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RESEARCH

# Detection of APC Gene Deletions in Colorectal Malignancies Using Quantitative PCR in a Chinese Population

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**Abstract** The adenomatous polyposis coli (APC) gene has been shown to be involved in genetic instability and to be downregluated in several human carcinomas. The chromosome locus of APC, 5q21-22, is frequently deleted in colorectal cancers (CRCs). The functional impact of such regions needs to be extensively investigated in large amount of clinical samples. Case-matched tissues of CRC and adjacent normal epithelium ($n$=134) were included in this study. Quantitative PCR was carried out to examine the copy number as well as mRNA expression of APC gene in colorectal malignancies. Our results showed that copy number deletions of APC were present in a relatively high percentage of colorectal cancer samples (26.1%, 35 out of 134). There was a positive correlation between copy number decrease of APC and tumor progression in CRCs. Furthermore, copy number loss of APC was correlated with decreased mRNA expression. However, mRNA levels of APC were also impaired in CRC samples with unaltered copy numbers, indicating that sporadic CRCs exhibit different mechanisms of APC regulation.

**Keywords** Colorectal cancer · APC · Copy number variation · Gene expression

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## Introduction

The tumor suppressor gene adenomatous polyposis coli (APC) is expressed constitutively within the normal colonic epithelium. The *APC* gene product is a 310-kDa-homodimeric protein, which is localized in the cytoplasm and the nucleus [1–5]. Appropriate levels of wild-type APC are critical to cytoskeletal integrity [1, 6], cellular adhesion [7], and Wingless/Wnt signaling [5, 8, 9]. Truncation of APC results in the disruption of complex formation and ultimately increased cytoplasmic levels of β-catenin, which up-regulates TCF-responsive genes critical for the proliferation and transformation of colonic epithelial cells [10, 11].

Germline mutations in the tumor-suppressor APC gene are associated with hereditary familial adenomatous polyposis (FAP) and somatic mutations are common in sporadic colorectal cancer [12, 13]. The colorectal cancer (CRC) is currently the third most common cancer and in Chinese population, responsible for about 130,000 deaths per year. Both genetic and environmental factors contribute to disease etiology, with about one-third of disease variance attributed to inherited genetic factors [14]. It is estimated that >85% of colorectal tumors have somatic mutations of the APC gene [15].

Recent studies have identified new genetic and epigenetic changes in CRCs. Studies using SNP arrays and aCGH have suggested that DNA deletions at chromosome position 5q21 are common in CRCs [16–18], which is also

**Table 1** Comparison of CNVs of APC between adjacent normal tissues (ANT) and healthy normal controls(HNC)[a]

| Samples | n | Copy number | | | | | *p* (vs. HNC) |
|---|---|---|---|---|---|---|---|
| | | Deletion | | | Amplification | | |
| | | 0 | 1 | 2 | 3 | >3 | |
| HNC | 152 | 2 | 7 | 135 | 5 | 3 | – |
| ANT | 134 | 1 | 4 | 125 | 2 | 2 | 0.868 |

[a] ANT represents adjacent normal tissues; HNC represents healthy normal controls

the chromosome locus of APC. Inactivation of APC in CRCs might be also attributed to copy number variation (CNV) of the gene. Most of the aCGH experiments focused on the genome-wide screening of CNVs, and the data obtained are generally informative but not definitive. Thus, a study comprehensively examining CNVs in relation to APC expression or prognosis should be performed using a large number of tumors.

In the present study, we collected 134 sporadic CRC samples with matched adjacent normal tissues from Chinese population for CNV analysis. Copy number loss of APC was present (26.1%) in CRC samples. There was a positive correlation between copy number loss of APC and mRNA down-regulation. These findings suggested the potential role of CNVs of APC in sporadic CRCs.

## Materials and Methods

### Patients and Tissue Collection

Colorectal cancer samples were obtained from 134 surgical patients of the Department of Gastroenterology, Shenzhen Hospital, and Peking University. The samples were collected from patients undergoing bowel resection. Adjacent normal mucosa samples located at least 2 cm far from the macroscopically unaffected margins of the tumor (polyp or carcinoma) were defined as normal controls. All tumors were adenocarcinomas and mucinous carcinomas (when >50% of the tumor volume was composed of mucin) were excluded. Colorectal cancers were staged according to the Dukes classification system: Dukes A (T1–T2, $N_0$, and M0; $n$=41), Dukes B (T3–T4, $N_0$, and M0; $n$=36), Dukes C (any T, N1–2, M0; $n$=43) and Dukes D (any T and any N and M1; $n$=14). Matched samples of colorectal adenocarcinomas ($n$=134) and normal colonic mucosa ($n$=134) were stored in liquid nitrogen. All patients were informed about the aims of specimen collection and gave signed written consent in accordance with the ethical guidelines of Peking University. Peripheral blood samples from 152 healthy controls were collected at Peking University People's Hospital. The study was approved by the ethical committee of Peking University Shenzhen Hospital.

### DNA Extraction and Quantification of Copy Numbers

Genomic DNA was isolated from the tissues using the Genomic DNA Extraction Kit (Innocent, Shenzhen, China) according to the manufacturer's instruction. Quantitative PCR was performed through BioRad Chromo4 real-time PCR system. The primers for RNAse P are: forward: 5'-AGA CTA GGG TCA GAA CRCA A-3' and reverse: 5'-CAT TTC ACT GAA TCC GTT C-3'.The primers for APC are: forward: 5'- TCRC AGT CTC TTC GAG CRCT T' and reverse: 5'-TCRC CCT AAC ATA CAG GGT GA-3'.

Average copy numbers of RNAse P in normal candidates (copy numbers = 2) were used as control [19]. The copy

**Table 2** CNVs of APC in CRC tissues and matched adjacent normal tissues (ANTs)

| CNVs, population | | n | Copy numbers | | P (vs. ANT) | P (vs. DukesA&B) |
|---|---|---|---|---|---|---|
| | | | ≧2 | Deletion <2 | | |
| Total | ANT | 134 | 129 | 5 | – | – |
| | CRC | | 99 | 35 | 2.71E-7 | – |
| DukesA&B | ANT | 77 | 75 | 2 | – | – |
| | CRC | | 62 | 15 | 8.29E-4 | – |
| DukesC&D | ANT | 57 | 54 | 3 | – | – |
| | CRC | | 37 | 20 | 7.26E-5 | 9.28E-3 |

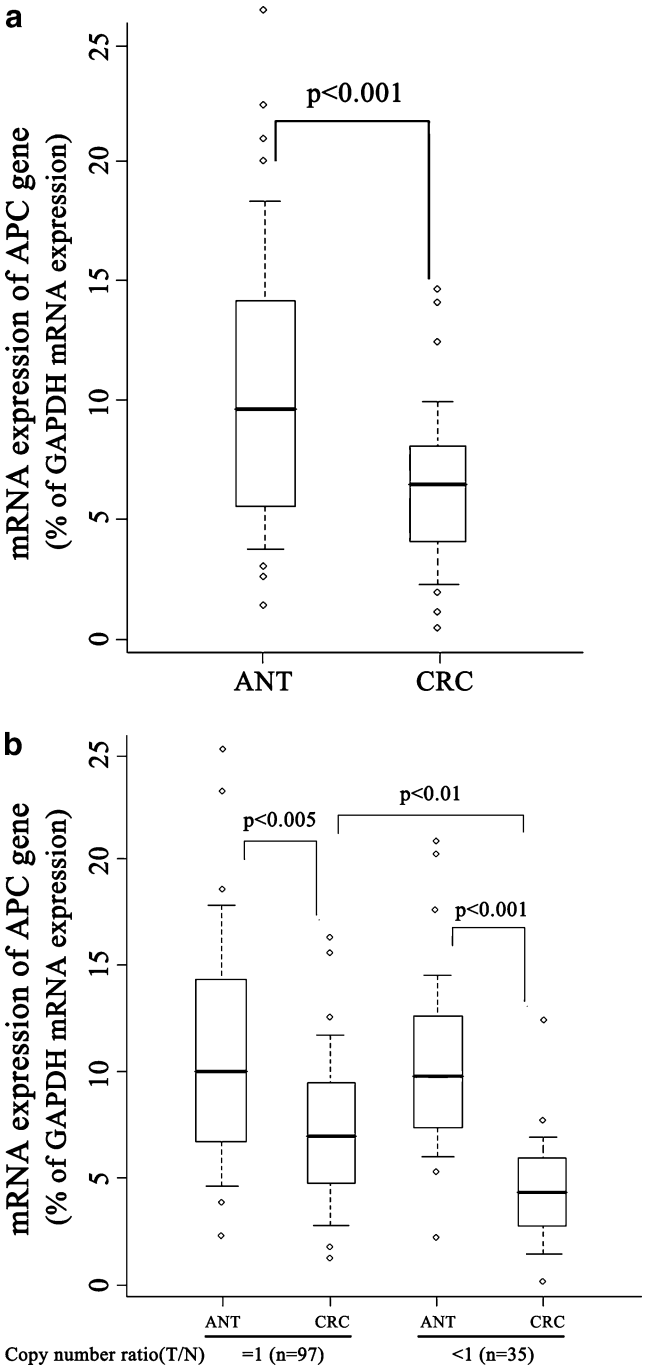


**Fig. 1** Real-time PCR assay was carried out as described under "Materials and Methods" section, and the results were obtained from indicated group of samples. *Boxplots* of relative copy number of APC mRNA measured with Real-Time PCR analysis showing median; *box*: 25th–75th percentile; *bars*: largest and smallest values within 1.5 box lengths; *little circles*: outliers. **a** mRNA expression level of APC in all the CRC samples compared with adjacent normal tissues. **b** mRNA expression level of APC in groups with unaltered ($n$=97) or deleted ($n$=35) DNA copies

numbers of APC were calculated by using the comparative Ct method. Cut-off values of 0.25, 0.75, 1.25, and 1.75 were used to define the copy numbers as 0, 1, 2, and 3, respectively. Fold change of each sample was presented as follows: fold change = relative expression level / average expression level in the group with two copies of DNA. A representative diagram for the DNA CNV analysis for one CRC/ANT pair was shown in Fig. S1.

A standard curve was prepared using 2 μl of crude DNA solutions, in which serially diluted samples (original, 2-, 4-, 8-, 16-diluted) were included. The slopes of Ct and efficiency of each primer were calculated by the BioRad Chromo4 real-time PCR system and Microsoft Excel 2007 for Windows. Relative quantification of APC was performed with the $2^{-\Delta\Delta Ct}$ method.

### RNA Extraction and Real-time PCR

Total RNA was isolated from tissues by using the AxyPrepTM Blood Total RNA MiniPrep Kit (Axygen) according to the manufacturer's instruction. First strand cDNA was synthesized with the RevertAidTM First Stand cDNA Synthesis Kit (Fermentas). Quantitative PCR was performed through the BioRad Chromo4 real-time PCR system. At the end point of PCR cycles, melt curves were made to check product purity. Since the efficiencies of amplification of targeted genes were very near to 100% (Fig. S2), we used $2^{-\Delta ct}$ method to calculate real-time PCR results. The mRNA level of APC was expressed as a ratio relative to the GAPDH mRNA in each sample (one of the representative calculations was shown in Fig. S2). Exploratory data analysis using box plot was applied to visually identify the expression level of target mRNA.

### Statistical Analysis

Statistical analysis was performed with the SPSS Software (version 12). Data were analyzed by the chi-square test or Fisher exact test. P-values less than 0.05 were considered statistically significant. Results of the APC mRNA expression for normal and tumor tissue samples were compared using two-way repeated measurement ANOVA. One-way, repeated measures analysis of variance (ANOVA-RM) was performed at a significance level of $p$=0.05 to determine differences from controls within each group. Two-way analysis of variance (ANOVA-2) was performed after baseline subtraction, at a significance level of $p$=0.05 to determine differences between the groups with deleted and unaltered APC copy number.

## Results

### Gene Copy Number Loss of APC in CRC Samples

As shown in Table 1, no statistical differences of copy number distribution between adjacent normal tissues

(ANTs) and healthy normal controls (HNCs) were observed, thus the ANT could be used as controls for the CRC tissues in the present study.

Table 2 shows CNVs of APC in paired samples of CRCs and ANTs. A total of 134 CRC samples were examined. A relatively high percentage of CRC samples showed deletions of APC (26.1%, 35 out of 134). The colorectal cancer tissues from patients with early-stage CRCs (Dukes A&B) contained on average less than 20% of the samples that had either zero or one copy of the APC gene, whereas more than 20% of samples that had either zero or one copy of APC was observed in advanced (Dukes C&D) CRCs. There is a statistical correlation between gene copy number loss and the CRC phenotypes ($p<0.001$).

### Positive Correlation Between Copy Number Increases and mRNA Over-expression of APC in CRCs

To find whether CNVs of APC have genotype-phenotype correlation, we compared the mRNA expression levels of APC between the CRC samples and paired adjacent normal tissues by quantitative real-time RT-PCR. As shown in Fig. 1a, decreased mRNA expression level of APC was observed in the CRC tissues compared with the ANTs ($p<0.001$). The obtained results were consistent with previous findings [20].

Gene CNVs can contribute to qualitative and quantitative diversities to their gene products. Next, we selected the samples with decreased or unaltered copies of APC and tested whether the APC mRNA expression was correlated with the copy numbers. The samples with increased copies of APC (2 out of 134) were not included due to the small sample size. As shown in Fig. 1b, the CRC samples in the group with deleted or unaltered copies of APC both showed an impaired expression of mRNA compared with adjacent normal tissues ($p<0.005$). There was a significant statistical difference between the CRC samples in the groups with deleted and unchanged copies of APC ($p<0.01$). Thus the DNA copy loss plays at least a partial role in the down-regulation of APC in CRCs.

## Discussion

CNVs identified by CGH and array technology have been clearly shown to have the potential to directly or indirectly influence a healthy individual's susceptibility to cancer, for example by varying the gene dosage of tumor suppressors or oncogenes. Examination of the CNVs for such genes is a starting point for investigations into the role of gene alteration in the colorectal carcinogenic process. However, there are many discrepancies among previous studies which used high-resolution approaches to screen CNVs. Thus, validation of such CNVs by a large amount of clinical samples is required.

In the present study, we collected 134 CRC (adenocarcinomas) samples for CNV analysis of APC. Since there was no statistical difference of CNVs between the healthy normal controls and normal tissues from CRC patients, the CNVs of APC were more likely acquired DNA aberreations in sporadic CRCs. The deletions of APC (26.1%, 35 out of 134) was found in the collected CRC samples, and the frequency of gene copy loss was consistent with previous studies where loss of chromosomes 5q21 were reported in 20–70% of colorectal cancers, respectively [16, 21, 22]. The results from the present study showed that the frequency of DNA copy number loss of APC in advanced CRCs was significantly more than early-stage CRC, suggesting that copy number loss played a role in CRC progression and might contribute to tumor aggressiveness. However, we found that copy numbers of APC were also increased in a small percentage of samples (2 out of 134). This discrepancy might be due to the different races and populations used in the studies. Another reason could be the different methodologies used in the different studies. We used gene-specific strategy to target short fragments (several hundred basepairs), and the sensitivity was increased.

It is expected that the CNVs do have genotype-phenotype correlation. Phenotypic effects of genetic differences, such as CNVs, are supposedly brought about by changes in expression levels [23, 24]. In the present study, we investigated the correlation between the APC mRNA expression and the copy numbers of its DNA. Contrary to our expectation, the correlation was not as positive as expected, although a statistical difference was obtained. And mRNA expression of APC was increased in both the groups of deleted and unchanged DNA copies. There was a statistical difference of mRNA expression between the groups of deleted and unaltered DNA copies. Thus CNVs did play a role of over-expression of the APC mRNA in CRCs, while there were also other mechanisms involved (e.g. site mutations [25] and CpG island hyper-methylation [26]).

In general, plausibly, our findings showed that the CNVs of APC have the potential to serve as diagnostic indicators, alone or in combination with other markers, for colorectal malignancies. However, the functional consequences of CNVs, the different feature of CNVs between colorectal and other colorectal malignancies and the underlying mechanisms of the heterogeneous expression levels need to be extensively investigated in the future.

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