the mouse myosin light chain 2V (*MLC2V*) locus. We demonstrated that *Hand1/eHAND* enhanced expansion of chamber walls and that absence of *Hand1/eHAND* expression in the IVG may be critical in the proper formation of the IVS.

MATERIALS AND METHODS

Gene targeting. From a 129SvJ bacterial artificial chromosome library, 3-kb upstream and 6-kb downstream fragments of the coding region of the *MLC2V* gene were isolated. The upstream 3-kb fragment, FLAG-tagged mouse *Hand1/eHAND* cDNA, the human growth hormone poly(A) signal, and the 6-kb downstream fragment were ligated into pPNTloxPneo (15). The targeting vector was linearized with NotI for transfection.

RW4 embryonic stem (ES) cells (Incyte Genomics, St. Louis, Mo.) isolated from the 129SvJ strain were cultured on mouse embryonic fibroblast feeder layers in high-glucose Dulbecco's modified Eagle medium containing 20% fetal calf serum and 10^3 U of leukemia inhibitory factor/ml. ES cells (1.0×10^7) were electroporated with 30 μg of the linearized targeting vector. Electroporated ES cells were cultured on neomycin-resistant feeder cells with 300 μg of G418/ml and 2 μM ganciclovir for 8 days. Two hundred eight drug-resistant colonies were isolated, and Southern hybridization demonstrated that four clones contained the correctly targeted allele at the *MLC2V* locus.

These clones were electroporated with 5 μg of the Cre-expressing vector pCre-Pac (KURABO, Osaka, Japan). After electroporation, cells were cultured on feeder cells with 1.7 μg of puromycin/ml for 2 days. Single colonies were picked up in duplicate, and neomycin-sensitive colonies were amplified and genotyped by Southern blotting. Two correctly targeted clones were injected into blastocysts from C57BL/6J mice. Male chimeras were bred with female C57BL/6J mice to test for germ line transmission. All animal procedures were approved by the Animal Research Committee, Graduate School of Medicine, Kyoto University.

Genotyping of progeny. DNA was isolated from tail biopsy specimens of weaned mice, yolk sacs, or placentas. PCR and Southern hybridization were performed to genotype embryos and mice. The primers used for detection of the targeted allele were 5′-TCCGCCTCACCTACAACTGC-3′ and 5′-ACAGAAG GGGGTCACCGTGG-3′.

Generation of transgenic mice. The 250-bp rat MLC2V promoter (10) was synthesized by PCR and was ligated to FLAG-tagged mouse *Hand1/eHAND* cDNA with the human growth hormone poly(A) signal. The identity of the synthesized promoter was confirmed by DNA sequencing. The creation of transgenic mice was done in a standard manner. F_0 embryos were dissected at embryonic day 11.5 (E11.5), and genotyping was performed by PCR on DNA isolated from the yolk sacs. PCR primer pairs used for detection of the transgenes were 5′-TGCTGTCAGCCCAATTAG-3′ and 5′-GGCTGCAGTCCTC CTCTTCCTCCCCCTC-3′.

In situ hybridization. In situ hybridization was performed as described previously (23). Briefly, embryos were fixed in 4% paraformaldehyde at 4°C overnight, dehydrated through graded ethanol and xylene, and embedded in paraffin wax. Sections of 6-μm thickness were hybridized with [^{35}S]CTP-labeled riboprobe at 55°C overnight. After hybridization, they were treated with RNase A, washed, and dehydrated through graded ethanol, and emulsion autoradiography was performed. Probes for α-cardiac actin, atrial natriuretic factor (*ANF*), *Hand1/eHAND*, *Hand2/dHAND*, *MEF2C*, *TEF-1*, *Nkx2.5*, and N-*myc* were described previously (23). An EagI-EcoRI fragment of the 3′ untranslated region (3′ UTR) of *Hand1/eHAND* was used to detect endogenous *Hand1/eHAND* expression. A probe for *Tbx5* (3) was kindly provided by Benoit G. Bruneau (University of Toronto, Toronto, Canada). A probe for *Chisel* (16) was synthesized by reverse transcription-PCR. The identity of the probe was confirmed by DNA sequencing.

Immunohistochemistry. After rehydration, paraffin sections of embryos were autoclaved in 10 mM EDTA (pH 8.0) at 121°C for 10 min, blocked with an avidin-biotin blocking kit (Vector, Burlingame, Calif.), and incubated with biotinylated mouse anti-FLAG M2 monoclonal antibody (Sigma, St. Louis, Mo.) (1:200) overnight at 4°C. After incubation, sections were washed and incubated with streptavidin-horseradish peroxidase (Nichirei, Tokyo, Japan), and peroxidase activity was detected with 3,3′-diaminobenzidine.

RESULTS

Generation of *Hand1/eHAND* KI mice. To investigate a role of *Hand1/eHAND* in the DV patterning of the embryonic heart, we generated mice expressing *Hand1/eHAND* in the whole ventricles. For this purpose, we employed a knock-in (KI) strategy to place *Hand1/eHAND* cDNA into the genomic locus of *MLC2V*, since this gene is expressed in ventricular myocytes throughout development, and heterozygous knockout mice for *MLC2V* were reported to display no obvious phenotype (5). After the first round of homologous recombination, the FLAG-tagged *Hand1/eHAND* cDNA and the *pgk-neo* cassette flanked by two *loxP* sites were inserted into the *MLC2V* locus (Fig. 1A). Four correctly targeted clones were identified (Fig. 1B). We then removed the *pgk-neo* cassette by transiently expressing the Cre recombinase (Fig. 1C). After the second round of recombination, two ES clones were injected into C57BL/6 blastocysts. We crossed male chimeras with female C57BL/6 to check for germ line contribution of ES cells by screening for the presence of agouti offspring. Two germ line chimeras were obtained, but none of their offspring carried the KI allele (0 of 20 agouti offspring), indicating that *Hand1/eHAND* KI mice were embryonically lethal.

Morphological and histological analysis of *Hand1/eHAND* KI embryos. To investigate the timing of lethality, we examined litters from a germ line chimera, all of whose offspring had agouti coat color. At E9.5 and E10.5, *Hand1/eHAND* KI embryos were indistinguishable from wild-type littermates. However, *Hand1/eHAND* KI embryos showed slight growth retardation at E11.5 and were severely retarded at E12.5, and PCR analysis of the placenta of absorbed embryos at E14.5 revealed that all absorbed embryos carried the KI allele. Viable embryos at E14.5 were all wild type. These results indicated that *Hand1/eHAND* KI embryos died between E12.5 and E14.5.

Histological examination at E9.5 revealed that trabeculation and endocardial cushion formation occurred normally in the hearts of *Hand1/eHAND* KI embryos. *Hand1/eHAND* KI and wild-type embryos were indistinguishable except that there was no IVG in *Hand1/eHAND* KI hearts (Fig. 2A and B). At E10.5, ventricular chambers, particularly the RVs, balloon out more markedly in *Hand1/eHAND* KI embryos, although their ventricles were single chambers, lacking the IVG and IVS (Fig. 2D and F). In contrast, IVS formation was clearly observed in wild-type littermates (Fig. 2C and E). The morphology of the inner curvature, AVC, and OFT was comparable between *Hand1/eHAND* KI and wild-type embryos (Fig. C, D, G, and H). At E11.5, no IVG or IVS formation was observed in *Hand1/eHAND* KI embryos (Fig. 2J and L), whereas the IVS was well developed in wild-type hearts (Fig. 2I and K). The compact zone myocardium was thinner in *Hand1/eHAND* KI embryos, suggesting that the embryonic lethality may be due to heart failure caused by poor development of the compact zone myocardium.

Gene expression in *Hand1/eHAND* KI heart. We first examined expression of *Hand1/eHAND* in *Hand1/eHAND* KI and wild-type embryos. In wild types, *Hand1/eHAND* was expressed in the outer curvature of the LV and the OFT at E9.5 and E10.5 (Fig. 3A and C). Weak expression of *Hand1/eHAND* was also observed in the outer curvature of the RV. At E11.5, *Hand1/eHAND* expression was down-regulated (Fig. 3E). Notably, *Hand1/eHAND* expression was absent at the IVG and IVS throughout development in wild-type embryos (Fig. 3A, C, and E). In contrast, *Hand1/eHAND* was expressed in the whole ventricle as well as in the AVC and OFT in *Hand1/eHAND* KI embryos (Fig. 3B, D, F, and H). The expression level was still

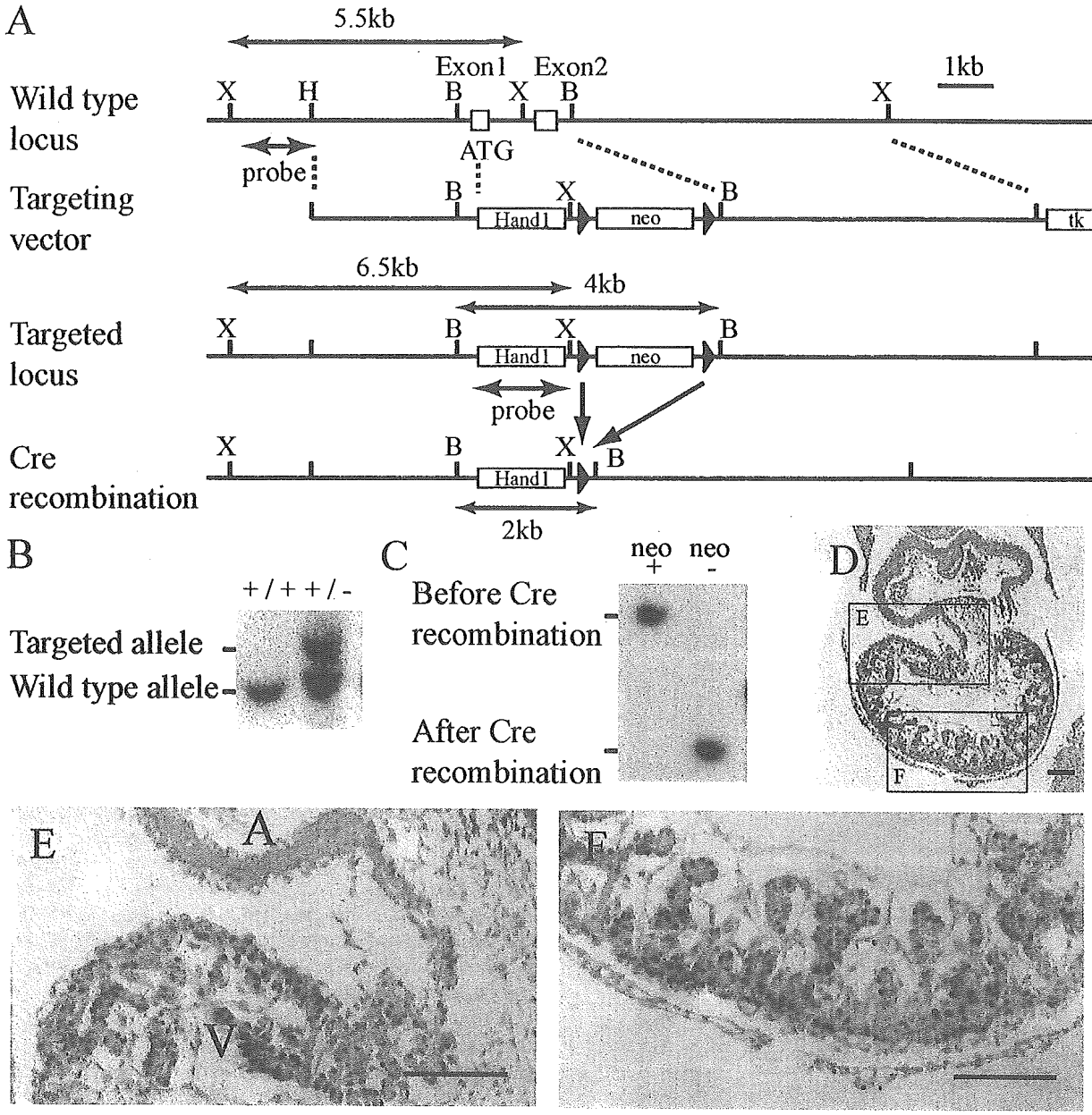

FIG. 1. (A) Targeting strategy. The structure of the *MLC2V* locus and the targeting vector are shown first and second, respectively. The mutated locus after homologous recombination is shown third, and the modified locus by Cre recombination is shown at the bottom. ATG is the transcriptional start site. The closed arrowheads represent the *loxP* sites. B, BamHI; H, HindIII; X, XbaI. (B) Genotyping of ES cell clones after homologous recombination. Genomic DNA was digested with XbaI and analyzed by Southern blotting. The 5′ probe (a BamHI-HindIII fragment) was used. Hybridization with the 5′ probe revealed the expected 5.5- and 6.5-kb fragments from the wild-type and targeted alleles, respectively. (C) Genotyping of ES clones after Cre recombination. Genomic DNA was digested with BamHI and analyzed by Southern blotting. *Hand1/eHAND* cDNA was used as a probe. The expected 4-kb fragment from the original targeted allele and the 2-kb fragment from the Cre mutated allele were revealed. Fragments from the wild-type allele for *Hand1/eHAND* were also detected (not shown on this figure). (D, E, and F) Immunohistochemistry with an anti-FLAG antibody. FLAG-tagged Hand1/eHAND protein was expressed in the nuclei of ventricular cells, whereas the expression was not detected in atrial cells in *Hand1/eHAND* KI embryos (E). FLAG-tagged Hand1/eHAND protein was expressed in the whole ventricle (F). A, atrium; V, ventricle. Bars, 100 μm.

high at E11.5 (Fig. 3F). To confirm the expression of the FLAG-tagged Hand1/eHAND protein, we also performed immunohistochemistry with an anti-FLAG antibody. The FLAG-tagged Hand1/eHAND protein was detected in cardiac myocytes of the whole ventricle in *Hand1/eHAND* KI embryos (Fig. 1D, E, and F) but not in wild types (data not shown).

We next examined endogenous *Hand1/eHAND* expression using the 3′ UTR of *Hand1/eHAND* as a probe, since the 3′ UTR is not included in the FLAG-tagged *Hand1/eHAND* cDNA. Endogenous *Hand1/eHAND* expression was confined to the left side of the single ventricle in *Hand1/eHAND* KI embryos, and the expression level was comparable to that in

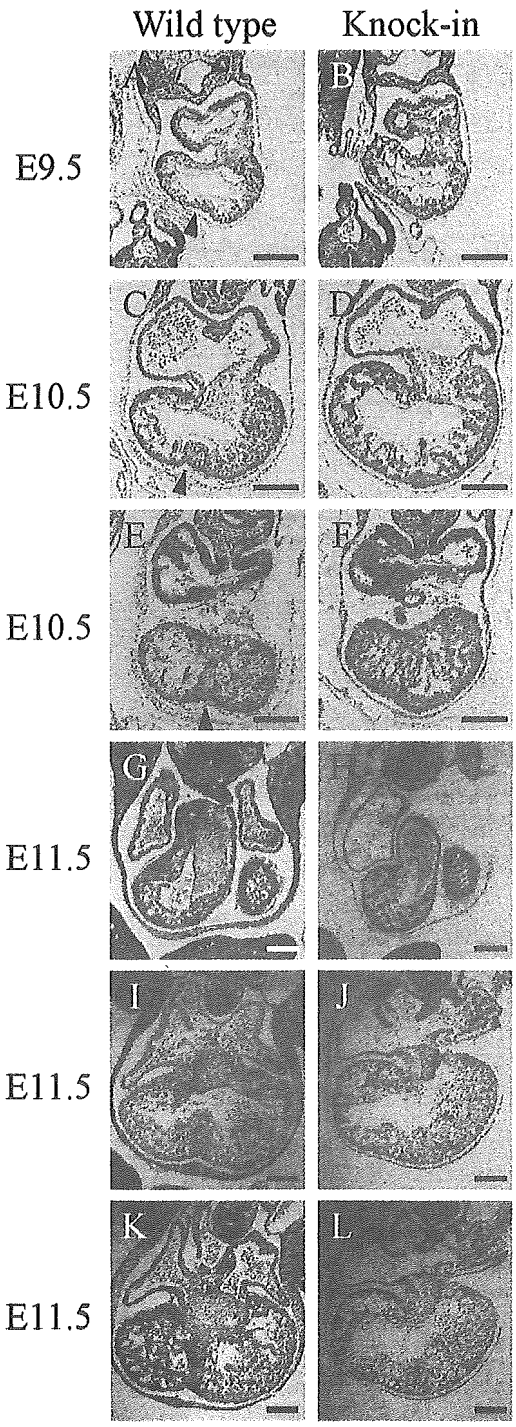

FIG. 2. Histological analysis of wild-type and *Hand1/eHAND* KI embryos from E9.5 to E11.5. Hematoxylin- and eosin-stained sections of wild-type (A, C, E, G, I, and K) and *Hand1/eHAND* KI (B, D, F, H, J, and L) embryos are shown. At E9.5, trabeculation, endocardial cushion formation, and looping normally occurred in *Hand1/eHAND* KI embryos (B). Note the absence of the IVG in *Hand1/eHAND* KI embryos. The IVG can be observed in wild-type embryos (arrowhead in panel A). At E10.5, the outer curvature expanded more markedly in *Hand1/eHAND* KI embryos (C and D). The difference was more evident in the RV. There was no IVG or IVS formation in *Hand1/eHAND* KI embryos (D and F). At E11.5, *Hand1/eHAND* KI embryos exhibited a single ventricle with complete absence of the IVS and IVG (J and L). Endocardial cushion formation in the OFT was comparable between wild-type (G) and *Hand1/eHAND* KI (H) embryos. In KI embryos, the development of the AVC was disturbed (J and L). The arrowheads in panels A, C, and E indicate the IVG. Bars, 200 μm.

wild-type embryos (Fig. 4A and B), indicating that there was a clear distinction between the left and right sides of the ventricle at the molecular level, although there was no IVG or IVS formation. Moreover, the left-side expression of *Tbx5* was not disturbed in *Hand1/eHAND* KI embryos (Fig. 4C and D), further supporting the observation that the right and left sides of the ventricle were molecularly distinctive in *Hand1/eHAND* KI embryos. Furthermore, endogenous *Hand1/eHAND* expression was detected in the AVC in *Hand1/eHAND* KI embryos (Fig. 4B), suggesting that a positive feedback regulation of *Hand1/eHAND* may exist in the AVC.

Chisel and *ANF* are regarded as molecular markers for the working myocardium (7). *Chisel* was expressed in the atrium and the outer curvature of the ventricle but was absent in the inner curvature and the AVC in wild-type embryos (Fig. 4I) (7, 16). Interestingly, in *Hand1/eHAND* KI embryos, *Chisel* was also expressed in the inner curvature and AVC, suggesting that *Chisel* expression was dependent on *Hand1/eHAND* (Fig. 4J). In wild-type embryos, *ANF* was strongly expressed in the trabecular layer of the LV and weaker expression was observed in the atrium and the trabecular layer of the RV. *ANF* expression was not observed in the inner curvature, the AVC, or the IVG in wild-type embryos (Fig. 4K). In *Hand1/eHAND* KI embryos, *ANF* expression in the RV was up-regulated and the expression was also detected in the inner curvature but not in the AVC, indicating that *ANF* expression in the RV and the inner curvature was regulated by *Hand1/eHAND* (Fig. 4L).

We further examined expression of transcription factors known to play critical roles in cardiac development. While expression of *Nkx2.5* (Fig. 4G and H) and *MEF2C* (data not shown) was comparable, *Hand2/dHAND* expression in the RV was down-regulated in *Hand1/eHAND* KI embryos (Fig. 4E and F), suggesting that *Hand1/eHAND* may suppress *Hand2/dHAND* expression. What is the molecular mechanism for thin myocardium in *Hand1/eHAND* KI embryos? Inactivation of N-*myc* or *TEF-1* in mice resulted in thin myocardium (4, 6, 14), but these genes were normally expressed in *Hand1/eHAND* KI embryos (data not shown). Homozygous Splotch mutant mice that lack the transcription factor *Pax3* also showed thin myocardium. *p57*, a cyclin-dependent kinase inhibitor normally expressed in the trabecular layer, was also expressed in the compact zone layer in the mutant embryos, suggesting precocious cardiomyocyte differentiation in Splotch mutants (11). We thus investigated expression of *p57* in *Hand1/eHAND* KI embryos, but *p57* expression was detected only in the trabecular layer both in *Hand1/eHAND* KI and wild-type embryos (Fig. 4M and N).

Normal IVS formation in transgenic embryos overexpressing *Hand1/eHAND* in the RV. The defect in the IVS formation in *Hand1/eHAND* KI embryos may be due to nonspecific effects of *Hand1/eHAND* overexpression. To further examine the significance of the absence of *Hand1/eHAND* expression in the boundary region, we generated and analyzed transgenic em-

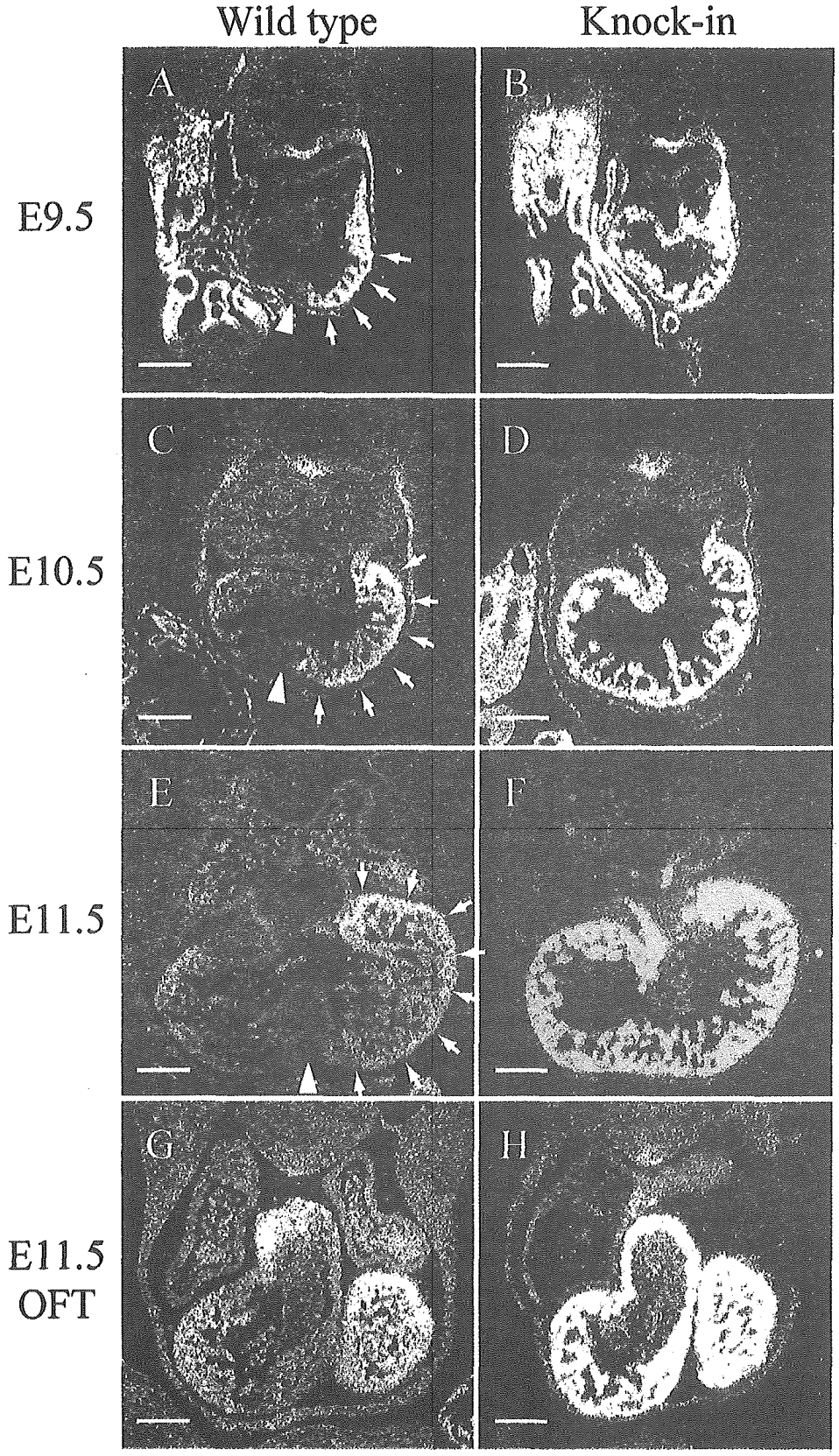

FIG. 3. Expression of *Hand1/eHAND* in wild-type and *Hand1/eHAND* KI embryos. In wild-type embryos, *Hand1/eHAND* was expressed in the outer curvature of the LV at E9.5 (arrows in panel A). Weak expression was observed in the outer curvature of the RV at E10.5 and E11.5 (C, E, and G). *Hand1/eHAND* expression was also detected in the distal part of the OFT (G). Note the absence of *Hand1/eHAND* expression in the IVG (arrowheads in panels A, C, and E). In *Hand1/eHAND* KI embryos, *Hand1/eHAND* was expressed in the whole ventricle, including the inner curvature, as well as in the AVC and the proximal part of the OFT (B, D, F, and H). In spite of the ectopic *Hand1/eHAND* expression, the inner curvature, AVC, or OFT did not expand outwards in *Hand1/eHAND* KI embryos (D, F, and H). Bars, 200 μm.

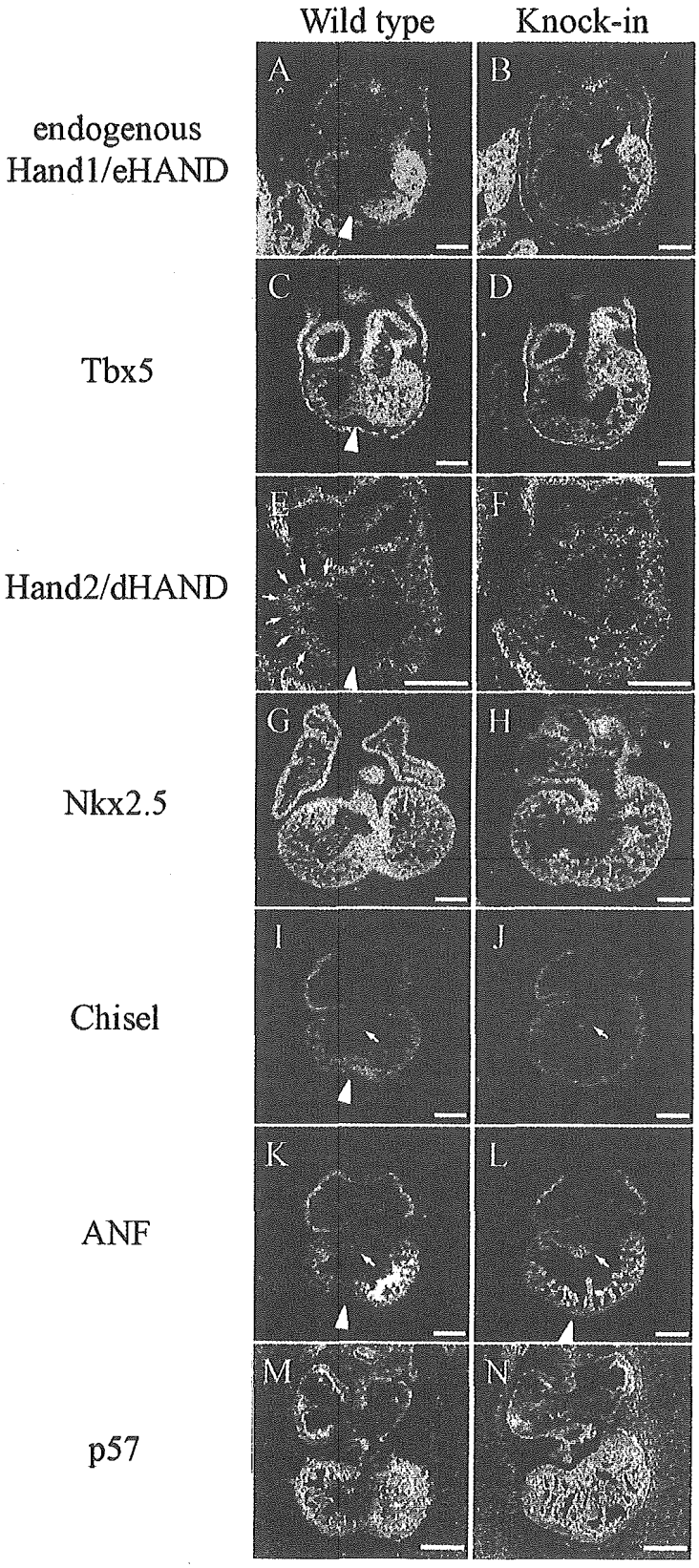

FIG. 4. Expression of cardiac transcription factors and molecular markers for the chamber myocardium. Expression of endogenous *Hand1/eHAND* (A and B), *Tbx5* (C and D), *Hand2/dHAND* (E and F), *Nkx2.5* (G and H), *Chisel* (I and J), *ANF* (K and L), and *p57* (M and N) are shown. Endogenous *Hand1/eHAND* expression was only detected in the left half of the ventricle in *Hand1/eHAND* KI embryos (B). The *Tbx5* expression

bryos overexpressing *Hand1/eHAND* in the RV by using the *MLC2V* promoter (Fig. 5A and B) (10, 18). As expected, the IVS formed normally in these transgenic embryos (Fig. 5D). Immunohistochemistry revealed FLAG-tagged Hand1/eHAND expression in the RV but not in the boundary region (Fig. 5C). These results indicated that the absence of *Hand1/eHAND* expression in the boundary region was critical for the proper formation of the IVS.

DISCUSSION

Septum formation is one of the critical steps in the transformation of a linear heart tube into a four-chambered heart. Morphologically, it has been pointed out that the boundary region between the LV and RV does not expand during the formation of the muscular IVS (Fig. 6A) (19). When the outer curvatures on each side of the narrow boundary region keep expanding, the two walls will eventually fuse, forming a septum (19). However, the molecular mechanism for expansion of the ventricular walls has never been elucidated. *Hand1/eHAND* KI embryos had a morphologically single ventricle, but there were distinctive LV and RV at the molecular level. Therefore, forced expression of *Hand1/eHAND* in the whole ventricle resulted in expansion of the entire ventricular wall including the boundary region (Fig. 6B). Although it is possible that overexpression of *Hand1/eHAND* may have affected the phenotype, the absence of *Hand1/eHAND* expression in the boundary region was critical in the development of the IVG and IVS (Fig. 6A) because transgenic embryos expressing *Hand1/eHAND* in the RV and LV, but not in the boundary region, exhibited normal formation of the IVS (Fig. 6C).

Ectopic expression of *Hand1/eHAND* in the entire RV resulted in more marked expansion of the outer curvature of the RV. Together with the result that *Hand1/eHAND* expression caused expansion of the boundary region, it is likely that *Hand1/eHAND* is involved in expansion of the ventricular walls. Then, which gene(s) regulate ballooning of the RV during normal cardiac development? Specific hypoplasia of the RV soon after cardiac looping in *Hand2/dHAND* knock-out embryos suggested a role of *Hand2/dHAND* in the expansion of the RV (21). Notably, *Hand2/dHAND* expression was also absent in the boundary region (Fig. 4E), thus suggesting a possibility that absence of *Hand1/eHAND* and *Hand2/dHAND* expression in the boundary region may be essential for the IVG and IVS formation (Fig. 6A).

Does *Hand1/eHAND* control the DV patterning of the embryonic heart? Interestingly, *Chisel* and *ANF*, molecular markers for the working myocardium (7), were ectopically expressed in the inner curvature and/or the AVC in *Hand1/eHAND* KI embryos. However, the inner curvature or the AVC did not expand morphologically. These results indicated that *Hand1/*

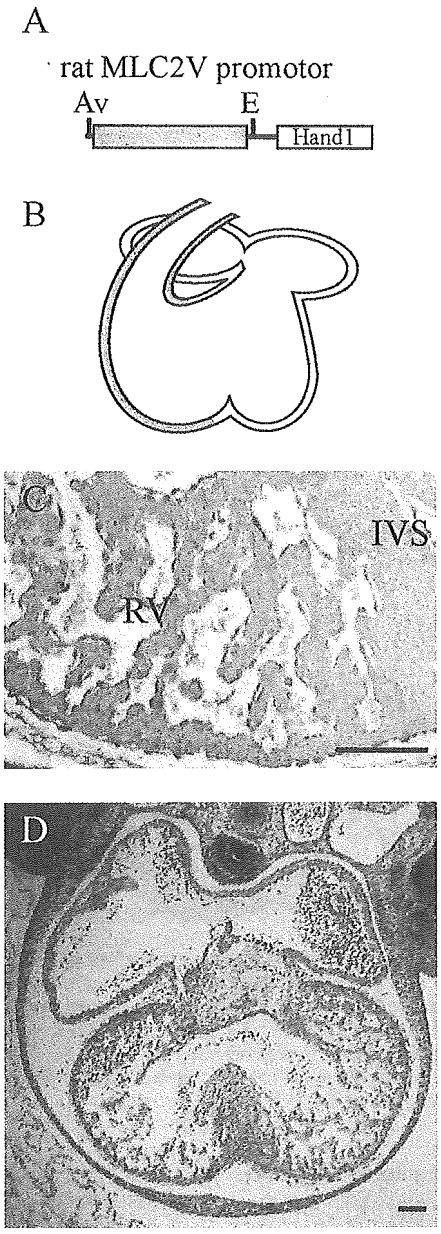

FIG. 5. *MLC2V-Hand1/eHAND* transgenic mice. (A) Schematic representation of the transgene. (B) The *MLC2V* promoter drives transgene expression in the RV and OFT but not in the boundary region. (C) Immunohistochemistry with an anti-FLAG antibody revealed FLAG-tagged Hand1/eHAND protein expression in the RV but not in the IVS. (D) In *MLC2V-Hand1/eHAND* transgenic embryos, the IVS formed normally. Av, AvaII; E, EcoRI. Bars, 100 μm.

gradient with higher expression in the LV was not disturbed in *Hand1/eHAND* KI embryos (D). Expression of *Hand2/dHAND* in the RV (E) was almost abolished in *Hand1/eHAND* KI embryos (F). Note the absence of *Hand2/dHAND* expression in the IVG in wild-type embryos (arrowhead in panel E). *Nkx2.5* expression was comparable between wild-type (G) and *Hand1/eHAND* KI (H) embryos. *Chisel* expression was also detected in the inner curvature and AVC in *Hand1/eHAND* KI embryos (J). Note the absence of *ANF* expression in the IVG (arrowhead in panel K) and inner curvature (arrow in panel K) in wild-type embryos (K). *ANF* expression was up-regulated in the RV and inner curvature in *Hand1/eHAND* KI embryos (L). *ANF* was also expressed at the region where the IVS was expected to form (arrowhead in panel L). Expression of *p57* was detected only in the trabecular layer both in wild-type and *Hand1/eHAND* KI embryos (M and N). Bars, 200 μm.

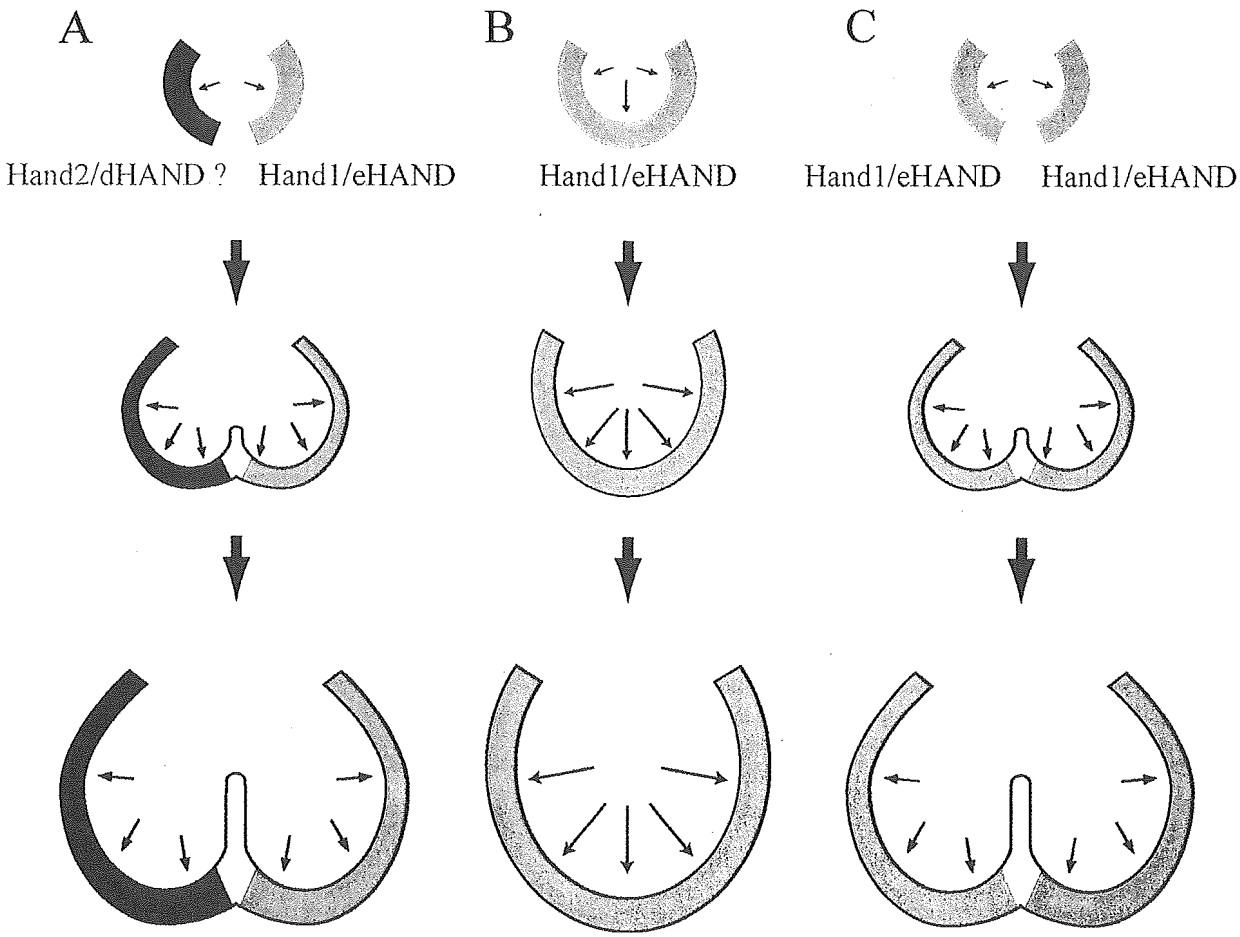

FIG. 6. Schematic presentation of ventricular expansion and IVS formation. (A) IVS formation in normal hearts. The outer curvatures of the LV and RV expand outwards. For the proper formation of the IVS, the boundary region should not expand. *Hand1/eHAND* and *Hand2/dHAND* may regulate expansion of the LV and RV, respectively. Note the absence of *Hand1/eHAND* and *Hand2/dHAND* expression in the boundary region. (B) In *Hand1/eHAND* KI hearts, the boundary region also expanded outward. As a result, the IVS did not form properly. (C) In *MLC2V-Hand1/eHAND* transgenic embryos, while the *Hand1/eHAND* transgene was expressed in the RV and endogenous *Hand1/eHAND* was expressed in the LV, *Hand1/eHAND* expression was absent in the boundary region. In these transgenic embryos, the boundary region did not expand outwards and the IVS formed normally.

eHAND regulated expression of molecular markers for the working myocardium but that additional gene(s) normally expressed in the outer curvature may be required for expansion of the chamber walls together with *Hand1/eHAND*.

The results of this study also gave insight into the hierarchical and combinatorial molecular cascade that controls cardiac development. Forced expression of *Hand1/eHAND* in the RV down-regulated *Hand2/dHAND* expression. It is possible that expression of *Hand2/dHAND* in the LV may be suppressed by high expression of *Hand1/eHAND* in the normal embryonic heart. However, *Hand1/eHAND* expression in the whole ventricles did not disturb the *Tbx5* expression gradient or endogenous *Hand1/eHAND* expression. Therefore, it is unlikely that *Hand1/eHAND* is the most upstream gene that specifies the LV myocyte lineage.

Between E10.5 and E11.5, cardiac myocytes undergo rapid cell division, resulting in doubling of cardiac mass (11). By E10.5, *Hand1/eHAND* KI embryos were indistinguishable from wild-type embryos except that they lacked the IVG and IVS. At E11.5, the compact zone layer of the *Hand1/eHAND* KI hearts were thin, suggesting that heart failure due to poor development of the compact zone layer may have caused the embryonic lethality. What is the mechanism for thin myocardium in *Hand1/eHAND* KI embryos? N-*myc*, *TEF-1*, and *p57* were normally expressed in *Hand1/eHAND* KI embryos, suggesting that there may be other mechanism(s). At E11.5, *Hand1/eHAND* expression was obviously down-regulated in wild-type embryos, while strong expression of *Hand1/eHAND* persisted in *Hand1/eHAND* KI embryos. Thus, down-regulation of *Hand1/eHAND* at the mid-stage of cardiac development may be important for the proper formation of the compact zone myocardium. Although it may seem inconsistent that *Hand1/eHAND* enhanced expansion of the ventricular chambers at E10.5 but that overexpression of *Hand1/eHAND* at E11.5 disturbed proliferation of the compact zone myocardium, fine-tuning of *Hand1/eHAND* expression at each stage may be required for the proper development of the embryonic heart. It is also possible that different mechanisms may exist to regulate expansion of the ventricular chambers and thickening of ventricular walls.

Recently, Takeuchi et al. reported that *Tbx5* may determine the position of the IVS in chicken and mouse embryonic hearts (22). When *Tbx5* was overexpressed in the whole ventricles, the *Hand1/eHAND* expression domain was expanded to the RV, resulting in a lack of IVS formation. Their study suggested that *Tbx5* may control *Hand1/eHAND* expression and that in the chicken heart, the boundary of the *Tbx5* and *Tbx20* expression domains may determine the position of the IVS (22). Together with the results of our study, it was likely that the function of *Tbx5* in the expansion of the ventricular walls and the IVS formation in murine hearts was mediated through eHAND. Moreover, since *Tbx20* is uniformly expressed in the LV and RV (12) and *Tbx5* is not expressed in the boundary region between the LV and RV in the normal murine hearts, the absence of *Tbx5* and *Hand1/eHAND* expression in the boundary region may be critical in the proper formation of the IVS in murine cardiac development.

In summary, expression of *Hand1/eHAND* enhanced expansion of chamber walls, and absence of *Hand1/eHAND* expression in the boundary region may be essential for the proper formation of the IVG and IVS. Moreover, additional factors normally expressed in the outer curvature may determine the DV patterning of the embryonic heart in concert with *Hand1/eHAND*.

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Coronary heart disease risk in Japan – an East/West divide?

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KEYWORDS
Coronary heart disease;
Japanese;
Hypercholesterolaemia;
Cardiovascular risk;
Western;
Diabetes

Lower rates of coronary heart disease (CHD) and other cardiovascular disease in the Japanese population compared with the US and other Western populations suggest the possibility of genetic differences that confer some protection from such disease in Japanese people. However, lifestyle changes in Japan in recent decades have been accompanied by the increasing prevalence of hypercholesterolaemia and diabetes, and recent data indicate an increase in prevalence of ischaemic heart disease. Studies in Japan have indicated a strong relationship between hypercholesterolaemia and CHD. Close attention must be devoted to what appears to be a growing risk for cardiovascular disease in the Japanese population.

Introduction

Although it is increasing, the prevalence of coronary heart disease (CHD) in the Japanese population remains lower than that in the US and other Western populations. However, with changes in Japanese lifestyle since World War II, the prevalence of such risk factors as hypercholesterolaemia and diabetes has increased, possibly predicting further increases in the incidence of CHD and other cardiovascular disease in Japan.

East/West differences?

It is well known that there are lower rates of cardiovascular mortality and CHD mortality among Japanese men, even those living in Hawaii, than among US Caucasians. There may be genetic differences between Japanese people and Western people that underlie some of the observed differences in risk. However, it is also the case that the prevalence of CHD risk factors has increased and continues to increase in Japan, and it is likely that rates of CHD and other cardiovascular disease will therefore increase. Fig. 1 shows mortality due to CHD, heart failure and other diseases of the heart from 1990 to 1998 in white men in the US, male Japanese Americans living in Hawaii and male Japanese living in Japan as reported by Sekikawa et al.[1] The relatively small percentage of mortality attributed to CHD and the large proportion attributed to heart failure in Japanese men likely represent some degree of misclassification of CHD deaths as heart failure deaths.

Risk factors

While total cholesterol levels have been decreasing in the US population, a marked increase in the Japanese population began in the 1980s, such that by the later part of the decade, mean levels in Japanese women exceeded those in US adults. These increases appear to be associated with the marked increase in ischaemic heart disease mortality observed in Japanese men and women in the early 1990s. Fig. 2 shows mean serum total cholesterol levels in American people and Japanese people by de-

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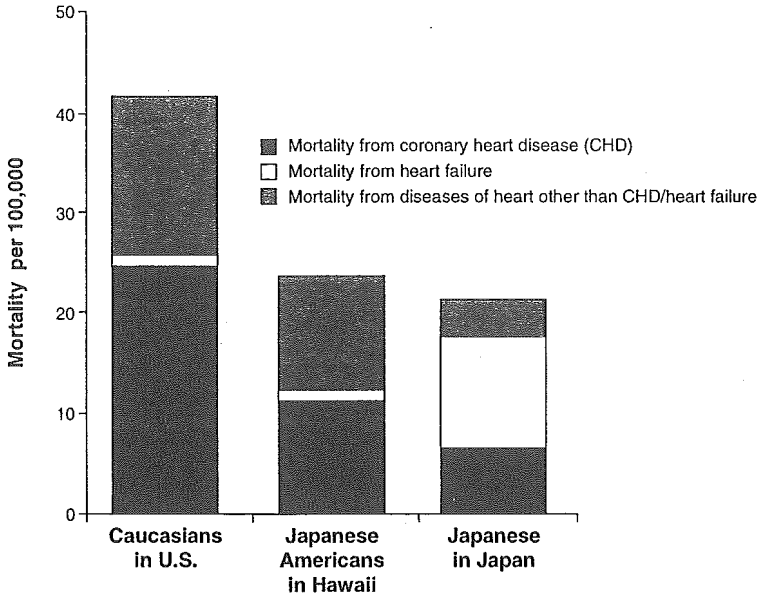

Fig. 1 Cardiovascular mortality from 1990 to 1998 in Japanese and US men. (Adapted with permission from Sekikawa et al.[1])

cade of life according to 1972–1976 data from the US and 1980 data from Japan. Japanese men and women who were around 20 years of age at the time of the study had higher cholesterol levels than did their US counterparts.[2] This age group is now around 40 years of age and there is concern that the elevated cholesterol levels may be associated with increased cardiovascular disease as this segment of the population ages. Also shown in Fig. 2 is the distribution of Japanese adults with total cholesterol levels $\geqslant$ 5.69 mmol/l (220 mg/dl) by decade of age in surveys performed in 1980, 1990, and 2000.[3] In addition to an increasing prevalence of elevated total cholesterol between 1980 and 1990 for each decade of age, these data also suggest a high prevalence of elevated cholesterol in older, post-menopausal women.

The frequency of diabetes is also increasing in Japan, with survey data indicating a doubling of the prevalence of diabetes between 1980 and 2000 (Fig. 3).[4] According to Ministry of Health, Labour and Welfare statistics, the estimated population of diabetic patients in Japan is expected to increase from 6.9 million in 1997 to 10.8 million in 2010. These changes in cardiovascular risk profiles likely represent changes in lifestyle in Japanese society, including consumption of increasing amounts of animal products and reduced physical activity.

Association of cardiovascular risk and hypercholesterolaemia

Studies in Japan have shown a strong relationship between hypercholesterolaemia and cardiovascular risk. In a 13-year cohort study of cause-specific mortality reported by Okamura et al.,[5] 9215 community-dwelling individuals aged at least 30 years without a history of cardiovascular disease were stratified according to baseline serum total cholesterol levels of <4.14, 4.14–5.16, 5.17–6.20, and $\geqslant$ 6.21 mmol/l (<160, 160–200, 200–240, and $\geqslant$ 240 mg/dl). Overall, 1206 deaths occurred during follow-up, of which 462 were attributed to cardiovascular disease and 79 to CHD. Total cholesterol levels >6.21 mmol/l (240 mg/dl) were significantly associated with coronary mortality; the relative risk for coronary mortality in this group was 2.93 (95% confidence interval [CI] 1.52–5.63) compared with the reference group defined by total cholesterol of 4.14–5.16 mmol/l (160–200 mg/dl). No significant relationship between total cholesterol and stroke was observed. On multivariate analysis, the attributable risk percentage of hypercholesterolaemia for CHD mortality was 66%.

The Japan Lipid Intervention Trial was a 6-year nationwide study in which 47,294 subjects without CHD and 5127 patients with CHD, all with serum total cholesterol $\geqslant$ 5.69 mmol/l (220 mg/dl), received open-label treatment with simvastatin 5–10 mg under standard clinical conditions.[6,7] Subjects were men aged 35–70 years and post-menopausal women aged <70 years. In the primary prevention cohort, simvastatin treatment reduced total cholesterol by 18.4%, low-density lipoprotein cholesterol (LDL-C) by 26.8% and triglycerides by 16.1%, and increased high-density lipoprotein cholesterol (HDL-C) by 4.5% (average changes during treatment). In the secondary prevention cohort, simvastatin treatment reduced total cholesterol by 19.8%, LDL-C by 28.6% and triglycerides by 15.9%, and increased HDL-C by 4.7% (average changes during treatment). In the primary prevention cohort, relative risk of CHD according to lipid values during treatment was significantly increased, with:

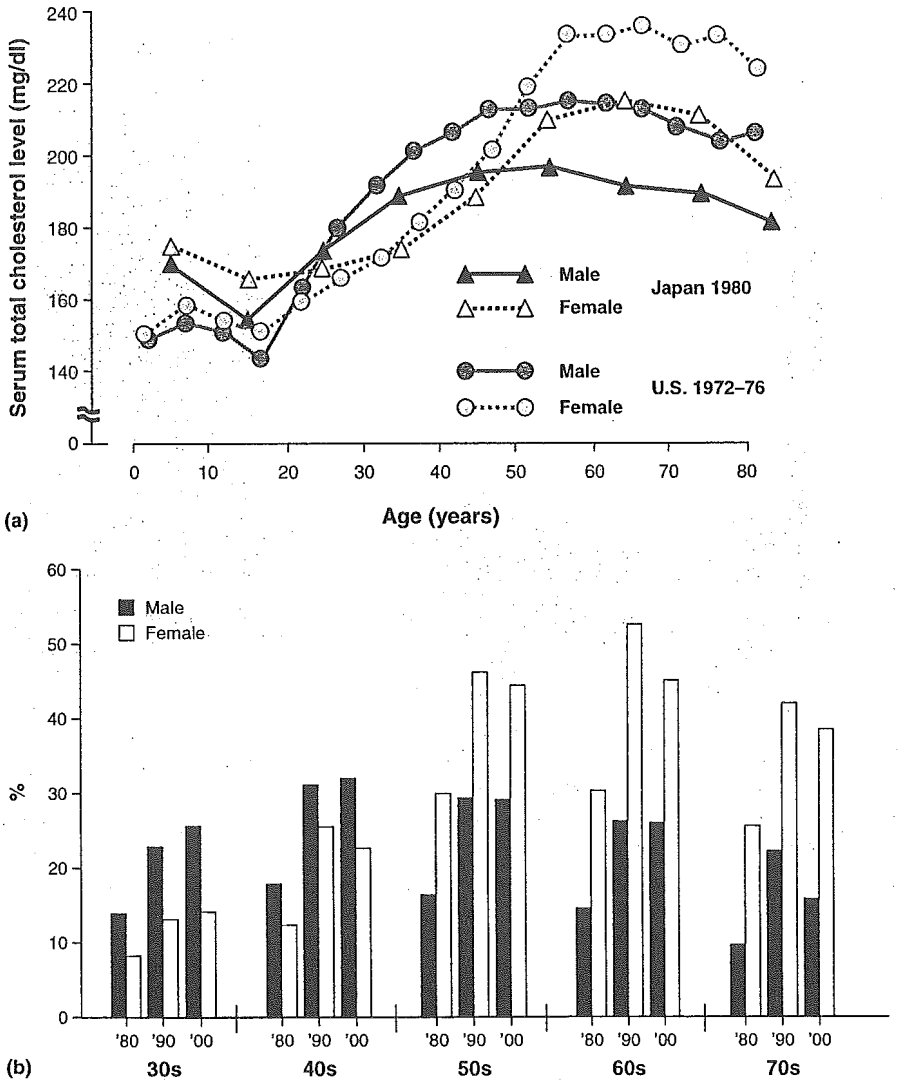

Fig. 2 Top: Mean serum total cholesterol levels according to age in Japanese (1980) and US (1972–1976) populations. (Adapted with permission from Sekimoto et al.[2]) Bottom: Proportion of individuals in Japan with total cholesterol ⩾5.69 mmol/l (220 mg/dl) by decade of age (from 30s to 70s) in surveys in 1980 ('80), 1990 ('90), and 2000 ('00). Data from the Ministry of Health, Labour and Welfare.[3]

- Total cholesterol ⩾ 6.21 mmol/l (240 mg/dl)
  - 6.21–6.70 mmol/l (240–259 mg/dl) (relative risk 2.63, 95% CI 1.68–4.12)
  - ⩾ 6.70 mmol/l (260 mg/dl) (relative risk 4.03, 95% CI 2.55–6.38)
- LDL-C ⩾ 4.14 mmol/l (160 mg/dl)
  - 4.14–4.63 mmol/l (160–179 mg/dl) (relative risk 2.59, 95% CI 1.62–4.15)
  - ⩾ 4.65mmol/l (180 mg/dl) (relative risk 5.71, 95% CI 3.64–8.97)
- Triglycerides ⩾ 3.39 mmol/l (300 mg/dl) (relative risk 2.16, 95% CI 1.38–3.37)
- HDL-C <1.03 mmol/l (40 mg/dl) (relative risk 1.45, 95% CI 1.01–2.07)

In the secondary prevention cohort, there was a trend for total cholesterol ⩾ 6.21 mmol/l (240 mg/dl) (relative risk 1.65, 95% CI 0.92–2.94) and HDL-C <1.03 mmol/l (40 mg/dl) (relative risk 1.60, 95% CI 0.99–2.58) to predict CHD events; LDL-C ⩾ 3.62 mmol/l (140 mg/dl) significantly increased relative risk (3.62–4.11 mmol/l [140–159 mg/dl], relative risk 1.95, 95% CI 1.06–3.58; ⩾ 4.14 mmol/l [160 mg/dl], relative risk 2.27, 95% CI 1.19–4.32). In the primary prevention cohort, each 0.26 mmol/l (10 mg/dl) decrease in average total cholesterol, LDL-C, and triglycerides reduced CHD risk by 11.3%, 15.8%, and 1.2%, and each 0.26 mmol/l (10 mg/dl) increase in HDL-C reduced risk by 37.5%. In the secondary prevention cohort, each 0.26 mmol/l (10 mg/dl) decrease in average LDL-C reduced relative risk of CHD by 8.0% (95% CI 3.8–12.0) and each 0.26 mmol/l (10 mg/dl) increase in HDL-C reduced relative risk by 28.3% (95% CI 13.9–40.3).

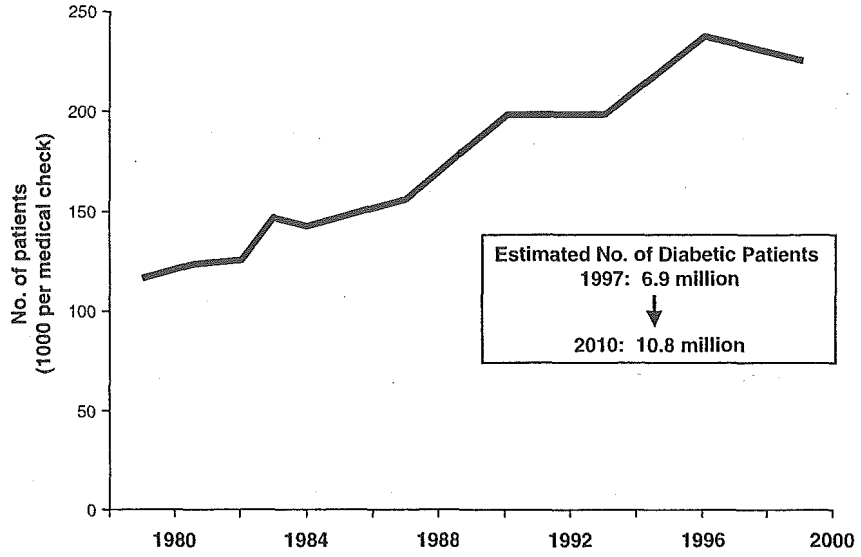

Fig. 3 Increase in prevalence of diabetes in Japan between 1980 and 2000. Data from the Ministry of Health, Labour and Welfare.[4]

Conclusion

The prevalence of coronary disease is increasing in the Japanese population, although it remains lower than in the US and other Western populations. Nevertheless, the prevalence of lipid risk factors in younger Japanese people is now similar to that in the US population, and there has been a continuous increase in the frequency of diabetes in Japan. As in Western populations, hypercholesterolaemia is associated with increased CHD risk. There is thus concern regarding what coming years will bring in terms of continued increases in coronary disease.

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Original Articles

Mutations in Japanese Subjects with Primary Hyperlipidemia — Results from the Research Committee of the Ministry of Health and Welfare of Japan since 1996 —

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Primary hyperlipidemia is caused by various molecular defects in lipid metabolism. The Research Committee on Primary Hyperlipidemia organized by the Ministry of Health and Welfare of Japan (present: the Ministry of Health, Labour and Welfare) has investigated reported mutations in Japanese patients with primary hyperlipidemia and related disorders (including hypolipidemia), and has created a database based on the questionnaire sent to the members of council board of the Japan Atherosclerosis Society. Mutations in the following genes were investigated: low density lipoprotein receptor, lecithin: cholesteryl acyltransferase, lipoprotein lipase (LPL), hepatic lipase, apolipoproteins A-I, A-II, A-IV, B, C-II, C-III and E, microsomal triglyceride transfer protein, and cholesterol ester transfer protein (CETP). Until 1998, 922 patients with primary hyperlipidemia and related disorders has been registered with the Research Committee, and 190 mutations in 15 genes had been reported, showing a marked variation in Japanese patients with primary hyperlipidemia and related disorders. So-called "common mutations" have been described in Japanese patients with familial hypercholesterolemia, LPL deficiency and CETP deficiency. The genetic defect of familial combined hyperlipidemia (FCHL) is still unknown although FCHL is speculated to be the most prevalent genetic hyperlipidemia, and further investigations should be performed to elucidate the molecular mechanisms of FCHL. *J Atheroscler Thromb, 2004; 11: 131–145.*

Key word: Apolipoprotein, Hyperlipoproteinemia, Mutational spectrum, Receptor

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Introduction

Hyperlipidemia is one of the important bases for atherosclerotic cardiovascular diseases, and it is crucial to clarify the molecular mechanism of its pathophysiology and establish treatments of hyperlipidemia. Primary hy-

perlipidemia, caused by various molecular deficits of enzymes, receptors, transfer proteins or apolipoproteins (apo) involved in lipid metabolism, is generally hard to treat and causes various complications, such as coronary artery disease in patients with familial hypercholesterolemia (FH) due to mutations in the low-density lipoprotein receptor (LDLR) gene (1). To clarify the molecular mechanism of primary hyperlipidemia, the Ministry of Health and Welfare of Japan (present: the Ministry of Health, Labour and Welfare) organized the Research Committee in 1983. In the present study, in order to evaluate the current status of mutational analysis for primary hyperlipidemia in Japan, we investigated the reported mutations for primary hyperlipidemia and related disorders and created a database for mutations in Japanese patients based on the work of the Research Committee on Primary Hyperlipidemia of the Ministry of Health and Welfare of Japan (Chairman: Professor Toru Kita, Kyoto University).

Methods

Registered mutations

Reported mutations in the following genes were registered with the Research Committee: LDLR, lecithin: cholesterol acyltransferase (LCAT), lipoprotein lipase (LPL), hepatic lipase (HL), apo A-I, apo A-II, apo A-IV, apo B, apo C-II, apo C-III, apo E, microsomal triglyceride transfer protein (MTP) and cholesteryl ester transfer protein (CETP). Some of these molecular defects, which cause hypolipidemia, were also registered with the Research Committee. Furthermore, mutations in genes recently identified as causes of primary hyperlipidemia and related disorders were also investigated in this report.

Registration of the mutations

A questionnaire was sent to the members of the council board of the Japan Atherosclerosis Society and the related institutes and/or hospitals of the councilors, and the database was built up based on the returned questionnaires until 1998. In this questionnaire, the following items were investigated: clinical diagnosis, age, sex, height, weight, blood pressure, corneal ring and/or opacification, xanthoma, history of smoking, hypertension, diabetes mellitus, coronary artery disease, cerebral vessel disease, carotid artery stenosis, peripheral artery disease and pancreatitis, serum levels of total cholesterol, triglycerides, high density lipoprotein (HDL)-cholesterol, apo A-I, A-II, A-IV, B, C-II, C-III and E, activities of CETP, LPL, HL, LCAT and LDLR, phenotype of apo E, phenotype of hyperlipidemia, and mutation (if identified). Furthermore, mutations reported in the academic meetings including the Annual Meetings of the Japan Atherosclerosis Society, as well as the published mutations in the journals until 2003, were added to the database as thoroughly as possible.

Results

Until 1998, 922 patients with primary hyperlipidemia had been registered with the Research Committee (2). Patients with FH were the largest in number, followed by those with apo E abnormality (deficiency/variant), CETP deficiency, LPL deficiency and apo A-I abnormality (deficiency/variant) (Table 1).

Disorders in cholesterol metabolism

LDLR is the most intensively analyzed gene in patients with primary hyperlipidemia. To date, approximately 80 mutations have been reported in Japanese patients with FH (Table 2), including large rearrangements, small deletions and/or insertions and point mutations throughout the gene, and a marked variation was observed in the mutations in the LDLR gene. Some of these mutations have been reported to be common among Japanese patients with FH (3, 4), such as C317S in exon 7 (5), P664L in exon 14 (6) and K790X in exon 17 (3).

Familial defective apo B-100 (FDB), of which the clinical characteristics are similar to those of FH, have been reported to be common in the Western population (7). FDB is caused by mutations in the binding domain of apo B-100 to LDLR, and to date, 3 mutations have been identified as responsible for FDB, R3,500Q (8), R3,500W (9) and R3,531C (10). However, no patients have been diagnosed with FDB in hundreds of clinically diagnosed Japanese FH patients (11).

Recently, mutations have been identified in patients with autosomal recessive hypercholesterolemia (ARH), characterized by severe hypercholesterolemia, xanthoma and premature atherosclerosis without any impairment of LDLR, in a gene encoding a putative adaptor protein, and a novel insertion mutation was discovered in the ARH gene (insertion of an extra C at positions 599–606 in exon 6, creating a permature stop codon at 657–659) in Japanese siblings with ARH (12).

FCHL is characterized by increased levels of apo B-100 and elevated levels of very low density lipoprotein (VLDL), LDL, or both, in plasma, and is speculated to be the most prevalent disorder of primary hyperlipidemia with an increased risk of atherosclerotic diseases (7), however, the molecular basis of FCHL is still unknown. Fourteen patients have been registered (2) with the Research Committee according to the diagnostic criteria published by the committee in 1987 (13).

On the other hand, hypobetalipoproteinemia and abetalipoproteinemia have been known to cause genetic hypocholesterolemia. The molecular basis of familial hypobetalipoproteinemia is the truncated form of apo B-100, and various mutations causing a premature stop codon have been identified in the Western population

(7). In Japanese patients, 3 mutations, apo B-38.7, B-54.4 and B-54.8 (Table 3) have been reported (14), each of which results in the appearance of a premature stop codon.

Abetalipoproteinemia, which is characterized by the absence of VLDL and LDL in plasma, fat malabsorption and acanthocytosis, is caused by a functional deficit of MTP (7). To date, 4 mutations have been reported as causes of abetalipoproteinemia in Japanese patients (Table 4) (15).

Disorders in triglyceride metabolism

LPL is a lipolytic enzyme and is essential for the hydrolysis of chylomicron and VLDL triglycerides, and a functional defect of LPL causes marked hyperchylomicronemia and hypertriglyceridemia (type I or type V hyperlipidemia) (16). To date, 25 mutations have been identified in Japanese patients with LPL deficiency (Table 5). Among these mutations, S447X (17) in exon 9 and 916delG (LPL-Arita) (18) have been reported to be common in Japanese patients with LPL deficiency.

Apo C-II is a coenzyme of LPL, and its genetic defect causes hyperchylomicronemia and hypertriglyceridemia, similar to LPL deficiency (16). Three mutations have been identified in Japanese patients with apo C-II deficiency (Table 6). In these patients, serum levels of triglycerides were not so high as those in LPL deficiency, and some patients had no history of pancreatitis, suggesting that the clinical features of apo C-II deficiency are mild compared with those of LPL deficiency.

Familial HL deficiency is a rare autosomal recessive disorder characterized by moderate hypertriglyceridemia and premature atherosclerotic cardiovascular diseases (15). One mutation (C53G) in the HL gene has been reported to be responsible for HL deficiency in a Japanese subject (Table 7) (19)

Type III hyperlipidemia (dysbetalipoproteinemia) is characterized by elevated concentrations of both plasma cholesterol and triglycerides, the presence of β-migrating VLDL, and accumulation of atherogenic cholesterol-enriched remnants, such as intermediate density lipoprotein and chylomicron remnants (20). The genetically determined polymorphism of apo E has a significant impact on lipid metabolism: the wild type of apo E is E3, and there are the common variants E2 (Arg158Cys) and E4 (Cys112Arg). The primary molecular defect in most patients with type III hyperlipidemia is homozygous ε2/ε2 (20). Other than the common variants E2 and E4, there have been some mutations causing apo E deficiency/variants (Table 8) in Japanese patients. Some of these mutations are responsible for type III hyperlipidemia with autosomal dominant inheritance. Apo E5 (21) and apo E7 (22) have been reported to be relatively common in Japanese patients. Patients with apo E7 have been re-

Table 1. Resistered numbers of patients with primary hyperlipidemia.

		Clinical Diagnosis			Genetic Diagnosis		
	Total	Homozygote	Heterozygote	Undetermined	Homozygote	Compound Heterozygote	Heterozygote
FH	636	17	617	2	12	3	28
ApoE abnormality	103	32	71	0	32	6	65
CETP deficiency	77	26	45	6	22	30	6
LPL deficiency	23	16	7	0	5	3	0
ApoA-I abnormality	18	3	15	0	3	12	2
FCHL	14	0	0	14	0	0	0
FH-like syndrome	12	0	0	12	0	0	0
LCAT deficiency	6	6	0	0	4	0	0
ApoC-II deficiency	6	4	2	0	3	2	0
LPG	5	0	5	0	0	0	5
Abetalipoproteinemia	3	3	0	0	3	0	0
Hypobetalipoproteinemia	1	1	0	0	1	0	0
HL deficiency	1	0	0	0	0	0	0
Others	17	8	3	6	1	0	0

Apo: apolipoprotein, CETP: cholesteryl ester transfer protein, FCHL: familial combined hyperlipidemia, FH: familial hypercholesterolemia, HL: hepatic lipase, LCAT: lecithin cholesterol acyltransferase, LPG: lipoprotein glomerulopathy, LPL: lipoprotein lipase.

Table 2. Mutations in the low-density lipoprotein receptor gene in Japanese patients with familial hypercholesterolemia.

Position	Mutation	Name	Nucleotide Change	Effect on Coding Sequence	Class	Author	References
intron 1	68-1 G→C		G→C at 68-1	3' splice signal	class 1	Maruyama T	Hum Mutat 11: 480–481, 1998
exon 2	W23X	Nanao	G→A at 132	Trp→Stop at 23	NR	Yu W	Atherosclerosis 165: 335–342, 2002
exon 2	C25Y†		G→A at 137	Cys→Tyr at 25	NR	Yu W	Atherosclerosis 165: 335–342, 2002
exon 2	D26N		G→A at 139	Asp→Asn at 26	NR	Varret M	Nucl Acid Res 26: 248–252, 1998
exons 2–3	In1-In3 del		deletion of 4 kb	deletion of exons 2,3	class 3	Yamakawa K	Hum Genet 82: 317–321, 1989
exons 2–3	In1-In3 del		deletion of 5 kb	deletion of exons 2,3	NR	Kigawa K	J Biochem (Tokyo) 113: 372–376, 1993
exons 2–3	In1-In3 del	Tonami-2	deletion of 10 kb	deletion of exons 2,3	class 3	Kajinami K	Circulation (Suppl) 80: II278, 1989
exons 2–4	In1-In4 del	Kanazawa-1	deletion of 12 kb	deletion of exons 2-4	NR	Kajinami K	J Intern Med 227: 247–251, 1990
exon 3	C54S		T→A at 223	Cys→Tyr at 54	class 2	Emi M	Jpn Heart J 39: 785–789, 1998
exon 3	230delG†		deletion of G at 230	frame shift	NR	Yu W	Atherosclerosis 165: 335–342, 2002
exon 3	C61F†		G→T at 244	Cys→Phe at 61	NR	Yu W	Atherosclerosis 165: 335–342, 2002
exon 3	C74X		C→A at 285	Cys→Stop at 74	class 1	Hirayama T	J Hum Genet 43: 250–254, 1998
exon 3	C74F†		G→T at 288	Cys→Phe at 74	NR	Yu W	Atherosclerosis 165: 335–342, 2002
exon 4	327insC†		insertion of C at 327	premature stop at codon 158	NR	Hirota R	Ann Clin Biochem 39 (Pt 5): 526–530, 2002
exon 4	R94H	Fukuoka	G→A at 344	Arg→His at 94	NR	Varret M	Nucl Acid Res 26: 248–252, 1998
exon 4	355del7bp†		deletion of GGGAAGT at 355	premature stop at codon 183	NR	Hattori H	Hum Mutat 14: 87, 1999
exon 4	C100G†		T→G at 361	Cys→Gly at 100	NR	Yu W	Atherosclerosis 165: 335–342, 2002
exon 4	S109T†		T→A at 388	Ser→Thr at 109	NR	Yu W	Atherosclerosis 165: 335–342, 2002
exon 4	389insC†		insertion of C at 389	frame shift	NR	Yu W	Atherosclerosis 165: 335–342, 2002
exon 4	E119K	Phillipines	G→A at 418	Glu→Lys at 119	class 2B	Hobbs HH	Hum Mutat 1: 445–466, 1992
exon 4	C134R†		T→C at 463	Cys→Arg at 134	NR	Yu W	Atherosclerosis 165: 335–342, 2002
exon 4	C139R†		T→C at 478	Cys→Arg at 139	NR	Yu W	Atherosclerosis 165: 335–342, 2002
exon 4	C163R†		T→C at 550	Cys→Arg at 163	NR	Yu W	Atherosclerosis 165: 335–342, 2002
exon 4	V168M†		G→A at 565	Val→Met at 168	NR	Yu W	Atherosclerosis 165: 335–342, 2002
exon 4	C183S†		G→C at 611	Cys→Ser at 183	NR	Yu W	Atherosclerosis 165: 335–342, 2002
exon 5	D245N†	Naha	G→A at 796	Asp→Asn at 245	NR	Yu W	Atherosclerosis 165: 335–342, 2002

Table 2. Mutations in the low-density lipoprotein receptor gene in Japanese patients with familial hypercholesterolemia (continued).

Position	Mutation	Name	Nucleotide Change	Effect on Coding Sequence	Class	Author	References
exon 5	D245G†		A→G at 797	Asp→Gly at 245	NR	Yu W	Atherosclerosis 165: 335–342, 2002
exon 6	D280Y	Tsuruga	G→T at 901	Asp→Tyr at 280	NR	Yu W	Atherosclerosis 165: 335–342, 2002
exon 7	C317S	Wakayama	T→A at 1012	Cys→Ser at 317	class 2	Funahashi T	J Intern Med 239: 187–190, 1996
exon 7	C317R	Gifu	T→C at 1012	Cys→Arg at 317	NR	Varret M	Nucl Acid Res 26: 248–252, 1998
exon 7	R329X†		C→T at 1048	Arg→Stop at 329	NR	Yu W	Atherosclerosis 165: 335–342, 2002
exons 7–14	In6–In14 del	Osaka-2	deletion of 12 kb	deletion of exons 8-14	class 5	Miyake Y	J Biol Chem 364: 16584–16590, 1989
exons 7–14	Ex7–In14 del	Okayama	deletion of 13 kb	deletion of exons 8-14	NR	Kajinami K	J Intern Med 227: 247–251, 1990
exon 8	1061insT†		insertion of T at 1061	frame shift	NR	Yu W	Atherosclerosis 165: 335–342, 2002
exon 8	D335H	Kanagawa	G→C at 1066	Asp→His at 335	NR	Varret M	Nucl Acid Res 26: 248–252, 1998
exon 8	1115del9bp, ins6bp		deletion of 9 bp, insertion of 6 bp from 1115	GlyGlyGlyTyr→ AlaLeuAsn from 351	NR	Yamakawa K	Hum Genet 93: 625–628, 1994
exon 8	C358Y		G→A at 1136	Cys→Tyr at 358	class 5	Hirayama T	J Hum Genet 43: 250–254, 1998
exon 9	F382L		T→A at 1207	Phe→Leu at 382	NR	Varret M	Nucl Acid Res 26: 248–252, 1998
exon 9	1246ins5bp		insertion of 5 bp at 1246	frame shift after Arg395	NR	Hattori H	Hum Mutat 14: 87, 1999
exon 9	R395W§	Morioka	C→T at 1246	Arg→Trp at 395	defective	Yagi K	J Jpn Atheroscler Soc 22: 100, 1994
exon 9	E397K†		G→A at 1252	Glu→Lys at 397	NR	Yu W	Atherosclerosis 165: 335–342, 2002
exon 9	V408M	Afrikaner-2	G→A at 1285	Val→Met at 408	NR	Varret M	Nucl Acid Res 26: 248–252, 1998
exon 9	A410T†		G→A at 1291	Ala→Thr at 410	NR	Hattori H	Hum Mutat 14: 87, 1999
exon 9	D412H	Osaka-3	G→C at 1297	Asp→His at 412	class 2	Miyake Y	Eur J Biochem 210: 1–7, 1992
exon 10	A480E§		C→A at 1502	Ala→Glu at 480	class 2B	Miyake Y	J Jpn Atheroscler Soc 24: 733, 1997
exon 10	V502M†		G→A at 1567	Val→Met at 502	class 2A	Yu W	Atherosclerosis 165: 335–342, 2002
intron 10	1587 – 1 G→A†		G→A at 1587 – 1	3' splice signal	NR	Yu W	Atherosclerosis 165: 335–342, 2002
exon 11	W512X†		G→A at 1599	Trp→Stop at 512	NR	Hattori H	Hum Mutat 14: 87, 1999
exon 11	1687insC†		insertion of C at 1687	premature stop at codon 559	NR	Hattori H	Hum Mutat 14: 87, 1999
exon 11	L547V†		C→G at 1702	Leu→Val at 547	NR	Hattori H	Hum Mutat 14: 87, 1999
intron 11	1705 + 1 G→C		G→C at 1705+1	5' splice signal	class 1	Miyake Y	Int Symp on Lipoprotein Metabolism & Atherogenesis, Kyoto

Table 2. Mutations in the low-density lipoprotein receptor gene in Japanese patients with familial hypercholesterolemia (continued).

Position	Mutation	Name	Nucleotide Change	Effect on Coding Sequence	Class	Author	References
exon 12	1773insG		insertion of G at 1773	frame shift	class 1	Hirayama T	J Hum Genet 43: 250–254, 1998
exon 12	R574Q		G→A at 1784	Arg→Gln at 574	NR	Varret M	Nucl Acid Res 26: 248–252, 1998
exon 12	P587S		C→T at 1822	Pro→Ser at 587	class 2	Hirayama T	J Hum Genet 43: 250–254, 1998
intron 12	1845 + 2T→C	Niigata	T→C at 1845 + 2	5' splice signal	class 1	Maruyama T	Eur J Biochem 232: 700–705, 1995
exon 13	1963delT		deletion of T at 1963	frame shift	class 1	Hirayama T	J Hum Genet 43: 250–254, 1998
exon 13	1867delATC†		deletion of ATC at 1867–1869	deletion of Ile at 602	NR	Yu W	Atherosclerosis 165: 335–342, 2002
exon 13	L621S†		T→G at 1925	Leu→Ser at 621	NR	Yu W	Atherosclerosis 165: 335–342, 2002
exon 14	G655S†		G→A at 2026	Gly→Ser at 655	NR	Yu W	Atherosclerosis 165: 335–342, 2002
exon 14	2035insT†		insertion of T at 2035	premature stop at codon 696	NR	Hattori H	Hum Mutat 14: 87, 1999
exon 14	P664L	Kanazawa-2	C→T at 2054	Pro→Leu at 664	class 2B	Soutar AK	Proc Natl Acad Sci USA 86: 4166–4170, 1989
exon 14	2055delG†		deletion of G at 2055	frame shift	NR	Yu W	Atherosclerosis 165: 335–342, 2002
exon 14	E693K†		G→A at 2140	Glu→Lys at 693	NR	Hattori H	Hum Mutat 14: 87, 1999
exon 15	In14–In15 del	Tonami-1	deletion of 6 kb	deletion of exon 15	NR	Kajinami K	Arteriosclerosis 8: 187–192, 1988
exon 15	2199delCA	Mishima	deletion of CA at 2199 or 2201	frame shift after Thr 713	NR	Tashiro J	Eur J Clin Invest 28: 712–719, 1998
exon 15	Q718X§	Yokote	C→T at 2215	Gln→Stop at 718	NR	Higashikata T	J Jpn Atheroscler Soc 23: 847, 1996
exons 15–16	In14–In16 del		deletion of 5.5 kb	deletion of exons 15, 16	NR	Yamakawa K	Hum Genet 82: 317–321, 1989
intron 15	2312–3 C→A†		C→A at 2312–3	3' splice signal	NR	Yu W	Atherosclerosis 165: 335–342, 2002
exon 16	1655delT		deletion of T at 1655	frame shift after Asp 530	NR	—	—
exons 16–17	In15–In17 del		deletion of 4 kb	deletion of exons 16, 17	NR	Yamakawa K	Hum Genet 82: 317–321, 1989
exons 16–18	In15–In18 del	Osaka-1	deletion of 7.8 kb	deletion of exons 16-18	class 4B	Miyake Y	Proc Natl Acad Sci USA 78: 5151–5155, 1981
exon 17	2412insG§		insertion of G after 2412	frame shift after Gly 784	NR	Miyake Y	J Jpn Atheroscler Soc 22: 646, 1995
exon 17	K790X		A→T at 2431	Lys→Stop at 790	class 1, 4	Maruyama T	Arterioscler Thromb Vasc Biol 15: 1713–1718, 1995

†: Mutation reported after the closing of registration to the Research Committee in 1998.
§: Mutation reported in an abstract form in Japanese.

ported to be susceptible to coronary artery disease.

Lipoprotein glomerulopathy (LPG) is a newly recognized renal disease characterized by abnormal lipoprotein deposition in the glomeruli, dysbetalipoproteinemia, and a high level of plasma apo E, and is caused by mutations in the apo E gene (23). Some mutations in the apo E gene have been described in Japanese Patients with LPG (Table 8) (24).

Disorders in HDL metabolism

Hyperalphalipoproteinemia (HALP) is a common disorder in the Japanese population, and approximately 60% of marked HALP with serum HDL-cholesterol levels of 100 mg/dl or over is caused by CETP deficiency (25). CETP is a key protein in the reverse cholesterol transport system, which transfers cholesteryl ester from HDL particles to apo B-containing lipoproteins, and its genetic defect results in marked HALP (26). Nine mutations have been described in Japanese patients with CETP deficiency (Table 9), and two mutations, 1451 + 1G > A in intron 14 (27) and D442G in exon 15 (28), are known to be common in patients with CETP deficiency.

Genetic hypoalphalipoproteinemia results from mutations in the apo A-I/C-III/A-IV gene complex, the LCAT gene the ATP-binding cassette transporter 1 (ABCA1) gene, and other unknown molecules (26). LCAT is a plasma enzyme that esterifies free cholesterol in serum lipoproteins, and LCAT deficiency leads to marked reduction in serum HDL-cholesterol levels (29). To date, 13 mutations have been reported in Japanese patients with LCAT deficiency (Table 10). Patients with the M293I mutation, with partially residual LCAT activity, have been reported to show mild renal dysfunction (30). The R99C and T123I mutations have been identified in patients with fish eye disease (partial deficiency of LCAT activity).

Eighteen mutations have been described in the apo A-I gene (Table 11), including apo A-I variants and deficiency. Patients with apo A-I deficiency show marked hypoalphalipoproteinemia and susceptibility to premature atherosclerosis (26). In contrast, in patients with an apo A-I variant, hypoalphalipoproteinemia is relatively rare.

Apo A-II deficiency is a rare disorder caused by mutations in the apo A-II gene (31), and only 1 mutation (Apo A-II Hiroshima) has been reported in a Japanese patient with apo A-II deficiency (Table 12). In these patients, the apo A-II level was undetectable. Although the serum apo A-I level was slightly decreased, hypoalphalipoproteinemia was not observed in a patient with apo A-II deficiency (31).

So far, no mutations have been identified in the apo A-IV gene or the apo C-III gene in Japanese subjects.

Mutations in the ABCA1 gene have recently been identified as causes of Tangier disease and familial HDL deficiency (FHD) (32). Some mutations in the ABCA1 gene have been described in Japanese patients with Tangier disease and FHD (Table 13).

Table 3. Mutations in the apolipoprotein B gene in Japanese patients with hypobetalipoproteinemia.

Mutation	Name	Nucleotide Change	Effect on Coding Sequence	Author	References
Q1755X	ApoB-38.7	C→T at 5472	Gln→Stop at 1755	Ohashi K	Arterioscler Thromb Vasc Biol 18: 1330–1334, 1998
7612insA†§	ApoB-54.4	insertion of A at 7612	Trp→Stop at 2468	Ohashi K	J Jpn Atheroscler Soc 29: 259, 2001
R2486X†§	ApoB-54.8	C→T at 7665	Arg→Stop at 2486	Ohashi K	J Jpn Atheroscler Soc 24: 142, 1998

†: Mutation reported after the closing of registration to the Research Committee in 1998.
§: Mutation reported in an abstract form in Japanese.

Table 4. Mutations in the microsomal triglyceride transfer protein gene in Japanese patients with abetalipoproteinemia.

Position	Mutation	Nucleotide Change	Effect on Coding Sequence	Author	References
intron 2	G1237A§	G→A at 1237	3' splice signal	Ohashi K	—
intron 9	(–1) G→A§	G→A at (–1)	3' splice signal	Yo S	J Jpn Atheroscler Soc 24: 734, 1997
exon 11	1389delA	deletion of A at 1389	frame shift after Glu462	Ohashi K	J Lipid Res 41: 1199–1204, 2000
exon 16	N780Y	A→T at 2338	Asn→Tyr at 780	Ohashi K	J Lipid Res 41: 1199–1204, 2000

§: Mutation reported in an abstract form in Japanese.

Table 5. Mutations in the lipoprotein lipase gene in Japanese subjects.

Position	Mutation	Name	Nucleotide Change	Effect on Coding Sequence	Class	Author	References
exon 1	W(−14)X		G→A at 216	Trp→Stop at (−14)		Nakaura T	J Atheroscler Thromb 3: 17–24, 1996
exon 2	N43S		A→G at 383	Asn→Ser at 43		Kobayashi J	Biochem Biophys Res Commun 205: 506–515, 1994
intron 2	423 + 1 G→A		G→A at 423+1	5' splice signal	class 1	Gotoda T	J Biol Chem 226: 24757–24762, 1991
exon 3	Y61X		T→A at 432	Tyr→Stop at 61	class 1	Gotoda T	Biochim Biophys Acta 1138: 353–356, 1992
exon 3	G105R†		G→A at 568	Gly→Arg at 105	class 2	Ikeda Y	Clin Sci (Lond) 99: 569–578, 2000
exon 5	G154V†		G→T at 716	Gly→Val at 154	class 1	Ikeda Y	J Lipid Res 42: 1072–1081, 2001
exon 5	G188E		G→A at 818	Gly→Glu at 188	class 1	Emi M	J Biol Chem 265: 5910, 1990
exon 5	I194T§		T→C at 836	Ile→Thr at 194		Kobayashi J	Domyakukoka 25: 131, 1997
exon 5	K198R		A→G at 848	Lys→Arg at 198		—	—
exon 5	V200A		T→C at 854	Val→Ala at 200		Takagi A	Athrosclerosis 134: 27–28, 1997
exon 5	D204E		C→G at 867	Asp→Glu at 204	class 2	Gotoda T	J Clin Invest 88: 1856–1864, 1991
exon 5	916delG	Arita	delection of G at 916	frame shift after Glu220		Takagi A	J Clin Invest 89: 581–591, 1992
exon 6	C239X§	Obama	C→A at 972	Cys→Stop at 239		Ikeda Y	J Jpn Atheroscler Soc 25: 141, 1998
exon 6	R243C§		G→T at 983	Arg→Cys at 243		Ikeda Y	J Jpn Atheroscler Soc 25: 141, 1998
exon 6	R243H		G→A at 983	Arg→His at 243	class 2	Gotoda T	J Clin Invest 88: 1856–1864, 1991
exon 6	A261T§		C→A at 1036	Ala→Thr at 261		Takagi A	J Jpn Atheroscler Soc 23: 872, 1996
exon 6	F270L	Mima	T→G at 1065	Phe→Leu at 270	class 2	Takagi A	Biochim Biophys Acta 1502: 433–446, 2000
exon 6	C278R		T→C at 1087	Cys→Arg at 278		Takagi A	Atherosclerosis 134: 27–28, 1997
exon 6	L303F†		G→C at 1069	Leu→Phe at 303	class 1	Saika Y	Eur J Clin Invest 33: 216–222, 2003
exon 7	del A§		deletion of A at codon 291	frame shift after codon 303		Kobayashi J	J Jpn Atheroscler Soc 25: 131, 1997
exon 7	A334T		G→A at 1255	Ala→Thr at 334		Kobayashi J	Biochem Biophys Res Commun 191: 1046–1054, 1993
exon 8	1400delG		deletion of G at 1400	frame shift after Trp 382	class 2 or 3	Gotoda T	J Clin Invest 88: 1856–1864, 1991
exon 8	W382X†		G→A at 1401	Trp→Stop at 382	class 1	Takagi A	Clin Chim Acta 285: 143–154, 1999
intron 8	1496 + 2 T→C†		T→C at 1496+2	5' splice signal	class 1	Ikeda Y	J Lipid Res 42: 1072–1081, 2001
exon 9	S447X		C→G at 1595	Ser→Stop at 447		Kobayashi J	Biochem Biophys Res Commun 182: 70–77, 1992

†: Mutation reported after the closing of registration to the Research Committee in 1998.
§: Mutation reported in an abstract form in Japanese.