

Apolipoprotein E4 is Associated with Primary Localized Cutaneous Amyloidosis

To the Editor:

Apolipoprotein E (apoE) is a lipid transport protein, which is a component of lipoproteins, such as very low density lipoprotein, intermediate density lipoprotein, high density lipoprotein, and chylomicron. ApoE is also produced and secreted in the skin, and is implicated to play roles in epidermal differentiation and proliferation (Barra *et al*, 1994).

We have reported that apoE was a component of amyloid deposits of primary localized cutaneous amyloidosis (PLCA) using immunohistochemistry, immunogold electron microscopy, and immunoblotting (Furumoto *et al*, 1998). PLCA, lichen amyloidosis, and macular amyloidosis are characterized by the finding that the amyloid deposits are limited to the papillary dermis (Kumakiri and Hashimoto, 1979). There is a hypothesis that the precursor protein of PLCA might be keratin protein from epidermal keratinocytes (Hashimoto *et al*, 1990). The pathogenesis of PLCA, however, remains undetermined and the amyloid fibril protein has not been identified. There are three common alleles for apoE, *e2*, *e3*, and *e4*, and their gene products are apoE2, apoE3, and apoE4, respectively (Mahley, 1988). In this study, we investigated whether the phenotypic variation of apoE is associated with PLCA.

Fourteen Japanese patients (four females and 10 males) with PLCA were studied (mean age, 53 years; range, 26–82 years, **Table I**). The control group consisted of 100 healthy unrelated Japanese individuals randomly chosen from volunteers (mean age, 34.4 years; range, 21–83 years). Cutaneous amyloidosis was diagnosed clinically and histopathologically. Sera obtained from the peripheral venous blood samples were frozen at -80°C until use. Phenotypes of apoE were examined using analytical isoelectric focusing followed by immunoblotting with goat anti-apoE antibody and alkaline phosphatase-conjugated rabbit antigoat IgG (Furumoto *et al*, 1997). Differences in apoE phenotype frequencies between the patients and control group were tested by the Chi-squared test with Yates' correction.

There are six of the most common phenotypes of apoE, E4/4, E3/3, E2/2, E4/3, E4/2, and E3/2. As shown in **Table I**, nine of 14 patients (64.3%) with PLCA have apoE4/4 or E4/3 phenotype, whereas 18 of 100 (18%) of controls have apoE4/4 or E4/3 phenotype. The phenotype frequency of apoE4/3 in PLCA was significantly higher than in healthy controls (57% in PLCA vs. 17% in control, $p < 0.01$), and this elevation was based on the increased frequency of *e4* allele (0.357 in PLCA vs. 0.095 in control; **Table II**).

Our results indicate that the *e4* allele is increased in frequency in PLCA. The *e4* allele frequency in the Japanese population is 0.11, which is similar to that of Caucasians (Breslow, 1988). ApoE4 is linked to the pathogenesis of Alzheimer's disease (AD), and the

Table I. Details of patients with primary localized cutaneous amyloidosis. Clinical data and phenotypes of apolipoprotein E

No.	Type of amyloidosis ^a	Sex	Age ^b	apoE phenotypes
1	LA	Male	45	E3/3
2	LA	Male	47	E3/3
3	LA	Male	54	E3/3
4	LA	Male	73	E3/3
5	LA	Male	82	E3/3
6	MA	Male	31	E4/3
7	MA	Male	36	E4/3
8	LA	Male	52	E4/3
9	LA	Male	64	E4/3
10	MA	Female	26	E4/3
11	MA	Female	62	E4/3
12	MA	Female	65	E4/3
13	LA	Male	71	E4/3
14	LA	Male	44	E4/4

^aLA, lichen amyloidosis; MA, macular amyloidosis.

^bAge, age at onset.

Table II. ApoE phenotypes and allele frequencies in PLCA

	PLCA		Controls	
Total (number/ females:males)	14	4:10	100	50:50
Phenotype (number/ females:males)				
E4/4	1	0:1	1	0:1
E3/3	5	0:5	76	39:37
E2/2	0		0	
E4/3	8 ^a	4:4	17	9:8
E4/2	0		0	
E3/2	0		6	2:4
Allele frequency $\epsilon 4$	0.357 ^a		0.095	
$\epsilon 3$	0.643		0.875	
$\epsilon 2$	0.0		0.030	

^a $p < 0.01$ vs. control.

allele frequency in the AD population is significantly higher (3-fold) than that of controls (0.38 vs. 0.122) (Poirier *et al*, 1993).

ApoE is associated with amyloid plaque of the many amyloid-forming diseases (Namba *et al*, 1991; Wisniewski and Frangione, 1992; Strittmatter *et al*, 1993). Recently, it has been reported that carboxyl-terminal-truncated fragments of apoE4, especially apoE4 (272–299), which were generated inside cultured neurons and in AD brains resulted in large, filamentous intracellular inclusions resembling neurofibrillary tangles in AD brain (Huang *et al*, 2001). The apoE4 fragments interact with phosphorylated tau and phosphorylated neurofilaments of high molecular weight. Therefore, it is suggested that apoE4 molecule plays an important

Manuscript received October 18, 2001; revised February 11, 2002; accepted for publication February 19, 2002.

Reprint requests to: Prof. Kazuyuki Nakamura, Department of Biochemistry, Yamaguchi University School of Medicine, Minami-kogushi, Ube 755–8505, Japan. Email: nakamura@po.cc.yamaguchi-u.ac.jp

Abbreviations: ApoE, apolipoprotein E; PLCA, Primary localized cutaneous amyloidosis.

role for the development and maintaining of amyloid deposit in AD brain. The function of apoE in epidermis is unknown, but it is naturally secreted by keratinocytes (Fenjes *et al*, 1989; Gordon *et al*, 1989). Our result indicates that apoE4 molecule synthesized by epidermal keratinocytes is strongly related to the pathogenesis of cutaneous amyloidosis, especially formation of amyloid fibrils.

We thank Dr. Y. Hamamoto, Dr. K. Nagai, Dr. K. Inoue, and Prof. C. Asagami for their cooperation providing us the blood samples.

Hiroko Furumoto, Takahiro Shimizu,† Masahiko Muto, Yumiko Hashimoto,* Kazuyuki Nakamura
Departments of Biochemistry and *Dermatology,
Yamaguchi University School of Medicine, Ube, Japan
†Division of Dermatology,
Saiseikai Yamaguchi General Hospital,
Yamaguchi, Japan

REFERENCES

- Barra RM, Fenjes ES, Taichman LB: Secretion of apolipoprotein E by basal cells in cultures of epidermal keratinocytes. *J Invest Dermatol* 102:61–66, 1994
- Breslow JL: ApoE. *Physiol Rev* 68:85–126, 1988
- Fenjes ES, Gordon DA, Pershing LK, Williams DL, Taichman LB: Systemic distribution of apolipoprotein E secreted by grafts of epidermal keratinocytes: Implications for epidermal function and gene therapy. *Proc Natl Acad Sci USA* 86:8803–8807, 1989
- Furumoto H, Nakamura K, Imamura T, Hamamoto Y, Shimizu T, Muto M, Asagami C: Association of apolipoprotein allele e2 with psoriasis vulgaris in Japanese population. *Arch Dermatol Res* 289:497–500, 1997
- Furumoto H, Shimizu T, Asagami C, Muto M, Takahashi M, Hoshii Y, Ishihara T, Nakamura K: Apolipoprotein E is present in primary localized cutaneous amyloidosis. *J Invest Dermatol* 111:417–421, 1998
- Gordon DA, Fenjes ES, Williams DL, Taichman LB: Synthesis and secretion of apolipoprotein E by cultured human keratinocytes. *J Invest Dermatol* 92:96–99, 1989
- Hashimoto K, Ito K, Taniguchi Y, Yang F, Youngberg G: Keratin in cutaneous amyloidosis. *Clin Dermatol* 8:55–65, 1990
- Huang Y, Liu XQ, Wyss-Coray T, Brecht WJ, Sanan DA, Mahley RW: Apolipoprotein E fragments present in Alzheimer's disease brains induce neurofibrillary tangle-like intracellular inclusions in neurons. *Proc Natl Acad Sci USA* 98:8838–8843, 2001
- Kumakiri M, Hashimoto K: Histogenesis of primary localized cutaneous amyloidosis: Sequential change of epidermal keratinocytes to amyloid via filamentous degeneration. *J Invest Dermatol* 73:150–162, 1979
- Mahley RW: Apolipoprotein E. Cholesterol transport protein with expanding role in cell biology. *Science* 240:622–630, 1988
- Namba Y, Tomonaga M, Kawasaki H, Otomo E, Ikeda K: Apolipoprotein E immunoreactivity in cerebral amyloid deposits and neurofibrillary tangles in Alzheimer's disease and kuru plaque amyloid in Creutzfeldt-Jakob disease. *Brain Res* 541:163–166, 1991
- Poirier J, Davignon J, Bouthillier D, Kogan S, Bertrand P, Gauthier S: Apolipoprotein E polymorphism and Alzheimer's disease. *Lancet* 342:697–699, 1993
- Strittmatter WJ, Saunders AM, Schmechel D, Pericak-Vance M, Enghild J, Salvesen GS, Roses AD: Apolipoprotein E. High-avidity binding to β -amyloid and increased frequency of type 4 allele in late-onset familial Alzheimer disease. *Proc Natl Acad Sci USA* 90:1977–1981, 1993
- Wisniewski T, Frangione B: Apolipoprotein E. A pathological chaperone protein in patients with cerebral and systemic amyloid. *Neurosci Lett* 135:235–238, 1992

Real-Time, *In Vivo* Quantification of Melanocytes by Near-Infrared Reflectance Confocal Microscopy in the Guinea Pig Animal Model

To the Editor:

Guinea pigs are widely used animal models in studying photo-dermatology research (Imokawa *et al*, 1986; Horio *et al*, 1991). The skin of pigmented guinea pigs contains both active interfollicular melanocytes and active follicular melanocytes. To investigate melanocyte biology, it is important to quantitate melanocytes and their characteristics. Previous studies on melanocytes in guinea pigs have utilized the 3,4-dioxyphenylalanine (DOPA) reaction in biopsied tissues that permanently changes the native tissue (Bischitz and Snell, 1959; Wolff and Winkelmann, 1967).

Advances in reflectance confocal microscopy (RCM) have resulted in the development of real-time near-infrared confocal scanning laser microscopes that produce real-time high resolution optical sections of human or animal skin in its native state *in vivo* (Rajadhyaksha *et al*, 1999). Optical reflectance imaging is based on the natural variations in refractive indices of tissue microstructures, and it has been shown that melanin is the best endogenous contrast source for RCM (Rajadhyaksha *et al*, 1995). Thus, RCM does not require any exogenous contrast agents.

Reflectance laser scanning confocal microscopy is an optical imaging technique that tightly focuses a laser beam on a specific spot within a turbid object (Wilson, 1990; Pawley, 1995; Webb,

1996). The focused laser spot is then scanned in two dimensions to yield images of horizontal or en-face optical sections that are parallel to the skin surface. Back-scattered (re-emitted) light from the illuminated point is detected through a small spatial filter (pinhole) that is located in an optically conjugate plane in front of the detector. The pinhole diameter is matched to the illuminated spot diameter through the intermediate optics. The pinhole thus rejects back-scattered light from out-of-focus planes, which allow high-resolution, high-contrast images. Unlike conventional histology, RCM allows imaging of thin sections with high resolution and contrast entirely noninvasively (without the need of excisional biopsies, processing, section, and staining the tissue). During the last decade, real-time RCM has imaged human and animal skin *in vivo* (New *et al*, 1991; Corcuff *et al*, 1993). Imaging is obtained at videorate (30 frames per second), allowing high temporal resolution (33 ms per frame) for visualizing dynamic processes, such as the time sequence of histologic events during an eczematous reaction (e.g., spongiosis with microvesicle formation and dermal vasodilation) (Gonzalez *et al*, 1999a), and those occurring after laser treatment of vascular lesions (Gonzalez *et al*, 1999b; Agashi *et al*, 2000). In this correspondence, we report on the noninvasive, quantitative evaluation of guinea pig melanocytes by *in vivo*, near-infrared RCM. This methodology makes possible the real-time analysis of melanocytes *in vivo*.

Outbred pigmented guinea pigs were selected for this study. The skin of the concave surface of the outer ear and back were examined in real-time by a commercially available confocal reflectance microscope (Vivascope, Lucid, Henrietta, NY) that uses an 830 nm diode laser and a $\times 30$, 0.9 numerical aperture

Manuscript received November 20, 2001; revised February 19, 2002; accepted for publication February 27, 2002.

Reprint requests to: Dr. Salvador González, Wellman Laboratories of Photomedicine, BAR 814, Massachusetts General Hospital, 55 Blossom Street, Boston, MA 02114, U.S.A. Email: gonzalsa@helix.mgh.harvard.edu

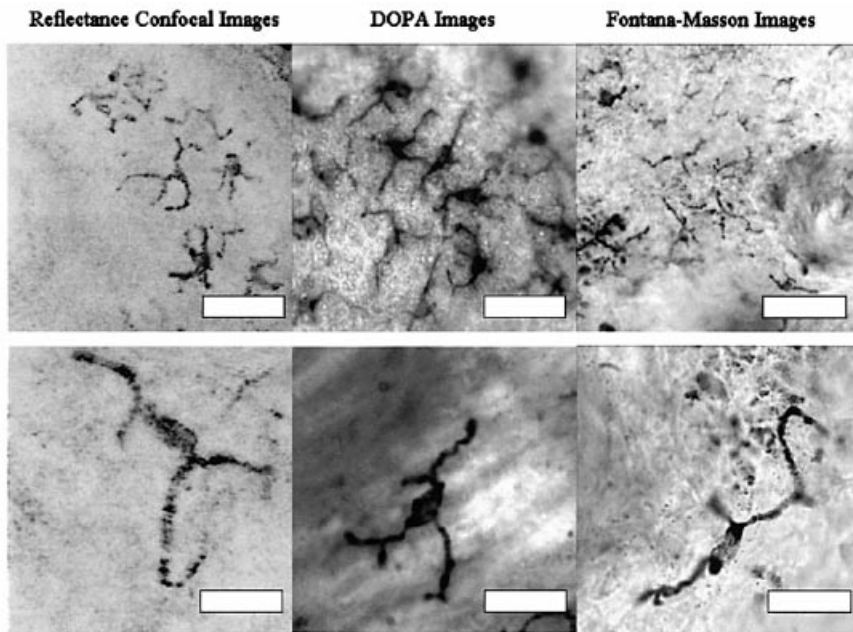


Figure 1. Comparison of images from the back. Images of melanocytes are shown. *Left panels, RCM; middle panels, DOPA staining; right panels, Fontana–Masson staining. Scale bar: top panels, 50 μm; bottom panels, 20 μm.*

Table I. Summary of melanocyte counts and features in the ear and back^a

	Melanocytes per mm ²		Melanocyte features		
	Ear	Back	Body area (μm ²)	Dendrite length (μm)	No. of dendrites
DOPA	702	201 ± 26.3	81.4 ± 18.4	26.1 ± 6.0	3.7 ± 0.80
RCM	–	173 ± 29.9	84.5 ± 12.3	29.4 ± 8.9	4.0 ± 0.62

^aFour guinea pigs participated in this study. In each guinea pig, melanocytes were counted in two sites on the back by RCM and DOPA images. On the concave surface of the outer ear, one site from one animal was studied. Melanocyte features were assessed in 24 melanocytes from the back. Data from the back represent mean ± SD.

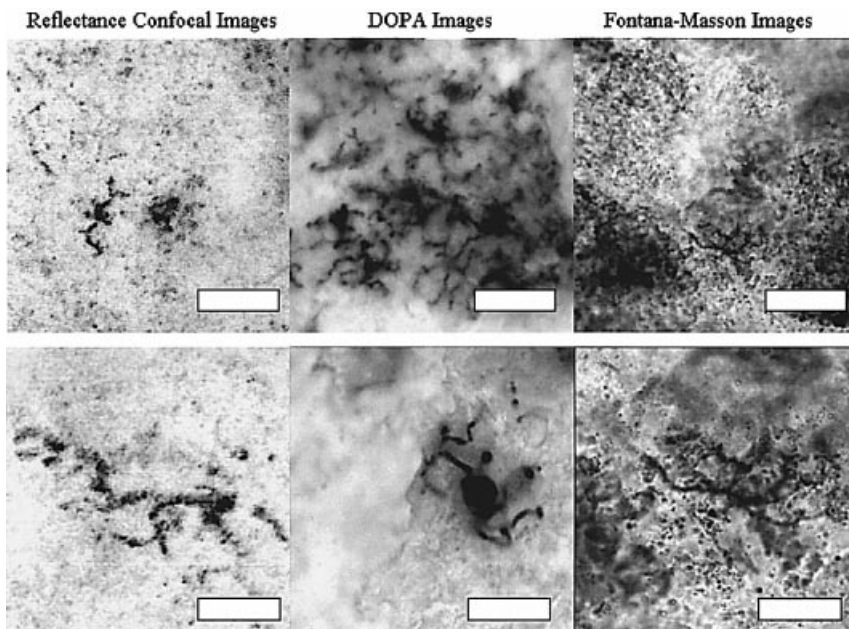


Figure 2. Comparison of images from the ear. Images of melanocytes are shown. *Left panels, RCM; middle panels, DOPA staining; right panels, Fontana–Masson staining. Scale bar: top panels, 50 μm; bottom panels, 20 μm.*

water immersion lens. After examination by RCM, skin specimens from the back and ear were obtained by 4 mm punch biopsies. Skin-splitting was done by previously reported methods (Staricco and Pinkus, 1957). Epidermal sheets were stained with either DOPA or Fontana–Masson and examined with × 40 and × 100

objective lens. In RCM images, melanocytes were counted in nine adjacent fields of view measuring 250 μm × 187 μm for a total area of 0.421 mm². In DOPA images, melanocytes were counted in six adjacent fields of view measuring 300 μm × 225 μm for a total area of 0.405 mm². The number of cells counted ranged from

50 to 90. The melanocyte density was expressed as the average number of melanocytes per mm^2 .

Quantitative studies on melanocytes have been done in both humans and animals using the DOPA reaction (Szabo, 1954; Breathnach, 1957; Staricco and Pinkus, 1957). We report on the direct *in vivo* examination of melanocytes. *In vivo* examination avoids potential artifacts introduced by biopsy, the removal of the dermis from the epidermis, and staining with DOPA or Fontana–Masson. *In vivo* examination also avoids potential artifact induced by fixation with the associated shrinkage of cells and tissue. Melanocytes in the wing and interfemoral membranes of the bat and in the ear of the mouse have been examined *in vivo* by transmitted light (McGuire, 1966; Snell *et al.*, 1966). RCM may be used to investigate melanocytes on any body part in any animal.

Melanin provided strong contrast in RCM images. Therefore, pigmented cells appeared bright. For the purpose of comparison, reflectance confocal images were inverted to print a “negative.” **Fig 1** shows images from RCM, DOPA, and Fontana–Masson staining from the back.

We compared our results from reflectance confocal images with conventional DOPA staining. The data are summarized in **Table I**. Melanocyte characteristics such as cell body area, number of dendrites, and length of longest dendrite were measured in RCM and DOPA images using an image processing and analysis program. Melanocyte characteristics exhibited a large SD, reflecting inherent variation. This was consistent with previously reported data (Bischitz and Snell, 1959). Using Student’s *t* test, there was no statistically significant difference between melanocyte features from RCM and DOPA images.

On the back, melanocyte counts by RCM images were consistently lower than by DOPA images. Our criteria for counting a melanocyte required visualization of a cell body and at least one dendrite. At times, we identified structures that were likely dendrites in RCM, but were unable to visualize the associated cell body. Therefore, RCM melanocyte counts were underestimated.

In the ear, only scattered melanocytes were visualized on RCM images. **Figure 2** shows RCM, DOPA, and Fontana–Masson images from the ear. It was not possible to assess melanocyte density in RCM images of the ear. From inspection of the Fontana–Masson images, there are more melanin granules in ear keratinocytes than back keratinocytes. We hypothesized that melanin granules are more efficiently transferred to keratinocytes in the ear compared with the back. Therefore, melanocytes were not well demarcated in the ear. We assessed the melanin content of RCM images by examining the distribution of gray values using image analysis. RCM images of the ear had higher gray values compared with images from the back. These data suggested that keratinocytes from the ear contained more melanin than those from the back.

DOPA images of the ear showed that melanocyte cell counts were about three times greater than on the back, consistent with previously reported data (Bischitz and Snell, 1959; Wolff and Winkelman, 1967).

Lawrence T. Wang, John T. Demirs, Madhu A. Pathak,
Salvador González

Wellman Laboratories of Photomedicine, Dermatology
Department, Massachusetts General Hospital, Harvard Medical
School, Boston, Massachusetts, U.S.A.

REFERENCES

- Agashi D, Anderson RR, González S: Time–sequence histologic imaging of laser-treated cherry angiomas using *in vivo* confocal microscopy. *J Am Acad Dermatol* 43:37–41, 2000
- Bischitz PG, Snell RS: A study of the melanocytes and melanin in the skin of the male guinea-pig. *J Anat* 93:233–245, 1959
- Breathnach AS: Melanocyte counts on human forearm skin. *J Invest Dermatol* 29:181–184, 1957
- Corcuff P, Bertrand C, Leveque JL: Morphometry of human epidermis *in vivo* by real-time confocal microscopy. *Arch Dermatol Res* 285:475–481, 1993
- González S, White WM, Rajadhyaksha M, Anderson RR, González E: Confocal imaging of sebaceous gland hyperplasia *in vivo* to assess efficacy and mechanism of pulsed dye laser treatment. *Lasers Surg Med* 25:8–12, 1999a
- González S, González E, White WM, Rajadhyaksha M, Anderson RR: Allergic contact dermatitis: Correlation of *in vivo* confocal imaging to routine histology. *J Am Acad Dermatol* 40:708–713, 1999b
- Horio T, Miyauchi H, Asada Y: The hairless guinea pig as an experimental animal for photodermatology. *Photodermatol Photoimmunol Photomed* 8:69–72, 1991
- Imokawa G, Kawai M, Mishima Y, Motegi I: Differential analysis of experimental hypermelanosis induced by UVB, PUVA, and allergic contact dermatitis using a brownish guinea pig model. *Arch Dermatol Res* 278:352–362, 1986
- McGuire J: Examination of mammalian melanocytes *in vivo*: a new approach. *J Invest Dermatol* 46:311–312, 1966
- New KC, Petroll WM, Boyde A, *et al*: *In vivo* imaging of human teeth and skin using real-time confocal microscopy. *Scanning* 13:369–372, 1991
- Pawley J, ed.: *Handbook of Biological Confocal Microscopy*, 2nd edn. New York: Plenum Press, 1995
- Rajadhyaksha M, Grossman M, Esterowitz D, Webb RH, Anderson RR: *In vivo* confocal scanning laser microscopy of human skin: melanin provides strong contrast. *J Invest Dermatol* 140:946–952, 1995
- Rajadhyaksha M, González S, Zavislan J, Anderson RR, Webb RH: *In vivo* confocal scanning laser microscopy of human skin II. advances in instrumentation and comparison to histology. *J Invest Dermatol* 113:293–303, 1999
- Snell RS, Silver AF, Chase HB: New method for studying mammalian melanocytes *in vivo*. *Nature* 210:219–220, 1966
- Staricco RJ, Pinkus H: Quantitative and qualitative data on the pigment cells of adult human epidermis. *J Invest Dermatol* 28:33–45, 1957
- Szabo G: The number of melanocytes in human epidermis. *Br Med J* 4869:1016–1017, 1954
- Webb RH: Confocal optical microscopy. *Rep Prog Phys* 59:427–471, 1996
- Wilson T, ed.: *Confocal Microscopy*. San Diego: Academic Press, 1990
- Wolff K, Winkelman RK: Quantitative studies on the Langerhans cell population of guinea pig epidermis. *J Invest Dermatol* 48:504–513, 1967

Exclusion of Candidate Genes and Loci for Multiple Lentiginos Syndrome

To the Editor:

Manuscript received January 14, 2002; revised March 15, 2002; accepted for publication March 19, 2002.

Reprint requests to: Dr. Theresa R. Pacheco, Assistant Professor, Department of Dermatology, University of Colorado Health Sciences Center, PO Box 6510, Mail Stop F703, 1665 N. Ursula, Aurora, CO 80010, U.S.A. Email: theresa.pacheco@uchsc.edu

Multiple lentiginos syndrome (MLS) is an autosomal dominant disorder of variable penetrance and expressivity. MLS is associated with generalized skin and mucosal pigmentation abnormalities called lentiginos (Arnsmeier and Paller, 1996; Uhle and Norvell, 1988). Lentiginos manifest histologically as a hyperproliferation of melanocytes along the dermal–epidermal junction. MLS has also appeared with other noncutaneous manifestations, mainly cardiac, auditory, and developmental abnormalities. It is thought to be a variant of the autosomal dominant LEOPARD syndrome (MIM151100) (Arnsmeier and Paller, 1996), which includes Lentiginos, ECG

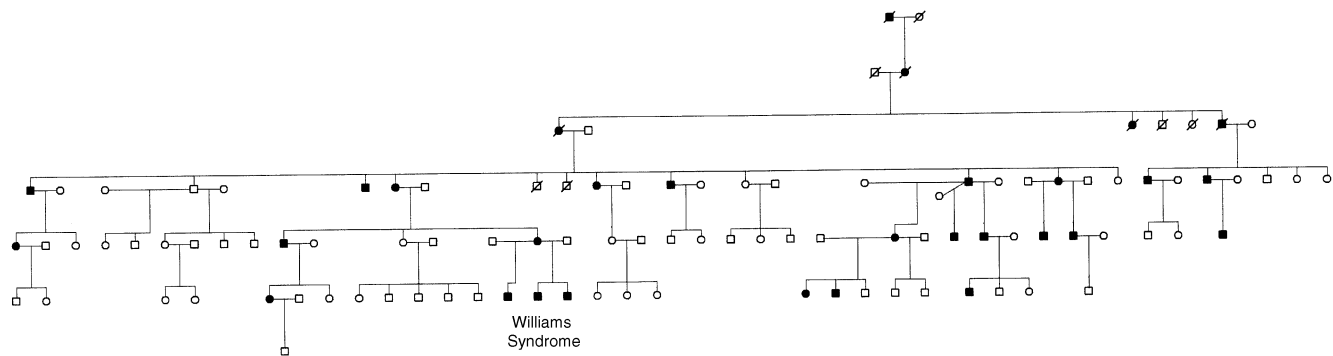


Figure 1. Pedigree of the family showing an autosomal dominant inheritance.

Table I. Disease loci, candidate genes, and their maximum LOD scores for MLS

Disease locus	Gene symbol, chromosome localization	Protein	Function	Markers (Marshfield location)	Maximum multipoint LOD
Neurofibromatosis 1 D17S1857 (43.01)	NF 1, -3.51 17q11.2	Neurofibromin	involved in the RAS signal transduction pathway	Tumor suppressor NF1 (51.9–58.8) D17S798 (53.41) D17S1868 (64.16)	-4.13 -6.60
Carney locus 1	N/A, 2p16		D2S337 (80.69)	D2S2259 (64.29) D2S391 (70.31)	-4.53 -4.09
Carney locus 2	PRKAR1A 17q23–q24	Type I regulatory α subunit of cyclic adenosine monophosphate dependent protein kinase (protein kinase A)	Dominant negative regulator of transcription in somatic cell hybrids	D17S944 (82.59) PRKAR1A (90.2) D17S949 (93.27)	-6.37 -4.52
Peutz–Jeghers locus 1	STK11/LKB1 19p13.3	Serine/threonine kinase 11	Cytosolic protein that has growth inhibitory activity	D19S886 (0) STK11/LKB1 (0–31.9) D19S209 (10.97) D19S216 (20.01)	-3.45 -3.76 -6.46
Peutz–Jeghers locus 2	N/A 19p13.4			D19S418 (92.56) D19S210 (100.01)	-3.29 -4.05
Cowden/ Bannayan–Zonana locus	PTEN 10q23	Dual specificity phosphatase (tensin homolog)	Putative tumor suppressor	D10S537 (91.13) PTEN (107.3–114.3) D10S185 (116.34)	-4.66 -5.30
Noonan syndrome locus	PTPN11 12q24.1	Protein tyrosine phosphate SHP-2	Contains two Src homology 2 (SH2) domains	D12S78 (111.87) PTPN11 (114.3–125.3) D12S79 (125.31)	-3.81 -6.40

abnormalities, Ocular hypertelorism/Obstructive cardiomyopathy, Pulmonary valve stenosis, Abnormalities of genitalia in males, Retardation of growth, and Deafness (Gorlin *et al*, 1969).

Clinically, MLS is commonly grouped with the lentiginosis syndromes. These syndromes combine the cutaneous findings of lentigines with a variety of developmental defects of the cardiovascular, endocrine, gastrointestinal, and nervous systems as well as increased susceptibility to tumorigenesis (Stratakis, 2000). The other lentiginosis syndromes include Peutz–Jeghers syndrome (MIM175200), the Carney complex (MIM160980), Cowden syndrome (MIM158350), and Bannayan–Zonana syndrome (MIM153480). Multiple lentigines have also been associated with neurofibromatosis type 1 (NF-1) (MIM162200) and Noonan syndrome (MIM 163950). MLS and NF-1 share café-au-lait patches and growth retardation and these two disorders should be included in the differential diagnosis of a patient with pigmentation anomalies and development defects (Coppin and Temple, 1997). Noonan syndrome and MLS overlap with certain clinical mani-

festations such as lentigines, café-au-lait patches, and developmental cardiac defects, such as pulmonary valve dysplasia and cardiomyopathies. Some researchers have suggested that both syndromes may be part of a common spectrum (Blieden *et al*, 1983; Mendez and Opitz, 1985; Coppin and Temple, 1997; Tullu *et al*, 2000).

Few detailed molecular studies of the MLS have been performed. A *de novo* mis-sense mutation in neurofibromin was identified in a patient with clinical overlapping features of NF1 and MLS (Wu *et al*, 1996); however, another study in a small family with MLS demonstrated no evidence of linkage to the NF1 locus (Ahlbom *et al*, 1995). To identify the gene(s) responsible for MLS, we performed a linkage study in a large affected family. Candidate genes or loci were studied that may be involved in other phenotypes that share some similarity to the disorder under study.

SUBJECTS AND METHODS

The Colorado Multiple Institutional Review Board approved the project. Diagnosis of MLS in affected family members was based on

the presence of multiple small 1–5 mm hyperpigmented macules on the entire body sparing the palmar and plantar surfaces. Family members were deemed unaffected if no hyperpigmented macules were present.

DNA was prepared from peripheral blood samples using the Qiagen Maxi kit (Qiagen, Valencia, CA). Twenty-four family members were genotyped for microsatellite markers flanking each of the candidate genes on chromosomes 2, 10, 12, 17, and 19. Markers were amplified using a PE Biosystems 877 Catalyst (Applied Biosystems), pooled in panels of 10–20 markers, and products were separated by electrophoresis in 5% polyacrylamide gels using a PE Biosystems 377 semiautomated sequencer. Allele sizing was carried out using GENESCAN 3.1 (Applied Biosystems), and individual genotypes were assigned using GENOTYPER 2.5 (Applied Biosystems), with manual checking to minimize data errors.

Two-point parametric LOD scores were calculated using MLINK (Cottingham *et al*, 1993) under assumptions of an autosomal dominant trait, disease gene frequency of 0.0001 and penetrance of 1.0 or 0.8. Multipoint parametric LOD scores were calculated using GENEHUNTER 2.1 (Kruglyak *et al*, 1996).

RESULTS

As shown in **Fig 1**, 13 members of this Hispanic family in four generations exhibited multiple lentigines inherited in a monogenic, autosomal dominant pattern.

Age of onset of the lentigines in family members is typically 2 y of age. Self-reported medical histories of examined affected family members revealed one relative affected with alopecia areata, two relatives affected with hearing impairments (one patient has Williams syndrome and the hearing loss in the other was attributed to a premature birth), and three relatives affected with undefined learning disabilities. Biopsies of select lesions revealed the histopathologic presence of increased numbers of melanocytes along the dermal–epidermal junction. No evidence of other phenotypic features of LEOPARD syndrome were observed in any of the observed affected family members.

A linkage screen of chromosomes 2, 10, 12, 17, and 19 was performed using markers within and/or flanking MLS candidate genes or loci (**Table I**). Two-point and multipoint parametric linkage analysis provided no evidence for linkage to a number of chromosomal regions on chromosomes 2, 10, 12, 17, and 19. The LOD scores for genetic markers flanking PTEN, NF1, STK11/LKB1, PRKAR1A, PTPN11, Carney locus 1, and Peutz–Jeghers locus 2 are ≤ 3 . Therefore, these genes and loci can be excluded as the cause of MLS in this family.

There are no published reports of Williams syndrome and MLS manifesting in the same patient. Although Williams syndrome and MLS share some similar phenotypic features (e.g., dental and craniofacial anomalies, pulmonary stenosis, cardiovascular anomalies, short stature, and developmental delay), linkage analysis showed LOD scores of -3.98 and -1.12 for the flanking markers, D7S502 and D7S630 of the Williams–Beuren syndrome (Online Mendelian, MIM194050) locus (Wang *et al*, 1999). Homozygosity for polymorphic markers flanking the Williams syndrome region at 7q11.2 confirmed the presence of a *de novo* deletion in the affected patient.

DISCUSSION

We describe an autosomal dominant disorder characterized by the early onset of multiple cutaneous lentigines. Linkage excluded candidate genes or loci for this disorder to chromosomal loci associated with similar clinical and pathologic features. The cause of MLS or LEOPARD syndrome is unknown. The spectrum of noncutaneous abnormalities associated with MLS is vast. Predominant noncutaneous abnormalities include obstructive cardiomyopathies, sensorineural deafness, and developmental deficiencies. The true incidence of noncutaneous features of MLS is

difficult to assess because the clinical descriptions are often incomplete. Family histories are not always provided and when included, often only discuss individuals with lentigines. The literature suggests that MLS manifests in three ways: (i) sporadic with no family history; (ii) autosomal dominant with affected family members with cutaneous and variable noncutaneous manifestation; and (iii) autosomal dominant with affected family members with only cutaneous manifestations (Voron *et al*, 1976; Arnsmeier and Paller, 1996; Uhle and Norvell, 1988). Additionally, numerous clinical reports identify a patient with LEOPARD syndrome and associate other family members with lentigines (Matthews, 1968; Polani and Moynahan, 1972; Seunaz *et al*, 1976; Peixoto *et al*, 1981; Loyd *et al*, 1982; Bleiden *et al*, 1983; Malathi *et al*, 1985; Choi *et al*, 1998). These reports of families with only generalized lentigines highlight the clinical dilemma of monitoring patients and their families for internal associations, especially cardiac and auditory abnormalities (Arnsmeier and Paller, 1996; Uhle and Norvell, 1988). Further molecular genetic studies are needed to determine whether MLS and LEOPARD are the same syndrome or distinct entities. The current family is being studied to establish linkage and to identify the responsible gene.

Theresa R. Pacheco, Nicole M. Oreskovich, Gary A. Bellus,*
Janet Talbert,† William Old,‡ Pamela R. Fain§
Departments of Dermatology and *Dermatology & Pediatrics,
†Human Medical Genetics Program, ‡Human Medical Genetics
Program & Computational Pharmacology,
§Department of Medicine, University of Colorado Health
Sciences Center, Denver, Colorado, U.S.A.

REFERENCES

- Ahlbom BE, Dahl N, Zetterqvist P, Anneren G: Noonan syndrome with café-au-lait spots and multiple lentigines syndrome are not linked to the neurofibromatosis type 1 locus. *Clin Genet* 48:85–89, 1995
- Arnsmeier SL, Paller AS: Pigmentary anomalies in the multiple lentigines syndrome: Is it distinct from LEOPARD syndrome? *Pediatr Dermatol* 13:100–104, 1996
- Blieden LC, Schneeweiss A, Shem-Tov A, Feigel A, Neufeld HN: Unifying link between Noonan's and Leopard syndromes? *Pediatr Cardiol* 4:168–169, 1983
- Choi JG, Chang SN, Kim SC, Park WH, Kwon HJ: Multiple lentigines syndrome, a comparison of normal skin and lentiginous skin by electron microscopy and immunohistochemical staining. *J Dermatol* 25:400–405, 1998
- Coppin BD, Temple IK: Multiple lentigines syndrome (LEOPARD syndrome or progressive cardiomyopathic lentiginosis). *J Med Genet* 34:582–586, 1997
- Cottingham RW Jr, Idury RM, Schaffer AA: Faster sequential genetic linkage computations. *Am J Hum Genet* 53:252–263, 1993
- Gorlin RJ, Anderson RC, Blaw M: Multiple lentigines syndrome. *Am J Dis Child* 117:652–662, 1969
- Kruglyak L, Daly MJ, Reeve-Daly MP, Lander ES: Parametric and nonparametric linkage analysis: a unified multipoint approach. *Am J Hum Genet* 58:1347–1363, 1996
- Loyd DW, Tsuang MT, Bengt JW: A study of a family with leopard syndrome. *J Clin Psychiatry* 43:113–116, 1982
- Malathi KE, Pal P, Mukherjee A: Leopard syndrome. *Indian Pediatr* 21:741–743, 1984
- Matthews NL: Lentigo and electrocardiographic changes. *N Engl J Med* 278:780–781, 1968
- Mendez HM, Opitz JM: Noonan syndrome: a review. *Am J Med Genet* 21:493–506, 1985
- O'Connell JR, Weeks DE: PedCheck: a program for identification of genotype incompatibilities in linkage analysis. *Am J Hum Genet* 63:259–266, 1998
- Online Mendelian Inheritance in Man, *Omim* (TM); Baltimore, MD: Mckusick–Nathans Institute for Genetic Medicine, Johns Hopkins University; Bethesda, MD: National Center for Biotechnology Information, National Library of Medicine, 2000. World Wide Web URL hyperlink: <http://www.ncbi.nlm.nih.gov/omim/> <http://www.ncbi.nlm.nih.gov/omim/>
- Peixoto MA, Perpetuo FO, de Souza RP, Miranda D, Loures CG: Leopard syndrome, a neural crest disorder: a case report. *Arq Neuropsiquiatr* 39:214–222, 1981
- Seunaz H, Mane-Garzon F, Kolski R: Cardio-cutaneous syndrome (the "LEOPARD" syndrome). Review of the literature and a new family. *Clin Genet* 9:266–276, 1976
- Stratakis CA: Genetics of Peutz–Jeghers syndrome, Carney complex and other familial lentiginoses. *Horm Res* 54:334–343, 2000
- Tullu MS, Muranjan MN, Kantharia VC, Parmar RC, Sahu DR, Bavdekar SB, Bharucha BA: Neurofibromatosis–Noonan syndrome or LEOPARD Syndrome? A clinical dilemma. *J Postgrad Med* 46:98–100, 2000

Uhle P, Norvell SS Jr: Generalized lentiginosis. *J Am Acad Dermatol* 18:444-447, 1988

Voron DA, Hatfield HH, Kalkhoff RK: Multiple lentiginos syndrome. Case report and review of the literature. *Am J Med* 60:447-456, 1976

Wang MS, Schinzel A, Kotzot D, *et al*: Molecular and clinical correlation study of Williams-Beuren syndrome: No evidence of molecular factors in the deletion

region or imprinting affecting clinical outcome. *Am J Med Genet* 86:34-43, 1999

Wu R, Legius E, Robberecht W, Dumoulin M, Cassiman JJ, Fryns JP: Neurofibromatosis type I gene mutation in a patient with features of LEOPARD syndrome. *Hum Mutat* 8:51-56, 1996