Identification of a Genetic Defect in the Hairless Gene in Atrichia with Papular Lesions: Evidence for Phenotypic Heterogeneity among Inherited Atrichias

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Summary

Recently, we showed that atrichia with papular lesions (APL), a rare inherited form of alopecia, is transmitted as an autosomal recessive trait in a large inbred kindred of Israeli-Arab origin. Furthermore, we mapped the APL locus to a 5-cM region of chromosome 8p12 in this family. The human "hairless" gene is a candidate target gene for the disease mutation because it maps to the APL locus and because it was recently found to be mutated in a related but clinically distinct form of alopecia known as "alopecia universalis" or "congenital alopecia." In the present study, the coding sequence of the hairless gene was compared by reverse transcription–PCR in fibroblast cell lines derived from an affected patient and an unrelated individual. We identified a single-base deletion (3434delC) in the hairless gene that cosegregated with the disease phenotype in the family. This deletion is predicted to cause a frameshift mutation in the highly conserved C-terminal part of the hairless protein, a region putatively involved in the transcription factor activity of the hairless gene product. The present results are indicative of phenotypic heterogeneity in inherited atrichias caused by mutations in the hairless gene, suggesting different roles for the regions mutated in APL and in other forms of congenital atrichia during hair development.

Introduction

Inherited universal alopecia without associated anomalies is considered rare (Bertolino and Freedberg 1993). To date, genetic defects have been described for only two heritable forms of isolated alopecia. Monilethrix (MIM 252200) is thought to result from a mutation in hair keratins (Winter et al. 1997*a*, 1997*b*; Korge et al. 1998; Zlotogorski et al. 1998*b*), whereas alopecia universalis (MIM 203655), also named "universal congenital alopecia" (Nöthen et al. 1998), was found to be associated with a missense mutation in the human "hairless" gene (W. Ahmad et al. 1998*a*, 1998*b*).

Atrichia with papular lesions (APL) (MIM 209500) is a rare congenital ectodermal form of alopecia. Fewer than 15 cases have been reported worldwide (Damste and Prakken 1954; Loewenthal and Prakken 1961; Del Castillo et al. 1974; Ischii et al. 1979; Czarnecki and Stingl 1980; Kanzler and Rasmussen 1986; Misciali et al. 1992; Delprat et al. 1994). This disease is characterized by hair loss soon after birth and the development, years later, of a diffuse papular rash over the entire skin surface (Misciali et al. 1992). The absence of those papules distinguishes congenital alopecia from APL (M. Ahmad et al. 1993; Kenue and Al-Dhafri 1994).

We recently described the largest family affected by APL reported to date and provided compelling evidence for an autosomal recessive mode of inheritance of this rare disorder in this family (Sprecher et al. 1998). Furthermore, on the basis of homozygosity mapping of this kindred, we mapped the APL locus to a 5-cM region on chromosome 8p12 that contains the human "hairless" gene (Sprecher et al. 1998). In the present report, we show the segregation, throughout this kindred, of a frameshift mutation in a highly conserved region of the human hairless gene.

Subjects and Methods

Subjects

We studied a large consanguineous APL-affected kindred of Israeli-Arab origin, which we recently described
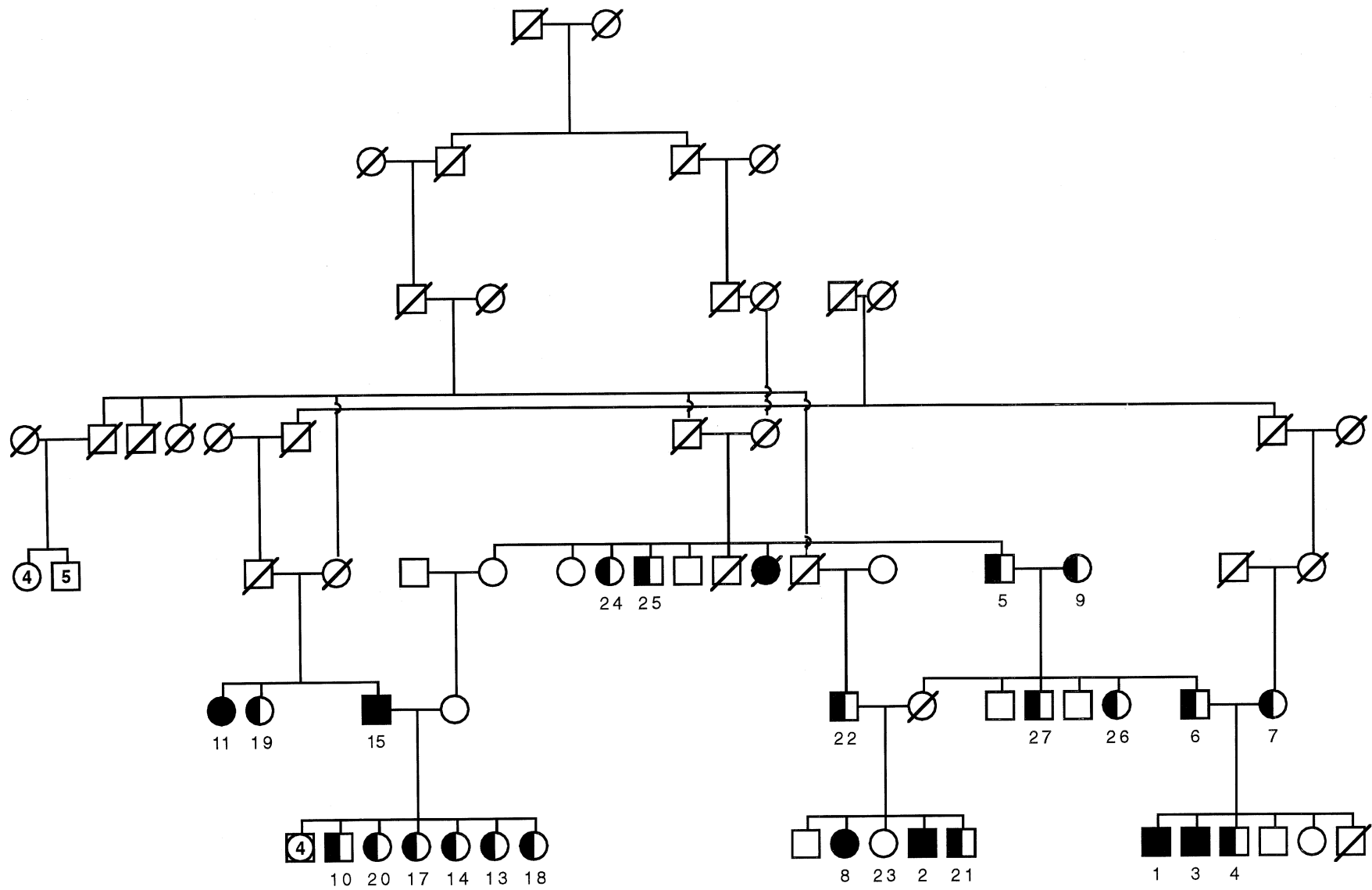
Figure 1 Pedigree of the APL-affected consanguineous kindred. Blood samples were obtained from all numbered individuals. Carrier status was determined for numbered individuals as described elsewhere (Sprecher et al. 1998).

Table 1

Primer Sequences for RT-PCR and Sequencing

Primer Pair	Position	Sequence
EX2AF	1–305	ATGGAGAGTACGCCCAGC
EX2AR		GCCTCCTTCCAGCGCAGT
EX2BF	260–609	AGGTCAACTGGCTGGGCA
EX2BR		GCTGCCTAAGCTGAAGGC
EX2CF	447–991	CAAGATCCTGGAGCGAGC
EX2CR		G GTAGGATGAACAGCAGC
1457-1	620–1142	CTACAAGGATCCGAGCATTC
1457-2		TTCTTCAGCTTGGTGTGGTG
EX3AF	1081–1400	GAAGTGAACAAGGCCTCTGG
EX3AR		TGCTCATCATGCTGTCCCGA
EX3BF	1193–1800	CTGAGGTCGAGGAGAGG
EX3BR		GCAGCAGCGTGGAATGC
EX5F	1602–2030	CAACTCCAGCTCTGAGGAA
EX7R		ATTGCAGTGCTCAGCTCTG
EX7F	1961–2550	ACGCTGCCTGTTCCCTGA
EX10R		CTCCTGCAGCCACAGCAA
EX10F	2275–3206	TGCGAACTGCTGGCTTCTA
EX15R		AGAAAGCGGCGGATGCG
EX15F	3006–3565	GACCAAGAACCTCTGTGT
EX18R		TGGCCTCCTGTAATGTCC

elsewhere (Sprecher et al. 1998). It originates from a village of Galilee and manifests an autosomal recessive pattern of APL inheritance affecting seven members over five generations (fig. 1). The kindred consists of three subfamilies. Its clinical and histopathological features are characteristic of the APL syndrome (Misciali et al. 1992). Total hair loss was observed at the age of 3–24 mo, with subsequent development of a diffuse papular eruption at the age of 5–7 years. Psychomotor development and general health were normal. On examination, all patients displayed complete hair loss, except for sparse eyelashes and eyebrows. Small follicular skin-colored or white papules were present on the entire skin surface. The histologic examination showed well-developed infundibula but absence of the remaining parts of the hair follicle and no hair-shaft formation. All unaffected siblings were clinically normal.

EDTA blood samples for genetic analysis were collected after we obtained informed consent from the subjects or their guardians according to a protocol reviewed and approved by the local Helsinki committee. DNA was prepared by standard methods (Sambrook et al. 1989). Altogether, we had access to DNA samples from six patients and 21 healthy family members. Primary fibroblast cell cultures were established by trypsinization from skin-punch biopsy specimens and maintained in MEM Alpha (Gibco BRL) in the presence of 15% fetal calf serum.

Reverse Transcription (RT)–PCR

We extracted mRNA from primary fibroblast cell cultures by use of an mRNA isolation kit (Boehringer Mannheim) according to the manufacturer's instruc-

tions. The primers used for RT-PCR are listed in table 1 and were designed according to the hairless cDNA sequence reported by W. Ahmad et al. (1998*a*) (GenBank accession number AF039196). We subjected RNA to RT-PCR in a PTC-100 thermocycler (MJ Research) by use of a one-tube RT-PCR kit (Boehringer Mannheim) according to the manufacturer's instructions. RT was performed at 50°C for 30 min. Denaturation was performed at 94°C for 2 min with 10 subsequent amplification cycles performed at 94°C for 30 s, 55°C for 30 s, and 68°C for 1 min, followed by 25 similar cycles with 5 s added to each elongation step. A prolonged elongation step of 7 min terminated the reaction. PCR products were analyzed on 2% agarose gels and purified with a QIAquick gel extraction kit (QIAgen) before sequencing.

Sequence Analysis

RT-PCR fragments were directly sequenced by capillary electrophoresis on an ABI PRISM 310 automated sequencer (Perkin-Elmer) with a Big Dye dye terminator cycle sequencing kit (Perkin-Elmer) and the primers shown in table 1. To determine the intron-exon boundaries of exon 18, we used the Expand Long Template PCR system (Boehringer), according to the manufacturer's instructions, to amplify genomic DNA with primers EX17F-5′-GGCGTGAGCTGCTGGAC-3′ and EX17R-5′-CAGAGCTGAGCAGAGAGG-3′, as well as with primers EX18F-5′-CTGGTGAGCACAGTCAGC-3′ and EX18R-5′-TGGCCTCCTGTAATGTCC-3′. Resulting DNA fragments were sequenced after purification by use of the same protocols described earlier. Subsequently, direct sequencing analysis of the family and of unrelated controls was performed by use of the following designed primers: INT17F2-5′-GCGCCAG-AGTCCACTCC-3′ and INT18R1-5′-GATCTGCTAT-GTCCACTGC-3′.

PCR-RFLP in Exon 18

We confirmed the mutation and its segregation in the family by means of *Bsa*I (New England Biolabs) digestion of a 405-bp genomic fragment encompassing exon 18 and amplified with primers INT17F1-5′-CTCTCC-AGCAGTTCTGAGTC-3′ and INT18R1-5′-GATCTG-CTATGTCCACTGC-3′ and *Taq* DNA polymerase (Promega). PCR conditions were as follows: denaturation at 94°C for 2 min; 30 subsequent amplification cycles performed at 94°C for 30 s, at 55°C for 30 s, and at 72°C for 30 s; and one final step at 72°C for 4 s. After their digestion, DNA fragments were analyzed by electrophoresis on 2% agarose gels.

Results

Evidence of linkage of the APL locus to a region on chromosome 8p encompassing the human hairless gene
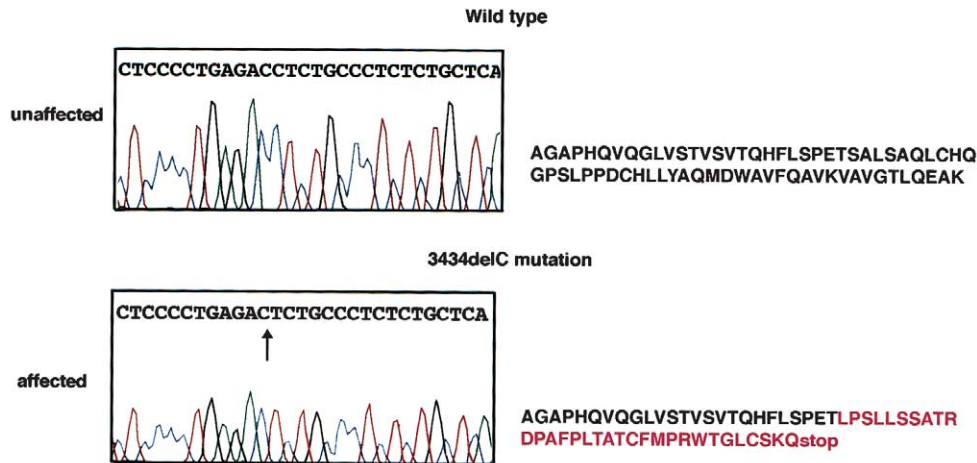
Figure 2   Sequence analysis of exon 18 comprising electropherograms that show part of the exon 18 sequence in a healthy individual and in her affected brother (*boxed, left*). A cytosine deletion at nucleotide 3434 is marked with an arrow. The mutation is predicted to create a frameshift mutation at codon 1146 with a resulting premature termination of the hairless gene product (*right*).

(Sprecher et al. 1998) led us to perform a mutational analysis of the gene in an APL-affected kindred of Israeli-Arab origin. We extracted mRNA from primary fibroblast cultures established from an individual affected with APL (individual 1, fig. 1) and from an unrelated individual. Fibroblast cells were used because no gene transcription was detectable in peripheral lymphocytes (data not shown). The entire coding sequence of the hairless gene was amplified by RT-PCR analysis with 10 overlapping primer pairs (table 1). Sequence analysis of the RT-PCR products showed a deletion of a cytosine residue at cDNA position 3434 in the APL-affected patient that was not found in the unaffected individual. The 3434delC deletion, at codon 1146 of the C-terminal end of the hairless protein, is predicted to create a frameshift mutation with a resulting premature termination (fig. 2).

We determined, on the basis of the homology existing between the coding regions of the murine and human hairless genes (W. Ahmad et al. 1998a) and on the basis of the location of the gene exon-intron boundaries in the mouse (Cachon-Gonzalez et al. 1994; GenBank accession number Z32675), the location of the mutation in exon 18. We subsequently defined the sequence of the intron-exon boundaries of this exon and designed a primer pair with which we determined the genomic sequence of exon 18 in all members of the kindred. The 3434delC mutation was found to segregate with the disease trait throughout the affected kindred. All affected family members were found to carry the mutation, homozygous by descent from related heterozygous parents. The deletion was present in the heterozygous state in all obligate heterozygous carriers and in unaffected carriers within the family. Sequence electropherograms from an

affected child homozygous for the 3434delC mutation and from his homozygous unaffected sister are shown in figure 2.

Since the mutation was found to abolish a *Bsa*I restriction site in the amplified exon, we confirmed the results discussed earlier by analyzing the amplified PCR fragments after their digestion with *Bsa*I. Two fragments consistent with the predicted sizes of 158 and 247 bp were observed in healthy homozygous subjects, whereas only one fragment of 405 bp was detected in homozygous patients. Accordingly, all three fragments were obtained in unaffected heterozygous relatives (fig. 3). The mutation was not detected, by use of either direct sequencing or PCR-RFLP, in 40 unrelated individuals of Israeli-Arab origin (80 chromosomes).

Discussion

A deleterious mutation was found in a large consanguineous APL-affected kindred of Israeli-Arab origin. In this family, a frameshift mutation cosegregated with the disease phenotype. The mutation was not observed in normal chromosomes. Furthermore, our data suggest phenotypic heterogeneity in inherited atrichias caused by mutations in the human hairless gene. The region affected by the frameshift mutation described herein is highly conserved in the human, mouse, and rat genomes (W. Ahmad et al. 1998a). This suggests that it may be of importance to the protein function. Interestingly, Thompson and Bottcher (1997) recently suggested that the C-terminal part of the hairless protein might be involved in the control of thyroid hormone receptor gene activity.

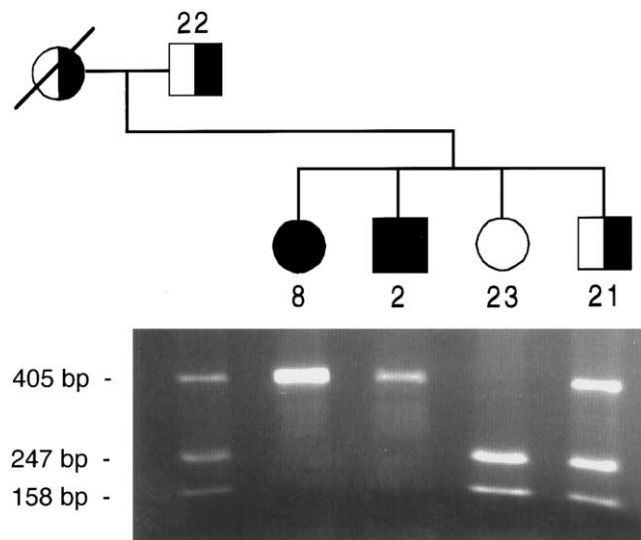Apart from this report, other mutations in the hairless

Figure 3   Screening of the 3434delC mutation by PCR-RFLP in APL-affected subfamily. Restriction digest with *Bsa*I of a 405-bp PCR product spanning exon 18 shows a 158- and a 247-bp fragment in an unaffected homozygous sib (individual 23) and an additional 405-bp band in heterozygous carriers of the mutation (individuals 22 and 21). Affected patients exhibit the 405-bp fragment only (individuals 8 and 2).

gene have been described in various forms of inherited alopecia (W. Ahmad et al. 1998*a,* 1998*b;* Cichon et al. 1998; Zlotogorski et al. 1998*a*). W. Ahmad et al. (1998*b*) have proposed, on the basis of these observations, that APL and alopecia universalis, or congenital alopecia, should be viewed as a single clinical entity. The diagnosis of APL is based on three well-defined clinical and histopathological criteria (Del Castillo et al. 1974; Misciali et al. 1992; Sprecher et al. 1998). First, total hair loss occurs after birth. Second, keratinous follicular cysts appear years later over extensive areas of the skin, especially on the face, scalp, neck, and extremities, in contrast with the absence of papules (Cichon et al. 1998) or their restricted distribution (W. Ahmad et al. 1998*b*) in other forms of inherited alopecia. This striking papular eruption is the most distinctive feature of APL, as reflected in the very name of the syndrome (Damste and Prakken 1954; Loewenthal and Prakken 1961; Del Castillo et al. 1974; Ischii et al. 1979; Czarnecki and Stingl 1980; Kanzler and Rasmussen 1986; Misciali et al. 1992; Delprat et al. 1994). Third, histopathological examination shows numerous follicular keratinous cysts. Most studies also report the presence of well-defined infundibula in skin biopsy specimens, with abnormal development of the germinal end of the follicle leading to the absence of a hair shaft (Del Castillo et al. 1974; Misciali et al. 1992; Sprecher et al. 1998). Follicular cysts presumably develop from these malformed follicles

and are often surrounded by normal sebaceous glands and smooth-muscle bundles. The presence of follicular cysts and residual infundibula instead of mature hair follicles in APL has been viewed as evidence for a differentiation arrest late in the process of hair follicle development, after initial follicular differentiation but before formation of the hair bulb (Misciali et al. 1992). The conspicuous papular rash of APL represents the clinical correlate of those follicular cysts (Misciali et al. 1992).

Thus, these three characteristics should be present to diagnose APL. Clearly, the kindred reported in this study, as well as the family reported by Zlotogorski et al. (1998*a*), belong to the APL type of inherited atrichia. In contrast, other families studied to date with mutations in the hairless gene fail to fulfill all APL criteria. No papules were reported in the families described by M. Ahmad et al. (1993), W. Ahmad et al. (1998*a*), Kenue and Al-Dhafri (1994), or Cichon et al. (1998), and the groups of papules on the elbows and knees of the patients described by W. Ahmad et al. (1998*b*) can hardly be considered equivalent to the diffuse papular rash of APL. Three types of mutations have been described in the hairless gene: missense mutations (W. Ahmad et al. 1998*a,* 1998*b;* Cichon et al. 1998), 5′ splice-site mutations (Cichon et al. 1998), and deletion mutations that lead to a frameshift and downstream premature termination codon (Zlotogorski et al. 1998*a;* the present report). Although the 5′ splice-site mutation reported by Cichon et al. (1998) could theoretically cause a frameshift mutation with premature termination, the use of a cryptic downstream splicing acceptor site is predicted to result in an insertional mutation at amino acid position 925 (Cichon et al. 1998). Thus, deletion mutations resulting in a premature termination codon in the hairless gene seem to be associated with the papular rash of APL and missense mutations with its absence. Although additional reports of mutations in families with APL are required to confirm this preliminary hypothesis, these observations do not seem to support recent attempts to group into a single entity all forms of congenital atrichia caused by mutations in the human hairless gene (W. Ahmad et al. 1998*b*). In fact, the well-established distinctions between APL and other forms of congenital atrichia may be both biologically and clinically relevant.

Indeed, the 3434delC mutation in exon 18, reported in the present article, indicates that a 136-bp region at the 3′ end of the gene coding sequence is of functional importance only in the later phases of hair follicle development, since, in its absence, hair follicle infundibula can still develop. Other regions of the gene in which mutations have been reported, such as exons 6, 12, 15, and 17, may be associated with the regulation of earlier phases of hair follicle development. In addition, deletion of the 3′ end region of the gene may be associated with

the papular rash of APL. Indeed, the deletion mutation in exon 9 described by Zlotogorski et al. (1998*a*) affects both the 136-bp-length 3′ end of the hairless cDNA and upstream regions affected by missense mutations described in congenital alopecia. Interestingly, Zlotogorski et al. (1998*a*) describe a characteristic papular rash in their patients but do not report remaining infundibula on examination of their skin biopsy specimens. Thus, this mutation may combine clinical and molecular features of both types of atrichia.

Clinically, APL can easily be distinguished from other forms of inherited alopecia when one relies on solid clinical and histopathological criteria. This distinction is far more than semantic. Indeed, APL, but not congenital alopecia, has been described in association with three clinical entities, namely, mental retardation, extensive polyposis throughout the gastrointestinal tract, and combined immunodeficiency (Del Castillo et al. 1974; Ischii et al. 1979; Czarnecki and Stingl 1980). Investigations should be conducted to exclude those conditions in patients with APL.

In conclusion, the fact that distinct clinical forms of inherited atrichia are caused by mutations in the same gene is not sufficient to justify their reclassification into a single entity. On the contrary, those observations should lead to further investigation of the molecular basis of phenotypic heterogeneity among the congenital atrichias. In fact, because the hairless gene product is thought to function as a multifunctional transcription factor (Thompson and Bottcher 1997), mutations in selected regions of the gene may have different effects on the development of the hair follicle and may generate distinct disease phenotypes. Delineating the relationship between phenotype and genotype in inherited atrichias, therefore, is likely to contribute in the near future to the deciphering of important molecular aspects of hair follicle development. Meanwhile, both prenatal diagnosis and carrier detection are now available to the affected families.

Acknowledgments

Electronic-Database Information

Accession numbers and URLs for data in this article are as follows:

GenBank, http://www.ncbi.nlm.nih.gov/Web/Search (accession numbers Z32675 for the mouse hairless gene and AF039196 for the human hairless gene)

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim (for APL [MIM 209500], alopecia universalis [MIM 203655], and monilethrix [MIM 252200])

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