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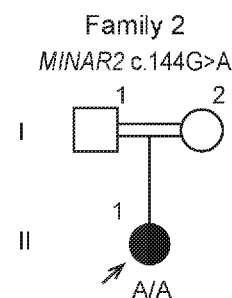
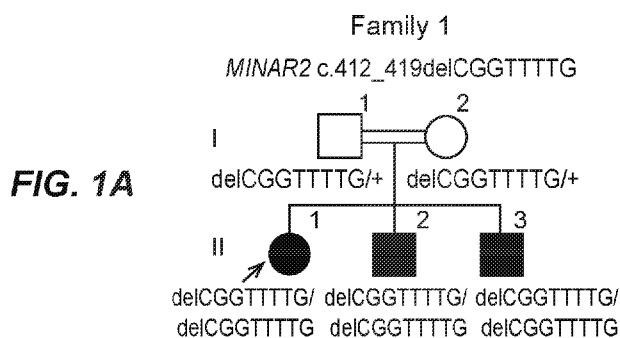
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(54) Title: TREATMENT OF SENSORINEURAL DEAFNESS



(57) Abstract: The present disclosure relates to a method of treating sensorineural deafness. A method of characterizing hearing loss in a human subject also is provided.

TREATMENT OF SENSORINEURAL DEAFNESS

GRANT FUNDING DISCLOSURE

[0001] This invention was made with government support under grant number DC009645 awarded by the National Institutes of Health (NIH). The government has certain rights in the invention.

CROSS REFERENCE TO RELATED APPLICATION AND INCORPORATION BY
REFERENCE OF MATERIAL SUBMITTED ELECTRONICALLY

[0002] This application claims priority to U.S. Provisional Patent Application No. 63/062,922, filed August 7, 2020, which is hereby incorporated by reference in its entirety.

[0003] Incorporated by reference in its entirety is a computer-readable nucleotide/amino acid sequence listing submitted concurrently herewith and identified as follows:

55776PC_Seqlisting.txt; Size: 20,678bytes; Created: August 6, 2021.

FIELD OF THE INVENTION

[0004] The present disclosure relates to methods of characterizing and treating sensorineural deafness.

BACKGROUND

[0005] Hearing loss is one of the most common sensory deficits, affecting 1 in 500 newborns globally (Mehl and Thomson 2002). Genetic factors are implicated in majority of cases, with more than 80% of the inherited form exhibiting autosomal recessive transmission (Morton and Nance 2006). No additional findings are present in over 70% of the cases, which are then classified as non-syndromic hearing loss (Morton and Nance 2006).

[0006] High genetic heterogeneity has made identification of gene and gene variants challenging, development in next-generation sequencing technologies has provided speed and accessibility required for analyzing a wide array of genes, to precisely locate the genomic cause of a disease. Identification and functional characterization of deafness-associated genes represent the most efficient way to enhance knowledge in the field of sensory physiology and hearing loss (Atik et al. 2015; Vona et al. 2015).

SUMMARY

[0007] The disclosure provides a method of treating sensorineural deafness in a mammalian subject (e.g., human) in need thereof. The method comprises administering to a subject having a mutation in a Membrane Integral NOTCH2 Associated Receptor gene (*MINAR2*) a composition that comprises a polynucleotide that encodes a *MINAR2* peptide, a *MINAR2* peptide, a small molecule that increases expression of *MINAR2*, an agent that blocks expression of a mutant *MINAR2* gene, an agent that corrects the mutation in the *MINAR2* gene. Administration of a combination of any of the foregoing is also contemplated. In various aspects, the method comprises detecting the presence of a mutation in the *MINAR2* gene in a sample from a subject. In various embodiments, the *MINAR2* mutation is a DNA variant classified as pathogenic or likely pathogenic according to American College of Medical Genetics and Genomics (ACMG) guidelines. The disclosure also contemplates a method comprising diagnosing the subject with sensorineural deafness when the presence of a mutation in the *MINAR2* gene is detected.

[0008] It should be understood that, while various embodiments in the specification are presented using "comprising" language, under various circumstances, a related embodiment may also be described using "consisting of" or "consisting essentially of" language. The disclosure contemplates embodiments described as "comprising" a feature to include embodiments which "consist of" or "consist essentially of" the feature. The term "a" or "an" refers to one or more. As such, the terms "a" (or "an"), "one or more," and "at least one" can be used interchangeably herein. The term "or" should be understood to encompass items in the alternative or together, unless context unambiguously requires otherwise.

[0009] All methods described herein can be performed in any suitable order unless otherwise indicated herein or otherwise clearly contradicted by context. The use of any and all examples, or exemplary language (e.g., "such as") provided herein, is intended merely to better illuminate the disclosure and does not pose a limitation on the scope of the disclosure unless otherwise claimed. Only such limitations which are described herein as critical to the invention should be viewed as such; variations of the invention lacking limitations which have not been described herein as critical are intended as aspects of the invention.

[0010] It is understood that each feature or embodiment, or combination, described herein is a non-limiting, illustrative example of any of the aspects of the disclosure and, as such, is meant to

be combinable with any other feature or embodiment, or combination, described herein. For example, where features are described with language such as “one embodiment,” “some embodiments,” “various embodiments,” “related embodiments,” each of these types of embodiments is a non-limiting example of a feature that is intended to be combined with any other feature, or combination of features, described herein without having to list every possible combination. Such features or combinations of features apply to any of the aspects of the invention.

[0011] The headings herein are for the convenience of the reader and not intended to be limiting. Additional aspects, embodiments, and variations of the invention will be apparent from the Detailed Description and/or drawings and/or claims.

BRIEF DESCRIPTION OF THE DRAWINGS

[0012] Figures 1A-1D: Families and the *MINAR2* variants. Fig. 1A: Pedigrees and segregation of the *MINAR2* variant in families. Filled symbols denote affected individuals and double lines indicate first cousin consanguinity. Fig. 1B: Electropherograms showing the identified variant. The wild type traces are from an unrelated individual. Hom: Homozygous mutant, Het: Heterozygous mutant. Fig. 1C: A diagram outlining the positions of the variants p.Arg138ValfsTer10 and p.Trp48Ter in the intracellular region of the *MINAR2* protein and the mapping to in exons 1-3 of the *MINAR2* gene. Fig. 1D: Alignment of the amino acid sequences for human *MINAR1* and *MINAR2*. Alignments derived from Clustal Omega. *MINAR1* positions = 273; *MINAR2* positions = 190. 56 identical positions, 20.217% identity, and 81 similar positions according to UniProt Align software.

[0013] Figure 2: Table of parameters used for detecting homozygous runs (Enlis software).

[0014] Figure 3: Table of homozygous runs >2 MB in the affected siblings.

[0015] Figure 4: Table of primers used for the Sanger DNA sequencing.

[0016] Figures 5A-5C: Families, *MINAR2* variants, and the effects of the c.393G>T variant on splicing. Fig. 5A: Pedigrees and segregation of the *MINAR2* variant in families. Filled symbols denote affected individuals and double lines indicate first cousin consanguinity. Electropherograms show the identified variant. The wild type traces are from an unrelated individual. Hom: Homozygous mutant. Fig. 5B: Locations of the identified variants. Fig. 5C:

Exon trap assay for variant c.393G>T. *MINAR2* exon 2 was inserted into a vector consisting of 5' and 3' exons. PCR products show larger and smaller bands in c.393G>T compared to wildtype. Sanger sequencing confirmed insertion of 85 bp at the donor site of exon 2 and skipping of exon 2.

[0017] Figures 6A-6E: Effects of *MINAR2* overexpression and silencing on angiogenesis and multiple pathways. Fig. 6A: Top four representative images show angiogenic potential of non-transfected (NT) human umbilical vascular endothelial cells (HUVEC) compared to cells transfected with *MINAR2* wild type (WT) plasmid construct, *MINAR2* plasmid construct bearing p.Trp48* and p.Arg138Valfs*10 variants, respectively. Right graph shows analysis of total length of angiogenic vessels formed after incubation of 12 hrs on Matrigel coated wells; images were analyzed with imageJ angioanalyzer and results are expressed as Mean±SEM. Significant differences were shown as (***) $p \leq 0.001$ when compared to control and as (##) $p \leq 0.01$ when compared to *MINAR2*(WT). Fig. 6B: Left: Western blot images of effect of *MINAR2* (WT) transient overexpression on NOTCH2 and VEGFA protein levels in HUVEC cells; Right: statistical analysis of GAPDH normalized relative protein expressions of NOTCH2 and VEGFA. Results are expressed as mean±SEM and significant differences are shown as (* $p \leq 0.05$) when compared to control. Fig. 6C: Left: western blot images of effect of SiRNA induced *MINAR2* silencing on NOTCH2 protein levels in PC12; Right: Beta-Actin normalized relative protein expression of NOTCH2. Results are expressed as Mean±SEM and significant differences are shown as (** $p \leq 0.01$) when compared to control or (## $p \leq 0.01$) when compared to groups other than control. Fig. 6D: Left: western blot images of effect of *MINAR2* wild type (WT), *MINAR2* p.Trp48* and *MINAR2* p.Arg138Valfs*10 variants transient overexpression on phosphorylation of Extracellular signal-regulated protein kinases 1 and 2 (Erk1/2); Right: statistical analysis of GAPDH-normalized relative protein expressions of pErk1/2 and Erk1/2. Results are expressed as mean±SEM and significant differences are shown as (***) $p \leq 0.001$ when compared to control and as (###) $p \leq 0.001$ when compared to *MINAR2*(WT). Fig. 6E: Left: Immunoblot analysis of S6 Ribosomal Protein and its phosphorylation at Ser235/236; Akt and its phosphorylation at Ser 473 in PC12 cells transfected with either scrambled negative control siRNA or *MINAR2* siRNA. Transfected cells were serum starved and treated with 50 ng/ml of NGF for 0 to 12 hours. Cell lysates were harvested after 48 hrs of SiRNA incubation and at 0,1,3 and 12 hours of NGF treatment. In addition, cells were treated with 100 nM of rapamycin (Rap) for 30 min after 12

hours of NGF treatment. GAPDH was used as a loading control. Right: Quantitative analysis of p-S6 levels in PC12 cells transfected with scrambled and MINAR2 SiRNA respectively at 0,1,3 and 12 hours of NGF treatment. The p-S6/S6 ratios are calculated from three biological repeats. Results are expressed as mean $\pm$ SEM and significant difference between scrambled SiRNA and MINAR2 SiRNA are shown as (* $p\leq 0.05$) when compared for any time point.

[0018] Figure 7: Table of next generation sequencing statistics

[0019] Figure 8: Table providing WGS homozygous runs >1 MB in three affected siblings of Family 1 and 2.

[0020] Figure 9: Table of audiological phenotype in affected individuals.

[0021] Figure 10: Table of characteristics of the identified *MINAR2* variants.

[0022] Figure 11: qRT-PCR analysis of effect of scrambled and three predicted MINAR2 SiRNA sequences namely A, B, and C at concentrations of 10nM and 80nM on MINAR2 mRNA expression in PC12 cells after 48 hrs of transfection respectively. The results are expressed as Fold change in mRNA levels $\pm$ SEM.

[0023] Figure 12: Relative expression pattern of *Minar2* (previously known as A730017C20Rik) in mouse inner ear (data from Elkon et. al., 2015) by using gene Expression Analysis Resource (gEAR; umgear.org/).

[0024] Figure 13: Table of ten longest homozygous runs in the proband of Family 2 individual II:1 via WGS.

DETAILED DESCRIPTION

[0025] The disclosure provides a method of characterizing and treating deafness. To better map the landscape of autosomal recessive sensorineural hearing loss, samples from families with parental consanguinity were collected. Genome sequencing (GS) was performed on a subset of these families and revealed loss of function variants in *MINAR2*. Variants in *MINAR2*, a frameshift 8bp deletion (p.Arg138ValfsTer10) and a nonsense variant (p.Trp48Ter) were identified in two unrelated Turkish families co-segregating with autosomal recessive sensorineural hearing loss. A third variant also was identified, p.Lys131Asn, in two additional families.

[0026] MINAR2 (previously known as uncharacterized protein KIAA1024L and mouse gene *A730017C20Rik*) shares some identity with MINAR1, which has been found to be involved in controlling neurite formation during neuronal differentiation through DEP Domain Containing MTOR Interacting Protein (DEPTOR) and angiogenesis through modulation of Notch2. Notch pathway is a highly conserved intercellular signaling cascade that is activated by the interaction of transmembrane ligands (Delta and Jagged) with Notch receptors, which are usually expressed on the surface of neighboring cells. Binding of Notch Ligand to receptor induces cleavage of the Notch receptors intracellular domain (NICD) and ensuing nuclear translocation of the NICD, where it binds to multiple DNA-binding proteins (Andersson et al. 2011; Chillakuri et al. 2013; Lu and Lux 1996).

[0027] Notch signaling plays a very delicate role in angiogenesis. In the initial stages of angiogenesis, Notch activation is generally repressed to allow proliferation of endothelial cells in response to VEGF stimulation, and its expression is later upregulated when endothelial cells stop proliferating and the vessels begin to stabilize (Henderson et al. 2001; Taylor et al. 2002). Disruption or interference with the Notch pathway in mice resulted in the development of vascular tumors and lethal hemorrhage (Liu et al. 2011) and its activity in cell culture inhibited the angiogenic functions of endothelial cells such as capillary tube formation, migration, and proliferation (Itoh et al. 2004; Leong et al. 2002; Nosedá et al. 2004; Williams et al. 2011).

[0028] Disclosed herein is the identification of mutations in the *MINAR2* gene (wild-type sequence found in NCBI Reference Sequence: NM_001257308.2; SEQ ID NO: 1). *MINAR2* encodes major intrinsically disordered NOTCH2-binding receptor 1-like (also known as Membrane integral NOTCH2-associated receptor 2 or MINAR2) (UniProtKB/Swiss-Prot Reference sequence: P59773; SEQ ID NO: 2).

[0029] In one aspect, the disclosure provides a method of characterizing sensorineural deafness in a subject. The method comprises detecting a mutation in *MINAR2* in the subject, as described further herein.

[0030] The disclosure also provides methods of treating sensorineural deafness. The method comprises administering to a subject having a mutation in the *MINAR2* gene a composition that comprises one or more of the following: a polynucleotide that encodes a MINAR2 peptide, a MINAR2 peptide, a small molecule that increases expression of MINAR2, an agent that blocks

expression of a mutant *MINAR2* gene, and an agent that corrects the mutation in the *MINAR2* gene. In various embodiments, the method comprises administering a combination of the foregoing agents, in a single composition or in separate compositions (optionally administered at different time points). In various aspects, the method comprises detecting the presence of a mutation in the *MINAR2* gene in a sample from the subject. In various embodiments, the *MINAR2* mutation is preferably a DNA variant classified as pathogenic or likely pathogenic according to American College of Medical Genetics and Genomics (ACMG) guidelines. In various aspects of the disclosure, a method is provided which comprises diagnosing the subject with sensorineural deafness when the presence of the mutation in the *MINAR2* gene is detected.

[0031] In various embodiments, the method comprises detecting the *MINAR2* gene mutations p.Arg138ValfsTer10, p.Trp48Ter, or any protein truncating mutation and/or mutation that leads to a “loss of function” or a hypomorphic function of the protein. In various aspects, the method comprises detecting the *MINAR2* gene mutation p.Lys131Asn. In related embodiments, mutations in the *MINAR2* gene may be detected using next-generation sequencing such as a gene panel, whole exome sequencing (WES), whole-genome sequencing (WGS), Sanger sequencing, and related DNA sequencing methods, polymerase chain reaction (PCR), real-time PCR (RT-PCR), microarray or Multiplex Ligation-dependent Probe Amplification (MLPA) assays for sequence and copy number variants, DNA restriction enzyme digestion and gel electrophoresis, and other DNA mutation detection methods known in the art. Also mutant *MINAR2* may be detected via next-generation sequencing or PCR amplification of *MINAR2* mRNA or western blotting of *MINAR2* protein.

[0032] In various aspects, the *MINAR2* mutation is detected by examining proteins using western blotting (immunoblot), High-performance liquid chromatography (HPLC), Liquid chromatography–mass spectrometry (LC/MS), antibody dependent methods such as enzyme-linked immunosorbent assay (ELISA), protein immunoprecipitation, protein immunostaining, protein chip methods or other protein detection methods suitable for mutation detection.

[0033] The sample may be any biological sample taken from the subject, including, but not limited to, any tissue, cell, or fluid (e.g., blood) which can be analyzed for a trait of interest, such as the presence or amount of a nucleic acid (e.g., *MINAR2* mRNA) or a protein (e.g., *MINAR2*

protein). In various embodiments, the biological sample is a plasma, saliva, urine, or skin sample.

[0034] A “subject” as referred to herein, can be any mammal, such as a human. In various embodiments, the subject is an infant, a child, an adolescent or an adult. In various embodiments, the subject is a human aged 5 years or younger. In other aspects, the subject is 13 years or younger, 18 years or younger, 30 years or younger, or over 30 years old. In various embodiments, the subject exhibits hearing loss from the inability to hear sounds ranging from 20 decibels (dB) to 1000 dB, or >1000 dB. In related aspects, the subjects hearing loss is an inability to hear sounds at 20, 30, 40, 50, 60, 70, 80, 90, 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000 or >1000 dB. In various aspects, the subjects hearing loss may be absent at the time of performing the methods described herein. In various embodiments, subjects may have other organ dysfunctions.

[0035] In some embodiments, MINAR2 peptide is administered to the subject. As such, the therapy supplements MINAR2 peptide levels where endogenous, functional MINAR2 levels are inadequate or absent. An exemplary MINAR2 peptide is provided in SEQ ID NO: 2. The disclosure contemplates use of a peptide that comprises at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, or 100% identity to SEQ ID NO: 2. For example, the peptide may comprise at least 80%, at least 85%, at least 90%, at least 95%, or 100% identity to SEQ ID NO: 2.

[0036] In various embodiments, the method comprises administering to the subject a polynucleotide (e.g., a polynucleotide that encodes the MINAR2 peptide/protein (i.e., the MINAR2 peptide described herein), an agent that blocks expression of a mutant *MINAR2* gene, and/or an agent that corrects the mutation in *MINAR2* gene). Polynucleotides are typically delivered to a host cell via an expression vector, which includes the regulatory sequences necessary for delivery and expression. In some aspects, the constructs described herein include a promoter (e.g., cytomegalovirus (CMV) promoter), a protein coding region (optionally with noncoding (e.g. 3'-UTR) regions that facilitate expression), transcription termination sequences, and/or regulator element sequences. In various aspects, tissue specific or regulatable expression may be desired. In this regard, for example, the Cre-loxP system may be utilized to express a polynucleotide of interest (e.g., *MINAR2* gene).

[0037] Expression vectors may be viral-based (e.g., retrovirus-, adenovirus-, or adeno-associated virus-based) or non-viral vectors (e.g., plasmids). Non-vector based methods (e.g., using naked DNA, DNA complexes, etc.) also may be employed. Optionally, the vector is a viral vector, such as a lentiviral vector or baculoviral vector, and in various preferred embodiments the vector is an adeno-associated viral vector (AAV). The expression vector may be based on any AAV serotype, including AAV-1, AAV-2, AAV-3, AAV-4, AAV-5, AAV-6, AAV-7, AAV-8, AAV-9, AAV-10, AAV-11, AAV-12, or AAV-13. Polynucleotides also may be delivered via liposomes, nanoparticles, exosomes, microvesicles, hydrodynamic-based gene delivery, or via a “gene-gun.”

[0038] In various embodiments, the agent is a polynucleotide that encodes a MINAR2 peptide. The amino acid sequence of the MINAR2 peptide is provided as SEQ ID NO: 2. The polynucleotide used in the method optionally encodes the amino acid sequence of SEQ ID NO: 2 or a sequence that is at least 80%, at least 85%, at least 90%, at least 95%, or 100% identical to the amino acid sequence of SEQ ID NO: 2 (which retains the function of MINAR2). Optionally, the polynucleotide comprises SEQ ID NO: 1 or a sequence that is at least 80%, at least 85%, at least 90%, at least 95%, or 100% identical to the polynucleotide sequence of SEQ ID NO: 1 (and which encodes MINAR2).

[0039] In various embodiments, the agent is a small molecule therapeutic (i.e., drug) that increases expression of MINAR2. As such, administration of the small molecule increases MINAR2 protein levels. It will be appreciated that “increasing” expression of MINAR2 does not require 100% increase of expression of MINAR2 protein levels in a subject; any level of increased expression of MINAR2 may be beneficial to a subject. In related embodiments, administration of the small molecule increases the expression of MINAR2 protein by about 1, 5, 10, 15, 20, 25, 30, 40, 50, 60, 70, 80, 90 or 100%.

[0040] In various embodiments, an agent which blocks the expression of a mutant *MINAR2* gene is administered, optionally in combination with a polynucleotide that encodes functional MINAR2 or MINAR2, itself. An agent that blocks expression of a mutant *MINAR2* gene refers to an agent that interferes with expression of a mutant *MINAR2* gene so that mutant *MINAR2* gene expression and/or mutant MINAR2 protein levels are reduced compared to basal/ wild-type levels. It will be appreciated that “blocking” expression of a mutant *MINAR2* gene does not

require 100% abolition of expression and MINAR2 production; any level of reduced expression of aberrant MINAR2 may be beneficial to a subject. Exemplary agents include, but are not limited to, antisense oligonucleotides (ASO), short hairpin RNA (shRNA), small interfering RNA (siRNA), or micro RNA (miRNA). In related aspects, the agent is an antisense oligonucleotide (ASO) used to knock-down (i.e., reduce) the expression of aberrant (i.e. mutant) *MINAR2*. It will be appreciated that “blocking” expression of the *MINAR2* gene does not require 100% abolition of expression of MINAR2 protein; any level of reduced expression of MINAR2 may be beneficial to a subject. For example, in various aspects, the *MINAR2* antisense oligonucleotide reduces the expression of MINAR2 protein by 1, 5, 10, 15, 20, 25, 30, 40, 50, 60, 70, 80 90 or 100%. An ASO is a single-stranded deoxyribonucleotide, which is complementary to an mRNA target sequence. In various aspects, the *MINAR2* antisense oligonucleotide targets an exonic or intronic sequence of the *MINAR2* gene.

[0041] In some embodiments, an agent that corrects the mutation in the *MINAR2* gene is employed. In this regard, the agent may comprise components employed in genome-editing techniques, such as designer zinc fingers nucleases (ZFNs), transcription activator-like effectors nucleases (TALENs), or CRISPR-Cas (clustered regularly interspaced short palindromic repeats-CRISPR associated) systems. In some embodiments, the agents are used to modify the sequence of the *MINAR2* coding region or a regulatory element and/or non-coding region associated with the *MINAR2* gene. In various aspects, genome editing may be used to replace part or all of the *MINAR2* gene sequence or alter MINAR2 protein expression levels. An exemplary agent for use in the method of the disclosure is, DNA encoding Cas9 molecules and/or guide RNA (gRNA) molecules. Cas9 and gRNA can be present in a single expression vector or separate expression vectors. Adenoviral delivery of the CRISPR/Cas9 system is described in Holkers et al., *Nature Methods* (2014), 11(10):1051-1057, which is incorporated by reference in its entirety.

[0042] The terms “treating” or “treatment” refer to reducing or ameliorating sensorineural deafness and/or associated disorders and/or symptoms associated therewith. These terms include reducing the severity of the disorder or any symptoms associated therewith. It is appreciated that, although not precluded, “treating” or “treatment” of a disorder or condition does not require that the disorder, condition, or symptoms associated therewith be completely eliminated; any increase in sensitivity to sound or change in a similar sensory perception is contemplated. A change in the ability of a subject to detect sound is readily accomplished through administration of simple

hearing tests, such as a tone test commonly administered by an audiologist. In most mammals, a reaction to different frequencies indicates a change in sensory perception. In humans, comprehension of language also is appropriate. A change in perception is indicated by the ability to distinguish different types of acoustic stimuli, such as differentiating language from background noise, and by understanding speech. Speech threshold and discrimination tests are useful for such evaluations. To detect a change in sensory perception (e.g., hearing), a baseline value is recorded prior to treatment using any appropriate sensory test. A subject is reevaluated at an appropriate time period following the method (e.g., 1 day, 3 days, 5 days, 7 days, 14 days, 21 days, 28 days, 2 months, 3 months or more), the results of which are compared to baseline results to determine a change.

[0043] A dose of an active agent (e.g., a polynucleotide that encodes a MINAR2 peptide; a MINAR2 peptide; a small molecule that increases expression of MINAR2; an agent that blocks the expression of a mutant *MINAR2* gene; an agent that corrects a mutation in *MINAR2* gene) will depend on factors such as route of administration (e.g., local vs. systemic), patient characteristics (e.g., gender, weight, health, side effects), the nature and extent of the sensorineural deafness or associated disorder, and the particular active agent or combination of active agents selected for administration.

[0044] Suitable methods of administering a physiologically-acceptable composition, such as a pharmaceutical composition comprising an agent described herein, are well known in the art. In various aspects, more than one route can be used to administer one or more of the agents disclosed herein. A particular route can provide a more immediate and more effective reaction than another route. For example, in certain circumstances, it will be desirable to deliver the composition orally; through injection or infusion by intravenous, intraotic, intraperitoneal, intracerebral (intra-parenchymal), intracerebroventricular, intramuscular, intra-ocular, intraarterial, intraportal, intralesional, intramedullary, intrathecal, intraventricular, transdermal, subcutaneous, intraperitoneal, intranasal, enteral, topical, sublingual, urethral, vaginal, or rectal means; by controlled, delayed, sustained or otherwise modified release systems; by implantation devices; using nanoparticles; or as a conjugate.

[0045] In various aspects, the agent described herein is administered to the inner ear. The most direct routes of administration entail surgical procedures which allow access to the interior

of the structures of the inner ear. Inoculation via cochleostomy allows administration of the agent directly to the regions of the inner ear associated with hearing. Cochleostomy involves drilling a hole through the cochlear wall, e.g., in the otic capsule below the stapedial artery as described in Kawamoto et al., *Molecular Therapy*, 4(6), 575-585 (2001), and release of a pharmaceutical composition. Administration to the endolymphatic compartment is particularly useful for administering an agent to the areas of the inner ear responsible for hearing. Alternatively, the agent can be administered to the semicircular canals via canalostomy. Canalostomy provides for exposure to the vestibular system and the cochlea, whereas cochleostomy does not provide as efficient transduction in the vestibular space. The risk of damage to cochlear function is reduced using canalostomy in as much as direct injection into the cochlear space can result in mechanical damage to hair cells (Kawamoto et al., *supra*). Administration procedures also can be performed under fluid (e.g., artificial perilymph), which can comprise factors to alleviate side effects of treatment or the administration procedure, such as apoptosis inhibitors or anti-inflammatories.

[0046] Another direct route of administration to the inner ear is through the round window, either by injection or topical application to the round window. Administration via the round window is especially preferred for delivering agents to the perilymphatic space.

[0047] The agent can be present in or on a device that allows controlled or sustained release, such as a sponge, meshwork, mechanical reservoir or pump, or mechanical implant. For example, a biocompatible sponge or gelfoam soaked in a pharmaceutical composition is placed adjacent to the round window, through which the agent permeates to reach the cochlea. Mini-osmotic pumps provide sustained release of an agent over extended periods of time (e.g., five to seven days), allowing small volumes of composition to be administered, which can prevent mechanical damage to endogenous sensory cells.

[0048] A polynucleotide can be introduced *ex vivo* into cells previously removed from a given subject. Such transduced autologous or homologous host cells can be progenitor cells that are reintroduced into the inner ear of the subject to express, e.g., functional MINAR2. One of ordinary skill in the art will understand that such cells need not be isolated from the patient, but can instead be isolated from another individual and implanted into the patient.

[0049] The agent is preferably administered as soon as possible after it has been determined that the subject is at risk for hearing loss (e.g., because of family history or detection of mutant

MINAR2 prior to clinical manifestation of hearing impairment) or has demonstrated hearing loss.

[0050] It is contemplated the two or more active agents described herein may be administered as part of a therapeutic regimen. Alternatively or in addition, one or more of the active agents may be administered with other therapeutics as part of a therapeutic regimen. The active agent(s) may be administered as a monotherapy or as a combination therapy with other treatments administered simultaneously or metronomically. The term "simultaneous" or "simultaneously" refers to administration of two agents within six hours or less (e.g., within three hours or within one hour each other). In this regard, multiple active (or therapeutic) agents may be administered the same composition or in separate compositions provided within a short period of time (e.g., within 30 minutes). The term "metronomically" means the administration of different agents at different times and at a frequency relative to repeat administration. Active agents need not be administered at the same time or by the same route; preferably, in various embodiments, there is an overlap in the time period during which different active agents are exerting their therapeutic effect.

[0051] Additional aspects and details of the disclosure will be apparent from the following examples, which are intended to be illustrative rather than limiting.

EXAMPLES

Example 1

[0052] This Example describes a study of subjects with sensorineural hearing loss.

[0053] *Subjects:* The diagnosis of sensorineural hearing loss was established via standard audiometry in a sound-proof room according to the current clinical standards. Clinical evaluation included a thorough physical examination and otoscopy in all cases. DNA was extracted from peripheral blood leukocytes of probands according to the standard procedures.

[0054] *Sequencing and Bioinformatics Analysis:* Genome sequencing was performed via BGISEQ-500 and Illumina X Ten instruments in Family 1 individual II:1 and II:2, respectively (Figure 1A). Whole-genome sequencing also was performed in Family 1 individual II:3 and Family 2 individual II:1 (Figure 7). Reads were mapped to human genome reference (NCBI build37/hg 19 version) with Burrows Wheeler Aligner (BWA). Genome Analysis Toolkit

(GATK) was used for the variant calling. Copy number variants (CNVs) were called using the CNVnator. Enlis Genome Research software (www.enlis.com/) was used to identify runs of homozygosity from genome sequencing data (Figure 7).

[0055] For the population allele frequency, gnomAD (Broad Institute) and dbSNP (NCBI) databases, as well as an internal exome/genome database that includes > 4,000 exomes from different ethnicities 100 genomes, were used. Minor allele thresholds of 0.005 for recessive and 0.001 for dominant variants were used. For the missense variants, a combination of criteria from the following databases were used: damaging for Sorting Intolerant From Tolerant (SIFT), Polyphen2, disease-causing for MutationTaster and ≥ 0.7 for REVEL score. American College of Medical Genetics (ACMG) and ClinGen hearing loss expert panel guidelines were used for variant interpretation. Sanger sequencing was used to evaluate co-segregation of the variant with hearing loss. Enlis Genome Research software was used to identify homozygous regions from GS data (Figure 2). After excluding variants in all previously recognized deafness genes, the focus became variants that mapped to shared runs of homozygosity, > 1 MB regions, in siblings (II:1 and II:2) of family 1 (Figure 3). Later variant filtering in shared autozygous regions in siblings, was applied.

[0056] *Screening cohort by genotyping:* Following identification of a *MINAR2* variant in a family, 685 probands with autosomal recessive deafness were screened. These probands either had additional affected family members and/or parental consanguinity. Thus, probands were sought which had *MINAR2* within a homozygous run determined by rs6885164, rs25810, rs9327540, and rs6888996. Genotypes were determined by using TaqMan assays (Applied Biosystems). For each TaqMan reaction, 20 ng DNA was mixed with TaqMan Universal PCR Master Mix (2×, Applied Biosystems) and SNP Genotyping Assay (20×, Applied Biosystems). PCR conditions were 50 °C for 2 min, 95 °C for 10 min, 40 cycles of 95 °C for 15 s, and 60 °C for 1 min. Plates were read on the 7900HT Fast Real-Time PCR instrument (Applied Biosystems, Foster City, CA). Finally, data were analyzed by using the SDS v2.4 software (Applied Biosystems). All three coding exons and exon–intron boundaries of *MINAR2* were further screened via Sanger sequencing in those individuals who were homozygous for selected SNPs (Figure 4).

[0057] *Statistics:* Single locus two-point LOD scores were calculated using Superlink Online SNP 1.1 with a disease allele frequency of 0.001 under fully penetrant autosomal recessive model assuming autozygosity.

Non-syndromic sensorineural hearing loss in affected individuals

[0058] Proband in family 1 (II:1) was a 17-year-old female with bilateral progressive sensorineural hearing loss. She was diagnosed with hearing loss during infancy, which progressed to profound deafness after age 10. Otoacoustic emissions were negative. High resolution temporal bone CT was normal. At age 12 she received unilateral cochlear implant improving her oral communication.

[0059] Individual II:2 was a 15-year-old male with progressive sensorineural hearing loss leading to profound deafness, which was treated with unilateral cochlear implant at age 10. Otoacoustic emissions were negative. Temporal bone CT did not show inner ear malformations.

[0060] Individual II:3 was a 3-year-old male who was diagnosed with sensorineural hearing loss during newborn screening. He was diagnosed with bilateral profound hearing loss and received a unilateral cochlear implant at age 1. Otoacoustic emissions were negative.

[0061] Individual II:1 in family 2 was a 42-year-old female with bilateral profound sensorineural hearing loss, which was either congenital or prelingual-onset.

[0062] None of the affected individuals had additional clinical findings for a known form of syndromic deafness. Their neurodevelopmental skills were on target except for speech development.

Genome sequencing reveals a frameshift variant in MINAR2

[0063] On average, read depth was 47.32x and 36.19x; 1x coverage of the genome was 99.2% and 99.6%, 4x coverage of the genome was 98.88% and 99.6% for family 1 individual II:1 and II:2.

[0064] A homozygous NM_001257308.2:c.412_419delCGGTTTTG (p.Arg138ValfsTer10) variant was identified in two siblings in family 1 by using genome sequencing. The cohort screening revealed a homozygous NM_001257308.2:c.144G>A (p.Trp48Ter) variant in a simplex family (family 2) in *MINAR2*. Both variants were present on the intracellular domain of

Minar2 corresponding to the regions of exon 3 and 1 respectively (Figure 1). Single locus two-point LOD score was 3.6.

Example 2

[0065] This Example describes further study of subjects with sensorineural hearing loss. Individuals in the first two families were included in Example 1. This Example reports on variants in *MINAR2*, membrane integral NOTCH2 associated receptor 2, in four families underlying autosomal recessive non-syndromic deafness. *MINAR2* is a recently annotated gene. Functional aspects of *MINAR2* and consequences of *MINAR2* dysfunction in humans remain largely uncharacterized. Three loss-of-function *MINAR2* variants, p.Trp48*, p.Arg138Valfs*10, and p.Lys131Asn, were identified as described herein in 13 individuals with congenital or prelingual-onset severe to profound sensorineural hearing loss. The Examples provided herein show that *Minar2* is expressed in the mouse inner ear, with the protein localizing mainly in the hair cells, spiral ganglia, the spiral limbus, and the stria vascularis. Via *in vitro* studies, the results described below show that *MINAR2* suppresses angiogenesis, as well as NOTCH2, MAPK, and mTOR pathways. The data suggest that *MINAR2* is essential for hearing in humans and its disruption leads to sensorineural hearing loss.

Materials and Methods

[0066] *Subjects:* The diagnosis of sensorineural HL was established via auditory brainstem response or standard audiometry in a sound-proof room according to the current clinical standards. Clinical evaluation included reviews of past medical history with an emphasis on environmental causes of HL and syndromic deafness and a thorough physical examination including an eye exam and otoscopy in all cases. DNA was extracted from peripheral blood leukocytes of participants according to the standard procedures.

[0067] *DNA Sequencing and Bioinformatics Analysis:* Whole-genome sequencing was performed in Family 1 individuals II:1, II:2, II:3 and Family 2 II:1 (Figure 7; Figure 5A). Reads were mapped to human genome reference (NCBI build37/hg 19 version) with Burrows Wheeler Aligner (BWA). Genome Analysis Toolkit (GATK) was used for variant calling (DePristo et al. 2011; Li and Durbin 2010; McKenna et al. 2010). Copy number variants (CNVs) were called using CNVnator (Abyzov et al. 2011). Enlis Genome Research software (www.enlis.com/) was used to identify runs of homozygosity from genome sequencing data (Figure 7).

[0068] Exome sequencing was performed on a HiSeq 2000 platform (Illumina), in individuals IV:3 and IV:5 in Family 3 and IV:1 in Family 4 (Figure 7). In addition, Illumina Infinium Global Screening Array (GSA) v2 (Illumina) kit used for genotyping in 16 members of Family 3 and three members of Family 4 to map the shared homozygous regions in affected individuals.

[0069] Using the NSHL-specific American College of Medical Genetics (ACMG) guidelines, minor allele frequency (MAF) thresholds of 0.005 for recessive and for recessive and 0.001 for dominant variants were used. Population allele frequencies were obtained from gnomAD (gnomad.broadinstitute.org/) and dbSNP (www.ncbi.nlm.nih.gov/projects/SNP/) databases, as well as from our internal exome/genome database that includes > 7,000 samples from different ethnicities. ACMG and ClinGen HL expert panel guidelines were used for variant interpretation (Oza et al. 2018; Richards et al. 2015).

[0070] Whole-genome and exome sequencing data in affected individuals were first analyzed for variants (SNVs, indels, and CNVs) in recognized HL genes retrieved from (hereditaryhearingloss.org/), Online Mendelian Inheritance in Man (omim.org/), University of Miami Molecular Genetics Laboratory HL gene panel, and a virtual gene panel for HL (v2.176) from PanelApp (www.genomicsengland.co.uk). This analysis did not reveal a plausible variant under any inheritance model. After excluding variants in previously recognized deafness genes, we focused on variants that mapped to shared runs of homozygosity > 1 MB regions, in affected members of each family (Figures 2 and 8).

[0071] Sanger sequencing was used to evaluate co-segregation of candidate variants with HL.

[0072] *Site directed mutagenesis:* MINAR2 (OHu01804, NM_001257308.2) cDNA cloned in pcDNA3.1+/C-(K)DYK was procured from Genscript Biotech (NJ, USA). Site directed mutagenic changes to incorporate c.412_419delCGGTTTTG, and c.144G>A were done using Quick Change Lightning Multi Site-Directed Mutagenesis Kit (Agilent Technologies, USA). Briefly, 40 ng plasmid was subjected to PCR amplification as per standard kit guidelines using mutagenic primers 5'-agtgtacatgtctccaaccaggtcattaggattttcc-3' (SEQ ID NO: 14), 5'-ggaaaatcctaatacctggttgggagacatgtact-3' (SEQ ID NO: 15), and 5'-gagtagacaagggtttgtcagtggtgtgcagcagg-3' (SEQ ID NO: 16), 5'-cctgctgcacaacactgacaaaacctgtctactc-3' (SEQ ID NO: 17) for c.412_419delCGGTTTTG and c.144G>A variants, respectively. Following PCR, 10µl of the product was subject to DpnI

digestion for 5min at 37°C and transformed into chemically competent DH5α cells (NEB, USA) by heat shock at 42 °C for 30sec. Resulting transformants were grown in SOC media for 1hr at 37 °C and selected overnight on LB agar plates containing 100μg/ml Ampicillin. Following day, single colonies were selected and further grown in LB broth containing 100μg/ml Ampicillin for 12 hours. Plasmid isolation and purification was done using plasmid miniprep kit (Qiagen, Germany). Sanger sequencing for confirmation of variant incorporation was done Genewiz, USA; by using cytomegalovirus virus forward primer (5'-CGCAAATGGGCGGTAGGCGTG-3'; SEQ ID NO: 18).

[0073] *Minigene assay for MINAR2 c.393G>T and transfections in HEK293:* The preparation of minigene was carried out using the pET01 vector (Mobitec, GmbH). This Exontrap system is based on a shuttle vector (pET01), which already contains 5' and 3' exon separated by a 600 bp intron sequence, including a multiple cloning site (MCS). To include MINAR2 c.393G > T, a total of 528bp regions including exon2 and flanking introns was amplified using primers 5'-CCGCTCGAGAGAGAACCCTAGAAATCCTTTT-3' (SEQ ID NO: 19) and 5'-TCCCCGAGAGAAAAGCCAG-3' (SEQ ID NO: 20), which contain overhangs for XhoI and SacII restriction sites. After amplification, bands were excised, digested, and ligated with T4 DNA ligase into pET01. After ligation, the resulting recombinant pET01 was transformed and selected on ampicillin plates 100g/ml.

[0074] Positive colonies were identified, and isolated-recombinant vectors were confirmed using Sanger sequencing primer 5'-GCGAAGTGGAGGA_tCCACAAG-3 (SEQ ID NO: 21). Furthermore, HEK-293 cells were transfected with 2μg of empty vector pET01, pET01 containing control fragment, and Mutant MINAR2 c.393G>T fragments. RNA isolation was done 48 hours after the initial transfection, and reverse transcription was done using SuperScript™ IV First-Strand Synthesis System (ThermoFisher, US) and cDNA primer 5'-GATCCACGATGC-3' (SEQ ID NO: 22) specific to pET01. RT-PCR was then performed with 5 'GATGGATCCGCTTCCTGCCCC-3,' (SEQ ID NO: 23) and 5 'CTCCCGCCACCTCAGTGCC-3' (SEQ ID NO: 24), and the resulting product was analyzed using agarose (1.5%) gel electrophoresis.

[0075] *In vitro angiogenesis assay of HUVEC cells:* HUVEC cells were cultured in gelatin coated flasks using EndoGRO endothelial cell growth kit (SCME002, Sigma-Aldrich). For

transient transfections with plasmid constructs 3×10^5 cells were plated with gelatin coated 6 wells plates. After 12 hours of initial plating HUVEC cells were transiently transfected with 2 μ g of *MINAR2* wildtype or *MINAR2* mutant constructs (c.412_419delCGGTTTTG and c.144G>A) using Jetprime transfection reagent.

[0076] For comparative analysis of angiogenic potential of transiently transfected HUVEC cells with *MINAR2* constructs, 7×10^3 cells were plated on matrigel coated 6 well plates as per user instructions of In Vitro Angiogenesis Assay Kit (ECM625, Millipore Sigma). Plates were incubated at 37°C in humidified CO₂ incubator for 12hrs. Images were acquired at 200 μ m with Leica DMIL inverted microscope, and analysis of the total length of angiogenic vessels was performed with Angiogenesis Analyzer for ImageJ, as described by (Carpentier et al. 2020).

[0077] *Cell culture and SiRNA induced silencing in PC12 cells:* PC12 cells were grown in poly-D-lysine coated T-75 tissue culture flasks at 37°C in 5% CO₂ using RPMI-1640 supplemented with 5% horse serum, 5% fetal bovine serum and 1X penicillin and streptomycin. Cells were fed 3 times in a week and were passaged at 80%-85% confluency. After 3 passages 2.7×10^5 cells were plated per well on poly-D-lysine coated 6 well plates and allowed to attach for 6-8 hrs, cells were then washed using 1X Dulbecco's PBS and incubated with antibiotics free fresh growth media containing 80nM *MINAR2* siRNA (GUCAUGCUGGUGGAUUGAUAGAAAC; SEQ ID NO: 25) complexed with 7.5 μ l/well of RNAiMAX transfection reagent for 24hrs. After 48hrs of transfection cells were subjected to serum starvation in RPMI containing 1% horse serum, 50ng/ml NGF (2.5S, Gibco Thermo Scientific) and 1X antibiotics.

[0078] To check the efficacy of *MINAR2* siRNA in silencing *MINAR2* mRNA levels, cells were harvested in buffer RLT after 48hrs of initial transfection and mRNA isolation was done as per user instructions of RNeasy Micro kit (Qiagen, Germany). cDNA was synthesized using qScript XLT cDNA SuperMix (Quanta Biosciences). The qRT-PCR reaction and cycle durations were set up as per user instructions of PowerUp SYBR Green Master mix using primer 5'-ATGCTGCCCTTTAGAC-3' (SEQ ID NO: 26); 5'-GACAGACCCAGACTGGAATAAC-3' (SEQ ID NO: 27) for *MINAR2* and 5'-ACTCCATTCTTCCACCTTTG-3' (SEQ ID NO: 28); 5'-CCCTGTTGCTGTAGCCATATT-3' (SEQ ID NO: 29) for GAPDH. Efficiency of silencing is shown in Figure 11.

[0079] *Western blotting:* Cells were harvested in RIPA buffer containing 1X protease and phosphatase inhibitor (Halt, Thermo Scientific). Protein estimation of samples was done using commercially available BCA kit (Pierce, Thermo Scientific). Equal concentration of protein was reduced and loaded on 4-20% Tris-Glycine gradient gel and protein separation was done as per the method of Laemmli et al. (Laemmli 1970). Following separation protein was transferred onto 0.22 micron using Turbo-trans Blot system (Biorad, USA). The resulting PVDF membrane were blocked in 5% BSA for 1.5hr and incubated overnight at 4°C in primary antibodies diluted (1:1000) in TBST (TBS+Tween 0.5%). Blots were washed with TBST and incubated in HRP conjugated Anti-Rabbit Goat secondary antibody (1:3000) for 1.5hr at room temperature. On termination of antibody reactions, blots were washed three times with TBST and developed and visualized using west pico supersignal ECL substrate (Pierce, Thermo, USA) and FluorChemE (ProteinSimple, USA) respectively.

[0080] *Minar2 mRNA expression in Mice:* To check the expression of *Minar2* in different tissues, lung, liver, kidney, brain and cochlea were dissected from P30 wildtype mice. In addition, the cochlear expression of *Minar2* was assessed in E18.5 and P0 samples. Total RNA was isolated with TRIzol Reagent (Invitrogen) according to manufacturer's instructions. Prior to reverse transcription, RNA samples were treated with rDNase I (DNA-free kit, Applied Biosystems). cDNA was synthesized using qScript XLT cDNA SuperMix (Quanta Biosciences). The primers used to amplify a 117 bp fragment of the *Minar2* transcript were: forward 5'-TGGACCATTGAGGAGTATGACA-3' (SEQ ID NO: 30) and reverse 5'-GTCGAAGCCAGGAGTGTACG-3' (SEQ ID NO: 31). For *Gapdh*, a 171 bp fragment was amplified with 5'-ACCCAGAAGACTGTGGATGG-3' (SEQ ID NO: 32) forward primer and 5'-CACATTGGGGGTAGGAACAC-3' (SEQ ID NO: 33) reverse primer.

[0081] *Distortion Product OtoAcoustic Emission (DPOAE) measurements in Mice:* Following ABR measurements DPOAEs were measured. The microphone probe tip of an Etymotic ER10B+ low noise system was positioned next to the opening of the left ear canal. Using Tucker Davis Technologies BioSigRZ software and MF1 loudspeakers, f2 tones were presented at 6, 12, 18, 24 and 30 kHz at increasing level -10 to 75 dB SPL in 5 dB steps, whilst f1 tones were presented at 10dB above the f2 (0 to 85 dB SPL), at a frequency ratio of 1 : 1.2 for f1 : f2 tones. For each f2 frequency the DPOAE threshold was determined at the lowest f2 stimulus level (dB

SPL) where the 2f1-f2 DPOAE rose in amplitude above two standard deviations from the mean noise floor.

[0082] *Endocochlear Potential (EP) recording in Mice:* EP was measured in urethane-anaesthetised mice (0.1ml/10g bodyweight of a 20%w/v solution) aged P30-32 using 150mM KCl-filled glass pipette microelectrodes, as described previously (Ingham et al. 2016; Steel and Barkway 1989). EP was recorded as the potential difference (mV) between the tip of a glass microelectrode inserted into scala media via a fenestration in the cochlea basal turn lateral wall and a reference Ag-AgCl pellet electrode inserted under the skin of the dorsal surface of the neck.

[0083] *Wholemout dissection, immunohistochemistry, and confocal imaging of synapses:* Cochleae of postnatal day (P)14 and P30-32 mice were fixed in 4% paraformaldehyde (PFA) in PBS for 2h and decalcified with 0.1M ethylenediaminetetraacetic acid (EDTA) overnight at room temperature (RT). After dissection of the organ of Corti, samples were permeabilised with 5% Tween in PBS for 30 min and blocked in 0.5% Triton X-100 and 10% Normal Horse Serum (NHS) in PBS for 2h. Then, the tissues were incubated overnight with the primary antibodies diluted in 8% NHS at RT. The primary antibodies used were rabbit anti-Myosin VIIa (diluted 1:200; 25-6790, Proteus), mouse IgG2 anti-glutamate receptor 2 (GluR2) (diluted 1:2000; MAB397, Emd Millipore), and mouse IgG1 anti-C-terminal-binding protein 2 (CtBP2) (diluted 1:200; 612044, BD Transduction Laboratories). Samples were incubated with the secondary antibodies for two cycles of 1h at 37°C. The secondary antibodies used were Alexa Fluor 647-conjugated chicken anti-rabbit (1:200; #A21443, Thermo Fisher Scientific), Alexa Fluor 488-conjugated goat anti-mouse (IgG2a) (diluted 1:1000; #A21131, Thermo Fisher Scientific) and Alexa Fluor 568-conjugated goat anti-mouse (IgG1) (1:1000; #A21124, Thermo Fisher Scientific). Specimens were mounted using ProLong Gold Antifade Mountant with DAPI (P36931, Life Technologies) and stored at 4°C. Samples were imaged with a Zeiss LSM 700 inverted confocal microscope interfaced with ZEN 2011 software (v14.0.17.201). All the images were captured with the plan-Apochromat 63x/1.40 oil DIC objective and 2.0 optical zoom. Confocal z-stacks were obtained with a z-step size of 0.25 µm, ensuring that all the synaptic puncta were imaged. Images were acquired at the 12 kHz and 24 kHz best-frequency regions and the ImageJ measure line plug-in (Eaton Peabody Laboratories) was used to map cochlear length to cochlear best frequencies. Brightness and contrast levels were adjusted for whole images using

Fiji. Synaptic puncta counts were performed manually using the cell-counter plugin in Fiji software. All the images containing puncta were merged in a z-stack and the z-axis maximum intensity projection was used for quantification of synapses, defined by colocalization of CtBP2- and GluR2-labelled puncta. The total number of ribbon synapses was divided by the number of Myo7a-labelled IHCs present in the image to determine the number of ribbon synapses per IHC. In the cases where an IHC was not completely visible in the image, the synapses corresponding to that cell were not counted. After counting of synapses, whole images were subjected to the smoothing function of Fiji for presentation. Two non-overlapping images were acquired at each best-frequency region, counting between 10 to 14 IHCs at each frequency. A one-way ANOVA with multiple comparisons was used to compare the different experimental groups.

[0084] *Immunofluorescence:* Tympanic bullae containing the cochleae were dissected from P0 mice under the microscope and locally perfused with 4% PFA through the round and oval windows. Samples were kept in 4% PFA at 4 °C overnight and rinsed in 1X PBS. Cochlea whole mounts were permeabilized with 0.5% Triton X-100 and blocked in 5% BSA for 1 hour at room temperature, followed by overnight incubation at 4°C with primary antibodies. A mouse anti-Myo7a (MYO7A 138-1, DSHB), a chicken anti-NF (AB5539, Millipore), a mouse anti-Tuj1 (MMS-435P), and a chicken anti-Beta Galactosidase (ab9361) were utilized as a primary antibody. Specimens were washed with PBS, and then incubated with anti-mouse Alexa Fluor 568 and 488, and anti-chicken 647 were applied. DAPI (Calbiochem) were used to counterstain a nuclear DNA. Specimens were washed with PBS and mounted in fluorescence mounting medium (Dako). Images were taken using a Zeiss LSM 710 fluorescence microscope.

[0085] To perform additional experiments, mice were culled by decapitation (P1) or by cervical dislocation (P14 and P30). The skull was cut in two halves and the brain was removed to facilitate the penetration of the fixative. Samples were washed with cold PBS, fixed overnight in 4% PFA at 4°C and then washed twice with cold PBS.

[0086] *X-gal staining:* Whole mount cochlea were incubated overnight at 37°C in X-gal staining solution (0.02% NP40, 0.01% Sodium Deoxycholate, 2mM MgCl₂, 5mM K₃Fe(CN)₆, 5mM K₄Fe(CN)₆, 0.5 mg/ml X-gal), washed twice with PBS 1-X and mounted in mounting medium (Dako).

Results

[0087] *Non-syndromic sensorineural hearing loss is diagnosed in affected individuals:* A summary of the auditory phenotype of 13 affected individuals is shown in Figure 9. Ages ranged from 3 to 80 years old at the last examination. Each affected individual was diagnosed with HL either at birth or during infancy.

[0088] Families 1 and 2 were of Turkish ancestry, recruited in Turkey. Parents of Family 1 stated that in II:1 and II:2, HL was milder in younger ages and progressed to profound degree around age 10. Otoacoustic emissions were absent in these individuals. These siblings received unilateral cochlear implants at ages 12 and 10, respectively, which improved their oral communication. Individual II:3 in Family 1 was diagnosed with profound sensorineural HL after failing the newborn hearing screening test. Otoacoustic emissions were absent at diagnosis. He received a unilateral cochlear implant at age 1 and communicates orally.

[0089] Families 3 and 4 were of Indian ancestry, recruited in India. While there is no known consanguinity in any of marriages in these two pedigrees, they are all from the same small town belonging to the same Hindu caste (Mali). All the affected individuals in Family 3 were born deaf and used signs, simple words, or sounds to be able to communicate. Severity of HL appeared to have remained the same in all the affected individuals from the beginning of life. Individuals IV:1 and IV:2 in Family 4 were diagnosed with profound sensorineural HL at the age of about 3 years via auditory brainstem response studies. Their mode of communication at that age was predominantly non-verbal.

[0090] A high-resolution temporal bone CT scan was normal in at least one affected member of each family. None of the affected individuals had additional clinical findings for a syndromic form of deafness. Their neurodevelopmental skills were on target except for speech delay. None of the affected individuals showed impaired balance on tandem walking and Romberg test. They did not have bradykinesia, tremor, or rigidity on neurological examination.

[0091] *MINAR2 variants co-segregate with autosomal recessive deafness:* Homozygosity mapping via genome sequencing revealed a shared 9.4 Mb region, chr5: 123,576,980-132,980,451 (hg19), in three affected children of Family 1. In Family 3, from SNP arrays the longest autozygous region in all 7 affected individuals is flanked by markers rs13174854 and rs377767449, which is 2.96 Mb chr5:128,738,407-131,705,915 (hg19). In Family 4, two affected

children share a 5.76 Mb autozygous region chr5: 126,978,108- 132,742,450 (hg19) flanked by markers rs11241936 and rs11242152.

[0092] Within the autozygous segments, genome sequencing revealed the *MINAR2* NM_001257308.2:c.412_419delCGGTTTTG (p.Arg138Valfs*10) variant in the three affected members of Family 1 and the NM_001257308.2:c.144G>A (p.Trp48*) variant in the proband of Family 2. Exome sequencing revealed *MINAR2* NM_001257308.2:c.393G>T (p.Lys131Asn) variant in 3 affected members of Families 3 and 4. These variants are absent in gnomAD and internal databases and are predicted to disrupt protein function (Figure 10).

[0093] Sanger sequencing confirmed the variants and showed that each variant co-segregates with ARNSHL in 4 families (Figure 5).

[0094] *Overexpression of MINAR2 suppresses angiogenesis and MINAR2 variants abolish this effect:* As MINAR1 has been shown to inhibit angiogenesis (Ho et al. 2018), the study investigated whether MINAR2 shows the same effect on angiogenesis in HUVEC. These studies revealed that overexpression of MINAR2 suppresses angiogenesis (Figure 6A). *MINAR2* variant detected in Families 1 and 2 was then overexpressed in HUVEC and angiogenesis was compared with the effects of wildtype *MINAR2*. *MINAR2* variants rescued angiogenesis, suggesting that they are loss-of-function variants (Figure 6A).

[0095] *MINAR2 c.393G>T (p.Lys131Asn) leads to aberrant splicing:* The c.393G>T variant substitutes the last nucleotide of exon 2 and is predicted to abolish a splice donor site. Exon trapping experiments show that this variant leads to insertion of 85 intronic nucleotides into exon 2, which alters the amino acid composition of the rest of the protein (Figure 5C). The same variant also leads to skipping of exon 2 entirely (Figure 5C).

[0096] *Expression levels of MINAR2 are inversely correlated with intracellular NOTCH2 abundance:* MINAR2 is named based on its structural similarity to MINAR1, which is shown to be involved in NOTCH2 signaling (Ho et al. 2018). MINAR2 is predicated to have a cytoplasmic domain, a single transmembrane domain with a few amino acids at the extracellular domain. However, MINAR2 encodes a significantly smaller protein (190 vs 917 amino acids) (Ho et al. 2018). The study explored if NOTCH2 abundance in cells is correlated with *MINAR2* expression. Furthermore, the study explored the effect of *MINAR2* expression on VEGF. The data show that overexpression of wildtype *MINAR2* is associated with reduced NOTCH2 and

VEGFA (a prominent VEGF protein in vascular endothelial cells) abundance (Figure 6B). Silencing of *MINAR2* in PC12 cells, which endogenously express *MINAR2*, shows an increase in NOTCH2 and confirms the suppressor effect of *MINAR2* on NOTCH2 abundance (Figure 6C). These results suggest that the suppressor effects of *MINAR2* on angiogenesis is via NOTCH2 and VEGF signaling pathways.

[0097] *MINAR2 is involved in MAP kinase and mTOR pathways:* The study tested the effect of *MINAR2* on the MAPK pathway by transiently overexpressing wildtype *MINAR2* on PC12 cells and detecting levels of ERK1/2 and pERK1/2, a crucial kinase of the MAPK signaling pathway. These studies show that overexpression of wildtype *MINAR2* reduces the abundance of pERK1/2. On the other hand, overexpression of the two *MINAR2* deafness variants in Families 1 and 2 does not show this effect, again supporting their loss-of-function properties (Figure 6D).

[0098] When *MINAR2* was silenced in PC12 cells, an increase was observed in a functional protein in mTORC1 activity, P-S6, at 12 hours (Figure 6E). However, there was no difference in pAKT, a component of mTORC2 activity (Figure 6E).

[0099] *Minar2 is expressed in the mouse cochlea:* The presence of *Minar2* transcript in different mouse tissues was assessed, including the inner ear, and specifically the cochlea. Total RNA was isolated from wildtype at E18.5, P0, and P30. RT-PCR with a forward primer located in exon 2 and a reverse primer in exon 3 of the *Minar2* gene (NM_173759) produced a unique band of 171bp corresponding to the wildtype mRNA in all analyzed tissues, with the exception of the liver. *Minar2* is highly expressed in the inner ear, and specifically in the cochlea, at E18.5, P0, and P30 (data not shown).

Discussion

[00100] This study presented three loss-of-function variants in *MINAR2* in four unrelated families co-segregating with non-syndromic sensorineural HL. Hearing loss started at birth or in early childhood in all affected individuals, and in the majority of cases it was already severe or profound at the time of diagnosis. A progressive HL reaching to profound degree during childhood was noted in some affected individuals. In addition to ABRs showing sensorineural HL, otoacoustic emissions were absent in three children tested, suggesting dysfunction of both inner hair cell/neurons and outer hair cells.

[00101] The studies described herein show that MINAR2 suppresses NOTCH2 signaling, VEGF, and angiogenesis. The Notch pathway is a highly conserved intercellular signaling cascade that is activated by the interaction of transmembrane ligands (Delta and Jagged) with Notch receptors, which are usually expressed on the surface of neighboring cells. Binding of the Notch Ligand to receptor induces cleavage of the Notch receptors intracellular domain (NICD) and ensuing nuclear translocation of the NICD, where it binds to multiple DNA-binding proteins. In the initial stages of angiogenesis, Notch activation is generally repressed to allow proliferation of endothelial cells in response to VEGF stimulation, and its expression is later upregulated when endothelial cells stop proliferating and the vessels begin to stabilize (Henderson et al. 2001; Taylor et al. 2002). Disruption or interference with the Notch pathway in mice resulted in the development of vascular tumors and lethal hemorrhage (Liu et al. 2011) and its activity in cell culture inhibited the angiogenic functions of endothelial cells such as capillary tube formation, migration, and proliferation (Itoh et al. 2004; Leong et al. 2002; Nosedá et al. 2004; Williams et al. 2011).

[00102] MINAR2 is structurally similar to MINAR1, which has been reported to be involved in controlling neurite formation during neuronal differentiation through DEP Domain Containing MTOR Interacting Protein (DEPTOR). In this study, we show that similar to MINAR1, MINAR2 downregulates mTOR signaling (Figure 6E), which may contribute to the abnormal hair cell innervation observed in the *Minar2* mutant mice. On the other hand, we found persistent *Minar2* mRNA expression in mouse inner ear at postnatal day 0 and showing a gradual decline up to day 30. Many fold increase in the early days of life and depleting over time suggests that Minar2 play a role in the development of the inner ear.

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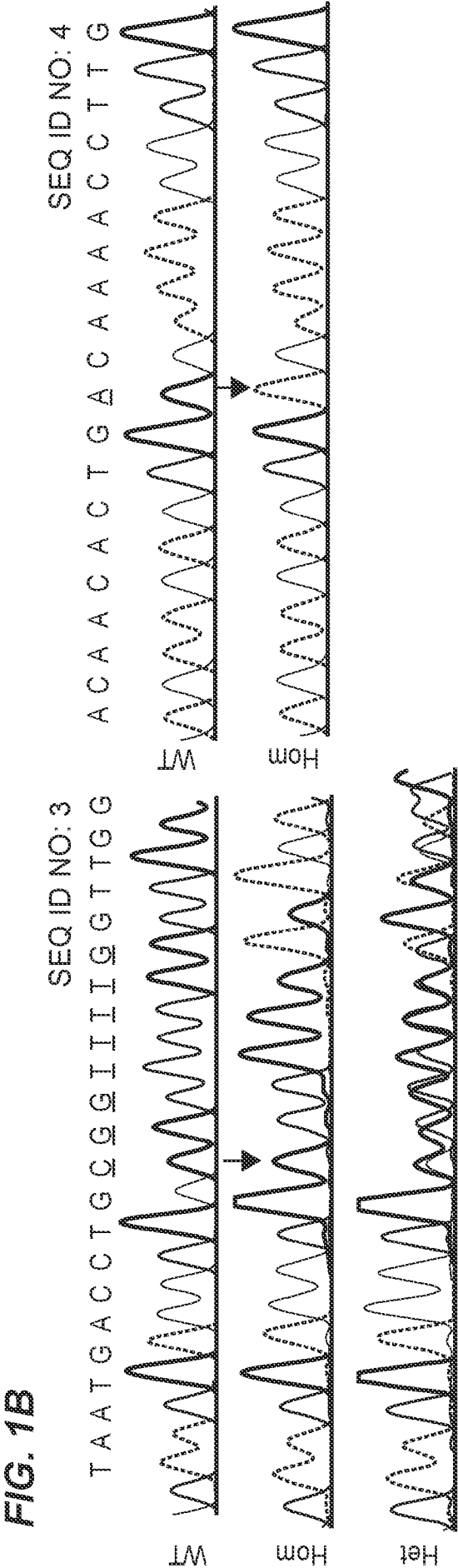
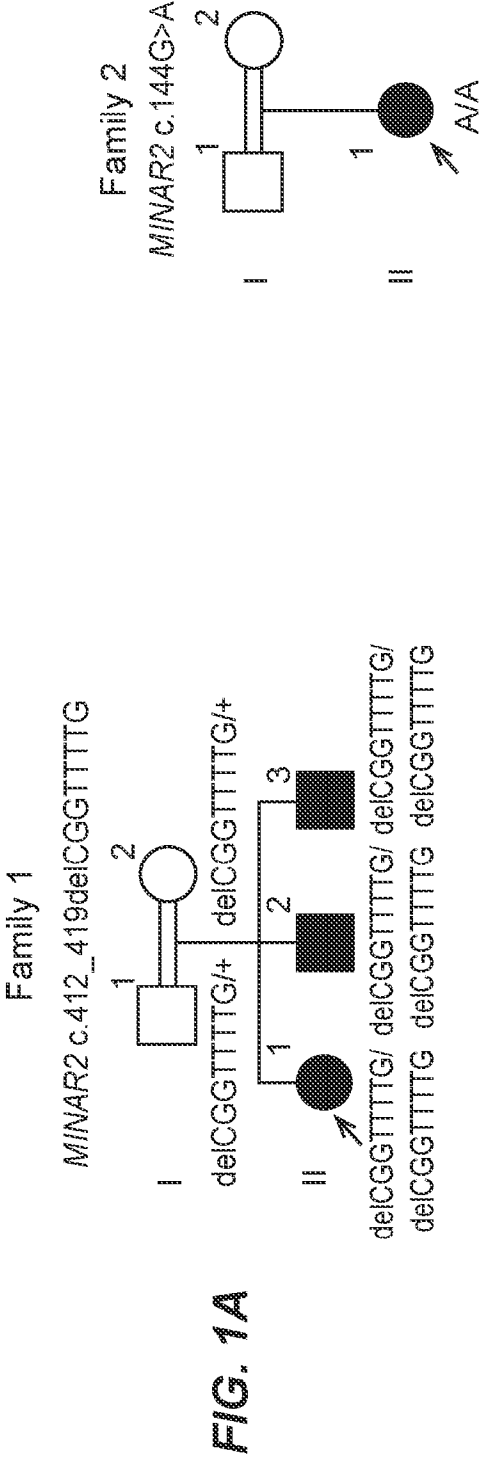
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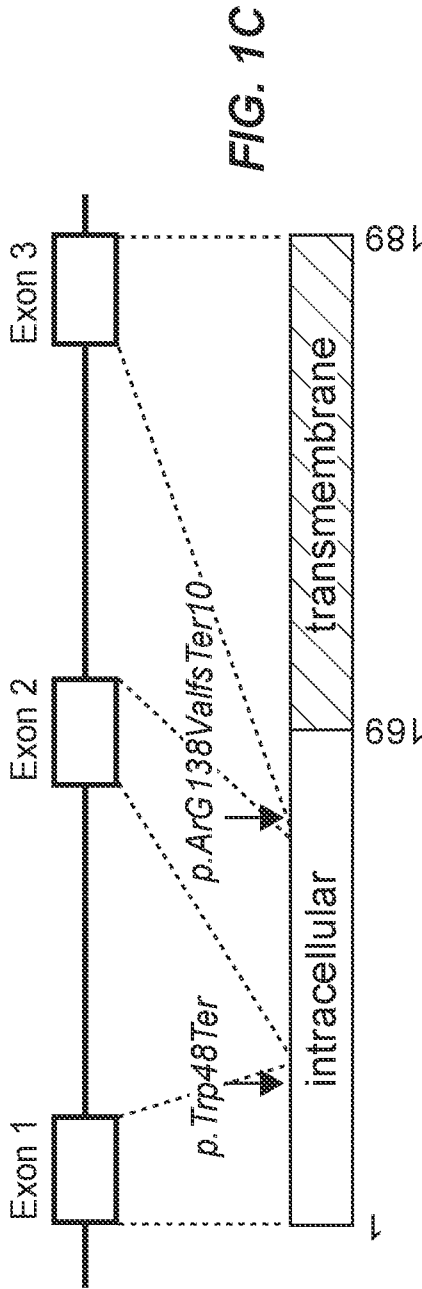
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What is claimed:

1. A method of treating sensorineural deafness in a human subject in need thereof, the method comprising:
administering to the subject having a mutation in a Membrane Integral NOTCH2 Associated Receptor gene (*MINAR2*) a composition that comprises a polynucleotide that encodes a *MINAR2* peptide; a *MINAR2* peptide; a small molecule that increases expression of *MINAR2*; an agent that blocks the expression of a mutant *MINAR2* gene; an agent that corrects a mutation in *MINAR2* gene, or a combination of any of the foregoing.
2. The method of claim 1, wherein the mutation in the *MINAR2* gene is p.Arg138ValfsTer10.
3. The method of claim 1, wherein the mutation in the *MINAR2* gene is p.Trp48Ter.
4. The method of claim 1, wherein the agent is a polynucleotide encoding the *MINAR2* peptide.
5. The method of claim 1, wherein the agent is a *MINAR2* peptide.
6. The method of claim 1, wherein the agent that blocks the expression of a mutant *MINAR2* gene is an *MINAR2* antisense oligonucleotide or CRISPR Cas9 protein and one or more guide RNA molecules, TALEN or zinc finger nuclease (ZFN).
7. The method of claim 1, wherein the agent that corrects the mutation in *MINAR2* gene is a CRISPR Cas9 protein and one or more guide RNA molecules, TALEN or zinc finger nuclease (ZFN).
8. The method of any one of claims 1-7, wherein the method comprises, prior to the administration step, detecting the presence of a mutation in the *MINAR2* gene in a sample from the subject.

9. The method of claim 8, wherein the mutation in the *MINAR2* gene is p.Arg138ValfsTer10.
10. The method of claim 8, wherein the mutation in the *MINAR2* gene is p.Trp48Ter.
11. A method of characterizing hearing loss in a human subject, the method comprising detecting a mutation in the *MINAR2* gene in a sample from the subject.
12. The method of claim 11, wherein the mutation in the *MINAR2* gene is p.Arg138ValfsTer10.
13. The method of claim 11, wherein the mutation in the *MINAR2* gene is p.Trp48Ter.





spIQ9UPX6	IMINAR1_HUMAN	IDLSLTSEGPSDDASPRMFHAHSGHGPKLENNPDWCCSDASGSNSESRLVKALKKSL	60
spIP59773	IMINAR2_HUMAN	MDLSVLPN-----NNHP-----DK	14
spIQ9UPX6	IMINAR1_HUMAN	FTRPSSRSLTEENSATESKI-----ASISNSPRDWRITTYTNRVGLNEEEIKDTGPGDNKD	116
spIP59773	IMINAR2_HUMAN	FLQLDVKSLTRSSALLQASLVRFPGGNYPAAQHWNQNLVYSQREK-----	58
spIQ9UPX6	IMINAR1_HUMAN	WHRKSKEADRRQYDIPPOHRLPKQPKDGFVEQVFSHPYPASLKAHMKSNPLYTDMRLTE	176
spIP59773	IMINAR2_HUMAN	-----KNJAAQRIRGSSADSLVTADSPPPSMSSVMKNNPLYGDLSLEE	101
spIQ9UPX6	IMINAR1_HUMAN	LAEVKRGQPSWTIEEYARNAGDKKLTALDLQTQESLNPNNLEYWMEDIYTPGYDSLLKR	236
spIP59773	IMINAR2_HUMAN	AMEERKKNPSTWITIEEYDKHSLHT-----NLSGHLKENPNDLRFVLGDMYTPGFDTLKK	155
spIQ9UPX6	IMINAR1_HUMAN	KEAEFRRAKVCKIAALIAAAACTVILVIVPICTMKS	273
spIP59773	IMINAR2_HUMAN	EEKQEKHSKFCRMGLLLVISIL--VTIVTITFT	190

FIG. 1D

Parameter	WGS
Variation types to test	SNPs only
Ignore rare or common variations	<5% and >95%
Ignore variations that have a quality score less than	20
Consolidate nearby regions	Checked
Minimum region length	50000 bp
Window scan size	50 variations
Maximum number heterozygous variants in window scan	1
Percent of windows for homozygous position call	5%
Number of consecutive positions to call a homozygous region	100
Maximum gap between variations in a region	100000 bp

FIG. 2

Shared homozygous regions	Region length	2131-102 (Family I, individual II:1)			781-102 (Family I, individual II:2)		
		1x coverage %	5x coverage %	avg read depth x	1x coverage %	5x coverage %	avg read depth x
chr5: 123,576,980-132,980,451	9403471	100	99.9	45.02	100	100	36.72
chr6: 137,615,271-144,367,344	6752074	100	99.9	43.81	100	99.9	36.64
chr3: 189,848,205-195,174,420	5326216	99.7	99.7	46.13	99.7	99.7	36.8
chr4: 62,238,278-67,116,175	4877898	100	99.8	38.76	100	99.9	35.61
chr8: 73,282,863-76,190,651	2907789	99.9	99.8	44.83	99.9	99.8	36.99
chr5: 8,090,121-10,645,871	2555751	99.9	99.8	47.58	99.9	99.9	37.84
chr6: 146,304,258-148,498,792	2194535	100	100	43.33	100	100	36.69

FIG. 3

Primer Name	Primer sequence 5' to 3'	SEQ ID NO:	Amplicon	Description
MIN_ex1_F	CGTGCAGCTGAGAGACTTGG	6	400 bp	Primers used for the screening cohort
MIN_ex1_R	GCAGCTACCTTGTTACTCTGTTC	7		
MIN_ex2_F	TCACATTGCATTCCCTACTATCA	8	397 bp	
MIN_ex2_R	GCGCCTAGTGAAGAGTACCTG	9		
MIN_ex3_F	CCATCAGTCCCCCATGACAGT	10	400 bp	Primers used for the c.412_419delCGGTTTG
MIN_ex3_R	CAACAAATCCATTCACTGC	11		
MINAR2_L137_F	CATCAGTCCCCCATGACAGTG	12	221 bp	
MINAR2_L137_R	AAAATCAGACCCCATACGACAGAA	13		

FIG. 4

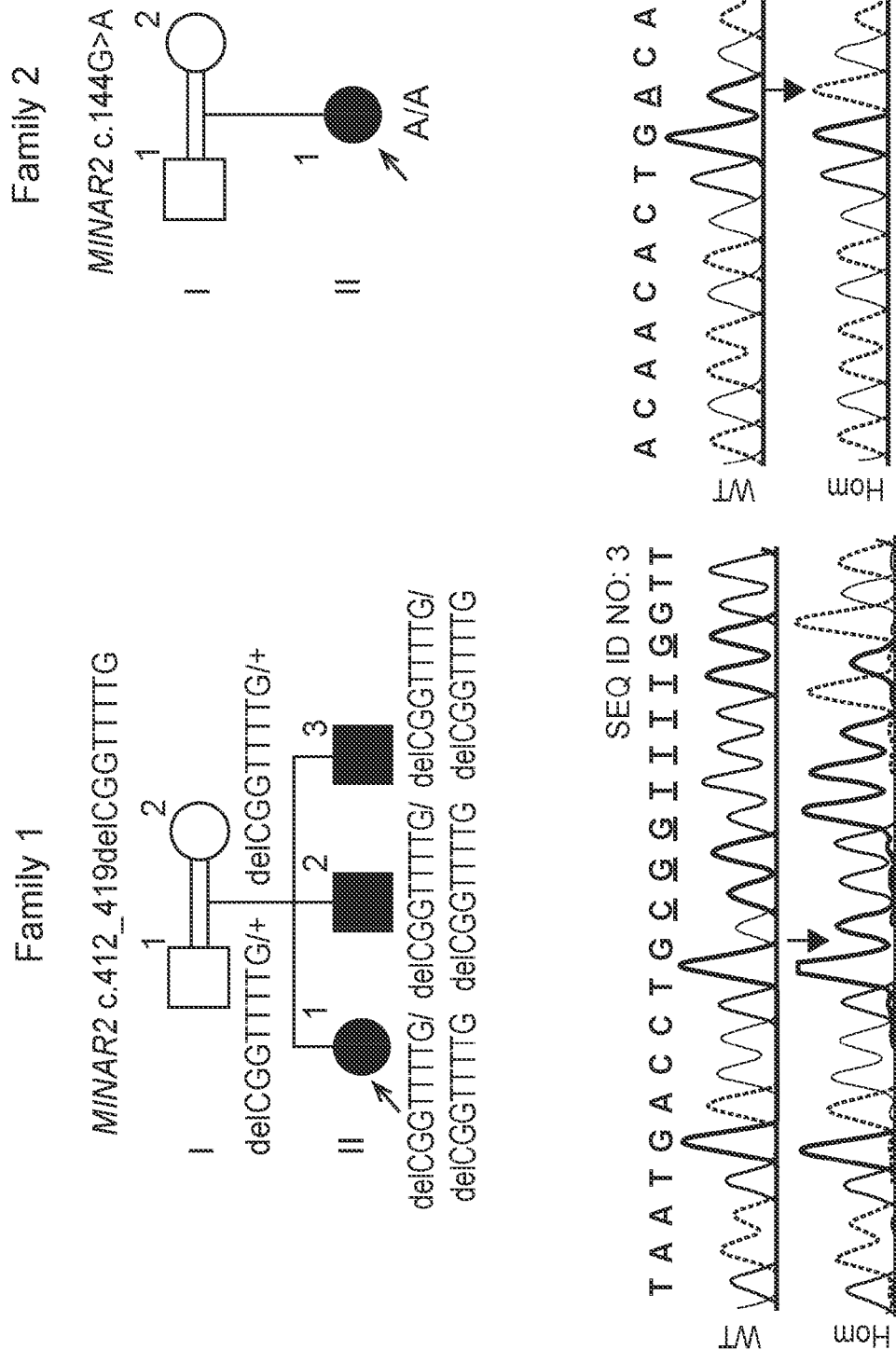
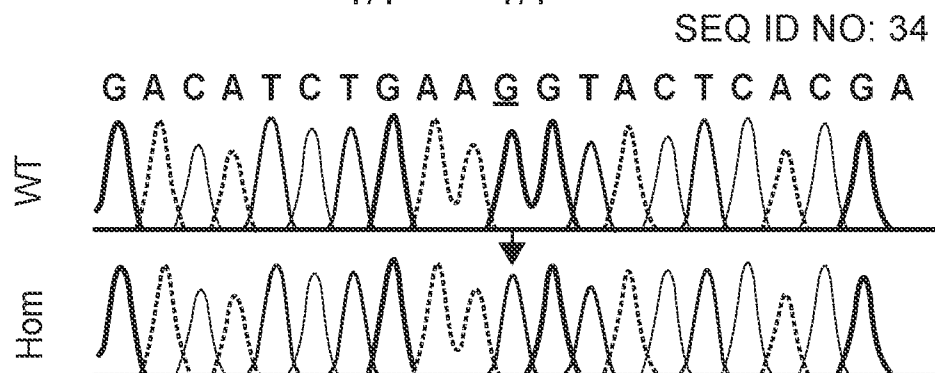
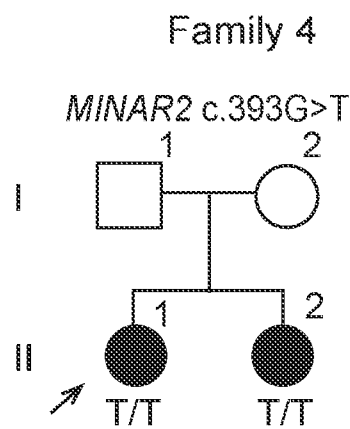
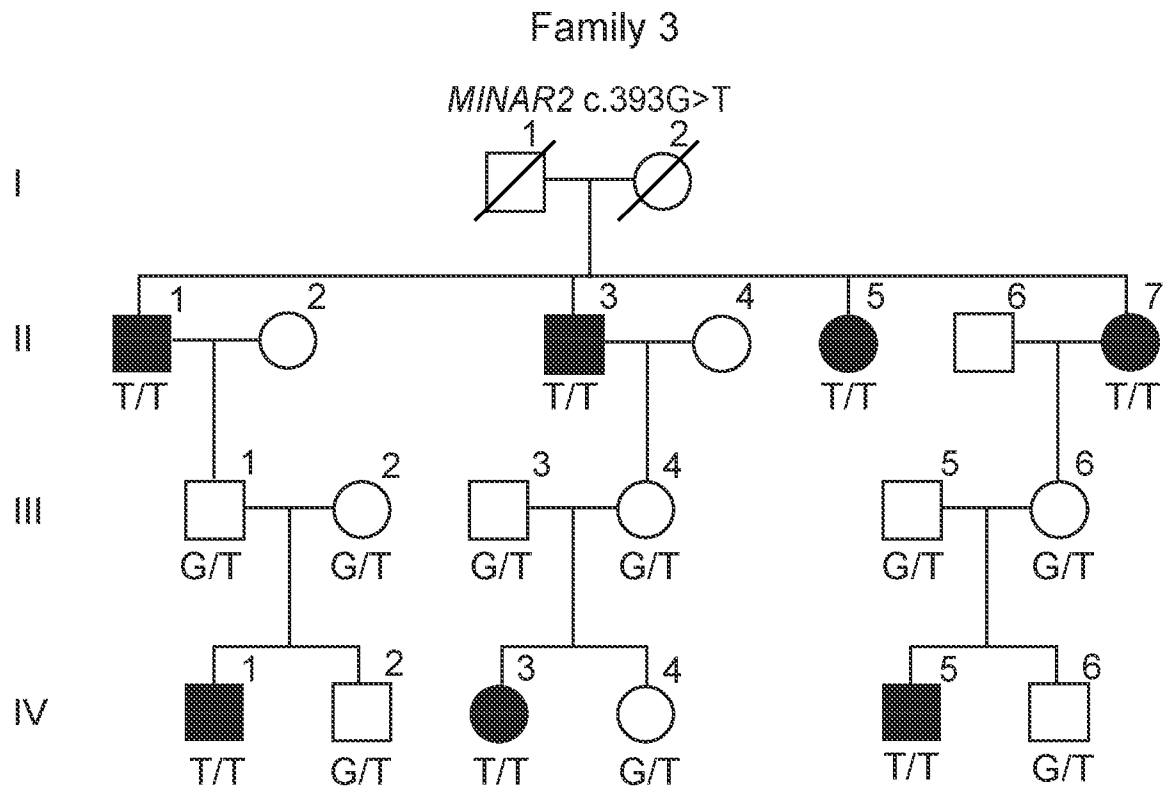


FIG. 5A

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**FIG. 5A, cont.**

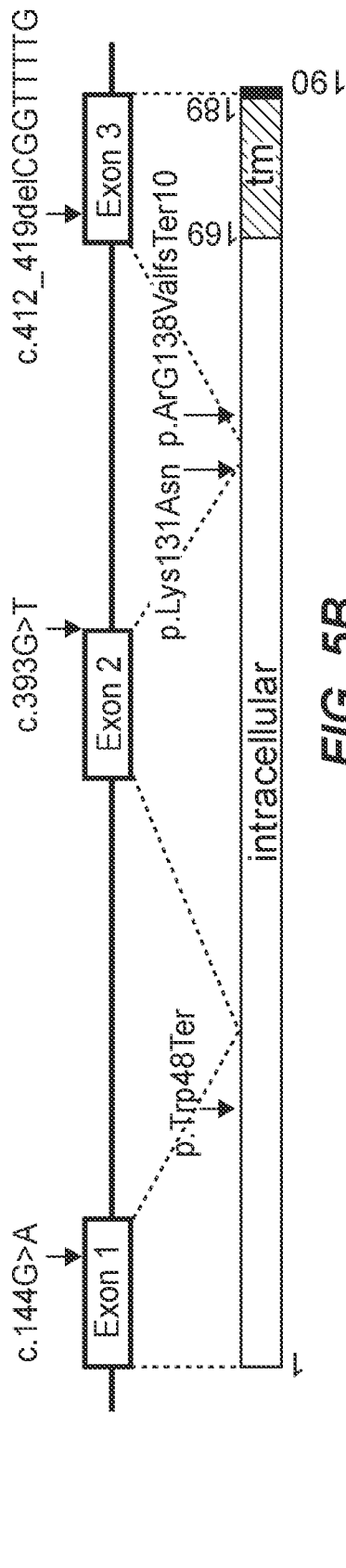


FIG. 5B

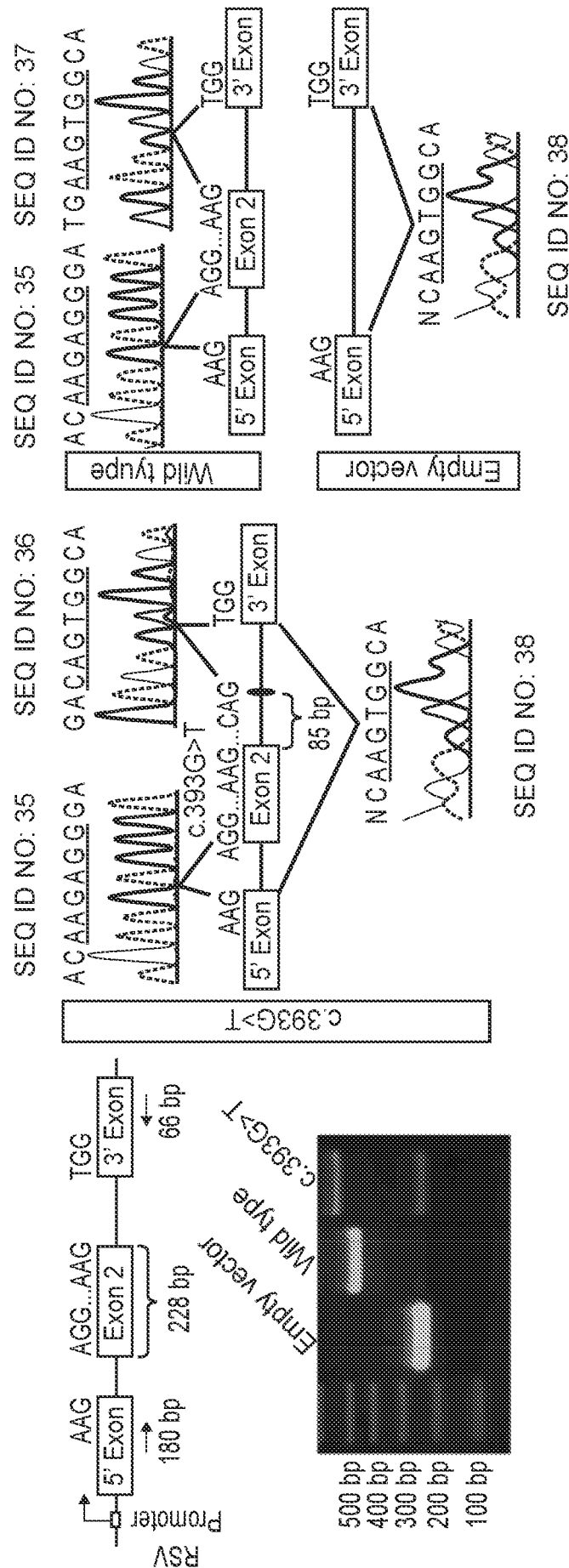


FIG. 5C

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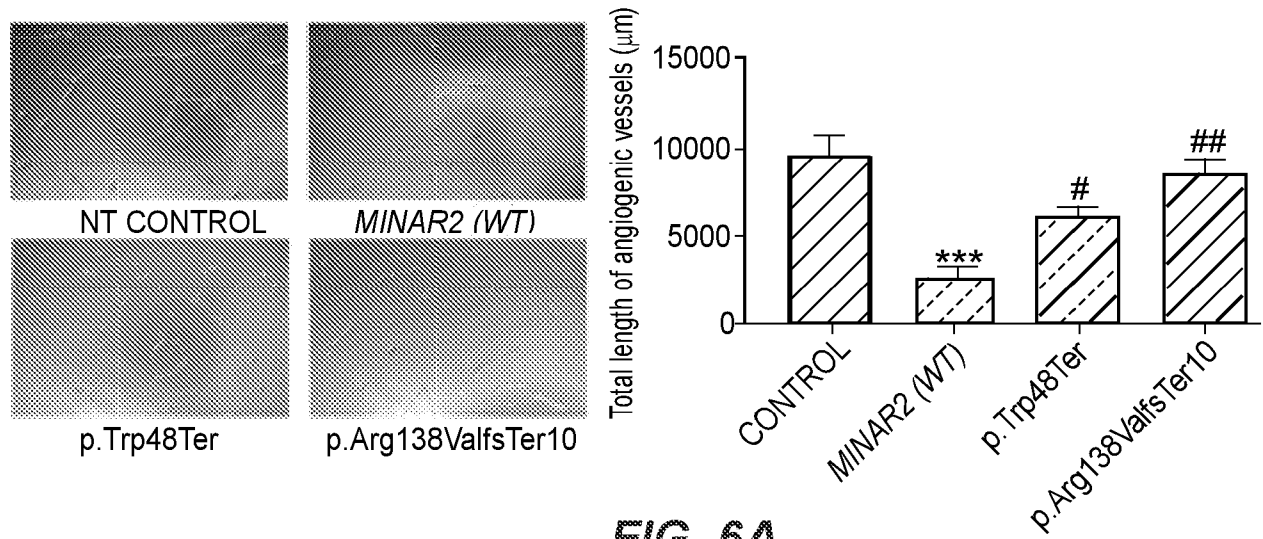


FIG. 6A

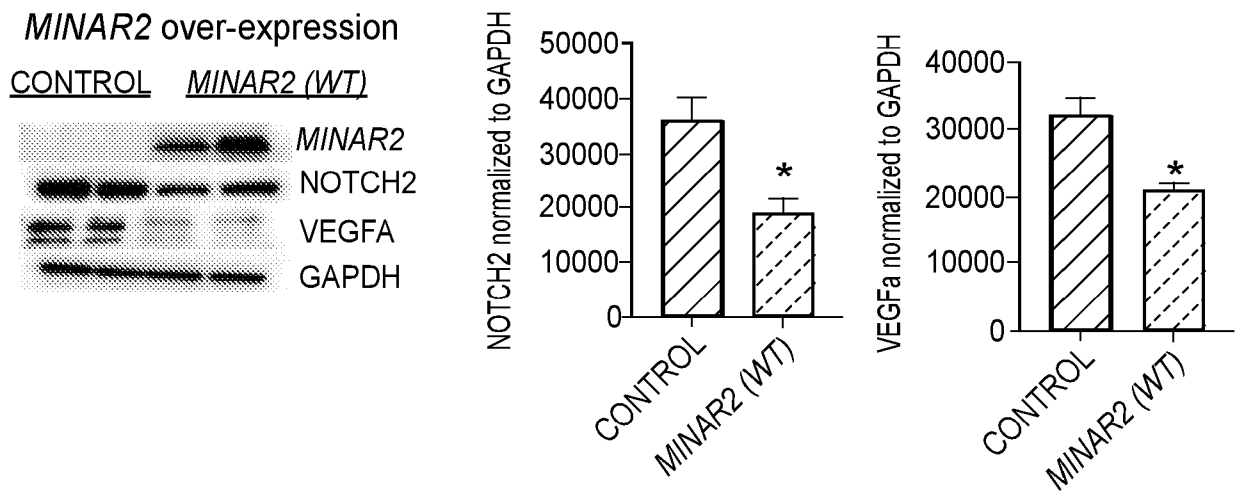


FIG. 6B

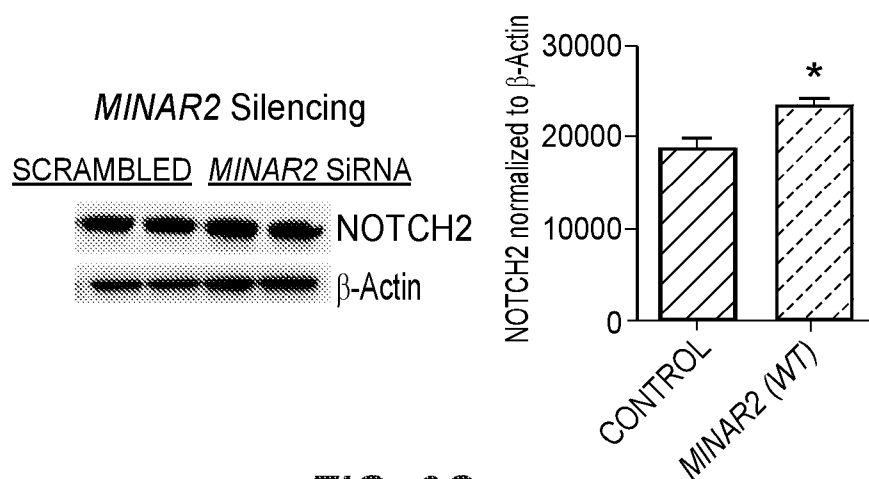


FIG. 6C

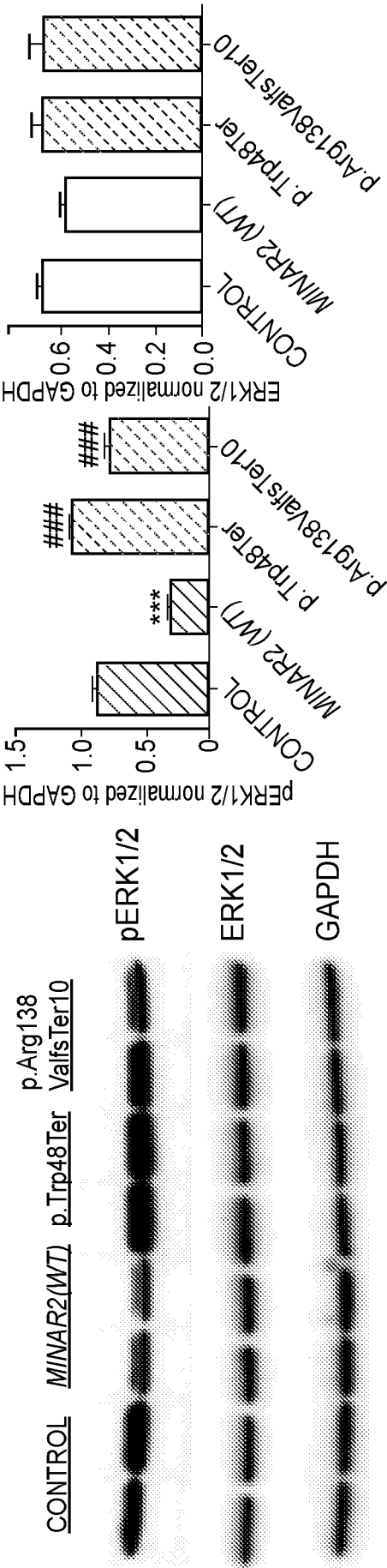


FIG. 6D

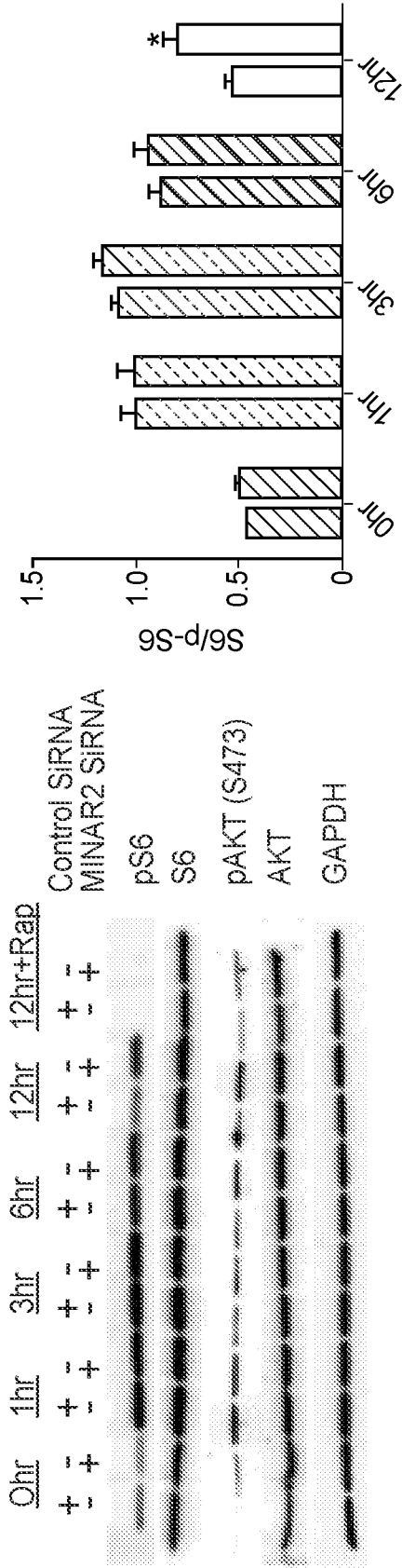


FIG. 6E

Family and individual	NGS performed	Platform	Average read depth	1X coverage	4x coverage
Family 1 Individual II:1	Genome sequencing	BGI BGISEQ-500	47.32x	99.20%	98.88%
Family 1 Individual II:2	Genome sequencing	Illumina X Ten	36.19x	99.60%	99.60%
Family 1 Individual II:3	Genome sequencing	BGI BGISEQ-500	35.17x	99.60%	99.20%
Family 2 Individual II:1	Genome sequencing	Illumina NovaSeq 6000	38.73x	99.20%	99.10%
Family and individual	NGS performed /Capture kit	Platform	Average throughput depth of target regions	Q20	Q30
Family 3 Individual IV:3	Exome Sequencing /Agilent SureSelect V7	Illumina HiSeq 2000	209.1x	97.74%	93.68%
Family 3 Individual IV:5	Exome Sequencing /Agilent SureSelect V7	Illumina HiSeq 2000	182.7x	97.77%	93.7%
Family 4 Individual II:1	Exome Sequencing /Agilent SureSelect V7	Illumina HiSeq 2000	216.7x	97.84%	93.91%

FIG. 7

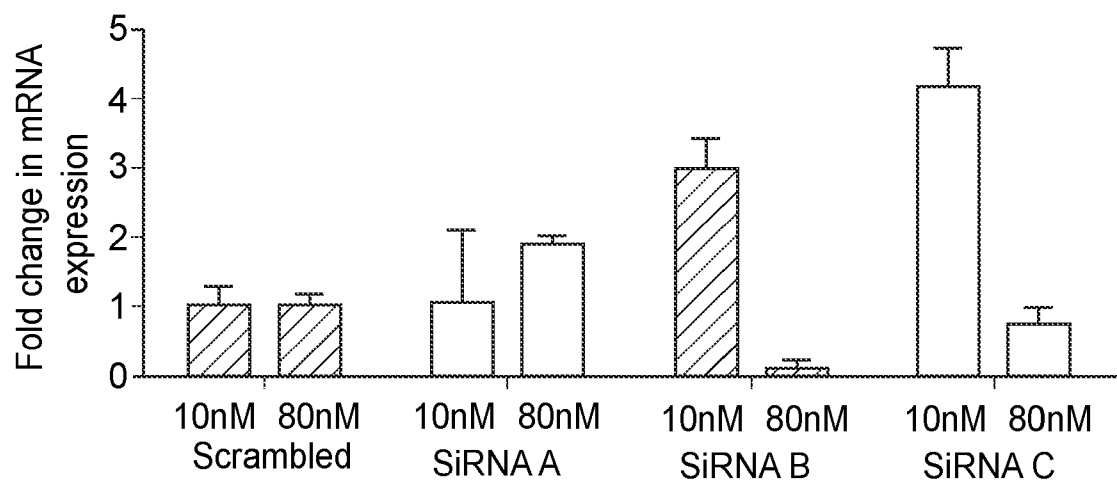
		Family 1, individual II:1			Family 1, individual II:2			Family 1, individual II:3		
	Region length	1x coverage %	5x coverage %	avg read depth	1x coverage %	5x coverage %	avg read depth	1x coverage %	5x coverage %	avg read depth
Shared homozygous region										
chr5: 123,576,980- 132,980,451	9,403,471	100	99.9	45.02	100	100	36.72	100	99.9	35.64

FIG. 8

ID	Age at last exam (years)	Sex	Age at HL onset	Severity	Progressive HL	Laterality
Family 1 Individual II:1	17	Female	Prelingual	Profound	yes	Bilateral
Family 1 Individual II:2	15	Male	Congenital	Profound	yes	Bilateral
Family 1 Individual II:3	3	Male	Congenital	Profound	no	Bilateral
Family 2 Individual II:1	28	Female	Congenital	Profound	no	Bilateral
Family 3 individual II:1	80	Male	Congenital	Profound	no	Bilateral
Family 3 individual II:3	74	Male	Congenital	Severe	no	Bilateral
Family 3 individual II:5	70	Female	Congenital	Severe	no	Bilateral
Family 3 individual II:7	68	Female	Congenital	Severe	no	Bilateral
Family 3 individual IV:1	13	Male	Congenital	Severe	no	Bilateral
Family 3 individual IV:3	19	Female	Congenital	Severe	no	Bilateral
Family 3 individual IV:5	17	Male	Congenital	Severe	no	Bilateral
Family 4 individual II:1	21	Female	Prelingual	Severe to profound	yes	Bilateral
Family 4 individual II:2	15	Female	Prelingual	Severe to profound	yes	Bilateral

FIG. 9

	Variant 1	Variant 2	Variant 3
Family ID	Family 1	Family 2	Families 3 and 4
c. DNA position	c.412_419delCGGTTTTG	c.144G>A	c.393G>T
a.a. notation	p.Arg138Valfs*10	p.Trp48*	p.Lys131Asn
Variant type	Frame-shift	Nonsense	Missense-Splice
gnomAD global MAF	absent	absent	absent
dbSNP	absent	absent	absent
GERP score	3.608 (Mean)	4.7199	4.7899
Revel score	NA	NA	NA
TraP Score	NA	NA	0.937

FIG. 10**FIG. 11**

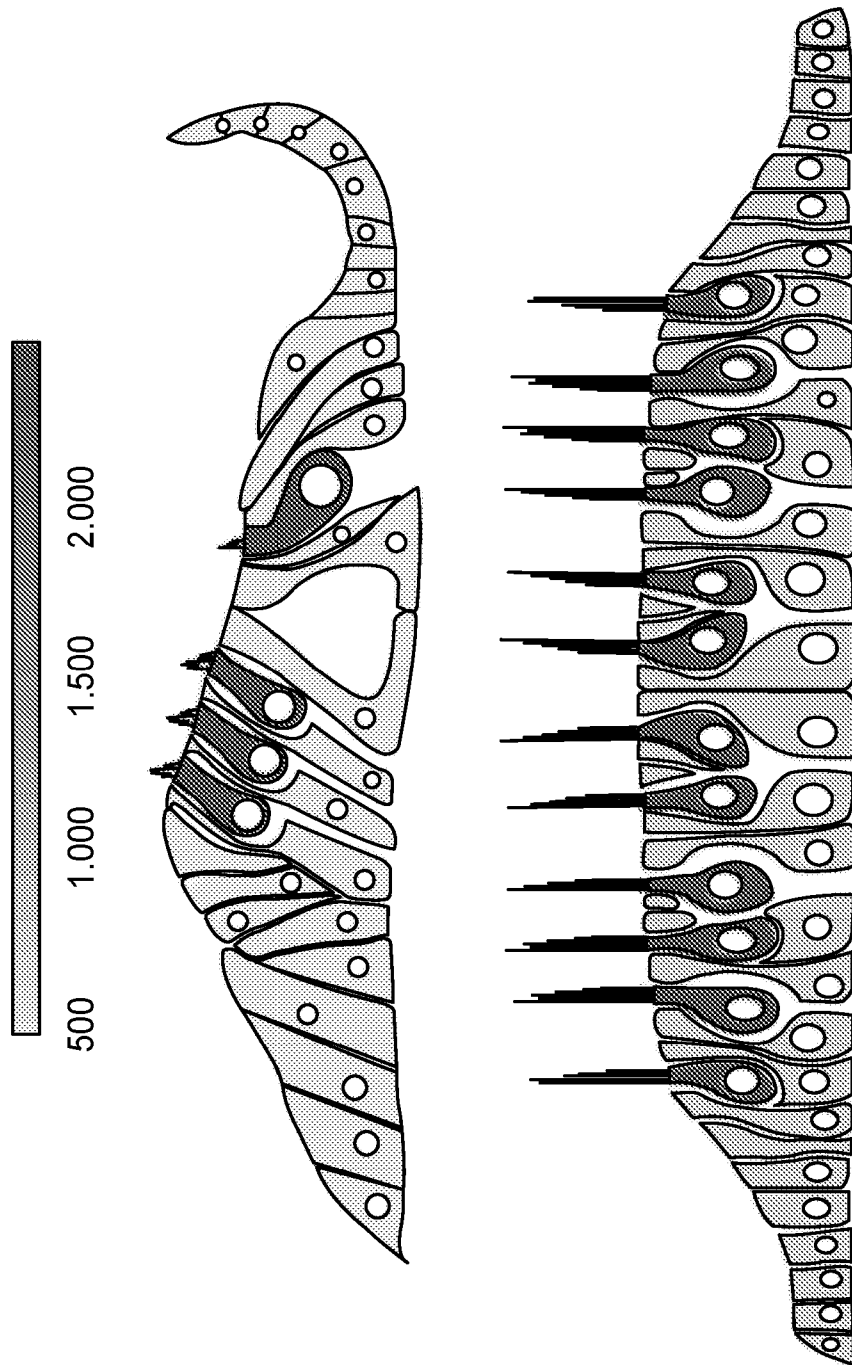


FIG. 12

Shared homozygous region	Region length	1x coverage %	5x coverage %	avg read depth x
chr1: 69,420,375-83,464,725	14,044,351	99.9	99.8	36.3
chr5: 128,253,080-141,730,596	13,477,517	100	99.9	37.2
chr10: 64,139,582-73,149,445	9,009,864	100	99.7	35.4
chr8: 118,676,625-126,694,576	8,017,952	100	100	34.4
chr10: 82,594,703-90,233,592	7,638,890	100	100	44.6
chr9: 214,706-6,553,054	6,338,349	99.9	99.8	35.3
chr10: 74,262,292-79,466,258	5,203,967	100	100	35.7
chr8: 46,870,230-52,072,125	5,201,896	100	100	36.8
chr1: 22,348,587-26,579,223	4,230,637	100	100	42.5
chr1: 33,482,763-37,403,014	3,920,252	100	100	41.5

FIG. 13

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2021/045031

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - A61K 9/00; A61K 38/17; A61P 27/16; C07H 21/04; C12N 15/09; C12N 15/11 (2021.01)

CPC - A61K 9/0046; A61K 9/0053; A61K 38/1709; A61P 27/16; C12Q 1/6883; C12Q 2600/156; C12Q 2600/16 (2021.08)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

see Search History document

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

see Search History document

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

see Search History document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 2017/0326254 A1 (MASSACHUSETTS EYE AND EAR INFIRMARY) 16 November 2017 (16.11.2017) entire document	1, 4-8, 11
Y	INGHAM et al. "Mouse screen reveals multiple new genes underlying mouse and human hearing loss," PLoS Biology. 11 April 2019 (01.04.2019), Vol. 17, Issue 4, Pgs. 1-30. entire document	1, 4-8, 11
A	US 2018/0258424 A1 (CODA BIOTHERAPEUTICS INC) 13 September 2018 (13.09.2018) entire document	1-13
A	DOHERTY et al. "GPSM2 Mutations Cause the Brain Malformations and Hearing Loss in Chudley-McCullough Syndrome," The American Journal of Human Genetics. 08 June 2012 (08.06.2012), Vol. 90, Issue 6, Pgs. 1088-1093. entire document	1-13
A	US 2018/0369414 A1 (MASSACHUSETTS EYE AND EAR INFIRMARY et al) 27 December 2018 (27.12.2018) entire document	1-13
A	WO 2019/078684 A2 (SEOUL NATIONAL UNIVERSITY HOSPITAL et al) 25 April 2019 (25.04.2019) entire document	1-13
P, A	WO 2020/237088 A1 (UNIVERSITY OF MIAMI) 26 November 2020 (26.11.2020) entire document	1-13

☐ Further documents are listed in the continuation of Box C.☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"D" document cited by the applicant in the international application

"E" earlier application or patent but published on or after the international filing date

"I" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

15 October 2021

Date of mailing of the international search report

NOV 26 2021

Name and mailing address of the ISA/US

Mail Stop PCT, Attn: ISA/US, Commissioner for Patents

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2021/045031

Box No. 1 Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:
 - a. ☒ forming part of the international application as filed:
 - ☒ in the form of an Annex C/ST.25 text file.
 - ☐ on paper or in the form of an image file.
 - b. ☐ furnished together with the international application under PCT Rule 13ter.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.
 - c. ☐ furnished subsequent to the international filing date for the purposes of international search only:
 - ☐ in the form of an Annex C/ST.25 text file (Rule 13ter.1(a)).
 - ☐ on paper or in the form of an image file (Rule 13ter.1(b) and Administrative Instructions, Section 713).
2. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.
3. Additional comments: