article

Whole blood RNA offers a rapid, comprehensive approach to genetic diagnosis of cardiovascular diseases

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Purpose: Long QT Syndrome, Marfan Syndrome, hypertrophic and dilated cardiomyopathy are caused by mutations in large, multi-exon genes that are principally expressed in cardiovascular tissues. Genetic testing for these disorders is labor-intensive and expensive. We sought to develop a more rapid, comprehensive, and cost-effective approach. **Methods:** Paired whole blood samples were collected into tubes with or without an RNA-preserving solution, and harvested for whole blood RNA or leukocyte DNA, respectively. Large overlapping cDNA fragments from *KCNQ1* and *KCNH2* (Long QT Syndrome), *MYBPC3* (hypertrophic and dilated cardiomyopathy), or *FBN1* (Marfan Syndrome) were amplified from RNA and directly sequenced. Variants were confirmed in leukocyte DNA. **Results:** All 4 transcripts were amplified and sequenced from whole blood mRNA. Six known and 2 novel mutations were first identified from RNA of 10 probands, and later confirmed in genomic DNA, at considerable savings in time and cost. In one patient with MFS, RNA sequencing directly identified a splicing mutation. Results from RNA and DNA were concordant for single nucleotide polymorphisms at the same loci. **Conclusion:** Taking advantage of new whole blood RNA stabilization methods, we have designed a cost-effective, comprehensive method for mutation detection that should significantly facilitate clinical genetic testing in four lethal cardiovascular disorders. ***Genet Med* 2007:9(1):23–33.**

Key Words: *Genetic testing, cardiomyopathy, sudden death, fibrillin-1, PaxGene genetic testing, cardiomyopathy, sudden death, fibrillin-1, PaxGene*

Many forms of heart disease have a genetic basis.[1] The clinical value of screening for many of these disorders is well established,[2,3] offering opportunities for pre-symptomatic intervention, parental counseling, and risk stratification. Unfortunately, access to genetic testing in cardiac diseases is limited by several factors. First, many of these disorders are recognized only in adulthood, reducing diagnostic suspicion for heritable disease, as well as limiting opportunities for gene identification through linkage analysis, since family members are often unavailable for testing. A second major problem is access: many of the tests are costly and labor-intensive, and only a few laboratories offer testing.

There are multiple technical reasons for this lack of access. Many cardiovascular disorders are caused by multiple mutations in very large genes, with mutations distributed throughout their length. For example, Marfan Syndrome (MFS) is associated with over 600 mutations throughout the 65-exon fibrillin-1 (*FBN1*) gene.[4] Long QT Syndrome is linked to more than 300 individual mutations in 7 different genes that together include over 70 exons; no single mutation accounts for a significant proportion of cases.[3,5–8] Hypertrophic cardiomyopathy (HCM) is caused by one of nearly 200 mutations in at least 10 genes encoding sarcomeric proteins; dilated cardiomyopathy (DCM) is associated with mutations in 16 different genes, and these account for only a fraction of familial instances of the disease.[9,10] Thus, high-throughput solutions such as microsphere assays are not feasible and/or would miss a large number of new mutations. Common approaches are therefore either brute force sequencing of all exons amplified from lymphocyte genomic DNA, or screening of the exons using a chromatographic method (e.g. single strand conformation polymorphism (SSCP) or denaturing high-performance liquid chromatography (DHPLC)) followed by sequencing of exons with abnormal mobility.[3,6,11,12] These tests are rarely covered by insurance, and the cost and effort associated with either strategy prices them out of reach for most patients.

An alternative solution to these difficulties is to sequence the messenger RNA (mRNA) encoded by these genes. Because mRNA is processed to remove most noncoding sequences, the amount of genetic material to be tested is available in larger fragments that can be efficiently analyzed by direct sequencing.

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Genetic testing for cardiovascular diseases could thus be greatly facilitated by mRNA-based screening. However, use of mRNA has traditionally required substantial additional cost, resources and time. Because of their accessibility, peripheral blood leukocytes or fibroblasts are preferred sources of mRNA, but in order to obtain sufficient material for genetic testing these cells must be purified or expanded in culture, sometimes with the assistance of immortalizing viruses. Another limitation is that the gene of interest must be transcribed at detectable levels in available tissue samples. This can be a particular problem for cardiac-restricted genes, since myocardial tissue from the proband is usually unavailable. Ectopic transcription has been noted for a number of genes[13] and "leaky" expression of the HCM-linked genes beta myosin heavy chain (*MYH7*), cardiac troponin T (*TNNT2*), and MYBPC3 in lymphocytes[14,15,16] has not proven robust enough to serve as a source of genetic testing material.

In the last two years, the demand for microarray-based gene expression analysis[17,18] has driven the development of new methods for harvesting and preserving RNA. To date, these methods have not been exploited for genetic testing, although their potential is clear. Here we describe the incorporation of improved RNA purification technology into a genetic screening method that permits RNA amplification and direct sequencing of cardiovascular disease-associated genes from <3 mL of unfractionated peripheral blood. We show that whole blood RNA is a robust, efficient and considerably less expensive source of sequence information compared with RNA from expanded or immortalized cell sources. Finally, we demonstrate the utility of the method by detecting 2 novel and 6 previously reported disease-causing mutations in 4 cardiovascular disease-associated genes, from 10 prospectively tested probands referred for genetic testing of LQTS, MFS and HCM. We propose the use of RNA from unfractionated blood as part of a rapid and simple method for screening mutations in complex cardiovascular genetic disorders.

METHODS

Human subjects

Subjects were recruited and evaluated in accordance with regulations set forth by the University of Miami Committee for the Protection of Human Subjects and under human subjects research protocols approved by the UM Institutional Review Board. Informed consent was obtained from all blood donors, including those providing control samples.

Preparation of nucleic acids

Genomic DNA was prepared from peripheral blood lymphocytes with the Puregene DNA Isolation kit (Gentra Systems, Inc., Minneapolis, MN), according to the manufacturer's recommendation. RNA was prepared from 2.5 mL of whole peripheral blood collected into PAXgene blood collection tubes, containing a mixture of quaternary amine surfactants (PAXgene Blood RNA Validation kit, Qiagen, Inc., Valencia, CA). Blood samples were stored at 4°C and processed within 4 days of collection according to the manufacturer's instructions. Of note, both the PAXgene Blood RNA Validation Kit and Versagene RNA purification kit (Gentra Systems) worked well in this application; the PAXgene protocol is given here. In brief, after warming for 2 hours to room temperature, the tubes were centrifuged for 10 minutes at 3,000*g*, and the supernatant decanted. The pellet was rinsed in 5 mL RNase-free water, and then re-suspended in 360 μL of buffer BR1 by vortexing. The pellet was then digested by addition of 300 μL BR2 lysis buffer and 40 μL proteinase K solution, vortexing and incubation at 55°C for 10 minutes, and centrifugation for 3 minutes at 16,000*g*. Supernatants were carefully pipetted into RNase-free microcentrifuge tubes, mixed with 350 μL of 100% ethanol, and applied to PAXgene Blood RNA Kit spin columns. Columns were centrifuged at 10,000*g* for 1 minute and the flow-through discarded. Bound RNA was then washed with 350 μL of buffer BR3, centrifuged at 16,000*g*, and digested for 15 minutes at room temperature with 80 μL DNase (RNase-free DNase, Qiagen). Samples were washed once with 350 μL of BR3 and twice with 500 μL of BR4. RNA was then eluted with 2 successive applications of 40 μL of buffer BR5, denatured at 65°C for 5 minutes and then either processed immediately or stored at −80°C. Nucleic acid concentrations were determined by UV absorption.

Reverse transcription and PCR

Reverse transcription was done in a 20-μL reaction using either cloned M-MLV RTase (Ambion, Inc., Austin, TX), or Transcriptor RTase (Roche Applied Science, Indianapolis, IN), according to the manufacturer's recommendations. Random hexamers were used as primers for each enzyme. First-round PCR was performed in a 25-μL reaction containing 2 μL of an RTase reaction as template, 1 μM each primer, 1.5 mM MgCl2, 250 μM each dNTP, 1 M betaine, 1× reaction buffer, and 1 unit of Taq polymerase (Continental Lab Products, San Diego, CA). When needed, second-round PCR was performed using nested primers, 2 μL of the first round PCR as a template, and otherwise identical ingredients. The primers and PCR conditions are shown in Table 1. Primers were designed using VectorNTI software (Invitrogen Corp., Carlsbad, CA).

DNA sequencing

PCR products were purified using a Qiaquick PCR Purification Kit (Qiagen). Purified products were quantified by gel electrophoresis, and used with reagents from Applied Biosystems, Inc. (ABI), in cycle sequencing reactions. These products were then resolved on an ABI 3100 Genetic Analyzer, and analyzed using ABI Sequence Analysis software. Reference sequences were obtained from Genbank and are listed in Table 1.

Concordance and allele expression analysis

To establish that RNA amplification detected both alleles, and that genotypes from RNA and DNA were concordant, we sought to detect common heterozygous single nucleotide polymorphisms (SNPs) from each of the genes tested in

Table 1
Primers and PCR conditions for blood RNA amplification

	Primer sequences		
PCR	Primer	Sequence	Region amplified
KCNQ1, KVLQT1, LQT1 (Genbank NM 000218, numbered 1–2031, ATG to stop)[a]			
A	LQT1-210S	CGCCGCGCCCCCAGTTGC	
A	LQT1-1312A	GCATCTTCTCTCCAGGAGT	201–1312, 1102 bp
B	LQT1-1105S	CCGGCGGCAGCCTCACTCA	
B	LQT1-2382A	CCAAGCTTGGTAACAACTCA	
		GACCTGAGAC	1105–2382, 1277 bp
KCNH2, *HERG*, LQT2 (Genbank NM 000238, numbered 1–3480, ATG to stop)			
A	LQT2-40S	GCCGCAGAACACCTTCCTGGACA	
A	LQT2-1258A	CAGGATGAGCCAGTCCCACACGG	40–1258, 1219 bp
B	LQT2-1075S	TGACCGTGAGATCATAGCACCTAAGAT AAAGG	
B	LQT2-2291A	TCTTGAACTTCATGGCCAGGGCC	1075–2291, 1217 bp
C	LQT2-2100S	GCCTCGAGGAGTACTTCCAGCACG	
C	LQT2-3602A	TTCCTCTCCCCTTCCACGGTCAG	2100–3602, 1503 bp
MYBPC3 (Genbank NM 000256, numbered 1–3825, ATG to stop)			
A	MBPC(-19S)	TGGTGTGACGTCTCTCAGGATGCC	
A	MBPC-1645A	GGTACACCTCCAGCTTCTTTTCCTGC	−19–1645, 1663 bp
A1	MBPC-23S	CAGTCTCAGCCTTTAGCAAGAAGCCA	
A1	MBPC-1016A	TGGAAGGCGATGCGCTCGTA	23–1016, 993 bp
A2	MBPC-891S	ACTGCTGAAAAAGAGAGACAGTTTCCGG	
A2	MBPC-1557A	CAGCATGGCCTCGTTGATGATCA	891–1557, 666 bp
B	MBPC-1353S	GCCCCCTGTGCTCATCACGC	
B	MBPC-2612A	CTGGGGGGACCGATAGGCATG	1353–2612, 1259 bp
B1	MBPC-1471S	GTGGAGCTGACCCGGGAGGAGA	
B1	MBPC-2567A	CCTGGACATGCCGATGGCGT	1471–2567, 1096 bp
C	MBPC-2421S	CCTGGAGCGCAAGAAGAAGAAGAGCTACC	
C	MBPC-3897A	CAACTTCCCTCCAGGCTCCTGGCA	2421–3897, 1476 bp
C1	MBPC-2504S	GCATGATCGAGGGCGTGGTGTACG	
C1	MBPC-3778A	CCTGTAAGTTGGTGGCCCTGCAGACATAG	2504–3778, 1274 bp
FBN1 (Genbank NM 000138, numbered 1–8616, ATG to stop)			
A	FB-116S	CGGTGGCGGCTCGGCATCAT	
A	FB-1595A	ACTCCCCACGGAGGTCCAGCTGG	−18–1462, 1479 bp
A1	FB-129S	GCATCATGCGTCGAGGGCGT	
A1	FB-1556A	CACACCGGCAACTCCCAGGAGT	−5–1423, 1427 bp
B	FB-1412S	GTCCCTCGACCACCAGTGGAATATCT	
B	FB-2581A	GCAAGGACTTGATTCGCATTCATCA	1279–2448, 1169 bp
B1	FB-1454S	CCACCAAGGGTGCTGCCAGTAA	
B1	FB-2513A	TGGGGCAGGTACAGACAAAACTTCC	1321–2380, 1059 bp
C	FB-2341S	TGGAATCTGTGAAAACCTTCGTGGG	
C	FB-3972A	TCTACACAAGTCTTCATGTCTTCAGATGCC	2208–3839, 1631 bp
C1	FB-2382S	GCAATTCAGGATATGAAGTGGATTCAACTG	

(*Continued*)

Table 1
Continued

Primer sequences

PCR	Primer	Sequence	Region amplified
C1	FB-3906A	GGGATATTTGTGCACTGACCACCATC	2249–3773, 1524 bp
D	FB-3785S	AGCTATGAATGTAGCTGTCAGCCGGG	
D	FB-5483A	TGATACACACTCCATTTTCACAGACCCC	3652–5350, 1698 bp
D1	FB-3825S	CTGACCAGAGATCATGCACCGACA	
D1	FB-5417A	CGGTATAAATGTCGATGACAAAGCCTG	3692–5284, 1592 bp
E	FB-5300S	TACAACATTGGCCGGGCGTGG	
E	FB-7067A	CACGGGTGTTGAGGCAGCGC	5167–6934, 1767 bp
E1	FB-5342S	CCCATCCCAAGTACAGATGAGTTTGC	
E1	FB-7002A	ACACAGCCTTCTCCATCAGGTCTCC	5209–6869, 1660 bp
F	FB-6825S	AATGTCCCGTGGGATATGTGCTCA	
F	FB-7790A	GGCATTCACAGGTGAAGCTTCCAG	6692–7657, 965 bp
F1	FB-6850S	AGAAGACCGTAGGATGTGCAAAGATGA	
F1	FB-7739A	CGCACAGATTGATGTCAGAGGTGC	6717–7606, 889 bp
G	FB-7642S	CATTGGCGGCTTCACATGCAAA	
G	FB-8831A	TGATGATTCTGATTGGGGGAAAATATAGTT	
G1	FB-7670S	CCGGATTTACCCAACACCATACG	
G1	FB-8766A	GGTCTCTGGATGGTGAATTAATGAAGC	7537–8633, 1096 bp

PCR conditions

PCR	Initial denaturation	Denaturation	Annealing	Polymerization	Number of cycles
KCNQ1					
A and B	4 min, 94°C	30 sec, 94°C	30 sec, 65°C	70 sec, 72°C	3535
KCNH2					
A, B and C	4 min, 94°C	30 sec, 94°C	20 sec, 62°C	60 sec, 72°C	
MYBPC3					
A	4 min, 94°C	30 sec, 94°C	30 sec, 61°C	80 sec, 72°C	35
A1	4 min, 94°C	30 sec, 94°C	30 sec, 61°C	50 sec, 72°C	35
A2	4 min, 94°C	30 sec, 94°C	30 sec, 61°C	50 sec, 72°C	35
B	4 min, 94°C	30 sec, 94°C	30 sec, 62°C	60 sec, 72°C	35
B1	4 min, 94°C	30 sec, 94°C	30 sec, 62°C	60 sec, 72°C	35
C	4 min, 94°C	30 sec, 94°C	30 sec, 65°C	90 sec, 72°C	35
C1	4 min, 94°C	30 sec, 94°C	30 sec, 65°C	70 sec, 72°C	35
FBN1					
A	4 min, 94°C	30 sec, 94°C	20 sec, 65°C	60 sec, 72°C	35
A1	4 min, 94°C	30 sec, 94°C	20 sec, 65°C	60 sec, 72°C	35
B	4 min, 94°C	30 sec, 94°C	20 sec, 65°C	60 sec, 72°C	35
B1	4 min, 94°C	30 sec, 94°C	20 sec, 65°C	60 sec, 72°C	35
C	4 min, 94°C	30 sec, 94°C	20 sec, 65°C	60 sec, 72°C	35
C1	4 min, 94°C	30 sec, 94°C	20 sec, 65°C	60 sec, 72°C	35
D	4 min, 94°C	30 sec, 94°C	20 sec, 65°C	60 sec, 72°C	35
D1	4 min, 94°C	30 sec, 94°C	20 sec, 65°C	60 sec, 72°C	35

(*Continued*)

Table 1
Continued

PCR conditions					
PCR	Initial denaturation	Denaturation	Annealing	Polymerization	Number of cycles
E	4 min, 94°C	30 sec, 94°C	20 sec, 65°C	60 sec, 72°C	35
E1	4 min, 94°C	30 sec, 94°C	20 sec, 65°C	60 sec, 72°C	35
F	4 min, 94°C	30 sec, 94°C	20 sec, 62°C	60 sec, 72°C	35
F1	4 min, 94°C	30 sec, 94°C	20 sec, 62°C	60 sec, 72°C	35
G	4 min, 94°C	30 sec, 94°C	20 sec, 62°C	60 sec, 72°C	35
G1	4 min, 94°C	30 sec, 94°C	20 sec, 62°C	60 sec, 72°C	35

[a]*KCNQ1* and *KCNH2* account for approximately 80% of known mutations in Long QT Syndrome. The primers shown here amplify all but the first 250 bp of coding sequence for *KCNQ1*, which has over 80% GC content and comparatively few disease-causing mutations, and which was not possible to amplify from whole blood. However this region can be readily amplified from genomic DNA in a single PCR reaction.

both RNA and DNA. The SNPs used for *KCNQ1* were 1638G>A (S546S, rs1057128) and 1986C>T (Y662Y, rs11601907). For *KCNH2*, we used 1956T>C (Y652Y, rs10226664) and 2690A>C (K897T, rs1805123). For *MYBPC3*, a SNP with 2 common alleles in exon 31, 3288A>G (E1096D, rs1052373), was used.

RESULTS

Our prospective series included 2 sets of blood samples from 14 individuals: 10 unrelated subjects referred for genotyping (6 suspected LQTS, 2 HCM, and 2 MFS) and 4 unaffected, unrelated controls. Table 2 provides clinical information on individual patients. Blood samples for RNA were collected directly into tubes containing quaternary ammonium surfactants (PAXgene Blood RNA Validation kit, Qiagen, Inc.), which simultaneously lyse cells and precipitate RNA. RNA was then isolated and purified using standard spin-column binding and elution methods. We routinely obtained a minimum of 5 μg of RNA from 2.5 mL of whole blood after 2–3 hours processing time. This amount of RNA is enough to perform at least 5 reverse transcription reactions, in turn yielding enough cDNA template for at least 50 primary PCRs.

Our PCR primer pairs were designed to amplify approximately 1–1.5 kb segments of continuous coding sequence, and produced products of the expected size from over 90% of samples. A single round of PCR was sufficient for *KCNQ1* and *KCNH2*, while *MYBPC3* and *FBN1* both required a second round of nested PCR (Table 1). RNA-derived sequence data were then validated, first by comparison to the reference gene sequence in GenBank, and then by analysis of the corresponding exons from genomic DNA. These studies confirmed that we were able to amplify the major disease-associated transcripts, including the nominally tissue-restricted LQTS-associated and *MYBPC3* transcripts, from whole blood RNA. Expression and amplification of both alleles were confirmed by identification of heterozygosity for previously reported or novel SNPs, or for disease-associated variants. In nearly all cases, genotypes determined from RNA and DNA were concordant.

Table 2
Characteristics of patients studied

Patient	Age	Sex	Diagnosis	Phenotype
1	1 mo	M	LQTS	QT_c of 570 ms
2	15 mo	M	LQTS	QT_c of 490 ms
3	26 yr	F	LQTS	QT_c of 480 ms, syncope, dyspnea
4	23 yr	F	LQTS	Multiple episodes of syncope after suddenly waking to a noise, AICD
5	11 yr	M	LQTS	QT_c of 520 ms, syncope/seizures
6	33 yr	F	LQTS	Heart attack at age 25, AICD
7	26 yr	M	HCM	Decreased exercise tolerance, IVSD of 1.9 cm
8	57 yr	M	HCM	Dyspnea, IVSD of 1.3 cm
9	17 yr	M	MFS	Dental crowding, mild scoliosis, arachnodactyly, 6'5" tall, 113 pounds, positive thumb and wrist signs
10	30 yr	M	MFS	Pectus carinatum, arachnodactyly, aortic root of 4.24 cm, father had aortic dissection at age 40

Long QT syndrome (*KCNQ1* and *KCNH2*)

From 10 probands and 4 healthy controls, all 3 single-locus genotypes for *KCNQ1* and *KCNH2* were observed, and genotypes determined by RNA and DNA were completely concordant, indicating that both alleles as well as the major RNA transcripts of *KCNQ1* and *KCNH2* are expressed and detectable in blood RNA. Our amplification strategy did not specifically detect two minor variant transcripts of KCNQ1 or KCNH2, each of which encodes an amino terminal truncated protein.[19,20] The variant tKVLQT1 is missing nucleotides encoding the cytoplasmic domain and one-third of its first transmembrane domain, but does not contain any unique exons, hence the primer set captures all of the coding information from both isoforms.[19] The HERG1b isoform replaces exons 1–5 of HERG1 with a unique 97 bp exon 1 encoding a truncated cytoplasmic domain.[20] This short unique sequence would be omitted by any exon amplification strategy that did not specifically target the HERG1b-specific exon 1. To our knowledge, this region of HERG1 has not yet been implicated in LQTS.

In two out of six patients referred for LQTS genotyping, three *KCNQ1* mutations were identified from blood RNA, and subsequently confirmed by DNA analysis. One proband, a male newborn with bradycardia and a QTc of 570 milliseconds, had a novel missense mutation, 1016T>C (F339S). The mutation, which was not detected in either parent, occurs at the second of 8 consecutive disease-implicated residues in the S6 transmembrane region. Functional studies of this mutant channel in a *Xenopus* oocyte expression system indicated the channel was unable to interact normally with the *KCNE1* beta subunit (unpublished data, 2005). The second proband, a 15-month-old male with a QTc of 490 milliseconds, had two mutations: 430A>G (T144A) and 1781G>C (R594P). Figure 1A,C show the amplified cDNA sequence for these mutations, confirmed on subsequent DNA sequencing (Figs. 1B,D). His asymptomatic mother, who had a QTc of 460 milliseconds, harbored both mutations, suggesting that these reside on the same allele and were co-inherited. Mutations at each of these three residues have been individually associated with LQTS, although to our knowledge R594P and F339S are novel, as is the existence of T144A and a second mutation on the same allele.[6,21,22]

Two other patients were found to have mutations in *KCNH2*. A 23-year-old woman had been treated for seizures since falling out of bed at age 16, and at age 20 lost consciousness when a maid knocked on her hotel room door. She was subsequently diagnosed with LQTS, and given an automatic implantable cardioverter defibrillator. Whole blood RNA genotyping revealed a 1704G>C mutation that was confirmed by sequencing exon 7 from genomic DNA. This creates a missense mutation, W568C, previously reported to cause LQTS[23] (Fig. 2). The second *KCNH2* mutation was found in an 11-year-old boy who had fainted a year before while playing soccer. His mother has experienced fainting spells throughout her life, and her father had died of a heart attack at age 42. A heterozygous base, 1838C>T, was found in the cDNA and confirmed by sequencing exon 7 from genomic DNA. This transition mutation leads to an amino acid change, T613M, also previously reported to cause LQTS.[6,21] Genetic testing of the boy's mother revealed that she was the source of his mutation.

Hypertrophic cardiomyopathy (*MYBPC3*)

Mutations in cardiac myosin binding protein C (*MYBPC3*) account for almost half the mutations associated with HCM, and >20% of clinically diagnosed HCM cases.[24] Nearly 100 mutations have been found, distributed throughout the length of the protein. We were able to successfully amplify *MYBPC3* from blood RNA, using a nested PCR strategy with primers shown in Table 1. In our group of 10 probands and 4 controls, SNP analysis revealed

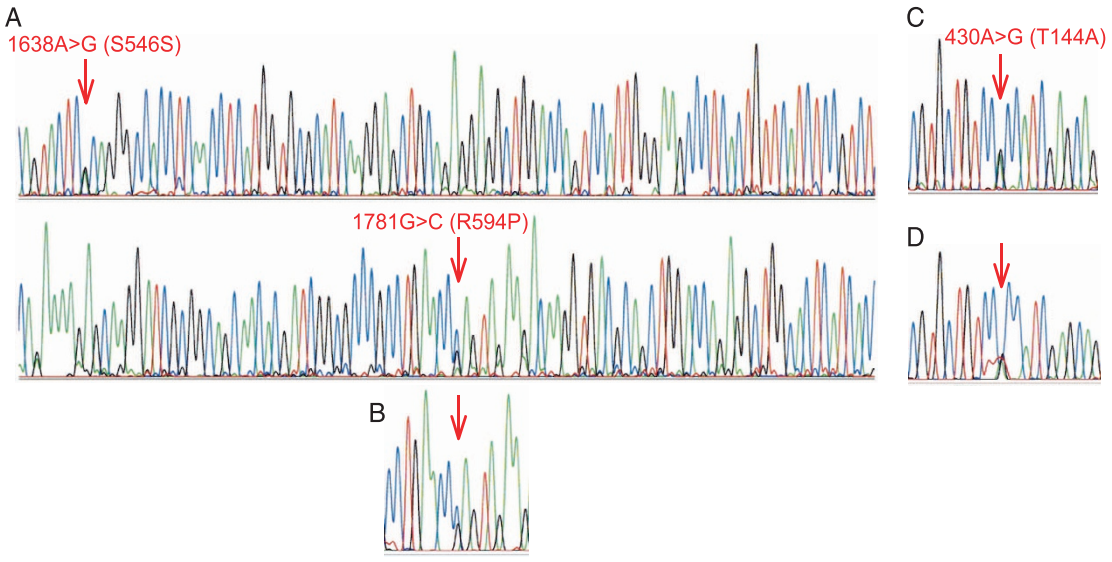

Fig. 1. LQT1 mutation detection using whole blood RNA: (A) Sequence of *KCNQ1* cDNA sense strand (nucleotides 1631–1829, including exons 13–16) showing a common SNP in exon 13, 1638A>G (S546S), and a presumptive LQTS mutation, 1781G>C (R594P) in exon 15. (B) Corresponding DNA sequence from exon 15 confirming the 1781G>C mutation. (C) *KCNQ1* cDNA sequence showing 430A>G (T144A), a previously reported LQTS mutation in exon 2 and (D) corresponding genomic DNA sequence.

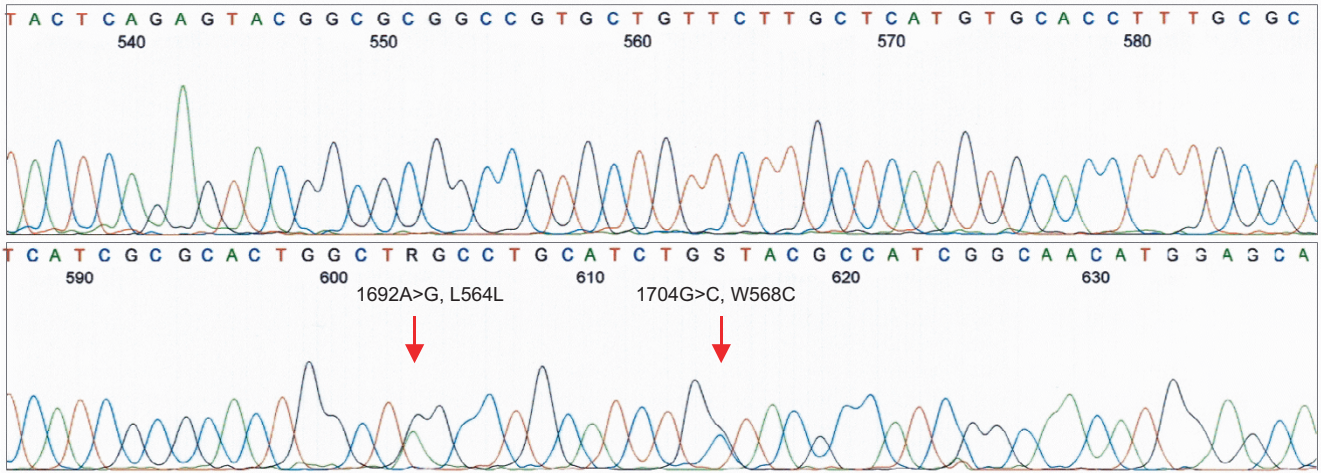

Fig. 2. LQT2 mutation detection using whole blood RNA: cDNA sequence of *KCNH2*, nucleotides 1624–1727, showing a common SNP, 1682A>G, and a mutation previously associated with LQTS, 1704G>C (W568C).

all 3 genotypes at this locus, with 100% concordance between RNA- and DNA-derived sequences (Fig. 3).

We then genotyped *MYBPC3* in a patient with a family history of cardiomyopathy and echocardiographic evidence of severe left ventricular hypertrophy localized to the septum and anterior wall. Whole blood RNA analysis revealed this patient to have a previously reported mutation, 1505G>A (R502Q).[25] Initial RNA analysis detected only the mutant 1505G>A sequence; however, both mutant and wild type alleles were observed in the corresponding DNA sequence, and we concluded that the proband is heterozygous for this autosomal dominant mutation, similar to the previous report.[25]

Marfan syndrome (*FBN1*)

Mutations in *FBN1* have been associated with MFS, a disease characterized by multiple connective tissue abnormalities, including lethal aneurysms of the ascending aorta. The entire 8.5-kb coding sequence of *FBN1* was successfully amplified from whole blood RNA using 7 overlapping nested PCR primer sets (Table 1).

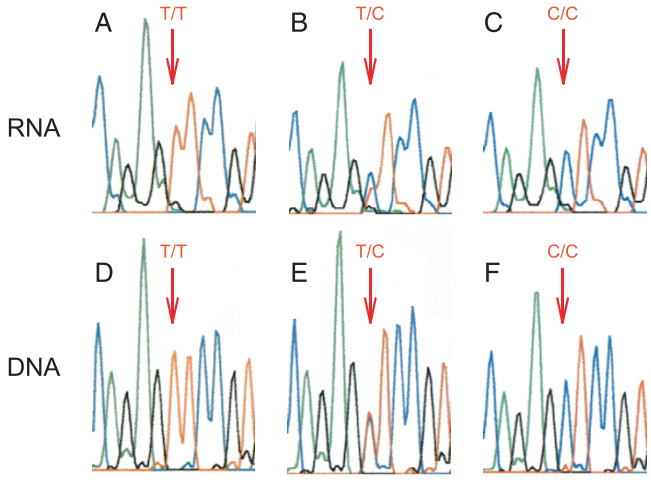

Fig. 3. *MYBPC3* SNP detection using whole blood RNA: Concordance between RNA- and DNA-determined genotypes for a SNP (3288A>G, E1096E, rs1052373) in exon 31 of *MYBPC3*. All sequences are antisense; (A–C) RT-PCR sequences, (D–F) genomic sequences. Panels A and D, B and E, and C and F are from 3 unrelated subjects. Arrows denote the polymorphic residue.

Using whole blood RNA, we detected disease-causing mutations in 2/2 probands suspected of MFS. The first was identified in one of two 17-year-old identical twins, both tall, with arachnodactyly, but no family history of MFS. A novel deletion in exon 39, 4905delC was identified and confirmed by analysis of the corresponding DNA sequence (Fig. 4A,B). The resulting frame shift created a premature stop codon 4 residues after the deletion.

The second proband, a 30-year-old male, had a mild Marfanoid habitus and an aortic root diameter of 4.24 cm. His father had recently undergone a second aortic root replacement for dissection of the ascending aorta, and had a clinical diagnosis of MFS. RNA sequence obtained from this patient was homozygous for loss of all exon 51 nucleotides (Fig. 4C). Sequencing of the corresponding DNA revealed heterozygosity for 2 previously identified variants, one in the intron preceding exon 51, IVS50–64A>G, and another in the coding region, 6354C>T (not shown, and Fig. 4D). The 6354C>T variant does not change the amino acid sequence (I2118I), but apparently disrupts a splicing enhancer,[26,27] resulting in an in-frame deletion of exon 51 in the mRNA. Exon 51 sequences encode part of a conserved cysteine-rich domain shared by many transforming growth factor-β binding proteins, and its loss could significantly impact fibrillin function.

Cost comparisons

The potential cost savings associated with whole blood RNA were considered using Marfan Syndrome (*FBN1*) as an example. A representative laboratory offering this test requires a skin biopsy and an additional fee to expand fibroblasts in culture.[28] Using our method, the entire coding sequence of *FBN1* could be amplified in 2–3 days from 2.5 mL whole blood, using 7 nested PCR amplifications. This represents a savings of weeks to months of process-

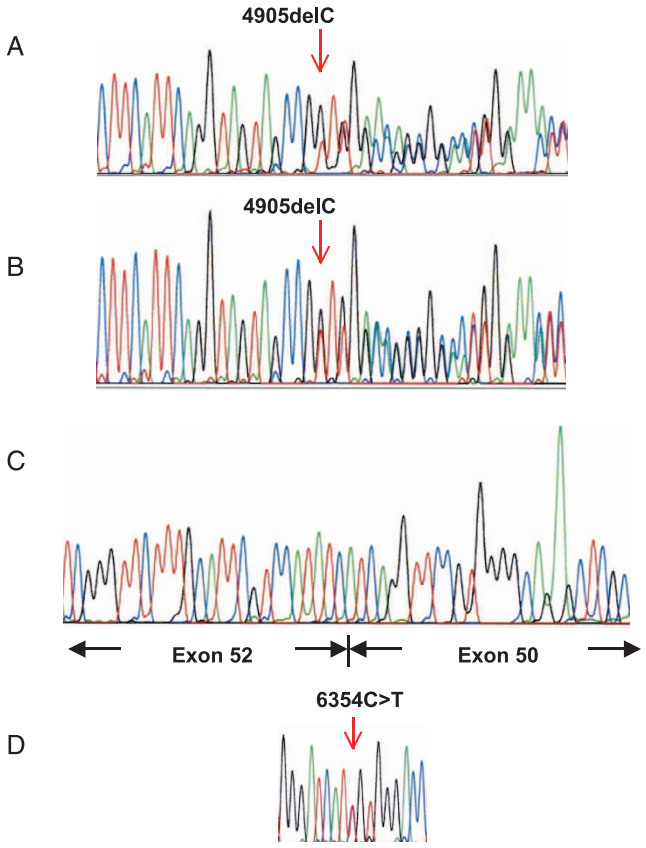

Fig. 4. Whole blood RNA analysis of MFS mutations: (A, B) Proband 1, (C, D) Proband 2. (A) Antisense cDNA sequence, nucleotides 4924–4881, of *FBN1* mutant 4905delC. Note presence of both alleles. (B) Corresponding DNA sequence. (C) Antisense cDNA sequence showing deletion of *FBN1*, exon 51. Note absence of wild type allele. (D) DNA sequence of *FBN1*, exon 51, 6354C>T, the point mutation that disrupts a splicing enhancer.

ing time, and avoids costs associated with skin biopsy and fibroblast culture, typically several hundred dollars per test. Tables 3 and 4 provide a cost analysis of mutation screening in MFS comparing the whole blood RNA method with existing DNA-based protocols as previously described,[29] using current prices for DNA sequence analysis on a capillary-type apparatus. Again, a significant cost advantage is seen for RNA.

DISCUSSION

The major new finding in this study is that tissue-restricted mRNAs associated with 3 genetically complex cardiovascular diseases (LQTS, MFS and HCM) can be directly amplified from whole, unfractionated peripheral blood, and used as a robust source for mutation detection. Although mRNA is often cited as the preferred starting material for mutation screening in large genes, obtaining sufficient material for study typically requires either purification of lymphocytes from up to 50 mL of blood, or skin biopsy followed by several weeks of tissue fibroblast culture.[30] Even with these approaches, mRNA is not currently used for diagnosis of HCM and LQTS, because expression of these genes is largely restricted to myocardium, and their mRNA transcripts cannot be reliably obtained from lymphocytes or fibroblast cultures. The method we describe here overcomes both obstacles, taking advantage of observations from recent microarray analyses that quaternary ammonium surfactants can greatly enhance the yield of rare mRNAs from whole blood, and increase the detection of tissue-restricted gene transcripts.[31,32] We show that cardiovascular-restricted mRNAs can be readily detected in peripheral blood without additional purification steps or cell culture, and then used as starting material for rapid, high-fidelity screening of coding and splicing mutations in genes associated with heart disease.

Sequencing of mRNA screen has the further advantage of being able to reveal functionally significant mutations that could be overlooked by genomic DNA analysis. Splicing consensus sequences are degenerate, and as such, are not unambiguously identifiable from genomic data. It is possible that cDNA sequencing protocols could miss certain large splicing variants that result in extremely large intron inclusions that cannot be amplified by PCR. However, such large inclusions

Table 3
Cost analysis of *FBN1* screening using whole blood RNA mutation detection

Method/Procedure	Supplies	Labor	Total
RNA extraction	$12.00	$19.89	$31.89
DNA extraction	$5.71	$9.95	$15.66
RTase reaction	$6.00	$5.00	$11.00
PCR, cleanup	$2.33	$2.32	$32.55
Sequencing	$1.75	$1.65	$95.20
Confirmation (assume 3 exons checked)			
PCR/cleanup	$2.33	$2.32	$13.95
Sequencing	$1.75	$1.65	$20.40
Subtotal for entire cDNA screen			$220.65
PCR licensing fee* (9% total)			$19.86
Supervision/analysis (7 hrs)			$256.20
Total cost per patient screened		$496.71	

Labor is calculated at $19.89/hr and supervision/analysis time at $36.60/hour. Sequencing is computed as cost per primer-template × 4 seqs/amplicon × 7 amplicons.

Table 4
Cost comparison of mutation detection methods

Method	Total for FBN-1	Total for LQT1/2
SSCP/HA analysis	$829.98	$461.72
Enzyme-mediated cleavage analysis	$1570.34	—
Direct sequencing of each exon	$1016.11	$580.21
Whole Blood RNA	$496.71	$291.76

Figures are taken from above cost analysis and from Yuan et al.[34] with normalization of DNA sequencing costs. Costs per screen assume batch sizes of 15 patient samples for SSCP and 22 samples for enzyme-mediated cleavage analysis.

are rare, since cryptic splice sites are frequently activated that limit the size of the intronic material included. On the other hand DNA sequencing will inevitably miss certain splicing mutations, such as those within remote or unknown splicing enhancers. This was the case with the FBN1 mutation described here (6354C>T) which was a synonymous base substitution remote from a splice junction, and could only have been identified as a splicing mutation by examining RNA directly.

Whole blood RNA cost advantages and comprehensiveness

As shown in Tables 3 and 4, whole blood RNA can be used to identify MFS mutations at a substantial cost advantage over existing methods; this advantage should further increase as DNA sequencing becomes less expensive. The use of whole blood RNA also saves time, as it can provide a complete initial mutation screen for any of the genes described here in less than a week. A well-equipped lab would require almost twice as long to perform the comparable DNA-based assays. Finally, whole blood RNA approach improves the logistics of screening. Almost all DNA-based assays require multiple samples and controls to be processed simultaneously for proper data interpretation, and to achieve cost efficiency. In contrast, the use of whole blood RNA is equally robust for one or multiple samples, and hence is particularly cost-effective when sample numbers are low.

Napolitano et al. recently described a hierarchical screening strategy for streamlining conventional DNA diagnosis of LQTS, by targeting 64 codons that have been found mutated more than once.[3] While representing an important advance, the method captured only 58% of probands carrying a mutation in their study, and would have identified smaller percentages (39% and 33%) of patients reported in two other large series.[5,6] A similar proportion of the estimated 4.5% of patients with more than one mutation would also be missed. For this reason, a more comprehensive sequencing approach may be necessary when a complete risk assessment is needed. The method we describe here can serve this need by providing complete determination of all protein-coding sequences at relatively low cost.

Our method offers equal or greater advantages for diagnosis of Marfan Syndrome and HCM. The major causative gene for MFS, *FBN1*, covers over 200 kb of DNA with more than 600 reported mutations. Currently available RNA-based approaches to this gene require weeks of fibroblast culture, adding greatly to the time and expense of testing; this step is obviated by use of whole blood RNA. For hypertrophic cardiomyopathy, detection and direct sequencing of cardiac-specific myosin binding protein-C, comprising more than 50% of known HCM mutations, can be performed efficiently using the same approach.

Applicability of whole blood RNA testing in other disorders

We predict that substantial efficiencies could be realized by adapting this method to other disorders involving multiple genetic loci and/or large, multi-exon genes. An obvious example is ARVD, a rare but lethal cardiomyopathy associated with mutations in one of the largest genes in the genome, the cardiac ryanodine receptor (ARVD2) (*RYR2*, 105 exons),[33] and with plakophilin 2 (ARVD9) (*PKP2*, 14 exons).[34]

Using RNA from purified or transformed lymphocytes, others have successfully amplified mRNA encoding beta-myosin heavy chain (*MYH7*), one of the proteins mutated in HCM,[14] as well as genes involved in cystic fibrosis (*CFTR*),[35] Duchenne muscular dystrophy (*DMD*),[36] and malignant hyperthermia (*RYR1*).[37] Differences in gene expression patterns between purified or transformed lymphocytes and unfractionated whole blood cells[38] may actually favor the detection of certain transcripts.[14,15] On the other hand, using whole blood RNA, we have successfully amplified mRNA from multiple genes not previously accessible to RNA sequencing, including those linked to LQTS/Brugada syndrome (*SCN5A*), ARVD (*PKP2*), dilated cardiomyopathy (lamin A/C, *LMNA*) and Barth Syndrome (tafazzin, *TAZ*; our unpublished data). These findings represent a useful starting observation for future technical improvements.

Potential limitations

Several technical issues should be considered in applying whole blood RNA sequencing, indeed any RNA-based method, to clinical diagnostics. Tissue-specific splicing could generate alternative transcripts in the test material that are not present in the target tissue, creating the appearance of exon-skipping or insertion mutations. We saw no evidence for this in the genes studied here, although many muscle transcripts are alternatively spliced, and these splicing patterns may not be preserved in heterologous cell types.[39] Nonsense-mediated mRNA decay,[48] which leads to degradation of transcripts with premature termination codons, could selectively deplete mutant RNA. However, this process is rarely complete, so that the mutant transcript is usually still detectable albeit at reduced levels. Consistent with this, we found that transcripts for both mutant and wild type alleles of the truncation mutation in *FBN1*, 4905delC, were nearly equally abundant. On two other occasions, only one allele from the gene of interest could be detected (one each for *MYBPC3* and *FBN1*), and in both cases the mutant allele was preferentially amplified. Although we cannot presently explain this finding, processes such as imprinting, allelic variation in expression,[40] inadvertent allele-specific PCR,[41] or preferential amplification[42] could lead to this result, and to a spurious homozygous genotype. Further analysis of the rate and cause of allelic dropout will be useful to guide further technical improvements in this method.

CONCLUSIONS

Our findings indicate that whole blood RNA sequencing can be a simple and convenient alternative to SSCP or DHPLC for missense mutation screening, and is inherently better for detection of splicing errors. Indeed, the use of whole blood RNA can replace these technologies as a robust initial screen of all coding regions of the 4 genes described here, followed by conventional DNA sequencing. Our limited data do not permit a quantitative determination of sensitivity or specificity. However, since we were able to detect mutations at frequencies similar to those reported for each

of these diseases, our data support the view that a diagnostic approach that begins with RNA testing may ultimately prove to have a sensitivity comparable to other mutation detection approaches such as SSCP and DHPLC.

As summarized recently,[3] prognosis and therapeutic response in LQTS are each strongly predicted by mutation locus. The risk of cardiac arrhythmias and sudden death can also be strongly influenced by genotype in HCM.[43] In each of these conditions, genetic information may be crucial in establishing the clinical diagnosis, particularly in presymptomatic or sporadic disease, and in adult patients without a positive family history.[30,44,45] As an added benefit, increasing the pool of genotype information will further improve understanding of genotype-phenotype relationships.[46] Our approach can reduce some of the practical barriers to DNA mutation screening, and can be immediately useful in research laboratories.

Additional studies will be needed to determine the appropriate place of RNA screening in clinical laboratories. Screening methods are often used in advance of DNA sequencing; reasons include the size and number of genes that need to be sequenced, the availability of labor and instrumentation required to achieve this, and the likelihood of diagnostic certainty within an affordable cost range. Screening tests have the potential to reduce the average labor and time required to identify a mutation, even when negative results are further pursued by DNA sequencing. Total costs for testing will depend greatly on the extent to which negative results are subjected to further testing, factoring in sequencing costs, pretest probability, and the desire for certainty. Our method adds another option to the menu of diagnostic prescreening tools that could have an impact on the logistics of DNA testing, increasing access to genetic testing and improving clinical management for these and other multi-gene disorders. In turn, a demonstrable health impact for genetic testing in these disorders could be persuasive for insurers.[47]

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