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Isolation of m-RNA from the Blood Samples of Neonatal Jaundice Patients and then Studied the Expression of Glucose 6-Phosphate Dehydrogenase (G6PD) Gene through cDNA Synthesis and Amplification

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Abstract: ***Diseases can be caused either due to genetic factors or physiological factors. But, there are some diseases which can occur due to both of these affects e.g. neonatal jaundice. Main characteristics of the disease are hyperbilirubinemia and yellowing of the skin of patient. Genes play important role in causing the disease which control the enzymes that are responsible in bilirubin excretion process and epigenetic factors are also responsible which regulate transcription of these genes. One enzyme that we studied was glucose 6-phosphate dehydrogenase which is responsible in causing neonatal jaundice. So, to study the role of epigenetic factors in glucose 6-phosphate dehydrogenase deregulation in neonatal jaundice patients present work was performed.***

Keywords: Hyperbilirubinemia, epigenetic factors, neonatal jaundice and, glucose 6-phosphate dehydrogenase (G6PD) gene, malaria

1. Introduction

Neonatal Jaundice is the disease caused due to both genetic factors and physiological factors. But, main cause is the sudden change in genes responsible. Often it is rare but, when disease is caused due to genetic factors then it is often considered very difficult to cure. Neonatal jaundice is characterized by yellowing of skin of newborn due to bilirubin deposition. When this bilirubin level is more than 85µmol/l then it causes jaundice in neonates, and when level is 34µmol/l, it results in yellowing of skin in adults. The disease is verified by dermal and subcutaneous tissue detection (Gartner and Herschel, 2001). Neonatal Jaundice patients have apparent yellowish sclera, and face extending down towards the chest.

It is a very common condition which affects nearly half of the babies in first week after birth. This is also known as Gilbert's Syndrome. Based on the causes Neonatal Jaundice is of two types; Physiological and Pathological Jaundice. In Physiological jaundice disease occurs due a minor defect during bilirubin excretion and, in Pathological jaundice disease occurs due to genetic defect i.e. any change in gene sequence due to mutation which in turn lead to partial or complete loss of activity of enzyme responsible in causing the disease. Main factors responsible for occurrence of jaundice are fetal hemoglobin breakdown and its replacement with that of adult hemoglobin, immature hepatic metabolic pathways.

Both the types are differentiated by the symptoms as presence of intrauterine retardation, scars of intrauterine infections, bruising, cephalhematoma, etc. family history of the patient is also noted for anaemia and jaundice (Barbara and Zhang et al, 2006).

Main point to focus here is bilirubin formation and its excretion process. If there is any defect in this process then jaundice can occur. In jaundice bilirubin play important role. Bilirubin gets conjugated with glucuronic acid in liver which make it soluble in water and allows its transport to small intestine. Here it is broken down to urobilinogen by intestinal bacteria and further into stercobilinogen which gets oxidized to stercobiln. This stercobiln gives brown color to feces.

2. Literature Survey

Nearly half of the neonates during their first week suffer from jaundice in which bilirubin does not conjugate with glucuronic acid due to which it becomes very difficult to excrete bilirubin from body and which in turn lead to bilirubin accumulation and rise in its level than normal. This is observed by appearance of symptoms of jaundice as yellowing of skin of patient and poor feeding and lethargy. This is also known as hyperbilirubinemia. It can occur due to physiological jaundice, breast milk jaundice, jaundice from hemolysis or jaundice due to abnormal functioning of liver. Tests performed are for direct and indirect bilirubin level, erythrocytes count, blood type and testing for Rh incompatibility (Coomb's test).

Breast feeding is considered important to prevent jaundice to occur in newborn. It has also been clinically proven that the babies which are breast fed have low occurrence of physiological jaundice than those which are artificially fed. So, to avoid physiological jaundice mothers are to be advised to feed their babies at 10-12 times a day (Hang and Chang et al, 2002).

Breast milk jaundice is another situation about which is very less known. For this condition babies which are about one week old are diagnosed. On 10-21 days interval level of occurrence rises and gain peak but, this may be only up to 2-3 months only. The babies with this condition have serum total bilirubin level of more than 10mg/dl in third week of their life. To avoid this condition there is only one treatment so far, and that is phototherapy. In phototherapy fluid uptake by babies is increased by either breast feeding or by using lactation aid to supplement.

So, from above mentioned details it is clear that jaundice can occur due to either of the causes as follows;

1. Pre hepatic cause
2. Hepatic cause
3. Post hepatic cause

Bilirubin level in blood is measure by various prescribed methods as Biochemical Method, Transcutaneous Bilirubinometer Method and, Bilimeter Method.

Gene which we studied and which is responsible in causing jaundice was glucose 6-phosphate dehydrogenase. This is very important gene as it is present in all cells of body and also it carries out first reaction of Pentose Phosphate Pathway. It is considered as protective measure against sickle-cell anaemia or malaria. G6PD enzyme play important role in protecting erythrocytes against oxidative stress condition. As Pentose Phosphate Pathway is the only source of energy in reduced form i.e. NADPH. So, any mutation in the gene produces protein variants which alters the functioning of the enzyme and result in generation of clinical and biochemical phenotypes (Ruwende and Hill et al, 1998).

3. Materials and Methods

3.1 Reagents;

a. Trizol Reagent**;**

Phenol;	3.8ml
Guanidium;	0.8M
Ammonium thiocyanate;	0.4M
Sodium acetate (3M);	0.1M
Glycerol;	500µl
Distilled water;	10ml

b. Chloroform (ice chilled)
c. Iso amyl alcohol
d. Iso propanol (ice chilled)
e. 70% ethanol
f. TE Buffer

3.2 Protocol for RNA Isolation

We had taken 500µl of blood sample and added 500µl of Trizol reagent to it. It was then kept on ice for 20 minutes. After 20 minutes of incubation it was centrifuged at 13,000 rpm for 10 minutes at 4°C. After centrifugation we collected supernatant in a fresh eppendroff in which we added 700µl of ice-chilled chloroform and 100µl of iso amyl alcohol. It was then mixed properly by inverting for 10 seconds. Then it was incubated at -20°C for 15 minutes and immediately centrifuged at 13,000 rpm for 15 minutes at 4°C. From that we collected upper aqueous layer in a fresh eppendroff. To this aqueous layer we added equal volume of ice chilled iso propanol and mixed thoroughly by inverting it several times. It was left undisturbed for 30-40 minutes at -20°C temperature. It was then centrifuged at 13,000 rpm for 10 minutes at 4°C. After centrifugation supernatant was discarded and the pellet was washed with ethanol. Then it was air dried and added 30-40µl of TE buffer to pellet. This was then stored at 4°C.

3.3 RNA Quantification

Total RNA isolated from blood was quantified by measuring the absorbance at 260nm in spectrophotometer (Helios α, thermo spectronic, double beam). It was calculated by formula;

Total RNA (µg/ml) = O.D AT 260nm * 40 * Dilution Factor

3.4 cDNA Synthesis

a) First Strand Synthesis

PCR was performed for synthesis of first strand of cDNA from RNA that was extracted from blood sample. Protocol for first strand synthesis is as follows;

8µl of RNA was taken in PCR vial. To it was added 3µl of random primer. Then added was 12µl of PCR water. It was heated in a thermo cycler at 65°C for 5 minutes. After that it was kept on ice for 5 minutes. Then vortex was done tone mix the samples. Then added was 4µl of 10X buffer and 2µl of di thio thretol (DTT) and 2µl of deoxyribonucleotide (dNTPs). Then added was 1µl of reverse transcriptase enzyme. After the cocktail was prepared, PCR was performed. First cycle was done at 42°C for one hour and second cycle was done at 70°C for 5 minutes. Then it was stored at -20°C temperature.

b) Second Strand Synthesis

On next day we proceeded with second strand synthesis. 17µl of PCR water was taken in a fresh PCR vial. To it added 2.5µl of 10X buffer. After that added 0.5µl of deoxyribonucleotide (dNTPs). To it added 1.5µl of magnesium chloride. After that we added 0.5µl of forward and then reverse primer of glucose 6-phosphate dehydrogenase gene. At last added was 0.5µl of Taq DNA Polymerase enzyme. And then PCR was performed.

95°C for 1 min (Initial denaturation)

Loop Open
94°C for 1 min (Denaturation)
63°C for 1 min (Annealing)
72°C for 2 min (Extension)

Loop Close after 35 cycles.
72°C for 5 min (Final extension)
4°C hold on forever.

4. Result and Discussion

Five samples were taken out of which two newborn were female and three were male. There was no mutated gene observed from the five samples taken. All were normal i.e. expressing glucose 6-phosphate dehydrogenase gene. So we can say that all the factors have no role in glucose 6-phosphate dehydrogenase gene expression although, the babies were marked positive for hyperbilirubinemia so, in that case we can say that the disease may be due to physiological factors and not due to pathological factors.

5. Conclusion

Through this study we came to some facts that only genes are not responsible in causing the disease but, external factors are also responsible in newborn.

6. Future Scope

The study gives future hope of research on the study of expression of G6PD gene in newborn through mutations which may help in finding new methods of its treatment.

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