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Research Paper

Identification of the Promoter of Human Carbonyl Reductase 3 (*CBR3*) and Impact of Common Promoter Polymorphisms on Hepatic *CBR3* mRNA Expression

Jianping Zhang[1] and Javier G. Blanco[1,2]

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Purpose. Recent studies suggest that polymorphisms in human carbonyl reductase 3 (*CBR3*) influence the pharmacodynamics of doxorubicin. First, we sought to identify the promoter of *CBR3*. Next, we examined whether two *CBR3* promoter polymorphisms (*CBR3* -725T>C and *CBR3* -326T>A) dictate promoter activity and hepatic *CBR3* mRNA levels.
Methods. The promoter activities of *CBR3* reporter constructs were investigated in HepG2 and MCF-7 cells. *CBR3* mRNA levels were documented in 95 liver samples from white (n=62) and black (n=33) donors. Genotype-phenotype correlation analyses were used to determine the impact of the *CBR3* -725T>C and *CBR3* -326T>A polymorphisms on hepatic *CBR3* mRNA levels.
Results. We identified the promoter of human *CBR3*. Liver samples from black donors showed higher relative *CBR3* mRNA levels than samples from whites (*CBR3* $mRNA_{blacks}$=3.0±3.1 relative fold *vs.* *CBR3* $mRNA_{whites}$=1.6±1.5 relative fold, p=0.021). The variant -725C and -326A alleles did not modify the gene reporter activities of engineered *CBR3* promoter constructs. In line, hepatic *CBR3* mRNA levels were not associated with *CBR3* -725T>C and *CBR3* -326T>A genotype status.
Conclusions. These studies provide the first insights into the regulation and variable hepatic expression of polymorphic *CBR3*.

KEY WORDS: carbonyl reductase 3 (*CBR3*); ethnicity; gene promoter; liver; single nucleotide polymorphism.

INTRODUCTION

In humans, there are three carbonyl reductases: carbonyl reductase 1 (*CBR1*), carbonyl reductase 3 (*CBR3*), and the recently identified carbonyl reductase 4 (*CBR4*) (1). Carbonyl reductase activity reduces the anticancer anthracyclines doxorubicin and daunorubicin into their corresponding C-13 alcohol metabolites doxorubicinol and daunorubicinol (2,3). Anthracycline alcohol metabolites have diminished tumor cell killing activity and exert cardiotoxicity in some cancer patients (4). New studies suggest that *CBR1* and *CBR3* single nucleotide polymorphisms (SNPs) influence the variable pharmacokinetics and pharmacodynamics of doxorubicin. For example, Fan *et al.* pinpointed associations between a *CBR3* non-synonymous SNP (*CBR3* C4Y, G>A, rs8133052) and the pharmacokinetics of doxorubicin in Southeast Asian breast cancer patients (5). *CBR3* C4Y genotype status was associated with doxorubicinol area under the concentration-time curve (AUC; GG *vs.* AA, p=0.004), and with the AUC ratio of doxorubicinol/doxorubicin (GG *vs.* AA, p=0.009). However, Lal *et al.*, failed to detect associations between *CBR3* polymorphisms and the pharmacokinetics of doxorubicin in 101 breast cancer patients, but the authors identified significant associations between *CBR1* polymorphisms (e.g. *CBR1* 627C>T, rs20572 and *CBR1* 967G>A, rs9024) and doxorubicin's clearance, exposure, and peak plasma concentration of doxorubicinol (6). Recently, we showed that a *CBR3* non-synonymous SNP (*CBR3* V244M, G>A, rs1056892) results in *CBR3* protein isoforms with distinctive catalytic activities towards doxorubicin. Recombinant *CBR3* V244 (G allele) synthesized 2.6-fold more doxorubicinol than *CBR3* M244 (A allele; *CBR3* V244: 8.3±3.6 nmol doxorubicinol/h.mg *vs.* *CBR3* M244: 3.2±0.7 nmol doxorubicinol/h.mg, p=0.010). In line, multivariate analyses on 30 cases of anthracycline-related congestive heart failure (CHF) and 115 matched controls pinpointed a trend towards an association between *CBR3* V244M genotype status and the risk of anthracycline-related CHF among pediatric cancer survivors (GG *vs.* AA, odds ratio: 8.16, p=0.056) (7).

[1] Department of Pharmaceutical Sciences, The State University of New York at Buffalo, 545 Cooke Hall, Buffalo, New York 14260-1200, USA.
[2] To whom correspondence should be addressed. (e-mail: jgblanco@buffalo.edu)

ABBREVIATIONS: AHR, aryl hydrocarbon receptor; AIM, ancestry informative markers; AP1 or JUN, activator protein 1; *CBR1*, Carbonyl reductase 1; *CBR3*, Carbonyl Reductase 3; *CBR4*, Carbonyl reductase 4; CHF, congestive heart failure; CI, confidence interval; OCT-1 or POU2F1, octamer binding transcription factor 1; PCR, polymerase chain reaction; SNP, single nucleotide polymorphism; SP1, Sp1 transcription factor; XRE, xenobiotic response elements.

Watanabe *et al.* mapped C*BR3* to chromosome 21q22.1 during the annotation of genes potentially involved in the pathogenesis of Down syndrome (8). There is a paucity of reports focused on the functional characterization of the *CBR3* gene promoter. Thus, our first aim was to identify the promoter of human *CBR3* by performing gene-reporter experiments with constructs encompassing up to 1.3 kilobases upstream from the translation start site of *CBR3*. Our second aim was to determine the extent of variability in the hepatic expression of *CBR3* mRNA by analyzing 95 liver samples from white (n=62) and black (n=33) donors. To further characterize the molecular bases of variable *CBR3* expression, we investigated the impact of two common *CBR3* promoter polymorphisms (*CBR3* -725T>C, rs2239566 and *CBR3* -326T>A, rs8132243) by performing gene reporter assays with engineered *CBR3* promoter constructs in HepG2 and MCF-7 cells. These studies were complemented by analyzing whether *CBR3* -725T>C and *CBR3* -326T>A genotype status correlate with hepatic *CBR3* mRNA levels.

MATERIALS AND METHODS

Human Liver DNA and RNA Samples

The Institutional Review Board of the State University of New York at Buffalo approved this research. Human liver tissue from cadaveric donors (whites, n=62; and black, n=33) was processed through Dr. Mary Relling's laboratory at St. Jude Children's Research Hospital, and was provided by the liver Tissue Cell Distribution System, which was funded by NIH Contract #N01-DK-7-0004/HHSN267200700004C. Liver DNA, RNA, and cytosols were isolated by following standardized procedures as described (9,10). In most cases, no information was available regarding the exact geographical origin of the donors, and no information was available on the ethnicity of the donor's parents. Correlation between self-reported ethnicity (i.e. black and white) and geographical ancestry (i.e. African and European) was determined by genotyping 176 ancestry informative markers as described (10).

CBR3 Reporter Gene Studies

DNA sequence upstream (1297 bp) from the translation start codon of *CBR3* (ATG) was amplified by PCR from human DNA sample HD17249 (Coriell Institute for Medical Research, Camden, NJ). The amplicon was cloned into a pGL3 basic firefly luciferase vector (Promega, Madison, WI), and six 5′ progressive deletion constructs were made by PCR. The $_{-1297}$*CBR3* reporter construct was modified with the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) to generate two single nucleotide variants (−725C/−326T and −725T/−326A), as well as the double nucleotide variant −725C/−326A. The identities of each construct and the absence of cloning artifacts were verified by direct sequencing.

Cultures of HepG2 and MCF-7 cells were co-transfected with *CBR3* reporter constructs (2.0 μg) and the pRLTK plasmid (0.3 μg for HepG2 cells and 20 ng for MCF-7 cells; Promega). The pRLTK renilla luciferase plasmid was used to correct for differences in transfection efficiencies. The pGL3-basic empty vector was transfected into control cultures to correct for background luciferase activity. Luciferase reporter gene activities were determined 48 h after co-transfections with the Dual-Luciferase Reporter Assay System (Promega). Light intensities were measured in a Synergy HT luminometer (BioTek, Winooski, VT). Corrected luciferase activity values were expressed relative to the activity of construct $_{-1297}$*CBR3* which was set arbitrarily as 100%.

CBR3 -725T>C and *CBR3* -326T>A Genotyping

CBR3 -725T>C (rs2239566) and *CBR3* -326T>A (rs8132243) were genotyped by direct sequencing after PCR amplification with the following primers: 5′-ATGTCAT GGTTCTCCCCAAA-3′ (forward) and 5′-CTGTGCCCTC TGTTCCAACT-3′ (reverse). Trace files were analyzed with the PolyPhred package (http://lpgws.nci.nih.gov/perl/snp/snp_cgi.pl).

Hepatic *CBR3* mRNA Expression

Total RNA from liver (100 ng) was reverse-transcribed and amplified by using one-step QuantiTect SYBR Green RT-PCR kits (Qiagen, Valencia, CA) with the following primers: 5′-GCTTCCACCAACTGGACATC-3′ (forward) and 5′-GGGCATTGGATCATCACTCT-3′ (reverse). RT-PCR reaction mixtures were incubated in a MX3050P thermal cycler equipped with proprietary software for data analysis (MxPro v3.00, Stratagene, La Jolla, CA). Relative *CBR3* mRNA levels were determined by the comparative quantitation method with individual β-actin mRNA levels as normalizers (11,12). Cycling parameters for the amplifications in parallel of *CBR3* and β-actin mRNAs were 50°C for 30 min (reverse transcription), 95°C for 10 min (*Taq* polymerase activation); 40 cycles of 95°C for 15 s (denaturation), 54°C for 30 s (annealing), 72°C for 30 s (extension), and 84°C for 30 s (fluorescence collection). Melting curve analyses demonstrated a unique PCR amplification product. Amplification efficiencies for *CBR3* and β-actin mRNAs were comparable. Standard curves for both mRNAs were run in parallel to ensure accurate quantifications (20-fold dynamic range, $r^2 \geq 0.96$). Experimental samples and standards for calibration curves were analyzed in quadruplicate. The relative amount of *CBR3* mRNA in each liver sample was automatically calculated with the comparative quantitation algorithm by using individual β-actin mRNA levels as normalizers. *CBR3* mRNA values were expressed relative to the normalized *CBR3* mRNA content of liver sample 237. Liver sample 237 was randomly selected for data normalization (11–13).

Statistical Analysis

Data are presented as mean ± standard deviation. Student's T test and one-way ANOVA were used to compare relative luciferase activities of reporter gene constructs. The normality of data sets was analyzed with the Kolmogorov-Smirnov test. The Mann-Whitney and Kruskal-Wallis tests were used to compare means from data sets with non-normal distributions. The Chi-square test was used to compare genotype distributions. Correlation analyses were performed with Spearman's test. Differences were considered to be significant at $p<0.05$. Analyses were performed with SPSS version 15.0 (SPSS, Inc.,

Chicago, IL), and GraphPad Prism version 5.00 (GraphPad Software Inc, La Jolla, CA). Linkage disequilibrium analyses were performed with Haploview (version 4.1).

RESULTS

Annotation and Functional Analysis of the Human *CBR3* Promoter

A fragment of 1297 bp upstream the translation initiation codon (ATG) of *CBR3* was cloned into a pGL3 basic firefly luciferase vector to perform gene-reporter studies. Direct sequencing of the insert revealed 100% of identity with a segment of nucleotide sequence from locus NT_011512 (Homo sapiens genomic DNA, chromosome 21q, Entrez Nucleotide Database). The proximal promoter of *CBR3* has the configuration of a typical CpG island promoter with an initiator element TTAGTC [Inr, Py-Py-A+1-N-T/A-Py-Py], several conserved motifs for the Sp1 transcription factor (SP1), a proximal GC-box, no TATA box, and no core promoter element (DPE, A/G_{+}28-G-A/T-C/T-G/A/C; Fig. 1). The CpG island encompasses 361 bp of 5′-flanking region sequence and extends 839 bp downstream of the ATG codon (14,15). The Database of Transcriptional Start Sites (DBTSS, release 6.0.1) reports several transcription start sites (TSS) for *CBR3* (e.g. −95, −107, −108, −228 and −281 bp). The TSS at −107 and −108 bp contain the Inr element. Further screening of the −1297 bp region with the web tools TESS and AliBaba2.1 revealed potential consensus motifs for a number of transcription factors including octamer binding transcription factor 1 (OCT-1 or POU2F1), activator protein 1 (AP1 or JUN), and additional SP1 sites (Fig. 1).

Reporter gene experiments demonstrated that the −1297 bp region exerts significant promoter activity in HepG2 and MCF-7 cells. Similar experiments with 5′ progressive deletion constructs pinpointed areas on the *CBR3* promoter that may regulate transcription in a cell-dependent manner (Fig. 2). For example, deletion of the −1297/−1148 bp region resulted in a construct ($_{-1148}$*CBR3*) with relatively diminished gene reporter activity in HepG2 cells ($_{-1297}$*CBR3 vs.* $_{-1148}$*CBR3* $p<0.001$). In contrast, $_{-1148}$*CBR3* showed 49% higher promoter activity in MCF-7 cells than the reference $_{-1297}$*CBR3* construct ($_{-1148}$*CBR3 vs.* $_{-1297}$*CBR3*, $p<0.001$). Similarly, deletion of the −935/−726 bp fragment ($_{-726}$*CBR3*) did not modify luciferase activity in HepG2 cells, but reduced gene reporter activity by 70% in MCF-7 cells ($p<0.001$). Furthermore, the $_{-313}$*CBR3* construct exhibited the highest and lowest promoter activities in HepG2 and MCF-7 cells, respectively. Experiments also showed that the −313/−172 bp segment is essential to sustain *CBR3* promoter activity in both cell lines because of the negligible gene reporter activity of the $_{-172}$*CBR3* construct.

CBR3 mRNA Expression in Livers from Black and White Donors

The hepatic expression of *CBR3* mRNA varied widely in samples from blacks and whites (*CBR3* $mRNA_{blacks}$ range: 0.4 – 12.2 relative fold, 95% CI of mean: 2.0 – 4.1 relative fold; *CBR3* $mRNA_{whites}$ range: 0.1 – 7.6 relative fold, 95% CI of mean: 1.2 – 1.9 relative fold). In both groups, relative *CBR3* mRNA levels did not correlate with the donors' ages (blacks: $r_s=-0.106$, $p=0.605$; whites: $r_s=-0.147$, $p=0.259$). On average, samples from blacks showed 1.9-fold higher relative *CBR3* mRNA levels than samples from whites (*CBR3* $mRNA_{blacks}$: 3.0±3.1 relative fold *vs.* *CBR3* $mRNA_{whites}$: 1.6±1.5 relative fold; Mann-Whitney test, $p=0.021$; Fig. 3). Differences between group means remained significant after the elimination of outlier values located above and/or below the 5th and 95th percentiles (blacks, $n=2$ outliers, and whites, $n=6$ outliers; Mann-Whitney test, $p=0.010$; Fig. 3).

Common Single Nucleotide Polymorphisms on the *CBR3* Promoter

The functional impact of the *CBR3* −326T>A and *CBR3* −725T>C polymorphisms was first investigated by introducing the variant −326A and −725C alleles into the $_{-1297}$*CBR3* reporter construct. In HepG2 cells, the variant −326A and −725C alleles did not modify gene reporter activities (Fig. 4). In MCF-7 cells, introduction of the −725C variant resulted in a modest 20% decrease in the gene reporter activity of $_{-1297}$*CBR3* ($p<0.001$), whereas the double −326A/−725C variant increased gene reporter activity by 36% ($p<0.001$; Fig. 4).

We also examined whether *CBR3* −326T>A and *CBR3* −725T>C genotype status dictate hepatic *CBR3* mRNA levels. The variant *CBR3* −326T>A and *CBR3* −725T>C alleles were relatively common in liver DNA samples from blacks and whites, and genotype distributions were in Hardy-Weinberg equilibrium in both groups (Table I). *CBR3* −725T>C and *CBR3* −326T>A genotypes were in linkage disequilibrium (LD) in whites ($D'=1.0$, $r^2=0.707$) and blacks ($D'=1.0$, $r^2=0.512$). Correlation analysis failed to demonstrate significant associations between *CBR3* −326T>A and *CBR3* −725T>C genotype status and relative hepatic *CBR3* mRNA levels in samples from blacks and whites (Fig. 5).

DISCUSSION

Recent studies suggest that *CBR1* and *CBR3* polymorphisms impact on the pharmacodynamics of doxorubicin in various cancer settings (5–7). The biological role of *CBR3* remains to be elucidated, and the functional annotation of the promoter is a necessary step towards the understanding of the bases that govern variable *CBR3* expression. Here, we identified the promoter of *CBR3* and examined the impact of two common promoter polymorphisms on the expression of hepatic *CBR3* mRNA. First, our results indicate that the region encompassing up to 1.3 kb upstream from the translation start site of *CBR3* exerts significant gene promoter activity in two different cellular contexts. Experiments with progressive deletion constructs pinpointed regions on the *CBR3* promoter that may regulate transcription in a cell specific manner. For example, the −539/−313 bp segment appears to bear regulatory element/s with contrasting actions in HepG2 and MCF-7 cells (Fig. 2). The experiments also showed that the −313/−172 bp segment is essential for promoter activity in both cell lines. The lack of activity of the $_{-172}$*CBR3* construct is consistent with the location of two major TSS at −228 and −281 bp, respectively. The −313/−172 bp region contains a GC box at −217 bp with an embedded consensus sequence for the binding of SP1 (Fig. 1).

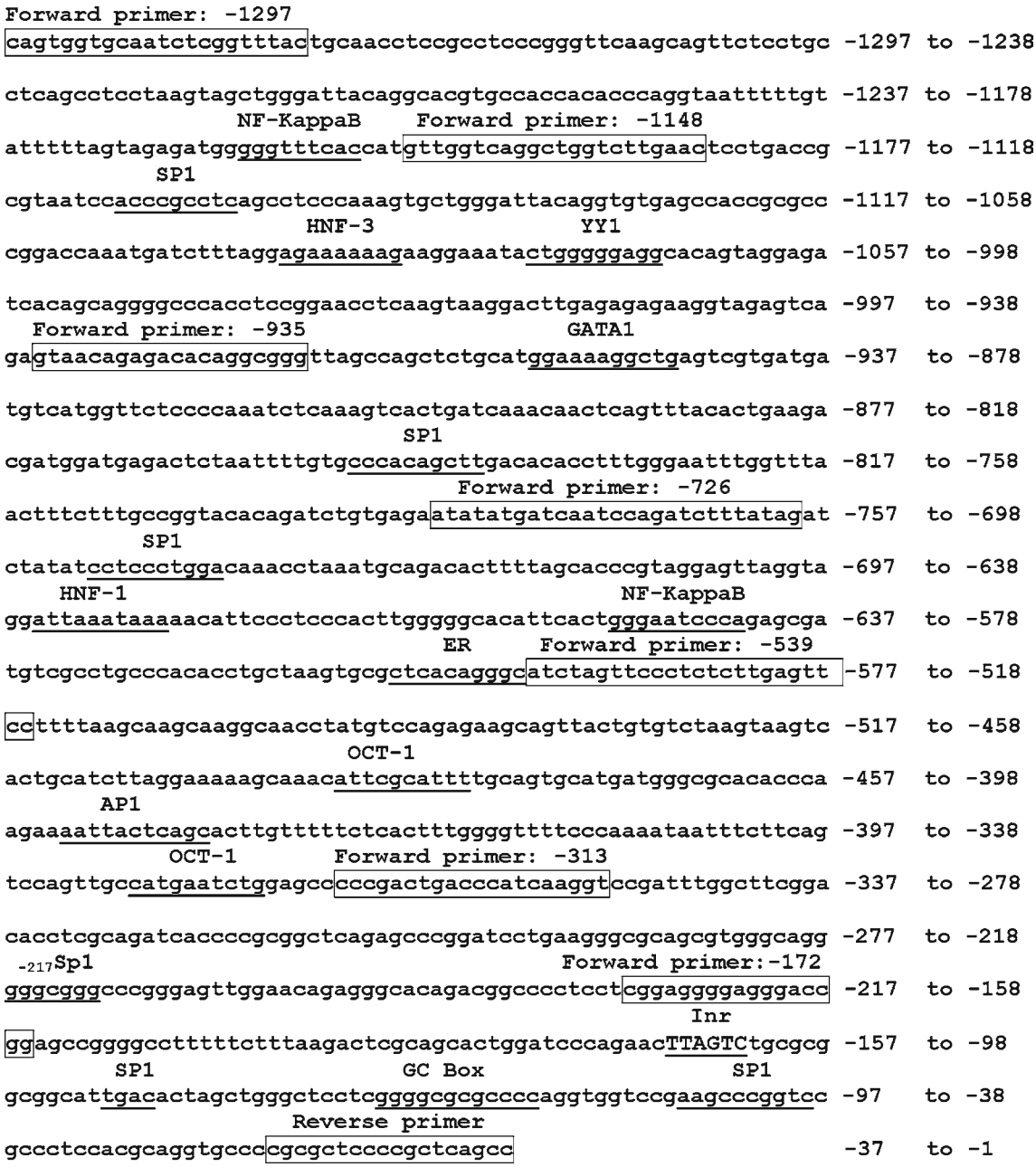

Fig. 1. Annotated sequence from the 5′-flanking region of human *CBR3*. The initiator element (Inr) is highlighted. Putative transcription factor binding sites are underlined. The location of primers is enclosed in boxes. NF-KappaB, nuclear factor kappa B; SP1, Sp1 transcription factor; HNF-3, hepatic nuclear factor 3; YY1, Ying Yang 1; GATA1, GATA binding protein 1 (globin transcription factor); HNF-1, hepatic nuclear factor 1; ER, estrogen receptor; OCT-1, octamer binding transcription factor; AP1, activator protein 1.

SP1 activates the transcription of several TATA-less genes, and it will be of interest to test whether the SP1 consensus is key to the constitutive activity of the *CBR3* promoter (16). Recently, we have shown that two functional xenobiotic response elements (*XRE*) on the promoter of the homologous *CBR1* activate transcription in response to ligands of the aryl hydrocarbon receptor (AHR)(15). DNA sequence comparisons of up to 1.1 kb by global alignment positioning showed limited identity and similarity (~40%) between the proximal promoter regions of *CBR1* and *CBR3.* In addition, computer assisted searches failed to identify conserved *XREs* on the *CBR3* promoter (6 kb screened). This finding suggests a potential functional distinction between the mechanisms that control the transcription of human *CBR1* and *CBR3*, respectively.

Our second aim was to document *CBR3* mRNA expression in liver samples from black and white donors. Our data suggest that hepatic *CBR3* mRNA levels are higher in samples from black donors (Fig. 3). Recent analysis of ancestry informative markers (AIM) on this set of liver samples demonstrated excellent concordance between the donors' ethnicity (i.e. black and white) and geographical ancestry (i.e. African and European) (10). Thus, the observed differences may in fact reflect distinctive patterns of *CBR3* mRNA

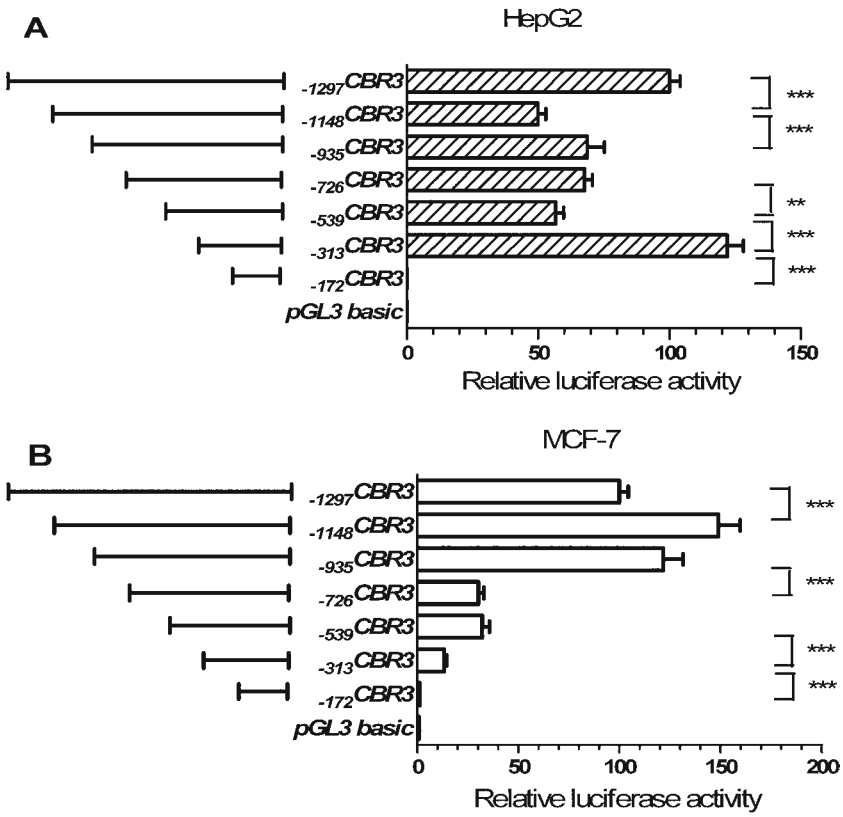

Fig. 2. Functional analysis of human *CBR3* promoter constructs in HepG2 cells (A) and MCF-7 cells (B). Panels show schematic representations of each *CBR3* promoter construct (left) and its corresponding luciferase activity from gene reporter experiments (right). Corrected luciferase activity values were expressed relative to the activity of construct $_{-1297}$*CBR3*, which was set arbitrarily as 100%. Each value represents the mean±SD of six independent experiments (*** $p<0.001$, ** $p<0.01$).

expression among individuals with different geographical ancestries, and further confirmatory studies are warranted. It will be interesting to examine whether differences in *CBR3* mRNA expression translate into distinct CBR3 protein levels in livers from black and white donors. Biochemical studies have shown that the intracardiac formation of anthracycline alcohol metabolites is relevant to the pathogenesis of anthracycline-related cardiotoxicity (17–19). On the other hand, epidemiological studies in pediatric cancer survivors showed that black ethnicity constitutes a risk factor for the development of

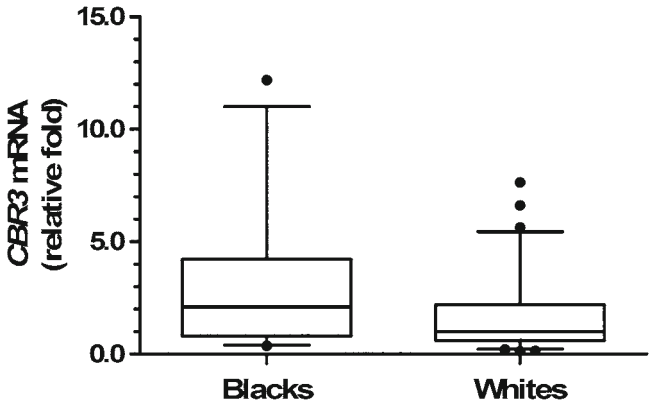

Fig. 3. Hepatic *CBR3* mRNA expression in samples from black ($n=33$) and white ($n=62$) donors. Relative *CBR3* mRNA levels were determined with the comparative quantitation method (see text for details). The whiskers indicate the 5th and 95th percentiles. Horizontal lines indicate the medians. Black circles indicate outliers. Samples and standards for calibration curves were analyzed in quadruplicate. (*CBR3* $mRNA_{blacks}$ *vs.* *CBR3* $mRNA_{whites}$, Mann-Whitney test, $p=0.021$).

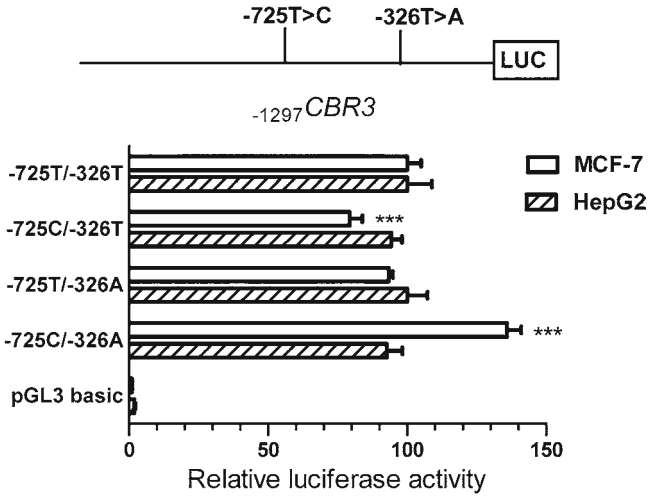

Fig. 4. Impact of *CBR3* -725T>C and *CBR3* -326T>A on the gene reporter activity of $_{-1297}$*CBR3*. In both cell lines, luciferase activities are expressed relative to the activity of the "wild type" $_{-1297}$*CBR3* construct (-725T/-326T), which was set arbitrarily as 100%. Each value represents the mean±SD of six independent experiments (***$p<0.001$).

Table I. *CBR3* -326T>A and -725T>C Genotype Distributions in DNA Samples from White and Black Liver Donors

	Whites (n=62)		Blacks (n=33)	
SNP	Genotypes	p and q*	Genotypes	p and q*
-725T>C	TT:TC:CC=50:9:3 (80.6%:14.5%:4.8%)	0.879: 0.121	TT:TC:CC=20:12:1 (60.6%:36.4%:3.0%)	0.788: 0.212
-326T>A	TT:TA:AA=53:7:2 (85.5%:11.3%:3.2%)	0.911: 0.089	TT:TA=25:8 (75.8%:24.2%)	0.879: 0.121

*p denotes the major allele and q denotes the minor allele

anthracycline-related cardiotoxicity (20,21). Thus, it is possible to speculate that potential interethnic differences in the cardiac expression of *CBR3* may influence the risk of anthracycline-related cardiotoxicity.

The experiments with engineered reporter constructs in breast cancer-derived MCF-7 cells showed that the double *CBR3* −326A/-725C promoter variant resulted in a modest increase (36%) in gene reporter activity (Fig. 4). The study by Fan *et al*. reported that *CBR3* C4Y (*CBR3* 11 G>A) genotype status was associated with basal *CBR3* mRNA levels in 66 breast tumor samples (GG *vs*. AA, p=0.001) (5). Thus, it will be of interest to test whether specific *CBR3* genotype combinations (i.e. −326A/−725C/11G) are associated with distinctive *CBR3* mRNA expression levels in breast tumors. On the other hand, we found no associations between *CBR3* −326T>A and *CBR3* −725T>C genotype status and *CBR3* mRNA expression in liver samples from black and white donors (Fig. 5). In agreement, the variant *CBR3* −326A and *CBR3* −725C alleles did not significantly modify the gene reporter activities of engineered *CBR3* promoter constructs in HepG2 cells (Fig. 4). Thus, our data do not support a significant role for the common *CBR3* −326T>A and *CBR3* −725T>C polymorphisms in dictating hepatic *CBR3* mRNA levels.

This study describes the first insights into the transcriptional regulation of *CBR3* and provides a platform to further elucidate the molecular bases that govern variable *CBR3* expression in humans.

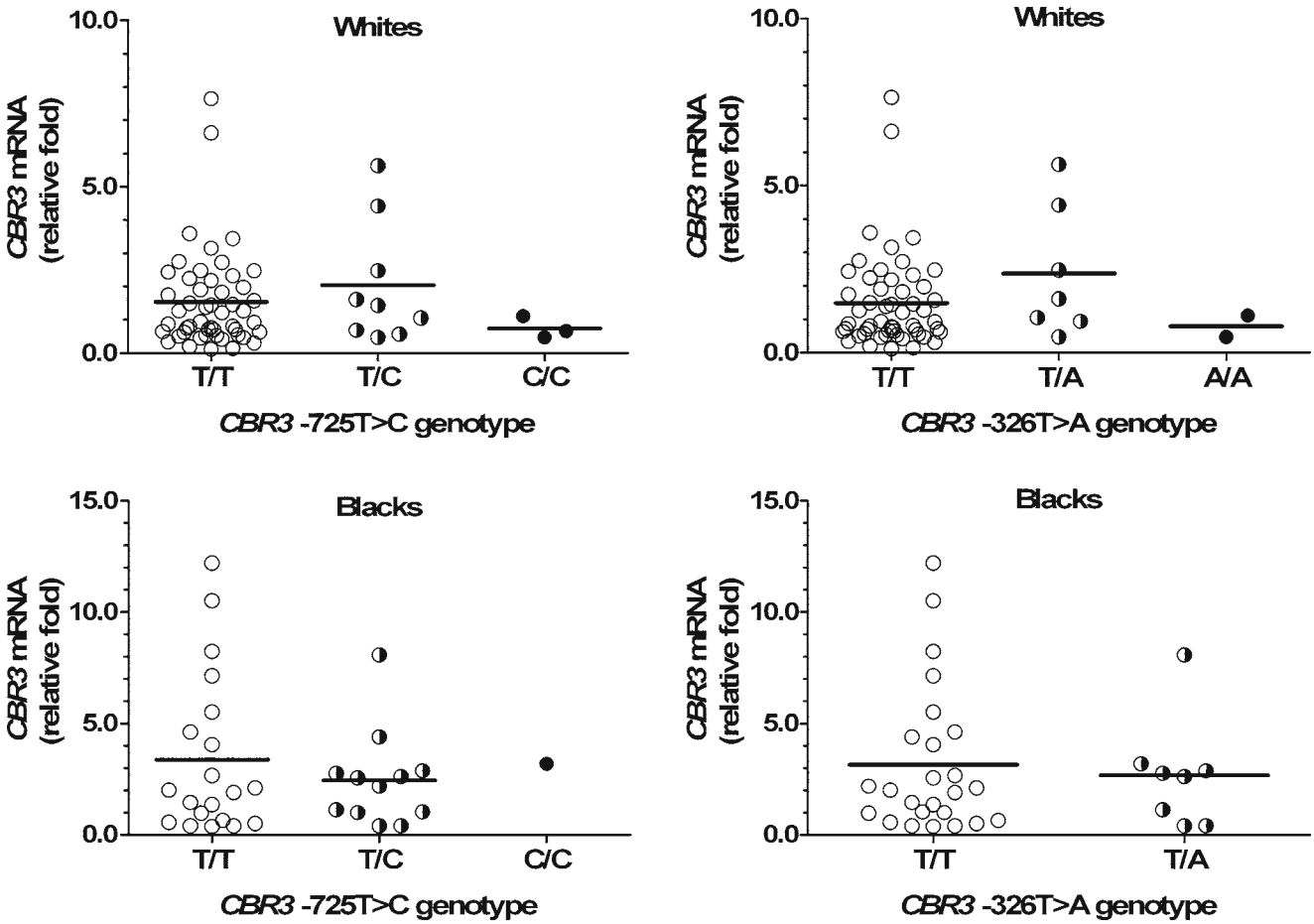

Fig. 5. Impact of *CBR3* -725T>C and *CBR3* -326T>A genotypes on the expression of *CBR3* mRNA in liver samples from white (top) and black (bottom) donors. Each circle represents individual liver samples (quadruplicate measurements). Horizontal lines indicate group means.

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