Absence of somatic alterations of the EB1 gene adenomatous polyposis coli-associated protein in human sporadic colorectal cancers

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Summary The human EB1 gene product was recently found, by a yeast two-hybrid screening, to be associated with the carboxy terminus of the APC (adenomatous polyposis coli) protein, the product of a tumour-suppressor gene thought to act as a gatekeeper in colorectal carcinogenesis. Because virtually all of the APC mutations result in the synthesis of carboxy-terminal truncated proteins, mutant APC proteins are expected to lose their ability to interact with EB1 gene product. Thus, the interaction between APC and EB1 proteins may be important for the tumour-suppressor activity of APC protein, and raises the hypothesis that EB1 is also involved in sporadic colorectal tumorigenesis. To investigate this hypothesis, somatic mutations in the entire coding sequence of EB1 cDNA were searched by reverse transcriptase single-strand conformational polymorphism (SSCP) analysis in 21 sporadic colorectal cancers and seven adenomas. None of these tumours contained somatic mutation, whereas a silent cDNA variant was identified in 14% of alleles. Furthermore, to investigate whether EB1 locus was included within a region subjected to losses of heterozygosity, four polymorphism markers surrounding EB1 locus were surveyed. Only one out of 28 colorectal tumours contained a loss of heterozygosity at the D20S107 marker. In conclusion, the present findings strongly suggest that EB1 gene is not involved in somatic colorectal carcinogenesis.

Keywords: EB1: APC: SSCP: familial adenomatous polyposis

The adenomatous polyposis coli gene (APC) on chromosome 5q21 encodes a tumour suppressor which is assumed to act as a gatekeeper in colorectal carcinogenesis (Kinzler et al. 1996). The APC gene is mutated in about 75% of colon cancer cell lines (Smith et al. 1993) and 60% of sporadic colorectal cancers (Miyoshi et al. 1992; Powell et al. 1992; Miyaki et al. 1994). In these tumours, APC mutations are thought to be an early event as they have been found in small benign adenomas (Powell et al. 1992), as well as in putative precursor of colorectal adenomas, i.e. aberrant crypt foci (Smith et al. 1994a). In addition, inherited mutations of APC gene are responsible for familial adenomatous polyposis, an autosomal dominant disorder that predisposes to early development of colorectal cancer (Groden et al. 1991; Kinzler et al. 1991). Both germline and somatic mutations are almost exclusively nonsense or frameshift mutations encoding for truncated APC proteins lacking their carboxy-terminal half (Nagase et al. 1993; De Vries et al. 1996).

The APC gene encodes a cytoplasmic 284-amino-acid protein which is believed to act as a tumour suppressor, blocking the cell cycle progression in G1 (Baeg et al. 1995) and precipitating entry into apoptosis of the colorectal epithelial cells (Morin et al. 1996). In addition to these functional features, several biochemical interactions between APC gene product and other proteins have been demonstrated. As a matter of fact, the APC protein can form stable homodimer with its amino-terminal domains (Joslyn et al. 1993; Su et al. 1993), and can associate with several other proteins, including β-catenin (Rubinfeld et al. 1993; Su et al. 1993), plakoglobin (γ-catenin) (Shibata et al. 1994), tubulin (Munemitsu et al. 1994; Smith et al. 1994b), glycogen synthase kinase-3β (a mammalian homologue of ZW-3 kinase) (Rubinfeld et al. 1996), hDLG (a homologue of the Drosophila disc large tumour-suppressor gene) (Matsumine et al. 1996) and a human protein named EB1 (Su et al. 1995).

EB1 gene on chromosome 20q11.2 encodes for a novel 268-amino-acid protein which has been found to associate with the carboxy-terminus of APC protein (cdons 2167–2843) through a yeast two-hybrid screening (Su et al. 1995). Recent immunoprecipitation assay have shown that EB1 protein can also associate with β-catenin (Morin et al. 1996), which has been found to participate in the Wg/Wnt cell proliferation pathway. EB1 protein shares little sequence similarity to other proteins except a calcium channel from carp, the bacterial RNA polymerase δ-chain, the Saccharomyces cerevisiae gene product Yer016p (Su et al. 1995) and the closely related gene RPI (Renner et al. 1997). Although its function remains unknown, the recent characterization of a putative EB1 homologue in urochordate marine invertebrates suggests that EB1 protein has an important conservative cellular function (Pancer et al. 1996). Because virtually all of the APC mutations result in carboxy-terminal truncated products, APC mutant proteins are expected to lose their ability to interact with EB1 gene product. Thus, the interaction between APC and EB1 gene products may be important for the tumour-suppressor activity of APC protein, and raises the hypothesis that EB1 may also be involved in sporadic colorectal tumorigenesis. In order to investigate this hypothesis, we analysed 21 sporadic colorectal cancers and seven adenomas for EB1 point mutations and losses of heterozygosity.

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MATERIALS AND METHODS

Tumour specimens

Twenty-one sporadic colorectal adenocarcinomas, seven adenomas, and normal corresponding tissue specimens were obtained from 21 patients (11 males and ten females) with mean age 67.5 years (range 53–84 years) at the time of surgery at the Institut Gustave Roussy. According to Astler–Coller’s staging, adenocarcinomas were classified as A in one case, B1 in two cases, B2 in five cases, C1 in one case, C2 in four cases and D in eight cases. Nine adenocarcinomas were located in the right colon, eight were located in the left colon and four were located in the rectum. Seven tubular colorectal adenomas containing severe dysplasia in five cases and moderate dysplasia in two other cases were also analysed. Five adenomas were located in the right colon, and two were located in the left colon. The sporadic nature of these tumours was supported by the following criteria: (1) absence of tumour microsatellite instability searched with three A-monomonucleotide repeat loci (BATII, BAT26 and BAT40) essentially as described elsewhere (Markowitz et al. 1995; Liu et al. 1996); (2) presence of fewer than five colorectal adenomas; (3) and absence of Amsterdam’s criteria in the patient’s pedigree (Vasen et al. 1991).

All the tissues were snap frozen and stored in liquid nitrogen until analysis. Tumour tissues were dissected directly from the surrounding normal areas by light microscopically directed scraping of the specimens.

Reverse transcription–polymerase chain reaction (RT–PCR)

Total RNAs were isolated and purified using the modified guani-
dium phosphate buffer method (Chomczynski et al. 1987) from tumour-dissected specimens. Two micrograms of total RNA was reverse transcribed using 200 ng of random hexamers, 20 units of ribonuclease inhibitor, 200 μM of deoxyxucleoside triphosphate and 20 units of avian myeloblastosis virus enzyme (Perkins-Cetus, Foster City, CA, USA) according to manufacturer’s instructions.

Figure 1 Schematic location of primers pairs used for RT-SSCP analysis of the EB1 cDNA coding sequence. The open box represents the coding region of EB1 cDNA, whereas the 5' and 3' untranslated regions are shown as shaded boxes. The location of the different primers are indicated in brackets. The amplification was successively performed by amplifying the entire coding sequence using the primers S1 and AS3, then re-amplifying the PCR product into three overlapping segments using the primer pairs S1–AS1, S2–AS2 or S3–AS3

Figure 2 SSCP autoradiograph (A) and sense strand sequence (B) of the S3–AS3 fragment in normal (N) and tumour specimens (T). Specimens 1 and 3 are homozygous for 191th polymorphism, whereas 2 is heterozygous.
gels or without glycerol for 4°C gels. Electrophoresis was carried out for 14 and 18 h at 8 W constant power for 4°C and room temperature gels respectively. Gels were transferred to Whatman 3MM paper, dried on a vacuum-slab dryer and autoradiographed for 12–24 h with an intensifying screen. Base pair changes were identified by shifts in the pattern of single-stranded DNA conformers.

Sequence analysis

PCR products displaying abnormal pattern were purified using Microspin columns (Pharmacia, Uppsala, Sweden), and directly sequenced with the deoxytermination method using fluorescently tagged dideoxyribonucleoside (Applied Biosystems, Foster City, CA, USA) on the Applied Biosystems model 373A DNA sequencer. The sequences were compared with the published human EBI cDNA sequence (Genbank accession number U24166) using the Sequence Navigator package (Applied Biosystems).

Analysis of allelic loss

To determine whether EBI was included within a region undergoing losses of heterozygosity in colorectal cancers. DNA from dissected frozen tumour specimens and normal corresponding tissues were extracted using standard methods (Maniatis et al. 1989) and subjected to PCR amplification of the polymorphic microsatellite markers surrounding EBI at loci D20S112 (AFM197xb12), D20S195 (AFM321xc1), D20S107 (AFM142xb4) and D20S178 (AFM240vd6) (Chumakov et al. 1995). After amplification for 40 cycles with annealing temperature at 50°C and incorporation of [α-32P]dATP. PCR products were separated on 6% denaturing polyacrylamide gels (acylamide: N,N'-bisacrylamide: 28:2), and autoradiography was performed. Allelic loss was scored if the autoradiographic signal was at least 50% reduced when compared with the corresponding normal allele.

RESULTS AND DISCUSSION

To improve the specificity of the reaction. EBI cDNA was amplified in two steps. In the first step, the entire coding sequence was amplified using the primers S1 and AS3 in 28 sporadic colorectal tumour specimens and normal corresponding tissues. Amplification gave a unique product of 897 bp, indicating the lack of abnormal splicing of EBI mRNA. In the second step, SSCP analysis of both tumour and normal specimens was performed by reamplifying the previous PCR products with the primer pairs S1–AS1, S2–AS2 or S3–AS3. No change in the electrophoretic mobility was found in colorectal cancer specimens only, indicating the lack of somatic mutation. Seven tumours and normal corresponding specimens contained sequence alterations of the S3–AS3 fragment as shifts in the electrophoretic mobility of single-stranded conformers (Figure 2A). Sequence analysis found a silent polymorphism changing a C to T in the third base of codon 191 (191^C to 191^T) (Figure 2B). No additional difference with the published sequence was found when sequencing the entire coding sequence of EBI.

To determine whether EBI locus, which maps on chromosome 20q11.2 (Su et al. 1995), was subjected to losses of heterozygosity, four polymorphic microsatellite markers surrounding the EBI locus were amplified in both tumour and normal DNAs. The
normal tissues were heterozygous for all four markers in the 28 analysed tumours. Allelic loss at the locus D20S107 on the 20q11.2 region was found in only 1 out of the 28 tumours (Figure 3). This finding is consistent with previous allelotype analysis of colorectal cancers showing the low frequency of 20q chromosome losses (Vogelstein et al. 1989; Thorstensen et al. 1996). Incidentally, microsatellite instability was found in two cancers with only one dinucleotide marker.

In the present study, we have tested the hypothesis that EB1 alterations may occur alternatively to APC mutations in human sporadic colorectal cancers. Although APC mutations were not searched for in the 28 tumours of this study, we probably analysed several tumours containing wild-type APC gene, since no APC mutations are found in about 40% of colorectal cancers (Miyouli et al. 1992; Powell et al. 1992; Miyaki et al. 1994). Regardless of the APC gene status, the results described above provide evidence that EB1 is not subjected to point mutations and is not included within a region undergoing losses of heterozygosity in a significant number of human sporadic colorectal cancers. However, it remains possible that more subtle alterations, such as mutations in the promoter region or abnormal DNA methylation, could inactivate EB1 alleles in human colorectal cancers. As a matter of fact, changes in the methylation status have been shown to affect various genes involved in sporadic colorectal carcinogenesis, such as APC (Hiltunen et al. 1997), and hMLH1 (Kane et al. 1997).

In conclusion, the present data suggests that EB1 is not involved in the development of human sporadic colorectal cancers.

Note added in proof

Since the submission of this article, EB1 product has been shown to localize to microtubules in vivo, in both fission (Beinhauer et al. 1997) and budding yeast (Schwartz et al. 1997). In addition, the function of EB1 product has been recently clarified in yeast (Muhua et al. 1998). In those cells, mutation of EB1 homologue has been shown to abolish the cell-cycle delay induced by misalignment of the mitotic spindle. These findings suggest that EB1 may be necessary to maintain neutral ploidy through this cell-cycle checkpoint mechanism.

ABBREVIATIONS

APC. adenomatous polyposis coli; PCR, polymerase chain reaction; SSCP, single-strand conformational polymorphism; RT, reverse transcriptase; SDS, sodium dodecyl sulphate.

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