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Genetic polymorphisms of VKORC1, CYP2C9, CYP4F2 in Bai, Tibetan Chinese

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Background: VKORC1, CYP2C9 and CYP4F2 are three critical genes associated with inter-individual variation of warfarin dose. Many dosing algorithms containing these gene polymorphisms and demographic characteristics have been set up for better use of warfarin. However, with distinct gene mutation frequencies among different ethnics, dosing algorithms differ greatly. For Chinese, related research just concentrate on Han Chinese, ignoring other Chinese ethnicities. This study aims to detect the popular polymorphisms in these three critical genes in Bai, Tibetan Chinese, to start the exploration of better use of warfarin in Chinese minorities. *Methods*: PCR-based methods were used to analyze VKORC1 3673G > A, CYP2C9*3, CYP4F2 rs2108622 C > T in Han, Bai and Tibetan Chinese. *Results*: The differences among the mutation frequencies of the studied genes in three ethnicities were not statistically significant. The frequency of A-allele of VKORC1 3673G > A was 92.8%, 90.2%, 90.8% in Bai, Tibetan, Han Chinese, respectively. The frequency of *3-allele in CYP2C9*3 was low in Bai (4.5%), Tibetan (2.8%) and Han Chinese (4.6%). Approximately one fourth of each ethnic had the mutant T-allele of CYP4F2 rs108622. However, Bai Chinese got statistically higher A-allele frequency of VKORC1 3673G > A than previously studied Han Chinese did. *Conclusions*: Bai Chinese got significant higher A-allele frequency of VKORC1 3673G > A.

1. Introduction

Warfarin is quite commonly used as oral anticoagulation in patients with thrombosis. But due to its narrow therapeutic index and wide inter-individual variation, INR is monitored as a target to control the dose. Many genes are involved in the action and metabolism of warfarin, including vitamin K epoxide reductase complex subunit 1 gene (VKORC1), cytochrome P450 2C9 gene (CYP2C9), cytochrome P450 4F2 gene (CYP4F2), epoxide hydrolase 1 gene (EPHX1), etc. (Lee et al. 2009). VKORC1, CYP2C9 and CYP4F2 are three major ones.

VKOR is the target of warfarin to operate anticoagulation. VKOR catalyzes vitamin K 2,3-epoxide reducing to vitamin K hydroquinone, which is elementary to the activation of vitamin K-dependent clotting factors (FII, FVII, FIX, and FX). The mutation of VKORC1 lead to alert sensitivity to warfarin, accounting for a major portion of dose inter-individual variance (D'Andrea et al. 2005; Wadelius et al. 2005). A popular SNP in the promoter, 3673G > A has been reported to be associated with inter-individual variation of warfarin dose and lead to changed activity, accounting for 20%–40% contribution to warfarin dose variability (Lee et al. 2009; Wadelius et al. 2009; Zhang et al. 2009; Cen et al. 2010). The A-allele, with 44% decreased promoter activity compared to the G-allele, need lower warfarin dose (Yuan et al. 2005).

CYP2C9 is a member of the cytochrome P450 family which metabolizes warfarin. Its common variants CYP2C9*2 and CYP2C9*3 are of 12%, 5% of activity compared to the wild-types, respectively (Rettie et al. 1994; Stubbins et al. 1996; Tanbe et al. 2000), leading to rising concentration of warfarin in blood, and dose–reduced adjustment. The mutation can explain 3%-26% wafarin dose variability (Lee et al. 2009; Wadelius et al. 2009; Yoshizawa et al. 2009; Zhang et al. 2009; Cen et al. 2010). The mutation frequencies of CYP2C9*2, CYP2C9*3 among ethnics are distinct. Compared to Caucasian, Asian frequency of CYP2C9*2 is almost nil, (Scordo et al. 2001), but frequency of CYP2C9*3 is 4–18%, partly higher than Caucasian (Yang et al. 2003; Lee et al. 2006). The distinct enzyme activity reduction caused by CYP2C9*2, *3, and their different frequencies among ethnics result in dosing algorithms' inter-ethnic inapplicability, which make CYP2C9 mutation an essential factor in setting up different ethnic-targeting dosing algorithms.

Another important warfarin dose variation-related factor is CYP4F2, which is associated with the ω-hydroxylation of arachidonic acid and vitamin E (Powell et al. 1998; Sontag and Parker 2002). CYP4F2 has been recently reported to be involved in the metabolism of vitamin K1, and one SNP, CYP4F2 rs2108622C > T can lead to changed activity of vitamin K oxidase. In human liver microsomes, genotype TT displays a 75% enzyme activity reduction compared to genotype CC. CYP4F2

Table 1: Genotype and allele frequencies of VKORC1, CYP2C9, CYP4F2 in healthy Bai, Tibetan, Han Chinese

Polymorphism		Han Chinese	Bai Chinese	Tibetan Chinese	Total
VKORC1 3673 G > A	GG	1/131 (0.8)	1/132 (0.7)	1/107 (0.9)	3/370 (0.8)
	GA	22/131 (16.8)	17/132 (12.9)	19/107 (17.8)	58/370 (15.7)
	AA	108/131 (82.4)	114/132 (86.4)	87/107(81.3)	309/370 (83.5)
	GA + GG	130/131 (99.2)	131/132 (99.3)	106/107 (99.1)	367/370 (99.2)
	Alleles				
	G	24/262 (9.2)	19/264 (7.2)	21/214 (9.8)	64/740 (8.6)
	A	238/262 (90.8)	245/264 (92.8)	193/214 (90.2)	676/740 (91.4)
CYP2C9*3	*1/*1	119/131 (90.8)	120/132 (90.9)	101/107 (94.4)	340/370 (91.9)
	*1/*3	12/131 (9.2)	12/132 (9.1)	6/107 (5.6)	30/370 (8.1)
	*3*3	0/131 (0)	0/132 (0)	0/107 (0)	0/370 (0)
	*1/*3 + *3*3	12/131 (9.2)	12/132 (9.1)	6/107 (5.6)	30/370 (8.1)
	Alleles				
	*1	250/262 (95.4)	252/264 (95.5)	208/214 (97.2)	710/740 (95.9)
	*3	12/262 (4.6)	12/264 (4.5)	6/214(2.8)	30/740 (4.1)
CYP4F2 rs2108622 C > T	CC	77/131 (58.8)	66/132 (50.0)	61/107 (57.0)	204/370 (55.1)
	CT	49/131 (37.4)	57/132 (43.2)	44/107 (41.1)	150/370 (40.5)
	TT	5/131 (3.8)	9/132 (6.8)	2/107 (1.9)	16/370 (4.4)
	CT + TT	54/131 (41.2)	66/132 (50.0)	46/107 (43.0)	166/370 (44.9)
	Alleles				
	C	203/262 (77.5)	189/264 (71.6)	166/214 (77.6)	558/740 (75.4)
	T	59/262 (22.5)	75/264 (28.4)	48/214 (22.4)	182/740 (24.6)

rs108622 T-allele carriers tend to have a higher hepatic level of Vitamin K1, and require a higher warfarin dose (McDonald et al. 2009). CYP4F2 rs2108622 can explained about 2%-7% of dose variability in Caucasian, Han Chinese (Caldwell et al. 2008; Borgiani et al. 2009; Lee et al. 2009; Zhang et al. 2009; Cen et al. 2010). Previous studies in our lab has also proved that CYP4F2 rs2108622C > T contributes about 4% to warfarin dose variation in Han Chinese (Cen et al. 2010). The effect is minor but significant, which shouldn't be neglected for warfarin dose adjustment.

The above mentioned three major genes have been studied in many ethnicities, like Caucasian, Black, Asian, to set up ethnic-targeting dosing algorithms for dose prediction (Schelleman et al. 2008; Lee et al. 2009; Zhang et al. 2009; Cha et al. 2010; Scott et al. 2010). In Chinese, similar research is just focused on Han Chinese (Ngow et al. 2009; Sandanaraj et al. 2009). Due to dosing algorithms' inter-ethnic inapplicability, the remaining 55 Chinese minorities shouldn't be ignored. That distinct inter-ethnic mutation frequencies among Chinese minorities lead to greatly alert enzyme activity has been reported, such as TPMP (Zhang et al. 2006), GSTT1 and GSTP1 (Zhong et al. 2005), CYP2D6 (Guan et al. 2006). For better use of warfarin, gene mutation frequencies of related receptors and enzymes need examination in minorities before hastily using Han Chinese dosing algorithms.

In Yunnan Province, Bai, Tibetan Chinese are two major minorities. To achieve adequate samples, Tibetan, Bai and Han Chinese are selected in this study. Frequencies of VKORC1 3673G > A, CYP2C9*3 and CYP4F2 rs2108622C > T are detected. Comparison will be carried out between Bai, Tibetan Chinese and Han Chinese.

2. Investigations and results

2.1. Subjects

The subjects were 370 healthy, unrelated individuals including 131 Han Chinese (71 males and 60 females, age range 19∼28 years, mean 24.3 years), 132 Bai Chinese (67 males and 65 females, age range 8∼85 years, mean 26.4 years) and 107 Tibetan Chinese (53 males and 54 females, age range 18∼70 years, mean 27.2 years). Samples of Han Chinese were obtained from Guangzhou, Bai and Tibetan Chinese from Yunan Xianggelila and Dali. Written informed consent was obtained from all subjects, and the institutional ethics committee approved the protocol.

2.2. DNA extracting and genotyping

Blood was collected into an EDTA-containing tube. DNA was extracted. Allelic variants of CYP2C9, VKORC1 and CYP4F2 were differentiated from the wild type allelic by the polymerase chain reaction-restriction fragment length (PCR-RFLP) method.

2.3. Statistical analysis

Statistical analysis was performed using the SPSS software. Hardy–Weinberg equilibrium was tested by χ^2 test to compare the observed gene mutation frequencies within groups. The statistical significance of the differences between groups was calculated by the χ^2 test or Fisher's exact test (two sided).

2.4. Results

The present study evaluated the genotype and the allele frequencies of VKORC13673G > A, CYP2C9*3, CYP4F2 rs2108622C > T in 370 healthy individuals from Bai, Tibetan, Han Chinese in mainland China. Table 1 shows the results.

The frequency of the A-allele of VKORC1 3673G > A was high in Bai (92.8), Tibetan (90.2) and Han Chinese (90.8), respectively, where Bai Chinese got the highest. Genotype GG was rare in all three ethnicities.

For CYP2C9*3, none of the individuals was homozygous of *3-allele. The frequency of *3-allele was low in Bai (4.5), Tibetan (2.8) and Han Chinese (4.6), respectively. The frequency of *3-allele in Tibetan Chinese was the lowest among three ethnics.

Table 2: Comparison of allele frequencies (%) of three studied ethnicities in the current study with those in previous studies

i. VKORC1 3673G > A

	VKORC1 3673G > A							
	G	A	n	P_0*	P_1~P_3*	P_4*	P_5*	P_6*
Han Chinese	24 (9.2)	238 (90.8)	262	< 0.05	> 0.05	0.071	0.245	0.208
Bai Chinese	19 (7.2)	245 (92.8)	264	< 0.05	> 0.05	0.008	0.050	0.030
Tibetan Chinese	21 (9.8)	193 (90.2)	214	< 0.05	> 0.05	0.153	0.386	0.372
Asian (healthy) (Scott et al. 2010)	68 (33.3)	136 (66.7)	204					
Han_1 Chinese (South China, healthy) (Gu et al. 2010)	26(9.8)	240(90.2)	266					
Han_2 Chinese (Han and Han_1 combined)	50(9.5)	478(90.5)	528					
Han_3 Chinese (South China patients) (Gu et al. 2010)	21(8.3)	233(91.7)	254					
Han_4 Chinese (Our previous patients) (Cen et al. 2010)	61 (13.7)	383 (86.3)	444					
Han_5 Chinese (Taiwan patients) (Lee et al. 2009)	29(12.2)	205(87.8)	234					
Han_6 Chinese (Han and Han_4 combined)	85(12.0)	621(88.0)	706					

*ii. CYP2C9 *3*

	CYP2C9 *3						
	*1	*3	n	P_1*	P_2*	P_3*	P_4~P_6*
Han Chinese	250 (95.4)	12 (4.6)	262	0.030	0.183	0.062	> 0.05
Bai Chinese	252 (95.5)	12 (4.5)	264	0.028	0.175	0.059	> 0.05
Tibetan Chinese	208 (97.2)	6 (2.8)	214	0.003	0.026	0.008	> 0.05
Han_1 Chinese (South China, healthy) (Gu et al. 2010)	241 (90.6)	25 (9.4)	266				
Han_2 Chinese (Han and Han_1 combined)	491 (93.0)	37 (7.0)	528				
Han_3 Chinese (South China patients) (Gu et al. 2010)	232 (91.3)	22 (8.7)	254				
Han_4 Chinese (Our previous patients) (Cen et al. 2010)	424 (95.5)	20 (4.5)	444				
Han_5 Chinese (Taiwan patients) (Lee et al. 2009)	188 (96.9)	6 (3.1)	194				
Han_6 Chinese (Han and Han_4 combined)	674 (95.5)	32 (4.5)	706				

iii. CYP4F2 rs2108622 C> T

	CYP4F2 (rs2108622)			
	C	T	n	P_4~P_6*
Han Chinese	203 (77.5)	59 (22.5)	262	> 0.05
Bai Chinese	189 (71.6)	75 (28.4)	264	> 0.05
Tibetan Chinese	166 (77.6)	48 (22.4)	214	> 0.05
Han_4 Chinese (Our previous patients) (Cen et al. 2010)	322 (72.5)	122 (27.5)	444	
Han_5 Chinese (Taiwan, patients) (Lee et al. 2009)	179 (76.2)	56 (23.8)	235	
Han_6 Chinese (Han and Han_4 combined)	525 (74.4)	181 (25.6)	706	

* P_0, P_1, P_2, P_3, P_4, P_5, P_6 value represented the statistically significance of allele frequencies between Bai, Tibetan, Han Chinese and Asian, Han_1, Han_2, Han_3, Han_4, Han_5, Han_6 Chinese in previous study, respectively.

For CYP4F2 rs2108622C > T, the frequency of the mutated allele T was approximately one third of that of C-allele in Bai (28.4), Tibetan (22.4) and Han Chinese (22.5), respectively, where Bai Chinese got the highest.

No statistically significant difference was found among three ethnics in each gene. The distributions of the genotypes of the three genes in all ethnics were in Hardy-Weinberg equilibrium.

We also compared our data with those from previous studies. For VKORC1 3673 G > A (Table 2i), Obviously significant differences were found between Asian American from US healthy donors, and Han, Bai, Tibetan Chinese, respectively. The A-allele frequency in Asian American were approximately 20% lower than those in the current three Chinese ethnicities. When comparing to healthy Han Chinese from previous studies

Table 3: Primers, PCR products and the restriction enzymes used in the characterization of polymorphic sites of the studied genes

	Forward primer	Reverse primer	Amplified fragment size (bp)	Restriction enzyme
VKORC1 3673G> A	5'-ATCCCTCTGGGAAGTCAAGC-3'	5'-CACCTTCAACCTCTCCATCC-3'	636	Bcn I
CYP2C9*3	5'-AATAATAATATGCACGAGGTCCAGA GGTAC -3'	5'-GATACTATGAATTTGGGACTTC-3'	141	Kpn I
CYP4F2 rs 2108622	5'-CGGAACTTGGACCATCTACA-3'	5'-CCTACTCTCCCACAGGCATTA-3	439	Pvu II

(Gu et al. 2010), or the combination of Han Chinese from current and previous studies, no significant difference was found. But Bai Chinese kept higher mutation frequency. As for the comparison with Han Chinese patients (Lee et al. 2009; Cen et al. 2010), Bai Chinese presented significantly higher A-allele frequency ($P \leq 0.05$), while no significant difference was found in Han, Tibetan Chinese.

With respect to CYP2C9*3 (Table 2ii), Tibetan Chinese showed significantly lower frequency of *3-allele comparing to Han Chinese healthy volunteers and patients ($P < 0.05$). As for CYP4F2 rs2108622 C > T (Table 2iii), no significant difference was found between the three ethnics and Han Chinese from previous studies

3. Discussion

The warfarin doing algorithms' inter-ethnic inapplicability, and the absence of related mutation frequency data in the Chinese minorities prompted us investigating VKORC1, CYP2C9, CYP4F2—three principle genes involved in inter-individual warfarin dose variation in Bai, Tibetan Chinese. In many ethnicities, such as Caucasian, African-American, Asian, Jewish, etc., CYP2C9, VKORC1 variant allele frequencies have been extensively reported among treated patients and healthy people. CYP4F2 increasingly draws researchers' attention for its disputed effect on warfarin dose variation (Lee et al. 2009; Zhang et al. 2009; Cen et al. 2010; Cha et al. 2010), and frequencies have been detected in Caucasian, Asian. However, most research done on Chinese just refer to Han Chinese. No related study surveys healthy donors or treated patients in Chinese minorities on even one of the three principle genes, not to mention three all together. Chinese minorities, due to long history and geographic isolation, the mutation frequencies may be greatly different compared to Han Chinese. Problems, like prolonged adjusted time caused by dose misprediction, probably come out in hasty application of dosing algorithms, multiple linear regression models set up for Han Chinese. That's what this study worked out to solve, prevent and improve.

In this study, VKORC1 3673G > A in three ethnicities (A-allele 91.4%) was much higher than those in American Asian (A-allel, 66.7%), but quite close to those of East Asian, like Japanese, Han Chinese from previous studies (A-allele 89%-92%) (Geisen et al. 2005; Yuan et al. 2005; Marsh et al. 2006; Takahashi et al. 2006; Yoshizawa et al. 2009; Cen et al. 2010; Gu et al. 2010). As for American Asian, the mutation frequency is probably due to the diverse sub-ethnic composition, and obviously different frequencies among sub-ethnics. Similarly, sub-ethnics in Chinese probably do not share similar mutation frequencies, and this makes studies like ours necessary.

When comparing to Han Chinese from previous studies, we combined our data with those released, whose subjects were healthy donors or treated patients, to enlarge the sample size, in order to make the comparison more credible. In this study, A-allele frequency (92.8%) of VKORC1 3673G > A in Bai Chinese was not only the highest among the three ethnics, but also statistically not lower than those of Han Chinese from previous studies, no matter if healthy donors or patients. Bai Chinese also got the highest mutated T-allele frequency (28.4%) of CYP4F2 rs2108622C > T. VKORC1 3673A leads to lower dose, while CYP4F2 rs2108622T causes higher dose. However, considering that VKORC1 3673G > A contributes the most to dose variation, but CYP4F2 rs2108622C > T just contributes a small proportion, Bai Chinese may benefit more from lower warfain dose than Han, Tibetan Chinese, and probably need a separate dosing algorithm for better use of warfarin in the future guidance.

In this study, Tibetan Chinese showed significantly lower CYP2C9*3 frequency than combined Han Chinese, which indicated a lower warfarin dose. However, A-allele frequency of VKORC1 3673G > A in Tibetan Chinese was statistically similar to those of Han Chinese. Dose variation of Tibetan Chinese is probably not influenced so complicated as with Han Chinese. And due to CYP2C9*3's medium contribution to warfarin dosage variation, moderate adjustment of dosing algorithms or multiple linear regression models are needed in the future.

4. Experimental

4.1. DNA extracting

Blood was collected into a EDTA-containing tube. In a 1.5 ml centrifuge tube:

(1) Every 100 μl blood was added 200 μl sterile water, 200 μl 6 mol/l NaI, 400 μl chloroform/isopropanol (24:1, v/v), centrifugalized at 13314 × g for 10 min. The supernatant was reserved.
(2) Isopropanol (300 μl) was added to the supernatant, stewing for 3 min at room temperature, then centrifugalize at 13314 × g for 10 min, The residual was reserved.
(3) Ethanol (70%, 500 μl) was added to the residual, centrifugalized at 13314 × g for 10 min. Then ethanol was removed. Repeated once.
(4) The centrifuge tube was inverted to dry. 40 μl TE buffer was added to make DNA dissolved.

4.2. Genotyping

Allelic variants of the CYP2C9, VKORC1 and CYP4F2 were differentiated from the wild type allelic by the polymerase chain reaction-restriction fragment length (PCR-RFLP) method. PCR amplification was carried out in a total reaction volume of 25 μl containing 50 ng genomic DNA, 2 μl dNTPs (0.25 mmol/l), 1 μl each of primer (10 μmol/l), 2.5 μl 10 × Ex Taq buffer, 0.75 U Ex Taq DNA polymerase. The PCR-amplified products of CYP2C9, VKORC1 and CYP4F2 were digested with restriction enzymes and analyzed after gel electrophoresis. Details on primer sequences, amplified fragment sizes and restriction enzymes are shown in Table 3.

PCR detecting VKORC1 3673G > A was performed as described (Obayashi et al. 2006). The cycling profile contained the first step held at 95 °C for 5 min, followed by 35 cycles at 95 °C for 1 min, 60 °C for 30 s, and 72 °C for 2 min, a final extension at 72 °C for 10 min. PCR products were digested by Bcn I at 37 °C for 5 h, and analyzed by 2.5% agarose gel electrophoresis. For CYP2C9*3, primers were based on what described (Sullivan-Klose et al. 1996). The cycling profile contained the first step held at 94 °C for 5 min, followed by 30 cycles at 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 1 min, a final extension at 72 °C for 7 min. PCR products were digested by Ava III and Kpn I at 37 °C for 3 h, and analyzed by 3% agarose gel electrophoresis. For CYP4F2 rs2108622, primers were designed by Primer premier 5.0. The cycling profile contained the first step held at 94 °C for 5 min, followed by 35 cycles at 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 1 min, a final extension at 72 °C for 7 min. PCR products were digested by Pvu II at 37 °C over night, and analyzed by 2.5% agarose gel electrophoresis.

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