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mRNA expression of minichromosome maintenance2 (MCM2) in colonic adenoma and adenocarcinoma

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Abstract

Since proliferation is essential for progression from normal cells to tumor, certain markers specific to proliferating cells may permit detection of premalignant lesions. Here, we aimed to evaluate the possible value of a proliferation marker, minichromosome maintenance2 (MCM2), in the early diagnosis of colorectal cancer, by analyzing the difference of MCM2 among normal mucosa, adenoma and adenocarcinoma, and investigating the relationship of MCM2 expression in adenomas with clinicopathologic variables. We observed that the expression of MCM2 protein was present on basal third to half of colonic crypts in normal mucosa, while throughout epithelium in adenomas and adenocarcinomas, and the expression of MCM2 mRNA in adenocarcinomas was significantly higher than that in adenomas (P=0.001), while the difference between adenoma and normal mucosa was not significant ($P=0.184$), and also found that the expression of MCM2 mRNA tended to be increased in the adenomas with high grade dysplasia, or older age of patients, respectively comparing with those with low grade dysplasia, and younger age of patients. These results suggested the potential value of MCM2 in early diagnosis of colorectal cancer.

Key words: minichromosome maintenance protein 2; colonic adenocarcinoma

adenoma; real-time reverse transcription- PCR

Introduction

MCM2 is one of six members of the minichromosome maintenance protein. It serves as a 'licensing factor' which is essential for initiation of DNA replication and for limiting replication to one round per cell cycle.[1,2] Some studies showed that MCM2 was a specific marker of the cell cycle state in tissues and its expression was observed during all phases of the cell cycle, however, MCM2 was lost after exiting from the cell cycle, with rapid loss after differentiation and slower loss in quiescent (G0) cells.[2-4] As a result of the sensitivity of antibody to MCM2 as marker of cell cycle state and because of the aberrant entry into the cell cycle in dysplasia and malignancy, MCM2 protein has been proposed as a candidate marker for cancer screening, surveillance, and as prognostic marker.[6-10] Williams et al. [5,9] also demonstrated that MCM2 surface positivity correlated with the severity of cell dysplasia. These observations have led to the rationale for using surface sampling techniques in combination with MCM2 staining to detect dysplasia and malignancy. [3,10] Furthermore, Tan DF et al.[1,5] showed that, comparison with two proliferative markers, proliferating cell nuclear antigen (PCNA) and Ki-67, the MCM2 was more sensitive proliferative marker in evaluation of premalignant lesions.

In 2002 Justin et al.[3] revealed that MCM_2 was confined to the proliferative compartmant in the basal third to half of the crypts of normal large bowel mucosa, By contrast, MCM_2 was expressed throughout the epithelium, including in surface mucosa

when colorectal adenomas or adenocarcinoma are present. Through analyzing the MCM2 expression of the colonocytes retrieved from stool, they detected MCM2-positive cell in all patients with colorectal cancer, and no one case was expressed with MCM2 in all control individuals. [3] As a result, in 2003´ colorectal surgical conference of England and North Ireland, they advocated to early screen colorectal cancer by detection of MCM2 expression in stool. However, since MCM2 is expressed in all epithelia throughout the crypts of adenoma and adenocarcinoma, MCM2-positive cells can also be detected in patients with colorectal adenomas. Therefore, all those patients whose stool was detected with MCM2-positive cells can´t be directly diagnosed as colorectal adenocarcinoma because of exception of colorectal adenoma. Then, how can we know those patients with MCM2-positive cells is adenoma or adenocarcinoma? To answer this question, we firstly should find out whether the expression of MCM2 between colorectal adenoma and adenocarcinoma has quantitatively difference. Consequently, the quantitative difference of MCM2 expression between colorectal adenoma and adenocarcinoma has become the considerable subjects and this difference perhaps offer experimental foundation to identify adenomas from MCM2-positive cells in stool and present a supplement to enhance the practicability of MCM2 as a marker of early detection for colorectal cancer. But a quantitative comparison of MCM2 expression between colorectal adenomas and adenocarcinoma has not previously been reported.

Materials and methods

Study population

The study included 12 patients with colonic adenocarcinomas, 33 with colonic adenomas and 12 accompanying normal colonic mucosa for comparison (taken from uninvolved morphologically normal colonic tissue distant from colonic cancer above 10cm). All specimens were taken from patients underwent operation or colonoscopy excision at the West China Hospital of Sichuan University from June 2004 to December 2004. None of patients had received preoperative chemo-and/or radiotherapy. The specimens were flash-frozen in liquid nitrogen and stored at -150℃. Each specimen was divided into two parts, one for immunohistochemistry and the other for real-time RT-PCR. Polyp size and morphological character, as reported by the endoscopist, was categorized as small (1-9mm) or large (≥10mm), and classified morphological character of adenomas without pedicel tissue as sessile adenoma or as petiolate adenoma with pedicel tissue. 'Advanced' adenomas were defined as the presence of adenomas with high grade dysplasia, villous histology, sessile morphology, a polyp size of at least 1cm, or older age of patients. All specimens, according to the histological diagnose criteria of the World Health Organization, were examined by pathologist at the Department of Pathology in the West China Hospital of Sichuan University to confirm their histopathologic type.

Immunohistochemistry

For immunohistochemical staining, from each sample block, 5 serial 4-um sections were cut and place on glass slides. Sections 1-4 were used for immunostaining, and

section 5 was stained with hematoxylin and eosin, this latter section was reviewed and compared with the original slide of department of pathology used for diagnostic purposes.

Sections were stained with polyclonal goat antibody against MCM2 (SC-9839, Santa Cruz Biotechnology, California, USA). Briefly, sections were deparaffinized and those to be stained with MCM2 were subjected to microware antigen retrieval in citrate buffer for 15 minutes, twice. The sections were incubated with a monoclonal MCM2 antibody of goat at 1/400 dilution over night at 4℃. The avidin-biotin detection method was employed on a DAKO autostainer universal system (DAKO ,Ely, United Kingdom). A negative control was performed by omission of the primary antibody of goat.

RNA extraction and cDNA preparation

The specimens was taken from -150℃ refrigerator and pick up 100mg tissues to put into a 1ml polypropylene tube. Total RNA was isolated using Trizol LS reagent (Jinmei Biotech Co., Ltd, Shenzhen, China) as described previously [11]. The quality of the RNA was determined by electrophoresis through agarose gels and staining with ethidium bromide, and the 18s and 28s RNA bands were visualized under UV light. To generate cDNA, 5 ul RNA was first denatured at 70℃ with 2.5uM random hexamers (TaKaRa Biotechnology Co., Ltd, Dalian China) for 5 min before quenching on ice; then 10mM final of each of the four deoxynucleotide triphosphates, 20U ribonuclease inhibitor, 100U M-MLV and 5 × M-MLV buffer (TaKaRa Biotechnology Co.,

Ltd ,Dalian, China) were added together to make up a final volume of 20 μ l reaction

mix. The reaction mix was incubated for 10 min at 20℃ and 1h at 42℃. The reverse

transcriptase was inactivated at 95℃ for 10 min and cooling at 5℃ for 5 min.

Real-time PCR

Theoretical basis real-time PCR was performed using relative quantification

protocol on an iCycler iQ System (Bio-Rad, USA). Eq (1) [12] was applied to calculate

the relative expression ratio of the target gene (MCM2) in a sample vs a control in

comparison to a reference gene (glyceraldehydes-3 phosphate dehydrogenase,

GAPDH):

$$\text{Ratio} = \frac{(E_{target})^{\triangle CT_{target(control-sample)}}}{(E_{reference})^{\triangle CT_{ref\,(control-sample)}}}$$

E_{target} and E_{ref}, respectively represents the real-time PCR efficiency of target gene and

reference gene transcript. $^{\triangle}CT_{target(control-sample)}$, $^{\triangle}CT_{ref(control-sample)}$is the Ct (threshold

cycle) deviation of control minus sample of the target or reference gene transcript.

Real-time PCR efficiencies (E) was 2, approximately, by calculated.

Primers and probes

Specific primers and probes for MCM2 and CAPDH were designed based on

sequence data from the ensemble database (http://www.ensembl.org). They were

purchased from TaKaRa Biotechnology Co. Ltd, Dalian, China (Table 1).

Table 1: Specific primers and probes for MCM2 and GAPDH gene

	MCM2	GAPDH
Forward primer	5'-CACATCGAGTCCATGATCC-3'	5'-CCTCAAGATCATCAGCAAT-3'
Reverse primer	5'-CAAAAGTCTTGCGCATGCT-3'	5'-CCATCCACAGTCTTCTGGGT-3'
Probe	5'-FAM-CTCTCCAGCATCACGCGGA-TAMRA-3'	5'-FAM-ACCACAGTCCATGCCATCAC-TAMRA-3'

PCR amplification

All PCR reactions were performed using an icycler iQ System (Bio-Rad USA). For each PCR run, briefly, a mastermix was prepared on ice with 1×PCR buffer, 2.5mM MgCl2, 0.3mM dNTP, 0.16uM Taqman probe, 0.33uM each primer, 1.25U of AmpliTaq Gold DNA polymerase (TakaRa Biotechnology Co., Ltd, Dalian, China) and 1ul of cDNA add as PCR template. The following iCycler iQ run protocol was used: denaturation program (95℃, 5min), amplification and quantification programs repeated 45 times (95℃ for 20s, annealing temperature for 30s, 56℃ for 30s), in addition, a non-template control (ddH2O control)was analysed for each mastermix. All of the samples were amplified simultaneously in triplicate in a one assay-run.

Statistical analysis

In our experiment, the relative expression analysis of target gene was performed using a software, named REST-XL©[13] (relative expression software tool, available at http://www.wzw.tum.de/gene-quantification/), which compares the expression of target gene in sample relative to control on the basis of Eq (1), and tests the group differences for significance with a newly developed pair wise fixed reallocation randomization test (http://www.bioss.ac.uk/smart/unix/mrandt /slides/frames.htm). P values less than 0.05

were regarded as statistically significant.

Results

Immunohistochemistry

In normal mucosa, the MCM_2 expression was confined to basal third to half of crypts and no expression was on surface mucosa (Fig.1A). However, MCM_2 was expressed in all epithelia throughout the crypts of adenoma and adenocarcinoma (Fig. 1, B and C). There was no difference on the location of MCM2 expression between adenoma and adenocarcinoma, but the intensity of staining for MCM2 in adenocarcinoma seemed greater than that in adenomas.
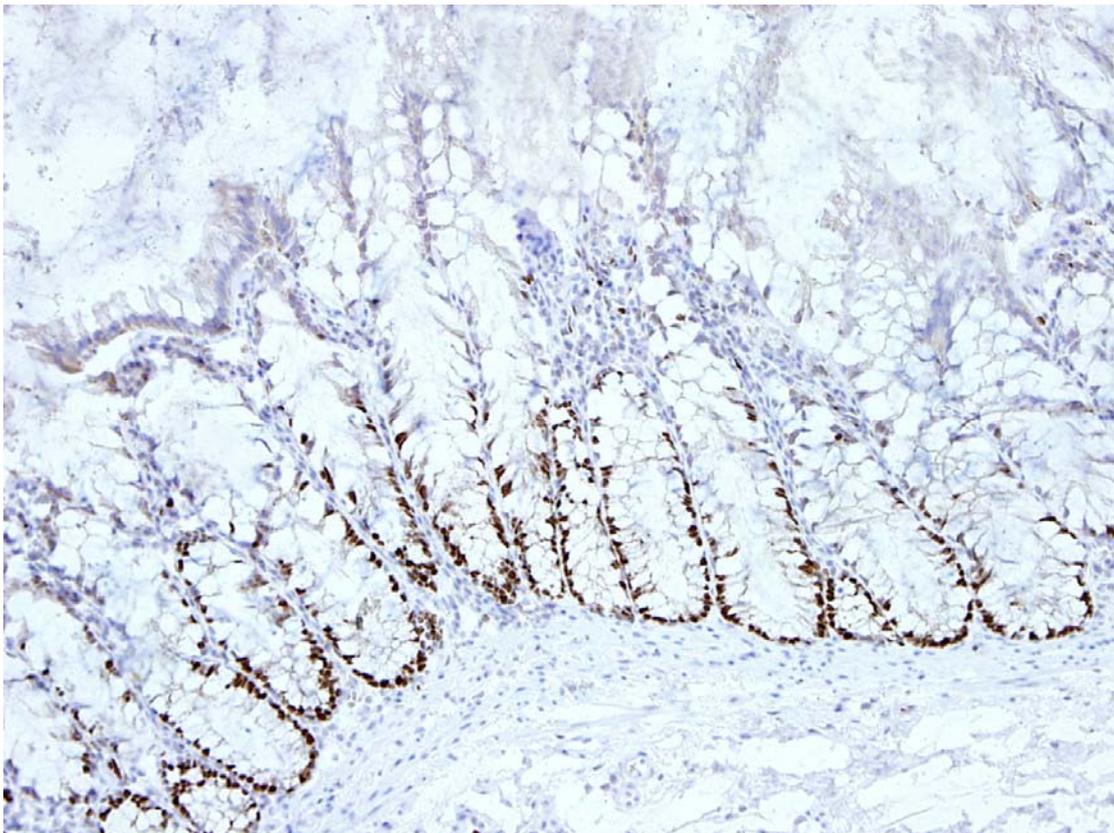

Figure 1: A normal colonic mucosa ($\times$200 magnification) MCM2 expression in healthy colon is confined to basal third to half of colonic crypts with no expression in surface mucosa (brown staining).
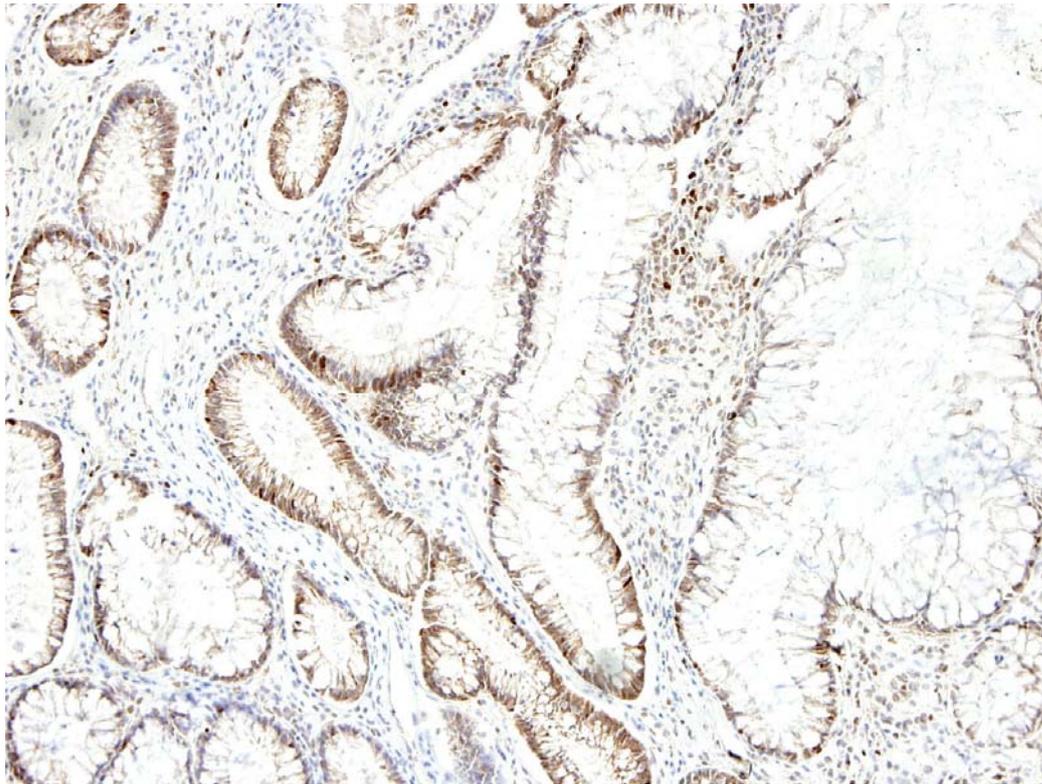
Figure 1: B tubular adenomas (×200 magnification) In colonic adenoma, MCM2 expression is seen throughout epithelium, including in surface mucosa.

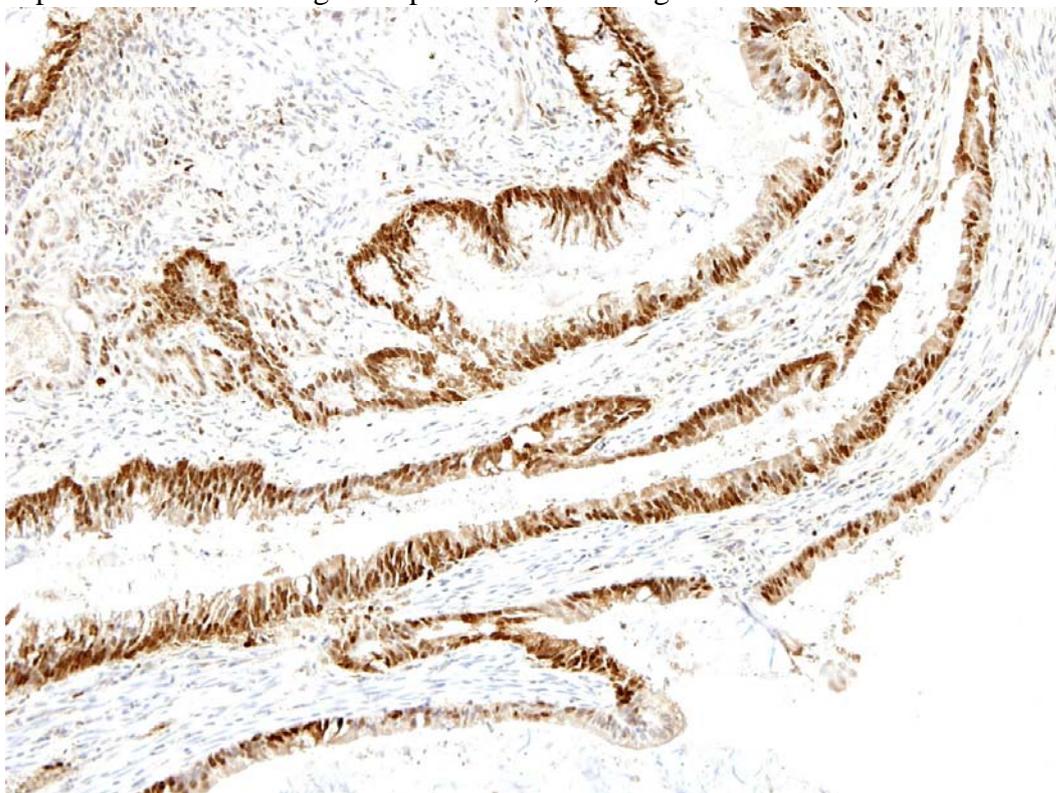**Figure 1: C descending colon adenocarcinoma** (×200 magnification) In colonic adenocarcinoma, MCM2 expression is also seen throughout epithelium. But, the intensity of cell staining for MCM2 in adenocarcinomas seemed greater than that in adenomas.

Real-Time RT-PCR

Comparison of MCM2 mRNA levels between adenocarcinoma and adenoma

To further compare the general expression level of MCM2 in adenocarcinoma relative to adenomas and test the group difference for significance, we put the Ct values for reference and target genes both in sample group (adenocarcinomas) and control group (adenomas) into the REST-XL$^{©}$ software to run it, and chose 2000 as the randomization number. The Ct values for reference and target gene were jointly reallocated to control and sample groups (=pair wise fixed reallocation), and the expression ratios were calculated on the basis of the Eq (1), then the randomization test was performed to tests the group difference for significance, the numeric results of the randomization test were given in the randomization data output box. The relative amounts of MCM2 mRNA to GAPDH mRNA in the adenocarcinoma group were significantly higher than those in the adenoma group (P=0.001), and MCM2 was up-regulated in adenocarcinoma group (in comparison to adenoma group) by the factor 463.38 (Table 2).

Table 2: Randomisation data output in comparison of MCM2 mRNA expression between adenomas and adenocarcinomas

Type of Specimen	Number of Specimens	Means of MCM2 gene	Means of Reference gene	Expression Ratios	P
Adenoma	33	39.92	17.49	—	
Adenocarcinoma	12	30.29	16.71	463.38[a]	0.001

[a] The ratio of MCM2 mRNA expression, presented as the fold change in adenocarcinomas normalized to an endogenous reference gene and relative to the group of adenoma.

Comparison of MCM2 mRNA levels between adenoma and normal mucosa

Briefly, to adopt previous way described in the test, we put the Ct values of reference and target genes both in normal bowel mucosa (control group) and adenomas (sample group) into the REST-XL$^{©}$ software, and also chose 2000 as the randomization number. To run the software, we obtain that expression of MCM2 mRNA was up-regulated in sample group, in comparison to control group, by the factor 5.73, but the difference did not reach a statistical difference ($P=0.184$, Table 3).

Table 3: Randomisation data output in comparison of MCM2 mRNA expression between colonic normal mucosa and adenoma

Type of Specimen	Number of Specimens	Means of MCM2 gene	Means of Reference gene	Expression Ratios	P
Normal mucosa	12	45.00	20.04	—	
Adenoma	33	39.92	17.49	5.73[b]	0.184

[b] The ratio of MCM2 mRNA expression, presented as the fold change in adenomas normalized to an endogenous reference gene and relative to the group of normal mucosa.

MCM2 mRNA expression in adenomas in relation to clinicopathological variables

We examined the relationship of MCM2 mRNA expression in adenomas with patients' age, tumor size, morphologic type, grade of dysplasia and histopathological type. As shown in Table 4, the expression of MCM2 mRNA was up-regulated in sample group (advanced adenomas, those adenomas with clinicopathological characteristics indicative of malignance) in comparison to respective control group, However, all of the above differences were not statistically significant (P>0.05, Table

4), although the P values of the two variables, age and grade of dysplasia, are extremely close to 0.05 (P=0.059, 0.068, respectively).

Discussions

MCM2, as a specific marker for cell proliferation, has been extensively studied in some of adenocarcinomas, but little is known about the diversification of MCM2 expression during colorectal adenoma-carcinoma sequence. In the present study, we firstly analyzed the localization of MCM2 protein expression by using immunohistochemical assay and quantified MCM2 RNA expression by means of real-time quantitative RT-PCR in a series of 12 normal mucosa samples, 33 colonic adenomas and 12 adenocarcinomas. We found that MCM2 protein was confined to the proliferative compartment in the basal third to half of the crypts in normal mucosa; by contrast, MCM2 protein was expressed throughout the epithelium in adenomas or adenocarcinoma. These results of expressional location of MCM2 protein are in accordance with the results from Freeman et al. [3, 10, 14] Interestingly, our data firstly showed that MCM2 mRNA was significantly up-regulated in adenocarcinomas in comparison to adenomas. However, there was no significant difference in the expression level of MCM2 mRNA between adenoma and normal mucosa. Taken together, the findings suggest that colonic adenocarcinoma could surpass adenomas in the capability of cell proliferation or numbers of cell entry into the cell cycle, and possibly imply that, compared the sequence of normal mucosa-adenoma, the cell

proliferation take an important role in phase of adenoma malignant to adenocarcinoma. Similar results are reported by Hiroshi Kawasaki et al,[15-17]who showed that the proliferative activity was significantly elevated during adenoma-carcinoma sequence by analyzing the expression of Ki-67 with immunohistochemistry. Therefore, this finding could give us a possibility and experimental foundation to identify colonic adenomas from MCM2-positive cells in stool and enhance the practicability of MCM2 as a marker of early detection for colonic cancer.

Adenomas are the precursors of most sporadic colorectal cancers. The adenoma-to-carcinoma sequence is characterized by recognizable histological changes. Lesions, starting with dysplastic aberrant crypt foci and benign tubular adenomas, have the potential to progress to advanced adenomas, which have a significant potential to transform into invasive adenocarcinomas.[18] In this study, according to the difference of macroscopic size, histopathologic type, morphologic type, grade of dysplasia and patients' age,[15, 16] we divided all samples of adenomas into different group, and further compared the difference of MCM2 mRNA expression in each group. Although statistically significant links were not observed between MCM2 mRNA expression and different clinicopathologic characters, we also amazedly observed that the expression level of MCM2 mRNA in 'advanced' adenomas with villous type, high grade dysplasia, larger size, sessile morphology and older age of patients was up-regulated in comparison to their respective control group. One of the reasons why the difference had no statistical significance may be due to a small number of the samples. Rocha Ramirez et al,[15] also showed that patient age, polyp size and morphology were the

more statistically significant risk factors for malignication in their patient group by the way of follow-up. Our finding also demonstrated that adenomas with risk factors for malignication, whose expression of proliferation marker-MCM2 was up-regulated, have potential of cell proliferation or more cells entry into the cell cycle, and might be more likely to progress down the malignant pathway than those adenomas whose expression level of MCM2 mRNA is lowness. Furthermore, Jonkers et al [19] reported that, in their study of endoscopic follow-up of 383 patients with colorectal adenomas, 14 were discharged from the follow-up programme by their treating physician because of their age or their severe co-morbidity, and 10 had not been compliant. Considering some of patients' age, co-morbidity, and compliance, quantitative detecting for proliferation markers may also provide an estimate of the frequency of potentially cancerous conditions in the patient with colonic adenoma, and this estimate may be helpful for determining whether to frequently surveil those patients with dysplastic lesion after colonoscopy excision and initiate other more costly evaluations for colonic cancer detection, although the histopathological diagnosis will remain the gold standard for malignant tumor.

In our study, we employed real-time RT-PCR method for the quantification of MCM2 mRNA expression and compared MCM2 mRNA expression between colonic adenomas and adenocarcinomas. The method, based on real-time analysis of PCR amplification and Taq-Man methodology, does not require post-PCR sample handling, thereby avoiding problems related to carry-over; it possesses a wide dynamic range and has a high sample throughput.[20, 21] Finally, and above all, real-time PCR makes

RNA quantization much more accurate, sensitive and reproducible, because it is based on Ct values established in the early exponential phase of the PCR reaction rather than end point measurement of the amount of accumulated PCR product. Real-time PCR has good intraassay and interassay reproducibility and yields statistical confidence values. [22, 23]

In this study, we also utilized a new software tool, named REST-XL$^{©}$, to test the group difference for significance with a newly developed randomization test. [24, 25]In our study, where the quantities of MCM2 mRNA are derived from ratios and variances can be high, normal distributions would not be expected, and it is unclear how a parametric test could best be constructed. Randomization test, which makes no assumptions about the distribution of observations in populations, is a useful alternative to more standard parametric tests for analyzing experimental data. It is more flexible than non-parametric tests based on ranks (Mann–Whitney, Kruskal–Wallis, etc.) and do not suffer a reduction in power relative to parametric tests (t-tests, ANOVA, etc). [25] Therefore, according up-mentioned reason, we think that randomization test with a pair-wise reallocation was the most appropriate approach for this application and choose it as a statistical way to use in this paper.

In summary, even though MCM2 was expressed in all epithelia throughout the crypts of adenoma and adenocarcinoma, our data firstly demonstrated that the level of MCM2 expression in adenocarcinoma was significantly higher than that in adenoma. This difference of the expression was an important first step to give us a possibility

and experimental foundation to identify adenoma from MCM2-positive cells in stool and estimate patients with adenoma at risk for subsequent development of adenocarcinoma, as well as present a supplement to enhance the practicability of MCM2 as a marker of early detection for colorectal cancer. Further studies are needed to clarify the prognostic value of MCM2 in adenoma, to explore MCM2 expression in different histopathologic type of colonic adenomas in a larger number of samples, and to detect patients at risk for subsequent development of adenocarcinoma.

Conflicts of interest

The authors declared no conflicts of interest.

Acknowledgements

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