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Early biomarkers of doxorubicin-induced heart injury in a mouse model

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ABSTRACT

Cardiac troponins, which are used as myocardial injury markers, are released in plasma only after tissue damage has occurred. Therefore, there is a need for identification of biomarkers of earlier events in cardiac injury to limit the extent of damage. To accomplish this, expression profiling of 1179 unique microRNAs (miRNAs) was performed in a chronic cardiotoxicity mouse model developed in our laboratory. Male B6C3F$_1$ mice were injected intravenously with 3 mg/kg doxorubicin (DOX; an anti-cancer drug), or saline once a week for 2, 3, 4, 6, and 8 weeks, resulting in cumulative DOX doses of 6, 9, 12, 18, and 24 mg/kg, respectively. Mice were euthanized a week after the last dose. Cardiac injury was evidenced in mice exposed to 18 mg/kg and higher cumulative DOX dose whereas examination of hearts by light microscopy revealed cardiac lesions at 24 mg/kg DOX. Also, 24 miRNAs were differentially expressed in mouse hearts, with the expression of 1, 1, 2, 8, and 21 miRNAs altered at 6, 9, 12, 18, and 24 mg/kg DOX, respectively. A pro-apoptotic miR-34a was the only miRNA that was up-regulated at all cumulative DOX doses and showed a significant dose-related response. Up-regulation of miR-34a at 6 mg/kg DOX may suggest apoptosis as an early molecular change in the hearts of DOX-treated mice. At 12 mg/kg DOX, up-regulation of miR-34a was associated with down-regulation of hypertrophy-related miR-150; changes observed before cardiac injury. These findings may lead to the development of biomarkers of earlier events in DOX-induced cardiotoxicity that occur before the release of cardiac troponins.

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Introduction

The long-term clinical use of a potent anthracycline, doxorubicin (DOX), is limited because of a cumulative dose-dependent cardiotoxicity (Singal and Iliskovic, 1998). Several invasive and non-invasive approaches have been utilized to monitor the cardiotoxic effects of DOX. These techniques have limitations. An invasive technique, such as endomyocardial biopsy (a sensitive indicator of chronic cardiotoxicity) is associated with risk of serious life-threatening procedural complications (Sethi and Copeland, 1997; From et al., 2011). Radionuclide angiocardiography, a non-invasive technique, is widely used to assess cardiac function. However, the resting left ventricular ejection fraction as determined by this method is a relatively insensitive means for detecting early changes of anthracycline-induced cardiotoxicity (Gharib and Burnett, 2002). Cardiac troponins are routinely used for the clinical detection of DOX-related heart damage in patients. However, these biomarkers are released in the blood only after tissue damage has occurred (Lipshultz et al., 1997; Herman et al., 1998, 1999; Bertinchant et al., 2003; Wallace et al., 2004). Thus, there is a critical need to identify biomarkers of early events of DOX-induced cardiac tissue damage. Availability of early biomarkers could provide the means to modify treatment strategies aimed at reducing or preventing DOX-induced adverse effects in the heart.

In recent years, expression profiling of miRNAs has emerged as an important screening tool for the discovery of potential biomarkers of various pathologies. MicroRNAs are small (~22 nucleotides), non-coding RNAs that regulate gene expression primarily by inhibiting the translation of a specific messenger RNA (mRNA) target into a functional protein and/or promoting mRNA degradation (Ambros, 2004; Bartel, 2004). It appears that miRNAs perform a crucial regulatory role in cardiac physiology and pathogenesis (Ikeda et al., 2007; Rao et al., 2009; Corsten et al., 2010; Small et al., 2010; Xiao and Chen, 2010; Boon et al., 2013). At present, there is a growing interest in recognizing the potential of miRNAs both as biomarkers and therapeutics for cardiovascular diseases

Abbreviations: cTnT, cardiac troponin T; DAB, diaminobenzidine; DOX, doxorubicin; DSBs, double strand breaks; FDR, false discovery rate; IgG, immunoglobin G; IHC, immunohistochemistry; qRT-PCR, quantitative real time polymerase chain reaction; miRNA, microRNA; RT, room temperature; SAL, saline; TBST, Tris-buffered saline with Tween20; TUNEL, Terminal deoxynucleotidyl transferase (TdT) mediated d-UTP nick end labeling.

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(van Rooij et al., 2008; Corsten et al., 2010; D'Alessandra et al., 2010; Bernardo et al., 2012; Matsumoto et al., 2013). The present study was intended to determine whether early biomarkers of cardiac injury could be identified by monitoring changes in the expression of miRNAs in the hearts of mice treated chronically with DOX (Desai et al., 2013). Results indicate a dose-related increase in the number of differentially expressed miRNAs that are associated with various cardiac events. Among these, miR-34a and miR-150 may be of particular importance as their expression was significantly altered before the occurrence of observable cardiac tissue injury as indicated by a significant release of cardiac troponin T (cTnT) in plasma (at 18 mg/kg) or cardiac pathology by light microscopy (at 24 mg/kg). These early miRNA changes in the heart could help in the development of translational biomarkers that will allow the identification of patients at risk for DOX-induced cardiotoxicity.

Materials and methods

Animal treatments. In our previous study utilizing a chronic cardiotoxicity mouse model, DOX-induced cardiac tissue injury was observed after exposure to 12 mg/kg and higher cumulative DOX doses as indicated by higher concentration of cTnT in plasma (Desai et al., 2013). A dose-related increase in cardiac lesions was also seen in mice after exposure to 24 mg/kg and higher cumulative DOX doses (Desai et al., 2013). In the present study, to facilitate the identification of early biomarkers of cardiac tissue injury in a chronic DOX cardiotoxicity mouse model, the DOX regimen was modified by including two cumulative DOX doses lower than 12 mg/kg. Male $B6C3F_1$ mice were obtained from the breeding colony at the National Center for Toxicological Research (NCTR) and were raised and treated as described previously (Desai et al., 2013). In brief, beginning at 8 weeks of age, mice were treated (via a tail-vein) with a weekly dose of 3 mg/kg DOX (Chempacific, Baltimore, MD) or an equivalent volume of 0.9% sterile saline (SAL; Sigma-Aldrich, St. Louis, MO) for 2, 3, 4, 6, and 8 weeks (cumulative DOX doses of 6, 9, 12, 18, and 24 mg/kg, respectively). Each DOX treatment group consisted of 12 male mice whereas each SAL treatment group consisted of 10 male mice. It is well established in humans that DOX cardiotoxicity may occur one year to decades after completion of the treatment (Lipshultz et al., 1991). Also, delayed cardiotoxicity has been shown in laboratory animals that developed cardiotoxicity weeks after exposure to DOX (Imondi et al., 1996; Herman et al., 1998; Lebrecht et al., 2007). In the present study therefore, mice were euthanized a week after each cumulative DOX exposure to mimic the clinical situation. All animals were observed daily for abnormal signs during the course of the study. The protocol for this study was approved by the NCTR Animal Care and Use Committee. All experimental procedures were performed in compliance with the Guide to the Care and Use of Laboratory Animals.

Collection of tissues. A week following the last dose, mice were anesthetized by the inhalation of 2.0% isoflurane mixed with 100% oxygen using a Euthanex E-Z Anesthesia system (Euthanex, Palmer, PA) and blood was collected by retro-orbital puncture into Microtainer® tubes coated with EDTA. Blood was immediately centrifuged at 1000 ×*g* for 10 min at 4 °C to separate plasma for measurements of cTnT concentration. Following retro-orbital bleeding, mice were euthanized by exsanguination and the heart was immediately excised and separated from the pericardium. A transverse section of the heart was collected midpoint between the base and apex and fixed in 4% paraformaldehyde for examination by light microscopy. The remaining heart tissue was immediately frozen in liquid nitrogen for storage at −80 °C for expression profiling of miRNAs.

Measurement of plasma cTnT concentration. Plasma cTnT concentrations were measured using the fourth-generation immunoassay (Elecsys® Troponin T STAT; Roche Diagnostics, Indianapolis, IN) on the Elecsys 2010 instrument (Roche Diagnostics). The lower limit of detection of this assay is 0.01 ng/ml.

Histological examination of heart. The 4% paraformaldehyde-fixed transverse section of the heart was processed and embedded in a glycol methacrylate-based polymer. Approximately 2-μm sections were stained with toluidine blue and examined by light microscopy. The hearts were scored for the severity and extent of cardiomyopathy (cytoplasmic vacuolization). Grade 0 = normal histological appearance and grade 1 (minimal) = one to three cardiomyocytes with vacuolated cytoplasm in the entire tissue section. The number of cardiomyocytes with vacuolated cytoplasm did not exceed three in any examined tissue section.

Isolation of RNA from hearts. Individual mouse heart was insufficient to perform different investigations. Therefore, for each sample, two mouse hearts from the same treatment group and with comparable plasma cTnT concentrations were combined together and ground to powder by pestle in a mortar chilled on dry ice. Total RNA was extracted from approximately 30 mg of heart powder using Qiagen miRNeasy Mini Kit (Qiagen Inc., Valencia, CA) according to the manufacturer's protocol. The yield and purity of all RNA samples were determined by measuring the optical density at 260 and 280 nm on a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE) and the quality was evaluated using the RNA 6000 LabChip and Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., Palo Alto, CA). The RNA samples with 260/280 ratios higher than 1.8 and RNA integrity numbers greater than 8.0 were used for microarray experiments.

Transcriptional profiling of miRNAs and data analysis. A total of 40 samples (2 treatments × 5 cumulative exposures × 4 samples per group) were used for miRNA microarray experiment. One hundred nanogram total RNA was processed for single color (Cy3) Agilent mouse 8 × 60 K miRNA microarrays v2, according to the manufacturer's protocols (Agilent Technologies, Inc.). An Agilent one-color spike-in kit was used as a positive control for both labeling and hybridization efficiency. Labeled probes were cleaned up using BioRad Micro Bio-Spin 6 columns according to the manufacturer's instructions (BioRad Life Science Research, Hercules, CA). Hybridized microarrays were washed and scanned using the Agilent High Resolution C Scanner (Agilent Technologies, Inc.). The images were analyzed using Agilent's Feature Extraction software. All raw data files were then uploaded to an in-house microarray data management and analysis software system, ArrayTrack™ (Tong et al., 2003; Fang et al., 2009).

Each miRNA microarray consisted of 49,324 features, representing forty replicates for each of 1179 unique miRNA probes and multiple replicates for each of 21 control probes. The average signal intensity of all forty probes (gTotalGeneSignal) for each miRNA provided by the Agilent Feature Extraction Software was used for data analysis. In the raw data files, non-expressed miRNAs were designated by a value of 0.1. Since 0.1 is not a real intensity value all 0.1 values were replaced by the minimum intensity value of 0.934099 across all the arrays before further processing of the data. All the controls and redundant miRNA probes were filtered out, resulting in 1179 unique features (miRNAs). Also, features which were not expressed (intensity = 0.934099) in any of the arrays were excluded, leaving 487 unique features (miRNAs). These 487 features (miRNAs) were expressed in at least one of the four arrays in any treatment group. Intensity data for all arrays were then transformed to $\log_2$ values to create a normal probability plot. Based on this plot, miRNAs with an average $\log_2$ intensity of 0 (very low expressed) were filtered out, resulting in a final list of 414 unique features (miRNAs). Following an array-wise 75th percentile normalization on 414 features (miRNAs), generalized linear model procedure (proc glm) in SAS was used to measure statistical significance of the contrast between DOX- and SAL-treated mice to determine differentially