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Advances in Brief

Three Secretory Phospholipase A_2 Genes That Map to Human Chromosome 1P35–36 Are Not Mutated in Individuals with Attenuated Adenomatous Polyposis Coli[1]

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Abstract

Mutation of *Pla2g2a*, a secretory phospholipase A_2 gene, dramatically increases the number of intestinal polyps that develop in the multiple intestinal neoplasia (Min) mouse, a murine model for adenomatous polyposis coli in humans. We tested the hypothesis that mutation of the human homologue(s) of this gene might be responsible for the more severe phenotype (hundreds of polyps) seen in a subset of individuals with attenuated adenomatous polyposis coli (AAPC). DNA sequence analysis demonstrated that alterations of *PLA2G2A*, as well as related genes *PLA2G2C* and *PLA2G5*, were evenly distributed between three classes of AAPC subjects: those with small, intermediate, and large numbers of adenomatous colonic polyps. Among 67 additional unrelated AAPC subjects, a stop mutation in *PLA2G2C* did not correlate with an increased burden of adenomatous polyps. Therefore, mutation of the human homologue(s) of murine *Pla2g2a* does not appear to be responsible for phenotypic variation among subjects with AAPC.

Introduction

The multiple adenomatous polyps of the colon and rectum that are characteristic of familial APC[3] precede the onset of colorectal cancer, usually early in life (1). In a less severe form of APC known as AAPC (2–4), the number of polyps may vary markedly among affected members of the same family, ranging from 1 or 2 to over 100, even though all patients sustain an elevated risk of colon cancer. The overall decrease in the average number of adenomatous polyps that develop in patients with AAPC appears to be determined by the 5′ position of the mutation within the *APC* gene, but the extreme variation in polyp number shown in sibships with AAPC supports the effects of other, unlinked loci (2–4). A murine model of this autosomal dominant disease, Min mice, develop numerous intestinal tumors that resemble the APC phenotype in human patients (5). Su *et al.* (6) have shown that Min mice carry a mutation at codon 850 in the murine *Apc* gene, which converts a leucine (TTG) to a stop (TAG) codon. Variation in the numbers of adenomatous polyps among human carriers of AAPC mutations (4) is similar to variations in the numbers of intestinal neoplasms demonstrated by Moser *et al.* (7) in backcrosses of the *Min* allele into AKR and MA strains of mice. These authors hypothesized that the overall reduction in the numbers of intestinal neoplasms and an observed increase in the life spans of Min mice were due to a single modifier locus unlinked to *Min*. Dietrich *et al.* (8) subsequently mapped this modifying locus to murine chromosome 4, and termed it *Mom-1*. The *Mom-1* locus lies in a region of the murine genome that is syntenic to human chromosome lp35–36, a region that shows loss of heterozygosity in colorectal tumors (40–50%) (9). Recently, MacPhee *et al.* (10) identified a candidate gene for *Mom-1*, by showing that mouse strains that carry the *Min* mutation and bearing large numbers of polyps, termed Mom sensitive, also carry a frameshifting mutation in one of the genes that codes for a secreted form of type II nonpancreatic PLA_2 *(Pla2g2a)*. Furthermore, in Min mice that develop only small numbers of polyps, termed Mom resistant, *Pla2g2a* is normal. The human homologue of the *Pla2g2a* gene reported by MacPhee *et al.* (10), *Pla2g2a*, shows a high degree of homology to two additional genes encoding secreted M_r 14,000 PLA_2, *PLA2G2C* (11), and *PLA2G5* (12, 13); these three human genes are tightly linked at chromosome lp35–36 (14). We examined all three PLA_2 genes in patients with AAPC.

Materials and Methods

The genomic DNA sequence of human *PLA2G2A* for the design of PCR primers was derived from Kramer *et al.* (15) and Seilhamer *et al.* (16). The DNA sequence of the human *PLA2G2C* and *PLA2G5* genes was derived from the reports of Chen *et al.*(11–13). The primers used for PCR-DNA sequencing are shown in Fig. 1.

The DNA samples chosen for analysis had been obtained from 20 AAPC patients with previously identified germline mutations in *APC* (4). The 20 subjects were subdivided into three clinical categories: those with 0–3 polyps by ages 40–54 years; those who had developed 30–65 polyps by ages 40–65 years; and those whose polyp burden was more than 100 polyps by ages 30–54 years. For one experiment (exon 2 of *PLA2G2C*), we used DNA samples from an additional 67 individuals in three similar clinical categories (0–18 polyps by ages 35–76 years; 26–77 polyps by ages 38–57 years; and >95 polyps by ages 30–77 years).[4]

Total genomic DNA from each patient was used as a template for the PCR. Two μl (400 ng) of genomic DNA were amplified in a 100-μl reaction mixture containing 1.5 mM $MgCl_2$,50 mM KCl, 10 mM Tris-HCl (pH 8.7), 10 pmol of each primer, and 2.5 units Taq DNA polymerase. Amplification conditions were as follows: 1 cycle at 94°C for 2 min; 30 cycles at 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min, followed by cooling to 4°C. After a 10-μl aliquot of each PCR product was analyzed for efficient amplification on an agarose gel, the remaining product was purified using a Centricon-100 column (Amicon, Inc., Beverly, MA). The PCR products were sequenced using an ABI sequencer, as previously described (4), with the following modification: AmpliTaq DNA polymerase, CS+ (Perkin Elmer/Cetus, Emeryville, CA), was used instead of AmpliTaq DNA polymerase.

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[3] The abbreviations used are: APC, adenomatous polyposis coli; AAPC, attenuated APC, Min, multiple intestinal neoplasia; Mom-1, modifier of Min-1; PLA_2, phospholipase A_2.

[4] R. W. Burt and M. F. Leppert, personal communication.

PLA2G2A:

2A1-F: 5'-TGTAAAACGACGGCCAGT-GAGCTAGGCCAGTCCATCTG-3'

2A1-R: 5'-CAGGAAACAGCTATGACC-CAAGAGTGCTTCCCTTCTGG-3'

2A2-F: 5'-TGTAAAACGACGGCCAGT-GAGGGCACAGAACCCCATG-3'

2A2-R: 5'-CAGGAAACAGCTATGACC-GGTAGGGAGGGATAGGTGG-3'

2A3-F: 5'-TGTAAAACGACGGCCAGT-GACAAGAGCCCCAACAGGG-3'

2A3-R: 5'-CAGGAAACAGCTATGACC-TAAACAAATGAGGGCCACTCG-3'

2A4-F: 5'-TGTAAAACGACGGCCAGT-GTTCCCACAAGAAGCCACTG-3'

2A4-R: 5'-CAGGAAACAGCTATGACC-GAGAGGGAAATTCAGCACTGG-3'

PLA2G2C:

2C1-F: 5'-TGTAAAACGACGGCCAGT-CCCTCACACACAGGTGAAGG-3'

2C1-R: 5'-CAGGAAACAGCTATGACC-CCTCCCACTGACATAAACAGG-3'

2C2-F: 5'-TGTAAAACGACGGCCAGT-AATGCCTCAGCACTCTAGTAAGG-3'

2C2-R: 5'-CAGGAAACAGCTATGACC-TAAGTGGCCTTAGAGCCTCTG-3'

2C3-F: 5'-TGTAAAACGACGGCCAGT-CCTCCAGCAGATTCCTGAGG-3'

2C3-R: 5'-CAGGAAACAGCTATGACC-GAGGTGAACGTTAAGGAAAGG-3'

2C4-F: 5'-TGTAAAACGACGGCCAGT-AGCCTTAGAACCAAACCCTGG-3'

2C4-R: 5'-CAGGAAACAGCTATGACC-GGATGCTGGATGATGTGAGG-3'

PLAG5:

50-F: 5'-TGTAAAACGACGGCCAGT-GGGTTTGTCCTCATCATCGG-3'

50-R: 5'-CAGGAAACAGCTATGACC-CACAACCCCCAATTTATGCAG-3'

51-F: 5'-TGTAAAACGACGGCCAGT-CACGGGGCATTGCCTGATAG-3'

51-R: 5'-CAGGAAACAGCTATGACC-GTGTCCTTTTCACCACCCTG-3'

52-F: 5'-TGTAAAACGACGGCCAGT-GCAGTGGGCCTAAAGCAGG-3'

52-R: 5'-CAGGAAACAGCTATGACC-AACATGGGTGCAGAGCCTGG-3'

53-F: 5'-TGTAAAACGACGGCCAGT-GTTGAATTTTTGCTCCTATTAGG-3'

53-R: 5'-CAGGAAACAGCTATGACC-GCAGCTGAACTTAAAGTCAGG-3'

54-F: 5'-TGTAAAACGACGGCCAGT-TCCATGGGGTCTCTGCTGAG-3'

54-R: 5'-CAGGAAACAGCTATGACC-GACTGAGGTCTGTGACTTAGG-3'

Fig. 1. Primers used to amplify PLA2 genes.

Table 1 *Polymorphisms in PLA2G2A, PLA2G2C, and PLA2G5 among 20 AAPC subjects*

Gene	Nucleotide-exon	Base change-type of mutation	Frequency among subjects
PLA2G2A	96-2	ACG/ACC-thr/silent	1 /20
PLA2G2A	132-2	TAC/TAT-tyr/silent	2 /20
PLA2G2C	894-2	CGA/TGA-arg/stop	6/20 + 22/67
		TGA/TGA-stop/stop	1/20 + 0/67
PLA2G2C	1185-4	CGG/CGG arg/arg	6 /20
		CGG/CAG arg/his	11 /20
		CAG/CAG his/his	3 /20
PLA2G5	−5 or −6-5′ to 1	Deletion of 1 "T"-No change	1 /20
PLA2G5	9-1	GGC/GGT thr/silent	9 /20
		GGT/GGT	1 /20

Results

Because Min mice and APC patients show many similarities in genotype and phenotype (5–7), we began our mutational study by sequencing the four exons of the coding region of the human homologue of the murine *Pla2g2a* gene, *PLA2G2A* (15, 16). After testing every AAPC subject in our three groups (total = 20), we identified two silent polymorphisms in exon 2 at codons 32 (ACG/ACC) and 44 (TAC/TAT) that were present in 5 and 10%, respectively, of our 20 subjects (Table 1).

Tischfield *et al.* (14) have found with 16% frequency a nonsense mutation in exon 2 of *PLA2G2C* among 30 normal individuals they examined. This made *PLA2G2C* a good candidate for a modifier of *APC* because it lies in the chromosomal region syntenic to *Mom-1*, presumably has a function similar to *PLA2G2A*, and occurs at a high enough frequency to account for the severe phenotype in a subset of subjects with AAPC. After examining the first set of AAPC subjects ($n = 20$), we expanded our data set to include an additional 67 AAPC subjects who also had developed different numbers of adenomatous polyps. We measured the frequency of this mutation in our AAPC patients to determine whether segregation of the stop codon corresponded with an increase in the number of adenomatous colonic polyps. Sequence chromatographs of the relevant region of exon 2 of *PLA2G2C* from a single heterozygous patient are shown in Fig. 2, and a summary of the results in our subject set is shown in Table 2. This putative nonsense mutation does not correlate with an increased number of polyps when individuals with very small numbers of polyps are compared to those with intermediate or large numbers ($P > 0.5$, Fischer's exact test). This finding is consistent with the conclusion of Tischfield *et al.* (14) that *PLA2G2C* is a pseudogene in humans; part of exon 3 is missing from the genomic sequence, and no mRNA transcript is detectable in human tissues corresponding to sites where its homologue is expressed in rat and mouse. We have been unable to detect expression of the putative *PLA2G2C* gene product in any of several epithelial cell lines.[5] The DNA sequence of exon 4 of the same gene in the 20 original subjects revealed amino acid changes at codon 395 that were present in similar frequencies among subjects with 0–5 polyps *versus* those with greater than 100 (data not shown). We sequenced the two other potential exonic regions of *PLA2G2C* and were unable to find any additional changes in the putative coding sequence (data not shown).

The third member of the *PLA2* gene family, *PLA2G5*, shows 57% nucleotide homology in the coding region to *PLA2G2A*. The four exons of *PLA2G5* (12) also were examined for mutation by DNA sequencing. We found two polymorphisms: one in the intron preceding exon 1 in a single patient with large numbers of adenomatous colonic polyps that do not appear to affect RNA splicing (data not shown), and the other, a silent mutation in the same exon, was present

[5] W. Kutchera, C. Kaplan, and S. Prescott, unpublished data.

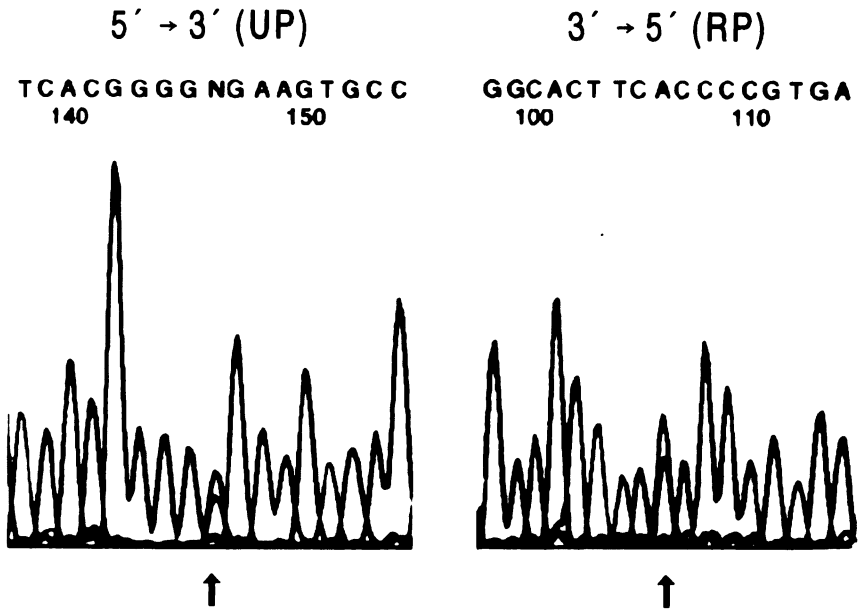

Fig. 2. Forward (5′-3′) and reverse (3′-5′) sequence chromatographs of the relevant region of exon 2 of *PLA2G2C* in a patient who is heterozygous for the base-pair change that would generate a stop codon. The arrows indicate the position of the nucleotide that is altered.

in numerous individuals (Table 1). We did not detect any additional bp changes in the other three exons of *PLA2G5*.

Discussion

The numerical distribution of adenomatous polyps among carriers of *AAPC* mutations resembles the numerical distribution of intestinal neoplasms in Min mice (7). It now appears that a single mutation in *Pla2g2a* is responsible for much of this strain-specific variation in mice (10). Therefore, it was of considerable interest to examine the human homologue of this gene and the other members of the PLA_2 gene family in humans carrying *APC* mutations responsible for AAPC. We found no mutation in any of the three phospholipase genes that appeared to correspond to a specific phenotypic group in our set of subjects; therefore, the variations in phenotype seen within AAPC pedigrees cannot be explained by alterations in these PLA_2 genes. The absence of functional mutations in *PLA2G2A* is consistent with a recent report by Riggins *et al.* (17) concerned with sporadic colorectal cancers.

Products of PLA_2 genes hydrolyze the *sn*-2 fatty acyl bond of phospholipids to produce free fatty acids and lysophospholipids (18). One mechanism by which PLA_2s might be related to the risk of colon cancer is through the generation of prostaglandins, since one of the free fatty acids that can be liberated by the action of PLA_2s, arachidonic acid, is a precursor for their biosynthesis (19). Colonic tumors in rat, mouse, and human colon tumors contain high levels of prostaglandins, which are implicated in the processes of cell proliferation and tumor growth (20). Nonsteroidal anti-inflammatory drugs, which inhibit prostaglandin synthesis, can reduce both the incidence and size of existing colorectal adenomas in rats, mice, and humans, including patients with APC and Gardner syndrome (21). However, it is not certain that secreted forms of PLA_2 are the relevant phospholipases for arachidonate release (22). Furthermore, the observation of MacPhee *et al.* (10) is contrary to what this explanation would predict.

The high degree of homology among genes that encode secreted, M_r 14,000 PLA_2 proteins suggests that they have at least one function in common, but they may also possess distinctive properties that have not been described yet. Nonetheless, our experiments provided no evidence that alterations in any of the secreted phospholipases examined here influence the risk of developing adenomatous colonic polyps in individuals who carry attenuated *APC* mutations.

Table 2 *Stop mutation in exon 2 of PLA2G2C does not correlate with increased numbers of adenomatous colorectal polyps among the original 20 AAPC subjects and 67 additional individuals*

No. of polyps	Age (yr)/no. of individuals	Nucleotide sequence at codon 298		
		CGA/CGA Arg/Arg	CGA/TGA Arg/Stop	TGA/TGA Stop/Stop
0–5	39–65/8	6	1	1
0–18	39–76/26	16	10	0
Total	34	22	11	1
26–65	34–65/4	3	1	0
26–77	38–57/13	10	3	0
Total	17	13	4	0
>100	34–59/8	4	4	0
>95	30–77/28	19	9	0
Total	36	23	13	0

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References

1. Burt, R. Polyposis Syndromes. *In:* T. Yamada, D. H., C. Alpers, D. W. Owyang, Powell, and F. E. Silverstein (eds.), Textbook of Gastroenterology, Vol. 2, pp. 1944–1966. Philadelphia: J. B. Lippincott Company, 1995.
2. Leppert, M., Burt, R., Hughes, J., Samowitz, W., Nakamura, Y., Woodward, S., Gardner, E., Lalouel, J-M., and White, R. Genetic analysis of an inherited predisposition to colon cancer in a family with a variable number of adenomatous polyps. N. Engl. J. Med., *322:* 904–908, 1990.
3. Spirio, L., Otterud, B., Stauffer, D., Lynch H., Lynch, P., Watson, P., Lanspa, S., Smyrk, T., Cavalieri, J., Howard, J., Burt, R., White, R., and Leppert, M. Linkage of a variant or attenuated form of adenomatous polyposis coli to the adenomatous polyposis coli *(APC)* locus. Am. J. Hum. Genet, *51:* 92–100, 1992.
4. Spirio, L., Olschwang, S., Groden, J., Robertson, M., Samowitz, W., Joslyn, G., Gelbert, L., Thliveris, A., Carlson, M., Otterud, B., Lynch, H, Watson, P., Lynch, P., Laurent-Puig, P., Burt, R., Hughes, J., Gilles, T., Leppert, M., and White, R. Alleles of the APC gene: an attentuated form of familial polyposis. Cell, *75:* 951–957, 1993.
5. Moser, A. R., Pitot, H. C., and Dove, W. F. A dominant mutation that predisposes to multiple intestinal neoplasia in the mouse. Science (Washington DC), *247:* 322–324, 1990.
6. Su, L-K., Kinzler, K. W., Vogelstein, B., Preisinger, A. C., Moser, A. R., Luongo, C., Gould, K., and Dove, W. F. Multiple intestinal neoplasia caused by a mutation in the murine homolog of the *APC* gene. Science (Washington DC), *256:* 668–670, 1992.
7. Moser, A. R., Dove, W. F., Roth, K. A., and Gordon, J. I. The MIN (multiple intestinal neoplasia) mutation: its effects on gut epithelium cell differentiation and interaction with a modifier system. J. Cell Biol., *116:* 517–526, 1992.
8. Dietrich, W., Lander, E. Smith, J., Moser, A., Gould, K., Luongo, C., Borenstein, N., and Dove, W. Genetic identification of Mom-1, a major modifier locus affecting Min-induced intestinal neoplasia in the mouse. Cell, *78:* 111–118, 1993.

9. Leister, I., Weith, A., Bruderlein, S., Cziepluch, C., Kangwanpong, D., Schlag, P., and Schwab, M. Human colorectal cancer: high frequency of deletions at chromosome lp35. Cancer Res., *50:* 7232–7237, 1990.
10. MacPhee, M., Chepenik, K., Liddell, R., Nelson, K., Siracusa, L., and Buchberg, A. The secretary phospholipase A2 gene is a candidate for the *Mom1* locus, a major modifier of APCMin-induced intestinal neoplasia. Cell, *81:* 957–966, 1995.
11. Chen, J., Engle, S., Seilhamer, J., and Tischfield, J. Cloning and characterization of novel rat and mouse low molecular weight Ca^{2+}-dependent phospholipase A_2s containing 16 cysteines. J. Biol Chem., *37:* 23018–23024, 1994.
12. Chen, J., Engle, S., Seilhamer, J., and Tischfield, J. Cloning and recombinant expression of a novel human low molecular weight Ca^{2+}-dependent phospholipase A_2. J. Biol. Chem., *269:* 2365–2368, 1994.
13. Chen, J., Engle, S., Seilhamer, J., and Tischfield, J. Cloning, expression and partial characterization of a novel rat phospholipase A_2. Biochim. Biophys. Acta, *1215:* 115–120, 1994.
14. Tischfield, J. A., Xia, Y-R., Shih, D. M., Klisak, I., Chen, J., Engle, S. J., Siakotos, A. N., Winstead, M. V., Seilhjamer, J. J., Allamand, V., Gyapay, G., and Lusis, A. J. Low molecular weight, calcium-dependent phospholipase A_2 genes are linked and map to homologous chromosome regions in mouse and man. Genomics, in press, 1996.
15. Kramer, R., Hession, C., Johansen, B., Kayes, G., McGray, P., Chow, E., Tixard, R., and Pepinsky, R. Structure and properties of a human non-pancreatic phosphlipase A2. J. Biol. Chem., *264:* 5768–5775, 1989.
16. Seilhamer, J., Pruzanski, W., Vadas, P., Plant, S., Kmiller, J., Kloss, J., and Johnson, L. Cloning and recombinant expression of phospholipase A2 present in rheumatoid arthritic synovial fluid. J. Biol Chem., *264:* 5335–5338, 1989.
17. Riggins, G. J., Markowitz, S., Wison, J. K., Vogelstein, B., and Kinzler, K. Absence of *$sPLA_2$* alterations in human colorectal cancer. Cancer Res., *76:* 433–434, 1995.
18. Dennis, E. Diversity of group types, regulation, and function of phospholipase A2. J. Biol Chem., *269:* 13057–13060, 1994.
19. Glaser, K., Mobilio, D., Chang, J., and Senko, N. Phospholipase A_2 enzymes: regulation and inhibition. TIPS Rev., *14:* 92–98, 1993.
20. Marnett, L. Aspirin and potential role of prostaglandins in colon cancer. Cancer Res., *52:* 5575–5589, 1992.
21. Rao, C., Rivenson, A., Simi, B., Zang, E, Kelloff, G., Steele, V., and Reddy, B. Chemoprevention of colon carcinogenesis by sulindac, a nonsteroidal anti-inflammatory agent. Cancer Res., *55:* 1464–1472, 1995.
22. Lin, L-L., Wartmann, M., Lin, A. Y., Knopf, J. L., Seth, A., and Davis, R. J., $CPLA_2$ is phosphorylated and activated by MAP kinase. Cell, *72:* 269–278, 1993.