

Alterations of the PPP2R1B gene located at 11q23 in human colorectal cancers

Y Takagi, M Futamura, K Yamaguchi, S Aoki, T Takahashi, S Saji

Abstract

Background/claims—In 1998 the PPP2R1B gene encoding the A subunit of the serine/threonine protein phosphatase was identified as a putative tumour suppressor gene in lung and colon cancer in the chromosome region 11q22–24. The aim of the present study was to determine the type of alterations in primary rectal cancers as well as colon cancers and the correlation between these alterations and clinicopathological data.

Methods—Mutation analyses of the PPP2R1B gene sequence encoding the binding sites of the catalytic C subunit (Huntington elongation A subunit TOR (HEAT) repeats 11–15) and partial binding sites of the regulatory B subunit were carried out on cDNA samples from 30 primary colorectal cancer specimens and corresponding normal tissues using a combination of the polymerase chain reaction and subsequent direct DNA sequencing.

Results—Five missense mutations producing amino acid substitutions were detected in the four colon cancer cases (13.3%; four of 30 colorectal cancers): ¹⁵glycine (GGT) to alanine (GCT) and ⁴⁹⁹leucine (TTA) to isoleucine (ATA) in the same case, and ⁴⁹⁸valine (GTG) to glutamic acid (GAG), ⁵⁰⁰valine (GTA) to glycine (GGA), and ³⁶⁵serine (TCT) to proline (CCT). Of these five mutations, three (60%) were located in HEAT repeat 13 and four (80%) showed T to other nucleotide substitutions. In addition, a normal polymorphism, ⁴⁷⁸leucine, was found. No correlation was found between these mutations and clinicopathological data.

Conclusion—Our results suggest that the PPP2R1B gene is one of the true targets at 11q23, and its inactivation is involved in the development of all types of colorectal cancers.

(Gut 2000;47:268–271)

Keywords: PPP2R1B gene; colorectal cancer; tumour suppressor gene; protein phosphatase

Loss of heterozygosity (LOH) is one of the most frequent alterations found in solid tumours and recognition of areas with high rates of loss of genetic material can lead to identification of chromosome regions harbouring putative tumour suppressor genes.

The region 11q22–24 has moderate to high frequencies of LOH in colorectal cancer (29–34%),^{1,2} breast cancer (40–50%),³ and lung cancer (63%).⁴ The PPP2R1B gene was

recently identified as a candidate tumour suppressor gene in lung and colon cancer in this chromosome region.⁵ The PPP2R1B gene encodes the β isoform of the A subunit of the serine/threonine protein phosphatase 2A (PP2A). The PP2A holoenzyme consists of three subunits: a 65 kD structural/regulatory A (PR65/A) subunit, a 36 kD catalytic C (PP2Ac) subunit, and a variable regulatory B subunit.⁶ The holoenzyme, in forming a trimer, regulates diverse activities such as differentiation, proliferation, or apoptosis, by reversible phosphorylation.⁷ The PR65/A subunit displays a series of 15 tandem internal repeats, termed a HEAT (Huntington elongation A subunit TOR) motif.^{8,9} The HEAT repeat motif is characterised by a pair of antiparallel α helices (termed A and B) that are stacked in a consecutive array to generate a regular repetitive protein architecture, reflecting the internal repeats of the protein sequence. The A and B helices of the HEAT motif form convex and concave faces, respectively. The parallel stacking of adjacent HEAT repeats aligns adjacent intrarepeat turns that form a continuous ridge, providing a platform for mediating the protein-protein interactions between PR65/A subunit and PP2Ac and B subunits.⁸ The PR65/A subunit binds the PP2Ac subunit through HEAT repeats 11–15 at the C terminus¹⁰ and binding between the PR65/A and the PP2Ac subunit was abolished by sequence alterations of HEAT repeats 13–15.⁵

Here we report mutational analysis of the sequence of this functionally important domain (HEAT repeats 11–15), and in addition HEAT repeats 1–4 and 9–10, in 30 primary colorectal cancers. The purpose of this study was to determine the type of alterations in primary colon and rectal cancers, and to assess the correlation between mutations of PPP2R1B and clinicopathological factors.

Materials and methods

PATIENT SAMPLES

Thirty primary colorectal cancers and corresponding normal tissue samples were obtained from 18 male and 12 female patients, aged 24–86 years (mean age 63 years at diagnosis) during surgery at the Gifu University School of Medicine from July 1997 to November 1997. According to the TNM (tumour, node, and metastasis) classification,¹¹ tumour stage

Abbreviations used in this paper: LOH, loss of heterozygosity; RT, reverse transcriptase; PCR, polymerase chain reaction; HEAT, Huntington elongation A subunit TOR; Ala, alanine; Val, valine; Gly, glycine; Glu, glutamic acid; Ile, isoleucine; Leu, leucine; Ser, serine; Pro, proline; PP2A, protein phosphatase 2A.

Second Department of Surgery, Gifu University School of Medicine, Tsukasa-machi, Gifu 500–8705, Japan
Y Takagi
M Futamura
K Yamaguchi
S Aoki
T Takahashi
S Saji

Correspondence to:
Dr Y Takagi. Email:
yutakagi@ email.msn.com

Accepted for publication
8 February 2000

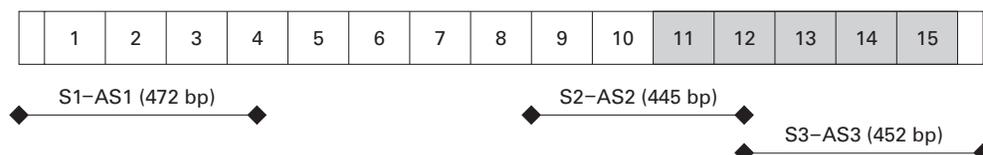


Figure 1 Schematic view of PPP2R1B cDNAs. Three sets of polymerase chain reaction primers were used to amplify a portion of the gene. Numbers indicate the internal repeats (HEAT repeats) and shaded numbers indicate the sequence of binding sites for the PP2Ac subunit on the PR65/A subunit.

showed four stage I, seven stage II, 12 stage III, and seven stage IV cases. With regard to tumour site, there were 15 cases in the ascending colon, two in the transverse colon, five in the sigmoid colon, and eight in the rectum. The tumours were diagnosed histologically as colorectal cancers. In all cases tissue samples were rapidly frozen in liquid nitrogen and stored at -80°C until analysis.

REVERSE TRANSCRIPTION-POLYMERASE CHAIN REACTION

Total RNA (2 μg), isolated and purified by standard procedures, was reverse transcribed using 0.3 μg of random primers, 20 U of RNase inhibitor, 10 mM of deoxynucleoside triphosphates, and 20 U of reverse transcriptase (Takara Biomedicals Co., Kyoto, Japan) under the manufacturer's suggested reaction conditions. The polymerase chain reaction (PCR) was performed in 25 μl of reaction mixtures containing the following: 1 μl of the complementary DNA mix, 2.5 μl of 10 X PCR buffer (50 mM KCl, 10 mM Tris HCl, pH 8.3, and 1.5 mM MgCl_2), 4.0 mM deoxynucleoside triphosphates, 1.25 U of Taq polymerase (Takara), and 0.5 mM of each PCR primer. Primers for PPP2R1B were designed to amplify three regions (fig 1). The primer pairs used were as follows: HEAT repeats 1-4, S1 (5'-CAG CAG GAG GAG AAA GAA CAT G) and AS1 (5'-ATC CCC ACT TGC TAA GCG TTT C) (472 bp); HEAT repeats 9-12, S2 (5'-GCC CAT TGA AGA TAG AGA GAC C) and AS2 (5'-AGC TTC TCG TAG GGC GTA TAC A) (445 bp); HEAT repeats 12-15,

S3 (5'-TTA TGT ATG GCT TGG CTC GTG G) and AS3 (5'-TTC CCT CCT GCT CCT CAT TAT G) (452 bp). The reactions were programmed for thermal cycling as follows (Takara, PCR thermal cycler MP): the initial denaturing step was for one minute at 94°C , followed by 35 cycles of 30 seconds at 94°C for denaturation, 30 seconds at 55°C for annealing, and one minute at 72°C for extension. The final extension for all PCR reactions was at 72°C for 10 minutes.

SEQUENCE ANALYSIS FOR MUTATIONS

PCR amplified products were electrophoretically separated on a 2% agarose gel. Bands of appropriate size were cut from the gel, purified with the GENE Clean III Kit (Bio101, Vista, California, USA), and sequenced using the ABI PRISM Dye Termination Cycle Sequencing Kit (Perkin-Elmer, Foster City, California, USA) and an ABI Prism 377 automated DNA sequencer (ABI, Foster City, California, USA). Sequences from tumour cases were aligned and compared with the normal PPP2R1B sequence. When changes were identified they were confirmed by repeating the PCR and sequencing the new amplification product.

CLINICOPATHOLOGICAL DATA

Data, including sex, age, stage of disease, and histopathological findings were available from the clinical and pathological records. The clinicopathological staging was carried out according to the UICC staging system.

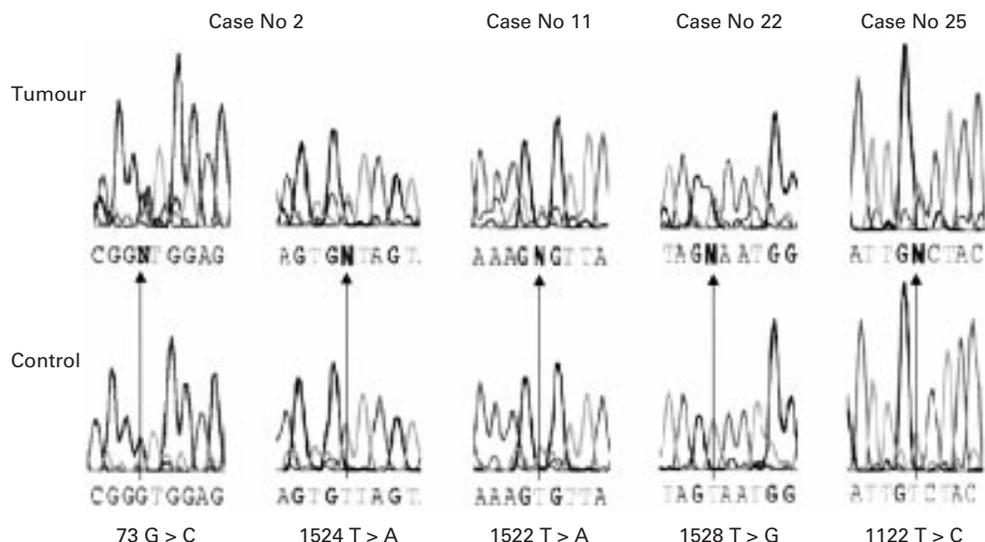


Figure 2 DNA sequence electropherograms showing the PPP2R1B gene mutations identified in patients with colorectal cancer. Individual electropherograms for the tumour are shown in the upper panels and the corresponding normal tissues (controls) are shown in the lower panels. The relevant nucleotide is indicated by an arrow.

Table 1 PPP2R1B sequence changes in colorectal cancers

Tumour specimen	Stage	Codon	Predicted effect	Nucleotide substitution
Case No 2	III	15	Gly to Ala	GGT to GCT
Case No 2	III	499	Leu to Ile	TTA to ATA
Case No 11	I	498	Val to Glu	GTG to GAG
Case No 22	III	500	Val to Gly	GTA to GGA
Case No 25	IV	365	Ser to Pro	TCT to CCT

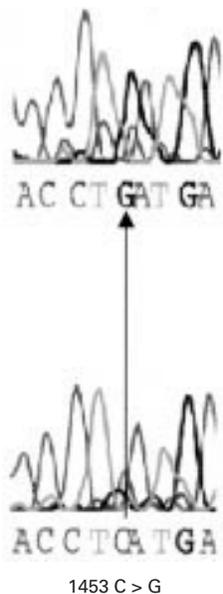


Figure 3 DNA sequence showing a single base substitution without amino acid substitution of the PPP2R1B gene. The consensus sequence is shown in the upper panel and an identified substitution is shown in the lower panel.

STATISTICAL ANALYSIS

Fisher's exact probability test was used for statistical analyses of the relation between detection of mutation and traditional clinical pathological parameters. A p value <0.05 was considered to indicate statistical significance.

Results

DNA SEQUENCING OF PCR PRODUCTS

Each HEAT repeats fragment of the PPP2R1B gene was successfully amplified by reverse transcription-polymerase chain reaction (RT-PCR) for all 30 primary colorectal cancer specimens and corresponding normal colorectal tissues. Sequence analysis showed that distinct PPP2R1B gene alterations were present in only four colon cancer specimens and not in the corresponding normal colon tissues (fig 2). These four cases showed five missense mutations predicted to result in amino acid changes. Case No 2 carried a glycine (Gly) to alanine (Ala) (GGT to GCT) at codon 15 and leucine (Leu) to isoleucine (Ile) (TTA to ATA) at codon 499. Case Nos 11, 22, and 25 had a valine (Val) to glutamic acid (Glu) (GTG to GAG) at codon 498, Val to Gly (GTA to GGA) at codon 500, and serine (Ser) to proline (Pro) (TCT to CCT) at codon 365, respectively (table 1). A single C to G transversion at nucleotide 1453 position (HEAT repeat 12) without amino acid substitution, ⁴⁷⁸Leu (CTC to CTG), was identified in the tumours of case Nos 10, 16, 18, 19, 23, 26, and 27 (fig 3).

CORRELATION BETWEEN MUTATIONS AND CLINICOPATHOLOGICAL STATUS

There was no significant association between mutations and age, sex, pathological factors (histological type, depth of invasion, lymph node metastasis, stage) or tumour site (table 2).

Discussion

Of five mutations detected in the present analysis, three (60%) occurred consecutively at

Table 2 Clinical and pathological characteristics of the patient population

	Mutation (-) (n=26)	Mutation (+) (n=4)*	p Value
Age (y)			NS
< 64	13	3	
≥64	13	1	
Sex			NS
Female	17	1	
Male	9	3	
Histological type			NS
Well	10	0	
Moderately	13	4	
Poorly	2	0	
Mucinous	1	0	
Depth of invasion			NS
Intramural	21	3	
Extramural	5	1	
Lymph node metastasis			NS
Positive	14	3	
Negative	12	1	
Stage			NS
I	3	1	
II	7	0	
III	10	2	
IV	6	1	
Tumour site			NS
Ascending colon	13	2	
Transverse colon	1	1	
Descending colon	0	0	
Sigmoid colon	4	1	
Rectum	8	0	

*Two mutations were found in case No 2. NS, not significant.

HEAT repeat 13. HEAT repeats 11–15 were found to be involved in the binding site for the PP2Ac subunit,¹⁰ and two other experiments showed that the PP2Ac subunit interaction with the PR65/A subunit was abolished by sequence alterations of the HEAT repeats 13–15.^{5,12} Furthermore, one of the three mutations, ⁴⁹⁸Val to Glu, was a change from a hydrophobic to a hydrophilic amino acid at highly conserved hydrophobic residues and was within the ridge of highly conserved residues (fig 4). The possibility that, due to one or more of the mutations, a highly conserved region of the PR65/A subunit necessary to form a functional trimer with PP2Ac and regulatory B subunits might be compromised should be considered. The role of the PP2A holoenzyme in forming the trimer makes it essential for diverse activities: downregulation of the mitogen activated protein kinase (MAPK) cascade, cell cycle checkpoint control,¹³ and nuclear telomerase activity inhibition.¹⁴ These

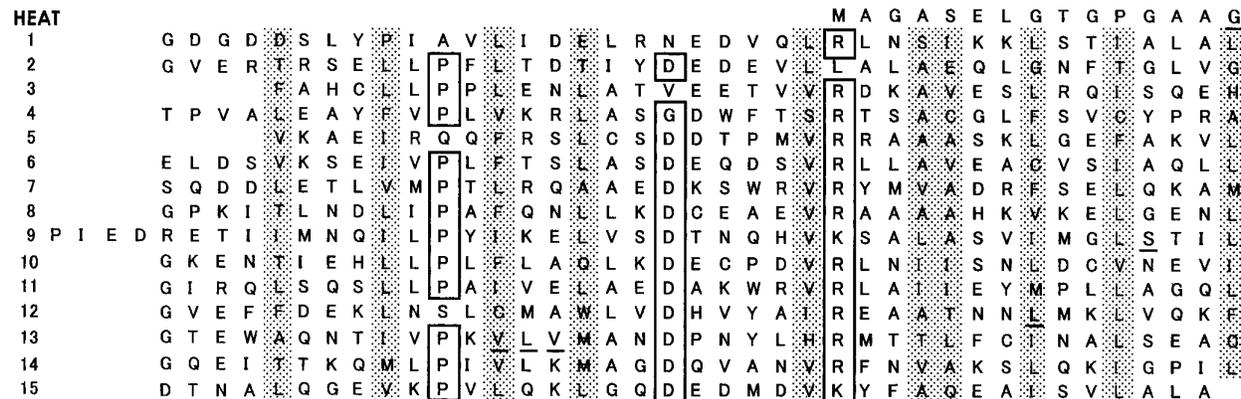


Figure 4 Multiple sequence alignment of HEAT motif sequences and location of the mutations and polymorphism. Boxed amino acids represent highly conserved residues and shaded amino acids indicate hydrophobic residues. Amino acids underlined are mutated except for L (⁴⁷⁸Leu) in HEAT repeat 12.

findings support the significant involvement of PPP2R1B mutation in colorectal carcinogenesis. Interestingly, of the five single base pair substitutions, four (80%) showed thymine to other nucleotide substitutions; in a previous study all mutations detected in colon cancers were thymine to cytosine,⁵ and the frequency of thymine to other nucleotide substitutions was much higher than that of p53 (6.3%) and APC mutations (1.1%) previously reported in colorectal cancers.¹⁵ These findings suggest that there might be a mechanism or factors causing PPP2R1B mutations different from those causing p53 and/or APC mutations in colorectal cancers. In addition, the present study showed a single C to G transversion without amino acid substitution in HEAT repeat 12,⁴⁷⁸ Leu, in some colorectal cancers and also in some normal colorectal tissues. An explanation for this might be that the single C to G transversion at codon 478 is a normal polymorphism (fig 3).

To clarify the correlation of these mutations with the clinicopathological factors, the mutations were analysed in each category according to clinicopathological factors. There was no significant correlation between any of the PPP2R1B mutations detected in the present analysis and clinicopathological factors. This finding is consistent with the combined findings of two previous studies: tumours with PPP2R1B alterations revealed a high frequency of LOH on chromosome 11q23⁵ and there was no association between LOH on chromosome 11q23 and clinical data (patient sex, age, tumour site, or Dukes' stage).^{1,2} However, because of the small number of tumours with mutations, definitive conclusions cannot be drawn.

Comparison of the moderate frequencies (29–34%) of LOH at 11q23^{1,2} with the low frequency of mutations detected in the present study as well as in the previous study⁵ suggest the following. Microdissection techniques followed by direct sequence analysis may show higher mutation frequencies of the PPP2R1B gene. In addition to the PPP2R1B gene, there may be another gene or genes involved in the development of colorectal cancers at 11q23. So far, several genes involved in tumorigenesis at 11q23–24 have been reported, such as ATM (ataxia-telangiectasia disorder gene at 11q23.1),¹⁶ MLL1 (a gene at 11q23 frequently rearranged in acute leukaemia),¹⁷ DDX10 (a putative RNA helicase gene at 11q23.1),¹⁸ LOH112CR2A (a potential tumour suppressor gene at 11q23),¹⁹ Chk1 (a gene at 11q24 encoding a protein kinase required for the DNA damage checkpoint function),²⁰ and APOC3 (apolipoprotein C-3 at 11q23).²¹ More recently, Wang and colleagues²² reported a second tumour suppressor gene involved in lung

cancers located telomeric to PPP2R1B. To our knowledge, mutations of these genes in human colorectal cancers have not yet been identified. Further investigation of chromosome region 11q23 may contribute to the understanding and elucidation of the molecular mechanism that underlies the aetiology of colorectal cancer.

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