Report

Common Deletion of *SMAD4* in Juvenile Polyposis Is a Mutational Hotspot

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Juvenile polyposis (JP) is an autosomal dominant syndrome in which affected patients develop upper- and/or lower-gastrointestinal (GI) polyps. A subset of families with JP have germline mutations in the *SMAD4* (*MADH4*) gene and are at increased risk of GI cancers. To date, six families with JP have been described as having the same *SMAD4* deletion (1244–1247delAGAC). The objective of the present study is to determine whether this deletion is a common ancestral mutation or a mutational hotspot. DNA from members of four families with JP, from Iowa, Mississippi, Texas, and Finland, that had this 4-bp deletion was used to genotype 15 simple tandem repeat polymorphism (STRP) markers flanking the *SMAD4* gene, including 2 new STRPs within 6.3 and 70.9 kb of the deletion. Haplotypes cosegregating with JP in each family were constructed, and the distances of the closest markers were determined from the draft sequence of the human genome. No common haplotype was observed in these four families with JP. A 14-bp region containing the deletion had four direct repeats and one inverted repeat. Because no common ancestor was suggested by haplotype analysis and the sequence flanking the deletion contains repeats frequently associated with microdeletions, this common *SMAD4* deletion in JP most likely represents a mutational hotspot.

Juvenile polyps were first recognized as comprising a distinct histopathologic entity in 1957 (Horrilleno et al. 1957), and a heritable form was described as "juvenile polyposis coli" in 1964 (McColl et al. 1964). In 1974, juvenile polyposis (JP [MIM 174900]) was divided into three different subtypes, including JP coli, JP of infancy, and generalized JP (Sachatello et al. 1974). In 1975, it was established that there was a significant risk of gastrointestinal (GI) malignancy in members of a large family with generalized JP, suggesting that the prevalent belief that these polyps were benign with no malignant potential was incorrect (Stemper et al. 1975). In 1998, genetic linkage to markers on chromosome 18q21.1 was

found in the same family (Howe et al. 1998*b*), and germline mutations were identified in the *SMAD4* gene (MIM 600993) (Howe et al. 1998*c*). In the latter report, three patients with JP were described who had the same 4-bp deletion in *SMAD4* exon 9. In 1999, three additional unrelated patients with JP were reported as having this same deletion (Friedl et al. 1999; Roth et al. 1999).

Common ancestry has been implicated as the cause for the high prevalence of specific *MLH1* (MIM 120436) mutations in Finland (Moisio et al. 1996), *BRCA1* (MIM 113705) mutations in Quebec (Tonin et al. 1998), and *BRCA2* (MIM 600185) mutations in northern Europe (Neuhausen et al. 1998). Another mechanism for common mutations in apparently unrelated families is that they result from mutational hotspots within genes (Cooper and Krawczak 1990; Krawczak and Cooper 1991). Among 60 unrelated patients with familial adenomatous polyposis of the colon (MIM 175100), 5 patients shared the same 5-bp deletion (3183–3187del-ACAAA), and 5 additional patients shared a different 5-bp deletion (3926–3930delAAAAG). Since 3 of these

10 patients' parents did not have these mutations, the high frequency of these specific changes could not be attributed entirely to founder mutations. The latter mutation occurred within a tandem repeat of 5 nt (AAAAG-AAAAG), with one repeat being lost, which may have resulted from slippage of DNA polymerase (Groden et al. 1993). In multiple endocrine neoplasia type 2A (MIM 171400), 16 families with a C634R missense mutation were determined to have 11 separate haplotypes by use of six markers from a 318-kb region containing the *RET* gene (MIM 164761), suggesting that this common substitution was not due to a founder effect (Gardner et al. 1994). The objective of the present study is to apply haplotype analysis to the families with JP that shared a 4-bp deletion, to determine whether this is the result of either an ancestral mutation or a mutational hotspot.

Informed consent was obtained from family members for genetic studies, as well as for review of medical records and pathology specimens. Each of the four families in this report are white, have had GI cancers, and do not share surnames nor have known common relatives. The Iowa kindred with JP is an extended white family comprising 29 members with JP or GI cancer (Stemper et al. 1975). Eleven individuals had developed colorectal cancer and six had developed upper-GI (four stomach, one duodenum, and one pancreas) cancers (Howe et al. 1998*a*). The Mississippi kindred with JP has 11 members with documented JP, of whom one developed gastric cancer and another developed colorectal cancer (Subramony et al. 1994; Scott-Conner et al. 1995). Three other members of this family died prior to the present study and had had a history of unspecified GI malignancies. The Finnish kindred with JP consists of 11 affected individuals from five generations. Four individuals are known to have upper-GI polyps, one died of gastric cancer, and one died of metastatic colorectal cancer. One other member died of metastatic adenocarcinoma of the liver of unknown primary site and had gastric, esophageal, and colonic juvenile polyps. Three additional affected family members each had one of the following: leukemia, goiter, and a benign ovarian neoplasm of unknown histology. The Texas kindred has five known affected members from three generations. The matriarch of the family had rectal, colonic, gastric, and duodenal polyps and has four affected offspring who had colonic polyps but no history of upper-GI polyps. One of these offspring developed colon cancer at age 30 years.

DNA was extracted from peripheral blood by use of a salting-out technique (Miller et al. 1988). DNA from 2 affected members (IV-22 and V-2) of the Iowa kindred (Howe et al. 1998*b*) were amplified by PCR, along with DNA from 10 members (5 affected, 4 at risk, and 1 spouse) of the Mississippi kindred, 2 affected siblings from the Finnish kindred, and 7 members (4 affected and 3 unaffected) of the Texas kindred with JP. PCR

was performed in a 10-μl volume that contained 25 ng DNA from each individual, 0.5 pmol of each primer, buffer (1.65 mM MgCl$_2$, 50 mM KCl, 10 mM Tris, and 5% glycerol), 100 μmol each dNTP, and 0.2 U *Taq* DNA polymerase. Amplification was performed in a thermocycler for 30 s at 94°C, 30 s at 55°C, and 30 s at 72°C, for a total of 35 cycles. Samples were electrophoresed through 6% denaturing polyacrylamide gels for 2–3 h at 60 W, the gels were silver stained (Bassam et al. 1991), and genotypes were determined from the gels.

SMAD4 exons and their adjacent intron-exon boundaries were used to perform BLAST searches of the draft sequence of the human genome (International Human Genome Sequencing Consortium 2001) and the Celera genome sequence (Venter et al. 2001). The genomic structure of the *SMAD4* gene was determined from the draft sequence by alignment of exons through use of the Sequencher program (Gene Codes). The genome-sequence segments that contained *SMAD4* were then searched for the sequences of previously identified chromosome 18q21 STRPs. New STRPs were then sought by alignment of 20-bp sequences of different of dinucleotide-, trinucleotide-, and tetranucleotide-repeat elements. When repeats were identified in the genome sequence, flanking primer pairs were selected using the Primer3 program. New candidate STRPs were amplified from 51 control patients, to determine their allele frequencies and heterozygosity, and were then tested in families with JP. Genetic-mapping data for each known marker were obtained from the Center for Medical Genetics sex-averaged map and complemented physical-mapping data from the Whitehead Institute Center for Genome Research human physical-mapping project (Human BAC Mapping Data). Distances between each marker and the *SMAD4* gene were determined by alignment of the human-genome sequences through use of the Sequencher program. Two novel polymorphic STRPs were identified from BAC clone RP11-729G3 (GenBank accession number AP001374) and Celera scaffold segments GA_x2KMHMRU853 and GA_x2KMHMRU8FE. *SMAD4GATA* (GenBank accession number AF364127) consists of 11 repeats in the BAC sequence and has three alleles (table 1). The other STRP is an AT/CA repeat (*SMAD4AT/CA* [GenBank accession number AF364126]), with 18 ATs, followed by 23 CAs in the BAC sequence, and has 14 alleles.

The markers used to determine haplotypes are listed in table 1 according to their physical- and genetic-map positions. In addition, five STRPs mapped to BACs where human-genome draft sequence data were available, and their distances from *SMAD4* could be calculated with precision. Uncertainties created by gaps and

Table 1

Haplotypes and Map Positions of Chromosome 18 Markers in Kindreds with JP

| | ALLELES IN KINDRED FROM | | | | DISTANCE[a] | DISTANCE FROM SMAD4 EXON 1 | |
MARKER	Iowa	Mississippi	Texas	Finland	(cM)	(kb)	HETEROZYGOSITY
D18S970	3	6	3	3	93.9	…	.67[b]
D18S1099	4	3	3	3, 5[c]	98.0	…	.58[d]
D18S474	8	4	1	8, 10[c]	98.0	121.2	.82[d]
D18S1110	3	3	5	6	98.0	37.0	.75[d]
SMAD4 exon 11						31.4	…
SMAD4AT/CA	4	7	9	13		26.3	.90
Deletion	4 bp	4 bp	4 bp	4 bp		20.2	…
SMAD4GATA	2	3	2, 3[c]	3		50.7	.55
D18S46	7	5	6	10	<99.2[e]	132.9	.80[d]
GATA06	3	2	5	4	104.1	…	.80[b]
D18S1156	3	2	2	2	104.1	…	.54[d]
D18S851	3	2	5	4	105.5	…	.55[b]
D18S484	1	1	3	3	105.5	…	.72[d]
D18S539	3	2	5	4	105.5	…	.63[b]
D18S487	5	5	6	6	106.3	…	.81[d]
D18S846	2	2	2	2	107.5	…	.38[b]
D18S977	1	4	4	4	~110	…	.92[b]

[a] Based on the Center for Medical Genetics sex-averaged map.
[b] From the Cooperative Human Linkage Center.
[c] The specific allele cosegregating with JP could not be determined.
[d] From the Genome Database.
[e] Map position of D18S363, which is 165.5 kb telomeric to D18S46 (see text).

the lack of ordered segments in the BAC sequences were checked against the Celera sequence (Venter et al. 2001). *SMAD4AT/CA* was within the intron that is between exons 9 and 10, just 6.1 kb from the 4-bp deletion found in these four families. *D18S363* was found to be 298 kb upstream from exon 1, which is consistent with the finding by Hahn et al. (1996) but discordant with that by Eppert et al. (1996), who placed *D18S363* centromeric to *SMAD4*. The last 50 kb of Celera scaffold GA_x2KMHMRU853 contained exons 1–11 of the *SMAD4* gene, and the following genes were more telomeric: *SMAD4GATA* (50.7 kb from exon 1), *D18S46* (133 kb), and *D18S363* (298 kb). The centromeric STRP *D18S1110* was present on both RP11-729G3 and Celera scaffold GA_x2KMHMRU8FE; *D18S474* was also present on GA_x2KMHMRU8FE but not on RP11-729G3. The 11 exons of the *SMAD4* gene were distributed among 31,445 bp of genomic DNA, with the largest intron spanning 9,479 bp between exons 9 and 10, which contained the *SMAD4AT/CA* STRP. No matches were found for *D18S1099, D18S470, D18S473, D18S970, D18S1156,* and *D18S851.*

The results of haplotype analysis for the four families are summarized in table 1. For the five markers centromeric to the deletion, there was no shared haplotype between the families. The alleles in each family are all different for the *SMAD4AT/CA* marker, which is only 6.1 kb downstream from the common deletion. There

was also no evidence of a shared haplotype with the markers that were telomeric to the deletion.

We evaluated the possibility that the 4-bp deletion could have been commonly inherited despite the absence of a shared haplotype. The decrease in disequilibrium over time decays as a function of $(1 - \theta)^n$ (Sham 1997), so that when a common haplotype is responsible for the mutations that are found in two families, the probability that both cases share the same haplotype is $(1 - \theta)^{2n}$. The probability that none of the six possible haplotype pairs from four families are shared is then $[1 - (1 - \theta)^{2n}]^6$. We set this probability to be .05 (the usual significance criterion) and consider the marker *SMAD4AT/CA* to be 0.006 cM from the 4-bp deletion. Solving for the number of generations, *n*, we find that, with ≤5% probability, ≥7,781 generations (233,430 years if one assumes 30 years per generation) must have passed for there not to be a shared haplotype among the cases if they had a common ancestor. Another mechanism for changes in alleles that originate from an ancestral allele is mutation at the microsatellite loci, which may vary in frequency from 0.2 to 3.3×10^{-3} (Sajantila et al. 1999). With the varied haplotypes flanking the common mutation seen in the present study, both recombination and mutation of microsatellites are less likely explanations than a mutational hotspot in exon 9 of the *SMAD4* gene. Owing to the proximity of these markers, we conclude that any common ancestor would likely date from the
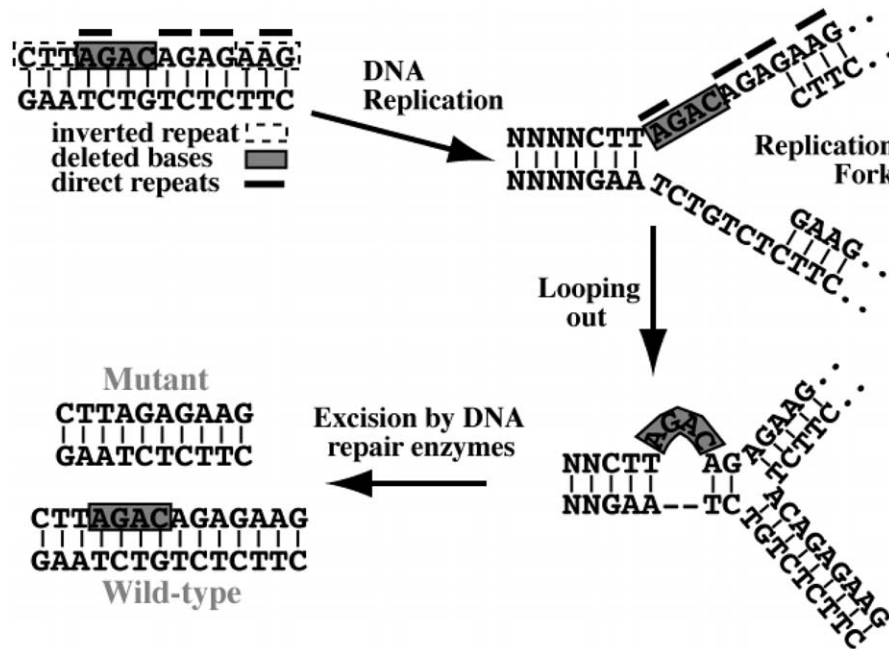
Figure 1 Possible mechanism of the 4-bp deletion through slipped mispairing. At the replication fork, the second AG direct repeat can mispair with the complement of the first AG repeat, causing a loop on the upper strand that is excised by DNA repair enzymes. The resultant upper-strand copies will have the 4-bp deletion, whereas the lower-strand copies will be the wild-type sequence.

dawn of the modern human species, which has been estimated to be between 171,000 and 479,000 years ago (Ingman et al. 2000).

Of the four families that we report, all include members with upper-GI juvenile polyps and colorectal cancer. In three of the four families, there are individuals with gastric cancer, and consistent extraintestinal phenotypic features have not been noted in these families. Friedl et al. (1999) also found two families with the same 4-bp deletion, one of which included three affected individuals with stomach cancer and two with colon cancer (Hofting et al. 1993). The other family was described as having two affected brothers and an asymptomatic carrier father. These findings suggest that this 4-bp deletion is associated with a more aggressive JP phenotype, with generalized JP and upper- and lower-GI cancers.

Ancestral mutations are relatively common in some diseases, such as hereditary nonpolyposis colorectal cancer (Moisio et al. 1996), cystic fibrosis (Morral et al. 1994), and breast/ovarian cancer (Neuhausen et al. 1998; Tonin et al. 1998). However, in many other diseases, new mutations appear to arise from an increased susceptibility due to specific DNA sequences. In this 4-bp deletion, the same mutant sequence could result if the deletion began at any of four consecutive bases. Krawczak and Cooper (1991) examined 60 deletions of <20 bp that were known to cause human diseases, to identify sequence characteristics that might predispose genes to microdeletion. They found that direct repeats flanked or spanned the deletion in two-thirds of cases, with the most common repeat length being 2–4 bp. In the deletion that we report, a direct repeat of AG is present in the first 2 bp of the deletion, and another follows the deleted AGAC. There is another AG repeat immediately after the second AG, and there is a fourth AG repeat 2 bp downstream from this. One proposed mechanism of this deletion would be slippage of DNA polymerase through these repeat sequences. Another proposed mechanism is slipped mispairing, in which the second repeat pairs with the complement of the first repeat at a DNA replication fork, which results in the repeat and intervening sequence looping outward and being excised by repair enzymes (fig. 1). Palindromic (i.e., inverted repeat) sequences are another mechanism that causes loops on a single DNA strand and were present flanking the deletion in this case (CTT 2 bp 5' and AAG 2 bp 3' to the deletion).

In the present study, the finding of different haplotypes in four families with markers in close proximity to the *SMAD4* deletion effectively excludes the possibility of a common ancestral mutation. The presence of direct and inverted repeats flanking the 4-bp deletion, which are features commonly observed in microdeletions, suggest that this area is instead a mu-

tational hotspot. This mutation leads to a more virulent form of JP, with a high incidence of gastric and colonic polyposis, as well as to gastric and colorectal cancer.

Acknowledgments

Electronic-Database Information

Accession numbers and URLs for data in this report are as follows:

BLAST, http://www.ncbi.nlm.nih.gov/BLAST/

Celera Publication Site, http://public.celera.com/cds/login.cfm (for human genome sequence)

Center for Medical Genetics, http://research.marshfieldclinic.org/genetics/Map_Markers/maps/IndexMapFrames.html (for comprehensive human genetic maps)

Cooperative Human Linkage Center, The, http://www.chlc.org/

GenBank, http://www.ncbi.nlm.nih.gov/Genbank/ (for *SMAD4* cDNA [accession number NM_005359], BAC clone RP11-729G3 [accession number AP001374], *SMAD4GATA* [accession number AF364127], and *SMAD4AT/CA* [accession number AF364126])

Genome Database, The, http://gdbwww.gdb.org/

Human BAC Mapping Data, http://www-genome.wi.mit.edu/seq/mapping.html (for the Whitehead Institute Center for Genome Research human physical-mapping project)

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/ (for JP [MIM 174900], *SMAD4* [MIM 600993], MLH1 [MIM 120436], *BRCA1* [MIM 113705], *BRCA2* [MIM 600185], adenomatous polyposis of the colon [MIM 175100], multiple endocrine neoplasia type II [MIM 171400], and *RET* [MIM 164761])

Primer3, http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi/

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