Clinical Chemistry

Neutral-Coating Capillary Electrophoresis Coupled with High-Resolution Mass Spectrometry for Top-Down Identification of Hemoglobin Variants

Article:
Ruben Yiqi Luo, Carolyn Wong, James Qiangwei Xia, Bertil E Glader, Run-Zhang Shi, and James L Zehnder.
Neutral-Coating Capillary Electrophoresis Coupled with High-Resolution Mass Spectrometry for Top-Down Identification of Hemoglobin Variants
Clin Chem 2023; 69(1): 56–67. https://doi.org/10.1093/clinchem/hvac171

Guest: Dr. Ruben Luo, an Assistant Professor of Pathology at Stanford University and the Associate Director of the Clinical Chemistry and Immunology Laboratory at Stanford Health Care.

Bob Barrett: This is a podcast from *Clinical Chemistry*. I'm Bob Barrett. Hemoglobin, the primary oxygen transport protein contained in red blood cells is essential for the body to produce energy and support key metabolic processes. About 1,400 hemoglobin variants with altered amino acid sequences have been documented, many of which cause hemoglobinopathies, conditions marked by atypical red blood cells counts, fatigue, pain, or organ damage. Rapid and accurate diagnosis of hemoglobinopathies is of utmost clinical importance, but test methods routinely used in most clinical laboratories are unable to differentiate between certain hemoglobin variants. Gene sequencing may be used in these cases, but this often adds substantial cost, is available only at specialized laboratories, and may not evaluate all hemoglobin subunit genes.

A new article appearing in the January 2023 issue of *Clinical Chemistry* examines this issue and describes a potential solution. We're pleased to have the lead author of this article in this podcast. Dr. Ruben Luo is an Assistant Professor of Pathology at Stanford University and the Associate Director of the Clinical Chemistry and Immunology Laboratory at Stanford Healthcare. So, Dr. Luo, could you start by telling us what are hemoglobin variants and how they're measured in most clinical laboratories?

Ruben Luo: Sure. Hemoglobin variants are the hemoglobin molecules with modified amino acid sequences as a result of genetic mutations, and they constitute a main type of hemoglobinopathy. So far, over 1,400 hemoglobin variants have been discovered and reported. Identification of hemoglobin variants, particularly the variant forms of subunits alpha, beta, gamma, and delta, are of significant value in the clinical diagnosis of hemoglobinopathy.

In clinical laboratories, the conventional methods to identify hemoglobin variants include gel electrophoresis, capillary electrophoresis, or CE, and high-performance liquid chromatography, or HPLC. These methods differentiate hemoglobin variants by their size and charge properties,

however, the resulting power of the conventional methods can be inadequate due to the lack of structural characterization resulting in ambiguity and identification of hemoglobin variants.

Bob Barrett: So, let's talk about capillary electrophoresis coupled with high-resolution mass spectrometry and just for brevity, we'll call that CEHRMS. How will this measure protein targets?

Ruben Luo: Well, as we know, mass spectrometry, particularly high-resolution mass spectrometry or HRMS has been a key technology for structural characterization of proteins. It provides superior resolution to unravel the primary structures of peptides or protein analytes. Here, the primary structure means just the amino acid sequence of the protein or peptide, and it results in accurate identification of clinical diagnostic targets when we apply HRMS in clinical laboratories.

To fully utilize the analytical power of HRMS, it is necessary to couple a separation technology so that analytes can be separated from interference to minimize ionization suppression. As CE has demonstrated superior performance in the separation of hemoglobin molecules, it can be coupled with HRMS through a specifically designed nano electrospray ion source to utilize the advantages of both technologies. The ion source merges the eluent from CE with a sheath liquid and ionize the analyte molecules immediately through electrospray ionization. By this means, we can achieve detailed structure characterization of hemoglobin variants to further enhance the system performance. We use neutral coating capillaries to enhance the CE separation efficiency for the analytes, so the performance can be very high in this application.

Bob Barrett: And how do we apply CEHRMS to the analysis of hemoglobin variants?

Ruben Luo: The CEHRMS method achieves identification of hemoglobin variants in two steps. Step one, intact protein analysis that preliminarily identifies hemoglobin subunits by precursor ions, and step two, top down analysis that characterizes the primary structures of hemoglobin subunits by fragment ions.

We have constructed a database of hemoglobin variants to which the CEHRMS analytical results can be matched to identify what hemoglobin variants are present in clinical specimens. And it should be noted that the neutral-coating CE plays an essential role in this application. Its excellent separation efficiency not only allows for a simple dilute and shoot sample preparation procedure, but it also facilitates the analysis of particular heterozygous samples that can take a normal hemoglobin subunit as well as a variant with a very small mass change.

For example, two relatively common hemoglobin subunit beta variants: one is beta-C C and the other is beta-E. They both have an amino acid mutation from glutamic acid to lysine, which results in a decrease of 0.948 Dalton in monoisotopic mass compared to the normal submitted beta. This trivia mass difference cannot be differentiated in mass spectrometry due to multiple charges on protein molecules. Therefore, the normal subunit beta and the beta variant must be separated by CE before the HRMS analysis.

Since the beta-C and the beta-E variants have different electrophoretic mobility than a normal subunit beta, they can be baseline separated and successfully identified using the CEHRMS method. Similarly, differentiation between the normal subunit beta and the beta-D-Punjab was also achieved, where the variant has an amino acid mutation from glutamic acid to glutamine with a monoisotopic mass change of 0.984 Dalton.

Bob Barrett: So, should the new CEHRMS method be considered a replacement for currently available methods such as conventional electrophoresis and gene sequencing, or can this be used in synergy with these other methods?

Ruben Luo: Well, this is a great question. As we know, in clinical laboratory practice, the conventional electro fragment methods have limited resolving power to identify hemoglobin variants due to the lack of structural characterization, especially for those rare hemoglobin variants. While gene sequencing can be used as a confirmatory test, the high cost limits its availability in clinical laboratories, and usually the clinical gene sequencing service does not cover all the hemoglobin subunit genes, especially gamma and the delta genes are commonly not covered.

With the ability to characterize the primary structures of hemoglobin, the CEHRMS method can well complement the conventional methods. It can be positioned as a reflex test method, which means a primary screening method which can be electrophoresis-based is first implemented, and then the CEHRMS test will be triggered if hemoglobin variants are suspected in the primary screening. In these settings, additional separation methods and gene sequencing may no longer be necessary.

Bob Barrett: Dr. Luo, since most hemoglobin variants don't really cause severe symptoms, why is it important to determine exactly which hemoglobin variant a patient carries?

Ruben Luo: Well, again, this is a very good question. Besides a series of hemoglobin variants that may result in severe clinical manifestations, for example, the famous hemoglobin S, C,

and E, there are indeed a large number of hemoglobin variants not causing severe symptoms in patients, especially when the patients are heterozygotes. So in those cases, we sometimes call these silent hemoglobin variants. Although accurately identifying the silent hemoglobin variants does not provide benefit to improve the patient conditions. It can help find ideologies of hemoglobin-related mild symptoms. For example, hemoglobin Tarrant and hemoglobin Malmo may cause polycythemia, and hemoglobin Raleigh may cause low hemoglobin without physiological anemia and incorrect A1C results.

In these cases, accurately identifying the hemoglobin variants can help clinicians rule out other hematological disorders and eventually save the medical resources that may be consumed otherwise. In addition, if carriers of silent or mild hemoglobin variants need to take medications that interact with hemoglobin, the accurate information of the hemoglobin variants will help physicians determine and optimize the application of such medications. For example, hemoglobin Koln, hemoglobin Seattle, and some other hemoglobin variants cause unstable hemoglobin molecules and need to be considered in the application of certain medications.

Bob Barrett: How frequently is hemoglobin variant identification performed as part of genetic counseling?

Ruben Luo: Well, it is not uncommon to find patients of certain ethnic groups with compound heterozygous mutations, which by themselves are clinically benign but when paired, could cause problems for the patients. Often, the patient's parents may not even know they have these variants because they are heterozygous with the silent hemoglobin variants. It becomes more difficult in multicultural areas where mixed race couples are being counseled.

In these cases, definitive identification of hemoglobin variants may be needed to give correct risk assessment even when hemoglobin variants are silent as we mentioned earlier. Considering the cost to accurately identify hemoglobin variants, the CEHRMS method is probably the most cost-effective solution for this need.

Bob Barrett: Well, from what you've told us, this CEHRMS method certainly sounds like a powerful tool. So, in closing, is there any other information you can tell us that might be used to guide patient care?

Ruben Luo: Yeah, absolutely. In addition to the amino acid sequence, the CEHRMS method can figure out the posttranslational modifications of a hemoglobin subunit. So far, acetylation of a lysine residue and glutathionylation of a cysteine residue in hemoglobin subunits have been accurately identified. These

posttranslational modifications have been reported to be related with oxidative stress, drug uses, and other pathophysiological conditions. When the CEHRMS method is employed for hemoglobin analysis, the information of those posttranslational modifications can be obtained and may potentially assist with the diagnosis and treatment of hematological disorders. So, we are in the process of obtaining those data and do study of those data to find out if they can help with the clinical practice of the physicians.

Bob Barrett: That was Dr. Ruben Luo from the Department of Pathology at Stanford University. He served as the lead author for a new article in the January 2023 issue of *Clinical Chemistry* on the identification of hemoglobin variants by high-resolution mass spectrometry and he's been our guest in this podcast on that topic. I'm Bob Barrett, thanks for listening.