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Original Article

Diagnostic test assessment. Validation study of an alternative system to detect microsatellite instability in colorectal carcinoma

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Summary

The American Society for Clinical Pathology (ASCP), College of American Pathologists (CAP), Association for Molecular Pathology (AMP), and the American Society of Clinical Oncology (ASCO) have been recently strongly recommended the evaluation of mismatch repair status (MMS) as molecular biomarkers in colorectal cancer for a better prognostic stratification of patients. This recommendation is emphasized by the recent evidence of Microsatellite Instability (MSI) as a predictive marker for chemotherapy and immunotherapy. In this scenario, the validation of molecular biomarker testing methods seems to be essential to design the most appropriate tailored therapy and the most suitable care strategy, respectively.

In this study, we validated an alternative method based on capillary electrophoresis system label-free PCR (Qiaxcel system) to evaluate the MSI Bethesda Panel. We also parallel the results with a standard approach.

Our data showed total concordance with the standard approach, with a highly time-efficient and easy procedure combined with high sensitivity for MSI detection.

Alternative capillary electrophoresis based on label-free PCR such as the Qiaxel system is a very sensitive and specific method to detect MSI for the management of patients with colorectal cancer. This procedure is adequate and suitable in diagnostic routine for the evaluation of microsatellite repeats compared to standard procedures.

Key words: colorectal cancer, tumour markers, molecular pathology, DNA

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Introduction

Different studies recently focused on selecting molecular tests for the best clinical and therapeutic management of patients with colorectal cancer (CRC) [1]. Improvements in earlier cancer detection together with an increased understanding of the molecular and genetic basis of the disease have been leading to the reduction in death rates for CRC, such as the selection of targeted and conventional therapies [2].

Microsatellite instability (MSI) is due to defect in the mismatch repair (MMR) pathway and accounts for approximately 15% of CRC [3]. The discrimination between MSI from microsatellite-stable (MSS) CRC is clinically important for different reasons: (i) detection of Lynch Syndrome since MMR is a reliable genetic marker which may guide clinicians towards informative, cost-effective genetic testing and increased surveillance [4];

(ii) prognosis, since patients whose tumours have MSI show a favourable prognosis in terms of both Overall Survival (OS) and Disease-Free Survival (DFS) [5]; (iii) the presence of MSI in CRC seems to be predictive of non-response to adjuvant 5-fluorouracil-based chemotherapy of early-stage disease [6]. In addition, emerging data indicate that MMR status has a predictive role for anti-programmed cell death protein-1 (PD-1)/ programmed cell death ligand protein-1 (PD-L1) immune checkpoint inhibitor therapy in patients with advanced tumours [7,8]. Recently, the American Society for Clinical Pathology (ASCP), College of American Pathologists (CAP), the Association for Molecular Pathology (AMP), and the American Society of Clinical Oncology (ASCO) have been strongly recommended to test MMR status in patients with CRCs for the identification of patients at high risk for Lynch syndrome and/or prognostic stratification [9].

The identification of deficient MMR (dMMR) can be performed by immunohistochemistry (IHC) for the four MMR proteins and/or by MSI DNA-based testing [10-12]. IHC is performed to detect MMR protein expression by the absence or loss of a particular protein within the nucleus of tumour cells; whereas diagnosis of MSI via polymerase chain reaction (PCR) consists of amplification of specific microsatellite repeats regions, where the most widely used panel, the Bethesda panel, consists of five microsatellite repeats, including 2 mononucleotide repeats (BAT25 and BAT26) and 3 dinucleotide repeats (D2S123, D5S346, and D17S250). Although IHC is extensively available in general pathology laboratories and does not require both tumour and normal tissue samples for testing, it could miss rare missense mutations affecting protein function and antigenicity, and it may be influenced by tissue fixation conditions [13]. In these cases, PCR-based MSI testing could be more sensitive to identify true functional MMR protein defects through mutational status.

In this study we conducted a retrospective validation of an automated capillary electrophoresis device, the QIAxcel system, to detect MSI in CRC at all five loci known as the Bethesda panel compared to the standard technology, aiming at establishing if the QIAxcel system could be useful for diagnostic use and its feasibility in patient care.

Materials and methods

Tumour samples

A total of 20 cases with a diagnosis of CRC were selected based on routine request and sufficient leftover tissue from primary tumours for MSI molecular analyses. The cases were retrospectively collected from two institutes (Cannizzaro Hospital, Catania, Italy and Ospedali Riuniti Villa Sofia-Cervello, Palermo, Italy) and analysed at the same time with both technologies and standard laboratory workflow.

Molecular testing

DNA extraction and quantification. At Ospedali Riuniti Villa Sofia-Cervello, Palermo, Italy, neoplastic genomic DNA (gDNA) was obtained from FFPE tissue whereas normal gDNA was obtained from blood. MagCore (Diatech) instrument and Qubit V2.2 Fluorometer (Thermofisher Scientific, USA) were used for the extraction and quantification of gDNA according to the manufacturer's protocols. At Cannizzaro Hospital normal and neoplastic gDNA was obtained from FFPE tissues, after evaluation and manual macrodissection of neoplastic as well as non-tumour counterpart in the same sample [14]. The gDNA was extracted using QIAamp MinElute spin columns (Qiagen, GmbH, Hilden, Germany) and quantified by Qubit V2.2 Fluorometer (Thermofisher Scientific, USA), according to the manufacturer's protocols.

Standard Procedure. MSI was assessed by using five highly polymorphic microsatellites chosen according to Bethesda guidelines: BAT25, BAT26, D5S346, D2S123, D17S250. Commercial fluorophore-labelled primers (Applied Biosystem, CA, USA) were used to amplify the set of microsatellites, in five different PCR. Primer sequences are listed in Table I. Microsatellite loci were genotyped by capillary electrophoresis on an ABI PRIMS 310 platform by GeneScan software (Applied Biosystems). Loss of Heterozygosity (LOH) was scored as positive when the degree of reduction in allelic signal intensity was greater than 70% in one of the alleles of the tumour population compared with control DNA, as previously described [15]. The presence of novel alleles in tumour cells combined with their ab-

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

Marker	Primer sequences	Products size (bp)
BAT25	Fw 5'- CTCGCCTCCAAGAATGTAAGT-3' Rv 5'- TCTGGATTTTAACTATGGCTC -3'	113
BAT26	Fw 5'-TGACTACTTTTGACTTCAGCC-3' Rv 5'-AACCATTCAACATTTTTAACCC-3'	121
D2S123	Fw -5' AAACAGGATGCCTGCCTTTA -3' Rv- 5' GGACTTTCCACCTATGGGAC-3'	197-227
D5S346	Fw 5'-ACTCACTCTAGTGATAAATCGGG Rv 5' AGCAGATAAGACAGTATTACTAGTT-3'	96-122
D17S250	Fw 5'-GGAAGAATCAAATAGACAAT 3' Rv 5'-GCTGGCCATATATATATTTAAACC-3'	151-169

Abbreviations: Fw, forward; Rv, reverse; bp, basepair.

sence in normal cells was referred to as MSI, whereas at least a double intensity signal of one of the alleles in tumour cells was defined as genomic amplification. *Qiaxcel Procedure*. The set of microsatellite loci BAT-25, BAT-26, D2S123, D5S346 and D17S250 was amplified with five different PCR using custom primers as previously reported [16] and listed in Table I, for both normal and tumour DNA separately. Briefly, 50 ng of DNA was used in a final reaction volume of 20/µl containing 10 pmol/µl of each primer, 2x Type-it Multiplex PCR Master Mix (Type-it Microsatellite PCR Kit, Qiagen) and underwent to a thermal cycling profile consisted of 1 cycle of 5 minutes at 95°C followed by 35 cycles of 30 seconds at 95°C, 90 seconds at 57°C, 30 seconds at 72°C, before a final extension for 15 minutes at 72°C, using GeneAmp PCR System 9700 (ThermoFisher Scientific, USA). The PCR products were analysed on the QIAxcel Advanced system using the QIAxcel DNA High-Resolution Kit, QX Alignment Marker 15 bp/600bp and the DNA size marker 25 bp-500bp at a concentration of 10ng/µl, without needing purification. The separation was performed using the OM800 method and the following parameters: 4 kV and 5 seconds for alignment marker injection, 5 kV and 10 seconds for the sample injection and 3 kV for 800 seconds for separation.

Results

Microsatellite testing was performed in a set of 20 retrospectively collected CRC samples, previously tested with known MSI status. In total 10 PCRs were performed per patient, as for every microsatellite tumor DNA was compared to DNA from healthy control tissue, respectively. In case of MSS, the electropherogram shows the same pattern in healthy tissue as in the tumor, even if it may vary in the overall intensity (Fig. 1). In MSI cases the differences are associated with a different peak pattern between tumour/healthy tissue, mostly characterised by additional peaks or a peak-shift to the right side of the diagram (which in turn indicates longer MS-sequences). According to the Bethesda agreement a sample is considered as High-Instable (MSI-H) if two or more markers of the Bethesda panel are alterated, Low-Instable if only one of the markers is alterated (SSI-L) and Stable (MSS) if all five loci are not different from the normal counterpart.

Among all CRC cases tested in this validation, all were successfully amplified and resolved using both procedures. The analysis was able to identify 9 (45%) cases with instable-high MSI (MSI-H), whereas the remain 11 (55%) cases showed a microsatellite stable status (MSS) profile at electrophoresis (Tab. II).

The comparison between the two procedures showed a very high concordance (100%) for the final result, with all positive cases correctly identified and genotyped; whereas we identified an overall concordance of 96% considering single loci tested. The four discordant loci were both mono and dinucleotide markers, mainly defined instable by the Qiaxcel system and scored as LOH by the standard method (Tab. II).

Table II. MSI status defined by the Bethesda panel of markers of CRC cases analyzed using Qiaxcel and Standard procedures.

Loci analyzed with Qiaxcel/Standard Procedure						
Case	**BAT25**	**BAT26**	**D2S123**	**D5S346**	**D17S250**	**MSI status**
#1*	ins/ins	ins/ins	ins/ins	ins/ins	ins/ins	MSI-H/ MSI-H
#2*	ins/ins	ins/ins	ins/ins	ins/ins	ins/ins	MSI-H/ MSI-H
#3*	ins/ins	ins/ins	ins/ins	ins/ins	ins/ins	MSI-H/ MSI-H
#4*	st/ins	ins/ins	ins/ins	ins/ins	ins/ins	MSI-H/ MSI-H
#5*	st/st	st/st	st/st	st/st	st/st	MSS/MSS
#6*	st/st	st/st	st/st	st/st	st/st	MSS/MSS
#7*	st/st	st/st	st/st	st/st	st/st	MSS/MSS
#8*	st/st	st/st	st/st	st/st	st/st	MSS/MSS
#9*	ins/st	ins/ins	ins/ins	ins/ins	ins/ins	MSI-H/ MSI-H
#10*	ins/ins	ins/ins	ins/ins	ins/ins	ins/ins	MSI-H/ MSI-H
#11*	st/st	st/st	st/st	st/st	st/st	MSS/MSS
#12*	st/st	st/st	st/st	st/st	st/st	MSS/MSS
#13*	st/st	st/st	st/st	st/st	ins/st	MSS/MSS
#14*	st/st	st/st	st/st	st/st	ins/st	MSS MSS
#15**	ins/ins	ins/ins	ins/ins	ins/ins	ins/ins	MSI-H/ MSI-H
#16**	st/st	st/st	st/st	st/st	st/st	MSS/MSS
#17**	st/st	st/st	st/st	st/st	st/st	MSS/MSS
#18**	ins/ins	ins/ins	ins/ins	ins/ins	ins/ins	MSI-H/ MSI-H
#19**	ins/ins	ins/ins	ins/ins	ins/ins	ins/ins	MSI-H/ MSI-H
#20**	st/st	st/st	st/st	st/st	st/st	MSS/MSS

Abbreviation: MSI-H, microsatellite status instable-high; MSS, microsatellite status stable; MSI, microsatellite status instability.
Note: ins, indicates instability at this marker; st, indicates stability at this marker. *Ospedali Riuniti Villa Sofia-Cervello samples; **Cannizzaro samples.

Discussion

These results are in accordance with the literature, where different studies have shown comparable performance between standard procedures and alternative methods for detection of MSI, with a range of con-

cordance around 95-98% [17-19]. Previous works have demonstrated the use of different technologies and their applicability for MSI detection in the past [17], even if fluorochrome-based PCR assays linked to capillary electrophoresis using a sequencing platform were most commonly used. Similarly, our results underlined the possibility to use alternative procedures to detect MSI status in the clinical practice and management of CRC. We decided to validate capillary electrophoresis based on a different platform, the Qiaxcel system, obtaining reproducible data and an easier procedure than the standard one. This was further supported by using, in this validation study, samples the underwent different extraction methods, but equally processed for the MSI status. The good concordance between the two technical approaches so is irrespective of DNA extraction technology, such as the type of specimens used (both FFPE and blood samples).

Using Qiaxcel capillary electrophoresis, all five microsatellites loci of the Bethesda panel tested were

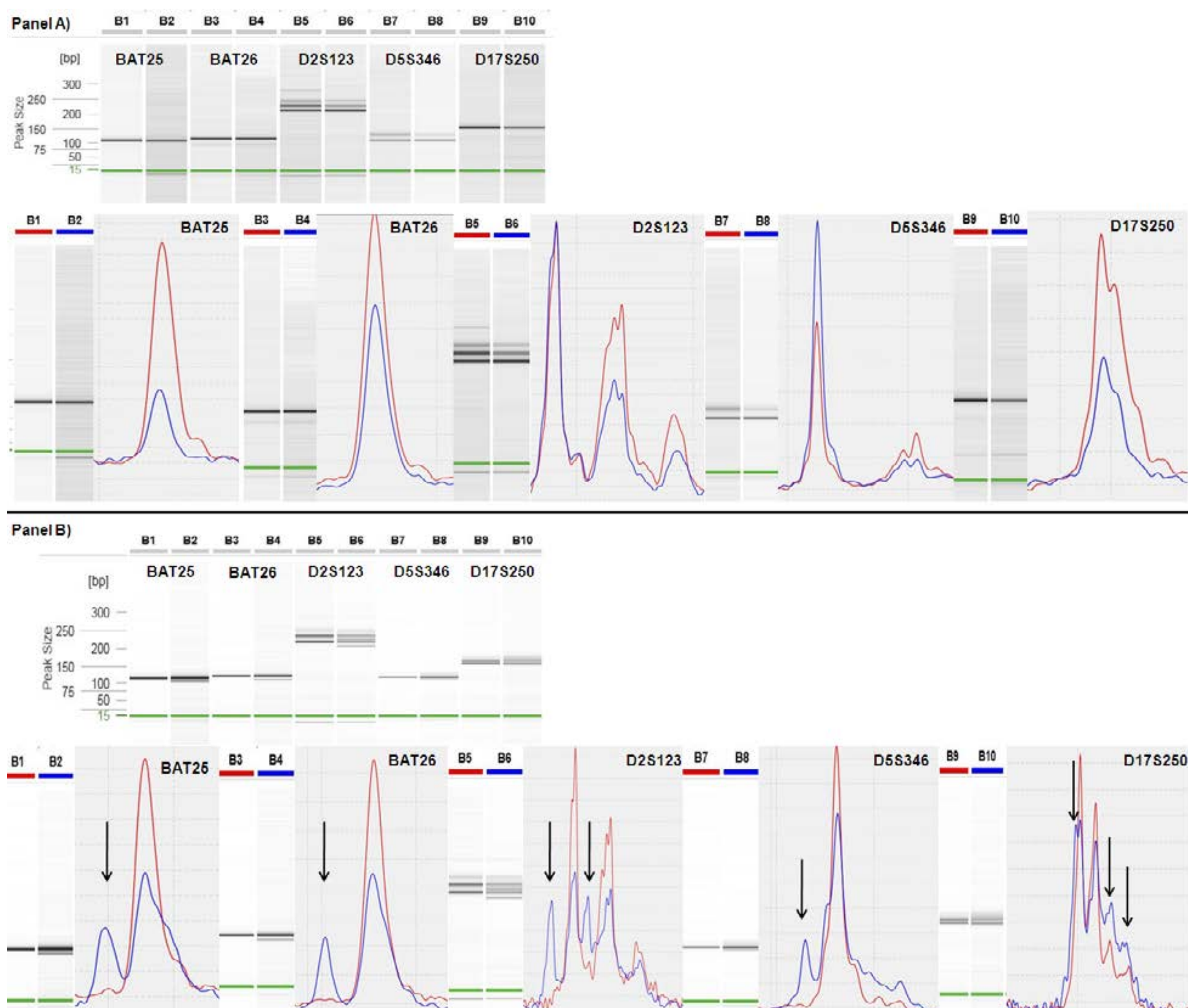

Figure 1. Representative illustration of the electropherograms of the five Bethesda loci (BAT-25, BAT-26, D2S123, D5S346 and D17S250) obtaining using Qiaxel system. In the figure electropherograms derived from a stable case (MSS, Panel A) and from an instable case (MSI-H, Panel B) are shown respectively. The patterns were obtained overlaying the spectrum derived from tumour (blue) and corresponding normal DNA (red) for each locus analyzed, with visible discrepancies (arrows), simplifying the identification of microsatellite instability. The corresponding gel electrophoresis migration for all the cases and loci are reported.

informative for these cases with well-resolved electropherograms starting from both tumour and matched normal counterpart, with label-free PCR (Fig. 1). The software allowed to overlay the patterns derived from tumour and corresponding normal DNA, highlighting the discrepancies between both spectrums and simplifying identification of MSI status. This is very useful in a diagnostic procedure to help in the interpretation of the spectrum obtained after electrophoresis and to define the correct genotype of samples tested, especially in cases with doubts and noising electropherograms [19]. In addition to previous studies, we obtained the corresponding gel electrophoresis migration for all the cases and loci, which was further helpful for the MSI analysis. The analysis with Qiaxcel system performed took a few time for sample preparation (2 hours) just as for gel migration (30-40 minutes) compared with standard procedure (4-6 hours), obtaining electropherograms with an adequate resolution for all loci tested with a reduction in costs and turn-around time (TAT) for the final report.
Although the reference panel is made of 5 loci (as evaluated in the present paper), several criticisms have been raised for its application in the detection of MSI. The difficulties to the interpretation of dinucleotide microsatellite loci has suggested to enlarge the original Bethesda panel (“pentaplex”) adding other 3 more mononucleotide loci [20,21]. However the use of mono- rather than dinucleotide repeats had been advised for false-positives associated to potential low-frequency polymorphisms of BAT26 or BAT25, as well as potential negative case considering the lost of BAT26 in cases with biallelic deletion of hMSH2 [21]. The clinical utility of introducing these new loci is a moot point. A recent study showed that no differences were found in terms of specificity to detect the MSI-H group of patient using the two MSI panels (the original and the enlarged) [22].
For this reason the original Bethesda panel remains the gold-standard for MSI-PCR detection, even if the “pentaplex” panel is also recommended and easily applicable, considering that all added loci are mononucleotide. This is also confirmed by the use of alternative electrophoresis methods for MSI detection, applied by other groups [18,23].

Conclusion

Our data demonstrated that the Qiaxcel system is not only fast and reliable but is also a very sensitive procedure for MSI analyses in clinical studies. The advantages of this procedure are free-labelling of PCR amplicon, not requiring specialised procedures or equipment to detect the label, such as laser-associated sequencing platforms. In conclusion, the approach used in this study for MSI analyses is a highly time-efficient and easy procedure combined with high sensitivity for MSI detection.

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