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Sensitive detection of microsatellite instability in tissues and liquid biopsies: Recent developments and updates

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A B S T R A C T

Microsatellite instability (MSI), a phenotype displayed as deletions/insertions of repetitive genomic sequences, has drawn great attention due to its application in cancer including diagnosis, prognosis and immunotherapy response prediction. Several methods have been developed for the detection of MSI, facilitating the MSI classification of cancer patients. In view of recent interest in minimally-invasive detection of MSI via liquid biopsy samples, which requires methods with high sensitivity to identify small fractions of altered DNA in the presence of large amount of wild type copies, sensitive MSI detection approaches are emerging. Here we review the available MSI detection methods and their detection limits and focus on recently developed next-generation-sequencing based approaches and bioinformatics algorithms available for MSI analysis in various cancer types.

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Contents

1. Introduction

Microsatellites, also known as short tandem repeats or short interspersed elements, are repetitive DNA sequences with repeating units of 1–6 bases spread throughout human genome, that are prone to DNA replication errors [1]. Due to polymerase slip-

page, deletions or insertions, ('indels') are introduced at these sites during the process of DNA replication, most of which are normally corrected by the DNA mismatch repair system (MMR). When MMR is impaired, indels accumulate leading to widespread length-polymorphisms of microsatellites referred to as microsatellite instability (MSI). As inactivation of MMR genes occurs frequently in tumors through somatic mutation, epigenetic silencing or microRNA regulation [2–4], MSI comprises an important prognostic or predictive cancer biomarker.

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MSI has been reported across multiple cancer types, and is common in gastric, urinary tract, endometrial and colorectal cancer. Approximately 15–20% sporadic colorectal carcinoma (CRC) displays high level of MSI and in Lynch syndrome, an inherited disorder that cause high risk of CRC, MSI is found in 90% of cases [5,6]. Depending on the frequency of unstable microsatellites, tumors are divided into three types: high MSI (MSI-H, >30% MSI), low MSI (MSI-L, 0–30% MSI) and microsatellite stable (MSS, 0% MSI) [7]. MSI-L and MSS tumors show similar pathologic and molecular features, and therefore are classified as a single type in clinics in comparison to MSI-H tumors [8].

Recently, interest in MSI detection in cancer is rising rapidly as increasing evidence indicate the association of MSI with clinical outcome and the benefit from immunotherapy. MSI-H CRC have better prognosis, higher survival rate and less risk of metastasis and recurrence relative to MSS tumors [9–11]. Beyond that, high level of MSI is predictive for efficacy of immune checkpoint blockade therapy across all solid tumors [12–14]. Two monoclonal antibodies that block the immune checkpoint component programmed cell death-1 protein (PD-1), pembrolizumab and nivolumab, and recently the combination of nivolumab with CTLA4 inhibitor ipilimumab have been granted approval by the Food and Drug Administration for the treatment of metastatic CRC with MMR deficiency or MSI-H [13,15,16].

Given its clinical potential, reliable analysis of MSI status is in demand. Several approaches have been developed for MSI detection (Fig. 1); however, efforts are still being devoted to improving sensitivity and specificity. The possibility of assessing MSI via liquid biopsies using cell-free plasma-circulating DNA (cfDNA) [17,18] for serial detection and tumor-monitoring applications contributes to the need for higher sensitivity. The emerging next-generation sequencing and bioinformatic algorithms for MSI classification have shown great potential in improving detection limit and reforming MSI detection strategy. This review describes methods and progress in MSI detection in cancer with substantial focus on approaches employing high-throughput assessment of microsatellites via next generation sequencing.

2. Immunohistochemistry (IHC)

Pathogenic mutations or aberrant methylation in MMR proteins usually diminish their expression, therefore MSI status is commonly assessed in clinics by IHC of four MMR proteins (hMLH1, hPMS2, hMSH2 and hMSH6) in tumor tissue specimens (Fig. 1). Tumors that have at least one MMR protein missing or inactivated are defined as MMR deficient (dMMR) and are often MSI-H. The IHC method is convenient with reported sensitivity and specificity > 90% [19]. However, its accuracy is subject to several factors including focal staining, quality of staining antibody and interpretation [20,21]. More importantly, dMMR and MSI-H are not always equivalent. Around 5–10% MSI-H tumors display intact MMR staining and localization [7]. This might result from the loss of other unidentified MMR proteins or missense mutations in MMR genes,
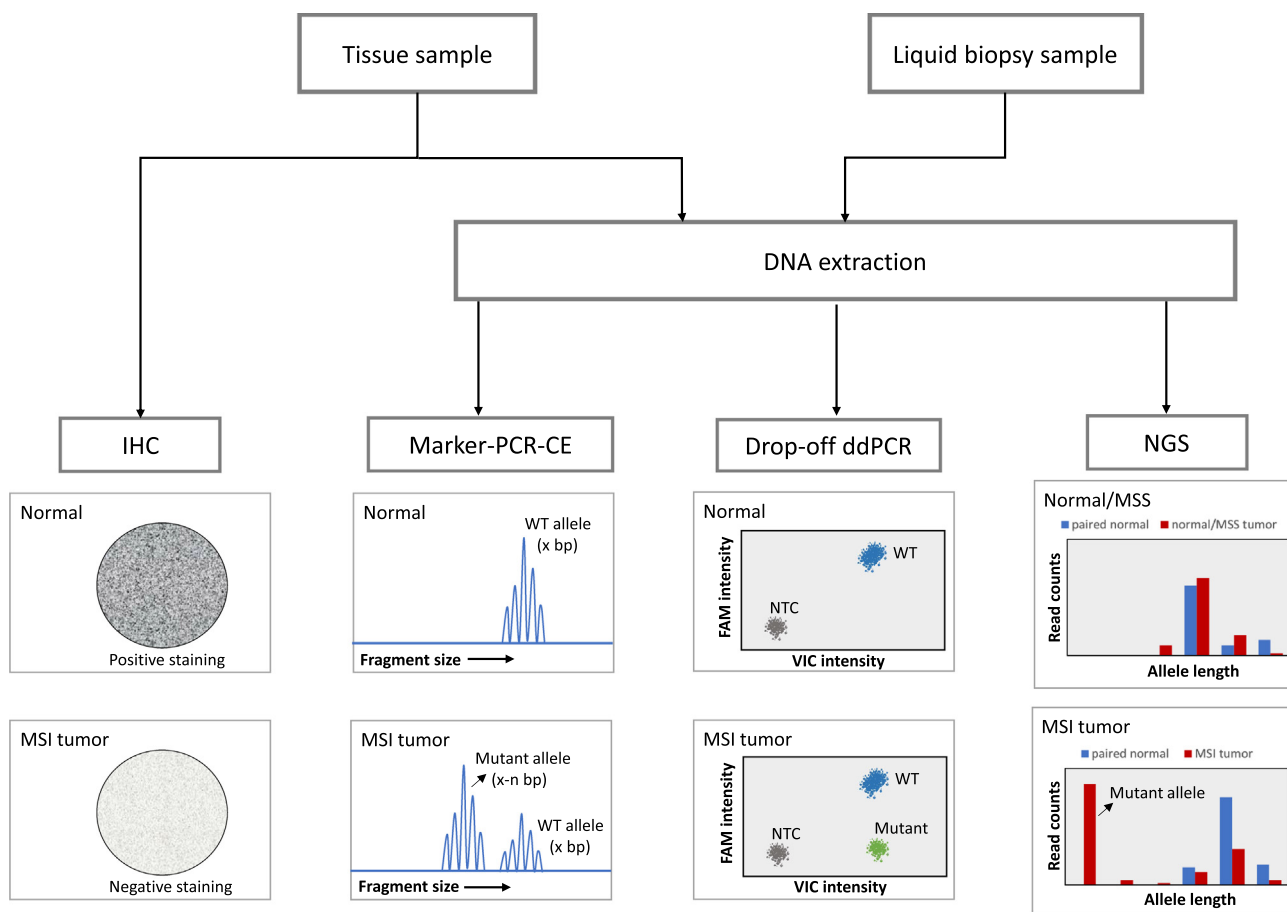

Fig. 1. Overview of MSI detection methods. IHC: Immunohistochemistry of MMR proteins is performed, and absence of MMR proteins is used for MSI calling. Marker-PCR-CE: peak size of target PCR amplicon from tumor sample is compared to that from normal sample or reference, and target with peak size shift is regarded as unstable site. ddPCR: wild type copies retain dual signal from reference probes and drop-off probes, while mutant copies retain only signal from reference probes. NGS: multiple microsatellite loci are sequenced, and the length distribution of target loci is compared between tumor sample and matched normal sample (or reference genome) to identify unstable loci.

hMLH1 for example, which allow normal translation of non-function protein [1,22].

3. Multiplex PCR of markers and capillary electrophoresis (CE)

A second established approach for MSI testing is based on PCR of specific microsatellite makers followed by fragment length analysis. A number of microsatellites were studied with the aim to identify the most reliable and sensitive loci for MSI detection and in 1997 agreement was achieved by National Cancer Institute upon a census panel which includes two mononucleotide (BAT25 and BAT26) and three dinucleotide (D5S346, D2S123 and D17S250) MS loci, known as the Bethesda/NCI panel [23]. Tumors are classified as MSI-H, MSI-L and MSS when bearing two or more, one or none unstable markers respectively [23]. Although this panel has served as reference and is still in use for MSI detection, the individual polymorphisms and the requirement of paired normal tissue for the analysis has practical disadvantages. Therefore, the pentaplex panel was later developed. Relying uniquely on mononucleotide microsatellites which are believed to be more sensitive than dinucleotide repeats, this panel includes five monomorphic markers BAT25, BAT26, NR-21, NR-22 and NR24 avoiding the demand for a corresponding normal [24] and is now regarded as a gold standard for MSI detection in colorectal cancer [25]. More recently, additional combinations of microsatellite markers have also been tested and might be beneficial for expanding MSI detection to cancer types other than colorectal cancer [26–28]. As an alternative to using multiplex PCR of selected monomorphic markers coupled with CE, information on MSI indels may also be obtained using inter-Alu-PCR [29], which amplifies thousands of microsatellites from ALU-elements using a single pair of primers. Since this approach requires next-generation sequencing (NGS) and bioinformatics to derive MSI information, it is described in more detail in the NGS section of this review.

Following multiplex PCR, the analysis of amplified products is reported via different techniques, including high-resolution melting (HRM) analysis, denaturing high-performance liquid chromatography (DHPLC) and most widely accepted fragment length analysis [30–32]. Initially, fragment analysis was performed by running polyacrylamide gel electrophoresis and then autoradiography. However, due to the low resolution in fragment size and time-consuming pitfall, it was substituted by a new procedure, in which makers are amplified with fluorescent primers and subsequently examined by capillary electrophoresis for microsatellite allele size [25,33]. This approach is practical and allows the inspection of multiple targets at the same time with a resolution up to single base difference, thereby becoming the gold standard protocol for MSI detection (Fig. 1). While these PCR-based approaches have demonstrated high reliability, there is one main drawback. During PCR, slipped strand mispairing ('stutter') is introduced by polymerases, leading to the presence of amplicons in a distribution pattern with mixed length that contains both original and undesirable frameshifted products. The longer microsatellites tend to have greater stutter. The presence of multiple peaks obstructs the ability to distinguish between wild type and altered microsatellites, especially for samples carrying low level mutations or small 1–2 bp indels. Therefore, this approach is subject to false negatives due to artifacts associated with polymerase slippage, tumor heterogeneity or low mutational burdens in situations such as in circulating DNA where altered DNA coexists with large amount of wild type DNA. The fragment length analysis method has shown to have a limit of detection (LOD) of ~10% tumor content [33].

Several modifications of protocol have been adopted to provide the highest degree of sensitivity and accuracy for MSI detection in cancer. Daunay et al. replaced PCR with low temperature isothermal amplification using recombinase polymerase amplification (LT-RPA) and successfully reduced the formation of stutter artifacts on several microsatellite markers, facilitating allele identification [34]. This rapid and simple method shows better sensitivity than regular PCR notably for small deletions and has an LOD ~5% indel-versus-wild type DNA fraction. Other groups have aimed to improve the LOD via incorporating indel/mutation enrichment techniques [35] prior to PCR (Fig. 2A). How-kit et al. [36] were able to detect MSI down to 0.05% of indel frequency on the monomorphic site HT17 using E-ice-COLD-PCR, a modification of the original COLD-PCR technology [37–41]. E-ice-COLD-PCR employs WT-complement poly T locked nucleic-acid (LNA) blocker probes that allow selective amplification of mutant alleles due to the preferential denaturation of mutant-probe heteroduplex over WT-probe homoduplex at critical denaturation temperature. Recently we developed nuclease-assisted minor allele enrichment with probe-overlap (NaME-PrO) for MSI detection [42]. NaME-PrO relies on the thermostable duplex-specific nuclease (DSN) and mutation-overlapping oligonucleotide-probes that interrogate WT DNA [43,44]. DSN digests perfectly matched dsDNA with high preference compared to single strand DNA or mismatched dsDNA [45]. Upon denaturation and subsequent annealing, WT DNA forms double-strand with probes leading to its removal in the presence of DSN, while in the case of mutation-containing DNA the mismatch compromises DSN digestion, thereby enabling the enrichment of mutant alleles after subsequent PCR. In combination with HRM, this approach yielded a detection limit of 0.01% [42]. NaME-PrO can be performed in multiplex manner [46], hence probes addressing 5 microsatellite markers BAT25, BAT26, NR21, NR24 and NR27 were applied simultaneously for indel enrichment, followed by multiplexed plus nested PCR, then HRM or capillary electrophoresis [42]. The method can be applied for diverse clinical specimens, including tissue, liquid biopsies, or FFPE samples of varying age [47]. In a modification of NaME-PrO, a method nuclease-assisted microsatellite instability enrichment (NaMSIE) was also developed using LNA-modified probes with an LOD of 0.5% mutant frequency using capillary electrophoresis [48].

4. Droplet digital PCR (ddPCR) for MSI detection

As a robust quantitative approach, the application of ddPCR is growing in genetic alteration detection including copy number variation, DNA methylation, gene rearrangement, minimal residual disease tracing [49–51], and lately MSI detection. A drop-off ddPCR assay was proposed by Silveira et al. [52] and was shown to quantify MSI loci with sensitivity around 0.1% mutant frequency [53]. This drop-off ddPCR is based on two fluorescent hydrolysis probes within the same fragment. The reference probe binds to the sequences present in the flanking region, while the drop-off probe binds to target microsatellite region. When MSI occurs, the mismatch between drop-off probe and the variant alleles compromises the hydrolysis of probe whereas the wild type alleles remain unaffected. As a result, droplet containing WT DNA is separated from those containing mutant DNA, enabling the quantification of mutant copies (Fig. 1 and Fig. 2B). The performance of drop-off ddPCR in MSI detection was assessed in both tumor and liquid biopsy samples via three microsatellite makers (BAT26, ACVR2A, DEFB105A/B) and concordance with gold standard PCR-CE method was observed in 100% of CRC and 93% of other tumor samples [53]. A commercial drop-off ddPCR Microsatellite Instability Assay system targeting pentaplex panel is now commercially available and shows sensitivity and specificity comparable to standard methods [54]. This ddPCR method, which is also applicable to formalin samples of varying degradation [47,54], provides a possibility to perform a one-step quantitative screening of MSI.
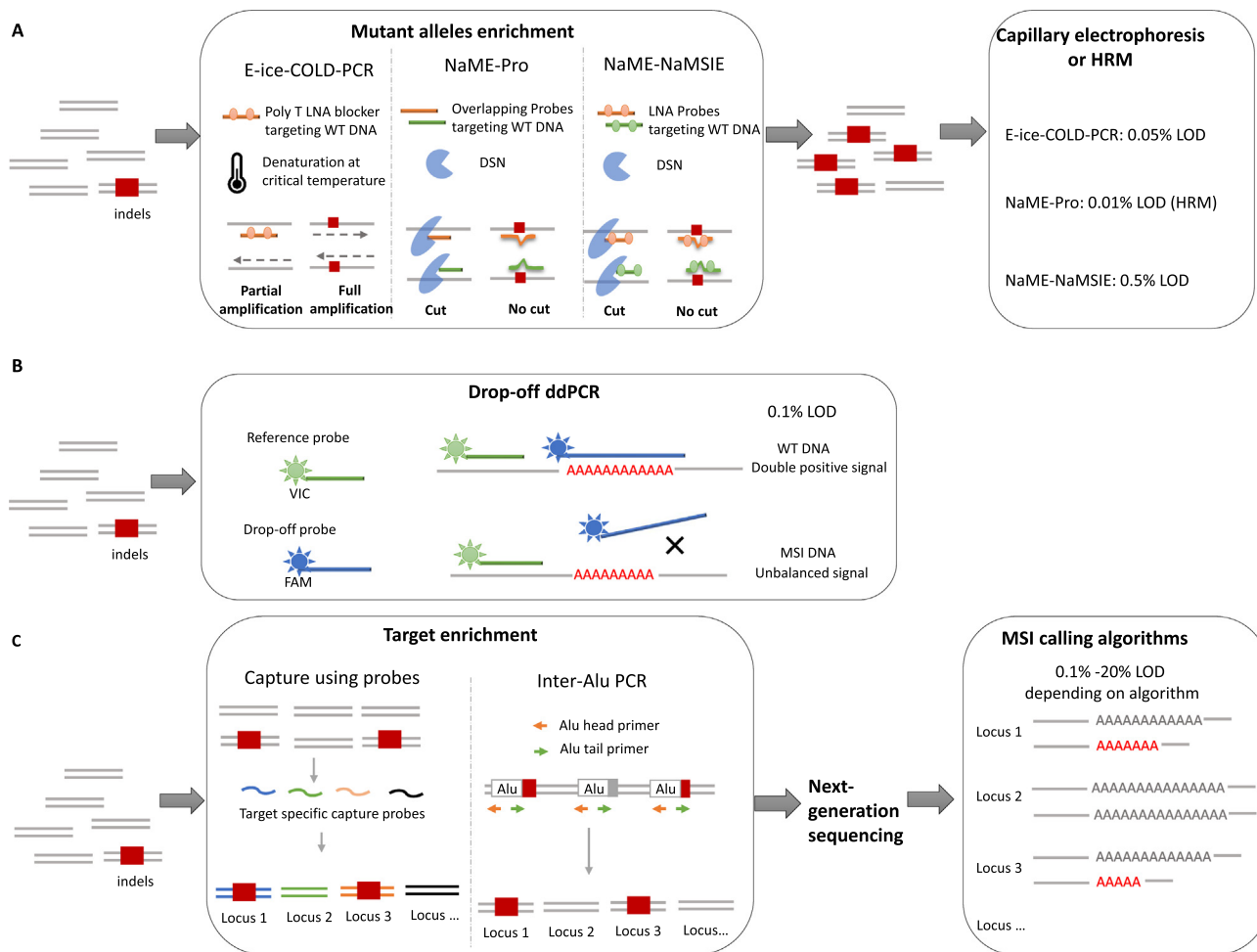
Fig. 2. Development of sensitive MSI detection approaches. (A) Mutant enrichment strategies improve MSI detection limit. (B) ddPCR enables sensitive quantification of unstable microsatellite loci. (C) NGS examines numerous microsatellites enriched by capture approaches or inter-Alu-PCR and determines MSI status based on collective information from a number of loci.

5. NGS approaches

Massively parallel next generation sequencing (NGS) approaches show great potential for MSI testing (Fig. 1 **and** Fig. 2**C**). Although approaches that use pre-selected microsatellite markers like PCR-CE are routinely recommended for MSI screening in colorectal cancer, their application in other cancers is less practical due to the relatively rare prevalence of instability at the same markers. The high throughput feature of NGS allows the examination of microsatellites at thousands of loci simultaneously while also obtaining the somatic mutation profile across targeted regions in a single assay [55–57]. Thus, NGS not only offers more information over conventional methods via scanning vast numbers of microsatellites, but may also extract additional biomarkers such as tumor mutational burden (TMB), which is valuable for diagnosis and treatment selection in common cancers [58–62]. Resource efficiency is another advantage of NGS, as sequencing libraries can be prepared with flexible amount of DNA including low input [29,63,64]. Moreover, NGS can quantify MSI with reduced noise and reach a sensitivity down to ~0.05%, compatible with MSI detection in specimens such as cfDNA [29,63,65], through coupling with computational tools. Several bioinformatic algorithms using different strategies for MSI detection have been reported (Table 1). Considering the surging interest in NGS, additional development of bioinformatic algorithms for MSI is foreseeable.

5.1. Algorithms for MSI status determination from NGS data

Most MSI-calling tools are developed by comparing the read-count length distribution of microsatellite sites, from which two district types are further distinguished. The first type, which includes MSIsensor, MANTIS, Cortes-Ciriano-MSI-event and NovoPM-MSI requires paired normal DNA for comparison. MSIsensor is a C++ program that determines the MSI status by comparison to microsatellite loci in a reference genome [66]. After an initial screening of a reference genome, a microsatellite list which records homopolymers of at least 5 bp and repeat units with maximum length of 5 bp is generated. Microsatellite regions with enough coverage (default: 20) from tumor/normal are then interrogated using Pearson's Chi-Squared Test to identify unstable loci that have significantly different distribution from the paired normal genome, then the program derives an 'MSI-score' to distinguish between MSI- and MSS samples [67,68]. MANTIS is a python program. Like MSIsensor it also relies on a reference genome to define microsatellite sites. After filtering reads for sequence length (longer reads > 100 bp preferred), minimum average base quality score and coverage (default: 30), the normalized read distribution from normal and tumor samples on individual mono- to penta-nucleotide repeat locus is compared to calculate a per-locus distance score [69]. To reflect the aggregate instability the average score of all loci is finally generated in a range from 0 to 2 represent-

Table 1
Summary of algorithms developed for NGS-based MSI detection.

Type of algorithm	Algorithms	Reference	Use of paired normal data	Statistics/models	Sequencing depth	Threshed for MSI calling	Sample type tested	Reported LOD
Comparing length distribution of microsatellite sites	MSI-sensor	2014 [66]	Yes	Chi-squared test	20	3.5%	Tissue	10%–20%
	MANTIS	2017 [69]	Yes	Average distance	30	0.4	Tissue	10%–20%
	Cortes-Ciriano-MSI-event	2017 [55]	Yes	Kolmogorov-Smirnov statistic	5	NA	Tissue	NA
	NovoPM-MSI	2020 [70]	Yes	Mann-Whitney U Test	30	0.2	Tissue	NA
	mSINGS	2014 [72]	No	Average allele number	30	0.2		10%–20%
	MSI-ColonCore	2018 [74]	No	coverage ratio	NA	MSI-H > 40% MSI-L 15%–40%	Tissue	NA
	MIAmS	2019 [73]	No	machine learning	NA	NA	Tissue	NA
	MSI NGS caller	2019 [75]	No	Euclidean distance	500	NA	Tissue	7%–9%
	Willis method	2019 [63]	No	Akaike Information Criterion statistic	NA	NA	cfDNA	0.1%–0.4%
	Georgiadis method	2019 [17]	No	Peak distance	NA	20 sites	Tissue, cell line cfDNA	1.2%–4.6%
	smMIP-MSIclassifier	2020 [76]	No	naïve Bayes approach	NA	0	Tissue, cell line	3%
	MSIsensor-pro	2020 [71]	No	MND model	5	NA	Tissue	10%–20%
	MSI-tracer	2020 [29]	No	Minimal distance	20	NA	Tissue, cfDNA	0.15–1.5%
	MSIsensor-ct	2021 [65]	No	Machine learning frameworks	3000	20%	Tissue, cfDNA	0.05%–0.2%
Counting Indel burden or somatic burden	MSIseq Index	2013 [77]	No	DINDEL	NA	PI/PD > 0.9	Tissue	NA
	MSIseq NGSclassifier	2015 [78]	Yes	R package RWeka	30	S.ind > 0.395/Mb	Tissue	NA
	Nowak method	2017 [79]	No	Indelocator	NA	microsatellite indel burden > 5/Mb	Tissue	NA
	MSIpred	2018 [80]	Yes	Support vector machine	NA	NA	Tissue	NA

ing entirely stable and entirely unstable respectively, and a score threshold 0.4 is set for MSI determination [62,69]. The Cortes-Ciriano method is an in-house pipeline that quantifies the absolute number of unstable sites as 'MSI event'. It compares the length distribution from tumor and matched normal per mono- to tetra-nucleotide repeat locus with at least 5 coverage by Kolmogorov-Smirnov statistic and use an FDR value < 0.05 to decide statistical significance [55]. No cutoff threshold is suggested for this software, but it can work as a binary classifier to distinguish MSI-H tumors from MSS/MSI-L. Different from these three methods, NovoPM-MSI only focuses on mononucleotide loci [70]. This approach evaluates the count distribution from tumor and paired normal on each locus with minimal coverage 30 via a Mann-Whitney U Test and uses the pass P-value coverage to define MSI with an empirical cut-off threshold at 0.2 [70].

The second type of algorithms, which include MSIsensor-pro, MSIsensor-ct, mSINGS, MIAmS, MSI-ColonCore, MSI NGS caller, Willis method, Georgiadis method, smMIP-MSIclassifier and MSI-tracer are matched-normal-free using reference information or baseline normal. MSIsensor-Pro is an updated version of MSIsensor [71]. It uses an MND (multinomial distribution) model to describe polymerase slippage and evaluates the instability via probability of deletion (p). An unstable site is scored when its p value extends certain range of the baseline p value built from 1532 TCGA normal samples from three cancer types, and the fraction of unstable sites within all detected sites is used for MSI determination. Albeit no cutoff for MSIscore is proposed, MSIsensor-pro has showed reliable performance in differentiating MSI-H and MSS samples even with low sequencing coverage ($5\times$) or low tumor purities (10%), especially when the analysis is confined to discriminative sites [71]. MSIsensor-ct is also developed based on MSIsensor. It is restricted to selective microsatellite sites that have an unstable distribution

portion > 20% and are present in > 10% samples in a 1565 tumor training cohort and employs a machine learning model to further exclude inefficient classifiers, leading to a final pool of 1476 classifiable sites. MSIscore is recorded as the ratio of unstable sites among these 1476 sites with a cut-off value 20% for MSI classification [65]. mSINGS inspects only mononucleotide repeats [72]. It pivots on a population of MSI-negative samples to calculate the mean and standard deviation (SD) of number of alleles for each locus as baseline and performs MSI-calling by comparing tumor samples to the baseline on sites with coverage above 30. Therefore, the custom baseline file is critical for the analysis. Tumor classification is achieved through the percentage of unstable site with a cut-off threshold 0.2 for MSI-H samples [72]. On top of mSINGS, MIAmS was further developed by employing machine learning to establish the length distribution model corresponding to MSI and MSS per locus from training set [73]. It then annotates sample MSI status using two independent classifiers, mSINGS and SVM (support vector machine) and outputs a final report for overall evaluation. MSI-ColonCore is designed in a similar principle as mSINGS. It uses a set of normal samples to obtain an average coverage ratio, which depicts the fraction of reads covering the reference lengths, and the SD level on each mononucleotide repeat [74]. By referring to these two numbers, the length-unstable locus is scored if the coverage ratio from interrogated sample is above the limit. However, instead of using random data to set up baseline, the baseline value and instability calling limit of MSI-ColonCore is established on 20 normal and paired tumor samples. According to the frequency of unstable loci, tumors are identified as MSI-H, MSI-L or MSS if the value is > 40%, 15–40% or < 15%, respectively. MSI NGS caller is a binary classifier that use a 'k-means' clustering model build on the collective information of mononucleotide microsatellites from a 94-sample training cohort for MSI identifica-

tion [75]. The clustering is based on two factors, the number of peaks and average indel length at each sample, which can separate MSS from MSI-H. For a tested sample, the classification is assigned based on the Euclidean distance of interrogated sample to MSS or MSI-H cluster centroid. In a method developed by Willis *et al*, the Akaike Information Criterion statistic is performed on independent target microsatellites to generate a score which indicates the probability of the length distribution variation at a given locus due to instability versus noise [63]. The locus score above trained baseline is record as unstable and sample that has enough unstable loci above trained threshold is considered as MSI-H, whereas the trained baseline and threshold are computed from a group of healthy donors. Another method proposed by Georgiadis *et al* checks mononucleotide loci above 8 bp and use a peak-finding algorithm c compare with the reference length from hg19 [17]. When deletion of more than 3 bp is observed, a locus is unstable. The number of unstable loci is adopted to call MSI with a cutoff at 20. In smMIP-MSIclassifier, target microsatellites are assessed via two traits, deletion frequency that describes the ratio of reads with deletion in compared to genome reference length and allelic bias of deletion that indicates the preferentially presence of deletion in SNP alleles [76]. By comparing to a training dataset containing 51 MSI-H and 47 MSS sample, the probability of a sample being MSI-H or MSS is predict from these two traits using a naïve Bayes approach. Sample with probability score above 0 is classified as MSI-H, while sample with score below 0 is MSS [76]. Finally, a recent method MSI-tracer investigates mononucleotide repeat loci longer than 10 bp that have sufficient coverage (default: 20) [29]. MSI-tracer aims to identify large deletions in the interrogated sample versus a reference sample by employing empirically-set thresholds. It employs two input datasets, one for the interrogated and another for a reference sample, respectively. The MSIsensor software is initially used to obtain microsatellite distribution files and to creates length histogram for each microsatellite. Sites are defined as unstable if the interrogated sample has distinct deletion peaks corresponding to microsatellites N bases (default: N = 2 bp) smaller than the shortest peak in the normal sample and there are at least M (default: M = 5) support read counts for these deletions. The fraction of unstable loci is then used to identify MSI status.

Another strategy to assess MSI depends on somatic mutation burden or microsatellite indel burden in sequences, including MSI-seq Index, MSIseq NGSclassifier, Nowak method and MSIpred. MSI-seq Index is a binary classifier for analysis that investigates mono- to hexa-nucleotide microsatellites extracted from human Refseq script [77]. It quantifies deletion across all aligned sites microsatellites including microsatellites using DINDEL, a software calling small indels in reference to genome from short-read sequence data, therefore a matched normal is not required. The fraction of insertions (PI) or deletions (PD) specific to microsatellites over all insertions and deletions are calculated separately, while the ratio of PI/PD with the cut-off value 0.9 is applied to discriminate MSI and MSS. MSIseq NGSclassifier scans the indels of mononucleotide bigger than 5 bp and other repeats with maximal length of 4 bp and uses decision tree framework based on R package to call MSI according to the number of indels per Mb with cutoff at 0.395 [78]. In a method proposed by Nowak *et al.*, sequence data is subject to MuTect version 1 0.27200 and Indelocator software to identify somatic mutations as well as insertions and deletions. MSI is defined when sample has a total mutation burden > 40 per Mb and microsatellite indel burden > 5 per Mb [79]. MSIpred is a python package that analyzes the mutation annotation derived from paired tumor-normal exome sequencing data. It extracts the mutation load information in 22 features and detect MSI automatically in different tumor types by a machine learning approach using support vector machine framework [80].

MSI classification using the various available algorithms has been accomplished in different types of cancer using WGS, WES, RNAseq, or targeted sequencing data [81]. The performance of some algorithms has been inter-compared in some studies. For example, the accuracy of MSIsensor, MANTIS and mSINGS using their default setting were evaluated in COAD/READ, UCEC and STAD cohorts downloaded from Cancer Genomics Hub. MSIsensor and MANTIS displayed equally good sensitivity, which was clearly better than mSINGS in all cohorts [69]. In another study from Jia et al. MSIsensor and MANTIS outperformed mSINGS but has similar accuracy as MSIsensor-pro using 1532 TCGA normal-tumor paired whole-exome sequencing data from 3 cancer types [71]. So far, no guidance is available regarding the choice of algorithm for MSI detection. The decision depends largely on type of sequencing data, availability of paired normal sample, type of cancer as well as type of DNA specimen. MSI detection has been achieved on NGS data obtained from fresh-frozen tissue [72,82,83], FFPE tissue [72,82–84] and liquid biopsy samples [17,29,63,65,85]. Most algorithms were designed based on NGS data obtained from tissue samples which usually contain high tumor purity. MSIsensor, MANTIS, mSINGS, MSIsensor-pro and MSI NGS calling algorithms show reduced power for calling MSI at tumor purities < 10% [71], thereby restricting their application in liquid biopsy where mutant DNA can be masked by presence of excessive wild-type alleles. In contrast, some of the algorithms aim to detect samples bearing low mutation levels and can push the limit of MSI detection down to 0.05% tumor purity (Table 1).

5.2. MSI detection in circulating-DNA obtained from liquid biopsies

The application of liquid biopsies for early cancer detection, monitoring cancer dynamics during treatment, identification of treatment resistance and minimal residual disease detection is growing rapidly. Mutated circulating DNA fragments provide early indications of relapse in melanoma patients during immunotherapy [86] and lung cancer treatments [87,88]. cfDNA can be useful as a biomarker in metastatic breast cancer [89,90] and colon cancer [91] and can monitor patients for remission/relapse [92]. The dynamics of tumor-circulating DNA (ctDNA) in plasma following initiation of therapy [93] can be prognostic [94], as an initial ctDNA rise followed by a ctDNA decrease is an indication for tumor response to treatment [95]. The ctDNA changes have been studied following uniform external beam radiation therapy [96], while the ctDNA-release time-course resulting from tumor brachytherapy and other forms of radiation exposure that deliver highly non-uniform radiation- induced, lethal DNA damage [97,98] in their immediate vicinity [99–101] remain to be explored.

Given the proliferation of liquid biopsy applications, interest in detecting MSI detection using liquid biopsy samples is also growing. For example, MSI detection from blood can be a practical and non-invasive approach to monitor the dynamics of tumors already known to be MSI-H. This is still technically challenging due to the large excess of circulating-DNA originating from normal tissues such as hematopoietic cells. To overcome this limitation, several MSI-detection algorithms have been tailored for low-level indel detection and used in combination with exome sequencing or targeted re-sequencing panels, which enable the assessment of MSI in cfDNA samples.

In a study from Willis *et al.*, the authors applied their in-house algorithm on sequencing data from 90 selected loci captured by Guardant360 panel [63]. By scanning these sites, they could assess MSI status of cfDNA with LOD down to 0.4% using 5 ng cfDNA input and 0.1% using 30 ng input [63]. In another study from Georgiadis *et al.*, a 98-kb pan-cancer panel was employed to capture target microsatellites across 58 genes, the analysis of which by their custom-made algorithms allows the detection of MSI alleles at

MAFs of 1.2% to 4.6% with 25 ng cfDNA input [17]. Lately, Han *et al.* [65] presented MSIsensor-ct for cfDNA applications. They perform the MSI classification on 1476 informative microsatellite loci that are filtered out by machine learning and show that MSIsensor-ct can detect ctDNA in the range of 0.1%–0.4% using simulated cfDNA sequencing data. LOD down to 0.05% or 0.2% was achieved with $3000\times$ or $1000\times$ sequencing depth respectively. They also demonstrated the accuracy of MSIsensor-ct in 39 real cfDNA samples carrying various circulating tumor DNA content using sequencing data captured by a custom-designed 599-Gene Panel [65]. Detection of cfDNA in these studies involves hybridization-based capture to ensure enrichment and high sequencing depth for the targeted loci (Fig. 2**C**). The procedure requires substantial investment, as hybrid capture is relatively time consuming as compared to PCR-based enrichment.

5.3. Inter-Alu-PCR

A multiplex-PCR-based approach for MSI detection that contains a single step inter-Alu-PCR for microsatellite target enrichment, coupled with bioinformatic analysis was recently developed by our group [29]. Alu is a ~300 bp DNA stretch, dispersed throughout the genome with a copy number of more than 1 million, amounting to ~11% of the human genome. It contains a consensus body sequence and a poly-adenine region at the 3′ end (A-tails). The A-tails are variable in length at each locus and are prone to accumulation of mutation and shrinkage, thereby forming variant microsatellite-like structures at the end of Alu elements [102]. Inter-Alu-PCR uses a pair of primers that extend outwards from head and tail of Alu elements to capture A-tail microsatellites between two adjacent Alu elements (Fig. 2**C**). The products from inter-Alu-PCR are followed by a library index PCR

and subjected to sequencing. This simple and rapid approach enables a 2–3 h sequencing-ready sample preparation protocol. The technique combines a large DNA target footprint, like the one obtained by hybrid capture panels, with the convenience of PCR-based enrichment and provides an LOD of 0.15%–0.5% for detecting MSI in liquid biopsies [29]. Given the high prevalence of Alu repeats which comprises over 1 million copy number across the human genome, multiple adjacent microsatellites are enriched in a single PCR without the need for hybrid capture. Due to the short PCR extension time used for inter-Alu-PCR (30 s) and the 150 bp pair-end sequencing applied, the majority of the A-tail mononucleotides successfully amplified and sequenced originate from closely-spaced, neighbor Alu elements, lying within ~150 bp of each other. The number of microsatellites obtained from inter-Alu-PCR after NGS sequencing depends on several factors, including sequencing depth, type of DNA (gDNA or fragmented DNA) and amount of DNA input. The number of microsatellites captured by inter-Alu-PCR increases with sequencing depth, but the increase saturates at higher depth. Under same sequencing depth, higher input DNA was associated with increased number of district microsatellite sites (Fig. 3**A**), while compared with fragmented DNA, more microsatellite sites were enriched from same amount of intact gDNA. Inter-Alu-PCR was able to retrieve 6000–14000 distinct microsatellite loci from 0.1 to 1 ng cfDNA. Under these conditions, 2000–4000 loci have a coverage above 20 which meets analysis criteria for several MSI algorithms (Fig. 3**A**). The ability to perform MSI analysis from 0.1 ng input cfDNA represents a ~2 orders of magnitude decrease relative to other platforms including WEX and capture-based target sequencing. This highlights the value of inter-Alu-PCR for sensitive MSI detection in cfDNA, in view of the limited amount of DNA available in liquid biopsies. The major type of microsatellites recovered from
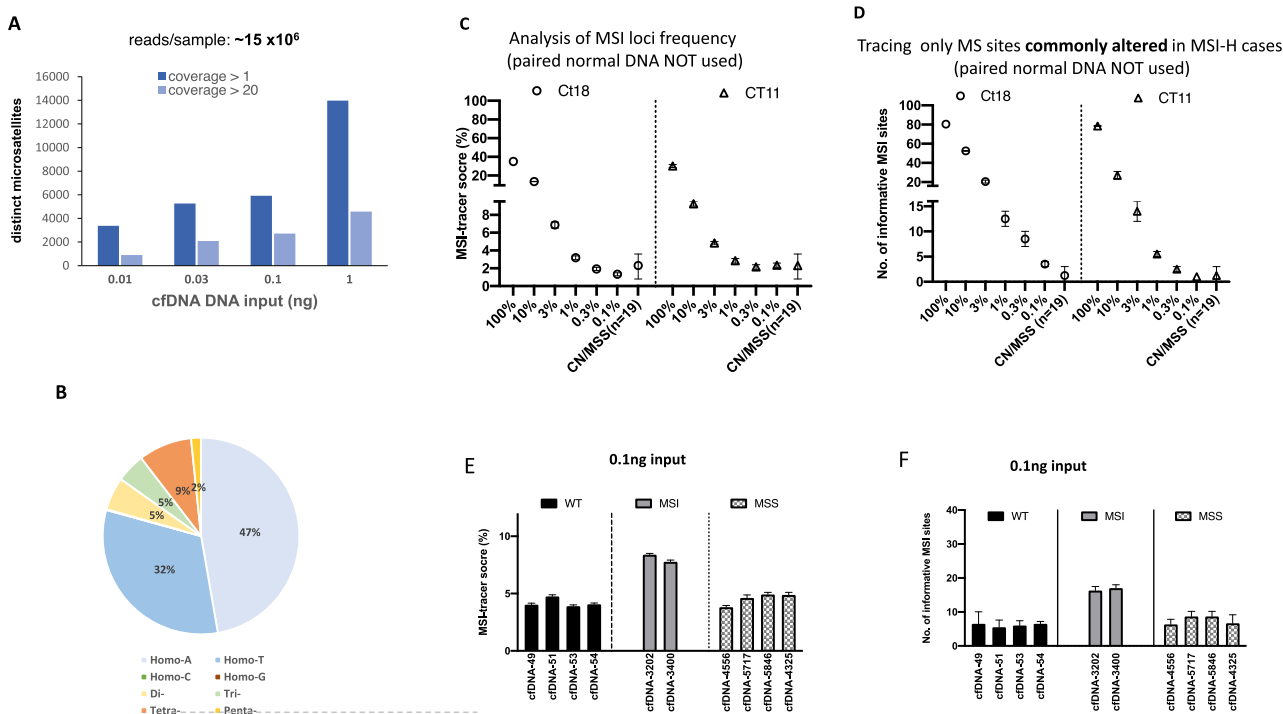

Fig. 3. Inter-Alu PCR coupled with MSI-tracer enables sensitive MSI detection from minute DNA samples. (A) Number of distinct microsatellite sites captured from 0.01 to 1 ng cfDNA at coverage 1 or 20 using ~15 × 10⁶ sequencing reads per sample. The data shown are from a single patient that is representative of the patient cohort examined. (B) Distribution of MS repeat types (1–5-mer) amplified by inter-Alu-PCR from 1 ng cfDNA. (C) inferred MSI status using inter-Alu-PCR and MSI-tracer on serial dilution of two MSI-H tumor samples CT18 and CT11 (each has ~50% tumor fraction) analyzed against non-matched normal tissue. (D) Analysis for MSI classification based on 115 MS loci commonly altered in the examined MSI-H tissue samples. (E) inferred MSI status using inter-Alu-PCR and MSI-tracer at 0.1 ng cfDNA input. (F) Analysis for MSI classification on 0.1 ng cfDNA using 115 MS informative loci.

inter-Alu-PCR is A/T homopolymers (Fig. 3B), which is more prone to MSI-induced indels [103], making inter-Alu-PCR highly sensitive for MSI detection. Through coupling inter-Alu-PCR with MSI-tracer algorithms, samples containing somatic indels at 0.5%–1.5% levels were detected when paired normal DNA or unpaired DNA were used, respectively (Fig. 3C) [29]. The detection limit when paired DNA is not available and unpaired normal DNA is used, a common scenario in clinical settings, was further improved to 0.15–0.5% by focusing the analysis solely to a group of 115 informative sites that are frequently mutated in MSI-H samples (Fig. 3D). Bearing such a low sensitivity, Inter-Alu-PCR coupled with MSI-tracer has been successfully applied to cfDNA samples. MSI-H tumor patients were clearly distinguished from MSS tumor patient or healthy donor using as low as 0.1 ng cfDNA input (Fig. 3E and F). The combined technical and practical advantages of inter-Alu-PCR coupled with MSI-tracer algorithm provide a powerful approach for MSI detection.

6. Conclusion

Recent progress in clinical studies performed with new detection technologies have increased understanding of the pathological role of MSI and created additional opportunities in cancer diagnostics and therapeutics. This in turn has stimulated demand for even more sensitive and specific tools for reliable MSI detection in diverse clinical samples including tissues, liquid biopsies, and others. While there are still technical limitations in MSI detection, the use of new technologies such as targeted NGS, ddPCR or traditional capillary electrophoresis coupled with mutation enrichment has provided significant improvements and is anticipated to enable further growth in the next few years.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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