ARTICLE

Mutations in *SLC34A2* Cause Pulmonary Alveolar Microlithiasis and Are Possibly Associated with Testicular Microlithiasis

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Pulmonary alveolar microlithiasis (PAM) is a rare disease characterized by the deposition of calcium phosphate microliths throughout the lungs. We first identified a PAM locus by homozygosity mapping to 4p15, then identified, by a candidate-gene approach, the gene responsible for the disease as *SLC34A2* (the type IIb sodium-phosphate cotransporter gene), which is involved in phosphate homeostasis in several organs. We identified six homozygous exonic mutations in the seven unrelated patients with PAM we studied. Three of the mutations were frameshifts, one was a chain termination, one was an amino acid substitution, and one was a deletion spanning the minimal promoter and the first exon. Absence of functional protein product of the gene is compatible with calcium phosphate deposition in alveolar airspaces. We show that impaired activity of the phosphate transporter is presumably responsible for the microliths and that PAM is a recessive monogenic disease with full penetrance. Testicular microlithiasis (TM) is a disease that is more common than PAM. It is often associated with cancer and infertility. Since the gene we identified is also expressed in testis, we searched for mutations in subjects with TM. In 2 of the 15 subjects with TM we studied, we identified two rare variants, one synonymous and the other noncoding, that are possibly associated with the condition.

Pulmonary alveolar microlithiasis (PAM [MIM 265100]) is a rare disease characterized by the deposition of calcium phosphate microliths throughout the lungs. Most patients are asymptomatic for several years or even for decades, and, generally, the diagnosis is incidental to clinical investigations unrelated to PAM. Cases with early onset or rapid progression are rare. A "sandstorm-appearing" chest roentgenogram is a typical diagnostic finding. The onset of this potentially lethal disease varies from the neonatal period to old age, and the disease follows a long-term, progressive course, resulting in a slow deterioration of lung functions. A variety of environmental factors have been suggested as the etiology, and about one-third of the reported cases are familial (reviewed by Ucan et al.,[1] Castellana et al.,[2] and Mariotta et al.[3]). A report on six related, affected individuals was the best support for autosomal recessive inheritance of the disease.[4] Testicular microlithiasis (TM), in contrast, is not rare; it has a prevalence of 0.6%–9% in the population.[5] It was found to be associated with the majority of primary testicular malignancies[6] and with ~1% of male idiopathic infertility cases.[7] We identified the gene responsible for PAM by positional cloning and subsequent candidate-gene approach. Here, we describe (1) the localization of the gene in the family with six affected individuals mentioned above and (2) the screening of *SLC34A2* (the type IIb sodium-phosphate cotransporter gene) for mutations in patients with PAM and subjects with TM.

Material and Methods

Subjects

A large consanguineous family[4] was used for linkage analysis. A total of 7 unrelated patients with PAM and 15 men with diffuse bilateral TM were included in the *SLC34A2* mutation screening. Informed written consent was obtained from all subjects or their parents. The study was approved by the Committee on Research with Human Participants at Boğaziçi University.

Linkage Analysis

A genome scan of three brothers with PAM in family 1 (individuals 501, 503, and 504 in the pedigree shown in fig. 1) was performed using the CHLC/Weber Human screening set version 8a. The set contained 156 polymorphic microsatellite markers that spanned the human autosomes with an average density of 1 per 25 cM. The positions of the markers were retrieved from the STS map of GenBank (NCBI Map Viewer and UCSC Genome Browser). Those loci exhibiting shared homozygosity were further analyzed with more densely spaced markers in the family members available for study. Marker alleles were resolved on 8% denaturing polyacrylamide gels and were visualized by staining with silver nitrate.[8] Linkage analysis was performed under the assumption of autosomal recessive inheritance, full penetrance, a disease gene frequency of 1 in 100,000, consanguinity, equal recombination frequencies in both sexes, and equal frequencies of marker alleles. PedCheck version 1.1 was used to detect any Mendelian or genotyping errors in the linkage data.[9] SimWalk2 version 2.91 was used for the calculation of multipoint LOD scores and for the construction of haplotypes, with allowance for the minimum number of recombination events.[10]

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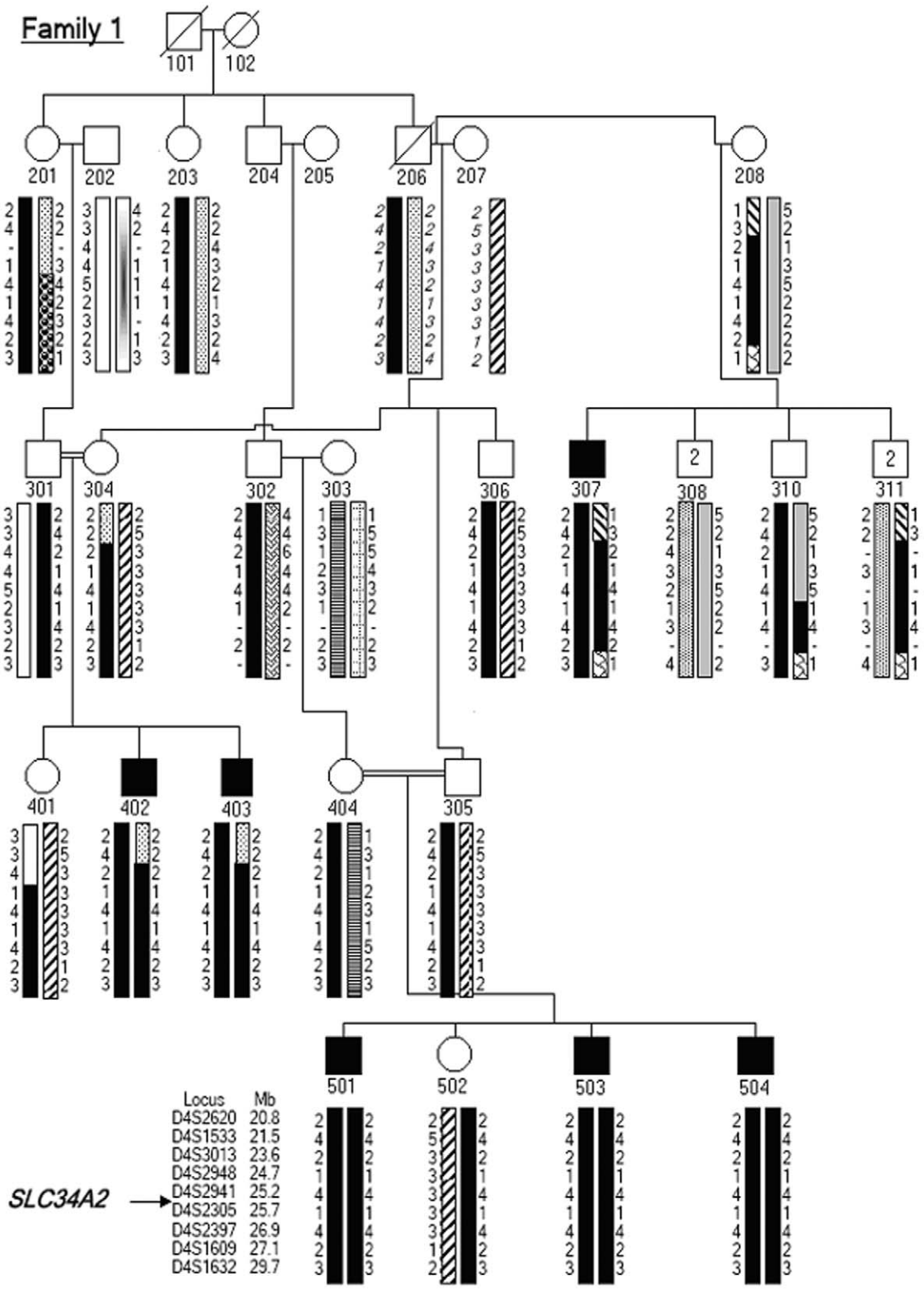

Figure 1. Partial pedigree diagram and haplotype analysis at 4p15.31-p15.1 for family 1. Haplotypes are shown by differently shaded bars. Deduced alleles are shown in italics.

Mutation Analysis

All 12 coding exons of *SLC34A2* and the flanking intronic sequences were amplified with intronic primers (table 1) designed using Primer3 software. Exon 13 was amplified as three overlapping fragments: 13a, 13b, and 13c. Each fragment was amplified in a 25-μl volume containing 125 ng of genomic DNA, PCR buffer with 2 mM $MgCl_2$, deoxynucleoside triphosphates (125 mM of each), *Taq* DNA polymerase (0.8 U/reaction), and 0.4 mM of the primer pair under the following cycle conditions: an initial denaturation step at 95°C for 5 min; followed by 35 cycles for 30 s at 94°C, 30 s of annealing at the appropriate temperature (given in table 1), and 2 min of elongation at 72°C; and a final extension step for 10 min at 72°C. The noncoding exon 1 was amplified for the family's patients and parents only. Because of the GC-rich nature of the region, a GC-Rich PCR System (Roche) containing 0.5-M GC-rich resolution buffer and GC-rich enzyme mix (2 U/reaction) were used. The 462-bp product was amplified using the 522-bp product as a template (table 1). PCR amplification for the 522-bp product was performed using the protocol stated by the manufacturer, whereas the cycling conditions for the 462-bp product were as follows: an initial denaturation step at 95°C for 5 min; followed by 35 cycles for 30 s at 94°C, 30 s of annealing at 55°C for 5 cycles and 52°C for the remaining 30 cycles, and 50 s of elongation at 72°C; and a final extension step for 10 min at 72°C.

The amplified fragments were subjected to SSCP, and any fragment exhibiting an aberrant pattern was further investigated with sequence analysis. SSCP analysis was performed on 8% polyacrylamide gels with crosslinking ratios of 2%, both with glycerol (10%) and without glycerol. Sequencing was performed with an ABI 310 analyzer (Applied Biosystems) at the Department of Molecular Biology and Genetics, Boğaziçi University.

Table 1. Primers for the Amplification of *SLC34A2* Exons

The table is available in its entirety in the online edition of *The American Journal of Human Genetics*.

Results

Linkage Analysis

The genome scan of the three affected brothers in the large consanguineous family and the subsequent genotyping at homozygous loci with additional markers in all family members available for study pointed to a single candidate locus at chromosome 4p15. We narrowed the gene locus to a 4.2-Mbp region at 4p15.31-15.2, flanked by markers *D4S1533* and *D4S2305*. The haplotype data are given in figure 1. LOD scores were calculated to assess the significance of the results. The multipoint LOD score peaked to 6.0 between *D4S3013* and *D4S2305* (fig. 2).

Mutation Analysis

The gene locus contained 16 genes, according to National Center for Biotechnology Information (NCBI) build 35.1. *SLC34A2* stood out as the likely disease gene, since it was a phosphate transporter (reviewed by Murer et al.[11]) expressed strongly in lung.[12,13] We analyzed all 12 coding exons in the patients with PAM by SSCP and performed subsequent DNA sequence analysis for samples displaying aberrant patterns. A total of five homozygous mutations in the six unrelated patients were identified (table 2), but no mutation was detected in the family. All mutations were predicted to result in loss of function of the protein

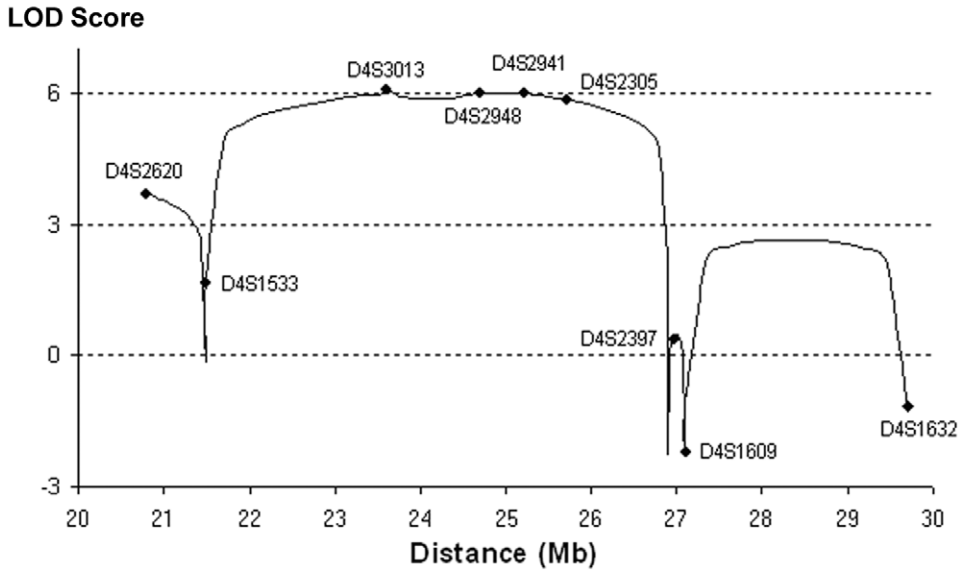

Figure 2. Multipoint linkage analysis at 4p15.31-p15.1 for family 1. The microsatellite markers used in this study are plotted on the graph.

Table 2. Novel *SLC34A2* Sequence Variants Identified in Patients with PAM, Subjects with TM, and a Normal Control

Phenotype and Subject(s)	Mutation	Location	Effect on Translation	Predicted Consequence on Protein
PAM:				
1	c.[−6773_−6588del]+[−6773_−6588del]	Promoter exon 1	...	Not synthesized
3 and 5	c.[114delA]+[114delA]	Exon 3	Frameshift	Truncation
7	c.[226C→T]+ [226C→T]	Exon 3	p.Q76X	Truncation
6	c.[316G→C]+[316G→C]	Exon 4	p.G106R	Substitution
2	c.[1328delT]+[1328delT]	Exon 11	Frameshift	Truncation
4	c.[1342delG]+[1342delG]	Exon 12	p.V448X	Truncation
TM:				
4	c.[T552C]+[-]	Exon 6	p.I184I	Aberrant splicing?
6	c.[*27G→T]+[-]	3′ UTR	Unknown	Unknown
Normal:				
30	c.[989C→T]+[-]	Exon 9	p.T330M	Substitution

product of the gene. Mutations c.114delA and c.1328delT resulted in the shift of the translational reading frame and the truncation of the protein 7 and 5 codons downstream, respectively, whereas c.1342delG led to immediate truncation. c.226→T (p.Q76X) created a premature termination codon. The remaining mutation was c.316G→C, which substituted an arginine for glycine—a basic residue for a polar, uncharged one. The glycine residue is conserved across species (chimpanzee, mouse, dog, and chicken). While analyzing the normal controls, we identified the novel variant c.989C→T (p.T330M) in a single chromosome in an unaffected female. Threonine is replaced with methionine (substituting a polar residue with a nonpolar one), most likely inactivating the protein. The residue is conserved in chimpanzee and mouse but is substituted with serine in dog and chicken.

Having failed to identify the putative mutation in the family despite sequence analysis of the coding exons, we analyzed exon 1 and the flanking sequences by direct sequence analysis of a seminested PCR product. A deletion of 186 nt was identified. The sequences flanked by copies of hexamer GGCAGG, together with one of the copies, had been lost. The deletion spanned the 40-bp noncoding exon and the minimal promoter.[14]

Moderate expression of *SLC34A2* in testis[12,13] prompted us to search for mutations in men with diffuse bilateral TM. We identified two rare variants in the heterozygous state in 2 of the 15 subjects with TM studied (table 2). The first variant was c.552T→C, a synonymous T→C conversion in exon 5. The nucleotide residue is conserved in chimpanzee but, intriguingly, is a C in mouse, dog, and chicken. The second variant was noncoding: c.*27G→T, a G→T transversion 27 nt downstream of the termination codon. The residue is conserved in chimpanzee; however, the sequences downstream of termination are not conserved at all among other species. Although the second variant was not found in any other individual studied, the first was carried by two individuals in the normal group.

None of the mutations or rare variants we identified had been reported previously. All identified variants that were not predicted to truncate the protein product (i.e., c.316G→C, c.989C→T, c.552T→C, and c.*27G→T) were screened in 7 unrelated patients with PAM, 15 subjects with TM, and a control group of 105–123 individuals, to achieve at least 80% power to distinguish a normal sequence variant.[15] This population control group comprised anonymized, unrelated individuals randomly chosen from our Turkish DNA collection.

Discussion

We localized the gene responsible for PAM in a large family and identified homozygous mutations in *SLC34A2* in all patients studied. Six mutations presumably affected the protein product, whereas the remaining mutation abolished gene expression. That mutation was a 186-bp deletion that possibly resulted from an ancestral unequal crossover at two copies of a hexamer. There are four copies of the hexamer within a 424-bp region around the exon, all with the same orientation, but no function has been proposed for it.

In contrast with the predicted severe effects of the identified mutations on the protein product of the gene or on the expression of the gene, the patients with PAM had mild clinical phenotypes, with the exception of the smokers (table 3). The lack of a genotype-phenotype correlation was supported also by the variation in age at onset among the affected members of the large family. The general clinical course of PAM seems to be that microliths begin forming early in childhood, but clinical symptoms arise much later, and lung deterioration is very slow in nonsmokers.

SLC34A2 has 13 exons, the first one noncoding, and encodes a 2,280-nt mRNA and a 690-aa protein. It is a member of the solute carrier family SLC34A that plays a major role in the homeostasis of inorganic phosphate. The gene is expressed most strongly in fetal and adult lung; therefore, it has been suggested that the gene has an important physiological function in lung. It was shown to be expressed in lung only in alveolar type II cells, which

Table 3. Clinical Features of Patients with PAM

Family and Patient	Sex	Age (years) at Appearance of Symptoms	Age (years) at Last Visit	Clinical Course	Comment(s)
Family 1:					
307, or 1	M	25	34	Severe	Heavy smoker
403	M	7	17	Moderate	Smoker; progressive roentgenographic changes
402	M	3	13	Slow	...
501	M	...	17	Asymptomatic	Received diagnosis at age 11 years[4]
503	M	...	15	Asymptomatic	Received diagnosis at age 9 years[4]
504	M	...	11	Asymptomatic	Received diagnosis at age 5 years[4]
Family 2:					
2	M	21	24[a]	Slow	Died of lung infection
Family 3:					
3	F	...	38	Asymptomatic	PAM diagnosed incidentally
Family 4:					
4	M	26	39	Slow	...
Family 5:					
5	F	9	17	Improving	Receiving treatment with disodium ethidronate[16]
Family 6:					
6	F	5	22	Stable	Receiving treatment with disodium ethidronate[16]
Family 7:					
7	F	29	35	Slow	...

[a] Deceased.

are responsible for surfactant production.[17,18] This finding led to the proposal that the function of the gene protein was to uptake liberated phosphate from the alveolar fluid for surfactant production, the major components of which are phospholipids. This hypothesis is in line with the observation that the microliths are located in alveolar airspaces.[19] In addition, calcifications along interlobular septa, bronchovascular bundles, and pleura were observed.[19] The finding that mutations in the gene are responsible for the disease in all our patients with PAM suggests that phosphate uptake in lung is performed mainly by this gene's protein product.

In light of our findings, it is certain that PAM is a recessively inherited disease and is not caused by environmental factors. It has full penetrance, since none of the unaffected members of the large family was homozygous for the disease haplotype. Genetic heterogeneity is not likely for this disease, since we identified mutations in all seven unrelated patients studied. The highest incidence of the disease has been reported in Turkey.[1] We did not find a common mutation among our patients. Only the mutations in patients 3 and 5 were the same, and those patients originated from different parts of the country. Thus, it is more likely that the mutation is recurrent rather than identical by descent. We propose that the high incidence of the disease in Turkey is due to the high proportion of consanguineous marriages. Indeed, all patients carried homozygous mutations, in accordance with identity by descent.

Moderate expression of *SLC34A2* in testis[12,13] prompted us to search for mutations in men with diffuse bilateral TM, to investigate any role of the gene in the etiology of the condition. The two rare variants we identified could not be assigned as mutations as readily as could those found in patients with PAM. Further studies are needed to determine whether they have any effect on the expression of the gene or on its protein product. Both variants were associated with infertility, but only c.*27G→T was associated with a tumor. Variant c.552T→C created a hexamer that has been proposed to be an exonic splicing enhancer that plays an important role in constitutive or alternative splicing.[20,21] Alternative splicing would decrease the number of full transcripts in the cell and, subsequently, decrease the activity of the gene. The other TM variant (c.*27G→T) was noncoding, located downstream of the translational termination codon. The residue is conserved in chimpanzee; however, the sequences downstream of termination have not been conserved at all among other species. We investigated whether the variant had an effect on the conformation of the mRNA, using the program RNA2 (GeneBee Molecular Biology Server), but we did not obtain a strongly supportive result. Therefore, we are unable to propose any possible effect of the variant on mRNA stability. In addition, the fact that testis has a lower temperature than the body temperature raises the question of whether the altered forms of the mRNA and the protein would have less effective conformations at the lower temperature. Whether the variants contribute in any way to susceptibility to TM, a common condition, needs to be investigated. We also mention that none of our seven male patients with PAM had positive findings when investigated for TM.

So far, calcium ions have been blamed for the pathogenesis. Now, it is clear that microlith formation is the result of phosphate-chelating calcium in the extracellular fluid. Microliths from both patients with PAM and subjects with TM had been found to be composed of calcium and phosphate.[22,23] The rare variants carried by two of our sub-

jects with TM indicated that *SLC34A2* could be responsible for the condition, at least in those subjects. It should be noted that the efficiency of SSCP is not 100%; thus, probable variants in other subjects with TM might have escaped detection. Also, variants could possibly be located in those regions of the gene we did not analyze. Thus, >2/15 of the subjects could possibly be carrying variants. The prevalence of TM in the Turkish population is reported as 2.4% in 17–42-year-old healthy men, which is within the range reported for other populations.[24]

This is the first report of a pathological role of *SLC34A2*. Although defects in the gene lead to calcium phosphate deposits, defects in the other members of the gene family—namely, *SLC34A1* and *SLC34A3*—result in hypophosphatemia, because those genes are responsible for phosphate reabsorption in kidney.[25,26] Interestingly, *SLC34A2* is also expressed in kidney. We were able to investigate phosphate uptake in one of our patients (patient 5). The observed value of 92% maximal renal tubular reabsorption was within the normal range. Thus, all our observations together indicate that *SLC34A2* does not play a role in renal reabsorption as important as the role of its paralogs. Also, serum phosphate levels were normal in all 9 of our 12 patients with PAM, and none of the 10 patients investigated had calcifications in the kidneys.

The identification of the gene responsible for calcium phosphate deposition in lung has broad implications, because the same gene might be responsible also for calcifications in several other tissues. This hypothesis is based on two observations. First, *SLC34A2* is expressed also in kidney, pancreas, prostate, ovary, small intestine, mammary gland, liver, and placenta,[12,13] and idiopatic isolated calcifications of some of these organs are well documented. Second, calcium deposits were reported in additional organs in some patients with PAM: pleura and kidneys,[27] seminal vesicles,[28] urethra,[29] and gall bladder.[29] The possible role of the gene in calcification in various tissues as well as in diseases with clinical manifestations resembling PAM and TM can now be investigated. Particularly interesting would be prostate microlithiasis and mammary calcifications, since malignancies are associated with them. Microcalcifications associated with malignant breast lesions are more commonly calcium phosphate[30] than calcium oxalate. The former was reported to enhance mitogenesis in both mammary epithelial cells and breast cancer cell lines.[31]

The identification of the gene responsible for PAM will facilitate genetic diagnosis of isolated cases. Also, early diagnosis in asymptomatic individuals in affected families would be most conveniently performed by a genetic test. Several therapeutic approaches have been applied with no knowledge of the molecular basis of PAM. Now, there is hope for the development of gene therapy. Since the gene encodes an integral membrane protein, as does the cystic fibrosis transmembrane regulator gene, strategies developed for gene therapy for cystic fibrosis might benefit patients with PAM in the future.

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Web Resources

Accession numbers and URLs for data presented herein are as follows:

GenBank, http://www.ncbi.nlm.nih.gov/Genbank/ (for sequences from human [accession numbers NM_006424.1 and NP_006415.1], chimpanzee [accession numbers XM_526805.1 and XP_526805.1], mouse [accession numbers NM_011402.2 and NP_035532.2], dog [accession numbers XM_545968.2 and XP_545968.2], chicken [accession numbers NM_204474.1 and NP_989805.1], and *Caenorhabditis elegans* [accession numbers NM_076180.3 and NP_508581.2])

GeneBee Molecular Biology Server, http://www.genebee.msu.su/genebee.html (for RNA secondary-structure prediction program)

NCBI Map Viewer, http://www.ncbi.nlm.nih.gov/mapview/

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/ (for PAM)

UCSC Genome Browser, http://genome.ucsc.edu/

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