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H．知的財産権の出願・登録状況

1．特許取得
なし
2．実用新案登録
なし
3．その他
なし

厚生労働科学研究費補助金（難治性疾患克服研究事業）
分担研究報告書

カテコラミン誘発性多形性心室頻拍における疾患特異的ヒトiPS細胞を用いた解析

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研究要旨：カテコラミン誘発性多形性心室頻拍（CPVT）は、運動や情動などのカテコラミン刺激によって、心室頻拍・細動による突然死を引き起こす遺伝性不整脈疾患である。原因遺伝子として約50-60%に筋小胞体からのCa放出に関わるリアノジン受容体（RyR2）遺伝子異常が検出される。今回、RyR2遺伝子異常が同定されているCPVT患者より、ヒト誘導多能性幹（iPS）細胞を作製し分化心筋の解析を行った。分化開始後3ヵ月における解析では、コントロールに比べて、RyR2遺伝子発現の減少、IP3R2発現の増大を認めた。Ca transient解析では、頻拍ペーシング負荷で不整脈誘発性の差はなく、現在解析中である。

A．研究目的

カテコラミン誘発性多形性心室頻拍（CPVT）は、運動や情動などのカテコラミン刺激によって、心室頻拍・細動による突然死を引き起こす遺伝性不整脈疾患である。原因遺伝子として約50-60%に筋小胞体からのCa放出に関わるリアノジン受容体（RyR 2）遺伝子異常が検出される。今回、CPVTの病態解明を目的とし、患者よりヒト人工多能性幹（induced pluripotent stem: iPS）細胞を作製し分化心筋の解析を行った。

B．研究方法

運動時の失神既往、二方向性心室頻拍を認めるRyR2遺伝子異常（p.I4587V）が検出されているCPVT患者において、皮膚を採取し、皮膚線維芽細胞を樹立した。ヒトiPS細胞の作製は、高橋、山中らの方法（Cell 131：861-872）を用い、レトロウイルスにて以下の4遺伝子（OCT3/4、SOX2、KLF4、c-MYC）を導入し、iPS細胞を得た。心筋分化は胚様体形成法（Yang et al. Nature 2008）にて行った。

（倫理面への配慮）

本研究は、京都大学医の倫理委員会にて承認済みである。

C．研究結果

CPVT患者から皮膚生検を行い、線維芽細胞を樹立した。続いて、レンチウイルスにてマウスSlc7a1遺伝子を導入し、その後、レトロウイルスにて山中4因子（OCT3/4、SOX2、KLF4、c-MYC遺伝子）を導入した。約3週間にてiPS細胞colonyが出現し、複数のcolonyを樹立した。胚様体形成法による心筋分化を行い、約8日ごろより自己拍動する胚様体が観察された。心筋分化開始後、1、3カ月の自己拍動する胚様体よりRNAを抽出し、Ca動態に関わる遺伝子発現の解析を行った。1→3カ月において、コントロール、患者由来分化心筋ともCASQ2（筋小胞体内のCa結合蛋白）の遺伝子発現増加を認めた。3ヵ月における解析では、コントロールに比べて、患者由来分化心筋におけるRyR2遺伝子発現の低下（コントロール 1.36±0.41 vs. CPVT 0.87±0.18 $p<0.05$, cTnT補正）、IP3R2（筋小胞体からのCa放出）発現の増大（1.03±0.99 vs. 4.06±2.95 $p=0.06$）を認めた。続いて、胚様体を酵素処理にてsingle cellにし、接着培養後、Fluo-8を用いたCa transient解析を行った。頻拍ペーシング負荷でtriggered activityは、有意差ない結果であった（コントロール83%（n＝35）v.s. CPVT 68%（n＝37），24℃，pacing rate 85/分）。

D．考　察

本研究にて、RyR2遺伝子異常を持つCPVT患者から疾患特異的ヒトiPS細胞を作製した。幼若な心筋では、筋小胞体のCa放出にはRyR2よりIP3R2がより大きく関与していることが知られており、Ca transient解析で不整脈誘発性に差が出にくい一因と考えられた。また、患者由来分化心筋におけるRyR2遺伝子発現の低下、IP3R2の増加もさらにphenotypeが出にくい要因と考えられる。カテコ

ラミン負荷や長期培養後の心筋による実験にて病態を再現できるか解析を進めている。

E．結　論

CPVTは、カテコラミン刺激により、心室頻拍・細動による突然死を引き起こす難治性疾患であり、疾患特異的ヒトiPS細胞を用いた解析が病態解明や薬効評価に役立つと期待される。

F．健康危険情報

なし。

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H．知的財産権の出願・登録状況（予定を含む）

1．特許取得

なし

2．実用新案登録

なし

3．その他

なし

Ⅲ．研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表

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Impact of out-stent plaque volume on in-stent intimal hyperplasia: Results from serial volumetric analysis with high-gain intravascular ultrasound ☆

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ABSTRACT

Background: Changes in out-stent plaque volume can be related to in-stent intimal hyperplasia. However, few data exist regarding the impact of out-stent plaque volume on in-stent intimal hyperplasia.
Methods: We prospectively performed volumetric intravascular ultrasound in 46 stable coronary patients (34 males, mean age of 66 years) immediately as well as 18 months after stenting. From the high-gain ultrasound images, out-stent plaque volume was calculated by extracting the stent volume from the external elastic membrane volume. Volumes of in-stent intimal hyperplasia and reference plaque were also evaluated.
Results: Out-stent plaque volume increased from 177.3 ± 100.8 mm^3 to 190.7 ± 111.1 mm^3 ($p<0.05$) in correlation with increases in-stent intimal hyperplasia ($r=0.536$, $p<0.05$). Under these conditions, changes in reference plaque volume correlated with those in LDL-C, which decreased from 121.2 ± 48.0 mg/dl to 103.3 ± 48.9 mg/dl ($r=0.43$, $p<0.05$). Interestingly, increases in out-stent plaque volume in the silorimus-eluting stent ($2.7 \pm 1.2\%$) were lesser than those in the bare-metal stent ($14.0 \pm 11.0\%$, $p<0.05$).
Conclusions: These results indicate that irrespective of LDL-C level, changes in out-stent plaque volume correlate with those in in-stent intimal hyperplasia. We suggest that silorimus-eluting stent can suppress in-stent intimal hyperplasia partially by affecting out-stent plaque, although further large-scale studies are required to define the role of out-stent plaque in the occurrence of in-stent intimal hyperplasia.

1. Introduction

Although development of in-stent intimal hyperplasia is known to be closely related to pre-existing atherosclerotic plaque [1], controlling this hyperplasia particularly in bare metal stents (BMS) is still difficult [2]. The lack of data regarding the changes in out-stent plaque possibly associated with in-stent intimal hyperplasia may be the cause for this difficulty. Furthermore, although reference coronary plaque volume could be reduced by drug therapies whether drug manipulations could effectively prevent progression of out-stent plaque is unclear [3–10].

In addition, it is interesting to examine whether drug-eluting stents can affect the out-stent plaque [11,12]. However, few data exist regarding the impact of the out-stent plaque volume (OSPV) on the development of in-stent intimal hyperplasia probably due to technical difficulty in determining OSPV in clinical settings. In the present study, we used high-gain intravascular ultrasound (IVUS) to investigate the relationship between out-stent plaque and in-stent intimal hyperplasia after stenting in stable coronary artery disease under moderate lipid-lowering therapy.

2. Methods

2.1. Study patients

This study was approved by the ethical review board at Kanazawa University. Written informed consent was given by all subjects. Forty-six patients (34 males, mean age of 66 years) with stable coronary artery disease who underwent percutaneous coronary stenting for de novo lesions between 2007 and 2009 in our institute were included in this study. Exclusion criteria included acute coronary syndrome, planned coronary artery surgery, renal failure, hepatic dysfunction, and coronary bypass grafting for total occlusion and complicated lesions including a procedure with the side branch.

2.2. Imaging procedures

After accessing the lesions with a standard 0.014-inch guidewire, they were pre-dilated by 2.0-mm-diameter balloon catheters, and pre-stenting IVUS examination was performed to determine the accurate size and length of the stents. The subjects received at least one stent implantation, and overlapping stents were placed serially to facilitate the use of multiple stents.

Sirolimus-eluting stents (SES; Cypher, Cordis, FL, USA) with $2.5–3.5 \times 13–23$ mm and BMS (Bx Velocity, Cordis, FL or Multi-link Vision; Abbott Vascular, IL, USA) with $2.5–4.0 \times 10–24$ mm were used. We implanted SES and BMS for 24 and 22 subjects, respectively. There were no patients receiving SES and BMS simultaneously. The target

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vessel was right coronary artery in 19 subjects and left coronary artery in 27 subjects. The average stent length was 23.6 ± 10.8 mm and the average maximum pressure of balloon inflation for stenting, including post-dilatation, was 16.8 ± 2.3 atm. Stent malapposition was not observed on IVUS examination immediately after the procedure and at follow-up. In the actual follow-up period of 18.4 ± 5.0 months, subacute closure of stents was not observed.

We used a mechanical IVUS imaging catheter (40 MHz, 2.5 Fr, Cardiovascular Imaging System; Boston Scientific Corp., MA, USA). All IVUS examinations were performed after intracoronary injection of 2–3 mg of isosorbide dinitrate to prevent catheter-induced spasm on conclusion of the interventional procedure. The IVUS catheter was introduced over the previously positioned 0.014-inch guidewire and advanced as distally as possible in one of the target vessels to visualize the entire coronary artery, and withdrawn automatically using a motorized pullback device at the speed of 0.5 mm/s. To accurately trace the external elastic membrane (EEM) behind the stent struts of all the stents, we adjusted both overall gain and depth gain of IVUS images higher than that usually used to trace that the EEM of reference plaque (Fig. 1A, B). Moreover, we used contrast agents or saline to distinguish the intimal hyperplasia from the lumen as clearly as possible.

A reference plaque located at least 5 mm distally or proximally from the target lesion of intervention was also evaluated. Reference plaque is the segment at non-intervention site of culprit vessel with a reproducible index, usually a branch site, on the same vessel. Spotty calcification, side branch, and distances from side branch bifurcation, and stent edge were also used as references.

2.3. Ultrasound volumetric analyses

IVUS images were analyzed at Kanazawa University Analysis Center for off-line quantitative analysis. Volumetric measurement was performed by experienced IVUS investigators (H.T. and S.T.) oblivious of the clinical data of the subjects while visualizing the baseline and follow-up IVUS images. Traces of IVUS images were obtained in accordance with the American College of Cardiology and European Society of Cardiology standards [13]. Cross sections in the IVUS images were measured with an

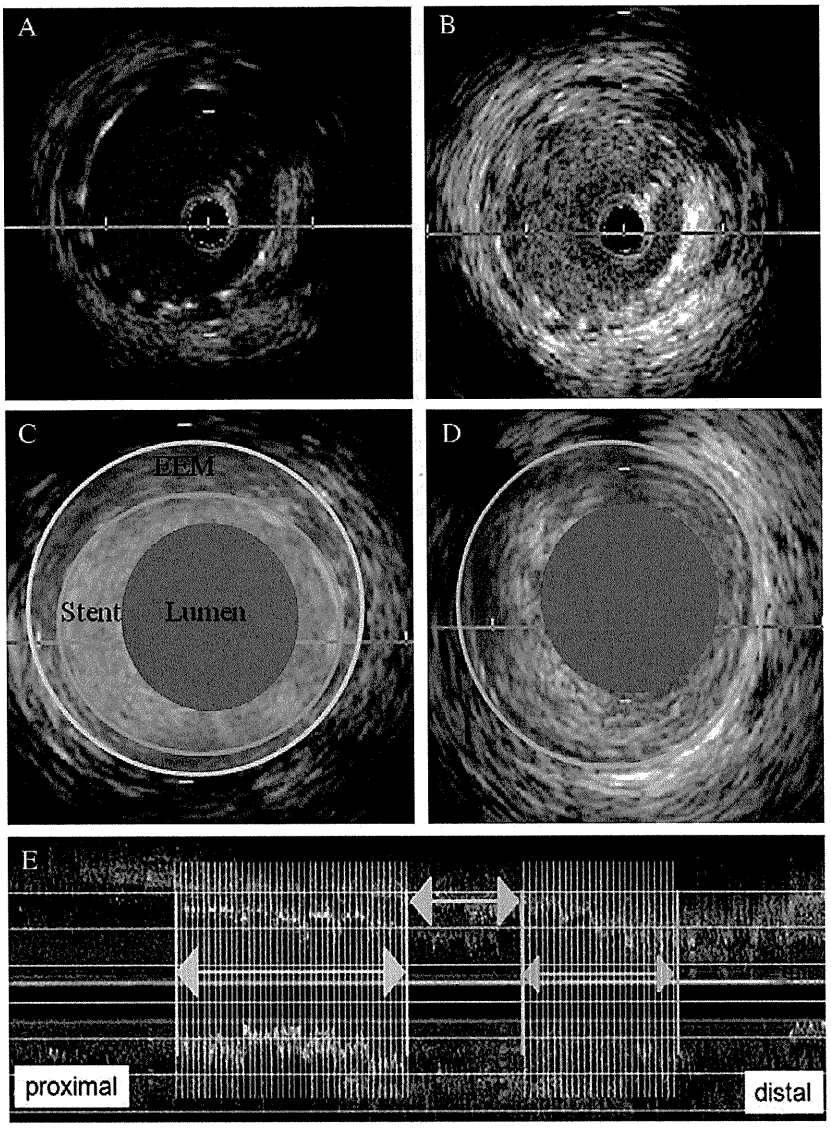

Fig. 1. Images of conventional and high-gain intravascular ultrasound and definitions of each plaque area. Although intravascular ultrasound (IVUS) with conventional gain could not detect external elastic membrane (EEM) appropriately (A), this could be traced by increasing overall and depth gain (B).The definitions of each area are EEM area as yellow, stent area as blue and lumen area as red. (C). Reference plaque area was determined by subtracting lumen from EEM areas (D). Volumes of each parameter were calculated by summing each area throughout stent and reference plaque, with a distance of >5 mm (pink arrow, E). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

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integrated software of EchoPlaque2 (Indec Co., Sunnyvale, CA, USA) at 0.067-mm intervals from distal edge of the stents to their proximal edge. Areas of EEM, stent, and lumen were traced (Fig. 1C and D), and their volumes were determined by summing each area throughout the measured stent (Fig. 1E).

Then, OSPV was calculated by subtracting stent volume from EEM volume, in-stent hyperplasia volume by subtracting lumen volume from stent volume, and reference plaque volume (RPV) was calculated by subtracting lumen volume from EEM volume. The primary endpoints were the percent change in OSPV (=OSPV (follow-up) − OSPV (baseline)/OSPV (baseline) × 100), percent volume of intimal hyperplasia (=Intimal hyperplasia volume (follow-up)/Stent volume (follow-up) × 100), and percent change in RPV (=RPV (follow-up) − RPV (baseline)/RPV (baseline) × 100). To assess the intraobserver and interobserver reproducibility of IVUS measurements, images at baseline and follow-up of 10 cases were randomly selected and re-analyzed at least 4 weeks after the initial analyses.

2.4. Statistical analysis

Values are expressed as mean ± SD unless otherwise stated. Differences of changes were compared using an unpaired *t*-test. Correlations between the percent change in each atheroma volume and the clinical parameters were analyzed by linear regression analysis and correlation coefficient. The level of statistical significance was set at $p<0.05$. Statistical analysis was performed using StatView 5.0 (SAS Institute Japan, Tokyo, Japan).

3. Results

3.1. Patients' backgrounds

Of 46 patients, 2 patients with BMS were excluded because of severe restenosis, due to which introduction of IVUS catheter, without any procedure or modification, into the interventional lesion was difficult; and 2 patients with SES were also excluded due to inaccurate IVUS measurements because of severe calcifications or branch vessels at the follow-up. Accordingly, measurements of OSPV and volume of intimal hyperplasia were completed in 42 patients (34 males with mean age of 66 years). For analyses, among the 25 left and 17 right coronary arteries of the patients, 22 had SES whereas 20 had BMS.

The baseline characteristics of the study subjects are given in Table 1. Before intervention, hypertension was observed in 63.3%, diabetes mellitus in 46.7%, and a history of smoking in 76.7% patients. Each patient received angiotensin II-converting enzyme inhibitor or receptor blockers (80%), statins (83.3%), β-blocker (33.3%), and aspirin (100%). After intervention, all patients further received 75 mg clopidogrel or 200 mg ticlopidine for 6 months after the procedure, and in addition to these antiplatelet treatments, administration of angiotensin II-converting enzyme inhibitor or receptor blockers and β-blockers was retained in all patients. They were also administered statins such as atorvastatin (5–20 mg/day) or rosuvastatin (2.5–5 mg). In the present study, we did not use intensive doses of these drugs, because the primary end point was to observe changes in OSPV and RPV under regular risk control with optimal blood pressure and LDL-C recommended for Japanese patients [14,15]. As a result, the LDL-C level was reduced from 121.2 ± 48.0 mg/dl to 103.3 ± 48.9 mg/dl (i.e., 15% reduction) from that of baseline, although the final level of LDL-C was somewhat greater than the recommended level (<100 mg/dl) [15].

Table 1
Characteristics of the study subjects.

	Baseline	Follow-up
Gender (male/female)	34/8	
Age (yr)	66.4 ± 12.7	
BMI (kg/m^2)	24.5 ± 2.7	
Hypertension (%)	63.3	
Diabetes (%)	46.7	
Smoking (%)	76.7	
ACE-I or ARB (%)	80.0	80.0
Statin (%)	83.3	93.3
β-blocker (%)	33.3	33.3
Insulin (%)	11.1	11.1
TC (mg/dl)	187.9 ± 51.8	158.5 ± 38.4*
TG (mg/dl)	130.6 ± 108.9	127.0 ± 66.1
HDL-C (mg/dl)	44.8 ± 12.4	44.6 ± 11.2
LDL-C (mg/dl)	121.2 ± 48.0	103.3 ± 48.9*
HbA1c (%)	6.2 ± 1.1	6.1 ± 1.0
hs-CRP (mg/dl)	0.20 ± 0.11	0.19 ± 0.14

BMI, body mass index; ACE-I, angiotensin converting enzyme inhibitor; ARB, angiotensin II receptor blocker; TC, total cholesterol; TG, triglyceride; LDL-C, LDL cholesterol; HDL-C, HDL cholesterol; hs-CRP, high-sensitive CRP.
Values are mean ± SD.
* $p<0.05$.

3.2. Plaque measurements

The intraobserver correlation coefficients for OSPV, RPV, and intimal hyperplasia were 0.997, 0.998, and 0.998, respectively; the coefficients of variation were 3.1%, 0.6%, and 1.7%, respectively. The interobserver correlation coefficients for OSPV, RPV, and intimal hyperplasia were 0.996, 0.998, and 0.997, respectively; the coefficients of variation were 5.4%, 1.3%, and 2.2%, respectively. When we performed the Bland–Altman analysis, all the data points were well within the mean ± 2 SD range.

Changes in each plaque volume are given in Table 2. During the follow-up period, EEM volume at stenting sites significantly increased from 325.2 ± 174.6 mm^3 to 341.4 ± 179.2 mm^3 ($p<0.05$), although stent volume was substantially unchanged from 151.1 ± 80.1 mm^3 to 152.7 ± 79.8 mm^3 (p = NS). Thus, OSPV significantly increased from 177.3 ± 100.8 mm^3 to 190.7 ± 111.1 mm^3 or by 9.3% ($p<0.05$). Lumen volume significantly decreased from 151.1 ± 80.1 mm^3 to 134.9 ± 72.9 mm^3 ($p<0.05$), yielding the increase in intimal hyperplasia to 18.7 ± 19.7 mm^3 ($p<0.05$). Under these conditions, RPV substantially unchanged from 40.2 ± 17.4 to 40.3 ± 18.7 mm^3 (p = NS).

Among the several risk factors, only percent change in LDL-C level had significant impact on the changes in RPV ($p<0.05$) because percent change in RPV correlated with that in LDL-C level ($r=0.43$, $p<0.05$) whereas percent change in OSPV and percent volume of intimal hyperplasia did not correlate with percent change in LDL-C level (Fig. 2). No significant correlations were observed between these parameters even in the subgroup who could achieve <70 mg/dl of LDL-C level (n = 5). Under these conditions, the level of high-sensitive C-reactive protein tended to be higher in increased OSPV>0% (0.21 ± 0.18 mg/dl) than in unchanged or decreased OSPV≤0% (0.16 ± 0.13 mg/dl), although overall level did not change before and during the follow-up period (Table 1). This suggests that changes in OSPV might be related to changes in inflammatory activity rather than lipid levels.

Interestingly, a significant correlation was observed between percent change in OSPV and percent volume of intimal hyperplasia ($r=0.536$, $p<0.05$; Fig. 3). Apparently, increases in OSPV and in-stent intimal hyperplasia were greater in patients with BMS than in those

Table 2
Baseline and follow-up IVUS results.

	Baseline	Follow-up	Percent change (%)	P value
Culplit plaque (mm^3)				
EEM volume	325.2 ± 174.6	341.4 ± 179.2	1.9 ± 5.0	< 0.05
Stent volume	151.1 ± 80.1	152.7 ± 79.8	1.2 ± 10.3	NS
Lumen volume	151.1 ± 80.1	134.9 ± 72.9	−10.0 ± 14.8	< 0.05
OSPV	177.3 ± 100.8	190.7 ± 111.1	9.3 ± 12.9	< 0.05
Intimal hyperplasia	0	18.7 ± 19.7	12.5 ± 10.2	< 0.05
Reference plaque (mm^3)				
EEM volume	90.8 ± 37.9	89.4 ± 36.1	−0.7 ± 11.5	NS
Lumen volume	40.2 ± 17.4	40.3 ± 18.7	0.3 ± 15.0	NS
RPV	50.7 ± 26.0	48.7 ± 24.2	−2.1 ± 15.0	NS

OSPV, out-stent plaque volume; RPV, reference plaque volume.
Values are mean ± SD.

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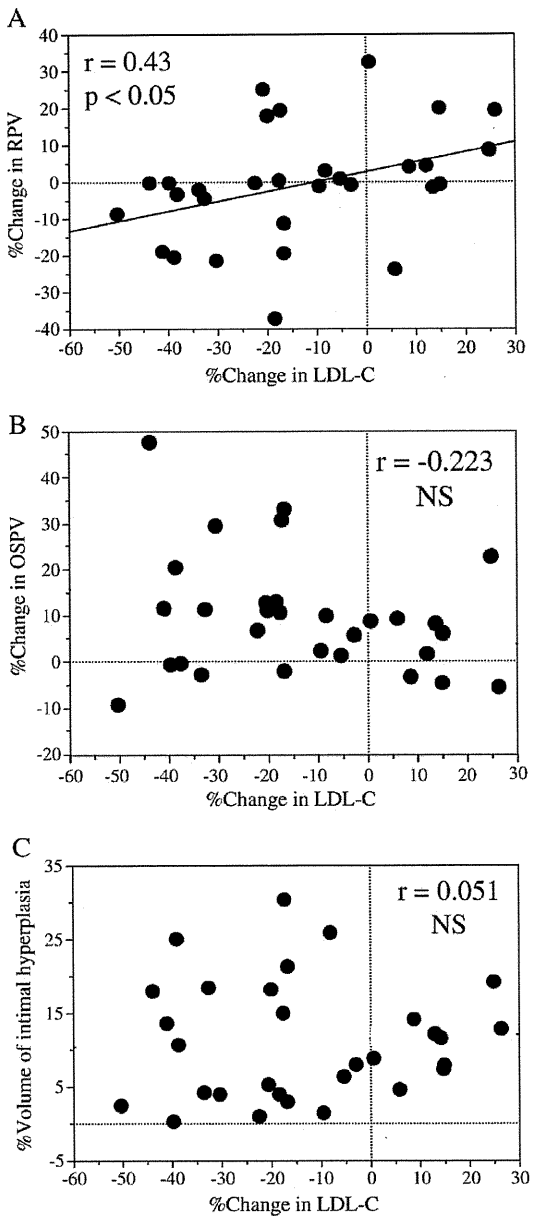

Fig. 2. Correlation between the change in plaque volume and the change in low density lipoprotein-cholesterol.Percent change in reference plaque volume (RPV) significantly correlated with the percent change in low density lipoprotein-cholesterol (LDL-C) (A), although percent change in out-stent plaque volume (OSPV) (B) as well as percent volume in intimal hyperplasia (C) did not correlate with change in LDL-C.

with SES. When the subjects were divided into two groups based on the stents used, in-stent intimal hyperplasia and percent change in OSPV of the SES group was significantly lower than that of the BMS group (7.3 ± 6.1% vs. 15.7 ± 7.8%, $p<0.05$ for intimal hyperplasia; 2.7 ± 1.2% vs. 14.0 ± 11.0%, $p<0.05$ for OSPV; Fig. 4).

Examining the effect of stent overlapping on intimal hyperplasia is important. When we compared the percent changes of each volume between the overlapping lesions and single lesions in 10 patients

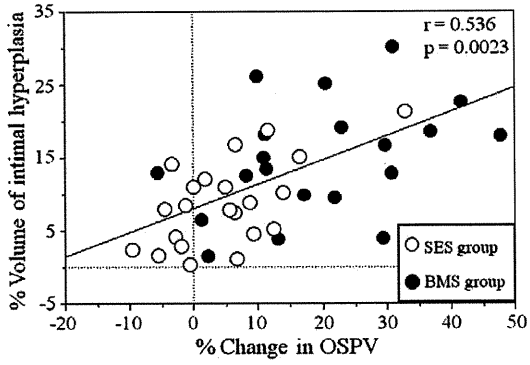

Fig. 3. Correlation between the change in out-sent plaque volume and the volume of intimal hyperplasia.Percent change in OSPV (horizontal axis) significantly correlated with percent volume of intimal hyperplasia (vertical axis). Note that increase in OSPV and intimal hyperplasia seemed to be greater in bare-metal stents (BMS) than those in sirolimus-eluting stents (SES).

receiving multiple stents implantation (6 for SES and 4 for BMS), no significant differences were observed between the calculated values of these lesions (Table 3).

Complications related to the high-gain IVUS procedure in both initial and follow-up examinations were not observed.

4. Discussion

The main findings of the present study are: 1) during the 18-month follow-up period, OSPV significantly increased, although RPV was substantially unchanged with 15% reduction in LDL-C level; 2) percent change in OSPV did not correlate with that of LDL-C level, whereas percent change in RPV correlated with LDL-C value and 3) under these conditions, percent change in OSPV, which had a significant correlation with the volume of intimal hyperplasia of the SES group was significantly lower than that of the BMS group. These results highlight the difficulty of controlling in-stent intimal hyperplasia associated with progression of OSPV with conventional medical therapy.

Measurement of the plaque volume behind the stent struts is considered to be difficult because of the presence of mechanical artifacts of the stent struts. Therefore, in this study, we adjusted the echo gain of IVUS images higher than that usually used to accurately trace EEM behind the stent struts. As a result, fully analyzable images could be obtained from 94% of the study subjects that could be followed up for 18 months.

Progression/regression of out-stent plaque is independent of LDL-C level, whereas those of reference plaque are related to LDL-C level.

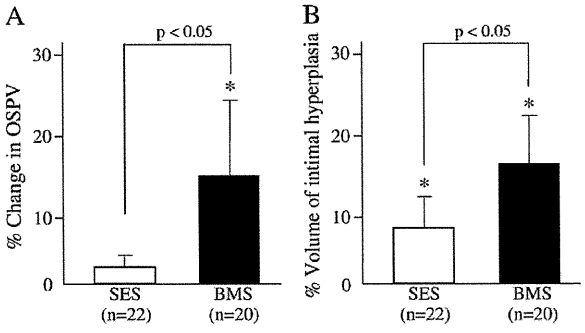

Fig. 4. Comparison of changes in out-stent plaque volume and intimal hyperplasia in different types of stents. Changes in both OSPV (A) and intimal hyperplasia (B) were greater in BMS compared with SES.

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Table 3
Comparison of stent overlapped lesion and single lesion.

	Overlapped lesion	Single lesion	P value
Percent change (%)			
EEM volume	2.0 ± 5.9	2.2 ± 5.0	NS
Stent volume	0.8 ± 10.0	1.4 ± 8.5	NS
Lumen volume	−12.4 ± 10.8	−11.5 ± 10.0	NS
OSPV	11.4 ± 10.6	10.4 ± 11.1	NS
Intimal hyperplasia	12.5 ± 11.3	13.4 ± 10.1	NS

EEM, external elastic membrane; OSPV, out-stent plaque volume.
Values are mean ± SD.

The occurrence of inflammation after the interventional procedure may contribute to increases in OSPV under the conventional medical therapy. Indeed, use of SES could suppress the increase in OSPV as well as in-stent intimal hyperplasia. This hypothesis was supported because positive vessel remodeling after stenting is derived from the increase in OSPV [16,17]. This remodeling may be derived from the post-procedural inflammation and impairment of endothelial function associated with this morphological change [18–20]. Actually, increased OSPV during the follow-up period was associated with increased high-sensitive C-reactive protein level in comparison with stable OSPV. Effective penetration of silorimus all over the out-stent plaque instead of only into the adjacent stent struts may be possible, thus resulting in decreased in-stent intimal hyperplasia. Examining the changes in OSPV under intensive lipid-lowering therapy, which can significantly decrease high-sensitive C-reactive protein level [6,7], may be useful, although moderate doses of statins were used to obtain different levels of LDL-C in this single-arm study.

This study has several limitations. First, a small sample size was used in this study because of the first trial demonstrating the significance of OSPV in man as a feasibility study. Even in this case, the changes in OSPV associated with intimal hyperplasia were evidently independent of LDL-C level. Second, we excluded cases of total occlusion or severe restenosis that needed re-intervention to re-evaluate the out-stent plaque and intimal hyperplasia at the follow-up period. Third, IVUS may not detect a thin intimal hyperplasia, particularly on the SES struts, despite careful examination of the intimal hyperplasia using some contrast agent. We did not evaluate the plaque components that are reportedly associated with vascular remodeling [21]. Use of optical coherent tomography may overcome IVUS shortcomings [22], although the observation of entire plaque using current OCT device having low axial penetration depth is still somewhat difficult for detecting the far wall of reference and out-stent plaques. Fourth, the stent material of BMS is not uniform in this study. This might affect a part of in-stent intimal hyperplasia results, although stent-biology interactions seem to be similar [23]. Further large-scale studies will demonstrate the clinical impact of the OSPV in the development of in-stent intimal hyperplasia.

5. Conclusions

This study demonstrates that there is a close relationship between out-stent plaque and in-stent intimal hyperplasia and that progression/regression of out-stent plaque is different from that of reference plaque in terms of response to changes in LDL-C. This may explain why in-stent intimal hyperplasia could not be reduced by simple LDL-C lowering therapy. We suggest that a new kind of drug targeted to reduce OSPV could possibly control in-stent intimal hyperplasia.

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A novel gain-of-function *KCNJ2* mutation associated with short-QT syndrome impairs inward rectification of Kir2.1 currents

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Aims Short-QT syndrome (SQTS) is a recently recognized disorder associated with atrial fibrillation (AF) and sudden death due to ventricular arrhythmias. Mutations in several ion channel genes have been linked to SQTS; however, the mechanism remains unclear. This study describes a novel heterozygous gain-of-function mutation in the inward rectifier potassium channel gene, *KCNJ2*, identified in SQTS.

Methods and results We studied an 8-year-old girl with a markedly short-QT interval (QT = 172 ms, QTc = 194 ms) who suffered from paroxysmal AF. Mutational analysis identified a novel heterozygous *KCNJ2* mutation, M301K. Functional assays displayed no Kir2.1 currents when M301K channels were expressed alone. However, co-expression of wild-type (WT) with M301K resulted in larger outward currents than the WT at more than −30 mV. These results suggest a gain-of-function type modulation due to decreased inward rectification. Furthermore, we analysed the functional significance of the amino acid charge at M301 (neutral) by changing the residue. As with M301K, in M301R (positive), the homozygous channels were non-functional, whereas the heterozygous channels demonstrated decreased inward rectification. Meanwhile, the currents recorded in M301A (neutral) showed normal inward rectification under both homo- and heterozygous conditions. Heterozygous overexpression of WT and M301K in neonatal rat ventricular myocytes exhibited markedly shorter action potential durations than the WT alone.

Conclusion In this study, we identified a novel *KCNJ2* gain-of-function mutation, M301K, associated with SQTS. Functional assays revealed no functional currents in the homozygous channels, whereas impaired inward rectification demonstrated under the heterozygous condition resulted in larger outward currents, which is a novel mechanism predisposing SQTS.

Keywords Arrhythmia (mechanisms) • Short-QT syndrome • K-channel • Atrial fibrillation • Inward rectification

1. Introduction

Short-QT syndrome (SQTS) is a recently recognized disorder, characterized by a shortened QT interval in the electrocardiogram (ECG), and associated with a high incidence of atrial fibrillation (AF), syncope, and sudden death due to ventricular tachyarrhythmias without structural cardiac abnormalities. The syndrome was first described by Gussak *et al.*[1] in 2000 within the context of a familial AF case associated with short-QT interval. SQTS is a genetically heterogeneous disease, and five ion channel genes (SQT1-6) have been identified as causative genes thus far: *KCNH2* encoding the α-subunit of the rapidly activating delayed rectifier potassium channels, I_{Kr} (SQT1)[2]; *KCNQ1* encoding the α-subunit of the slowly activating delayed rectifier potassium channels, I_{Ks} (SQT2)[3]; *KCNJ2* encoding

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the Kir2.1 channels that underlie the inward rectifier potassium currents, I_{K1} (SQT3)[4]; *CACNA1C*, *CACNB2b*, and *CACNA2D1*, which encode the α1C, β2b, and α2δ-1-subunits of cardiac L-type calcium channels (SQT4, SQT5,[5] and SQT6[6]), respectively. SQT4 and SQT5 are considered clinical entities with the combined phenotypic characteristics of SQTS and Brugada syndrome, manifesting in a J point and ST-segment elevation in the right precordial ECG leads.

Regardless of the extensive genetic screening carried out on SQTS patients, genetic mutations have been identified in a small number of cases.[2–5,7,8] In 2005, Priori *et al.*[4] first reported that a *KCNJ2* mutation was responsible for SQTS (SQT3); however, no additional SQT3 variants have been reported thus far. This lack of progress has significantly hindered our advances in understanding the mechanisms underlying this disease. In the present study, we describe a novel *KCNJ2* mutation which impaired the inward rectification of Kir2.1 currents. This is a novel *KCNJ2* gain-of-function mechanism leading to SQTS.

2. Methods

2.1 Genetic analysis

Genetic analysis was performed after written informed consent in accordance with the study protocol approved by the Kyoto University ethical committee. The investigation conforms to the principles outlined in the Declaration of Helsinki. Genomic DNA was isolated from blood lymphocytes, and screened for the entire open-reading frames of *KCNQ1*, *KCNH2*, *KCNE1-3*, *KCNJ2*, *CACNA1C*, and *SCN5A* by denaturing high-performance liquid chromatography using a WAVE System Model 3500 (Transgenomic, Omaha, NE, USA). Abnormal conformers were amplified by polymerase chain reaction and sequencing was performed on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA), and compared with 400 Japanese control alleles.

2.2 Neonatal rat ventricular myocyte isolation

This investigation was performed in accordance with the Guide for the Care and Use of Laboratory Animals, published by the National Institutes of Health (NIH Publication No. 85-23, revised 1996), and was approved by the Kyoto University Animal Experimentation Committee. A standard trypsin dissociation method was used to prepare neonatal rat ventricular myocytes (NRVMs).[9] The hearts were removed from 1- to 2-day-old Wistar rats euthanized by decapitation. The ventricles were minced, and the myocytes were dissociated with trypsin. Dispersed cells were pre-plated on 100 mm culture dishes for 1 h at 37°C in 5% CO_2 to remove fibroblasts. Non-attached, viable myocytes were collected, and placed on 35 mm culture dishes.

2.3 Mutagenesis and transient transfection of *KCNJ2* plasmids

The entire coding region of the *KCNJ2* was subcloned into the pCMS-EGFP vector (Clontech, Palo Alto, CA, USA) using methods previously described.[10] The mutation was introduced by site-directed mutagenesis using the QuikChange Mutagenesis Kit (Stratagene, La Jolla, CA, USA). We sequenced the entire plasmid to confirm the presence of the mutation and the absence of any unwanted variations. To assess the functional modulation of mutant channels, human embryonic kidney (HEK) 293 cells were transiently transfected with *KCNJ2* WT and/or mutant plasmids using FuGENE 6 (Roche, Indianapolis, IN, USA) as directed in the manufacturer's instructions. In order to investigate the mutant's effects on myocyte action potentials, plasmids were transfected 1 day after plating NRVMs, using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA).[11]

2.4 Cell surface expression of *KCNJ2*

Immunofluorescence microscopy was used to detect the presence of KCNJ2 channels on the plasma membrane of HEK 293 cells. A haemagglutinin (HA) epitope (YPYDVPDYA) was introduced into the pCMS-EGFP-KCNJ2 [wild-type (WT) and mutant] construct between residues Ala-115 and Ser-116 (extracellular loop between TM1 and TM2).[10,12] HEK 293 cells were transfected with 1.0 μg of WT or mutant plasmids, or 0.5 μg of each WT and mutant plasmids to assess a heterozygous condition in 35 mm glass-bottom dishes. Two days later, the cells were fixed with 4% paraformaldehyde solution, and images were taken at ×40 magnification on an LSM 510 confocal microscope (Carl Zeiss, Jena, Germany).

2.5 Electrophysiological analysis

For voltage-clamp experiments, a total of 0.75 μg of WT and/or mutant *KCNJ2* plasmids were transfected in HEK 293 cells; 48–72 h after transfection, functional assays were conducted on GFP-positive cells by a conventional whole-cell configuration of patch-clamp techniques at 37°C, using an Axopatch 200A patch clamp amplifier and a Digidata 1322A digitizer (Axon Instruments, Foster City, CA, USA).[10] Pipettes were filled with a solution (in mM): 140 KCl, 2 $MgCl_2$, 1 EGTA, and 10 HEPES (pH 7.3 with KOH). The bath solution was composed of (in mM): 135 NaCl, 5 KCl, 1 $MgCl_2$, 10 glucose, and 10 HEPES (pH 7.4 with NaOH).

In order to record action potentials on NRVMs, 3 μg of WT, or a mixture of 1.5 μg WT and 1.5 μg mutant *KCNJ2* plasmids, were transfected; 48–72 h after transfection, functional assays were conducted on non-transfected or transfected cells that were recognized by their obvious green fluorescence, using a whole-cell patch-clamp technique at 37°C with the same devices. Action potentials were evoked by 2 ms supra-threshold current pulses at 10 Hz in a current-clamp mode. The pipette solution contained (in mM): KCl 140, $MgCl_2$ 1, MgATP 4, NaCl 10, and HEPES 10 (pH 7.2 with KOH). Tyrode solution contained (in mM): NaCl 140, KCl 4, $CaCl_2$ 2, $MgCl_2$ 1, HEPES 10, and glucose 10 (pH 7.4 with NaOH). Action potential duration (APD) was measured as the time from the overshoot to 90% repolarization (APD_{90}).

2.6 Statistics

All the data are shown as mean ± standard error of the mean. For mean value and comparisons between two sample groups, an unpaired Student's *t*-test was used to evaluate statistical significance. For comparisons between multiple groups, we applied a Steel–Dwass test. For either evaluation, a *P*-value <0.05 was considered significant.

3. Results

3.1 Clinical features

An 8-year-old girl with a markedly shortened QT interval (QT = 172 ms, QTc = 194 ms; *Figure 1A*) had been suffering from multiple disorders, such as severe mental retardation, abnormal proliferation of oesophageal blood vessels, epilepsy, and Kawasaki disease. Upon presentation during a routine check-up, her treating physician noticed an irregular heart rhythm. Her 12-lead ECG showed AF (*Figure 1B*), and she underwent external electrical cardioversion because intravenous infusion of procainamide (15 mg/kg) failed to recover sinus rhythm. The echocardiography revealed no significant abnormality. During further evaluation with right-heart catheterization, the Swan–Ganz catheter induced supra-ventricular tachycardia when it was inserted in the right atrium, and ventricular fibrillation occurred at the position of the right ventricular outflow tract, which suggested the presence of increased myocardial irritability.