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MicroRNAs as potential biomarkers for doxorubicin-induced cardiotoxicity

Gustav Holmgren [a,b,*], Jane Synnergren [a], Christian X. Andersson [c], Anders Lindahl [b], Peter Sartipy [a,d]

[a] Systems Biology Research Center, School of Bioscience, University of Skövde, Box 408 Kanikegränd 3A, SE-541 28, Skövde, Sweden
[b] Department of Clinical Chemistry and Transfusion Medicine, Institute of Biomedicine, University of Gothenburg, Sahlgrenska University Hospital, SE-413 45, Gothenburg, Sweden
[c] Takara Bio Europe AB, Arvid Wallgrens Backe 20, SE-413 46, Gothenburg, Sweden
[d] AstraZeneca Gothenburg, CVMD GMed, GMD, Pepparedsleden 1, SE-430 51, Mölndal, Sweden

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ABSTRACT

Anthracyclines, such as doxorubicin, are well-established, highly efficient anti-neoplastic drugs used for treatment of a variety of cancers, including solid tumors, leukemia, lymphomas, and breast cancer. The successful use of doxorubicin has, however, been hampered by severe cardiotoxic side-effects. In order to prevent or reverse negative side-effects of doxorubicin, it is important to find early biomarkers of heart injury and drug-induced cardiotoxicity. The high stability under extreme conditions, presence in various body fluids, and tissue-specificity, makes microRNAs very suitable as clinical biomarkers. The present study aimed towards evaluating the early and late effects of doxorubicin on the microRNA expression in cardiomyocytes derived from human pluripotent stem cells. We report on several microRNAs, including miR-34a, miR-34b, miR-187, miR-199a, miR-199b, miR-146a, miR-15b, miR-130a, miR-214, and miR-424, that are differentially expressed upon, and after, treatment with doxorubicin. Investigation of the biological relevance of the identified microRNAs revealed connections to cardiomyocyte function and cardiotoxicity, thus supporting the findings of these microRNAs as potential biomarkers for drug-induced cardiotoxicity.

1. Introduction

Recent years of research have shown the great potential of using microRNAs as clinical biomarkers (Li and Zhang, 2015; Schulte and Zeller, 2015). MicroRNAs show a highly evolutionary conservation; they are stable in various body fluids, and can therefore easily be measured in clinical samples (Cheng et al., 2007; Kim, 2005; Lewis et al., 2005; Moss and Tang, 2003; Pasquinelli et al., 2000; Raitoharju et al., 2013; Zhang, 2008). MicroRNAs play important roles in many biological processes, including cell differentiation, replication and regeneration, and they have been associated with several diseases (Hwang and Mendell, 2006; Jovanovic and Hengartner, 2006).

Doxorubicin is an efficient chemotherapeutic agent for treatment of a variety of cancers, including breast cancer (Gennari et al., 2008), leukemia (Fernandez et al., 2009), lymphomas (Luminari et al., 2011), and many other tumor types. Treatment with anthracycline-based chemotherapy is however, associated with severe cardiotoxicity, often resulting in early discontinuation (Carvalho et al., 2009; Ferreira et al., 2008; Minotti et al., 2004; Zhang et al., 2009). The exact mechanisms responsible for doxorubicin-induced cardiomyopathy are not completely known, but the fact that the cardiotoxicity is dose-dependent and that there is a variation in time-to-onset of toxicity, and gender- and age differences suggests that several mechanisms may be involved (Lipshultz et al., 1991; Wallace, 2003; Yeh et al., 2004). An increased understanding of the potential roles of microRNAs in these processes may help to shed more light on the mechanisms, and provide means to prevent or treat negative side effects of doxorubicin.

The usefulness of human pluripotent stem cell-derived cardiomyocytes (hPSC-CMs) to study anthracycline-induced cardiotoxicity has been demonstrated in previous studies (Chaudhari et al., 2015; Eldridge et al., 2014; Holmgren et al., 2015). We and others have assessed the global mRNA expression in cardiomyocytes treated with doxorubicin and identified several differentially expressed mRNA that potentially could serve as relevant biomarkers for doxorubicin-induced cardiotoxicity (Chaudhari et al., 2015; Holmgren et al., 2015). In addition, a report by Eldridge et al. (2014) also demonstrated the use of hPSC-CMs for mechanistic studies of the cardiotoxic events. However, none of these studies investigated the effects of doxorubicin on the microRNA level.

Towards this aim, our present study focused on the investigation of the microRNA expression pattern in cardiomyocytes exposed to doxorubicin using a similar experimental set up as we reported previously (Holmgren et al., 2015). The effects on the microRNA expression in cells treated with doxorubicin were investigated in pure cardiomyocyte cultures derived from human embryonic stem cells (hESC). An evident effect of doxorubicin on the cells was observed,

Abbreviations: hESC, human embryonic stem cells; hPSC-CM, human pluripotent stem cell-derived cardiomyocytes; SAM, significance analysis of microarray.

* Corresponding author at: Systems Biology Research Center, School of Bioscience, University of Skövde, Box 408 Kanikegränd 3A, SE-541 28, Skövde, Sweden.
E-mail addresses: gustav.holmgren@his.se (G. Holmgren), jane.synnergren@his.se (J. Synnergren), Christian.andersson@takara-clontech.eu (C.X. Andersson), anders.lindahl@clinchem.gu.se (A. Lindahl), peter.sartipy@his.se (P. Sartipy).

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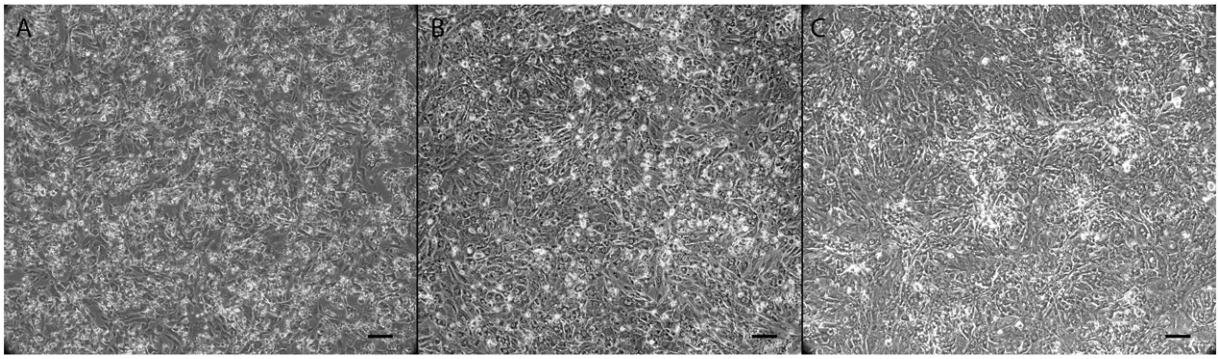

Fig. 1. Representative light microscopy images of Cellartis® Pure hES-CM two (A), three (B), and four (C) days post thawing. Scale bar represents 100 μm.

even after the wash-out period, and a distinct influence on the global microRNA expression profile was detected. A number of microRNAs that showed a significant differential expression, both during and after the acute exposure, were identified. Notably, a pathway over-representation analysis of the identified microRNAs revealed several over-represented pathways related to cardiomyocyte functions, toxic responses, and cell differentiation.

2. Materials and methods

2.1. Cell culture

Human cardiomyocytes, Cellartis® Pure hES-CM, were obtained from Takara Bio Europe AB (Gothenburg, Sweden) and cultured under serum free conditions according to the Cellartis Cardiomyocyte User Manual (Cat. No. Y10075, www.clontech.com). The cells were thawed and seeded (200,000 cells/cm^2) in 12 well culture dishes. After a recovery period of 6 days with medium changes every second day, the cells were incubated with or without doxorubicin (D1515, Sigma-Aldrich, Sweden) at various concentrations (50 nM, 150 nM, or 450 nM) for up to 2 days. The incubation with doxorubicin was followed by a 12-day wash-out period without drug exposure. The experiment was performed in triplicate, i.e. cells were thawed at three separate time points to ensure a true biological variability.

2.2. Cell harvest

At selected time points during the study (day 0, 1, 2, 7, and 14 from the start of compound exposure), cells were harvested for subsequent RNA and protein isolation. Total RNA was extracted using GenElute RNA/DNA/Protein Plus Purification Kit (E5163, Sigma-Aldrich, Sweden, http://www.sigmaaldrich.com). Quantification of nucleic acids and total protein content was performed on a NanoDrop ND-1000 (NanoDrop, http://www.nanodrop.com).

2.3. MicroRNA array profiling

MicroRNA array profiling was conducted at Exiqon Services, Denmark. The quality of the RNA was verified by an Agilent 2100 Bioanalyzer profile. Total RNA from both samples and reference was labeled with Hy3™ and Hy5™ fluorescent label, respectively, using the miRCURY LNA™ microRNA Hi-Power Labeling Kit, Hy3™/Hy5™ (Exiqon, Denmark) following the instruction manual provided by the manufacturer (http://www.exiqon.com/microrna-microarray-analysis-power-labeling-kits). The Hy3™-labeled samples and a Hy5™-labeled reference RNA sample were mixed pair-wise and hybridized to the miRCURY LNA™ microRNA Array 7th Gen (Exiqon, Denmark). The hybridization was performed according to the miRCURY LNA™ microRNA Array Instruction manual using a Tecan HS4800™ hybridization station (Tecan, Austria). The miRCURY LNA™ microRNA Array slides were scanned using the Agilent G2565BA Microarray Scanner System (Agilent Technologies, Inc., USA) and the image analysis was carried out using the ImaGene® 9 (miRCURY LNA™ microRNA Array Analysis Software, Exiqon, Denmark). The quantified signals were background corrected (Normexp with offset value 10, see Ritchie et al. (Ritchie et al., 2007)) and normalized using the global LOWESS (locally weighted scatterplot smoothing) regression algorithm. The threshold of detection was calculated for each individual microarray slide as 1.2 times the 25th percentile of the overall signal intensity of the slide. MicroRNAs with intensities above threshold in less than 20% of the samples were removed from the final data set used for the expression analysis. For the present data set, a total of 1420 probes were discarded and 679 probes passed this filtering procedure.

2.4. Low and high level analysis of microarray data

Statistical analysis of the microarray data was performed using R (version 3.2.2). Hierarchical clustering was performed using the *hclust* function with Pearson correlation as distance measurement and unweighted pair group method with arithmetic averaging (UPGMA) as linkage method. Principal component analysis was executed using the *prcomp* function with zero-centered data scaled to unit variance. In order to identify differentially expressed microRNA between the treatment groups, the multiclass significance analysis of microarray (SAM)

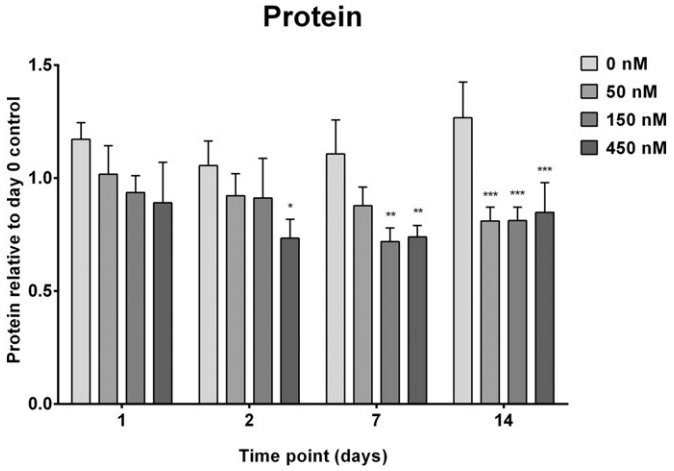

Fig. 2. Protein content per sample related to day 0 control set to 1. The error bars represent standard deviation of the three biological replicates. *, **, and *** indicate p-value <0.05, <0.01, and <0.001 respectively, from a one-way ANOVA at each time point.