RESEARCH ARTICLE

Analysis of Small Fragment Deletions of the APC gene in Chinese Patients with Familial Adenomatous Polyposis, a Precancerous Condition

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Abstract

Background: : Familial adenomatous polyposis (FAP) is an autosomal dominant inherited disease mainly caused by mutations of the adenomatous polyposis coli (*APC*) gene with almost complete penetrance. These colorectal polyps are precancerous lesions that will inevitable develop into colorectal cancer at the median age of 40-year old if total proctocolectomy is not performed. So identification of *APC* germline mutations has great implications for genetic counseling and management of FAP patients. In this study, we screened *APC* germline mutations in Chinese FAP patients, in order to find novel mutations and the *APC* gene germline mutation characteristics of Chinese FAP patients. Materials and Methods: The FAP patients were diagnosed by clinical manifestations, family histories, endoscope and biopsy. Then patients peripheral blood samples were collected, afterwards, genomic DNA was extracted. The mutation analysis of the *APC* gene was conducted by direct polymerase chain reaction (PCR) sequencing for micromutations and multiplex ligation-dependent probe amplification (MLPA) for large duplications and/or deletions. Results: We found 6 micromutations out of 14 FAP pedigrees, while there were no large duplications and/or deletions found. These germline mutations are c.5432C>T(p. Ser1811Leu), two c.3926_3930delAAAAG (p.Glu1309AspfsX4), c.3921_3924delAAAA (p.Ile1307MetfsX13), c3184_3187delCAAA(p.Gln1061AspfsX59) and c4127_4126delAT (p.Tyr1376LysfsX9), respectively, and all deletion mutations resulted in a premature stop codon. At the same time, we found c.3921_3924delAAAA and two c.3926_3930delAAAAG are located in AAAAG short tandem repeats, c3184_3187delCAAA is located in the CAAA interrupted direct repeats, and c4127_4128 del AT is located in the 5'-CCTGAACA-3' ,3'-ACAAGTCC-5 palindromes (inverted repeats) of the *APC* gene. Furthermore, deletion mutations are mostly located at condon 1309. Conclusions: Though there were no novel mutations found as the pathogenic gene of FAP in this study, we found nucleotide sequence containing short tandem repeats and palindromes (inverted repeats), especially the 5 bp base deletion at codon 1309, are mutations in high incidence area in *APC* gene,.

Keywords: Familial adenomatous polyposis - *APC* gene - germline mutation - colorectal cancer

Introduction

Familial adenomatous polyposis (FAP, OMIM ID: 175100) is a kind of autosomal dominant disorder characterized by hundreds to thousands of colorectal adenomatous polyp (Keiko et al., 2014). FAP is a precancerous disease, which almost all patients will develop into colorectal cancer (CRC) without identifying early and colorectal surgery in time (Song et al., 2013). FAP patients will present with multiple colorectal polyps of phenotypes, various from dozens to thousands, such as classic FAP (more than 100 colorectal polyps) and attenuated FAP (usually 20-100 colorectal polyps) (Yousef et al., 2006; Half et al., 2009). The FAP patients may have

also extracolonic manifestations, including osteomas, congenital hypertrophy of the retinal pigment epithelium (CHRPE), upper gastrointestinal polyps (in the stomach or duodenum), desmoid tumors, dental abnormalities, and tumors in other organs (thyroid, pancreas, liver, and adrenal gland) (Jang et al., 2010). FAP is estimated to have a prevalence of 2 to 3 per 100,000 individuals (Sarah et al., 2013) and is found in all ethnic groups. Now, adenomatous polyposis coli (APC) gene (OMIM#175100; http://www.ncbi.nlm.nih.gov) inactivation is recognized as the main pathogenesis cause of FAP. The APC gene located on chromosome 5q21~q22, meanwhile it contains 15 exons and encodes a commonest 10.7 kb transcript, whose open reading frame (exons 1–15) can translate into

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a 2843 amino acid and 312 kDa protein (Kittiyod et al., 2012; Keiko et al., 2014). The APC protein containing multiple domains is a multi-function protein, which has an important role in Wnt signal transduction, cell adhesion, cytoskeleton stability, and transcription-independent-mediated apoptosis (Rustgi, 2013; Zhang et al., 2013). Up to now, more than 1400 different APC mutations have been identified in FAP patients, which contains more than 800 kinds of germline mutations, and the rest is somatic mutations (Zhang et al., 2014). Close to 95% of these pathogenic alterations in APC gene are nonsense or frameshift mutations that lead to a premature stop codon formation, resulting in truncated APC proteins (Stenson et al., 2003). About 60-80% of FAP families can be detected APC mutations (van der et al., 1997), and 20-25% of them are de novo mutations. The diagnosis standard for FAP is an individual with more than 100 colorectal adenomas occurring prior to 40 years of age, or one who has less than 100 adenomatous polyps but has family history of FAP or with congenital hypertrophy of the retinal pigment epithelium (CHRPE) (Stekrova et al., 2007; Kittiyod et al., 2012). As these polyps are precancerous lesions, patients will inevitable progress into colorectal cancer in almost all patients at the median age of 40 years if total proctocolectomy is not performed (Sarah et al., 2013). So it is important to identify APC germline mutations for genetic counseling and management of FAP patients.

The occurrence of gene mutations is not only affected by the exogenous causes, such as radiation or chemical mutagens, but also has its endogenous mechanisms, such as the mutation sequence base surroundings (Krawczak et al., 1991). The mutational "hotspots" detected in phage T4 indicated that the local DNA sequence environment may be strongly influence the frequency of mutational lesions (Michael et al., 1991). Moreover, two mutation hotspots in APC gene: codon 1309 and codon 1061 were found in 10 % and 5 % FAP patients respectively (Half et al., 2009). Therefore, studying of gene mutation would help us to understand the molecular mechanisms underlying mutagenesis, so far as to construct explanatory models which have predictive value for the candidate gene. Up to now, there has been formed a variety of hypothesis to explain the genetic mutations, such as 5-methyl cytosine deamination and replication-based model of mutagenesis. To some extent, these explanations can well explain certain genetic mutations, but all of them failed to accurately predict the possibility of mutation for a specified gene and no of them are universally applicable for interpretation of all the mutations.

In this study, in order to provide genetic counseling infected pedigrees, and to find novel mutations of APC gene and research endogenous mechanisms of gene mutation we used direct DNA sequencing and multiplex ligation-dependent probe amplification (MLPA) detected the APC germline mutations in 14 FAP pedigrees.

Materials and Methods

Patients

There were 14 unrelated pedigrees which were diagnosed with FAP included in our study. Among the 14 probands, 6 are males, and 8 are females, with the mean age of 32 years old (aged 27 ~ 47 years old). All of the FAP families were confirmed based on history collection, genetics investigation and propositus colonoscopy examination and pathological biopsy. After informed consent for genetic testing was received from all patients, all of the participators were tested for APC gene mutations in Jiangsu Cancer Hospital affiliated to Nanjing Medical University (NJMU) between 2005 to 2014. In this study, We had collected as much as possible the FAP family members peripheral blood. Soon afterwards, each sample was placed into the EDTA anticoagulant tube by 5ml in peripheral blood after 1500 r/min (2500×g) centrifuge for 10 min, then we got the white cell layer, and stored in minus 20°C refrigerator as a standby. As the probands without germline mutation is not necessary for the detection of the rest family members, so the total number of samples of all the family by screening was 29, including male 21 female samples and 8 female samples, and among them, a total of 8 patients with FAP.

Mutation analysis

The peripheral blood genomic DNA was extracted using QIAGEN DNA Blood Midi Kit (IAGEN, Germany) according to the instruction, quantified by spectrophotometer at 260 nm and stored at -20 °C until use. Then we used PCR amplification of all APC exons. There are a total of 34 primer pairs used in our study, including primer pairs covering exons 1-14 and 20 primer pairs covering exon 15 of the APC gene, and each of amplified fragment size of 200 ~ 500 bp. These primers were designed and synthesized as previously reported by Wang(Wang et al., 2008), and synthesized in Takara Biotechnology (Dalian) Co., Ltd.. Polymerase chain reaction (PCR) was performed in a total volume of 50 μl containing 2 μl of each primer (10 mmol/L),2μl DNA sample (60-80 ng/mL), 4 μl dNTPs (each 2.5 mmol/L), Taq synthetase 0.5μl and 30μl of double distilled water, according Takara Taq Hot Start Version kit (Takara,Japan). The following thermal profile was applied, using a PTC-200 thermal cycler (MJ Research, USA): 5 minutes at 94°C for initial denaturation followed by 40 cycles at 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 30 seconds, with a final extension at 72°C for 5 minutes. Afterwards, the amplification products were sequenced in Shanghai Huada Gene Technology Co., Ltd (China).

For the APC micromutations negative pedigrees, we performed multiplex ligation-dependent probe amplification (MLPA) to detect if there were large duplications and/or deletions or not, for some FAP occurrence is caused by large duplications and/or deletions of the APC gene. APC gene detection kit MLPA—P043 (MRC. Holland, Amsterdam, the Netherland) is a mixture, containing probes covering 20 exons, the promoter region, and 11 controls. Detailed operation steps have been reported (Wang et al., 2008).

Finally, the results of sequencing analysis in Chromas2.4.1 and Clustalx1.8, and the MLPA results were performed in ABI 3100 Avant Sequencer (Applied Biosystmes, Foster City, CA). Gene mutation nomenclature rules see reference (den Dunnen et la.,2003;

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Table 1. Germline Mutations in the APC gene of 6 Unrelated FAP Pedigrees

Nucleotide change	DNA sequence around the mutation site	Amino acid change
c5432C>T	AGACAACAAAGATTCAAAGAAACAGAATTT	p. Ser1811Leu
c3926_3930 del AAAAG	AGCAGAAATAAAAGAAAAGATTGGAACTAGGTC	p.Glu1309AspfsX4
c3926_3930 del AAAAG	AGCAGAAATAAAAGAAAAGATTGGAACTAGGTC	p.Glu1309AspfsX4
c3921_3924 del AAAA	CAAATAGCAGAAATAAAAGAAAAGATTGGAACT	p.Ile1307MetfsX13
c3184_3187 del CAAA	TAAAACAAAGTGAGCAAAGACAATCAAGGAAT	p.Gln1061AspfsX59
c4127_4128 del AT	AAAAGTCCACCTGAACACTATGTTCAGGAGACCCA	p.Tyr1376LysfsX9

*The smaller letters indicates mutation points, the underlined indicates reverse complementary sequence, contiguous and interrupted direct repeats.
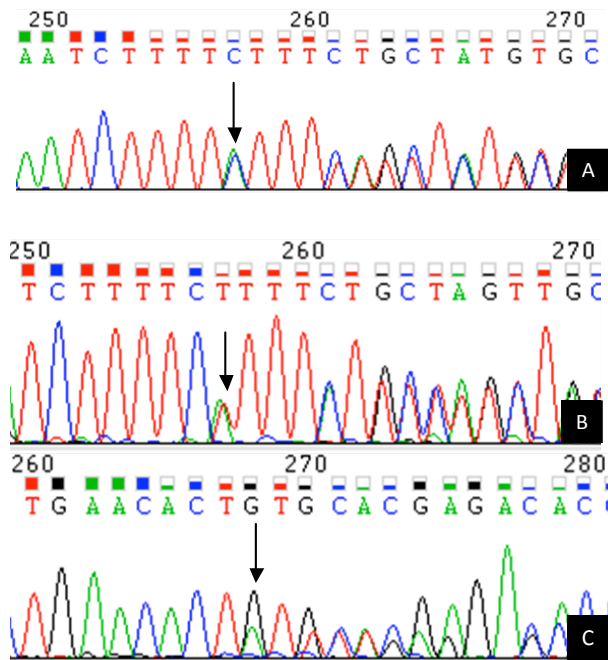

Figure 1. The sequencing map of pedigree 3, 4 and 6. (A) The reversed sequencing map of pedigree 3, and the arrow indicates AAAAG deletion at c3926_3930, that is c3926_3930 del AAAAG. (B) The reversed sequencing map of pedigree 4, and the arrow indicates AAAA deletion at c3921_3924, that is c3921_3924 del AAAA. (C) The sequencing map of pedigree 6, and the arrow indicates AT deletion at c4127_4128, that is c4127_4128del AT.

den Dunnen et al., 2001), and nucleotide sequence of APC gene came from genomic library of cDNA sequence (GenBank#NM00038).

Results

In this study, the results of direct DNA sequencing revealed 6 heterozygous micromutations from the 14 FAP pedigrees were found, and they are c.5432C>T (p. Ser1811Leu), two c.3926_3930 del AAAAG (p.Glu1309AspfsX4), c.3921_3924 del AAAA(p. Ile1307MetfsX13), c3184_3187 del CAAA (p.Gln1061AspfsX59) and c4127_4126del AT(p. Tyr1376LysfsX9), respectively (see table 1), and all of them are frameshift mutations or missense mutation. Among them, c.5432C>T, c3184_3187 del CAAA and one of the c.3926_3930 del AAAAG had been reported previously (Wang et al., 2008), and the rest pedigrees'

sequence map can be seen in Figure 1. Nevertheless, the MLPA results denoted no large duplications and/or deletions had been detected. At the same time, we found all of the mutation points were located in the 15 exon, the 5' half of APC gene. When take attention into the mutation points surrounding base, we found that c.3921_3924 del AAAA and two c.3926_3930 del AAAAG are locate in the AAAAG short tandem repeats sequence, c3184_3187 delCAAA is also locate in the CAAA short tandem repeats sequence, and c4127_4128 del AT is locate in the 5'-CCTGAACA-3', 3'-ACAAGTCC-5 palindromes (inverted repeats) of the APC gene, furthermore, deletion mutations are mostly located at condon 1309.

Discussion

Germline mutations of the APC tumor suppressor gene are the causative reason for FAP, and somatic mutations are constantly found in colorectal cancer, a disease with high incidence rate in Jiangsu China (Chen et al., 2013; Chen et al., 2014; Yang et al., 2013). In contrast with proto oncogene, tumor suppressor genes are suffered "loss-of-function" mutations in the carcinogenic effect (Yeo, 1999). Most of the mutations are nonsense mutations or frameshift mutations which is consist with our results, as a result protein truncating mutations formed (Genevie ve et al., 2005), and rare whole-gene deletions or -exon deletions have been described. Germline mutations are considered spread over APC gene entire coding region, but about 60% of the APC gene variants are concentrated in the central region of APC (amino acids 1284-1580), the so called mutation cluster region (MCR) (Miyoshi et al., 1992; Sun et al., 2014). Deletions at the codons 1061 (c.3183_3187delACAAA) in 5 % of the cases and 1309 (c.3927_3931delAAAGA) in 10 % of the cases are found to be the most frequent germline mutations (Half et al., 2009; Caspari et al., 1994). The positive correlations between clinical manifestations and the location of the APC mutation in the gene have been observed in some study. Classical FAP is thought to be associated with mutations between codons 1250 and 1464 in the APC gene are, whereas mutations apart from this area (3' end, 5' end or exon 9, especially codons between 1020 and 1169) are related to attenuated FAP (Sieber et al., 2006; Newton et al., 2012; Jaehoon et al,. 2-13),at the same time, deletion mutation at the condon 1309 is thought to be responsible for a severe clinical phenotype of FAP. However, the clinical manifestations are not always consist with mutations in the APC gene, even FAP patients with identical variants (Crabtree et al., 2002; Liao et

al., 2014), for this may caused by the conjunct results of environments influences and/or other endogenous factors, such as modifying genes (e.g., phospholipase A2), methylation of the promoter region, mismatch repair gene mutation (MMR) and variation in splicing region, etc (van Es et al., 2001; Yan et al., 2014).

As a critical gene for the epithelial cell progression and differentiation, APC has shown a multifunctional protein involved in a wide variety of processes, including cell adhesion, cell migration, cytoskeletal reorganization, regulation of cell proliferation, and chromosomal stability(Johan et al., 2001). There are multiple domains have been mapped to APC, including the homodimerization domain, the homology domain, the armadillo repeats, the three imperfect 15-amino-acid repeats and seven 20-amino-acid repeat, the SAMP repeats, the basic domain, and the C-terminal region (Fearnhead, 2001). By inhibiting the Wnt signal pathway, APC play an important role in the regulation of proliferation and differentiation of epithelial cells (Aoki et al., 2007). Through downregulation of β-catenin, APC prevent the excessiveβ-catenin enters the nucleus and activates transcription factor family members with TCF/LEF, involved in cell proliferation and apoptosis regulation (Aoki et al., 2007). The progression of FAP is caused by loss of function normally correlated with the deleted domains of APC. At the same time, germline mutation study of the APC gene has revealed that the most mutations identified in FAP patients is predicted to result in a C-terminally truncated protein product by introduce a premature stop codon or frame shifts (van Es et al., 2001). In this study, we detected 6 germline mutations from 14 pedigrees, and as many as 5 pedigrees' germline mutations introduced a premature stop codon by forming frame shifts in exon 15 of APC gene. This is consistent with the reported in domestic and abroad.

The vast majority of mutations causing human genetic disease are point mutations and deletions, nevertheless, a majority of mutations are not accidental occurrence, mostly influenced by environmental effects and endogenous mechanism. The majority of well-characterized gene mutations endogenous mechanism appear to be between one and a few base pairs change. Deamination of 5-methylcytosine in the CpG dinucleotide has been implicated as a mechanism for point mutation from C to T (Cooper et al., 1990; Miyoshi et al., 1992), and this phenomenon is just like methylation of promoter region. However, so far there are no mutation hotpoints have been found involved CpG sites (Miyoshi et al., 1992), just like pedigree 1 in our study, for the C>T mutation is not located in CpG sites. The specific mechanism for CpA to TpA and CpT to TpT change is not known by now. However, as one of the most common DNA polymerase error is thought to be a G mispairing with T with a lack of repair at this mismatch, the CpA to TpA mutation we observed might have been generated in this manner (Miyoshi et al., 1992).

As for the 5 remaining mutation positive pedigrees, all of them are small fragment deletions. It is well known that deletions most frequently occur at repeat sequence, followed by palindromes (inverted repeats),

symmetrical sequence and homologous sequences. Direct repeats including separated direct repeats and overlapping direct repeats of between 2 bp and 8 bp are the most common found in the immediate vicinity of broken link points of mutation sites (Michael ET AL., 1991). A number of deletion mutagenesis models, such as recombination, replication or repair-based models are characterized by direct repeats. However, findings were found to be more compatible with a replication-based slipped mispairing model than others, first proposed by Streisinger et al. and developed in detail by Kunkel (Michael et al., 1991). This hypothesis can be simply summarized as deletions might occur during DNA replication as a result of slippage misalignment of the template strand and subsequent, through the formation of a number of different secondary structure intermediates which are mediated by direct repeats, palindromes (inverted repeats), symmetric elements, symmetrical sequence and homologous sequences. In this study, short tandem repeats and separated direct repeats were found surrounding the deletion breakpoint junction regions of the pedigree 2 to pedigree 5, including AAAAG in pedigree 2 to pedigree 4, and CAAA in pedigree 5. And the mechanism of nucleotide deletions can be assumed to be as follows. When replication proceeds, the DNA duplex becomes single-stranded at the replication fork permitting illegitimate pairing between the sequence of AAAAG or CAAA and the complementary sequence of TTTTC or GTTT located in the new synthetic chain. As a result, a single-stranded loop is formed containing the AAAAG or CAAA repeat and sequence lying between direct repeats AAAAG or CAAA. Subsequently, DNA repair enzymes may excise this loop and rejoin the broken ends of the DNA strand, and one wild-type and one deleted duplex would generate in the next round of replication (Michaelet al., 1991). As for the pedigree 6, the palindromes (inverted repeats) 5'-CCTGAACA-3', 3'-ACAAGTCC-5 can be found surrounding the deletion breakpoint junction regions. A palindrome is defined as possesses self-complementarity within the same single DNA strand, and it allows this strand to fold back on itself to form a cruciform or hairpin structure. The misaligned secondary structures is formed mediated by imperfect or perfect (self-) complementarity of (quasi-) palindromic sequences (David teal., 1991). Subsequently, the unpaired bases will remove by DNA repair enzymes. When this single strand replicated as template, gene deletions is formed mediated by a palindrome.

Summarily, there are 6 micromutations detected out of 14 FAP pedigrees, including 1 point mutation and 5 small fragment deletions. This is consistent with the previously reports. At the same time, the small fragment deletions are matched well with replication-based slipped mispairing model. So nucleotide sequence containing short tandem repeats and palindromes (inverted repeats), especially the 5 bp base deletion at codon 1309, are mutations in high incidence area in APC gene. However, this model can only well explain 25% deletion mutations, otherwise this model cannot be used to predict the specific nucleotide deletion of a gene. In order to prevent and control FAP and other genetic diseases, further research is therefore needed to

elucidate the mechanisms of gene deletion mutant.

Acknowledgements

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Newly diagnosed without treatment