A comprehensive contribution of genes for aryl hydrocarbon receptor signaling pathway to hypertension susceptibility

[a,b]Alexey V. Polonikov, [a,b] Olga Yu. Bushueva, [a] Irina V. Bulgakova, [c] Maxim B. Freidin, [d] Mikhail I. Churnosov, [a,b] Maria A. Solodilova, [a] Vladimir P. Ivanov

[a] Department of Biology, Medical Genetics and Ecology, Kursk State Medical University, 3 Karl Marx St., Kursk 305041, Russian Federation

[b] Research Institute for Genetic and Molecular Epidemiology, Kursk State Medical University, 18 Yamskaya St., Kursk 305041, Russian Federation

[c] Population Genetics Laboratory, Research Institute for Medical Genetics, 10 Nabereznaya Ushaiki, Tomsk 634050, Russian Federation

[d] Department of Medical Biological Disciplines, Belgorod State University, 85 Pobeda St., Belgorod 308015, Russian Federation

Author for correspondence: Alexey Polonikov (Professor, M.D., Ph.D.) Department of Biology, Medical Genetics and Ecology, Kursk State Medical University, 3 Karl Marx St., Kursk 305041, Russian Federation.

E-mail: polonikov@rambler.ru Telephone/Fax: +7(4712) 58-81-47

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Abstract

Objective The present study was designed to investigate whether genetic polymorphisms of the aryl hydrocarbon receptor (AHR) signaling pathway are involved into the molecular basis of essential hypertension (EH).

Methods A total of 2160 unrelated Russian individuals comprising 1341 EH patients and 819 healthy controls were recruited for the study. Seven common AHR pathway single nucleotide polymorphisms (SNP) such as rs2066853, rs2292596, rs2228099, rs1048943, rs762551, rs1056836 and rs1800566 have been genotyped by TaqMan-based allele discrimination assays.

Results We found that SNP rs2228099 of *ARNT* is associated with increased risk of EH (OR=1.20 95%CI 1.01-1.44, *P*=0.043) at a dominant genetic model, whereas polymorphism rs762551 of *CYP1A2* showed an association with decreased disease risk at a recessive genetic model (OR=0.68 95%CI 0.52-0.89, *P*=0.006). A log-likelihood ratio test allowed identifying epistatic interaction effects on EH susceptibility for all SNPs. MB-MDR analysis revealed that cigarette smoking, rs1048943, rs762551, rs1056836, rs2228099 were significant contributing factors in 19, 18, 13, 13 and 11 interaction models, respectively. The best MDR model associated with EH risk included rs1048943, rs762551, rs1056836 and cigarette smoking (cross-validation consistency 100%, prediction error 45.7%, P_{perm}<0.0001). The mRNA expression and *in silico* function prediction analyses have confirmed a regulatory potential for a majority of SNPs associated with EH susceptibility.

Conclusion Our pilot study was the first to show that gene-gene and gene-environment interactions in the AHR signaling pathway represent important determinants for the development of essential hypertension, and the pathway may become an attractive target for pharmacological intervention in hypertensive patients in the future.

Introduction

Hypertension is a major health burden due to its high prevalence and associated increased rates of morbidity, mortality, and disability from cardiovascular disease and stroke worldwide [1, 2]. It has been estimated that almost 28% of the world's adult population has uncontrolled hypertension [3] and the global burden of disease will increase to more than 1.5 billion by 2025 [4]. In most cases, the etiology of hypertension remains unclear, that is the reason to define the disease as essential hypertension (EH). The mechanisms involved in the regulation of blood pressure in human populations are complex and are likely modulated by tight interactions between genetic and environmental factors suggesting a multifactorial nature of hypertension [5, 6].

Genome-wide association scans (GWAS) and candidate gene studies have successfully identified a number of common genetic variants influencing blood pressure (BP) variation and hypertension susceptibility in ethnically diverse populations [7, 8, 9, 10]. Despite the progress in hypertension genomics, the difficult task remains in the bridging of genetic findings into the clinic. Such a translation, on the one hand, takes a considerable time to move from a identified gene target to an approved marketed drug, on the other hand, the effect sizes of GWAS-identified BP loci are relatively small and the merit of their utilization in the clinical practice is not clear [11]. Although adequate drug treatment and control of hypertension result in reduced morbidity and mortality [12, 13], the findings obtained by pharmacogenomic studies of antihypertensive drugs are also far from being utilized in the clinic [14, 15, 16]. Thus much of the heritability of BP, hypertension and efficacy of antihypertensive treatment remains unexplained, highlighting the need for further identification of major genetic and environmental factors responsible for the global epidemic of the disease.

A huge number of studies have shown a positive relationship between incident hypertension and ambient air pollution [17, 18, 19, 20, 21, 22, 23, 24]. A rapidly growing body of evidences suggests that airborne polycyclic aromatic hydrocarbons (PAHs) may represent an important group of organic toxic chemicals with potentially causative role for hypertension [25, 26, 27, 26, 29]. PAHs are a group of pollutants widely prevalent in the environment, formed during incomplete combustion of organic

materials such as coal and petroleum product combustion, cigarette smoking, food cooking and industrial activities [30]. Individuals exposed to PAHs defend themselves against intracellular damage by activating the transcription of genes involving in the aryl hydrocarbon receptor (AHR) signal transduction cascade that defends the host, removing and metabolizing the toxicant [31, 32]. Figure 1 summarizes the organization and functions of AHR signaling pathway. AHR is a ligand-dependent transcription factor that regulates the induction of the phase I and II xenobiotics-metabolizing enzymes (XMEs) and, thus mediating most of the toxic and carcinogenic effects of PAHs as well as polyhalogenated hydrocarbons (dioxins, furans) and polychlorinated biphenyls [37, 38]. The basic helix-loop-helix (bHLH) proteins AHR, AHR nuclear translocator (ARNT) and AHR repressor (AHRR) and regulated XMEs represent the AHR signaling pathway, the adaptive xenobiotic stress system which recognizes putatively toxic compounds and triggers their detoxification and elimination [39].

Several common single nucleotide polymorphisms (SNPs) in genes involved in the AHR signaling pathway have been identified and demonstrated to determine interindividual differences in ability to activate and detoxify PAHs [40, 41, 42, 43, 44]. A single study, which investigated two SNPs of the AHR pathway, observed that an interaction between the *CYP1A1* T3801C and *AHR* G1661A polymorphisms is associated with blood pressure [45]. A few studies in humans have revealed an association between genes encoding AHR regulated XMEs such as and *CYP1A1*, *CYP1A2* and *CYP1B1* and hypertension susceptibility [46, 47, 48]. To our knowledge, no studies have so far been done to investigate a comprehensive contribution of AHR pathway genes to the development of essential hypertension. Therefore, the aim of our pilot study was to investigate whether common polymorphisms of AHR signaling pathway are comprehensively involved into the molecular basis of essential hypertension.

Methods

Study population

The study protocol was approved by Ethical Review Committee of Kursk State Medical University and written informed consent was obtained from each participant before enrollment. A total of 2160 unrelated individuals of Russian origin from of the Central Russia (predominantly from Kursk region) comprising 1341 EH patients and 819 healthy subjects with normal blood pressure were recruited at Cardiology Clinics of Kursk Regional Clinical Hospital and Neurology Clinics of Kursk Emergency Medicine Hospital as described previously [49, 50]. Essential hypertension was diagnosed by qualified cardiologists. Patients were defined as hypertensive according to WHO criteria or if they had a history of receiving any antihypertensive drug. All EH patients had no clinical signs, symptoms, and laboratory findings suggestive of secondary hypertension. Study patients completed a questionnaire concerning conventional demographic characteristics and also smoking status which was considered as a measure of individual exposure to PAHs. The baseline characteristics of the study participants are given in Table 1. EH patients were matched to healthy controls on sex and age (P>0.05).

Genetic analysis

Whole blood samples were collected by venipuncture from all study subjects in EDTA-coated tubes and maintained at −20°C until processed. Genomic DNA was isolated from thawed blood samples using a standard phenol/chloroform procedure. Candidate genes involved into the AHR signaling pathway were selected based on their involvement in the pathway using KEGG PATHWAY (www.genome.jp/kegg/pathway.html), Reactome Pathway (www.reactome.org) and PharmGKB (www.pharmgkb.org) databases. The selected AHR pathway genes included *AHR* (aryl hydrocarbon receptor, Gene ID 196), *ARNT* (aryl hydrocarbon receptor nuclear translocator, Gene ID 405); *AHRR* (aryl-hydrocarbon receptor repressor, Gene ID 57491), *CYP1A1* (cytochrome P450 family 1 subfamily A member 1, Gene ID 1543), *CYP1A2* (cytochrome P450 family 1 subfamily A member 2, Gene ID 1544), *CYP1B1* (cytochrome P450 family 2 subfamily A member 1, Gene ID 1545), and *NQO1* (NAD(P)H dehydrogenase, quinone 1, Gene ID 1728). Most commonly studied and potentially

functional SNPs in these genes (minor allele frequency >5%) such as *AHR* R554K (rs2066853), *AHRR* P185A (rs2292596), *ARNT* 567C>G (rs2228099), *CYP1A1* I462V (rs1048943), *CYP1A2* -163C>A (rs762551), *CYP1B1*, V432L (rs1056836) and *NQO1* P187S (rs1800566) were selected for the study. Detailed information on biological function of the genes and their polymorphisms is present in Supplementary Table 1. The polymorphisms were genotyped by TaqMan-based allele discrimination assays on the CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA) based on the protocols published in the literature (sequences of TaqMan-probes and primers sets with references used in this study are listed in Supplementary Table 2. The average call rate for genotyping was 97.6%. As quality controls, about 5% of the samples were randomly selected blindly to case-control status and their repeated genotyping yielded 100% reproducibility.

To evaluate genotype-phenotype correlations we used the genotype and mRNA expression data available for 60 HapMap European subjects and SNPexp v1.2 online tool (http://app3.titan.uio.no/biotools/tool.php?app=snpexp). The functionality of selected SNPs was also assessed *in silico* by the SNP Function Prediction tool developed by Xu and Taylor [51] and available online at the SNPinfo Web Server (https://snpinfo.niehs.nih.gov/snpinfo/snpfunc.htm).

Data analysis

The sample size for the study groups was estimated by the use of statistical power calculations for a chi-square test based on allele and genotype frequencies of AHR pathway SNPs in European populations. An association analysis of the AHR pathway SNPs with EH risk was able to detect a difference of 4-7% in the genotype distributions between the cases and controls assuming 77-97% power and 5% Type I error ($\alpha = 0.05$) based on the sample sizes of 1341 hypertensives and 819 healthy subjects.

Allele frequencies were estimated by the gene counting method, and the chi-squire test was used to identify significant departures from Hardy-Weinberg equilibrium. The distribution of alleles was analyzed by 2x2 contingency tables, and the distributions of the genotypes and their combinations between patients and controls were evaluated by logistic regression analysis. Categorical variables such

gender, smoking status and family history of hypertension were also compared by using the chi-squire test. These statistics were calculated by using STATISTICA software for Windows 10.0 (StatSoft Inc., Tulsa, OK, USA). The association between genotypes and EH risk was measured by multiple logistic regression analysis to calculate odds ratios (OR) with 95% confidence intervals (CI) and adjusted for age and gender. Pairwise gene–gene interactions were evaluated by log-likelihood ratio test (LRT) assuming codominant, dominant, recessive and overdominant models and adjusted for age and gender. The calculations were preformed using the SNPassoc package for R [52].

Since the dimensionality of the data is known to be the central problem in statistical analysis for gene–gene interactions in common disease [53, 54], we used the multifactor dimensionality reduction method (MDR) [55, 56, 57], a data-mining bioinformatic approach, to investigate high-order gene–gene and gene-environment interactions in essential hypertension. We applied the model-based multifactor dimensionality reduction method (MB-MDR) [58] implemented into the mbmdr package for R [59] to our dataset. It is an extension of the popular MDR method which allows measuring the association between multi-locus genotypes and the phenotype, and provides a set of statistically significant interactions instead of a single best model. In the first step of the MB-MDR algorithm, association tests of each multilocus genotype combination, environment risk factor (cigarette smoking) with EH risk are performed using logistic regression. Then, each multilocus genotype was assigned into three risk categories: high, low and no-risk, accordingly. In the second step of the algorithm, association of pooled genotypes in low-risk and high-risk categories was evaluated through logistic regression analysis. Wald statistics was used to explore the significance of results and the interaction models were ranked by adjusted *P*-values in the third step. Then, the most significant high-order interaction between the predictors and EH risk was considered the best model and adjusted for multiple testing through 1000 permutations. Finally, we selected the best most promising interaction for further evaluation by the conventional MDR analysis (MDR 3.0.2, http://sourceforge.net/projects/mdr/) with a purpose to assess model's cross-validation consistency and prediction error. Permutation testing was used to test the significance of the reported measure of prediction accuracy and cross-validation

consistency. Generalized linear model (GLM) was used for the genotype-phenotype correlation analysis to evaluate the differences in the relative mRNA expression levels among carriers with different genotypes.

Results

Association analysis of AHR pathway SNPs with essential hypertension

A percentage of positive family history of hypertension was significantly greater in EH patients versus healthy controls (Table 1). In contrast, the number of smokers was greater in the controls than in EH patients. No differences were found between the groups regarding to other demographic characteristics. A departure from Hardy-Weinberg equilibrium (HWE) was observed for rs2066853 in both cases (P=0.03) and controls (P=0.03) and also for rs762551 in controls (P=0.05). The rest SNPs were in agreement with HWE in the study groups.

Table 2 shows genotype and allele frequencies of AHR pathway SNPs. Allele and genotype frequencies in the studied groups were compatible with those reported in European populations. SNP rs2228099 showed an association with increased risk of EH (OR=1.20 95%CI 1.01-1.44, P=0.043) at a dominant model, whereas polymorphism rs762551 was associated with decreased disease risk at a recessive genetic model (OR=0.68 95%CI 0.52-0.89, P=0.006). The association of rs762551 with EH risk remained significant after correction for multiple testing (P_{cor}=0.05). Meantime, association of SNP rs2228099 with EH did not reach statistical significance after correction for multiple testing (P_{cor}= 0.39).

Epistatic interactions between AHR pathway SNPs and the risk of essential hypertension

In order to assess gene-gene interactions determining hypertension susceptibility, first we explored associations between AHR pathway pairwise genotype combinations and disease risk. The analysis identified fourteen combinations of AHR pathway genotypes associated with EH risk at $P \leq 0.05$. Supplementary Table 3 shows overall genotype combinations associated with EH risk. Figure 2 summarizes plots of AHR pathway genotype combinations significantly associated with EH risk. Carriers of the 462IV *CYP1A1* × 432VL *CYP1B1* genotypes (Figure 2, A) had a significantly

decreased risk of hypertension, compared with carriers of the rest genotypes (OR=0.49, 95% CI 0.22-0.75, *P*=0.001), demonstrating epistatic interaction between the loci at an overdominant genetic model. An overdominant model of gene-gene interaction was also observed for the *CYP1A2* and *ARNT* loci (Figure 2, B). Genotype combination *NQO1* 187PP × *ARNT* 567CG (Figure 2, C) was associated with increased EH risk (OR=1.23, 95% CI 1.01-1.50, *P*=0.04), whereas the *NQO1* 187PP × *ARNT* 567CC/CG genotype combination showed association with decreased disease risk (OR=0.81, 95% CI 0.67-0.98, *P*=0.03).

In addition, log-likelihood ratio test was performed to look for epistatic interaction effects between AHR pathway SNPs on hypertension susceptibility. As can be seen from Table 3, SNPs rs2228099 and rs762551 showed significant individual effects on the risk of essential hypertension. The analysis identified epistatic interactions between *ARNT* and *CYP1A2* (overdominant model, $P_{interaction}$=0.008), *CYP1A1* and *CYP1B1* (dominant model, $P_{interaction}$=0.001), *AHR* and *NQO1* (recessive model, $P_{interaction}$=0.004), *ARNT* and *NQO1* (overdominant model, $P_{interaction}$=0.013), *AHRR* and *CYP1A1* (recessive model, $P_{interaction}$=0.041).

High-order gene-gene and gene-environment interactions in hypertension susceptibility

The MB-MDR method was applied to the dataset with a purpose to investigate high-order gene–gene and gene–environment interactions contributing to hypertension. Two, three and four-order interaction models among seven SNPs and smoking status were analyzed and then adjusted for age and sex. Table 4 shows best AHR pathway gene-gene and gene-smoking interactions significantly associated with hypertension risk. Cigarette smoking, rs1048943, rs762551, rs1056836 and rs2228099 were included as significant contributing factors in 19, 18, 13, 13 and 11 interaction models respectively (detailed list of all 27 interaction models is given in Supplementary Table 4).

High-order gene-gene and gene-environment interaction obtained by conventional MDR method are shown in Table 5 and Figure 3. The best interaction model associated with the risk of essential hypertension included rs1048943 (*CYP1A1*), rs762551 (*CYP1A2*), rs1056836 (*CYP1B1*) and cigarette smoking (Wald statistic=31.51, permutation *P*<0.001). The interaction between rs1048943, rs762551,

rs1056836 and cigarette smoking showed the highest cross-validation consistency (100%) and lowest prediction error (45.7%). The dendrogram (Figure 3,B) illustrates a complex pattern of gene-gene and gene-environment interactions determining EH susceptibility. Cigarette smoking and rs762551 had the highest degree of redundancy in their interactions and also found to interact with rs1056836 in the same manner, but to a lesser degree. In the interaction graph (Figure 3,C), cigarette smoking and rs762551 eliminate 0.48% and 0.36% of class entropy, respectively, thereby having the largest univariate effects. A substantial percentage of entropy (0.11%) was explained by rs762551 and smoking, indicating a redundant (antagonistic) interaction between them. SNPs rs1048943 and rs1056836 showed relatively small percentages of entropy when considered independently (0.03% and 0.11%, respectively), while a large percentage of entropy was explained by their interactions with smoking and rs762551.

Then we performed MB-MDR analysis stratified by smoking status which allowed identifying specific combinations of SNPs influencing disease susceptibility in exposed and unexposed individuals. Table 6 shows best models of gene-gene interactions associated with essential hypertension in cigarette smokers and non smokers. Detailed data on genotype combinations associated with EH risk in smokers and non-smokers are shown in Supplementary Table 5. There are substantial differences in gene-gene interactions between smokers and non smokers, suggesting that exposure to PAHs is an important factor modifying association between AHR pathway genes and hypertension susceptibility.

Genotype-phenotype correlation analysis in AHR pathway genes

Data on both mRNA expression levels and genotypes for AHR pathway gene polymorphisms were available from 60 HapMap individuals of European descent. The levels of *ARNT* mRNA were correlated with the rs2228099 locus (P=0.0003, Figure 4,C). *AHRR* mRNA expression levels showed an increased trend for rs2292596 (P=0.007, Figure 4,B). An increased trend was also seen in *CYP1B1* mRNA expression levels and rs1056836 (P=0.01, Figure 4,F). A board-line correlation (P=0.08) of *NQO1* mRNA expression levels occurred with the rs1800566 (Figure 4,G). No significant correlations were found between both *AHR* and *CYP1A2* expression levels and SNPs rs2066853 and rs762551,

respectively (Figure 4,A and E). Moreover, *in silico* functional analysis performed by the SNP Function Prediction tool has confirmed a regulatory potential for the *ARNT*, *AHRR*, *CYP1B1* loci (Supplementary Table 6). The *CYP1A1* and *NQO1* loci also showed a regulatory potential with a probably damaging effect for rs1800566. A SNP rs762551 is located at binding sites for transcription factors such as, for instance, general transcription factor IIIA (V$AP2ALPHA_01), paired box gene 2 (V$PAX2_01), suggesting a functional significance of the *CYP1A2* polymorphism.

Discussion

In our pilot study, we investigated whether common polymorphisms of the aryl hydrocarbon receptor signaling pathway, an inherited determinant for PAH-mediated cardiovascular toxicity, are comprehensively involved into the molecular basis of essential hypertension. The study showed for the first time that polymorphic genes for the AHR pathway are important determinants of genetic susceptibility to essential hypertension. We found that SNP rs762551 of *CYP1A2* is associated with decreased risk of EH. CYP1A2 is a PAHs-induced cytochrome P450 enzyme metabolizing xenobiotics such as PAHs, caffeine, aflatoxin B1, and acetaminophen [33]. Polymorphism rs762551 is known to influence caffeine metabolism and has been found to be associated with the risk of myocardial infarction [60], blood pressure variation and hypertension [61]. This polymorphism is known to be in a linkage disequilibrium with a SNP rs1378942 (r^2=0.63, HapMap CEU), located in the gene cluster including the *CYP1A2* gene, showed the strongest association (P=1×10^{-23}) with diastolic blood pressure in a sample of 34433 subjects of European ancestry [46]. Furthermore, the relationship between rs762551 and hypertension risk was demonstrated in the study of Guessous with coworkers who observed that this negative association occurred in non smokers and is modified by reported caffeine intake [47]. Thus, the present study provided additional evidence that *CYP1A2* is an important susceptibility gene for essential hypertension.

The present study was the first to show that polymorphism rs2228099 of the *ARNT* gene could be a novel susceptibility gene to hypertension. Although association of the SNP with the risk of essential hypertension did not reach statistical significance after correction for multiple testing, rs2228099 in

combinations with other AHR pathway SNPs showed join effects on EH risk. Like AHR, the aryl hydrocarbon receptor translocator is a member of the bHLH transcription protein superfamily which is necessary for dimerization with AHR [37, 39]. ARNT associates with ligand-bound AHR to form a protein complex for binding to the xenobiotic response element (XRE) in enhancers of target genes such as those encoding xenobiotic-metabolizing enzymes as well as genes associated with oxidative stress, fat metabolism and transport, and cell proliferation [62]. Besides participation in the AHR signaling, ARNT is also known as hypoxia inducible factor-1β, transcriptional factor for vascular endothelial cells that regulates genes involved in response to hypoxia [63], a pathological process that has a role in hypertension pathogenesis. SNP rs2228099 represents a synonymous change Val to Val (C>G) at codon 189 in exon 7 of the *ARNT* gene. No functional information is available for this polymorphism in dbSNP (http://www.ncbi.nlm.nih.gov/projects/SNP/). Although this silent SNP is not accompanied by the amino acid change in the ARNT protein, genotypes CG and GG are associated with increased expression of ARNT mRNA compared with genotype CC, as it has been demonstrated by the genotype-phenotype correlation analysis in our study. It can be assumed that the carriers with genotypes CG and GG of *ARNT* may have favorable conditions for chronic and persistent activation of the AHR-ARNT complex resulted in the induction of XMEs. It is not excluded that this polymorphism is in linkage disequilibrium (LD) with another yet unidentified functional SNP of *ARNT* which could be related to blood pressure variation and/or hypertension risk. For instance, SNP rs2228099 is in strong LD with rs12410394 which has been found to be associated with the risk of colorectal neoplasia [64], the finding pointing out, on the one hand, the functionality of this SNP, on the other hand, the link of this locus with PAHs-related cancer susceptibility.

The present study did not observe association between essential hypertension and polymorphism of AHR, the main player and initiator of the signaling cascade. It was not surprisingly, since AHR like many of such proteins, induced as part of the stress-response to environmental toxicants, is evolutionarily conserved, and any functional alterations in the AHR cascade appear to be critical to the evolution, it least for humans. Apparently, a relatively low rate of mutations and functional

polymorphisms in the *AHR* gene [32, 40] confers advantages in the bridging between AHR and its regulated XMEs in maintaining the optimal setting of the host for adaptive responses to PAHs and other chemical compounds upon the constantly changing environment.

The MB-MDR method provided additional evidences for a) the integrated function of the AHR pathway genes may promote a coordinated metabolism of PAH xenobiotics; and b) the AHR signaling pathway loci and their related XMEs are collectively involved into the molecular basis of essential hypertension. A majority of the modeled gene-gene interactions associated with EH risk comprises genes such as *ARNT*, *CYP1A1*, *CYP1A2*, *CYP1B1* and *NQO1*, the findings consistent with results obtained at previous stages of our study. The analysis for gene-gene interactions performed by SNPassoc package allowed identifying SNPs possessing significant effects on disease risk only in combinations. Overall 27 statistically two-, three-, and four-order interaction models have been identified to influence the risk of essential hypertension. In particular, significant gene-gene interactions were found between *CYP1A1* and *CYP1B1*, *AHR* and *NQO1*, *ARNT* and *NQO1*, *ARNT* and *CYP1A2*, *AHRR* and *CYP1A1*. These findings point out epistasis, the effect of one gene may not be disclosed if the effect of another gene is not considered [57]. Interactions between the loci suggest that the gene–gene effect on disease risk may be driven by a true interaction, rather than by the main effect from each gene alone. Notably, AHR pathway SNPs showed complex hierarchic interactions, as identified the by the MDR method (Figure 3). The observed gene–gene interactions make mechanistic sense, because these genes may be collectively involved in the pathogenesis of essential hypertension through the same detoxification pathway.

It is known that cigarette smoking is a model of chronic AHR activation in man [65]. Notably, a majority of interaction models identified by MDR included cigarette smoking as a covariate indicating an importance of gene-environment interactions for the penetration of hypertension phenotype. Best gene-smoking MB-MDR interaction model associated with EH risk comprised cigarette smoking and *CYP1A1*, *CYP1A2*, *CYP1B1*. Furthermore, MDR analysis stratified by smoking status allowed identifying specific SNPs combinations influencing hypertension susceptibility in exposed and

unexposed individuals. Interactions between *ARNT*, *CYP1A1*, *CYP1A2* and *CYP1B1* were significantly associated with disease susceptibility in smokers, whereas *ARNT*, *AHRR*, *CYP1B1* and *NQO1* gene polymorphisms contributed to the disease in non smokers. Differences in the spectrum of interacted genes between smokers and non smokers apparently reflect that the molecular mechanisms by which AHR pathway SNPs contribute to hypertension may be distinguished substantially depending on whether the individual is exposed or not exposed to PAHs. It is permissible to assume that the mechanisms of hypertension in smoker individuals are related to an enhanced metabolic activation of PAHs by CYP1 family of enzymes such as 1A1, 1A2 and 1B1. For instance, a carriage of common "high-risk genotype" combinations such as *CYP1A1* 462II × *CYP1B1* 432VV and *AHR* 554RR × *CYP1A1* 462II × *CYP1A2* 164AA × *CYP1B1* 432VV (Supplementary table 5, A) in cigarette smokers could promote xenobiotics toxification (the conversion of a chemical compound into a more toxic form than a parent molecule). In contrast, a carriage of common genotype combinations, e.g. *ARNT* 567CC × *CYP1A2* 164CC, *CYP1A1* 462II × *CYP1A2* 164CC × *CYP1B1* 432VL or *ARNT* 567CC × *CYP1A1* 462II × *CYP1A2* 164CC (Supplementary table 5, A), is associated with decreased EH risk. This association may be explained by decreased activation of the AHR cascade and CYP1A2 induction in PAH-exposed individuals ("low-activity genotypes" 567CC and 164CC are associated with decreased mRNA levels of *ARNT* and *CYP1A2*, respectively).

The allele 462Val of the *CYP1A1* gene is known to be associated with significant increase in the enzyme activity and induction [66]. In this context, it is unclear why genotype 462IV showed a protective effect against EH risk even in the carriers of "high-risk genotypes" such as *CYP1A2* 164AA or *CYP1B1* 432VV. It should be noted that similar "protective effect" of allele 462Ile *CYP1A1* was found against the risk of lung cancer [67, 68]. An interesting finding is that non-smoker individuals with most common genotype combination, i.e. *ARNT* 567CG × *CYP1A2* 164AC, were at higher risk of essential hypertension. Apparently, a carriage of these "high-activity genotypes" (Figure 4 and Supplementary Table 6) promotes an enhanced activation of the AHR cascade and, therefore the increased risk of hypertension could be related to CYP1A2-mediated cardiovascular toxicity due to an

exposure to the background levels of PAHs present in the environment. In non smokers, protective effects of genotype combinations *AHRR* 185PA × *ARNT* 567CC × *CYP1B1* 432VV and *AHRR* 185PA × *ARNT* 567GG × *CYP1B1* 432LL against hypertension risk can be explained by the fact that "the high-activity" of genotype *CYP1B1* 432VV could be compensated by "the low-activity" of genotype *ARNT* 567CC and vice versa, thus, decreasing AHR pathway activation and associated cardiovascular toxicity.

The present study has some limitations. A majority of the associations of AHR pathway SNPs with hypertension susceptibility were not strong, thereby showing small-to-modest effects of these genes on disease phenotype. The study focused only on major XMEs genes regulated by the pathway, whereas genes being under transcriptional regulation from the AHR-ARNT heterodimer also include at least: *GSTA2*, *UGT1A1*, *UGT1A6*, and *NFE2L2* [35, 69]. Because of not all AHR pathway SNPs were selected for this study, our findings do not allow any definitive conclusion yet to be made on the comprehensive contribution of the genes to hypertension susceptibility. It is safe to assume that simultaneous examination of all tag-SNPs within these genes may provide more comprehensive genetic profiling of the AHR pathway in essential hypertension. Therefore, the hypothesis that AHR pathway genes are collectively involved into the molecular basis of essential hypertension requires further confirmation in other studies. Nevertheless, based on the study findings, it is plausible to assume that individuals with increased activity of the AHR cascade and enhanced toxification of xenobiotics are at increased risk for essential hypertension related to PAH exposure. Undoubtedly, a complete understanding of the causative role of environmental PAHs in the development of hypertension will require conducting experimental and clinical studies to answer the question whether the toxicogenomic mechanisms is an important part of disease pathogenesis.

Despite the exact role of AHR signaling in the regulation of blood pressure remains to be elucidated, undoubtedly, the pathway could serve as a target in the treatment and prevention of hypertension and related diseases. In particular, pharmacological approaches that antagonize AHR signaling pathway with a focus on the adverse effects of toxic AHR-ligands could decrease

cardiovascular toxicity and benefit patients with hypertension and associated diseases. For instance, Resveratrol, a dietary antioxidant supplement with a natural substance, would be a potential candidate as the means of prevention of the AHR-mediated toxicity of smoking and environmental pollution on a wide spread scale [65]. Further ecological and pharmacological genomics studies are required to provide deeper insights into the roles of the AHR pathway genes in responses to environmental xenobiotics and will identify effective therapeutic options for management of hypertension at population and individual levels.

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Legends to Figures

Figure 1 The organization of AHR signaling pathway and regulated xenobiotic metabolizing enzymes [32, 33, 34, 35, 36].

The ligand-binding to and followed activation of AHR is the initial step in the mode of action for a variety of biological and toxicological responses to TCDD and dioxin-like compounds of the environment. AHR recognizes the presence of xenobiotics in the cytoplasm, and then acts to induce XMEs to facilitate the elimination of the foreign compounds. Main genes of the AHR-pathway include the ligand-binding receptor AHR, the AHR nuclear translocator (ARNT), and the AHR regulator (AHRR). Normally, AHR exists in a dormant state within the cytoplasm in association with proteins Hsp90, XAP2, and p23 which help to correctly fold and stabilize the AHR and prevent inappropriate trafficking to the nucleus. Upon ligand binding, AHR in the complex is activated by a conformation change and migrates to the nucleus, where it forms a heterodimer with ARNT, thereby forming a protein complex capable of binding to DNA. The AHR-ARNT complex binds to the xenobiotic response element (XRE) motifs in enhancers of target genes, thereby inducing the transcription of XMEs such as CYP1A1, CYP1A2, CYP1B1 and others. AHRR, sharing structural similarities with AHR and ARNT, may compete with the AHR to bind XRE. The AHRR-ARNT heterodimer is capable of binding with XRE, but without transactivate gene expression. However, AHRR may enhance the release of AHR-ARNT complex from the XRE sequence, resulting in inhibition of AHR-mediated signal transduction and, therefore, protecting against XME-mediated cardiotoxicity. AHR, aryl hydrocarbon receptor; TCDD 2,3,7,8, tetrachlorodibenzo-p-dioxin; ARNT, aryl hydrocarbon receptor nuclear translocator; AHRR, aryl hydrocarbon receptor repressor; PolI, DNA polymerase I.

Figure 2 Plots of interactions between AHR pathway genotypes associated with essential hypertension at different genetic models.

NS, not significant; OR, odds ratio; 95% CI, 95% confidence intervals.

Figure 3 High-order gene-gene (G×G) and gene-environment (G×E) interaction analyses for the AHR pathway SNPs in essential hypertension (Data obtained by Multifactor Dimensionality Reduction package, version 3.0.2).

(A) Cross-validation statistics for the best G×E interaction models underlying essential hypertension susceptibility. The best *four*-order interaction model with maximum cross-validation consistency and minimum prediction error is indicated in bold. (B) Interaction dendrogram. The lines comprise a spectrum of lines representing a continuum from synergy to redundancy of G×G and G×E interactions with a variable strength. Brown lines represent the midway point between synergy and redundancy (additive interaction). On the redundancy end of the spectrum, the highest degree is represented by blue with a lesser degree represented by green. The synergy lines rang from red, representing a high degree of synergism (not present in dendrogram), to orange, representing a lesser degree of synergism. (C) Interaction entropy graph. Each SNP is shown in a rectangle box with the percent of entropy (main effect). Two-way G×G and G×E interactions are depicted as color lines accompanied by a percent of entropy (interaction effect). OR (95% CI), odds ratio with 95% confidence intervals; P_{perm}, permutation *P*-value for the interaction model. SNP, single-nucleotide polymorphism.

Figure 4 The relative expression levels of AHR pathway genes mRNA by different genotypes in 60 HapMap subjects of European descent.

Expression profiles were analyzed by the HumanWG-6 Expression BeadChip. The effects of SNPs such as A) AHR rs2066853, (B) AHRR rs2292596, (C) ARNT rs2228099, (D) CYP1A1 rs1048943, (E) CYP1A2 rs762551, (F) CYP1B1 rs1056836 and (G) NQO1 rs1800566 on mRNA levels of corresponding genes are evaluated by generalized linear models (GLM). Absence of carriers for

462VV CYP1A1 genotypes in this HapMap sample did not allow evaluating the correlation analysis for rs1048943. NA, not available.

Table 1 Baseline characteristics of the study groups

Baseline characteristics	Controls, n=819 n (%)	EH patients, n=1341 n (%)	*P*-value
Age, mean ± standard deviation	56.2±8.9	56.4±10.2	0.63
Males	393 (49.1%)	675 (52.9%)	0.09
Body mass index (kg/m^2), mean ± standard deviation	27.1 ± 7.4	27.7 ± 6.8	0.06
Antihypertensive medication use	-	979 (73.0%)	-
Positive family history of hypertension	422 (57.3%)	717 (64.0%)	**0,003***
Number of smokers (ever/never)	314 (39.0%)	407 (31.5%)	**0,001***

* means a significant difference between the groups.

Table 2 Genotype and allele frequencies of AHR pathway genes in EH patients and controls

Gene, polymorphism	Genotype, allele	Controls, n=819 n (%)[1]	EH patients, n=1341 n (%)[1]	*P*-value OR (95% CI)[2]	*P*-value $_{adj}$OR (95% CI)[3]
AHR, R554K (rs2066853)	554RR	658 (80.6)	1044 (79.0)	0.63	0.64
	554RK	142 (17.4)	252 (19.1)	1.12 (0.89-1.40)	1.12 (0.89-1.40)
	554KK	16 (2.0)	26 (2.0)	1.02 (0.55-1.92)	1.00 (0.53-1.88)
	554K	0.107	0.114	0.50 1.07 (0.88-1.31)	-
AHRR, P185A (rs2292596)	185PP	254 (31.4)	403 (31.2)	0.75	0.76
	185PA	408 (50.4)	636 (49.3)	0.98 (0.80-1.20)	0.98 (0.81-1.20)
	185AA	147 (18.2)	251 (19.5)	1.08 (0.83-1.39)	1.08 (0.83-1.39)
	185A	0.565	0.558	0.69 0.98 (0.86-1.11)	-
ARNT, 567C>G (rs2228099)	567CC	344 (43.2)	501 (38.8)	0.14	0.12
	567CG	351 (44.0)	618 (47.9)	1.21 (1.00-1.46)	1.21 (1.00-1.47)
	567GG	102 (12.8)	172 (13.3)	1.16 (0.87-1.53)	1.17 (0.88-1.55)
	567G	0.348	0.373	0.11 1.11 (0.98-1.27)	-
CYP1A1, I462V (rs1048943)	462II	691 (85.6)	1145 (86.6)	0.82	0.83
	462IV	112 (13.9)	171 (12.9)	0.92 (0.71-1.19)	0.92 (0.71-1.19)
	462VV	4 (0.5)	6 (0.5)	0.91 (0.25-3.22)	0.97 (0.27-3.46)
	462V	0.074	0.069	0.50 0.92 (0.72-1.17)	-
CYP1A2, -163C>A (rs762551)	-163AA	387 (47.3)	635 (47.6)	0.02	**0.015**
	-163AC	322 (39.3)	571 (42.8)	1.08 (0.90-1.30)	1.09 (0.90-1.31)
	-163CC	110 (13.4)	128 (9.6)	0.71 (0.53-0.94)	0.71 (0.53-0.94)
	154C	0.330	0.311	0.19 0.92 (0.80-1.05)	-
CYP1B1, V432L (rs1056836)	432VV	278 (33.9)	424 (31.8)	0.56	0.60
	432VL	390 (47.6)	651 (48.8)	1.09 (0.90-1.33)	1.09 (0.90-1.33)
	432LL	151 (18.4)	260 (19.5)	1.13 (0.88-1.45)	1.12 (0.87-1.44)
	432L	0.422	0.439	0.30 1.07 (0.94-1.21)	-
NQO1, P187S (rs1800566)	187PP	506 (63.0)	836 (62.7)	0.51	0.51
	187PS	252 (31.4)	437 (32.8)	1.05 (0.87-1.27)	1.04 (0.86-1.26)
	187SS	45 (5.6)	61 (4.6)	0.82 (0.55-1.22)	0.82 (0.55-1.22)
	187S	0.212	0.208	0.75 0.98 (0.84-1.14)	-

[1] Absolute number and percentage of individuals with particular genotype.

[2] Odds ratio with 95% confidence intervals (codominant genetic model).

[3] Odds ratio with 95% confidence intervals adjusted for age and gender.

Bolded is statistically significant *P*-value with two degrees of freedom.

Table 3 Epistatic interactions between AHR pathway SNPs in essential hypertension

(Gene-gene interactions are evaluated by SNPassoc package for R [52])

SNPs	Genetic models	*AHR* rs2066853	*AHRR* rs2292596	*ARNT* rs2228099	*CYP1A1* rs1048943	*CYP1A2* rs762551	*CYP1B1* rs1056836	*NQO1* rs1800566
AHR rs2066853	Codominant	0.644	0.746	0.838	0.320	0.483	0.874	**0.017**
	Dominant	0.379	0.738	0.577	0.476	0.863	0.753	0.166
	Recessive	0.944	0.643	0.281	-	0.886	-	**0.004***
	Overdominant	0.348	0.346	0.706	0.468	0.589	0.433	0.065
AHRR rs2292596	Codominant	0.802	0.759	0.970	0.255	0.257	0.587	0.466
	Dominant	0.967	0.930	0.606	0.960	0.383	0.494	0.102
	Recessive	0.992	0.468	0.966	**0.041**	0.105	0.252	0.279
	Overdominant	0.647	0.626	0.674	0.523	0.320	0.699	0.319
ARNT rs2228099	Codominant	0.824	0.750	0.124	0.584	**0.041**	0.696	**0.041**
	Dominant	0.605	0.753	**0.043**	0.161	**0.012**	0.287	0.409
	Recessive	0.874	0.949	0.702	0.805	0.134	0.500	0.157
	Overdominant	0.545	0.752	0.083	0.496	**0.008***	0.829	**0.013***
CYP1A1 rs1048943	Codominant	0.760	0.736	0.807	0.833	0.944	**0.002**	0.530
	Dominant	0.470	0.923	0.521	0.549	0.795	**0.001***	0.380
	Recessive	0.999	0.977	0.973	0.974	0.416	0.210	0.641
	Overdominant	0.459	0.465	0.501	0.547	0.983	**0.003**	0.267
CYP1A2 rs762551	Codominant	0.672	0.665	0.122	0.798	**0.014**	0.247	0.120
	Dominant	0.929	0.999	0.915	0.701	0.912	0.418	0.969
	Recessive	0.936	0.414	0.679	0.927	**0.005**	0.058	0.411
	Overdominant	0.375	0.518	0.080	0.552	0.097	0.980	0.879
CYP1B1 rs1056836	Codominant	0.705	0.808	0.448	0.855	0.510	0.599	0.864
	Dominant	0.435	0.908	0.207	0.574	0.861	0.321	0.778
	Recessive	0.987	0.582	0.678	0.970	0.457	0.600	0.597
	Overdominant	0.607	0.712	0.467	0.678	0.648	0.605	0.451
NQO1 rs1800566	Codominant	0.599	0.753	0.500	0.748	0.550	0.640	0.502
	Dominant	0.941	0.967	0.967	0.882	0.965	0.893	0.915
	Recessive	0.909	0.497	0.697	0.690	0.333	0.697	0.278
	Overdominant	0.561	0.563	0.554	0.624	0.590	0.564	0.539

The upper part of the matrix contains the *P*-values for epistatic interactions evaluated by log-likelihood ratio (LRT) test. The diagonal contains the *P*-values from LRT for the crude effect of each SNP. The lower triangle contains the *P*-values from LRT comparing the two-SNP additive likelihood to the best of the single-SNP models. Bolded are statistically significant *P*-values for SNP-SNP interactions (* means most significant *P*-values for a particular model). *P*-values are adjusted for age and gender.

Table 4 Best gene-gene and gene-smoking interactions significantly associated with the risk of essential hypertension*

(Models are obtained by Model-Based Multifactor Dimensionality Reduction method, MB-MDR package for R [59])

	G×G / G×E interaction models	NH	*beta* H	WH	NL	*beta* L	WL	P_{perm}
	Two-order interaction models							
1	*CYP1A1* rs1048943× smoking	1	0.263	7.79	2	-0.342	12.28	0.003
2	*CYP1A2* rs762551 × smoking	2	0.323	11.83	2	-0.390	12.08	0.003
3	*CYP1A2* rs762551 × *ARNT* rs2228099	1	0.422	12.53	1	-0.598	7.66	0.011
	Three-order interaction models							
1	*CYP1A1* rs1048943 × *CYP1B1* rs1056836 × smoking	2	0.277	8.10	2	-0.608	22.12	<0.001
2	*CYP1A1* rs1048943 × *CYP1A2* rs762551 × *CYP1B1* rs1056836	0	NA	NA	4	-0.636	21.26	0.001
3	*ARNT* rs2228099 × *NQO1* rs1800566 × smoking	1	0.264	4.42	4	-0.567	19.04	0.003
	Four-order interaction models							
1	*CYP1A1* rs1048943 × *CYP1A2* rs762551 × *CYP1B1* rs1056836 × smoking	2	0.345	7.49	4	-0.896	31.51	<0.001
2	*ARNT* rs2228099 × *CYP1A1* rs1048943 × *CYP1B1* rs1056836 × smoking	1	0.537	5.02	4	-0.617	22.07	0.007
3	*ARNT* rs2228099 × *CYP1A1* rs1048943 × *NQO1* rs1800566 × smoking	1	0.274	4.21	3	-0.845	19.15	0.008

NH - number of significant High risk genotypes in the interaction.

beta H - regression coefficient for High risk exposition in the step2 analysis. NA – not available.

WH - Wald statistic for High risk category.

NL - number of significant Low risk genotypes in the interaction.

beta L - regression coefficient for Low risk exposition in the step2 analysis.

WL - Wald statistic for Low risk category.

P_{perm} - Permutation *P*-value for the interaction model. The models were adjusted for age and gender.

* Full list of statistically significant models for gene-gene and gene-smoking interactions are present in Supplementary table 1.

Table 5 Cross-validation statistics for best models of gene-gene and gene-smoking interactions in essential hypertension (Models are obtained by Multifactor Dimensionality Reduction method, version 3.0.2)

	G×G / G×E interaction models	OR (95%CI)	Testing Balanced accuracy	Cross-validation Consistency	P_{perm}
	Two-order interaction models				
1	*CYP1A1* rs1048943× smoking	1.53 (1.28-1.83)	0.543	10/10	< 0.0001
2	*CYP1A2* rs762551 × smoking	1.46 (1.23-1.74)	0.539	10/10	< 0.0001
3	*CYP1A2* rs762551 × *ARNT* rs2228099	1.38 (1.16-1.63)	0.513	10/10	0.0003
	Three-order interaction models				
1	*CYP1A1* rs1048943 × *CYP1B1* rs1056836 × smoking	1.86 (1.52-2.27)	0.530	10/10	< 0.0001
2	*CYP1A1* rs1048943 × *CYP1A2* rs762551 × *CYP1B1* rs1056836	2.18 (1.70-2.79)	0.503	10/10	< 0.0001
3	*ARNT* rs2228099 × *NQO1* rs1800566 × smoking	1.66 (1.40-1.97)	0.529	10/10	< 0.0001
	Four-order interaction models				
1	*CYP1A1* rs1048943 × *CYP1A2* rs762551 × *CYP1B1* rs1056836 × smoking	1.89 (1.59-2.26)	0.525	10/10	< 0.0001
2	*ARNT* rs2228099 × *CYP1A1* rs1048943 × *CYP1B1* rs1056836 × smoking	1.94 (1.63-2.31)	0.512	10/10	< 0.0001

3	*ARNT* rs2228099 × *CYP1A1* rs1048943 × *NQO1* rs1800566 × smoking	1.85 (1.56-2.20)	0.515	10/10	< 0.0001

[1]Odds ratio with 95% confidence intervals
[2]P_{perm} - Permutation *P*-value for the interaction model.

Table 6 Best models of gene-gene interactions associated with essential hypertension stratified by cigarette smoking

(Models are obtained by Model-Based Multifactor Dimensionality Reduction method)

	G×G interaction models	NH	*beta* H	WH	NL	*beta* L	WL	P_{perm}
	Two-, three- and four-order interaction models in smokers							
1	*CYP1A1* rs1048943 × *CYP1B1* rs1056836	1	0.434	5.98	2	-0.499	9.93	0.014
2	*ARNT* rs2228099 × *CYP1A1* rs1048943	0	NA	NA	1	-0.952	8.20	0.03
3	*ARNT* rs2228099 × *CYP1A2* rs762551	0	NA	NA	1	-1.232	8.25	0.05
4	*CYP1A1* rs1048943 × *CYP1A2* rs762551 × *CYP1B1* rs1056836	1	0.440	3.08	4	-0.795	20.02	< 0.002
5	*ARNT* rs2228099 × *CYP1A1* rs1048943 × *CYP1A2* rs762551	0	NA	NA	2	-1.520	15.04	0.004
6	*CYP1A1* rs1048943 × *CYP1B1* rs1056836 × *NQO1* rs1800566	1	0.364	3.04	3	-1.344	14.77	0.01
7	*AHR* rs2066853 × *CYP1A1* rs1048943 × *CYP1A2* rs762551 × *CYP1B1* rs1056836	1	0.507	3.75	3	-1.637	19.81	0.004
8	*ARNT* rs2228099 × *CYP1A1* rs1048943 × *CYP1A2* rs762551 × *CYP1B1* rs1056836	0	NA	NA	3	-1.988	16.02	0.03
9	*ARNT* rs2228099 × *AHRR* rs2292536 × *CYP1A1* rs1048943 × *NQO1* rs1800566	1	0.631	4.401	4	-0.951	15.25	0.034
	Two-, three- and four-order interaction models in non smokers							
1	*ARNT* rs2228099 × *CYP1A2* rs762551	1	0.460	8.45	1	-0.254	2.83	0.043
2	*CYP1A2* rs762551 × *NQO1* rs1800566	0	NA	NA	2	-0.658	8.61	0.049
3	*ARNT* rs2228099 × *CYP1A2* rs762551× *CYP1B1* rs1056836	3	0.580	10.45	3	-0.826	12.39	0.096
4	*ARNT* rs2228099 × *AHRR* rs2292536 × *CYP1B1* rs1056836	0	NA	NA	3	-0.697	11.80	0.126
5	*AHR* rs2066853 × *ARNT* rs2228099 × *AHRR* rs2292536 × *CYP1B1* rs1056836	0	NA	NA	5	-0.847	21.41	0.016

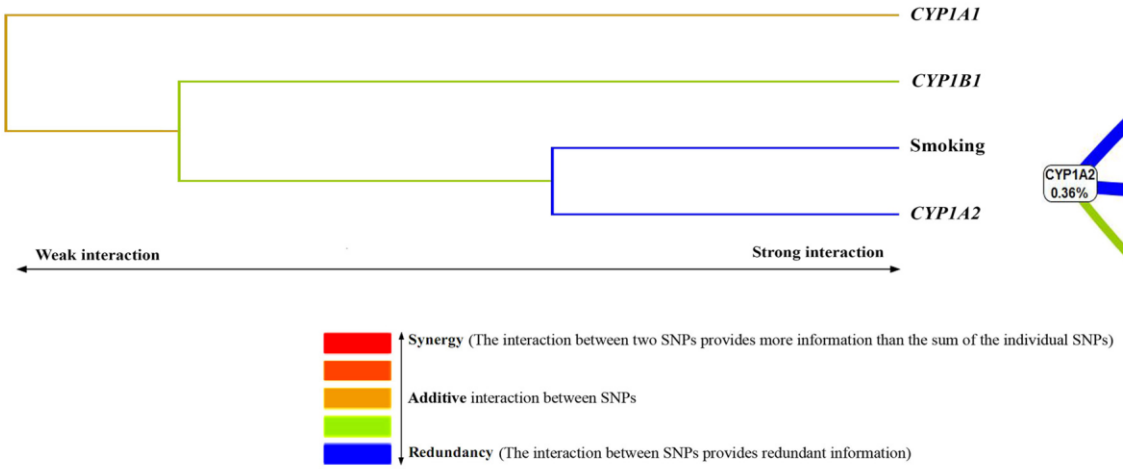
6	*ARNT* rs2228099 × *CYP1A2* rs762551× *CYP1B1* rs1056836 × *NQO1* rs1800566	2	1.216	7.41	6	-0.769	22.00	0.022
7	*AHR* rs2066853 × *ARNT* rs2228099 × *CYP1A2* rs762551× *CYP1B1* rs1056836	2	0.772	6.27	5	-0.786	19.84	0.04

NH - number of significant High risk genotypes in the interaction.
beta H - regression coefficient for High risk exposition in the step2 analysis. NA – not available.
WH - Wald statistic for High risk category.
NL - number of significant Low risk genotypes in the interaction.
beta L - regression coefficient for Low risk exposition in the step2 analysis.
WL - Wald statistic for Low risk category. P_{perm} - Permutation *P*-value for the interaction model.

A

No factor	Best G×E interaction models obtained by the MDR analysis	OR (95%CI)	Prediction error	Cross-validation consistency	P_{perm}
2n	*CYP1A1* rs1048943 × smoking	1.53 (1.28-1.83)	47.5%	10/10	<0.0001
3n	*CYP1A1* rs1048943 × *CYP1B1* rs1056836 × smoking	1.86 (1.52-2.27)	47.0%	10/10	<0.0001
4n	***CYP1A1* rs1048943 × *CYP1A2* rs762551 × *CYP1B1* rs1056836 × smoking**	**1.89 (1.59-2.26)**	**45.7%**	**10/10**	**<0.0001**

B



C