Epidemiology and Prevalence of Patients with Congenital Disorders of Glycosylation in Malaysia

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Abstract
Congenital disorders of glycosylation (CDG) are rarely, inherited metabolic diseases in which the glycosylation process is severely impaired. The prevalence has not been well determined and the aims of this study were to describe the epidemiology and calculate the birth prevalence of CDG in the Malaysian population. From 2018 to 2022, 548 patients were suspected with CDG. Sample were collected and analysed using isoelectric focusing (IEF) and capillary zone electrophoresis (CZE) methods. Qualitative measurement of sialo pattern was determined to categorised CDG Type I, II and abnormal transferrin pattern. Transferrin level was quantitated using automated BN ProSpec® System as well as demographic and clinical features were documented. The distribution of CDG was 0.4% and 2.9% was diagnosed as abnormal transferrin types. The prevalence of CDG among Malaysian was 0.22 per 100,000 live births and the combined prevalence of abnormal pattern was 0.85 per 100,000 live births. Positive cases were found in infants of Malay ethnicity. Overall, the prevalence of CDG in Malaysia was low and may be underestimated yet consistent with other reported in other countries.

Keywords
Capillary Zone Electrophoresis, Congenital Disorder of Glycosylation, Genetic Disease, Isoelectric focusing, Rare Disease.

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Introduction

Congenital disorders of glycosylation (CDG) are rare, inherited metabolic diseases in which the glycosylation process is severely impaired. Glycosylation process is the multiple steps of adding sugar residues to proteins and lipids of cellular pathways [1]. These protein and lipid glycosylation process are involving many genes, and the defect along the steps can caused more than 130 diseases [2].

Patients with CDG manifest multi-systemic clinical indications where most commonly are developmental delay, failure to thrive, hypotonia, neurologic abnormalities, hepatopathy, and coagulopathy. Some patients present with eye, skin, and cardiac disease, as well as facial dysmorphisms [3]. Moreover, neurologic changes and cognitive delays are seen in the majority of affected individuals, but there are also certain cases and types that do not have neurologic manifestations [4]. Given the broad clinical and genetic etiology of CDG, clinical diagnosis relies on high index of suspicion in multi-systemic disease [1].

First-line screening test in patients with suspected CDG should be by isoelectric focusing (IEF) of serum transferrin [5]. Transferrin is one of the most abundant glycoproteins in human plasma and has been widely used as a rapid biomarker for detection of N-glycosylation defects [6]. Other methods, such as agarose electrophoresis, capillary electrophoresis (CE), micro-column separation combined with turbidimetry, enzyme-(EIA) and radioimmunoassay (RIA) have also been used for screening [7]. More recently, high-pressure liquid chromatography (HPLC) and capillary zone electrophoresis (CZE) have been introduced as well as mass spectrometry (MS) [8,9].

The prevalence of CDG in the general population has not been well established, although patients have been reported worldwide from almost every ethnic background and both sexes. Available data based on reports for PMM2-CDG and the estimated prevalence was 1:20,000 in Dutch populations and 1/77,000 in Estonia. The prevalence of CDG in the Polish population was estimated at approximately 1 per million while that of PMM2 was at 0.4 per million. The annual incidence of CDG was estimated at 0.013 per 100,000 people in 2020 [10]. Moreover, the combined carrier frequency of CDG in the Saudi population is 11.5 per 10,000, which translates to a minimum disease burden of 14 patients per 1,000,000 [11].

In Malaysia, the only reported CDG case was published on 2009 by Thong et al. The case was identified in a Malaysian infant female at 2 days of life. The diagnosis was suspected on the basis of abnormalities of clinical manifestations and was supported by abnormal serum transferrin isoform pattern that showed elevated levels of the disialotransferrin isoform and trace levels of the asialotransferrin isoform. Following that, enzyme testing showed decreased level of phosphomannomutase (PMM) activity indicating a diagnosis of CDG type Ia [12].

The current prevalence of CDG in Malaysia is still unknown and over the years, there are increasing requests for analysis of CDG from both public and private hospitals. The aim of this study were to describe the epidemiology and to calculate the birth prevalence of CDG in the Malaysian population.

Materials and Methods

Study design

This is a laboratory records-based analysis of all patients diagnosed with CDG at Institute for Medical Research (IMR) between 2018 to 2022.

Ethical statement

Ethical approval was obtained from the National Medical Research Centre (NMRR) [NMRR/21064]. The procedures followed were in accordance with the ethical principles of Helsinki Declaration 1975, as revised in 1983.
Study population
Malaysia is located at South East Asia and divided into two parts, namely Malaysian Borneo and Peninsular Malaysia. Region of Sabah and Sarawak as well as one federal territory (Labuan) are located at Malaysian Borneo while other 11 states and two federal territories (Kuala Lumpur and Putrajaya) are situated at peninsular Malaysia. In 2020, the population of Malaysia was estimated to be 32 700 286 million people [13]. Malaysian is ethnically diverse, with majority of Malay, Chinese, Indian, Orang Asli (aborigines’ people) and the natives from Sarawak and Sabah. The minorities include Sikh, Punjabi, Portuguese and others.

Variable and measurement
Variable measurand in this study includes gender, ethnicity, age at diagnosis, CDG type, polymorphism and clinical manifestation.

Data collection
Laboratory findings including results interpretation were retrieved from the laboratory records and reviewed. Patients were included in the study only if the diagnosis of CDG was confirmed with both screening or/and confirmatory testing.

Laboratory process and tests
This study is conducted at Unit Protein Khas (UPK) of IMR, Kuala Lumpur. All laboratory diagnoses of screening and confirmatory were done in IMR which is the reference centre providing tests for CDG. We received samples from all healthcare facilities including private hospitals throughout Malaysia. There were two screening tests for LSD: i) isoelectric focusing electrophoresis (IEF), ii) capillary zone electrophoresis (CZE). The confirmatory diagnosis of CDG was by neuraminidase treatment by IEF method. Total of 548 patient results were studied. Three milliliter of the serum sample were collected and subjected to IEF or CZE.

Control
'Normal CDT Control' and 'High CDT Control' (SEBIA, France) were obtained from a pool of normal human sera in a lyophilized form, with High CDT Control has an increased CDT fraction and Normal CDT Control has normal range CDT fraction. Briefly, both CDT Control vial were reconstituted with one milliliter of Calibrators Diluent (SEBIA, France). The mixture was allowed to stand for 30 minutes and were mixed gently. Both controls were used and treated as human serum.

Isoelectric focousing (IEF)
Semi-automated IEF was performed using PhastSystem™ and PhastGel™ separation media (GE Healthcare, Sweden) according to the manufacturer’s instructions. Briefly, the gel was hydrated using distilled water and SERVALYT (GE Healthcare, Sweden) prior sample preparations. Ten microliters of serum sample was mixed with 200 microliters ferric citrate and 100 microliters sodium bicarbonate(NaHCO₃) followed by IEF on Phast system at 15°C. Then, 60 microliters of antibody of polyclonal rabbit anti human transferrin (DAKO, USA) was spread evenly by rolling a roller spreader/ test tube onto gel. The gel was put in a covered glass container and was incubated for 30 minutes at room temperature. 0.9% saline was poured into the glass container until the gel is fully covered and was soaked for 10 minutes at room temperature. For gel processing steps, the saline was discarded. The gel was treated with 20% TCA solution (200 g TCA + 1000 mL dH₂O), staining solution (1 tablet Phast Gel Blue R + 80 mL dH₂O + 120 mL ethanol 96%) and destaining solution (300 mL Ethanol 96% + 100 mL Acetic Acid + 600 mL dH₂O).

If banding pattern shows Type II, neuraminidase treatment using mixture of 6.5 µl neuraminidase solutions, 200 µl ferric citrate stock, 100 µl NaHCO₃ stock and 3 µl of sample patient was added. The mixture was then incubated overnight at 37°C. Prior to the procedure, 100 µl dH₂O was added to the mixture. Interpretation was based on the different phenotype of transferrin according to the positions of the separated isoforms fraction.
Capillary zone electrophoresis (CZE)

CZE was performed using commercially available system (Capillarys 2™) together with Capillarys 2™ CDT assay kit which include Buffer, Sample Diluent, Wash Solution, Capillarys/Minicap CDT Wash Solution and Reagents(SEBIA, France). The preparations were according to the manufacturer’s instructions. Briefly, 200 µL of patient serum was mixed with 50 µL of Capillarys 2™ CDT samples treatment solution in the microtube. The mixture was briefly vortexed and centrifuged at 600 g for 10 minutes. The supernatant was collected and put in new microtube for the analysis on the Capillarys 2™system. The procedures were continued with automatic electrophoretic migration and direct detection of protein. The protein migration was under constant voltage for about eight minutes and the temperature was controlled by Peltier effect. Seven silica capillaries were operated in parallel (effective length 17.5 cm × 25 µm I.D.) and detection of the sialo transferrin occurs with UV detection at 200 nm wavelength. Data analysis was performed using the software package Phoresis 8.6.3 (SEBIA, France).

Measurement level of transferrin

Fully automated nephelometry analysis was performed according to the manufacturer's instructions using automated BN ProSpec® System, Internal Quality Control and N Protein Standard(SIEMENS, Germany). Briefly, three milliliters of patients’ serum sample were put in sample segment and were automatically diluted 1:20 with N Diluent (SIEMENS, Germany) before measurement. The serum transferrin level (µg/L) was automatically calculated based on the intensity of the scattered light passed through the sample.

Prevalence estimation

Disease birth prevalence was expressed as number of patients per 100,000 live births and calculated using the method reported by Pinto, 2003[14]. Prevalence was calculated by dividing the total number of diagnosed cases by the total number of live births that occurred between the years of birth of the older and the younger patients (birth period). Data on the number of live births per year in Malaysia were collected from Department of Statistics Malaysia 2020 (https://www.dosm.gov.my).[13].

Results

Distribution of CDG types

We diagnosed two (0.4%) positive cases consisted of Type I and Type II. Thirteen samples (2.9%) were diagnosed as for abnormal transferrin types consisted of complement, variant, polymorphism and interference. Figure 1 shows representative of CDG Type I, Type II and polymorphism pattern of patient samples using IEF. Meanwhile, Figure 2 shows representative of CDG Type I, Type II, complement, variant and interference sialo pattern of patient samples using CZE method. The prevalence of CDG in Malaysia is shown in Table 1. The prevalence of CDG among Malaysian was 0.22 per 100,000 live births. The prevalence of abnormal transferrin type was 0.85 per 100,000 live births.

Demographic and clinical data

Demographics of Malaysian patients is presented in Table 2. Out of 548 patients who were clinically suspected of having CDG, two (0.4%) were diagnosed with two different CDG subtypes. One case of Type I CDG was found in female age 4 years old and one case of Type II CDG was found in 4 months old male patients. Both were infants and none was found in adults patients of 18 years at diagnosis. Based on the ethnicity, both positive cases were Malay. Transferrin level in positive cases showed within normal range. It is well known that CDG were presented with wide range of clinical heterogeneity [1,4]. Although the presumptive diagnosis was varied, both of the patients were diagnosed with muscle weakness and respiratory symptoms.

Thirteen (2.4%) showed abnormal transferrin types i.e. polymorphism, interference, variant or complement which were found in patient age of 4 months to 80 years old. Patients of these diagnosis showed similar clinical heterogeneity as CDG patients. Two patients showed low transferrin level and one with high level. Based on the ethnicity, mostly were Malay and one was Chinese.
Table 1: Birth prevalence of CDG in Malaysia

<table>
<thead>
<tr>
<th>CDG type</th>
<th>No of patient</th>
<th>Years of birth</th>
<th>No of live birth</th>
<th>Birth prevalence (per 100,000)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TYPE I</td>
<td>1</td>
<td>2018</td>
<td>501945</td>
<td>0.02</td>
</tr>
<tr>
<td>TYPE II</td>
<td>1</td>
<td>2019</td>
<td>487957</td>
<td>0.2</td>
</tr>
<tr>
<td>Total CDG</td>
<td>2</td>
<td></td>
<td>989902</td>
<td>0.22</td>
</tr>
<tr>
<td>Complement</td>
<td>4</td>
<td>2018-2020</td>
<td>1460097</td>
<td>0.34</td>
</tr>
<tr>
<td>Variant</td>
<td>4</td>
<td>2019-2021</td>
<td>1411884</td>
<td>0.28</td>
</tr>
<tr>
<td>Interference</td>
<td>4</td>
<td>2018-2021</td>
<td>1899841</td>
<td>0.21</td>
</tr>
<tr>
<td>Polymorphism</td>
<td>1</td>
<td>2018</td>
<td>501945</td>
<td>0.02</td>
</tr>
<tr>
<td>Total abnormal transferrin</td>
<td>13</td>
<td></td>
<td>5273767</td>
<td>0.85</td>
</tr>
</tbody>
</table>

**Figure 1:** Transferrin isoelectric focusing pattern. (A) 1; Normal, 2; Type II CDG before confirmatory test by neuraminidase enzyme treatment, 3; Type I CDG. (B) Lane 1,3,5; Type-II CDG before neuraminidase treatment. Lane 2,6; Polymorphism type after neuraminidase treatment with presence of two bands at 0-sialo, 4; Type II CDG after neuraminidase treatment with presence of one band at 0-sialo.
### Table 2: Demographic and clinical features of patients with CDG in Malaysia

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age at diagnosis</th>
<th>Gender</th>
<th>Ethnicity</th>
<th>Transferrin level (g/l)</th>
<th>Clinical features</th>
<th>CGD type</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4Y</td>
<td>Female</td>
<td>Malay</td>
<td>2.33</td>
<td>-Muscle weakness, -Respiratory symptoms</td>
<td>TYPE I</td>
</tr>
<tr>
<td>2</td>
<td>4M</td>
<td>Male</td>
<td>Malay</td>
<td>2.56</td>
<td>-Developmental delay, -Hepato/Splenomegaly, -Muscle weakness, -Respiratory symptoms</td>
<td>TYPE II</td>
</tr>
<tr>
<td>3</td>
<td>3Y</td>
<td>Male</td>
<td>Chinese</td>
<td>2.25</td>
<td>-Global developmental delay -Seizures -Progressive unsteady gait -Hyperkinetic movement</td>
<td>Variant</td>
</tr>
<tr>
<td>4</td>
<td>80Y</td>
<td>Male</td>
<td>Malay</td>
<td>1.04</td>
<td>-Recurrent episode of encephalopathy with memory loss</td>
<td>Polymorphism</td>
</tr>
<tr>
<td>5</td>
<td>4M</td>
<td>Female</td>
<td>Malay</td>
<td>2.66</td>
<td>-Muscle weakness -Hepatomegaly -Splenomegaly</td>
<td>Interference</td>
</tr>
<tr>
<td>6</td>
<td>7M</td>
<td>Female</td>
<td>Malay</td>
<td>2.61</td>
<td>-Epilepsy -Global developmental delay -Having younger sibling with similar problem</td>
<td>Variant</td>
</tr>
<tr>
<td>7</td>
<td>4Y</td>
<td>Female</td>
<td>Malay</td>
<td>2.35</td>
<td>-Bruising -Global developmental delay -Seizures -Progressive unsteady gait -Hyperkinetic movement -Microcephaly with progressive cerebellar atrophy by medical imaging</td>
<td>Complement</td>
</tr>
<tr>
<td>8</td>
<td>1Y</td>
<td>Male</td>
<td>Malay</td>
<td>2.8</td>
<td>-Unexplained global dermatology</td>
<td>Interference</td>
</tr>
<tr>
<td>9</td>
<td>2Y</td>
<td>Male</td>
<td>Malay</td>
<td>3.08</td>
<td>-Developmental delay -Intellectual delay -Having brother with similar problem</td>
<td>Complement</td>
</tr>
<tr>
<td>10</td>
<td>2Y</td>
<td>Male</td>
<td>Malay</td>
<td>1.41</td>
<td>-Uncontrolled seizures</td>
<td>Variant</td>
</tr>
<tr>
<td>11</td>
<td>1Y</td>
<td>Male</td>
<td>Malay</td>
<td>3.9</td>
<td>-Muscle weakness -Hepatomegaly -Splenomegaly</td>
<td>Interference</td>
</tr>
<tr>
<td>12</td>
<td>3Y</td>
<td>Female</td>
<td>Malay</td>
<td>2.76</td>
<td>-Developmental delay -Intellectual delay</td>
<td>Complement</td>
</tr>
<tr>
<td>13</td>
<td>2Y</td>
<td>Male</td>
<td>Malay</td>
<td>2.26</td>
<td>-Hepatomegaly -Splenomegaly -Multisystem malformation</td>
<td>Variant</td>
</tr>
<tr>
<td>14</td>
<td>4Y</td>
<td>Male</td>
<td>Malay</td>
<td>2.79</td>
<td>-Swallowing problem -Frequent falling but able to stand up</td>
<td>Complement</td>
</tr>
<tr>
<td>15</td>
<td>1Y</td>
<td>Female</td>
<td>Malay</td>
<td>3.08</td>
<td>-Developmental delay -Intellectual delay</td>
<td>Interference</td>
</tr>
</tbody>
</table>
Discussion

Our analysis suggested that the prevalence of CDG in Malaysia is low yet parallel with the other countries\cite{10,11,15,16}. Ten years study (2008-2017) from Southern Brazil reported only four cases of CDG Type I namely PMM2-CDG, MPDU1-CDG and SLC35A2-CDG \cite{15}. Moreover, the prevalence of CDG in the Polish population was estimated at approximately 1 per million while the annual incidence was estimated at 0.013 per 100,000 people in 2020. Similar with European population, the estimated prevalence of combined N-linked protein glycosylation defects was 1/22,000 and 1/24,000 populations\cite{10}. In East-Asians, the total CDG prevalence was 1 in 121,935\cite{11,16}.

The low prevalence of CDG may be due to short period of data retrieval (2018–2022). The study of prevalence of CDG in other countries took more than 6 and up to 10 years data collection\cite{10,11,15,16}. Clinical suspicion of CDG in patients among health worker especially general clinician and pediatrician in Malaysia was still low and should be addressed by the respective authorities. Sufficient laboratory facilities and increase in awareness on CDG can help in detecting this underdiagnosed patients. Geographical factor also plays some role as some patients from very remote areas may have difficulties to access the medical facilities. This may had underestimated the prevalence of CDG in this region.

In Malaysia, we cannot determine the most prevalent CDG type since only one case for each CDG types. There were phenomenon of homozygosity or compound heterozygosity in some variants as in the case of p.Arg141His homozygosity in PMM2, FUK and MAN2B2 variants in which can only be identified by whole-exome sequencing. One particular test cannot be used to detect most CDG group since CDG group involves at least 137 defects\cite{11,17}. In this study, IEF and CZE techniques were used to demonstrate hypoglycosylation of transferrin. However, these methods do not recognize all CDG defects, so other approaches including analysis of membrane-linked markers and urine oligosaccharides should be taken as well as confirmation of diagnosis and detailed of CDG subtyping were required \cite{18,19}. To improve diagnostics of CDG, it is suggested to use mass spectrometric methods for glycoprotein analysis because of their higher sensitivity and specificity that can cover most of CDG variants \cite{20}.

By looking at demographic data, the positive cases were infants of 4 months and 4 years old of age. Equally, one was female and male from Malay ethnicity. Given wide range of clinical heterogeneity of CDG, both patients develop muscle weakness and respiratory symptoms.

In this study, we also observed abnormal transferrin pattern consisted of complement, interference, polymorphism and variants. The prevalence of this abnormal pattern was 0.83 per 100,000 live births. There were factors contributed to these issues, for example presence of immunoglobulin, other subtypes of CDG (CDG Ia, CDG Ib, CDG Ic, CDG Ix), low transferrin quantitation value, degradation of the sample and newborn sample less than three months\cite{20-23}. These results were consistent with other studies in which interferences by hemoglobin and fibrinogen, transferrin polymorphisms, complement fraction C3b, and unknown compounds have been described in CZE techniques\cite{21,24}. In this abnormal pattern of transferrin, the clinical features were similar with the positive CDG cases which could be split into three: neurological symptoms, digestive symptoms and dysmorphic features systems. Neurological symptoms include developmental and intellectual delay, progressive unsteady gait, hyperkinetic movements, hypertonia and muscle weakness. Digestives symptoms are as like having swallowing problems, hepatomegaly and splenomegaly. Features systems were patients with having atrophy microcephaly, outgrowth, unexplain globalderm and dysmorphic flat nasal and unplumed nose. Similar with other reported studies, the clinical presentation were broad \cite{1,25}.

During the last 10 years, our institution is the only laboratory offering screening and confirmatory test for CDG in Malaysia. All clinically suspected cases from all part in Malaysia are referred to IMR for diagnosis. Thus, our data highly reflective of the status of CDG in Malaysia.
Conclusion
These data are the first to describe the epidemiology and prevalence of patients with CDG in Malaysia. In summary, CDG can be considered as uncommon disease in Malaysia and study that is more comprehensive may provide valuable information.

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Conflict of Interest Disclosure
None to declare

References