including nursing science, nursing care, study of social welfare, social science, psychology, economics, religion and ethics, as well as medical sciences;
2 Promotion of gerontology, reform and enhancement of geriatrics in undergraduate, postgraduate and lifelong education;
3 Building geriatric medical centers in each area, and accumulating large-scale evidence of geriatric diseases and geriatrics; and
4 Structural development and promotion of home-based care and multidisciplinary care.
Through implementation of the above measures, Japan is expected to function as a successful example for the rest of the world. **Geriatr Gerontol Int 2012; 12: 16–22.**

Keywords: education, elderly, geriatrics, gerontology, multidisciplinary approach.

1. Preface

Over the past 50 years, the percentage of elderly people in the population of Japan has increased fourfold from 5.7% in 1960 to 23.1% in 2010. Japanese society is aging at an unprecedented rate. According to the National Institute of Population and Social Security Research, the percentage the elderly population is estimated to continue increasing, reaching 26.0% in 2015 and further increasing rapidly. After 2020, the percentage of elderly people in the population is expected to stabilize; however, as a result of a decrease in the total population, the percentage will further increase to 40.5%, peaking in 2055. Japan will face a super-aged society, in which 40% of the population will be over 65 years-of-age. Unless appropriate countermeasures are taken, such as a rapid improvement in clinical skills and knowledge among physicians involved in geriatrics, marked advances in the prevention of lifestyle-related diseases, prevention of geriatric syndromes including dementia, and marked expansion of home-based care or local-care, we cannot avoid a situation where many frail elderly people have to live with no support. However, many issues remain; that is, a marked reduction of long-term care facilities, a reduction in length of hospital stay in acute hospitals and a delay in expanding home-based care system, and whether thanatology reflects a social change. We should also consider social issues, such as ageism, caregiver burnout, dignified death and the appropriateness of placing gastrostomy tubes in elderly patients with dementia. To provide dignified care, particularly for older people, appropriate care should be carried out in not only the terminal phase, but also during the last few years before death.

However, despite the challenge, little is known about gerontology and geriatrics in Japan, and they are not fully used in clinical settings or education. To solve this problem, a macroscopic integration and cooperation are needed, using an interdisciplinary approach involving medical science, nursing science, nursing care, study of social welfare, social science, engineering, jurisprudence, economics, psychology and ethics. Furthermore, along with the reform and enhancement of geriatrics in undergraduate and postgraduate education, fostering specialists who can practice geriatrics is needed. Also, for non-geriatricians or general practitioners who currently and prospectively provide care in clinical settings, an educational system should be prepared to deepen their understanding of geriatric medicine.

2. Current situation and measures

(1) Social contribution of the elderly and the medical economy

As a result of the low birth rate, the percentage of the total labor force (aged 20–64 years) is expected to decrease in Japan. Elderly people are usually divided into two groups based on age: 65–84 years (young-old) and 75 years and older (old-old). Although many elderly people, particularly the young-old, have sufficient physical strength to fulfil their job duties and a make social contribution though productive activity, they are not fully utilized. The promotion of social participation and the contribution of the elderly is expected to contribute to creating purpose in their lives, as well as an increase of a substantive productive population, financial stability and self-sustainability for the elderly, and an upturn of national economic activity through an increase of total consumption. Therefore, for elderly people to be engaged in various social activities, strategies for developing a social structure for re-education, volunteer activity, various employment statuses and employment opportunities should be prepared using an interdisciplinary approach involving study of social welfare, social science and economics. However, as the total number of jobs is fixed, consideration should also be given to young workers.

Life expectancy in Japan is the highest in the world. Japan also has the highest healthy life expectancy. In 2008, USA health expenditures accounted for 16% of the nation's gross domestic product (GDP), twice the Japanese rate. Compared with other countries, Japanese health expenditures as a percentage of GDP accounted for two-thirds of that of France and Germany, suggesting that we have the most cost-effective health-care

systems. In addition, the annual cost of health care has been approximately 670 000 yen per elderly person for the past 10 years. However, the aging of the population is expected to impact on future spending growth. Sasaki compared life-long medical costs between the longevity and non-longevity groups, and found that longevity decreases medical costs and has positive economic impacts.[1] Thus, it is important to enhance preventive medicine to achieve longevity, make continuous efforts for cost-effective medicine and improve satisfaction with the health-care systems. Discussion of geriatric medicine should be made after disclosing the aforementioned facts to the public.

Problems in geriatric medicine are closely linked to social structures, including care, welfare and dwelling surrounding the health-care system. To reveal and solve problems regarding the elderly and an aged society, the promotion of gerontology using an interdisciplinary approach is increasingly needed.

Regarding employment opportunities for older workers and future directions of medicine, care and welfare, discussion should be made among specialists from various health-care specialties. The Japan Geriatrics Society and the Japan Gerontological Society, as a core organization, should expand their activities to achieve a "society where elderly people can enjoy their lives" with the cooperation of the National Center for Geriatrics and Gerontology, Tokyo Metropolitan Geriatric Hospital and Institute of Gerontology, the Institute of Gerontology the University of Tokyo, and J. F. Oberlin University.

(2) The current state of geriatric medicine and its direction

Geriatric disorders have several features.

First, diseases occur as a result of a decline in organ systems associated with aging. Therefore, even if a disease is not so severe, a patient might have been developing an unexpectedly marked decline in organ systems. In addition, homeostatic function with aging, biophylaxis capacity and nutritional absorption capacity often decrease, and symptoms become chronic and refractory.

In terms of clinical symptomatology, older people often complicate many diseases together with a geriatric syndrome with multiple etiologies. Signs and symptoms vary according to each individual, and are often atypical. Response to drugs is different in elderly compared with non-elderly people.

Older people are more likely to develop multiple diseases, and visit different hospitals and receive many screening tests and prescriptions at the same time;[2] thus, total expenditures on the elderly become inevitably high, which has been said to cause financial collapse of the Japanese health insurance system. However, regarding this issue, we should focus on the medical cost required for a single disease between elderly and non-elderly people, and we should be aware that restricting the increasing financial burden on patients to receive screenings or prescriptions for each disease would be ageism for elderly people and uncontroversial. However, unnecessary duplication of the screening given at each hospital should be avoided. To achieve this, an effective screening system carried out by primary-care physicians, and privacy-preserving medical data sharing of test results and medication among hospitals and clinics are needed. Regarding medications, the Japan Geriatrics Society has prepared the "Guidelines for medical treatment and its safety in the elderly" as an outcome of the sponsored research in Japan Foundation on Aging and Health.[3] The guideline explained standard medical treatments mainly for the elderly by giving examples of low priority, such as making an easy prescription or non-evidence-based prescription to prevent deterioration of chronic disease. In either retrospective fee-for-service or a prospective payment system (fixed amount), physicians should provide the same level of prescription to each patient. To carry out effective screening for the elderly or evidence-based medical treatment, a constructive research system should be developed separately from health-care reform in terms of medical economy. The Japanese government has decided to abolish the existing medical insurance system for those aged 75 years and older; however, the following principles stated in the existing medical insurance system should be included in the next system for the elderly: (i) elderly disease prevention; (ii) comprehensive geriatric assessment; and (iii) incentives to promote discharge planning.

Older people often develop functional disorders associated with chronic disease or aging. Functional disorders not only jeopardize the independence of people and pose social disadvantage, but also lead to secondary disease. This often makes elderly people fully dependent, resulting in lower quality of life. Therefore, in the treatment of geriatric disorders, priority should be given to functional outcomes, as well as life expectancy and the prognosis of organ systems. In addition, because a psychological change associated with an environmental change often leads to a deterioration of symptoms in elderly people, treatment policy and discharge planning should be prepared with a holistic consideration of the patient using the comprehensive geriatric assessment (CGA). In geriatric medicine, it is important not only to protect organ systems, but also to maintain physical function to prevent assisted living.

To maintain independent living, a person needs to have sustained function, including daily life functions, cognitive function, emotion and sociality (family, friends, job). CGA is used to determine the aforementioned functional status both comprehensively and systematically. The results of CGA give us a clue of what kind of

support can help maintain independent living or assisted living with minimum care for elderly people. However, CGA is not a popular tool. Therefore, we should examine ways of increasing the awareness of CGA to promote its use for the improvement of geriatric medicine.

End-of-life care for elderly patients is an extremely important issue in geriatric medicine; however, very few elderly people in Japan have made advance directives to show their wishes about their health care during the end-of-life period. In geriatrics, there are so many issues to discuss, including confirmation of patient's wishes, the need of a health-care representative, and the relationship between the patient and their physician. Therefore, we should investigate the awareness of end-of-life care for elderly patients among health-care professionals, including physicians and nurses, people involved in care, patients, and their families, to discuss future direction of care. Regarding end-of-life care in elderly people, "Attitudes toward end-of-life care in elderly patients",[4] which was announced in 2000 by the ethics committee of the Japan Geriatrics Society and is currently under revision, and a proposal prepared by the end-of-life care research group,[5] should be referred.

(3) Fostering health-care professionals involved in geriatric medicine

Despite the growth of the elderly population, physicians with special geriatric training are not expected to increase under the present system of medical education. In order to solve the problem of care for the growing elderly population, the educational system should be restructured to provide an understanding of geriatric medicine for non-geriatricians, general practitioners and physicians working at care facilities that provide care for elderly patients. This might be an effective and practical approach for fostering physicians taking care of the elderly. To provide sufficient geriatric knowledge to general practitioners and non-geriatricians, the education program should include basic geriatrics contents to retain quality of geriatric care, which would be required even for non-geriatricians. The Japan Geriatrics Society has published *Clinical Handbook for Active Aging and Geriatric Care* for physicians, which aims to provide basic knowledge of elderly-specific symptoms, assessment, treatment and care. It is expected that using this handbook for students, residents, practitioners and non-geriatricians might contribute to the expansion of geriatric medicine. In the USA, in order to deal with a shortage of geriatric specialists, medical students are required to receive a minimum geriatrics education.[6]

(4) Promotion of geriatric disease clinical research

In Japan, a system for making diagnosis and providing treatment and care for patients with elderly diseases, including dementia, has not been fully developed. In elderly care, it is important to make an accurate diagnosis and collect clinical evidence to reflect diagnosis and evidence in clinical settings. To accumulate evidence of geriatric medicine and nursing, the promotion of clinical research and a marked expansion of geriatric medical centers with high-level medical services are eagerly awaited.

Currently, there are just two geriatric medical centers in Tokyo and Nagoya. Therefore, the number of centers should be increased and should be placed in each district (Hokkaido, Tohoku, Hokuriku, Kanto, Koshinetsu, Tokai, Kinki, Chugoku, Shikoku and Kyushu). The National Center for Geriatrics and Gerontology, as a core facility, is required to examine the efficacy of geriatrics-related activities and consistency with countermeasures, supervise multicenter studies and clinical research projects, and strive to enhance geriatric medicine through the standardization of geriatric medicine and care, and preparation of medical guidelines. In this process, each center, as a platform of geriatric medicine, should accumulate clinical data, and is also required to function as a facility to educate non-geriatricians.

The Japan Geriatrics Society has been carrying out clinical research on the treatment of hyperlipemia involving the elderly aged 75 years and over. An establishment of a support system for such clinical research and an accumulation of evidence on the efficacy of nutrition and exercise are also considered important.

(5) Promotion of home-based care and multidisciplinary care

Based on the demand of older people who prefer to remain at home, and a government policy that aims to shorten the length of hospital stay and the number of beds to decrease the growing burden of health-care expenditure, the promotion of home-based care has been provided. However, the medical structure of home-based care has not been fully devised, requiring further development of a medical and nursing structure where older people can receive continuing treatment and care, including rehabilitation, within the local community, while not being too dependent on the hospital stay, or not being forced to choose home-based care. Enhancement of home-based care might contribute to reducing the burden on physicians and nurses at acute hospitals, and might also compensate for other care services, such as emergency care and obstetrics.

One of the concerns of home-based care among physicians, patients and their families is the difficulty with hospital admissions in the event of sudden illness or deterioration. To solve this problem, the National Center for Geriatrics and Gerontology has established a "Home-based care unit". Preregistration from both a general practitioner and the patient is necessary for

admission to this unit, with the intention to continue home-based care. The patient can be admitted any time by referral of a general practitioner. The outcome of this program is eagerly awaited.

In home-based care settings, a group of professionals from diverse disciplines mutually cooperate to provide care for a patient. For such a multidisciplinary approach, it is important to choose appropriate professionals according to the condition and disease stage of the elderly patient. However, this multidisciplinary approach involves some problems. One is the legislative "gap" between health-care providers registered under the Medical and Dental Practitioners Acts and the Act on Public Health Nurses, Midwives and Nurses, and nursing care providers registered under the Long-Term Care Insurance. The other is the discrepancy in the principle between health-care and nursing-care providers. To solve these problems, it is essential to examine them along with the legislative issues, and promote home-based care, particularly at universities offering courses in geriatrics and local community hospitals where there are accumulating results of a multidisciplinary approach to caring for elderly patients, to further promote the cooperation between medical-care and social-welfare services.

3. Proposals

We make the following proposals as countermeasures against various issues in geriatrics:

(1) Development and promotion of a system that enables elderly people to participate socially and make a contribution using an interdisciplinary approach among the various areas, including nursing science, nursing care, study of social welfare, social science, engineering, psychology, economics, religion and ethics, as well as medical sciences.

Promotion of social participation and contribution of the elderly, while considering the total number of jobs and young workers, is expected to contribute to creating purpose in their lives, and reduce the growing number of older people who become frail or in need of care. It is also expected to bring about an increase in a substantial productive population, financial stability and self-sustainability for the elderly, and an upturn of the national economic activity through an increase of total consumption.

(2) Promotion of gerontology, reform, and enhancement of gerontology and geriatrics in undergraduate, postgraduate and lifelong education.

To solve problems associated with elderly people or an aged society, gerontological and geriatric research and education should be enhanced. By fostering medical professionals who understand the physical and mental traits of older adults, and those who can provide a holistic approach with consideration to organic integration with nursing care, provision of reliable care and nursing services is expected.

(3) Build geriatric medical centers in each area, and accumulate large-scale evidence of geriatric diseases and geriatrics.

For system reform of diagnosis, treatment and nursing care, evidence should be accumulated through large-scale clinical studies.

(4) Structural development and promotion of home-based care and multidisciplinary medicine and care.

Promotion of home-based care and multidisciplinary medicine and care, particularly at universities offering courses in gerontology and local community hospitals where there are accumulating results of a multidisciplinary approach to care for elderly patients, can be expected to help reduce the burden of physicians and nurses, and meet the demand of older people.

Through implementation of the aforementioned measures, Japan is expected to function as a successful model for the rest of the world.

4. Summary

The phenomenon of an aging population is often considered within a negative spectrum; however, elderly people in need of care only account for 13% of the total elderly population, and this is not being expected to further increase. We should rather focus on the fact of an increasing number of "healthy elderly individuals with rich experience and knowledge", which would not become a negative factor in the future. The restructuring of these healthy elderly resources for social development is believed to bring a permanent bright future, and it is expected that medical-care and social-welfare services will make a significant contribution within this framework. The realization of healthy longevity in society is possible; however, we should be aware that it is only possible by the integration of geriatric medicine and social welfare.

To cope with the problems that come with a rapidly aging society as the world-leading model, the development of elderly-friendly medical devices and nursing-care equipment to avoid a labor shortage is considered essential. Taking the lead in the development of medical equipment for elderly people enables us to provide other countries with aging populations with a model for success, and is also expected to contribute to the creation of new employment and an increase in export as one of the main industrial products in Japan.

The task given to the country with the longest healthy life expectancy is to try to achieve the highest level of elderly satisfaction. As a result of a community change, "roles" and "presence with respect" of the elderly have become weakened, and a medical- and nursing-care "burden" for the younger population has been casting

a dark shadow over the society. As the baby boomer generation ages into elderly status, new roles, including a future health-care workforce and volunteer activities, and community satisfaction should be rebuilt. Gerontology and geriatrics ought to take the lead in showing a practical approach to the industry and the administration to create new images of the elderly.

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Disclosure statement

The authors declare no conflict of interest.

References

1 Sasaki H. Medical Economy, Geriatrics Textbook (Japanese), 2008; 248–249.
2 Akishita M, Teramoto S, Arai H *et al.* Incidence of adverse drug reactions in geriatric wards of university hospitals. *Jpn J Geriatr (Jpn)* 2004; **41**: 303–306.
3 Guidelines for medical treatment and its safety in the elderly, edited by the Japan Geriatrics Society, 2005.
4 Iguchi A. Terminal care of the elderly. *Jpn J Geriatr (Jpn)* 2005; **42**: 285–287.
5 Proposal by the end-of-life care research group. The Scientific Council of Japan (Japanese), 2008.
6 Ito H. Perspective of geriatric medicine. *Kagaku (Jpn)* 2010; **80**: 68–72.

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

Tetsuhisa Hattori[1], Takeru Makiyama[1]*, Masaharu Akao[2], Eiji Ehara[3], Seiko Ohno[1], Moritake Iguchi[2], Yukiko Nishio[1], Kenichi Sasaki[1], Hideki Itoh[4], Masayuki Yokode[5], Toru Kita[6], Minoru Horie[4], and Takeshi Kimura[1]

[1]Department of Cardiovascular Medicine, Kyoto University Graduate School of Medicine, 54 Shogoin Kawahara-cho, Sakyo-ku, Kyoto 606-8507, Japan; [2]National Hospital Organization Kyoto Medical Center, Kyoto, Japan; [3]Department of Pediatric Cardiology, Osaka City General Hospital, Osaka, Japan; [4]Department of Cardiovascular and Respiratory Medicine, Shiga University of Medical Science, Otsu, Japan; [5]Clinical Innovative Medicine Translational Research Center, Kyoto University Hospital, Kyoto, Japan; and [6]Kobe City Medical Center General Hospital, Kobe, Japan

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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

* Corresponding author. Tel: +81 75 751 3196; fax: +81 75 751 3289, Email: makiyama@kuhp.kyoto-u.ac.jp

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.

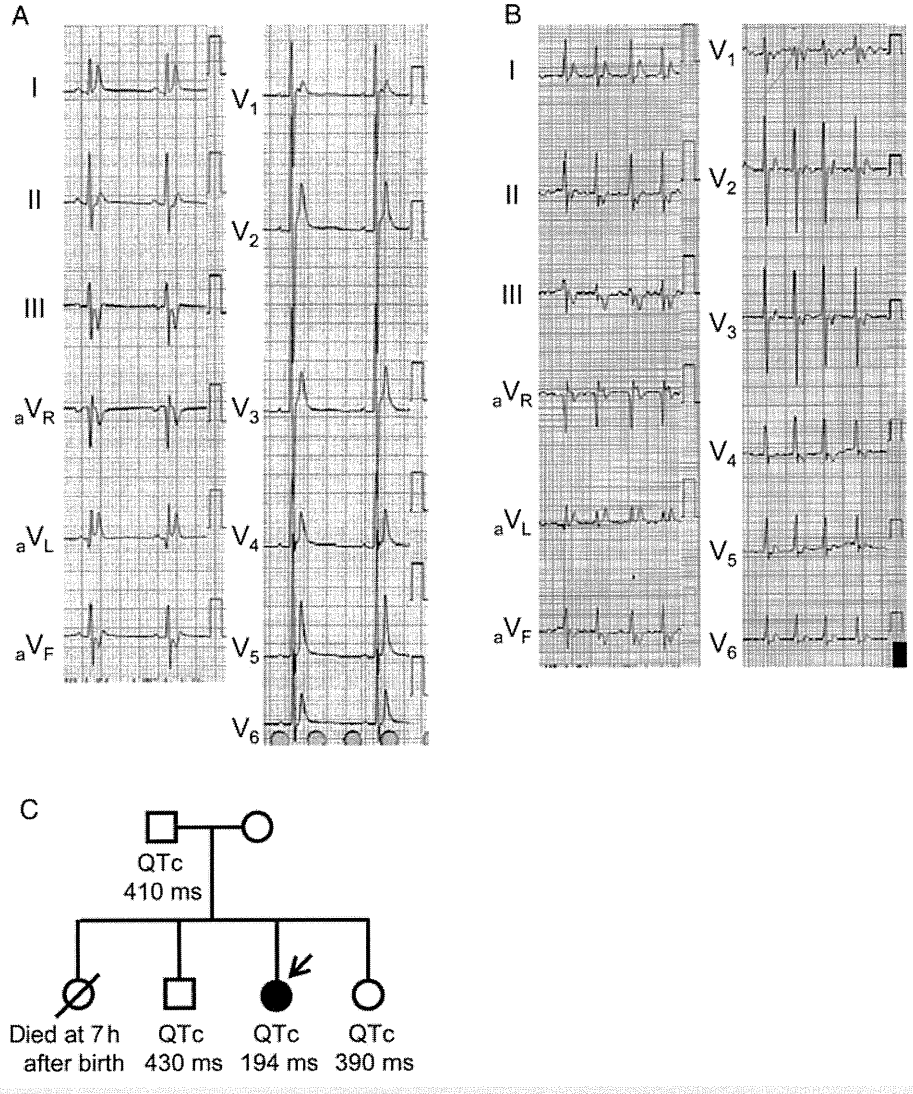

Figure 1 ECG of the proband and family pedigree. ECG shows sinus rhythm (*A*) and AF (*B*). The QT and QTc intervals were 172 and 194 ms, respectively. (*C*) Family pedigree. Arrow indicates the proband; a filled symbol indicates clinically and genetically affected individual.

She was diagnosed with SQTS from these clinical features (i.e. a markedly shortened QT interval, paroxysmal AF, and VF inducibility).

The proband had a family history of perinatal death in her elder sister (*Figure 1C*), but her family did not undergo genetic investigation or further clinical evaluation with the exception of ECGs taken for her father, elder brother, and younger sister. Genetic investigations could not be carried out due to a lack of informed consent. The ECGs for the family members displayed normal QTc intervals (410, 430, and 390 ms, respectively; *Figure 1C*).

3.2 Genetic analysis

In this patient, we screened for candidate cardiac ion channel genes (*KCNQ1*, *KCNH2*, *KCNE1-3*, *KCNJ2*, *CACNA1C*, and *SCN5A*). As a result of the genetic analysis, we identified a novel heterozygous mutation, a single-base substitution at nucleotide 902 (c.902T>A) in the *KCNJ2* gene, resulting in an amino acid change from methionine to lysine at 301 in the Kir2.1 potassium channel (*Figure 2A*). Met-301 is located in the C-terminal cytoplasmic domain of the channel (*Figure 2B*).[13] The amino acid at codon 301 (methionine) is highly conserved among different species (*Figure 2C*). Furthermore, this mutation was absent in 400 Japanese control alleles. We failed to identify mutations in any other candidate genes.

3.3 Cell surface expression of KCNJ2 mutants

In order to investigate whether the M301K mutations affect intracellular Kir2.1 trafficking, we introduced an HA epitope into the extracellular domain of KCNJ2, and examined the subcellular distribution of channels in transfected HEK 293 cells using confocal microscopy[10] (*Figure 2D*). *Figure 2D* illustrates the typical results of confocal imaging. HEK 293 cells were successfully transfected with either HA-*KCNJ2 WT*, *KCNJ2* WT/HA-M301K, or HA-M301K (*Figure 2D*, upper panels). All types of HA-tagged Kir2.1 proteins exhibited red fluorescence at the plasma membrane (*Figure 2D*, middle and lower panels), indicating that both homo- and heterozygous mutant channels were trafficking-competent.

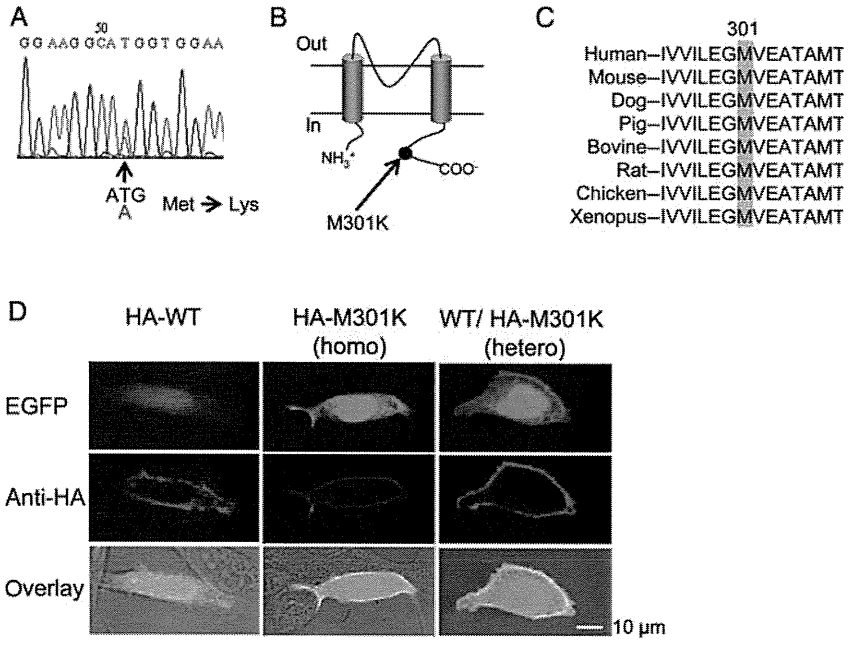

Figure 2 DNA sequence, topology, and homology. (*A*) Mutated DNA sequences derived from patient's genomic DNA. The trace shows a heterozygous substitution of thymine to adenine resulting in the amino acid change M301K. (*B*) Topology of the Kir2.1 channel showing localization of M301. (*C*) Amino acid sequence alignment of Kir2.1 channels from various species in the region surrounding codon 301 (highlighted). (*D*) Cellular localization of WT and mutant Kir2.1 channels. HA-WT indicates HA-tagged KCNJ2-WT, HA-M301K;HA-tagged KCNJ2-M301K, and WT/HA-M301K;KCNJ2-WT without HA-tagging and HA-tagged KCNJ2-M301K. The upper panel shows GFP, the middle panel shows the red fluorescence of the secondary anti-HA antibody, and the bottom panel is a mergence of the green fluorescence, red fluorescence, and transmission.

3.4 Cellular electrophysiology

We performed a functional characterization of the mutant channels in HEK 293 cells. *Figure 3A* shows representative current traces from cells expressing *KCNJ2* WT, M301K, or WT/M301K, elicited by voltage-clamp steps (duration 400 ms) from −120 to +100 mV (10 mV step), applied from a holding potential of −60 mV. The currents were normalized to cell capacitance and were plotted as a function of test potentials (*Figure 3B*). As previously reported, expression of the *KCNJ2* WT in HEK 293 cells resulted in normal inward rectifying potassium currents (*Figure 3A* left panel and blue symbols in *Figure 3B*). When M301K mutant channels were expressed alone, they were entirely non-functional (*Figure 3A* middle panel and green symbols in *Figure 3B*). In contrast, when cells were co-transfected with both equimolar WT and M301K, ample potassium currents showing a very weak inward rectification could be recorded (*Figure 3A* right panel and red symbols in *Figure 3B*). Average current densities were significantly smaller than those of WT Kir2.1 channels at potentials between −120 and −90 mV ($P < 0.05$), and significantly larger at potentials between −30 and +100 mV ($P < 0.05$).

3.5 Contribution of amino acid charge at residue 301 to Kir2.1 currents

Methionine at 301 is located within the G-loop that forms the narrowest segment of the cytoplasmic pathway,[13,14] and negatively charged amino acids on the inner wall of the cytoplasmic pore, where the G-loop is located, are known to be important for the strength of the inward rectification.[13–15] We therefore speculated that the amino acid charge at this position may be crucial for the inward rectification of Kir2.1 channels, and that its change from methionine (neutrally charged) to lysine (positively charged) may result in functional changes in Kir2.1 currents. In order to analyse the contribution of the amino acid charge at 301 to inward rectification, we changed the amino acid at M301 to another positively charged amino acid, arginine, and to another neutral amino acid, alanine, for comparison. *Figure 4A* illustrates the whole-cell Kir2.1 currents in homo- and heterozygous mutant conditions for M301R (left panel) and M301A (right panel). Homozygous M301R mutant channels displayed no functional currents, whereas WT/M301R attenuated the inward rectification (*Figure 4A* left panel). These observations suggest that the currents through the M301R channels are similar to those of the M301K channels (*Figure 3*) under both homo- and heterozygous conditions. On the other hand, in the M301A channels—in which the residual charge remained neutral—the currents showed normal inward rectification in both homo- and heterozygous conditions similar to those produced by WT Kir2.1 channels (*Figure 4A* right panel). In order to evaluate the intensity of inward rectifying properties, we assessed the rectification index, along with the ratio of the current amplitudes at 0 and −100 mV.[15] *Figure 4B* shows the rectification indexes obtained from WT, M301A (0.10 ± 0.02, $n = 10$), WT/M301A (0.073 ± 0.015, $n = 11$), WT/M301K (1.12 ± 0.16, $n = 11$), and WT/M301R (0.99 ± 0.14, $n = 11$). Although the rectification indexes for WT/301A and M301A showed no significant difference, the indexes for both WT/M301K and WT/M301R were significantly increased in comparison with WT (0.061 ± 0.01, $n = 15$, $P < 0.001$, left-most bar in *Figure 4B*).

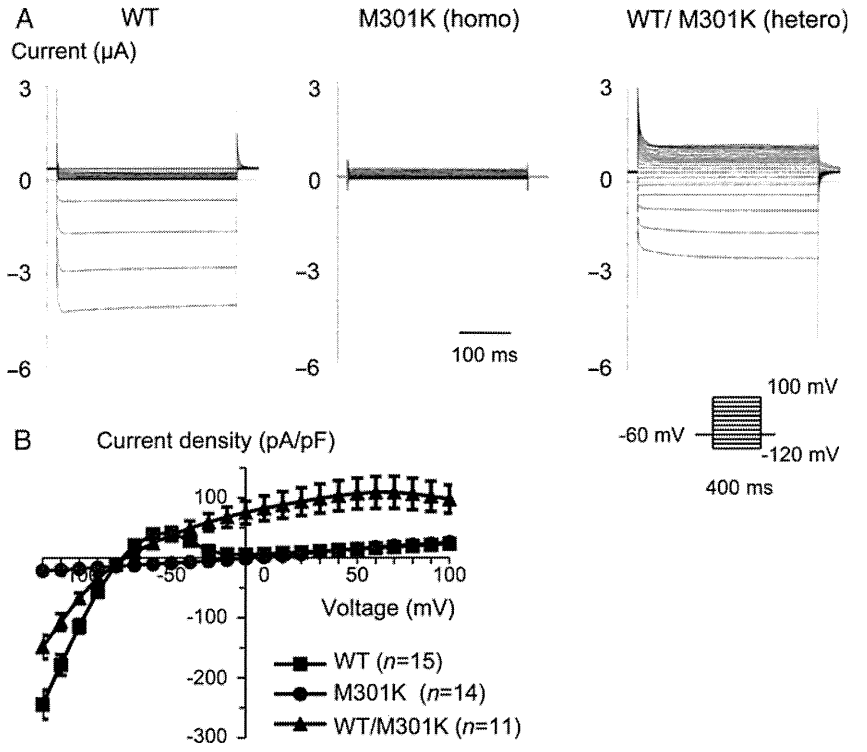

Figure 3 Voltage-clamp recordings from transfected HEK 293 cells. (*A*) Representative current traces of WT, M301K, and WT/M301K. Currents were elicited by 400 ms depolarizing voltage steps from −120 to +100 mV and from a holding potential of −60 mV. (*B*) Current–voltage relationships are plotted as the current. Current density was calculated by dividing the whole-cell current amplitude by cell capacitance. No functional currents were recorded in the homozygous M301K channels. On the other hand, the mean current densities of the WT/M301K channels are significantly larger than the WT ($P < 0.05$) at each voltage from −30 to +100 mV, and smaller at each voltage from −120 to −90 mV ($P < 0.05$).

3.6 Action potentials recording in *KCNJ2*-M301K-transfected NRVMs

We investigated the impacts of M301K mutant Kir2.1 channels on NRVMs' action potentials using a transient transfection method. *Figure 5A* shows typical action potentials recorded for non-transfected (control) NRVMs (*Figure 5A*, left panel), and NRVMs transfected with *KCNJ2* WT or WT/M301K (*Figure 5A* middle and right panels, respectively). Phase 3 repolarization was accelerated in the *KCNJ2* WT- and WT/M301K-overexpressed groups (*Figure 5A* middle and right panels, respectively) and we could further note that the dome is nearly lost in the WT/M301K group. APD_{90} was significantly abbreviated in the *KCNJ2* WT-overexpressed group (28.2 ± 3.4 ms, $n = 10$, $P < 0.001$, *Figure 5A*, middle panel) in comparison with the control group (123.3 ± 12.2 ms, $n = 11$, *Figure 5A*, left panel; bar graphs in *Figure 5B*). Additionally, APD90 was significantly shorter in the WT/M301K mutant-overexpressed group (9.4 ± 2.1 ms, $n = 16$, $P < 0.001$, *Figure 5A*, right panel; bar graph in *Figure 5B*) than in the WT-overexpressed group.

4. Discussion

4.1 Major findings

In the present study, we identified a novel heterozygous *KCNJ2* mutation, M301K, in a patient with a markedly shortened QT interval. The QT interval, 172 ms, of this patient is the shortest among previous SQTS reports,[2–7,16] to our knowledge. The methionine at position 301 is located in the C-terminus of Kir2.1 channel, and is considered to form a pore-facing loop region.[13] Functional assays using a heterologous expression system revealed that homozygous M301K Kir2.1 channels carried no currents with preserved plasma membrane expression; however, heterozygous WT/M301K Kir2.1 channels attenuated inward rectifying properties, which resulted in increased outward currents for positive voltages and negative voltages down to −30 mV. Significant increases in outward currents within the voltage range of the action potentials shortened APD by accelerating membrane repolarization as shown in *Figure 5*, which is implicated in increased cardiac vulnerability.

4.2 Impaired inward rectification of Kir2.1 currents: a novel mechanism predisposing SQTS

In 2005, Priori *et al.*[4] first reported a heterozygous gain-of-function *KCNJ2* mutation, D172N, in a patient with SQTS. In the report, homozygous D172N Kir2.1 channels displayed larger outward currents compared with WT Kir2.1 alone, and heterozygous channels yielded intermediate results. In both homozygous and heterozygous D172N mutant channels, the inward rectification properties of Kir2.1 currents were preserved. In heterozygous M301K mutant channels identified in our patient, however, the inward rectification was significantly reduced, allowing ample outward potassium currents at positive potentials. In addition, it should be emphasized that the homozygous M301K mutant channels were non-functional. These functional changes, such as the impaired inward rectification of the

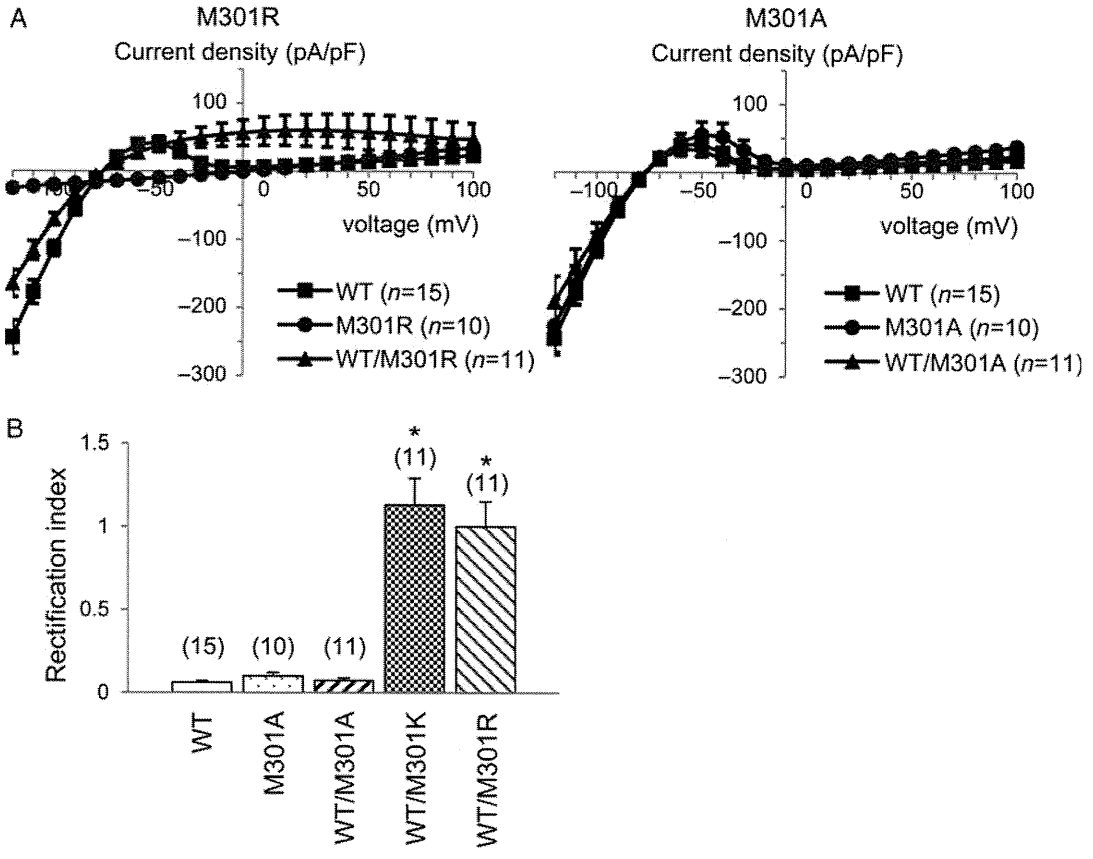

Figure 4 Comparison of macroscopic currents through WT Kir2.1 and mutants. (*A*) Current–voltage relationships for WT, M301R, and M301A are shown. M301R mutant channels displayed no functional currents and WT/M301R mutant channels displayed decreased inward rectification. On the other hand, the currents recorded in the homozygous M301A and heterozygous WT/M301A mutant channels showed no significant difference from WT. (*B*) Rectification index for WT ($n = 15$), M301A ($n = 10$), WT/M301A ($n = 11$), WT/M301K ($n = 11$), and WT/M301R ($n = 11$) channels. The rectification index was calculated by dividing the value of the outward currents measured at 0 mV by the absolute value of the inward currents measured at −100 mV. $^*P < 0.001$.

Kir 2.1 currents resulting in increased outward currents, are a novel *KCNJ2* gain-of-function mechanism predisposing SQTS.

The phenotypic characteristics of our index patient somewhat differ from those of the *KCNJ2*-D172N mutation carriers.[4] No apparent arrhythmias were recorded with D172N mutation carriers. On the other hand, our M301K patient showed paroxysmal AF and multiple disorders. Additionally, mechanical stimulation by a Swan–Ganz catheter induced paroxysmal supraventricular tachycardia and VF. Moreover, the QTc interval in our patient was much shorter (QTc = 194 ms, *Figure 1*) than that of the D172N carriers (QTc = 315 and 320 ms).[4] Another gain-of-function *KCNJ2* mutation, V93I, was reported in a familial AF case.[17] Their functional analysis showed a similar result with D172N, but the affected members had normal QT intervals. These diverse clinical manifestations may be related to the extent and the different gain-of-function mechanisms of the Kir2.1 currents.

4.3 Relationship between impaired inward rectification and charged amino acid residues at 301

Kir currents exhibit strong inward rectification, which is thought to be due to pore blocking induced by multivalent ions from intracellular Mg^{2+}.[18–20] Channel blockade by physiological concentrations of Mg^{2+} is influenced by the electrostatic negativity within the cytoplasmic pore.[15] Negative charges on the inner wall of the cytoplasmic pore are therefore key determinants of the strength of the inward rectification. Many amino acid residues inside the pore demonstrate interactions with the ion over long distances, suggesting that mutations potentially affect ion or blocker energetics over the entire pore profile.[14,21] The M301K mutation causes the change of the amino acid residue at 301 from a non-charged amino acid residue, methionine, to a positively charged residue, lysine. In order to evaluate the importance of the charge at 301, additional whole-cell patch-clamp recordings were carried out on M301A (remained neutral) and M301R (neutral to positive) (*Figure 4*). Inward rectification of Kir2.1 currents was well preserved in both homozygous and heterozygous M301A channels. Heterozygous M301R channels, however, attenuated inward rectification, and homozygous M301R channels were non-functional similar to that of the M301K channels. These electrophysiological results indicate that the neutral amino acid residue at 301 plays an important role in generating Kir2.1 inward rectification. The decrease in the net negative charge within the cytoplasmic pore may facilitate the reduction in both the susceptibility of the channel to Mg^{2+} block and the voltage dependence of the blockade. It

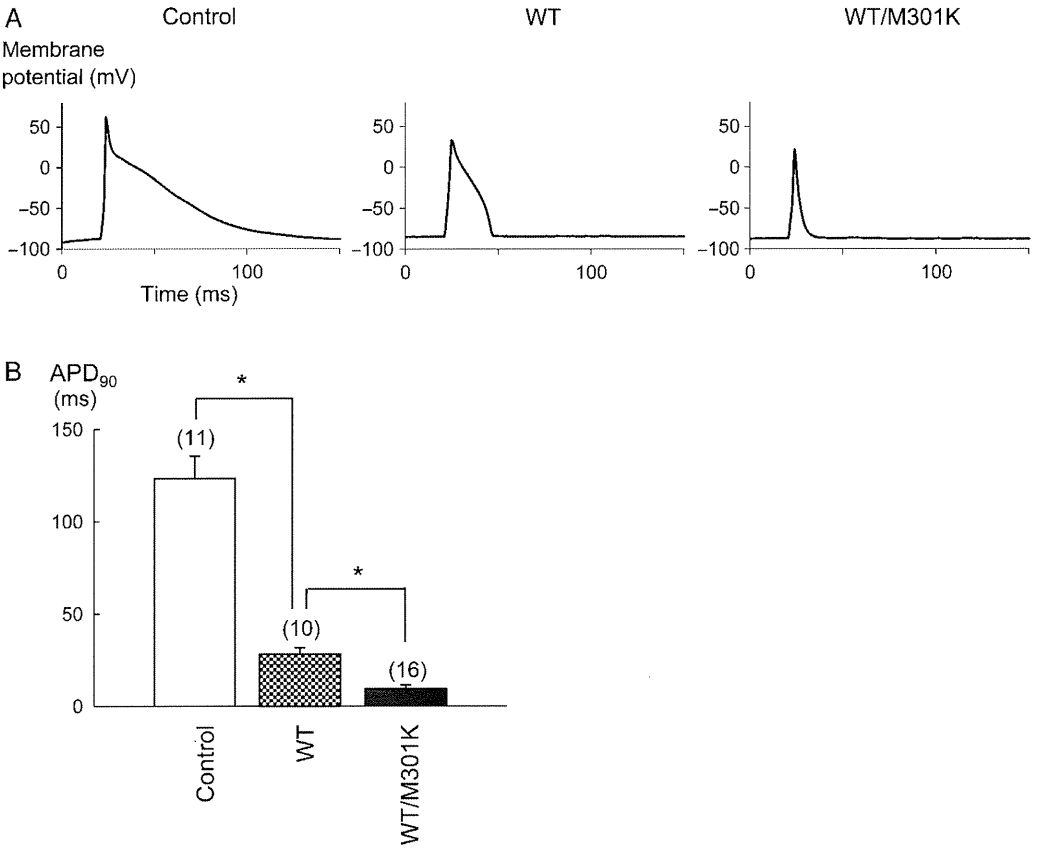

Figure 5 Effects of the M301K mutation on NRVM action potentials. Typical action potentials were demonstrated in a non-transfected cell (*A*), in a WT-overexpressed cell (*B*), and in a heterozygous overexpressed cell (*C*). Graphs show APD at 90% repolarization from the overshoot (*D*). In WT-overexpressed NRVMs, the plateau phase of the cardiac AP was markedly abbreviated, resulting in short repolarization. Under the heterozygous overexpressed condition, the results exhibited virtually no plateau phase, and the mean APD_{90} was significantly shorter in comparison with WT overexpressed alone. $^*P < 0.001$.

remains unknown why only tentative hetero-multimers of WT and M301K are active and lose their inward rectification properties. In homozygous M301K channels, all of the tetrameric subunits must have a positively charged lysine at 301, which may impair potassium ion permeation due to a conformational change in the near-pore region.

4.4 Heterozygous *KCNJ2*-WT/M301K overexpression shortened APD in NRVMs

In cardiomyocytes, Kir2.1, Kir2.2, and Kir2.3 channels are supposed to be able to co-assemble in order to modulate their channel properties.[22] Thus, there can be a multitude of Kir2.x heteromultimers, and to date a wide range of single-channel conductances of inward rectifier channels have been reported in studies conducted on various mammalian myocytes, including human.[23–25] This variety at the individual channel level may contribute to the different stoichiometry of the tetrameric channels.[26] Because Kir2.1 is a major component of IK1 in the myocardium, we overexpressed the *KCNJ2* M301K mutant channels in NRVMs to examine the effects of the mutation on APD. Overexpression with WT alone resulted in shorter APD in comparison with non-transfected myocytes (*Figure 5B*). These results are consistent with a previously published report.[27] Notably, heterozygous overexpression with WT and M301K further amplified the shortened APD (*Figure 5C*). These results were compatible with the electrophysiological changes assessed in HEK 293 cells, because the heterozygous WT/M301K channels showed a larger outward current than WT Kir2.1 channels under the physiological range of membrane potentials (*Figure 3*). Weak inward rectification observed in the heterozygous WT/M301K channels suggests that potassium ion can get through Kir2.1 channel at depolarized potential, probably resulting in loss of the action potential dome recorded in the *KCNJ2* WT/M301K-overexpressed group. The experiments were performed using a transient overexpression system that was different from the patient's heart, and the amount of overexpressed channels was difficult to be estimated accurately. But, these results are beneficial in understanding that the heterozygous *KCNJ2* M301K mutation could abbreviate APD and cause an extremely short-QT interval in the patient's ECG.

4.5 Clinical features of the index patient with *KCNJ2*-M301K

Regarding the clinical criteria for the diagnosis of SQTS, they have yet to be defined. However, we should consider SQTS in a patient presenting with a QTc <340 ms and other factors suggestive of arrhythmia (such as syncope or family history of sudden death).[28] A prominent clinical manifestation of SQTS is arrhythmias, such as AF

and VF.[1–5,7] In this patient, however, additional medical histories not limited to arrhythmias, such as severe mental retardation, abnormal proliferation of the oesophageal blood vessels, epilepsy, and Kawasaki' disease, were also documented. Because *KCNJ2* is known to be expressed in a variety of tissues, such as cardiac and skeletal muscle, the brain, arterial smooth muscle cells and developing bony structures of the craniofacial region, extremities, and vertebrae,[29–31] some of her compound disorders may be attributed to the *KCNJ2* mutation. In fact, loss-of-function mutations in *KCNJ2* cause Andersen–Tawil syndrome, which is characterized by prolonged repolarization, dysmorphic features, and periodic paralysis.[10,32] In the family of our female patient, we could not perform extensive genetic testing. We cannot exclude the possibility of the presence of other affected genes. Further analyses using knock-in mice or induced pluripotent stem cells would culminate monumental insight into the relationship between the *KCNJ2* M301K mutation and the patient's extra-cardiac phenotypes.

4.6 Conclusions

We described a novel *KCNJ2* gain-of-function mutation, M301K, in a patient with SQTS. Functional assays revealed no functional currents in the homozygous channels, whereas impaired inward rectification in the heterozygous channels manifested in larger outward currents, which is a novel mechanism predisposing SQTS.

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Conflict of interest: none declared.

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References

1. Gussak I, Brugada P, Brugada J, Wright RS, Kopecky SL, Chaitman BR *et al.* Idiopathic short QT interval: a new clinical syndrome? *Cardiology* 2000;**94**:99–102.
2. Brugada R, Hong K, Dumaine R, Cordeiro J, Gaita F, Borggrefe M *et al.* Sudden death associated with short-QT syndrome linked to mutations in HERG. *Circulation* 2004; **109**:30–35.
3. Bellocq C, van Ginneken AC, Bezzina CR, Alders M, Escande D, Mannens MM *et al.* Mutation in the KCNQ1 gene leading to the short QT-interval syndrome. *Circulation* 2004;**109**:2394–2397.
4. Priori SG, Pandit SV, Rivolta I, Berenfeld O, Ronchetti E, Dhamoon A *et al.* A novel form of short QT syndrome (SQT3) is caused by a mutation in the KCNJ2 gene. *Circ Res* 2005;**96**:800–807.
5. Antzelevitch C, Pollevick GD, Cordeiro JM, Casis O, Sanguinetti MC, Aizawa Y *et al.* Loss-of-function mutations in the cardiac calcium channel underlie a new clinical entity characterized by ST-segment elevation, short QT intervals, and sudden cardiac death. *Circulation* 2007;**115**:442–449.
6. Templin C, Ghadri JR, Rougier JS, Baumer A, Kaplan V, Albesa M *et al.* Identification of a novel loss-of-function calcium channel gene mutation in short QT syndrome (SQTS6). *Eur Heart J* 2011;**32**:1077–1088.
7. Hong K, Piper DR, Diaz-Valdecantos A, Brugada J, Oliva A, Burashnikov E *et al.* De novo KCNQ1 mutation responsible for atrial fibrillation and short QT syndrome in utero. *Cardiovasc Res* 2005;**68**:433–440.
8. Garberoglio L, Giustetto C, Wolpert C, Gaita F. Is acquired short QT due to digitalis intoxication responsible for malignant ventricular arrhythmias? *J Electrocardiol* 2007; **40**:43–46.
9. Akao M, Ohler A, O'Rourke B, Marban E. Mitochondrial ATP-sensitive potassium channels inhibit apoptosis induced by oxidative stress in cardiac cells. *Circ Res* 2001; **88**:1267–1275.
10. Haruna Y, Kobori A, Makiyama T, Yoshida H, Akao M, Doi T *et al.* Genotype-phenotype correlations of KCNJ2 mutations in Japanese patients with Andersen-Tawil syndrome. *Hum Mutat* 2007;**28**:208.
11. Ma D, Tang XD, Rogers TB, Welling PA. An Andersen-Tawil syndrome mutation in Kir2.1 (V302M) alters the G-loop cytoplasmic K+ conduction pathway. *J Biol Chem* 2007;**282**:5781–5789.
12. Ballester LY, Benson DW, Wong B, Law IH, Mathews KD, Vanoye CG *et al.* Trafficking-competent and trafficking-defective KCNJ2 mutations in Andersen syndrome. *Hum Mutat* 2006;**27**:388.
13. Pegan S, Arrabit C, Zhou W, Kwiatkowski W, Collins A, Slesinger PA *et al.* Cytoplasmic domain structures of Kir2.1 and Kir3.1 show sites for modulating gating and rectification. *Nat Neurosci* 2005;**8**:279–287.
14. Tai K, Stansfeld PJ, Sansom MS. Ion-blocking sites of the Kir2.1 channel revealed by multiscale modeling. *Biochemistry* 2009;**48**:8758–8763.
15. Fujiwara Y, Kubo Y. Functional roles of charged amino acid residues on the wall of the cytoplasmic pore of Kir2.1. *J Gen Physiol* 2006;**127**:401–419.
16. Gollob MH, Redpath CJ, Roberts JD. The short QT syndrome: proposed diagnostic criteria. *J Am Coll Cardiol* 2011;**57**:802–812.
17. Xia M, Jin Q, Bendahhou S, He Y, Larroque MM, Chen Y *et al.* A Kir2.1 gain-of-function mutation underlies familial atrial fibrillation. *Biochem Biophys Res Commun* 2005;**332**:1012–1019.
18. Matsuda H, Saigusa A, Irisawa H. Ohmic conductance through the inwardly rectifying K channel and blocking by internal Mg2+. *Nature* 1987;**325**:156–159.
19. Horie M, Irisawa H, Noma A. Voltage-dependent magnesium block of adenosine-triphosphate-sensitive potassium channel in guinea-pig ventricular cells. *J Physiol* 1987;**387**:251–272.
20. Hille B. *Ion Channels of Excitable Membranes*. Sunderland: Sinauer Associates, 2001.
21. Robertson JL, Palmer LG, Roux B. Long-pore electrostatics in inward-rectifier potassium channels. *J Gen Physiol* 2008;**132**:613–632.
22. Liu Y, Fowler CD, Wang Z. Ontogeny of brain-derived neurotrophic factor gene expression in the forebrain of prairie and montane voles. *Brain Res Dev Brain Res* 2001; **127**:51–61.
23. Nakamura TY, Artman M, Rudy B, Coetzee WA. Inhibition of rat ventricular IK1 with antisense oligonucleotides targeted to Kir2.1 mRNA. *Am J Physiol* 1998;**274**: H892–H900.
24. Picones A, Keung E, Timpe LC. Unitary conductance variation in Kir2.1 and in cardiac inward rectifier potassium channels. *Biophys J* 2001;**81**:2035–2049.
25. Wible BA, De Biasi M, Majumder K, Taglialatela M, Brown AM. Cloning and functional expression of an inwardly rectifying K+ channel from human atrium. *Circ Res* 1995;**76**: 343–350.
26. Preisig-Muller R, Schlichthorl G, Goerge T, Heinen S, Bruggemann A, Rajan S *et al.* Heteromerization of Kir2.x potassium channels contributes to the phenotype of Andersen's syndrome. *Proc Natl Acad Sci USA* 2002;**99**:7774–7779.
27. Miake J, Marban E, Nuss HB. Functional role of inward rectifier current in heart probed by Kir2.1 overexpression and dominant-negative suppression. *J Clin Invest* 2003;**111**:1529–1536.
28. Viswanathan MN, Page RL. Short QT: when does it matter? *Circulation* 2007;**116**: 686–688.
29. Raab-Graham KF, Radeke CM, Vandenberg CA. Molecular cloning and expression of a human heart inward rectifier potassium channel. *Neuroreport* 1994;**5**:2501–2505.
30. Karkanis T, Li S, Pickering JG, Sims SM. Plasticity of KIR channels in human smooth muscle cells from internal thoracic artery. *Am J Physiol Heart Circ Physiol* 2003;**284**: H2325–H2334.
31. Karschin C, Karschin A. Ontogeny of gene expression of Kir channel subunits in the rat. *Mol Cell Neurosci* 1997;**10**:131–148.
32. Plaster NM, Tawil R, Tristani-Firouzi M, Canun S, Bendahhou S, Tsunoda A *et al.* Mutations in Kir2.1 cause the developmental and episodic electrical phenotypes of Andersen's syndrome. *Cell* 2001;**105**:511–519.

患者まで届く

再生医療 生体吸収性担体を用いた重症下肢虚血に対する血管新生療法

Novel therapeutic angiogenesis for ischemic limb using biodegradable gelatin hydrogel

丸井　晃[1)2)]，田畑 泰彦[3)]，柳　茂樹[1)2)]，山本 雅哉[3)]，米澤　淳[4)]，桂　敏也[4)]，手良向 聡[2)]，横出 正之[2)]，清水　章[2)]，中尾 一和[2)5)]，坂田 隆造[1)]

Marui, Akira[1)2)] / Tabata, Yasuhiko[3)] / Yanagi, Shigeki[1)2)] / Yamamoto, Masaya[3)] / Yonezawa, Atsushi [4)] / Katsura, Toshiya[4)] / Teramukai, Satoshi[2)] / Yokode, Masayuki[2)] / Shimizu, Akira[2)] / Nakao, Kazuwa[2)5)] / Sakata, Ryuzo[1)]

1）京都大学大学院医学研究科 心臓血管外科
Department of Cardiovascular Surgery, Kyoto University Graduate School of Medicine
2）京都大学医学部附属病院 探索医療センター
Translational Research Center, Kyoto University Hospital
3）京都大学再生医科学研究所
Institute for Frontier Medical Sciences, Kyoto University
4）京都大学医学部附属病院 薬剤部
Department of Pharmacy, Kyoto University Hospital
5）京都大学大学院医学研究科 内分泌代謝内科
Department of Medicine and Clinical Science, Kyoto University Graduate School of Medicine

Key words
血管新生療法／塩基性線維芽細胞増殖因子（bFGF）／ゼラチンハイドロゲル／重症下肢虚血

Summary

Critical limb ischemia remains a challenge. To overcome shortcomings or limitations of gene therapy or cell transplantation, a sustained release system of basic fibroblast growth factor (bFGF) using biodegradable gelatin hydrogel has been developed. A phase I–IIa study was performed, in which 7 patients had critical limb ischemia. They were intramuscularly injected with 200μg of bFGF-incorporated gelatin hydrogel microspheres into the gastrocnemius of the ischemic limb. Significant improvements were observed in the distance walked in 6 min, transcutaneous oxygen pressure, pain scale, and non-healing foot ulcer. The blood levels of bFGF were undetected or within the normal level in all patients. The sustained release of bFGF from gelatin hydrogel might be simple, safe, and effective to achieve therapeutic angiogenesis because it did not need genetic materials or collection of implanted cells. To commercialize and systemize for Health Insurance of bFGF-incorporated gelatin hydrogel, we are conducting a novel clinical study approved by the system for advanced medicine with use of gelatin hydrogel made under the regulation of good manufacturing practice (GMP).

はじめに

再生医療はトランスレーショナルリサーチであり，患者さんまで届くことを最終目標とする分野であることは言うまでもない。血管新生療法は再生医療の中でもパイオニア的な領域であり，下肢血管の動脈硬化を原因とする閉塞性動脈硬化症（arteriosclerosis obliterans：ASO）に対する血管内皮増殖因子（vascular endothelial growth factor：VEGF）の遺伝子治療が1995年に米国タフツ大学のIsner教授らにより発表されて以来，さまざまなサイトカインや増殖因子を使った血管新生療法が試みられている[1]。また，自己骨髄細胞などを血管新生に応用する細胞移植治療もさまざまな試みがなされている。しかし現時点で製品化，もしくは保険診療化されている血管新生療法が存在しないのは残念なことである。多くの手法では動物実験や第Ⅰ～Ⅱ相の臨床試験での有効性は確認されているが，第Ⅲ相臨床試験や治験では十分な有効性が認められておらず，今後のさらなる試みが期待されるところである。

再生医療にはさまざまな手法があり，人工多能性幹細胞（iPS細胞）・胚性幹細胞（ES細胞）などは将来的には，臓器移植に代わる治療法として再生医療の大きな分野を切り開く可能性を秘めているが，血管新生という比較的シンプルな再生医療については，臨床効果が同等であれば手技が簡便でかつ低コストである方法であることが「患者まで届く再生医療」を達成するために非常に重要であると考えている。ここでは下肢の血管新生療法について我々の試みを紹介する。

重症虚血下肢

近年，食生活の欧米化による糖尿病患者の増加，高齢化社会に伴い，動脈硬化を基礎とするASOは増加している。特に，安静時下肢痛や潰瘍を有する重症下肢虚血患者については，欧米の報告では，人口100万人当たり毎年500～1,000人の新たな患者が発生するとされている[2]。血行改善薬の開発，経皮的血管形成術およびバイパス術などの外科治療はその治癒に大きく寄与しているが，ASOやバージャー病のために下肢切断を余儀なくされる患者も多く，現存の治療法が十分であるとは言い難い。下腿切断以上の大切断が必要な患者は年間100万人当たり120～500人との報告があり，下肢切断に至った患者の精神的，肉体的苦痛は計り知れないものがあり，また日常活動性やQOLの低下は著明である。そのため重症下肢虚血に対する新しい治療の開発は非常に重要なテーマである。

血管新生療法

血管新生療法には，主として①血管新生蛋白を局所または全身に投与する蛋白治療，②血管新生蛋白を発現する遺伝子を投与する遺伝子治療，③細胞移植治療，の3種類がある。血管新生蛋白は通常生体内での半減期が極めて短いため，蛋白療法では血管新生蛋白の全身への大量・反復投与を必要とした。そのため大量反

表1．血管新生療法の課題

蛋白治療	遺伝子治療	細胞移植治療
・極めて短い半減期 ・全身・反復・大量投与による副作用 腫瘍増殖 網膜増殖 動脈硬化促進	・遺伝子発現のコントロールが困難 ・免疫・炎症反応 ・長期的な安全性	・細胞種の適否 ・細胞採取法（全身麻酔・G-CSF） ・細胞分化法・培養法 ・免疫反応 ・倫理的問題 ・複雑な手技 ・高コスト

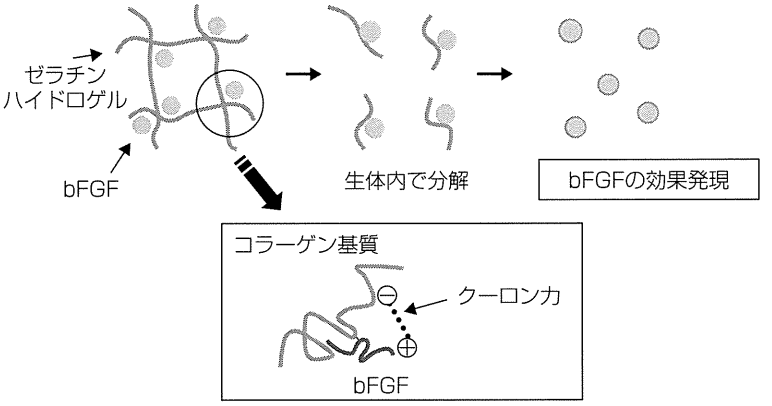

図1　bFGF徐放化ゼラチンハイドロゲルの作用機序

復投与による腫瘍形成・網膜増殖・動脈硬化の悪化などの副作用が問題となった（**表1**）。そこで蛋白の有効濃度を治療部位に一定期間保つ方法として遺伝子治療が行われているが，ウイルスベクターなどの遺伝材料の免疫・炎症反応などの安全性の問題はいまだ解決されておらず，人体での遺伝子の発現を自由にコントロールできる方法も十分ではない[3)4)]。また細胞移植治療については，自己骨髄または末梢血単核球細胞による血管新生療法では，日本で行われた骨髄単核球細胞移植による第Ⅱ相試験では有効性が報告されており[5)6)]，また，造血性蛋白である顆粒球コロニー刺激因子（G-CSF）によって末梢血に単核球細胞を動員し血管新生療法を行う治療法についても，第Ⅱ相試験で有効性が報告されている[7)]。しかし，自己骨髄幹細胞を大量に採取する際の全身麻酔や採取手技による患者侵襲は決して少なくなく，また末梢血幹細胞を動員するためのG-CSFは一時的に白血球数を数万レベルにまで増加させ，それによる心筋梗塞・脳梗塞・脾臓破裂などの報告があり，安全性の面での改善の余地が残っている。

bFGF徐放化ゼラチンハイドロゲル

そこで我々は従来の血管新生療法の課題を解決すべく，遺伝材料や細胞移植を用いず，かつ血管新生蛋白を十分かつ必要期間作用させるためのドラッグデリバリーシステム（DDS）を生体吸収材料（ゼラチンハイドロゲル）にて確立し，安全性・有効性を両立させる新たな血管新生療法を開発した[8)]。豚皮ゼラチンを化学架橋して作製するゼラチンハイドロゲルは生体吸収性材料であり，さまざまな蛋白をその生理活性を保った状態で物理化学的相互作用（主に静電的相互作用）によって固定化包含する（**図1**）。蛋白を包含したゼラチンハイドロゲルが生体内で分解されると，それとともに包含された蛋白がハイドロゲルから徐放化される。ハイドロゲルの分解期間は数日～数ヵ月と調整可能で，その期間，ほぼ一定して蛋白が局所で徐放化され，その生物活性が発揮されるという極めて優秀なDDSである。

この方法の最大の利点はゼラチンハイドロゲルの生体吸収性であり，臨床における安全性が期待できる点である。ゼラチンはすでに臨床応用されている材料であり，生体内で分解された後，生体に毒性のないアミノ酸になる。またゼラチンハイドロゲルは，蛋白の発現の時期，濃度をきめ細かくコントロールでき，シート状・ディスク状・粒子状などの成形が可能で応用箇所の幅が広く，さまざまな蛋白を組み合わせての徐放投与が可能である（DDSにおける幅広い応用性）。さ

らに，細胞移植・遺伝子治療と比べて患者投与までの手技が非常に簡便で低コストであり，実施可能性という点でも臨床向きと言える。

基礎研究および先行臨床試験

我々は，このゼラチンハイドロゲルから優れた血管新生・組織再生因子である塩基性線維芽細胞増殖因子（basic fibroblast growth factor：bFGF）を徐放させることにより，さまざまな動物実験で有効性を証明してきた。例をあげると，虚血性心疾患における血管新生・心機能回復[9)-14)]，細胞移植治療前の血管新生誘導[15)]，胸骨感染後の骨再生促進[16)17)]，重症下肢虚血に対する血管新生[18)-27)]，肺高血圧症治療[28)]，人工臓器感染予防[29)30)]など，非常に多くの領域での有効性を確認してきた。

さらに，これらの結果を踏まえてASO 3名，バージャー病4名，計7名の重症下肢虚血患者に対して第

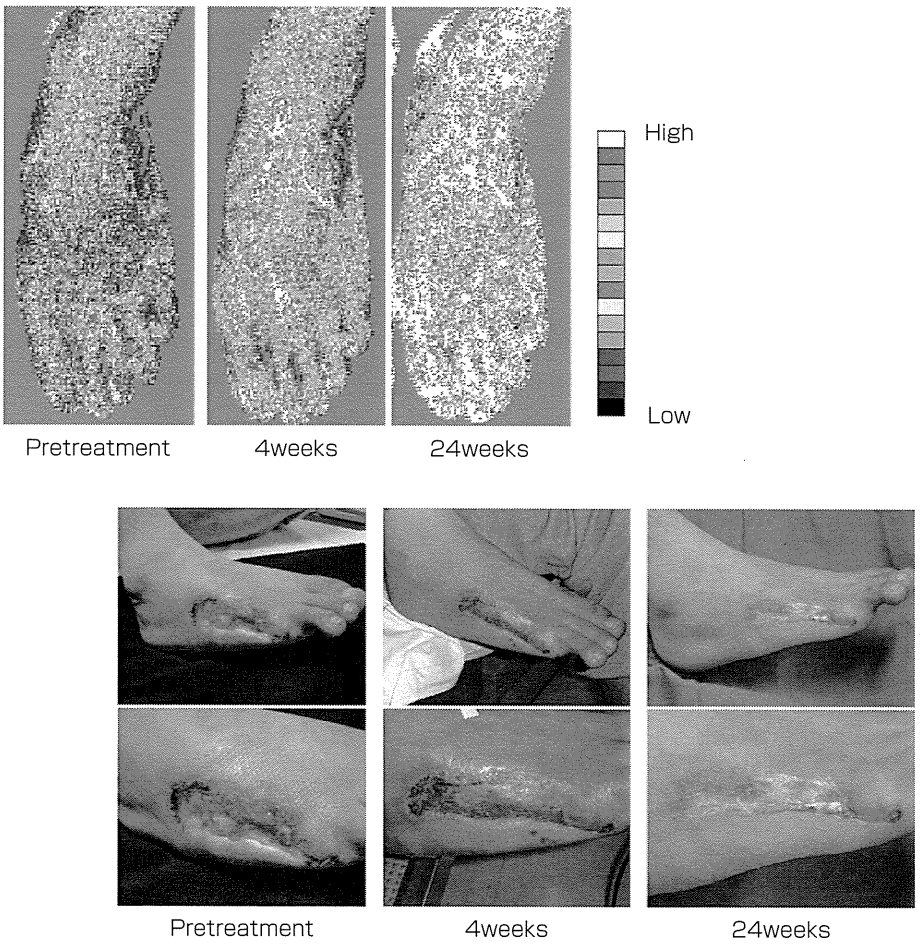

図2　（➔巻末Color Gravure参照）
上：レーザードップラー血流計による血流改善効果の確認
下：下肢潰瘍の著明な改善

（文献31より引用・改変）

Ⅰ～Ⅱ相臨床試験を行った[31]。bFGFは皮膚科領域で褥瘡治療薬として製造販売されているbFGFスプレー製剤(フィブラスト®スプレー)を使用した。bFGF徐放化ゼラチンハイドロゲル200μgを腰椎麻酔下に重症下肢虚血患者の下腿を中心に40ヵ所筋肉内投与を行い(単回投与)，4週後・24週後に臨床効果の評価を行った。実際の投与時間はbFGF徐放化ゼラチンハイドロゲルの準備時間を含めて約1時間程度であった。有効性評価としては，潰瘍を有した6例中3例で潰瘍消失，1例で縮小を認めた。また6分間歩行距離・疼痛スコアおよび経皮的酸素分圧TcO_2・レーザードップラー血流計での評価血流は4週後・24週後ともに有意に改善した(**図2**，**表2**)。

高度医療評価制度と治験薬GMP

これらの結果から，bFGF徐放化ゼラチンハイドロゲルの有効性が示唆されたが，「患者さんまで届く」にはまだ多くのハードルが存在するのも事実である。製剤の市販化を目指すためには医師主導治験もしくは企業治験が必要であるが，相応の臨床実績および有効性のデータがなければ，一朝一夕に企業と合意に至ることは現実には困難である。そこで我々は厚生労働省の「第三項先進医療(高度医療評価制度)」に着目した。薬事法の承認などが得られていない医薬品・医療機器の使用を伴う先進的な医療技術については，現時点では原則として保険との併用が認められていない。しかし医療の高度化や，これらの医療技術を安全かつ低い負担で受けたいという患者のニーズは少なからず存在している。そこで高度医療評価制度は，これらの医療技術のうち一定の要件下に行われるものについて，先進医療の一類型として保険診療との併用を認め，薬事法による申請等に繋がる科学的評価可能なデータ収集の迅速化を図ることを目的として2008年4月に創設された。

そこで今回，前述の重症下肢虚血患者に対する第Ⅰ-Ⅱ相臨床試験をさらに進め，bFGF(フィブラスト®スプレー)の効能外使用を含む第三項先進医療(高度医療評価制度)を承認取得し，保険医療に向けた臨床試験を開始した。対象患者は先行試験同様に重症下肢虚血のASOおよびバージャー病患者で，bFGF徐放化ゼラチンハイドロゲルの安全性および有効性を評価することを目的としている[32]。また今回の試験では，将来実施する予定の治験を勘案し，ゼラチンハイドロゲ

表2．血流改善効果

	治療前	治療4週後	24週後
上肢下肢血圧比	0.62±0.12	0.73±0.14*	0.68±0.11
6分間歩行距離(m)	295±42	448±81*	491±85*
疼痛スケール†	3.5±0.2	1.3±0.4*	1.0±0.6*
TcO_2 (mmHg)	53.5±5.2	66.5±5.0*	65.5±4.0*
レーザードップラー(relative unit)	436±66	520±80*	614±61*
サーモグラフィー(℃)	27.2±0.54	27.9±0.44*	27.4±0.48

*$p < 0.05$ vs. 治療前

疼痛スケール†

0：疼痛を認めない
1：非ステロイド性抗炎症剤を必要としない軽度の疼痛がある
2：非ステロイド性抗炎症剤の使用にて消失する疼痛がある
3：非ステロイド性抗炎症剤を使用しても自制内の疼痛がある
4：非ステロイド性抗炎症剤を使用しても我慢しがたい疼痛がある

(文献31より引用・改変)

ルの製造を「治験薬の製造管理，品質管理等に関する基準（治験薬GMP）」に準じて調製を行っている。治験薬GMPとはgood manufacturing practiceの略で，医薬品や医療用具，食品などの安全性を含む品質保証の手段として，工場などの製造設備（ハード）およびその品質管理・製造管理（ソフト）について，事業者が遵守しなければならないことを明確にした基準のことであり，臨床治験に使用する薬剤は，治験薬GMP基準を満たしていることが要求される。今回の試験では京都大学薬剤部にGMP基準に則った特殊無菌製剤室を設置し，製造の手順書および記録書の整備・無菌試験や安定性試験などの品質管理項目を設定し，安全で高品質な試験薬を製造している。高度医療評価制度下での新規臨床研究は2010年9月より患者登録を開始し現在試験を遂行中である。

おわりに

再生医療の先駆けとして脚光を浴びた虚血下肢に対する血管新生療法であるが，残念ながら製品化・保険診療化されたものは現時点では存在しない。血管新生療法にはさまざまなアプローチがあるが，組織再生や臓器再生と比較して血管新生というシンプルな現象を誘導するには，同等の効果が得られるなら細胞移植や遺伝子治療よりも安全・簡便かつ低コストの方法はより臨床応用に近いと思われる。実際，筆者のような一臨床医としても手技的な煩雑さは可能な限り回避したいのが本音で，シンプルな方法は非常に魅力的である。将来的に製剤がキット化され，かつ保険診療化されれば，虚血が進行性の患者さんには定期的かつ簡便に血管新生療法を行うことが可能になると思われ，患者負担からも簡便な方法がより現実的と考えている。

また血管新生療法はそれだけで重症下肢虚血の患者を救済することは困難であり，カテーテル治療や外科的血行再建と併用することによってより効果を発揮すると考える。たとえるならカテーテル治療や外科的バイパスは新幹線であり，血管新生療法は在来線や地下鉄であろう。どちらが欠けても目的地に到着するのは困難であり，症例によって両者をうまく融合させた集学的治療が必要であろう。下肢の血管新生療法に関してはわが国からの研究成果，治験例が数多く報告されており，わが国の技術が世界に先駆けて製品化されることを願ってやまない。

●文　献

1）Isner JM, Walsh K, Symes J, et al: Arterial gene therapy for therapeutic angiogenesis in patients with peripheral artery disease. Circulation **91**: 2687-2692, 1995
2）Norgren L, Hiatt WR, Dormandy JA, et al: Inter-Society Consensus for the Management of Peripheral Arterial Disease (TASC II). J Vasc Surg **45**(Suppl): S5-67, 2007
3）Epstein SE, Fuchs S, Zhou YF, et al: Therapeutic interventions for enhancing collateral development by administration of growth factors: basic principles, early results and potential hazards. Cardiovasc Res **49**: 532-542, 2001
4）Simons M, Bonow RO, Chronos NA, et al: Clinical trials in coronary angiogenesis: issues, problems, consensus: An expert panel summary. Circulation **102**: E73-86, 2000
5）Tateishi-Yuyama E, Matsubara H, Murohara T, et al: Therapeutic angiogenesis for patients with limb ischaemia by autologous transplantation of bone-marrow cells: a pilot study and a randomised controlled trial. Lancet **360**: 427-435, 2002
6）Matoba S, Tatsumi T, Murohara T, et al: Long-term clinical outcome after intramuscular implantation of bone marrow mononuclear cells (Therapeutic Angiogenesis by Cell Transplantation [TACT] trial) in patients with chronic limb ischemia. Am Heart J **156**: 1010-1018, 2008
7）Huang P, Li S, Han M, et al: Autologous transplantation of granulocyte colony-stimulating factor-mobilized peripheral blood mononuclear cells improves critical limb ischemia in diabetes. Diabetes Care **28**: 2155-2160, 2005
8）Tabata Y: Tissue regeneration based on growth factor release. Tissue Eng **9**(Suppl 1): S5-15, 2003
9）Tambara K, Tabata Y, Komeda M: Factors related to the efficacy of skeletal muscle cell transplantation and future approaches with control-released cell growth factors and minimally invasive surgery. Int J Cardiol **95**(Suppl 1): S13-

bFGF徐放化ゼラチンハイドロゲルによる臨床研究(第三項先進医療)の患者紹介のお願い

京都大学心臓血管外科では安静時疼痛もしくは下肢潰瘍のある閉塞性動脈硬化症・バージャー病の患者さんに対してbFGF徐放化ゼラチンハイドロゲルによる血管新生療法の第三項先進医療(高度医療評価制度)での臨床試験を行っています。詳細は下記当科ホームページに記載しております。

適応基準にあてはまると予想される患者さん，あるいは適応基準判定が難しい患者さんがいらっしゃいましたら大変お手数とは存じますが，まずは下記にご一報いただければ幸いに存じます。当方で最終的な判定を行いたいと思いますので何卒よろしくお願い申し上げます。

京都大学心臓血管外科ホームページ：http://www.kyoto-cvs.com/

＜ご連絡先＞ 京都大学心臓血管外科：担当 丸井 晃・柳 茂樹
心臓血管外科外来受付：075-751-4460/4461（8時30分～17時）
E-mail：cvs@kuhp.kyoto-u.ac.jp

15, 2004
10) Nakajima H, Sakakibara Y, Tambara K, et al: Therapeutic angiogenesis by the controlled release of basic fibroblast growth factor for ischemic limb and heart injury: toward safety and minimal invasiveness. J Artif Organs **7**: 58-61, 2004
11) Sakakibara Y, Tambara K, Sakaguchi G, et al: Toward surgical angiogenesis using slow-released basic fibroblast growth factor. Eur J Cardiothorac Surg **24**: 105-111; discussion 112, 2003
12) Takaba K, Jiang C, Nemoto S, et al: A combination of omental flap and growth factor therapy induces arteriogenesis and increases myocardial perfusion in chronic myocardial ischemia: evolving concept of biologic coronary artery bypass grafting. J Thorac Cardiovasc Surg **132**: 891-899, 2006
13) Ueyama K, Bing G, Tabata Y, et al: Development of biologic coronary artery bypass grafting in a rabbit model: revival of a classic concept with modern biotechnology. J Thorac Cardiovasc Surg **127**: 1608-1615, 2004
14) Iwakura A, Fujita M, Kataoka K, et al: Intramyocardial sustained delivery of basic fibroblast growth factor improves angiogenesis and ventricular function in a rat infarct model. Heart Vessels **18**: 93-99, 2003
15) Sakakibara Y, Nishimura K, Tambara K, et al: Prevascularization with gelatin microspheres containing basic fibroblast growth factor enhances the benefits of cardiomyocyte transplantation. J Thorac Cardiovasc Surg **124**: 50-56, 2002
16) Iwakura A, Tabata Y, Koyama T, et al: Gelatin sheet incorporating basic fibroblast growth factor enhances sternal healing after harvesting bilateral internal thoracic arteries. J Thorac Cardiovasc Surg **126**: 1113-1120, 2003
17) Iwakura A, Tabata Y, Tamura N, et al: Gelatin sheet incorporating basic fibroblast growth factor enhances healing of devascularized sternum in diabetic rats. Circulation **104** (12 Suppl 1) :I325-329, 2001
18) Huang Y, Marui A, Sakaguchi H, et al: Sustained release of prostaglandin E1 potentiates the impaired therapeutic angiogenesis by basic fibroblast growth factor in diabetic