

Special Article - Red Blood Cells

Potential Diagnostic Application of the Quantitative Assessment of Red Blood Cell Membrane Protein Expression

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Abstract

We have recently developed an efficient flow cytometry-based method which allows to quantitatively determine the expression levels of selected Red Blood Cell (RBC) membrane proteins. Interestingly, RBCs express several hundred membrane proteins, the expression of which is determined by the individual genetic background and complex regulatory effects. Many of these membrane proteins are closely linked to the development of human diseases or disease-susceptibilities, thus the determination of specific RBC protein expression levels may help to perform a patient-specific, stratified diagnosis. In this technology we generate RBC ghosts with a simple fixation procedure, then use monoclonal antibodies optimized to recognize specific RBC membrane proteins. The data obtained in selected groups of patients and control subjects are compared, and the genetic basis of the alterations in membrane protein expression is further examined by performing specific SNP and mutation analysis from genomic DNA samples. In this mini-review we summarize our recent studies related to changes in RBC protein expression in gout patients, and describe an interesting discovery based on the RBC expression studies of a plasma membrane calcium pump.

Keywords: Red blood cells; Flow cytometry; ABCG2; Gout; PMCA; Malaria; Biomarkers

Abbreviations

FACS: Fluorescence-Activated Cell Sorter; GWAS: Genome-Wide Association Studies; RBC: Red Blood Cell; SNP: Single Nucleotide Polymorphism; WGA: Wheat Germ Agglutinin

Introduction

Methodology for assessing RBC membrane protein expression and its medical role

Relevant RBC membrane proteins are selected based on the literature (red blood cell CD antibodies, GWA and medical diagnostic studies), and these data are compared to Mass Spectrometry (MS) measurements and the data in the erythrocyte database [1-3]. For Flow Cytometry (FACS) measurements, RBC ghosts (hemoglobin depleted erythrocyte membranes) are prepared from a small drop of blood and these ghosts are labeled by a fluorescent lectin derivative (e.g. wheat germ agglutinin-AlexaFluor647, WGA-A647) to allow selective gating. Thereafter, the ghosts are labeled with specific monoclonal antibodies, then with suitable fluorescent secondary antibodies (for details see [3,4]). The measurements are carried out in a flow cytometer, preferably equipped with automatic high throughput plate sampler.

In order to obtain quantitative results in the FACS assay, each membrane protein and each selective first antibody has to be carefully titrated to give maximum level interactions. Still, the results of the FACS measurements do not provide an actual number of the given membrane protein per RBC, and data have to be evaluated based on

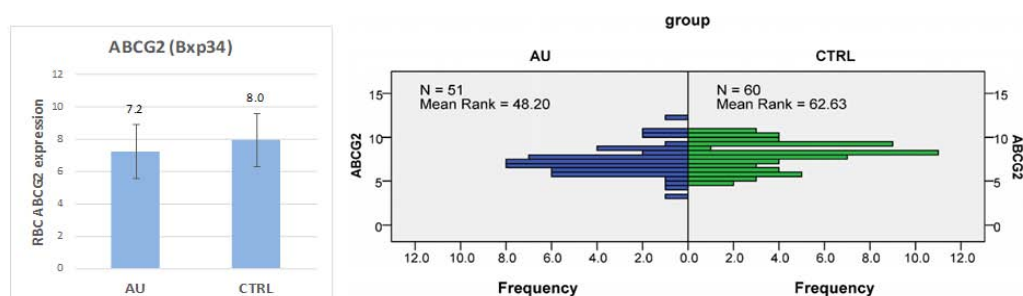
the protein levels obtained in a certain population or under specific disease conditions.

The advantage of the FACS assay lies in the quick and cost-efficient method that requires only a small amount of blood. However, a major issue in this work is the selection of proper antibodies for trustworthy, quantitative membrane protein measurements. In most cases no information is available about the use of commercially available antibodies in flow cytometry, and the quantities of most of the red blood cell membrane proteins are also unknown. In this latter regard, recently improving quantitative mass spectrometry measurements may provide proper estimates [1,2,4]. Still, for the quantitative detection of a particular RBC membrane protein, the use of at least two different antibodies, favorably recognizing different epitopes of the same protein, are preferred. In our research projects we have examined numerous commercially available monoclonal antibodies, potentially suitable for the characterization of RBC membrane proteins that is showing specific and sensitive detection of membrane protein levels even at low protein concentrations (Table 1).

Parallel with the FACS assays, genomic DNA is prepared from the same small blood samples, in order to study relevant individual polymorphisms and/or mutations. The alterations in the membrane protein levels found in individual samples thus can be further examined by genetic testing. As a first approach, disease-related Single Nucleotide Polymorphisms (SNPs) in the genes of the selected membrane proteins are searched for in related GWA studies, and the identified, sufficiently frequent variants are tested by PCR, e.g. using

Table 1: Examples of membrane proteins with medical relevance and with established antibody-based quantitative assays in RBCs.

Protein	Gene	Molecular function	Biological function
ABCA1	<i>ABCA1</i>	ATP binding cassette transporter, cholesterol / lipid transport	role in cellular efflux of cholesterol and lipids
ABCG2	<i>ABCG2</i>	ATP binding cassette transporter, xenobiotic and drug transport, urate transporter	role in drug ADME-tox, urate metabolism, cancer drug resistance
GLUT-1	<i>SLC2A1</i>	glucose transmembrane transporter activity in many cell types	glucose uptake, role in ascorbic acid metabolism
GLUT-3	<i>SLC2A3</i>	glucose transmembrane transporter activity mainly in neurons	glucose uptake, role in ascorbic acid metabolism
GLUT-4	<i>SLC2A4</i>	glucose transmembrane transporter activity in many cell types, mainly in adipocytes	glucose uptake
IR	<i>INSR</i>	insulin receptor, insulin binding, protein tyrosine kinase activity	glucose homeostasis
PMCA4b	<i>ATP2B4</i>	transmembrane calcium transport and ATPase activity, main Ca^{2+} exporter in many tissues	role in Ca^{2+} dependent signal transduction
SGLT2	<i>SLC5A2</i>	Sodium/glucose symporter activity	role in carbohydrate metabolic processes
URAT-1	<i>SLC22A12</i>	urate/anion antiporter	role in urate re-absorption in the kidney, regulates blood urate levels, response to drugs

**Figure 1:** Left panel: average ABCG2 levels in the healthy and gout patients, respectively. The right panel shows the distribution frequency of the ABCG2 levels in the two groups. AU: gout patients, CTRL: control group.

TaqMan assays. In cases of individuals showing a large difference in a given RBC protein level from the mean level in the population, the relevant genes are sequenced to search for potential rare mutations. In these cases, both the variants in the coding sequences and/or in the promoter/enhancer regions may give relevant information about the source of protein expression differences. Missense variations in the coding regions may cause major folding or trafficking problems, while mutations in the promoter/enhancer regions may significantly affect membrane protein expression levels through regulatory pathways [5-7].

Based on these experiments, the main goal is to connect the red blood cell membrane protein alterations with disease susceptibility, drug toxicity, treatment sensitivity or other relevant medical variables. The connection between the SNPs, the membrane protein levels, and the medical variables has to be statistically analyzed to uncover important correlations, providing a diagnostic value. The aim of our recent studies described below as examples, has been to uncover connections between RBC membrane protein changes and related genetic variations, which may play a role in the development or treatment possibility of diseases.

Selected RBC membrane protein studies with potential diagnostic applications

A. Gout and the expression of the ABCG2 protein in RBCs: Gout is a painful disorder of purine metabolism, with deposition of urate crystals in intra-articular and peri-articular areas. The basis of this deposition is an increased uric acid concentration in the blood, either because of a hyperproduction, or a reduced excretion of this

physiological metabolite. The major site of the uric acid secretion is the kidney, but extrarenal secretion, especially in the intestine, is also important [8]. Urate deposition in the joints result in inflammation, episodic gout flares, gouty arthropathy, tophi formation, and often appears together with urolithiasis [9,10].

The ABCG2 protein is a multidrug transporter, which plays an important physiological role in the transport of xeno- and endobiotics and, when overexpressed, may cause multidrug resistance in cancer. This protein is mostly expressed in barrier tissues (e.g. the gut and the blood-brain barrier), in the liver and the kidney, and significantly modulates drug absorption, distribution, excretion and toxicity (ADME-Tox) properties [11]. Interestingly, it has been revealed, that ABCG2 is also an important transporter for uric acid excretion, especially in the intestine [12]. A relatively frequent ABCG2 polymorphism, Q141K (C421A), resulting in reduced protein expression and function, has been found in GWA studies as a major factor in gout formation [13].

It has been relatively recently (in 2012) discovered that ABCG2 is expressed in the red cell membrane, and the lack of this expression results in a rare blood group variant, J⁻ [14]. In our related experiments we have analyzed the expression of this protein by our flow cytometry methods, coupled with a genetic test. This study [5], showed that the ABCG2 protein can be quantitatively measured in the RBC membrane, the Q141K polymorphism in a heterozygous form results in a decreased RBC-ABCG2 level, and an even lower protein expression is seen in homozygous ABCG2-Q141K individuals, or in individuals (and their relatives) carrying a stop or a frameshift

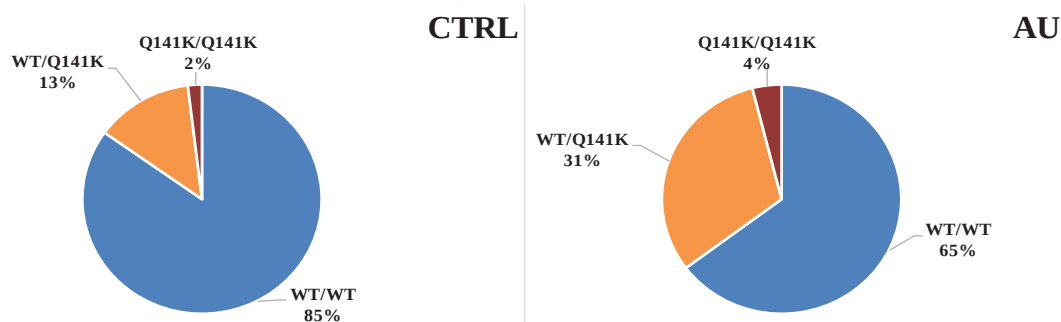


Figure 2: Differences between the frequencies of the Q141K polymorphism in healthy control (CTRL) individuals and in the gout patients (AU).

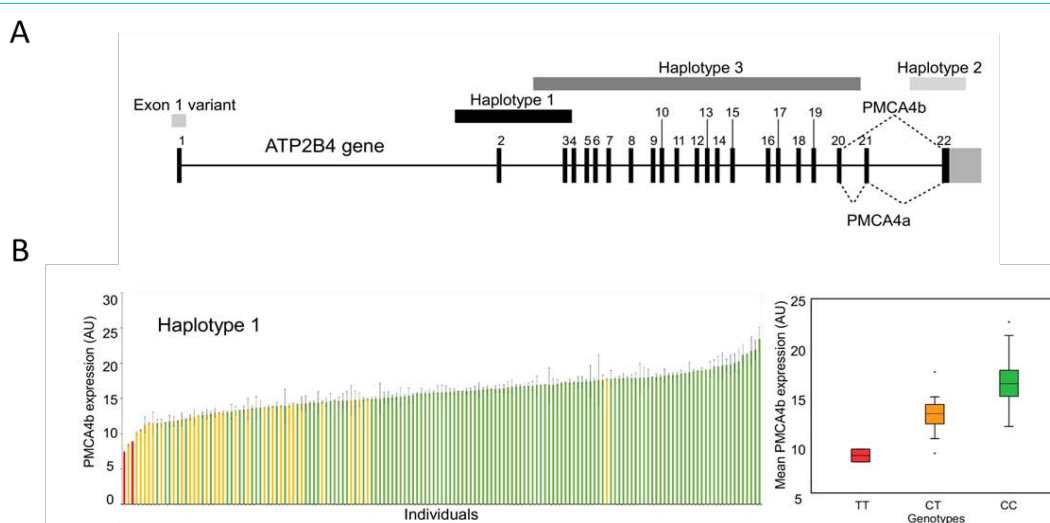


Figure 3: PMCA expression level correlation with genetic analysis. A, Important frequent SNPs and haplotypes of the ATP2B4 gene. B, The relationship of protein expression and the SNPs of the haplotype of ATP2B4 gene. Minor allele homozygotes: red, minor allele heterozygotes: yellow, major alleles: green. Right side: Mean values $\pm$ SD values (at least two independent parallels), of the PMCA4b expression levels versus the ATP2B4 genotypes (Kruskal-Wallis tests). Modified from Zámbo et al. [7].

mutation of ABCG2 in a heterozygous form. In a further work [6], by using the same approach and analyzing individual samples with lower expression of ABCG2 in the RBC membrane, we found a relatively frequent new mutant variant, ABCG2-M71V, which also has a major expression and trafficking problem.

Recently we have initiated a study to investigate the correlation between lower RBC membrane ABCG2 expression and gout formation. In these, as yet unpublished experiments, we found a correlation between the RBC membrane expression levels of the ABCG2 and its variants and the appearance of gout at the clinic (Figures 1 and 2). In the gout patients we also found several less frequent mutations, which probably also contribute to the lower ABCG2 expression levels. Thus, lower RBC expression of the ABCG2 protein may guide further diagnostic and therapeutic approaches in these patients.

B. Expression of the calcium pump protein, PMCA4b in RBC membranes - a potential role in malaria susceptibility: The Plasma Membrane Ca^{2+} -ATPases (PMCA) are important active calcium exporter proteins in the human cells. The main function of these proteins is to maintain cellular calcium homeostasis by ensuring

the low concentration of cytoplasmic calcium and participating in calcium dependent signal transduction. The PMCA proteins in human have four major isoforms, coded by four different genes. In addition, they have numerous tissue-specific splice variants, and the human red blood cells mostly express the PMCA4b variant [15-18].

When measuring the expression levels of PMCA4b in the RBC with a monoclonal antibody, we found several individuals with much lower expression levels than the mean value in the population. As measured in independent calcium transport experiments, the RBCs of individuals with lower PMCA4 expression had a much lower calcium extrusion capacity, thus reduced PMCA expression correlated with reduced transport function [7].

In order to find out the potential genetic background of the lower protein expression, we sequenced in these individuals the exons of the ATP2B4 gene coding for the PMCA4b protein. Interestingly, we did not find any mutations in the coding sequences of any of the samples. To further explore the potential genetic background of the reduced protein expression, we determined numerous SNPs in the potential promoter and/or enhancer regions of the PMCA4 gene. As shown in Figure 3, we found that the SNPs characteristic for a minor

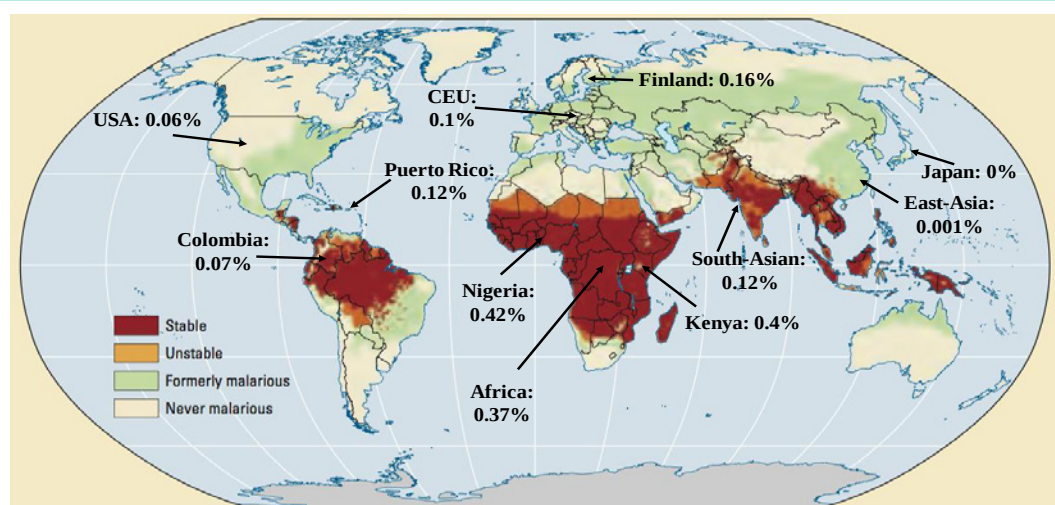


Figure 4: World map of past and current malaria prevalence with MAF% of SNPs in haplotype 1. Red: stable malaria infection, orange: periodic malaria infection, green: previously infection, white: free from infection. Modified from: https://www.reddit.com/r/MapPorn/comments/65zpt5/os_malaria_past_and_current_prevalence_all_around/?utm_source=share&utm_medium=web2x

haplotype around exon 2 (Haplotype 1, which includes a potential alternative promoter in erythroid cell lines [19], an enhancer, and the translational start site), correlated with the reduced PMCA4b expression level. No other haplotypes or SNPs in the gene showed such a correlation with membrane protein expression levels.

It has been also suggested, that this region can be a potential alternative promoter in erythroid cells [19]. A recent study, analyzing the potential enhancer of the *PMCA4* gene, found an erythroid-specific small enhancer region, corresponding to an area within Haplotype 1 [20].

Most interestingly, in a GWA study, the SNPs in Haplotype 1 have been found to correlate with a protection against malaria [21]. Moreover, based on CDC and NCBI data, this Haplotype occurs at much higher frequency in malaria infected regions – see Figure 4. These data suggest that malaria infection susceptibility is much lower in individuals with lower RBC PMCA4b protein, and this expression difference is a selection force in the major malaria-infected regions.

The potential mechanism behind this effect is that *Plasmodium* proliferates in the so called parasitophore vacuole within the red blood cells, and Gazarini et al. [22], suggested that the parasite may use the PMCA4b of the inverted human erythrocyte membrane to maintain elevated calcium levels within this vacuole. Therefore, an active calcium RBC pump is required to allow parasite survival and growth, and a reduced PMCA4b expression in the erythrocytes may decrease the survival of this infectious agent.

Conclusion

A large number of membrane protein alterations are closely linked to the development of various human diseases, while the quantitative estimation of these proteins in the human tissues is practically unavailable. Interestingly, the determination of membrane protein expression levels in the RBCs in many cases reflects overall genetic or regulatory alterations, and may help to perform a patient-specific, stratified diagnosis. There could be several limitations in this approach, as tissue-specific regulation of membrane protein

expression may not appear in the RBC samples. Still, our approach may help personalized medical diagnostics, and we are currently involved in clinically oriented RBC protein expression studies in major metabolic diseases, including gout and diabetes. The results of the combined flow cytometry and molecular genetic assay platform presented here may efficiently help to explore further diagnostic and therapeutic possibilities in these multifactorial diseases.

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