Research Article

CLINICAL INVESTIGATION

Failure to replicate a previously reported GWAS association between KCNJ1 gene and co-amoxiclav-induced liver injury

Background: Co-amoxiclav is associated with Drug-Induced Liver Injury (DILI). HLA genotype is an important predictor of DILI susceptibility but it is likely that non-HLA risk factors also contribute. This study aimed to characterize non-HLA risk factors in larger cohorts than previously. Although KCNJ1 is known for its regulation of kidney activities through modulation of potassium homeostasis, the association seen between this gene and co-amoxiclav DILI, detected in a previous Genome-Wide Association Study (GWAS), which was close to p-value threshold significance, was stimulating to investigate its role in DILI susceptibility.

Methods: A variant (rs2855790) in KCNJ1 was genotyped to extend the previous findings. 73 co-amoxiclav adjudicated DILI cases and 75 community controls were genotyped using RFLP-PCR assay. Drug causality of the cases was assessed using the RUCAM method. The data obtained were analyzed for its Odds Ratio (OR) value and Fisher's exact test was used to generate a p-value.

Results: Although, variant allele frequency in cases were much lower (15.8%) than in controls (24.7%); however, the difference was not statistically significant (OR=0.58, 95% CI=0.29-1.1; P=0.13). Hence, the previously reported association with KCNJ1 (rs2855790) could not be confirmed in this new cohort of co-amoxiclav DILI.

Conclusion: Given the biological role of KCNJ1 in the kidney, not the liver, this gene was not a very biologically plausible candidate as a DILI gene so the final result obtained for this is not too surprising.

Keywords: KCNJ1 • GWAS • Co-amoxiclav • Liver Injury • DILI • Pharmacogenetics

Submitted: 22 November 2020; Accepted: 07 December 2020; Published online: 23 December 2020

Mohammad A. Alshabeeb[1,2*]

[1]Developmental Medicine Department, King Abdullah International Medical Research Center (KAIMRC), Riyadh, Saudi Arabia,

[2]King Saud Bin Abdulaziz University for Health Sciences of the university (KSAU-HS), Riyadh, Saudi Arabia.

*Author for correspondence: shabeebonline@hotmail.com

Introduction

The liver is particularly susceptible to drug toxicity due to its vital role in xenobiotic metabolism and elimination. Drug-Induced Liver Injury (DILI) involves a widely variable range of toxicity comprising alteration in liver biochemical tests and different levels of liver damage; for example, hepatitis, necrosis, steatosis, cirrhosis, fulminant liver failure, and blood clots of the veins within the liver which probably leads to significant patient morbidity and mortality [1]. The majority of DILI cases, particularly mild and moderate, return to normal condition upon cessation of the causative hepatotoxic agent though cholestatic reactions tend to be prolonged compared to hepatocellular [2].

A range of medications can induce hepatic toxicity in susceptible individuals, in particular, antibiotics are believed to be the most common hepatotoxic agents [3]. Co-amoxiclav is a well-tolerated drug with acceptable safety records; however, various trials have confirmed its toxicity [4,5]. Co-amoxiclav was reported as the leading drug causing DILI in the largest two prospective registries conducted in Spain [6], and in the USA; Drug-Induced Liver Injury Network (DILIN) study [7].

GWAS approach has been increasingly used by pharmacogenetic researchers to identify predisposing genetic factors associated with particular drug toxicities and to detect susceptibility markers related to therapeutic failure in certain patients versus others who achieved satisfactory drug response [8-10]. Strong associations with particular HLA alleles have been detected by GWAS for co-amoxiclav-induced liver injury [11], this supports previous findings and confirms the major role of HLA markers in co-amoxiclav DILI development [12,13,4,5]. Additional non-HLA SNPs also show associations that are close to genome-wide significance. Since using HLA genotype alone to predict susceptibility to these reactions shows relatively low

predictive value, it is likely that additional genetic factors contribute to susceptibility though the observed effect sizes may be lower than those for the HLA risk factors.

This study aimed to investigate the role of KCNJ1 as a Non-HLA marker detected as a risk factor for drug-induced hepatotoxicity at a level close to genome-wide significance using the data from the previously published GWAS study conducted by Lucena et al [14]. KCNJ1 (potassium inwardly-rectifying channel, subfamily J, member 1) is located on chromosome 11 and encodes the Renal Outer Medullary Potassium Channel (ROMK). The main function of this protein is to regulate cellular potassium homeostasis.

Methods

SNP selection

As summarised in Table 1, many SNPs outside the Major Histocompatibility Complex (MHC) region of chromosome 6 gave relatively low p values in the GWAS study conducted by Lucena et al [14], which involved 66 co-amoxiclav DILI. Additional cases of DILI relating to co-amoxiclav were used in this study to further investigate the association of DILI with the potential markers. It was decided to select a subset of SNPs based on the following criteria: (i) SNP is located within a gene (ii) the p-value for the previous UK cases was <0.001 and in Spanish <0.05, (iii) more than one SNP in the region showed this level of significance. Four SNPs in the KCNJ1 gene fulfilled these criteria and therefore the gene was chosen for further study. The detected polymorphisms were rs3016776, rs2855798, rs2847381, and rs2855790; they were suggested as risk factors for co-amoxiclav DILI in the GWAS study performed by Lucena et al [14], although the association was not genome-wide significant ($p=4.26x10^{-5}$, $p=6.57x10^{-5}$, $p=3.93x10^{-5}$ and $p=3.7x10^{-5}$, respectively), the commonly agreed genome-wide significant threshold is p

Table 1. Top markers detected from the GWAS study after removal of chromosome 6 SNPs

			UK (fisher, 66 cases, 291 controls)			Spain (Fisher, 53 cases, 167 controls)		
Gene	**SNP**	**Chr**	**P**	**OR**	**MAF**	**P**	**OR**	**MAF**
AKAP6	rs17522991	14	$2.3x10^{-6}$	2.47	0.266	0.2478	1.32	0.350
	rs17523067	14	$2.3x10^{-6}$	2.47	0.266	0.2478	1.32	0.350
CACNA1E	rs4282766	1	0.0063	3.12	0.028	0.0269	2.45	0.054
CADM2	rs7619493	3	0.0069	1.87	0.151	0.1847	1.47	0.159
	rs9816329	3	0.0072	1.85	0.153	0.3292	1.33	0.189
	rs9310001	3	0.0103	1.82	0.155	0.4069	1.28	0.195
DOK6	rs11662320	18	0.0176	0.59	0.294	0.0053	0.45	0.296
	rs17081109	18	0.0327	0.58	0.230	0.0010	0.32	0.225
	rs12969411	18	0.0330	0.59	0.229	0.0010	0.32	0.225
DPP9	rs2109069	19	0.0025	1.78	0.311	0.0003	2.34	0.240
FAM107A	rs13088795	3	0.0012	2.21	0.115	0.0963	1.70	0.114
FREM1	rs12236053	9	0.0044	0.36	0.136	0.0578	0.40	0.111
GADD45G	rs16905942	9	$3.6x10^{-5}$	3.02	0.081	0.0723	1.966	0.072
	rs2890109	9	0.0002	2.91	0.065	0.0023	4.181	0.027
	rs2890110	9	$2.5x10^{-5}$	3.00	0.084	0.0509	2.037	0.075
	rs620311	9	0.0027	2.27	0.089	0.0413	2.572	0.039
INPP4B	rs1497393	4	0.0005	1.92	0.330	0.0365	1.62	0.329
	rs7666932	4	0.0015	1.84	0.328	0.0269	1.67	0.323
KCNJ1	rs2855798	11	0.0006	0.39	0.213	0.0060	0.39	0.213
	rs2847381	11	0.0006	0.39	0.213	0.0018	0.31	0.207
	rs2855790	11	0.0006	0.39	0.213	0.0012	0.31	0.210
	rs3016774	11	0.0008	0.39	0.212	0.0018	0.31	0.207
KCNJ3	rs1823003	2	0.0100	0.58	0.341	0.0123	0.50	0.305
ODZ2	rs7715979	5	0.0052	1.99	0.122	0.0499	1.87	0.117
PARP4	rs9511249	13	0.0048	1.73	0.318	0.1255	1.46	0.319
	rs1050112	13	0.0048	1.73	0.318	0.1002	1.47	0.317
RBPMS	rs6988150	8	0.0245	1.56	0.263	0.0001	2.53	0.240
SIPA1L3	rs8107385	19	0.0012	0.54	0.473	0.0186	0.58	0.482
	rs2304132	19	0.0016	0.54	0.469	0.0186	0.58	0.482
SLC30A2	rs3121763	1	0.0078	0.34	0.112	0.0003	0.19	0.168
SMOX	rs1741327	20	0.0003	2.03	0.273	0.0604	1.56	0.320
	rs1051904	20	0.0007	1.92	0.278	0.0162	1.74	0.356
SYNPO2	rs6828669	4	0.0101	1.63	0.349	0.0139	1.78	0.317
TRIM63	rs7553840	1	0.0079	0.33	0.113	0.0003	0.22	0.183

< $5x10^{-8}$. We decided to study the SNP rs2855790 in this project based on its location at position 112 on exon 1 close to the promoter region of the KCNJ1 gene. In addition, the SNP rs2855790 was found in strong Linkage Disequilibrium (LD) with the other 3 variants (r^2=0.97 with both rs3016776 and rs2847381 while r^2=0.94 with rs2855798).

Cases and controls

The cases studied (n=73) were described in detail in a previous publication [15]. Adjudication of cases was performed using the RUCAM scoring method; an international consensus criterion [16,17]. Institutional Review Boards (IRB) approval for this study was obtained from the Leeds East Research Ethics committee (ref 04/Q1206/9).

Extraction of DNA from peripheral blood leukocytes was performed using a perchlorate-chloroform as described by Daly et al [18]. As controls for the DILI cases, a community group consisting of healthy individuals (n=75) from North-East England, supplied by the late Dr. Peter Donaldson, was used. The control group was previously described in detail by Velaga et al [19].

An additional control data on the Population Reference Samples (POPRES) control group (n=282) was used. UK individuals from this cohort were described by Nelson et al [20]. POPRES group is a valuable resource for population, disease, and pharmacological genetics research which have already been shown to be a good genetic match for co-amoxiclav cases included in previous GWAS studies for DILI [14].

The control groups complied with Hardy-Weinberg Equilibrium (HWE); a web-based calculator was used (http://www.husdyr.kvl.dk/htm/kc/popgen/genetik/applets/kitest.htm). HWE results confirm that the selected controls met the standard quality criteria. Due to the insufficient information about the control participants, we were unable to match them with cases for age, sex, or body weight. The population controls used were not necessarily drug-treated. It is widely accepted to use drug non-exposed controls in genetic studies involving comparisons with very rare diseases such as co-amoxiclav DILI which can be induced in a very small portion of users (< 1%) due to the very low likelihood that controls would ever develop the disease. Also, recruitment of controls for the study who had been prescribed co-amoxiclav and following them up would be costly and time-consuming and was not feasible given limited resources. However, achieving satisfactory statistical power to determine significant association does not necessarily rely on matching the comparable groups [20]. The data obtained were analyzed for its Odds Ratio (OR) value and Fisher's exact test was selected to generate a p-value and 95% Confidence Intervals (CIs) using GraphPad PRISM version 5.0.

RFLP-PCR Assay for KCNJ1

PCR-restriction fragment length polymorphism analysis was used for KCNJ1 (rs2855790) genotyping. Figure 1 shows the assay which was developed in Newcastle University laboratories using unique primers (5'-TCACTCACTTAACTGCCACG-3' and 5'-GAGGTGTTTCTCCTCTTACC-3', as forward and reverse primers, respectively). PCR cycling conditions used for KCNJ1 amplification were 35 cycles of 1 min at 94°C denaturation, 1 min at 54 °C annealing and 72 °C elongation for 1 min, followed by a 7 min extension at 72 °C resulting in 290 bp product. A 20 µl PCR product was digested overnight at 37 °C using 2 U of BseYI enzyme which was predicted to digest the common allele (C), whereas DNA fragments carrying the minor (T) allele remained uncut. When digested homozygous wild-type DNA was separated by electrophoresis on a 10% polyacrylamide gel, two fragments of 141 bp and 149 bp were predicted to be seen.

Results

The entire 4 SNPs identified by the GWAS study are located in one block (2nd block) on the KCNJ1 gene Haploview (Figure 2) which indicates the strength of LD

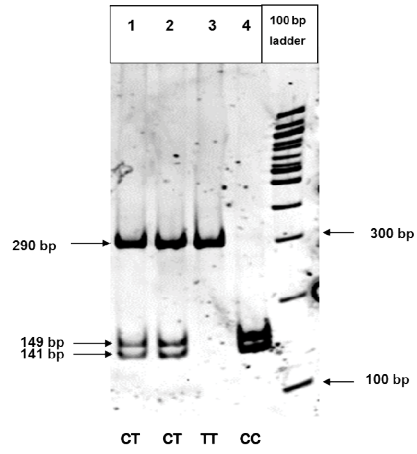

Figure 1: 10 % polyacrylamide gel showing KCNJ1 gene digested with BseYI enzyme. Lane 1 and 2 are heterozygous, lane 3 is a homozygous mutant, and lane 4 is homozygous wild-type.

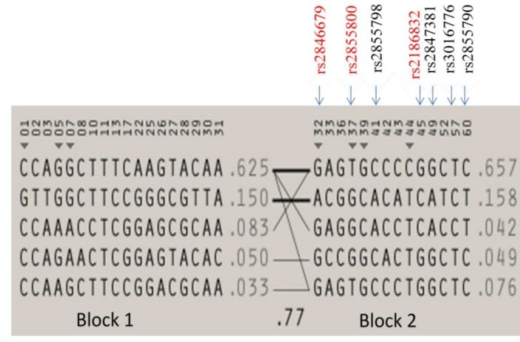

Figure 2: Block 1 and 2 from Haploview for the KCNJ1 gene showing 4 GWAS suggested SNPs in Black and 3 SNPs in Red associated with lower blood pressure. The numbers below the arrows indicate the SNP number on Haploview. SNP number 60 (rs2855790) was chosen to study in this project.

Table 2. KCNJ1 (rs2855790) genotyping results in co-amoxiclav DILI cases and two community controls

		Samples		
		Co-amoxiclav cases (n=73)	Community controls (n=75)	POPRES controls (n=282)
Genotypes	CC (%)	50 (68.5)	42 (56)	169 (60)
	CT (%)	23 (31.5)	29 (38.7)	104 (36.8)
	TT (%)	0	4 (5.3)	9 (3.2)
MAF (%)		15.8	24.7	20
P value		0.13		0.22
OR		0.58		0.67
95% CI		0.29-1.1		0.4-1.2

between variants. The genotyping results of the tested SNP (rs2855790 C/T) indicated a lower frequency of the variant allele (T) in cases (15.8%) than in controls (24.7%) however, the difference was not statistically significant (OR=0.58, 95% CI=0.29-1.1; p=0.13) (Table 2). Further comparison to a larger control group (POPRES, n=282) also failed to replicate the GWAS findings (p=0.22 for this study versus $p=3.7x10^{-5}$ for GWAS study).

Discussion

Multiple polymorphisms in KCNJ1 were previously detected as risk markers for several cardiovascular dysfunctions as a result of induced electrolyte imbalances. Tobin et al [21] found that 5 SNPs located in KCNJ1 showed significant associations with mean 24-hour systolic or diastolic blood pressure. The authors also reported an association between several SNPs in KCNJ1 and left ventricular mass as assessed by measuring the voltage signal on electrocardiograms (ECG). The effect was in the same direction as seen with BP and the strongest association noted was with rs675759. Brochard et al [22] have also detected ten rare mutations in the KCNJ1 gene associated with antenatal Bartter syndrome, type 2 (Hyperprostaglandin E syndrome), which is characterized by salt wasting, hypokalemic alkalosis, hypercalciuria, and low blood pressure. The relevance of KCNJ1 to a disease affecting the liver is slightly unclear but because of the relatively strong signal seen in the GWAS suggested by Lucena et al [14], it was decided to perform genotyping for the SNP showing the lowest p-value.

Using GWAS as a powerful tool to identify novel genetic associations with complex diseases and drug-induced toxicities is increasingly feasible. This valuable approach was able to detect a significant effect of HLA loci on the emergence of drug-related hepatic injury, particularly associations of HLA-B*57:01 with flucloxacillin DILI [23,24] and HLA-A*02:01 with co-amoxiclav hepatotoxicity [25]. This study has focused on non-HLA markers as candidates possibly affecting patients' response to co-amoxiclav. Genotyping co-amoxiclav DILI cases for KCNJ1 selected mutation showed no evidence for an association between the cases and the SNP rs2855790, despite a slightly lower variant allele frequency noticed in the DILI cases. This gene, which encodes the potassium channel known as ROMK, has a biological role in moderating renal function and seemed to have no impact on liver toxicities related to exposure to co-amoxiclav.

Conclusion

This gene was not a very specific biological target as a DILI gene given the biological function of KCNJ1 in the kidney, not the liver, so the achieved outcome in this study seems to be not too unusual.

Acknowledgment

I would like to express my sincere thanks to Ann Daly and Munir Pirmohamed for coordinating patient recruitment and adjudication. My gratitude goes to the late Pete Donaldson for providing the DNA of the control samples.

References

1. Farmer AD, Brind A. Drug-induced liver injury. *Medicine* 39: 536-540 (2011).
2. Padda MS, Sanchez M, Akhtar AJ, et al. Drug-induced cholestasis. *Hepatology* 53: 1377-1387 (2011).
3. Chang CY, Schiano TD. Review article: Drug hepatotoxicity. *Aliment Pharmacol Ther* 25: 1135-1151 (2007).
4. O'Donohue J, Oien KA, Donaldson P, et al. Co-amoxiclav jaundice: Clinical and histological features and HLA class II association. *Gut* 47: 717-720 (2000).
5. Donaldson PT, Daly AK, Henderson J, et al. Human leucocyte antigen class II genotype in susceptibility and resistance to co-amoxiclav-induced liver injury. *J Hepatol* 53: 1049-1053 (2010).
6. Andrade RJ, Lucena MI, Fernández MC, et al. Drug-induced liver injury: An analysis of 461 incidences submitted to the Spanish registry over a 10-year period. *Gastroenterology* 129: 512-521 (2005).
7. Chalasani N, Fontana RJ, Bonkovsky HL, et al. Causes, clinical features, and outcomes from a prospective study of drug-induced liver injury in the United States. *Gastroenterology* 135: 1924-1934 (2008).
8. Hsu YH, Xu X, Jeong S. Genetic determinants and pharmacogenetics of osteoporosis and osteoporotic fracture. *Osteoporosis.* 91: 485-506 (2020).
9. Szostak B, Machaj F, Rosik J, et al. Using pharmacogenetics to predict methotrexate response in rheumatoid arthritis patients. *Expert Opin Drug Metab Toxicol* 16: 617-626 (2020).
10. Kan M, Himes BE. Genetics and pharmacogenetics of asthma. Precision in Pulmonary, Critical Care, and Sleep Medicine. *Respiratory Med* 54: 25-37 (2020).
11. Singer JB, Lewitzky S, Leroy E, et al. A genome-wide study identifies HLA alleles associated with lumiracoxib-related liver injury. *Nat Genet* 42: 711-714 (2010).
12. Hautekeete ML, Horsmans Y, Van Waeyenberge C, et al. HLA association of amoxicillin-clavulanate--induced hepatitis. *Gastroenterology* 117: 1181-1186 (1999).
13. Andrade RJ, Lucena MI, Alonso A, et al. HLA class II genotype influences the type of liver injury in drug-induced idiosyncratic liver disease. *Hepatology* 39: 1603-1612 (2004).
14. Lucena MI, Molokhia M, Shen Y, et al. Susceptibility to amoxicillin-clavulanate-induced liver injury is influenced by multiple HLA class I and II alleles. *Gastroenterology* 141: 338-347 (2011).
15. Alshabeeb MA, Aithal GP, Daly AK. Investigation of oxidative stress-related candidate genes as risk factors for drug-induced liver injury due to co-amoxiclav. *DNA Cell Biol* 39: 349-354 (2020).
16. Benichou C. Criteria of drug-induced liver disorders: Report of an international consensus meeting. *J Hepatol* 11: 272-276 (1990).
17. Aithal GP, Watkins PB, Andrade RJ, et al. Case definition and phenotype standardization in drug-induced liver injury. *Clin Pharmacol Ther* 89: 806-815 (2011).
18. Daly AK, Fairbrother KS, Andreassen OA, et al. Characterization and PCR-based detection of two different hybrid CYP2D7P/CYP2D6 alleles associated with the poor metabolizer phenotype. *Pharmacogenetics* 6: 319-328 (1996).
19. Velaga MR, V Wilson, Jennings CE, et al. The codon 620 tryptophan allele of the lymphoid tyrosine phosphatase (LYP) gene is a major determinant of Graves' disease. *J Clin Endocrinol Metabol* 89: 5862-5865 (2004).
20. Nelson MR, Bryc K, King KS, et al. The Population Reference Sample, POPRES: A resource for population, disease, and pharmacological genetics research. *Am J Hum Genet* 83: 347-358 (2008).
21. Tobin MD, Tomaszewski M, Braund PS, et al. Common variants in genes underlying monogenic hypertension and hypotension and blood pressure in the general population. *Hypertension* 51: 1658-1664 (2008).
22. Brochard K, Boyer O, Blanchard A, et al. Phenotype-genotype correlation in antenatal and neonatal variants of Bartter syndrome. *Eur Renal Assoc* 24: 1455-1464 (2009).
23. Daly AK, Day CP. Genetic association studies in drug-induced liver injury. *Semin Liver Dis* 29: 400-411 (2009).
24. Daly AK, Donaldson PT, Bhatnagar P, et al. HLA-B*5701 genotype is a major determinant of drug-induced liver injury due to flucloxacillin. *Nat Genet* 41: 816-819 (2009).
25. Ma Q, Yang W, Wang L, et al. Research advances in the association of drug-induced liver injury with polymorphisms in human leukocyte antigen. *Int Immunopharmacol* 81: 106037 (2020).