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Analysis of variants in the HCN4 gene and in three single nucleotide polymorphisms of the CYP3A4 gene for association with ivabradine reduction in heart rate: A preliminary report

Short title: *HCN4* and *CYP3A4* variants related to ivabradine

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Abstract

Background: Ivabradine, a selective bradycardic drug, inhibits the I_f. In patients with heart failure (HF), ivabradine reduces the risk of rehospitalization and mortality. The average heart rate (HR) reduction is 8–10 beats, although clinical trials reveal interindividual variability. The aim of the study is to identify variants associated with HR reduction produced by ivabradine in genes involved in the drug metabolism (*CYP3A4*) or related to the drug target (*HCN4*).

Methods: In an exploratory cohort (n = 11), patients started on ivabradine were genotyped and the HR reduction was studied.

Results: The mean HR reduction after the treatment was 18.10 ± 12.26 bpm. The HR reduction was ≥ 15 bpm in 3 patients and > 5 and < 15 bpm in 7 patients. Four synonymous variants, L12L, L520L, P852P, and P1200P, were detected in the *HCN4* gene (frequency = 0.045, 0.045, and 0.681, respectively). Moreover, the *CYP3A4*1F* and *CYP3A4*1B* were found in one patient each and *CYP3A4*1G* was presented in 3 patients.

Conclusions: This is the first study using an exploratory pharmacogenetic approach that attempts to explain interindividual variability in ivabradine HR reduction. However, more research must be undertaken in order to determine the role of variants in *HCN4* and *CYP3A4* genes in response to ivabradine.

Key words: heart failure, ivabradine, *HCN4*, *CYP3A4*, pharmacogenetic

INTRODUCTION

Heart failure (HF) is defined as a syndrome in which patients show typical symptoms (e.g. breathlessness, ankle swelling, and fatigue) and signs (e.g. elevated jugular venous pressure, pulmonary crackles, and displaced apex beat) resulting from an abnormal cardiac structure or function [1]. Approximately 1–2% of the adult population in developed countries has HF, with the prevalence rising to ≥ 10% among individuals aged 70 or over.

HF remains a disabling disorder that can severely affect patients' quality of life. However, there is a crucial need for the development of innovative therapeutic approaches [2].

One strategy is the control of the heart rate (HR) in HF patients. Ivabradine, which slows the heart by selective I_f current inhibition with no other cardiovascular effects, has been approved by the European Medicines Agency for this purpose, following the BEAUTIFUL and SHIFT trials [3, 4]. The SHIFT trial showed that HR reduction with ivabradine significantly reduced adverse clinical outcomes in a population with symptomatic HF and HR ≥ 70 bpm [4]. However, the magnitude of HR reduction achieved with ivabradine displays considerable interindividual variability [4]. In fact, in a pre-specified subgroup of SHIFT, the reduction in HR at 28 days was ≤ 5

bpm in 21% of patients, > 5 and < 15 bpm in 34% of patients, and ≥ 15 bpm in 41% of patients [5]. The effect of ivabradine is known to be influenced by the HR at baseline. However, other factors, such as genetic variations, may play a role in the magnitude of HR reduction achieved with ivabradine, although to date this hypothesis has not been addressed.

On the basis of mechanism of action and pharmacokinetics of ivabradine, *HCN4*, which encodes isoform 4 of hyperpolarization-activated cyclic Nnucleotide-gated channels, whose proteins are the α-subunits of the channel generating the I_f current [6], and *CYP3A4*, which encodes the isoform 3A4 of the cytochrome P450, the main enzyme responsible for the metabolism of ivabradine [7], could be candidate genes to present variants that could be associated with differential responses to ivabradine.

The ivabradine binding site has recently been located within the inner cavity of HCN4 channels (Y506, F509, and I510), where the bound ivabradine is stabilized by several Van der Waals and hydrophobic interactions [6]. Several *HCN4* variants modifying the I_f current have been described [8–10]. Moreover, the importance of *HCN4* variants has been recently described because a new *locus* near *HCN4* gene has been associated with HR [11]. However, the effect of *HCN4* variants as modifiers of the response to ivabradine has not been studied.

It is known that 80% of ivabradine metabolic clearance is done through the cytochrome P450 isoform 3A4 (CYP3A4) [7]. Nowadays, more than 40 single nucleotide polymorphisms (SNPs) in the *CYP3A4* gene have been identified with varying functional effects [12]. The CYP3A4*1G SNP (rs2242480), also known as IVS10+12G>A, can increase the activity of the CYP3A4 enzyme [12, 13], whereas CYP3A4*22 (rs35599367) has been associated with reduced CYP3A4 activity [14, 15]. The functional effect of CYP3A4*1B (rs2740574) is controversial [14] because it has been associated with an enhanced CYP3A4 expression due to the reduced binding of a transcriptional repressor [16] as well as a reduction in *CYP3A4* activity [17].

Therefore, it is important to determine the potential role of variants in the *HCN4* and *CYP3A4* genes in response to treatment with ivabradine. Thus, the objective of this study was to screen for variants in the entire codified region of the *HCN4* gene and in three particular SNPs in the *CYP3A4* gene which could affect the interindividual variability found in the magnitude of HR reduction achieved with ivabradine.

METHODS

Patients

The study was carried out on 11 patients from the Advanced Heart Failure and Transplant Unit of the *Complejo Hospitalario Universitario de A Coruña* (CHUAC) with HF, reduced ejection fraction (EF ≤ 35%), sinus rhythm, HR > 70 bpm and New York Heart Association (NYHA) class II–IV, and in whom ivabradine therapy had been initiated (Table 1). Blood samples for DNA analysis were taken concomitantly with blood tests for clinical monitoring. Two 24 h Holter electrocardiogram (ECG) recordings were taken, the first prior to ivabradine therapy and the second 15 days later. Mean HR reduction between the 2 Holter studies was calculated. The study was approved by the "*Comité ético de investigación de Galicia*" (Reference: 2012/323) and conforms to the ethical guidelines of the Declaration of Helsinki. Informed consent was obtained for both the samples and the genetic screening tests.

Genetic study

Genomic DNA was isolated from peripheral blood samples using Illustra™ DNA Extraction Kit BACC3 (GE Healthcare), as previously described [18, 19]. The variant analysis was carried out by a polymerase chain reaction (PCR) followed by direct sequencing [18, 19]. The primers were designed using Primer3 software (http://bioinfo.ut.ee/primer3-0.4.0/) (Table 2). The entire codifying sequence and the flanking intronic regions of the *HCN4* gene were amplified by PCR. Three CYP3A4 SNPs, previously described with a functional effect (*1B,*1G,*22), were analyzed. A further SNP, *1F (rs11773597), was analyzed due to its inclusion in the amplicon design for the screening of CYP3A4*1B. The sequences were compared with the reference genomic sequence of the genes using Variant Reporter 1.0 (Applied Biosystem).

***In silico* tools**

Localization: the topological placement of the mutations was carried out using the Swiss-Prot database (http://ca.expasy.org/uniprot/).

Splice site score predictions

The NNSplice (http://www.fruitfly.org/seq_tools/splice.html [20]), NetGene2 (http://www.cbs.dtu.dk/services/NetGene2/ [21]), and HSF (http://www.umd.be/HSF/ [22]) programs were used to predict whether the exon changes could affect splice-enhancing sequences.

RESULTS

Clinical characteristics of the patients and response to ivabradine

A summary of the patients' clinical characteristics is given in Table 1. Two patients discontinued the treatment with ivabradine due to adverse drug-related events. One patient (No. 3) described gastrointestinal disorders, a stifling sensation, dyspnea and rubefaction on day eleven of the treatment. Another patient (No. 6) stopped the treatment after 2 months due to low HR-related asthenia.

The mean reduction of HR after treatment with ivabradine was 18.10 ± 12.26 bpm. Using the subgroup classification applied to a sub-study of the SHIFT study, the HR reduction was shown to be ≥ 15 bpm in 3 patients and between 5–15 bpm in 7 patients. None of our patients showed an HR reduction of ≤ 5 bpm.

Variants found in the *HCN4* gene

As shown in Table 3, Figures 1A and 2A–D, 4 synonymous single nucleotide variants were found in the *HCN4* gene. c.36C>G (L12L), c.1558C>T (L520L) and c.2256G>A (P852P) were each found in heterocygosity in one patient. Thus, the minor allele frequency (MAF) in our cohort of each variant is 0.045. The other variant found was c.3600A>G (P1200P), and it was present in 3 patients in heterocygosity and in 6 patients in homocygosity. Thus, the P1200P MAF in our cohort is 0.682.

In silico analysis of the variants found in the *HCN4* gene

Although 3 programs used failed to detect any impact on the corresponding natural splice site for the 4 variants studied, these variants could modify the exonic splicing enhancer and the silencer motifs (Table 4).

The variant c.36C>G (L12L) is predicted to create a new site as an exonic splicing enhancer (ESE) to link the Ser/Arg-rich (SR) protein SF2/ASF. Moreover, using the Sironi method, L12L is predicted to produce a 'site broken' of a silencer motif.

The ESE finder software predicted a 'site broken' when the variant c.1558C>T (L520L) was present for the Srp40 and SF2/ASF SR proteins. The putative exonic splicing enhancer (PESE) software predicted a new site and a 'site broken' in the presence of the L520L, but the motif values, in both cases, were low. Analysis of silencer motifs revealed a 'site broken' when the L520L was present.

Two programs used in the *in silico* analysis detected a new site as ESE for the c.2256G>A (P852P)variant.

Moreover, two of the programs predicted that the variant c.3600A>G (P1200P) could create a new site for binding the SC35 and 9G8 proteins, whereas the RESCUE ESE predicted a 'site broken' of an exonic splicing enhancer.

The prediction for the variants L12L, L520L, and P1200P were the presence of several 'sites broken' in silencer motifs.

SNPs distribution in the *CYP3A4* gene

Figure 1B shows the SNPs analyzed in our patients. Table 3 and Figure 2E–G show the genotypes of the 11 patients for the 4 variants analyzed. CYP3A4*1F SNP was found in 1 patient; CYP3A4*1B SNP in another; and the variant CYP3A4*1G in 3, 2 of them presented the variant in heterocygosity and one in homocygosity. The MAF in our cohort for *1F, *1B, and *1G are 0.045, 0.045, and 0.182, respectively.

DISCUSSION

Knowledge regarding the genetic basis of differential therapeutic drug response has generated hope for individually tailored drug therapy. Interindividual differences in drug metabolism, distribution and excretion, and drug targets (receptors) are important considerations in assessing drug efficacy, safety, and dose [23]. Thus, in the case of ivabradine, it is important to study the variants in the *CYP3A4* and *HCN4* genes, as they both codify the proteins involved in its metabolism and relate to the drug target. This study identifies four synonymous variants in the *HCN4* gene (L12L, L520L, P852P, and P1200P) and two SNPs in the *CYP3A4* gene (CYP3A4*1F and CYP3A4*1G).

Clinical data

This exploratory analysis studies the effect of ivabradine in 11 patients. The distribution of no responders (n = 0), poor responders (n = 7), and high responders (n = 3) is not homogeneous. In a number of studies [4, 5], the effects of ivabradine were found to be greater in patients with higher baseline HR due to the use-dependent block. Our results also showed that it is important to analyze the baseline HR in order to study the HR reduction produced by ivabradine. As shown in Table 1, in 5 out of 11 patients, the baseline HR was 70–80 bpm; in 4 out of 11, the baseline HR was 81–90 bpm; and in 2 out of 11, the baseline HR was higher than 91 bpm. In line with the above, the biggest

reduction was obtained in patients with the highest baseline HR; in our cohort, these were patients no. 7 and 11, in whom the HR reduction after ivabradine treatment was 23 and 53 bpm, respectively.

Two out of 11 patients (Table 1) were withdrawn from the treatment during the study; one prior to the second Holter recording and the second after 2 months due to low HR-related asthenia. In previous trials [3, 4, 24], withdrawal rates were between 4% and 28%. If we focus on withdrawal rates due to an adverse event, the figures range between 1.7% and 13.2% [3, 4, 24, 25]. The percentage of withdrawals in our exploratory study was 18.2%, within the range given above, although it is higher than the adverse event withdrawal rate.

However, it is important to note that this is an exploratory prospective study on consecutive patients. A large-scale multicenter study would be required in order to analyze in greater depth the effects of polymorphism in ivabradine treatment and reach relevant conclusions about relation of variants in *CYP3A4* and *HCN4* genes on ivabradine HR.

Distribution of mutations in the cohort

The synonymous variants found in the *HCN4* gene in our cohort are included in the Single Nucleotide Polymorphism Database (dbSNP) and the MAFs described in dbSNP were 0.011, 0.053, 0.023, and 0.131 for the variants L12L (rs201193660), L520L (rs12909882), P852P (rs117819825), and P1200P (rs529004), respectively. In our cohort, the variants L12L, L520L, and P852P were found in one patient each. Thus, the MAFs for these variants were 0.045, similar to those previously described. However, the MAF in our cohort for the variant P1200P is 0.682, which is far higher than that described in dbSNP. In fact, in our cohort, the minor allele is c.3600A and not c.3600G, as is described in literature and in the sequence.

Point variants in the coding regions of genes were traditionally assumed to act by altering single amino acids in the encoded proteins. Consequently, the synonymous mutations detected in genetic screens are presumed to be neutral. However, translationally silent mutations can disrupt ESEs or exonic splicing silencers (ESSs) and cause an alteration in the splicing machinery, with dramatic effects on the structure of the gene product [26–28]. ESEs represent binding sites for SR proteins, which are believed to play a role in the initial steps of spliceosome assembly, whilst ESSs have been shown to bind negative regulators belonging to the heterogeneous nuclear

ribonucleoprotein (hnRNP) family [27, 28]. The function of ESEs and ESSs appears to be especially important for the regulation of alternative splicing events, yet these sequences probably also play a relevant role in defining constitutive exons [27, 28]. Therefore, in this study, we performed an *in silico* analysis in order to detect the two major classes of *cis*-regulator of splicing, ESEs and ESSs.

Two variants found in this study, L12L and P852P, were predicted to produce an ESE creation, where predicted ESEs are present in the variant but not in the wild-type sequence. The other two variants, L520L and P1200P, were predicted to produce ESE creation and ESE disruptions, where one or more predicted ESEs present in the wild-type sequence are disrupted by the variants. Moreover, the software used predicted a 'site broken' for a silencer motif for the variants L12L, L520L, and P1200P. Consequently, and as addressed by the biocomputational approach, the four variants found in the *HCN4* gene could alter the splicing machinery. However, the presence of a score motif in a sequence does not necessarily identify that sequence as a functional ESE or ESS, which indicates not a very strict quantitative correlation between numerical score and ESE or ESS activity [26]. Until stronger predictive algorithms are available, direct experimental evidence will remain necessary before drawing a safe conclusion that a particular sequence can act as an ESE or ESS in its natural context [26]. In addition, assessing the clinical impact of these variants is a complex task since each three of the variants are present in one patient only, whilst P1200P is presented in 9 of 11 patients studied. However, it has been shown that ivabradine reduced the HR by 13 bpm in a patient carrying the L12L variant. A patient with L520L discontinued the treatment due to adverse events; and in the case of a patient carrying P852P variant the HR was reduced by 11 bpm. It is important to note that patients carrying the L12L and the P852P variants also carried the P1200P variant and the presence of more than one variant further complicates the interpretation of the data.

In the case of SNPs studied in the *CYP3A4* gene, we found the CYP3A4*1F in one patient in heterocygosity (MAF = 0.045); CYP3A4*1B in one patient in heterocygosity (MAF = 0.045); and CYP3A4*1G in 2 patients in heterocygosity and in 1 patient in homocygosity (MAF = 0.182). The frequencies of CYP3A4*1F are similar to those previously described in dbSNP (0.036). However, the frequencies found in our cohort for the variants CYP3A4*1B and CYP3A4*1G are lower than those previously described in dbSNP, being 0.201 and 0.334, respectively.

In our cohort, CYP3A4*1F is present in 1 patient who experienced an 11 bpm reduction in HR after 15 days of treatment with ivabradine. CYP3A4*1F is a variant in the 5' regulatory region of the gene and it leads to a new CpG island that can be methylated [29]. This potential methylation position could be significant for the function of this enzyme. To date, no particular phenotype has been associated with this allele [29, 30]. Further research is therefore necessary in order to assess the role of CYP3A4*1F in the function of the enzyme.

CYP3A4*1B was found in heterocygosity in one patient whose HR was reduced by 11 bpm following treatment with ivabradine. The functional effect of this SNP has not been established because it has been associated not only with an enhanced CYP3A4 expression due to reduced binding of a transcriptional repressor [16] but also with a reduced level of *CYP3A4* activity [17]. Thus, further clinical and basic research is required in order to assess the role of the CYP3A4*1B.

CYP3A4*1G SNP has been described as a variant that can increase CYP3A4 activity [12, 13]. In our cohort, 2 patients have this variant in heterocygosity and 1 in homocygosity. The reduction after treatment with ivabradine was 14 bpm in 1 patient with the variant in heterocygosity, and 23 bpm in a patient with the variant in homocygosity. The other patient with the variant discontinued the treatment due to adverse events. CYP3A4*1G therefore appears to have insufficient impact on the metabolism of ivabradine as the 2 patients analyzed are good responders.

The role of variants in the response to ivabradine

Inherited genetic differences may result in the identification of sub-groups of patients, including those who are good responders, poor responders and those likely to present adverse drug reactions [23]. In our exploratory cohort, no poor responders were detected. HR was ≥ 15 bpm in 3 patients and > 5 and < 15 bpm in 7 patients, and 2 patients discontinued the treatment due to adverse events. Variant distribution in this small-size cohort of this exploratory study does not allow the variants to be correlated with these sub-groups. Consequently, further research is required in order to assess the role of variants in the *CYP3A4* and *HCN4* genes in ivabradine treatment.

CONCLUSIONS

This study has identified 4 synonymous variants in the *HCN4* gene and 3 SNPs in the *CYP3A4* gene. None of the variants appear to have a major effect on the reduction

of HR produced by ivabradine. However, and due to the limited size of the cohort, further research must be carried out in order to determine the role of different variants in *HCN4* and *CYP3A4* genes in response to ivabradine.

Moreover, the *in silico* analysis must be given due consideration, as when isolated from other experimental data, it fails to provide sufficient information in terms of genetic counselling.

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Conflict of interest: none declared

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Tables:

Table 1. Clinical characteristics of patients included in the study.

	Demographic characteristics			Cardiac parameters						Medical history	Treatment before the study (dosage in mg per day)
Patient no.	Age [years]	Sex	Body mass index [kg/m^2]	SBP [mm Hg]	DBP [mm Hg]	LVEF [%]	NYHA class	HR-1 [bpm]	HR reduction [bpm]		
1	56	M	25.98	87	59	20	II	89/78	11	HT, dyslipidemia	Atorvastatin (10), carvedilol (12.5), enalapril (10), furosemide (120)
2	60	M	27.36	101	68	30	II	73/61	12	HT, DM, MI, dyslipidemia	Aspirin (150), carvedilol (100), furosemide (40), enalapril (3.75), rosuvastatin (10), spironolactone (25).
3*	31	M	32.49	107	70	24	II	78/–	–	HT	Aspirin (100), bisoprolol (10), candesartan (8), furosemide (120), spironolactone (50)
4	59	M	40.26	140	90	30	II	89/75	14	HT, DM	Bisoprolol (10), enalapril (20), eplerenone (25), torasemide (10)
5	72	M	28.23	170	106	32	II	89/76	13	HT, dyslipidemia, hypercholesterolemia	Aspirin (100), carvedilol (50), furosemide (80), ramipril (10)
6*	54	M	37.42	134	78	20	II	76/65	11	COPD, HT	Atorvastatin (40), bisoprolol (5), enalapril (5), furosemide (40), spironolactone (25)
7	31	M	20.24	130	100	22	II	115/92	23	HT, MI, DM	Amlodipine (5), aspirin (100), atorvastatin (20), carvedilol (50), furosemide (240), losartan (100).
8	30	F	19.84	96	64	25	IV	80/60	20	HT	Bisoprolol (2.5), enalapril (2.5), eplerenone (25), furosemide (40)
9	45	M	23.72			34	II	86/73	13	MI, dyslipidemia, hypercholesterolemia	Aspirin (100), bisoprolol (2.5), enalapril (5), eplerenone (25), furosemide (120), rosuvastatin (10)
10	56	M	26.85	98	55	23	II	75/64	11	DM, dyslipidemia	Carvedilol (25), clopidogrel (75), ezetimibe (10), furosemide (80), simvastatin (40),spironolactone (12.5)
11	58	M	28.35	93	57	35	II	106/53	53	HT, DM, dyslipidemia	Aspirin (100), bisoprolol (2.5), enalapril (5), eplerenone (25), furosemide (80), pravastatin (40)

COPD — chronic obstructive pulmonary disease; DBP — diastolic blood pressure; DM — diabetes mellitus; F — female; HR-1 — heart rate before/after ivabradine; HT — hypertension; LVEF — left ventricular ejection fraction (%); M — male; MI — myocardial infarction; NYHA — New York Heart Association; SBP — systolic blood pressure; *Patients that discontinued the treatment

Table 2. Primers used in the study.

Fragment	Primer sequence (5'□3')	Amplicon size
HCN4 Ex1 Fw	GACTCGGAGCGGGACTAGGAT	1078 nt
HCN4 Ex1 Rv	CCAGCGCAAGGCAGGAAAGTT	
HCN4 Ex2_F	CCAGATGCTGTCCCTCAGAT	576 nt
HCN4 Ex2_R	CCAGTTCCTCACTCCCTCTG	
HCN4 Ex3_F	CAGAGTCCAGGCAGAGCAGT	377 nt
HCN4 Ex3_R	GGTCCTACATGCTGGAACTCA	
HCN4 Ex4_F	CTTTCAGCCAACAGCAAGGT	496 nt
HCN4 Ex4_R	TTCCCTCACACTGGGAGTTC	
HCN4 Ex5+6_F	GGAACCAAGTTTAGCCAGGA	695 nt
HCN4 Ex5+6_R	GCCTCTGTCCCCTCGGTAT	
HCN4 Ex7_F	TTCTGTGCCAGGCAGTCA	361 nt
HCN4 Ex7_R	GGAAGGAGATCAGGTGCAGA	
HCN4 Ex8A Fw	CTTTATGCCTAAGCCAGGTCT	847 nt
HCN4 Ex8A_R	CTAGATGACGGGGATCTGGA	
HCN4 Ex8B1_F	AACAGCTGGCTGGATTCTCTGC	578 nt
HCN4 Ex8B1_Rv	CAAGGATCCGTGGGAGCCAGA	
HCN4 Ex8C_F	CTTCCCCTGTAGGCTTTACTC	724 nt
HCN4 Ex8C_Rv	CCTGGTTATTTTCTGCTGTCTT	
CYP3A4*1B_F	CCAACAGAATCACAGAGGACCAGC	908 nt
CYP3A4*1B_R	CTCTGAGTCTTCCTTTCAGCTCTGTGT	
CYP3A4*22 Ex7_F	CCCATCTTGTATCATCCACAA	466 nt
CYP3A4*22 Ex7_R	TGAGAGAAAGAATGGATCCAAAA	
CYP3A4*1G Ex10_F	AGGGATTTGAGGGCTTCACT	399 nt
CYP3A4*1G Ex10_R	TTTCTTTTCAGAGCCTTCCTACA	

These primers were designed using the reference sequence from GenBank-GRCh37.p9 [HCN4: NC_000015.9 (73612200..73661605); CYP3A4: NC_000007.13 (99354583…99382811)] and Primer3 software.

Table 3. Variants found in the cohort in *HCN4* gene and variants studied in *CYP3A4* gene.

Patient no.	**Variants found in *HCN4* gene**
1	g.46772A>G (p.P1200P)
2	g.46772G (p.P1200P)
3	g.39660C>T (p.L520L)
4	g.46772G (p.P1200P)
5	g.1030C>G (p.L12L), g.46772A>G (p.P1200P)
6	g.45728G>C (p.P852P), g.46772G (p.P1200P)
7	g.46772G (p.P1200P)
8	g.46772G (p.P1200P)
9	g.46772G (p.P1200P)
10	None
11	g.46772A>G (p.P1200P)

CYP3A4	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
CYP3A4*1F	CC	CC	CC	CC	CC	CG	CC	CC	CC	CC	CC
CYP3A4*1B	AA	AA	AA	AA	AA	AA	AG	AA	AA	AA	A A
CYP3A4*22	CC	CC	CC	CC	CC	CC	CC	CC	CC	CC	CC
CYP3A4*1G	GG	GG	GA	GA	GG	GG	AA	GG	GG	GG	G G

Table 4. *In silico* analysis of exonic splicing enhancer (ESE) and silencer motifs of the variants found in the *HCN4* gene.

***ESE finder matrices for SRp40, SC35, SF2/ASF and SRp55**					
Variant	**Reference motif**		**Mutant motif**		**Variation**
	Linked SR protein	Reference motif (value 0–100)	Linked SR protein	Reference motif (value 0–100)	
p.L12L			SF2/ASF (IgM-BRCA1)	cggct**g**t (78.23)	New site
			SF2/ASF	cggct**g**t (73.44)	New site
p.L520L	SRp40	tcc**c**tgg (84.13)			Site broken
	SF2/ASF (IgM-BRCA1)	cc**c**tgga (84.13)			Site broken
p.P1200P			SC35	aactgcc**g** (76.21)	New site
***RESCUE ESE hexamers**					
Variant	**Reference motif**		**Mutant motif**		**Variation**
p.P852P			**a**tcttc		New site
p.P1200P	**a**tccaa				Site broken
***Predicted PESE Octamers from Zhang & Chasin**					
Variant	**Reference motif**		**Mutant motif**		**Variation**
	Reference motif	Motif value (0–100)	Reference motif	Motif value (0–100)	
p.L520L			ccagtcc**t**	38.75	New site
	tcc**c**tgga	42.96			Site broken
p.P852P			acacc**a**tc	29.64	New site
***ESE motifs from HSF**					
Variant	**Reference motif**		**Mutant motif**		**Variation**
	Linked ESE protein	Reference motif (value 0–100)	Linked ESE protein	Reference motif (value 0–100)	
p.P1200P			9G8	gcc**g**tc (62,82)	New site
Silencer motifs from Sironi *et al.					
Variant	**Reference motif**		**Mutant motif**		**Variation**
	Sironi Motif Reference	Reference silencer (value 0–100)	Sironi Mutant Motif	Mutant silencer (value 0–100)	
p.L12L	Motif 1: CTAGAGGT	**c**tacagcc (67.64)			Site broken (13.49)
p.L520L	Motif 3: TCTCCCAA	agtcc**c**tg (69.14)			Site broken (–0.16)
p.P1200P	Motif 3: TCTCCCAA	actgcc**a**t (72.52)			Site broken (11.26)

Figure 1. A. Schematic structure of the HCN4 channel showing the variants identified in the present study; **B.** Schematic structure of the human *CYP3A4* gene showing the

four single nucleotide polymorphisms **(**SNPs) analyzed in the present study, which produce variations in normal functioning of cytochromes.

Figure 2. A–D. Electropherograms of the variants identified in the *HCN4* gene; **E, F.** Electropherograms of two of the four single nucleotide polymorphisms **(**SNPs) analyzed where minor alleles are presented.

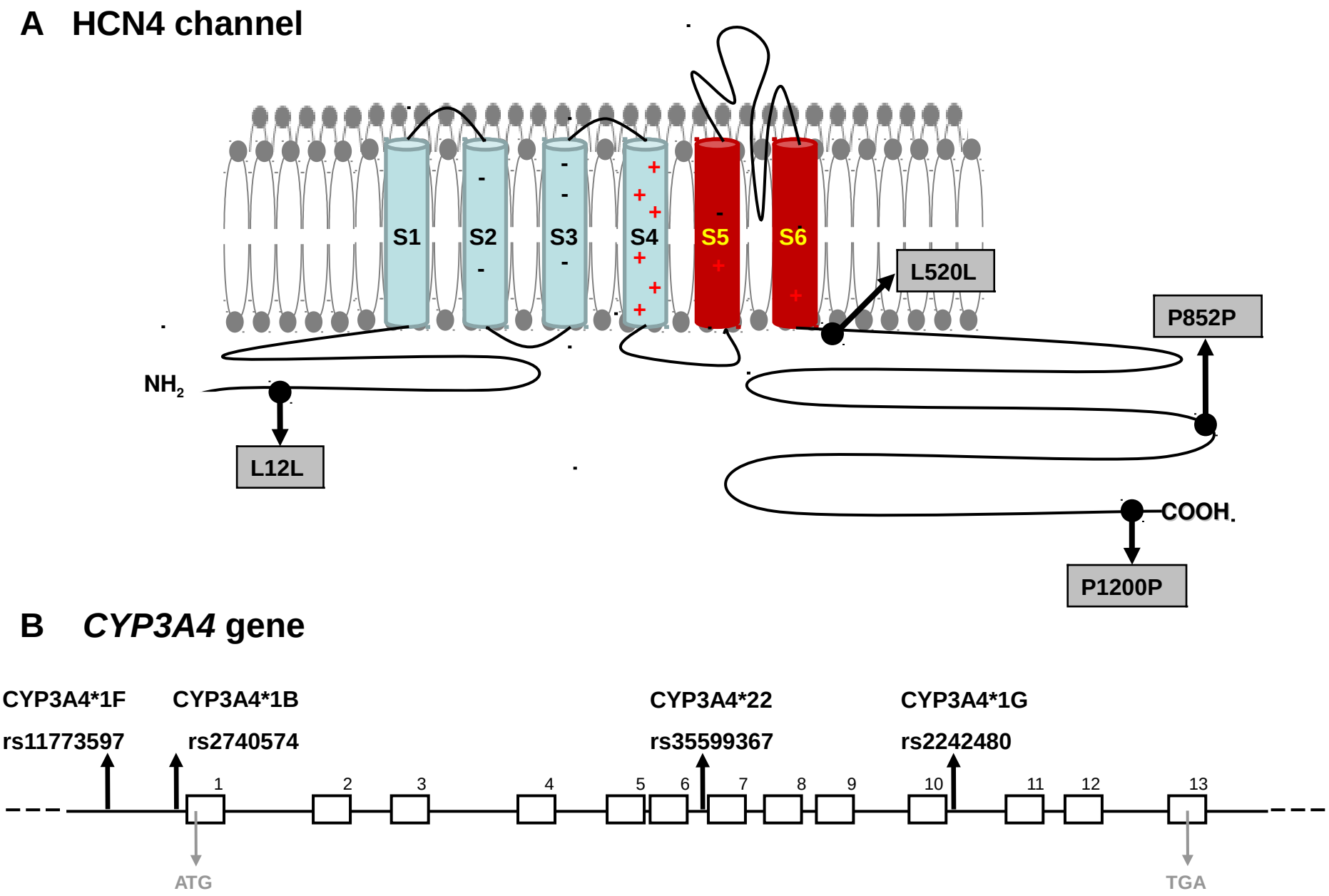
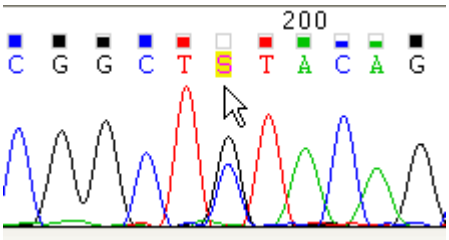

A HCN4 channel
S1
S2
S3
S4
S5
S6
L520L
P852P
NH2
L12L
COOH
P1200P
B CYP3A4 gene
CYP3A4*1F
rs11773597
CYP3A4*1B
rs2740574
CYP3A4*22
rs35599367
CYP3A4*1G
rs2242480
1
2
3
4
5
6
7
8
9
10
11
12
13
ATG
TGA

Figure 1

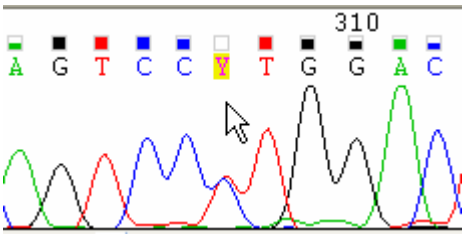
A p.L12L

(c.36C>G, g.1030C>G)



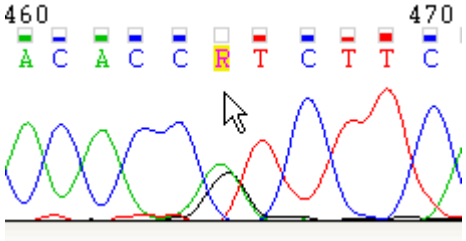
B p.L520L

(c.1558C>T, g.39660C>T)



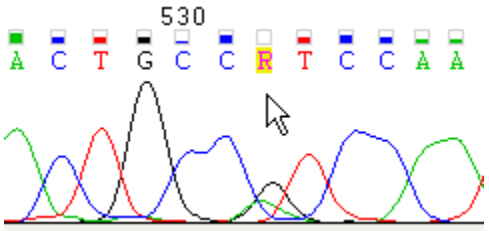
C p.P852P

(c.2256G>A, g.45728G>A)



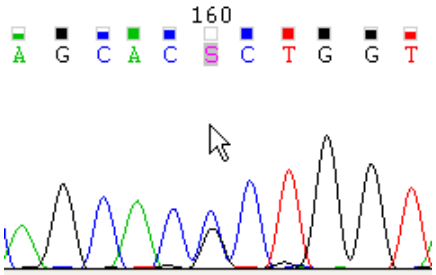
D p.P1200P

(c.3600A>G, g.46772A>G)

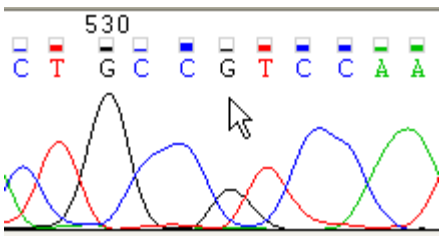


E CYP3A4*1F

(c.1-748G>C, g.361G>C)



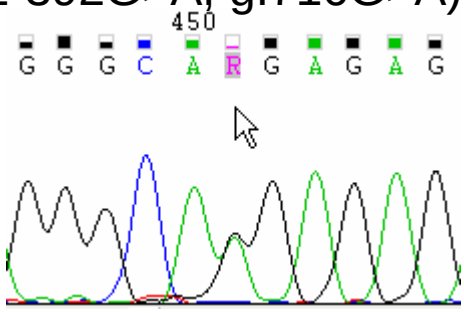
c.3600A>G



c.3600G

F CYP3A4*1B

(c.1-392G>A, g.716G>A)



G CYP3A4*1G

(c.1026+12G>A, g.21346G>A)

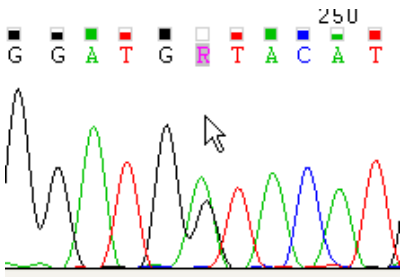


Figure 2