Original Article

Impact of maternal DNA contamination of fetal DNA in chorionic villi on prenatal diagnosis of sickle cell anemia

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ABSTRACT

Objective: The study aim was to determine the hemoglobin genotypic and allelic distributions in fetal population, and to quantitatively evaluate the effect of heterozygous maternal DNA contamination of homozygous fetal DNA in chorionic villi, on fetal hemoglobin genotypes.

Materials and Methods: A descriptive, cross sectional study of amplification refractory mutation system-polymerase chain reaction (ARMS-PCR) analysis of fetal hemoglobin genotype using DNA from chorionic villi and maternal venous blood. The primary fetal hemoglobin genotypes were obtained in the first phase and the secondary hemoglobin genotypes after contaminating homozygous primary genotypes (HbAHbA and HbSHbS) with varying proportions of heterozygous maternal DNA (HbAHbS). Data analysis was done with Micosoft Excel 2010 statistical package and Chi-square (goodness-of-fit).

Results: There was no statistically significant deviation in the hemoglobin genotypic and allelic counts between the observed and the expected counts in the fetal population based on Mendelian expectation. Contaminating homozygous fetal DNA with >11.1% (0.5 µl) of heterozygous maternal DNA produced significant change in fetal hemoglobin genotype results. Homozygous hemoglobin genotypes HbAHbA were affected more than HbSHbS.

Conclusion: Study established Mendelian distribution in the fetal population and the levels of heterozygous maternal contamination of homozygous fetal DNA that resulted in significant risk of misdiagnosis.

Key words: ARMS-PCR; fetal; maternal contamination; sickle cell anemia.

Introduction

Sickle cell anemia (SCA) is a common single gene defect in sub-Saharan Africa, India, Saudi Arabia, and Mediterranean countries and also the first molecular disease diagnosed through the application of molecular techniques.[1-3] It is an autosomal recessive condition that most commonly follows mating between heterozygous couples and less commonly after mating between homozygous and heterozygous couple. The distribution in the fetal population is expectedly Mendelian. The gold standard for early prenatal diagnosis is chorionic villous sampling (CVS) for molecular diagnosis with polymerase chain reaction (PCR) based technique.[4] The sample consists of fetal cells in chorionic villi that are potentially contaminated with maternal decidua and blood. Complete separation of cells and DNA of chorionic villi origin from those of maternal decidua remains a challenge,

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because of the anatomical relationship between the maternal decidua and the chorionic villi. This contributes significantly to prenatal misdiagnosis of SCA, particularly with PCR-based protocols that amplify DNA.[5,6] Misdiagnosis due to co-amplification of maternal HbAHbS DNA sequences is of serious medical and genetic consequence, especially, with homozygous fetal hemoglobin genotype (HbAHbA and HbSHbS) that may be misreported as heterozygous (HbAHbS) fetal hemoglobin genotype.[5-7]

The aim of the study was to determine the fetal hemoglobin genotypes distribution in early pregnancy and the quantitative effect of maternal DNA contamination of homozygous fetal DNA on hemoglobin genotype result.

Materials and Methods

This was a descriptive cross sectional study of controlled molecular laboratory experiment designed to determine the fetal hemoglobin genotypes distribution and evaluates the quantitative effect of heterozygous maternal DNA contamination of homozygous fetal DNA obtained from chorionic villi sample.

Participants and approval

The study ethical approval was obtained from the Human Ethics and Research Committee of the Olabisi Onabanjo University Teaching Hospital, Sagamu, Ogun State. The subjects were pregnant women attending antenatal clinics in Olabisi Onabanjo University Teaching Hospital, Sagamu, while subject samples were the chorionic villi that were analyzed at the molecular laboratory units of department of Cell Biology and Genetics, University of Lagos and High Rocks Fetal Medicine and Genetic Diagnosis Center. Lagos non-directional counseling was provided and signed informed consent was obtained from the pregnant women between 11^{+0} weeks and 13^{+6} weeks. Seventy six pregnancies were studied based on Cochran formula for determination of sample size.[8]

Chorionic villous sampling and sample dissection

Transabdominal CVS using aspiration method was used to obtain sample. The aspirated chorionic villi were dissected free of maternal contaminants and further examined under inverted microscope to ensure complete dissection of maternal decidua contaminant [Figure 1].

DNA extraction and assessment

DNA was extracted from 5 mg to 15 mg of chorionic villi and 0.5 ml of parental venous blood using modification of boiling methods.[9] DNA was assessed using NanoDrop (ND-1000) spectrophotometer. The $A^{260}/_{280}$ was used to assess the purity of DNA and >1.8 was considered satisfactory. The A_{260} was used to determine concentration. Samples for analysis were >50 ng/μl.

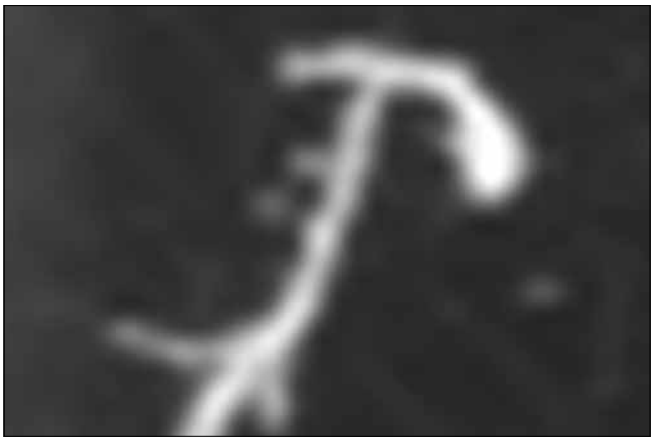

Figure 1: Dissected chorionic villi

Amplification refractory mutation system-polymerase chain reaction analysis

The molecular analysis was done using modification of ARMS-PCR protocol described by Newton *et al*.[10] The PCR MasterMix was prepared with PCR mix β\{deoxynucleotides (dNTPs), distilled water (dH_2O), $MgCl_2$, 10X buffer\}, ARMS Primers {Forward (Mutant & Normal) and Reverse (Common C)}, and Taq DNA polymerase enzyme. The forward primers contains 30 nucleotide base each (HbS M: 5'- CCC ACA GGG CAG TAA CGG CAG ACT TCT GCA-3: and HbS N: 5' CCC ACA GGG CAG TAA CGG CAG ACT TCT GCT-3') while the reverse primer has 20 nucleotide base (HbS Com C: 5'-ACC TCA CCC TGT GGA GCC AC-3'). The PCR products were loaded into 16 gel wells representing 8 reactions. The first three reactions were positive controls which were confirmed HbSHbS, HbAHbS, and HbAHbA genotypes respectively. The positive controls served as internal controls to confirm the optimum workings of the PCR. The next two reactions are parental samples previously confirmed as HbAHbS genotypes. The following two reactions are fetal samples of unknown hemoglobin genotypes, while the last reaction is the negative control. The negative control was to rule out contamination.

The secondary fetal hemoglobin genotypes that were obtained after heterozygous maternal DNA contamination of homozygous fetal sample were determined in the second phase. Each reaction was made up of positive controls (HbAHbA, HbAHbS, and HbSHbS), maternally contaminated homozygous fetal DNA , 0% (0.00 μl), 5.9% (0.25 μl), 11.1% (0.50 μl), 15.8% (0.75 μl), and 20.0% (1.00 μl) respectively and the negative control. The PCR programme used was 28 cycles of denaturation (93°C/3 minutes), annealing (67°C/1 minute), and extension (72°C/1 minute).

Gel documentation/ultraviolet transillumination

PCR products were electrophoresed in a 1% agarose gel stained with ethidium bromide and the gel was visualized using the ultraviolet light filter on the Gel Imager (Alpha innotech 3400).

Data management and analysis

Data was analyzed with Microsoft Excel 2010 statistical software package. The statistical significance between observed and expected values was evaluated with χ^2 (goodness-of-fit) test and result considered significant at $P < 0.05$. Degree of freedom ($df_{0.05}$) = number of genotypes – number of alleles. The observed hemoglobin genotypes in the study population were derived from counts before maternal contamination (primary fetal hemoglobin genotypes) and after maternal contamination (secondary fetal hemoglobin genotypes). The observed count of primary and secondary fetal hemoglobin genotypes at each proportion of maternal DNA contamination were counted and statistical significance of the change in genotype observed was assessed with χ^2, at $P < 0.05$.

Results

The gel images of hemoglobin genotypes (HbAHbA, HbAHbS, and HbSHbS), observed under ultraviolet transillumination are shown in Figure 2a, b, and c, respectively. In each sample analyzed, the hemoglobin genotype is shown by the presence of band in the left pair of a gel well (mutant allele), both left and right pair of gel well (mutant and normal allele) or right pair of the gel well (normal allele).

The observed and expected hemoglobin genotype distributions based on the Mendelian ratio from heterozygous mating in Table 1a, shows that the observed count (13) of HbSHbS was lower than the expected count of 19, while observed counts of both HbAHbA (22), and HbAHbS (41) were above the expected counts of 19 and 38 respectively. These differences in distribution are; however, not statistically significant ($P = 0.26$). The distributions of the two alleles responsible for the genotypes are shown in Table 1b. The observed allelic counts were higher for HbS (85) and lower for HbS (67) compared with the expected counts of 76 based on Mendelian principle. Chi-square analysis shows statistically insignificant difference ($P = 0.18$). Table 1b shows the genotypic and allelic frequencies in the fetal population. The frequency of HbA allele is higher (0.56) than the expected while that of HbS (0.44) lower than the expected frequency of (0.50) based on Mendelian principle. The frequencies of HbAHbA (0.29) and HbAHbA (0.54) were more, while that of HbSHbS (0.17) less than the expected frequencies based on Mendelian principle.

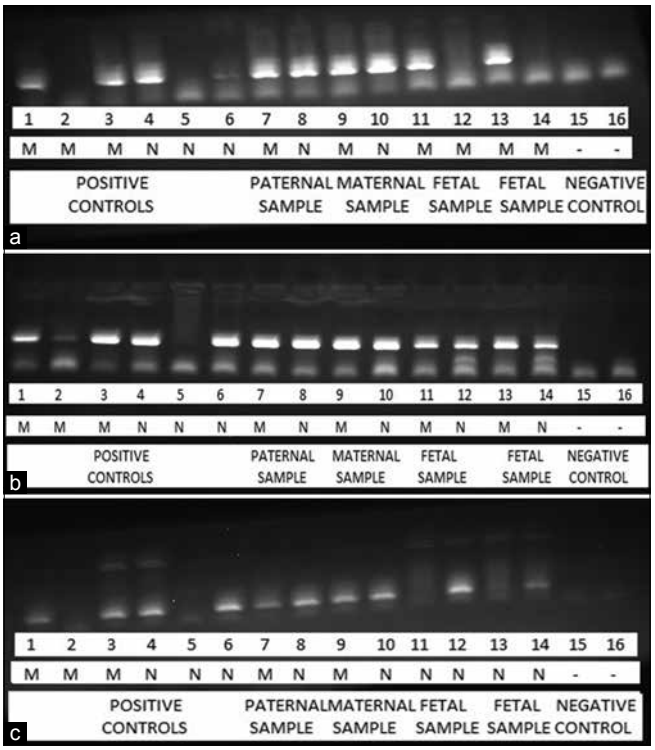

Figure 2: Ultraviolet Documentation of Gel Images of Primary haemoglobin Genotypes. The positive controls samples in wells 1-6, were confirmed by the display of bands that implies the presence of an allele complementary to the specific primer in the reaction (right well lane band for HbSHbS, both wells lane bands for HbAHbS and left well lane band for HbAHbA). The parental samples in wells 7-10 show bands in the lanes of both pairs of well, confirming HbAHbS. (a) Fetal sample in gel wells 11-14 reveals bands in the lanes of only the left wells containing mutant primer as confirmation HbSHbS. Absence of band from the lanes of wells 15 and 16 rules out contamination. (b) foetal sample in gel wells 11-14 reveal bands in the lanes of the right and left wells containing mutant and normal primers respectively, as confirmation HbAHbS samples. Absence of bands in lanes of gel wells 15 and 16 ruled out DNA contamination. (c) Fetal sample in gel wells 11-14 reveals bands in the lanes of right tubes containing normal primers, as confirmation HbAHbA samples. Absence of bands in lanes of gel wells 15 and 16 ruled out DNA contamination

Table 1: Foetal haemoglobin genotypic and allelic distribution and frequency

(a) Fetal haemoglobin genotypes and alleles distribution				
Haemoglobin genotypes and allelic count				
Haemoglobin	**HbAHbA**	**HbAHbS**	**HbSHbS**	**Statistical significance**
Observed counts	22	41	13	χ^2=2.60
Expected counts	19	38	19	P=0.27
Allele	HbA	HbS		
Observed counts	85	67		χ^2=2.12
Expected counts	76	76		P=0.18

(b) The fetal genotypic and allelic frequencies					
	Alleles		**Genotypes**		
	HbA	**HbS**	**HbAHbA**	**HbAHbS**	**HbSHbS**
Observed	0.56	0.44	0.29	0.54	0.17
Expected	0.50	0.50	0.25	0.50	0.25

The outcome of contaminating fixed amount of fetal DNA with varying proportions of heterozygous maternal

DNA on the 35 homozygous fetal hemoglobin genotypes (HbAHbA and HbSHbS) are shown in Tables 2a and 2b. With respect to HbAHbA samples [Table 2a], contaminating fetal DNA in chorionic villi sample with 5.9% (0.25 µl) of maternal DNA sample did not result in change of hemoglobin genotype result in any of the samples. Eight (36.4%) primary fetal hemoglobin genotype results changed, when fetal DNA sample was contaminated with 11.1% (0.5 µl) and 15.8% (0.75 µl) of maternal DNA sample, while 18 (82.3%) primary fetal hemoglobin genotype results changed when fetal DNA sample was contaminated with 20.0% (1.0 µl) of maternal DNA sample.

The results after heterozygous maternal DNA contamination of homozygous fetal DNA in respect of primary fetal hemoglobin genotype HbSHbS results were as follows [Table 2b]: There was no change observed in primary fetal hemoglobin genotype result when fetal DNA in chorionic villi sample was contaminated with 5.9% (0.25 μl) of maternal DNA sample. Two (11.1%) fetal primary fetal haemoglobin genotype results changed, when fetal DNA was contaminated with 11.1% (0.5 µl) and 15.8% (0.75 µl) respectively of maternal DNA, while eleven (84.6%) fetal primary fetal hemoglobin genotype results changed when foetal DNA in chorionic villi samples was contaminated with 20.0% (1.0 µl) of maternal DNA sample.

The statistical significance of the changes in primary homozygous fetal hemoglobin results are shown in Table 3a and b. The analysis confirms that all the changes observed, were statistically significant.

Discussion

Human hemoglobin polymorphism with respect to sickle cell anemia is of considerable interest in fetal medicine practice as well as in molecular genetics. The study observed that heterozygous maternal DNA contamination of 11.1% of homozygous fetal DNA in chorionic villi sample was sufficient to produce statistically significant change in the primary fetal hemoglobin genotype results. This is against the 30% maternal contamination previously reported using restriction fragment polymorphism (RFLP).[5] This finding confirms the fact that PCR-based protocol is relatively more sensitive to the presence of DNA in comparison with RFLP technique.

This study is the first report available to us in literature on the outcome of heterozygous maternal DNA contamination of homozygous fetal DNA on fetal hemoglobin genotype result using a PCR-based technique in Nigerian population. This can be explained by the fact that the ARMS-PCR technique for molecular identification of different mutations only became increasingly applied for molecular diagnosis of SCA especially in Nigeria. The possibility of misdiagnosis which was however first reported in 1986, was based on the use of RFLP technique and more recently reported to occur with the use of PCR based molecular protocol.[5,7,11]

This study finding is presented as a reliable and accurate data of primary fetal hemoglobin genotype because the extracted DNA were of high quality and also the molecular tool used (ARMS-PCR) is very sensitive to the presence of low proportions of DNA contamination. The observed fetal hemoglobin genotype distribution was not statistically different from expected distribution based on Mendelian principle. This could have implications for the understanding of the roles of possible selections pressures that operate during this gestational period. In particular, the heterozygous advantage for HbAHbS observed in the study population at this early gestation could be

Table 2: Change in foetal haemoglobin genotype results after contaminating fixed (4.0 ul) foetal DNA with varying proportions of maternal DNA

(a) Change from HbAHbA to HbAHbS		
Amount of Maternal DNA Sample (µl)	**Proportion of Maternal DNA Contamination of Foetal DNA in Chorionic Villi Sample (%)**	**Change in foetal genotype (%)**
0.00	0.0	0
0.25	5.9	0
0.50	11.1	36.4
0.75	15.8	36.4
1.00	20.0	82.3
(b) Change from HbSHbS to HbAHbS		
Amount of Maternal DNA Sample (µl)	**Proportion of maternal DNA Contamination of Foetal DNA in Chorionic Villi Sample (%)**	**Change in foetal genotype (%)**
0.00	0.0	0
0.25	5.9	0
0.50	11.1	11.1
0.75	15.8	11.1
1.00	20.0	84.6

Table 3: Statistical analysis of changes in primary foetal haemoglobin genotypes

(a) Primary haemoglobin genotype HbAHbA			
Haemoglobin genotype (*n*=22)	**Maintained (%)**	**Changed (%)**	**Statistical significance**
4.00+0.25	22 (100.0)	(0)	
4.00+0.50	14 (63.6)	8 (36.4)	χ^2=19.17
4.00+0.75	14 (63.6)	8 (36.4)	*P*=0.0003
4.00+1.00	4 (18.8)	18 (1.2)	
(b) Primary haemoglobin genotype HbSHbS			
Haemoglobin genotype (*n*=13)	**Maintained (%)**	**Changed (%)**	**Statistical significance**
4.00+0.25	13 (100.0)	(0)	
4.00+0.50	11 (88.9)	2 (11.1)	χ^2=19.39
4.00+0.75	11 (88.9)	2 (11.1)	*P*=0.0002
4.00+1.00	2 (15.4)	11 (84.6)	

postulated to be due to the effect of severe malaria parasitization from maternal blood and through the placenta barrier to the fetus. The possibility is further enhanced by the fact that malaria is endemic in the population, while the use of intermittent preventive treatment (IPTp) is not instituted at this gestational age.[3] In addition, the barrier function of the placenta appears to be most effective from the second trimester, thus leaving the fetus exposed in the first trimester, when sequestration of *P. vivax* in the placenta, has been demonstrated.[12,13]

The boiling protocol for DNA extraction and ARMS-PCR technique for molecular analysis has been shown in this study to be feasible methods that is appropriate, especially in low resource settings to produce satisfactory quality and quantity of DNA for prenatal diagnosis of SCA. Other studies have also shown their suitability for extracting DNA from small sample quantity such as the chorionic villi.[14-16]

The implication of the study finding is that a smaller proportion of heterozygous DNA contaminant is enough to produce significant change in homozygous primary DNA results. Although both groups of homozygous hemoglobin genotypes were affected, it is more pronounced in HbAHbA results, thus suggesting that a misdiagnosed HbAHbS result is more likely to have been HbAHbA instead of HbSHbS.

Conclusion

The study demonstrated the fetal hemoglobin genotypes distribution and also established the levels of heterozygous maternal contamination of homozygous fetal DNA that resulted in significant risk of misdiagnosis. The fetal hemoglobin genotype distribution is a justification for studies into the operations of selection pressures *in-utero*, especially the preciserole of congenital malaria. The study would also stimulate awareness of the possibility of prenatal misdiagnosis and should justify researches into methods for reducing maternal contamination in fetal sample obtained from CVS.

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Nil.

Conflicts of interest

On behalf of all authors, the corresponding author states that there is no conflict of interest.

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