Employing CRISPR for editing of *G6PD Viangchan*

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Glucose-6-phosphate dehydrogenase (G6PD)

Glucose $\rightarrow$ Glucose-6-Phosphate $\rightarrow$ 6-Phoshogluconate

Hexokinase

G6PD

Defeciency

NADP $\rightarrow$ NADPH

GSH $\rightarrow$ GSH DEF.

Hemolysis

No protection For RBC’s Against Oxidative substances
Background & Epidemiology

Incidence/prevalence of G6PD deficiency & What is known about the disease burden in Malaysia?

- Studies in Kelantan and Selangor

- Frequency distribution
  - General population 3.4% (184/5362)
    - Male 5.3% (160/2992)
    - Female 1.05% (25/2370)
  - Malay
    - Male 4.6% (86/1852)
    - Female 1.3% (19/1530)
  - Chinese
    - Male 7.2% (69/956)
    - Female 0.7% (5/726)
  - Indians
    - Male 2.7% (5/184)
    - Female 0.7% (1/141)
  - Negrito (Orang Asli)
    - Male 11%
    - Female 7%
• What have we known?
G6PD DEFICIENCY

Most common symptom - haemolytic anaemia – drugs, food (fava beans), other substances, triggers (Cappellini and Fiorelli, 2008)

Neonatal jaundice (NNJ) → kernicterus & permanent neurological damage/death (Doxiadis and Valaes, 1964; Luzzatto, 2006)

Most severe outcome AHA → acute renal failure (Beutler, 2008)

Some mutation caused lifelong congenital non-spherocytic hemolytic anemia (CNSHA)

217 mutations - G6PD gene deficiency (Gómez-Manzo et al., 2016b, Gómez-Manzo et al., 2017)

The most common - Malay ethnics - G6PD Viangchan (37.2%) - G6PD Mediterranean (26.7%) - G6PD Mahidol (15.1%) (Ainoon et al., 2003, Yusoff et al., 2004).
Molecular epidemiology
G6PD deficiency individuals are generally asymptomatic throughout their life.

Acute anaemia haemolytic (AHA), neonatal jaundice (NNJ) and chronic non-sphaerocytic anaemia (CNSA).

AHA is the most common manifestation.

Thus critical to understand how such episodes can be prevented.

Public health problem.
Issues and challenges

Determination of mechanism of pathogenesis of NNJ in G6PD deficiency

Development of yet simpler and more accurate kits for determination of G6PD levels that can be used in developing countries

Rapid methods for sequencing genes, including G6PD, at low cost

*Philip J Mason, Jose M Batista, Florida Gilsanz 2007
G6PD at crossroads of haematology, pharmacogenetics and malariology

* Lucio Luzzatto, Caterina Nannelli, Rosario Naotaro, 2016
In the interest of global health

We now have the means to protect G6PD deficient individuals from exposure to fava beans and to iatrogenic risks.

If known, we might be able to mimic the mechanism to protect others.

How G6PD protects against malaria – not fully understood.

* Lucio Luzzatto, Caterina Nannelli, Rosario Naotaro, 2016
Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/CRISPR-associated protein (Cas) system is a newly emerging mutagenesis (gene-editing) tool in genetic engineering.
CRISPR/Cas9

- Found in bacteria and involved in immune defence.
- Bacteria use CRISPR/Cas9 to cut up the DNA of invading bacterial viruses.
- Correct a disease-causing error.
- First introduced into mammalian organisms in 2013.

RNA-guided genome editing tool
CRISPR/Cas9 for gene editing - advantages

Simple-to-design, easy-to-use and multiplexing

Cost-effective and convenient tool for various genome editing purposes (e.g. gene therapy studies).

In cell lines or animal models, CRISPR-Cas9 can be applied for therapeutic purposes

Can correct the causal mutations in monogenic disorders and thus rescue the disease phenotypes, (currently represents the most translatable field in CRISPR-Cas9-mediated gene therapy).

CRISPR-Cas9 can also engineer pathogen genome such as HIV for therapeutic purposes, or induce protective or therapeutic mutations in host tissues.

CRISPR-Cas9 has shown potentials in cancer gene therapy such as deactivating oncogenic virus and inducing oncosuppressor expressions.
Mechanisms of CRISPR-Cas9-mediated genome editing and epigenome modulation.

CRISPR for editing of $G6PD$ Viangchan
JUSTIFICATIONS OF STUDY

Correction - molecular level – offers options for treatment for deficiency.

No defined treatment - avoiding the triggers for their whole life.

CNSHA patients - blood transfusion

G6PD gene - unlock the pathway to treat more other diseases as well

CRISPR for editing of G6PD Viangchan
Methodology

1. Production of wild type and Viangchan human G6PD protein and activity assay (for comparison)
2. Production of lentiviral virus particles that carries G6PD Viangchan (871 G>A)
4. Gene deficient correction by using ribonucleic CRISPR/Cas9 method
Methodology 1: Production of full length human G6PD Wildtype and G6PD Viangchan protein

1. The full length G6PD gene was produced by PCR.
2. G6PD PCR product was purified and digested.
3. Expression vector was digested and purified.
4. 1:3 ratio of ligation was performed.
5. Transformation into competent cells.
6. Positive colonies were isolated and digested.
7. Sequencing.
8. Retransformed into BL21 cells.
Methodology 2: Production of lentiviral particles

Production of G6PD-deficient gene in 3rd generation lentiviral vector
(pLJM-eGFP+ΔG6PD)

Transfection of plasmid DNAs (pLJM-eGFP+ΔG6PD, pMDLg/pRRE, pRSV/REV and pMD2) into HEK293T by using Lipofectamin 3000 to produce viral particles.

Viral particles were collected and concentrated by using ultracentrifugation system

Measurement of lentiviral titer by GFP selection
Methodology 3: Generation of G6PD Viangchan in THP-1 cell line

1. Transduction of the lentiviral particles into THP-1 cells
2. Selection of the positive colony by using GFP selection by using cell sorting
3. Protein isolation from transduced cells and non-transduced cells
4. Western blot against G6PD protein
5. G6PD enzyme activity assay and peroxidase activity assay analysis
Methodology 4: G6PD deficient correction by using CRISPR/Cas9 system

1. Primer design
2. Production of CRISPR gRNA by PCR
3. In-vitro transcription
4. Transfection by using Lipofectamine 3000
5. T7 Endonuclease assay
6. G6PD enzyme activity analysis
Results

G6PD enzyme activity after CRISPR transfection

Data of G6PD enzyme activity in bar chart. Result shows a slight elevation of enzymes activity in corrected THP-1 cell compared to negative control.
• G6PD WT and Viangchan was successfully produced with comparable G6PD enzyme activity.
• G6PD deficient THP-1 cell line that produces stable G6PD Viangchan was successfully created with approximately 50% of reduction.
• G6PD deficient correction was carried out with very little success.
• Only 8.8% of G6PD correction was detected.
• Significant progress on G6PD deficiency – better understanding of genetic controls, abnormalities in the different forms, improvement in treatment
• However, G6PD deficiency still poses a challenge
• Gene editing using CRISPR/Cas 9, show correction though not optimal
• Different approach to create G6PD disease model
• Study in different cell lines (haematopoietic progenitor cells etc)
• G6PD Mahidol and Mediterranean?
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