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Loss of the *PLA2G2A* gene in a sporadic colorectal tumor of a patient with a *PLA2G2A* germline mutation and absence of *PLA2G2A* germline alterations in patients with FAP

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Abstract The *Min* (multiple intestinal neoplasia) mouse with a germline mutation in the adenomatous polyposis coli gene serves as an animal model for familial adenomatous polyposis coli (FAP). The number and age at onset of colorectal adenomas varies in the offspring of *Min* mice crossed with other strains. The murine gene for the secretory phospholipase A2 (*PLA2G2A*) was found to be the main candidate for these variations. To test the hypothesis of a correlation between *PLA2G2A* gene alterations and human tumor development, we screened 14 patients with FAP and 20 patients with sporadic colorectal cancer for germline and somatic *PLA2G2A* gene mutations. None of the individuals with FAP showed *PLA2G2A* germline alterations. However, a germline mutation was observed in one patient with an apparently sporadic colorectal tumor; the wildtype allele was somatically lost in the tumor of this patient.

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Introduction

Nearly 70% of all colorectal tumors carry mutations in the tumor suppressor gene *APC* (adenomatous polyposis coli; Nishisho et al. 1991; Powell et al. 1992). Germline mutations in the *APC* gene lead to familial adenomatous polyposis coli (FAP), an autosomal dominant predisposition to cancer (Kinzler et al. 1991; Groden et al. 1991).

Patients with a 5-bp deletion that lies at codon 1309 and that occurs in about 10% of all FAP patients manifest on average an earlier onset and a more severe course of the disease, compared with patients having mutations at other regions of the *APC* gene (Caspari et al. 1994). Nevertheless, large phenotypic variation occurs even among patients of the same families or in patients from different families but with the same mutation (Leppert et al. 1990; Paul et al. 1993; Giardiello et al. 1994). Although environmental factors may be partially responsible, modifier genes have been proposed to account for some of this phenotypic variation.

A germline mutation in the murine *APC* gene was identified in the inbred *Min* mouse (multiple intestinal neoplasia), which serves as an animal model for FAP (Moser et al. 1990; Su et al. 1992). Young *Min* mice develop numerous adenomatous polyps in the small and large intestine. The number of polyps decreases dramatically in the F1 generation when *Min* mice are crossed with mice of inbred strains carrying the wildtype locus of "modifier of *Min*" (*Mom1*; Moser et al. 1992; Dietrich et al. 1993). Thus, at least one other dominant gene modifies the influence of the *Min* mutation on polyp susceptibility. The *Mom1* allele has been mapped (Dietrich et al. 1993), and the non-pancreatic Group IIA phospholipase A2 (*PLA2G2A*) localized in this chromosomal region has been identified as the main candidate gene of the *Mom1* locus (MacPhee et al. 1995). *Min* mice showing severe FAP-like symptoms carry a single base insertion in exon 3 of the phospholipase A2 gene and this leads to a truncated gene product.

The human phospholipase A2 gene spans over 4141 bp and has been cloned by Seihamer et al. (1989); it maps to

the chromosomal region 1p35-p36.1 (Praml et al. 1995b). Another phospholipase gene (*PLA2G5*) and one putative pseudogene (*PLA2G2C*) have been mapped to the same chromosomal region (Spirio et al. 1996). Somatic losses of heterozygosity (LOHs) of this chromosomal region have been described for a large proportion of human sporadic colorectal tumors (Leister et al. 1990). The chromosomal region is flanked by the two microsatellite markers D1S199 and D1S436 (Praml et al. 1995a).

The results obtained with the *Min* mouse have led to the assumption that *PLA2G2A* may also function as a modifier gene in human tumor progression. In this study, we have analyzed patients with FAP and patients with sporadic colorectal cancer for germline or somatic *PLA2G2A* mutations in order to examine whether this gene is responsible for tumor progression or phenotypic variation.

Materials and methods

Patients

Twelve patients with the 5-bp germline deletion in codon 1309 of the *APC* gene from 6 families were examined for germline mutations in the *PLA2G2A* gene. Family FAP-240 with a 4-bp deletion at position 1068 was also included in this study. The ages at diagnosis of symptoms or during presymptomatic endoscopic examination in call-up patients are shown in Fig. 1. In addition to the affected patients, four unaffected relatives of two FAP families were examined.

DNA from blood and normal and tumor tissue specimens of 20 patients with sporadic colorectal carcinomas were analyzed. The detected somatic DNA alterations, including six different microsatellite markers and the tumor relevant genes *APC*, *p53*, K-*ras* and *DCC*, together with the pathological classification of these tumor samples, have recently been described in detail (Deuter et al. 1996).

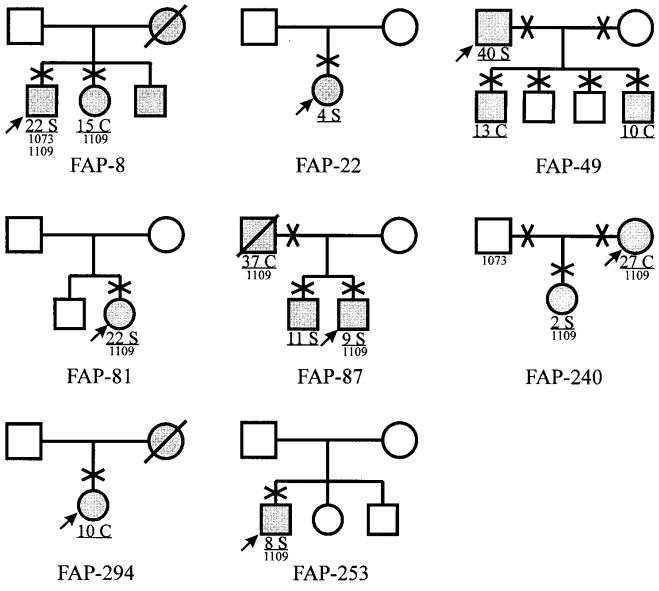

Fig. 1 Simplified pedigrees of the FAP families examined in this study. *Squares* Males, *circles* females, *grey* affected individuals, *crosses* individuals examined in this study, *arrows* index patients. The ages at diagnosis of FAP because of bowel symptoms (*S*) or during prophylactic endoscopic screening in call-up patients (*C*) are *underlined*. Affected individuals of family FAP-240 showed a 4-bp deletion at position 1068 of the *APC* gene; all other affected individuals showed a 5-bp deletion at position 1309. Individuals showing heterozygous sequences at position 1073 or 1109 of the *PLA2G2A* gene are indicated

DNA purification and polymerase chain reaction

Paired tumor and normal tissue specimens were frozen in liquid nitrogen directly after surgical removal and stored at –80°C. Genomic DNA from blood or tissue was isolated by the salting out procedure (Miller et al. 1988) or by the use of DNA spin columns (Qiagen, Hilden, Germany).

The following primers specific for the *PLA2G2A* genomic sequence were used for standard polymerase chain reaction (PCR) of exons 2 through 5 of the *PLA2G2A* gene: 5′-GCCAGTCCATCTGCATTTGTC-3′/5′-GCTGTCCCCCATGCTCAGA-3′ (exon 2), 5′-CCAGACAACTCCCAAATTTCC-3′/5′-b-AGGGATAGGTGGCCTCACCG-3′ (exon 3), 5′-CCTCCTGGAGCTGTGGGACA-3′/5′-CCAGCACTGTCTAAACAAATGAG-3′ (exon 4), 5′-TTCCACAAGAAGCCACTG-3′/5′-AAGGTTTCCAGGGAAGAG-3′ (exon 5). The downstream primers for PCR of exons 3 and 5 were 5′ biotinylated to enable strand separation after amplification. Primers 5′-GCCTACTGCAGGCCCATGG-3′ and 5′-TAATAAAGTCCCATCTTGTG-3′ were used for sequencing the complete exon 3 and exon 5, from its beginning to the stop codon. PCR and sequencing analysis of those samples showing sequence alterations were repeated twice to exclude PCR errors.

Single-strand conformation polymorphism and sequence analyses

After mixing 5 µl PCR product with 10 µl denaturing buffer (95% formamide, 20 mM EDTA, 10 mM NaOH, 0.05% bromphenolblue, 0.05% xylenecyanol in 90 mM TRIS-borate, 2 mM EDTA, pH 8.3), the mixture was heat-denatured and chilled on ice. The DNA single strands were separated on non-denaturing polyacrylamide minigels at room temperature and detected by silver staining.

The amplified exon 3 and exon 5 fragments of all samples were sequenced by using ^{35}S-dATP. Single-strand DNA preparation was performed by using streptavidin coupled magnetic beads and following a standard protocol (Thomas et al. 1994).

LOH analysis

In this study, LOH analysis was performed by two different methods: the analysis of recently identified sequence variations or microsatellite analysis (Seilhamer et al. 1989; Masharani et al. 1988; Riggins et al. 1995). For microsatellite analysis, a new non-radioactive method was developed based on a recently described method (Praml et al. 1995a). The microsatellite markers D1S436 and D1S199 flanking the *PLA2G2A* gene were amplified by using the PCR primers described (Gyapay et al. 1994). PCR products were electrophoresed on denaturing polyacrylamide minigels. The samples were blotted onto a nylon membrane by capillary transfer. The membrane was blocked and hybridized overnight with a $(CA)_{15}$ oligonucleotide labeled with fluorescein. After incubation with anti-fluorescein antibody coupled to horseradish peroxidase, bands were detected by enhanced chemoluminescence (Amersham, Braunschweig, Germany). Data interpretation and identification of LOHs were performed by comparing the relative intensities of signals (Praml et al. 1995a).

Results

To determine whether phenotypic variation in FAP is caused by the *PLA2G2A* gene, which is a strong candidate

for a modifier gene, we examined patients from FAP families and some of their relatives for germline mutations in the coding region of the *PLA2G2A* gene (Fig. 1). Blood and normal and tumor tissue of 20 unrelated patients with sporadic colorectal cancer were also examined for germline or somatic *PLA2G2A* gene mutations. Analysis of somatic LOH was performed in all tumor tissue samples either by examination of intragenic polymorphisms or by analysis of the two microsatellite loci D1S436 and D1S199, which flank the *PLA2G2A* gene.

The coding regions of exons 2 and 4 of all samples were analyzed by single-strand conformation polymorphism (SSCP) analysis. Exon 3 of all samples was sequenced to detect all putative sequence alterations exactly at, or close to, the position of the human *PLA2G2A* gene, which corresponds to the position of the murine *Mom1* mutation. Exon 5 of all samples was sequenced, too.

Interestingly, in the blood sample and in the normal colorectal tissue of patient Do13, an 80-year-old female patient with an apparently sporadic colorectal tumor, a heterozygous deletion of 2 bp at genomic position 1119 (codon 48) in exon 3 of the *PLA2G2A* gene was detected (Fig. 2). Assuming normal splicing of the gene, this frameshift deletion is predicted to result in a premature stop at codon 67 in exon 4. We detected a somatic loss of the wildtype *PLA2G2A* sequence in the tumor of the patient (Fig. 2). No LOH could be detected by means of the flanking microsatellite markers: D1S436 showed heterozygosity, whereas D1S199 appeared to be non-informative. The tumor of patient Do13 was diagnosed as being a late metastazising carcinoma carrying a heterozygous *p53* point mutation and a somatic monoallelic loss of the *DCC* gene (Deuter et al. 1996). The *PLA2G2A* germline mutation identified in patient Do13 had not been transmitted to her normal 58-year-old son (Fig. 2).

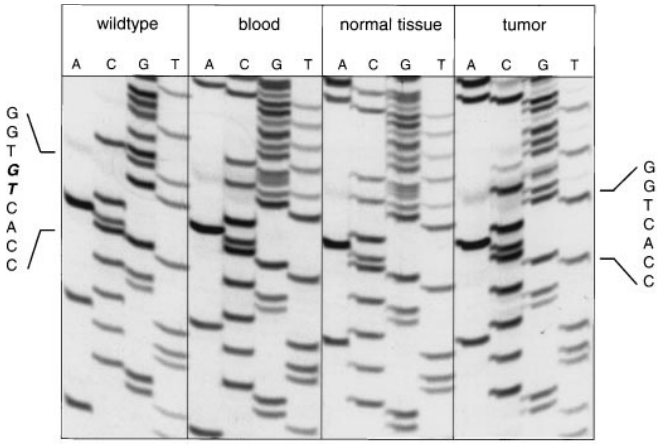

Fig. 2 Sequencing gels showing the heterozygous germline mutation and the somatic loss of the normal *PLA2G2A* allele in patient Do13. The wildtype *PLA2G2A* sequence detected in the son of patient Do13 is shown (*wildtype*). The DNA from *blood* and colorectal *normal tissue* of patient Do13 shows the heterozygous 2-bp deletion at position 1119. The normal sequence is somatically lost in the *tumor* of patient Do13

No variation was observed in the SSCP banding patterns of exons 2 and 4 (not shown). All samples from FAP families and from patients with sporadic colorectal tumors showed the normal sequence from the beginning of exon 5 to the stop codon. All individuals of FAP families including the unaffected family members showed the normal sequence in exon 3 of the *PLA2G2A* gene.

Two silent sequence variations in exon 3 of the human *PLA2G2A* gene at genomic positions 1073 or 1109, corresponding to codons 32 and 44, were identified at a frequency of 7.5% or 21.3% for the rare allele, respectively. These frequencies, which were determined in 80 independent chromosomes, are similar to those previously described (Spirio et al. 1996; Riggins et al. 1995). Individuals from FAP families showing heterozygosities are shown in Fig. 1.

The detected germline mutation and the intragenic polymorphisms were used as parameters to detect the somatic loss of the *PLA2G2A* gene in sporadic colorectal carcinomas of 20 unrelated individuals. A total of 8 patients was informative, including patient Do13 (Table 1). Two of these (25%) showed somatic loss of the *PLA2G2A* gene.

Of the analyzed sporadic colorectal tumors, 19 were informative for at least one microsatellite marker (Fig. 3). Six samples (32%) showed LOH of at least one mi-

Table 1 LOHs of the analyzed 20 sporadic tumors. LOHs of the *PLA2G2A* gene itself or the flanking microsatellite markers were detected either by the loss of the heterozygous gene sequence or the loss of one microsatellite allele, respectively

	LOH/informative cases
Gene position	
1073 (ACG→ACC)	0/1
1109 (TAC→TAT)	1/6
1119 (GTGTCA→GTCA)	1/1
Microsatellite markers	
D1S199	4/11 (36%)
D1S436	4/16 (25%)
At least one marker	6/19 (32%)
Both markers	2/8 (25%)

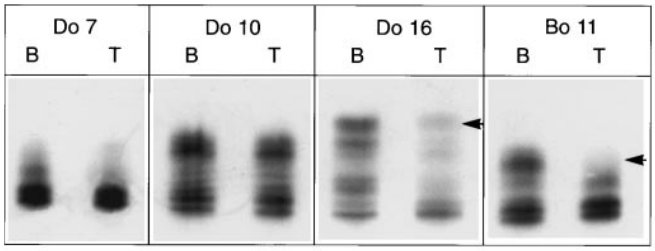

Fig. 3 Examples of LOH analysis by use of the microsatellite marker D1S436. DNA samples from blood (*B*) and tumor (*T*) were examined. Examples from a non-informative signal (patient *Do7*), from a retention of heterozygosity (*Do10*), and from two LOHs (*Do16*, *Bo11*) are shown. *Arrowheads* Alleles lost in the tumors. Background bands of very low intensities were interpreted as being normal alleles from contaminating DNA originating from surrounding normal tissue

crosatellite region (Table 1). Eight patients were informative for both microsatellites; two of them showed the loss of both markers.

Discussion

The heterozygous *PLA2G2A* mutation was found in DNA from the blood and normal tissue of patient Do13 (Fig. 2); thus, it is unlikely to have resulted from a somatic mutation event. The tumor of this patient showed the somatic loss of the wildtype *PLA2G2A* sequence, indicating complete loss of the *PLA2G2A* gene function. Remarkably, this LOH did not include the neighboring microsatellite region D1S436. Our results indicate that the loss of the *PLA2G2A* gene itself and not the loss of other neighboring chromosomal regions harboring yet unidentified genes may contribute to tumor progression. The biallelic loss of the *PLA2G2A* gene may play a role in tumor progression. Patient Do13 developed a single colorectal carcinoma at the age of 80. The history of her family showed no malignant disease. Therefore, our results give no indication for an increased predisposition to cancer in individuals with heterozygous *PLA2G2A* germline mutations. This finding is consistent with a recently described heterozygous germline transition close to the 3′-end of the *PLA2G2A* gene; this transition was not accompanied by a somatic loss of the non-mutated allele in the corresponding tumor (Riggins et al. 1995).

Whereas a severe course of the disease FAP has been described for patients with the 5-bp deletion at codon 1309 of the *APC* gene, considerable interfamilial or even intrafamilial variation has been noticed. To examine whether this phenotypic variation is related to mutations in the *PLA2G2A* gene, we selected several families with the 1309 mutation and very early onset, in addition to families with a large variation in clinical phenotype. For comparison, only the ages at diagnosis of bowel symptoms were considered (Fig. 1). In family FAP-87, for example, the diagnosis of FAP was established first in the youngest son and only later in his father. The daughter of family FAP-240 showed a hepatoblastoma at the age of 2. The age at diagnosis of FAP in all analyzed patients ranged between 4 and 40 years. However, no *PLA2G2A* germline alteration was detected in the analyzed FAP patients. Therefore, in contrast to the murine gene in *Min* mice, the human *PLA2G2A* gene does not seem to function as a major modifying gene of tumor progression in FAP patients. These results are consistent with the findings of others who have not detected *PLA2G2A* germline mutations in patients with attenuated adenomatous polyposis coli, a less severe form of FAP (Spirio et al. 1996).

The results of the LOH analysis (Table 1) are comparable with the findings of others. One report describes an LOH frequency of 31% because of the loss of heterozygous sequences (Riggins et al. 1995), whereas another report describes the somatic loss of the microsatellite markers D1S199 and D1S436 in 42% and 48% of all examined sporadic tumors (Praml et al. 1995a), respectively.

In summary, we have shown that inherited *PLA2G2A* sequence mutations occur rarely, whereas somatic LOHs of the chromosomal *PLA2G2A* locus are common in patients with human sporadic colorectal carcinomas. Based on these results, we suggest that the *PLA2G2A* gene plays a suppressing role in the development and progression of human tumors. Additionally, the finding that some tumors show LOH but no *PLA2G2A* mutation indicates that at least one other tumor-relevant gene is located close to the *PLA2G2A* gene at the human chromosomal region that corresponds to the murine *Mom1* locus.

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