Polymorphism

RESEARCH

Gene-gene interactions of *CYP2D6 (*2, *4, *10)* and *GST (T1, M1, P1)* variants in essential hypertensive *Jat Sikh* patients

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ABSTRACT

Objective: To investigate interactions, if any, among six functional SNPs of metabolic genes viz. *CYP2D6 (*2, *4, *10)* and *GST (T1, M1, P1)* in essential hypertensive *Jat Sikh* north Indian patients.

Methods: Unrelated 200 essential hypertensive patients and 200 normotensive healthy individuals were genotyped for *CYP2D6 (*2, *4, *10)* and *GST* (T1, M1, P1; 313A>G) polymorphisms using PCR-RFLP analysis. Association of disease-risk with SNPs was ascertained by logistic regression analysis. High order gene–interactions were ascertained by performing multifactor dimensionality reduction (MDR) and classification and regression tree (CART) analyses.

Results: The patient and control groups differed significantly in the genotype frequencies of *GSTP1* polymorphisms ($p<0.0001$). The crude odds ratio analysis divulged that individuals with the heterozygous genotypes in *CYP2D6*4* ($p=0.0280$), *10 ($p=0.0002$) and *GSTP1* ($p=0.0001$) genes have 1.60 to 4.50 folds, and those with the homozygous mutant genotypes in *CYP2D6*4* ($p=0.0019$) and *GSTP1* ($p=0.0001$) genes have3.21-7.02 folds likelihood for hypertension. MDR analysis revealed the best predictive epistatic interaction among *CYP2D6*4*, *10 and *GSTP1* SNPs for disease as 24% patients were heterozygous for these genotypes (OR=7.3889; 95% CI= 4.7417-11.5141). The decision tree by CART analysis further revealed *GSTP1* as a major predictor for hypertension risk.

Conclusion: Interactions of heterozygous genotypes of *CYP2D6*4*, *10 and *GSTP1* were revealed as significantly contributing towards hypertension with *GSTP1* as a major predictor for hypertension risk in *Jat Sikh* patients.

KEYWORDS: Hypertension, Jat Sikh, CART, metabolic genotypes, Epistatic interactions.

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INTRODUCTION

The complex interplay of gene-gene and gene-environmental interactions is challenging for the understanding of complex diseases, but which, if deciphered, can have clinical importance in diagnosis, prognosis and treatment of the disease. An important disease condition is essential hypertension, a complex multifaceted chronic disease which requires deeper understanding because although often symptomless (Baradaran *et al.*, 2010), it is a major risk for cardiovascular, renovascular and cerebrovascular diseases (Drozdz and Kawecka-Jaszcz, 2014; Yuan*et al.*, 2017). Some of the well-known pathological factors leading to hypertension are vasculature abnormalities, endothelial dysfunction, vascular remodeling and increased oxidative stress (Sun, 2015; Cunha *et al.*, 2017; Oparil *et al.*, 2018), which are modulated by a complex interplay of many environmental and genetic factors. Besides the lifestyle, nearly 837 genes and 255 single nucleotide polymorphisms (SNPs) are associated with hypertension (Dai *et al.*, 2013). Of these, the predispositional genes include Angiotensinogen (*AGT*), Angiotensin I converting enzyme (*ACE*), Angiotensiogen II receptor subtype I (*AGTRI*), Alpha -I- Antichymotrypsin (*ACT* or *SERPINA3*) (Whitfield *et al.*, 2009). Others are important as components of Renin-angiotensin system (RAS), which plays a key role in vascular homeostasis. The angiotensin I converting enzyme (ACE) is also involved in the therapeutic management of hypertension (Tchelougou et al., 2015). The modifiable risk factors, primarily lifestyle (alcohol intake, smoking habits), excess salt intake, being overweight, obesity and physical inactivity (Pilakkadavath and Shaffi, 2016; Arora *et al.*, 2017) in combination with genetic variability and the non-modifiable factors of age, gender, family history and ethnicity can strongly predispose an individual to the risk of developing hypertension. Increased oxidative stress has an important pathophysiological role in the development of hypertension (Rodrigo *et al.*, 2016) and although not exhaustively explored, alterations in the metabolizing enzyme detoxification pathways could be significant co-players in disease-condition. In fact, disease-propensity can be influenced by metabolic genotypes (Ma *et al.*, 2011). Among these variants of the metabolic genotypes of Cytochrome P450 (http://www.ncbi.nlm.nih.gov/gene/1565 accessed on March 21, 2021) and Glutathione S–Transferase (http://www.ncbi.nlm.nih.gov/gene/2950 accessed on March 21, 2021) have shown an association with hypertension, probably from ineffective homeostasis and reduced/altered free radical scavenging activities, while treatment modalities can also be affected from inter-individual variation to drug response by the *CYP2D6* and *GST* gene polymorphisms. The cytochrome CYP2D6 enzyme is one the seven members of the CYP450 family of monooxygenases involved in the metabolism of more than 25% of drugs and environmental and endogenous substances (Zhou *et al.*, 2008; Zhou *et al.*, 2009; Zanger and Schwab 2013) and its activity is maintained by *CYP2D6* genetic variants (Ingelman-Sundberg *et al.*, 2007). According to the Pharmacogene Variation (PharmVar) Consortium the *CYP2D6* gene (Chr22q13.1) has 100 allelic variants (Gaedigk *et al.*, 2018). The Glutathione-S-transferase multigene family of metabolic enzymes carry out detoxification of endogenous and exogenous electrophilic compounds, by making them water-soluble and favouring their elimination (Hayes *et al.*, 2005). In *GST* gene cluster there are eight gene classes (alpha, Kappa, mu, omega, pi, sigma, theta and zeta (http://www.ncbi.nlm.nih.gov/gene/2950 accessed on March 19, 2021), of these *GSTA1* (6p12), *GSTT1* (22q11.2), *GSTM1* (1p13.3) and *GSTP1* (11q13) gene variants are highly polymorphic and confer differential enzyme activity, ranging from reduced

activity to complete loss-of-activity (Matic *et al.*, 2013).

Documentations in literature on the metabolic genotypes of *CYP2D6* and *GST* studied in relation to hypertension are sparse from this region. But antihypertensive effect of drug-therapy in relation to *CYP2D6* polymorphism has been widely studied in hypertensive patients (Bijl *et al.*, 2009; Blake *et al.*, 2013; Ayyappadhas *et al.*, 2015; Wu *et al.*, 2015; Chen *et al.*, 2018). *CYP2D6* polymorphisms have also been documented in different population sub-groups (Teh and Bertilsson, 2012) but only one study has come to attention, directly relating *CYP2D6* polymorphism with hypertension (Chen *et al.*, 2018). In the case of *GST* polymorphisms, a large number of studies exist on association with hypertension (Teh and Bertilsson, 2012; Dhameja *et al.*, 2013; Ge *et al.*, 2015; Han *et al.*, 2015) but on meta-analysis, inconsistent results have emerged (Ge *et al.*, 2015; Rong *et al.*, 2019). Considering sparse reports from north India and to avoid bias from population stratification, in view of the prevalence of hypertension in the state of *Punjab* even in the rural areas, the present case-control study investigated interactions between genetic variants of *CYP2D6* (**2, *4* and **10*) and *GSTT1, GSTM1* and *GSTP1* for hypertension risk in *Jat Sikh* hypertensive patients (n=200) and normotensive (n=200) healthy participants (controls) from rural areas of Amritsar district (31°38'11.8"N, 74°52'29.14"E). Gene-gene interactions of these six genetic variants for association analysis for risk to hypertension were statistically analyzed. Multifactor dimensionality reduction (MDR) is an effective non-parametric statistical method for detecting at-risk gene-gene interactions in causing diseases (Li *et al.*, 2016) by considering the ratio between the percentage of cases in each genotype combination and percentage of controls in genotype combination. The classification and regression tree (CART) analysis was performed to study further combinational effect of genes. CART analyses the interaction of factors for a particular trait based upon explanatory power and variance (Breiman *et al.*, 1984).

MATERIALS & METHODS

Study design- The case-control study design was adopted to directly compare differences in the polymorphic nature of *CYP2D6 (*2, *4, *10)* and *GST (T1, M1, P1)* genes in essential hypertensive patients and normal control groups to find an association, if any, with hypertension. The study was carried out under informed consent after approval from the Institutional Ethics Committee (IEC) of Guru Nanak Dev University, Amritsar.

Sample size calculation- The statistical validity of results is ensured by appropriate sample size. The sample size for the present study was calculated (Power calculated for various gene variants= 86.25%) based upon the global minor allele frequencies of these SNPs (www.snpedia.com), which gave the range of 131 to 177 and therefore, 200 patients and 200 healthy participants were considered sufficient for the study.

Study Group- After written informed consent a total of 400 unrelated participants belonging to *Punjabi Jat Sikh* population sub-group from rural areas of Amritsar district of *Punjab* were included in the study. The inclusion criteria of patient group (n=200) were: more than 40 years of age, physician diagnosed essential hypertensive patients and those on mono drug therapy (atenolol-a beta blocker). Age-, sex-, socioeconomic status- and area-matched healthy normotensive adults belonging to the same population sub-group comprised the control group. Participants belonging to other sub-groups or those having secondary hypertension, cardiovascular, renal or cerebrovascular complications and patients on antihypertensive treatment other than with atenolol, were excluded from the study. Patients were contacted from the local hospitals and controls from the general population.

Demographic and disease related information was recorded on a predesigned questionnaire. General obesity (Body Mass Index, BMI) was determined considering height and weight measurements taken using standard methodology (Weiner and Lourie, 1981), and for DNA isolation 2ml of intravenous blood was drawn from each participant into vials containing the anticoagulant, ethylene diamine tetra acetic acid (EDTA). The samples were transported to the laboratory on ice.

Amplification of DNA- Genomic DNA was isolated by the organic method (Gill et al., 1987) with minor modifications. Quantity and quality of DNA was checked on 2% agarose gel and samples having high molecular weight genomic DNA was used for amplification. *CYP2D6*2*, *CYP2D6*4*, *CYP2D6*10* and of *GST P1* (rs1695) polymorphisms were detected by PCR-RFLP method; the *GSTT1* and *GSTM1* variants were determined by multiplex PCR. The details of primers used for amplification and references of methods followed are given in Table 1.

Table 1: Amplification details of *CYP2D6 (*2, *4* and **10)* and (*GSTT1, M1* and *P1*)

Gene variant	Primer sequence	Method of detection	Reference
GSTT1M1	For *GST* T1 F 5'TTCCTTACTGGTCCTCACATCTC 3' R 5'TCACCGGATCATGGCCAGCA3'	Multiplex PCR	Girisha *et al.*, 2004
	For *GST* M1 F 5'GAACTCCCTGAAAAGCTAAAGC3' R5' GTTGGGCTCAAATATACGGTGG-3'		
	Internal Control F 5'TGCCAAGTGGAGCACCCAA3' R 5' GCATCTTGCTCTGTGCAGATT3'		
GSTP1 (rs 1695)	F 5' ACCCCAGGGCTCTATGGGAA3' R5'TGAGGGCACAAGAAGCCCC3'	PCR-RFLP	Theophilius *et al.*, 2006
*CYP2D6*2*	F5'-GCTGGGGCCTGAGACTT3' R5'-GGCTATCACCAGGTGCTGGTGCT3'		
*CYP2D6*4*	F5'TGCCGCCTTCGCCAACCACT3' R5'TCGCCCTGCAGAGACTCCTC3'		
*CYP2D6*10*	F'5GTGCTGAGAGTGTCCTGCC3' R'5 CACCCACCATCCATGTTTGC3'		

PCR-RFLP Polymerase Chain Reaction-Restriction Fragment Length Polymorphism

Statistical Analysis- The Statistical Package for the Social Sciences (SPSS) software for Windows version 16.00 (SPSS Inc., Chicago, Illinois, USA) was used for statistical analysis. The data on continuous variables are presented as mean ± standard error of mean (S.E.M.) and on categorical variables, as numbers and percentages. The statistical significance was set at $p \leq 0.05$. The allelic and genotypic frequencies were manually calculated by gene counting method and Chi-squared analysis was used to determine whether there were any significant differences in allelic and genotypic frequencies in patient and control groups. The genotypic frequencies were tested for Hardy-Weinberg equilibrium. Haplotype analysis was performed using the Haploview program to check for gene linkage. Odds ratio (OR) and 95% confidence interval were calculated for the relative risk of SNPs of *CYP2D6* and *GST* in disease variation. Adjusted odds ratio was calculated to rule out effects of potential risk factors like gender, age,

alcohol consumption, socioeconomic status, BMI, Family history of the disease. Gene-gene interaction predisposing to hypertension were analyzed using a multistep approach. In the first phase, multiple regression and odds ratio analyses were carried out to look for the effect of genotypes on the disease condition. In the second phase Multifactor Dimensionality Reduction (MDR) analysis was performed for gene-gene combinations that may be predictable of disease. MDR is a non-parametric, model-free data mining method to detect, characterize and interpret disease susceptible gene-gene epistatic interactions (Jason et al., 2015). In MDR analysis, cross-validation and permutation testing defines the status of disease. Of all the genotype combinations generated by MDR analysis, only the combinations with highest testing accuracy and cross validation consistency were considered as best predictor combinations. To further examine for high order SNP-SNP interactions Classification and Regression Tree analysis (CART) was performed. CART is a binary-recursive-partitioning approach that partitions the data, based upon risk associated with independent variables. The most significant predictor, which contributes maximum to disease susceptibility, splits first in the tree. In the tree-formation, splitting process continues until the terminal nodes do not have subsequent significant values. CART analysis is similar to traditional regression techniques but has the advantage that data are easy to interpret. The terminal nodes with minimum number of patients are used as a reference to calculate the odds ratio and the 95% confidence interval (CI) for all other nodes with different genotype combinations.

RESULTS

Study Participants- Hypertensive patients (200) and normal healthy (n=200) individuals with rural background residing in Amritsar District, of Jat Sikh population sub-group were studied for their demographic/ lifestyle patterns. The patient and control group individuals were matched for age (61.59±0.80y patients, 60.36±0.89y controls) and gender representation (Table 2). Despite antihypertensive treatment blood pressure indices, were significantly elevated in the patient group (systolic blood pressure p≤0.001; diastolic blood pressure p≤0.001; pulse pressure p≤0.001; mean arterial pressure p≤0.001). The BMI derived from the anthropometric measurements is considered as a validated marker for obesity (Maffeis et al., 2001). In the studied group 60.50% of patients were obese. In literature also increased prevalence of hypertension has been reported in obese females (Fujita and Hata, 2014) and Shihab et al. (2012) have observed a direct association of weight gain and increased risk of hypertension. In the presently studied population sub-group, 77.50% controls were obese; these individuals are at increased risk for predisposition to hypertension and its co-morbidities (Landsberg et al., 2013).

Table 2: Demographic and Disease-related variables of Patients and Controls

Characteristics	Patients (n=200)	Controls (n=200)	p-value	
			Chi-squared/students t-test	Mann-Whitney U test
Gender (M/F)	99/101	106/94	NS[a]	-
Age (y)	61.59±0.80	60.36±0.89	NS[b]	NS
BMI (kg/m^2)	26.60±0.32	29.39±0.35	p≤0.001[b]	p≤0.001
Disease-related indices				
SBP (mmHg)	145.52±1.39	130.00±0.38	p≤0.001[b]	p≤0.001

DBP (mmHg)	86.86±0.66	76.79±0.32	p≤0.001[b]	p≤0.001
PP (mmHg)	58.67±1.03	53.22±0.42	p≤0.001[b]	p≤0.01
MAP (mmHg)	106.22±0.84	94.53±0.28	p≤0.001[b]	p≤0.001

BMI body mass index, SBP systolic blood pressure, DBP diastolic blood pressure, PP pulse pressure, MAP mean arterial pressure, a Chi squared value, b Students' t-test, NS non-significant

Individual genotype-variants and hypertension susceptibility- Allelic and genotypic frequencies were calculated for all the genes under study. The allele frequencies for *CYP2D6*2* polymorphism showed significant deviation from Hardy-Weinberg equilibrium (HWE) in both, the patient (p=0.000) and control (p=0.000) groups. However, the allele frequencies of *CYP2D6*10* and *GSTP1* showed deviation from HWE only in the control group (Table 3). The minor allele frequencies for *CYP2D6*4* (0.38), *CYP2D6*10* (0.46) and *GSTP1* (0.57) were higher in the patient group compared to the respective values in controls (0.27, 0.43, 0.27). Statistically significant differences were observed for both *CYP2D6*4* (p=0.0009) and *GSTP1* (p<0.001), implying their association with hypertension. Crude odds ratio revealed that individuals from patient and control groups with heterozygous genotypes for *CYP2D6*4* (GA; OR=1.5949; 95% CI= 1.0516-2.4187), *CYP2D6*10* (CT; OR= 2.4140; 95% CI= 1.5165-3.8427) and *GSTP1* (AG; OR= 4.4906; 95% CI= 2.7548-7.3201) had ~1.6, ~2.4 and ~4.5 times higher risk for hypertension, respectively. The risk for disease was also increased in individuals of both patient and control groups with homozygous variant genotype for *CYP2D6*4* (AA; OR=3.2121; 95% CI= 1.5368-6.7136) and *GSTP1* (GG; OR=7.0174; 95% CI= 4.0108-12.2779), ~3.2 and ~7.01 times, respectively. On adjustment for gender, age, alcohol consumption, socioeconomic status, BMI, family history of the disease statistical significance was lost for all the gene variants. Chi-squared analysis for *GST*M1 and *GST*T1 revealed no effect of these polymorphisms on hypertension susceptibility. For the *CYP2D6* *10 alleles also, the individuals with heterozygous genotype (CT) had ~2.4 folds higher likelihood (OR=2.4140; 95% CI= 1.5165-3.8427; p=0.0002) for hypertension. For *GST* (T1 and M1) no association was observed with disease in this population sub-group.

Haplotypes and Inheritance Models- The pair-wise linkage disequilibrium plot and haplotype analysis revealed that there was no linkage between gene variants under study. Also, none of the SNPs had the tendency to be inherited together in the group under study.

On analyzing different models of inheritance for the *CYP2D6*2* (OR=7.02; 95% CI=4.01-12.28; p=0.000), *CYP2D6*10* (OR=3.21; 95% CI=1.54-6.71; p=0.019) and *GSTP1* (OR=7.02; 95% CI=4.01-12.28; p=0.000) variants, the additive model of inheritance was the best fit. The dominant model, on the other hand was best fit for *CYP2D6*4* (OR=1.08; 95% CI=1.21-2.68; p=0.004), *CYP2D6*10* (OR=1.76; 95% CI=1.17-2.66; p=0.007) and *GSTP1* (OR=5.36; 95% CI=3.45-8.33; p=0.000) variants. In addition to these the co-dominant and recessive model of inheritance were best fit for *CYP2D6*4* (OR=1.71; 95% CI=1.25-2.33; p=0.001, OR=2.55; 95% CI=1.26-5.17; p=0.007, respectively) and *GSTP1* (OR=2.81; 95% CI=2.12-3.73; p=0.000, OR=3.38; 95% CI=2.06-5.54; p=0.000, respectively). Overall, 1.08-7.02 times higher risk of hypertension inheritance was posed by various models for different SNPs (Table 3).

Table 3: Distribution of *CYP2D6 (*2, *4 and *10)* and *GST (P1, M1 and T1)* genotypes and alleles in patients and controls

Genotype Frequencies	Patients n=200 (%)	Controls N=200 (%)	Chi-squared (p- value)	Crude OR (95% CI)	Adjusted OR (95% CI)

				(p)	(p)
*CYP2D6 *2* (rs16947)					
Homozygous wild N (%)	92 (56.00)	85(42.50)	1.359 (p=0.082)	Reference	Reference
Heterozygous N (%)	66(33.00)	59(29.50)		1.0335 (0.6534-1.6348) (0.8879)	4.121 (0.38-53.455) (p=4.12)
Homozygous variant N (%)	42(21.00)	56(28.00)		0.6929 (0.4215-1.1390) (p=0.1480)	0.589 (0.013-25.893) (p=0.784)
Allele frequencies					
A	0.63	0.57	2.08 (p=0.1491)	-	
G	0.37	0.43			
Hardy-Weinberg equilibrium	P=0.000	P=0.000	-		
Genetic Models	Additive Model (AA vs. GG): OR, 7.02; 95% CI, (4.01-12.28); P=0.000				
	Dominant Model (AA vs. AA+AG); OR, 0.87; 95% CI, (0.58-1.29); p= 0.481				
	Co-Dominant Model (AA Vs. AG); OR, 0.85; 95% CI, (0.67-1.09); p=0.192				
	Recessive Model (GG vs. AG+AA); OR, 0.68; 95% CI, (0.43-1.08); p=0.103				
*CYP2D6 *4* (rs3892097)					
Homozygous wild N (%)	77 (38.50)	106 (53.00)	5.740 (p=0.167)	Reference	Reference
Heterozygous N (%)	95 (47.50)	82 (41.00)		1.5949 (1.0516-2.4187) (p=0.0280)	5.018 (0.255-98.762) (p=0.289)
Homozygous variant N (%)	28 (14.00)	12 (06.00)		3.2121 (1.5368-6.7136) (p=0.0019)	30.157 (0.184-4939.519) (p=0.190)
Allele frequencies					
G	0.62	0.73	11.10 (p=0.0009)	-	
A	0.38	0.27			
Hardy-Weinberg equilibrium	P=0.880	P=0.458			
Genetic Models	Additive Model (GG vs. AA): OR, 0.69; 95% CI, (0.42-1.14); p=0.148				
	Dominant Model (GG vs. GG+GA): OR, 1.80; 95% CI, (1.21-2.68); p=0.004				
	Co-Dominant Model (GG vs. GA): OR, 1.71; 95% CI, (1.25-2.33); p=0.001				
	Recessive Model (AA vs. GA+GG): OR, 2.55; 95% CI, (1.26-5.17); p=0.007				
*CYP2D6*10* (rs1065852)					
Homozygous wild N (%)	60(30.00)	86 (43.00)	8.539 (p=0.202)	Reference	Reference

Heterozygous N (%)	96(48.00)	57 (28.50)		2.4140 (1.5165-3.8427) (**p=0.0002**)	51.515 (0.695-3818.262) (p=0.073)
Homozygous variant N (%)	44(22.00)	57 (28.50)		1.1064 (0.6623-1.8485) (p=0.6993)	1.534 (0.066-35.668) (p=0.790)
Allele frequencies					
C	0.54	0.57	0.73 (p=0.3931)	-	
T	0.46	0.43			
Hardy-Weinberg equilibrium	P= 0.632	**P= 0.000**			
Genetic Models	Additive Model (CC vs. TT): OR, 3.21; 95% CI, (1.54-6.71); **p= 0.019**				
	Dominant Model (CC vs. CC+CT): OR, 1.76; 95% CI, (1.17-2.66); **p=0.007**				
	Co-Dominant Model (CC vs. CT): OR, 1.11; 95% CI, (0.87-1.43); p=0.403				
	Recessive Model (TT vs. TC+CC): OR, 0.71; 95% CI, (0.45-1.11) ; p=0.134				
GST P1 (rs1695)					
Homozygous wild N (%)	43(21.50)	119(59.50)	62.166 (**p=0.0001**)	Reference	Reference
Heterozygous N (%)	86(43.00)	53(26.50)		4.4906 (2.7548-7.3201) (**p=0.0001**)	1274.711 (0.243-6676634.518) (p=0.102)
Homozygous variant N (%)	71(35.50)	28(14.00)		7.0174 (4.0108-12.2779) (**p=0.0001**)	150299.099 (0.452-499.710) (p=0.066)
Allele frequencies					
A	0.43	0.73	71.39 (**p<0.0001**)		
G	0.57	0.27			
Hardy-Weinberg equilibrium	P=0.082	P=**0.000**			
Genetic Models	Additive Model (AA vs. GG): OR, 7.02; 95% CI, (4.01-12.28); **p=0.000**				
	Dominant Model (AA vs. AA+AG); OR, 5.36; 95% CI, (3.45-8.33); **p=0.000**				
	Co-Dominant Model (AA Vs. AG); OR, 2.81; 95% CI, (2.12-3.73); **p=0.000**				
	Recessive Model (GG vs. AG+AA): OR, 3.38; 95% CI, (2.06-5.54); p=**0.000**				
GSTT1 (rs17856199)					
Present	105(52.50)	120(60.00)	1.991 (p=0.1582)		
Null	95(47.50)	80(40.00)			
GSTM1 (rs366631)					
Present	46 (23.00)	53(26.50)	0.483 (p=0.4870)		
Null	154 (77.00)	147(73.50)			

OR odds ratio, CI confidence interval, Adjusted for Gender, Age, Alcohol consumption, socioeconomic status, Body Mass Index, Family history of disease, p≤0.05 was taken as significant, values in bold are significant.

Combinatorial effect of polymorphic variants on hypertension risk- The pathogenesis of hypertension results from the cumulative action of many genes. The gene-gene interactions are capable of identifying genes with very weak or no association individually. Thus, the genotypic combinations of the six functional polymorphisms viz. *CYP2D6*2* (rs16947), *CYP2D6*4* (rs3892097), *CYP2D6*10* (rs1065852) and *GST* (T1, M1 and P1; rs1695) were studied to predict the risk for hypertension.

Multifactor Dimensionality Reduction (MDR) analysis- MDR analysis was performed on the data set of study participants, with or without hypertension. Based upon the higher cross validation consistency (CVC) and testing accuracy (TA), the best epistatic interaction model was a three - factor model comprising *CYP2D6*4*, *CYP2D6*10* and *GSTP1* with a CVC of 10/10 and TA of 0.7025 (Table 4). This three-factor interaction indicated ~7.4 times higher risk for developing hypertension (OR= 7.3889; 95% CI= 4.7417-11.5141). The other interaction was a two-factor interaction viz. *CYP2D6*4* and *GSTP1* with CVC of 8/10 and TA of 0.67 indicating ~6.7 times higher risk for hypertension (OR = 6.6818; 95% CI= 4.1763-10.6905). The details of the two-factor and three-factor risk combinations are shown in Figures 1 and 2, respectively. Considering the three-factor combination, 12% of patients and 2% of controls with heterozygous genotypes for *CYP2D6*4*, *CYP2D6*10* and *GSTP1* are at increased risk for hypertension. Similarly in the two-factor combination, (22%) patients and (16.50%) normal control individuals with heterozygous genotypes of *CYP2D6*4* and *GSTP1* are at increased risk for hypertension.

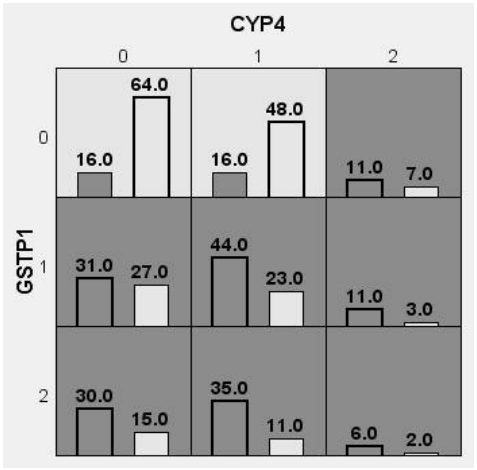

Fig 1: Two gene combination model for hypertension phenotype in Jat Sikh samples. High-risk combinations are in dark shading. The number of individuals with hypertension in each cell is at the left-hand bar of the histogram and the number of controls (normotensives) is at the right-hand bar. (0 represents homozygous wild, 1 resents heterozygous and 2 represents homozygous variant for both the genotypic variants).

Table 4: Gene-Gene combination models for Hypertension risk

Models	Training Accuracy	Testing Accuracy (TA)	OR (95% CI)	χ2 (p-value)	CV Consistency	p-value permutation
*CYP2D6*4, GSTP1*	0.7006	0.67	6.6818 (4.1763-10.6905)	69.4444 (<0.0001)	8/10	0.674-0.675

*CYP2D6*4, CYP2D6*10, GSTP1*[a]	0.7311	0.7025	7.3889 (4.7417-11.5141)	84.9458 (<0.0001)	10/10	0.091-0.096

[a] Best model predicted by MDR analysis, CV cross validation consistency, OR odds ratio, CI confidence interval

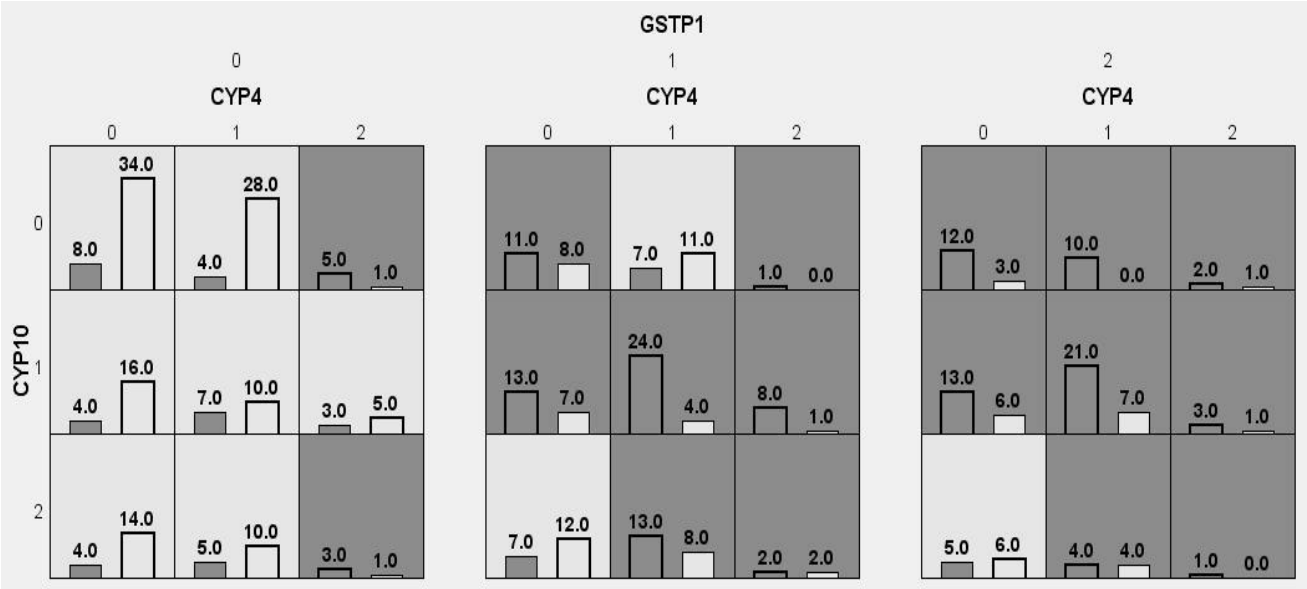

Fig 2: Three gene combination model for hypertension phenotype in Jat Sikh samples. High-risk combinations are in dark shading. The number of individuals with hypertension in each cell is at the left hand bar of the histogram and the number of controls (normotensives) is at the right hand bar. (0 represents homozygous wild, 1 resents heterozygous and 2 represents homozygous variant for both the genotypic varianats)

Classification and Regression Tree (CART) analysis- CART analysis was carried out for high order SNP-SNP interactions and decision tree generated is shown in Figure 3. There are four terminal nodes (Table 5) of the tree (Node 1, Node 4, Node 5, Node 6). The first split on the decision tree has *GSTP1* indicating *GSTP1* as the main risk factor for hypertension. The results were in accordance with those obtained by MDR analysis. *GSTP1* showed significant association with increased risk for hypertension in both the two-factor and three-factor epistatic interactions evaluated by MDR analysis. Individually also, there was a significant association of *GSTP1* polymorphism with hypertension. The further split in the regression tree was based on *CYP2D6**10, followed by *CYP2D6*4*. These results are also consistent with the results from MDR analysis. The Node 1 with lowest rate of hypertensive patients (26.50%) and highest rate (73.50%) of normal control individuals is considered as "the reference" to calculate the risk for other respective genotypic combinations made in regression tree at different nodes as there are the least number of patients with homozygous wild genotype *GSTP1* (AA) and most of 73.5% are normotensive individuals. The individuals with heterozygous and homozygous mutant genotypes for *GSTP1* (AG and GG, respectively), heterozygous and homozygous wild genotypes for *CYP2D6*10* (CT and CC, respectively) and *CYP2D6*4* (GA and

GG, respectively) have ~9.7 times higher risk for developing hypertension (Node 5; OR= 9.7373; 95% CI= 5.6085-16.9054; p=0.0012). The risk decreased to ~3.9 times for hypertension when in the combination heterozygous and homozygous wild genotypes of *CYP2D6*4* were replaced with homozygous mutant (AA) genotype (Node 6; OR= 3.9535; 95% CI =2.0480-7.6318; p<0.0001). The individuals with heterozygous and homozygous mutant genotypes of *GSTP1* (AG and GG, respectively) and the homozygous mutant genotype for *CYP2D6*10* (TT) had ~2.7 times higher risk for developing hypertension (Node 4; OR= 2.6836; 95% CI= 1.4751-4.8820; p<0.0001).

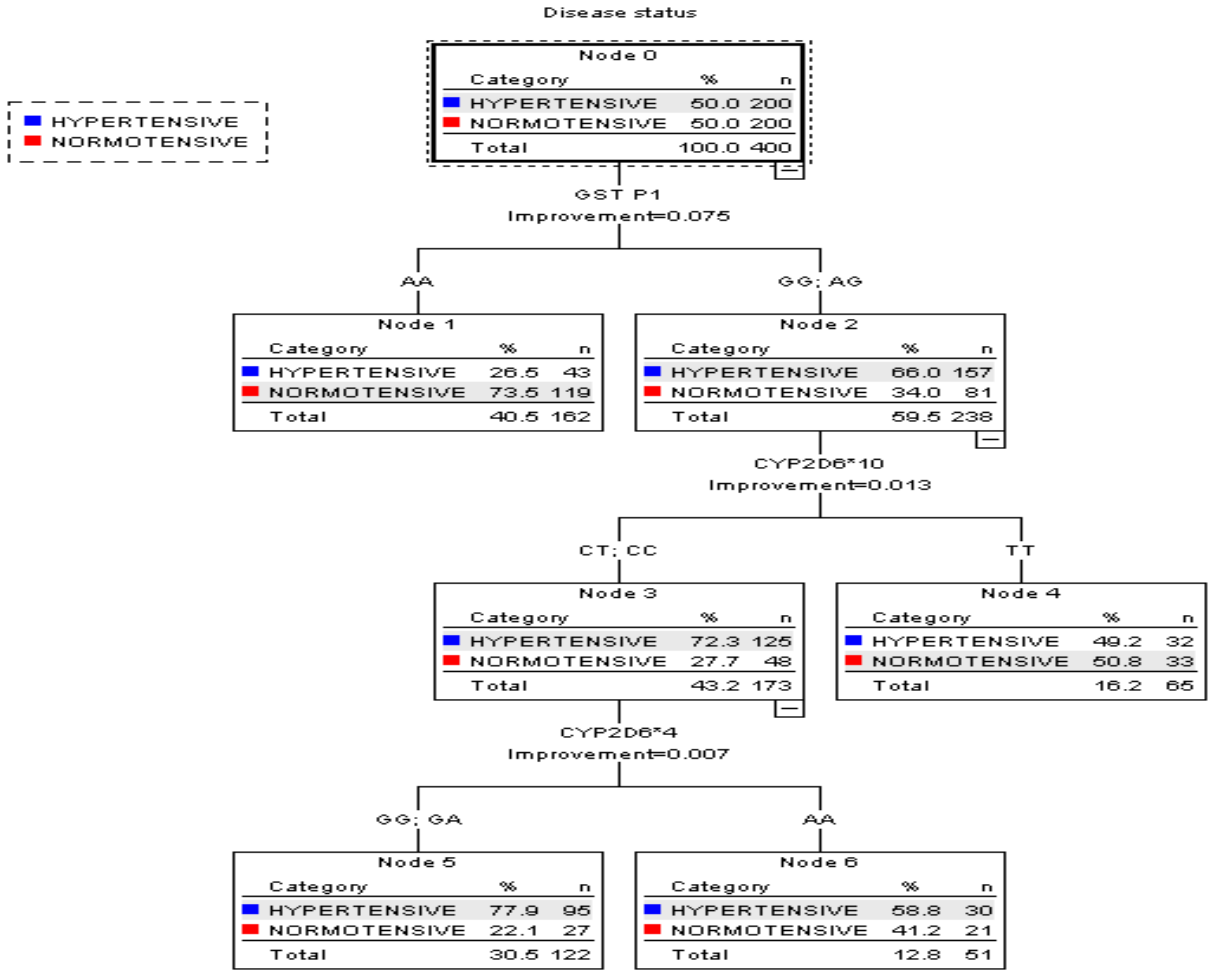

Fig 3: Classification and regression tress formed after CART analysis of *CYP2D6*2*, *CYP2D6*4*, *CYP2D6*10*, *GSTP1*, *GSTT1* and *GSTM1* for hypertension prediction

Table 5: Risk estimation for hypertension based upon CART analysis

Terminal Node	Genotype for the Node	Patients N (%)	Controls N (%)	OR (95% CI)	p-value
Node 1	*GSTP1* AA	43 (26.5)	119 (73.5)	Reference	

Node 4	*GSTP1* GG;AG/ *CYP2D6*10* TT	32 (49.2)	33 (50.8)	2.6836 (1.4751-4.8820)	0.0012
Node 5	*GSTP1* GG; AG/ *CYP2D6*10* CT; CC/ *CYP2D6*4* GG; GA	95 (77.9)	27 (22.1)	9.7373 (5.6085-16.9054)	<0.0001
Node 6	*GSTP1* GG; AG/ *CYP2D6*10* CT; CC/ *CYP2D6*4* AA	30 (58.8)	21 (41.2)	3.9535 (2.0480-7.6318)	<0.0001

OR odds ratio, CI confidence interval

DISCUSSION

The escalating incidence of hypertension and its related co-morbidities was the background to the present study, which was undertaken to study whether gene-gene combinations and SNP-SNP interactions of metabolic genes in a stratified population are associated with essential hypertension. Association studies of metabolic genotypes and essential hypertension exclusively in Jat Sikh population sub-group have not came to attention. Genetic variations in detoxifying or metabolizing systems can lead to increased oxidative stress and may contribute to hypertension. Heritability of hypertension varies from 30-50%. However, considering that all the high-frequency variations detected by genome wide association studies together account for 2-3% of blood pressure variability only, the part of missing contribution can therefore be attributed to rare variants (Russo et al., 2018) or to interaction between different genetic variants.

The Jat Sikhs, an endogamous group, are the majority (60%) among Sikhs in the state of *Punjab* and comprise a single largest group, generally residing in villages. As there is significant effect of socioeconomic status, family income and education level on hypertension-onset (Holmes et al., 2013), differing in rural areas from urban, the present case-control study as a first of its kind on rural Jat Sikh population sub-group was carried out to study interaction among metabolizing enzyme gene SNPs in essential hypertension. To reduce gender bias, both males and females were included. Also, because biological differences between genders are differently affected by interactions between genetic and environmental components (Ngun et al., 2011) and so can influence disease status.

The CYP2D and *GST* are two important phase-I and phase-II metabolizing enzymes, respectively which are involved in elimination of many therapeutics agents, toxin and even oxidative stress (Zanger and Schwab 2013; Hayes et al., 2015). Variation in genes encoding metabolizing enzymes result in altered enzyme activity affecting inter-individual variability to drug response, environmental toxins, increased oxidative stress and disease-predisposition (Ahmed et al., 2016). On the assumption that CYP2D and *GST* may have functional relevance, either with blood pressure regulation or drug metabolizing response or oxidative stress control, six functional polymorphisms viz. *CYP2D6* *2 (rs16947), *CYP2D6* *4 (rs3892097), *CYP2D6*10* (rs1065852) and *GST* (T1, M1 and P1; rs1695) were selected for analysis. Variation in these genes has been known to modify disease-susceptibility and drug response (Ali et al., 2013; Rafee et al., 2014).

Allele frequencies of *CYP2D6*4* alleles in present study were different from previously reported allele frequencies where minor allele frequency was significantly higher in normal controls compared to hypertensive patients. The genetic polymorphisms of *CYP2D6* gene have not been studied for hypertension-risk but for clinical outcome of various antihypertensive drugs (Lymperopoulos et al., 2015; Chen et al., 2018) depending on the observed

enzyme activity in different persons viz. poor/intermediate/extensive/ or ultra-rapid metabolizers (UM). Inter-ethnic differences for *CYP2D6* phenotypes have also been reported (Lerena et al., 2014; Gaedigk et al., 2016). Only two studies associating *GSTP1* and hypertension have come to attention: in Han adult males (Lin et al., 2009) and in the Italian population (Polimanti et al., 2011) with results contradictory to those of the present study as no association was reported for hypertension and *GSTP1* polymorphism. Association was observed for the first time for *Punjabi Jat Sikh* population sub-group in the present study. In literature, inconsistent results have been reported: some having positive association of hypertension with *GST*T1 (Polimanti et al., 2011; Lee et al., 2012) with *GST*M1 (Han et al., 2017; Lee et al., 2018) or with both (Abbas et al., 2015; Kumar et al., 2017). Others found no association of *GST*T1 and *GST*M1 with hypertension (Dhameja et al., 2013; Rizvi et al., 2017). On meta-analysis of 13 studies relating *GST*T1 and of 14 studies relating *GST*M1, Ge and co-workers (2015) also did not report any association with state of hypertension (Ge et al., 2015). The inconsistent results in different studies can be attributed to the association of multiple genes with hypertension.

The deviation from Hardy-Weinberg equilibrium can be attributed to natural selection, mutation, migration, non-random mating and finite population size (Rodriguez et al., 2009; Andrews, 2010). In the present study, deviation can be because of one or more of these factors; bias in sampling may also be important because it is a hospital-based study and further; the patients were under treatment restrictive to mono therapy with (Theophilus et al., 2006; Zihlif et al., 2012). According to Major Todd, Jats belong to major Rajput tribe, which in turn are descendants of Aryan tribe. However, according to General Cunningham, Jats are progenitors of Scythian race and have immigrated from north-west (Ibbetson, 1916). As per the first Persian account "Mujmat ul-tawarikh (1026)" Jats are a primordial tribe of Sind while the drug atenolol. Controls were unrelated healthy normotensive adults from the general population. Non-random mating could be another factor for HWE deviation as Jat Sikhs practice endogamy. The *CYP2D6*10* alleles in South African healthy citizens also showed deviation from HWE (Dodgen et al., 2013). On pursuing the phase I and II enzymes, there is observed a delicate balance between them. The phase I enzymes bioactivate many carcinogens and environment pollutants by converting them into electrophilic compounds which are conjugated by phase II enzymes and are eliminated. The polymorphism in the metabolic genes result in altered metabolic activities with susceptibility to disease. The *CYP2D6* enzyme (debrisoquine 4-hydroxylase) is involved in phase I metabolism of most of the drugs (Zanger and Schwab, 2013) and genetic changes coding *CYP2D6* enzyme leads to variable enzymatic activity. The GST enzyme, a phase II class enzyme is involved in detoxification of many endogenous and exogenous compounds, including the products of oxidative stress. The enzyme efficiency varies for different *GST* alleles and the ethnic-dependent polymorphism has been reported in literature (Sharma et al., 2014). The *CYP2D6* and *GST* genes studied in the present study are important in the metabolism of xenobiotics (Hayes et al., 2015; Tredici et al., 2018) and may have a role in susceptibility to hypertension by restrictive elimination of reactive oxygen species and in the metabolism of antihypertensive drugs; the present study aimed to study such interactions.

The frequency of minor alleles of *CYP2D6*2*, *4 and *10 was higher in the presently studied Jat Sikh population compared to other populations
according to "Firishta" the Persian chronicler, Jats started their colonization in *Punjab* near Multan under one of the Jat rulers, "Jit Salindra. But Jats were considered a Central Asian nomadic group by Fuchs (1974) who immigrated into north-west India where they become Sikh followers in the 17th century (Puri, 2003). Some common haplotypes have also been reported among Jat Sikhs and other

populations from Indus valley (https://www.jatland.com/home/Jat_History accessed on February 21, 2021). There are limited investigations on Jat Sikhs. The present study as a comprehensive study of its kind, hence purports to provide novel information on Jat Sikhs. In literature only one study has come to attention investigating the association of *CYP2D6* (*1 and *10) and hypertension (Aliet al., 2013) in a group showing a significant association between *CYP2D6* genotype and hypertension. For *CYP2D6*10, inconsistent results for hypertension treatment response have been reported in literature (Ota et al., 2015; Jung et al., 2018). Levinsson and coworkers (2014) reported significant association of three *GSTP1* gene SNPs (rs1871042, rs749174 and rs762803) with hypertension. For *GSTP1* rs1695 polymorphism (Ile105Val), 105Val allele has significantly reduced enzyme activity (Watson et al., 1998) and therefore can result in increased oxidative stress, thereby predisposing individuals to hypertension (Oparil et al., 2003). *GST*T1 and *GST*M1 did not exhibit association with hypertension in the presently studied group. The results are similar to those in other population sub-groups (Dhameja et al., 2013; Abbas et al., 2015; Kumar et al., 2017).

The present study results for association of these genes with hypertension in the Jat Sikh group can be attributed to the combinational effect of such genes in causation of hypertension. The genotypic interactions obtained both from MDR and CART analyses show consistency with each other. The best at-risk interaction is among *CYP2D6*4*, *CYP2D6*10* and *GSTP1*, however individually, *CYP2D6*10* has shown no association with hypertension. These results are similar to other studies where individual SNPs showed no association with hypertension; however, combination analysis showed a strong risk for hypertension (Wang et al., 2014; Meng et al., 2017). The interactions found in this study are new for association with hypertension. The interactions on MDR analysis are epistatic interactions and imply that the genes may not be linked directly (Lippert et al., 2013; Yang et al., 2015).

CONCLUSION

Individually the *CYP2D6*4* and *GSTP1* (rs1695) gene variants, and the interaction among *CYP2D6*4*, *CYP2D6*10* and *GSTP1* gene polymorphisms have shown susceptibility for hypertension in the *Punjabi Jat Sikh* group though some alleles were not in HWE. The present work is a pioneer study relating polymorphisms in metabolizing genes of *CYP2D6* and *GST* enzymes in *Punjabi Jat Sikh* population sub-group. In future studies, the interactions should be validated in this and other population sub-groups with a larger sample size.

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Author's contribution

GG conceived the idea; TK collected the samples and performed experiments; TK and GG wrote the manuscript. All authors have read and approved the final version of the manuscript.

Conflict of interest

Authors have no conflict of interest.

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Declaration of originality

The authors have declared that the data/text presented in this manuscript is original and no text, figure or data has been copied from any other source without appropriate citation.

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