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Expression of Micro RNA 144 in sickle cell disease

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Abstract

Sickle cell is a point mutation, individual homozygous Sickle disease (HbSS) shows the same genetic mutation but the severity and manifestation of this disease are extremely heterogeneous .The major pathology of sickle cell diseases is haemolysis and vaso-occlusion. This study is aim at quantifying the MiRNA144 in steady and crisis state in sickle cell diseases. D'Agostino & pearson Ormnibus normality test, Kruskal Wallis and Dunns multiple comparison test were computed on all the patients recruited. 40 HbSS patients were enrolled, 20 at steady state and 20 at crisis state, then 20 AA controls and 20 AS controls. The mean value of microRNA-144 of HbSS at crisis state was 551.80 ± 503.40 copies/5µl cDNA while HbSS at steady state was 1481 ± 2043.00 copies/5µl cDNA, the mean value of miRNA-144 of HbAA was 4.10 ±7.58 copies/5µl cDNA, while the mean value of miRNA144 of HbAS was 22.15 ± 15.49 copies/5µl cDNA. There was no statistical significant difference ($p>0.05$) in the mean value values of miRNA144 in HbSS at crisis and steady state , there was a statistical significant difference ($P <0.001$) in the miRNA 144 of HbSS at crisis state and HbAA , there was statistical significant difference ($P < 0.001$) in the miRNA144 at steady state and HbAA and HBAS. In conclusion there is no statistical significant difference between miRNA144 in sickle cell diseases.

Keywords: Sickle cell, HbSS, MiRNA144, cDNA .

Introduction

Sickle Cell Diseases (SCD) affects millions of people throughout the world and particularly common among those whose ancestors came from Sub-Saharan Africa, Spanish –Speaking regions in the western Hemisphere (South America, The Caribbean and central Africa) Saudi Arabia, India and Mediterranean countries such as Turkey, Greece and Italy (CDC, 2011;Obeagu et al.,2014; Okoroiwu and Obeagu, 2015). Sickle Cell disease is of major public health concern. From 1989 through 1993, an average of 75,000,hospitalisation due to the sickle cell disease occurred in the United States, costing approximately and 475 million dollars(Ashley-Koch *et al.,* 2000).Sickle cell related death among black African-American children younger than

4years of age fell by 42% from 1999 through 2002. This drop coincided with the introduction in 2000 of a vaccine that protects against invasive pneumococcal disease (Yanni *et al.*, 2009)

In 2006 the World Health Organisation (WHO) pronounced Nigeria as the country with the highest number of sufferer of sickle cell anaemia in the world .The global health watch dog put the annual number of sickle cell anaemia suffers in African at about 200,000noting that Nigerian accounts for 150,000 sickle cell anaemia every year (WHO, 2006). In most countries, where sickle cell anaemia is a major public health concern, its management has remained inadequate, national control programmes do not exist. The basic facilities to manage the patients are usually absent. Systematic screening is not a common practice and the diagnoses is usually made when a patient present with a severe complication .Simple cheap and very cost effective procedure such as the use of penicillin to prevent infections are not widely available in many countries (WHO, 2010).The governing bodies of WHO have adopted two resolutions on haemoglobin disorder. The resolution on sickle cell disease from the 59th world health assembly in May 2006 and the resolution on thalassemia from the 118th meeting of the WHO executive board call upon affected countries and the secretariat of WHO to strengthen their response to these conditions. In addition a resolution on the prevention and management of birth defects, including sickle cell disease and thalassemia were adopted by the 63th world health assembly in May 2010 (WHO, 2011).

In a resolution of World Health Assembly (WHA) 57. 13, the Health Assembly urged member states to mobilizes resources for action on genomics and in May 2005 the executive Board took note of the secretariat's report on control of genetics disease subsequently, the Assembly of the African Union at its fifth ordinary session supported the inclusion of sickle cell anaemia in the list of public health priorities (WHA 57.13, 2005).

With afore mention facts about sickle diseases and owning to the economic, psychological and health burden and with WHA 57:13 resolution which *urges* member states to mobilizes resources for action on genomics. There is serious need to carry out research on sickle cell diseases and bring out lasting solution. Sickle cell does not affect only red blood cells but the entire haemopoietic system therefore Micro RNA 144 which directly regulate Nuclear factor erythroid-2-Related factor 2, a central regulator of cellular response to stress and is associated with anaemia severity in sickle cell diseases is to be assayed (Obeagu,2015; Obeagu et al., 2015).

Aim

The overall aim of the study is to investigate the link between steady and crisis state in sickle cell disease and regulatory RNA.

The specific objective is:

To quantify micro RNA 144 in steady state and crises in Sickle cell diseases using real time PCR Taqman chemistry.

Materials and Methods

Ethical Approval

Favourable ethical approval was obtained from the UNTH Health Research Ethics Committee.

Sample size

Sample size was calculated using StatMate version 2.0 (www.graphpad.com **).** A sample size of 80 has a 99% power to detect a difference of 0.006 the means miRNA 144 in steady state compared with crises with a significance level (alpha) of 0.05 (two-tailed).

Sampling

About 4 ml of whole Blood was collected into commercial EDTA tubes at a concentration of 1.5mg of K_3EDTA per ml of blood for the analysis of Micro RNA 144

Laboratory procedures

Extraction of microrna using gene JET RNA purification kit (spin colunm method)

Components of Gene JET RNA Purification kit

Gene Jet RNA purification kit	250 preps #k0732
Proteinase K	5x600µl
Lysis Buffer	200ml
Wash buffer 1(concentrated)	200ml
Wash buffer 2(concentrated)	100ml
GeneJET RNA purification columns pre-assembled with collection tubes	250
Collection tubes(2ml)	50
Collection tubes(1.5ml)	50

Human blood cells total RNA purification

Before starting

- Supplement the required amount of lysis Buffer with β-mercaptoethanol or DDT.20µl of 14.3 M β-mercaptoethanolor 2 M DDT was added to each 1ml volume of lysis buffer required.
- Blood sample collection and RNA purification from blood cells was carried out within the same day. Samples were stored at 40c until used.

Procedure

- Blood cells were collected by centrifugation of 0.5mls of whole blood at 400g for 5 min at 4^0C.Blood cells generated a pellet of approximately 60% of the total sample volume; cl ear supernatant (plasma) was removed from the pellet with a pipette.
- The pellets were suspended in 600µl of lysis buffer supplemented β-mercaptoethanol or DTT vortex to mixed thoroughly.
- 450µl of ethanol (96-100%) was added and mixed by pipetting.
- 700µl of lysate was transferred to the GeneJET RNA purification column inserted in a
- collection tube.The column was centrifuged for 1 min at 12000g.The flow through was discarded and the purification column was placed back into the collection tube. This step was repeated until all of the lysate has been transferred into the column and centrifuged. The collection tube containing the flow through Solution was discarded .The GeneJet RNA purification column was discarded into a new 2ml collection tube.
- 700µl of wash buffer 1(supplemented with ethanol) was added to the Gene JET RNA purification column and centrifuged for 1 min at 12000g. The flow through was discarded and the purification column was placed back into the collection tube.
- 600µlof wash buffer 2 was added to the GeneJET RNA purification column and centrifuged for 1 min at 12000g.The flow through was discarded and the purification column was placed back into the collection tube.
- 250µl wash buffer 2 was added to the GeneJET RNA purification column and centrifuge for 2 min at 1200g.The collection tube containing the flow through solution was discarded and transfer the GeneJET RNA purification column to a sterile 1.5ml RNase free micro centrifuge tube.

- 50µl of water, nuclease free was added to the centre of the geneJET RNA purification column membrane. It was centrifuged for 1 min at12000g to elute RNA.
- The purification column was discarded. The purified RNA was used for Micro RNA 144 using Real-time PCR system.

Reagents and materials

- cDNA mix (210µl)- thaw the cDNA mix. One tube is sufficient for 10 samples.
- RNAse (RNAse inhibitor-40 U/µl)
- M-MuLV-Reverse Transcriptase.
- Probe sequence ABL-146F GATACGAAGGGAGGGTGTACCA
- Primer sequence ABL-240R CTCGGCCAGGGTGTTGAA
- Control gene ABL-183P FAM-TGCTTCTGATGATGGCAAGCTCTACGTCTCCT-BHQ

Sample: Total RNA (Place on ice)

Protocol for cDNA synthesis

1. The RNA samples were placed on the water bath or dry blocks set at 65^0C for 10minutes.
2. AcDNA cocktail was prepared by adding 6µl of RNasin and 12µl of M-MuLV Reverse Transcriptase to the 210 µl of cDNA mixed in a 2ml tube, the cocktail was mixed by brief vortex.
3. 21 µl of the cDNA cocktail was added into the 40µl of RNA elute of samples or control (placed on ice during pipetting) and incubate at 37^0C for 2hours in the dry thermal block.
4. After 2hours, the tube was transferred to dry block set at 65^0C for 10minutes.
5. After 10minutes, the tubes was pulse-centrifuged and labelled the cDNA accordingly for storage/testing.

The microRNA was reverse transcribed into complimentary DNA (cDNA) the cDNA is more stable for quantitation.

Quantitation of MicroRNA using the cDNA in a Real time PCR

The reaction was set up in a 48 well plate by pipetting as follows:

2x Real time probe master mix 12.5 µl
Primer mix for microRNA 144 7.5 µl
cDNA 5µl
Total reaction volume was 25µl

The plate was sealed with film and placed on the plate holder in the real time PCR machine.
The ABL one step plus Real Time PCR was operated according to the manufacturer's instructions and lab procedures,briefly described:

- The stabilizer was turned on that turns the Q-PCR machine on.
- The button for laptop was pressed on.
- The Password word was entered same as username.
- The step one software v2.2 on the desktop was double clicked
- The template folder was double clicked, the appropriate test was selected.
- It opens the experiment window, on the left menu is "set up" which has two pages.

(a) Define targets and samples**.**
(b) Assign targets and samples.

- On Define targets and samples**,** enter sample details were entered; note standard are already in the template. Samples were entered according to worksheets, and clicked on "assign target and samples".
- On this new page check that standards are correct or modify by clicking on **"define and set up standards",** select and deselect appropriate wells according to worksheet, ensure sample wells are assigned targets. Ensure that every step is completed in **"set up"**
- At the run method, The reaction volume was checked to be 25µl, and the number of cycles : 40
- The machine was opened and the plate was put inside.
- Run was clicked, click on start, the machine loads software and opens a save page.
- Save run as run number, target and date for example 001 MiRNA 04-07-13.

- The machine starts, that plate compartment closes and the software drives the QPCR machine.
- The estimated run time. The amplification plot is the default window during run.

Statistical methods

Most data from the study were numerical, therefore following normality test by using D'Agostino and Omnibus test, appropriate post hoc ANOVA tests were used to test for significant difference and correlation. The Graph Padprism software version 5.02 was used.

Results

Eighty (80) subjects were selected for this research, 48 were female while 32 were males. 40 SS patients were attending UNTH Ituku- Ozzala Enugu, they were divided into 20 at crisis state and 20 at steady state. AA and AS control subjects, 20 each were used. They have average age between 2- 45 years.

MicroRNA144 was quantified using ABI one step plus Real Time PCR. The statistical variables obtained for the research is as follow;

Table 1: The mean value of microRNA 144 of HbSS at crisis state was 551.80±503.40cps/5ul cDNA, SS at steady state 1481.00±2043.00cps/5ul cDNA, HbAS22.15±15.49cps/5ulcDNA and HbAA control 4.10±7.58cps/5µl Cdna

Table 2: summarizes the comparison of MiRNA144 in patients with SCD in crisis and steady state , in AA and AS controls.There was no statistical significant difference (P>0.05) in the mean values of microRNA 144 of HbSS at steady and crisis state,HbSS at Crisis vsHbAS control and HbAS control with HbAA control. While there was a statistical significant difference (p< 0.0001) in the microRNA 144 of HbSS at Crisis and HbAA,SS steady with AS control and HbAA withHbAS.

Table 1: MicroRNA144 of HbSS at Crisis, HbSS Steady, HbAS and HbAA.

MicroRNA144 Parameter	SS Crisis	SS Steady	AA Control	AS control
Mean2±SD	**551.8±503.4**	1481±2043	4.10±7.58	22.15±15.49
Median	649.0	727.5	1.00	33.0
Confidence interval of mean.	316.1-787.4	525.0-2438.0	0.55-7.65	14.90-29.40
Passed D' Agostino Normality Test(α=0.05)?	No	NO	NO	NO

This table shows Mean, Median, SD, Confidence Interval of mean of MicroRNA144 in HbSS Crisis, HbSS Steady, HbAS and HbAA.

Table 2: P Value for MicroRNA144 Using dunns multiple comparison test.

Dunns multiple comparison Test for microRNA144	Difference in rank sum	P Value<0.05?	Summary
SS-Crises vs SS-Steady	-13.15	NO	NS
SS-Crises vs AS Control	17.83	NO	NS
SS-Crises vs AA control	31.93	YES	0.0001
SS-Steady vs AS Control	30.98	YES	0.0001
SS-Steady vs AA Control	45.08	YES	0.0001
AS Control vsAAControl	14.10	NO	NS

Discussion

The morbidity of Sickle cell diseases varies with genotype as well as within the same genotype, some of the established predictors of this phenotypic heterogeneity are the levels of HbF present, presence of α-thalassaemia and the β-haplotype associated with the Hbs gene (Steinberg, 2005)

In 2006, the world Health organisation (WHO) pronounced Nigeria as the country with the highest number of sufferer of sickle cell anaemia in the world. In a resolution, world Health Assembly urge member states to mobilizes resources for action on genomics, the executive board took note of the secretariat report on control of genetics disease and subsequently supported the inclusion of sickle cell anaemia in the list of public health properties (WHA 57.13)

This study is therefore particularly interested to investigate the link between steady and crisis state in sickle cell disease and some adhesion molecules with regulatory RNA, which is microRNA 144.

The microRNA 144 showed a statistical significant difference between HbSS steady state and HbAS control; HbSS steady state and HbAA; HbSS crisis and AA control ($P < 0.001$). There was no statistical significant difference ($P > 0.05$) in the mean values between HbSS Crisis state and HbSS steady state. MicroRNA 144 is linked to anaemia in Sickle cell diseases subjects(Sangokoya et al., 2010) This study shows a high level miRNA144 in HbSS compared to AS and AA. This agrees with a previous study that compared miRNA144 in HbSS and AA controls in red cells, which stated that "Increase in miRNA 144 is associated with decrease glutathione regeneration and increased anaemia severity in HbSS erythrocytes (Sangokoya *et al.*, 2010) However, no significant difference was found in miRNA144 levels between HbSS in crisis and steady state ($P > 0.05$), there is no report of such difference in the Literature. It is plausible that several factors are required to modulate severity of crises that might not be microRNA dependent.

In conclusion, this study shows that, there was no statistical significant difference between miRNA144 at steady and crisis state. However more work is needed to be done on miRNA144 especially in Africa to compare our results with the Caucasians, and patients used should be free from hyroxyurea which lowers adhesion molecules and affect our findings. This will help to suggest if miRNA144 can serve as a genetic modifier of HbSS related anaemia and can provide novel insight into the heterogeneity and pathophysiology of sickle cell disease.

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