表 1　非症候群性難聴の原因遺伝子[2)]

常染色体優性遺伝形式

Locus(OMIM)	Gene(OMIM)	Reference
	CRYM	Abe ら(2003)
DFNA1	*DIAPH1*	Lynch ら(1997)
DFNA2A	*KCNQ4*	Kubisch ら(1999)
DFNA2B	*GJB3*	Xia ら(1998)
DFNA3A	*GJB2*	Kelsell ら(1997)
DFNA3B	*GJB6*	Grifa ら(1999)
DFNA4	*MYH14*	Donaudy ら(2004)
	CEACAM16	Zheng ら(2011)
DFNA5	*DFNA5*	Van Laer ら(1998)
DFNA6/14/38	*WFS1*	Bespalova ら(2001), Young ら(2001)
DFNA8/12	*TECTA*	Verhoeven ら(1998)
DFNA9	*COCH*	Robertson ら(1998)
DFNA10	*EYA4*	Wayne ら(2001)
DFNA11	*MYO7A*	Liu ら(1997)
DFNA13	*COL11A2*	McGuirt ら(1999)
DFNA15	*POU4F3*	Vahava ら(1998)
DFNA17	*MYH9*	Lalwani ら(2000)
DFNA20/26	*ACTG1*	Zhu ら(2003), van Wijk ら(2003)
DFNA22	*MYO6*	Melchionda ら(2001)
DFNA25	*SLC17A8*	Ruel ら(2008)
DFNA28	*GRHL2*	Peters ら(2002)
DFNA36	*TMC1*	Kurima ら(2002)
DFNA44	*CCDC50*	Modamio-Hoybjor ら(2007)
DFNA48	*MYO1A*	Donaudy ら(2003)
DFNA50	*MIRN96*	Mencia ら(2009)
DFNA51	*TJP2*	Walsh ら(2010)
DFNA64	*SMAC/DIABLO*	Chen ら(2011)

常染色体劣性遺伝形式

Locus(OMIM)	Gene(OMIM)	Reference(OMIM)
DFNB1A	*GJB2*	Kelsell ら(1997)
DFNB1B	*GJB6*	Del Castillo ら(2002)
DFNB2	*MYO7A*	Liu ら(1997), Weil ら(1997)
DFNB3	*MYO15A*	Wang ら(1998)
DFNB4	*SLC26A4*	Li ら(1998)
DFNB6	*TMIE*	Naz ら(2002)
DFNB7/11	*TMC1*	Kurima ら(2002)
DFNB8/10	*TMPRSS3*	Scott ら(2001)
DFNB9	*OTOF*	Yasunaga ら(1999)
DFNB12	*CDH23*	Bork ら(2001)
DFNB15/72/95	*GIPC3*	Ain ら(2007), Rehman ら(2011), Charizopoulou ら(2011)
DFNB16	*STRC*	Verpy ら(2001)
DFNB18	*USH1C*	Ouyang ら(2002), Ahmed ら(2002)
DFNB21	*TECTA*	Mustapha ら(1999)
DFNB22	*OTOA*	Zwaenepoel ら(2002)
DFNB23	*PCDH15*	Ahmed ら(2003)
DFNB24	*RDX*	Khan ら(2007)
DFNB25	*GRXCR1*	Schraders ら(2010)
DFNB28	*TRIOBP*	Shahin ら(2006), Riazuddin ら(2006)
DFNB29	*CLDN14*	Wilcox ら(2001)
DFNB30	*MYO3A*	Walsh ら(2002)
DFNB31	*WHRN*	Mburu ら(2003)
DFNB35	*ESRRB*	Collin ら(2008)
DFNB36	*ESPN*	Naz ら(2004)
DFNB37	*MYO6*	Ahmed ら(2003)
DFNB39	*HGF*	Schultz ら(2009)
DFNB42	*ILDR1*	Borck ら(2011)
DFNB49	*MARVELD2*	Riazuddin ら(2006)
DFNB53	*COL11A2*	Chen ら(2005)
DFNB59	*PJVK*	Delmaghani ら(2006)
DFNB61	*SLC26A5*	Liu ら(2003)
DFNB63	*LRTOMT/COMT2*	Ahmed ら(2008), Du ら(2008)
DFNB66/67	*LHFPL5*	Tlili ら(2005), Shabbir ら(2006), Kalay ら(2006)
DFNB74	*MSRB3*	Waryah ら(2009), Ahmed ら(2011)
DFNB77	*LOXHD1*	Grillet ら(2009)
DFNB79	*TPRN*	Rehman ら(2010), Li ら(2010)
DFNB82	*GPSM2*	Walsh ら(2010)
DFNB84	*PTPRQ*	Schraders ら(2010)
DFNB91	*GJB3*	Liu ら(2000)
	SERPINB6	Sirmaci ら(2010)

X 連鎖性

Locus(OMIM)	Gene(OMIM)	Reference
DFNX1(DFN2)	*PRPS1*	Liu ら(2010)
DFNX2(DFN3)	*POU3F4*	De Kok ら(1995)
DFNX4(DFN6)	*SMPX*	Schraders ら(2011), Huebner ら(2011)

メイド医療が実現可能となってきた[10)].

2. 進行性や変動の有無の予測

通常の聴力検査ではその後の難聴の進行や変動の有無を予測することは困難であるが，遺伝子診断を行うことにより進行の有無や変動の有無を予測することが可能である．たとえば，同じ常染色体劣性遺伝形式をとる難聴原因遺伝子変異であっても，*GJB2* 遺伝子変異症例では難聴の進行を認めることは稀である[4,11)]のに対して，*SLC26A4* 遺伝子変異による難聴症例ではめまい発作を伴い，変動しながら難聴が増悪することが明らかとなっている[5)]．また，常染色体優性遺伝形式をとる

KCNQ4, *TECTA*, *WFS1* などの遺伝子変異による難聴症例およびミトコンドリア遺伝子変異による難聴症例も進行性の難聴を呈することが知られている[12-15]．進行性の難聴を特徴とする原因遺伝子変異が同定された場合には定期的に聴力を測定するとともに，補聴器・人工内耳などの機器の調整を行い，十分な聴取能を確保することが必要である．また，高度難聴への進行が予測される遺伝子変異の場合には将来的に人工内耳を視野に入れた経過観察が必要となる．

3. 随伴症状の有無の予測

Pendred症候群やUsher症候群のように症候群性の難聴であっても随伴症状の発症時期が小児期以降発症の場合には，生後～小児期にかけては難聴以外の症状を呈さないため非症候群性難聴と区別がつかない．このような遅発性の随伴症状を伴う症候群性難聴の場合，遺伝子診断が予後の予測や適切な介入法選択のための有用な情報となる．*SLC26A4* 遺伝子変異は難聴と甲状腺腫を伴うPendred症候群の原因遺伝子でもあるため，*SLC26A4* 遺伝子変異が認められた場合には甲状腺機能を含めた経過観察が重要である[5]．

また，*MYO7A*，*CDH23*，*PCDH15* などの遺伝子変異により発症するUsher症候群では先天性の高度難聴＋後天性の網膜色素変性症を発症することが知られており，10歳前後で夜盲を自覚するまでは視覚症状に気がつかない場合が多い．遺伝子診断を行うことで，網膜色素変性症を予測可能となるため，両側人工内耳を行うなど将来の視覚障害に対応するために聴覚を積極的に活用するなどの治療計画を立てることが可能となる[16]．

4. 発症・増悪の予防

近年，分子遺伝学的・分子生物学的解析より，ミトコンドリア1555 A＞G変異，1,494C＞T変異をもつ場合にはアミノ配糖体抗菌薬に高感受性となることが明らかとなってきた．この変異を伴う難聴が診断された場合には，①罹患者の難聴の進行予防，および②非罹患の同胞の発症予防，が可能となる．いったん難聴を発症すると非可逆的であるが，アミノ配糖体抗菌薬を避けることで，罹患者の場合には難聴の進行を，非罹患の同胞の場合には高度難聴の発症を予防できるというメリットがあるため，薬物カードを配布して予防に努めている[15,17]．

遺伝性難聴のターゲットリシークエンシング解析

保険収載された先天性難聴の遺伝子診断は，正確な診断を行うという意味だけでなく，予後の予測，随伴症状の予測，難聴の進行予防，治療法選択に有用な情報が得られるなど多くのメリットのある検査である．遺伝子診断に基づいた難聴のサブタイプ分類と，サブタイプに応じたオーダーメイド医療の提供はこれからの難聴医療に必須となると思われるが，現在保険診療で行われているインベーダー法を用いたスクリーニング検査の診断率は30～40%程度であり，今後の診断率の向上のためには新規原因遺伝子変異の追加が必要である．しかし，①難聴の原因遺伝子として100以上の遺伝子が関与するため，原因となる遺伝子変異の探索は容易ではない，②日本人難聴患者に高頻度で認められる主要な変異はすでにインベーダー法によるスクリーニング検査に取り込まれているため，遺伝子変異を追加することによるコストの増加に対し診断率向上の程度はわずかであるため，費用対効果に乏しい，という問題点があった．しかし，次世代シークエンサーが実用化され，多数の原因遺伝子を網羅的に解析することが可能となり，また解析費用の低下により，現実的にすべての難聴原因遺伝子を網羅的に解析することが可能となってきた．

難聴の原因を探索する際の効率を考えると，①インベーダー法に搭載されている日本人難聴患者に高頻度で認められる変異のスクリーニング検査，②既報告の難聴原因遺伝子のターゲットリシークエンシング解析，③新規の難聴原因遺伝子の探索を目的としたエクソーム解析，④エクソン領域以外に原因のある難聴遺伝子変異を見出すためのゲノム解析，の順に検索を進めるのがよいと考えられる．インベーダー法によるスクリーニング検査などで遺伝子変異が同定されないケースでは，難聴の原因遺伝子であることが報告されている54遺伝子を網羅的に解析するターゲットリシークエンシングが有用である．とくに，劣性遺伝形式を

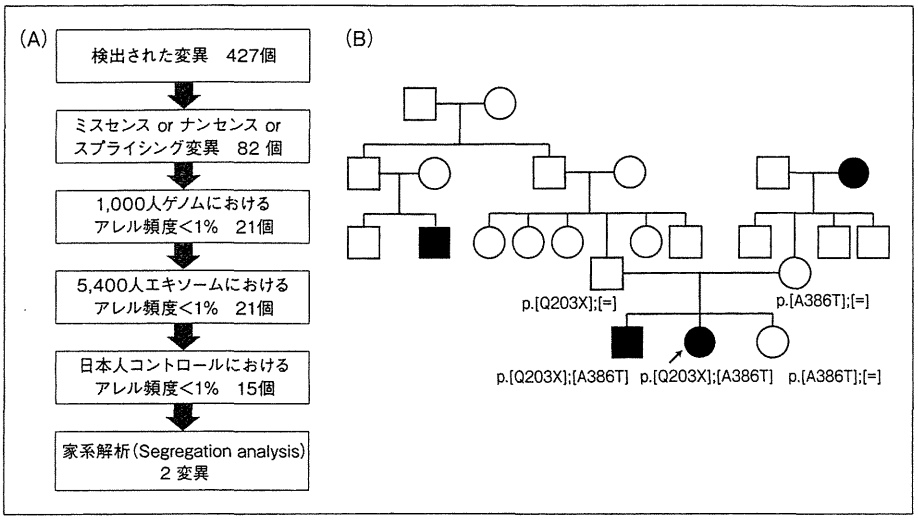

図 2　難聴のターゲットリシークエンシング解析による遺伝子変異の絞込み
実際に行われた遺伝子解析による遺伝子変異の絞込みの様子(A)と遺伝子 X の複合ヘテロ接合体変異の見出された非症候群性難聴家系(B)を示す．(Miyagawa, et al., submitted)

とる遺伝性難聴では，founder effect によると考えられる民族共通の遺伝子変異が高頻度で認められるため，インベーダー法や TaqMan Genotyping 法による診断が有効であるが，優性遺伝形式をとる遺伝性難聴の場合，家系ごとに変異の部位が異なる場合が多く，効率的なスクリーニング法が確立していないため，既知遺伝子の網羅的ターゲットリシークエンシング解析が非常に有用である．

現在，わが国も含めて既知難聴遺伝子を網羅的に解析するターゲットリシークエンシングが行われており，成果が得られつつある[18-20]．現在までに報告されている難聴の原因遺伝子をすべて合わせるとおおよそ 0.5〜0.6 Mbase となるため，エンリッチメントの方法としては SureSelect などのシークエンスキャプチャーによるものが多く用いられている．また最近では，micro droplet PCR や HaloPlex，IonAmpliSeq などの Amplicon ベースのエンリッチメント法も徐々に用いられはじめている．

Shearer ら(2010)は，既知の難聴原因遺伝子 54 種類の全エクソン領域を SureSelect により網羅的にキャプチャーする手法を利用して，難聴患者 8 例(陽性コントロール 2 例を含む)の遺伝子解析を行った．陽性コントロール 2 例とも既報告の変異が同定され，また，原因不明であった 6 家系中 5 家系より難聴の原因と考えられる遺伝子変異を同定し報告している[18]．

また著者らが，難聴患者 216 例(陽性コントロール 62 例を含む)を対象に，既知の非症候群性難聴の原因遺伝子 54 遺伝子および既知の症候群性難聴原因遺伝子および内耳で高発現の認められる遺伝子を含めた 121 遺伝子の全エクソン領域を SureSelect Custom＋Illumina GA Ⅱx により網羅的に解析を行ったところ，陽性コントロールにおける変異検出率は 93%以上と非常に高効率で検出可能であり，また，全体の約 70%より何らかの遺伝子変異が見出されることが明らかとなった．このことから，新規の原因遺伝子探索手法としても非常に有用であることが明らかとなってきている(Miyagawa ら．submitted)．

その後，著者らのグループでは新規の原因遺伝子変異を同定する目的で，前述 216 名に加え 296 名の追加の解析を SureSelect Custom＋Illumina HiSeq2000 で行い，合計 512 名のターゲットリシークエンシング解析を完了している．ターゲットリシークエンシング解析においては多数の SNPs が検出されるため，見出された変異が病的変異かまれな多型かを判断するのは非常に困難である．見出された遺伝子変異に関して，①1,000 人ゲノムおよび 5,400 エキソームのアレル頻度によ

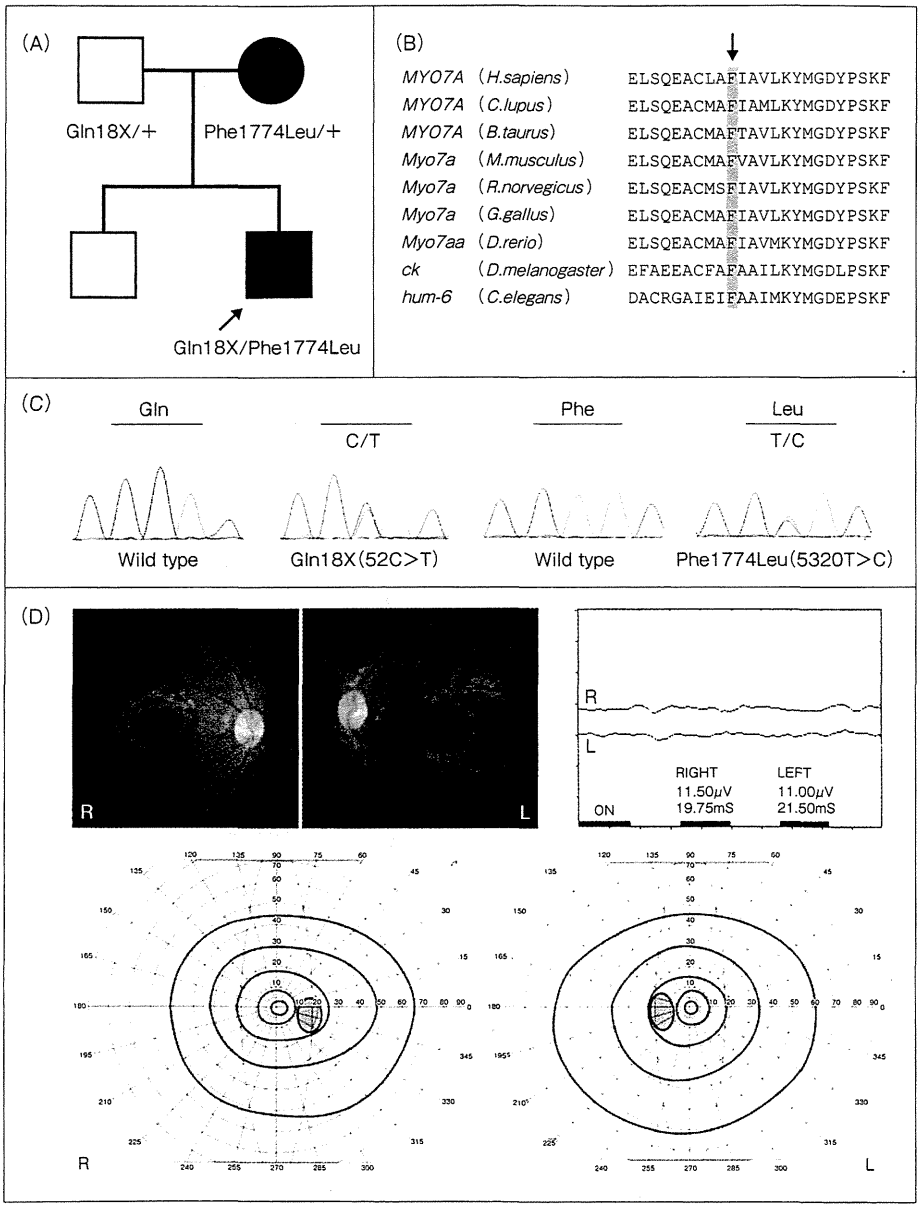

図 3　遺伝子診断により網膜色素変性症自覚前にUsher症候群を診断された症例[16]

A：遺伝子診断により網膜色素変性症発症前に遺伝子診断された症例の家系図および見出された *MYO7A* 変異.

B：変異部位の種間の保存性.

C：サンガーシークエンスによるクロマトグラム.

D：眼底所見，網膜電図検査(ERG検査)の結果および視野検査(Goldman visual field examination)の結果.

るフィルター，②日本人コントロールにおけるアレル頻度によるフィルターをかけることにより候補となる遺伝子変異が10個以下のレベルまで絞り込まれる．とくに，遺伝性難聴の場合，極少数のケースを除き浸透率100%の単一遺伝子のMendel遺伝形式をとるため，③家系サンプルによるセグリゲーション解析を行うことにより原因候補遺伝子変異は数個レベルまで絞り込むことが可能であり，原因遺伝子変異を同定できる場合も多い(図2)．

また著者らは，平成22年度～24年度厚生労働科学研究「Usher症候群に関する調査研究班」と

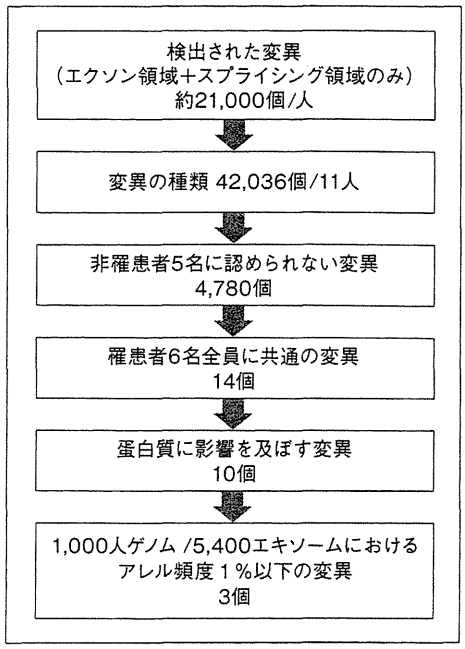

図 4　難聴のエクソーム解析による遺伝子変異の絞込み

優性遺伝形式をとる遺伝性難聴家系（罹患者 6 名，非罹患者 5 名）を対象に行ったエクソーム解析による遺伝子変異の絞り込みの様子を示す．十分に大きな家系の場合には同一家系内の複数サンプルのエクソーム解析を行うことにより候補遺伝子を数個レベルまで絞り込むことが可能である．

して，全国の共同研究施設より収集した Usher 症候群症例 46 例中，Usher 症候群 type 1 症例 16 例を対象に IonAmpliSeq を用いて，Usher 症候群の原因遺伝子として知られる 9 遺伝子（*MYO7A*, *CDH23*, *PCDH15*, *USH1C*, *USH1G*, *USH2A*, *GPR98*, *DFNB31*, *CLRN1*）を増幅し，Ion-Torent を用いての網羅的解析を行ったところ，14 例（87.5％）よりホモ接合体または複合ヘテロ接合体で原因遺伝子変異を検出し原因を同定することができた．また，検出された変異の種類は欧米人の変異とは異なるものであり民族特異性があることが明らかとなった[16]．興味深いことに，変異の検出された遺伝子は *MYO7A* が 7 例（50％），*CDH23* が 4 例（28％），*PCDH15* が 3 例（21％）であり，海外と同程度の頻度であることが明らかとなった．このことより Usher 症候群においても難聴と同様，原因遺伝子の種類や頻度は民族間で共通しているが，変異の種類に関しては民族特異性があることが明らかとなった．Usher 症候群の原因として知られる 9 遺伝子は数多くのエクソンからなる大きな遺伝子が多く，エクソンの合計が 326 にもなる．従来のサンガーシークエンス法を用いた解析では膨大な作業量が必要であったが，パーソナル型の次世代シークエンサーを用いた遺伝子解析により大幅に効率化を行うことが可能となってきた．

また，Usher 症候群は先天性高度難聴に後天性の網膜色素変性症を随伴するため，生下時～幼少期には非症候群性の難聴と診断されている症例がいるものと考えられる．そこで，非症候群性難聴症例を対象に Usher 症候群の原因遺伝子を網羅的に解析したところ，網膜色素変性症を発症する前に遺伝子変異が同定され，その後眼底検査や網膜電図検査（ERG 検査）により網膜色素変性症と診断された症例を経験した[16]．このように，遺伝子診断は Usher 症候群の早期診断および早期介入のために非常に有用な情報となりうることが期待される（図 3）．

遺伝性難聴のエクソーム解析の実際

難聴の原因であることが報告されている遺伝子を網羅的に解析するターゲットリシークエンシング解析を行ってもなお原因遺伝子変異が同定されない場合には，全遺伝子のエクソン領域を対象としたエクソーム解析を行うことにより難聴の原因が特定可能である．

海外でも新規難聴原因遺伝子の探索の一環としてのエクソーム解析が行われはじめているが，現時点では連鎖解析や homozygous mapping などの他の遺伝子解析手法と組み合わせて実施することで，変異の絞り込みを行うなどの工夫を行った解析が行われている[21-23]．

Walsh ら（2010）は，連鎖解析により遺伝子座 DFNB85 が同定されていた劣性遺伝形式をとる近親婚難聴家系 1 家系を対象に，SureSelect＋IlluminaGA Ⅱx を用いたエクソーム解析を行い，DFNB85 座近傍の遺伝子に関して homozygous mapping と組み合わせることにより，新規の難聴原因遺伝子 GPSM2 を同定し報告している．また，内耳における局在を調べるとともに発生段階での

遺伝子発現量を調べることにより，GPSM2が難聴の原因となりうる可能性を検討している．

著者らの研究室では連鎖解析が可能な規模の大家系を対象にエクソーム解析を用いた難聴遺伝子の解析を行っており，新規変異の候補が見出されている状況である．実際に解析を行った優性遺伝形式をとる遺伝性難聴1家系を例にあげて説明を行う．優性遺伝形式をとる遺伝性難聴家系のうち罹患者6名，非罹患者5名の11名を対象にIllumina TrueSeq Whole Exome kit+Illumina HiSeq2000を用いたエクソーム解析を行った(図4)．

一般的なエクソーム解析で利用されるパイプライン(Duplication Readの除去>QC値でのフィルタリング>BWAによるhg19へのマッピング>GATK toolsによる変異の検出)を用いて変異の検出を行ったところ，11名それぞれに約20,000カ所の変異が同定された．同定された変異を対象に，ANNOVARを用いてアノテーションを付けたファイルをcsvファイルとして用意し，①蛋白質に影響を及ぼす変異(ミスセンス変異，ナンセンス変異，スプライシング変異，欠失・挿入変異)，②1,000人ゲノムおよび5,400エクソームにおける頻度が0.01以下，③家系内罹患者に共通かつ非罹患者に認められない，の3条件を組み合わせて候補遺伝子の絞り込みを行ったところ3変異まで絞り込むことが可能であった．3変異とも過去に難聴との関連が報告されていない新規の遺伝子に存在するため，内耳における遺伝子発現や発現部位に関して検討を行っている．

おわりに

従来，難聴の多くは原因不明で治療法もなかったため，難聴の治療は画一的に行われていたが，遺伝子解析技術の進歩と人工内耳の登場により正確な診断に基づいた個別化医療が実現しつつある．遺伝子診断はこの根幹をなす技術であり今後の発展が期待されている．先天性難聴の遺伝子診断に関しては平成24年(2012)4月より保険診療として実施されており，今後ますます遺伝子診断の重要性が高まっていくものと思われる．また，保険診療で実施されているスクリーニング検査において原因遺伝子変異が見出されない場合には，ターゲットリシークエンシングやエクソーム解析が有用であることが明らかとなってきている．現時点ではターゲットリシークエンシングが主流であるが，解析コストとデータ量に大きな違いがあるものの解析手技には大きな違いはないため，日本人のコントロールデータの充実と解析コストの低下に従いエクソーム解析が普及してくると予測される．しかし，どのようなエンリッチメント手法を用いても全エクソン領域のカバー率が100%になることはないため，将来的には比較的均質なデータが得られるゲノム解析へとシフトすると考えられる．今後，難聴の臨床現場でこのような新しい手法を用いた正確な診断に基づいた難聴医療が定着していくことを期待している．

文献/URL

1) Morton, C. C. and Nance, W. E.: *N. Engl. J. Med.*, **354**: 2154-2164, 2006.
2) Van Camp, G. et al.: Hereditary Hearing loss Homepage. (http://hereditaryhearingloss.org)
3) Ohtsuka, A. et al.: *Hum. Genet.*, **112**: 329-333, 2003.
4) Oguchi, T. et al.: *J. Hum. Genet.*, **50**: 76-83, 2005.
5) Suzuki, H. et al.: *Acta Otolaryngol.*, **127**: 1292-1297, 2007.
6) Wagatsuma, M. et al.: *Clin. Genet.*, **72**: 339-344, 2007.
7) Usami, S. et al.: *Acta Otolaryngol.*, **128**: 446-454, 2008.
8) Usami, S. et al.: *PLoS ONE*, **7**: e31276, 2012.
9) Hildebrand, M. S. et al.: *Laryngoscope*, **119**: 2211-2215, 2009.
10) Usami, S. et al.: *Acta Otolaryngol.*, **132**: 377-384, 2012.
11) Tsukada, K. et al.: *Clin. Genet.*, **78**: 464-470, 2010.
12) Akita, J. et al.: *J. Hum. Genet.*, **46**: 355-361, 2001.
13) Moteki, H. et al.: *J. Hum. Genet.*, **57**: 587-592, 2012.
14) Fukuoka, H. et al.: *J. Hum. Genet.*, **52**: 510-515, 2007.
15) Lu, S. Y. et al.: *Clin. Genet.*, **75**: 480-484, 2009.
16) Yoshimura, H. et al.: *Int. J. Pediatr. Otorhinolaryngol.*, **77**: 298-302, 2013.
17) Usami, S. et al.: *J. Hum. Genet.*, **44**: 304-307, 1999.
18) Shearer, A. E. et al.: *Proc. Natl. Acad. Sci. USA*, **107**: 21104-21109, 2010.
19) Lin, X. et al.: *Hear Res.*, **288**: 67-76, 2012.
20) De Keulenaer, S. et al.: *BMC Med. Genomics*, **5**: 17, 2012.
21) Walsh, T. et al.: *Am. J. Hum. Genet.*, **87**: 90-94, 2010.
22) Gao, J. et al.: Whole exome sequencing identifies a novel DFNA9 mutation, C162Y. *Clin. Genet.*, 2012, Aug. 29. (Epub ahead of print)
23) Diaz-Horta, O. et al.: *PLoS ONE*, **7**: e50628, 2012.

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Diverse spectrum of rare deafness genes underlies early-childhood hearing loss in Japanese patients: a cross-sectional, multi-center next-generation sequencing study

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Abstract

Background: Genetic tests for hereditary hearing loss inform clinical management of patients and can provide the first step in the development of therapeutics. However, comprehensive genetic tests for deafness genes by Sanger sequencing is extremely expensive and time-consuming. Next-generation sequencing (NGS) technology is advantageous for genetic diagnosis of heterogeneous diseases that involve numerous causative genes.

Methods: Genomic DNA samples from 58 subjects with hearing loss from 15 unrelated Japanese families were subjected to NGS to identify the genetic causes of hearing loss. Subjects did not have pathogenic *GJB2* mutations (the gene most often associated with inherited hearing loss), mitochondrial m.1555A>G or 3243A>G mutations, enlarged vestibular aqueduct, or auditory neuropathy. Clinical features of subjects were obtained from medical records. Genomic DNA was subjected to a custom-designed SureSelect Target Enrichment System to capture coding exons and proximal flanking intronic sequences of 84 genes responsible for nonsyndromic or syndromic hearing loss, and DNA was sequenced by Illumina GAIIx (paired-end read). The sequences were mapped and quality-checked using the programs BWA, Novoalign, Picard, and GATK, and analyzed by Avadis NGS.

Results: Candidate genes were identified in 7 of the 15 families. These genes were *ACTG1*, *DFNA5*, *POU4F3*, *SLC26A5*, *SIX1*, *MYO7A*, *CDH23*, *PCDH15*, and *USH2A*, suggesting that a variety of genes underlie early-childhood hearing loss in Japanese patients. Mutations in Usher syndrome-related genes were detected in three families, including one double heterozygous mutation of *CDH23* and *PCDH15*.

Conclusion: Targeted NGS analysis revealed a diverse spectrum of rare deafness genes in Japanese subjects and underscores implications for efficient genetic testing.

Keywords: Hereditary hearing loss, Target gene capture, Deafness gene, Heterogeneity

Background

Hearing loss is a common sensory defect, affecting approximately one in 500 to 1000 newborns [1]. Approximately 50% of congenital hearing loss cases and 70% of childhood hearing loss cases are attributed to genetic mutations [1]. The remaining 50% of congenital cases are attributable to other factors such as prenatal exposure to measles, cytomegalovirus, premature birth, and newborn meningitis. Genetic tests for hereditary hearing loss assist in the clinical management of patients and can provide the first step in the development of therapeutics [2]. For example, early diagnosis of Usher syndrome, which comprises congenital hearing loss and late-onset retinitis pigmentosa, provides important information to choose communication modalities. However, causes of hereditary hearing loss are highly heterogeneous; more than 60 genes have been identified as responsible for nonsyndromic

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hearing loss [3], and several hundreds of syndromic diseases, such as Pendred syndrome and Usher syndrome, are accompanied by hearing loss. *GJB2* mutations are the most common cause of childhood hearing loss worldwide [1], followed by *SLC26A4* mutations [4]. *OTOF* mutations are common in patients with auditory neuropathy, which is characterized by normal outer hair cell function and abnormal neural conduction [5]. The prevalence of childhood hearing loss patients with mutations in other deafness-related genes is likely to be less than 1% [1]. Such high heterogeneity of hearing loss makes it impractical to perform genetic tests by Sanger sequencing. This is also the case for some types of syndromic hearing loss. For example, nine genes have been reported to cause Usher syndrome, and all are large and difficult to analyze using Sanger sequencing.

Next-generation sequencing (NGS) technology has been applied to genetic diagnosis of nonsyndromic hearing loss [6-8] and exploring the causes of hearing loss [9-11]. These studies have revealed that it is technically feasible to identify causative genes for nonsyndromic and syndromic hearing loss using targeted NGS [6,8]. In this study, we used targeted NGS to identify the genetic basis of hearing loss in Japanese families.

Methods

Subjects

This was a multi-center study of 58 subjects (36 subjects with hearing loss and 22 subjects with normal hearing) from 15 unrelated Japanese families in which at least two family members had bilateral hearing loss. All subjects were patients at the National Hospital Organization Tokyo Medical Center or a collaborating hospital. Medical histories were obtained and physical, audiological, and radiological examinations were carried out for the subjects and family members. Subjects with hearing loss related to environmental factors were excluded. Subjects with *GJB2* mutations or mitochondrial m.1555A>G or 3243A>G mutations were excluded. Subjects with enlarged vestibular aqueduct, which is often associated with *SLC26A4* mutations, and subjects with clinical features that suggested syndromic hearing loss were excluded. Subjects with auditory neuropathy were tested for *OTOF* mutations, which are associated with auditory neuropathy [12], and subjects with *OTOF* mutations were excluded. The Ethics Review Committees of the National Hospital Organization Tokyo Medical Center and all collaborating hospitals approved the study procedures. All procedures were conducted after written informed consent had been obtained from each subject or their parents.

Targeted capture and DNA sequencing

We selected coding exons and proximal flanking intronic sequences of 84 genes, including 17 genes responsible for autosomal dominant nonsyndromic hearing loss (DFNA), 32 genes responsible for autosomal recessive nonsyndromic hearing loss (DFNB), 8 genes responsible for both DFNA and DFNB, one gene responsible for auditory neuropathy, 3 genes responsible for X-linked hearing loss, and 23 genes responsible for syndromic hearing loss. A list of the targeted genes responsible for nonsyndromic or syndromic hearing loss is provided in the supporting material [Additional file 1]. More than 90% of the target genomic sequences were successfully designed to be captured by the SureSelect Target Enrichment System (Agilent Technologies, CA, USA) (data not shown). Genomic DNA was extracted from whole blood using the Genetra Puregene DNA isolation kit (QIAGEN, Hilden, Germany) and checked for quality using Qubit (Life technologies, CA, USA). Genomic DNA (3 μg) was fragmented into approximately 150 base pairs and used to capture the targeted genomic sequences. The captured DNA was subjected to the paired-end read sequencing system (GAIIx system; Illumina, CA, USA).

Sequence analysis

Sequence analysis initially focused on the 61 genes responsible for nonsyndromic hearing loss. If no candidate mutations were detected among these genes, the 23 genes responsible for syndromic hearing loss were subjected to sequence analysis.

The sequences were mapped and quality-checked with the programs BWA, Novoalign, Picard, and GATK using the human reference sequence hg19/GRCh37. Single and multiple nucleotide variants, including small insertion or deletions that would affect amino acid sequences or could affect splice sites, were annotated by Avadis NGS v.1.4.5 (Strand Life Sciences, Bangalore, India). Variants already known as pathogenic mutations or detected with <1% frequency in public databases (dbSNP135 [13], 1000GENOME [14], NHLBI Exome Variant Server [15]) were extracted and further subjected to segregation analysis within each family. If no candidate variants were found, the 23 genes responsible for syndromic hearing loss were subjected to the same procedures.

Selected variants were classified as known mutations, possible pathogenic mutations, or variants with unknown pathogenicity; the latter classification was made if there were reports of a controversial finding of pathogenicity or >1% allele frequency in the in-house database of 95 (up to 189) Japanese subjects with normal hearing. Conservation of the corresponding mutated amino acid was compared across nine primate, 20 mammal, and 13 vertebrate species by UCSC Conservation [16]. Functional pathogenic effects of the variants were predicted by PolyPhen-2 [17] and PROVEAN [18]. Effect on splice-site mutations was predicted by NNSPLICE [19].

All the variants and their segregation in each family were confirmed by Sanger sequencing. The specific primer sets were selected from the resequencing amplicon probe sets (NCBI) or designed originally by Primer-BLAST (NCBI). The genotype of each individual and segregation in the family was characterized using DNASIS Pro (Hitachisoft, Tokyo, Japan).

Structural modeling

To find sequences homologous to ACTG1 and MYO7A that could be used as the structural templates for the modeling exercise, we searched the Protein Data Bank (PDB) using Gapped BLAST [20] and PDBsum [21]. The crystal structure of *Limulus polyphemus* filamentous actin (PDB: 3B63) and the 4.1 protein-ezrin-radixin-moesin (FERM) domain of *Mus musculus* myosin VIIa in complex with Sans protein (PDB: 3PVL) were utilized as the templates to model ACTG1 with the p.G268S mutation and MYO7A with the p.W2160G mutation, respectively. The models were built using SWISS-MODEL [22-24] in the automatic modeling mode and with default parameters. The quality of the models was evaluated using the Verify_3D Structure Evaluation Server [25,26]. The α-carbon frames and ribbon models were superimposed using Chimera [27].

Results

Pedigrees of the seven families are shown in Figure 1; clinical features are described in Table 1 and supplemental materials [Additional file 2 and Additional file 3]. In this targeted NGS study, the mean read depth of the target regions was more than 100× for all subjects (data not shown). Table 2 summarizes the number of variants detected from the 61 or 84 targeted genes for each subject. The number of variants was consistent across subjects (339–435 variants per subject for 61 genes, 539–607 variants per subject for 84 genes), which supported the reproducibility and reliability of our technical procedures and analytical pipeline. After excluding frequent variants (>1%) in public databases, 12 variants of

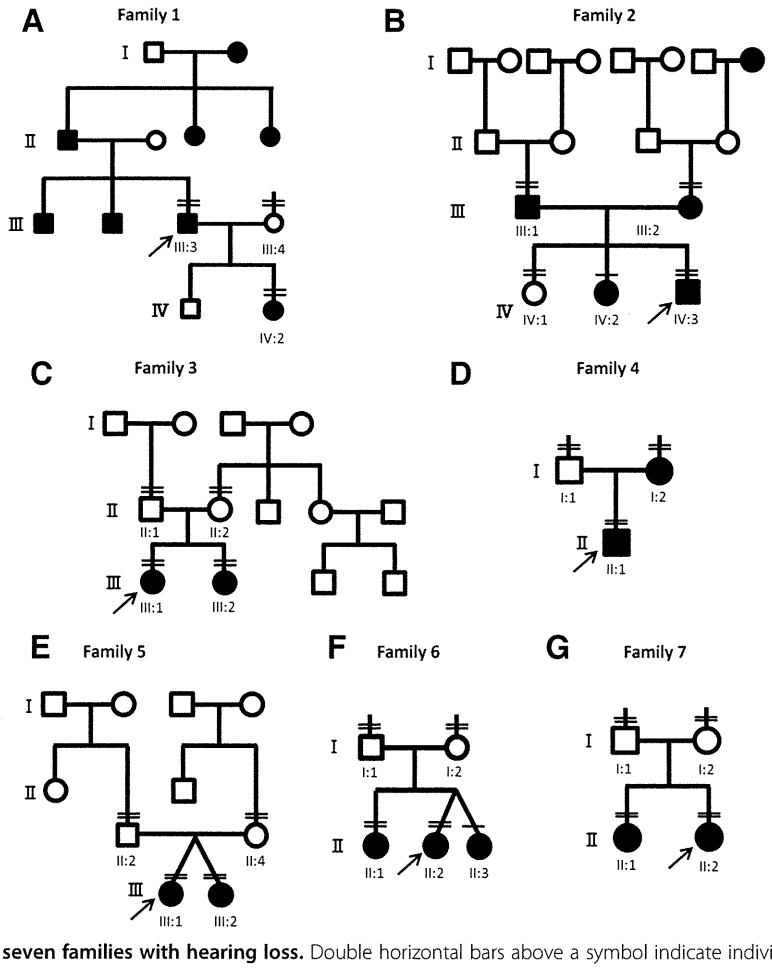

Figure 1 Pedigrees of the seven families with hearing loss. Double horizontal bars above a symbol indicate individuals who underwent genetic analysis by targeted next-generation sequencing. Single horizontal bars above a symbol indicate individuals who underwent analysis by Sanger sequencing. **A**-**G** denote pedigrees of family 1-7, respectively.

Table 1 Summary of subjects with hearing loss

Family	Subject	Age at onset (years)	Age at the time of the study (years)	Hearing loss severity (left/right)*	Progression
1	III:3	45	53	Moderate/Moderate	Yes
	IV:2	10	16	Mild/Normal	No
2	III:1	unknown	no data	Profound/Profound	Unknown
	III:2	unknown	no data	Moderate/Severe	Unknown
	IV:3	0	1	Severe**	Yes
3	III:1	0	9	Severe**	Unknown
	III:2	0	6	Moderate/Moderate	Unknown
4	I:2	0	30s	Profound/Profound	No
	II:1	0	2	Profound/Profound	No
5	III:1	0	2	Severe**	No
	III:2	0	2	Profound**	No
6	II:1	5	14	Profound/Severe	Yes
	II:2	0	12	Profound/Profound	Yes
7	II:1	0	3	Moderate (ASSR***)	Unknown
	II:2	0	0	Severe (ASSR)	Unknown

*Hearing loss severity was evaluated based on average hearing level at 500, 1,000, 2,000, and 4,000 Hz (mild, 20–40 dB; moderate, 41–70 dB; severe, 71-95dB; profound, >95 dB) according to recommendations [3]. **Binaural hearing level. ***ASSR, auditory steady state responses.

9 genes co-segregated with symptoms and were selected as possible pathogenic mutations (Table 3) or variants with uncertain pathogenicity in 7 families (Table 4).

Candidate mutations in each family

In family 1 (Figure 1A), subjects III:3 and IV:2 with hearing loss had a unique heterozygous missense mutation of *ACTG1* (c.802G >A; p.G268S), whereas subject III:4 with normal hearing did not. *ACTG1* encodes actin gamma 1 and is responsible for DFNA20/26 (OMIM 604717) [28]. The glycine residue at 268 of actin gamma 1 is located on a hydrophobic loop that has been suggested to be critical for polymerization of the actin monomers into a filament (Figures 2A and 2B) [29]. Molecular modeling predicted that the p.G268S mutation would disrupt the hydrophobic interactions that are important for polymerization of actin gamma 1 (Figures 2C and Figure 2D). The p.G268S mutant would weaken polymerization of actin gamma 1, which could result in destabilized cytoskeletal structure of stereocilia and dysfunction of the sensory hair cells.

Family 2 (Figure 1B) had two candidate genes with possible pathogenic mutations: A unique heterozygous *POU4F3* frameshift mutation, c.1007delC (p.A336Vfs), was detected in subjects III:1 and IV:3 with hearing loss, and a unique heterozygous *DFNA5* nonsense mutation, c.781C >T (p.R261X), was detected in subjects III:2 and IV:3 with hearing loss, whereas subject IV:1 with normal hearing had neither of these mutations. Sanger sequencing revealed that subject IV:2 with hearing loss had both the heterozygous mutations. *POU4F3* is responsible for DFNA15 (OMIM 602459) [30,31], and *DFNA5* is responsible for DFNA5 (OMIM 600994) [32]. A frameshift mutation in *DFNA5*, which would lead to decreased expression, has been reported not to cause hearing loss [33]; therefore, the cause of hearing loss in subjects IV:2 and IV:3 is more likely to *POU4F3* with the p.A336Vfs mutation derived from subject III:1, rather than *DFNA5* with p.R261X mutation derived from subject III:2.

In family 3 (Figure 1C), subjects III:1 and III:2 with hearing loss had compound heterozygous *SLC26A5* with c.209G >A (p.W70X) and c.390A >C (p.R130S) mutations, whereas subjects II:1 and II:2 with normal hearing had a heterozygous p.W70X mutation and a heterozygous p.R130S mutation, respectively. *SLC26A5* encodes prestin, a member of the SLC26A/SulP transporter family, and is responsible for DFNB61 (OMIM 613865) [34].

In family 4 (Figure 1D), subjects I:2 and II:1 with hearing loss did not have candidate mutations in the first 61 genes. Analysis of the additional 23 genes indicated a heterozygous *SIX1* mutation, c.328C >T (p.R110W), in the subjects with hearing loss but not in subject I:1 with normal hearing. *SIX1* is responsible for DFNA23 (OMIM 605192) and Branchio-otic syndrome 3 (BOS3, OMIM 608389). The p.R110W mutation was previously reported in two BOS3 families [35]. To make the clinical diagnosis of branchiootorenal syndrome or branchiootic syndrome, major and minor criteria of these syndromes must be present [36]. In the affected subjects of the present study, clinical histories were thoroughly evaluated and physical examination of the ear, nose, throat, head and neck, and audiological tests were performed. In addition, CT of the temporal bone was evaluated in subject II:1. With these examinations, the affected subjects did not

Table 2 Summary of the number of variants detected in each subject

Family	Subject	Number of genes analyzed	No.SNV/MNV*	No. non-synonymous SNV/MNV
1	III:3	61	414	84
	III:4	61	370	74
	IV:2	61	391	82
2	III:1	61	386	81
	III:2	61	422	87
	IV:1	61	435	82
	IV:3	61	400	84
3	II:1	61	383	82
	II:2	61	339	70
	III:1	61	350	74
	III:2	61	398	86
4	I:1	84	570	138
	I:2	84	569	126
	II:1	84	546	131
5	II:2	61	388	72
	II:4	61	374	87
	III:1	61	361	84
	III:2	61	396	85
6	I:1	61	429	96
	I:2	61	371	81
	II:1	61	378	86
	II:2	61	375	84
7	I:1	84	607	139
	I:2	84	554	126
	II:2	84	582	132
	II:1	84	539	117

*SNV, single nucleotide variant; MNV, multiple nucleotide variant.

present clinical features of the major and minor criteria other than hearing loss. Therefore, family 4 was considered to have non-syndromic hearing loss, DFNA23, based on the clinical information available at the time of this study.

In family 5 (Figure 1E), subjects III:1 and III:2 with hearing loss had compound heterozygous *MYO7A* mutations, c.6439-2A >G (intron 51) and c.6478T >G (p.W2160G). Subjects II:2 and II:4 with normal hearing had a heterozygous c.6439-2A >G mutation and a heterozygous p.W2160G mutation, respectively. *MYO7A* is responsible for DFNA11 (OMIM 601317) [37], DFNB2 (OMIM 600060) [38], and Usher syndrome 1B (OMIM 276900) [39]. Tryptophan 2160 in myosin 7A was found to be located in a carboxyl-terminal FERM domain in the myosin-tail (Figures 3A and Figure 3B); this domain reportedly associates with filamentous actin [40] and contributes to hair bundle formation. Molecular modeling predicted that the p.W2160G mutation would reduce hydrophobic interactions among residues in the center of the F3 subdomain of the FERM domain (Figures 3C and 3D). The p.W2160G mutation would destabilize the structure of the F3 domain and could result in disrupted protein interaction and stereocilial degeneration of the sensory hair cells [41,42].

In family 6 (Figure 1F), subjects II:1 and II:2 with hearing loss had a heterozygous *CDH23* mutation, c.719C>T (p.P240L), and a heterozygous *PCDH15* mutation, c.848G >A (p.R283H). Sanger sequencing revealed that the other subject with hearing loss (subject II:3) also had both heterozygous *CDH23* and *PCDH15* mutations. A p.P240L mutation in*CDH23* has been reported to be pathogenic [43]. Subject I:1 with normal hearing had a heterozygous mutation in *CDH23* (p.P240L), and subject I:2 with normal hearing had a heterozygous mutation in *PCDH15* (p.R283H). *CDH23* is responsible for both DFNB12 (OMIM 601386) and Usher syndrome 1D (OMIM 601067) [44], whereas *PCDH15* is responsible for both DFNB23 (OMIM 609533) and Usher syndrome 1F (OMIM 602083) [45]. Double heterozygous mutations of *CDH23*

Table 3 Summary of possible pathogenic mutations

Gene	Nucleotide change	Amino acid change	NCBI ID	dbSNP135	Allele frequency in 1000GENOME	Allele frequency in ESP6500	Allele frequency in Japanese control	PolyPhen-2 prediction (score)	PROVEAN prediction (score)	Pathogenicity	Family	Reference
ACTG1	c.802G>A	p.G268S	NM_001199954.1	None	-	0	0/192	Probably damaging (0.998	Deleterious (-4.504)	Possible	1	
POU4F3	c.1007delC	p.A336Vfs	NM_002700.2	None	-	0	0/192	-	-	Possible	2	
SLC26A5	c.390A>C	p.R130S	NM_198999.2	None	-	0	0/192	Benign (0.443)	Deleterious (-4.813)	Possible	3	
SLC26A5	c.209G>A	p.W70X	NM_198999.2	None	-	0	n.t*.	-	-	Possible	3	
SIX1	c.328C>T	p.R110W	NM_005982.3	rs80356459	No info	0	n.t.	Probably damaging (1.000)	Deleterious (-7.775)	Causative	4	35
MYO7A	c.6478T>G	p.W2160G	NM_000260.3	None	-	0	0/192	Probably damaging (1.000)	Deleterious (-12.649)	Possible	5	
MYO7A	c.6439-2A>G (intron 51)	Splice mutation	NM_000260.3	None	-	0	0/192		-	Possible	5	
CDH23	c.719C>T	p.P240L	NM_022124.5	rs121908354	1/2183	0	n.t.	Probably damaging (1.000)	Deleterious (-3.051)	Causative	6	43
PCDH15	c.848G>A	p.R283H	NM_001142763.1	None	-	1/13005	0/192	Probably damaging (0.998)	Neutral (-1.918)	Possible	6	
USH2A	c.12431delC	p.A4144GfsX23	NM_206933.2	None	-	0	0/190		-	Possible	7	

*n.t. = not tested

Table 4 Summary of variants with uncertain pathogenicity

Gene	Nucleotide change	Amino acid change	NCBI ID	dbSNP135	Allele frequency in 1000GENOME	Allele frequency in ESP6500	Allele frequency in Japanese control	PolyPhen-2 prediction (score)	PROVEAN prediction (score)	Pathogenicity	Family	Reference
DFNA5	c.781C>T	p.R261X	NM_004403.2	None	-	0	0/192	-	-	Uncertain	2	
USH2A	c.1346G>A	p.R449H	NM_206933.2	None	-	0	5/378	Benign (0.017)	Neutral (-0.880)	Uncertain	7	

and *PCDH15* have been reported to be a digenic cause of hearing loss [46].

In family 7 (Figure 1G), subjects II:1 and II:2 with hearing loss did not have candidate mutations in the first 61 genes. Analysis of the additional 23 genes indicated a compound heterozygous *USH2A* variant or mutation, c.1346G >A(p.R449H) and c.12431delC (p.A4144GfsX23), in subjects with hearing loss, whereas subjects I:1 and II:2 with normal hearing had a heterozygous p.R449H variant and a heterozygous p.A4144GfsX23 mutation, respectively. *USH2A* is responsible for Usher syndrome 2A (OMIM 276901) [47]. Although *USH2A* with the p.R449H variant was not found on dbSNP135, 1000GENOME, or the Exome Variant Server, the allele frequency in Japanese control subjects with normal hearing was 1.3% (5/378).

In the remaining eight families, none of the detected variants co-segregated with hearing loss in the pedigrees (data not shown).

Discussion

In the present study we selected Japanese subjects that had hereditary hearing loss without *GJB2* mutations, mitochondrial mutations, enlarged vestibular aqueduct or auditory neuropathy-associated *OTOF* mutations, and we aimed to detect the spectrum of rare deafness genes in these patients. Targeted NGS for 84 deafness genes resulted in identification of candidate genes in 7 of 15 families and revealed the diverse spectrum of rare deafness genes in Japanese subjects with nonsyndromic hearing loss for the first time. This is the first report of mutations in *ACTG1*, *POU4F3*, and *SLC26A5* in Japanese families with hearing loss. Families 5, 6, and 7 appeared to have candidate mutations or variants in *MYO7A*, *CDH23*, *PCDH15*, and *USH2A*, all of which are associated with Usher syndrome [39,44,45,47]. Our results are in contrast to an NGS study of a different ethnic group [48], which showed *TMC1* mutations to be the prevalent candidate cause of hearing loss.

For the eight families without candidate genes, hearing loss could be attributable to mutations in non-captured regions including regulatory domains of the 84 genes, other unidentified deafness genes, unknown multigenic causes, copy number variations, or chromosomal structural change.

Double heterozygous mutations

In family 5, double heterozygous mutations of *CDH23* and *PCDH15* were detected as a candidate cause. This combination of double heterozygous mutations has been reported [46]. Cadherin 23 and protocadherin 15 consist of the upper and lower part of tip link, respectively, which is critical for proper function of mechanotransduction channels on the stereocilia of the sensory hair cells [49]. In addition, P240 of CDH23 is on the extracellular cadherin 1 domain, and R283 of PCDH15 is on the extracellular cadherin 2 domain, which are considered to interact with each other for tip-link bound [49], raising the possibility that the double heterozygous mutations could lead to a destabilized tip-link.

Additional findings of double heterozygous mutations associated with hereditary hearing loss have been reported for *KCNJ10* and *SLC26A4* [50] and for *FOXI1* and *SLC26A4* [51], and some mutated genes may have a modifying effect [52]. Although most NGS pipelines, including ours, focus on identifying monogenic causes of disease, development of a detection strategy for digenic and oligogenic causes of disease should be considered in the future.

Discrimination of mutations from variants

The key challenge for the diagnostic application of NGS is to distinguish causal alleles from the numerous nonpathogenic variants present in each individual. In the present study, for example, the high allele frequency of *USH2A* with the p.R449H variant in Japanese control subjects implied that pathogenicity of this variant was unlikely. Ethnic diversity of genetic variance has been reported in deafness genes such as *OTOF* [12] and *CDH23* [43,53], and integration of a database of genetic variants with allele frequencies in a specific ethnic group would increase the certainty of the causative nature of genetic mutations by filtering out variants that occur with high frequency. This would facilitate targeted NGS analysis for genetic diagnosis of hearing loss.

Additional files

Additional file 1: The 84 genes that were targeted for next-generation sequencing.

Additional file 2: Clinical features of family members.

Additional file 3: Audiograms of subjects with hearing loss in the seven families in which candidate genes were detected. Figure legend: Hearing level as a function of frequency in subject IV:2 from family 1 (A), subject III:3 from family 1 (B), subject IV:3 from family 2 (C), subject III:1 from family 2 (D), subject III:2 from family 2 (E), subject III:1 from family 3 (F), subject II:1 from family 4 (G), subject III:1 from family 5 (H), subject II:2 from family 6 (I), subject II:3 from family 6 (J), and subject II:2 from family 7 (K). Open circles with solid lines represent air conduction thresholds of the right ear; crosses with dotted lines represent air conduction thresholds of the left ear; [symbols represent bone conduction thresholds of the right ear;] symbols represent bone conduction thresholds of the left ear; arrows pointing to the bottom left represent scale-out hearing level of the right ear; arrows pointing to the bottom right represent scale-out hearing level of the left ear.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

HM and NS carried out capturing and sequencing the DNA samples, interpreted the data, and drafted the manuscript. CT carried out capturing and sequencing the DNA samples. AS and JK worked on DNA sequencing and interpreting the data. KN carried out molecular modeling of gene

products. KKosaki and TM designed the study and interpreted the data. NM, KKaga, and TM contributed to accumulation and interpretation of clinical data. TM finalized the manuscript. All authors read and approved the final manuscript.

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References

1. Morton CC, Nance WE: **Newborn hearing screening–a silent revolution.** *N Engl J Med* 2006, **354**:2151–2164.
2. Kral A, O'Donoghue GM: **Profound deafness in childhood.** *N Engl J Med* 2010, **363**:1438–1450.
3. *Hereditary hearing loss homepage.* http://hereditaryhearingloss.org.
4. Hutchin T, Coy NN, Conlon H, Telford E, Bromelow K, Blaydon D, Taylor G, Coghill E, Brown S, Trembath R, Liu XZ, Bitner-Glindzica M, Mueller R: **Assessment of the genetic causes of recessive childhood non-syndromic deafness in the UK - implications for genetic testing.** *Clin Genet* 2005, **68**:506–512.
5. Matsunaga T, Kumanomido H, Shiroma M, Goto Y, Usami S: **Audiological features and mitochondrial DNA sequence in a large family carrying mitochondrial A1555G mutation without use of aminoglycoside.** *Ann Otol Rhinol Laryngol* 2005, **114**:153–160.
6. Shearer AE, DeLuca AP, Hildebrand MS, Taylor KR, Gurrola J 2nd, Scherer S, Scheetz TE, Smith RJ: **Comprehensive genetic testing for hereditary hearing loss using massively parallel sequencing.** *Proc Natl Acad Sci USA* 2010, **107**:21104–21109.
7. Shearer AE, Smith RJ: **Genetics: advances in genetic testing for deafness.** *Curr Opin Pediatr* 2012, **24**:679–686.
8. Brownstein Z, Bhonker Y, Avraham KB: **High-throughput sequencing to decipher the genetic heterogeneity of deafness.** *Genome Biol* 2012, **13**:245.
9. Delmaghani S, Aghaie A, Michalski N, Bonnet C, Weil D, Petit C: **Defect in the gene encoding the EAR/EPTP domain-containing protein TSPEAR causes DFNB98 profound deafness.** *Hum Mol Genet* 2012, **21**:3835–3844.
10. Schraders M, Haas SA, Weegerink NJ, Oostrik J, Hu H, Hoefsloot LH, Kannan S, Huygen PL, Pennings RJ, Admiraal RJ, Kalscheuer VM, Kunst HP, Kremer H: **Next-generation sequencing identifies mutations of SMPX, which encodes the small muscle protein, X-linked, as a cause of progressive hearing impairment.** *Am J Hum Genet* 2011, **88**:628–634.
11. Zheng J, Miller KK, Yang T, Hildebrand MS, Shearer AE, DeLuca AP, Scheetz TE, Drummond J, Scherer SE, Legan PK, Goodyear RJ, Richardson GP, Cheatham MA, Smith RJ, Dallos P: **Carcinoembryonic antigen-related cell adhesion molecule 16 interacts with alpha-tectorin and is mutated in autosomal dominant hearing loss (DFNA4).** *Proc Natl Acad Sci USA* 2011, **108**:4218–4223.
12. Matsunaga T, Mutai H, Kunishima S, Namba K, Morimoto N, Shinjo Y, Arimoto Y, Kataoka Y, Shintani T, Morita N, Sugiuchi T, Masuda S, Nakano A, Taiji H, Kaga K: **A prevalent founder mutation and genotype-phenotype correlations of OTOF in Japanese patients with auditory neuropathy.** *Clin Genet* 2012, **82**:425–432.
13. *dbSNP.* http://www.ncbi.nlm.nih.gov/projects/SNP/.
14. *1000GENOME.* http://www.1000genomes.org/.
15. *NHLBI exome variant server.* http://evs.gs.washington.edu/EVS/.
16. *UCSC conservation.* http://genome.ucsc.edu/index.html.
17. *PolyPhen-2.* http://genetics.bwh.harvard.edu/pph2/.
18. *PROVEAN.* http://provean.jcvi.org/index.php.
19. *NNSPLICE.* http://www.fruitfly.org/seq_tools/splice.html.
20. Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ: **Gapped BLAST and PSI-BLAST: a new generation of protein database search programs.** *Nucleic Acids Res* 1997, **25**:3389–3402.
21. *PDBsum.* http://www.ebi.ac.uk/pdbsum/.
22. Arnold K, Bordoli L, Kopp J, Schwede T: **The SWISS-MODEL workspace: a web-based environment for protein structure homology modelling.** *Bioinformatics* 2006, **22**:195–201.
23. Kiefer B, Riemann M, Buche C, Kassemeyer HH, Nick P: **The host guides morphogenesis and stomatal targeting in the grapevine pathogen plasmopara viticola.** *Planta* 2002, **215**:387–393.
24. Peitsch MC, Tschopp J: **Comparative molecular modelling of the Fas-ligand and other members of the TNF family.** *Mol Immunol* 1995, **32**:761–772.
25. Bowie JU, Luthy R, Eisenberg D: **A method to identify protein sequences that fold into a known three-dimensional structure.** *Science* 1991, **253**:164–170.
26. Luthy R, Bowie JU, Eisenberg D: **Assessment of protein models with three-dimensional profiles.** *Nature* 1992, **356**:83–85.
27. Pettersen EF, Goddard TD, Huang CC, Couch GS, Greenblatt DM, Meng EC, Ferrin TE: **UCSF chimera–a visualization system for exploratory research and analysis.** *J Comput Chem* 2004, **25**:1605–1612.
28. Morin M, Bryan KE, Mayo-Merino F, Goodyear R, Mencia A, Modamio-Hoybjor S, del Castillo I, Cabalka JM, Richardson G, Moreno F, Rubenstein PA, Moreno-Pelayo MA: **In vivo and in vitro effects of two novel gamma-actin (ACTG1) mutations that cause DFNA20/26 hearing impairment.** *Hum Mol Genet* 2009, **18**:3075–3089.
29. Shvetsov A, Musib R, Phillips M, Rubenstein PA, Reisler E: **Locking the hydrophobic loop 262–274 to G-actin surface by a disulfide bridge prevents filament formation.** *Biochemistry* 2002, **41**:10787–10793.
30. Collin RW, Chellappa R, Pauw RJ, Vriend G, Oostrik J, van Drunen W, Huygen PL, Admiraal R, Hoefsloot LH, Cremers FP, Xiang M, Cremers CW, Kremer H: **Missense mutations in POU4F3 cause autosomal dominant hearing impairment DFNA15 and affect subcellular localization and DNA binding.** *Hum Mutat* 2008, **29**:545–554.
31. Vahava O, Morell R, Lynch ED, Weiss S, Kagan ME, Ahituv N, Morrow JE, Lee MK, Skvorak AB, Morton CC, Blumenfeld A, Frydman M, Friedman TB, King MC, Avraham KB: **Mutation in transcription factor POU4F3 associated with inherited progressive hearing loss in humans.** *Science* 1998, **279**:1950–1954.
32. Bischoff AM, Luijendijk MW, Huygen PL, van Duijnhoven G, De Leenheer EM, Oudesluijs GG, Van Laer L, Cremers FP, Cremers CW, Kremer H: **A novel mutation identified in the DFNA5 gene in a Dutch family: a clinical and genetic evaluation.** *Audiol Neurootol* 2004, **9**:34–46.
33. Van Laer L, Meyer NC, Malekpour M, Riazalhosseini Y, Moghannibashi M, Kahrizi K, Vandevelde A, Alasti F, Najmabadi H, Van Camp G, Smith RJ: **A novel DFNA5 mutation does not cause hearing loss in an Iranian family.** *J Hum Genet* 2007, **52**:549–552.
34. Liu XZ: **Prestin, a cochlear motor protein, is defective in non-syndromic hearing loss.** *Hum Mol Genet* 2003, **12**:1155–1162.
35. Ruf RG, Xu PX, Silvius D, Otto EA, Beekmann F, Muerb UT, Kumar S, Neuhaus TJ, Kemper MJ, Raymond RM Jr, Brophy PD, Berkman J, Gattas M, Hyland V, Ruf EM, Schwartz C, Chang EH, Smith RJ, Stratakis CA, Weil D, Petit C, Hildebrandt F: **SIX1 mutations cause branchio-oto-renal syndrome by disruption of EYA1-SIX1-DNA complexes.** *Proc Natl Acad Sci USA* 2004, **101**:8090–8095.
36. Smith RJH, *et al*: **Branchiootorenal spectrum disorders.** In *Gene reviews.* Edited by Pagon RA, Adam MP, Bird TD. http://www.ncbi.nlm.nih.gov/books/NBK1380/.
37. Liu XZ, Walsh J, Tamagawa Y, Kitamura K, Nishizawa M, Steel KP, Brown SD: **Autosomal dominant non-syndromic deafness caused by a mutation in the myosin VIIA gene.** *Nat Genet* 1997, **17**:268–269.
38. Liu XZ, Walsh J, Mburu P, Kendrick-Jones J, Cope MJ, Steel KP, Brown SD: **Mutations in the myosin VIIA gene cause non-syndromic recessive deafness.** *Nat Genet* 1997, **16**:188–190.

39. Weil D, Kussel P, Blanchard S, Levy G, Levi-Acobas F, Drira M, Ayadi H, Petit C: **The autosomal recessive isolated deafness, DFNB2, and the Usher 1B syndrome are allelic defects of the myosin-VIIA gene.** *Nat Genet* 1997, **16:**191–193.
40. Yang Y, Baboolal TG, Siththanandan V, Chen M, Walker ML, Knight PJ, Peckham M, Sellers JR: **A FERM domain autoregulates drosophila myosin 7a activity.** *Proc Natl Acad Sci USA* 2009, **106:**4189–4194.
41. Adato A, Michel V, Kikkawa Y, Reiners J, Alagramam KN, Weil D, Yonekawa H, Wolfrum U, El-Amraoui A, Petit C: **Interactions in the network of usher syndrome type 1 proteins.** *Hum Mol Genet* 2005, **14:**347–356.
42. Wu L, Pan L, Wei Z, Zhang M: **Structure of MyTH4-FERM domains in myosin VIIa tail bound to cargo.** *Science* 2011, **331:**757–760.
43. Wagatsuma M, Kitoh R, Suzuki H, Fukuoka H, Takumi Y, Usami S: **Distribution and frequencies of CDH23 mutations in Japanese patients with non-syndromic hearing loss.** *Clin Genet* 2007, **72:**339–344.
44. Bork JM, Peters LM, Riazuddin S, Bernstein SL, Ahmed ZM, Ness SL, Polomeno R, Ramesh A, Schloss M, Srisailpathy CR, Wayne S, Bellman S, Desmukh D, Ahmed Z, Khan SN, Kaloustian VM, Li XC, Lalwani A, Riazuddin S, Bitner-Glindzicz M, Nance WE, Liu XZ, Wistow G, Smith RJ, Griffith AJ, Wilcox ER, Friedman TB, Morell RJ: **Usher syndrome 1D and nonsyndromic autosomal recessive deafness DFNB12 are caused by allelic mutations of the novel cadherin-like gene CDH23.** *Am J Hum Genet* 2001, **68:**26–37.
45. Ahmed ZM, Riazuddin S, Ahmad J, Bernstein SL, Guo Y, Sabar MF, Sieving P, Riazuddin S, Griffith AJ, Friedman TB, Belyantseva IA, Wilcox ER: **PCDH15 is expressed in the neurosensory epithelium of the eye and ear and mutant alleles are responsible for both USH1F and DFNB23.** *Hum Mol Genet* 2003, **12:**3215–3223.
46. Zheng QY, Yan D, Ouyang XM, Du LL, Yu H, Chang B, Johnson KR, Liu XZ: **Digenic inheritance of deafness caused by mutations in genes encoding cadherin 23 and protocadherin 15 in mice and humans.** *Hum Mol Genet* 2005, **14:**103–111.
47. Eudy JD, Weston MD, Yao S, Hoover DM, Rehm HL, Ma-Edmonds M, Yan D, Ahmad I, Cheng JJ, Ayuso C, Cremers C, Davenport S, Moller C, Talmadge CB, Beisel KW, Tamayo M, Morton CC, Swaroop A, Kimberling WJ, Sumegi J: **Mutation of a gene encoding a protein with extracellular matrix motifs in usher syndrome type IIa.** *Science* 1998, **280:**1753–1757.
48. Brownstein Z, Friedman LM, Shahin H, Oron-Karni V, Kol N, Abu Rayyan A, Parzefall T, Lev D, Shalev S, Frydman M, Davidov B, Shohat M, Rahile M, Lieberman S, Levy-Lahad E, Lee MK, Shomron N, King MC, Walsh T, Kanaan M, Avraham KB: **Targeted genomic capture and massively parallel sequencing to identify genes for hereditary hearing loss in middle eastern families.** *Genome Biol* 2011, **12:**R89.
49. Sotomayor M, Weihofen WA, Gaudet R, Corey DP: **Structure of a force-conveying cadherin bond essential for inner-ear mechanotransduction.** *Nature* 2012, **492:**128–132.
50. Yang T, Gurrola JG 2nd, Wu H, Chiu SM, Wangemann P, Snyder PM, Smith RJ: **Mutations of KCNJ10 together with mutations of SLC26A4 cause digenic nonsyndromic hearing loss associated with enlarged vestibular aqueduct syndrome.** *Am J Hum Genet* 2009, **84:**651–657.
51. Yang T, Vidarsson H, Rodrigo-Blomqvist S, Rosengren SS, Enerback S, Smith RJ: **Transcriptional control of SLC26A4 is involved in pendred syndrome and nonsyndromic enlargement of vestibular aqueduct (DFNB4).** *Am J Hum Genet* 2007, **80:**1055–1063.
52. Riazuddin S, Castelein CM, Ahmed ZM, Lalwani AK, Mastroianni MA, Naz S, Smith TN, Liburd NA, Friedman TB, Griffith AJ, Riazuddin S, Wilcox ER: **Dominant modifier DFNM1 suppresses recessive deafness DFNB26.** *Nat Genet* 2000, **26:**431–434.
53. Miyagawa M, Nishio SY, Usami S: **Prevalence and clinical features of hearing loss patients with CDH23 mutations: a large cohort study.** *PLoS One* 2012, **7:**e40366.

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ORIGINAL ARTICLE

Genetic analysis of *PAX3* for diagnosis of Waardenburg syndrome type I

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Abstract

Conclusion: *PAX3* genetic analysis increased the diagnostic accuracy for Waardenburg syndrome type I (WS1). Analysis of the three-dimensional (3D) structure of PAX3 helped verify the pathogenicity of a missense mutation, and multiple ligation-dependent probe amplification (MLPA) analysis of *PAX3* increased the sensitivity of genetic diagnosis in patients with WS1. *Objectives:* Clinical diagnosis of WS1 is often difficult in individual patients with isolated, mild, or non-specific symptoms. The objective of the present study was to facilitate the accurate diagnosis of WS1 through genetic analysis of *PAX3* and to expand the spectrum of known *PAX3* mutations. *Methods:* In two Japanese families with WS1, we conducted a clinical evaluation of symptoms and genetic analysis, which involved direct sequencing, MLPA analysis, quantitative PCR of *PAX3*, and analysis of the predicted 3D structure of PAX3. The normal-hearing control group comprised 92 subjects who had normal hearing according to pure tone audiometry. *Results:* In one family, direct sequencing of *PAX3* identified a heterozygous mutation, p. I59F. Analysis of PAX3 3D structures indicated that this mutation distorted the DNA-binding site of PAX3. In the other family, MLPA analysis and subsequent quantitative PCR detected a large, heterozygous deletion spanning 1759–2554 kb that eliminated 12–18 genes including a whole *PAX3* gene.

Keywords: *Mutation, MLPA, clinical diagnosis, hearing loss, dystopia canthorum, pigmentary disorder*

Introduction

Waardenburg syndrome (WS) is a hereditary auditory pigmentary disorder that is responsible for 1–3% of congenital deafness cases [1]. WS is classified into four types based on symptoms other than the auditory and pigmentary disorder. Type I WS (WS1) includes dystopia canthorum, and this feature distinguishes WS1 from type II WS. Type III WS is similar to WS1 but is associated with musculoskeletal anomalies of the upper limbs. Type IV WS is similar to type I but is associated with Hirschsprung disease. Diagnostic criteria for WS1 have been proposed [2]. The clinical features of WS1 demonstrate incomplete penetrance and highly varied expression [3,4], which makes diagnosis in individual patients challenging. For example, WS1 patients may present only one isolated symptom. Diagnosis of high nasal root and medial eyebrow flare can be difficult when they are mild. Hearing loss and early graying are relatively common in the general population and are not specific to WS1. Thus, the accuracy of WS1 diagnosis needs to be improved by the use of additional diagnostic procedures.

It is reported that more than 90% of patients with WS1 harbor point mutations in *PAX3* [5], and an additional 6% of WS1 patients harbor partial or complete *PAX3* deletions [6]. This high frequency of *PAX3* mutation in WS1 suggests that clinical diagnosis of WS1 could be facilitated by *PAX3* genetic analysis. To date, more than 80 *PAX3*

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mutations are reported to be associated with WS1 [5]. A de novo paracentric inversion on chromosome 2 in a Japanese child with WS1 provided a clue for identification of *PAX3* in the distal part of chromosome 2 [7]. However, only a few *PAX3* mutations including the chromosomal inversion have been reported in Japanese patients with WS1 since then [8,9].

In the present study, we conducted *PAX3* genetic analysis to facilitate diagnosis of WS1 in two Japanese families. In one family, to verify the pathogenicity of an identified missense mutation, we analyzed the effect of the mutation on the three-dimensional (3D) structure of PAX3. In the other family, no mutations were identified by direct sequencing, so multiple ligation-dependent probe amplification (MLPA) analysis was used to search for large deletions in *PAX3* and thereby increase the sensitivity of genetic diagnosis.

Material and methods

Patients and control subjects

Two Japanese families with WS1 were included in the study. The diagnosis of WS1 was based on criteria proposed by the Waardenburg Consortium [2]. The normal-hearing controls comprised 92 subjects who had normal hearing according to pure tone audiometry. This study was approved by the institutional ethics review board at the National Tokyo Medical Center. Written informed consent was obtained from all subjects included in the study or from their parents.

Clinical evaluation

A comprehensive clinical history was taken from subjects who were examined at our hospitals or from their parents. During physical examination, special attention was given to the color of the skin, hair, and iris, and to other anomalies such as dystopia canthorum, medial eyebrow flare, limb abnormalities, and Hirschsprung disease. After otoscopic examination, behavioral audiometric testing was performed. The test protocol was selected according to the developmental age of the subject (conditioned orientation response audiometry, play audiometry, or conventional audiometric testing, from 125 to 8000 Hz), and testing was performed using a diagnostic audiometer in a soundproof room. Auditory brainstem response (ABR) and otoacoustic emission were also evaluated in some subjects.

Direct sequencing

Genomic DNA from the subjects was extracted from peripheral blood leukocytes using the Gentra Puregene® Blood kit (QIAGEN, Hamburg, Germany). Mutation screening of *PAX3* was performed by bidirectional sequencing of each exon (exons 1–11) together with the flanking intronic regions using an ABI 3730 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Primer sequences for *PAX3* are listed in Table I. Mutation nomenclature is based on the genomic DNA sequence of [GenBank accession no. NG_011632.1], with the A of the translation initiation codon considered as +1. Nucleotide conservation between mammalian species was evaluated using ClustalW (http://www.ebi.ac.uk/Tools/msa/clustalw2/). PolyPhen-2 software (http://genetics.bwh.harvard.edu/pph2/) was used to predict the functional consequence(s) of each amino acid substitution.

MLPA

MLPA analysis was performed using an MLPA kit targeting *PAX3*, *MITF*, and *SOX10* (SALSA MLPA Kit P186-B1, MRC-Holland, Amsterdam, The Netherlands) according to the manufacturer's protocol. Exon-specific MLPA probes for exons 1–9 of *PAX3* and control probes were hybridized to genomic DNA from the subjects and normal controls and ligated with fluorescently labeled primers. A PCR reaction was then performed to amplify the hybridized probes. The amplified probes were fractionated on an ABI3130xl Genetic Analyzer (Applied Biosystems) and the peak patterns were evaluated using GeneMapper (Applied Biosystems).

Real-time PCR

To determine the length of each deleted genomic region, 100 ng of genomic DNA from the subjects and a normal control were subjected to quantitative PCR (Prism 7000, Applied Biosystems) using Power SYBR® Green Master Mix (Life Technologies, Carlsbad, CA, USA) and 12 sets of primers designed to amplify sequence-tagged sites on chromosome 2 (GenBank accession nos: RH46518, RH30035, RH66441, GDB603632, 1988, RH24952, RH47422, RH65573, RH26526, RH35885, RH16314, and RH92249).

Homology modeling of the PAX3 paired domain

The DNA-binding site of the paired domain of PAX3 was modeled using SWISS-MODEL [10] with the crystal structure of the PAX5 paired domain-DNA complex (PDB ID:1PDN_chain C) as the template because PAX3 and PAX5 are functionally and structurally similar [11]. The amino acid

Table I. Primer sequences for *PAX3*.

Exon 1	Forward	5′-TGTAAAACGACGGCCAGTAGAGCAGCGCGCTCCATTTG-3′
	Reverse	5′-CAGGAAACAGCTATGACCGCTCGCCGTGGCTCTCTGA-3′
Exon 2	Forward	5′-TGTAAAACGACGGCCAGTAAGAAGTGTCCAGGGCGCGT-3′
	Reverse	5′-CAGGAAACAGCTATGACCGGTCTGGGTCTGGGAGTCCG-3′
Exon 3	Forward	5′-TGTAAAACGACGGCCAGTTAAACGCTCTGCCTCCGCCT-3′
	Reverse	5′-CAGGAAACAGCTATGACCGGGATGTGTTCTGGTCTGCCC-3′
Exon 4	Forward	5′-TGTAAAACGACGGCCAGTAATGGCAACAGAGTGAGAGCTTCC-3′
	Reverse	5′-CAGGAAACAGCTATGACCAGGAGACACCCGCGAGCAGT-3′
Exon 5	Forward	5′-TGTAAAACGACGGCCAGTGGTGCCAGCACTCTAAGAACCCA-3′
	Reverse	5′-CAGGAAACAGCTATGACCGGTGATCTGACGGCAGCCAA-3′
Exon 6	Forward	5′-TGTAAAACGACGGCCAGTTGCATCCCTAGTAAAGGGCCA-3′
	Reverse	5′-CAGGAAACAGCTATGACCGGTGTCCATGGAAGACATTGGG-3′
Exon 7	Forward	5′-AACTATTATTTCATCAGTGAAATC-3′
	Reverse	5′-ATTCACTTGTATAAAATATCCACC-3′
Exon 8	Forward	5′-TGTAAAACGACGGCCAGTTGAAGCCAGTAGGAAGGGTGGA-3′
	Reverse	5′-CAGGAAACAGCTATGACCTGCAGGTTAAGAAACGCAGTTTGA-3′
Exon 9a	Forward	5′-TGTAAAACGACGGCCAGTTTGATACCGGCATGTGTGGC-3′
	Reverse	5′-CAGGAAACAGCTATGACCTGCAGTCAGATGTTATCGTCGGG-3′
Exon 9b	Forward	5′-TGTAAAACGACGGCCAGTCACAACTTTGTGTCCCTGGGATT-3′
	Reverse	5′-CAGGAAACAGCTATGACCGGGACTCCTGACCAACCACG-3′
Exon 10–11	Forward	5′-TGTAAAACGACGGCCAGTGCAAATGGAATGTTCTAGCTCCTCG-3′
	Reverse	5′-CAGGAAACAGCTATGACCGGTCAGCTCCAGGATCATATGGG-3′

sequences of the PAX3 and PAX5 paired domains were 79% homologous. The predicted PAX3 structure and the p.I59F mutation structure were superimposed on the backbone atoms of the PAX5 paired domain-DNA complex and displayed using the extensible visualization system, UCSF Chimera [12].

Results

In family 1, the proband, a 9-month-old male, was the first child of unrelated Japanese parents. Abnormal responses were found upon newborn hearing screening in the left ear, and left hearing loss was diagnosed by ABR. On physical examination, dystopia canthorum was noted, with a W-index of 2.77. The patient's mother also had dystopia canthorum, with a W-index of 2.68. She also had a history of early graying that started at age 16 years. She had not been diagnosed with WS1. According to the parents, 10 members of this family, including the proband and the mother, showed clinical features consistent with WS1 (Figure 1). ABR performed in the proband

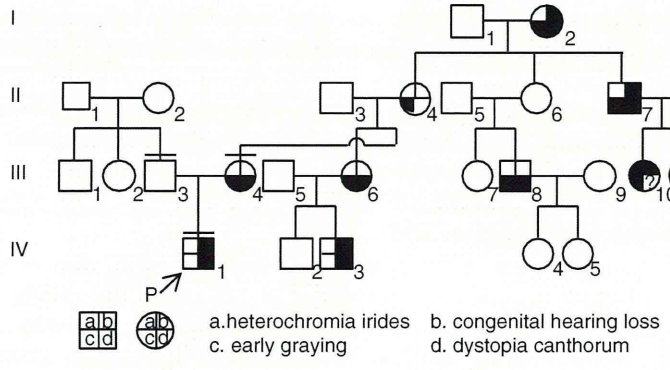

Figure 1. Pedigree of family 1. The proband is indicated by an arrow. The individuals we examined personally are indicated by a bar over the symbol. Phenotypes observed in this family are indicated symbolically as detailed below the pedigree.

revealed normal hearing in the right ear and no responses to 105 dB click stimuli in the left ear. Computed tomography (CT) of the temporal bone showed normal structures in the inner, middle, and outer ears.

Genetic analysis of *PAX3* was conducted in this family, and direct sequencing of *PAX3* revealed a heterozygous mutation, c.175A>T, in the proband and his mother. This mutation resulted in a missense mutation, p.I59F (Figure 2A). The proband's father did not harbor this mutation. p.I59F is located within exon 2 and is part of the paired domain of *PAX3*, which is a critical region for interaction between transcription factors and target DNA (Figure 2B). A multiple alignment of *PAX3* orthologs at this region demonstrated that I59 was evolutionarily conserved among various species (Figure 2C). The p.I59F mutation was not identified in any of the 184 alleles from the normal control subjects. This mutation was predicted to be 'probably damaging' according to PolyPhen-2 software.

The predicted 3D structures of the paired domain of the PAX3-DNA complex indicated that the PAX3 paired domain binds to the corresponding DNA (white double helixes) via hydrogen bonds (pink lines) at the N-terminal of α-helix1 (H1), α-helix2 (H2), and α-helix3 (H3) (indicated in blue; Figure 3A). I59 is located in the middle of H1, H2, and H3 and is surrounded by hydrophobic residues (green) protruding from H1, H2, and H3. Because the van der Waals radius of phenylalanine (Figure 3C; white arrows) is larger than that of isoleucine (Figure 3B, white arrowheads), F59 repels the surrounding hydrophobic residues by van der Waals forces and increases the distance between F59 and the surrounding hydrophobic residues, resulting in structural distortion of the DNA-binding site of PAX3. Since this site is precisely shaped for maximal binding to the corresponding DNA, this mutation is likely to reduce the binding ability of the paired domain of PAX3 and cause WSI. A mutational search found the same mutation in another Japanese family [8].

In family 2, the proband, a female aged 4 years and 4 months, was the first child of unrelated Japanese parents. Abnormal responses were found upon newborn hearing screening in the right ear, and right hearing loss was diagnosed by ABR. On physical examination, dystopia canthorum, medial eyebrow flare, and a white forelock were noted. She was admitted to hospital suffering from ketotic hypoglycemia of unknown cause when aged 4 years. Her mother presented with heterochromia iridis, dystopia canthorum, and medial eyebrow flare, and her grandmother presented with early graying that started at around 20 years of age, dystopia canthorum, and

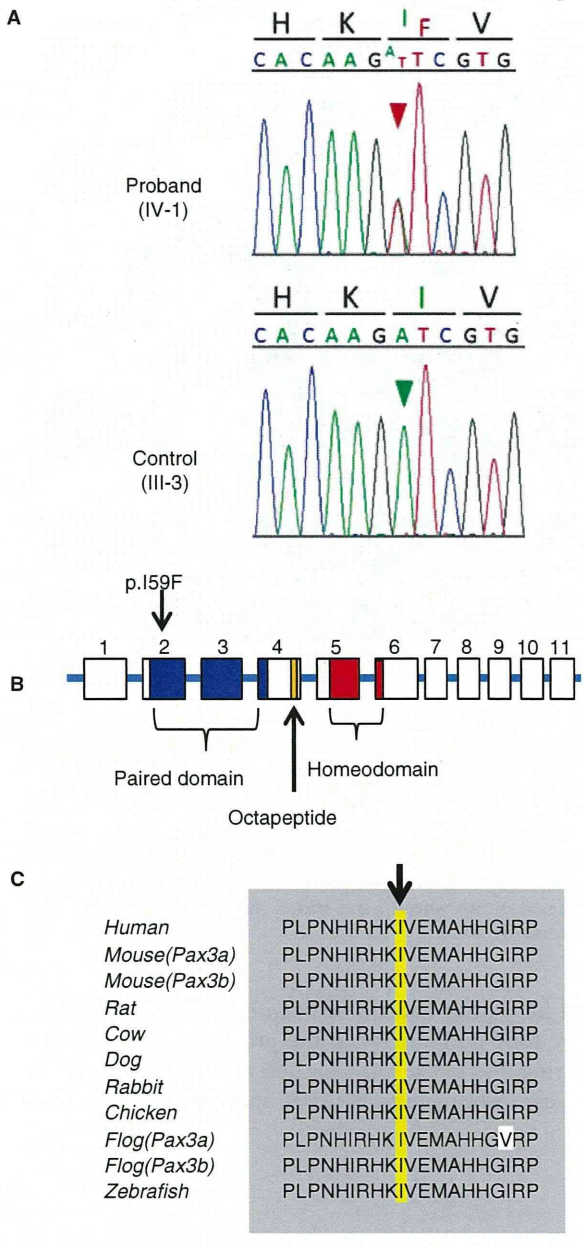

Figure 2. The p.I59F mutation of *PAX3* detected in family 1. (A) Sequence chromatogram for the proband and unaffected control. A heterozygous A to T transversion (red arrowhead) that changes codon 59 from ATC, encoding isoleucine (I), to TTC, encoding phenylalanine (F), was detected in the proband but not in the control (green arrowhead). (B) Localization of the p.I59F mutation and functional domains of PAX3. (C) A multiple alignment of PAX3 orthologs. Regions of amino acid sequence identity are shaded gray. The position of I59 is indicated by an arrow and shaded yellow.

medial eyebrow flare. According to the grandmother, the father of the grandmother also had dystopia canthorum and medial eyebrow flare. The pedigree of family 2 is shown in Figure 4. The grandmother