FUNCTIONAL ANALYSIS OF THE 5’ REGULATORY REGION OF THE 5-AMINOLEVULINATE SYNTHASE (ALAS1) GENE IN RESPONSE TO ESTROGEN

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Abstract – Genetic defects in the heme synthesis enzymes lead to a group of heterogeneous disorders termed the porphyrias. Numerous factors influence the clinical expression of porphyrias, primarily by altering the rate of heme synthesis. To date, no genotype-phenotype correlation has been made to explain the variable penetrance observed in variegate porphyria (VP) and other acute hepatic porphyrias. As first and rate determining gene in the heme pathway, 5-aminolevulinate synthase-1 (ALAS1), appears to be an ideal candidate modifier. Previous studies established critical mechanisms for ALAS1 regulation and a direct transcriptional response to drugs by defined drug-responsive enhancer sequences (ADRES). To identify possible functional variants within the 5’ region of ALAS1, selected regulatory regions, including the ADRES elements, were screened by DNA sequencing analysis in 26 VP patients heterozygous for the causative R59W mutation in the protoporphyrinogen oxidase (PPOX) gene. Two novel variants, -853C>T and -1253T>A were identified. In silico analyses indicated that the -853C>T transition is located immediately 5’ to a half-palindromic putative estrogen receptor binding site. Co-transfection experiments with an estrogen receptor-α (ERα) expression vector in HepG2 cells, suggest that this region mediates an increased transcriptional response in the presence of estrogen (E2) and ERα. The wild-type -853C/-1253T allele induced a 47% increase in transcription, while the -853T/-1253A double mutant allele showed a 35% increase in transcription compared to expression in the absence of E2. The highest induction was observed for the mutant -853T/1253T allele that generated an increase of 66%. We conclude that the -853T variant functions as an enhancer in the presence of estrogen and speculate that the -1253A variant reduces transcription activity.

Key words: aminolevulinate synthase gene, estrogen, gene regulation, heme, porphyria, ADRES

INTRODUCTION

The importance of heme is underscored by its involvement in numerous vital cellular functions such as energy transfer, drug metabolism and enzyme biosynthesis. In mammals, heme synthesis is most active in the bone marrow, where hemoglobin is produced, and in the liver, for the formation of heme proteins such as the drug-metabolizing cytochrome P450 (CYP450) enzymes.

Abbreviations: ALAS1, 5-aminolevulinate synthase-1 gene; PPOX, protoporphyrinogen oxidase gene; AHP, acute hepatic porphyria; ERα, estrogen receptor alpha; Sp1, stimulating protein-1; UTR, untranslated region; DMEM, Dulbecco’s modified Eagle’s medium; FCS, fetal calf serum; PCR, polymerase chain reaction; RLU, relative light unit; SEM, standard error of the mean.
apparent evidence for a genotype-phenotype correlation. In the AHPs, various environmental precipitating factors, such as sunlight and hormones have been identified, but none sufficiently explain the penetrance of the dominant disease-causing mutation. This suggests that a combination of environmental and additional modifier loci modulate the clinical expression of the disease-causing mutation. A classic model of an AHP with numerous additive factors contributing to the phenotypic outcome, is variegate porphyria (VP). The typical clinical appearance of VP include skin symptoms and/or acute attacks that are proposed to be directly attributable to the build-up of cytotoxic heme precursors (26). The high occurrence of VP and the prevalence of the R59W disease-causing mutation in the protoporphyrinogen oxidase gene (PPOX), make South African VP patients an ideal cohort to study the consequence of other genetic factors that, in conjunction with R59W, may possibly influence the severity or outcome of the disease. While investigations into possible modifying variants within the promoter and coding regions of PPOX itself, revealed an abundance of variants, none of these could explain the variance in clinical expression observed in VP patients (14,48,49). These studies proposed that the contributions of other candidate trans-acting loci should be investigated (3,48).

A likely candidate modifier gene is the first and rate-determining gene of the hepatic heme synthesis pathway, 5-aminolevulinate synthase-1 (ALAS1). A previous study investigated the existence of modifying variants within the coding- and splice-site regions of ALAS1, however, only one variant was detected and no correlation was observed between this previously identified silent mutation (4713C>T; NCBI refSNP ID: rs352168) and the variable VP phenotype (44).

The mechanisms for the basal and drug-induced regulation of ALAS1 are currently intensively investigated. Initially it was believed that ALAS1 enzyme production was exclusively controlled by heme given that expression of this gene in the liver has been shown to be under negative feedback control of heme in various species (6,10,32). The regulatory pool of unassigned heme destabilizes ALAS1 mRNA (19), blocks mitochondrial import of the precursor protein (31) and represses mRNA transcription (32). Various reports have, however, suggested that the direct inhibition of ALAS1 transcription by heme, appears to be unlikely at physiological heme concentrations (19) and that the precise molecular mechanism by which heme regulates ALAS1 mRNA levels, remains poorly characterized and controversial (7,34).

In a study by Podvinec et al. (36), the gene activity of the -1249 bp to -1 bp human ALAS1 promoter region was assayed in chicken hepatoma cells (LMH). The promoter fragment exhibited basal promoter activity, but did not cause an increase in transcription in response to prototypic inducers of ALAS1. They subsequently used an in silico method to analyse sequences further upstream in the ALAS1 5' region and identified two sequence elements in the 30 kb 5' flanking region of the gene that directly activate human ALAS1 gene transcription in response to drugs. These two delta-aminolevulinate acid drug-responsive enhancer sequences (ADRES), located 16 and 20 kb upstream of the transcription initiation site, was tested in vitro and found to be responsive to the compounds phenobarbital (PB), metyrapone, propylisopropylacetamide (PIA) and glutethimide. They concluded that drug mediated induction of ALAS1 is due to a direct effect of the inducing compound and not a consequence of the increased synthesis and accumulation of cytochrome P450 and subsequent depletion of the heme pool.

In this study, focus was directed to the 5' regulatory region of ALAS1, to identify variants possibly affecting the transcription rate of this gene, and consequently the rate of heme synthesis. In particular, we aimed to examine the hypothesis that variants in the ADRES elements and/or regulatory regions in the proximal promoter region, will affect the transcriptional regulation of ALAS1,
by altering the amount of heme precursor build-up prior to a defective heme synthesis enzyme. An alteration in ALAS1 gene expression in combination with a R59W PPOX allele may have a significant effect on the amount of heme presursor build-up prior to the defective PPOX enzyme, influencing the clinical outcome of VP even in siblings sharing the same disease-causing mutation.

**MATERIALS AND METHODS**

**Subjects**

The study cohort included 26 R59W heterozygous South African VP patients (21 Caucasian and 5 Mixed Ancestry individuals) incorporating both randomly chosen individuals along with 1 Mixed Ancestry sibling pairs and a descendant (Supplementary Figure B). The group consisted of 2 male and 24 female VP patients with a mean age of 46 years. Biochemical analyses and medical diagnoses for characteristic VP symptomatic expression were available [5]. Patients were classified into 4 symptomatic groups: asymptomatic (10), acute attacks (4), skin symptoms (6) and skin symptoms and acute attacks (6). All patients, family members of patients, and controls (90 Caucasian and 88 Mixed Ancestry individuals) gave written informed consent for the use of their genomic DNA. The study was approved by the Ethics Review Committee for Human Research, Stellenbosch University.

**Mutation analysis**

According to the reference sequence (NCBI accession number AC006252), four oligonucleotide primers were designed, incorporating NheI and BglII restriction sites, to amplify the -1286 bp to +6 bp ALAS1 upstream region, encompassing the most 5' transcription start site (TSS) as described by Roberts and Elder (40). Furthermore, two fragments in the upstream distal promoter region, each containing an ADRES element, were amplified (36) with primers containing Kpn1 and NheI restriction sites. The most 5' ADRES element was termed ADRES1 (5'-GGGACTCCAGTGACCTCCTTGCACA-3') and was contained in the region from position -21016 to -20607. The second ADRES element, termed ADRES2 (5'-GGGTGAGCTAAGTTCA-NN-TGTGCTGCCGTGACCT-3'), was contained in the region from position -15686 to -16118. In total, four primer sets (see Supplementary Tables A & B) were designed for PCR amplification of the ALAS1 promoter and ADRES elements in standard PCR reactions containing 2mM MgCl2 and annealing temperatures as indicated in the supplementary tables. Successfully amplified PCR products were subjected to semi-automated DNA sequencing analysis on an ABI 3130XL Genetic Analyser (Applied Biosystems), using 11 primers (see Supplementary Tables A & B and Figure A) to create bidirectional sequenced, overlapping fragments that were compared to the reference sequence.

**Genotyping**

Allele frequencies of the detected variants were established in the two population-matched control groups (90 Mixed Ancestry; 88 Caucasian individuals) and family members (31) of the individuals in the study cohort heterozygous for the detected variants. The variants included a -853CT transition and -1253TA transversion and will be discussed further in the results section. These variants were respectively genotyped by restriction analysis using the enzymes AcII and Bsal. Primer sequences and conditions are available in Supplementary Tables C & D.

**In silico analysis**

The reference sequence was subjected to bioinformatic analysis to predict transcription factor binding sites (TFBS) by comparing data obtained from five different software packages, including TREs (25), MatInspector (39), Alibaba2 (15), Signal Scan (30) and Match (27). Default program parameters were used as search criteria. The rVISTA computational platform (http://genome.lbl.gov/vista/index.shtml) was used for multi-species comparative genomic analysis. The reference sequence (approximately 1.3 kb) was compared to the ALAS1 promoter sequences from chimpanze (Pan troglodytes), macaque (Macaca mulatta), dog (Canis familiaris), cow (Bos taurus), mouse (Mus musculus), rabbit (Oryctolagus cuniculus), rat (Rattus norvegicus), chicken (Gallus gallus) and zebrafish (Danio rerio). Promoter sequences (2 kb upstream and 5'-UTR) were extracted from the Ensembl Genome Browser (archive version at http://jul2008.archive.ensembl.org/index.html). Estrogen responsive (ER) binding sites were predicted using the TRANSFAC database at default settings for “matrix similarity and core similarity cut-off” via the rVISTA platform.

**Plasmid construction**

To test the functional significance of the -853C>T and -1253T>A variants, constructs of the ALAS1 5'-UTR region was amplified by PCR and subcloned into the pGL2-Basic vector (Promega). Three constructs were generated, as illustrated in Figure 1, consisting of the -1286 bp to +6 bp wild-type ALAS1 fragment [denoted pGL(ALAS-WT)]; a fragment containing the -1253A and -853T variants [denoted pGL(ALAS-ERE/Sp1)] and a fragment containing only the -853T variant [denoted pGL(ALAS-ERE)]. A standard protocol primer-mediated mutagenesis technique was used to create the pGL(ALAS-ERE) from the pGL(ALAS-ERE/Sp1) construct. All constructs were sequenced to confirm authenticity.

**Figure 1**

(A) pGL(ALAS-WT), contains the wild-type ALAS1 promoter fragment; (B) pGL2(ALAS-ERE/Sp1), contains the ALAS1 promoter fragment with the -853T and -1253A variants; (C) pGL2(ALAS-ERE), contains the ALAS1 promoter with the -853T variant. The putative Sp1- and ERE half-sites are indicated with grayed boxes and the transcription start site (TSS) of the Luc gene indicated with arrowed boxes.
**Cell culture and transient transfections**

Human hepatocellular carcinoma HepG2 cells were cultured in steroid-free culture conditions devoid of steroid hormones (phenol red-free DMEM (Sigma) supplemented with 10% (v/v) charcoal-striped FCS (Highveld) and L-glutamine (2mM)) at 37°C and 5% CO₂. At 24 hours prior to transfection, cells were seeded in 6-well plates at 40-60% confluence. Cells were transfected with a mixture containing the respective ALAS1 reporter constructs (1 μg/well), the normalizing pSV-β-galactosidase expression plasmid (25 ng/well) and the human estrogen receptor-α (ER-α) expression plasmid, pCDNA3-ER-α (25 ng/well), using the transfection reagent FuGENE®6 (Roche). Parallel transfections with the promoterless pGL2-Basic plasmid were used as a negative control. The medium was discarded 24 hr following transfection and fresh medium added, containing either estradiol [E₂ (Sigma), 3 x 10^(-8)M final concentration] or vehicle (EtOH, 0.03% final concentration) (23). Cells were lysed in 250 μl reporter lysis buffer (Promega) and light emitted quantified with a Veritas Microplate Luminometer (Turner Biosystems) using the luciferase assay kit (Promega). The β-glo assay kit (Promega) was used for quantification of β-galactosidase activity. Both kits were used according to the manufacturer’s instructions.

Data were presented as the ratio of luciferase activity (RLU) normalized to β-galactosidase activity (RLU), from triplicate wells from three representative experiments. The values obtained for the pGL(ALAS-WT) construct was assigned a relative activity of 1 and expression of the other constructs indicated as a value relative to this.

**Statistical analysis of data**

The normalized values for reporter gene expression results were analyzed (SAS software, SAS Institute, Inc., Cary, NC) and only values within the ±20% range of one another were used to determine arithmetic means and S.E. values of the means. The significance of differences between multiple group means was determined by analysis of variance (ANOVA). In those cases in which significant differences (p < 0.05) were detected by ANOVA, pair-wise t tests were performed, using the SAS software package and Microsoft Excel. Results were presented as means ± SEM or as a percentage of the reference.

**RESULTS**

**Identification and characterization of ALAS1 promoter variants**

Sequence analyses of the PCR fragments detected two novel variants in the proximal promoter; a C>T transition at nucleotide position -853 and a T>A transversion at nucleotide position -1253. In the study population, three related female individuals of Mixed-Ancestry were found to be heterozygous for both variants (Table 1).

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Although the variant alleles were only detected in individuals of Mixed Ancestry in our patient cohort, subsequent genotyping of control populations indicated that both alleles were present in the Mixed Ancestry and Caucasian populations. The allele frequencies were calculated for both variants in the Mixed Ancestry- [(<<-853C:T = 0.956:0.044); (-1253T:A = 0.956:0.044)] and the Caucasian control groups [(<<-853C:T = 0.989:0.011); (-1253T:A = 0.943:0.057)]. The -853T allele is more prevalent in the Mixed Ancestry population, while the frequency of the -1253A allele was found to be slightly higher in the Caucasian population. Pedigree analysis of the 31 family members using 4 generations revealed that, in this family, the two variant alleles -853 and -1253 are co-inherited (supplementary Figure B). No variants were detected in the previously described ADRES elements.

**Predicted Regulatory Sites**

Computer-aided analyses of the -1286 to +6 bp ALAS1 promoter region revealed several motifs resembling consensus binding sites of many known nuclear factors. The in silico survey was therefore directed by comparing results from the TFBS prediction programs using the sequences in and around the -853C/T and -1253T/A variants. Results from three of the five software programs indicated that the -853 variant is located immediately 5' to a half-palindromic estrogen response element (ERE), with the binding site (TGACCT) for the estrogen receptor-α (ER-α) as shown in Figure 2. The -1253 variant was located 3' to a non-consensus stimulatory protein-1 (Sp-1) TFBS (Figure 2). These observations prompted an examination of the rest of the amplified promoter region for other possible ERE sites/half-sites. In total, five putative perfect and imperfect ERE half-sites were predicted (Figure 2). ALAS1 comparative promoter analysis between species (conserved area in brackets) revealed the human reference sequence to be 99.5 % conserved to chimpanzee (1291 bp), 93.1 % to macaque (1294 bp), 72.8 % to dog (371 bp), 70.3 % to cow (390 bp), 72 % to mouse (236 bp), 77.2 % to rabbit (145 bp) and 73.3 % to rat (116 bp). ALAS-1 promoter...
sequences of chicken and zebrafish showed no conservation (at 70% default level) to the human reference sequence. Several putative estrogen responsive (ER) binding sites were detected in the conserved genomic areas (Figure 3) between species. The ERE half-site containing the core sequence 5'-TGACCT-3' specifically adjacent to the -831C/T variant was detected in the ALAS-1 promoter sequences of the two primates (chimpanzee and macaque – Figure 3). Subsequently, functional analysis of the 1292 bp fragment was initiated to establish the estrogen responsiveness of the ALAS1 promoter. Also, the effect of the -853T variant adjacent to the putative ERE half-site on ALAS1 transcription in response to estrogen was determined. The possible effect of the -1253A variant was further speculated.

Figure 2. A section of the reference nucleotide sequence of the ALAS1 5’ flanking region stretching from position -756 to -1273. The sequence was retrieved from the NCBI Entrez Nucleotide Database (accession number AC006252), with the first base of the most 3’ TSS denoted as +1, and the nucleotide directly 5’ to this set as -1 (according to [17]). A complete optimal ERE site has a consensus sequence of 5’-AGGTCAnnnTGACCT-3’. The optimal perfect and imperfect ERE half-sites are highlighted and overlined with their directions indicated with an arrowhead; the predicted Sp1-like site at -1262 to -1252 bp is double-underlined. The wild-type nucleotides of the detected variants (-853C/T and –1253T/A) are identified in.

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Figure 3. Multi-species comparative analysis of the ALAS1 proximal (~ 1.3 kb) promoter region. Putative ER binding sites (using TRANSFAC) were identified in conserved promoter regions. Only EREs (dark grey triangles) detected in the majority of conserved areas between species (indicated by dashed lines) are shown.

Differential expression of the ALAS1 promoter region in response to estrogen

In order to test the involvement of estrogen in the differential expression profile of the wild type ALAS1 construct [pGL(ALAS-WT)], HepG2 cells, known to express little or no estrogen receptor-α (30,46), were co-transfected with the pGL(ALAS-WT) construct and an ER-α expression vector (pCDNA-ER-α) and induced with E2. In a single E2-dose-response experiment (Figure 4), normalised luciferase expression values indicated a dose-responsive increase in ALAS1 promoter expression in response to augmented concentrations of estrogen. Since estrogen at a concentration of 3 x 10⁻⁶M induced the highest luciferase expression values for the pGL(ALAS-WT) construct, this concentration was chosen as the most favorable to be used for subsequent induction experiments.

Figure 4. HepG2 cells transiently transfected with the wtALAS1 promoter reporter construct (pGL(ALAS-WT)), an expression vector for ERα (pCDNA3-ERα) and a galactosidase normalizing plasmid (pSV-β-galactosidase) were induced with increasing concentrations of E2 as indicated. Luciferase values were normalized with β-galactosidase values and a dose response curve of the normalized data is presented displaying the mean ± SEM of the expression values of one experiment performed in triplicate.
To establish the effect of the detected promoter variants on E2-induced ALAS1 expression, the wild type ALAS1 construct (pGL(ALAS-WT)) plus the mutated constructs [pGL(ALAS-ERE/Sp1) and pGL(ALAS-ERE)] were co-transfected with pCDNA-ER-α in HepG2 cells, grown under steroid hormone deprived conditions, and induced with either vehicle or E2 (Figure 5). The normalized expression value of the pGL(ALAS-WT) construct co-transfected with ERα and induced with EtOH, was used as reference and assigned a value of 1. Comparisons between the three constructs induced with EtOH, indicated that pGL(ALAS-WT) and pGL(ALAS-ERE) had approximately the same expression levels, with the expression of pGL(ALAS-ERE/Sp1) slightly lower (11%), though not significantly so (p = 0.49), than these two. Comparisons between the three constructs in the presence of E2, demonstrated a 23% decrease in expression in pGL(ALAS-ERE/Sp1) compared to pGL(ALAS-WT). Although not statistically significant (p = 0.104), such a decrease may have a significant influence in vivo, or at more physiologically relevant concentrations of E2. During E2 stimulation, the expression of pGL(ALAS-ERE) was 41% lower than that of pGL(ALAS-ERE) and was also statistically significant with a p value of 0.0087. The increase in expression of pGL(ALAS-ERE) compared to pGL(ALAS-WT) was noticeable in E2 induced conditions (17% increase), and even though this difference in expression was not statistically significant, in individual experiments the increase seen in pGL(ALAS-ERE) was much more prominent. Transfections induced with E2 resulted in a statistically significant increase in expression of all constructs, when compared to the respective constructs treated with the solvent, EtOH. The pGL(ALAS-WT) construct induced with E2, demonstrated a 47% increase in expression compared to pGL(ALAS-WT) in the absence of E2 (induced with EtOH), with a p value of 0.0033. pGL(ALAS-ERE/Sp1) had a 35% increased expression level in the presence of E2 compared to its expression in EtOH (p = 0.038). The pGL(ALAS-ERE) construct displayed the highest expression: a 66% increase in the presence of E2, compared to its expression in the absence of E2 (p = 0.0004).

DISCUSSION

Genetic variants within, or adjacent to, gene regulatory elements may alter the binding of transcription factors and affect gene expression (reviewed in 9,29). Prior studies have defined several critical regulatory elements in the 5' regions of the human, mouse, chicken and rat ALAS1 genes that regulate expression patterns of this gene (5,12,13,36,40). The main objective of our study was to describe the nature and extent of nucleotide variation in the human ALAS1 5' region and to analyze the influence of naturally occurring promoter variants on the modulation of ALAS1 gene expression in VP patients. In this regard, we have identified two novel, functional promoter variants at nucleotide positions -853C/T and -1253T/A, and no variants in either of the two ADRES regions, indicating a high degree of sequence conservation of the ADRES elements in our study cohort. In silico analyses predicted five putative ERE half-sites and indicated that the -853C/T transition is located 5' to one half of an optimal consensus binding site for ER-α while the -1253T/A transversion is located 3' to a putative non-consensus Sp1 site. Comparing conserved promoter sequences between species may elucidate cis-motifs that are more likely to be functional due to evolutionary conservation. In this investigation, we identified several ER cis-motifs shared in the conserved areas of ALAS-1 promoter between species. With
special emphasis on the ERE half-site adjacent to the -831C/T variant, although present in the conserved regions of the chimpanzee and macaque ALAS-1 promoter, no additional information (i.e. retained functionality between other species) could be retrieved. Although it is recognized that TFBS software programs provide only a prediction of possible binding sites when unconfirmed by functional studies, our computational search results prompted further investigation into the effect of these variants on nearby predicted regulatory elements. In a study similar to ours, Harendza et al. (20) identified a transition 1 bp from an ERE half-site in the Gelatinase A gene which mediated a decrease in transcriptional activity in response to estrogen, reinforcing the hypothesis that a closely situated variant may influence ERE function.

Co-transfection of reporter constructs with a human ER-α expression vector in hepatocytes, maintained in steroid-free conditions, and induced with E₂, demonstrates that all three ALAS1 promoter constructs are estrogen responsive. Furthermore, the presence of the -853T variant mediated a moderate increase in transcription, while the addition of the -1253A variant reduced the effect of the -853T variant. Initially we speculated that the increased expression observed for all our ALAS1 promoter constructs in response to estrogen, was due to the elevated demand for heme to metabolize the high levels of estrogen (32). However, several reports indicate that heme regulates ALAS1 by decreasing its mRNA half-life (19,34) or by influencing the intracellular translocation of the protein via the heme responsive regions, situated in the coding region of this gene (7). Since these heme-mediated regulatory mechanisms all occur post-transcriptionally, the results observed in our transfection assays, can be attributed to the transcriptional effect of estrogen-activated ER-α on the ALAS1 promoter. Microarray experiments by Stuckey et al. (45) reflected our findings and verified earlier studies that reported the induction of the ALAS enzyme upon stimulation with sex steroids and their metabolites (16,17,24), when they demonstrated an increase in ALAS1 production in mouse uterine tissues upon estrogen stimulation. Our results suggest that the increase in ALAS1 activity in response to estrogen, may be consistent with the functionality of either one or more of the predicted ERE half-sites in the ALAS1 promoter region. If these EREs are indeed functional, the elevated expression of the pGL(ALAS-ERE) construct may therefore signify an increased effect of estrogen-activated ERα on transcription, mediated by the -853T variant.

It is known that estrogen regulation can be mediated by imperfect half-sites and modulated by the sequences flanking ERE half-sites (42). Anolik et al.(2) and Driscoll et al. (11) demonstrated that AT-rich sites adjacent to ERE sites increase the binding affinity of ERα-ERE binding with a subsequent enhanced transcriptional activity. They postulated that these sequences immediately flanking ERE sites, might share a common feature that stabilizes E₂-ERα DNA binding, conferring enhanced E₂-ERα-ERE binding and facilitating the cooperative functionality of multiple ERE sites. They suggested that the rationale behind this phenomenon is that, since AT-rich sequences are characterized by low melting temperatures and DNA bending, such a change in chromatin structure may bring DNA binding sites and their bound proteins in closer proximity to each other and to the transcription initiation complex. Since ERα-ERE function by creating a DNA bend, it can be hypothesized that the -853T variant, which has two A nucleotides at its 5’ side, may create an area that is more easily bent, assisting in recruiting ER-α to the ERE site/s and components of the initiation complex. In view of these findings, it is therefore possible that the -853T variant may influence the stability of ERα-ERE binding, resulting in an enhanced transcriptional effect.

Another consideration is that ERα may act indirectly on the ALAS1 promoter region, with the -853T variant increasing its effect. The indirect interaction of ERα to ERE half-sites have been extensively reported (1,4,28). In humans, indirect binding of ERα can indeed result in estrogen regulation, predominantly via Sp1 (37,41). It has been shown that in a number of cases, Sp1/ERα interactions mediate estrogen inducibility of promoters, especially where half-sites are involved (35).

Estrogen levels fluctuate in an organism and vary greatly between sexes and at different stages of life. In females, the plasma levels of estrogens increase at puberty and also vary throughout the menstrual cycle, with estrogen levels being at its highest prior to ovulation (reviewed by 18). At menopause, depletion of the ovarian follicles leads to a steady decline in ovarian estrogen production. In males, estrogens also play an important physiological role, although little is
known about the regulation of estrogen production by extragonadal tissues (reviewed by 43). Additionally, numerous dietary- and medicinal supplements may deliver additional estrogen or estrogen-like molecules to the body. Results obtained from our study imply that an individual will, during periods of augmented estrogen levels, experience elevated levels of ALAS1 transcription, which will cause a subsequent increase in the rate of the entire heme synthesis pathway. Our findings may have an important impact on individuals suffering from acute hepatic porphyria. Acute porphyric attacks, such as observed in VP and other porphyrias, are primarily caused by the accumulation of heme precursors in the presence of a defective enzyme in the heme pathway. Consequently, a porphyria patient may experience an added build-up of these heme precursors during episodes of high estrogen levels, and subsequent enhanced porphyria phenotypic consequences. Since this estrogenic effect seems to be augmented by the presence of the -853T variant, porphyria patients with this variant, and other variants with similar effects, may experience even further aggravating phenotypic consequences during periods of increased estrogen levels. This theory is consistent with the occurrence of VP symptoms primarily in women and predominantly at the onset of puberty, with acute attacks often precipitated by contraceptive use and the frequency of the acute attacks decreasing with age (47, http://www.porphyria-europe.com/01-for-patients/EN/for-patients). In other types of porphyrias, reports also exist of the association of acute attacks with the menstrual cycle (8).

The enhanced transcriptional effect caused by estrogen is, however, lessened by the addition of the -1253A variant. From the results obtained in our study, we speculate that a porphyria patient with the -853T/-1253A allele may be protected against severe acute symptoms, since the decrease in ALAS1 transcription mediated by this variant, will reduce the rate of heme synthesis with a resultant reduction in the build-up of cytotoxic precursors that will curb disease penetrance. The dissimilarity in phenotypic expression between the three patients with both variants in our cohort, may be due to differences in their ages and in their contraceptive usage. The clinical history of these patients indicates no previous use of hormone replacement therapy. The patient data indicated that the asymptomatic patient heterozygous for -853C/T and -1253T/A, is 22 years of age with no previous history of hormonal contraceptive use. The other two patients heterozygous for the two variants, were both 50 years and older, classified as having only skin symptoms and a history of contraceptive use.

The localization of both variants on the same allele therefore presents an intriguing scenario where the same allele may have either a protective or precipitating effect on disease penetrance under differing physiological conditions. In VP for example, an individual with the –1253A variant could therefore experience a decrease in disease penetrance under normal cellular conditions, but during periods of elevated estrogen levels, the –853T variant could enhance VP penetrance by amplifying the normal response to estrogen.

Additional ALAS1 reporter gene assays in other cell lines will indicate if the observed estrogen-responsiveness of this gene is liver-specific or operational in other tissues. Further studies analysing larger porphyria patient groups may provide insight into possible association of the detected variants with a specific phenotypic outcome. Specifically, analyses of acute intermittent porphyria patients (AIP) might prove to be more informative when considering that it occurs at a higher frequency in other populations and the fact that that AIP patients present with recurrent acute attacks that was found to be more frequent in females (21). Although the limited sample size and other precipitating factors confounded the effect of association on a clinical level, this study forms a basis for further analysis of the ALAS1 promoter to confirm the mechanism and involvement of ER-α and the putative ERE half-sites in the estrogen-mediated up-regulation of ALAS1. These findings offer a description of the mechanism involved in heme regulation via ALAS1 in response to estrogen and contribute to unraveling the mode of regulation of this gene, providing the groundwork for further studies concerning heme homeostasis.

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REFERENCES


