Genetic Aetiology of Autosomal Recessive Non-Syndromic Hearing Loss in Sub-Saharan African Patients: Evaluation Using Targeted and Whole Exome Sequencing.

By: Kamogelo Lebeko

LBKKAM001

SUBMITTED TO THE UNIVERSITY OF CAPE TOWN

In fulfilment of the requirements of the PhD degree in Human Genetics

University of Cape Town, Faculty of Health Sciences, Department of Pathology, Division of Human Genetics

Supervisor:	Ambroise Wonkam
Co-Supervisors:	Collet Dandara
	Shaheen Mowla

Date of Submission: 11 February 2019

Declarations

I, Kamogelo Lebeko, hereby declare that this work presented herein this dissertation/thesis is based on my original work (unless where acknowledgement declares otherwise). I also declare that neither the whole work nor any part of it has been, or is being, or is to be submitted for another degree in this or any other university.

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1. Bosch J, **Lebeko K**, Nziale JJN, Dandara C, Makubalo N, Wonkam A. 2014. In search of genetic markers for nonsyndromic deafness in Africa: a study in Cameroonians and Black South Africans with the GJB6 and GJA1 candidate genes. OMICS 18: 481–5

2. Wonkam A, Bosch J, Noubiap JJN, **Lebeko K**, Makubalo N, Dandara C. 2015. No evidence for clinical utility in investigating the connexin genes GJB2, GJB6 and GJA1 in non-syndromic hearing loss in black Africans. South African Med. J. 105: 23–26

3. **Lebeko K**, Bosch J, Nzeale JJ, Dandara C, Wonkam A. 2015. Genetics of hearing loss in Africans: use of next generation sequencing is the best way forward. Pan Afr. Med. J. 20: 1–14

4. **Lebeko K**, Sloan-Heggen CM, Noubiap JJN, Dandara C, Kolbe DL, Ephraim SS, Booth KT, Azaiez H, Santos-Cortez RLP, Leal SM, Smith RJH, Wonkam A. 2016. Targeted genomic enrichment and massively parallel sequencing identifies novel nonsyndromic hearing impairment pathogenic variants in Cameroonian families. Clin. Genet. 90: 288–290

5. **Lebeko K**, Manyisa N, Chimusa ER, Mulder N, Dandara C, Wonkam A. 2017. A Genomic and Protein–Protein Interaction Analyses of Nonsyndromic Hearing Impairment in Cameroon Using Targeted Genomic Enrichment and Massively Parallel Sequencing. Omi. A J. Integr. Biol. 21: 90–99.

Date: 11 February 2019 Student Number: LBKKAM001

Signed by candidate

Signature: Student Name: Kamogelo Lebeko

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Preface on the thesis organisation plus list of publications

Hearing loss (HL) imposes a great burden on affected patients and their families, particularly when there is little to no intervention in early life. HL has progressed from the 11th leading cause of disability globally in 2010, to the fourth-leading cause in 2015. More than 80% of people affected by HL live in low and middle-income countries, specifically in sub-Saharan Africa (SSA) with up to 6 per 1000 newborn affected per year. Very little is known of the genetic causes of hearing loss in African populations, which accounts for up to 50% of aetiology of congenital HL. The availability of such data in European and Asian populations has greatly contributed towards the decrease in the burden of the disease. It has made it possible to provide diagnostic services as early as possible, leading to early intervention and ultimately an increase in the quality of life of the patients and their families. To address this dearth of genetic data on African populations with HL, the research work relevant to and proposed for my PhD thesis has been, in part, written and published during my registration for this higher degree. Therefore, we have adopted this thesis format i.e.: "with inclusion of publications", for most parts of the thesis. The fourth chapter of the thesis will be presented in the traditional format and includes an introduction, materials and methods, results and discussion sub sections.

The inclusion of published work also aimed to: (i) demonstrate the candidate's proficiency and output of new knowledge in the relevant field which also aided in reinforcing the academic career of the candidate and enhance her candidacy for the financial support received from the National Research Foundation (NRF), (ii) enable participation in and attendance at academic conferences and training workshops: the candidate effectively attended a total of five specialised meetings in several cities including Cape Town, Pretoria, Livingstone, Washington DC, Dar Es Salaam and Cairo; and lastly (iii) the published works included in this thesis fulfil the encouragements put forth by the Faculty of Health Sciences and the University of Cape Town (UCT) which seek to disseminate the knowledge generated by scholars within the faculty, particularly at PhD level. These publications result in a complete body of work that represents a holistic project that effectively enhances the profile of

the institution. With the advice and guidance of the supervisors, the candidate's contributions to the included publications ranged from conceiving and designing the experiments; execution of experiments; analysis of experimental results; drafting the full manuscripts and incorporating revisions from co-authors and journal reviewers. The candidate's contributions to this project and thesis can be detailed as follows:

Conception of research:

The study and all experiments were conceived by the candidate in conjunction with the primary supervisor Professor Ambroise Wonkam and jointly refined with the advice of the co-supervisors: Dr Shaheen Mowla and Professor Collet Dandara.

Data collection:

The candidate made use of already available biological material and clinical data with appropriate informed consent. Additional DNA extractions were performed entirely by the candidate and appropriately catalogued.

Experimentation and analysis:

All experiments; molecular analysis by genotyping and sequencing; and functional analysis by tissue culture and expression analysis of protein product was executed in full by the candidate. The primary analysis of all data was performed by the candidate and when additional bioinformatics analysis was required, a collaborator was included by the primary supervisor and their contribution to the work is clearly indicated in each publication.

Publications:

Synthesis of all the works and drafting of all manuscripts of the first author publications included in this thesis were executed in full by the candidate, after which revisions from all co-authors were similarly incorporated before submission to the journal by the primary supervisor. After review, all reviewer comments were addressed by the candidate in conjunction with the primary supervisor. The detailed role of the candidate in the publications included in this thesis is clearly defined in each chapter. Publications of co-authorship were contributed to by the candidate by experimental work and analysis and in part drafting of relevant sections of the manuscripts.

This thesis is presented with three cohesive elements; (i) sub-Saharan perspective of genetic hearing loss; (ii) application of targeted gene panels in resolution of hearing loss in sub-Saharan African patients and lastly, (iii) application of whole exome sequencing in the resolution of hearing loss in sub-Saharan African patients.

We have met all requirements and abided by the UCT's Doctoral Degrees Board, under Rules GP6.7 as follows:

(i) The candidate's proposal to include publications in the current thesis was approved by the UCT Faculty of Health Sciences Doctoral Degrees Board.

(ii) The thesis contains an adequate Introduction; a chapter on the Aims and Objectives; a comprehensive Academic Discussion of the results, forming the basis of the Conclusions and Perspectives drawn from this research.

(iii) Each Results chapter with publications included, is preceded by a synopsis of how the publications directly tie to the aims and objectives of the project, as well as to the thesis.

(iv) All included publications, listed below, were written and published during the candidate's tenure as a PhD student.

List of publications included (all co-authors agreed to the inclusion of these manuscripts)

1. Bosch J, **Lebeko K**, Nziale JJN, Dandara C, Makubalo N, Wonkam A. 2014. In search of genetic markers for nonsyndromic deafness in Africa: a study in Cameroonians and Black South Africans with the GJB6 and GJA1 candidate genes. OMICS 18: 481–5

2. Wonkam A, Bosch J, Noubiap JJN, **Lebeko K**, Makubalo N, Dandara C. 2015. No evidence for clinical utility in investigating the connexin genes GJB2, GJB6 and GJA1 in non-syndromic hearing loss in black Africans. South African Med. J. 105: 23–26

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5. **Lebeko K**, Manyisa N, Chimusa ER, Mulder N, Dandara C, Wonkam A. 2017. A Genomic and Protein–Protein Interaction Analyses of Nonsyndromic Hearing Impairment in Cameroon Using Targeted Genomic Enrichment and Massively Parallel Sequencing. Omi. A J. Integr. Biol. 21: 90–99.

The candidate,

Kamogelo Lebeko

Abstract

Hearing Loss (HL) is one of the highest contributors to disability worldwide. The highest incidence of the disease is seen in developing countries, such as those in sub-Saharan Africa (SSA). Patients affected with disabling HL are reported to be more than 466 million worldwide. The causes of HL can either be environmental or genetic with each contributing about 50% towards all cases, in many settings. In developing countries, the environment might contribute more due to poor health services and infrastructure available to the population. In the absence of environmental causes, there is a genetic component at play, that is largely unknown in African populations. Up to 70% of HL of genetic origin are non-syndromic (NS). The mode of inheritance is recessive in nearly 77% of non-syndromic HL. Up to date, more than 100 genes have been associated with HL harbouring more than 1000 causative variants. In many populations of European and Asian descent, pathogenic variants in *GJB2* (connexin gene 26) and *GJB6* (connexin gene 30) are a major contributor to autosomal recessive non-syndromic hearing loss (ARNSHL). Comprehensive hearing health care programs should cover genetic causes by providing molecular testing, and genetic counselling, specifically SSA where genes and mutations causing HL remain largely unknown.

The aim of this project was thus to uncover the genetic causes of HL among patients' cohorts from Cameroon and South Africa. This was addressed by 1) sequencing common variants in the most relevant genes in other populations (*GJB*2 and *GJB6*), 2) using a targeted gene panel to resolve HL in 10 multiplex families from Cameroon presenting with ARNSHL and negative for *GJB*2 and *GJB6* mutations screening, 3) screening novel variants found in known genes in a cohort of 82 singleplex HL cases from Cameroon and South Africa, and lastly, 4) using Whole Exome sequencing to explore the two unresolved multiplex cases with and subsequent findings confirmed by functional studies, and also screened in 80 singleplex HL cases.

The following findings are reported:

GJB6, GJA1 mutations screening and literature review

No GJA1 or *GJB6* mutation was not found in multiplex and simplex cases of HL in both Cameroonians and South Africans. The review of the literature confirms that the prevalence of *GJB2*- or *GJB6*-related NSHL is approximating to zero in most sub-Saharan African populations.

Targeted Exome Sequencing (OtoSCOPE)

The targeted genes, panel that included 116 genes, was able to resolve 7 of 9 families (77.8%) which were successfully sequenced, with one family failing to be sequenced. The causative variants identified in the 7 resolved families were : 1) compound heterozygous c.5806_5808delCTC and c.5880_5882delCTT in *MYO7A*; 2) compound heterozygous c.646T>A (p.Phe216Ile) and c.38G>A (p.Arg13His) in *LOXHD1*; 3) homozygous c.766-2A>G in *OTOF*; 4) a deletion and a complex copy number variation in *STRC*; 5) compound heterozygous c.1678G>A (p.Asp560Asn) and c.2007C>A(p.Asp669Glu) in *SLC26A4*; 6) Homozygous c.1996C>T(p.Arg666Stop) in *MYO7A*; 7) compound heterozygous c.6399C>A(p.Asp2133Glu) and c.2000T>C (p.Met667Thr) in *CDH23*. Five out of 12 variants were novel. Screening of these causative variants in known genes, in 82 singleplex HL cases from Cameroon and South Africa was unable to resolve any of the cases: the variants were in either heterozygous in low frequency or absent.

Bioinformatic pathways exploration of SNP data of known HL genes revealed an extensive network within the HL genes, with 10 identified as important nodes, including *MYO7A.* Most HL genes were found to be involved in two biological processes which were sensory perception of mechanical stimulus (GO: 0050954, p= 1.430e-8) and sound (GO: 0007605, p = 1.246e-8). The molecular functions of variants found within these genes were found to mostly fall within the binding (GO: 0005488) and/or structural molecule activity (GO: 0005198).

Whole Exome sequencing

Whole exome sequencing was performed on four of the nine multiplex families: the two families that were unresolved by targeted panel sequencing, and two previously resolved families that were used as positive controls for the variant annotation and filtering pipeline. The results were the resolution of 3/4 families, including the two-

positive control. The previously unresolved “family 8” was found to harbour a novel variant within the *GRXCR2* gene, a gene only associated with HL once before. The c.251delC variant was revealed through *in silico* studies to cause a premature stop codon at position 116 due to its frameshift effect. The screening of this variant in our cohort of 80 singleplex cases revealed one other unrelated HL patient harbouring this causative variant. Due to the limited literature on the gene and its protein*, in silico* studies were used to show the predicted secondary structure folding of the protein as well as potential protein binding regions. Analysis showed that the predicted loss of a stable region of the protein as well as that of a putative binding domain could explain the pathogenic nature of the variant. *In vitro* studies showed that the variant hindered the detection of the protein by way of a DDK tag downstream in the plasmid. Additionally, GFP-Tagged GRXCR2 showed altered expression pattern in the variant when compared to the wildtype.

In summary, our data has revealed the efficacy of using next generation sequencing tools in resolving HL among sub-Saharan African patients as opposed to the single candidate gene approach. In our quest, we have employed two widely used strategies, targeted panel and whole exome sequencing (WES), both of which have had great successes in various populations. The targeted approach was able to resolve 77.8% of our families but did not detect variants for two of the families revealing the presence of other variants harboured in rarely associated gene not captured or included on the panel. This prompted for the use of a more comprehensive approach such as WES. These results corroborated with those of two families previously resolved by targeted exome sequencing. Additionally, one of the previously unresolved family was now resolved. This showed that WES was sensitive enough to detect variants in known HL genes but comprehensive enough to detect variants in other regions of the exome which have not been associated with HL or rarely associated with HL. The benefit of WES also extends to the contribution of exomic data from patients of African descent as there is an underrepresentation of this group in exome repositories as well as genomic or SNP databases. To the best of our knowledge, this is the first study to use WES to resolve HL in patients of African descent. The other benefit of such a venture is the use of this data not only for patients in SSA but also those in the diaspora.

In conclusion, we have successfully demonstrated the feasibility of using NGS tools in identifying causative variants in HL patients in SSA. Additionally, we have shown that WES is a more suitable approach to trying to resolve HL in Africa. Therefore, the data strongly support that genetic studies on families segregating HL in SSA could be the next frontier of HL genetic research, of global importance through discovering novel variants in known genes, and potentially novel genes. These studies will improve HL genetic diagnosis, retrospective counselling and testing, prevention and care including future prediction of treatment outcomes in sub-Saharan Africans, and in people of African descent.

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Chapter 1: Introduction

Hearing Loss (HL) is the number one communication disorder in the world. It is defined by the WHO as the inability to detect sound greater than 25dB in the better hearing ear of adults and 30dB in the better hearing ear of children (World Health Organisation, 2012). The incidence rate of congenital HL is about 0.5-1/1000 live births in developed countries and is as high as 7/1000 live births in developing countries (Olusanya and Newton, 2007). The disparities seen is thought to be due to the high burden of environmental factors which plague developing countries due to poor health services and lack of resources and infrastructure (Olusanya and Newton, 2007). Generally, these factors contribute to 50% of HL cases with the remainder being genetic. Due to the high burden of environmental factors in developing countries and lower resources, these figures have not been observed, and many cases remain classified as cases of unknown origin.

Hearing loss is highly heterogeneous in both its manifestation and its heritability. The most common manifestation of the disease is HL on its own and is referred to as nonsyndromic (NSHL). This happens in about 70% of all HL cases. Syndromic HL accounts for up to 30% of cases observed where the HL is accompanied by another ailment. Such syndromes included Usher Syndrome, Pendred syndrome and Waardenburg syndrome just to mention a few. Of the NSHL cases, the mode of inheritance can be autosomal, or sex linked, dominant or recessive as well as mitochondrial. The most common mode of inheritance is autosomal recessive nonsyndromic hearing loss (ARNSHL) which accounts for upwards of 80% of all genetic cases.

The genetic causes of HL have been uncovered across various populations. The connexin gene *GJB2* is by far the most frequently associated gene with ARNSHL. It accounts for up to 50% of HL cases among patients of European descent, particularly the c.35delG variant (Tsukada *et al*., 2015). However this variant has been shown to be founder variant and is not present in patients of different ethnicities (Tsukada *et al*., 2015). Nonetheless, screening of this gene has been extensive in different populations and has seen the identification of population specific variants for the Asian (Dzhemileva *et al*., 2010), Jewish and Ghanaian (Hamelmann *et al*., 2001) population

groups. Outside of these above mentioned groups and their region/group specific founder variants, the gene does not seem to play a significant role, particularly in sub-Saharan African populations (Gasmelseed *et al*., 2004; Kabahuma *et al*., 2011; Bosch *et al*., 2014a). This has been demonstrated several times over.

Initial efforts to uncover causative genes and their variants was done through homozygosity mapping and direct Sanger sequencing. This led to the identification of more than 80 genes in over 100 loci (Van Camp and Smith, 2017). These genes harboured more than 1000 variants associated with HL. This was performed on populations from Asia, Europe, the Americas and the Middle East, but did not included sub-Saharan African populations (Rudman *et al*., 2017). In order to facilitate the identification of causative genes and their variants specific to the sub-Saharan region as well as contribute new knowledge to the field, a more rapid approach is needed (Lebeko *et al*., 2015). One that would see a large amount of data being generated and made available to patients of this region.

Given the genetic heterogeneity of the disease, next generation sequencing (NGS) tools are best suited to be utilized in the effort of trying to resolve HL in the sub-Saharan African region. These tools allow for multiple parallel enrichment and sequencing of specific regions of the genome. These can be targeted to genes known to contribute to HL as uncovered in other population groups. The benefit of this approach is that there is a shorter route when it comes to filtering of results and their analysis as all the genes are known to cause HL and thus their variants are potentially pathogenic and causative in nature. There is also often no need to have added evidence towards the annotation of identified variants as the genes have been validated as causative. Several panels have been developed which capture coding regions of genes which have already been associated with HL. These include OtoSCOPE (Shearer *et al*., 2010), MiamiOtoGenes (Tekin *et al*., 2016), and OtoSeq (Shahzad *et al*., 2013; Sivakumaran *et al*., 2013) to name a few which are available for use to screen between 116-248 known HL genes.

Alternatively, given the Mendelian nature of the disease, whole exome sequencing (WES) is available and suitable to explore a wider region of the genome (Teer and Mullikin, 2010). It is still only 1% of the genome and is thus manageable in terms of data analysis. One would need a lot more phenotypic information when applying a

filtering strategy as to allow for accurate selection of potentially pathogenic variants. This includes mode of inheritance as well as the exclusion of other ailments to rule out SHL. With the above-mentioned tools available, it is thus feasible to start the journey to identifying which genes contribute to HL in this vast, diverse region of Sub-Saharan Africa.

1.2. Aim:

The overall aims of this project were:

(i) To investigate the most prevalent genes associated with ARNSHL in a cohort of HL patients from Cameroon and South Africa.

(ii) To investigate the utility of one of the available targeted gene enrichment panels (OtoSCOPE) in resolving multiplex cases of ARNSHL and uncovering a common variant which is contributing towards the disease.

(iii) To investigate the efficacy of using a wider approach such as WES in resolving multiplex cases from Cameroon and discovering new genes and their variant(s) which are contributing towards the disease in the region.

1.3. Objectives

The aims were carried out through the following objectives:

a) Recruitment of multiplex and singleplex cases from Cameroon and South Africa with putatively genetic causes of ARNSHL.
b) Screening of the most common genes (*GJB2, GJB6 and GJA1)* in this cohort.
c) Selection of multiplex families for resolution through OtoSCOPE.
d) Exploration of HL gene annotation and network of genes included on OtoSCOPE
e) Screening of identified variants in simplex cases.
f) Selection of multiplex families for resolution through whole exome sequencing
g) Screening of WES uncovered variants in the cohort of singleplex cases
h) Bioinformatics analysis of newly uncovered genes and variants
i) Functional studies to explore and validate pathogenicity of variants

Chapter 2: *GJB6, GJA1* mutations screening and literature reviews

Synopsis: The chapter presents three published articles: an original research which explored the most commonly associated genes in sub-Saharan Africa and two peer-reviewed review articles on the advocacy of using next generation sequencing technologies in resolving hearing loss among patients of African descent.

2.1. Bosch J, **Lebeko K**, Nziale JJN, Dandara C, Makubalo N, Wonkam A. 2014. In search of genetic markers for nonsyndromic deafness in Africa: a study in Cameroonians and Black South Africans with the *GJB6* and *GJA1* candidate genes. *OMICS* 18: 481–5.

Abstract

Deafness is the most common sensory disability in the world and has a variety of causes. Globally, variants in *GJB2* have been shown to play a major role in nonsyndromic deafness, but this has not been seen in Africans. Two other connexin genes, *GJB6* and *GJA1*, have been implicated in hearing loss but have seldom been investigated in African populations. We set out to investigate the role of genetic variation in *GJB6* and *GJA1* in a group of Cameroonian and South African Blacks with nonsyndromic recessive hearing loss. A subset of 100 patients, affected with nonsyndromic hearing loss, from a cohort that was previously shown not to have *GJB2* variant, was analysed by Sanger sequencing of the entire coding regions of GJB6 and *GJA1*. In addition, the large-scale *GJB6*-D3S1830 deletion was also investigated. No pathogenic variant was detected in either *GJB6* or *GJA1*, nor was the *GJB6*-D3S1830 deletion detected. There were no statistically significant differences in sequence variants between patients and controls. Variants in *GJB6* and *GJA1* are not a major cause of nonsyndromic deafness in this group of Africans from Cameroon and South Africa. Currently, there is no sufficient evidence to support their testing in a clinical setting for individuals of African ancestry.

Nature of Publication: Original Full Journal Article

Journal/Publisher: OMICS, Journal of Integrative Biology, Mary Anne Liebert Inc.; Peer reviewed; Online ISSN: 1557-8100

Candidate contribution: Performed experiments pertaining to *GJB6* and analysis of the results. Contributed to writing in part with relation to *GJB6* analysis

Co-Authors contributions:

JB: Conceived and designed experiments for the project, performed the experiments and analysed data, wrote and revised the manuscript.

JJNN: Performed the recruitment of patients,

NM: Performed the recruitment of patients

CD: Contributed reagents and materials for the experiments, revised manuscript

AW: Conceived and designed the experiments, analysed data, wrote and revised and approved manuscript.

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In Search of Genetic Markers for Nonsyndromic Deafness in Africa: A Study in Cameroonians and Black South Africans with the *GJB6* and *GJA1* Candidate Genes

Jason Bosch,[1] Kamogelo Lebeko,[1] Jean Jacques Noubiap Nziale,[2] Collet Dandara,[1] Nomlindo Makubalo,[1] and Ambroise Wonkam[1]

Abstract

Deafness is the most common sensory disability in the world and has a variety of causes. Globally, mutations in *GJB2* have been shown to play a major role in nonsyndromic deafness, but this has not been seen in Africans. Two other connexin genes, *GJB6* and *GJA1*, have been implicated in hearing loss but have seldom been investigated in African populations. We set out to investigate the role of genetic variation in *GJB6* and *GJA1* in a group of Cameroonian and South African Blacks with nonsyndromic recessive hearing loss. A subset of 100 patients, affected with nonsyndromic hearing loss, from a cohort that was previously shown not to have *GJB2* mutation, was analyzed by Sanger sequencing of the entire coding regions of *GJB6* and *GJA1*. In addition, the large-scale *GJB6*-D3S1830 deletion was also investigated. No pathogenic mutation was detected in either *GJB6* or *GJA1*, nor was the *GJB6*-D3S1830 deletion detected. There were no statistically significant differences in sequence variants between patients and controls. Mutations in *GJB6* and *GJA1* are not a major cause of nonsyndromic deafness in this group of Africans from Cameroon and South Africa. Currently, there is no sufficient evidence to support their testing in a clinical setting for individuals of African ancestry.

Introduction

Deafness is one of the leading causes of disability globally and is most severe in the developing world (Stevens et al., 2013). While it is seen in fewer than 2 per 1000 births in Europe (Parving, 1999), it occurs in approximately 7 per 1000 births in Nigeria (Olusanya and Somefun, 2009) and 5.5 per 1000 births in South Africa (Swanepoel et al., 2009). Deafness is a highly variable and extremely heterogeneous condition that can range from mild to total hearing loss and present either as a single symptom or as one of many clinical features. Deafness can be caused by environmental conditions, genetics, aging, or a combination of these factors.

Currently, 65 different genes have been identified that contribute to nonsyndromic deafness and there are many more causative mutations (Van Camp and Smith, 2012). Genetic deafness can be inherited in a dominant, recessive, or mitochondrial manner, with some genes displaying more than one type of inheritance, depending on the change involved (Van Camp and Smith, 2012). Mutations in *GJB2* (connexin 26) have been shown to be a major contributor to deafness globally, but not in sub-Saharan Africa, with the exception of Ghana (Chan and Chang, 2014). Other potential candidate genes that could lead to nonsyndromic deafness in Africans are *GJB6* (connexin 30) and *GJA1* (connexin 43).

The second biggest genetic cause of nonsyndromic deafness in the European population is the *GJB6*-D13S1830 deletion identified by del Castillo et al. (2002, 2003) and present in up to 9.7% of patients in some European countries. Although originally considered to be a case of digenic inheritance, as connexins 26 and 30 are known to interact, other evidence suggests that the deletion includes an unidentified cis-regulatory region for *GJB2* (Rodriguez-Paris and Schrijver, 2009).

GJA1 emerged as a possible candidate gene for hearing loss in Black Africans when mutations in this gene were associated with nonsyndromic hearing loss in African Americans (Liu et al., 2001). However, subsequent analysis has shown that those results were due to failure to differentiate between *GJA1* and its pseudogene, and that the reported mutations occurred

[1]Division of Human Genetics, Department of Clinical Laboratory Sciences, Faculty of Health Sciences, University of Cape Town, Cape Town, South Africa.
[2]Faculty of Medicine and Biomedical Sciences, University of Yaoundé I, Yaoundé, Cameroon.

only in the pseudogene (Paznekas et al., 2003). Subsequent studies of *GJA1* and hearing loss have failed to provide convincing evidence of an association, either finding no causative variants or variants at very low frequencies (Uyguner et al., 2003; Yang et al., 2007, 2010; Kooshavar et al., 2012).

The mutation del(*GJB6*-D13S1830) in *GJB6* (Kabahuma et al., 2011) and variations *GJA1* are screened in some clinical settings in South Africa, especially in Black South African, but with insufficient evidence of their clinical utility. We aimed to validate the utility of testing for *GJB6* and *GJA1* in two carefully selected groups of Africans from Cameroon and South Africa, affected by nonsyndromic recessive hearing loss.

Methods

Ethical considerations

Recruitment of patients from Cameroon was approved by Cameroon's National Ethics Committee, authorization number N°123/CNE/SE/2010. Ethics approval for the *GJB6* and *GJA1* research was granted by the University of Cape Town's Human Research Ethics Committee, reference numbers 042/2013 and 080/2011, respectively. Written informed consent was obtained from all participants, if they were 18 years or older, or from the parents/guardians with verbal assent from the children.

Patient selection

Cameroonian patients were recruited from seven of the ten regions of Cameroon, mainly from schools for the deaf, and those procedures including participants' medical and family history general systemic and otological examination have been reported previously (Wonkam et al., 2013). South African patients, all from the Xhosa ethnic group, were recruited from Efata School for the Blind and Deaf in the Eastern Cape Province, South Africa.

For the present study, a subset of 100 patients was chosen in order to maximize the probability of finding a genetic cause of nonsyndromic deafness, as revealed by one or more affected family members or consanguinity, or deafness of unknown origin, that were shown not to have mutations in *GJB2* (unpublished data). This group of patients selected for the present study included six Cameroonian patients from consanguineous marriages, 52 familial Cameroonian cases (patients coming from families that had more than one patient affected with nonsyndromic hearing loss), five familial South African cases, two patients with heterozygous *GJB2* mutations, 15 prelingual Cameroonian sporadic cases, and 20 South African sporadic cases. All individuals, with the exception of four Cameroonian patients, had been previously genotyped for *GJB2*. The inclusion of Cameroonian patients was carefully recruited throughout the country, with the intended attempt to serve as a proxy for the multiple ethnolinguistic background found in Africa. Indeed, Cameroon is called "Africa in miniature" as the Cameroonian population has been shown, not only at the cultural level but also at the population genetic and linguistic level, to mimic the genetic diversity that is observed in Africa (Tishkoff et al., 2009). Ethnically matched controls from Cameroon and South Africa were recruited from data-based DNA, in the Division of Human Genetics, Faculty of Health Sciences, University of Cape Town, South Africa.

Molecular methods

At the Molecular Diagnosis Laboratory of the Gyneco-Obstetric and Paediatric Hospital of Yaoundé, Cameroon, genomic DNA samples were extracted from peripheral blood of the patients, following instructions on the available commercial kit [Puregene Blood Kit® (Qiagen, USA)]. At the Division of Human Genetics, Faculty of Health Sciences, University of Cape Town, DNA was purified from saliva (Oragene® kit; DNA Genotek®, USA) according to the manufacturer's instructions.

Detection of del(*GJB6*-D13S1830) was performed using the method and primers described by del Castillo et al. (2002, 2003). The entire coding region of *GJB6* was amplified using the method described by Chen et al. (2012). A 1348 bp fragment consisting of the entire *GJA1* coding region was amplified using the F1 (5′ – GAA ATA CGT GAA ACC GTT GG – 3′) and R3 (5′ – CCT GGT GCA CTT TCT ACA GC – 3′) primers described by Huang et al. and which were designed to distinguish between *GJA1* and its pseudogene (Huang et al., 2011). Amplified products were sequenced, using both the forward and reverse primers, on an ABI 3130XL Genetic Analyser (Applied Biosystems, Foster City, CA). The same primers were used for amplification and sequencing.

Bioinformatic and statistical analyses

Chromatogram files were manually checked using FinchTV 1.3.1 (GeoSpiza) and aligned in BioEdit 7.0.5.3 to the *GJB6 and GJA1* reference sequence (Ensembl transcripts, retrieved 31 August 2012). Detected variations were checked against dbSNP (Sherry et al., 2001), and the effects of non-synonymous mutations were predicted using Polyphen-2 (Adzhubei et al., 2010). Differences in allele, genotype, and haplotype frequencies between cases and controls were assessed using SHEsis (http://analysis2.bio-x.cn/myAnalysis.php) (Shi and He, 2005; Li et al., 2009). The Chi-square test and the Fisher's exact test were used to compared SHEsis results, and a p value of less than 0.05 was considered statistically significant.

Results

Patients

The Cameroonian cohort was evenly distributed in terms of gender and well phenotyped. Full sociodemographic data of the participants is presented in Table 1. Ten patients in the Cameroonian cohort were from consanguineous marriages and all presented with severe to total (≥71 db) bilateral hearing loss (Table 2). The majority of the Cameroonian patients (85%) had sensorineural deafness, one had mixed hearing loss, and the rest were undetermined.

GJB6

GJB6 amplification was unsuccessful in two Cameroonian patients, and none of the remaining 98 patients presented with the *GJB6*-D13S1830 deletion. Only one variant (rs145762940) was detected, in the heterozygous state, in the coding region of *GJB6,* leading to the synonymous c.480G > A change. No variations in *GJB6* were detected in 31 controls (12 South African and 19 Cameroonian).

Table 1. Patient Sociodemographic Information

	South Africa Case (frequency)	*Cameroon Case (frequency)*
Gender		
Male	20 (0.80)	39 (0.52)
Female	4 (0.16)	35 (0.47)
Unknown	1 (0.04)	1 (0.01)
Age		
Average	13.95	12.11
Unknown	4 (0.16)	2 (0.03)
Age of Onset		
Prelingual (<2 Years)	3 (0.12)	70 (0.93)
Perilingual (2–4 Years)	6 (0.24)	0
Postlingual (>4 years)	3 (0.12)	4 (0.05)
Unknown	13 (0.52)	1 (0.01)
Transmission		
Familial	5 (0.20)	52 (0.69)
Unknown/unknown	20 (0.80)	23 (0.31)
	N=25	*N*=75

GJA1

It was not possible to amplify the *GJA1* gene in 10 participants (two South African and eight Cameroonian). Five variants were detected in *GJA1* (Table 3), one of which occurred in the intron, but none of which are known to be pathogenic. Forty-one controls (17 South African, 24 Cameroonian) were also sequenced, but only the synonymous c.717G>A change was detected. In addition, there were no statistically significant differences between cases and controls.

Discussion

Identification of genetic markers of deafness may lead to early detection and advise the choice of intervention. However, it has become apparent that the genetic variants associated with deafness in different populations are not the same. Thus, this study aimed to fully sequence and characterize, for the first time to our knowledge, the role of variation in *GJB6* and *GJA1* in a group of sub-Saharan Africans with nonsyndromic hearing loss.

As in previous studies amongst Chinese (Chen et al., 2012), Indians (Padma et al., 2009), Turkish (Tekin et al., 2003), and both African American and Caribbean Hispanics

Table 2. Audiological Data from the Cameroonian Cohort

Severity of deafness	*Left ear (frequency)*	*Right ear (frequency)*
Severe 1 (71–80)	1 (0.01)	2 (0.03)
Severe 2 (81–90)	3 (0.04)	4 (0.05)
Profound 1 (91–100)	24 (0.32)	22 (0.29)
Profound 2 (101–110)	22 (0.29)	23 (0.31)
Profound 3 (111–119)	10 (0.13)	11 (0.15)
Total (120)	3 (0.04)	1 (0.01)
Unknown	12 (0.16)	12 (0.16)
	N=75	*N*=75

Table 3. Genotypes of Patients and Controls with Respect to Variations in *GJA1*

				South Africa					*Cameroon*					
				Case		*Control*			*Case*			*Control*		
Transcript	*Protein*	*Domain*	*RS number*	*Het. (freq.)*	*WT (freq.)*	*Het. (freq.)*	*WT (freq.)*	P *Value*	*Hom. (freq.)*	*Het. (freq.)*	*WT (freq.)*	*Het. (freq.)*	*WT (freq.)*	P *Value*
c.-67A>G	NA	Intron	rs189167598	0	23 (1.000)	0	17 (1.000)	NA	1 (0.015)	0	66 (0.985)	0	24 (1.000)	0.547
c.189T>C	p. =	EC1	rs139688042	1 (0.043)	22 (0.957)	0	17 (1.000)	0.384	0	0	67 (1.000)	0	24 (1.000)	NA
c.366T>C	p. =	IC2	Novel	1 (0.043)	22 (0.957)	0	17 (1.000)	0.384	0	0	67 (1.000)	0	24 (1.000)	NA
c.717G>A	p. =	IC3	rs57946868	2 (0.087)	21 (0.913)	3 (0.176)	14 (0.824)	0.397	0	11 (0.164)	56 (0.836)	2 (0.083)	22 (0.917)	0.332
c.758C>T	p.(A253V)	IC3	rs17653265	1 (0.043)	22 (0.957)	0	17 (1.000)	0.384	0	0	67 (1.000)	0	24 (1.000)	NA

EC, extracellular domain; freq, frequency; Het., heterozygous; Hom., homozygous; IC, intracellular domain; WT, Wildtype.

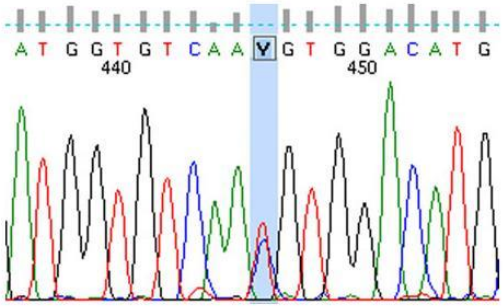

FIG. 1. Novel *GJA1* variant, c.366T>C (p.=) detected in one South African patient.

without *GJB2* mutations (Samanich et al., 2007), this study did not find either the *GJB6*-D13S1830 deletion or coding region variations. This supports the hypothesis that the *GJB6*-D13S1830 deletion is the result of a founder effect (del Castillo et al., 2003).

Although variants were detected in *GJA1*, there were no significant differences between patients and controls. We report a novel c.366T>C (p.=) *GJA1* variant (Fig. 1) which has not, to our knowledge, been described before. Only one variant, the c.758C>T (p.(A253V)) change, was nonsynonymous. However, c.758C>T is a known change that is not considered to be pathogenic. It has been reported before in both cases and controls in various studies on *GJA1* (Alexandrino et al., 2009; Paznekas et al., 2009; Kooshavar et al., 2012; Van Norstrand et al., 2012), but has been suggested to modify disease severity in certain cases (Cella et al., 2006).

We are aware of several limitations in this study, including that our cohort is too small to provide reliable information on the contribution of rare variants. In addition, although the diversity of Cameroonian patients provides strength to the study, by allowing us to examine variations that may occur throughout Africa, it also introduces a weakness by possibly introducing too much variation. This is not the case with the Xhosa population from South Africa, and results from other African studies should always be kept in mind. Despite these limitations, the data presented here support the conclusion that neither *GJA1* nor *GJB6* is a major cause of deafness in Africans.

As the two major genetic causes of global nonsyndromic deafness, *GJB2* and *GJB6,* as well as *GJA1,* have not been shown to be associated with nonsyndromic deafness in Africans studies here, the focus should turn to the other 65 candidate genes (Van Camp and Smith, 2012). The most effective approach would be to use methods such as massively parallel sequencing that can screen multiple genes at once (Shearer et al., 2010) or Whole Exome Sequencing (WES), which has proven successful at elucidating the causes of deafness in a variety of genes and populations, even in small families (Diaz-Horta et al., 2012). The use of targeted massively parallel sequencing approach or the WES approach offers the best chance of uncovering the genetic causes of deafness in a setting with the genetically diverse populations found in Africa.

Conclusion

Our results do not support a link between mutations in either *GJB6* or *GJA1* and nonsyndromic deafness in sub-Saharan Africans from Cameroon and South Africa. At present, there is no sufficient evidence to support their testing in a clinical setting for individuals of African ancestry.

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Author Disclosure Statement

The authors declare that no competing financial interests exist.

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Address correspondence to:
Dr. Ambroise Wonkam
Division of Human Genetics
Faculty of Health Sciences
University of Cape Town
Anzio Road
Observatory
7925 Cape Town
South Africa

E-mail: ambroise.wonkam@uct.ac.za

2.2.: Wonkam A, Bosch J, Noubiap JJN, **Lebeko K**, Makubalo N, Dandara C. 2015. No evidence for clinical utility in investigating the connexin genes *GJB2*, *GJB6* and *GJA1* in non-syndromic hearing loss in black Africans. South African Med. J. 105: 23–26.

Abstract:

Background. Deafness is the most common sensory disability in the world. Globally, variants in *GJB2* (connexin 26) have been shown to play a major role in non-syndromic deafness. Two other connexin genes, *GJB6* (connexin 30) and *GJA1* (connexin 43), have been implicated in hearing loss, but these genes have seldom been investigated in black Africans. We aimed to validate the utility of testing for *GJB2, GJB6* and *GJA1* in an African context. **Methods.** Two hundred and five patients with non-syndromic deafness from Cameroon and South Africa had the full coding regions of *GJB2* sequenced. Subsequently, a carefully selected subset of 100 patients was further sequenced for *GJB6* and *GJA1* using Sanger cycle sequencing. In addition, the large-scale *GJB6*-D3S1830 deletion was investigated. **Results.** No pathogenic variants that could explain the hearing loss were detected in *GJB2*, *GJB6* or *GJA1*, and the *GJB6*-D3S1830 deletion was not detected. There were no statistically significant differences in genomic variations in these genes between patients and controls. A comprehensive literature review supported these findings. **Conclusion.** Variants in *GJB2*, *GJB6* and *GJA1* are not a major cause of non-syndromic deafness in black Africans and should not be investigated routinely in clinical practice.

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Candidate contribution: Performed experiments pertaining to *GJB6* and analysis of the results. Contributed to writing in part with relation to *GJB6* analysis

Co-Authors contributions:

JB: Performed experiments and analysed data Wrote and revised the manuscript.

JJNN: Performed the recruitment of patients

NM: Performed the recruitment of patients

CD: Contributed reagents and materials for the experiments, revised manuscript

AW: Conceived the idea of the project, analysed data, wrote and revised and approved manuscript.

GENETICS IN MEDICINE

No evidence for clinical utility in investigating the connexin genes *GJB2*, *GJB6* and *GJA1* in non-syndromic hearing loss in black Africans

A Wonkam, J Bosch, J J N Noubiap, K Lebeko, N Makubalo, C Dandara

Assoc. Prof. Ambroise Wonkam is a medical geneticist trained at the University of Geneva, Switzerland, whose research interest is reflected in more than 70 publications with a focus on monogenetic diseases of people of African descent. He was the principal investigator on this project. Jason Bosch completed his MSc in human genetics on the project, with molecular analysis of the GJB2 *and* GJA1 *genes, and Kamogelo Lebeko did all the experiments on the* GJB6 *gene for her honours degree in human genetics. Nomlindo Makubalo, a paediatrician, spent two years in the medical genetics unit and did all the clinical phenotyping of the cohort from the Eastern Cape, South Africa, where she is currently practising. Assoc. Prof. Collet Dandara, a molecular geneticist, supervised all the molecular experiments in the project. All these authors were affiliated to the Division of Human Genetics, Department of Clinical Laboratory Sciences, Faculty of Health Sciences, University of Cape Town, South Africa, during the course of the project. Jean Jacques Noubiap, of the Faculty of Medicine and Biomedical Sciences, University of Yaoundé I, Yaoundé, Cameroon, is a general practitioner with strong research interests. He performed all the clinical phenotyping of the Cameroonian cohort for his MD degree.*

Corresponding author: *A Wonkam (ambroise.wonkam@uct.ac.za)*

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Results. No pathogenic mutations that could explain the hearing loss were detected in *GJB2*, *GJB6* or *GJA1*, and the *GJB6*-D3S1830 deletion was not detected. There were no statistically significant differences in genomic variations in these genes between patients and controls. A comprehensive literature review supported these findings.

Conclusion. Mutations in *GJB2, GJB6* and *GJA1* are not a major cause of non-syndromic deafness in black Africans and should not be investigated routinely in clinical practice.

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Deafness is one of the leading causes of disability in the world. The prevalence of hearing loss is highest in South Asia and sub-Saharan Africa, attributable to poor healthcare systems where complications at birth as well as infections result in loss of hearing in the newborn.[1]

Screening for hearing loss in newborns is standard practice in many countries and is the most effective way of detecting hearing problems and reducing the negative effects of hearing loss. South Africa (SA) does not have a national screening programme. At best, only 7.5% of public hospitals provide screening for hearing loss, with only 1% providing universal screening.[2] The situation is even worse in the rest of sub-Saharan Africa.[3] Clinical presentation of hearing loss is extremely heterogeneous, ranging from mild to total hearing loss and presenting either as a single symptom or as one of many clinical features. The causes of hearing loss can be genetic or environmental. A recent review of the aetiology of childhood hearing loss showed that 48.3% of cases of hearing loss were of unknown cause, 30.4% were genetic and 19.2% were acquired.[4] In developing communities, the environment contributes significantly more to congenital hearing loss than in the developed world. In Africa, bacterial meningitis contributes to cases of hearing loss in infants and young children.[3] With improved healthcare there will be a reduction in cases of hearing loss caused by disease and an increase in the proportion attributable to genetics, the majority of which are non-syndromic.[1]

To date, 65 different genes, with many different causative mutations, have been identified that contribute to non-syndromic deafness.[5] Mutations in gap junction (GJ) genes, specifically *GJB2* (connexin 26), have been shown to be the major contributors to deafness globally.[6]

GJ proteins (connexins) regulate functions of the cochlea

GJs are intercellular channels that allow ions, second messengers and small metabolites to be exchanged by adjacent cells. Connexins form intercellular channels by combining in groups of six to form a structure called a connexon.[7] Connexons from adjacent cells join together to form GJs (Fig. 1, A). The connexin proteins are named according to their weight; connexin 26 is a protein with a molecular weight of 26 kDa. The two major groups of connexins are the alpha and beta connexins, based on sequence similarity of the cytoplasmic loop. *GJB2* was the second beta connexin gene to be identified. *GJB2* is located on chromosome 13 and codes for the GJ protein connexin 26. Connexin 26 is involved in the transport of potassium ions and other small molecules and is expressed in the cochlea (Fig. 1, B).[8] It

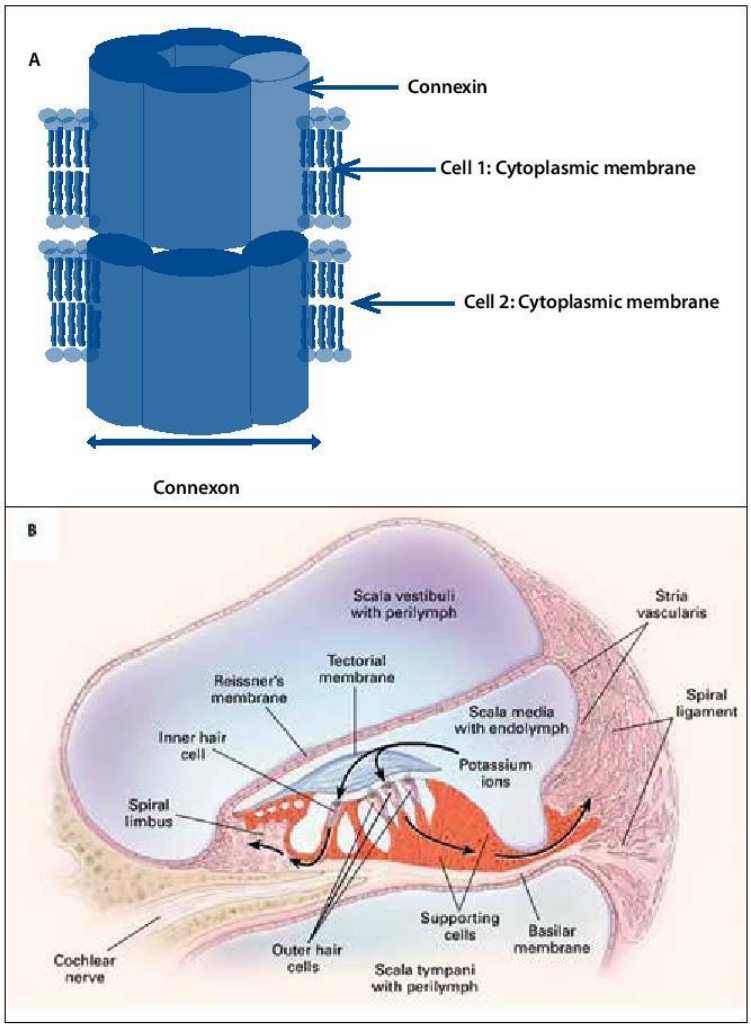

Fig. 1. GJ proteins (connexins) and the inner ear. A: Connexins form intercellular channels by combining in groups of six to form a structure called a connexon; two connexons from adjacent cells join together to form GJs. B: Cross-section through the cochlea. Red cells express connexin 26 (adapted from Willems et al.[8]*). (GJ = gap junction.)*

is believed that mutations in *GJB2* (connexin 26 gene) affect its ability to transport potassium ions and therefore regulate the endocochlear potential required for nerve impulses involved in hearing, but the mode of action is not definitively elucidated.[9]

No mutations in connexin genes *GJB2*, *GJB6* or *GJA1* among Cameroonians and Xhosa South Africans

With the exception of Ghana,[10] mutations in *GJB2* (connexin 26) have not been shown to be a major contributor to deafness in sub-Sahara Africa.[6] Could other potential candidate genes, *GJB6* (connexin 30) and *GJA1* (connexin 43), lead to non-syndromic deafness in Africans? The *GJB6*-D13S1830 deletion is present in up to 9.7% of people of European descent,[11] and represents the second leading genetic cause of non-syndromic deafness. When *GJA1* mutations were detected in African Americans,[12] *GJA1* emerged as a possible candidate for hearing loss in indigenous Africans. However, failure by investigators to differentiate between *GJA1* and its pseudogene led to this hypothesis being discarded.[13]

We performed a series of molecular investigations and reviewed the literature with the aim of validating the clinical utility of testing for *GJB2*, *GJB6* and *GJA1* in the African context. As part of this validation, we recruited a total of 205 patients affected with non-syndromic hearing loss from a well-described Cameroonian cohort[3] and newly recruited black South Africans of Xhosa ancestry, the majority (85%) of whom had sensorineural deafness. All 205 patients were investigated for *GJB2* gene, as previously reported.[14] A subset of 100 selected patients, with deafness likely to be of genetic cause (mostly familial cases) and who did not have any mutation in *GJB2* gene, were investigated for mutations in the *GJB6* and *GJA1* genes.[15]

All the coding regions of *GJB2, GJB6* and *GJA1* were amplified and detection of del(*GJB6*-D13S1830) was also investigated.[14,15] In the *GJB2* gene, two likely pathogenic mutations were detected in two unrelated Cameroonian participants, g.3741_3743delTTC (p.F142del) and g.3816G>A (p.V167M) in a single individual each and in the heterozygous state (Table 1). No pathogenic mutation was detected among the SA patients.[14] Phylogeny analysis of the sequence data from the Cameroonian and SA controls, together with that of various populations extracted from the 1000 Genomes Project, shows as expected that the SA patients and Cameroonian controls grouped with the other African populations. There was a low variance when comparing sequences in *GJB2* in Africans with that of other population groups: the principal component analysis explains only 40% of the variations.[14] Specific sequence variants in the *GJB2* gene in Africans could therefore not explain the low occurrence of mutations associated with non-syndromic deafness in this population.

In the *GJB6* gene, none of the patients had the *GJB6*-D13S1830 deletion. Only one variant (rs145762940) was detected, in the heterozygous state, in the coding region of *GJB6*, leading to the c.480G>A (p.G160=) change.[15] Equally, in the *GJA1*, five variants were detected; one of these occurred in the intron, but none were known to be pathogenic.[15]

Few mutations in *GJB2*, *GJB6* or *GJA1* genes among other populations of African ancestry

Our report and review of the literature confirmed that *GJB2* gene is of little significance in non-syndromic hearing loss in populations of African descent (Table 1).[10,14,16-20] In addition, by combining data from previously unstudied deaf Xhosa patients in SA, Cameroonian patients, previous studies in Africans and the 1000 Genomes Project,

Table 1. Comparison of pathogenic mutations found in *GJB2* in a few populations of African ancestry

Variations		Country (observed/total alleles)				
Genomic	Coding	Cameroon	Ghana[¶]	Kenya/Sudan[§]	South Africa[†]	USA (African Americans)
g.3352_3353insG	c.35dupG	-	1/730	-	-	-
g.3352delG	c.35delG	-	-	10/1 178	-	7/100[**]
g.3396C>T	c.79G>A	-	-	-	-	2/46[‖], NA[‖]
g.3419T>C	c.101T>C	-	-	-	-	NA[‡]
g.3426G>A	c.109G>A	-	-	1/1 178	-	-
g.3455_3460del	c.138_143del	-	-	1/1 178	-	-
g.3512C>A	c.195C>A	-	-	1/1 178	-	-
g.3553T>C	c.236T>C	-	1/730	-	-	-
g.3566C>G	c.249C>G	-	-	-	-	1/100[**]
g.3586_3587insT	c.269_270insT	NA[*]	-	-	-	-
g.3658A>G	c.341A>G	-	-	-	-	NA[‡]
g.3697G>A	c.380G>A	-	-	1/1 178	-	-
g.3741_3743delTTC	c.424_426delTTC	1/360[†]	-	-	-	-
g.3744C>T	c.427C>T	-	110/730	-	-	1/100[**]
g.3795G>A	c.478G>A	-	-	1/1 178	-	NA[‡]
g.3816G>A	c.499G>A	1/360[†]	-	4/1 178	-	NA[‡]
g.3850T>C	c.533T>C	-	4/730	-	-	-
g.3868G>A	c.551G>A	-	1/730	-	-	-
g.3906G>T	c.589G>T	-	1/730	-	-	-
g.3925-3926delinsAA	c.608_610delinsAA	-	2/730	-	-	-
g.3958C>T	c.641T>C	-	1/730	-	-	-

NA = variations found during the study, but only in the control group. Variant information was obtained through the relevant paper's own results and a combination of the Deafness Variation Database (http://deafnessvariationdatabase.org/) and the Connexin-Deafness Homepage (http://davinci.crg.es/deafness/index.php).

Study references *Trotta *et al.*,[16] †Bosch *et al.*,[14] ‡Kabahuma *et al.*,[17] §Gasmelseed *et al.*,[18] ¶Hamelmann *et al.*,[10] ‖Samanich *et al.*[19] and **Pandya *et al.*[20] indicate that the mutation was found neither in patients nor in controls.

the analysis further supported the limited contribution of *GJB2* genes in non-syndromic hearing loss in Africans. Interestingly, we reported two cases of keratitis-ichthyosis-deafness (KID) syndrome in two Cameroonian patients,[21] caused by mutations in *GJB2*. In both cases the mutation found (p.Asp50Asn) was the most common in many populations globally.[5,22] Adding to the established founder effect of the *GJB2* mutations reported in European and Asian populations,[5] the data indicate that the high frequency of *GJB2* mutations in non-syndromic hearing loss have evolved in Eurasian populations after their migration out of Africa, and spread with population migrations. Finally, at the genetic level, the Cameroonian population diversity mimics that of various ethnolinguistic groups in African populations;[23] it is anticipated that results from a carefully selected sample in this population could capture those of many other populations on the African continent.

As in previous studies in Africans,[17] African Americans and Caribbean Hispanics with *GJB6* mutations,[19] we did not find either the *GJB6*-D13S1830 deletion or coding region changes. Similarly, no pathogenic variants were detected in *GJA1*, suggesting their non-implication in hearing loss among the Cameroonians and black South Africans studied,[15] as has been reported in African Americans.[13]

Clinical implications and research perspectives

From our analysis, there is no evidence that mutations in *GJB2*, *GJB6* or *GJA1* are associated with non-syndromic deafness in sub-Saharan African patients. We therefore recommend against routine use of either gene for clinical testing in patients of African ancestry. We suggest that future research should take advantage of the power of massively parallel sequencing to screen multiple genes at once. This approach has previously been shown to offer the best chance of uncovering the genetic causes of deafness in settings with a genetically diverse populations.[24]

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2.3. **Lebeko K**, Bosch J, Nzeale JJ, Dandara C, Wonkam A. 2015. Genetics of hearing loss in Africans: use of next generation sequencing is the best way forward*. Pan Afr. Med. J.* 20: 1–14.

Abstract:

Hearing loss is the most common communication disorder affecting about 1-7/1000 births worldwide. The most affected areas are developing countries due to extensively poor health care systems. Environmental causes contribute to 50-70% of cases, specifically meningitis in sub-Saharan Africa. The other 30-50% is attributed to genetic factors. Nonsyndromic hearing loss is the most common form of hearing loss accounting for up to 70% of cases. The most common mode of inheritance is autosomal recessive. The most prevalent variants associated with autosomal recessive nonsyndromic hearing loss (ARNSHL) are found within connexin genes such as *GJB2*, mostly in people of European and Asian origin. For example, the c.35delG variant of *GJB2* is found in 70% of ARNSHL patients of European descent and is rare in populations of other ethnicities. Other *GJB2* variants have been reported in various populations. The second most common variants are found in the connexin gene, *GJB6*, also with a high prevalence in patients of European descent. To date more than 60 genes have been associated with ARNSHL. We previously showed that variants in *GJB2*, *GJB6* and *GJA1* are not significant causes of ARNSHL inpatients from African descents, i.e. Cameroonians and South Africans. In order to resolve ARNSHL amongst sub-Saharan African patients, additional genes would need to be explored. Currently at least 60 genes are thought to play a role in ARNSHL thus the current approach using Sanger sequencing would not be appropriate as it would be expensive and time consuming. Next Generation sequencing (NGS) provides the best alternative approach. In this review, we reported on the success of using NGS as observed in various populations and advocate for the use of NGS to resolve cases of ARNSHL in sub-Saharan African populations.

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JB performed the molecular analysis of *GJB2* and *GJA1* genes among Cameroonians and South Africans; Read and agreed to the final manuscript

JJNN performed the review of the aetiology of Hearing loss in Africa and analysis of Cameroonian clinical data, Read and agreed to the final manuscript

CD: supervised the molecular analysis, Read and agreed to the final manuscript

AW: Conceived and supervised the project and compiled the revisions. Read and agreed to the final manuscript

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Review

Genetics of hearing loss in Africans: use of next generation sequencing is the best way forward

Kamogelo Lebeko[1], Jason Bosch[1], Jean Jacques Nzeale Noubiap[2], Collet Dandara[1,3], Ambroise Wonkam[1]

[1]Division of Human Genetics, Faculty of Health Sciences, University of Cape Town, Cape Town, South Africa, [2]Faculty of Medicine and Biomedical Sciences, University of Yaoundé I, Yaoundé, Cameroon, [3]Institute for Infectious Disease and Molecular Medicine (IDM),Faculty of Health Sciences, University of Cape Town, Cape Town, South Africa

[&]Corresponding author: Ambroise Wonkam , Division of Human Genetics, Faculty of Health Sciences, University of Cape Town, Anzio Road, Observatory, 7925, Cape Town, South Africa

Key words: ARNSHL, Sub-Saharan Africa, next generation sequencing, whole exome sequencing, targeted exome sequencing, hearing loss, OTOScope®

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Abstract

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Introduction

Hearing loss is defined as disabling when the loss of hearing is greater than 40dB in the better hearing ear [1]. It is listed as the 12th most common contributor to disease burden globally [2]. Hearing loss is the number one communication disorder in the world affecting about 360 million individuals, 32 million of whom are children under the age of 15 [1]. It affects about 1 in 1000 live births in developed countries and about 3 in 1000 births in developing countries [1]. Prevalence of hearing loss is highest in South Asia and sub-Saharan Africa [3] which are part of thelow income regions or the developing world [1]. This is attributed to poor health care systems where complications at birth as well as infections could result in loss of hearing in the new-born [4]. In the Philippines, a prevalence rate of 6 per 1000 was reported which is comparable to the 7 per 1000 and 6 per 1000, in Nigeria and South Africa, respectively [5].

There are various classifications used to describe the clinical manifestation of hearing loss. The American Speech-Language-Hearing Association (ASHA) classifies hearing loss according to the following characteristics [6]: **pathophysiology** refers to the component of the hearing system which is non-functional. This can be any of the outer, middle or inner ear components. Conductive hearing loss refers to the non-functionality of the outer or middle ear. Sensorineural refers to hearing loss due to non-functionality of the inner ear. When both the inner and outer or middle ear is not functioning, it is referred to as mixed hearing loss; **severity** is determined by measuring the threshold needed for sound to be perceived. It is determined by generating an audiogram. The higher the threshold where sound is perceived, the more severe the loss of hearing; **onset** is either pre-lingual, which is before the development of speech as seen in congenital cases, or post-lingual. If the hearing loss in both ears then it is referred to as bilateral and unilateral if only one ear is affected.

We reviewed the available studies on the aetiology and genetics of hearing loss in Africa, including our previous publications on this topic that have shown the non-implication in Africansof the genes most commonly associated with ARNSHL among patients of European and Asian descent: GJB2 and GJB6. In this article,we review the success of using NGS as done so by other research groups in various populations and advocate for the use of NGS to resolve cases of ARNSHL in sub-Saharan African populations. This will provide rapid results which will contribute towards building a genetic profile on ARNSHL amongst sub-Saharan Africans.

Methods

The present article is a systematic review of most recent publications of hearing loss with focus on relevant articles on aetiology of hearing loss in sub-Saharan Africa and those that involved NGS to study the causes of hearing loss. The key word used was hearing loss, aetiology, genetics, Africa. We used the following search engines: PubMed® and Google Scholar® (August 2014). Only publications in English, were retrieved and included in the manuscript.

Current status of knowledge

Aetiology of hearing loss: environmental causes

The causes of hearing loss can either be genetic or environmental. In developing communities, the environment contributes significantly more to the incidence of congenital hearing loss than in the developed world [2]. This is attributed to limited access to healthcare systems that are not always adequately equipped to assist and monitor pregnancy and birth. Malnutrition during pregnancy may lead to low birth weight which may result in complications which could lead to hearing loss [2]. The lack of gestational vitamin A has been suggested to contribute to hearing loss development in developing countries [7]. Other environmental factors which contribute to cases of non-genetic congenital hearing loss are trauma which is most common in areas where mothers give birth unassisted or by poorly trained staff and infection of viral diseases which affect the unborn child such as infection with Cytomegalovirus [8].

Specifically in Africa, an infection which contributes to cases of hearing loss in infants and young children is that of bacterial meningitis. In a study in Kenya [9] and many other African countries [10-14], it was observed that there was a high prevalence of sensorineural hearing loss amongst children treated for bacterial meningitis (**Table 1**). Children living in developing countries who fail to get vaccinated are at a greater risk of developing hearing loss sequel to bacterial meningitis infection [15].

There has been some investigation into genes that are thought to be candidates for differential susceptibility of certain individuals to noise-induced hearing loss (NIHL) while others seem unaffected [16]. A more complex incident of hearing loss is age related hearing loss (ARHL). The elderly account for the highest proportion of hearing-impaired people in the world. Within the age group of 65 and older, one in three individuals will experience some degree of hearing loss[1]. The complexity of ARHL is due to the interaction between environment factors, clinical history i.e. medication, social behaviour e.g. smoking, drinking, and genetic factors [17]. Much like with NIHL, there haven't been many genes isolated as candidate genes for susceptibility to ARHL. In cases of congenital hearing loss, the absence of known environmental influence leads to the assumption that a genetic factor is the cause of hearing loss

Aetiology of hearing loss: genetics of congenital hearing loss

The genes

There is high variability in the genes causing hearing loss as well as their causative mutations.To date, there are over 60 genes implicated in cases of hearing loss [18].

Clinical features and heredity

The manifestation of the hearing loss may be syndromic whereby there are other clinical features associated with the loss of hearing.On the other hand, it might be nonsyndromic whereby hearing loss is the only observed symptom.Nonsyndromic hearing loss accounts for up to 75% of cases of putative genetic origin [19]. Syndromic and nonsyndromic cases of hearing loss can be caused by mutations which act in a dominant or recessive manner. They can be autosomal or X-linked and some have been identified on mitochondrial DNA. Fifty percent of the cases are genetic with autosomal recessive non-syndromic hearing loss (ARNSHL) being the most common [20]. **Figure 1** illustrates that the most common mode of inheritance in nonsyndromic hearing loss.

Syndromic hearing loss

Usher syndrome

This syndrome is characterised by hearing loss and retinitispigmentosa. It accounts for 50% of cases of combined deafness and blindness worldwide. It has three clinical subtypes classified according to the severity of the hearing loss [21]. The most prevalent mode of inheritance for Usher syndrome is autosomal recessive [22]. Up to 11 genes to date have been identified with causative mutations which lead to the development of profound congenital hearing loss or progressive hearing loss. Genes involved in Usher syndromes include *MYO7A, CDH23,* and *USH1C* to name a few. *MYO7A* has been implicated in various populations as harbouring causative mutations for ARNSHL [23, 24]. There is a scarcity of epidemiological data in Sub-Saharan African populations with only one case reported in Cameroon in 2013 [14].

Pendred syndrome

This is an autosomal recessive syndrome with symptoms such as sensorineural hearing loss andpartially defective iodine organification, a process of adding iodine to thyroglobin for the production of thyroid hormone[25]. It accounts for up to 10% of all cases of genetic hearing loss [26].Mutations in SLC26A, which encodes for Pendrin, have been associated with Pendred syndrome. It is a homogeneous syndrome as all patients with biallelicmutations in SLC26A have Pendred syndrome. Pendrin functions as an anion exchanger and is expressed in the kidney, thyroid and inner ear. Loss of hearing is caused by increased calcium concentrations in the endolymph disrupting signalling transduction [26].

Keratitis-Ichthyosis-Deafness syndrome

This is a congenital disorder characterised by profound hearing loss, hyperkeratosis (thickening of the skin) and erythrokeratoderma (scaly skin) (**Figure 2** (A)). It affects the eye as well: Keratitis in the name refers to the inflammation of the cornea.The major genes implicated in KID are connexin genes *GJB2* and *GJB6* [27]. The most prevalent mutation associated with KID is p.Asp50Asn in *GJB2* [28]. A recent publication has also reported two cases in sub-Saharan Africa of sporadic origin carrying the above mentioned mutation [29]. This indicates that the mutation is not population specific.

Waardenburg syndrome

It is described as an auditory-pigmentory disorder which affects the iris, hair and skin's pigmentory deposits (**Figure 2** (B)) [30]. Hearing loss associated with Waardenburg syndrome is usually of congenital sensorineural type with about 40% of cases displaying progressive hearing loss. It is inherited in an autosomal dominant manner [31]. Major genes in Waardenburg syndrome are PAX3 and SOX10 [30]. Waardenburg has been noted as the most frequent hearing loss syndrome amongst sub-Saharan African patients [32].

Oculo-auriculo-vertebral (OAV) spectrum (Goldenhar Syndrome)

This is a rare congenital disorder that affects the development of the ear, nose and soft palate [33]. There are other anomalies which might present within the spectrum. The cause is largely unknown though it is thought to have a genetic component [34].Given the rarity of the syndrome and its genetic heterogeneity, it is not unexpected that there is little data freely available on cases of Goldenhar Syndrome amongst sub-Saharan patients. Following aPubMed search for "Goldenhar Syndrome" "Africa", three papers appeared with only one being freely accessible.It was a case report in 1998 of a 15-month old from South Africa with ameloblastic fibroma associated with OAV [35].

Nonsyndromic hearing loss

Connexin genes and ARNSHL in the global population

Autosomal recessive nonsyndromic hearing loss (ARNSHL) is the most common type of hearing loss [36]. The most common mutations associated with ARNSHL are found within connexin genes. They have been implicated amongst European populations of Caucasian descent, as well as in Mediterranean populations such as Spain, Italy, France, and also among Arab populations in Middle East and North Africa. Mutations in GJB2, which encodes for connexin 26, are the most common cause of hearing loss amongst this population. The most common GJB2 mutation is c.35delG which is seen in up to 70% of cases [37]. The second most common gene associated with hearing loss is GJB6 which encodes for connexin 30 [38]. Outside of the Caucasian European population, the implication of c.35delG in hearing loss has been rare leading to the hypothesis that this is a founder mutation amongst populations of Caucasian descent [39]. Mutations uncovered in these two connexin genes have allowed for a rapid diagnostic approach within this population. Up to 70% of the cases of hearing loss are resolvedby screening for the most common mutations followed by screening the entire gene.

Screening of GJB2 has been conducted in other populations [40] such as in Asian populations where 35delG has not been detected, which further corroborates the founder effect hypothesis. Instead, a different founder mutation for this population group was identified as 235delC [41]. Amongst Mediterranean populations and Ashkenazi Jews, 167delT is the most prevalent GJB2 mutation, also hypothesized to be due to a founder mutation [36, 42]. **Figure 3** shows the most common GJB2 mutations within their respective populations.

Connexin genes and ARNSHL in africans

In Sub-Saharan Africa, *GJB2* has been investigated in various countries. It was only implicated in Ghana with p.R43W being the most common mutation for this population [43]. Screening in Cameroon [44], South Africa [45] and Kenya [46] did not reveal any significant *GJB2* mutations. *Wonkamet al* reported two cases of KID syndrome due to mutations in *GJB2* and illustrated that these cases have the most common mutation in the global population [29]. In addition Bosch et al extracted data for the 1000 Genome project and compared it to that of the GJB2 sequences from both Cameroon and South Africa and showed that there was a low level of variance[47]. Together, with the founder effect of the GJB2 mutation reported in Caucasian population [37, 48, 49], these data indicated that the African DNA are not "immune" of GJB2 mutations, which seem to havein Eurasian populations after their migration out of Africa, and spread with population migrations.

GJB6 has also been investigated in other populations and its variants are yet to be identified outside of the European Caucasian population [45, 50-55]. We recently showed that major *GJB6* mutations are not of significance in sub-Saharan African populations and that there is little evidence to suggest that rare causative mutations might be harboured by this population group [56]. We also screened the coding region of *GJB6* to try and uncover any rare point mutations [56]. The results suggested that *GJB6* genetic variation is not a significant factor amongst sub-Saharan African patients with ARNSHL.

There has been some contention on the role of *GJA1* in hearing loss with initial reports being subsequently discredited as the researchers

had initially failed to distinguish between the gene from the pseudogene; no pathogenic mutations in *GJA1* coding region in Africans from Cameroon and South Africa [56].

Other genes involved in nonsyndromic hearing loss

Outside of connexin genes, there have been mutations reported in various other genes.**Table 2** summarises a few of these in according to their role or function. Many of the mutations were found amongst Caucasian, Asian and Middle-Eastern populations [57]. To the best of our knowledge, there are neither data reported from sub-Saharan populations with ARNSHL nor any recurrent mutations in sub-Saharan populations.

Due to the number of different genes and mutations that have been implicated in hearing loss, it has become imperative that methods that are able to interrogate many gene positions at the same time be employed to find the relevant variants in African populations. Thus, the obvious choice becomes sequencing, especially using next-generation sequencing (NGS).

Exploration of hearing loss genes using Next-Generation sequencing

Whole exome sequencing

Massive parallel sequencing allows for the interrogation of multiple genes at the same time. This can be whole genome sequencing where by the entire 3 billion base pairs of the individual's genome are sequenced. With most monogenic disorders having been caused by exonic mutations, whole exome sequencing is often favoured. Moreover, exonic regions represent only 1% of the entire genome which makes this interrogation both time and financially feasible as compared to whole genome sequencing [58]. This involves selecting only the exons of the genome to sequence and screen for mutations or variations. This approach was recommended for autosomal recessive monogenic disorders' mutation screening [59].

The use of consanguineous families also helps narrow the search for such mutations as one has to look for homozygosity and the close relatedness of the family members' helps in distinguishing between variations and disease related mutations. Other approaches involve using unrelated individuals whom present with the same clinical symptoms and comparing their captured variants to decide whether or not they are significant [58].

The use of gene mapping has long been a standard principle to try and identify the gene associated with a disease. In 2010 Walsh Et al. used homozygosity mapping and whole exome sequencing to identify the causative gene on locus DFNB82 in a Palestinian family with nonsyndromic hearing loss [60]. The region of interest contained 5 genes and spun 3.1Mb on chromosome 1p13.1. The design used to capture data from exonic regions covered 38Mb of the human gene which encompassed 23, 739 genes. Two of the 5 genes could not be evaluated as they lay on segmental duplications. 80 variants which passed the quality threshold were reported on the remaining three genes. Only 7 had not been previously reported on thedbSNP database. Ultimately p.R127X of GPSM2was identified as the causative mutation on this locus [60].

In the Middle East, 30 individuals from 20 consanguineous families were selected for whole-exome sequencing analysis [61]. The families were from Iran and Turkey. On average each sample yielded more than 90 000 point mutations from whole-exome sequencing before any filters were applied [61]. There after only 12 homozygous mutations from known deafness genes were reported in 12 families [61]. They also report 4 novel heterozygous mutations in the 12 families where homozygous mutations had already been reported. They recommend the exploration of heterozygous mutations within known implicated genes when seeking causative mutations in small families or sample sizes [61].

The drawback of using Whole exome sequencing is that you select for the entire exome which will also carry genes not related to your disease of interest. One might also enrich for areas of less importance while losing genes which could be of interest or importance. The filtering of variants leaves a few variants to be interrogated. There is also the risk of losing rare variants. If one does not have family members' or parents' DNA to also work with, the efficiency is also negatively affected. A better approach would be to select for genes or specific areas of interest to screen rather than the entire exome, especially if associations for the disease have already been made.

Targeted gene sequencing

When the genes associated with a particular disorder have already been identified, targeted exome sequencing could be the most appropriate subsequent step in clinical settings. This involves using DNA chips and arrays with the desired genes already selected to

capture DNA from those selected regions [58]. This fine tunes the approach as only specific genes are interrogated. This setting also provides a way for diagnostic tools to be developed to be used in clinical settings [62].

Genes associated with ARNSHL have been identified in many populations around the world [57]. These results further demonstrated the heterogeneity of ARNSHL thus using a targeted increases the likelihood of finding causative mutations amongst other populations. A tool has been developed which targets genes known to be associated in ARNSHL for exome sequencing [62]. The tool is named OTOScope and is reported to have the sensitivity and feasibility to rapidly diagnose and resolve cases of ARNSHL [62]. It is a DNA Microarray Chip which enriches exonic data of more 67 genes which have been implicated in nonsyndromic hearing loss and in Usher syndrome [62]. Massive parallel sequencing of these regions allows for the resolution of ARNSHL and Usher Syndrome. It has also shown to be sensitive and specific enough for use in clinical settings.

The developers of the tool demonstrated the sensitivity and specificity of their tool by screening three positive controls and one negative control [62]. Upon validation of their results (by Sanger sequencing) to the known genotypes of the controls, they screened 6 unknowns i.e. unresolved cases [62]. Pathogenic mutations were identified in 5 of the unknowns, three of which had not been previously reported [62]. Subsequently, 100 new samples with an apparent genetic deafness were screened using OTOScope. An overall diagnostic rate of 42% was reported for this second study [63].

Another next generation sequencing tool has been developed called OtoSeq [64]. It targets about 24 genes associated with sensorineural hearing loss.The targeted genes are enriched using microdroplet polymerase chain reaction [65]. It was used to resolve hearing loss in a sub-set of a large Pakistani cohort of 243 families. Thirty four families were selected for the screening using OtoSeq based on co-segregating with markers for *MYO7A, SLC26A4* and *CDH23*. Twenty fourmutations were identified in 28 families. Eleven of these mutations were novel *MYO7A* mutations [66].

This method of targeted enrichment and massively parallel sequencing has been adopted by other research groups to resolve cases hearing loss. Amongst them investigators of the Chinese population, Yang et al, have demonstrated the success of using targeted exome sequencing. Their technique used exonic data from 79 deafness genes [67]. The 125probands used were excluded of mutations in the most common deafness genes amongst this population namely *GJB2, SLC26A* and a mitochondrial mutation in *MT-RNR1*. They report 45 novel recessive mutations and three novel dominant mutations. They also put emphasis on the fact that17.4% of these novel mutations were found in less commonly screened genes advocating for more genetic testing to be done on these genes [67]. Amongst the Japanese, 216 patients with bilateral sensorineural hearing loss were recruited. One hundred and twelve genes were selected for targeted genesequencing. They report 57 genes to have been identified as responsible for the loss of hearing in their cohort [68].Amongst the top candidate genes with the highest number of mutations were *GJB2,SLC26A4, USH2A, GPR98, MYO15A, COL4A5 and CDH23* [68]. Eighty six point six percent (86.6%) of the patients carried at least one mutation and 69 patients' hearing loss was resolved [68]. More than 250 mutations were confirmed by Sanger sequencing.

Conclusion

These are a few examples of the use of NGS to uncover mutations responsible for cases of hearing loss across various population groups.Upon the identification of such mutations, there can be a step towards population specific diagnostic tool development. There is very little data available on the genetic profile of hearing loss patients in Sub-Saharan Africa; with the current investigatory route being NGS in resolving this matter, it is the only way forward.

Competing interests

The authors declare no competing interest.

Authors' contributions

KL performed the articles search and drafted the manuscript and performed the molecular analysis of *JGB6* gene among Cameroonians and South Africans, JB performed the molecular analysis of *GJB2* and *JGA1* genes among cameroonians and South

Africans; JJNN performed the review of the aetiology of Hearing loss in Africa and analysis of Cameroonian clinical data, CD supervised the molecular analysis, AW conceived and supervised the project and compiled the revisions. All the authors read and agreed to the final manuscript.

Tables and figures

Table 1: Comparison of aetiological studies on hearing loss in sub-saharan Africa

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Figure 1: Proportion of syndromic, nonsyndromicand mode of inheritance of hearing loss. (adapted from [21])

Figure 2: Clinical phenotype of selected syndromes: (a) cameroonian patient presenting with erythrokeratoderma, a symptom of KID; (B) *Heterochromia iridis* in a cameroonian patient with Waardenburg syndrome

Figure 3: The most common *GJB2* mutations found amongst various World populations. Countries shown in grey do not have published data on GJB2 mutations, the majority in sub-Saharan Africa (adapted from [40])

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Table 1: Comparison of aetiological studies on hearing loss in sub-Saharan Africa

Country	**Gambia**	**Nigeria**	**Sierra Leone**	**Ghana**	**Cameroon**
Year of Study	1985 [10]	1982 [11]	1998 [12]	1988 [13]	2013 [14]
Number of Patients	259	298	354	105	582
Hereditary	8.1%	13.1%	-	-	14.8%
Meningitis	30%	11%	23.9%	8.5%	34.4%
Measles	1.9%	13%	4.1%	30%	4.3%
Rubella	1.5%	2%	-	3.5%	0.5%
Mumps	-	3%	16.7%	3.5%	2.1%
Ototoxicity	-	9%	20.8%	-	6%

Table 2: Genes implicated in cases of autosomal recessive non-syndromic hearing loss

Function	**Genes**
Cochlear Homeostasis	Gap Junctions: *GJB2, GJB6, GJB3*
	Tight Junctions: *CLDN14, TRIC*
	SLC26A4
Cellular Organization	Myosins: *MYO3A, MYO6, MYO7A, MYO15A*
	TRIOBP, WHRN, USH1C, CDH23
Tectorial Membranes Associated Proteins	*TECTA, COL11A2, STRC*
Neural transmission	*OTOF, PJVK*
Other or Unknown function	*TMC1, TMPRSS3, LOXHD1, PDZD7, GIPC3*
Adapted from **[57]**	

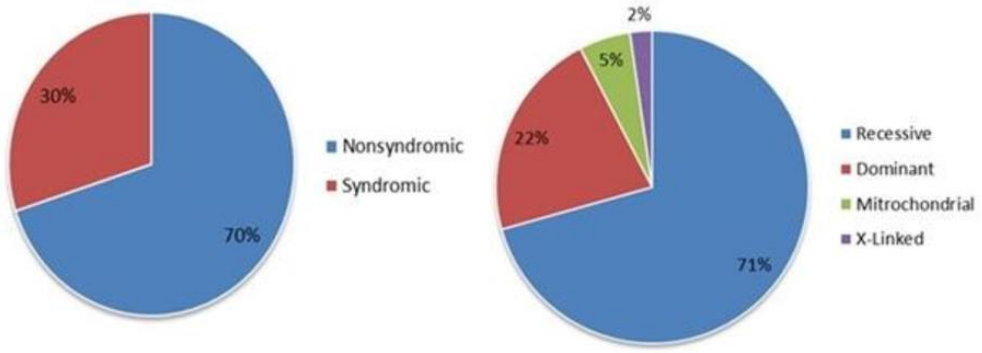

Figure 1: Proportion of syndromic, nonsyndromicand mode of inheritance of hearing loss. (adapted from [21])

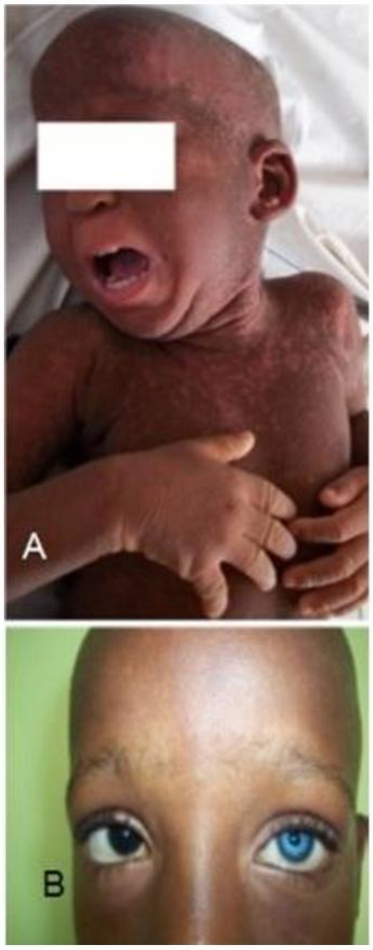

Figure 2: Clinical phenotype of selected syndromes: (a) cameroonian patient presenting with erythrokeratoderma, a symptom of KID; (B) *Heterochromia iridis* in a cameroonian patient with Waardenburg syndrome

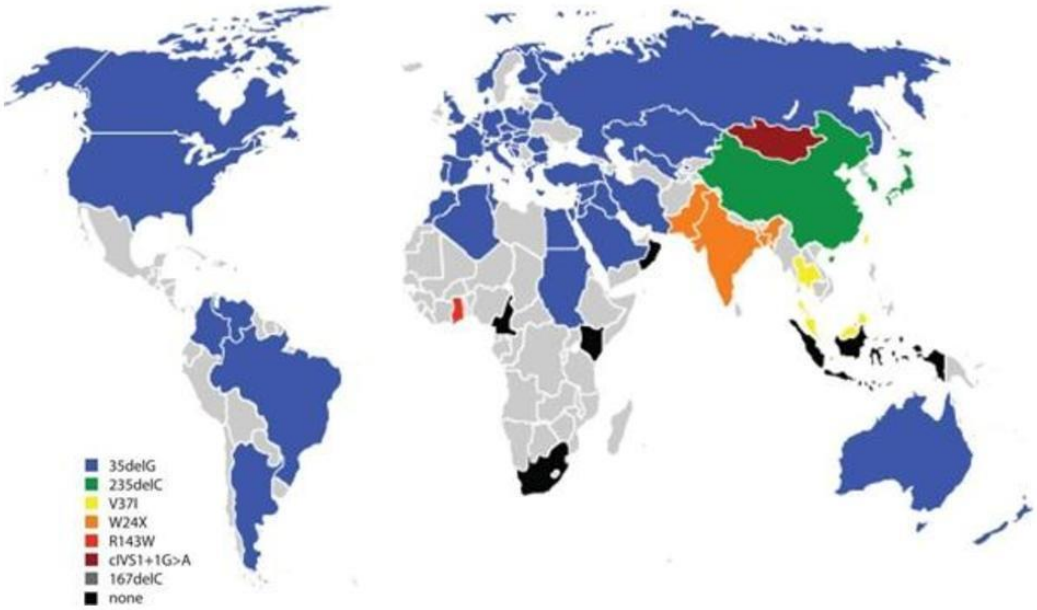

Figure 3: The most common *GJB2* mutations found amongst various World populations. Countries shown in grey do not have published data on GJB2 mutations, the majority in sub-Saharan Africa (adapted from [40])

Chapter 3: Application of targeted exome sequencing in ARNSHL in Sub-Saharan African patient

Synopsis: In order to timeously identify the causative variants which contribute to HL in sub-Saharan African patients, NGS tools should be employed. This chapter includes two original publications that: 1) Report on the findings of using a comprehensive HL panel called OtoSCOPE to resolve HL in 10 selected multiplex families, and 2) The prevalence of identified variants in a cohort of singleplex cases as well as the bioinformatics network profile of HL genes.

3.1: **Lebeko K**, Sloan-Heggen CM, Noubiap JJN, Dandara C, Kolbe DL, Ephraim SS, Booth KT, Azaiez H, Santos-Cortez RLP, Leal SM, Smith RJH, Wonkam A. 2016. Targeted genomic enrichment and massively parallel sequencing identifies novel nonsyndromic hearing impairment pathogenic variants in Cameroonian families. *Clin. Genet.* 90: 288–290.

Abstract

This article explored the use of a targeted gene enrichment panel called OtoSCOPE in the resolution of 10 selected multiplex families from Cameroon presenting with ARNSHL. The resolution rate after screening 66 known HL genes was 77%. Twelve different variants (5 of which were novel) were identified in 6 different genes, majority of which contributed to the disease as a result of compound heterozygotes. The identified variants were not significantly present in a cohort of 80 Singleplex cases and thus they remain unresolved. The unresolved multiplex cases highlight that there are other genes which contribute to HL which can still be identified as significant contributors to the disease for this cohort.

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Candidate Contribution: Designed and performed the experiments, analysed data, wrote, revised and approved the manuscript.

Co-Authors' contributions

CMSH performed experiments, analysed data, reviewed and approved manuscript.

JJNN performer analysis of Cameroonian clinical data, read and agreed to the final manuscript

CD: supervised the molecular analysis, Read and agreed to the final manuscript

RHJS contributed materials/reagents/analysis tools, revised and approved the manuscript

DLK, SSE, KTB, HA, RLPSC, SML revised and reviewed the manuscript

AW: Conceived and supervised the project and compiled the revisions. Read and agreed to the final manuscript

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Letter to the Editor

Targeted genomic enrichment and massively parallel sequencing identifies novel nonsyndromic hearing impairment pathogenic variants in Cameroonian families

To the Editor:

In contrast to many world populations, in sub-Saharan Africa *GJB2*-related nonsyndromic hearing impairment (NSHI) is rare (1), and little is known about the contribution to hearing impairment (HI) by other known genes. Targeted genomic enrichment (TGE) and massively parallel sequencing (MPS), using a platform called OtoSCOPE®, have been shown as an efficient tool for comprehensive genetic testing for NSHI (2). In this study, we have investigated the clinical utility of this panel to resolve the genetic causes of autosomal recessive nonsyndromic hearing impairment (ARNSHI) in familial cases from Cameroon.

The study was approved by the Cameroon National Ethics Committee (REF 123/CNE/SE/2010), the Human Research Ethics Committee of the University of Cape Town (REF 455/2014), and the University of Iowa IRB (approval number 1035709). Patients were recruited from schools of the deaf in Cameroon (3). Clinical evaluation included a comprehensive questionnaire (exposure to noise, ototoxic agents, and familial history) and the diagnosis of sensorineural HI according to the current clinical standards (3). All probands and affected individuals were examined for syndromic features by a medical geneticist, and an ophthalmologist. Families with at least two individuals with ARNSHI who are negative for pathogenic variants in *GJB2* and *GJB6* were studied.

DNA samples of affected individuals were analyzed at the Molecular Otolaryngology and Renal Research Laboratories (MORL) at the University of Iowa's Carver College of Medicine; TGE and MPS were completed as previously described (2). The Exome Aggregation Consortium (ExAC) database was used to remove high frequency variants (i.e. minor allele frequency $\geq 0.1\%$) which were unlikely to be pathogenic and to obtain variant frequencies in Europeans, Asians and Africans. The conservation and deleteriousness of the variants were evaluated using the following bioinformatic tools: Likelihood ratio test (LRT), Mutation Assessor (MA), Mutation Taster (MT), PolyPhen2 (PP2), PROVEAN (PR), and SIFT (4). Population-specific allele frequencies were obtained by genotyping 250 ethnically matched Cameroonian control, using direct Sanger sequencing (Applied Biosystems, Foster City, CA). For the five novel variants identified in Cameroonian families, molecular modeling was performed using SWISS-MODEL and Phyre2; Specific templates were used for each protein or domain within which the novel variant lies (Table S1).

A total of 26 individuals from 10 families with congenital ARNSHI were studied. All the affected individuals had nonsyndromic, pre-lingual, bilaterally symmetric profound sensorineural HI, with hearing thresholds between 81 and 119 dB (Table S2, Supporting Information). In seven out of 10 families (70%), 12 putatively pathogenic variants were identified in six NSHI genes (*CHD23*, *LOXHD1*, *MYO7A*, *SLC26A4*, *OTOF*, and *STRC*) (Table 1). Five of the 12 variants (41.6%) have not been previously implicated in HI etiology whereas the remaining seven variants were shown to be involved in NSHI in populations outside of sub-Saharan Africa (Table 1). Most of the variants are not present or ultra-rare in the ExAC database, which has data on 60,706 individuals (Table 1). All identified variants segregate with the HI phenotype (Fig. S1). In three families (30%), no pathogenic variants were identified. For each of these five novel variants (Tables S1), molecular modeling revealed potential disruption of folding or inter-domain binding due to these variants, which are expected to result in changes to residue interactions within the same protein or with other proteins (Table S1, Fig. S2) and may explain the protein dysfunction that leads to hearing impairment.

This report is the first from sub-Saharan Africa to reinforce the value of TGE and MPS to determine the genetic cause of HI in this population (Table S3) (2, 5). The use of a comprehensive deafness-specific panel is state of the art and offers high diagnostic high sensitivity and specificity (5). Nevertheless, with small families, future studies in Cameroon should consider the use whole exome sequencing, in order to validate the co-segregation of an allele and a specific phenotype using the logarithm of the odds (LOD) score method. The absence of pathogenic variants in 30% of families suggests that novel hearing loss genes may be discovered among Africans, as supported by a recent report describing the lowest diagnostic

Table 1. Variants identified in Cameroonian families using OtoSCOPE[a]

Family	#Aff	GT	Gene	Variant		ExAC all MAF	ExAC Afr MAF	Cameroonian controls MAF	Deleterious by bioinformatics	Known or novel
1	3	Het	*MYO7A*	c.5809_5811delCTC	p.Leu1937del	0	0	0	MT	Novel
				c.5886_5888delCTT	p.Phe1963del	0.00005	0	0	MT	Known (6)
2	3	Het	*CDH23*	c.6399C>A	p.Asp2133Glu	0	0	0	LRT, MA, MT, PP2, PR, SIFT	Novel
				c.8720T>C	p.Met2907Thr	0	0	0.002	LRT, MT, SIFT	Novel
3	2[b]	Het	*LOXHD1*	c.3371G>A	p.Arg1124His	0.00005	0	0.002	LRT, MA, MT, PP2, PR, SIFT	Novel
				c.3979T>A	p.Phe1327Ile	0.0001	0.0008	0	LRT, MA, MT, PP2, PR, SIFT	Novel
4	3	Hom	*OTOF*	c.766-2A>G	NA	0	0	0	MT	Known (7)
5	4	Het	*STRC*	20-kb del	NA	NA	NA	NA	NA	Known (8)
				CNV	NA	NA	NA	NA	NA	Known (8)
6	3	Het	*SLC26A4*	c.1678G>A	p.Asp560Asn	0.00002	0	0	LRT, MT, PP2, SIFT	Known (9)
				c.2007C>A	p.Asp669Glu	0	0	0	LRT, MA, MT, PP2, PR, SIFT	Known (10)
7	3	Hom	*MYO7A*	c.1996C>T	p.Arg666	0.00002	0	0.002	MT	Known (11)

Aff, affected; Afr, African; CNV, copy number variation; ExAC, exome aggregation consortium; GT, genotype; Het, heterozygous; Hom, homozygous; MAF, minor allele frequency; LRT, likelihood ratio test; MA, Mutation Assessor; MT, Mutation Taster; NA, not available; PP2, PolyPhen2 HVAR; PR, PROVEAN.

[a]RefSeq#: *MYO7A*, NM_000260.3; SLC26A4, NM_000441.1; *OTOF*, NM_194248.2; *LOXHD1*, NM_144612.6; *CDH23*, NM_022124.5.

[b] Hearing-impaired individuals from family 3 are second-cousins, the rest are siblings.

rate for ARNSHI in African Americans (5). In aggregate, these results confirm the efficiency of comprehensive genetic testing in defining the causes of NSHI in Cameroon and highlight the value of African populations for the identification of novels genes associated with NSHI.

Supporting Information

Additional supporting information may be found in the online version of this article at the publisher's web-site.

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K. Lebeko[a†]
C. M. Sloan-Heggen[b†]
J. J. N. Noubiap[c]
C. Dandara[a]
D. L. Kolbe[b]
S. S. Ephraim[b]
K. T. Booth[b]
H. Azaiez[b]
R. L. P. Santos-Cortez[d]
S. M. Leal[d]
R. J. H. Smith[b]
A. Wonkam[a,c]

[a]Division of Human Genetics, Department of Pathology, Faculty of Health Sciences University of Cape Town, Cape Town, South Africa

[b]Department of Otolaryngology, Molecular Otolaryngology and Renal Research Laboratories, The University of Iowa, Iowa City, IA, USA

[c]Department of Medicine, Faculty of Health Sciences University of Cape Town, Cape Town, South Africa, and

[d]Center for Statistical Genetics, Department of Molecular and Human Genetics, Baylor College of Medicine, Houston, TX, USA

[†]Co-first authors.

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Correspondence:
Prof Ambroise Wonkam,
Division of Human Genetics,
Faculty of Health Sciences,
University of Cape Town,
Anzio Road,
Observatory, 7925 Cape Town,
South Africa.
Tel.: +(0027) 21 406 6307;
Fax: +(0027) 21 406 6826;
e-mail: ambroise.wonkam@uct.ac.za

Table S1. Molecular Modeling Results for Novel Variants Identified in Cameroonian Families*

Protein	Variant	Domain	Template	PDB #	Ref.	Predicted Effect on Protein
MYO7A	p.Leu1935del	αA helix of F1 lobe of FERM2	MYO7A MyTH4-FERM1-SH3 in complex with SANS CEN1	3PVL	[8]	Disrupts folding of FERM2 domain, which is predicted to result in poor contact of F1 lobe residues with the CEN1/central domain of SANS protein
CDH23	p.Asp2133Glu	EC20	CDH23 EC2	2WD0	[9]	Loss of Ca^{++}-binding site, which would likely affect tip link rigidity, unfolding and *trans*-EC binding
CDH23	p.Met2907Thr	EC27	CDH8 EC3	2A62	[10]	H-bond change is expected to result in structural changes in linker regions
LOXHD1	p.Arg1124His	β8 strand of PLAT 8	11R-lipoxygenase	3VF1	[11]	Longer β9 strand and malpositioned residues in the linker region between strands β8-β9, limiting access to β8 strand residues that might be involved in binding
LOXHD1	p.Phe1327Ile	β2 strand of PLAT 10	11R-lipoxygenase	3VF1	[11]	Might affect gating of the β-sandwich or binding of other proteins

PDB, RCSB Protein Data Bank; Ref, reference number; CDH, cadherin; EC, extracellular domain.

* For the five novel variants identified in Cameroonian families, molecular modeling was performed using SWISS-MODEL [6] and Phyre2 [7].

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Table S2: Description of Study Subjects

Characteristics	Categories	*n* (%)
Sex	Male	14 (53.8)
	Female	12 (46.2%)
Genetics	Autosomal Recessive	10[1] (100)
	Nonsyndromic	26 (100)
	GJB2-, GJB6-negative	26 (100)
Age of Onset of Hearing Impairment	Congenital / Prelingual	26 (100)
Type of Hearing Impairment	Profound I (91-100dB)	12 (46.2)
	Profound II (101-110dB)	11 (42.3)
	Profound III (111-119dB)	3 (11.5)
	Bilateral Symmetric	26 (100)
	Sensorineural	26 (100)
Audiogram Shape	Flat	9 (34.6)
	Sloping	17 (65.4%)

[1] Number of families

Supplementary Table S3: Genes included on the targeted genomic enrichment panel used in this study

Gene Name	Gene Name		Location	REFSEQ IDs	Inheritance
ACTG1	actin, gamma 1	DFNA20/26	*17q25*	NM_001614	dominant
ADGRV1	G protein-coupled receptor v1	USH2C	*5q14.3*	NM_032119	recessive
CCDC50	coiled-coil domain containing 50	DFNA44	*3q28-q29*	NM_178335	dominant
CDH23	cadherin-related 23	DFNB12, USH1D	*10q21-q22*	NM_022124	recessive
CLDN14	claudin 14	DFNB29	*21q22*	NM_012130	recessive
CLRN1	clarin 1	USH3	*3q25.1*	NM_174878	recessive
COCH	coagulation factor C homolog, cochlin	DFNA9	*14q12-q13*	NM_004086	dominant
COL11A2	collagen, type XI, alpha 2	DFNB53/DFNA13	*6p21.3*	NM_080680	both
CRYM	crystallin, mu	…	*16p12.2*	NM_001888	dominant
DFNA5	deafness, autosomal dominant 5	DFNA5	*7p15*	NM_004403	dominant
DFNB31/WHRN	whirlin/autosomal recessive deafness type 31 protein	USH2D	*9q32*	NM_015404	recessive
DFNB59/PJVK	pejvakin/autosomal recessive deafness type 59 protein		*2q31.2*	NM_001042702	recessive
DIAPH1	diaphanous homolog 1	DFNA1	*5q31*	NM_005219	dominant
DSPP	dentin sialophosphoprotein	DFNA39	*4q21.3*	NM_014208	dominant
ESPN	espin	DFNB36	*1p36.3*	NM_031475	recessive
ESRRB	estrogen-related receptor beta	DFNB35	*14q24.1-24.3*	NM_004452	recessive
EYA4	eyes absent homolog 4	DFNA10	*6q22-q23*	NM_004100	dominant
GIPC3	GAIP C-terminus interacting protein 3	DFNB15/DFNB95	*19p13.3*	NM_133261	recessive
GJB2	gap junction protein, beta 2	DFNB1/DFNA3	*13q12*	NM_004004	both
GJB3	gap junction protein, beta 3	DFNA2	*1p34*	NM_024009	both
GJB6	gap junction protein, beta 6	DFNB1/DFNA3	*13q12*	NM_006783	both
GPSM2	g-protein signalling modulator 2	DFNB82	*1p13.3-22.1*	NM_013296	recessive
GRHL2	grainyhead-like 2	DFNA28	*8q22*	NM_024915	recessive
GRXCR1	glutaredoxin cysteine-rich 1	DFNB25	*4p13*	NM_001080476	recessive
HGF	hepatocyte growth factor	DFNB39	*7q21.1*	NM_000601	recessive

ILDR1	immunoglobulin-like domain containing receptor 1	DFNB42	*3q21.1*	NM_175924	recessive
KCNQ4	potassium voltage-gated channel, KQT-like subfamily, member 4	DFNA2	*1p34*	NM_004700	dominant
LHFPL5	lipoma HMGIC fusion partner-like 5	DFNB66/67	*6p21.2-p22.3*	NM_182548	recessive
LOXHD1	lipoxygenase homology domains 1	DFNB77	*18q21.1*	NM_144612	recessive
LRTOMT	leucine rich transmembrane and O-methyltransferase	DFNB63	*11q13.4*	NM_145309	recessive
MARVELD2	MARVEL domain containing 2	DFNB49	*5q12.3-q14.1*	NM_001038603	recessive
miR-96	microRNA 96	DFNA50	*7q32.2*	NR_029512	microRNA
miR-182	microRNA 182	…	*7q32.2*	NR_029614	microRNA
miR-183	microRNA 183	…	*7q32.2*	NR_029615	
MTRNR1	mitochondrially encoded 12S RNA	…	*chrM*		
MTTS1	mitochondrially encoded tRNA serine 1	…	*chrM*		
MYH14	myosin, heavy chain 14, non-muscle	DFNA4	*19q13.33*	NM_024729	dominant
MYH9	myosin, heavy chain 9, non-muscle	DFNA17	*22q13.1*	NM_002473	dominant
MYO15A	myosin XVA	DFNB3	*17p11.2*	NM_016239	recessive
MYO1A	myosin IA	DFNA48	*12q13-q15*	NM_005379	dominant
MYO3A	myosin IIIA	DFNB30	*10p11.1*	NM_017433	recessive
MYO6	myosin VI	DFNA22, DFNB37	*6q14.1*	NM_004999	both
MYO7A	myosin VIIA	DFNA11, DFNB2	*11q13.5*	NM_000260	both
OTOA	otoancorin	DFNB22	*16p12.2*	NM_144672	recessive
OTOF	otoferlin	DFNB6/9	*2p23.1*	NM_194248	recessive
PCDH15	protocadherin-related 15	DFNB12, USH1F	*10q21.1*	NM_033056	recessive
POU3F4	POU class 3 homeobox 4	DFN3	*Xq21.1*	NM_000307	x-linked recessive
POU4F3	POU class 4 homeobox 3	DFNA15	*5q31*	NM_002700	dominant
PRPS1	phosphoribosylpyrophosphate synthetase 1	DFN2	*Xq22*	NM_002764	recessive

PTPRQ	protein-tyrosine phosphatase receptor, type Q	DFNB84	*12q21.2*	NM_001145026	recessive
RDX	radixin	DFNB24	*11q23*	NM_002906	recessive
SLC17A8	solute carrier family 17, member 3	DFNA25	*12q21-24*	NM_01098486	recessive
SLC26A4	solute carrier family 26, member 4	DFNB4	*7q31*	NM_000441	recessive
SLC26A5	solute carrier family 26, member 4	…	*7q22.1*	NM_198999	recessive
STRC	stereocilin	DFNB16	*15q21-q22*	NM_153700	recessive
TECTA	tectorin alpha	DFNB21/DFNA8/DFNA12	*11q*	NM_005422	both
TJP2	tight junction protein 2	DFNA51	*9q21*	NM_001170416	recessive
TMC1	transmembrane channel-like 1	DFNB7/DFNB11/DFNA36	*9q13-q21*	NM_138691	both
TMIE	transmembrane inner ear	DFNB6	*3p14-p21*	NM_147196	recessive
TMPRSS3	transmembrane protease, serine 3	DFNB8/DFNB10	*21q22*	NM_024022	recessive
TPRN	taperin	DFNB79	*9q34.3*	NM_001128228	recessive
TRIOBP	TRIO and F-actin binding protein	DFNB28	*22q13*	NM_001039141	recessive
USH1C	Usher syndrome 1C homolog	DFNB18/USH1C	*11p14-p15.1*	NM_153676	recessive
USH1G	Usher syndrome 1G	USH1G	*17q25.1*	NM_173477	recessive
USH2A	Usher syndrome 2A	USH2A	*1q41*	NM_206933	recessive
WFS1	Wolfram syndrome 1 (wolframin)	DFNA6/DFNA14	*4p16.3*	NM_006005	dominant

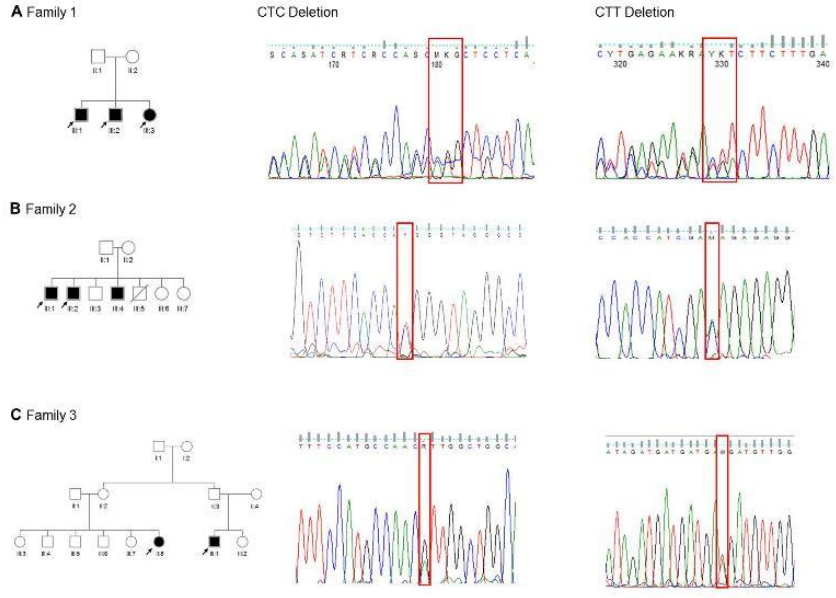

Fig. S1

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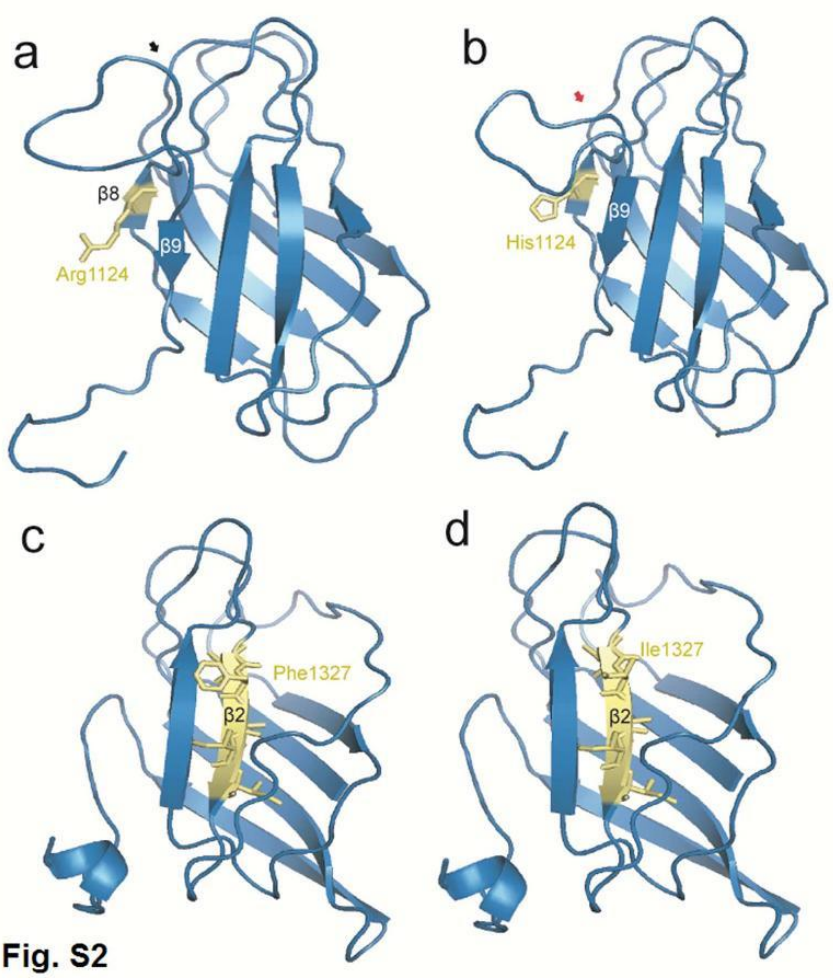

Fig. S2

61x71mm (300 x 300 DPI)

3.2. **Lebeko K**, Manyisa N, Chimusa ER, Mulder N, Dandara C, Wonkam A. 2017. A Genomic and Protein–Protein Interaction Analyses of Nonsyndromic Hearing Impairment in Cameroon Using Targeted Genomic Enrichment and Massively Parallel Sequencing. *Omi. A J. Integr. Biol.* 21: 90–99.

Abstract

Hearing impairment (HI) is one of the leading causes of disability in the world, impacting the social, economic, and psychological well-being of the affected individual. This is particularly true in sub-Saharan Africa, which carries one of the highest burdens of this condition. Despite this, there are limited data on the most prevalent genes or variants that cause HI among sub-Saharan Africans. Next-generation technologies, such as targeted genomic enrichment and massively parallel sequencing, offer new promise in this context. This study reports, for the first time to the best of our knowledge, on the prevalence of novel variants identified through a platform of 116 HI genes (OtoSCOPE), among 82 African probands with HI. Only variants *OTOF* NM_194248.2:c.766-2A>G and *MYO7A* NM_000260.3:c.1996C>T, p.Arg666Stop were found in 3 (3.7%) and 5 (6.1%) patients, respectively. In addition, and uniquely, the analysis of protein–protein interactions (PPI), through interrogation of gene subnetworks, using a custom script and two databases (Enrichr and PANTHER), and an algorithm in the igraph package of R, identified the enrichment of sensory perception and mechanical stimulus biological processes, and the most significant molecular functions of these variants pertained to binding or structural activity. Furthermore, 10 genes (*MYO7A, MYO6, KCTD3, NUMA1, MYH9, KCNQ1, UBC, DIAPH1, PSMC2,* and *RDX*) were identified as significant hubs within the subnetworks. Results reveal that the novel variants identified among familial cases of HI in Cameroon are not common, and PPI analysis has highlighted the role of 10 genes, potentially important in understanding HI genomics among Africans.

Candidate contribution: Participated in the study design, Carried out genetic studies and data analysis, and assisted drafting of the article and revision.

Co-Authors contributions:

AW conceived the study, participated in its design, interacted with the patients, coordinated the blood sample collection, and drafted the article.

NM carried out the genetic studies, performed the data analysis, and contributed to drafting the article, performed the bioinformatics analysis, coordinated the data interpretation and statistical analysis,

CD carried out the genetic studies, performed the data analysis, and contributed to drafting the article, performed the bioinformatics analysis, coordinated the data interpretation and statistical analysis,

EC performed the bioinformatics analysis, coordinated the data interpretation and statistical analysis.

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Research Article

A Genomic and Protein–Protein Interaction Analyses of Nonsyndromic Hearing Impairment in Cameroon Using Targeted Genomic Enrichment and Massively Parallel Sequencing

Kamogelo Lebeko,[1] Noluthando Manyisa,[1] Emile R. Chimusa,[1] Nicola Mulder,[2] Collet Dandara,[1] and Ambroise Wonkam[1,3]

Abstract

Hearing impairment (HI) is one of the leading causes of disability in the world, impacting the social, economic, and psychological well-being of the affected individual. This is particularly true in sub-Saharan Africa, which carries one of the highest burdens of this condition. Despite this, there are limited data on the most prevalent genes or mutations that cause HI among sub-Saharan Africans. Next-generation technologies, such as targeted genomic enrichment and massively parallel sequencing, offer new promise in this context. This study reports, for the first time to the best of our knowledge, on the prevalence of novel mutations identified through a platform of 116 HI genes (OtoSCOPE®), among 82 African probands with HI. Only variants *OTOF* NM_194248.2:c.766-2A>G and *MYO7A* NM_000260.3:c.1996C>T, p.Arg666Stop were found in 3 (3.7%) and 5 (6.1%) patients, respectively. In addition and uniquely, the analysis of protein–protein interactions (PPI), through interrogation of gene subnetworks, using a custom script and two databases (Enrichr and PANTHER), and an algorithm in the igraph package of R, identified the enrichment of sensory perception and mechanical stimulus biological processes, and the most significant molecular functions of these variants pertained to binding or structural activity. Furthermore, 10 genes (*MYO7A*, *MYO6*, *KCTD3*, *NUMA1*, *MYH9*, *KCNQ1*, *UBC*, *DIAPH1*, *PSMC2*, and *RDX*) were identified as significant hubs within the subnetworks. Results reveal that the novel variants identified among familial cases of HI in Cameroon are not common, and PPI analysis has highlighted the role of 10 genes, potentially important in understanding HI genomics among Africans.

Keywords: nonsyndromic hearing impairment, genomics, protein–protein interaction, Africans, Cameroon

Background

Hearing impairment (HI) is one of the leading causes of disability in the world with the highest rate for age-standardized disability-adjusted life-years (DALYs). For example, the HI-related DALYs is approximately one and a half time to twice greater than congenital heart disease (Murray et al., 2015). It has a global prevalence of about 1 per 1000 live births in developed countries, with a much higher rate in the developing world, as high as 6 per 1000 in sub-Saharan Africa (Olusanya et al., 2014).

Genetic factors are estimated to account for 50% of variability in the congenital HI phenotype with a mostly nonsyndromic and autosomal recessive (AR) mode of inheritance (Hutchin et al., 2005). To date, 90 genes have been implicated in nonsyndromic hearing impairment (NSHI) (http://hereditaryhearingloss.org/main.aspx?c=.HHH&n=86163, accessed December 1, 2016). In many populations, pathogenic variants in *GJB2* (connexin 26 gene) are a major contributor to autosomal recessive NSHI (ARNSHI) (Chan and Chang, 2014). However, the prevalence of *GJB2*- or *GJB6*-related NSHI is approximating to zero in most sub-Saharan African populations and little is known about the contribution to HI by other known NSHI genes (Bosch et al., 2014a, 2014b; Gasmelseed et al., 2004; Javidnia et al., 2014; Kabahuma et al., 2011; Lasisi et al., 2014).

[1]Division of Human Genetics, Department of Pathology, Faculty of Health Sciences - University of Cape Town, Cap Town, South Africa.
[2]Division of Computational Biology, Faculty of Health Sciences - University of Cape Town, Cap Town, South Africa.
[3]Department of Medicine, Faculty of Health Sciences - University of Cape Town, Cap Town, South Africa.

Targeted genomic enrichment (TGE) and massively parallel sequencing (MPS), for example, with OtoSCOPE®, include 116 HI genes, and has been shown to be an efficient tool for genetic testing for NSHI (Shearer et al., 2010). Using OtoSCOPE, genetic testing of ten multiplex Cameroonian families segregating NSHI identified pathogenic variants in 7 out of 10 families (70%) (Lebeko et al., 2016); within the 7 families, 12 pathogenic variants in 6 genes previously implicated in NSHI were identified, of which 5 were novel (41.6%). The report of five novel pathogenic variants highlights the importance of ethnic-specific filtering (Sloan-Heggen et al., 2016) and underscores the need to further investigate these variants among nonfamilial Cameroonian cases of NSHI.

This study investigated the prevalence of these newly identified mutations in a cohort of HI patients from Cameroon and South Africa. In addition, to explore the interaction of various HI genes that could potentially play a role in clinical penetrance and variable expression of the phenotype, as well the epidemiology of HI in the general population, we investigated the relationships between variants identified by OtoSCOPE using bioinformatics analysis of a human protein–protein interaction (PPI) network.

Materials and Methods

Ethics approval and consent to participate

This study was approved by the Human Research Ethics Committee of the University of Cape Town (ethics approval HREC REF: 455/2014) and the Cameroon National Ethics Committee (ethics approval N°123/CNE/SE/2010). Written informed consent was obtained from participants 18 years or older and from parents/guardians of minors with verbal assent from the children.

Patients and inclusion criteria

Patients were recruited from (1) schools of the deaf and outpatient clinics in Cameroon, as previously reported (Wonkam et al., 2013), and (2) from South Africa at the Effata School of the Deaf in Umtata Eastern Cape and were self-identified as Black Xhosa. As previously described, we included, in this study, patients of Black African descent with NSHI of either (1) putative genetic origin, as revealed by one or more affected family members or consanguinity, or (2) unknown origin. We excluded patients with syndromic HI and those with obvious environmental causes such as meningitis, rubella, mumps, measles, severe prematurity and/or birth weight less than 1500 g, neonatal hyperbilirubinemia, neonatal asphyxia, ototoxicity, or severe head trauma (Bosch et al., 2014a).

Clinical evaluation included a comprehensive questionnaire (exposure to noise, ototoxic agents, and familial history) and the diagnosis of sensorineural HI according to current clinical standards, as previously reported (Wonkam et al., 2013). All probands and affected individuals were examined for syndromic features by a medical geneticist and an ophthalmologist. All probands were negative for pathogenic variants in *GJB2*, *GJB6*, and *GJA1*. The total number of patients included in this study was 25 Black Xhosa South Africans and 57 Cameroonians.

Control participants

A total of 250 ethnically matched Cameroonian controls without HI and negative for a family history were recruited, from apparently healthy blood donors in Yaoundé.

Molecular analysis

DNA preparation and polymerase chain reaction amplification. DNA was extracted from whole blood as previously described (Bosch et al., 2014a, 2014b; Wonkam et al., 2013). Polymerase chain reaction (PCR) primers were designed to amplify between 150 and 400 bps encompassing each mutation. Patients' samples were genotyped through singleplex snapshot PCR using a SNaPShot Multiplex Ready Reaction Mix (Applied Biosystems, Foster City, CA, USA). Fragments were resolved on an ABI 3130xl Genetic Analyzer (Applied Biosystems) with the data being analyzed on GeneMapper, Bioedit Sequence Alignment Editor v 7.2.5 23, and Finch TV v 1.4.0 (Washington, DC, USA). Results of genotypes were confirmed by direct Sanger sequencing using a BigDye Terminator v 3.1 Cycle Sequencing Kit (Applied Biosystems) (Fig. 1), at the Division of Human Genetics, Faculty of Health Sciences, University of Cape Town, South Africa.

Sequencing analysis and identification of causative mutations. From the previously reported study using OtoSCOPE, the average coverage for the 350,160 targeted base pairs was 99.60% at a greater than 10X depth of coverage. Sequencing reads were aligned to the reference Human genome (Hg19) using the Burrows-Wheeler Aligner (BWA) algorithm as reported (Shearer et al., 2010). The Exome Aggregation Consortium database was used to remove high-frequency variants (i.e., minor allele frequency ≥0.1%), which were unlikely to be pathogenic and obtain variant frequencies in Europeans, Asians, and Africans (Lebeko et al., 2016). The conservation and deleteriousness of the variants were evaluated using the following tools: likelihood ratio test (LRT), mutation assessor, mutation taster, PolyPhen2, PROVEAN, and sorting intolerant from tolerant (SIFT) (Wang et al., 2010).

Molecular modeling

For the five novel variants identified in Cameroonian families, molecular modeling was performed using SWISS-MODEL (Biasini et al., 2014) and Phyre2 (Kelley et al., 2015) (Fig. 2; Supplementary Fig. S1). Specific templates that were used for each protein or domain within which the novel variant lies were previously reported (Lebeko et al., 2016).

Network analysis

Network and enrichment analysis. We used a comprehensive human PPI network (Chimusa et al., 2016; Wu et al., 2009) to understand how the set of casual variants are layered in a biological network. We performed enrichment analysis to examine how these variants and genes within the identified subnetwork are associated with human phenotypes and determine their potential biological processes, pathways, and molecular functions. For this, we used a custom script and two databases: Enrichr (www.ampharm.mssm.edu/Enrichr) and PANTHER (http://pantherdb.org).

To identify potential gene hubs in the network of HI, we queried our reported genes (Lebeko et al., 2016) alongside all

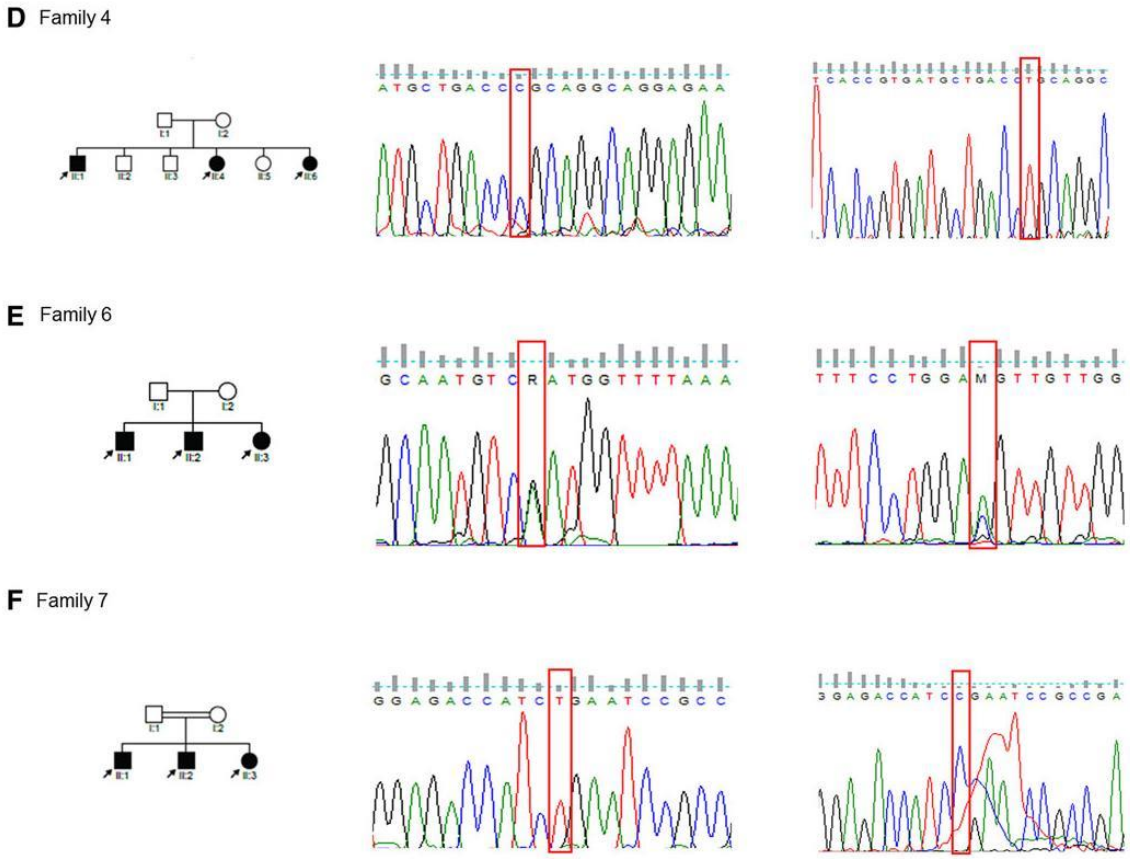

FIG. 1. Pedigrees of selected families (4, 6 and 7) and sequence data showing variants that are causal for hearing impairment. Detected variants include the following: homozygous *OTOF* NM_194248.2:c.766-2A>G **(panel D)**; compound heterozygous *SLC26A4* NM_000441.1:c.1678G>A, p.Asp560Asn and NM_000441.1:c.2007C>A, p.Asp669Glu **(panel E)**; and homozygous *MYO7A* NM_000260.3:c.1996C>T, p.Arg666Stop **(panel F)**.

other known genes associated with HI in the PPI network; these are the genes which were screened through the OtoSCOPE panel (116 in total). We applied a clustering algorithm in the igraph package of R (www.r-project.org) to determine community structure from the obtained subnetwork of hearing loss interactive genes.

Prioritizing variants on the merged data from all family members. We additionally prioritized variants using the aggregated data of all interrogated family members. We merged all single variants calling files (vcf) from each family member and annotated the resulting merged vcf using ANNOVAR (Wang et al., 2010). Based on prediction approaches implemented in ANNOVAR, we filtered and retained only variants (Supplementary Table S1) that have a functional prediction using 10 approaches (SIFT, PolyPhen 2, MutationTaster, MutationAssessor, LRT, FATHMM, MetaSVM, MetaLR, GERP++, and PhyloP). Functional prediction status included ''Deleterious/probably or damaging/disease_causing'' (D) or ''disease_causing_automatic'' (A). Enrichment analysis was performed using two databases, including Enrichr (www.ampharm.mssm.edu/Enrichr) and PANTHER (http://pantherdb.org). We estimated and aggregated the minor allele frequencies (MAF) of single nucleotide polymorphisms (SNPs) downstream and upstream (0 kb, respectively) within each of these retained genes using exome data from 105 Cameroonian control population, all African, European, and East Asian populations from the 1000 Genome Phase 3 Project.

Results

Patients' description

The patients' descriptions are summarized in Table 1. The total number of samples analyzed were from 82 patients with a skew toward male participants (58.5%, $n=48$). The majority of affected individuals had nonsyndromic, prelingual, bilaterally profound sensorineural HI, with hearing thresholds between 81 and 119 dB.

Prevalence of previously identified pathogenic variants

Mutations identified through OtoSCOPE in our previous study were not prevalent in this group of patients with NSHI

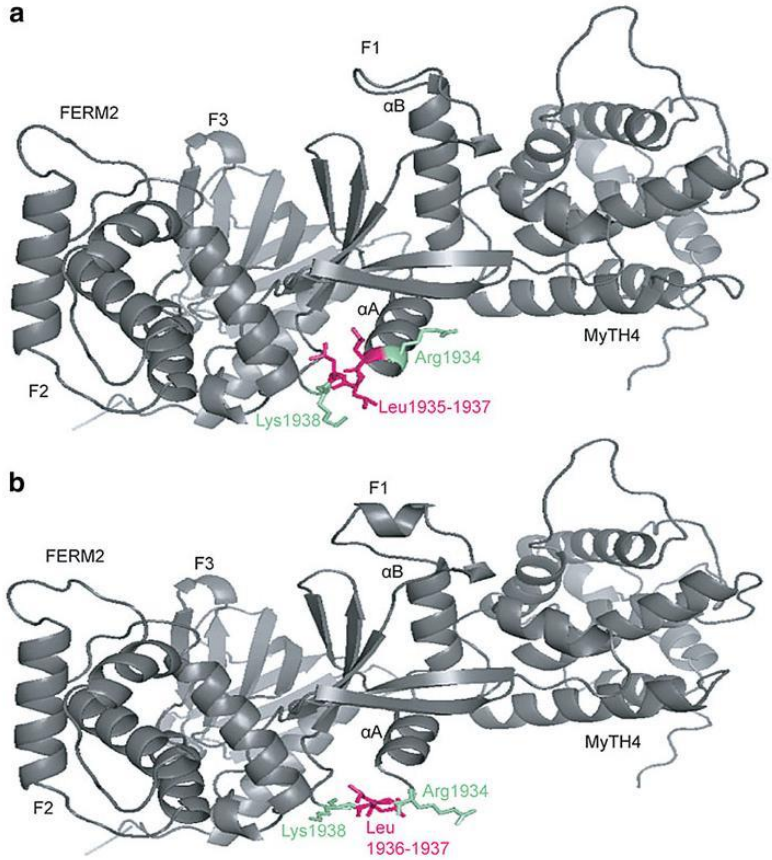

FIG. 2. Molecular modeling of MYO7A p.Leu1935del comparing wild-type **(a)** and mutant **(b)** proteins. Loss of one out of three leucine residues (*pink*) at the edge of the αA helix of the F1 lobe within the FERM2 domain of the MYO7A protein is predicted to result in unraveling not just of the αA_{F1} helix but also of the αB_{F1} helix. The variant is expected to cause misfolding of the F1 lobe and poor contact between F1 and the central/CEN1 domain of SANS protein.

(Table 2). Among Cameroonian individuals with HI, only *OTOF* NM_194248.2:c.766-2A>G was found in 3 (3.7%) patients with an MAF = 0.02, and *MYO7A* NM_000260 .3:c.1996C>T, p.Arg666Stop was found in 5 (6.1%) patients with an MAF = 0.04 (Table 2). All these variants were in a heterozygous state in all the cases and are thus unlikely to explain, alone, the cause of HI in these patients. None of these variants were found among South African patients. None of the variants previously reported in *MYO7A*, *CDH23*, *LOXHD1*, and *SLC26A4* were identified in this group of patients with HI (Table 2).

Molecular modeling

Previous molecular modeling data reported changes to the LOXHD1 protein that affected binding (Lebeko et al., 2016). The effect of the novel MYO7A variant results in a disruption of the folding of the FERM2 domain needed for binding to the SANS protein central domain disrupting their dimerization (Fig. 2) (Wu et al., 2011). The p.Asp2133Glu change in CDH23 results in a loss of calcium binding site which is integral to the functioning of the protein (Supplementary Fig. S1b) (Sotomayor et al., 2010). Coupled with the change in linker regions caused by the p.Met2907Thr substitution (Supplementary Fig. S1d) (Patel et al., 2006), the protein is rendered nonfunctional.

Gene network analysis and enrichment of reported genes

Querying the genes reported in Table 2 in the human PPI network, we identified 16 pairs of interactive genes (Fig. 3A). Interestingly, the novel mutation identified in *MYO7A* appears to be the central hub of the subnetwork, indicating a major functional role that the variant may play in HI. The subnetwork (Fig. 3A) was not found to be significantly associated with any biological pathway; however, from the OMIM (Online Mendelian Inheritance of Man database) disease database (www.omim.org), the whole subnetwork is enriched with Usher Syndrome and deafness variants ($p = 4.134e\text{-}7$ and 0.0001138, respectively). Two biological processes were found to be significantly associated with the

Table 1. Summary of Participants' Demographics

Demographic	*Category*	n *(%)*
Sex	Male	48 (58.5)
	Female	33 (40.2)
	N/A	1 (1.22)
Age of onset	Congenital/prelingual	56 (68.3)
	Perilingual	6 (7.32)
	Postlingual	7 (8.54)
	N/A	13 (15.85)
Cause/transmission	Familial	37 (45.1)
	Sporadic (unknown)	37 (45.1)
	N/A	8 (9.75)
Type of hearing loss (dB)	Severe I (71–80)	1 (1.22)
	Severe II (81–90)	1 (1.22)
	Profound I (91–100)	12 (14.63)
	Profound II (101–110)	9 (10.98)
	Profound III (111–120)	5 (6.1)
	Total HL (>120)	1 (1.22)
	Asymmetric	23 (28.05)
	N/A	31 (37.08)
Previous testing	*GJB2*, *GJB6*, *GJA1*, and variants identified by OtoSCOPE®	82 (100)

N/A, no data available.

subnetwork, including sensory perception of mechanical stimulus (GO: 0050954, $p = 1.430e\text{-}8$) and sensory perception of sound (GO: 0007605, $p = 1.246e\text{-}8$). Furthermore, 60% of the molecular functions (Fig. 3B) of these variants are in binding (GO: 0005488) and/or structural molecule activity (GO: 0005198) pathways.

From the compiled gene network of 116 hearing loss genes, we identified 10 hubs (the most import gene node, harboring the major connectivity within the subnetwork), including *MYO7A*, *MYO6*, *KCTD3*, *NUMA1*, *MYH9*, *KCNQ1*, *UBC*, *DIAPH1*, *PSMC2*, *and RDX* (Fig. 4A). The subnetwork (Fig. 4B) is significantly associated with known biological pathways such as proteasome degradation, Tumor necrosis factor (TNF)-alpha signaling pathway, and transforming growth factor (TGF)-beta signaling pathway (Fig. 4B). Sixty-three percent of the genes within the subnetwork (Table 3) have a molecular function in binding (GO: 0005488) and/or catalytic activity (GO: 0003824) pathways, and are significantly enriched with deafness ($p = 4.22e\text{-}15$).

Leveraging the combined data in prioritizing variants and gene enrichment

We merged the data of nine Cameroonian family members, and filtered and prioritized variants using the approach and criteria described above. We replicated OtoSCOPE findings in two genes, *OTOF* and *LOXHD1* (Supplementary Fig. S2 and Supplementary Table S1), and identified other genes in linkage disequilibrium (LD) with those reported in Table 2. Furthermore, we used a comprehensive human PPI network (Chimusa et al., 2016; Wu et al., 2009) to derive their biological network positions. We identified 29 pairs of interacting genes (Supplementary Fig. S2A). From the OMIM disease database (www.omim.org), the whole subnetwork is enriched with HI variants ($p = 0.000001870$ and adjusted $p = 0.000013$).

The highest proportion of variants in the subnetwork (Supplementary Fig. S2A) is associated with endoplasmic reticulum pathways (Supplementary Fig. S2B) and 60% of the molecular functions (Supplementary Fig. S2C) of these variants are related to binding (GO: 0005488) and/or catalytic activity (GO: 0003824). Using exome data of 105 Cameroonian controls, as well as data extracted from the 1000 Genomes project, results displayed in Supplementary

Table 2. Prevalence of Mutations Identified by OtoSCOPE Among Cameroonian Patients with Nonsyndromic Hearing Impairment

Gene	*Biological function/pathway*	*Chromosomes*	*Exon/ Intron*	*cDNA change*	*Protein change*	*Allele frequency in patients (no. of chromosomes)*	
						Cameroon	*South Africa*
MYO7A	Intracellular protein transport	11q13.5	Exon 40	c.5806_5808 delCTC	p.Leu1935del	0 (0/114)	0 (0/25)
			Exon 40	c.5880_5882 delCTT	p.Phe1963del	0 (0/114)	0 (0/25)
CDH23	Calcium-dependent cell adhesion	10q22.1	Exon 46	c.6399C>A	p.Asp2133Glu	0 (0/114)	0 (0/25)
			Exon 59	c.8720T>C	p.Met2907Thr	0 (0/114)	0 (0/25)
LOXHD1	Targeting of proteins to plasma membrane	18q21.1	Exon 22	c.3371G>A	p.Arg1124His	0 (0/114)	0 (0/25)
			Exon 26	c.3979T>A	p.Phe1327Ile	0 (0/114)	0 (0/25)
OTOF	Calcium ion binding	2p23.3	Intron 6–7	c.766-2A>G	N/A	0.02 (3/114)	0 (0/25)
SLC26A4	Regulation of membrane potential	7q22.3	Exon 15	c.1678G>A	p.Asp560Asn	0 (0/114)	0 (0/25)
			Exon 17	c.2007C>A	p.Asp669Glu	0 (0/114)	0 (0/25)
MYO7A	Intracellular protein transport	11q13.5	Exon 17	c.1996C>T	p.Arg666	0.04(5/114)	0 (0/25)

RefSeq#: MYO7A, NM_000260.3; SLC26A4, NM_000441.1; OTOF, NM_194248.2; LOXHD1, NM_144612.6; CDH23, NM_022124.5.
cDNA, complementary DNA.

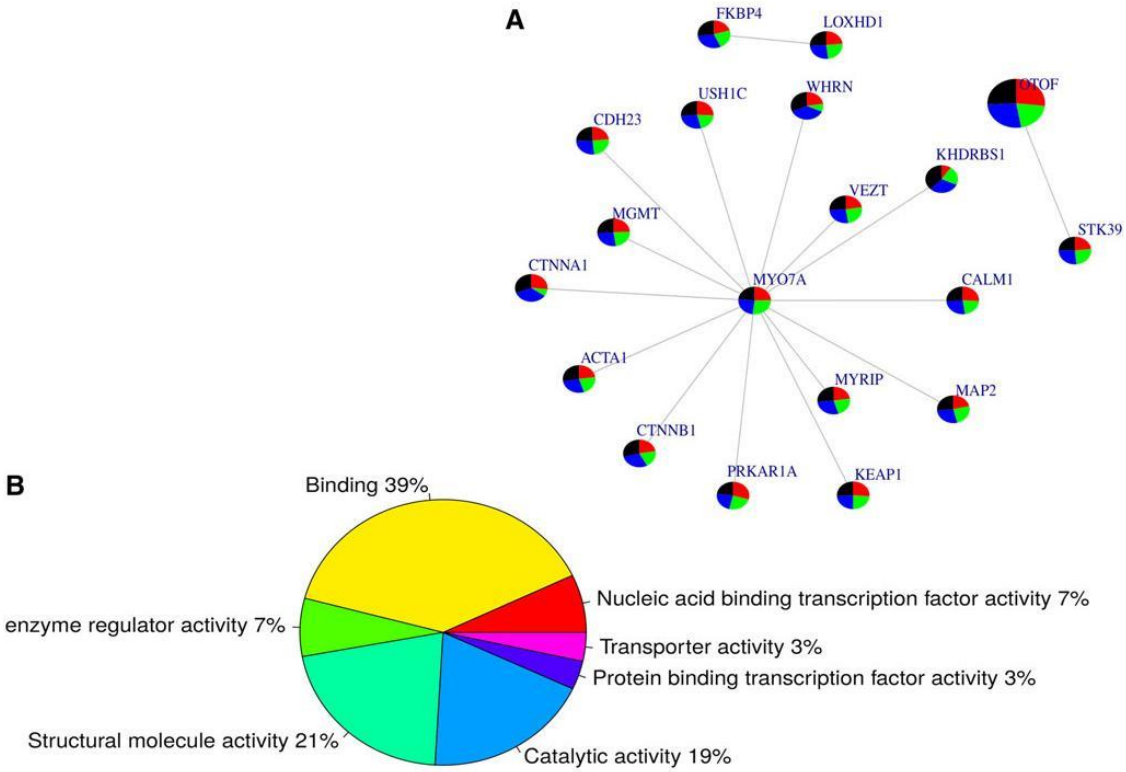

FIG. 3. Subnetwork of interactive genes with potential mutations associated with hearing loss. From the identified causal mutations occurring in genes displayed in Table 2 and Supplementary Table S1, we used a human protein–protein interaction network to interrogate how these genes are positioned in a biological network: we identified 16 pairs of interacting genes **(panel A).** The whole subnetwork is enriched with Usher syndrome and deafness gene variants ($p = 4.134e\text{-}7$ and 0.0001138, respectively). Interestingly, the *MYO7A* gene that had a novel identified mutation appears to be the central hub of that subnetwork, suggesting a major role that variant may play in hearing impairment. Two biological processes were found to be significantly associated with the subnetwork, including sensory perception of mechanical stimulus (GO: 0050954, $p = 1.430e\text{-}8$) and sound (GO: 0007605, $p = 1.246e\text{-}8$); furthermore, 60% of the molecular functions **(panel B)** of these variants are annotated to binding (GO: 0005488) and/or structural molecule activity (GO: 0005198).

Table S1 indicate that the variants in the majority of these hub genes displayed almost similar MAFs across various populations, with the exception of variants in *MYO1A,* which were significantly more prevalent among East Asians, and variants in *GIPC3,* which were relatively rare among the East Asian populations.

Discussion

HI is one of the highest contributors to disability worldwide, impacting on the social, economic, and psychological well-being of the affected individual (Olusanya, 2011). This is particularly true in low-income regions such as sub-Saharan Africa, which also carries one of the highest burdens of this disease (WHO, 2012). Despite this, there are limited data to support the most prevalent genes or mutations that cause HI among patients from this region. Using TGE and MPS in 10 Cameroonian families recently demonstrated the importance of this approach in the resolution of genetic aetiology of HI in Africa and it role in uncovering novel variants (Lebeko et al., 2016). Follow-up research among nonfamilial cases of HI, potentially of genetic origin as presented in this study, revealed that the aforementioned variants are not common in other nonfamilial cases of HI in Cameroon, and absent from a small sample of Xhosa patients from South Africa.

The findings of the heterozygous state of *OTOF* NM_194248.2:c.766-2A>G found in three patients, and *MYO7A* NM_000260.3:c.1996C>T, p.Arg666Stop found in 5 (6.1%) patients (Table 2) suggest that these eight individuals are most likely compound heterozygotes, with second mutations to be investigated in these genes. The relatively high number of NSHI genes and novel variants among a relatively small sample of Cameroonians (Lebeko et al., 2016) demonstrates the high level of genetic heterogeneity, and could reflect genetic diversity found in Cameroons, which mimics many populations in sub-Saharan Africa (Lambert and Tishkoff, 2009).

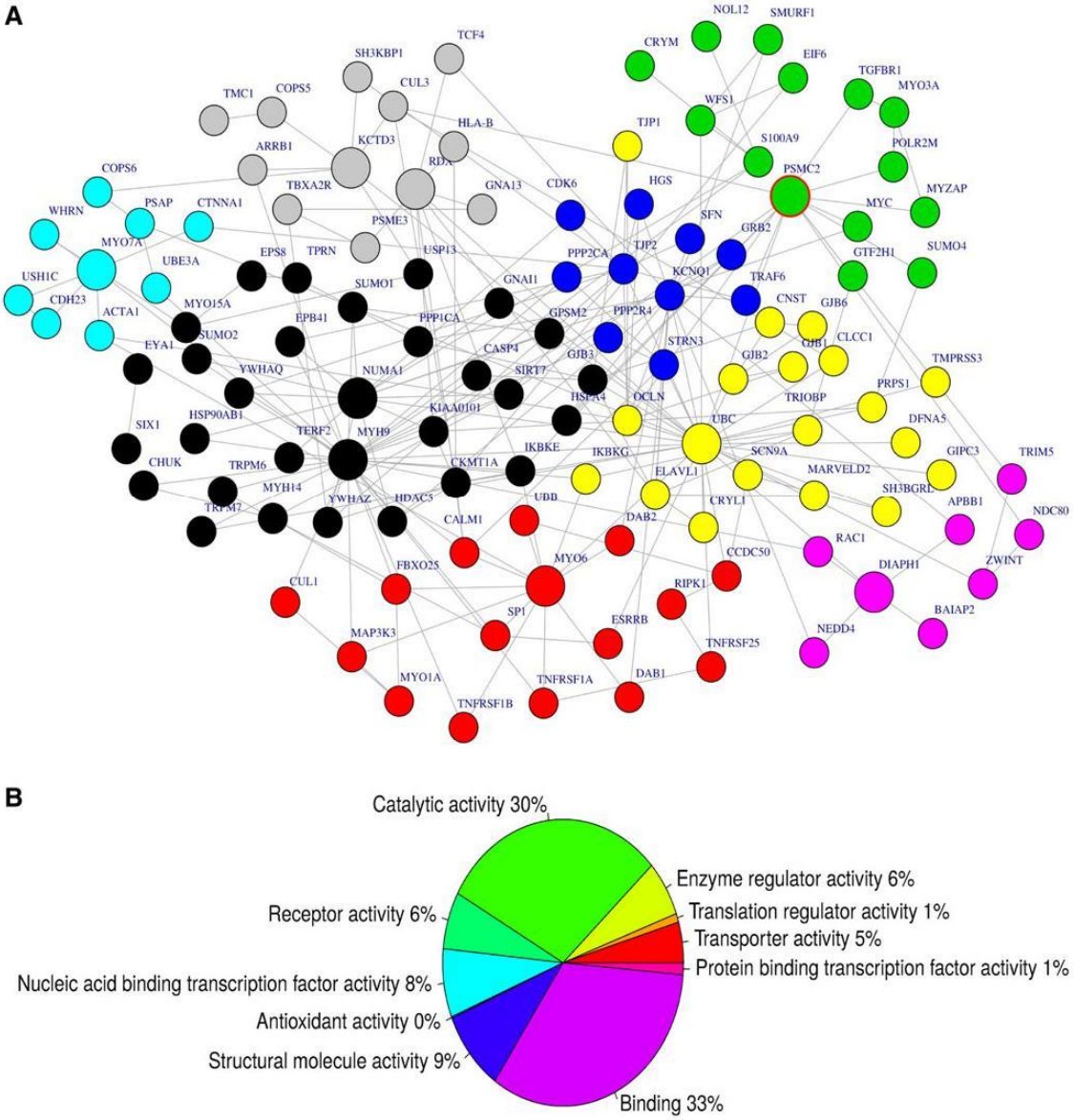

FIG. 4. Subnetwork of interacting genes with mutations potentially associated with hearing loss. We identified 10 hubs from the subnetworks extracted from protein–protein interaction database **(panel A)**, including *MYO7A*, *MYO6*, *KCTD3*, *NUMA1*, *MYH9*, *KCNQ1*, *UBC*, *DIAPH1*, *PSMC2*, *and RDX*. The molecular functions of the subnetwork are significantly associated with known biological pathways such as Proteasome Degradation, TNF-alpha signaling pathway, and TGF-beta signaling pathway **(panel B)**. TNF, tumor necrosis factor; TGF, transforming growth factor.

TABLE 3. TOP ASSOCIATED BIOLOGICAL PATHWAYS FOR THE IDENTIFIED SUBNETWORK

Term	*Overlap*	p	*Adjusted* p	*Z-score*
Proteasome degradation	25/62	5e-13	7e-11	−1.90
TNF-alpha signaling pathway	28/92	5e-12	5e-10	−1.8
TGF-beta signaling pathway	30/132	4e-10	2e-08	−1.8
Calcium regulation in the cardiac cell	29/149	1e-08	7e-05	−1.8

Pathway enrichment using Enrichr (www.ampharm.mssm.edu/Enrichr), based on genes in the subnetwork in Figure 4A; 63% of the genes within the subnetwork are annotated to the molecular functions binding (GO: 0005488) and/or catalytic activity (GO: 0003824) and are significantly enriched with deafness ($p=4.22e-15$).

TNF, tumor necrosis factor; TGF, transforming growth factor.

The absence of a mutation in 30% of Cameroonian families previously studied indicates the possibility of discovering new HI-associated genes in Africans (Lebeko et al., 2016). Supporting this, recent studies using MPS that looked at the exons of 180 genes in various populations across the globe, including two sub-Saharan African populations, showed the lowest number of resolved cases, reported at 4% and 17%, among Nigerian and South African populations, respectively (Yan et al., 2016). A similar trend with only 26% resolution rate was reported among African-Americans, the lowest when compared to that of other ethnic groups (Sloan-Heggen et al., 2016). Thus, in an effort to address the genetic causes of HI among patients of African descent, the use of whole-exome sequencing should be the next approach. Estimates indicate that at least 1000 NSHI genes remain to be identified (OMIM) (Hertzano and Elkon, 2012), and African populations could be important in this endeavor.

The *MYO7A* variant c.5880_5882 delCTT (p.Phe1963del) was previously identified in a French patient with Usher syndrome (Roux et al., 2006), and bioinformatics analysis performed in this study also revealed that the subnetwork associated with this *MYO7A* variant is enriched with Usher syndrome annotation ($p = 4.134e\text{-}7$). While surveillance will determine whether retinitis pigmentosa will develop in the Cameroonian hearing-impaired probands and affected siblings, it is possible that the compound heterozygous variants in *MYO7A* are causal of NSHI. Two previously reported MYO7A tail variants caused NSHI rather than Usher syndrome (Riazuddin et al., 2008), like the novel *MYO7A* p.Leu1935del variant reported in this study.

Likewise, for *CDH23* compound heterozygous variants (Table 2), the occurrence of an NSHI variant *in trans* with an Usher allele is predicted to cause NSHI only (Schultz et al., 2011). Similarly, the compound heterozygous *SLC26A4* variants identified in one of the Cameroonian families (family 6) (Fig. 1; Table 2) were previously identified in Chinese NSHI probands (Jiang et al., 2015; Yasunaga et al., 2000; Yuan et al., 2012) and are unlikely to cause Pendred syndrome. A homozygous splice mutation, *OTOF* [c.766-2A>G], identified in this study (family 4; Fig. 1) was previously reported in a southwest Indian family segregating NSHI (Yasunaga et al., 2000).

The bioinformatics interrogation of genes in the human PPI network performed in this study could give us new insight into biological process involved in HI, in understanding variable clinical phenotypes, and hopefully, investigating new routes for therapeutic interventions. As expected, there was a significant association between the reported subnetwork and the biological processes' sensory perception of sound and mechanical stimulus. Specifically, the network analysis identified 10 hubs that involved genes that could happen in future studies to be of importance in Cameroon. In addition, the analysis has reaffirmed the importance of *MYO7A* in HI. *MYO7A* is a large gene that spans 87 kb of the genome and plays an important role in nonsyndromic as well as syndromic HI (i.e., Usher syndrome) (Gibbs et al., 2010). OtoSCOPE interrogates the exons of genes known to be associated with HI, thus any variants identified within the genes could potentially have clinical implications (Shearer et al., 2013).

The above PPI analysis results on the variants reported by OtoSCOPE, in addition to analysis of data extracted from the 1000 Genomes with a community network analysis across the different populations, have also shown that for some targeted HI genes, the overall MAFs at the gene level were population-specific common (Supplementary Table S1). Last, prioritization of genes and their variants from merged data replicated *OTOF* and *LOXHD1* as important genes in HI, which also appeared to be occurring in LD with other reported variants. Much like *MYO7A*, *OTOF* carries variants that are reported in multiple populations (Table 2) and could prove to be important genes in HI across different populations, and their roles and interactions should be further investigated.

Conclusions and Outlook

The result reveals that novel variants recently identified among selected familial case of HI in Cameroon variants are not common in isolated cases of NSHI. PPI network analysis has highlighted the potential role of 10 genes in HI genomics and phenotypes among Cameroonians that need further investigation. It is anticipated that future ramifications of the study will include study of exomes data of families segregating HI, isolated probands with HI, as well as control populations without HI from Africa, with the anticipation that the genes highlighted by the present studies could play prominent roles in the etiology and epidemiology of HI in sub-Saharan Africa. Such knowledge will assist in establishing appropriate molecular diagnosis from this condition, as well as clinical and molecular clues to be used for anticipatory guidance in clinical settings, based on genotypes to phenotypes correlation studies.

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A.W. conceived the study, participated in its design, interacted with the patients, coordinated the blood sample collection, and drafted the article. K.L., M.N., E.C., N.M., C.D., and A.W. carried out the genetic studies, performed the data analysis, and contributed to drafting the article. K.L. and C.D. participated in its design, interacted with the patients, coordinated the blood sample collection, and helped to draft the article. E.C., N.M., and A.W. performed the bioinformatics analysis, coordinated the data interpretation and statistical analysis, and helped to draft the article. All authors read and approved the final article.

Author Disclosure Statement

The authors declare that no competing financial interests exist.

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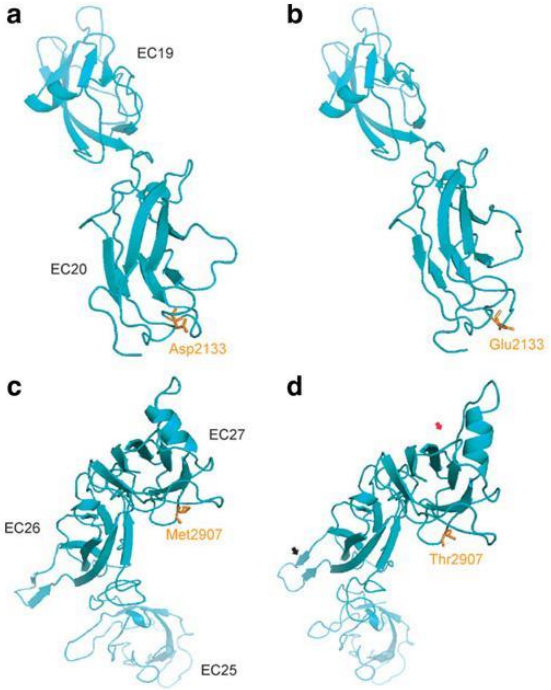
Address correspondence to:
Ambroise Wonkam, MD, PhD
Division of Human Genetics
Faculty of Health Sciences - University of Cape Town
Anzio Road, Observatory
Cape Town 7925
South Africa

E-mail: ambroise.wonkam@uct.ac.za

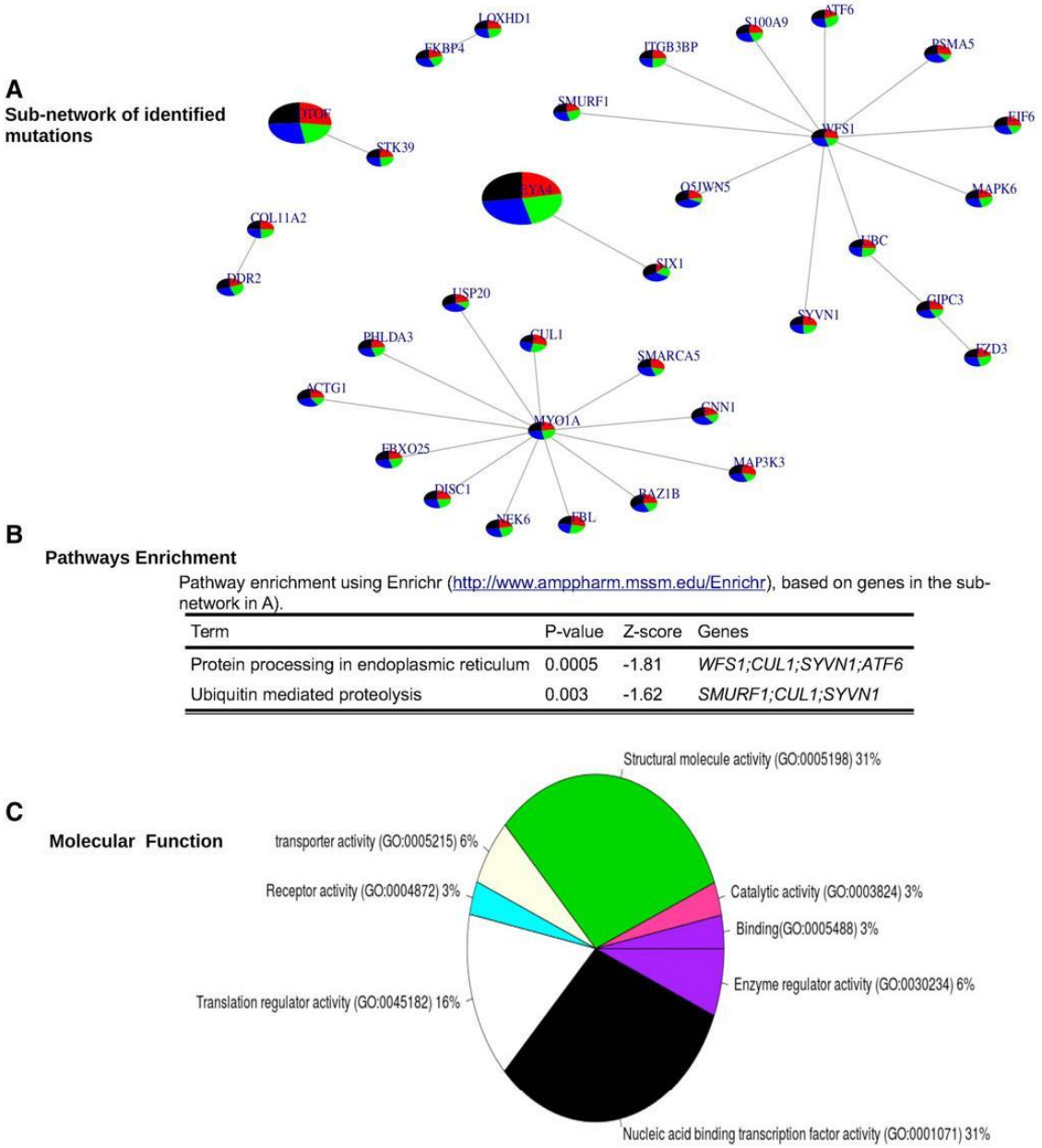
Abbreviations Used

AR = autosomal recessive
ARNSHI = autosomal recessive nonsyndromic hearing impairment
cDNA = complementary DNA
DALYs = disability-adjusted life-years
ExAC = Exome Aggregation Consortium
HI = hearing impairment
LD = linkage disequilibrium
LRT = likelihood ratio test
MAF = minor allele frequencies
MPS = massively parallel sequencing
N/A = no data available
NSHI = nonsyndromic hearing impairment
OMIM = Online Mendelian Inheritance of Man database
PCR = polymerase chain reaction
PPI = protein–protein interaction
TGE = targeted genomic enrichment
vcf = variants calling files

Supplementary Data



SUPPLEMENTARY FIG. S1. Molecular modeling of CDH23 p.Asp2133Glu and p.Met2907Thr. **(a)** Using CDH23 extracellular cadherin repeat domains EC1-2 as template, the Asp2133 residue occurs at a putative Ca^{++} binding site that is highly conserved between β strands E′ and F′ within EC20. **(b)** The additional methylene group in glutamic acid compared to aspartic acid confers higher affinity for calcium, which would likely affect stiffness of stereociliary tip links that are formed by CDH23 (i.e., increased rigidity), degree of unfolding when exposed to stretching forces, and the *trans*-binding strength between EC20-21 within the same CDH23 protein. **(c)** Based on CDH8 structure, the Met2907 residue is predicted to lie within EC25 of CDH23. **(d)** The p.Met2907Thr variant is predicted to affect H-bond formation, resulting in the gain of a β-sheet within the linker region between EC26-27 (*black arrow*) and a more open configuration for EC27 (*red arrow*) that is linked to the transmembrane region of the CDH23 protein.



Pathway enrichment using Enrichr (http://www.ampharm.mssm.edu/Enrichr), based on genes in the sub-network in A).

Term	P-value	Z-score	Genes
Protein processing in endoplasmic reticulum	0.0005	-1.81	*WFS1;CUL1;SYVN1;ATF6*
Ubiquitin mediated proteolysis	0.003	-1.62	*SMURF1;CUL1;SYVN1*

SUPPLEMENTARY FIG. S2. Subnetwork of interacting genes with mutations potentially associated with hearing loss based on the merged data from family members. **(panel A)** Subnetwork extracted from the protein–protein interaction database, **(panel B)** *Top* two associated biological pathways to the identified subnetwork, and **(panel C)** molecular functions of the related subnetwork.

Supplementary Table S1. Other Variants Identified in Cameroonian Families Using Otoscope and Related Gene-Specific Variant Frequency in Exome Data 105 Cameroonian Control, All African, European, and East Asian from 1000 Genome Project Phase 3

Gene	*95% CI frequency Cameroonian*	*95% CI frequency in African from 1000G*	*95% CI frequency in European from 1000G*	*95% CI frequency in East Asian from 1000G*	*Gene name*	*Location*	*Inheritance*
MYO3A	0.21 (0.004–0.46)	0.12 (0.0008–0.71)	0.19 (0.001–0.72)	0.14 (0.001–0.63)	Myosin IIIA DFNB30	10p11.1	Recessive
COL11A2	0.14 (0.004–0.48)	0.18 (0.0008–0.91)	0.18 (0.001–0.72)	0.22 (0.001–0.97)	"collagen type XI alpha 2" DFNB53/DFNA13	6p21.3	Both
MYO1A	0.06 (0.002–0.36)	0.052 (0.0008–0.15)	0.06 (0.001–0.15)	0.11 (0.001–0.28)	Myosin IA DFNA48	12q13-q15	Dominant
LOXHD1	0.18 (0.004–0.47)	0.095 (0.0008–0.64)	0.18 (0.001–0.86)	0.20 (0.001–0.88)	Lipoxygenase homology domains 1 DFNB77	18q21.1	Recessive
ADGRV1	0.19 (0.0–0.47)	0.126 (0.0008–0.86)	0.14 (0.001–0.76)	0.25 (0.001–0.90)	G protein-coupled receptor v1 USH2C	5q14.3	Recessive
GIPC3	0.14 (0.004–0.45)	0.11 (0.0008–0.64)	0.12 (0.001–0.54)	0.06 (0.001–0.23)	GAIP C-terminus-interacting protein 3 DFNB15/DFNB95	19p13.3	Recessive
OTOF	0.16 (0.004–0.47)	0.12 (0.0008–0.8)	0.16 (0.001–0.94)	0.24 (0.001–0.99)	Otoferlin DFNB6/9	2p23.1	Recessive
CLDN14	0.23 (0.004–0.49)	0.17 (0.0008–0.8)	0.29 (0.001–0.90)	0.28 (0.001–0.68)	Claudin 14 DFNB29	21q22	Recessive
WFS1	0.21 (0.004–0.5)	0.17 (0.0008–0.8)	0.25 (0.001–0.83)	0.451 (0.001–0.99)	Wolfram syndrome 1 (wolframin) DFNA6/DFNA14	4p16.3	Dominant

The frequency was obtained by aggregating the frequencies from all SNPs in LD (>0.75) within each gene.
95% CI, 95% confidence interval; LD, linkage disequilibrium; SNPs, single nucleotide polymorphisms.

Chapter 4: Whole exome sequencing reveals novel frameshift mutation in *GRXCR2* that alters protein expression and localisation in a multiplex family from Cameroon

Introduction

Hearing Loss (HL) is one of the most common sensroy disorders that affects both the elderly and young alike (Atik *et al*., 2015). With congenital cases of HL, it can be a shock to families, particularly when there is no family history of HL. Factors resulting in congenital HL can be environmental or genetic or result from an interaction of the two. In sub-Saharan Africa (SSA), the contribution of the environment to HL is much higher than known genetic contributors. As a result, cases which have no known envirnomntal cause are classified as unknown. This differs greatly to developed countries where the identification of prevalent genes and their variants has allowed for the development of specific gene panels used in diagnostic services. These developments have been made possible by decades of single gene discovery within these population groups, but similar progress has been lacking in SSA. These panels may still be of relevance in unexplored population groups such as sub-saharan African populations as they offer a great starting point with the screening of known genes associated with HL. This is a far more cost effective approach than the traditional single candidate gene approach. However, the use of these panels has proven to be less effective in resolving patients of African descent (Shearer *et al*., 2014b; Yan *et al*., 2016). Previously we used OtoSCOPE®, a targeted gene enrichment (TGE) panel, to try and resolve cases of autosomal recessive nonsyndromic hearing loss (ARNSHL) from 10 multiplex families from Cameroon, of which 7 families (77.8%) were successfully resolved (Lebeko *et al*., 2016). This is a relatively high resolution rate compared to other literature where TGE was employed to resolved cases of ARNSHL in patients of sub-Saharan descent (Manzoli *et al*., 2013; Shearer *et al*., 2014b; Rudman *et al*., 2017), probably owing to the stringent inclusion criteria of only familial HL cases. These lower resolution rates among Africans in several studies (Shearer *et al*., 2014b; Yan *et al*., 2016), as well as the presence of at least two unresolved families in our previous study (Lebeko *et al*., 2016) suggests the presence of causative variants in novel genes which were not investigated through OtoSCOPE® and other TGE

platforms. It is evident that a wider approach is needed to elucidate the cause of HL in these unresolved families.

Whole exome sequencing (WES) provides a suitable strategy that allows for the interrogation of a select part of the human genome. The gene enrichment process captures the exons, which are protein coding regions that make up to 1% of the human genome, the 5'- and 3'-UTRs, which affect gene translation process and can affect gene functionality, and splice regions found within specific regions of introns. This all allows for a comprehensive coverage which can detect genetic variations that potentially alter the translation of the gene as well as the integrity of the gene product. With congenital HL mostly being of genetic aetiology and largely a monogenic Mendelian disorder, WES is appropriate to investigate specifically familial cases. This approach has been employed in various populations and has demonstrated its suitability in uncovering novel causative variants in genes previously associated with HL as well as in genes rarely or not yet associated with HL (Atik *et al*., 2015; Liu *et al*., 2015; Pandey and Pandey, 2015; Churbanov *et al*., 2016; Rudman *et al*., 2017). In the present study WES was therefore employed to identify the causative variants harboured in the unresolved multiplex families previously sequenced with OtoSCOPE® (Lebeko *et al*., 2016). Additionally, to show the sensitivity and accuracy of WES in detecting causative variants, we also sequenced two multiplex families which had previously been resolved through OtoSCOPE®.

Methodology

Ethical approval

The study was performed in accordance with the Declaration of Helsinki. This study was granted ethics approval by the Cameroon National Ethics Committee (ethics approval N°123/CNE/SE/2010 and N°033/CNE/DNM/07) as well as the university of Cape Town Human Research Ethics Committee (ethics approval HREC REF: 455/2014 and HREC REF: 132/2010). Written informed consent was obtained from patients 18 years or older or from parents/guardians for minors with (Wonkam *et al*., 2013; Bosch et al., 2014a,b) verbal assent from the children.

Patients' Clinical Data

Participants were recruited in Cameroon and South Africa as previously reported (Wonkam *et al*., 2013; Bosch *et al*., 2014b). In Cameroon, patients were recruited at various outpatient clinics in different regions of the country. In South Africa, self-identified black Xhosa participants were recruited at the Efata School of the deaf and Blind in Mthata, Eastern Cape (Bosch *et al*., 2014a). Patients were included in this study if they were of Black African descent presenting with ARNSHL of either putatively genetic origin or of unknown cause. Patients were excluded from this study if there was indication of an environmental cause or evidence of syndromic HL (Bosch *et al*., 2014a). As previously reported, clinical evaluation confirmed diagnosis of sensorineural HL according to current clinical standards alongside collection of qualitative data (including family history) through a comprehensive questionnaire (Wonkam *et al*., 2013). Syndromic HL was ruled out after examination by an ophthalmologist and a medical geneticist. All participants have been evaluated for causative variants in *GJB2, GJB6* and *GJA1.*

Familial Cases evaluated through Next Generation Sequencing

Four multiplex families from the cohort were selected for WES. Their recruitment was conducted as described above. Families included had at least 2 affected HL probands of the same generation with unaffected parents (Lebeko *et al*., 2016).

Control Population

Ethnically matched control samples were used in this study. They were 100 normal hearing patients from different regions of Cameroon, recruited at the same centres as the patients. Controls for the South African Population were taken from Black South African participants' DNA available to the Division of Human Genetics.

Whole Exome Sequencing

Four multiplex families were selected for WES, two of which had been previously resolved through OtoSCOPE® (Lebeko *et al*., 2016). DNA was extracted from blood samples as previously reported (Bosch *et al*., 2014b). Using the SureSelect Human All Exon 50 Mb kit (Agilent Technologies, Inc.), which covers the exonic sequences of

≈24 000 genes corresponding to 50 Mb of genomic DNA, library preparation and sequencing on the Illumina HiSeq 2000 were performed at the Institute of Human Genetics, Helmholtz Zentrum München, Germany. The Burrows-Wheeler Alignment tool (version 0.7.5) was used to align the reads to the human genome assembly hg19 (GRCh37). Variant calling was performed with SAMtools (v0.1.19) and PINDEL (v0.2.4t). Variant quality was determined using the SAMtools varFilter script: default parameters were applied, with the exception of the minimum *P* value for base quality bias (−2), which was set to 1e−400. Variant annotation was performed applying custom Perl scripts, including information about known transcripts (UCSC genes and RefSeq genes), known variants (dbSNP v135), type of variant, and amino acid change (where applicable). Variants were filtered and excluded if there was no AF information in DBSNP137, 1000KG201204, ESP6500AA, ESP6500EA databases. Coding and splicing variants were included, and potential genes and variants were searched through a PubMed search using the following terms: “deaf, deafness, sloping + audiograms, prelingual + sensorineural + profound, prelingual + sensorineural + symmetric.”

In Silico pathogenicity prediction of genomic variants

GRXCR2 cDNA sequence was extracted from the Ensemble genome browser (ENST00000377976.2) and manually edited by deleting the cytosine at position 251 of the cDNA sequence. The altered sequence was then interrogated using two online programs, namely ExPASy (web.expasy.org/translate/) and EMBOSS Transeq (http://www.ebi.ac.uk/Tools/st/emboss_transeq/). This was done to predict the effect of the identified novel frameshift deletion on the translated protein sequence. To infer the importance of this variant across species, a multiple sequence alignment of higher primates was extracted from Ensemble (http://ensembl.org).

Protein Prediction and Modelling

PSIPRED protein sequence analysis workbench online platform (Buchan *et al*., 2013) (http://bioinf.cs.ucl.ac.uk/psipred/) was used to determine the secondary structure of the translated protein sequence of *GRXCR2*. The platform has a variety of tools and algorithms which can be applied to the provided sequence. The PSIPREDv3.3 tool,

which predicts the secondary structures of the protein sequence inputted, and DISOPRED 3, which predicts intrinsically disordered regions (IDRs) and protein binding sites within them (i.e. IDRs), were selected to analyse the sequence. Protein-protein interactions and potential network of *GRXCR2* was investigated using STRING (https://string-db.org/).

Genotyping of patients' samples

To investigate any potentially causative and secondary variants harboured in this gene, the entire coding region of *GRXCR2* was screened using direct Sanger sequencing in 80 patients and 100 controls. Primers (Appendix Table A1) were designed to amplify all three exons of *GRXCR2.* To amplify the desired fragments, PCR was performed by denaturing at 95°C for 3 minutes followed by 35 cycles of 30 second denaturation at 94°C, 30 second primer annealing at 55°C and 1 minute elongation at 72°C. This was followed by a final elongation step at 72°C for 10 minutes. PCR amplification was confirmed by gel electrophoresis and products were cleaned up using FastAp and Endonuclease I. BigDye® Terminator v3.1 Cycle Sequencing mix (Life Technologies) was used for the sequencing reaction according to the manufacturer's guidelines. Sequencing products were resolved on 3130xl Genetic Analyser ABI Prism (Applied Biosystems) using capillary electrophoresis and resultant electropherograms were analysed using DNAStar software.

Functional analysis of the GRXCR2 Variant

Plasmid DNA construct Isolation and amplification

GRXCR2 open reading frame (ORF) cloned into a pCMVEntry vector was purchased from Origene Technologies (Cat no: RC213752). Within this vector, the GRXCR2 ORF contained a DDK-tag and myc-tag at the C-terminus end. (Appendix Figure 4 A1). Site directed mutagenesis (SDM) was performed in order to introduce the identified c.251delC variant within this construct. Custom designed SDM primers [Forward 5`-ACTGCTCAGAGATCAGTGTGTTTAGAGAGGG-3` and Reverse -5`-CCCTCTCTAAACACACTGATCTCTGAGCAGT-3`] incorporating the variant were produced (Whitehead Scientific) and used in PCR using the pCMV-GRXCR2 vector as template and the KAPA HiFi HotStart ReadyMix PCR Kit (Roche). The PCR reaction mix was prepared as a 50uL reaction according to the manufacturers

guidelines (Appendix). The PCR amplification was confirmed by resolving 10ul on a 1% agarose gel. The methylated parental plasmid which would not have the variant was digested by 1uL Dpn1 enzyme at 37°C for 1 hour. Confirmation of the presence of plasmid after digestion was again confirmed on a 1% agarose gel through electrophoresis. The plasmid was then inserted into competent XL1-Blue bacterial cells through transformation using the heat shock method. After 2hr incubation, 100 µL of bacterial culture was spread onto LB-Agar plates with a 50ug/ul concentration of kanamycin and incubated at 37°C O/N. Colonies on the plates indicated successful transformation. Plasmid DNA was harvested from O/N cultures grown in 5ml Luria Broth with 50ug/ul Kanamycin using the PureYield™ Plasmid Miniprep System (Promega) and integrity of the plasmid DNA was confirmed after resolution on a 1% agarose gel.

PCR primers [Forward GFP-HindIII: 5'-GCCGCCAAGCTTCCATGGAGG-3' and Reverse GFP-BamHI: 5'-CCGCGTGGATCCTTGATTGCA-3'] were then designed to amplify the wildtype and mutated ORFs with the introduction of the restriction enzyme site for HindIII at the 5' end and BamHI at the 3' end. This was done to allow for sub cloning of the ORFs into the selected pEGFP-C1 vector (Appendix Figure A2). This GFP expression vector allows for the cloning of the GRXCR2 ORF such that the resulting recombinant GFP-GRXCR2 fusion protein is expressed with the GFP tagged on the N-terminal side of GRXCR2. PCR amplification was performed as outlined and resolved on a 1% agarose gel. After successful amplification was observed, the PCR products were cloned into a pGEM®-T Easy vector (Promega) (Appendix Figure A3) using the T4 DNA ligase (Promega). This vector has T-overhangs which will bind to the A-overhangs left by Taq Polymerase during PCR and thus allows for cloning of PCR products. Following incubation O/N at 4°C, the ligation mixture was transformed into competent DH5α bacterial cells and plating onto agar plates with 100ug/uL ampicillin and an O/N incubation at 37°C. Positive colonies were confirmed using colony PCR followed by resolution on agarose, using the primers described earlier. A single positive colony per reaction was picked and inoculated in 5ml Luria Broth with 100ug/uL ampicillin O/N at grown at 37°C on a shaker. Plasmid DNA was isolated using the GenElute™ Plasmid Miniprep Kit (SigmaAldrich) and integrity of the plasmid DNA was observed after resolution through electrophoresis on a 1% agarose gel.

Following confirmation of successful ligation of the ORFs into the pGEM-T easy vector, double restriction enzyme (RE) digestion using BamHI (Promega) and HindIII (Promega) was performed on the recombinant pGEM T-GRXCR2WT and pGEM T-GRXCR2MT and the peGFP-C1 vector. To verify the success of the digestion by the REs, PCR products were resolved on a 1% agarose gel. The digested ORFs were extracted from the gel using the QIAquick Gel Extraction Kit (Qiagen) according to the manufacturer's guideline. pEGFP-C1 was cleaned up after digestion using GeneJET PCR Purification Kit (ThermoFischer) following the manufacturer's guidelines.

Ligation reactions were set up to ligate the digested ORF fragments into the digested pEGFP-C1 vector using T4 DNA ligase (Promega). Following O/N incubation at 4℃, ligation reactions were transformed into competent DH5α bacterial cells. Transformed cells were plated onto agar with 50ug/ul kanamycin and incubated O/N at 37°C. Positive colonies were confirmed using colony PCR and primers that allows for amplification of the cloned ORFs. Agarose gel electrophoresis confirmed the presence of the ORFs and thus success of ligation into the pEGFP-C1 vector. A single positive colony per reaction was picked and inoculated in 5ml Luria Broth with 50ug/uL kanamycin O/N at 37°C on a shaker. Plasmid DNA was harvested using the PureYield™ Plasmid Miniprep System (Promega) and integrity of the plasmid DNA was observed after resolution through electrophoresis on a 1% agarose gel. Cloning was further confirmed through direct sanger sequencing.

Expression and Detection of Ha-tagged GRXCR2 in HEK293 cells

Cell line selection

In an effort to model the behaviour of proteins expressed in stereocilia of the inner ear hair cells, researchers have turned to using cell lines that present with an apical border or a brush border (Loomis *et al*., 2003). These are cell lines that present with finger-like projections such as microvilli at their periphery or on their cellular membrane (Loomis *et al*., 2003). The similarities in cellular structure and architecture allow for inference to be made with regards to the behaviour of the proteins in the inner hair cells which play a role in stereocilia formation and maintenance. This is important for proteins thought to be involved in the structure of the cilia or their development as is postulated with GRXCR2. Human Embryonic Kidney (HEK) 293 is a widely used cell line across various disciplines and topics. It is generally easy to maintain in tissue

culture and to transfect using conventional techniques. It was selected for this study due to its apical structures and cellular architecture as well as its relative ease of growth and transfection. Cells were grown from passage number 14 in Dulbecco's Modified Eagle's medium [DMEM] (Sigma-Aldrich) supplemented with 10% Foetal Bovine Serum [FBS] (Gibco, Thermo Fisher) and 1% Penicillin Streptomycin (Pen Strep) (Gibco, Thermo Fisher) and incubated at 37°C in a humidified incubator with 5% CO_2.

Transfection, protein isolation and Western Blotting

HEK293 cells were counted and 3.5 x 10^4 cells were plated per well in 6 well plates and grown until 50-60% confluent under the conditions described above. In order to introduce plasmid DNA into HEK293 cells, 1 ug of pCMV-GRXCR2WT or pCMV-GRXCR2MT was added to 100ul of complete medium as well as 3ul of X-tremeGENE™ HP DNA Transfection Reagent (Roche) and incubated at room temperature for 20 minutes. This was then added to the plated cells and gently agitated to allow even distribution of the reaction mix. This was further incubated for 30 hours at 37°C in a humidified incubator with 5% CO_2.

Following a 30-hour incubation period post-transfection, total protein was harvested from the cells using boiling blue buffer (Appendix). PBS (Appendix) was used to wash cells twice after medium had been removed. Fifty microliters (50ul) of boiling blue buffer was added onto the cells and the cells scrapped using the plunger of a 1 ml syringe. If consistency of the extract was too thick, more boiling blue buffer was added (up to 150ul total) in order to achieve maximal cell lysis. The extract was then transferred to a 1.5ml sterile microtube and boiled at 95°C for 15min in a heating block. The lysate was stored at -20°C until use.

For total protein resolution on SDS-PAGE, a 1 mm thick 12% resolving gel (Appendix) and a 5% stacking gel (Appendix) were prepared in a Bio-Rad Mini PROTEAN© 3 casting apparatus as per the manufacturer's instructions (Bio-Rad). The prepared gel was placed into a Bio-Rad running tank containing 1X running buffer (Appendix). Thirty microliters (30ul) of each sample were loaded into the wells. Also included as in one of the wells was 5 µL of the PageRuler™ Prestained Protein Ladder (Thermo Scientific, Europe). The set-up apparatus was connected to the Bio-Rad Power pack

200 and the gel was electrophoresed at 100 V/cm for 75 minutes. Following resolution, the protein was transferred onto a nitrocellulose membrane using the set up illustrated in Figure 4.1 below which was placed in the transfer unit of the Bio-Rad Mini PROTEAN© and the protein transfer took place for 90min at 100V.

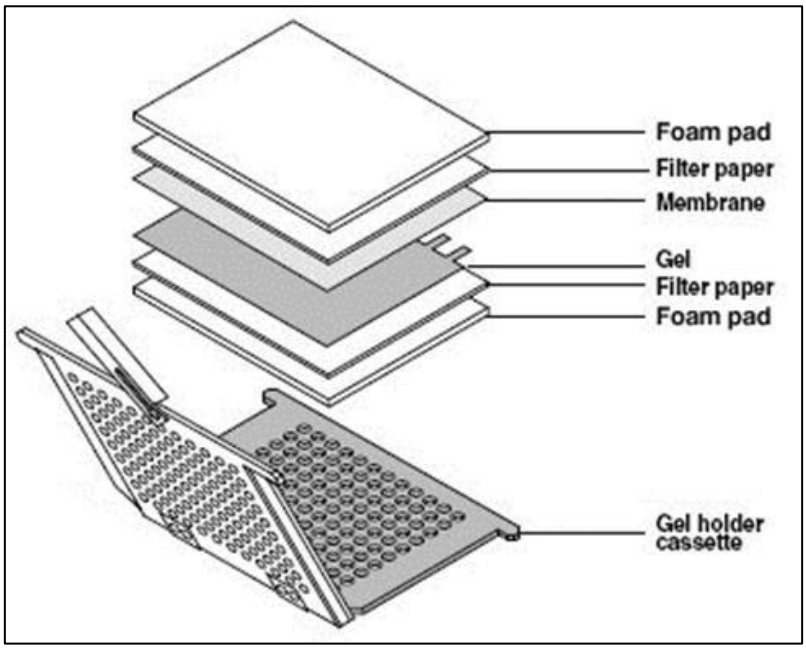


Figure 4.1: Illustration of set up used to in transfer of protein onto a nitrocellulose membrane

Following transfer, the membrane was carefully removed from the "sandwich" and was washed briefly with PBS-Tween (Appendix). Ponceau S stain (source) (Appendix) was used to verify that the transfer was successful.

For detection of GRXCR2 protein, the membrane was blocked in blocking buffer (Appendix) for 1 hour at room temperature with gentle agitation. This was followed by O/N incubation at 4°C with gentle agitation in blocking buffer containing a 1:1000 dilution of the mouse monoclonal antiFLAG M2 primary antibody (Sigma Aldrich). Following O/N incubation, the membrane was washed twice for 10 min and twice for 5 min with cold PBS Tween. Thereafter, the membrane was incubated in blocking buffer containing a 1:5000 dilution of the secondary polyclonal anti-Mouse goat antibody (Sigma Aldrich) for 1 hour at room temperature with gentle agitation. The membrane was then washed as described before and the protein detected by incubating in the dark in the Bio-Rad Clarity Western ECL Substrate (Thermo

Scientific, Europe) followed by exposure to x-ray film and developing and fixing of the exposed x-ray.

Expression of GFP-tagged GRXCR2 protein in HEK293 cells and visualization using confocal microscopy.

Cells were counted and $3.5x10^4$ cells were plated per well in 35mm plates with 4 chambers and a glass bottom, in order to achieve 50 – 60% confluence the next day which was the day they were transfected. The chamber allows for live cell viewing and imaging using the confocal microscope. 2ug of pEGFP-C1-GRXCR2WT, pEGFP-C1-GRXCR2MT and pEGFP-C1 were incubated, respectively, with 4ul of X-tremeGENE™ HP DNA Transfection Reagent (Roche) in 100ul of complete DMEM (10% FBS+1%Pen Strep) for 20 minutes at room temperature. Media was removed from cells and fresh 2ml complete media was added followed by the addition of plasmid-transfection reagent complex in a dropwise manner. After 48 hours incubation at 37°C with 5% CO2, live cells were viewed using a Zeiss LSM8800 with Airyscan confocal microscope (Zeiss, Germany). The detector of the confocal was the photo multiplier tube (PMT) and allowed detection of the green fluorescence signal through the Argon laser at 488nm. Images were visualised and processed using the ZEN Black Software (latest version) provided by Zeis.

Results

Patients' demographics

Information regarding the age, gender as well as the age of onset of hearing loss for the South African and Cameroonian patient participants are summarised in Table 4.1. Sixty percent (60%) of the patients were males (n=48) with 70% of patients reported to have a prelingual onset of hearing loss. When investigating the transmission of hearing loss, 46,25% (n=37) were reported to have a family history of hearing loss, with most of this group being from Cameroon. The average age of the participants at recruitment was 12 years old and 14 years old for the Cameroonian and South African patients respectively.

Table 4.1: Summary of Socio-Demographic data of patients

		South African (n= 23)	Cameroon (n=57)	Total (n=80)
Sex	**Male**	16	32	48
	Female	7	25	32
Age of Onset	**Prelingual**	3	53	56
	Perilingual	6	0	6
	Postlingual	3	3	6
	N/A	11	1	12
Transmission	**Familial**	5	32	37
	Sporadic	13	22	35
	N/A	5	3	8

Prelingual – Before the development of speech; Perilingual - Onset before speech development is completed; Postlingual – Onset occurred after the development of speech; N/A – No data is available in records.

Thorough examination of Cameroonian patients at recruitment identified the cases to be sensorineural where the inner ear component is affected whereas the South African patients did not have this data available. Furthermore, the degree of hearing loss of the Cameroonian patients was determined using a sloping audiometric graph and is summarised in Table 4.2. Twenty nine (29) Cameroonian patients exhibited symmetrical loss of hearing, with 23 patients determined to have asymmetrical exhibition. No data was available 5 Cameroonian patients as well as the South African patients.

Table 4.2: Degree of Hearing loss of Patients

Degree of hearing loss (dB)	Cases	%
Severe 1 (71 – 80)	1	1.25
Severe 2 (81 – 90)	1	1.25
Profound 1 (91 – 100)	12	15
Profound 2 (101 – 110)	9	11.25
Profound 3 (111 – 120)	5	6.25
Total HL (>120)	1	1.25
Asymmetric	23	28.75
Not determined	28	35
Total	80	100

Degree of hearing loss categories as provided by WHO. Patients are classified according the degree of hearing loss observed in the better hearing ear in cases of non-symmetrical hearing loss.

Description of families selected for WES

The multiplex families selected for WES are illustrated as pedigrees in Figure 4.2. The probands are all born to unaffected parents, strengthening the hypothesis that the mode of inheritance for the families is autosomal recessive.

Family 4 (Figure 4.2A) has 3 affected siblings (out of 6) reported with prelingual onset of HL. The siblings were at varying ages at the time of recruitment with the youngest affected sibling being a 9 years old male (4.2A - II:1) presenting with non-symmetrical HL with the highest degree observed in the left ear at P2 and the right ear at P1. The other siblings are both female, aged 18 (A II:4) and 21 (A II:6) at the time of recruitment, presenting with symmetrical HL with a severity of P2 and P1 respectively. Following TGE, the family was resolved and showed that the affected siblings all carried an intronic variant in *OTOF*. The c.766-2A>G variant has been reported before as the causative variant resulting in ARNSHL (Yasunaga *et al*., 2000).

Family 6 (Figure 4.2B) has unaffected parents with all three of their children reported to have congenital HL. During recruitment, only two of the three siblings were available for collection of blood samples for DNA extraction (B II:2 and II:3). Demographic data

was only collected for B II:2 which was a 3-year-old male, but the severity of HL was not recorded. Nonetheless, the availability of DNA from two of the three siblings allowed for the resolution of the family through TGE. The previously reported result showed that two novel variants, c.1678G>A and c.2007C>A, were identified in both probands in compound heterozygosity in the *SLC26A4* gene. Also reported as a contributor to the HL was a complex copy number variation of the *STRC* gene.

Family 8 had two affected children, a 6-year-old male and 9-year-old female, born to unaffected parents. Degree of hearing loss was measured at P2 for the female and appeared to be symmetrical however no audiological data was available for the male proband. Through TGE, no causative variant was picked up and the family remained unresolved. Secondary variants were reported in other genes but were not significant enough to be causative.

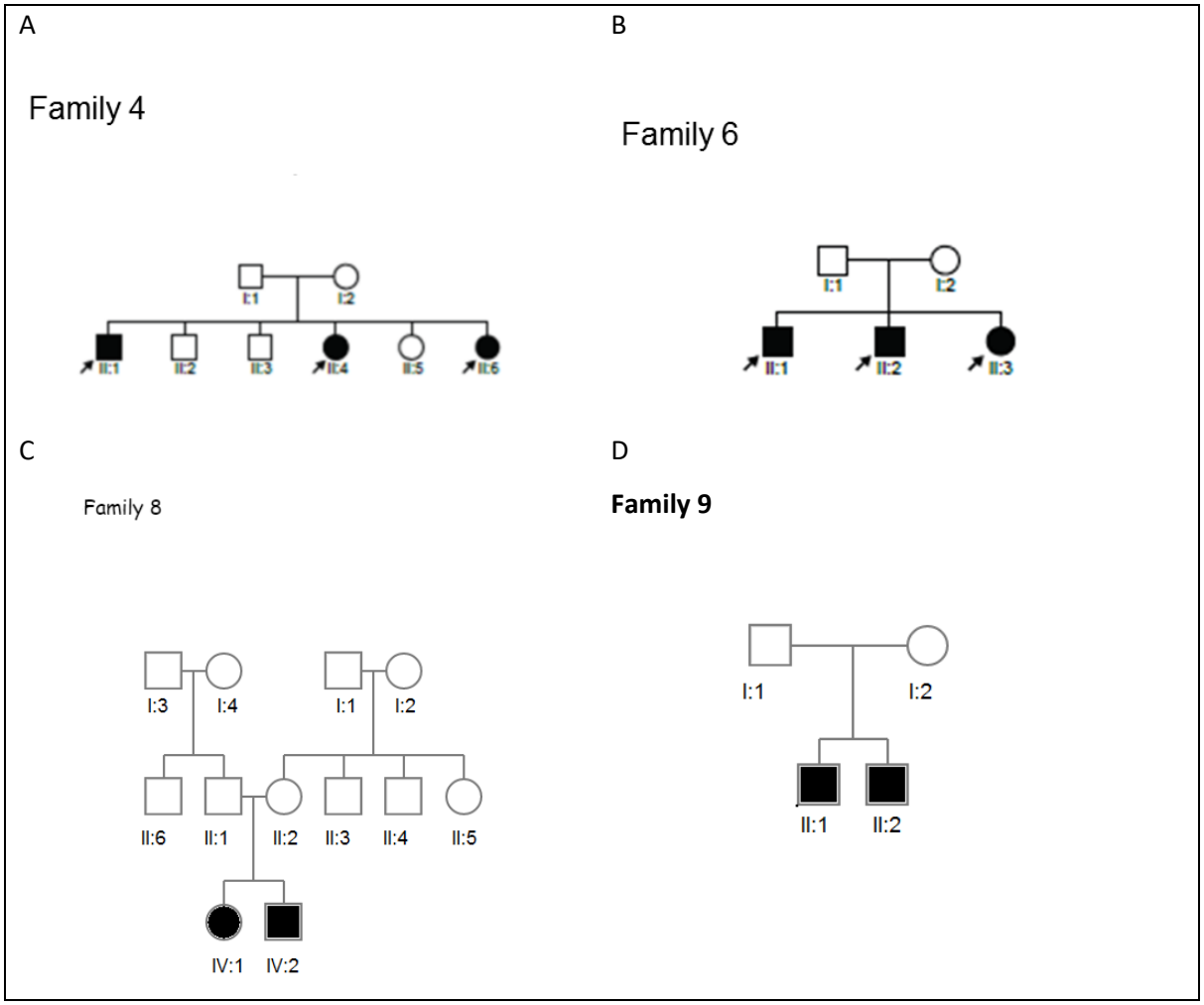


***Figure 4.2: Pedigrees of multiplex families selected for WES following TGE**. All families show at least two affected children born to unaffected parents. This strengthens the genetic nature of HL as well as the mode of inheritance as recessive. This was important to minimise or rule out unknown or unaccounted for environmental factors which plague the region.*

The fourth family, Family 9, has two affected brothers, aged 15 and 17, born to unaffected parents. Their audiological data shows a symmetrical degree of hearing loss of P1 in the 15-year-old and a non-symmetrical presentation of hearing loss with a degree of P1 in the right ear and P2 in the left year. This family, too, remained unresolved after TGE analysis through OtoSCOPE®.

Table 4.3: Summary of findings of each family from TGE through OtoSCOPE® and WES

Family		Otoscope findings			Whole exome sequencing findings		
		Gene	Nucleotide change	Protein change	Gene	Nucleotide change	Protein Change
4	Causative Variants	*OTOF*	c.766-2A>G	intronic	*OTOF*	c.766-2A>G	intronic
	Secondary findings	*CDH23*	c.3361A>T	p.Ile1121Phe			
		DFNA5	c.658G>A	p.Gly220Ser			
		TMPRSS3	c.715T>C	p.Try239His			
		USH2A	c.14804G>A	p.Arg4935Gln			
		USH2A	c.12883A>G	p.Ile4295Val			
		USH2A	c.4796G>A	p.Gly1599Asp			
6	Causative Variants	*SLC26A4*	c.1678G>A	p.Asp560Asn		c.1678G>A	p.Asp560Asn
			c.2007C>A	p.Asp669Glu	*SLC26A4*	c.2007C>A	p.Asp669Glu
		STRC	Complex Copy Number Variation	-			
	Secondary findings	*CDH23*	c.3038A>C	p.Asp1013Ala	*PAX3*	c.1320C>T	p.Asp440Asn
		GJB3	c.300B>C	p.Glu100Asp	*MYO7A*	c.4878_4879insT	p.Ser1628frameshift
		MYO7A	c.6002C>T	p.Thr2001Met	*MYO7A*	c.6002C>T	p.Thr2001Met
		TRIOBP	c.3068C>T	p.Ala1023Val			
		WFS1	c.854G>A	p.Arg285His			
8	Causative Variants	-			GRXCR2	c.251delC	p.Arg84Frameshift
	Secondary findings	*CDH23*	c.6329C>T	p.Ala2110Val	-		
		CDH23	c.6596T>A	p.Ile2199Asn			
		GPR98	c.463A>G	p.Ile155Val			
		MYO7A	c.5065G>A	p.Asp1689Asn			
		TPRN	c.1259C>T	p.Pro420Leu			
		TRIOBP	c.202A>G	p.Thr68Ala			
		USH2A	c.13409C>T	p.Pro4470Leu			
9	Causative Variants	-			-		
	Secondary findings	*GPR98*	c.6383G>A	p.Arg2128Gln	-		
		LRTOMT	c.*1867T>A	intronic			
		MYH14	c.262C>T	p.Arg88Trp			
		MYO15A	c.3359G>A	p.Arg1120His			
		MYO7A	c.*546C>T	intronic			
		USH1C	c.1069C>T	p.Arg35Tro			
		USH2A	c.8575C>T	p.Arg2859Cys			
		WFS1	c.482G>A	p.Arg161Gln			

Panel sequencing findings are replicated on WES

In Family 4, 2 of the 3 affected siblings were subjected to WES and confirmed to carry the c.766-2A>G in the *OTOF* gene identified previously through OtoSCOPE® (Table 4.3). This intronic variant results in a splicing error that skips exon 9 and introduces a premature stop codon in exon 10 resulting in a truncated protein. This variant is automatically disease causing as called by mutation taster, which is the highest category given by the programme.

Similarly, in Family 6, WES reported 5 novel variants as possible candidate causative variants, 2 of which were the same variants reported through TGE (Table 4.3) as causative variants in the *SLC26A4* gene. The other novel variants identified through WES were in *PAX3* and *MYO7A* but were excluded on account of their possible syndromic manifestation as well as their suspected dominant pattern of inheritance, both of which did not fit the ARNSHL phenotype observed in the family.

The causative variant for Family 8 was identified through WES as the homozygous deletion of a cytosine at position 5:145252279 in the GRCh37 human reference genome build.

No causative variants were identified for Family 9 through WES. A summary of the findings in these families is presented in Table 4.3.

Confirmation of a novel HI gene *(GRXCR2)* with a novel causative variant

The novel variant identified at position 5:145252279 corresponds to a homozygous deletion within a gene called Glutaredoxin and Cysteine Rich Domain Containing 2 (*GRXCR2*). It is located on chromosome 5 (5q32) and spans about 14.49Kb and is transcribed from the reverse strand. It has 3 exons, all of which are coding, and translates into a 248-residue long protein of a size of about 28kDa. The novel c.del251C variant was categorised as pathogenic by Mutation taster the highest pathogenicity category by the programme. The novel c.251del_C variant is in exon 1 and was confirmed by Sanger sequencing (Figure 4.3.).

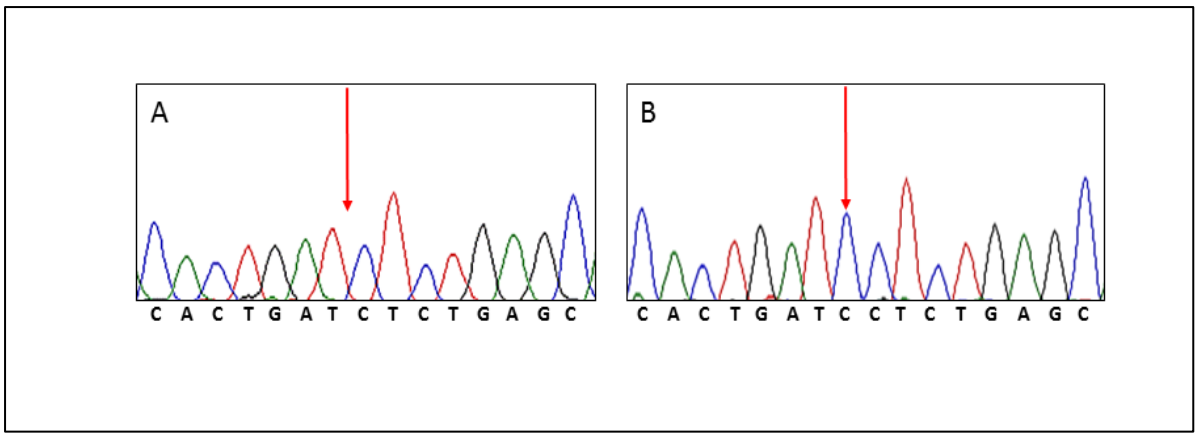


Figure 4.3.: Partial chromatograms of exon one: Sanger sequencing results validating the presence of the c.251del_C variant in patients (A) and the retention of the cytosine in a control sample (B). There is a homozygous deletion as identified in whole exome sequencing.

Upon validation of the presence of the variant in our probands, we observed evolutionary conservation of this base across 8 primate species when aligned against one another (Figure 4.4.).

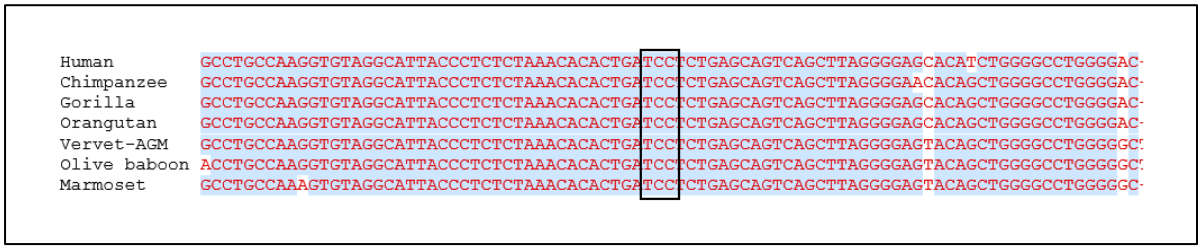


Figure 4.4: Evolutionary conservation of portion of GRXCR2 in higher primate species with affected codon showing conservation: The sequence of the codon and surrounding codons have been maintained across these higher primates which can infer the biological and functional importance of the base.

GRXCR2 protein

The *GRXCR2* gene is expressed in the inner ear during development and trace amount of RNA has been detected in the foetal heart tissues and in adult testes (Schraders *et al*., 2010). The GRXCR2 protein belongs to the glutaredoxin domain containing family. This is due to the presence of region with sequence similarities to glutaredoxin proteins. It is also a paralog to GRXCR1 to which it has a protein sequence similarity of 33.06%. Other features of the protein are the cysteine-rich

domain at the C-terminus end which has a predicted zinc-finger motif. Experiments conducted have shown that the N-terminus domain is responsible for localization of the protein which seems to be prominent on the cell membrane, particularly on cellular projections such as stereocilia bundles of the inner and outer hair cells of the inner ear (Avenarius, 2012). The identified variant contains a deletion of a cytosine base which corresponds to position 251 in the cDNA sequence.

The pathogenicity of the variant was further explored by the predicted change it would have on the amino acid sequence due to the frameshift nature of the c.251del_C variant. Protein sequence prediction by ExPASy and EMBOSS Trans revealed the altering of the amino acid sequences after p.R84 as well as the introduction of a premature stop codon at position 116 of the amino acid sequence (Figure 4.5.).

MEDPEKKLNQKSDGKPRKVRFKISSSYSGRVLKQVFEDGQELESPKEEYPHSFLQESLET
MDGVYGSGEVPRPQMCSPKLTAQRSVCLERVMPTPWQAASLGSTITRRMTISPYLL*

*Figure 4.5: Protein translation sequence of GRXCR2 in the presence of the c.251del_C variant. Yellow highlighted residues indicate residues which are altered in exon 1. Grey highlighted bases are altered in exon 2 and the * denotes the premature stop codon.*

In order to begin understanding how the alteration of the protein sequence and size affect functionality, we first sought to investigate, *in silico*, the secondary structure of the wildtype GRXCR2 protein. PSIPRED returned a profile which depicts the predicted secondary structures and their position along the protein sequence. GRXCR2 is a relatively small protein with its secondary structure depicted mostly random coiling and few helices and beta sheets (Figure 4.6.). Comparison of the wildtype and the mutated sequence was not possible as the 115 residues long sequence is too short to be interrogated in PSIPRED.

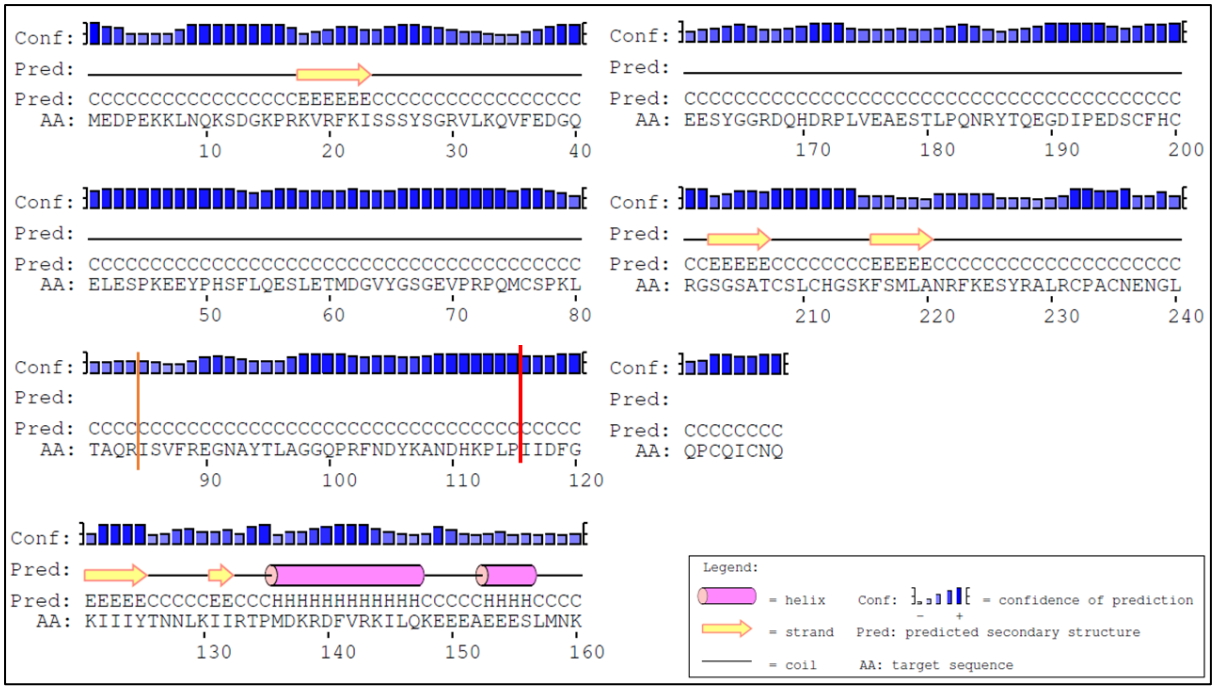


Figure 4.6.: Secondary structures of GRXCR2 by PSIPRED. Orange vertical line denotes the site of the base deletion and alteration of protein sequence. The red vertical line denotes the position of the premature stop codon.

The predicted stability of the protein post translation can also give insight into the behaviour of the protein in the cell. Shown in Figure 4.7 is the disorder profile of the protein according to identified Intrinsic Disorder Regions (IDRs) which are regions in the protein that can alter their state from structured to unstructured as a protein prepares to or carries out it functions, particularly in binding with other proteins. GRXCR2 was found to have three distinct protein binding regions corresponding with high confidence of IDRs. Truncation of the protein leads to the loss of the C-terminus IDR which includes the cysteine rich region which is predicted to fold into a zinc-finger structure known to act in protein-protein interactions.

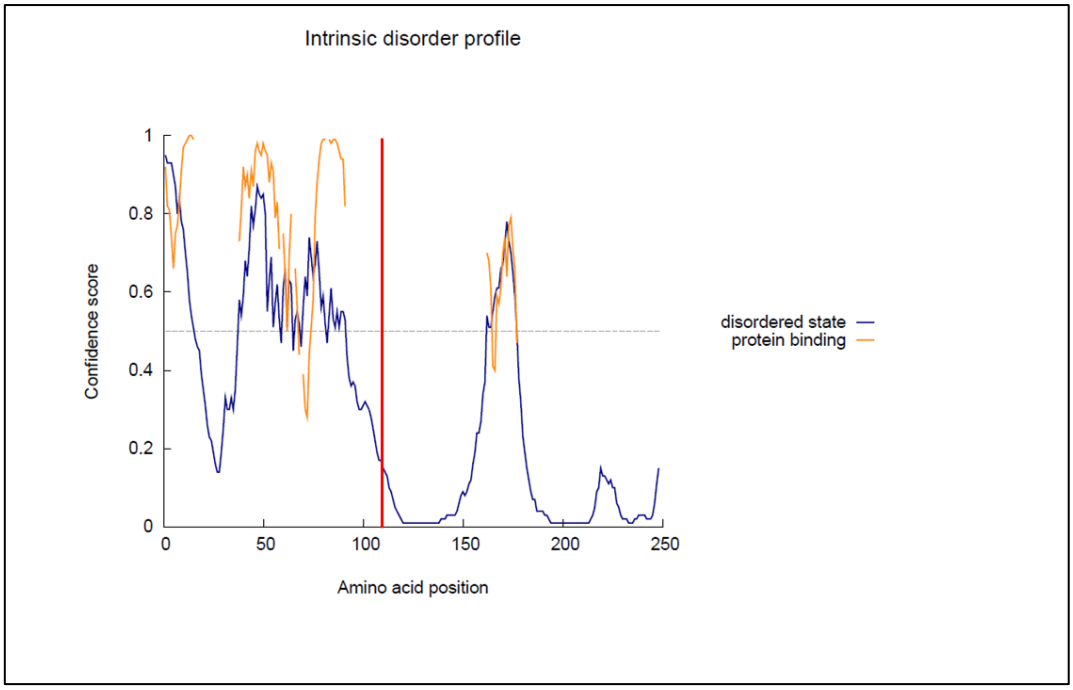


Figure 4.7: DISOPRED3 Intrinsic Disorder profile of GRXCR2 with protein binding regions. Red vertical line denotes truncation of protein.

Given the presence of protein binding sequences or regions within GRXCR2, potential predicted protein-protein interactions (PPI) were investigated using STRING database, but no data was available for GRXCR2.

Exploration of *GRXCR2* for additional variants in HL cohort through Sanger Sequencing

We investigated the frequency of this variant in our patient and control cohort of both South Africans and Cameroonians. The variant was identified in one other proband (patient 34), unrelated to the members of Family 8 (Figure 4.8.).

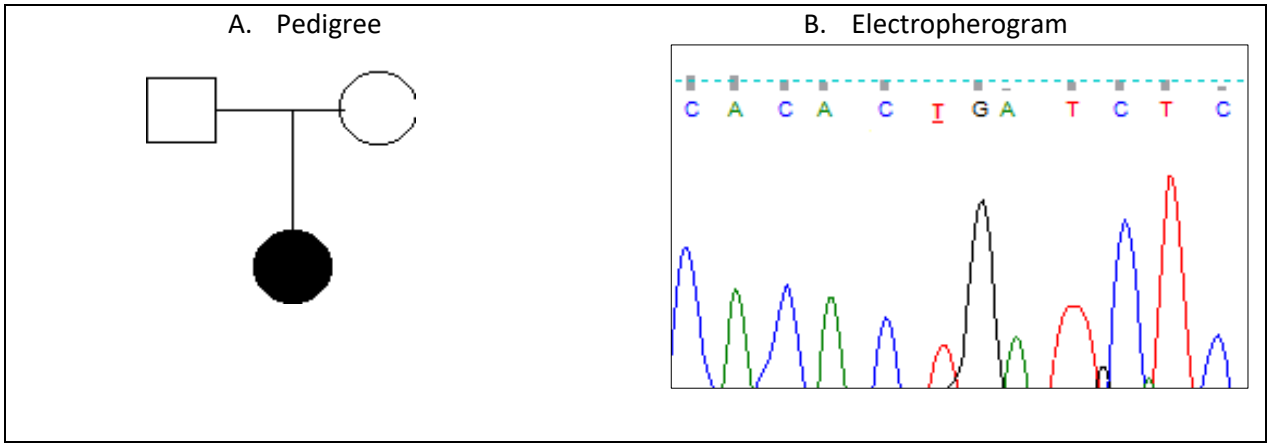


Figure 4.8.: Pedigree of simplex family (A) and Electropherogram showing presence of c.251del_C in the probands (B).

This isolated unrelated patient is a female who was 8 at the time of recruitment. She exhibited non-symmetrical hearing loss with a severity of P1 in the left ear and S2 in the right ear.

Identification of the identified novel variant in a second unrelated case prompted the sequencing of the other 2 exons to identify any other variants harboured within *GRXCR2* by this cohort. No other novel variants were observed in the rest of *GRXCR2*. Two polymorphisms were identified, both of which do not seem to be disease causing (Table 4.4). Despite c.543A>C being a missense variant, it carries a high minor allele frequency and is tolerated in the gene product and is thus not disease causing.

Table 4.4.: Variants identified in exon 2 and exon 3 of GRXCR2 from sequencing of the entire patient cohort.

Genomic position	Nucleotide change	Protein change	Pathogenicity (According to Mutation taster, Mutations tester and SWIFT)	Cameroonian Alleles (homozygous)	South African Alleles (homozygous)
5:145866522	c.543A>C	p.Leu181Phe	Tolerated	10/114 (2)	5/23 (0)
5:145866555	c.510C>T	p.His170=	Benign	4/114 (0)	0/23

Functional Studies

Confirmation of premature stop codon through western blotting

To be able to visualise the effect of the variant on the translation of the ORF, western blotting was used. The detection of the DDK-tag downstream of the GFXCR2 ORF in

the pCMV vector allowed for visual confirmation of a premature stop codon in the GRXCR2MT ORF. In the case of the WT protein, the tag will be expressed and therefore detected, while in the case of the variant, the protein will be prematurely truncated and hence not express a DDK-tagged protein. HEK293 cells were transfected with plasmids expressing either the WT or the mutant protein. Total protein was isolated, separated using SDS-PAGE, and then transferred onto nitrocellulose membrane. To confirm the successful transfer of protein from the SDS-PAGE gel to the nitrocellulose membrane, a Ponceau S stain was performed (Figure 4.9), which shows successful transfer of equal amounts of protein for the two lanes which carry the pCMV-GRXCR2WT and pCMV-GRXCR2MT protein lysates.

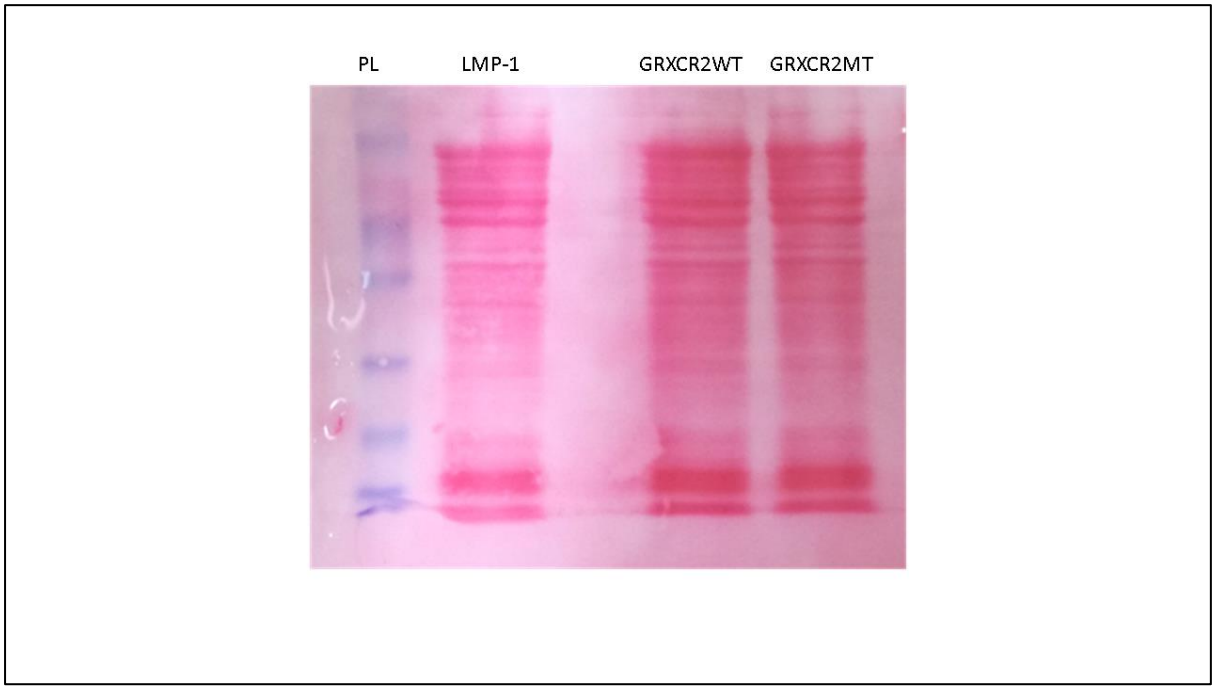


Figure 4.9: PonceauS staining showing successful transfer of protein from gel to nitrocellulose. Lane PL is the pre-stained protein ladder PageRuler (Thermo Scientific, Europe). Lane LMP-1 is a positive control for the DDK\FLAG tag antibody. Lane GRXCR2WT is loaded with total protein lysate from cells transfected with wildtype ORF. Lane GRXCR2Mt is the protein lysate from cells transfected with the mutated GRXCR2 ORF.

Following western blotting using an anti-body specific to the DDK/Flag tag, a clear dominant band of the predicted size was observed for the WT protein, which was absent for the mutant protein (Figure 4.10.).

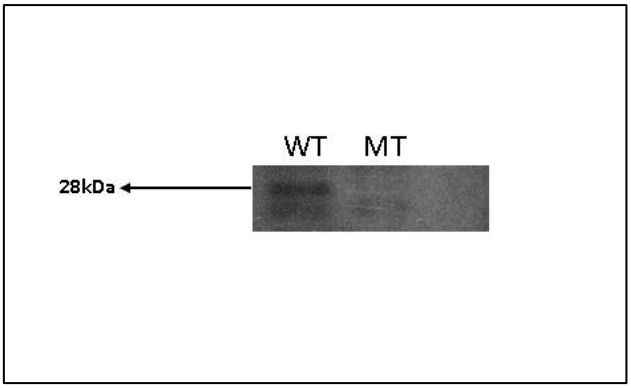


Figure 4.10.: The c.del251C GRXCR2 mutation prevents detection of DDK/FLAG: HEK293 cells were transfected with the pCMV-GRXCR2WT or GRXCR2MT expression constructs. Western analysis of whole cell protein lysates from the transfected cells indicated GRXCR2WT steady state levels were significantly higher than GRXCR2MT levels.

This demonstrated the detection of the DDK/FLAG tag downstream of the GRXCR2 in the wild type and the inability to detect it in the mutant at the corresponding band size, confirming a disruption in the translation of the DDK/FLG due to the predicted premature stop codon.

Live imaging HEK293 cells transfected with WT or MT GFP-GRXCR2 proteins using confocal microscopy

Visualisation of the protein in the cell was made possible by fusion of the WT and mutant protein to GFP through sub-cloning into the pEGFP-C1 vector, which inserts the GFP tag in frame into the ORF on the N-terminal end of the protein. Following transfection into HEK293 cells of vectors expressing GFP-GRXCR2WT or GFP-GRXCR2MT or empty vector (GFP expressing only), visualization was done using the confocal microscope at 48 hours post-transfection. As is shown in Figure 4.11, cells transfected with the empty vector showed strong and uniform pattern of expression throughout the cells. Transfection of HEK293 cells with the empty pEGFP-C1 also served as verification of the GFP expression and detection.

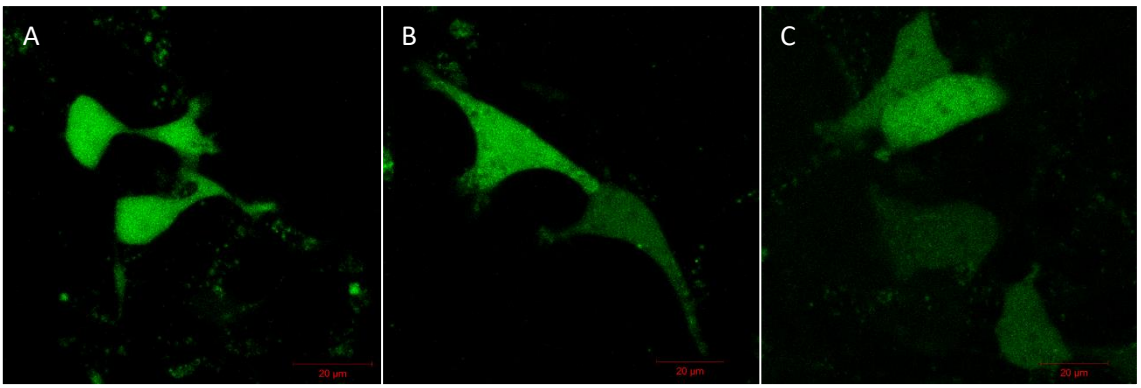


Figure 4.11.: Representative cells showing expression of GFP in HEK293 cells: (A-C) HEK293 Cells showing different intensities of uniform GFP expression when transfected with the GFP-Empty vector.

For WT-GTP tagged GRXCR2, the protein seemed to be preferentially localized outside of the nucleus and predominantly in the cytoplasm, with some punctate staining close to the periphery of the cell membranes (Figure 4.12 – top panels). This suggests that the WT protein is expressed in the cytoplasm, where it is confined, and potentially shuttled to the periphery close to the membrane. Since these cells were visualised live, a DNA specific stain could not be used to locate the nuclei. Staining of the DNA required fixation of the cells which led to leakage of GFP outside of the cells and therefore could not be performed. Interestingly, and in contrast to the GFP-GRXCR2WT protein, the mutant GRXCR2 protein lacked any particular localization within the cells and showed a distribution similar to that observed for the empty vector, although at a seemingly lower intensity (Figure 4.12 lower panel).

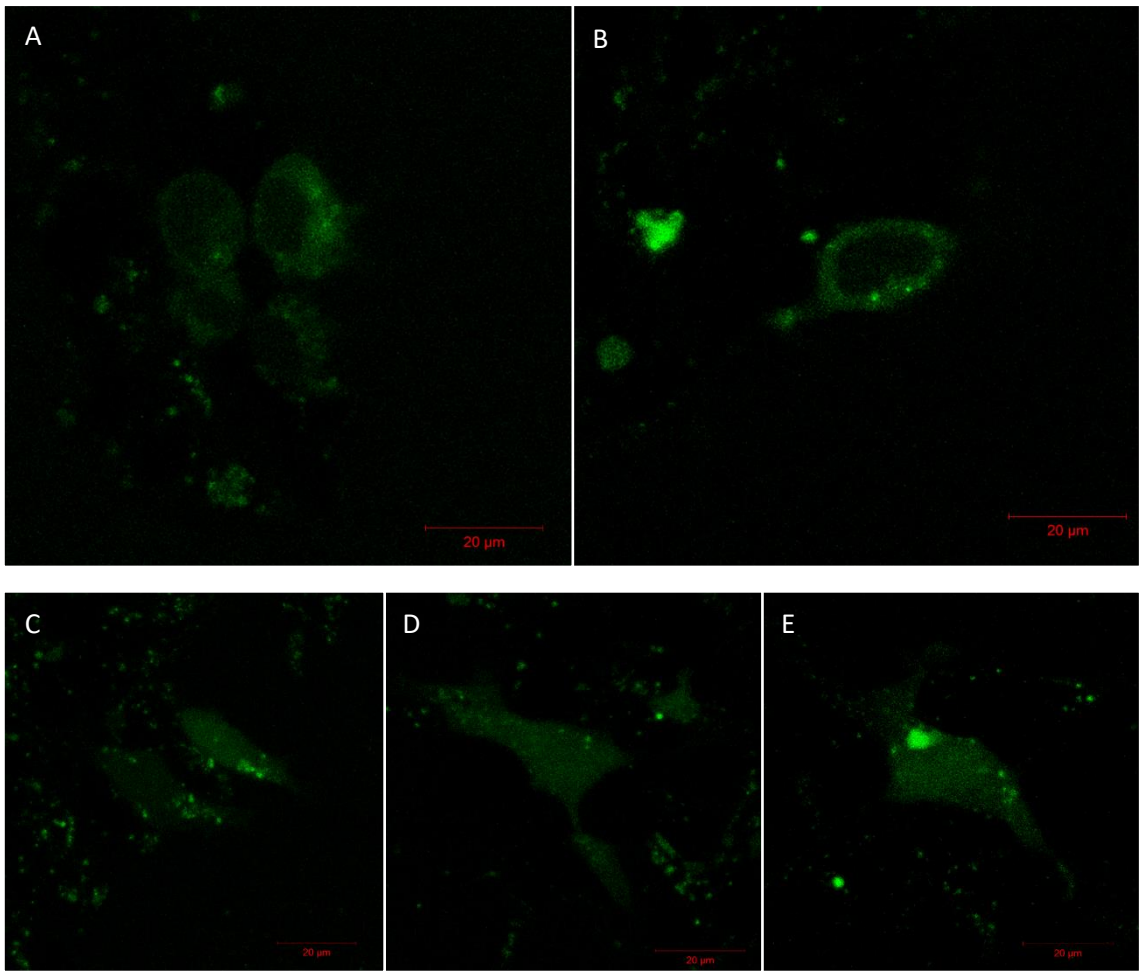


Figure 4.12:Represntive cells showing expression of GFP-GRXRC2WT(Top) and GFP-GRXCR2MT(bottom) in HEK293 cells: (A-B) Both panels show distinct darker regions owing to shuffling of recombinant protein out of the nucleus.(C-E) GFP with mutant GRXCR2 shows uniform expression with decreased intensity. No darker regions seen in top panel were not observed in this construct.

Discussion

Originality and Strengths

Next generation sequencing has increased the rate of discovery of variants associated with various diseases, including HL (Atik *et al*., 2015). However, accessibility to infrastructure and funds for such ventures are not always available in developing countries, specifically those in SSA. To date, few papers have been released with data on NGS used to resolve cases of HL in this population group, and more specifically, the use of TGE (Shearer *et al*., 2014b; Bademci *et al*., 2016; Lebeko *et al*., 2016; Rudman *et al*., 2017). Results of these studies show that there is still a lot to be uncovered about the genetic spectrum of HL in sub-Saharan African populations. Our study is the first to use WES to resolve cases of ARNSHL in multiplex families from SSA (Cameroon). It has demonstrated the efficacy of this approach in being specific enough to identity variants in known genes but equally sensitive enough to identify variants in unknown genes, or genes not yet associated with HL. This is seen by the low number of newer genes being discovered as contributors to the disease even when using WES in Caucasian, Asian and Middle Eastern Populations (Atik *et al*., 2015). Only 22 new genes have been uncovered through WES compared to the already 180+ known genes. If there is an increase in the number of WES analysis performed on Sub-Saharan African groups, it is likely that this will lead to an increase in the discovery of new genes associated with HL.

Targeted Gene vs Whole Exome sequencing

In our study cohort of 10 families, we were able to resolve 7 of the families using the then latest version of the OtoSCOPE® (Lebeko *et al*. 2016). This high-resolution rate of 77% can be attributed to the clinical selection of only families with multiple members and therefore highly likely to be display a HI of genetic origin, that could not be the case with simplex cases, as reported in previous studies with lower pick-up rate among Africans (Sloan-Heggen *et al*., 2016; Shearer *et al*., 2014b; Rudman *et al*., 2017; (Yan *et al*., 2016). Indeed, the overall trend seen among patients of African descent is that of having the lowest resolution rates when screening for variants in known HL genes and comparing the results to other populations group using the same panel (Sloan-Heggen *et al*., 2016; Shearer *et al*., 2014b; Rudman *et al*., 2017) and other TGE HL panels (Yan *et al*., 2016). We reported five novel variants in 7 families, while the

number of new variants identified within different European and Asian populations groups is not showing an increase (Shearer *et al*., 2014b; Rudman *et al*., 2017). The variants identified in our multiplex families through TGE were not present in the rest of our singleplex cases. This could be due to multiple reasons: 1) the limited number of families studied that may not have captured the most common genes in the studied population, 2) the extreme genetic diversity of HL that will required hundreds of families studies a have an appropriate population profile in a given country, 3) the great genetic diversity of the population in Cameroon that will favour private or regional mutations prevalence (Lambert and Tishkoff, 2009). In addition, while data suggest that the discovery of new HL genes and variants is reaching saturation in the more widely screened populations (Caucasian and East Asian), our study suggests the possibility of discovering novel genes through 2/9 Cameroonian families investigated. The data advocates for the inclusion of a more diverse population group i.e. Africans, and also the relevance of using a wider approach such as WES. Indeed, the selection of 4/9 families for WES, two of which we had identified causative variants through targeted panel support the use of WES rather than targeted panel in under studied population such as sub-Saharan Africans. Through WES in this sub-set, we saw a 75% resolution rate among the families which is higher than the 50% seen through OtoSCOPE® for the same four families. The identification of the same variants in two previously resolved families and the resolution of a previously unresolved family has demonstrated that, with appropriate coverage, WES is sensitive enough to detect these variants in known genes while simultaneously being comprehensive enough to identify causative variants in novel genes. The efficacy of this approach was also demonstrated in a multi-ethnic cohort from different regions around the world which saw a 56% resolution rate using WES (Bademci *et al*., 2016). Additionally the authors confirmed that copy number variations' contribution to HL can also be explored through WES when building the filtering pipeline (Bademci *et al*., 2014; Shearer *et al*., 2014a). There remains an unresolved multiplex family in our cohort which could point to the need of a more comprehensive filtering pipeline. This be could due to a complex structural change which was not detected by WES or a variant in other parts of the genome which was not captured by WES and would be discovered through Whole genome sequencing (WGS), though this presents with challenges of its own. The closest approach used to WGS was whole genome SNP mapping which was able to resolve 100% of 30 families from Pakistan (Shafique *et al*., 2014). This panel captures

and genotypes more than 700 000 SNPs across the human genome which can be filtered similarly to a WES approach. When working with patients or populations of African descent, this might not be as effective due to the data that informed the development of such panels usually has a very low representation of data from individuals of African ancestry.

However, in order to build a comprehensive genetic profile for African patients with HL, the present data indicate that WES is the best next approach and should be the go-to step for clinical and research settings to allow for the identification of new variants as well as new genes which are contributing to the loss of hearing.

Identification of variants in *GRXCR2*.

Whole exome sequencing identified a novel variant in *GRXCR2* in the probands of *"Family 8"* which was previously unresolved through TGE. The c.251delC was identified in homozygous state in the affected members and confirmed by Sanger sequencing. *In silico* investigation of this variant showed that this frameshift variant results in the introduction of a premature stop codon and ultimately the truncation of the protein, and is predicted disease causing by several tools including mutation taster. Furthermore, we identified this variant in a singleplex HL case following screening of our cohort and matched controls. The presence of the variant in another unrelated patient strengthens the hypothesised contribution of this variant to HL. When looking at the background of our probands and the singleplex case, we found that they come from distinct regions of Cameroon of different ethnolinguistic group. The multiplex family is from a Northern region of Cameroon known as Ngaoundere and are from the Fulani ethno-linguistic group. The second patient found to be carrying this variant is from Bafoussam in the western Cameroon, on and is of the Bamiléké group. However, this variant could be a founder variant and compels one to theorise that the variant is not restricted to one group or region and could be found elsewhere within the greater West African region if not other parts of the continent. Indeed, the NHIBI Exome Sequencing Project reports a single heterozygous carrier of this variant whom is reported as an African American individual (http://evs.gs.washington.edu/EVS/) further highlighting the potential presence of this variant within the African population from the diaspora. One can thus recommend that this gene be further explore in multiple African populations with ARNSHI. Notably, the *GRXCR2* c.714dupT mutation

was identified in a Pakistani family with HL (Imtiaz *et al*., 2014); therefore the present report in an African family and a patients confirm the implication of *GRXCR2* in ARNSHI in humans.

Focus on *GRXCR2*

The glutaredoxin and cysteine rich domain containing 2 (*GRXCR2*) gene is found on chromosome 5 and has three exons which encode a cysteine rich protein that is 248 residues long. It is a paralog of *GRXCR1* and both have been shown to be required for stereocilia bundle development, organization and maintenance (Avenarius, 2012). *GRXCR2* is expressed in the sensory hair cells of the inner ear, with trace amounts detected in the heart and testes. Pirouette mouse models showed variants in *GRXCR1* to result in hearing loss with vestibular dysfunction (Odeh *et al*., 2010). A similar syndromic phenotype was observed in humans who carried pathogenic variants in this gene (Schraders *et al*., 2010). Importantly, null mutations in *GRXCR2* in mice results in early onset progressive hearing loss without vestibular dysfunction (Avenarius, 2012), a phenotype observed in our present study as well in previously reported family (Imtiaz *et al*., 2014). The differences observed in the phenotype between variants in *GRXCR1* and *GRXCR2* suggests the proteins might have slightly distinct roles in the stereocilia maturation pathway. This is further seen by differences in the organisation and structure of stereocilia in the presence of variant in *GRXCR2* or *GRXCR1* (Avenarius, 2012).

GRXCR2 has three functional domains, one of which has a sequence similarity to glutaredoxin. Members of this protein family are enzymes that use glutathione as an electron donor in the reduction of oxidized cysteines of other proteins (Holmgren, 1989). However, conserved residues required for this action are absent in the GRXCR2 sequence thus, this protein is unlikely able to carry out this function (Holmgren, 2000). Other domains within GRXCR2 include a cysteine rich domain ($Cx_2Cx_7Cx_2Cx_{20}Cx_2Cx_7C$) at the C-terminus end with a zinc-finger motif. This conformation has been extensively shown to be needed in protein-protein interactions. The disruption of this domain, as result of the c.714dupT results in an extension of the C-terminus, creating an N-glycosylation motif that signals the protein for degradation, leading to the HI phenotype observed in the affected members of the Pakistani family (Imtiaz *et al*., 2014). The novel c.251delC variants we found introduces a premature

stop codon at position p.116 which results in a truncated protein without a (functional) C-terminus cysteine rich domain (Figure 4.7.). It is most likely that PPIs mediated by this domain are essential for the GRXCR2 functionality, particularly in the formation of homodimers or other protein complexes. This too was investigated by Avenarius and showed strong evidence for GRXCR2 homodimerization, as well as GRXC1 homodimerization. However, experiments relating to their interaction with one another were not conclusive. Improvement on these experiments as suggested by Avenarius could give further insight to these two paralogues interactions. One could compare this hypothesis to the interaction seen in the Connexin family between GJB2 and GJB6 in their heterodimerization needed for functionality while still showing evidence of distinct roles seen by their different disease manifestations (Xu and Nicholson, 2013)

Nonetheless, the evidence is enough to provide the mechanism by which these two *GRXCR2* variants affect this protein with regards to the Cysteine rich domain and how this could contribute to the destabilization of stereocilia in the absence of functional GRXCR2 multimers.

Gene ontology for *GRXCR2* described the molecular function as being a heat shock protein as well as being involved in unfolded protein binding. When looking at the intrinsic disorder profile, we identified three regions showing IDR properties with inferred protein binding. The IDRs are most likely essential sites in the protein that carry out the chaperone function. That is to help new proteins stabilize and fold correctly as well as to refold proteins that were damaged by cellular stress. Given the disease onset and progression of HL, it is possible that GRXCR2 is responsible for the development, maturation and maintenance of stereocilia bundles in this mechanism. In its absence, inner and outer hair cells of the inner ear have an incorrect arrangement due to errors during the developmental phase, owing to the absence of a chaperone to help stabilize and ensure correct folding of components of the bundle. Additionally, the mechanism of action on the hair cells is a stress that affects the stability of stereocilia bundles and without the refolding of proteins, this structure deteriorates over time and is seen by the progressive nature of HL seen in patients with causative variants in *GRXCR2*. And if indeed tetramers of GRXCR2 are needed for functionality, abolishment of the cysteine rich domain would result in a null effect causing the above described scenarios. Establishment of the definitive PPI of GRXCR2 will give great insight to its role in the stereocilia bundle maturation as well as its position within the

network of HL genes. Potentially, gene therapy could be explored for this type of HL as the stereocilia could be maintained and repaired even if they were poorly organised and hearing could be maintained for much longer periods.

Functional studies were used to elucidate the mechanism of the pathogenic effect of the c.251delC variant *in vivo*. We selected the HEK293 cell line to express the protein and used it as a model for the inner ear hair cells due to its apical surface which can be a model for the stereocilia. This means the cellular architecture is similar such that the mechanism by which proteins are localised to the apical surface are similar to those used for proteins localised to the stereocilia of the inner ear hair cells.

Expression of a c-terminal tag was not detected in western blot analysis of the mutated GRXCR2 protein confirming the presence of a premature stop and aligned with result from the *in silico* prediction of the translation of the mutated sequence. The effect of the variant on cellular localisation was explored through creating fusions with GFP of both the WT and the mutant protein on the N-terminal side. The role of the N-terminus domain in localisation of the GRXCR2 protein was well demonstrated by Avenarius in his PhD thesis (Avenarius, 2012). This showed proteins lacking either the C-terminus domain or the central glutaredoxin-like domain were still able to localise to the apical surface and showed protein lacking the N-terminus domain failing to localise to the apical surface and were detected in the cytoplasm and nucleus (Avenarius, 2012). The wildtype GRXCR2 protein seems to allow for transportation towards the periphery and is more concentrated towards the cellular membrane where we find the apical structures with similar architecture as the stereocilia of inner ear hair cells. This could be due to the PPIs between GRXCR2 proteins which allow for its localisation to the cellular membrane. Thus, we see the GRXCR2 protein able to carry out its function even with the GFP protein fused to it. In the mutated GRXCR2 protein, we observed a decreased intensity in the GFP fluorescence when compared to the empty pEGFP-C1 vector and also did not observe a migration towards the periphery of the cell as seen in the wildtype GRXCR1 protein construct. This could be due to that the pEGFP-C1 plasmid is small and more of it is transported into cell as compared to the plasmid with the ORF. Another factor could be the degradation of the complex due to the truncated GRXCR2 or an expression inefficiency also due to the presence of the

truncated GRXCR2 protein. The presence of the GFP upstream of the GRXCR2 protein is not enough to justify the lack of migration in the mutated GRXCR2 as this function was still carried out in the wildtype. In order to elucidate the effect of the variant on the protein in the absence of GFP upstream, GRXCR2 specific antibodies can be used in an immunohistochemistry experiment as well as adding markers/staining for various organelles to paint a full picture as to the expression and migration pattern of the truncated GRXCR2. Nonetheless, the evidence presented supports the argument of this variant alters the functionality of the protein such that it is not able to carry out normal functions as in the wildtype which lead to the support of stereocilia bundles during growth and maturation as well as their maintenance during functioning.

Limitations

Though we have shown the efficacy of WES in resolving cases of ARNSHL in African patients and have shown sufficient evidence to the pathogenicity of the c.251delC variant in GRXCR2 as well as postulated to the mechanism of action of the protein and its contribution to hearing and thus HL in its absence, there is room for improvement in this presented study. The presence of the variant in a second case points towards the possibility of a founder effect with regards to this variant. We were not able to explore this further due to lack of DNA material from the probands and the affected patient. In our functional studies, the use of GRXCR2 specific antibodies could have increased accuracy of both localisation and protein expression levels. Further exploration of this could have given a bit more clarity in terms of the exact mechanism that is disrupted by the truncated protein. DNA specific stains to visualise the nucleus could not be used in this experimental design as fixing of HEK293 cells onto glass slides resulted in leakage of GFP which prevented detection.

Conclusions

We have demonstrated the efficacy and accuracy of using WES in clinical setting to resolve HL in a multiplex family from Cameroon, by identifying a novel c.251delC variant harboured in *GRXCR2,* in two unrelated patients, and confirming the implication of this genes in HL in Human, as it was only reported one in a different

population. Additionally, we have shown that WES is able to detect variants in known HL genes, by replicating the results uncovered through TGE on the OtoSCOPE® panel. This further strengthens the advocacy of using WES in further exploring genetic causes of HL in African patients instead of using available TGE panels. *In silico* tools as well as functional studies demonstrated the pathogenicity of our novel c.251delC variant harboured in *GRXCR2*, as well as provided some insight to the possible role of the protein in the stereocilia maturation pathway. Identification of the variant in a second patient strengthens the association of the variant and HL as well as points towards a possible founder effect which needs to be further investigated.

Chapter 5: Overall Discussion, Conclusions and Perspectives

Burden of Hearing Loss

Hearing loss is sometimes called the silent disease as it is not visibly obvious who suffers from it or is affected by it and often times, ironically are not heard. This is well articulated by Olusanya and Newton as they demonstrate the oversight of the burden of disease of congenital HL in comparison to other ailments in the DALYs report (Olusanya and Newton, 2007). In the developing world, the burden of HL is felt more heavily due to its concurrence with unfavourable economic circumstances (Olusanya and Newton, 2007). This, coupled with stigma and isolation of both the patient and their family, contributes to a decreased quality of life. Patients have decreased access to education and ultimately employment and end up being a burden to the state for much of the duration of their life (Ruben, 2000; Schroeder, 2006). Early detection through rapid diagnostics is one way to try and mitigate these negative consequences. It allows for families and communities to be better prepared for the presense of hearing impaired individuals. In developed countries, screening programmes for newborns greatly assist in the management of HL and sees a lesser lifelong burden (Morton and Nance, 2006). This has partially been made possible by knowledge on the genetic causes of HL specific to their respective population groups (Atik *et al*., 2015). The extensive investigation of causative genes and their variants has allowed for the development of various HL panels such as OtoSCOPE® (Shearer *et al*., 2010), MiamiOtoGenes (Tekin *et al*., 2016) and OtoSeq (Shahzad *et al*., 2013). Outside of these vast panels, researchers have also been able to narrow down their screening to few a specific HL genes and variants known to be more prevalent in their respective population groups. The refinement of the number of genes and variants screened from those available on the above mentioned panels saves costs and analysis time. This makes the tools more easily accessible, thus increasing their feasibility in being used in a routine clinical setting. Such advancement is a result of continuous research spanning decades within predominantly Caucasian and Asian population groups which lead to the most common genes and variants being uncovered. In order to work towards the alleviation of the burden of HL, particularly in the most affected and vulnerable developing regions, there needs to be a continuous effort towards the discovery, building and refining of the genetic profile of HL genes across the greater sub-Saharan region, home predominantly to patients of African descent. Knowing that

this population group is vastly diverse (Lambert and Tishkoff, 2009), it is unlikely that one gene panel would suffice, but rather region specific panels would ultimately be the most useful. A strategy that could see improvement in diagnosis capacity, early intervention and ultimately of the improvement in basic healthcare services across the regions. However, we see that even in a relatively more developed country such as South Africa, the implementation of screening, be it at birth or during school going years is still far from where it needs to be (Petrocchi-Bartal and Khoza-Shangase, 2016). This could mean there are potentially far more cases of HL that go by unreported and undetected until much later in the affected child's life in South Africa (Gardiner *et al*., 2018). There is terefore an urgent needs to provide early screening and detection in SSA, including the incorporation of genetic screening that would also prepare families and potentialy alleviating anxiety and increase quality of life (Gardiner *et al*., 2018).

Most prevalent hearing loss genes and variants are absent in sub-Saharan Africans

The most prevalently reported and associated HL genes are connexin genes *GJB2, and GJB6*. These genes have been extensively screened across various populations and the causative variants identified have shown to be region and populations specific (Rodriguez-Paris and Schrijver, 2009; Chan and Chang, 2014). An example of such findings is with c.35delG of *GJB2* and del(GJB6-D13S1830) of *GJB6* that are able to resolve HL in up to 50% of HL cases among patients of European descent (Chan and Chang, 2014). Additionally, the founder variants c.235delC and c.167delT within GJB2 have been identified among patients in East Asia and among patients of Ashkenazi Jews ancestry respectively (Dzhemileva *et al*., 2010). However, exploration of these genes in 100 patients from South Africa and Cameroon showed them to not be significant (Bosch et al., 2014a, 2014b). These findings were also demonstrated in other regions of SSA (Samanich *et al*., 2007; Shan *et al*., 2010; Trotta *et al*., 2011; Javidnia *et al*., 2014; Lasisi *et al*., 2014); the present thesis has contributed to support this (Lebeko *et al*, 2015). This is with the exception of a population group in Ghana which revealed a *GJB2* founder variant have been found to be prevalent (Hamelmann *et al*., 2001). With such a rate of high genetic variation in Africans, and high genetic heterogeneity of HL, a wider comprehensive approach is more useful when trying to uncover genes that will be useful in clinical screening (Lebeko *et al*, 2015).

Next Generation Sequencing Technologies in Hearing loss

The NGS approach has been used to explore different diseases, both Mendelian and complex. Given that the most prevalent mode of inheritance for HL is autosomal recessive, identifying variants contributing to HL through NGS technology is relatively easy as one expects to detect a homozygous or a compound trans heterozygous change in both copies of the gene that alters or abolishes protein functionality. Initially, the recruitment of consanguine families from North Africa through to the Middle East presenting with ARNSHL allowed for the elucidation of several genes which have subsequently been investigated in other populations around the world (Hilgert *et al*., 2009). The high homozygosity between family members helped to narrow down regions where variants were co-segregated with HL. However, the occurrence of consanguine families is not common in many other parts of the world and thus homozygosity mapping and candidate gene screening is not always ideal (Ropers, 2007). Within the African setting, making use of NGS to explore both known and unknown genes is the most ideal approach as there already exists a plethora of associated genes. This approach also ramifies the process and will help cover as many regions in a short space of time as it is very likely that there will be several candidate genes within smaller regions and even more within the entire sub-Saharan African population group (Atik *et al*., 2015; Rudman *et al*., 2017). As mentioned before NGS technologies have varying degrees of coverage. From whole genome sequencing, where the entire human genome of 3.5 billion bases are sequenced to WES where only about 1% of the genome is captured to TGE where a selected number of genes are investigated. Each of these approaches have their own pros and cons.

Whole genome sequencing would allow for the interrogation of the entire human genome. This would most certainly resolve the genetic cause of HL in “family 9” which remains unresolved. As straight forward as this sounds, there are still a few challenges which need to be overcome before this approach can be implemented or even become the bread and butter of genetics research and personal medical care. Currently the cost of sequencing the entire human genome at a sufficient depth of coverage for accurate diagnosis is still quite high compared to WES. There is also no consensus on the format for the storage of this genetic data and the bioinformatics training required to analyse these large data sets is still lagging, especially on the African

continent. Currently no study has attempted to sequence the entire human genome in the investigation of genetics causes of HL. In the meantime, other NGS approaches such as WES and TGE are readily available as stepping stones to grow and perfect the exploration of the human genome in diagnostics and personal medical care in all diseases.

Targeted Enrichment Panels in Hearing loss

Realising that HL genetic spectrums are probably population and regions specific, several research groups have begun the quest of creating diagnostic panels for HL relevant to their population groups. The criteria behind which genes are included on these panels and which ones are left out are different for each panel. Nonetheless, all the panels are following the principle of targeted gene enrichment of known HL genes in order to elucidate the causative variant of patients presenting with HL. More resources are spent on getting high coverage of the selected genes thus increasing the accuracy of the reads and results. Analysis is also greatly simplified as the genes and their function are mostly known

Several HL panels are currently used in clinical practice, such as OtoSCOPE® (Shearer *et al*., 2010, 2013; Moteki *et al*., 2016; Sloan-Heggen *et al*., 2016) , MiamiOtoGenes (Tekin *et al*., 2016; Yan *et al*., 2016), and OtoSeq (Shahzad *et al*., 2013; Sivakumaran *et al*., 2013). These panels have a varying number of genes on the panels with the latest released panel (MiamiOtoGenes) having 180 genes (Yan *et al*., 2016). A larger custom panel included both mice and human genes which brought the gene number up to 246 genes (Brownstein *et al*., 2011). The resolution rates across these panels varies across the populations on which they are used, with the middle eastern populations consistently displaying the highest detection rates and the African populations, showing the lowest (Sloan-Heggen *et al*., 2016; Yan *et al*., 2016).

The drawback of the panels could be seen when one looks at the selection criteria of the genes themselves. Are they comprehensive in that every gene reported to have an associated with HL gene is included? Do they include only selected genes which have been validated by functional studies, strengthening their association? Are the selected genes filtered by ethnicity i.e. the population group in which they were discovered or for which the panel is designed for? MiamiOtoGenes as well as

OtoSCOPE seem to aim to cater for as many ethnic groups as possible, with OtoSeq seemingly targeting populations within the Mediterranean region, seen by the 24 genes selected for the panel (Shahzad *et al*., 2013). At the time of selecting a suitable panel to use to explore ARNSHL in sub-Saharan Africa, these questions were considered, and OtoSCOPE was selected for our study. It was the most comprehensive panel at the time and not limiting in the genes included based on the ethnicity of the population in which the gene was identified, and MiamiOtoGenes was not available. We selected 10 multiplex families from Cameroon presenting with ARNSHL and utilized OtoSCOPE (v4) to elucidate the causative variants in 116 known HL genes. Subsequent versions of the panel have seen more genes being added with the latest available version screening 152 genes. The panel considers both syndromic and nonsyndromic presentations of HL and investigates genes which are associated with both. Through OtoSCOPE, we were able to resolve 7 families, with 1 of the 10 failing to enrich adequately. We identified 12 different variants in 6 different genes. We also saw 5 novel variants and found that most of the causative variants were found to be contributing to the disease as compound heterozygous (Lebeko *et al*., 2016). We demonstrated the utility of a comprehensive panel such as OtoSCOPE, and the potential it has to elucidate patients from SSA. The variants identified in our study were subsequently screened in our cohort of 80 singleplex cases and were found to not be significant in the cohort and unable to resolve any of the cases. With the exception of 2 previously reported variants identified in family 5 and 7, the other variants were not detected in the controls nor the singleplex cases indicating that they might be rare variants in the population. We do however recognize that our data was limited by the small sample size of families segregating HL, as well as the number of isolated cases investigated (Lebeko *et al*, 2016). Nevertheless, the study illustrated the potential of discovering a high proportion of novel mutations in known genes and as well as novel genes in population of African ancestry.

Other studies have explored patients of African descent, particularly in the diaspora and the ability of these panels to resolve these cases. The findings show a much lower detection rate than in our cohor. This could be due to the difference in the inclusion criteria as we focused only on familial cases, with highest likelihood of genetic aetiology (Lebeko *et al*, 2016). African Americans and admixed populations from Brazil

and the Caribbean islands show the lowest resolution and detection rates across platforms and panels where multi-ethnic patients were screened using the same panel. The rates vary from 4% using MiamiOtoGenes (Yan *et al*., 2016) to 26% using OtoSCOPE (Sloan-Heggen *et al*., 2016). It is worth noting that the same filtering and annotation pipelines were used for all the populations in the above-mentioned studies respectively. This has an influence on the accuracy of the annotation, particularly when considering minor allele frequency. This was demonstrated by Shearer *et al,* that ethnicity has a significant influence on the detection but moreover the MAF thresholds used (Shearer *et al*., 2014b). This consideration will need to be applied to data from patients of African descent should they make use of these technologies in order to get the most accurate results on which variants are likely causative. The results of the two above mentioned panels with regards to resolution of African patients with HL, along with the presence of two unresolved families in our study (Lebeko *et al*, 2016) are strong evidence to advocate for the use of whole exome sequencing when seeking to elucidate causative variants for patients of African descent.

Whole Exome Sequencing in Hearing loss

The human genome has about 3,5 billion base pairs. Of these, only 1% are known to be part of the exome; that is exonic regions of genes which code for regions that make up protein products. With Mendelian disorders, it's most likely an exonic variant that will be the causative variant leading to an altered or absent protein. When using WES in selected 4 families, 2 resolved by targeted exome sequencing (OtoSCOPE) and two that remained unresolved after OtoSCOPE, and we were able to find a deleterious variants in three out of the four families, replicating the result obtained from the targeted panel (Lebeko *et al*, 2016), and discovering a novel c.251delC homozygous variant GRXCR2, *GRXCR2,* a novel HL genes only reported one in a family from Pakistan (Imtiaz *et al*., 2014). One of the families remained unresolved and could indicate the presence of a causative variant in a part of the genome not captured by the WES. To our knowledge this is the this is the first study that use of WES to resolve HL in SSA.

When looking at studies that have utilised WES to resolve HL across the world, we noticed that there is a higher resolution rate than when using TGE. The rate of resolution when analysing large number of samples range from 43% (Choi *et al*., 2013)

to 56% (Bademci *et al*., 2016). Moreover, when we investigated the rate of gene discovery within the studies that have used WES or NGS since 2010, there has been a great increase in the discovery of new HL genes and variants (Atik *et al*., 2015). Interestingly, we were not able to find papers where WES was used on Caucasian patients. Perhaps this is due to observed genetic saturation in that this population group in which the genes have been discovered have no more diversity to offer thus we will constantly find the same genes being implicated in HL. This is an additional suggestion that novel genes and variants are likely to be found in the largely understudied populations of African ancestry.

The rate of resolution when using WES is much higher than TGE but does not come close to completion. This shows that there is still a lot that needs to be done in improving WES platforms from capturing, to variant calling, filtering and sorting. Improvement of these steps within WES could see an increase in resolution rates across all diseases.

GRXCR2 in Hearing Loss

Herein, we report a second variant within *GRXCR2* that cases ARNSHL in humans with the first being found in a Pakistani family (Imtiaz *et al*., 2014). Furthermore, this novel variant was found to be harboured by another patient within our cohort. Exploration of databases has shown one individual of African American descent having one copy of this variant, showing that it could potentially be present in some patients in the diaspora. This gene has been shown to contribute to HL in a mouse model (Avenarius, 2012). These are the only reported consequences of variants in this gene. The protein is believed to be involved in the maturation and organization of stereocillia bundles of the inner ear hair cells (Avenarius, 2012). The exact mechanism is yet to be elucidated. Given the genetic heterogeneity of HI, and the different roles that already implicated genes and their proteins play, it is worth exploring the kind of interactions these genes and proteins have with one another. This exploration of interactions between genes and their proteins was a task we undertook *in silico* (Lebeko *et al*., 2017). When studying the biological processes these known genes are involved in more than 60% is attributed to binding and catalytic activity (Lebeko *et al*.,

2017). This gives insight into the intricate and unique roles each gene has in the development and maintenance of normal hearing in humans. GRXCR2 has three distinct protein binding domains and is postulated to function in a complex or as homo or heteromers (Avenarius, 2012). The exact proteins to which GRXCR2 binds have yet to be elucidated (Lebeko *et al*., 2017). Given the morphology of hair cells in the absence of GRXCR2, it might be an important chaperone protein that does not itself make up the bundles but perhaps deliver to the structural components needed to mature and subsequently maintain the integrity of the stereocillia post-partum when mechanical stimuli is introduced (Avenarius, 2012). Nonetheless, there is enough evidence to show the importance of this gene and its protein in the development and maintenance of normal hearing. Our exploration of the entire coding region of *GRXCR2* did not yield other novel variants which could be contributing to HL in our cohort. However, this might not be the case for other African populations affected by HL.

Building an African-specific hearing loss Genetic profile

To date, we have resolved 8/9 families presenting with ARNSHL. Of the 13 variants we identified in our families (Lebeko *et al*, 2016), we were only able to resolve one other case from our cohort of 80 singleplexes. The studies indicate the need to recruit larger number of families in different regions of the African continent, and to use WES to truly capture the nature of the genes and mutations involved in HL in people of African ancestry. As more families and patients are recruited, we are able to continue the exploration of the most prevalently reported genes. Even though these connexin genes are unlikely candidate genes for diagnostics of HL in SSA as already discussed, there remains the possibility that there might be other founder variants harboured by smaller regions within the population. This allows for the expansion of the frontier of new knowledge of HL genes while simultaneously testing and/or fortifying the current knowledge. This is one benefit of WES. Another benefit is the ability to retrieve data and reanalyse it using different pipelines as well as using the data in other studies not related to HL. Indeed, WES databases will benefit from the diversification of data with the inclusion of data from a diverse group such as African patients. In building and expanding a genetic profile of HL for SSA, there is no doubt that pooled resources

would increase the rate at which new regions are covered. The H3Africa initiative is a great example of efforts on the continent to increase capacity but also centralize and share resources. Such initiatives will see the actualization on biorepositories for different regions allowing for retrieval of data as suggested above. Another consideration on the road to building an accurate genetic profile is the development of a genetic testing algorithm specific to SSA patients with HL. This is especially important as we have seen how different considerations need to be made according to ethnic groups when calling variants (Shearer *et al*., 2014b).

Policy and Counselling services implications

The ultimate goal for these research efforts is to allow for the implementation of early detection and screening services. It would be duly beneficial to tackle the HL burden in Africa from both environmental and genetic ends. The development of infrastructure and an increase in resources should consider the need to accommodate genetic screening and testing through the information which will be readily and increasingly available as research efforts continue. This includes pre- and postnatal genetic screening. For this to become a reality, there needs to be an intention to increase training with regards to genetic counsellors on the continent (Gardiner *et al*, 2018). They are an intricate part of the service provided in helping patients and their families understand the intention and the limitations of the screening services. In a region where communicable diseases and other ailments often take precedent, this might not be an easy task, but one that needs to be advocated for. Genetic counsellors play an essential role, and with the increase in availability of genetic testing and information, one cannot imagine an improved and reformed health care system across the continent and not envision their services. This is a service that is already relevant in other more commonly known genetic diseases such as down syndrome and sickle cell disease. The benefit is alleviation of burden of various diseases through counselling and education which ultimately leads to increased quality of life (Abou Tayoun *et al*., 2016).

The availability or provision for prenatal screening might not be warranted as HL is not life threatening, but the availability of the service within the first 12 months of life of the infant are crucial as they allow for intervention before the development of speech. This allows for various options which might restore hearing and allow for speech

development in some cases where hearing aids can be employed (progressive hearing loss) or cochlear implants can be provided for those who would benefit from them (Eppsteiner *et al*., 2012).

Conclusions

In this thesis we have contributed to show that variants in *GJB2*, *GJB6* and *GJA1* are not significant causes of ARNSHL inpatients from African descents. We have subsequently demonstrated the efficacy and accuracy of using both targeted exome panel sequencing and WES in clinical setting to resolve HL in a multiplex family from Cameroon. Additionally, we have shown that WES is able to detect variants in known HL genes and were able to replicate results uncovered through TGE on the OtoSCOPE panel. This further strengthens the advocacy of using WES in further exploring genetic causes of HL in African patients instead of using available TGE panels. *In silico* tools as well as functional studies demonstrated the pathogenicity of our novel c.251delC variant harboured in *GRXCR2*, as well as provided some insight to the possible role of the protein in the stereocilia maturation pathway. Identification of the variant in a second patient strengthens the association of the variant and HL as well as points towards a possible founder effect which needs to be further investigated. Overall therefore, the data strongly supports that genetic studies on families segregating HL in sub-Saharan Africa could be the next frontier of HL genetic research. This region could prove to be of global importance through the discovery of novel variants and genes, and of local relevance by improving HL genetic diagnosis, retrospective counselling and testing, as well as prevention and care. This will positively impact the future prediction of treatment outcomes in sub-Saharan Africans and in people of African descent globally.

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Appendix

Table 4.A1: Primers used to amplify coding region of GRXCR2

Primer name	Primer sequence (5' – 3')	Product size (bp)
GRXCR2-Exon 1 Forward	AAC TTC CCA TTT GGT GCA CC	631
GRXCR2-Exon 1 Reverse	TGT ATG GGT GTC AGT TGC TG	
GRXCR2-Exon 2 Forward	TTC TCT GCT GTT TCC TGG TAT G	381
GRXCR2-Exon 2 Reverse	CTT GGA GAC CAT TGC TGT AGG	
GRXCR2-Exon 3 Forward	CAG CCT TTG CTG TAG TCT AGT	328
GRXCR2-Exon 3 Reverse	AGA AAT AAC TTT AGG GAG GGT AAG A	

SDM PCR reaction set up and Parameters

Table 4.A2: SDM PCR reaction Mix set up

Component	50ul reaction volumes	Final concerntration
Nuclease free H_2O	Up to 50uL	N/A
2X KAPA HiFi HotSart ReadyMix	25uL	1X
10uM Forward primer	1.5 ul	0.3uM
10uM Reverse Primer	1.5ul	0.3uM
pCMV6-Entry-GRXCR2	1ul	20ng

Table 4.A3.: PCR Cycling Parameters for SDM

Step	Temperature (°C)	Duration	Cycles
Initial Denaturation	95	3 min	1
Denaturation	98	20 sec	15
Annealing	56	15 sec	
Extension	72	8 min	
Final Extension	72	8 min	1

Vectors

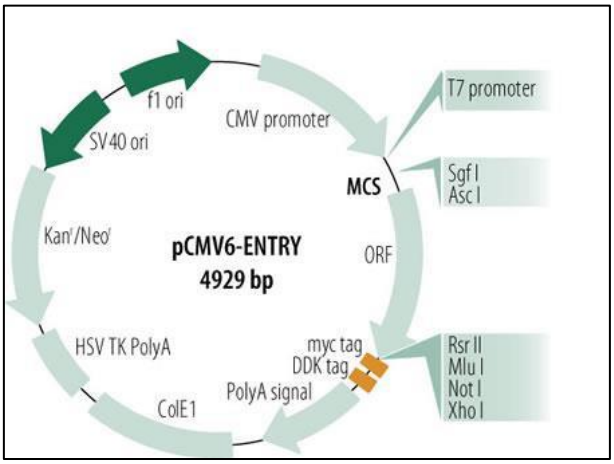

Figure 4.A1: pCMV6-Entry Vector with GRXCR2 ORF purchased from Origene

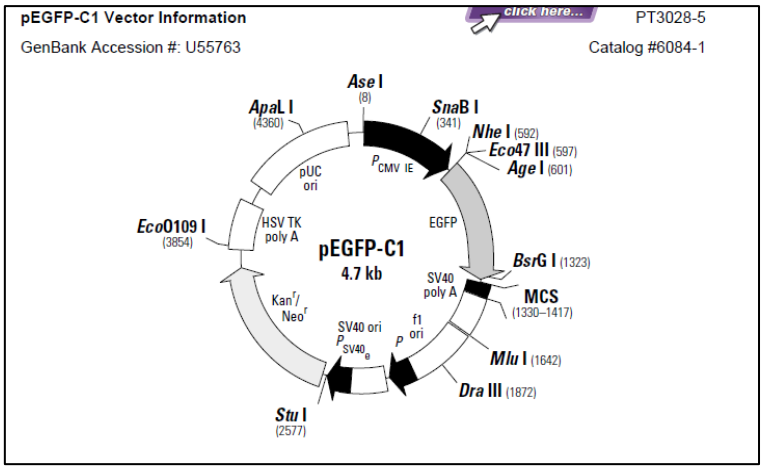

Figure 4.A2: pEGFP Vector Map: GFP is upstream of MCS

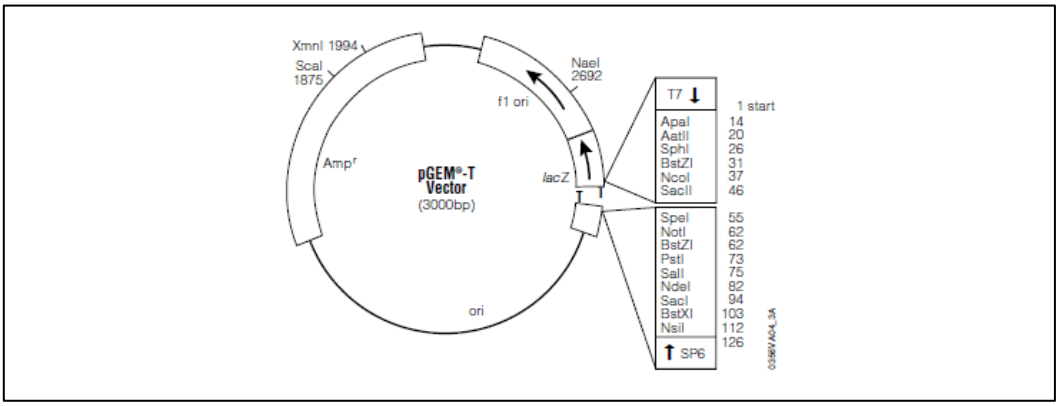

Figure 4.A3: pGEM-T Vector- T-overhang allows for easy cloning of PCR products

B

Buffer compositions

Boiling blue buffer (10ml)

Component	Volume (ml)
1.5 M Tris-HCL (pH 6.8)	0.83
20% SDS	2
B-mecarptoethanol	1
Glycerol	2
H_2O	4.166
Coomassie Brilliant Blue R-250	A pinch amount

PBS (10X)

Components	Volume/Amount
NaCl	80g
KCl	2g
Na_2HPO_4 (anhydrous)	14.4g
KH_2PO_4(anhydrous)	2.4g
H_20	Add 800ml and mix thoroughly and fill up to 1L

Use 1:10 dilution with H_2O to get 1X

PBS-TWEEN

- Dissolved 1ml Tween 20 in 1L 1X PBS

10% Ammonium Persulfate (100ml)

- Dissolve 1g ammonium persulphate (APS) in 8 ml H_2O and adjust volume to 100ml

30% acryl-bisacrylamide mix (100ml)

- 29g acrylamide
- 1g N,N'-methylenebisacrylamide
- 60 ml H_2O

C

- Heat at 37°C to dissolve the chemicals
- Adjust volume to 100ml with H_2O

12 % Resolving gel (7.5ml)

Component	Volume (ml)
H2O	2.4
30% acryl-bisacrylamide mix	3
1.5M Tris (pH 8.8)	1.95
10% SDS	0.075
10% ammonium persulfate	0.075
TEMED	0.003

5% Stacking gel (2ml)

Components	Volume (ml)
H_2O	1.4
30% acryl-bisacrylamide mix	0.33
1.5M Tris (pH 6.8)	0.25
10% SDS	0.02
10% ammonium persulfate	0.02
TEMED	0.002

Running buffer (10X)

Components	Volume/Amount
SDS	10g
Tris-base (Trizma)	30g
Glycine	144g
H_2O	Add 800ml to mix thoroughly then fill up to 1L

Dilute 1:10 with H_2O for 1X

Transfer Buffer (10X)

Components	Volume/amount
Tris	38g
Glycine	144g
H_2O	Add up to 1L

To make 1X:

10X Transfer buffer	100ml
Isopropanol	200ml
H_2O	700ml

Blocking solution

- 2.5g dried milk in 50ml PBS

Ponceau S Stain Solution (100ml)

- 2g Ponceau S
- 30g trichloracetic acid
- 30g sulfosalicylic acid
- Dissolve in 80 ml H_2O

Adjust volume to 100ml with H_2O

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