C-Window Peaks on CE-HPLC are Extremely Rare in Northern India, and Only Infrequently Represent HbC

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Abstract Hemoglobin C (HbC, *HBB:c.19G > A*) is a structural variant that has been reported rarely from India. This was a retrospective review of all high performance liquid chromatography (HPLCs) submitted over a 14 year period to a tertiary care center in North India with an aim of finding hemoglobins that elute in the C-window. Of the 32,364 HPLCs screened, 6 cases showed peaks in the C-window. Of these 6 cases, only two cases contained hemoglobin C. These were one case each of HbC/β thalassemia and compound heterozygosity for HbC and HbD. There were 4 cases which showed very similar red cell indices and chromatograms with multiple peaks eluting in D-window, C-window and an additional peak with a retention time of 4.74 min. These four cases were compound heterozygous for an α chain variant HbQ-India and a β-chain variant HbD.

Keywords C-window · Hemoglobin C · India · D-Q hybrid

Introduction

Hemoglobin C (HbC) is a structural variant of normal adult hemoglobin which occurs due to a point mutation at position 6 of the β-globin chain (βGlu-Lys, *HBB:c.19G > A*). HbC is asymptomatic in heterozygous state, causes a mild hemolytic anemia in homozygous state but is related to sickling crises when inherited in combination with Hemoglobin S (HbS, *HBB:c.20A > T*) [1]. It is

common in Africa [1] but is exceedingly rare in India and other South-east Asian countries. [2–5] The first Indian family of HbC/β thalassemia was reported from our institution [6]. There are only a few cases of HbSC disease reported from India [4, 7]. Large series of patients describe only an occasional case of HbSC [4] or do not contain any case of HbC at all [2, 3, 5].

Hemoglobin high performance liquid chromatography (HbHPLC) using the cation exchange principle is the most frequently employed modality to detect variant hemoglobins. Detection of hemoglobin variants is based upon the peak area and the retention times. The β-thalassemia Short[®] program is a commonly used program at most centers and it divides the run into windows dependent on retention times. C-window comprises of all hemoglobins that elute between a retention time of 4.90–5.30 min [2, 8]. The hemoglobin variant HbO-Arab (*HBB:c.364G > A*) is also known to elute in the C-window on the Biorad Variant II High performance liquid chromatography (HPLC) system [Bio-Rad laboratories, Hercules, USA] with the β-thalassemia Short[®] program [8]. An α-globin chain variant, HbO-Indonesia (*HBA1:c.349G > A*) with a similar electrophoretic mobility as HbO-Arab also elutes at a retention time of ~4.90 min and a single case has been reported from India [9].

In addition, there are a few case reports of double heterozygous Q-India/D-Punjab cases in which the D-Q hybrid eluted in the C-window with presence of additional peaks of HbQ-India, HbD-Punjab and HbA [10–15].

With this background, we performed a retrospective analysis on all HPLCs over a 14 year period to look for cases with C-window peaks of >5% to determine the frequency of variants eluting in the C-window on HbHPLC using the β-thalassemia short program in North India.

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Materials and Methods

This is a retrospective analysis conducted in the Department of Hematology of a tertiary care hospital in New Delhi. All HPLCs requested between 1st January 2002 and 31st December 2015 were reviewed for presence of C-window peaks of >5%. This cut-off was taken as 5% to exclude small insignificant peaks. The Biorad Variant I system (BioRad laboratories, California, USA) with variant β -thalassemia short program was used from 2001 to 2009 and the rest of the analyses were done on the Biorad Variant II system (BioRad laboratories, California, USA) using the same program.

Results

Overall a total of ~32,364 HPLCs were done during this 14-year period. From these, 6 cases of hemoglobin peak >5% eluting in the C-window were isolated with an overall prevalence of 0.019% (6/32,364) of all HPLCs. The details of these 6 cases follow.

Case 1 and 2. A 22 year old female was referred to Medical Genetics for prenatal counseling. Her husband was a known case of β -thalassemia trait with a frameshift 8/9 mutation. The patient's HbHPLC showed a predominant peak in C-window of 81.6% with a retention time of 5.24 min along with elevated HbF and HbA2 levels [Figure 1a, case 1]. The RBC indices were thalassemic. The red blood cell (RBC) indices and HbHPLC peak characteristics are presented in Table 1. The findings were confirmed on hemoglobin electrophoresis at alkaline and acid pH [Fig. 2, lane 1]. Parental HbHPLC was performed as differentials being considered were homozygous HbC and HbC/ β thalassemia. Her father showed thalassemic

RBC indices with HbA2 of 5.6% and was a heterozygous β -thalassemia and genetic analysis revealed a frameshift 8/9 mutation. Mother's Mentzer index was 12.6 and the indices were also thalassemic. The HbHPLC showed two predominant peaks corresponding to HbD-Punjab and HbC with values of ~41.9 and ~47.5% and retention times of ~4.27 and ~5.20 min respectively [Figure 1b, case 2]. Hence she was a compound heterozygote for HbD-Punjab/HbC which was again confirmed hemoglobin electrophoresis at alkaline and acid pH [Fig. 2, lane 4]. The band was confirmed as HbC as its electrophoretic mobility on alkaline pH was in CEOA2 region and in acid electrophoresis, the mobility matched the C-region. This is important a HbO-Arab also has a similar mobility on alkaline electrophoresis but on acid electrophoresis it moves with HbA. This confirmed the propositus as a case of HbC/ β thalassemia. The red blood cell (RBC) indices and HbHPLC peak characteristics of the propositus and the mother are presented in Table 1. The entire family hailed from Punjab. This family was published as the first report of HbC from India in 2007 [6]. The overall frequency of HbC in our cohort was 0.006%.

Case 3–6 showed very similar RBC indices and HPLC findings. Case 3 and 4 were 26 year and 28 year old females who underwent HPLC as a part of antenatal screening. Both patients had a normal CBC. The red cell indices are presented in Table 1. The HPLC revealed a dominant HbA peak with presence of ~31.3 and ~31.5% peaks in D-window, both with a retention time of 4.11 min. Both patients had an unknown peak with a retention time of 4.74 and 4.76 min respectively, corresponding to HbQ-India. In addition, there was a peak in the C-window with retention times of 4.97 and 4.99 min respectively with a peak amount of ~7.2%. This peak is the D-Q hybrid which is known to elute in the C-window.

Fig. 1 **a** The HPLC chromatogram from the propositus shows a large peak in C-window with retention time 5.24 min with a second prominent peak corresponding to HbF indicating a compound heterozygous HbC/ β thalassemia. **b** HPLC chromatogram from the

mother shows two peaks corresponding to HbD-Punjab and HbC indicating a double heterozygous state for HbD-Punjab and HbC. The small peak just before HbC in both the chromatograms likely represents post-translationally modified HbC

Table 1 Red cell indices and peak characteristics of all cases with hemoglobins eluting in C-window

	Case 1	Case 2	Case 3	Case 4	Case 5	Case 6
Age (years)/sex (M/F)	22/F	48/F	26/F	28/F	6/M	8/M
Hb (g/dL)	9.4	14.1	13.4	13.1	12.7	12.3
MCV (fL)	60.9	72.3	84.4	84.4	75.5	69.8
MCH (pg)	20.1	24.7	28.7	29.3	25.2	23.1
MCHC (g/dL)	33.0	34.2	34.1	34.8	33.4	33.1
RBC count (Mill/ μ L)	4.94	5.72	4.65	4.47	5.02	5.33
HbA0 (%)	1	2.9	43.4	43.8	46.3	45.8
HbF (%)	10.1	1	0.2	0.2	0.2	0.2
HbA2 (%)	5.8	1.9	2.0	1.5	1.9	1.6
C-Window with RT (%; min)	81.6; 5.24	47.5; 5.20	7.2; 4.97	7.2; 4.99	6.0; 4.98	6.0; 4.97
D-Window with RT (%; min)		41.9; 4.27	31.5; 4.11	31.3; 4.11	29.7; 4.14	28.4; 4.13
Unknown peaks with RT (%; min)			9.3%; 4.74	9.1%; 4.76	9.0%; 4.75	9.4%; 4.75

Fig. 2 **a** Hemoglobin electrophoresis at alkaline pH, lane 1 from case 1 shows a predominant band in CEOA2-region and a smaller band in the F region. Lane 4 is the case 2, mother of patient 1 and shows two bands- one in CEOA2-region and other in SDG region when going

from cathode to anode. **b** Hemoglobin electrophoresis at acid pH from the same patients applied in the corresponding lanes confirms the diagnosis

Case 5 and 6 were male siblings aged 6 and 8 years respectively. Both the siblings underwent HPLC to rule out a possibility of thalassemia trait as they had microcytic hypochromic red cell indices. However, the Mentzer index was 15.0 and 13.1 respectively. The red cell indices and peak characteristics are presented in Table 1. Similar to case 3 and 4, HbA constituted the predominant hemoglobin. There were peaks corresponding to HbD-Punjab of ~29.7 and ~28.4%, an unknown peak at ~4.75 min amounting to ~9.0 and ~9.4% and corresponding to peak characteristics of HbQ-India. In addition, there was a peak in C-window of ~6.0% in both the siblings due to elution of the D-Q hybrid [Fig. 3a, b]. Hemoglobin electrophoresis at alkaline pH (SAS1, Helena Biosciences, Great Britain) showed 3 prominent bands in the HbA region, HbS region due to elution of HbD-Punjab and HbQ-India and in HbC-region due to the D-Q hybrid [Fig. 4, lanes 7 and 8]. Parental HbHPLC was also performed. The father's complete blood count was normal.

HbHPLC showed a peak in the D-window of ~33.7% with a retention time of 4.14 min and thus was a heterozygous HbD-Punjab. His hemoglobin electrophoresis at alkaline and acid pH findings also confirmed the above findings [Fig. 4, lane 9]. Mother's complete blood count was also normal but the HbHPLC revealed an unknown peak of ~17.9% at a retention time of 4.76 min. On hemoglobin electrophoresis at alkaline pH, the suspicion of HbQ-India heterozygosity was confirmed [Fig. 4, lane 10]. Thus, both the children were double heterozygotes for HbQ-India and HbD-Punjab.

Discussion

Only a handful of cases of HbC variant have been reported from India. Of these, an earlier report from our center described case 1 and case 2, the HbC/ β thalassemia and compound heterozygous HbD and HbC [6]. There are two

Fig. 3 a The HPLC chromatogram from the case 5 shows 4 predominant peaks- HbA, HbD-Punjab, HbQ-India and D-Q hybrid. The *solid arrow* marks the peak causes possibly by combination of α Q-India chains with δ -chains leading to variant HbA2 formation. **b** Chromatogram of the father of case 5 and 6 shows features

consistent with heterozygous HbD-Punjab. **c** Chromatogram of the mother of case 5 and 6 shows features consistent with heterozygous HbQ-India and the *dashed arrow* points to trails of HbQ-India peak that occurs possibly due to variant HbA2

Fig. 4 a Hemoglobin electrophoresis at alkaline pH of case 5 and case 6 (lanes 7 and 8) showed 3 bands from anode to cathode. These are- HbA2 + D-Q hybrid that co-migrate in the CEOA2-region, a band corresponding to SDG region and a third band corresponding to HbA. Lane 9 represents heterozygous HbQ-India seen in the mother while lane 10 represents the heterozygous HbD-Punjab state in father.

b Hemoglobin electrophoresis at acid pH of case 5 and 6 (lanes 7 and 8) shows two bands- one corresponding to migration of HbD-Punjab together with HbA and the other anodal to it due to HbQ-India and D-Q hybrid. Lane 10 belongs to the mother and shows two bands- HbA and a band anodal to HbA corresponding to HbQ-India

cases of HbSC reported, one as a case report [7] and another as a single case in a series of 543 abnormal chromatograms [4]. Most other large series do not describe even a single case of HbC alone or in combination with another hemoglobinopathy. [2, 3, 5] Our study confirms the fact that HbC is vanishingly rare in India with a total frequency of $\sim 0.006\%$ in all HPLCs over a 14 year period.

Rest of the cases where HbHPLC showed a peak in the C-window were actually cases of compound heterozygous HbQ-India/HbD-Punjab where the D-Q hybrid hemoglobin eluted in the C-window of HbHPLC and also had a similar electrophoretic mobility on alkaline pH. However, its mobility was different on the acid electrophoresis with no band corresponding to HbC. The first case of double heterozygous HbD-

Punjab/HbQ-India was described only in 2012 by Higgins et al. in a 39 year old female of Indian origin [9]. The subsequent series of two cases was published from New Delhi [10]. Since then, there have been a few case report describing this interesting co-inheritance of the α -chain variant HbQ-India and the β -chain variant, HbD-Punjab [11–15]. All these cases have been reported from India which is largely explained due to the geographical pattern of inheritance of these hemoglobins. HbQ-India is reported commonly in Sindhi community of India [15] and the reported prevalence is $\sim 0.43\%$ in various studies [16, 17]. HbD-Punjab has been reported from North-Western India is commonest in the Sikh community. The frequency reported from North India is $\sim 0.55\%$ [18].

Double heterozygosity for HbD-Punjab and HbQ-India produces an interesting pattern on HbHPLC with four predominant peaks corresponding to HbA, HbD-Punjab, an unknown peak corresponding to HbQ-India and a peak in the C-window corresponding to the D-Q hybrid. In all the cases reported till date on HbHPLC, the D-Q hybrid ranged between 6 and 8% while the HbQ-India varies between 9 and 10.5% and HbD-Punjab varies from 32 to 34% including our 4 cases [10–15]. It is important to note that, normally HbQ-India constitutes ~ 15 –20% in heterozygous state if not associated with iron deficiency or a concomitant β -thalassemia trait [16, 17] and in cases with double heterozygous HbD-Punjab/Q-India, the total amount of HbQ-India and D-Q hybrid also reaches an almost similar value. The same is true for HbD-Punjab that constitutes ~ 36 –40% in heterozygous state [18] and in the rare state described above, the sum of HbD-Punjab and D-Q hybrid constitutes an almost similar value. It is interesting to note that due to Q-India being an α -chain variant, the abnormal α Q-India chains combine with δ -chains and this variant HbA2 causes small peaks just at the point where Q-India merges with the baseline on HbHPLC. Similar small peaks are observed where HbQ-India peak ends and the D-Q hybrid starts as pointed in Fig. 3.

Double heterozygous state for HbD-Punjab/HbQ-India causes multiple abnormal peaks on HbHPLC but with the advent of capillary electrophoresis to diagnose hemoglobinopathies, it is necessary that differences between the two techniques should be understood. A double heterozygous HbD-Punjab/HbQ-India if resolved by capillary zone electrophoresis showed 4 peaks corresponding to HbA ($\sim 47.5\%$), HbD (42.3%), HbA2 + unknown peak in the HbC zone ($\sim 9.6\%$) and a small Z1 peak ($\sim 0.5\%$) [15]. This presumably occurs due to separation of D-Q hybrid with the HbA2 and the small Z1 peak observed could be due to combination of abnormal α -chains with δ -chains leading to formation of variant HbA2.

We did not find any cases of HbO-Arab or HbO-Indonesia in our series. These are also known to elute in the C-window on HbHPLC using the β -thalassemia short

program. While HbO-Arab is a β -globin chain variant, HbO-Indonesia is an α -chain variant with similar electrophoretic mobility as HbO-Arab and only a single case has been reported from India so far [9].

Conclusions

HbC is strikingly rare in India. If a C-window peak is seen in Indian patient, it should instigate further tests for confirmation. It is important to be cognizant of the fact that when α and β globin chain mutations are co-inherited, they produce intriguing patterns on HbHPLC as in addition to the β chain variant, α chain variant, HbA, there occurs a formation of heterodimer of abnormal α and β chains that may elute at different location than the original variant hemoglobins.

Compliance with Ethical Standards

Conflict of interest All authors declare that they have no conflict of interest.

Ethical Approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Human and Animal Rights This article does not contain any studies with animals performed by any of the authors.

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