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ARTICLE

Non-synonymous alterations in AKR7A3 and ABCA6 correlate with bleeding in aged patients treated with rivaroxaban

Ming Zhao[1] | Qiang Zhang[2] | Xizi Wang[2] | Qianqian Zhang[2] | Conghui Tian[2] | Rongrong Li[2] | Xiaodong Jia[2] | Mingliang Gu[2] | Liping Yang[1]

[1]Department of Pharmacy, Beijing Hospital, National Center of Gerontology, Institute of Geriatric Medicine, Chinese Academy of Medical Sciences, Beijing Key Laboratory of Assessment of Clinical Drugs Risk and Individual Application (Beijing Hospital), Beijing, P.R. China

[2]Joint Laboratory for Translational Medicine Research, Liaocheng People's Hospital, Liaocheng, P.R. China

Correspondence

Liping Yang, Department of Pharmacy, Beijing Hospital, National Center of Gerontology, Institute of Geriatric Medicine, Chinese Academy of Medical Sciences, Beijing Key Laboratory of Assessment of Clinical Drugs Risk and Individual Application (Beijing Hospital), Beijing, P.R. China.
Email: yanglp_2000@hotmail.com

Mingliang Gu, Joint Laboratory for Translational Medicine Research, Liaocheng People's Hospital, Liaocheng, P.R. China.
Email: minglianggu@hotmail.com

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Abstract

Rivaroxaban is an oral anticoagulant that inhibits thrombin and blocks coagulation cascade through directly inactivating factors Xa. Despite rivaroxaban is widely used for prevention and treatment of venous thrombosis, and its common adverse reactions have been reported, including abnormal coagulation, mucosal hemorrhage, hematuria, and intracranial hemorrhage. To explore potential drivers of individual differences in adverse reactions induced by rivaroxaban, we performed whole-exome sequencing and found that *AKR7A3* rs1738023/rs1738025 and *ABCA6* rs7212506 are susceptible sites for rivaroxaban-related bleeding in aged patients treated with rivaroxaban. Gene functional annotation and signaling pathway enrichment indicated that homozygous mutations in *AKR7A3* and *ABCA6* might alter normal rivaroxaban transport and metabolism, and lead to continuous accumulation of activated drugs and toxic substances in vivo. Our results suggested that interindividual differences in bleeding events induced by rivaroxaban may be potentially driven by genetic alterations related to abnormal metabolism and transport of rivaroxaban.

Study Highlights

WHAT IS THE CURRENT KNOWLEDGE ON THE TOPIC?

Although rivaroxaban has been wildly used for the prevention and treatment of prevent of deep vein thrombosis without requiring routine coagulation monitoring, the adverse events, such as bleeding following rivaroxaban treatment, has not been fully addressed.

WHAT QUESTION DID THIS STUDY ADDRESS?

The correlation between genetic variations and rivaroxaban treatment-induced side effects (e.g., bleeding).

Ming Zhao, Qiang Zhang, and Xizi Wang contributed equally to this work.

WHAT DOES THIS STUDY ADD TO OUR KNOWLEDGE?

The *AKR7A3* rs1738023/rs1738025 and *ABCA6* rs7212506 confer susceptibility to adverse reactions caused by rivaroxaban.

HOW MIGHT THIS CHANGE CLINICAL PHARMACOLOGY AND TRANSLATIONAL SCIENCE?

This study identified *AKR7A3* and *ABCA6* genes involved in drug metabolism and transport associated with susceptibility to rivaroxaban-related bleeding events, and provided supporting evidence for the prevention and treatment of anticoagulant-caused adverse effects.

INTRODUCTION

Rivaroxaban, a novel oral anticoagulant, is widely used to prevent and treat deep vein thrombosis and pulmonary embolism (PE) as well as to reduce the risk of stroke in patients with nonvalvular atrial fibrillation (NVAF).[1] Rivaroxaban directly inactivates factor Xa and blocks common pathway of endogenous and exogenous coagulation cascades, by which to inhibit thrombin and prevent fibrin clot formation.[2] Although routine coagulation monitoring is unnecessary, rivaroxaban has been attributed to an increased risk of bleeding, such as gastrointestinal bleeding, compared with vitamin K antagonists. Potential risks for bleeding include abnormal coagulation phase (prolonged PT or elevated INR), subcutaneous or mucosal bleeding, hematuria, gastrointestinal bleeding, and intracranial hemorrhage, which has been associated with the risk of hospitalization and death.[3,4] NVAF patients treated with rivaroxaban had a higher risk of gastrointestinal bleeding in the first 90 days.[5] The incidence rates of major bleeding and intracranial hemorrhage induced by rivaroxaban were reported to reach 3.6% and 0.5%, respectively, in patients with NVAF.[6] The expected range of plasma levels for standard dose of rivaroxaban were 12–137 ng/ml at peak and 178–343 ng/ml at trough, respectively, reported in clinical pharmacokinetic studies.[7] In a prospective study, rivaroxaban plasma levels in patients admitted for bleeding events ranged from 12 to 251 ng/ml.[8] Currently, it is a challenge to directly correlate blood concentration of rivaroxaban with bleeding events.

Genetic variations in drug metabolism enzymes and transporters largely affect drug disposal, and differential treatment responses among individuals. A pharmacokinetic study has demonstrated that two-thirds of rivaroxaban is metabolized, and the remaining one-third is directly excreted from urine in the form of active drug (including active renal secretion and glomerular filtration).[9] CYP3A4/5 and CYP2J2 were mainly responsible for metabolism of rivaroxaban, whereas ABCB1 and ABCG2 are involved in active renal secretion of rivaroxaban.[10] Pharmacogenomic studies on rivaroxaban mainly focus on single nucleotide polymorphisms (SNPs) of *CYP3A4/5* and *ABCB1* genes. It has been reported that *ABCB1* polymorphisms or haplotypes are correlated with peak concentration of rivaroxaban in a steady-state,[11] and have been associated with rivaroxaban-induced thrombotic events.[12] However, genetic basis of rivaroxaban-induced bleeding events has not been fully addressed.

Considering the complexity of rivaroxaban metabolism, this retrospective case-control study was conducted based on whole-exome sequencing in order to discover candidate genes associated with the potential risk of bleeding caused by rivaroxaban. In this study, we performed whole-exome sequencing on 45 patients treated with rivaroxaban and determined the correlation between genetic variations and bleeding events after treatment. Using gene functional annotation, protein structure change prediction and pathway analysis, we demonstrated how SNPs collectively affect biological functions of drug metabolism enzymes and transporters and cause bleeding events after rivaroxaban administration in aged patients.

MATERIALS AND METHODS

Research subjects

We randomly selected 15 patients (case group) with bleeding events after taking rivaroxaban and 30 patients with no adverse reactions (control group) within 1-year follow-up. The case group consists of 11 patients with atrial fibrillation, and four patients with cerebral infarction, PE, or arterial occlusion. The bleeding event among the patients in the case group are two cases of skin ecchymosis, one case of oral bleeding, one case of hemoptysis, seven cases of gastrointestinal bleeding, one case of vaginal bleeding, two cases of hematuria, and one case of cerebral hemorrhage. In the control group, 29 patients had atrial fibrillation, whereas one patient underwent orthopedic surgery. Although different doses of rivaroxaban were administered according to disease status and renal functional level, all patients received medications at commonly used dosages (5–20 mg). There

was no significant drug-drug interaction observed among comedications taken by all patients included in this study. The 5 ml of peripheral blood was collected with EDTA anticoagulation tube. This study was approved by the Ethical Review Committee of Beijing Hospital. Informed consent was signed by each subject before blood sample collection.

Whole exome sequencing

Genomic DNA was extracted from whole blood by using QIAamp DNA blood MIDI Kit (Qiagen), and quality control was performed by nanodrop (Thermo Fisher Scientific), with an OD 260/280 ration of 1.8–2.0.[13] Approximately 200 bp DNA fragmentation was conducted using Covaris S220 (Gene Company Limited). Breaking parameters were setup as follows: duty factor 10%; peak incident power 175; cycles per burst 200; treatment time 360s; and bath temperature 4°C–8°C. Agilent 2100 quality control was performed on fragmented DNA.

Library preparation was performed using the Agilent Sureselect DNA Targeting Sequence Capture Kit, following manufactory protocol, including: (1) end repair was performed on fragmented DNA, where A was added to the 3′ end, and all gaps were connected with adapters. After each step, AMPure XP beads were used for purification. (2) Polymerase chain reaction (PCR) was performed with an amplification volume of 50 ul. The program was set up as follows: 98°C pre-denaturation for 2 min; 98°C denaturation for 30 s, 65°C annealing for 30 s, 72°C extension for 1 min, totally 10 cycles; 72°C extension 10 min; and 4°C hold. The product was purified with AMPure XP beads. (3) Amplified DNA was hybridized and placed at 65°C for 16–24 h. (4) After hybridization, stranded penicillin magnetic beads were applied for probe capture and PCR amplification. The amplification volume was 50 μl. The program was set up as follows: pre-denaturation at 98°C for 2 min; denaturation at 98°C for 30 s, annealing at 57°C for 30 s, and extension at 72°C for 1 min; totally 12 cycles; 72°C extension for 10 min; and 4°C hold. (5) AMPure XP beads were used for purification, whereas Aglinet 2100 was used for quality control. The fragment size was about 250bp–350bp, and, thus, library preparation was completed.

PE75 sequencing was performed using Nextseq 500 (Illumina).

Quality control and allelic association

Trimmomatic was used to remove the original sequencing connector and low-quality sequence. Filtered sequence was aligned to Thousands Genome Reference Sequence (GRCh37) with BWA. The output data were converted to the BAM file and sorted with Samtools. Sequences were deduplicated with Picard. Using GATK software, single nucleotide variation and indel mutation (In/Del) were analyzed and filtered. Then, called variants were mapped to dbSNP database and annotated with ANNOVAR.

To obtain high-quality data for association analysis, SNPs were trimmed using the following criteria: (1) the call rate of sample or SNP greater than 90%; (2) a threshold of 0.0001 for Hardy-Weinberg equilibrium. Generally, SNPs with minor allele frequencies greater than 0.01 were included in association analysis. Using quality control (QC)-passed SNPs to calculate differences in allele frequency and allele superiority ratio between case and control, SNPs related to bleeding after rivaroxaban were included ($p < 0.05$). QC and Allelic association were completed through Plink.

STRING and HPRD were used to query interaction proteins. The Swiss-model was used to construct 3D configuration of encoded proteins based on amino acid sequences. Then, the SwissPDB viewer was used to identify structural changes before and after a mutation was introduced and to estimate alterations in force fields. Pfam was used to predict protein domains. TMpred was performed to predict protein transmembrane regions.

RESULTS

Allelic and genotypic analysis of bleeding events induced by rivaroxaban

To discover candidate genes that are associated with the potential risk of bleeding post-rivaroxaban treatment, we collected the blood samples from 15 patients with diverse bleeding events within 1 year after received rivaroxaban treatment, and 30 patients who were bleeding free for over 1 year. There was no significant difference in age, medical history, and creatinine clearance rate (renal function) between case and control groups by one-way analysis of variance; $p > 0.05$; Table 1).

Whole exome sequencing revealed a total of 62 SNPs that showed a significant difference in mutational frequency between case and control groups ($p < 0.05$; Table S1), including three SNPs (ABCA6 rs7212506 and AKR7A3 rs1738023/rs1738025) were located in genes of rivaroxaban transporter and metabolic enzymes (Table 2). A pharmacokinetic study demonstrated that two-thirds of rivaroxaban was metabolized,[9] and mutations in genes encoding metabolic enzymes or transporters modulate potential risk of adverse events caused by rivaroxaban treatment.

We therefore calculated genotype distribution of these three SNPs by comparing homozygous variant

TABLE 1 Basic characteristics of patients in case and control groups

Index	ADR	Control	*p* value
Gender, male/female, *n* %	8/7	14/16	—
Age, years[a]	82.7 ± 4.9	79.2 ± 7.2	0.104
BMI, kg/m^2	25.9 ± 3.5	24.1 ± 2.9	0.119
Creatinine clearance rate, ml/min	56.9 ± 17.2	65.7 ± 21.8	0.222
History of stroke, yes/no	2/13	7/23	0.356
History of CAD, yes/no	11/4	16/14	0.167
History of diabetes mellitus, yes/no	4/11	8/22	0.645
History of hyperlipidemia, yes/no	8/7	13/17	0.375

Abbreviations: ADR, adverse drug reaction; BMI, body mass index; CAD, coronary artery disease.
[a]Values were presented as mean ± SD.

TABLE 2 SNPs with significantly different allele frequencies related to drug transport and metabolism

CHR	Gene	Exon	SNP	Allele	Case	Control	*p* value
17	ABCA6	20	rs7212506	C>T	0/0/15	1/11/18	0.03289
1	AKR7A3	5	rs1738023	T>C	0/0/15	1/8/21	0.00536
1	AKR7A3	7	rs1738025	T>C	0/0/15	1/8/21	0.00536

Abbreviations: ABCA6, ATP Binding Cassette Subfamily A Member 6; AKR7A3, Aldo-keto reductase family 7 member A3; CHR, chromosome; SNP, single nucleotide polymorphism.

TABLE 3 Differential locus genotype distribution and risk of bleeding events

				Case		Control			
CHR	Gene	SNP	Genotype	*n*	%	*n*	%	OR (95% CI)	*p* value
17	ABCA6	rs7212506	CT + CC	0	0	12	40	1	—
			TT	15	100	18	60	1.667 (1.244–2.232)	0.004
1	AKR7A3	rs1738023	TC + TT	0	0	9	30	1	—
			CC	15	100	21	70	1.429 (1.130–1.806)	0.02
1	AKR7A3	rs1738025	TC + TT	0	0	9	30	1	—
			CC	15	100	21	70	1.429 (1.130–1.806)	0.02

Abbreviations: CHR, chromosome; CI, confidence interval; OR, odds ratio; SNP, single nucleotide polymorphism.

versus heterozygous variant and non-mutated genotypes, and found that homozygotes *ABCA6* rs7212506 and *AKR7A3* rs1738023/rs1738025 served as susceptible sites for bleeding events when taking rivaroxaban (Table 3).

Protein structure prediction and function analysis

These SNPs in coding regions of genes were predicted to change proteins' structures, functions, and signal transduction, to assess the correlation among these SNPs and biological dysfunction. We performed protein structure analysis and found the MET to ILE mutation on the 875th amino acid of ABAC6 altered the minimum free energy from −1358.7 KCAL/mol to −1460.5 KCAL/mol. The ASN to ASP mutation on the 215th amino acid of AKR7A3 caused the minimum free energy change from −500.8 KCAL/mol to −499.1 KCAL/mol, while the minimum free energy of AKR7A3 T323A altered from −500.8 KCAL/mol to −502.5 KCAL/mol. According to analysis on protein structure and force field, the force fields were affected by AKR7A3 N215P, AKR7A3 T323A, and ABCA6 M875I affect (Figure 1), whereas the protein stability decreased followed the increasing of the minimum free energy. The decreased protein stability alters the spatial conformation of protein and in turn causes abnormal function and the disorder in biological processes, results in a series of adverse reactions.

The 875th amino acid of ABAC6 is located on the ABC2 membrane three domain, whereas the 215th amino acid of AKR7A3 is located on Aldo/Keto/Red domain (Figure 2). The ABCA6 has 13 transmembrane domains, whereas the

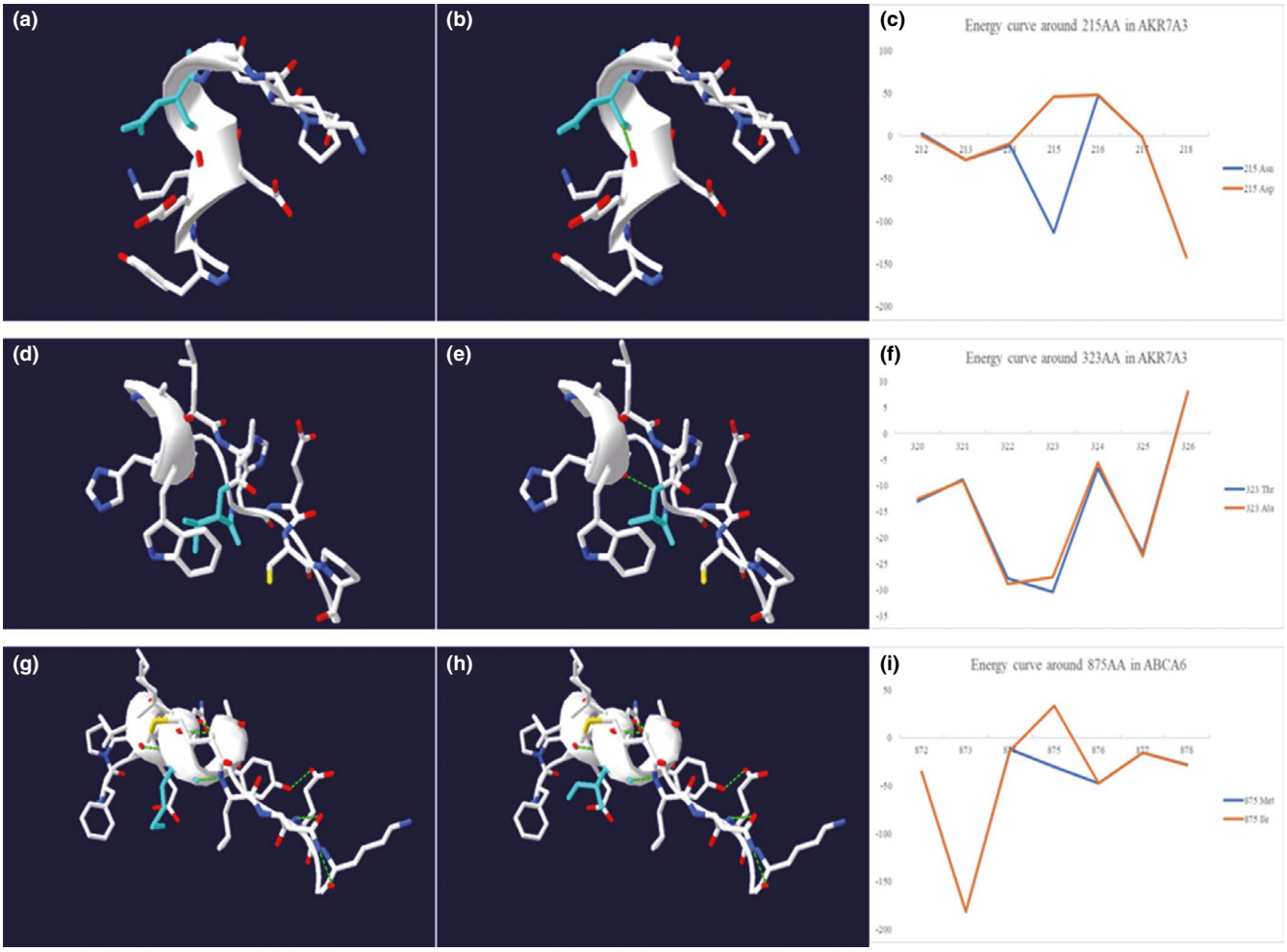
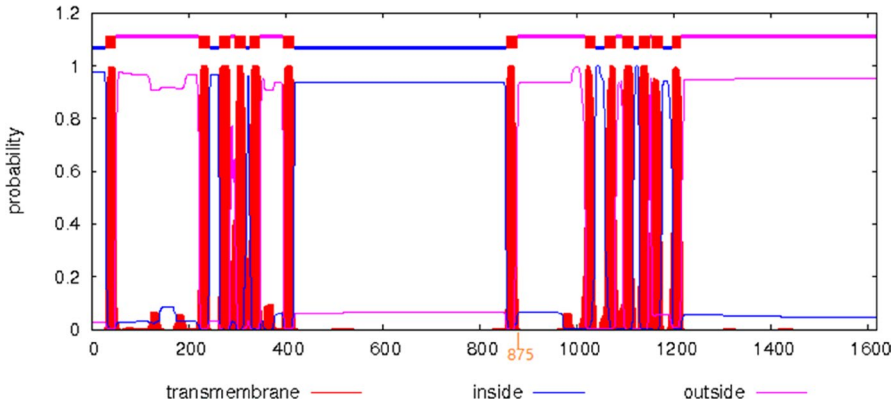


FIGURE 1 Schematic diagram of 3D protein structure and force field curves. (a–i) The protein structure and force field changes of AKR7A3 N215P, AKR7A3 T323A, and ABCA6 M875I were, respectively, demonstrated. The blue represents the amino acids at the variant site; the green dotted line represents hydrogen bonds; the blue line in the force field curve represents the energy before the amino acid change at this point; and the red line represents the energy after the amino acid change

FIGURE 2 Schematic diagram of the prediction results of ABCA6 transmembrane domain. The 875th amino acid of ABCA6 that triggers adverse bleeding reactions is on the seventh transmembrane structure



875th amino acid is located within the seventh transmembrane domain (Figure 3). The MET to ILE mutation on the 875th amino acid of ABAC6 might reduce the capacity of transmembrane transportation, resulting in disturbance of ABC transporter signaling pathway, whereas the ASN to ASP mutation on the 215th amino acid of AKR7A3 affects drug metabolism of rivaroxaban. Collectively, these mutations on AKR7A3 and ABCA6 might contribute to the accumulation of active parent drug and metabolic toxic substances.

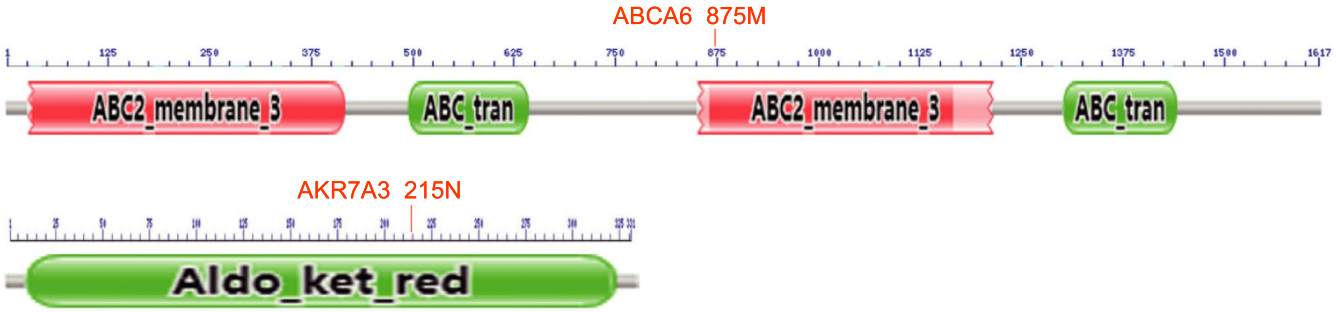


FIGURE 3 Schematic diagram of the prediction results of ABCA6 and AKR7A3 protein domains

DISCUSSION

Although rivaroxaban, a new oral anticoagulant, has potential advantages in terms of pharmacokinetics without requiring routine coagulation monitoring, adverse reactions (such as bleeding) caused by rivaroxaban has not been fully addressed.[14,15] We performed whole exome sequencing on 15 patients who suffered from bleeding events after rivaroxaban treatment and 30 patients without adverse effects. *AKR7A3* rs1738023, rs1738025, and *ABCA6* rs7212506 were associated with bleeding events. For the first time, whole exome sequencing was applied in the Chinese population to screen genes related to drug metabolism and transport involved in rivaroxaban-induced bleeding events. Our results highlight the potential of using these SNPs to predict the risk of bleeding caused by rivaroxaban treatment.

The metabolism of rivaroxaban is mainly dependent on CYP3A4, CYP2J2, and non-CYP components. The AKR7A3 is a member of AKR family that involves in detoxification or biological activation of aldehydes or ketones, and plays an important role in drug metabolism, hormone metabolism, and carcinogen decomposition. AKR7A3 is involved in exogenous drug metabolism. STRING and HPRD databases were used to query interaction proteins of AKR7A3. We selected that there are mutual relations in both databases (score > 0.9 in STRING database), and found that AKR7A3 interacted with AKR7A2. They are involved in biological oxidations, pharmacokinetics, aflatoxin activation, and detoxification signaling pathway. We predicted that the homozygous variant of AKR7A3 blocked the normal metabolic process of rivaroxaban, leading to accumulation of the active parent drug in vivo. The 215th amino acid of AKR7A3 is located within the Aldo/ Keto/Red domain, and the ASN to ASP mutation on the 215th amino acid of AKR7A3 disturbs drug metabolism, including rivaroxaban. Therefore, *AKR7A3* mutation reduced scavenging capability of CYPS to metabolize foreign matters (e.g., exogenous active drugs), resulting in the accumulation of toxic substances of drug metabolites and adverse reactions, such as bleeding.

ABCA6 is a member of ATP-binding cassette transporter superfamily that transports both extracellular and intracellular substrates including drugs or metabolites. The 875th amino acid of ABAC6 is located within the ABC2 membrane three domain, as well as the seventh transmembrane domain. We speculated that the homozygous variant of *ABCA6* might affect transmembrane stability, resulting in disorder of ABC transporter signaling pathway, which in turn modulates disposal process of rivaroxaban. When the 875th amino acid of ABAC6 changed from MET to ILE, the transmembrane transporting capacity of ABAC6 was reduced, which resulted in disturbance of ABC transporter signaling pathway. In patients with the homozygous variant of ABCA6, the drug transport of rivaroxaban may get disturbed leading to a prolonged existence of active parent drug and metabolites, which further results in bleeding events.

Notably, homozygous variant of ABCA6 and AKR7A3 also existed in the control group, which did not show bleeding phenotype due to different dosage and duration of administration. In addition, there are differences between individuals, and many variant genes associated with adverse drug reactions. Due to the nature of a retrospective hospital-based case-control study at a single center, as well as a limited sample size, we could not determine the combination effects of multiple SNPs on adverse effects using limited size of samples. Moreover, there was no significant difference in age, medical history, and creatinine clearance rate between case and control groups. We could not collect a complete set of clinical information that are required for a comprehensive multiple regression analysis.

Nevertheless, for the first time, our study demonstrated concordance of AKR7A3 N215P, AKR7A3 T323A, and ABCA6 M875I in modulating rivaroxaban-induced blooding events among the aged Chinese patients, which warrants the future multicenter study to develop a comprehensive predictive model of bleeding events caused by rivaroxaban treatment, and tailor individual administration of clinical medication.

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CONFLICT OF INTEREST

The authors declared no competing interests for this work.

AUTHOR CONTRIBUTIONS

M.Z., Qiang Z., and X.W. wrote the manuscript. L.Y. and M.G. designed the research. M.Z., Qiang Z., Qianqian Z., C.T., and R.L. performed the research. X.W. and X.J. analyzed the data.

ORCID

Ming Zhao https://orcid.org/0000-0002-2273-4641

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

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