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EXPRESSION OF THE ANTHRACYCLINE METABOLIZING ENZYME CARBONYL REDUCTASE 1 (CBR1) IN HEARTS FROM DONORS WITH DOWN SYNDROME

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Running title: CBRI expression in heart samples from donors with Down syndrome.

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Abbreviations: DS, Down syndrome; CBRI, carbonyl reductase 1; AML, acute myeloid leukemia; SNP, single-nucleotide polymorphism; UTR, untranslated region; QRT-PCR, quantitative reverse transcription-polymerase chain reaction; NDRI, National Disease Research Interchange; aCGH, array comparative genomic hybridization; BAC, bacterial artificial chromosome; UPLC-MS/MS, ultra pressure liquid chromatography coupled to tandem mass spectrometry; OR, odds ratio; CI, confidence interval; miRNA, microRNA.
ABSTRACT

Cancer patients with Down syndrome (DS) are susceptible to developing anthracycline-related cardiotoxicity. The pathogenesis of anthracycline-related cardiotoxicity has been linked to the intracardiac synthesis of alcohol metabolites by carbonyl reductase 1 (CBR1). CBR1 is located in the DS critical region (21q22.12). The expression of CBR1 in the DS heart has not been characterized. This study documented CBR1 expression in hearts from DS donors ($n = 4$) and non-DS donors ($n = 15$). DS hearts showed 1.8-fold higher CBR1 mRNA levels than non-DS hearts (DS: 3.3-relative fold vs. non-DS: 1.8-relative fold, $p = 0.012$). CBR1 protein levels were 1.9-fold higher in DS hearts compared to non-DS hearts (DS: 13.5 ± 7.7 nmol/g cytosolic protein vs. non-DS: 7.2 ± 3.9 nmol/g cytosolic protein; $p = 0.029$). CBR1 activity for daunorubicin was 1.7-fold higher in DS hearts than in non-DS hearts (DS: 3.8 ± 0.1 nmol daunol/min·mg vs. non-DS: 2.3 ± 0.2 nmol daunol/min·mg; $p = 0.050$). CBR1 1096G>A (rs9024) impacts CBR1 activity, and one heart trisomic for the variant A allele (A/A/A) exhibited low enzymatic activity. These findings suggest that increased CBR1 expression in the DS heart may contribute to the risk of anthracycline-related cardiotoxicity.
INTRODUCTION

Down syndrome (DS) is the most common genetic cause of mental retardation and originates from the presence of an extra copy of chromosome 21, although in roughly 5% of documented DS cases a partial trisomy leads to the condition (Amano et al., 2003; Patterson, 2009). Children with DS have a 10-20-fold increased risk of developing acute myeloid leukemia (AML) compared to sex- and age-matched children without DS. Treatment regimens for pediatric AML rely heavily on the use of the anthracycline daunorubicin. However, the therapeutic benefit of chemotherapy with daunorubicin is mitigated by the development of cardiotoxicity in some patients. Risk factors for anthracycline-related cardiotoxicity include total cumulative exposure, younger age at cancer diagnosis, radiation therapy to the heart region, and female sex. Epidemiological studies have identified pediatric cancer patients with DS as a population at particularly greater risk for both acute and chronic anthracycline-related cardiotoxicity (Krischer et al., 1997; Grenier and Lipshultz, 1998; Ravindranath et al., 2005). For example, Krischer et al. found that Trisomy 21 was associated with an increase in the relative risk for anthracycline-related cardiomyopathy of 3.4-fold (Krischer et al., 1997). A report from the Children’s Oncology Group documented clinically symptomatic cardiomyopathy in 17.5% of DS-AML patients treated with anthracyclines (O’Brien et al., 2008).

The pathogenesis of anthracycline-related cardiotoxicity has been linked to the synthesis of anthracycline C-13 alcohol metabolites by cardiac CBR1 activity (Minotti et al., 2004). Transgenic mice overexpressing human CBR1 in heart exhibited high levels of intracardiac doxorubicinol and increased signs of myocardial damage after doxorubicin administration (Forrest et al., 2000). Conversely, mice with a null Cbr1 allele (Cbr1<sup>+/−</sup>) had decreased doxorubicinol plasma levels and significantly decreased incidence of anthracycline-related
cardiotoxicity compared to mice with two active Cbr1 alleles (\(Cbr1^{+/+}\)) (Olson et al., 2003). Interestingly, the \(CBRI\) gene is located in the DS critical region (21q22.12), and an early report noted that \(CBRI\) expression is subjected to gene-dosage effects in lymphoid cells with Trisomy 21 (Lemieux et al., 1993). Elevated CBR1 protein levels have been noted in several regions of the brain in DS individuals (Balcz et al., 2001). There is a paucity of reports documenting cardiac \(CBRI\) expression in DS donors. Thus, the goal of the present study was to examine cardiac \(CBRI\) expression in samples from DS and non-DS donors. \(CBRI\) mRNA and CBR1 protein levels were investigated by quantitative real-time reverse transcription-polymerase chain reaction (QRT-PCR) and quantitative immunoblotting, respectively. Cardiac CBR1 activity was measured for the substrate daunorubicin.

Recently, we identified a relatively common single-nucleotide polymorphism (SNP) in the 3’-untranslated region (UTR) of \(CBRI\) (\(q = 12.5\%\) in whites; \(CBRI\) 1096G>A, rs9024) that impacts the synthesis of doxorubicinol in liver. Liver samples with \(CBRI\) 1096G>A homozygous G/G genotype exhibited 1.5-fold higher doxorubicinol synthesis rates than samples with the heterozygous G/A genotype (Gonzalez-Covarrubias et al., 2009). Thus we explored whether \(CBRI\) 1096G>A genotype status affects \(CBRI\) expression in the DS heart.
METHODS
Heart samples

The Institutional Review Board of the State University of New York at Buffalo approved this research. Heart samples (20 - 100 g, left ventricle) from DS \((n = 4)\) and non-DS \((n = 15)\) donors were procured from the National Disease Research Interchange (NDRI). DS status (yes/no) was obtained from medical records. Tissue procurement protocols have been open since 12/2007 and 2/2008 for non-DS and DS hearts, respectively, and included the following criteria: no current diagnosis of cancer, myocardial infarction, and/or congestive heart failure, no evidence of sepsis, and no history of chemotherapy and/or radiation within the last year. Post mortem to tissue recovery interval was \(\leq 10\) h. Heart samples were frozen immediately after recovery and stored in liquid nitrogen until further processing. Heart samples were processed following standardized procedures to isolate RNA and DNA as described (Gonzalez-Covarrubias et al., 2009). Cytosols were obtained by differential ultracentrifugation as described (Mordente et al., 2003).

Array CGH

Genomic DNA was used to probe high resolution comparative genomic hybridization arrays (aCGH) as described (Snijders et al., 2001; Cowell et al., 2004). Arrays contained \(~ 6,000\) sequenced bacterial artificial chromosome (BAC) clones which provided a resolution of 700 kb across the genome. Changes in DNA copy number were determined by evaluating log\(_2\) ratios across whole chromosomes.

Quantitative real-time PCR

Total RNA from liver (200 ng) was reverse-transcribed and amplified by using one-step QuantiTect SYBR Green RT-PCR kits (Qiagen, Valencia, CA). \(CBR1\) mRNA levels were
determined by the comparative quantitation method as described (Gonzalez-Covarrubias et al., 2009). Individual β-actin mRNA levels were used for normalization. Experimental samples and standards for calibration curves were analyzed in quadruplicate. The relative amount of CBR1 mRNA in each sample was automatically calculated with a comparative quantitation algorithm (iQ5 Optical System Software version 2.0, Bio-Rad, Hercules, CA).

**Quantitative immunoblotting**

Cytosolic CBR1 protein levels were quantitated as described (Gonzalez-Covarrubias et al., 2009). Briefly, cytosols (150 μg) and recombinant CBR1 standards (Abcam, Cambridge, MA; 0.03, 0.05, 0.10, 0.15, and 0.30 μg) were loaded into 12% protein gels and separated by electrophoresis. Proteins were probed with a specific polyclonal anti-human CBR1 antibody (1:2500; Santa Cruz, Santa Cruz, CA) and a secondary rabbit anti-IgG antibody conjugated with horseradish peroxidase (1:10,000; Sigma-Aldrich). Immunoreactive bands were visualized with the ECL Plus Western blotting detection system (GE Healthcare). CBR1 band intensity values (pixels/mm²) were quantified with a ChemiDoc XRS gel documentation system (Bio-Rad). Cardiac CBR1 levels were estimated by extrapolation from the calibration curves of recombinant CBR1. Detection of CBR1 was linear (range: 0.03 - 0.30 μg; $r^2 > 0.93$).

**Kinetic analysis**

Validation experiments with pooled heart cytosols showed that 400 μM daunorubicin [S] ensured conditions of $V_{\text{max}}$ and/or maximal CBR1 activity (zero-order kinetics). Thus, maximal CBR1 activities (reaction rates) were directly proportional to the amount of cytosolic CBR1 enzyme (Gonzalez-Covarrubias et al., 2009). Maximal CBR1 activities for the substrate daunorubicin were measured in cardiac cytosols essentially as described (Gonzalez-Covarrubias et al., 2009). Incubation mixtures (1.0 ml) contained potassium phosphate buffer (0.1 M; pH
7.4), NADPH (200 μM; Sigma-Aldrich), and daunorubicin (400 μM; Sigma-Aldrich). Kinetic reactions were started by the addition of cytosols (100 μl, total protein concentration: 7.0 ± 2.1 mg/ml; range, 4.5 - 11.7 mg/ml). Reaction mixtures were flash frozen with liquid nitrogen and stored at -70°C until daunorubicinol quantification. Daunorubicinol was quantitated with a validated UPLC-MS/MS method as described (Kassner et al., 2008; Gonzalez-Covarrubias et al., 2009).

**CBR1 genotyping**

The CBR1 1096G>A (rs9024) polymorphism was investigated by allelic discrimination with fluorescent probes and real-time PCR (Assays-by-Design; Applied Biosystems, Carlsbad, CA). The two possible heterozygous conditions (G/G/A and G/A/A) in DS samples were detected by allele quantification. DNA samples with known CBR1 1096G>A genotypes were mixed to generate standards for calibration curves. The theoretical ratios of the variant A allele were 0, 12.5, 20.0, 33.3, 50.0, 66.7, 80.0, 87.5, and 100% (Cheng et al., 2005). Genotyping reactions were run as previously described (Gonzalez-Covarrubias et al., 2009). CBR1 1096G>A genotype status was extrapolated from the calibration curves depicting normalized fluorescent signal vs. percentage of allele A.

**Data analysis**

Statistics were computed with Excel 2007 (Microsoft, Redmond, WA) and GraphPad Prism (GraphPad Software Inc., version 4.03, San Diego, CA). The Mann-Whitney test was used to compare group means. Differences were considered to be statistically significant at p < 0.05.
RESULTS

First, aCGH analysis was performed on DNA isolated from heart samples from DS donors to confirm Trisomy 21. Analysis of log₂ ratios demonstrated that all DNA samples from DS donors exhibited a third copy of chromosome 21 (figure 1A). Further examination revealed that all DS DNA samples carried 3 copies of CBR1 (figure 1B). Next, cardiac CBR1 mRNA expression in DS and non-DS hearts was examined by QRT-PCR. On average, cardiac CBR1 mRNA levels were 1.8-fold higher in DS samples than in non-DS samples (CBR1 mRNA<sub>DS</sub>: 3.3 ± 0.5 relative fold vs. CBR1 mRNA<sub>non-DS</sub> : 1.8 ± 0.2 relative fold, \( p = 0.012 \); figure 1C). Increased cardiac CBR1 mRNA expression dictated higher CBR1 protein levels in cytosols from DS samples compared to non-DS samples (CBR1 protein<sub>DS</sub>: 13.5 ± 7.7 nmol/g cytosolic protein vs. CBR1 protein<sub>non-DS</sub>: 7.2 ± 3.9 nmol/g cytosolic protein, \( p = 0.029 \); figure 1D).

To further examine the impact of Trisomy 21 on cardiac CBR1 expression, we measured CBR1 activity for the anthracycline substrate daunorubicin. Cardiac CBR1 activity demonstrated typical one-site Michaelis-Menten kinetics for daunorubicin (\( V_{\text{max}} \): 1.8 ± 0.2 nmol/min·mg; \( K_m \): 202.0 ± 50.6 µmol; figure 1G). In line with the CBR1 mRNA and CBR1 protein expression data, maximal CBR1 activity, expressed as the rate of synthesis of daunorubicinol, was 1.7-fold higher in heart samples from DS donors than in samples from non-DS donors (CBR1 activity<sub>DS</sub>: 3.8 ± 0.1 nmol daunol/min·mg vs. CBR1 activity<sub>non-DS</sub>: 2.3 ± 0.2 nmol daunol/min·mg, \( p = 0.050 \); figure 1H). CBR1 expression in DS hearts varied between individuals at the mRNA (13.3-fold, range: 0.3 – 4.0 relative fold), protein (5.5-fold, range: 3.0 – 16.4 nmol/g cytosolic protein) and activity levels (3.5-fold, range: 1.1 – 3.9 nmol daunol/min·mg). Similarly, non-DS heart samples showed variable CBR1 expression at the mRNA (1.9-fold, range: 2.2 – 4.1 relative fold), protein
(3.9-fold, range: 6.0 – 23.6 nmol/g cytosolic protein) and activity levels (3.1-fold, range: 1.6 – 4.9 nmol daunol/min·mg).

Next, we examined CBR1 1096G>A genotype status in the DS samples. The calibration curve of normalized fluorescent signal vs. ratio of the variant allele A showed a linear relationship ($r^2 = 0.83$). Table 1 shows cardiac CBR1 expression stratified by CBR1 1096G>A genotype status.
DISCUSSION

This study documents increased CBR1 expression in heart samples from DS donors. On average, DS hearts showed increased CBR1 mRNA (1.8-fold), CBR1 protein (1.9-fold) and CBR1 activity (1.7-fold) compared to non-DS hearts. These observations are in agreement with the expected 1.5-fold increase in CBR1 expression dictated by the gene dosage effect (Lemieux et al., 1993; Amano et al., 2004). Our findings may provide insights concerning the higher incidence of anthracycline-related cardiotoxicity in DS cancer patients compared to non-DS patients (Krischer et al., 1997; Ravindranath et al., 2005; O’Brien et al., 2008). Studies have demonstrated that the development of cardiomyopathy correlates with myocardial accumulation of alcohol metabolites (Forrest et al., 2000; Olson et al., 2003; Minotti et al., 2004). Thus, increased CBR1 expression in the DS heart would impact on the pathogenesis of cardiotoxicity due to the synthesis of relatively high levels of cardiotoxic daunorubicinol.

The relatively small number of DS heart samples is the main limitation of this study. Heart tissue donors with DS are scarce, and procurement rates are low (≈ 1 sample/7.3 months) even for cooperative resources such as the NDRI. Stringent criteria were used for tissue procurement and analysis to minimize experimental variability. Thus, our findings may provide a representative picture of cardiac CBR1 expression in the DS heart.

Recently, we identified a relatively common polymorphism in the 3’- UTR of CBR1 (CBR1 1096G>A, rs9024) that impacts hepatic CBR1 expression at the protein and activity levels. CBR1 protein expression levels were 1.7-fold higher in liver samples with homozygous G/G genotype compared to samples with one copy of the A allele and CBR1 activity, assessed as the rate of synthesis of doxorubicinol, was 1.5-fold higher in liver samples that were G/G compared to those that were G/A (Gonzalez-Covarrubias et al., 2009). In agreement, a recent
report described an association between CBR1 1096G>A genotype status and the risk of cardiomyopathy in pediatric cancer survivors without DS exposed to low-dose [1 - 250 mg/m²] anthracyclines (OR = 5.33, 95% CI, 1.4 - 20.6; p = 0.01; G/G vs. GA/AA) (Blanco et al., 2010). Here, we aimed to extend these observations to DS heart samples harboring an extra copy of CBR1. The DS heart with CBR1 1096G>A genotype homozygous for the variant A allele (A/A/A) showed the lowest CBR1 activity for daunorubcin (Table 1). This individual finding highlights the potential contribution of the polymorphism in dictating variable CBR1 expression in the DS heart. We hypothesized that CBR1 1096G>A impacts the binding of specific microRNA (miRNA) to the polymorphic site at the 3’-UTR of CBR1. Functional studies are ongoing to evaluate whether candidate miRNAs regulate CBR1 expression in various cell types. Additional genotype-phenotype studies with larger sample sizes are needed to define the impact of CBR1 1096G>A on cardiac CBR1 expression.

This pilot study represents the first characterization of cardiac CBR1 expression in DS samples. Our findings suggest that increased CBR1 expression and activity dictated by the gene dosage effect would impact on the risk of anthracycline-related cardiotoxicity in DS cancer patients.
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FOOTNOTES

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Figure Legends

Figure 1
Characterization of cardiac CBR1 expression. A) Array comparative genomic hybridization (aCGH) of a representative DS heart sample. B) Segment of chromosome 21 encompassing CBR1. C) Cardiac CBR1 mRNA expression in samples from non-Down syndrome (n = 15) and Down syndrome (n = 4) donors. Relative CBR1 mRNA levels were determined with a comparative quantitation method. Individual β-actin mRNA levels were used as normalizers. Each circle depicts the average of four replicates. Horizontal lines indicate group means (p = 0.012). D) Cardiac CBR1 protein expression in samples from non-Down syndrome (n = 14) and Down syndrome (n = 4) donors. Horizontal lines indicate group means (p = 0.029). E) Immunodetection of β-actin and recombinant CBR1 standards from a typical calibration curve (range = 0.03 - 0.3 μg). F) Immunodetection of CBR1 in heart cytosols. G. Maximal CBR1 activities with the substrate daunorubicin in heart cytosols from non-Down syndrome (n = 15) and Down syndrome (n = 4) donors. Each circle depicts the average of duplicates for individual samples. Group means are indicated by horizontal lines (p = 0.050). H) Typical one-site Michaelis-Menten kinetics for cardiac CBR1 activity with the substrate daunorubicin.
**Table 1**

*Cardiac CBR1 expression in DS samples stratified by CBR1 1096G>A genotype status*

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Figure 1