microRNA analysis for the detection of autologous blood transfusion in doping control

Application note

Doping Control

Authors

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Introduction

Blood transfusion (BT) as blood doping practice is banned by the World Anti-Doping Agency (WADA) and can be abused by cheating athletes to increase the rate of oxygen transport to tissues with the aim to improve sport performance. At present, a method for the detection of Homologous Blood Transfusion (HBT) has been implemented by the WADA accredited antidoping laboratories worldwide, while no internationally recognized method has been finalized so far for the direct detection of autologous blood transfusions (ABT), which can at present be only detected indirectly by targeting longitudinal profiling of key hematological parameters. In this perspective, several researches approaching different fields are underway to find reliable biomarkers to be suitable in the development of a method to directly detect autologous blood transfusion.

In this work the role and the variability of microRNA (miRNAs) as biomarkers related to the practice of an autologous blood transfusion practice has been investigated. miRNAs (figure 1) are a class of 18-24 nucleotides long non coding RNAs acting as post-trancriptional modulators of mammalian gene expression. They are produced in the nucleus by a coding gene as pri-miRNA and the exported to the cytoplasm as pre-miRNA, then cleaved by a Dicer enzyme and processed by a RISC complex to the mature functional miRNA. Mature miRNAs are involved in the regulation of many physiological processes (such as erythropoiesis), since they can have effect in the cleavage of a target mRNA or function as transcriptional repressor or involved in mRNA deadenylation. Recent evidences shown that miRNAs can be useful biomarkers in the diagnosis certain diseases such as cancer and heart malignancies [1] and also an emerging role as long term biomarkers for the detection of erythropoiesis-stimulating agents and ABT abuse [2-3]

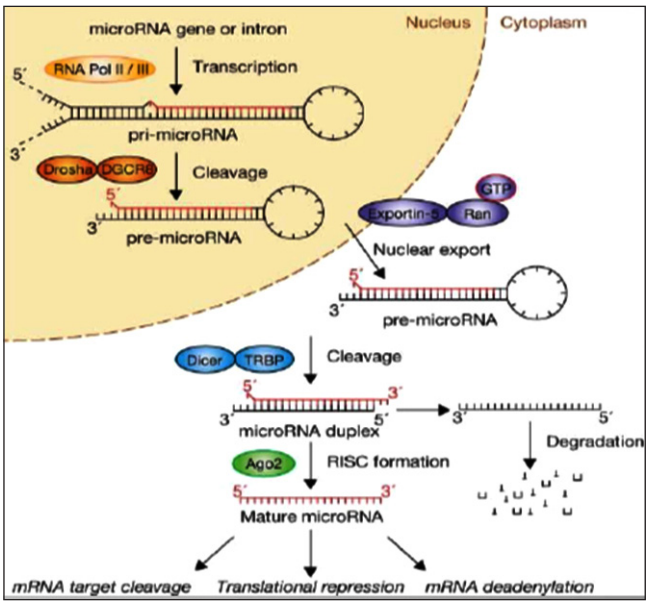


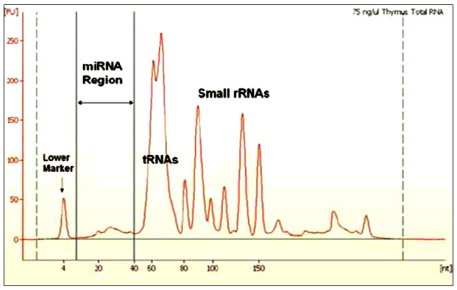
Figure 1. Biogenesis of miRNA

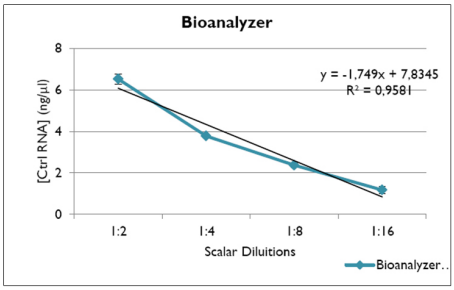
Experiment

Sample acquisition and preparation

A total of eight selected miRNAs (mi923, mi150, mi144, mi96, mi196a, mi30b, mi197, mi451) have been extracted and quantified from six whole blood samples from healthy athletes at three different times (T=0 within 24h from sample collection, T=1 after 15 days, T=2 after 30 days). Another blood sample was withdrawn fresh from a donor, then stored as concentrated erythrocytes and, after 30 days, used to get an ex-vivo autologous blood transfusion with new fresh blood from the same donor. All miRNAs were extracted with a specific kit (miRneasy, Qiagen), and quantified with a specific Chip Electrophoresis System (Agilent Bioanalyzer 2100). 5ng miRNA each sample were then retrotranscribed to cDNA using TaqMan® microRNA reverse transcription kit (Applied Biosystems) and using specific stem-loop primers for each system analyzed. cDNA were then used as template for quantitative PCR (qPCR) using TaqMan® microRNA specific kit and real-time PCR 7500Fast instrument from Applied Biosystems. Data analysis was made with the technique of «relative quantification» that was chosen as the more suitable for the aims of these experiments [4].

Figure 2. Agilent Bioanalyzer 2100 Chip electrophoresis system





Controllo ng/µl	DS	CV%
6,53	0,24	3,70
3,78	0,01	0,37
2,37	0,09	3,85
1,17	0,16	13,89

Figure 2. Agilent Bioanalyzer 2100 Chip electrophoresis system

miRNA quantification

We considered that a sensitive and reliable quantification of extracted miRNAs is a key step for getting an optimal reproducibility of the following quantification step in qPCR. Agilent Bioanalyzer 2100 Chip electrophoresis system (figure 2) was chosen for quantification. It is in fact specific to quantify miRNAs from all other RNAs and it is high sensitive and reproducible. Serial dilutions starting from a control miRNA sample with known concentration were used to monitor instrumental performances.

qPCR and data analysis

For the aim of this study, relative quantification with normalization to an housekeeping gene was chosen as the most appropriate method. To calculate the relative expression of all miRNAs considered in the study, threshold cycles (Cts), obtained for each sample, were used. Delta Ct (ΔCt) was first calculated as normalization of the target gene with the housekeeping gene. ΔΔCt value was obtained as normalization of ΔCt sample to the ΔCt of a sample used as calibrator. Expression of miRNAs was calculated as «relative quantities» (RQ) estimated as 2-ΔΔCt. mi150 was selected as the most appropriate housekeeping gene since its variability was found to be the most stable having it the lower coefficient of variation (4.5%) among all miRNAs studied (figure 3).

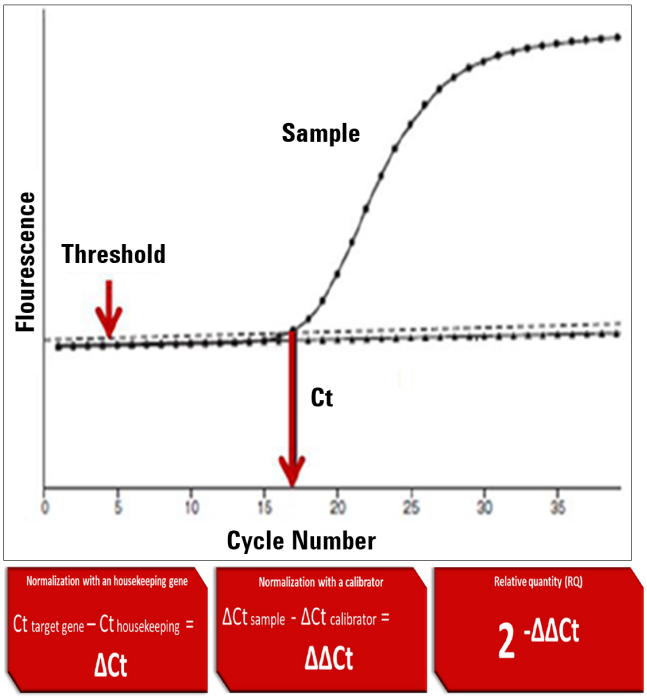


Figure 3. Analysis of miRNA expression with the method of relative quantification.

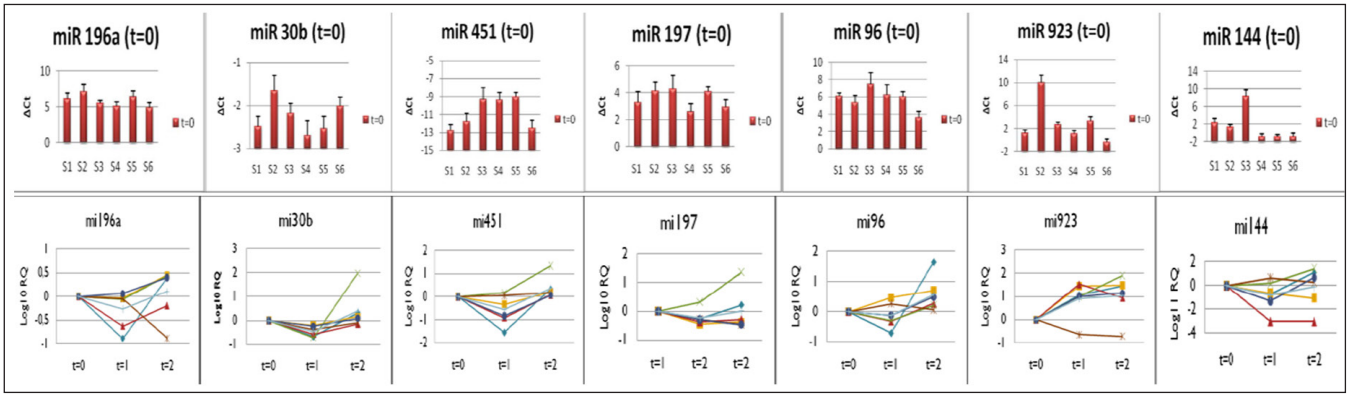


Figure 4. Variability of miRNA expression in blood samples (high). Longitudinal variability of miRNAs in 30 days of storage (low)

Results

Variability of miRNA expression in blood samples at T=0 (fresh blood): mi144, mi923 and mi451 have shown the most consistent variability in the RQ among the samples while mi30b, mi196a, mi197, mi196 have shown less variability (figure 4, high)

Longitudinal variability of miRNA during 30 days of blood storage: we observed a gradual tendency of the miRNAs to increase their expression in samples at t=2 compared to t=0. mi197, mi96, mi196a, mi30b, and mi451 shown their variability to be restrained in 2 to 4 grade of magnitude while mi144 and mi923 have shown the most consistent differences in the expression from t=0 to t=2 however with great variability among samples. We also detected some outlier sample moving away from this general pattern (figure 4, low)

Erythrocytes concentrated and ex-vivo autologous transfusion analysis: a marked difference has been observed in erythrocytes concentrate sample (analyzedafter 30 days of storage) where expression levels of mi923, mi30b, mi197, mi96 and mi451 resulted higher compared to the fresh samples at the time of the withdrawal. Interestingly, the expression of some miRNAs (such as mi197, mi30b, mi451, mi96 and mi923) is very high in the erythrocytes concentrated sample and it is detectable also in the ex-vivo transfused sample with levels higher compared to the fresh non-transfused sample (even though lower compared to the concentrated erythrocytes sample because of the post-transfusion dilution effect). Moreover, two miRNAs (mi144 and mi923) show the most significant increasing in the transfused sample compared to the fresh one. As result, it is very important to note that, from the data obtained, the possibility to use miRNAs expression both as biomarkers of storage and biomarkers of effect in blood doping detection emerges (figure 5).

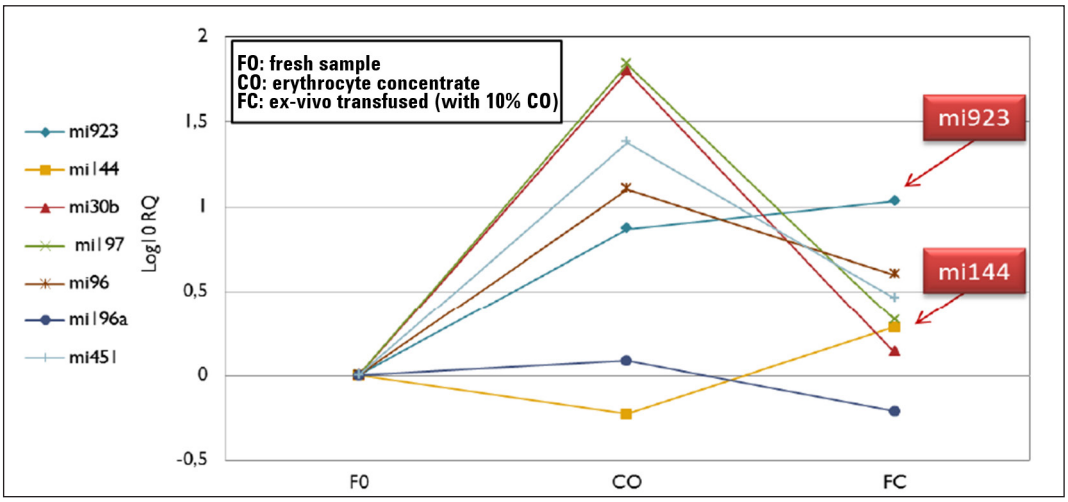


Figure 5. Results of variation in the expression of miRNA after an ex-vivo autologous transfusion.

Conclusions

Nowadays, one of the major challenges in the fight against doping in sport is the development of a direct method for the detection of autologous blood transfusions. The approach of the use of miRNAs expression seems to be very promising. Techniques of analytical gene expression have suitable sensibility and reproducibility to be applied in this direction. However, one of the key points to be better focused in the nearly future is related to the intra-individual variability in the expression of the diagnostic miRNAs. Broadening the panel of miRNAs to be assayed and carrying out a solid population study, associated to a statistical and forensic analysis of the variability, will be the next step to be attempted by our research group.

References

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