INTRODUCTION

Microsatellite instability (MSI) is defined as a hypermutable phenotype with alteration in repetitive DNA sequence length. Microsatellites or short tandem repeat (STR) markers are DNA sequences, including repetitive units with 1 to 6 nucleotides.

This is an open access journal, and articles are distributed under the terms of the Creative Commons Attribution-NonCommercial-ShareAlike 4.0 License, which allows others to remix, tweak, and build upon the work non-commercially, as long as appropriate credit is given and the new creations are licensed under the identical terms.

For reprints contact: WKHLRPMedknow_reprints@wolterskluwer.com

How to cite this article: Miar P, Tabatabaiefar MA, Abdollahi Z, Noruzi M, Kazemi M, Naimi A, et al. BAT25, ACVR2, and TGFBR2 mononucleotide STR markers: A triplex panel for microsatellite instability testing in colorectal tumors. Adv Biomed Res 2022;11:79.
Moreover, MSI-H is predominantly associated with long-term adjuvant chemotherapy in patients with MSI-CRCs is used when fewer than 30% of STR markers are unstable, while MSI-low (MSI-L) is used when more than 30% of STR markers are unstable, MSI-high (MSI-H) is defined when the NCI panel.

Lynch syndrome (LS) is the most common inherited colorectal cancer (CRC) due to a germline mutation in one of the MMR genes. In this autosomal dominant syndrome, there is also a high rate of cancer incidence in the endometrium, stomach, ovary, small bowel, hepatobiliary tract, upper urologic tract, glioblastoma, pancreas, and breast. MSI feature has been reported in more than 90% of LS tumors and about 15% of sporadic CRCs. MSI tumors have distinct clinicopathological features such as mucinous cells, signet-ring cells, and poorly differentiated cells with relatively good prognoses. There are three clinical criteria for LS diagnosis, including Amsterdam I, Amsterdam II, and Bethesda with 60%, 80%, and 96% sensitivity, respectively.

Different microsatellite markers can be used to detect MSI. In 1997, the National Cancer Institute (NCI) established a five-microsatellite-marker panel for MSI testing containing two dinucleotide and three mononucleotide markers (BAT25, BAT26, D2S123, D5S346, D17S250). After a while, studies showed higher sensitivity and specificity for mononucleotide markers compared with dinucleotide, trinucleotide, tetra-nucleotide, and pentanucleotide markers, significantly. Accordingly, the Promega company established a pentaplex panel including five quasi-monomorphic mononucleotide markers (BAT-25, BAT-26, MON0-27, NR-21, and NR-24) with higher sensitivity and specificity than the NCI panel. Three categories have been recommended for the MSI description. MSI-high (MSI-H) is defined when more than 30% of STR markers are unstable, MSI-low (MSI-L) is used when fewer than 30% of STR markers are unstable, and if there are no unstable markers, the tumor is classified as microsatellite-stable (MSS). Studies have demonstrated a better prognosis in MSI-CRCs in comparison to MSS ones. Moreover, the therapeutic response to Fluorouracil-based adjuvant chemotherapy in patients with MSI-CRCs is less than MSS-CRCs, according to some studies. Moreover, MSI-H is predominantly associated with long-term immunotherapy-related responses in CRC and non-CRC malignancies treated with immune checkpoint inhibitors.

The BAT25 selection was done according to the different previous studies and Promega standard kit panel. ACVR2 and TGFBR2 mononucleotide markers were also selected from molecular studies on MSI in 2016.

Based on the previous studies, the BAT25 mononucleotide marker that locates in the c-kit gene with A repetitive sequence has high sensitivity and specificity for MSI detection in different populations. Furthermore, two other mononucleotide markers, including ACVR2 in Actin receptor type 2 gene, and TGFBR2 in transforming growth factor-beta (TGF-β) receptor type 2 gene with A and A repetitive sequences, have also presented instability in MSI-CRCs.

Although the Promega kit is commonly used for MSI testing, the evaluation of other markers in different populations may help increase the accuracy of the MSI detection kit. Moreover, the cost of the Promega kit is high in Iran, and it is inaccessible due to political sanctions. Accordingly, this pilot study was run to evaluate the sensitivity and specificity of a novel triplex panel including three mononucleotide markers for MSI testing in colorectal tumors at-risk for LS among the Iranian population compared to the Promega standard kit. The main question is, what percentage of the diagnostic results of the test with the triplex panel produced in the laboratory of the Department of Genetics, Isfahan University of Medical Sciences, are compatible with the Promega kit?

Materials and Methods

Patients and specimens

Altogether, 280 CRC patients with a positive family history of cancer were enrolled in this study from Iranians Cancer Control Charity Institute (MACSA), a referral charity-based service provider for cancer patients and their families in central Iran, Isfahan, and Pourina Hakim Gastrointestinal Research Center, Isfahan, Iran. Thirty-seven tumors and their adjacent normal Formalin-Fixed Paraffin-Embedded (FFPE) tissue specimens were collected based on Amsterdam II criteria in the patients [Table 1]. The fresh samples or those less than five years old were given priority. Tumor tissues and their adjacent FFPE were separated by a pathologist for each patient. This study was supported by an MSc grant from Isfahan University of Medical Sciences (397055) with IR.MUI.RESEARCH.REC.1397.131 as ethical number (Research ethics certificate has been attached).

Primers and PCR

DNA extraction was done for 37 pairs of FFPE tissue samples from both tumors and their adjacent normal tissues, separately, using the Salehi et al., 2008 protocol. The extracted DNA was used for three mononucleotide markers as a template in the Polymerase Chain Reaction (PCR). Primers were designed using Primer3 (V.0.4.0), and their fluorescent labeling was performed based on Schuelke’s method [Table 2].

Table 1: Amsterdam II criteria for clinical screening of colorectal cancer patients at-risk for Lynch syndrome

| Amsterdam II criteria |
|-----------------------|
| Three or more relatives with HNPCC-associated cancer, one of whom is a first-degree relative of the other two. |
| FAP should be excluded. |
| Cancer can be observed in at least two generations. |
| One or more cancer cases diagnosed ≤50 years. |
| HNPCC: Hereditary Non-polyposis colorectal cancer, FAP: Familial adenomatous polyposis |

Advanced Biomedical Research | 2022
The final volume of PCR reagents for each marker was considered 10 µl, containing 5 µl Biofact Master Mix (2×), 0.2 µl forward primer (10 µM), 0.2 µl reverse primer (10 µM), 0.3 µl NED*M13 (10 µM), 3.3 µl dH₂O, and 1 µl DNA (50 ng). The Touch-down PCR was run on the extracted DNA and the designed primers according to Table 3.

Moreover, a multiplex PCR was set up as a three-monomonucleotide marker panel according to Table 4. The Touch-down PCR condition was performed as mentioned before.

**MSI analysis**

All three mononucleotide markers were labeled with NED; hence to avoid overlapping graphs, we did not pool all PCR products in one tube in the first step. PCR products of ACVR2 and TGFBR2, and BAT25 were analyzed in separate tubes for fragment analysis.

For multiplex PCR, amplified sequences were finally sent for fragment analysis. The amplified fragments were detected by ABI PRISM 3100 Genetic Analyser. Moreover, all extracted DNA from tumors and their adjacent normal FFPE tissue specimens were evaluated by MSI Analysis System kit (Promega), as a gold standard based on the MSI Analysis System protocol, Version 1.2.

The instability of more than one marker is considered as MSI-H, and no instability is defined as MSS. Moreover, if just one marker presents instability, the tumor is categorized as MSI-low. [Figures 1 and 2].

**Data analysis**

The fragment analysis is performed by capillary electrophoresis that can detect different fluorescent colors in a microtube and separate the different fragments in terms of size with precision in pairs. The presence of MSI, which leads to the changes in the length of the duplicate sequence, can be detected by fragment analysis. As mentioned before, fragment analysis was performed by capillary electrophoresis ABI PRISM 3100 to reduce or increase the length of repetitive sequences.

Furthermore, raw files extracted from the device were analyzed by Gene marker software (Version 1.85). The tumor sample of each patient was compared with the normal one, and the changes or non-changes in the number of repetitive sequences of each marker were examined and recorded. The sensitivity and specificity of each marker and triplex panel were calculated.

Furthermore, the Kappa statistics that determine what percentage of the two tests (regardless and after eliminating the possibility of chance agreements) agree are calculated, too. Kappa statistics analysis was performed using STATA software version 14.

## RESULTS

Altogether, 37 CRC cases with Amsterdam II criteria were finally included in this study from 280 CRC patients with a positive family history of cancer. The instability state of three selected markers on tumor DNA was evaluated in comparison with the Promega kit as a gold standard. A marker was considered sensitive when the results of its usage were the same as the gold standard. The MSI testing results obtained from the innovative three-monomonucleotide marker panel and MSI Analysis System kit (Promega) have been summarized in Table 5.

Overall, 10 of 37 (27%) samples were defined as MSI-H, and 27 of 37 (72%) samples were reported as MSS by gold standard Promega kit. Based on sensitivity and specificity calculation, among three innovative markers, BAT25 was also unstable in all 10 MSI-H cases (100% sensitivity). ACVR2 and TGFBR2 markers showed instability with 76.9% and 83.3% sensitivity, respectively. Moreover, in all 27 MSS samples, according to the Promega, the BAT25 marker showed stability (100% specificity). ACVR2 and TGFBR2 were also stable in all 27 MSS cases (100% specificity). Meanwhile, no samples were considered as MSI-L by the MSI Analysis System kit (Promega) [Table 5].

Moreover, these three mononucleotide markers were used as a three-monomonucleotide marker panel for MSI detection with multiplex PCR [Figures 3 and 4]. Considering instability in ≥ one marker as MSI-H, the results were the same as the Promega kit, with no MSI-L sample. Furthermore, the sensitivity and specificity of the triplex panel are calculated with 100% sensitivity and 100% specificity based on Table 6.
Miar, et al.: BAT25, ACVR2, and TGFBR2 for MSI testing in colorectal tumors

The Kappa statistics for the triplex panel are calculated (Kappa statistics: 1.00; *P* value <0.0001). Most experts classify the Kappa statistics above 0.75 as “excellent agreement beyond chance”.

### DISCUSSION

MSI status as a good prognostic and predictive molecular marker in colorectal tumors could be evaluated using different markers. In 2004, a pentaplex panel was suggested for MSI testing with high accuracy, which is currently used worldwide. Meanwhile, other different markers with diverse accuracy have been suggested by several studies on different populations. To obtain a more cost-effective panel for the Iranian population, we evaluated three mononucleotide markers separately and optimized a triplex panel for MSI testing.

These three mononucleotide markers were analyzed in DNA extracted from tumors and their adjacent normal FFPE tissues of 37 CRC patients with Amsterdam II criteria. The amplification of markers was performed in both separate PCRs and a multiplex PCR, and the results were compared with the Promega MSI testing kit. Our study indicated 100% sensitivity for the BAT25 marker. Also, both ACVR2 and TGFBR2 presented 76.9% and 83.3% sensitivity with 100% specificity, respectively.

According to the findings, the innovative triplex panel could detect all MSI-H cases, but when the analysis was according to panel 2, it demonstrated one case as MSI-L that had not been detected with the gold standard. Meanwhile, when we defined tumors for panel 1, the MSI-L case was detected as MSI-H. According to the currently accepted protocols, MSI-H status is defined when more than 30% of the markers present instability. Thus, considering one marker as a cut-off

---

**Table 4: PCR reagents for BAT25, ACVR2, and TGFBR2 markers**

| No. | Reagent Name                                      | Concentration | Volume (µl) | Description                               |
|-----|--------------------------------------------------|---------------|-------------|-------------------------------------------|
| 1   | Master Mix (2×) (Biofact Company)                | 2×            | 5           |                                          |
| 2   | Primer Forward + M13 (Eurofins Company)         | 10 µM         | 0.2 per primer (0.15 for TGFBR2)          |
| 3   | Primer Reverse (Eurofins Company)               | 10 µM         | 0.2 per primer (0.15 for TGFBR2)          |
| 4   | M13 + Flourescent dye (Eurofins Company)        | 10 µM         | 0.3         |                                          |
| 5   | DNA                                              | 50 ng/µl      | 1           |                                          |
| 6   | ddH2O                                            |               | Up to 10   |                                          |

**Table 5: Performance of each three mononucleotide markers separately in comparison to the gold standard (Promega kit)**

| Marker          | Instability | Stability | Sensitivity | Specificity |
|-----------------|-------------|-----------|-------------|-------------|
| BAT25           | 10/10       | 27/27     | 100%        | 100%        |
| ACVR2           | 7/10        | 30/27     | 76.9%       | 100%        |
| TGFBR2          | 8/10        | 29/27     | 83.3%       | 100%        |
| MSI Analysis System kit (Promega) | 10/37      | 27/37 MSS | 100%        |

MSI-H: Microsatellite instability-high, MSS: Microsatellite stable

---

Figure 1: An example of Promega kit fragment analysis in an MSI-H patient (above the line: Normal, below the line: Tumor). MSI-H: Microsatellite instability-high

---

**Figure 1**: An example of Promega kit fragment analysis in an MSI-H patient (above the line: Normal, below the line: Tumor). MSI-H: Microsatellite instability-high.
for MSI-H in the triplex kit, as the current guidelines, may lead to misclassification of MSI-L tumors to MSI-H, an issue that could be prevented by adding more markers to this panel. The Promega Kit with five STR markers is used commonly for MSI evaluation, but the high price of the kit and its poor accessibility due to the imposed sanctions have limited its application in Iran. Although reducing the number of markers can reduce the kit price, adding more markers would be more reliable for MSI testing. Meanwhile, these three suggested markers can be included in an innovative MSI kit. As mentioned, the primers design technique in this study is affordable and will reduce the price. In this Primer design technique, no extra fluorescent labels are needed, and M13 sequences can play a critical role in one fluorescent label entry into target sequences for all three markers. All PCR products for these three markers were designed in different sizes and detected by one fluorescent label (NED) easily, and all Fragment analysis graphs in multiplex PCRs were detected without overlapping. This technique will reduce the kit price dramatically and can be used for the MSI evaluation.

In addition, the Promega MSI panel is considered the gold standard for MSI detection. However, the utility of the Promega kit is challenging due to the variable degree of sensitivity and specificity as well as the polymorphic features of its markers. Therefore, current guidelines strongly recommended developing new panels with a relatively small number of highly sensitive mononucleotide repeat markers with monomorphic features across the different populations. Integrating the previously identified MSI marker loci with acceptable sensitivity and specificity is a promising approach to developing informative panels. To the best of our knowledge, there is no study to apply BAT25, ACVR2, and TGFBR2 as a triplex panel to evaluate MSI in CRC tumors. Our results of validity measuring were encouraging compared to the gold standard. The accuracy of these three markers is shown both separately and as a triplex panel. Altogether, fast and easy performance (due to the multiplex PCR we

| Record of gold Standard (Promega kit) | Record of triplex panel | Total |
|--------------------------------------|-------------------------|-------|
| MSS                                  | 27                      | 0     | 27   |
| MSI-H                                | 0                       | 10    | 10   |
| Total                                | 27                      | 10    | 37   |

MSI-H: Microsatellite instability-high, MSS: Microsatellite stable
developed in our study), acceptable accuracy, and simplified interpretation (due to the relatively small number of stutter) of this triplex panel would introduce it as an informative candidate panel for MSI testing.

**Conclusion**

The current study demonstrated high accuracy for the three-monomonucleotide marker (BAT25, ACVR2, and TGFBR2) as a triplex panel in MSI testing. Moreover, this study reconfirms the importance of molecular screening of tumor DNA through MSI testing in CRC patients at-risk for LS. Given the results, this triplex panel can be considered in more evaluations with more samples for investigating the feasibility of using it as an alternative diagnostic kit for MSI testing.

**Acknowledgments**

We appreciate the patients and their families for permitting us to use their samples. We also appreciate the health workers in the Iranian Cancer Control Charity Institute (MACSA), Isfahan, Iran, and Poursina Hakim Digestive Diseases Research Center, Isfahan, Iran.

**Financial support and sponsorship**

Nil.

**Conflicts of interest**

There are no conflicts of interest.

**References**

1. Sinicrope FA. Lynch syndrome-Associated colorectal cancer. N Engl J Med 2018;379:764-73.
2. Pellat A, Netter J, Perkins G, Cohen R, Coulet F, Paré Y, et al. [Lynch syndrome: What is new?]. Bull Cancer 2019;106:647-55.
3. Vilar E, Gruber SB. Microsatellite instability in colorectal cancer—The stable evidence. Nat Rev Clin Oncol 2010;7:153-62.
4. Kelkar YD, Srubczewski N, Hile SE, Chiaromonte F, Eckert KA, Makova KD. What is a microsatellite: A computational and experimental definition based upon repeat mutational behavior at A/T and GT/AC repeats. Genome Biol Evol 2010;2:620-35.
5. Koh W-J, Abu-Rustum NR, Bean S, Bradley K, Campos SM, Cho KR, et al. Uterine neoplasms, version 1.2018, NCCN clinical practice guidelines in oncology. J Natl Compr Canc Netw 2018;16:170-99.
6. Biller LH, Syngal S, Yurgelun MB. Recent advances in Lynch syndrome. Fam Cancer 2019;18:211-9.
7. Kastrinos F, Mukherjee B, Tayob N, Wang F, Sparr J, Raymond VM, et al. The risk of pancreatic cancer in families with Lynch syndrome. JAMA 2009;302:1790-5.
8. Bauer CM, Ray AM, Halstead-Nussloch BA, Dekker RG, Raymond VM, Gruber SB, et al. Hereditary prostate cancer as a feature of Lynch syndrome. Fam Cancer 2011;10:37-42.
9. Win AK, Young JP, Lindor NM, Tucker KM, Ahnen DJ, Young GP, et al. Colorectal and other cancer risks for carriers and noncarriers from families with a DNA mismatch repair gene mutation: A prospective cohort study. J Clin Oncol 2012;30:958-64.
10. Lynch HT, Snyder CL, Shaw TG, Heinen CD, Hitchins MP. Milestones of Lynch syndrome: 1895-2015. Nat Rev Cancer 2015;15:181-94.
11. Vasen HF, Möslin G, Alonso A, Bernstein I, Bertario L, Blanco I, et al. Guidelines for the clinical management of Lynch syndrome (hereditary non-polyposis cancer). J Med Genet 2007;44:353-62.
12. Umar A, Boland CR, Terdiman JP, Syngal S, de la Chapelle A, Rüschoff J, et al. Revised Bethesda guidelines for hereditary nonpolyposis colorectal cancer (Lynch syndrome) and microsatellite instability. J Natl Cancer Inst 2004;96:261-8.
13. Giardiello FM, Allen JI, Axilbund JE, Boland CR, Burke CA, Burt RW, et al. Guidelines on genetic evaluation and management of Lynch syndrome: A consensus statement by the us multi-society task force on colorectal cancer. Gastroenterology 2014;147:502-26.
14. Dietmaier W, Wallinger S, Bocker T, Kullmann F, Fishel R, Rüschoff J. Diagnostic microsatellite instability: Definition and correlation with...
mismatch repair protein expression. Cancer Res 1997;57:4749-56.
15. Bachr JW, Flanagan LA, Smalley RL, Nassif NA, Burgart LJ, Halberg RB, et al. Development of a fluorescent multiplex assay for detection of MSI-high tumors. Disease Markers. IOS Press; 2004. p. 237-50.
16. Hegde M, Ferber M, Mao R, Samowitz W, Ganguly A. ACMG technical standards and guidelines for genetic testing for inherited colorectal cancer (Lynch syndrome, familial adenomatous polyposis, and MYH-associated polyposis). Genet Med 2014;16:101-16.
17. Boland PM, Yurgelun MB, Boland CR. Recent progress in Lynch syndrome and other familial colorectal cancer syndromes. CA Cancer J Clin 2018;68:217-31.
18. Yurgelun MB, Hampel H. Recent advances in Lynch syndrome: Diagnosis, treatment, and cancer prevention. Am Soc Clin Oncol Educ Book 2018;38:101-9.
19. Popat S, Hubner R, Houlston RS. Systematic review of microsatellite instability and colorectal cancer prognosis. J Clin Oncol 2005;23:609-18.
20. Zhao P, Li L, Jiang X, Li Q. Mismatch repair deficiency/microsatellite instability-high as a predictor for anti-PD-1/PD-L1 immunotherapy efficacy. J Hematol Oncol 2019;12:54.
21. Buhard O, Saraweer N, Lectard A, Duval A, Hamelin R. Quasimonomorphic mononucleotide repeats for high-level microsatellite instability analysis. Dis Markers 2004;20:251-7.
22. Alhopuro P, Sammalkorpi H, Niittymäki I, Biström M, Raitila A, Saharinen J, et al. Candidate driver genes in microsatellite-unstable colorectal cancer. Int J Cancer 2012;130:1558-66.
23. Cortes-Ciriano I, Lee S, Park WY, Kim TM, Park PJ. A molecular portrait of microsatellite instability across multiple cancers. Nat Commun 2017;8:15180.
24. Hause RJ, Pritchard CC, Shendure J, Salipante SJ. Classification and characterization of microsatellite instability across 18 cancer types. Nat Med 2016;22:1342-50.
25. Pagnin A, Zertimech F, Leclerc J, Wacrenier A, Lejeune S, Descarpentries C, et al. Evaluation of a new panel of six mononucleotide repeat markers for the detection of DNA mismatch repair-deficient tumours. Br J Cancer 2013;108:2079-87.
26. Salehi R, Tabanifar B, Asgarani E, Faghhi M, Allame T. An efficient method for DNA extraction from paraffin wax embedded tissues for PCR amplification of human and viral DNA. Iran J Pathol 2008;3:173-8.
27. Schuelke M. An economic method for the fluorescent labeling of PCR fragments. Nat Biotechnol 2000;18:233-4.
28. Chang L, Chang M, Chang HM, Chang F. Microsatellite instability: A predictive biomarker for cancer immunotherapy. Appl Immunohistochem Mol Morphol 2018;26:e15-21.
29. Storojeva I, Boulay JL, Heinimann K, Ballabeni P, Terracciano L, Laffer U, et al. Prognostic and predictive relevance of microsatellite instability in colorectal cancer. Oncol Rep 2005;14:241-9.
30. Xirola RM, Llor X, Pons E, Castells A, Alenda C, Pihol V, et al. Performance of different microsatellite marker panels for detection of mismatch repair-deficient colorectal tumors. J Natl Cancer Inst 2007;99:244-52.
31. Goel A, Nagasaka T, Hamelin R, Boland CR. An optimized pentaplex PCR for detecting DNA mismatch repair-deficient colorectal cancers. PLoS One 2010;5:e9393.
32. Raedle J. Bethesda guidelines: Relation to microsatellite instability and MLH1 promoter methylation in patients with colorectal cancer. Ann Intern Med 2001;135:566-76.
33. Bianchi F, Galizia E, Catalanì R, Belvederesi L, Ferretti C, Corradini F, et al. CAT25 is a mononucleotide marker to identify HNPPC patients. J Mol Diagnostics 2009;11:248-52.
34. Farahani N, Nikpour P, Emami MH, Hashemzadeh M, Zeinalian M, Shariatpanahi SS, et al. Evaluation of MT1XT20 single quasi-monomorphic mononucleotide marker for characterizing microsatellite instability in persian Lynch syndrome patients. Asian Pac J Cancer Prev 2016;17:4259-65.
35. Steinke V, Holzapfel S, Loeffler M, Holinski-Feder E, Morak M, Schackert HK, et al. Evaluating the performance of clinical criteria for predicting mismatch repair gene mutations in Lynch syndrome: A comprehensive analysis of 3,671 families. Int J Cancer 2014;135:69-77.
36. Cicek MS, Lindor NM, Gallinger S, Bapat B, Hopper JL, Jenkins MA, et al. Quality assessment and correlation of microsatellite instability and immunohistochemical markers among population- And clinic-based colorectal tumors: Results from the colon cancer family registry. J Mol Diagnostics 2011;13:271-81.
37. Baudrin LG, Deleuze JF, How-Kit A. Molecular and computational methods for the detection of microsatellite instability in cancer Front Oncol 2018;8:621.
38. Salipante SJ, Scroggins SM, Hampel HL, Turner EH, Pritchard CC. Microsatellite instability detection by next generation sequencing. Clin Chem 2014;60:1192-9.