Hemoglobin D-Punjab Homozygotes and Double Heterozygotes in Premarital Screening: Case Presentations and Minireview

Srdjan Denic and Abdul-Kader Souid

ABSTRACT

Hemoglobin D-Punjab is the most common variant of hemoglobin D. In premarital screening, molecular testing is often unavailable, and diagnosis (and marriage guidance) often relies on the hemoglobin analysis, family studies and epidemiological facts. The use of latter methods sometimes results in hemoglobin D-Punjab/ β -thalassemia double heterozygote being mistaken for its homozygote, which could be costly. We present the clinical and laboratory characteristics of hemoglobin D-Punjab phenotypes/genotypes in 15 individuals and review similar reports in the literature. We find that the quantity of hemoglobin D-Punjab in homozygotes is higher than in hemoglobin D-Punjab/ β -thalassemia double heterozygotes, its fraction > 92% being consistent with homozygosis. The limitations of this diagnostic criterion are discussed, and clinical severity of this and other hemoglobin D-Punjab double heterozygotes reviewed.

Keywords: Hemoglobinopathies; Sickle cell anemia; Thalassemia major; Population genetics; Prevention genetics; Community health services.

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I. INTRODUCTION

Hemoglobin D (Hb D) has more than a dozen variants among which Hb D-Punjab is most common [1], [2]. The variants differ at molecular level, but genetic tests are often unavailable in premarital screening. In practice, the diagnosis and marriage guidance often rely on the results of hemoglobin analysis, family studies and epidemiological knowledge. The use of latter methods has recently resulted in misdiagnosis of Hb D-Punjab/ β -thalassemia double heterozygotes [3], [4]. In addition, some uncertainty might exist about other Hb D-Punjab double heterozygotes due to marked geographic variations in frequency of the Hb D and other major hemoglobinopathies, small number of reports, evolving methodology of analysis of hemoglobinopathies, and sometimes confusing terminology [1], [5]. In this article, we present Hb D-Punjab carrier phenotypes, review similar reports in the literature, and categorize the phenotypes/genotypes into three clinical groups:

- i) Benign
- ii) Afflicted
- iii) Uncertain

II. MATERIALS AND METHODS

This is a retrospective study of individuals with Hb D-Punjab from a single hematology clinic in Al Ain, Abu Dhabi, United Arab Emirates (UAE). The study subjects

were either referred from the national premarital screening program (Abu Dhabi, UAE) or by members of their family. Laboratory and clinical information were collected from electronic medical records. The complete blood count and hemoglobin analysis were performed using a Cell-DYN Sapphire hematological analyzer (Abbott Diagnostics, Santa Clara, CA, USA) and high-performance liquid chromatography (HPLC; VARIANT IITM, Bio-Rad Laboratories, Hercules, CA, USA), respectively. Hemoglobin- β locus (*HBB*; MIM#141900) analysis was performed by ViennaLab Diagnostics GmbH (Vienna, Austria) and involved the 22 most common deletions within the Indian and Middle Eastern populations. Hemoglobin- α locus 1 (*HBA1*; MIM#141800) and hemoglobin- α locus 2 (*HBA2*; MIM#141850), which encompass common deletions/duplications, Hb Constant Spring, and α -Saudi alterations, were analyzed by Mayo Clinic Laboratories (Rochester, MN, USA). *HBB* was not analyzed for the presence of Hb D.

The diagnosis of Hb D-Punjab (*HBB*: c.364G>C, p.Glu122Gln) was deduced from the results of HPLC hemoglobin analysis, taking the following aspects into consideration:

- i) All subjects were members of the same (Balushi) kinship, and tribal allegiance was determined from the family name.
- ii) The group migrated to the Arabian Peninsula (UAE and Oman) from Baluchistan (Pakistan and Iran) two

centuries ago, i.e., originates from areas close to the Indian regions of Punjab and Gujarat, which have the highest recorded frequency of Hb D carriers (1.5%–3%) [2], [6], [7].

iii) They practice intra-family (close cousin or consanguineous) and intra-tribal (endogamous) marriages, which limit gene flow. The literature was queried using the PubMed, Google Scholar, and ResearchGate databases using multiple Hb D-related search terms and by cross-referencing the articles.

III. RESULTS

A. Case Presentations

The results of 13 subjects with Hb D-Punjab and 2 with β -thalassemia are shown in Tables 1–3. All subjects were asymptomatic and had normal findings on physical examination.

TABLE 1: LABORATORY RESULTS OF TWO PROSPECTIVE COUPLES WITH Hb D-PUNJAB

Couple A		Sex-age	Couple B	
F-36	M-27		F-36	M-35
4.9	7.2	RBC ($\times 10^{12}/L$)	5.3	5.6
-	-	Reticulocytes ($\times 10^9/L$)	61.8	-
110	140	Hemoglobin (g/L)	114	130
69.1	58.2	MCV (fL)	63.3 ^a	73.2
16.0	16.9	RDW (%)	21.6	17.2
12	146	Ferritin ($\mu g/L$)	38	-
61.8	3.6	Hb A (%)	0	94.3
2.8	5.8	Hb A2 (%)	3.8 ^a	4.5
0	0.8	Hb F (%)	0.7	1.2
35.4	89.8	Hb D (%)	95.5	0
c.92+5G>C		HBB analysis	Negative	c.92+5G>C
$\alpha_1 \alpha_2 / -^{3.7} \alpha_2$	$\alpha_1 \alpha_2 / -^{3.7} \alpha_2$	HBA1/HBA2 analysis	Negative	$-^{3.7} \alpha_2 / -^{3.7} \alpha_2$
Hb D trait	Hb D/ β^+	Phenotype	Hb D/D	β^+ -Thal trait
Silent α -Thal trait	Silent			
	α -Thal trait			

TABLE 2: LABORATORY RESULTS OF TWO FAMILIES WITH Hb D-PUNJAB

Family 1					Family 2				
Mother	Father	Daughter-7	Daughter-17	Daughter-19	Mother	Father	Son-6 ^a	Son-7	Daughter-9
5.8		6.3	5.8	5.7	RBC ($\times 10^{12}/L$)	5.2	5.99	6.3	4.8
-		33.8	80.0	86.1	Reticulocytes ($\times 10^9/L$)	42.3	57.8	59.6	59.9
114		125	121	114	Hemoglobin (g/L)	130	133	117	117
57.1		58.3	64.0	58.0	MCV (fL)	77.8	72.6	56.2	73.4
16.3		13.8	14.1	13.9	RDW (%)	13.2	14.1	16.6	12.4
63.5		69		29	Ferritin ($\mu g/L$)			40	
0/0		0	0	0	Hb A (%)	61.8	94.3	3.5	60.6
3.8/3.7		2.5 ^a	2.8	3.0	Hb A2 (%)	3.0	5.2	6.3	3.1
0.4/0.4		2.2	0.5	0.6	Hb F (%)	0	0.5	1.7	0.5
95.8/95.8		95.3	96.7	96.4	Hb D (%)	35.2	0	88.5	35.8
Hb D/D		Hb D/D	Hb D/D	Hb D/D	Phenotype	Hb D trait	β -Thal trait	Hb D/D β^+	Hb D trait
α -Thal trait	Unknown	α -Thal trait	α -Thal trait	α -Thal trait					

TABLE 3: LABORATORY RESULTS OF TWO BIOLOGICALLY UNRELATED CHILDREN WITH Hb D-PUNJAB

F-7	Sex-age	M-4
6.6	RBC ($\times 10^{12}/L$)	5.62
109	Reticulocytes ($\times 10^9/L$)	-
121	Hemoglobin (g/L)	107
62.0	MCV (fL)	61.4
13.1	RDW (%)	14.6
-	Ferritin ($\mu g/L$)	32.8
0	Hb A (%)	68.3
2.6	Hb A2 (%)	2.3
4.8	Hb F (%)	0.4
92.6	Hb D (%)	29.0
5.6	Hb Bart (%)	9.7
Hb DD		Hb D trait
α -Thal trait	Phenotype	α -Thal trait

IV. DISCUSSION

All subjects with Hb D-Punjab – heterozygotes (n=5), homozygotes (n=6), and Hb D-Punjab/ β -thalassemia double heterozygotes (n=2) – were members of the same Balushi kinship group.

A. Hb D-Punjab Heterozygote

In five subjects with Hb D trait (Tables 1–3), the total hemoglobin level was normal or slightly decreased, MCV was decreased, and the fraction of Hb D was between 29% and 39%. This is similar to results of other studies and low MCV is often related to coinheritor of α -thalassemia [8]–[11]; in population without α -thalassemia Hb D-Punjab heterozygotes have normal hemoglobin levels and MCV [12]. In short, the Hb D-Punjab trait is clinically silent [8],

[13].

B. Hb D-Punjab Homozygote

Five of the six of our subjects with Hb D-Punjab homozygosis have normal hemoglobin comprising of high Hb D, absent Hb A, and normal Hb A2 fraction (Tables 1–3). In all six subjects, however, Hb D-Punjab fraction was >92%. This high fraction was reported by others in Hb D-Punjab homozygotes (Table 4). As mentioned above, low MCV is likely a result of coinherited α -thalassemia, which is common in our and other studied populations [14]–[16]. We excluded the results of one report due to issues with selection of study subjects [17]. Only one of our six subjects had possible hemolysis solely based on a borderline high reticulocyte count (Table 3, F-7). Similar mild hemolysis was previously reported in one subject [18]. In contrast, two studies that evaluated blood donated by two Hb D-Punjab homozygotes showed that it could be used for transfusion purposes [15], [19].

This benign clinical phenotype of Hb D-Punjab heterozygote and homozygote explains the gene's wide distribution and relatively high frequency in many human groups [1], [2]. The *HBB*:c.364G>C, p.Glu122Gln variant has independently originated in the Mediterranean region and Asia approximately 40,000 years ago [20]. This occurred much earlier than the emergence of endemic malaria (approximately 10,000 years ago) and malaria-linked hemoglobinopathies [21]. Thus, Hb D-Punjab could either be a neutral mutation at drift or an adaptation to unknown environmental challenge.

TABLE 4: FRACTIONS OF Hb D-PUNJAB IN Hb D-PUNJAB HOMOZYGOTES AND Hb D-PUNJAB/ β -THALASSEMIA DOUBLE HETEROZYGOTES

N	Hb D/D	Hb D/ β	N	Method	Reference
6	92.6%–96.7%	88.5%, 89.8%	2	HPLC	This study
		81.9%	1	CE	[24]
		89.2%	1	HPLC	[3]
3	96.9%–97.0%	90.9%, 91.8%	2	HPLC	[25]
1	96.2%			Unknown	[22]
		82.0%, 83.9%,	3	HPLC	[4]
		85.3%			
		79.0%	1	HPLC	[26]
		85.7%, 86.3%	2	HE, LPLC	[26]
1	96.6%			HE	[17]
		88.0%	1	HPLC	[6]
		94.2%, 91.0%,	3	HE	[23]
		94.5%*			
11	Total		16		

HPLC, high-pressure liquid chromatography; LPLC, low-pressure liquid chromatography; CE, capillary electrophoresis; HE, hemoglobin (cellulose acetate) electrophoresis.

* In all three subjects, the Hb A2 fraction was elevated and Hb A was absent.

C. Hb D-Punjab/ β -thalassemia Double Heterozygotes

In our study, two individuals with the Hb D-Punjab/ β genotype had normal total hemoglobin levels, comprising high Hb D fraction (89% and 90%), low Hb A fraction (3.5% and 3.6%), and elevated Hb A2 fraction (6.3% and 5.8%); both subjects had low MCV (Tables 1 and 2). Other studies have reported similar findings in Hb D-Punjab/ β -thalassemia double heterozygotes [3], [4], [6], [16], [22]–[26]. Overall, we find that 14 of 16 subjects with this genotype/phenotype have Hb D fraction < 92% (Table 4).

The Hb D-Punjab homozygote and its β -thalassemia double heterozygotes are often confused. In one report, reliance on low MCV for the diagnosis of Hb D/ β -thalassemia heterozygosis has resulted in misdiagnosis of Hb D-Punjab homozygote [27]. In general, low MCV is of little value for diagnosis of Hb D-Punjab/ β double heterozygote because of a high prevalence of the α -thalassemia in many populations. In other reports, Hb D-Punjab/ β -thalassemia double heterozygotes were misdiagnosed as homozygotes because of normal Hb A2 level [3], [4]. In general, the Hb A2 level is affected by co-inherited α -thalassemia, iron deficiency, β -globin gene haplotype, and environmental factors [28], [29]. A review of at least one report suggests that administrative and technical inaccuracies should also be considered [3]. In this context, our finding of an Hb D fraction of >92% in Hb D-Punjab homozygotes could be useful in performing accurate diagnosis if molecular study is unavailable. This finding requires further study as two individuals with Hb D-Punjab/ β -thalassemia had Hb D-Punjab fractions of 94% (Table 4) and were shown to be exceptions to this rule; however, they were the only subjects in whom hemoglobin was analyzed using hemoglobin gel electrophoresis [22].

In one subject (F-36; Table 1, Couple B), *HBB* analysis for Hb D-Punjab was needed but was unavailable. She had a high Hb D fraction (95.5%), absence of Hb A, slightly elevated Hb A2 fraction (3.6%, 3.7%, and 3.8% on repeated examinations), and low MCV. She intends to marry a non-cousin with the β -thalassemia trait from the same tribe and it is essential that the Hb D-Punjab/ β -thalassemia genotype is excluded in her. The study of hemoglobin in this subject's parents could not be performed and the analyses of *HBB* and

HBA1 as well as *HBA2* did not result in detection of α - and β -thalassemia deletions/mutations. However, low MCV is consistent with the presence of the thalassemia trait. We think our results are most likely due to a limited spectrum of variants of *HBB* and *HBA* covered by our investigation and a high diversity of thalassemia alleles in our population [11], [30], [31]. In this context, Hb D fraction of >92% in our subject supports the diagnosis of Hb D-Punjab homozygosis.

D. Hb D-Punjab/S Double Heterozygote

The patients with Hb D-Punjab/S genotype/phenotype have diversity of clinical presentations, ranging from asymptomatic to severe anemia [32]–[40]. Their prognosis is better than that of patients with Hb S/S, and the unpredictability of presentation is affected by the Hb F level, α -thalassemia, *HBB* haplotype, age, and environmental factors [37], [41].

E. Hb D-Punjab/E Double Heterozygote

The benign phenotype of Hb D-Punjab/E double heterozygotes has been reported in at least six healthy individuals [42]–[44]. They had either a normal or a slightly decreased hemoglobin with Hb D fraction always higher than that of Hb E.

F. Hb D-Punjab/C Double Heterozygote

The only report of Hb D-Punjab/C double heterozygotes, published in 2020, describes a young adult with an initial misdiagnosis of mild sickle cell disease [45]. As only one case has been reported to date, the generalization regarding its clinical phenotype is limited.

G. Hb D-Punjab Carriers as Prospective Parents

In addition to the six most common Hb D-Punjab phenotypes/genotypes reviewed above, Hb D-Punjab carriers could produce offspring with many other genotypes (Table 5). In premarital counseling, each possible genotype must be considered for each couple. The fetal risk of a clinically significant hemoglobinopathy has been graded in a proposed guide (Table 5):

- Green indicates absence of such a risk in the Hb D-Punjab carrier genotypes/phenotypes, as discussed above.
- Yellow indicates a gray zone in decision-making owing to insufficient knowledge.
- Red indicates high fetal risk due to one or more possibly deleterious genotypes/phenotypes in offspring.

V. CONCLUSION

All Hb D-Punjab double heterozygotes are clinically benign except Hb D-Punjab/S. Fraction of Hb D in Hb D-Punjab homozygotes is higher than in Hb D-Punjab/ β double heterozygotes and value of 92% could be used as the diagnostic cutoff.

TABLE 5: SUGGESTED PREMARITAL GUIDE FOR HEMOGLOBIN D-PUNJAB CARRIERS: GENOTYPES OF PROSPECTIVE COUPLES AND FETAL RISK OF CLINICALLY SIGNIFICANT HEMOGLOBINOPATHY^a

		Spouse B													
Genotypes		A/A	A/β	A/S	D/A	D/D	D/β	D/S	D/E	D/C	A/E	E/E	A/C	C/C	β/β ^b
Spouse A	D/A							D/S		D/C			D/C	D/C	
	D/D							D/S					D/C	D/C	
	D/β		β/ β	S/β			β/β	S/β	E/β	C/β	E/β	E/β	C/β	C/β	β/β
	D/S		S/ β	S/S	D/S	D/S	S/β	S/S ^c	D/S ^c	S/C	E/S	E/S	S/C	S/C	S/β
	D/E						E/β	D/S ^c		C/E			C/E	C/E	E/β
	D/C					D/C	C/β	D/S ^c	C/E		C/E	C/E		D/C	C/β

^a Some genotypes have marked phenotype heterogeneity.^b Thalassemia intermedia.^c More than one possible genotype resulting in a clinically significant disease.

Legend

Alert	Odds of clinically significant hemoglobin disease in offspring
	>0
	→ 0
	Unknown, small sample, limited information, or high phenotype heterogeneity
C/E	>0 for shown genotype

Genotype	Reference
E/β	[46], [47]
C/β	[48]-[50]
S/C	[51]-[53]
C/E	[54]-[56]
E/S	[45], [57], [58]

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