**NT5E Genetic Mutation Is a Rare But Important Cause of Intermittent Claudication and Chronic Limb-Threatening Ischemia**

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**Background:** NT5E genetic mutations are known to result in calcification of joints and arteries (CALJA), and worldwide, 14 patients from 7 families have been reported.

**Methods and Results:** A total of 5 patients from 2 independent families with CALJA were found in Japan. Of them, 3 complained of intermittent claudication (IC), and 1 suffered from bilateral chronic limb-threatening ischemia (CLTI). Whole-exome sequencing analysis revealed an identical mutation pattern (c.G3C on the exon 1 start codon) that was unique compared with NT5E mutations reported in other countries.

**Conclusions:** Vascular specialists need to recognize CALJA as a rare cause of ischemic IC and CLTI.

**Key Words:** Calcification of joints and arteries; Chronic limb-threatening ischemia; Intermittent claudication; NT5E gene; Peripheral artery calcification

Heavy calcification of aneurysmally dilated arteries combined with joint calcification, so-called calcification of joints and arteries (CALJA), was first described in 1912 by Magnus-Lavy, however, the molecular and genetic background of CALJA was unknown until St. Hilaire’s report in 2011. Since they unveiled mutations of the NT5E (5’-ectonucleotidase) gene as a cause of CALJA, 14 patients from 7 families have been reported around worldwide (Table). Heavily calcified occluded arteries of the lower extremities caused intermittent claudication (IC) as an ischemic symptom in many of these patients, but there is no literature on CALJA patients needing vascular reconstruction for limb salvage. Herein, we report 2 families with identical genetic mutation patterns, and 3 patients needed distal bypass surgeries, including 1 patient with chronic limb-threatening ischemia (CLTI) who had already lost her lower extremity before being diagnosed with CALJA. This report provides information on rare genetic disorders of arterial calcification caused by a deficiency of CD73 (ACDC) and addresses key issues that will contribute to a better understanding of the pathogenesis of arterial calcification and aneurysmal dilatation, both of which are still unsolved but very important vascular pathologies.

**Methods**

To clinically diagnose CALJA, we performed the following examinations to indicate calcification of the arteries and joints: X-ray imaging of the hands and lower extremities, and computed tomography (CT) scans. To evaluate ischemia of the lower extremities, measurement of the ankle-brachial pressure index (ABI) or skin perfusion pressure of the foot was performed.

For genetic analysis, genomic DNA was extracted from whole blood cells using a QIAamp DNA Blood Mini Kit (QIAGEN, Hilden, Germany). We performed whole-exome sequencing analysis as previously described. In brief, we used the SureSelect Human All Exon V6 kit (Agilent Technology, Santa Clara, CA, USA) for capture and a HiSeq2500 (Illumina, San Diego, CA, USA) for sequencing. Reads were aligned to GRC37 using Burrows-Wheeler Alignment Tool.
extremity (Figure 1C–E) and a partially calcified artery around the elbow and hand joints. Metacarpal phalangeal and interphalangeal joint calcification was also found (Figure 1F, G). CT and digital subtraction angiography demonstrated extensive arterial occlusions between the external iliac artery and popliteal artery, as well as the malleolar arteries (Figure 1H). Left common iliac artery-posterior tibial artery (PTA) bypass using a great saphenous vein (GSV) was performed (Figure 1H). Although minor amputations (her 1st and 4th toe) were needed, complete ulcer healing was achieved (Figure 1B). She regained her ability to walk with a prosthesis and has continued walking for 2 years after bypass surgery.

Because the findings from the series of examinations were clearly distinguished from those usually observed with peripheral artery disease (PAD), we investigated the X-ray screening and measuring the ABIs of her family members: 2 siblings of the proband had arteriomegaly with severe calcification in their lower extremities (Figure 1I, J). Patient II-1 was asymptomatic and had an ABI of 0.75. Patient II-8 had IC with an ABI of 0.6. Neither patient had atherosclerotic risk factors and so they received conservative treatment. They were finally diagnosed with CALJA based on NT5E mutation by genetic testing.

### Results

In this IRUD project, we found 2 independent families with clinically diagnosed CALJA arising from NT5E mutations.

**Family 1**

Family 1 (Family no. 8 in Table) included 3 patients diagnosed with CALJA (Figure 1A). The proband, Patient II-10 was a 70-year-old woman with a 20-year history of IC. Her right lower leg was amputated below the knee in a previous hospital due to infection of ischemic ulcers of the forefoot. During the postoperative term, ulcers appeared on multiple toes of the left foot (Figure 1B). Her ABI was unmeasurable with no pulse wave and skin perfusion pressure was 16mmHg on the dorsum and 9mmHg on the plantar surface. Plain radiography demonstrated heavy calcification with areas of arteriomegaly in the lower extremity (Figure 1C–E) and a partially calcified artery around the elbow and hand joints. Metacarpal phalangeal and interphalangeal joint calcification was also found (Figure 1F, G). CT and digital subtraction angiography demonstrated extensive arterial occlusions between the external iliac artery and popliteal artery, as well as the malleolar arteries (Figure 1H). Left common iliac artery-posterior tibial artery (PTA) bypass using a great saphenous vein (GSV) was performed (Figure 1H). Although minor amputations (her 1st and 4th toe) were needed, complete ulcer healing was achieved (Figure 1B). She regained her ability to walk with a prosthesis and has continued walking for 2 years after bypass surgery. Because the findings from the series of examinations were clearly distinguished from those usually observed with peripheral artery disease (PAD), we investigated the X-ray screening and measuring the ABIs of her family members: 2 siblings of the proband had arteriomegaly with severe calcification in their lower extremities (Figure 1I, J). Patient II-1 was asymptomatic and had an ABI of 0.75. Patient II-8 had IC with an ABI of 0.6. Neither patient had atherosclerotic risk factors and so they received conservative treatment. They were finally diagnosed with CALJA based on NT5E mutation by genetic testing.

**Family 2**

Family 2 (Family no. 9 in Table) had sisters with CALJA aged 81 and 74 years, respectively (Figure 2A). The 81-year-old woman presented with a 50-year history of joint pain in her legs and a history of left femoro-PTA bypass surgery performed 5 years ago for IC. Although she regularly visited an outpatient clinic for moderate aortic
Metacarpal phalangeal joint calcification was also found (Figure 2D). The 74-year-old woman (Patient II-11) reported a 10-year history of bilateral IC in her calves. At 71 years of age, she was diagnosed with severe AS, and AVR was performed. During the follow-up, she continued to have IC. The ABI value was 0.39 for the right leg and 0.32 for the left leg. Radiographs and CT revealed similar findings to those observed in her sister, such as occluded aneurysmal femoropopliteal arteries with extensive heavy calcification. Right lower limb was amputated previously.

Both patients were diagnosed with CALJA based on NT5E mutation by genetic testing.
vascular calcification through tissue-nonspecific alkaline phosphatase (TNAP). Jin et al proved that increased activity of TNAP compensated for reduced adenosine production in an experimental disease model using CALJA patient-specific induced pluripotent stem cell technology. TNAP is known to hydrolyze inorganic pyrophosphate (PPi), a calcification inhibitor, and lower cellular PPi concentrations to promote calcification. Although the molecular mechanism of aneurysmal dilatation due to NT5E deficiency is not well understood, Markello et al reported their histological findings of arterial specimens that showed calcification and disruption of the internal elastic lamina, with clusters of osteoclast-like cells. Endothelial CD73 plays an important role in the prevention of inflammation, atherosclerosis and calcification of vascular tissues. This evidence suggests that CD73 dysfunction results in arterial calcification, aneurysmal dilatation, and artery occlusion through reduction of adenosine and increase of TNAP with mineralization-related osteoclastogenesis in the affected arterial wall.

Of the 8 variants of NT5E reported in CALJA patients, 5 are frameshift, splice junction or nonsense mutations, which may disrupt the production of intact protein. The disease is thought to be caused by the loss of enzyme.

Figure 2. Pedigree and the clinical and radiographic findings in Family 2. (A) Pedigree. Open symbols indicate unaffected family members, and solid symbols are affected members. Arrows indicate the probands. Squares indicate male family members, circles female members, and slashed symbols are for deceased members. (B) Heavily calcified femoral artery on plain radiographs of Patient II-4. (C) CT angiography image of the same patient after bypass surgeries of both lower limbs. Bilateral femoral arteries are aneurysmally dilated and occluded with extensive heavy calcification. (D) Metacarpal joint calcification (arrowhead). Patient II-6 also has extensive calcification in the bilateral superficial and deep femoral arteries shown on plain radiography (E) and CT angiography (F).

Genetic Analysis
After filtering the variants between patients and non-patients, we identified a homozygous variant of NT5E, NM_002526: c.3G>C (p.Met1?), in the patients (Figure 3). The homozygous variant was not found in the Japanese genome variant database (HGVD; http://www.hgvd.genome.med.kyoto-u.ac.jp) or in-house data of whole-exome sequencing. The variant was confirmed by Sanger sequencing. The homozygous variant and patients were cosegregated in these families (unpublished data).

Discussion
We found a homozygous variant of c.3G>C in NT5E in 2 families with CALJA. According to the Genome Aggregation Database (gnomAD: https://gnomad.broadinstitute.org/), the allele frequency of the variant in the East Asian population is 1/1,560. Thus, the prevalence of affected patients with the homozygous variant is estimated to be approximately 1/2,500,000 in the East Asian population.

The NT5E gene encodes the cell surface protein CD73, which enzyme catalyzes conversion of adenosine monophosphate (AMP) to adenosine. Dysfunction of CD73 leads to reduced adenosine production, which results in vascular calcification through tissue-nonspecific alkaline phosphatase (TNAP). Jin et al proved that increased activity of TNAP compensated for reduced adenosine production in an experimental disease model using CALJA patient-specific induced pluripotent stem cell technology. TNAP is known to hydrolyze inorganic pyrophosphate (PPi), a calcification inhibitor, and lower cellular PPi concentrations to promote calcification. Although the molecular mechanism of aneurysmal dilatation due to NT5E deficiency is not well understood, Markello et al reported their histological findings of arterial specimens that showed calcification and disruption of the internal elastic lamina, with clusters of osteoclast-like cells. Endothelial CD73 plays an important role in the prevention of inflammation, atherosclerosis and calcification of vascular tissues. This evidence suggests that CD73 dysfunction results in arterial calcification, aneurysmal dilatation, and artery occlusion through reduction of adenosine and increase of TNAP with mineralization-related osteoclastogenesis in the affected arterial wall.

Of the 8 variants of NT5E reported in CALJA patients, 5 are frameshift, splice junction or nonsense mutations, which may disrupt the production of intact protein. The disease is thought to be caused by the loss of enzyme.
activity, which is consistent with genetically recessive inheritance. The variant c.3G>C, found in the current 2 families, is in the translation start site of NT5E. This type of variant disrupts the initiation start codon and may lead to the use of alternative start site upstream or downstream of the transcript, or to be loss of translation. The variant c.3G>C, found in the current 2 families, is in the translation start site of NT5E. This type of variant disrupts the initiation start codon and may lead to the use of alternative start site upstream or downstream of the transcript, or to be loss of translation. The identified c.3G>C displays homozygous and maps within NT5E exon 1. The mean coverage of the region is 63. In the transcript, there is no ATG codon in the 5′-UTR, but is at 172-bp downstream of the original start site, which could be used as an alternative start codon. However, its product might be a truncated CD73 protein with partial lack of the nucleotidase domain. Thus, we speculate that the variant causing CALJA is a loss-of-function mutation.

The symptoms in the lower extremities of the affected members of these families were more severe, especially in the patient with CLTI in Family 1, than is usually seen in other patients. However, the genotype-phenotype relationship is still unknown because only a few cases have been reported. Because the disease is late-onset, other factors might also contribute to the severity and timing of onset. Based on the allele frequency, there might be undiagnosed patients with the homozygous variant of NT5E. In order to elucidate the pathophysiology of this disease and vascular calcification (medial calcification) and to develop treatment and prevention, the awareness of CALJA to vascular specialists and accumulation of such patients are important.
Conclusions
In the present study, we identified members of 2 families in Japan with the same NT5E mutation who were diagnosed with CALJA. The genetic mutation pattern we found was unique compared with NT5E mutations reported in other countries. These CALJA patients also had relatively severe disease in terms of ischemic symptoms of the lower limbs, and 3 patients needed tibial artery bypass. Based on our estimation, many patients who suffer from PAD may be undiagnosed with CALJA, so vascular specialists need to recognize CALJA as a cause of lower limb ischemia, including CLTI.

Disclosures
N.A. is a member of Circulation Journal’ Editorial Team.

Conflict of Interest / Funding
None.

IRB Information
Approved by the Ethics Committee of the Asahikawa Medical University (17091-2) followed by Yamagata University.

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