Hematological and cytogenetic factors modulating the severity of the Sickle-cell disease at the University Hospital Sourô Sanou

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Summary

Introduction

Sickle cell disease is a monogenic disease with variables clinicals manifestations and various complications. This variability is related to the phenotype, the intracellular hemoglobin concentration, the importance of fetal hemoglobin, the quality of the Therapeutic outlet, and association with others pathologies: membrane abnormalities, G6PD's deficiency, Gilbert 's disease etc. ...it is a prospective study of twelve major sickle cell disease affected children on hematological and genetic factors modulating the severity of their illness.
Materials and Methods

Twelve major sickle cell disease affected children were included in this study. The following tests were made for each of them: -A full blood count with manual blood smears reading. -Identification of their beta's Haplotypes by RFLP followed by PCR. -A search of the mutation of the sixth codon of the beta gene and a search of rearrangements of alpha locus were executed.

The most common factors influencing the synthesis of fetal hemoglobin in the African context were sought by molecular biology. They were: BCL11A (rs10189857, rs1427407) HMIP (rs9399137) Xmn1 (rs7482144). Some specific mutations responsible for the disease of Gilbert and G6PD deficiency negatively modulate the evolution of sickle cell disease were studied by molecular biology also.

Results

The full blood counts with blood smear revealed regenerative microcytic hypochromic: anemia; aplastic microcytic hypochromic anemia and regenerative normochromic normocytic anemia

The Giants' polynuclears; larges platelets and even macropolycytes probably related to a deficiency in folic acid.

The attendance of hypercytoses (Polynucléosis, thrombocytosis, hyperréticulocytose) and erythroblastosis are amenable to treatment with hydroxyuré.
The presence of Howell Jolly's body witness functional asplenia, mononucleosis and trophozoïte

of Plasmodium means that the anti-infection prevention must be rigorous.
The beta loci Genotyping showed: six compounds heterozygous S / C with five Haplotypes Benin / Cameroon and one haplotype Senegal / Cameroon; six homozygous s / s including four Haplotypes Benin / Benin, two Haplotypes Benin / Senegal.
No patient has alone three mutations (BCL11A, HMIP, and Xmn1) support for synthesis of fetal hemoglobin.

Four patients have $\alpha^{-3,7}$ type of thalassemia .For G6PD's deficiency, a hemizygous A- and a conductor A- were diagnosed. The research for favorable mutations of UGTA1 to Gilbert's disease found three cases.

Conclusion
Sickle cell disease is modulated by several genetic factors that it is useful to know for adequate therapeutic management.

Key words: sickle cell disease, genetic variants at ɣ loci modifiers, Haplotypes, genotype β, SNPs, G6PD deficiency, UGTA1 mutations.

Résumé

Introduction

La maladie drépanocytaire est une maladie monogénique à manifestations cliniques variables et complications variées. Cette variabilité est liée au phénotype, à la concentration intra-cellulaire d'hémoglobine, à l'importance de l'hémoglobine fœtale, à la qualité de la prise thérapeutique, et de l'association à d'autres pathologies : anomalies de membrane, déficit en G6PD, maladie de Gilbert etc.…c'est une étude prospective sur douze enfants drépanocytaires majeurs portant sur les facteurs hématologiques et génétiques modulant la gravité de leur maladie.

Matériels et méthodes

Douze enfants drépanocytaires majeurs ont été inclus dans l'étude.les examens suivants ont été faits pour chacun d'eux :

Un hémogramme avec lecture manuelle du frottis sanguin.

Une identification de leurs Haplotypes betas par RFLP suivi de PCR.

Une recherche de la mutation du sixième codon du gène beta PCR et une recherche de la délétion du locus alpha par Gap-PCR.

Les facteurs les plus fréquents et influençant la synthèse de l'hémoglobine fœtale dans le contexte Africain ont été recherché par biologie moléculaire. Ce sont : BCL11A (Rs10189857, Rs1427407),

HMIP(Rs9399137), XMN1 (rs7482144). Certaines mutations spécifiques responsables de la maladie de Gilbert et du déficit en G6PD modulant négativement l'évolution de la drépanocytose ont été étudiées par biologie moléculaire également.

Résultats

Les hémogrammes ont mis en évidence : des anémies microcytaires hypochromes régénératives et arégénératives.

Des anémies normocytaires normochromes régénératives.

La présence de corps de Howell Joly témoin d'une asplénie fonctionnelle, d'un syndrome mononucléosique et de trophozoïte de plasmodium signifie que la prévention anti-infectieuse doit être de rigueur.

Des polynucléaires géants, des macroplaquettes, des macropolycytes probablement liés à un déficit en acide folique.

Des hypercytoses (Polynucléoses, hyperthrombocytoses, hyperréticulocytose) et érythroblastoses qui sont justiciables d'un traitement par l'hydroxyuré.

Le Génotypage beta a mis en évidence six hétérozygotes composites S/C dont cinq Haplotypes Benin/Cameroun, un Haplotype Sénégal/Cameroun et six homozygotes S/S dont quatre Haplotypes Benin/Benin, deux Haplotypes Benin/Sénégal.

Aucun patient ne présente à lui seul, trois mutations (BCL11A, HMIP, Xmn1) favorables pour la synthèse de l'hémoglobine fœtale.

Quatre patients ont une thalassémie de type $\alpha^{-3,7}$ Pour le déficit en G6PD, un hémizygote A- et une conductrice A- ont été diagnostiqués. La recherche de mutation d'UGTA1 favorable à l'apparition de la maladie de Gilbert a trouvé trois Cas.

Conclusion

La drépanocytose est une maladie monogénique qui est modulée par des facteurs polygéniques en cis et trans dont il est important de connaitre, pour une prise en charge thérapeutique adaptée.les examens hématologiques de routine et la biochimie sont insuffisants pour la mise en évidence de certains facteurs modulant la gravité de la drépanocytose.la biologie moléculaire est la solution ultime pour résoudre ces situations compliquées.

Mots clés : Drépanocytose, Haplotypes, génotype β, Variants génétiques modificateurs du locus ɣ, Thalassémie $\alpha^{-3,7}$, déficit G6PD, mutants UGTA1.

Introduction

The globin of hemoglobin is synthesized from embryo to adult by nine different genes: ξ^1(Zeta),ξ^2,α^1(alpha),α^2,ε(epsilon),γ^A(gamma),γ^G,δ(Delta),β(beta) **(1)** . Two types of γ^G exist among the adult by the difference of the 75th amino acid. The hemoglobin S at the molecular level is due to a replacement of the second nucleotide which is a adenine (GAG) by a nucleotide thymine (GTG) at the level of the Sixth codon of the gene β of the Hemoglobin **(2)** . The consequence is the replacement in position 6 of the chain β wich is the glutamic acid by valine, giving it the property of sickling under specific conditions **(3)**. For hemoglobin C the first nucleotide in the sixth codon beta (GAG) is replaced by an adenine (AAG). The severity of the sickle cell disease varies depending on the phenotype, the hematological factors (cellular, plasma), the intra cellular concentration of hemoglobin S, the importance of the fetal hemoglobin and enzyme factors. The phenotypic variability is linked to the haplotype variability which is linked to the importance of fetal hemoglobin (hereditary persistence or Beta Thalassemia). The Sickle cell disease is a monogenic disease but modulated by polygenic factors.

The objective of this prospective study is:

- To assess the therapeutic monitoring

- Research of certain genetic factors modulating the severity of the Sickle cell disease.

The study was approved by the Ethics Committee of the hospital and informed consent has been obtained by the parents of the children.

Materials and Methods

It is about twelve children with sickle cell disease (six composite heterozygous SC and six forms homozygous SS) aged one to ten years of which seven girls and five boys. Two blood samples of 2 or 4,5ml on Vacutainer blood collection tubes (EDTA) for large children and infants, in the fold of the Elbow have been collected to: - carry out a CBC with a concentration of reticulocytes on the PLC Mindray BC-6800.A manual blood smears reading have been conducted for each of the patients.

-Research the more frequent and influencing molecular factors to the evolution of the Sickle cell anemia. The factors selected for the study were: the study of the gene β, the identification of sickle cell disease's Haplotypes, research of the disease of Gilbert, the Alpha Thalassemia, the glucose 6 phosphate dehydrogenase (G6PD) deficiency; and other factors responsible for the increase in fetal hemoglobin: BCL11A and HMIP who are repressors of the synthesis of hemoglobin F at birth, the

polymorphism of the promoter of the gene ɣG (Xmn1) regulator of the switching of genes of globin.

For the various examinations of Molecular Biology: An extraction of DNA has been made using the phenol-chloroform method the for:

- Study of the gene β-globin:

It consisted in the search of the mutation at codon 6 of the gene β globin (GAG>GTG (HBS) and HBB: c.20A>T) by direct sequencing of DNA after a real-time PCR **(4)** .

-The research of the mutations of the sequences of reference (RS or SNP in English) of BCL11A and HMIP: **(5)** , [rs1427407 **(6, 7, 8)**] ,[rs10189857 **(9, 10, 11)**]and [rs9399137 **(12, 13)**].It was conducted by PCR in real-time, followed by a direct sequencing of DNA.

-For the research of the polymorphism in -158 of the promoter of the gene G gamma(Xmn1)[rs7482144], the technique of TaqMan genotyping assay has been applied **(14, 15)**.It consisted of researching for the substitution of the 27th nucleotide 5' of this sequence which is a cytosine (C) thymine (T) [Single Nucleotide Polymorphisms, or SNP in English]. The presence of the thymine (T) is associated with the increase of the hemoglobin F. The research on the deletion of the Genes α-globin was conducted using GAP-PCR in search of a thalassemia of type $\alpha^{-3,7}$ and/or a triplication $\alpha^{(anti\ \alpha\text{-}3,7)}$ **(16, 17, 18)**

.The study of sickle cell disease's Haplotypes was conducted using PCR of the allele beta and digestion by a restriction enzyme of the frame pre G gamma on gel electrophoresis of polyacrylamide and autoradiography. Method of RFLP Haplotypes frameworks" pre Gɣ" **(19)**

-The study of the G6PD's gene (NM_001042351.1) has consisted in the research of the three mutants of G6PD, the most frequently encountered in the population of African origin responsible for G6PD's deficiency:

*Variant A-: P. Val 68 puts (G6PD: c .202G>A) +p.Asn 126ASP

* Variant B-:p.Ser 188Phe (G6PD: c.563C>T)

* Variant Betica: P.Leu323 Pro (G6PD:C.968T>C) +p.Asn126ASP by PCR followed by sequencing of exons 3 to 6 and of the exon 9 **(16, 17)** .

*The typing pattern $(TA)_n$ of the promoter of the gene UGTA1 was made by PCR with radio-isotopes and the amplicons were separated in a gel electrophoresis of polyacrylamide and detected by autoradiography.

The confirmation of the type of pattern has been carried out by purification of PCR products on tracking column of sequencing **(20, 21)** .

Results

Table I Hémogrammes

Patient number	Sex	AGE	Complete Blood count	Blood smears	Reticulocytes
1	Male.	3 years 9 months	HB9,3g/dl VGM74,7Fl TGMH 24.8 pg	Body of Howell Jolly; Erythro-myélémie ; large platelet; thrombocytosis.	202000/mm3
2	female.	5Years 10 Months	HB10g/dl VGM73,4 fl TGMH 25,2pg	large platelet, thrombocytosis, platelet clusters, Erythroblastosis.	117000/mm3
3	Male.	6years 8months	HB 3,9g/dl VGM 81,2Fl 26,8TGMH pg	Erythroblastosis	53200/mm3
4	female.	2 years 4 months	HB 8,2g/dl VGM 79,7fl TGMH 25.4pg	Lymphocytosis with Hyperbasophilic lymphocytes.	313800/mm3
5	female.	6years 7 months	HB 7,5g/dl VGM 76,8fl TGMH PG26,1	Polynucléosis; large platelet ; thrombocytosis	228100/mm3
6	female.	9 years 5months	HB13,2g/dl VGM 66 ,2fl TGMH 22,7pg	thrombocytosis	191100/mm3
7	female.	5 years 3 months	HB 10.8g/dl VGM70,4fl TGMH 24pg	Schizocytes, thrombocytosis; Erythroblastosis.	99400/mm3
8	Male.	5months	HB 10.4g/dl VGM68,9 fl TGMH23,6pg	Dacryocytes; platelet clusters.	70500/mm3
9	female.	5 Years 10 Months	HB 7,6g/dl VGM 95,8fl TGMH31,1pg	Erythroblastosis (14%). Trophozoïte of Plasmodium falciparum	346900/mm3
10	female.	9years 7months	HB 9,2g/dl VGM86,8fl TGMH 28,4pg	Body of Howell Jolly, acidophilic erythroblast.	171100/mm3
11	Male.	5 Years 10 Months	HB 8,2g/dl VGM 82.2 fl TGMH 27,2pg	Erythroblastosis; leukocytosis, thrombocytosis.	297700/mm3
12	Male.	6 years	HB 11,4g/dl VGM 67,4fl TGMH23,1pg	Polynucléosis, giants polynuclears , thrombocytosis, large platelet, platelet clusters.	70,200/mm3

The number 3 which showed no signs of hemorrhage has been transfused immediately after the sample collection because of the severity of the anemia. The numbers 1 and 10 have a functional asplenia and must be strictly followed on the infectious level. The folic acid supplementation of numbers 1, 2, 4 and 12 must be assessed to ensure that the treatment is followed and with the correct dose. All of these patients must in principle be put under hydroxyuré because of hypercytoses: leukocytosis, thrombocytosis high réticulocytosis and erythroblastosis. A case of mononucleosis syndrome which must be explored. The presence of the trophozoïte of Plasmodium falciparum on the smear of one of the patients recalls that the prevention of malaria must be of rigor in the sickle cell disease.

Table II Molecular Study

On the rs1427407 the mutation of the 26 th nucleotide G>T is favorable to the lifting of the repressor of the synthesis of the hemoglobin F. Patients numbers1, 2, 4, and 12 have a heterozygous mutation while the numbers 9 have the mutation in the homozygous

On the rs10189857 the mutation of the 26th nucleotide A>G is favorable to the lifting of the repressor of the synthesis of the hemoglobin F. Patients numbers 1, 2.3 and 12 have the mutation in heterozygous, whereas the numbers 5.6 have the mutation in homozygous.

On the rs9399137 the mutation of the 27th T nucleotide>C is favorable to the lifting of the repressor of the synthesis of the hemoglobin F. Only patient number 10 has a mutation in the heterozygous.

Patient number	Sex	Genotype Beta	BCL11A Rs10189857	BCL11A Rs1427407	HMIP Rs9399137	(Xmn1) Rs7482144	Alpha genotype
1	Male	S/S	A/G	G/T	T/T	-/-	-3.7/αα
2	female	S/C	A/G	G/T	T/T	-/-	Aα/αα
3	Male	S/C	A/G	G/G	T/T	-/-	Aα/αα
4	female	S/S	A/A	G/T	T/T	-/+	-3.7/αα
5	female	S/S	G/G	G/G	T/T	-/-	Aα/αα
6	female	S/C	G/G	G/G	T/T	-/-	-3.7/αα
7	female	S/C	A/A	G/G	T/T	-/-	-3.7/αα
8	Male.	S/C	A/A	G/G	T/T	-/+	Aα/αα
9	female.	S/S	A/A	T/T	T/T	-/-	Aα/αα
10	female	S/S	A/A	T/T	C/T	-/-	Aα/αα
11	Male	S/S	A/A	G/G	T/T	-/+	Aα/αα
12	Male	S/C	A/G	G/T	T/T	-/-	Aα/αα

The mutation in -158 on the promoter of the gene ɣG(C>G) rs7482144 (Xmn1) is associated with a persistence of the hemoglobin F. Patients bearing the numbers 4.8, and 11 are heterozygous for a hereditary persistence of fetal hemoglobin.

Patients numbers 1, 4, 6.7 have an Alpha Thalassemia of type $\alpha^{-3,7,}$ which means that they have a concentration intra-Erythroid of hemoglobin S lesser compared to a patient of the same phenotype without Alpha Thalassemia. It is a contributing factor to the decrease of the sickling. They all have a microcytosis (Table I), which must not be placed under the account of a iron's deficiency or of an inflammation even if the association is not excluded. The peripheral smear did not guide us to the presence of the RBCS pitted. The gap-PCR has solved the problem.

Table III molecular study (continued)

Patient number	Sex	Genotype Beta	Haplotype	G6PD Deficiency	UGTA typing1
1	Male	S/S	Benin/Benin	A- Hemi	5/7
2	female	S/C	Benin/Cameroun	Normal	5/7
3	Male	S/C	Benin/Cameroun	Normal	7/7
4	female	S/S	Benin/Sénégal	Normal	6/6
5	female	S/S	Benin/Benin	A- hetero	6/7
6	female	S/C	Benin/Cameroun	Normal	6/6
7	female	S/C	Benin/Cameroun	Normal	6/8
8	Male	S/C	Sénégal/Cameroun	Normal	6/7
9	female	S/S	Benin/Benin	Normal	7/8
10	female	S/S	Benin/Benin	Normal	7/8
11	Male	S/S	Benin/Sénégal	Normal	5/7
12	Male	S/C	Benin/Cameroun	Normal	6/7

Patients numbers 2, 3, 6,7 and12 which are composite heterozygous S/C are the haplotype Benin for the S and haplotype Cameroon for the C. The patient number 8 which S/C also has a haplotype Senegal for the S and Cameroon for the C. No haplotype Benin for the C. An origin of the Central Africa has not been found among relatives in the ascending line of the propositus.

Patients homozygous S/S (4 and11) are composite heterozygous at the level of their haplotype: Senegal / Benin.

Patients1, 5, 9, 10 are homozygous S/S and homozygous Benin/Benin for the haplotype. We have not encountered cells ghosts or hémighosts on the peripheral smear but the patient (number1) has a G6PD's deficiency and the patient (number 5) is a driver of the tare. This means that the peripheral smear does not contribute when the spleen is functional. The systematic biochemical dosage of the G6PD can be negative because of the highréticulocytosis among sickle cell disease patients in general. The best strategy for diagnosis is therefore the molecular biology which also allows to detect the carriers.

About the disease of Gilbert, the patients 3, 9, and 10 carry the favorable mutations of the disease **(20, 21)**

Discussion

The analysis of the CBC, allows us to say that it would be wise for these patients to be put under hydroxyuré to reduce the hypercytosis ,the erythroblastosis, and the dense cells which are factors favoring the occurrence vaso-occlusive crises. The folic acid supplementation should be reviewed because some patients present on their smear giants polynuclear and larges platelets even macropolycytes. The anti-infectious monitoring must be more rigorous in other (patients 1, 4, 9, and10) because of the functional asplenia, the mononucléosique syndrome and the presence of the trophozoïte of Plasmodium falciparum. The anemia's are either microcytic hypochromic regenerative or aregeneratives, or normocytic normochromic regenerative and must be explored.

Different SNPS have been described either cis: Xmn1 in 11p15 and other, or Trans: 8q, XP22, 2p15, 6Q23.investigations are continuing to find other SNP. The study focused only on the most frequent and significant in our context. The SNP act in synergy and it takes at least three variants of mutation in sequence of reference to act on the expression of the hemoglobin F and the frequency of painful crises with sickle cell disease **(5, 22)** .None of the patients studied met three variants of mutation, they have at the maximum two mutations: Patients 1, 2,5,10 and 12 have in association deleterious factors. This is explained because the Haplotypes encountered are of type Benin who has not spontaneously has a high rate of fetal hemoglobin **(23)** . There is a positive correlation between the increase in the hemoglobin F due to variants of SNPs with the hemoglobin concentration, erythrocyte indices and a negative correlation with the leucocytes and platelets. The total concentration of hemoglobin would increase by increase of cells F without increase of their concentration intra-cellular of hemoglobin, and the number of leukocytes and platelets would decrease. Some variants would increase the erythrocyte indices. **(24)** .This would explain may be the mechanism of action of the hydroxyuré which is until now incompletely understood. The Bantu Haplotypes and Cameroon which are very rarely accompanied without treatment of hemoglobin F are modified by the hydroxyuré **(25)** . If the SNPs do not decrease the concentration intra-cellular hemoglobin levels, this is not the case of the Alpha Thalassemia. It decreases the concentration intra-cellular of hemoglobin S and therefore the severity of the disease. Four of the patients have a thalassemia $\alpha^{-3,7}$ of which two are associated with two SNPS favorable to the synthesis of hemoglobin F. among these two, one to a deleterious association which is the G6PD deficiency. The G6PD deficiency, associated with the homozygous sickle cell anemia S/S, is two different causes of hemolysis different constitutional. The preventive treatment of secondary of these two pathologies is different and this association is responsible for the occurrence of syndrome of Moyamoya **(26, 27, 28)** if patients are not put early under hyper transfusion regime or under hydroxyuré **(25)** . A communication (not published) of the Clinical Network of sickle-cell disease says that these two treatments are equivalent. The typing of the promoter of UGTA1 **(21)** has highlighted a disease of Gilbert by homozygous mutation (TA)7/(TA)7 in a patient composite heterozygous S/C and two cases of heterozygous mutation composite (TA)7/(TA)8 in two patients homozygous S/S. The Gilbert's disease if it is benign, associated with sickle cell disease, she is a purveyor of vesicular lithiasis with its possible complications. Even if the precipitating factors are

often superimposable for the two disorders, there are preventive specificities monitoring **(20)** and therapeutic of The Gilbert's disease which must be taken into account **(29)**.Traditionally it is the chronic elevation of bilirubinate free as the diagnosis of Gilbert's disease is suspected. The Sickle cell anemia is a hemolytic disease therefore the increase of the free bilirubinate does not direct systematically toward Gilbert's disease but it placing on the account of the Sickle cell disease. The molecular biology at the research of the Gilbert's disease should be systematic in the syndromes Major with sickle cell disease.

Conclusion

The Sickle cell anemia is a monogenic disease which is modulated by factors polygenic in cis and Trans which are important to know for an adapted therapeutic care. Routine hematological examinations and biochemistry are insufficient for highlighting some factors modulators of the gravity of the Sickle cell disease. Molecular biology is the ultimate solution to resolve these complicated situations.

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