STUDY OF SPLICING VARIANTS IN THE USH GENES THROUGH MINIGENE ASSAY AND TRANSCRIPT ANALYSIS FROM EPITHELIAL NASAL CELLS

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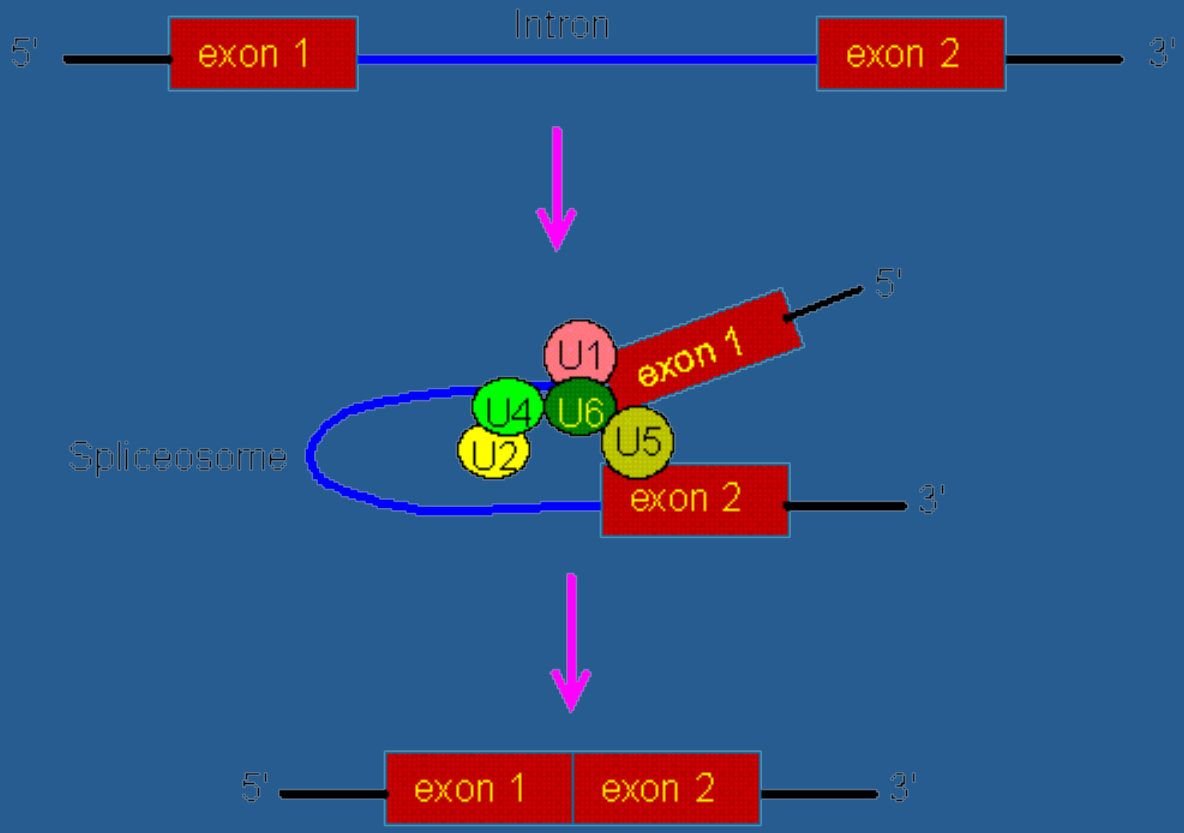
CENTRO DE INVESTIGACIÓN BIOMÉDICA EN RED DE ENFERMEDADES RARAS

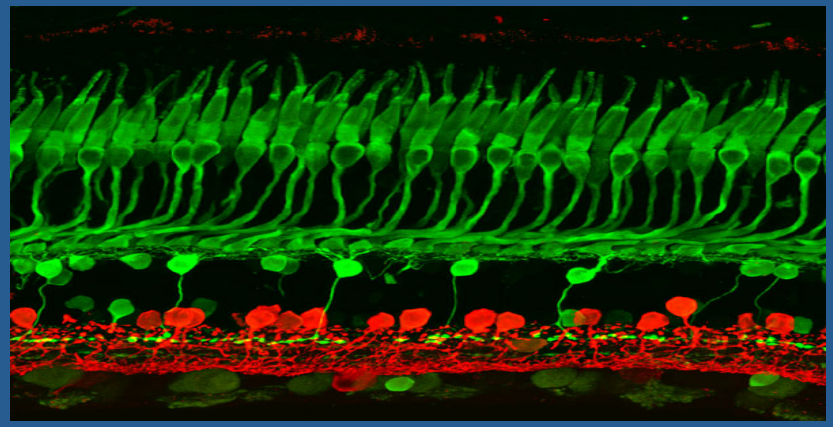
Introduction

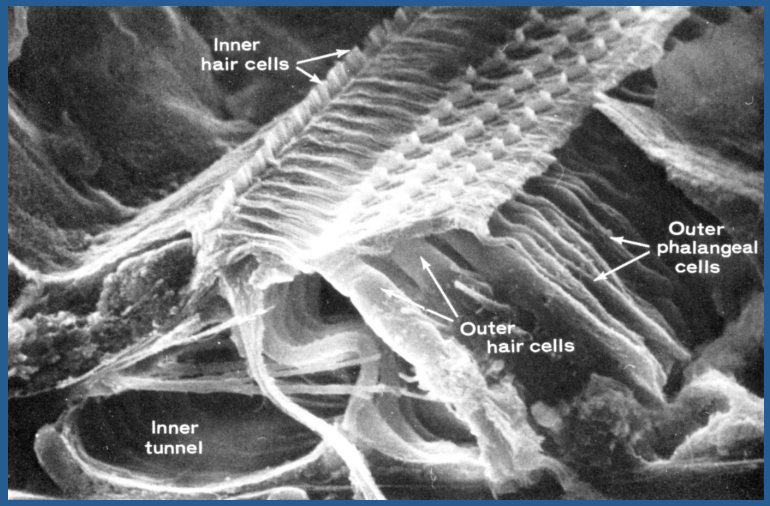
- **splicing** is a modification of the nascent pre-messenger RNA (pre-mRNA) transcript in which introns are removed and exons are joined.



Introduction

- USH genes express in photoreceptors in the retina and in the hair cells in the choclea. Also, in other tissues with limited accesibility. Most of them not in blood cells.





Nasal epithelial cells

- Cohn et al., (2006). 8 USH proteins present in nasal ciliated epithelium
- Vaché et al., (2010). mRNA from USH genes obtained from it

Introduction

- Mutation screening

The consequences of nonsense mutations are usually clear. However, the consequence of missense, silent and intronic changes many times are unknown and additional studies are needed to know the pathogenicity of these variants.

Alteration of *splicing mechanisms* ?

Expression functional studies

Objectives:

1) to determine the pathogenic nature of selected USH1 variants and their effect in the splicing process by minigene assays.

2) to analyze the USH1 transcripts, obtained from the nasal epithelium cells of our patients, in order to corroborate the observed effect of mutations by minigenes in patient's tissues.

Material and Methods

Selection of variants

variants (118) found in a homozygous state or in trans with a disease-causing mutation, cosegregation with the disease, not found in 200 control chromosomes or sequence variation located in exon or introns that may affect the mRNA processing.

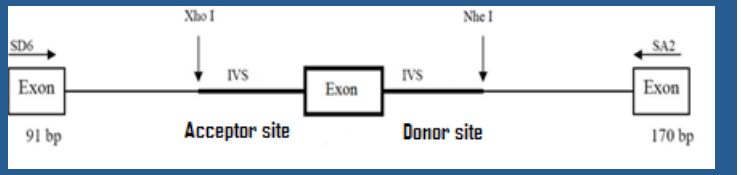
***In silico* analysis** :

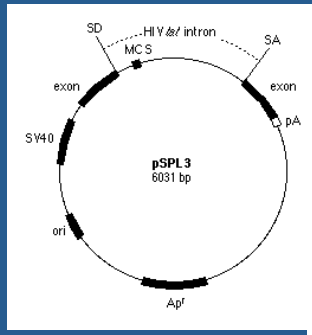
variants identified en USH1 genes were analyzed with the following bioinformatic programs: NNSplice, SpliceView, HSF and NetGene2.

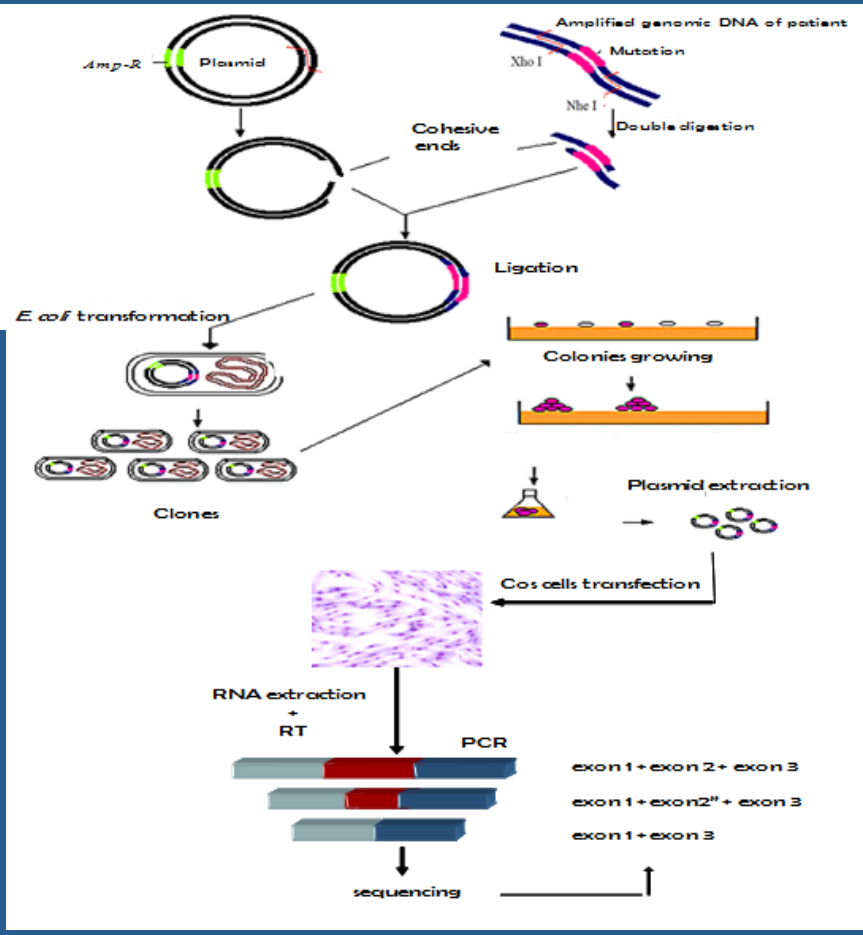
Gene	Variants probably involved in splicing								
MYO7A	c.6_9dup (p.L4DfsX39)	c.470G>A (p.S157N)	c.640G>A (p.G214R)	c.721C>G (p.R241G)	c.1097T>C (p.L366P)	c.1342_1343delAG (p.S448LfsX2)	c.3508G>A (p.E1170K)	c.3652G>A (p.G1218R)	c.5581C>T (p.R1861X)
USH1C	c.1086-12G>A								
CDH23	c.2289+1G>A				c.6049G>A (p.G2017S)		c.8722+1delG		
PCDH15	c.521A>G (p.N174S)		c.1304_1305insC (p.T436YfsX12)		c.1737C>G (p.Y579X)		c.2868+5G>A	c.3717+2dupT	

Material and Methods

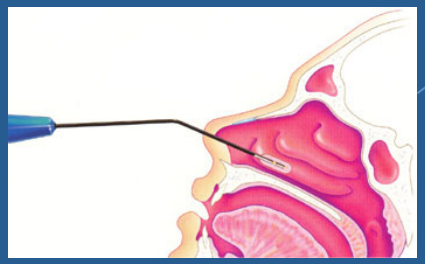
- **Minigenes**:







- **Analysis of mRNA from epithelial nasal cells**:



Obtention of epithelial nasal cells

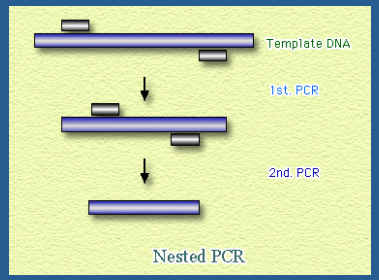
RNA Extraction

↓

cDNA

↓

Nested PCR



Results and discussion

Sequence variants	Type of splice site	*NetGene2*	*HSF*	*NNSplice*	*Splice View*	Score
c.6_9dup (p.L4DfsX39) *MYO7A* [14]	Acceptor	Score for acceptor site increases from 77 to 82	The WT consensus sequence is not recognized	One donor site is not recognized	New acceptor sites are created and other acceptor sites are not recognized	3
c.470G>A (p.S157N) *MYO7A* [14]	Donor	Score for the main donor site decreases from 93 to 60	Score for donor site decreases and a new acceptor site is created	The main donor site is not recognized	The main donor site is not recognized	4
c.640G>A (p.G214R) *MYO7A* [22]	Acceptor	Neutral	The WT consensus sequence is not recognized	A new acceptor site is created	Neutral	1
c.721C>G (p.R241G) *MYO7A* [15]	Donor	Three new donor site are created	A new acceptor site is created	Score for the main acceptor site decreases from 81 to 59	A new donor site is created	4
c.1097T>C (p.L366P) *MYO7A* [15]	Acceptor	Score for the main acceptor site decreases from 83 to 77	Score for the acceptor site decreases	A new acceptor site is created	Neutral	3
c.1342_1343delAG (p.S448LfsX2) *MYO7A* [14]	Donor	The main donor site is not recognized	The main donor site and the acceptor site are not recognized	The main donor site is not recognized	The main donor site is not recognized	4

Nine *MYO7A*, three *CDH23*, five *PCDH15* and one *USH1C* variants were predicted to alter the splicing mechanism creating or eliminating donor/acceptor splice sites.

Eight out of the eighteen variants showed the highest score (4), five of them showed a score of 3, two of the variants were observed to show a score of 2 and the three remaining changes showed a score of 1.

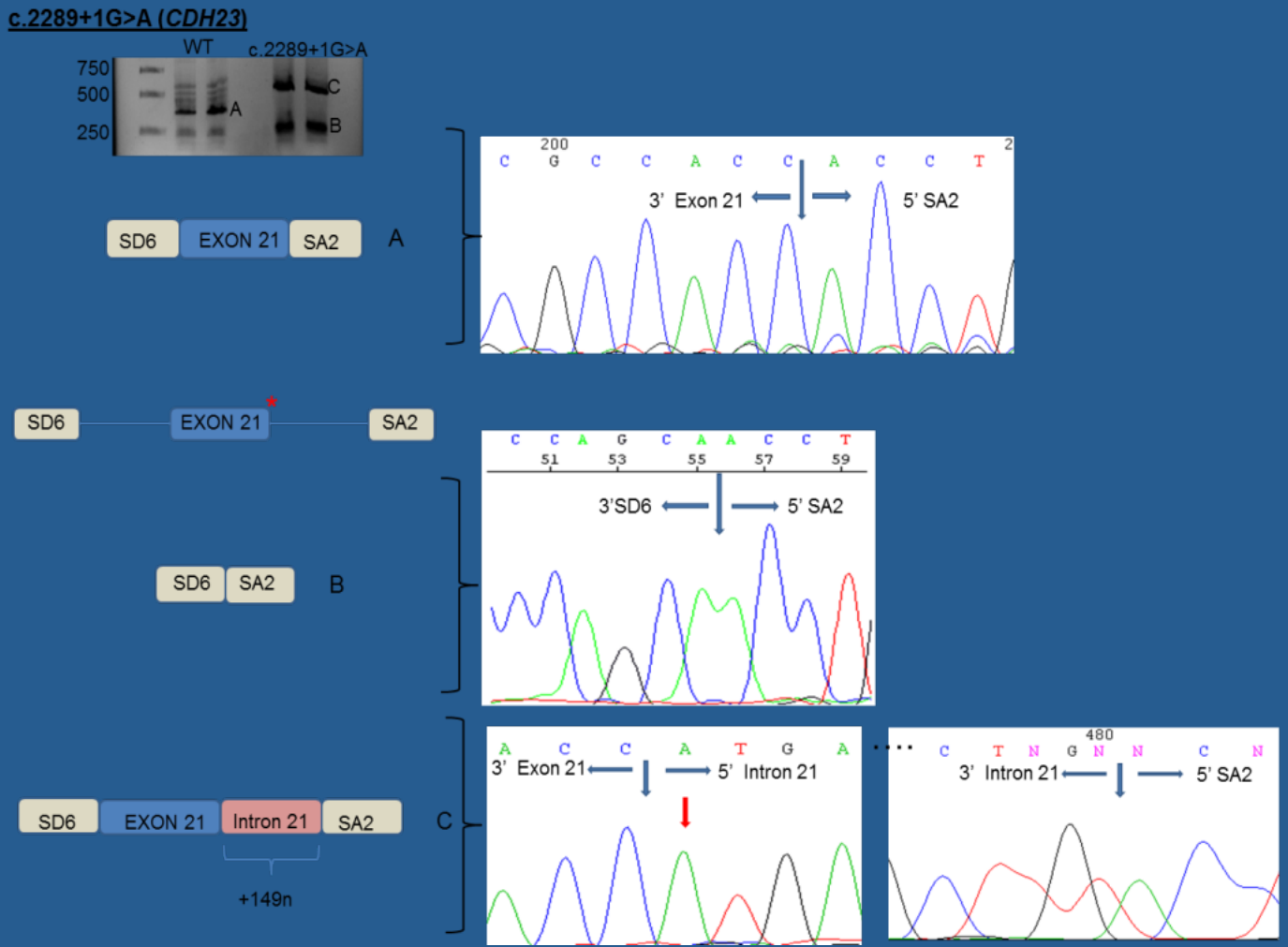
Results and discussion

The minigene assays showed that only seven of them were affecting the mRNA processing

Variant	Effect at RNA level	Efect at protein level
c.470G>A (p.S157N) *MYO7A (4)*	Exon skipping	p.T96WfsX29
c.6049G>A (p.G2017S) *CDH23 (3)*		p.T1976_G2017del
c.1342_1343delAG (p.S448LfsX2) *MYO7A (4)*	Partial deletion of the involved exon	p.N443_E450del
c.3652G>A (p.G1218R) *MYO7A (4)*		p.Y1211AfsX18
c.8722+1delG *CDH23 (3)*		p.S2909AfsX43
c.2289+1G>A *CDH23 (4)*	Insertion of part of the intron adjacent to the involved exon + exon skipping	p.N765SfsX35 + p.E727KfsX9
c.3717+2dupT *PCDH15 (4)*		p.V1242RfsX2 + p.A1168_L1239del

Minigenes: example

Variant	Effect at RNA level	Effect at protein level
c.2289+1G>A CDH23	Insertion of part of the intron adjacent to the involved exon + exon skipping	p.N765SfsX35 + p.E727KfsX9



Results and discussion: mRNA analysis from nasal epithelial cells

We only could obtain samples from 8/18 variants analyzed in vitro by minigenes

Patient	Gene	Allele 1/Allele 2
RP-1481	*MYO7A*	**c.6_9dup (p.L4DfsX39)** (exon2)/+ (3)
RP-1546	*MYO7A*	**c.640G>A (p.G214R)** (exon7)/+ (1)
RP-115	*MYO7A*	**c.3508G>A (p.E1170K)** (exon 28)/c.3238A>T (p.K1080X) (exon 25) (1)
RP-1479	*MYO7A*	**c.5581C>T (p.R1861X)** (exon 40)/ **c.5581C>T (p.R1861X)** (exon 40) (1)
RP-280	*MYO7A*	**c.5856G>A (p.K1952K)** (exon 42)/c.1190C>A (p.A397D) (exon 11) (4)
RP-1534	*CDH23*	**c.2289+1G>A** (intron 21)/**c.6049G>A (p.G2017S)** (exon 46) (3)
RP-928	*CDH23*	**c.8722+1delG** (intron 60)/c.6511delC (exon48) (3)

Table Genotypes of ther five USH1 patients and the two family healthy carriers of USH1 mutations presented in this study.

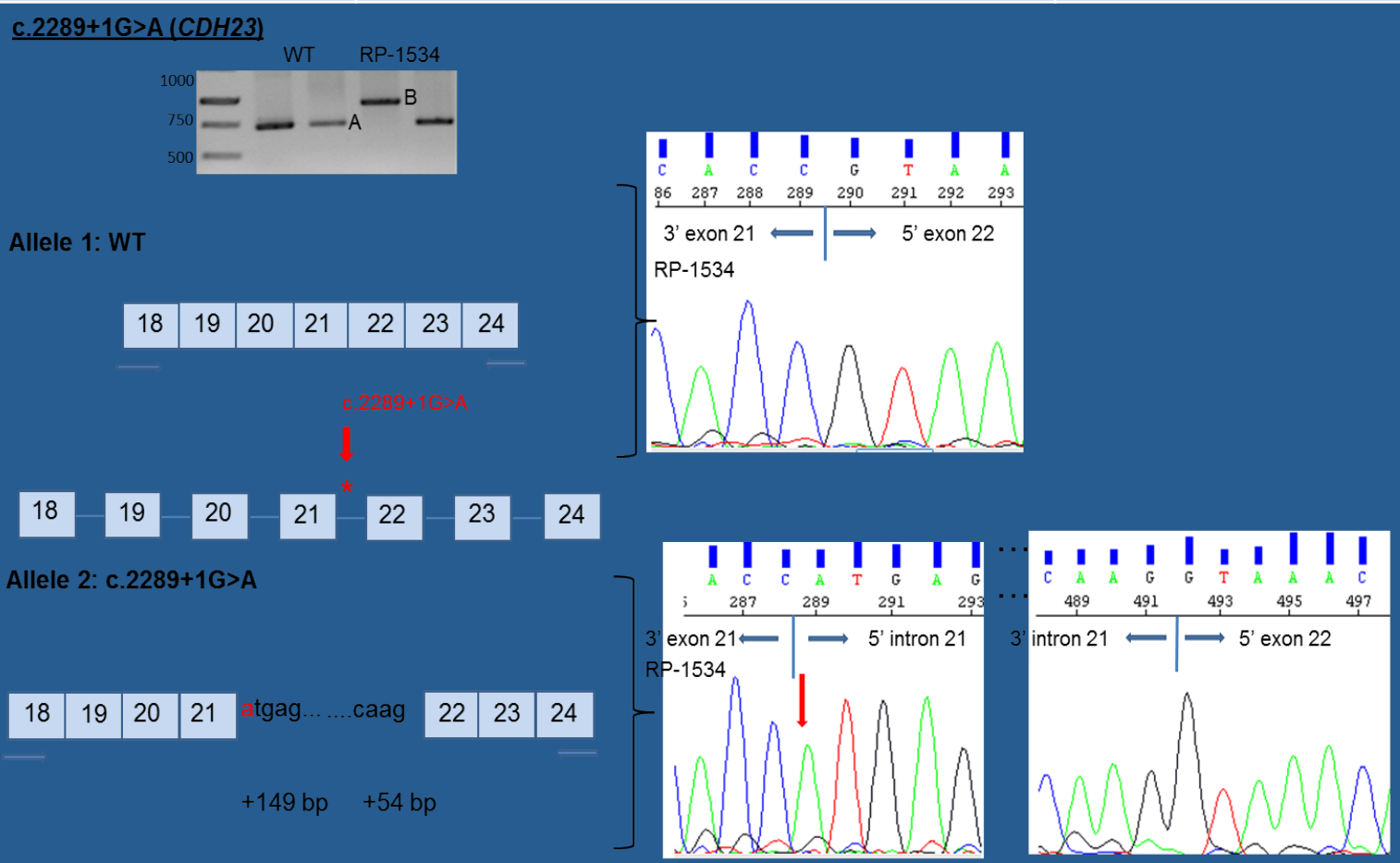
Results and discussion: mRNA analysis from nasal epithelial cells

USH1 Transcript	Effect at RNA level	Effect at protein level
c.5856G>A (p.K1952K) *MYO7A* (4)	Exon skipping	p.A1915_K1952del
c. 2289+1G>A CDH23 (4)	New donor site. Insertion of the first 149nt of intron 21 and the last 54 nt of the same intron	p.N765SfsX35
c.8722+1delG CDH23 (3)	Deletion of the last base of exon 60	p.S2909AfsX43

Only in 3/8 studied variants we could observe an abnormal splicing process ex vivo. In the remaining 5 variants, both WT and mutant alleles were amplified showing that the presence of mutations did not affect the splicing process

Results and discussion: mRNA analysis from nasal epithelial cells

USH1 Transcript	Effect at RNA level	Effect at protein level
c.2289+1G>A *CDH23*	New donor site. Insertion of the first 149nt of intron 21 and the last 54 nt of the same intron	p.N765SfsX35



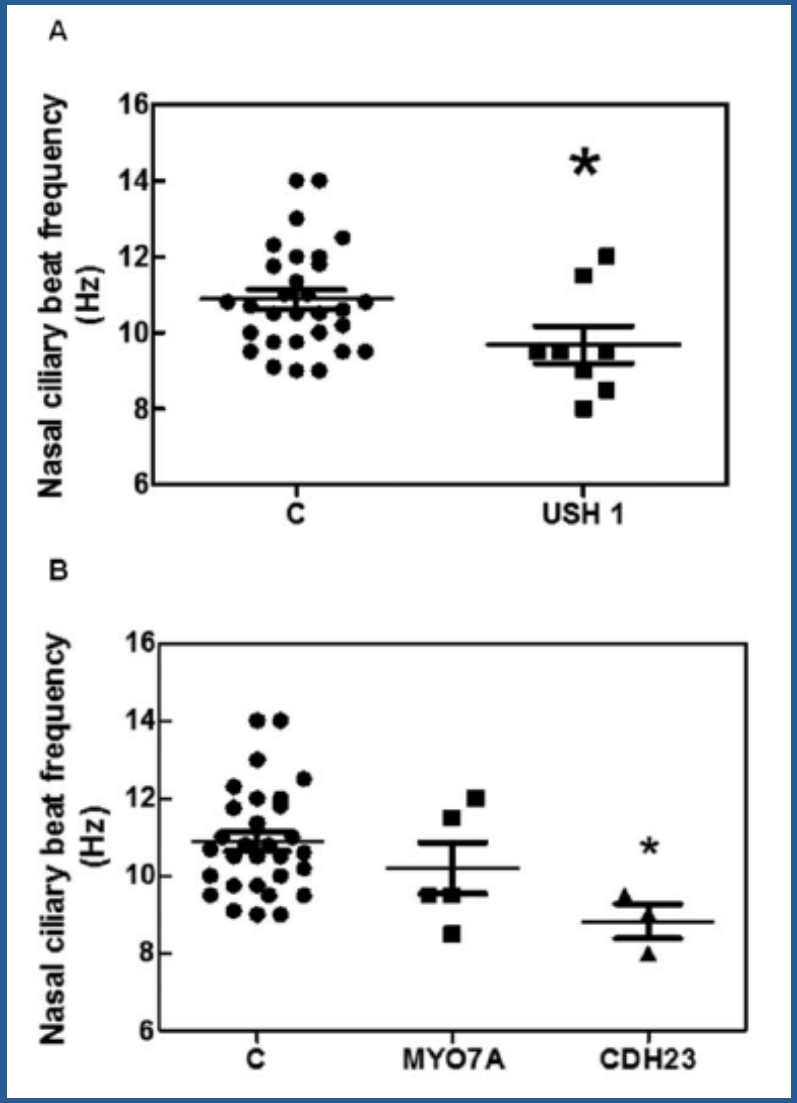
Results and discussion

Comparison of the results with minigenes mRNA analysis from nasal cells in the 8 USH1 transcripts

Variants	Effect at RNA level Minigenes	Effect at protein level Minigenes	Effect at protein level Nasal cells	Effect at protein level Nasal cells
c.6_9dup (p.L4DfsX39) MYO7A (3)	Neutral	p.L4DfsX39	Neutral	p.L4DfsX39
c.640G>A (p.G214R) MYO7A (1)	Neutral	p.G214R	Neutral	p.G214R
c.3508G>A (p.E1170K) MYO7A (1)	Neutral	p.E1170K	Neutral	p.E1170K
c.5581C>T (p.R1861X) MYO7A (1)	Neutral	p.R1861X	Neutral	p.R1861X
c.5856G>A (p.K1952K) MYO7A (4)	Exon skipping	p.A1915_K1952del	Exon skipping	p.A1915_K1952del
c.2289+1G>A CDH23 (4)	New donor site. Insertion of the first 149 nt of intron 21	p.N765SfsX35	New donor site. Insertion of the first 149 nt of intron 21 and the last 54 nt of the same intron	p.N765SfsX35
	Exon skipping	p.E727KfsX9		
c.8722+1delG CDH23 (3)	Deletion last base exon 60	p.S2909AfsX43	Deletion last base exón 60	p.S2909AfsX43

Results and discussion

Nasal ciliary beat frequency in five *MYO7A* and three *CDH23* patients and in 30 controls



Ciliary beat frequency		
controls	10.88±0.25 Hz	p= 0.031
USH1	9.68±0.49 Hz	

Ciliary beat frequency		
controls	10.88±0.25 Hz	p<0.05
CDH23	8.83±0.44 Hz	
USH1	10.22±0.66 Hz	

Conclusions

In silico analysis is a good first step to determine the effect of mutations in the splicing but not absolutely reliable.

Minigenes are a good approach to ascertain the pathogenic nature of splice site variants when is difficult to obtain RNA from patients' tissues, as in the case of USH genes.

The analysis mRNA from nasal epithelial cells is an alternative method to discriminate neutral Usher variants from those with a pathogenic effect on the splicing process.

The nasal ciliated epithelium of USH1 patients has a lower ciliary beat frequency than control subjects. However, the ciliary activity is sufficient to operate normally and no clinical consequences were observed in these patients.

Thank you for your attention