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Mutational screening of (*CLN6*), (*CLN7*) and (*CLN14*) genes in Egyptian patients with Neuronal Ceroid Lipofuscinosis

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Abstract: Neuronal ceroid lipofuscinoses (NCL), the commonest autosomal recessive neurodegenerative disorder, is marked by an accumulation of auto-fluorescent storage material, primarily in neurons. NCL affects the neurons and causes damage due to accumulation of auto-fluorescent lysosomal storage material inside the neuronal cytoplasm. Although it is a rare fatal childhood disorder, childhood, patients born with it suffer life threatening complications and disabilities that deteriorates their quality of life. The current study aims to screen for mutations in CLN 6,7, and 14 genes in twenty Egyptian patients clinically diagnosed with seizures, Retinal degeneration, progressive mental deterioration and ataxia, using direct Sanger sequencing. Two reported mutations have been detected in CLN6 gene, homozygous missense (c.299T>G) and 3-bp deletion (c.711_713delCTT) in two different cases who are not siblings, and the other eighteen patients had no genetic defects in the enrolled genes. Further studies on larger number of participants are required in order to further clarify the causative CLN genes, particularlyCLN6, CLN7 and CLN14 among Egyptian patients.

Keywords Mutation, Neuronal ceroid lipofuscinoses (NCL),autosomal recessive, neurodegenerative, disorders, Sanger sequencing.

1. INTRODUCTION

Neuronal ceroid lipofuscinoses is neurodegenerative, lysosomal storage disorder, affecting from newborn to adults, with NCL prevalence varies from 1.3 to 7 per 100 000 live births [1]. They are distinguished by the cumulation of lysosomal storage substance in brain cells, which causes neurodegeneration, seizures, vision loss, and early death [2]. The great majority of NCL types have variable symptom initiation within the same genetic form. Among patients, there are differences in the onset age and course of the disease which leads to difficulty in categorizing NCL subtypes [3]. NCL-affected patients have been classified according to the clinical onset of symptoms, into six categories (Classic Adult-Onset Neuronal Ceroid Lipofuscinosis, Finnish variant Neuronal Ceroid Lipofuscinosis, variant Late Infantile Neuronal Ceroid Lipofuscinosis, Turkish variant Neuronal Ceroid Lipofuscinosis, and Congenital Neuronal Ceroid Lipofuscinosis), however, the overlap of clinical and genetic traits prevents certain patients from being easily categorized into a particular group [4]. However, ultrastructural pattern classified NCL types into granular osmiophilic deposits, curvilinear profiles, rectilinear profiles and fingerprint profiles [5]. The function conflicts are split into two categories; genes encoding lysosomal enzymes and genes encoding membrane proteins. This process involves a number of genes, including genes encoding lysosomal enzymes (CLN1/PPT1, CLN2/TPP1, CLN10/ CTSD, CLN13/CTSF), genes encoding a soluble lysosomal protein (CLN5) and genes encoding a protein in the secretory pathway (CLN11/GRN).

In addition, genes encoding cytoplasmic proteins (CLN4/DNAJC5, CLN14/ KCTD7) and transmembrane proteins (CLN3, CLN6,

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CLN7/MFSD8, CLN8, CLN12/ATP13A2) are also associated with NCLs [6].Therefor, 13 genes have been identified with over 430 pathogenic variations (*CLN1* to *CLN14*) NCL is frequently identified late because of a low level of awareness and non-specific clinical features. The age at onset and kind of clinical presentation ought to be taken into account while determining the first diagnosis of NCL disease. This would be followed by enzyme testing for CTSD (CLN10), PPT1 and TTP1. Genetic testing, such as Sanger sequencing and whole exome sequencing for CLN5, CLN6, CLN7/MFSD8, CLN8, and CLN14/KCTD7, should be recommended when enzyme testing for PPT1 and TTP1 yielded negative results and electron microscopy revealed typical storage material. [7]. In 2017, Food and Drug administration gave an approval for Cerliponase alfa, a recombinant human tripeptidyl peptidase (TPP1) as a treatment of NCL2 [8]. In this study, we molecularly screened pathogenic mutations in *CLN6,7,14* genes using direct Sanger sequencing.

2. METHODS

2.1. Patients:

Twenty patients were enrolled and referred from the Neurogenetic clinic at the National Research Centre (NRC), Cairo, Egypt's Medical Research of Excellence Center. All of them were born from two consanguineous couplings.

The informed consent has been signed by patients' parents The NRC and El Al-Azhar University's medical research ethics committee accepted this consent. This study was approved by Al-Azhar Faculty of Pharmacy (Girls) scientific research ethical committee (no. 180, 2018).All twenty patients had symptoms and indications that were compatible with NCL including seizures, ataxia, delayed language development, visual loss, motor and cognitive regression. Their ages ranged from 18 month to 8 years. The patients had to meet a number of criteria to be enrolled in this study, including their age at disease beginning, symptoms, disease progression, and, in some cases, ultrastructural pathology. EEG and MRI brain examination are done to all patients.

2.2. Mutation diagnosis of *CLN* genes:

Targeted gene sequencing was used for genetic testing. Genomic DNA was isolated from peripheral blood using the Thermo Scientific Gene JET Genomic DNA Purification Kit (Thermo Scientific, US) and as per the instructions provided by the manufacturer. of the 20 patients. Using Nano Drop, the purity and quantity of the DNA were determined and stored in aliquots. at Minus 20°C. The Qiagen Taq PCR Core kit was used to carry out polymerase chain reactions for the coding area (exons) and locations representing flanking splice of CLN genes (CLN6, 7 and 14). These primers were generated using the genomic sequence (GenBank accession numbers) and kit instructions. The primer sequences can be requested. The following PCR cycling protocol was used in Biorad Cyler (USA): 95 °C for ten minutes, followed by 35 cycles of 95 °C for one minute, 57 °C for one minute, and 72 °C for one minute, with the last cycle being extended to 72 °C for ten minutes, before being stored at 4 °C. After being treated with exonuclease/shrimp alkaline phosphatase, purified PCR amplicons were stored in (Perkin Elmer) for 1 cycle at 37°C for sixty minutes and 80°C for fifteen minutes (Sigma, USA). It was possible to find mutations in each gene's coding region by comparing the sequence charts with the predicted sequence using the large dye terminator kit from Applied Biosystem in Foster City, California, public datasets (dpSNAP and 1000 genome project), and SIFT (Sorting Intolerant From Tolerant) analytical software. Finch TV 1.4.0, public datasets (dpSNAP and 1000 genome project), and SIFT (Sorting Intolerant From Tolerant) analytic software.

3. RESULTS

3.1. Clinical investigations

The clinical criteria finding were detected in the pre-diagnosed patients; psychomotor and mental regression, lost/or abnormal speech, dysarthria, seizures, cognitive regression, ataxia, vision loss, and ataxia. All patients had anomalies on their MRIs. EEG and brain **(Table1).**

3.2. Molecular analysis

PCR amplification was carried for the coding region and sites representing flanking splice of the three genes*; CLN6*, *CLN7* and *CLN14* in all patients (P1 to P20). A representative sample of amplified products from each of the three genes for one patient is shown on a 2% agarose gel (Figure 1).

Sanger sequencing of the *CLN 6*, 7 and *14* genes for all 20 patients showed two distinct earlier reported pathogenic mutations within *CLN6* gene in two unrelated patients. In the other 18 cases, there were no pathogenic mutations in the enrolled genes. Amongst the two patients, one was a female, and had a homozygous missense mutation in exon 4 (c.299T>G), which resulted in a leucine to arginine residue substitution. The second male patient carried 3-bp deletion (c.711_713delCTT) in exon 7 that caused phenylalanine deletion (Tabe1, Figure 2,3).

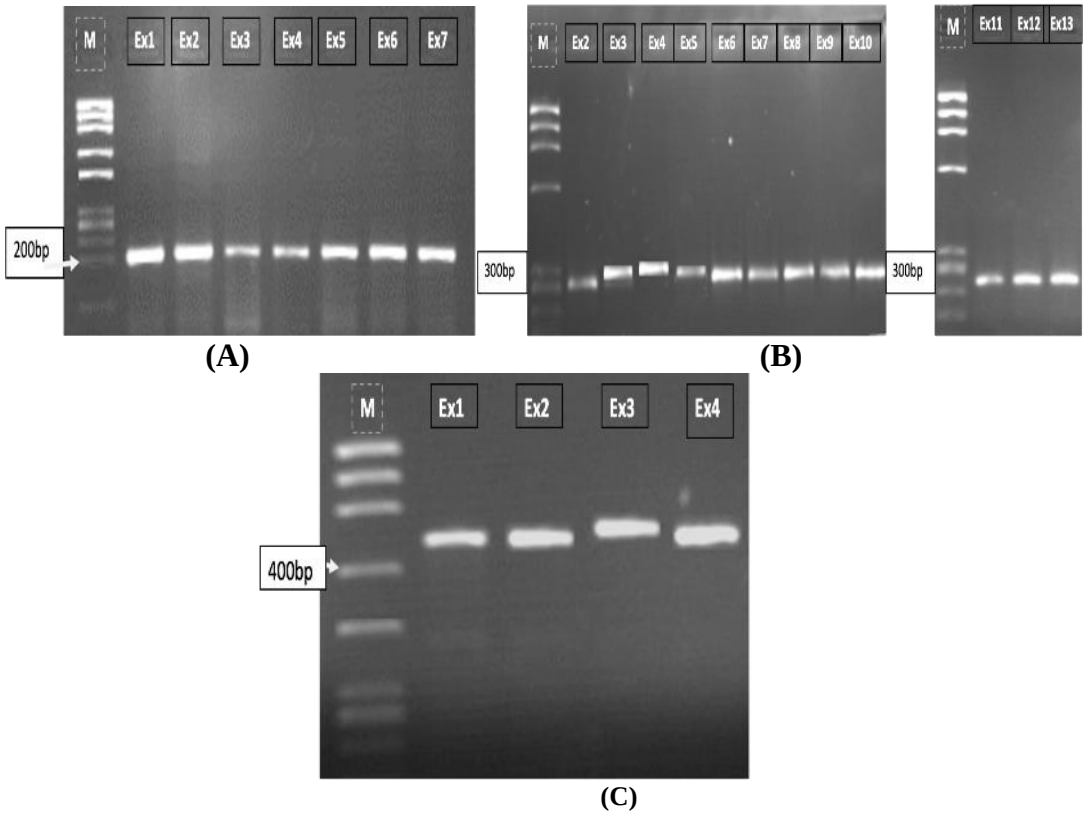


Figure 1: 2% Agarose gel electrophoresis illustrates PCR products of *CLN 6, 7* and 14. **A:** 13 Fragments of *CLN7* gene. **B**: 4 Fragments of *CLN14* gene. **C:** 7 Fragments of *CLN6* gene. **M:** DNA marker.

Table 1. Clinical and Molecular findings detected in the two unrelated patients

Patient	Gender	Age	Clinical Criteria	EEG	MRI	DNA changes	A.A	Mutation type
1	Female	4.5 years	Clinical features include loss of previously aqiured mile stone, motor delay, tendon reflexes, epilepsy, loss of speech, vision loss, and seizures.	Generalized Epileptogenic dysfunction	Cerebraland cerebellar atrophic changes	c.299T>G	L100R	Missense in Exon 4 of *CLN6* gene
2	Male	6.5	Clinical manifestations include motor delay, epilepsy, delayed speech, dysarthria, ataxia, vision loss, and seizures.	Generalized Epileptogenic dysfunction	Cerebral and cerebellar atrophic changes	c.712_713 delCTT	F238	3-bp deletion in Exon7 of *CLN6* gene
3-20	Males & Female		Psychomotor and mental regression, lost/or abnormal speech, dysarthria, seizures, cognitive regression, ataxia, vision loss, and ataxia.	EEG showed abnormalities in all patients	Brain MRI Cerebral and cerebellar atrophic	No pathogenic mutations in the enrolled genes in patients 3 to 20		

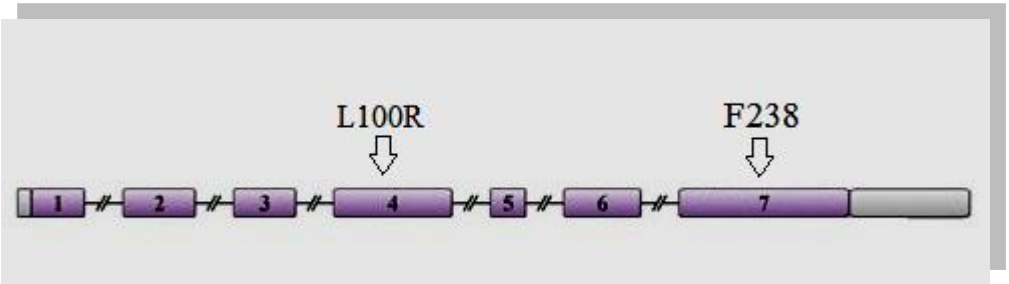
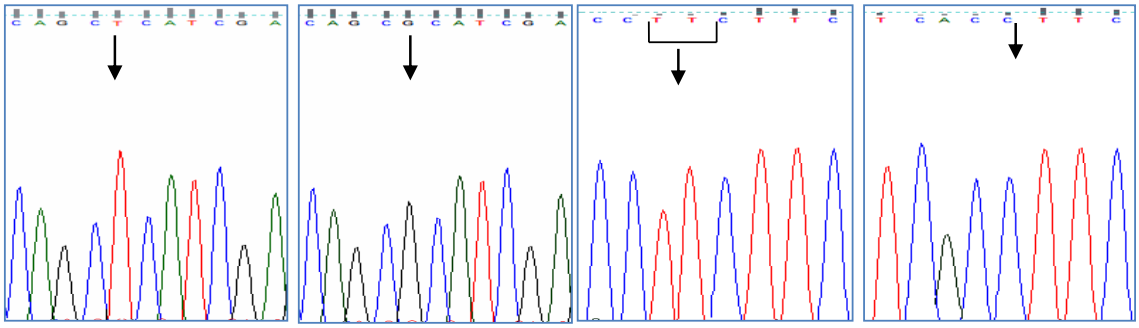


Figure 2: Schematic representation of the *CLN6* gene showing the Missense mutation (L100R) identified in exon4 of patient1. (B) 3-bp deletion mutation (F238) detected in exon7 of patient2.



(A) **(B)**

Figure 3: Electropherograms showing comparison charts of normal and pathogenic mutations described in *CLN6* gene mutations. (A) Missense mutation (L100R) identified in exon4 of patient1. (B) 3-bp deletion mutation (F238) detected in exon7 of patient2.

4. DISCUSSION

The Neuronal Ceroid Lipofuscinoses is heterogeneous group of lysosomal storage disorders (LSD) mostly influencing children. Epidemiological data indicates that the incidence of NCL disease is 1–3/100.000 world-wide [9].The current study enrolled twenty patients, pre-diagnosed with symptoms consistent with those of NCL including, mental regression, seizures, ataxia, delayed language development, visual loss, motor decline, spasticity and cognitive regression [10]. Ages of patients ranged from 18 month to 8 years. Correlation of the clinical features and the age at onset of patients' help to identify the specific type of NCL, which could be confirmed molecularly. According to clinical and pathological criteria, NCL forms were divided to two major categories: age of onset and the ultrastructural features [11]. EEG and brain MRI of all patients demonstrated various abnormalities. The role of neuropathology in defining this category of disorders is described by the selective involvement of the cerebral and cerebellar0 cortices and the topography of abnormalities in the brain. ultrastructural features included, fingerprint profiles (FPP) and rectilinear profiles (RLP) [12]. Clinical perspective of NCL enabled a new gene-based classification, giving a potent diagnostic tool of NCL-types and studying both phenotypic variability and heterogeneity within most NCL forms. Most mutations in common genes are typically linked to the conventional phenotypes; however, even within one family, there can be phenotypic variation. The 20 patients had common disease patterns ranged from motor regression or seizures to cognition regression and visual loss, those features are common to Infantile and Late-infantile (LINCL) forms and intern *CLN6* and *CLN7* genes were prior to be suspected as causative genes of mutations in different genes, since both genes are the most common forms of late-infantile (LINCL) form of the NCL disease [13]. Progressive myoclonic epilepsy was investigated in few patients that suggested screening for mutations in KCTD7 [14]. The CLN6 gene has two separates previously known pathogenic mutations in two unrelated cases, according to Sanger sequencing of the coding area and splice sites for all 20 patients. The remained eighteen patients showed no pathogenic mutations in the enrolled genes. The female patient possessed a missense mutation that is homozygous in exon 4 (c.299T>G), which caused leucine to arginine residue substitution, meanwhile, the other male patient showed 3-bp deletion (c.711_713delCTT) in exon 7 that caused phenylalanine deletion.*CLN6* gene is found on15q23 and encodes a 311 amino acid transmembrane endoplasmic reticulum (ER) protein that functions in the ER to Golgi transfer of lysosomal enzymes. Mutations in *CLN6*gene result in probably damage protein residues of the transmembrane domain and this might significantly disrupt the CLN6 protein's structure and lead to ER retention loss.[15]. Algorithms of homozygous

missense mutation (c.299T>G) according to (PolyPhen-2 software) showed the probably damage of protein structure and though affect its function. Meanwhile, 3-bp deletion mutation (c.711_713delCTT) was investigated by Arsov *et.al.*, in one of three families showed CLN6 mutations. It was a reported to be associated with variant late-infantile NCL and suggested to be possibly degrading ER protein rapidly. Patient with this mutation suffered from epilepsy, one tonic-colonic seizure, ataxia, and cognitive [16].Despite the several indicated genes for CLN6, MFSD8, and KCTD7 sickness, patients in this study had comparable clinical outcomes. This could be attributed to the variability of NCL forms. On 15q23, CLN7/MFSD8 is situated. The protein CLN7 is a member of the MFS major facilitator superfamily of secondary active transporters. The cerebral cortex and cerebellum exhibit the highest amounts of MFSD8 expression, which is present everywhere. The characteristic of CLN7 disease is the buildup of proteins and other substances in nerve cells, which results in cell damage and ultimately cell death. Although sporadic cases of retinopathy have also been documented, individuals who have biallelic mutations in MFSD8 frequently have typical NCL symptoms like seizures, visual loss, mental regression, and ataxia [17]. CLN14/KCTD7 Potassium Channel Tetramerization Domain-containing Protein 7 is ultrarare childhood form of NCL, this is a result of disease databases being used to disseminate knowledge. Patients with KCTD7 mutations exhibit movement disorders and progressive myoclonic epilepsy, but how KCTD7 controls neural development and function remains unclear. Future research will be very interested in figuring out the substrate(s) of the projected KCTD7- and Cullin-3-containing ubiquitin-ligase complex and their possible significance in NCL pathogenesis[18]. A number of therapeutic modalities are being researched as prospective treatments for these fatal illnesses; one has already achieved clinical approval as a therapeutic drug for neuronal ceroid lipofuscinosis, or CLN2 sickness (Cerliponase alfa, a lysosomal enzyme infused into the brain ventricles of patients with CLN2 disease). Other varieties of NCL can be treated using a range of small compounds, stem cell therapy, gene therapy, immunosuppressive drugs to treat neuroinflammation brought on by neurodegeneration, and other methods [19]. Our research increased understanding of varied late infantile NCL in the Arab ethnic group and provided information that could be used in future genotype-phenotype correlation studies. The significance of early discovery of this phenotype, together with swift and accurate confirmation by molecular diagnostics, was underlined; this would allow for a reevaluation of NCL epidemiology and show how widespread NCL disorders are globally. To increase understanding of these diseases, the effectiveness of suitable genetic counselling, and through future family planning, particularly in areas in-depth epidemiological studies are required as a result of the rise in consanguinity marriage.

5. CONCLUSIONS

The screening of mutation in CLN 6,7,14 genes in twenty patients using sangar sequencing technique showed two early reported mutations have been detected in *CLN6* gene, homozygous missense (c.299T>G) and 3-bp deletion (c.711_713delCTT) in two different cases who are not siblings, and the other eighteen patients had no genetic defects in the enrolled genes.

Limitations: In our study, the comparison with healthy children was only in the meaning of clinical and pathological symptoms. We could not do a full DNA screening to the healthy patients as well to detect any variability or gene mutations. Also, there was information shortage in the data regarding the demographic characteristics for all studied children which limited our study to extrapolate or correlate them with the detected gene mutations**.** In our study, the comparison with healthy children was only in the meaning of clinical and pathological symptoms. We could not do a full DNA screening to the healthy patients as well to detect any variability or gene mutations. Also, there was information shortage in the data regarding the demographic characteristics for all studied children which limited our study to extrapolate or correlate them with the detected gene mutations**.** We were not able to do a full DNA screening to patients as well to detect any variability or other gene mutations. Also, there was information shortage in the data regarding the demographic characteristics for all studied children which limited our study to extrapolate or correlate them with the detected gene mutations. Further studies are mandatory to link demographic data of the Egyptian patients with the mutations detected in NCL subtypes.

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Conflicts of Interest: The authors declare no conflicts of interest.

Ethical Statement: this study was approved by the ethical committee of faculty of pharmacy(Girls), AL-Azhar University (code:180; session19; date 5/3/2018).

Author Contribution: M.S. contributed to performing experiments, data collection and

interpretation, manuscript writing. M.Z. contributed to conception and experimental design. A.A contributed to conceptualization of the study, supervising the experiments. supervised plan of the work and reviewed the manuscript. A.S contributed to revising the molecular work and reviewed the manuscript. M.I contributed to obtaining blood samples, and revising the manuscript. M.R. contributed to experimental design, performing experiments and interpreting results, and revising the manuscript.

List of Abbreviations: NCL: Neuronal ceroid lipofuscinosis; vLINCL:Variant late infantile neuronal ceroid lipofuscinosis; LINCL: late infantile neuronal ceroid lipofuscinosis; NRC: National Research Center; MRI: Magnetic resonance imaging; EEG: Electroencephalygraphy; PCR: Polymerase Chain Reaction; LSD: Lysosomal storage disease; FPP: Fingerprint profile; RLP: Rectilinear profile; GRN): Granulin; PPT1: Palmitoyl-protein thioesterase-1; TPP1: Tripeptidyl peptidase 1

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