

Some cautionary notes on the petite “Holy Grail” of molecular diagnostics

Jo Vandesompele,^{1,2} and Pieter Mestdagh¹

¹Center for Medical Genetics, Ghent University; and ²Biogazelle, Zwijnaarde, Belgium

E-mail: joke.vandesompele@ugent.be doi:10.3324/haematol.2013.103127

The “Holy Grail” of molecular diagnostics is the sensitive and specific detection of a disease-associated stable biomarker in non-invasively-acquired patient material. Without realizing it, we may actually have found it.

Since the seminal papers by Mitchell *et al.*¹ and Chim *et al.*² in 2008, many studies have shown that small RNA molecules called microRNAs circulate in the blood in cell-free mode. They appear to be quite abundant in serum and plasma, are stable, and their levels are correlated to disease state, prognosis or response to treatment.

So far, 2578 human mature microRNAs are known (miRBase release 20). Many of these are expressed in a cell type-specific manner, making them ideal candidate biomarkers. MicroRNAs are also useful therapeutic targets; one drug is already in clinical trials and several more are waiting to enter clinical phases.³ MicroRNAs display exquisite stability in serum or plasma because they are packaged in membrane-encapsulated vesicles or protected by RNA-binding proteins. MicroRNAs also survive in unfavorable physiological conditions, such as repeated freeze-thawing, and long-term storage at room temperature.⁴

Today, different technologies are available for microRNA expression profiling, such as RT-qPCR, microarray or bead-based hybridization, or massively parallel sequencing. While there are a few reports comparing some of these technologies, no study has systematically addressed the relative merits of each technology using objective performance criteria over a wide range of sample types and microRNA molecules. One of the goals of the international microRNA quality control study (miRQC) was to develop an analytical framework to enable cross-platform evaluations (Mestdagh *et al.*, submitted manuscript, 2014). Spiked-in human serum samples were also included in this study, and the conclusions are that the different platforms tested have different detection rates, specificity and reproducibility. The most appropriate method depends on the specific research question.

In the current issue of *Haematologica*, Kubiczkova *et al.*⁵ used RT-qPCR as quantification platform, and report for the first time the differential expression of five microRNAs in serum from patients with multiple myeloma and monoclonal gammopathy of undertermined significance. Their expression pattern can be used to discriminate healthy controls from patients with high sensitivity and specificity. Furthermore, several of these microRNAs are correlated to known disease-associated biochemical parameters and to patient survival, suggesting their possible involvement in disease etiology or progression.

While the study by Kubiczkova *et al.*⁵ definitely raises our hopes of reaching the goal of non-invasive diagnostics, we should add a few cautionary notes to microRNA-based biomarker analysis in general. First, it is not entirely clear where the circulating microRNAs are coming from; are they exclusively secreted from the malignant cells, are they coming from immune system cells, are they signs from other host-related physiological processes, or a combination of these? The authors attempted to address this issue by comparing

patient sera and matching bone marrow plasma cells, but were unable to draw any strong conclusions. Comparing expression levels of very different samples types (serum vs. tissue/cells) is a technical challenge and involves the integration of spike-in controls and a dedicated normalization strategy. The current gold standard for microRNA normalization is the use of the average expression level of all expressed microRNAs, the so-called global mean normalization method^{6,7} (see also blog on <http://www.biogazelle.com/how-find-stably-expressed-micrnas>). This works well within one sample type, but may not be adequate to compare very different types. The authors also compared exosomal and non-exosomal fractions of serum. Interestingly, different exosome purification methods may yield microvesicle fractions of different purity and nature, impacting the observed microRNA repertoire.⁸ Also our own unpublished work indicates that the catalog of longer RNA molecules can be quite different depending on the particular exosome isolation method used (Van Deun *et al.*, submitted manuscript, 2014).

A second cautionary note is that the biological function of extra-cellular microRNAs remains a matter of debate. It has been suggested that microRNAs merely reflect byproducts of cellular activity with only a few extracellular microRNAs having a signaling function.⁴ In contrast, microRNAs have been referred to as a new class of hormones, actively secreted for distant cell-to-cell communication.⁹ In the context of cancer, it is hypothesized that such excreted microRNAs help prepare future metastatic niches. In the context of a useful biomarker, this discussion is not so important, as long as the biomarker is fit for the purpose, i.e. results in accurate classification. Nevertheless, to what extent cell-free microRNAs are part of an active (hormonal) communication system still needs to be investigated. This information may prove to be helpful in prioritizing anti-cancer targets.

A third cautionary note concerns sample quality assessment, not so much in terms of integrity (as we know that microRNAs are quite resistant to degradation because of their protected environment), but more in terms of purity and composition. Heme and other factors in the blood (and hence serum and plasma) are known to be potent enzymatic inhibitors. It is, therefore, recommended to spike-in artificial RNA molecules at known concentration to verify the absence of inhibitors. Furthermore, dedicated standard operating procedures for serum or plasma preparation are required in order not to introduce sample-specific differences in RNA content due to non-homogeneous sample handling. Hemolysis during sample preparation can alter the microRNA content of plasma or serum.¹⁰ In addition, small differences in duration and centrifugal force can create differences in the presence of platelets in plasma, ranging from platelet rich to platelet poor or free plasma, which has an impact on the microRNA repertoire.¹¹ Future studies on circulating microRNAs should, therefore, explicitly state the centrifugal protocol and blood storage time to ensure reproducibility. Even better, it would be helpful if standardized and proven protocols were used.

Finally, as for any biomarker study, it is crucially important

to have independent validation of the established biomarker results. In this respect, it still remains to be seen whether the 5 prioritized biomarkers from Kubiczikova *et al.*⁵ can be confirmed in an independent cohort. The models or gene signatures from biomarker studies easily suffer from being over fit, as typically many more genes are measured than samples. In these circumstances, multiple hypothesis-testing correction measures need to be taken (e.g. checking for the false discovery rate), along with internal cross-validation or preferably external blinded validation. Furthermore, challenging the novel biomarker in a multi-variate analysis along with established diagnostic markers or risk predictors is also crucial in order to establish the added value of the new marker; if not, it's JAM (just another marker)! Only when a biomarker test has withstood the challenge of independent multi-variate validation will it have a chance of actually being used in the clinic and providing benefits for the patients. And that is the ultimate goal of this type of work.

Despite the cautionary notes outlined here, there is a bright future for using circulating RNAs, and in particular microRNAs, because of their tissue specificity and stability, as an embodiment of the long-sought for "Holy Grail" of non-invasive molecular diagnostics.

Jo Vandesompele is a Professor and Senior Lecturer at the Center for Medical Genetics, Ghent University, Belgium, where his main fields of interest are non-coding RNA in cancer, biomarker research, functional genomics, and applied bio-informatics. He is also co-founder and CEO of Biogazelle, Zwijnaarde, Belgium, where he works in the fields of transcriptome analysis, and RNA-based diagnostics and therapeutics. Pieter Mestdagh is a postdoctoral researcher at the Center for Medical Genetics, Ghent University, Belgium. His main fields of interest are non-coding RNA in cancer, biomarker research, and functional and integrative genomics.

Financial and other disclosures provided by the author using the ICMJE (www.icmje.org) Uniform Format for Disclosure of Competing Interests are available with the full text of this paper at www.haematologica.org.

References

1. Mitchell PS, Parkin RK, Kroh EM, Fritz BR, Wyman SK, Pogosova-Agadjanyan EL, et al. Circulating microRNAs as stable blood-based markers for cancer detection. *Proc Natl Acad Sci USA*. 2008;105(30):10513-8.
2. Chim SSC, Shing TKF, Hung ECW, Leung T-Y, Lau T-K, Chiu RWK, et al. Detection and characterization of placental microRNAs in maternal plasma. *Clin Chem*. 2008;54(3):482-90.
3. Hydbring P, Badalian-Very G. Clinical applications of microRNAs. *F1000Research*. 2013;2:136.
4. Turchinovich A, Weiz L, Burwinkel B. Extracellular miRNAs: the mystery of their origin and function. *Trends Biochem Sci*. 2012;37(11):460-5.
5. Kubiczikova L, Kryukov F, Slaby O, Dementyeva E, Jarkovsky J, Nekvindova J, et al. Circulating serum microRNAs as novel diagnostic and prognostic biomarkers for multiple myeloma and monoclonal gammopathy of undetermined significance. *Haematologica*. 2014;99(3):511-8.
6. Fan J-B, editor. miRNA expression profiling: from reference genes to global mean normalization. *Methods Mol Biol*. Totowa, NJ: Humana Press; 2012;822(Chapter 18):261-72.
7. Mestdagh P, Van Vlierberghe P, De Weer A, Muth D, Westermann F, Speleman F, et al. A novel and universal method for microRNA RT-qPCR data normalization. *Genome Biol*. 2009;10(6):R64.
8. Rekker K, Saare M, Roost AM, Kubo A-L, Zarovni N, Chiesi A, et al. Comparison of serum exosome isolation methods for microRNA profiling. *Clin Biochem*. 2014;47(1-2):135-8.
9. Cortez MA, Bueso-Ramos C, Ferdin J, Lopez-Berestein G, Sood AK, Calin GA. MicroRNAs in body fluids—the mix of hormones and biomarkers. *Nature Publishing Group*. 2011;8(8):467-77.
10. Kirschner MB, Kao SC, Edelman JJ, Armstrong NJ, Vallely MP, van Zandwijk N, et al. Haemolysis during Sample Preparation Alters microRNA Content of Plasma. *PLoS ONE*. 2011;6(9):e24145.
11. Page K, Guttery DS, Zahra N, Primrose L, Elshaw SR, Pringle JH, et al. Influence of plasma processing on recovery and analysis of circulating nucleic acids. *PLoS ONE*. 2012;8(10):e77963-3.

Post-translational arginylation as a novel regulator of platelet function

Markus Bender and Hervé Falet

Brigham and Women's Hospital, Harvard Medical School, Boston, MA, USA

E-mail: hfalet@rics.bwh.harvard.edu doi:10.3324/haematol.2013.103119

In this issue of *Haematologica*, Lian *et al.* show that megakaryocyte-specific loss of arginyltransferase (ATE1)-mediated post-translational arginylation leads to enhanced clot retraction and *in vivo* thrombus formation in mice, due to enhanced myosin regulatory light chain (RLC) phosphorylation in platelets.¹

Platelets are small, discoid-shaped cells circulating in the bloodstream. After vascular injury, platelets are recruited to the exposed subendothelial extracellular matrix that triggers platelet activation to seal wound sites by the formation of a hemostatic plug. Consequently, excessive bleeding is prevented under normal circumstances. However, if thrombus formation is uncontrolled, it may lead to vessel occlusion and to life-threatening events, such as myocardial infarction and stroke.²

Platelet activation involves a large number of platelet sur-

face receptors, signaling molecules, and cytoskeletal-modifying proteins, as well as their complex interactions. Platelet signaling requires a cascade of intracellular protein post-translational modifications, of which phosphorylation is probably the best studied. Mass spectrometry analysis revealed that more than 270 proteins are phosphorylated in human platelets.³ The number of studies on how post-translational modifications influence platelet biology is increasing, demonstrating that these modifications constitute an emerging, biologically significant field.

Post-translational arginylation, or tRNA-dependent addition of the amino acid arginine to proteins, was discovered more than 40 years ago. However, it remained a comparatively less-known post-translational modification. Arginylation was once thought to play a singular role in the N-end rule pathway of protein degradation. However,