

Original Research

Genes and Genetic Variations Involved in the Development of Hypertension: Focusing on a Greek Patient Cohort

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Introduction: Essential hypertension (HTN) is a multifactorial disease involving environmental, genetic and other factors. Over the past years, genetic studies of essential HTN have increased dramatically but the molecular mechanisms involved are still unknown. As part of a research program coordinated by Boston university (USA), we studied the role of various genes and single nucleotide polymorphisms (SNPs) in the inheritance or the onset of HTN in African-American, Caucasian-American and Greek families.

Methods: Among 128 Greek families with a history of HTN, we studied 1474 people. Of the total examined, 273 men and 286 women were hypertensive. Based on 410 DNA samples from the hypertensive subjects, different SNPs were examined. An overall meta-analysis of the results from the Greek families, as well as a comparison with the 2 other groups (African-Americans and Caucasian-Americans), was performed.

Results: We report SNPs that are associated with the inheritance of HTN and are located either at the promoters of N-methyltransferase and catalase genes, or within the coding region of NEDD4L ubiquitin ligase gene, or SNPs in mitochondrial DNA of hypertensive probands. Furthermore, we clarified the role of hereditary predisposition in the development of HTN, showing that the presence of maternal HTN was significantly higher in African-Americans and Greeks compared to Caucasian-Americans (81.7%, 84.8%, and 65%), while the paternal HTN showed no such difference (50%, 48.3% and 44.9%), respectively.

Conclusions: Although genetic factors that were correlated with HTN were identified, it was not possible to identify a single gene that should be targeted for the treatment of HTN. Nevertheless, the important role of the maternal hereditary predisposition to HTN in the Greek patients and the responsible genetic factors involved should be further examined.

Essential hypertension (HTN) is a common multifactorial disorder that involves complex genetic, vasoconstrictive, environmental and other risk factors.¹ The hereditary nature of essential HTN has been established in many family studies: up to 30% of blood pressure variability is considered to be genetically determined and an individual's genetic predisposition to hypertensive dis-

ease ranges from 15 to 35%.² The risk of familial heritability of essential HTN is positively correlated with the number of hypertensive relatives. When a person has a first-degree hypertensive relative, then the risk of developing HTN is twice that of the general population. The risk quadruples when the number of first-degree relatives of hypertensive subjects rises.^{3,4}

Individuals belonging to the same

family share a common genetic background and common environmental conditions. In an effort to distinguish the genetic from the environmental influences, studies were conducted in families with biological and adopted children. The correlation for HTN prevalence is strongest among biological brothers (or between natural parents and children) and weakest among adopted children (or between parents and adopted children), proving that the observed incidence of HTN among biological members of a family has a significant genetic predisposition. Finally, studies in twin siblings showed a greater correlation between blood pressure values among monozygotic twins compared with dizygotic, further emphasizing the role of genes in blood pressure phenotype.^{5,6} These studies are considered to fully reflect genetic predispositions, since the twins, whether monozygotic or dizygotic, are born at the same time and are likely to be exposed to similar environmental conditions.⁶

There is increasing evidence that complex interactions between genes and environment play an important role in determining the risk of various common diseases such as HTN.⁷ It is now accepted that certain environmental factors act as regulators of gene expression, promoting the development of a disease and speeding or slowing its course in persons with a clear genetic predisposition.^{8,9}

Over the past few years, genetic studies of essential HTN have increased dramatically; nevertheless, their conclusions allowed only a partial understanding of the molecular mechanisms involved in its development. These studies mostly concern correlation analyses between HTN development and specific gene loci, either by examining the entire genome (genome-wide association studies, GWAS), or focusing on specific genes or polymorphisms (candidate gene association studies, CGAS).

It should be noted that most of these studies failed to detect any genes or genetic polymorphisms that could be definitively associated with the development of disease. This could be attributed to the fact that HTN seems to be the outcome of many genetic changes, which act cumulatively leading to a pathological phenotype.¹⁰ However, both GWAS and CGAS have steadily led to the identification of certain genetic loci, or even pathological alleles, which are directly related to the development of essential HTN.¹¹

In the context of genetic testing for the identification of any genes involved in the development of HTN, the antihypertensive clinic of the Cardiology

Clinic of the Tzaneio Hospital (Piraeus, Greece) participated in a major research program coordinated by the Laboratory of Hypertension and Atherosclerosis of Boston University Medical School (USA), funded by the National Institutes of Health (NIH-SCORE, USA). Our antihypertensive clinic participated as an associate centre in this research program, but also as an independent study centre because of the large number of hypertensive participants, under the supervision of Dr H. Gavras, professor at Boston University Medical School.

These patients' samples are part of larger cohort study, which included families of Greek Caucasians, African-Americans and Caucasian-Americans. The sample of Greek patients was very important, as it included a large number of families with children, many of whom were hypertensive and whose genetic material has been studied extensively.¹² Evidence from the Greek families was analyzed separately and also compared with the data from African-Americans and Caucasian-Americans. This study presents all the genes and polymorphisms of nuclear DNA, the corresponding polymorphisms in mitochondrial DNA that could be related to the development of essential HTN, as well as a statistical analysis of maternal and paternal contributions to the inheritance of HTN.

Methods

Subjects

More than 500 families from the USA, Greece, Brazil, South Africa and other countries were included in the research protocol. In this study, our sample included 128 families of Greek origin (Caucasians), regardless of family history, who gave their written consent to participate in this research protocol, according to research regulations and ethics. Subsequently, patients underwent a full clinical examination and all the information required by the protocol regarding them and their family members was recorded. All blood samples from the hypertensive subjects were sent on dry ice to Boston University for genetic analysis, along with copies of the patients' medical records. Boston Medical Center Institutional Review Board for Human Subjects extensively reviewed and approved this study.

Analysis

To study the polymorphisms of the NEDD4L ubiqu-

itin ligase gene, total DNA was isolated from whole blood cells and quantitated. Nuclear DNA sequencing was performed (Applied Biosystems) after exon target amplification by polymerase chain reaction (PCR) and purification of the product (AMPure solid-phase reversible immobilisation, Agencourt). Subsequently, analysis of single nucleotide polymorphisms (SNPs) was performed in an ABI 3730 DNA analyser (Applied Biosystems).¹³

For the study of the PMNT and B1 & B2 receptors of bradykinin genes, as well as catalase gene promoter, total DNA was isolated from whole blood cells (Puregene kit, Gentra Systems). As previously reported,^{14,15} different genotypes were detected and identified with the Homogenous MassEXTEND test (Sequenom).

For the analysis of mitochondrial DNA, again total DNA was isolated from whole blood cells and quantitated, and mitochondrial DNA (mtDNA) was amplified by PCR, using 58 pairs of reference oligonucleotides designed at the National Institute of Standards and Technology, USA.¹⁶ The methodology for isolating total DNA and PCR conditions has been described previously.¹⁷ The double-stranded DNA products were sequenced, compared with reference mtDNA sequences using the BLAST 2 SEQUENCES program, and specific gene mutations were identified. The analysis was based on mutations of the MITOMAP database¹⁸ using the MitoAnalyzer program, which was also developed in the National Institute of Standards and Technology, USA.

Statistical analysis

Continuous variables with normal distribution were expressed as mean \pm standard deviation, or in the case of asymmetric distribution as median and range. The categorical data are presented as absolute and relative frequencies. Comparisons between groups were performed using the Student t-test or χ^2 test, as appropriate. Covariance analysis was used to examine any differences between groups after adjustment for confounding factors. For the survival part of the study, survival analysis was conducted to compare the risk of HTN between mothers and fathers, and among the offspring of parents suffering from HTN. All statistical analyses were performed using the statistical package SPSS 15.0 (SPSS Inc., Chicago, IL, USA), and a p-value <0.05 was considered as statistically significant.

Results

Meta-analysis of Greek families with hypertensive probands

A total of 1474 people belonging to Greek families with hypertensive members were examined. Of the total examined, 273 men, aged 58 ± 8 years, were diagnosed with HTN, and 85.71% of them were receiving antihypertensive therapy. Among the examined females, 286 women, aged 63 ± 6 years, were diagnosed with HTN and 90.5% of them were being treated. Of the treated patients, 28.6% received monotherapy while 71.4% received combined antihypertensive therapy. Of the monotherapy recipients, 48.2% reached their target blood pressure values, compared to 36% of the combined therapy recipients. Of all the treated patients, 26% of men and 28% of women reached their target blood pressure values (140/90 mmHg). Of the 559 hypertensive patients, 74.8% had a body mass index (BMI) >24.9 , 25% had hyperlipidaemia and 15.5% were diabetic. Of the 863 non-hypertensive probands, 41% had BMI >24.9 , 6.4% had hyperlipidaemia and 4% were diabetic. In the 128 families with hypertensive offspring that were examined, we analysed the parents' data collectively and found that only 9.4% of the fathers were hypertensive. In contrast, we observed that a very high proportion (44.5%) of the examined families included hypertensive mothers; in 36.7% of the families both parents were hypertensive, while in 8.6% none of the two parents had elevated blood pressure levels. A rough analysis of the parents' data revealed a strong maternal influence on the inheritance of essential HTN.

Blood samples were obtained from 410 individuals (all members of the 128 families) who met the protocol's inclusion criteria, and genetic analysis was performed. For the present study, we analysed the results of genetic analysis collectively and the results for the Greek population individually only in those cases where there were significant findings, and always in comparison with the other patient groups (white Americans and African-Americans). The reported polymorphisms are associated with the inheritance of HTN and are located either in the promoters of N-methyltransferase and catalase genes, or within the coding region of NEDD4L ubiquitin ligase gene, or polymorphisms in mitochondrial DNA of hypertensive probands. Furthermore, it has been shown that polymorphisms of the A2b adrenergic receptor gene are not significantly associated with the occurrence of

essential HTN. Finally, a thorough statistical analysis of maternal and paternal contributions to the inheritance of HTN was performed. These results have been presented separately in studies already published in international journals^{12-15,19-22} and have contributed substantively to the study of inheritance and development of essential HTN.

Identification of polymorphisms in the promoter of the N-methyltransferase gene

As for the genetic loci implicated in essential HTN, several studies have suggested that a region on human chromosome 17 may be linked to blood pressure regulation.^{19,23,24} The genetic analysis from samples of hypertensive patients in this study revealed a correlation between a region of human chromosome 17 and the development of essential HTN. Subsequently, a positive linkage between HTN and a specific region between D17S1814 and D17S800 of the 17th chromosome was reported, in which the N-methyltransferase (PNMT) gene is located (position 17q21), approximately 38cM from the chromosome's telomere.²² A recent study focused on the association of two single nucleotide polymorphisms (SNPs) in the region of the PNMT promoter, PNMT-148 and PNMT-343 and the development of essential HTN in a patient cohort of three different ethnicities: African-Americans, white Americans and Greeks.¹⁴ It should be emphasised that these polymorphisms were previously found to be positively associated with the development of HTN only in African-Americans.²⁵

Identification of gene polymorphisms in NEDD4L ubiquitin ligase gene

The NEDD4L gene encodes a ubiquitin ligase that is important for the downregulation of kidney epithelial Na⁺ channels. Numerous polymorphisms located on the first exon of this gene have been associated with the development of HTN in African-Americans, as well as white Americans and Greeks. Of the 26 SNPs identified, 10 were associated with orthostatic HTN. In African-Americans, 7 SNPs (rs10515976, rs4149591, rs4149601, rs513563, rs182383, rs7228980, rs9953409) showed a significant allelic association or genotypic association. One SNP (rs4149601) was associated with HTN and is known to result in abnormal splicing and non-functional protein. Two (rs513563 and rs3865418) were also found to be associated with HTN in white Americans and two other

SNPs (rs4149589 and rs3865418) were associated with HTN in Greek whites. The fact that polymorphisms were identified and related to blood pressure regulation in 3 different cohorts further strengthens the hypothesis that the genetic background is a key factor in essential HTN.¹³

Identification of polymorphisms in the promoter region of catalase gene

We found that the combination of 2 SNPs, the CAT-844 AA and CAT-262 CT or TT at the promoter region of catalase (CAT) gene, was significantly associated with the development of essential HTN in Greek hypertensive patients. In the sample of African-Americans who were examined there was no statistically significant association.¹⁵

The gene polymorphisms of A2b adrenergic receptor gene are not related to the occurrence of essential HTN

Among the genetic determinants of essential HTN, the genes that encode proteins involved in blood pressure regulation, such as genes of alpha- and beta-adrenergic receptors, are obvious candidates. It is known that the signalling pathway of the A2b adrenergic receptor plays an important role in the sympathetic nervous system and mediates the action of epinephrine and norepinephrine. To evaluate the potential association between the A2b receptor and essential HTN, genetic variants of the A2b gene in 108 pairs of brothers who had HTN, including Greek patients, were analysed.²⁰ Extensive genetic and molecular analysis revealed that there are two main forms of the receptor, which differ in the presence of either nine or twelve glutamic acid residues in the acidic region of the third cytoplasmic loop of the receptor protein. However, none of these variants was found to contribute substantially to a genetic predisposition to essential HTN.

Maternal but not paternal history of HTN is associated with the offspring's blood pressure level

A previous study by our research group²¹ showed that relatives of hypertensive patients are predisposed to the development of essential HTN, supporting the genetic basis of the disease. In this study, it was reported that the incidence of HTN was higher for the parents and the brothers of patients compared with their wives. Moreover, the probability of stroke was

statistically significantly higher among hypertensive parents and brothers compared with normotensive patients, suggesting that these two pathologic conditions are likely to be genetically controlled.

A more detailed statistical analysis of the same sample of Greek patients showed that when the mother is hypertensive, then the chance of having hypertensive offspring is greater than for normotensive mothers.¹² The examined patients' sample was first categorised according to the known or unknown HTN status of their parents. The proportion of fathers with unknown hypertensive status was greater than that of the mothers, but this difference was not statistically significant (3.3% vs. 5%, $p=0.05$). Importantly, a statistically significantly higher incidence of hypertensive mothers compared with the proportion of hypertensive fathers was reported (84.8% vs. 48.3%, $p=0.01$), further supporting the hypothesis that maternal heredity plays a significant role in the inheritance of HTN.¹²

At this point it should be noted that the same observation was also reported in hypertensive African-American and Caucasian-American patients¹², which strengthens our hypothesis. In the same study, it was shown that the proportion of hypertensive mothers ranged from 65-85% while the corresponding figure for fathers was only 50%.¹² The results from all three patient groups (African-Americans, Caucasian-Americans and Greeks) were studied collectively in a survival meta-analysis. The cumulative lifetime risk distribution for HTN was significantly different for mothers and for fathers ($p<0.001$). Although the risk was similar at early ages, after the age of 55 years mothers had a higher risk of developing HTN. The mean age of HTN onset for the mothers was not significantly different from that of the fathers (56.7 years vs. 55.7, $p>0.25$).¹²

To further study the maternal influence on the familial aggregation of HTN, the hypertensive status of the probands was examined, again drawing information from the questionnaires. For Greek Caucasians, 58.3% of the offspring of the affected mothers were hypertensive, while the corresponding rate for fathers was only 30% ($p=0.0192$). Similar effects, although marginal and not statistically significant, were observed in African-Americans and Caucasian-Americans. Our colleagues assessed the lifetime risk for all three patient groups, ignoring their ethnicity. As expected, the risk of developing HTN is greater when both parents are affected and lower for those with neither parent affected.¹² The offspring of hyperten-

sive mothers had a significantly higher risk of HTN ($p<0.01$) compared to offspring of hypertensive fathers.¹²

Mitochondrial genome mutations in hypertensive patients

This statistically significant correlation between blood pressure levels in mother and offspring led us to study and identify mtDNA polymorphisms that could be associated with HTN. As the mitochondrial genome is inherited through the mother, a hallmark of mitochondrial disorders is excess maternal transmission.²⁶ A complete mtDNA sequencing of 20 probands with HTN likely due to mitochondrial involvement (10 African-Americans and 10 whites, one of them Greek) allowed us to detect novel and previously reported mtDNA variants.¹⁷ Their comparison with reference RNA sequences, known as Cambridge or CRS, revealed 297 point mutations, including 24 in rRNA genes, 15 in tRNA genes and 46 amino acid variants, while the rest were detected in non-coding regions or were synonymous mutations.¹⁷ Since the CRS was derived from an individual of European descent, the number of variants observed in African-Americans was greater than that of whites, as expected. Among the coding region mutations, 30 variants appear to be novel, as they are not registered in the MITOMAP database, the most comprehensive mitochondrial database that catalogues all reported mtDNA variants identified in populations worldwide.¹⁸ Of 20 hypertensive probands, 13 carried at least one novel variant, usually in combination with already documented mutations. Two probands (CG2 and CG3) carried only silent mutations.^{17,26}

Discussion

Essential HTN is a complex disorder that results from the interplay of genetic and environmental risk influences. It affects about 20% of the population and is one of the main risk factors for stroke, myocardial infarction and end-stage renal failure.¹² Despite the fact that a genetic predisposition to HTN has been recorded for decades,²¹ the mode of its inheritance remains unclear; the nature and the number of the underlying susceptibility variants have not yet been fully determined.

The genetic studies of our group have successfully led to the detection and correlation of specific polymorphisms that are associated with blood pres-

sure regulation^{14,19,22-24,27} and possibly with essential HTN. There are numerous candidate genetic factors that could be identified, based on dysfunctions of the cardiovascular system and kidneys of hypertensive patients, and in the already known pathway of HTN physiology. The effort to detect genes associated with essential HTN has several major limitations: the large number of genes that can regulate blood pressure, the combined effect of their expression, the potential genetic polymorphisms of each gene, the phenotypic heterogeneity of patients, as well as different environmental factors that affect blood pressure.^{10,28,29}

Several studies based on hypertensive patients have reported polymorphisms in both mitochondrial¹⁷ and nuclear^{12,14,15,19,20,22,30} genes. In the present study, we performed a detailed meta-analysis of several genetic regions that have been implicated in blood pressure regulation as potential genetic factors for essential HTN in Greek hypertensive patients and compared our observations with those from white American and African-American patients. A limitation of this study is the use of family questionnaire data to determine the HTN status of the parents. Although other studies have proven that this method is reasonably accurate,³¹ the results of this study are not as rigorous as a study in which medical history is verified for all relatives.

Our first finding was a polymorphism in the promoter region of the N-methyltransferase (PNMT) gene, which was found to be significantly associated with the development of essential HTN in a sample of hypertensive African-Americans, but not in Greek white or American white populations. The PNMT gene is located on a specific region of chromosome 17, which has been found in previous studies to influence blood pressure.^{19,23,24} This polymorphism may affect the transcriptional activity of the PNMT gene or may be in linkage disequilibrium with another functional polymorphism. There may be many possible reasons for this significant association in African-Americans but not in the two white populations. It is possible that the PNMT gene plays a smaller role in HTN susceptibility in whites, or that the polymorphism is not functional—it is known that linkage disequilibrium patterns differ between ethnic groups.¹⁴

The next recorded single-nucleotide polymorphisms are located on the NEDD4L ubiquitin ligase gene, which is directly associated with the development of HTN in US whites, African-Americans and Greek whites. One of these SNPs (rs4149601A allele) is associated with elevated blood pressure and

leads to the abnormal maturation of the transcribed mRNA encoding a non-functional protein. This SNP was predicted to reduce the ubiquitination and degradation of proteins that function as ion pumps of Na⁺ in epithelial cells. A higher density of epithelial Na⁺ channels or a longer residence on the cell surface leads to increased transmembrane Na⁺ ion transport in epithelial cells, thus shifting to a positive Na⁺ balance and a rise in blood pressure.¹³ It should be noted, though, that only some transcripts are affected by this SNP and contain this alternatively spliced exon.

Another interesting genetic factor is the strong association between two different SNPs in the promoter of catalase gene and HTN in the cohort of Greek patients.¹⁵ The CAT-844AA SNP that was identified in this study¹⁵ has also been reported in a previous study conducted in Chinese hypertensive patients; high blood pressure levels were observed in patients homozygous for CAT-844AA.³² The CAT-844 SNP is located in a promoter region recognized by numerous transcription factors, thus affecting the transcriptional activity of the catalase gene. More specifically, the CAT-844G allele establishes the recognition and commitment of MZF1 and AP2 transcription factors, while the CAT-844A allele affects the binding sites of Ikaros-2 and LYK-1 transcription factors. This was the first study to implicate genetic variation of the catalase gene in the susceptibility to essential HTN.³² More importantly, a second SNP (CAT-262T allele) of the catalase promoter region was reported for the first time to be significantly associated with high blood pressure levels in Greek patients. This SNP promotes the binding of AP-1 and SP-1 transcription factors to the catalase promoter, leading to increased expression of the catalase enzyme.³³

Efforts to identify genetic determinants of HTN have been directed primarily at the nuclear genome, whereas the role of the mitochondrial genome remained unexplored for years. The hypothesis that maternal heredity plays a significant role in the inheritance of HTN was supported by statistical but not genetic data. As already mentioned, it appears that the offspring of hypertensive mothers have a statistically significant higher risk of developing HTN compared to the offspring of hypertensive fathers.¹² A limitation of this study was that the environmental and heritable influences could not be distinguished. For example, it is possible that a common environmental factor shared between mother and offspring, but not shared between the parents, could potentially result in the

pattern observed. On the other hand, the offspring usually develop HTN many years after leaving their parents' house, so the common environment is less likely to be of great significance.

Genetic data on mtDNA polymorphisms of hypertensive patients that are likely to contribute to the inheritance and development of the disease further support this hypothesis. It is now widely accepted that mtDNA mutations contribute decisively to the development of genetic diseases,³⁴ some of which are associated with HTN. For example, the A10398G mutation in NADH dehydrogenase subunit 3 gene identified in 12 hypertensive individuals has been shown to occur with increased frequency in African Americans with HTN-associated end-stage renal failure.³⁵ Therefore, all mitochondrial mutations presented in the study of Schwartz and colleagues¹⁷ can serve as a starting point for further studies based on hypertensive patients, as it seems they have the potential to contribute, individually or synergistically, to the development of essential HTN.

Collectively, our meta-analysis shows that the development as well as the inheritance of essential HTN is directly related to the genetic diversity in the promoter region of specific nuclear genes and the diversity in mitochondrial DNA. It was also concluded that single SNPs might not be as important as the synergistic effect of the interaction between different SNPs. The scientific community is now turning from the simplistic single SNP association to more complex haplotype-based association studies, in an effort to elucidate the entire spectrum of genetic factors that cause essential HTN.

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