Identification of Gene Variants Related to Malignant Hyperthermia in Han Chinese People by Next-Generation Sequencing.

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Research

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Abstract

Background: Malignant hyperthermia (MH) is a pharmacogenetic disease triggered by anesthesia. In recent years, next generation sequencing(NGS) technology, targeted sequencing approaches have been used to identify regions related to MH and can not only detect known MH variant sites but also allow the exploration of novel MH-related variants.

Methods: Twenty-four volunteer's blood samples were collected, genomic DNA was then extracted, target regions were amplified, and through targeted sequencing of these target regions, both known and novel variants were identified, and analysis of these variants identified some that may damage protein function. Online prediction tools were also used to determine the possibility of variants that may cause MH.

Results: We performed next-generation sequencing of 53 positions on 4 genes, in which 36 variants of the *RYR1* gene were found. Three of these caused a change in the amino acid sequence. Additionally, 11 variants of the *CACNA1S* gene were found, of which 3 led to amino acid sequence changes. Five variants of the *JSRP1* gene were found, and four variants caused the amino acid sequence to change.

Conclusions: In healthy volunteers, variants in *RYR1* and *CACNA1S* were more common, and variants in *JSRP1* more commonly had amino acid sequence changes. Whether a variant is pathogenic can be predicted using a prediction website, but the sensitivity and specificity still need to be improved.

Introduction

Malignant hyperthermia (malignant hyperthermia or malignant hyperpyrexia, MH) is a pharmacogenetic disease triggered by anesthesia in patients who are susceptible. MH presents as a hypermetabolic response to volatile halogenated anesthetics, such as halothane, sevoflurane, desflurane, isoflurane and the depolarizing muscle relaxant succinylcholine(1). The typical signs include muscle rigidity, hyperthermia, tachycardia, tachypnea, acidosis, hyperkalaemia, and rhabdomyolysis(1). The incidence of MH ranges from 1:10,000 to 1:250,000, and the syndrome is inherited in an autosomal-dominant pattern(1, 2). Although the incidence of MH is low, the mortality rate can be high without specific treatment.

Calcium channels in skeletal muscle cell membranes of MH susceptible patients have been found to be defective, and the abnormal release of ions leads to an abnormal tetanic contraction of skeletal muscles, resulting in a hypermetabolic state. This can eventually lead to death in MH patients due to organ failure. MH is linked to variants in the reticulum calcium channel protein (*RYR1*) and the α1S subunit of the skeletal muscle dihydropyridine receptor (*CACNA1S*)(3). Over 400 variants have been founded in the *RYR1* gene, and at least 48 may cause MH(1). Less than 1% of variants have been found in *CACNA1S*, but not all of these are pathogenic(1). The Junctional sarcoplasmic reticulum protein 1 (*JSRP1*) and SH3 and cysteine-rich domain-containing protein 3 (*STAC3*) genes may also be linked to MH according to the literature. *JSRP1* is linked to sarcoplasmic reticulin 1, and *STAC3* is an essential component of the skeletal muscle excitation–contraction coupling machinery, which have both been reported to be related to MH(4, 5).

The *in vitro* muscle contracture test (IVCT) is the standard test used to diagnosis MH. Some research has supported the notion that DNA screening may be a viable alternative primary diagnostic approach to IVCT(6). The advantage of genetic testing is that it avoids invasive surgical operations. The clinical value of genetic testing as a diagnostic tool for MH remains to be sufficiently explored. There is a bottleneck in the DNA-based diagnosis of MH because of the large number of uncharacterized variants and the technical difficulty involved in the functional analysis of MH. In recent years, with the development of targeted next-generation sequencing technology, it has become possible to detect known MH variant sites and explore more novel MH-related variants. In this report, we sequenced MH-related variants in 24 healthy Han Chinese and predicted whether these variants were pathogenic.

Methods

Blood samples were obtained from 24 volunteers after written consent. The research was approved by the Affiliated Hospital Academy of Military Medical Sciences Clinical Research Ethics Committee (Reference KY-2018-6-56; Beijing, China). DNA was extracted from peripheral blood lymphocytes, and total genomic DNA was sequenced using a targeted panel, which included known variants, novel variants, and other variants that may damage protein function. Genomic DNA was extracted using a Qiagen kit (Qiagen, Hilden, Germany) according to the manufacturer protocol, randomly fragmented into 150–250 bp, and DNA libraries were prepared using the KAPA LTP Library Preparation kit and polymerase chain reaction (PCR) amplification of the fragmented DNA. Genetic variants in *RYR1*

(NM_000540), *CACNA1S* (NM_000069), *STAC3* (NM_145064), and *JSRP1* (NM_144616) were screened in all of the samples. The variant IDs and chromosome locations were obtained from the literature, as well as from the PHARMKB website and the EMHG web page.

Biotin-labeled primers were required in sequencing. First, the corresponding primers were designed based on established primer design principles, and then labeled with biotin on the 5-'end. The PCR primers and sequencing primers used in this work are shown in Table 1. Next-generation sequencing technology was then used to determine the DNA sequence of the target region to find the variant site and variant frequency of given pathogenic genes from each sample. The raw sequencing data were deposited in the Sequence Read Archive (SRA) at the National Center for Biotechnology Information (accession number: PRJNA613550).

Table 1
Primer Sequences Covering All Exons for Pyrophosphate Reaction

Primer	Primer sequence (5'→3')
12S-2-F	ACACACACCGCCCGTCACCCTC
12S-2-R	Biotin-CCAGTATGCTTACCTTGTTACGAC
12S-2-sequence	CACCGCCCGTCACCC

Prediction of the impact of amino acid changes on protein function were compiled from data from the literature on variants associated with MH and we then used seven prediction websites to predict the effect of variants on each protein's function. We used PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/)(7), PANTHER (http://www.pantherdb.org/tools/csnpScoreForm.jsp)(8), PhD-SNP (http://snps.biofold.org/phd-snp/phd-snp.html)(9), Pmut (http://mmb.pcb.ub.es/PMut/)(10), SIFT (http://sift.jcvi.org)(11), MutPred (http://mutpred.mutdb.org/)(12), and Mutation Taster (http://www.mutationtaster.org/)(13) to evaluate the possibility of using these online prediction tools to determine variants that cause MH (all last accessed February 2020). However, the accuracy of predictions varied from program to program. All of the bioinformatic programs used in this study are available online. We calculated the probability of identifying true deleterious variants as the sensitivity and the specificity as the probability of identifying true neutral variants. PolyPhen-2 classifies variants as 'benign,' 'possibly damaging,' or 'probably damaging'(7). PANTHER classes variants as either 'neutral' or associated with 'disease'(8). PhD-SNP predicts variants as 'neutral' or 'disease' causing(9). Pmut calculates a reliability index ranging from 0 to 10 and a prediction of either 'neutral' or 'pathological'(10). SIFT predicts the impact of an amino acid change on protein function by calculating a 'SIFT score,' with 0–0.05 being classified as 'damaging' and 0.05–1 as 'tolerated' (11). MutPred outputs a general score, with $g > 0.75$ ($P < 0.01$) representing a variant that is very confident to have a phenotypic effect(12). Mutation Taster predicts variants by score(13).

Results

After 24 healthy Han volunteers signed the informed consent documents, we collected blood samples and performed targeted sequencing of MH-related genes. Detection of known variants and novel variants, as well as variants that may damage protein function, was then performed. (Fig. 1). We performed sequencing analysis on 53 positions in four genes, of which 36 nucleotides in the *RYR1* gene were found to be mutated, with three causing a change in the amino acid sequence. About 11 nucleotides in the *CACNA1S* gene were mutated, of which three nucleotide changes led to amino acid sequence changes. Five nucleotides of the *JSRP1* gene were mutated and four nucleotide variants caused the amino acid sequence to change. One nucleotides in the *STAC3* gene were found to be mutated without altering the amino acid sequence (Table 2).

Table 2
Variants detected in genes from 24 patients

Gene	Exon	Nucleotide mutation	Amino acid sequence change	SNP ID	Rate	Number
CACNA1S	Exon 6	c.C735T	p.C245C	rs768251314	0.04	1
CACNA1S	Exon 22	c.C2748T	p.H916H	rs2297902	0.33	8
CACNA1S	Exon 38	c.C4615T	p.R1539C	rs3850625	0.13	3
CACNA1S	Exon 11	c.C1491T	p.N497N	rs16847674	0.25	6
CACNA1S	Exon 11	c.T1551C	p.G517G	rs4915477	0.38	9
CACNA1S	Exon 11	c.C1564T	p.L522L	rs4915476	0.13	3
CACNA1S	Exon 13	c.C1881T	p.Y627Y	rs145689664	0.04	1
CACNA1S	Exon 5	c.C597T	p.I199I	rs2296383	0.67	12
CACNA1S	Exon 31	c.C3822T	p.I1274I	rs56183942	0.25	5
CACNA1S	Exon 44	c.T5399C	p.L1800S	rs12139527	0.29	7
CACNA1S	Exon 11	c.G1530T	p.E510D	rs758354862	0.04	1
JSRP1	Exon 4	c.G198A	p.M66I	rs189529380	0.04	1
JSRP1	Exon 7	c.C864T	p.H288H	rs77817818	0.04	1
JSRP1	Exon 2	c.G3C	p.M1I	rs7250822	0.42	10
JSRP1	Exon 6	c.G449C	p.G150A	rs80043033	0.5	9
JSRP1	Exon 5	c.T275C	p.V92A	rs10426549	0.13	3
RYR1	Exon 79	c.C11266G	p.Q3756E	rs4802584	0.13	3
RYR1	Exon 51	c.T8190C	p.D2730D	rs2915951	0.54	10
RYR1	Exon 85	c.T11754A	p.T3918T	rs45613041	0.13	3
RYR1	Exon 2	c.G79A	p.V27M	rs143481004	0.04	1
RYR1	Exon 51	c.T8118C	p.I2706I	rs2960340	0.54	10
RYR1	Exon 44	c.C7098T	p.P2366P	rs2229147	0.17	4
RYR1	Exon 45	c.C7281T	p.A2427A	rs77310009	0.17	4
RYR1	exon66	c.G9690A	p.L3230L	rs2304151	0.17	4
RYR1	exon49	c.C7863T	p.H2621H	rs2229142	0.46	10
RYR1	exon46	c.C7362T	p.R2454R	rs369428665	0.04	1
RYR1	exon98	c.A14256C	p.T4752T	rs1468571	0.33	7
RYR1	exon27	c.C3618T	p.D1206D	.	0.04	1
RYR1	exon62	c.A9186G	p.P3062P	rs2071089	0.54	10
RYR1	exon39	c.G6365A	p.S2122N	.	0.04	1
RYR1	exon47	c.G7527A	p.V2509V	rs2071088	0.29	6
RYR1	exon49	c.C7872T	p.R2624R	rs1469698	0.17	4
RYR1	exon7	c.A594G	p.L198L	rs2229139	0.71	11
RYR1	exon41	c.C6705T	p.F2235F	.	0.04	1
RYR1	exon44	c.C7209T	p.R2403R	rs78795178	0.17	4

Gene	Exon	Nucleotide mutation	Amino acid sequence change	SNP ID	Rate	Number
RYR1	exon50	c.G7977A	p.T2659T	rs2229144	0.54	10
RYR1	exon45	c.C7260T	p.H2420H	rs12973632	0.04	1
RYR1	exon18	c.C2004T	p.D668D	rs78835441	0.13	2
RYR1	exon24	c.C2979T	p.N993N	rs2228070	0.83	11
RYR1	exon19	c.C2286T	p.P762P	rs3745847	0.75	13
RYR1	exon55	c.T8589C	p.S2863S	rs2229146	0.54	10
RYR1	exon26	c.C3456T	p.I1152I	rs11083462	0.83	10
RYR1	exon47	c.C7584T	p.P2528P	rs1465698	0.17	4
RYR1	exon11	c.T1077C	p.A359A	rs10406027	1	0
RYR1	exon24	c.G2943A	p.T981T	rs2228069	0.58	10
RYR1	exon67	c.C10218T	p.Y3406Y	rs41274330	0.33	7
RYR1	exon44	c.C7089T	p.C2363C	rs2228071	0.17	4
RYR1	exon47	c.G7500A	p.A2500A	rs2228072	0.29	6
RYR1	exon96	c.G14061A	p.L4687L	rs201629205	0.04	1
RYR1	exon53	c.G8337A	p.E2779E	rs2915952	0.54	10
RYR1	exon15	c.G1668A	p.S556S	rs2288888	0.75	13
RYR1	exon3	c.G255T	p.V85V	rs532101469	0.04	1
STAC3	exon6	c.G570A	p.K190K	rs76667525	0.25	5

In these results, the levels of variation in *RYR1* and *CACNA1S* were quite high, and exome sequencing data from the ClinSeq dataset was then evaluated in 24 health volunteers. In addition, using variant data from *RYR1*, *CACNA1S*, *JSRP1*, and *STAC3* for unselected populations, we evaluated the prevalence of MH susceptibility in the general population. Among these mutants, 10 variants that led to changes in amino acid sequences were predicted by prediction software to be pathogenic. Poly-Phen2 suggested that T5399C and G1530T in *CACNA1S* are probably damaging. Variant G79A in *RYR1* was probably damaging and G6365A was possibly damaging, with the rest of the variants being benign. Panther predicted that the variants of *CACNA1S* and *RYR1* were both probably damaging, and that G3C and T275C in *JSRP1* were probably damaging. PhD-SNP predicted that the C4615T variant in *CACNA1S* was pathogenic, while the rest of the variants identified were neutral. Both Pmut and PROVEAN pointed out that the C4615T and G1530T variants in *CACNA1S* were pathogenic, and the rest were benign. SIFT indicated that the C4615T and T5399C variants in *CACNA1S* were pathogenic, but the rest of the identified variants were tolerated. MutPred considers all of the identified variants to be benign. Mutation Taster considered G1530T in *CACNA1S* and G6365A in *RYR1* as diseasing causing. In this study, G1530T in *CACNA1S* was suggested to be pathogenic by 6 prediction software, and C4615T was suggested to be pathogenic by 5 prediction software. (Table 3)

Table 3
Prediction tool predicts variants results

Gene	Mutation	PolyPhen-2	Panther	PhD-SNP	Pmut	PROVEAN	SIFT	Mutpred2 score	Mutation taster
CACNA1S	c.C4615T	benign	probably damaging	disease	disease	deleterious	damaging	0.575	polymorphism
CACNA1S	c.T5399C	probably damaging	probably damaging	neutral	neutral	neutral	damaging	0.632	polymorphism
CACNA1S	c.G1530T	probably damaging	probably damaging	disease	disease	deleterious	tolerated	0.697	disease causing
JSRP1	c.G198A	benign	possibly damaging	neutral	neutral	neutral	tolerated	0.046	polymorphism
JSRP1	c.G3C	benign	probably benign	neutral	neutral	neutral	tolerated	0.114	polymorphism
JSRP1	c.G449C	benign	possibly damaging	neutral	neutral	neutral	tolerated	0.194	polymorphism
JSRP1	c.T275C	benign	probably benign	neutral	neutral	neutral	tolerated	0.017	polymorphism
RYR1	c.C11266G	benign	probably benign	neutral	neutral	neutral	tolerated	0.067	polymorphism
RYR1	c.G79A	probably damaging	probably benign	neutral	neutral	neutral	tolerated	0.18	polymorphism
RYR1	c.G6365A	possibly damaging	probably damaging	neutral	neutral	neutral	tolerated	0.395	disease causing

Discussion

MH is an autosomal-dominant genetic disease that affects calcium release from the sarcoplasmic reticulum (SR) by skeletal muscle. It is characterized by a cause of hypermetabolism when exposed to irritating agents, such as anesthesia. European Malignant Hyperthermia Group (EMHG) guidelines indicate that to classify a pathogenic variant, variants need to be genetically and functionally characterized(3, 14, 15). Recent evidence has also confirmed the foresight of requiring functional analysis of missense variants before their adoption for diagnostic use(14). Currently, the golden standard for diagnosing MH is an *in* IVCT that is based on the contraction of muscle fibers in the presence of halothane or caffeine (16, 17). According to the EMHG guidelines, an individual is under suspicion to MH when both caffeine and halothane test results are positive. However, IVCT is expensive, confined to specialized testing centers, and requires a surgical procedure. Some research has supported DNA screening as a viable alternative primary diagnostic approach to IVCT(6). The potential expense in these additional variants has been more than compensated by the advances in genetic technology.

As the cost of targeted DNA sequencing decreases, next-generation sequencing (NGS) has become the method of choice for variant detection and has been used for the diagnosis of many disease causing variants(3). However, one of the bottlenecks in data analysis is the large number of variants identified in each individual. Common variants can be filtered based on their minor frequencies, but all of the other rare variants need to be investigated.

Genetic analysis of healthy volunteers in this study found that among variants in the *RYR1*, *CACNA1S*, and *JSRP1* genes, variants in *RYR1* had the largest number, with three identified variants causing amino acid sequence changes. Four *JSRP1* gene variants caused amino acid sequence changes, and three *CANA1S* gene variants caused amino acid sequence changes. There were only 2 site changes in *STAC3*, and none of them caused amino acid sequence changes.

Combined with variants in the literature, there are more than 400 variants of unknown significance in *RYR1*, and 48 have been shown to be associated with MH(18–20). Changes in the amino acid sequences in proteins may disrupt the binding sites of other proteins, catalyzing residues and protein folding (structure), but not all of the amino acid sequence changes result in changes in protein function. Experimental evidence clearly indicates that the signs and symptoms of MH are associated with the uncontrolled release of intracellular Ca^{2+} from the skeletal muscle SR(21). Enhanced intracellular Ca^{2+} leads to abnormal metabolism of skeletal muscle, manifested by

activation of muscle contraction, oxygen consumption and CO2 production, ATP hydrolysis and heat generation. Other proteins that have a potential or known role in the function of RyR1 include intact SR membrane proteins and proteins that link between the stable plasma membrane and the sarcoplasmic reticulum(22). Because RyR1 plays a vital role in maintaining Ca^{2+} homeostasis and excitation–contraction coupling in skeletal muscle cells, MH susceptible individuals carrying *RYR1* variants may have skeletal muscle metabolism abnormalities even without triggering by anesthesia(23, 24).

The second gene related to MH sensitivity is *CACNA1S*, which encodes one of the dihydropyridine receptor (DHPR) subunits of skeletal muscle, and is found at locus 1q32(25). Currently, there were a number of variants (T1354S, A1086H, A1086S, R174W, and R1086H) that have been found in the *CACNA1S* gene in MH patients(26–30), but only two *CACNA1S* variants have been sufficiently characterized to be regarded as pathogenic for MH(20). These variants have not been functionally characterized as MH pathogenic factor by EMHG criteria(25).

JP-45 encoded by *JSPR1* is another intact SR protein that has been shown to co-localize with RyR1. Some studies suggest that individuals with *JSPR1* variants and pathogenic *RYR1* variants will have a lower overall phenotype than those expressing *RYR1* variants alone(4, 31). These observations highlight the possibility that polymorphic variants regulate *RYR1* function and may help explain observed MH-sensitive variable phenotypes(32).

There are many free prediction tools that can be used to predict the pathogenicity of variants. Although pathogenic and common sequence variants in certain genes have been used to test the accuracy of computer-based prediction methods, variantal genes for this disease has not been performed yet. There have been reports in the literature that the prediction tools of the website used here have a total sensitivity of 84%–100% and a specificity of 25%–83%(3). Prediction is very useful for selecting variants for further functional analysis. There is always a certain degree of computer analysis in variant identification, but it is too early to begin using it for clinical diagnosis of MH sensitivity. No program yet can correctly predict all MH pathogenic variations. According to the literature, MutPred, SNPs & GO, PhD-SNP and CADD have the highest sensitivities (true positives), and SIFT and MutPred have the highest specificities (true negatives)(3). In this study, G1530T in *CACNA1S* was suggested to be pathogenic by 6 prediction tools, and C4615T was suggested to be pathogenic using 5 prediction tools. The results of the different prediction software were different, and how to increase the sensitivity and specificity of each prediction software by expanding the database and improving the algorithm is an issue that also needs attention.

Numerous *RRY1* or *CACNA1S* variants have been identified by next-generation sequencing (33–35). However, none of the currently available platforms for sequencing, chemistry for sample preparation, or analysis software, are able to yield 100% coverage of all exons in the human genome(36). Individuals carrying one of the MH pathogenic variants are considered to be susceptible to MH, meaning that they have an increased risk of developing MH. When a familial pathogenic variant is identified, genetic testing can be used on extended family members, and all of the members of a family carrying the variant should be considered MH susceptible(30). However, the susceptibility to MH in individuals who do not carry familial variations cannot be ruled out because multiple pathogenic variations may exist in the same family(37, 38). There is evidence that there are multiple genetic factors that affect the MH-sensitive phenotype, because defects in more than one gene work together to produce a phenotype, or actually work opposite each other to modify or mask the MH phenotype(38).

It is worth noting that the variant of *RYR1* also causes a variety of congenital myopathies with different clinical manifestations, but it is characterized by histological changes in muscle morphology(39). For clinical safety, these patients should be considered as having MH. Although variant screening of MH genes using the latest techniques has become feasible and cost-effective, the genetic heterogeneity of MH still prevents a‘quick and cheap’ MH test that can be done the night before anesthesia to prevent manifestation of MH(40).

Conclusion

In Han Chinese healthy volunteers, variants in *RYR1* and *CACNA1S* were the most common, and variants in *JSRP1* were more commonly amino acid sequence changes. Whether the gene variant was pathogenic could be predicted using a prediction website, but the sensitivity and specificity need to be improved before widespread use of this approach for MH identification. When the above variation occurs, further verification by IVCT is needed.

Declarations

Study design observational study

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Ethics approval and consent to participate

The study was approved by the Affiliated Hospital Academy of Military Medical Sciences Clinical Research Ethics Committee (Approved Document Number: KY-2018-6-56) and all volunteers provided written informed consent.

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Figures

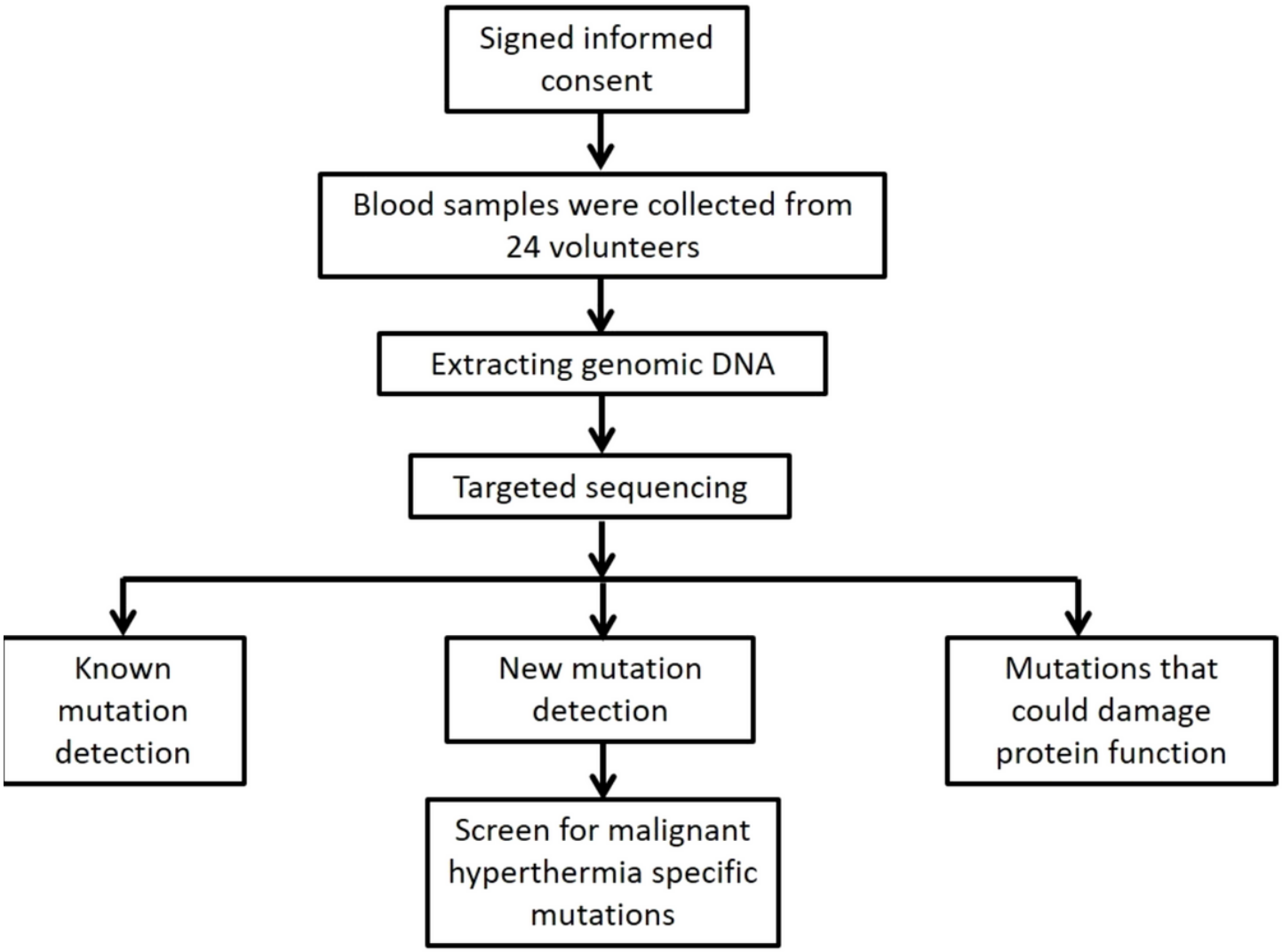

Figure 1

Flow diagram of the study design and conduct Flow diagram of the study design and conduct.

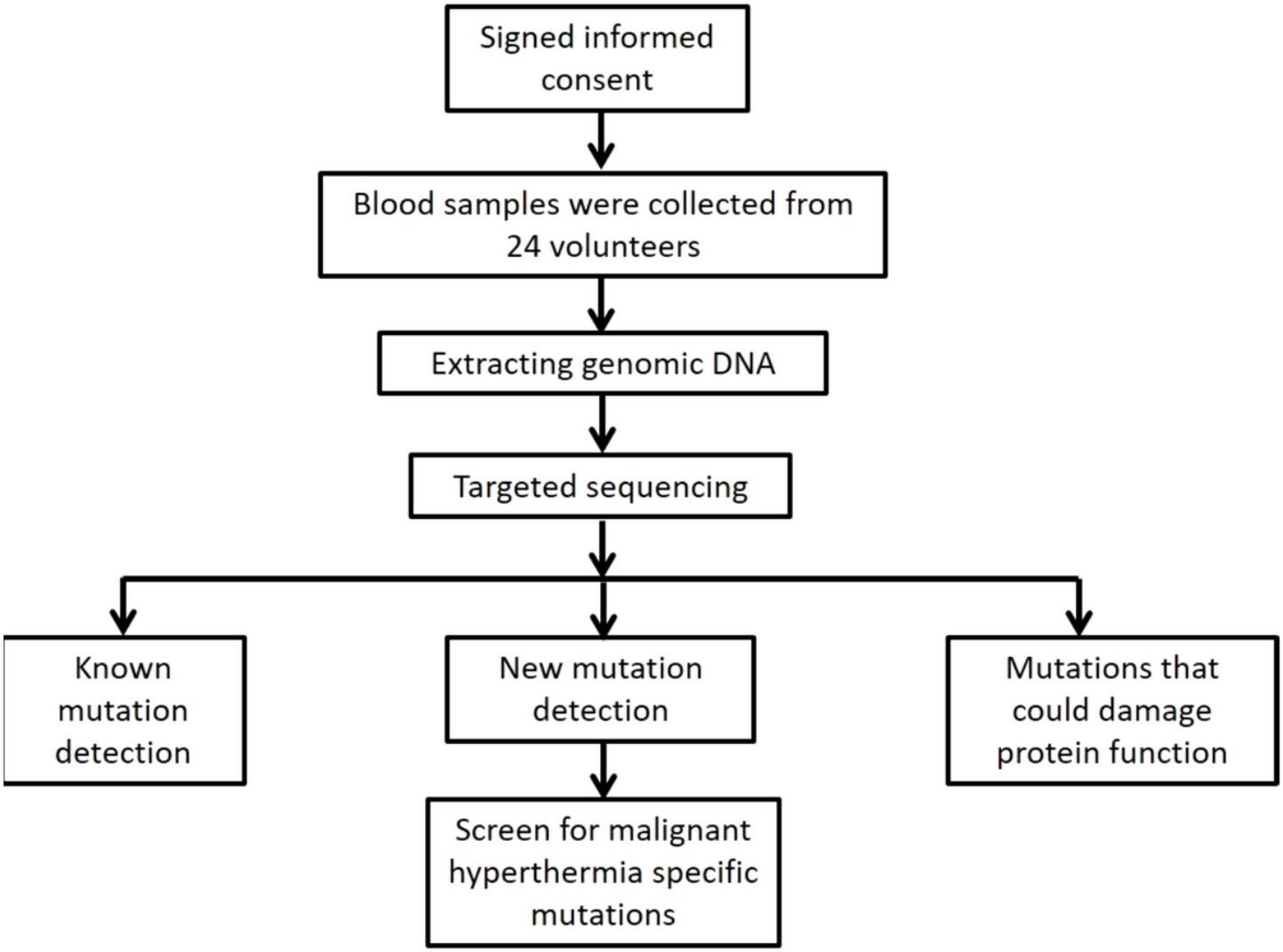

Figure 1

Flow diagram of the study design and conduct Flow diagram of the study design and conduct.