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ORIGINAL ARTICLE

CREBBP IS A MAJOR PROGNOSTIC BIOMARKER FOR RELAPSE IN CHILDHOOD B-CELL ACUTE LYMPHOBLASTIC LEUKEMIA: A NATIONAL STUDY OF UNSELECTED COHORT

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ABSTRACT

Although the identification of disease subtypes conveying prognostic significance along with minimal residual disease (MRD) assessment represent cornerstones for stratification in childhood acute lymphoblastic leukemia (ALL), approximately half of the relapses occur in patients from standard-risk groups. Identification of the drivers of treatment failure is crucial for detection of high-risk clones at diagnosis. We evaluated clinical variables and the most common genetic alterations in an unselected cohort of 55 patients with B-ALL treated according to the ALL-IC-BFM 2002 protocol, with a median follow-up of 46 months. Matched diagnosis-relapse samples underwent screening for additional alterations using whole-exome sequencing. Mutations in the *CREBBP* gene were found in 80% (4/5) of the patients with relapse, either present from the disease onset or acquired at relapse, while none of the examined patients in remission presented alterations in this gene. Deletions in *TP53* and *EBF1* (present in 2/5 and 1/5 of the patients with relapse, respectively) were infrequent or absent in the patients in remission, respectively. Screening for alterations in the *CREBBP* gene at diagnosis and/or at multiple time-points during chemotherapy could be incorporated into treatment protocols, as it may contribute to the identification of significant number of patients with predefined or acquired chemoresistant clones.

Keywords: acute lymphoblastic leukemia, relapse, molecular biomarkers, *CREBBP*

INTRODUCTION

Acute lymphoblastic leukemia (ALL) is the most prevalent form of cancer among children, comprising 25% of all childhood malignancies, with a consistently increasing incidence rate over the years [1, 2]. It arises following the clonal proliferation of immature B and/or T lymphoid cells, with around 80% of the cases being of B lineage origin [3, 4]. The most common initial genetic lesions are chromosomal loss (hypodiploidy), gain (hyperdiploidy), or fusion genes, leading to a pre-leukemic clone. A subsequent second hit, either a copy number alteration (CNA) or single nucleotide variant (SNV), is believed to be the cause of lymphoid arrest and the development of symptomatic disease [4, 5].

Conventional (karyotyping, fluorescence in situ hybridization - FISH) and molecular (reverse transcription quantitative polymerase chain reaction - qRT-PCR, multiplex ligation-dependent probe amplification - MLPA) tech-

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niques are routinely used for the identification of numerical and structural chromosomal abnormalities, allowing for the detection of several disease subtypes with different prognostic and therapeutic associations [3, 5, 6]. Among these, high-hyperdiploidy is the most common subtype in childhood B-ALL (25-30%), which is associated with favorable prognosis [6-8]. The structural chromosomal abnormalities involve genes that regulate hematopoiesis and lymphoid development (*RUNX1, ETV6*), activate oncogenes (*MYC*), or constitutively activate tyrosine kinases (*ABL1*). In particular, recurring translocations leading to different subtypes in B-ALL include t(12;21)(p13;q22) encoding ETV6::RUNX1 associated with favorable prognosis, t(1;19)(q23;p13) encoding TCF3::PBX1 with intermediate prognosis, t(9;22)(q34;q11) encoding BCR::ABL1, and rearrangements of *MLL* at 11q23 with different partner genes, both associated with poor prognosis [7, 8]. More recently, genomic profiling has led to the identification of new abnormalities that are not detectable by conventional methods, resulting in more than 20 disease subtypes [9, 10].

The risk classification at diagnosis of patients with ALL varies among different treatment protocols, but in general this includes the patient's age, white blood cell (WBC) count, and presence of specific disease subtypes. In general, patients are classified as standard-risk if diagnosed at the age 1-5, presenting a WBC count of < 20 x 10^9/L and the absence of iAMP21, *IKZF1* deletion or *CRLF2* overexpression. The high-risk group includes patients with either poor prednisolone response, hypodiploidy, BCR::ABL1, TCF3::HLF or MLL::AFF1 subtypes, or any other *MLL* rearrangement in patients younger than 1 year, while all other patients are considered intermediate-risk. Although the identification of disease subtypes conveying prognostic significance along with minimal residual disease (MRD) assessment represent cornerstones for disease stratification, approximately half of the relapses occur in patients from standard-risk groups [11]. Additionally, it has been shown that more than half of the relapse samples have at least one genetic alteration originating either from the leukemic or the pre-leukemic clone, potentially affecting disease progression and therapy response [12,13]. Many of these alterations affect transcription factors (*ETV6*, *PAX5*, and *IKZF1*), epigenetic regulators (*ATF7IP*, *SETD2*, *KMT2D*, and *CREBBP*), cell cycle regulators (*CDKN2A/B*, *BTG1*, and *RB1*), RAS pathway genes (*KRAS*, *NRAS*, and *PTPN11*), and the tyrosine kinase *FLT3* [14, 15]. Identification of the drivers of treatment failure is crucial for detection of high-risk clones at the time of diagnosis, which can also contribute to uncovering new therapeutic targets, personalization of the treatment protocol and reduction of the short- and long-term adverse effects of intensified chemotherapy.

In this prospective observational national study, we present the clinical variables, identify the most common molecular biomarkers and the individual therapy response (MRD) data, as well as their relation to the clinical status in a cohort of 55 children with B-ALL. Additionally, we conduct a more comprehensive analysis of the patients who experienced disease relapse using whole exome sequencing to detect other alterations that may prove useful in risk stratification and to potentially discover new altered pathways that could be targeted therapeutically.

MATERIALS AND METHODS

Clonal rearrangements detection and MRD analysis

All patients (a total of 55) included in the study were diagnosed with B-ALL at the University Clinic for Pediatric diseases in Skopje over a period of 6 years (2018-2023). For the molecular clonality analyses, nucleic acids were extracted from bone marrow mononuclear cells using an automated nucleic acid extractor (MagCore Super, RBC Bioscience, New Taipei City). The clonal immunoglobulin (Ig) gene rearrangements were identified using multiplex PCR according to the BIOMED-2 protocol [16]. The dominant rearrangements (from the diagnostic and relapse samples) were subsequently sequenced with the specific family primers using the Sanger sequencing method, and the sequences were analyzed using the IMGT/V-QUEST and IgBLAST tools. The initial clonal rearrangements were also detected using Next-Generation Sequencing (NGS) method with the LymphoTrack Dx IGH-FR3 assay (Invivoscribe Technologies, San Diego, CA, USA) according to the manufacturer's protocol. These clones were tracked for MRD analysis at two time-points, on day 33 and day 78 from therapy onset, with a sensitivity threshold at 10^(-4) [17].

Hybrid transcripts and copy number alterations

The most common gene rearrangements, including t(12;21)(p13;q22) ETV6::RUNX1, t(1;19)(q23;p13) TCF3::PBX1, t(9;22)(q34;q11) BCR::ABL1, and t(4;11)(q21;q23) MLL::AFF1 were detected using qRT-PCR according to the BIOMED-1 protocol [18]. Aneuploidy was assessed using the SALSA MLPA P036 and P070 subtelomeric probe-mix kits (MRC-Holland, Amsterdam, the Netherlands) following the manufacturer's recommended procedures. High-hyperdiploidy or hypodiploidy were defined if >50 or <44 chromosomes were present, respectively. Identification of copy number alterations in specific regions and genes with prognostic significance in B-ALL was performed using the P327, P329, P335, and P038 SALSA MLPA kits, which allowed for the detection of intrachromosomal amplification of chromosome 21 (iAMP21), *EGR* deletion, deletions or duplications in the

PAR1 region (*CRLF2*, *CSF2RA*, *IL3RA*), deletion of *TP53*, deletion of *IKZF1*, and deletions or duplications in genes associated with B-cell differentiation and cell cycle control (*CDKN2A/2B*, *PAX5*, *ETV6*, *RB1*, *BTG1*, and *EBF1*).

Whole exome sequencing

Targeted, massively parallel sequencing of exons in >99% of protein-coding genes (Whole Exome Sequencing, WES) was performed in all patients with relapse (samples obtained both at diagnosis and at relapse) and in five additional patients in remission. The reactions were conducted on an Illumina NovaSeq 6000 sequencer, using the Twist Human Core + RefSeq + Mitochondrial Panel kit (Twist Bioscience, San Francisco, CA, USA) with a mean read depth of targeted regions across samples of approximately 100X. Variant annotation, filtering, and classification of the detected variants were done according to ACMG guidelines, utilizing multiple databases and tools (ClinVar, HGMD, dbSNP, ExAC, gnomAD, OMIM, Varsome, Franklin Genoox).

The patients were treated according to the ALL-IC-BFM 2002 protocol, and the median follow-up was 46 months (range: 11-79). Since this was an observational study, the MLPA analyses and the NGS-MRD data were not used for patient stratification or clinical decisions. The study was approved by the Ethics Subcommittee of the Macedonian Academy of Sciences and Arts, and written informed consent was obtained from the patients' guardians in accordance with the Declaration of Helsinki.

RESULTS

Clinical characteristics and treatment response

All clinical data of the patients included in the study are summarized in Figure 1. The median age at diagnosis was 4 years (range: 0-11). The white blood cell (WBC) count at diagnosis was higher than 20 x 10^9/L in 34% of the patients, while CNS infiltration was a less frequent event (10%). The percentage of patients with poor prednisolone response (PPR; absolute blast count >1000 in pe-

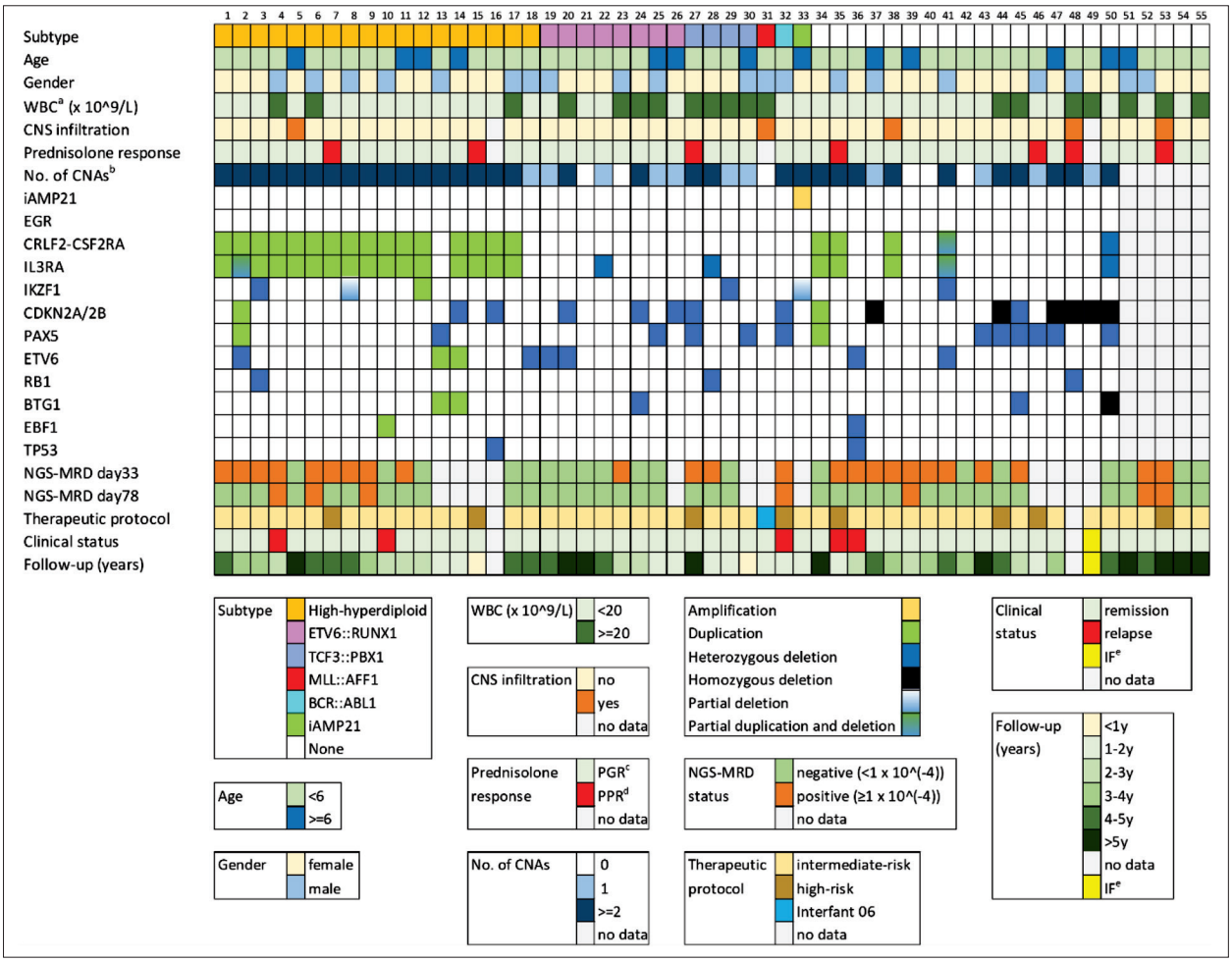

Figure 1: Heatmap of the clinical, molecular and treatment response data in a cohort of 55 pediatric patients with B-ALL
[a] white blood cell count; [b] copy number alterations; [c] prednisolone good response; [d] prednisolone poor response; [e] induction failure

ripheral blood on day 8) was 13%. Following the ALL-IC-BFM 2002 classification [19], 15% of the patients (8/52) were stratified into the high-risk group. One patient (aged <1 year) was enrolled in the Interfant-06 trial, while two patients moved out of the country after diagnosis and were lost for follow-up. The remaining patients received treatment according to the intermediate-risk protocol. NGS-MRD data for two time-points from therapy onset (day 33 and day 78) were available for 43 patients. A positive MRD status (MRD ≥ 1 x 10^(-4)) was detected in 24/43 (56%) and 7/43 (16%) of the patients on days 33 and 78, respectively [17]. During a median follow-up of 46 months, five patients relapsed. All relapses occurred between 17 and 32 months after diagnosis.

Molecular alterations at diagnosis

The most common genetics subtype in the study population was high-hyperdiploidy, present in 33% (18/55) of the patients, followed by the ETV6::RUNX1 subtype in 15% (8/55). Four other patients (7%) were identified with the TCF3::PBX1 subtype, and one patient each (1,8%) with the MLL::AFF1, BCR::ABL1 and iAMP21 subtypes. At diagnosis, at least one copy number alteration in the selected genes was detected in 88% (44/50) of the patients, with 75% of them showing more than two alterations (Figure 1). The most common alteration was the duplication of the PAR1 region (*CRLF2, CSF2RA, IL3RA*), while PAR1 deletion was observed in one patient. Deletions in the *IKZF1* gene were present in 10% (5/50) of the patients, in two of which partial, and none of them presented with the IKZF1^{plus} profile [20]. *CDKN2A/2B* gene deletion was detected in 28% (14/50) of the patients (8 heterozygous, 6 homozygous), while *PAX5* gene deletion in 22% (11/50), co-occurring in six patients. Deletion and duplication of the *ETV6* gene was detected in 6 (12%) and 2 (4%) patients, respectively. *BTG1* gene deletion was observed in three patients (one of them homozygous), while duplication was found in two other patients. *RB1* gene deletion was present in three patients, while the *EBF1* gene was deleted in one patient and duplicated in another. Deletion of *TP53* gene was present in two patients (4%).

CNAs and SNVs in the matched diagnosis-relapse samples

Clinically relevant alterations in the five patients with relapse (No. 4, 10, 32, 35 and 36 from Figure 1), including the findings from the WES analysis, are summarized in Table 1. Among them, two patients were identified with high-hyperdiploidy, one of which had an additional *EBF1* gene duplication; one patient had a BCR::ABL1 hybrid transcript along with *CDKN2A/2B* and *PAX5* gene partial deletions, and one patient was found to have *TP53*, *EBF1*, and *ETV6* gene deletions at diagnosis. The aneuploidy, hybrid transcript, and the *EBF1* and *ETV6* gene alterations were preserved in the matched relapse samples. The deletion of *TP53* was present in both diagnosis and relapse samples in one patient and gained at relapse in another. By contrast, the *CDKN2A/2B* deletions were either gained or lost in the relapse samples, and *PAX5* gene deletion was lost in the relapse sample. Additionally, single nucleotide variants in three genes were involved in the relapse samples, including the epigenetic regulator *CREBBP*, the RAS pathway gene *NRAS*, and the DNA mismatch repair gene *MSH2*. Notably, alterations in these genes were not observed in the five diagnostic samples from patients in remission that were analyzed by WES. Mutations affecting the histone acetyltransferase (HAT) domain of the *CREBBP* gene were persistent in the diagnostic and re-

Table 1. Clinically relevant alterations in the patients with relapse

			Copy number alterations					Single nucleotide variants			
No.[a]	**Sample type**	**Aneuploidy/ Hybrid transcript**	***TP53***	***EBF1***	***CDKN2A/2B***	***PAX5***	***ETV6***	***CREBBP***	***NRAS***	***MSH2***	***FLT3***
4	Dg	high-hyperdiploidy	-/-	-/-	-/-	-/-	-/-	-	-	-	+
	Relapse	high-hyperdiploidy	-/-	-/-	-/-	-/-	-/-	+	+	-	-
10	Dg	high-hyperdiploidy	-/-	-/dup	-/-	-/-	-/-	+	-	-	-
	Relapse	high-hyperdiploidy	-/del	-/dup	-/-	-/-	-/-	+	-	-	-
32	Dg	BCR::ABL1	-/-	-/-	-/del[b]	-/del[b]	-/-	-	-	-	-
	Relapse	BCR::ABL1	-/-	-/-	-/-	-/-	-/-	+	-	+	-
35	Dg		-/-	-/-	-/-	-/-	-/-	+[c]	+	-	-
	Relapse		-/-	-/-	-/del	-/-	-/-	+[c]	-	-	-
36	Dg		-/del	-/del	-/-	-/-	-/del	-	-	-	-
	Relapse		-/del	-/del	-/-	-/-	-/del	-	-	-	-

[a] Patient identification number from Figure 1
[b] Partial deletion
[c] Different amino acid change detected at Dg and relapse

lapse clones in a single patient, acquired at relapse in two patients or involved a change in the same amino acid in this domain in one patient (from R1446H in the diagnostic clone to R1446C in the relapse clone). The p.G12D mutation in the *NRAS* gene was the second most common alteration in these patients, either gained or lost in the relapse samples of two patients, while the mutation in *MSH2* was present in the relapse sample only, of one patient. Additionally, a mutation in *FLT3* was observed in the diagnostic clone in one patient who relapsed, but mutations in this gene were also found in 2 out of 5 patients in remission. The type of SNV for each gene is presented in Table S1.

Clonal evolution of the relapses

Fragment analysis and Sanger sequencing of the immunoglobulin heavy chain genes in the matched diagnosis and relapse samples revealed the presence of the same clonally rearranged sequences at relapse as those observed at diagnosis in four patients (No. 4, 10, 35 and 36) (Table S2). In two of these patients (No. 10 and 36), one of the clonal rearrangements was lost at relapse. Nevertheless, in these two patients, we observed the same genetic alterations in both the diagnosis and relapse samples, with additional gain of *TP53* deletion in one of them. In the other two patients with the same clonal rearrangements, we observed gains (in *CDKN2A/2B*, *CREBBP*, and *NRAS*) and losses (in *FLT3* and *NRAS*) of genetic alterations at relapse. The last patient (No. 32) retained only the BCR::ABL1 hybrid transcript at relapse, lost the *CDKN2A/2B* and *PAX1* deletions, and gained alterations in *CREBBP* and *MSH2*. This patient also showed different clonally rearranged sequences at relapse, presenting three rather than five clonal sequences detected at diagnosis, of which only one was same in both samples (Table S2).

DISCUSSION

This study presents data from all pediatric patients diagnosed with B-ALL in our country over a period of six years. All patients were treated according to the ALL-IC-BFM 2002 protocol, which was escalated to high-risk protocol in eight patients. After a median follow-up of 46 months, five patients (9%) experienced disease relapse. In general, the patients with relapse were diagnosed before the age of 6; none presented with CNS infiltration at diagnosis, and the WBC count was slightly higher than 20 x 10^9/L in only one patient. Initial high-risk features (poor prednisolone response and BCR::ABL1 hybrid transcript) were detected in only two of the five patients with relapse. These findings support the need for inclusion of new molecular biomarkers to help identify the high-risk clones at diagnosis and redefine the stratification [6,21].

We identified the presence of alterations in the *CREBBP* gene in 80% (4 out of 5) of the patients with relapse in our cohort, all of which occurred in the HAT domain of the gene. These alterations were found at the time of initial diagnosis in 2 and at relapse in another 2 patients. Notably, none of the specimens from patients in remission featured alterations in this gene. The *CREBBP* gene has been recognized as one of the most common relapse-enriched genes in ALL [22-24], and its association with relapse is particularly evident in the high-hyperdiploid subtype [10, 25]. Alterations in this gene affect the response to one of the key components of the treatment protocol, glucocorticoids, leading to a treatment failure [22]. The mechanisms through which CREBBP contribute to glucocorticoid resistance are considered to be associated with its activity as a transcriptional co-activator, which interacts with the glucocorticoid receptor (GR) and potentially modulates its transcriptional activity. When glucocorticoids bind to the GR, the receptor undergoes a conformational change allowing it to interact with co-activators like CREBBP, which in turn can acetylate histones and other chromatin regulators, promoting a more accessible chromatin structure and facilitating gene transcription of target genes involved in processes like apoptosis. Therefore, alterations in this gene can lead to disruption of normal cellular processes including transcriptional regulation, chromatin remodeling and apoptosis. Importantly, mutations in the *CREBBP* gene also have a therapeutic significance, as it has been found that different CREBBP inhibitors and histone deacetylase inhibitors can alleviate chemotherapy resistance and may become a successful approach for the treatment of relapsed ALL [26].

We also detected alterations in several other genes with potential prognostic value. These include deletions in *TP53* in two patients with relapse (in one of them relapse-specific) which were infrequent in the rest of the patients, and deletion in *EBF1* gene in one patient with relapse which was absent in the rest of the patients in our cohort. Previous studies have also associated these alterations with disease progression and reduced overall survival rates [3, 5, 22, 27, 28]. Furthermore, *NRAS* gene mutation was found at relapse only, in one of the patients. Mutations in this gene have been observed in high-risk ALL by others and have been reported as important biomarkers for poor relapse-free survival [23, 29, 30]. However, *NRAS* mutation was also lost in the relapse clone in another patient from our cohort, indicating its sub-clonal nature and uncertain role in clonal chemoresistance. By contrast, deletions of *IKZF1* and *CDKN2A/2B* genes, individually reported as high-risk markers for disease progression and correlated with poor outcome in several studies [31-34], were not found to independently influence prognosis in our study. Their prognostic significance has been further refined with the detection of the

IKZF1^{plus} profile [20] which, however, was not present in our cohort. Additionally, the occurrence of the *IKZF1* deletion in the high-hyperdiploid subtype, which was also associated with an increased relapse risk in a recent large prospective study [10], was detected in one patient in our cohort, who is still in remission after a follow-up of 46 months.

Concerning the evolutionary mechanisms of the clones from diagnosis to relapse, we found that none of the patients with relapse in our cohort experienced expansion of a novel clonal population completely distinct from the population present at diagnosis. Rather, in most of them (4 out of 5), in addition to the same clonal rearrangement and initiating genetic event (CNA, hybrid transcript), we observed novel alterations at relapse that were not detected in the primary clone/s. This either indicates that they were present in minor sub-clones, not detectable with the applied method, survived chemotherapy, and arose as dominant clone/s due to the presence of chemotherapy resistance mutations, or that they were acquired during chemotherapy (treatment-induced) [13, 22]. However, the absence of other alterations in three of these patients suggests that the clonal evolution from an ancestral sub-clone was probably the mechanism for relapse, which has also been described as the most frequent event by others [13, 24, 35]. Only one patient retained all the alterations within the diagnostic and relapse clones, suggesting a linear evolution [35].

The strengths of this study are that it includes an unselected cohort of pediatric patients with B-ALL who were uniformly treated, and that it provides comprehensive data for all patients along with detailed molecular characterization for those with relapse. The limitations of the study include the lack of complete knowledge of the frequency of SNVs at diagnosis in patients without relapse, the insufficient depth of the WES analysis to detect mutations present in minor sub-clones (<10%), and the relatively small patient cohort.

In conclusion, we identified that alterations in the epigenetic regulator *CREBBP* were the most frequent event in the patients with relapse, either appearing at diagnosis or being acquired at relapse. Screening for alterations in this gene at the beginning, and/or at multiple time-points during chemotherapy, could be incorporated into treatment protocols, as they may contribute to the identification of significant number of patients with predefined or acquired chemoresistant clones. In addition, detection of deletions in the *TP53* and *EBF1* genes in the *CREBBP*-negative patients could further help identify patients at increased risk for relapse. Finally, screening for clinically actionable alterations in these and other pathways and genes (RAS, MMR genes), could be of substantial significance for patients with relapse in the coming years by offering a more individualized targeted therapy or immunotherapy therapeutic approach.

SUPPLEMENTARY MATERIALS

Table S1: Type of the single nucleotide variants detected in the patients with relapse.

Table S2: The fragment lengths and Sanger sequencing details (V-D-J genes and CDR3 sequences) of the clonal immunoglobulin heavy chain gene rearrangements present in the matched diagnosis-relapse samples.

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Author Contributions

Conceptualization, A.D., S.K. and N.M.G.; Methodology, E.K.B., M.S.S. and E.G.; Software, E.K.B; Formal Analysis, E.K.B., M.S.S and E.G; Investigation, A.D., S.K., N.M.G. and E.K.B.; Resources, S.K. and A.J.; Writing – Original Draft Preparation, E.K.B.; Writing – Review & Editing, A.D., S.K. and N.M.G.; Supervision, A.D., S.K. and N.M.G.

Conflicts of Interest

The authors declare no conflict of interest.

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Institutional Review Board Statement

The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Ethics Subcommittee of the Macedonian Academy of Sciences and Arts (date of approval 03.04.2023).

Informed Consent Statement

Informed consent was obtained from all subjects involved in the study.

Data Availability Statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

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ORIGINAL ARTICLE

CHROMOSOMAL MICROARRAY IN CHILDREN BORN SMALL FOR GESTATIONAL AGE – SINGLE CENTER EXPERIENCE

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ABSTRACT

The association between small for gestational age birth and chromosomal abnormalities identified through karyotyping is well-established. Notably, advancements in cytogenetic techniques have shifted from routine karyotyping to the recommended use of microarray technology. This transition allows higher resolution and the detection of sub-microscopic copy number variants (CNVs).

Our study included 49 patients born small for gestational age, 27 males and 22 females. Clinical data were gathered from reports by clinical genetic specialists, and a questionnaire was included in the referral list to our laboratory. All participants were of pediatric age, ranging from neonatal to 12 years old. Chromosomal microarray testing was conducted by the Agilent SurePrint G3 Human CGH Microarray 8×60K.

The application of molecular karyotyping yielded clinically significant results in 16 cases (32.65%), which included 13 deletions and 6 duplications. Three patients presented with two clinically significant CNVs (csCNVs). In ten cases, we identified recurrent microdeletion or microduplication syndromes well-documented in the literature: Williams syndrome as the most commonly identified (three patients), and others like Koolen de Vries, Prader-Willi, Miller-Dieker, Dryer, DiGeorge syndrome, 7q11.23 microduplication, 16p13.11 microdeletion, and 1q21.1 microdeletion syndrome. Six patients had rare non-recurrent pathological CNVs. There was no statistically significant difference between patients with csCNVs and those without regarding the presence of intellectual disabilities, central nervous system, cardiac or skeletal malformations.

Chromosomal microarray proves to be a useful diagnostic tool in the etiology diagnosis of children born small for gestational age.

Keywords: chromosomal microarray, CNVs, small for gestational age

INTRODUCTION

Fetal growth restriction (FGR), or intrauterine growth restriction, refers to a condition where a fetus fails to reach its full growth potential [1]. Small for gestational age (SGA) is a term usually used to describe newborns (or fetuses) who weigh less than the 10th percentile of their population or customized growth charts based on gestational age [2, 3]. It is estimated that FGR impacts up to 10% of pregnancies while SGA is seen in at least 11% of newborns. It is important to note that around 40% of fetuses diagnosed as SGA do not have any underlying pathology and are simply constitutionally small in contrast to FGR where pathological mechanisms are frequently described. Therefore, SGA fetuses are not always growth-restricted and some fetuses with FGR could be appropriate for their gestational age but have not reached their maximum growth potential [2]. While there is considerable overlap between the two terms and despite existing inconsistencies in definition, most specialists use the term SGA to describe newborn size, which may or may not be linked to an underlying pathological cause. In contrast, FGR is generally caused by an antenatal pathologic disease [4].

FGR/SGA may have significant prenatal and postnatal consequences, such as increased risk of perinatal death, neurodevelopmental abnormalities, metabolic syndrome, and cardiovascular disease [5, 6]. Although the etiology and pathophysiological mechanisms can overlap, utero-placental

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dysfunction is the cause in the vast majority of cases of FGR [2]. However, multiple gestation, maternal disease, and structural and genetic fetal abnormalities are all possible causes [7]. Among these factors, fetal genetic defects, particularly chromosomal abnormalities, emerge as significant contributors.

The association between fetal growth impairment and chromosomal abnormalities identified through karyotyping is well-established. However, the strength of this association is significantly influenced by the gestational age at which growth impairment is identified [8], and the presence of structural fetal anomalies [9].

Over the past decades, the landscape of prenatal and postnatal screening has undergone a transformative shift, marked by advancements in technology and methodology. The introduction of the first-trimester combined test, along with other ultrasound exams during early pregnancy, has revolutionized the ability to screen for both structural and genetic abnormalities in fetuses [10]. The enhanced quality of imaging and expertise in ultrasound further contribute to the precision of assessing fetal phenotypes. Additionally, genetic testing has evolved from routine karyotyping to the recommended use of chromosomal microarray technology, enabling higher resolution and the detection of submicroscopic copy number variants (CNVs) [11]. CNVs are usually 1 kb to several Mb in length, include both duplications and deletions, and can affect single exons, one or several genes as well as regulatory sequences [12].

Through the postnatal application of CNV microarray technology, this research aims to clarify the complexities associated with small-for-gestational-age infants. It explores their phenotypic and genotypic spectrum, enhancing our knowledge of prenatal growth failure and paving the way for informed clinical decision-making and parental counseling.

MATERIAL AND METHODS

Patients

Our retrospective study included 49 patients born small for gestational age (27 males and 22 females). All patients were of pediatric age, ranging from newborn to 12 years. Their measured birth weights were below the 10th percentile for gestational age. Each patient was examined by clinical genetic specialists who provided detailed phenotypic reports. Clinical data were collected based on specialists' reports and the questionnaire included in the laboratory referral list. All guardians of the patients provided informed consent. The study was approved by the Ethics Committee Faculty of Medicine, University of Belgrade (1322/VII-4).

CNV detection and interpretation

Patients' DNA was isolated from 3-5ml of peripheral blood by the standard salting-out method [13]. The array-CGH method was performed using Agilent microarray oligonucleotide slides (SurePrint G3 Human CGH Microarray 8 × 60K) (Agilent Technologies, Santa Clara, CA, USA) according to manufacturer's protocol. Microarray slides were scanned with a DNA Microarray Scanner and data were obtained by Cytogenomic software (Agilent Technologies). Genomic positions were based on human genome reference sequence GRCh 37/hg19.

All identified copy number variations (CNVs) were analyzed and classified according to the most recent guidelines from the American College of Medical Genetics and Genomics (ACMG) [14]. The significance of these variants was evaluated based on several factors, including type (gain or loss), size, gene content (particularly dosage sensitivity), and inheritance pattern, all considered to the patient's clinical phenotype. To ensure proper classification of detected CNVs, a comprehensive review of relevant peer-reviewed literature and accessible databases such as PubMed, the Database of Genomic Variants (DGV), DECIPHER, ClinGen, and Online Mendelian Inheritance in Man (OMIM) has been performed. Benign CNVs were not reported. Pathogenic and likely pathogenic CNVs are considered clinically significant (csCNV). The diagnostic yield in our study was determined by detecting at least one csCNV in a patient.

Statistical analysis

Statistical analysis was performed by Pearson's chi-squared (χ^2) or Fisher's exact test using SPSS v.20.0 (SPSS Inc., Chicago, IL, USA).

RESULTS

The clinical characteristics of 49 patients who were born small for their gestational age are summarized in Table 1.

Molecular karyotyping yielded clinically significant results in 16 cases, resulting in a detection rate of 32.65%. We identified 13 deletions, ranging in size from 442 kb to 15480 kb, and six duplications, 404 kb to 64280 kb in size. Additionally, three patients had two csCNVs. In ten cases, we identified CNVs linked to well-known syndromes (see Table 2). The most common was Williams syndrome,

Table 1. Overview of the phenotypic characteristics of the patient group.

Feature	Patients n=49, n (%)
male/female	27 (55.1)/22 (44.9)
DD/ID	46 (93.9)
facial dysmorphism	40 (81.6)
microcephaly	14 (28.6)
cardiac anomalies	13 (26.5)
skeletal malformations	11 (22.4)
urogenital tract anomalies	8 (16.3)

DD – developmental delay; ID – intellectual disability

Table 2. Description of the genomic imbalances classified as pathogenic/likely pathogenic

Case	Gender	Age	GW	Birth weight (g)	Region involved	Boundaries	Size (kb)	MD syndromes, OMIM #	Additional phenotype
1	f	21	≥37	2500	1q21.1-q21.2	chr1:(146564743-147786706)x1	1200	1q21.1 microdeletion syndrome, #612474	failure to thrive, microcephaly, DD
2	m	19	38	2080	2p25.3	chr2:(1842071-2246200)x3	404		Microcephaly, DD
3	f	1	38	1860	3q22.1-q29	chr3:(133562250-197840339)x3	64280		congenital heart anomaly, cleft palate
4	m	1	≥37		7p15.3-p14.3	chr7:(20993642-30739239)x1	9750		severe FGR, renal hypoplasia, ASD, shortened long bones, facial dysmorphism
5	m	32	≥37	2350	7q11.23	chr7:(72726578-74139390)x1	1412	Williams, #194050	DD, facial dysmorphism
6	m	29	≥37	2360	7q11.23	chr7:(72726578-74139390)x1	1412	Williams, #194050	DD, gastroesophageal reflux disease, pulmonary artery stenosis
7	f	2	≥37		7q11.23	chr7:(72726578-74139390)x1	1412	Williams, #194050	pulmonary artery stenosis
8	m	24	38	2700	7q11.23 22q11.21	chr7:(72726578-74139390)x3 chr22:(18919942-21440514)x1	1412 2520	7q11.23 microduplication, #609757 DiGeorge, #188400	DD, palatoschisis, hydronephrosis
9	m	1	39	2230	7q35-q36.3 16q24.1-q24.3	chr7:(143425418-158909738)x1 chr16:(86743412-90111263)x3	15480 3370		Microcephaly, hypotropia, facial dysmorphism
10	m	1	≥37		9p24.3-p22.3 19q13.33-q13.43	chr9:(271257-14956477)x1 chr19:(50380618-59092570)x3	14685 8710		facial dysmorphism, shortened long bones,cryptorchidisam CRYPTORCHIDISM hypospadias
11	f	29	31/32	1150	15q26.2-q26.3	chr15:(94447479-102383473)x1	7940	Drayer sy, #612626	DD, ASD, VSD, VUR, short stature
12	m	1	≥37	1850	16p13.11	chr16:(14910205-16525348)x1	1600	16p13.11 microdeletion syndrome	Hypotrophy, hypotonia, facial dysmorphism
13	f	121	≥37	2650	15q11.2 - q13.1	chr15:(22765628-29085896)x1	6300	Prader-Willi, #176270	DD/ID, obesity, brachydactyly
14	f	1	39	2020	17p13.3-p13.2	chr17:(51885-3882130)x1	3830	Miller-Dieker, #247200	VSD, aberrant brain MRI findings
15	m	11	38	2080	17q21.31	chr17:(43717703-44159862)x1	442	Koolen de Vries, #610443	DD, neonatal hypothroidism microcephaly, colon perforation
16	f	132	≥37		19p13.2-p13.12	chr19:(12474346-14485846)x3	2010	;	microcephaly, ASD, short stature, learning difficulties

GW – gestational week; MD – microdeletion/microduplication, DD – developmental delay; FGR – fetal growth restriction; ASD – atrial septal defect; VSD – ventricular septal defect; VUR – vesicoureteral reflux; ID – intellectual disability; MRI – magnetic resonance imaging

diagnosed in three patients (18.7%). Other syndromes previously associated with intrauterine growth restriction include Koolen de Vries syndrome, Prader-Willi syndrome, Miller-Dieker syndrome, Dryer syndrome, and 1q21.1 microdeletion syndrome. In one patient CNVs typical for DiGeorge and 7q11.23 microduplication were detected. However, in patients with either syndrome, SGA is not one of the phenotypic characteristics. Another detected microdeletion that typically does not include SGA is the 16p13.11 microdeletion. We identified unique CNVs in six cases. The main phenotypic characteristics and microarray findings of all patients with csCNVs are summarized in Table 2. Most of the patients were in neonatal age (6 of them) and besides SGA they had additional clinical features. Older children had mild to moderate developmental delay/intellectual disabilities (DD/ID) and other comorbidities.

To determine if additional specific phenotypic characteristics could predict the detection of csCNV, we analyzed the frequency of these phenotypes in two groups: those with positive molecular karyotype findings and those without. All phenotypes except skeletal malformations were more common in a group with pathogenic CNVs compared to the group with normal molecular karyotype. It was pronounced for urogenital anomalies and microcephaly, although those differences did not reach statistical significance (Table 3).

Table 3. Differences in phenotypic characteristics between patients with pathogenic CNVs and those with normal molecular karyotype

Features	Pathogenic CNVs n=16	Normal molecular karyotype n=33	p-value
Facial dysmorphism	15 (93.7)	25 (75.7)	0.238
Microcephaly	7 (37.5)	7 (18.2)	0.176
Cardiac anomalies	6 (37.5)	7 (21.2)	0.304
Skeletal malformations	2 (12.5)	9 (27.3)	0.300
Urogenital tract anomalies	4 (25.0)	4 (12.1)	0.132

DISCUSSION

The purpose of our study was to enhance the understanding of SGA in the context of chromosomal abnormalities, encompassing advancements in diagnostic methodologies with a specific focus on SGA infants. The nature of growth disturbances is highly heterogeneous making it crucial to comprehend the complex relationship between fetal growth restrictions and genetic abnormalities. Accurate diagnostic testing is vital, as a genetic diagnosis significantly influences prognosis.

The majority of our patients presented with complex forms of SGA with a lot of comorbidities, which may explain the high detection rate of positive findings: 36.4%. The detection rate is significantly higher than the 16% found in other cases tested in our laboratory. These different cases involved individuals with DD/ID, congenital anomalies, autism, and epilepsy, but without intrauterine growth restriction. It highlights the importance of SGA as a key predictive phenotype in the diagnostic yield of molecular karyotyping. Chen et al. reported a chromosomal structural copy number variation (csCNV) detection rate of 33.3% in cases of FGR associated with structural anomalies, which aligns closely with our findings [15]. In contrast, FGR cases without structural malformations, which correspond to small for gestational age (SGA) infants without additional complications, have a lower incidence of genetic abnormalities. Wu et al. conducted an analysis of 488 fetuses diagnosed with FGR but without structural malformations. They found that the diagnostic yield for classic and molecular karyotypes was 3.9% [16]. Additionally, one meta-analysis indicated a 4% increased yield of chromosomal microarray analysis (CMA) compared to classic karyotyping in non-malformed growth-restricted fetuses. Furthermore, the incremental yield of CMA in cases of FGR associated with fetal malformations was 10% [17].

In our patient group, we most frequently observed Williams syndrome (P5, P6, P7), caused by a microdeletion on chromosome 7q11.23. This syndrome is characterized by a unique combination of clinical features, including distinctive facial characteristics, cardiovascular anomalies, intellectual disability, and a remarkably sociable personality [18]. The association between Williams syndrome and intrauterine or postnatal growth failure has been well documented, highlighting its importance among different types of fetal growth restrictions. At least 82% of fetuses with typical 7q11.23 deletion have intrauterine growth retardation [19]. Our study supports these findings, emphasizing that this deletion should be considered during prenatal assessments of FGR and in cases of SGA birth.

Our study unveiled several other genetic syndromes previously associated with FGR and SGA. In a two-year-old girl (P1) born small for gestational age, with microcephaly, failure to thrive, and facial dysmorphism, we

detected a clinically significant microdeletion of 1.2 Mb in 1q21.1-q21.2 region, as well as 5p14.1-p13.3 microdeletion of 3.1 Mb, classified as a variant of unknown significance (VUS). A microdeletion detected on chromosome 1 is the recurrent deletion of distal region 1q21.1 located between breakpoints BP3-BP4 and includes the *GJA5* gene. Liu et al. summarized prenatal phenotypes characteristic for 1q21.1 microdeletions and observed IUGR in 26.7% of the cases [20]. This microdeletion has low penetrance and variable expressivity. In many cases, it is inherited from healthy parents. The second CNV, a deletion in the region 5p14.1-p13.3, encompasses ten genes, none of which are protein-coding. To our knowledge, there are no reports on the phenotype of patients with similar deletions.

One well-known syndrome associated with prenatal and postnatal growth failure is Drayer syndrome (MIM #612626), caused by a deletion in the 15q26-qter region. Patient P11 exhibited a 5.6 Mb deletion in this region (15q26.2-q26.3) and presented with developmental delay, mild facial dysmorphia, short stature, and skeletal dysplasia. Microcephaly, congenital heart disease, epilepsy, diaphragmatic hernia, renal anomalies, neonatal lymphedema, and aplasia cutis congenita could be additional characteristics of this syndrome [21]. Haploinsufficiency of the insulin-like growth factor-1 receptor (IGF1R) gene, located in this region, has been previously associated with the growth pathway and linked to impaired prenatal and postnatal growth [22]. More proximally on chromosome 15 is a region frequently linked to benign but also pathogenic CNVs, 15q11.2, which contains imprinted genes. Deletion of paternal copy of *SNRPN* and the *NDN* genes in this region cause Prader-Willi syndrome (PWS; MIM #176270). P13, from our cohort, is a 12-year-old girl with DD/ID, obesity, brachydactyly, and a 6 Mb deletion characteristic of PWS. Her obstetric history includes intrauterine growth restriction beginning in the third trimester. During her first year of life, she experienced failure to thrive but subsequently became overweight, which is typical for individuals with PWS. This syndrome is rarely diagnosed prenatally due to the lack of well-defined fetal phenotypes, which would warrant prenatal molecular genetic testing [23]. In the study by Dudley et al. focusing on prenatal, perinatal, and postnatal complications in PWS, it was observed that 29.4% (10 out of 34) of patients with PWS caused by uniparental disomy (UPD) and 42.3% (22 out of 52) of PWS patients resulting from deletion were classified as small for their gestational age [24]. The reasons for SGA in these PWS cases remain unexplained.

We identified other recurrent syndromes linked to fetal and postnatal growth restriction. This includes a 3.8 Mb microdeletion in the 17p13.3-p13.2 region, causing Miller-Dieker syndrome, and a 425 kb microdeletion in the 17q21.31 region, associated with Koolen-de Vries syndrome. Haploinsufficiency of the *PAFAH1B1* and *YWHAE* genes in Miller-Dieker syndrome is believed to cause intractable seizures, severe developmental delays, lissencephaly, facial dysmorphisms, intrauterine growth restriction, and involvement of other organ systems. Growth restriction often persists during the postnatal period [25]. In our sample, this syndrome was diagnosed in a one-month-old infant with FGR identified from the 20th week of gestation, along with facial dysmorphia and abnormal neuroimaging findings observed after birth (P14). In the case of an 11-month-old boy (P15) born SGA, with DD, microcephaly, congenital hypothyroidism, and surgically corrected colon perforation postnatally, diagnosis of Koolen-de Vries syndrome was established by CMA. This condition is multisystemic and characterized by DD/ID, epilepsy, distinct facial features, and congenital malformations affecting multiple organ systems. Research conducted by Koolen et al. on a cohort of 45 children with this syndrome revealed that 26% of cases experienced intrauterine growth retardation, 30.4% presented with low birth weight, and 41.7% also had proportionate short stature postnatally [26].

A rare and interesting example of microduplication of the 19p13.2-p13.12 region associated with impaired growth was detected in a 12-year-old girl born SGA (P16), later followed by short stature treated with growth hormone therapy. She also had microcephaly, atrial septal defect, mild ID with learning difficulties, and autistic features. Previously, in Trimouille et al., ten patients with 19p13 duplications were reported. Common clinical features included short stature, small head circumference, delayed bone age, and ID (mild to severe). Unfortunately, birth parameters were unknown for six patients, and only one had a birth weight below the 10th percentile. The research indicates that the phenotype linked to 19p13 duplication may, in some respects, be regarded as the reciprocal phenotype of Malan syndrome (MIM # 614753, previously known as Sotos syndrome-2), which is caused by heterozygous mutations, including deletions of the *NFIX* gene [27, 28]. This syndrome is characterized by DD/ID, overgrowth, macrocephaly, prominent forehead, high anterior hairline, up-slanted palpebral fissures, and prominent chin. The observed phenotype in all patients with 19p13 microduplications that include *NFIX* indicates that the phenotypes associated with both 19p13 microdeletions and microduplications may result from the contrasting effects of *NFIX* haploinsufficiency and overexpression.

Patient P3, a 1-month-old infant, has a 64 Mb duplication of the region 3q22.1-q29. The patient presented with cleft lip, and a congenital heart anomaly. In most cases, this condition is diagnosed only after birth. Individuals with this syndrome may exhibit a range of defects, including

abnormalities of the central nervous system, facial dysmorphia, congenital heart defects, urogenital tract anomalies, intellectual disabilities, and growth disturbances [29].

Our cohort revealed some recurrent or non-recurrent syndromes not previously linked to SGA. In the first group it is interesting to mention a two-year-old boy (P8) with mild DD, palatoschisis and hydronephrosis, with two recurrent CNVs: 22q11.2 deletion characteristic for DiGeorge syndrome, and 7q11.23 microduplication, reciprocal to Williams syndrome chromosomal region. In patients with DiGeorge syndrome, FGR/SGA has been noted at a rate close to the population incidence [30]. There is insufficient prenatal information available regarding 7q11.23 microduplication. In the study conducted by Wang et al., fetuses diagnosed with 7q11.23 microduplication syndrome presented with several intrauterine phenotypes, including low-lying conus medullaris, dilated ascending aorta, cleft palate, anencephaly, hydronephrosis, and other renal anomalies [31]. While the other characteristics of the phenotype could be accounted for by the presence of the two different CNVs, associated with two well-described syndromes, intrauterine growth restriction is not typically observed in these cases.

Similarly, a one-month-old infant with SGA (P12) exhibited a 1.6 Mb microdeletion in the 16p13.11 region. This CNV has been previously described and reported as a predisposition to neurodevelopmental disorders and different congenital anomalies. Short stature has been observed in several cases [32]. This deletion can occur *de novo*; however, due to its incomplete penetrance and variable expressivity, it is often inherited from a parent who is either mildly affected or presents with a completely normal phenotype. In a study by Cai et al. on the 16p13.11 microdeletion/microduplication, it was found that fetuses with CNVs in this region typically do not exhibit any characteristic features on intrauterine ultrasound and are generally healthy after birth. [33]. Therefore, the SGA observed in our case cannot be attributed to the detected CNV.

Additionally, our study identifies some csCNVs in children with SGA that have not been linked to growth restriction before, either prenatal or postnatal. A 19-month-old boy (P2), who experienced FGR, developmental delay, and microcephaly, exhibited a microduplication at chromosome 2p25.3. This microduplication includes part of the *MYT1L* gene, previously associated with neurodevelopmental disorders. *MYT1L* acts as a transcriptional repressor in neuronal progenitor cells, inhibiting Notch signaling and promoting neuronal differentiation [34]. However, there is no possible explanation for FGR in patients with deletion of this gene. In the study by Coursimaults et al., which investigated 40 children with pathogenic variants of *MYT1L* and 22 previously published patients, FGR was not frequently observed (6-8% of the patients) [35].

Patient P4 was referred for CMA due to severe FGR, born small for gestational age, with atrial septal defect, renal hypoplasia, and shortened long bones. The analysis revealed a deletion of 9.75 Mb in the region 7p15.3-p14.3, which includes 60 protein-coding genes, 16 linked to various human diseases. Crippa et al described a patient with *de novo* deletion of 7.5 Mb in the same region. This patient experienced both prenatal and postnatal growth restriction and was part of a cohort exhibiting features consistent with Silver-Russell syndrome. The authors suggested that the growth restriction might be attributed to the insulin-growth factor 2 mRNA binding protein 3 (IGF2BP3) gene (OMIM*608259) deletion and confirmed its decreased expression. This gene looks like a promising candidate for FGR since it regulates the amounts of IGF2 transcripts by encoding an RNA-binding factor unique to the 5′UTR of IGF2 mRNA [36].

We had three patients with two pathogenic CNVs on different chromosomes. In these cases, it is challenging to determine the impact of a single region or specific gene because the phenotype often results from the interaction between the two variants from different genomic regions. A newborn (P9) was found to have a 15.5 Mb deletion in the region 7q35-q36.3. This presented with microcephaly, SGA, and a progeroid appearance characterized by a "bird-like face." Fan et al. summarized the phenotypes of 17 previously reported patients with terminal deletions in the 7q35-q36.3 region, noting that 16 of these patients experienced growth restriction. Additionally, multiple congenital malformations were observed, including abnormalities in brain and facial structures, DD/ID, limb and sacral anomalies [37]. The patient in our cohort also had a 3.37 Mb microduplication in the 16q24.1-q24.3 region, which has previously been linked to mild-to-moderate ID, speech delay, and slight dysmorphic features [38]. However, there are no reports connecting this microduplication with FGR or SGA. It appears that terminal deletion on chromosome 7q has a more significant impact on growth restriction.

A newborn patient (P10) was born small for gestational age and presented with facial dysmorphism, cryptorchidism, and hypospadias. Genetic analysis revealed a terminal deletion of 14.7 Mb in the region 9p24.3-p22.3 and a terminal duplication of 8.71 Mb in the region 19q13.33-q13.43. The deleted region contains 44 protein-coding genes and 14 disease-associated. The phenotype of patients with a distal deletion of chromosome 9 includes trigonocephaly, DD/ID, and genitourinary malformations (MIM# 158170). The duplicated region on chromosome 19 contains 290 protein-coding genes, with 29 of these genes associated with diseases. Distal duplications of chromosome 19 are linked to various conditions, including low birth weight, short stature, craniofacial dysmorphia, and psychomotor delay. Additionally,

individuals may exhibit hypotonia, epilepsy, congenital heart defects, as well as urogenital and gastrointestinal malformations [39]. In this case, the expression of both pathogenic CNVs overlaps; however, the distal duplication of 19q may better explain the observed features related to SGA.

Identifying these syndromes has significant clinical implications beyond merely confirming a diagnosis. It includes early interventions to improve long-term outcomes for individuals affected by these conditions. Key management strategies for individuals with genetic syndromes include cardiac and other organ systems examination and monitoring, developmental support, and behavioral interventions. This underscores the importance of early detection and timely intervention in their care.

Most patients born SGA experience catch-up growth until they are 2 to 4 years old; however, 10% to 15% of them do not. Six of our patients with pathogenic CNVs were at neonatal age when the CMA was performed. At the same time, only five were older than two years. As a result, we have limited information regarding the postnatal growth of our patients. Among the five children older than two, short stature was documented only in two: one girl with Dryer syndrome (P11) and another with duplication at 19p13.2-p13.12 (P16). The latter patient was already receiving recombinant human growth hormone therapy (rhGH). For the past twenty years, rhGH has been approved for use in children SGA and short stature aged 2 in the USA and 4 in Europe. The response to this treatment is not always optimal, and it depends on the underlying cause of the SGA, which highlights the importance of genetic testing before starting the therapy [40].

In cases with detected csCNVs but without a clear association with SGA, it is possible that SGA may have been caused by different external environmental factors. The limitation of our study is that we did not have information about possible factors present during pregnancy that could lead to SGA. Other significant limitations are the small number of patients that were available for our research since this was a single-center study and the lack of follow-up data on the growth in the case of the children who were tested as newborns. Increasing the size of the study group and collecting information regarding the growth of children born small for gestational age would be of great importance for the following research.

In conclusion, integrated genetic testing that combines chromosomal microarray analysis with a thorough assessment of phenotypes provides valuable insights into the genetic causes of growth restriction. This method allows for the identification of clinically significant copy number CNVs and supports the development of personalized management strategies tailored to the specific needs of individuals affected by this condition.

Ethics Committee approval

The study was approved by the Ethics Committee Faculty of Medicine, University of Belgrade (1322/VII-4).

Conflict of interest

The authors declare no conflict of interest.

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ORIGINAL ARTICLE

DELINEATION OF PARTIAL CHROMOSOMAL ABNORMALITIES IN EARLY PREGNANCY LOSSES

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ABSTRACT

Pregnancy loss (PL), particularly early pregnancy loss (EPL), is a prevalent reproductive complication, with approximately 15% of confirmed pregnancies affected. Chromosomal abnormalities are implicated in more than half of EPLs, with trisomies being the most prevalent. Partial abnormalities, including segmental deletions, duplications, and unbalanced translocations, are detected in up to 10% of EPL cases. This study focuses on the precise characterization of partial chromosomal abnormalities, previously identified by Quantitative fluorescent polymerase chain reaction (QF-PCR) and multiplex ligation probe amplification (MLPA) analyses. By employing an array comparative genomic hybridization (aCGH), we analyzed 20 EPL samples, identifying 32 partial chromosomal abnormalities, including 18 deletions and 14 duplications, with an average size of 33.2 Mb. Notably, two abnormalities previously undetected by QF-PCR and MLPA were revealed (deletions in 7q36, and 1p36.32p36.31regions), emphasizing the necessity of high-resolution genomic tools. Chromosomes 1, 18, and 13 emerged as frequently involved, aligning with previous associations with recurrent pregnancy loss. Recurrent abnormalities were identified in six chromosomal regions, with chromosome 1p36.33-p36.32 exhibiting the highest frequency. Gene Ontology (GO) enrichment analysis of recurrent regions highlighted disruptions in critical biological processes, including molecular binding, enzymatic activity, and cellular development. Many genes in these regions are linked to multisystem syndromes, suggesting their involvement in early embryonic development and pregnancy viability.

Our findings underscore the complexity of EPL's genetic landscape, demonstrating that large CNVs, may disrupt multiple genes critical for development. Although, subtelomeric MLPA reliably detects telomeric partial chromosomal abnormalities in EPLs, aCGH is essential for detection and precise characterization of all CNVs, thus enhancing diagnostic and counseling strategies in affected couples.

Key words: Partial fetal chromosomal abnormalities, early pregnancy loss, EPLs, aCGH

INTRODUCTION

Pregnancy loss (PL), encompassing spontaneous abortion or miscarriage, refers to the premature termination of a pregnancy before fetal viability, typically before the 20th gestational week. Early pregnancy loss (EPL) denotes losses occurring within the first trimester (<12 weeks) [1]. Approximately 15% of couples with confirmed pregnancies experience EPL, with recurrent PL (RPL) affect around 2% of them [2]. The etiology of PL is complex, involving a confluence of maternal and fetal factors. Maternal factors include endocrine disturbances, uterine anomalies, implantation issues, and infections [3]. In the event of fetal cause of EPL, chromosomal abnormalities account for roughly half of EPL cases, whereas the underlying cause remains elusive in the other half. Chromosomal trisomies, are the most prevalent fetal chromosomal aberrations, constituting up to 56% of abnormal EPLs, with trisomy 16 being the most common [4, 5]. Triploidy and monosomy, each affecting about 15% of abnormal conceptuses, follow in frequency. Rarer chromosomal anomalies comprise a smaller proportion of these cases. Lately, with the development of the molecular

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genetic technologies, especially with the widespread application of the whole exome sequencing (WES), embryonic and fetal monogenic conditions, were also reported in EPLs. Genes linked to fetal death, such as *CPLANE1, CHD7, FBN1, FGFR3, NIPBL*, and *SOS1*, are often associated to multisystem disorders. Others are associated or related with specific conditions like cardiac anomalies (*CSRP3, GATA4, SCN5A*), skeletal dysplasia (*COL1A1, FGFR2*), kidney diseases (*GREB1L, NPHS1*), and CNS abnormalities (*PIK3R2*). This diversity suggests that EPL has varied etiologies [6-11].

Partial chromosomal aberrations, involving gain or loss of chromosomal content, represent a significant factor contributing to EPL. Our previous research identified these aberrations in 8% of abnormal EPLs, demonstrating heterogeneity across different chromosomes [4].

Quantitative fluorescent polymerase chain reaction (QF-PCR) and multiplex ligation probe amplification (MLPA) have been established as valuable screening tools for chromosomal abnormalities in the context of EPL. These techniques have enabled detailed analysis of chromosomal aberrations, including partial chromosome abnormalities, revealing their diverse nature and impact on pregnancy outcome [4, 12-14]. Consequently, while these techniques provide a valuable first step in the diagnostic process, they may not yield a definitive genetic diagnosis in all cases since they cannot detect all abnormalities, such as interstitial chromosomal abnormalities or determine the size and gene content of the detected partial chromosomal abnormalities. Understanding these abnormalities is crucial for improving diagnostic accuracy, genetic counseling, and potentially developing preventive strategies for recurrent pregnancy loss. Given the profound implications of partial chromosomal imbalances for fetal development and pregnancy outcome, a comprehensive and in-depth characterization of these aberrations is imperative.

In this study, we employed array comparative genomic hybridization (aCGH) to delineate the genomic architecture of previously detected partial chromosomal imbalances, including their size, location, and gene content. This granular level of analysis is expected to provide deeper insights into the pathogenic mechanisms associated with these aberrations and contribute to a more comprehensive understanding of the complex fetal etiology of early pregnancy loss.

MATERIALS AND METHODS

Study group and selection of samples

In a prior investigation, partial chromosomal abnormalities were identified in 16 out of 900 analyzed EPL samples [4]. More recently, an additional four samples exhibiting partial chromosomal abnormalities were detected and incorporated into the current analysis, bringing the total number of EPL samples included in this study to 20. These samples consisted of single chromosomal abnormalities in half, while the other half exhibited double chromosomal abnormalities.

All EPL samples referred to the Research Center for Genetic Engineering and Biotechnology "Georgi D. Efremov", at the Macedonian Academy of Sciences and Arts, Skopje for analysis of chromosomal aneuploidies were selected by a collaborative team of gynecologists and pathologists. Standard histopathological analysis was performed on all EPLs, as described previously [14, 15]. To ensure ethical compliance, written informed consent was obtained from all study participants, and the research protocol received approval from the institutional ethics committee (Macedonian Academy of Sciences and Arts, 09-1047/6 from 04.05.2016).

All EPL samples were processed using standard phenol-chloroform or automated extraction methods to obtain genomic DNA for further analysis. To initially exclude maternal contamination and to screen for common aneuploidies, we employed quantitative fluorescent polymerase chain reaction (QF-PCR) with a panel of short tandem repeat (STR) markers targeting chromosomes 13, 18, 21, and the sex chromosomes [16]. This method allowed for rapid detection of trisomies and monosomies involving chromosomes 13, 18, 21 and X, as well as triploidies. To complement QF-PCR, multiplex ligation-dependent probe amplification (MLPA), using P036-Subtelomere Mix 1 and P070-Subtelomere Mix 2B probemixes, was implemented to identify chromosomal imbalances on all chromosomes.

Array comparative genomic hybridization (aCGH)

To further elucidate the genomic architecture of the observed partial chromosomal abnormalities, array comparative genomic hybridization (aCGH) analysis was conducted following Agilent Technologies' SureTag Complete DNA Labeling Kit (Agilent Technologies, Santa Clara, CA, USA). Genomic DNA extracted from the analyzed samples, along with sex-matched control DNA, was enzymatically fragmented using the restriction enzymes AluI and RsaI to ensure uniform DNA fragment sizes. Subsequently, the fragmented DNA samples were fluorescently labeled: the experimental DNA was labeled with Cyanine 5 (Cy5), while the control DNA was labeled with Cyanine 3 (Cy3). After purification, the labeled experimental and control samples were combined and co-hybridized onto Agilent Technologies' CGH Constitutional G3 4x180k microarrays. Following hybridization, data acquisition was performed using Agilent Technologies' DNA microarray scanner with surescan technology, ensuring accurate cap-

ture of fluorescence signals. The resulting data were subsequently processed and analyzed using Agilent's Genomic Workbench software platform. This analysis provided a comprehensive visualization of CNVs, with detailed genomic annotations based on the UCSC hg19 genome assembly, facilitating precise mapping and interpretation of the chromosomal imbalances observed in the study.

Identifying Recurrent Chromosomal Regions and Gene Ontology (GO) enrichment analysis

To gain insights into the genetic factors underlying early pregnancy loss, we conducted a comprehensive analysis of a total of 32 chromosomal abnormalities detected by aCGH in 20 EPLs with partial chromosomal abnormalities. We have established a criterion for identifying recurrent chromosomal regions: a chromosomal region, included in deletion or duplication, had to be observed in more than two EPLs. We determined the smallest overlapping region (SOR) for each recurrent chromosomal region. This approach enabled us to pinpoint the specific genes and pathways that are likely involved in the pathogenesis of early pregnancy loss.

To gain a deeper understanding of the biological functions and processes associated with the identified genes, we conducted a Gene Ontology (GO) enrichment analysis utilizing the Panther software [12]. This analysis compares the frequency of specific GO terms within a group of genes involved in SORs. Significantly enriched GO terms were visualized using a bar plot, thus providing a clear and intuitive way to interpret the results of the enrichment analysis and identify the biological functions and processes and that are most likely to be involved in EPL.

RESULTS

General findings

To investigate the genomic landscape of the previously detected partial chromosomal abnormalities in EPLs with QF-PCR and subtelomeric MLPA methods, we have performed array CGH analysis on a total of 20 samples.

All 30 previously detected chromosomal abnormalities among the 20 studied EPLs were confirmed and 2 additional chromosomal abnormalities previously not detected with QF-PCR and subtelomeric MLPA methods were revealed. These additional abnormalities were out of the ligation sites of the used subtelomeric MLPA probes, therefore they were not detected previously.

Among the 20 investigated samples, the total number of detected partial chromosomal abnormalities was 32, of which 18 (56% of all detected abnormalities) were deletions and 14 were duplications (44%). Nine samples had single partial chromosomal abnormality, 10 samples carried double abnormalities, while one sample carried three partial abnormalities. All the characterized abnormalities were on distal chromosomal sites, except one sample which had an additional aberration located proximally (7q22.1q34) of the expected single chromosomal abnormality on chromosome 7q36, previously not detected with the QF-PCR and subtelomeric MLPA methods. Also, another sample with deletion on 1p region and duplication on 1q region detected previously by MLPA, was found to carry an additional duplication involving the 1p36.32p36.31 chromosomal region. Schematic presentation of the detected chromosomal abnormalities among the affected chromosomes is shown on Figure 1. The sizes of

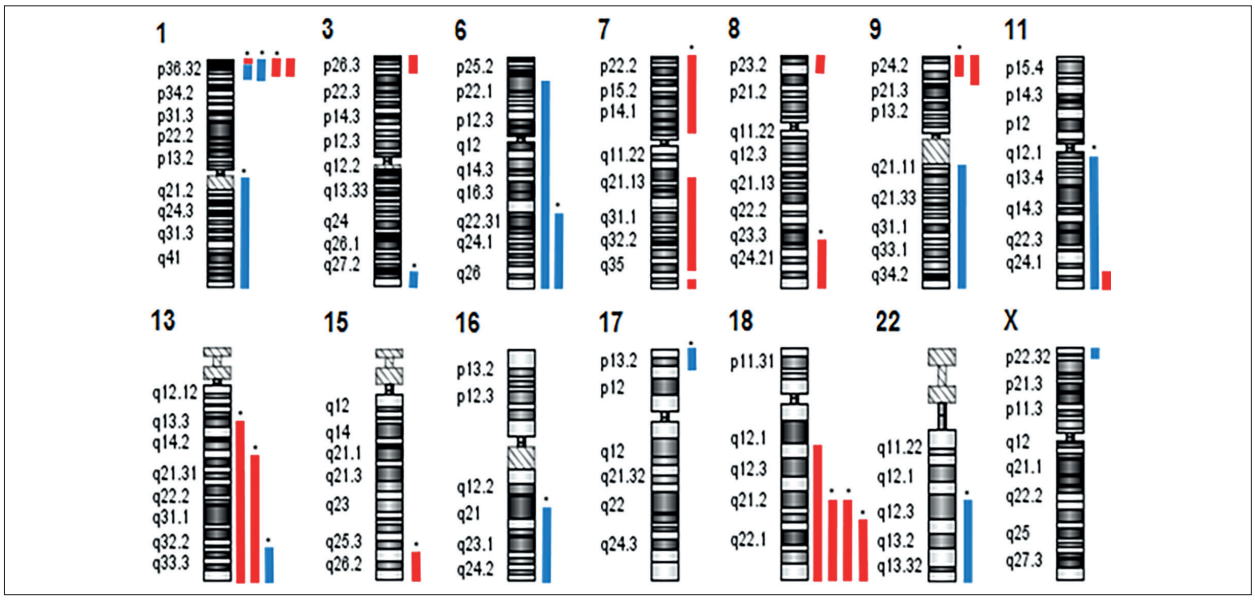

Figure 1. Figure 1. Schematic presentation of the detected chromosomal abnormalities among the affected chromosomes. (Red bars-deletions; blue bars-duplications), *chromosomal abnormalities detected together with other abnormality, see Table 1.

Table 1. Detailed genomic locations of the detected partial chromosomal abnormalities

	Sample	Chromosome band	Start-stop positions	Deletion/Duplication	Length (Mbp)
1	Abp-24	3p26.3-p26.1	73914-7761159	Deletion	7.68
2	Abp-118	6p22.3-q27	20695423-170911240	Duplication	150.21
3	Abp-141	17p13.3-p13.2	59739-4424713	Duplication	4.36
		18q21.2-q23	49010709-78010032	Deletion	28.99
4	Abp-154	8p23.3-p23.1	176814-12686483	Deletion	12.51
5	Abp-264	Xp22.33	169064-2344976	Duplication	2.17
6	Abp-359	11q24.2q25	127546842-134927114	Deletion	7.38
7	Abp-498	9p24.3-p23	204193_9092170	Deletion	8.88
8	Abp-565	8q24.13-q24.3	123993466-146294098	Duplication	22.3
		13q14.3-q34	54627609-115089535	Deletion	60.46
9	Abp-612	3q25.33-q29	160109099-197845254	Duplication	37.73
		9p24.3-p22.3	204193-14453418	Deletion	14.25
10	Abp-627	7q22.1-q34	101658846-140064713	Deletion	38.4
		7q36.3	158074913-159118566	Deletion	1.04
11	Abp-760	18q12.1-q23	25698937-78010032	Deletion	52.31
12	Abp-805	1p36.33-p36.32	564424-2352253	Deletion	1.78
		1p36.32p36.31	2394396-5710339	Duplication	3.31
		1q21.1q44	142617943-249212668	Duplication	106.59
13	Abp-815	1p36.33-p36.13	564424-17886109	Deletion	17.32
14	Abp-840	13q13.3-q34	39536567-115093155	Deletion	75.55
		16q21-q24.3	64826408-90148400	Duplication	25.32
15	Abp-882	1p36.33-p36.11	564424-24570889	Deletion	24.01
		11q11-q25	55361638-134904063	Duplication	79.54
16	Abp-914	13q32.3-q34	101077865-115105297	Duplication	14.02
		18q21.31-q23	55906486-78010032	Deletion	22.1
17	Abp-941	9q21.11-q34.3	70984337-141087916	Duplication	70.1
18	Abp-942	6q22.31-q27	122649716-170911237	Duplication	48.26
		7p22.3-p13	45064-45032669	Deletion	44.98
19	Abp-990	1p36.33-p35.2	746608-31284865	Duplication	30.53
		18q21.2-q23	49862572-78010032	Deletion	28.14
20	Abp-998	15q26.1-q26.3	93296596-102388476	Deletion	9.09
		22q13.1-q13.33	37984158-51219009	Duplication	13.23

the abnormalities ranged from 1.04 Mb to 150.21 Mb, with average size of 33.2 Mb. Detailed genomic locations of the detected partial chromosomal abnormalities is presented in Table 1 and the gene content of the detected abnormalities is described in Supplementary Table 1.

Chromosome 1 exhibited the highest frequency of abnormalities, with six of the detected alterations mapped to this chromosome. Chromosome 18 was the second most affected, harboring four abnormalities, followed by chromosome 13 with three abnormalities.

Recurrent chromosomal regions and GO enrichment analysis

To identify significant CNV regions and to determine the molecular functions and biological processes of the genes contained in these regions who are associated with early pregnancy loss (EPL), we first focused on recurrent regions (>2) among the 32 detected partial chromosomal alterations determining the SOR regions.

With this approach, we identified a total of six recurrent events. The most common recurrent event was on the terminal part of the chromosome 1p, with SOR of 1.78 Mb in the 1p36.33-p36.32 regions, shared by five deletions/duplications Common recurrent events were found on the terminal part of chromosomes 18q (SOR of 22.1 Mb in the 18q21.31-q23 region) and chromosome 13q (SOR of 14.02 in the 13q32.3-q34 region). With two recurrent events were chromosomes 6q, 9p and 11q, with SOR of 48.26 Mb in the 6q22.31-q27 region, SOR of 8.88 Mb in the 9p24.3-p23 region and SOR of 7.38 Mb in the 11q24.2q25 region.

Schematic presentation of the SOR regions is presented on Figure 2. The OMIM genes in every recurrent abnormality were identified and the respective diseases caused by those genes were paired. The most common disorders caused by the identified genes were multisystem syndromes, which may explain their pathogenicity in early human development. The OMIM genes and the associated disorders are shown in Supplementary Table 2.

Gene Ontology (GO) enrichment analysis was conducted using Panther, and significantly enriched GO terms were visualized through bar plots (Figures 3 and 4). The analysis revealed that the genes are predominantly associated with molecular binding activities, enzymatic functions as catalytic molecules, or ATPase activity.

In terms of biological processes, the recurrent genes identified in this study primarily encode proteins involved in essential functions such as biological regulation, cellular processes, and metabolic processes (Figure 4). Furthermore, several genes within the affected regions were linked to developmental processes, indicating that their dysfunction could contribute to early developmental abnormalities, potentially leading to pregnancy loss.

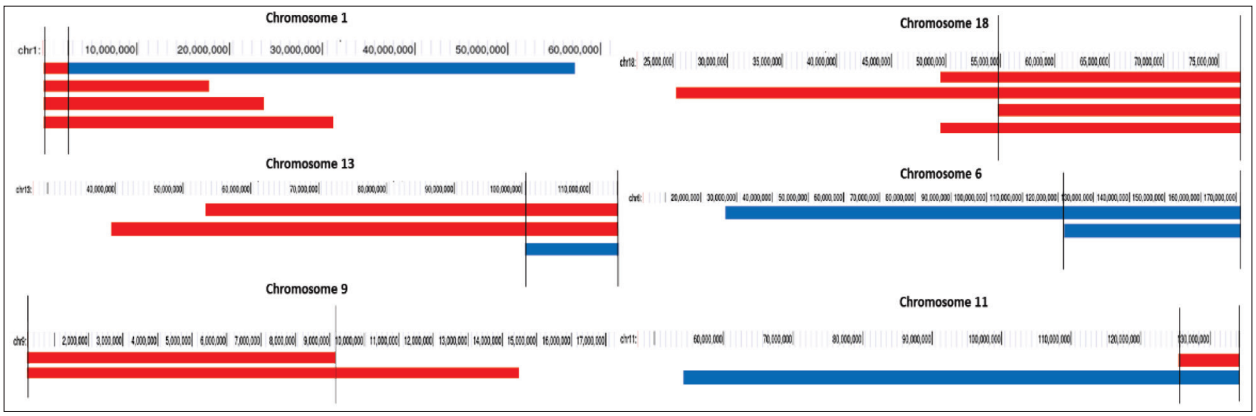

Figure 2. The detected smallest overlapping regions (SORs), (Red bars-deletions; blue bars-duplications).

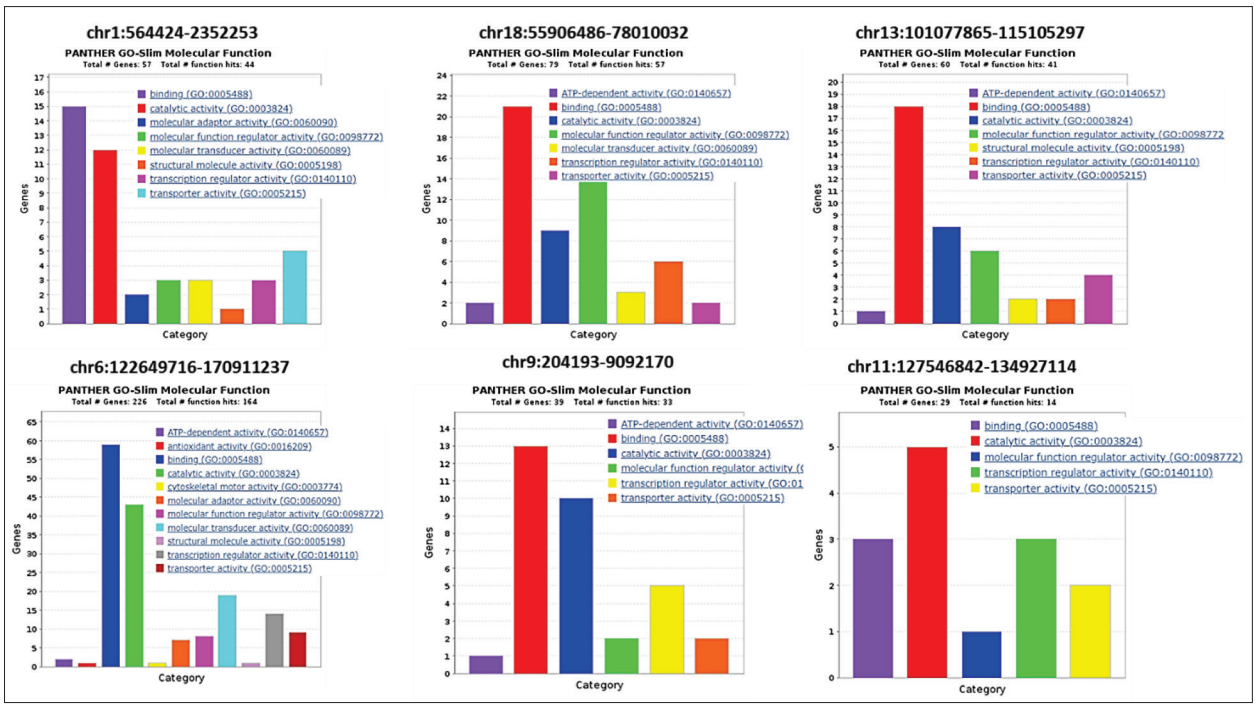

Figure 3. Molecular function profiling of recurrent genes in the studied regions.

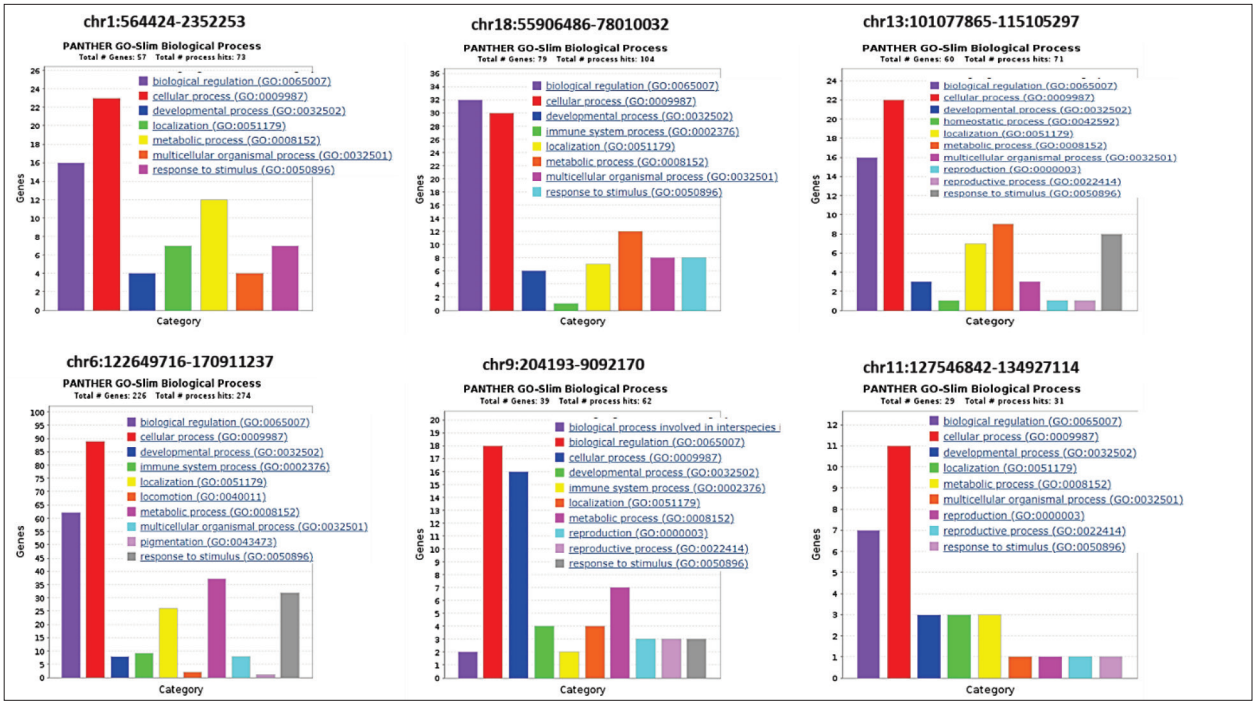

Figure 4. An analysis of the biological processes associated with frequently occurring genes within the specified recurring regions.

DISCUSSION

This study aimed to refine the characterization of previously identified partial chromosomal abnormalities in EPLs from early pregnancy losses (EPLs) by employing aCGH. Our findings significantly expand upon the initial QF-PCR and subtelomeric MLPA data, providing a detailed understanding of the genomic landscape in these cases. The identification of multiple abnormalities within individual samples, unidentified by the previous analyses underscores the complex genetic etiology of EPLs. For instance, in one sample we identified an additional abnormality located proximally to the expected single chromosomal abnormality on chromosome 7q36, which had not been previously detected using the QF-PCR and subtelomeric MLPA. In another sample, we discovered an additional duplication on the 1p36.32p36.31 chromosomal regions, despite the initial distal deletion and duplication on 1p and 1q regions respectively. Interstitial chromosomal abnormalities account for approximately 5–10% of chromosomal anomalies in early pregnancy losses (EPLs) [18, 19]. These abnormalities cannot be detected using methods such as the previously used QF-PCR and subtelomeric MLPA, underscoring the limitations of these techniques. This highlights the critical need for high-resolution approaches, such as aCGH in EPLs with no chromosomal abnormality detected by subtelomeric MLPA.

Our study's findings are particularly noteworthy given the large size of the CNVs detected (averaging 33.2 Mb), suggesting that these genetic alterations may disrupt multiple genes and are essential for normal embryonic development. The frequent involvement of chromosomes 1, 18, and 13 in these abnormalities is in line with their established association with pregnancy loss [20, 21]. These chromosomes have previously been implicated in recurrent pregnancy loss (RPL), particularly in studies of aneuploidy and large chromosomal rearrangements [22]. Chromosomal copy number variations (CNVs) in key regions such as 1p36.33-p36.32, 9p24.3-p23, 11q24.2-q25, 13q32.3-q34, and 18q21.31-q23 have been strongly implicated in early pregnancy loss (EPL). These regions host genes critical for apoptosis, placental development, and cellular signaling. For example, 1p36.33-p36.32 harbors genes essential for apoptosis, while abnormalities in 11q24.2-q25 involve genes like *ETS1*, crucial for connective tissue integrity. Disruptions in 18q21.31-q23 affect placental function, and 13q abnormalities interfere with vascular development. Advances in next-generation sequencing have enhanced the identification of these CNVs in EPL cases, confirming their significance in embryonic viability [23-26]. In live-born individuals, similar abnormalities may result in congenital disorders, developmental delays, and physical malformations, but often allow for survival past the prenatal period. The key difference lies in the severity of the

genetic disruption and the associated clinical outcomes, with EPL cases often involving more pronounced, lethal alterations. By contrast, live-born individuals may exhibit milder phenotypes due to mosaicism or less severe genetic disruptions [27].

Around 1/3 of all genes in the recurrent regions are associated with multisystem syndromes, as shown by the OMIM genes enrichment analysis (Supplementary Table 2). Some of the genes are strongly associated with EPLs due to their critical roles in embryonic development and placental function. For instance, *PEX10* and *PEX3*, linked to peroxisomal biogenesis disorders, often result in embryonic lethality due to metabolic dysfunction [28]. *COL4A1* and *COL4A2*, involved in vascular integrity, are associated with placental abnormalities and pregnancy complications [29]. Additionally, *CITED2* plays a vital role in placental development, and its deficiency is linked to embryonic lethality [30]. The results of our Gene Ontology (GO) enrichment analysis provide additional insights into the biological processes potentially disrupted by these chromosomal abnormalities. Our data suggest that the recurrent genes identified are primarily involved in essential biological processes such as molecular binding, enzymatic activity, ATPase activity, growth, metabolism, reproduction, and developmental processes. Dysregulation of these genes could plausibly lead to early embryonic developmental abnormalities, contributing to pregnancy loss. This finding is consistent with previous studies highlighting the role of genetic dysregulation in critical pathways such as apoptosis, cell cycle control, and placental development in EPL [31-36].

It is essential to acknowledge the limitations of this study, including the retrospective design and relatively small sample size. These factors may restrict the generalizability of our findings. The majority of the detected chromosomal abnormalities could have arisen from parental balanced chromosomal translocations [37]. Unfortunately, this cannot be confirmed at the time, since we were unable to perform parental karyotypes. The probabilities of different reproductive outcomes for carrier individuals of reciprocal balanced translocations are commonly based on an estimate of the likelihood of a fetus to survive with chromosomal imbalances resulting from the adjacent-1 segregation, while conceptions with other unbalanced segregations may not be viable [38]. This can lead to a more precise risk assessment and proper genetic counselling for the next pregnancies of the couples.

The focus on CNVs also does not exclude the possibility of other genetic factors contributing to EPLs, such as single nucleotide variants (SNVs) or epigenetic changes that were not assessed in this study. Furthermore, while aCGH offers higher resolution than many traditional methods, it does not capture all types of genomic variation [39]. Future studies should aim to integrate CNV data with whole-genome sequencing and epigenetic profiling to develop a more comprehensive understanding of the genetic landscape of EPLs. Functional validation studies using in vitro and in vivo models are critical to elucidating how these genetic changes affect cellular and developmental processes [40-42]. This comprehensive research approach will be essential for understanding the underlying mechanisms of EPLs.

CONCLUSION

In conclusion, our study provides valuable insights into the partial chromosomal abnormalities associated with EPLs. The identification of recurrent CNV regions and their associated genes represents a significant step towards understanding the complex genetic etiology of this condition. Although, subtelomeric MLPA reliably detects telomeric partial chromosomal abnormalities in EPLs, aCGH is essential for detection and precise characterization of all CNVs, thus enhancing diagnostic and counseling strategies in affected couples.

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Ethics Committee Approval

The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Ethics Committee of the Macedonian Academy of Science and Arts (09-1047/6 from 04.05.2016). Written informed consent was obtained from all study participants.

Author Contributions

Concept-Gj.B.; D.P.K; Design-D.P.K; Supervision-D.P.K; Materials-K.K.S.; Data Collection-Gj.B, M.T, K.K.S.; Analysis and Interpretation-Gj.B, M.T.; Literature Review- Gj.B, D.P.K.; Writing-Gj.B, D.P.K.; Critical Review-Gj.B, M.T., K.K.S., D.P.K.

Conflict of Interest

The authors declared no conflict of interest.

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ORIGINAL ARTICLE

A PILOT STUDY OF *ANXA2*, *MED12*, *CALM1* AND *MAPK1* GENE VARIANTS IN PRIMARY HYPERPARATHYROIDISM

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ABSTRACT

Primary hyperparathyroidism (PHPT) is a common endocrine disorder characterized by the overactivity of the parathyroid glands. While a few genes have been linked to a predisposition for PHPT, the genetic foundation of the disease remains unclear, despite it being the third most prevalent endocrine disorder. This pilot study aimed to investigate, for the first time, the potential association between specific variants in Annexin A2 (*ANXA2*-rs7170178, rs17191344, rs11633032), Mediator Complex Subunit 12 (*MED12*-rs1057519912), Calmodulin 1 (*CALM1*-rs12885713), and Mitogen-Activated Protein Kinase 1 (*MAPK1*-rs1057519911) genes with PHPT. Previous expression analyses have indicated that the proteins related to these genes are involved in parathyroid adenomas or PTH signaling. Fifty unrelated PHPT patients and an equal number of healthy controls were enrolled in the study. Genotyping was conducted using the polymerase chain reaction - restriction fragment length polymorphism assay. Statistical analysis was performed to assess the connection between genetic variants and PHPT. Our results revealed no significant differences in genotypes' or alleles' distributions of any of the studied variants between PHPT patients and controls. These findings suggest that these variants may not be linked to PHPT in the studied population. This pilot study, focusing on a Caucasian group of PHPT patients, contributes to the existing genetic data for future meta-analyses, which will provide a more precise definition of the genetic factors associated with PHPT susceptibility worldwide.

Keywords: *ANXA2*, *CALM1*, *MAPK1*, *MED12*, genetic variants, primary hyperparathyroidism

INTRODUCTION

Parathyroid hormone (PTH) acts as an important regulator of calcium homeostasis in the human body [1]. The importance of PTH is reflected by the wide range of functions that calcium performs, as it participates in cell signaling, neural and muscular function, hormone release and regulation, and bone metabolism [2]. PTH increases the reabsorption of calcium in the kidney and the gastrointestinal tract while at the same time enhances the release of calcium from the bone reservoir by indirectly stimulating osteoclasts. Finally, PTH stimulates the conversion of 25-hydroxy vitamin D into 1,25-dihydroxy vitamin D (calcitriol), which is the active form of vitamin D and is released into the circulation [1].

Primary hyperparathyroidism (PHPT) is a prevalent endocrine disorder distinguished by the independent secretion of PTH as a result of overactivation of the parathyroid

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glands [3]. It is the third most common endocrine disorder after diabetes and thyroid disease, with prevalence between 0.1-0.4%. The number of diagnoses increases with age, peaking at 50-60 years, and it is more likely to occur in females. For the time being, PHPT is diagnosed by abnormalities in PTH and blood calcium levels [4].

Almost 90% of the patients with PHPT are found to have sporadic, non-familial, and non-syndromic disease. Sporadic PHPT is usually caused by a single gland adenoma (85%) but may also be caused by hyperplasia of all four glands (10%). Double adenomas (2-5%) and parathyroid carcinomas (<1%) account for the least common causes of the disease. Several genes and pathways have been implicated in PHPT. These include genes involved in cell cycle regulation, Wnt/β-catenin signaling pathway, cellular growth, proliferation, tissue repairing, homeostasis, and apoptosis [5]. However, the genetic basis of PHPT is still under investigation.

In the present study, four genes previously reported to be involved in parathyroid adenomas or PTH signaling were analyzed for their association with PHPT. These genes are Annexin A2 (*ANXA2*), Mediator Complex Subunit 12 (*MED12*), Calmodulin 1 (*CALM1*), and Mitogen-Activated Protein Kinase 1 (M*APK1*).

The *ANXA2* gene (15q22.2) encodes the ANXA2 protein, a calcium-regulated phospholipid-binding protein that has been found upregulated in some tumor cells, affecting cell survival and mediating interactions between intercellular and extracellular microenvironments. It performs crucial roles in tumor progression, especially in the invasion and metastasis of tumor cells [6]. In addition, increased expression of ANXA2 has been reported in parathyroid adenomas [7, 8].

The *MED12* gene (Xq13.1) is involved in gene regulation, as it serves as an essential component of the transcription mechanism of RNA polymerase II [9]. In general, *MED12* variants are common in neoplasms and benign tumors, while upregulation of MED12 has been observed in parathyroid adenomas [10,11].

CALM1 (14q32.11) encodes one of the three calmodulin proteins, which are small calcium-sensitive proteins that rapidly transmit information about changes in calcium concentration, regulating gene expression in neurons and potentially shaping cardiac action in heart cells [12]. In parathyroid adenomas, calmodulin has been reported to inhibit PTH secretion [13].

Finally, the *MAPK1* gene (22q11.22) encodes a member of the MAP protein kinase family. It is also known as extracellular signal-regulated kinase 2 (ERK2) and has been strongly associated with proliferation, differentiation, and signaling regulation in osteoblasts [14]. The MAPK1/ERK2 protein is a key component of the Ras-Raf-MEK-ERK and c-Jun N-terminal kinases (JNK) signaling pathways, which are downstream targets of PTH [14,15].

All these genes may have a role in PHPT predisposition due to their involvement in tumorigenesis in parathyroid glands and PTH signaling pathways. No studies were reported to test the association of genetic variants of *ANXA2*, *MED12*, *CALM1*, and *MAPK1* genes with PHPT predisposition, which is the reason why this pilot study was conducted. Genetic variants in *ANXA2* (rs7170178 A>G, rs17191344 A>G, and rs11633032 G>A; all downstream of the gene), *MED12* (rs1057519912; exonic: C>G, T), *CALM1* (rs12885713; intronic: C>T), and *MAPK1* (rs1057519911, exonic; C>T), previously described as variants with clinical relevance in several diseases, were studied as predisposing factors to PHPT pathogenesis.

MATERIALS AND METHODS

Fifty unrelated patients with primary hyperparathyroidism (PHPT) (2 males and 48 females, 56.1 ± 13.9 years) and an equal number of ethnically matched healthy volunteers (8 males and 42 females, 50.6 ± 18.4 years) were recruited for the study. The diagnosis of PHPT was confirmed by the elevated levels of parathyroid hormone (PTH) and calcium in blood serum, as well as through imaging methods such as sonography, 99mTc-sestamibi scintigraphy, and 4D-CT validated by histological examinations [16]. The control group had no personal or family history of chronic autoimmune or neoplastic diseases. Since this study on the association of the studied variants with PHPT was conducted for the first time, it was not possible to determine the standardized effect size to be used before the pilot trial. Therefore, the sample size was calculated with a 90% confidence level and a probability of 0.05 [17] following the suggested standards for pilot studies [18]. The study protocol was approved by the Ethics Committees of the Aristotle University of Thessaloniki, and written informed consent was obtained from each patient.

Genomic DNA was extracted from peripheral blood lymphocytes using the PureLink Genomic DNA Kit (Invitrogen) following the manufacturer's protocol. The samples were genotyped using the polymerase chain reaction - restriction fragment-length polymorphism (PCR-RFLP) assay. The primer pairs used for amplification of each region are shown in Table 1. Amplified fragments were then digested with appropriate restriction enzymes (New England Biolabs - Table 1), following the manufacturer's instructions, and visualized after electrophoresis on 3% agarose. All samples were run twice using RFLP analysis confirming the credibility of the results. This methodology is both time- and cost-saving for a pilot study like the pre-

Table 1. Primer sequences, restriction enzymes, and restriction digestion patterns were used for genotyping of the studied genetic variants.

Gene	Variant	Primer sequence (5´-> 3´)	Amplicon size	Restriction Enzyme	Restriction digestion pattern
ANXA2	rs7170178	F: 5'-TTCACAGCAGTTCAAAATAC-3' R: 5'-CTGGGTTTCCAGAGATGGAA-3'	550bp	HpyCH4V	G: 338bp/106bp/72bp/34bp A: 195bp/143bp/106bp/72bp/34bp
	rs17191344	F: 5'-TGCAAACAGGCGCCACTAAA-3' R: 5'-CAGACATGAGGCCAAGGAACT-3'	280bp	Hpy99I	A: 94bp/186bp G: 139bp/94bp/47bp
	rs11633032	F: 5'-CAACAAGCATGGGGTTGC-3' R: 5'-GTTGACATTTGCCCTTCGCTT-3'	131bp	BceAI	G: 123bp/ 8bp A: 131bp
MED12	rs1057519912	F: 5'-ACAAGCCTACAGTAGGAATCC-3' R: 5'-TGGCACCACTCCCTTCCTAC-3'	143bp	SmlI	C: 94bp/49bp G/T: 143bp
CALM1	rs12885713	F: 5'-GGGATACGGCGCACCATATAT-3' R: 5'-GGTACCTCCGATGCCGCTG-3'	179bp	HpyCH4V	T: 144bp/35bp C: 179bp
MAPK1	rs1057519911	F: 5'-TGGCTGATCTATGTCCCTGA-3' R: 5'-CACACAAGAGGATTGAAGTAG-3'	122bp	MnlI	C: 63bp/59bp T: 122bp

Table 2. Statistical analysis of rs7170178 (*ANXA2* gene) and rs12885713 (*CALM1* gene) variants between PHPT patients and controls.

Gene: Genotypes of the studied variant	Patients	Controls	Statistical model	OR (95%CI)	*p*-Value	HWE in control group (*p*-value)
***ANXA2:* rs7170178**						
AA	18	22	Additive (AA *vs*. AG *vs*. GG)		0.45	0.78
AG	29	23				
GG	3	5				
AA	18	22	Homozygous (GG *vs*. AA)	0.73 (0.15-3.49)	1	
GG	3	5				
AA	18	22	Heterozygous (AG *vs*. AA)	1.54 (0.67-3.53)	0.31	
AG	29	23				
AA	18	22	Dominant (AG+GG *vs*. AA)	1.4 (0.62-3.11)	0.41	
AG+GG	32	28				
AA+AG	47	45	Recessive (GG *vs*. AA+AG)	0.57 (0.13-2.55)	0.71	
GG	3	5				
A	65	67	Allelic (G *vs*. A)	1.09 (0.61-1.96)	0.76	
G	35	33				
***CALM1:* rs12885713**						
CC	20	14	Additive (CC *vs*. CT *vs*. TT)		0.39	0.98
CT	19	25				
TT	11	11				
CC	20	14	Homozygous (TT *vs*. CC)	0.7 (0.24-2.06)	0.52	
TT	11	11				
CC	20	14	Heterozygous (CT *vs*. CC)	0.53 (0.21-1.32)	0.17	
CT	19	25				
CC	20	14	Dominant (CT+TT *vs*. CC)	0.58 (0.25-1.35)	0.21	
CT+TT	30	36				
CC+CT	39	39	Recessive (TT *vs*. CC+CT)	1 (0.38-2.57)	1	
TT	11	11				
C	59	53	Allelic (T *vs*. C)	0.78 (0.45-1.37)	0.39	
T	41	47				

sent one. However, other genotyping methods can be utilized in subsequent larger-scale studies if the results of the present study indicate the feasibility of such an approach. Pearson's chi-square test was used to examine possible deviations of genotype distributions from the Hardy-Weinberg equilibrium (HWE) in the control group. Differences in variant distribution between PHPT patients and controls were tested under six models of genetic association: homozygote, heterozygote, dominant, recessive, allelic, and additive using Pearson's chi-square test. Fisher's exact test was used when expected values were less than 5. Additionally, the odds ratio (OR) with a 95% confidence interval (CI) was calculated (reference allele vs variant allele). A difference at $p \leq 0.05$ was considered statistically significant in all statistical tests. All analyses were performed using the SPSS statistical package (SPSS Inc.).

RESULTS

The study group mainly included female PHPT patients, which can be excused by the female preponderance of the primary PHPT adenoma [19].

Three variants (*ANXA2*: rs17191344, rs11633032, *MAPK1*: rs1057519911) were found to be monomorphic for the wild-type alleles in both patients and controls. Additionally, the genotype distribution of the *MED12* rs1057519912 variant did not differ between PHPT patients and controls, with 2 patients and 2 control subjects being heterozygous. As a result, these four variants were not included in further statistical analyses.

The genotypic distribution of *ANXA2* rs7170178 and *CALM1* rs12885713 variants in PHPT patients and controls is displayed in Table 2. The distribution of genotypes was in line with Hardy-Weinberg equilibrium in the control group. No statistically significant different distributions of rs7170178 and rs12885713 genotypes or alleles were found between PHPT patients and controls (Table 2).

DISCUSSION

PHPT is a prevalent endocrine disorder characterized by the excessive functioning of the parathyroid glands [3]. The genetic basis of PHPT is only partially understood, and genetic variants have emerged as potential contributors to the development and progression of the disease affecting the genes' expression. This comprehensive study aimed to investigate the association between specific variants of *ANXA2*, *MED12*, *CALM1*, and *MAPK1* genes with PHPT.

The genes investigated in this study have been implicated in tumorigenesis and have shown associations with various types of cancer and parathyroid malignancies. ANXA2 is involved in tumor progression, invasion, and metastasis and its upregulation has been reported in parathyroid adenomas and several types of cancer [7, 8]. *MED12* variants were frequently observed in various cancer types, while overexpression has been related to parathyroid adenomas [11, 20]. CALM1 was reported to participate in calcium signaling [12], making it a potential candidate for association with PHPT. Moreover, CALM1 has been related to an inhibitory effect on PTH secretion in parathyroid adenoma [13]. Finally, MAPK1/ERK2 has been described as a key component of signaling pathways regulated by PTH secretion and has been related to cell proliferation, differentiation, and survival [15].

In previous studies, the minor alleles of *ANXA2* rs7170178, rs17191344, and rs11633032 gene variants have been reported to reduce *ANXA2* gene expression and to down regulate ANXA2 signaling [21, 22]. Specifically, the minor alleles create repressor-binding protein sites for transcription factors that contribute to reduced *ANXA2* gene expression [21, 22]. The variants rs17191344 and rs11633032 have been associated with coronary disease risk in Caucasians through increasing low-density lipoprotein cholesterol levels, while rs7170178 has been associated with osteonecrosis in sickle cell disease in Latin and Indian patients [21–23]. Moreover, *MED12* rs1057519912 and *MAPK1* rs1057519911 have been identified as hotspots in cancer [24]. In addition, rs12885713 in the promoter region of the *CALM1* gene has been reported to affect the transcription of the gene [25]. This variant has been studied for its association with osteoarthritis, but the results were contradictory [26, 27]. The meta-analysis including studies stratification by ethnicity in the analyses revealed that the rs12885713 variant increases the risk of osteoarthritis among Asians [26]. Furthermore, rs12885713 has also been associated with double curve and lumbar curve adolescent idiopathic scoliosis in Chinese patients [28, 29].

In a previous study, we have reported that ANXA2, MED12, and MAPK1 proteins have positive staining in the immunohistochemical study of sporadic parathyroid adenomas in varying intensity and allocation percentages [30]. Due to technical issues in protocol establishment the protein CALM1 has not been included in that study. In the present pilot genetic association study, a total of 50 unrelated PHPT patients and an equal number of healthy controls were genotyped for *ANXA2* (rs7170178, rs17191344 and rs11633032), *MED12* (rs1057519912), *CALM1* (rs12885713) and *MAPK1* (rs1057519911) genetic variants. The variants rs17191344 and rs11633032 of the *ANXA2* gene and the rs1057519911 of the *MAPK1* gene were found to be monomorphic which is in accordance with the very low frequency of their minor alleles reported in the NCBI database for Caucasians. However, these variants were initially selected to be studied based on

their reported positive association with transcription levels of *ANXA2* gene and as a cancer variant hotspot of *MAPK1* gene [21, 22, 24]. Regarding the variants rs7170178 *ANXA2*, rs1057519912 *MED12*, and rs12885713 *CALM1* no significant association was observed in genotypes or alleles distributions between PHPT patients and controls.

Due to the reported female preponderance of PHPT adenoma [19], the study group mainly included female PHPT patients. The present study is a pilot one and sets the initial step in exploring a novel intervention. Pilot results can inform about the feasibility and identify modifications needed in the design of a larger study testing the same hypothesis [31, 32]. It is worth mentioning that it was reported that power analyses should not be presented in an application in case of a pilot study which does not propose inferential tests. Instead, a pilot sample size is based on the pragmatics of recruitment and the necessity for examining the feasibility [31, 32].

The lack of significant associations between the studied genetic variants and PHPT of our study may be attributed to several factors, including the genetic heterogeneity of PHPT [33]. PHPT is a complex disorder influenced by both genetic and environmental factors, and multiple genetic variants likely contribute to its development. It is estimated that 60% of the variation in PTH concentration is genetically determined [34], and therefore several genetic variants have thus far been associated with PHPT pathogenesis causing among others disturbances in calcium regulation or cell signaling [35–38]. The sample size of our study is small, but it follows the suggested standards for pilot studies, which try to find preliminary evidence and tendencies of the studied variants' associations [18]. Additionally, the lack of significant genetic associations in this study is restricted to the studied genetic variants and does not reject the possible association between other variants of *ANXA2*, *MED12*, *CALM1*, and *MAPK1* genes with PHPT predisposition.

However, the publication of negative findings is as important as publishing statistically significant findings to overcome the issue of publication bias, which results from the preferential publication of positive associations and the reduced likelihood of negative findings being reported [39–41]. Even though the reported associations between gene variants and disease could have tremendous importance for the prevention, prediction, and treatment of diseases, commonly there is an irreproducibility of the results, and the majority of these associations are not robust [42]. Preliminary studies based on random small sample groups have been proved of great importance as many times the results of genome-wide association studies have limited clinical predictive value and other limitations [43,44]. Consequently, the negative associations, as these of the present study, offer to decide if it is advantageous to investigate the above-mentioned variants as risk factors in disease predisposition especially in complex disorders such as PHPT [34]. It is worth mentioning that given the small sample size, the study may be underpowered to detect subtle associations. However, there are many reasons for the significance of pilot studies' results in the scientific community such as assessing the feasibility of a survey, assessing whether the research protocol is realistic and workable, and developing a research question and research plan [45].

Undoubtedly, understanding the genetic basis of PHPT can provide valuable insights into disease mechanisms and potentially guide the development to personalized treatment strategies. Genetic association studies of both positive and negative results can be proven a valuable resource in the struggle to understand and treat diseases since the conclusions should not be drawn from a single report. Positive and negative associations of other similar studies add to the pool of genetic data for their future meta-analyses concluding with more accuracy.

Declaration of Interest: The authors report no conflicts of interest.

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ORIGINAL ARTICLE

INVESTIGATION OF TLR4 POLYMORPHISM IN CHILDREN WITH VESICOURETERAL REFLUX AND RENAL SCARRING

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ABSTRACT

Vesicoureteral reflux (VUR) is an important factor in the etiology of recurrent urinary tract infections (UTIs). Permanent kidney damage may develop in children with high-grade VUR in the long term. This damage may progress with the development of scar tissue in some patients. The *TLR4* gene is an important resistance mechanism, especially against UTIs. *TL*R4 gene polymorphism is associated with recurrent UTIs and kidney scar development in the long term. This study aimed to examine the relationship between scar development and *TLR4* gene polymorphism in children with VUR. This cross-sectional study included 49 patients with recurrent UTIs and primary vesicoureteral reflux. Patients were divided into two groups (26 patients with the scar, and 23 patients without scar) according to the presence of scar tissue. *TLR4* gene polymorphisms of the patients were evaluated by Next Generation Sequencing. The *TLR4* gene polymorphism was significantly higher in the compound heterozygous group with scarring than in the group without scarring (p=0.03). Gene polymorphisms, c.958T>C, c.942A>G, c.776A>G, c.1076C>T, c.896A<G, c.1196C>T, c.1078C>T were presented more commonly in the group with scarring. Moreover, gene polymorphisms c.942A>G and c.776A>G were defined for the first time in this study among patients with scar tissue. The higher incidence of some *TLR4* gene polymorphisms in patients with scarring suggested that these variations might cause permanent kidney damage. In addition to genetic predisposition, environmental factors such as untreated UTIs might also contribute to scar formation.

Keywords: Next Generation Sequencing; Toll-Like Receptor 4; Urinary tract infection; Vesico-ureteral reflux

INTRODUCTION

Urinary tract infection (UTI) is an important health problem that is commonly seen in children and has short and long-term complications. One of the common causes of the disease is vesicoureteral reflux. Vesicoureteral reflux (VUR) is a pathology characterized by reflux of urine accumulated in the bladder from one or both ureters to the kidney as a result of anatomical disorders [1]. Primary VUR occurs due to poor development and dysfunction of the congenital ureterovesical junction whereas secondary VUR occurs due to increased intravesical pressure [2].

Primary VUR is seen in 1-2% of the pediatric population; however, this rate rises to 30-40% in the presence of urinary tract infections, and recurrent UTI is an important cause of renal parenchymal damage [3]. Bacterial infection in the renal parenchyma causes an acute inflammatory reaction, accelerating scar formation and progression to chronic kidney disease (CKD) [4].

Many studies reported that resistance to bacterial UTIs is controlled by some genes. Among these genes, toll-like receptors (TLR) are transmembrane proteins involved in the innate immune response. Single gene defects or variations in genes encoding TLR, chemokines, and chemokine receptors alter the susceptibility of the host to urinary pathogen invasion [5]. *TLR4* is the first toll-like receptor identified in humans and is expressed in monocytes and dendritic cells. *TLR4* recognizes bacterial

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lipopolysaccharides and contributes to host defense against Gram-negative bacteria [6].

Different polymorphisms in the *TLR4* gene cause UTI facilitates the emergence of the disease, and may pave the way for CKD in the long term. Identification of these polymorphisms will enable accurate genetic counselling and rapid screening of individuals with risk factors. This study aimed at analyzing *TLR4* gene polymorphisms in pediatric patients with recurrent UTI and VUR using Next Generation Sequencing (NGS).

MATERIALS AND METHOD

This cross-sectional study was carried out with 49 patients who were followed up due to primary vesicoureteral reflux at Duzce University, Faculty of Medicine, Department of Pediatric Nephrology, Duzce, Turkey.

Patients under 18 years of age with VUR and recurrent UTIs were included in the study. Oral and written informed consent was obtained from all individual participants and their families included in the study. Those who did not give consent and those who had additional renal or other system anomalies and patients with stage 5 CKD were excluded. The study protocol was approved by the Institutional Ethics Committee of Duzce University School of Medicine (Ethics No: 2019/285). The study was conducted by the ethical principles set forth in the Declaration of Helsinki. This project was supported by the Scientific Research Project Department of Duzce University (Grant number: 2021.04.03.1194).

Diagnosis of UTI was made based on history and exam findings and confirmed with appropriately collected urine. The presence of VUR was confirmed by voiding cystourethrography (VCUG) and the severity of VUR was graded according to the International Reflux Study in Children (IRSC) (I-V) [7]. A DMSA scan was performed 6 months after the last UTI. Patients were divided into two groups according to the presence of any kidney scars determined in the DMSA scan. Office blood pressure was measured by the auscultation method. Before starting blood pressure measurements, the patient rested in a sitting position for at least 3-5 minutes, relaxed and rested. The arm was outstretched, in line with the mid-sternum and supported. An appropriately sized cuff was wrapped around the upper arm and connected to a manometer and blood pressure was measured.

Genomic DNA was isolated from 200 µl peripheral leukocytes of the cases using DNA isolation kits (Anatolia Diagnostics and Biotechnology Products Inc., Istanbul, Turkey). Polymerase chain reaction (PCR) pools generated before the NGS reaction were purified by the NucleoFast 96 PCR (MACHEREY-NAGEL GmbH) kit. Then the quantification of the PCR products was standardized on NanoDrop 1000 (Thermo Fisher Scientific Inc.) and the *TLR4* gene was sequenced by NGS (MISEQ-Illumina). Serum and urine biochemical parameters were also recorded.

Statistical Analysis

The data were analyzed via IBM SPSS Statistics 22.0 (IBM Corp. Released 2013. IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY: IBM Corp.). The Shapiro-Wilk test was performed to examine the distribution of data. All quantitative variables were reported by mean±standard deviation (SD) and median (interquartile range: IQR), as categorical variables were summarized by frequency and %. The Mann-Whitney U test was performed to compare the patients with and without scares with respect to the quantitative variables. Pearson chi-square test, Fisher's exact test and Fisher-Freeman-Halton test were used to reveal the differences between two groups for categorical variables. A p-value≤0.05 was considered statistically significant.

RESULTS

A total of 49 individuals, 26 (53.1%) of them with kidney scars and 23 (46.9%) without scars were included in the current study. Both groups were similar in terms of age. The distribution of cases with scars was as follows: Seven (14.3%) with bilateral multiple scars, four (8.2%) with one scar on the right, eight (16.3%) with multiple scars on the right, one (2%) with multiple scars on the right and renal atrophy on the right, three (6.1%) with one scar on the left, and three (6.1%) with multiple scars on the left. The age of patients with kidney scars was found to be significantly higher than that of patients without kidney scars ($p<0.001$). Two groups were similar with respect to the gender distribution and the level of serum urea ($p=0.786$ and $p=0.667$, respectively). The levels of systolic and diastolic blood pressures and serum creatinine were significantly higher in patients with scars compared to those without any scar (for all, $p<0.001$). However, although the estimated glomerular filtration rate was lower in the group with scars, no statistically significant difference was observed between the groups ($p>0.05$), (Table 1).

The distribution of VUR severity within each group is presented in Figure 1. There was a significant difference between the two groups with respect to the severity of VUR ($p<0.001$). The patients with grade 4 VUR were significantly more frequent in the group with scars, while the patients with grade 1 VUR were significantly more frequent in the group without any scar ($p<0.05$).

Compound heterozygous variations were more common in patients with kidney scarring ($p<0.05$) (Table 2).

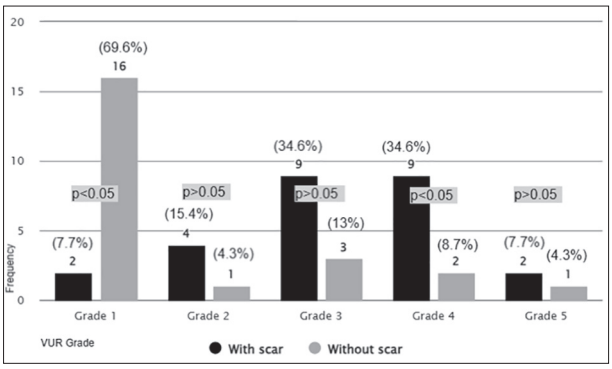

Figure 1. The distribution of VUR severity within groups (VUR: Vesicoureteral reflux)

Furthermore, although not statistically significant, the variations, heterozygous c.942A>G p.Lys314 in Ex4 rs56070048, heterozygous c.896A<G p.Asp299Gly in Ex3 rs4986790, heterozygous c.1196C>T p.Thr399Ile rs4986791 in EX3 and heterozygous c.1078C>T p.Ser360Pro in Ex3 were found more commonly in patients with scarring compared to those without scarring (p>0.05) (Table 2).

DISCUSSION

One of the mechanisms in the body that resist disease agents and develop resistance is the recognition and identification of the pathogen. TLRs play an important role in the healthy progress of this process. The coordinated activity of TLRs on the cell surface or inside the phagosomes enables the release of cytokines, recruitment of neutrophils, and release of free radicals and phagocytosis in the immune system, [8]. These responses determine the severity of the disease. Signaling disorders in the immune system resulting from polymorphisms in receptors and cytokines affect the susceptibility to infectious pathogens and the development of complications [9]. In this study, it has been shown that VUR increases the susceptibility to scar formation with the effect of *TLR4* gene variations. These variations were determined by the NGS method [10]. To our knowledge, this is the first study in which the associations of *TLR4* gene polymorphisms with UTI were investigated by NGS screening.

Pyelonephritis is an important risk factor for CKD in children. Although anatomical anomalies such as VUR are associated with recurrent UTIs in the majority of patients,

Table 1. Comparison of anthropometric characteristics and blood values of cases with and without scarring

	Renal scar (+) (n=26)	**Renal scar (-) (n= 23)**	p
Age, year (Mean±SD)	10.5±4.37	9.91±3.43	0.3
Gender (boy/girl) (n)	8/18	6/17	0,9
Systolic blood pressure, mmHg (Mean±SD)	114.84±12.05	93.91±6.74	**0.000**
Diastolic blood pressure, mmHg (Mean±SD)	68.88±8.86	54.26±6.29	**0.000**
Serum urea (mg/dL) (Mean±SD)	25.33±6.87	24.22±6.46	0.667
Serum creatinine (mg/dL) (Mean±SD)	0.56±0.19	0.37±0.13	**0.000**
e-GFR (mL/min/1.73m^2) (Mean±SD)	108.72±23.54	121.53±32.35	0.11

e-GFR: estimated glomerular filtration rate

Table 2. TLR4 gene variation distribution status in cases with and without scarring

Variation Status in *TLR4* gene	**Renal scar (+), n(%)**	**Renal scar (-), n(%)**	**p**
Heterozygous	17 (65.4)	20 (87)	>0.05
Compound heterozygous	9 (34.6)	2 (8.7)	**<0.05**
Normal	0 (0)	1 (3)	>0.05
c.958T>C p.Ser320Pro in Ex4	25 (96.2)	22 (95.7)	>0.05
c.942A>G p.Lys314 in Ex4 (rs56070048)	2 (7.7)	0 (0)	>0.05
c.776A>G p.Asp259Gly in Ex4 (rs4986790)	2 (7.7)	1 (4.3)	>0.05
c.1076C>T p.Thr359Ile in Ex4 (rs4986791)	4 (15.4)	1 (4.3)	>0.05
c.896A<G p.Asp299Gly in Ex3 (rs4986790)	2 (7.7)	0 (0)	>0.05
c.1196C>T p.Thr399Ile rs4986791 in EX3	1 (4.3)	0 (0)	>0.05
c.315C>T p.Pro105 rs5030711 in Ex4	0 (0)	1 (4.3)	>0.05
c.1078C>T p.Ser360Pro in Ex3	1 (4.3)	0 (0)	>0.05

the urinary system is usually normal both anatomically and functionally in patients with recurrent UTIs [11]. This suggests that some other factors related to host defense may also be involved in pyelonephritis and scar formation [12]. Successful defense against bacterial infection requires coordinated work of the innate and adaptive immune responses. TLRs are important for the recognition of microorganisms by the innate immune system as well as for laying a bridge between innate and adaptive immune responses [8]. These receptors act as critical sensors of microbial attack and also serve as effectors of the innate defense that ensures the elimination of pathogens [13]. It was suggested that TLR gene polymorphisms may affect an individual's ability to respond to TLR ligands, resulting in altered susceptibility to infections or inflammation [14]. This altered susceptibility may clinically emerge as decreased inflammatory response, protection against pyelonephritis, as in asymptomatic bacteriuria, or, conversely, recurrent UTIs [9, 12].

On the other hand, the effects of the *TLR4* gene on kidney damage were addressed through different mechanisms. It is thought that endogenous molecules that accumulate at non-physiological amounts or sites during cellular damage can bind to *TLR4* and trigger inflammation [15]. It was reported that there is a relationship between the *TLR4* expression and the degree of kidney damage in progressive CKD due to inflammation-induced fibrosis [6]. Cellular debris which is a product of the degradation of extracellular matrix as a result of cellular damage and increased matrix cycle and endogenous ligands such as heat shock proteins could be activated *TLR4* [16]. Endogenous *TLR4* ligands such as fibrinogens, heparan sulfate, hyaluronan, and fibronectins are overproduced during progressive renal fibrosis and tubulointerstitial damage and bind to *TLR4* on macrophages. Then, with the activation of antigen-producing cells, NF-κB dependent gene expression occurs [17]. Interstitial inflammation and fibrosis occur continuously in the process [6].

In this study, carriage of compound heterozygous variation in the *TLR4* gene was much more common in the group with kidney scarring than in the group without scarring. Some studies showed that *TLR4* gene polymorphism affects cellular immune response and cytokine production in vitro and that this paves the way for the deterioration of resistance against microorganisms. It was also noted that each polymorphism has different effects in defense against different microorganisms [18]. In a study conducted by Svanborg et al., it was found that C3H/Hej mice inoculated with virulent Escherichia coli strains could not eliminate Escherichia coli infection and developed UTI. The researchers stated that the response of these animals to bacteria was weakened and the resistance to infection decreased because of impaired neutrophil migration due to the defective *TLR4* gene [19]. In another study, it was shown that some *TLR4* gene polymorphisms increased the prevalence of Gram-negative infections and that these polymorphisms facilitated the progression to sepsis and septic shock [20]. The fact that *TLR4* polymorphism decreases resistance, especially against Gram-negative bacteria and that UTIs occur frequently due to Gram-negative microorganisms may explain the increase in the frequency of kidney scar tissue development in these patients. Scar development is more common in the presence of frequent and complicated UTIs. Therefore, the higher incidence of *TLR4* polymorphism in patients with scar tissue suggests that this group of patients more frequently have complicated UTIs.

One of the variations detected in the study group was c.942A>G. This variation was not detected in the group without scarring whereas it was determined at a rate of 6.9% in the group with scarring. Torices et al. reported that this variation can be seen at a low rate in patients with rheumatoid arthritis; however, there is no information about its clinical significance in the literature [21]. On the other hand, the relationship between kidney scar development and the same variation has not yet been defined. The absence of this variation in the control group was suggested that c.942A>G variation may be a factor that increases the susceptibility to scar development. Furthermore, another variation found at a rate of 10.3% in the patient group, but not seen in the control group, was the c.776A>G variation. It was reported that this variation reduces *TLR4* response to lipopolysaccharides and leads to less inflammatory cytokine production. As a result, it was stated that an adequate inflammatory response could not be given and that the resistance to infections decreased in the presence of this variation [22]. It could be concluded that the risk of permanent damage increases with the decreased inflammatory response and insufficient clearance of infectious agents from the environment. However, why this condition resulted in scarring in some patients has not been clarified yet. Perhaps, the infection may be difficult to eliminate and scarring may be easier due to multiple polymorphisms. However, some inflammatory cytokines are known to be associated with the development of scar tissue and resistance to infections. One of the most important cytokines is TNF-alpha. It was reported that TNF-alpha causes tissue damage. This damage could also be seen in the kidneys; however, *TLR4*-mediated blockade of TNF-alpha production is also associated with improvement in kidney functions in experimental models [23]. On the other hand, it is known that different doses of cytokines have different effects. Although the release of low-dose cytokine is an important factor in resistance to infections, high-dose releases can cause kidney damage. Therefore, the variations found in this study may trigger the development of

scar tissue by reducing the block in TNF-alpha production. However, we thought that further detailed experimental studies are required to reveal the effects of cytokines on permanent kidney damage since they are released locally as well as systemically.

Some genetic polymorphisms identified might be directly associated with the increased frequency of certain infections in the body. In particular, the decrease in the resistance of the urinary system to infections was associated with the presence of these polymorphisms. One of the reasons for the susceptibility to UTIs might be c.896A<G variation. In the study conducted by Agnese et al., it was pointed out that the risk of Gram-negative bacterial infection increased in people with *TLR4* c.896A<G polymorphism (ASP299Gly) [24]. Similarly, the c.1196C>T variation detected in this study was shown to increase susceptibility to invasive Gram-negative bacterial infections [25]. Karoly et al. reported the frequency of *TLR4* c.896A<G polymorphism as 13% with VUR and 2% without VUR in patients with UTI. The authors stated that this allele is a risk factor for recurrent UTIs independent of urinary anomalies [26]. In another study, the frequency of *TLR4* c.896A<G polymorphism was found to be 12.5% in patients with UTIs. The researchers also reported that this variation was more common in children with scar-positive pyelonephritis than in children with scar-negative pyelonephritis [12]. Another variation, which was more common in patients with scar tissue, was c.1076C>T. The rate of this variation was 13.8% in the patient group and 5% in the control group. Although some studies in the literature showed that this variation increased the susceptibility to UTI, there were no data regarding its effects on scar tissue development. In a meta-analysis conducted by Huang et al., many data were evaluated showing that the rs4986791 variation increases the susceptibility to UTI. The researchers stated that this variation is unlikely to be associated with the frequency of UTIs since the current studies have been carried out with a small number of patients [27]. Although there is no clear information about this variation, we thought that it may possibly increase the development of scar tissue in the presence of recurrent UTIs.

The c.958T>T gene encodes the T6SS protein. T6SS protein is one of the main contact-dependent delivery system proteins responsible for interactions between bacterial cells. At least one type of this protein was found in gram negative bacteria [28]. Gene mutations are also thought to play a role in the etiology of chronic damage due to infectious agents. Therefore, c.958T>T polymorphism may contribute to the development of scar tissue. However, the fact that this mutation was demonstrated in both groups in our study suggests that gram-negative infections, although frequent, do not contribute to the development of scar tissue.

c.315C>T is a polymorphism shown in some parasitic infections and some bacterial infections that might be associated with cancer [29, 30]. However, there is no data in the literature related with scar tissue development. We think that it is coincidental that this polymorphism was found in only one patient in our study.

Moalem et al. identified a mutation, suggesting that the c.1078>T polymorphism may be associated with ciliary dysgenesis [31]. Ciliary functions play an important role in the elimination of infectious agents. Disruption of these functions may trigger scar development secondary to infections. In our study, this polymorphism was detected in only one patient who developed scar tissue. Therefore, based on this result, it is difficult to claim that c.1078>T polymorphism may be associated with scar tissue.

It should not be ignored that genetic predisposition combined with environmental effects is an important factor in the development of scar tissue. It could not be stated that scar tissue develops based on genetic factors alone. This may explain the lack of scarring in some patients, even in the presence of genetic variation. Additionally, kidney scar tissue could develop without genetic variation. On the other hand, vesicoureteral reflux could also be genetically transmitted on its own. The prevalence of VUR has been reported as 27-51% in siblings, 80-100% in monozygotic twins, and 35-50% in dizygotic twins [32]. In a study, it was reported that the rate of VUR development in children whose parents had VUR was 66% [33]. Therefore, it could be said that genetic variations also contribute to scar development significantly. On the other hand, there may be an association between the increase in the degree of VUR and the development of scar tissue. It has been reported that scar tissue develops in 89% of children with high-grade VUR after an episode of pyelonephritis[4]. In our study, there was no relationship between genetic mutation and the degree of VUR.

There were some limitations in this study. Compound heterozygous mutation was found to be significantly higher in patients with scarring, but the study sample size was small. To confirm these results, the relationship between genetic and clinical findings can be clearly demonstrated by studies including a larger number of patients. Another limitation of the study is the lack of a healthy control group or a group with UTI but not VUR.

CONCLUSION

It is known that *TLR4* gene variations increase the frequency of infection and consequently the susceptibility to the development of scar tissue. This is the first study investigating *TLR4* gene variations by NGS method. Variations of c.958T>C, c.776A>G, c.1076C>T, c.896A<G,

c.1196C>T, c.315C>T, c.1078C>T were found at high rates in patients with kidney scarring. Studies with many patients are needed for revealing the effects of both genetic and environmental factors on the development of kidney scarring.

STATEMENTS AND DECLARATIONS

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Competing interests

The authors declare no competing interests and have no financial interests to declare

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ORIGINAL ARTICLE

NEXT-GENERATION SEQUENCING INFERTILITY PANEL IN TURKEY: FIRST RESULTS

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ABSTRACT

Background

Male infertility is a complex pathophysiological disorder. At least 2000 genes are implicated in the etiology of male infertility, making it a very complex genetic condition. In cases of male infertility, genetic testing using next-generation sequencing (NGS) technology may be useful for diagnosis. Thus, the purpose of this investigation was to apply the diagnostic offer for genetic variant identification using an NGS panel.

Methods

We developed an NGS gene panel that we used in 85 infertile male patients. The panel consisted of 132 genes exploring the genetic causes of male infertility; namely spermatogenesis failure due to single-gene mutations, central hypogonadism, androgen insensitivity syndrome, congenital hypopituitarism, and primary ciliary dyskinesia etc.

Results

A total of 85 patients (85 males) between 21 year and 45 years old were included in the study group. NGS analysis had been applied in all the primary infertility cases. As a result of NGS analysis, 58 clinical variants in 28 genes were detected in 41 patients (%48.23- 41/85)

Conclusion

Consequently, pre-diagnostic genes included in a custom-made NGS panel test can enhance genetic diagnostic testing and have an impact on the clinical management of male infertility.

Keywords: Male infertility; NGS diagnosis; Genetic causes of male infertility

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INTRODUCTION

The way that male infertility is treated has undergone a significant transformation as a result of our growing understanding of the physiology of male reproduction, fertilization, and the development of increasingly potent assisted reproductive procedures. A physical exam and medical history gathering are currently part of the diagnostic procedure provided to infertile male patients. This is followed by a mix of laboratory tests specifically chosen for each case, including a thorough genetic laboratory analysis. At least a year of infertility should precede the administration of diagnostic testing. Accordingly, a couple is considered infertile if they are unable to conceive following a year of regular, unprotected sex. 15% of male patients who are infertile have genetic issues. Chromosome abnormalities or single gene mutations are examples of them. The Online Mendelian Inheritance in Man (OMIM) database contains information on more than 200 genetic conditions associated with male infertility (1-4).

Many disorders, most notably Mendelian or uncommon diseases where having causal variants significantly reduces reproductive fitness, have had exceptional results using NGS (5).

The candidate gene approach in model animals and whole genome investigations using single-nucleotide polymorphism microarray and next-generation sequencing (NGS) technologies, such as exome or whole-genome sequencing, are the two main methods for identifying the genes responsible for infertility. The reason of male infertility is still unknown in up to 70% of instances, despite extensive diagnostic testing, because traditional genetic tests sometimes fall short of making a diagnosis. Recent studies appear to address how NGS technology is increasing the rate of male infertility diagnosis. Accordingly, it has already been established that several diagnostic genes have a role in the pathophysiology of male infertility. It may be possible to make a diagnosis with the use of pre-

diagnostic genes, such as those that have been linked to male infertility but do not yet have solid proof of a causal relationship (6, 7, 8).

To do this, the current study was conducted to assess a number of pre-diagnostic genes by contrasting the outcomes with those obtained using our standard NGS custom-made gene panel for the diagnosis of male infertility, which consists of 132 genes. The genes included in the gene panel are composed of genes that have been associated with infertility to date.

METHODS

Patients and Samples

The research included 85 individuals with a clinical diagnosis of male infertility who had tested negative on diagnostic genetic testing. 84 individuals were thought to have primary spermatogenic failure, while one individual was thought to have central hypogonadism. Following the elimination of female factor infertility and acquired reasons of male infertility, main spermatogenic failure was suspected with a history of couple infertility longer than two years (e.g. male accessory gland infection, varicocele, testicular trauma, etc.). All patients are cases of infertility for two or more years. The patient group consists of patients who do not have any known additional disease or malignancy.

Additionally, individuals included in this study tested negative for early genetic anomalies such karyotype abnormalities, Y chromosome AZF microdeletions. MLPA technique was performed using the SALSA MLPA probemix P360 version B1 (MRC Holland, Amsterdam, The Netherlands) kit following the manufacturer's instructions. The kit contained 55 probes, of which 12 were located in autosomal chromosomes (for internal control reaction), and 43 were located in Y-chromosome AZF regions (16 AZFa, 15 AZFb, and 12 AZFc regions).

Each patient provided written consent after being fully briefed. The study was conducted in accordance with the principles outlined in the Declaration of Helsinki, and the local Ethics Committee approved the study.

Conventional G-banded karyotype analysis from peripheral blood was performed as part of the initial screening tests. The study included peripheral lymphocyte culture by a standard method using the Leishman-banding technique, centromere-banding (C-banding) and nucleolar organizing region staining performed as needed according to the AGT Cytogenetics Laboratory Manual. The best metaphases were karyotyped, and the total chromosome count was usually determined in 25 cells. The International System for Human Cytogenetic Nomenclature (ISCN) was used for the nomenclature of human chromosomes. Patients with no anomalies as a result of karyotype analysis were included in the NGS analysis.

Eighty five samples were sequenced using QIAseq Targeted DNA Custom Panel (Qiagen, Hilden, Germany). 2 mL of peripheral blood were collected and then preserved in anticoagulation tubes. Genomic DNA was isolated from peripheral whole blood using the EZ1 DNA Investigator Kit (Qiagen, Hilden, Germany). After DNA extraction, target sequences were enriched by using customized capture probes chips (Illumina, San Diego, CA). This kit included 132 genes targeting disease. Libraries covering the target genes were prepared according to the QIAseq Targeted DNA Panel protocol (Qiagen, Hilden, Germany). Following the target enrichment process, libraries were sequenced on the MiSeq System (Illumina, San Diego, CA, USA). OCI analysis (Qiagen, Hilden, Germany) was used for Quality control and Variant Call Format file generation. In silico evaluation of the pathogenicity of nucleotide changes in exons was performed using Polymorphism Phenotyping v2 (PolyPhen-2, http:// genetics.bwh.harvard.edu/pph2/), Sorting Intolerant from Tolerant (SIFT, https://sift.bii.a-star.edu.sg/), and MutationTaster (http://www.mutationtaster.org). Minor allele frequencies (MAF) were checked in the Genome Aggregation Database gnomAD (http://gnomad.broadinstitute.org/).Variant analysis was performed with Ingenuity software (Qiagen, Hilden, Germany). Variants were interpreted according to the American College of Medical Genetics and Genomics (ACMG) recommended standard. Sanger sequencing was performed for confirmation when the target region coverage was less than 15 reads. Nucleotide alterations were analyzed and validated by Sanger sequencing. After confirmation, each variant was classified as a pathogenic, likely pathogenic, variant of unknown significance (VUS), likely benign, or benign, according to the American College of Medical Genetics (ACMG) guidelines. Coding genomic regions (CDS) that were sequenced with coverage less than 15X were eventually re-sequenced using Sanger technology.

RESULTS

A total of 85 patients (85 males) between 21 years and 45 years old were included in the study group. NGS analysis had been applied in all the primary infertility cases. As a result of NGS analysis, 58 clinical variants in 28 genes were detected in 41 patients (%48,23- 41/85) (Table 1). Thirty-two of these variants are unknown clinical significance (VUS), 11 of them likely pathogenic, and 15 of these variants are classified as pathogenic in according to the Varsome, The Human Genomic Variant Search Engine, Franklin by Genoox, Clinvar and American College of Medical Genetics and Genomics (ACMG) databases.

Table 1. NGS panel for the diagnosis of male infertility

1.	NPHP4	23.	DAZL	45.	FKBPL	67.	DDX25	89.	ZMYND15	111.	SYCP2
2.	C1orf167	24.	DNAH1	46.	PLG	68.	C1RL	90.	GP1BA	112.	CBS
3.	MTHFR	25.	PROS1	47.	ZPBP	69.	C1RL-AS1	91.	KLHL10	113.	DNMT3L
4.	CLCA4	26.	BOC	48.	C7orf61	70.	DPY19L2	92.	ITGB3	114.	POFUT2
5.	BRDT	27.	CFAP44	49.	SERPINE1	71.	CHPT1	93.	TEX14	115.	GP1BB
6.	F3	28.	CFAP44-AS1	50.	POLR2J3	72.	SYCP3	94.	ACE	116.	POLR2F
7.	SPAG17	29.	GP9	51.	CFTR	73.	CCDC62	95.	PGS1	117.	SOX10
8.	F5	30.	SPATA16	52.	TEX15	74.	PIWIL1	96.	DNAH17	118.	PICK1
9.	SERPINC1	31.	CCDC39	53.	PLAT	75.	CPB2	97.	DNAH17-AS1	119.	MEI1
10.	F13B	32.	GP5	54.	CHD7	76.	F7	98.	TAF4B	120.	ADGRG2
11.	MTR	33.	CEP135	55.	TMEM70	77.	F10	99.	GGN	121.	MAGEB4
12.	LHCGR	34.	SPINK2	56.	CCIN	78.	TDRD9	100.	PLAUR	122.	MAGEB1
13.	FSHR	35.	BMP3	57.	NR5A1	79.	CATSPER2	101.	LHB	123.	NR0B1
14.	DNAH6	36.	FGB	58.	ASS1	80.	TERB2	102.	NLRP7	124.	TBC1D25
15.	NPAS2	37.	FGA	59.	UPF2	81.	NME4	103.	NLRP2	125.	AR
16.	LOC101927142	38.	KLKB1	60.	CFAP43	82.	FAHD1	104.	AURKC	126.	TEX11
17.	PROC	39.	F11	61.	NANOS1	83.	MEIOB	105.	SIRPG	127.	USP26
18.	TFPI	40.	MTRR	62.	SYCE1	84.	SEPT12	106.	SIRPA	128.	F9
19.	STRADB	41.	PRDM9	63.	FSHB	85.	PRM1	107.	THBD	129.	F8
20.	C2CD6	42.	ITGA2	64.	F2	86.	TERB1	108.	SUN5	130.	FUNDC2
21.	LOC100129175	43.	F2R	65.	MAJIN	87.	SERPINF1	109.	E2F1	131.	SRY
22.	CFAP65	44.	F13A1	66.	CATSPER1	88.	CXCL16	110.	PROCR	132.	USP9Y

The most frequently observed variants are those observed in the CFTR gene. 18 CFTR gene variants were detected in 16 different patients. Among these, 7 variants are pathogenic, 4 variants are likely pathogenic and 7 variants are VUS. The remaining 40 variants are distributed among the other 27 genes in the panel. Among these, 8 variants were evaluated as pathogenic, 7 variants as likely pathogenic, and 25 variants as VUS. Segregation analyses could not be performed in patients with VUS. Pathogenic and likely pathogenic variants were detected de novo. Among the variants considered as VUS, the most frequently observed variants clustered in the *DNAH1* gene. Among the pathogenic variants, the most common variants after *CFTR* were detected in the *CBS* and *F11* genes (Table 2). We also detected a compound heterozygous *CFTR* variant in one of our patients. This condition of the patient was evaluated clinically.

Table 2. Variants detected in the patient group as a result of NGS

Patient n.	VUS - inheritance	Likely pathogenic- inheritance	Pathogenic- inheritance
1.	NM_000130.5(F5):c.1128G>T p.R376S **(PP3) (AD,AR)** NM_001370.2(DNAH6):c.8422G>A (p.V2808I) **(PP3)** **(n/a)**		
2.	NM_000313.4(PROS1): c.1021G>T (p.A341S) **(PM1,PM2,PP3) (AD)**	NM_000492.4(CFTR):c.1516A>G (p.Ile506Val) **(PM1,PM2,PM3,PP3,BP6) (AR)**	
3.	NM_015512.5(DNAH1):c.8885A>C (p.Lys2962Thr) **(PP3) (AR)**		
4.	NM_000492.4(CFTR):c.443T>C (p.Ile148Thr) **(PS3,M1,PM2,PM3,PP3,BS2, BS3,BP2,BP6) (AR)**		

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5.	NM_000492.4(CFTR):c.2981T>G (p.Phe994Cys) **(PM1,PM2,PP3) (AR)**		NM_000128.4(F11):c.1556G>A (p.Trp519Ter) **(PVS1,PS4,PM2,PM3,PP5)** NM_000128.4(F11):c.403G>T (p.Glu135Ter) **(PA1,PVS1,PS3,PS4,PM3,PP5,BS1,BS2) (AD,AR)**
6.	NM_001242805.2 (BRDT):c.163C>T (p.Pro55Ser) **(PP3) (AR)**		
7.	NM_001350162.2 (TEX15):c.2580_2583del (p.Asp860GlufsTer15) **(PS3,PP3) (AR)**	NM_000173.7(GP1BA): c.1235_1298delAGCCCAC... (p.E412fs*39) **(PVS1,PM2) (AD,AR)**	
8.	NM_000789.4(ACE):c.2299G>A (p.Glu767Lys) **(PM2,PP3) (AR)**		
9.		NM_000312.4(PROC):c.982C>T (p.Arg328Cys) **(PM1,PM2,PP3,PP5) (AD,AR)**	NM_002203.4(ITGA2):c.981_985del (p.Lys327AsnfsTer6) **(PVS1,PM2) (n/a)**
10.			NM_000492.4 (CFTR):c.2491G>T (p.Glu831Ter) **(PVS1,PS3,PS4,PM2) (AR)**
11.			NM_000492.3(CFTR):c.1521_1523del (p.Phe508del) **(PA2,PS3,PM1,PM4,PP3,BS3, BS4,BP2,BP5) (AR)**
12.			NM_000492.4(CFTR):c.1210-11T>G **(PA2,PS3,PM3,PP5,BS1,BS2,BP2) (AR)**
13.	NM_000492.4(CFTR):c.2991G>C (p.Leu997Phe) **(PS1,PM1,PM2,PM3,PP3,BS2,BP2,BP6) (AR)**		
14.			NM_054012.4(ASS1):c.535T>C (p.Trp179Arg) **(PS3,PS4,PM1,PM2,PM3,PP3,PP5) (AR)**
15.	NM_144605.4(SEPT12): c.208T>C (p.Phe70Leu) **(PM2,PP3)**	NM_000492.4(CFTR): c.1397C>T (p.S466L) **(PM1,PM2,PM3,PP3) (AR)**	
16.	NM_000492.4(CFTR):c.2973A>G (p.Ile991Met) **(PM1,PM2) (AR)**		
17.	NM_015512.5(DNAH1):c.10164G>T (p.K3388N)**(PP3) (AR)**		
18.		NM_015102.5(NPHP4):c.224G>A (p.Trp75Ter) **(AR)**	
19.	NM_000492.4(CFTR): c.1043T>A (p.M348K) **(PM1,PM2,PM3,PP3,BP2,BP6) (AR)** NM_012128.4 (CLCA4): c.760dupA (p.T254fs*3) **(PVS1) (n/a)**	NM_000492.4 (CFTR) : c.3038C>T (p.P1013L) **(PM1,PM2,PM3,PP3)**	
20.			NM_000071.3(CBS):c.833T>C (p.Ile278Thr) **(PS3,PS4,PM1,PM2,PM3,PM5,PP3) (AR)**
21.			NM_000128.4(F11):c.325G>A (p.Ala109Thr) **(AD,AR) (PS3,PS4,PM1,PM2,PM3,PP3,PP5)** NM_000492.4(CFTR):c.1516A>G (p.Ile506Val) **(PM1,PM2,PM3,PP3,BP6) (AR)**
22.		NM_173812.5(DPY19L2): c.247C>T (p.Q83*) **(PVS1,PM2) (AR)**	NM_000492.4(CFTR):c.1521_1523delCTT (p.F508del) **(PA2,PS3,PS4,PM1,PM3,PM4, PP3,PP5,BS1,BS2,BS4,BP2) (AR)**
23.		NM_000492.3(CFTR):c.3872A>G (p.Q1291R) **(PM1,PM2,PM3,PM5,PP3,PP5) (AR)**	
24.			NM_000071.3(CBS):c.833T>C (p.I278T) **(PS3,PS4,PM1,PM2,PM3,PM5,PP3) (AR)**
25.	NM_000492.4(CFTR):c.3256A>G (p.Thr1086Ala)**(PP3) (AR)**		

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26.	NM_000894.2 (LHB):c.169T>C NP_000885.1:p.Tyr57His **(PM2,PP3) (AR)**		
27.		NM_000301.5(PLG):c.2384G>A (p.Arg795His) **(PS4,PM2,PP3) (AD,AR)**	
28.	NNM_000789.4(ACE): c.3490G>A (p.G1164R) **(PM2,PP3) (AR)**		
29.			NM_000492.4(CFTR): c.350G>A (p.R117H) **(PA2,PS3,PM1,PM2,PM3,PM5,PP1,PP3, PP5,BS2,BS4,BP2) (AR)**
30.	NM_015512.5 (DNAH1): c.8976C>G p.F2992L**(PP3) (AR)**		
31.		NM_000131.4 (F7): c.805+3_805+6delGGGT (-) **(PVS1,PM2) (AR)**	
32.	NM_001994.3(F13B): c.209A>C (p.Q70P) **(PM2,PP3) (AR)**		NM_000492.4 (CFTR) : c.1521_1523delCTT (p.F508del) **(PA2,PS3,PM1,PM3,PM4,PP3,PP5,BS1, BS2,BS3,BS4,BP2) (AR)**
33.		NM_017780.4 (CHD7) :c.5995G>A (p.A1999T)**(PS4,PM2,PP2,PP3) (AD)**	
34.	NM_012128.3 (CLCA4):c.575C>A NP_036260.2:p.Ser192Cys **(PP3) (n/a)**		
35.	NM_015512.5(DNAH1): c.9495G>A (p.Thr3165) **(PM2,PP3) (AR)** NM_152467.5 (KLHL10): c.1226A>G (p.E409G) **(PM2,PP3) (AD)** NM_144605.4 (SEPTIN12) :c.611G>T (p.Arg204Leu) **(PP3) (AD)**		
36.	NM_001330438.2 (DDX25): c.110C>T (p.Ala37Val) **(PP3) (n/a)**		
37.			NM_000071.3(CBS):c.833T>C (p.Ile278Thr) **(PS3,PM1,PM5,PP3)**
38.	NM_001312675.1 (F10):c.202C>T NP_001299604.1:p.Arg68Cys **(PM2,PP3) (AR)** NM_000212.2 (ITGB3):c.1576G>C NP_000203.2:p.Glu526Lys **(PP3) (AD,AR)** NM_015512.5 (DNAH1): c.4642C>G (p.L1548V) **(PM2,PP3) (AR)**		
39.	NM_000301.5(PLG):c.2134G>A (p.Gly712Arg) **(PP3) (AD,AR)**		
40.	NM_000492.4(CFTR):c.890G>A (p.Arg297Gln) **(PS3,PM1,PP3,BS3,BS6) (AR)** NM_173812.5(DPY19L2): c.2221C>A (p.P741T) **(PM2,PP3) (AR)**		
41.	NM_173628.3(DNAH17):c.7752+2T>A **(PVS1) (AR)**		
Total	**32**	**11**	**15**

AD: autosomal dominant; **AR:** autosomal recessive

DISCUSSION

Highly diverse phenotypic representation and a complicated multifactorial etiology, including environmental and genetic factors, characterize the condition of male infertility. In most cases, it is challenging to identify a genetic cause of infertility due to the large number of candidate genes (9, 10). In any case, a multi-disease gene panel can help identify the cause of male infertility. In order to categorize genetic variants, a multifactorial likelihood model can be used to assess the likelihood that a variant is pathogenic based on a previous likelihood of patho-

genicity based on in silico research and the genetic and epidemiological data that are currently available (11-13). Genetic variants can be categorized into five categories according to the American College of Medical Genetics and Genomics' references: pathogenic, likely pathogenic, variant of unknown consequence, likely benign, or benign (14). A genetic alteration known as a VUS has ambiguous effects on gene function. The interpretation of VUS is a challenging task for the clinical management of infertile male patients and genetic counseling. Since VUS are not clearly related with a phenotype currently, but could be categorized as pathogenic in the future, it is crucial to detect and assess them. An example of this situation is the NM_000071.3(*CBS*):c.833T>C (p.Ile278Thr) variant detected in our patient group. This variant, which was evaluated as VUS in the databases at the beginning of our study, was later classified as pathogenic.

Variants in *PROS1* and *CFTR* were detected in our patient number 2. *PROS1* variants show autosomal dominant inheritance. Even though the detected variant was evaluated as VUS, the classification of this variant should be followed in the future.

Similarly, in our patient number 5, 2 pathogenic variants belonging to the F11 gene and a variant evaluated as VUS in *CFTR* were detected. F11 variants show autosomal dominant inheritance. The detected variants were evaluated as pathogenic. OMIM has been associated with Factor XI deficiency.

In another patient, case number 7, variants were detected in 2 separate genes. VUS evaluation was performed for *TEX15*. *TEX15* is associated with the Spermatogenic failure 25 phenotype in the OMIM database, and exhibits an autosomal recessive pattern of inheritance. The other variant detected in the patient is a possible pathogenic variant belonging to the *GP1BA* gene. This gene, which is associated with different types of diseases in the OMIM database, can show autosomal dominant and recessive inheritance. Particularly notable among these diseases are Bernard-Soulier syndrome, type A2 (dominant) and von Willebrand disease, platelet-type (dominant).

PROC variant was detected in patient number 9 and was reported as possibly pathogenic. PROC variants have been associated with autosomal recessive and dominant forms of Thrombophilia 3 due to protein C deficiency in OMIM. Another variant in the patient is the pathogenic variant in the *ITGA2* gene. The inheritance pattern and phenotype of variant of this gene have not yet been elucidated.

We detected variants in *SEPT 12* and *CFTR* genes in patient number 15. *SEPT* 12 variants cause autosomal dominant Spermatogenic failure 10.

In patient number 19, we detected compound heterozygous variants of the *CFTR* gene and an additional variant of the *CLCA4* gene. While *CFTR* gene variants exhibit autosomal recessive inheritance, there are no entries in the databases yet for the *CLCA4* gene.

We detected *F11* and *CFTR* pathogenic variants in patient number 21. *F11* was found to be associated with autosomal dominant and recessive forms of Factor XI deficiency in the OMIM database.

We detected *DPY19L2* and *CFTR* pathogenic variants in patient number 22. *DPY19L2* was found to be associated with autosomal recessive forms of spermatogenic failure 9 in the OMIM database. Similarly, patient number 40 has variants in these two genes that were evaluated as VUS.

F13B and *CFTR* variants were detected in another of our patients, case number 32. *F13B* has been associated with Factor XIIIB deficiency in OMIM. *F13B* variants show autosomal recessive inheritance.

Interestingly, variants in 3 different genes were detected in our last 2 patients. Variants considered to be VUS were detected in the *DNAH1, KLHL10* and *SEPT12* genes in the first patient. *KLHL10* and *SEPT12* variants have been associated with autosomal dominant spermatogenic failure. DNAH1 has been found to be associated with autosomal recessive Ciliary dyskinesia, primary and Spermatogenic failure. These findings were associated with the patient's phenotype. In the second patient, variants considered to be VUS were detected in the *DNAH1, F10* and *ITGB3* genes. The *F10* gene has been associated with autosomal recessive Factor X deficiency. *ITGB3* has been associated with autosomal recessive Bleeding disorder, platelet-type and autosomal dominant Glanzmann thrombasthenia.

Although it is difficult to reconcile those with recessive inheritance in the detected variants with the patient clinics, those with dominant inheritance were compatible with the patient clinics.

DNAH1 gene is the most frequently detected VUS variant in our patient group. A diverse range of patients with aberrant flagellar structures have been shown to have mutant *DNAH1* in the majority of current research. Male infertility has been linked to numerous morphologic defects of the sperm flagella caused by mutations in *DNAH1*. After intracytoplasmic sperm injection, patients with multiple morphologic abnormalities of the flagella (MMAF) caused by mutations in the *DNAH1* gene have a favorable prognosis. These investigations have demonstrated that dysplasia of the sperm fibrous sheath (DFS) and infertility are directly related to abnormalities in the *DNAH1* gene (15-18).

The most frequently detected *CFTR* variants in our patient group were seen in all 3 groups (pathogenic, likely pathogenic, VUS). One of the most researched genes for male infertility, the *CFTR* gene, has 27 exons and more

than 180,000 base pairs of DNA. A membrane ion channel protein called CFTR, which is encoded on chromosome 7p, controls the vas deferens in the male genital tract. Considered a moderate type of cystic fibrosis (CF), CBAVD is a major contributing factor to obstructive azoospermia (OA) and is one of the primary factors leading to male reproductive abnormalities. Previous research has shown a positive correlation between *CFTR* mutations and CBAVD. There are about 1,500 variants listed in the *CFTR* database. Mutations in the F508 and IVS8-5T genes may be important in nonobstructive male infertility disorders such oligozoospermia and nonobstructive azoospermia (NOA). According to our findings, there is a chance that the genetic variant IVS8-5T could serve as a biomarker for nonobstructive male infertility. Three of the pathogenic variants we detected in the patient group are IVS8-5T. We also detected a compound heterozygous *CFTR* variant in one of our patients. The condition of the patient was evaluated clinically. *CFTR* variant rates are also significantly higher than the carrier rate reported in our patient group (19-22).

Another gene we detected among pathogenic gene variants is the *CBS* gene. The *CBS* gene is the most common locus for mutations associated with homocystinuria. Cystathionine-β-synthase, also known as CBS, is an enzyme that is encoded by the CBS gene in humans. The trans-sulfuration pathway enzymes cystathionine β-synthase (CBS) and cystathionine γ-lyase (CSE) are recognized for their non-specific substrate recognition. These enzymes provide their substrates an alternate CBS and CSE pathway, allowing them to function somewhat in reverse. In addition, these enzymes are involved in the synthesis of hydrogen sulphide (H_2S). This is a gaseous transmitter with antioxidant and anti-inflammatory properties. Although both CSE and CBS are frequently present in the testes—CSE is mostly found in immature germ cells and Sertoli cells, while CBS is extensively distributed in Leydig cells, germ cells, and Sertoli cells—it is unclear how much each kind of cell contributes to the production of H_2S in the testes. Numerous research studies have demonstrated that male infertility is associated with failure of one carbon metabolism, namely the imbalance of CBS and CSE enzymes in the trans-sulfuration pathway, and a specific deficit in H_2S output is documented (23, 24).

Another gene we detected as a pathogenic variant in our patient group was *FXI*. Compound heterozygous *FXI* pathogenic variant was detected in one of our patients. In another patient, we detected a heterozygous *FXI* variant of *CFTR* accompanied by pathogenic variant. *FXI*, or coagulation factor XI, inhibits fibrinolysis and encourages the production of fibrin. One measurement only elevated plasma FXI levels are linked to an increased risk of thrombosis. Hemophilia C, another name for factor XI deficiency, is an autosomal recessive condition mostly affecting Ashkenazi Jews. It is typically linked to variants in bleeding characteristics. The majority of transmission is autosomal recessive. Couples who are considered at-risk (both individuals carry a mutation that causes the disease) should be informed through genetic counseling that there is a 25% chance that each pregnancy will result in a homozygous child who is affected. There have also been reports of heterozygous patients with bleeding symptoms, pointing to an autosomal dominant mode of transmission with varying penetrance (25, 26).

Genes that code for hormones and hormone receptors which are involved in the functioning of the human reproductive system are included in the fertility panel design. Numerous investigations have demonstrated the association between specific polymorphisms in genes encoding receptors, including those that bind to FSH and LH, and the results of an ovarian hyperstimulation cycle under control and in vitro fertilization treatment. The intended genetic panel's results will yield the data required to ascertain the frequency of these variants in our community and assess the panel's usefulness in clinical settings.

The involvement of the clinicians who seek this genetic investigation needs to be emphasized. If the gene panel is able to pinpoint the underlying reason of infertility, clinicians will need to have a comprehensive picture of the patient's phenotype. "Idiopathic infertility" affects a large number of individuals, and while a genetic component may be identified in certain cases, the absence of a distinct phenotype may make it more difficult to interpret the data, particularly variants with unclear significance. Clinicians should also be aware that three factors play a major role in how these investigations are interpreted: the patient's phenotypic characteristics, their medical history, and any pertinent family history. For the diagnostic laboratory to properly interpret variants found through testing, it is imperative that they have information about all observable traits as well as the family's medical history (27-30).

The first unique gene sequencing panel intended for the diagnosis of hereditary infertility in males is presented here, for the first time in Turkey. The use of this panel will advance knowledge of the genetic causes of infertility, enhance genetic and reproductive counseling, and eventually lead to more accurate assisted reproductive techniques.

CONCLUSION

Consequently, pre-diagnostic genes included in a custom-made NGS panel test can enhance genetic diagnostic testing and have an impact on the clinical management of male infertility. There are currently no comprehensive

systematic studies or meta-analyses on the epidemiology of male infertility, and it is unknown how common male infertility is. The need of diagnosing hereditary infertility is further supported by the epidemiological data that show infertile patients have greater morbidity and a shorter life expectancy. Finally, we demonstrated the effectiveness of NGS-based methods that additionally use pre-diagnostic genes. This gene panel may aid in determining the disorder's underlying etiology and directing clinical treatment.

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Data Availability

The datasets generated and analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of Interest

The authors have no conflicts of interest to declare.

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ORIGINAL ARTICLE

INTERLEUKIN-1β AND *TUMOR NECROSIS FACTOR-α* GENE POLYMORPHISMS IN SYSTEMIC SCLEROSIS

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ABSTRACT

The complex cytokine network plays an important role in disease susceptibility and development, therefore single-nucleotide polymorphisms (SNPs) in or near cytokine genes may be relevant to development of systemic sclerosis (SSc). We in this study investigated 22 SNPs in 13 cytokine genes of SSc patients, and their association with disease susceptibility. Twenty-three clinically diagnosed SSc patients were enrolled for this purpose along with 80 healthy volunteers for comparisons. Aseptically collected 2ml of peripheral venous blood from each subject was processed for DNA extraction. Cytokine genotyping was carried out using the extracted genomic DNA by PCR employing sequence-specific primers and data was analyzed for any association with SSc susceptibility. Variations in allele, genotype, or haplotype distribution between patients and healthy volunteers were observed for the following SNPs: *IL-1β* –511 C/T (rs16944) and +3962 T/C (rs1143634); *IL-4Rα* +1902 G/A (rs1801275); *IL-12* –1188 C/A (rs3212227); *TGF-β1* codon 25 G/C (rs1800471); *TNF-α*–308 G/A (rs1800629) and –238 G/A (rs361525); *IL-4* –1098 T/G (rs2243248) and –590 T/C (rs2243250); *IL-6* –174 G/C (rs1800795) and nt565 G/A (rs1800797); and *IL-10* –1082 G/A (rs1800896), –819 C/T (rs1800871) and –592 C/A (rs1800872). However, only the SNPs in *IL-1β* –511 and +3962, and *TNF-α* –308 and –238 were found to be significantly associated with SSc susceptibility. Our findings suggest that *IL-1β* and *TNF-α* gene SNPs may play a role in development of SSc, although large observational and experimental studies are needed to substantiate these findings.

Keywords: Autoimmunity; Cytokines; Disease development; Genotyping; Predisposition

INTRODUCTION

Systemic sclerosis (SSc) is a generalized disorder of small arteries, microvessels, and connective tissue. It is a disease of unknown origin, with the highest incidence occurring between 45 to 55 years of age [1]; the frequency is three to eight times higher in females [2]. Several studies have demonstrated that the extent of skin involvement directly correlates with internal organ involvement and prognosis in SSc patients [3, 4]. Manifestations associated with SSc have been found to negatively impact the quality of life in affected individuals [5].

Long-term occupational exposure to environmental toxins is a common finding in SSc patients [6]. However, the effect of these environmental toxins on immune system of these genetically susceptible patients is unclear. Recent studies have raised the possibility that both genetic and environmental factors act synergistically at several stages of autoimmunity pathogenesis. These studies predict that individuals susceptible to spontaneous autoimmunity should be more susceptible following xenobiotic exposure by virtue of the presence of predisposing background genes [7]. Studies have shown that genetic predisposition plays an important role in susceptibility and the development

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of autoimmune diseases. This is likely due to functional polymorphisms within multiple genes, each of which, by modulating corresponding protein expression, influences disease susceptibility.

Cytokines may promote the deposition of collagen and fibrosis [8] and many studies have focused on the role of these mediators in SSc, enlisting alterations in their concentrations [9, 10] or in the balance between Th1 and Th2 cytokine levels [11]. Because cytokine production is regulated at the genetic level [12, 13], it has been hypothesized that single-nucleotide polymorphisms (SNPs) in or near cytokine genes may be relevant to the development of SSc.

Numerous studies examining patients with SSc from diverse ancestral backgrounds have identified SNPs in various cytokine genes. Among these, the *IL-1* cytokine family has emerged as particularly significant in the pathogenesis of SSc. There is compelling evidence linking polymorphisms within the *IL-1* family to the disease, as summarized by Xu et al. [14]. Specific gene variants in *IL-1α* [15], *IL-1β* and *IL-2* [16,17], as well as *IL-10* [18,19], have been observed across different populations. However, some associations reported in individual studies [19] have not been consistently replicated in others [17], highlighting the complexity and variability of these genetic links.

These and several other contradictions motivated us to investigate the commonly studied cytokine gene SNPs among our SSc patients, and compare our findings with those previously reported. In this study we evaluated the presence of 22 SNPs in 13 cytokine genes in SSc patients and attempted to associate the significant SNPs with SSc disease susceptibility in our population.

MATERIALS AND METHODS

Samples

Patients clinically diagnosed with SSc, confirmed through established laboratory investigations and meeting the American College of Rheumatology (ACR) criteria [20], were consecutively enrolled at a tertiary care hospital in North India. The rarity of SSc, its genetic heterogeneity, and strict diagnostic criteria present significant challenges in participant recruitment for SNP studies. Additionally, specific disease subtypes, comorbidities, drug exclusions, geographic barriers, and ethical concerns further limit the eligible patient pool.

Our study involved 23 SSc patients and 80 age-matched healthy volunteers of Asian ethnicity. Patients and healthy volunteers were unrelated, and the latter had no clinical history of skin disease, minimizing potential confounders and ensuring clear group distinction. Peripheral venous blood (2ml) was collected aseptically from each patient and healthy volunteers into EDTA vacutainer tubes and used for DNA extraction. The study was approved by the Institutional Ethical Committee-Human Research, and written informed consent was obtained from each patient and healthy volunteer before enrollment in the study.

Genomic DNA Extraction from Blood

Genomic DNA was extracted from blood samples of 23 SSc patients and 80 healthy controls for cytokine genotyping of 22 SNPs in 13 cytokine genes using PCR with sequence-specific primers. DNA extraction was performed using the HiPurA™ blood genomic DNA extraction kit (HiMedia Laboratories) as per the manufacturer's protocol. Briefly, 200µl of blood was mixed with 20µl Proteinase K solution, vortexed, then treated with 20µl RNase A solution. After incubation, 200µl of lysis buffer (C1) was added, followed by a 10-minute incubation at 55°C. Ethanol (200µl) was added, and the lysate was transferred to a spin column for centrifugation. The column was washed with prewash and wash buffers, then eluted with 100µl elution buffer after a 5-minute incubation. DNA was stored at -20°C for PCR analysis.

Cytokine Genotyping by PCR

Cytokine genotyping was carried out from genomic DNA by PCR with sequence-specific primers using commercially available Cytokine Genotyping Kit (Invitrogen Corporation, USA). Twenty two SNPs (*IL-1α* –889 T/C; *IL-1β* –511 C/T and +3962 T/C; *IL-1R* pst1 1970 C/T; *IL-1RA* mspa1 11100 T/C; *IL-4Rα* +1902 G/A; *IL-12* –1188 C/A; *IFN-γ* +874 A/T; *TGF-β1* codon 10 T/C and codon 25 G/C; *TNF-α* –308 G/A and –238 G/A; *IL-2* –330 T/G and +166 G/T; *IL-4* –1098 T/G, –590 T/C, and –33 T/C; *IL-6* –174 G/C and nt565 G/A; *IL-10* –1082 G/A, –819 C/T, and –592 C/A) in thirteen cytokine genes were assessed in all the patients and healthy volunteers using the kit according to the included instructions.

For 48 reactions/wells for each sample, 140µl of PCR buffer was mixed with 3.3µl of Taq DNA polymerase, 329µl of water and 50µl of 75-125ng/µl concentrated DNA template. The reaction mixture (10µl) was dispensed into each well and the following thermal cycler profile was used for amplification. Step 1 was denaturation for 2 minutes at 94°C; Step 2 comprised 10 cycles of 94°C for 15 seconds and 65°C for 60 seconds with no separate extension step; Step 3 (20 cycles) consisted of 94°C for 15 seconds, 61°C for 50 seconds, and 72°C for 30 seconds. The profile was set on hold at 4°C.

The PCR products were loaded onto a 2 percent agarose gel in a specific order for electrophoresis and run at 150 volts for 20-25 minutes for separating the DNA. After electrophoresis, the ethidium bromide stained gel was photographed and interpreted for specific amplification patterns using the worksheet provided with the kit.

Presence of a control band in each lane was ascertained. Wells identifying the *IL-2*, *IL-4*, *IL-6*, and *IL- 10* cytokines contained an 89 bp fragment of the β-globin gene as an internal control. Wells identifying the *IL-1α*, *IL-1β*, *IL-1R*, *IL-1Rα*, *IL-4Rα*, *IL-12*, *IFN-γ*, *TGF-β*, and *TNF-α* cytokines contained a 440 bp fragment of the human C-reactive protein gene as an internal control.

Statistical Analysis

Two-sided Fisher's exact test was used to compare allele, genotype and haplotype frequencies between patients and controls. The threshold for significance was $p<0.05$, and the relative risks associated with rare alleles, genotypes and haplotypes were estimated as odds ratios (ORs) with 95% confidence intervals (CIs). The deviation from Hardy-Weinberg equilibrium (HWE) was determined using a goodness-of-fit Chi-square test to compare the observed genotype frequencies with the expected frequencies among the patients and healthy volunteers. The polymorphisms were excluded if they deviated from HWE. All statistical analyses were performed by SPSS 16.0 (SPSS Inc).

RESULTS

Patients and controls

A total of 23 patients with SSc (4 males, 19 females; mean age 35.5 years) and 80 healthy volunteers (32 males, 48 females; mean age 36 years) were analyzed for 22 SNPs in 13 cytokine genes using cytokine genotyping with sequence-specific primers. The duration of SSc disease ranged from 2 months to 14 years. Common presentations included Raynaud phenomenon, skin sclerosis, and pigmentation, along with finger contractures, digital ulcers, dyspnea, restricted mouth opening, joint issues, and dysphagia. The Rodnan skin score ranged from 9 to 51. The higher proportion of females in the SSc patient group (82.6%) compared to the control group (60%) reflects the well-established female predominance in systemic sclerosis (SSc), with the disease being more prevalent in women. The higher number of healthy volunteers was driven by the rarity of SSc, its strict diagnostic criteria, and the challenges in recruitment, while healthy volunteers are more readily available. This larger control group ensures a robust comparison, minimizes biases, and enhances the study's ability to detect genetic associations, especially given SSc's genetic heterogeneity and clinical variability. All SNPs, except the *IL-12* -1188 C/A ($p<0.05$ for patients and $p<0.01$ for controls), were in Hardy-Weinberg equilibrium ($p>0.05$) for both groups.

Significance of Cytokine Gene Polymorphisms

Distribution of allelic or genotypic frequencies of *IL-1α* –889 T/C (rs1800587); *IL-1RI* pst11970 C/T (rs2234650); *IL-1RA* mspaI11100 T/C (rs315952); *IFN-γ* +874 A/T (rs2430561); *TGF-β1* codon 10 T/C (rs1982073); *IL-2* –330 T/G (rs2069762) and +166 G/T (rs2069763); and *IL-4* –33 T/C (rs2070874) cytokine gene polymorphisms was similar in patients and controls. No statistically significant associations of these SNPs with the disease could be found (data not shown). However, variations in allele, genotype or haplotype distribution were observed in *IL- 1β* –511 C/T (rs16944) and +3962 T/C (rs1143634); *IL-4Rα* +1902 G/A (rs1801275); *IL-12* –1188 C/A (rs3212227); *TGF-β1* codon 25 G/C (rs1800471); *TNF-α*–308 G/A (rs1800629) and –238 G/A (rs361525); *IL-4* –1098 T/G (rs 2243248) and –590 T/C (rs2243250); *IL-6* –174 G/C (rs1800795) and nt565 G/A (rs1800797); and *IL-10* –1082 G/A (rs1800896), –819 C/T (rs1800871) and –592 C/A (rs1800872) gene polymorphism (Table 1).

Table 1. Single nucleotide polymorphisms showing allele and genotype frequencies in patients with systemic sclerosis and healthy controls.

Cytokine polymorphism			SSc (n=23)	HC (n=80)	*p*-value
IL1β -511 (rs16944)	Alleles	T	33 (71.7)	80 (50.0)	**0.011***
		C	13 (28.3)	80 (50.0)	**0.011***
	Genotypes	TT	14 (60.9)	29 (36.3)	0.054
		TC	5 (21.7)	22 (27.4)	0.789
		CC	4 (17.4)	29 (36.3)	0.128
IL1β +3962 (rs1143634)	Alleles	C	28 (60.9)	129 (80.6)	**0.01***
		T	18 (39.1)	31 (19.4)	**0.01***
	Genotypes	CC	11 (47.8)	56 (70.0)	0.081
		CT	6 (26.1)	17 (21.2)	0.585
		TT	6 (26.1)	7 (8.8)	0.068
IL4Rα +1902 (rs1801275)	Alleles	G	9 (19.6)	23 (14.4)	0.488
		A	37 (80.4)	137 (85.6)	0.488
	Genotypes	GG	2 (8.7)	0 (0.0)	**0.048***
		GA	5 (21.7)	23 (28.8)	0.602
		AA	16 (69.6)	57 (71.2)	1.000

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IL12 -1188 (rs3212227) [deviation from HWE, therefore excluded]	Alleles	C	16 (34.8)	94 (58.8)	**0.005***
		A	30 (65.2)	66 (41.2)	**0.005***
	Genotypes	CC	0 (0.0)	35 (43.8)	**0.000***
		CA	16 (69.6)	24 (30.0)	**0.001***
		AA	7 (30.4)	21 (26.2)	0.791
TGFβ1 codon10 (rs1982073)	Alleles	C	23 (50.0)	81 (50.6)	1.000
		T	23 (50.0)	79 (49.4)	1.000
	Genotypes	CC	0 (0.0)	1 (1.2)	1.000
		CT	23 (100.0)	79 (98.8)	1.000
		TT	0 (0.0)	0 (0.0)	-
TGFβ1 codon25 (rs1800471)	Alleles	G	35 (76.1)	96 (60.0)	0.056
		C	11 (23.9)	64 (40.0)	0.056
	Genotypes	GG	12 (52.2)	16 (20.0)	**0.006***
		GC	11 (47.8)	64 (80.0)	**0.006***
		CC	0 (0.0)	0 (0.0)	-
TGFβ1 codon10, codon25	Haplotypes	CG	12 (26.1)	17 (10.6)	**0.014***
		TG	23 (50.0)	79 (49.4)	1.000
		CC	11 (23.9)	64 (40.0)	0.056
TNFα -308 (rs1800629)	Alleles	G	42 (91.3)	103 (64.4)	**0.000***
		A	4 (8.7)	57 (35.6)	**0.000***
	Genotypes	GG	19 (82.6)	23 (28.8)	**0.000***
		GA	4 (17.4)	57 (71.2)	**0.000***
		AA	0 (0.0)	0 (0.0)	-
TNFα -238 (rs361525)	Alleles	G	27 (58.7)	137 (85.6)	**0.000***
		A	19 (41.3)	23 (14.4)	**0.000***
	Genotypes	GG	4 (17.4)	57 (71.2)	**0.000***
		GA	19 (82.6)	23 (28.8)	**0.000***
		AA	0 (0.0)	0 (0.0)	-
TNFα -308, -238	Haplotypes	GG	23 (50.0)	80 (50.0)	1.000
		AG	4 (8.7)	57 (35.6)	**0.000***
		GA	19 (41.3)	23 (14.4)	**0.000***
IL4 -1098 (rs 2243248)	Alleles	T	35 (76.1)	138 (86.2)	0.112
		G	11 (23.9)	22 (13.8)	0.112
	Genotypes	TT	14 (60.9)	58 (72.5)	0.309
		TG	7 (30.4)	22 (27.5)	0.796
		GG	2 (8.7)	0 (0.0)	**0.048***
IL4 -590 (rs2243250)	Alleles	C	38 (82.6)	109 (68.1)	0.065
		T	8 (17.4)	51 (31.9)	0.065
	Genotypes	CC	15 (65.2)	43 (53.7)	0.353
		CT	8 (34.8)	23 (28.8)	0.611
		TT	0 (0.0)	14 (17.5)	**0.036***
IL4 -33 (rs2070874)	Alleles	T	9 (19.6)	52 (32.5)	0.102
		C	37 (80.4)	108 (67.5)	0.102
	Genotypes	TT	3 (13.05)	15 (18.8)	0.757
		TC	3 (13.05)	22 (27.4)	0.180
		CC	17 (73.9)	43 (53.8)	0.098
IL6 -174 (rs1800795)	Alleles	G	38 (82.6)	144 (90.0)	0.193
		C	8 (17.4)	16 (10.0)	0.193
	Genotypes	GG	18 (78.3)	64 (80.0)	1.000
		GC	2 (8.7)	16 (20.0)	0.350
		CC	3 (13.0)	0 (0.0)	**0.01***
IL6 nt565 (rs1800797)	Alleles	G	40 (87.0)	145 (90.6)	0.580
		A	6 (13.0)	15 (9.4)	0.580
	Genotypes	GG	20 (87.0)	65 (81.2)	0.757
		GA	0 (0.0)	15 (18.8)	**0.021***
		AA	3 (13.0)	0 (0.0)	**0.01***

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IL10 -1082 (rs1800896)	Alleles	A	32 (69.6)	88 (55.0)	0.091
		G	14 (30.4)	72 (45.0)	0.091
	Genotypes	AA	9 (39.1)	8 (10.0)	**0.002***
		AG	14 (60.9)	72 (90.0)	**0.002***
		GG	0 (0.0)	0 (0.0)	-
IL10 -819 (rs1800871)	Alleles	C	23 (50.0)	103 (64.4)	0.088
		T	23 (50.0)	57 (35.6)	0.088
	Genotypes	CC	6 (26.1)	23 (28.8)	1.000
		CT	11 (47.8)	57 (71.2)	**0.047***
		TT	6 (26.1)	0 (0.0)	**0.000***
IL10 -592 (rs1800872)	Alleles	A	19 (41.3)	57 (35.6)	0.493
		C	27 (58.7)	103 (64.4)	0.493
	Genotypes	AA	2 (8.7)	0 (0.0)	**0.048***
		AC	15 (65.2)	57 (71.2)	0.611
		CC	6 (26.1)	23 (28.8)	1.000
IL10 -1082, -819, -592	Haplotypes	ATA	17 (37.0)	50 (31.2)	0.479
		ACC	15 (32.6)	38 (23.8)	0.252
		GCC	14 (30.4)	72 (45.0)	0.091

Allele, genotype and haplotype frequencies are presented as absolute numbers with percentage in parentheses; rs - refSNP cluster ID number;
*Mean difference is significant at the indicated *p*-value

As polymorphism in *IL-12* –1188 C/A did not obey HWE, it was excluded from the study. While HWE deviations can signal genotyping errors or population stratification, in our case, they are likely due to the small sample size and the specific characteristics of the SSc population.

IL-1β Cytokine Gene Polymorphism

Significant differences in allele distributions were found between the patients with SSc and controls for *IL-1β* +3962. The T allele was significantly more common in patients than in controls (OR 2.675, 95% CI 1.315-5.442; $p<0.05$). The T allele of *IL-1β* -511 also showed a trend towards association with SSc (OR 2.538, 95% CI 1.245-5.177; $p<0.05$). No significant difference in their genotype distribution was found between patients with SSc and controls (all $p>0.05$) (Table 2).

TNF-α Cytokine Gene Polymorphism

The *TNF-α* -308 G allele frequency was significantly increased in patients with SSc compared with the healthy volunteers (91.3% vs 64.4%; $p<0.001$; OR 5.811, 95% CI 1.982- 17.032). The GG genotype at *TNF-α* -308 was also significantly increased in the SSc patients (82.6% vs 28.8%; $p<0.001$; OR 11.772, 95% CI 3.61-38.384). Carriage of the *TNF-α* -238 A allele was significantly more common among patients with SSc than among control subjects (41.3% vs 14.4%; $p<0.001$; OR 4.192, 95% CI 2.011–8.737). The GA genotype frequency at *TNF-α* -238 was also significantly higher in patients than in controls (82.6% vs 28.8%; $p<0.001$; OR 11.772, 95% CI 3.61-38.384). Haplotype analysis showed that specific (-308G -238A) haplotype was observed more often in the SSc patients than in controls ($p<0.001$) (Table 3).

Table 2. IL-1β Cytokine Gene Polymorphism association with systemic sclerosis.

Cytokine polymorphism			SSc (n=23)	HC (n=80)	*p*-value	Odds Ratio	95% CI
IL-1β -511 (rs16944)	Alleles	T	33 (71.7)	80 (50.0)	**0.011***	2.538	1.245 - 5.177
		C	13 (28.3)	80 (50.0)	**0.011***	0.394	0.193 - 0.803
	Genotypes	TT	14 (60.9)	29 (36.3)	0.054	2.736	1.054 - 7.098
		TC	5 (21.7)	22 (27.4)	0.789	0.732	0.242 - 2.213
		CC	4 (17.4)	29 (36.3)	0.128	0.370	0.115 - 1.194
IL-1β +3962 (rs1143634)	Alleles	C	28 (60.9)	129 (80.6)	**0.01***	0.374	0.184 - 0.760
		T	18 (39.1)	31 (19.4)	**0.01***	2.675	1.315 - 5.442
	Genotypes	CC	11 (47.8)	56 (70.0)	0.081	0.393	0.152 - 1.013
		CT	6 (26.1)	17 (21.2)	0.585	1.308	0.447 - 3.828
		TT	6 (26.1)	7 (8.8)	0.068	3.681	1.096 - 12.36

Allele and genotype frequencies are presented as absolute numbers with percentage in parentheses;
rs - refSNP cluster ID number; 95% CI - 95% confidence interval; *Mean difference is significant at the indicated *p*-value

Table 3. TNF-α Cytokine Gene Polymorphism association with systemic sclerosis.

Cytokine polymorphism			SSc (n=23)	HC (n=80)	*p*-value	Odds Ratio	95% CI
TNF-α -308 (rs1800629)	Alleles	G	42 (91.3)	103 (64.4)	**0.000***	5.811	1.982 - 17.032
		A	4 (8.7)	57 (35.6)	**0.000***	0.172	0.059 - 0.504
	Genotypes	GG	19 (82.6)	23 (28.8)	**0.000***	11.772	3.61 - 38.384
		GA	4 (17.4)	57 (71.2)	**0.000***	0.085	0.026 - 0.277
		AA	0 (0.0)	0 (0.0)	-	-	-
TNF-α -238 (rs361525)	Alleles	G	27 (58.7)	137 (85.6)	**0.000***	0.239	0.114 - 0.497
		A	19 (41.3)	23 (14.4)	**0.000***	4.192	2.011 - 8.737
	Genotypes	GG	4 (17.4)	57 (71.2)	**0.000***	0.085	0.026 - 0.277
		GA	19 (82.6)	23 (28.8)	**0.000***	11.772	3.61 - 38.384
		AA	0 (0.0)	0 (0.0)	-	-	-
TNF-α -308, -238	Haplotypes	GG	23 (50.0)	80 (50.0)	1.000	1.000	0.519 - 1.927
		AG	4 (8.7)	57 (35.6)	**0.000***	0.172	0.059 - 0.504
		GA	19 (41.3)	23 (14.4)	**0.000***	4.192	2.011 - 8.737

Allele, genotype and haplotype frequencies are presented as absolute numbers with percentage in parentheses;
rs - refSNP cluster ID number; 95% CI - 95% confidence interval; *Mean difference is significant at the indicated *p*-value

Table 4. IL-10 Cytokine Gene Polymorphism association with systemic sclerosis.

Cytokine polymorphism			SSc (n=23)	HC (n=80)	p-value	Odds Ratio	95% CI
IL-10 -1082 (rs1800896)	Alleles	A	32 (69.6)	88 (55.0)	0.091	1.870	0.928 - 3.77
		G	14 (30.4)	72 (45.0)	0.091	0.535	0.265 - 1.078
	Genotypes	AA	9 (39.1)	8 (10.0)	**0.002***	5.786	1.904 17.577
		AG	14 (60.9)	72 (90.0)	**0.002***	0.173	0.057 - 0.525
		GG	0 (0.0)	0 (0.0)	-	-	-
IL-10 -819 (rs1800871)	Alleles	C	23 (50.0)	103 (64.4)	0.088	0.553	0.285 - 1.073
		T	23 (50.0)	57 (35.6)	0.088	1.807	0.932 - 3.504
	Genotypes	CC	6 (26.1)	23 (28.8)	1.000	0.875	0.306 - 2.497
		CT	11 (47.8)	57 (71.2)	**0.047***	0.370	0.143 - 0.957
		TT	6 (26.1)	0 (0.0)	**0.000***	0.739	0.580 - 0.942
IL-10 -592 (rs1800872)	Alleles	A	19 (41.3)	57 (35.6)	0.493	1.272	0.651 - 2.485
		C	27 (58.7)	103 (64.4)	0.493	0.786	0.402 - 1.537
	Genotypes	AA	2 (8.7)	0 (0.0)	**0.048***	0.913	0.805 - 1.036
		AC	15 (65.2)	57 (71.2)	0.611	0.757	0.282 - 2.026
		CC	6 (26.1)	23 (28.8)	1.000	0.875	0.306 - 2.497
IL-10 -1082, -819, -592	Haplotypes	ATA	17 (37.0)	50 (31.2)	0.479	1.290	0.650 - 2.560
		ACC	15 (32.6)	38 (23.8)	0.252	1.553	0.759 - 3.179
		GCC	14 (30.4)	72 (45.0)	0.091	0.535	0.265 - 1.078

Allele, genotype and haplotype frequencies are presented as absolute numbers with percentage in parentheses;
rs - refSNP cluster ID number; 95% CI - 95% confidence interval; *Mean difference is significant at the indicated *p*-value

IL-10 Cytokine Gene Polymorphism

There were significant differences in the genotype frequencies of the *IL-10* -1082 A/G, - 819 C/T and -592 C/A polymorphisms between SSc patients and healthy volunteers. We found a higher frequency of the AA genotype at *IL-10* -1082 (39.1% vs 10%; $p<0.01$), TT genotype at *IL-10* -819 (26.1% vs 0%; $p<0.001$) and the AA genotype at *IL-10* -592 (8.7% vs 0%; $p<0.05$) in SSc patients than in the healthy volunteers. We also found that the ACC and ATA haplotypes were more frequent in SSc patients compared to healthy volunteers, but the difference was not statistically significant (Table 4).

In addition to these results, significantly higher frequencies of genotypes were also observed in SSc patients as compared to controls in *TGF-β1* codon25 GG genotype (52.2% vs 20.0%; $p<0.01$) and *IL-6* nt565 AA genotype (13% vs 0%; $p<0.05$). *IL-6* gene polymorphism showed near complete linkage disequilibrium between the –174 G and nt565 G alleles.

DISCUSSION

Cytokine production and release are key events in SSc pathogenesis as they are involved in T and B cell activa-

tion leading to inflammation, auto-antibodies production, microvascular damage and fibrosis [21]. The Th1/Th2/Th17/Treg balance is one of the hallmarks of SSc pathogenesis, as the Th2 and Th17 cytokines response leads to tissue fibrosis, whereas Th1 and Th17 cytokines promote inflammation in SSc patients.

IL-1α and *IL-1β* are proinflammatory cytokines involved in a number of autoimmune diseases. Patients who have SSc have increased circulating levels of *IL-1α* and *IL-1β*. Genetic associations with *IL-1β* have been investigated in patients with SSc and significant associations of the *IL-1β* -31 C and *IL-1β* -511 T alleles have been found [17]. Our results provide evidence suggesting that the T alleles of *IL-1β* -511 and *IL-1β* +3962 are associated with SSc in our population. Polymorphism in the human *IL-1β* gene has been reported to influence cytokine expression [22]. *IL-1β* stimulates the production of prostaglandin E2, which is an important cofactor for the induction of T-helper lymphocyte activity towards Th2 direction. A shift towards the Th2 system has been indicated in SSc [11].

Changes in *IL-1β* expression levels may reflect the genetic variation in *IL-1β* gene. The findings on biological roles of *IL-1β* polymorphisms, however, have not been consistent across studies. TT genotype of *IL-1β* -511 has been associated with higher gastric mucosa *IL-1β* levels in *Helicobacter pylori* positive population [23]. On the other hand, subjects with CC genotype showed an increased release of *IL-1β* from mononuclear cells after stimulation with lipopolysaccharide [24]. Recent studies suggest that the functional role of *IL-1β* -511 may depend on *IL-1β* promoter region haplotypes including *IL-1β* -511 [25]. Although the findings are inconsistent, these previous studies suggest that *IL-1β* -511 could affect the expression levels of *IL-1β*. On the other hand, the influence of *IL-1β* +3962 on *IL-1β* expression levels has not been previously reported. Polymorphisms in *IL-1β*, particularly SNPs *IL-1β* +3962 and *IL-1β* -511, have been identified as risk factors for susceptibility, progression, and severity of periodontal disease across various populations [26,27]. Elevated *IL-1β* levels in gingival crevicular fluid, saliva, and serum of periodontitis patients further support these associations [27]. Additionally, existing literature links inflammation associated with SSc etiology to the development of oral conditions like periodontitis [28]. Together, these findings suggest that *IL-1β* gene polymorphisms may contribute to the development of SSc in our study population.

A significant association of *TNF-α* -308 G allele and *TNF-α* -238 A allele with SSc was observed in this study. *TNF-α*, a member of *TNF*-superfamily, is a potent pro-inflammatory cytokine which affects different aspects of immune response, cell growth, differentiation and activation [29]. Due to its broad spectrum of pro- inflammatory functions *TNF-α* has been implicated in the pathogenesis of many immune disorders including all connective tissue diseases [29]. Increased production of *TNF-α* by PBMCs as well as elevated serum concentrations of *TNF-α* have been demonstrated SSc patients [30]. The enhanced production of *TNF-α* by PBMCs of SSc patients is associated with increased synthesis of *TNF-α* mRNA indicating increased expression of the *TNF-α* gene in SSc patients [31]. Moreover, elevated concentrations of *TNF-α* have been demonstrated in bronchoalveolar lavage fluid of SSc patients with interstitial lung disease [32]. Similarly, elevated serum *TNF-α* concentration in SSc patients were found in SSc patients with pulmonary fibrosis [33]. Recent studies have revealed inconsistent results regarding correlation of *TNF-α* polymorphisms with periodontitis susceptibility [34].

In our study, despite the patients and controls being of the same ethnic origin and from the same geographic region, we identified a strong association between the *TNF-α* -308 G allele and SSc. The functional significance and transcriptional impact of this allele remain a topic of debate, as some studies have found no direct link between *TNF-α* -308 polymorphisms and *TNF-α* production [35]. However, it is possible that an unidentified gene in linkage disequilibrium with the *TNF-α* -308 G allele may play a role in the increased susceptibility to SSc observed in individuals carrying this allele. In contrast, the *TNF-α* -238 A allele and GA genotype have also been previously associated with SSc [36]. Collectively, these findings suggest that *TNF-α* gene polymorphisms contribute to the pathogenesis of SSc in our study population.

The *IL-10* gene promoter region contains several SNPs including –1082 G/A, –819 C/T and –592 C/A in the transcription factor-binding region. Alleles of all three polymorphisms are in linkage disequilibrium, giving rise to only three major allele combinations out of possible eight in Caucasian populations: the GCC haplotype is responsible for higher *IL-10* secretion, whereas ACC and ATA haplotypes are associated with its lower production. Although we observed significant differences in the genotypes of the three *IL-10* SNPs, no significant difference in haplotype (GCC/ATA/ACC) distribution between SSc patients and healthy individuals was observed. The G allele at −1082, and haplotypes containing this allele, have been associated with high *IL-10* production, while the A allele and the ATA haplotype have been associated with low *IL-10* production [37]. Our study does not show a possible correlation between *IL-10* SNPs and its production in SSc patients. However, a significant association of *IL-10* SNPs have recently been shown with chronic periodontitis [38].

The importance of *TGF-β* in SSc pathogenesis has been demonstrated well. *TGF-β*, its receptor, and down-

stream signaling molecules are expressed at increased levels in affected organs in SSc. *TGF-β* activates dermal fibroblasts leading to increased production of extracellular matrix. Given the importance of *TGF-β* in SSc, it has been hypothesized that polymorphisms in its gene may contribute to SSc susceptibility. However, there is a paucity of studies in this direction and the findings have been conflicting [39-41]. In this study we found no association between the SNP in *TGF-β1* codon10 and SSc. However, significant differences were observed in *TGF-β1* codon25 genotypes between patients and controls. Given the strong linkage disequilibrium among the SNPs in this gene, it is difficult, if not impossible, to assess which, if any, of these SNPs is truly responsible for the quantitative variation in *TGF-β1* level. Possibly, particular alleles at these loci additively (or interactively) influence the quantitative (and possibly qualitative) expression of this cytokine.

The significant association between *IL-1β* (-511, +3962) and *TNF-α* (-308, -238) SNPs with SSc suggests a genetic predisposition to the disease, highlighting their potential as biomarkers for early diagnosis, risk assessment, and targeted therapies. These SNPs may contribute to SSc pathogenesis by dysregulating key inflammatory cytokines, promoting fibrosis and vascular damage. Additionally, they could help explain disease heterogeneity, offering insights into the severity of SSc and enabling better patient stratification. Given their role in other autoimmune conditions, these findings also provide broader implications for shared pathogenic mechanisms and therapeutic strategies, with the potential for inclusion in genetic screening panels for at-risk individuals.

Our study is limited by a small sample size and a restricted number of SNPs, as genetic susceptibility to SSc likely involves a broader combination of genes, along with environmental and epigenetic factors. Additionally, we did not adjust *p*-values for multiple testing, which complicates the determination of statistical significance and requires further investigation. While our findings suggest a potential association between *IL-1β* and *TNF-α* SNPs and SSc, larger studies are needed to confirm these results. The higher proportion of females in the SSc patient group compared to the control group reflects the well-known female predominance in systemic sclerosis [42], which is more common in women, especially in younger and middle-aged adults. While this sex distribution aligns with existing epidemiological data, the small sample size and sex imbalance may reduce the study's statistical power and affect the generalizability of the results. Larger, more balanced studies are needed to confirm these findings and explore potential sex-specific genetic associations.

Future research should explore the functional roles of these SNPs through *in vitro* expression studies to evaluate their effects on cytokine production and immune cell activation. Employing genome-editing technologies like CRISPR-Cas9 could also provide insights into how these SNPs influence gene expression and cellular responses in relevant immune cells. Longitudinal cohort studies in diverse populations would further elucidate how these variants correlate with disease progression and treatment outcomes. These approaches would offer valuable insights into the mechanisms underlying SSc and help identify potential therapeutic targets, providing clearer directions for future studies in this field.

CONCLUSION

Our study found a significant association between *IL-1β* -511, +3962, and *TNF-α* -308, -238 SNPs and SSc in our population, consistent with previous research linking these SNPs to SSc susceptibility and progression. Larger, more balanced studies are needed to confirm these associations. Future research should focus on the functional roles of these SNPs through *in vitro* studies, genome editing, and longitudinal cohort studies to better understand their impact on disease progression and treatment outcomes.

Author Statement

The manuscript represents original, unpublished material not under editorial consideration elsewhere, and that ethical guidelines were followed in the conduct of the research.

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None

Conflict of Interest

The authors have no conflict of interest to disclose.

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ORIGINAL ARTICLE

IN VITRO ANALYSIS OF AKR1D1 INTERACTIONS WITH CLOPIDOGREL: EFFECTS ON ENZYME ACTIVITY AND GENE EXPRESSION

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ABSTRACT

Clopidogrel, a P2Y12 receptor antagonist, is widely used to prevent cardiovascular events, but significant variability in its efficacy persists among patients. AKR1D1, involved in bile acid synthesis and regulation of CYP enzymes, may contribute to this variability. This study aims to investigate whether clopidogrel and its inactive metabolite, 2-oxoclopidogrel, interact with AKR1D1 at the enzymatic or transcriptional level. Enzymatic activity assays demonstrated that neither clopidogrel nor 2-oxoclopidogrel acts as a substrate or inhibitor of AKR1D1. Expression studies in HepG2 cells further revealed no significant changes in *AKR1D1* mRNA levels following treatment with these compounds. These findings indicate that clopidogrel does not directly influence AKR1D1's metabolic functions, including bile acid synthesis, steroid hormone clearance, or the production of 5β-reduced steroids, which regulate CYP enzyme expression. From a physiological perspective, the absence of interaction minimizes the risk of adverse effects on CYP-mediated drug metabolism, nutrient absorption, lipid digestion, and the absorption of lipophilic drugs. Future research should explore AKR1D1's broader substrate specificity, particularly focusing on non-steroidal compounds, and investigate the clinical implications of AKR1D1 polymorphisms in clopidogrel-treated patients to enhance personalized therapeutic strategies.

Keywords: AKR1D1, clopidogrel, drug metabolism, enzyme interaction, pharmacogenomics, gene expression.

INTRODUCTION

Clopidogrel, a P2Y12 receptor antagonist, is a cornerstone of dual antiplatelet therapy alongside aspirin, widely used to prevent major cardiovascular events in patients with acute coronary syndromes or undergoing percutaneous coronary interventions (1, 2). While clopidogrel has been shown to be more effective than aspirin in reducing the risks of myocardial infarction, ischemic stroke, and vascular death, there remains significant variability in treatment outcomes among patients (3, 4). This variability can range from reduced efficacy or resistance to therapy, affecting 5–44% of patients, to increased risk of bleeding due to excessive antiplatelet activity (5, 6). The phenomenon of "clopidogrel resistance" presents a major clinical challenge, particularly because the underlying mechanisms are not fully understood (7–9).

The metabolism of clopidogrel is complex. As a prodrug, it undergoes two primary processes: hydrolysis by carboxylesterase 1 (CES1), which inactivates the majority of the drug, and a two-step activation mediated by several cytochrome P450 (CYP) enzymes, which convert clopidogrel to its active form (10, 11). Among these, CYP2C19 is the most crucial, with polymorphisms in this enzyme (*CYP2C19*2, *3*, and **17*) being major determinants of clopidogrel's variable pharmacokinetics and treatment outcomes (12–14). However, these genetic variations explain only about 12% of the observed variability, leaving much of the interindividual differences unexplained (12, 15).

The variability in CYP enzyme activity and its effects on drug metabolism extend beyond clopidogrel, as CYP enzymes play a pivotal role in the biotransformation of many medications (16). Aldo-Keto Reductase 1D1 (AKR1D1), an enzyme critical in bile acid synthesis and steroid clearance (17–19), has been identified by Chaudhry et al. (2013) as a key trans-regulator of the CYP enzyme network, suggesting a broader regulatory

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mechanism. *AKR1D1* regulates *CYP2C19*, *CYP3A4*, and *CYP2C9* expression via its metabolic products. Specifically, the 5β-reduced steroids generated by AKR1D1 activity act as ligands for nuclear receptors such as farnesoid X receptor (FXR), pregnane X receptor (PXR), and constitutive androstane receptor (CAR), which in turn regulate CYP enzyme expression (20, 21). The *AKR1D1*36* polymorphism (*rs1872930*), in particular, leads to increased expression of *AKR1D1*, resulting in the upregulation of these CYP enzymes (21).

Kapedanovska et al. (2019) explored this hypothesis in the context of clopidogrel and demonstrated that the *AKR1D1*36* allele is associated with an increased risk of major adverse cardiovascular and cerebrovascular events (MACCE) in patients treated with clopidogrel, establishing *AKR1D1* as an independent risk factor (22).

Building on previous findings linking *AKR1D1*36* to the regulation of the cytochrome P450 enzyme network, this study seeks to elucidate the potential role of AKR1D1 in clopidogrel metabolism. Specifically, the research aims to evaluate whether clopidogrel and its inactive metabolite, 2-oxoclopidogrel, act as substrates or inhibitors of the AKR1D1 enzyme, thereby elucidating potential interactions at the protein level. Furthermore, the study investigates the influence of clopidogrel and its metabolite on *AKR1D1* gene expression in HepG2 cells to explore potential mechanisms that could modulate clopidogrel metabolism. These investigations are intended to provide a deeper understanding of AKR1D1's involvement in drug metabolism and its broader implications for pharmacological research.

MATERIALS AND METHODS

Reagents and Chemicals

– Molecular Cloning Reagents:

- Full-length *AKR1D1* cDNA (OriGene, Rockville, MD, USA; Cat# RC223056): Used for amplifying and cloning the *AKR1D1* gene into an expression vector.
- pET28b+ vector (Novagen (Merck KGaA), Darmstadt, Germany; Cat# 69865-3): Expression vector used for recombinant protein production.

– Enzyme Assay Reagents:

- Testosterone (Sigma-Aldrich, St. Louis, MO, USA; Cat# T1500): Substrate for AKR1D1 activity assays.
- Clopidogrel Bisulfate (Sigma-Aldrich, St. Louis, MO, USA; Cat# C0612): Evaluated for interactions with AKR1D1 at both enzymatic and transcriptional levels.
- 2-oxoclopidogrel (Cayman Chemical, Ann Arbor, MI, USA; Cat# 20394): Metabolite of clopidogrel tested for interactions with AKR1D1 at both enzymatic and transcriptional levels.

– Cell Culture Reagents:

- HepG2 Hepatocyte Cell Line (ATCC, Manassas, VA, USA; Cat# HB-8065): An in vitro model system used to study AKR1D1 expression and hepatic metabolism.
- Dulbecco's Modified Eagle Medium (DMEM; Sigma-Aldrich, St. Louis, MO, USA; Cat# D6429): Basal medium for HepG2 cell maintenance.
- Fetal Bovine Serum (FBS) (Capricorn Scientific, Ebsdorfergrund, Germany; Cat# FBS-12A): Supplemented in DMEM to support cell growth and viability.
- Trypsin-EDTA Solution (Lonza, Walkersville, MD, USA; Cat# CC-5012): Used for cell detachment during subculturing.

– MTT Assay Reagents:

- MTT Solution (5 mg/mL) (Sigma-Aldrich, St. Louis, MO, USA; Cat# M2128): Used to evaluate cell viability through formazan crystal formation.
- Dimethyl Sulfoxide (DMSO) (Thermo Fisher Scientific, Waltham, MA, USA; Cat# D12345): Used to dissolve formazan crystals for spectrophotometric analysis.
- NADPH (Sigma-Aldrich, St. Louis, MO, USA; Cat# N7505): Essential cofactor for spectrofluorometric enzyme activity assays.

–Expression Assay Reagents

- HpaI Restriction Enzyme (New England Biolabs, Ipswich, MA, USA; Cat# R0105S): Used for linearizing the AKR1D1 plasmid to generate a calibration curve for qRT-PCR quantification.
- Shrimp Alkaline Phosphatase (Takara Bio, Shiga, Japan; Cat# 2650A): Used for dephosphorylating the linearized plasmid DNA to prevent self-ligation.
- TRI Reagent® (Sigma-Aldrich, St. Louis, MO, USA; Cat# T9424): Used for total RNA extraction from HepG2 cells.
- ProtoScript® II Reverse Transcriptase (New England Biolabs, Ipswich, MA, USA; Cat# M0368): Used to synthesize complementary DNA (cDNA) from isolated RNA.
- XCEED qPCR SG 2x Mix Lo-ROX (Institute of Applied Biotechnologies, Prague, Czech Republic; Cat# QR0100): Used for quantitative real-time PCR analysis of AKR1D1 gene expression.

- Forward and Reverse Primers (GeneriBiotech, Hradec Králové, Czech Republic): Specific primers designed for the amplification of *AKR1D*1 during quantitative real-time PCR.

METHODS

Cloning, Expression, and Purification of AKR1D1

The coding sequence for the wild-type *AKR1D1* (*AKR1D1*1*) was amplified and subcloned into the pET28b+ vector using standard molecular cloning techniques (23,24). *E. coli* BL21 (D3) cells were transformed with the prepared pET28b+_*AKR1D1* vector, and overexpression of the enzyme was induced using isopropyl β-D-1-thiogalactopyranoside (IPTG). The enzyme was subsequently purified using the NGC™ Discover™ 100 Pro chromatography system (Bio Rad, USA). The cloning, expression, and purification methods for AKR1D1, including optimization steps, have been comprehensively described in a recent publication (25).

Standard Spectrofluorometric Assay and Enzyme Studies

Substrate Evaluation: The enzymatic reduction of testosterone to 5β-dihydrotestosterone (5β-DHT) was monitored using a spectrofluorometric assay. The reaction mixture contained AKR1D1 (7.8 μM), NADPH (15 μM), and testosterone (10 μM) in 100 mM potassium phosphate buffer (pH 6.0). The reduction was followed by measuring NADPH fluorescence, with an excitation wavelength of 340 nm and an emission wavelength of 460 nm, on an Infinite M200 Tecan spectrophotometer (Tecan, Switzerland) over a 45-minute period. For substrate evaluation, testosterone was replaced with either clopidogrel or 2-oxoclopidogrel at a final concentration of 50 μM.

Inhibition Studies: For inhibition studies, AKR1D1 (7.8 μM) was pre-incubated with clopidogrel or 2-oxoclopidogrel (10 μM or 50 μM) in the same reaction conditions as described above. Reactions were initiated by the addition of NADPH, and fluorescence changes were monitored over 45 minutes.

The concentrations of clopidogrel and 2-oxoclopidogrel (10 μM and 50 μM) were chosen based on established practices in enzymatic studies involving related AKR1C enzymes (26). The 50 μM concentration was selected to ensure sufficient substrate availability for detecting potential enzymatic activity, while 10 μM and 50 μM were used in inhibition studies to observe potential dose-dependent effects. This approach allows for exploratory assessment of AKR1D1 interactions with these compounds under conditions aligned with prior enzymatic studies.

Cell Culture Preparation and Viability Assessment

HepG2 cells, chosen for their similarity to *AKR1D1* expression in primary human liver cells (27), were maintained in Dulbecco's Minimum Essential Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37°C in a 5% CO_2 atmosphere.

An MTT assay was performed to determine non-toxic concentrations of clopidogrel and 2-oxoclopidogrel for expression studies. HepG2 cells were treated with concentrations ranging from 0.1 μM to 50 μM for 48 hours, and cell viability remained >90% at all tested concentrations. Based on these findings 5 μM was selected as a safe and physiologically relevant concentration for further experiments.

Expression Studies

HepG2 cells were seeded into 12-well plates (1,600,000 cells per well) and allowed to adhere for 24 hours. Cells were then treated with 5 μM clopidogrel or 2-oxoclopidogrel for 24 hours. Following treatment, total RNA was extracted using TRI Reagent®, and RNA quality and concentration were assessed spectrophotometrically. Complementary DNA (cDNA) was synthesized using reverse transcriptase, and 80 ng of cDNA was utilized for quantitative real-time PCR (qRT-PCR) analysis.

qRT-PCR was conducted in triplicate to quantify *AKR1D1* expression, with amplification specificity validated by dissociation curve analysis. Absolute quantification was achieved using a calibration curve generated from serial dilutions of linearized *AKR1D1* cDNA.

To prepare the plasmid for the calibration curve, the *AKR1D1* plasmid was linearized using the HpaI restriction enzyme and subsequently dephosphorylated with shrimp alkaline phosphatase to prevent self-ligation.

The concentration of 5 μM aligns with previous studies involving AKR1D1-related compounds, such as anabolic steroids, where 5 μM approximates intracellular levels after cellular uptake (28). While plasma concentrations of clopidogrel and its metabolites are in the nanomolar range due to rapid metabolism (12), the chosen concentration ensures sufficient exposure to detect potential regulatory effects on *AKR1D1* expression in vitro (29).

Statistical Analysis

Statistical comparisons between treated and control groups were conducted using a two-tailed Student's t-test. Data are presented as mean ± standard deviation (SD), with significance set at $p < 0.05$. All experiments were performed in triplicate unless otherwise stated.

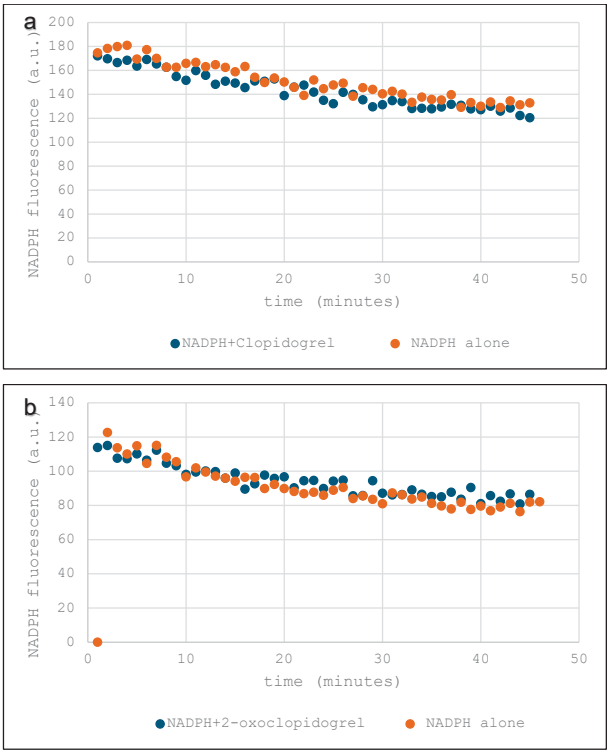

Figure 1. Evaluation of AKR1D1 substrate specificity using NADPH fluorescence assays for: (a) clopidogrel (50 µM) and (b) 2-oxoclopidogrel (50 µM). Changes in NADPH fluorescence over time were measured in the presence (blue) and absence (orange) of the substrate candidates. No significant differences were observed ($p > 0.05$).

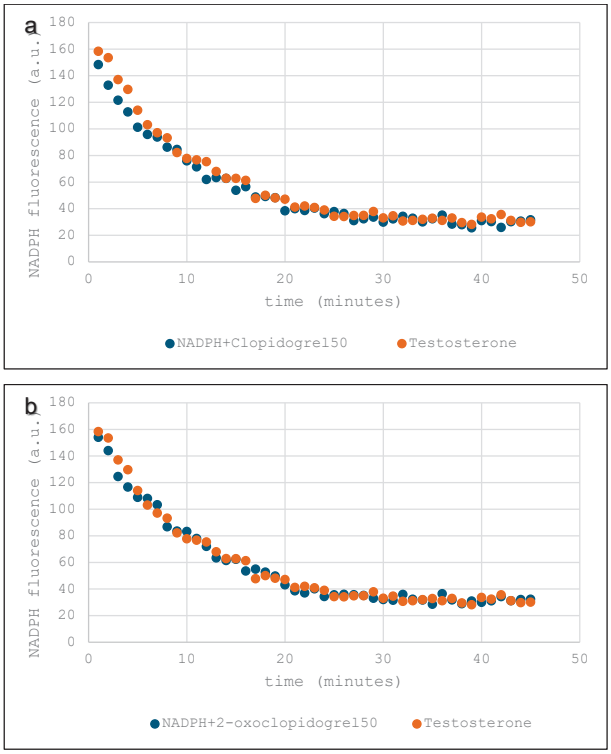

Figure 2. Evaluation of the inhibitory activity of (a) clopidogrel (50 µM) and (b) 2-oxoclopidogrel (50 µM) on AKR1D1. NADPH fluorescence was measured in the presence of the candidate inhibitor and testosterone (blue) and compared to the response to testosterone alone (orange). No significant differences were observed ($p > 0.05$).

RESULTS

Enzyme Substrate and Inhibition Studies

The potential role of clopidogrel and 2-oxoclopidogrel as substrates or inhibitors of AKR1D1 was investigated through a set of enzymatic activity assays using spectrofluorometric measurements. The enzymatic activity was monitored by measuring NADPH fluorescence, and no significant changes were observed in the presence of either clopidogrel or 2-oxoclopidogrel, suggesting that neither compound acts as a substrate (Fig. 1) or an inhibitor (Fig. 2) of AKR1D1. The fluorescence remained unchanged across all treatment groups, supporting the conclusion that these compounds do not interact directly with AKR1D1 ($p > 0.05$).

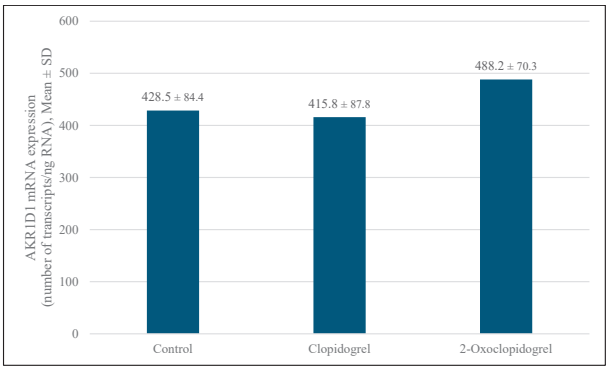

Figure 3. AKR1D1 mRNA expression levels (mean ± SD) in HepG2 cells following treatment with clopidogrel and 2-oxoclopidogrel (5 µM each). No significant changes were observed compared to the control group ($p > 0.05$).

Expression Studies

The impact of clopidogrel and 2-oxoclopidogrel on *AKR1D1* expression was assessed by conducting qRT-PCR on HepG2 cells treated with each compound. The absolute quantification approach, employing a calibration curve generated from linearized *AKR1D1*, revealed no statistically significant changes in *AKR1D1* mRNA levels in response to treatment with either clopidogrel or 2-oxoclopidogrel compared to control samples ($p > 0.05$; Fig. 3). These results suggest that these compounds do not induce or repress *AKR1D1* expression at the transcriptional level in HepG2 cells.

DISCUSSION

The study provides valuable insights into the interaction of clopidogrel and its metabolite, 2-oxoclopidogrel, with the enzyme AKR1D1. The enzymatic assays demonstrate that neither clopidogrel nor its metabolite, 2-oxoclopidogrel, acts as a substrate or inhibitor of AKR1D1. This aligns with the known specificity of AKR1D1, which predominantly interacts with steroidal compounds and not with non-steroidal drugs like clopidogrel. The absence of inhibition is significant because it suggests that clopidogrel does not interfere with AKR1D1's essential role in bile acid synthesis and steroid hormone clearance, thus preserving critical metabolic processes (17, 18, 20, 30). Disruption of this processes could impair digestion, absorption, and drug solubilization, which further emphasizes the physiological importance of AKR1D1's functional integrity.

While AKR1D1's substrate specificity appears to favor steroidal compounds, its structural homology with AKR1C enzymes - which can interact with both steroidal and non-steroidal substrates - raises the possibility that AKR1D1 could be flexible under certain conditions (19, 30, 31). Although studies have demonstrated that certain non-steroidal compounds, including indomethacin, mefenamic acid, and 4-benzoylbenzoic acid, which are potent inhibitors of AKR1C enzymes, do not inhibit AKR1D1 (31), only three non-steroidal compounds have been evaluated to date. Given the limited scope of these investigations, the possibility remains that AKR1D1 could interact with other non-steroidal substrates or inhibitors, warranting further exploration.

The potential flexibility in AKR1D1's substrate specificity is an intriguing aspect of the enzyme's functionality, particularly when considered in the context of genetic polymorphisms, such as the *AKR1D1*36* variant that has been is associated with an increased risk of major adverse cardiovascular and cerebrovascular events (MACCE) in patients treated with clopidogrel (22). While the *AKR1D1*36* polymorphism has been linked to altered expression levels and downstream effects on CYP enzyme regulation (21), its direct impact on enzymatic function and substrate specificity remains unexplored. By contrast, engineered mutations, such as the E120H substitution, have demonstrated that single amino acid changes can significantly alter AKR1D1's enzymatic activity and substrate specificity (32). These findings highlight the potential for naturally occurring genetic variants, like *AKR1D1*36*, to similarly influence enzyme functionality, warranting further investigation.

Gene expression analyses further demonstrated that clopidogrel and 2-oxoclopidogrel do not modulate *AKR1D1* expression at the transcriptional level. This finding implies that clopidogrel does not interfere with the production of 5β-reduced steroids, metabolic products of AKR1D1 that regulate CYP enzyme expression via nuclear receptor pathways such as FXR, CAR, and PXR (21). Consequently, the lack of *AKR1D1* modulation by clopidogrel reduces the likelihood of indirect effects on CYP enzyme activity, which could otherwise alter the metabolism of co-administered drugs.

This study's findings should be interpreted with caution regarding clinical relevance. In vivo systems involve a complex interplay of metabolic factors that may not be fully represented in the in vitro model used here. Therefore, further research is necessary to validate these observations and assess the broader implications of AKR1D1 and its genetic variations on drug metabolism as well as their potential impact on clinical outcomes particularly in the context of personalized medicine.

CONCLUSION

This study provides foundational insights into AKR1D1's interactions with clopidogrel and its metabolite, suggesting that they do not directly influence AKR1D1 activity or expression. However, further research, particularly in vivo and genetic studies, is necessary to fully elucidate AKR1D1's role in drug metabolism and its potential implications for clinical practice.

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ORIGINAL ARTICLE

ASSOCIATION OF *CYP2C19*2* C.681G>A (RS4244285) LOSS-OF-FUNCTION ALLELE WITH CARDIOVASCULAR DISEASE RISK IN THE KOSOVO POPULATION

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ABSTRACT

The *CYP2C19*2 c.681G>A (rs4244285)* loss-of-function (*LOF*) allele has been associated with reduced clopidogrel efficacy and increased risk of major adverse cardiovascular events (MACE). PGx-guided treatment, despite the recommendations, is not fully implemented in routine clinical practice. The primary aim of this hybrid retrospective-prospective study was to determine whether identifying *CYP2C19* LOF patients may benefit the antiplatelet drug prescribing decisions made in Kosovo. The study cohort consisted of clopidogrel treated patients presenting at the University Clinical Center in the period from December 2023 to May 2024. To evaluate the correlation between *CYP2C19* LOF and the treatment outcome in a follow-up period of 2 years, we first assessed the *CYP2C19*2* genotype using the Taq Man Real Time PCR method. Among 150 patients, 58 (19.33%) were identified as carriers *CYP2C19*2 LOF* allele. The observed allele distribution was significantly different when compared with the one reported for a healthy Kosovar population (13.03%). *CYP2C19*2* LOF carriers exhibited a 1.6-fold higher probability of developing cardiovascular disease compared to non-carriers, based on allelic and codominant model of statistical analysis (OR=1.60; 95% CI=1.08-2.37; p=0.018 and OR=1.64; 95% CI=1.04-2.57; p=0.031, respectively). The median observation time of follow up was not reached until this analysis was conducted. Our data supports the potential association of the *CYP2C19*2 LOF* allele with an increased risk for CVD in the population of Kosovo. Our data add to the evidence advising careful consideration of *CYP2C19* genetic diversity when recommending PGx-guided clopidogrel therapy, particularly in populations, such the Kosovar, where genetic determinants are not yet fully elucidated.

Keywords: clopidogrel, *CYP2C19*2*, Kosovo population, pharmacogenetics, risk for cardiovascular disease.

INTRODUCTION

Clopidogrel remains a cornerstone in dual antiplatelet therapy for reducing the risk of cardiovascular events in patients with coronary artery disease (CAD), as well as those undergoing percutaneous coronary interventions (PCI). It is particularly beneficial in preventing thrombotic events in patients at risk for or who have experienced acute coronary syndromes (ACS), including myocardial infarction (MI) with or without ST-elevation (STEMI or NSTEMI) or unstable angina [1]. Clinical trials (such as CAPRIE, PLATO, CLARITY-TIMI 28, COMMIT/CCS-2), have consistently demonstrated clopidogrel's efficacy in various settings, including in secondary prevention and acute coronary syndromes. However, these clinical trials also emphasize the emergence of newer antiplatelet agents, such as prasugrel and ticagrelor that may demonstrate superior efficacy in certain clinical scenarios, challenging the traditional use of clopidogrel in some patient groups [2]. Clopidogrel is the only irreversible $P2Y_{12}$ (purinergic P2Y, G-protein coupled 12) inhibitor to have a class I

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indication in patients with stable CAD undergoing stent implantation and is recommended in those with a contraindication to ticagrelor or prasugrel or those taking an oral anticoagulant [3]. Despite its proven benefits, clopidogrel's effectiveness as a prodrug is influenced by genetic and non-genetic factors, with genetic variations in the *CYP2C19* gene being a key determinant of its therapeutic outcomes. *CYP2C19* genetic variants, particularly loss-of-function (LOF) alleles like *2, *3, *4, and *5, have been shown to affect clopidogrel metabolism significantly. Individuals carrying two LOF-alleles exhibit reduced enzyme activity, leading to lower levels of the active metabolite and diminished antiplatelet effects. This reduction in efficacy has been associated with a 2-4 fold increased risk of ischemic events, stent thrombosis, and major cardiovascular and cerebrovascular events, particularly in high-risk patient populations [4-9]. As a result, genotype-guided clopidogrel therapy has gained attention, with clinical guidelines from the American College of Cardiology (ACC) and American Heart Association (AHA) recommending its consideration, especially for patients with CAD or those undergoing PCI [10-13]. Despite promising results from studies, such as from the POPular Genetics trial [12], conflicting findings from other studies (such as CURE, TRITON-TIMI 38, EAST-AFNET 4, GRAVITAS, ISAR-REACT 5) have raised concerns about the generalizability of genotype-guided therapy [14]. These inconsistencies in the evidence contribute to the challenges in achieving widespread adoption of routine *CYP2C19* testing. The uncertainty surrounding the benefits of genotype-guided therapy has led to divergent recommendations from regulatory bodies, professional societies and pharmacogenetics consortia [14-16]. For example, The U.S. Food and Drug Administration (FDA) highlights reduced clopidogrel effectiveness in individuals with *CYP2C19* LOF-alleles and suggests alternative therapies for poor metabolizers, whereas the European Medicines Agency (EMA) adopts a more cautious approach, advising against co-administration with *CYP2C19* inhibitors but not mandating genetic testing [3]. Similarly, pharmacogenetic guidelines, such as those from the Clinical Pharmacogenetics Implementation Consortium (CPIC) and the Dutch Pharmacogenetics Working Group (DPWG), recommend *CYP2C19* genotype-guided antiplatelet therapy, particularly in specific high-risk populations, but these recommendations differ in certain aspects and have not been universally integrated into clinical practice [3, 17, 18]. The ESC's 2020 guidelines for ACS management propose genotype-guided therapy as an alternative to standard dual antiplatelet therapy with prasugrel or ticagrelor but refrain from endorsing routine testing for all PCI patients due to limited evidence. The AHA also calls for more research to establish the role of genotyping in improving clinical outcomes, particularly for ACS patients and secondary stroke prevention.

In addition to *CYP2C19*, other genetic polymorphisms such as those in *CES1, PON1, ABCB1*, and *P2RY12* also influence clopidogrel's pharmacokinetics and pharmacodynamics by affecting drug absorption, metabolism, and platelet receptor activity and further complicating the variability in clopidogrel response [19, 20]. Non-genetic factors, including age, comorbidities (e.g., chronic kidney disease and diabetes mellitus), and drug interactions (co-administration of medications that act as *CYP2C19* substrates, inhibitors, or inducers) also play a significant role in modulating drug efficacy [21, 22]. Beyond cardiovascular outcomes, *CYP2C19*2* variants have been associated with altered susceptibility to gastrointestinal disorders, such as peptic ulcers and gastroesophageal reflux disease, as well as certain neurological conditions, highlighting their multifaceted impact [21, 23, 24]. Inter-ethnic variability in the distribution of *CYP2C19* genotypes adds another layer of complexity. Certain alleles, such as *CYP2C19*2*, are more prevalent in specific populations, with significant differences observed across ethnic groups [25, 26]. The lack of comprehensive data on the intra-ethnic distribution of *CYP2C19* variants, coupled with challenges such as cost-effectiveness, logistical barriers, and limitations in the local healthcare infrastructure, restrict integration of PGx testing into routine clinical practice, particularly in resource-limited or under-researched settings like Kosovo, where the integration of genetic testing into routine clinical practice remains an ongoing problem.

This study aims to assess the frequency of the *CYP2C19*2* genotype in clopidogrel-treated patients with cardiovascular disease indications (CVDI) in Kosovo and evaluate its association with major adverse cardiovascular events (MACE), including cardiovascular death, nonfatal myocardial infarction, stroke, stent thrombosis, and revascularization.

MATERIAL AND METHODS

Study population

The study population for the current hybrid observational study with retrospective data collection and prospective follow up was comprised of 150 adult patients, 18 years and older, presenting at the cardiology clinic at the University Clinical Center of Kosovo in the period from December 2023 to May 2024. Major inclusion criteria were the use of clopidogrel therapy because of established cardiovascular diseases (CVD), coronary heart disease (CHD), coronary artery disease (CAD), and cerebrovascular disease, particularly patients with acute coronary syndromes (ACS), including myocardial

infarction (MI) with or without ST-elevation (STEMI or NSTEMI) or unstable angina undergoing percutaneous coronary intervention (PCI) with or without stent. Patients were excluded from the study if they had: 1) a prior history of bleeding (e.g., peptic ulcer, intracranial hemorrhage, menstrual bleeding), 2) clinically significant abnormalities in platelet function or severe hepatic insufficiency, 3) drug addiction or alcohol use disorder, or 4) had donated blood within the last 2 months before starting clopidogrel therapy. Female patients on hormone replacement therapy or using an intrauterine contraceptive device were also excluded. These exclusion criteria align with those in previously conducted Phase 3 randomized, double-blind clinical studies of clopidogrel, ensuring a reliable, homogeneous study population while minimizing the risk of confounding variables and adverse effects. Demographic data (age and gender) and clinical data, including indications for clopidogrel therapy (e.g., acute myocardial infarction, stroke, cardiac catheterization for coronary artery disease, cardiac artery bypass graft surgery, carotid angiography, carotid stenting, carotid endarterectomy, and extremity arteriography), as well as risk factors and co-morbidities (such as heart disease, ischemic heart disease, heart failure, hypertension, diabetes mellitus, stroke, and chronic ischemic heart disease), were manually abstracted from the medical health records (MHR). From each participant previously written informed consent was obtained. The follow-up period for Event-Free Survival (EFS) analysis was 24 months. The study complied with the principles of Helsinki Declaration and was approved from Committee Ethics of Alma Mater Europaea Campus College "REZONANCA" (Protocol No: AD-762/23) and Chamber of Pharmacists of Kosova (No.105).

Genotyping procedures

Genomic DNA was extracted from 2 ml of peripheral blood using the NX-48S Genomic DNA Kit according to the manufacturer's recommended protocol (Genolution Inc, Seoul, Republic of Korea) and stored at -20°C until further analysis. DNA purity and concentrations were verified by UV absorption at 260/280 nm using the NanoDrop 2000™ spectrophotometer (Thermo Fisher Scientific). *CYP2C19*2* c.681G>A (rs4244285) polymorphism was genotyped by allele discrimination PCR on a Stratagene Mx3005P (Agilent Technologies, Santa Clara, CA, USA) real-time PCR system using TaqMan® DME genotyping assay (C__25986767_70; Thermo Fisher Scientific). The genotypes were determined in a reaction mix containing 20 ng DNA in a total volume of 25 µL according to the manufacturer's recommended protocol. Positive and negative controls were included on each plate and reproducibility was checked by re-genotyping 15% of the cases. In accordance with the Clinical Pharmacogenetics Implementation Consortium (CPIC)–recommended genotype-to-phenotype classifications, *CYP2C19*2* metabolizer phenotypes were assigned and reported as follows: Poor Metabolizer (PM with 2 no function alleles; **2/*2* genotype), Intermediate Metabolizer (IM with 1 no function allele; **1/*2* genotype), normal metabolizer (NM; **1/*1* genotype) [16]. All genotyping procedures were performed at the Center for Biomolecular Pharmaceutical Analysis, Faculty of Pharmacy-University Ss. Cyril and Methodius, Skopje, Republic of North Macedonia.

Statistical analysis

The study population was analyzed descriptively, with demographic, clinical, and genetic data presented as counts and frequencies (percentages). Allele and genotype frequencies were assessed for Hardy-Weinberg equilibrium using the Chi-squared (χ^2) test. *CYP2C19*2* data from patients were compared with a historical genotype control of the healthy Kosovo population as reported by Krasniqi et al. (2017) [27] utilizing Fisher's exact test. Stratified analyses examined the association between patients *CYP2C19*2* status and risk factors. Odds ratios (OR) with 95% confidence intervals (CI) were calculated, with statistical significance set at $p \leq 0.05$. All analyses were conducted using MedCalc Software v22.026 (Med Calc Software Ltd, Ostend, Belgium).

RESULTS

Baseline characteristics of the 150 Kosovo patients treated with clopidogrel are summarized in Table 1. The mean age was 68 years, 78.7% of patients being older than 61 year and 40% being female. Among the patients, 43.3% presented with an ACS indication (STEMI/NSTEMI). All patients had at least one comorbidity including common conditions such as hypertension, diabetes, and dyslipidemia. Most patients had arterial hypertension (67.3%) and 35.3% of patients presented with more than one comorbidity.

Allele, genotype, and phenotype frequencies of *CYP2C19*2* polymorphism are detailed in Table 2. A total of 34.0% of patients were *CYP2C19* intermediate or poor metabolizers (29.33% IMs and 4.67% PMs). The frequency of *CYP2C19*2* LOF allele was 19.33%. The observed genotype distributions did not significantly deviate from Hardy–Weinberg equilibrium (χ^2=0.532; p=0.465).

Table 3 provides a comparison of *CYP2C19*2* allele and genotype frequencies observed in our cohort of patients with those reported for the healthy Kosovo population. According to the results, *CYP2C19*2 LOF* allele carriers have approximately 1.6 times higher probability

Table 1. Demographic and Clinical Characteristics of the Study Patient Cohort.

		Full cohort N=150	Frequency
Demographic parameters		n	(%)
Age group	< 40 years	2	1.3
	41-60 years	30	20.0
	> 61 years	118	78.7
Gender	Male	90	60.0
	Female	60	40.0
Clinical parameters			
Indication for clopidogrel therapy	STEMI/NSTEMI*	65	43.3
	Other	85	56.7
Co-morbidities	One	97	64.7
	More than one	53	35.3
Diabetes mellitus	With	65	43.3
	Without	85	56.7
Hypertension	With	101	67.3
	Without	49	32.7
Dyslipidemia	With	6	4.0
	Without	144	96.0

* STEMI/NSTEMI - ST segment elevated myocardial infarction / NonST segment elevated myocardial infarction

Table 2. Distribution of the CYP2C19*2 Allele and Genotype/Phenotype Frequencies in the Patient Population.

CYP2C19*2 polymorphism [rs4244285]	**Patient cohort (N=150)**	**Frequency (%)**	
	n	***observed***	***expected***
Genotype (phenotype)#			
***1/*1 (NM)**	99	66.0	65.07
***1/*2 (IM)**	44	29.33	31.19
***2/*2 (PM)**	7	4.67	3.74
Allele			
***1**	242	80.67	*NA*
***2**	58	19.33	

*1/*1 (NM) – Normal Metabolizer; *1/*2 (IM) – Intermediate Metabolizer; *2/*2 (PM) – Poor Metabolizer; NA- non applicable

Table 3. Association of CYP2C19*2 polymorphism and risk for CVD in Kosovo population.

Model of Statistical Analysis	**Patients (N=150)**		**Healthy population# (N=234)**		OR	95 % CI	p- value
	n	Frequency (%)	n	Frequency (%)			
Co – dominant							
*1/*1 (NM)	99	66	178	76.07	1.00		
*1/*2 (IM)	44	29.33	51	21.79	1.295	0.991 - 1.694	0.067
*2/*2 (PM)	7	4.67	5	2.14	1.632	0.896 - 2.701	0.112
Dominant							
*1/*1(NM)	99	66	178	76.07	1.00		
*2/*2+*1/*2 (PM+IM)	51	34	56	23.93	1.334	1.035 - 1.719	0.031
Allelic							
*1 allele	242	80.67	407	86.97	1.00		
*2 allele	58	19.33	61	13.03	1.307	1.06 - 1.612	0.018

Historical genotype control group according to Krasniqi et al., 2017 [27]

Table 4. Distribution of CYP2C19*2 Allele, Genotype, and Phenotype Frequencies in the Patient Population (N=150), According to Age, Gender, and the Most Common CVD Risk Factors.

CYP2C19*2 polymorphism [rs4244285]		Genotype (phenotype)[#]			Allele	
		*1/*1 n (%)	*1/*2 n (%)	*2/*2 n (%)	*2 n (%)	*1 n (%)
Group age	< 40 years	1 (50)	1 (50)	0 (0)	1 (25)	3 (75)
	41-60 years	18 (60)	10 (33.33)	2 (6.67)	14 (23.33)	46 (76.67)
	> 61 years	80 (67.80)	33 (27.96)	5 (4.24)	43 (18.22)	193 (81.78)
Gender	Male	61 (67.78)	25 (27.78)	4 (4.44)	33 (18.33)	147 (81.67)
	Female	38 (63.33)	19 (31.67)	3 (5)	25 (20.83)	95 (79.17)
Diabetes mellitus	With	45 (69.23)	16 (24.62)	4 (6.15)	24 (18.46)	106 (81.54)
	Without	54 (63.53)	28 (32.94)	3 (3.53)	34 (20)	136 (80)
Hypertension	With	67 (66.34)	29 (28.71)	5 (4.95)	39 (19.31)	163 (80.69)
	Without	32 (65.31)	15 (30.61)	2 (4.08)	19 (19.39)	79 (80.61)
Dyslipidemia	With	4 (66.67)	2 (33.33)	0 (0)	16 (61.54)	10 (38.46)
	Without	95 (65.97)	42 (29.16)	7 (4.87)	42 (15.33)	232 (84.67)

*1/*1 (NM) – Normal Metabolizer; *1/*2 (IM) – Intermediate Metabolizer; *2/*2 (PM) – Poor Metabolizer.
All p-values were greater than 0.05, indicating no statistically significant differences.

for developing CVD compared to non-carriers (OR=1.6; 95% CI=1.08-2.37; p=0.018). The association between *CYP2C19*2* allele and increased probability of developing CVD was further confirmed in dominant (NM vs. IM+PM) model of statistical analysis (OR=1.64; 95% CI=1.04-2.57; p=0.031) as well. Additionally, differences in the genotype/phenotype distribution were observed between the patient and historical healthy control groups (66.0%, 29.33% and 4.67% vs 76.07%, 21.76% and 2.14% for NM, IM and PM, respectively), although these differences did not reach statistical significance (codominant analysis: p=0.067 IM, p=0.112 PM; NM as reference). The present analysis does not allow a stratified assessment of association based on age and gender, since it is based on previously published data concerning *CYP2C19*2 LOF* allele in healthy Kosovo population. No statistically significant difference between stratified groups of patients (according to indication for clopidogrel treatment, coexistence of one or more risk factors) with respect to a *CYP2C19*2* variant allele (Table 4; all p>0.05). The median observation time of follow up was not reached until this analysis was conducted.

DISCUSSION

The worldwide implementation of pharmacogenetics highlighted population-specific differences in allelic and genotype/phenotype frequencies of genes coding for drug-metabolizing enzymes. One significant challenge in translating treatment-associated polymorphisms into routine clinical use is the lack of knowledge regarding its frequency in the targeted population in comparison to the population frequency. In the context of clopidogrel treatment, understanding the prevalence of *CYP2C19 LOF* variants within a population is critical for assessing their clinical implications. Careful consideration is necessary when interpreting studies on the association between *CYP2C19* metabolizer phenotype and clopidogrel treatment outcomes. Evidence supporting this association primarily stems from studies involving ACS patients (with at least 50% undergoing PCI) and settings where clopidogrel was compared with an alternative $P2Y_{12}$ inhibitor [28]. Conversely, studies opposing this association often focus on lower risk, non-PCI patients or data that did not strongly justify the utility of PGx guided clopidogrel treatment for secondary stroke prevention or ACS patients [13, 16]. The presented study primarily aimed to determine the frequency of *CYP2C19*2 LOF* allele in clopidogrel treated patients with cardiovascular disease indications (CVDI) and other concurrent risk factors in Kosovo and to evaluate the prognostic value and association of *CYP2C19* PM phenotype with the risk of MACE in this cohort during two-year term follow-up under routine clinical care. The prevalence of the *CYP2C19*2* allele in this cohort of Kosovo patients (19%) and the historical genotype control group of healthy population (13%) was consistent with the range reported in other European populations [29, 30], with latter falling within the 9-18% range observed across the population from the Balkan region, including Turkey (12%), Greece (13%), Macedonia (14.4%), Serbia (11%), Republic of Srpska (16%) in Bosnia and Hercegovina) Croatia (15%) and Slovenia (16%). Intra-population comparisons, however revealed a significantly higher frequency of the *CYP2C19*2 LOF* allele in the patient cohort compared to the healthy Kosovo population reported by Krasniqi et al. (2017), suggesting a possible association between the *CYP2C19*2 LOF* allele and increased overall

probability of CVD. This association appeared to be independent of the type and number of comorbidities as well as patients' previous history on MI (STEMI or NSTEMI). Notably, the proportion of poor metabolizers (PMs) identified in our patient cohort was significantly higher than the reported in studies of other European populations (4.67% vs. 2.4%, respectively) [11, 29, 31].

This study is the first to report this correlation within the Balkan population. Comparable research conducted in Macedonia [32], Serbia [33] and Croatia [34] has demonstrated that *CYP2C19*2* functions as an independent risk factor for adverse treatment outcomes, defined by the occurrence of MACE in patients receiving dual antiplatelet therapy with aspirin and clopidogrel. Notably, this is the first investigation that explores the relationship between the *CYP2C19*2* allele and the risk of CVD among the Balkan population. The potential mechanism by which *CYP2C19*2* may contribute to an increased risk of cardiovascular disease (CVD) extends beyond its pharmacogenetic role, encompassing disruptions in the metabolism of critical endogenous substrates, such as eicosanoids (arachidonic acid derivatives) and steroids. These substrates are integral to maintaining vascular homeostasis, modulating inflammatory processes, and regulating oxidative stress. Impaired CYP2C19 activity could alter the metabolism of these molecules, leading to endothelial dysfunction, compromised vascular function, and dysregulated blood pressure. Such metabolic disruptions may exacerbate conditions like hypertension and atherosclerosis, ultimately contributing to the pathophysiology of CVD [35, 36].

These preliminary findings highlight the importance of investigating intra-population genetic variations to identify specific genetic markers or health risks prevalent within a particular ethnic group. They supplement the growing body of evidence linking the *CYP2C19*2 LOF* allele with the occurrence and development of CAD, CVD and CHD. The results align with previously published studies suggesting that the *CYP2C19 LOF* allele may be involved in CVD susceptibility [37-41]. Rothenbacher et al. (2013) reported that stable CHD patients, carriers of the homozygous *CYP2C19*2 LOF* allele are at increased risk for subsequent CVD events during the long follow up, independent of other risk factors [37]. Similarly, Zhang et al. (2019) found that *CYP2C19*2* not only increased the risk of CHD, but also worsened clinical outcomes in CHD patients during an extended follow-up period [38]. Cai et al. (2023) observed that among the Hakka population, carriers of the *CYP2C19 LOF* alleles, both heterozygous and homozygous, exhibited increased susceptibility to hypertension [39]. In Martínez-Quintana's study (2017), patients with an acute coronary event and poor or normal CYP2C19 metabolizer phenotype were more likely to have insulin-dependent diabetes mellitus than those with rapid or ultrarapid metabolizer phenotypes [40]. More recently, Chen et al. (2024) reported that carriers of *CYP2C19 LOF* alleles are at increased risk for premature coronary artery disease, particularly when combined with other risk factors such as overweight, smoking, hypertension and diabetes mellitus [41].

When addressing the observed intra-population differences in *CYP2C19*2* frequencies, it is important to acknowledge that relying on previously published data may introduce potential variability due to differences in study design, methodology, or population characteristics and recruitment between historical genotype controls and the study cohort might affect the validity of direct comparisons. However, we recognize this limitation arising from the lack of a contemporaneously recruited healthy control group, as well as the small sample size and absence of demographic data for the healthy population. Despite these limitations, the internal validity of our findings remains intact. Importantly, there were no differences in the sample collection, handling and storing, DNA extraction or genotyping procedures, and both studies used DNA extraction kits comparable in terms of sensitivity and specificity and employed the same PCR-based methods and genotyping assays. Additionally, both the patient cohort and the historical control group primarily consist of ethnic Albanians from all parts of Kosovo. Nevertheless, it is essential to consider that differences in exposure to environmental factors, diet, or healthcare access between the cohorts could act as confounding variables, potentially masking genetic associations. Overall, these results provide valuable insights into the allele, genotype, and phenotype frequencies of the *CYP2C19* drug-metabolizing enzyme in Kosovar patients indicated for clopidogrel treatment. They add to the growing evidence advising the need for careful consideration of *CYP2C19* genetic diversity as a population-specific factor when recommending PGx-guided clopidogrel therapy [25, 26], particularly in populations like Kosovo's, where genetic determinants are not yet fully elucidated. The observed association between the *CYP2C19*2 LOF* allele and increased probability of developing CVD for clopidogrel treatment in our study hinders the ability to conclusively demonstrate a clinical benefit of *CYP2C19*2* PGx guided treatment in the population of Kosovo. Variability in treatment outcome, if identified during the study follow up period, could potentially be attributed to additional patient-specific determinants (such as comorbidities, concomitant drug treatments or as-yet undiscovered population-specific genetic factors). Future studies incorporating a broader range of demographic and clinical variables are needed to better elucidate the role of *CYP2C19*2* PGx guided therapy in optimizing clopi-

dogrel treatment strategies for this population. Moreover, additional investigations should evaluate whether the carriage of the *CYP2C19*2* allele itself is associated with an adverse outcome in patients not taking clopidogrel.

CONCLUSION

This study identifies a possible association between the *CYP2C19*2* allele and an increased probability of CVD within the Kosovo population, offering novel insights into the intra-ethnic variability of this LOF allele and its clinical relevance for PGx-guided therapy within the region. The findings should be considered when optimizing the implementation of the clopidogrel PGx testing in the routine clinical practice at the national level. Further studies examining the relationship between the *CYP2C19* PGx data, patient-specific follow up outcomes, and plasma concentration of clopidogrel's active metabolite are necessary to validate these observations and assess the utility of PGx-guided clopidogrel treatment in Kosovo population.

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DECLARATION OF INTEREST

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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CASE REPORTS

VALUE OF OPTICAL GENOME MAPPING (OGM) FOR DIAGNOSTICS OF RARE DISEASES: A FAMILY CASE REPORT

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ABSTRACT

Optical genome mapping (OGM) is a novel method enabling the detection of structural genomic variants. The method is based on the laser image acquisition of single, labeled, high-molecular-weight DNA molecules and can detect structural genomic variants such as translocations, inversions, insertions, deletions, duplications, and complex structural rearrangements. We aim to present our experience with OGM at the Clinical Institute of Genomic Medicine, University Medical Centre Ljubljana, Slovenia. Since its introduction in 2021, we have used OGM for the testing of facioscapulohumeral muscular dystrophy 1, characterization and resolution of variants identified by other technologies such as microarrays, exome and genome next-generation sequencing, karyotyping, as well as testing of rare disease patients in whom no genetic cause could be identified using these methods.

We present an example family case of two previously undiagnosed male siblings with an overlapping clinical presentation of thrombocytopenia, obesity, and presacral teratoma. After karyotyping, microarray analysis and next-generation sequencing, by using OGM, a maternally inherited cryptic translocation t(X;18)(q27.1;q12.2) was identified in both brothers. Despite an extended segregation analysis, based on strictly applied ACMG criteria and ClinGen guidelines, the identified translocation remains a variant of unknown significance. Despite the remaining limitations of OGM, which will hopefully be resolved by improvements in databases of known benign SV variation and the establishment of official guidelines on the clinical interpretation of OGM variants, our work highlights the complexity of the diagnostic journey, including this novel method, in rare disease cases.

Keywords: Optical genome mapping, OGM, structural variants (SV), genomic variants, rare disease genetic testing

INTRODUCTION

Optical genome mapping (OGM) is a novel technology enabling the detection of structural genomic variants (SV) at a resolution and in size range previously not readily available by other methods, opening new fields of research[1,2]. In human diagnostics, OGM has so far been applied to cancer genetics / haematology[3–6], constitutional molecular genetics[1,2], quality control assurance in genome modification (such as detection of off-target effects in genetically modified cell lines)[7], and in routine clinical genomic diagnostics of facioscapulohumeral dystrophy (FSHD)[8–11].

OGM has been in use at the Clinical Institute of Genomic Medicine (CIGM), University Medical Centre Ljubljana (UMCL), Slovenia since 2021 for research and diagnostic purposes. Our research focus (ARIS Programme P3-0326) involves discovering mechanisms of unexplained recurrent spontaneous pregnancy loss, male infertility, and integration and co-interpretation of whole genome sequencing (WGS) and OGM data (ARIS Programme J3-4517). The planned integration of OGM data with WGS will hopefully further increase the yield of diagnostics in such cases. In routine genetic diagnostics at CIGM, OGM is currently used for diagnostic testing of FSHD[11], characterization and resolution of variants identified by other technologies, and undiagnosed rare disease patients. In this way, we have so far successfully used OGM to characterize variants identified by other

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technologies, such as microarray, NGS, and karyotyping, to resolve the clinical significance of various SVs[12].

Herein, we present a family case report of rare disease patients tested using OGM that was performed in collaboration with the Center for Medical Genetics and Immunology (CMGI), Clinical Center of Montenegro (CCM) (BI-ME/21-22-016). In addition to the challenges faced in interpretation of SVs based on strictly applied ACMG criteria and ClinGen guidelines, our work serves to highlight the complexity of the diagnostic journey in rare disease cases.

MATERIALS AND METHODS

Patients

Two undiagnosed male siblings with an overlapping clinical presentation of thrombocytopenia, sacro-coccygeal teratoma, hydronephrosis/reflux vesicoureteral and obesity, who were referred to the CGMI, CCM, Montenegro, were enrolled in this family case-report.

Clinical data was collected during the patients' in-person appointments and evaluation by clinical geneticist at the CGMI, CCM, and all specialist examinations, were performed as part of standard routine clinical care. Before genetic testing, pre-test genetic counseling was provided by a clinical geneticist, followed by obtaining written parental consent at the CGMI, CCM. All procedures in the study were conducted according to the routine standard of care and in accordance with the principles of the Declaration of Helsinki. Karyotyping was performed at the CGMI, CCM, while microarray analyses, exome sequencing and optical genome mapping were performed at the CIGM, UMCL, Slovenia.

Karyotype

Chromosome analysis was performed for both probands, by using G-bended karyotyping (bend resolution 400 – 470, according to ISCN), after 72 hours of peripheral blood cultivation.

Microarray analyses

Microarray analysis was initially performed on the probands and their parents by using oligonucleotide array Agilent Technologies 4×180K (AMADID:035689), according to the manufacturer's instructions. Agilent CytoGenomics 5.1.2.1 software was used to visualize and report the CNVs, as previously described [12].

Exome sequencing

Exome sequencing of proband 1 and proband 2 with parents in trio setup was performed as previously described [13,14], and included the analysis of a total of >2000 genes associated with the clinical phenotype of the probands. The full list of genes for each of the included gene panels is available in the Supplement.

Optical genome mapping

Optical genome mapping was performed as previously described[12]. Briefly, high-weight molecular DNA was extracted from 1.5 million lymphocytes from whole blood (EDTA collected) using the SP Blood & Cell Culture DNA Isolation Kit or the SP-G2 Blood & Cell Culture DNA Isolation Kit following manufacturer instructions (Bionano Genomics Inc., San Diego USA). The following day, DNA molecules were labeled with the DLE-1 enzyme using the Direct Label and Stain (DLS) Kit or Direct Label and Stain-G2 (GLS-G2) kit (Bionano Genomics Inc.). Labeled DNA was loaded on the three-flowcell Saphyr Chip® G2.2 or G2.3 (Bionano Genomics Inc.) and ran on the Saphyr instrument (Bionano Genomics Inc.) to reach a minimum yield of 500 Gbp (DLE-1 label, [GRCh38] reference genome). The de novo assembly and Variant Annotation Pipeline were executed on Bionano Solve3.7_20221013_25 while reporting and direct visualization of SVs was done on Bionano Access 1.7.2.

Variant interpretation

We reported only those genomic variants that have statistical support based on the adequate genomic coverage and chosen analysis type for the detection of CNV, duplications, deletions, and other SVs such as insertions, inversion, intra- and inter-chromosomal translocations, as determined by internal Access QC parameters. The method does not enable the analysis of regions that do not contain DLE-1 labeling sites (centromeres, telomeres, and other heterochromatin regions). According to the ACMG and ClinGen guidelines[15], CNV variants are classified into one of five classes of pathogenicity based on the sum of points in each category of assessment, and were classified by comparison with their overlap with SV and CNV variants contained in the DGV (Database of Genomic Variants - http://dgv.tcag.ca/gb2/gbrowse/dgv2_hg19/)[16], gnomAD (genome Aggregation Database - https://gnomad.broadinstitute.org/), OGM (Bionano Genomics Inc. internal Access® database), ClinGen (Clinical Genome Resource Consortium) (https://dosage.clinicalgenome.org/), DECIPHER (https://www.deciphergenomics.org/), and/or ClinVar (https://www.ncbi.nlm.nih.gov/clinvar/) public databases and the CIGM genomic variant database. OGM results are given according to the genome mapping nomenclature as specified in the ISCN guidelines[17].

Visualization and figure preparation

Figure 1 was prepared from original visualizations generated by Bionano Access 1.7.2 software (Bionano), segregation, and optical genome mapping, respectively.

The final composite Figure 1 was technically prepared in terms of size, layout, format, and type of file with no modification to the original data, from the original visualizations, by using GIMP 2.10[18]. The pedigree was constructed and drawn using (Progeny Clinical Version N/Progeny Lab Version N) (Progeny Genetics LLC, Aliso Viejo, CA, www.progenygenetics.com).

RESULTS

Clinical characteristics

Characteristics of two male siblings (proband 1 and proband 2) with an overlapping clinical presentation of thrombocytopenia, sacrococcygeal teratoma, hydronephrosis/reflux vesicoureteral and obesity, are shown in Table 1.

Both parents and three sisters of the probands were healthy and without any of the clinical signs and symptoms shown in the probands, except for a few asymptomatic episodes of borderline platelet values in the mother.

Karyotype analyses

Normal male karyotypes were detected for both male siblings (Proband 1: 46, XY; Proband 2: 46, XY), with no clonal abnormalities (30 metaphases), at the stated band level of resolution. Karyotypes of the parents were also normal (Mother: 46, XX; Father: 46, XY).

Microarray analyses

Microarray analyses of the proband 1 showed an interstitial single copy gain of 18q12.2 region, approximately 459,7 kb in size in a male profile: arr[GRCh38] 18q12.2(38880911_39340584)×3 (arr[GRCh37] 18q12.2(36460875_36920548)×3). The identified duplication did not overlap with any known disease-causing genes and was not present in the databases containing variants from healthy individuals (DGV), nor in the medical literature or databases ClinGen, ClinVar, or DECIPHER. Due to its size and rarity, the copy number gain was interpreted as a variant of unknown significance, and segregation analysis using arrays was recommended. Segregation testing using microarrays in the mother and father of the proband showed the presence of the same 18q12.2 copy number gain in the mother of the proband. As the molecular karyotyping showed the presence of the same interstitial duplication of the 18q12.2 region in the proband and the mother; arr[GRCh38] 18q12.2(38880911_39340584)×3mat, and the duplication did not affect clinically important genes, it was interpreted as a likely benign genomic variant, and so clinical testing was continued to determine the cause of the clinical presentation in the proband and his brother.

Exome sequencing

Exome sequencing was initially performed for proband 1, as previously described[13,14]. The original gene panel included >100 genes associated with thrombocytopenia and hereditary thrombocytopenia including Wiskott-Aldrich syndrome. The analysis did not identify any variants that could explain the phenotype and therefore a reinterpretation of the exome sequencing data of proband 1 was performed with an expansion to genes associated with the additional phenotypes observed (Table 1). Despite adding over 1500 genes to the analysis, no causative variants were identified. Finally, exome sequencing in trio setup was

Table 1. Clinical characteristics of the probands

Clinical characteristics	Proband 1	Proband 2	Onset
Age at first visit	11 yr. & 11 mo.	3yr. & 6 mo.	
Early development	Hyperactivity	Normal	
Teratoma regio sacro-coccigealis	+	+	Neonatal
Hydronephrosis / *RVU	+ (right side)	+ (left side)	Neonatal
Thrombocytopenia	+	+	Neonatal
Purpura, petechiae, bruises	+	+	Infancy
Premaxillary prominence	+	+	Toddler
Juvenile palmoplantar dermatosis	+	+	Toddler
Obesitas	+	+	Toddler
Hypo-imunoglobulinaemia	+		10 yr.
Ameloblastoma mandibulae	+		10 yr.
Intellectual disability, mild	+		6 – 7 yr.
Epilepsia	+		14 yr.
Cerebral dysmyelination (MRI)	+		17 yr.
Frontoparietal polymicrogyria (MRI)	+		17 yr.

* Reflux vesico-ureteral

performed for proband 2 and both parents with updated gene panels. Despite including >2000 genes associated with the clinical phenotypes, no conclusively causative SNV variants or small indels could be identified. While the duplication observed on the microarray analysis was apparent from the coverage, no breakpoints could be identified by exome sequencing. The full list of genes included in the exome sequencing analysis is available in the Supplement.

Optical genome mapping

Optical genome mapping showed a translocation between chromosomes X and 18, accompanied by a duplication of the 18q12.2 region, of maternal origin in both probands; ogm[GRCh38] t(X;18)(q27.1;q12.2)(140408784~140427850;38878133~39396298)mat,dup(18)(18q12.2)(38927193_39426970)mat. The translocation breakpoints and the associated duplication of the 18q12.2 region did not overlap any clinically significant genes and are unlikely to be visible using classic karyotyping methods. The accompanying 18q12.2 duplication was approximately 499,7 kb in size, and was consistent with the previously observed duplication in the proband 1, 2 and their mother using microarray analysis: arr[GRCh38] 18q12.2(38880911_39340584)×3mat (Figure 1).

Interpretation and segregation analysis

The translocation and accompanying duplication of maternal origin do not directly affect genes known to cause disease in humans. However, several genetic mechanisms are known to influence the expression of nearby genes by influencing regulatory regions or by topological means, some promoting and some inhibiting expression [19–22]. Therefore, as described previously, we used the UCSC Genome Browser Viewer to visualize our region of interest in the context of neighboring genomic regions [12,23], however no obvious regulatory regions explaining the phenotype could be identified as being affected by the detected translocation and accompanying duplication. However, literature search revealed that the region of chromosome 18 involved in the rearrangement has previously been described in the context of germinal translocation t(11;18)(q22.1;q12.2), (ClinVar ID: 599287), where the translocation carriers also had age-dependent hypertension linked to 11q22.1, as well as obesity [24]. Additionally, somatic translocations between chromosome X and chromosome 18 involving different breakpoints have been previously described in synovial sarcomas (t(X;18)(p11.2;q11.2)), including in a rare case with submandibular presentation [25,26], however, the exact breakpoints of the critical region of 18q11.2 do not correspond to those identified in our patients. Therefore, because of the lack of direct evidence of pathogenicity, but because of the clinical match of the probands, the involvement of the chromosome X in males and a female with a very slight phenotype of transient thrombocytopenia, and the size of regions possibly affected indirectly, the translocation was classified as a variant of unknown clinical significance. When we are unable to provide final conclusions, extensive segregation may prove beneficial to clarifying the classification of the variant, as recently described in case of a *PLP1* duplication by our group[12]. In case of variants involving chromosome X in males, testing additional male family members may provide additional information helpful to clinical clarification, which is why we expanded the segregation to include healthy brothers of the carrier mother. The results of the segregation analysis are shown in Figure 2. None of the four maternal uncles were carriers of this rare familial translocation, that remains a variant of unknown significance.

Limitations

OGM requires a special isolation/extraction step, producing ultra-long/high molecular weight DNA (hmwDNA) molecules, that are typically in the 200 kilobases (kb) to megabase (Mb) range, in contrast to typical DNA isolation protocols where the resulting DNA is usually up to 20 kb in size. Therefore, archival DNA samples cannot be used for OGM, and fresh extraction is needed. After extraction, DNA is labeled across specific motifs using the DLE-1 enzyme, while the backbone DNA is also labeled using a special stain. The current technical limitations of OGM concern the size of DNA required, DLE-1 labeling limitations, and interpretation challenges. As large DNA molecules are needed for this method, it currently cannot be performed from archived DNA or FFPE, and therefore fresh samples are needed. Furthermore, the method cannot detect SV within regions that do not contain the DLE-1 labeling motif, such as Robertsonian translocations. Similarly, regions spanning segmental duplications, e.g. on the chrY chromosome can result in several alternative assemblies. The interpretation of genomic variants in terms of pathogenicity is currently based on recommendations from ACMG and the joint consensus of the American College of Medical Genetics and Genomics (ACMG) and the Clinical Genome Resource (ClinGen)[15,27]. However, many of the different SVs detected by the OGM method, for example, balanced translocations, inversions, etc. lack clear guidelines for classification, and so the interpretation of these SVs needs to be carefully considered on a case-by-case basis. The limited size of known normal OGM genetic variation at the moment means, that many identified variants remain variants of unknown significance. Finally, because of its novelty, there is a need to establish a larger database of normal human genomic variation detected us-

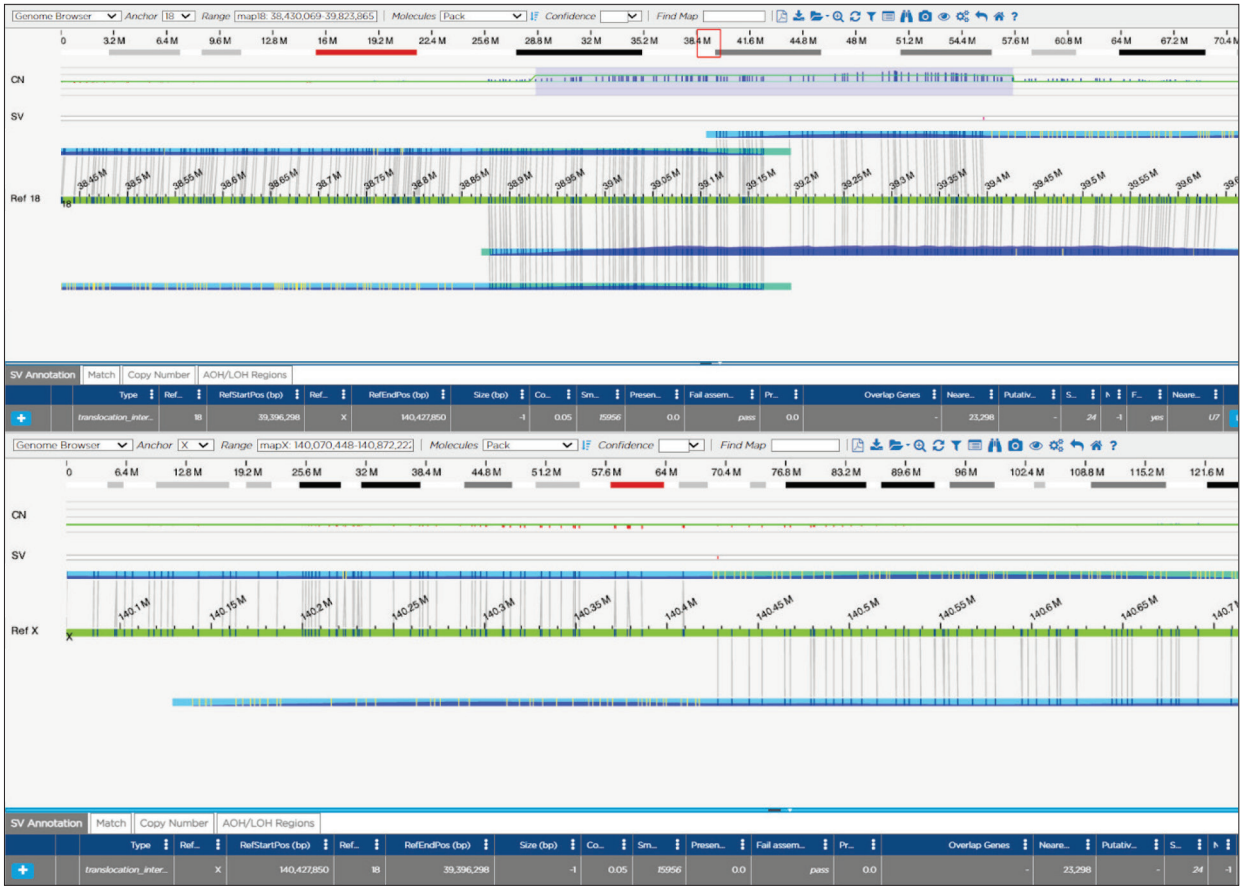

Figure 1. Optical genome mapping results showing molecules involved in the translocation mapping to chromosome 18 and chromosome X.

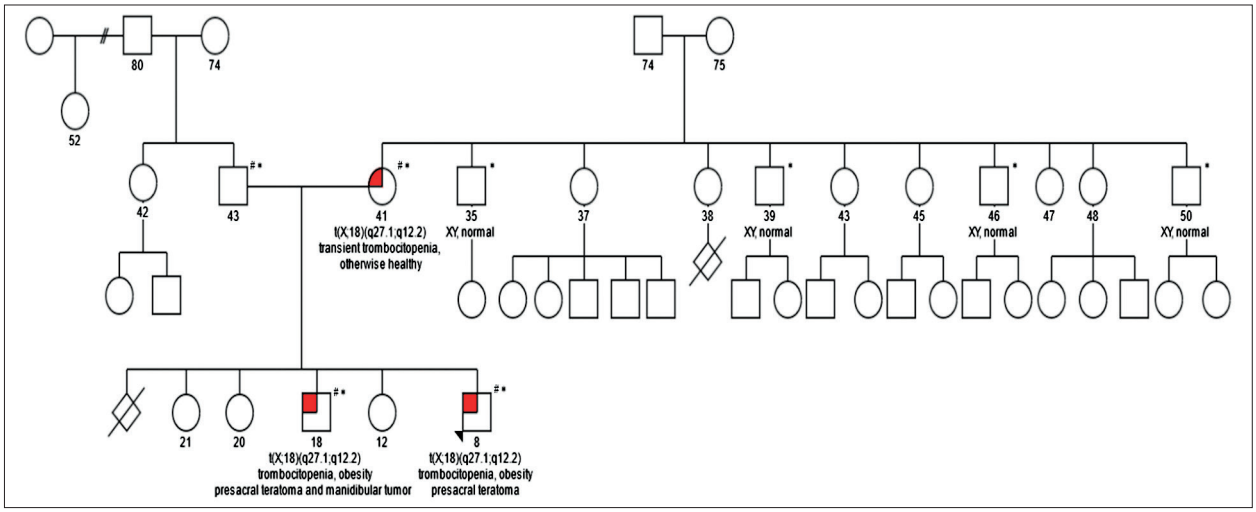

Figure 2. Family pedigree and segregation analysis results.
family members in whom karyotyping, microarray and NGS were performed.
* family members in whom OGM was performed.

ing OGM. When possible, the results should be confirmed by using an independent method, while for many rare disease cases, a trio setup is preferable to resolve causality.

Despite an extended segregation analysis, based on strictly applied ACMG criteria and ClinGen guidelines, the identified translocation remains a variant of unknown

significance, highlighting the complexity of diagnostic results in rare disease cases as well as the remaining limitations of this technology. Hopefully, the future increase in healthy control population OGM variants and the establishment of official guidelines on the clinical interpretation of OGM variants will resolve many current interpretation challenges.

DISCUSSION

The diagnostic journey in case of rare disease is often complex and consist of many steps. In our case, the traditional karyotyping was negative and was followed by microarray. While microarray identified a duplication of 18q12.2 that was initially classified as a variant of unknown significance, after segregation showed it to be of maternal origin, it was reclassified as likely benign, and the patients were referred for exome sequencing. After initial exome sequencing was negative, reinterpretation with additional gene panels was performed in the proband, and following another negative result, was followed up by panel exome sequencing of over 2000 genes in trio setup, which also failed to identify causative variants, and the probands were referred for OGM. OGM showed a t(X;18)(q27.1;q12.2) translocation, that was confirmed to be of maternal origin and had the previously observed duplication as an accompanying event: ogm[GRCh38] t(X;18)(q27.1;q12.2)(140408784~140427850;38878133~39396298)mat,dup(18)(18q12.2)(38927193_39426970)mat. Despite extended segregation, we did not identify any additional healthy male carriers of the translocation in the family. Therefore, pending reinterpretation and possible functional assessments that may become possible in the future with additional technologies, the identified familial translocation currently remains a variant of unknown significance.

CONCLUSION

In conclusion, OGM represents a useful new method in the repertoire of genomic diagnostics available at CIGM UMCL, however applying ACMG criteria and ClinGen guidelines to SVs remains demanding, highlighting the complexity of the modern genomic diagnostic approach to rare disease testing. In our experience, currently, a major limitation of the method remains the difficulty of interpretation due to its novelty and the lack of healthy control population variants, which will hopefully be resolved and will increase the diagnostic yield of this method in the future. To circumvent this limitation, in the testing of rare disease patients, OGM in a trio setup is currently advised.

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Declaration of interest
The authors report no conflicts of interest.

Supplement
Gene panels included in the exome sequencing analysis.

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CASE REPORT

PHENOTYPIC VARIABILITY OF COWDEN SYNDROME WITHIN A SINGLE FAMILY: IMPACT ON DIAGNOSIS, MANAGEMENT AND GENETIC COUNSELLING

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ABSTRACT

Cowden syndrome (CS) represents a rare autosomal dominant disorder caused by mutations in the *PTEN* gene located on chromosome 10q23.3. This entity belongs to the PTEN hamartoma tumor syndrome (PHTS) spectrum. The *PTEN* gene encodes a tumor suppressor protein crucial for regulating cell growth, survival, and apoptosis. Pathogenic mutations in *PTEN* result in dysregulated cell proliferation, manifesting clinically as benign and malignant growths across various tissues.

CS is characterized by a predisposition to multiple hamartomas and an elevated risk of cancers, most notably in the skin, soft tissues, thyroid, breast, and gastrointestinal tract. In pediatric patients, macrocephaly is frequently the earliest feature, often accompanied by developmental delays and neurological deficits. This case series details the clinical evolution and multidisciplinary management of two siblings with CS and normal psychomotor development. Genetic testing identified a familial *PTEN* mutation, with multiple affected relatives, including the siblings' father, paternal aunt and paternal grandfather, each displaying distinct phenotype.

This familial clustering highlights the autosomal dominant inheritance of CS and points out the critical importance of early genetic testing, vigilant surveillance, and tailored counselling for at-risk relatives. Phenotypic variability observed between members of the same family points out the difficulties in predicting transgenerational outcomes and complicates genetic counselling.

Keywords: Cowden syndrome (CS); *PTEN* gene; Phenotype; Genetic counselling;

INTRODUCTION

Cowden syndrome (CS), also known as PTEN hamartoma tumor syndrome (PHTS), is a rare autosomal dominant disorder caused by germline mutations in the *PTEN* gene located on chromosome 10q23.3 (1). The *PTEN* gene encodes a tumor suppressor protein that plays a critical role in maintaining genomic stability, regulating cell proliferation, and facilitating apoptosis (2). Loss-of-function mutations in *PTEN* disrupt these processes, leading to the unregulated growth of cells and the development of hamartomas and malignancies across multiple tissues (3). The *PTEN* gene negatively regulates the PI3K/AKT signalling pathway, essential for cell growth and metabolism. Germline *PTEN* mutations are heterogeneous, including nonsense, missense, and splice-site variants, with over 300 mutations described (4). These mutations cause variable expressivity and incomplete penetrance, even among family members (5). De novo mutations are identified in 10–20% of cases (1). CS is part of the PTEN hamartoma tumor spectrum (PHTS), which includes Bannayan-Riley-Ruvalcaba syndrome (BRRS) and Lhermitte-Duclos disease (5). The clinical manifestations of CS are diverse, involving hamartomas in the skin, thyroid, gastrointestinal tract, and other tissues (1). Genotype-phenotype correla-

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tions in PTEN disorders show significant variability caused by mutations and phenotypic interactions (4,5).

CASE SERIES

This case series involves members of a nonconsanguineous Serbian family. Detailed exploration of the family tree revealed that at least five family members exhibited clinical presentations consistent with CS. These manifestations included recurrent benign tumors, atypical vascular malformations, and hamartomatous lesions involving soft tissues and internal organs (Figure 1).

Patient 1

The first family member brought to medical attention was an 8-year-old girl, the third child from a regularly monitored pregnancy. She was born at term via natural delivery, with a birth weight of 3200 g and Apgar scores of 9 and 10. The patient initially presented in December 2016 to the surgical outpatient clinic with a noticeable lesion in the right axillary region. On examination, the lesion measured 4 cm in diameter and was circular and localized. Ultrasound revealed a prominent mass in the right anterior axillary line consistent with fatty tissue, with no involvement of underlying bone or muscle structures.

The lesion was closely monitored, but after significant growth was noted, it was surgically excised in March 2017. The excised mass measured 6 × 5 cm, and histopathological (HP) findings confirmed a lipoma. For the next four years, the patient was lesion-free until December 2021, when swelling in the medial aspect of the right knee was reported following an injury. An MRI of the knee region revealed a tumor within the vastus lateralis and medialis muscles, raising concerns for a myofibroblastic or synovial tumor, with sarcoma considered as a differential diagnosis.

A subsequent evaluation by a pediatric hematologist included full blood count, biochemical tests, coagulation screening, immunoglobulins, and ultrasound of the left inguinal region due to regional lymphadenopathy. A chest CT scan in May 2022 showed no evidence of pulmonary consolidation or lymphadenopathy but revealed a 1.2 cm hypodense lesion in the spleen, prompting further monitoring.

In May 2022, the patient underwent excision of the right thigh tumor. Histopathological evaluation identified the tissue as benign soft-tissue angiomatosis. However, in February 2023, follow-up imaging suggested tumor recurrence in the right knee region. The recurrence was surgically removed in November 2023, and HP analysis confirmed identical findings to the prior assessment.

In June 2024, the patient presented with pain in the left inguinal region and thigh. Examination revealed two freely mobile subcutaneous masses, measuring 8 × 4 cm near the left iliac crest and 5 × 3 cm in the anterior inner thigh. Both masses were surgically excised. Macroscopic examination of the subcutaneous tissue showed hyperlobulated adipose tissue with densely packed vascular components forming a cribriform structure. Histopathological analysis revealed a complex soft tissue lesion composed of disorganized mesenchymal tissues, including atypical combined vascular malformations, proliferative lipomatous components, and fibrous elements. Immunohistochemistry (CD31, CD34, D2-40, smooth muscle actin, desmin, S-100 protein, Ki-67) supported the diagnosis of PHOST (Figure 2).

Genetic testing via whole exome sequencing identified a heterozygous pathogenic variant c.48T>A (p.Tyr16Ter) in the *PTEN* gene, confirming a diagnosis of CS type 1. Following diagnosis, regular multidisciplinary monitoring by a plastic surgeon, hematologist, and geneticist was initiated. There have been no new lesions detected to date.

Patient 2

The patient's 9-year-old older sister presented in December 2023 with a soft tissue mass in the lumbar region. MRI revealed a lipomatous lesion involving muscle and aponeurotic structures. In May 2024, the mass was surgically excised, preserving muscle and nerve function. Postoperative HP analysis confirmed a benign lipomatous lesion. However, follow-up imaging revealed recurrence of the tumor at the initial site.

Genetic testing confirmed the presence of the familial *PTEN* variant c.48T>A (p.Tyr16Ter), and the diagnosis of CS was extended to the older sister. She is now under regular multidisciplinary surveillance.

Patient 3

Clinical examination of the children's father revealed plantar keratosis. Genetic testing confirmed the same *PTEN* variant (c.48T>A, p.Tyr16Ter) as in his daughters. Given the genetic findings, the father was referred for endocrine and hematological evaluations, as well as regular cancer surveillance. To date, he has not developed malignancies but remains under continuous monitoring.

Patient 4

The paternal grandfather of the siblings, now a 78-year-old man, was identified as Patient 4. Based on anamnesis and photographic analysis, he was noted to have two large soft tissue masses located in the neck and interscapular regions. Although genetic testing was not performed, these findings, along with the confirmed familial PTEN variant in his descendants, strongly suggest that he is affected by CS. To date, he has not reported any significant health issues potentially associated with the CS

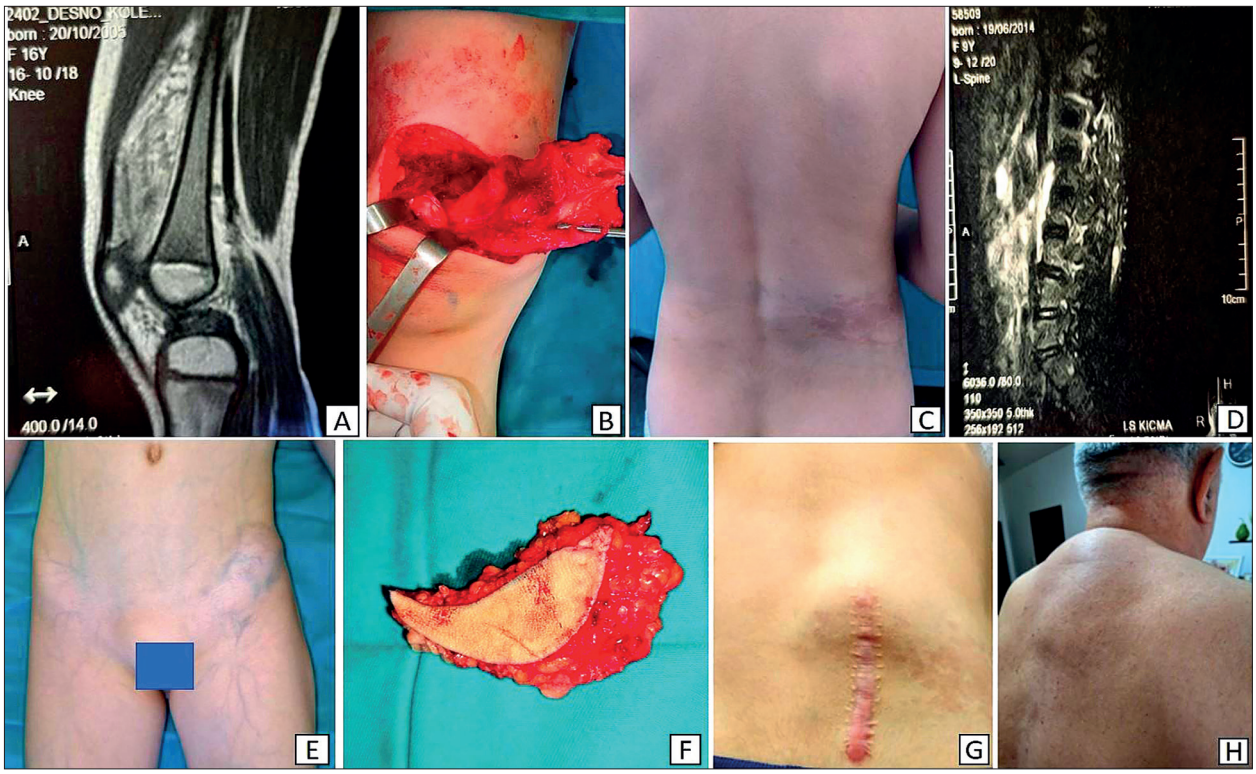

Figure 1. A Magnetic resonance imaging (MRI) of the thigh showing a tumor within the vastus lateralis and medialis muscles, Patient 1; **B** Intraoperative findings during excision of the tumor from the medial aspect of the distal thigh, Patient 1; **C** Tumor in the left inguinal region, Patient 1; **D** Completely excised tumor from the left inguinal region, Patient 1; **E** Tumor located in the left paraspinal region, Patient 2; **F** Magnetic resonance imaging (MRI) of the lumbar region reveals a paraspinal lipomatous lesion involving the musculature and aponeurotic structures, Patient 2; **G** Tumor recurrence in the left paraspinal region at the site of the postoperative scar, Patient 2; **H** Presence of subcutaneous soft tissue tumors in the siblings' grandfather, Patient 4.

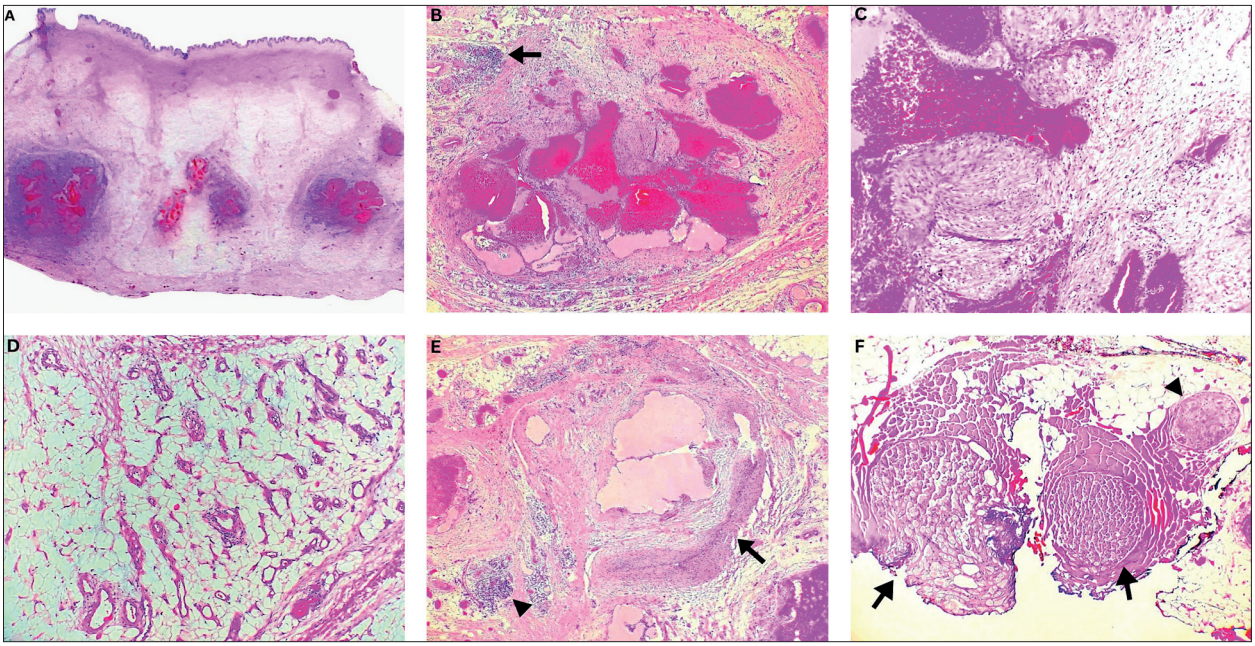

Figure 2. A. At scanning magnification the lesion were composed of a variable admixture of adipose tissue and abnormal vessels;
B. Dominant adipocytic component containing clusters of back-to back thin-walled veins and lymphocytic aggregate (arrow);
C. Fibromyxoid stroma in vascular clusters; **D.** Indeterminate small vessels reminiscent of veins dispersed within adipose tissue;
E. Arterial component with coiled thick-walled artery with tiny lumen (arrow) and lymphocytic aggregate (arrowhead);
F. Hypertrophic nerves within lesion (arrows) compared to normal looking nerve (arrowhead).

spectrum. As a precautionary measure, he was advised to undergo regular surveillance, including monitoring for potential malignancies, in alignment with current recommendations for individuals with CS.

Patient 5

Patient 5 is the 70-year-old paternal great-aunt of the two sisters. She has a long-standing history of medical follow-up due to benign central nervous system tumor, adrenal tumors, and diffuse intestinal polyposis. Anamnesis indicates that she has undergone multiple surgical interventions over the years for these conditions. While no documentation confirms whether genetic testing has been performed, the clinical presentation aligns with features of CS, particularly given the familial history of a confirmed *PTEN* pathogenic variant. She has been advised to continue regular monitoring under the care of a hematologist, with emphasis on multidisciplinary surveillance for the potential CS complications.

Anamnestic data further suggests that the great-grandfather of the proband passed away in his late 70s due to liver cancer. However, no reliable medical records are available to confirm the diagnosis or precisely assess contributing risk factors such as alcohol consumption or underlying conditions. While this observation could be relevant to the familial context, its significance remains uncertain.

DISCUSSION

Clinical Manifestations

The clinical manifestations of CS are diverse, involving hamartomas in the skin, thyroid, gastrointestinal tract, and other tissues (1). Macrocephaly, often present at birth or early infancy, is a key diagnostic feature in children, accompanied by facial dysmorphism such as a high anterior hairline and hypertelorism (6). Developmental delays and ASD, seen in up to 25% of patients, are frequent neurological findings, with brain imaging sometimes revealing structural anomalies, often white matter lesions (6, 7). The clinical manifestations of CS are diverse, involving hamartomas in the skin, thyroid, gastrointestinal tract, and other tissue (8–10). Recognizable dermatological traits include trichilemmomas around the nose and mouth, oral papillomas, and acral keratoses (9, 10). Sclerotic fibromas and lipomas, presenting as soft, mobile masses on the torso or extremities, further define the dermatological profile (8, 9). Gastrointestinal polyps affect up to 80% of patients, causing symptoms like abdominal pain or rectal bleeding. Though mostly benign, routine colonoscopic surveillance is recommended due to a small malignancy risk (11, 12). Less common vascular anomalies, such as arteriovenous malformations, can cause functional issues depending on their size and location (3, 13). In the case of our female patients, there were no elements of macrocephaly or dyscrania, and psychomotor development was normal. The characteristic skin changes associated with the syndrome were also absent. The only findings that contributed to the diagnosis were the presence of characteristic tissue tumor changes. Screening of other organs and organ systems revealed no signs of visceral involvement typically seen in CS. At this point, it is challenging to speculate on the genotype-phenotype correlation, given that this particular genotype, to our knowledge, has not been phenotypically described in any previously published case report in the available literature.

Genotype-Phenotype Correlation and Penetrance in CS

The PTEN variant identified in this family, c.48T>A (p.Tyr16Ter), represents a nonsense mutation associated with a truncated, non-functional protein. We confirmed by the thorough review of the available literature that the PTEN variant c.48T>A (p.Tyr16Ter) is a previously recognized pathogenic mutation. However, a detailed search of medical articles available online has not identified any publications to date presenting the clinical features of patients harboring this specific genotype. Genotype-phenotype correlations in CS demonstrate significant variability in clinical presentations, with features ranging from benign hamartomas to malignant transformations (14, 15). For example, catalytic domain mutations are strongly associated with severe neurodevelopmental outcomes, including ASD and intellectual disability (6, 14). Beyond genotypic effects, traits such as macrocephaly, vascular anomalies, and lipomatous changes suggest potential phenotypic synergy, where certain characteristics amplify others (15). This interplay may result from shared developmental pathways disrupted by PTEN dysfunction. Macrocephaly, for instance, often correlates with altered brain architecture, predisposing to neurodevelopmental disorders (7). Similarly, vascular anomalies like angiomatosis or arteriovenous malformations may exacerbate tissue overgrowth, contributing to complex lesions like PTEN hamartoma of soft tissue (PHOST) (8, 13). Incomplete penetrance and variable expressivity are important characteristics of PTEN-related disorders, with up to 15% of carriers remaining asymptomatic throughout life (16). Modifier genes, environmental influences, and epigenetic factors likely contribute to this variability, as suggested by studies in other hamartomatous syndromes such as Peutz-Jeghers and juvenile polyposis syndrome(15)

Malignant Alterations in CS and Age-Related Observations

CS is associated with an elevated lifetime risk of malignancies, particularly breast, thyroid, and endometrial cancers, with risk estimates reaching 85% for breast cancer in females and 35% for thyroid cancer (6, 17). However, in this family, two older members (the paternal grandfather and the great-aunt) have not developed malignancies despite their advanced age. This observation could be explained by the incomplete penetrance and suggests possible protective factors that warrant further investigation.

Age at onset of malignancies in CS varies, with breast cancer often developing by the fourth decade and thyroid malignancies potentially occurring earlier (14, 17). Other common malignancies include thyroid (especially follicular carcinoma) and endometrial cancers, warranting regular thyroid ultrasounds and gynecological evaluation (5, 18). Risks of renal cell carcinoma and colorectal cancer further emphasize the need for comprehensive cancer surveillance protocols (19). Interestingly, hepatic tumors have been reported in older individuals with CS, particularly in the context of non-alcoholic steatohepatitis (NASH) (20). The absence of malignancies in older family members suggests the need for personalized surveillance plans tailored to individual risk factors. Regular screening, including breast MRI and thyroid ultrasound, is therefore essential, even in asymptomatic carriers (5, 17).

Therapeutic and Surveillance Considerations

While surgical management of benign tumors provides effective short-term relief, recurrence is common, as seen in this case. The use of pharmacological approaches, including mTOR inhibitors such as sirolimus and everolimus, offers a promising strategy for reducing tumor burden by targeting dysregulated cell growth pathways in PTEN-mutant cells. Hormonal therapies, such as tamoxifen, may also be considered for risk reduction in individuals predisposed to breast and endometrial cancers, though their use in pediatric populations remains under investigation (21–24).

In cases where malignancies arise, targeted therapies, such as tyrosine kinase inhibitors, may complement standard oncological treatment, particularly when molecular pathways contributing to tumor growth are identified (25).

Genetic Counseling and Family Segregation Analysis

The autosomal dominant inheritance of CS puts imperative on genetic counselling for affected families. In this case, segregation analysis confirmed the familial *PTEN* mutation in the proband's sister and father. Early identification of mutation carriers enables the implementation of individualized surveillance programs, which are critical for minimizing cancer risk and improving outcomes. For at-risk family members, genetic analysis is indicated. In case of positive findings, regular monitoring and imaging are essential components of the clinical approach (1, 17, 26).

CONCLUSION

This familial case of CS exemplifies the complex interplay of genetic factors, phenotypic variability, and clinical vigilance required in managing PTEN hamartoma tumor syndrome (PHTS). Beyond confirming a pathogenic *PTEN* variant in multiple family members, it illustrates the broader implications of integrating genetic diagnostics into routine clinical practice. The diverse presentations within this family, ranging from benign soft tissue tumors to vascular anomalies, highlight the unpredictable clinical evolution of CS, necessity of personalized management strategies and flexibility needed in genetic counselling.

Importantly, this case emphasizes the value of early identification and interdisciplinary collaboration in the management of a long-term risks associated with CS. As the understanding of PTEN-related pathways evolves, the potential for targeted therapies continues to grow, offering opportunities not only to address tumor progression but also to enhance the quality of life for affected individuals and their families.

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