Risk of sudden coronary death based on genetic background in Chinese Han population

NENGHUA ZHANG[1*], XIAOCHUN LV[2*], XIAOJUAN CHENG[3], JIAQI WANG[3], JINDING LIU[3], JIE SHI[3], JIE LIU[1], BO HU[1], DEQING CHEN[4] and GENGQIAN ZHANG[3]

[1]Department of Clinical Laboratory and Pathology, Municipal Key-Innovative Discipline of Molecular Diagnostics, Jiaxing Hospital of Traditional Chinese Medicine, Jiaxing University, Jiaxing, Zhejiang 314001; [2]Department of Cardiovascular Medicine, Fenyang Hospital of Shanxi Province, Fenyang Hospital Affiliated to Shanxi Medical University, Fenyang, Shanxi 032200; [3]Department of Forensic Biology, School of Forensic Medicine, Shanxi Medical University, Jinzhong, Shanxi 030619; [4]Department of Pathology, Forensic and Pathology Laboratory, Judicial Expertise Center, Jiaxing University Medical College, Jiaxing, Zhejiang 314001, P.R. China

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Abstract. Associations between gene variations and sudden cardiac arrest or coronary artery disease have been reported by genome-wide association studies. However, the implication of the genetic status in cases of sudden coronary death (SCD) from the Chinese Han population has remained to be investigated. The present study established a mini-sequencing system to examine putative death-causing single nucleotide polymorphisms (SNPs) using multiplex PCR, single base extension reaction and capillary electrophoresis techniques. A total of 198 samples from the Chinese Han population (age range, 34-71 years; mean age, 53.86 years) were examined using this method. Samples were classified into three groups: Coronary heart disease (CHD, n=70), SCD (n=53) and control (n=75) group. Significant associations were identified for 10, 4 and 6 SNPs in CHD, SCD and sudden death from CHD, respectively, using the χ^2 test. The SNPs obtained by binary logistic regression may be used to assess and predict the risk of disease. The predictive accuracy of the SNPs in each prediction model and their area under the receiver operating characteristic curve (AUC) values were determined. The AUC of the four SNPs (rs12429889, rs10829156, rs16942421 and rs12155623) to predict CHD was 0.928, the AUC of the six SNPs (rs2389202, rs2982694, rs10183640, rs597503, rs16942421 and rs12155623) to predict SCD was 0.922 and the AUC of the four SNPs (rs16866933, rs4621553, rs10829156 and rs12155623) to predict sudden death from CHD was 0.912. The multifactor dimensionality reduction values were as follows: 0.8690 (prediction model of CHD), 0.7601 (prediction model of SCD) and 0.7628 (prediction model of sudden death from CHD). Taken together, the results of the present study suggested that these SNPs have considerable potential for application in genetic tests to predict CHD or SCD. However, further studies are required to investigate the putative functions of these SNPs.

Correspondence to: Dr Deqing Chen, Department of Pathology, Forensic and Pathology Laboratory, Judicial Expertise Center, Jiaxing University Medical College, 118 Jiahang Road, Jiaxing, Zhejiang 314001, P.R. China
E-mail: dqchen@zjxu.edu.cn

Professor Gengqian Zhang, Department of Forensic Biology, School of Forensic Medicine, Shanxi Medical University, 55 Wenhua Street, Jinzhong, Shanxi 030619, P.R. China
E-mail: gengqianzhang@sxmu.edu.cn

*Contributed equally

Abbreviations: AUC, area under the receiver operating characteristic curve; CAD, coronary artery disease; CE, capillary electrophoresis; CHD, coronary heart disease; GWAS, genome-wide association study; MDR, multifactor dimensionality reduction; NGS, next-generation sequencing; OR, odds ratio; rSAP, recombinant shrimp alkaline phosphatase; SBE, single base extension; SCD, sudden coronary death; SNP, single nucleotide polymorphism; WES, whole-exome sequencing; WGS, whole-genome sequencing

Key words: sudden coronary death, SNaPshot, single nucleotide polymorphism, coronary heart disease, capillary electrophoresis

Introduction

Sudden coronary death (SCD) is the most common type of sudden death in adults, particularly in middle-aged and elderly individuals (1). SCD primarily occurs when coronary atherosclerosis results in acute myocardial ischemia and there is a sudden interruption to coronary blood flow (2-4). SCD frequently occurs after an inducement, such as drinking alcohol, fatigue, smoking or exercise, and may be fatal within a small number of hours, whereby certain individuals pass away during their sleep (5). Previous studies have reported that ~80% of SCDs are associated with the existence of coronary artery disease (CAD), which is the most common underlying cause in adults (6,7). Several epidemiological studies have demonstrated that a family history of SCD is an

independent risk factor of SCD and patients with different gene mutations may have similar symptoms (8,9). Genetic studies in the past three decades have highlighted the genetic changes of patients with inherited cardiac disease that cause SCD (10,11). Genome-wide association studies (GWAS) are a systemic tool used to evaluate the whole genome to determine the association between gene variants and diseases (12,13). Sudden cardiac arrest caused by CAD has been identified in GWAS (14,15). However, whether these single nucleotide polymorphisms (SNPs) obtained by GWAS that are associated with sudden cardiac arrest or coronary heart disease (CHD) are directly associated with SCD has remained elusive.

Studies on SCD have not been performed in terms of risk gene variations in the Chinese Han population and the underlying genetics that contribute to the SCD single nucleotide variations are of significant interest. When using comparable population groups, it is important to determine the genetic background of an alive individual with CHD and that of individuals who experienced SCD with CHD, which may help understand the predictive value and association between the genetic changes and incidence of SCD to improve SCD prevention measures. Forensic practitioners frequently encounter cases of sudden death for unknown reasons during autopsy. Thus, genetic predictive studies will help determine the cause of death, particularly for those patients who died of CHD.

The majority of biological tissue specimens degrade due to external exposure prior to examination and improper treatment, such as implementation of paraffin-embedded tissues and formalin-fixed human tissues (16,17). Degraded biological specimens are not used for GWAS, whole-genome sequencing (WGS) or whole-exome sequencing (WES), which impedes the application of these techniques in forensic examinations of cases of SCD (18-21). It has been reported that SCD-related SNPs may contribute to SCD, which is easily detected in degraded samples.

The present study developed an assay with mini-sequencing techniques based on the GWAS results of clinical samples reported by Aouizerat *et al* (14) to investigate the genetic background of SCD in the Chinese Han population. This assay applies to most degraded tissues and is yet to be confirmed for use in other populations to identify risk factors, as well as provide accurate diagnoses and prevention advice for first-degree relatives.

Materials and methods

Sample collection. A total of 198 samples were collected from the Chinese Han population. The clinical characteristics of all participants are presented in Table SI. Samples were classified into three groups: The CHD (n=70), SCD (n=53) and control (n=75) groups. The CHD group consisted of blood samples from 70 patients with CHD. The SCD group consisted of 53 cases in which SCD was confirmed following autopsy after death. The control group consisted of 24 cases that died from other causes (such as trauma, poisoning, suffocation, etc.) and 51 volunteers who had no family history of SCD and CHD. The clinical characteristics were not available for the anonymous healthy controls. Sample data are presented in Table I. The sex ratio (male/female) was 3.67 (55/15), 4.89 (44/9) and 3.69 (59/16) in the CHD, SCD and control groups, respectively, with respective median ages (range) in each group of 52 (40-69), 52.5 (37-71) and 53 (34-69) years for the males, and 58 (45-70), 61 (47-68) and 55.5 (48-68) years for the females. Comparisons between the three groups demonstrated no significant differences in sex, age and other complicated diseases. Genomic DNA was extracted from venous blood using the E.Z.N.A™ SE Blood DNA kit (Omega Bio-Tek, Inc.). Tissues were treated using the Mag-Blind® Tissue DNA kit M6223 (Omega Bio-Tek, Inc.) according to the manufacturer's instructions. DNA samples were quantified at an optical density of 260 nm using a BioSpectrometer (22331; Eppendorf AG). The extracted DNA samples were subsequently diluted with highly purified water to the final concentration of 10 ng/μl and stored at -20˚C until subsequent analysis.

Establishment of the SNaPshot assay. The SNaPshot kit (ABI PRISM® SNaPshot™ Multiplex kit; Thermo Fisher Scientific, Inc.) was used to establish a mini-sequencing method for SNP screening in a combination of the multiplex PCR, single base extension (SBE) and capillary electrophoresis (CE) techniques.

Construction of a multiplex PCR system

Candidate SNPs. The sudden cardiac arrest-related gene variations were selected from GWAS results. To determine whether the polymorphisms of these SNPs are prone to induce SCD in the Chinese Han population, 21 SNPs with $P<1x10^{-7}$ were selected from GWAS, based solely on previous studies (22-25). The results revealed that the 21 SNPs were independent and did not exhibit any linkage disequilibrium cluster. Data on the SNPs are presented in Table II.

Multiplex PCR. The multiplex PCR primers were designed using the online software Primer 3.0 (http://primer3.ut.ee). The specificity of the primers was confirmed using National Center for Biotechnology Information Primer Blast (https://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=BlastHome). Auto Dimer v1 software was used to assess the primer-dimer and hairpin structures (26,27). In forensic medicine, most samples experience a period of *ex vivo* degradation prior to the DNA test and even during the fixation procedure with formaldehyde (28,29). Thus, PCR with short fragment amplification (132-280 bp) was performed, which was amenable to type-degraded DNA samples in forensic casework. The primer sequences are listed in Table III. PCR amplification was performed using the GeneAmp PCR System 9700 (Applied Biosystems; Thermo Fisher Scientific, Inc.). The PCR mixture had a total volume of 10 μl, containing 5 μl 2X Multiplex PCR mix (M5 HiPer Multiplex PCR MasterMix; Mei5 Bioservices Co., Ltd.), 1 μl PCR primer mix (the final concentration of each primer was 0.2 μM) and 5 ng DNA template. The following thermocycling conditions were used: Initial denaturation at 95˚C for 10 min, followed by 30 cycles of 94˚C for 20 sec, 58˚C for 20 sec and 72˚C for 30 sec, and a final extension at 72˚C for 5 min (30).

Construction of the mini-sequencing system

SBE primers. SBE primers were designed to pair the bases adjacent to the expected position of the SNP. To isolate

Table I. Sample data of the present study.

Group	Sex	N (%)	Sex ratio (M/F)	Age, years	Patients complicated with other diseases, %
CHD	M	55 (78.57)	3.67:1	52 (40-69)	20
	F	15 (21.43)		58 (45-70)	20
SCD	M	44 (83.02)	4.89:1	52.5 (37-71)	25
	F	9 (16.98)		61 (47-68)	22
Control	M	59 (78.67)	3.69:1	53 (34-69)	None recorded
	F	16 (21.33)		55.5 (48-68)	None recorded

Age is expressed as median (range). M, male; F, female.

Table II. Information of 21 SNPs in the present study.

SNP	Allele	Context	Gene	Location (GRCh38.p12)	P-value from GWAS	Population by GWAS
rs2389202	A/T	Intergenic	MIR1973, TRMT112P1	Chr4: 116333133	$4.000x10^{-7}$	European
rs11624056	A/T	Intergenic	FLRT2, GALC	Chr14: 87039904	$3.000x10^{-8}$	European
rs2982694	G/T	Intron	ESR1	Chr6: 151964552	$7.000x10^{-10}$	European
rs4665058	A/C	Intron	BAZ2B	Chr2: 159333698	$2.000x10^{-10}$	European
rs17718586	G/T	Intergenic	CALM2P1, CASC17	Chr17: 70648048	$2.000x10^{-8}$	European
rs12429889	T/C	Intergenic	KLF12, RNY1P5	Chr13: 74168185	$5.000x10^{-20}$	European
rs16866933	G/A	Intron	ZNF385B	Chr2: 179701951	$6.000x10^{-14}$	European
rs7307780	C/T	Intergenic	RPL10P13, PHLDA1	Chr12: 75826838	$5.000x10^{-15}$	European
rs17291650	A/G	Cds-synon	ATF1	Chr12: 50819650	$3.000x10^{-7}$	European
rs9581094	T/C	Intron	PARP4	Chr13: 24508492	$7.000x10^{-7}$	European
rs10183640	G/A	Intergenic	ACVR1, UPP2	Chr2: 157923692	$5.000x10^{-7}$	European
rs12189362	C/T	Intron	GRIA1	Chr5: 153677988	$3.000x10^{-10}$	European
rs11187837	A/G	Intron	PLCE1	Chr10: 94276223	$4.000x10^{-7}$	European
rs4621553	G/A	Intergenic	YTHDC2, KCNN2	Chr5: 113694467	$4.000x10^{-8}$	European
rs1559040	C/T	Intron	ACYP2	Chr2: 54120613	$4.000x10^{-8}$	European
rs10829156	A/G	Intron	ARL5B	Chr10: 18661626	$4.000x10^{-7}$	European
rs2281680	G/A	Intron/ncRNA	AP1G2	Chr14: 23563861	$6.000x10^{-8}$	European
rs597503	G/A/C	Intergenic	SCML2P1, LAMA1	Chr18: 6939948	$2.000x10^{-8}$	European
rs16942421	G/A/T	Intron	KCTD1	Chr18: 26576461	$8.000x10^{-10}$	European
rs12155623	A/C/T	Intergenic	EFCAB1, SNAI2	Chr8: 48899642	$3.000x10^{-7}$	European
rs2251393	G/A/C	UTR-3	10-Mar	Chr17: 62701571	$4.000x10^{-7}$	European

Chr, chromosome; SNP, single nucleotide polymorphism; GWAS, genome-wide association study; ncRNA, non-coding RNA.

each SBE product in the following CE, -$(CT)_n$ or -$(AGCT)_n$ ('n' indicates the number of repetitions) tails were added at the 5'-end of each SBE primer, according to the length to be detected. In this experiment, the length of expected SBE products ranged between 20-82 bp. SBE primer sequences are listed in Table III.

Purification and SBE reaction. Recombinant shrimp alkaline phosphatase (rSAP; New England BioLabs, Inc.) was adopted to remove excessive deoxyribonucleoside triphosphate (dNTP) from the PCR product and exonuclease I (Exo I; New England BioLabs, Inc.) for excessive PCR primers. The purification reaction was performed in a total volume of 5 μl, containing 3.5 μl PCR product, 0.5 U rSAP and 4 U Exo I. The reaction mixture was incubated at 37˚C for 1 h, followed by 95˚C for 15 min. The purified PCR products were subjected to an SBE reaction in a total volume of 5 μl, containing 2 μl purified PCR products, 2.5 μl SNaPshot reaction mix (SNaPshot™ Multiplex kit; Applied Biosystems; Thermo Fisher Scientific, Inc.) and 0.5 μl SBE primer mixtures (the concentration of the primers was displayed in Table III). The SBE reaction conditions were as follows: 96˚C for 5 sec, 50˚C for 10 sec and 60˚C for 15 sec for 35 cycles. rSAP was adopted to remove excessive dideoxyNTP (ddNTP) prior to CE.

Table III. PCR and SBE primers used in the study.

SNP	PCR primer sequence (5'-3')	Product length, bp	SBE primer sequence (5'-3')	SBE primer concentration, μM
rs2389202	F: TGAACTTCATTGCCATAGTCTCC R: GAAGAGACACTGGCCCTCT	185	TTGGAAAAGATAAAGTCACA	0.20
rs11624056	F: TGTACACTGCTCGGTGATGG R: ACGTCTCTCAGGCTTCTCCA	170	$(GACT)_1$AAATCTCAGAAATCACCACT	0.01
rs2982694	F: CCAAGTATTTTGCTGTTGTTGCT R: CTGGGTGACAGAGTGAGACT	280	$(GACT)_2$TTACTGCATTTGTTTATCAG	1.65
rs4665058	F: CGCGACATGTAACCAGAAATCA R: CCGACCATTTTAGACTTTCCCAG	145	$(CT)_5$TCTTAAAAACAAAATAGCTT	1.25
rs17718586	F: CCATGTCTTCAGCTACACACAG R: GCAGCATATACAACACCTAGCATAG	198	$(GACT)_3$CTACCTGTATCAAAGTAAAT	0.02
rs12429889	F: AGCGTGCATCTTTCATTTCCT R: GGCAAAGAATGGCTCACAGATAC	178	$(GACT)_4$GCTTTGAAACGGTGGCTGTT	0.50
rs16866933	F: CGTGGAAAGGAATGGGCAC R: GCAATCTGGTCTCTTTGGGC	143	$(CT)_9$TCCATCCTAAGCCTCCCAGA	0.03
rs7307780	F: CCCAGAGTGTTTGCTGTTCC R: GACATGCCTTTCACCTTCCAC	176	$(GACT)_5$ATTAGTCTGTTCTCTCATTG	0.35
rs17291650	F: AGTGACCACGGAAAATTACTGAAG R: TTTTCCAGGACTGCAACTCG	166	$(CT)_{11}$TTTAGAGAAGCTGCTCGAGA	0.06
rs9581094	F: GTGTTTCCTGGAAAAGTGACTCAT R: GCCTAAGTGACAAAAGCGAGA	205	$(GACT)_6$ATCTTGTTTTGCATTTTTCT	0.40
rs10183640	F: CGATCAGTTTGGCTGGAGAGA R: AAGCCTGGACAACATAGCGA	177	$(CT)_{13}$AGGAATTTGAACTTTATCTT	0.10
rs12189362	F: CTCTTGGGGCTCCTGTAGAT R: TCTTGCTGTGCTGGTTTGTC	200	$(GACT)_7$TGCTAGAGAAGCTGTATTTC	0.50
rs11187837	F: AGTTGCCCTTGAGTCAGCC R: CACAAGTGGCCAGGTTTCA	190	$(CT)_{15}$CACTCTGGGAAATGCAGGCT	0.55
rs4621553	F: GTTCAGATGCCTTTAGTTGCTGA R: TGCTCATCTTGCCCAGATTTC	174	$(CT)_{17}$TAGTTATACATTACTCAAGG	0.10
rs1559040	F: CGCATTGTGACTATCTGTTGGTA R: CAGACCAGTAGCACAGCCT	234	$(GACT)_9$TTGCCAGCCAGAAATCTCCA	0.04
rs10829156	F: GCCAGTCTTCAGAGTTTAGCATA R: ACACGTCCCTTCTATTCGGT	244	$(CT)_{19}$TCTCGTTTATTGATGTTTGA	0.25
rs2281680	F: GAGGGCAGGACTCCAGAAAG R: TGAGGCATGGACCAGGATG	150	$(CT)_{21}$CATGGAAACCTCTTTCTCCT	0.02
rs597503	F: GGAGATGAATGGTAGTGGTTGC R: TGGTGCCAAAAGTCCTTGTT	164	$(CT)_{23}$TGAATTTCATGGAAATGTAC	0.15
rs16942421	F: CCCTTGCTGAGATTTGGGTG R: CGTTCGAAATGGCTGCTAGG	203	$(CT)_{27}$AAAATACATTTGAATGTACT	0.30
rs12155623	F: GTAGGGCTGAAGAACATGCAAT R: GCTTCAGCACCCCACAAAAC	132	$(CT)_{29}$GGCTTTGGGTGGAAAAGAAC	0.04
rs2251393	F: GCTGCCCATAGATGCTCAAG R: AGCCCTTCTTTCTACGTCCC	134	$(CT)_{31}$ACCCCAAAAGAGAGTGGCAC	0.05

F, forward; R, reverse; SBE, single base extension; SNP, single nucleotide polymorphism.

Separation and visualization by CE. To visualize the SBE products, 1.5 μl SNaPshot reaction products were mixed with 10 μl Hi-Di formamide (Applied Biosystems; Thermo Fisher Scientific, Inc.) and 0.1 μl of GeneScan™ 120 LIZ™ size standard (Applied Biosystems; Thermo Fisher Scientific, Inc.). The mixture was denatured at 95˚C for 5 min, followed by incubation at 0˚C for 3 min. A 3130 genetics analyzer (Applied Biosystems; Thermo Fisher Scientific, Inc.) with a 36-cm

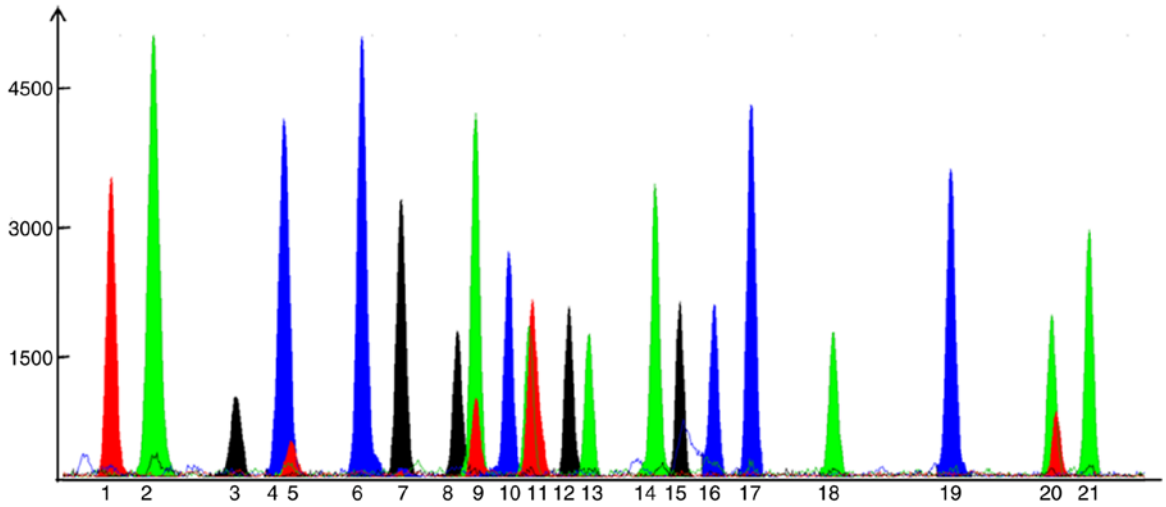

Figure 1. Results of 21 single nucleotide polymorphisms from a random individual from the control group. Blue represents guanine, green represents adenine, black represents cytosine and red represents thymine. Signals: 1, rs2389202; 2, rs11624056; 3, rs4665058; 4, rs17718586; 5, rs2982694; 6, rs16866933; 7, rs12429889; 8, rs7307780; 9, rs17291650; 10, rs10183640; 11, rs9581094; 12, rs12189362; 13, rs11187837; 14, rs4621553; 15, rs1559040; 16, rs10829156; 17, rs2281680; 18, rs597503; 19, rs16942421; 20, rs12155623; and 21, rs2251393.

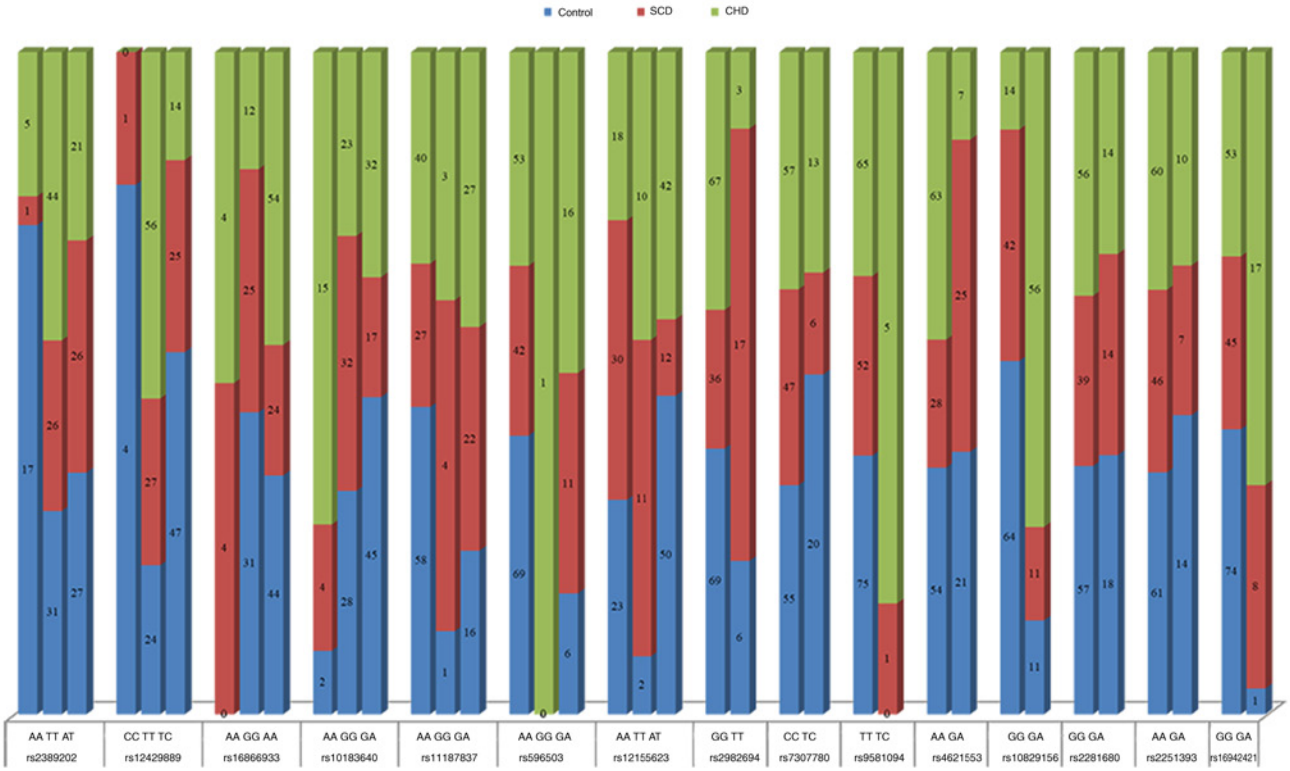

Figure 2. Results of 15 SNPs in each group. Blue represents the control group, red represents the SCD group and green represents the CHD group. Genotype data for these 15 SNPs are presented in Table SII. SNP, single nucleotide polymorphism; CHD, coronary heart disease; SCD, sudden coronary death.

capillary filled with POP-4 gel (Applied Biosystems; Thermo Fisher Scientific, Inc.) was used to detect the SNP genotype. Mutated or normal SNPs were analyzed using GeneMapper ID v3.2 software (Applied Biosystems; Thermo Fisher Scientific, Inc.), based on different fluorescent signals.

Sensitivity of the SNaPshot assay. To further evaluate the detection sensitivity of the mini-sequencing system, multiplex PCR and SBE procedures were performed using 10, 1, 0.1 and 0.01 ng template DNA, respectively. The results are presented in Fig. S1. All experiments were performed in triplicate. Full SNP profiles were detected with 0.1 ng human genomic DNA.

Accuracy of the mini-sequencing system. Samples were randomly sampled for single PCR and sent to Thermo Fisher Scientific, Inc. for Sanger sequencing to verify the accuracy of the experiment. The results were consistent with those of the mini-sequencing assay established in this experiment (Fig. S2).

Table IV. Results of 15 SNPs compared with the χ^2 test among three groups of samples.

A, Comparison between control group and CHD group

SNP	Allele	P-value	Risk allele	Odds ratio	95% CI
rs2389202	A>T	0.001	A	1.837	1.274-2.648
rs12429889	T>C	<0.0001	C	3.667	2.138-6.290
rs16866933	G>A	0.008	G	1.268	1.059-1.519
rs10183640	G>A	0.042	G	1.209	1.004-1.454
rs11187837	A>G	0.010	A	1.151	1.032-1.284
rs597503	G>A	0.006	A	1.102	1.026-1.183
rs12155623	A>T	0.150	A	1.149	0.950-1.390
rs2982694	G>T	0.190	T	1.867	0.720-4.839
rs7307780	C>T	0.278	T	1.436	0.743-2.776
rs9581094	T>C	0.020	T	1.037	1.004-1.071
rs4621553	G>A	0.010	G	2.800	1.229-6.382
rs10829156	G>A	<0.0001	G	1.544	1.339-1.781
rs2281680	G>A	0.587	G	0.978	0.902-1.060
rs2251393	G>A	0.499	G	1.307	0.600-2.845
rs16942421	G>A	<0.0001	G	1.131	1.062-1.204

B, Comparison between control group and SCD group

SNP	Allele	P-value	Risk allele	Odds ratio	95% CI
rs2389202	A>T	0.018	A	1.540	1.061-2.233
rs12429889	T>C	0.059	C	1.440	0.977-2.121
rs16866933	G>A	0.883	G	1.012	0.861-1.190
rs10183640	G>A	0.114	G	0.881	0.756-1.028
rs11187837	A>G	0.001	A	1.227	1.074-1.403
rs597503	G>A	0.044	A	1.071	0.996-1.152
rs12155623	A>T	0.515	A	0.942	0.789-1.125
rs2982694	G>T	<0.0001	T	0.249	0.136-0.459
rs7307780	C>T	0.045	T	2.356	0.979-5.666
rs9581094	T>C	0.233	T	1.010	0.991-1.028
rs4621553	G>A	0.049	G	0.594	0.351-1.003
rs10829156	G>A	0.392	G	1.034	0.956-1.119
rs2281680	G>A	0.774	G	1.014	0.922-1.115
rs2251393	G>A	0.433	G	1.413	0.591-3.382
rs16942421	G>A	0.003	G	1.074	1.016-1.136

C, Comparison between CHD group and SCD group

SNP	Allele	P-value	Risk allele	Odds ratio	95% CI
rs2389202	A>T	0.437	A	1.193	0.765-1.860
rs12429889	T>C	0.001	C	2.547	1.406-4.614
rs16866933	G>A	0.024	G	1.253	1.032-1.521
rs10183640	G>A	0.001	G	1.372	1.144-1.645
rs11187837	A>G	0.400	A	0.938	0.807-1.091
rs597503	G>A	0.550	A	1.028	0.939-1.126
rs12155623	A>T	0.052	A	1.219	1.001-1.485
rs2982694	G>T	<0.0001	T	7.484	3.262-17.171
rs7307780	C>T	0.292	T	0.610	0.240-1.551
rs9581094	T>C	0.186	T	1.027	0.990-1.066
rs4621553	G>A	<0.0001	G	4.717	2.121-10.490

Table IV. Continued.

SNP	Allele	P-value	Risk allele	Odds ratio	95% CI
rs10829156	G>A	<0.0001	G	1.494	1.286-1.735
rs2281680	G>A	0.433	G	0.964	0.879-1.058
rs2251393	G>A	0.869	G	0.925	0.364-2.349
rs16942421	G>A	0.237	G	1.052	0.969-1.142

SNP, single nucleotide polymorphism; CHD, coronary heart disease; SCD, sudden coronary death; CI, confidence interval.

Statistical analysis. Microsoft Excel 2010 (Microsoft Corp.) and SPSS 23.0 software (IBM Corp.) were used to record and analyze the data. The prediction data of CHD were obtained by comparing the control and CHD groups, while the prediction data of SCD were obtained by comparing the control and SCD groups and the prediction data of sudden death from CHD were obtained by comparing the CHD and SCD groups. Odds ratio (OR) values were obtained using the χ^2 test. $P<0.05$ was considered to indicate a statistically significant difference. To evaluate the contribution of SNPs to CHD, SCD or sudden death from CHD, two types of prediction models were established: i) The statistically significant SNPs obtained via the χ^2 test was added into the prediction model to assess the risk of disease. The predicted probabilities were compared with observed disease status, whereas the area under the receiver operating characteristic curve (AUC) was derived as an overall measurement of prediction accuracy, including sensitivity and specificity measured at different probability thresholds, and the true area was set at 0.5 (an AUC value of 0.5 signified complete lack of prediction and 1.0 perfect prediction). Multifactor dimensionality reduction (MDR) was used to assess the potential effect of whether the model improved prediction accuracy and SNP-SNP interactions. ii) All polymorphic SNPs as variables or covariates evaluated by binary logistic regression and obtained association SNPs were incorporated into the prediction model. The AUC and MDR were used to evaluate the ability of the multi-SNPs to identify and predict diseases.

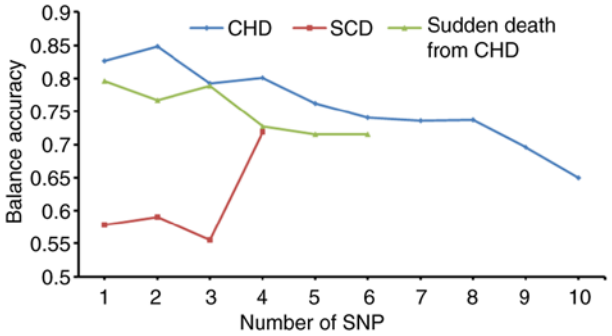

Figure 3. MDR results of the prediction models that added SNPs obtained via the χ^2 test. Blue represents the prediction model of CHD, red represents the prediction model of SCD and green represents the prediction model of sudden death from CHD. The statistical results of MDR are presented in Table SIII (balance accuracy testing). The results demonstrated that as the number of predictive model SNPs increased and the testing accuracy of models exhibited a downward trend, particularly the models of CHD and sudden death from CHD. SNP, single nucleotide polymorphism; CHD, coronary heart disease; SCD, sudden coronary death; MDR, multifactor dimensionality reduction.

Results

Establishment of the mini-sequencing assay. The present study established a mini-sequencing system for screening SCD-related SNPs following extensive adjustment of the multiplex PCR and primers. PCR with short fragment amplification (132-280 bp) was performed, which is easily detected even in degraded samples in forensic casework. All degraded samples were fully typed in the present study. Fig. 1 presents the results of genotyping of 21 SNPs from a random individual sample in the control group.

Genotype data in the Chinese Han population. The present study focused on 21 candidate SNPs from difference loci previously reported by Aouizerat *et al* (14) via GWAS. A total of six SNPs (rs11624056, rs4665058, rs17718586, rs17291650, rs12189362 and rs1559040) that were reported as disease-prone in the GWAS did not exhibit any polymorphism in the population assessed, suggesting a different genetic background between Chinese and European populations. A total of 15 SNPs were polymorphic in the Chinese Han population, including rs2389202, rs12429889, rs16866933, rs10183640, rs11187837, rs597503, rs12155623, rs2982694, rs7307780, rs9581094, rs4621553, rs10829156, rs2281680, rs2251393 and rs16942421. Genotype data for these 15 SNPs are presented in Fig. 2 and Table SII. A total of 10 SNPs (rs2389202, rs12429889, rs16866933, rs10183640, rs11187837, rs597503, rs9581094, rs4621553, rs10829156 and rs16942421) were associated with CHD and four SNPs (rs2389202, rs11187837, rs2982694 and rs16942421) were associated with SCD. Furthermore, six SNPs (rs12429889, rs16866933, rs10183640, rs2982694, rs4621553 and rs10829156) were identified as risk factors for sudden death in patients with CHD. The OR values of the 15 SNPs in the three groups are presented in Table IV. These genotype data contribute to the accumulating evidence on the influence of genetic variation on the risk of CHD and SCD.

Prediction model analysis via the χ^2 test and binary logistic regression. The statistically significant SNPs were sequentially added into the prediction model to evaluate the accuracy by the AUC and MDR. The results demonstrated that, as the number

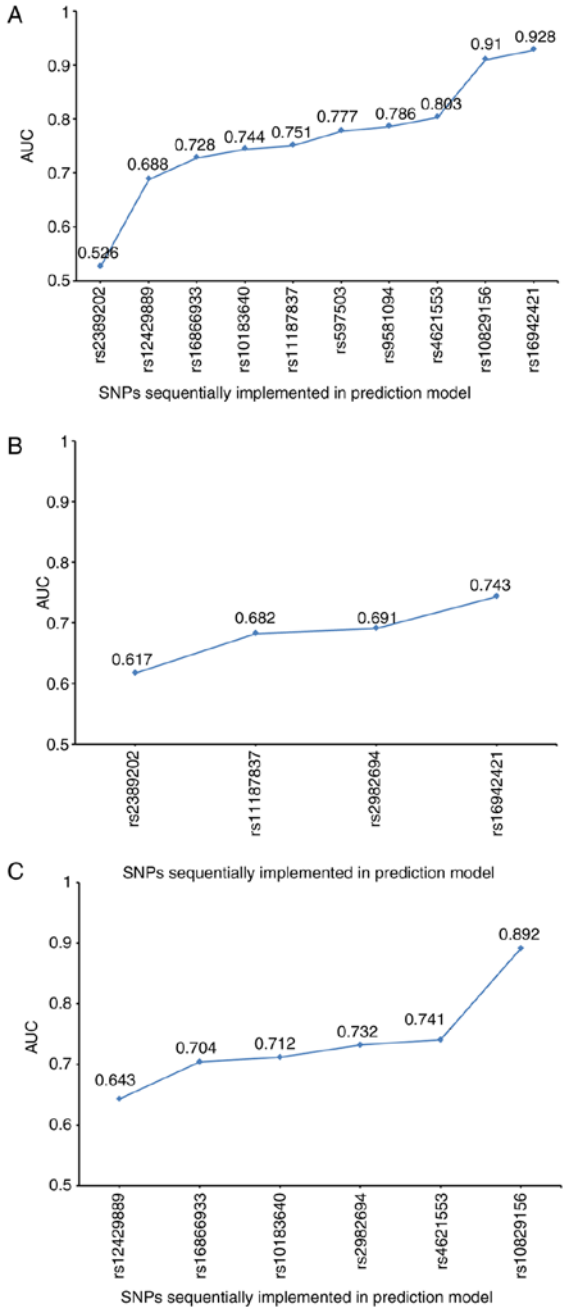

Figure 4. Results of the AUC of the prediction models that added SNPs obtained via the χ^2 test with 10, 4 and 6 SNPs, respectively. (A) AUC values of the prediction model of CHD. (B) AUC values of the prediction model of SCD. (C) AUC values of the prediction model of sudden death from CHD. The statistical results of AUC are presented in Table SIV (Region). The results demonstrated that, as the number of predictive model SNPs increased, the testing accuracy of models exhibited an upward trend. SNP, single nucleotide polymorphism; CHD, coronary heart disease; SCD, sudden coronary death; AUC, area under the receiver operating characteristic curve.

of predictive model SNPs increased, the testing accuracy of models exhibited a downward trend by MDR (Fig. 3); however, the AUC exhibited an upward trend (Fig. 4). MDR data are presented in Fig. 5 and Table SIII. According to entropy-based analysis, this interaction is redundant (redundant information from both factors). Rs10829156 and rs12429889 treated independently explains 33.62 and 18.60% of entropy (it removes 33.62 and 18.60% of 'uncertainty' in CHD determination), respectively. The entropy of interaction between these two SNPs was -18.60%, suggesting that this part of the variation in the determination of CHD is common for both SNPs (Fig. 5A). Similarly, rs2982694 and rs11187837 treated independently explains 6.89 and 6.00% of entropy (it removes 6.89 and 6.00% of 'uncertainty' in the SCD determination), respectively. The entropy of interaction between these two SNPs was -7.45%, suggesting that this part of the variation in the determination of SCD is common for both SNPs (Fig. 5B). The AUC values of the prediction models for CHD, SCD and sudden death from CHD are presented in Table SIV. Taken together, these results suggested that feasibly incorporated statistically significant SNPs in the prediction models may be used to predict the occurrence of CHD or SCD.

To identify a better prediction model, the role of the 15 polymorphic SNPs in CHD or SCD was assessed via binary logistic regression. The predicted probabilities were compared with the observed disease status and the AUC was derived as an overall measurement of prediction accuracy. These SNPs were added to the prediction model and interactions were analyzed by MDR. The results demonstrated that as the number of SNPs increased, the testing accuracy of models exhibited a steady or slight upward trend (Fig. 6), which achieved an improved prediction effect. MDR data are presented in Fig. 7 and Table V. The AUC values of these prediction models determined via binary logistic regression were >90% (Fig. 8). The SNPs of each prediction model are listed in Table VI. Taken together, these results suggested that the high efficiency of these models may provide prediction results for certain individuals.

Discussion

Despite extensive research on the etiology, pathogenesis and risk factors of SCD in recent years, strategies for its prevention and treatment remain insufficient. In addition, the interpretation of GWAS remains difficult. A key limitation of GWAS is that it only depicts associations, not causation. Furthermore, their clinical utility remains to be evaluated in prospective studies together with established risk factors in the present study. In the present study, a mini-sequencing detection system was established containing 21 putative SNPs that have been reported to be associated with sudden cardiac arrest via GWAS. The association of these SNPs with the risk of CHD or SCD in the Chinese Han population was assessed. The results confirmed that 15 SNPs were polymorphic in the Chinese Han population. Different prediction models were constructed for these 15 SNPs to evaluate the risk of CHD, SCD and sudden death from CHD. These models may provide a prediction for a significant proportion of individuals and may be useful to provide a novel detection and evaluation method for the prevention and treatment of CHD and SCD in the future.

The genetic architecture of human complex traits substantially differs. Given that the majority of GWAS were performed in European-descent individuals, SNPs from these GWAS may not be transferrable to individuals from other populations, highlighting the importance of diversification of GWAS into non-European populations. No polymorphism was detected in six SNPs in the Chinese Han population, including rs11624056, rs4665058, rs17718586, rs17291650, rs12189362 and rs1559040. In addition, 10 SNPs were associated with CHD, four SNPs were associated with SCD and six SNPs were

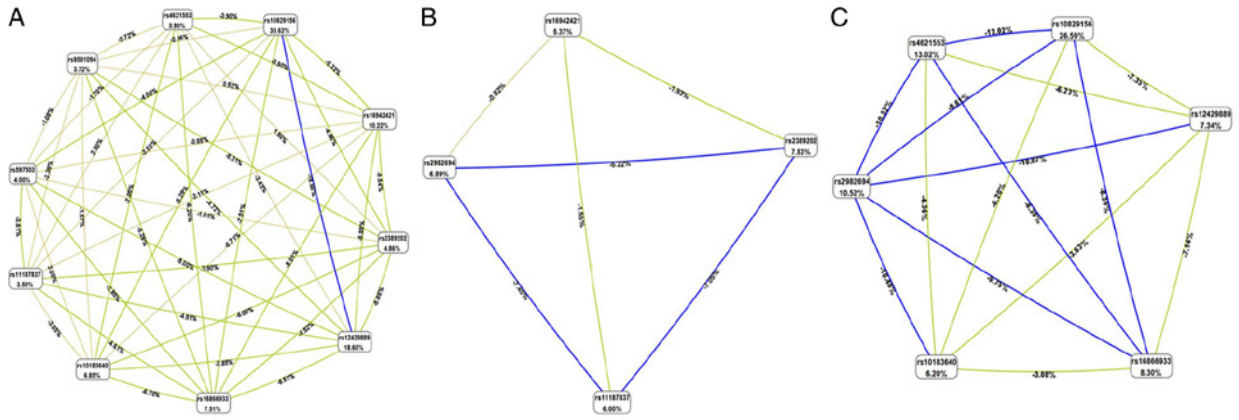

Figure 5. Entropy-based interaction graph from MDR analysis. MDR results of the prediction models that added SNPs obtained via the χ^2 test, with 10, 4 and 6 SNPs, respectively. (A) Prediction model of CHD. (B) Prediction model of SCD. (C) Prediction model of sudden death from CHD. Entropy values in the cells of individual SNPs indicate the main independent effects. Entropy values marked on the lines connecting two SNPs represent the entropy of interaction. Blue lines indicate a high degree of redundancy, green lines indicate a reduced degree of redundancy and yellow lines represent independence or additivity. SNP, single nucleotide polymorphism; CHD, coronary heart disease; SCD, sudden coronary death; MDR, multifactor dimensionality reduction.

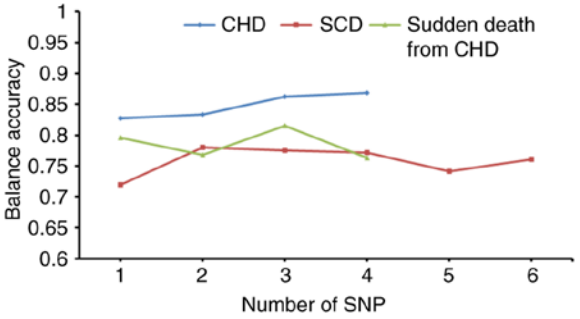

Figure 6. MDR results of the prediction models that added SNPs via binary logistic regression. Blue represents the prediction model of CHD, red represents the prediction model of SCD and green represents the prediction model of sudden death from CHD. The statistical results of MDR are presented in Table V (balance accuracy testing). The results demonstrated that as the number of SNPs increased, the testing accuracy of models exhibited a steady or slight upward trend, which achieved a better prediction effect. SNP, single nucleotide polymorphism; CHD, coronary heart disease; SCD, sudden coronary death; MDR, multifactor dimensionality reduction.

associated with sudden death from CHD. This confirmed that gene variations exert different effects in different populations. Furthermore, whether these results represent an association of genetic variation with the phenotype CHD or SCD requires further investigation.

In the present study, the prediction model obtained via the χ^2 test was required to be combined with additional SNPs to achieve the same prediction probability as the prediction model obtained via binary logistic regression. Furthermore, the prediction model obtained via the χ^2 test was less capable of predicting SCD compared with the prediction model obtained via binary logistic regression. Thus, the prediction model obtained via binary logistic regression is more effective to assess and predict the risk of CHD, SCD or sudden death from CHD.

Previous studies have demonstrated that differences in gene expression result in different physical and pathological traits (31). The rs11187837 locus is situated in the intron region of the phospholipase C ε 1 (PLCE1) gene (GRCh38.p12). A previous study reported that differentially expressed genes of PLCE1 may have a critical role in atrial myocyte hypertrophy (32). The rs10183640 variation is located at the junction of activin A receptor type 1 (ACVR1) and the uridine phosphorylase 2 gene (GRCh38.p12). Previous studies have demonstrated that the ACVR1 gene was closely associated with cardiac fibroblasts, aortic valve development and endocardia cushion formation (33-35). Differentially expressed genes of ACVR1 may be predictors for decreased left ventricular ejection fraction and help diagnose congenital heart defects (36-38). The rs4621553 locus is located at the junction of the YTH domain containing 2 and potassium calcium-activated channel subfamily N member 2 (KCNN2) genes (GRCh38.p12). Previous studies reported that KCNN2 gene variation may have an important role in the development of coronary artery aneurysms and may act as adjunctive markers for risk stratification in patients susceptible to SCD (39,40). The rs12429889 locus is located at the junction of the Kruppel-like factor 12 (KLF12) and RNY1 pseudogene 5 genes (GRCh38.p12). GWAS reported that KLF12 genes are associated with an increased risk of ventricular arrhythmia, syncope and SCD (41,42). The rs597503 locus is located at the junction of the SCML2 pseudogene 1 and laminin subunit α 1 genes (GRCh38.p12). A study on 1,414 Hispanics demonstrated that LAMA1 gene variants are strongly associated with cardio-metabolic traits (43). In addition, Aouizerat *et al* (14) confirmed that rs2389202, rs16942421, rs16866933, rs9581094 and rs10829156 are risk factors of CAD. Of note, rs2982694 was reported to be associated with SCD rather than CAD in the present study. Several genetic association studies of estrogen receptor 1 (ESR1) gene variants concerning CAD have been published (44,45). It has been suggested that ESR1 is a potential candidate gene during acute coronary events, such as acute thrombotic cardiovascular diseases and atherosclerosis to plaque rupture (46-48). In addition, the ESR1 gene regulates the expression of multiple genes following activation by estrogen in cardiovascular disease (49-51). Previous studies have demonstrated that ESR1 polymorphism was associated with atherosclerosis in coronary arteries, the presence of coronary thrombosis and myocardial infarction, and was

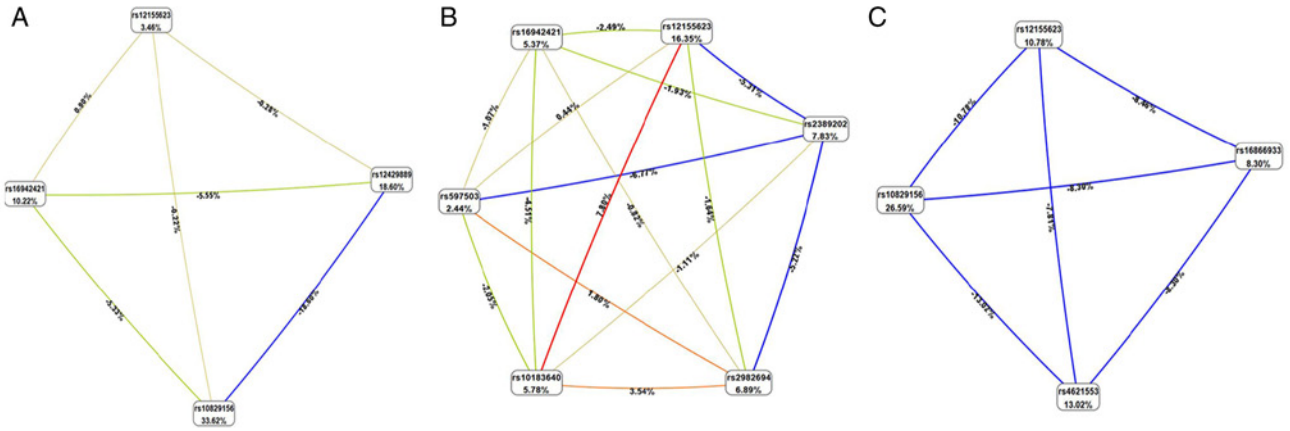

Figure 7. Entropy-based interaction graph from MDR analysis. MDR results of the prediction models that added SNPs via binary logistic regression, with 4, 6 and 4 SNPs, respectively. (A) Prediction model of CHD. (B) Prediction model of SCD. (C) Prediction model of sudden death from CHD. Entropy values in the cells of individual SNPs indicate the main independent effects. Entropy values marked on the lines connecting two SNPs represent the entropy of interaction. Blue lines indicate a high degree of redundancy, green lines indicate a reduced degree of redundancy, yellow lines represent independence or additivity and red lines represent an increased degree of independence. SNP, single nucleotide polymorphism; CHD, coronary heart disease; SCD, sudden coronary death; MDR, multifactor dimensionality reduction.

associated with an elevated risk of CHD in males (46,52,53). Whether rs2982694 is associated with CAD or other cardiac diseases requires further investigation.

CHD and SCD are associated with genetic complexities. The results of the present study indicated that the four SNPs, rs12429889, rs10829156, rs16942421 and rs12155623, are able to predict CHD, the six SNPs, rs2389202, rs2982694, rs10183640, rs597503, rs16942421 and rs12155623, are able to predict SCD and the four SNPs, rs16866933, rs4621553, rs10829156 and rs12155623, are able to predict sudden death from CHD. In the present study, the AUC values of these prediction models were >90% (P<0.0001; Table VI). Taken together, the results of the present study have achieved considerable progress in assessing and predicting CHD, SCD and sudden death from CHD. However, improving the prediction accuracy for CHD or SCD will need to rely on the identification of more associated DNA variants and additional sample sizes, which will be a continuous and accumulative effort, but is certainly achievable in the future.

These SNPs have so far not been used in clinical and forensic prediction and diagnosis of CHD, SCD or sudden death from CHD. The 15 polymorphic SNPs were confirmed in the Chinese Han population (n=198), which may help further understand the pathogenesis of SCD. The purpose of establishing this detection system and prediction models was to achieve a forensic assistant identification and genetic diagnosis of SCD, and to provide a basis for gene therapy. The screening of susceptible genes of CHD or SCD in relatives may provide guidance for their lifestyle and medication, as well as a basis for precision medicine.

A key limitation of the present study is that the CHD and SCD samples were collected from different individuals. In addition, the prediction model is only based on 198 samples from the North Chinese Han population and certain patients with CHD and those who passed away from SCD cannot be traced. Furthermore, it is difficult to predict whether patients with CHD may experience sudden death in the future.

The present study focused on SNPs associated with SCD and the SCD samples collected had an uneven sex ratio (4:1). Thus, CHD and normal control samples of patients whose clinical characteristics [with an uneven sex ratio (4:1)] were consistent with those of the SCD group were collected to control for the variables. No statistically significant differences were observed in terms of age or sex distribution and the presence of any underlying diseases among the three groups, suggesting that these variables were evenly distributed among the groups. The purpose of this process is to minimize the interference of other factors with the prediction model to improve the accuracy of prediction.

In the present study, the SNaPshot assay was performed using multiplex PCR, SBE and CE techniques. The mini-sequencing technology uses a dideoxynucleotide termination method to ligate a fluorescent ddNTP to the 3'-end of an SBE primer to obtain a fluorescent deoxynucleotide sequence. This technique is widely adopted in DNA laboratories due to its high accuracy and sensitivity. There are several technical methods for screening SNPs, such as Sanger sequencing of exons, WGS and WES based on next-generation sequencing (NGS) techniques. Sanger sequencing and mini-sequencing methods are considered more accurate compared with NGS. NGS is expensive and time-consuming and is not applicable to degraded DNA. In clinical and forensic practice, delay-examination and environmental exposure of formaldehyde-fixed samples frequently lead to degradation of tissues, which results in decreased DNA quality for WGS or WES detection (54). In the present experiment, tissues from the SCD group and subjects with death for other causes were degraded due to formaldehyde fixation (fixation period varies from 1 month to 3 years). Not every DNA laboratory is equipped with an NGS instrument to detect this prevailing disease. Using the method of the present study is more feasible to detect degraded DNA samples and more compatible with regular DNA laboratories to meet the increasing requirement of detecting this global disease.

In the present study, 15 polymorphic SNPs associated with CHD or SCD were identified and their predictive value

Table V. Multifactor dimensionality reduction results of the prediction models which added SNPs obtained by binary logistic regression.

A, Prediction model of CHD

Step	SNPs	Balance accuracy training	Balance accuracy testing	CV consistency	χ^2 (P-value)
1	rs10829156	0.8267	0.8267	10/10	62.1767 (<0.0001)
2	rs10829156, rs16942421	0.8482	0.8338	8/10	70.2788 (<0.0001)
3	rs10829156, rs16942421, rs12155623	0.8629	0.8629	10/10	76.3077 (<0.0001)
4	rs12429889, rs10829156, rs16942421, rs12155623	0.8757	0.869	10/10	81.8735 (<0.0001)

B, Prediction model of SCD

Step	SNPs	Balance accuracy training	Balance accuracy testing	CV consistency	χ^2 (P-value)
1	rs12155623	0.7201	0.7201	10/10	24.0998 (<0.0001)
2	rs10183640, rs12155623	0.8066	0.7796	10/10	50.2438 (<0.0001)
3	rs10183640, rs597503, rs12155623	0.8392	0.7757	8/10	58.5882 (<0.0001)
4	rs2982694, rs10183640, rs597503, rs12155623	0.8634	0.7718	7/10	64.916 (<0.0001)
5	rs2389202, rs10183640, rs597503, rs16942421, rs12155623	0.8851	0.7413	6/10	73.5285 (<0.0001)
6	rs2389202, rs2982694, rs10183640, rs597503, rs16942421, rs12155623	0.899	0.7601	10/10	83.8712 (<0.0001)

C, Prediction model of sudden death from CHD

Step	SNPs	Balance accuracy training	Balance accuracy testing	CV consistency	χ^2 (P-value)
1	rs10829156	0.7962	0.7962	10/10	42.6898 (<0.0001)
2	rs16866933, rs10829156	0.7975	0.7677	8/10	42.6898 (<0.0001)
3	rs16866933, rs4621553, rs10829156	0.8245	0.8151	9/10	50.9111 (<0.0001)
4	rs16866933, rs4621553, rs10829156, rs12155623	0.8364	0.7628	10/10	53.857 (<0.0001)

SNP, single nucleotide polymorphism; CHD, coronary heart disease; SCD, sudden coronary death; CV, cross validation.

was determined from 198 samples from the Chinese Han population. Prediction accuracies were expressed as AUC values >0.9. Although these prediction models have not been introduced in clinical practice, the preliminary genetic model

Table VI. SNPs included in each prediction model.

Predicted disease	Prediction model obtained by χ^2 test			Prediction model obtained by binary logistic regression		
	SNP	AUC	MDR	SNP	AUC	MDR
CHD	rs2389202, rs12429889, rs16866933, rs10183640, rs11187837, rs597503, rs9581094, rs4621553, rs10829156, rs16942421	0.928	0.65	rs12429889, rs10829156, rs16942421, rs12155623	0.928	0.869
SCD	rs2389202, rs11187837, rs2982694, rs16942421	0.743	0.7191	rs2389202, rs2982694, rs10183640, rs597503, rs16942421, rs12155623	0.922	0.7601
Sudden death from CHD	rs12429889, rs16866933, rs10183640, rs2982694, rs4621553, rs10829156	0.892	0.7156	rs16866933, rs4621553, rs10829156, rs12155623	0.912	0.7628

SNP, single nucleotide polymorphism; CHD, coronary heart disease; SCD, sudden coronary death; AUC, area under curve; MDR, multifactor dimensionality reduction.

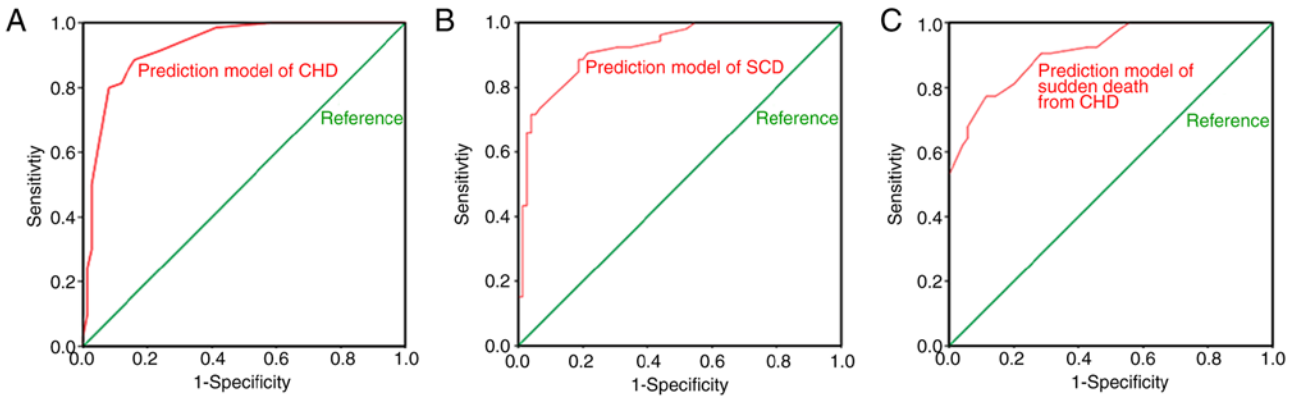

Figure 8. AUC values of the prediction models that added SNPs via binary logistic regression. (A) Prediction model of CHD. (B) Prediction model of SCD. (C) Prediction model of sudden death from CHD. The SNPs of each prediction model and their AUC values were as follows: Four SNPs (rs12429889, rs10829156, rs16942421 and rs12155623) that predict CHD, 0.928; six SNPs (rs2389202, rs2982694, rs10183640, rs597503, rs16942421 and rs12155623) that predict SCD, 0.922; and four SNPs (rs16866933, rs4621553, rs10829156 and rs12155623) that predict sudden death from CHD, 0.912. SNP, single nucleotide polymorphism; CHD, coronary heart disease; SCD, sudden coronary death; MDR, multifactor dimensionality reduction; AUC, area under the receiver operating characteristic curve.

may assist decision-making on CHD, SCD or sudden death from CHD for preventative actions and forensic investigations of the cause of death. Furthermore, the results of the present study suggest that with more genome-wide associated SNPs identified in the future and included in the prediction model together with the SNPs presented here, CHD, SCD or sudden death from CHD will become predictable from DNA, with high accuracy to allow routine practical applications, such as in medicine and forensics.

In conclusion, the present study established a mini-sequencing detection system containing 21 putative SNPs that have been reported to be associated with sudden cardiac arrest. The results of the χ^2 test demonstrated significant associations for 10, 4 and 6 SNPs, in CHD, SCD and sudden death from CHD, respectively. Furthermore, prediction models were established to assess and predict the risk of CHD, SCD or sudden death from CHD. The combination of SNP-associated loci in each group enables the development of a test model with a good predictive performance. Taken together, these results confirm the influence of genetic variation on the risk of SCD in patients with CHD.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

GZ and DC conceived and designed the study. GZ and NZ administratively supported the present study. DC and XC performed the majority of the experiments and drafted the manuscript. XL, JW, JDL, JS, JL, BH and DC provided, selected, assembled, analyzed and interpreted the data. GZ and DC confirm the authenticity of all the raw data. All authors contributed toward data analysis, drafting and critically revising the manuscript, and agree to be accountable for all aspects of the work. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of Shanxi Medical University (Jinzhong, China; approval no. ZX201601) and written informed consent was provided by all participants or their family members prior to the start of the study.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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