Genetic investigations in rare genodermatoses with pigmentaional abnormalities

Ph.D. Thesis

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LIST OF PUBLICATIONS

Publications providing the basis of the dissertation

I. Tóth L, **Fábos B**, Farkas K, Sulák A, Tripolszki K, Széll M, Nagy N.: Identification of two novel mutations in the SLC45A2 gene in a Hungarian pedigree affected by unusual OCA type 4. *BMC Med Genet.* 2017 Mar 15;18(1):27. **IF: 2.198**

II. **Fábos B,** Farkas K, Tóth L, Sulák A, Tripolszki K, Tihanyi M, Németh R, Vas K, Csoma Z, Kemény L, Széll M, Nagy N.: Delineating the genetic heterogeneity of OCA in Hungarian patients. *Eur J Med Res.* 2017 Jun 19;22(1):20. **IF: 1.414**

Impact factors of the publications providing the basis of the dissertation: **3.612**

Publications indirectly related to the subject of the dissertation

I. Szabó K, Gáspár K, Dajnoki Z, Papp G, **Fábos B**, Szegedi A, Zeher M.: Expansion of circulating follicular T helper cells associates with disease severity in childhood atopic dermatitis. *Immunol Lett.* 2017 Apr 18. pii: S0165-2478(17)30085-8. **IF: 2.860**

II. Sulák A, Tóth L, Farkas K, Tripolszki K, **Fábos B,** Kemény L, Vályi P, Nagy K, Nagy N, Széll M. One mutation, two phenotypes: a single nonsense mutation of the CTSC gene causes two clinically distinct phenotypes. *Clin Exp Dermatol.* 2016 Mar;41(2):190-5. **IF: 1.315**

III. Nagy N, Vályi P, Csoma Z, Sulák A, Tripolszki K, Farkas K, Paschali E, Papp F, Tóth L, **Fábos B**, Kemény L, Nagy K, Széll M.: CTSC and Papillon-Lefèvre syndrome: detection of recurrent mutations in Hungarian patients, a review of published variants and database update. *Mol Genet Genomic Med.* 2014 May;2(3):217-28.

Impact factors of all publications: **7.787**

1. INTRODUCTION

1.1. Rare monogenic skin diseases

From ancient times to the present, the basic approach for diagnosing skin diseases has been to classify the diseases according to their visible signs and symptoms. This approach highlights that dermatology is still a highly morphology-orientated specialty (Shelley *et al.,* 1976; Nagy *et al.,* 2015). Since that time, the desire to understand the nature of observed skin lesions constantly drives the development of dermatology and the incorporation of novel investigative methods into its everyday practice (Shelley *et al.,* 1976; Nagy *et al.,* 2015).

Breeding agricultural plants and animals characterized the pre-Mendel era of genetics (Stern *et al.,* 1950; Hansen *et al.,* 2014). After Gregor Mendel established the basic rules of heredity in the nineteenth century (Mendel *et al.,* 1993), several major discoveries, such as the identification of DNA as the material encoding inheritable information, of the genetic code and of the mechanisms of gene expression, have initiated the era of molecular genetics (Watson *et al.,* 1953; Min Jou *et al.,* 1972). Very recently, the enormous technical development of sequencing methods and platforms has resulted in large-scale genomic projects, which produce amounts of data that were unimaginable a few decades ago (Sanger *et al.,* 1977; Stoneking *et al.,* 2011).

These discoveries and techniques have been used to identify several normal genetic variations, as well as candidate genes and their disease-causing mutations, accelerating the elucidation of the genetic background of several monogenic skin diseases (genodermatoses).

Genodermatoses are defined as life-threatening or chronically debilitating skin conditions whose prevalence is less than 5 in 10 000 of the general population (Baldovino *et al.,* 2016). In opposite with common diseases, which are considered as the consequence of multifactorial – life style, environmental and genetic – etiological factors; genodermatoses are usually monogenic disorders and they are mostly determined by the presence or the absence of any causative genetic alteration which can cause the consequential failure of the certain protein and can lead to the development of the disease (Aronson *et al.*, 2006).

Although dermatology and genetics are considered separate disciplines, the combination of these two fields has already resulted in enormous improvement in the understanding of monogenic skin diseases (Nagy *et al.,* 2015). Investigations on genodermatoses are essential for the development of the knowledge about the genetic background on these diseases and the research can help family planning in the affected families and can also provide a novel therapeutic modality for the affected patients in the future (Aymé *et al.,* 2015). This latter one has also great importance, since currently, int he majority of the cases, only symptomatic treatment is available for patients with genodermatosis (Nagy *et al.,* 2015).

The following clinical entities are classified as genodermatoses: epidermolysis bullosa, keratotic disorders, disorders of skin color, ectodermal genodermatoses, genodermatoses associated with connective tissue, vascular genodermatoses and genodermatoses with skin manifestation and elevated cancer risk (Aymé *et al.,* 2015).

1.2. Genodermatoses with abnormal pigmentation

One of the most clinically heterogenous group of genodermatoses, is the one with pigmentational abnormalities.

The pigmentation of the skin is, except in rare pathological instances, the result of three pigments or chromophores: melanin, a brown/black (eumelanin) or red/yellow polymer (pheomelanin) produced by melanocytes; hemoglobin in red blood cells in the superficial vasculature; and dietary carotenoids (Rees *et al.,* 2003; Chatzinasiou *et al.,* 2015). Melanin, the most important, is formed from tyrosine, via the action of tyrosinase in the lysosome-related organelles of melanocytes, called melanosomes. Melanocytes are dendritic cells, arising from the neural crest during embryonic development and located in the basal layer of the epidermis. The melanosomes are transferred from a melanocyte to a group of 36 keratinocytes called the epidermal melanin unit, to which they provide melanin (Chatzinasiou *et al.,* 2015).

Interest in the genetics of human pigmentation is longstanding. Variation in human pigmentary form - of skin, hair, and eyes - is one of the most striking polymorphic human traits (Rees *et al.,* 2011; Chatzinasiou *et al.,* 2015). More than

150 genes have now been identified that affect pigmentation of the skin, the hairs and the eyes (Chatzinasiou *et al.,* 2015).

The availability of largescale DNA analysis and genome-wide scans, together with our existing knowledge of the genes involved in pigmentation, have contributed to the interpretation of the mechanism of skin pigmentation (Yamaguchi *et al.,* 2009; Rees *et al.,* 2011).

Disorders of pigmentation can result from migration abnormalities of melanocytes from the neural crest to the skin during embryogenesis (Plensdorf *et al.,* 2009; Fistarol *et al.,* 2010). In addition, impairment of melanosome transfer to the surrounding keratinocytes, an alteration in melanin synthesis and a defective degradation or removal of melanin may lead to abnormal skin pigmentation (Plensdorf *et al.,* 2009; Fistarol *et al.,* 2010). Immunologic or toxic mediated destructions of melanocytes can also cause pigmentation abnormalities (Plensdorf *et al.,* 2009; Fistarol *et al.,* 2010).

Disorders of pigmentation are can occur as a genetic or acquired disease (Plensdorf *et al.,* 2009; Fistarol *et al.,* 2010). In my thesis, I have focused on the ones with genetic background, which considered as genodermatoses with abnormal pigmentation.

These pigmentational abnormalities can affect the whole body of the patient and therefore can be generalized, but can manifest only in one body part and considered as localized abnormality (Chatzinasiou *et al.,* 2015). The previous one is usually the consequence of germline mutations of genes playing key role in the regulation of pigmentation, the latter ones can be the consequence of somatic mosaicizm. In these latter cases, the mutation is present in a mosaic form and only affect the body part with altered pigmentation. In my thesis, I have investigated the putative underlying germline mutations of the genes involved in the development of the pigmentation of the skin, the hairs and the eyes.

Pigmentational abnormalities either can be associated with increased amount of pigments resulting in hyperpigmented lesions or with decreased or complete lack of pigments leading to the development of hypopigmented lesions (Chatzinasiou *et al.,* 2015).

In my thesis, I have summarized the results of my genetic investigations in very stigmatizing, rare monogenic skin disease characterized with decreased or complete lack of pigments, the oculocutaneous albinism. The complexity of this

genodermatoses is increased by the fact that the abnormalities of the color might not only affect the skin of the patients, but also the hairs and the eyes as well (Figure 1).

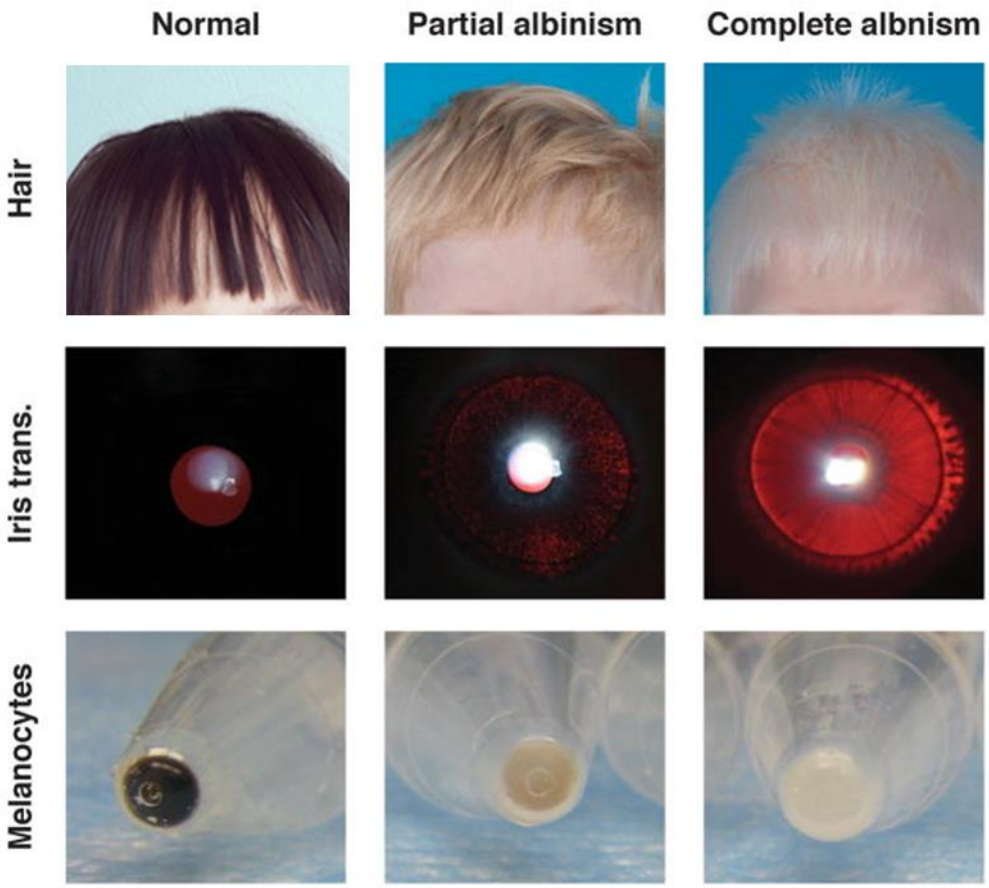

Figure 1. *The clinical* **s***pectrum of the albinism incudes partial and complete pigment loss and can affect the hairs, the skin and the eyes of the patients (Isabel et al., 2007).*

Reseach of this topic have great importance for the patients themselves either in helping family planning and also with contributing to the bases of future studies aming to the develop causative treatment modalities for the affected patients.

1.3. The oculocutaneous albinism

Oculocutaneous albinism (OCA) is a clinically and genetically heterogenic group of rare monogenic diseases characterized by diffuse reduced melanin production in the skin, hair and/or eyes (Mártinez-García *et al.,* 2013). OCA affects one in 20,000 individuals worldwide; however, the prevalence of its subtypes varies

among different populations (Gargiulo *et al.*, 2011). To date, six genes have been implicated in the development of the isolated OCA forms, and an additional 24 genes have been associated with syndromic OCA variants (Simeonov *et al.,* 2013). I have focused my scientific work to the investigation of the isolated (non-syndromic) OCA variants, in which besides the pigmentational abnormalities of the hairs, skin and eyes there is no further affected organ.

Four genes have been implicated in the etiology of the most common isolated OCA forms (Table I.): the tyrosinase gene (*TYR*; OMIM 606933), which is responsible for the development of OCA type 1 (OCA1) (King *et al.*, 2003; Ghodsinejad Kalahroudi *et al.,* 2014), mutations of the oculocutaneous albinism two gene (*OCA2*; OMIM 611409), which are associated with OCA type 2 (OCA2) (Durham-Pierre *et al.,* 1994), pathogenic variants of the tyrosinase-related protein gene (*TYRP*; OMIM 115501), which are linked with OCA type 3 (OCA3) (Rooryck *et al.,* 2008), and mutations in a membrane-associated transporter gene (*SLC45A2*; OMIM 606202), which are implicated in OCA type 4 (OCA4) (Inagaki *et al.*, 2004). Although OCA2 and OCA4 are present in Caucasian populations, OCA1 is the most common form and OCA3 is very rare (Rooryck *et al.,* 2008).

Gene	**Gene product**	**Disease name**
TYR	Tyrosinase enzyme (TYR)	OCA1 (Yellow albinism)
OCA2	Oculocutaneous albinism type two protein (OCA2)	OCA2 (Brown albinism)
TYRP	Tyrosinase-related protein (TYRP)	OCA3 (Rufous albinism)
SLC45A2	Solute carrier family 45 member 2 protein (SLC45A2)	OCA4 (Brown albinism, clinically similar to OCA2)
Unknown	Unknown	OCA5
SLC24A5	Solute carrier family 24 member 5 protein (SLC24A5)	OCA6

C10ORF11	Leucine-rich melanocyte differentiation-associated protein (LRMDA)	OCA7

Table I. *Presentation of the subtypes of the isolated (non-syndromic) OCA variants.*

Rare isolated OCA variants listed in Table I. include OCA type 5 (OCA5), OCA type 6 (OCA6) and OCA type 7 (OCA7). In OCA5 the causative gene has not yet been elucidated, there is only one reported Pakistanian family affected with OCA5 (Kausar *et al.*, 2013). OCA6 (OMIM 606574) results as the consequence of mutations in the solute carrier family 24 member 5 *(SLC24A5)* gene. OCA6 patients have been reported from the Faroe Islands (Gronskov *et al.*, 2013). OCA7 (OMIM 615179) develops as the results of mutations in the *C10ORF11* gene encoding the leucine-rich melanocyte differentiation-associated protein (LRMDA), OCA7 has only been reported in one Faroese family so far (Gronskov *et al.*, 2013).

Since OCA3 common in South Africans and OCA5, OCA6 and OCA7 are extremely rare variants reported only in a few patients, I have focused my scientific work for the investigations of the OCA1, OCA2 and OCA4 non-syndromic variants.

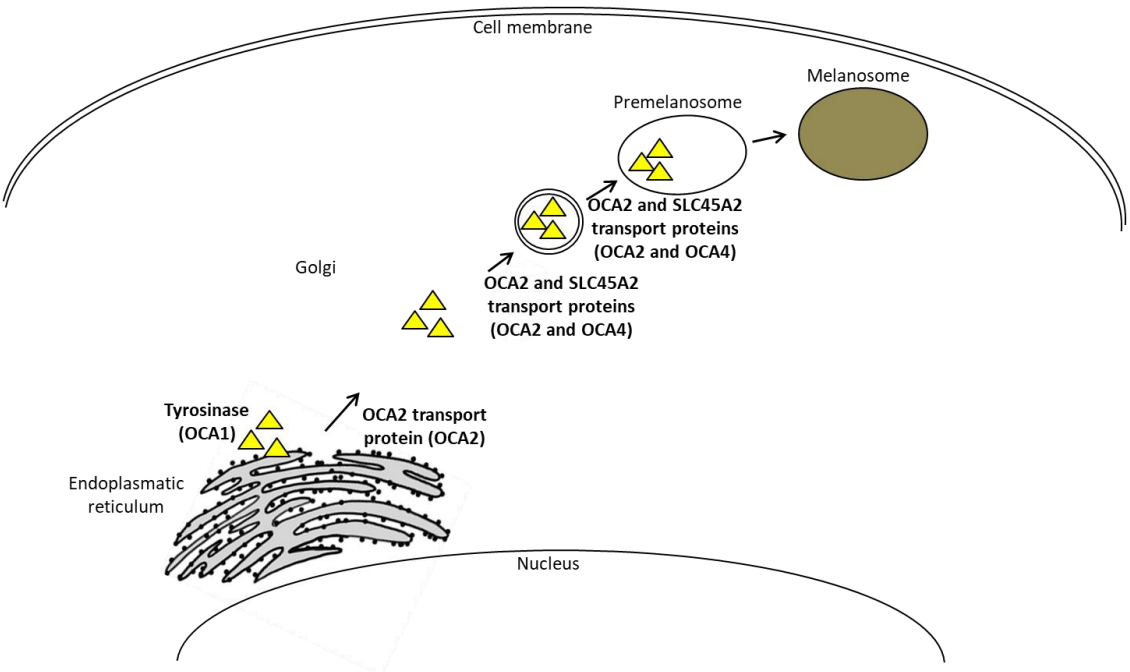

Figure 2. *The scematic presentation of the interactions of the TYR, OCA2 and SLC45A2 proteins during pigmentation. The tyrosinase enzyme catalyzes the first and second steps in melanin synthesis: the hydroxylation of tyrosine to L-DOPA and*

the oxidation of L-DOPA to DOPA-quinone. The OCA2 and SLC45A2 transporter proteins are implicated in the trafficking of tyrosinase to melanosomes.

The *TYR* gene in OCA1 encodes the tyrosinase enzyme, which catalyzes the first and second steps in melanin synthesis: the hydroxylation of tyrosine to L-DOPA and the oxidation of L-DOPA to DOPA-quinone (King *et al.,* 2003). The *OCA2* and *SLC45A2* genes in OCA2 and OCA4 encode transporter proteins, which are implicated in the trafficking of tyrosinase to melanosomes (Figure 2.).

1.4. Aims

Genodermatoses represent a major challenge for health care organizations due to the small number of patients and the lack of the relevant knowledge and expertise of the specific rare disease. Among genodermatoses, one of the most complex disease group is the genodematoses with pigmentational abnormalities. Therefore, my thesis focuses on the elucidation of the genetic background of genodermatoses with pigmentational abnormalities.

Within the group of genodermatoses with pigmentational abnormalities, I have focused my scientific work for the investigation of OCA, which is characterized by variable hair, skin and ocular hypopigmentation. In order to identify the causative mutations in the investigated Hungarian OCA patients (n=12), I have performed the genetic screening of the *TYR, OCA2* and *SLC45A2* genes, which have been implicated in the etiology of the most common isolated OCA forms (OCA1, OCA2 and OCA4).

With the performed clinical workup and mutation screening of the *TYR, OCA2* and *SLC45A2* genes, the main aims of my scientific work were the followings:

1. to promote the understanding of the heterogeneity of OCA,
2. to assess the independent and cumulative contributions of the *TYR, OCA2* and *SLC45A2* genes to the development of OCA,
3. to compare relative and cumulative frequencies of the clinical variants of OCA in a representative Hungarian OCA population.

My results revealed rare (mutation) and common (polymorphism), novel and recurrent genetic variants of the investigated genes and contributed to the understanding of the genotype-phenotype correlations in this clinically and

genetically heterogenic group of genodermatoses. My results also demonstrated that the concomitant analysis of OCA genes is critical, providing new insights to the phenotypic diversity of OCA and expanding the mutation spectrum of OCA genes in Hungarian patients.

The proposed genetic, molecular biology investigations might also lead to the identification of novel therapeutic target molecules and, eventually, to the development of novel therapeutic modalities for patients with OCA.

These investigations have been performed in accordance with the current trends of biomedical research of the European Union, which supports the investigation of rare, so-called “neglected” diseases, since the mechanisms revealed in rare monogenic skin diseases would also lead to the further understanding of the mechanisms of common diseases. As my investigation on OCA might provide further insights into mechanisms of common skin diseases with pigmentational abnormalities such as vitiligo or melasma.

2. PATIENTS AND METHODS

2.1. Patients

The individuals (n=12) participating in this study were recruited at the Mór Kaposi Teaching Hospital of the Somogy County (Kaposvár, Hungary), at the Hospital of Zala County (Zalaegerszeg, Hungary) and at the Department of Dermatology and Allergology, University of Szeged (Szeged, Hungary). The enrolled patients fulfilled the clinical criteria for OCA (Table II.).

Patient	Gender	Age	Skin Color	Hair Color	Iris pigmentation
1	Female	3	White	White	Hypopigmented
2	Female	31	White	White	Hypopigmented
3	Female	28	White	White	Hypopigmented
4	Male	4	White	White	Hypopigmented
5	Female	57	White	White	Hypopigmented
6	Female	60	White	White	Hypopigmented
7	Male	6	White	White	Hypopigmented
8	Male	21	White	White	Hypopigmented
9	Male	7	White	White	Hypopigmented
10	Male	11	White	White	Hypopigmented
11	Male	15	White	White	Hypopigmented
12	Male	48	White	White	Hypopigmented

Table II. *Clinical features of the investigated Hungarian OCA patients (n=12).*

The investigation was approved by the Internal Ethical Review Board of the University of Szeged. Written informed consent was obtained from the patients and the healthy controls, and the study was conducted according to the Principles of the Declaration of Helsinki.

2.2. Methods

2.2.1. DNA isolation

The performed genetic investigations got ethical approved by the Hungarian National Public Health and Medical Officer Service. After written informed consent was obtained from all investigated individuals, peripheral blood samples were collected from the investigated patients (n=12) and from unrelated controls for genetic analysis (n=100). Genomic DNA was extracted from the whole blood samples by a BioRobot EZ1 DSP Workstation (QIAGEN, Hilden, Germany). Genomic DNA was dissolved in 100 µl distilled water.

2.2.2. Polymerase chain reaction amplification

The coding regions and flanking introns of the investigated *TYR*, *OCA2* and *SLC45A2* genes were amplified by polymerase chain reaction (PCR) with specific primers. Amplifications were carried out in 20 µl volumes containing 4 µl sample DNA, 9 µl Dream Taq Green PCR Master Mix (Fermentas), 4 µl distilled water and 1,5 - 1,5 µl of each primers.

The using primer sequences were obtained from the UCSC Genome Browser (www.genome.ucsc.edu) and Primer3 (http://bioinfo.ut.ee/primer3-0.4.0/). Primer sequences used to amplify the coding regions of the *TYR* gene are represented in Table III, the ones of the *OCA2* gene in Table IV and Table V, and the primers of the *SLC45A2* gene in Table VI.

***TYR* gene primer sequence 5' to 3'**
Exon 1a Forward: CAAACTGAAATTCAATAACATATAAGG
Exon 1a Reverse: GTGGACAGCATTCCTTCTCC
Exon 1b Forward: TTCAGAGGATGAAAGCTTAAGATAAA
Exon 1b Reverse: CGTCTCTCTGTGCAGTTTGG
Exon 1c Forward: CTGGCCATTTCCCTAGAGC
Exon 1c Reverse: CCACCGCAACAAGAAGAGTC
Exon 1d Forward: CATCTTCGATTTGAGTGCCC
Exon 1d Reverse: CCCTGCCTGAAGAAGTGATT
Exon 2 Forward: CCAACATTTCTGCCTTCTCC
Exon 2 Reverse: TCAGCTAGGGTCATTGTCGAT
Exon 3 Forward: AGTTATAAATCAAATGGGATAATCA
Exon 3 Reverse: ACATTTGATAGGCACCCTCT
Exon 4 Forward: CTGTTTCCAATTTAGTTTTATAC
Exon 4 Reverse: TACAAAATGGCCTATGTTAAGC
Exon 5 Forward: TGTCTACTCCAAAGGACTGT
Exon 5 Reverse: GGCACTTAGCTGGATGTGTT

Table III. Primer sequences used to amplify the coding regions and the flanking introns of the *TYR* gene.

The PCR conditions were the following: after an initial denaturation step at 95 °C for 10 min, 40 cycles of amplification was performed consisting of 30 sec at 95 °C (denaturation), 30 sec at the optimal annealing temperatures of the primers (~59°C) and 45 sec at 72°C (synthesis). The annealing temperature and the number of the cycles were depended on the primers, the synthesis reaction time was determined according to the length of the reaction product.

***OCA2* gene primer sequence 5' to 3'**
Exon 2 Forward: CAAACGTTAGTCTCAGTGTGCC
Exon 2 Reverse: GGAAACCCAATCTGTGTGAAG
Exon 3 Forward: CACCTGAGTGCTGGGAACAC
Exon 3 Reverse: GCCAGGTGCAATGCTCAG
Exon 4 Forward: AGAGGAAAGCTTGCTTTGTAGC
Exon 4 Reverse: AGATGGAGGGGCCATGTAG
Exon 5 Forward: GCTGTGGGTTTACTGGTCAC
Exon 5 Reverse: ACAACCCTCAGCATCTCCTC
Exon 6 Forward: CAGTAGCCCCATCATCACATC
Exon 6 Reverse: GTCACAACCGTCTGCAAGTG
Exon 7 Forward: CGCATTTCTTCACACACTGTC
Exon 7 Reverse: GACTAAGAATGGTGTCCTCGC
Exon 8 Forward: TACCTAGACCGAGCAGTGCC
Exon 8 Reverse: TTAAACGCACGTGTCCCAG
Exon 9 Forward: GCCATGGCTGATACAGAGG
Exon 9 Reverse: TCAAGCCTCCCTGACTGTG
Exon 10 Forward: TCATGTCCACACAGGCTTTC
Exon 10 Reverse: TCTTTGAGCTGACATCCCAC
Exon 11 Forward: AGGCAAGTGGATGGTGAGAT
Exon 11 Reverse: ACACTTCTCAGTCAAGCCCT
Exon 12 Forward: CAGAGGCCAGAGCTCAAATG
Exon 12 Reverse: CTGCCCTGCAGAAGCAAC

Table IV. Primer sequences used to amplify the coding regions and the flanking introns of the *OCA2* gene (from exon 2 to exon 12).

***OCA2* gene primer sequence 5' to 3'**
Exon 13 Forward: GAGGATCAGAGGGGTGACAG
Exon 13 Reverse: AGGCAGTGCAGGCAGAG
Exon 14 Forward: ATCTCTGGGTTGCATGTGG
Exon 14 Reverse: TCTAACTAAGTGGAGGTGTGCG
Exon 15 Forward: CGGAAAGTGCTGGGATTACA
Exon 15 Reverse: CAGCAACCCATCAACAGATAC
Exon 16 Forward: ATCGACTGTGTGGGGAACAG
Exon 16 Reverse: AGGCCCATGGAATGTTCTG
Exon 17 Forward: GAGTGAGCACCTTTTCCAGC
Exon 17 Reverse: AGAAACGGCATTCAGTCACG
Exon 18 Forward: CGTAGGTTATGACACGCTGC
Exon 18 Reverse: GTCAGTGTCTGGGAACAGGC
Exon 19 Forward: GGTTAAAGAAATGAATCGGTGTG
Exon 19 Reverse: AGATGTAGGCTTTCTTCATTCACC
Exon 20 Forward: CTCCATGAATCTTCGTTGTGCA
Exon 20 Reverse: ATTAATGGGACCTGTTCTTACCAG
Exon 21 Forward: GGCCCCTCTGAGTCTCG
Exon 21 Reverse: TCCTCTACACCTGTGAGTGC
Exon 22 Forward: CACAGTATGGCAGCTTCTCTG
Exon 22 Reverse: CTAACTGTTGCTTTGGGCTG
Exon 23 Forward: GAGAACAGAAGCTTACCACCAAG
Exon 23 Reverse: ATCTCCCCTACACCACAGTCTC
Exon 24 Forward: GGTGCTAAGGCCATGTTCTC
Exon 24 Reverse: TTCTTCAAACAGTGGGGTCAG

Table V. Primer sequences used to amplify the coding regions and the flanking introns of the *OCA2* gene (from exon 13 to exon 24).

Finally a 10 min terminal elongation step was followed at 72°C in a MyCycler PCR machine (BioRad).

***SLC45A2* gene primer sequence 5' to 3'**
Exon 1 Forward: ACACAGACCCTAGGACCACG
Exon 1 Reverse: TCCTCCTGCAGAGGTACACAC
Exon 2 Forward: ACGCGGATGATTCTAAAACAGG
Exon 2 Reverse: TGGAAGTGCCTCATTGTCTG
Exon 3 Forward: ACTTGAACCCACATTGCCTG
Exon 3 Reverse: TCTTCTCGTCAAACAGACAAAAC
Exon 4 Forward: TGTCTGTGTGTTCTGGCTCC
Exon 4 Reverse: AGGTGTTAATGGAGGAAATGATG
Exon 5 Forward: CAGAGGTGGAGAAGCAGAGT
Exon 5 Reverse: GAACCCACTGATTCCAAGAGC
Exon 6 Forward: ACCAAGGCAATTTCAAGCTGT
Exon 6 Reverse: GCAGTTGGTTGGGCATTTGA
Exon 7 Forward: GCTGACCTGTGCCCTAAATG
Exon 7 Reverse: TAACTTCCTGCCATGTGCTTC

Table VI. Primer sequences used to amplify the coding regions and the flanking introns of the *SLC45A2* gene.

2.2.3. Gel electrophoresis and gel documentation

The PCR products were checked on 2% agarose gel (SeaKem LE agarose, Lonza) using TBE buffer (Lonza) and visualized by 2,5 µl GelRed (Biotium) staining. The gel was analyzed by BioRad Molecular Imager® GelDoc™ XR gel documentation system with QuantityOne software.

2.2.4. Sequencing

After amplifying the coding regions and flanking introns of the investigated *TYR, OCA2* and *SLC45A2* genes, DNA sequencing was performed on the purified amplification products. The suitable PCR products were sequenced by a traditional capillary sequencer in an ABI Prism 3100 (Applied Biosystems) sequencing machine with Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Bio systems). The service of the sequencing was provided in scientific cooperation by the Delta Bio 2000 company (Szeged, Hungary).

2.2.5. Analysis of the sequencing results

Sequencing data was compared with the wild-type gene sequences using the Ensemble Genome Browser (http://ensemble.org).

In case of novel genetic variants, to predict their putative functional effects, they were assessed with *in silico* prediction programs, including SIFT (http://sift.bii.a-star.edu.sg/), Polyphen-2 (http://genetics.bwh.harvard.edu/pph2) and MutationTaster (http://mutationtaster.org). In case of novel variants, it was also determined whether they affect any known functional domain on the encoded protein. Moreover, it was investigated whether the identified novel variant is located in an evolutionary conserved region of the gene.

Recurrent genetic variants were checked in the literature using Pubmed (https://www.ncbi.nlm.nih.gov/pubmed/).

2.2.6. Summary of the applied methods

The genetic workflow contained the following steps (Figure 3.): Blood samples were obtained from all enrolled individuals. Genomic DNA was isolated from the obtained samples. PCR reactions were prepared in order to amplify the coding regions and the flanking introns of the investigated *TYR, OCA2* and *SLC45A2* genes. After PCR reactions were done, their successfullness were checked by gel

electrophoresis. All the PCR products gave specific bands on agarose gel running were sequenced. Sequencing data was analyzed using in silico softwares.

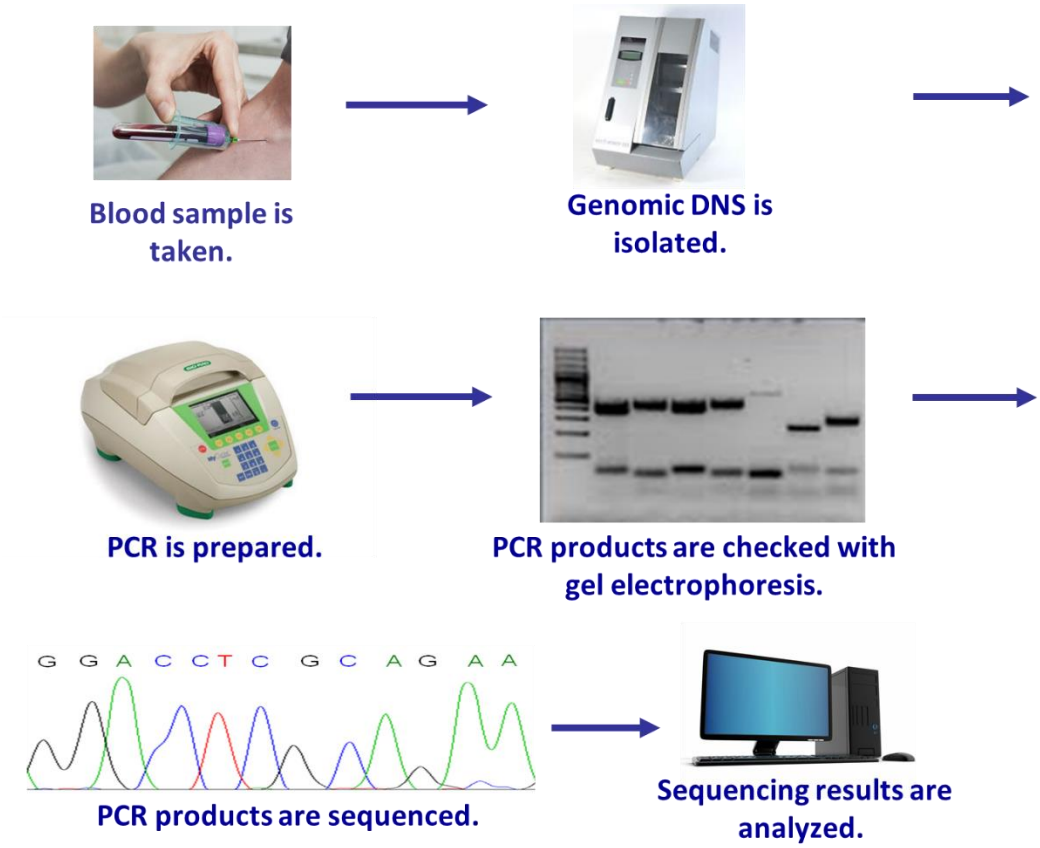

Figure 3. *Scematic representation of the applied workflow of the performed genetic investigations.*

3. RESULTS

3.1. Overview of the sequencing results of the investigated Hungarian OCA patients

In order to identify the causative mutations in the investigated Hungarian OCA patients (n=12), I have performed the genetic screening of the *TYR*, *OCA2* and *SLC45A2* genes, which have been implicated in the etiology of the most common isolated OCA forms (OCA1, OCA2 and OCA4).

Patient	Mutation 1	Mutation 2	Polymorphisms	Molecular diagnosis
1	*TYR* gene: p.Arg217Gln (hetero)	–	–	OCA1
2	*SLC45A2* gene: p.Gly411Asp (hetero)	*SLC45A2* gene: p.Gln487X (hetero)	*TYR* gene: p.Ser192Tyr (homo) Global MAF: 0.1234	OCA4
3	*SLC45A2* gene: p.Gly411Asp (hetero)	*SLC45A2* gene: p.Gln487X (hetero)	*TYR* gene: p.Ser192Tyr (hetero) Global MAF: 0.1234	OCA4
4	*TYR* gene: p.Pro406Leu (hetero)	–	*SLC45A2* gene: p.Leu374Phe (homo) Global MAF: 0.2750	OCA1
5	*TYR* gene: p.Pro406Leu (hetero)	–	*SLC45A2* gene: p.Leu374Phe (homo) Global MAF: 0.2750	OCA1
6	*TYR* gene: p.Arg402X (hetero)	*TYR* gene: p.Pro406Leu (hetero)	*SLC45A2* gene: p.Leu374Phe (homo) Global MAF: 0.2750	OCA1
7	*TYR* gene: p.Pro406Leu (hetero)	–	*TYR* gene: p.Arg402Gln (hetero) Global MAF: 0.0813 *SLC45A2* gene: p.Leu374Phe (homo) Global MAF: 0.2750	OCA1
8	*SLC45A2* gene: p.Val367Ile (hetero)	–	*TYR* gene: p.Ser192Tyr (hetero) Global MAF: 0.1234 *SLC45A2* gene: p.Leu374Phe (homo) Global MAF: 0.2750	OCA4
9	*TYR* gene: p.Pro406Leu (hetero)	–	*SLC45A2* gene: p.Leu374Phe (homo) Global MAF: 0.2750 *OCA2* gene: p.Arg305Trp (hetero) Global MAF: 0.0790	OCA1
10	*TYR* gene: p.Arg217Gln (hetero)	*TYR* gene: p.Pro406Leu (hetero)	*SLC45A2* gene: p.Leu374Phe (homo) Global MAF: 0.2750	OCA1
11	*TYR* gene: p.Arg217Gln (hetero)	*TYR* gene: p.Pro406Leu (hetero)	*SLC45A2* gene: p.Leu374Phe (homo) Global MAF: 0.2750	OCA1
12	*TYR* gene: p.Pro406Leu (hetero)	–	*SLC45A2* gene: p.Leu374Phe (homo) Global MAF: 0.2750	OCA1

MAF = mutant allele frequency

Table VII. *Summary of the results of the genetic investigations in the OCA patients.*

Molecular genetic investigation of Hungarian OCA patients identified pathogenic mutation in all of the patients (Table VII.): five patients carried a combination of two heterozygous pathogenic mutations, whereas only one heterozygous pathogenic mutation was identified in seven patients. In six of these patients, non-pathogenic variants of the *TYR, OCA2* and *SLC45A2* genes were also detected. None of these variants alone is expected to result in the development of OCA; however, these variants were also included in the subsequent analysis, as they might provide further insight into modifying mechanisms of disease development and might lead to the establishment of genotype–phenotype correlations in OCA.

3.1.1. Recurrent pathogenic and recurrent non-pathogenic variants were identified on the *TYR* gene

Direct sequencing of the *TYR* gene revealed pathogenic mutations in 75% (n=9) of the investigated patients (Figure 4.).

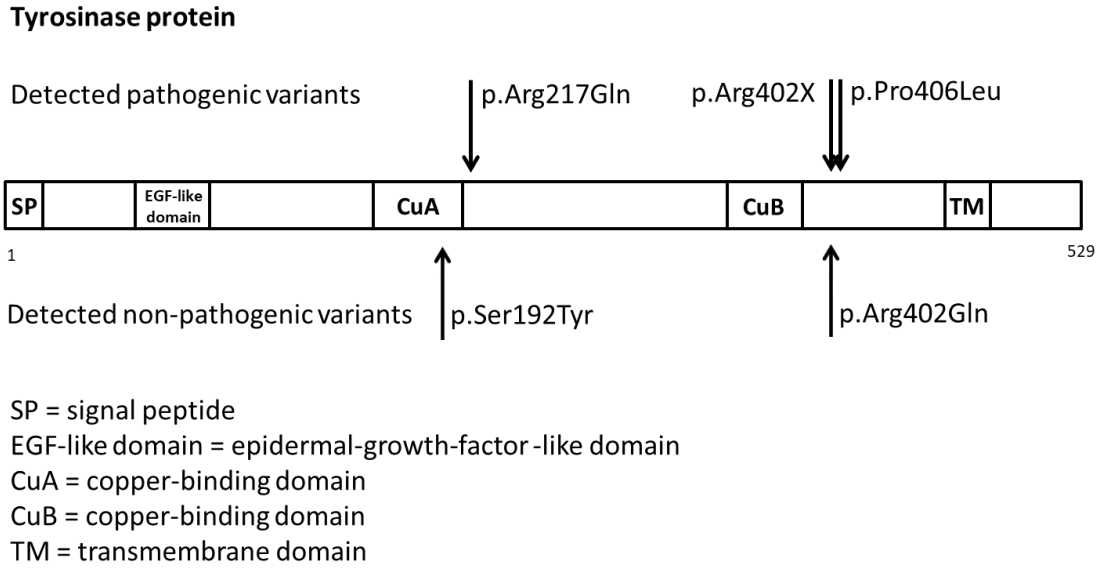

Figure 4. *Distribution of the detected TYR variants on the tyrosinase protein.*

The most frequently detected mutation was the p.Pro406Leu missense mutation, which was present in heterozygous form in 66% (n=8) of the patients. Two patients carried the p.Pro406Leu mutation in combination with the p.Arg217Gln

missense mutation, and one patient carried it with the p.Arg402X nonsense mutation. In five patients carrying the p.Pro406Leu mutation in heterozygous form, no further pathogenic mutation could be identified, and this was confirmed by screening the *TYR, OCA2* and *SLC45A2* genes. However, all five patients carrying the p.Pro406Leu pathogenic mutation also carried common polymorphisms, such as the p.Arg402Gln polymorphism of the TYR gene, the p.Arg305Trp variant of the OCA2 gene, and the p.Leu374Phe polymorphism of the SLC45A2 gene. These polymorphisms are considered as benign variants, which are unable to cause OCA by themselves, but might contribute to the development of OCA in combination with other pathogenic mutations (Fukai *et al.,* 1995; Simeonov *et al.,* 2013). In one patient, only the p.Arg217Gln heterozygous pathogenic missense mutation was identified. No further pathogenic mutations or associated common polymorphism could be detected in this patient. The p.Ser192Tyr polymorphism of the *TYR* gene affects a copper-binding domain of the protein; all the other pathogenic and non-pathogenic variants are located outside of the known functional domains of the enzyme.

3.1.2. Only one recurrent non-pathogenic genetic variant was identified on the *OCA2* gene

No pathogenic *OCA2* mutation was identified in the investigated OCA individuals (Figure 5.). However, one patient with the pathogenic p.Pro406Leu *TYR* mutation also carried the common p.Arg305Trp polymorphism of the *OCA2* gene in heterozygous form (Figure 5.). This variant does not affect any known functional domains of the OCA2 protein.

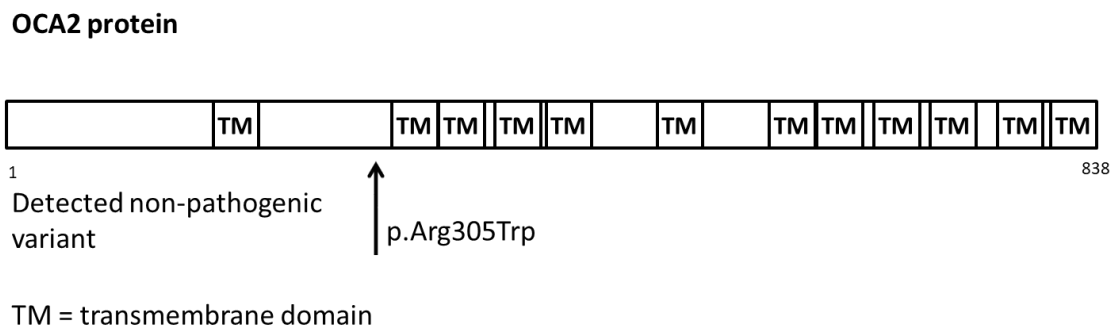

Figure 5. *Distribution of the detected OCA2 variant on the transporter protein.*

3.1.3. Two novel pathogenic mutations and recurrent non-pathogenic variants were identified on the *SLC45A2* gene

Based on our results, six of 12 patients carried SLC45A2 variations: two patients carried the combination of the newly identified p.Gly411Asp pathogenic missense mutation (Figure 6.) and also novel the p.Gln487X nonsense mutation (Figure 7.).

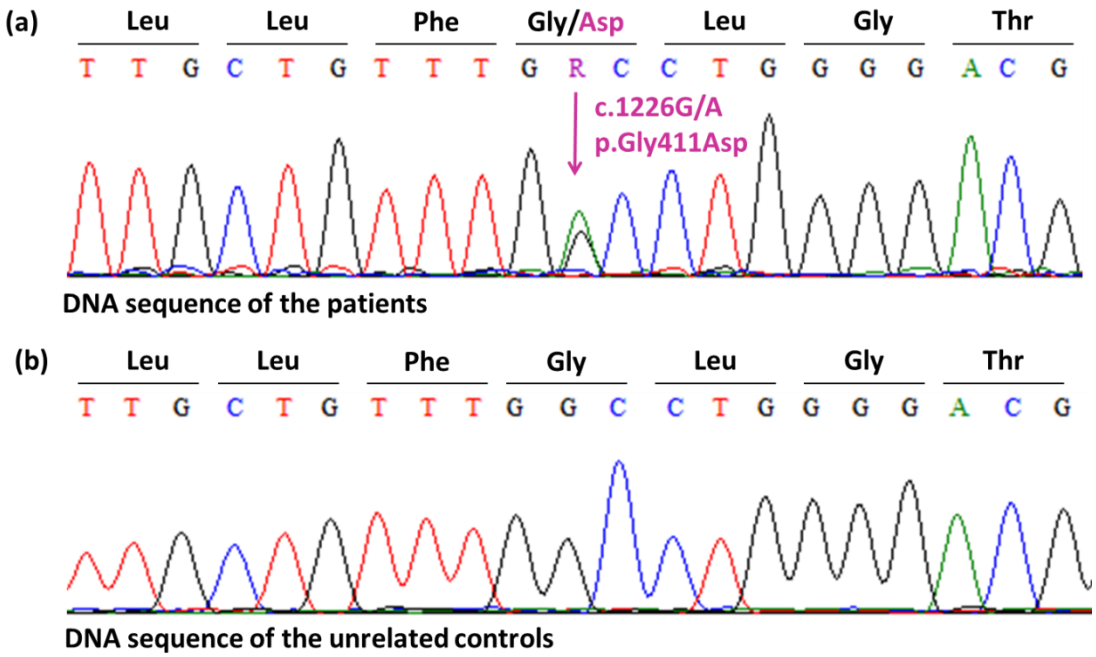

Figure 6. *Direct sequencing revealed a heterozygous missense mutation (c.1226G/A p.Gly411Asp) in the sixth exon of the SLC45A2 gene in Patient 2 and Patient 3. (a) DNA sequence of the patients (b) DNA sequence of the unrelated controls.*

These two mutations are novel mutations, which have not been reported in the literature previously, therefore here I gave detailed phenotypic descriptions of the patients and detailed genetic results.

The two affected OCA patients (Patient 2 and Patient 3 in Table II summarizing clinical data and in Table VII summarizing the genetic investigations) have complete absence of pigment in their hair, pale skin, pink nevi and blue eyes

with nystagmus. Patient 2 has been suffering from Crohn's disease for 9 years and hypothyreosis for 4 years. Regarding Patient 3, she is not aware of any known concomitant diseases. The parents of the affected siblings are clinically unaffected by OCA4.

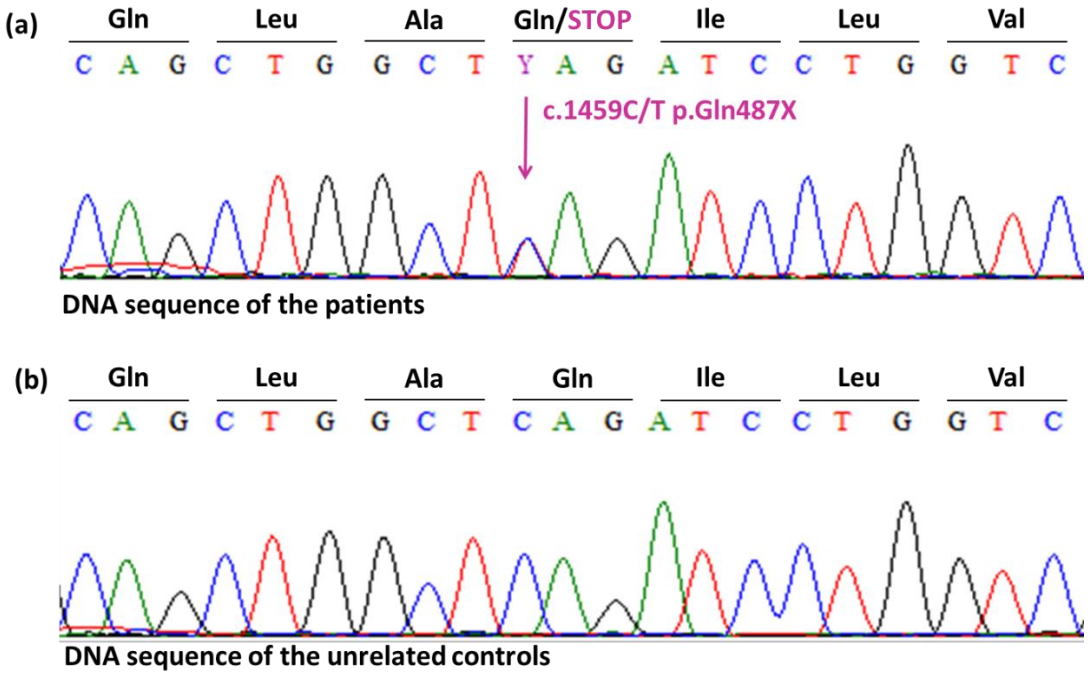

Figure 7. *Direct sequencing revealed a heterozygous nonsense mutation (c.1459C/T p.Gln487X) in the seventh exon of the SLC45A2 gene in Patient 2 and Patient 3. (a) DNA sequence of the patients (b) DNA sequence of the unrelated controls.*

Both Patient 2 and Patient 3 carry both novel mutations: the c.1226G/A p.Gly411Asp missense and the c.1459C/T p.Gln487X nonsense ones.

Regarding their location on the SLC45A2 protein (Uniprot: Q9UMX9), they are situated within transmembrane domains (Figure 8.). The c.1226G/A p.Gly411Asp missense mutation is located within the ninth and the c.1459C/T p.Gln437X nonsense mutation within the tenth transmembrane domains of the SLC45A2 protein.

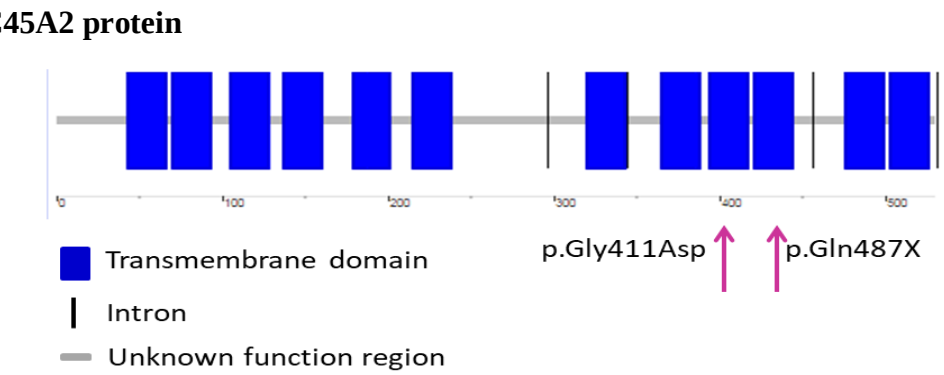

Figure 8. *The identified novel mutations are located within the transmembrane domains of the SLC45A2 protein.*

Concerning the location of the identified mutations, it can be hypothesized that they impair the transport function of the SLC45A2 protein. The dysfunctional SLC45A2 might cause acidic melanosomal lumen, which leads to improper incorporation of copper into typrosinase and thus to reduced tyrosinase activity and the development of the OCA phenotype (Bin *et al.*, 2015). The c.1226G/A p.Gly411Asp missense mutation affects an evolutionary conserved region of the MATP protein (Figure 9.) further emphasizing the putative pathogenic role of this mutation in the development of the observed pigmentation abnormalities of the affected patients.

Figure 9. *The identified novel missense mutation (c.1226G/A p.Gly411Asp) is situated within an evolutionary conserved region of the SLC45A2 protein.*

In four patients, only the non-pathogenic p.Leu374Phe missense polymorphism was detected. All the detected pathogenic and non-pathogenic variants are located within trans-membrane domains of the encoded protein (Figure 10.).

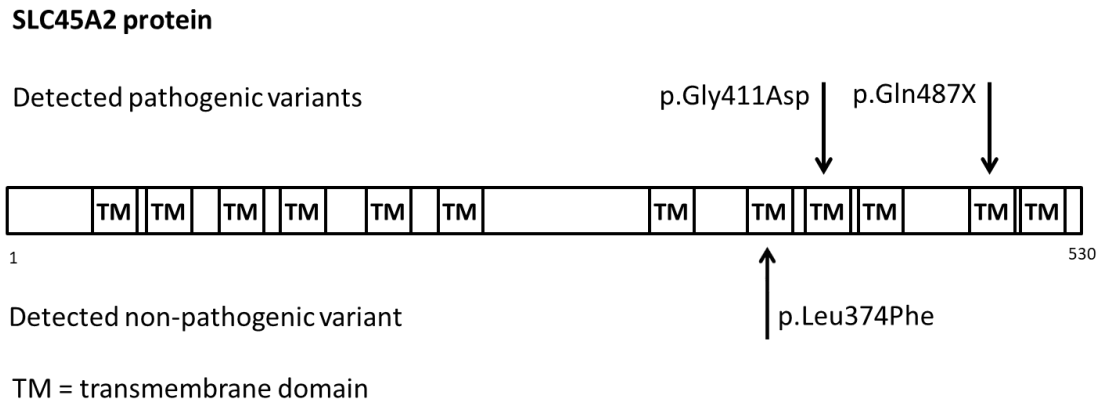

Figure 10. *Distribution of the detected SLC45A2 variants on the transporter protein.*

3.2. Summary of the results of the genetic investigations

Concomitant analysis of OCA genes is critical, providing new insights to the phenotypic diversity of OCA and expanding the mutation spectrum of OCA genes in Hungarian patients. Molecular genetic investigation of Hungarian OCA patients (n=12) identified pathogenic mutation in all of the patients, however, six of these patients also carry non-pathogenic variants of the *TYR, OCA2* and *SLC45A2* genes. However, none of these variants alone is expected to result in the development of OCA; these variants might provide further insight into modifying mechanisms of disease development and might lead to the establishment of genotype–phenotype correlations in OCA.

4. DISCUSSION

4.1. Delineating the genetic heterogeneity of OCA in Hungarian patients

With the performed clinical and genetic workup, I have investgated clinical and genetic heterogenity of OCA1, OCA2 and OCA4 in Hungarian OCA patients (n=12) in order to promote the understanding of the heterogeneity of OCA, to assess the independent and cumulative contributions of the *TYR, OCA2* and *SLC45A2* genes to the development of OCA and to compare relative and cumulative frequencies of the clinical variants of OCA in a representative Hungarian OCA population.

4.1.1. *TYR* mutations accounts for approximately 75% of the isolated OCA cases in the investigated Hungarian OCA population

Pathogenic *TYR* mutations were present in 75% (n=9) of the patients. This result correlates well with previous findings that OCA1 is the most common isolated OCA subtype and *TYR* mutations account for approximately 50% of the isolated OCA cases worldwide (Rooryck *et al.,* 2008; Simeonov *et al.,* 2013). Among the approximately 320 *TYR* mutations identified to date, missense mutations are the most common (Ghodsinejad Kalahroudi *et al.*, 2014). In our study, four missense and one nonsense variants were detected for the TYR gene. Two of these missense mutations are considered pathogenic and two are considered benign polymorphisms. In contrast, the protein carrying the p.Arg402Gln polymorphism exhibits reduced tyrosinase activity at physiological temperature and is considered a temperature-sensitive variant (Berson *et al.,* 2000; Halaban *et al.,* 2000; Toyofuku *et al.,* 2001; Tripanthi *et al.,* 1992). The contribution of the p.Arg402Gln TYR polymorphism to the OCA phenotype is still unknown. By itself, this variant is unable to cause OCA; however, its increased frequency in OCA patients with one heterozygous pathogenic TYR mutation suggests that it can contribute to the development of OCA in combination with a pathogenic mutation (Hutton and Spritz, 2008; Chiang *et al.,* 2009). One Hungarian patient carried the p.Arg402Gln polymorphism in combination with the p.Pro406Leu pathogenic variant. Previous reports (Hutton and Spritz, 2008; Chiang *et al.*, 2009) suggest this combination might contribute to the development of the OCA symptoms of the patient. It is important to emphasize that

certain variants might not cause a disease phenotype in isolation, but contribute to the development of the disease in combination with other pathogenic variants. The difficulty of assessing the impact of these variants highlights the importance of the databases providing information about OCA gene variants, detailed phenotypic descriptions and delineation of OCA genetic heterogeneity.

Three of the nine Hungarian OCA patients with *TYR* mutations carry a combination of two pathogenic mutations. In six of the nine, only one heterozygous pathogenic mutation was identified. These results correlate well with the recently reported investigation of an Iranian OCA population: pathogenic *TYR* variants were identified in 19 of 30 patients (Ghodsinejad Kalahroudi *et al.*, 2014). In this study, six patients carried only one pathogenic TYR mutation, and no pathogenic mutation was identified in five patients (Ghodsinejad Kalahroudi *et al.*, 2014).

All of the pathogenic *TYR* mutations detected in the Hungarian OCA patients have previously been identified in OCA patients of different ethnicity. The p.Pro406Leu mutation was detected in Caucasians from Iran, the p.Arg217Gln mutation in Caucasians from USA, Canada and Northern-Europe, and the p.Arg402X in Caucasians from Lebanon (Hutton and Spritz, 2008; Simeonov *et al.*, 2013). The frequency of pathogenic mutations differs in different populations, and, therefore, these populations might vary in their genetic susceptibility to certain diseases. Based on our results and the results of previous studies, the identified pathogenic TYR mutations are not specific to the Hungarian population, as they have been detected worldwide in OCA patients (Hutton and Spritz, 2008; Simeonov *et al.*, 2013).

4.1.2. *OCA2* mutation was not detected in the investigated Hungarian OCA population

No pathogenic OCA2 mutation was identified in the investigated Hungarian individuals, although one patient carried the benign p.Arg305Trp polymorphism in heterozygous form. This variant has been associated with human eye color and might be an inherited biomarker of cutaneous cancer risk (Rebbeck et al., 2002; Jannot et al., 2005). This also suggest that the genetic screening of the TYR and SLC45A2 genes should preceed the genetic investigation of the OCA2 gene, since based on my results, the mutations of this gene is probably less frequent than the mutations of the TYR and SLC45A2 genes among Hungarian OCA patients.

4.1.3. Two novel mutations in the *SLC45A2* gene identified in two Hungarian OCA patients are associated with unusual OCA4 phenotype

Among the investigated OCA patients, there were two patients who carry two novel heterozygous pathogenic variants of the *SLC45A2* gene: the p.Gly411Asp missense and the p.Gln487X nonsense mutations. The OCA4 symptoms of the affected patients is highly possibly the consequence of the identified mutations of the *SLC45A2* gene. However, it is still uncovered, whether the concomitant diseases of Patient 2 (Crohn's disease for 9 years and hypothyreosis for 4 years) are related to the identified *SLC45A2* mutations. Regarding Crohn's disease, there is one previous study in the literature, which reports a Canadian patient affected by both OCA4 and Crohn's diseases (Fernandez *et al.,* 2012). High throughput genetic investigations of this Canadian patient identified two pathogenic homozygous mutations, one in the *SLC45A2* gene and another in the *G6PC3* gene encoding the third subunit of the glucose-6-phosphatase enzyme (Fernandez *et al.,* 2012). The authors concluded that the patient suffers from two distinct diseases: OCA4 and severe congenital neutropenia type 4 (SCN4). The Crohn's disease of the patient was concerned as a manifestation of the SCN4 (Fernandez *et al.,* 2012). In case of the investigated Hungarian OCA patients the mutation screening of the *SCN4* gene was not performed, since their clinical symptoms do not support this diagnosis. However, we hypothesize that the concomitant diseases (Crohn's disease and hypothyreosis) of Patient 2 are not related to the identified *SLC45A2* mutations.

Mutations of the SLC45A2 gene can either cause complete or partial loss of pigmentation and thus contribute to the development of several different OCA phenotypes (Simeonov *et al.,* 2013). However, with the comparison of the *SLC45A2* mutations and the patients' clinical symptoms, genotype–phenotype correlations have not yet been established in OCA4 (Simeonov *et al.,* 2013). Mutations of the *SLC45A2* gene typically associated with the so called "brown OCA" phenotype referring to partial loss of pigmentation in the affected patients (Kamaraj *et al.,* 2014). In contrast with this, here we report two Hungarian OCA patients with unusual OCA4 phenotypes, since the investigated patients have developed complete absence of pigmentation, which is more common in OCA1 caused by mutations in the *TYR* gene. To rule out any other putative genetic modifier variant, which might

be responsible for the identified unusual phenotype, the mutation screening of the *TYR* and the *OCA2* genes have also been performed, but mutation has not been identified, however both patients carry the p.Ser192Tyr common, non-pathogenic polymorphism of the *TYR* gene, which might have any phenotype modifying role if pathogenic *SLC45A2* mutations are present. Our report further contributes not only to the mutation spectrum of the *SLC45A2* gene but also to the spectrum of the observed unusual clinical symptoms and hopefully it will add novel data to future studies characterizing genotype-phenotype correlations in OCA4.

OCA has been considered for many years as a group of monogenic rare diseases without cure. However, in case of OCA4, accumulating knowledge regarding the underlying mechanism of the disease might alter this viewpoint. It has been recently demonstrated in MNT-1 cell lysates that the reduced tyrosinase activity, induced by the knockdown of the *SLC45A2* can be recovered by exogenously introduced copper treatment (Bin *et al.,* 2015).

In conclusion, two novel heterozygous mutations are detected, a missense and a nonsense one, in the *SLC45A2* gene in two Hungarian patients affected by OCA4. The location of the mutations and the evolutionary conservation of the missense one suggest their putative pathogenic role in the development of OCA4 in the investigated patients. Our study provides new insights to the genetic background of OCA4 and might serve as a basis for future studies aiming to develop novel therapeutic approaches to OCA patients.

4.1.4. European recurrent mutation of the *SLC45A2* gene identified in the investgated Hungarian OCA population

In addition to the pathogenic *SLC45A2* mutations (the p.Gly411Asp missense and the p.Gln487X nonsense mutations) described above, the p.Leu374Phe polymorphism was detected in nine Hungarian OCA patients. This latter variant has a striking population distribution and exists almost exclusively in Europeans (Graf *et al.,* 2005). Additionally, this variant has also been implicated in the development of different shades of hair, skin and eye color (Graf *et al.,* 2005).

4.2. Clinical relevance of the concomitant investigations of the *TYR, OCA2* and *SLC45A2* genes in the investigated Hungarian OCA population

My results emphasize the importance of the parallel investigation of multiple genes for studying disease phenotypes. The OCA cases presented in this study and many other cases reported in the literature call our attention to the fact that clinical symptoms — which may overlap in many cases — are not sufficient for a diagnosis of different OCA forms: the molecular genetic investigation of all OCA genes is required to determine the subtype of the disease. The Hungarian OCA patients in this study exhibited identical clinical features (Table II.); however, molecular genetic investigation identified the OCA1 subtype in nine cases and the OCA4 subtype in three cases (Table VII.). Even with this data, the genetic basis of the disease in seven patients carrying only one pathogenic *TYR* or *SLC45A2* mutation is still not completely explained. Screening of the non-pathogenic variants, which alone could not lead to the development of OCA, should be carried out in association with pathogenic variants that might have clinical significance. Further targeted sequencing of the genes involved in the syndromic OCA variants, including the Hermansky-Pudlak, Chediak-Higashi and Griscelli syndromes, as well as genes involved in human pigmentation, is hoped elucidate the underlying disease-causing variant(s) (Simeonov *et al.*, 2013).

Our results suggest that, among the investigated genes, the majority of the mutations are located within the *TYR* gene. This result correlates well with the results obtained with other populations, as *TYR* mutations are the most common for OCA worldwide (Martinez-Garcia *et al.*, 2013). Screening of the *TYR* gene is, therefore, of primary importance for diagnostics. Mutations in the investigated Hungarian OCA patients were found most frequently in exons 1 and 4 of the *TYR* gene. In light of the fact that the majority of the identified *TYR* mutations are located within exon 1 and 4 (Kalahroudi *et al.*, 2014), we recommend screening these exons first.

According to our current knowledge, 10–25% of the isolated and syndromic OCA cases are not explained by paired, trans-oriented mutations in known genes (Chaki *et al.*, 2006; Gronskov *et al.*, 2009; Wei *et al.*, 2010). Based on our results and the results of previous studies, we suggest that screening non-Mendelian OCA-associated genes might elucidate the causative genetic variant for these cases (Simeonov *et al.*, 2013). The genetic heterogeneity of OCA is extremely complex:

both rare mutations of Mendelian genes and common variants of non-Mendelian genes can contribute to the development of the disease. Our multi-gene study provides novel data for the genetic diversity of OCA in Hungarians and indicates that approaches that take this complexity into account, including large-scale studies, are needed to complete our understanding of the genetic heterogeneity of this disease.

5. SUMMARY

In my PhD dissertation, my aim was to elucidate the genetic background of a clinically and genetically heterogeneous group of genodermatoses, the OCA. OCA is characterized by decreased pigmentation or by complete loss of pigmentation in the skin, the hairs and the eyes. Here I have performed complete clinical and genetic workup and summarized the results of my investigations in OCA. Regarding its genetic heterogeneity, since all the patients were considered as having non-syndromic OCA variants, I have performed the concomitant analysis of multiple genes - *TYR, OCA2* and *SLC45A2* -, which are implicated in the majority of the isolated OCA variants. After the study got ethical approval, I have enrolled and investigated a Hungarian OCA population, which involved 12 patients. The number of the enrolled patients might seem to be small, but considering that OCA is a rare disease, the enrollment of 12 individuals with similar clinical phenotype was already enormous work.

During my investigations, I would like to highlight, that I have identified two novel *SLC45A2* mutations in two Hungarian OCA patients with unusual phenotype of OCA4 (Tóth *et al.,* 2017). These novel heterozygous mutations were the followings: a missense one (c.1226G/A p.Gly411Asp) and a nonsense one (c.1459C/T p.Gln437X). These mutations were present in both patients suggesting their compound heterozygous states. The identified novel mutations affect the transmembrane domains of the protein suggesting that they might impair its transport function leading to decreased melanosomal pH and decreased tyrosinase activity. My investigations provide new insights to the genetic background of OCA4 by reporting an unusual OCA4 phenotype and expanding the mutation spectrum of the *SLC45A2* gene.

Besides the identification of novel mutations, the other significant results of my study are the followings: Although, the clinical features of the investigated Hungarian OCA patients (n=12) were identical, the molecular genetic data suggested an OCA1 subtype in nine cases and an OCA4 subtype in three cases (Fábos *et al.,* 2017). In five patients, two different heterozygous pathogenic mutations were present, whereas seven patients had only one pathogenic mutation and associated non-pathogenic variants (Fábos *et al.,* 2017). Therefore, my results suggest that the concomitant screening of the non-pathogenic variants — which alone do not cause

the development of OCA, but might have clinical significance in association with a pathogenic variant — is important (Fábos *et al.*, 2017).

Based on the results of my scientific work, these investigations has promoted the understanding of the heterogeneity of OCA, assessed the independent and cumulative contributions of the *TYR, OCA2* and *SLC45A2* genes to the development of OCA, and compared relative and cumulative frequencies of the clinical variants of OCA in a representative Hungarian OCA population.

My results confirm that the concomitant analysis of OCA genes is critical, providing new insights to the phenotypic diversity of OCA and expanding the mutation spectrum of OCA genes in Hungarian patients.

6. ACKNOWLEDGEMENT

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7. ELECTRONIC DATABASE INFORMATION

Ensemble Genome Browser (for the wild type sequencing data of the human genome, for the gene variation database regarding disease-causing and non-causing alterations and for the taxonomy analysis of the identified mutation) www.ensemble.org

Online Mendelian Inheritance in Man (for the detailed informations on the genetics, inheritance, clinical features and identified mutations in monogenic disorders) www.omim.org

UCSC Genome Browser, Primer3 (for the design of specific primers used to amplify the sequenced regions of the genes) http://genome.ucsc.edu/; http://bioinfo.ut.ee/primer3-0.4.0/

PubMed (for the literature search to identify the previously published cases) http://www.ncbi.nlm.nih.gov/pubmed

UniProt, SMART (for the detailed information of protein sequences and functional information) www.uniprot.org; smart.embl-heidelberg.de/

MutationTaster (for the pathogenicity prediction of the identified genetic variants) www.mutationtaster.org/

SIFT Home (for the pathogenicity prediction of the identified genetic variants) sift.jcvi.org

PolyPhen-2: prediction of functional effects of human nsSNPs (for the pathogenicity prediction of the identified genetic variants) www.genetics.bwh.harvard.edu/pph2/

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9. APPENDIX

Abbreviations

C10ORF11 gene: chromosome 10 open reading frame 11 gene

GPR143 gene: G protein-coupled receptor 143 gene

MATP: membrane-associated transport protein

OCA: oculocutaneous albinism

OCA1: oculocutaneous albinism type 1

OCA2 gene: oculocutaneous albinism two gene

OCA2: oculocutaneous albinism type 2

OCA3: oculocutaneous albinism type 3

OCA4: oculocutaneous albinism type 4

OCA5: oculocutaneous albinism type 5

OCA6: oculocutaneous albinism type 6

OCA7: oculocutaneous albinism type 7

SLC24A5 gene: sodium/calcium/potassium exchanger 5 gene

SLC45A2 gene: solute carrier family 45, member 2 gene

TYR gene: tyrosinase gene

TYRL gene: tyrosinase-like gene

TYRP gene: tyrosinase-related protein gene

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Delineating the genetic heterogeneity of OCA in Hungarian patients

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Abstract

Background: Oculocutaneous albinism (OCA) is a clinically and genetically heterogenic group of pigmentation abnormalities characterized by variable hair, skin, and ocular hypopigmentation. Six known genes and a locus on human chromosome 4q24 have been implicated in the etiology of isolated OCA forms (OCA 1–7).

Methods: The most frequent OCA types among Caucasians are OCA1, OCA2, and OCA4. We aimed to investigate genes responsible for the development of these OCA forms in Hungarian OCA patients ($n = 13$). Mutation screening and polymorphism analysis were performed by direct sequencing on *TYR*, *OCA2*, *SLC45A2* genes.

Results: Although the clinical features of the investigated Hungarian OCA patients were identical, the molecular genetic data suggested OCA1 subtype in eight cases and OCA4 subtype in two cases. The molecular diagnosis was not clearly identifiable in three cases. In four patients, two different heterozygous known pathogenic or predicted to be pathogenic mutations were present. Seven patients had only one pathogenic mutation, which was associated with non-pathogenic variants in six cases. In two patients no pathogenic mutation was identified.

Conclusions: Our results suggest that the concomitant screening of the non-pathogenic variants—which alone do not cause the development of OCA, but might have clinical significance in association with a pathogenic variant—is important. Our results also show significant variation in the disease spectrum compared to other populations. These data also confirm that the concomitant analysis of OCA genes is critical, providing new insights to the phenotypic diversity of OCA and expanding the mutation spectrum of OCA genes in Hungarian patients.

Keywords: Oculocutaneous albinism, Concomitant analysis, *TYR* gene, *SLC45A2* gene, *OCA2* gene

Background

Oculocutaneous albinism (OCA) is a clinically and genetically heterogenic group of rare monogenic diseases characterized by diffuse reduced melanin production in the skin, hair, and/or eyes [1]. Eye symptoms including photophobia, nystagmus, strabismus, foveal hypoplasia, reduced iris, and retinal pigmentation and reduction in visual acuity are present in all types of albinism. To date, six genes and a locus on 4q24 human chromosomal region have been implicated in the development of the isolated OCA forms (OCA 1–7) [2]. Tyrosinase gene (*TYR*; OMIM 606933) is responsible for the development of OCA type 1 (OCA1) [3, 4]. Mutations of the oculocutaneous albinism two gene (*OCA2;* OMIM 611409) are associated with OCA type 2 (OCA2) [5]. Pathogenic variants of the tyrosinase-related protein gene (*TYRP*; OMIM 115501) are linked with OCA type 3 (OCA3) [6]. Mutations in a membrane-associated transporter gene (*SLC45A2*; OMIM 606202) are implicated in OCA type 4 (OCA4) [7]. OCA5 phenotype is linked to an unknown gene on human chromosome 4q24 [8]. Mutations of the sodium/calcium/potassium exchanger 5 gene (*SLC24A5*; OMIM 609802) encoding a solute carrier protein are associated with a new form of OCA, named as OCA6 [9]. The mutations of chromosome 10 open reading frame 11 gene (*C10ORF11*; OMIM 614537) are responsible for OCA7 type of albinism [10]. Furthermore, an additional

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10 genes have been associated with syndromic OCA variants such as Hermansky–Pudlak syndrome (HPS) and Chediak–Higashi syndrome (CHS) [2]. Noted, there is a form of albinism, ocular albinism (OA1), affecting the eyes, but does not affect the hair and skin, which is caused by the mutation in G protein-coupled receptor 143 gene (*GPR143*; OMIM 300808) [11].

Oculocutaneous albinism affects approximately one in 20,000 individuals worldwide; however, the prevalence of its subtypes varies among different populations [12]. Although OCA1, OCA2, and OCA4 are present in Caucasian populations, the most common form is OCA1 [6]. OCA3 is present in mainly Africans and is rarely seen in other populations [6], OCA5 has been found in one Pakistani family to date [8], OCA6 is recently discovered in one Chinese family [9] and OCA7 has been explored in several Faroese families (Denmark) [10].

Since the most frequent forms of OCA in Caucasian population are OCA1, OCA2, and OCA4, we performed mutation screening of the *TYR*, *OCA2*, and *SLC45A2* genes to promote the understanding of disease heterogeneity, to assess the independent and cumulative contributions of these three genes to the disease development, and to compare relative and cumulative frequencies of disease variants in a representative Hungarian OCA population.

Table 1 Clinical features of the Hungarian OCA patients

Patient	Gender	Age	Skin	Hair color	Iris color
1	Male	75	Hypopigmented, no tanning ability	Snow-white	Blue
2	Male	7	Hypopigmented, no tanning ability	Snow-white	Blue
3	Female	57	Hypopigmented, no tanning ability	Snow-white	Blue
4	Male	48	Hypopigmented, no tanning ability	Snow-white	Blue
5	Female	60	Hypopigmented, no tanning ability	Snow-white	Blue
6	Male	11	Hypopigmented, no tanning ability	Snow-white	Blue
7	Male	15	Hypopigmented, no tanning ability	Snow-white	Blue
8	Female	3	Hypopigmented, no tanning ability	Snow-white	Blue
9	Female	31	Hypopigmented, no tanning ability	Snow-white	Blue
10	Female	28	Hypopigmented, no tanning ability	Snow-white	Blue
11	Male	21	Hypopigmented, no tanning ability	Snow-white	Blue
12	Male	6	Hypopigmented, no tanning ability	Snow-white	Blue
13	Male	4	Hypopigmented, no tanning ability	Snow-white	Blue

Patients and methods

Examined individuals

The individuals ($n = 13$) participating in this study were recruited at the Mór Kaposi Teaching Hospital of the Somogy County (Kaposvár, Hungary), at the Hospital of Zala County (Zalaegerszeg, Hungary) and at the Department of Dermatology and Allergology, University of Szeged (Szeged, Hungary). In the enrolled patients, the diagnosis of OCA was established in the presence of skin and hair hypopigmentation and distinctive ocular changes such as nystagmus, reduced iris pigmentation, reduced retinal pigmentation, and foveal hypoplasia (Table 1). All investigated individuals were Hungarians.

The investigation was approved by the Internal Ethical Review Board of the University of Szeged. Written informed consent was obtained from the patients and the healthy controls, and the study was conducted according to the Principles of the Declaration of Helsinki.

Genetic investigation

Blood was drawn from the enrolled individuals, and genomic DNA was isolated using a BioRobot EZ1 DSP Workstation (QIAGEN; Godollo, Hungary). The entire coding regions and the flanking introns of the *TYR*, *OCA2*, and *SLC45A2* genes were amplified (primer sequences used were taken from the UCSC Genome Browser). Since a pseudogene of *TYR*, tyrosinase-like gene (*TYRL*; OMIM 191270) is known, which shows 98.55% identity with the 3′region of *TYR* (exon 4 and 5), specific primers were used for amplification of these regions [13]. Direct sequencing of PCR products was performed on an ABI 3100 sequencer and compared with the wild-type gene sequences using the Ensemble Genome Browser.

Pathogenicity predictions

As in previous study [14], in silico tools were applied to identify the functional impact of the newly detected missense mutations. Here we used SIFT (Sorting Intolerant from Tolerant, PolyPhen 2.0 (Polymorphism Phenotyping), Mutation Taster, PROVEAN (Protein Variation Effect Analyzer) and PANTHER (Protein ANalysis THrough Evolutionary Relationships) tools. SIFT is based on the evolutionary conservation and predicts whether an amino acid substitution affects protein function. SIFT prediction score ranges from 0 to 1, and the amino acid substitution is predicted damaging if the score is less than an equal to 0.05, and tolerated if the score is greater than 0.05 [15]. PolyPhen 2.0 is based on structural and comparative evolutionary considerations and predicts the possible impact of an amino acid substitution on the

stability and function of a protein. PolyPhen 2.0 uses the same range than SIFT (0–1) and the substitution is predicted to be possibly/probably damaging at greater than an equal to 0.5 value [16]. Mutation Taster is a prediction software based on the physicochemical properties of amino acids and scores substitutions according to the degree of difference between the original and the new amino acid (0–215) [17]. PROVEAN prediction is based on the sequence homology. If the PROVEAN score is equal to or below the default score threshold (−2.5), the protein variant is predicted to be deleterious and if the score is above the threshold, the variant is predicted to be neutral [18]. PANTHER program is a library of protein family and subfamily, which predicts the occurrence frequency of an amino acid in evolutionary conserved protein sequences. If the score is −3 or less, the variant is predicted has deleterious effect [19].

Results

During the investigation of *TYR*, *OCA2*, and *SLC45A2* genes, we have identified pathogenic mutations in 84% ($n = 11$) of the examined individuals ($n = 13$), as shown in Table 2. In 4 cases, two heterozygous mutations have been found, suggesting a compound heterozygous state. Seven patients carried only one disease-causing mutation. Furthermore, in 11 cases out of 13, we have detected one or more common polymorphisms.

Direct sequencing of the *TYR* gene revealed pathogenic mutations in 69% ($n = 9$) of the investigated patients. Only one patient carried two heterozygous mutations, a Thymin-base duplication (c.74dupT, p.Ser26Leufs*2) and a nonsense (c.346C>T, p.Arg116*) mutation in the first exon of *TYR* gene, suggesting a compound heterozygous state. Three patients carried the c.1037−7T>A splice site mutation in homozygous form. Out of these three patients, two (Patient 2 and 3) are related to each other and one (Patient 4) is not aware of any relationship with the other two mutation carriers. We have detected the c.1204C>T p.Arg402* nonsense mutation heterozygously in one case and the c.650G>A p.Arg217Gln missense mutation heterozygously in three patients. The heterozygous c.1366+4A>G splice site mutation has been detected in one patient. This patient was additionally heterozygous for the *SLC45A2* c.1099G>A p.Val367Ile mutation. Considering the common polymorphisms of *TYR* gene, the c.575C>A p.Ser192Tyr, and c.1205G>A p.Arg402Gln were detected in seven patients. The p.Ser192Tyr variant affects a copper-binding domain of the protein; all the other exonic pathogenic and non-pathogenic variants are located outside of the known functional domains of the enzyme (Fig. 1a).

No pathogenic *OCA2* mutation was identified in the investigated OCA individuals. However, one patient with the pathogenic c.1037−7T>A *TYR* mutation carried the common c.913C>T p.Arg305Trp polymorphism of the *OCA2* gene in heterozygous form. This variant does not affect any known functional domains of the OCA2 protein (Fig. 1b).

Based on our results, 3 of 13 patients carried *SLC45A2* pathogenic mutation. Two patients carried the combination of two novel mutation previously described by our workgroup: the c.1226G>A p.Gly409Asp missense mutation and the c.1459C>T p.Gln487* nonsense mutation. These mutations are not present in any SNP database (ExAC, 1000 Genome Project, ClinVar). Prediction analyses were performed to identify the functional role of the missense mutation. All prediction software suggested that the p.Gly409Asp mutation is deleterious (SIFT score: 0.002, damaging; PolyPhen 2.0 score: 0.996, probably damaging; Mutation Taster score: 94, disease causing; PROVEAN score: −3.25, deleterious; PANTHER score: −4.26, deleterious). The nonsense mutation was deemed to be pathogenic. One patient carried the c.1099G>A p.Val367Ile missense mutation beside the c.1366+4A>G splice site mutation on the *TYR* gene. In eight patients, only the non-pathogenic c.1122G>C p.Leu374Phe missense polymorphism was detected. All the detected pathogenic and non-pathogenic variants are located within transmembrane domains of the encoded protein (Fig. 1c).

Discussion

This study reports the concomitant investigation of three genes (*TYR*, *OCA2*, and *SLC45A2*) in 13 Hungarian OCA patients, which have been implicated in the development of isolated OCA forms. The *TYR* gene encodes the tyrosinase enzyme, which catalyzes the first and second steps in melanin synthesis: the hydroxylation of tyrosine to L-DOPA and the oxidation of L-DOPA to DOPA-quinone [3]. The *OCA2* and *SLC45A2* genes encode transporter proteins, which are implicated in the trafficking of tyrosinase to melanosomes [20, 21].

Pathogenic *TYR* mutations were present in 69% ($n = 9$) of the patients. However, the sample size of this study is small, our results correlate well with previous findings that OCA1 is the most common isolated OCA subtype and *TYR* mutations account for approximately 25–50% of the isolated OCA cases worldwide [6, 22].

In our study, three missense variants were detected for the *TYR* gene. One of these variants is considered pathogenic (p.Arg217Gln) and two (p.Ser192Tyr, p.Arg402Gln) are considered common polymorphisms. The p.Arg217Gln mutation is located in a non-conservative region of tyrosinase protein, at this amino acid position two other known missense mutations are described (p.Arg217Gly, p.Arg217Trp) [23]. The polymorphisms of *TYR* gene were not directly related to

Table 2 Detected *TYR*, *SLC45A2*, and *OCA2* mutations and polymorphisms

Patient	Mutation 1	Mutation 2	Polymorphisms	Molecular diagnosis
1	*TYR* gene: c.74dupT p.Ser26Leufs*2 (hetero)	*TYR* gene: c.346C>T p.Arg116* (hetero)	–	OCA1
2	*TYR* gene: c.1037−7T>A (homo)	–	*SLC45A2* gene: c.1122G>C p.Leu374Phe (homo) Global MAF: 0.2750 Caucasian MAF: 0.0616 *OCA2* gene: c.913C>T p.Arg305Trp (hetero) Global MAF: 0.0790 Caucasian MAF: 0.0650	OCA1
3	*TYR* gene: c.1037−7T>A (homo)	–	*SLC45A2* gene: c.1122G>C p.Leu374Phe (homo) Global MAF: 0.2750 Caucasian MAF: 0.0616	OCA1
4	*TYR* gene: c.1037−7T>A (homo)	–	*SLC45A2* gene: c.1122G>C p.Leu374Phe (homo) Global MAF: 0.2750 Caucasian MAF: 0.0616	OCA1
5	*TYR* gene: c.1204C>T p.Arg402* (hetero)	–	*TYR* gene: c.575C>A p.Ser192Tyr (homo) Global MAF: 0.1234 Caucasian MAF: 0.3718 *SLC45A2* gene: c.1122G>C p.Leu374Phe (homo) Global MAF: 0.2750 Caucasian MAF: 0.0616	OCA1
6	*TYR* gene: c.650G>A p.Arg217Gln (hetero)	–	*TYR* gene: c.1205G>A p.Arg402Gln (hetero) Global MAF: 0.0813 Caucasian MAF: 0.2525 *SLC45A2* gene: c.1122G>C p.Leu374Phe (homo) Global MAF: 0.2750 Caucasian MAF: 0.0616	OCA1
7	*TYR* gene: c.650G>A p.Arg217Gln (hetero)	–	*TYR* gene: c.1205G>A p.Arg402Gln (hetero) Global MAF: 0.0813 Caucasian MAF: 0.2525 *SLC45A2* gene: c.1122G>C p.Leu374Phe (homo) Global MAF: 0.2750 Caucasian MAF: 0.0616	OCA1
8	*TYR* gene: c.650G>A p.Arg217Gln (hetero)	–	–	OCA1
9	*SLC45A2* gene: c.1226G>A p.Gly409Asp (hetero)	*SLC45A2* gene: c.1459C>T p.Gln487* (hetero)	*TYR* gene: c.575C>A p.Ser192Tyr (homo) Global MAF: 0.1234 Caucasian MAF: 0.3718 *TYR* gene: c.1205G>A p.Arg402Gln (hetero) Global MAF: 0.0813 Caucasian MAF: 0.2525	OCA4
10	*SLC45A2* gene: c.1226G>A p.Gly409Asp (hetero)	*SLC45A2* gene: c.1459C>T p.Gln487* (hetero)	*TYR* gene: c.575C>A p.Ser192Tyr (hetero) Global MAF: 0.1234 Caucasian MAF: 0.3718	OCA4
11	TYR gene: c.1366+4A>G (hetero)	*SLC45A2* gene: c.1099G>A p.Val367Ile (hetero)	*TYR* gene: c.575C>A p.Ser192Tyr (hetero) Global MAF: 0.1234 Caucasian MAF: 0.3718 *SLC45A2* gene: c.1122G>C p.Leu374Phe (homo) Global MAF: 0.2750 Caucasian MAF: 0.0616	OCA1/ OCA4
12	–	–	*TYR* gene: c.575C>A p.Ser192Tyr (homo) Global MAF: 0.1234 Caucasian MAF: 0.3718 *TYR* gene: c.1205G>A p.Arg402Gln (hetero) Global MAF: 0.0813 Caucasian MAF: 0.2525 *SLC45A2* gene: c.1122G>C p.Leu374Phe (homo) Global MAF: 0.2750 Caucasian MAF: 0.0616	Unknown
13	–	–	*SLC45A2* gene: c.1122G>C p.Leu374Phe (homo) Global MAF: 0.2750 Caucasian MAF: 0.0616	Unknown

MAF minor allele frequency

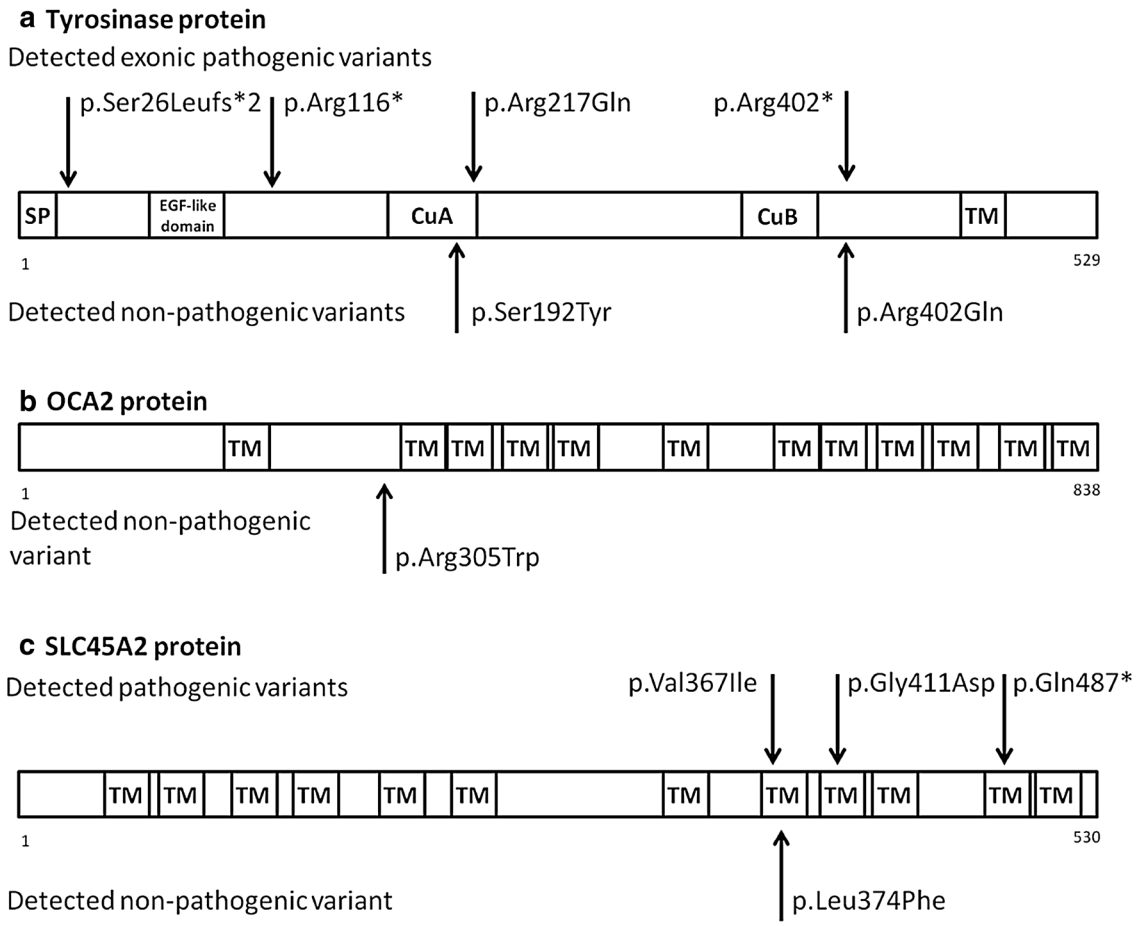

Fig. 1 **a** Distribution of the detected *TYR* variants on the tyrosinase protein, **b** *OCA2* variant on the transporter protein, and **c** *SLC45A2* variants on the transporter protein (*SP* signal peptide, *EGF-like domain* epidermal-growth-factor-like domain, *CuA* copper-binding domain, *CuB* copper-binding domain, *TM* transmembrane domain)

pigmentation phenotypes in normal Caucasians, but their impact should be taken into account as an important modifier of human skin, hair, and eye color [24]. Functional studies reported that 192Tyr allele reduced tyrosinase activity, significant reduction in heterozygous and consistent decrease in homozygous form were observed, and the presence of 402Gln allele resulted significantly less TYR protein, displayed altered trafficking and glycosylation, with reduced DOPA oxidase [24]. The p.Arg402Gln polymorphism exhibits reduced tyrosinase activity at physiological temperature and is considered a temperature-sensitive variant [25–27]. This variant alone is unable to cause OCA; however, its increased frequency in OCA patients with one heterozygous pathogenic *TYR* mutation suggests that it can contribute to the development of OCA in combination with a pathogenic mutation [28]. Two Hungarian patients carried the p.Arg402Gln polymorphism in combination with the p.Arg217Gln pathogenic variant. Previous report suggests this combination might contribute to the development of the OCA symptoms of the patient [26].

In one case, compound heterozygosity for two mutations was found. A heterozygous T-base duplication (c.74dupT p.Ser26Leufs*2) and a nonsense mutation (c.346C>T, p.Arg116*) were identified on *TYR* gene. Both mutations lead to the development of a premature termination codon. Due to these changes, the translated mutant TYR protein is highly truncated and we assume that these enormous truncations of the mutant TYR protein may lead to its dysfunction.

Two of the nine Hungarian OCA patients with *TYR* mutations carry a combination of two pathogenic mutations. In three of nine, a splice site mutation was identified in homozygous form. In four of nine, only one heterozygous pathogenic mutation was identified. These results correlate well with the recently reported investigation of an Iranian OCA population: pathogenic *TYR*

variants were identified in 19 of 30 patients, and in this study, six patients carried only one pathogenic *TYR* mutation, and any pathogenic mutation were not identified in five patients [4].

The pathogenic *TYR* mutations detected in the Hungarian OCA patients have been previously identified in OCA patients of different ethnicity. The p.Arg217Gln mutation was detected in Caucasians from USA, Canada, and Northern-Europe; the p.Arg402* in Caucasians from Lebanon; the c.1037−7T>A in Caucasians and Japanese; the c.1366+4A>G in Caucasians; and the c.346C>T in Caucasians, Japanese, and Germans [22, 29]. The frequency of pathogenic mutations differs in different populations, and, therefore, these populations might vary in their genetic susceptibility to certain diseases. Based on our results and the results of previous studies, the identified pathogenic *TYR* mutations are not specific to the Hungarian population, as they have been detected worldwide in OCA patients [22, 29].

No pathogenic *OCA2* mutation was identified in the examined Hungarian individuals, although one patient carried the common p.Arg305Trp polymorphism in heterozygous form. This variant has been associated with human eye color and might be an inherited biomarker of cutaneous cancer risk [28, 30].

Three mutation and a common polymorphisms were detected on the *SLC45A2* gene. Two patients carried two heterozygous variants of the *SLC45A2* gene previously described by our workgroup: the p.Gly409Asp missense and the p.Gln487* nonsense mutations [31]. Both mutations are situated in transmembrane domains of the MATP protein (Uniprot: Q9UMX9). The locations of the mutations suggest that they impair the transport function of the MATP protein. MATP dysfunction might cause an acidic melanosomal lumen, leading to incorrect incorporation of copper into tyrosinase. The reduced tyrosinase activity could lead to the development of the OCA phenotype [32]. Besides, the p.Leu374Phe polymorphism was detected in nine Hungarian OCA patients. This variant has a striking population distribution, exists almost exclusively in Europeans, and has also been implicated in the development of different shades of hair, skin, and eye color [33].

Our results emphasize the importance of the parallel analysis of multiple genes for studying disease phenotypes. The OCA cases presented in this study and many other cases reported in the literature call our attention to the fact that clinical symptoms—which may overlap in many cases—are not sufficient for a diagnosis of different OCA forms: the molecular genetic investigation of all OCA genes is required to determine the subtype of the disease. The Hungarian OCA patients in this study exhibited identical clinical features (Table 1); however, molecular genetic investigation identified the OCA1 subtype in eight cases, the OCA4 subtype in two cases, and the molecular diagnosis was not clearly defined in three patients (Table 2). Even with these data, the genetic basis of the disease in seven patients carrying only one pathogenic *TYR* or *SLC45A2* mutation is still not completely explained. We wish to emphasize the screening of the non-pathogenic variants, which alone could not lead to the development of OCA, should be carried out in association with pathogenic variants that might have clinical significance. Further targeted sequencing of the genes involved in the syndromic OCA variants, including HPS and CHS, as well as genes involved in human pigmentation, is hoped elucidate the underlying disease-causing variant(s) [22].

Our results and previously reported studies suggest that, among the investigated genes, the majority of the mutations are located within the *TYR* gene [3, 4]. This result correlates well with the results obtained in other populations, as *TYR* mutations are the most common for OCA worldwide [1]. Screening of the *TYR* gene is, therefore, of primary importance for diagnostics. Mutations in the investigated Hungarian and in other previously reported OCA patients were found most frequently in exons 1 and 4 of the *TYR* gene [4]. In light of the fact that the majority of the identified *TYR* mutations are located within exon 1 and 4, we recommend screening these exons first.

According to our current knowledge, 10–25% of the isolated and syndromic OCA cases are not explained by paired, trans-oriented mutations in known genes [34, 35]. Based on our results and the results of previous studies [22], we suggest that screening non-Mendelian OCA-associated genes might elucidate the causative genetic variant for these cases.

Conclusions

The genetic heterogeneity of OCA is extremely complex: both rare mutations of Mendelian genes and common variants of non-Mendelian genes can contribute to the development of the disease. Our multi-gene study provides novel data for the genetic diversity of OCA in Hungarians and indicates that approaches that take this complexity into account, including large-scale studies, are needed to complete our understanding of the genetic heterogeneity of this disease.

Abbreviations

OCA: oculocutaneous albinism; OCA1: oculocutaneous albinism type 1; OCA2: oculocutaneous albinism type 2; OCA3: oculocutaneous albinism type 3; OCA4: oculocutaneous albinism type 4; OCA5: oculocutaneous albinism type 5; OCA6: oculocutaneous albinism type 6; OCA7: oculocutaneous albinism type 7; *TYR*: tyrosinase gene; *OCA2*: oculocutaneous albinism two gene; *TYRP*: tyrosinase-related protein gene; *SLC45A2*: solute carrier family 45, member 2 gene; *SLC24A5*: sodium/calcium/potassium exchanger 5 gene; *C10ORF11*: chromosome 10 open reading frame 11 gene; HPS: Hermansky–Pudlak syndrome; CHS: Chediak–Higashi syndrome; OA1: ocular albinism; *GPR143*:

G protein-coupled receptor 143 gene; *TYRL*: tyrosinase-like gene; MATP: membrane-associated transport protein.

Authors' contributions
BF and KF performed the mutation analysis and wrote the manuscript. LT, AS, and KT participated in the mutation analysis. BF, MT, RN, KV, and ZC cared for patients. LK and MS were mentors who guided the research study. NN designed the study and helped in drafting the manuscript. All authors read and approved the final manuscript.

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Competing interests
The authors declare that they have no competing interests.

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All available data are included within the article.

Consent for publication
All the reported patients gave their informed consent to the publication.

Ethics approval and consent to participate
The investigation was approved by the Internal Review Board of the University of Szeged. Written informed consent was obtained from the patients and unrelated healthy individuals. The study was conducted according to the Principles of the Declaration of Helsinki.

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RESEARCH ARTICLE Open Access

Identification of two novel mutations in the *SLC45A2* gene in a Hungarian pedigree affected by unusual OCA type 4

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Abstract

Background: Oculocutaneous albinism (OCA) is a clinically and genetically heterogenic group of pigmentation abnormalities. OCA type IV (OCA4, OMIM 606574) develops due to homozygous or compound heterozygous mutations in the *solute carrier family 45, member 2* (*SLC45A2*) gene. This gene encodes a membrane-associated transport protein, which regulates tyrosinase activity and, thus, melanin content by changing melanosomal pH and disrupting the incorporation of copper into tyrosinase.

Methods: Here we report two Hungarian siblings affected by an unusual OCA4 phenotype. After genomic DNA was isolated from peripheral blood of the patients, the coding regions of the *SLC45A2* gene were sequenced. In silico tools were applied to identify the functional impact of the newly detected mutations.

Results: Direct sequencing of the *SLC45A2* gene revealed two novel, heterozygous mutations, one missense (c.1226G > A, p.Gly409Asp) and one nonsense (c.1459C > T, p.Gln437*), which were present in both patients, suggesting the mutations were compound heterozygous. In silico tools suggest that these variations are disease causing mutations.

Conclusions: The newly identified mutations may affect the transmembrane domains of the protein, and could impair transport function, resulting in decreases in both melanosomal pH and tyrosinase activity. Our study provides expands on the mutation spectrum of the *SLC45A2* gene and the genetic background of OCA4.

Keywords: Oculocutaneous albinism type 4, Unusual phenotype, *SLC45A2* gene, Compound heterozygous state, Novel mutations

Background

Oculocutaneous albinism (OCA) is a clinically and genetically heterogenic group of rare monogenic diseases characterized by reduced melanin production in the skin, hair and/or eyes [1]. OCA symptoms can include poor visual acuity, nystagmus, iris transillumination, strabismus, photophobia, foveal hypoplasia and misrouting of optic nerve fibers at the chiasm [2]. All OCA forms exhibit autosomal recessive inheritance [1].

OCA type 4 (OCA4, OMIM 606574) is a rare form of OCA caused by mutations in the *solute carrier family 45, member 2* (*SLC45A2*) gene on chromosome 5p13 [3]. The *SLC45A2* gene encodes a membrane-associated transport protein (MATP), which is located in melanosomes and shows high sequence and structural similarity to *Drosophila melanogaster* and plant sucrose transporters containing an RXGRR motif [4, 5]. *SLC45A2* knockdown reduced melanin content and tyrosinase activity by acidifying the pH of melanosomes in a human melanoma cell line, MNT-1 [6]. It has been suggested that, as a proton/sugar symporter, MATP transports sugars from the melanosomes to the cytoplasm using a proton gradient generated by a proton pump. Thus, normal protein function ensures elevated melanosomal pH, allowing proper binding of copper to tyrosinase and resulting in normal tyrosinase activity [6].

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To date, 78 of the mutations identified in the *SLC45A2* gene are related to OCA4 [7]. In this study, we report a Hungarian family with two members affected by OCA4. Our genetic investigation identified that these members carried two novel heterozygous mutations in a compound heterozygous state, expanding the mutational spectrum of OCA4.

Methods

Patients

A Hungarian family with two affected siblings was investigated (Fig. 1). The affected individuals were 30 (Patient II/1) and 27 years (Patient II/2) old at the time of investigation. Both exhibited pale skin, complete absence of hair pigment, pink nevi and blue eyes with nystagmus. This complete absence of pigmentation is unusual for OCA4. Patient II/1 has been suffering from Crohn's disease for 9 years and hypothyreosis for 4 years. Patient II/2 was not aware of any known concomitant diseases. The parents (I/1 and I/2) of the affected siblings are clinically unaffected by OCA4. The investigated patients declined publication of their clinical pictures.

Genetic investigation

Blood was taken from the affected patients as well as from unrelated, healthy Hungarian individuals without pigmentation abnormality ($n = 30$), and genomic DNA was isolated using a BioRobot EZ1 DSP Workstation (QIAGEN; Godollo, Hungary). The entire coding region of the *SLC45A2* gene and the flanking introns were amplified and sequenced (primer sequences used were taken from the UCSC Genome Browser www.genome.ucsc.edu). The investigation was approved by the Internal Review Board of the University of Szeged. Written informed consent was obtained from the patient and the study was conducted according to the Principles of the Declaration of Helsinki. After identifying the causative mutations in the patients, further genetic screening of the parents was declined.

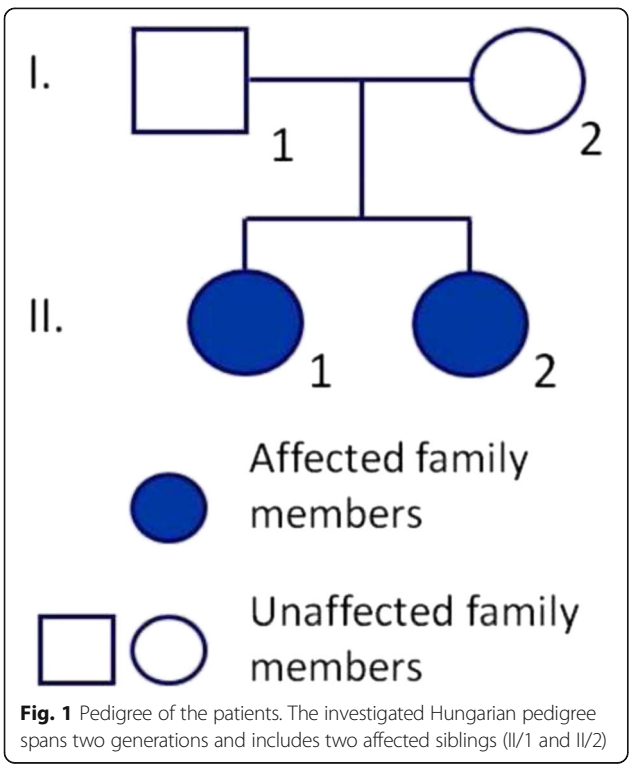

Fig. 1 Pedigree of the patients. The investigated Hungarian pedigree spans two generations and includes two affected siblings (II/1 and II/2)

Pathogenicity predictions for missense variants

In silico tools were applied to identify the functional role of the newly found variants. Here we used SIFT (Sorting Intolerant from Tolerant, http://sift.jcvi.org/), PolyPhen2 (Polymorphism Phenotyping, http://genetics.bwh.harvard.edu/pph2), Mutation Taster (http://www.mutationtaster.org/), PredictSNP (http://loschmidt.chemi.muni.cz/predictsnp/) PROVEAN (Protein Variation Effect Analyzer, http://provean.jcvi.org/index.php) and PANTHER (Protein ANalysis THrough Evolutionary Relationships, http://www.pantherdb.org/) tools.

Results

Direct sequencing of the coding regions and the flanking introns of the *SLC45A2* gene revealed two heterozygous mutations, one missense mutation (c.1226G > A, p.Gly409Asp) in the sixth exon (Fig. 2a) and one nonsense mutation (c.1459C > T, p.Gln437*) in the seventh exon (Fig. 2b). Both patients carried both mutations, suggesting a compound heterozygous state. Unrelated healthy controls carried the wild type sequence. To decide, whether the detected missense mutation is pathogenic, we use in silico analysis tools (SIFT, PolyPhen2, Mutation Taster, PredictSNP, PROVEAN and PANTHER). All prediction tools suggested that the p.Gly409Asp mutation is deleterious. The nonsense mutation was deemed to be pathogenic. It causes the development of a premature termination codon at 487 amino acid position thereby the MATP protein truncated and it may lead to its dysfunction.

Discussion

Both mutations are situated in transmembrane domains of the MATP protein (Uniprot: Q9UMX9): the p.Gly409Asp missense mutation is located within the ninth domain and the p.Gln437* nonsense mutation within the tenth (Fig. 2c). The locations of the mutations suggest that they impair the transport function of the MATP protein. MATP dysfunction might cause an acidic melanosomal lumen, leading to improper incorporation of copper into tyrosinase. The reduced tyrosinase activity could, in turn, lead to the development of the OCA phenotype [6]. The p.Gly409Asp missense mutation affects an evolutionary conserved region of the MATP protein (Fig. 2d), further emphasizing the

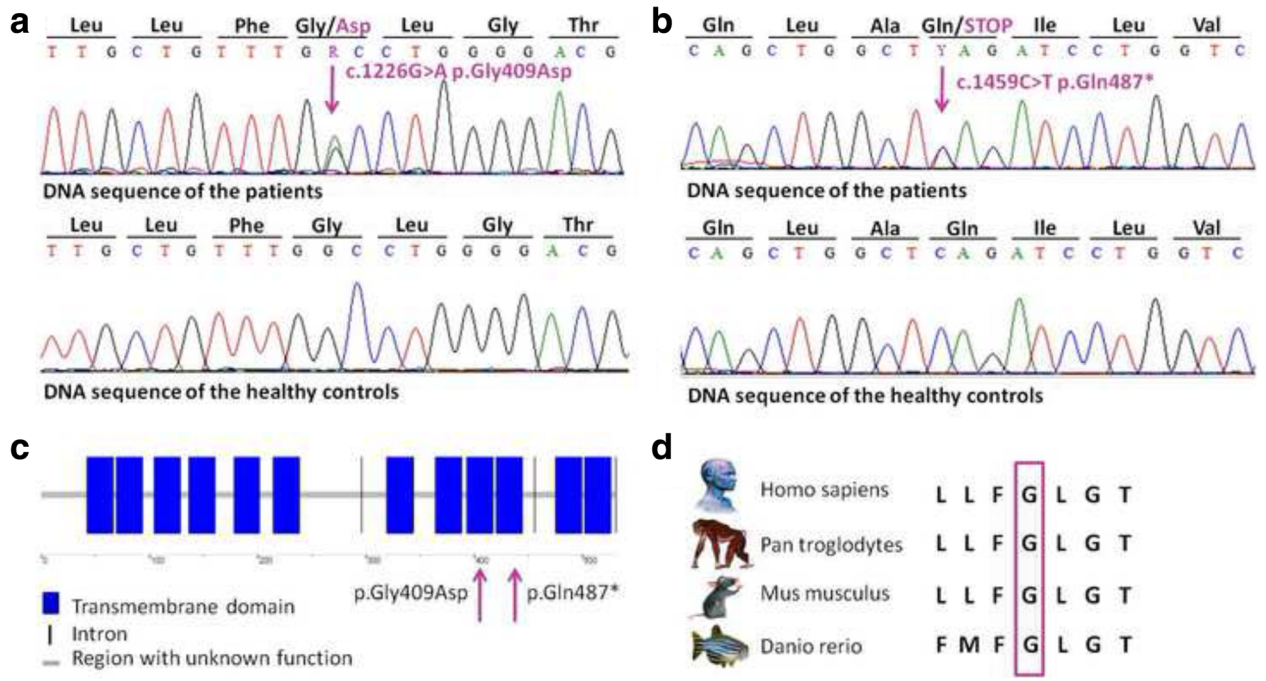

Fig. 2 Identification of two novel mutations of the *SLC45A2* gene. **a** Direct sequencing revealed a heterozygous missense mutation (c.1226G > A, p.Gly409Asp) in the sixth exon and **b** a heterozygous nonsense mutation (c.1459C > T, p.Gln437*) in the seventh exon of the gene. Both mutations were present in both affected patients. Unrelated controls ($n = 30$) carried the wild type sequence. **c** The identified mutations are located within the transmembrane domains of the MATP protein. **d** The identified missense mutation is situated within an evolutionary conserved region

putative pathogenic role of this mutation in the development of the observed pigmentation abnormalities of the affected patients.

Mutations of the *SLC45A2* gene have been reported to cause complete or partial loss of pigmentation, thus contributing to the development of several different OCA phenotypes. However, a genotype–phenotype correlation based the *SLC45A2* mutations and the patients' clinical symptoms has not yet been established for OCA4 [8]. Mutations of the *SLC45A2* gene are typically associated with partial loss of pigmentation, referred to as the "brown OCA" phenotype [7]. The two siblings reported here exhibited an unusual OCA4 phenotype, as they developed the complete absence of pigmentation. This phenotype is more common in type 1 OCA, which is caused by mutations in the *tyrosinase (TYR)* gene. To rule out the influence of other putative genetic-modifier variants responsible for the unusual phenotype, the mutation screening of the *TYR* and *OCA2* genes was also performed. Common polymorphisms of *TYR* gene were detected, neither pathogenic nor non-pathogenic variants of the *OCA2* gene were identified. Patient II/1 carried the p.Ser192Tyr variant homozigously and the p.Arg402Gln variant heterozigously. Heterozygous p.Ser192Tyr polymorphism was identified in Patient II/2. These two common variants of *TYR* gene occur at high frequency (p.Ser192Tyr: Global MAF: 0.1234, Caucasian MAF: 0.3718; p.Arg402Gln: Global MAF: 0.0813, Caucasian MAF: 0.2525) but were not directly related to pigmentation phenotypes in normal Caucasians [9]. However, functional studies reported that 192Tyr and 402Gln alleles have reduced TYR enzyme activity. Heterozygous p.Ser192Tyr and p.Arg402Gln variants caused significant reduction in TYR expression, and a consistent decrease in TYR protein levels was observed in homozygous p.Ser192-Tyr cells [9].

Since one of our investigated OCA4 patients (II/1) is also affected by Chron's disease, it is possible that the mutations of the *SLC45A2* gene could be susceptibility factors for the development of Chron's disease. This hypothesis is further supported by the literature, since a previous study reporting a sister and a brother affected by congenital neutropenia and oculocutaneous albinism identified a nonsense mutation in the *G6PC3* gene (c.829C > T, p.Gln277*) responsible for the development of congenital neutropenia and frameshift mutation in the *SLC45A2* gene (c.986delC, p.T329Rfs*68), which could explain the OCA phenotype [10]. In this previous study, the investigated brother is also affected by Chron's disease, suggesting a putative association between the mutations of the *SLC45A2* gene and Chron's disease [10].

OCA has been considered for many years as a group of monogenic rare diseases without cure. Accumulating knowledge regarding the underlying mechanism of the OCA4 might alter this viewpoint: it has been recently

demonstrated in MNT-1 cell lysates that exogenously applied copper recovers reduced tyrosinase activity resulting from *SLC45A2* knockdown [6].

Conclusions

In conclusion, we report two novel heterozygous mutations, one missense and one nonsense, of the *SLC45A2* gene in two Hungarian sisters affected by OCA4. The prediction analysis and the location of the mutations as well as the evolutionary conservation of the missense mutation suggest a pathogenic role in the development of OCA4. Our report, which further contributes to the mutation spectrum of the *SLC45A2* gene as well as to the spectrum of the observed unusual clinical symptoms, will hopefully contribute to future studies characterizing genotype-phenotype correlations in OCA4. This study provides expands to the genetic background of OCA4 and might serve as a basis for future studies aiming to develop novel therapeutic approaches for OCA patients.

Abbreviations

G6PC3: Glucose 6 phosphatase, catalytic, 3 gene; MATP: Membrane-associated transport protein; MNT-1: Human melanoma cell line; OCA: Oculocutaneous albinism; *OCA2*: Oculocutaneous albinism II gene; OCA4: Oculocutaneous albinism type IV; RXGRR: A conserved amino acid motif; SCN4: Severe congenital neutropenia type 4; *SCN4*: Sodium channel, voltage gated, type IVgene; *SLC45A2*: Solute carrier family 45, member 2 gene; *TYR*: Tyrosinase gene

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Availability of data and materials

All of the data are included in the manuscript.

Authors' contributions

LT carried out the mutation analysis and the first draft of the manuscript. BF cared for patients, carried out the mutation analysis and the first draft of the manuscript. KF, AS and KT participated in the mutation analysis. MS and NN were mentors who designed and guided the research study. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

Consent for publication

All the reported patients gave their informed consent to the publication.

Ethics approval and consent to participate

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