The retinol acid receptor B gene is hypermethylated in patients with familial partial lipodystrophy

Rene Cortese, Florian Eckhardt, Marianne Volleth¹, Manfred Wehnert², Uwe Koelsch³, Peter Wieacker¹ and Thomas Brune³

Epigenomics AG Berlin, Kleine Präsidentenstr. 1, 10178 Berlin, Germany

¹Institute of Human Genetics, Otto-von-Guericke University, Leipziger Str. 44, 39120 Magdeburg, Germany

²Institute for Human Genetics, Ernst-Moritz-Arndt University, Fleischmannstrasse 42/44, 17489 Greifswald, Germany

³University Children's Hospital, Department of Perinatology: Gerhard-Hauptmann-Str. 35, 39108 Magdeburg, Germany

(Requests for offprints should be addressed to T Brune who is now at Universitätsklinikum Magdeburg, Zentrum für Kinderheilkunde, Gerhard-Hauptmann-Str. 35, D-39108 Magdeburg, Germany; Email: thomas.brune@med.ovgu.de)

(R Cortese and F Eckhardt contributed equally to this work)

Abstract

Mutations in the *LMNA* gene cause various phenotypes including partial lipodystrophy, muscular dystrophies, and progeroid syndromes. The specific mutation position within the *LMNA* sequence can partially predict the phenotype, but the underlying mechanisms for the development of these different phenotypes are still unclear. To investigate whether different DNA methylation patterns contribute to the development of different phenotypes caused by *LMNA* mutations, we analyzed a panel of ten candidate genes related to fat metabolism, aging, and a tendency to different methylation patterns: *CSPG2*, *ESR1*, *IGF1R*, *IGFR2*, *LMNA*, *MLH1*, *RANBP1*, *RARB*, *ZMPSTE24*, and *TGFBR1*. We studied two independent families each comprising three individuals affected by familial partial lipodystrophy type 2 (FPLD2). Affected members in each family carried two different mutations of the *LMNA* gene (R482L and R471G respectively). In addition, we analyzed four progeria patients (2 × *LMNA*/C G608G, 1 × *LMNA*/C S143F, and 1 × *ZMPSTE24* IVS9-Ex10) and seven healthy adults. The gene encoding retinoic acid receptor B (*RARB*) showed a higher methylation in all six patients with FPLD2 when compared with the progeria patients with other *LMNA* mutations as well as the healthy controls ($P < 0.05$). All other investigated genes showed no difference in the methylation patterns between the groups. A drug-induced inhibition of the retinol pathway is discussed as the key pathway for developing HAART-associated lipodystrophy and our data support a possible role of the retinol pathway in the development of lipodystrophy phenotypes.

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Introduction

Mutations within the *LMNA* gene cause many diseases with different phenotypes such as different muscular dystrophies, progeroid syndromes, mandibuloacral dysplasia, dilated cardiomyopathies, restrictive dermopathy, and lipodystrophy syndromes. The mutation position within the *LMNA* sequence can partially predict the phenotype, but the underlying mechanisms for the development of these different phenotypes are still unclear (Hutchison *et al.* 2001, Hegele 2005). One possible reason could be specific epigenetic alterations of other genes.

Epigenetic alterations such as histone modifications and DNA methylation have been proposed to be involved in the etiology of a number of diseases and to modify their pathological phenotype (Schumacher & Petronis 2006). Specifically, the best-studied epigenetic modification, DNA methylation, is believed to be involved in human pathologies such as cancer (Baylin 2005), diabetes (Maier & Olek 2002), atherosclerosis (Dong *et al.* 2002),

inflammatory bowel disease (Kim *et al.* 1996), inflammatory arthritis (Petronis & Petroniene 2000), and autoimmune disease (Richardson 2003).

To investigate if different DNA methylation patterns contribute to the development of different phenotypes caused by *LMNA* mutations, we analyzed a panel of ten candidate genes related to fat metabolism and aging including genes that display an age-dependent differential methylation. We analyzed the selected genes in six familial partial lipodystrophy (FPLD) patients from two independent families and four patients suffering from progeria, all bearing different mutations in the *LMNA* gene. The results were compared with those of five healthy adults and one healthy member of both FPLD2 families.

Patients, materials and methods

The Independent Ethics Committee of the University of Magdeburg, Germany, approved the protocol and

the study was performed according to the guidelines of the Declaration of Helsinki. Written informed consent was obtained from all patients.

Patients

Ten patients from two independent families bearing different mutations in the *LMNA* gene were enrolled in the study. Five healthy adults and one healthy member of each of the FPLD2 families served as controls. The patients and the controls were grouped and evaluated as displayed in Table 1.

FPLD group

Patients 1–3 belong to the same family (Family A) and exhibit the typical phenotype of familial partial lipodystrophy type 2 (FPLD) including acanthosis nigricans, partial lipodystrophy with sparing of the face, severe hypertriglyceridemia, insulin-resistant diabetes, and severe hypertriglyceridemia. Patient 1 is a 36-year-old female and the mother of Patient 2, a 12-year-old female. Patient 3 is an 8-year-old female and a cousin of patient 2. All three carry the *LMNA* mutation

R482L (Ambrosch *et al.* 1996, Capanni *et al.* 2003). One healthy member of the family, an 11-year-old female cousin of patient 2 with no mutation, serves as a family internal control (Patient 7).

Patients 4–6 belong to the same family (Family B) and carry the *LMNA* R471G mutation. Patient 4, a 14-year-old girl, is affected by an overlapping syndrome including partial lipodystrophy, insulin-resistant diabetes, acanthosis nigricans, liver steatosis, muscle weakness, and contractures. Patient 5 is her 18-year-old sister and exhibits only a partial lipodystrophy. Patient 6 is their father carrying the same mutation but he does not show a lipodystrophy phenotype. Their healthy mother (Patient 12) shows no *LMNA* mutation and serves as a family internal control (Muschke *et al.* in press).

Progeria group

Patients 7 and 8 are a 1-year-old girl and a 5-year-old boy, both affected by typical symptoms of a Hutchinson–Gilford syndrome (progeria) reported by de Busk (1972). Both carry the *LMNA* G608G mutation typical of progeria (Eriksson *et al.* 2003, De Sandre-Giovannoli *et al.* 2003). Patient 9 is a 5-year-old girl

Table 1 List of all investigated patients and the controls

	Patient number	Sex	Age (years)	Phenotype	Lamin mutation	Additional mutation
FPLD group	Patient 1	Female	36	Familial partial lipodystrophy	LMNA A/C R482L	None
	Patient 2	Female	12	Familial partial lipodystrophy	LMNA A/C R482L	None
	Patient 3	Female	8	Familial partial lipodystrophy	LMNA A/C R482L	None
	Patient 4	Female	14	Familial partial lipodystrophy	LMNA A/C R471G	None
	Patient 5	Female	18	Familial partial lipodystrophy, mild phenotype	LMNA A/C R471G	None
	Patient 6	Male	46	No phenotype	LMNA A/C R471G	None
Laminopathies group	Patient 7	Female	1	Hutchinson–Gilford syndrome	LMNA A/C G608G	None
	Patient 8	Male	7	Hutchinson–Gilford syndrome	LMNA A/C G608G	None
	Patient 9	Female	5	Early onset myopathy with progeria like symptoms	LMNA A/C S143F	None
	Patient 10	Male	3	Progeria-like symptoms, myopathy, and cardio myopathy were absent	LMNA A/C R654X	ZMPSTE24 IVS9-Ex10
Healthy control group	Patient 11	Female	11	Healthy family control	None	None
	Patient 12	Female	42	Healthy family control	None	None
	Patient 13	Female	30	Healthy control	None	None
	Patient 14	Female	32	Healthy control	None	None
	Patient 15	Female	29	Healthy control	None	None
	Patient 16	Female	33	Healthy control	None	None
	Patient 17	Female	24	Healthy control	None	None

with an overlapping syndrome including early onset myopathy with progeroid features. She carries a *de novo* heterozygous *LMNA* mutation (S143F) and during the first year of life displayed myopathy with marked axial weakness; progeroid features including growth failure, scleroderma-like skin, and osteolytic lesions developed later (Kirschner *et al.* 2005). Patient 10 is a three and a half-year-old boy suffering from scleroderma-like lesions of the skin which emerged at 3 months of age. Poor weight gain with loss of subcutaneous fat, prominent scalp veins, progressive hair loss, and lentigo senilis were noted shortly afterwards. X-rays revealed the absence of the lateral regions of clavicles as well as acroosteolysis of the fingers, myopathy, and cardiomyopathy. Sequencing of *LMNA* gene revealed a heterozygous stop mutation leading to a protein which is elongated by seven additional amino acids at the C terminal. The mother and a healthy brother of the patient carry the same mutation. Sequencing of the *ZMPSTE24* gene showed a homozygous deletion of an acceptor splice site (IVS9-Ex10) leading to the complete loss of function of the mutated *ZMPSTE24* (Denecke *et al.* 2006).

Control group

Five healthy females (Patients 13–17) served as controls.

Lymphoblastoid B-cell lines

Ten milliliters of venous blood was drawn from each patient and controls, and placed in a heparin blood collection tube. The blood samples were fractionated by the standard Ficoll-Hypaque method (Ficoll Biocoll,

1-077 g/ml Nycomed Pharma AS, Oslo, Norway, Biochrom AG, Berlin, Germany). After washing twice with phosphate buffered saline (PBS), the cells were infected with Epstein–Barr virus (EBV) containing supernatant. After 30 min, culture medium (RPMI 1640) supplemented with 12% fetal bovine serum (FBS) containing cyclosporin A was added. Six to eight weeks after setting-up, an aliquot of the cells was used for further investigations. To rule out an impact of the immortalization process on the methylation pattern of the investigated genes, we compared DNA from freshly prepared lymphocytes (A) and the corresponding immortalized lymphocytes (B) of three healthy probands.

Cryopreservation

After washing once with serum-free culture medium, the cells were resuspended in cryomedium, i.e. complete culture medium containing 10% DMSO, transferred to cryotubes, and deposited in a Nalgene Cryo 1 °C freezing container at –80 °C for 24 h. The next day, cryotubes were transferred to a liquid nitrogen container.

Thawing

The cryotubes were placed in a 37 °C water bath until the cell suspension was thawed. After washing once with culture medium, the cells were transferred to a culture vessel containing fresh culture medium.

DNA preparation

DNA was isolated from B-lymphoblastoid cells. Cells were pelleted by centrifugation, washed twice in 1 × PBS

Table 2 Panel of the analyzed genes related to differential methylation, aging, and progeria selected from the literature

Genes full name		Corresponding reference
Abb.		
Genes selected due to their age-associated differential methylation		
<i>CSPG2</i>	Versican, chondroitin sulfate proteoglycan core protein 2	Ahuja <i>et al.</i> (2001)
<i>ESR1</i>	Estrogen receptor 1	Li <i>et al.</i> (2004)
<i>IGF2R</i>	Insulin-like growth factor II receptor	Yang <i>et al.</i> (2000)
Genes selected due to their association with aging		
<i>IGF1R</i>	Insulin-like growth factor I receptor	Liang <i>et al.</i> (2003)
<i>LMNA</i>	<i>Lamin A/C</i>	Mattout <i>et al.</i> (2006)
<i>MLH1</i>	Homolog of <i>Escherichia coli</i> MutL	Kim <i>et al.</i> (2006)
<i>RANBP1</i>	Ran-binding protein 1	Pujol <i>et al.</i> (2002)
<i>RARB</i>	Retinoic acid receptor-β	Si <i>et al.</i> (1995)
Genes selected due to their association with progeria		
<i>LMNA</i>	<i>Lamin A/C</i>	Mattout <i>et al.</i> (2006)
<i>ZMPSTE24</i>	<i>Zinc metalloprotease STE24</i>	Denecke <i>et al.</i> (2006)
<i>TGFBRI</i>	Transforming growth factor-β receptor, type I	There are some unpublished reports that transforming growth factor B in the therapy of children suffering from Hutchinson–Gilford syndrome is able to slow the occurrence of symptoms and delay their life span

and resuspended in 2 ml OLD-T buffer (40 mM Tris-HCl, 150 mM NaCl, 25 mM EDTA, pH 7.5). After addition of 50 µl proteinase K and 100 µl 20% SDS, incubation was carried out at room temperature overnight, followed by phenol/chloroform extraction. The DNA was precipitated using two volumes of absolute ethanol.

Candidate genes

The panel of the analyzed genes consisted of ten candidate genes related to differential methylation, aging and progeria selected from the literature. All genes and the corresponding references are shown in Table 2.

Bisulfite sequencing and evaluation

The described methods and algorithms have been published previously (Lewin *et al.* 2004, Rakyan *et al.* 2004, Eckhardt *et al.* 2006). The entire workflow is shown in Fig. 1. Bisulfite conversion of genomic DNA was performed as previously described, followed by a direct quantification of DNA methylation. Briefly, the bisulfite reaction was carried out using genomic DNA that was treated with a 2.5 M sodium bisulfite solution (Berlin *et al.* 2005), converting unmethylated cytosines into uracil, whereas methylated cytosines remained unaltered. Upon PCR amplification, the uracil was converted into a thymine and the cytosine:thymine ratio was used for the methylation quantification as described below. PCR amplification using primers (Table 3) flanking the region of interest was performed with Qiagen hot-start PCR reagents using the following PCR profile: hot-start at 95 °C for 15 min (95 °C for 1 min, 55 °C for 45 s, 72 °C for 1 min) × 40 cycles, and an elongation step of 72 °C for 10 min. PCR amplicons were controlled by agarose gel electrophoresis, and excess nucleotides and primers were subsequently removed using ExoSAP-IT (USB Corporation, Cleveland, OH, USA).

The PCR product (without prior subcloning) was subsequently sequenced on an ABI 3730 capillary sequencer using the same PCR primers and a 1/20 dilution of the ABI Prism Big Dye terminator V3.1 kit (Applied Biosystems, Foster City, CA, USA). The sequencing thermo profile included an initial incubation at 96 °C for 120 s followed by 25 thermo cycles (96 °C for 30 s, 55 °C for 15 s, 60 °C for 240 s).

Quantitative cytosine methylation levels were calculated from the ABI sequencing trace files as previously described (Lewin *et al.* 2004, Rakyan *et al.* 2004, Eckhardt *et al.* 2006). In brief, after trace curve normalization, the methylation of each individual cytosine-phosphate-guanine site (CpG) was quantified

Figure 1 Workflow of bisulfite sequencing and evaluation of the candidate genes. After the bisulfite treatment, methylation values are calculated from the sequence trace file using the C:T signal intensities. Methylation values for each sample and CpG position are color coded ranging from 0% methylation (yellow) to 100% methylation (blue).

Table 3 Primers used for PCR amplification

	Location ^a	Primer 1 sequence	Primer 2 sequence	Fragment length
Gene symbol				
<i>IGF2R</i>	5' UTR	ATAGAAGGTTTGAGAGGTTTGT	CTTCCCAAATTAACCACTATTC	207
<i>TGFBP1</i>	5' UTR	GGAGGAAATAGGAGAGTGAGT	AAATCTACCAACCCCAACA	345
<i>LMNA</i>	Exon 1	GTTGTAGGAGAAGGAGGATTTG	TCCCAAAAACCCACTT	486
<i>CSPG2</i>	5' UTR	GTGGGGAATATGTAAGGAAA	CCCAACAACCTAAACCTTA	460
<i>hMLH1</i>	5' UTR	ATTGATTGGATAGTTTGAATGTT	CCAAATCACCTCAACAAAAA	240
<i>IGF1R</i>	Intron 1	TTATTGGTATATTTAAGATGGTAGGG	AAAAATAAAAACCCCTCTTTATC	478
<i>ZMPSTE24</i>	Exon 1	GGAGGGATTAGGGAGAGTTT	CCTCCTCAAAACCACAATC	495
<i>RANBP1</i>	5' UTR	TTAAAGTGGTTGGGGGTT	TCCCCACCTATTCTCTAACA	492
<i>ESR1</i>	5' UTR	TTGTTGGATAGAGGTTGAGTTT	CTATCAATTCGCCCACTACT	349
<i>RARB</i>	Intron 1	GTTGTTTAGAGGTAGGAGGGTT	ACAATCAAACATAATCTCCCTT	458

^a5' UTR, 5'-untranscribed region.

by C/T signal peak intensities ratios at the cytosine position located within a CpG (the dinucleotide where DNA cytosine methylation occurs). Only sequences displaying a conversion rate higher than 98% were used for analysis. Methylation values for individual Cs within each amplicon and sample were calculated and visualized by color coding ranging from dark blue (100% methylation) to yellow (0% methylation). The rows of the matrix correspond to the methylation levels of individual CpG sites, and each column represents an individual sample.

To minimize the risk of possible methylation variation caused by the immortalization process, our cell culture methodology or the freezing and thawing process (Liu *et al.* 2005), we compared the methylation pattern DNA from freshly prepared lymphocytes and the corresponding immortalized lymphocytes before and after thawing of three healthy probands.

Statistical analysis

Differential methylation was tested for significance using Wilcoxon's rank sum test. Significant results ($P < 0.05$) were visualized as a green box on the right of the figures.

Results

Figure 2 displays the methylation profiles of all individuals investigated beside the two healthy family controls. In all 14 samples, the ten genes analyzed showed a low methylation level of 0–30%. Within the FPLD group, the *RARB* gene showed a significantly higher methylation ($P < 0.05$) in all six patients when compared with both patients with other *LMNA/C* mutations and to healthy controls. In addition, we observed methylation differences between the two families in the FPLD group with, for example, *LMNA* being highly methylated in patients 1–3 (Family A) than

in patients 4–6 (Family B). However, these findings were not statistically significant when tested against the control group or the progeria group.

The methylation profile of the *RARB* gene is shown in detail in Fig. 2b. The figure shows that the healthy members of each affected FPLD family also displayed a significantly lower methylation of the *RARB* gene when compared with the affected family members. No difference in methylation could be detected between the healthy members of the FPLD family, the progeria patients, and unrelated healthy controls.

Noteworthy, all methylation changes were detected in B-lymphoblastoid cells isolated from whole blood samples. This fact enables the study with minimal intervention, which is a very desirable feature in a diagnostic procedure. In order to control possible changes in the methylation patterns introduced during culture and freezing–thawing of the cells (Liu *et al.* 2005), we compared DNA from freshly prepared lymphocytes and the corresponding immortalized lymphocytes of three healthy probands (Fig. 3). The methylation profiles of our marker did not change during the immortalization process or different steps of culture suggesting that the immortalization process had no effect on the methylation pattern of these genes.

Discussion

FPLD patients have a normal fat distribution in early childhood, but with the onset of puberty, subcutaneous adipose tissue gradually disappears from the upper and lower extremities as well as from the gluteal and truncal regions, resulting in a muscular appearance with prominent superficial veins. Simultaneously, the adipose tissue accumulates on the face and neck, causing a double chin, fat neck, or cushingoid appearance. Adipose tissue may also accumulate in the axillae, back, labia majora, and intra-abdominal region (Kobberling & Dunnigan 1986). The lipodystrophies

Figure 2 (a) Methylation profiles of all investigated individuals. The ten genes of all samples analyzed showed a low methylation level of 0–30%. The methylation profile of the *RARB* gene is shown in detail in (b). The *LMNA* gene shows a higher methylation level in patients 1–3 (Family A) than in patients 4–6 (Family B). This result is not statistically significant when tested against the control group or the progeria group. (b) The methylation profile of the *RARB* gene in detail. Within the FPLD group, the *RARB* gene showed a significantly higher methylation ($P < 0.05$) in all six patients when compared with both patients with other *LMNA/C* mutations and when compared with healthy controls.

Figure 3 Methylation pattern of the *LMNA* and the *RARB* genes from freshly prepared lymphocytes and the corresponding immortalized lymphocytes before and after thawing of three healthy probands. No change of the methylation pattern is detectable in immortalized lymphocytes.

are familial or acquired disorders characterized by variable loss of fat tissue. In FPLD, loss of fat occurs during puberty. In a subgroup of FPLD, Dunnigan type or FPLD2, subcutaneous fat is lost from the limbs and partially from the trunk. Later on, the patients may show acanthosis nigricans, hirsutism, polycystic ovaries, insulin-resistant diabetes, dyslipidemia, and liver steatosis. Although FPLD2 is caused by mutations of *LMNA* (Hegele *et al.* 2000a,b,c), its manifestation is markedly dependent on sex with female carriers more severely affected (Vigouroux *et al.* 2000). This may indicate that epigenetic factors also play a role in the development of the lipodystrophic phenotype.

In the present study, we investigated the relation of DNA methylation and the underlying mutation as a reason for the development of the differential phenotypes in patients bearing *LMNA* mutations. For this purpose, we analyzed the methylation patterns of ten candidate genes in patients with FPLD2, with other laminopathies and controls. Out of the ten gene methylation profiles, we found that the studied amplicon of the *RARB* gene is significantly hypermethylated in FPLD when compared with the patients with other laminopathies, indicating an epigenetic component in this disease. The studied amplicon is located in intron 1 of the annotated *RARB* gene (Ensembl v42).

The *RARB* gene encodes the RAR- β and mediates retinoid effects in controlling cell growth, differentiation, apoptosis, and carcinogenesis. *RARB* has been shown to be differentially methylated in several cancers

(Paz *et al.* 2003) such as malignant melanoma (Furuta *et al.* 2004), testicular tumors (Kawakami *et al.* 2003), and carcinoma of cervix (Narayan *et al.* 2003). Although the function of *RARB* in the etiology of FPLD2 is currently not known, it is noteworthy that protease inhibitor therapy of human immunodeficiency virus (HIV) is frequently associated with a FPLD-like condition characterized by lipodystrophy of the limbs, fat accumulation of abdomen, breasts and cervical region ('buffalo hump'), hyperlipidemia, and insulin resistance. This symptoms complex is called highly active anti-retroviral therapy (HAART)-related lipodystrophy syndrome (Carr 2000). Protease inhibitors including indinavir have been shown to reduce adipocyte cell differentiation and increase apoptosis of adipocytes *in vitro*. The HAART-related lipodystrophy syndrome may be a result of the inhibition of two proteins involved in lipid metabolism, which have significant homology to the catalytic site of HIV protease, namely cytoplasmic retinoic acid-binding protein (cRABP) and low-density lipoprotein receptor-related protein (Lenhard *et al.* 2000). cRABP is an intracellular protein involved in the transmission of the vitamin A-derived signal, which regulates genes responsible for lipid metabolism and adipocyte differentiation (Carr *et al.* 1998).

Although the phenotypes of HAART-induced lipodystrophy and FPLD seem distinct in the sense that FPLD patients have fat deposition in face and neck whereas HAART lipodystrophic individuals have facial fat loss,

both the phenotypes have multiple clinical and metabolic features in common (Behrens *et al.* 2000). Our results and the above-mentioned role concerning the inhibition of CRABP in the etiology of HAART-related lipodystrophy suggest a link between the retinoic acid pathway and lipodystrophy phenotypes and support a role for changes in the methylation of the retinoic acid receptor gene in the development of lipodystrophic phenotypes.

The different DNA methylation of the *LMNA* gene between the two FPLD families may play an additional role in the different development of the FPLD phenotype. Although the difference in *LMNA* is visible in the plot, the *P* values after the Wilcoxon's test do not show statistical significance. As we compared only three patients in each group, marginal *P* values are expected. Thus, these results would suggest a trend towards differential methylation of *LMNA* gene between both families carrying different mutations. Additional studies focusing on the link between the observed differential methylation and the different mutation in *LMNA* will allow an understanding of whether they have a synergetic effect on the development of the phenotype.

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