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Cloud-based data collection and multidisciplinary approach in diagnosis of naevoid basal cell carcinoma syndrome

PhD Thesis

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LIST OF PAPERS RELATED TO THE SUBJECT OF THE THESIS

I. Pál M, **Vetró É**, Nagy N, Nagy D, Horváth E, Bokor AB, Varga A, Seres L, Oláh J, Piffkó J, Széll M.: Whole-Exome Sequencing Identified Two Novel Pathogenic Mutations in the *PTCH1* Gene in BCNS.
Curr Issues Mol Biol. 2023;45(7):5293-5304.
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II. **Vetró É**, Oláh J, Kalmár T, Maróti Z, Horváth E, Széll M, Piffkó J, Seres L.: Multidisciplinary approach for mapping genetic variants in naevoid basal cell carcinoma syndrome. Newly identified patched 1 mutation in half-sisters.
Asian J. Dent. Sci. 2022;5,7–14.

III. **Vetró É**, Oláh J, Nagy D, Széll M, Piffkó J, Seres L.:
A Gorlin–Goltz-szindróma genetikai aspektusai [Genetic aspects of Gorlin-Goltz syndrome]
Orv Hetil. 2020;161:2072-2077. [Hungarian]
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Introduction

Naevoid basal cell carcinoma syndrome (NBCCS) is a rare, hereditary condition that is characterized by a wide range of developmental disorders and a predisposition to different malignancies.

Epidemiology

To obtain precise information regarding its incidence and prevalence is difficult, due to its rarity and the fact that individuals with milder features may not be recognized. The estimated prevalence varies over a wide range, the most quoted one is 1:57.000 but in Italy, it was reported as 1:256.000. Its incidence at birth was assessed to be 1:19.000. NBCCS affects both women and men in a rather equal distribution. As for races, all are affected and have been reported worldwide, although African Americans and Asians represent only 5% of the cases.

Etiopathogenesis - Hedgehog signaling pathway

NBCCS is an autosomal dominant disorder which can be caused by mutations in the protein patched homolog 1 (PTCH1) gene on chromosome 9q22.32., and protein patched homolog 2 (PTCH2) gene on chromosome 1p34.1. or suppressor of fused homolog (SUFU) gene on chromosome 10q24.32.

In most cases, mutations of the PTCH1 gene are responsible for the development of NBCCS. PTCH1 is a tumour suppressor gene which encodes the patched-1 transmembrane receptor (Ptch1) that recognizes the hedgehog (Hh) protein. The lack of ligand Hh, PTCH1 exerts an inhibitory effect on Smoothened (SMO), a downstream protein in the Sonic Hedgehog signaling pathway (SHH). This inhibition is ceased upon Hh binding to Ptch1, during a catalytic process that does not demand a direct interaction between Ptch1 and Smo.

SUFU gene is a negative regulator of the SHH pathway and it encodes a cytoplasmic protein, which inhibits the GLI1 protein. Thus, mutations in the SUFU gene result in activating the SHH pathway. All the above-mentioned genes and proteins play an essential role in the SHH signaling pathway. Malfunctions of the signaling pathway were proven to be involved in over 50% of cancer types and may lead to developmental disorders, affecting the differentiation processes of embryogenesis, tissue regeneration, and cell division processes.

Clinical features

Signs and symptoms of NBCCS are extremely variable; the integumentary-, central nervous-, genitourinary-, cardiovascular-, ocular-, auditory- and even skeletal systems may be affected. Most patients suffering from NBCCS experience the development of multiple jaw keratocyst (KCs) and/or basal cell carcinomas (BCCs).

Genetic background

70 to 80% of patients with NBCCS have a familial aggregation of symptoms and de novo pathogenic variants are responsible for 20-30% of cases. In rare cases, gonadal or somatic mosaicism may occur. In approximately 50-85% of NBCCS cases sequence variations of the *PTCH1* gene, in 6–21% duplication or deletion of exon(s) of the *PTCH1* gene, in approximately 5% sequence variations of the *SUFU* gene, and in 1% deletion or duplication of exon(s) of the *SUFU* gene play role in the development of the disease. In 15-27% of patients, the genetic mechanism causing NBCCS remains unknown.

Diagnosis

The diagnosis of NBCCS is based on the recognition of the aforementioned characteristic clinical findings and the identification of a pathogenic genetic variant. Diagnostic criteria have been modified several times.

Currently, a diagnosis of NBCCS requires (1) one major criterion and molecular confirmation or (2) the presence of two major diagnostic criteria or (3) one major and two minor diagnostic criteria. Major criteria include (a) multiple BCCs or one BCC by the age of 20 years, (b) odontogenic keratocysts of the jaw (as proven by histology), (c) palmar or plantar pitting, (d) bilamellar calcification of the falx cerebri, (e) medulloblastoma and (f) first-degree relatives with NBCCS. Minor criteria include macrocephaly, congenital malformations, skeletal abnormalities, radiologic abnormalities and ovarian or cardiac fibromas

Genetic aspects of diagnosis

In NBCCS the suggested stepwise sequence of performing a serial test of a single gene is the following: [1] PTCH1 sequence analysis; [2] PTCH1 deletion/ duplication analysis; [3] SUFU sequence analysis; [4] SUFU deletion/ duplication analysis; [5] PTCH1 ribonucleic acid (RNA) analysis. The importance of testing the PTCH2 gene remains controversial, due to the insufficient evidence of its causative role.

However, formerly Sanger sequence analysis was considered the gold standard, aiming at identifying the exact order of nucleotide bases, but this method is time- and cost-consuming. Next-generation sequencing (NGS) techniques, including whole exome sequencing (WES), are more eligible for high-throughput and large-scale sequencing and detecting point mutations. By nature, WES is not able to identify large deletions or duplications. This situation can be solved by the usage of multiplex ligation-dependent probe amplification (MLPA) which is a specific PCR assay and an advantageous tool to scan large deletions and duplications, however, it cannot detect point mutations or mutations not reported before.

If all the above-mentioned tests fail to detect alterations, but the symptoms are suspicious for NBCCS, then DNA samples from two different BCCs can be tested for PTCH1 and SMO to reveal the possibility of postzygotic mosaicism in the background.

Objectives

Our aim was to conduct a retrospective and prospective cohort study to evaluate patients affected by NBCCS. The main objectives of the study are the following:

1) set up a multidisciplinary team with experts in the diagnosis and management of NBCCS
2) retrospective and prospective, cloud-based data collection of NBCCS patients
3) unveil the major and minor characteristics of the probands
4) subject to probands genetic investigation
5) find a correlation between phenotypic and genotypic values
6) examine the utility of MLPA end WES in mapping the genetic background of NBCCS
7) organize the management and surveillance of affected probands.

Materials and methods

Data collection

A multidisciplinary team, including oral and maxillofacial surgeons, dermatologists, radiologists, and clinical geneticists worked out a diagnostic protocol for all patients suspected of NBCCS.

Patients

Sixteen Hungarian patients from 11 families fulfilling the diagnostic criteria of NBCCS were enrolled in this study during an eight-year period.

The inclusion criteria of the study were those updated by Bree et al. in 2011 regarding the diagnosis of NBCCS:

(1) one major criterion and molecular confirmation
(2) the presence of two major diagnostic criteria
(3) one major and two minor diagnostic criteria.

All those patients who did not display any symptoms of NBCCS were excluded from the study.
No attempt was made to confirm the possible presence of postzygotic mosaicism.

Radiological examination

Panoramic radiography, chest x-ray, posterior-anterior skull radiography, and abdominal and pelvic ultrasound are performed in all cases.
If a genitourinary or a central nervous system disorder is suspected in radiology or upon clinical symptoms, magnetic resonance imaging of the region of interest is carried out, too.

DNA Extraction

Genomic DNA was extracted from venous blood mixed with the anticoagulant EDTA using the DNeasy® Blood & Tissue Kit (QIAGEN, Germany), as described in the manufacturer's instructions.

Whole-Exome Sequencing

Genotypes of patients were determined using next-generation sequencing. Library preparation was carried out using the SureSelectQXT Reagent Kit (Agilent Technologies, Santa Clara, CA, USA). Pooled libraries were sequenced on an Illumina NextSeq 550 NGS platform using the 300-cycle Mid Output Kit v2.5 (Illumina, Inc., San Diego, CA, USA).

Multiplex-Ligation-Dependent Probe Amplification

To assess larger genetic aberrations, we used SALSA MLPA Probemix P067 PTCH1 (MRC-Holland, Netherlands) containing probes for 23 of the 25 exons in the *PTCH1* gene (LRG_515; no probes are included for exons 1 and 9), according to the manufacturer's instructions.

Data processing

The process of gathering data and sharing between specialists was implemented by applying Google Sheets (Google LLC, CA, USA).

Statistical analysis

Statistical analyses were carried out using VassarStats.

Results

In our Hungarian NBCCS cohort, the male/female ratio was 7:9. The clinical phenotypes of the affected patients are summarized in. The most frequent clinical manifestations were histologically proven odontogenic keratocyst of the jaw (13 patients, 81%), multiple BCCs (nine patients, 56%) and congenital malformations (seven patients, 43%) including hypertelorism (3 patients, 18%), cleft lip- and palate (3 patients, 18%), bifid, fused ribs (1 patient, 6%) and polydactyly (1 patient, 6%). Bilamellar calcification of the falx cerebri and macrocephaly were detected in four patients (25%). Palmar and plantar pits were present in three patients (18%). Fifty per cent of the investigated patients were aware of a first-degree relative affected by NBCCS.

WES identified three novel likely pathogenic mutations and three recurrent pathogenic variants of the *PTCH1* (NM_000264.5) gene.

All mutations identified via WES were validated with Sanger sequencing.

From the MLPA results, a pathogenic deletion of the second exon of the *PTCH1* gene was identified in one patient. WES did not identify pathogenic variants of the *PTCH1* gene for this patient.

Discussion

16 patients with NBCCS were enrolled in our Hungarian cohort study. Taking the prevalence of NBCCS into account in this study, we performed a closely thorough examination of the tertiary health care region of South-East Hungary, which covers 1.5 million inhabitants.

By comparing our results with the most quoted authors regarding the phenotypic manifestations of NBCCS, we found that in our study the prevalence of hypertelorism (18%), calcification of falx cerebri (25%), macrocephaly (25%) and skeletal abnormalities were lower than in the other studies. However, the prevalence of cleft lip and palate (18%) was higher than that found in any other surveys. Two previous studies found a higher prevalence of this characteristic than the average. In Japan, the ratio of cleft patients was 9%, while in Switzerland it was 11%. The frequency of the manifestation of BCCs and jaw keratocysts in the affected patients did not show differences compared to previous studies.

Should we have applied the criteria proposed by Kimonis et al. for the diagnosis of NBCCS, one patient would not have been recognized in our cohort. This proband fulfilled one major and one minor criterion. On the other hand, in seven patients, no pathogenic variations in the tested genes were found even after WES and MLPA. Interestingly four out of these patients had three major signs of NBCCS based on either diagnostic criteria and they should have been evidently considered NBCCS. These findings unequivocally highlight the deficiencies of the up to now proposed diagnostic approaches.

In addition, we reviewed the cohort studies on the investigation of NBCCS. In 2007, Rupprecht et al. published their observations on 8 patients affected with NBCCS collected over a 12-year period. The study retrospectively analysed the epidemiological, clinical, radiological and histological data on the probands, but the research did not intend to investigate the genetic aspects of NBCCS. In 2016, Rehefeldt

and her colleagues systematically examined the clinical signs of the syndrome in the context of Zurich register, without aiming at clarifying the genetic background of the examined 30 patients. One year later an Australian study was published which showed the most similarity with our cohort in terms of the method of genetic investigation. The recruited 19 patients underwent Sanger sequencing and MLPA or CGH-array-based tests. However, at the beginning of the study they applied Sanger sequencing and just the latter samples were examined by next-generation sequencing. In this study, from the tested 11 patients 6 (54.5%) were found to have a pathogenic PTCH1 mutation. In the same year, the Maastricht University Medical Center published the results of a multicentre retrospective cohort study, which examined the period between 1999 and 2015. In this study, patients with characteristic signs of NBCCS were genetically tested for PTCH1 mutations by Sanger sequencing. With the sole application of Sanger, the mutation detection rate was 21%. Also, in that year, Gianferante et al. shared their observations on 18 well-characterized NBCCS families. WES and aCGH techniques were used to detect mutations of all genes which can be affected in NBCCS and as well as 155 genes that play a role in the SHH pathway. 89% of the families were affected with PTCH1 mutations, but the other genes tested showed no alterations.

As a consequence of the above, cohort studies, which examine the manifestations of NBCCS frequently report a low number of cases, use different methods in terms of genetic testing and are not able to find genotype-phenotype correlations in NBCCS.

In our cohort study, we recognized three novel and three recurrent variants of the *PTCH1* gene using the WES and MLPA methods.

The MLPA technique was used to investigate the possibility of large deletions in the *PTCH1* gene. The method identified a deletion of exon 2. As WES did not identify any pathogenic variants of the *PTCH1* gene in the MLPA-positive patient, our result suggests the importance of this combination of screening methods for the genetic diagnosis of NBCCS.

These results further emphasize that even high-throughput genetic screening methods, such as WES, have limitations and that missing heritability is an issue for rare diseases such as NBCCS.

To resolve this phenomenon in rare diseases, novel technological approaches, such as the promising techniques of whole-genome sequencing (WGS) or epigenetic analysis, should be implemented in clinical practice.

To understand this complicated disorder better, information on a large number of patients is necessary. Insomuch as NBCCS is a rare hereditary condition, researchers are under the necessity of assembling small pieces of information gained from long-run cohort studies and genetic databases. In this genetic puzzle, newly identified pathogenic mutations can help to elucidate the genetic background and the genotype-phenotype correlation of NBCCS.

Based on our results, we hypothesize that the application of WGS and/or an epigenetic approach should be applied to unsolved NBCCS cases to attempt to resolve missing heritability for NBCCS.

Summary of new findings

1.) We have successfully introduced a cloud-based, multidisciplinary, real-time data collection method to obtain clinical and genetic data on a rare hereditary disease.

2.) According to our best knowledge, in Europe, our survey was the first genetic cohort study in which all the probands underwent genetic testing with the application of multiplex ligation-dependent probe amplification and whole exome sequencing.

3.) With the combined application of whole exome sequencing and multiplex ligation-dependent probe amplification we have successfully recognized three novel pathogenic variants of the PTCH1 gene.

4.) The prevalence of cleft lip and palate patients in our cohort is the highest ever has been reported.

5.) Our findings justify the assumption that all of the currently applied diagnostic systems for NBCCS have their shortcomings and they are incapable of setting up the diagnosis conclusively.

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