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Haemoglobin Noah Mehmet Oeztuerk (α_2 $\delta_2$143 (H21)His → Tyr: A novel δ-chain variant in the 2,3-DPG binding site

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ABSTRACT

A new δ-chain variant, δ143 (H21) His → Tyr or Hb Noah Mehmet Oeztuerk, was discovered during the investigation of the cause of hemolytic anaemia in a 6-month-old infant of Turkish descent. It was detected by Cation exchange high-performance liquid chromatography (CE-HPLC) using PolyCAT A column. P_{50} was 20.6 ± 0.60 mmHg and 29.3 ± 0.40 mmHg for the carrier and the wild-type, respectively. This suggests an increase in oxygen affinity. On routine CE-HPLC Hb A_2 was low (1.2%) and the variant was not detected. An extended family study revealed that the variant was not associated with the anaemia or with any other clinical abnormality.

1. Introduction

Haemoglobin A_2 or $\alpha_2\delta_2$ is a naturally occurring haemoglobin which has electrophoretically less negative charge than the adult haemoglobin Hb A. The primary sequence of the δ-chain differs from the β-chain of Hb A in 10 out of 146 residues [1]. Haemoglobin A_2 (Hb A_2) is expressed at low concentration and its concentration in red cells from normal adults is about 2–3%. In patients with β-thalassemia Hb A_2 levels are often increased (>3.5%). The role of Hb A2 in the human normal red cells is not yet known. But, its increase is used as a criterion for diagnosis of β-thalassemia. The methods commonly used to determine Hb A_2 include elution following cellulose acetate electrophoresis, DE-52 micro-chromatography and Cation exchange high-performance liquid chromatography (CE-HPLC). CE-HPLC is emerging as the method of choice for quantification of Hb A_2 and Hb F. δ-globin gene mutations, can, however, mask the presence of β-thalassemia when the diagnosis is based only on the determination of Hb A_2. This depends on whether the variant co-chromatographs with Hb A2 or not. In this report, we have characterized a new δ-chain variant in eight members of a Turkish family with a spurious Hb A_2 level of 1.2% as measured by a routine, 7.5 min, CE-HPLC analyzer (Tosoh HLC-723 GHb VII).

2. Materials and methods

2.1. Blood samples

Venous blood was collected in tubes containing disodium salt of ethylenediaminetetraacetic acid (EDTA) as anticoagulant. Samples from 7 family members, living in Turkey were shipped by air to Freiburg, Germany. Informed consent was obtained.

2.2. Haematology and haemoglobin analyses

Haematological evaluations were carried out on routine equipment. The presence of a possible abnormal Hb variant was assessed by electrophoretic and chromatographic procedures routinely used in our laboratory [2]. Haemoglobin components were separated and quantified by high-resolution cation exchange HPLC using PolyCAT A column [2].

Isolation of normal and abnormal Hb A_2 required a preparative chromatography. It was performed on diethylaminoethyl-cellulose as described before [3]. A column (30 mm × 250 mm) of DEAE-cellulose equilibrated with 0.2 M glycine −0.01% KCN buffer was used. Hemolysate containing 200 mg haemoglobin is dialyzed against water overnight at 4 °C and applied to the column. The

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wild-type Hb A_2 and the variant δ-chain were eluted with a linear gradient of NaCl going from 0.0 to 0.2 M in the equilibrating buffer. The purity was checked by PolyCAT A HPLC.

2.3. Mass spectrometry

2.3.1. Material and reagents

All chemicals and reagents were of analytical grade and purchased from Sigma–Aldrich (Steinheim, Germany). Tris–HCl was from Euromedex (Souffelweyersheim, France). Porcine trypsin was from Promega (Madison, WI, USA). Distilled water produced from a Milli-Q Water System™ (Millipore, Guyancourt, France) was used for the preparation of the buffer and solvents.

2.3.2. Mass spectrometry analysis

Mutated haemoglobin (5 μl) fraction isolated by DEA-cellulose chromatography was denaturated with 5 μl of 2,2,2-trifluoroethanol. Then 30 μl of Tris–HCl (50 mM, pH 8.5) buffer and porcine trypsin in the ratio 1/20 (w/w) were added. The sample was left at 39 °C for twenty hours. The digestion was stopped with 1 μl of 10% formic acid. Before LC–MS or LC–MS/MS analysis, the solution was diluted (1:50) with 0.1% trifluoroacetic acid in water. One microliter of this solution was finally injected by nano-liquid chromatography–mass spectrometry (nano-LC/MS) and nano-LC–MS/MS. The analyses were performed using an Agilent 1100 series HPLC-Chip/MS system (Agilent Technologies, Palo Alto, USA) coupled to an HCT Plus ion trap (Bruker Daltonics, Bremen, Germany). The column used was a chip C18 SB-ZORBAX, 300 Å (75 μm × 43 mm, 5 μm). The solvent system consisted of 2% acetonitrile, 0.1% formic acid in water (solvent A) and 2% water, 0.1% formic acid in acetonitrile (solvent B). Elution was performed at a flow rate of 300 nl/min with a 8–40% gradient (solvent B) over the first 7 min followed by a 70% stage (solvent B) over 3 min before the reconditioning of the column at 92% of solvent A.

The voltage applied to the capillary cap was optimized to −1750 V. For tandem MS experiments, the system was operated with automatic switching between MS and MS/MS modes. The three most abundant peptides, preferring doubly charged ions, were selected on each MS spectrum for further isolation and fragmentation. The MS/MS scanning was performed in the ultrascan resolution mode at a scan rate of 26,000 *m*/*z* per second. A total of 6 scans were averaged to obtain a MS/MS spectrum. The complete system was fully controlled by ChemStation (*Agilent* Technologies) and EsquireControl (Bruker Daltonics) softwares.

Mass data collected during nano-LC–MS/MS analysis were processed, converted into .mgf files, and interpreted using a local Mascot (Matrix Science, London, U.K.) server. Searches were performed against the National Center for Biotechnology Information nonredundant (NCBI) database without any taxonomic, molecular weight, or isoelectric point restrictions. Searches were performed with a tolerance on mass measurements of 0.25 Da in both MS and MS/MS modes. For peptides not corresponding to expected masses for normal delta haemoglobin, the corresponding MS/MS spectrum was submitted to manual de novo sequencing.

2.4. DNA analysis

Genomic DNA was extracted from peripheral blood using the QIAamp DNA Mini Kit (Qiagen) according to the manufacturer's protocol.

The three exons of the δ-globin gene were amplified by PCR using the oligonucleotides C, F, H and I, as published by De Angioletti et al. [4]. The amplified products were sequenced directly with the Dye Primer Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems) according to the manufacturer's recommendations (oligonucleotides as for amplification). Sequencing reactions were analyzed on an ABI 3730 automated sequencer.

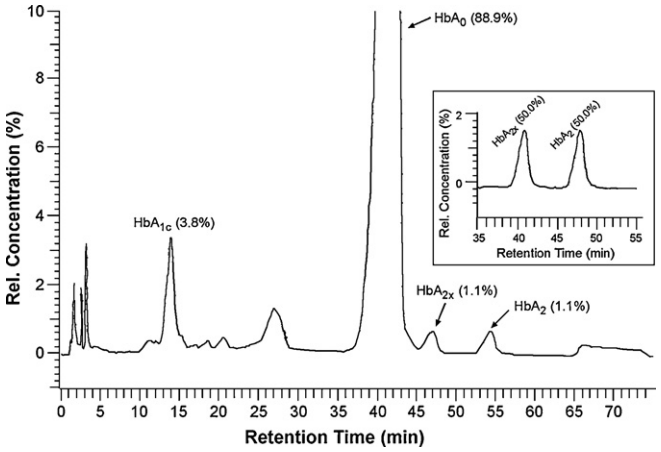

Fig. 1. CE-HPLC separation of the Hb components in the whole red cell lysate from a carrier of Hb Noah Mehmet Oeztuerk and in the Hb fraction isolated by DEA-cellulose chromatography (inset).

2.5. Oxygen affinity

The oxygen binding was assessed on the isolated Hb A2 fraction containing 2.5 g/dl haemoglobin. The determination of the oximetric parameters was performed on a ABL720 blood gas analyzer (Radiometer Copenhagen). P_{50}, the partial oxygen pressure for a given blood sample at which the haemoglobin is half-saturated, was calculated using the oxygen status algorithm, version 3, of Siggaard-Andersen and Siggaard-Andersen [5]. Catalase (20 mg/l) was added to the effluent fractions to limit methhaemoglobin formation.

3. Results

3.1. Family

The propositus, a 6-month-old infant of Turkish descent was admitted to the hospital for an investigation of the cause of the anaemia. He had a significant haemolytic anaemia with hypochromia and anisocytosis and was transfusion-dependent. The Hb level was 6.8 g/dl, mean corpuscular volume (MCV) 97.6 fl, mean corpuscular Hb (MCH) 27.3 pg, mean corpuscular Hb concentration (MCHC) 28.0 g/dl, and packed cell volume (PCV) 0.244 l/l. There is the suspicion that the propositus has a Diamond-Blackfan syndrome, a red cell aplasia in which the bone marrow is of normal cellularity but in which there is a relative absence of erythroid precursors.

He was found to have a spurious low level of Hb A2. Thirteen members of his family were evaluated; eight including her father showed inconspicuous low Hb A2 concentration (1.0–1.2%) but were not anaemic. Hb ranged from 13.0 to 15.8 g/dl.

3.2. Haemoglobin analyses

Electrophoresis on agarose gel and DE-52 micro-chromatography failed to reveal the presence of an abnormal haemoglobin, the level of Hb A_2 was 1.47 ± 0.22% and 2.10 ± 0.17%, respectively. The samples from the carriers run on a routine, 7.5 min, CE-HPLC (Tosoh HLC-723 GHb VII) showed a mean level of Hb A_2 of 1.23 ± 0.11% and the variant was not detected. CE-HPLC with PolyCAT A column (Fig. 1) demonstrated the presence of minor abnormal peak of 1.0% with a retention time of 48 min eluting

between Hb A0 and Hb A2. Hb F (2.3%) was detectable for the propositus. Lysate from affected subjects showed the normal HbA_2 (1.08 ± 0.05) and the abnormal component (1.06 ± 0.04%) in equal proportions. The Hb variant was masked after blood transfusion for the propositus, but the isolated total Hb A2 fraction comprised approximately an equal proportion of the normal and abnormal components after chromatography on PolyCAT A HPLC (Fig. 1 inset). The purity of Hb A_2 isolated from a control was 99%. Due to the low amount of Hb variant LC–MS experiments performed on the tryptic digest of the whole red cell lysate did not permit the detection of the variant peptide. Therefore, abnormal HbA_2 was purified by DEA-cellulose chromatography to perform further mass spectrometry experiments.

3.3. DNA analysis

Analysis of the DNA sequence of the amplified δ-globin gene fragment from the propositus and his father revealed the presence of single CAC → TAC base change at nucleotide 430 codon 143 in DNA that leads to a replacement of histidine by tyrosine (Fig. 2).

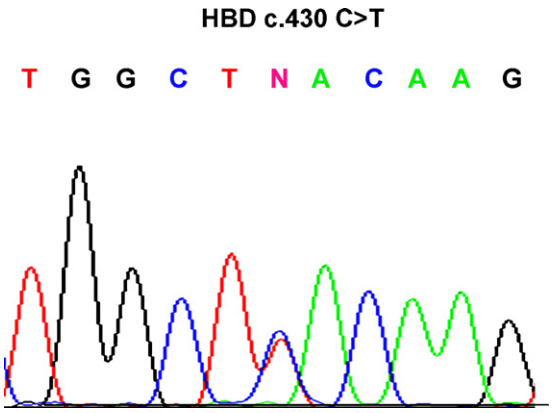

Fig. 2. DNA sequencing of the δ-globin gene. Portion of sequence printout showing location of a transitional mutation that results in replacement of arginine by tyrosine in codon 143, heterozygosity for c.430 C > T.

3.4. Mass spectrometry

The mutant HbA_2 isolated form DEA-cellulose chromatography was submitted to trypsin digestion and the mixture of peptides was analyzed by nano-LC–MS, in order to have the very best chromatographic time resolution to detect any shoulder in the chromatogram given by the ion current (Fig. 3). Then a nano-LC–MS/MS experiment was performed in exactly the same chromatographic conditions allowing the identification of peptides. Results of identifications from molecular masses and fragmentations are summarized on Table 1. The chromatographic peak at 6 min displays a mass of 1174.6 Da which does not correspond to any expected mass for a tryptic peptide from HbA_2. Its fragmentation spectrum shows it has a sequence identical to that of normal peptide delta T15 with a mutation of His 143 to Tyr. The fragments showing the loss of Tyr 143 display a ΔM of 163.01 Da for a theoretical ΔM of 163.06 Da. This mutation deduced from the MS/MS spectrum (Fig. 4) matches the difference in molecular mass observed (measured: 1175.6 Da; calculated: 1149.7; $\Delta M = 25.9$ Da).

3.5. Oxygen affinity

The data represent the mean ± standard deviation (S.D.) from isolated Hb A_2 from 3 carriers and 3 control persons. On the isolated Hb A_2 fractions from the carriers the mean oximetric readings were: pH 7.56 ± 0.02; COHb = 0.58 ± 0.20%; Hb = 2.23 ± 0.06 g/dl; methhaemoglobin = 1.1 ± 0.15%; sO_2 (oxygen saturation) = 92.3 ± 0.52%; $pO_2 = 50.70 \pm 2.9$ mmHg; $pCO_2 = 3.8 \pm 0.02$ mmHg. They were for the control persons as follows: pH 7.30 ± 0.04; COHb = 0.40 ± 0.14%; Hb = 3.2 ± 0.25 g/dl; methhaemoglobin = 0.9 ± 0.10%; sO_2 (oxygen saturation) = 92.0 ± 0.45%; $pO_2 = 69.40 \pm 2.5$ mmHg; $pCO_2 = 3.8 \pm 0.02$ mmHg. The mean calculated P_{50} was lower in the Hb A_2 fraction from the carrier than in the Hb A2 fraction from the control persons (20.6 ± 0.60 mmHg versus 29.3 ± 0.40 mmHg).

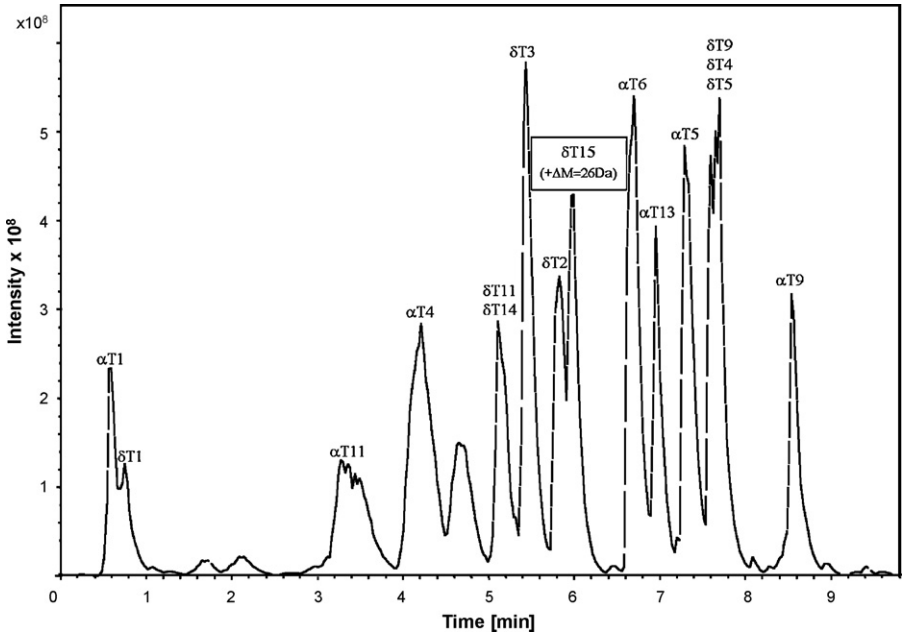

Fig. 3. Base peak chromatogram (BPC) obtained from nano-LC–MS analysis of tryptic HbA_2 mutant. The peak at 6 min exhibited a mass of 1148.7 Da which does not correspond to any tryptic peptide expected for normal HbA_2.

Table 1
Peptides identified by nano-LC–MS and nano-LC–MS/MS analyses of tryptic digest from isolated mutant HbA_2

Fragment	Peptide sequence	Theoretical mass ($M+H^+$)	Experimental mass ($M+H^+$)
αT1	V_1LSPADK$_7$	729.41	729.22
δT1	V_1HLTPEEK$_8$	952.50	952.41
αT11	V_{93}DPVNFK$_{99}$	818.43	818.38
αT4	V_{17}GAHAGEYGAEALER$_{31}$	1529.73	1529.60
δT11	L_{96}HVDPENFR$_{104}$	1126.56	1126.42
δT14	E_{121}FTPQMQAAYQK$_{132}$	1441.67	1441.52
δT3	V_{18}NVDAVGGEALGR$_{30}$	1256.65	1256.54
δT2	T_9AVNALWGK$_{17}$	959.52	959.49
δT15	δT15 with mass excess of 26 Da: V_{133}VAGVANALA(Y)K$_{144}$	1149.67 1175.67 (ΔM = 26.00)	1175.56 (ΔM = 25.89)
αT13	F_{128}LASVSTVLTSK$_{139}$	1252.71	1252.58
αT5	M_{32}FLSFPTTK$_{40}$	1071.55	1071.42
δT9	V_{67}LGAFSDGLAHLDNLK$_{82}$	1669.88	1669.72
δT4	L_{31}LVVYPWTQR$_{40}$	1274.72	1274.56
δT5	F_{41}FESFGDLSSPDAVMGNPK$_{59}$	2044.92	2044.60
αT9	V_{62}ADALTNAVAHVDDMPNALSALSDLHAHK$_{90}$	2996.48	2996.38

Sequence coverage from MS/MS data for this mutant δ-globin chain was 68%.

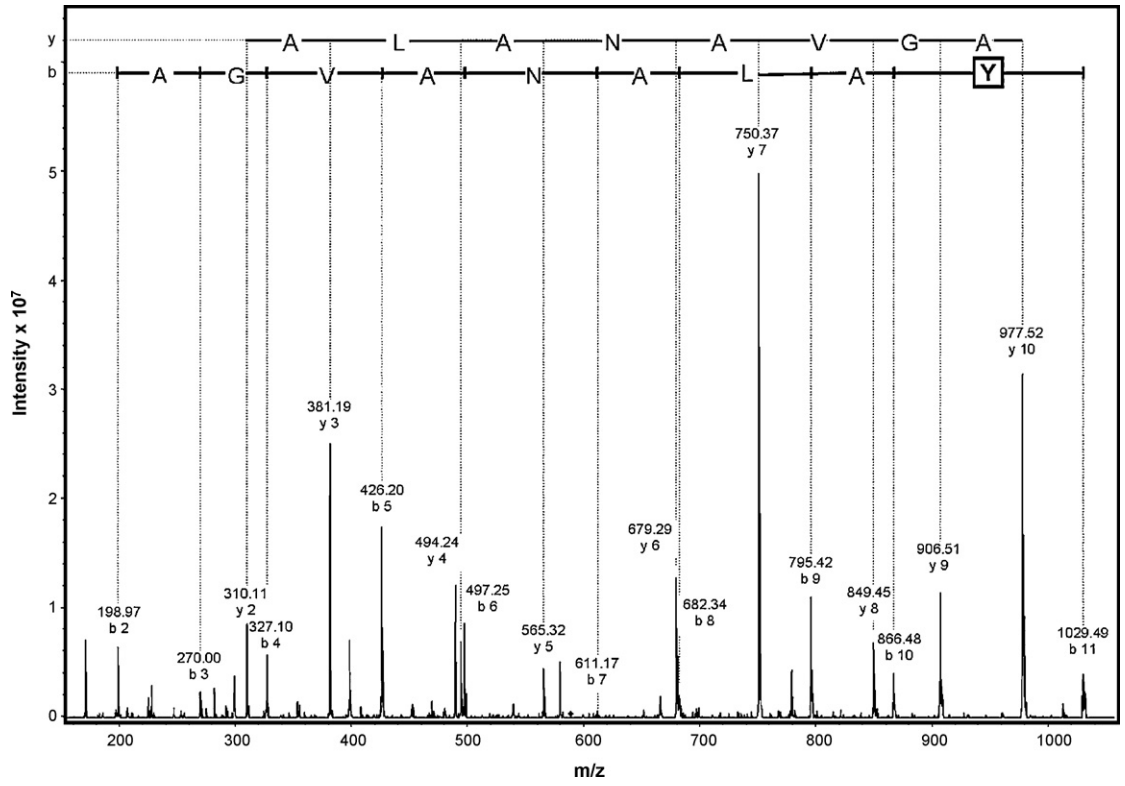

Fig. 4. MS/MS fragment ion spectrum obtained from the 2^+ molecular ion at m/z 588.4 from the 12 amino acids mutated tryptic peptide δT15 ($_{133}$VVAGVANALAYK$_{144}$).

On account of this significant difference, it is concluded that the variant has markedly increased oxygen affinity.

4. Discussion

Nearly 80 δ-globin variants have been characterized so far [6]. In this study we describe the characterization of a novel variant in which His is replaced by Tyr at or δ143 (H21). The first δ-variant with the substitution at 143 (H21) corresponds to that found in Hb A_2 Abruzzo or δ143 (H21) His → Arg, which was recently described in Italian patients [7] and is homologous to that of the ß-chain, Hb Abruzzo [8–10]. Because Hb A_2 represents only a minor portion of the total haemoglobin, δ-variants are of little clinical importance. However, in ß-thalassemia, Hb A2 levels are often increased and this characteristic increase is used as criterion for diagnosis of ß-thalassemia. Hb Noah Mehmet Oeztuerk did not appear to give rise to appreciable changes in the haematological picture. Indeed all carriers displayed normal values except the propositus who was anaemic and required transfusion. However, we found no association between his anaemia and the δ143 His → Tyr mutation. The presence of this mutation results in decreased expression of Hb A_2 wild-type, and therefore, could lead to a misdiagnosis of ß-thalassemia based on the level of Hb A_2. Electrophoresis, DE-52 micro-chromatography, and CE-HPLC are routinely used for quantifying Hb A_2. In both electrophoresis and DE-52 micro-chromatography the variant peak and the normal peak co-eluted as a single peak. Densitometric evaluation of Hb A_2 after electrophoresis was not an accurate and reliable method for quantifying Hb

A_2 as the sum of wild-type and the variant in samples from the carriers of the δ143 His → Tyr mutation. Indeed the Hb A_2 level was still low. Therefore, the detection of ß-thalassemia trait may be compromised using this method. However, on DE-52 microchromatography the measurement of Hb A_2 as the sum of two δ-peaks was accurate. This method could, therefore, be used for the diagnosis of ß-thalassemia in carriers of the δ143 His → Tyr mutation. On routine CE-HPLC (e.g., Tosoh HLC-723 GHb VII) short ß-thalassemia programs no abnormal peak was detected but Hb A_2 was still low, and therefore, the diagnosis of ß-thalassemia based on the Hb A_2 level only will be compromised. Two peaks are detected by CE-HPLC using PolyCAT A column with extended gradient. In this case attention should be paid to the additional peak when the diagnosis of ß-thalassemia is based only on the determination of Hb A_2. However, the delta variant also induces a risk of missing ß-thalassemia carrier ship when a diagnosis is based only on the retention time of the Hb A_2 peak. In general mutations which cause a low Hb A_2 could lead to misdiagnosis when inherited together with a β-thalassemia.

The replacement of His by Tyr at δ143 (H21) has an equivalent on the ß-globin gene, named Hb Old Dominion/Burton-upon-Trent [11]. This ß-variant is associated with a slight increase in oxygen affinity but without hematologic effect. We did not carry out the functional studies on isolated Hb Noah Mehmet Oeztuerk, but molecular modelling of the Hb A_2 molecule (data not shown) with the δ143 His → Tyr mutation shows that the hydrogen bond between $\delta_1$143Tyr and $\delta_2$139Asn, which is present in the template 1SI4 is conserved in Hb Noah Mehmet Oeztuerk. This hydrogen bond has been reported to stabilize the R conformation of the molecule, and therefore, increases its oxygen affinity [12]. Histidine at ß143 (H21) is involved in the binding of diphospho-glycerate (DPG) and five ß Hb variants at this position have been reported having an increased oxygen affinity, due to reduced DPG binding [13]. Considering the close structural similarity of the ß- and δ-globins, it is expected that Hb Noah Mehmet Oeztuerk will have increased oxygen affinity. Indeed in the Hb A_2 fractions containing 50% of the variant the oxygen affinity was increased with a P_{50} of 20.6 ± 0.60 mmHg compared with 29.3 ± 0.40 mmHg for the wild-type Hb A_2. This significant difference suggests that Hb Noah Mehmet Oeztuerk has markedly increased oxygen affinity.

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