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The Role of *MMAC1* Mutations in Early-Onset Breast Cancer: Causative in Association with Cowden Syndrome and Excluded in *BRCA1*-Negative Cases

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Summary

Cowden syndrome (CS) is an autosomal dominant disorder associated with the development of hamartomas and benign tumors in a variety of tissues, including the skin, thyroid, breast, endometrium, and brain. It has been suggested that women with CS are at increased risk for breast cancer. A locus for CS was recently defined on chromosome 10 in 12 families, resulting in the identification of the CS critical interval, between the markers *D10S215* and *D10S541*. More recently, affected individuals in four families with CS have been shown to have germ-line mutations in a gene known as "*PTEN*," or "*MMAC1*," which is located in the CS critical interval on chromosome 10. In this study, we report three novel *MMAC1* mutations in CS and demonstrate that *MMAC1* mutations are associated with CS and breast cancer. Furthermore, we also show that certain families and individuals with CS do not have mutations in the coding sequence of *MMAC1*. Finally, we did not detect *MMAC1* mutations in a subpopulation of individuals with early-onset breast cancer, suggesting that germ-line mutations in this gene do not appear to be common in this group.

Introduction

Cowden syndrome (CS) (Lloyd and Dennis 1963), or multiple hamartoma syndrome (Weary et al. 1972), is an autosomal dominant disorder associated with the development of hamartomas and benign tumors in a variety of tissues, including the skin, thyroid, breast, endometrium, and brain. It has been suggested that women with CS are at increased risk for breast cancer (Brownstein et al. 1978), and, as in other susceptibility syndromes, they appear to develop breast cancer at an early age (Schrager et al., in press). CS is also associated with a specific skin lesion, trichilemmoma (tumor of the follicular infundibulum), and thus this breast cancer–susceptibility syndrome can be recognized by the presence of a cutaneous biomarker (Brownstein et al. 1977, 1978). We have studied in detail the clinical and pathological findings (Schrager et al., in press) in this syndrome and have demonstrated that the mean age at presentation with malignant breast disease in CS is 46 years, with the age range at presentation with breast cancer in affected women being 33–74 years (Schrager et al., in press). Moreover, very few of the women with CS whom we studied had a family history of breast cancer (Schrager et al., in press). Of interest is that men with CS appear not to be at increased risk for the development of breast cancer (Brownstein et al. 1978; Schrager et al., in press). We have also shown that women with CS develop exuberant benign breast disease and frequently report a history of multiple breast biopsies prior to the development of breast cancer. The history of skin disease and benign breast disease can therefore allow identification of affected individuals prior to the development of breast cancer in this high-risk population.

It has been demonstrated in a previous study that a locus for CS exists on chromosome 10 (Nelen et al. 1996). In that study, a total of 12 families were examined, resulting in the identification of the CS critical interval as being between markers *D10S215* and *D10S564*. Certain affected individuals in these families had CS and Lhermette-Duclos disease (LDD) (Nelen et al. 1996; Liaw et al. 1997), a rare brain disorder characterized by a dysplastic gangliocytoma of the cerebel-

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lum (Albrecht et al. 1992). Fine mapping of this area refined this initial result (Liaw et al. 1997), supporting a location, for the CS gene, between markers *D10S215* and *D10S215*. More recently, affected individuals in four families with CS have been shown to have germline mutations (Liaw et al. 1997) in a gene known as "*PTEN*" (Li et al. 1997), "*MMAC1*" (Steck et al. 1997), or "*TEP1*" (Li et al. 1997), which is located in the CS critical interval on chromosome 10. Of interest is that the predicted *MMAC1* protein contains sequence motifs with significant homology to the catalytic domain of protein phosphatases and to the cytoskeletal proteins, tensin and auxillin (Li et al. 1997; Steck et al. 1997). Moreover, coding-region mutations in *MMAC1* have been observed in human tumors or tumor cell lines of the breast, brain, prostate, and kidney (Li et al. 1997; Steck et al. 1997). Although the function of this gene is unknown, it is likely that *MMAC1* plays a role in the control of cell proliferation and that its loss of function is important in the development of human tumors.

Material and Methods

Clinical Material

Approval for this study was obtained by the Investigation Review Board of Columbia Presbyterian Medical Center. Blood samples were obtained after informed consent was obtained from individuals with CS. An aliquot was used for DNA extraction, while peripheral-blood mononuclear cells were purified from a second sample and were used to generate an Epstein-Barr virus–transformed lymphoblastoid cell line. The diagnosis of CS was made on the basis of the International Cowden's Consortium CS diagnostic criteria (Nelen et al. 1996). For individuals with early-onset breast cancer, the sample consists of 63 women who developed breast cancer at age <35 years (average age at diagnosis 27.7 years), who did not have a clinical diagnosis of CS, and who had previously been shown not to carry clearly deleterious mutations in *BRCA1* (5 women in the sample carried missense polymorphisms of unknown significance) (Shattuck-Eidens et al. 1997). These women are a subset of a sample of 798 unrelated individuals from 20 collaborating institutions, chosen from families that were generally at an elevated risk of carrying *BRCA1* mutations. Most families were chosen because of multiple cases of breast cancer, early age at diagnosis of breast cancer, and incidence of ovarian cancer, since these conditions have been previously shown to be associated with germ-line mutations of *BRCA1*. Some of the families were extended to second-degree relatives. All samples from institutions in the United States were collected from individuals participating in research studies on the genetics of breast cancer. Each individual read and signed informed-consent documents approved by the local institutional review board. All samples from institutions outside the United States were collected according to the appropriate guidelines, concerning research involving human subjects, imposed by the institution's equivalent authorities. Only one representative from each family was included in the sample, and no families in which genetic markers showed linkage to *BRCA1* were included. All the samples used in the *MMAC1* study were stripped of identifiers. This is a heterogeneous sample that represents the diversity among patients who present at high-risk clinics, as opposed to the more controlled sampling done for family or population studies. This has directed our analyses toward methods that do not require that sample frequencies of subgroups reflect frequencies in the general population. Therefore we can assess, for example, the probability that a woman with breast cancer diagnosed at age 30 years carries a deleterious *BRCA1* or *MMAC1* mutation, but we cannot estimate the frequency of such women in the general population.

DNA Extraction

After informed consent was obtained, patients' genomic DNA was extracted from whole blood or lymphoblastoid cell lines by use of the QIAamp Blood Maxi Kit. Concentration was measured by optical density 260 (OD_{260}), and purity was checked by the ratio OD_{260}: OD_{280}.

Genotyping

Primer pairs for the chromosome 10 locus were obtained from Research Genetics. The forward-strand primer was end-labeled in the presence of ^{33}P-γATP and polynucleotide kinase. PCR reactions were performed in a total reaction volume of 30 μl. The reactions consisted of 10 mM each primer, 200 mM deoxynucleotides, 1.5 units of *Taq* DNA polymerase, and 50 ng of genomic DNA. PCR was performed for 35 cycles, with 45 s denaturation at 94°C, 45 s annealing at 55°C, and 1 min elongation at 72°C; a final 10-min elongation was used. PCR reactions were stopped by addition of 20 μl of stop solution (95% formamide, 1 mM EDTA, 0.25% bromophenol blue, and 0.25% xylene cyanol). Then reactions were denatured for 5 min at 94°C, and the products were separated on an 8% denaturing polyacrylamide gel. Allele sizes were determined by comparison to SequaMark (Research Genetics), which was included as a size standard on the gels.

Linkage Analysis

Two-point linkage analysis was performed by use of MLINK. The status of individuals of age <20 years was considered as unknown. Disease-gene frequency was set equal to .000001, and marker-allele frequencies were

Table 1

Two-Point Analysis of CS Families with CA-Repeat Markers

Family and Marker	LOD Score for Recombination Fraction of						
	.0	.01	.05	.1	.2	.3	.4
A:							
D10S579	.00	.00	.00	.00	.00	.00	.00
D10S215	.30	.30	.28	.26	.20	.15	.08
D10S541	.00	.00	.00	.00	.00	.00	.00
D10S1739	.30	.30	.28	.26	.20	.15	.08
D10S564	.30	.30	.28	.26	.20	.15	.08
B:							
D10S579	.00	.00	.00	.00	.00	.00	.00
D10S215	.30	.29	.26	.21	.13	.06	.02
D10S541	.00	.00	.00	.00	.00	.00	.00
D10S1739	.30	.29	.26	.21	.13	.06	.02
D10S564	.30	.29	.26	.21	.13	.06	.02
C:							
D10S579	.00	.00	.00	.00	.00	.00	.00
D10S215	$-\infty$	−3.40	−2.00	−1.40	−.80	−.44	−.19
D10S541	.00	.00	.00	.00	.00	.00	.00
D10S1739	−.05	−.06	−.09	−.13	−.16	−.15	−.09
D10S564	$-\infty$	−3.40	−2.00	−1.40	−.80	−.44	−.19
D:							
D10S579	$-\infty$	−1.52	−.28	.11	.28	.19	.05
D10S215	$-\infty$	−1.58	−.33	.07	.25	.18	.05
D10S541	$-\infty$	−1.44	−.39	.01	.22	.18	.06
D10S1739	−2.20	−.45	.14	.32	.35	.23	.08
D10S564	−.03	.08	.30	.38	.35	.22	.07

estimated by use of ILINK. Both MLINK and ILINK are from the LINKAGE package, version 5.2 (Lathrop et al. 1984). Reconstruction of the most probable haplotypes in family D was accomplished by use of GENEHUNTER (Kruglyak et al. 1996). Pedigrees were drawn by use of Cyrillic, version 2.02.

Mutation Detection

We performed nested PCR amplifications on genomic DNAs and screened the resulting amplicons for sequence variants, as described elsewhere, with several modifications (Steck et al. 1997). First, exon 6 was screened as a single secondary amplicon amplified by use of the exon 6 FB-RR primer pair. Second, exon 8 was screened as two secondary amplicons by use of the following FB-RQ and FC-RR primers: CA6.HB (5′-GTTTTCCCAGTCACGACGAGGTGACAGATTTTCTTTTTTA-3′) and CA6.HQ (5′-AGGAAACAGCTATGACCATTCGGTTGGCTTTGTCTTTA-3′); and CA6.HC (5′-GTTTTCCCAGTCACGACGCATTTGCAGTATAGAGCGT-3′) and CA6.HR (5′-AGGAAACAGCTATGACCATAGCTGTACTCCTAGAATTA-3′). Third, since mononucleotide runs in certain introns caused poor dye-primer sequencing, we obtained dye-terminator sequence data on secondary amplicons exon8 FB-RQ and exon 9 FB-RR, using the nested primers 5′-TTTTTTTTTAGGACAAAATGTTTC-3′ and 5′-AATTCAGACTTTTGTAATTTGTG-3′. We obtained >95% double-stranded coverage of the *MMAC1* coding sequence, for all genomic DNAs screened; all mutations were confirmed by sequencing of a newly amplified product.

Results

Linkage Analysis and Mutation Screening In CS Kindreds

In order to extend the observations indicating a CS locus on chromosome 10, we performed a two-point linkage analysis using five markers located in the CS critical interval, on four families with clinical evidence of CS (Nelen et al. 1996). All families were examined in detail, and the diagnosis of this syndrome was made on the basis of the International Cowden's Consortium CS diagnostic criteria (Nelen et al. 1996). Two small families displayed positive LOD scores that could not exclude linkage to three loci on chromosome 10 (see data on families A and B; table 1). Two other families, with clinical findings identical to those described above, showed significant negative LOD scores for some of the markers in this region (families C and D; table 1). A heterogeneity test was also performed, which gave nonsignificant results (data not shown). These findings were confirmed by the haplotypes' construction (fig. 1). In particular, in family C, individual 2 transmits to both her affected children the haplotype inherited from her

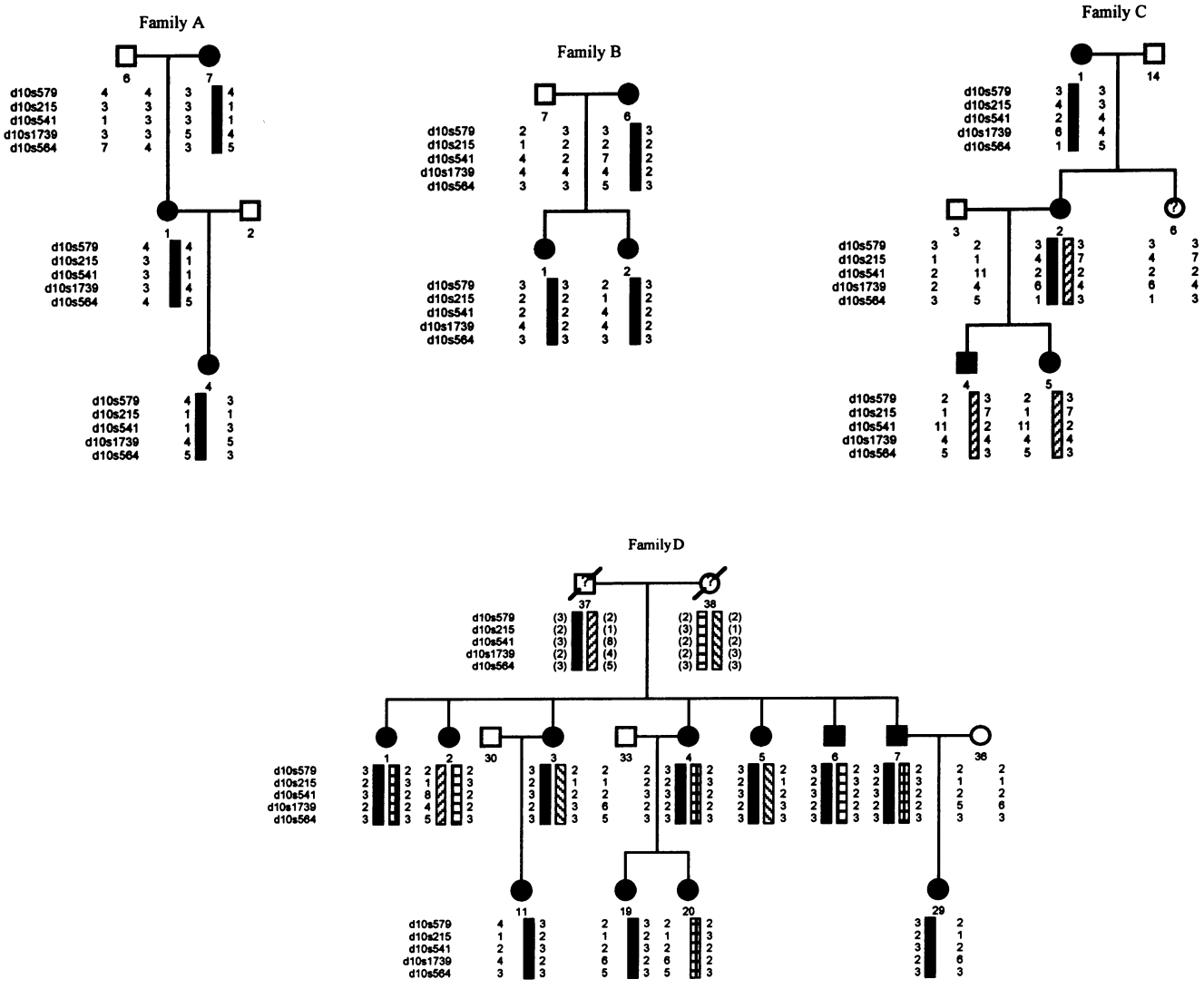

Figure 1 Haplotype construction with markers on chromosome 10 in four families with CS

unaffected father. Finally, in family D, individuals 2 and 20 have inherited a haplotype different from that of their affected relatives.

Using a PCR- and sequencing-based approach, we examined the nine exons and associated splice junctions of *MMAC1*, using the described primers (Steck et al. 1997), in 16 affected individuals from these four families. Of interest is that 4 of these 16 individuals had breast cancer and that 2 of these 4 had breast cancer at age <40 years. We detected no mutations in the coding sequence in these 16 individuals from these four families with the classic symptoms and signs of CS.

Table 2

CS Mutations in Present Study

Mutation	Exon/Intron	Predicted Effect
791insAT	Exon 7	Frameshift
915del13	Exon 8	Frameshift
137ins3	Exon 2	One-amino-acid insertion (Asn)

Mutational Analysis in Individuals with CS

We then screened a set of 31 affected individuals from 23 families with CS whose kindreds had not been used in our linkage studies (see table 2). Of the 31 individuals, 13 were related individuals from five families. Thus, a total of 23 unrelated probands were screened. A single affected female (Walton et al. 1986) demonstrated a frameshift mutation in exon 7 of the coding sequence (see fig. 2). Specifically, we demonstrated an AT insertion

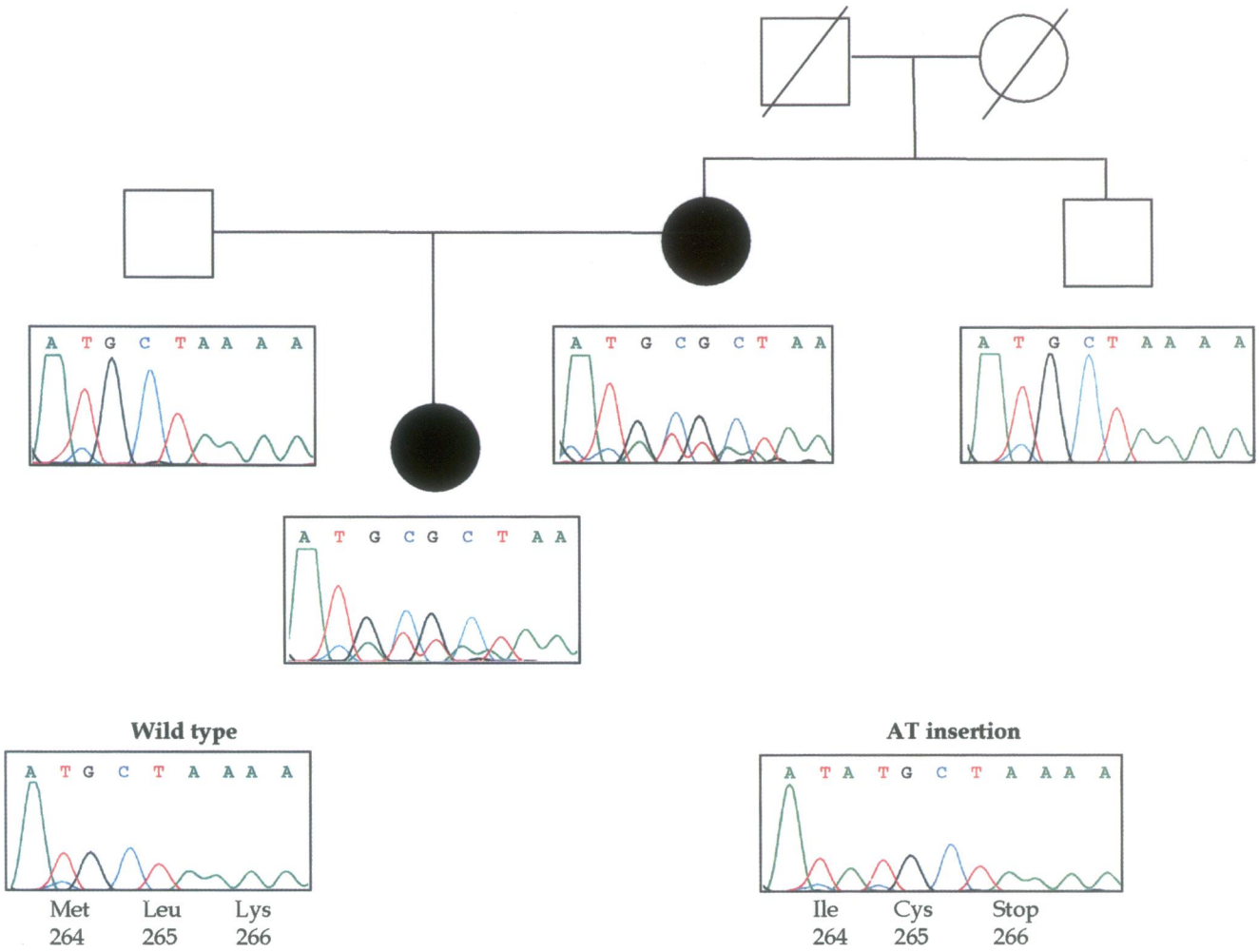

Figure 2 DNA Sequencing of *MMAC1* in a family with CS and early-onset breast cancer. The affected mother (*blackened circle*) demonstrates a 2-bp insertion (AT) in exon 5, which is not seen in her unaffected brother (*unblackened square*). Her affected daughter has inherited the AT insertion.

after nucleotide 791 (791insAT), which resulted in a frameshift and downstream premature termination codon. Of interest is that this woman developed mammogram-negative breast cancer at age 36 years, which was discovered at the time of prophylactic mastectomy (Walton et al. 1986). The proband had an unaffected brother as well as an affected daughter. Direct sequencing of exon 7 in these individuals demonstrated both the presence of the identical mutation in the affected daughter (fig. 2) and the absence of the mutation in the unaffected brother. In studying a second individual with CS and early-onset (age 33 years) breast cancer, we demonstrated a 3-bp insertion in exon 2 (137ins3), resulting in the insertion of a single amino acid (Asn). Finally, in another woman, who had bilateral breast cancer (Schrager et al., in press) and endometrial cancer, we identified a 13-bp frameshift deletion in exon 8 (915del12). These data demonstrate three additional mutant alleles of *MMAC1* that are associated with CS (Liaw et al. 1997) and, in particular, with CS and breast cancer (Brownstein et al. 1978; Schrager et al., in press). However, in 27 individuals from 20 families, we did not detect mutations in the coding sequences of *MMAC1*. In this population, seven of these individuals had breast cancer, although all of these women developed breast cancer at age >40 years. One of these seven individuals had bilateral breast cancer. In total, therefore, combining the family data and the data from these individuals, we detected coding-sequence mutations in 4 individuals from 3 CS families but did not detect coding-sequence alteratons (i.e., missense or silent variants) in 43 other individuals from 24 families with CS.

Mutational Analysis in Women with Early-Onset Breast Cancer

A strong case has been made for the existence of a genetic mechanism regulating breast-tumor formation in early-onset breast cancer (i.e., the development of breast cancer at age <40 years) (Claus et al. 1990). Since CS is inherited in an autosomal dominant fashion, the genetic mechanisms regulating the development of breast cancer in this population may also play a role in the development of early-onset breast cancer. Since we detected germ-line *MMAC1* mutations in CS associated with early-onset breast cancer, and since mutations in this gene occur at relatively high frequency in breast tumors and in breast-tumor cell lines (Li et al. 1997; Steck et al. 1997), we wanted to further investigate the role of germ-line *MMAC1* mutations in early-onset breast cancer. In an effort to bias ourselves toward a sample set potentially enriched in germ-line *MMAC1* mutations, we sequenced the gene in 63 women who had developed breast cancer at age <35 years (average age at diagnosis 27.7 years) and who had previously been shown not to carry clearly deleterious mutations in *BRCA1* (5 women in the sample carried missense polymorphisms of unknown significance). No coding-sequence alterations were detected in the nine exons of *MMAC1* in this sample set. In contrast, using the exact same mutation-detection and analysis criteria on a similarly ascertained set of non-Ashkenazi individuals with breast cancer (without exclusion of *BRCA1* carriers), we would expect to detect seven deleterious mutations and five missense polymorphisms of unknown significance in *BRCA1* (Shattuck-Eidens et al. 1997). Furthermore, other than the four CS patients carrying germ-line mutations in *MMAC1* who have been described above, we detected no sequence polymorphisms in the coding sequence of this gene in >200 germ-line chromosomes, and, in fact, we found only one sequence difference (silent) between the human and chimpanzee sequences. If the frequency of coding and proximal splice-junction sequence variants in *MMAC1* were 5% in the population from which this sample was drawn, then we would have had a 95% chance of detecting one or more such variants.

Discussion

CS is distinct among autosomal dominant genetic syndromes that predispose to the development of breast cancer, since it has a unique cutaneous biomarker, the trichilemmoma (Brownstein et al. 1977, 1978). Furthermore, women with CS frequently give a history of multiple breast biopsies for benign breast disease prior to the development of breast cancer (Schrager et al., in press). Most of these women did not have a family history of breast cancer. To date, the most well-described association of CS with organ-specific cancer susceptibility is that for the female breast (Brownstein et al. 1977; Schrager et al., in press). Other organs that appear to develop cancer with increased frequency in CS include the thyroid and the endometrium. In contrast to other autosomal breast cancer–susceptibility syndromes, such as the one associated with mutations in *BRCA1* (Ford et al. 1995), the development of ovarian cancer in this syndrome is quite rare. However, CS shares with these syndromes an earlier age at onset of breast cancer, as well as an increased likelihood of bilateral breast cancer (Schrager et al., in press).

Previous observations demonstrated linkage of CS to chromosome 10q22-23 (Nelen et al. 1996). Furthermore, it is also now evident that mutations in a gene (Liaw et al. 1997) known as "*PTEN*" (Li et al. 1997), "*MMAC1*" (Steck et al. 1997), or "*TEP1*" (Li and Sun 1997), found in the CS critical interval on chromosome 10, are associated with CS individuals (Liaw et al. 1997). In the observations reported here, we have identified three new germ-line mutations in the coding sequence of *MMAC1* that are associated with CS, specifically in individuals with CS and breast cancer. In two related individuals with CS, we have described a frameshift mutation in exon 7, resulting in a premature termination codon, that is identical in an affected mother and her affected daughter. This *MMAC1* mutation appears to be associated with early-onset breast cancer, since one of the two affected individuals developed breast cancer at age 36 years. In a third affected individual, we identified a 13-bp deletion in exon 8. Although this individual did not develop breast cancer at an early age, she had a history of bilateral breast cancer. Of interest is that she also developed endometrial cancer while on tamoxifen. Given that endometrial cancer has been associated with CS (Starink et al. 1986) and with tamoxifen use (Fornander et al. 1989), the contribution of both risk factors to the development of disease in this one women is unknown. However, this raises the possibility that the subpopulation of women who develop endometrial cancer while on tamoxifen may have CS and/or mutations in *MMAC1*. Finally, we identified a 3-bp insertion in exon 2 in another woman, who developed breast cancer at age 33 years.

In the set of CS individuals that we have studied, we have detected germ-line *MMAC1* mutations in 4 individuals from 3 families but have not observed any coding-sequence alterations in the remaining 43 individuals from 24 unrelated families. These data support our limited linkage information, suggesting that all CS families may not show linkage to the locus identified on chromosome 10. Although the experiments that we performed do not rule out the possibility that either (*a*) mutations in the 5′ regulatory regions or 3′ UTR of *MMAC1* or (*b*) other mechanisms (e.g., methylation si-

lencing) that alter its expression level are associated with CS, both the linkage data and the DNA-sequencing results support the idea that CS may be genetically heterogeneous. Tuberous sclerosis, another autosomal dominant disorder associated with the formation of hamartomas in the skin and other organs, has been shown to be genetically heterogeneous, with distinct loci located at chromosomes 9q34 (Haines et al. 1991) and 16p13.3 (Kandt et al. 1992). Our results suggest that this may also be true for CS. Why this was not demonstrated in the initial observations is not clear, but it could be due to the ethnic backgrounds of the initial families examined (Nelen et al. 1996; Liaw et al. 1997). Moreover, certain of these individuals presented with CS and LLD, which we have yet to identify in a CS proband or in a CS family (Nelen et al. 1996; Liaw et al. 1997).

A strong case has been made for the existence of a genetic mechanism regulating breast-tumor formation in early-onset breast cancer (Claus et al. 1990). Indeed, early-onset breast cancer has been associated with mutations in *BRCA1* (Miki et al. 1994) and *BRCA2* (Wooster et al. 1995). CS is associated with early-onset breast cancer, and the cancer is usually ductal carcinoma (Brownstein et al. 1977, 1978). Rachel Cowden, for whom the syndrome is named, apparently died of breast cancer at age 31 years (Lloyd and Dennis 1963; Brownstein et al. 1978). Herein we have identified *MMAC1* mutations in two CS individuals with early-onset breast cancer, as well as in one CS individual with bilateral breast cancer. However, when we searched for germ-line *MMAC1* mutations in a subgroup of women with early-onset breast cancer who lacked signs of CS and who previously had been shown to have wild-type sequences of *BRCA1*, we failed to detect any sequence variants. These data suggest that germ-line mutations in *MMAC1* occur infrequently, at least in this subpopulation of early-onset breast cancer cases.

In summary, we have extended the observation that *MMAC1* mutations are associated with CS (Liaw et al. 1997), and we have demonstrated that *MMAC1* mutations appear to be associated with CS and breast cancer. However, we also have shown that certain families and individuals with CS do not have mutations in the coding sequence of *MMAC1*. Finally, we have failed to detect *MMAC1* mutations in a subpopulation of individuals with early-onset breast cancer, which suggests that germ-line mutations in this gene do not appear to be common, at least in a subpopulation of breast cancer cases who also do not demonstrate mutations in *BRCA1*.

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