Altered erythrocyte membrane protein composition mirrors pleiotropic effects of hypertension susceptibility genes and disease pathogenesis

Alexey V. Polonikov[a], Dmitry V. Ushachev[b], Vladimir P. Ivanov[a], Mikhail I. Churnosov[c], Maxim B. Freidin[d], Alexander V. Ataman[e], Victoria Yu Harbuzova[e], Marina A. Bykanova[a], Olga Yu Bushueva[a], and Maria A. Solodilova[a]

Objective: The study was designed to assess the effects of polymorphisms in genes associated with essential hypertension on the variation of erythrocyte membrane proteins (EMPs) in hypertensive patients.

Methods: Major EMPs content was analyzed in blood from 1162 unrelated Russians (235 hypertensive patients, 176 healthy controls, and 751 random individuals from the Central Russia population). Essential hypertension patients were genotyped for 11 polymorphisms of essential hypertension susceptibility genes including *ADD1* (rs4961), *GNB3* (rs5443, rs16932941), *NOS3* (rs1799983, rs2070744), *ACE* (rs5186), *AGTR1* (rs5186), *AGT* (rs699, rs4762), *MR* (rs5534), and *TGFB1* (rs1800471). EMP contents and their relationship with the genetic loci were analyzed using various statistical tests.

Results: Sex-specific differences in EMP contents between the cases and controls were observed. Regardless of sex, hypertensives exhibited mainly decreased levels of alpha (SPTA1) and beta-spectrin (SPTB) and increased levels of glucose transporter (GLUT1) as compared with healthy subjects ($P \leq 0.001$). EMP correlated differently in essential hypertension patients and controls. Almost 70% of the joint variation in the EMP levels is explained by five gender-specific principal components. The essential hypertension susceptibility genes showed considerable effects on the levels of spectrins and glucose transporter. A joint variation of the genes explained about half the total polygenic variance in the GLUT1, SPTA1, and SPTB levels in hypertensives.

Conclusions: The study showed that essential hypertension susceptibility genes are the important factors of the inherited EMP variation, and their pleitropic effects may be mirrored in the altered expression of genes encoding cytoskeletal proteins and those related to intracellular glucose metabolism.

Keywords: cytoskeleton, erythrocyte membrane proteins, essential hypertension, genetic polymorphism, genetic susceptibility, glucose metabolism, glucose transporter, molecular mechanisms, quantitative variation, spectrin

INTRODUCTION

Hypertension is a global public health issue and is responsible for at least 45% of deaths owing to heart disease and 51% of deaths owing to stroke [1]. The number of deaths attributable to hypertension rose from 7.6 to 9.4 million estimated for a period between 2000 and 2013 [2,3]. Hypertension is known to be a multifactorial disease arising from a complex interplay between multiple genetic and environmental factors jointly contributing to disease susceptibility [4,5]. Despite decades of intensive basic and clinical research, intrinsic mechanisms underlying essential hypertension (essential hypertension), the common form of hypertension, remain far from being completed [6]. There is a substantial progress toward detection of genes underpinning distinct molecular mechanisms of the disease [5,7,8], and wide spectrum of intermediate phenotypes underlying high BP and essential hypertension has been identified [6,9].

A number of studies have shown that alterations in structural and functional properties of the cell membrane may represent disease-associated intermediate phenotypes reflecting the particular mechanisms of essential hypertension [10–18]. An elevated erythrocyte Na^+-Li^+ countertransport is one of the best known intermediate phenotypes of the membrane abnormalities found in hypertensive humans and animals [19]. Further studies resulted in the discovery of generalized structural and functional alterations in erythrocyte membrane mirrored the similar phenomenon in other cell types in essential hypertension

Journal of Hypertension 2015, 33:000–000

[a]Department of Biology, Medical Genetics and Ecology, Kursk State Medical University, [b]Division of Cardiology, Kursk Regional Clinical Hospital, Kursk, [c]Department of Medical Biological Disciplines, Belgorod State University, Belgorod, [d]Population Genetics Laboratory, Research Institute for Medical Genetics, Tomsk, Russian Federation and [e]Department of Physiology, Pathophysiology and Medical Biology, Sumy State University, Sumy, Ukraine

Correspondence to Alexey Polonikov (Prof, MD, PhD), Department of Biology, Medical Genetics and Ecology, Kursk State Medical University, 3 Karl Marx St., Kursk 305041, Russian Federation. Tel/Fax: +7 4712 58 81 47; e-mail: polonikov@rambler.ru

Received 16 February 2015 **Revised** 10 June 2015 **Accepted** 10 June 2015

DOI:10.1097/HJH.0000000000000699

[20,21]. Functional alterations in Na^+-Li^+ countertransport and other membrane ion transporters are thought to be responsible for abnormal intracellular distribution of calcium ions required for contraction of smooth muscle cells, leading to increased cytosolic free calcium and arterial contractility leading to hypertension [12,22,23]. These observations made erythrocytes an attractable and biologically plausible model for studying the role of membrane alterations in the molecular mechanisms of hypertension [12,14,24,25].

It is important to note that abnormalities in ion transporters' functions are considered as a genetically determined feature of primary hypertension, rather than the consequence of long-term elevation of BP [12,23]. The altered activity of monovalent ion transporters in essential hypertension, in contrast to monogenic hypertensions, may be because of abnormalities of systems involved in the regulation of their expression and/or function [23]. Familial aggregation and higher concordance of increased Na^+-Li^+ countertransport in monozygotic twins than in dizygotic twins suggest multifactorial nature of membrane disorders in essential hypertension [24]. This means that the ion transport abnormalities of cell membrane in essential hypertension are attributable to the effects of multiple genes, which have the potential to modify expression of genes whose products form the structure of the cell membrane.

Numerous studies have shown that the intracellular distribution of monovalent ions in hypertension is correlated with alterations in protein composition of the erythrocyte membrane [14,15,26–31]. Despite incredible advances in genome research, a little concern so far is given to uncovering the pathogenetic link between well characterized genetic determinants of essential hypertension and altered properties of the cell membrane. Although a large number of polymorphic genes were found to be associated with susceptibility to essential hypertension, no study has been done to investigate whether these genes contribute to alterations in content of cell membrane proteins in the disease. Pursuing this interest, the present study was designed to test the hypothesis that alterations in the levels of erythrocyte membrane proteins in essential hypertension are related to the effects of polymorphic genes associated with disease susceptibility.

METHODS

Study participants

The study was carried out in four stages (Fig. 1). A total of 1162 unrelated individuals were involved into the study. All the participants were Russians from Central Russia (predominantly from Kursk region). The Ethical Review Committee of the Kursk State Medical University has approved the study protocol, and each participant signed informed consent at the recruitment. A random sample of 751 (371 males and 380 females) was enrolled to estimate the population means of the EMPs for statistical genetic analysis. The study group also included 235 patients with doctor-diagnosed essential hypertension and 176 healthy subjects with normal BP. Mean age of essential hypertension patients (67 men and 168 women) was 47.8 ± 9.6 years, and the mean age of the healthy subjects (75 men and 132 women) was 45.9 ± 11.2 years. Patients with essential hypertension were recruited from the Cardiology Clinics of Kursk Regional Clinical Hospital and Kursk Emergency Medicine Hospital between 2003 and 2007 [32]. Diagnosis of essential hypertension was verified by qualified cardiologists. Patients were defined as hypertensive according to the World Health Organization criteria. All these subjects underwent both molecular genetic analysis for hypertension susceptibility genes and biochemical investigations for erythrocyte membrane proteins contents.

Molecular genetic analysis

On the basis of supportive peer-reviewed scientific publications, we selected genes involved in the regulation of vascular homeostasis with a focus on well recognized hypertension susceptibility loci that have been reported to be associated with the risk of essential hypertension or/and with the level of BP in Russian populations [33–38]. A total of 11 single nucleotide polymorphisms (SNPs) of hypertension susceptibility genes including transforming growth factor beta 1 (*TGFB1*) R25P (rs1800471), adducin 1 (*ADD1*) G460W (rs4961), guanine nucleotide-binding protein beta polypeptide 3 (*GNB3*) C825T (rs5443) and G272S (rs16932941), nitric oxide synthase 3 (*NOS3*) E298D (rs1799983) and $-786T > C$ (rs2070744), angiotensin I-converting enzyme (*ACE*) I/D (rs5186), angiotensin II receptor type 1 (*AGTR1*) A1166C (rs5186), angiotensinogen (*AGT*) M235T (rs699) and T174M (rs4762), mineralocorticoid receptor or nuclear receptor subfamily 3 group C member 2 (*NR3C2*) A4582C (rs5534) were selected for this study.

Approximately 5 ml of venous blood were collected from the patients in K3-EDTA tubes and genomic DNA was isolated by standard phenol-chloroform extraction. Genetic polymorphisms were typed by PCR-RFLP assays as described elsewhere [33–35,39–44]. Each PCR reaction contained internal controls, and random retesting of about 10% of samples resulted in a 100% concordance rate with initial genotyping data.

Biochemical analyses

Approximately 5 ml of fasting whole blood samples were collected from each study participant in the morning. Erythrocytes were precipitated from fresh heparinized whole blood samples by the method of Beutler *et al.* [45]. Ghosts of erythrocytes were prepared according to a modified method described by Dodge *et al.* [46]. Washed ghosts of erythrocytes were lyophilized, frozen, and stored at $-20^{\circ}C$. Fractionation of major erythrocyte membrane proteins was performed by one-dimensional SDS polyacrylamide gel electrophoresis (SDS-PAGE) with gradient concentration 5–25% according to Laemmli's method [47]. The membrane proteins were detected by immunological methods as described elsewhere [48] and classified accordingly as proposed by Steck and Fairbanks *et al.* [49,50]. The identification and molecular weight calculations of red blood cell proteins were performed using standards of protein markers as previously reported [48]. The levels of erythrocyte membrane proteins were quantified through known concentration of

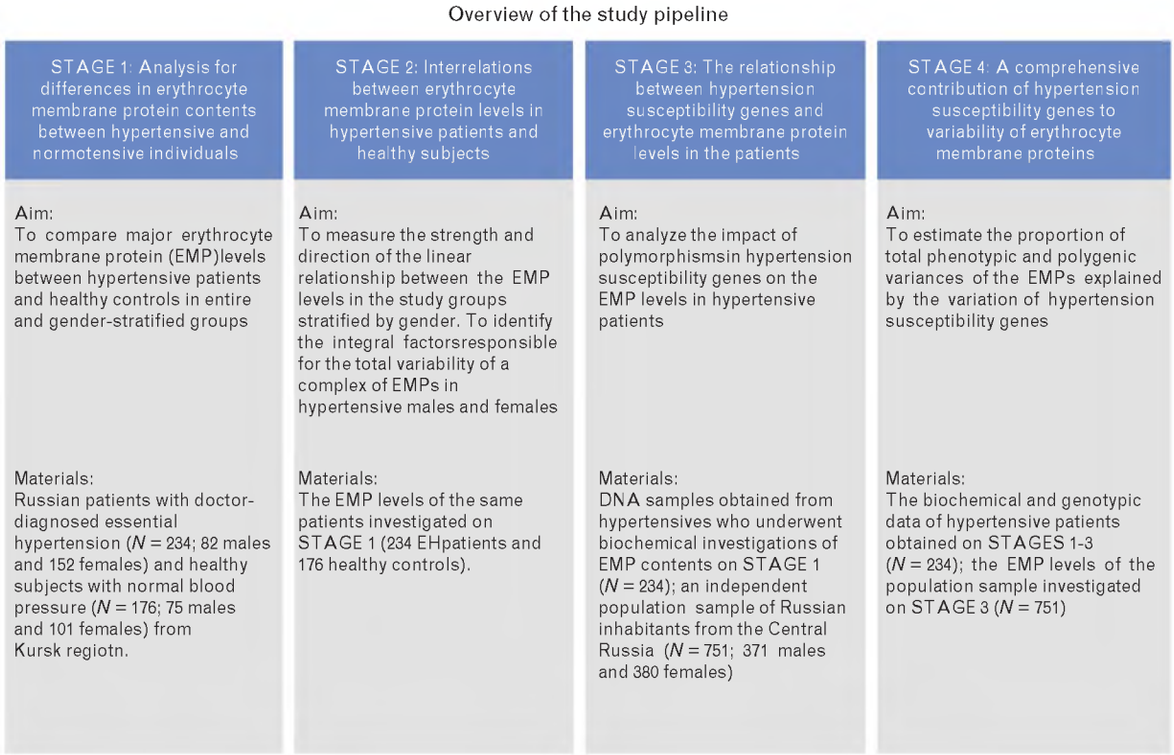

FIGURE 1 Overview of the study pipeline. initial analysis was done to establish differences in contents of 14 major erythrocyte membrane proteins (EMPs) between hypertensive and normotensive subjects (STAGE 1). Then, the strength and direction of alterations in the EMP levels were assessed and the integral factors explaining the joint variation of membrane proteins in essential hypertension patients were estimated (STAGE 2). Subsequently, the analysis of relationships between 11 common polymorphisms in hypertension susceptibility genes and EMP contents was carried out (STAGE 3) and the estimates of the proportion of total phenotypic and polygenic variances of the proteins explained by the variation of a group of the genetic loci were obtained (STAGE 4).

bovine serum albumin (μg) using the program ONE-Dscan (Scanalytics Inc., Fairfax, VA, USA).

Statistical analysis

The distribution of the quantitative traits was evaluated for normality by Kolmogorov–Smirnov test. Differences in EMP contents between the case and control groups were tested using modifications of Student's t test for the traits with equal or unequal variances as required. The Brown-Forsythe's test was applied to assess the equality of EMP variances between the groups [51]. A P value ≤ 0.05 was considered significant. Pearson correlation coefficients were calculated to measure the linear relationships between the EMP levels in the study groups. The principal component analysis was applied to identify the integral factors responsible for the total variation of EMPs [52]. The varimax-normalized rotation was used to simplify the interpretation of the principal components. Allele frequencies for the polymorphisms were estimated by the gene counting method. Hardy-Weinberg equilibrium for genotype frequencies was assessed by χ^2 test. A one-way and two-way analysis of variance (ANOVA) was performed for the EMP levels to test the hypothesis that their phenotypic variation is affected by the genetic polymorphisms alone and their combinations, respectively [53]. The coefficient of determination (R^2) was used to evaluate the degree of relationship between genetic polymorphisms and EMP levels.

The average effects of the particular allele (α_i, $i = 1, 2$) on EMP variability in hypertensive patients were estimated using algorithms described in [54]:

$$a_1 = \frac{f_{11} \cdot \mu_{11} + 0.5 \cdot f_{12} \cdot \mu_{12}}{f_1}$$

$$a_2 = \frac{f_{22} \cdot \mu_{22} + 0.5 \cdot f_{12} \cdot \mu_{12}}{f_2} - \mu,$$

where f_i is frequency of allele i; f_{ij} is the frequency of genotype ij expected under the assumption of Hardy–Weinberg equilibrium; μ_{ij} is the average of the trait values for individuals with the genotype ij; μ is the estimate of the population mean.

The variance attributable to genotypic differences was computed as [54]:

$$G = \sum f_{ij}(\mu_{ij} - \mu)^2,$$

where the values are summed for each genotype of the locus.

The variance attributable to the average effects of the alleles at the locus (additive genetic component) was calculated as:

$$A = 2\sum f_i \cdot \alpha_1^2,$$

where the values are summed up for each allele of the locus.

The variance attributable to the nonadditive interactions (dominant genetic component) between the alleles of the locus was calculated as:

$$D = G - A$$

Additive and dominant components of the variance attributable to a complex of the loci were calculated as weighted averages of the trait over all polymorphisms. A percentage of the average value of the genotypic variance attributable to the interloci variability to heritability (h^2) was considered as the contribution of genetic variance (G_{pg}) to the total polygenic variance of the traits. Estimates of EMP heritability were computed in our previous study as a doubled parent–child correlation coefficients for each trait [55]. The statistical calculations were done using Statistica for Windows v10.0 (StatSoft; Tulsa, OK, USA) and Microsoft Excel 2007 (Microsoft Corp., USA).

RESULTS

Alterations in contents of erythrocyte membrane proteins in patients with essential hypertension

All erythrocyte membrane proteins in the study subjects were normally distributed ($P > 0.05$). Table 1 shows a comparison of EMP levels between patents with essential hypertension and healthy subjects. The increased levels of protein band 4.1 (EPB41), glucose transporter (GLUT1) and actin (ACTB), and decreased levels of α-spectrin (SPTA1), β-spectrin (SPTB), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were found in hypertensives as compared with controls. Sex-stratified analysis revealed differences in the EMP levels between males and females. The decreased SPTA1 levels and increased GLUT1 levels were found in hypertensive males in comparison with healthy controls. Meanwhile, the levels of cytoskeletal proteins SPTA1, SPTB, dematin (DMTN), and GAPDH were significantly lower in hypertensive females than in healthy females, whereas the GLUT1 and ACTB levels were higher in hypertensive then in normotensive females. Protein bands 2.1 (ankyrin 1, ANK1), anion exchanger (AE1), pallidin (EPB42), GLUT1, and GAPDH showed differences in variances between the study groups (Supplementary Table 1, http://links.lww.com/HJH/A511).

Joint variability of erythrocyte membrane proteins in hypertensive patients

Statistically significant correlations ($P \leq 0.05$) between the levels of erythrocyte membrane proteins were observed in both hypertensive patients and healthy controls (Fig. 2). Notably, paired correlation coefficients between EMPs, whose contents were altered in hypertensives in comparison with healthy subjects, showed considerable differences between the case and control groups. Correlations between such EMPs in males and females were of interest. For instance, SPTA1 levels were negatively correlated with GAPDH contents in hypertensive females and healthy males in contrast to hypertensive males and healthy

TABLE 1. Comparative analysis of erythrocyte membrane proteins levels between patents with essential hypertension and healthy subjects: entire groups and groups stratified by gender

Membrane proteins	Entire groups (I)		Males (II)		Females (III)		Student's t (p-level)		
	essential hypertension patients (N = 234)	Controls (N = 176)	essential hypertension patients (N = 82)	Controls (N = 75)	EH patients (N = 152)	Controls (N = 101)	Entire groups (I)	Males (II)	Females (III)
	M ± SE	M ± SE	M ± SE	M ± SE	M ± SE	M ± SE			
SPTA1	106.25 ± 1.85	119.21 ± 2.42	105.56 ± 3.02	123.50 ± 3.53	106.63 ± 2.35	116.03 ± 3.29	**4.32 (2×10^{-5})**	**3.88 (2×10^{-4})**	2.39 (0.02)
SPTB	100.93 ± 1.93	112.60 ± 2.35	101.43 ± 2.87	110.23 ± 3.57	100.66 ± 2.54	114.35 ± 3.12	**3.87 (1×10^{-4})**	1.93 (0.06)	**3.40 (0.001)**
ANK1.1	31.96 ± 0.72	31.09 ± 1.00	29.86 ± 1.24	31.24 ± 1.37	33.09 ± 0.88	30.99 ± 1.41	0.70 (0.48)	0.75 (0.46)	1.26 (0.21)
ANK1.2	19.11 ± 0.56	17.91 ± 0.71	18.31 ± 0.96	17.24 ± 1.12	19.53 ± 0.68	18.40 ± 0.92	1.34 (0.18)	0.73 (0.47)	1.01 (0.32)
ANK1.3	14.07 ± 0.40	13.58 ± 0.53	13.40 ± 0.70	12.74 ± 0.77	14.43 ± 0.49	14.21 ± 0.73	0.74 (0.46)	0.63 (0.53)	0.26 (0.80)
AE1	199.87 ± 1.93	196.29 ± 2.70	201.19 ± 3.39	193.94 ± 4.10	199.16 ± 2.34	198.03 ± 3.60	1.08 (0.28)	1.37 (0.17)	0.26 (0.79)
EPB41	49.10 ± 0.70	46.88 ± 0.89	47.92 ± 1.27	45.61 ± 1.38	49.74 ± 0.84	47.82 ± 1.16	1.98 (0.05)	1.24 (0.22)	1.37 (0.17)
EPB42	54.07 ± 0.88	53.74 ± 1.29	52.41 ± 1.51	49.01 ± 2.24	54.97 ± 1.09	57.25 ± 1.42	0.21 (0.83)	1.26 (0.21)	1.29 (0.20)
GLUT1	151.90 ± 2.03	140.51 ± 2.62	154.16 ± 3.83	140.67 ± 3.90	150.68 ± 2.35	140.39 ± 3.54	**3.44 (0.001)**	**2.47 (0.01)**	2.42 (0.02)
DMTN	26.54 ± 0.52	26.96 ± 0.65	27.21 ± 0.97	25.21 ± 0.93	26.17 ± 0.61	28.26 ± 0.89	0.51 (0.61)	1.47 (0.14)	2.00 (0.05)
ACTB	54.80 ± 0.80	51.80 ± 0.91	55.21 ± 1.41	53.32 ± 1.23	54.58 ± 0.97	50.67 ± 1.29	**2.47 (0.01)**	1.00 (0.32)	**2.46 (0.01)**
GAPDH	34.80 ± 0.66	36.92 ± 0.88	34.25 ± 1.17	35.31 ± 1.29	35.10 ± 0.79	38.11 ± 1.20	1.92 (0.05)	0.61 (0.54)	2.10 (0.04)
TPM1	42.77 ± 0.85	43.20 ± 1.08	43.99 ± 1.42	43.55 ± 1.60	42.11 ± 1.05	42.95 ± 1.46	0.32 (0.75)	0.21 (0.84)	0.48 (0.63)
GSTM1	23.12 ± 0.89	24.51 ± 0.98	23.75 ± 1.58	23.62 ± 1.41	22.77 ± 1.08	25.18 ± 1.35	1.05 (0.30)	0.06 (0.95)	1.40 (0.16)

M, mean; SE, standard error of mean. bolded are statistically significant differences in the EMP levels between the groups.

Pairwise correlation matrix of erythrocyte membrane proteins in hypertensive patients and healthy controls stratified by gender

EMPs	SPTA1	SPTB	ANK1.1	ANK1.2	ANK1.3	AE1	EPB41	EPB42	GLUT1	DMTN	ACTB	GAPDH	TPM1	GSTM1
SPTA1		0.416 0.647				0.322 NS	−0.366 NS		−0.645 −0.557			NS −0.307	−0.266 −0.396	
SPTB	0.647 0.629		NS 0.257				−0.302 −0.401		−0.582 −0.658		−0.314 −0.285		NS −0.295	
ANK1.1	0.166 NS	0.235 NS		0.458 0.527	0.540 0.595		0.311 NS	0.287 NS	NS −0.359	−0.222 NS	−0.389 −0.379	−0.311 −0.373	−0.221 NS	−0.405 −0.261
ANK1.2			0.461 0.636		0.743 0.796	0.296 NS	NS 0.248	0.358 NS	−0.253 NS		−0.234 −0.319	−0.238 −0.250	−0.254 NS	NS −0.285
ANK1.3			0.454 0.673	0.758 0.791			0.325 0.288	0.391 0.294	NS −0.290		−0.407 −0.470	−0.239 NS	−0.219 NS	−0.317 NS
AE1	NS 0.199	NS 0.282	NS 0.318					0.296 NS	−0.479 −0.260		−0.306 NS	−0.243 −0.409	−0.471 −0.556	−0.243 −0.336
EPB41	NS −0.252	−0.185 −0.347	0.201 NS	0.238 NS	0.179 NS			0.358 0.387	NS 0.334			−0.297 NS	−0.251 NS	−0.498 −0.237
EPB42				0.207 NS	0.276 NS		0.269 0.365		−0.318 0.449				−0.317 −0.350	−0.233 −0.319
GLUT1	−0.660 −0.740	−0.652 −0.698	−0.264 −0.274			−0.238 −0.348	0.160 0.246				0.279 0.439			
DMTN			−0.233 −0.351	NS −0.320	NS −0.264						−0.236 0.351		0.266 NS	0.240 0.477
ACTB		−0.270 NS	−0.240 NS	−0.293 −0.337	−0.270 −0.468	NS −0.247	NS 0.255					0.292 NS		
GAPDH	−0.240 NS		−0.322 −0.355	−0.388 NS	−0.341 −0.221	−0.248 −0.480	−0.179 NS						NS 0.541	0.247 NS
TPM1	−0.364 −0.270	−0.297 −0.274	−0.256 −0.520	−0.283 −0.306	−0.330 −0.304	−0.272 −0.609	−0.318 NS							0.437 NS
GSTM1			−0.446 −0.456	−0.322 −0.270	−0.323 −0.211	−0.181 −0.334	−0.340 NS							

FIGURE 2 Pairwise correlation matrix of erythrocyte membrane proteins in hypertensive patients and healthy controls stratified by sex. Statistically significant correlation coefficients in hypertensive (numerator) and normotensive (denominator) males and females are shown above the central diagonal and below it, respectively. NS means a nonsignificant correlation coefficient.

females. The levels of GLUT were positively correlated with EPB41 levels in healthy males, but no such correlation was observed in hypertensive males.

Principal components analysis was applied to the dataset to reduce the dimensionality of a set of erythrocyte membrane proteins and to identify the integral factors responsible for their joint variability. A significant proportion of the joint variation of EMP levels (72.4% in males and 71.0% in females) was because of the five factors, which showed differences between sexes (Table 2). In males, substantial loadings to the principal component 1 (PC1) (explained 27% of total EMP variance) appeared for a majority of EMPs such as an anchoring membrane protein ankyrin (bands 2.1, 2.2, and 2.3), AE1, ACTB, TPM1, and membrane-associated enzymes GAPDH and membrane-associated GSTM1. In females, the PC1 (explained 24% of total EMP variance) had substantial loadings only for ankyrin, actin,

TABLE 2. Principal components analysis of erythrocyte membrane proteins levels in patents with essential hypertension

	Males					Females				
Membrane proteins	**PC1**	**PC2**	**PC3**	**PC4**	**PC5**	**PC1**	**PC2**	**PC3**	**PC4**	**PC5**
SPTA1	−0.189	−0.692	−0.500	−0.109	−0.049	−0.363	−0.750	0.276	0.125	−0.048
SPTB	−0.183	−0.751	0.032	−0.149	0.112	−0.315	−0.800	0.113	0.066	−0.263
ANK1.1	−0.711	0.089	0.139	−0.277	0.178	−0.711	0.016	−0.141	0.179	−0.365
ANK1.2	−0.676	0.124	0.384	0.122	0.440	−0.715	0.332	−0.383	−0.110	−0.076
ANK1.3	−0.723	0.256	0.450	0.030	0.204	−0.717	0.303	−0.328	−0.205	−0.073
AE1	−0.601	−0.297	−0.272	0.134	−0.115	−0.316	−0.017	0.059	0.117	0.875
EPB41	−0.509	0.472	−0.148	0.108	−0.449	−0.353	0.467	0.361	0.005	−0.029
EPB42	−0.576	−0.056	0.133	0.600	−0.158	−0.349	0.214	0.357	−0.643	0.006
GLUT1	0.396	0.819	−0.044	−0.124	−0.068	0.362	0.774	−0.084	0.114	−0.071
DMTN	0.171	−0.365	0.559	0.201	−0.520	0.096	−0.227	0.111	−0.798	0.095
ACTB	0.501	0.250	−0.424	0.437	0.348	0.317	0.315	0.638	0.306	−0.097
GAPDH	0.467	−0.154	0.178	0.596	0.123	0.572	0.047	0.254	−0.311	−0.252
TPM1	0.566	0.010	0.540	−0.258	−0.110	0.642	0.005	−0.480	−0.099	−0.111
GSTM1	0.552	−0.412	0.323	0.006	0.255	0.540	−0.393	−0.411	−0.071	0.118
Eigen values of principal components	3.785	2.506	1.657	1.185	1.009	3.379	2.575	1.515	1.388	1.096
The percentage of total variance explained by the respective number of factors	27.0	17.9	11.8	8.5	7.2	24.1	18.4	10.8	9.9	7.8

Gray cells represent factor loadings with a value $\geq \pm 0.500$, a threshold selected for inclusion of the trait in the interpretation of principal components.

tropomyosin, and GSTM1. The PC2 (responsible for almost 18% of total EMP variance) showed substantial factor loadings for major cytoskeletal proteins such as spectrin and protein 4.1, and also GLUT1 in both sexes and additionally for GSTM1 in males. The PC3 differed substantially between males and females. In males, the PC3 had substantial loadings for SPTA1, SPTB, ankyrin (band 2.3), DMTN, ACTB, and TPM1. In females, the PC3 was only associated with the variability of ACTB, TPM1, and GSTM1. The rest two principal components were responsible for only 15% and 17% of total EMP variance in males and females, respectively.

The average effects of alleles of hypertension susceptibility genes on the levels of erythrocyte membrane proteins

We estimated average effects of alleles on the levels of EMT in the hypertensives using methodology proposed by Sing and Davignon [54]. To calculate the average effects, we used population means for each EMP estimated in a population sample of Russians (Supplementary Table 2, http://links.lww.com/HJH/A511) and the frequencies of alleles of disease susceptibility genes in hypertensives (Supplementary Table 3, http://links.lww.com/HJH/A511). Figure 3 shows a graphical representation of the average effects of alleles on the EMP levels for genes associated with hypertension in our population (the average effects of alleles for other loci are given in Supplementary Figure 1, http://links.lww.com/HJH/A511). As expected, allelic variants of almost all of the investigated genes influence, though in varying degrees, the contents of EMP in hypertensive patients. In most cases, the average effects of alleles on the EMP levels were moderate, and the direction of deviations of the trait from the population mean was mutually inverted in the carriers of alternative alleles. Furthermore, the effects of alleles on the EMP levels were unequal in males and females. In particular, the 460W allele of the *ADD1* gene influenced the levels of spectrins, ankyrin 2.1, GLUT1, DMTN, and GSTM1 in males, and SPTA1, SPTB, ankyrin 2.2, AE1, GLUT1, ACTB, GAPDH, and GSTM1 in females. Notably, the effects of the allele 460W on the EMP contents were in opposite directions in hypertensive males and females; the allele increased the level of GLUT1 and was associated with a lower SPTA1 and SPTB in males, whereas in females, the allele decreased GLUT1 content and increased the levels of the spectrins. Higher effects on contents of GLUT1, SPTA1, and SPTB in females were seen for the 272S allele of the *GNB3* gene. The 25P allele of the *TGFB1* gene exhibited similar effects on GLUT1, SPTA1, and SPTB contents in both sexes, similarly to those found in males with the 460W allele. In males, the effects of the *AGT* 235 M allele on the levels of GLUTand spectrins were comparable with the effects of the *ADD1* 460W allele.

The contribution of the studied genes to quantitative variability of erythrocyte membrane proteins in hypertensive patients

Using ANOVA, we assessed the impact of hypertension susceptibility genes on the EMP levels in female and male patients (Fig. 4). Noteworthy, the EMPs, whose contents were changed in hypertensive patients in comparison with healthy controls, were associated with combinations of hypertension susceptibility genes. In particular, a combination of the *GNB3* C825T and *AGT* T174 M polymorphisms explained 24.3% ($P = 0.01$) of the total variation of SPTB and 17.6% ($p = 0.04$) of GLUT variation in males. The *AGT* M235T and *AGTR1* 1166A/C loci were responsible for 21.2% ($p = 0.01$) of SPTB variation, whereas a combination of *AGT* M235T with the *NOS3* E298D locus was responsible for 13.3% ($P = 0.05$) of the total variance of this cytoskeletal protein in males. In females, the *ADD1* G460W and *NOS3* E298D gene polymorphisms explained 7.4% ($P = 0.04$) of the total variation of SPTA1 and 11.0% ($P = 0.01$) of SPTB variation, whereas a combination of the *AGTR1* 1166A/C и *GNB3* G814S loci was responsible for 7.5% ($P = 0.02$) of SPTB variation. Moreover, combinations of the polymorphisms *GNB3* C825T and *GNB3* G814S, *GNB3* C825T and *NOS3* E298D, *GNB3* G814S and *NOS3* E298D in females explained on average from 5% to 9% of the variation of EMPs whose contents were altered in hypertensive patients (SPTA1, SPTB, and GLUT1).

As essential hypertension is a complex polygenic disease, there is a need for a comprehensive analysis where multiple genetic polymorphisms are evaluated simultaneously regarding their joint contribution to the variability of EMPs in hypertensive individuals. Therefore, we used methodology described by Sing and Davignon [54] to estimate the summary contribution of disease susceptibility genes to the variation of erythrocyte membrane proteins in hypertensive patients (Table 3; Supplementary Tables 4 and 5, http://links.lww.com/HJH/A511). The total genetic variance (G) associated with the studied genes ranges from 3.1 (DMTN) to 47.6% (GLUT1) in males, and from 1.1 (DMTN) to 27% (SPTA1) in females. Notably, the highest impact of the genetic variation was found on the variation of SPTA1 (24.4% in males and 27% in females) and SPTB (29.4% in males and 23.2% in females) and also integral membrane proteins such as AE1 (36.8% in males and 12% in females) and GLUT1 (47.6% in males and 23.1% in females). Taking into account heritability for SPTA1 (52%) and SPTB (60%) [55], the studied genes were estimated to be responsible for as much as 47 and 49% of their total polygenic variances in males and 50 and 39% in females. Moreover, these loci may also be responsible for as much as 64 and 31% of the total polygenic variance of GLUT in males and females, respectively.

DISCUSSION

Alterations in the levels of erythrocyte membrane proteins and their joint variation in essential hypertension

Consistently with previous studies [12,27,55,56], we demonstrated that essential hypertension is accompanied by alterations in the composition of major erythrocyte membrane proteins. In particular, hypertensive patients exhibited increased levels of protein 4.1, glucose transporter, and actin and also decreased levels of α and SPTB and GAPDH. Two independent studies conducted on Russian families from Kursk region [55,56] showed similar alterations in integral and peripheral membrane proteins (AE1, ACTB,

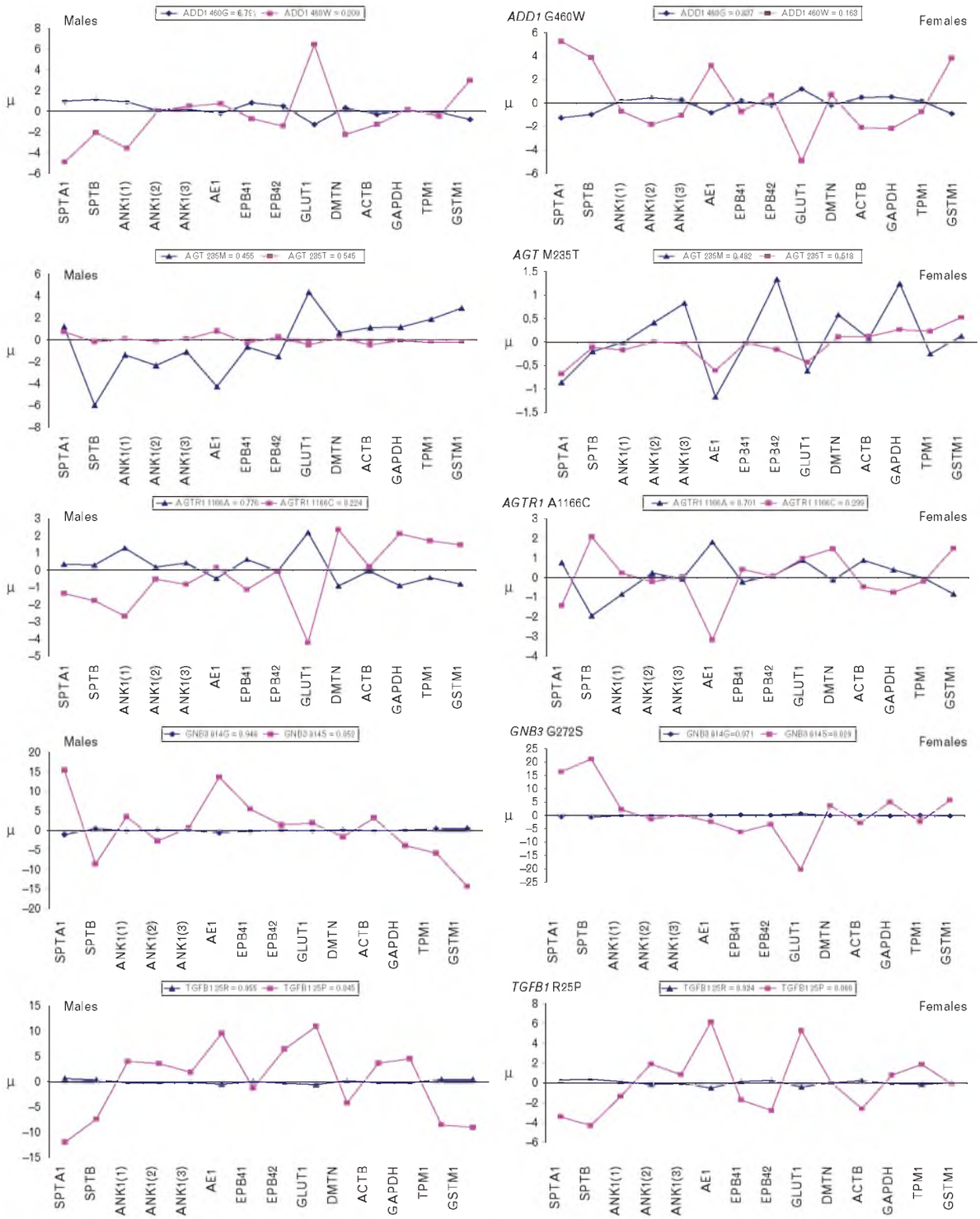

FIGURE 3 The average effects of alleles in hypertension susceptibility genes on the levels of erythrocyte membrane proteins in hypertensive patients. Line charts represent the average effects of major (blue lines) and minor (red lines) alleles on EMP variability, that is, a deviation of the trait from the population mean (μ) in hypertensive males (left charts) and females (right charts). Pleiotropic and sex-specific effects on the EMPs levels were noted for several hypertension susceptibility genes. For instance, the overall effect of the 460W *ADD1* allele was to decrease of the SPTA1 and SPTB levels and to increase of the content of GLUT1 in hypertensive males, whereas in hypertensive females the 460W allele associated with an increase of both spectrins and decrease of GLUT1.

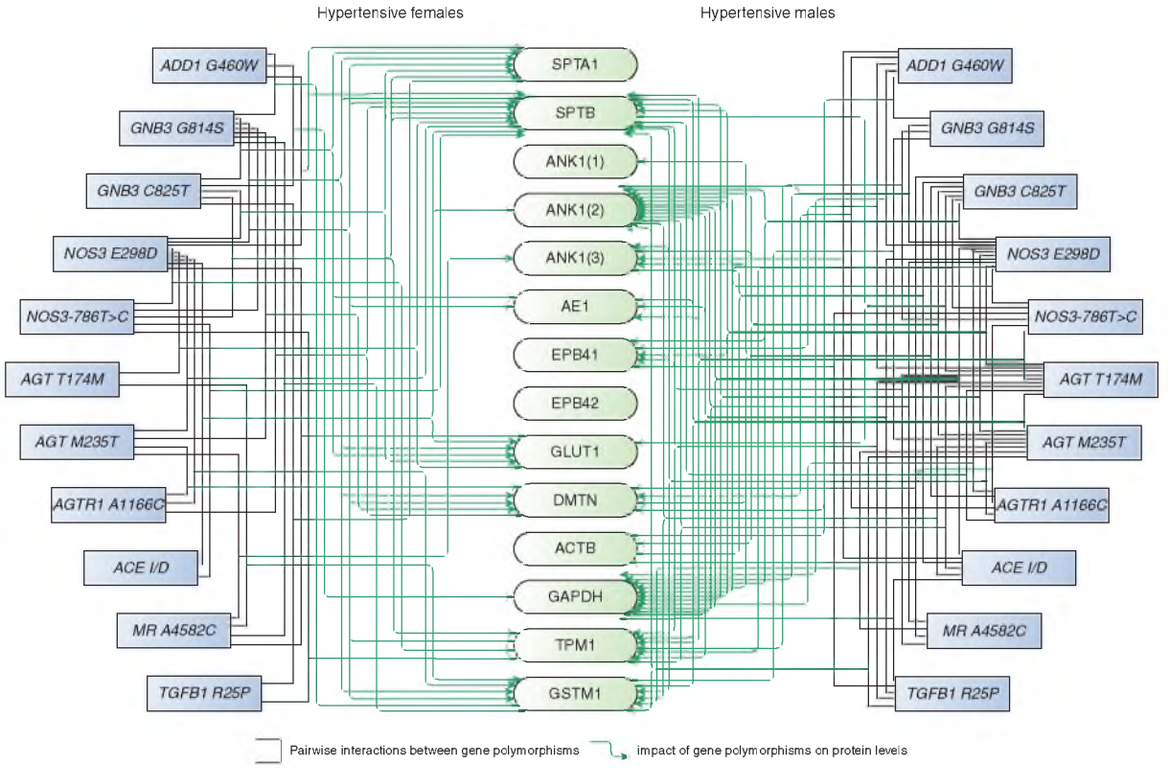

FIGURE 4 The effects of pairwise combinations of hypertension susceptibility genes on the levels of erythrocyte membrane proteins in hypertensive patients. The figure shows the significant effects ($P \leq 0.05$) of pairwise combinations of the genetic polymorphisms on the EMP levels in hypertensive females (left part) and males (right part). The estimates were performed by two-way ANOVA and expressed as R^2 values, which varied between 10 and 26% in males, and between 6 and 11% in females. The free yEd graph editor was used to draw the figure.

EPB41, and DMTN) and proteins related to glucose metabolism (GLUT1 and GAPDH) in patients with essential hypertension. Similarly, in a sample of Russian hypertensives from Moscow, alterations in GLUT1 and GAPDH levels in essential hypertension patients in comparison with healthy subjects have been also observed [27]. Furthermore, the present study demonstrated for the first time sex-specific alterations in major erythrocyte membrane proteins in hypertensive patients. Although similar alterations in the levels of spectrins and GLUT were found in both sexes, hypertensive females also exhibited decreased levels of DMTN and GAPDH in combination with increased levels of ACTB. Thus, patients with essential hypertension demonstrate altered expression and/or posttranslational

TABLE 3. The percentage of total phenotypic and genetic variances of the EMPs explained by a comprehensive contribution of disease susceptibility genes in hypertensive patients

Genetic components, %		Membrane proteins												
		SPTA1	SPTB	ANK1 (1)	ANK1 (2)	ANK1 (3)	AE1	EPB41	EPB42	GLUT1	DMTN	ACTB	GAPDH	TPM1	GSTM1
Males	*G*	**24.35**	**29.39**	**6.86**	**4.26**	**1.90**	**36.82**	**4.02**	**4.43**	**47.55**	**3.08**	**4.59**	**4.55**	**12.07**	**10.29**
	A	15.62	20.44	4.94	2.65	1.24	17.23	2.25	3.02	33.03	1.55	2.40	2.74	5.76	6.21
	D	8.73	8.95	1.92	1.61	0.66	19.60	1.77	1.41	14.51	1.53	2.19	1.81	6.31	4.08
	G_{pg}	**47.20**	**49.22**	**9.33**	**6.59**	**7.24**	**41.75**	**4.52**	**23.18**	**64.43**	**3.85**	**9.34**	**5.62**	**16.33**	**20.57**
Females	*G*	**26.99**	**23.20**	**1.79**	**1.85**	**0.71**	**12.02**	**0.99**	**2.56**	**23.12**	**1.11**	**1.79**	**2.21**	**2.65**	**3.70**
	A	20.20	19.07	1.00	1.05	0.44	6.94	0.57	1.59	15.56	0.45	1.25	1.70	0.57	2.08
	D	6.79	4.13	0.79	0.80	0.26	5.08	0.42	0.97	7.57	0.66	0.54	0.51	2.09	1.62
	G_{pg}	**50.31**	**38.87**	**2.44**	**2.87**	**2.69**	**13.63**	**1.11**	**13.42**	**31.33**	**1.39**	**3.64**	**2.73**	**3.59**	**7.39**
h^2, %		51.6	59.7	73.5	64.6	26.2	88.2	88.9	19.1	73.8	79.9	49.1	80.9	73.9	50.0

G (bold face values in white cells) denotes the percentage of total variance of the traits attributable to genotypic differences of the locus; *A* and *D* denote additive and dominant components of the *G*, respectively; G_{pg} (bold face values in gray cells) denotes the percentage of genotypic variance, attributable to differences between the loci, to the total polygenic variance of the traits; h^2 denotes heritability of the traits computed as doubled parent-child correlation coefficients for each trait in our previous study [55]. EMP, erythrocyte membrane protein; DMTN, dematin actin binding protein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GLUT1, glucose transporter 1; SPTA1, α-spectrin; SPTB, β-spectrin.

modifications of genes encoding cytoskeletal proteins and proteins directly involved in glucose metabolism.

Altered levels of erythrocyte membrane proteins in essential hypertension patients are correlated to each other, suggesting common sources of their co-variations, that is, shared molecular mechanisms underlying the regulation of expression of the corresponding genes. Principal components analysis identified several integral factors of joint variability of EMPs in hypertensives. The first factor mainly influenced by AE1, EPB41, EPB42, ACTB, TPM1, GAPDH, and GSTM1 reflecting both spatial and functional coupling of these proteins localized in the inner half of the plasma membrane. Based on the results of biochemical studies showed that the cytoplasmic domain of anion exchanger constitutes a major multiprotein complex responsible for erythrocyte membrane-peripheral protein interactions and attachment of the spectrin-actin network and cytoskeletal proteins EPB41, EPB42, and TPM1 to the inner surface of the membrane via anchoring protein ankyrin, we assume that this principal component may represent a 'factor of membrane-anchoring protein assembly' [57–59]. The multifunctional domain of AE1 is also a single binding site for GSTM1, GAPDH, and other glycolytic enzymes, providing the enzymatic bridge to cytoskeletal proteins and ion transport ATPases [60,61]. The second principal component with the highest loadings for α and β-subunits of spectrin and GLUT may represent major alterations in the EMP composition found in hypertensives. In general, the results obtained by PCA confirmed that alterations in the levels of particular erythrocyte membrane proteins in essential hypertension patients are closely correlated to each other forming distinct groups of jointly varying proteins, which could be attributable to pleitropic effects of different polygenic systems.

Possible relationship between altered protein composition of erythrocyte membrane and pathogenesis of essential hypertension

In Fig. 5, we summarized possible mechanisms through which alterations in the content of EMPs can be related to the pathogenesis of essential hypertension. It is well known that alterations in the levels of the cytoskeletal proteins such as spectrins, actin, protein bands 4.1 and 4.9

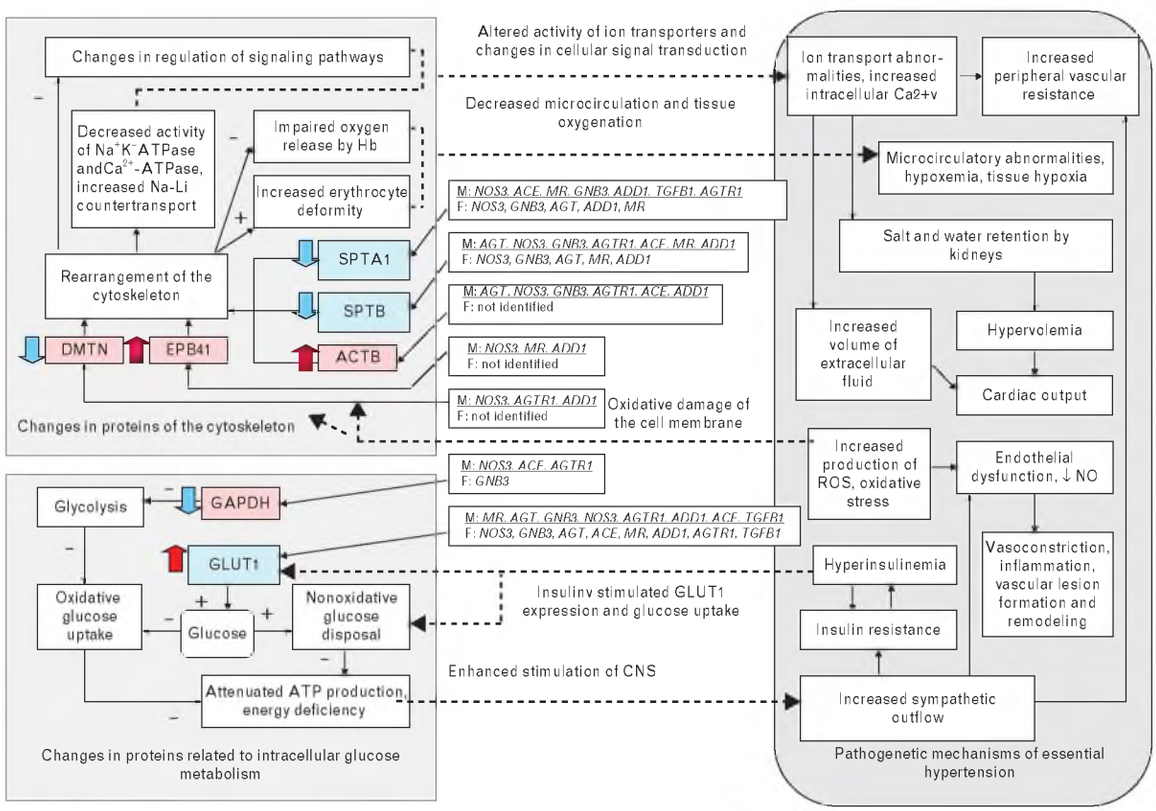

FIGURE 5 Possible molecular mechanisms by which alterations in contents of erythrocyte membrane proteins are involved in the pathogenesis of essential hypertension. The upper left gray box summarizes possible consequences of altered cytoskeletal protein contents in hypertensive patients and their relation to disease phenotypes. The lower left gray box summarizes proposed mechanisms by which membrane proteins related to intracellular glucose metabolism contribute to the development of hypertension. The right gray box represents well characterized mechanisms of essential hypertension [9,62]. Colored blocks indicate membrane proteins showed alterations in hypertensive patients: blue blocks indicate proteins altered only in both sexes, whereas pink blocks indicate proteins altered exclusively in females. Blue arrows indicate a decreased protein level, whereas red arrows indicate an increased protein level. The middle blocks indicate hypertension susceptibility genes influenced alterations in EMP contents in hypertensive males (M, numerator) and females (F, denominator). The dotted lines indicate pathogenetic links between altered contents of erythrocyte membrane proteins and the mechanisms of essential hypertension. See text for more details. ACE, angiotensin I-converting enzyme; ACTB, actin; ADD1, alpha-adducin; AGT, angiotensinogen; AGTR1, angiotensin II receptor type 1; DMTN, dematin (actin-binding protein); EPB41, erythrocyte membrane protein band 4.1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GLUT1, glucose transporter 1; GNB3, guanine nucleotide-binding protein beta polypeptide 3; Hb, hemoglobin; MR, mineralocorticoid receptor; NO, nitric oxide; NOS3, nitric oxide synthase 3; ROS, reactive oxygen species; SPTA1, α-spectrin; SPTB, β-spectrin; TGFB1, transforming growth factor beta 1.

affect rheological properties of red blood cells through the changes in the cytoskeletal architecture [58,63]. First, rearrangement of cytoskeletal proteins has a considerable impact on erythrocyte deformity increasing blood viscosity, which was found to affect vascular blood flow and exacerbate microcirculatory disorders in hypertension [64]. Notably, the increased membrane microviscosity in hypertensives is accompanied by altered kinetics of sodium-lithium countertransport [65], which is a major ion transport abnormality in essential hypertension. Second, rearrangement of cytoskeletal proteins influences the ability of intracellular hemoglobin to release oxygen leading to tissue hypoxia, as it has been shown in hypertensive patients [18]. Third, abnormal ratio between spectrins, actin, protein 4.1, and dematin may impair signaling pathways coupling extracellular regulatory stimuli to the cytoskeleton through spatial discoordination between receptors and channels involved into the regulation of membrane transport and signal transduction. Lastly, an alteration in the protein composition of the membrane cytoskeleton has the ability to influence the functioning of the ion transport ATPases of the membrane through conformational modifications of their active centers [58,66,67]. Animal studies showed that a removal of spectrin from erythrocytes results in two and three-fold decreasing in the activity of Na^+K^-ATPase and Ca^{2+}-ATPase, respectively [66,68], pointing out that reduced levels of spectrins has the potential to influence the activity of transport ATPases, a possible mechanism involved into the ion transport abnormalities in human hypertension [13,23,24].

The present study revealed that the proteins directly involved in glucose metabolism such as GLUT and glycolytic enzyme GAPDH were also significantly altered in hypertensive patients compared with healthy subjects. Abnormal glucose metabolism is a well characterized disorder found in essential hypertension [69,70]. Figure 5 shows potential mechanisms by which GLUT1 and GAPDH may contribute to the development of hypertension. Glucose transporter is a type III integral membrane protein that facilitates the transport of glucose over a plasma membrane and maintains the low level of basal glucose uptake required to sustain respiration in the cell [71]. As expression levels of GLUT1 in cell membranes are increased by reduced glucose levels [72], our finding of the increased GLUT1 levels in hypertensives may be attributable to the reduced glucose influx within the cell owing to insulin resistance in patients [73]. GAPDH is a membrane-bound enzyme catalyzing an important energy-yielding step in the glycolytic breakdown of glucose [74]. It is important to notify that the enzyme in erythrocytes represents an important part of the glycolytic machinery, which compartmentalizes ATP, allowing its direct consumption by ion pumps and transporters [75]. Perhaps the changes in the GLUT1 levels in hypertensives reflect the need of cells in a higher glucose uptake and ATP production. This assumption seems attractive in the light of the hypothesis that the cause of elevated BP in chronic arterial hypertension is considered to be a compensatory response to decreased ATP biosynthesis [76]. It was suggested that intracellular Ca^{2+} overload in hypertension can be a consequence of augmented ion permeability of the plasma membrane and/or decreased Ca^{2+}-ATPase activity because of partial ATP depletion [76]. These authors proposed that an attenuated intracellular ATP content enhances the stimulation of sympathic nerve system maintaining long-term elevation of BP. Thus, changes in expression levels of the GLUT1 and GAPDH genes in hypertensive patients may represent a part of insulin-mediated systemic impairment of glucose disposal in peripheral tissues, accompanied by decreased glucose uptake and utilization by the cells [70,73,77].

A summary effect of the genes on the variation of erythrocyte membrane proteins in patients with essential hypertension

We revealed that hypertension susceptibility genes contribute to the variation of erythrocyte membrane proteins in our patients. The strength of these effects was quite substantial (individual R^2 values varied between 6 and 24% in both sexes), indicating that the genes are the important factors of the inherited variation of EMP contents in hypertensives. This means that pleiotropic effects of these genes are not limited by the regulation of vascular tone, sodium, and water homeostasis, but also influence the manifestation of genes expressed in erythroid tissue. Genes associated with the risk of essential hypertension such as *ADD1*, *AGT*, *AGTR1*, *GNB3*, and *TGFB1* exhibited considerable effects on the levels of membrane proteins in hypertensive patients. In particular, combination of *GNB3* C825T and *AGT* T174M gene polymorphisms showed highest contribution to the variation of SPTB (24%) and GLUT1 (18%) levels in male patients. The studied genes account for about half the total polygenic variance of GLUT1, SPTA1, and SPTB levels in both hypertensive males and females. Undoubtedly, the effects of genes on the EMP variation can overlap. As it has been found in our previous study [55], about 5–10% of the total variance of spectrins and GLUT as well as 95% of variability in the levels of GLUT and protein band 4.1 in hypertensives (both proteins were increased in essential hypertension patients in comparison with controls) were explained by common underlying genes.

Finally, the results of our study are in line with findings observed by genetic mapping studies on the animal model of human hypertension. The comparative genome mapping allowed identifying the homologous chromosomal regions in rats and humans. In particular, the long piece of the human chromosomal region 1p34-pter corresponds to the rat locus 5(a)-5(b) [23]. The 5(a) region contains quantitative trait locus (QTL) for elevated SBP in spontaneously hypertensive rats and includes the Glutb gene [78], a rat homologue of human glucose transporter. In addition, in Dahl S rats, GLUT gene is located in the QTL that has also been reported to co-segregate with SBP [79]. Interestingly, except for GLUT1 located in a 1p34.2, a homologous region at human chromosome 1p contains or is nearby the loci for α-spectrin (1q21) and protein band 4.1 (1p33-p32), whose altered contents in essential hypertension may be related to the pleitropic effect of putative homologous QTL in hypertensive men. Moreover, another QTL identified in chromosome 2(a) is responsible for

increased BP in spontaneously hypertensive rats [80] and includes subunits of Na^+-K^+ pump and Spta1, a rat homologue of human SPTA1.

The present study has several limitations. We cannot exclude the possibility that changes in the levels of the EMPs in hypertension may be related with oxidative damage of the membrane due to increased production of reactive oxygen species and decreased antioxidant activity, the mechanisms that are important in hypertension pathogenesis [81–83]. Some caution in interpretation of the data on genotype-phenotype relationships is warranted because not all possible gene polymorphisms have been investigated and none of the parameters representing abnormal ion transport of red blood cell membrane have been analyzed.

To the best of our knowledge, this is the first study in which a summary contribution of disease susceptibility genes to alterations in contents of EMPs has been estimated in hypertension. Our study results extent and provide further support to the hypothesis that abnormalities in membrane structure and function represent an important part of hypertension pathogenesis. Rethinking the hypothesis of 'cell membrane alteration as a source of primary hypertension' [12], we can highlight that alterations in the levels of EMPs is not a causative factor of essential hypertension *per se*, but is rather a mirror of the multilevel (from organs to the cell) and multisystem (from a particular cell to various cell types/tissues) disorders related to the disease mechanisms. A better understanding of a nature of cell membrane disorders and their pathogenetic link with hypertension will contribute to a discovery of clinically useful biological markers and targets for the development of innovative drugs for management and prevention of the disease.

ACKNOWLEDGEMENTS

The study was supported in part (biochemical investigations of EMPs) by the Federal Targeted Program 'Scientific and Scientific-Pedagogical Personnel of the Innovative Russia in 2009–2013'. Molecular genetic investigations (genotyping of genes involved in the regulation of vascular homeostasis in hypertensive patients) were done with support of the Russian Research Foundation (№15–15–10010).

This manuscript has not been submitted to any other journal nor published fully or partially elsewhere.

Conflicts of interest

We declare that there is no conflict of interests concerning this manuscript.

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Reviewers' Summary Evaluations

Reviewer 1

Polonikov *et al.* analyzed the relationship between a number of hypertension susceptibility genes and erythrocyte membrane proteins. The results point towards a few specific genes and membrane proteins and provide some novel pathophysiological insights. The main strength of the article, however, is the integration of complex phenotypic, clinical, and genetic data into disease pathways in the spirit of a systems biology approach. The present work could be a template for the analysis and presentation of complex biological data – at least it is one possible approach.

Reviewer 2

In this interesting study, the authors report that hypertension susceptibility genes contribute to inherited erythrocyte membrane protein variation suggesting that abnormalities in membrane structure and function represent an important part of hypertension pathogenesis. The study is of interest. However, to explore the validity of the results, findings should be further validated in bigger and independent samples.