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Supplementary Files

Supplementary File 1

Methods

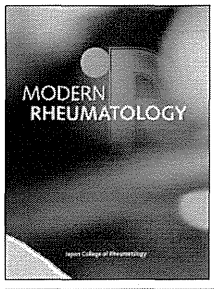
Figure S1. L-type calcium channel currents in HL-1 cells.

Figure S2. Expression of L-type calcium channel proteins in HL-1 cells.

Figure S3. mRNA expressions of uric acid transporters in human embryonic stem cell-derived cardiomyocytes.

Table S1. cDNA primer sequences of human urate transporters

Please find supplementary file(s);
http://dx.doi.org/10.1253/circj.CJ-15-0416



Modern Rheumatology

ISSN: 1439-7595 (Print) 1439-7609 (Online) Journal homepage: http://www.tandfonline.com/loi/imor20

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To cite this article: Hiroto Tsuboi, Takayuki Sumida, Hisashi Noma, Kazumasa Yamagishi, Ai Anami, Kotaro Fukushima, Hitoshi Horigome, Yasuki Maeno, Mitsumasa Kishimoto, Yoshinari Takasaki, Masahiro Nakayama, Masako Waguri, Haruhiko Sago & Atsuko Murashima (2015): Maternal predictive factors for fetal congenital heart block in pregnant mothers positive for anti-SS-A antibodies, Modern Rheumatology, DOI: 10.3109/14397595.2015.1106661

To link to this article: http://dx.doi.org/10.3109/14397595.2015.1106661

Accepted author version posted online: 12 Oct 2015.
Published online: 20 Nov 2015.

Japan College of Rheumatology
MODERN RHEUMATOLOGY

http://informahealthcare.com/mor
ISSN 1439-7595 (print), 1439-7609 (online)

Mod Rheumatol, 2015; Early Online: 1–7

DOI: 10.3109/14397595.2015.1106661

Taylor & Francis
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ORIGINAL ARTICLE

Maternal predictive factors for fetal congenital heart block in pregnant mothers positive for anti-SS-A antibodies

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Abstract

Objective: To determine the maternal predictive factors for fetal congenital heart block (CHB) in pregnancy in mothers positive for anti-SS-A antibodies.

Methods: The Research Team for Surveillance of Autoantibody-Exposed Fetuses and Treatment of Neonatal Lupus Erythematosus, the Research Program of the Japan Ministry of Health, Labor and Welfare, performed a national survey on pregnancy of mothers positive for anti-SS-A antibodies. We analyzed 635 pregnant mothers who tested positive for anti-SS-A antibodies before conception but had no previous history of fetal CHB. We performed univariate and multivariate analysis (models 1, 2, and 3 using different set of independent variables) investigated the relation between risk of fetal CHB and maternal clinical features.

Results: Of the 635 pregnant mothers, fetal CHB was detected in 16. Univariate analysis showed that fetal CHB associated with use of corticosteroids before conception (OR 3.72, $p = 0.04$), and negatively with use of corticosteroids (equivalent doses of prednisolone (PSL), at $\geq$10 mg/day) after conception before 16-week gestation (OR 0.17, $p = 0.03$). In multivariate analysis, model 1 identified the use of corticosteroids before conception (OR 4.28, $p = 0.04$) and high titer of anti-SS-A antibodies (OR 3.58, $p = 0.02$) as independent and significant risk factors, and model 3 identified use of corticosteroids (equivalent doses of PSL, at $\geq$10 mg/day) after conception before 16-week gestation as independent protective factor against the development of fetal CHB (OR 0.16, $p = 0.03$). Other maternal clinical features did not influence the development of fetal CHB.

Conclusion: The results identified high titers of anti-SS-A antibodies and use of corticosteroids before conception as independent risk factors, and use of corticosteroids (equivalent doses of PSL, at $\geq$10 mg/day) after conception before 16-week gestation as an independent protective factor for fetal CHB.

Keywords

Anti-SS-A antibodies, Congenital heart block, Risk factor

History

Received 19 March 2015
Accepted 4 October 2015
Published online 17 November 2015

Introduction

Anti-SS-A/Ro antibodies are associated with neonatal lupus erythematosus (NLE), which presents clinically with fetal congenital heart block (CHB), transient skin rash, and hematological and hepatic abnormalities [1]. Anti-SS-A antibodies are also commonly detected in patients with autoimmune diseases, such as Sjögren's syndrome (SS) (60–90%), systemic lupus erythematosus (SLE) (30–50%), and rheumatoid arthritis (RA) (11%) [1]. Importantly, anti-SS-A antibodies are also detected in between 1% and 2% of randomly tested pregnant women [2]. Child birth in Japan is about 1,000,000 per year; therefore, child birth from mothers with anti-SS-A antibodies is estimated at 10,000 per year. The prevalence of neonatal lupus rash was reported to be 10–20% in the offspring of anti-SS-A antibodies-positive women, while laboratory abnormalities in asymptomatic babies can be detected in up to 27% of anti-SS-A antibodies

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positive mothers [1]. Although fetal CHB is a rare manifestation among NLE features and the prevalence of fetal CHB was reported to be 1–2% in pregnant women with anti-SS-A antibodies [1,3–10], fetal CHB is associated with significant mortality (20–30%, primarily fetal/neonatal) and morbidity (67% require permanent placement of a pacemaker before adulthood) [5]. Because of the rarity of fetal CHB in pregnant women with anti-SS-A antibodies, neither randomized prospective clinical trials nor meta-analysis has yet been reported [9]. Previous fetal CHB has been confirmed as a definite maternal risk factor for fetal CHB in pregnant women with anti-SS-A antibodies, and the recurrence rate of fetal CHB in subsequent pregnancies after preceding offspring complicated with fetal CHB is reported to be approximately 12–20%, or 8- to 9-fold risk than in without previous fetal CHB [4,6,7,10]. Recently, Anami et al. [10] reported that anti-SS-A antibody titer of 1:32 or higher in the maternal sera by double immune-diffusion (DID) was an independent risk factor for fetal CHB, and that treatment with either prednisolone (PSL) or betamethasone during pregnancy lowered the risk of fetal CHB in these mothers. On the other hand, Tunks et al. [8] reported that fetal CHB was not associated with higher levels of maternal anti-SS-A antibodies but with that of anti-SS-B/La antibodies. Moreover, Ambrosi et al. [7] identified maternal older age and summer season of child birth as novel risk factors for fetal CHB. Observational studies suggested the possible effectiveness of intravenous gamma globulin (IVIG) and hydroxychloroquine (HCQ) in reducing fetal CHB-risk [9]. Other maternal factors such as health status and use of corticosteroids have not been confirmed as related factors to occurrence of fetal CHB in pregnant women positive for anti-SS-A antibodies [4,6].

Currently, there are no standard guidelines for surveillance, prevention, and treatment on fetal CHB associated with anti-SS-A antibodies. Because the presence of anti-SS-A antibodies can be detected after the diagnosis of fetal CHB or NLE in some cases, screening and management for asymptomatic women with anti-SS-A antibodies seem to be an important clinical issue. Thus, the Research Team for Surveillance of Autoantibody-Exposed Fetuses and Treatment of Neonatal Lupus Erythematosus, a Research Program sponsored by the Japan Ministry of Health, Labor and Welfare (MHLW) invited rheumatologists, obstetricians and gynecologists, pediatric cardiologists, epidemiologists, and biostatisticians to conduct a national survey on pregnancy of mothers positive for anti-SS-A antibodies to clarify the maternal predictive factors for fetal CHB. The present study documents this survey and reports the results.

Patients and methods

The national survey on pregnancy of mothers positive for anti-SS-A antibodies

The Research Team for Surveillance of Autoantibody-Exposed Fetuses and Treatment of Neonatal Lupus Erythematosus, the Research Program of the Japan MHLW conducted the national survey on pregnancy of mothers positive for anti-SS-A antibodies at the institutions affiliated with members of the research team (from 2008 to 2009), as well as 60 Departments of Rheumatology and Perinatal Medical Centers across Japan (from 2010 to 2011). The retrospective survey investigated maternal information such as age at delivery, history of conceptions, including history of NLE and fetal CHB, rheumatologic symptoms, clinical diagnosis, autoantibodies status, including anti-SS-A antibodies, anti-SS-B antibodies (DID and/or ELISA), anti-SS-A 52 kDa antibodies, and anti-SS-A 60 kDa antibodies (detected by western blotting and/or ELISA), other antibodies, treatment before and after conceptions, and interventions for CHB after diagnosis, as well as child-related information, such as gestational week of delivery, gender, birth weight, APGAR score, and presence or absence of NLE and fetal CHB. Approval for this study was obtained from the local ethics committees of the participating institutions.

Selection of cases for analysis among collected cases

Clinical data on 732 cases were collected. First, we excluded the cases with unknown fetal CHB status, as well as cases with previous fetal CHB. Next, we also excluded cases who tested positive for anti-SS-A antibodies after conception, and one case with ± status of anti-SS-A antibody by DID and unknown by ELISA. Thus, the study subjects were 635 pregnant mothers who tested positive for anti-SS-A antibodies before conception, and who had no past history of fetal CHB, including 16 with fetal CHB and 619 without fetal CHB (Figure 1).

Comparison of clinical features between cases with fetal CHB and without fetal CHB

We compared various maternal clinical features, including age at delivery, history of conceptions, rheumatologic symptoms, clinical diagnosis, anti-SS-A antibodies status, and treatment before and after conceptions, between 16 cases with fetal CHB and 619 cases without fetal CHB.

We divided the cases into those with high titer and low titer of anti-SS-A antibodies by DID using a cut-off value of 1:32. The selection of the cut-off value was based on the finding by Anami et al. [10] who reported that a titer of 1:32 or higher in the maternal serum by DID was an independent risk factor for fetal CHB. In cases with unknown DID status, 1:32 by DID was converted into equivalent titers by each ELISA kit [Miyano et al., Clin Rheumatol 24:247–259, 2012, in Japanese], and we regarded cases with high titer by ELISA equivalent to ≥1:32 by DID as high titer, and cases with low titer by ELISA equivalent to <1:32 by DID as low titer. Thus, 114 cases were classified as the high titer group (6 with CHB and 108 without CHB), 506 cases as the low titer group (10 with CHB and 496 without CHB), and 15 cases as the unknown titer group (15 without CHB).

Statistical analysis

Comparisons between groups were conducted using the Student's *t*-test for continuous variables and Fisher's exact test for binary variables. Univariate analysis investigated the relation between risk of fetal CHB and the following variables: maternal clinical features, such as age at delivery, history of conceptions, rheumatologic symptoms, clinical diagnosis, anti-SS-A antibodies status, and treatment before and after conceptions. We also performed multivariate analysis with fetal CHB as the dependent variable, and maternal clinical features that were indicated to have associations in the univariate analyses ($p<0.1$), and some maternal factors which could affect corticosteroids therapy considering their clinical relevancies as in the independent variables. We also performed logistic regression analysis, and calculated odds ratio (OR) for CHB, 95% confidence interval (CI), and p value. For missing data, we used the multiple imputation method; 200 imputed datasets were generated using the MICE (multiple imputation by chained equations) method and their results were synthesized by the Rubin's rule [11]. SPSS for Windows, version 18.0 (IBM Japan Inc., Tokyo, Japan) and R version 3.0.3 (R Foundation for Statistical Computing, Vienna, Austria) were used for statistical analyses. All quoted p values are two-sided and the significance level was set to 0.05.

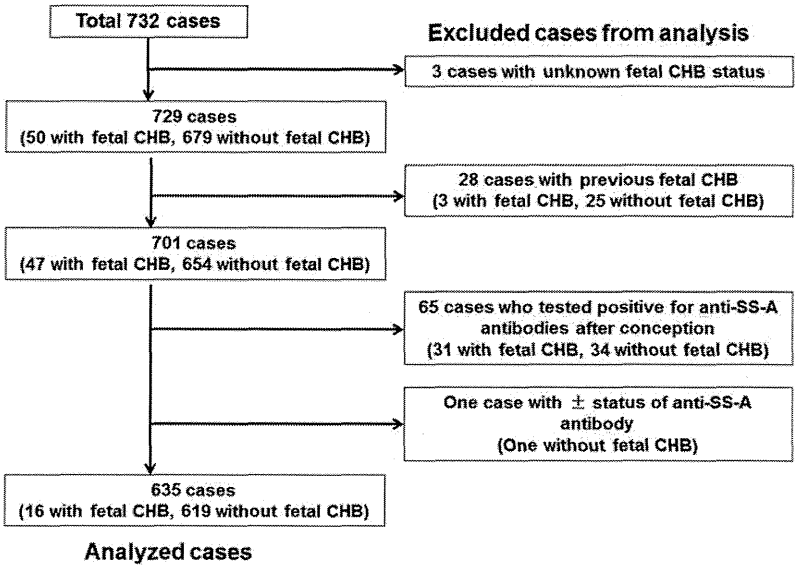

Figure 1. Flow chart showing selection of cases for analysis among 732 pregnant women who tested positive for anti-SS-A antibodies.

Results

Selected cases for analysis among collected cases

Clinical data on 732 cases (50 cases with fetal CHB, 679 cases without fetal CHB, and 3 cases with unknown fetal CHB status) were collected. First, we excluded the 3 cases with unknown fetal CHB status, as well as 28 cases with previous fetal CHB, which is a definite maternal risk factor for fetal CHB (3 with fetal CHB and 25 without fetal CHB) from analysis. Next, among the residual 701 cases, we also excluded 65 cases who tested positive for anti-SS-A antibodies after conception (31 with fetal CHB and 34 without fetal CHB), and one case with ± status of anti-SS-A antibody by DID and unknown by ELISA (without fetal CHB). Thus, the study subjects were 635 pregnant mothers who tested positive for anti-SS-A antibodies before conception, and who had no past history of fetal CHB, including 16 with fetal CHB and 619 without fetal CHB (Figure 1).

In these analyzed 635 cases, many cases (82.2%, 522/635 cases) had diagnosis of maternal connective tissue disease (CTD). We could speculate that these cases were tested for anti-SS-A antibodies in clinical practice before conception. However, many fetal CHB cases (31/50 cases) were tested positive for anti-SS-A antibodies after conception as described above. These fetal CHB cases had no opportunity to receive systemic examinations and corticosteroids therapy before conception or after conception before 16-week gestation (before onset of fetal CHB). Thus, we supposed that if we included these fetal CHB cases in the present analysis, these cases caused a serious selection bias for corticosteroids therapy.

Comparison of clinical features of mothers with and without fetal CHB

There was no significant difference in age at delivery, history of conception, rheumatologic symptoms, clinical diagnosis, and anti-SS-A antibodies status between patients with fetal CHB and without fetal CHB (Table 1). The frequency of treatment with corticosteroids (equivalent doses of PSL, at $\geq$10 mg/day) during pregnancy before 16 weeks of gestation was significantly lower in patients with fetal CHB (20.0%) than without fetal CHB (60.2%, $p = 0.03$). The frequency of treatment with corticosteroids before conception was higher (81.3%), and use of corticosteroids (equivalent doses of PSL, at $\geq$10 mg/day) before and after conception tended to be lower (11.1%) in patients with fetal CHB than without fetal CHB (54.2% and 49.5%, respectively), albeit statistically insignificant ($p = 0.06$ and $p = 0.053$, respectively) (Table 1).

Table 2 provides details on the number of patients who received corticosteroids treatment stratified according to the type of corticosteroids and time of administration in relation to conception. PSL was the most commonly used by many patients with and without fetal CHB before conception, while other types of corticosteroids were administered in only a few cases. In comparison, betamethasone was administered in 4 patients with fetal CHB and 28 cases without fetal CHB after conception. Regarding before conception, populations treated with PSL or other corticosteroids were comparable between cases with and without fetal CHB ($p = 0.135$). On the other hand, after conception, population treated with other corticosteroids than PSL was significantly larger in cases with fetal CHB than in without fetal CHB ($p = 0.0002$). We supposed that fetuses with CHB might be treated with placental transferable corticosteroids such as betamethasone and dexamethasone.

Because we did not investigate the reasons why the mothers took corticosteroids before and after conception in this retrospective survey, we further analyzed the association of the timing of corticosteroids use with onset of fetal CHB and diagnosis of maternal CTD. We divided the timing of corticosteroids use into three phases, such as before conception, after conception before 16-week gestation, and after 16-week gestation. We focused on 16-week gestation, because it has been reported that between 16 and 24 weeks of gestation was the period during which the fetus was at the highest risk of developing CHB [2]. Actually, in the present study, every fetal CHB developed between 18 and 30 weeks of gestation. Therefore, the period after conception before 16-week gestation might be a window of opportunity to modify the risk of fetal CHB.

Table 1. Comparison of clinical features of patients with and without fetal CHB.

Clinical features	With CHB (N = 16)	Without CHB (N = 619)	*p* value*
Age at delivery, mean (SD)	30.63 (4.08)	31.88 (4.35)	0.972
History of conception	6 (37.5%)	238 (38.8%)	1.000
Neonatal lupus erythematosus	1 (7.1%)	21 (3.6%)	1.000
Rheumatologic symptoms	14 (87.5%)	463 (77.8%)	0.537
Sicca symptom	4 (25.0%)	157 (25.4%)	1.000
Dry eye	4 (25%)	126 (20.4%)	0.888
Dry mouth	4 (25%)	125 (20.2%)	0.875
Erythema	6 (37.5%)	174 (28.1%)	0.588
Purpura	1 (6.3%)	16 (2.6%)	0.911
Raynaud's phenomenon	1 (6.3%)	84 (13.6%)	0.633
Fever	6 (37.5%)	127 (20.5%)	0.181
Arthralgia	5 (31.3%)	205 (33.1%)	1.000
Meningitis	0 (0%)	6 (1.0%)	1.000
Interstitial nephritis	0 (0%)	13 (2.1%)	1.000
Interstitial pneumonia	0 (0%)	13 (2.1%)	1.000
Pulmonary hypertension	0 (0%)	1 (0.2%)	1.000
Thrombosis	0 (0%)	5 (0.8%)	1.000
Clinical diagnosis of connective tissue disease	14 (87.5%)	508 (82.1%)	0.818
Sjögren's syndrome	7 (43.8%)	238 (38.4%)	0.865
Systemic lupus erythematosus	5 (31.3%)	259 (41.8%)	0.554
Mixed connective tissue disease	1 (6.3%)	37 (6.0%)	1.000
Rheumatoid arthritis	1 (6.3%)	23 (3.7%)	1.000
Anti-phospholipid antibody syndrome	0 (0%)	28 (4.5%)	0.800
High titer of anti-SS-A antibodies	6 (37.5%)	108 (17.9%)	0.094
Corticosteroids therapy before conception	13 (81.3%)	333 (54.2%)	0.059
Use of immunosuppressants before conception	1 (6.3%)	64 (10.4%)	0.900
Use of corticosteroids after conception	12 (75.0%)	369 (59.6%)	0.326
Use of immunosuppressants after conception	0 (0%)	11 (2.5%)	1.000
Use of anti-platelet drugs and/or anti-coagulants after conception	2 (15.4%)	162 (33.3%)	0.289
Plasma exchange after conception	1 (7.1%)	11 (2.4%)	0.815
Use of intravenous immunoglobulins after conception	0 (0%)	0 (0%)	1.000
Use of corticosteroids before and after conception	9 (56.3%)	318 (51.5%)	0.905
Use of corticosteroids (equivalent doses of PSL, at ≥10 mg/day) before and after conception	1 (11.1%)	157 (49.5%)	0.053
Use of corticosteroids after conception before 16-week gestation	10 (62.5%)	355 (57.5%)	0.888
Use of corticosteroids (equivalent doses of PSL, at ≥10 mg/day) after conception before 16-week gestation	2 (20.0%)	210 (60.2%)	0.026

CHB, congenital heart block; PSL, prednisolone.
*Student's *t*-test for continuous variables and Fisher's exact test for binary variables.

Table 2. Type of corticosteroids administered before and after conception.

Type of corticosteroids	With CHB (N = 16)	Without CHB (N = 619)
Before conception		
Total corticosteroids use	13 cases	333 cases
PSL	9	282
mPSL	0	8
Bet	0	2
Dex	0	0
Unknown	4	41
After conception		
Total corticosteroids use	12 cases	369 cases
PSL	5	328
PSL–Bet	2	10
PSL–Bet–Dex	1	0
PSL–Dex	2	2
PSL–Dex–Bet	0	1
mPSL	0	7
mPSL–PSL	0	1
Bet	1	15
Bet–PSL	0	2
Dex	1	1
Unknown	0	2

PSL, prednisolone; mPSL, methylprednisolone; Bet, betamethasone; Dex, dexamethasone.
Data are number of patients.

We identified seven groups according to the timing of corticosteroids use, as followings. Group A, only before conception (N = 27); Group B, continuous use before and after conception (N = 295); Group C, before conception and resumption after conception before 16-week gestation (N = 20); Group D, before conception and resumption after 16-week gestation (N = 4); Group E, only after conception before 16-week gestation (N=50); Group F, only after 16-week gestation (N = 12); and Group G, without use of corticosteroids (N = 227) (Figure 2). Among these seven groups, Groups B, C, and E (total 365 cases) took corticosteroids after conception before 16-week gestation. Of these, 80.8% (295/365 cases, Group B) had continuous use before and after conception, and many cases of Group B (95.6%, 282/295 cases) had diagnosis of CTD. Thus, in Group B, almost cases seemed to take corticosteroids for maternal rheumatologic manifestations. Similarly, in Group C, many cases had diagnosis of CTD (70%, 14/20 cases) and seemed to resume taking of corticosteroids for deterioration of maternal rheumatologic manifestations after conception. In Group C, only one case had developed a fetal CHB at 21-week gestation, in this fetal CHB case, corticosteroids might be resumed not for a fetal CHB but for maternal rheumatologic manifestations. On the other hand, in Group E, some cases did not have diagnosis of CTD (24%, 12/50 cases). We could speculate that corticosteroids might be added after conception before 16-week gestation for prevention of fetal CHB in some cases of Group E. Interestingly, only in Group E, there was no fetal CHB case (Figure 2).

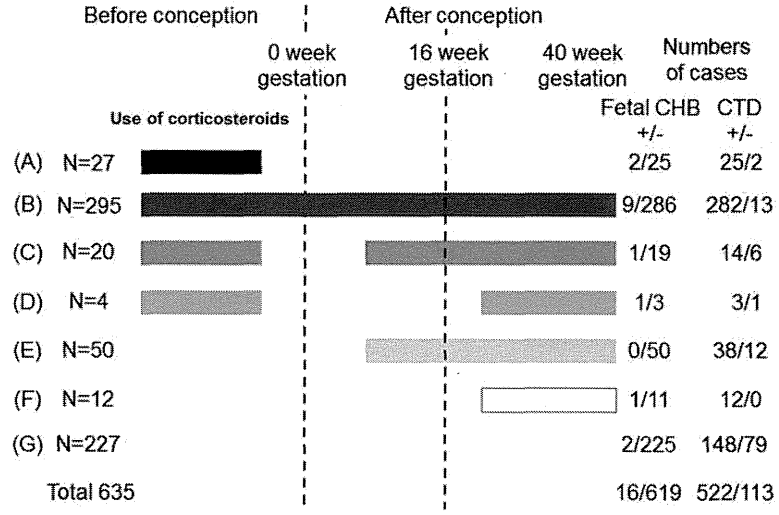

Figure 2. Association of the timing of corticosteroids use with onset of fetal CHB and diagnosis of maternal connective tissue disease. Group A, only before conception (N = 27); Group B, continuous use before and after conception (N = 295); Group C, before conception and resumption after conception before 16-week gestation (N = 20); Group D, before conception and resumption after 16-week gestation (N = 4); Group E, only after conception before 16-week gestation (N = 50); Group F, only after 16-week gestation (N = 12); and Group G, without use of corticosteroids (N = 227); corticosteroids therapy before conception, Groups A, B, C, and D (N = 346); use of corticosteroids after conception, Groups B, C, D, E, and F (N = 381); use of corticosteroids after conception before 16-week gestation, Group B, C, E (N = 365); CHB, congenital heart block; CTD, connective tissue disease.

Table 3. Results of univariate analysis of fetal CHB.

Parameters	Odds ratio for fetal CHB	95% CI	*p* value
Age at delivery	0.935	0.833, 1.050	0.256
History of conception	0.950	0.340, 2.654	0.923
Neonatal lupus erythematosus	1.943	0.243, 15.512	0.530
Rheumatologic symptoms	2.105	0.471, 9.407	0.329
Sicca symptom	0.981	0.311, 3.092	0.974
Dry eye	1.304	0.413, 4.121	0.650
Dry mouth	1.317	0.417, 4.163	0.638
Erythema	1.534	0.548, 4.295	0.414
Purpura	2.512	0.311, 20.278	0.387
Raynaud's phenomenon	0.425	0.055, 3.269	0.410
Fever	2.324	0.828, 6.528	0.109
Arthralgia	0.918	0.314, 2.682	0.875
Meningitis	-	-	-
Interstitial nephritis	-	-	-
Interstitial pneumonia	-	-	-
Pulmonary hypertension	-	-	-
Thrombosis	-	-	-
Clinical diagnosis of connective tissue disease	1.530	0.342, 6.844	0.578
Sjögren's syndrome	1.245	0.457, 3.394	0.668
Systemic lupus erythematosus	0.632	0.216, 1.844	0.400
Mixed connective tissue disease	1.049	0.134, 8.189	0.964
Rheumatoid arthritis	1.728	0.218, 13.699	0.604
Anti-phospholipid antibody syndrome	-	-	-
High titer of anti-SS-A antibodies	2.783	0.989, 7.837	0.053
Use of corticosteroids before conception	3.717	1.046, 13.206	0.042
Use of immunosuppressant before conception	0.571	0.074, 4.407	0.590
Use of corticosteroids after conception	2.033	0.647, 6.386	0.224
Use of immunosuppressants after conception	-	-	-
Use of anti-platelet drugs and/or anti-coagulants after conception	0.514	0.136, 1.944	0.326
Plasma exchange after conception	0.994	0.181, 5.474	0.995
Use of corticosteroids before and after conception	1.202	0.441, 3.273	0.719
Use of corticosteroids (equivalent doses of PSL, at ≥10 mg/day) before and after conception	0.268	0.036, 2.012	0.119
Use of corticosteroids after conception before 16-week gestation	1.223	0.438, 3.415	0.700
Use of corticosteroids (equivalent doses of PSL, at ≥10 mg/day) after conception before 16-week gestation	0.170	0.033, 0.878	0.034

CHB, congenital heart block; NLE, neonatal lupus erythematosus; PSL, prednisolone; CI, confidence interval.

Table 4. Results of multivariate analysis of predictive factors for fetal CHB.

Predictor variables	Odds ratio for fetal CHB	95% CI	*p* value
Analysis 1 (N = 635)			
Age at delivery	0.922	0.816, 1.042	0.194
History of conception	1.063	0.368, 3.071	0.910
Rheumatologic symptoms	1.238	0.240, 6.389	0.799
Clinical diagnosis of connective tissue disease	0.626	0.117, 3.348	0.583
High titer of anti-SS-A antibodies	3.581	1.214, 10.561	0.021
Use of corticosteroids before conception	4.284	1.097, 16.730	0.036
Analysis 2 (N = 635)			
Age at delivery	0.922	0.816, 1.041	0.191
History of conception	1.068	0.373, 3.059	0.902
Rheumatologic symptoms	1.746	0.349, 8.737	0.497
Clinical diagnosis of connective tissue disease	0.976	0.184, 5.180	0.977
High titer of anti-SS-A antibodies	2.950	1.022, 8.516	0.045
Use of corticosteroids before and after conception	1.038	0.360, 2.999	0.944
Analysis 3 (N = 635)			
Age at delivery	0.923	0.814, 1.047	0.212
History of conception	1.105	0.373, 3.276	0.857
Rheumatologic symptoms	1.430	0.272, 7.501	0.672
Clinical diagnosis of connective tissue disease	0.835	0.153, 4.553	0.835
High titer of anti-SS-A antibodies	3.591	1.193, 10.806	0.023
Use of corticosteroids (equivalent doses of PSL, at ≥10 mg/day) after conception before 16-week gestation	0.156	0.028, 0.859	0.033

CHB, congenital heart block; PSL, prednisolone; CI, confidence interval.

Relationship between fetal CHB and various clinical factors

Fetal CHB did not associate with age at delivery, history of conceptions, presence of rheumatologic symptoms, and clinical diagnosis (Table 3). On the other hand, the development of fetal CHB associated significantly with corticosteroid use before conception (OR 3.72, $p = 0.04$), whereas corticosteroids use (equivalent doses of PSL, at ≥10 mg/day) after conception before 16-week gestation was associated with significantly lower risk of fetal CHB (OR 0.17, $p = 0.03$). Further univariate analysis showed that fetal CHB tended to associate with high titer of anti-SS-A antibodies (OR 2.78, $p = 0.053$), albeit with border statistical significance (Table 3).

Multivariate analysis of predictive factors for fetal CHB

We considered three multivariate models in this analysis. First, the variables (1) age at delivery, (2) history of conception, (3) presence of rheumatologic symptoms, (4) clinical diagnosis of CTD, (5) high titer of anti-SS-A antibodies, and (6) corticosteroid use before conception were entered into multivariate analysis. The results identified high titer of anti-SS-A antibodies (OR 3.58, $p = 0.02$) and corticosteroid use before conception (OR 4.28, $p = 0.04$) as independent and significant risk factors for fetal CHB (Table 4, Analysis 1).

Next, variables 1–5 above and the use of corticosteroids before and after conception were entered into model 2 of multivariate analysis. The results identified high titer of anti-SS-A antibodies (OR 2.95, $p = 0.045$) as the only independent and significant risk factor for fetal CHB, but not the use of corticosteroids before and after conception (OR 1.04, $p = 0.94$) (Table 4, Analysis 2).

The last model (model 3) included variables 1–5 above and corticosteroids use (equivalent doses of PSL, at ≥10 mg/day) after conception before 16-week gestation. The results identified high titer of anti-SS-A antibodies as a significant and independent risk factor for fetal CHB (OR 3.59, $p = 0.02$), while corticosteroids use (equivalent doses of PSL, at ≥10 mg/day) after conception before 16-week gestation was an independent protective factor against the development of fetal CHB (OR 0.16, $p = 0.03$) (Table 4, Analysis 3).

Discussion

Our retrospective study of pregnant women with anti-SS-A antibodies reported three clinically important findings. First, univariate and multivariate analyses showed no significant association between the development of fetal CHB and various maternal parameters, such as age at delivery, history of conceptions, the presence of rheumatologic symptoms, and clinical diagnosis, in pregnant mothers who tested positive for anti-SS-A antibodies before conception and had no history of fetal CHB. These findings are somewhat in agreement with those of previous reports [4,6,12,13]. At the present time, only past history of fetal CHB seems to be a definite maternal risk factor for fetal CHB [4,6,7,10], while other maternal clinical features, such as high age [7,10], history of fetal cutaneous NLE [5], and summer season of child birth [7] are possible risk factors for fetal CHB. Considered together, the above results suggest that one cannot predict the development of fetal CHB based on maternal clinical features other than past history of fetal CHB in pregnant women positive for anti-SS-A antibodies.

Second, our multivariate analyses identified high titer of anti-SS-A antibodies (titer ≥1:32 by DID or equivalent by ELISA) as a significant and independent risk factor for fetal CHB. Although this finding was in agreement with previous report by Anami et al. [10], another study showed that fetal CHB did not associate with high levels of maternal anti-SS-A antibodies but with that of anti-SS-B antibodies [8]. These discrepancies could reflect differences in patient population and methods used for detection of antibodies. Moreover, anti-SS-B antibodies are usually detected together with anti-SS-A antibodies. Thus, anti-SS-A and SS-B antibodies might confound with each other. Standardization for antibodies assays and large cohort study are needed to determine the role of anti-SS-A/B antibodies in CHB.

Third, multivariate analyses identified corticosteroid before conception as a significant independent risk factor for fetal CHB, and that the use of corticosteroids (equivalent doses of PSL, at ≥10 mg/day) after conceptions before 16-week gestation was a significant independent protective factor against the development of fetal CHB, though the use of corticosteroid before and after conception had no effect on the development of fetal CHB.

The benefits of corticosteroids in preventing fetal CHB remain controversial [9,14]. Two separate retrospective studies reported possible corticosteroid protective effects during pregnancy against fetal CHB [8,10], while another study found that the use of corticosteroids did not affect recurrence of fetal CHB in mothers with past history of fetal CHB [4]. Moreover, the PR Interval and Dexamethasone Evaluation (PRIDE) study, a multicenter, open-label, nonrandomized study, found irreversibility of third-degree heart block and progression of second- to third-degree block despite treatment with dexamethasone [15]. These conflicting results could reflect differences between patients, the use of different types of corticosteroids and doses, and timing or duration of administration. In our study, we focused on the relationship of dose and timing of corticosteroid therapy with the incidence of fetal CHB. Based on our findings, we propose that anti-SS-A antibodies positive women with stable condition of CTD under the corticosteroids therapy before conception who continue the same therapy after conception are not at higher risk of fetal CHB, and that tapering or discontinuation of corticosteroids after conception in these women could increase the risk of fetal CHB. On the other hand, for women who were not treated with corticosteroids before conception, commencement of corticosteroids therapy (equivalent doses of PSL, at $\geq$10 mg/day) before 16-week gestation could reduce the risk of fetal CHB.

Although these three observations seem important clinically, our study has some limitations. First, the assays used to determine the titer of anti-SS-A antibodies were not standardized. Second, anti-SS-A antibodies were not investigated routinely in prenatal check-up, therefore many asymptomatic women with anti-SS-A antibodies could have been missed in this study. Third, this survey could have oversampled fetal CHB cases than non-fetal CHB cases, since anti-SS-A antibodies were more likely to be measured in women with a CHB child. For this reason, we excluded women confirmed to be positive for anti-SS-A antibodies after conception to reduce the possibility of selection bias.

In this study, we analyzed the maternal factors associated with the development of fetal CHB. The same data could be useful for analysis of the features of fetuses with CHB. Further studies using these data are planned in the near future.

In conclusion, the present study identified high titer of anti-SS-A antibodies as an independent risk factor for fetal CHB, and administration of corticosteroids (equivalent doses of PSL, at $\geq$10 mg/day) after conception before 16-week gestation to protect against the development of fetal CHB.

Acknowledgments

The authors thank Ms. Takagai for the excellent technical assistance, and Dr. F. G. Issa for editing the manuscript. We also thank Drs. Hironobu Hyodo, Tomonori Ishii, Mika Okuda, Kaneshige Sasaki, Yurika Mukai, Toshiyuki Sadou, Hiroaki Dobashi, Akio Morinobu, and Susumu Nishiyama for the kind cooperation in conducting the survey and the supply of clinical data from each of the participating institutions. We would also like to thank the many people and institutions for their valuable assistance in providing case records.

Conflict of interest

This work was supported in part by a grant from the Japan Ministry of Health, Labor and Welfare (MHLW) (#H22-Jisedai-Ippan-007).

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Journal of Arrhythmia ■ (■■■■) ■■■–■■■

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Journal of Arrhythmia

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Review

Molecular pathogenesis of long QT syndrome type 1

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ARTICLE INFO

Article history:
Received 12 August 2015
Received in revised form
16 November 2015
Accepted 17 December 2015

Keywords:
LQT1
KCNQ1
Genotype-phenotype correlations
Adrenergic regulation

ABSTRACT

Long QT syndrome type 1 (LQT1) is a subtype of a congenital cardiac syndrome caused by mutation in the *KCNQ1* gene, which encodes the α-subunit of the slow component of delayed rectifier K^+ current (I_{Ks}) channel. Arrhythmias in LQT1 are characterized by prolongation of the QT interval on ECG, as well as the occurrence of life-threatening cardiac events, frequently triggered by adrenergic stimuli (e.g., physical or emotional stress). During the past two decades, much advancement has been made in understanding the molecular pathogenesis underlying LQT1. Uncovering the genotype-phenotype correlations in LQT1 is of clinical importance to better understand the gene-specific differences that may influence the propensity for developing life-threatening arrhythmias under specific conditions. Elucidation of these mechanisms will also help to improve the diagnosis and management of this cardiac disorder based on gene-specific considerations. This review describes the current medical consensus and recent developments regarding the molecular pathogenesis of LQT1 and provides a novel insight into the adrenergic regulation of this disease.

Contents

1. Introduction

Long QT syndrome (LQTS) is a potentially life-threatening arrhythmia characterized by delayed myocardial repolarization that produces QT prolongation on ECG, and an increased risk of torsades de pointes (TdP)-triggered cardiac events, such as syncope, cardiac arrest, and sudden cardiac death (SCD) [1,2]. This syndrome, with an estimated incidence of 1/2000 and a mortality rate of 21% for symptomatic patients not receiving therapy within one year from the first syncope event [1,3], includes congenital and acquired (e.g., drug-induced) conditions.

Molecular genetic studies have revealed that congenital LQTS is linked to mutations in genes encoding subunits of cardiac ion channels or adapter proteins that modify the channel functions.

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http://dx.doi.org/10.1016/j.joa.2015.12.006
1880-4276/

Please cite this article as: Wu J, et al. Molecular pathogenesis of long QT syndrome type 1. J Arrhythmia (2016), http://dx.doi.org/10.1016/j.joa.2015.12.006i

There are two types of inherited syndromes: autosomal dominant Romano-Ward syndrome [4] and autosomal recessive Jervell and Lange-Nielsen syndrome [5,6] that is usually associated with deafness [7]. In 1991, the Keating group reported for the first time that a single genetic locus on chromosome 11p15.5 was associated with LQTS within a single family [8]. Based on subsequent pioneering work [9–11], at least 15 types of genes have been found to be linked to 15 different types of LQTS (LQT1-15) to date.

In 1996, Wang et al. confirmed that type 1 congenital LQTS (LQT1) is caused by mutations in the *KCNQ1* (*KvLQT1*) gene, which is highly expressed in the heart and encodes a protein with structural features of a voltage-gated potassium channel [11]. Of the fifteen LQTS types, LQT1 is the most common and present in approximately 40–50% of all genotyped patients [12,13]. The *KCNQ1* gene encodes the α-subunit of the slow component of delayed rectifier K^+ current (I_{Ks}) channel (Kv7.1). This protein, together with the β-subunit KCNE1 and an adapter protein Yotiao, forms a macromolecular complex (i.e., the functional potassium ion channel I_{Ks}) [14,15]. The channel carries the major outward repolarizing K^+ current during the plateau phase of cardiac action potentials (APs) and plays a critical role in maintaining repolarization reserve in the heart [16,17]. Mutations in *KCNQ1* can cause dysfunction in the I_{Ks} channel, such as a delay in channel opening or a reduction in the duration for which it is open [8,16,18,19]. This results in a decrease in repolarizing K^+ current or a loss-of-function during phase 3 of the cardiac AP, which eventually causes QT prolongation and serious arrhythmias.

LQT1 can have numerous clinical manifestations, ranging from no symptoms to sudden cardiac death, which reflects the heterogeneity in channel dysfunction. Mutation type, location, and even a patient's ethnic background, age, and gender are critical factors that affect the pathophysiology of the disease [1]. A variety of studies have shown that LQT1 is more frequently triggered by adrenergic stimuli (e.g., physical exertion or emotional stress) compared with other forms of LQTS, particularly by diving and swimming [20–22]. Under normal physiological conditions, sympathetic activation promotes I_{Ks}, which shortens ventricular repolarization against the activation of L-type Ca^{2+} current and thereby protects against Ca^{2+}-related arrhythmogenicity [17]. When I_{Ks} is defective because of a *KCNQ1* mutation, the ventricular repolarization or QT interval fails to shorten appropriately, thus creating a highly arrhythmogenic condition [1].

2. Molecular basis of LQT1

The LQT1-related *KCNQ1* gene is 404 kb long and located on chromosome 11p15.5. This gene codes for a 75-kDa protein containing 676 amino acids [11,23] and is mainly expressed in the heart, kidneys, small intestine, pancreas, prostate, and other non-excitable epithelial tissues [24]. It belongs to the Kv7 subfamily of voltage-gated K^+ channels (Kv) and shares a tetrameric architecture with all Kv channels. Each subunit contains six membrane-spanning segments (S1–S6 involving amino acid residues 122–348) connected by alternating intra- and extra-cellular loops, as well as a pore loop (amino acid residues 300–320) located between segments S5 and S6, with a cytosolic amino terminus (NH_2 terminus, residues 1–121) and a long cytosolic carboxyl terminus (COOH terminus, residues 349 to 676) (Fig. 1) [19,25,26]. The four subunits form a symmetrical alignment for the channel molecule together with KCNE1 (protein containing 129 amino acids with a single transmembrane segment) and Yotiao proteins, and construct a specialized pathway that allows for the conduction of potassium ions through water-filled pores located in the center of the complex. S1-S4 segments of the potassium channel form a voltage-sensing domain (VSD).

The S4 helix of KCNQ1 consists of a peculiar sequence of positively charged amino acids forming a region that is involved in sensing the membrane voltage and controlling the open probability of the channel [27]. In the resting state of the channel, these positively charged side chains are expected to be closer to the intracellular side of the membrane. Upon depolarization, effective charge motion within the membrane electric field toward the extracellular side of the membrane is accomplished through a series of conformational changes in the VSDs that lead to opening of the channel [28]. The pore region is composed of two transmembrane segments (S5 and S6) joined together by a linker (including a pore loop) that contains the conserved amino acids of the selectivity filter (residues 312–317) and affects the channel current amplitude, selectivity among ions, and channel blockade [29,30]. KCNQ1 possesses a large COOH terminus that is important

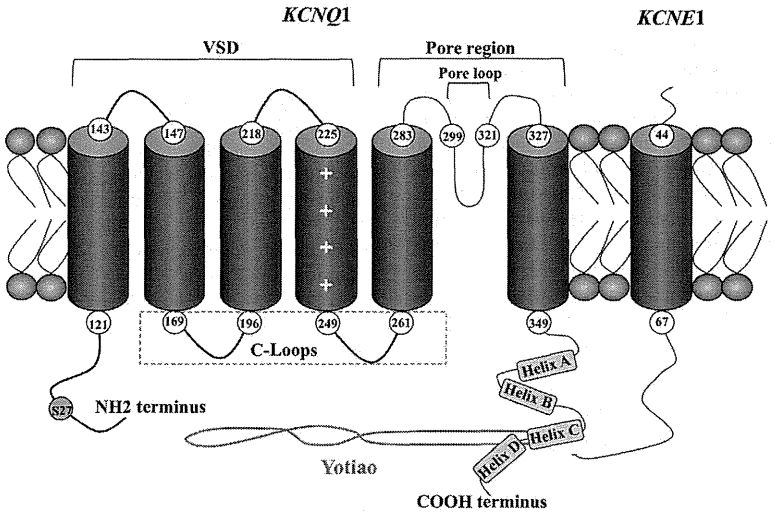

Fig. 1. Predicted topology of the I_{Ks} channel, which is formed by KCNQ1 and KCNE1 subunits and an adapter protein Yotiao. Each KCNQ1 subunit contains a NH_2 terminus, six membrane-spanning segments (S1–S6) including a pore loop and a voltage-sensing domain (VSD) (S1-S4), two cytoplasmic loops (S2–S3 and S4–S5), and a COOH terminus domain. The KCNE1 subunit contains a single α-helical transmembrane domain with an extracellular NH_2 terminus and a cytoplasmic COOH terminus domain.

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for channel gating, assembly, and trafficking [19,31]. The COOH terminus is comprised of four amphipathic α-helices, coiled-coils, and clusters of basic amino acids. A and B proximal helices form sites for calmodulin (CaM) binding, whereas the distal coiled-coil helix C and helix D are responsible for tetramerization [19,31]. Helix C interacts with the KCNE1 distal COOH terminus and is thought to be a crucial region for modulation by phosphatidylinositol-4,5-bisphosphate (PIP_2), which acts to stabilize the open state of the channel [32]. A domain near the COOH terminus (residues 589–620) of KCNQ1 is responsible for subunit assembly specificity, and deletion of a part of this domain leads to impaired assembly of the channel complexes, followed by mistrafficking [33]. In the COOH terminus tail, a leucine zipper motif (residues 588–616) has been identified as the unique site through which A-kinase anchoring protein 9 (AKAP9, or Yotiao) targets protein kinase A (PKA) and protein phosphatase 1 (PP1) to the KCNQ1 complex [15]. Although the NH_2 terminus is relatively short, it contains an important residue (S27) that is critical for mediating the phosphorylation of KCNQ1 [15].

To date, over 250 mutations in *KCNQ1* have been found to be linked to LQT1 [34] and new LQT1 causing mutations continue to be identified. The vast majority of *KCNQ1* mutations are single nucleotide substitutions (missense) or small insertion/deletions that localize to the S1-S6 transmembrane domains [5,18,35,36]. One study assessing 600 LQT1 patients found that approximately 66.2% of *KCNQ1* mutations (75.3% of mutation carriers) were identified in the membrane-spanning segments (approximately 1/3 in the pore loop or adjacent transmembrane regions), 31.2% (24.3% of mutation carriers) in the C terminus, and only 2.6% (0.4% of mutation carriers) in the N terminus [18]. Importantly, these data are consistent with the results from another clinical study [25]. Mutations in the transmembrane, linker, and pore region of *KCNQ1* are usually defined as high-probability disease-causing mutations that tend to cause severe cardiac events in patients at younger ages compared to mutations in the COOH terminal region [37–41].

3. Genotype-phenotype correlations

Existing evidence to date indicates that genetic background may influence the severity of the disease. The mutation type, specific location, and degree of dysfunction play a critical role in the clinical course of LQT1. Moss et al. reported that LQT1 patients with transmembrane mutations and dominant-negative ion current effects had a longer corrected QT (QTc) interval and a higher frequency of cardiac events than individuals with mutations in other regions or mutations resulting in haploinsufficiency, and these genetic risks were independent of traditional clinical risk factors and drug therapy [18]. More recently, a retrospective study assessing genotype-phenotype correlations in 110 infant mutation carriers from LQT1 families also reported that carriers of the dominant negative Y111C mutation presented with a tendency towards more severe heart rate reduction and postnatal QTc prolongation than carriers of the R518X nonsense mutation [42].

Shimuzu et al. studied 95 patients carrying 27 *KCNQ1* mutations (19 in transmembrane regions and eight in the COOH terminus) [39]. They found that patients with transmembrane mutations had longer QTc, higher T-wave alterations, and more frequent LQTS-related cardiac events (including syncope, cardiac arrest, or sudden cardiac death) than those with C-terminal mutations, though the frequency of TdP was not different between the two study groups. In addition, most of the first cardiac events occurred before the age of 15 years in the LQT1 patients (particularly in males) with transmembrane mutations, whereas only half of the LQT1 patients with C-terminal mutations suffered their first cardiac events before the age of 15.

Other retrospective data also indicate that missense cytoplasmic-loop mutations [43], pore mutations [36], and some specific point mutations, such as A341V, in *KCNQ1* [44,45] are associated with a longer QT interval and result in an increased risk of cardiac events and severe clinical phenotypes. In contrast to these studies, however, a study assessing 294 LQT1 patients with *KCNQ1* gene mutations demonstrated that there were no significant differences in clinical presentation, ECG parameters, and cardiac events among LQT1 patients by 40 years of age with *KCNQ1* mutations in different locations [46]. One possible explanation for this discrepancy is that the criteria for *KCNQ1* mutation type and position were different between their studies. LQT1 patients with transmembrane mutations (including those in the C-loop) were also found to be more sensitive to sympathetic stimulation and achieved a pronounced benefit from treatment with β-blockers compared to the patients with C-terminal mutations [43,47]. Therefore, the avoidance of strenuous exercise, in particular swimming, diving, or competitive sports, is recommended for LQT1 patients, especially younger males.

Silent mutations and compound mutations are also important genetic factors that affect the phenotype of LQT1. Approximately 25–36% of genetically positive patients with LQT1 may have a normal QTc range (defined as < 440 ms) without any clinical symptoms at rest [47,48]. Although these silent mutation-positive patients have a significantly lower risk of life-threatening cardiac events compared to those with phenotypic patterns, it should not be assumed that such a phenotype-negative individual who has a normal QTc is not affected by the cardiac disease. A number of risk assessments have confirmed that lethal arrhythmias can occur in these apparently healthy silent mutation carriers without any premonitory sign, especially during emotional stress or physical exertion [49–51]. There is also growing evidence that compound mutation carriers have a more severe cardiac phenotype compared with individuals carrying a single mutation [35,42,52–54]. Compound mutations were found to be associated with longer QTc, more frequent cardiac events, and earlier onset of cardiac events. Therefore, the management of patients with such mutations should be tailored to their increased risk for arrhythmias [55].

4. Genotype-I_{Ks} correlation

In 1996, Sanguinetti et al. and Barhanin et al. independently found that, when coassembled with the accessory subunit KCNE1, the KCNQ1 and KCNE1 complex could form a channel that very closely exhibited conductive and kinetic properties similar to that of cardiac I_{Ks} [14,56]. Kass and coworkers subsequently found that the targeting protein Yotiao, as a component of the macromolecular complex, is required to reconstitute cAMP-dependent regulation of I_{Ks} and provides a mechanistic link between the sympathetic nervous system and modulation of the cardiac action potential duration (APD) [15]. The KCNQ1 and KCNE1 subunits coassemble with Yotiao adapter into the cardiac I_{Ks}, and mutation in *KCNQ1*, *KCNE1*, or *AKAP9* (Yotiao) can cause functional reduction of I_{Ks} channels, leading to life-threatening cardiac arrhythmias corresponding to LQT1, LQT5, and LQT11, respectively.

Previous studies indicate that two distinct biophysical mechanisms mediate the reduced I_{Ks} current in patients with *KCNQ1* mutations: (1) coassembly or trafficking defects in which mutant subunits are not transported properly to the cell membrane and fail to incorporate into the tetrameric channel, with the net effect being a less than 50% reduction in channel function (haploinsufficiency); and (2) formation of defective channels involving mutant subunits with the altered channel protein

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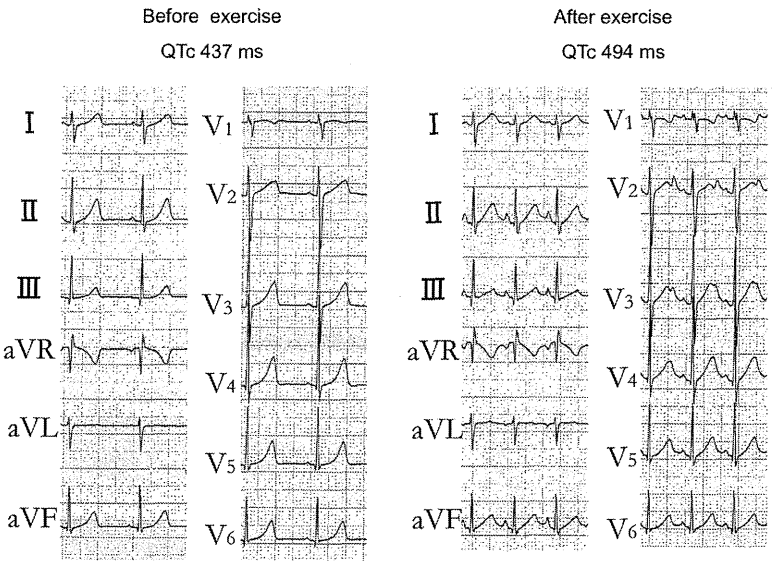

Fig. 2. Twelve-lead electrocardiograms (left: resting, and right: after exercise) in a 7-year-old boy carrying a *KCNQ1*-G269S mutation. QTc=corrected QT interval.

transported to the cell membrane, resulting in a dysfunctional channel having a greater than 50% reduction in channel current (dominant-negative effect) [18]. Recently, Mousavi et al. evaluated the functional properties of eight *KCNQ1* mutations that were identified in the S4 and S4-S5 linker (D242N, R243C, L250H), pore loop (G306V, D317N), and COOH terminus (L374fs+43X, N586D, L619M), respectively [7]. The results showed that D317N and L374fs+43X mutations exhibited a strong dominant-negative effect on KCNQ1-WT channel functions, which is consistent with previous findings for *KCNQ1* mutations located in the NH_2 terminus (Y111C), S2–S3 linker (R174C, A177P, Ala178fs/105, R190Q), S3-S4 (S225L), S4 domain (R243H), S5 domain (G269D, G269S, L272F), P-loop (Y281C, T311I, G314S, Y315S, Y315C, P320H, and P320A), S6 domain (ΔF339, L342F), and COOH terminus (R317N, R533W, R539W, R555H, K557E) [37,42,57–63]. The other mutations analyzed in that study were haploinsufficient for KCNQ1 channel function. Other reports have also indicated that membrane expression of the KCNQ1 channel protein can be reduced by trafficking defects in mutations located in the S2-S3 linker (A178T), S5 domain (ΔS276), pore loop (T322M), S6 domain (A336fs+16X), and COOH terminus (Y461X, R518X, A525T, Q530X, E543fs+107X, T587M, G589D, R594Q) [7,42,51,64–66].

The above data indicate that the correlation between the genotype and channel function in LQT1 is complicated and diversified. Even different mutations at the same position (e.g., *KCNQ1*-R243C and *KCNQ1*-R243H) cause different degrees of channel dysfunction. Moreover, not only do mutations with a dominant-negative effect occur in almost every location of the *KCNQ1* gene, but those with a trafficking defect exist in the main domains of the gene as well. However, the number of KCNQ1 pore-loop mutations causing a dominant-negative effect is much great than the number of mutations causing haploinsufficiency, suggesting that the pore-loop mutations are more commonly associated with severe electrophysiological and clinical phenotypes. Interestingly, Aizawa et al. found that the *KCNQ1* mutation Ala178fs/105 not only forms a hetero-multimer and causes a dominant-negative effect on the I_{Ks} channel, but that it also gives rise to a trafficking defect in the channel protein [63]. It is possible that both defective channel trafficking and defective channel formation mechanisms exist for some *KCNQ1* mutations simultaneously. Another correlation between genotype and channel function has been described whereby some compound *KCNQ1* mutations (e.g., T391I/Q530X, A525T/R518X, and A178T/K422fs39X) severely disrupt channel trafficking [67].

In addition to inducing I_{Ks} dysfunction through dominant-negative loss-of-function effects and defective channel trafficking, mutations in *KCNQ1* suppress I_{Ks} channel function by reducing the channel affinity of interacting proteins [68,69]. Phosphatidylinositol-4,5-bisphosphate (PIP_2) is a cofactor necessary for the activity of KCNQ1 channels [32,68,69]. It has been shown that intracellular PIP_2 regulates KCNQ1 channel activity in such a way that PIP_2 stabilizes the open state of the channels, which leads to an increased current amplitude, slowed deactivation kinetics, and a shift in the activation curve toward negative potentials. Park et al. showed that mutations in the S4 domain (R243H) and COOH terminus (R539W and R555C) increased the rate of dissociation of PIP_2 from the KCNQ1 channel, which decreased the number of open-state channels in the membrane [68]. Coyan et al. confirmed that R243H and R555C mutations cause an acceleration of KCNQ1 current rundown when membrane PIP_2 levels are decreasing. By observing the interaction of the *KCNQ1* R539W mutant with cholesterol, this group further suggested that the channel-cholesterol interaction might overcome the channel-PIP_2 interaction and stabilize the channel open-state [69].

5. Regulation by PKA

It is well known that cardiac events in LQT1 syndrome patients are more frequently triggered by adrenergic stimuli (e.g., physical or emotional stress) than those in other forms of LQTS. A clinical study of 371 LQT1 patients found that cardiac events were most common during exercise (62%) and emotional arousal (26%), while occasional during sleep or rest (3%) and from other triggers (9%) [20]. Approximately 35–36% of genotype-confirmed LQT1 patients have a normal QTc range without any clinical symptoms at rest [47,48], but lethal arrhythmias can occur in these apparently healthy silent mutation carriers without any premonitory sign, especially during adrenergic stimuli [49–51]. Recently, a heterozygous missense *KCNQ1* mutation G269S was identified in 11

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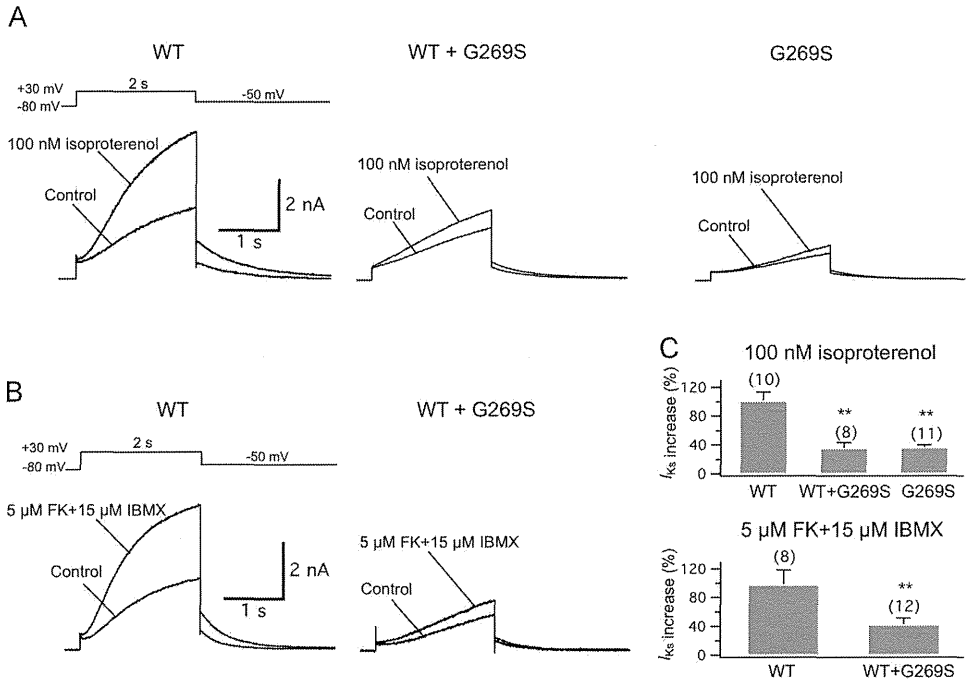

Fig. 3. I_{Ks} reconstituted with *KCNQ1*-G269S reduced responses to PKA stimulation. Superimposition of I_{Ks} traces recorded from human embryonic kidney 293 (HEK293) cells expressing Yotiao+KCNE1 with KCNQ1-WT, WT+G269S, and G269S before and after bath application of 100 nmol/L isoproterenol (A) or 5 mmol/L forskolin (FK)+15 mmol/L 3-*isobutyl*-L-*methyl*-*xanthine* (*IBMX*) (B). (C) The *percentage increase in tail* I_{Ks} *after bath application of 100 nmol/L isoproterenol (upper) and 5 mmol/L* FK+15 *mmol/L* IBMX (lower). $^{**}p < 0.01$ *w.r.t.* KCNQ1-WT. PKA=protein kinase A.

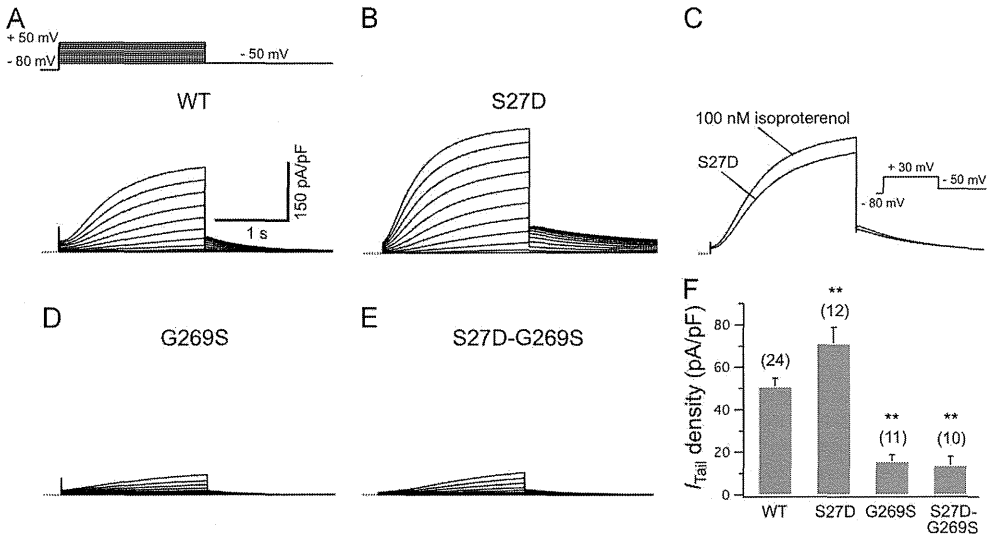

Fig. 4. G269S prevents the increase in I_{Ks} caused by the phosphomimetic S27D mutation. Representative current traces recorded from HEK 293 cells expressing Yotiao+KCNE1 with KCNQ1-WT (A), S27D (B), and exposure to 100 nmol/L isoproterenol (C), G269S (D), and S27D-G269S (E), respectively. (F) Bar graphs show effects of G269S on tail I_{Ks} densities recorded on repolarization to −50 mV following a 2-s depolarization to 30 mV for the different transfection conditions.$^{**}p < 0.01$ *w.r.t.* KCNQ1-WT.

patients from four unrelated families. Most of the 11 patients had normal to borderline QTc intervals at rest, but had a significant QTc prolongation after exercise (Fig. 2). One family member had died suddenly and another one experienced syncope while dancing. *Functional characterization of the* I_{Ks} *channel reconstituted with* G269S in mammalian cells showed that the mutation modestly affected I_{Ks}, but severely blunted the increase in I_{Ks} after treatment with isoproterenol, pharmacological activators of PKA (Fig. 3), or in the PKA phosphomimetic mutation KCNQ1-S27D (Fig. 4), which mimics PKA-mediated phosphorylation of I_{Ks} channels. These findings provide important insight into the molecular mechanisms underlying adrenergic-induced LQTS and may explain why

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patients with silent mutations exhibit an excessive prolongation of QT intervals during exercise. The results also suggest that beta-blocker therapy may have a beneficial effect in these patients.

In human ventricular myocytes, the I_{Ks} (outward current), rapid component of delayed rectifier K^+ current I_{Kr} (outward current), and L-type Ca^{2+} current $I_{Ca,L}$ (inward current) play a dominant role in the repolarization of APs and are the most important determinants of APD. Under physiological conditions, I_{Kr} and $I_{Ca,L}$, but not I_{Ks}, normally play a crucial role in controlling the ventricular AP at rest [70]. Therefore, *KCNQ1* mutations (e.g., G269S) that cause a mild-to-moderate functional defect in I_{Ks} might ordinarily have little effect on the ventricular AP, which may explain why some *KCNQ1* mutation carriers have normal to borderline QTc intervals with no or mild clinical symptoms at rest. In addition, the reason why individuals carrying a *KCNQ1* mutation display a silent phenotype at rest may also be due to the "repolarization reserve" mechanism [71].

On the other hand, I_{Ks} plays a major role in regulating the ventricular AP after adrenergic stimuli (that upregulates I_{Ks} through cAMP-dependent PKA pathway) to prevent excessive ventricular APD or QT prolongation due to an $I_{Ca,L}$ increase [15, 72]. It is possible that the slow deactivation kinetics of I_{Ks} also contribute to the current upregulation through adrenergic stimuli. Due to the incomplete deactivation of I_{Ks}, there is residual activation at the onset of the succeeding AP that accumulates at fast rates, thus increasing the probability of the channel being in an open state [73].

Any abnormality causing a loss-of-function in the I_{Ks} macromolecular complex may lead to adrenergic-induced imbalance in ventricular repolarization currents and consequent QTc prolongation, which is identified based on the defective response of I_{Ks} to PKA stimuli due to mutations in *KCNQ1* (-G269S, -A341V, and -K557E) [51,61,63], *KCNE1*-P127T [32], and Yotiao-S1570L [74]. Importantly, the role that I_{Ks} plays during adrenergic stimulation may explain why 88% of the cardiac events in LQT1 patients in the above study occurred during exercise and emotional stress [20].

6. Acquired LQT1

In addition to congenital pathology, LQTS can also be induced by a variety of stimuli, such as QT-prolonging medications, emotional stress, and strenuous exercise, especially under certain circumstances (risk factors). Of all triggers, QT-prolonging medications (e.g., antiarrhythmics, antihistamines, antibiotics, antidepressants, antipsychotics, and antiemetics) are the most common cause of acquired LQTS (aLQTS), which is believed to be related to drug-induced I_{Kr} channel block [71]. Due to unique pore structural properties (spacious inner cavity and aromatic drug-binding sites in the S6 domain facing the inner cavity), the I_{Kr} channel displays an unusual susceptibility to a wide range of structurally diverse compounds that interact with the pore.

Risk factors for aLQTS include electrolyte disturbances (e.g., hypokalemia, hypomagnesemia), bradycardia, gender, heart disease, and liver insufficiency. Moreover, genetic mutations in major LQTS-related genes including *KCNQ1* have also been shown to be involved in aLQTS [75–78]. Siebrands et al. reported that the *KCNQ1*-A344V mutation increased the susceptibility of I_{Ks} channel to a local anesthetic bupivacaine, while the mutation *per se* did not cause a severe clinical phenotype of LQT1 [77]. Veerman et al. reported that the *KCNQ1*-K422T mutation *per se* had a mild clinical phenotype of LQT1, but additional fluoxetine or norfluoxetine resulted in more prominent QTc prolongation in the mutation carriers [78]. Electrophysiological study demonstrated that both fluoxetine and norfluoxetine inhibited *KCNQ1/KCNE1* currents in HEK293 cells [78]. The above studies suggest that loss-of-function in I_{Ks} caused by *KCNQ1* mutation not only can predispose patients to congenital LQT1, but can be also associated with acquired LQT1. Normal cardiac repolarization critically depends on the interplay of multiple ion currents, and these provide some redundancy or "reserve", which protects against excessive QT prolongation and allows for an LQTS mutation to remain clinically silent or mild. The lesions in these repolarizing mechanisms can reduce "repolarization reserve" and therefore increase the risk for aLQTS [71]. The loss-of-function in I_{Ks}, which is a major repolarization current, occurs due to a *KCNQ1* mutation and decreases the repolarization reserve [16,17,71,77]. However, this may be insufficient to elicit a full-blown LQT1 phenotype, especially at rest. When a pathologic trigger such as an I_{Ks}-blocking and/or I_{Kr}-blocking medication is present, the superimposition of lesions will produce marked AP prolongation and lead to acquired LQT1. In fact, the adrenergic-induced latent LQT1 is a type of aLQTS, which is triggered by sympathetic stimuli.

7. Conclusions

Uncovering the molecular pathogenesis of LQT1 is helpful, and even mandatory, for precise diagnosis, risk stratification, and management of LQT1 patients. Although some progress has been achieved in investigating the genotype-phenotype correlation through protracted and unremitting efforts, our current understanding of the molecular pathogenesis remains incomplete and sometimes fails to allow for translating the genotype-phenotype correlation into clinical reality. Moreover, neither the localization of a *KCNQ*1 mutation nor its cellular electrophysiological effect is sufficient to predict the impact on clinical manifestations.

The reasons why individuals (even from the same family) carrying the same mutation (e.g., *KCNQ1*-A341V and *KCNQ1*-R231C) exhibit diverse cardiac phenotypes clinically remain unknown. The findings to date indicate that mechanisms underlying LQTS are not only multifactorial, but are also involved in pathway crosstalk. Some recent studies show that protein kinase C and the parasympathetic nervous system are also involved in the control of clinical phenotypes in LQT1 [79,80], which brings in a new view to uncovering pathogenic mechanisms underlying the inherited arrhythmia. In addition, the use of induced pluripotent stem cells may better elucidate the clinical heterogeneity in LQTS, especially in cases that have compound mutations.

Conflict of interest

All authors declare no conflict of interest related to this study.

Acknowledgments

This study was supported by the National Natural Science Foundation of China (Nos. 81273501 and 81470378).

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Please cite this article as: Wu J, et al. Molecular pathogenesis of long QT syndrome type 1. J Arrhythmia (2016), http://dx.doi.org/10.1016/j.joa.2015.12.006i

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Please cite this article as: Wu J, et al. Molecular pathogenesis of long QT syndrome type 1. J Arrhythmia (2016), http://dx.doi.org/10.1016/j.joa.2015.12.006i

CLINICAL GENETICS An International Journal of Genetics, Molecular and Personalized Medicine

Clin Genet 2015: 87: 279–283
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CLINICAL GENETICS
doi: 10.1111/cge.12357

Short Report

Mosaic *KCNJ2* mutation in Andersen–Tawil syndrome: targeted deep sequencing is useful for the detection of mosaicism

Hasegawa K., Ohno S., Kimura H., Itoh H., Makiyama T., Yoshida Y., Horie M. Mosaic *KCNJ2* mutation in Andersen–Tawil syndrome: targeted deep sequencing is useful for the detection of mosaicism.
Clin Genet 2015: 87: 279–283. © John Wiley & Sons A/S. Published by John Wiley & Sons Ltd, 2014

Andersen–Tawil syndrome (ATS) is an inherited disease characterized by ventricular arrhythmias, periodic paralysis, and dysmorphic features. It results from a heterozygous mutation of *KCNJ2*, but little is known about mosaicism in ATS. We performed genetic analysis of *KCNJ2* in 32 ATS probands and their family members and identified *KCNJ2* mutations in 25 probands, 20 families who underwent extensive genetic testing. These tests revealed that seven probands carried *de novo* mutations while 13 carried inherited mutations from their parents. We then specifically assessed a single proband and the respective family. The proband was a 9 year old girl who fulfilled the ATS triad and carried an insertion mutation (p.75_76insThr). We determined that the proband's mother carried a somatic mosaicism and that the proband's younger brother also carried the ATS phenotype with the same insertion mutation. The mother, who exhibited mosaicism, was asymptomatic, although she exhibited Q(T)U prolongation. Mutant allele frequency was 11% as per TA cloning and 17.3% as per targeted deep sequencing. Our observations suggest that targeted deep sequencing is useful for the detection of mosaicism and that the detection of mosaic mutations in parents of apparently sporadic ATS patients can help in the process of genetic counseling.

Conflict of interest

All authors declare no conflict of interest.

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Key words: Andersen–Tawil syndrome – *KCNJ2* – mosaicism – sequencing

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Received 25 December 2013, revised and accepted for publication 5 February 2014

Andersen–Tawil syndrome (ATS) is a rare, hereditary, multisystem disorder that presents with the classic triad of ventricular arrhythmias with Q(T)U prolongation on electrocardiograms (ECGs), periodic paralysis, and dysmorphic features (1, 2). It is inherited in an autosomal–dominant trait, but ATS occurs sporadically resulting from a heterozygous mutation of *KCNJ2*. The gene encodes the inward rectifying potassium channel Kir2.1, ubiquitously expressed in the myocardium, skeletal muscle, and brain (3). We have previously reported variable ATS phenotypes, including a substantial number of sporadic cases (4, 5).

Mosaicism is a biological phenomenon describing the development of an individual from a single fertilized egg with two or more assemblies of cells with distinct genotypes (6). Somatic mosaicism has been identified as the cause of cancer (7) and has been found in patients with inheritable diseases (8). In cardiac diseases, mosaicism was identified to cause disorders such as hypertrophic cardiomyopathy (HCM) (9), long QT syndrome (LQTS) (10, 11), and catecholaminergic polymorphic ventricular tachycardia (CPVT) (12). The importance of genetic counseling in families with mosaicism has been argued upon; in these previous

reports, mosaicism was detected by direct sequencing of genomic DNA obtained from lymphocytes, hair, buccal epithelium, or skin fibroblasts. It is, however, difficult to detect the low-grade mosaicism by conventional Sanger sequencing methods.

Here we provide molecular evidence for the presence of mosaicism in ATS. Furthermore, we validated the frequency of mutant alleles using the TA cloning method followed by direct sequencing as well as the deep sequencing method using a bench-top next-generation sequencer. The detection rates of mutant alleles were then compared.

Materials and methods

Study subjects

The study was approved by our Institutional Ethics Committees, and all patients provided informed consent. Thirty-two consecutive ATS probands with at least two features of ATS were included in the study.

Mutational analysis using Sanger sequencing

Genomic DNA was isolated from the peripheral blood lymphocytes of the patients as well as family members. We performed direct sequencing analysis of genomic DNA from the proband for the entire open reading frames of *KCNJ2* (NM_000891.2) using an ABI PRISM 3130 DNA sequencer (Applied Biosystems, Wellesley, MA). In addition to this gene, we examined *KCNQ1*, *KCNH2*, *KCNE1-3* and *KCNE5*, and *SCN5A* to exclude the presence of compound mutations related to LQTS. When a mutation was detected, we examined its presence in >400 Japanese control alleles to exclude the possibility of polymorphisms. The probands' family members were then screened for the mutation identified in the probands. When a genetic mosaicism in somatic cells was suspected because of abnormal electropherograms, we validated the electropherograms using two other primer pair sets. To estimate the frequency of mutant alleles, we amplified the target region in the genomic DNA and subcloned into a TA vector. We transformed the TA vector to DH5α-competent cells, picked up 100 colonies in each carrier, and confirmed the sequence of the target region in each colony using the Sanger's method.

Mutational analysis using next generation sequencing

The probes for the target region were constructed by DESIGN STUDIO software (Illumina, San Diego, CA), and the samples were prepared using a TruSeq Custom Amplicon Kit (Illumina). We loaded them to MISEQ (Illumina), analyzed by the MISEQ REPORTER software attached to the MISEQ, and analyzed the data by AMPLICON VIEWER software (Illumina) to evaluate the mutation frequency.

Results

Clinical features

We identified *KCNJ2* mutations in 25 of 32 (78%) ATS probands with ≥2 clinical features. We were able to conduct genetic surveys of the families of 20 of these probands (80%). We found that seven probands (35%) carried *de novo* mutations while 13 (65%) inherited them from one of their parents.

In a family with an insertion mutation, we suspected mosaicism in the mother of the proband (Fig. 1a). The proband was a 9 year old girl with dysmorphic features, including an inward bending of the fifth finger, a single transverse palmar crease on both sides, and a broad nose. She had complained of palpitations at a school health check-up and was found to have multifocal premature ventricular contractions (PVCs). Her 12-lead ECG showed prominent U waves in leads V2–V4 (Fig. 1b, arrows; QT/QTc/QUc, 350/373/693 ms). Ventricular tachycardia with bidirectional PVCs (Fig. 1c) at rest was detected on Holter ECG (Fukuda Denshi, Japan). Therefore, she was treated with oral flecainide (100 mg/day), following which her PVCs were largely suppressed. She also reported a history of repetitive generalized muscle weakness since the age of 2 years.

None of her relatives had a history of periodic paralysis, syncope, or sudden cardiac death. However, her 38 year old mother (Fig. 1a, I-2) also exhibited QT prolongation with U waves (Fig. 1d; QT/QTc/QUc, 400/478/693 ms). The younger brother of the proband (Fig. 1a, II-3) also exhibited frequent premature beats on late-term fetal ultrasound scans, and he was delivered by Cesarean section at a gestational age of 36 weeks. On the first day after birth, his ECG showed frequent PVCs (Fig. 1e), and he exhibited a single transverse palmar crease on both sides.

Mutational analysis using Sanger sequencing

Sanger sequencing of the proband showed a novel heterozygous duplication of *KCNJ2* (c.222_224dupCAC), causing the insertion of threonine (p.T75_C76insThr; Fig. 2b) in a highly conserved amino acid position. The mutation was absent in 400 Japanese control alleles and has not been reported according to the National Heart, Lung, and Blood Institute (NHLBI) Exome Sequencing Project (ESP), Exome Variant Server (http://evs.gs.washington.edu/EVS/). We found no mutations in the other candidate genes assessed. The mutation was also detected in her younger brother, but not in her father and sister (Figs 1a and 2). The sequence electropherogram of her mother suggested a mosaicism because signals with small amplitudes starting from nucleotide 225 were clearly detectable (Fig. 2b); these were absent in the control sample and asymptomatic father and elder sister (data not shown). Furthermore, we validated the sequence electropherogram by using two other independent primer pair sets to rule out an asymmetric PCR allele. One pair (Primer set C) was both outside of the previous primer (Fig. 2a,c), the other (Primer set D) was both inside of the previous primer

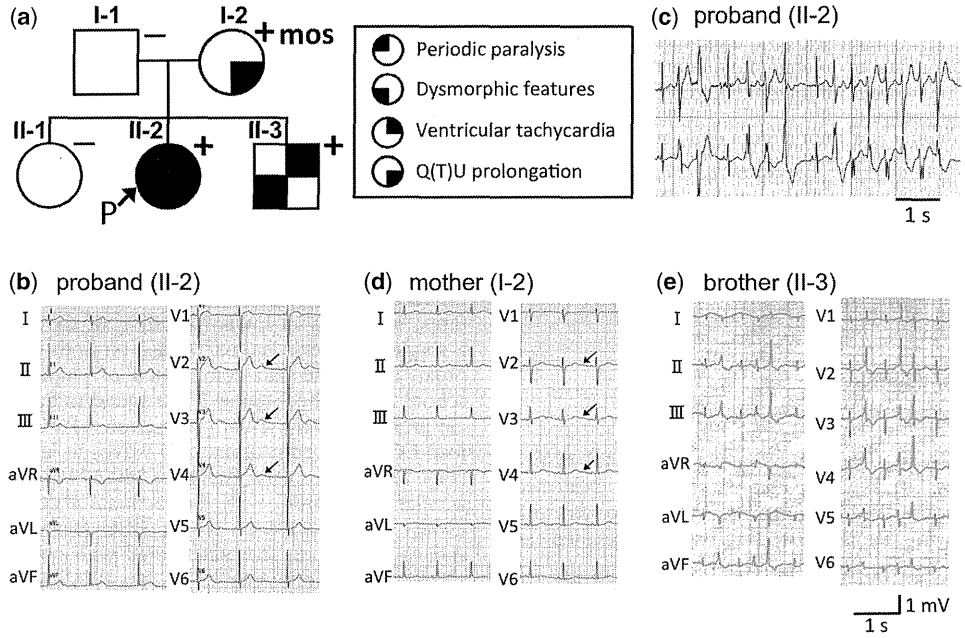

Fig. 1. Clinical characteristics. **(a)** Family pedigree. Males and females are represented as squares and circles, respectively. An arrow indicates the proband. The +/− symbols indicate the presence/absence of KCNJ2 p.75_76insThr. The filled symbols indicate phenotypes. **(b)** A 12-lead ECG of the proband. The arrows indicate the U waves. **(c)** A Holter electrocardiogram (ECG) of the proband shows frequent premature ventricular contractions (PVCs) at rest. **(d)** A 12-lead ECG of the mother. The arrows indicate the U waves. **(e)** A 12-lead ECG of the brother shows frequent PVCs.

(Fig. 2a,d). These results confirmed that the mother carried a mutant allele in low frequency.

On TA cloning and direct sequencing, we detected 11 mutant alleles (11%) from the mother, 52 mutant alleles (52%) from the proband, and 48 mutant alleles (48%) from the brother (Fig. 3).

Mutational analysis using next generation sequencing

We validated the percentage of mutant alleles using targeted deep sequencing methods. The percentage of mutant alleles was 17.3% in the mother and 46.2% in the proband, with 249 and 476 coverages, respectively.

Discussion

In this study, we describe a novel *KCNJ2* duplication mutation in an ATS family. The proband had repetitive periodic paralysis since the age of 2 and ventricular arrhythmias since 9. Her newborn brother was suspected to be ATS because of premature contracts during late-term fetal stage. He showed frequent PVCs on the first day after birth. Asymptomatic mother showed Q(T)U prolongation on her ECG, although she had no dysmorphic feature. In the genetic analysis, the mother was suspected to be a carrier of the same mutation as the proband, but in mosaicism. This was suspected because of the presence of small peaks on the mother's sequence electropherogram. We then confirmed mosaicism and the frequency of mutant alleles using both TA cloning and targeted deep sequencing methods. These independent methods yielded similar mutant allele frequencies.

The frequency of the mother's mutant allele in genomic DNA from lymphocytes is less than 20%. Therefore the reduction in normal Kir 2.1 channel function is assumed to be insufficient to cause ATS phenotype. However, the mosaicism in somatic cell lines can be affected depending on the germ layers and organs (6), and we could not confirm the allele frequency in cardiomyocytes, because it is ethically impossible to conduct endomyocardial biopsy for asymptomatic mother. From the Q(T)U prolongation in mother's ECG, we hypothesized that the mutant allele frequency would be higher in her cardiomyocytes than that in lymphocytes.

Among the inherited heart diseases, mosaicism was first noted in association with familial HCM in 2000 (9), in a female with germline mosaicism. Germline transmission from an asymptomatic mother to an infant with a severe LQTS phenotype has also been reported, where the mother was identified with mosaicism of *SCN5A* (10). In a report on Timothy syndrome, a recognized severe subtype of LQTS, a parent with mosaicism was shown to have less severe manifestations (11). More recently, Roux-Buisson and colleagues (12) identified a mosaicism in the mother of a CPVT proband carrying an *RYR2* mutation. Although mosaicism resulted in no symptoms in those parents, their children developed lethal arrhythmias