Original Article

The Role of Micro-RNA 466i on Vaso-occlusive Complications of Sickle Cell Anaemia

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ABSTRACT

Vaso-occlusion in sickle cell anaemia (SCA) is mediated via increased expression of adhesion molecules. Micro-RNA 466i up regulates the expression of interleukin-10 and may contribute to the pathogenesis of the complications in SCA. We sought to investigate the relationship between changes in the level of micro-RNA 466i and the frequency of vaso-occlusive complications in SCA patients. Red blood cells were lysed using ammonium chloride. The resulting white blood cell pellets were lysed in guanidiniumisothiocyanate (GITC) lyses buffer. From the GITC lysate, total RNA was extracted. Thereafter, the amount of micro-RNA 466i was quantified. There were no relationships between microRNA 466i and the frequency of complications in SCA patients. ($p=0.066$) There was also no significant difference in the levels of micro-RNA 466i between patients who had vaso-occlusive complications and those that did not. ($p=0.9307$) Micro-RNA 466i increased with age ($p=0.03$) but there was no significant difference between the males and the females patients. ($p=0.370$) Micro-RNA 466i (a pro-inflammatory nucleotide) play little or no role in the pathogenesis of vaso-occlusive complications in SCA. Its assay in this group of patients is not useful in the overall management of these patients.

Keywords: Complications, Micro-RNA 466i, Sickle Cell Anaemia, Vaso-Occlusion.

INTRODUCTION

Sickle cell anaemia is an inherited cause of chronic haemolytic anaemia whose clinical manifestations arise from the ability of haemoglobin S (HbS) to polymerize and deform red blood cells into the characteristic sickle shape.¹ This disease affects many systems in the body causing episodes of acute illness and progressive organ damage. The molecular basis of sickle cell anaemia is due to a single nucleotide substitution (adenine to thymine) in the 6th codon of the β -globin gene.^{2,3} As a result, the hydrophilic amino acid - glutamic acid- coded by GAG in the 6th position of the β -chain is replaced with the hydrophobic

amino acid-valine-coded by GTG, to give the sickle haemoglobin (HbS).⁴ Human haemoglobin is a tetrameric molecule that consists of two pairs of identical polypeptide α and β subunits, each, encoded by a different family of genes. The association of two mutant β -globin subunits forms haemoglobin SS (HbSS). Under low oxygen concentration, the absence of a polar acid at position six of the β -globin chain promotes the non-covalent polymerization of haemoglobin, which distorts the red blood cell into a sickle shape decreasing its deformability.⁵ Normal red blood cells are quite deformable,

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allowing the cells to change shape in order to pass through capillaries. In sickle cell anaemia, low oxygen tension occurring in the end organs and tissues due to cellular anaerobic metabolism, promotes red blood cell sickling. Repeated episodes of sickling distort the cell membrane and decrease the cellular elasticity. These cells fail to return to normal shape when oxygen tension is restored. As a consequence, these rigid blood cells are unable to change their shape as they pass through narrow capillaries, leading to vessel occlusion and ischaemia.

The resultant anaemia of this illness is caused by haemolysis, from the removal of the deformed red cells in the spleen. Although the bone marrow attempts to compensate by increasing its proliferation of erythroid cells, it is not commensurate with the rate of destruction of the red blood cells. A normal red blood cell typically lives 90-120 days but sickle red blood cells only survive 10-20 days.⁶ This is because, the distorted sickle cells are susceptible to sequestration and destruction within the spleen by macrophages. There are three main mechanisms of sickle cell anaemia. The first is red blood cell sickling leading to continual haemolysis and anaemia. Secondly, there is vaso-occlusion leading to tissue infarction and pain. Thirdly, the patients have increased risk of infection,⁷ as a result of a defect in the alternative pathway of complement system,^{8,9} resulting in inability of the individual to deal with encapsulated organisms.¹⁰

Vaso-occlusion results from both passive mechanical obstructions of blood vessels by sickled red blood cells and active adhesion of sickled and unsickled red cells, to other blood cells and vascular endothelium.^{11,12} The current concept of vaso-occlusive crisis in sickle cell anaemia views the process as being the result of interaction between white cells, red cells, platelets, plasma proteins and vascular endothelium.¹³

Cytokines activate vascular endothelial cells and white cells, resulting in increased expression of their adhesion molecules which have important role in the genesis of vaso-occlusion.¹⁴⁻¹⁷ Platelets express P-selectins and white blood cells L-selectins which mediate interaction with the vascular endothelium. The expression of these adhesion molecules is increased during inflammatory processes as a result of release of cytokines. High levels of these adhesion molecules predispose to vaso-occlusion. The adhesion of white blood cells occurs mostly in the endothelium of post-capillary venules. Vessel occlusion can lead to ischaemia, infarction, and variable degrees of pain and organ dysfunction. Microvascular occlusion in the bone

marrow leads to hypoxia, tissue ischaemia and infarction, usually characterized by excruciating pains in the long bones, sternum and spine.¹⁸

Vaso-occlusive complications occur due to tissue damage and organ dysfunction results from repeated episodes of blood vessel occlusion by blood cells. These complications include cerebro-vascular accident (stroke), which manifests as motor or sensory deficit; visual impairment or blindness. Other complications include acute chest syndrome, which is an acute lung injury, defined as the development of a new alveolar pulmonary infiltrates involving at least one lung segment. Vaso-occlusion in the renal vasculature result in renal impairment and eventually renal failure a condition known as sickle cell nephropathy. Priapism, another complication, is a painful erection of the penis caused by vaso-occlusion of venous drainage from the penis. Also included is avascular necrosis of the head of femur or humerus, which can lead to pain and disability.

MicroRNAs are short nucleotides that regulate gene expression either by degrading the messenger RNA or by inhibiting the translation of messenger RNA into protein.¹⁹ They are essential regulators of hematopoiesis, immune cell development, immune responses, inflammation, and autoimmunity, providing a new therapeutic window.²⁰ When a gene is switched on, its sequence is converted into messenger RNA which carries the information to make a protein. MicroRNA recognizes and binds to the messenger RNA and prevents it from making its protein.¹⁹

Vaso-occlusion in sickle cell anaemia is mediated via increased expression of adhesion molecules and is thought to be associated with recurrent or continual inflammation.²⁰ There is increased level of pro-inflammatory cytokines such as interleukin-2,^{4, 6, 8} and tumor necrosis factor alpha (TNF- α) during inflammation.²¹ These cytokines stimulate adhesive interaction between blood cells (white blood cells, platelets, red blood cells) and activates vascular endothelial cells. We therefore undertook this study to investigate the relationship between changes in the level of micro-RNA 466i and the frequency of vaso-occlusive complications in SCA patients.

MATERIALS AND METHODS

This study was carried out on patients with sickle cell anaemia that attended sickle cell clinic in a tertiary hospital in Nigeria. This hospital is located in a state in the south east geo-political region of the country. The population of the state according to

2006 census was 3,267,837. The calculated sample size for this study was 45. Subjects between the ages of two to fifty years old were recruited from the Paediatrics and Adult sickle cell clinics. The consecutive, non-randomized sampling method was used to select participants. There were two groups of patients involved in the study: group A and B. Group A were SCA patients who had vaso-occlusive complications e.g. nephropathy, avascular necrosis of the bone, acute chest syndrome, stroke or priapism. Group B were SCA patients who do not have any of these vaso-occlusive complications. After recruitment into the different groups, two and half (2.5ml) of blood was collected into an Ethylene diamine tetra acetic acid (EDTA) bottle from each patient during their steady state after taking informed consent. Patients with co morbidities e.g. diabetes or that were on hydroxyurea therapy and on blood transfusion programme were excluded. The Health Research Ethics Committee of the hospital gave approval for the study.

Detailed clinical, pertinent personal history and other important information were collected from clinic records and from the SCA patients and their guardians for children with a 'case record form' (proforma) which was designed for this study. The data included, enrolment identity number, gender, age, residence, ethnicity, occupation, steady state haemoglobin, number of vaso-occlusive complications and history of blood transfusion. The sample collected in EDTA bottles was within 24 hours of collection used for microRNA 466i quantitation. Micro-RNA 466i extraction was done by lysing the red blood cells using ammonium chloride red cell lysis buffer. The resulting white blood cell pellets were lysed in guanidiniumisothiocyanate (GITC) lysis buffer. From the GITC lysate total RNA was extracted. Thereafter the amount of microRNA 466i was quantified.

Total RNA (messenger RNA and micro-RNA) was isolated using PAX Gene blood RNA tube (BD Diagnostic) to stabilize RNA for up to 5 days following which samples were batched. RNA was isolated from 2.5ml of blood using PAX gene blood micro RNA (QIAGEN) following the manufacture protocol. The quantity of micro-RNA 466i was determined in each patient and correlation sought with the number of vaso-occlusive complications.

Data generated were analyzed using the statistical package for social science (SPSS) computer software version 16.0 for windows. The findings were presented in tables. Comparison of quantities of microRNA 466i in patients with or without vaso-occlusive complications was done using unpaired student t-test. The correlation between the levels of microRNA 466i and

frequency of vaso-occlusive complications was done using one-way Analysis of Variance (ANOVA). The relationship between microRNA 466i and age, sex were determined using unpaired student t-test. Statistical significance was considered to be at p value < 0.05 .

RESULTS

Out of the fifty SCA patients, twenty five had vaso-occlusive complications (group A) and twenty five had no vaso-occlusive complications (group B). The mean age for group A was 28 ± 9.87 years, while it was 17 ± 10.69 years for group B. The males were 16(64%) in group A and 11(44%) in group B, while the females were 9(36%) in group A and 14(56%) in group B. The other socio-demographic characteristics of the study population are shown in Table 1

Table 1: Socio-demographic characteristics of study population based on presence or absence of vaso-occlusive complications

Socio-demographic variables	SCA patients without complications (n=25) n (%)	SCA patients with complications (n=25) n (%)
Age (years)		
0-19	15(60)	5 (20)
20-29	9(36)	17 (68)
>40	1(4)	3 (12)
Mean $\pm$ SD	17 ± 10.69	28 ± 9.87
Sex		
Male	11 (44)	16 (64)
Female	14 (56)	9 (36)
Educational level		
Nursery	2 (8)	0 (0)
Primary	7 (28)	0 (0)
Secondary	9 (36)	9 (36)
Tertiary	7 (28)	16 (64)
Occupation		
Pupil/student	20 (80)	10 (40)
Unemployed	1 (4)	8 (32)
Civil/public servant	2 (8)	5 (20)
Trading	2 (8)	1 (4)
Clergy	0 (0)	1 (4)

In Table 2, Twenty five patients with complications (group A) had a mean normalized micro-RNA 466i of 99.9 ± 15.9 while the other 25 patients without complications group B, had a mean normalized micro-RNA 466i of 76.14 ± 17.5 . The t-test value was 0.9307 ($P = 0.3567$) indicating that there was no significant difference in the quantity of micro RNA 466i between the two groups.

Table 2: Levels of normalised micro-RNA 466i in SCA patients with or without vaso-occlusive complications

Parameters	Number	Mean	Test statistics (student t-test)	p-value
Normalized MicroRNA 466i				
With complications	25	78.14 ± 17.5		
Without complications	25	99.90 ± 15.9	0.9307	0.3567

Considering the number of complications and the levels of microRNA 466i, result showed that, twenty five (25) patients without vaso-occlusive complication had a mean value of micro RNA 466i of 5568 ± 596 copies/ 5ul of cDNA, twenty (20) patients with one complication each had a mean value of 7529 ± 563 copies/5ul of cDNA and five (5) patients with two complications each had a mean value of 5575 ± 1458 copies /5ul of cDNA.(P =0.66) This shows that there was no relationship between the number of vaso-occlusive complications and the level of microRNA 466i. Patients that were twenty-five years and below had a mean microRNA 466i of 5458 ± 531 copies/5µl of cDNA and those twenty-six to fifty years had a mean microRNA 466i of 7231 ± 595copies/5µl of cDNA. The student t-test was 2.225(P = 0.370). Sex had no significant influence on the level of micro RNA 466i in these patients.

Table 3: The relationship between the quantity of micro RNA466i and number of vaso-occlusive complications, age and sex of the patients

Parameters	Freq. (n)	Mean (miRNA 466i copies/5µl of cDNA)	Test statistics one way Anova	p-value
Number of vaso-occlusive complication				
0	25	5568 ± 596		
1	20	7529 ± 563		
2	5	5575 ± 1458	2.879	0.066
Age (years)				
1-25	25	5458 ± 531		
26-50	25	7231 ± 595	2.225	0.031
< 25 with complications	12	7271 ± 723		
< 25 without complications	12	6946 ± 991	0.2658	0.793
> 25 with complications	13	7015 ± 834		
> 25 without complications	13	4298 ± 505	2.787	0.010
Sex				
Male	27	7735 ± 1186		
Female	23	6447 ± 660	0.905	0.370

DISCUSSION

The age range of sickle cell anaemia patients that were recruited for the study was 2-50 years. Out of the patients that had complications only 5 (20%) were below the age of 20years while 20 (80%) were above 20 years. The mean age for patients with complications was 28 ± 9.87 years. This was higher than the mean age of those without complications (17 ± 10.69 years).

This supports the observation that the tendency to develop vaso-occlusive complications of sickle cell anaemia was more after the age of 20 years. This slightly differs from previous studies by Ebong²³ and Iwegbe²⁴ who indicated that the onset of complications in sickle cell anaemia were higher after the age of 14years. This difference can be explained by improvement in availability of treatment modalities as well as accessible healthcare facilities, which is now available to sickle cell anaemia patients. This being a cross-sectional study, though involving patients of various ages, a cohort study with a follow-up from childhood would be the best to substantiate our observation.

From this study more males (64%) had complications than females (36%). The reason why complications were more in males than females could be the fact that transcription factor for gamma (γ) chain haemoglobin (Hb) has been mapped to X-chromosome (F-locus). Females have two X-chromosomes and therefore have more opportunities of expressing HbF, while males have one X-chromosome and therefore less opportunity to express HbF.²⁵ High HbF levels in patients with sickle cell anaemia has been shown to ameliorate the severity of the disease.²⁶ It could also be that the males having a higher tendency to engage themselves in stressful activities such as sports and alcohol, which could expose them to dehydration which might predispose them to having more frequent crisis and eventually complications.

Patients with complications were found only in secondary and tertiary institutions while patients without complication were in all the educational levels. Although there were no previous studies to support or disagree with this finding, it seems that the reason why majority of the patients with complications were seen during their secondary and higher schools is because of the level of independence from their parents. It is probable that their parent take greater care (healthcare) of their children when they were in the Nursery and Primary schools compared to when the patients live independently in higher schools. Also the finding of an increasing tendency to develop vaso-occlusive crisis may also be responsible for this observation.

About eighty percent (80%) of patients without complication were students while majority of the patients with complications were made of students, unemployed and civil servants in that order. One could infer that the unemployed may not be keen to attend to their health needs due to lack of finance, which on a long run could predispose them to repeated episodes of vaso-occlusive crises and ultimately vaso-occlusive complications. Also the older age group (working class) patients are more

likely to have complications due to increasing frequency of end organ compromise.²⁷

The patients with complications had a mean microRNA 466i of 7138 ± 545 copies/5 μ l of cDNA while those without complication had a mean of 5568 ± 596 copies/5 μ l of cDNA. Although the mean quantity of microRNA 466i in those with complications was higher than those without complication, it was not statistically different.

In this study, the group with only one complication had the highest mean value of 7529 ± 563 copies/5 μ l cDNA, followed by those with two vaso-occlusive complications that had a mean value of 5575 ± 1458 copies/5 μ l of cDNA while 25 patients without vaso-occlusive complications had the least mean value of 5568 ± 596 copies /5 μ lcDNA. Although there was no significant difference between the number of vaso-occlusive complications and the quantity of microRNA 466i, it is evident that the patients with one or two complications had a higher quantity of microRNA 466i compared to those patient without complications.

The study showed that the levels of microRNA 466i increases with the age of the patients. This could explain the observation made in this study, that older patients had more vaso-occlusive complications than the younger patients. The relationship between the levels of microRNA 466i and sex, showed that males had higher mean quantity of microRNA 466i than the females. This might also contribute to the findings of males having more complications than the females, which was observed in this study.

CONCLUSION

This study showed that there was no significant difference in the levels of microRNA 466i in patients with and without vaso-occlusive complications of sickle cell anaemia. Also there was no significant difference in the quantity of micro-RNA 466i among the patients who had no, 1 or 2 vaso-occlusive complications. Micro-RNA 466i (a pro-inflammatory nucleotide) therefore plays little or no role in the pathogenesis of vaso-occlusive complications in SCA. Its assay in this group of patients or treatments that will target it may not be necessary in the overall management of these patients.

Conflict of interest

None declared.

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