



How will next generation sequencing (NGS) improve the diagnosis of congenital hemolytic anemia?

Paola Bianchi, Cristina Vercellati, Elisa Fermo

UOC Ematologia, UOS Fisiopatologia delle Anemie, Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico Milano, Milan, Italy

Correspondence to: Paola Bianchi, BSc, PhD. UOC Ematologia, UOS Fisiopatologia delle Anemie, Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico Milano, Via Francesco Sforza, 35, 20122 Milan, Italy. Email: paola.bianchi@policlinico.mi.it.

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Congenital hemolytic anemias are a group of very heterogeneous and rare disorders caused by alterations in structure, transport functions or metabolism of red blood cells (RBC).

Because the pathophysiology of some rare forms is poorly understood, these disorders represent a group of diseases that still lack easy-to-apply tools for diagnosis, clinical management, and patient stratification.

Moreover, epidemiological data in the international literature are generally still incomplete, and the estimated prevalence of some defects varies widely among countries.

The advent of next generation sequencing (NGS) technologies make these new approaches useful tools to investigate the genetic basis of these disorders and to identify new nosological entities. Moreover, the reduction in cost of these techniques allowed the development of targeted NGS-based panels of known genes and their market development.

From this perspective, an article recently published by Xue and colleagues (1) in *Annals of Translational Medicine* highlights the importance of NGS in the diagnosis of congenital hemolytic anemia. The study focused on ten Chinese patients affected by hereditary spherocytosis (HS) and compared the results with clinical features and laboratory examinations.

HS is considered to be the most common disorder associated with congenital hemolytic anemia, and reported to have a homogeneous worldwide distribution with an estimate prevalence of about 1:2,000 in individuals from

Europe and North America (2,3). In Chinese population however, HS prevalence seems to be lower and calculated to be about 1 in 100,000 people (1).

The diagnosis of HS is currently based on clinical and family history, evaluation of biochemical markers of hemolysis, RBC morphology, and functional testing, including the osmotic fragility test and eosin-5-maleimide (EMA) binding test; the combination of acidified lysis glycerol test (AGLT50) and EMA binding test has been reported to have the better sensitivity and specificity in diagnosing HS (4). Other more specific tests, such as the new generation ektacytometer laser-assisted optical rotational cell analyzer (LoRRca) Osmoscan and SDS-PAGE analysis, are available only in specialized laboratories and, when included in the diagnostic workflow, may provide additional information on the specific membrane defect (5-7).

Despite the availability of a battery of diagnostic tests, none of the above mentioned methods can detect all cases of HS; moreover, the concomitance of different defects (for example the co-presence of a RBC membrane defect with a β -thalassemia trait or a RBC enzymopathy) (8-10) or blood cell contamination due to red cell transfusion in the more severe patients, may interfere with the interpretation of the results, therefore representing a possible cause of misdiagnosis (9).

The molecular basis of HS are complex, because it is caused by alterations in genes encoding for one or more of the major RBC cytoskeleton and transmembrane proteins:

Table 1 Recent studies performed by targeted NGS panels in patients with hemolytic anemias. Number of the genes included in the panel, patients studied, overall sensitivity and, when available, sensitivity of panels in patient with undefined hemolytic anemia are reported

Studies	No. of genes analysed	No. of cases	Overall sensitivity	Sensitivity in hemolytic patients with no previous diagnosis
Chonat <i>et al.</i> , 2019 (21)	32 (membrane defects)	11 (HS)	100%	Not studied
van Vuren <i>et al.</i> , 2019 (16)	7 (membrane defects)	95 (HS)	89%	Not studied
Xue <i>et al.</i> , 2019 (1)	10 (membrane defects)	10 (HS)	90%	Not studied
Peng <i>et al.</i> , 2018 (20)	n.a.	51 (HS)	72%	Not studied
Li <i>et al.</i> , 2018 (17)	217	46 (CHA)	60.9%	n.a.
Russo <i>et al.</i> , 2018 (14)	34 and 71	74 (CHA)	64.9%	45.8%
Agarwal <i>et al.</i> , 2016 (18)	28	17 (CHA)	70%	70%
Roy <i>et al.</i> , 2016 (13)	33	57 (CHA)	38.6%	11%

CHA, chronic hemolytic anemias; HS, hereditary spherocytosis; n.a., not available.

ankyrin-1 (*ANK1*), band-3 (*SLC4A1*), α -spectrin (*SPTA1*), β -spectrin (*SPTB*) and protein 4.2 (*EPB42*) (11,12), resulting in a wide clinical heterogeneity, ranging from asymptomatic patients with compensated anemia, to severely anemic, transfusion-dependent cases.

Molecular testing in HS has not been considered for a long time except in particular cases, due to the consistent number and size of involved genes; however, this approach is now moving increasingly towards customized multi-gene panels (i.e., targeted-NGS) and whole-exome sequencing (13–15) for diagnosis of RBC defects, making easier, cheaper and faster to sequence and analyze a large number of genes (1).

The genes involved in HS in fact, are now usually included in the targeted panels specifically designed for the diagnosis of hereditary hemolytic anemias (14–20).

In the papers published in the last years, about 70–100% of patients with a previous diagnosis of HS were found to have a causative variants in the genes known to be associated with this disease (*Table 1*), suggesting that the use of targeted NGS has a high diagnostic efficiency in HS when used in combination with conventional diagnostic techniques; moreover, the molecular characterization of the defect allowed a definition of genotype-phenotype correlation (16,21), and in some cases the clarification of phenotypic variability sometime observed also inside the same family (19).

Xue and colleagues used the targeted capture and sequencing technique to confirm the diagnose in ten HS patients at molecular level. The authors detected pathogenetic variants in nine cases. Although the analyzed

cohort is very limited to give information on the sensitivity of their approach, the detection rate obtained is comparable with previous reports; in particular, they found four mutations in *SPTB*, one in *ANK1* and 1 in *SLC4A1* gene, all at the heterozygous level and with dominant transmission in the affected families.

Given the wide spectrum of disorders associated with hemolytic anemias, NGS approaches offer a powerful diagnostic tool also for the cases who didn't reach a definitive diagnosis with conventional methods, or for patients affected by ultra rare disorders. However, in these cases the diagnostic yield of targeted-NGS approach drastically drops down (*Table 1*), depending on the design of the platform, on the number of genes included in each panel (14,15), but possibly also on the kind of the patients studied; actually, a complete and detailed phenotyping and clinical classification of the patients is often mandatory to reach a definitive diagnosis in these cases.

The report of numerous variants of unknown significance (VUS) (22) to whom it is difficult to assign a certain pathogenetic significance may further reduce the sensitivity and specificity of results; in fact, not every mutation detected by DNA analysis should be classified as a disease-causing variant, even after *in silico* analysis by mutation prediction programs, until their pathogenic nature is confirmed with the functional analysis, such as protein quantification, Western Blot, RT-PCR analysis, or gene reporter assays. This may be particularly true for some variants that may have a mild effect on the phenotype accounting also for intrafamily variability as in the case reported by Xue and colleagues (1) (the same pathogenic

variant identified in the affected mother and in the apparently healthy daughter).

The interpretation of NGS-derived data related to RBC membrane defects is also challenging because patients with inherited anemias can have multiple mutations with complex genotype-phenotype interactions (23), or may display low expression polymorphisms that modulate the phenotype [i.e., SPTA1LeLy (apics) or SPTA1Lepra (apics)] (21).

It would be desirable to have a common approach to standardize variability in current practice, including technical aspects, the number of genes analyzed, the diseases covered by the analysis, how to report variants and what specific functional tests are needed to validate a new pathogenetic mutation.

Although guidelines for variant reporting [e.g., American College of Medical Genetics and Genomics (ACMG)] (24) provide excellent advice on how to interpret the likely pathogenicity of genetic variants, no disease-specific guidance exists to assist in the clinical interpretation of NGS findings for individuals with rare inherited anemias (25). Given the rarity and the complexity of these disorders a shared scheme assessing specific external quality controls and interpretation of NGS could be of value.

As Xue and colleagues well underline in their study, one of the main causes of misdiagnosis in the past years could be also attributed to the lack of knowledge of these group of rare disorders by clinicians; the introduction of NGS techniques allowed in the past 2 years to double the number of diagnosed HS patients among Chinese population (1), further confirming the usefulness of this approach, and also its possible role in a more appropriate evaluation of rare disease prevalence among different populations.

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Footnote

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