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ORIGINAL ARTICLE

Significant prevalence of sickle cell disease in Southwest Germany: results from a birth cohort study indicate the necessity for newborn screening

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Abstract Children with sickle cell disease (SCD) benefit from newborn screening, because life-threatening complications can be prevented by pre-symptomatic diagnosis. In Germany, the immigration of people from endemic countries is steadily growing. Comprehensive data about the epidemiology and prevalence of SCD in Germany are however lacking, and SCD is not included in the national newborn screening program. We provide data on the prevalence of SCD in a population from both urban and rural areas in Southwest Germany. Anonymized dried blood spots from 37,838 unselected newborns were analyzed by allele-specific PCR for the HbS mutation. Samples tested positive were subjected to Sanger sequencing of the entire β-globin coding sequence firstly to validate the screening and secondly to identify compound heterozygous SCD patients with other mutations of the β-globin gene. We identified 83 carriers of the sickle cell trait, three compound heterozygous SCD patients (two with sickle cell-β-thalassemia, one with sickle cell-Hb Tianshui) but no homozygous SCD patients. The novel molecular method and strategy for newborn screening for SCD presented here compares favorably in terms of sensitivity (1.0 for homozygous HbS, 0.996 for heterozygous HbS), specificity (0.996), practicability, and costs with conventional biochemical screening. Our results demonstrate a significant prevalence of SCD of approximately 1:12,000 in an unselected urban and rural population in Southwest Germany. Together with previously published even higher results from exclusively urban populations in Berlin and Hamburg, our data provide the basis for the decision on a newborn screening program for SCD in Germany.

Keywords Sickle cell disease · Prevalence · Newborn screening · Allele-specific PCR

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Introduction

Sickle cell disease (SCD) results in significant morbidity and mortality already in infancy due to infections, splenic sequestration, and gradual deterioration of organ functions [1–3]. Early diagnosis by newborn screening has been shown to prevent fatal complications in about 20 % of affected children by appropriate prophylactic measures [4]. Consequently, screening for SCD has been established in many countries throughout the world [5–11]. In Germany, newborn screening for SCD has so far only been offered in regionally confined pilot projects [12–14]. Data on the epidemiology of SCD in Germany are scarce and have not been obtained in an unbiased systematic fashion. One German laboratory specialized in the analysis of hemoglobinopathies identified 3085 SCD patients between 1971 and 2007 [15]. Based on data on global migration, the number of HbS carriers has been estimated at approximately 85,000 in Germany in the year 2000 [16]. Lobitz et al. performed a newborn screening study on a cohort

of 34,084 neonates born in Berlin and identified 14 babies with SCD and 265 HbS carriers resulting in a prevalence of 1:2400 in this urban population with a high proportion of immigrants. Grosse et al. found a comparable frequency in the metropolitan area of Hamburg [14]. These studies demonstrate that in Berlin and Hamburg, SCD is already more common than any of the disorders targeted by the national German newborn screening program. However, they are likely not representative for the entire country, because SCD in Germany affects the immigrant population, which is distributed heterogenously: while cities such as Berlin or Hamburg harbor large communities of immigrants that are predicted to be at risk for SCD, in rural areas, the population at risk for SCD can be expected to be smaller.

Therefore, we aimed to determine the prevalence of SCD in newborns who are analyzed by the screening laboratory Heidelberg, a cohort that represents more than 16 % of all births in Germany and originates from both urban and rural areas. In order to avoid bias introduced by a selective screening of risk populations, we analyzed anonymized dried blood samples that had been archived after the general newborn screening was completed.

Materials and methods

Study design and population

Between 01.10.2012 and 01.02.2013, 38,447 dried blood samples of neonates were sent to the newborn screening laboratory in Heidelberg/Germany for regular newborn screening targeting 14 endocrine and metabolic disorders. Samples originated from the states of Baden-Württemberg (n=21,917), Rheinland-Pfalz (n=10,166), Nordrhein-Westfalen (n=3691), Saarland (n=2633), Hessen (n=11), and others (n=21). For 374 samples, the parents had denied consent for storage. After completion of the regular screening, 37,886 samples were archived and later on used for this study. Samples were anonymized by removing two 1.6 mm punches from all cards with sufficient material. The first punch was used for allele-specific PCR and Sanger sequencing. The second punch was stored and only used if the analysis of the first punch was inconclusive. Forty-eight samples could not be analyzed because too little material was available. All other samples were subjected to allele-specific PCR. If the HbS allele was detected, the sample was subjected to PCR amplification and the β-globin coding sequence re-analyzed by Sanger sequencing. We used this DNA-based screening strategy instead of the more commonly used high-performance liquid chromatography [17] or capillary electrophoresis [18], because samples had been stored for more than 12 months and only insufficient amounts of hemoglobin can be eluted from older samples thus precluding a biochemical analysis [19]. Furthermore, DNA-based screening enables an unequivocal identification of both, the homozygous and the compound heterozygous genotypes of SCD, which allowed us to consider the entire spectrum of SCD in this study.

All procedures followed were in accordance with the ethical standards of the responsible institutional ethics committee and with the Declaration of Helsinki in its current revised version. The study was approved by the Ethics Committee of the Medical Faculty, Heidelberg University.

DNA extraction and allele-specific PCR

1.6 mm punches were incubated in sealed 96-well plates with 13 μl lysis reagent (DNA Extract All Reagents Kit, Cat. Number 4402599, Applied Biosystems, Foster City, CA, USA) for 3 min at 95 °C. Lysis was stopped by adding 13 μl of stabilizing reagent (DNA Extract All Reagents Kit, Cat. Number 4402599, Applied Biosystems, Foster City, CA, USA).

For genotyping, a custom single nucleotide polymorphism (SNP) genotyping assay (Applied Biosystems, Foster City, CA, USA) that contained the two probes (β-globin wild type probe: VIC®-CTGACTCCTGAGGAGAA, β-globin HbS probe: FAM™-CTGACTCCTGTGGAGAA) and a primer pair (β-globin forward primer: TCAAACAGACACCATGGTGCAT, β-globin reverse primer CCCCACAGGGCAGTAACG) was used. In each well of a 96-well plate 2 μl of extracted DNA were mixed with 8 μl PCR reaction mix consisting of 5 μl TaqMan®GTXpress™ Master Mix (Cat. Number 4403311, Applied Biosystems, Foster City, CA, USA), 0.12 μl probe mix (80× custom SNP genotyping assay, Applied Biosystems, Foster City, CA, USA), and 2.9 μl water. On each plate, 88 samples were analyzed along with the following controls: two wells with no DNA added (no template controls: one well with extract from empty filter paper, one well with just water), two wells with DNA extracted from a healthy control not carrying HbS trait, two wells with DNA extracted from a heterozygous HbS carrier control, and two wells with DNA extracted from a homozygous HbS patient.

PCR was run on a StepOnePlus™ Real-Time PCR System (Cat. Number 4376600, Applied Biosystems, Foster City, CA, USA) with the following cycling program: 20 s at 95 °C, followed by 43 cycles with 1 s 95 °C+20 s 60 °C.

Results from a 96-well plate were only analyzed if the control samples were within the expected range for wild type, heterozygous, and homozygous HbS, respectively. Samples that were not analyzable for technical reasons were repeated, for instance if amplification was insufficient because of low DNA content. Samples were regarded as wild type if the signal intensities were below a discriminating line (see Fig. 1). Samples that were suspected to carry the HbS allele because the

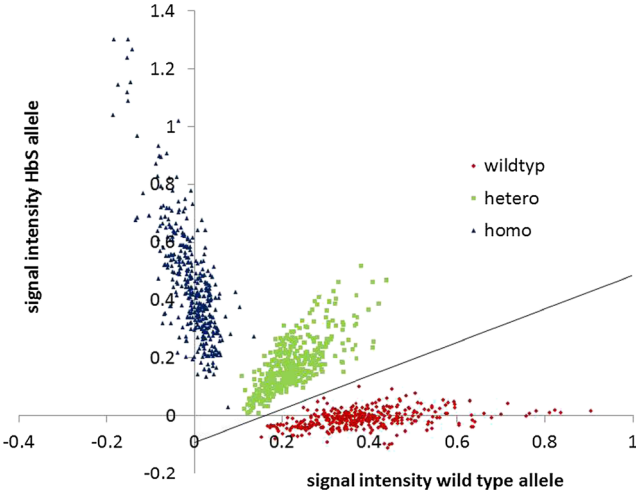

Fig. 1 Discrimination of heterozygous and homozygous carriers of HbS from wild type controls by allele-specific PCR. A test set of control samples was subjected to allele-specific PCR and signal intensities for the HbS allele were plotted over signal intensities for the wild type allele. HbS carriers are well separated from wild type controls by a line $y=0.58\times-0.093$

coordinates for signal intensities were above the discriminating line were subjected to Sanger sequencing.

PCR amplification of β-globin coding sequence and Sanger sequencing

Exons 1 and 2 of β globin were amplified by nested PCR (primers for first amplification: forward TATGCTTACCA AGCTGTGATTCCA; reverse AACGATCCT GAGACTTCCACA; primers for second amplification forward ATGGTATGGGGCCAAGAGAT; reverse CCCCT TCCTATGACATGAACTTAA). Exon 3 was amplified by nested PCR (primers for first amplification: forward CATAATCTCCCTACTTTATTTTCTTTT; reverse ACAGCATAGCAAAACTTTAACCTC; primers for second amplification forward ACAATGTATCATGCCTCTTTGCAC; reverse TCCCAAGGTTTGAACTAGCTCTTC).

Two microliters of DNA extracted from dried blood spots were used as a template for the first amplification. Two microliters of unpurified first round PCR product were further amplified by a second round of PCR. Cycling program 10 min at 95 °C followed by 39 cycles with 95 °C/30 s, 60 °C/45 s, 72 °C/75 s, and a final step with 72 °C/10 min. The PCR product was purified (NucleoSpin Gel and PCR Clean-up Kit, Cat. Number 740609250, Macherey-Nagel) and sent to GATC (Konstanz, Germany) for Sanger sequencing from both ends of the PCR fragment. For sequencing, the same primers were used as for the second step of PCR amplification. The sequences were analyzed by visual inspection of electropherographs.

Results

Sensitivity and specificity of allele-specific PCR

The efficiency of DNA extraction and the signal intensities after allele-specific PCR varied between experiments. In order to discriminate carriers of the HbS allele from normal controls, we subjected a pilot set of dried blood spots ($n=396$ for each condition) from volunteers that were either heterozygous carriers of the HbS allele, homozygously affected by SCD, or individuals with a normal genotype to allele-specific PCR. Signals for the HbS allele were plotted over the wild type allele and a line ($y=0.58\times-0.093$) was calculated with the method of linear discriminant analysis that separated HbS carriers from normal controls (Fig. 1). Samples resulting in coordinates above this line were regarded to carry the HbS allele, all other samples were considered to be normal for both alleles.

In order to test the sensitivity of this screening method, we subjected a validation set of dried blood spots to allele-specific PCR and applied the algorithm outlined above to determine the HbS carrier status. Two hundred thirty-five of 236 heterozygous and all of 234 homozygous samples were identified correctly, corresponding to a sensitivity of 0.996 and 1.0, respectively. Two hundred thirty-three of 234 normal samples were identified correctly, corresponding to a specificity of 0.996.

Samples that were diagnosed to carry the HbS allele based on allele-specific PCR were either subjected to Sanger sequencing or, if low signal intensity implied a low DNA extraction, re-analyzed by allele-specific PCR.

Prevalence of sickle cell trait and sickle cell disease

We analyzed 37,838 samples for the HbS allele by allele-specific PCR. Ninety-four samples were tested positive by allele-specific PCR and subjected to Sanger sequencing for validation (Fig. 2). Eighty-six of the 37,838 samples were found to carry the HbS allele in a heterozygous state, resulting in a positive predictive value of 0.91 for the allele-specific PCR in our patient cohort. This corresponded to a frequency of heterozygous carriers of 1:440 (95 % confidence interval 1:550 to 1:356).

Next, we performed DNA sequence analysis of the β-globin gene including the complete coding sequence, the promoter region, and both untranslated regions of all carriers of the HbS allele. We identified two newborns with sickle cell-β-thalassemia who were compound heterozygous for the HbS mutation and a β-thalassemia mutation. Further, we identified one newborn who was compound heterozygous for the HbS mutation and the rare unstable abnormal hemoglobin Hb Tianshui (Table 1). We did not identify newborns with homozygous SCD in this cohort.

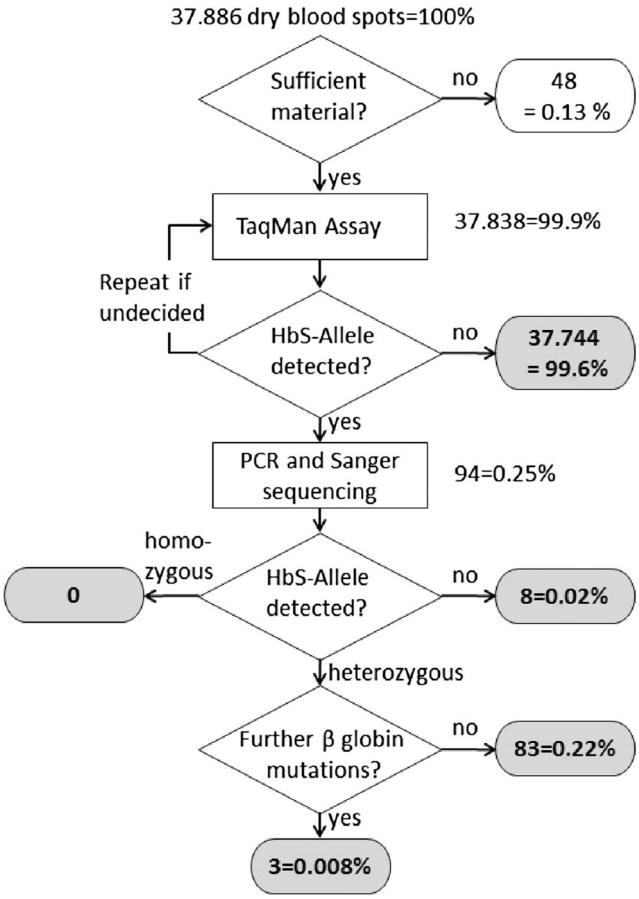

Fig. 2 Algorithm for newborn screening for sickle cell disease and results from a birth cohort. Dried blood samples from 37,886 neonates that were sent to the laboratory for newborn screening Heidelberg between 01.10.2012 and 01.02.2013 were subjected to the analysis for SCD by allele-specific PCR and, if positive, by Sanger sequencing. Reportable results are in shaded *gray*

In sum, 3 out of 37,838 newborns were identified with a form of SCD predicted to require treatment (Table 1), corresponding to a frequency of 1:12,613 (95 % confidence interval 1:61,159 to 1:4316). In a real screening setting, 48 children would have been recalled because of insufficient material on the screening card as well as the three children with relevant sickling hemoglobinopathies, i.e., only 1.3 ‰. This figure compares favorably with other screening tests. In Germany, the overall recall rate amounts to 8‰ [20].

Economical considerations

Based on current list prices for reagents, the consumables for DNA extraction and allele-specific PCR are approximately €1.03 per sample. Sequencing amounts to €25.34 for each sample that is found to be heterozygous for HbS by allele-specific PCR. Taking into account that approximately 1 % of samples require repetition of the allele-specific PCR, the average material costs for each sample amount to €1.10. An experienced technician can process up to 528 samples with one real-time PCR device per working day, thus amounting to a cost for personnel of approximately €0.46. Assuming an average life span of the required machinery (calculated at €17.950) of 5 years and an average sample number of 100, 000 p.a., we estimate the costs for the machinery at approx. €0.04. The total cost is thus estimated at €1.60 per sample. This compares favorably to approximately €2.10 for the biochemical analysis.

Discussion

We report on a molecular genetic method of newborn screening for SCD that requires a minimal amount of dried blood, can even be applied to samples that have been stored for more than 12 months and is highly accurate. The expenditures of time and money for the analysis compare favorably with chromatographic methods. Although the application of molecular genetics to newborn screening is restricted by legislation in some countries, we propose that the procedure described here can be suitable both, as a primary screening method or as a secondary method for samples that do not give unequivocal results after conventional biochemical analysis. Specifically, molecular genetic screening may be superior to biochemical screening if sample quantity or quality is low. Further, DNA-based screening can be reliably applied in premature infants or in newborns who have received blood transfusions.

Reflecting the proportion of immigrants among the population, the frequency of HbS carriers (1:440) was significantly lower in our cohort than in Berlin (1:129) and Hamburg (1:189) [13, 14]. At the same time, we observed an HbS

Table 1 Findings prompting recall: in three patients, besides the HbS mutation (HBB c.20A>T) further pathogenic mutations in the β globin gene were identified by Sanger sequencing

Mutations	Functional effect	Expected phenotype in compound heterozygosity with HbS	Reference
T>C nt +1570 relative to cap site/-12 relative to AATAAA polyA signal	Impaired polyadenylation, decreased β globin mRNA stability	SCD HbS/β thal	[27]
T>C nt −1 relative to cap site	Impaired mRNA transcription	SCD HbS/β thal	dbSNP rs386134236
c.119 A>G (39 Gln>Arg, Hb Tianshui)	Decreased hemoglobin stability	Mild SCD Similar electrophoretic behavior as HbS	[28, 29] [30]

carrier frequency that greatly exceeds the estimate of 1:1000 for Germany in the year 2000, which was based on data from the World Bank's Global Bilateral Migration Database [16]. The discrepancy between the estimate based on the influx of immigrants and the observed frequency of HbS carriers in our cohort may be explained by continuously ongoing immigration since 2000 and by the relatively high proportion of immigrants among the reproducing population.

The ratio of heterozygous HbS carriers over newborns with a clinically relevant SCD was 28.7 in our cohort and comparable to results from screening programs in the Netherlands [7], Belgium [8], and Spain [21], but higher than in Berlin (ratio 18.3, [13]) and Hamburg (ratio 13.1, [14]). This may indicate that in Southwest Germany, the immigrant population is spread more evenly and marriages within the population at risk for SCD are less likely than in Berlin or Hamburg. If the Hardy-Weinberg principle would have been applicable, the observed rate of heterozygotes would result in a prevalence of homozygous SCD of approximately 1 in 774,440, corresponding to less than one newborn in Germany per year. However, both the number of patients with SCD in our outpatient clinic and the results of this pilot newborn screening project suggest that the true prevalence of sickling conditions is much higher than calculated based on the HbS allele frequency. The two main reasons for this deviation from the Hardy-Weinberg principle are the contribution of additional non-HbS alleles to sickling conditions and preferred partnerships among populations at risk.

The primary genetic analysis implemented here compares favorably with primary biochemical testing with regard to false positive rates, costs, and recalls. Our findings document that SCD is a significant health problem in Germany with a frequency that exceeds that of most metabolic diseases that are integral part of the general newborn screening program in Germany. In several populations, newborn screening for SCD has been shown to allow for life-saving interventions and to reduce morbidity and mortality [4, 22–24]. With the detection of a significant prevalence of SCD in an unselected, mixed population from both urban and rural areas, a newborn screening program that is restricted to metropolitan areas with a high proportion of immigrants does not appear to be justifiable. A directed newborn screening targeting only children at risk is not feasible in the multicultural society of Germany. Mating between partners from different ethnicities is increasingly taking place. Data on the ethnic affiliation of newborns would need to be surveyed with separate written informed consent from both parents and transmitted to the screening laboratory [25]. Many parents will not be able to judge on their risk of transmitting the HbS trait, and a directed screening based on ethnicity would be considered as discriminating. The World Health Organization has urged its member states to reinforce a systematic screening program "tailored to the specific socioeconomic context" [26]. We believe that the burden of disease documented here and by others [13, 14] provides the basis for the decision on how to add SCD to the newborn screening program in Germany.

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Compliance with ethical standards All procedures followed were in accordance with the ethical standards of the responsible institutional ethics committee and with the Declaration of Helsinki in its current revised version. The study was approved by the Ethics Committee of the Medical Faculty, Heidelberg University.

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

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