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ORIGINAL ARTICLE

May the APC gene somatic mutations in tumor tissues influence the clinical features of Chinese sporadic colorectal cancers?

XIAORONG LIU[1], XIANGNIAN SHAN[1], WALTRAUT FRIEDL[3], SIEGFRIED UHLHAAS[3], PETER PROPPING[3], JINTIAN LI[4] & YAPING WANG[2]

[1]*Department of Genetics, Medical School, South East University, 210009, Nanjing, P. R. China,* [2]*Laboratory of Medical Genetics, Medical School, Nanjing University, 22 Hankou Road, 210093, Nanjing, P. R. China,* [3]*Institute of Human Genetics, University Hospital Bonn, Wilhelmstrasse 31, D-53111 Bonn, Germany and* [4]*Department of Molecular Biology, Jiangsu Institute of Cancer Research, 210000, Nanjing, P. R. China.*

Abstract

The *APC* gene plays an important role in colorectal carcinogenesis. The impact of *APC* mutations on the clinical features in sporadic CRC remains to be uncovered. The *APC* gene was screened for mutations with systematic analysis techniques including DHPLC, PTT, MLPA and DNA sequencing in 43 Chinese sporadic CRC patients. Twenty nine somatic mutations (in 17 different types) in *APC* gene were found in 18 of 43 sporadic CRC patients. Of those, nine were novel mutations. Higher frequency of somatic *APC* mutations was found in younger CRC patients than that in elder ones. The biallelic somatic mutations of *APC* gene were identified in four CRC patients whose tumors had more invasive clinical features. The nonsense mutation Arg1114X in *APC* gene was found in five of 43 CRC tumor tissues. A higher cancer metastasis rate was uncovered in CRC patients with this mutation. The somatic mutations of *APC* gene may influence the clinical features of sporadic CRC. Arg1114X in APC gene, as a hot spot mutation in Chinese CRC, may predispose to the cancer metastasis of sporadic CRC.

The *APC* gene acts as a gate-keeper for the development of colorectal cancers (CRC). Most of CRC are thought to follow a genetic pathway involving APC. Mutations in the *APC* gene are considered as one of the earliest events both in the initiation and progression of sporadic CRC and FAP-associated disease [1,2].

The *APC* has been regarded as a classical Knudson-type tumor suppressor gene. It is generally accepted that tumors start to grow in the colorectum of the FAP patients when an appropriate cell acquires a somatic mutation at the *APC* locus to accompany a preexisting germline mutation. In part of the sporadic tumors of the colorectum, two inactivating APC mutations often occur, one leading to a truncated protein and the other being a similar or changed mutation resulting in allelic loss (so called biallelic *APC* mutation) [3,4]. However, whether the biallelic *APC* mutations have impact on the progression of the tumors in sporadic CRC remains to be proved.

It has been reported that the clinical variability in FAP might be related to the positions of germline mutations in *APC* gene [5–9]. However, the relationship between somatic mutations of *APC* gene in tumor tissues and clinical features of sporadic CRC has seldom been investigated. It is necessary to uncover the mutation spectrum of the APC gene in sporadic CRC if a clue about the "genotype-phenotype" correlation of *APC* gene in sporadic CRC were expected.

The mutation frequency and spectrum of *APC* gene in sporadic CRC varies among studies [10]. Two reasons might be considered, the first is that the *APC* gene is composed of 8 529 base pairs from 15 exons in genomic DNA. It is comprehensively difficult to perform a completed sequence screening of this gene. The second, mutation cluster region

Correspondence : Wang Yaping, Department of Medical Genetics, Medical School, Nanjing University, 22 Hankou Road, 210093, Nanjing, P. R. China. Tel: +86 25 83686495. Fax: +86 25 83686559. E-mail: wangyap@nju.edu.cn

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(MCR) reported in *APC* gene has understandably conducted some studies to concentrate mutation checking in this region. Here we scan the *APC* gene in sporadic CRC by several techniques to show the frequency and spectrum of *APC* mutations and compare the advantages of different techniques in mutation screening in the *APC* gene. And at the same time, correlation of clinical features and *APC* mutations in sporadic CRC would also be analyzed.

Materials and methods

Subjects

A total of 43 index patients with sporadic CRC had undergone tumor resection before receiving chemotherapy or radiotherapy. Ages of these patients ranged from 31 to 77 years old (mean age 57 years). Thirteen of them were diagnosed at a younger age ($<$50 years old), 23 around mean age (from 50 to 69 years old) and seven were older ($\geq$70 years old). All of CRC patients in this study had been diagnosed by histopathology.

With regard to tumor localization, two anatomical sites were considered: Sixteen of them were colon cancers and 27 rectal cancers. Tumor grading was defined according to WHO directives, 35 of them presented middle differentiated adenocacinoma (II) and eight low differentiated (III). All cases were staged using a modified Dukes' system: A, cancer limited to bowel wall (4 cases); B, with extramural spread (24 cases); C, with lymph node metastasis (14 cases); D, with distant metastasis (1 case). We also evaluated the pattern of cancer growth for the samples studied: expanding (17 cases) or infiltrating (26 cases).

Informed consent was obtained from all patients included in this study.

Sample collection

Genomic DNA was extracted from both normal colorectal tissues and tumor tissues of sporadic CRC patients for screening both germline and somatic mutations in *APC* gene.

Mutation analysis

Three techniques were used to analyze different kinds of mutations in the *APC* gene.

Denaturing high-performance liquid chromatography (DHPLC). DHPLC is a sensitive technique with high throughout-put to detect small mutations (small deletions or insertions leading to frameshifts, splice site alterations, nonsense and missense mutations) in DNA fragments with length of 100–500 bp. The exons 1 to 14 of *APC* gene, each of them in range of 100–400 bp, were analyzed by DHPLC (WAVE system, Transgenomic). Oligonucleotide primers used in PCR for DHPLC had been reported previously [11]. Assay conditions (acetonitrile gradient and temperature profile) were optimized for DHPLC of DNA fragment with a bank of samples previously characterized by DNA sequence. The PCR products which showed variant peaks in DHPLC were analyzed by direct sequencing (ABI 3100, Applied Biosystems).

Protein truncation test (PTT). PTT can sensitively detect a truncating mutation in gene fragments of up to 2 kb in one performance and is commonly used to check mRNA for mutations. Exon 15 of *APC* gene is composed of 6 571 bp. PTT that allows the detection of truncated protein was performed with genomic DNA to screen mutation in this exon of *APC* gene in higher efficiency. Exon 15 was amplified in four overlapping fragments from genomic DNA templates by primers that had been reported [11,12]. Resultant PCR products were used in a coupled transcription-translation reaction (Promega) incorporating ^{35}S-labeled methionine. Labeled protein products were analyzed by 8% SDS/PAGE. The samples showing truncated protein products were analyzed by direct sequencing for characterization of the mutations (ABI 3100, Applied Biosystems).

Multiplex ligation-dependent probe amplification (MLPA). MLPA was used to detect the large fragment deletion or duplication in *APC* gene in the checked samples on the reason that the large fragment variations in *APC* gene were suggested to represent a frequent cause of FAP [7,13]. The mixture of probes in MLPA contains 20 probes for test *APC* gene (two of them for the promotor region, one for the 5'untranslated sequence, 14 for exon 1–14, and three for exon 15 of *APC* gene), and 11 control probes specific for DNA sequences outside the *APC* gene (MRC-Holland). MLPA was performed according to the protocol supplied with the kit.

Statistic analysis

The statistic evaluations were performed with Nonparametric test (Wilcoxon rank test) and χ^2 test (Fisher's exact test). A significant difference was considered if p-value (two-tail) was less than 0.05.

Results

The combined use of DHPLC, PTT, MLPA and DNA sequencing for mutation analysis in *APC* gene allowed the detection of 29 somatic mutations in 18 samples of 43 sporadic CRC patients. No germline mutation of *APC* gene was discovered in sporadic CRC patients.

Somatic mutations of APC gene in sporadic CRC

In this study, we identified 29 somatic mutations (in 17 different types) in the tumors of 18/43 (42%) patients with sporadic CRC (Table I). Among the 18 patients with *APC* gene somatic mutations, 11 of them have only one mutation and seven have 2–4 mutations in *APC* gene. The mutations detected in this study were distributed in exons 3, 9, and 15. Of 17 mutation types detected in this study, eight (about 48%) were deletions or insertions (one of them was 91 bp deletion uncovered by PTT in exon 15 of *APC* gene), and nine (52%) were single-base pair substitutions resulting in a nonsense mutation. Interestingly, near 80% of the single-base pair substitutions showed a transition of C:G to T:A. All of the mutations would introduce premature termination signals from which a truncated protein of *APC* gene was produced. Nine of the mutations detected in this study had not been presented in the *APC* mutation database so far (http://www.hgmd.cf.ac.uk).

No large genomic deletion was detected in *APC* gene in tumor tissues checked by MLPA. We also checked the DNA samples from normal colon tissues of these patients in whom somatic mutation had been unveiled in their tumor tissues. No germline mutation was found in them.

Relationship between somatic mutations in APC gene and the clinical features of sporadic CRC

Different frequencies of somatic *APC* gene mutations were uncovered among the groups on the age of CRC diagnosis (Table II). Statistical analysis showed that a significantly higher frequency of somatic mutations in *APC* gene existed in the tumors of younger CRC patients (Wilcoxon rank test: $p = 0.048$). A negative correlation was unveiled between the diagnosis age of patients disease onset and the mutation frequency found in *APC* gene (Pearson correlation coefficients: $r = -0.382$, $p = 0.012$).

Table I. Somatic *APC* mutations detected in tumors of sporadic CRCs.

Nr	Exon	Nucleotide change	Target sequence of mutation	Consequence
CRC2	15	c.4348C >T	CGA →TGA	p.Arg1450X
CRC4	3	c.421_422delAG*	AGAGAGGT →AGAGGT	Framshift
	9	c.994C >T	CGA →TGA	p.Arg332X
	15	c.2626C >T	CGA →TGA	p.Arg876X
	15	c.4663insA	AAAAAA →AAAAAAA	Framshift
CRC5	15	c.2560A >T*	AGA →TGA	p.Arg854X
CRC7	15	c.4270_4360del 91bp*	CTTCCA…AAAAAT →CCT…AAAAT	Frameshift
CRC9	9	c.994 C >T	CGA →TGA	p.Arg332X
	15	c.4663insA	AAAAAA →AAAAAAA	Framshift
CRC13	15	c.4446_4455del 10bp*	TCTTCC…GCTGAT →TCTGAT	Frameshift
CRC15	15	c.4393_4394delAG	AGAGAGAGAGTG →AGAGAGAGTG	Frameshift
CRC16	9	c.1143insG*	CGGGCCAGGGCC →CGGGCGCAGGGCC	Frameshift
	15	c.3340C >T	CGA →TGA	p.Arg1114X
CRC18	15	c.2626C >T	CGA →TGA	p.Arg876X
	15	c.3340C >T	CGA →TGA	p.Arg1114X
	15	c.3566C >A*	TCA →TAA	p.Ser1189X
CRC26	15	c.2872A >T*	AGA →TGA	p.Arg958X
CRC29	15	c.2872A >T*	AGA →TGA	p.Arg958X
CRC31	15	c.4132C >T	CAG →TAG	p.Gln1378X
CRC32	15	c.3340C >T	CGA →TGA	p.Arg1114X
	15	c.4348 C >T	CGA →TGA	p.Arg1450X
CRC33	3	c.421_422delAG*	AGAGAGGT →AGAGGT	Framshift
	15	c.2926insA	GGTAAAAGA →GGTAAAAAGA	Frameshift
	15	c.4393_4394delAG	AGAGAGAGAGTG →AGAGAGAGTG	Frameshift
CRC37	15	c.3340C >T	CGA →TGA	p.Arg1114X
CRC38	15	c.4234delG*	AGGGAATG →AGGAATG	Frameshift
CRC40	15	c.2555T >A*	TTG →TAG	p.Leu852X
	15	c.4348 C >T	CGA →TGA	p.Arg1450X
CRC43	15	c.3340C >T	CGA →TGA	p.Arg1114X

*novel mutations.

Table II. Correlation between *APC* mutations and the age of diagnosis of the CRC.

Age of diagnosis (yrs)	With 1 mutation	With ≥2 mutations	Without mutation	Ratio of mutation
~49	3	6	4	69.2%
50~69	7	1	15	34.7%
≥70	1	0	6	14.2%
	Wilcoxon rank test: p = 0.048			
	Pearson correlation Coefficients: r = −0.382, p = 0.012			

The correlation between the somatic mutations of *APC* gene and the clinical/pathological features of sporadic CRC patients were analyzed as well. Of 18 CRC patients with somatic mutation of *APC* gene, seven were detected to have two or more mutations. With the results of PTT, four of them (CRC4, 18, 32, 33) were identified as harboring biallelic mutations in the exon 15 of *APC* gene. However, the biallelic mutations could not be confirmed in the other three patients with two or more somatic mutations in *APC* gene. All of the four CRC patients with biallelic mutations of APC gene were at early diagnosis ages (<50 years) and have a similar infiltrating pattern of cancer growth (Table III). A higher frequency of somatic mutation in *APC* gene was found in the CRC patients with tumor of Dukes' stage C/D when comparing with the patients with tumor of Dukes' stage A/B (Fisher's exact test: p = 0.03). On the other hand, a less frequency of somatic mutation in *APC* gene was found in the tumor tissues with expanding growth pattern than that with infiltrating pattern (Fisher's exact test: p = 0.001). But, no significant difference of the frequency of somatic *APC* mutations was found between colon and rectal cancers (p = 0.207), and between different tumor grades (p = 1.000).

The nonsense mutation at codon 1114 (Arg1114X) in *APC* gene was frequently uncovered in this study. It was found in 5 of 43 (11.6%) tumor tissues. All of the patients with this mutation had their cancer metastasis (Table IV). Statistical analysis showed that a significant difference of cancer metastasis rate existed between the CRC patients with and without this mutation in *APC* gene (p = 0.036).

Discussion

An effective analysis system is essential to complete mutation screening and molecular diagnosis due to the diversity of APC gene variations. In this study, combination of three methods (DHPLC, PTT and MLPA) for mutation analysis in *APC* gene is based on the characteristics of the three techniques and the structure of the *APC* gene. Variable frequencies of *APC* mutations have been reported in human CRC, ranging from 40% to 80% [9,10,14–16]. The differences of mutation frequency in the literatures were probably explained by variations in the methodologies used to screen mutations in *APC* gene.

In our results, nearly 30% of the somatic mutations were deletions, 18% and 52% were insertions

Table III. Correlation between *APC* mutations and the age/pathological features of tumors.

	With biallelic mutations	With allelic mutations	Without mutation	p-value*
Age of diagnosis				
<50 (yrs) (n = 13)	4	5	4	p = 0.004
≥50 (yrs) (n = 30)	0	9	21	
Tumor localization				
Rectum (n = 27)	2	7	18	p = 0.54
Colon (n = 16)	2	7	7	
Grading				
II (n = 35)	3	12	20	p = 1.000
III (n = 8)	1	2	5	
Dukes' stage				
A/B (n = 28)	1	7	20	p = 0.03
C/D (n = 15)	3	7	5	
Pattern of cancer growth				
Expanding (n = 17)	0	2	15	p = 0.003
Infiltrating (n = 26)	4	12	10	

*Fisher's exact test.

Table IV. Correlation between mutation at codon1114 and the metastasis status of the tumors.

	With metastasis	Without metastasis
With mutation at codon1114	5	0
With mutations at another loci	5	8
p-value*	0.03	

*Fisher's exact test.

and single-base pair substitutions respectively. Most of deletions and insertions (nearly 70%) in the *APC* gene were observed at positions containing repeated sequences and nearly 74% of single-base pair substitutions were C:G to T:A transitions. It is similar to the results on the western CRC patients [10,16–19]. As we know, the high frequency of deletion and insertion at the positions of repeated sequences suggested to be caused by DNA replication errors, and deamination of 5-methylcytosine in the CpG dinucleotide had been implicated as a cause for the transition of C:G to T:A. So a problem facing now is that whether the type of *APC* mutations in sporadic CRC is affected by some other genes, or only decided by the impact of environmental carcinogens. Further work need to be done to elucidate the mechanism of these mutations.

However, a difference in the pattern of distribution of *APC* mutations was observed between Chinese and western CRC patients (Figure 1). Compared with the results from western sporadic CRC patients, the truncating mutations at codon 958 and 1114 detected in this study were at a distinct high frequency in Chinese sporadic CRC patients. On the other hand, mutations at codon 1309, 1307 and 1465 that were frequently found as germline and somatic mutations in FAP and CRC patients had not been detected in this work. Lower frequencies of these mutations might impute to the small sample set here. Additionally, whether mutations at codon 958 and 1114 are the hot spots in Chinese CRC should be confirmed by work on a larger sample set in the future.

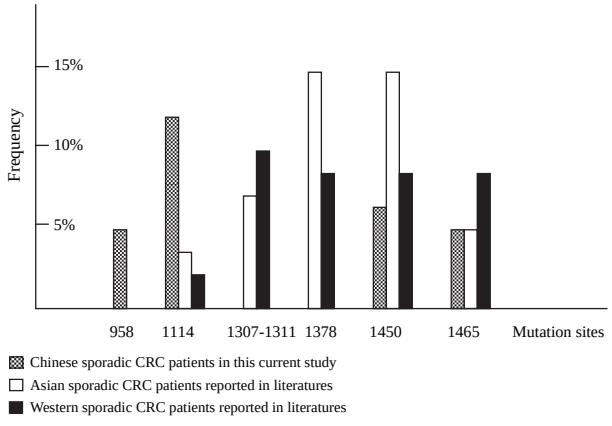

Figure 1. Hot spots of APC somatic mutations in western and Asian sporadic CRC patients.

It is generally accepted that inactivation of both alleles at the *APC* locus is required for development of most tumors in the colon and rectum. Inactivation of the *APC* gene by two (even more) mutations was also observed in sporadic CRC tumors in this study. Higher frequency of somatic *APC* mutations, even with more than one mutation, in the young CRC than that in the elder CRC patients suggested that a genomic instability might exist in the cells of young CRC patient. In this study, the patients with biallelic somatic mutations in tumor tissue had more invasive clinical features, including early age of disease onset, infiltrating pattern of cancer growth and Dukes' stage C/D of tumors. These indicated that the functional state of *APC* gene in CRC would affect the tumor biological characteristics.

Although accumulated works has been done on the somatic *APC* mutation analysis in CRC patients, a few of them were on the relation to clinical features of CRC and the mutations detected [19]. However similar work has seldom been done in Chinese CRC patients. The results in this study showed that somatic mutation at codon 1114 may be a hot spot in Chinese sporadic CRC and patients with this mutation showed a higher rate of cancer metastasis. As we know, the sequence between codon 1020 and 1169 of *APC* gene translated the first domain of APC protein for interaction with β-catenin which is important for activation of E-cadherin, a Ca(++)-dependent adhesion molecule that controls cell motility by affecting cell migration and morphogenesis [20,21]. Our result suggests that mutations occurring in this motif may expedite the tumor metastasis in sporadic CRC. Nevertheless, further work should be performed on the functional significance of this mutation to confirm whether it can be consider as a molecular marker for CRC metastasis.

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