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Clinical Utility of Molecular Autopsy in Fetal and Pediatric Patients with Suspected Genetic Disorders

Genetik Hastalık Şüphesi Olan Fetal ve Pediatrik Hastalarda Moleküler Otopsinin Klinik Faydası

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ABSTRACT

Molecular autopsy is defined as whole exome/genome sequencing (WES/WGS) performed on DNA samples in the postmortem period to clarify the genetic etiology in patients who demised without a diagnosis. With this study, we aim to present our experience with postmortem WES/WGS analysis, in a group of pediatric patients to increase the knowledge on the clinical utility of molecular autopsy and its effect on genetic counseling.

A retrospective cohort study was conducted on postmortem WES/WGS analysis records performed between 2017-2021 in Acibadem University Department of Pediatric Genetics. Clinical data and analysis results of patients who died in the perinatal/infancy period without a molecular diagnosis were collected from medical records.

A total of 16 cases were included in the study. In 56% of the cases, molecular autopsy revealed a diagnosis. In 10 genes (*BBS9, BRAF, SLC12A1, PIEZO1, WDR62, ERCC8, NDUFAF2, RAG1, MOGS, ETFB*), 12 variants were detected. Fifty percent of these variants were novel, reported for the first time with this study. Inborn diseases of metabolism (33%) and neurologic disorders (22%) were the most common disease groups.

Our results show that WES/WGS analysis yields a high diagnostic rate in the postmortem period. The diagnosis elucidated by molecular autopsy provides invaluable information for the family who has experienced a loss. The identification of the underlying genetic cause enables the family to plan for future pregnancies. Diagnosing a fetus or an infant who was lost without a specific diagnosis helps identify novel genes and further delineate perinatal lethal phenotypes related to known genes.

Keywords: Molecular outopsy, Pediatric, Genetic diseases, Postmortem, Next generation sequencing

ÖZ

Moleküler otopsi, herhangi bir tanı konulamadan kaybedilen hastalarda genetik etiyolojiyi aydınlatmak üzere postmortem dönemde gerçekleştirilen tüm ekzom/genom dizilemeyi (WES/WGS) kapsamaktadır. Çalışmamızın amacı pediatrik yaş grubunda postmortem WES/WGS tecrübemizi paylaşarak, moleküler otopsinin klinik kullanımdaki yararını ve genetik danışmanlık üzerindeki etkisini sunmaktır.

Çalışmamız retrospektif kohort çalışması olarak planlandı. Acıbadem Üniversitesi Pediatrik Genetik Anabilim Dalı'nda 2017-2021 yılları arasında, perinatal veya infant döneminde moleküler tanı konulamadan kaybedilen ve postmortem WES/WGS yapılan hastaların klinik verileri ve moleküler sonuçları hastalara ait tıbbi kayıtlar incelenerek toplandı.

Çalışmaya toplam 16 vaka dahil edildi. Vakaların %56'sında moleküler otopsi sonrası tanı konuldu. On gende (*BBS9, BRAF, SLC12A1, PIEZO1, WDR62, ERCC8, NDUFAF2, RAG1, MOGS, ETFB*), toplam 12 varyant tespit edildi. Bu varyantların %50'si daha önce bildirilmeyen, novel varyantlardı. Doğuştan metabolizma hastalıkları (%33) ve nörolojik bozukluklar (%22) en sık görülen hastalık grupları olarak öne çıktı.

Sonuçlarımız, WES/WGS analizinin özellikle ülkemiz gibi akraba evliliği oranının yüksek olduğu bölgelerde ölüm sonrası dönemde yüksek bir tanı oranına sahip olduğunu göstermektedir. Moleküler otopsi ile aydınlatılan tanı, kayıp yaşayan aile için gelecekteki gebeliklerin planlanması ve yönetimi açısından çok değerli bilgiler sağlamaktadır. Ayrıca, spesifik bir tanı olmadan kaybedilen fetüs veya infantların teşhisi, yeni genlerin tanımlanmasına ve bilinen genlerle ilgili perinatal ölümcül fenotiplerin belirlenmesine de olanak tanımaktadır.

Anahtar Kelimeler: Moleküler otopsi, Pediatrik, Genetik Hastalıklar, Postmortem, Yeni nesil dizileme

The local ethics committee approved the protocol of the study with an accession number of ATADEK -201914/10 on 12.09.2019.

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INTRODUCTION

Up to 40% of all under-five years child deaths are among the neonatal/infancy period and genetic anomalies contribute significantly to this mortality by causing serious congenital structural abnormalities as well as severe neurometabolic problems.[1] Chromosome analysis and microarray analysis are the first-tier diagnostic tests performed on fetuses and infants thought to be affected by genetic diseases. Since most of the monogenic disorders cannot be detected with these analyzes, the underlying molecular pathology can be elucidated in only 5% of the patients.[2, 3] Although tests for monogenic diseases can be planned following such an approach, some of the affected individuals are lost before further molecular analysis can be performed, as some of the monogenic disorders may have a fatal phenotype in utero or early in life. In such cases, the lack of the molecular diagnosis limits the genetic counseling to be given to the family and the preconceptional / prenatal diagnosis options that can be offered for future pregnancies.

Whole exome sequencing (WES) has been increasingly used in routine diagnostic settings since 2009, to elucidate the underlying molecular pathology in patients followed up for multiple structural anomalies and neurometabolic disorders.[4, 5] However, the use of WES analysis in patients who are demised in the prenatal or infancy period without being diagnosed is still limited. Molecular autopsy, a concept that has gained importance in this field in recent years, is defined as whole exome/whole genome sequencing (WGS) analysis performed on DNA samples in the postmortem period.[6, 7] Molecular autopsy, with the reverse genotyping approach, in patients who demised in the perinatal and infancy period without diagnosis, would illuminate the family's path in terms of planning and taking measures for the next pregnancy by providing the diagnosis. It also has importance as an invaluable tool for identifying new genes and/or determining prenatal lethal phenotypes of known genes.

Here we describe our experience with postmortem WES/WGS analysis in a group of prenatal /infancy period demised individuals to add information about the clinical utility of molecular autopsy and its effect on genetic counseling.

MATERIAL AND METHODS

Patients

The data for the postmortem WES/WGS analysis were retrospectively collected by reviewing the WES/WGS records, performed between 2017-2021 in the Acibadem University School of Medicine Pediatric Genetics Department. Among these cases, those who were demised in the perinatal/infancy/early childhood period without molecular/clinical diagnosis were included in the study. The clinical presentation, physical examination records, prenatal and postnatal imaging reports as well as the demographic data, family history were collected from the medical records of the patients. Statistical analysis was performed with SPSS.22 (IBM Company, Armonk, NY, USA) package software. Numeric mean and standard deviation were used for descriptive statistics.

WES/ WGS Analysis and Interpretation of Results

The DNA samples of index cases were isolated from either peripheral blood, fetal skin tissue, or formalin-fixed paraffin-embedded (FFPE) tissue samples. Parental DNA samples were isolated from peripheral blood leukocytes.

WES was performed on Illumina Next-Seq platform with Twist Human Core Exome Plus kit adapters with an average coverage depth of ~20X, and WGS was performed on HiSeqX platform with Illumina adapters with an average coverage depth of ~30X. End-to-end, in-house bioinformatics pipelines were

applied, which includes base calling, primary filtering of low-quality reads and probable artifacts, and annotation of variants. The DNA sequences were aligned to the NCBI Build37 (hg18) version of the human genome. Alignments were confirmed by using Integrative Genomics Viewer v.2.313. Variant evaluations were focused on coding exons along with flanking +/-20 intronic bases both for WGS and WES. However, in the WGS analysis, an expanded search to include non-coding regions was performed in the presence of a candidate gene or when a second variant had to be sought in an autosomal recessive (AR) inherited phenotype. All disease-causing variants reported in the Human Genome Mutation Database (HGMD), in ClinVar or in-house database in addition to all variants with minor allele frequency (MAF) of less than 1% in the GnomAD database were considered. The variants' effects on protein function were investigated using in-silico prediction tools such as SIFT, PolyPhen2, M-CAP, Mutation Taster, and MVP. The interpretation of the variants was performed according to the 2015 American College of Medical Genetics (ACMG) standards and guidelines.[8] Variants were categorized into classes 1–5 with respect to their pathogenicity and causality (Class-1: pathogenic (P), Class-2: likely pathogenic (LP), Class-3: variant of uncertain significance (VUS), Class-4: likely benign (LB), Class-5: benign (B)).

Ethical Aspect of Research

The local ethics committee approved the protocol of the study with an accession number of Acibadem Mehmet Ali Aydinlar University Medical Research Evaluation Committee (ATADEK) No: 201914/10. The study was conducted following the Declaration of Helsinki. Informed consent was obtained from each patient and their legal guardians for molecular analysis prior to the WGS/WES.

Limitation of Research

The limitation of our study is that copy number variation analysis could not be performed in 14 cases in which WES analysis was performed.

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RESULTS AND DISCUSSION

A total of 16 cases (5 fetuses, 11 postnatally demised) were included in the study (Table 1). In most of the cases (81%) trio WES (index and biparental) was performed. The mean gestational age in fetal cases was 23.8 ± 2.4 weeks (mean ± SD) (min-max.: 20- 27 weeks). There were three termination of pregnancy and three intrauterine fetal demises in this group. In the postnatal demised group, the mean age of death was 9.4 ± 11.6 months (mean ± SD) (min-max.: 1day- 37months). Skin tissues were used for DNA isolation in all fetal cases. In postnatal cases, DNA isolation was mainly performed from peripheral blood samples (82%), and in two cases, umbilical cord and FFPE tissue were used to obtain DNA samples. In 38% of the families, there was a history of consanguinity and a similarly affected sibling. In WES/WGS analyses, 12 variants in 10 genes were detected in 56% of the cases, which were responsible for the clinical findings (Table 2). In the light of medical records and detailed family history data, a preliminary diagnosis was reached in 38% of the patients before the molecular autopsy and molecular diagnoses were found to be compatible with these prior clinical diagnoses, in 50% of those patients. Metabolic diseases (33%) and neurologic disorders (22%) were the most common identified disease groups. All except one of the variants were in genes associated with AR inheritance patterns and the parents were found to be carriers.

Table 1. Clinical Findings of the Patients

Patient No	Gender	Gestational age at TOP/IUFD	Age (months)	Cons.	Affected Sibling	Clinical Findings	Clinical Prediagnosis
P1	F	NA	5.5 months	Yes	No	Recurrent infections, abnormal skin pigmentation, ichthyosis, suspected severe combined immunodeficiency	Omenn Syndrome
P2	M	NA	1 month	Yes	Yes	Premature birth severe polyhydramnios, hepatomegaly, pancytopenia, abnormality of coagulation, renal insufficiency, hyperuricemia, and hematuria	No PCD
P3	F	20 (TOP)	NA	Yes	Yes	Severe short limbs in prenatal USG, postmortem radiographs suggesting severe perinatal lethal osteogenesis imperfecta	Osteogenesis Imperfecta
P4	F	24 (TOP)	NA	Yes	Yes	Short limbs, persistent umbilical vein, and microcephaly in prenatal USG	No PCD
P5	F	20 (IUFD)	NA	No	Yes	Hydrops fetalis	No PCD
P6	F	27 (TOP)	NA	No	No	Lissencephaly, bilateral minimal ventriculomegaly, abnormal parieto-occipital fissure, cavum velum interpositum cyst in prenatal USG, ventriculomegaly and lissencephaly in fetal MRI	No PCD
P7	F	24 (TOP)	NA	No	Yes	Microcephaly, polymicrogyria, nodular heterotopia, cortical generalized thinning/atrophy in prenatal MRI	No PCD
P8	M	NA	3 months	No	No	Failure to thrive, neonatal hepatitis, renal tubular acidosis, renal insufficiency	No PCD
P9	F	NA	18 months	No	No	Decreased fetal movements in the prenatal period, severe hypotonia, need for ventilator support, swallowing impairment, severe mental and motor retardation	No PCD
P10	F	NA	5 months	No	No	Nuchal translucency, pleural effusion, and polyhydramnios in prenatal USG, hypertrophic cardiomyopathy, abnormal and curly hair, coarse facial appearance	Cardio-facio-cutaneous Syndrome
P11	M	NA	6 months	No	No	IUGR and polyhydramnios in prenatal USG, early-onset epileptic encephalopathy, dysmorphic features including wide nasal bridge, anteverted nostrils, wide nasal tip, dolichocephaly, vertical ridging and furrowing in the forehead, abnormal myelination in cranial MRI	No PCD
P12	F	NA	21 days	No	No	Tetralogy of Fallot, ventricular hypertrophy, cerebral calcification, abnormality of lateral ventricles, neuronal migration defects, corpus callosum agenesis, and schizencephaly in cranial MRI	No PCD
P13	F	NA	1 day	No	Yes	Oligohydramnios, enlarged polycystic kidneys, mega cisterna magna in prenatal USG, premature birth, renal insufficiency	Glutaric Acidemia IIA/ ARPKD
P14	F	NA	4.5 months	No	No	Recurrent severe pneumonia, CMV infections, lymphopenia, suspected severe combined immunodeficiency	SCID
P15	F	NA	24 months	Yes	No	Global developmental delay, hypotonia, spastic diplegia, microcephaly, postaxial polydactyly, dysmorphic facial features including narrow forehead, arched eyebrows,up-slanted palpebral fissures, tented upper lip vermilion, and hypoplasia of the corpus callosum, abnormal cerebellum morphology in cranial MRI	Bardet-Biedl Syndrome
P16	M	NA	37 months	Yes	No	Failure to thrive, global developmental delay, stroke, abnormal pons morphology, and mega cisterna magna in cranial MRI, atrial septal hypertrophy	Glutaric Acidemia

F: female, M: male, Cons.: consanguinity, TOP: termination of pregnancy, IUFD: intrauterine fetal demise, USG: ultrasonography, MRI: magnetic resonance imaging, PCD: prior clinical diagnosis, ARPKD: autosomal recessive polycystic kidney disease, SCID: severe combined immune deficiency, NA: not accessible

Table 2. Molecular Findings of the Patients

Patient No	Analysis Type	Gene(s)	Transcript No (NM_)	Variant Type	Variant(s)	Zygosity	Inheritance	ACMG Classification	OMIM Disease / Inheritance Pattern
P1	Trio WES	*RAG1*	000448.2	Missense	c.1331C>T	Hom.	Inherited	Pathogenic (PP5, PM2, PM1, PP3)	Omenn Syndrome (#603554) / AR
P2	Trio WES	*SLC12A1*	000338.2	Frameshift	c.2952_2955del	Hom.	Inherited	Pathogenic (PVS1, PM2, PP3)	Bartter Syndrome Type 1 (#601678) / AR
P3	Trio WES	ND	NA	NA	NA	NA	NA	NA	NA
P4	Trio WES	ND	NA	NA	NA	NA	NA	NA	NA
P5	Trio WES	*PIEZO1*	001142864	Nonsense/ Nonsense	c.3495C>G/ c.5793C>A	Comp. Het.	Inherited	Pathogenic/ Pathogenic (PVS1, PM2, PP3)/ (PVS1, PM2, PP3)	Hereditary Lymphedema Type III (#616843) / AR
P6	Trio WES	ND	NA	NA	NA	NA	NA	NA	NA
P7	Trio WES	*WDR62*	001083961	Nonsense/ Missense	c.1408C>T/ c.1531G>A	Comp. Het.	Inherited	Pathogenic/ Likely Pathogenic (PVS1, PM2, PP3, PP5) (PM2, PM3, PP3, PP5)	Primary Microcephaly Type 2 (#604317) / AR
P8	Trio WES	ND	NA	NA	NA	NA	NA	NA	NA
P9	Trio WES	*MOGS*	006302.2	Missense/ Missense	c.560G>C/ c.1693C>T	Comp. Het.	Inherited	VUS/ VUS (PM2, PP3) / (PM2, PP3)	CDG Type IIb (#606056) / AR
P10	Solo WES	*BRAF*	004333.4	Missense	c.736G>C	Het.	De-novo	Pathogenic (PM1, PM2, PP2, PP3, PP5)	CFC Syndrome (#115150) / AD
P11	Trio WES	ND	NA	NA	NA	NA	NA	NA	NA
P12	Solo WGS	ND	NA	NA	NA	NA	NA	NA	NA
P13	Trio WES	*ETFB*	001985.2	Intronic	c.57+9_57+19del	Hom.	Inherited	VUS (PM2)	Glutaric Acidemia Type IIA (#231680) / AR
P14	Solo WGS	ND	NA	NA	NA	NA	NA	NA	NA
P15	Trio WES	*BBS9*	001348041	Nonsense	c.841A>T	Hom.	Inherited	Pathogenic (PVS1, PM2, PP3)	BBS (#615986) / AR
P16	Trio WES	*ERCC8* and *NDUFAF2*	000082.4/ 174889.5	Gross Deletion	71Kb deletion at chr5:60170416-60241292	Hom.	Inherited	Pathogenic	Cockayne Syndrome (#216400) / Mitochondrial Complex I Deficiency (#618233) AR / AR

WES: whole exome sequencing, WGS: whole genome sequencing, ND: non-diagnostic, AR: autosomal recessive, AD: autosomal dominant, Het: heterozgous, Hom: homozgous, Comp.Het.: compound heterozygous, CDG: congenital disorders of glycosylation, CFC: cardio facio cutaneus, NA: not accessible.

In seven (44%) patients, a molecular diagnosis could not be reached in WES/WGS (2 WGS, 5 trio-WES) analysis. Among the negative trio-WES analyzes, 60% had been performed in fetal cases and there was no consanguinity history in the majority (71%).

Pathogenic/ Likely Pathogenic Variants

A total of nine different variants (1LP, 8P), in eight genes (*BBS9, BRAF, SLC12A1, PIEZO1, WDR62, ERCC8, NDUFAF2, RAG1*) were detected in seven (44%) patients. Four of these variants were novel (*BBS9*: c.841A>T, *SLC12A1*: c.2952_2955del, *PIEZO1*: c.3495C>G; c.5793C>A). Clinical pre-diagnoses (Omenn syndrome (P1), Cardio-facio-cutaneous syndrome (P10), and Bardet-Biedl syndrome (P15)) were found to be compatible with molecular diagnoses in three patients. Pre-diagnosis could not be determined in the other patients due to quite heterogeneous clinical findings. Most of the patients with P/ LP variants (71%) were in the postnatal period and 57% had a history of consanguinity. Detailed clinical and molecular findings of the patients are presented in Table 1 and Table 2.

VUS Variants

Three VUS variants were detected in two genes (*ETFB*, *MOGS*) in two patients. The first patient was a female deceased postpartum. She had been diagnosed prenatally with enlarged kidneys, polycystic kidney dysplasia, oligohydramnios, and enlarged cisterna magna. Although parents denied consanguinity, there was a history of having a similarly affected male sibling, which indicated a possible autosomal recessive inheritance pattern. In the light of all the findings, trio-WES was performed with the clinical prior clinical diagnoses of autosomal recessive multicystic dysplastic kidney disease and glutaric acidemia type 2. A homozygous intronic variant, *ETFB*: c.57+9_57+19del, was detected. *ETFB* was responsible for the glutaric acidemia type 2 and the variant was in proximity to the splicing donor site of exon 1 indicating a possible splice effect.[9] Since the consequence of this variant is not known, it was classified as VUS according to the recommendations of ACMG. However, considering the clinical findings and family history, we strongly considered that the variant might be responsible for the phenotypic findings.

The second patient was a female who died at the age of 18 months due to pneumonia. She had breech presentation, decreased fetal movements, hypotonia, and a weak cry at birth. For the first six months of life, she had been in the neonatal invasive care unit with ventilator support and had been discharged with tracheostomy. When she died at the age of 18 months, she had swallowing impairment necessitating oro-gastric tube feeding and was severely mental-motor retarded. Parents were not consanguineous and there was no history of a relative with a similar phenotype. Trio WES revealed two missense variants (c.560 G>C; c.1693 C>T) in *MOGS* and segregation analysis in parents confirmed compound heterozygosity. Pathogenic variants in *MOGS* were associated with congenital disorders of glycosylation type IIb. To date, only a few individuals had been described with severe generalized hypotonia, epilepsy, and dysmorphism.[10] According to ACMG, these variants were classified as VUS, however, considering the clinical overlap with the reported patients, these variants were considered as strong candidates in the index case.

Next generation sequencing technologies are rapidly becoming involved in daily diagnostic practice as a cost-effective and time-efficient strategy for identifying underlying genetic factors in patients with complex multi-systemic findings. The reported diagnostic rate for WES in the rare disease group, varies between 25% and 35% and reaches 49% in the case of consanguinity.[4, 5, 11, 12] However, the use of WES/WGS in postmortem cases is not yet widespread. In a limited number of studies on fetuses lost in the perinatal period due to structural anomalies, diagnosis rates are reported up to 50% with trio WES analysis and it is emphasized that the integration of

phenotype data into the analysis increases the diagnostic rate.[6, 7, 13-15] In our study, the diagnostic rate was determined as 56%, slightly higher than the reported rates in the literature. We also think that this high diagnosis rate could be achieved by the deep phenotyping we performed in our study and application of WES as trio in the majority of the cases. In addition, considering that most of the molecular diagnoses are associated with AR inherited diseases, the high rate of consanguinity (38%) in our study can be suggested as another reason for the higher diagnostic rate.

In AR inherited disorders, which constitute a large part of rare genetic diseases, there is often a history of more than one affected child in families. Thirty-eight percent of families included in this study also had a history of an affected sibling with similar findings. The recurrence of these diseases is increasing the psychosocial and financial burden of rare diseases for both families and society. Thus, elucidating the molecular pathology in the index case is very important in terms of determining the risk of recurrence for future pregnancies and the interventions for prevention. With this respect, molecular autopsy stands out as a precious tool in the postmortem period. In our study, for the case in which a de-novo variant was detected in BRAF (P10), informing the family that there was low risk in the next pregnancy, highly had relieved them. Additionally, in cases with inherited variants, sharing the recurrence risks and presenting the prenatal/preconception diagnosis options, contributed to the parent's decisions about family planning.

Neurometabolic diseases and congenital anomaly syndromes are reported as the most frequently diagnosed disease groups in cases solved by molecular autopsy.[6,13] Similarly, congenital metabolic disorders (33%) and neurological diseases (22%) were prominent among the cases diagnosed in our study. In neurometabolic disorders, the exact clinical diagnosis is usually challenging due to multisystemic, heterogeneous, and overlapping findings. WES/WGS mediated reverse phenotyping stands out as the most effective tool in the diagnosis of this group of diseases. Considering the low clinical diagnosis rates especially in this patient group, it is of great importance to archive tissue samples in the postmortem period and to consult families about molecular autopsy options.

We could not determine the underlying molecular pathology in 40% of the cases despite detailed phenotyping. In the literature, this rate is reported to be 40- 80 % in different patient populations. However, with the developments in bioinformatics programs and the detection of new disease-related variants and novel genes, negative diagnosis rates are expected to decrease gradually. In cases with negative WES/WGS results, it has been reported that re-analysis with updated data at regular intervals provides an increase up to 13% in molecular diagnosis.[7] Thus, it is of great importance to keep in mind the value of reanalysis in cases without diagnosis and to address this issue in genetic counseling to be given to the family.

CONCLUSION AND RECOMMENDATION

Our results clearly show that WES/WGS analyses in the postmortem period yield a high diagnostic rate in regions like Turkey where the rate of consanguineous marriage is high. The diagnosis to be elucidated by molecular autopsy not only provides unique information for the family for the planning and management of future pregnancies but also allows the identification of novel genes and previously unknown fetal lethal phenotypes related to rare diseases

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