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Warfarin resistance in *Rattus losea* in Guangdong Province, China

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Abstract

Control of rodent populations is performed worldwide with coumarin derivatives, such as warfarin. After widespread use, their effect has been diminished by the rapid spread of resistant rodents. Warfarin resistance in *Rattus loseas* in Jiangmen and Zhanjiang City, Guangdong Province, was investigated by lethal feeding tests. Twenty-three of 30 *R. loseas* trapped in Jiangmen City were assayed as warfarin-resistant individuals, whereas only 1 of 30 rodents in Zhanjiang was resistant. These results emphasize the need for thorough resistance monitoring as a basis for adequate control measures to prevent the use of ineffective rodenticides in Jiangmen City. The resistance mechanism mainly involves VKORC1, the molecular target for coumarin drugs. VKORC1 mRNA expression in wild-caught resistant animals showed no difference compared with that in susceptible individuals. Mutation screening of VKORC1 was carried out and an Arg58Gly mutation was identified as the prevailing type in *R. loseas* from Jiangmen City, which may constitute the genetic basis of anticoagulation resistance in *R. losea* in this resistance region.

Keywords: *Rattus losea*; Warfarin resistance; VKOR; VKORC1; Coumarin; Hemostasis; Mutation; SNP; Gene expression; Alignment

1. Introduction

Control of rodent populations is maintained worldwide with coumarin derivatives nowadays, which are also widely used for oral anticoagulant therapy in humans. Coumarin derivatives act as antagonists of vitamin K, which functions as a cofactor for the posttranslational carboxylation of glutamate residues to gamma-carboxyglutamate (Gla) in vitamin K-dependent proteins. Gla-containing proteins are mainly involved in hemostasis (coagulation factors II, VII, IX, X; proteins C, S and Z), [1] as well as in bone metabolism (osteocalcin), [2] cell proliferation and apoptosis (growth-arrest-specific proprotein 6, Gas6) [3]. The carboxylation modification is accomplished by the enzyme gamma glutamyl carboxylase (GGCX), which also functions as an epoxide synthase, because it converts the cofactor vitamin K hydroquinone into vitamin K 2,3-epoxide. Availability of vitamin K for this reaction requires prior reduction of vitamin K 2,3-epoxide by the vitamin K epoxide reductase (VKOR) complex in the endoplasmic reticulum (ER) membrane [4]. This cyclic interconversion of vitamin K metabolites is known as the vitamin K cycle [5]. Coumarin derivatives inhibit the vitamin K cycle, and consequently inhibit the coagulation reaction.

Many biochemical analyses have shown that the warfarin resistance mechanism mainly involves the VKOR complex, [6,7] though the components of the VKOR complex have not been identified yet. In 2004, the gene encoding an essential protein of VKOR enzymatic activity (named vitamin K epoxide reductase subunit 1, VKORC1) was cloned and sequenced in humans and rats [8,9]. The encoded protein was concomitantly identified as the molecular target for coumarin drugs. And the following studies showed that VKORC1 is a member of a large family of homologs that are represented among vertebrates and

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arthropods, as well as protists, plants, and bacteria [10,11]. Recent study showed that VKORC1 protein alone was able to catalyze the reduction of the vitamin K epoxide to vitamin K and vitamin K to vitamin KH_2 [12].

Rattus losea, mainly distributed in southern China, Lao People's Democratic Republic, Thailand, Viet Nam, and Taiwan Province of China, is one of the main pests in agriculture. After the widespread use of warfarin in some areas in Guangdong Province, China, effective *R. losea* control has become hampered by the rapid development of warfarin resistance in this rodent pest. Warfarin has also been widely used in Jiangmen and Zhanjiang cities, especially in Jiangmen City, where farmers have used warfarin six times every year for more than two decades. In the present report, warfarin resistance was detected in *R. losea* in Jiangmen City and Zhanjiang City, Guangdong Province, as well as mutations of VKORC1 that are probably responsible for the resistance.

2. Materials and methods

2.1. Animals for warfarin feeding tests

The study population consisted of 60 *R. loseas*, 30 each from farms in Jiangmen City (January 2006) and in Zhanjiang City (March 2006), in Guangdong Province, China. The sampling sites are shown in Fig. 1. All animals were healthy adults and females were not pregnant. They were confined in individual cages and received the same food and water *ad libitum*. All animals received humane care in compliance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health. Resistance to warfarin was assayed by feeding studies developed by the World Health Organization (WHO) with some modifications: an acclimatization period, followed by a pretest diet assessment of 10 days, then by a 9-day no-choice feeding schedule of 0.002% warfarin-containing corn (diet consumption was monitored daily), and 21 days of posttreatment observation. Survival during the test with the amount of active ingredient ingested (>10 mg/kg bodyweight) were considered as evidence of resistance [13].

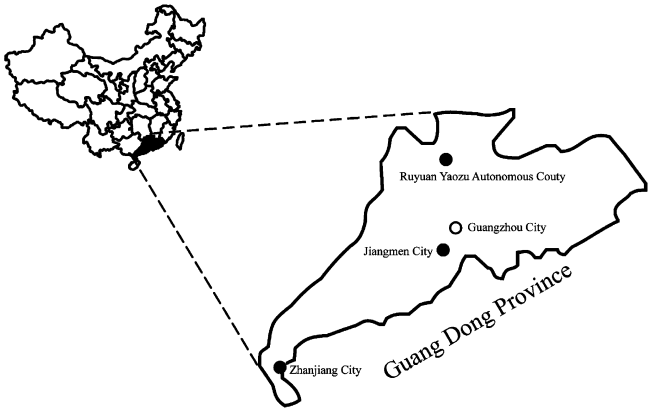

Fig. 1. The map of Guangdong Province, showing the wild *R. losea* collection sites: Jiangmen City, Zhanjiang City, and Ruyuan Yaozu Autonomous County.

2.2. Total RNA extraction and reverse transcription reaction

Total RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, CA,). RNA concentrations were evaluated spectrophotometrically at 260 nm. The 260/280 ratios were between 1.8 and 2. Reverse transcription was achieved using Oligo $(dT)_{15}$ primer (Promega, Madison, WI) and M-MuLV reverse transcriptase (New England Biolabs, Hitchin, UK) according to the supplier's instructions. Conditions for reverse transcription were as follows: 60 min at 42 °C, followed by 5 min at 98 °C.

2.3. VKORC1 amplification and sequencing

The ORF sequence of VKORC1 was identified using *R. loseas* trapped from Ruyuan Yaozu Autonomous County, Shaoguan City, Guangdong Province (Fig. 1), where anticoagulation rodenticides had never been used and rodents were all warfarin susceptible. *R. loseas* were killed under isoflurane anesthesia ($n = 4$). Livers were excised, rapidly frozen in liquid nitrogen, and stored at −80 °C. The total RNA was isolated and reverse transcribed to cDNA. VKORC1 in *R. losea* were amplified using primers based on corresponding sequences for *Rattus norvegicus* in GenBank (Accession No. NM_203335). The primers amplify a 511 bp fragment spanning the whole ORF of VKORC1 mRNA. Nucleotide sequences for the sense primer and antisense primer were 5′-GTGTCTGCGCTGTACT GTCG-3′ and 5′-CCTCAGGGCTTTTTGACCTT-3′, respectively. PCR was performed using 1 U of VentR DNA Polymerase (New England Biolabs) in a final concentration of 1× PCR buffer as formulated by New England Biolabs, 250 μM of dNTP, and 0.5 μM of each primer set in a total volume of 20 μL. PCR conditions consisted of 94 °C for 2 min, followed by 30 cycles of 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 40 s, with a final extension period of 72 °C for 5 min. PCR products were run on a 1.5% agarose gel containing ethidium bromide. The corresponding fragments were cut and purified using a QIAquick Gel Extraction Kit (Qiagen, Valencia, CA), and sequenced on a commercial ABI 3730 capillary sequencer.

2.4. Database and sequence analyses

The prediction of the amino acid sequence of *R. losea* VKORC1 was carried out using the ORF finder program in NCBI. Sequence alignments with its orthologs in other species were performed with program clustalW in EBI.

2.5. VKORC1 mRNA expression and statistical analysis

To compare the expression of VKORC1 mRNA in the two groups of animals, total RNA from susceptible

($n = 4$) and resistant ($n = 4$) rats was extracted from their livers and reverse transcribed. The resulting cDNA (1 μL) was amplified by PCR using specific primers for *R. losea* VKORC1 and GAPDH. The sense and antisense primer sequences for the GAPDH gene were 5′-AAACCCATCACCATCTTCCA-3′ and 5′-CCTGCTTCACCACCTTCTTG-3′, respectively, amplifying a 580 bp fragment. The cycle number within the exponential phase of the amplification curve was chosen. The amplification was performed at 94 °C for 2 min, 25 (for GAPDH) or 29 (for VKORC1) cycles of 94 °C for 30 s, 58 °C for 30 s, 72 °C for 40 s, followed by a final extension at 72 °C for 5 min. PCR products were run on a 1.5% agarose gel containing ethidium bromide and band densities were measured using Bandleader Software. The transcripts of *R. losea* VKORC1 from susceptible and resistant rat hepatic tissue samples were normalized to the abundance of GAPDH. All values are expressed as means ± SE. Two-tailed Student's *t* test was used to determine the statistical difference between the two groups. $P < 0.05$ was considered significant.

2.6. VKORC1 mutations and single nucleotide polymorphisms (SNPs) in potential warfarin resistance areas

In order to screen the mutations in the coding region of VKORC1 in resistance *R. losea*, hepatic RNA from six resistant animals, as well as 51 additional animals caught in Jiangmen City (39 animals) and Zhanjiang City (12 animals) on April 2007, was extracted and reverse transcribed to cDNA. Their nucleotide sequences were obtained by cDNA sequencing as described in 2.3. Mutation and polymorphism screens were then carried out by sequence alignment.

3. Results and discussion

3.1. Warfarin-resistant R. losea in Jiangmen City

Coumarin derivatives, e.g. warfarin, which can effectively repress blood coagulation, remain one of the main tools available to control rodent populations worldwide. They have been widely used since the 1950s in Europe and 1980s in China. However, after widespread use, their effectiveness is jeopardized by the evolution of resistance. Many resistant rodent strains have been identified so far in the world (in Europe, USA, Canada, Japan, and Australia) [14].

Thirty *R. loseas* trapped in Jiangmen City were assayed by a lethal feeding test with warfarin. Twenty-three of the 30 individuals survived. The resistance rate of these animals was 76.7%. Only one female animal out of 30 *R. loseas* from Zhanjiang survived the feeding test after ingesting a total of 14.15 mg/kg warfarin. The doses of active ingredient and mortality are listed in Table 1. Even rats from Jiangmen City that died consumed more active ingredient (12.90 ± 4.24 mg/kg warfarin) compared with those (7.60 ± 2.69 mg/kg warfarin) from Zhanjiang City ($P < 0.0001$), indicating that the rodents in Jiangmen City had higher warfarin resistance. Inappropriate use of anticoagulant at resistance foci can apply a selection pressure that tends to increase both the spread and severity of resistance among rodent populations [15]. Our results imply that the extensive resistance in Jiangmen City may enable resistant individuals to survive treatment with warfarin in the fields. And the results emphasize the need for thorough resistance monitoring as a basis for adequate control measures to prevent the use of ineffective rodenticides in this region.

3.2. Nucleotide and amino acid sequence of VKORC1 in R. losea

Though there are many factors that affect warfarin sensitivity, e.g., genetic variants in CYP2C9 (Cytochrome P450, subfamily IIC, polypeptide 9) (the principal drug-metabolizing enzyme that catalyzes the hydroxylation of warfarin [16,17]), warfarin resistance mainly involves the VKOR complex. We therefore identified the coding region sequence of VKORC1 mRNA in warfarin susceptible *R. loseas* trapped in Ruyuan Yaozu Autonomous County. The nucleotide sequence of VKORC1 was deposited in GenBank as Accession No. EF028346. Amino acid sequence alignment was carried out with VKORC1 from *R. norvegicus*, *Homo sapiens*, and other eukaryotes (Fig. 2). The deduced product had 161 amino acid residues, which is only one residue different from *R. norvegicus* VKORC1. The overall amino acid sequence identity com-

Table 1
Results of warfarin feeding test on *R. losea* trapped in Jiangmen and Zhanjiang City

Animals		Body weight (g)		Mortality (no)	Total consumption of active ingredient (mg/kg)			
					Survived		Died	
Site	Sex	Mean	Range		Mean	Range	Mean	Range
Jiangmen	Male, 13	83.04	52–117	4/13	15.01	11.90–19.08	11.90	8.5–14.56
	Female, 17	71.18	52–102	3/17	16.30	12.93–20.53	15.35	9.45–21.25
Zhanjiang	Male, 24	84.13	62–116	24/24	/	/	7.59	4.48–13.73
	Female, 6	70	56–78	5/6	14.15	/	7.67	4.85–12.50

pared with *H. sapiens* VKORC1 (GenBank Accession No. NP_076869) is 83%.

3.3. Expression of VKORC1 mRNA by PCR

It has been reported that a French strain of rat showed a lower expression of VKORC1 mRNA in resistant rat livers compared with susceptible ones [18]. And a similar phenomenon was also observed in a Danish strain of rat [19]. The expression of VKORC1 mRNA was also compared in our resistant and susceptible animals; the transcripts of *R. losea* VKORC1 from the two groups were normalized to the abundance of GAPDH. VKORC1 mRNA expression from warfarin-resistant *R. loseas* was similar to that from susceptible individuals via PCR (Fig. 3). It seems that the transcriptional difference of VKORC1 mRNA can not be the reason for resistance in the *R. losea* in Jiangmen City, Guangdong Province.

3.4. Mutations and SNPs in VKORC1 mRNA

Several studies have focused on mutations in VKORC1 in both human disorders and in laboratory strains and wild

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Gg  MAA--------RAALCAVGLALAGYALHVERAHERDPTYRASCDLGPSVSCTRVFASRWG 52
Tr  MAIPTWE-RKVRIFLCVFGLLLSVYALHVELSRERNPDYRAMCDLGESVSCSKVFSSRWG 59
Rl  MGTTWRSPGRLRLALCLAGLALSLYALHVKAARARNEDYRALCDVGTAISCSRVFSSRWG 60
Rn  MGTTWRSPGRLRLALCLAGLALSLYALHVKAARARNEDYRALCDVGTAISCSRVFSSRWG 60
Mm  MGTTWRSPGLVRLALCLAGLALSLYALHVKAARARDENYRALCDVGTAISCSRVFSSRWG 60
Hs  MGSTWGSPGWVRLALCLTGLVLSLYALHVKAARARDRDYRALCDVGTAISCSRVFSSRWG 60
Dm  -MEQAYSTASRLRGICVCGLAISVYSLYVKMKLKEDENYRPMCDVNDNISCSLVFKSGYG 59
                 :*  ** :: *:*:*:     .:  **. **:.  :**: ** * :*

Gg  RGLGLLEAFVGRDSAINVPNSVIGIAFYSLQHCSAQCRARASSAALLVTSVTSVLASLYL 112
Tr  RGFGLVQYFVDKDSPLNQPNSVLGIIFYTLQMCLGLSLSRKAALFLVFSSWVSVAGSLYL 119
Rl  RGFGLVEHVLGADSILNQSNSIFGCMFYTLQLLLGCLRGRWASILLILSSLVSVAGSLYL 120
Rn  RGFGLVEHVLGADSILNQSNSIFGCMFYTIQLLLGCLRGRWASILLILSSLVSVAGSLYL 120
Mm  RGFGLVEHMLGADSVLNQSNSIFGCLFYTLQLLLGCLRGRWASILLVLSSLVSVAGSVYL 120
Hs  RGFGLVEHVLGQDSILNQSNSIFGCIFYTLQLLLGCLRTRWASVLMLLSSLVSLAGSVYL 120
Dm  DGFG-----LGNITQVNAPNGAIGCAFYILYFLSSFFNHRWLCLVQLIVCTLTLLLCVYL 114
      *:*    :.   : :* .*. :*  ** :    .    * .   :. .  ::   .:**

Gg  ALVLAFGLHDLCLVCLSTYVVNAALLFLNWRRWRRCGQTAVRKRE- 157
Tr  ASILAFVLGDFCMVCVSTYLVNFVLLFTNLKRRR--AIEGLKEKSG 163
Rl  AWILFFVLYDFCIVCITTYAINAGLMLLSFQKV---PEHKVKKP-- 161
Rn  AWILFFVLYDFCIVCITTYAINAGLMLLSFQKV---PEHKVKKP-- 161
Mm  AWILFFVLYDFCIVCITTYAINVGLMLLSFQKV---PEHKTKKH-- 161
Hs  AWILFFVLYDFCIVCITTYAINVSLMWLSFRKVQE-PQGKAKRH-- 163
Dm  GFLLILVFYDFCLVCVTIYFIHTWLFQEVLRRYR-----RLYM--- 152
    . :* : : *:*:**:: * ::   *:    ::
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Fig. 2. Alignment of VKORC1 orthologs using the algorithm ClustalW. The numbers on the left indicate the amino acid positions of each protein. The "∗" and ":" indicate constitutive and semiconstitutive amino acids. Dashes represent gaps introduced to optimize alignment. The five completely conserved residues (Cys43, Cys51, Cys132, Cys135, and Ser57) are indicated in the hatched box. *Rn: Rattus norvegicus* (GenBank Accession No. NP_976080); *Hs: Homo sapiens* (GenBank accession no. NP_076869); *Rl: Rattus losea* (GenBank Accession No. ABK27271); *Gg: Gallus gallus* (GenBank Accession No. NP_996530); *Mm: Mus musculus* (GenBank Accession No. NP_848715); *Tr: Takifugu rubripes* (GenBank Accession No. AAR82912); *Dm: Drosophila melanogaster* (GenBank Accession No. NP_001014533).

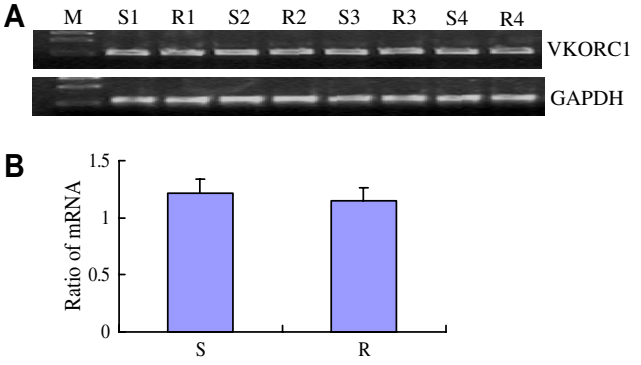

Fig. 3. VKORC1 mRNA expression in warfarin-resistant (R) and susceptible (S) *R. loseas* by PCR. PCR products were subjected to electrophoresis on a 1.5% agarose gel. A representative result is shown (A) "S1–S4" and "R1–R4" represent different individuals of susceptible and resistant rodents, respectively. "M" represents the DNA marker. Band densities were normalized for GAPDH levels, and no difference in the expression in the two groups was obtained statistically (B). Error bars represent SEM.

catches of *R. norvegicus*. Several mutations (Arg35Pro, Ser56Pro, Trp59Gly, Leu120Gln, Leu128Gln, Tyr139Ser, Tyr139Cys, and Tyr139Phe) have been identified in warfarin-resistant rats and four mutations in human beings resistant to warfarin (Val29Leu, Val45Ala, Arg58Gly, and Leu128Arg), suggesting that coding-region variants of VKORC1 are extremely detrimental [14,20]. However, no information is available on the genetic mechanism occurrence of resistance in *R. losea*.

Mutation screening was carried out across the ORF region of VKORC1 cDNA from six of our warfarin-resistant *R. losea* samples. A unique single nucleotide mutation was observed in all the resistant individuals. The single nucleotide difference leads to a mutation in the 58th residue of the translation product: the replacement of arginine in susceptible individuals by glycine (Fig. 4). The Arg58Gly mutation has been reported in warfarin-resistant patients by Rost and his colleagues [8]. Furthermore, they also detected the enzyme activity of this type of mutation, showing that recombination expression of the Arg58Gly mutation led to a lower functional efficiency of the enzyme complex. Our field studies showed that the missense mutation Arg58Gly was prevalent in resistance foci (Jiangmen City), with 36 individuals in 39 animals carried the Arg58-Gly mutation, and was probably the specific genetic basis for resistance in Jiangmen City.

Hydrophobicity plots and secondary structure predictions suggest a topology including 3–4 α-helical transmembrane segments in VKORC1 [11,21]. Bioinformatic analyses of VKORC1 homologous protein sequences identified Cys43, Cys51, Cys132, Cys135, and Ser57 as completely conserved residues in all species surveyed, and these residues should comprise catalytic site residues [11]. Especially, the C132–X–X–C135 motif, it is characteristic of the active site of many redox proteins [22]. And the mutation of either of the cysteine residues in this motif resulted in loss of VKORC1 activity [5]. Several residues, such as L120, L128 and Y139, which located in the predicted transmembrane domains [21], though not the highest conserved residues, were also important for warfarin resistance [14]. Serines are generally known to be important residues in active sites of reductases. The highly conserved Ser57 in VKORC1 is a potential candidate for the binding site of the substrate vitamin K epoxide. And the substitution of serine by alanine at 57 resulted in an almost complete loss of VKOR activity [23]. The Arg58Gly mutation in our resistant *R. loseas* lies next to the proposed substrate binding site Ser57, and probably leads to warfarin resistance by reducing the binding activity with anticoagulants.

Continuous inappropriate use of anticoagulant in Jiangmen City increased the proportion of higher resistant *R. losea* in the population. Our feeding test showed that even the animals which died during the test in Jiangmen City survived a longer time and consumed more warfarin than those from Zhanjiang City. We speculated that this phenomenon can partly be due to the heterozygous 58th mutation individuals. Heterozygous individuals carried this mutation possess higher resistant ability than wild type, though cannot survive the feeding test.

There were other types of mutations observed in the wild rodents, including two SNPs and an Arg35Cys mutation (Table 2) showing a high degree of sequence divergence in wild *R. loseas*. Though SNPs do not alter the amino acid sequence of the protein, it was shown that non-coding polymorphism in VKORC1 can also contribute to the variability in the maintenance dose of warfarin in patients [24–26]. The R35C substitution, which was only obtained in one individual from Jiangmen City, may lead to functional impairment of VKORC1 activity and/or

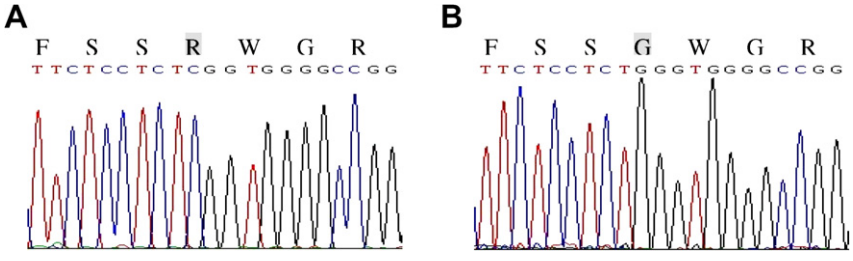

Fig. 4. The Ary58Gly mutation of VKORC1 in warfarin-resistant *R. losea* from Jiangmen City, Guangdong Province, China. The 58th amino acid in warfarin-susceptible animals is arginine (A), while in warfarin-resistant individuals is glycine (B).

Table 2
VKORC1 mutations and polymorphisms found in *R. loseas* from Jiangmen and Zhanjiang cities

Site	Amino acid substitution	No. of specimens	Neutral mutation (SNP)
Jiangmen	R58G (homozygous)	29/39	–
	R58G (heterozygous)	6/39	–
	R58G (heterozygous)	1/39	C96C (heterozygous)
	R35C (heterozygous)	1/39	–
Zhanjiang	–	1/12	A41A (homozygous)

– Indicates none detected.

warfarin sensitivity. Further studies are required to identify the frequency of these genetic variations in rodent populations and their association with warfarin dose required for pest control.

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