**Name of journal:** *World Journal of Gastroenterology*

**ESPS Manuscript NO: 12624**

**Columns: ORIGINAL ARTICLE**

***Retrospective Study***

**KRAS, BRAF gene mutations and DNA mismatch repair status in Chinese colorectal carcinoma patients**

Ye JX *et al.* KRAS, BRAF and MMR status in Chinese CRC patients

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**Supported by** grants from the Xinjiang Uygur Autonomous Region Natural Science Fund (partly), No. 201233146-14

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**Received:** July 17, 2014  **Revised:** October 28, 2014

**Accepted:** December 5, 2014

**Published online:**

**Abstract**

**AIM:** To investigate gene mutations and DNA mismatch repair proteins (MMR) abnormality of Chinese colorectal carcinoma (CRC) patients with clinicopathologic analysis.

**METHOD:** Clinical and pathological information for 535 patients including 538 tumors were reviewed and recorded. Mutation analysis for exon 2 of KRAS and exon 15 of BRAF genes were all performed by Sanger sequencing except in 9 tumors an amplification refractory mutation system (ARMS) PCR was used. MMR proteins expression including MHL1, MSH2, MSH6 and PMS2 was evaluated by immunohistochemistry. KRAS and BRAF mutation status, the expression status of MMR genes were correlated with age, gender, cancer stage, location, and histology. Correlations between KRAS or BRAF mutation and MMR proteins expression were also explored.

**RESULTS:** The overall frequencies of KRAS and BRAF mutation were 37.9% and 4.4%, respectively. KRAS mutation was more common in patients ≥ 50 year-old (39.8% *vs* 22% in patients < 50 year-old, *P <* 0.05). The frequencies of BRAF mutant was higher in tumors from female (6.6% *vs* male 2.8%, *P <* 0.05), located in right colon (9.6% *vs* 2.1% in left colon, 1.8% in rectum, *P <* 0.01), with mucinous differentiation (9.8% *vs* 2.8% without mucinous differentiation, *P <* 0.01), being poorly differentiated (9.5% *vs* 3.4% well/moderately differentiated, *P <* 0.05). MMR deficiency was strongly associated with proximal location (20.5% in right colon *vs* 9.2% in left colon and 5.1% in rectum, *P <* 0.001), early cancer stage (15.0% in stageⅠ-Ⅱ *vs* 7.7% in stage Ⅲ-Ⅳ, *P <* 0.05), and mucinous differentiation (20.2% *vs* 9.2% without mucin, *P <* 0.01). A higher frequency of MLH1/PMS2 lost was found in females (9.2% *vs* 4.4% in male, *P <* 0.05) and MSH2/MSH6 lost tended to be seen in younger (< 50 year-old) patients (12.0% *vs* 4.0% ≥ 50 year-old, *P <* 0.05). MMR deficient tumors were less likely to have KRAS mutation (18.8% *vs* 41.7% in MMR proficient tumors, *P <* 0.05) and tumors with abnormal MLH1/PMS2 tend to harbor BRAF mutations (15.4% *vs* 4.2% in MMR proficient tumors, *P <* 0.05).

**CONCLUSION:** The frequency of sporadic CRCs undergoing BRAF mutation, MLH1 deficiency and MSI pathway in Chinese population may be lower than that in western population.

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**Key words**: Colorectal carcinoma; KRAS; BRAF; DNA mismatch repair; MLH1; MSH2; MSH6; PMS2

**Core tip**: Tests for KRAS, BRAF and DNA mismatch repair proteins status were important for clinical management for patients with colorectal carcinoma (CRC). Investigation from large samples for these molecular markers were limited in Chinese CRC patients. In present study, we collected and summarized clinicopathological and molecular data of 535 CRC patients in our institution. These results would help to understand CRC molecular features, guide Lynch syndrome screening, CRC clinical management and individualized therapy in Chinese population.

Ye JX, Liu Y, Qin Y, Zhong HH, Yi WN, Shi XY. KRAS, BRAF gene mutations and DNA mismatch repair status in Chinese colorectal carcinoma patients. *World J Gastroenterol* 2014; In press

**INTRODUCTION**

Colorectal carcinoma (CRC) is one of the most common cancers in the world. Although in China, CRC is not as common as that in most western countries, its incidence has increased steadily in recent years. The reasons for this are thought to be associated with living standard improvement and lifestyle change[1].

Based on current knowledge on the carcinogenesis of CRCs, individualized clinical managements are recommended to CRC patients[2,3]. Testing tumor tissue for predictive or prognostic gene mutations to guide personalized therapy is a rapidly emerging field in pathology.

The epidermal growth receptor (EGFR) is a major therapeutic target in colorectal cancer[4]. Activating mutations of KRAS gene is thought to stimulate the RAS/RAF/MAPK pathway independent of EGFR activation, therefore CRCs with KRAS mutation are resistant to EGFR inhibitors[5]. Although the predictive value of BRAF mutation status for EGFR inhibitors is still uncertain[6,7], its prognostic value for CRCs is widely accepted, *i.e.*, patients with BRAF V600E mutation tend to have a poor prognosis[8-10]. Moreover, BRAF V600E mutation in a MLH1 deficient CRC indicates it is a sporadic rather than a Lynch syndrome associated carcinoma with high level of microsatellites instability (MSI-H)[11]. MSI-H CRCs are either caused by germ line mutation or epigenetic silencing of DNA mismatch repair (MMR) genes[2] and have distinct clinical and pathological features. Detection of mismatch repair protein deficiency or MSI status is not only useful for screening Lynch syndrome but also can serve as a prognostic marker of favorable outcome. In addition, it is also a negative predictive marker for fluoropyrimidine-based chemotherapy in patients with stage Ⅱ disease[12,13]. From 2010, mutation analysis for KRAS and BRAF as well as MSI/MMR testing have been suggested to be performed for CRC patients by National Comprehensive Cancer Network (NCCN) clinical practice guidelines[14].

Frequencies of KRAS, BRAF mutation and MSI-H in CRCs have been widely studied in western populations. Among them, KRAS mutation is the most frequent molecular changes, ranging from 22% to 46.7%[15-20], while BRAF mutation is less frequent, ranging from 5.0% to 21.8%[15,21-25]. Several studies have reported the frequencies of KRAS and BRAF mutations in Chinese CRC patients in English literatures. However, most of the studies performed with limited samples size and the results were controversial, with frequency of KRAS mutation ranging from 19.7% to 43.9% and BRAF mutation ranging from 1.7% to 25.4%[26-32]. MSI CRCs account for approximately 15-20% of all CRCs in western countries[17,19,20,33-35]. Limited report from China shows a lower frequency of MSI CRCs in Chinese patients (ranging from 9.6% to 13%) than that in western populations but close to reports from Korea[36-40].

Information from previous studies raises the possibility that geographic and/or racial difference may present between Chinese and Western populations. Therefore, more data are needed to further clarify the characteristics of these important molecular changes in Chinese CRC patients. In the present study, we collected data of CRC patients from 2010 to 2013 in our department and hope to provide more information of CRC in Chinese patients.

**MATERIALS AND METHODS**

***Patients and tissues***

We searched the pathology database of the Department of Pathology of Peking University Third Hospital from 2010 to 2013 for primary or metastasis colorectal adenocarcinomas. Five hundred and thirty-five patients including 538 tumors tested for KRAS, BRAF or MMR proteins expression were collected. The pathology records and clinical charts were reviewed, the following information was obtained: patient gender, age, anatomic site of tumor, morphological characteristics (histologic type, tumor grade, depth of tumor penetration, lymph node involvement, lymphatic or vascular invasion, perineural invasion), history of metastasis or other tumor. Base on clinical data, primary locations of tumors were defined as right colon (from the cecum through the transverse colon), left colon (from the splenic flexure through the rectosigmoid flexure) and rectum (15 cm above the anal verge). Tumors were staged according to the seventh edition of the American Joint Commission on Cancer (AJCC) TNM staging system. Well to moderate differentiated tumors were grouped together and tumors diagnosed as mucinous adenocarcinoma, signet-ring cell carcinoma and adenocarcinomas with mucinous or signet-ring cells differentiation were recorded as tumors with mucin-producing.

Data and tissue collection were approved by the Ethics Committee of Peking University Health Science Center, following the ethical guidelines of the 1975 Declaration of Helsinki.

***Immunohistochemistry for MMR proteins***

Sections of one representative block of each tumor were incubated with antibodies to MLH1, MSH2, MSH6 and PMS2. Standard heat-induced epitope retrieval in EDTA solution (pH 9.0 for MLH1, PMS2, and MSH2, pH 6.0 for MSH6), the Dako EnVision Detection System, and a Dako Autostainer (Dako North America Inc, California, USA) were employed. The sources, dilutions, and incubation time of each primary antibody are listed in Table 1. Any tumor cell with nuclear staining was defined as positive for that marker. Positive staining for all these proteins was regarded as proficient MMR (pMMR). Negative staining for any of these four proteins was regarded as deficient MMR (dMMR) (Figure 2). Since family history and genetic information were unavailable, no attempt was made to further classify patients into with Lynch syndrome or sporadic MSI-H CRCs.

***Analysis of KRAS or BRAF gene mutation by Sanger sequencing***

As previously described by Zhong *et al*[41,42], genomic DNA was extracted from FFPE sections tumor tissue with the Qiagen Blood and Tissue Kit (QiagenInc, Valencia, CA) according to the manufacturer’s protocol. The KRAS exon 2 and BRAF exon 15 were amplified by polymerase chain reaction (PCR) using PromegaGoTaq® Hot Start Colorless Master Mixes (Promega Corporation, Madison, WI, USA). The specific primers and sizes of the expected amplicons were presented in Table 2. Genomic DNA of 50-100 ng was amplified in a 50 μL reaction containing 25 μL of Hot Start Colorless Master Mix and 5 μL of 10 μM primer mix. The PCR reaction consisted of 2 min at 95°C, followed by 40 cycles of 94°C for 30 s; 55°C for 40 s and 72°C for 1 min, finished by 72°C for 7 min. Five microliter of the PCR product was analyzed by 1.2% agarose gel with 100 to 600 bp DNA marker. Gels were visualized on a BioRad Gel Doc 2000TM system and Quantity One software (BioRad, Hercules, CA, USA). The resulting PCR amplicons were purified and sequenced in both directions using the BigDye Terminator kit and an ABI Prism 3500 DNA Analyzer (Applied Biosystems, Foster City, CA) according to the manufacturer’s instructions. Mutant cases were validated by second independent PCR and sequencing. The sequencing results were observed by ABI Sequence Scanner software and compared with the reference sequence of BRAF and KRAS gene from NCBI database to mark the position of nucleotide change.

***Analysis of KRAS and BRAF gene mutation by amplification refractory mutation system (ARMS) PCR***

For nine cases, mutations in KRAS exon 2 and BRAF exon 15 were identified by ARMS-PCR. Briefly, FFPE tissue was digested using 20-mg/mL proteinase K in ATL buffer (Qiagen) overnight at 56 °C. DNA isolation was performed with the QIAamp DNA Mini Kit (Qiagen) according to the manufacturer’s protocol. KRAS and BRAF mutation status were assessed with Human KRAS Gene 7 Mutations Fluorescence Polymerase Chain Reaction Diagnostic Kit and Human BRAF Gene V600E Mutations Fluorescence Polymerase Chain Reaction Diagnostic Kit (Amoy Diagnostics Co. Ltd, Xiamen, China) on Agilent-Stratagene M × 3000P Q-PCR System (Agilent Technologies, Santa Clara, CA), according to the manufacturers’ instructions respectively. The 7 most common KRAS mutations (p.G12D, p.G12V, p.G12A, p.G12C, p.G12S, p.G12R, and p.G13D) in CRCs were detected. The reaction conditions included 1 cycle at 95°C for 5min; 15 cycles at 95°C for 25 s, 64°C for 20 s, 70°C for 20 s; 31 cycles at 93°C for 25 s, 60°C for 35 s, 72°C for 20 s, fluorescence signals were collected at 60°C.

***Statistical analysis***

Clinical and pathological characteristics were summarized by percentage. Mutation rates of KRAS, BRAF and overall or each MMR protein expression deficiencies were also calculated. Clinical and pathological characteristics were compared across different subgroups and analyzed with χ2 test. Correlations between KRAS or BRAF mutation and dMMR status were also explored. *P* values < 0.05 were considered statistically significant.

**RESULTS**

***Patient characteristics***

Of these 535 patients, males were slightly more than females with a male to female ratio of 1.34:1. Patient age at presentation ranged from 21 to 95 years (median 65 years) with 10.7% of patients < 50 years at diagnosis. Most patients (61.2%) presented with stage II or III disease. Almost all patients had solitary primary tumor except three patients had synchronous tumors (two patients had two tumors confined in the left colon, one had two tumors with one in the right colon and the other in the rectum). In 6 patients, only metastasis lesions were available for testing, and 3 of them received neoadjuvant therapy. Both preoperative biopsy and radical specimens were tested for KRAS and BRAF mutations in two patients, and both primary and metastasis lesions were tested in 5 patients. Since there was no discrepancy between biopsy and radical resection specimens or between primary and metastatic tumors, the results from two different tests were recorded as once. Clinical information of all studied patients was summarized in Table 3.

***KRAS gene mutation and correlation with clinicopathological features***

KRAS status was ascertained for 485 patients including 488 tumors. The over-all mutation rate was 37.9% (185/488). KRAS mutations identified in codon 12 included G12D (*n =* 90, 18.4%), G12V (*n =* 37, 7.6%), G12C (*n =* 9, 1.8%), G12A (*n =* 8, 1.6%), G12S (*n =* 7, 1.4%), G12R (*n =* 1, 0.2%). Mutations in KRAS codon 13 included G13D (*n =* 31, 6.4%), G13C (*n =* 1, 0.2%) and G13G (silent mutation) (*n =* 1, 0.2%). One patient was identified with concomitant KRAS mutations in codon 12 and codon 33 (G12D and D33N). Compared with patients < 50 year-old, KRAS mutations in patients ≥ 50 year-old were more common (39.8% *vs* 22%, *P <* 0.05). The rate of KRAS mutations was not significantly associated with gender, tumor location, tumor differentiation, stage and with/without mucin-producing (Table 4).

***BRAF mutation and correlation with clinicopathological features***

Twenty (4.4%) mutations were detected in 453 CRCsfrom 450 patients. Sixteen mutations were V600E and the other four were D594G, D594N, R603stop and S602Y, respectively. There were no concomitant KRAS and BRAF V600E mutations, but one patient had concomitant mutations of KRAS G12V (Figure 1A) and BRAF R603stop (Figure 1B), one with KRAS G13D (Figure 1C) and BRAF S602Y (Figure 1D). The proportion of mutant BRAF was higher in females (6.6% *vs* 2.8% in males, *P <* 0.05), in tumors located in right colon (9.6% *vs* 2.1% in left colon and 1.8% in rectum, *P <* 0.05), with poor differentiation (9.5% *vs* 3.4% with well/moderately-differentiation, *P <* 0.05) and with mucinous appearance (9.8% *vs* 2.8% without mucin, *P <* 0.05). BRAF mutations were not related with patient’s age and tumor stage (Table 4).

***MMR proteins expression and correlations with clinicopathological features***

Overall, 11.4% tumors showed loss of expression for at least one MMR protein, and the deficient rates of MLH1, MSH2, MSH6 and PMS2 in studied patients were 5.8% (28/481), 2.7% (13/481), 3.8% (18/480) and 6.5% (19/293), respectively. The proportion of dMMR tumors varied by site with markedly higher rates (20.5%) in tumors located in right colon compared to those in left colon (9.2%) and rectum (5.1%, *P <* 0.001). There were more stageⅠ-Ⅱ dMMR tumors than stage Ⅲ-Ⅳ dMMR tumors (15.0% *vs* 7.7%, *P <* 0.05) and dMMR tumors tended to show mucinous differentation. In females, a higher frequency of MLH1/PMS2 deficiency was found (9.2% *vs* 4.4% in male, *P <* 0.05). Loss of MSH2/MSH6 expression was more frequent in patients < 50 year-old than in those ≥ 50 year-old (12.0% *vs* 4.0%, *P <* 0.05). Although dMMR tumors were more often in those with poor differentiation than in those with well-moderate differentiation, it did not show significant statistical difference (16.5% *vs* 10.4%, *P* > 0.05) (Table 5).

***Correlations between KRAS, BRAF and dMMR status***

Less KRAS mutant were seen in dMMR tumors than in pMMR tumors (18.8% *vs* 41.7%, *P <* 0.05). BRAF mutation rate was higher in dMMR tumors than in pMMR tumors, although it did not show significant statistical difference (9.3% *vs* 4.2%, *P* > 0.05). Nevertheless, tumors with defected MLH1/PMS2 tend to harbor BRAF mutation comparing with pMMR tumors (15.4% *vs* 4.2%, *P <* 0.05). No BRAF mutation was detected in tumors with MSH2/MSH6 defection (Table 6).

**DISCUSSION**

As predictive and/or prognostic biomarkers, KRAS and BRAF mutation tests are important for predicting EGFR-targeted therapy and prognosis of CRC patients. However, regional and racial differences for mutation rates may present[15-19,38,43,44]. Marked differences between Chinese population and other countries were also observed[26-32], which needed to be further confirmed especially based on large sample size.

In present study, we found an over-all KRAS mutation rate of 37.9% in CRCs which was close to most of the previous reports either about Chinese or other ethnicities[15-18,20,26-30,43,45-49]. Studies based on clinical practices or CRC cell line models showed that CRCs with KRAS G13D mutation responded partly to cetuximab and panitumumab[50,51]. Therefore the subtypes of KRAS mutation may also have clinical implications. In our cases, the major mutant types were G12D, G12V and G13D, accounting for 85% of all mutations. 6.4% of CRC patients in our group were found to have KRAS G13D mutation, which was also consistent with other reports[15,16,52]. One uncommon finding in our cases was concomitant KRAS mutations in codon 12 (G12D) and codon 33 (D33N). The patient was a 59 year-old male with a poorly differentiated tumor located in the left colon. To our knowledge, the latter mutation is the firstly identified mutant in CRCs. Other non-hot mutations were also found in previous reports, however, clinical impact for these mutations is unknown[53-55]. CRCs or colorectal cancer cell lines with special KRAS mutation seemed to have different malignant potential and proliferative ability[50,56], which may lead to different responses to anti-EGFR agents. But detailed mechanism needs further exploration.

Correlations between KRAS status and clinicopathological features are controversial. Some of the previous reports showed the frequency of KRAS mutation was in association with age, gender, tumor grade or stage but some did not[15,16,19,26,28,30]. Reports from four independent Chinese groups[28,30,45,52] showed KRAS mutations were associated with patient gender, but not patient age. In a study including 966 CRCs, Gao *et al*[57] observed KRAS mutations were not only associated with patient gender and age, but also with tumor differentiation. Similar inconsistent results were also presented in studies on other ethnicities[46,55]. In our series of cases, we could only find that tumors in patients older than 50 years tended to harbor mutant KRAS. These diverse findings suggest the difference of KRAS mutation rate in different groups is too minute to be declared with limited samples. The different criteria for age division might also be a cause[46,58].

BRAF mutation rates are significantly different in previous studies of non-Chinese population[15,21-25]. The lowest mutation rate came from a study on Japanese CRC patients (3%), followed by reports from Russia and Israel (4.1% and 5%, respectively)[24,43,44]. On the contrary, reports about European and Americans showed significantly higher rates of BRAF mutation, which were mostly around 15%[19,20,23,59]. In most of the studies about Chinese population[30,31,48], low percentage of CRCs were found to harbor mutant BRAF, ranging from 1.7% to 7%. The only exception is a report from Mao *et al*[32], which showed the frequency of BRAF mutation was as high as 25.4% in a group of Chinese CRC patients. However, their report also showed that 24% of KRAS mutant cases had concomitant BRAF V600E mutation. This phenomenon was inconsistent with the general opinion that concomitant KRAS and BRAF V600E mutation was rare if not mutually exclusive[15,45,60,61]. Considering the relatively limited number of cases included in their study (69 cases), the result may be not representative. In our study, we also observed a low BRAF mutation rate of 4.4%, which was consistent with most of other reports in Chinese and was similar to that in Japanese[44]. Our finding together with other reports support the opinion that the frequency of BRAF mutation is varied among different races and/or regions[21,31,61]. In addition, no patient was found to have concomitant mutations for KRAS and BRAF V600E in our cases. However, of the four cases with non-V600E BRAF mutation, two had concomitant KRAS mutation. Although the significances for these uncommon mutations were uncertain[22], our findings further confirmed the rarity of concomitant KRAS and BRAF V600E mutations in CRCs. Also in accordance with previous reports[21,61], we found BRAF mutation to be more common in females, in proximal located poorly differentiated tumors, or in tumors with mucin-producing, although no significant association was found between BRAF mutation and patient age or tumor stage. This may partially due to the number of BRAF mutant cases is limited in our study.

CRCs with deficient MMR exhibit high frequency MSI and have distinctive clinicopathological features, biological behavior and clinical treatment comparing to CRCs with pMMR, which in most cases are microsatellite stable (MSS)[2]. dMMR CRCs accounted for about 15%-20% of all CRCs in reports from Western countries[12,13,17,19,20,33,34,62], and are more common in stage II tumors (up to 22%) than in stage III tumors (up to 14%)[15,35]. Information about dMMR CRCs in Chinese population is limited. Huang *et al*[36] and Jin *et al*[37] using PCR-based MSI testing showed the frequencies of MSI-H CRCs in their cases from Southeast China were 11.9% to 13%, which were slightly lower than most of the reports from Western population[15,17,19,20,33-35]. Detecting MMR protein lost by IHC showed similar efficiency to more complexed gene analysis for MSI or MMR gene mutation[63,64]. Current study is one of the largest series that analyzing the MMR status by IHC in Chinese CRCs. Again, our data showed a low freqeuncy of dMMR in Chinese CRCs. The over-all frequency of dMMR CRCs and the frequencies of dMMR CRCs in early stage (stage I-II) and advanced stage (stage III-IV) CRCs were 11.4%, 15.4% and 7.7%, respectively. Our finding in combined with Huang *et al[36]* and Jin *et al*[37] reports suggest that the genetic background of CRCs in Chinese might be different from that in Western populations, and was less likely to undergo MSI pathway.

Of notes, the majority of dMMR CRCs is sporadic and caused by inactivation of MLH1 (about 95%). Lynch syndrome, which is caused by germ-line mutations of MMR genes, accounts for about 20% of the dMMR CRCs and MLH1 mutation is found in approximately 40% of the cases[65]. Therefore, the vast majority of dMMR CRCs should have MLH1 deficiency. By using IHC, our study was able to inform which MMR proteins were lost in a MSI CRC, and could provide more information than previous reports in Chinese CRCs. Surprisingly, our data showed only 50.9% of dMMR CRCs with MLH1 loss, which was much lower than expected. Moreover, BRAF mutations have been reported in 33% to 60% of MSI-H tumors particularly in tumors with methylation of the MLH1 promoter[20,35,66,67]. Among our dMMR cases, only 15.4% had BRAF mutation, which was also much lower than previous reports. These findings suggest that the low frequency of dMMR CRCs in our series is likely caused by including less sporadic dMMR CRCs. Our finding further indicated that ethnic and geographic difference might present in Chinese dMMR CRCs, although further investigations, such as germ-line mutation anaylsis of MMR genes and/or analysis of MLH1 promoter methylation, are needed to clarify this possibility. Since Hampel *et al*[68]’s study has showed that the widely used Amsterdam or Bethesda screening guideline may miss as many as 22% of patients with Lynch syndrome, feasible and economic IHC technique was suggested to perform on all newly diagnosed CRCs to screen Lynch syndrome and guide clinical management for MSI patients[69,70] . In this case, there will be more and more data coming out to provide detailed information of Chinese CRCs.

In summary, our results show a low frequency of BRAF mutation and MMR deficiency, especially less MLH1 deficiency in a large series of Chinese CRC patients. It suggests that CRCs undergo MSI pathway maybe less in Chinese populations, and it is probably caused by there are less sporadic MMR deficient CRCs in Chinese. However, additional epidemiologic data and genetic investigations are needed to confirm the difference.

**COMMENTS**

***Background***

Mutation analysis for KRAS and BRAF as well as MSI/MMR (microsatellites instability/DNA mismatch repair) testing have been suggested to be performed for patients with colorectal carcinomas (CRCs). Information from previous studies raises the possibility that geographic and/or racial difference may present between Chinese and Western populations. Investigation from large samples for these molecular markers were limited in Chinese CRC patients.

***Research frontiers***

The current study demonstrated that a low frequency of BRAF mutation and MMR deficiency, especially less MLH1 deficiency in a large series of Chinese CRC patients.

***Innovations and breakthroughs***

Results in present study suggests CRCs undergo MSI pathway maybe less in Chinese populations, and it is probably caused by there are less sporadic MMR deficient CRCs in Chinese.

***Applications***

These results would help to understand CRC molecular features, guide Lynch syndrome screening, CRC clinical management and individualized therapy in Chinese population.

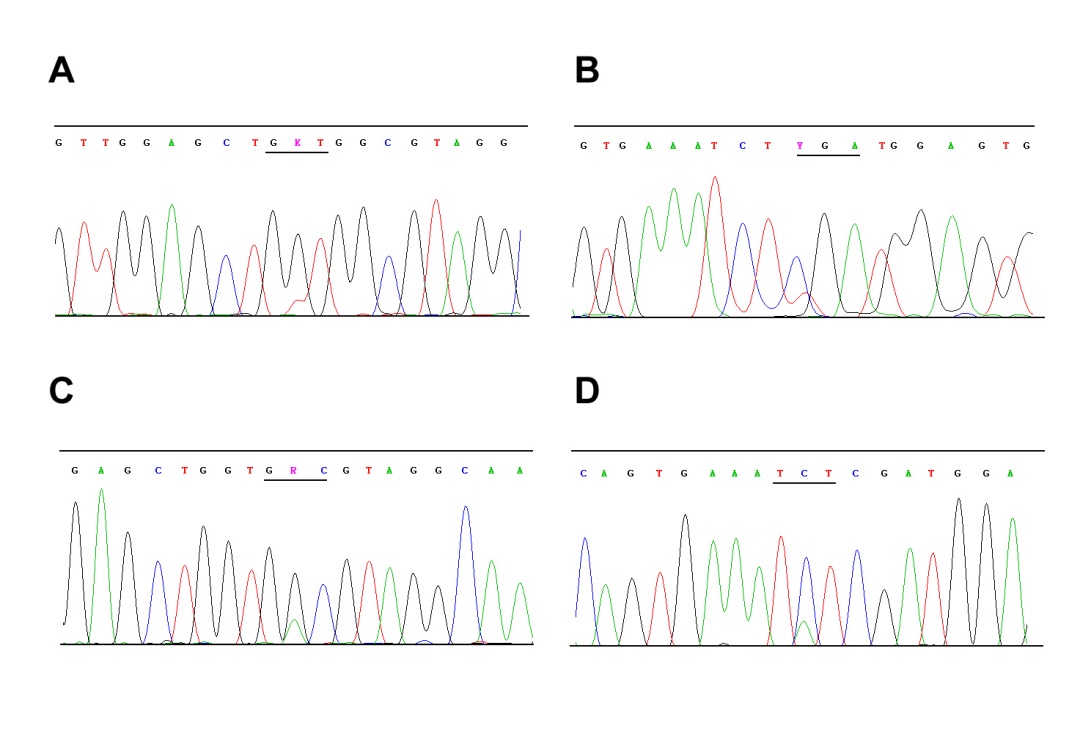
***Peer review***

The study reports KRAS, BRAF gene mutation and MMR protein expression status in large number of Chinese CRC patients. The results corroborates the earlier findings and shows high mutation in KRAS gene. Association of these genetic marker with various clinicopathological parameters have also been made. The study also show less MMR defect in these patients.

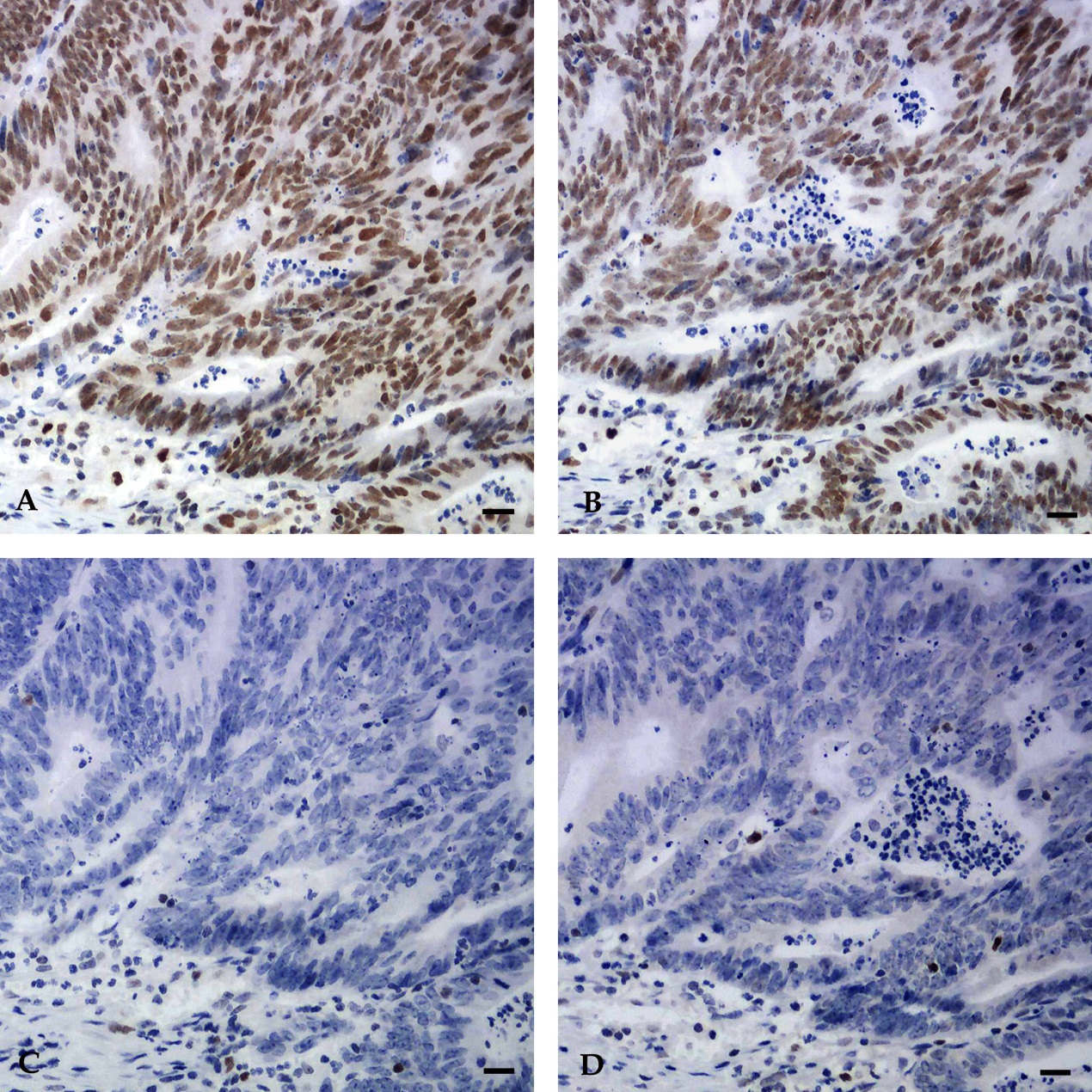
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**P-Reviewer:** Roychoudhury S **S-Editor:** Yu J **L-Editor: E-Editor:**



**Figure 1 Sequence chromatograms displaying concomitant mutations of KRAS and BRAF found in this study.** Concomitant mutations of KRAS G12V (1A) and BRAF R603stop were detected in CRC tissue of a 71 year-old woman (1B). Another patient, a 62 year-old woman, had mutations of both KRAS G13D (1C) and BRAF S602Y (1D). The amino acid changes were corresponded to the codon alternations as following: G12V (GGT→GTT) and G13D (GGC→GAC) in exon 2 of KRAS gene, S602Y (TCT→TAT) and R603stop (CGA→TGA) in exon 15 of BRAF gene.



**Figure 2 MMR proteins immunohistochemical stain for one case of colorectal carcinoma.** Tumor cells with retained MLH1 (A) and PMS2 (B) expression, and with absent MSH2 (C) and MSH6 (D) expression, which was regarded as deficient MMR (dMMR). Note stromal cells and lymphocytes serving as internal positive control.

**Table 1 Primary antibodies for mismatch repair proteins immunohistochemistry**

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **Antibody** | **Supplied by** | **Clone No.** | **Positive signal localization** | **Dilution** | **Incubation temperature (℃)** | **Incubation period (h)** |
| MLH1 | Novocastra, UK | 14 | nuclear | 1:50 | 37 | 2 |
| MSH2 | Novocastra, UK | FE11 | nuclear | 1:20 | 37 | 2 |
| MSH6 | Novocastra, UK | BC/44 | nuclear | 1:80 | 37 | 2 |
| PSM2 | Novocastra, UK | EP51 | nuclear | 1:30 | 37 | 2 |

**Table 2 Primer sequences for KRAS exon 2 and BRAF exons 15, corresponding annealing temperature and size of expected PCR products**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Exon** | **Primer No.** | **Primer sequence 5’-3’** | **TA (℃)** | **Product size (bp)** |
| KRAS 2 | 2-F | TAGTCACATTTTCATTATTTTTAT | 55 | 160 |
| 2-R | AGATTTACCTCTATTGTTGGAT |
| BRAF 15 | 15-F | ATCTACTGTTTTCCTTTACTTACT | 55 | 160 |
| 15-R | ATTCTTACCATCCACAAAATG |

TA: Annealing temperature.

**Table 3 Clinicopathological information of studied patients *n* (%)**

|  |  |  |
| --- | --- | --- |
| **Clinical feature** | | **Total cases** |
| Gender | Male | 306 (57.2) |
| Female | 229 (42.8) |
| Age(yr) | < 50 | 57 (10.7) |
| ≥ 50 | 478 (89.3) |
| Location | Right colon | 177 (32.9) |
| Left colon | 165 (30.7) |
| Rectum | 196 (36.4) |
| Tumor differentiation | Poor | 94 (17.5) |
| Well-moderate | 444 (82.5) |
| Tumor stage | Ⅰ | 95 (17.7) |
| Ⅱ | 168 (31.2) |
| Ⅲ | 215 (40.0) |
| Ⅳ | 50 (9.3) |
| Not available | 10 (1.9) |

**Table 4 Correlations between KRAS, BRAF status and clinicopathological features *n* (%)**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Clinicopathological features** | **KRAS** | | **BRAF** | |
| **Mutant/tested cases** | ***P*** | **Mutant/tested cases** | ***P*** |
| Gender | | | | |
| Male | 100/277 (36.1) | 0.336 | 7/254 (2.8) | **0.048** |
| Female | 84/208 (40.4) | 13/196 (6.6) |
| Age(yr) | | | | |
| < 50 | 11/50 (22) | **0.014** | 3/47(6.4) | 0.758 |
| ≥ 50 | 173/435 (39.8) | 17/403 (4.2) |
| Location | | | | |
| Right colon | 67/161 (41.6) | 0.318 | 14/146 (9.6) | **0.001** |
| Left colon | 50/150 (33.3) | 3/167 (2.1) |
| Rectum | 68/177 (38.4) | 3/140(1.8) |
| Mucin-producing | | | | |
| With | 41/108 (38) | 0.990 | 10/102 (9.8) | **0.006** |
| Without | 144/380 (37.9) | 10/351 (2.8) |
| Tumor differentiation | | | | |
| Poor | 25/84 (29.8) | 0.091 | 7/74 (9.5) | **0.045** |
| Well-moderate | 160/404 (39.6) | 13/379 (3.4) |
| Tumor stage | | | | |
| Ⅰ-Ⅱ | 85/232 (36.6) | 0.553 | 10/218 (4.6) | 0.918 |
| Ⅲ-Ⅳ | 97/247 (39.3) | 10/228 (4.4) |

**Table 5 Correlation between MMR proteins deficiency and clinicopathological features *n* (%)**

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| **Clinicopathological features** | | **dMMR** | | **MLH1/PMS2** | | **MSH2/MSH6** | |
| **Defective /tested** | ***P*** | **Defective /tested** | ***P*** | **Defective /tested** | ***P*** |
| Total | | 55/481 (11.4) |  | 31/481 (6.4) |  | 24/481 (5.0) |  |
| Gender | Male | 25/272 (9.2) | 0.095 | 12/272 (4.4) | **0.034** | 13/272 (4.8) | 0.970 |
| Female | 29/206 (14.1) | 19/206 (9.2) | 10/206 (4.9) |
| Age(yr) | < 50 | 9/50 (18.0) | 0.114 | 3/50 (6.0)) | 1.000 | 6/50 (12.0) | **0.031** |
| ≥ 50 | 45/428 (10.5) | 28/428 (6.5) | 17/428 (4.0) |
| Location | Right colon | 33/161 (20.5) | **0.000** | 23/161 (14.3) | **0.000** | 10/161 (6.2) | 0.446 |
| Left colon | 13/142 (9.2) | 5/142 (3.5) | 8/142 (5.6) |
| Rectum | 9/178 (5.1) | 3/178 (1.7) |  | 6/178 (3.4) |
| Mucin-producing | With | 20/99 (20.2) | **0.002** | 11/99 (11.1) | **0.034** | 9/99 (9.1) | 0.065 |
| Without | 35/382 (9.2) | 20/382 (5.2) | 15/382 (3.9) |
| Tumor differentiation | Poor | 13/79 (16.5) | 0.125 | 6/79 (7.6) | 0.649 | 7/79 (8.9) | 0.148 |
| Well-moderate | 42/402 (10.4) | 25/402 (6.2) | 17/402 (4.2) |
| Tumor stage | Ⅰ-Ⅱ | 37/246 (15.0) | **0.012** | 20/246 (8.1) | 0.127 | 17/246 (6.9) | **0.049** |
| Ⅲ-Ⅳ | 18/234 (7.7) | 11/234 (4.7) | 7/234 (3.0) |

**Table 6 Correlation between MMR proteins expression deficiency and KRAS or BRAF status *n* (%)**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **MMR status** | **KRAS (*n =* 432)** | | **BRAF (*n =* 424)** | |
| **Mutant/tested cases** | ***P*** | **Mutant/tested cases** | ***P*** |
| dMMR | 9/48 (18.8) | **0.0021** | 4/43(9.3) | 0.264 |
| MLH1/PMS2 deficiency | 3/29 (10.3) | **0.001**2 | 4/26(15.4) | **0.037**3 |
| MSH2/MSH6 deficiency | 6/19 (31.6) | 0.383c | 0/17(0) | 0.817c |
| pMMR | 160/384 (41.7) |  | 16/381(4.2) |  |

1dMMR *vs* pMMR; 2MLH1/PMS2 deficiency *vs* pMMR; 3 MSH2/MSH6 *vs* pMMR. MMR: DNA mismatch repair.