# Gene Transfer and Target Diseases

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## 1 Gene Transfer and Target Diseases

The first human gene-transfer protocol was developed in 1989, in an attempt to track lymphocytes in the immunologic treatment of melanoma and renal cell cancer. Although many attempts have been made in clinical trials of gene therapy, the FDA has not approved the marketing of any gene therapy agent.

For the successful therapeutic application of gene therapy, the delivery of several kinds of genes and nucleotides has been tested in animal models of genetic diseases as well as in patients to supply missing proteins to maintain cellular function, or to deliver proteins that induce proliferation or apoptosis of the cell, etc. For example, gene therapy has been applied to treating cardiovascular diseases, including coronary artery disease (CAD), peripheral artery disease (PAD), restenosis after vascular interventions and graft failure, hyperlipidemia, thrombosis, and cancer (e.g., lung, kidney, prostate, brain). In this chapter, the pathophysiology of several diseases and the application of gene delivery in their treatment will be described.

## 2 Cardiovascular Diseases

### *2.1 Coronary Artery Disease and Peripheral Artery Disease*

Atherosclerosis is the most prevalent process that affects adult coronary and peripheral arteries. Atherosclerotic lesions narrow arteries, leading to a reduction of the arterial blood supply to the myocardium and skeletal muscle. Stimulation of collateral vessel formation by the use of gene therapy will help to increase perfusion of the ischemic tissues. Genes encoding growth factors, such as vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF) and hepatocyte growth factor (HGF),

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have been successfully tested in animal models and clinical trials for therapeutic angiogenesis.

VEGFs are mitogenic and survival factors for endothelial cells, and promote angiogenesis and lymphangiogenesis. The VEGF family consists of six members, VEGF-A, -B, -C, -D, -E and placental growth factor (PLGF), which differ in their molecular masses and biological properties (Dvorak et al. 1995; Leung et al. 1989; Maglione et al. 1993; Joukov et al. 1996; Achen et al. 1998; Ogawa et al. 1998; Olofsson et al. 1996). The three receptors, VEGFR-1, VEGFR-2 and VEGFR-3, have tyrosine kinase activity. VEGF-A is the most well known member of the VEGF family, as it plays a crucial role in angiogenesis and vasculogenesis (Ferrara 2000, 2001). In addition, VEGF-A has several splice variants, of which two ($VEGF_{121}$ and $VEGF_{165}$) have been reported to be angiogenic in both animal models and clinical trials (Losordo et al. 1998; Vale et al. 2000). $VEGF_{121}$ is readily diffusible because it lacks a heparan-sulfate binding site, whereas $VEGF_{165}$ binds to the matrix after being secreted. VEGF-B, -C, -D, -E and PLGF also show angiogenic activity in animal models (Yoon et al. 2003; Rissanen et al. 2003; Kiba et al. 2003).

The FGF family has twenty-three members that share 30%–70% identical amino acid sequences. These growth factors act directly on vascular cells and induce endothelial cell growth and angiogenesis. Among the FGFs, FGF-1, -2, -4 and -5 have been tested for their angiogenic activity in animal models and are also the subjects of clinical trials in the therapy of cardiovascular disease and Atherosclerosis obliterans (ASO) (Ueno et al. 1997b; Javerzat et al. 2002; Grines et al. 2003). FGFs are multifunctional proteins that act through various alternatively spliced isoforms with four tyrosine kinase receptors, FGFR-1, -2, -3 and -4 (Galzie et al. 1997; Ornitz et al. 1996).

HGF is another multifunctional growth factor that stimulates the proliferation and migration of endothelial cells. In rabbit, rat and mouse ischemia models, HGF was reported to stimulate angiogenesis (Morishita et al. 1999, 2002; Morishita 2004; Hayashi et al. 1999). The efficacy and safety of intramuscular injection of naked human HGF plasmid was recently demonstrated in clinical trials in 22 patients with PAD or Berger's disease (Morishita 2004).

Angiopoietins (Angs) are also growth factors for vascular development. The Ang family has four members, Ang-1, -2, -3, -4, all of which bind to Tie-2, a tyrosine kinase receptor (Ward and Dumont 2002). Ang-1/Tie-2 and VEGF/VEGFR2 are crucial for the mobilization and recruitment of hematopoietic stem cells and the recruitment of circulating endothelial progenitor cells (Hattori et al. 2001). Ang-1 decreases the inflammatory response and promotes vascular maturity; thus, a combination of VEGF and Ang-1 may be a good strategy for therapeutic angiogenesis (Siddiqui et al. 2003; Yamauchi et al. 2003).

Hypoxia-inducible transcription factor (HIF)-1α can activate several genes involved in angiogenesis, such as VEGF, VEGFR-2, IGF-2 and erythropoietin (Levy et al. 1995). Adenovirus-mediated HIF-1α gene therapy is currently in clinical testing for the treatment of myocardial ischemia.

## 2.2 Restenosis After Vascular Interventions and Vein Graft Failure

The occlusion of arteries after balloon angioplasty, stenting, or the failure of bypass vein graft is a major factor that determines the prognosis of peripheral and coronary

artery disease. Smooth muscle cell proliferation, remodeling, matrix deposition, thrombosis, and platelet and leukocyte adhesion may all play a role in the development of arterial restenosis in these settings (Topol and Serruys 1998). In order to decrease vascular cell proliferation, various gene therapy strategies have been employed. Antiproliferative strategies designed for the treatment of experimental cardiovascular disease can be grouped into two main categories: (1) antisense approaches, ribozymes, and transcription-factor decoy strategies to inactivate positive cell cycle regulators; (2) overexpression of negative regulators of cell growth.

Transfection to arterial smooth muscle cells with thymidine kinase combined with gancyclovir, antisense oligonucleotides and ribozymes against cell cycle regulators, c-*myb*, c-*myc*, *cdc*-2, *cdk*-2, *ras*, *bcl*-x, and decoy constructs against transcription factors, such as E2F and NFκB, have all been shown to inhibit neointimal proliferation (Morishita et al. 1993, 1997; Indolfi et al. 1995; Pollman et al. 1998; Burgess et al. 1995; Suzuki et al. 1997). Inhibition of the cell cycle by transfection of genes encoding the non-phosphorylated forms of the retinoblastoma gene products p21, p27, p53, or of the growth arrest homeobox gene (*gax*) has been reported in animal models (Tanner et al. 1998; Yonemitsu et al. 1998; Chang et al. 1995a,b; Smith et al. 1997).

The transfection of genes encoding growth factors, including VEGF and HGF, results in decreased neointima formation in experimental animals (Hiltunen et al. 2000; Laitinen et al. 1997). The rapid regeneration of endothelial cells by growth factors restored the secretion of nitric oxide, C-type natriuretic peptide (CNP) and prostacyclin $I_2$, which have anti-proliferative effects on smooth muscle cells. The local expression of CNP suppressed neointimal formation in injured arteries of rats and vascular remodeling in porcine coronary arteries (Ueno et al. 1997a; Morishige et al. 2000).

## 2.3 Hyperlipidemia

Some hyperlipidemias are congenital, caused by a monogenic disorder. The strategies of gene therapy in hyperlipidemia are divided into three groups. (1) to supply the defective gene to correct the dyslipidemia, (2) to overexpress proteins involved in lipid metabolism, and (3) miscellaneous approaches.

Several studies have applied the first therapy strategy to the treatment of monogenic hyperlipidemias. Familial hypercholesterolemia (FH) is caused by defect in the LDL receptor, which results in severe hypercholesterolemia beginning at birth, cutaneous and tendon xanthomas, and atherosclerosis in childhood (Goldstein JL 2001). Trials of LDL or VLDL receptor gene delivery to the liver were carried out in WHHL rabbits and FH patients (Grossman et al. 1994, 1995; Kozarsky et al. 1994; Pakkanen et al. 1999; Lebherz et al. 2004; Kankkonen et al. 2004). The Apo A1 gene encoding a protein necessary for HDL synthesis and involved in reverse cholesterol transport, was delivered in patients deficient in this gene (Benoit et al. 1999). Apo E gene transfer was shown to be successful in treating *apoE* knockout mice (Cioffi et al. 1999; Okamoto et al. 2002; Gough and Raines 2003; Harris et al. 2002). Lipoprotein lipase (LPL), which catalyzes triglyceride formation in chylomicrons and VLDL, were delivered in LPL-deficient animals (Excoffon et al. 1997; Liu et al. 2000). Delivery of the gene encoding lecithin cholesterol acyl transferase (LCAT) corrected dyslipidemia in patients deficient in the gene and in hypoalphalipoproteinemia patients (Brousseau et al. 1998; Seguret-Mace et al. 1996).

The overexpression of proteins involved in the regulation of lipid metabolism has been tested in several systems. Hepatic expression of the catalytic subunit of apolipoprotein B mRNA editing enzyme (Apobec-1) reduced serum LDL-cholesterol levels in normal and WHHL rabbits (Greeve et al. 1996; Kozarsky et al. 1996). Expression of secreted "decoy" human-macrophage scavenger receptors (MSR) inhibited foam-cell formation in murine macrophages (Jalkanen et al. 2003a,b). Overexpression of LPL in transgenic WHHL rabbits improved hyperlipidemia and obesity (Koike et al. 2004). Finally, the long-term expression of human apo A-1 increased HDL size and inhibited atherosclerosis progression in LDLR knockout mice (Belalcazar et al. 2003).

## 2.4 Thrombosis

Gene transfer has a number of cardiac and systemic applications disease conditions, such as acute coronary syndromes, restenosis following percutaneous coronary intervention (PCI) and venous grafts, and thrombotic states. The strategies for gene therapy of thrombosis are: (1) to inhibit the coagulation pathway or platelet aggregation, (2) to activate fibrinolysis, and (3) to modulate endothelial function.

In order to target the coagulation cascade, transfer of the hirudin gene led to reduced intimal hyperplasia in a rat carotid-artery injury model (Rade et al. 1996). Gene delivery of thrombomodulin, a cell-surface glycoprotein of endothelial cells that binds thrombin, was reported to reduce thrombus formation in a rabbit model of stasis-induced arterial thrombosis (Waugh et al. 1999). In a rabbit carotid shear-stress-induced model of thrombosis, overexpression of the tissue-factor pathway inhibitor (TFPI) gene reduced thrombus formation (Nishida et al. 1999). Overexpression of cyclooxygenase-1, an inhibitor of platelet aggregation, increased production of the antiplatelet prostaglandin prostacyclin and reduced thrombus production in a porcine model of balloon-injury-induced carotid thrombosis (Zoldhelyi et al. 2001). Transfer of the nitric oxide (NO) synthase gene was reported to reduce arterial thrombosis in a rat carotid injury model and in a porcine coronary artery balloon-injury model (von der Leyen et al. 1995).

The targeted activation of fibrinolysis was achieved by overexpressing either recombinant tissue-type plasminogen activator (rTPA) or surface-anchored urokinase in endothelial cells and then seeding these cells into grafts in order to increase fibrinolytic activity. Reduced local platelet and fibrin deposition were observed while systemic markers of coagulation and fibrinolysis remained unchanged (Dichek et al. 1996).

Overexpression of NO synthase modulates endothelial function, inhibiting platelet adhesion after arterial injury (Yan et al. 1996).

## 2.5 Primary Pulmonary Hypertension

Primary pulmonary hypertension is a rare but life-threatening disease that causes right ventricular failure and death. The average survival from the time of diagnosis is 2.8 years (Nagaya 2004). In order to reduce pulmonary vascular resistance, the transfer of genes encoding eNOS, calcitonin gene-related peptide (CGRP), and prostacyclin synthase (PGIS) has been shown to be effective in model animals (Champion et al. 1999, 2000; Christman et al. 1992; Tuder et al. 1999; Nagaya et al. 2000).

## 2.6 Cerebral Vascular Disease

Several possible targets for gene therapy in treating cerebral vascular disease have been proposed: (1) prevention of vasospasm after subarachnoid hemorrhage (SAH), (2) protection against brain damage after ischemic stroke, (3) stimulation of collateral vessel formation in areas at risk of ischemia, (4) prevention of restenosis after angioplasty of the carotid and vertebrobasilar arteries, (5) inhibition of thrombosis.

Vasospasm is a serious problem after SAH and, currently, there is no effective method of prevention. Vasospasm is a great potential target of gene therapy because it occurs several days after the occurrence of SAH, so that there is sufficient time to deliver a gene (Toyoda et al. 2003). Moreover, the risk of vasospasm is highest during the 2 to 3 weeks after SAH, a short enough period of time to allow transient gene expression. The mechanisms of vasospasm after SAH may include impaired endothelium-dependent vasorelaxation, production of endothelium-derived contracting factors (endothelin, etc.), and impaired activity of potassium channels in cerebral vessels.

The in vivo transfer of the gene encoding endothelial NOS improved the NO mediated relaxation of the basilar arteries in vitro after experimental SAH (Onoue et al. 1998). Vasospasm in transgenic mice that overexpressed CuZn-SOD or EC-SOD was less severe after experimental SAH (Kamii et al. 1999); McGirt et al. 2002). Vascular contraction was inhibited after SAH by intracisternal administration of preproendothelin-1 antisense oligoDNA, which reduced production of endothelin peptide (Onoda et al. 1996).

CGRP has potent activity in opening potassium channels, hyperpolarizing arterial muscle, and dilating arteries. After SAH, CGRP was shown to depleted from nerves supplying cerebral arteries. The genetic transfer of prepro-CGRP prevented vasospasm in rabbits after experimental SAH (Nozaki et al. 1989; Edvinsson et al. 1991).

The therapeutic targets and genes used for gene therapy of vascular diseases are summarized in Table 1.

TABLE 1. Therapeutic target and genes used for gene therapy in vascular disease

| Therapeutic target | Treatment genes |
|---|---|
| Therapeutic angiogenesis | VEGF-A, -B, -V, -D, -E, FGF-1, -2, -4, -5, angiopoetin-1, HGF, MCP-1, PDGF, eNOS, iNOS, adrenomedullin |
| Restennosis, vein-graft failure | VEGF-A, C, eNOS, iNOS, COX, Thymidine kinase, CNP Fas ligand, p16, p21, p27, p53, NFkB and E2F decoys, cdk-2, cdc-2, c-myb, c-myc, ras, bcl-x, PCNA antisense oligonucleotides Ribozimes, Bloking PDGF or TGF-β expression or their receptors |
| Atherosclerosis, hyperlipidaemia | LDL receptor, VLDL receptor, apoA-1, Lipoprotein lipase, Hepatic lipase, LCAT, apoB, Lipid transfer proteins, Lp(a) inhibition, Soluble scavenger-receptor decoy, Soluble VCAM or ICAM, SOD, PAF-AH |
| Thrombosis | Hirudin, tPA, thrombomodulin, COX, TFPI |
| Pulmonary hypertension | Prepro-calcitonin gene related peptide, ANP, eNOS, prostacyclin syntase, VEGF-A, adrenomeullin |
| Vasospasm after SAH | Endothelial NOS, ECSOD, CuZnSOD, Antisense preproendothelin-1, Prepro-CGRP |

# 3 Cancer

Cancer is a genetic disease in which individual cells have mutations in genes related to growth control and apoptosis. In addition, cancer cells also have support systems to promote invasion and metastasis. Tumor growth is a result of the interaction of cancer cells with their microenvironment, including the extracellular matrix, immune system cells and cells involved in angiogenesis; therefore, each of these steps can serve as a target in controlling tumor growth. The strategies for cancer gene therapy can be directed at the tumor itself or at the host. Strategies directed at the tumor involve killing the tumor cells or slowing down their growth by, for example, introducing tumor suppressor genes, suppressing protooncogenes, and inducing prodrug/suicide, or apoptosis. Strategies direct at the host involve the inhibition of angiogenesis, the protection of normal tissues and increasing immunity.

## 3.1 Tumor Suppressor Genes

In order to target growth regulation in cancer cells, tumor suppressors, that inactivate the growth of some tumors, can be introduced (Bookstein et al. 1993). Since mutations in *p53* are widespread in human cancer, this gene may be the first target for the genetic therapy of cancer using tumor suppressors. The *p53* gene encodes a transcription factor involved in the regulation of the cell cycle and apoptosis. Gene transfer of *p53* to *p53*-defective cells resulted in cessation of cell growth or the induction of apoptosis (Yen et al. 2000; Horio et al. 2000). The transfer of other tumor suppressor genes, such as the retinoblastoma (*Rb*), *p16*, *pTEN* and *mda-7* genes, also effected suppression of tumor growth, (Demers et al. 1998; Jarrard et al. 1997; Lu et al. 1997).

## 3.2 Protooncogenes

Protooncogenes are activated by overexpression due to gene amplification, point mutations, modification of regulatory elements leading to increased transcription, and rearrangements. The products of these genes include growth factors or their receptors (EGF, EGFR), signal transduction proteins (ras, PI3 kinase), transcription factors (myc, fos, jun) (Isaacs et al. 1995; Konishi et al. 1997; Roylance et al. 1997), and suppressors of apoptosis (bcl-2). Gene therapy strategies to correct protooncogene activation include the transfer of dominant negative gene products, antisense oligodeoxynucleotides, ribozymes, and small interference RNAs. For example, disruption of overexpressed c-*myc* using anti-sense c-*myc* resulted in a 94.5% reduction of tumor size in prostate cancer (Steiner et al. 1998).

## 3.3 Prodrug/Suicide

The mechanism of prodrug/suicide gene therapy is based on the difference in mitotic activity between normal and cancer cells. The cells are transduced with the herpes simplex virus thymidine kinase (HSV-TK) gene, and are subsequently killed by treatment with the drug acyclovir. The transduced enzyme, HSV-TK, phosphorylates the prodrug into the antiviral compund gancyclovir triphosphate, an inhibitor of DNA

synthesis, which leads to cell death (Ayala et al. 2000; Koeneman et al. 2000; Shalev et al. 2000; Martiniello-Wilks et al. 1998; Hall et al. 1999).

## 3.4 Apoptosis Induction

The triggering of programmed cell death in tumor cells without affecting normal cells is an attractive therapeutic approach in cancer gene therapy. Ligands that induce apoptosis include tumor necrosis factor (TNF)-α, FasL (Hedlund et al. 1998; Hedlund et al. 1999), and TRAIL (Griffith and Broghammer 2001; Voelkel-Johnson et al. 2002). As TRAIL has the lowest activity in normal tissue, it is an especially promising new therapeutic approach to cancer therapy.

## 3.5 Inhibition of Angiogenesis

Regulation of the angiogenic switch depends on the local balance between activators and inhibitors. Angiogenesis is triggered by the release of angiogenic stimulators by tumor cells, either as a result of genetic alterations or through activation of the physiologic response to hypoxia, which activates various inducible factors, e.g., transcription factors that trigger the transcription of genes encoding angiogenic stimulators. Many angiogenic factors have already been identified, including VEGF (Ferrara 1999), vascular permeability factor (VPF), FGF, EGF, platelet-derived endothelial growth factor (PD-EGF), PDGF, insulin-like growth factors (IGFs) (Trojan et al. 1994), interleukin-8 (IL-8), transforming growth factor α and β (TGF-α and -β) (Maggard et al. 2001), heparin growth factor, granulocyte colony stimulating factor (GMCSF), E-selectin (Tang et al. 2004), and TNF-α (Claesson-Welsh 2003; Brieger et al. 2003), and used for anti-angiogenic gene therapy of cancers. The goal of this type of therapy is to switch the balance between angiogenic factors and angiogenic inhibitors in the tumor microenvironment to the anti-angiogenic phenotype.

## 3.6 Increased Immunity

Gene therapy targeted to the immune system has a good likelihood of success as a cancer therapy. In this approach, an antitumor immune response in the host is created, either by immunotherapy, vaccination with cytokine genes, including IL-2 (Belldegrun et al. 2001; Kawakita et al. 1997; Moody et al. 1994; Toloza et al. 1996), IL-4, IL-7, IL-12 (Sanford et al. 2001; Hull et al. 2000; Nasu et al. 1999), GM-CSF (Simons et al. 1999), M-CSF, and interferons.

# 4 Conclusions

The therapeutic target and genes used for gene therapy in cancer are summarized in Table 2.

TABLE 2. Therapeutic target and genes used for gene therapy in cancer

| Aim of Therapeutic Strategy | Target Genes |
|---|---|
| Induction of tumor suppressor genes | p53, Rb, p16, pTEN, mda-7 |
| Suppression of Protooncogene | EGF, EGFR, ras, PI3 kinase, c-myc, c-fos, jun, Bcl-2 |
| Prodrug/Suiside | HSV-tk (ganciclovir) |
| Apoptosis Induction | TNFa, Fas ligand |
| Induction of apoptosis | Bcl-2, c-myc, c-raf, MDM-2, IGF-II, STAT-3 p21, p53, Fas ligand, c-cam, Caspase7 TRAIL, Bax |
| Inhibition of angiogenesis | Suppression of VEGF, VPF, FGF, EGF, PD-EGF, IGFs IL-9, TGF-a and b, GM-CSF, E-selection, TNF-a |
| Immunotherapy | IL-2, IL-4, IL-7, IL-12, GM-CSF, Interferons |

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# Clinical Features and Genetic Analysis of Autosomal Recessive Hypercholesterolemia

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**Previously we have reported on siblings with severe hypercholesterolemia, xanthomas, and premature atherosclerosis without any impairment of low-density lipoprotein receptor in their fibroblasts as a first characterization of autosomal recessive hypercholesterolemia (ARH). Recently, mutations were identified for this disease in a gene encoding a putative adaptor protein. The purpose of this study was to examine the molecular pathogenesis of ARH in Japanese siblings. A novel insertion mutation was discovered in the *ARH* gene of the siblings. An insertion of an extra cytosine residue was identified in a locus comprising eight consecutive cytosines at positions 599 through 606 in exon 6, resulting in a sequence of nine cytosines and generating an early stop codon at 657–659. The mother was heterozygous for this mutation. Neither transcription product nor protein of ARH was detected in the fibroblasts of the homozygous patients. A single nucleotide polymorphism was discovered among the normal control subjects at position 604 (cytosine to thymine: *ARH-604C* to *ARH-604T*), which changes the proline residue at 202 to serine. Interestingly, ARH is caused by a mutation of cytosine to adenine at this same position. Both siblings exhibited fatty liver, which may also be related to this mutation. (*J Clin Endocrinol Metab* 88: 2541–2547, 2003)**

IN 1973 KHACHADURIAN and Kuthman (1) first described two categories of hereditary hypercholesterolemia, autosomal dominant and recessive. A few years later, extensive studies on hypercholesterolemia with autosomal dominant inheritance led to the discovery of the low-density lipoprotein (LDL) receptor. A genetic defect in this receptor protein characterizes familial hypercholesterolemia (FH). Patients with homozygous FH show severe elevation of plasma LDL, cutaneous and tendon xanthomas, and atherosclerotic vascular lesions during the first decade of life (2). On the other hand, severe hypercholesterolemia with autosomal recessive inheritance exhibits almost the same clinical features as those of FH homozygotes but is apparently rare and had never been characterized until our first reports of this disease (3, 4). We described the siblings as having severe elevation of plasma LDL levels despite normal LDL receptor activity in their fibroblasts as the first characterization of severe hypercholesterolemia with autosomal recessive inheritance. The binding, incorporation, and degradation of $^{125}$I-LDL by their fibroblasts were normal (3). Their LDL also bound, internalized, and degraded normally in their fibroblasts (3). The pulse-chase pattern for the LDL receptor protein in their fibroblasts was normal, suggesting that their LDL receptor protein was synthesized and processed normally (3). Their LDL receptor genes were shown to have different haplotypes, excluding the possibility that the disorder is caused by homozygosity of this gene. The fractional catabolic rates of plasma cholesterol were calculated from the rebound increase curve after LDL apheresis and were found to be severely impaired in both patients. This indicates a similarity between homozygous FH disorder and autosomal recessive hypercholesterolemia (ARH) with respect to the LDL metabolism in plasma (4).

Several authors subsequently reported severe hypercholesterolemia with autosomal recessive inheritance, and this disease was named autosomal recessive hypercholesterolemia (5–7). Using isotope-labeled LDL *in vivo*, ARH was shown to have selective reduction in hepatic LDL uptake (6, 7).

Recently, Garcia *et al.* (8) mapped the *ARH* locus to chromosome 1p35 and identified six mutations in a gene encoding a putative LDL receptor adaptor protein in the families with ARH. ARH protein contains a phosphotyrosine-binding domain, which in other cells binds NPXY motifs in the cytoplasmic tails of cell-surface receptors. This suggests that ARH has a tissue-specific role in LDL receptor function because it is required in liver but not in fibroblasts. However, the underlying mechanism linking gene mutations and impairment of LDL metabolism still remains unclear. Thus, it is very important to identify the nature of the mutations for the patients in whom the clinical characterization has been established to investigate a role of this new gene product in LDL metabolism. For this reason we undertook the analysis of *ARH* for the Japanese family with ARH. We report a novel insertion mutation in exon 6 of the LDL receptor adaptor protein gene in these homozygous siblings with ARH. We describe the detail clinical features of these patients as well

Abbreviations: ARH, Autosomal recessive hypercholesterolemia; FCS, fetal calf serum; FH, familial hypercholesterolemia; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; LDL, low-density lipoprotein; PTB, phosphotyrosine binding; S-MEM, minimum essential culture medium.

as a new single nucleotide polymorphism occurring at the same locus in healthy volunteers.

## Patients and Methods

### *Characteristics of the patients*

The proband is a 48-yr-old Japanese male who first noticed xanthomas in his elbows and knees at age 9 or 10 yr. He visited the National Cardiovascular Center Hospital for the first time when he was 30 yr old in 1982. Large cutaneous and tendinous xanthomas were identified in his fingers, elbows, and knees (Fig. 1, A and B). The thickness of his Achilles tendons was 23 mm in the right and 28 mm in the left. Before he began medical treatment, he was diagnosed as having fatty liver by abdominal computed tomography and ultrasonogram. Alcohol consumption was examined by a questionnaire. The kind of alcohol beverage, quantity per day, and frequency per week were asked. He was a light drinker (22 g alcohol/d). He has been treated with LDL-apheresis once every other week since 1983. In 1987 at the age of 35 yr, the patient developed coronary symptoms, and significant stenoses were found in the coronary artery lesions by angiography. Percutaneous transluminal coronary angioplasty was performed five times, and he has not experienced any cardiac symptom since then.

The elder sister of the proband was found to be deaf when she was 1 yr of age after suffering from severe high fever for unknown reasons. She had severe hypercholesterolemia and exhibited multiple cutaneous and tendinous xanthomas from age 14 yr. She developed muscle pain and weakness when she was 32 yr old and was diagnosed with polymyositis and fatty liver by muscle and liver biopsies, respectively. The muscle biopsy specimen showed infiltration of massive inflammatory cells. At the time of the diagnosis, no medication was being administered. She had no habit of alcohol intake. The thickness of her Achilles tendons was 18.5 mm in the right and 20 mm in the left. She was treated with LDL-apheresis for hypercholesterolemia and corticosteroid for polymyositis. When she was 45 yr old, the polymyositis became aggravated, and higher doses of corticosteroid were given to control the disease. Intestinal perforation and peritonitis followed, and the patient died of endotoxic shock.

The siblings were born to consanguineous parents of an uncle and a niece (4). Their father died of stroke at the age of 61 yr, never having any signs of hypercholesterolemia or xanthomas. Their mother is healthy with normal plasma cholesterol level (200 mg/dl) and has no xanthomas. No known relative has a record of coronary artery disease. The major clinical parameters are listed in Table 1.

### *Cell culture*

Fibroblasts were established as cell lines from skin biopsy specimens of the proband, the sister of the proband, their mother, and a normal control subject. Stock cultures were maintained as monolayers in a humidified incubator (5% $CO_2$) at 37 C in minimum essential culture medium (S-MEM) (Life Technologies, Inc., Rockville, MD) containing penicillin (100 U/ml), streptomycin (100 $\mu$g/ml), and 10% (vol/vol) fetal calf serum (FCS) (Hyclone Laboratories, Inc. AB, Lund, Sweden).

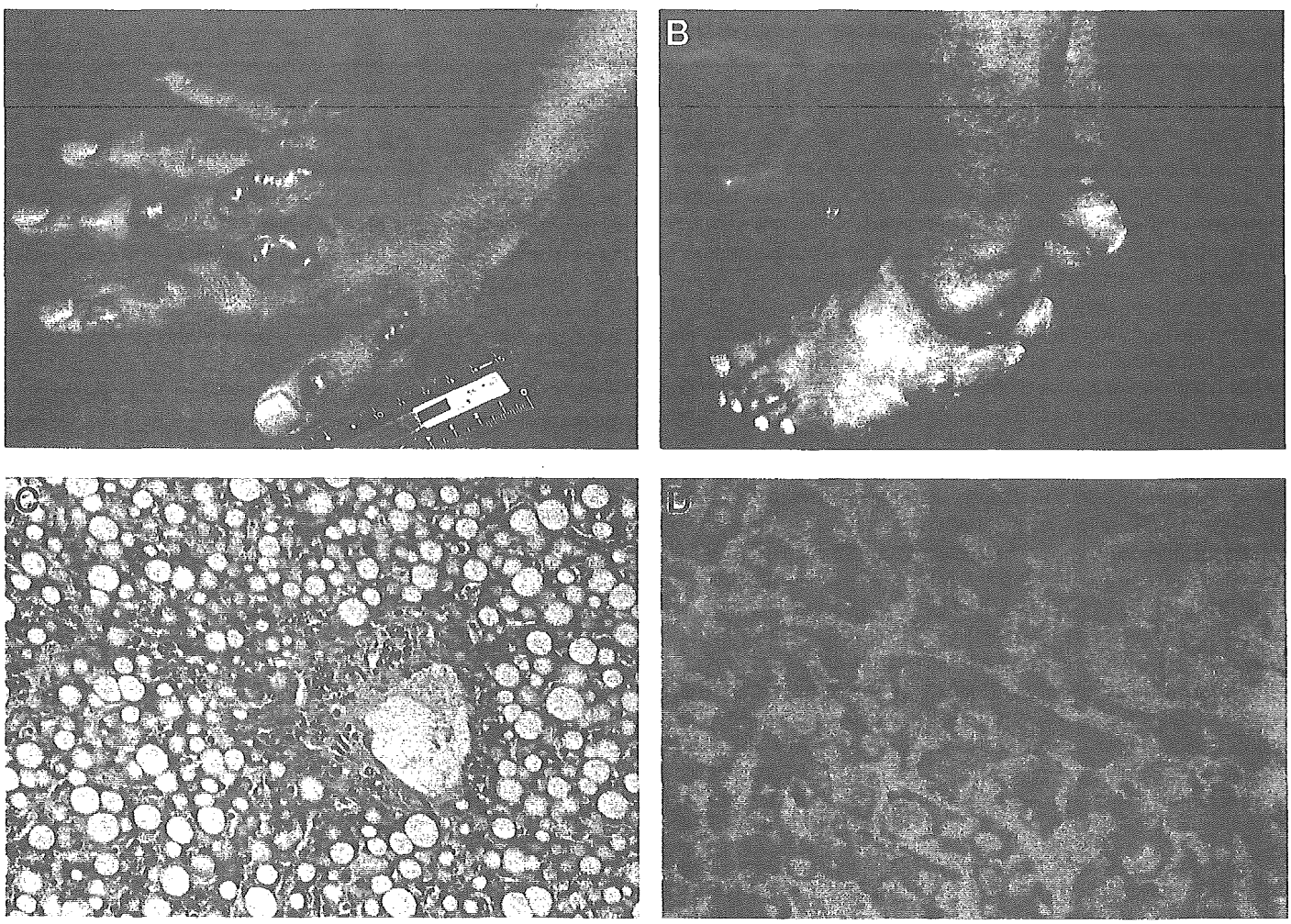


FIG. 1. Multiple extensive xanthomas in the proband's hand (A) and foot (B). C, Microscopic findings of the patient's (sister of the proband) liver. The specimen was stained by hematoxylin and eosin. D, The specimen was stained by Sudan III.

**TABLE 1.** Clinical data of the family member

| | Proband | Sister of the proband | Mother | Control[a] |
|---|---|---|---|---|
| Age (yr) | 48 | Dead (45) | 74 | |
| Body weight (kg) | 78 | 45 | 50 | |
| Height (cm) | 168 | 153 | 150 | |
| Body mass index (kg/m$^2$) | 27.6 | 19.2 | 22.2 | |
| Initial total cholesterol (mg/dl) | 600 | 533 | 200 | |
| Initial triglyceride (mg/dl) | 160 | 150 | 130 | |
| Xanthomas (cutaneous/tendon) | (+/+) | (+/+) | (−/−) | |
| Lipid profiles in liver specimens (% of wet weight) | | | | |
| Triglyceride | Not determined | 0.97 | Not determined | 0.47 ± 0.20 |
| Cholesteryl ester | Not determined | 0.16 | Not determined | 0.19 ± 0.08 |
| Free cholesterol | Not determined | 0.21 | Not determined | 0.17 ± 0.03 |
| Phospholipid | Not determined | 1.23 | Not determined | 2.95 ± 0.29 |

[a] Data are from Ref. 16.

### *Northern blot analysis*

Total cellular RNA was isolated from fibroblasts using the acid guanidium thiocyanate-phenol-chloroform method, described by Chomczynski and Sacchi (9). Total RNA (10 μg) was electrophoresed in 0.9% agarose gels containing 2.2 M formaldehyde and then transferred onto nylon membranes (Biodyne Nylon Membranes, Pall BioSupport, East Hills, NY). The membrane was hybridized at 68 C for 3 h, with the following probes labeled with [$^{32}$P]dCTP (Amersham Biosciences, Buckinghamshire, UK) using the multipriming method (10). The human *ARH* cDNA was purchased from RZPD (Berlin, Germany) as DKFZp586D0624 (GenBank accession no. AL117654), and the human LDL receptor cDNA was a kind gift from Dr. D. W. Russell (University of Texas). Full-length cDNAs were used as probes. The human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was obtained from CLONTECH Laboratories, Inc. (Palo Alto, CA). The membrane was washed and exposed to an imaging plate (Fuji Photo Film Co., Ltd., Tokyo, Japan). The radioactive bands were detected and quantitative analysis was done by a BAS 2500 image analyzer (Fuji Photo Film Co., Ltd.).

### *DNA sequence*

Genomic DNA was isolated from fibroblasts (the proband, the sister of the proband, and their mother) or peripheral mononuclear cells of normal control human subjects and used as a template for PCR. The sequence of the exon and intron boundaries of the *ARH* gene was obtained from Celera human sequence data except for exon 1 (11). DNA fragments containing each exon and exon-intron boundaries of the *ARH* gene were amplified from the genomic DNA by Gene Amp, PCR System 9700 (Applied Bio-System, Foster City, CA). PCR was performed using the Advantage 2 protocol (CLONTECH) with 25 pmol of each pair of primers listed in Table 2. The exon 1-intron 1 boundary information did not exist in the Celera human sequence data or the database of the Human Genome Project Consortium. To obtain the sequence of the proband's information, mRNA was reverse transcribed into cDNA using Superscript II reverse transcriptase (Life Technologies, Inc.). The primer used in reverse transcription is shown as exon 1-RT in Table 2. This primer is an antisense sequence located in exon 2. The cDNA was amplified by PCR using exon 1-F sense primer and a nested antisense primer exon 1-R of primer exon 1-RT. PCR products were purified by Microcon (Millipore Corp., Bedford, MA) and used as templates for direct sequencing. DNA sequencing was carried out according to the manufacturer's instructions using a dye terminator method (BigDye Terminator Cycles Sequencing Ready Reaction, Applied BioSystem).

For sequencing the sample of the mother, the amplified DNAs were cloned into pCR 2.1 vector (Invitrogen, Carlsbad, CA). Ten independent clones were isolated for each amplified DNA and sequenced.

### *Western blot analysis*

The fibroblasts from the proband, the sister of the proband, their mother, and a normal control subject were seeded into a 100-mm dish. On d 5 of cell growth, cells received S-MEM containing 10% of lipoprotein-deficient serum prepared from FCS. After incubation for 48 h, the cells were suspended in PBS and centrifuged at 5000 rpm for 5 min. The cell pellets were washed in buffer containing 100 mM NaCl, 50 mM HEPES, 10 mM EDTA, 10 mM EGTA, 2.2% dimethyl sulfoxide, and protease inhibitor mixture (Sigma, St. Louis, MO) and lysed in the same buffer containing 1% (vol/vol) triton X-100 (Sigma). Lysates were centrifuged in a microfuge for 5 min at 15,000 rpm, and the supernatant was collected. Ten micrograms protein were separated by electrophoresis (25 V, 3 h) on a 10% polyacrylamide-sodium dodecyl sulfate gel. Proteins were transferred to a nitrocellulose membrane (Bio-Rad Laboratories, Inc., Hercules, CA). The membrane was incubated with serum (1:100) from a rabbit immunized with a peptide containing the C-terminal 18 amino acids of human ARH. Horseradish peroxidase-conjugated goat antibody against rabbit IgG and the enhanced chemiluminescence Western blotting detection kit (Amersham Biosciences) were used for the signal detection.

**TABLE 2.** Oligonucleotide sequence of the PCR primers

| Primer | Sequence |
|---|---|
| Exon 2-F | GAGAGCTGTTGCTGGTGGTG |
| Exon 2-R | GGTCCTTGGTCCCTGGTTCC |
| Exon 3-F | TCAAGTGAGGCTGGCAGACT |
| Exon 3-R | CCAAGTGGCAGAGTGGATGG |
| Exon 4-F | CCCTGCAGGGCTTCCCACAT |
| Exon 4-R | CCACTGGACCCTGTCCCCGC |
| Exon 5-F | TCTGCCCTGGCTGACACTGC |
| Exon 5-R | ACCTCCCACCACACCCACAA |
| Exon 6-F | TGGGGTCTGAGGCTCCAACA |
| Exon 6-R | CCACACCGCATGGTCAATCC |
| Exon 7-F | GGGGGCCAGGAAGGAAGAGG |
| Exon 7-R | AGGCTCACCCAGAGGTCAGG |
| Exon 8-F | CCACCCTGAGCTTGTGTCCT |
| Exon 8-R | GCACACCAGGCCCCCTACCC |
| Exon 9-F | GTGCCCCCTCGCGTCTGACC |
| Exon 9-R | GATGCCCCCAGGGAAATTTG |
| Exon 1-RT | AACAGCATCCCCTCCAGCAGCGTCTCCCGC |
| Exon 1-F | CGCGTCCGCCGGAGCGGGCC |
| Exon 1-R | GTGTCTGTCCAGTTCTCAGGCAGCT |

### *Lipid analysis of liver specimens*

An autopsy liver specimen was obtained from the sister of the proband. The tissue was homogenized and the lipid fraction was extracted with chloroform:methanol, 2:1 (vol/vol). After the sample was filtered and washed using Folch's procedure three times, the extract was dried with $N_2$ (12). The lipid fraction was measured for triglyceride, total and free cholesterol, and phospholipid using enzymatic assay kits (13–15).

All procedures for the analysis of patients have been approved by the institutional ethics committee, and written informed consent was obtained from each subject or their legal representatives.

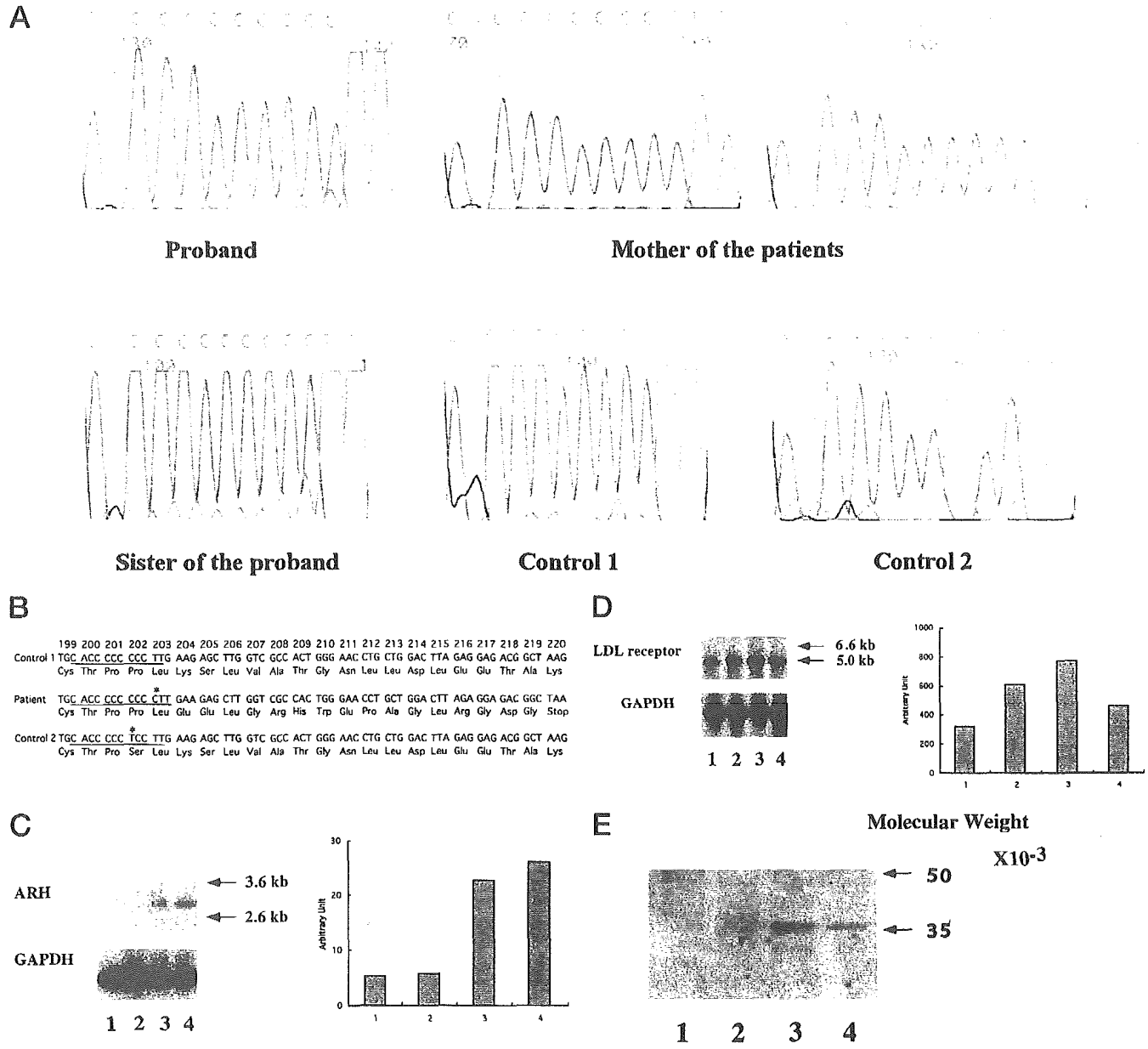


FIG. 2. A, DNA sequence data of the affected siblings, their mother, and control subjects. The mother of the patients has two alleles detected by subcloning. B, The predicted amino acid sequence in the patient and control subjects. The *asterisks* show the mutation locus of the DNA. C, Northern blot analysis of the *ARH* gene. On d 0, fibroblasts from each subject were seeded into 10-cm plastic dishes and cultivated in S-MEM containing 10% FCS. On d 7, cells were collected. Total RNA (10 $\mu$g) was electrophoresed in 0.9% agarose in the presence of 2.2 M formaldehyde, transferred onto nylon membrane, and hybridized with $^{32}$P-labeled probes. The membrane was rehybridized with human GAPDH as an internal standard. Quantitative analysis was done and is shown in the *right panel*. Lane 1, The proband; lane 2, the sister of the proband; lane 3, their mother; lane 4, normal control subject. D, Northern blot analysis of LDL receptor gene. On d 0, fibroblasts from each subject were seeded into 10-cm plastic dishes and cultivated in S-MEM containing 10% FCS. On d 5, the medium was changed to S-MEM containing 10% lipoprotein-deficient serum. On d 7, the cells were collected. Total RNA (10 $\mu$g) was electrophoresed in 0.9% agarose in the presence of 2.2 M formaldehyde, transferred onto nylon membrane, and hybridized with $^{32}$P-labeled probes. The membrane was rehybridized with human GAPDH as an internal standard. Quantitative analysis was carried out and is shown in the *right panel*. Lane 1, The proband; lane 2, the sister of the proband; lane 3, their mother; lane 4, normal control subject. E, Western blot analysis of ARH protein in cultured skin fibroblasts from each subject. Lane 1, The proband; lane 2, the sister of the proband; lane 3, their mother; lane 4, normal control subject.

## Results

### *Mutation in the ARH gene*

For each DNA segment of exon 2–9, an exon and exon-intron boundary was amplified with the primers described in *Patients and Methods*. Because the information for intron 1 was not found in the available data banks, a different strategy had to be used. The information for exon 1 was found by using RT-PCR, with primers of 5′-flanking sequence and from the sequence in exon 2. The PCR products of each exon of the siblings and mother of the patients were sequenced

and compared with their counterparts in control subjects. There are eight sequential cytosines between the nucleotide positions 599 and 606 (nucleotides are numbered from the first nucleotide that encodes the starting methyonine codon) in exon 6 of the normal *ARH* gene. An extra cytosine is inserted into this region of the *ARH* gene in the affected siblings. This insertion mutation causes a frame shift and results in a change of amino acid residue sequence at position 204 and generating a stop codon at position 220 (Fig. 2, A and B). For the mother's sample, the amplified DNAs were cloned and sequenced. From among 10 independent clones, four showed eight cytosines and six showed nine cytosines, suggesting that the mother is heterozygous for the mutation (Fig. 2A).

Among 20 healthy normolipidemic control subjects, four were shown to be homozygous for a nucleotide change of cytosine to thymine at nucleotide position 604 that then coded for a serine instead of proline at amino acid position of 202. Another 10 control subjects were heterozygous for the same mutation. No mutation detected in the affected siblings (extra cytosine-inserted mutation) was found in normolipidemic controls. Clinical features of control subjects are shown in Table 3. Alleles having the single nucleotide polymorphism were designated as *ARH-604C* and *ARH-604T*.

*Northern blot analysis*

Total RNA was isolated from fibroblasts of the affected siblings, their mother, and a control subject and was analyzed by Northern blot. When hybridizing with the full-length *ARH* cDNA as a probe, no band was detected for either patient (the proband or sister of the proband) (Fig. 2C). Quantitative analysis of the blotting bands is also shown in Fig. 2C. The *ARH* RNA band of the mother appeared a little fainter than that of the control subject. The RNA of the LDL receptor of the patients appeared normal both in size and expression level (Fig. 2D).

*Western blot analysis*

No immunodetectable ARH protein could be demonstrated for either patient (the proband or sister of the proband), but the ARH band was visualized significantly in the heterozygous patient (Fig. 2E).

*Findings in the liver*

Both homozygous patients were diagnosed with fatty liver by means of liver biopsy or abdominal computed tomography around age 30 yr. After the sister of the proband died, an autopsy sample of the liver was microscopically examined and analyzed for a lipid profile. The hepatocytes were swollen (Fig. 1C), and large lipid droplets were identified with Sudan III staining (Fig. 1D). Lipid was extracted from the liver. Triglyceride, cholesterol, and phospholipid levels were determined. An increase of triglyceride was prominent among other lipids, being consistent with the diagnosis of fatty liver (Table 1).

## Discussion

Here, we described clinical features and genetic analysis of an autosomal recessive hypercholesterolemia family. When this family had previously been described as the first clinical characterization of this disease (3, 4), other factors known at that time to be potentially involved in hypercholesterolemia were excluded. From the rebound curve after the LDL-apheresis treatment, the fractional catabolic rate of plasma cholesterol was calculated and found to have decreased (4). We, therefore, surmised that the patients had a catabolic disorder in the plasma cholesterol caused by a factor(s) other than defective LDL receptors.

Zuliani *et al.* (7) described Sardinian families in which the probands had a marked reduction in the catabolic rate of $^{125}$I-LDL and a reduction in the rate of hepatic uptake of LDL, demonstrated using the $^{99}$technetium-LDL biodistribution study. Schmidt *et al.* (6) reported a Turkish subject with clinical homozygous FH phenotype whose parents, siblings, and children all had normal cholesterol levels. An LDL turnover study of the patient showed a delayed catabolism despite a functionally intact LDL receptor and apolipoprotein B, suggesting that the genetic impairment involves plasma LDL metabolism. Thus, these patients, classified as ARH, have been categorized in the clinical entity of the genetic disorder of LDL catabolism by reason(s) other than dysfunction of the LDL receptor gene.

Recently, Garcia *et al.* (8) mapped the *ARH* locus to chromosome 1p35. After screening 13 genes within this locus, they identified six mutations in a gene encoding a putative adaptor protein in ARH families including Sardinian ones who had been reported to have retardation in LDL turnover (7). Notably, an LDL receptor adaptor protein contains a phosphotyrosine-binding (PTB) domain that interacts with the NPXY motif in the cytoplasmic tails of cell surface receptors including the LDL receptor. On the basis of this information, we analyzed this gene in the family we previously reported as ARH and identified a novel insertion mutation in the *ARH* gene in exon 6. There are eight sequential cytosines between nucleotide positions 599 and 606 in exon 6 of the normal *ARH* gene, and an extra cytosine is inserted into this region of the *ARH* gene in the affected siblings to cause a frame shift, generating a stop codon after amino acid position 219. A predicted, truncated ARH protein contains an intact PTB domain. In preparation of this manuscript, two reports of genetic analysis of ARH have been published (17, 18). Arca *et al.* (17) reported that two *ARH* mutations were

**TABLE 3.** Clinical profiles of control subjects

| | n | Age (yr) | TC (mg/dl) | TG (mg/dl) | BMI (kg/m$^2$) |
|---|---|---|---|---|---|
| ARH-604C (Homo) | 6 | 39.7 ± 7.2 | 186.7 ± 24.7 | 77.3 ± 24.4 | 22.1 ± 1.7 |
| ARH-604C/T (Hetero) | 10 | 40.2 ± 11.0 | 198.8 ± 26.8 | 82.2 ± 35.6 | 21.8 ± 2.9 |
| ARH-604T (Homo) | 4 | 38.3 ± 4.3 | 158.0 ± 72.0 | 70.8 ± 31.4 | 19.2 ± 10.0 |

TC, Total cholesterol; TG, triglycerides; BMI, body mass index.