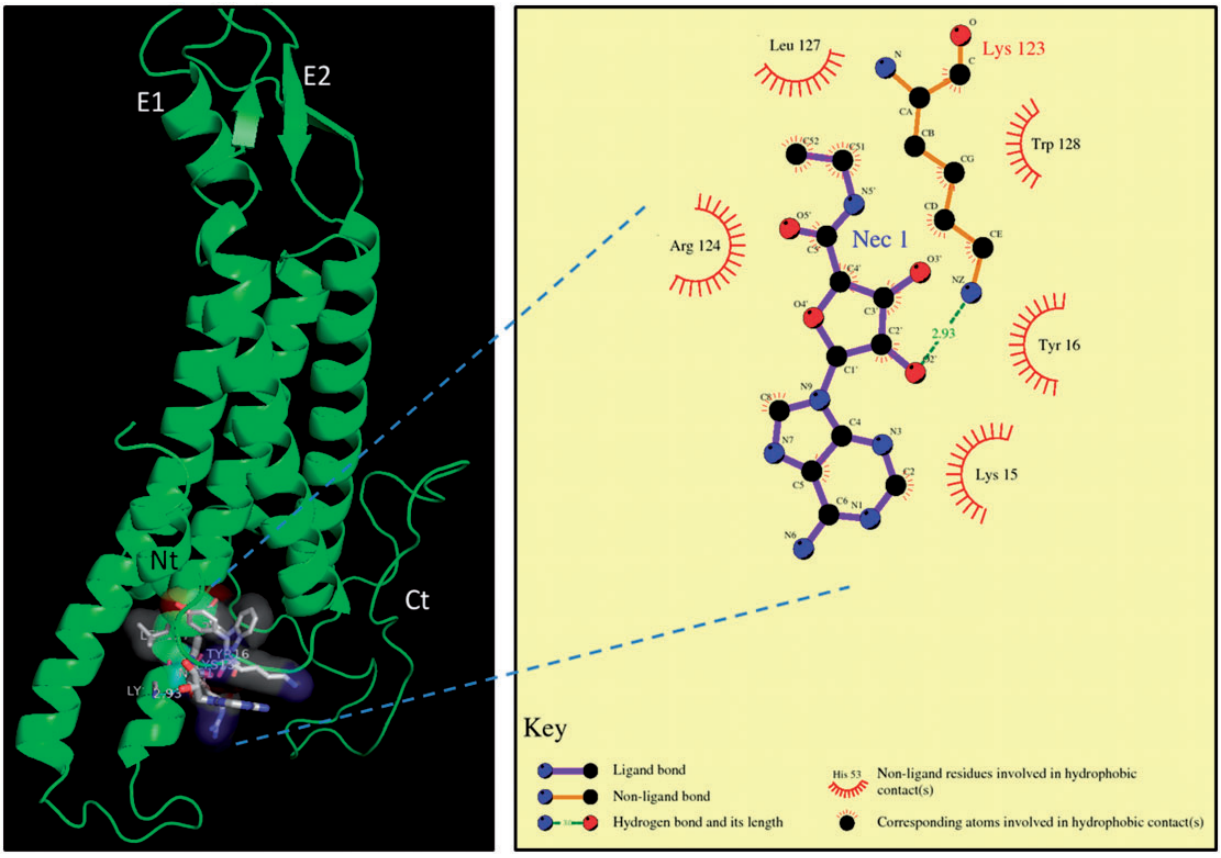

Figure 5. GJB4 mutant protein in complex with NEC. A LigPlus plot shows the interacting residues in detail.

the PER viewer,[71] it was observed to fall within a region of pathogenic missense burden for both gene family-wise and gene-wise analyses (Figure S2). PER sources disease-associated missense variants from ClinVar and the Human Gene Mutation Database (HGMD), retaining only "pathogenic" and/or "likely pathogenic" variants in ClinVar, and variants with "high confidence" calls in HGMD, all in the GRCh37.p13/hg19 coordinate. Interestingly, *GJB4* p.Asn119Thr (N_119) variant was observed to align with a *GJB2* variant (E_120) which is associated with sensorineural hearing loss.[71] Our study identified the variant in one patient with allele frequency less than 0.01 and none in the control population, but there was not enough evidence to conclude on its pathogenicity.

Analysis of *in silico* protein modeling revealed a striking difference between wildtype and mutant models of the p. Asn119Thr variant. The asparagine at position 119, which is on a cytoplasmic loop, forms random coils in the wild type model, whereas threonine in the same position forms a helix in the mutant model. It appears that the presence of Threonine at this position increases the overall propensity for a helix.

The ligand-binding property of the mutant p.Asn119Thr protein was slightly different from the wild type *GJB4* protein. An extra ligand, N-Ethyl-5′-Carboxamido Adenosine (NEC), was found to bind the *GJB4* p.Asn119Thr mutant protein and not the wild type. NEC (DB03719) is a non-carcinogenic purine nucleoside, a cAMP/cGMP phosphodiesterase (PDE) inhibitor[72] that doubles as a human adenosine A (2 A) receptor agonist.[73] PDE inhibitors are often used in the treatment of erectile dysfunction because of their adenosine A (2 A) receptor agonist role. Post-marketing and retrospective clinical trial analysis has shown that these PDE inhibitors have severe side effects such as hearing loss.[74] However, the above observation is inconclusive as there is no direct association established between hearing loss and PDE inhibitors.

Limitation of the study

The study identified a rare missense variant *GJB4*-p. Asn119Thr in a single hearing-impaired patient which makes it difficult to associate the variant to the hearing impairment phenotype. The pathogenicity of the variant was predicted using *in silico* predictive tools. Although these tools give a good prediction of the possible clinical effect of the variant which is very useful, they are not as accurate as functional assays. We therefore recommend the use of cell and animal models to confirm the pathogenicity of the *GJB4*-p.Asn119Thr variant.

Conclusions

In this study, only one possibly pathogenic *GJB4* variant (p.Asn119Thr) was identified in a hearing-impaired patient. The protein modeling and virtual screening identified differences in the protein structure and binding

properties of the mutant p.Asn119Thr *GJB4* protein compared to the wild type. There is a need for functional studies and investigations from larger populations to elucidate the pathogenicity of the variant (*GJB4*-p.Asn119Thr) predicted as "likely pathogenic". We did not identify any *GJC3* variant of clinical significance in the study population. Hence, *GJB4* and *GJC3* variants were found not to be significant contributors to non-syndromic autosomal recessive hearing impairment in Ghana. We therefore recommend the used of modern genomic approaches to investigate the associated HI gene variants in the study participants.

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DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article

ETHICAL APPROVAL

Ethical approvals were obtained from the Noguchi Memorial Institute for Medical Research Institutional Review Board (NMIMR-IRB CPN 006/16–17) and the University of Cape Town's Faculty of Health Sciences' Human Research Ethics Committee (HREC 104/2018).

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SUPPLEMENTAL MATERIAL

Supplemental material for this article is available online.

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