Sequencing of *GJB2* in Cameroonians and Black South Africans and comparison to 1000 Genomes Project Data Support Need to Revise Strategy for Discovery of Nonsyndromic Deafness Genes in Africans

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Abstract

Mutations in the *GJB2* gene, encoding connexin 26, could account for 50% of congenital, nonsyndromic, recessive deafness cases in some Caucasian/Asian populations. There is a scarcity of published data in sub-Saharan Africans. We Sanger sequenced the coding region of the *GJB2* gene in 205 Cameroonian and Xhosa South Africans with congenital, nonsyndromic deafness; and performed bioinformatic analysis of variations in the *GJB2* gene, incorporating data from the 1000 Genomes Project. Amongst Cameroonian patients, 26.1% were familial. The majority of patients (70%) suffered from sensorineural hearing loss. Ten *GJB2* genetic variants were detected by sequencing. A previously reported pathogenic mutation, g.3741_3743delTTC (p.F142del), and a putative pathogenic mutation, g.3816G>A (p.V167M), were identified in single heterozygous samples. Amongst eight the remaining variants, two novel variants, g.3318-41G>A and g.3332G>A, were reported. There were no statistically significant differences in allele frequencies between cases and controls. Principal Components Analyses differentiated between Africans, Asians, and Europeans, but only explained 40% of the variation. The present study is the first to compare African *GJB2* sequences with the data from the 1000 Genomes Project and have revealed the low variation between population groups. This finding has emphasized the hypothesis that the prevalence of mutations in *GJB2* in nonsyndromic deafness amongst European and Asian populations is due to founder effects arising after these individuals migrated out of Africa, and not to a putative “protective” variant in the genomic structure of *GJB2* in Africans. Our results confirm that mutations in *GJB2* are not associated with nonsyndromic deafness in Africans.

Introduction

DEAFNESS IS A GLOBAL PROBLEM that is most serious in the developing world, with 7 per 1000 children in Nigeria born with deafness (Olusanya and Somefun, 2009) and 5.5 per 1000 live births in South Africa (Swanepoel et al., 2009). These incidences are about five times higher than observed in the United States and Europe (Mehra et al., 2009; Parving, 1999).

Although deafness is a highly heterogeneous condition, it has been found that mutations in the Gap Junction Beta 2 gene (*GJB2*), encoding connexin 26, are responsible for up to 50% of cases in populations of European descent (Rabionet

et al., 2000). The major mutations in *GJB2* have been seen to be population specific, resulting from founder effects. These include c.35delG affecting Caucasians (Van Laer et al., 2001), c.167delT affecting those of Ashkenazi Jewish ancestry (Morell et al., 1998), and c.235delC affecting East Asians (Yan et al., 2003). In contrast, the few studies available suggest that *GJB2* is not a major contributor to deafness in some Africans (Gasmelseed et al., 2004) or African Americans (Rabionet et al., 2000). The aim of this study was to ascertain the significance of mutations in *GJB2* in a selected group of Cameroonian and South African Black patients with nonsyndromic deafness.

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Materials and Methods

Ethics approval statement

The study was approved by the Cameroon National Ethics Committee (ethics approval N°123/CNE/SE/2010) and the Human Research Ethics Committee of the University of Cape Town (ethics approval HREC REF: 080/2011). Written and signed informed consent was obtained from all participants aged 18 years old or more, or from the parents/guardians with verbal assent from the children.

Settings, patients, and controls

Cameroonian patients were recruited from seven of the ten regions of Cameroon, mainly from schools for the deaf, and those procedures 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.

During recruitment, information on participants' medical and family history was obtained from the participants themselves, their parents, and medical records, depending on which sources were available. In the majority of cases, general systemic and otological examination was performed, as well as an audiological evaluation using either pure tone audiometry or auditory brain stem response test. Audiological test results that were obtained before admission to schools for the deaf were also reviewed for some subjects. When syndromic deafness was suspected, additional tests, when possible, were later performed to confirm or exclude the diagnosis.

We included (1) patients of Black African descent with nonsyndromic hearing loss, as confirmed by a clinical or audiological report, with deafness of either (2) putative genetic origin, as revealed by one or more affected family members or consanguinity, or (3) unknown origin, and who consented to participate in the study. We excluded patients with syndromic hearing loss 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.

Following the above criteria, a total of 180 Cameroonian patients from a large cohort previously reported (Wonkam et al., 2013) and 25 South African patients were selected.

Normal hearing individuals from the same population background as patients were recruited: 17 South African and 64 Cameroonian controls.

DNA amplification and mutation analysis

Genomic DNA samples were extracted from peripheral blood of the patients, following instructions on the available commercial kit [Puregene blood Kit® (Qiagen, Alameda, CA)], at the Molecular Diagnosis Laboratory of the Gyneco-Obstetric and Paediatric Hospital of Yaoundé, Cameroon. While at the Division of Human Genetics, University of Cape Town, a modified version of the salting out method was used to extract DNA from peripheral blood specimen of South African patients, or DNA was purified from saliva (Oragene® kit; DNA Genotek®, USA) according to the manufacturer's instructions. Primers for the *GJB2* genes were validated using BLAST® and primer analysis software. The

complete coding region of exon 2 was amplified and then sequenced using an ABI 3130XL Genetic Analyzer® (Applied Biosystems, Foster City, CA), in the Molecular Research Laboratory in the Division of Human Genetics, University of Cape Town, South Africa.

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 *GJB2* reference sequence (Ensembl transcript ENST00000382848 (*GJB2*-001), retrieved 31 August 2012). Detected variations were checked against dbSNP, and the effects of non-synonymous mutations were predicted using Polyphen-2. SHEsis (<http://analysis2.bio-x.cn/>) was used to examine statistical differences in allele, genotype, and haplotype frequencies between the cases and controls, as well as linkage analysis. The Chi-square test and the Fisher's exact test were used to compare SHEsis results, and a *p* value ≤ 0.05 was considered statistically significant. Data for the sequenced region of *GJB2* was downloaded from the 1000 Genomes browser (<http://browser.1000genomes.org>) for all available populations. A phylogeny was constructed with PopTree software using the Neighbour Joining algorithm, Nei's DA genetic distance, and 1000 bootstraps. Principle Components Analysis (PCA) was performed in R (R Core Team 2013) using the FactorMineR Package and the same 1000 Genomes Project data. As the 1000 Genomes Project data is from apparently healthy individuals, only control data from Cameroon and South Africa were used for comparisons.

Results

Socio-demographics

Amongst the Cameroonian participants, 47 were familial cases (26.1%); our analysis includes two cases of two affected siblings, two cases of three affected siblings, and one case of four affected siblings. Consanguinity was not reported in the South African cohort, but 10 (5.6%) Cameroonian patients were from known consanguineous unions. Admixture with other non-African population groups was highly unlikely in all participants.

Clinical information

Audiological information was only available for patients from Cameroon (Table 1). The majority of patients (70%) suffered from sensorineural hearing loss and a small number (4.4%) suffered from mixed hearing loss.

Variations/pathogenic mutation analysis

Two pathogenic or probably pathogenic mutations were detected in two unrelated Cameroonian participants, g.3741_3743delTTC (p.F142del) and g.3816G > A (p.V167M). Both were detected in only a single individual in the heterozygous state (Table 2). There were no pathogenic mutations detected in South African patients. We also detected two novel variants, g.3318-41G > A and g.3332G > A, both in the heterozygous form that was likely non-pathogenic. The g.3318-41G > A change occurs in the first intron of *GJB2* and the g.3332G > A change leads to a synonymous mutation. The most common variants in both the South African and

TABLE 1. SEVERITY OF HEARING LOSS IN THE CAMEROONIAN COHORT

Level of Deafness ^a	Left Ear (Freq.)	Right Ear (Freq.)
Severe 1 (71–80)	6 (0.03)	7 (0.04)
Severe 2 (81–90)	19 (0.11)	20 (0.11)
Profound 1 (91–100)	53 (0.29)	54 (0.30)
Profound 2 (101–110)	49 (0.27)	46 (0.26)
Profound 3 (111–119)	17 (0.09)	22 (0.12)
Total (120)	7 (0.04)	2 (0.01)
Unknown	29 (0.16)	29 (0.16)
	N = 180	N = 180

^aThe level of deafness is given according to BIAP classification; <http://www.biap.org/en/recommendations/65-ct-2-classification-des-surdites/5-recommandation-biap-021-bis>.

Cameroonian cohorts were the intronic change g.3318-34C>T and two changes in the 5'UTR, g.3318-15C>T and g.3318-6T>A.

Phylogenetics and principal components analysis (PCA)

The phylogeny (Fig. 1) shows the populations from the 1000 Genomes Project clustering into Asians, Africans, and

Europeans with the admixed populations positioned closer to the base of the tree. As expected, the South African and Cameroonian controls grouped with the other African populations. The PCA explains 40% of the variation between population groups (Fig. 2) and different populations are characterised by different SNPs.

Discussion

This comprehensive report on the significance of *GJB2* mutations in deaf African populations (Table 1) offers a substantial contribution to the literature on the topic by examining previously unstudied Xhosa deaf patients in South Africa, expanding the studied population from Cameroon, pulling together the results of previous studies in Africa, and comparing it all to data extracted from the 1000 Genomes Project. The inclusion of a carefully selected group of Cameroonian patients is of major importance. Indeed, Cameroon is frequently referred to as “Africa in miniature,” because of its central location on the continent, its many geographical and cultural attributes, and the diversity of its population (there are more than 200 distinct local languages in the country). The country spans two main geographical zones of almost equal size: the equatorial rain forest in the south and the tropical savannah and the Sahel region in the

TABLE 2. COMPARISON OF RESULTS BETWEEN SELECTED STUDIES OF *GJB2* IN AFRICAN POPULATIONS

Variations			Country (observed/total alleles)				
Genomic	Coding	Pathogenicity	Cameroon	Ghana ^{&}	Kenya/ Sudan [§]	South Africa	African American
g.3318-41G>A	c.-41G>A	Polymorphism	1/360*				
g.3318-35T>G	c.-35T>G	Polymorphism			1/1178		
g.3318-34C>T	c.-34C>T	Polymorphism	100/360*		85/1178	19/50*, 119/364 [@]	
g.3318-15C>T	c.-15C>T	Polymorphism	15/360*		38/1178	7/50*, 56/364 [@]	1/46 ⁺ , NA ⁺
g.3318-6T>A	c.-6T>A	Polymorphism	4/360*		2/1178	1/50*	
g.3332G>A	c.15G>A	Polymorphism	1/360*				
g.3352_3353insG	c.35dupG	Pathogenic		1/730			
g.3352delG	c.35delG	Pathogenic			10/1178		7/100~
g.3395C>T	c.78C>T	Polymorphism			1/1178		
g.3396C>T	c.79G>A	Pathogenic					2/46 ⁺ , NA ⁺
g.3419T>C	c.101T>C	Pathogenic					NA ⁺
g.3426G>A	c.109G>A	Pathogenic			1/1178		
g.3455_3460del	c.138_143del	Pathogenic			1/1178		
g.3503C>T	c.186C>T	Polymorphism	NA*, 2/122 [#]		3/1178		
g.3512C>A	c.195C>A	Pathogenic			1/1178		
g.3542G>T	c.225G>T	Polymorphism	1/360*				
g.3553T>C	c.236T>C	Pathogenic		1/730			
g.3566C>G	c.249C>G	Pathogenic					1/100~
g.3586_3587insT	c.269_270insT	Pathogenic	NA [#]				
g.3627A>C	c.310A>C	Polymorphism			1/1178		
g.3658A>G	c.341A>G	Pathogenic					NA ⁺
g.3697G>A	c.380G>A	Pathogenic			1/1178		
g.3741_3743delTTC	c.424_426delTTC	Pathogenic	1/360*				
g.3744C>T	c.427C>T	Pathogenic		110/730			1/100~
g.3774G>A	c.457G>A	Polymorphism			2/1178	NA*	
g.3795G>A	c.478G>A	Pathogenic			1/1178		NA ⁺
g.3816G>A	c.499G>A	Pathogenic	1/360*		4/1178		NA ⁺
g.3850T>C	c.533T>C	Pathogenic		4/730			
g.3868G>A	c.551G>A	Pathogenic		1/730			
g.3906G>T	c.589G>T	Pathogenic		1/730			
g.3925-3926delinsAA	c.608_610delinsAA	Pathogenic		2/730			
g.3958C>T	c.641T>C	Pathogenic		1/730			

NA refers to variations that were found during the study but only in the control group. Variant information was obtained through the relevant article'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: *This study, [#]Trotta et al. (2011), [@]Kabahuma et al. (2011), [§]Gasmelseed et al. (2004), [&]Hamelmaan et al. (2001), ⁺Sammanich et al. (2007), [~]Pandya et al. (2003).

FIG. 1. Phylogeny constructed from 1000 Genomes and study control data. The phylogeny tree shows the various populations' clusters of the 1000 Genomes. As expected, the South African and Cameroonian controls grouped with the other African populations. Numbers indicate bootstrap values over 1000 iterations. Population abbreviations are: ASW, Americans of African Ancestry in SW USA; CAM_C, Cameroonian control; CEU, Utah Residents (CEPH); CHB, Han Chinese in Beijing; CHS, Southern Han Chinese; CLM, Colombians from Medellin Colombia; FIN, Finnish in Finland; GBR, British in England and Scotland; IBS, Iberian population in Spain; JPT, Japanese in Tokyo Japan; LWK, Luhya in Webuye Kenya; MXL, Mexican Ancestry from Los Angeles USA; PUR, Puerto Ricans from Puerto Rico; SA_C, South African control; TSI, Toscani in Italia; and YRI, Yoruba in Ibadan Nigeria.

north. At the genetic level, Cameroonian population diversity mimics that of various ethno-linguistic groups in African populations (Tishkoff et al., 2009) and it is anticipated that results from a carefully selected sample in this population could represent a snapshot of that of many African populations. Furthermore, we included patients from the Xhosa population of South Africa, a formerly unstudied offshoot of the Bantu population that migrated from areas around Cameroon (Tishkoff et al., 2009).

To our knowledge, the present study is the first to have conducted a comparison of African *GJB2* sequences with the data from the 1000 Genomes Project and revealed the variation between population groups. This unique finding has emphasized the hypothesis that the prevalence of mutations in *GJB2* in nonsyndromic deafness amongst European and Asian populations is due to founder effects arising after these individuals migrated out of Africa, and not to a putative "protective" variant in the genomic structure of *GJB2* in Africans. Indeed, the description of *de novo* p.Asp50Asn mutation, in two Cameroonian patients diagnosed with KID syndrome, also confirms that mutations in *GJB2* do occur in syndromic deafness in Africans (Wonkam et al., 2013), probably at a frequency that is comparable to that of other populations, since *de novo* p.Asp50Asn mutation is the most prevalent in KID syndrome globally (Mazereeuw-Hautier et al., 2007). The exception to this low prevalence of *GJB2* mutations in nonsyndromic deafness in African patients, is a specific mutation, p.R143W (c.427C>T), occurring at a high rate in the Ghanaian population from Adamarobe village (Hamelmann et al., 2001). This could also be attributed to a

founder effect as this mutation has not been reported in other African populations but has been reported only in few African Americans (Table 1), whose ancestors were probably brought from Ghana during the slave trade. Our results both build on the previously published studies of *GJB2* deafness in Africa while distinguishing itself through, to the best of our knowledge, the first comparison of *GJB2* sequences data from Cameroonian and South Africans with those extracted from the 1000 Genomes Project, that have allowed a global comparison and revealed low level of variance.

Similar to Africans, Caribbean Hispanics, probably due to their African ancestry, do not show a large contribution to deafness from *GJB2* variations (Samanich et al., 2007).

The mutation p.V167M has been also reported in three studies, only in patients of African ancestry. It has been detected in a heterozygous state in 4/406 (~1%) among Kenyans with prelingual, nonsyndromic hearing loss (Gasmelseed et al., 2004), and in 1/94 (~1%) among African American controls (Samanich et al., 2007). While it may be pathogenic, it has not been reported in a homozygous form and predictive tools give ambiguous results. Polyphen-2 predicts it as "possibly damaging with a score of 0.618 (sensitivity: 0.87; specificity: 0.91)" with HumDiv and "possibly damaging with a score of 0.537 (sensitivity: 0.82; specificity: 0.82)" with HumVar. The Polyphen-2 score shows the probability that a specific change will be pathogenic.

Three common nonpathogenic variants were detected in our cohort, all of which have previously been reported at high frequencies in African populations. These are the g.3318-34C>T, g.3318-15C>T, and g.3318-6T>A changes, which

FIG. 2. Principal component analysis (PCA) study and 1000 Genomes populations with respect to variation in *GJB2*. The PCA explains 40% of the variation between population groups and different populations are characterised by different SNPs. Population abbreviations are: ASW, Americans of African Ancestry in SW USA; CAM_C, Cameroonian control; CEU, Utah Residents (CEPH); CHB, Han Chinese in Beijing; CHS, Southern Han Chinese; CLM, Colombians from Medellin Colombia; FIN, Finnish in Finland; GBR, British in England and Scotland; IBS, Iberian population in Spain; JPT, Japanese in Tokyo Japan; LWK, Luhya in Webuye Kenya; MXL, Mexican Ancestry from Los Angeles USA; PUR, Puerto Ricans from Puerto Rico; SA_C, South African control; TSI, Toscani in Italy; and YRI, Yoruba in Ibadan Nigeria.

occurred in 60%, 28%, and 4% of South African patients, 47%, 8%, and 2% of Cameroonian patients, respectively. Our results are similar to those of Kabahuma et al. (2011) where g.3318-34C>T and g.3318-15C>T were the only mutations detected in 182 cases and 63 controls from the Limpopo province, South Africa. The authors described the g.3318-34C>T change in 46% and 42% of patients and controls, respectively, and the g.3318-15C>T change in 21% and 35% of patients and controls, respectively (Kabahuma et al., 2011). The same variants, including the g.3318-6T>A change, were also present in deaf individuals from both Kenya and Sudan (Gasmelseed et al., 2004); the g.3318-34C>T and g.3318-15C>T changes in 13% and 6% of patients, respectively. The g.3318-6T>A change was only found in 0.5% of Kenyan patients. These variants appear to be probably specific to African ancestry as PCA discloses these SNPs that appear to differentiate African populations from others included in the 1000 Genomes Project data.

From the present cohort shown not to have *GJB2* mutation, a subset of 100 patients affected with nonsyndromic hearing loss was analyzed by Sanger sequencing of the entire coding regions of *GJB6* and *GJA1* that have been implicated in hearing loss but have seldom been investigated in African populations. 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 (Wonkam et al., 2014). There were no statistically significant differences in sequence variants between patients and controls, confirming that mutations in *GJB6* and *GJA1* are not major causes of nonsyndromic deafness in this group of Africans from Cameroon and South Africa (Wonkam et al., 2014). The identification of the causal mutations in families with hearing loss, using classical single genes screening approaches, is difficult and expensive due to the heterogeneous nature of nonsyndromic hearing loss. Future research should then focus on high-throughput sequencing using platforms such as OtoSCOPE®, which combines targeted genomic enrichment and next generation sequencing to examine 66 deafness genes at once (Shearer et al., 2010), or to use whole exome sequencing, 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).

Conclusion

In conclusion, our results confirm that mutations in *GJB2* are not associated with nonsyndromic deafness in Africans and support the conclusion that investigation of *GJB2* is unnecessary in most African patients with nonsyndromic deafness.

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Author Disclosure Statement

The authors declare no conflict of interest.

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