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Using a Rapid, Accurate, and Cost-Effective QiaXcel Advanced Platform for Microsatellite Instability Detection in Colorectal Carcinoma in North of Iraq

Nigar A Abdalrahman[1*], DInya A Mohamad[2]

[1]Molecular Biologist at Hiwa Hematology/Oncology Hospital, Sulaymaniyah, Kurdistan Region, Iraq.
[2]Department of Biology, College of Science, University of Sulaimani, Sulaymaniyah, Kurdistan Region, Iraq.
*Correspondence to: Nigar A Abdalrahman (E-mail: nigarana82@gmail.com)
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Abstract

Objective: The objective of this investigation was to address the limitations of the most popular microsatellite instability (MSI) detection method, which uses fluorescent capillary sequencers.

Methods: Using the QiaXcel Advanced system (Qiagen, Hilden, Germany) based on capillary electrophoresis, the MSI status of 53 Iraqi Formalin Fixed Paraffin Embedded (FFPE) CRC samples was examined. BAT25, BAT26, BAT40, D2S123, D5S346, D17S250, NR21, NR22, NR27, MycI1, TPOX, and TH01 were among the panel of twelve polymorphic markers that were used.

Results: Using a QiaXcel Advanced platform was successfully established to determine the MSI status. Among 53 cases of CRC, MSI was observed in 12 cases (22.64%) who had MSI-H.

Conclusion: Due to MSI's significance in the progression of cancer, this quick and inexpensive PCR-based technique can enhance the clinical management of CRC, which may further alter the patient's outcome.

Keywords: Colorectal cancer, microsatellites, MSI, QiaXcel advanced

Introduction

Microsatellite (MS), also called Short Tandem Repeats (STRs), consists of 1–6 nucleotides repeated, typically repeated 15 to 65 times.[1] Compared to other genomic areas, these repeated sequences have higher rates of mutation accumulation, are mainly located near the end of the chromosome, and are distributed most widely in coding and non-coding region.[2] MS represents approximately 3% of the human genome.[3] It has two main parts: the core and the peripheral flank, variation in the number of repeating units mainly occurs in the core part and this gives MS specific characteristics.[2] MS is characterized as a powerful tool in genetic diversity, population analysis, and paternity tests, therefore it can be used in numerous fields including botany, genetics, zoology, and medical microbiology.[4,5]

During replication, mutations accumulated in these repetitive sequences mainly because DNA polymerases cannot bind effectively and cause mismatched nucleotides and DNA slippage which results in the insertion or deletion loops (IDLs).[6] Typically, the identification and correction of errors are carried out by mismatch repair (MMR) genes (MLH1, MSH2, MSH6, PMS2). Mutation in these genes led to an accumulation of errors in microsatellite sequence and cause microsatellite instability (MSI). In DNA coding regions, insertions or deletions in microsatellites cause frameshift mutations, which can result in protein truncations.[7]

Deficient Mismatch Repair (dMMR) arises from germline mutations in MMR genes and EPCAM among individuals with hereditary nonpolyposis CRC (HNPCC) or Lynch syndrome, or due to somatic hypermethylation of the MLH1 promoter.[8,9] dMMR occurs in many types of cancer such as colorectal cancers (CRCs), endometrial cancer (EC), gastric cancer, pancreatic cancer, ovarian, sebaceous carcinomas, glioblastoma and lymphomas.[10] Roughly (15–20)% of CRCs caused by dMMR.[11]

Microsatellite instability (MSI) is a phenotypic sign of dMMR caused by the inactivation of MMR genes.[12] Oncologists have gained awareness of microsatellites and their significance in various human cancers, particularly colorectal carcinomas. MSI are frequently observed in these cancers, indicating potential damage to the host cell's mismatch repair system. As a result, MSI testing has emerged as a valuable predictive and diagnostic marker.[13,14] A systematic study conducted by Popat et al.[15] revealed that patients with MSI exhibited significantly improved prognosis compared to individuals with MS-stable tumors, and they also displayed enhanced responsiveness to chemotherapy.

The detection of dMMR can be accomplished through two approaches: immunohistochemistry (IHC) that focuses on four MMR proteins including (MLH1, MSH2, MSH6 and PMS2), and/or MSI DNA-based testing.[16] IHC involves the assessment of MMR protein expression, enabling the observation of specific protein absence or loss within the nuclei of tumor cells. On the other hand, MSI diagnosis via polymerase chain reaction (PCR) involves amplifying specific microsatellite repeat regions.

In the current era of prevalent MSI testing, immunohistochemistry (IHC) has demonstrated commendable performance as an alternative approach for assessing MSI status, establishing itself as a fundamental tool for clinical evaluation of MSI status. It can be used widely, is affordable, and is practicable.[2]

However, this method detects the loss of MMR protein expression, specifically, MSH2, MSH6, MLH1, and PMS2, rather than direct DNA alterations, and it can only classify CRC as either MSI-H or MSS. It is unable to identify MSI-L or estimate the level of allelic instability, and its results can be influenced by tissue fixation conditions.[17]

Moreover, IHC may miss about five percent of MSI-H tumors with normal level of MMR proteins (these MMR proteins still express but aren't working properly). In this case of tumors would then be incorrectly categorized as MSS instead of MSI.[18] IHC's accuracy was assessed by Cheah et al. to be between 89 and 95 percent,[19] whereas CGE and fluorescent

multiplex PCR can both achieve accuracy levels of up to 100 percent.[20] Therefore, PCR-based MSI testing is a useful alternate method in these situations since it is more sensitive in identifying real functional MMR protein deficiencies through mutational status.

In Iraq, it appears that the MSI test for colorectal tumors that receives the most requests is the most important test in management of colorectal cancer patients. Nevertheless, analyzing microsatellites typically demands high-end laboratory equipment, such as a capillary sequencing instrument with the ability to differentiate multiple fluorescent dyes. As a result, MSI testing is restricted to highly specialized laboratories. Capillary electrophoresis platforms are a more widely accessible tool and are routinely used in diagnostic labs for precise DNA and RNA studies. Therefore, we aimed to build and establish MSI test based on CGE using QiaXcel Advanced system (Qiagen, Hilden, Germany) in Hiwa Hospital.

Methods

Patient Samples

A total of fifty-three patients with a diagnosed colorectal cancer were included in the study. In all cases normal tissues from the corresponding patients were utilized as control samples. Tumors and matched normal tissues (normal adjacent tissue) NAT (n = 106) were obtained from Hiwa Oncology and Hematology Hospital and private Pathology Centers from North of Iraq (Kurdistan- Sulaymaniyah).

DNA Extraction and Quantitation

Genomic DNA was isolated from formalin-fixed paraffin-embedded (FFPE) samples of both tumor and adjacent normal tissue (NAT) using the QIAamp® DSP DNA FFPE Tissue Kit (Qiagen, Hilden, Germany). For each FFPE block, five to seven 10 µm sections were utilized for DNA extraction, following the kit's manufacturer instructions. DNA quantification was conducted using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) for the obtained DNA samples.

Microsatellite Instability Analysis

Microsatellite instability analysis was conducted on both tumor tissue and adjacent normal tissue using a panel of twelve polymorphic markers including (BAT25, BAT26, BAT40, D2S123, D5S346, D17S250, NR21, NR22, NR27, Mycl1, TPOX and TH01), as outlined in Table 1. The selection of markers encompassed various criteria: the mononucleotide markers (NR21, NR22, BAT25 and BAT26) originated from the initial pentaplex panel.[21] While NR27 was adopted from the modified pentaplex panel,[22] and the (BAT40) which is a poly T marker and located on chromosome 1 was demonstrated to have a predominant sensitivity in both CRC and extra-colonic tumors.[23-25] The selection also involved three di-nucleotide markers, D2S123, D5S346, and D17S250, from the Bethesda panel. To ensure sample uniqueness and verify the origin of tumor and corresponding normal samples, two tetranucleotide markers (TPOX and TH01) were chosen for their substantial polymorphism and minimal MSI.[26] Furthermore, MYCL1, a highly polymorphic tetranucleotide repeat marker known for its relatively elevated instability rate, was also included. MYCL1 is frequently employed to assess MSI status and is among the frequently mutated markers used for detecting low-level MSI.[27-29]

The Polymerase chain reaction (PCR) was performed using previously existed primers with free labeled fluorescent. Amplification reactions (20 µl) were created by incorporating 50–100 ng of genomic DNA into AddStart Taq Master (2x Conc.) (KOREA), which contained 20 mM Tris-HCl (pH 8.8), 100 mM KCl, 0.2% Triton® X-100, and 4mM $MgCl_2$. The mix also contained protein stabilizer, sediment, loading dye, and 0.5 mM of each dATP, dCTP, dGTP, and dTTP. The amplification process involved incubation step for 10 minutes at 95°C, followed by 35 cycles of three steps including denaturation at 95°C for 30 seconds, annealing at 59°C for 30 seconds, and extension at 72°C for 30 seconds. The last incubation at 72°C

Table 1. **List of primer sequences used to amplify the set of microsatellite loci and corresponding product size obtained**

Name of microsatellite	Gene	Forward-primer 5′ to 3′	Reverse-primer 5′ to 3′	Average PCR fragment size (bp)
BAT-25	c-kit	TCGCCTCCAAGAATGTAAGT	TCTGCATTTTAACTATGGCTC	124
BAT-26	hMSH2	TGACTACTTTTGACTTCAGCC	AACCATTCAACATTTTTAACCC	122
BAT-40	HSD3B1	AGTCCATTTTATATCCTCAAGC	GTAGAGCAAGACCACCTTG	149
NR-21	SLC7A8	TAAATGTATGTCTCCCCTGG	ATTCCTACTCCGCATTCACA	104
NR-22	Transmembrane precursor protein B5	GAGGCTTGTCAAGGACATAA	AATTCGGATGCCATCCAGTT	143
NR-27	Inhibitor of apoptosis-protein 1	AACCATGCTTGCAAACCACT	CGATAATACTAGCAATGACC	89
D2s123	AFM093xh3	AAACAGGATGCCTGCCTTTA	GGACTTTCCACCTATGGGAC	211
D17s250	LASP1	GGAAGAATCAAATAGACAAT	GCTGGCCATATATATATTTAAACC	162
D5s346	APC	ACTCACTCTAGTGATAAATCGGG	AGCAGATAAGACAGTATTACTAGTT	137
MYCL1	MYCL1	TGGCGAGACTCCATCAAAG	CCTTTTAAGCTGCAACAATTTC	190
TH01	TH01	GTGGGCTGAAAAGCTCCCGATTAT	GTGATTCCCATTGGCCTGTTCCTC	162
TPOX	TPOX	ACTGGCACAGAACAGGCACTTAGG	GGAGGAACTGGGAACCACACAGGT	244

bp, (base-pair).

for 7 minutes was conducted for all biomarkers except for TH01 and TPOX with annealing step at 69°C for 30 seconds. The GeneAmp PCR System 9700 (ThermoFisher Scientific, USA) was employed for this amplification.

The PCR products were subjected for analysis using the QIAxcel Advanced System (QIAGEN GmbH, Hilden, Germany) with the QIAxcel DNA High-Resolution Kit (2400) (QIAGEN GmbH, Hilden, Germany). A 15 bp/600 bp QX Alignment Marker was employed alongside the DNA size marker 25 bp–500 bp at a concentration of 30 ng/µl, without necessitating purification steps. Separation was carried out using the OM400 method, utilizing a customized protocol: alignment marker injection at 5 kV for 10 seconds, sample injection at 5 kV for 10 seconds, and separation at 6 kV for 400 seconds.

The classification of samples was as follows: Microsatellite Stable (MSS) when no changes in microsatellites were detectable; Microsatellite Instability-Low (MSI-L) when one microsatellite locus displayed instability; and Microsatellite Instability-High (MSI-H) when more than one of the examined markers exhibited instability. All classifications were compared against the corresponding Normal Adjacent Tissue (NAT) for evaluation purposes.

Results

Microsatellite instability (MSI) testing was carried out on a collection of 53 retrospectively gathered colorectal cancer (CRC) samples. Among these cases, certain samples had previously undergone MSI testing through immunohistochemistry (IHC) and were subsequently validated in an external laboratory using fluorescent dye-based capillary electrophoresis. These samples were retested to ensure the precision of our findings.

A total of 24 PCR reactions were conducted per patient, encompassing twelve microsatellites (BAT25, BAT26, BAT40, D2S123, D5S346, D17S250, NR21, NR22, NR27, Mycl1, TPOX and TH01). For each microsatellite, the DNA from the tumor was compared to the isolated DNA from healthy control tissue.

Despite the possibility of variations in overall intensity, the electropherogram for microsatellite-stable (MSS) samples exhibits an identical pattern in both tumor and healthy tissue (Fig. 1A). In cases of microsatellite instability (MSI), differences between tumor and healthy tissue are typically indicated by the presence of either additional peaks (insertion) at the right side of the graph, indicating longer microsatellite (MS) sequences or deleted peaks (deletion) which show shorter microsatellite (Fig. 1B). The classification of samples as MSS or MSI-H depends on the number of unstable markers. A sample is labeled as MSS when none of the markers are unstable, MSI-L if one marker displays instability, and MSI-H if two or more markers are unstable. These instabilities are typically evident through distinct peak patterns.

The analysis successfully identified 12 cases (22.64%) with high-level microsatellite instability (MSI-H), while the remaining 41 cases (77.36%) exhibited a microsatellite-stable (MSS) profile in electrophoresis. None of the cases were classified as MSI-L, and Mycl1 did not exhibit any variations in any of the MSS samples; however, variations were observed in MSI-H samples.

Discussion

Previously, QiaXcel technology has been applied for microsatellite analysis in endometrial cancer and plant species, as documented in references.[5,30] Moreover, this technology has been successfully employed for microsatellite analysis in colorectal carcinoma by Forster et al.,[31] serving as a validated alternative method to detect microsatellite instability (MSI) in CRC.[32] Our research outcomes are consistent with the existing literature, which have revealed comparable performance levels between traditional approaches and alternative approaches for MSI recognition. Notably, various studies have reported concordance rates ranging from approximately 95% to 98%.[33,34] While previous investigations have explored diverse technologies for MSI detection,[33] fluorescent-based PCR assays coupled with capillary sequencing devices have commonly been used. Building upon these insights, our study underscores the viability of implementing this alternative system at Hiwa Hospital, given its accuracy in identifying MSI status and its potential significance in the clinical practice and management of colorectal cancer.

The Qiaxcel platform offers a more straightforward and reproducible procedure compared to the standard method, ensuring consistent and reliable data results. Additionally, is cost-effective and time-consuming compared with IHC and sequencing platforms.

To decrease the number of individual PCRs, it's feasible to combine microsatellite targets with varying sizes. While the variation in microsatellites is useful for phylogenetic analyses, like parental testing,[35,36] it poses a challenge for colorectal cancer testing, requiring tumor-free tissue controls for each patient. Furthermore, discrepancies in the overall intensity of electropherograms are probably attributed to variations in DNA quality and the preparation process from FFPE material.

The software provided a valuable feature to overlay patterns from tumor and normal DNA, making it easier to identify discrepancies between both spectra and simplify the detection of MSI status. This proved especially beneficial in cases with uncertain and noisy electropherograms, aiding in the accurate interpretation of electrophoresis results and defining the correct genotype of the samples tested.[34] Additionally we acquired corresponding migration data from gel electrophoresis for all MSI markers in all cases, providing additional support for the analysis with the Qiaxcel system, sample preparation time was notably reduced, and the electropherograms achieved adequate resolution for all tested loci. This led to cost savings and a quicker turnaround time for the final report, significantly enhancing the efficiency of the analysis process.

Conclusion

Our results strongly demonstrate that the Qiaxcel system is not only fast and reliable but also shows a remarkable sensitivity for the clinical analysis of MSI. A notable advantage is the label-free PCR amplicons, which eliminates the necessity for specialized methods or equipment, such as sequencing platforms. To sum up, it be concluded that the using of QiaXcel system for MSI analysis is remarkably time-efficient

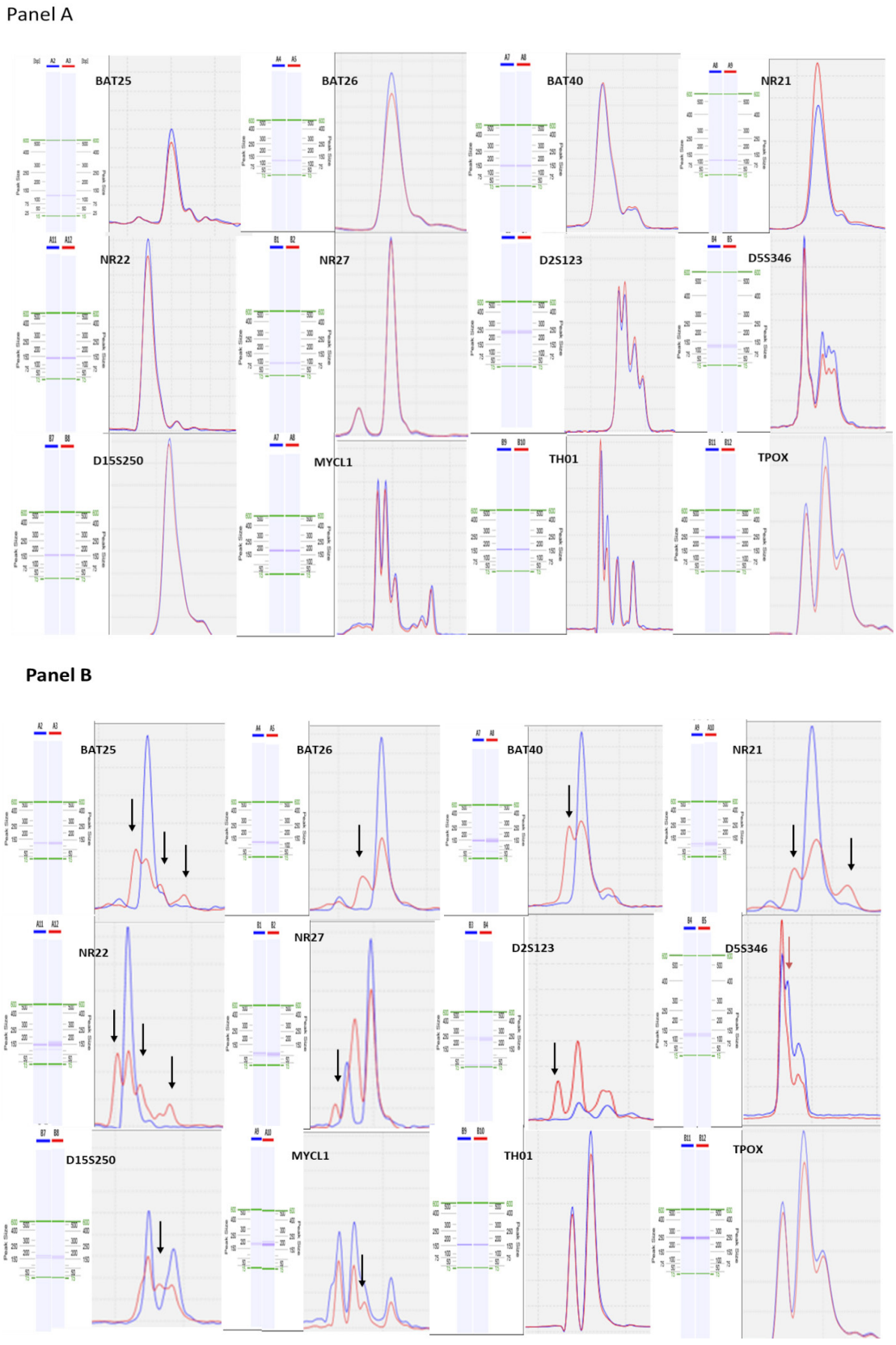

Fig. 1 **Representative illustration of the electropherograms of the encompassing twelve microsatellites (BAT25, BAT26, BAT40, NR21, NR22, NR27, D2S123, D5S346, D17S250, Mycl1, TPOX and TH01) obtaining using Qiaxel system. In the figure electropherograms derived from a stable case (MSS, Panel A) and from an instable case (MSI-H, Panel B) are shown respectively. The patterns were obtained overlaying the spectrum derived from tumor (red) and corresponding Normal Adjacent Tissue (blue) for each locus analyzed, with visible discrepancies arrows (the black arrows additional peaks and the red arrow is deleted peak), simplifying the identification of microsatellite instability. The corresponding gel electrophoresis migration for all the cases and loci are reported.**

and straightforward procedure, while still maintaining a high level of sensitivity. The approach we used is suitable for validating routine diagnostics of MSI testing in colorectal cancers and can be applied in pathology laboratories lacking a capillary sequencing device.

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Conflicts of Interest

This study was not funded by any organization, institute, or anybody.

Authors' Contribution

Both authors shared roles in the laboratory collecting samples, manuscript writing and statistical analysis.

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