Original Article
Comparison of microsatellite status detection methods in colorectal carcinoma

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Abstract: There are two commonly accepted methods for detecting microsatellite status. One is to detect amplified microsatellite loci by polymerase chain reaction (PCR) and the other is to detect mismatch repair gene (MMR) protein expression by immunohistochemistry (IHC). PCR detection is considered to be accurate in clinical operations while IHC is widely used due to ease of operation and lesser expense. In order to compare IHC with PCR in detecting microsatellite status in colorectal carcinoma, a total of 569 samples of colorectal carcinoma resection were collected in the Department of Pathology, Nanjing Drum Tower Hospital, between June 2014 and June 2017. In all samples, IHC and PCR was used to detect microsatellite status and the consistency of results between the two methods was compared. We found that 48 cases of microsatellite instability (MSI) were detected by PCR including 37 cases of microsatellite instability high (MSI-H), 11 cases of microsatellite instability low (MSI-L), and 521 cases of MSS. MSI accounted for 8.44% of all cases and MSI-H accounted for 6.50%. IHC results of the 569 patients showed that 69 cases were deficient mismatch repair (dMMR) and 500 cases were proficient mismatch repair (pMMR). dMMR accounted for 12.13% of all cases. Loss expression of PMS2 protein was the most common while MSH6 was rare. The coincidence rate of the two methods for detecting microsatellite states was 91.92%. IHC and the PCR method had high consistency in microsatellite status. Compared with PCR, the IHC method is more economical and more convenient for clinical operations. When the 4 repair proteins were without deficiency detected by IHC, it could be diagnosed as MSS/MSI-L and further PCR was not necessary. When any repair protein was found to be deficient, PCR detection was needed to determine whether MSI existed. Our conclusion will save a lot of time and costs in clinical work.

Keywords: Immunohistochemistry (IHC), polymerase chain reaction (PCR), colorectal carcinoma, microsatellite instability (MSI), mismatch repair gene (MMR)

Introduction

Colorectal carcinoma is a common gastrointestinal carcinoma. Its pathogenesis is complex and it can be divided into hereditary and sporadic colorectal carcinoma. Lynch syndrome is the most important etiology that relates to hereditary colorectal carcinoma. Incidence of colorectal carcinoma in East Asia is on the rise. In China, there were 376,300 new cases of colorectal carcinoma and 191,000 death cases in 2015 [1]. Incidence and mortality of colorectal carcinoma in Hong Kong ranks second in malignant tumors. In colorectal carcinoma, microsatellite instability (MSI) exists, caused by insertion or deletion of a microsatellite in a tumor that leads to change in microsatellite length, with new microsatellite alleles emerging. MSI correlates with development, prognosis, efficacy, and inheritance of colorectal carcinoma [2-9]. Recent studies have shown that MSI is instructive for anti-PD-1 immunotherapy. The ORR in dMMR and pMMR groups was 40% and 0%, respectively. DCR in the two groups was 78% and 11%, respectively, both with significant differences [10].

In previous studies, MSI was presented in both hereditary and sporadic colorectal carcinomas. Approximately 15% to 24.3% of sporadic colorectal carcinoma in Western countries presented MSI [11-14] with 7.75% to 13% in China,
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which was close to South Korea but lower than Western population [15-18]. Different incidences of MSI were likely due to different genetic backgrounds and testing techniques. In hereditary nonpolyposis colorectal carcinoma, limited reports from China showed the same result as Western countries, as MSI could be detected in 80 to 90% of patients [19, 20]. It is evident that detection of MSI in hereditary colorectal carcinoma is essential.

At present, there are two methods to detect the stability of microsatellites. One is to detect the amplified microsatellite loci by PCR. Commonly used detection markers are BAT25, BAT26, DSS346, D2S123, and D17S250, as recommended by the National Carcinoma Institute. The state of MSI is determined by comparing the shift of the markers in tumor tissue and normal tissue. High level of instability is gauged by tumors with a shift in at least two markers. At least 30 percent of interpretable markers were classified as having high levels of microsatellite instability (MSI-H), in accordance with international criteria. A low level of microsatellite instability (MSI-L) was defined as a shift in only one dinucleotide marker. Tumors without any shift in markers were categorized as microsatellite stable (MSS) tumors. Another method is to determine microsatellite status by immunohistochemistry (IHC) detection of proteins encoded relating to DNA mismatch repair genes (MMR) including MLH1, MSH2, MSH6, and PMS2. Deficient mismatch repair (dMMR) was defined by the presence of either MSI-H or by loss of MLH1, MSH2, MSH6, or PMS2 protein expression, as outlined above. Proficient mismatch repair (pMMR) was defined by presence of either MSS/MSI-L (i.e., instability at <30% of loci screened) or by intact MMR protein expression [21]. MSI-L has been shown to be biologically similar to tumors exhibiting MSS at all loci tested and these two molecular phenotypes can be grouped together. Detection of MSI status by PCR is the earliest established molecular detection to identify MSI in colorectal carcinoma and is considered to be the gold standard for detecting MSI [22].

Figure 1. Results of paired normal and colorectal cancer DNA tissues tested for MSI using Bethesda recommended 5 markers by PCR. A. MSS patient. B. MSI-H patient. Black arrows indicate the mutation sites.

Figure 1.
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In the present study, we have found that long experimental period, high labora-
tory conditions, and high cost. At present, with continuous understanding of the mechanism of MSI and popularity of commercial monoclonal antibody of MMR proteins, the simple IHC method is more often being applied for MSI screening in colorectal carcinoma. It has been reported that IHC detection of MSI had similar results with PCR [23, 24].

Materials and methods

Materials

Patients and tissues: Five hundred sixty-nine surgical resection samples pathologically diagnosed as colorectal carcinoma were collected from the Department of Pathology, Nanjing Drum Tower Hospital, between June 2014 and June 2017. All samples satisfied the following criteria: (1) sporadic colon or rectal cancer confirmed by pathological diagnosis, (2) no preoperative therapy, including preoperative radiotherapy, in rectal cancer patients, and (3) received radical resection or a palliative operation. Exclusion criteria were (1) tumors in the appendix and anal canal; (2) second primary tumor out of colorectal; (3) high-grade intraepithelial neoplasia. This study was approved by the Ethics Committee of Nanjing Drum Tower Hospital. Written informed consent was obtained from each individual.

Reagents: Anti-MLH1, mouse monoclonal, ES05, 1:100 dilution, Leica/Novocastra, UK; anti-PSM2, rabbit monoclonal, EP51, 1:100 dilution, Epitomics, USA; anti-MSH2, mouse monoclonal, FE11, 1:100 dilution, Dako, Denmark; anti-MSH6, rabbit monoclonal, EP49, 1:150 dilution, Epitomics, USA; MSI MIX 1, 140 μL, Yuanqi Bio-Pharmaceutical CO, Shanghai, China; MIX 2, 200 μL, Yuanqi Bio-Pharmaceutical CO, Shanghai, China; PCR Premix (containing AmpliTaq Gold DNA Polymerase buffer, magnesium chloride and dNTPs), Yuanqi Bio-Pharmaceutical CO, Shanghai, China.

Methods

PCR-capillary electrophoresis detection for MSI: DNA was extracted from formalin-fixed paraffin-embedded tissue with QIAamp Tissue kit (Qiagen), according to manufacturer instructions. MSI detection kit was used to amplify the mutations of BAT25, BAT26, D5S346, D2S123, and D17S250. DNA samples from tumor tissues and normal tissues were amplified in a 20 μL volume containing 100 ng of DNA, 1 μmol/L of dye-labeled forward and unlabeled reverse primers, 200 μmol/L of deoxynucleotide, 1.5 mmol/L of MgCl2, and 0.75 U of Taq DNA polymerase. PCR was performed under the following conditions: denaturation at 95°C for 5 minutes, 35 cycles of denaturation at 95°C for 30 seconds, annealing at 53°C for 30 seconds, and extension at 72°C for 30 seconds. Final extension was at 72°C for 10 minutes. PCR product was analyzed by a genetic analyzer (Applied Biosystems 3500, ABI). Raw data were analyzed using GeneMapper 4.1 software. In accordance with National Cancer Institute (NCI) guidelines, MSI at ≥ 2 loci was defined as MSI high (MSI-H) (Figure 1B), instability at a single locus was defined as MSI low (MSI-L), and no instability at any of the loci tested was defined as microsatellite stable (MSS) (Figure 1A). Because extensive data indicate that tumors with low frequency are biologically similar to
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Of the 569 patients with colorectal carcinoma that underwent PCR detection (Figure 3A), 521 were MSS, accounting for 91.56%, 48 were MSI, accounting for 8.44%, of which 37 were MSI-H and 11 were MSI-L, accounting for 6.50% and 1.93%, respectively.

IHC detection for microsatellite status

Of the 569 cases receiving IHC staining of MMR proteins (Figure 3B), there were 69 cases of dMMR and the remaining 500 cases were pMMR. Incidence of dMMR and pMMR was 12.13% and 87.87%, respectively.

Comparison of IHC and PCR detection

PCR is shown in Table 1 and Figure 3C. Except for one case in which MLH1, MSH2, MSH6, and PMS2 expression was deficient, expression of MMR protein was positive in the other 36 cases of MSI-H. It is worth noting that there were 7 cases determined as MSI-H by PCR while all of the MMR proteins were positively expressed,

Table 1. Expression of mismatch repair gene proteins in 37 cases of MSI-H colorectal carcinoma determined by PCR (n)

<table>
<thead>
<tr>
<th>MMR</th>
<th>-</th>
<th>+</th>
<th>2+/3+</th>
</tr>
</thead>
<tbody>
<tr>
<td>MLH1</td>
<td>12</td>
<td>17</td>
<td>8</td>
</tr>
<tr>
<td>MSH2</td>
<td>4</td>
<td>10</td>
<td>23</td>
</tr>
<tr>
<td>MSH6</td>
<td>3</td>
<td>8</td>
<td>2</td>
</tr>
<tr>
<td>PMS2</td>
<td>25</td>
<td>7</td>
<td>5</td>
</tr>
</tbody>
</table>

Note: PCR, polymerase chain reaction; MSI-H, microsatellite instability high; MMR, mismatch repair gene.

Results

PCR detection for microsatellite status

Of the 569 patients with colorectal carcinoma that underwent PCR detection (Figure 3A), 521 were MSS, accounting for 91.56%, 48 were MSI, accounting for 8.44%, of which 37 were MSI-H and 11 were MSI-L, accounting for 6.50% and 1.93%, respectively.

IHC detection for microsatellite status

Of the 569 cases receiving IHC staining of MMR proteins (Figure 3B), there were 69 cases of dMMR and the remaining 500 cases were pMMR. Incidence of dMMR and pMMR was 12.13% and 87.87%, respectively.

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Table 2. Comparison between IHC and PCR of microsatellite status in 569 cases of colorectal carcinoma (n)

<table>
<thead>
<tr>
<th>IHC</th>
<th>PCR</th>
<th>Total</th>
<th>Kappa consistency test</th>
</tr>
</thead>
<tbody>
<tr>
<td>MSI-H</td>
<td>MSI-L/MSS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MSI-H</td>
<td>30</td>
<td>39</td>
<td>69</td>
</tr>
<tr>
<td>MSI-L/MSS</td>
<td>7</td>
<td>493</td>
<td>500</td>
</tr>
<tr>
<td>Total</td>
<td>37</td>
<td>532</td>
<td>569</td>
</tr>
</tbody>
</table>

Note: PCR, polymerase chain reaction; IHC, immunohistochemistry; MSS, microsatellite stable; MSI-L, microsatellite instability low; MSI-H, microsatellite instability high.

Figure 4. Expression of MMR protein in 37 cases of MSI-H colorectal cancer determined by PCR. White: negative expression; gray: weak positive expression or “+”; black: strong positive expression or “+++/++++”.

Expression of MMR protein in colorectal carcinoma of MSI-L/MSS determined by PCR

In 11 cases of colorectal carcinoma determined as MSI-L by PCR, there were eight cases expressing positive of all four proteins, showing the same results with PCR. There were still 3 cases found with negative staining but they were dMMR. The error rate was 27.27%. Among 521 cases of colorectal carcinoma detected as MSS by PCR, 485 were judged as pMMR by IHC, consistent with results of PCR. However, there were also 36 cases of dMMR and the error rate was 6.91%.

There were 500 cases of pMMR (MSI-L/MSS) detected by IHC and just 7 cases were judged as MSI-H by PCR. The error rate was only 1.40%. There were 69 patients with dMMR (MSI-H), of which 39 were judged as MSI-L/MSS by PCR, accounting for 56.52%. The error rate was very high.

Expression of MMR proteins in colorectal carcinoma of MSI-H determined by PCR

In 37 cases of colorectal carcinoma with MSI-H determined by PCR, the rate of expression loss of MMR was 81.08% (30/37) (Figure 4). The vast majority of occurrences with dMMR were due to inactivation of PMS2 (67.57% (25/37)), similar to previous reports [24-26]. MSH2 and MSH6 account for a much smaller percentage (10.81% and 8.11) while MLH1 accounted for 32.43%. Negative staining was not found in the 7 MSI-H cases.

Discussion

Colorectal carcinoma remains an important risk factor for human life and health. Microsatellite status has had a great impact on its chemotherapy and immunotherapy [2-10], which is of great significance in determination of treatment, judgment of prognosis, and screening of family genetic diseases. In our experiment, PCR and IHC were used to detect microsatellite status of colorectal carcinoma. Results show that the coincidence rate was 91.92%, which was high in consistency. In our study, MSI cases accounted for approximately 8.44%-12.13% of all 569 cases, lower than that reported in Western populations [11-14] but...
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close to reports from Korea. This may be related to differences with race, genetics, and lifestyle [15-18].

Our study results showed that deletion rate of PMS2 was the highest among four MMR while MSH6 was the lowest. We saw that negative staining for PMS2 protein had higher sensitivity but lower specificity for detecting microsatellite status, while MSH6 showed the opposite.

With improvement of molecular biological experiments, accuracy and convenience of microsatellite detection has been greatly enhanced but with the sensitivity and specificity of IHC detection, 100% is still difficult to reach. It has been reported that detection of MSH6 mismatch repair protein expression in vitro or MSH6 mutant mice may be regarded as MSS by PCR [27-29] and even 14/18 (78%) cases were regarded as MSS or MSI-L when MSH6 expression was abnormal [30]. The results of our study also demonstrated that 39 out of 532 MSI-L/MSS cases were regarded as dMMR by IHC and 7 out of 37 MSI-H cases were regarded as pMMR by IHC. The reasons for different results between the two detection methods may include the following aspects: (1) PCR detection showed that some of the genes that affected microsatellite status were mutated but the antigenic determinant of expression production was not undermined. Although the production was not functional, IHC result was still positive, which may lead to false negatives; (2) Due to MMR protein's functional redundancy, it may not be enough to lead to occurrence of MSI-H when individual MMR protein was missing; (3) The time of tissue fixation and staining will also affect IHC results during the process; (4) Sensitivity of IHC is also dependent on its antibody type and other types of MMR genes can also lead to dMMR besides MLH1, MSH2, MSH6, and PMS2.

Because IHC detection of microsatellite status has the advantages of being a simple operation, short time of operation, low cost, low requirement of experimental instruments, and display of each repair protein, it has high application value. It can be used as a first-line screening method for detecting microsatellite status in colorectal carcinoma. However, Vasen et al. [31] indicated that IHC could not completely replace PCR method in determining microsatellite status until other dMMR genes were elucidated. Our study shows 500 cases of pMMR detected by IHC. Just 7 cases were judged as MSI-H by PCR and the error rate was only 1.40%. There were 69 patients with dMMR (MSI-H), 39 were judged as MSI-L/MSS by PCR, accounting for 56.52%. The error rate was very high. Therefore, we believe that when the 4 repair proteins were without deficiency detected by immunohistochemistry, it could be diagnosed as MSS/MSI-L and further PCR was not necessary. But when any repair protein was found to be deficient, PCR detection was needed to determine whether microsatellite instability existed. Our conclusion will save a lot of time and cost for clinical work.

In order to reduce false negatives and positive proportions detected by IHC method during our application, we strictly performed every step of the operation and results of IHC were confirmed by two experienced pathologists, independently. Errors caused by subjective factors should be avoided as much as possible. It is critical that we discover new genes that affect deletion of MMR proteins.

Acknowledgements

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Disclosure of conflict of interest

None.

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