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 amyloidosus, 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, *e*2, *e*3, and *e*4, 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 *e*4 allele (0.357 in PLCA vs. 0.095 in control; **Table II**).

Our results indicate that the *e*4 allele is increased in frequency in PLCA. The *e*4 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 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

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

[a]LA, lichen amyloidosis; MA, macular amyloidosis.
[b]Age, 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	8a	4:4	17	9:8
E4/2	0		0	
E3/2	0		6	2:4
Allele frequency ε4	0.357a		0.095	
ε3	0.643		0.875	
ε2	0.0		0.030	

[a]$p < 0.01$ vs. control.

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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 (Fenjves *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.

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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 photodermatology 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-dioxyphenyalanine (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 video-rate (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

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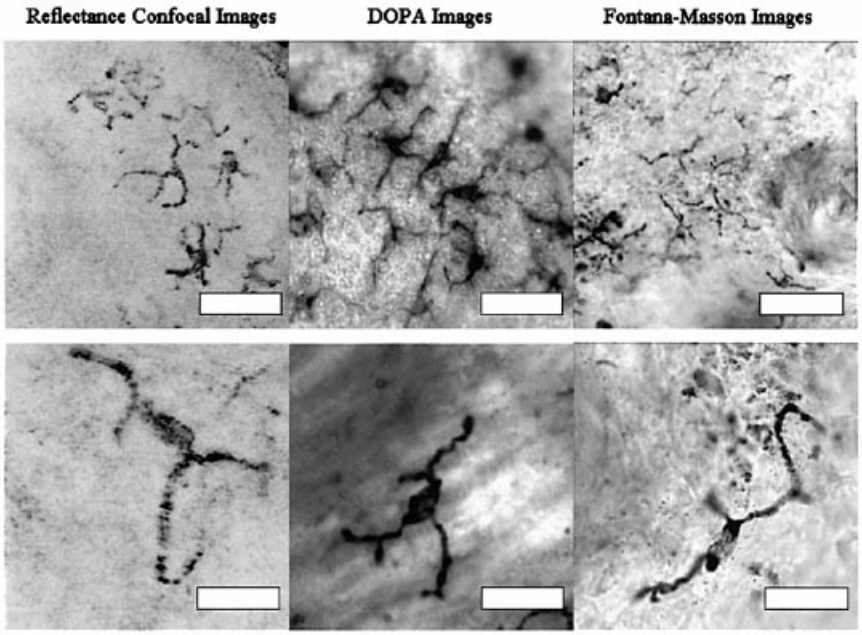

Figure 1. Comparison of images from the back. Images of melanocytes are shown. *Left panels*, RCM; *middle panels*, DOPA staining; *right panels*, Fontana–Masson staining. *Scale bar*: *top panels*, 50 μm; *bottom panels*, 20 μm.

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

[a]Four 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.

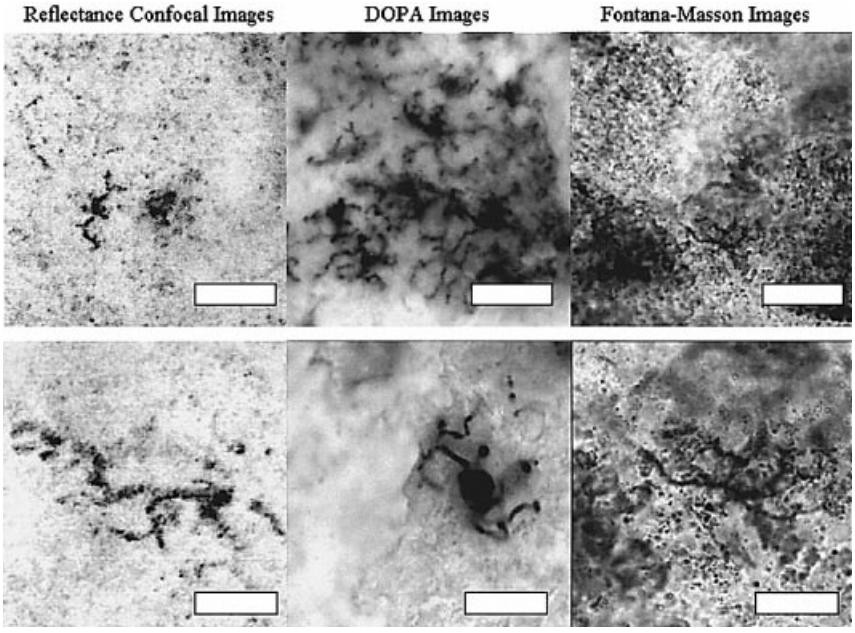

Figure 2. Comparison of images from the ear. Images of melanocytes are shown. *Left panels*, RCM; *middle panels*, DOPA staining; *right panels*, Fontana–Masson staining. *Scale bar*: *top panels*, 50 μm; *bottom panels*, 20 μm.

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 mm^2. In DOPA images, melanocytes were counted in six adjacent fields of view measuring 300 μm × 225 μm for a total area of 0.405 mm^2. 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 underestimates.

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).

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Exclusion of Candidate Genes and Loci for Multiple Lentigines Syndrome

To the Editor:

Multiple lentigines syndrome (MLS) is an autosomal dominant disorder of variable penetrance and expressivity. MLS is associated with generalized skin and mucosal pigmentation abnormalities called lentigines (Arnsmeier and Paller, 1996; Uhle and Norvell, 1988). Lentigines 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 *L*entigines, *E*CG

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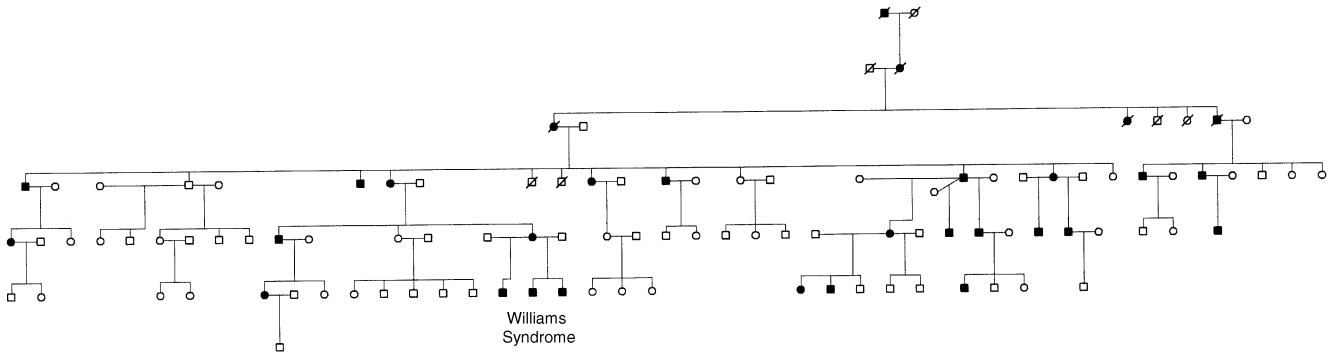

Figure 1. Pedigree of the family showing an autosomal dominant inheritance.

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	NF 1,	Neurofibromin	involved in the RAS	Tumor suppressor	–4.13
D17S1857 (43.01)	–3.51		signal transduction	NF1 (51.9–58.8)	–6.60
	17q11.2		pathway	D17S798 (53.41)	
				D17S1868 (64.16)	
Carney	N/A,		D2S337 (80.69)	D2S2259 (64.29)	–4.53
locus 1	2p16			D2S391 (70.31)	–4.09
				–3.75	
Carney	PRKAR1A	Type I regulatory	Dominant negative	D17S944 (82.59)	–6.37
locus 2	17q23–q24	α subunit of	regulator of	PRKAR1A (90.2)	
		cyclic adenosine	transcription in	D17S949 (93.27)	–4.52
		monophosphate	somatic cell hybrids		
		dependent protein			
		kinase (protein			
		kinase A)			
Peutz–Jeghers	STK11/LKB1	Serine/threonine	Cytosolic protein that	D19S886 (0)	–3.45
locus 1	19p13.3	kinase 11	has growth inhibitory	STK11/LKB1 (0–31.9)	–3.76
			activity	D19S209 (10.97)	–6.46
				D19S216 (20.01)	
Peutz–Jeghers	N/A			D19S418 (92.56)	–3.29
locus 2	19p13.4			D19S210 (100.01)	–4.05
Cowden/	PTEN	Dual specificity	Putative tumor	D10S537 (91.13)	–4.66
Bannayan–Zonana	10q23	phosphatase	suppressor	PTEN (107.3–114.3)	–5.30
locus		(tensin homolog)		D10S185 (116.34)	
Noonan syndrome	PTPN11	Protein tyrosine	Contains two	D12S78 (111.87)	–3.81
locus	12q24.1	phosphate SHP–2	Src homology	PTPN11 (114.3–125.3)	–6.40
			2 (SH2) domains	D12S79 (125.31)	

abnormalities, *O*cular hypertelorism/*O*bstructive cardiomyopathy, *P*ulmonary valve stenosis, *A*bnormalities of genitalia in males, *R*etardation of growth, and *D*eafness (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 manifestations such as lentigines, café-au-lait patches, and developmental cardiac defects, such as pulmonary valve dyplasia 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 $\leqslant 3$. 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; Seuanez *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.

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