# IDENTIFICATION OF

# PHENOTYPE MODIFIER GENETIC FACTORS

# IN MONOGENIC DISEASES

Ph.D. Thesis

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## LIST OF PUBLICATIONS

### Publications providing the basis of the dissertation

**1. Pap ÉM**, Farkas K, Tóth L, Fábos B, Széll M, Németh G, Nagy N.: Identification of putative genetic modifying factors that influence the development of Papillon-Lefévre or Haim-Munk syndrome phenotypes. **Clin Exp Dermatol**. **2020** Jul;45(5):555-559. **IF: 1.977**

**2. Pap ÉM**, Farkas K, Széll M, Németh G, Rajan N, Nagy N.: Identification of putative phenotype-modifying genetic factors associated with phenotypic diversity in Brooke-Spiegler syndrome. **Exp Dermatol**. **2020**.07.26. Accepted for publication. **IF: 3.368**

**3. Pap ÉM,** Széll M, Németh G, Nagy N: Fenotípus módosító genetikai faktorok azonosítása ritka monogénes betegségekben. **Orv Hetil.** Under review.

# 1. INTRODUCTION

## 1.1. Monogenic diseases

Monogenic diseases result from modifications in a single gene occurring in all cells of the body. Though they are relatively rare, they can affect millions of patients worldwide. Scientists currently estimate that over 10,000 of human diseases are known to be monogenic.

Monogenic diseases can vary greatly. They can cause mild, severe or very severe symptoms and can be associated with phenotypic diversity like common diseases. Their symptoms can impair the life quality of the patient significantly and they can also result in stigmatization and difficulties in socialization (Kelsall *et al*., 2013).

Due to the Human Genome Project (HGP) and to the development of next-generation sequencing technologies, nowadays we know more and more about monogenic diseases. In the last 30 years, many disease causing genes and mutations have been identified and the genetic background of many monogenic diseases have been elucidated. However it can occur that sequencing is unable to answer clinically relevant questions regarding phenotypic diversity and/or disease prognosis (Jarinova *et al.,* 2012; Timmerman *et al.,* 2014; Kiritsi *et al.,* 2015; Smith *et al.,* 2019). This limitation has been overcome in my thesis, since with the identification of putative phenotype modifier genetic factors these questions can be answered and the clinical associations can be explained.

In my thesis, I have summarized the results of my genetic investigations in monogenic diseases: the clinical variants of the cylindromatosis gene (*CYLD*) mutation-caused disease spectrum such as multiple familial trichoepithelioma type 1 (MFT1), familial cylindromatosis (FC) and Brooke-Spiegler syndrome (BSS) and the clinical variants of the cathepsin C (*CTSC*) mutation-caused disease spectrum such as the Papillon-Lefèvre syndrome (PLS) and the Haim-Munk syndrome (HMS). My investigations focused on the identification of putative phenotype modifier genetic factors in these rare monogenic diseases, which are responsible for the observed phenotypic differences among the affected patients carrying the same disease-causing *CYLD* or *CTSC* mutations.

### 1.2. Clinical variants of the *CYLD* mutation-caused spectrum

BSS is a rare monogenic skin disease characterized by the development of skin appendage tumors such as cylindromas, trichoepitheliomas and spiradenomas (Evans 1954; Bignell *et al.,* 2000). The gene responsible for the development of BSS is the *CYLD* localized on 16q12-q13 (Bignell *et al.,* 2000). To date, a total of 95 different diseases-causing mutations have been published for the *CYLD* gene (Nagy *et al.,* 2015). Concerning the so far reported phenotypes and the associated *CYLD* mutations, it is difficult to establish genotype-phenotype correlations in BSS. However, the elucidation of the genotype-phenotype correlations has significant clinical relevance in promoting the understanding of disease mechanism and contributing to the development of future therapeutic modalities. The picture is even more complex, since mutations of the *CYLD* gene have been identified in patients with phenotypic features of either BSS, FC or MTF1 suggesting that these syndromes are clinical variants of the *CYLD* mutation-caused spectrum (Rajan *et al.,* 2011; Nagy et al., 2015).

### 1.3. Clinical variants of the *CTSC* mutation-caused spectrum

PLS and HMS are characterized by overlapping dermatological and dental symptoms, including hyperkeratosis of the palms and soles as well as severe periodontitis (Selvaraju *et al.,* 2003; Nagy *et al.,* 2014). Patients with PLS can also develop mild mental retardation, calcification of the dura mater, hyperhidrosis and increased susceptibility to infections (Gorlin *et al.,* 1964; Haneke *et al.,* 1979; Dalgic *et al.,* 2011). Specific features of HMS include pes planus, arachnodactyly, acroosteolysis and onychogryphosis (Papillon *et al.,* 1924; Haim *et al.,* 1965; Hart *et al.,* 1999).

The prevalence of PLS is approximately four cases per million, and, to date, approximately 300 cases have been reported worldwide. Parental consanguinity has been noted in more than 50% of these cases (Hewitt *et al.,* 2004). The prevalence of HMS is approximately one case per million, and the majority of reported cases are descendants of a few consanguineous families from a religious isolate in Cochin, India. One unrelated Brazilian patient has also been reported. Fewer than 100 HMS cases have been reported in the literature to date (Papillon *et al.,* 1924; Haim *et al.,* 1965; Hart *et al.,* 1999).

The ratio of affected males to females is 1:1 for both syndromes. PLS and HMS are both inherited in an autosomal recessive manner and develop as a consequence of mutations of the *CTSC* gene (Toomes *et al.,* 1999; Adkison *et al.,* 2002).
Currently, 89 *CTSC* gene mutations have been identified (Sulák *et al.,* 2016). The majority of these mutations have been detected in PLS patients, whereas only 4% have been associated with HMS (Selvaraju *et al.,* 2003; Nagy *et al.,* 2014).
In light of the reported PLS and HMS phenotypes and the associated *CTSC* mutations, it was hypothesized that PLS and HMS are the same entity with different phenotypic appearances (Sulák *et al.,* 2016). Although it is difficult to establish genotype–phenotype correlations, the elucidation of these correlations is likely to have significant clinical relevance for the development of the different clinical variants (PLS and HMS), the disease mechanism and the development of future therapeutic modalities.

## 2. AIMS

Here we report an investigation of Hungarian and Anglo-Saxon BSS pedigrees, which have been chosen as they both carry the same recurrent germline mutation in the *CYLD* gene (c.2806C>T, p.Arg936X), yet show striking difference in their phenotypes (Nagy *et al.,* 2013). These two pedigrees provide an excellent opportunity to identify phenotype-modifying genetic factors that are responsible for the phenotypic diversity in BSS. In order to comprehensively profile coding variants and identify putative phenotype-modifying genetic factors - which could potentially explain the observed clinical differences between the Hungarian and the Anglo-Saxon BSS pedigrees carrying the same causative *CYLD* mutation - we performed whole exome sequencing (WES).
We have recently investigated two Hungarian patients, one with PLS and one with HMS, who nonetheless carry the same homozygous nonsense mutation (c.748C/T; p.Arg250X) of the *CTSC* gene (Sulák *et al.,* 2016). As there is currently no explanation for why one mutation can lead to these two different clinical variants (PLS and HMS), we became interested in the identification of phenotype-modifying genetic factors that could facilitate the understanding of the phenotypic differences between these patients. In order to identify putative phenotype-modifying genetic

factors - which could explain the observed clinical differences between the PLS and HMS patients carrying the same causative *CTSC* mutation – WES was performed.

## 3. PATIENTS AND METHODS

### 3.1. Patients

#### 3.1.1. Patients affected by BSS

A Hungarian pedigree from Bukovina (Romania) affected by BSS and an English BSS pedigree from Northern England were included in this study. The clinical phenotypes of the affected family members and the pedigrees are reported in detail in a previous paper from our research group (Nagy *et al.,* 2013). The patients of the Hungarian BSS pedigree were presented with severe symptoms: numerous, large, tumors arising from different skin appendages developing on the scalp, face and trunk mainly (Nagy *et al.,* 2013). The patients of the Anglo-Saxon pedigree showed moderate severity: few tumors, small size arising from different skin appendages developing primarily on the face (Nagy *et al.,* 2013).

#### 3.1.2. Patients affected by PLS and HMS

The clinical phenotypes of the affected patients are reported in detail in a previous paper from our research group (Sulák *et al.,* 2016). A Hungarian woman presented with a typical HMS phenotype: mild hyperkeratotic plaques were observed symmetrically on her palms and soles, onychogryphosis and arachnodactyly were noted on her fingers and pes planus on her soles. A Hungarian male patient presented with the classical PLS phenotype: moderate hyperkeratosis on his palms and soles. Both patients were missing all permanent teeth and using a permanent dental prosthesis. In our previous paper, we also reported haplotype analysis that raise the possibility that these patients are siblings (Sulák *et al.,* 2016). It was not possible to genotype unaffected relatives (Sulák *et al.,* 2016).

### 3.2. Methods

#### 3.2.1. DNA samples

Out of the previously reported patients, two Hungarian and two Anglo-Saxon ones, affected by the different phenotypes of BSS, but carrying the same disease-causing

mutation (c.2806C>T, p.Arg936X) in the *CYLD* gene, were investigated (Nagy *et al.,* 2013). DNA samples from the patients (n=4) were used for WES (performed by UD-GenoMed Medical Genomic Technologies Ltd., Debrecen, Hungary; http://www.ud-genomed.hu/).

Two previously reported Hungarian patients affected by PLS and HMS carrying the same disease-causing mutation (c.748C/T; p.Arg250X) of the *CTSC* gene were investigated (Sulák *et al.,* 2016). DNA samples from both patients were subjected to WES, which was performed by UD-GenoMed Medical Genomic Technologies Ltd. The quality of the DNA samples was evaluated by agarose-gel electrophoresis.

### 3.2.2. Whole exome sequencing

Briefly, 4 µg of DNA with a concentration of 100 ng/µL were used for library construction. A liquid chip capture system (Agilent Research Laboratories, Santa Clara, CA, USA) was used to efficiently enrich all human exon regions. High-throughput deep sequencing was subsequently performed on the Illumina (San Diego, CA, USA) platform. An exon kit (SureSelect Human All Exon V6 Kit; Agilent) was used for library construction and capture experiments, and a bioanalyser (Model 2100; Agilent) was subsequently used to verify the library insert size. The Illumina platform was used for sequencing according to the effective concentration of the library and the data output requirements. High-throughput paired-end sequencing (paired-end 150 bp; PE150) was performed.

### 3.2.3. Bioinformatics analysis

After WES was completed, bioinformatics analysis was performed, including quality assessment of sequencing data, single-nucleotide polymorphism (SNP) detection and whole exome association analysis.

The sequencing data quality control requirements were as follows: sequencing error rate of each base position < 1%, mean Q20 ratio > 90%, mean Q30 ratio > 80%, mean error rate < 0.1%, alignment rate for sequencing reads ≤ 95% and read depth of the base at one position ≥ 10X.

### 3.2.4. Single nucleotide polymorphism

SNP testing was performed as follows: high-quality sequences were aligned with the human reference genome (GRCh37/hg19) to detect sequence variants, and the detected variations were analysed and annotated. Variants were filtered according to read depth, allele frequency, and prevalence in genomic variant databases such as ExAc (v.0.3), ClinVar and Kaviar.
Variant prioritization tools (PolyPhen2, SIFT, LRT, Mutation Taster, Mutation Assessor) were used to predict the functional impact. All the identified candidate variants were confirmed by direct sequencing (Delta Bio 2000 Ltd., Szeged, Hungary; http://www.deltabio.hu/).

## 4. RESULTS

### 4.1. WES identified three putative phenotypic variants in BSS

A comparison of the WES data from the Hungarian and Anglo-Saxon patients carrying the same disease-causing mutation (c.2806C>T, p.Arg936X) in the *CYLD* gene identified 20 variants, which were all present in the Hungarian patients, but not in the Anglo-Saxon patients. Three of the 20 variants were suggested as putative phenotype-modifying polymorphisms by variant prioritization tools: the rs1053023 SNP of the *signal transducer and activator of transcription 3 (STAT3)* gene, the rs1131877 SNP of the *tumor necrosis factor receptor-associated factor 3 (TRAF3)* gene and the rs202122812 SNP of the *neighbor of BRCA1 gene 1 (NBR1)* gene. The rs1053023 polymorphism is located in the 3'UTR region of the *STAT3* gene, while the other two polymorphisms (rs1131877 and rs202122812) are common missense variants of the *TRAF3* and *NBR1* genes, respectively.

### 4.2. WES identified two putative phenotypic variants in HMS

A comparison of the WES data from the PLS and HMS patients carrying the same disease-causing mutation (c.748C/T; p.Arg250X) of the *CTSC* gene identified 36 variants, which were present in the HMS patient, but not in the PLS one. No mutation or polymorphism was found in PLS. Two out of the 34 variants are suggested as putative phenotype-modifying polymorphisms: the rs34608771 SNP of the *SH2 domain containing 4A (SH2D4A)* gene and the rs55695858 SNP of the

*odorant binding protein 2A (OBP2A)* gene. Both variants are common missense polymorphisms.

## 5. DISCUSSION

Although the identification of disease-causing mutations is still extremely important for therapy and family planning, direct sequencing is unable to answer clinically relevant questions regarding phenotypic diversity and disease prognosis (Jarinova *et al.,* 2012; Timmerman *et al.,* 2014; Kiritsi *et al.,* 2015; Smith *et al.,* 2019). This limitation of direct sequencing was encountered with the Hungarian and Anglo-Saxon BSS pedigrees reported previously by our workgroup (Nagy *et al.,* 2013). Comparing the WES data of the Hungarian and Anglo-Saxon BSS patients, we identified three putative phenotype-modifying genetic variants that potentially explain the striking phenotypic differences among patients carrying the same disease-causing *CYLD* mutation.

STAT3 is a transcription factor, which is constitutively activated in a variety of human cancers and plays critical roles in cancer cell survival, metastasis and angiogenesis (Yu *et al.,* 20009). STAT3 is activated by interleukin-6 (IL-6) and directly activates microRNAs (miRs), such as miR-21 and miR-181b-1 (Aggarwal *et al.,* 2009). MiR-21 and miR-181b-1 inhibit the enzymatic activity of CYLD, leading to increased nuclear factor-κB (NF-κB) activity. Thus, STAT3 is not only a downstream target of IL-6 but, with miR-21, miR-181b-1 and CYLD, is part of the positive feedback loop that underlies the epigenetic switch that links inflammation to cancer (Iliopoulos *et al.,* 2010). The B-cell CLL/lymphoma 3 (BCL3) protein, which directly interacts with the STAT3 protein, is deubiquitinated by the CYLD enzyme, and abnormal BCL3 ubiquitination has been associated with the development of basal cell carcinomas (Chaudhary *et al.,* 2015). It has been recently reported that *BCL3* serves as an oncogene in cervical cancer and its oncogenic effect is mediated by *STAT3* (Zhao *et al.,* 2016). Interestingly, the rs1053023 SNP of the *STAT3* gene has already been associated with multiple sclerosis (Lu *et al.,* 2005) and reported in B-cell non-Hodgkin lymphoma (Butterbach *et al.,* 2011). However, this is the first study that suggests a potential phenotype-modifying role for *STAT3* in BSS.

TRAF3 is a member of the TRAF family of proteins, which serve as both crucial intracellular adaptors and E3 ubiquitin ligases that mediate signaling after the

activation of various receptors. Receptors that signal through TRAF proteins include those involved in inflammation, innate immune responses, and cell death and, most notably, interact with the following: tumor necrosis factor receptors, Toll-like receptors, RIG-1-like receptors and interleukin-1 receptors (Wang *et al.,* 2010; Hacker *et al.,* 2011). The TRAF-interacting protein (TRAIP) interacts with TRAF3, while TRAIP is reported to interact with CYLD (Chapard *et al.,* 2012). TRAIP expression is increased in basal cell carcinomas and in multiple breast epithelial cell lines with oncogenic potentials ranging from non-malignant to highly invasive (Almeida *et al.,* 2011). Mutations in *TRAF3* and *CYLD* leading to constitutive activation of NF-κB have been identified in cancers, including multiple myeloma and solid tumors (Harhaj *et al.,* 2012). The rs1131877 SNP of the *TRAF3* gene is highly predictive for the development grade ≥2 acute esophageal postradiotherapy toxicity (De Ruyck *et al.,* 2011), and here we have demonstrated its association with the phenotypic diversity in BSS.

NBR1 is an autophagic adaptor protein involved in the efficient clearance of damaged mitochondria (Shi *et al.,* 2015). Briefly, upon mitochondrial damage, E3 ubiquitin ligases are recruited from the cytosol to depolarized mitochondria, where they target damaged mitochondrial proteins for ubiquitination and bulk degradation by autophagy (Shi *et al.,* 2014). NBR1 is a functional homolog of sequestosome 1 (SQSTM1), another autophagic adaptor protein, which is a selective autophagy substrate that also act as cargo receptors for degradation of other substrates (Svenning *et al.,* 2011). The interaction of CYLD with TRAFs is dependent upon SQSTM1, and the absence of SQSTM1 results in the reduction of the activity of the CYLD enzyme (Wooten *et al.,* 2008; Into *et al.,* 2010). The rs202122812 SNP of the *NBR1* gene has not been previously associated with any human diseases: this is the first study to indicate its clinical relevance in the development of the BSS-related phenotypic diversity.

The comparison of the WES data of the HMS and PLS patients identified a putative phenotype-modifying genetic variant (rs34608771 SNP) in the *SH2D4A* gene, which encodes a T-cell-expressed adapter protein that is expressed in T-cells, B-cells, macrophages and dendritic cells (Hashimoto *et al.,* 2000). SH2D4A regulates T-cell receptor (TCR) signal transduction in T-cells, and, in human T-cells, its expression increased in response to T-cell activation (Gonçalves *et al.,* 2018). SH2D4A is linked to cathepsin C via cystatin F. This latter protein is a cysteine-

protease inhibitor expressed selectively in immune cells, such as T-cells, NK cells and dendritic cells (Obata-Onai *et al.,* 2002). The rs34608771 polymorphism of the *SH2D4A* gene has not been associated previously with any human diseases: this is the first study which links it to the development of the HMS clinical variant and raises its putative association with the phenotypic differences between PLS and HMS patients.

The other putative phenotype-modifying genetic variant (rs55695858 SNP) is located within the *OBP2A* gene, which encodes an odorant-binding carrier protein that has a known environmental biosensor function (Lacazette *et al.,* 2000). The OBP2A protein is expressed in the nasal structures, salivary and lachrymal glands and lungs, and, thus, has an oral sphere profile (Lapinski *et al.,* 2009 and 2019). OBP2A interacts with the glycosyltransferase 6 domain containing 1 (GLT6D1) protein, encoded by the *GLT6D1* gene, which has been identified as a susceptibility locus for periodontitis by genome-wide association studies and this association has been confirmed by several previous studies (Li *et al.,* 2009; Hasim *et al.,* 2015). Although genetic variants of the *OBP2A* gene have been implicated in influencing the substrate-binding specificity of the encoded protein, none have previously been associated with the development of a human disease (Halfon *et al.,* 1998; Hamilton *et al.,* 2008). Since periodontitis is a major feature of the PLS and HMS phenotypes, we suggest that the rs55695858 SNP of the *OBP2A* gene might contribute to the phenotypic differences observed between PLS and HMS patients.

## 6. CONCLUSIONS

Our study aimed to explain the phenotypic differences in BSS patients carrying the same disease-causing *CYLD* mutation by identifying phenotype-modifying genetic polymorphisms and to elucidate the phenotypic differences in PLS and HMS patients carrying the same disease-causing *CTSC* mutation by identifying phenotype-modifying genetic polymorphisms. It should be noted that, in addition to genetic factors, environmental or lifestyle factors might also contribute to the observed phenotypic differences between the investigated patients. Further functional studies are needed to prove the clinical relevance of the identified phenotype-modifying genetic factors and to describe the underlying mechanism that explains their phenotype-modifying roles. Our study contributes to the accumulating evidence supporting the clinical importance of phenotype-modifying

genetic factors and their potential to facilitate the elucidation of genotype–phenotype correlations, phenotypic diversity and disease prognosis (Lee *et al.,* 2008).

## 7. SUMMARY

My investigations focused on the identification of putative phenotype modifier genetic factors in these rare monogenic diseases, which are responsible for the observed phenotypic differences among the affected patients carrying the same disease-causing *CYLD* or *CTSC* mutations.

We recently investigated a Hungarian and an Anglo-Saxon pedigrees affected by BSS. Despite carrying the same disease-causing mutation (c.2806C>T, p.Arg936X) of the *CYLD* gene, the affected family members of the two pedigrees exhibit striking differences in their phenotypes. To identify phenotype-modifying genetic factors, WES was performed and the data from the Hungarian and Anglo-Saxon BSS patients were compared.

Three putative phenotype-modifying genetic variants were identified: the rs1053023 SNP of the signal transducer and activator of transcription 3 gene, the rs1131877 SNP of the tumor necrosis factor receptor-associated factor 3 gene and the rs202122812 SNP of the neighbor of BRCA1 gene 1 gene.

We investigated two Hungarian patients suffering from different phenotypic variants (PLS and HMS) but carrying the same homozygous nonsense *CTSC* mutation (c.748C/T; p.Arg250X). To gain insights into phenotype modifying associations, WES was performed for both patients and the results were compared to identify potentially relevant genetic modifying factors.

WES revealed two putative phenotype-modifying variants: a missense mutation (rs34608771) of the *SH2 domain containing 4A (SH2D4A)* gene encoding an adaptor protein involved in the intracellular signaling of the cystatin F, a known inhibitor of the cathepsin protein, and a missense variant (rs55695858) of the *odorant binding protein 2A (OBP2A)* gene influencing the function of the cathepsin protein through the glycosyltransferase 6 domain containing 1 (GLT6D1) protein.

Our investigations contribute to the accumulating evidence supporting the clinical importance of phenotype-modifying genetic factors, which have high potential to aid the elucidation of genotype–phenotype correlations and disease prognosis.

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