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

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Abstract

The expression of some selected miRNAs extracted from human whole blood has been studied with the purpose of applying miRNAs gene expression in the detection of autologous blood transfusions in doping control. The variability among the physiological expression of miRNAs in human whole blood was evaluated, and a panel of miRNAs was preliminarily selected as possible target biomarker for autologous blood transfusions. Our results indicated that some of the miRNAs have the tendency to behave as “markers of storage” since their expression resulted in an increment in the erythrocytes concentrate compared to the fresh sample. Moreover some other miRNAs behave as “markers of effect” since their expression resulted poorly or null alterated in the erythrocytes concentrate but with higher expression in the ex-vivo simulation of autologous blood transfusion. This indicates the possibility of using specific miRNAs for the purpose of detecting autologous blood transfusion in doping control.

Introduction

In this work the role and the variability of microRNA (miRNAs) as potential biomarkers have been investigated, which are able to reveal the practice of an autologous blood transfusion [1].
We have considered the variability of a panel of miRNAs [2], in view of their selection as biomarkers of blood aging during storage, thus representing a potential diagnostic tool to detect the recourse to blood doping – including autologous blood transfusion [3] – by the athletes. Whole blood samples collected in the framework of routine anti-doping tests were anonymized, analyzed and used in an "*ex vivo*" autologous blood transfusion experiment.

Experimental

Eight miRNAs (mi923, mi150, mi144, mi96, mi196a, mi30b, mi197, and mi451) were extracted and quantified from six whole blood samples from healthy athletes, after anonymization, at three different times (T=0 within 24h from sample collection, T=1 after 15 days, T=2 after 30 days). A reference blood sample was withdrawn fresh from a donor, then stored (as concentrated erythrocytes) and, after 30 days, used to perform an *ex vivo*, simulated autologous blood transfusion, mixing an aliquot of the same sample with new, freshly withdrawn blood from the same donor. All miRNAs were extracted by a specific miRneasy kit (Qiagen SpA, Milano, Italy).
Quantification of the extracted miRNAs was made by a specific Chip Electrophoresis System (Bioanalyzer 2100, Agilent Technologies Italia SpA, Milano, Italy). Retrotranscription step to cDNA was performed with 5 ng RNA with a TaqMan® microRNA reverse transcription kit (Life Technologies Italia, Monza, Italy). cDNA were used as template for quantitative PCR (qPCR) using TaqMan® microRNA specific kit and real-time PCR 7500Fast instrument from Applied Biosystems (Life Technologies Italia, Monza, Italy) according manufacturer instructions. Data analysis of the PCR products was performed by the technique of “relative quantification” [4], chosen as the most suitable for the purposes of the present work. In brief, the difference between the threshold cycle of target miRNA and the endogenous control (Delta Ct) is subtracted to the Delta Ct of the calibrator sample (T=0) so to achieve a DeltaDelta Ct that is converted to Relative Quantity (RQ) of the target miRNA.

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Results and Discussion

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 inter-individual variability (Fig. 1).

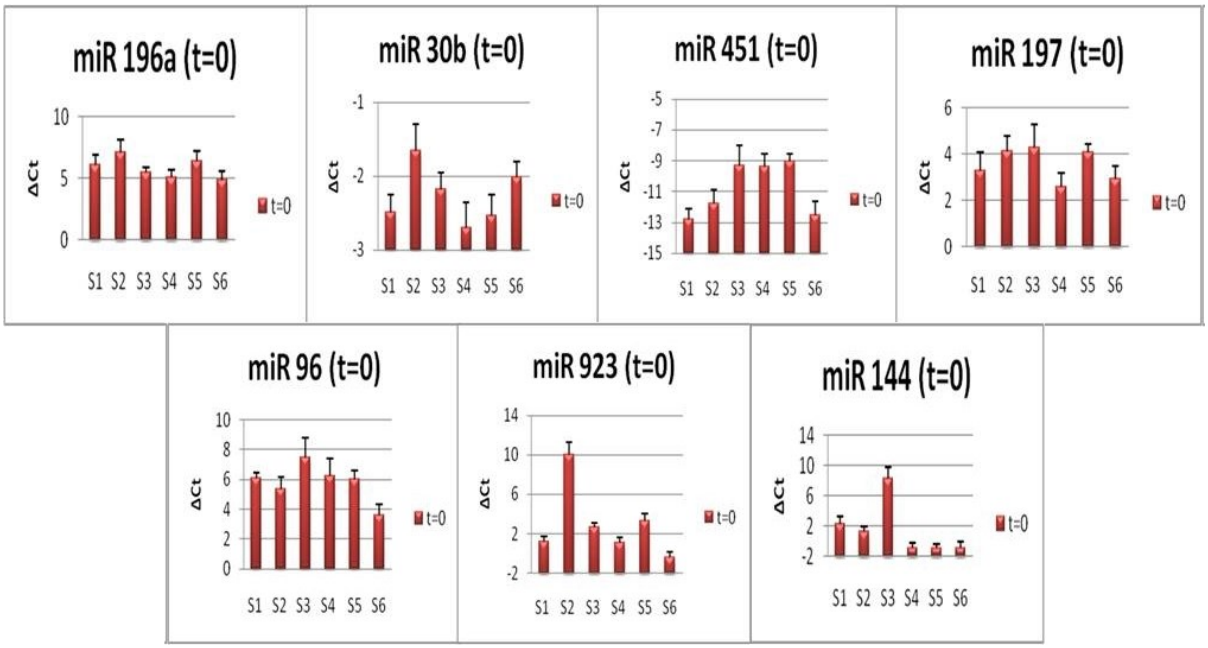

Figure 1: Variability of miRNA expression in blood samples (high).

Longitudinal variability of miRNA during 30 days of blood storage
miRNAs 96, 923 and 144 shown a tendency of increasing their expression in samples at T=2 compared to T=0 with slight differences among samples. Other miRNAs such as mi197, mi30b, and mi451 showed an opposite pattern from T=0 to T=1 and coming to basal levels at T=2. Also, mi144 and mi923 have shown the most consistent differences in the expression from T=0 to T=2 however with great variability among the samples. We also detected some outlier sample moving away from this general pattern (Fig. 2). The overall pattern seems to point to a great inter-individual differences.

Erythrocytes concentrated and *ex vivo* autologous transfusion analysis
A marked difference has been observed in erythrocytes concentrate sample (analyzed after 30 days of storage) where expression levels of mi923, mi30b, mi197, mi96 and mi451 were 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 increase in the transfused sample compared to the fresh one. As a result, from the data obtained, the possibility to use miRNAs expression both as biomarkers of storage and biomarkers of effect in blood doping detection emerges (Fig. 3).

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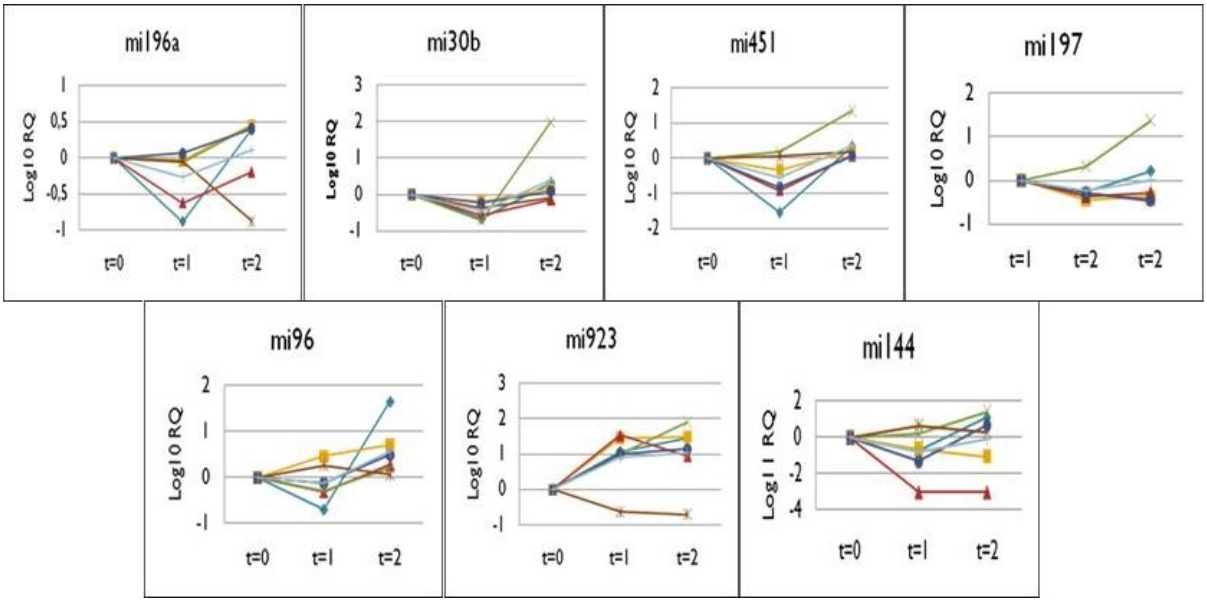

Figure 2: Longitudinal variability of miRNAs in 30 days of storage.

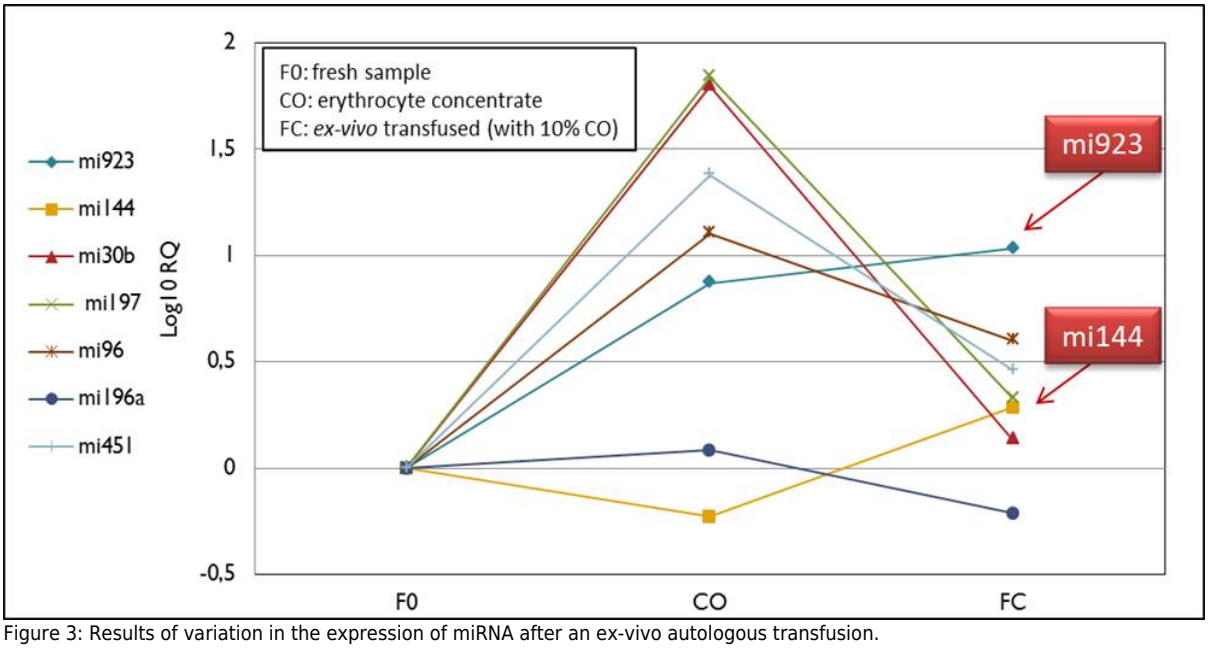

Figure 3: Results of variation in the expression of miRNA after an ex-vivo autologous transfusion.

Conclusions

The approach of using miRNAs expression seems to be very promising. The analytical techniques of gene expression have suitable sensitivity and reproducibility to be applied for this purpose. The choice of an appropriate gene to serve as endogenous control revealed to be a key step in the application this procedure. However, one of the key points to be

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focused in the nearly future is related to the intra-individual variability in the expression of the diagnostic miRNAs. Our data revealed that there is a consistent variability among different subjects in the expression of miRNAs in whole blood. This must be considered when developing a strategy for a method to detect ABT based on “decision limits” to one or more diagnostic miRNAs.

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Acknowledgements

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