Multiple Familial Trichoepithelioma Caused by Mutations in the Cylindromatosis Tumor Suppressor Gene

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ABSTRACT

The recessive oncogene cylindromatosis (CYLD) mapping on 16q12-q13 is generally implicated in familial cylindromatosis, whereas a gene region for multiple familial trichoepithelioma has been assigned to 9p21. Markers from both chromosome intervals were subjected to linkage analysis in a large family with multiple hereditary trichoepithelioma (TE) from Algeria. Linkage to 9p21 was excluded, whereas CYLD remained as a candidate. Mutation analysis identified a single bp germ-line deletion expected to result in truncation or absence of the encoded protein, which segregated with the multiple TE phenotype. In individual tumors, loss of heterozygosity at 16q or a somatic point mutation in the CYLD gene was detected. Hence, mutations of the tumor suppressor gene CYLD at 16q12-q13 may give rise to familial TE indistinguishable from the phenotype assigned to 9p21.

INTRODUCTION

Multiple familial trichoepithelioma (MFT; MIM 601606) is characterized by numerous, firm skin-colored papules that originate from hair follicles. Histopathologically, trichoepitheliomas (TE) contain branching nests of basaloid cells, horn cysts, and abortive hair papillae. The tumors represent benign hamartomas of the pilosebaceous apparatus. Guggenheim (1) reported that TE, which is also called epithelioma adenoides cysticum (2), may occur in a family or even in one patient together with cylindroma as described by Spiegl (3), which is why they postulated their genetic identity. This has been corroborated by multiple reports on the coexistence of cylindromas and TEs and, occasionally, spiradenomas (4). Therefore, an entity called Spiegl-Brooke syndrome (MIM 605041) has been created, and TEs and, occasionally, spiradenomas (4). Therefore, an entity called Spiegl-Brooke syndrome (MIM 605041) has been created, which is thought to be associated with defects in the regulation of the proliferation and differentiation of putative stem cells of the folliculosebaceous-apocrine unit giving rise to different adnexal skin tumors as well as to other neoplasms (5).

In 1996, Harada et al. (6) reported linkage of autosomal dominant multiple TE to chromosome 9p21. However, in contrast to the notion of genetic identity of cylindroma and TE, defects of a tumor repressor gene on chromosome 16, CYLD, were documented to cause familial cylindromatosis (7, 8). CYLD mutations were recently detected in Chinese families with MFT (9, 10). Because some families with CYLD mutations were affected with both cylindromas and TEs (11, 12), CYLD deficiency appears to be associated with Spiegl-Brooke syndrome (MIM 605041). In fact, familial cylindromatosis may represent one end of a clinical spectrum of a group of adnexal neoplasms of the skin (13).

However, there still remains the question whether MFT without associated cylindroma represents a separate genetic entity linked to 9p21 (MIM 601606), or whether it may likewise originate from mutations at the locus of Spiegl-Brooke syndrome (MIM 605041).

In some MFT tumors, structures reminiscent of basal cell carcinoma (BCC) may be found (14), although MFT clearly differs from Gorlin syndrome (multiple nevoid BCC syndrome) that is caused by mutations in the patched gene (PTCH1) on 9q22.3. In half of a series of sporadic TEs loss of heterozygosity (LOH) at 9q22.3, the region where PTCH1 maps was detected by Matt et al. (15) who postulated a common gatekeeper mechanism for both TE and BCC. Their observations corroborate a previous report that sporadic TE contain somatic mutations in the overexpressed PTCH gene, suggesting that a failure in the negative feedback mechanism in the sonic hedgehog-patched (SHH-PTC) signaling pathway may be an early and necessary event in TE development (16).

We describe a large family of Algerian origin with 21 individuals affected with multiple TE in four consecutive generations. All of these patients showed the typical clinical and histopathological features of multiple TE preponderantly involving the face and in some patients also the scalp. Tumors suggesting a diagnosis of cylindroma, spiradenoma, or BCC were absent. We exclude involvement of a locus on chromosome 9p as well as LOH in tumors on 9q and identify a germ-line mutation in the tumor suppressor gene CYLD on chromosome 16q12-q13 segregating with MFT in affected members of this family. We demonstrate LOH or a point mutation eliminating the function of the second copy of this gene. In view of the previous assignment to 9p21, these results suggest genetic heterogeneity of MFT.

MATERIALS AND METHODS

Subjects and Analyzed Samples. A 25-year-old woman and her 24-year-old sister presented for advice because of multiple facial tumors. Physical examination showed numerous, firm skin-colored papules and nodules involving the nasal root, the medial part of the eyebrows, and the nasolabial folds. The family history revealed that 21 individuals belonging to four consecutive generations were similarly affected, and this was confirmed in part by physical examination (Fig. 1A). In some of them, a few papules or nodules were likewise found on the scalp and in the periauricular areas. The tumors tended to become obvious after puberty but in several family members, some small papules had already appeared during childhood.

This large Algerian kindred in which MFT segregated was analyzed for the underlying genetic defect. Blood samples were obtained from 13 family members (Fig. 2) and from 100 unrelated, unaffected Caucasian-control individuals or of cases that were for other reasons not available for direct clinical evaluation was based on photographic documentation or family history.

Histopathological Examination. For histopathological examination, sections obtained from facial lesions of 3 family members (III, 4; IV, 3; and IV, 6) and from a scalp lesion of an individual (IV, 6) were stained with H&E and from tumor specimens that were DNA from individual tumor specimens that were

Mutation Analyses. DNA from individual tumor specimens that were separated macroscopically from surrounding tissue was extracted by use of a “QIAamp DNA Mini Kit” (Qiagen) following the “tissue protocol” of the
DNA from blood lymphocytes was extracted by standard procedures. Patient and control DNA was taken at a concentration of 50 ng/μl as template for PCR. Exons 4–19 of CYLD including intron-exon boundaries were amplified by PCR. We applied the primer combinations and, with minor modifications, the procedures described by Bignell et al. (8).

The coding exons 4 to 19 of CYLD were sequenced in lymphocyte DNA from individual III, 1 and in DNA from tumors (III, 1 tum1; IV, 6 tum3; and IV, 7 tum1) on an automated DNA sequencer model 377 (Applied Biosystems) by the dye terminator procedure. The sequences were compared with the normal control sequences and the published cDNAs (GenBank accession no. AJ250014) by use of the ABI Prism Sequencing Analysis Version 2.1.1 and Sequence Navigator software programs (Applied Biosystems). In all of the individuals tested from this family as well as from 100 unrelated controls, the PCR products of exons 15 and 19, in which genomic mutations have been found by sequencing, were subjected to single-strand conformation analysis by electrophoresis in 12% polyacrylamide gels in 0.5 Tris-borate EDTA buffer at 20°C or 10°C, respectively, for 10 h at 200 V or 90 min at 600 V, followed by silver staining. Bands deviating in mobility from the ones detected in unaffected individuals were eluted from the gel, reamplified by PCR, purified by a QIAquick PCR purification kit (Qiagen), and resequenced.

**Typing of Microsatellite Markers.** Microsatellite markers D9S925, D9S171, and D9S169 covering the region 9p2 in which, previously, linkage with the MFT phenotype had been detected (6), microsatellite markers D9S15, D9S252, D9S303, and D9S287 encompassing the PTCH1 locus on chromosome 9q22.3 and microsatellites encompassing the CYLD locus on chromosome 16 (Table 1) were genotyped on an automated DNA sequencer model 377 (Applied Biosystems) after PCR amplification by use of primers and conditions described in the Genome Database5 and analyzed with the Genotyper program (Applied Biosystems). Microsatellite markers CDRP28 and CDRP23 were amplified as described by Bignell et al. (8).

**Linkage and LOH Analysis.** Two-point limit of detection-score analyses were performed for linkage between disease status and every marker using the MLINK program of Linkage software package version 5.1 (17) assuming a monogenic effect in a dominant mode of inheritance, a disease allele frequency of 1:10,000, and a penetrance of 98%. Locus order was derived from the human genome working draft database6 for markers on chromosomes 9 and from Bignell et al. (8) for markers on chromosome 16. Allele frequencies were adopted from the Genome Database,5 where possible, or set to be equally distributed (CDRP23, 28, and D16S416). Genotypic and phenotypic information given in the pedigree displayed in Fig. 2 and in Table 1 was used for the analyses. Multipoint analysis was performed using GeneHunter software, version 2.1 (18). Several untyped individuals were removed from analysis because of time and memory constraints of the software. The disease model was assumed same as in pair-wise analysis, and the order of the markers was essentially as shown in Table 1. The constitutional genotype of each individual was determined from lymphocyte DNA. To establish the presence of LOH, an allelic imbalance was assessed by comparing signal intensity recorded by the Genotyper program (Applied Biosystems) of normal and tumor alleles from 6 patients.

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5 Internet address: http://www.gdb.org.
6 Internet address: http://genome.ucsc.edu.
RESULTS

Histopathological Features. Histopathological examination of lesions from both face and scalp consistently showed islands of small basophilic cells of basaloid appearance. Peripheral palisading and central formation of keratin cysts was noted. In the surrounding dermis, reticular proliferations of small basaloid cells were present, and occasionally abortive hair differentiation was found (Fig. 1B). In no part of the specimens, including those obtained from scalp lesions, was any structure reminiscent of cylindroma seen. In particular, there were no hyaline sheaths surrounding the islands of basaloid cells. In all of the specimens, the dermis was filled with circumscribed proliferative formations of basophilic basaloid cells surrounding numerous cysts with a fully keratinized center. The walls of these horn cysts contained some layers of cells with eosinophilic cytoplasm and large vesicular nuclei. Some of the basophilic cells were arranged in an adenoid network. All of these histopathological features were characteristic of TE.

The Multiple TE Phenotype Segregates with Markers on 16q12-q13 But Not on 9p. Both previously identified candidate loci for MFT and familial cylindromatosis were screened for linkage with the phenotype in this family. In the region that had shown previously linkage with MFT on chromosome 9p (6), linkage was excluded for the microsatellite markers D9S925, D9S171, and D9S169 (Table 1; Fig. 2). In contrast, analysis with markers encompassing the CYLD locus on chromosome 16 revealed a two-point limit of detection score above 2, suggesting linkage (Table 1). This analysis was corroborated by a multipoint linkage analysis for the phenotype MFT and the same microsatellite markers on chromosomes 16. Markers D16S753 to D16S673 encompassing the CYLD gene show limit of detection scores above 3.0 (Fig. 3). Hence, the CYLD gene was considered a strong candidate and subjected to mutation analysis.

Identification of a Disease-Associated Germ-Line Mutation. Three hundred-400 bp fragments encompassing exons and flanking intron sequences of the CYLD gene were amplified by PCR by use of

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Table 1 Two-point limit of detection scores for the phenotype familial multiple trichoepithelioma and microsatellite markers on chromosomes 16 and 9

<table>
<thead>
<tr>
<th>Marker</th>
<th>Marker</th>
<th>.00</th>
<th>.10</th>
<th>.20</th>
<th>.30</th>
<th>.40</th>
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<tr>
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<td>1.98</td>
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<td>1.02</td>
<td>0.49</td>
</tr>
<tr>
<td>CDRP 23</td>
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<td>1.27</td>
<td>1.01</td>
<td>0.71</td>
<td>0.37</td>
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<tr>
<td>D16S 146</td>
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<td>0.10</td>
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<td>0.04</td>
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<tr>
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Fig. 2. Partial pedigree of an Algerian family with multiple hereditary trichoepithelioma. Genotypes observed in DNA from blood lymphocytes and individual tumors are indicated for microsatellite markers on chromosomes 9 and 16; , tumors excised from the respective patients. In tumors showing loss of heterozygosity (III1, Tum1; III5, Tum1; II6, Tum1; and II8, Tum1), the missing alleles are indicated by a dash. The haplotype segregating with the phenotype multiple familial trichoepithelioma is marked with a vertical line. The individuals marked with a horizontal line above the symbol have been examined physically.

Fig. 3. Diagram showing the results of multipoint linkage analysis (18) for the phenotype familial multiple trichoepithelioma and microsatellite markers on chromosomes 16 listed in Table 1. Markers D16S753 to D16S673 encompassing the CYLD gene show limit of detection scores above 3.0.
genomic DNA from lymphocytes of one affected individual and screened for mutations by sequence analysis. The sequence of the exon 15 PCR product revealed an insertion c.2104_2105 ins A, resulting in a premature chain termination T02fSx723 (Fig. 1C). An abnormally migrating band was observed in single-strand conformational analysis with the product of exon 15 in all of the affected individuals but not in unaffected family members (data not shown). Cloning of the deviating band of exon 15 followed by sequence analysis of several clones confirmed the presence of the insertion mutation. Control single-strand conformational analysis of this CYLD exon in 100 chromosomes 16 from healthy, unrelated blood donors excluded that the observed electrophoretic pattern was because of a DNA polymorphism.

LOH or a Somatic Point Mutation Affecting CYLD Cause a Second Hit in Individual Tumors. To search for an inactivating mutation in the second allele in individual tumors, 9 TEs excised from 6 affected family members were screened for LOH of polymorphic markers CDRP28, CDRP23, and D16S416 from the chromosome interval encompassing CYLD (8) by comparing the amounts of allelic products amplified from blood or tumor tissue. LOH in the haplotype that did not contain the disease-associated germ-line mutation was observed in 6 tumors. Thealleles missing in these tumors (III1, Tum1; IIIS, Tum1 and Tum2; IV6, Tum1 and Tum2; IV8, Tum1) are indicated in Fig. 2 by dashes. The entire coding sequence of CYLD from the three remaining tumors was subjected to sequence analysis. In one of the samples that had not shown LOH, an intragenic somatic mutation (c.2541G>A) was detected, resulting in a stop codon (W847X; Fig. 1D).

The PTCH1 Locus on Chromosome 9q22.3 Is Not Affected by LOH. Microsatellite markers flanking the PTCH1 locus on chromosome 9q22.3 were analyzed for LOH in tumor samples and in the blood of the individuals from which the tumors had been excised. No indication for LOH at this location was detected (data not shown).

DISCUSSION

On the basis of ample evidence from clinical reports and histopathological studies, Chalstrey (19) postulated that the inherited, multiple, and benign tumors of the epithelial appendages, cylindromas, and TEs might be manifestations of mutations in the same gene. Cylindromas (3, 20, 21) are tumors with cords of basophilic cells surrounded by a hyaline membrane formed by polysaccharide secretory cells (22). They show differentiation toward sweat glands, whereas TEs (2, 23, 24) originate from the basal cell layer of the epidermis and from hair follicles and contain structures reminiscent of hair formation (25, 26). Numerous reports of coexistence of both types of tumors in the same family and even in the same individual were published (27). There are too many well-documented kindreds in which subjects were affected with both types of tumors to suggest the possibility of a chance association of two separate autosomal mutations in each case (1, 13, 28, 29). Moreover, syringomas that show differentiation toward sweat ducts may coexist with TEs (30). Therefore, multiple familial cylindroma as described by Spiegler (3) and TE as described (under the name “epithelioma adenoïdes cysticum”) by Brooke (2) have been lumped into one single entity, Spiegler-Brooke syndrome (MIM 605041).

On the other hand, a definite clinical and histopathological dichotomy between families with cylindromatosis (MIM 132700) and those with trichoepitheliomatosis (MIM 601606) has been well established and is supported at the genetic level because the two phenotypes have been assigned to two different loci on 9p21 (trichoepitheliomatosis) and 16q12-q13 (cylindromatosis; Ref. 6). However, in the present family with a classical TE phenotype, we have excluded linkage to 9p and demonstrated mutations in CYLD, the gene affected in familial cylindromatosis as well as in Spiegler-Brooke syndrome (8, 11). We conclude that there may be two forms of MFT, one that is linked to 9p, and another one that is linked to 16q12-q13 (MIM 601606), corroborating recent observations from Chinese families (9, 10). Remarkably, Matt et al. (15) who analyzed sporadic TE with polymorphic markers from 9p21 were unable to document LOH at this locus.

The clinical and molecular findings present in this family show that mutations in the CYLD gene may cause the classical phenotype of multiple TE without any clinical or histopathological overlapping with cylindroma. This finding may be explained either by allelic heterogeneity at the CYLD locus or by the action of a modifying gene. A modifier gene restricting the phenotype to TE would have to be closely linked to CYLD, because otherwise segregation with occurrence of cylindroma in this family would be expected. Alternatively, specific alleles of a modifier might determine the phenotype of cylindroma or even spiradenoma associated with TE. These alleles might not occur in the Algerian population sample from which this family originates.

A failure in the negative feedback mechanism in the SHH-PTCH signaling pathway may be an early and necessary event in skin tumorigenesis (31), possibly involving up-regulation of GLI1 gene expression (32–35). For adnexal skin tumors such as TE, a gatekeeper function may be associated with the functioning of PTCH1, a receptor for hedgehog proteins, which is mutated in BCCs (16). Malignant changes (BCC) have been noted occasionally in cylindromas (36) and TEs (14, 37). Experimentally induced BCC in mice tend to be associated with other hair follicle neoplasias such as trichoblastoma, TE, and cylindroma (32). Conversely, patients with Gorlin syndrome (MIM 109400) caused by mutations in the PTCH1 gene may have, in addition to BCCs, some skin lesions showing more benign structures in the form of TE (38, 39). Sporadic TEs are associated with deletions at 9q22.3, the site where PTCH1 maps (15), or they contain somatic mutations in the overexpressed PTCH1 gene (16).

We could not detect LOH at the PTCH1 locus in TEs from this Algerian family. Analogously, no PTCH mutations were detected by Vorechovsky et al. (16) in two patients with the familial form of multiple TE. It would be worthwhile to extend the LOH analysis at the PTCH1 locus to test whether a reduction in the amount of this receptor protein on top of a deficiency of CYLD might result in cylindromas or spiradenomas.

CYLD has been identified recently as a negative regulator of the transcription factor nuclear factor kB involved, among other tasks, in protection against apoptosis (40–43). Skin appendages require nuclear factor kB activity for their development (44, 45). Impairment of apoptosis resulting from mutations in CYLD might deregulate proliferation in the developing skin appendages in familial cylindromatosis as well as trichoepitheliomatosis (40). Other, functionally related proteins with ubiquinating activity might inhibit nuclear factor kB signaling in a similar manner (43, 46). A genetic defect in one of them functioning in hair follicles in parallel with CYLD might similarly shift the balance between proliferation and apoptosis toward proliferation, resulting in tumor growth. This would explain the genetic heterogeneity of the multiple TE phenotype.

Brummelkamp et al. (40) found recently that enhanced protection from apoptosis resulting from defective CYLD can be reversed by simple pharmacological agents such as sodium salicylate and prostaglandin A1. This observation raises hope for a therapy restoring normal growth control in patients with familial TE as well as other adnexal tumors.
ACKNOWLEDGMENTS

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