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(54) **MUTATION WITHIN THE CONNEXIN 26
GENE RESPONSIBLE FOR PRELINGUAL
NON-SYNDROMIC DEAFNESS AND
METHOD OF DETECTION**

Oct. 23, 2002, now Pat. No. 7,258,975, which is a continuation of application No. 09/485,415, filed on May 3, 2000, now Pat. No. 6,485,908, filed as application No. PCT/EP98/05175 on Aug. 14, 1998.

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(57) **ABSTRACT**

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A purified polynucleotide having a chain of nucleotides corresponding to a mutated sequence, which in a wild form encodes a polypeptide implicated in hereditary sensory defect, wherein said mutated purified polynucleotide presents a mutation responsible for prelingual non-syndromic deafness selected from the group consisting of a specific deletion of at least one nucleotide.

Related U.S. Application Data

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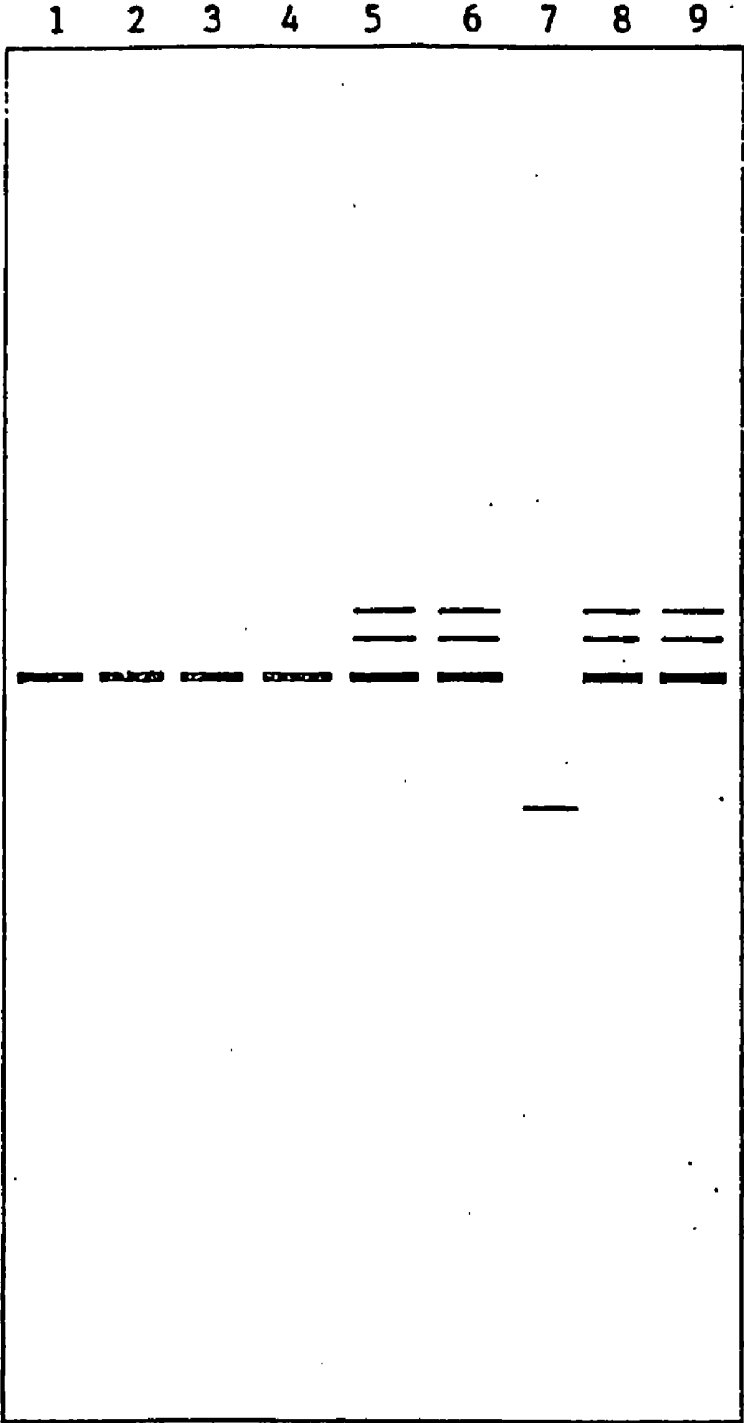


Figure 1

**MUTATION WITHIN THE CONNEXIN 26
GENE RESPONSIBLE FOR PRELINGUAL
NON-SYNDROMIC DEAFNESS AND
METHOD OF DETECTION**

[0001] This application is a continuation of application Ser. No. 10/278,089, filed Oct. 23, 2002, now U.S. Pat. No. 7,258,975, which is a continuation of Ser. No. 09/485,415, filed May 3, 2000, now U.S. Pat. No. 6,485,908, which is a 371 of PCT/EP98/05175, filed Aug. 14, 1998, which claims the benefit of priority to U.S. Provisional Application No. 60/055,863, filed Aug. 15, 1997, all of which are incorporated herein by reference.

BACKGROUND OF THE INVENTION

[0002] The present invention concerns a mutation responsible for autosomal prelingual non-syndromic deafness and a method for the detection of this hereditary sensory defect for homozygous and heterozygous individuals. The invention concerns more particularly a specific deletion of at least one nucleotide in the connexin 26 (Cx 26) gene and especially in a guanosine rich region, notably between the nucleotides 27 and 32. The invention is also directed to the use of polynucleotide, or fragments thereof, for example as tools useful for the in vitro detection of a mutation of a gene belonging to the Cx26 gene family.

[0003] Profound or severe prelingual deafness affects one child in a thousand in developed countries (Morton N E. Genetic epidemiology of hearing impairment. In *Genetics of hearing impairment*. (The New York Acad Sci, New York 1991; 630:16-31). It is a major handicap as it impedes language acquisition.

[0004] According to studies performed in a U.S. population of children with non-syndromic (isolated) prelingual deafness and in whom an obvious environmental cause has been excluded, it is estimated that up to two-thirds of the cases have a genetic basis (Marazita M L, Ploughman L M, Rawlings B, Remington E, Amos K S, Nance W E. Genetic epidemiological studies of early-onset deafness in the U.S. school-age population. *Am J Med Genet*. 1993; 46:486-91). These forms are mainly sensorineural and are almost exclusively monogenic. The major mode of inheritance is autosomal recessive (DFNB), involving 72% to 85% of cases, this fraction increasing to 90% when only profound deafness is taken into account. Autosomal recessive prelingual deafness is known to be genetically highly heterogeneous. Estimates of the number of DFNB loci vary from thirty to one hundred (Petit C. Autosomal recessive non-syndromal hearing loss. In *Genetics and Hearing Impairment*. Martini A, Read A P, Stephens D, eds (Whurr, London) 1996; 197-212), for a review), of which fourteen have so far been mapped to the human chromosomes (Petit C. Genes responsible for human hereditary deafness: symphony of a thousand. *Nature Genet*. 1996; 14:385-91) for review, (Verhoeven K, Van Camp G, Govaerts P J, et al. A gene for autosomal dominant non-syndromic hearing loss (DFNA12) maps to chromosome 11q22-24. *Am J Hum Genet*. 1997; 60:1168-74 and Campbell DA, McHale D P, Brown K A, et al. A new locus for non-syndromal autosomal recessive sensorineural hearing loss (DFNB16) maps to human chromosome 15q21-q22. *J Med Genet*. 1997; in press).

[0005] A majority of the families attending genetic counseling clinics consist of normal hearing parents with a single deaf child who wish to know the risk of recurrence of the

defect. In most cases, given the major role of environmental causes of prelingual deafness, it is not usually possible even to recognize whether the hearing loss is of genetic origin. Genetic counseling in such families would be greatly improved by an ability to detect DFNB mutations. In this respect, the high genetic heterogeneity of the condition represents a major obstacle.

[0006] After the initial identification of the DFNB1 locus on 13q11 in a large consanguineous Tunisian family (Guilford P, Ben Arab S, Blanchard S, et al. A non-syndromic form of neurosensory, recessive deafness maps to the pericentromeric region of chromosome 13q. *Nature Genet*. 1994; 6:24-8), two studies performed on New Zealand/Australian families (Maw M A, Allen-Powell D R, Goodey R J, et al. The contribution of the DFNB1 locus to neurosensory deafness in a Caucasian population. *Am J Hum Genet*. 1995; 57:629-35), and on Italian/Spanish families (Gasparini P, Estivill X, Volpini V, et al. Linkage of DFNB1 to non-syndromic neurosensory autosomal-recessive deafness in Mediterranean families. *Eur J Hum Genet*. 1997; 5:83-8) suggested that this locus might be a major contributor to prelingual deafness in these populations, although individual lod scores obtained in these families were not significant owing to the small size of these families.

[0007] Recently, the Cx26 gene, which encodes a gap junction protein, connexin 26, has been shown to underlie DFNB1 deafness. Two different G→A substitutions resulting in premature stop codons in three DFNB1 linked consanguineous Pakistani families have been reported (Kelsell D P, Dunlop J, Stevens H P, et al. Connexin 26 mutations in hereditary non-syndromic sensorineural deafness. *Nature* 1997; 387:80-3). These two substitutions were identified, respectively, at codon 77 and at codon 24. This result has offered the opportunity directly to assess this hypothesis.

[0008] The difficulties encountered in genetic counseling for prelingual non-syndromic deafness due to the inability to distinguish genetic and non-genetic deafness in the families presenting a single deaf child was one of the reasons that led the inventors to undertake a characterization of the spectrum and prevalence of mutations present in the Cx26 gene in 35 families from several parts of the world with autosomal recessive prelingual deafness.

SUMMARY OF THE INVENTION

[0009] The determination of a mutation in the Cx26 gene has notably rendered possible the use of a detection probe as a tool for the identification of a specific form of autosomal prelingual non-syndromic deafness, and more particularly the useful role of a newly identified 30delG (a G deletion at position 30; position 1 being the first base of the initiator codon) mutation in such families. This invention establishes that the contribution of the DFNB1 locus predominantly results essentially from the 30delG mutation. It is now believed that the 30delG accounts for about three-quarters of all recessive DFNB1 mutations.

[0010] The invention is thus intended to provide a purified polynucleotide having a chain of nucleotides corresponding to a mutated sequence, which in a wild form encodes a polypeptide implicated in hereditary sensory defect. The mutated purified polynucleotide presents a mutation responsible for prelingual non-syndromic deafness.

[0011] The invention also provides oligonucleotides comprising of 15 to 50 consecutive nucleotides of the mutated purified polynucleotide that are useful as primers or as probes.

[0012] In addition, the invention aims to supply a method and a kit for the detection of the hereditary sensory defect for homozygous as heterozygous individuals.

[0013] According to the invention, the purified polynucleotide having a chain of nucleotides corresponding to a mutated sequence, which encodes in a wild form a polypeptide implicated in hereditary sensory defect, presents a mutation responsible for prelingual non-syndromic deafness selected from the group consisting of a specific deletion of at least one nucleotide.

[0014] By mutation, according to the invention it means a specific deletion of at least one nucleotide. Thus, a mutated sequence means a polynucleotide sequence comprising at least a mutation.

[0015] A chain of nucleotides, according to the invention, means a polynucleotide, which encodes not necessarily a polypeptide, but which presents between 27 and 2311 nucleotides linked together.

[0016] The invention particularly concerns a purified polynucleotide wherein, the specific mutation is a deletion located in a region encoding connexin 26 of chromosome 13q11-12, preferably located in a guanosine rich region starting at nucleotide 27 preferably at nucleotide 30, and extending to nucleotide 32 or nucleotide 35, all the recited nucleotides being inclusive. More particularly according to the invention, the specific deleted purified polynucleotide encodes for a truncated polypeptide.

[0017] By truncated polypeptide, according to the invention it means a fragment of the polypeptide, which does not present the properties of the wild form of the polypeptide either in length, in amino acid composition, or in functional properties.

[0018] A preferred embodiment of a specific deletion is a guanosine deletion at position 30, also called "30delG mutation". Another preferred embodiment of the specific deletion is a 38 by deletion beginning at position 30.

[0019] The invention also includes a purified polynucleotide, which hybridizes specifically with any one of the polynucleotides as defined above under the following stringent conditions: at low temperatures between 23° C. and 37° C., in the presence of 4×SSC buffer, 5×Denhardt's solution, 0.05% SDS, and 100 µg/ml of salmon sperm DNA. (1×SSC corresponds to 0.15 M NaCl and 0.05M sodium citrate; 1×Denhardt's solution corresponds to 0.02% Ficoll, 0.02% polyvinylpyrrolidone and 0.02% bovine serum albumin).

[0020] The invention also concerns an oligonucleotide useful as a primer or as a probe comprising 15 to 50 consecutive nucleotides of the polynucleotide according to any one of the polynucleotides as defined above. The oligonucleotide sequence is selected from the following group:

[0021] A first couple:

(SEQ ID No. 1)
5' -TCTTTTCCAGAGCAAACCGCC-3'

(SEQ ID No. 2)
5' -TGAGCACGGGTTGCCTCATC-3'.

[0022] The length of the PCR product has been obtained from 285 by in length;

[0023] A second couple allowing to explore the other part of the reading frame:

(SEQ ID No. 3)
5' -GACACGAAGATCAGCTGCAG-3'

(SEQ ID No. 4)
5' -CCAGGCTGCAAGAACGTGTG-3'

[0024] A third couple:

(SEQ ID No. 9)
5' -CTAGTGATTCCTGTGTTGTGTGC-3';
and

(SEQ ID No. 10)
5' ATAATGCGAAAAATGAAGAGGA-3'
and

[0025] A fourth couple:

(SEQ ID No. 14)
5' -CGCCCCCGCGCCCCCGCCCCGCCCCCGCCCCCGCCCCCTAGTGA
TTCCTGTGTTGTGTGC-3';
and

(SEQ ID No. 10)
5' ATAATGCGAAAAATGAAGAGGA-3'.

[0026] Another oligonucleotide useful as a probe is selected from the following group:

(SEQ ID No. 5)
5' -AGACGATCCTGGGGGTGTAACAAA-3'

(SEQ ID No. 6)
5' -ATCCTGGGGGTGTA-3'

(SEQ ID No. 7)
5' -AGACGATCCTGGGGGCTCACCGTCTC-3'.

[0027] In addition, the invention concerns a method for the detection of an hereditary sensory defect, namely autosomal prelingual non-syndromic deafness, for homozygous as heterozygous individuals in a biological sample containing DNA, comprising the steps of

[0028] a) bringing the biological sample into contact with a oligonucleotide primers as defined above, the DNA contained in the sample having been optionally made available to hybridization and under conditions permitting a hybridization of the primers with the DNA contained in the biological sample;

[0029] b) amplifying the DNA;

[0030] c) revealing the amplification products;

[0031] d) detecting the mutation.

[0032] Step d) of the above-described method may comprise a Single-Strand Conformation Polymorphism (SSCP), a Denaturing Gradient Gel Electrophoresis (DGGE) sequencing (Smith, L. M., Sanders, J. Z., Kaiser, R. J., Fluorescence detection in automated DNA sequence analysis. *Nature* 1986; 321:674-9); a molecular hybridization capture probe or a temperature gradient gel electrophoresis (TGGE).

[0033] Step c) of the above-described method may comprise the detection of the amplified products with an oligonucleotide probe as defined above.

[0034] According to the invention, a biological sample can be a blood sample extracted from people suffering from any kind of deafness with any criteria as follows: neurosensory or mixed isolated deafness, advanced or not, at any degree of severity, concerning familial or sporadic case, or individuals exposed to noise, or individuals suffering from a low acoustic, or individuals susceptible to carry an anomaly in the gene, or from an embryo for antenatal diagnostic.

[0035] Another aim of the invention comprises a method for the detection of an hereditary sensory defect, the autosomal prelingual non-syndromic deafness, for homozygous and heterozygous individuals in a biological sample containing DNA, comprising the steps of:

[0036] a) bringing the biological sample into contact with an oligonucleotide probe according to the invention, the DNA contained in the sample having been optionally made available to hybridization and under conditions permitting a hybridization of the primers with the DNA contained in the biological sample; and

[0037] b) detecting the hybrid formed between the oligonucleotide probe and the DNA contained in the biological sample.

[0038] Step b) of the above-described method may consist in a single-strand conformation. Polymorphism (SSCP), a denaturing gradient gel electrophoresis (DGGE) or amplification and sequencing.

[0039] The invention also includes a kit for the detection of an hereditary sensory defect, the autosomal prelingual non-syndromic deafness, for homozygous as heterozygous individuals, said kit comprising:

[0040] a) oligonucleotides according to the invention;

[0041] b) the reagents necessary for carrying out DNA amplification; and

[0042] c) a component that makes it possible to determine the length of the amplified fragments or to detect a mutation.

BRIEF DESCRIPTION OF THE DRAWINGS

[0043] This invention will be more described in greater detail by reference to the drawings in which:

[0044] FIG. 1 depicts the results of temperature gradient gel electrophoresis for detection of mutants in which:

[0045] Lanes 1 and 2: DNA from normal patients.

[0046] Lanes 3 and 4: DNA from homozygous patients with 30delG mutation.

[0047] Lanes 5 and 6: DNA from heterozygous patients.

[0048] Lane 7: PCR control without DNA.

[0049] Lane 8: PCR fragment amplified from a normal DNA and hybridized with a standard DNA fragment harboring the 30delG mutation.

[0050] Lane 9: PCR fragment amplified from a mutant homozygous DNA and hybridized with a normal standard DNA fragment harboring the guanine 30.

DETAILED DESCRIPTION OF THE INVENTION

[0051] Prelingual non-syndromic (isolated) deafness is the most frequent hereditary sensory defect in children. The inheritance in most is autosomal recessive. Several dozens of genes might be involved, only two of which, DFNB1 and DFNB2, have so far been identified (Kelsell, D. P., et al., Connexin 26 mutations in hereditary non-syndromic sensorineural deafness. *Nature* 1997; 387:80-3; Liu, X-Z, et al., Mutations in the myosin VIIA gene cause non-syndromic recessive deafness, *Nature Genet.* 1997; 16:188-90; and Weil,

D., et al., The autosomal recessive isolated deafness, DFNB2, and the Usher 1B syndrome are allelic defects of the myosin-VIM. *Nature Genet.* 1997; 16:191-3). A search was made searched for mutations in the gene encoding connexin 26, Cx26, which has recently been shown to be responsible for DFNB1. Mutation analysis of Cx26 was performed by PCR amplification on genomic DNA and sequencing of the single coding exon.

Example 1

Patients

[0052] Thirty-five affected families from various geographical regions, mainly France, New Zealand and Australia, Tunisia and Lebanon, were studied. They could be classified into three categories: (1) consanguineous families each having a significant linkage to the DFNB1 locus; (2) small non-consanguineous families in which linkage analysis was compatible with the involvement of DFNB1; and (3) small families in which no linkage analysis had been undertaken.

[0053] The first category consists of six large families living in geographically isolated regions. Five were from Tunisia, two from the north and three from the south. Linkage to the DFNB1 locus in the two families from northern Tunisia (families 20 and 60) had previously been reported (Guilford P, Ben Arab S, Blanchard S, et al., A non-syndromic form of neurosensory, recessive deafness maps to the pericentromeric region of chromosome 13q. *Nature Genet.* 1994; 6:24-8); the three families from southern Tunisia (S15, S19 and ST) and the family from Lebanon (LH) comprise total of three, five, two, and five deaf children, respectively, the deafness being of severe or profound degree. The marriages were between first cousins (S15, ST and LH) and between first and second cousins (S19). Linkage analysis of these six families resulted in individual lod scores ranging from 2.5 to 10 with polymorphic markers from the DFNB1 region (D13S175, D13S141, D13S143 and D13S115).

[0054] The second category of patients comprises seven New Zealand families with at least two deaf siblings (families 51, 1160, 1548, 1608, 1773, 1873, 1877) and one Australian (9670) family. Family 1608 was atypical in that four siblings sharing the same DFNB1 marker haplotypes had a mild to moderate deafness (severe at high frequency), with the child of one of them being profoundly deaf. In family 1873, the unrelated parents (individuals 11.2 and 11.3) were deaf as well as their two children, and we have therefore considered this as two families, bringing to nine the total of independent families. Apart from families 1608 and 1873, no parent acknowledged any hearing impairment. These nine families showed cosegregation between deafness and polymorphic markers of the DFNB1 region with maximum individual lod scores ranging from 0.6 to 1.2. Ten other families in the original study of Maw et al. (Maw M A, Allen-Powell D R, Goodey R J, et al. The contribution of the DFNB1 locus to neurosensory deafness in a Caucasian population. *Am J Hum Genet.* 1995; 57:629-35) had shown no cosegregation and one other cosegregating family was not tested for Cx26 mutations. The New Zealand families were all of Caucasian origin with no known Polynesian admixture. According to the antecedent family names, the ancestral proportion among the families reflected that of the general Caucasian New Zealand population with the great predominance being of Anglo-Celtic patrimony and a small fraction due to migration from continental Europe. Neither parental consanguinity, nor links

between any of the families were recognized. In the Australian case, the father was from Northern Ireland and the mother from Yorkshire, England.

[0055] The third category is composed of nineteen families living in France and two in New Zealand, each with at least two children having a severe to profound deafness. No parent acknowledged any hearing impairment, except for the mother in family P16 and the father in family P17 who had moderate and progressive high-frequency hearing loss. Five of these families had foreign ancestors from Lebanon (family P3), Turkey (family P4), Portugal (family P9), Algeria (family P14) and Poland (father in family P16). In two of the families (P7 and P14), the parents were distantly related.

Example 2

[0056] Amplification of the coding exon of Cx26 PCRs were carried out on genomic DNA using a set of primers that allowed the amplification of the entire coding sequence of the Cx26 gene, which consists of a single coding exon (Kelsell D P, Dunlop J, Stevens H P, et al. Connexin 26 mutations in hereditary non-syndromic sensorineural deafness. *Nature* 1997; 387: 80-3). Primer sequences were as follows:

(SEQ ID No. 1)
5' - TCTTTTCCAGAGCAAAACCGCC - 3' and

(SEQ ID No. 2)
5' - TGAGCACGGTTCCTCATC - 3'.

[0057] PCR conditions were: 35 cycles of 95° C., 1 min; 58° C., 1 min; 72° C., 2 min. The PCR product obtained was 777 by in length.

Example 3

DNA Sequencing

[0058] Sequencing of the PCR products was performed as previously described (Smith L M, Sanders J Z, Kaiser R J, et al., Fluorescence detection in automated DNA sequence analysis, *Nature* 1986; 321:674-9) using the dideoxy chain terminator method on an Applied Biosystems DNA sequencer ABI373 with fluorescent dideoxynucleotides. The primers used were the same as those for the PCR amplification plus two internal primers

(SEQ ID No. 3)
5' - GACACGAAGATCAGCTGCAG - 3' and

(SEQ ID No. 4)
5' - CCAGGCTGCAAGAACGTGTG - 3'.

Example 4

Mutations in Consanguineous Tunisian and Lebanese DFNBI Families

[0059] In these families the involvement of the DFNBI locus could be demonstrated by linkage analysis. In four of the five families from Tunisia (S15, S19, 20, and 60) and in the Lebanese family (LH), the same mutation was detected in all affected children on both Cx26 alleles, namely, a deletion of a guanosine (G) in a sequence of six G extending from

position 30 to 35 (position 1 being the first base of the initiator codon) (Table 1). This mutation is hereafter referred to as 30delG mutation according to the nomenclature proposed by Beaudet and Tsui ((Beaudet A L, Tsui L-C. A suggested nomenclature for designating mutations, *Hum Mutation* 1993; 2: 245-8)). It creates a frameshift, which results in a premature stop codon at nucleotide position 38. The mutation segregating in the fifth family from Tunisia (ST) was identified as a G to T transversion at nucleotide position G39 creating a premature stop codon (GAG→TAG) at codon 47, and was designated E47X. In each family, normal hearing parents were found to be heterozygous for the corresponding mutation.

Example 5

Mutations in Small Nonconsanguineous New Zealand and Australian Families Consistent with DFNBI Linkage

[0060] In these families, segregation analysis has previously been reported as compatible with the involvement of the DFNBI locus (Maw M A, Allen-Powell D R, Goodey R J, et al. The contribution of the DFNBI locus to neurosensory deafness in a Caucasian population. *Am J Hum Genet.* 1995; 57: 629-35). The deaf individuals from five of the nine families (51, 1160, 1608 (III.20), 1873 (II.3) and 1877) were homozygous for the 30delG mutation. The deaf children from family 1773 were heterozygous for 30delG. Deaf individual II.2 from family 1873 (see "subjects" and Table 1) was heterozygous for a deletion of 38 by beginning at nucleotide position G30, designated 30del38. No other mutation was detected in the deaf children of family 1773 and the deaf individual (II.2) in family 1873. Nevertheless, in this last individual, a deletion of the polymorphic marker immediately proximal to the Cx26 gene (locus D13S175) had previously been observed (Maw M A, Allen-Powell D R, Goodey R J, et al. The contribution of the DFNBI locus to neurosensory deafness in a Caucasian population. *Am J Hum Genet.* 1995; 57: 629-35), which may indicate that a DNA rearrangement has impaired the functioning of the other Cx26 allele of the gene in cis. In family 9670, compound heterozygosity for a missense mutation (R184P) and an in frame single-codon deletion (delE138) was observed in affected siblings. In only one family (1548) was no Cx26 mutation detected. Results are summarized in Table 1.

Example 6

Mutations in Small Families Uncharacterized for DFNBI Linkage Living in France and New Zealand

[0061] Nineteen families (P1 to 17, L14190 and L13131) living in France and two in New Zealand (families 1885 and 2254) were studied. In these families, cosegregation of the deafness with polymorphic markers had not been analysed. Deaf children from six of the twenty-one families (P1, P3, P5, P9, P10, and P16) were found to be homozygous for the mutation 30delG. In five additional families (P6, P11, P14, P17, and 1885), deaf children were heterozygous for this mutation; no other mutation was detected in these families. In the ten remaining families, no mutation in the Cx26 gene was found.

Example 7

Molecular Hybridization Using Allele-Specific Capture Probes

[0062] Molecular hybridization capture probe (see, e.g., D. Chevrier et al. PCR product quantification by non-radioactive hybridization procedures using an oligonucleotide covalently bound to microwells. *Molecular and Cellular Probes* 1993; 7: 187-197 and D Chevrier et al. Rapid detection of *Salmonella* subspecies I by PCR combined with non-radioactive hybridization using covalently immobilized oligonucleotide on a microplate. *FEMS Immunology and Medical Microbiology* 1995; 10: 245-252 each of which is incorporated by reference herein) permit specific detection of the 30delG mutation. The technique has been adapted to permit rapid diagnosis of pre-lingual non-syndromic deafness caused by the 30delG mutation. The technique provides certain advantages in a clinical setting because it uses stable, nonradioactive molecules, it can be easily automated, and it is well adapted to large scale analysis.

[0063] Using primers designed for PCR amplification, the region of interest in the Cx26 gene is amplified from genomic DNA samples. The primer sequences are as follows:

(SEQ ID No. 9)
CONN3: 5' -CTAGTGATTCCTGTGTTGTGTGC-3'

(SEQ ID No. 10)
CONN4: 5' ATAATGCGAAAAATGAAGAGGA-3'

[0064] PCR is performed with the CONN3 (SEQ ID No. 9) and CONN4 (SEQ ID No. 10) primers (1 μ M each), an aliquot of the DNA to be analyzed (2 μ l, 100-300 ng), 1.5 mM MgCl₂, 200 μ M dNTP, and Taq polymerase. The amplification program consists of the following steps: 1) 95° C., 5 min; 2) addition of enzyme, 95° C., 1 min; 3) 60° C., 1 min (ramp rate=0.25° C./s); 4) 72° C., 1 min; 5) repeat steps 2 to 4 for 40 cycles; and 6) 72° C., 10 min. PCR products are verified by a rapid gel electrophoresis.

[0065] The amplified PCR product contains either the normal or the mutant Cx26 sequence. To distinguish between the normal and mutant sequence, two capture probes are designed. The sequences of these two capture probes are as follows:

[0066] For detection of normal sequence:

(SEQ ID No. 11)
CONN6: 5' -AAAAAAAATCCTGGGGGTGTG-3'

[0067] For detection of mutant sequence:

(SEQ ID No. 12)
CONN7: 5' -AAAAAAAATCCTGGGGGTGTGA-3'

Each capture probe must be 22 nucleotides long. Furthermore, to be efficient, the capture probe must include an A₇ spacer at its 5' end and a hybridization region of 15 bases. Such a capture probe is able to specifically differentiate the mutant sequence from the normal sequence. Thus, CONN6 (SEQ ID No. 11) is designed to specifically hybridize with the normal sequence, whereas CONN7 (SEQ ID No. 12) is designed to specifically hybridize with the mutant sequence.

[0068] Before attaching the capture probes to a microtiter plate, they are phosphorylated at their 5' ends. The phospho-

rylation is carried out for 1 hour at 37° C. in presence of 20 nmoles of CONN6 (SEQ ID No. 11) or CONN7 (SEQ ID No. 12) oligonucleotides, 100 μ M ATP, 10 units T4 polynucleotide kinase in 200 μ l of buffer (50 mM Tris-HCl pH 7.4; 10 mM MgCl₂ 5 mM dithiothreitol; and 1 mM spermidine). The mixture is heated for 10 min. at 68° C. to inactivate the T4 polynucleotide kinase, then the oligonucleotide is precipitated by adding 145 μ l of 10 M CH₃COONH₄, 15 μ l H₂O, and 800 μ l iced ethanol. After a 30 min. incubation in ice, the mixture is centrifuged for 20 min. at 12,000 \times g at 4° C. The resulting pellet is washed with 500 μ l iced ethanol (70%) and dissolved in 800 μ l of TE buffer. The phosphorylated oligonucleotide concentration is determined by optical density at 260 nm.

[0069] Before attaching the phosphorylated oligonucleotides to microplates, they are denatured by heating at 95° C. for 10 min. and rapidly cooled in ice to avoid the formation of secondary structure. 500 ng of phosphorylated CONN6 (SEQ ID No. 11) or CONN7 (SEQ ID No. 12) and 1 μ l of 1 M 1-methylimidazole, pH 7, is added to each well of a microplate, which is kept on ice. The total volume of each well is adjusted to 70 μ l with distilled water, before adding 30 μ l of a cold, 1-ethyl-3(3-dimethylaminopropyl) carbodiimide solution (167 mM). The microplate is covered and incubated for 5 hours at 50° C. in an incubator (Thermomix® from Lab-systems). After the 5-hour incubation, the microplate is washed three times with a warm solution (50° C.) of 0.4 N NaOH containing 0.25% SDS. The microplate is incubated for 5 min. with the same warm solution and washed again with warm NaOH/SDS (50° C.). Finally, the microplate is washed five times with TE buffer. The coated microplate can be kept several months at 4° C., if the wells are filled with TE buffer.

[0070] The amplified sequences from the genomic DNA samples are incubated with a biotinylated detection probe in the coated microplates. Unlike the capture probes, which are allele specific, the detection probe can hybridize with both the normal and mutant sequences. The sequence of the detection probe is:

(SEQ ID No. 13)
CONN12: 5' -CAGCATTGGAAAGATCTGGCTCA-3'

The amplified sequences and the detection probe, which is biotinylated at its 5' end, are denatured directly in the microplates by successively adding to each well: 95 μ l of water, 5 μ l of PCR reaction, 40 μ l of biotinylated probe (SEQ ID No. 13) at 22 nM diluted in water, and 14 μ l 1 N NaOH. After 10 min., 21 μ l of 1 M NaH₂PO₄ and 1% Sarkosyl is added to each well to bring the total volume to 175 μ l per well. The final concentration of the detection probe is 5 nM. The microplate is covered and incubated overnight at 40° C. in an incubator (Thermomix® from Lab-systems) and then extensively washed (5 times) with TBS-Tween to remove the excess biotinylated probe (SEQ ID No. 13).

[0071] An immunoenzymatic method is used to detect the hybridized probe. Each well receives 100 μ l of the conjugate (Extravidine—alkaline phosphatase, Sigma E-2636) diluted 1/4000 in TBS-BSA-Tween. The microplate is covered and incubated for 1 hour at 25° C. Following the incubation, the microplate is washed 5 times with TBS-Tween. Then 200 μ l of preheated (37° C.) substrate (7.5 mg para-nitro-phenylphosphate in 20 ml of the following buffer: 1 M diethanolamine pH 9.8 containing 1 mM MgCl₂) are added to each well.

hour. Following electrophoresis, the gel is incubated for 6 min. in 1.25×TAE containing 25 µg/ml ethidium bromide. Excess ethidium bromide is removed by a 20 min. wash in 1.25×TAE, and the DNA fragments are visualized with a UV transilluminator.

[0085] A typical TGGE result is represented in FIG. 1. The amplified DNA from homozygous patients (normal or mutant) produces only one band. The amplified DNA from heterozygous patients results in three different fragments in the polyacrylamide gel. The more intense band, which migrates more rapidly, corresponds to both homoduplexes, which cannot be separated in this gel. The other two bands, which migrate more slowly, correspond to both kinds of heteroduplexes.

[0086] The DNA of normal homozygous patients can be differentiated from the DNA of mutant homozygous patients by analyzing the PCR fragments that were subjected to the conditions that permitted heteroduplex formation. Heteroduplexes form when the PCR amplified fragment from a normal homozygous genome is mixed with sequences from a known, mutant homozygous genome, or when the PCR amplified

fragment from a mutant homozygous genome is mixed with sequences from a known, normal homozygous genome. These heteroduplexes are visible by TGGE analysis. Consequently, the DNA of normal and mutant homozygous patients can be easily differentiated by this technique using the primers described in the present study.

[0087] In all the known DFNB1 families (6/6), in all but one (8/9) of the putatively DFNB1-linked families, and in about half (11/21) of the families not tested for DFNB1 linkage, a mutation in Cx26 was detected. Furthermore, of the 44 chromosomes reckoned to be independent upon which a Cx26 mutant allele was identified or inferred, 33 (75%) were found to carry the same deletion of a guanosine, G, at position 30 (30delG).

[0088] Cx26 mutations represent a major cause of recessively inherited prelingual deafness and would be implicated in about half of cases in the examined populations. In addition, one specific mutation, 30delG, accounts for the majority (about three-quarters in our series) of the Cx26 mutant alleles.

[0089] The wild type connexin 26 gene published in LEE S. W. et al. (1992) *J. Cell Biol.* 118: 1213-1221 has the following sequence:

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1  GATTTAATCC TATGACAAAC TAAGTTGGTT CTGCTTTCAC CTGTTTTGGT
51  GAGGTTGTGT AAGAGTTGGT GTTTGCTCAG GAAGAGATT AAGCATGCTT
101 GCTTACCCAG ACTCAGAGAA GTCTCCCTGT TCTGTCTAG CTATGTTCTT
151 GTGTTGTGTG CATTTCGCTT TTCCAGAGCA AACCGCCAG AGTAGAAGAT
201 GGATTGGGGC ACGCTGCAGA CGATCCTGGG GGGTGTGAAC AAACACTCCA
251 CCAGCATTGG AAAGATCTGG CTCACCGTCC TCTTCAATTT TCGCATTATG
301 ATCCTCGTTG TGGCTGCAA GGAGGTGTGG GGAGATGAG AGGCCGACTT
351 TGCTGCAAC ACCCTGCAGC CAGGCTGCAA GAACGTGTGC TACGATCACT
401 ACTTCCCAT CTCCCACATC CGGCTATGGG CCCTGCAGCT GATCTTCGTG
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751 CCCACGGAGA AGACTGTCTT TCACAGTGTT CATGATTGCA GTGTCTGGAA
801 TTTGCATCCT GCTGAATGTC ACTGAATTGT GTTATTGCT AATTAGATAT
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1001 AACCATTTGA AACCCCTGTA GGCCTCAGGT GAAACTCCAG ATGCCACAAT
1051 GAGCTCTGCT CCCCTAAAGC CTCAAAACAA AGGCCTAATT CTATGCCTGT
1101 CTTAATTTTC TTTCACTTAA GTTAGTTCCA CTGAGACCCC AGGCTGTTAG
1151 GGGTTATTGG TGTAAGGTAC TTTCATATTT TAAACAGAGG ATATCGGCAT
1201 TTGTTTCTTT CTCTGAGGAC AAGAGAAAAA AGCCAGGTTT CACAGAGGAC

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1251 ACAGAGAAGG TTTGGGTGTC CTCCTGGGGT TCTTTTGGCC AACTTTCCCC
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 1351 CTAACAGTGG ACAAAAGTAC CAGTGCCTTA AACTCTGTTA CACTTTTTGG
 1401 AAGTGAAAAC TTTGTAGTAT GATAGGTAT TTTGATGTAA AGATGTTCTG
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 2251 AACTAAAATA ATTTTGTAAT GTATCAAATA CATTTAAAAC ATTAAATAT
 2301 AATCTCTATA AT

[0090] The wild type connexin 26 gene is published in D. T. Kiang et al. (1997) Gene 199 (1-2): 165-171.

TABLE 1

Mutations in the Cx26 coding exon in individuals affected with familial forms of prelingual deafness			
Family (geographical origin)	30delG mutation	Other mutation	Deafness
DFNB1-linked families			
S15 (sTu)	homozygous	—	profound
S19 (sTu)	homozygous	—	profound
ST (sTu)	—	homozygous E47X	profound
20 (nTu)	homozygous	—	profound
60 (nTu)	homozygous	—	profound
LH (Leb)	homozygous	—	severe-profound
Families consistent with DFNB1 linkage			
51 (NZ)	homozygous	—	severe-profound
1160 (NZ)	homozygous	—	moderate-severe*
1548 (NZ)	—	—	profound
1608 (NZ)	homozygous	—	profound**
1773 (NZ)	heterozygous	—	profound
1873 individual II.3 (NZ)	homozygous	—	moderate

TABLE 1-continued

Mutations in the Cx26 coding exon in individuals affected with familial forms of prelingual deafness			
Family (geographical origin)	30delG mutation	Other mutation	Deafness
1873 individual II.2 (NZ)	—	heterozygous 30del38	profound
1877 (NZ)	homozygous	—	profound
9670 (Aust)	—	delE118/R148P	moderate-severe
Families uncharacterized for DFNB1 linkage			
P1 (Fr)	homozygous	—	severe-profound
P2 (Fr)	—	—	profound
P3 (Leb)	homozygous	—	severe-profound
P4 (Tur)	—	—	severe-profound
P5 (Fr)	homozygous	—	profound
P6 (Fr)	heterozygous	—	severe-profound
P7 (Fr)	—	—	moderate
P8 (Fr)	—	—	moderate
L13131 (Fr)	—	—	profound
L14190 (Fr)	—	—	mild-moderate
P9 (Por)	homozygous	—	severe-profound
P10 (Fr)	homozygous	—	severe-profound
P11 (Fr)	heterozygous	—	moderate-severe
P12 (Fr)	—	—	severe-profound

TABLE 1-continued

Mutations in the Cx26 coding exon in individuals affected with familial forms of prelingual deafness			
Family (geographical origin)	30delG mutation	Other mutation	Deafness
P13 (Fr)	—	—	profound
P14 (Alg)	heterozygous	—	moderate-severe
P15 (Fr)	—	—	severe-profound
P16 (mother/Fr, father/Pol)	homozygous	—	severe**

TABLE 1-continued

Mutations in the Cx26 coding exon in individuals affected with familial forms of prelingual deafness			
Family (geographical origin)	30delG mutation	Other mutation	Deafness
P17 (Fr)	heterozygous	—	severe***
1885 (NZ)	heterozygous	—	profound
2254 (NZ)	—	—	moderate-severe

The analysis reported here concerns deaf children of the various families except for family 1873 (see patients and methods).

*moderate in one ear, severe in the other ear.

**moderate hearing loss in mother (severe at high frequencies),

***mild hearing loss in father, who are heterozygous carriers for the 30delG mutation.

Geographical origins: (Alg) Algeria, (Aust) Australia, (Fr) France, (Leb) Lebanon, (NZ) New Zealand, (Pol) Poland, (Por) Portugal, (nTu) North Tunisia, (sTu) South Tunisia, (Tur) Turkey

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1-28. (canceled)

29. A method for diagnosing autosomal prelingual nonsyndromic deafness in a human, the method comprising determining the presence of a mutation identified as a G to T transversion at nucleotide position G139 creating a premature stop codon (GAG→JAG) at codon 47 (designated E47X) of a connexin 26 gene in a DNA sample obtained from the human, wherein position 1 is the first base of the initiator codon, and diagnosing autosomal prelingual nonsyndromic deafness.

30. A method for diagnosing deafness in a human, wherein the deafness is associated with the presence of a mutation

identified as a G to T transversion at nucleotide position G139 creating a premature stop codon (GAG→JAG) at codon 47 (designated E47X) of a connexin 26 gene, wherein position 1 is the first base of the initiator codon, the method comprising determining the presence of the E47X mutation in a connexin 26 gene present in a DNA sample obtained from the human.

31. A method for detecting the presence of a mutation identified as a G to T transversion at nucleotide position G139 creating a premature stop codon (GAG→JAG) at codon 47 (designated E47X) of a connexin 26 gene in a biological sample containing DNA, wherein position 1 is the first base of the initiator codon, the method comprising:

- a) bringing the biological sample into contact with a pair of oligonucleotide primers consisting of 15 to 50 nucleotides, wherein the oligonucleotide primers hybridize under stringent conditions with a region of interest of the connexin 26 gene comprising a E47X mutation, under conditions permitting hybridization of the oligonucleotide primers with the DNA contained in the biological sample;
- b) amplifying the region of interest of the connexin 26 gene;
- c) detecting the amplification products; and
- d) detecting the mutation in the amplification products.

32. A method for detecting the presence or absence of a mutation identified as a G to T transversion at nucleotide position G139 creating a premature stop codon (GAG→JAG) at codon 47 (designated E47X) of a connexin 26 gene, wherein position 1 is the first base of the initiator codon, the method comprising:

- a) bringing a biological sample containing DNA from a human into contact with an oligonucleotide consisting of 15 to 50 consecutive nucleotides, wherein the oligonucleotide hybridizes with a connexin 26 gene comprising a E47X mutation, and does not hybridize with a wild-type connexin 26 gene, under conditions permitting hybridization of the oligonucleotide with the DNA contained in the biological sample; and
- b) detecting the presence or absence of hybrids formed between the oligonucleotide and the DNA contained in the biological sample, thereby indicating the presence or absence, respectively, of the mutation.

33. The method of claim 31, wherein step d) comprises:

- a) incubating the amplification products with a labeled detection probe that hybridizes with a region of interest of both a normal connexin 26 sequence and a mutant connexin 26 sequence comprising a first capture probe that hybridizes with a region of interest of the normal connexin 26 sequence but does not hybridize with a region of interest of the mutant connexin 26 sequence;
- b) incubating the amplification products with the labeled detection probe and a second capture probe that hybridizes with a region of interest of the mutant connexin 26

sequence but does not hybridize with a region of interest of the normal connexin 26 sequence;

- c) hybridizing the amplification products with the labeled detection probe and with the first capture probe or the second capture probe; and
- d) comparing the hybridization signal obtained from the first capture probe with the hybridization signal obtained from the second capture probe.

34. The method of claim 33, wherein the detection probe is labeled with biotin.

35. The method of claim 33, wherein the first capture probe and the second capture probe are bound to a microplate.

36. The method of claim 31, wherein the biological sample is blood.

37. The method of claim 31, wherein in step d) the mutation is detected by a technique selected from:

- single-strand conformation polymorphism (SSCP);
- denaturing gradient gel electrophoresis (DGG);
- sequencing; and
- temperature gradient gel electrophoresis (TGGE).

38. The method of claim 33, wherein the detection probe is non-radioactively labeled.

39. The method according to claim 33, wherein step d) comprises:

- a) detecting a first signal from the hybridization of the detection probe with the first capture probe and a second signal from the hybridization of the detection probe with the second capture probe; and
- b) calculating a ratio between the first signal and the second signal, wherein a ratio of at least 2 indicates that the biological sample contains DNA that is homozygous for the normal connexin 26 gene, a ratio of no more than 0.5 indicates that the biological sample contains DNA that is homozygous for the E47X mutation, and a ratio of greater than 0.5 and less than 2 indicates that the biological sample contains DNA that is heterozygous for the E47X mutation.

40. The method of claim 32, wherein before step a), the DNA contained in the biological sample is amplified using a pair of primers.

* * * * *

专利名称(译)	连接蛋白26基因内的突变负责语前非综合征性耳聋和检测方法		
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摘要(译)

一种纯化的多核苷酸，其具有对应于突变序列的核苷酸链，其以野生形式编码与遗传性感觉缺陷有关的多肽，其中所述突变的纯化的多核苷酸具有引起前语非综合征性耳聋的突变，所述突变性耳聋选自自由以下组成的组：特异性缺失至少一个核苷酸。

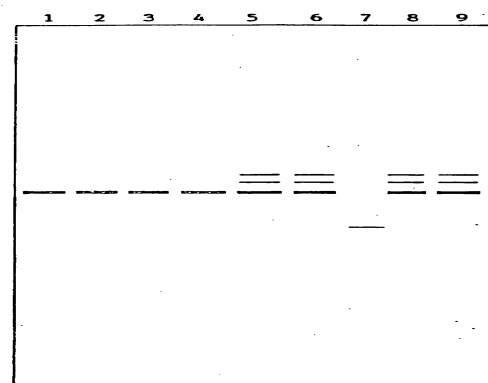


Figure 1