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• COLORECTAL CANCER •

Somatic mutations of APC gene in carcinomas from hereditary non-polyposis colorectal cancer patients

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Abstract

AIM: To investigate the mutational features of adenomatous polyposis coli (APC) gene and its possible arising mechanism in hereditary non-polyposis colorectal cancers (HNPCC).

METHODS: PCR-based *In Vitro* Synthesized Protein Test (IVSP) assay and sequencing analysis were used to confirm somatic mutations of whole APC gene in 19 HNPCC cases.

RESULTS: Eleven cases with 13 mutations were determined to harbor APC mutations. The prevalence of APC mutation was 58%(11/19). The mutations consisted of 9 frameshift and 4 nonsense ones, indicating that there were more frameshift mutations (69%). The frameshift mutations all exhibited deletion or insertion of 1-2 bp and most of them (7/9) happened at simple nucleotide repeat sequences, particularly within (A)n tracts (5/9). All point mutations presented C-to-T transitions at CpG sites.

CONCLUSION: Mutations of APC gene were detected in more than half of HNPCC, indicating that its mutation was a common molecular event and might play an important role in the tumorigenesis of HNPCC. Locations of frameshift mutations at simple nucleotide repeat sequences and point mutations at CpG sites suggested that many mutations probably derived from endogenous processes including mismatch repair (MMR) deficiency. Defective MMR might affect the nature of APC mutations in HNPCC and likely occur earlier than APC mutational inactivation in some patients.

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INTRODUCTION

Familial colorectal cancer (CRC) can be mainly divided into 2 distinct classes, hereditary non-polyposis colorectal cancers (HNPCC) and familial adenomatous polyposis (FAP). HNPCC is an autosomal dominant inherited disease characterized by an increased predisposition to colorectal and some extracolonic cancers and presents with particular clinicopathological and molecular features[1]. Affected individuals often carry germline alterations at mismatch repair (MMR) genes, such as hMsh2, hMlh1, hPsm1 and hPsm2, which lead to microsatellite instability (MSI) and replication errors (RER). FAP is caused by the germline mutation of tumor suppressor gene APC and manifested by the development of hundreds of colorectal adenomas, some of which will inevitably undergo malignant transformations if not removed. APC mutations can be detected in both FAP and non-FAP tumors including HNPCC and sporadic colorectal ones, somatic mutation of which is a common defect in sporadic colorectal tumor and FAP, both sharing similar mutational spectra and distribution[2,3-5]. It is accepted that adenoma-carcinoma sequence of CRC was initiated by APC mutation in majority of non-HNPCC[2,6]. Adenoma in HNPCC featured aggressiveness and followed by an alternative and rapidly evolving pathway. There was evidence that HNPCC lacked loss of heterozygosity (LOH) of APC gene, being quite different from FAP[7]. It showed that all adenomas from *Min* mice demonstrated LOH, had significantly less numbers of somatic APC mutations compared with those from Msh2 deficient Min (Apc+/-Msh2-/-), which had a 2-fold higher rate of somatic Apc mutations (10/adenoma) than the non-neoplastic intestinal mucosa (5/sample), and did not demonstrate LOH. The results proved that somatic APC mutation rather than LOH, was a likely mechanism in the development of adenomas in Apc+/-Msh2-/- mice[8].

Studies also revealed[9,10] that APC gene might play a role in chromosomal segregation, with mutations in APC disrupting this function and causing chromosomal (or karyotypic) instability (CIN). MSI and CIN were two different forms of genetic instability occurring in CRC. In MI^+ sporadic and HNPCC tumors, defective repair of mismatched bases resulted in an increased mutation rate at the nucleotide level and consequent widespread MSI with diploidy while a large fraction of others had CIN, leading to an abnormal chromosome number (aneuploidy). In HNPCC, mutations of APC, p53 and K-ras-2 genes and LOH of tumor-suppressor genes were significantly less frequent (P=0.03 or 0.0006), but transforming growth factor beta type II receptor mutation was significantly more frequent (P=0.000001) than those in non-HNPCC[7]. Summarized data from Jass[11] supported that the frequency of APC mutation was significantly lower in MSI-H colorectal tumors (39%, 36/92) and HNPCC (44%, 21/48) than in MSI-L (51%, 18/35) and MSS (58%, 241/417). However, the situation was quite different for 5q LOH in MSI-H tumors (3%;1/32) and HNPCC (10%;1/10), which were significantly less than MSI-L (47%;17/49) and MSS (57%;113/199). This suggested that MSI instead of APC mutation probably confered a growth advantage in the context of early tumorigenesis of HNPCC and MSI-H tumors. Here we focused on APC gene to analyze its mutational frequency and features in carcinomas from HNPCC, which would be helpful to understand the effect of MMR deficiency on APC mutation in the early stage of carcinogenesis.

Based on the fact that both somatic and germline mutations of APC gene were dispersed throughout the 5' half of the sequence, vast majority of which were premature ones and led to truncated proteins[3-5]. A Coupled In Vitro Synthesized

Protein Test (IVSP) was therefore used to selectively and rapidly detect any mutations leading to stop codons[12-14]. The assay was proved very efficient and only a small proportion of nontruncating APC mutations like missense mutations were reported to be missed[15]. The study aimed at exploring the possible arising mechanism of somatic APC mutation and its role through investigating frequency and mutational features of the gene in HNPCC.

MATERIALS AND METHODS

Sample collection and DNA extraction

HNPCC was determined according to ICG criteria based on the family history[16], fresh tumor samples of the patients were from resected and histologically verified colorectal carcinomas. Genomic DNA and RNA of tumors and matched normal-looking colonic mucosa were isolated by standard procedures. Nineteen samples were collected from paired carcinomas and adjacent normal tissues in HNPCC patients.

Determination of APC mutation

Whole APC gene with at least 15 exons (8.5Kb) was divided into 5 overlapping segments (seg.), Exon 1-14 was defined as seg. 1 and exon 15 amplified with four overlapping segments as seg. 2-5. The RT-PCR primer-pair sequence for seg. 1 and PCR primer-pairs for seg. 2-5 was similar to those reported[12,13,15]. IVSP assay, which selectively detected truncating mutations, was used to screen mutations of whole APC gene segment by segment. All samples showing producible truncated protein bands were sequenced using Sequenase 2 (United States Biochemical) with ^{35}S-labled dATP, dried sequence gels were exposed to XAR (Kodak) film at room temperature overnight.

Table 1 Spectra of APC mutations in carcinomas from HNPCC patients

Tumor ID	APC nt change	Codon	Target sequence
Point mutations			
T27	C>T	332	CpG
T533	C>T	1 338	CpG
K1, C×7*	C>T	1 450	CpG
Frameshift mutations			
C×7*	1 bp ins	847	TCTCaAAAAAGAT
C086*	1 bp ins	941	TCGGaAAAATTCA
K40	1 bp ins	1 454	CCTaAAAAATAAA
22	1 bp ins	1 554	GCAGaAAAAAACTAT
Cx10	1 bp ins	1 935	TTTCCCCaAGTCA
K10	1 bp del	907	TCTgGGTCT
C086*	2 bp del	1 464	AAGAGAGagAGTGG
K39	2 bp del	799	GATtaTGTT
T10	1 bp del	1 416	AGTGGcATTATA

Note: ID means tumor identification; nt stands for neocleotide; '>' for transition or transvertion; each tumor with more than 2 mutations marked by *; del for deletion; ins for insertion; small letter for nucleotide frameshift.

RESULTS

Spectra of APC mutations in HNPCC are listed in Table 1. Screening for truncating APC mutations was carried out using IVSP assay in carcinomas from 19 HNPCC cases. There were altogether 11 mutated cases with 13 mutations confirmed in the study, all of which were only found in the tumor tissues, indicating the somatic nature of mutation. The prevalence of APC mutation in HNPCC was 58%(11/19). Exhibiting mutations consisted of 9 frameshift ones and 4 nonsense, indicating the existence of more frameshift mutations. All of frameshift mutations were deletion or insertion of 1-2 bp and most of them (7/9) happened at simple nucleotide repeat sequences, particularly within (A)n tracts (5/9). Repeat number (n) of mononucleotide A ranged from 2 to 7. All of four detected point mutations presented C-to-T transitions at CpG sites in the study. Distributions of mutations were uneven and with no hot spot but hot region. Eight (61.5%) of thirteen mutations were located in the segment 3 (codon 1099-1701) and half of them in the mutation cluster region (codon 1286-1513).

DISCUSSION

Colorectal cancer is the fourth leading cause of cancer deaths and tends to increase in China, especially in big cities like Shanghai. The basic mechanism of reducing the mortality remains elusive and poses a big challenge for clinicians and researchers.

HNPCC is one of the most common inherited cancer syndromes, accounting for approximately 4-6% of all CRCs. It is characterized by defective MMR, which results in MSI and manifests hypermutable phenotype[17,18]. In our study, MSI was defined in all but one (T16) of HNPCC cases (data not shown).

In contrast to FAP, HNPCC was usually not associated with LOH of APC allele and therefore not develop extensive polyposis but multiple primary cancers. MMR gene responsible for HNPCC functioned as caretaker and indirectly inhibited the promotion of tumor growth, defective MMR was considered to be primary event because of its occurrence throughout the adenomas in HNPCC[19,20]. It had been reported that CRC with and without MSI involved different genes including APC[21]. APC gene, a FAP causing gene, was proven to be rate-limiting for tumor initiation and functioned as housekeeper of cellular proliferation. APC mutation provided cells with a selective advantage without known effect on the mutation rate, it suggested that both APC and MMR genes played an important role in the early carcinogenesis of familial CRC. Accumulating evidence actually revealed that the sequence of alteration of cancer genes in MMR deficient tumor was somewhat different from non-HNPCC[7,21]. The study was to investigate the mutational features of housekeeping gene APC and its potential arising mechanism in carcinomas with deficiency of caretaking gene MMR, in order to evaluate the role of APC gene alteration in HNPCC.

There were 11(58%) of the 19 HNPCC carcinomas exhibiting APC somatic mutations, which was a bit higher than that (50%) reported in a series of Japanese HNPCC adenomas[22] and somewhat lower than that (67%) in sporadic CRC[4,5]. The result implied that inactivation of APC gene also played an important role in the tumorigenesis of HNPCC. But there was evidence that the frequency of APC gene mutation in HNPCC was likely less than that in non-HNPCC (21% *vs* >70%; $P<0.05$)[23].

Analysis of the spectra of APC mutations revealed that APC frameshift mutations were more frequent than point ones in HNPCC (69.3% *vs* 30.7%; $P<0.05$). As with frameshift mutations, most of them (67%) occurred in the simple repeat sequences (microsatellite) of the APC coding regions, particularly at (A)n tracts (56%), which was consistent with the effect of mismatch repair deficiency on mutations of other target genes containing short repeated sequences in the coding regions[24]. It suggested that MMR deficiency might be one of the important pathway leading to inactivation of its tumor suppressor ability by altering microsatellite of the APC coding regions. Animal studies further supported the notion that APC mutations in Msh6(-/-)Apc1638N mice consisted predominantly of base substitutions (93%) creating stop codons, however, in Msh3(-/-)Msh6(-/-)Apc1638N tumors, a mixture of base substitutions (46%) and frameshifts (54%) was observed, indicating that Msh3 could suppress frameshift mutations of Apc gene in Msh6(-/-)Apc1638N mice[25]. The observation demonstrated that type of APC mutation was closely related

to the function of specific MMR gene while in Mlh1-/- Apc1638N animals, the prevailing mechanism of APC mutation in tumors was altered from allelic loss to intragenic mutation as a result of Mlh1 deficiency, the observed mutations were more frameshifts (73%) than base substitutions (27%), most frameshifts were located within dinucleotide repeats[26]. We therefore hypothesized that defective MMR might precede APC mutation in some HNPCC cases whereas for those frameshifts not showing microsatellite alterations, the initiation mechanism for these tumors was not clear yet and proposed to be similar to that for sporadic CRCs[2,3,5,12], which meant that APC mutation, rather than genomic instability, might be the initiating event in tumorigenesis.

With respect to point mutations of APC gene, they were all non-sense mutations and with pathogenic nature because of the generation of stop cordons. All these mutations in the study presented C-to-T transitions and affected Cog dinucleotides, suggesting that they probably derived from endogenous processes due to spontaneous deaminations of 5-methylcytosine. Further studies were therefore necessary to clarify the origin and relationship between endogenous and exogenous processes of APC mutations in CRC tumorigenesis.

In HNPCC cases without detected APC mutations, it was still of great importance to determine methylated status of APC gene or mutation of other genes like β-catenin, although more sensitive methods needed to be developed or used. Yamamoto reported that DNA methylation of APC was higher in HNPCC than in sporadic CRC with MSI-H (53% *vs* 35%)[27]. Miyaki observed that a notable frequency of β-catenin gene mutation (43%) was found to occur in HNPCC and detected in tumors without APC mutations[23]. These data suggested that activation of the β-catenin -Tcf signaling pathway, through either β-catenin mutation or silence (methylation) of APC, might aslo contribute to part of HNPCC colorectal carcinogenesis.

In conclusion, inactivation of APC gene played an important role in the tumorigenesis of HNPCC; Most of APC mutations probably derived from endogenous processes including MMR deficiency and presented some mutational features. Defective MMR might occur earlier than APC mutation in some patients with HNPCC.

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