BioResearch Open Access
Volume 2, Number 6, December 2013

DOI: 10.1089/biores.2013.0042

Three Nonsynonymous Single Nucleotide Polymorphisms in the *RhitH* Gene Cause Reduction of the Repression Activity That Leads to Upregulation of M-LPH, a Participant in Mitochondrial Function

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Abstract

Human Mpv17-like protein (M-LPH) has been suggested to play a role in mitochondrial function. In this study, we identified a RhitH (human regulator of heat-induced transcription) binding site in intron 1 of the *M-LPH* gene. Tissue distribution analysis showed that M-LPH was specifically distributed in tissues with high mitochondrial metabolism. Functional and genetic analyses of nonsynonymous single nucleotide polymorphisms (SNPs) in the *RhitH* gene revealed that p.Cys461Ser, p.Thr465Ala, and p.Leu495Gln, corresponding to substitutions in the zinc fingers, cause reductions in the repression activity that lead to upregulation of M-LPH expression. The analyses also showed that the minor allele frequencies of these SNPs are extremely low in worldwide populations.

Key words: cellular biology; molecular biology

Introduction

The Mpv17-like protein (*M-LP*) gene was initially identified through screening of genes that are age-dependently expressed in mouse kidney.[1–3] M-LP has high sequence homology with Mpv17, a mitochondrial inner membrane protein. It has been shown that the *Mpv17* gene is responsible for a recessive kidney disease in mouse, and that *Mpv17* gene–inactivated mice exhibit symptoms resembling those of human glomerulosclerosis or Alport syndrome.[4,5] Moreover, it has recently been reported that mutations in the human *Mpv17* gene cause mitochondrial DNA depletion syndrome,[6] over 10 pathogenic mutations having been identified so far.[7–10] The mouse *M-LP* gene is expressed as two distinct splicing transcripts, M-LP$_L$ and M-LP$_S$, generated by alternative usage of two distinct promoters localized upstream of exons 1 and 2 of the *M-LP* gene.[11] Of the two transcripts, M-LP$_S$ shows an obvious age-dependent pattern of expression. Subsequent analysis has revealed that the expression of M-LP$_S$ is transcriptionally regulated by at least two transcription factors: heat shock factor as an activator and Rhit (regulator of heat-induced transcription), a novel repressor, which binds to the Tramtrack (Ttk) 69K binding site within the M-LP$_S$ promoter.[12] The age-dependent expression of M-LP$_S$ is considered attributable to Rhit expression in view of the striking inverse relationship between them. Rhit belongs to the Krüppel-associated box (KRAB)-containing protein family, which is characterized by the presence of a KRAB domain of approximately 50–75 amino acids and multiple Cys_2His_2 zinc fingers. The KRAB domain is located near the N-terminus and is composed of either the KRAB-A box, involved in repression by binding to corepressor KRAB-associated protein 1,[13] or the KRAB-B box, which is considered to enhance repression, or both.[14,15] Rhit contains a KRAB domain comprising only the KRAB-A box, and a DNA binding domain composed of eight Cys_2His_2 zinc fingers in the C-terminus. Human homologs of M-LP (M-LPH) and Rhit (RhitH) have also been separately identified.[16,17] The *M-LPH* gene is expressed as two alternatively spliced variants generated by usage of the same promoter, which encode two distinct

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ABBREVIATIONS: GABP, GA-binding protein; KRAB, Krüppel-associated box; M-LPH, human Mpv17-like protein; PCR, polymerase chain reaction; RFLP, restriction fragment length polymorphism; RhitH, human regulator of heat-induced transcription; RPTECs, renal proximal tubule epithelial cells; SDS, sodium dodecyl sulfate; SNP, single nucleotide polymorphism; Ttk 69K, Tramtrack 69K

isoforms, M-LPH1 and M-LPH2. On the other hand, RhitH has a functional domain composition similar to that of Rhit, with which it shares 69% identity and 75% similarity. The molecular functions of M-LPH and RhitH have yet to be elucidated, but studies demonstrating that the expression of several antioxidant enzymes is affected by transfection with M-LPH have suggested that they participate in metabolism of reactive oxygen species. Moreover, it has been revealed that GA-binding protein (GABP) binds to specific elements in the promoter of the *RhitH* gene.[17] GABP is one of the key regulators of the mitochondrial electron transport system, regulating the expression of cytochrome *c* oxidase subunits IV, Vb, and VIIa and ATP synthase β.[18–21] Additionally, it has been demonstrated that overexpression of M-LPH increases resistance to mitochondrial dysfunction caused by an inhibitor of the respiratory chain.[17] These observations strongly suggest the involvement of M-LPH in mitochondrial function through regulation by RhitH. Although several single nucleotide polymorphisms (SNPs) that might affect the repressor activity are located in the *RhitH* gene, it remains unknown if these are functional.

In the present study, we first confirmed the involvement of RhitH in the regulation of M-LPH expression, and then analyzed the tissue distribution of M-LPH and RhitH to gain further clues to the function of M-LPH. In addition, we focused on functional nonsynonymous SNPs in the *RhitH* gene as possible genetic factors responsible for M-LPH dysfunction and examined nine SNPs in the functional domains for possible effects on the repression activity of RhitH.

Materials and Methods

Cells

The human breast cancer cell line MCF-7 (JCRB0134) was obtained from the Health Science Research Resources Bank (HSRRB, Osaka, Japan). We chose MCF-7 because a moderate level of ZNF205 expression is demonstrated on the website of the Human Protein Atlas Project (www.proteinatlas.org). MCF-7 cells were maintained in Eagle's minimal essential medium containing 10% (v/v) fetal calf serum, 10 μg/mL insulin, 0.1 mM nonessential amino acids, and 1 mM sodium pyruvate in a humidified incubator at 37°C with an atmosphere of 5% CO_2. Normal human renal proximal tubule epithelial cells (RPTECs) were purchased from Lonza (Walkersville, MD) and maintained in accordance with the manufacturer's instructions.

Biological samples

Genomic DNA was extracted from blood or bloodstain samples collected randomly from healthy subjects derived from 16 different populations (126 Ovambos, 105 Ghanaians, 75 Xhosas, 136 Turks, 68 Germans, 110 Japanese, 352 Koreans, 193 Chinese, 112 Mongolians, 153 Tibetans, 35 Tamils, 48 Sinhalese, 40 Tamangs, 51 Huicholes, 88 Nahuas, and 60 Mestizo) after obtaining written informed consent. The study was approved by the institutional Human Ethics Committee.

Generation of luciferase reporter constructs and dual luciferase assays

DNA segments containing different portions of the sequences from 5′-upstream to intron1 of the *M-LPH* gene were amplified by polymerase chain reaction (PCR) using the sets of primers shown in Table 1 and human genomic DNA as the template and cloned into the *Sac*I/*Bgl*II site of the pGL4.10[luc2] vector (Promega, Madison, WI). The first nucleotide of exon 1 is defined here as +1. Site-specific mutations of RhitH binding sites within the intron 1 sequence of the *RhitH* gene were carried out with a KOD-Plus-Mutagenesis kit (Toyobo, Osaka, Japan) using pGL4-345/589 as a template. MCF-7 cells were grown in 12-well plates to 60% confluency and transiently cotransfected with 1 μg of firefly luciferase reporter constructs and 20 ng of pRL-TK *Renilla* luciferase reporter (Promega) using 2.5 μL of Lipofectamine 2000 (Invitrogen, Carlsbad, CA). At 48 h after transfection, luciferase activities were assayed using a Dual-Luciferase Reporter Assay System (Promega). The pGL4.10[luc2] vector without the promoter sequence was used as a negative control. Values were obtained in relative light units, and the activities of the experimental reporter (firefly luciferase) were normalized to the activities of the internal control (*Renilla* luciferase). All assays were performed in triplicate.

Measurement of mRNA levels

The mRNA levels of cDNAs from various tissues (human multiple tissue cDNA panels, ages 17–78 years; human fetal multiple tissue cDNA panels, ages 18–36 weeks; BD Biosciences, Palo Alto, CA) were determined by quantitative real-time PCR with a StepOne plus real-time PCR System (Applied Biosystems, Foster City, CA) and QuantiFast Probe Assay containing primers for M-LPH and a labeled probe (Qiagen, Chatsworth, CA) in accordance with the manufacturer's instructions. The amount of mRNA was normalized to the internal control, β-actin. All PCR assays were performed in triplicate.

Overexpression of wild-type and substituted RhitH

A DNA fragment encoding the full-length RhitH was created by PCR amplification using the primer set RhitH-exp-S1/RhitH-exp-A1 (Table 1) and the first-strand cDNA synthesized using total RNA extracted from MCF-7 cells as the template. The PCR product was cloned into the *Hin*dIII/*Eco*RI site of pcDNA3.1 (Invitrogen). The resulting vector was designated pcDNA3.1/RhitH and used as a wild-type construct. Substituted constructs corresponding to the minor allele of nine SNPs (p.Leu133Val, p.Arg135Trp, p.Arg135Gln, p.Arg309Gln, p.Ser461Cys, p.Ala465Thr, p.Leu491Phe, p.Gln495Leu, and p.Ser517Thr) were created by introducing corresponding nucleotide substitutions into pcDNA3.1/RhitH with a KOD-Plus-Mutagenesis kit. The primers used for mutant strand synthesis are listed in Table 1. RPTECs were grown in six-well plates to 60% confluency and transiently transfected using Lipofectamine 2000. Two days after transfection, cell extracts were prepared from the transfected cells and subjected to Western blot analysis.

Detection of M-LPH

The extracts of the cells transfected with wild-type or substituted RhitH constructs were mixed with a one-fifth volume of 0.375 M Tris-HCl (pH 6.8) containing 10% (w/v) sodium dodecyl sulfate (SDS), 35% (v/v) glycerol, 0.6 M dithiothreitol, and 0.03% (w/v) bromophenol blue. After being left to

Table 1. Primers Used for the Generation of Expression Constructs

Experiment	*Sense/antisense*	*Positions*	*Sequence (5′-3′)*[a]
Reporter construction			
Sac-LAS1	Sense	−1511 to −1478	CCC gag ctc TAA GCC ACC GCA CCC AGA CCT AAT G
Sac-LAS2	Sense	−345 to −312	CCC gag ctc CGT GAT GGC GCA CGC GTG TAG TCC C
Bgl-LAAS1	Antisense	724 to 755	CCC aga tct AAA TTC CTG ACC TGG TGG ACC TC
Bgl-LAAS2	Antisense	335 to 367	CCC aga tct AGT TGA AGT TGG CGT GGA AGG TCA
Site-specific mutations of RhitH binding sites			
MLPH-6550mu-1	Sense	548 to 573	**T**CA GG**G** G**G**C TGG GGA CCC GGG CAG GA
MLPH-6550mu-2	Antisense	547 to 523	AGC GCC CTC CCT CCA GCC GCT TGA T
MLPH-6570mu-1	Sense	568 to 593	**T**CA GG**G** G**G**C GGG GCT CTG AGA CCG GA
MLPH-6570mu-2	Antisense	567 to 543	CCG GGT CCC CAG GCT CCT GCA GCG C
Expression vector construction			
RhitH-exp-S1	Sense		aag ctt AAA ATG TCT GCA GAC GGC GGA G
RhitH-exp-A1	Antisense		gaa ttc CTA GGT GGG AGC GGG TGG
RhitH mutant synthesis			
• p.Leu133Val			
M-133-S1	Sense		AC**G** TCT CCC GGG AGG AGT GGG GAC
M-133-A1	Antisense		ACA AGG CCA CAT CCT CGA AAG TCA C
• p.Arg135Trp			
M-135-1-S1	Sense		CC**T** GGG AGG AGT GGG GAC GGC TG
• p.Arg135Gln			
M-135-2-S1	Sense		CCC **A**GG AGG AGT GGG GAC GGC TG
M-135-1-A1	Antisense		AGA GGT ACA AGG CCA CAT CCT CGA A
• p.Arg309Gln			
M-309-S1	Sense		CC**A** GTG CGA GCA GTG CGG CAA GGG C
M-309-A1	Antisense		TAG CTC TTC CTG CCC ACC TCA CTG TC
• p.Ser461Cys			
M-461-S1	Sense		TT**G** CAA CCT CAT CGC GCA CAA CCG CAC
M-461-A1	Antisense		CGC TGG CTG AAG CAC TTG CCG CAC T
• p.Ala465Thr			
M-465-S1	Sense		TC**A** CGC ACA ACC GCA CAC ACA CAG G
M-465-A1	Antisense		TGA GGT TGG AAC GCT GGC TGA AGC A
• p.Leu491Phe			
M-491-S1	Sense		AC**T** TCA CCG CGC ACC AGC GCA CCC A
M-491-A1	Antisense		GCG AGC TGT GGC TGA AGC TCT TGC
• p.Gln495Leu			
M-495-S1	Sense		CC**T** GCG CAC CCA CCG TGG CGT G
M-495-A1	Antisense		TGC GCG GTG AGG TGC GAG CTG T
• p.Ser517Thr			
M-517-S1	Sense		GC**A** CCA ACC TGC ACC GGC ACG AGA A
M-517-A1	Antisense		GCC GGC TGA AGC TCT TGC CGC ACA A

[a]*Sac*I and *Bgl*II sites are shown in lowercase type, and nucleotides altered are shown in boldface type.

stand overnight at room temperature, an aliquot was subjected to 15% SDS polyacrylamide gel electrophoresis. Detection of M-LPH was performed by Western blotting using anti-M-LPH (Sigma-Aldrich, St. Louis, MO) as the primary antibody and horseradish peroxidase–conjugated goat antibody against rabbit IgG (Bio-Rad, Hercules, CA) as a secondary antibody. An immunoreaction-enhancing solution (Immuno shot; Cosmo bio, Tokyo, Japan) was used as the antibody diluent, and the signals were visualized using an ECL select Western blot detection system (GE Healthcare, Uppsala, Sweden).

PCR restriction fragment length polymorphism genotyping of SNPs

PCR/restriction fragment length polymorphism (PCR-RFLP) methods for respective genotyping of the nine SNPs were newly developed. Each set of primers for amplification of the DNA fragment containing the substitution site was designed on the basis of the nucleotide sequence of the *RhitH* gene (GenBank accession no. NT_010393.16; positions 3102563–3110518), as shown in Table 2. Since p.Cys461Ser and p.Ser517Thr neither suppressed nor created any known restriction enzyme recognition sites, a mismatched PCR-amplification method was employed for these SNPs. Genotyping of the SNPs was performed in accordance with our previously published descriptions.[22] The validity of the genotyping results obtained was confirmed by sequencing analysis of genomic DNAs derived from several representative subjects.

Results

Confirmation of RhitH involvement in expression of the M-LPH *gene*

In our previous study, we identified a Ttk 69K binding site as a negative *cis*-regulatory element in the 5′-flanking region of the mouse *M-LP* gene and isolated Rhit as a *trans*-element

Table 2. Primers Used for Polymerase Chain Reaction/Restriction Fragment Length Polymorphism Genotyping of Single Nucleotide Polymorphisms

SNP	*Primer*	*Sense/antisense*	*Sequences*[a]	*Annealing temperature (°C)*	*Restriction enzyme*
rs145558914 p.Leu133Val[b]	145558914-1	Sense	5′-TTC GAG GAT GTG GCC TTG TAC-3′	65	*Hpy*CH4IV
	145558914-2	Antisense	5′-AGG CGA GTG CTT ACC CAG TGA-3′		
rs143032070 p.Arg135Trp	143032070-1	Sense	5′-GAT GTG GCC TTG TAC CTC TCC-3′	65	*Msp*I
	143032070-2	Antisense	5′-AGT GCT TAC CCA GTG ACA GCC-3′		
rs145284053 p.Arg135Gln	145284053-1	Sense	5′-ATG TGG CCT TGT ACC TCT CCC-3′	65	*Msp*I
	145284053-2	Antisense	5′-CGA GTG CTT ACC CAG TGA CAG-3′		
rs146334748 p.Arg309Gln	146334748-1	Sense	5′-AGG TGG GCA GGA AGA GCT ACC-3′	65	*Msp*I
	146334748-2	Antisense	5′-AGT CAG TGC AGG CGT AGG GCT T-3′		
rs144096180 p.Ser461Cys	144096180-1	Sense	5′-GCA AGT GCT TCA GCC AGC GAT-3′	65	*Mbo*I
	144096180-2	Antisense	5′-TGA GGT GCG AGC TGT GGC TGA A-3′		
rs150778586 p.Ala465Thr	150778586-1	Sense	5′-AGC CAG CGT TCC AAC CTC ATC-3′	65	*Bst*UI
	150778586-2	Antisense	5′-GCT GTG GCT GAA GCT CTT GCC-3′		
rs147630313 p.Leu491Phe	147630313-1	Sense	5′-AGC TTC AGC CAC AGC TCG GGC-3′	65	*Hae*III
	147630313-2	Antisense	5′-CTG AAG CTC TTG CCG CAC AAC-3′		
rs61735886 p.Gln495Leu	61735886-1	Sense	5′-TTC AGC CAC AGC TCG CAC CTC-3′	65	*Hpy*CH4IV
	61735886-2	Antisense	5′-GGT GGT GTG GAT CTT CTC GTG-3′		
rs148859377 p.Ser517Thr	148859377-1	Sense	5′-AAG AGC TTC AGC CAC AGC TCG C-3′	65	*Hpy*188III
	148859377-2	Antisense	5′-TTC TCG TGC CGG TGC AGT CTG G-3′		

[a]The underlined residues indicate the mismatched nucleotide incorporated in each primer.
[b]The single nucleotide polymorphism (SNP) nomenclature is based on the guidelines suggested by the Human Genome Variation Society.

(transcriptional repressor) that binds to this site.[12] Ttk 69K is a zinc finger protein of *Drosophila* that has been identified as a repressor of the pair-rule genes *even-skipped* and *fushi tarazu*.[23] Ttk 69K shares homology with Rhit in the zinc finger DNA binding domain in the C-terminal region. Since Rhit and RhitH share extremely high homology (96.4% identity, 98.0% similarity) in the DNA binding domain, it is assumed that RhitH recognizes a Ttk 69K binding site in the *M-LPH* gene, in a manner similar to Rhit. Therefore, using TFSEARCH (www.cbrc.jp/research/db/TFSEARCHJ.html), we searched for potential Ttk 69K binding sites within the stretch from −1500 to 5033 in the *M-LPH* gene, which encompasses the 5′-flanking region, exon 1, and intron 1. In contrast to the mouse *M-LP* gene in which the Ttk 69K binding site exists in the 5′-flanking region, it was found that two Ttk 69K binding sites exist in intron 1 at positions 548 to 555 and 568 to 575. To examine whether these Ttk 69K binding sites have a negative regulatory role, several constructs were created for luciferase reporter assays (Fig. 1). The DNA fragment comprising nucleotides −345 to 589 was found to reduce the promoter activity significantly as compared with the fragment comprising nucleotides −1511 to 335 or −345 to 335, implying that a negative regulatory element is present in the sequence from positions 335 to 589. Next, we abolished the Ttk 69K binding sites of pGL4-345/589 one at a time by introducing three point mutations and evaluated the promoter activities of the resulting constructs. The activity of pGL4-345/589-M1 was considerably reduced, while that of pGL4-345/

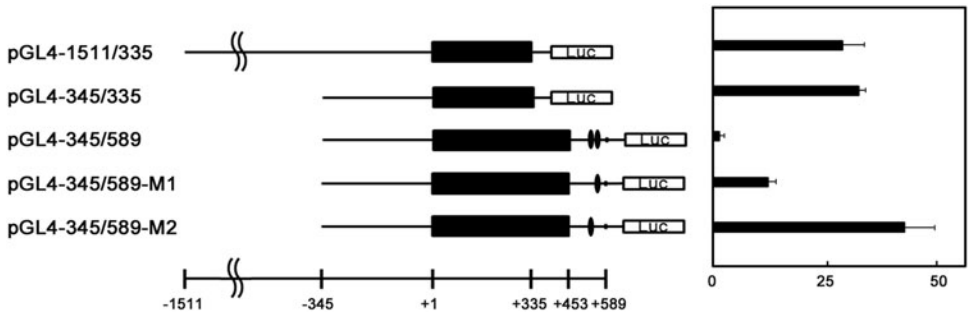

FIG. 1. Luciferase reporter assay of M-LPH promoter activity for identification of negative regulatory elements. Relative luciferase activities of the reporter constructs containing different portions of the *M-LPH* gene are shown. The constructs lacking the first (548–555) or the second (548–555) Ttk binding site were designated pGL4-345/589-M1 and pGL4-345/589-M2, respectively. +1 indicates the position of the transcription start site of exon 1. The results are expressed as ratios relative to the value for pGL4-345/589. The bars represent the mean ± SD of the results from three independent experiments. The shaded ovals (⬮) indicate the putative Ttk 69K binding sites. M-LPH, human Mpv17-like protein; SD, standard deviation.

589-M2 remained unchanged, indicating that the first Ttk 69K binding site is involved in negative transcriptional regulation. It has been shown previously that RNAi-mediated knockdown of RhitH causes an increase of M-LPH expression.[17] Together, these two results indicate that RhitH binds to the Ttk 69K binding site and functions as a transcription repressor in M-LPH expression, showing a relationship similar to that between mouse Rhit and M-LP$_S$.

Tissue distribution of RhitH and M-LPH

To investigate the relationship between RhitH and M-LPH in more detail, we analyzed their respective mRNA levels in adult and fetal (intermediate and late stages) tissues by quantitative real-time PCR. In adult tissues, RhitH was widely distributed except for digestive organs, whereas M-LPH was distributed specifically in tissues with high mitochondrial metabolism such as skeletal muscle, liver, kidney, and brain (Fig. 2A). Contrary to the expectation that the mRNA levels of M-LPH and its transcriptional repressor RhitH would be inversely correlated, M-LPH was, in fact, highly expressed in tissues with high levels of the RhitH mRNA. These results strongly suggested the participation of an additional transcriptional factor that activates M-LPH expression in tissues with high mitochondrial metabolism. In fetal tissues, by contrast, both RhitH and M-LPH showed relatively ubiquitous expression except for thymus and spleen, and a moderate inverse correlation was observed between the two, indicating that RhitH primarily regulates M-LPH expression in the fetus (Fig. 2B). Interestingly, a comparison of M-LPH expression levels revealed significant differences between fetal and adult tissues; the levels in adult tissues were 1.29- to 6.87-fold higher (3.09-fold on average) than those in fetal tissues (Fig. 2C). On the other hand, there was not so much difference in

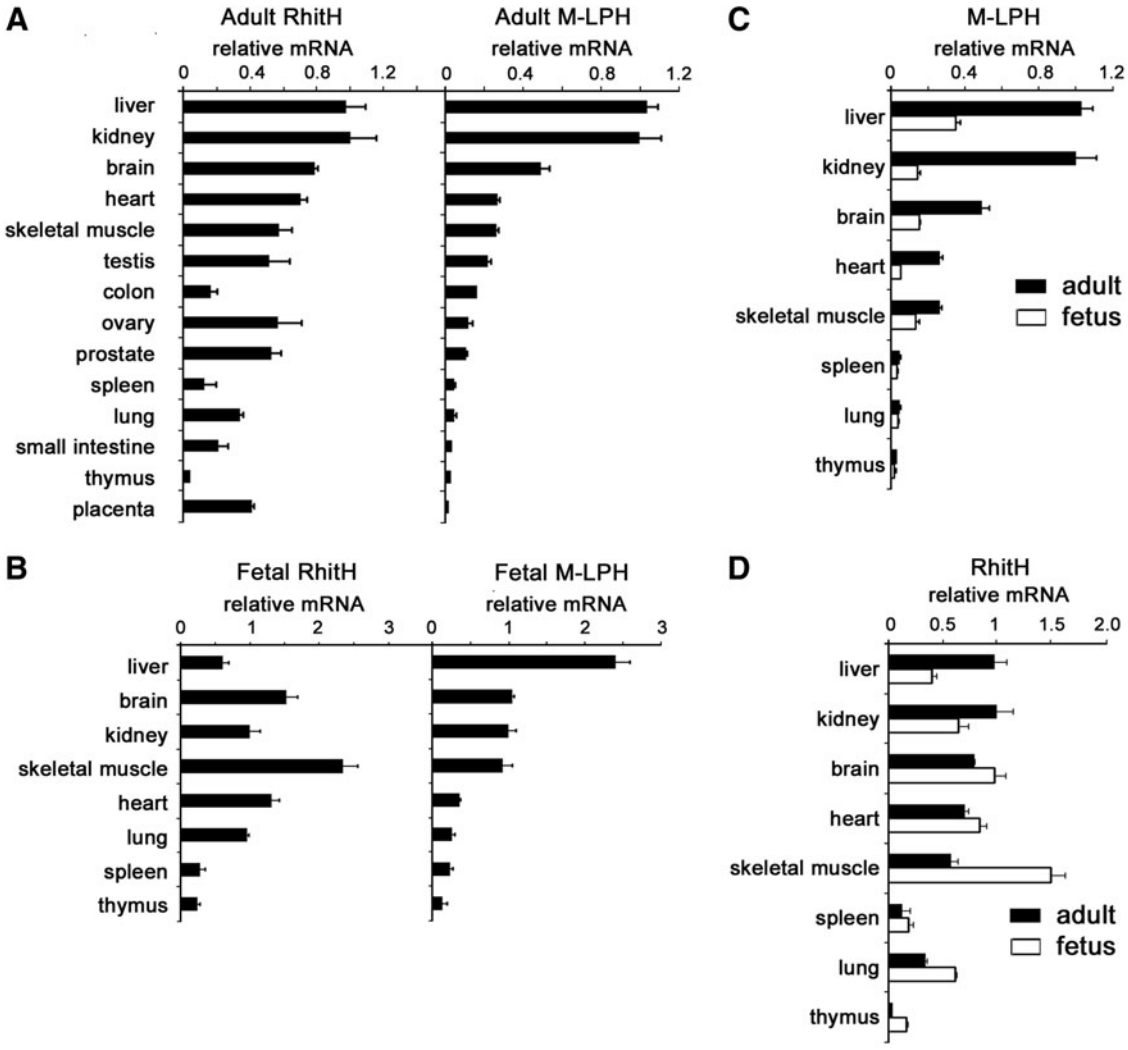

FIG. 2. mRNA levels of M-LPH and RhitH in adult and fetal tissues. **(A, B)** Expression of the mRNAs of M-LPH and RhitH in adult **(A)** and fetus **(B)** was detected by real-time PCR using β-actin as an internal control. The results are expressed as ratios relative to the respective value for the kidney. The bars represent the mean ± SD of results from three independent experiments. **(C, D)** Comparison of M-LPH **(C)** and RhitH **(D)** mRNA expression between adult and fetal tissues. The results are expressed as ratios relative to the value for adult kidney. The bars represent the mean ± SD of results from three independent experiments. RhitH, human regulator of heat-induced transcription; PCR, polymerase chain reaction.

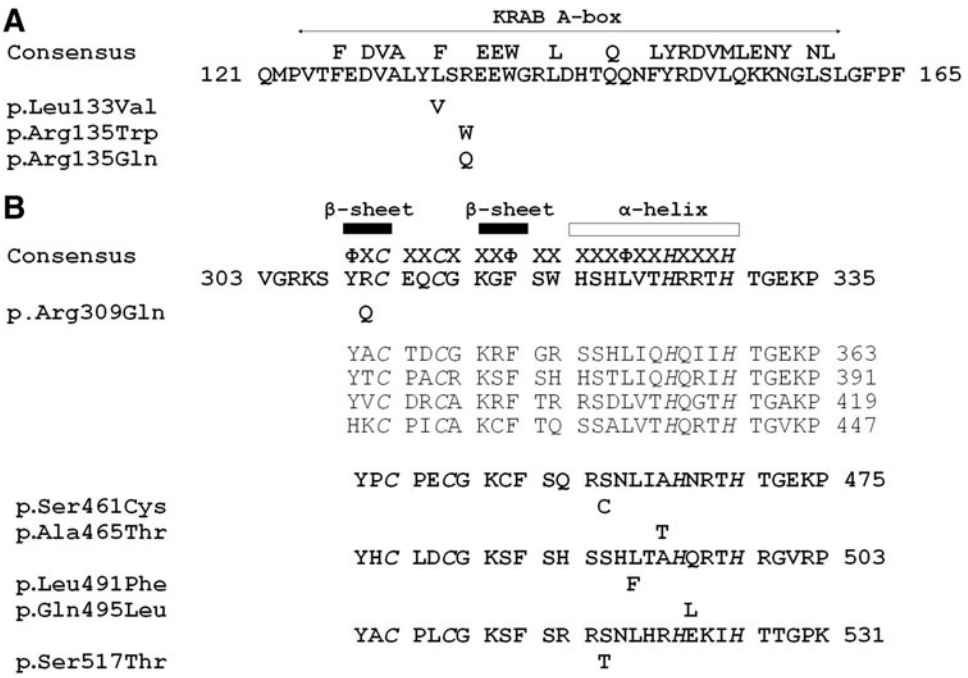

FIG. 3. Amino acid substitutions in the KRAB A-box **(A)** or in the zinc fingers **(B)** corresponding to nonsynonymous SNPs of the *RhitH* gene. **(A)** The position of the KRAB A-box is shown by an arrow and the consensus sequence retrieved from PROSITE database is presented. **(B)** The zinc finger consensus sequence is provided: X and Φ represent any amino acid and a hydrophobic amino acid, respectively, and Cys and His residues involved in zinc coordination are shown in italics. The position of the α-helix and β-sheets are shown by clear and solid rectangles, respectively. KRAB, Krüppel-associated box; SNP, single nucleotide polymorphism.

the levels of RhitH expression; those in adult tissues were 0.24- to 2.48-fold (0.94-fold on average) as high as those in fetal tissues (Fig. 2D).

Effect of nonsynonymous SNPs on the activity of RhitH as a transcriptional repressor

In order to evaluate the effect of genetic mutation on the repression activity of RhitH, we searched for nonsynonymous SNPs in the *RhitH* gene that cause amino acid substitutions in the KRAB domain or zinc finger domain and retrieved three SNPs in the KRAB A-box (p.Leu133Val, p.Arg135Trp, and p.Arg135Gln) and six in the zinc finger domain (p.Arg309Gln, p.Ser461Cys, p.Ala465Thr, p.Leu491Phe, p.Gln495Leu, and p.Ser517Thr) from the dbSNP database (www.ncbi.nlm.nih.gov/snp; Fig. 3). Substituted constructs corresponding to the minor allele of nine SNPs were expressed in RPTECs separately, and M-LPH expression in the cells was examined using Western blot analysis. This demonstrated levels of M-LPH similar to that in the wild type in the samples with substitutions in the KRAB-A box, indicating little alteration in the repression activity of RhitH (Fig. 4). Comparison of the KRAB-A box sequence of RhitH and the consensus sequence of the KRAB domain retrieved from the PROSITE database (http://prosite.expasy.org) demonstrated that Leu and Val at position 133 of RhitH, encoded by the major and minor alleles, are both different from the consensus Phe residue, and that the amino acid corresponding to position 135 of RhitH is not conserved. Accordingly, it appears reasonable that substitutions derived from p.Val133Leu, p.Arg135Trp, and p.Arg135Gln in the KRAB A-box are less effective. On the other hand, the levels of M-LPH were clearly increased by three substitutions in the zinc fingers (p.Ser461Cys, p.Ala465Thr, and p.Gln495Leu), while the remaining three (p.Arg309Gln, p.Leu491Phe, and p.Ser517Thr) had little effect. Thus, it was clarified that three substitutions in the zinc fingers caused reduction of RhitH repression activity that led to increased expression of M-LPH.

Genotyping of the nine nonsynonymous SNPs in the RhitH *gene for 16 different populations worldwide*

Using a newly developed PCR-RFLP method, we determined the distribution of the genotype and allele frequencies for all of the nine SNPs in 16 different populations ($n = 1752$). Consequently, all of the subjects were genotyped as homozygous for the predominant allele in each SNP; all SNPs were found to be distributed in a mono-allelic manner. From these results, the minor allele frequency of these SNPs was estimated to be less than 0.0003.

Discussion

To our knowledge, this is the first study to have identified functional SNPs in the *RhitH* gene. However, these nonsynonymous SNPs showed no polymorphism in any of the

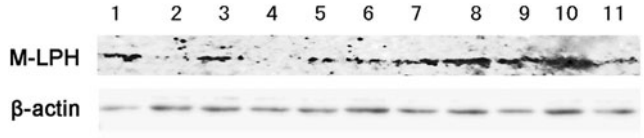

FIG. 4. Western blot analysis of M-LPH expression in RPTECs transfected with substituted constructs corresponding to the minor allele of nine SNPs. Lane 1, pcDNA3.1; lane 2, pcDNA3.1/RhitH (wild type); lane 3, p.Leu133Val; lane 4, p.Arg135Trp; lane 5, p.Arg135Gln; lane 6, p.Arg309Gln; lane 7, p.Ser461Cys; lane 8, p.Ala465Thr; lane 9, p.Leu491Phe; lane 10, p.Gln495Leu; lane 11, p.Ser517Thr. Expression of β-actin is shown as an internal control. RPTECs, renal proximal tubule epithelial cells.

worldwide populations examined. Thus, the *RhitH* gene shows remarkably low genetic diversity with regard to its functional nonsynonymous SNPs. These facts allow us to conclude that RhitH has been well conserved at the functional level during the evolution of human populations.

The structural details of how Cys_2His_2 zinc fingers bind specifically to dsDNA have been well studied.[24–26] In the presence of a single zinc ion, each zinc finger forms a compact $\beta\beta\alpha$ domain, composed of two antiparallel β-sheets and one α-helix. DNA is usually recognized by two to four tandemly arranged zinc fingers, and side-chains of residues located in the N-terminal portions of the α-helices form DNA base contacts. All three nonsynonymous SNPs affecting the repression activity are localized in the N-terminal portions of the α-helices. Considering that positions 461, 465, and 495 lie in zinc fingers 6 and 7, at least these two zinc fingers are assumed to participate in base contacts. In summary, it was demonstrated that p.Cys461Ser, p.Thr465Ala, and p.Leu495Gln serve as functional SNPs affecting the repressor function of Rhit.

Recently, Abo[27] proposed an intriguing hypothesis that a shift of energy production system occurs during aging. For energy production, humans use two systems: glycolysis and mitochondrial pathways. According to this theory, the mitochondrial pathway is dominant in early fetal life, but the glycolysis pathway gradually becomes dominant towards the late fetal stage; in childhood, the glycolysis pathway is slightly dominant but then is gradually replaced by the mitochondrial pathway; in adulthood, the ratio of the two pathways is proportionate; in old age, the mitochondrial pathway becomes dominant. In this study, we analyzed the tissue distribution of M-LPH and RhitH to gain further clues to the function of M-LPH and found that expression levels of M-LPH in fetal tissues are lower than those in adult tissues. From the viewpoint of the foregoing theory, this result is quite reasonable, considering that M-LPH is involved in mitochondrial function. Furthermore, alteration pattern of mouse M-LP_S, previously shown in our study, also fits the theory well: expression of mouse M-LP_S commenced at puberty, reached its highest level during maturation, and decreased with aging.[11] Therefore, investigation of alterations in M-LPH and RhitH during aging is of considerable interest.

Acknowledgments

DNA samples of bloodstain samples of the Ovambo and Turkish populations were kindly provided by Dr. B. Brinkmann (University of Münster, Münster, Germany). Blood samples of Korean and Mongolian populations were kindly provided Dr. K. Shiwaku (Shimane University). This study was supported in part by Grants-in-Aid from the Japan Society for the Promotion of Science (24659335 and 21390215 to R. I., 25460864 to M.U., and 22249023 to T.Y.).

Author Disclosure Statement

No competing financial interests exist.

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