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Revisiting the association of HLA alleles and haplotypes with *CYP21A2* mutations in a large cohort of patients with congenital adrenal hyperplasia

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Abstract

The *CYP21A2* gene encoding 21-hydroxylase is on chromosome 6p21.3 within the human leukocyte antigen (HLA) class III major histocompatibility complex and an association between congenital adrenal hyperplasia (CAH) due to 21-hydroxylase deficiency and HLA class I and II alleles has been shown in genetically isolated populations. One-third of CAH causing alleles are 30-kb deletions due to homologous recombination events between active and pseudogenes resulting in chimeric genes. The aim of this study was to re-visit the association between the *CYP21A2* variants and HLA polymorphisms in a large ethnically diverse cohort of patients with CAH who underwent comprehensive *CYP21A2* genotyping, including specification of chimeric gene subtypes (CAH CH-1 through CH-9 of *CYP21A1P/CYP21A2* chimeras; CAH-X CH-1 through CH-3 of *TNXA/TNXB* chimeras) in alleles with 30-kb deletions. The study population included 201 patients (86 males, 115 females, age 3–75 years) with CAH due to 21-hydroxylase deficiency (159 classic, 42 nonclassic) and 194 parents. Based on the availability of parental genotype, we determined the haplotypes of *CYP21A2* mutations and HLA types in 95 probands (190 alleles). Five prevalent haplotype associations were found: p.V281L and B*14-C*08 (P <0.0001); p.I172N and DQB1*03 (P = 0.035); and of the chimeric genes caused by 30-kb deletions: CH-1 and A*03 (P =0.033); CH-5 and C*06-DRB1*07 (P < 0.0001); and CAH-X CH-1 and DQB1*03 (P = 0.004). Our findings show that a number of associations between HLA alleles and haplotypes and *CYP21A2* mutations, including large 30-kb deletions, exist commonly across ethnicities. These HLA associations may have clinical implications for patients with CAH and may provide insight into the genetics of this highly complex region of the human genome.

1. Introduction

Congenital adrenal hyperplasia (CAH) is a group of autosomal recessive disorders that impair cortisol biosynthesis. About 95% of CAH cases are caused by a deficiency in the 21-hydroxylase enzyme, encoded by *CYP21A2* gene (OMIM 613815). Impairment of this enzyme causes alterations in cortisol, aldosterone and androgen biosynthesis in the

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steroidogenesis pathway (El-Maouche et al., 2017). The severe or classic form of CAH, characterized by excess androgen, severe cortisol deficiency with or without aldosterone deficiency, is estimated to occur in 1 in 10,000 to 1:20,000 births, is potentially life-threatening, and is part of the newborn screen in the United States and over 40 countries worldwide (Speiser et al., 2010). A mild or nonclassic form of CAH, characterized by mostly excess androgen, is estimated to be one of the most common autosomal recessive conditions affecting up to 1 in 200 Caucasians and may be asymptomatic (Hannah-Shmouni F, Genetics in Medicine 2017).

In 1977, genetic linkage between the human leukocyte antigen (HLA) complex and 21-hydroxylase deficiency was reported, providing a potential method for prenatal diagnosis of a CAH affected fetus (Dupont et al., 1977). In 1985, the *CYP21A2* gene was identified on chromosome 6p21.3 within the HLA class III major histocompatibility (MHC) complex as encoding 21-hydroxylase (White et al., 1985). Building off of this pre-established relationship, some studies have documented specific *CYP21A2* mutations to be associated with particular HLA alleles and haplotypes in genetically isolated populations. The most commonly reported HLA association amongst CAH patients has been the association between the nonclassic CAH mutation, p.V281L, and HLA-B14 in Ashkenazi Jewish, Hispanic, Italian, and Croatian subjects. (Pollack et al., 1981; Speiser et al., 1985; Israel et al., 2006; Grubic et al., 2016).

The human genome assembly GRCh38/hg38 maps *CYP21A2* with 7 other genes in tandem: *STK19-C4A-CYP21A1P-TNXA-STK19P-C4B-CYP21A2-TNXB*. *STK19* encodes serine/threonine kinase 19, *C4A* and *C4B* encode two isotopes of complement component 4 and *TNXB* encodes tenascin-X, an extracellular matrix protein. *STK19P*, *CYP21A1P* and *TNXA* are pseudogenes, highly homologous to their functional gene counterparts that make this region error prone, especially during meiosis. In fact, the majority of causative *CYP21A2* mutations originate from *CYP21A1P* due to gene conversion events (Speiser et al., 2010). The meiotic errors also result in 30-kb deletions that lead to pathogenic *CYP21A1P/CYP21A2* or *TNXA/TNXB* chimeric genes. These large deletions account for approximately one-third of CAH alleles (White et al., 1988; Stikkelbroeck et al., 2003; Vrzalova et al., 2011) and have been further classified into 9 subtypes of *CYP21A1P/CYP21A2* chimeras (CH-1 through CH-9) (Chen et al., 2012) and 3 subtypes of *TNXA/TNXB* chimeras (CAH-X CH-1 through CH-3) (Chen et al., 2016). In the case of *TNXA/TNXB* chimeras, the loss of *CYP21A2* extends to affect the 3' terminus of *TNXB*, resulting in a contiguous gene deletion syndrome termed CAH-X (Merke et al., 2013). Patients with CAH-X have an autosomal dominant connective tissue disorder, Ehlers-Danlos Syndrome, in addition to CAH. Given that chimeras are a significant index of the meiotic errors and sometimes highly relate to the CAH phenotype (e.g. CH-4, CH-9 correspond to a mild nonclassic CAH phenotype and CAH-X chimeras result in a CAH-X phenotype), determination of the chimera subtype by junction site analysis in CAH genetic tests may be clinically meaningful (Chen et al., 2012; Morissette et al., 2015). However, the specific type of chimera is not routinely identified in *CYP21A2* genotyping.

In this study, we re-visit the association between *CYP21A2* mutations and HLA polymorphisms in a large ethnically diverse population of patients with CAH due to 21-

hydroxylase deficiency who underwent comprehensive *CYP21A2* genotyping, including junction site analysis of 30-kb deletions.

2. Materials and methods

2.1 Study population and genotyping

We investigated 201 unrelated patients (86 males, 115 females, age 3–75 years) with CAH due to 21-hydroxylase deficiency (159 classic, 42 nonclassic) and 194 parents enrolled in a Natural History Study at the National Institutes of Health Clinical Center in Bethesda, MD (clinical trials no.). Written informed consent was obtained from all participants and a parent of participating children.

Genomic DNA was isolated from blood samples obtained from peripheral blood samples, and *CYP21A2* genotyping was performed as previously described (Finkielstain et al., 2011; Chen et al., 2012). DNA was extracted (Gentra Puregene kit; QIAGEN, Valencia, CA) and analyzed for the 12 most common *CYP21A2* mutations (p.P30L, IVS2–13A/C>G, exon 3, 8 bp deletion (p.G110Efs), p.I172N, exon 6 cluster (p.I236N, p.V237E, p.M239K), p.V281L, p.Leu307fs, p.Q318X, p.R356W, and p.P453S) (Esoterix Laboratories, Calabasas Hills, CA). The 30-kb deletions and junction sites of chimeras, as well as rare mutations, were determined by a validated Sanger sequencing (Prevention Genetics, Marshfield, WI) (Chen et al., 2012).

All subjects were studied to determine the distribution of HLA alleles and haplotypes. HLA haplotypes were unambiguously determined by family genetic segregation analysis where sufficient data was available. Genomic DNA was isolated from peripheral blood using the Qiagen BioRobot EZ1 and associated DNA isolation kits (Qiagen, Valencia, CA). Intermediate/high resolution typing for both Class I (A*/B*/C*) and Class II loci (DRB1*/DQB1*/DRB345) was performed using LABType sequence-specific oligonucleotide (SSO) Typing Kits obtained from One Lamba, Inc (Canoga Park, CA, USA) and a Luminex 100 analyzer (Luminex Corp, Austin, TX, USA). Some samples were further tested by sequence-based typing methodology (AlleleSEQR Sequenced Based Typing Kits, Atria Genetics, Hayward, CA, USA) for confirmation or to resolve ambiguities. The reaction products were analyzed on an Applied Biosystems (ABI) Prism* 3730xL DNA Analyzer (Adams et. al., 2005). While we did not have sufficient data to separate HLA haplotypes and CAH mutations by allele in all patients, we were able to separate out allele specific associations for many of the patients (n=95 patients, 190 alleles).

2.2 Statistical analysis

We analyzed HLA alleles among subjects in relation to *CYP21A2* mutations and evaluated allele-specific associations for those patients with parental data when possible. HLA type allele frequencies were calculated by directly counting individual alleles and comparisons were made by evaluating HLA type frequencies between CAH patients of a particular *CYP21A2* mutation and the rest of the CAH cohort. All statistical analysis was completed using the SPSS Statistics software package (IBM SPSS Statistics for Windows, Version

25.0. Armonk, NY). The *P*-values were calculated by the Chi-squared test or Fisher's exact test. A *P*-value of <0.05 was considered statistically significant.

3. Results

A variety of *CYP21A2* mutations were observed in our cohort (Figure 1). Overall, 131 (32.6%) alleles contained a 30-kb deletion, and 46 (11.4%) alleles carried the most common mutation responsible for nonclassic CAH, p.V281L. Our cohort was ethnically diverse (Figure 2) and no associations were found between ethnicity and HLA type or ethnicity and *CYP21A2* mutation. The reported ethnicities of our patients carrying the p.V281L mutation consisted of 25% Ashkenazi Jews, 9.1% Hispanics, 6.8% Italians, and the remainder with mixed ethnicities..

Based on the availability of parental genotype, we determined the haplotypes of *CYP21A2* mutations and HLA types in 95 probands. Amongst those 190 alleles, several prevalent haplotypes of *CYP21A2* mutations and HLA types were identified: p.V281L-HLA B*14-C*08 haplotype accounted for 67.3% (35/52) of the p.V281L alleles ($P<0.0001$); p.I172N-HLA DQB1*03 haplotype accounted for 39.3% (11/28) of the p.I172N alleles ($P=0.035$); CAH CH-1-HLA A*03, CAH CH-5-HLA C*06-DRB1*07 and CAH-X CH-1-HLA DQB1*03 haplotypes accounted for 41.2% (7/17, $P=0.033$), 76% (19/25, $P<0.0001$) and 58.3% (7/12, $P=0.004$) of the respective chimeric alleles (Figure 3). The allele frequency of each listed HLA type in general population, as calculated from an open HLA database (Gonzalez-Galarza et al., 2015), is 5.0% for B*14 (n=3,416,022), 3.8% for C*08 (n=124,132), 34.2% for DQB1*03 (n=87,254), 9.3% for A*03 (n=3,469,439), 9.0% for C*06 (n=122,341) and 12.8% for DRB1*07 (n=3,480,448).

Similar linkage disequilibrium patterns were observed when evaluating the entire cohort: 88.6% (39/44) of the probands with p.V281L mutation had an HLA B*14-C*08 haplotype ($P<0.0001$); 50% (20/40) of the probands with p.I172N mutation had an HLA DQB*03 allele ($P=0.01$); 69% (20/29) of probands with the CAH CH-1 chimera had an HLA A*03 allele ($P<0.001$); 83.8% (31/37) probands with the CAH CH-5 chimera had an HLA C*06-DRB1*07 haplotype ($P<0.0001$); 80.0% (12/15) probands with the CAH-X CH-1 chimera had an HLA DQB1*03 haplotype ($P = 0.002$).

4. Discussion

In our large cohort of ethnically diverse patients with CAH and comprehensive *CYP21A2* mutation analysis, we describe newly identified associations between *CYP21A2* and class I and II HLA alleles. The *CYP21A2* gene is located within the class III cluster of the MHC complex, while class I and class II HLA antigens play a central role in activating T-lymphocytes and are essential in adaptive immune responses. Our findings of significant associations between two point mutations and three types of chimeric 30-kb deletions with specific HLA alleles might have clinical implications and may provide insight into the complex genetics of the HLA region of the human genome.

The HLA complex was first found to be associated with alterations in the biosynthesis of steroid hormones in 1977. This initial study reported that the unknown affected gene

associated with CAH segregated with HLA types in three families. Based on a lod-score analysis, the CAH trait was closely linked with HLA and that the CAH trait was likely closer to the HLA-B locus than to the HLA-A locus (Dupont et al., 1977). Following the discovery of the *CYP21A2* gene on chromosome 6p21.3 within the HLA complex in 1985, some studies have found associations between specific *CYP21A2* mutations and HLA types. The strongest finding to date has been the association between the commonly found p.V281L nonclassic CAH mutation and HLA-B*14 (Speiser et al., 1985; Israel et al., 2006; Grubic et al., 2016).

Historically, the HLA-B*14 allele was found to be in genetic linkage disequilibrium with nonclassic CAH in a population of Ashkenazi Jews, Hispanics, and Italians (Speiser et al., 1985). Later studies confirmed an association between p.V281L, and the HLA-B*14 allele in Ashkenazi Jews, non-Ashkenazi Jews, Croatians, and Turks (Yarman et al., 2004; Israel et al., 2006; Grubic et al., 2016). In accordance with these findings, we found an association between p.V281L and the extended haplotype HLA-B*14-C*08 in our ethnically diverse population of CAH patients.

Although this has not been described previously in a CAH population, this is not surprising as population studies have shown the B*14 allele to be in linkage disequilibrium with the C*08 allele (Grubic et al., 2016). The implications of the HLA-B*14-C*08 HLA haplotype are unknown, but interestingly, this haplotype has been associated with hypersensitivity reactions to nevirapine, a non-nucleoside reverse transcriptase inhibitor used in the treatment of HIV (Littera et al., 2006).

In our cohort, we found an association between the point mutation, p.I172N, and the HLA-DQB1*03 allele. The DQB1*03 allele has been potentially associated with a variety of other disease susceptibilities and protections. In a Slovak population study, this allele was found to be protective against the development of multiple sclerosis (Michalik et al., 2015). An Israeli study observed that patients with lichen planopilaris expressed a significantly higher frequency of the DQB1*03 allele (Pavlovsky et al., 2015). Finally, an association between the DQB1*03 allele and a possible susceptibility to chronic Hepatitis C was reported in Japan (Miki et al., 2013).

HLA associations with the various *CYP21A1P/CYP21A2* and *TNXA/TNXB* chimeras have not been previously evaluated. In our study, we found HLA associations with three different chimeric genes, including CH-1 and CH-5, the two most common *CYP21A1P/CYP21A2* chimeras that are created through homologous recombination events (Chen et al., 2012). The *CYP21A1P/CYP21A2* CH-1 chimera (junction site p.G110fs ^ p.I172N) was found to be associated with the HLA-A*03 allele. The A*03 allele has been found to have a potential protective role in HIV-1 infection and viremia control in a Chinese population (Zhang et al., 2013). The *CYP21A1P/CYP21A2* CH-5 chimera (junction site p. L307fs ^ p.Q318X) was significantly associated with the HLA-C*06-DRB1*07 haplotype. The HLA-C*06-DRB1*07 haplotype has been found to be associated with less severe joint disease in patients who have psoriatic arthritis (Ho et al., 2007). The individual alleles that make up this haplotype have also been linked with certain disease susceptibilities. The HLA-C*06 allele, has been associated with a predisposition towards psoriasis in a Slovak population

(Shawkatova et al., 2013) and in South Indian Tamils (Indhumathi et al., 2016), and the DRB1*07 allele has been associated with type II autoimmune hepatitis (Baharlou et al., 2016).

Finally, we found an association between a CAH-X chimera and one particular HLA allele. This *TNXA/TNXB* chimera involves a deletion in *TNXB*, which continues into the neighboring *CYP21A2*, causing a contiguous gene deletion syndrome termed CAH-X (Merke et al., 2013). Patients with CAH and heterozygosity for a *TNXB* deletion have hypermobility-type Ehlers Danlos syndrome with variable symptoms of a connective tissue dysplasia (Miller and Merke, 2018). Our present study discovered an association between the CAH-X chimera type, CAH-X CH-1 (junction site *TNXB* exon 32 ^ exon 35 c. 11435_11524+30 del), and the HLA-DQB1*03. The association between CAH-X CH-1 and HLA-DQB1*03 finding is particularly interesting not only because it is the first discovery of a CAH-X chimera being linked with an HLA allele, but also because this particular HLA allele, DQB1*03, was also associated with a different *CYP21A2* mutation, p.I172N.

The major strength of our study was the comprehensive genotyping performed which included junction site analysis of 30-kb deletions. This allowed us to investigate possible HLA associations with various types of *CYP21A2* mutations, including chimeric genes. In addition, a major strength was the large cohort size and ethnic diversity. Previous studies have evaluated the relationship between HLA type and CAH within the confines of genetically isolated populations. In our study, strong associations were found, despite the diversity of our population. Although we were able to perform comprehensive genotyping, one shortcoming was our inability to segregate specific genotypes to specific alleles for our entire cohort due to a lack of parental data on every subject.

In conclusion, we identified a variety of associations between two *CYP21A2* point mutations and three chimeric genes with various HLA alleles and haplotypes. These findings were not limited to a single population as our cohort consisted of subjects of mixed ethnic backgrounds. Future studies looking at the link between *CYP21A2* mutations and HLA alleles and haplotypes are needed to determine the clinical implications and could provide insight into complexities of the major histocompatibility complex.

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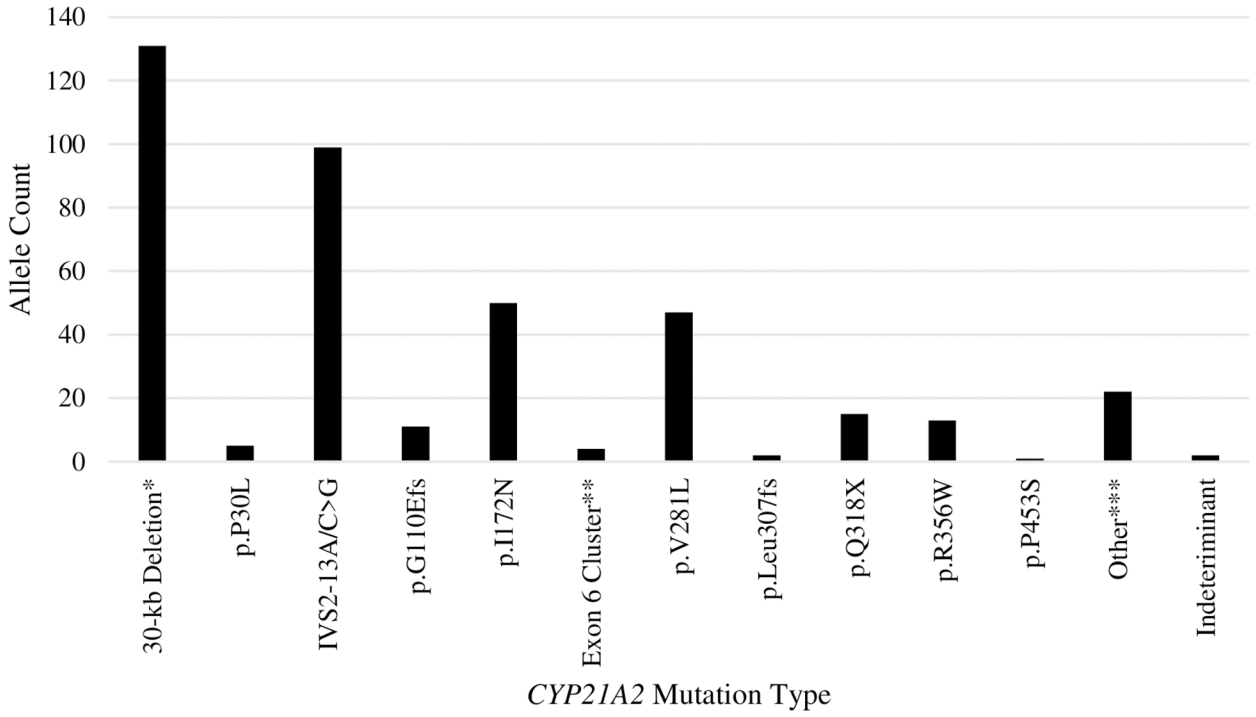

Fig. 1. Distribution of commonly found *CYP21A2* mutations in a cohort of patients with CAH due to 21-hydroxylase deficiency.

*30-kb deletion includes *CYP21A1P/CYP21A2* chimeras: CH-1 (24.4%), CH-3 (2.3%), CH-4 (2.3%), CH-5 (34.4%), CH-8 (6.1%), CH-9 (0.8%),CH-10 (0.8%); and *TNXA/TNXB* chimeras: CAH-X CH-1(11.5%), CAH-X CH-2 (5.3%), and undetermined (12.2%.)

**Exon 6 cluster: p.I236N, p.V237E, and p.M239K.

***Other includes the following rare mutations, IVS8+1G>A, p.W405X, p.C423fs, p.Y98D, p.R426P, p.Cys30fs, p.H120R, p.M284V, p.E351K, p.S170fs, p.R483fs, p.G464fs, p.H365Y, p.W405X, p.F306+1nt, p.R408H, and p.C423fs.

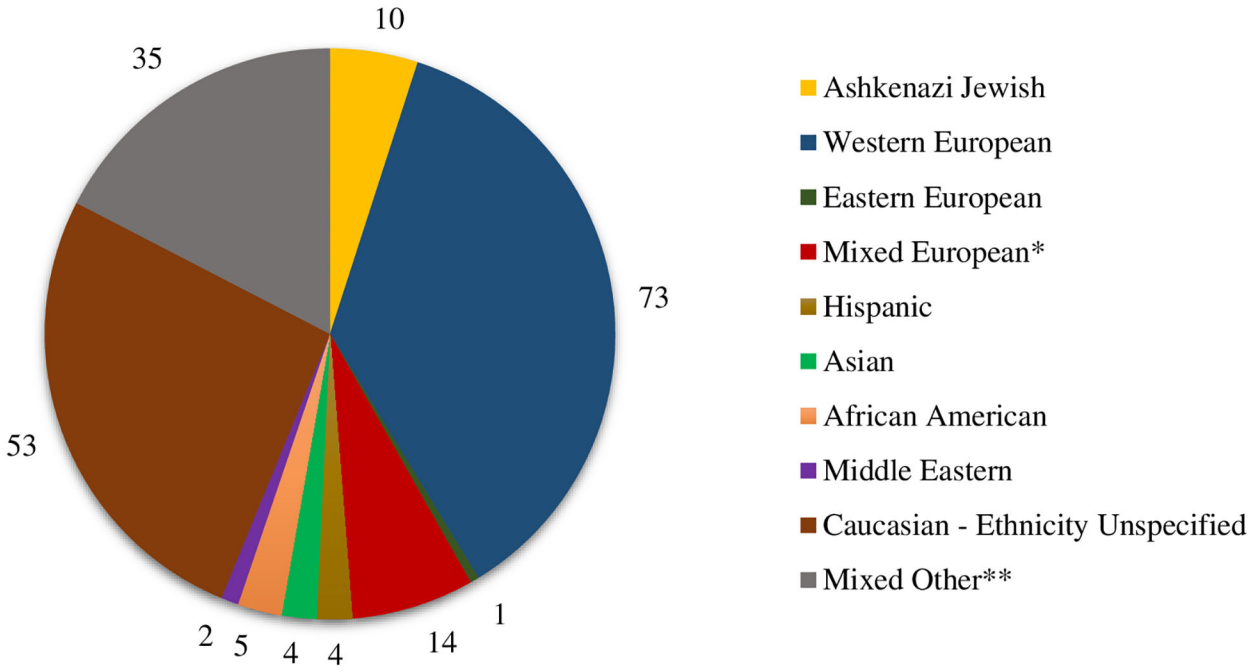

Fig. 2. Diverse ethnicity in a cohort of 201 unrelated patients with congenital adrenal hyperplasia due to 21-hydroxylase deficiency.

*Mixed European includes both Western and Eastern European ethnicities

**Mixed Other includes subjects with two or more unrelated ethnicities

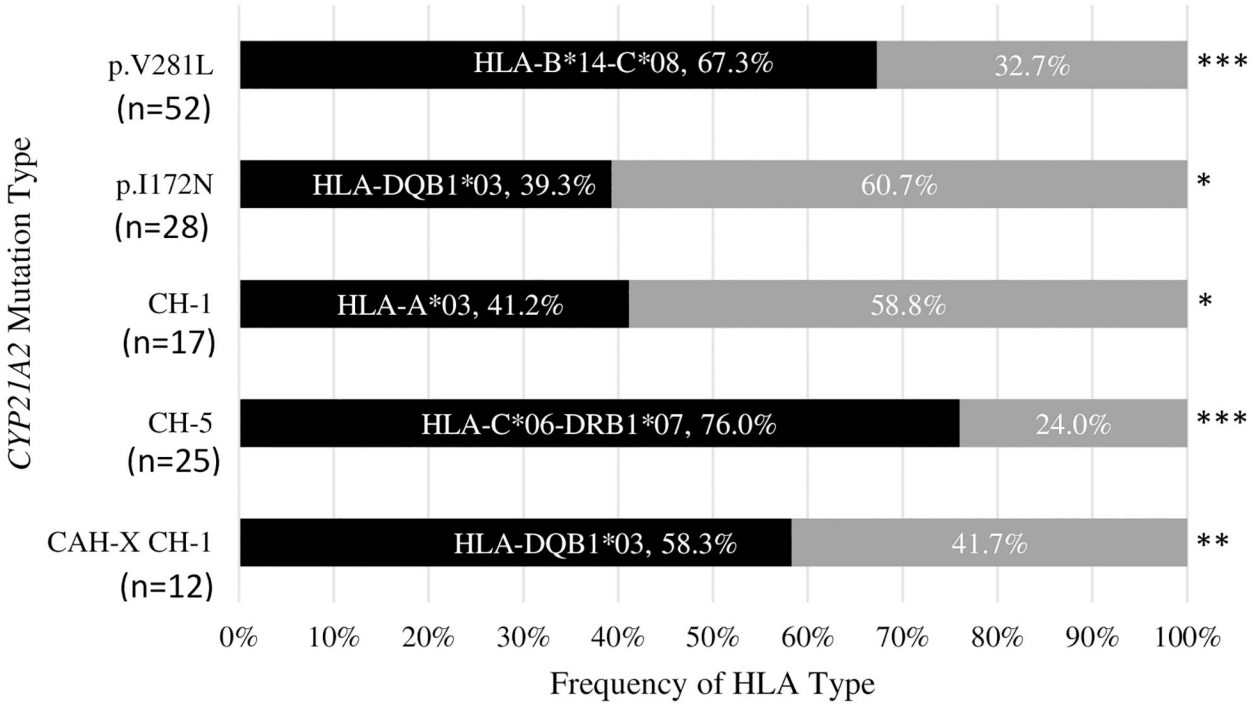

Fig. 3.

HLA type and haplotype frequencies amongst *CYP21A2* mutation alleles. Significant associations are shown. Total number (n) of each indicated *CYP21A2* mutation allele is shown.***p <0.0001; **p <0.01; *p <0.05.