Int J Legal Med (2012) 126:969–973
DOI 10.1007/s00414-011-0598-x

SHORT COMMUNICATION

Long QT syndrome mutation detection by SNaPshot technique

Jeanett Edelmann · Stefanie Schumann · Marina Nastainczyk · Daniela Husser-Bollmann · Rüdiger Lessig

Received: 10 March 2011 / Accepted: 24 June 2011 / Published online: 18 July 2011

Abstract Long QT syndrome (LQTS) is a cardiac disorder with an abnormality of cardiac rhythm associated with sudden death especially in younger, apparently healthy individuals. If there is no clear cause of death detectable during comprehensive coroner's inquest (autopsy-negative cases), you have to consider LQTS and other heritable arrhythmia syndromes. A molecular genetic screening regarding mutations in associated genes can help to ensure the cause of death and to protect affected family members. Genetic testing of LQTS, currently performed mainly by sequencing, is still very expensive and time consuming. With this study we present a rapid and reasonable method for the simultaneously screening of some of the most common mutations associated with LQTS, focused on the KCNQ1 and KCNH2 genes. With the method of SNaPshot minisequencing, a total of 58 mutations were analyzed in four multiplex assays which were successfully established and optimized. The comparison with samples previously analyzed by direct sequencing showed concordance. Furthermore, autopsy-negative cases were tested but no mutations could be observed in any of the specimen. The presented method is well suitable for LQTS mutation screening. An enhancement to further mutations and population-based investigations regarding mutation frequencies should be the aim of prospective studies.

Keywords Long QT syndrome · SNaPshot technique · KCNQ1 · KCNH2

Electronic supplementary material The online version of this article (doi:10.1007/s00414-011-0598-x) contains supplementary material, which is available to authorized users.

J. Edelmann (✉) · M. Nastainczyk
Institute of Forensic Medicine, University of Leipzig,
Leipzig, Germany
e-mail: jeanett.edelmann@medizin.uni-leipzig.de

S. Schumann
Institute of Forensic Medicine, University of Münster,
Münster, Germany

D. Husser-Bollmann
Department of Internal Medicine/Cardiology,
Heart Center University Leipzig,
Leipzig, Germany

R. Lessig
Institute of Forensic Medicine, University of Halle,
Halle, Germany

Introduction

Long QT syndrome (LQTS) is a cardiac disorder with an abnormality of cardiac rhythm. It is characterized by delayed ventricular repolarisation which is seen as a prolonged QT interval on electrocardiograms (ECG). Patients have an increased risk for arrhythmia-related syncope due to torsades de pointes as well as electrocardiographic abnormalities with prolonged QT values, furthermore cardiac arrest and sudden death caused by physical or emotional stress or occurring at rest and during sleep respectively, mainly in younger and otherwise healthy individuals [1]. LQTS can be acquired by commonly used drugs but primarily, it is a congenital disorder caused by mutations of cardiac ion channel genes.

Traditionally two clinical manifestations can be differentiated: Romano–Ward syndrome is the more common autosomal dominant form, and Jervell Lange-Nielsen syndrome is a less common autosomal recessive form which is more severe and accompanied by sensorineural deafness [2, 3].

Twelve genes have been identified in which more than 600 different mutations are associated with LQTS [4–6]. Probably there might be a lot more of rare and unidentified variants [7].

Mutations in the three major LQTS susceptibility genes namely KCNQ1, KCNH2, and SCN5A account for 75% [8] to 90% [7] of all congenital LQTS cases. LQT1 is the most common type of LQTS. It is caused by mutations in KCNQ1 gene on chromosome 11p15.5. LQT2, the second frequent form of LQTS, is caused by mutations in the KCNH2 (HERG) gene on 7q36.1. Both are encoding for subunits of potassium channels. The third common gene involved in LQTS is SCN5A which is located on chromosome 3p21 resulting in the genetic subtype LQT3 and encoding for sodium channel subunits [9–11].

The postmortem diagnosis of LQTS in cases of unexplained deaths is very important because the relatives of the deceased will be at risk and could obtain precautionary therapies to prevent sudden deaths [12]. The LQTS is normally without any macroscopic and microscopic findings. Therefore in cases of sudden cardiac deaths without structural cardiac abnormalities, screening for mutations associated with LQTS or other heritable arrhythmia syndromes is an approach to assign a distinctive cause of death [13] and detecting a risk for affected but asymptomatic relatives as well.

Due to the high costs and time-consuming sequencing methods, molecular genetic testing does not belong to the routine tasks of forensic and pathology laboratories. A further problem in forensic casework can be the time between death and the date of investigation, perhaps degradation of DNA disturbs the analysis. The present study is aimed to validate a rapid, sensitive, and reliable method by means of single-nucleotide primer extension analysis using multiplex SNaPshot assays and capillary electrophoresis for simultaneous screening of several common mutations in the KCNQ1 and KCNH2 genes.

Materials and methods

Samples

We analyzed 35 anonymous samples from individuals with uncertain cause of death (autopsy-negative cases) which were obtained from the Institute of Forensic Medicine, University of Leipzig once performing the autopsy. Genomic DNA was extracted from fresh or dried blood samples using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany).

Furthermore, 10 anonymous DNA samples from genotyped patients with confirmed LQTS, which have been previously analyzed by direct sequencing until the first point mutation was observed, were provided by the Department of Internal Medicine/Cardiology, University of Leipzig and used as positive controls. The majority of these individuals have mutations in one of the two genes KCNQ1 or KCNH2 (Table 1). In addition five DNA samples from known non-LQTS individuals (confirmed by sequencing) were used as negative controls.

We decided to investigate LQTS relevant point mutations, based on a study published by Napolitano et al. [2]. The adequate exons were previously amplified by polymerase chain reaction (PCR).

Multiplex PCR

Primarily, already published PCR primers for KCNQ1 gene exons 3, 5, 6, and 7 and KCNH2-gene exons 6, 7, 8, and 9 [14, 15] were tested in single-product reactions for correct sizing and absence of any secondary PCR products. Next we designed and optimized three multiplex reactions for a useful simultaneous amplification of the above-mentioned exons (Table S1a, b) using equivalent primer sequences. The final multiplex PCR reaction volume was 13 μl consisting of 6.25 μl 2× multiplex master mix (Qiagen, Hilden Germany), 0.77 μM of each primer, and 1 μl

Table 1 Summary of medical-referred cases of LQTS

Sample	Nucleotide change	Amino acid change	Gene	LQTS subtype
1	A>G	D673G	KCNQ1	LQTS1
2	C>T	R231C	KCNQ1	LQTS1
3	C>T	S641F	KCNH2	LQTS2
4	C>T	R1014X	KCNH2	LQTS2
5	C>T	R1014X	KCNH2	LQTS2
6	C>T	R1014X	KCNH2	LQTS2
7	C>T	R176W	KCNH2	LQTS2
8	G>A	R98E	KCNE1	LQTS5
9	G>A	R98E	KCNE1	LQTS5
10	G>A	R98E	KCNE1	LQTS5

genomic DNA template with about 1 ng DNA. A personal cycler Biometra T3 (Biometra, Göttingen, Germany) was used to perform thermal cycling according to the following conditions: 15 min at 95°C (initial denaturation) followed by 30 cycles at 94°C for 30 s (denaturation), 64°C for 50 s (annealing), 72°C for 40 s (extension), and a final extension of 20 min at 60°C. Subsequently, the resulting products, differing from 156 to 449 bp, were incubated with shrimp alkaline phosphatase (SAP) and exonuclease I (Exo I) enzymes for 60 min at 37°C to dephosphorylate unused dNTPs and degrade remaining single-stranded DNA. Inactivation of the enzymes was ensued by incubating for 15 min at 75°C.

SNaPshot analysis

Mutation detection was performed with the ABI SNaPshot Multiplex Kit followed by automated minisequencing on ABI 310 Genetic Analyzer. Therefore single-base extension primers (SNP primers) were designed to perform the SNP assays as multiplex reaction. The SNP primers (Tables S2a, b and S3a, b) were adjusted to anneal next to the nucleotide with potential mutation, on either the sense or antisense DNA strand. In order to produce different fragment lengths avoiding overlapping between the final SNaPshot products, different primer sizes were created by adding a variable nonhomologous polynucleotide tail at the 5′ end whereby the primers differ by two to four nucleotides. We used 25 SNP primers in two multiplex reactions to detect the mentioned mutations in the KCNQ1 gene (Table S2a, b). For KCNH2 in total 21 SNP primers were designed. Thereby the first reaction includes 12 primers for mutations in the exons 6 and 7 (Table S3a), the second reaction is encompassing nine more primers for mutations in the exons 7, 8, and 9 (Table S3b). Altogether, using 46 SNP primers, it is theoretically possible to detect 58 known and common mutations. The SNaPshot extension reactions were performed in a final volume of 10 µl containing 1 µl of purified PCR product, 3 µl of SNaPshot Ready Reaction Mix (ABI Prism SNaPshot Multiplex, Applied Biosystems, Darmstadt, Germany), and 0.4 µl of each single-base extension primer with an end concentration differing from

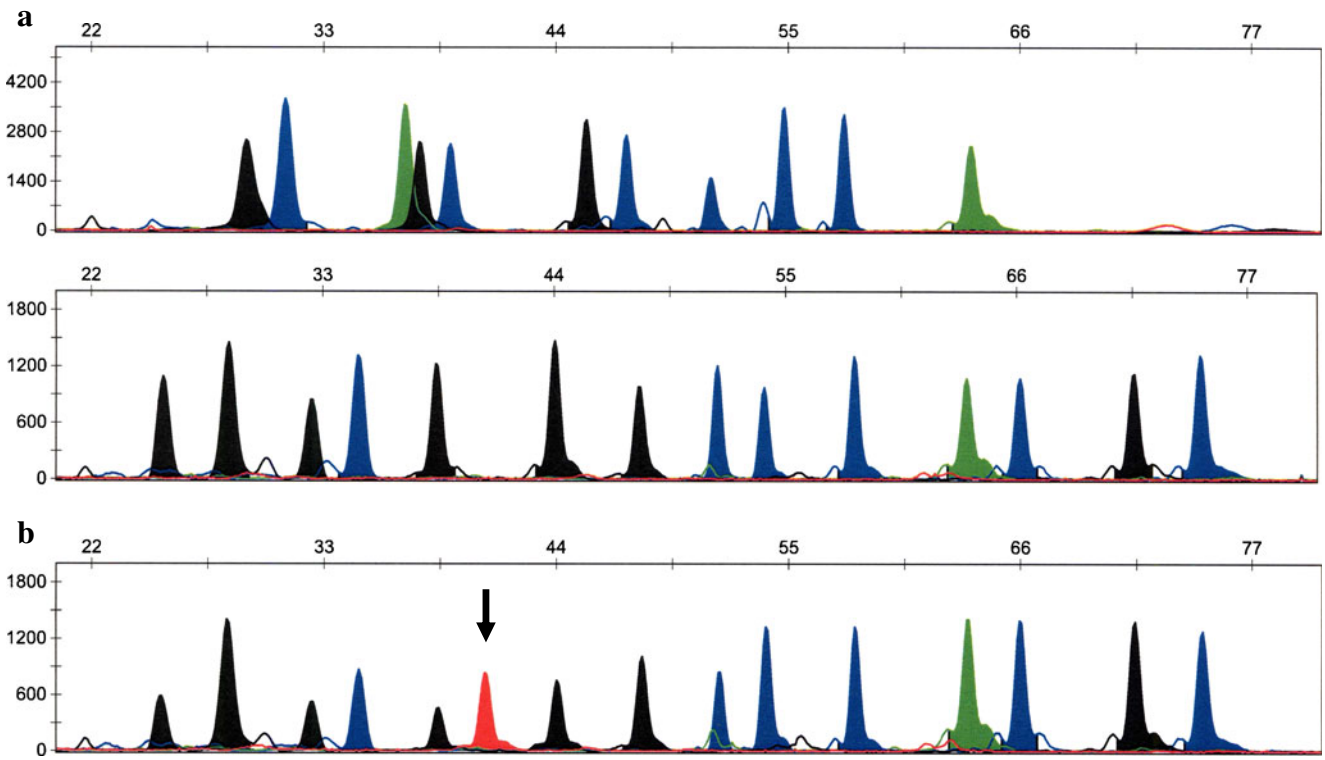

Fig. 1 **a** KCNQ1—electropherogram of GeneScan Analysis of the two multiplex primer combinations. *Upper panel* position and nucleotide of wild-type alleles are as follows (from *left* to *right*): 1022/C, 898/G, 839/T*, 784/C, 806/G, 830/C, 535/G, 569/G, 973/G, 1031/C*, 773/A (*reverse primers). *Lower panel* position and nucleotide of wild-type alleles are as follows (from *left* to *right*): 520/C, 521/G*, 568/C, 805/G, 691/C, 692/G*, 724/G*, 914/G, 772/C*, 941/G, 964/A, 965/C*, 1027/C, 1028/C* (*reverse primers). **b** KCNQ1—electropherogram of a genetic variant (positive control). Heterozygote nucleotide change at locus 691/C>T with coding effect R231C

0.007 to 2.0 μM. The amplification was realized for 25 cycles at 96°C for 10 s (denaturation), 50°C for 5 s (annealing), and 60°C for 30 s (extension). The SNaPshot products were purified with SAP (37°C—60 min, 75°C—15 min) and thereafter analyzed by capillary electrophoresis on the ABI 310 Genetic Analyzer (Applied Biosystems) using size standard GeneScan™120LIZ® and GeneMapper®ID Software v3.2 for genotyping.

Wild-type alleles or arising point mutations were identified by an expected specific fragment length resulting from the SNP primer tailing and a different peak color. In general the measured product sizes are differing by 2–4 bp from the designed sizes because the four fluorophors have a slightly varying mobility, however not affecting subsequent analysis [16, 17]. Finally we established four multiplex reactions using 46 SNP primers hence theoretically detecting 58 known and common mutations.

Results

We successfully developed four SNP multiplex assays, two for each of the genes, KCNQ1 and KCNH2 (Figs. 1a and 2a). This number was necessary because an overlapping of some primer sequences arises. The structure and concentration of all primers as well as analysis parameters of the assays were optimized. After validation of these tests, our DNA samples were investigated: autopsy-negative cases (n=35), LQTS-confirmed samples (n=10) and non-LQTS individuals (n=5).

Screening for KCNQ1 and KCNH2 candidate genes by the described SNaPshot assays resulted in the finding of two heterozygous single-nucleotide mutations within the LQTS-confirmed samples. One variation was in the KCNQ1 gene, exon 5, C691T causing a missense mutation at codon 231 (R231C, Fig. 1b) and another in the KCNH2 gene, exon 7, C1922T causing a missense mutation at codon 641 (S641F, Fig. 2b). Both mutations were present in the heterozygote form: one wild-type peak and one "mutant" peak (arrow) with a different color could be observed. The molecular screening performed with the shown multiplex assays could identify only these two variations, whereby a total number of 58 potential mutations in different exons were incorporated. No further point mutations were detected in any of the negative samples as well as in the 35 DNA probes from cases with autopsy-negative results.

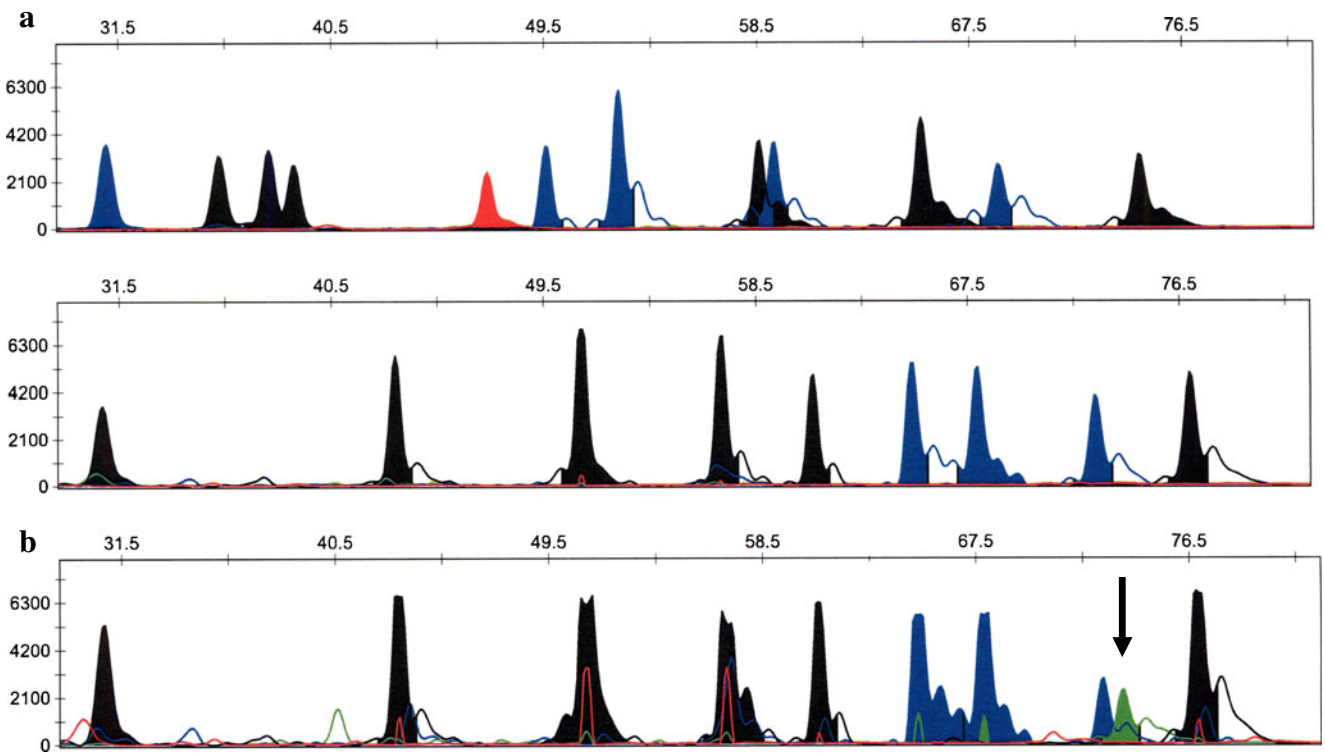

Fig. 2 **a** KCNH2—electropherogram of GeneScan Analysis of the two multiplex primer combinations. *Upper panel* position and nucleotide of wild-type alleles are as follows (from *left* to *right*): 1235/G, 1283/C, 1600/C, 1601/G*, 1655/T, 1681/G, 1682/C*, 1714/G*, 1810/G, 1825/G*, 1882/G, 1883G* (*reverse primers). *Lower panel* position and nucleotide of wild-type alleles are as follows (from *left* to *right*): 2254/C, 2255/G*, 1715/G*, 1838/C, 1979/C, 1841/C*, 1868/C*, 1922/C*, 1745/G* (*reverse primers). **b** KCNH2—electropherogram of a genetic variant (positive control). Heterozygote nucleotide change at locus 1922/C>T* with coding effect S641F (*reverse primer)

Discussion

It was shown that the SNaPshot technique is suitable to detect point mutations in the selected genes. The presented assays could be successfully established, optimized, and applied, getting reproducible results and are suitable for large scale screening. It is suggested that the multiplex SNaPshot technique is applicable, solid, flexible, cost efficient, and a good alternative to sequencing.

After all, the molecular genetic identification of a variant does not necessarily indicate abnormal function of the encoded protein. Otherwise the absence of mutations in a proportion of symptomatic individuals can be attributed to the fact that not all genes or genetic regions involved in the syndrome have been identified [9]. Although no mutation could be detected in the autopsy-negative samples of our study, we cannot preclude that other possible genetic variations are causing the death.

LQTS mutation carriers have a variable penetrance of the syndrome and so the occurring phenotypes show wide clinical heterogeneity [18]. Therefore, diagnosis can be quite difficult in some cases. For instance, about 36% of LQTS cases exhibit a normal QT interval on the surface ECG [19]. In other cases, affected members with same mutations display varying degrees of QT prolongation and can show highly variable symptoms. Phenotypes can range from no symptoms to sudden death [20]. A molecular genetic screening regarding mutations in associated genes can help to ensure the cause of death and to protect affected family members. Finally, the diagnosis of LQTS is an interaction between family history, clinical features, and molecular genetic mutation screening.

The list of LQTS-causing genes is continuously expanding, and indications for genetic analysis are increasing. Information about the type of genetic variation has a direct prognostic and therapeutic impact in LQTS; it is not only a diagnostic tool. So the identification of specific mutations has influence on the type of therapy and risk stratification [21]. A positive genetic testing can confirm the diagnosis of a patient, detect the cause of death for sudden deceased persons, and identify asymptomatic family members.

Perspectively, further assays for other LQTS-associated mutations can be added to the screening system. Studies with higher number of samples as well as population-based investigations are necessary to determine true frequencies of various LQTS mutations or to identify LQTS polymorphism.

Acknowledgments The authors would like to thank Ms. Manuela Pigors for her studies and support.

References

1. Schwartz PJ, Priori SG, Locati EH, Napolitano C, Cantu F, Towbin JA, Keating MT, Hammoude H, Brown AM, Chen LS (1995) Long QT syndrome patients with mutations of the SCN5A and HERG genes have differential responses to Na^+ channel blockade and to increases in heart rate. Circulation 92:3381–3386
2. Napolitano C, Priori SG, Schwartz PJ, Bloise R, Ronchetti E, Nastoli J, Bottelli G, Cerrone M, Leonardi S (2005) Genetic testing in the long QT syndrome. JAMA 294(23):2975–2980
3. Crotti L, Celano G, Dagradi F, Schwartz PJ (2008) Congenital long QT syndrome. Orphanet J Rare Dis 3:18
4. Allegue C, Gil R, Sanchez-Diz P, Torres M, Quintela I, Carracedo A, Brion M (2010) A new approach to long QT syndrome mutation detection by Sequenom MasARRAY system. Electrophoresis 31:1648–1655
5. Bhuiyan ZA, Al-Shahrani S, Al-Khadar AS, Al-Ghamdi S, Al-Khalaf K, Mannens MMAM, Wilde AAM, Momenah TS (2009) Clinical and genetic analysis of long QT syndrome in children from six families in Saudi Arabia: are they different? Pediatr Cardiol 30(4):490–501
6. Goldenberg I, Zareba W, Moss AJ (2008) Long QT syndrome. Curr Probl Cardiol 33:629–694
7. Rich SS, Hall IM (2011) DNA structural variants as genetic risk factors for long QT syndrome. J Am Coll Cardiol 57 (1):48–50
8. Kapa S, Tester DJ, Salisbury BA, Harris-Kerr C, Pungliya MS, Alders M, Wilde AAM, Ackerman MJ (2009) Genetic testing for long-QT syndrome: distinguishing pathogenic mutations from benign variants. Circulation 120:1752–1760
9. Bokil NJ, Baisden JM, Radford DJ, Summers KM (2010) Molecular genetics of long QT syndrome. Mol Genet Metab 101:1–8
10. Tester DJ, Ackermann MJ (2007) Postmortem long QT syndrome genetic testing for sudden unexplained death in the young. J Am Coll Cardiol 49(2):240–246
11. Modell SM, Lehmann MH (2006) The long QT syndrome family of cardiac ion channelopathies: a HuGE review. Genet Med 8(3):143–155
12. Wedekind H, Schulze-Bahr E, Debus V, Breithardt G, Brinkmann B, Bajanowski T (2007) Cardia arrhythmias and sudden death in infancy: implication for the medicolegal investigation. Int J Legal Med 121:245–257
13. Rodriguez-Calvo MS, Brion M, Allegue C, Concheiro L, Carracedo A (2008) Molecular genetics of sudden cardiac death. Forensic Sci Int 182:1–12
14. Itoh T, Tanaka T, Nagai R, Kamiya T, Sawayama T, Nakayama T, Tomoike H, Sakurada H, Yazaki Y, Nakamura Y (1998) Genomic organization and mutational analysis of HERG, a gene responsible for familial long QT syndrome. Hum Genet 102:435–439
15. Splawski I, Shen J, Timothy KW, Vincent GM, Lehmann MH, Keating MT (1998) Genomic structure of three long QT syndrome genes: *KVLQT1*, *HERG*, and *KCNE1*. Genomics 51:86–97
16. Hurst CD, Zuiverloon TC, Hafner C, Zwarthoff EC, Knowles MA (2009) A SNaPshot assay for the rapid and simple detection of four common hotspot codon mutations in the PIK3CA gene. BMC Res Notes 2:66
17. Filippini S, Blanco A, Fernández-Marmiesse A, Alvarez-Iglesias V, Ruíz-PonteC CA, Vega A (2007) Multiplex SNaPshot for detection of *BRCA1/2* common mutations in Spanish and Spanish related breast/ovarian cancer families. BMC Med Genet 8:40
18. Roden DM (2008) Clinical practice. Long-QT syndrome. N Engl J Med 358(2):169–176
19. Schimpf R, Veltmann C, Wolpert C, Borggrefe M (2009) Channelopathies: Brugada syndrome, long QT syndrome, short QT syndrome, and CPVT. Herz 34(4):281–288
20. Darbar D (2006) Screening for genomic alterations in congenital long QT syndrome. Heart Rhythm 3(1):56–57
21. Fowler SJ, Napolitano C, Priori SG (2010) When is genetic testing useful in patients suspected to have inherited cardiac arrhythmias? Curr Opin Cardiol 25:37–45