MASTER'S THESIS

Molecular genetic analyses of consanguineous families with hearing impairment and vision loss

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Submitted by,

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Statutory Declaration

I declare that this thesis is an original report of my research, which has been composed by myself, and has not been submitted, in whole or in part, for any previous application for a degree. The experimental work contained herein is my own, except where explicitly stated otherwise. Collaborative contributions have been indicated clearly and acknowledged and due references have been provided on all supporting literatures and resources. I declare that throughout this thesis I followed the “Standards of Good Scientific Practice” without exception.

Graz, August, 2020 Beatrice Brugger

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Statement of Contributes

This study was conducted with the contribution of the following people:

Institute of Human Genetics, Medical University of Graz:

- Christian Windpassinger, Assoc. Prof. PD Mag. Dr.rer.nat.: project leader and supervisor
- Jasmin Blatterer MSc.: supervisor

Gomal Centre of Biochemistry and Biotechnology, Gomal University D.I.Khan:

- Muzammil Khan: Recruitment of USHER1 and USHER2 family, patient examination, provision of patient related information, sample preparation and shipment

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Abbreviations

aa	Amino acid
ABI	Applied Biosystems
AH	Average heterozygosity
ASR	Allelic Size Range
ATP	Adenosine Triphosphate
bp	Base pair
CDH23	Cadherin Related 23
Chr	Chromosome
Da	Dalton
dB	Dezibel
DMSO	Dimethyl sulfoxide
DNA	Desoxyribonucleicacid
EGF	Epidermal Growth Factor
ExAC	Exome Aggregation Consortium
gnomAD	Genome Aggregation Database
GRCh37/hg19	Genome Reference Consortium Human Build 37/human genome 19
HGMD	Human Gene Mutation Database
IHC	Inner hair cell
kDA	Kilo Dalton
LRP2	Low density lipoprotein receptor-related protein 2
MB	Mega bases
MM	Master Mix
mV	Milli Volt
MYO6	Myosin VI
MYO7A	Myosin VIIA
OHC	Outer hair cell
OMIM	Online Mendelian Inheritance in Man
PCDH15	Protocadherin-15
PCR	Polymerase chain reaction
rcf	Relative centrifugal force
RP	Retinitis pigmentosa
RPE	Retinal pigment epithelium
rs- number	RefSNPs- number
SCN2A	Sodium voltage-gated channel alpha subunit 2
Shh	Sonic hedgehog
SNP	Single nucleotide polymorphism
STS Marker	Sequence-tagged site marker
TBS	Tris-buffered saline
UCSC	University of California Santa Cruz
USH1C	USH1 Protein Network Component Harmonin
USH1F	Protocadherin-15 alias PCDH15
USH1G	USH1 Protein Network Component Sans
USH2A	Usherin

USH3A	Clarin-1
UV	Ultra violet
vcf	Variant call format
VLGR1	Adhesion G Protein-Coupled Receptor V1
WES	Whole Exome Sequencing

Abstract

Usher syndrome is a widespread disease which can result in deafness and blindness. It is inherited autosomal recessively and belongs to one of about 7000 rare genetic diseases. Certain genes can be linked to one of three subtypes of Usher syndrome. Each form of the Usher syndrome, Usher type 1, Usher type 2 and Usher type 3, triggers a different phenotype.

Some forms of blindness occur in cause of DNA mutations. In case of Usher syndrome they can result in retinitis pigmentosa. First, perception of colours worsens, later on, bright dark vision deteriorates. After that, this disease can lead to complete blindness. Deafness manifests in all subtypes at a different time. Whilst with Usher type 1 a complete deafness can be expected already in the first years of life. Usher type 2 and 3 affected individuals show a later manifestation of hearing impairment. In addition, early hearing loss in Usher type 1 could lead to a diminished development of speech. Genetic defects, which end up in a disturbed development of stereocilia can also affect the vestibular organ and leads to vestibular dysfunction or a delay in independent sitting or walking. Nowadays, some forms of therapy are being researched, in some cases there are already possibilities to eradicate the phenotype. However, it requires molecular genetic methods which determine the exact causal mutation in a certain gene.

In this thesis, two mutations possibly associated to hearing impairment and vision loss could be found. An already known mutation in the MYO7A gene, NM_000260.4:c.1258A>T, which is already related to the Usher syndrome was identified in all affected individuals of one consanguineous family.

Additionally, we were able to detect the variant NM_004525.3:c.11972G>C in the *LRP2* gene in all affected individuals from a consanguineous family. Some mutations in this gene are known to cause Donnai-Barrow syndrome or Stickler like syndrome. Further analysis has to be performed, to confirm the relation of this mutation with the present disease.

Zusammenfassung

Usher-Syndrom ist eine weit verbreitete Krankheit, welche zu Taub und Blindheit führen kann. Sie wird autosomal rezessiv vererbt und gehört zu einer von etwa 7000 seltenen genetischen Erkrankungen. Das Usher-Syndrom wird je nach Phänotyp in einen von drei Subtypen eingeteilt. Diese können durch Mutationen in verschiedenen Genen ausgelöst werden.

Blindheit kann unter anderem durch Veränderungen in der DNA ausgelöst werden. Im Falle des Usher-Syndroms können sie zu einer Retinitis pigmentosa führen. Hierbei verschlechtert sich zunächst die Farbwahrnehmung, später das Hell-Dunkel-Sehen und einige Betroffene können auch an vollständiger Blindheit leiden. Taubheit manifestiert sich bei allen Subtypen zu einem anderen Zeitpunkt. Während bei Usher Typ 1 bereits in den ersten Lebensjahren mit einer völligen Taubheit gerechnet werden kann, zeigen Betroffene von Usher-Typ 2 oder 3 eine spätere Manifestation der Schwerhörigkeit. Der frühe Hörverlust beim Usher-Typ 1 kann zu einer verminderten Sprachentwicklung führen. Genetische Defekte, die in einer gehinderten Entwicklung der Stereozilien resultieren, können das Gleichgewichtsorgan betreffen und in einer vestibularen Dysfunktion, oder Verzögerung des unabhängigen Sitzens oder Gehens enden. Es wird an Therapien geforscht, in einigen Fällen gibt es bereits Möglichkeiten, den Phänotyp auszumerzen. Die molekulargenetische Analyse ist jedoch Voraussetzung für diese Therapien.

In dieser Arbeit konnten zwei Mutationen gefunden werden, die möglicherweise mit Hör- und Sehbehinderung in Verbindung stehen. Eine bereits bekannte Mutation im *MYO7A* Gen NM_000260.4:c.1258A>T, die mit dem Usher-Syndrom in Zusammenhang steht wurde bei allen betroffenen Personen einer konsanguinen Familie identifiziert.

Zusätzlich konnten wir die Mutation NM_004525.3:c.11972G>C im *LRP2* Gen bei allen betroffenen Personen aus einer konsanguinen Familie nachweisen. Es ist bekannt, dass einige Mutationen in diesem Gen das Donnai-Barrow-Syndrom oder Stickler-like Syndrom verursachen. Es müssen weitere Analysen durchgeführt werden, um den Zusammenhang dieser Mutation mit der vorliegenden Krankheit zu bestätigen.

1 Introduction

To our present knowledge 6000 to 7000 rare genetic diseases exist in total. For less than 2000 rare genetic diseases the underlying genetic defect is already found. (2, 3). A rare genetic disease is characterised by a mutation, which is located in protein coding regions or affect the transcription of a protein. Further characteristic is, that the aberration is very rare in the total population (3). Nearly all genetic diseases are rare, but not all rare diseases have a genetic origin (4). Rare genetic diseases are often inherited in a recessive way. In this inherited manner, affected patients have two mutated alleles and show the phenotype for the disease triggered by the mutation. Non affected parents are carrier and have one mutated allele. In the next generation, according to Mendelian inheritance, children have a disease risk of 25%. To identify a correlation between a mutation in a gene and the disease, two methods can be used.
A disease phenotype indicates an alteration in an already known disease gene. In this case, Sanger sequencing for each exon of the candidate gene can be performed. However, it is a time consuming way to analyse any alteration in a gene. If the mutation is known or is likely to be disease causing, Sanger Sequencing can be used to see whether the mutation segregates within the affected family members with the disease. If there is no indication as to which mutation in which gene could be the trigger for the disease, further methods can be combined to limit the possibilities. A powerful method could be positional mapping like karyotyping, linkage analysis, homozygosity mapping, copy number variation or SNP based analysis. By using these methods, the possible positions of a candidate gene can be further limited to a few. For traditional gene identification, it is also advantageous to know the underlying inherited mode e.g. dominant, recessive or X-linked. If a SNP Array analysis is available, this data can be used for homozygosity mapping. For instance, genes known for autosomal recessive inherited diseases are preferably located in large homozygous regions. An *in silico* analysis of these regions, for example with usage of the UCSC Genome Browser in combination with the OMIM (Online Mendelian Inheritance in Man) database can help to identify candidate genes. Protein function of an expressed gene may give an indication of whether changes in this gene, and possibly consequent changes in protein composition, could be a possible trigger for the underlying disease. In this case, Sanger sequencing of the whole coding sequence of the gene can be performed (3). Two strategies were pursued in this work, the linkage strategy and the homozygosity

strategy. Linkage strategy is dependent to the mode of inheritance during meiosis and is the result from crossing over between the homology regions on chromosomes. Genes, which are close to each other, have a lower risk to be separated. The separation risk is indicated in centimorgan (cM). The closer a gene is located to the neighbouring gene, the less likely they get separated during the process of crossing-over (5). The linkage analysis uses polymorphic sequences (e.g. microsatellites) in the genome to identify the genomic location of the disease gene in familial cases. Since microsatellites differ in the allele length between individuals, the origin of a particular allele can be deduced. For this analysis, parents, siblings, grandparents and other blood relatives can be analysed. Linkage mapping strategy can limit the possible loci where the causal mutation in a gene might be present (3). The homozygosity strategy is used to map recessive traits in consanguineous families. As described above, in a rare Mendelian disease, the causal mutation in a gene should be preferably found in a large homozygous region. To perform a homozygosity mapping, SNP- Array analysis has to be performed (3) Therefore, DNA from affected individuals is hybridized with specific oligonucleotide probes. Results are compared with a reference sample and is analysed with specific software to investigate homozygous region (6).

The gold standard for identifying new disease genes is Whole Exome Sequencing (WES). The entire exome and additionally important regions are sequenced. This method provides all variants in the sequenced region of an individual. Special filter strategies allow to reduce 40.000 variants to about 50. If there are additional WES data of family members, they can be compared to each other, after this step ~2-10 variants are left. These are analysed to see if they could correlate with the phenotype. When a candidate variant is found, Sanger sequencing has to be performed with the DNA of all family members. If the mutation is the cause of the recessive disease, all affected family members will be homozygous for the mutation and all non-affected family members are heterozygous or homozygous for the WT allele.

1.1 Usher Syndrome

Usher Syndrome is a rare, recessive genetic disease and can only definitely be diagnosed with genetic tests and methods named before (7–10). It is the leading genetic cause of hearing and vision loss. (11, 10) The phenotype can be pronounced from ten (12) main genes. Appearance of Retinitis pigmentosa (RP) and deafness were the first description for the phenotype about 150 years ago (12). Today, three types of the syndrome can be distinguished, Usher type 1, Usher type 2 and Usher type 3 (13, 9, 12). These three types can be differentiated by the severity and onset of deafness and vestibular dysfunction (13, 8, 9, 12, 11). While hearing impairment might start at birth, RP gets worse during lifetime (10). RP manifests itself by perception problems in the dark, or night blindness and a gradual loss of peripheral vision. Symptoms of RP vary with the manifestation of the age of the affected person and the genetically underlying mutation (11, 14). Hearing impairment differs from the three subtypes of Usher Syndrome. Usher type 1 shows hearing loss from birth. Further, patients with this type show vestibular areflexia with severe balance problems. (15, 11, 14). Additionally to these symptoms, also RP can occur in the first decade of life (11, 14). Usher type 2 results in a milder phenotype. Patients show a mild to severe hearing loss, normal vestibular function and an onset of RP in the second decade of life (11, 15, 16). In Usher type 3 patients the onset of RP is after the first decade of life. Affected people have variable vestibular function/dysfunction and progressive hearing loss (14).

1.2 Retinitis Pigmentosa

Retinitis Pigmentosa is a retinal disease with a loss of function of the sensory cells. At first the function of the rods decreases, which results in a limited vision in the dark and the dusk. The next step of the degeneration also pertains to the function of the rods and affects central vision, also called tunnel vision. With progression of the disease, cones lose their function as well. First the colour vision gets lost followed by total blindness (17–19, 12, 8). Usher syndrome represents with 10% of all Retinitis Pigmentosa patients one of the most common causes for the disease (17). Cell death of rods is caused by genetics, but for the decreasing functional capability of the cones, no underlying mechanism has been found yet. One hypothesis state that the cones receive too little nutrients, like glucose, to work properly. This happens due to oxidative stress, less energy support or the lack of supply of special proteins of the photoreceptor

cells, for example RdCVF. This protein is usually produced by the rods and helps the cones to import glucose into the cells. Therefore they produce less energy to survive, which triggers cell death (17). The subsequent figure 1 shows the structure of the retina and the spacial position of the rods and cones (1). Up to now there is no treatment which makes the disease reversible. Possible approaches are gene therapy, stem cell therapy, drugs like Unoproston-Isopropyl, cell protectors like growth factors, electric or sub-retinal retina transplants, or optogenetics. Regarding optogenetics, light sensitive ion channels or pumps are integrated into the photoreceptors to reconstruct their function (17). Vitamin A, given as a supplement, can also slow down the progression of RP (20, 21).

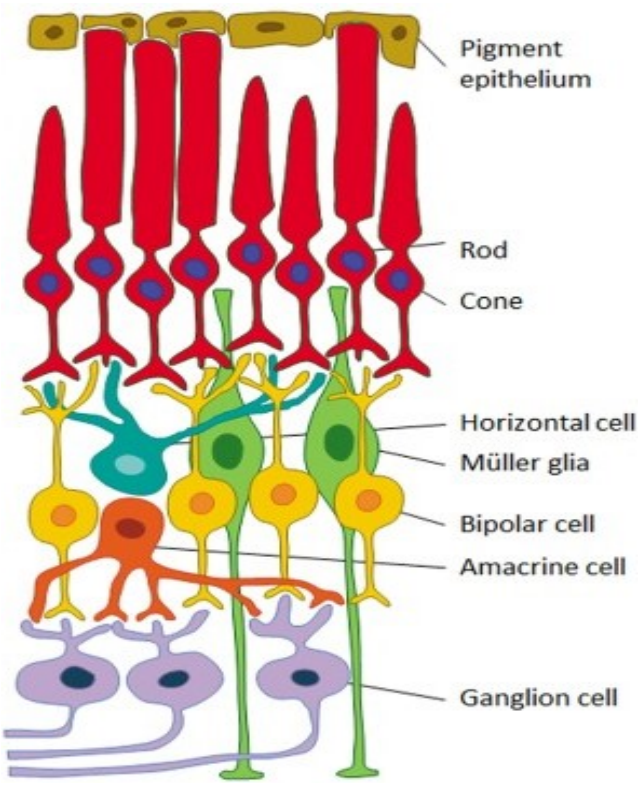

Figure 1: Structure of the Retina (1)

This figure shows the cellular organisation of the retina. In the outer layer of the retina are photoreceptors, which are the trigger of the RP in Usher syndrome, and the retinal pigment epithelium RPE cells as the outermost layer. Horizontal cells, bipolar cells, amacrine cells and ganglion cells represent the inner retina. They are downstream neurons of the photoreceptor. Müller cells are spanning through the outer and the inner cell layer.

1.3 Hearing impairment

Deafness or partial hearing loss is another impairment of Usher patients. Hearing in general can be differentiated in five classes. A healthy individual has a hearing range from 20 to 20.000 Hertz (Hz). The hearing threshold starts at 0 decibel (dB), a normal conversation has a value of about 50 dB and the pain threshold is at 120 dB. This classification represents the WHO Hearing Classification International Standard in 2005. In table 1, the values of dB are shown, in between which individuals with a hearing impairment are categorised into different classes of severity. The range of decibels shown represents the volume, when the patients start to hear noise. (22)

Table 1: WHO Hearing Classification International Standard in 2005

Severity of hearing impairment	[dB]
mild	20-40
moderately severe	56-70
severe	71-95
extremely severe	>95

The organ of Corti is positioned in the cochlea and it is the actual organ which enables hearing. More accurately, the inner hair cells render hearing possible. Inner hair cells (IHC) and outer hair cells (OHC) are secondary sense cells. IHCs change the mechanic signals of sound waves to electrochemical signals, which then go through synapses and reach the nervous system (10, 23). OHCs provide qualitative and quantitate sensitivity, they enhance the sound in the cochlea. In the cochlea are about 15.000 receptors, 3.000 IHCs and 12.000 OHCs (23). IHCs are organised in one row, while the OHCs are organised in three rows, representing the structure of a ‘W’ and are anchored within the tectorial membrane. (23, 10, 11). On these cells are hair bundles, they represent the area of the mechanosensitivity. One bundle includes one kinocilium and some stereocilia. Stereocilia are linked with “Tip-Links”. Hair cells are stabilised with four supporting cells. In this particular area there are only differentiated cells, therefore the organ of Corti cannot regenerate itself. The function of the hair bundle is to open and close ion channels. This happens due to the mechanical stimulation of the hair bundle. For a non-impaired healthy individual a stimulation of the hair follicle of only one nanometre (nm) and one grade at most is sufficient to hear.

A movement into the direction of the longest stereocilia results in an opening of the ion channel and a movement into the direction of the shortest stereocilia results in the closing of the ion channel. Cation influx, such as Calcium (Ca^+) and Potassium (K^+), leads to a change in membrane potential. This is the step when the mechanical stimuli are converted into an electrical impulse. Due to the presence of tip links, all stereocilia move in one direction. In the stereocilia the molecule myosin, a transcript from the myosin VI gene, is highly expressed. It is also highly expressed at the apical site of the cuticular plate. The proteins protocadherin 15 (*PCDH15*) and cadherin 23 (*CDH23*) build the link from the kinocilia to the stereocilia, furthermore the tip links the stereocilia in-between. The ankle links are built from the transcripts of the *VLGR1* (G-protein associated receptor 1) and *USH2A* (Usherin) genes. These links are predominantly built in vestibular hair cells. One important protein is myosin VIIa. The *MYO7A* gene is expressed in stereocilia and in the region of ankle links, which is shown in Figure 2: Anatomy of a hair bundle (10, 11). Genes involved in the most prominent Usher Type 1 are *MYO7A*, *CDH23, PCDH15, USH1G, USH1F* and *USH1C*. *MYO7A* is the most prominent Usher Typ 1 gene and encodes for the unconventional myosin type VII. It is important for the transport of organelles by binding to the actin filaments throughout the cell. Myosin needs adenosine triphosphate (ATP) hydrolysis to be able to move on the filaments (12).

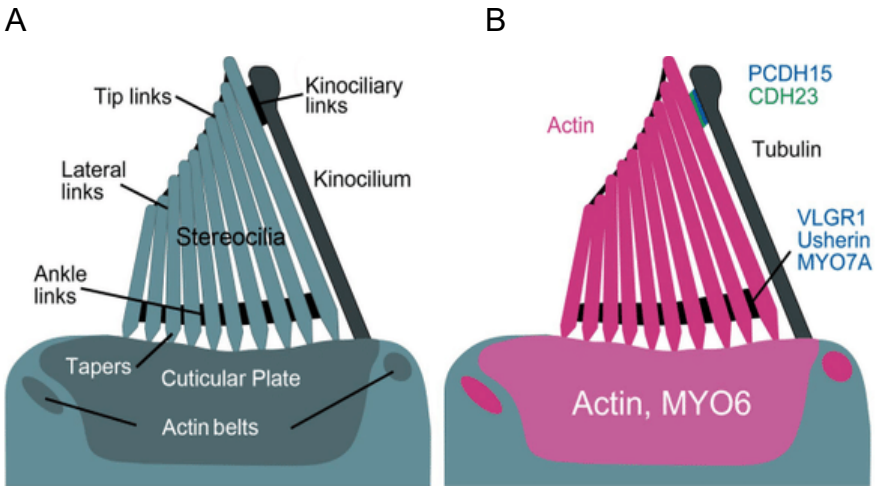

Figure 2: Anatomy of a hair bundle (7)

(a) Hair bundles include stereocilia, kinocilia, ankle links, lateral links, tip links, cuticular plate actin bonds and tapers. In hair bundles only one kinocilium is connected with the stereocilia.

(b) The most important genes expressed in the region of the inner ear hair cells are PCDH15, CDH32, VLGR1, USH2, MYO6 and MYO7A.

2 Materials and Methods

2.1 Materials

Kits	Company
Infinium Global Screening Array-24 Kit	Illumina®
Nextera® Rapid Capture Exomes	Illumina®
SureSelect V6 human All Exon libraries	Agilent

Reagents	Company
HotStarTaq®	Quiagen
ABI PRISM® Linkage Mapping SetVersion 2.5	Applied Biosystems
LiChrosolv® water	Merck
DMSO	Strategene®
BigDye Terminator v3.1	Applied Biosystems
5x Sequencing Buffer	Applied Biosystems
Sephadex™ G-50 Superfine	GE Health Care
LE Agarose	Biozym
GelRed™	Biotium
6x TriTrack DNA Loading Dye	Thermo Fisher Scientific

Instrumentals	Company
9800 Fast Thermal Cycler	Applied Biosystems
ABI PRISM™ 3130xl Genetic Analyzer	Thermo Fisher Scientific

Software	Company
VariantStudio 3.0	Illumina®
Chromas	Technelysium
GenomeStudio 2.0	Illumina®
Peak Scanner Software v1.0	Thermo Fisher Scientific

2.2 Methods

2.2.1 Sample collection

To find rare autosomal recessive disease it the most feasible way is to recruit consanguineous families with a probably genetic disease. In Pakistan 70% of all marriages are consanguineous, therefore their children have a higher risk to have a rare disease. Since 2015 we work with Dr. Muzammil Khan from the Gomal Centre of Biochemistry and Biotechnology, Gomal University D.I. Khan, who recruits the consanguineous families. His team isolated the DNA from blood via standard procedure (FavorPrep™ Genomic DNA Extraction Blood DNA Kit as well as Phenol-Chloroform DNA isolation) and sent the DNA Samples to our laboratory at the Medical University in Graz, Austria. For the USHER1 (IRB number: GU/GCBB/IRB/BIOMED-03) family we got DNA from three affected (USHER1-1, USHER1-2, USHER1-3) and three unaffected individuals (USHER1-7, USHER1-8, USHER1-9). In this branch, four of eight siblings are affected and one of these siblings is in a consanguineous marriage; out of this marriage one affected individual was born. The parents of the eight affected children live in a consanguineous marriage as well.

For the USHER2 (IRB number: GU/GCBB/IRB/BIOMED-03) family we got DNA from six individuals, two of them are affected. The DNA is from the father of the children and the five siblings.

The ethics applications were processed by Gomal University, D.I.Khan, Pakistan. Consent was granted to perform a full genetic analysis, including SNP analysis, exome analysis, Sanger sequencing and further molecular genetic analyses.

2.2.2 SNP Array

SNP Array data from individuals USHER1-1, USHER1-2 and USHER1-3 were generated in Canada. The array was performed at the Neurogenetics lab (PI: Prof. John Vincent), Centre for Addiction and Mental Health CAMH), Toronto, Canada. They used an Infinium Global Screening Array-24 Kit to perform SNP Array data (Core Exome). In this kit 435.238 markers are being detected. We received the raw data and then analysed it with homozygosity mapping.

2.2.3 Homozygosity Mapping

With SNP Array data from USHER1-1 USHER1-2 and USHER1-3 a homozygosity mapping was performed. For the analysis we utilised the Illumina Genome Viewer from the software Illumina Genome Studio2.0. To identify homozygous regions, shared with the affected individuals of the USHER1 family, the B- Allele Frequency of the SNP data was visualised. To choose the size of the homozygous regions, the data (RefSNP number and position number) from the last heterozygous area and the first rs and position number of the next heterozygous region was noted. The identified areas were analysed using the information from the whole exome sequencing.
For the USHER2 family we had no SNP Array data, so we performed a homozygosity mapping with the HomozygosityMapper Homo sapiens (24) on the internet (http://www.homozygositymapper.org/), with the individual USHER2-2. In the application on the Internet a smaller number of markers from WES data has been available for performing homozygosity mapping. It is therefore not as accurate as homozygosity mapping with SNP array data.

2.2.4 Microsatellite Marker analysis

Marker analysis was performed to analyse and corroborate homozygous regions. By using this method, all individuals of the families were analysed to confirm segregation of the disease locus within the family. Therefore, special primers, herein named markers, were used. These primers hybridise to specific regions on the genomic DNA, flanking microsatellites. The length of these microsatellites differs in all individuals, therefore, by analysing the unaffected as well as the affected individuals, an inheritance pathway can be established. All affected individuals should be homozygous regarding these specific loci on the genome, whereas the alleles of all the unaffected individuals should be presented in a heterozygous way or should be homozygous for the WT allele. To analyse segregation of a homozygous region between the individuals USHER1-1 to USHER1-3 and USHER1-7 to USHER1-9 sequence-tagged site (STS) polymorphic microsatellite markers were selected via UCSC Genome Browser (GRCh37/hg19). STS microsatellites were amplified via PCR according to the conditions in Table 2. A Master Mix with the first three ingredients was pipetted.

The reaction mix has a total volume of 11,5µL with following composition:

- 6µL HotStarTaq® Master Mix (Quiagen)
- 1µL Marker Mix (forward and reverse Primer, Applied Biosystems)
- 4µL LiChrosolv® water (Merck)
- 0.5µL genomic sample DNA

Table 2: PCR Program microsatellite marker analysis

Temperature	Time	Cycles
94°C	15min	1
94°C	30sec	∞34
57°C	30sec	
72°C	45sec	
72°C	7min	1
4°C	hold	

For the USHER1 family we performed a marker analysis for the homozygous region in chromosome 2, at position 159093577 to 177349591 (Table 3). Therefor we used one fluorescent marker from ABI PRISM® Linkage Mapping Set Version 2.5 Applied Biosystems (25).

Table 3: Polymorphic microsatellite markers for marker analysis. Position according to GRCh37/hg19

Marker	Position	ASR [bp]	AH	Dye
D2S335	171709986-171710207	183-205	0,79	NED

ASR= allelic size range bp=base pair AH=average heterozygosity

For USHER2 we found a gene which is known to cause Usher syndrome. Furthermore, it lies in a homozygous region, therfore we performed marker analysis to compare, if all affected individuals have an identical homozygous haplotypeat the same chromosomal position and the unaffected are heterozygous at this locus (Table 4). We used two markers from ABI PRISM® Linkage Mapping Set Version 2.5 Applied Biosystems (40).

Table 4: Polymorphic microsatellite markers for marker analysis. Position according to GRCh37/hg19

Marker	Position	ASR [bp]	AH	Dye
D11S937	77854318-77854608	144-180	0.88	FAM
D11S901	81844549-81844974	311-327	0.82	FAM

ASR= allelic size range bp=base pair AH=average heterozygosity

2.2.5 Sanger Sequencing

To investigate segregation of a mutation with the disease for the family Sanger sequencing is performed. If a mutation represents the underlying cause, then, it should not be found or at least be heterozygous in the unaffected individuals. However, affected individuals should be homozygous for the mutation. For Sanger sequencing two PCR steps were performed. The first one to amplify the specific sequence in a genome harbouring the mutation. The subsequent sequencing reaction is used to amplify one strand, forward or reverse with a fluorescence dye to detect the DNA sequence of the amplified segment. For the first amplification we used the following approach:

- 6µL HotStarTaq® (Quiagen)
- 3.5µL LiChrosolv® Water (Merck)
- 1µL DMSO (Stratagene®)
- 0.5µL primer forward [10ng/µL] (Microsynth)
- 0.5µL primer reverse [10ng/µL] (Microsynth)
- 0.5µL DNA.

The compounds were mixed and spun down and the samples were put in the Thermocycler 9800 Fast Thermal Cycler from Thermo Fisher Scientific. The program for the amplification in the Thermal Cycler is shown in Table 7.

Table 5: PCR Programme, amplification of the searched part of the DNA

Temperature	Time	Cycles
94°C	15min	1
94°C	30sec	x34
57°C	30sec	
72°C	45sec	
72°C	7min	1
4°C	hold	∞

The sequencing approach is as follows:

- 0.5µL BigDye Terminator v3.1 (Applied Biosystems)
- 1.4µL 5x Sequencing Buffer (Applied Biosystems)
- 0.4µL Sequencing Primer [10µM] (Microsynth)
- 6.2µL LiChrosolv® water (Merck)
- 1µL PCR Product

We mixed the samples with the pipet and spun them down. The PCR was performed in the Thermocycler 9800 Fast Thermal Cycle, the protocol is shown in Table 6.

Table 6: PCR Programme, amplification of forward or reverse strand of the first amplified PCR product

Temperature	Time	Cycles
96°C	30sec	∞25
50°C	15sec	
60°C	4min	
4°C	hold	

Purification of the PCR Product was done via size exclusion chromatography:
At first, Sephadex™ G-50 Superfine (GE Health Care) is used to fill CentriStep columns to ¾. CentriStep tubes were put into PCR tubes and centrifuged for 15sec at 750rcf to eliminate superfluous water. After this step, CentriStep tubes were centrifuged a second time for 2 minutes at 750 rcf to eliminate extended superfluous water. The second PCR products were diluted with 10µL LiChrosolv® water and 20µL

of the sample was pipetted onto the column. Then, samples were centrifuged a third time for 2 minutes at 750rcf to receive purified DNA in new tube. 10µL of the sample was filled on a 96-well sequencing plate and analysed with the ABI PRISM™ 3130xl Genetic Analyzer using POP-7™ Polymer (Thermo Fisher Scientific) as a separation matrix. The generated raw data was analysed using Chromas (Technelysium) and the UCSC Genome Browser.

All primer were design in the software Primer3 Input (version4.0) and ordered via the company Microsynth. Primer for the mutation in the *LRP2* gene are exhibited in Table 7. Primer, used for analysis of the mutation in the *MYO7A* gene are shown in Table 8.

2.2.5.1 Sequencing Primer USHER1 (*LRP2*)

Table 7: Sequencing Primer LRP2 (NM_004525.3:c.11972G>C)

Primer	Sequence [5'-3']	len[nt]	Tm[°C]	GC[%]	Product size
LRP2_E46.f	TAACAAAGGGGAGTGGGTGG	20	59.23	55.00	350
LRP2_E46.r	GCCCTCTGTCCTCTCCAAG	19	59.10	63.16	

2.2.5.2 Sequencing Primer USHER2 (*MYO7A*)

Table 8: Sequencing Primer MYO7A (NM_000260.4:c.1258A>T)

Primer	Sequence [5'-3']	len[nt]	Tm[°C]	GC[%]	Product size
MYO7A_E12_f	AGTGGCTGATCACTGCCTTT	20	59.87	50.00	198
MYO7A_E12r	GAAATTCCCATGAGCAGAGC	20	59.78	50.00	

2.2.6 Gel Electrophorese

5µL of the PCR product from the first PCR of the Sanger sequencing were picked and mixed with 1µL TriTrack Loading Dye (Thermo Fisher Scientific). A 1% gel was prepared according to the following protocol:

- 1g Agarose (Biozym LE Agarose)
- 100ml TAE buffer 1x
- 10µL GelRed™ Biotium (Biotium)

After 30 minutes, the gel can be used, and samples can be applied (Table 9). Gel electrophoresis is conducted for 30min with 130V and 400mV. Pictures of the gel were taken under UV Light.

Table 9: Sample concentration

Material	Concentration
DNA	5µL
TriTrack Loading Dye	1µL

2.2.7 Whole Exome Sequencing

To detect all variants in the exome and special regions from an affected individual in the USHER1 family, we forwarded DNA sample from Usher1-2 to a Whole Exome Sequencing in our diagnostic laboratory, which was conducted with the Nextera® Rapid Capture Exome Kit (Illumina) according to protocol. The received raw data was transformed into a vcf. file and opened with the Illumina VariantStudio Software 3.0. The data included 10.590 variants. After applying a filter to receive the heterozygous variants, 6.301 Variants were left, to improve the quality we used the Pass Filter, after that, 6.301 variants were left and at last we wanted to show all variants, which are missense, frameshift, stop gained, stop lost, initiator codon, in frame insertion, in frame deletion, and splice site affected variants (Figure 10). Then 2.170 variants were left. By setting the population frequency under 1%, 19 variants were left. For USHER1 and USHER2 we used a gene list (Table 10), which includes eight characteristic Usher

genes. For USHER1 no Variant was left anymore, therefore, we reset this filter application. Regarding 8 remaining Variants, two were in homozygous regions.
For USHER2 we received WES data from Macrogen. Macrogen prepared the DNA from human blood samples with SureSelect V6 human All Exon libraries. Regarding the sequencing Macrogen used NovaSeq 6000 2 x 150 bp and 100 x coverage by Macrogen. Raw data (FASTQ and BI files) was processed by us and was transformed into vcf files. After annotation of the vcf file we received 38.694 variants. At first, we sorted out all heterozygous variants, then, 15.157 variants are left. After this step we improved quality, so we activated the pass filter (15.157 variants). The next step was to select all variants from a drop-down menu (Figure 10: Filter setting in Variant Studio, Select all). 5.205 variants were left. To set the parameters for the maximum allele frequency to filter for a rare disease, we set it to <1%, after this filter step, 109 variants were left. Afterwards, we used a list (Development of a genotyping microarray for Usher syndrome, Cremers et. al) with the most prevalent USHER genes (Table 10).

Table 10: Characteristic Usher Genes (13)

Gene	Protein coding exons	Known pathologic variants
CDH23	69	52
MYO7A	48	118
PCDH15	32	7
USH1G	2	4
USH1C	28	8
USH2A	72	88
VLGR1	90	6
USH3A	6	9

3 Results

3.1 USHER1 Family

3.1.1 Phenotype

At first sight the Usher 1 Family shows a typically Usher syndrome phenotype, which is autosomal recessively inherited. The pedigree also shows that female individuals are affected more frequently. However, the male individual Usher 1-8 in generation four rules out an X-linked recessive inheritance. A dominant inheritance was excluded due to unaffected previous generations and the frequent occurrence of the disease in the fifth generation. Regarding the phenotype description, it is important to know that both parents (Usher1-4 mother and Usher1-8 father) are healthy, which deepens the assumption that the disease follows an autosomal recessive inheritance pattern. The two healthy parents are both over 55 years old and show no symptoms of age-related vision or hearing loss. No physiological, morphological, neurological, dermatological, radiological or metabolic abnormality was found in either parent.

Phenotype of Usher1-1 is disparate from the parents. The patient is female, who is now about 35 years old. During her embryonic development, no suspicion for a disease arose. The birth occurred via spontaneous vertex delivery. After birth, the patient had congenial symptoms of vision and hearing loss. The vision loss was progressive, at this time the patient suffers from low vision, near to blindness. She can recognise light and dark through light perception. Furthermore, the patient has a severe bilateral sensorineural hearing loss but normal vestibular functions. She is able to speak normally and understands when spoken to her. The rest of the physiologic, morphologic, neurologic, dermatologic and radiologic exam was normal. Besides, the patient is able to sit and walk independently.

The other affected individual, Usher1-2 is also female and is about 28 years old. Like her sister, the antenatal history was normal and birth was at full term via spontaneous vertex delivery. She was born with congenital symptoms of vision loss and deafness. The patient shows a complete loss of vision, only light perception is present. In addition, she shows deep bilateral sensorineural hearing loss. The vestibular functions are normal. Due to deafness, she never developed the ability to speak. The rest of the physiological, morphological, neurological, dermatological and radiological examination was inconspicuous. She can sit and walk independently as well.

Usher1-3 is also female and about 24 years old. Like her affected siblings, the antenatal history was normal. She was born via spontaneous delivery. At birth, she had congenital symptoms of vision loss and progressive post lingual hearing loss. Currently she has intensively low vision and moderate bilateral sensorineural hearing loss but normal vestibular functions (she has a less severe condition than her sisters USH1-1 and USH1-2). She can speak normally and can understand when being spoken to. The individual sits and walks independently. Remaining analysis of the physiologic, morphologic, neurologic, dermatologic and radiologic exam was normal. Disease appeared in first decade of life in all patients with variations in the magnitude of the phenotype. Symptoms of all affected patients is consistent with Usher type 2. An additional survey has shown that affected individuals do not show any facial dysmorphia.

3.1.2 Homozygosity mapping

Homozygosity mapping was analysed for individuals Usher1-1, Usher1-2, Usher1-3 with a cut off from >2MB. The Results are summarised in Table 11.

Table 11: Homozygosity Mapping USHER1
Data of the SNP Array (Infinium Global Screening Array-24 Kit, 435.238 markers) were analysed with the GenomeViewer from the Illumina GenomeStudio Software 2.0

Chromosome	rs-Number	Start-Position	End-Position	bp
1	954145-1146268	90498540	95220414	4.721.874
2	309152-6742749	136657252	139537261	2.880.009
2	1608037-13403148	159093577	177349591	18.256.014
3	1513121-1401070	101765067	116427291	14.662.224
3	rs10935515-rs4681371	144556283	147432019	2.875.736
7	rs4345498-rs1917113	118301283	120735062	2.433.779
8	rs16938118-rs10102468	49514893	52054326	2.539.433
21	rs2826676-rs410349	22506099	28235194	5.729.095

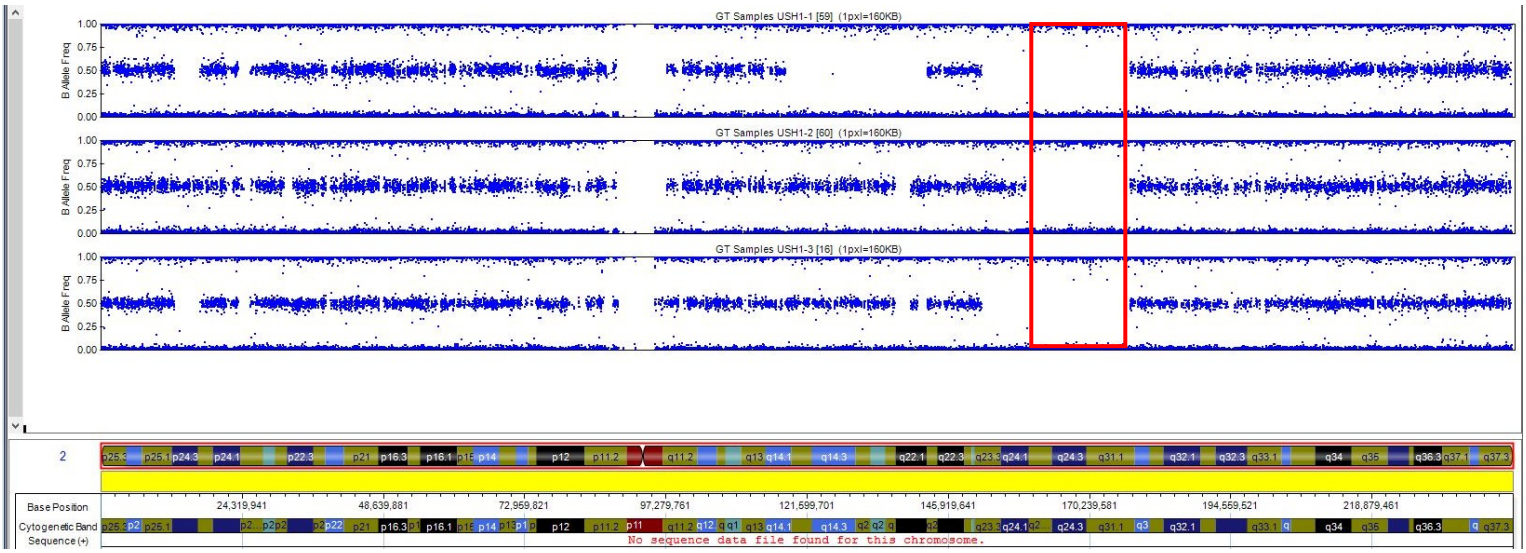

Figure 3: Homozygosity Mapping GenomeStudio Illumina 2.0

Homozygosity mapping from USHER1-1, USHER1-2 and USHER1-3. The red area represents the common homozygous region, which harbours the LRP2 gene (Chr.2:169983619-170219122). The gene is located on chromosome 2, at position 159093577-17734959.

3.1.3 OMIM Gene search

All genes with their known function and occurring phenotype accompanying a loss of function are stored in the OMIM data base.(26) The cause for most of autosomal recessive diseases are located within a homozygous region, so we activated the OMIM icon in the UCSC Genome Browser (27), to filter all genes, which are located in homozygous regions resulting from homozygosity mapping approach (Table 11). With this analysis no candidate gene could be found, consequently the DNA was sent to another institute to perform WES. Thereafter, vcf files were generated and all genetic variants from the individuals were retrieved.

3.1.4 VariantStudio analysis

Candidate genes for autosomal recessive inherited diseases can often be found in large homozygous regions. After analysis with VariantStudio software, two known disease genes were located in one of the common homozygous areas, *LRP2* and *SCN2A*. *SCN2A* strongly correlates with epilepsy and autism (31). Some mutations in *LRP2* gene are known to cause Donnai-Barrow syndrome. Disease phenotype is characterized by facial dysmorphism, myopia, other ocular findings and hearing loss.

The variant, NM_004525.3:c.11972G>C, in the *LRP2* gene is located in the homozygous region on chromosome 2 at position 170013928 (GRCh37/hg19), where a guanine is replaced by a cytosine. This results in an amino acid change from cysteine to serine, p.C3991S.

3.1.5 *In silico* analysis

Due to the results of the HGMD® (28), in total 51 mutations are listed for the *LRP2* gene. The phenotype ranges from Stickler like syndrome to Donnai-Barrow syndrome and retinal dystrophy. Mutation taster browser (29) gave a prediction for this variant, the result was possibly disease causing. ExAc (41) and gnomAD (42) database did not list the variant NM_004525.3:c.11972G>C. According to the UCSC Genome Browser (GRCh37/hg19), amino acid AA3991 changed from a cysteine to a serine due to the mutation (p.C3991S). InterPro database analysis showed two domains in the *LRP2* gene, which are affected by the mutation according to the protein sequence analysis and classification. Those affected domains are the EGF-like domain IPR000742 and the EGF-like calcium-binding domain IPR026823. One domain in IPR000742 ranges from amino acid 3969 to 4006 and the other domain in IPR026823 ranges from amino acid 3949 to 4006. Because of the possible adverse effect of the mutation on the function of LRP2 in combination with the phenotype present in the affected individuals we further performed segregation analysis with STS marker, shown in Figure 4.

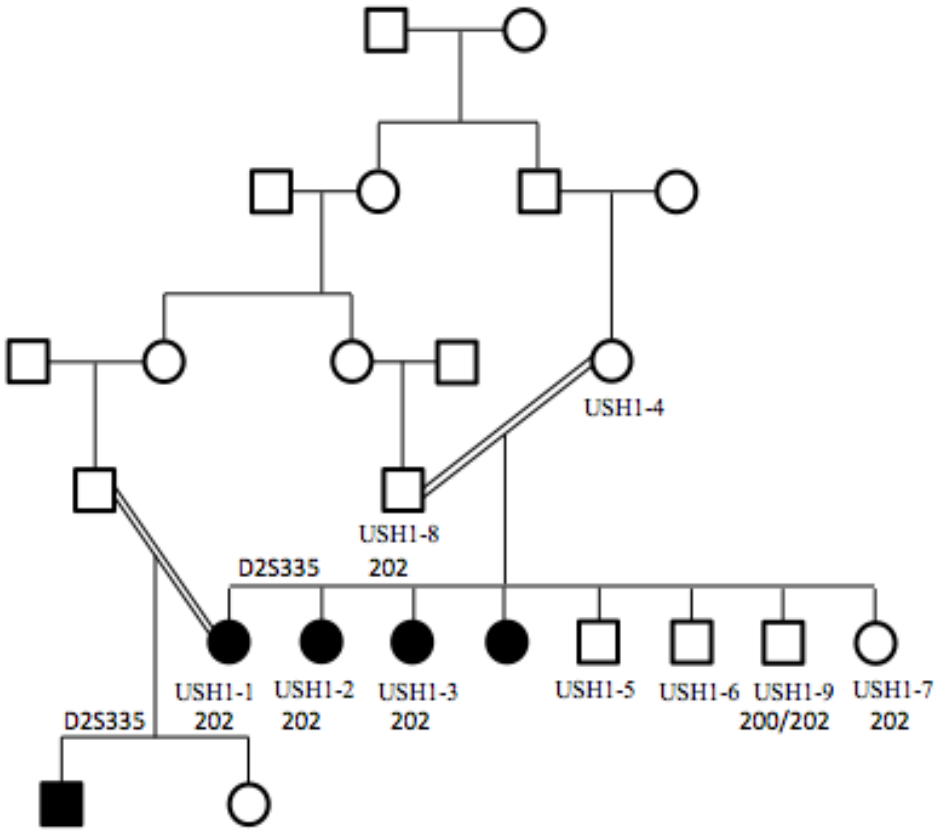

Figure 4: Pedigree of Pakistani family USHER1 showing 23 Individuals from six generations. The squares represent men and the circles women. Shapes, which were filled, represent affected individuals. Results of the marker analysis shows that the marker D2S335 did not segregate with the disease in the family.

Marker analysis with one marker was not informative. To obtain substantial results, a minimum of two informative marker are needed. It is possible that two individuals with identical alleles for a microsatellites harbour different DNA sequences. Next step was Sanger sequencing to see if the mutation segregates in the family with the disease. For this step, we created primers, which were located left and right from the mutation to amplify this part of the DNA sequence. To get reliable results, DNA was sequenced in forward and reverse to find the mutation NM_004525.3:c.11972G>C in the *LRP2* gene on chromosome 2. Figure 5 presents the result of the Sanger sequencing.

A

B

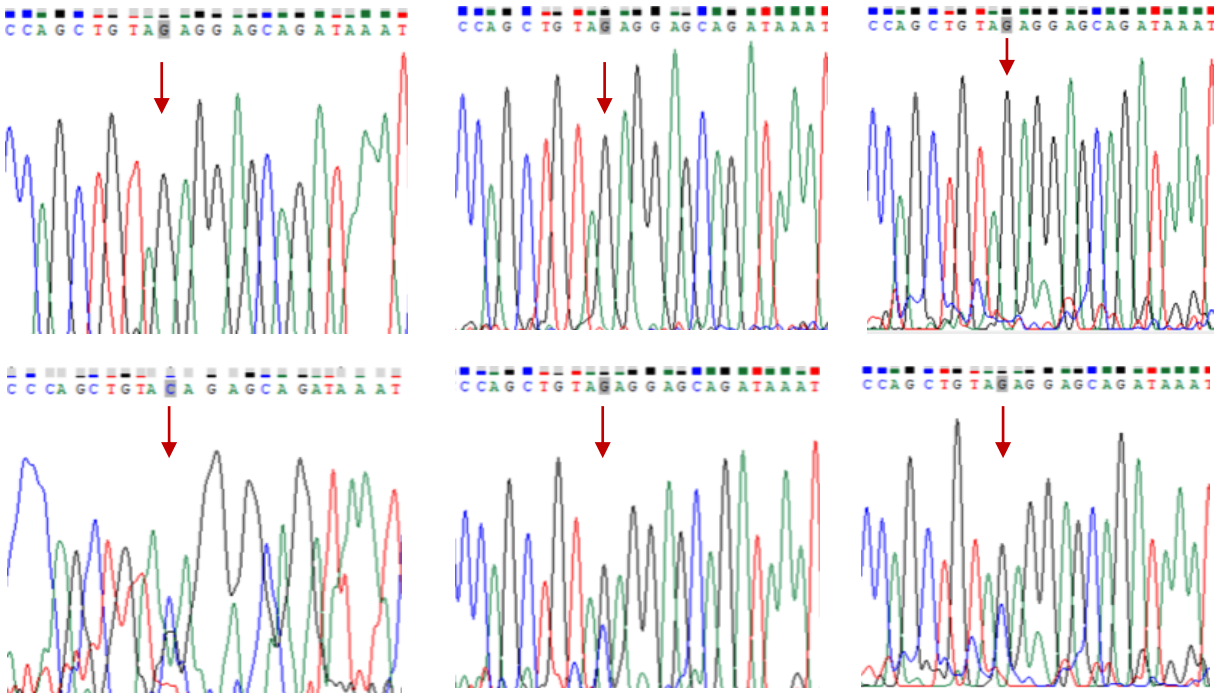

Figure 5: Sequencing results of the position from the variant NM_004525.3:c.11972G>C

Figure 5 represents sequence results from affected individuals in A and results from unaffected individual in B. In A it is clearly to see, that the G, pointed with arrowhead, is represented homozygously in individuals USHER1-1, USHER1-2, and USHER1-3 Sequencing results in B show heterozygous alleles at the same position in all three individuals, where the mutation occurs in the affected patients. The mutation segregates in the family with the disease.

3.2 USHER2 Family

3.2.1 Phenotype

The main phenotypic features in affected individuals of Usher 2 family are hearing impairment and night blindness. Both parents are healthy and asymptomatic with perfect hearing and vision perception. No age-related hearing loss was observed in either parent. Symptoms of all affected patients were consistent with Usher syndrome. Affected individual USH2-1 is 30 years old and show congenial symptoms of vision and hearing loss (bilateral sensorineural hearing loss) but normal vestibular functions. The rest of physiologic, morphologic, neurologic, dermatologic and radiologic exam was normal. The patient is able to sit and walk independently. He cannot speak due to pre-lingual deafness, cannot hear and has weak eyesight and night blindness. This individual shows no photophobia and no structural abnormality in eye.
Affected individual, USH2-2 is 23 years old. Antenatal history is inconspicuous, like the one from USH2-2, and the birth was at full term via spontaneous vertex delivery. She was born with congenial symptoms of vision loss and deafness (bilateral sensorineural hearing) but normal vestibular functions. The patient can sit and walk independently. She cannot speak due to pre-lingual deafness. Physiologic, morphologic, neurologic, dermatologic and radiologic exam was normal. To summarise, she cannot speak nor hear, she has a weak eyesight and night blindness, but no photophobia was observed and no structural abnormality in the eye was found.

3.2.2 Homozygosity Mapping

WES data was used for a homozygosity mapping with the HomozygosityMapper database (36). It isn’t as accurate as a SNP Array, because in this approach fewer number of polymorphism were analysed. SNP Array provides data through the whole genome, while WES provide data through the exome and small parts of the introns. Nevertheless, for a rough assessment of the homozygous regions it is applicable. For homozygosity mapping, a cut off from 2MB was used. 13 homozygous regions in DNA of individual USHER2-2 were detected, which are listed in Table 12.

Table 12: Homozygosity mapping via Homozygosity Mapper Homo sapiens (GRCh37/hg19) (24)

Chromosome	Start-Position	End-Position	bp
1	6.947.717	12.854.068	5.906.351
1	13.052.871	16.383.742	3.330.871
1	17.087.582	20.097.822	3.010.240
2	220.046.975	223.084.833	3.037.858
5	1.882.129	21.752.050	19.869.921
7	855.854	11.022.230	10.166.376
9	122.001.000	127.101.875	5.100.875
11	46.893.108	49.196.490	2.303.382
11	76.506.888	89.486.362	12.979.474
11	99.690.461	107.646.894	7.956.433
12	18.234.256	29.819.161	11.584.905
12	118.828.755	122.359.397	3.530.642
18	56.024.396	66.504.351	10.479.955

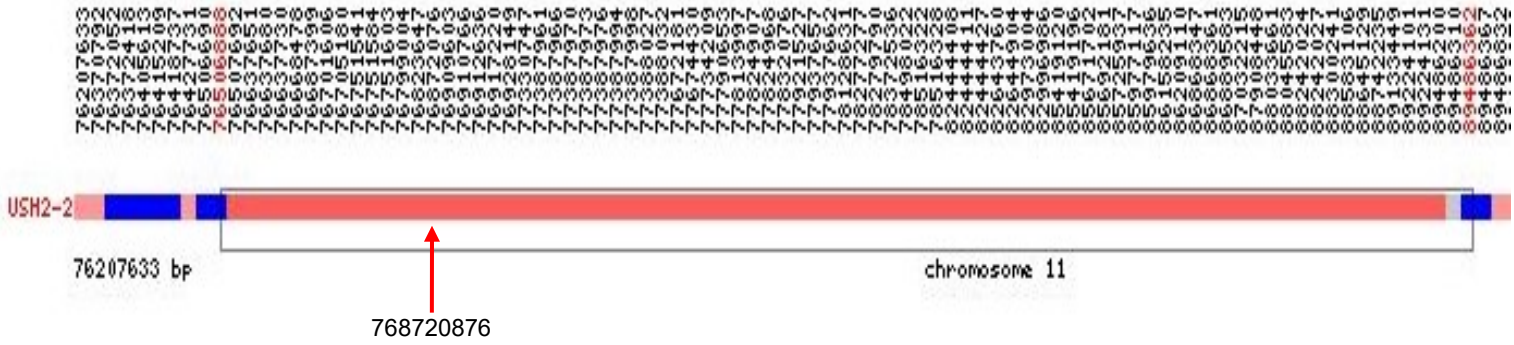

Figure 6: Homozygosity Mapping USHER2-2

The figure represents a part of the homozygosity mapping from chromosome 11 (HomozygosityMapper, 18/06/20). The mutation is located in close proximity to the polymorphic site at 768720876bp, which is marked by an arrowhead.

3.2.3 Variant Studio analysis

After the filtering steps, described in part 2.2.7 of this master thesis, two possible candidate variants were found in a homozygous region (Table 13).

Table 13: Result, filter analysis vcf file, with VariantStudio Software3.0 (Illumina) and homozygous region search (GRCh37/hg19)

Gene	Position	cDNA Mutation	Protein mutation
MYO7A	Chromosome 11: 76872076	c.1258A>T	p.K420*
MYO7A	Chromosome 11: 76909603	c.4505A>G	p.D1520G

The second mutation occurs downstream from the first one, hence, it will not be translated. For the mutation NM_000260.4:c.1258A>T an entry in HGMD® exists (CM071018 Usher Syndrome) (28). It is not unlikely that this mutation and the resulting shorter MYO7A protein is causal for the disease. In ClinVar this mutation (NM_000260.4:c.1258A>T) is described to cause deafness and classified as pathogenic (52). The first marker D11S937 (Chr.11: 77854318-77854608), targeting the homozygous region from chromosome 11 on position 76506888 to 89486362, did not segregate in the family with the disease. The first marker is targeted 365188 base pairs away from the mutation. The second marker is at the end of the homozygous region (Table 12), and also did not segregate with disease in the family (Figure 7). Homozygous regions can have a different length in different individuals. Due to the results of the marker analysis, Sanger sequencing was performed to analyse, if the mutation segregates in the family with the disease. Therefore, we sequenced all available family members from USHER2-1 to USHER2-6. The results are shown in Figure 8, results from the segregation analysis are shown in Figure 9.

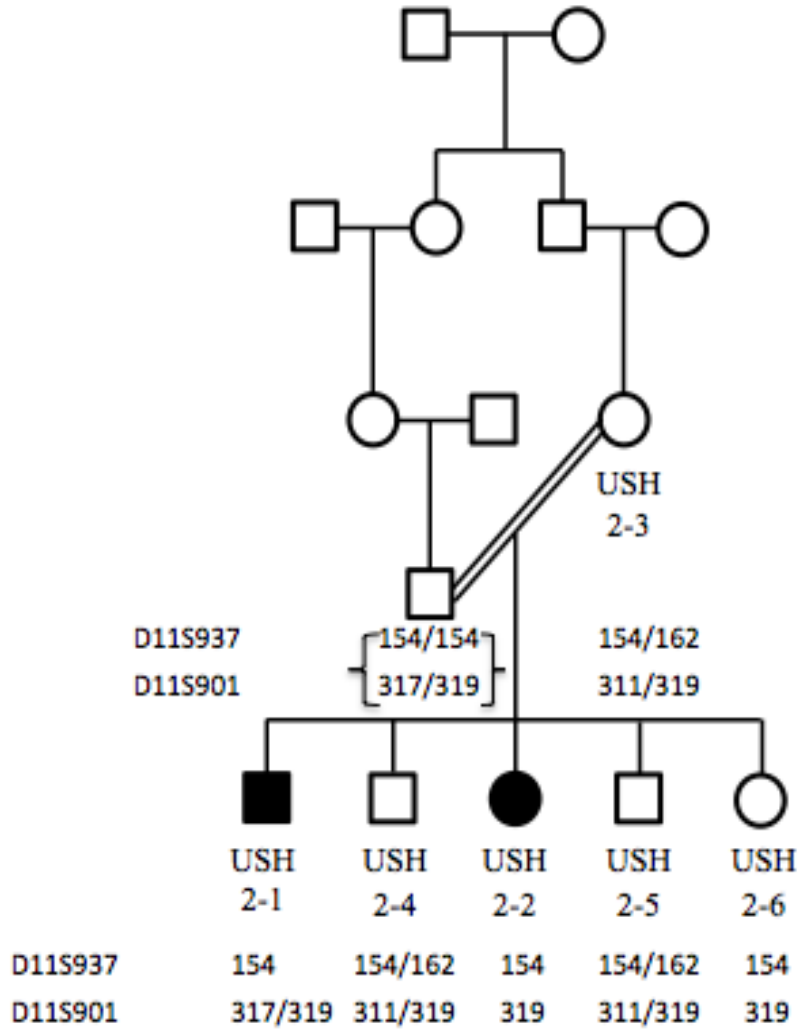

Figure 7: Results marker analysis Usher2 family:

Square figures represent male and circles represent female individuals. Affected individuals have a black filling, the unaffected are framed. The first marker D11S937 (chr11:77854318-77854608) is at the proximal end of the homozygous region, marker D11S901 (chr11:81844549-81844974) is at the distal end of the homozygous region. The first marker is homozygous in all affected individuals and in USHER2-6. Sanger sequencing results showed that the mutation NM_000260.4:c.1258A>T on chromosome 11:76872076 is located in a homozygous region in all affected individuals. Therefore, the heterozygous marker in USHER2-6 can come from an affected allele from the father and one unaffected allele with an ASR of 154 from the mother.

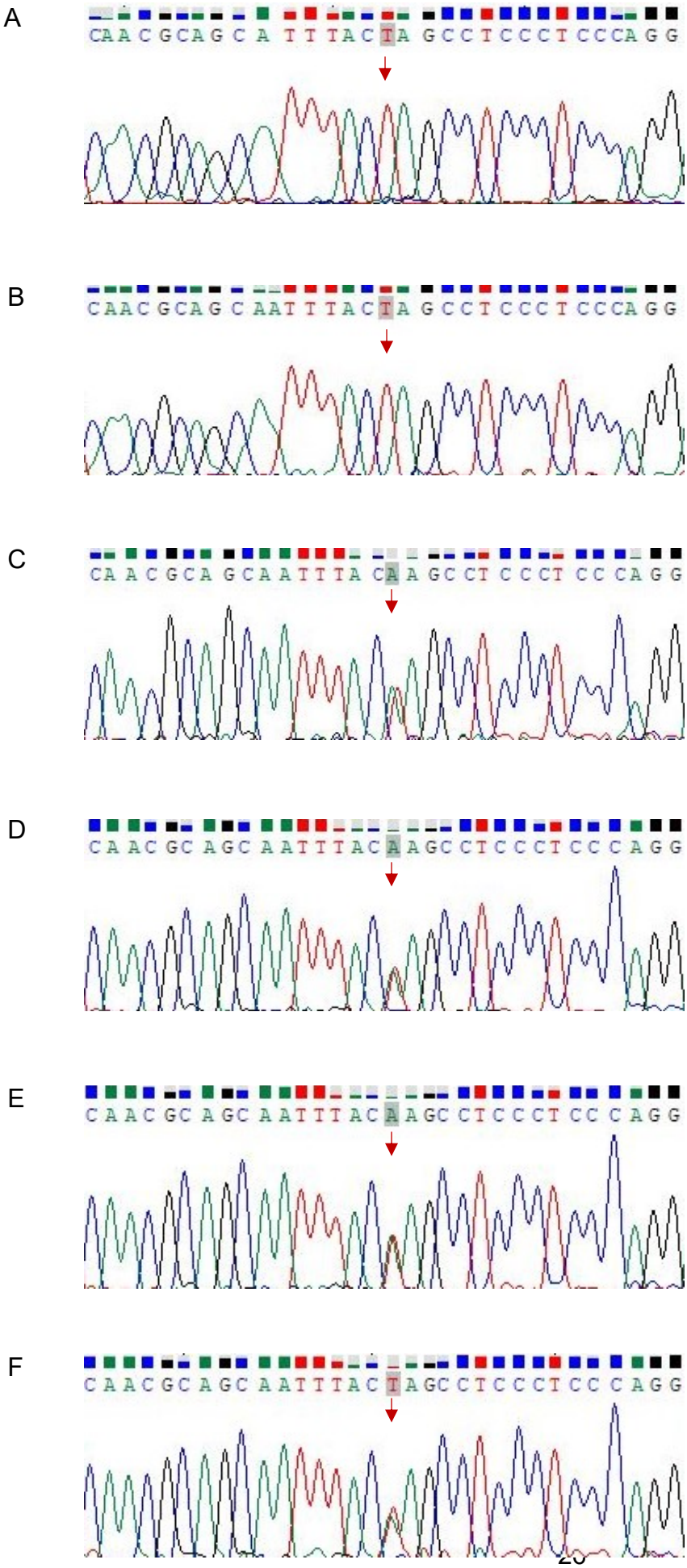

Figure 8: Sanger Sequencing results of the mutation NM_000260.4:c.1258A>T

Electropherogram A and B represent sequencing data of individuum USHER2-1 and USHER2-2. Electropherogram C, D, E and F represent sequencing data of individuum USHER2-3, USHER2-4, USHER2-5 and USHER2-6. The arrowhead points to thymidine. It is present homozygously in A and B. Individuals USHER2-3, USHER2-4, USHER2-5 and USHER2-6 are unaffected. C, D, E and F show a heterozygous status of the variant, with an adenine and a thymidine. The mutation segregates in the family with the disease.

Mutation NM_000260.4:c.1258A>T segregates in the family with the disease. All affected individuals carry a thymidine (T) in *MYO7A* NM_000260.4:c.1258A>T on both alleles. Unaffected individuals present one mutated allele. The mutation NM_000260.4:c.1258A>T in *MYO7A* is a known mutation for Usher syndrome with an allele frequency of 0,003654% (30). The mutation results in a premature stop codon (p.K420*) (27).

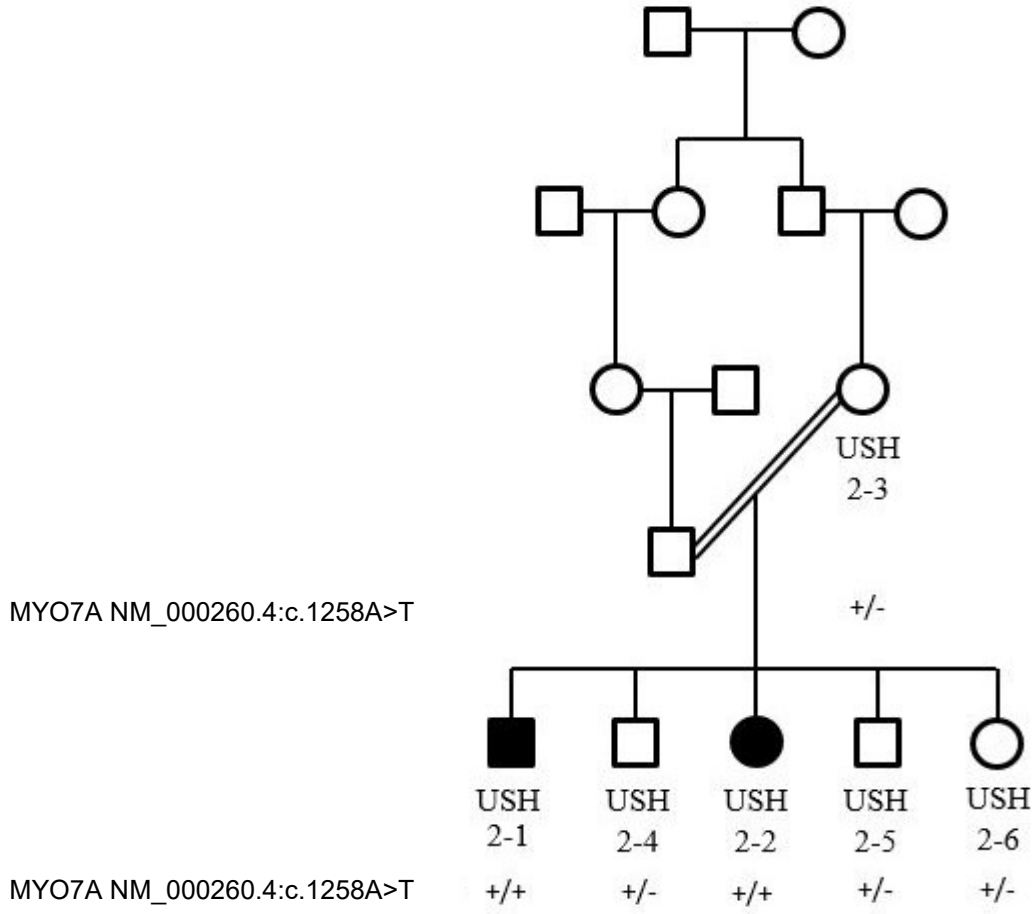

Figure 9: Sanger sequencing analysis MYO7A NM_000260.4:c.1258A>T Usher2 family
Mutation NM_000260.4:c.1258A>T *in the gene MYO7A segregates in the family with the disease. All unaffected individuals are carrier for the mutation and the affected individuals have two mutated alleles.*

4 Discussion

Usher syndrome is a rare genetic disease, 400.000 people worldwide are affected. It causes progressive vision loss due to RP, severity of hearing impairment depends on the underlying genetic defect and therefore the type of the syndrome. While people with Usher Type 1 are usually born with deafness, affected from Usher type 2 show moderate to severe hearing loss in. Usher type 3 is the mildest form of Usher syndrome there individuals are born with normal hearing, hearing loss occurs with adolescence, also RP manifests in youth years. Additionally to hearing impairment and vision loss affected individuals with Usher type 1 show vestibular dysfunctions, which occurs in affected with Usher type 2 or III rarely (14).

In this thesis the genetic cause of Usher syndrome in two independent families was analysed. For one family, herein named Usher 2 family, an already known mutation in a known Usher gene could be identified. The mutation NM_000260.4:c.1258A>T is located in the *MYO7A* gene, most of Usher type 1 cases, 39-55%, occur in cause of mutations in this gene (14) and all cases of Usher type 1B (31). It has an allele frequency of 0.003654% (30). To recall, a rare genetic disease is characterized by an allele frequency under 0.01 (32). In the UK National Collaborative Usher Study they analysed 188 probands with Usher syndromes and 456 family members. 381 European, 48 Caucasian and additionally 57 individuals from Pakistan were included in the study as control group. In this study, the mutation NM_000260.4:c.1258A>T was classified as pathogenic (33).

The Myosin 7A gene is located on chromosome 11 spanning from 76.839.310 to 76.926.286 base pairs (GRCh37/hg19). The RefSeq of this gene is NM_000260. It is the longest transcript of the MYO7A gene and consists of 50 exons. (34). The protein called unconventional myosin-VIIa isoform 4 (35), has an important role in intracellular movements. There are eight different isoforms known for this gene (36). The gene encodes 2215 amino acids (37) which forms twelve protein domains. The largest domain is the myosin head motor domain (IPR001609) (38, 39), and is a member of the myosin family. Proteins transcribed by *MYO7A* are characterised by a motor domain, actin-binding domain, neck domain that interacts with other proteins and an anchor, which consists of a tail. They can move along actin filaments by ATPase activity and thus transport other molecules (40, 36). *MYO7A* encodes, in contrast to other myosin genes, a short tail. In humans, a defect in this gene is related to Usher

Type 1b and in mouse to the shaker-1 phenotype (41, 42). The gene is expressed in the inner ear and the retina, the light sensitive layer at the back of the eye. It is involved in the renewal of the outer photoreceptor disks. It also plays an important role in the regulation of opsin transport into retinal photoreceptors. Additionally, it is responsible for the distribution and migration of melanosomes and phagosomes of the retinal pigment epithelium (36, 40, 22). Hearing loss in Usher syndrome is caused by differentiation, morphogenesis and organisation of a genetic defect in *MYO7A* gene in hair cell bundles in the cochlea. Proteins of the *MYO7A* gene are important for maintaining the stereocilia. Stimulation of stereocilia plays an important role because the signals are transmitted from the vestibular system to the brain. A defect in this gene can lead to vestibular dysfunction. There are 19 probable pathogenic mutations in the *MYO7A* gene recorded for Usher type 1 as shown in Table 14: Mutations in MYO7A known to cause Usher type 1 (37).

Table 14: Mutations in MYO7A known to cause Usher type 1 (37)

Mutation	Amino acid change	Exon	Domain
Missense			
c.77C>A*	p.A26E	3	Motor
c.395C>T	p.P132L	5	Motor
c.721C>G	p.R241G	7	Motor
c.1097T>C	p.L366P	11	Motor
c.3134T>C/ c.5507T>C	p.I1045T/p.L1836P	25/40	Tail
c.3652G>A	p.G1218R	29	Tail
c.3719G>A	p.R1240Q	29	Tail
c.4475C>T	p.A1492V	34	Tail
c.6610G>C	p.A2204P	49	Tail
Nonsense			
c.1884C>A	p.C628X	16	Motor
c.5581C>T	p.R1861X	40	Tail
Insertion/deletion			
c.655_660del	p.I219_H220del	7	Motor
c.986dupG	p.N330QfsX5	9	Motor
c.3764delA	p.K1255RfsX8	30	Tail
c.4297delC	p.Q1433SfsX116	32	Tail
c.5835_5838delCTTT	p.F1946SfsX23	42	Tail
c.6025delG*	p.A2009PfsX32	44	Tail
Putative splice site mutation			
c.2283-1G>T	—	20	Neck

Some mutations causing Usher type 1B are predicted to occur null mutations resulting in loss of function proteins. Hearing impairment can be addressed with cochlear implants, in cause of this, researchers focus on therapy for progressive vision loss (31). Different forms of therapy are currently tested in clinical studies. Gene therapy approaches use a lenti virus to introduce *MYO7A* cDNA in subretinal cells. It shows positive effects on infected cells, which produce functional proteins after therapy (31). Two trials for gene therapy products for Usher type 1B patients is were performed in France (43, 44). Result of the trial was non or less worsened in severity during the course of the study with injection of a low dose of the product. With higher dose, the disease get worse (44). Additionally, a gene therapy trial for mutations in *USH2A* exon 13 is currently being implemented (45). Gene therapy could be the method of choice for genetic diseases, it fixes the cause of the problem, not only the symptoms.

For the other family, herein named Usher 1 family, an unknown variant in *LRP2* gene NM_004525.3:c.11972G>C, in a potential candidate gene could be identified. *LRP2* is encoding for lipoprotein-related protein 2 and is also called megalin (46, 47). The gene is located on the q-arm of chromosome 2 at position 169.983.619-170.219.122 and encodes for 4566 amino acids comprising 79 exons and the protein itself has a molecular weight of 521,958 kDa. The RefSeq number is NM_004525.3 (48). It is mainly expressed in absorbent epithelial tissue. The protein represents a transmembrane protein and consists of a membrane domain, an N-terminal domain for binding ligands on the extracellular side of the cell, and a C-terminal domain in the cytoplasm. The compounds, which enter the cell by endocytosis, include lipoproteins, sterols, vitamin binding proteins and hormones. *LRP2 gene* is not directly related to the Usher syndrome, but mutations in this gene can lead to a clinically overlapping phenotype. According to HGMD®, changes in this gene lead to Donnai-Barrow syndrome or Stickler-like syndrome. Donnai-Barrow syndrome is characterised by symptoms such as facial dysmorphia, myopia, ocular findings, hearing loss, retinal dystrophy, cataract and vision loss. Additionally, affected people suffer from proteinuria. The severity of the phenotype can vary but there are no patients know without facial dysmorphia (49). *LRP2* is important for the development of optic nerves. Sonic hedgehog SHH dependent migration and proliferation of oligodendrocyte progenitor cells. *LRP2* protein eliminates SHH in the retinal margin and excretes it

again, protecting retinal progenitor cells from mitogenic stimuli. Furthermore, the correct expression of the *LRP2* gene is necessary for hearing. This is probably due to the inertia with estrogen in the inner ear (50). Stickler's syndrome shows high myopia, esotropy, changes in hearing and cataract (42). Both, Donnai-Barrow and Stickler syndrome, have other phenotypic manifestations additional to hearing and vision loss, but the phenotypes vary, hence both syndromes can be mistaken for with the Usher syndrome.

Two siblings from Iraq show symptoms of Stickler syndrome, but a milder form. At the first sight, they have high myopia and other various forms of vision impairment and an episodic conductive hearing loss later in age. Both have missense mutation in *LRP2* gene. Like Usher 1 family, there is a possibility that affected in both families show significantly milder phenotype of the Donnai-Barrow syndrome. For the siblings in Iraq urine was collected to analyse, if they additionally suffer from proteinuria. The test was positive and in this paper they propose that it is a milder form of Donnai-Barrow syndrome. Herein described family could also have a milder form of Donnai-Barrow syndrome (51). To decide, if it is a milder form of this syndrome, a test for proteinuria has to be performed. With this aspect, it would be very risky to say, that this gene could be a new gene loci for Usher Syndrome. Are results from the test of proteinuria negative, this hypothesis can be pursued again.

5 Literature Cited

1. Figure 1: Structure of the retina. A: Microphotograph of a…; 18/08/01 [cited 19/08/20]. Available from: URL: https://www.researchgate.net/figure/Structure-of-the-retina-A-Microphotograph-of-a-cross-section-through-the-retina-of-an_fig4_259915517.

2. INSERM US14 -- ALL RIGHTS RESERVED. Orphanet: About rare diseases [cited 19/08/07]. Available from: URL: https://www.orpha.net/consor/cgi-bin/Education_AboutRareDiseases.php?lng=EN.

3. Gilissen C, Hoischen A, Brunner HG, Veltman JA. Disease gene identification strategies for exome sequencing. Eur J Hum Genet 2012; 20(5):490–7.

4. INSERM US14 -- ALL RIGHTS RESERVED. Orphanet: About rare diseases [cited 19/08/07]. Available from: URL: https://www.orpha.net/consor/cgi-bin/Education_AboutRareDiseases.php?lng=EN.

5. Lobo I, Shaw K. Discovery and Types of Genetic Linkage. Scitable. Available from: URL: http://www.nature.com/scitable/topicpage/discovery-and-types-of-genetic-linkage-500.

6. Daniel Schober. Microarrays, Genexpressionsanalyse und Bioinformatik.

7. Usher syndrome, type 1 (Concept Id: C1568247) - MedGen - NCBI [cited 19/07/25]. Available from: URL: https://www.ncbi.nlm.nih.gov/medgen/C1568247.

8. Frequent questions about Usher syndrome [cited 19/08/08]. Available from: URL: https://www.usher-syndrome.org/what-is-usher-syndrome/frequent-questions.html/title/what-is-usher-syndrome-type-i-.

9. Usher syndrome, type 1 | Genetic and Rare Diseases Information Center (GARD) – an NCATS Program [cited 19/08/07]. Available from: URL: https://rarediseases.info.nih.gov/diseases/5435/usher-syndrome-type-1.

10. Stöver T, Diensthuber M. Molekularbiologie des Gehörs. Laryngorhinootologie 2011; 90 Suppl 1:S22-34.

11. Mathur P, Yang J. Usher syndrome: hearing loss, retinal degeneration and associated abnormalities. Biochim Biophys Acta 2014; 1852(3):406–20.

12. Williams DS. Usher syndrome: animal models, retinal function of Usher proteins, and prospects for gene therapy. Vision Res 2007; 48(3):433–41.

13. Cremers FPM, Kimberling WJ, Külm M, Brouwer AP de, van Wijk E, te Brinke H et al. Development of a genotyping microarray for Usher syndrome. J Med Genet 2007; 44(2):153–60.

14. Frequent questions about Usher syndrome [cited 19/08/20]. Available from: URL: https://www.usher-syndrome.org/what-is-usher-syndrome/frequent-questions.html/title/what-is-usher-syndrome-type-i-.

15. Types; 19/08/08 [cited 19/08/20]. Available from: URL: https://stanfordhealthcare.org/medical-conditions/ear-nose-and-throat/usher-syndrome/types.html.

16. Lentz J, Keats B. Usher Syndrome Type II: University of Washington, Seattle; 2016.

17. RP [cited 19/08/12]. Available from: URL: undefined.

18. Lentz J, Keats BJB. Usher Syndrome Type I: University of Washington, Seattle; 2016.

19. Usher-Syndrom: Ursachen, Symptome, Therapie | audibene.de [cited 19/08/08]. Available from: URL: https://www.audibene.de/usher-syndrom/.

20. INSERM US14 -- ALLE RECHTE VORBEHALTEN. Orphanet: Retinitis pigmentosa [cited 19/08/08]. Available from: URL: https://www.orpha.net/consor/cgi-bin/OC_Exp.php?Lng=DE&Expert=791.

21. Usher Syndrome; 2015 [cited 19/08/08]. Available from: URL: https://www.nidcd.nih.gov/health/usher-syndrome.

22. Chen Y, Lu Y, Kuyaxi P, Cheng J, Zhao J, Zhao Q et al. Identification of Pathogenic Genes of Nonsyndromic Hearing Loss in Uyghur Families Using Massively Parallel DNA Sequencing Technique. Dis Markers 2018; 2018:5298057.

23. DocCheck Medical Services GmbH. Haarzelle - DocCheck Flexikon: DocCheck Medical Services GmbH; 19/06/24 [cited 19/08/13]. Available from: URL: https://flexikon.doccheck.com/de/Haarzelle.

24. HomozygosityMapper; 18/06/20 [cited 19/08/07]. Available from: URL: http://www.homozygositymapper.org/.

25. GarbCL. LMS_V25.book.

26. Online Mendelian Inheritance in Man (OMIM) [cited 19/08/07]. Available from: URL: https://www.omim.org/.

27. UCSC Genome Browser Gateway [cited 19/08/07]. Available from: URL: https://genome-euro.ucsc.edu/cgi-bin/hgGateway?redirect=manual&source=genome.ucsc.edu.

28. HGMD gene search [cited 19/08/07]. Available from: URL: https://portal.biobase-international.com/hgmd/pro/search_gene.php?

29. MutationTaster; 18/05/23 [cited 19/08/07]. Available from: URL: http://www.mutationtaster.org/.

30. ExAC Browser [cited 19/08/07]. Available from: URL: http://exac.broadinstitute.org/.

31. Lopes VS, Williams DS. Gene Therapy for the Retinal Degeneration of Usher Syndrome Caused by Mutations in MYO7A. Cold Spring Harb Perspect Med 2015; 5(6).

32. Allele frequency, penetrance, and disease; 2019 [cited 2020 Jun 11]. Available from: URL: https://www.nature.com/scitable/content/allele-frequency-penetrance-and-disease-21642/.

33. Le Quesne Stabej P, Saihan Z, Rangesh N, Steele-Stallard HB, Ambrose J, Coffey A et al. Comprehensive sequence analysis of nine Usher syndrome genes in the UK National Collaborative Usher Study. J Med Genet 2012; 49(1):27–36.

34. Homo sapiens myosin VIIA (MYO7A), transcript variant 4, mRNA - Nucleotide - NCBI [cited 19/08/21]. Available from: URL: https://www.ncbi.nlm.nih.gov/nuccore/NM_001369365?report=GenBank.

35. RefSeq Gene [cited 19/08/21]. Available from: URL: https://genome-euro.ucsc.edu/cgi-bin/hgc?hgsid=233179450_VJZd4UYwk6hE6zERQK4ts58KlEB4&c=chr11&l=76839309&r=76839541&o=76839291&t=76926286&g=refGene&i=NM_001369365.

36. MYO7A - Unconventional myosin-VIIa - Homo sapiens (Human) - MYO7A gene & protein; 19/07/31 [cited 19/08/21]. Available from: URL: https://www.uniprot.org/uniprot/Q13402.

37. BMJ Publishing Group. MYO7A mutation screening in Usher syndrome type I patients from diverse origins; 07/01/01 [cited 19/08/21]. Available from: URL: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2598023/.

38. EMBL-EBI I. InterPro protein sequence analysis & classification < InterPro < EMBL-EBI [cited 19/08/07]. Available from: URL: https://www.ebi.ac.uk/interpro/.

39. EMBL-EBI I. Myosin head, motor domain (IPR001609) < InterPro < EMBL-EBI [cited 19/08/21]. Available from: URL: http://www.ebi.ac.uk/interpro/entry/IPR001609.

40. Reference GH. MYO7A gene; 19/08/20 [cited 19/08/21]. Available from: URL: https://ghr.nlm.nih.gov/gene/MYO7A#resources.

41. MYO7A myosin VIIA [Homo sapiens (human)] - Gene - NCBI [cited 19/08/21]. Available from: URL: https://www.ncbi.nlm.nih.gov/gene/4647.

42. UniProt; 19/07/31 [cited 19/08/07]. Available from: URL: https://www.uniprot.org/.

43. vital_usher_syndrome_induced_by_myo7a_mutation_and_retinotosis_pigmentosa [cited 2020 Jun 11].

44. Study of SAR421869 in Participants With Retinitis Pigmentosa Associated With Usher Syndrome Type 1B - Full Text View - ClinicalTrials.gov; 2020 [cited 2020 Jun 11]. Available from: URL: https://www.clinicaltrials.gov/ct2/show/NCT01505062?term=jos%C3%A9+alain+sahel&rank=3.

45. Study to Evaluate Safety and Tolerability of QR-421a in Subjects With RP Due to Mutations in Exon 13 of the USH2A Gene - Full Text View - ClinicalTrials.gov; 2020 [cited 2020 Jun 11]. Available from: URL: https://clinicaltrials.gov/ct2/show/study/NCT03780257?term=PROQR.

46. Schrauwen I, Sommen M, Claes C, Pinner J, Flaherty M, Collins F et al. Broadening the phenotype of LRP2 mutations: a new mutation in LRP2 causes a predominantly ocular phenotype suggestive of Stickler syndrome. Clin Genet 2014; 86(3):282–6.

47. Database GHG. LRP2 Gene - GeneCards | LRP2 Protein | LRP2 Antibody [cited 19/08/23]. Available from: URL: https://www.genecards.org/cgi-bin/carddisp.pl?gene=LRP2.

48. Human Gene LRP2 (uc002ues.3) Description and Page Index [cited 19/08/23]. Available from: URL: https://genome-euro.ucsc.edu/cgi-bin/hgGene?hgg_gene=uc002ues.3&hgg_prot=uc002ues.3&hgg_chrom=chr2&hgg_start=169983618&hgg_end=170219122&hgg_type=knownGene&db=hg19&hgsid=233195352_gPnJx4YbVbwuTTGpMHwWp90VFKYq.

49. Online Mendelian Inheritance in Man (OMIM) [cited 19/08/23]. Available from: URL: https://www.omim.org/entry/222448.

50. Human hg19 chr11:76872074-76872078 UCSC Genome Browser v385 [cited 19/08/07]. Available from: URL: https://genome.ucsc.edu/cgi-bin/hgTracks?db=hg19&lastVirtModeType=default&lastVirtModeExtraState=&virtModeType=default&virtMode=0&nonVirtPosition=&position=chr11%3A76872074%2D76872078&hgsid=747481705_D4KV1r48X4roAWLJbPMk46hNSDCd.

51. Kantarci S, Ragge NK, Thomas NS, Robinson DO, Noonan KM, Russell MK et al. Donnai-Barrow syndrome (DBS/FOAR) in a child with a homozygous LRP2 mutation due to complete chromosome 2 paternal isodisomy. Am J Med Genet A 2008; 146A(14):1842–7.

52. ClinVar NM_000260.4(MYO7A):c.1258A>T (p.Lys420Ter) AND Deafness, autosomal recessive 2 Available from: URL: https://www.ncbi.nlm.nih.gov/clinvar/RCV000770845.1/

Supplementary

Figure 10: Filter setting in Variant Studio, Select all