High-Accuracy Determination of Microsatellite Instability Compatible with Liquid Biopsies

Amanda Bortolini Silveira, François-Clément Bidard, Amélie Kasperek, Samia Melaabi, Marie-Laure Tanguy, Manuel Rodrigues, Guillaume Bataillon, Luc Cabel, Bruno Buecher, Jean-Yves Pierga, et al.

Clinical Chemistry

Manuscript Title:	High-accuracy determination of microsatellite instability compatible with liquid biopsies
Manuscript No:	CLINCHEM/2019/309807 [R2]
Manuscript Type:	Article
Date Submitted by the Author:	23 Dec 2019
Complete List of Authors:	Amanda Bortolini Silveira, Francois-Clement Bidard, Amélie Kasperek, Samia Melaabi, Marie-Laure Tanguy, Manuel Rodrigues, Guillaume Bataillon, Luc Cabel, Bruno Buecher, Jean-Yves Pierga, Charlotte Proudhon, and Marc-Henri Stern
Keywords:	droplet digital PCR; liquid biopsy; microsatellite instability; mismatch repair deficiency

Dear Reviewer,

Thank you for reviewing this manuscript, please remember the following:

1. The attached manuscript is confidential and should not be circulated or shared with anyone.

2. Authors of this manuscript should never be contacted until after its publication.

3. If you have a conflict of interest regarding this work, contact the editorial office immediately.

4. Be sure to review the authors' potential conflicts of interest by following the "Author Disclosures" link in your reviewer area. Contact the editorial office if you need assistance.

High-accuracy determination of microsatellite instability compatible with liquid biopsies

Running title: MSI detection in tissue and plasma

Amanda Bortolini Silveira[1], François-Clément Bidard[1,2,3], Amélie Kasperek[1], Samia Melaabi[4], Marie-Laure Tanguy[5], Manuel Rodrigues[2,6], Guillaume Bataillon[4], Luc Cabel[2,3], Bruno Buecher[2], Jean-Yves Pierga1[2,7], Charlotte Proudhon[1]*, and Marc-Henri Stern[6]*

[1] Circulating Tumor Biomarkers Laboratory, Institut Curie, PSL Research University, INSERM CIC 1428, Paris, France.

[2] Department of Medical Oncology, Institut Curie, PSL Research University, Paris, France.

[3] Versailles Saint Quentin en Yvelines University, Paris Saclay University, Saint Cloud, Paris, France.

[4] Department of Biopathology, Institut Curie, PSL Research University, Paris, France.

[5] Biostatistics unit, Institut Curie, Saint Cloud, Paris, France.

[6] Inserm U830, DNA Repair and Uveal Melanoma (D.R.U.M.) team, Equipe labellisée par la Ligue Nationale Contre le Cancer, Institut Curie, PSL Research University, Paris, France.

[7] Paris Descartes University, Paris, France.

***Corresponding authors:**

Dr. Charlotte Proudhon, Tel: +33 1 56 24 62 82. Email: charlotte.proudhon@curie.fr

Dr. Marc-Henri Stern, Tel: +33 1 56 24 66 46. Email: marc-henri.stern@curie.fr

Keywords: microsatellite instability, mismatch repair deficiency, droplet digital PCR, liquid biopsy

List of abbreviations:

Microsatellite instability (MSI)

Circulating tumor DNA (ctDNA)

Droplet digital PCR (ddPCR)

DNA mismatch repair (MMR)

Microsatellite stable (MSS)

Next-generation sequencing (NGS)

Immunohistochemistry staining (IHC)

Cell-free DNA (cfDNA)

Formalin-fixed, Paraffin-embedded (FFPE)

Limit of detection (LOD)

Limit of blank (LOB)

Mutant Allele Frequency (MAF)

Colorectal cancer (CRC)

Wild-type (WT)

Human genes:

KRAS (KRAS proto-oncogene, GTPase)

BRAF (B-Raf proto-oncogene, serine/threonine kinase)

EGFR (epidermal growth factor receptor)

ACVR2A (activin A receptor type 2A)

DEFB105A/B (defensin beta 105A/B)

PIK3CA (phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha)

MLH1 (mutL homolog 1)

MSH2 (mutS homolog 2)

49 *MHS6* (mutS homolog6)

50 *PMS2* (PMS1 homolog 2)

Abstract

Background: Microsatellite instability (MSI) has recently emerged as a predictive pan-tumor biomarker of immunotherapy efficacy, stimulating the development of diagnostic tools compatible with large-scale screening of patients. In this context, non-invasive detection of MSI from circulating tumor DNA stands as a promising diagnostic and post-treatment monitoring tool.

Methods: We developed drop-off droplet-digital PCR (ddPCR) assays targeting BAT-26, activin A receptor type 2A (*ACVR2A*) and defensin beta 105A/B (*DEFB105A/B*) microsatellite markers. Performances of the assays were measured on reconstitution experiments of various mutant allelic fractions, on 185 tumor samples with known MSI status, and on 72 blood samples collected from 42 patients with advanced colorectal or endometrial cancers before and/or during therapy.

Results: The three ddPCR assays reached analytical sensitivity <0.1% variant allelic frequency and could reliably detect and quantify MSI in both tumor and body fluid samples. High concordance between MSI status determination by the three-marker ddPCR test and the reference pentaplex method were observed (100% for colorectal tumors and 93% for other tumor types). Moreover, the three assays showed correlations with $r \geq 0.99$ with other circulating tumor DNA markers and their dynamic during treatment correlated well with clinical response.

Conclusions: This innovative approach for MSI detection provides a non-invasive, cost-effective and fast diagnostic tool, well suited for large-scale screening of patients that may benefit from immunotherapy agents, as well as for monitoring treatment responses.

Introduction

Tumors with DNA mismatch repair (MMR) deficiency accumulate high numbers of DNA replication errors, particularly deletions at repetitive DNA sequences termed microsatellites. This phenotype, referred to as microsatellite instability (MSI), can occur in several human cancers, but is more frequently observed in colorectal, endometrial and gastric tumors *(1, 2)*. Until recently, MMR status testing was performed in the context of newly diagnosed colorectal cancers and as a first screening tool in cancers suspected to be related to Lynch syndrome, an inherited cancer predisposition condition associated with MMR deficiency and MSI cancer formation *(3-5)*. Following the approval of pembrolizumab (anti-programmed cell death-1) in 2017 for the treatment of unresectable or metastatic MSI solid tumors, MSI testing became critical for all advanced cancers regardless of their tissue of origin *(6-9)*.

Routine clinical testing relies on immunohistochemistry of MMR machinery components (mutL homolog 1 [MLH1], mutS homolog 2 [MSH2], mutS homolog 6 [MSH6], and PMS1 homolog 2 [PMS2]) and PCR amplification of reference microsatellite markers, followed by capillary electrophoresis *(4)*. The presence of alleles in the tumor that are not found in matched normal tissue are indicative of MSI. The most widely used commercial kit, referred to as the "pentaplex assay", interrogates 5 microsatellite markers (BAT-25, BAT-26, NR-21, NR-24, and MONO-27), comprising stretches of 21 to 27 poly-A repeats. Tumors with instability in two or more markers are classified as MSI, whereas those with one or no detectable alterations are classified as microsatellite stable (MSS). This panel has good clinical sensitivity for MSI status characterization in Lynch-spectrum tumors *(10, 11)*. However, its relevance for MSI status characterization in other tumor types is largely unknown. Recently, algorithms that can detect MSI using next-generation sequencing (NGS) data were developed *(1, 2, 11-13)*. Such approaches showed that MSI is much more prevalent than previously thought. For example, using exome sequencing data to investigate more than

200,000 microsatellites, Hause et al. identified MSI in 14 different cancer types, including those with low MSI incidence *(1)*. Moreover, these studies have also identified new highly informative MSI markers, opening avenues for the development of panels more relevant for cancer types not classically associated with MSI.

The current trend towards systematic detection of MSI in metastatic cancer patients has also stimulated the development of non-invasive diagnostic methods capable of detecting MSI in circulating tumor DNA (ctDNA). Such strategies require methodologies with high sensitivity, capable of detecting abnormal MSI alleles highly diluted in fragmented DNA from normal cells. Here, we describe for the first time a quantitative droplet-digital PCR (ddPCR) approach for highly sensitive detection of MSI (<0.1% mutant allelic frequency or MAF). The method is compatible with both tissue and body fluid samples and provides a cost-effective, simple and fast diagnostic tool for the high-throughput screening of patients and the monitoring of responses to therapy.

Materials and methods

Cell lines and clinical samples

Human cancer cell lines HCT-116, HCT-15 and LoVo were used as MSI positive controls. SW480, SW1116, PSN1, and buffy coat from healthy donors were used as MSS controls. Genomic and cell-free DNA (cfDNA) were extracted using the QIAamp DNA mini and Circulating Nucleic Acid Kits, respectively. Archived clinical samples were retrieved from Institut Curie Pharmacogenomics. A waiver of informed consent was obtained given the retrospective nature of the study. Plasma samples from healthy donors were obtained from *Etablissement Français du Sang*, under French and European ethical practices.

ddPCR conditions and data analysis

Primers and probes are described in online Supplemental Table 1. The *KRAS* proto-oncogene (*KRAS*) drop-off assay is described elsewhere *(14)*. The $KRAS^{Q61L}$ assay was ordered from BioRad. ddPCR reactions were performed using the Bio-Rad QX100, with 900 nM of each primer, 250 nM of each probe, up to 5000 genome equivalents of DNA, and under the following conditions : 95°C for 10 min followed by 40 cycles of 94°C for 30 s, 61°C for 3 min (BAT-26) or 59°C for 3 min (defensin beta 105A/B [*DEFB105A/B*]) or 55°C for 3 min (activin A receptor type 2A [*ACVR2A*]) or 60°C for 1 min (*B-RAF* proto-oncogene [$BRAF^{V600E}$] and phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha [$PIK3CA^{H1047R}$]) or 63°C for 1 min ($KRAS^{G12/13}$) or 55°C for 1 min ($KRAS^{Q61L}$); final hold at 98°C for 10 min. Cluster thresholding and quantification were performed with QuantaSoft v1.7.4. For the drop-off assays, droplets were manually assigned as wild type (WT) or mutant based on their fluorescence amplitude: WT=VIC^{+}/FAM^{+}; MSI mutant=$VIC^{+}/FAM^{-/low}$ and mutant KRAS=$VIC^{-/low}/FAM^{+}$. Samples were run in one or multiple replicates depending on sample availability, cfDNA concentration and ctDNA fraction. Samples were considered to be

positive if at least one replicate presented ≥2 positive MSI droplets and if the mean MAF was higher than the defined limit of detection (LOD) of each assay.

Limit of blank (LOB) and LOD

The false-positive rate of each assay was estimated using ≥53 replicates of WT DNA. The LOB was defined as the upper 95% confidence limit of the mean false-positive measurements. The analytical sensitivity was estimated using serial dilutions of HCT-116 in WT DNA, with MAFs ranging from 10% to 0.01%. The number of replicates per dilution point ranged from 3 to 8. LOD was estimated as the lowest mutant concentration that could be reliably distinguished from the LOB *(14, 15)*.

Statistical analysis

The discriminatory power of each marker was investigated using receiver operating characteristic analyses. Areas under the curve were: 0.95 (95% confidence interval, 0.9-0.99) for BAT-26 and 0.99 (0.99-1) for ACVR2A and DEFB105A/B. Optimized cut-offs were calculated to minimize classification errors. Analytical sensitivity and specificity for the chosen cut-offs were: 99% [confidence interval, 88%-100%] and 95% [96%-99%] for BAT-26, 99% [96%-100%] and 100% for ACVR2A, and 99% [96%-100%] and 96% [91%-100%] for DEFB105A/B, respectively. Global error rates were 3.2% for BAT-26, 0.8% for ACVR2A and 2.4% for DEFB105A/B.

Results

Proof of principle

We recently described drop-off ddPCR strategies for the accurate detection and quantification of all nucleotide variations present in a given region, for example in *KRAS* exon 2 and *EGFR* exon 19 hotspots *(14)*. The drop-off ddPCR method relies on the design of two hydrolysis

probes within the same PCR fragment. The reference probe (REF) is complementary to a non-variable region, and the target probe (DROP-OFF) is complementary to the WT sequence of the hotspot region where mutations are frequently observed. The presence of variants in the hotspot region results in a mismatch with the DROP-OFF probe and the absence of hydrolysis during amplification. Therefore, in a drop-off ddPCR assay three clusters of droplets can be observed: droplets with no template (REF^-/$DROP\text{-}OFF^-$), droplets containing WT alleles (REF^+/$DROP\text{-}OFF^+$) and droplets containing mutant alleles (REF^+/$DROP\text{-}OFF^{-/low}$).

We predicted that such drop-off ddPCR strategy could be instrumental to detect the microsatellite length variations that characterize MSI tumors. To determine the feasibility of this approach, we first developed an assay targeting the BAT-26 reference microsatellite, one of the five markers of the pentaplex assay *(10, 16)*. The BAT-26 assay included a VIC-labeled REF probe and a FAM-labeled drop-off probe (MSI probe), which covered the entire A27 homopolymer plus 4 and 2 bases upstream and downstream of the repeat, respectively (Figure 1A). After adjustments of the annealing temperature and extension time, we reached a clear separation between WT and MSI droplet clouds (Figure 1). The assay could specifically detect MSI in DNA from the MSI colorectal cancer (CRC) cell lines HCT-116 and HCT-15, which carry shortened BAT-26 alleles (Figure 1B and online Supplemental Figure 1A). Although the separation of the WT and MSI-positive clouds was clear in both cell lines, it was less pronounced for HCT-15 (3 nucleotide [nt] deletion) than for HCT-116 (12 nt deletion), suggesting a low level of non-specific binding of the MSI probe to BAT-26 alleles containing short deletions (Figure 1B). It is noteworthy that no instability was observed in DNA from MSS cell lines, such as the CRC cell lines SW480 and SW1116 or the pancreatic cancer cell line PSN1.

To demonstrate the flexibility of the MSI-ddPCR approach, we next developed assays targeting the two most discriminatory microsatellite markers associated with MSI-H cancers

identified by Hause *et al. (1)*. The two loci comprised the A8 and A9 repeats located within the *ACVR2A* gene and *DEFB105 A* and *B* paralogous genes, respectively. These shorter markers usually show deletions of only 1 or 2 nt and are difficult to interrogate with the PCR-capillary electrophoresis approach. Similar to what was observed for BAT-26, the ACVR2A and DEFB105A/B assays specifically detected MSI in HCT-116 and HCT-15, while no instability was observed in MSS cell lines (Figure 1B and online Supplemental Figure 1). Moreover, both assays detected MSI in the LoVo CRC cell line, which carries a homozygous deletion encompassing the BAT-26 marker (online Supplemental Figure 1B and C). The DEFB105A/B assay discriminates MSI alleles with different deletion sizes, as observed for HCT-116 and LoVo, which harbor MSI alleles shortened by 1 nt and 2 nt (online Supplemental Figure 1C). For *ACVR2A*, MSI alleles shortened by a single nt were observed in the three cell lines (Supplemental Figure 1C). Remarkably, the three assays accurately quantified MSI in 1/2 and 1/10 dilutions of HCT-116, HCT-15 and LoVo DNA in WT DNA (online Supplemental Figure 1D). Indeed, MAFs quantified by MSI-ddPCR assays correlated with $r \geq 0.99$ ($p<0.001$) with the ones reported by a commercial ddPCR assay that targets $KRAS^{G13D}$, a heterozygous mutation shared by the three cell lines. Taken together, these results demonstrate the feasibility of developing ddPCR assays targeting long and short repetitive regions of the genome and its applicability to MSI status determination and accurate quantification.

Analytical sensitivity for MSI detection and quantification accuracy

We subsequently used serial dilutions ranging from 10% to 0.01% of HCT-116 DNA in WT DNA and pure WT DNA to estimate the analytical sensitivity of each MSI-ddPCR assay. The LOB was estimated at 0.01% for the three assays (online Supplemental Figure 2A, online Supplemental Table 2). The LOD was found to be 0.04% for BAT-26 and 0.08% for both

213 *ACVR2A* and *DEFB105A/B* markers (online Supplemental Figure 2B-D). For the three assays,
214 the Pearson's correlations were r=0.99 p<0.0001 between the expected and observed MAFs,
215 suggesting the accurate quantification of MSI alleles at a wide range of allelic frequencies.
216 This *in vitro* performance of the MSI ddPCR assays prompted us to assess their efficiency in
217 clinical samples.
218
219 *Comparison MSI-ddPCR versus pentaplex-PCR*
220 BAT-26, *ACVR2A* and *DEFB105A/B* markers are recurrently unstable among MSI CRC
221 samples *(1, 11, 16)*. To test the clinical sensitivity of this 3-marker panel for detection of MSI
222 in this cancer type, we blindly tested a series of 126 formalin-fixed paraffin-embedded
223 (FFPE) samples previously classified as MSI (n=70) or MSS (n=56) using the pentaplex test
224 and immunohistochemistry (IHC) for the 4 major MMR machinery components (online
225 Supplemental Table 2, FFPE-1 to 126). As expected, MSI MAFs estimated by MSI-ddPCR
226 assays showed a bimodal distribution, concordant with the MSI status reported for these
227 samples (Figure 2A and online Supplemental Tables 3 and 4, FFPE-1 to 126). Median MAFs
228 obtained for MSI and MSS samples were 41% (interquartile range, 30%-54%) and 0% (0%-
229 0%) for BAT-26; 36% (23%-48%) and 0.08% (0%-0.2%) for *ACVR2A*; and 21% (17%-29%)
230 and 0.13% (0%-0.4%) for *DEFB105A/B*, respectively. Conversely, MAFs estimated by
231 ddPCR assays targeting known *BRAF* (n=34), *KRAS* (n=23) or *PIK3CA* (n=1) tumor
232 mutations displayed a unimodal distribution among MSI and MSS samples, with median
233 values of 22% (16%-29%). Using receiver operating characteristic analyses, we next
234 determined optimized MAF cut-offs of ≥ 6% for BAT-26 and ≥ 1.2% for both *ACVR2A* and
235 *DEFB105A/B* to classify these markers as unstable. Considering a sample as MSI when at
236 least 2 markers were reported as unstable, the 3-marker MSI-ddPCR test achieved 100%
237 clinical sensitivity and 100% clinical specificity in comparison with the pentaplex

classification (online Supplemental Table 3). This criterion was used in order to avoid misclassification of MSS samples as MSI due to germline polymorphisms, as observed for BAT-26 in samples FFPE-55, 65 and 91 (online Supplemental Table 3). Finally, we tested an additional sample (FFPE-127) corresponding to a metastatic tumor highly infiltrated by lymphocytes (cellularity < 10%). This sample was classified as dMMR by IHC (loss of expression of MSH2 and MSH6), but as MSS by pentaplex PCR. MSI-ddPCR results supported the MSI phenotype with MAFs close to the defined thresholds (BAT-26, *ACVR2A* and *DEFB105A/B* markers at 2.9%, 2.5% and 0.8% MAF, respectively; online Supplemental Tables 3 and 4), which could be explained by the higher analytical sensitivity of this approach.

Based on these results, we concluded that the 3-marker MSI-ddPCR performed as well as the pentaplex-PCR for MSI status characterization in CRC, with the additional advantage of allowing MSI testing in tumor samples highly contaminated with WT DNA.

We next evaluated the assay ability to detect MSI in other tumor types. We screened a collection of 59 previously characterized FFPE samples (25 MSI and 34 MSS), including endometrial (20 MSI and 28 MSS), ovarian carcinomas (1 MSI and 3 MSS), sebaceoma (1 MSI), cholangiocarcinoma (1 MSI), gastric carcinoma (1 MSI), small bowel adenocarcinoma (1 MSI), pancreatic ductal adenocarcinomas (2 MSS) and appendix carcinoma (1 MSS) (Figure 2B and online Supplemental Tables 3 and 4, FFPE-128 to 186). Using the criteria described above, we found concordant results between the pentaplex-PCR and the 3-marker MSI-ddPCR. 92% of the samples defined as MSI by the pentaplex test were classified as MSI by MSI-ddPCR (23/25), whereas all the MSS samples were correctly classified as MSS. We further evaluated 8 additional samples (endometrial carcinoma, n=4; ovarian carcinoma, n=1; chordoma, n=1; glioma, n=1; glioblastoma, n=1) that were classified as dMMR by IHC but MSS by pentaplex PCR. As expected, BAT-26 was reported stable by MSI-ddPCR in these 8

samples. However, MSI was detected at *ACVR2A* and *DEFB105A/B* markers in 4 and 5 samples, respectively (online Supplemental Tables 3 and 4). Taken together, these results supported the development of assays targeting additional markers identified by cancer exome sequencing data to improve the clinical sensitivity of MSI testing for different cancer types.

Quantification of tumor content by MSI-ddPCR

One of the advantages of ddPCR over the standard pentaplex-PCR is its quantitative capability. Using the data obtained for the 40 MSI CRC samples mutated for *BRAF* (n=31), *KRAS* (n=8) or *PIK3CA* (n=1) oncogenes (Figure 2A; online Supplemental Tables 3 and 4), we evaluated the correlation between MAFs estimated by MSI-ddPCR assays and the ones reported by ddPCR assays targeting these mutations. Pearson's correlations of r=0.78 and r=0.7, respectively; $p<0.0001$ (Figure 2C) were observed for the BAT-26 and *ACVR2A* markers, which accurately estimated the tumor fraction. No correlation was found for *DEFB105A/B* across the different samples (Pearson's r=0.25 p=0.12; Figure 2C), which may be explained by frequent variations in the number of copies of the *DEFB105* genes among individuals (2 to 12 per haploid genome) *(17),* or by clonal heterogeneity of *DEFB105* instability. Taken together, these results supported the utility of the MSI-ddPCR assays for accurate quantification of tumor fractions in patient samples.

MSI status characterization and tumor fraction in blood samples by MSI-ddPCR

MSI-ddPCR is a highly clinically sensitive and quantitative assay. We thus hypothesized that it would be relevant as a liquid biopsy tool to simultaneously detect MSI and quantify ctDNA. We therefore characterized 30 archival blood samples (plasma or serum) collected at baseline and/or during treatment from 14 patients with locally advanced or metastatic CRC or endometrial tumors, defined as MSI by the pentaplex assay. The three ddPCR assays detected

MSI in blood samples from all the patients tested (online Supplemental Figure 3, online Supplemental Tables 5 and 6). Moreover, for patients with *BRAF* or *PIK3CA* mutated tumors (P1, P2, P4, P5, and P8), ctDNA fractions quantified by the MSI-ddPCR assays were validated by ddPCRs specifically targeting these mutations (*BRAF*^{V600E} and *PIK3CA*^{H1047R}, Pearson's r=0.99 p<0.0001, Figure 3B, online Supplemental Tables 5 and 6). These results supported the reliability of the MSI-ddPCR assays for accurate ctDNA quantification. Indeed, for patients with sequential samples collected at baseline and at different time points during treatment, ctDNA kinetics estimated by MSI-ddPCR assays mirrored the tumor response to therapy (Figure 3A). Of note, patients P1, P4, P5, P6, and P7 were treated with pembrolizumab. Strikingly, patients P1, P4, and P7 responded to pembrolizumab and showed undetectable or dramatically decreased ctDNA concentrations (>90% MAF decrease) over the first weeks of treatment. On the other hand, patients P5 and P6 did not respond to therapy and showed increased or stable ctDNA concentrations. Similarly, decreased ctDNA concentrations for the patient P9 followed well the patient's partial response to chemo-radiotherapy (Figure 3A). Finally, when the MSI-ddPCR assays were applied to 38 serum and 4 plasma samples collected from 28 patients with advanced MSS tumors, none of the samples were positive for MSI, even though 74% (29/39) of the samples from patients with *KRAS* (n=21) or *BRAF* (n=5) mutated tumors were positive for ctDNA (*KRAS* or *BRAF* median MAF 4.2%, min=0.2%, max=71.4%). Similarly, plasma samples collected from healthy donors (n=48) were all negative for MSI, yielding a clinical specificity of 100% (online Supplemental Tables 5 and 6). Based on these data, we concluded that the MSI-ddPCR assays were both highly clinically sensitive and specific, as well as suitable for the quantitative detection of ctDNA in longitudinal studies.

Discussion

Here we present a new ddPCR test for fast, clinically sensitive and cost-effective detection of MSI. Most importantly, the high analytical and clinical sensitivity reached by this method allows for its use as a liquid biopsy tool to simultaneously detect MSI and quantify ctDNA. The method is also well suited to screen FFPE tumors and does not require matched normal samples for reliable MSI identification. As a proof-of-concept, we set up ddPCR assays targeting the microsatellites located inside *MSH2* (BAT-26), *ACVR2A* and *DEFB105A/B* genes. While BAT-26 is one of the five NCI reference markers widely used in clinical practice, *ACVR2A* and *DEFB105A/B* are novel discriminatory markers identified as frequently unstable among MSI cancers *(1, 2)*. As expected based on the frequent instability of these markers in CRC *(1, 11, 16)*, the MSI-ddPCR test showed 100% concordance with the reference pentapex assay for MSI status characterization in this cancer type. The concordance decreased to 90% for endometrial cancers, with both panels failing to classify all dMMR samples as MSI (20/24 correctly classified as MSI by pentaplex and 21/24 by MSI-ddPCR). Indeed, pan-cancer identification of MSI remains challenging when using reduced panels of markers. This limitation is currently being addressed by more comprehensive NGS-based approaches *(1, 2, 11-13)*. A recent study reported the use of a 98-kb pan-cancer panel, which included microsatellites, to detect MSI in blood *(18)*. With a panel of 99 microsatellite sequences, Willis *et al.* described MSI detection across 16 cancer types *(19)*. However, NGS assays are costly and time-consuming when compared to targeted approaches such as MSI-ddPCR. Moreover, MSI-ddPCR achieved 100% clinical sensitivity in blood samples compared to 87% *(19)* and 78% *(18)* for the above studies. MSI-ddPCR is therefore more suitable for routine clinical practice screening and monitoring of response to treatment. Systematic investigation of MSI in cancer exomes has uncovered shared and cancer-type specific patterns of instability and therefore is setting the ground for future development of

more sensitive panels of markers *(1, 2, 11-13)*. In this context, MSI-ddPCR stands as a promising approach for the next generation of panel-based MSI tests. Indeed, the method provides robust direct measurements of MSI for both long non-coding markers (such as BAT-26), as well as for coding microsatellites (such as *ACVR2A*), which usually show indels of only a single nucleotide. Therefore, MSI-ddPCR should be easily adapted to different panels of markers, depending on the clinical need, and could help to improve MSI testing in extracolonic tumors for which the reference pentaplex panel has more limited clinical sensitivity. Moreover, high order multiplexing of ddPCR assays should become feasible with the release of multicolor digital PCR platforms.

The analytical sensitivity of MSI-ddPCR is at least 2 orders of magnitude higher than the detection threshold of the reference pentaplex method (≥10% MAF) *(20, 21)*, opening up the possibility of non-invasive MSI detection in ctDNA. CtDNA has demonstrated its clinical value as a liquid biopsy tool *(22, 23)*, and has been approved in metastatic non-small cell lung cancer as a non-invasive surrogate to tumor biopsies for the detection of activating *EGFR* mutations *(24)*. In face of the unprecedented approval of anti-checkpoint inhibitors for the treatment of metastatic solid tumors regardless of tumor site, a similar situation could be envisaged for MSI *(6-9)*. Recently, an alternative strategy was proposed for highly sensitive MSI detection *(21)*. The method uses targeted nuclease digestion to enrich for altered microsatellite sequences prior to PCR amplification. This procedure reduces confounding stutter bands generated by amplification of unaltered microsatellites, therefore increasing the resolution of fragment analysis. However, pre-analytic enrichment renders this approach non-quantitative. MSI-ddPCR in contrast provides absolute quantification of MSI alleles and therefore is also suitable for other clinical applications of ctDNA, including patient follow-up *(25)*. We anticipate that MSI-ddPCR will prove its usefulness for the monitoring of immune

checkpoint inhibitor efficacy. Early detection of non-responders should improve patient management and significantly reduce costs for the healthcare system.

In summary, MSI-ddPCR allows robust, non-invasive detection of MSI with high clinical sensitivity. The method is straightforward and has the potential to be routinely applied clinically in laboratories equipped with a digital PCR platform. MSI-ddPCR is compatible with high throughput screening of patients, including those with cancers with low MSI prevalence, promising increased access to personalized treatments.

Acknowledgements: We thank Aurore Rampanou, Caroline Hego, Chloe Derouet, Pascal Portois and Frederique Maraone for invaluable technical support. We thank Charles Decraene and Olivier Lantz for discussions and Christopher R Muller for critical reading of the manuscript. We also thank the group of Valérie Taly for providing us with MSI cell lines (LoVo, HCT-15).

Author contributions: ABS, FCB, CP and MHS designed the study. ABS and AK performed experiments. ABS, FCB, CP and MHS analyzed data. MLT performed the statistical analysis. ABS, FCB, SM, GB, LC, BB and JYP contributed with identification of clinical samples. ABS wrote the manuscript with support of FCB, CP and MHS.

Disclosure/Conflict of interest: ABS, FCB, AK, CP and MHS are inventors on a patent application submitted by the Institut Curie (PCT/ EP 2018/068760). The other authors declare no competing financial interests. This work was supported by SiRIC1 Curie (grant INCa-DGOS-Inserm_4654), SiRIC2 Curie (grant INCa-DGOS-Inserm_12554), and the Innovative Medicines Initiative Joint Undertaking under grant agreement no. 115749 (project Cancer-ID).

References

1. Hause RJ, Pritchard CC, Shendure J, Salipante SJ. Classification and characterization of microsatellite instability across 18 cancer types. Nat Med 2016;22:1342-50.
2. 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.
3. Umar A, Risinger JI, Hawk ET, Barrett JC. Testing guidelines for hereditary non-polyposis colorectal cancer. Nat Rev Cancer 2004;4:153-8.
4. Buza N, Ziai J, Hui P. Mismatch repair deficiency testing in clinical practice. Expert Rev Mol Diagn 2016;16:591-604.
5. Latham A, Srinivasan P, Kemel Y, Shia J, Bandlamudi C, Mandelker D, et al. Microsatellite instability is associated with the presence of lynch syndrome pan-cancer. J Clin Oncol 2019;37:286-95.
6. Le DT, Durham JN, Smith KN, Wang H, Bartlett BR, Aulakh LK, et al. Mismatch repair deficiency predicts response of solid tumors to pd-1 blockade. Science 2017;357:409-13.
7. Overman MJ, McDermott R, Leach JL, Lonardi S, Lenz HJ, Morse MA, et al. Nivolumab in patients with metastatic DNA mismatch repair-deficient or microsatellite instability-high colorectal cancer (checkmate 142): An open-label, multicentre, phase 2 study. Lancet Oncol 2017;18:1182-91.
8. Overman MJ, Lonardi S, Wong KYM, Lenz HJ, Gelsomino F, Aglietta M, et al. Durable clinical benefit with nivolumab plus ipilimumab in DNA mismatch repair-deficient/microsatellite instability-high metastatic colorectal cancer. J Clin Oncol 2018;36:773-9.

9. Kim ST, Cristescu R, Bass AJ, Kim KM, Odegaard JI, Kim K, et al. Comprehensive molecular characterization of clinical responses to pd-1 inhibition in metastatic gastric cancer. Nat Med 2018;24:1449-58.

10. Bacher 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. Dis Markers 2004;20:237-50.

11. Maruvka YE, Mouw KW, Karlic R, Parasuraman P, Kamburov A, Polak P, et al. Analysis of somatic microsatellite indels identifies driver events in human tumors. Nat Biotechnol 2017;35:951-9.

12. Bonneville R, Krook MA, Kautto EA, Miya J, Wing MR, Chen HZ, et al. Landscape of microsatellite instability across 39 cancer types. JCO Precis Oncol 2017;2017.

13. Middha S, Zhang L, Nafa K, Jayakumaran G, Wong D, Kim HR, et al. Reliable pan-cancer microsatellite instability assessment by using targeted next-generation sequencing data. JCO Precis Oncol 2017;2017.

14. Decraene C, Silveira AB, Bidard FC, Vallee A, Michel M, Melaabi S, et al. Multiple hotspot mutations scanning by single droplet digital pcr. Clin Chem 2018;64:317-28.

15. Bidshahri R, Attali D, Fakhfakh K, McNeil K, Karsan A, Won JR, et al. Quantitative detection and resolution of braf v600 status in colorectal cancer using droplet digital pcr and a novel wild-type negative assay. J Mol Diagn 2016;18:190-204.

16. Suraweera N, Duval A, Reperant M, Vaury C, Furlan D, Leroy K, et al. Evaluation of tumor microsatellite instability using five quasimonomorphic mononucleotide repeats and pentaplex pcr. Gastroenterology 2002;123:1804-11.

17. Hollox EJ, Armour JA, Barber JC. Extensive normal copy number variation of a beta-defensin antimicrobial-gene cluster. Am J Hum Genet 2003;73:591-600.

18. Georgiadis A, Durham JN, Keefer LA, Bartlett BR, Zielonka M, Murphy D, et al. Noninvasive detection of microsatellite instability and high tumor mutation burden in cancer patients treated with pd-1 blockade. Clin Cancer Res 2019;25:7024-34.

19. Willis J, Lefterova MI, Artyomenko A, Kasi PM, Nakamura Y, Mody K, et al. Validation of microsatellite instability detection using a comprehensive plasma-based genotyping panel. Clin Cancer Res 2019;25:7035-45.

20. Lee HS, Kim WH, Kwak Y, Koh J, Bae JM, Kim KM, et al. Molecular testing for gastrointestinal cancer. J Pathol Transl Med 2017;51:103-21.

21. Ladas I, Yu F, Leong KW, Fitarelli-Kiehl M, Song C, Ashtaputre R, et al. Enhanced detection of microsatellite instability using pre-pcr elimination of wild-type DNA homo-polymers in tissue and liquid biopsies. Nucleic Acids Res 2018;46:e74.

22. Alix-Panabieres C, Pantel K. Clinical applications of circulating tumor cells and circulating tumor DNA as liquid biopsy. Cancer Discov 2016;6:479-91.

23. Siravegna G, Marsoni S, Siena S, Bardelli A. Integrating liquid biopsies into the management of cancer. Nat Rev Clin Oncol 2017;14:531-48.

24. Douillard JY, Ostoros G, Cobo M, Ciuleanu T, Cole R, McWalter G, et al. Gefitinib treatment in egfr mutated caucasian nsclc: Circulating-free tumor DNA as a surrogate for determination of egfr status. J Thorac Oncol 2014;9:1345-53.

25. Cabel L, Proudhon C, Romano E, Girard N, Lantz O, Stern MH, et al. Clinical potential of circulating tumour DNA in patients receiving anticancer immunotherapy. Nat Rev Clin Oncol 2018;15:639-50.

Figure Captions

Figure 1. Microsatellite instability (MSI)-ddPCR drop-off assays. A. Schematic representation of the principle of the method. B. 2-D scatter plots of BAT-26, *ACVR2A* and *DEFB105A/B* assays using 10% dilutions of HCT-116 or HCT-15 MSI CRC cell lines in WT DNA. Droplets containing wild type (WT), MSI alleles and no DNA template are indicated as blue, green and gray clusters, respectively.

Figure 2. Microsatellite instability (MSI) status characterization in tumor specimens by MSI-ddPCR. Distribution of MSI allelic frequencies measured by BAT-26, *ACVR2A* and *DEFB105A/B* assays in colorectal carcinoma (CRC) (A) or non-CRC tumors (B). Black and red dots represent samples classified as MSI or microsatellite stable (MSS) by the pentaplex test, respectively. Optimized thresholds were calculated using receiver operating characteristics analysis. (C) Correlation between mutant allele frequencies (MAFs) measured by MSI-ddPCR and $BRAF^{V600E}$, $KRAS^{G12/13}$ or $PIK3CA^{H1047R}$ dPCR assays.

Figure 3. Microsatellite instability (MSI) detection in blood samples of advanced cancer patients by MSI-ddPCR. A. ctDNA dynamics in blood samples at baseline (B) and during treatment (M: month, W: week). Arrows indicate associated responses based on CT scans (PR: partial response, SD: stable disease, PD: progressive disease). B. Correlation between ctDNA fractions estimated by MSI-ddPCR assays and ddPCR assays specifically targeting $BRAF^{V600E}$ or $PIK3CA^{H1047R}$ mutations for patients (P) 1, 2, 4, 5, and 8.

Fig. 1

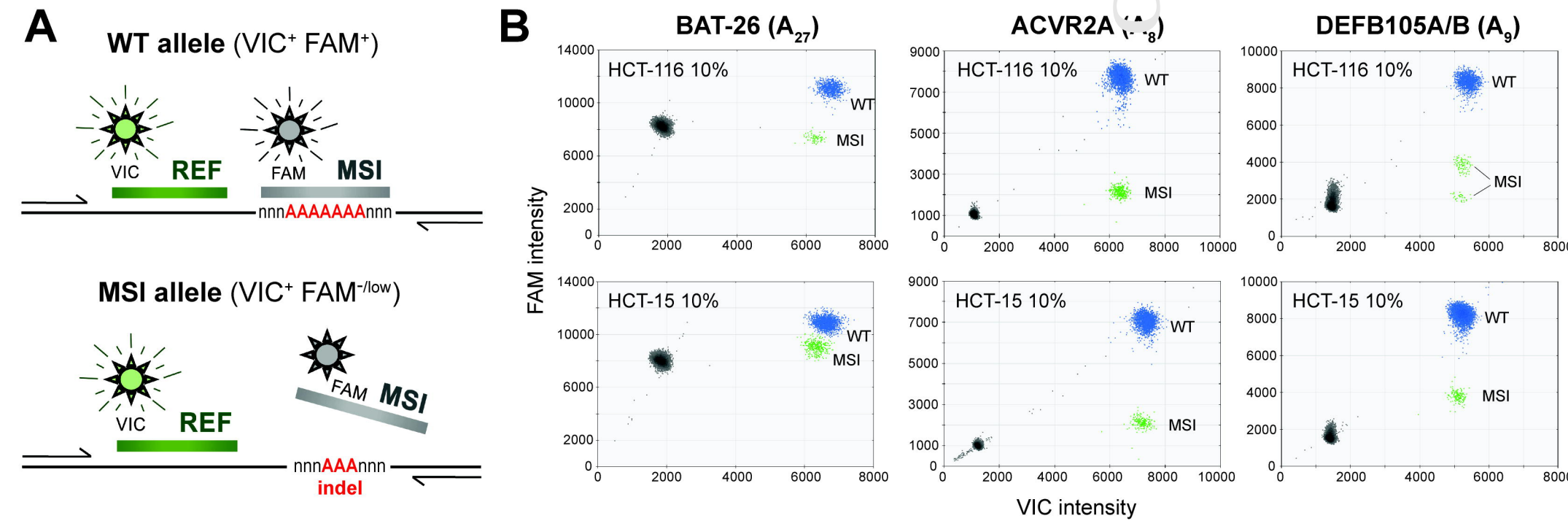

A
WT allele (VIC+ FAM+)
VIC
REF
FAM
MSI
nnnAAAAAAAnnn
MSI allele (VIC+ FAM-/low)
VIC
REF
FAM
MSI
nnnAAAnnn
indel
B
BAT-26 (A27)
ACVR2A (A8)
DEFB105A/B (A9)
HCT-116 10%
HCT-15 10%
WT
MSI
FAM intensity
VIC intensity

Fig. 2

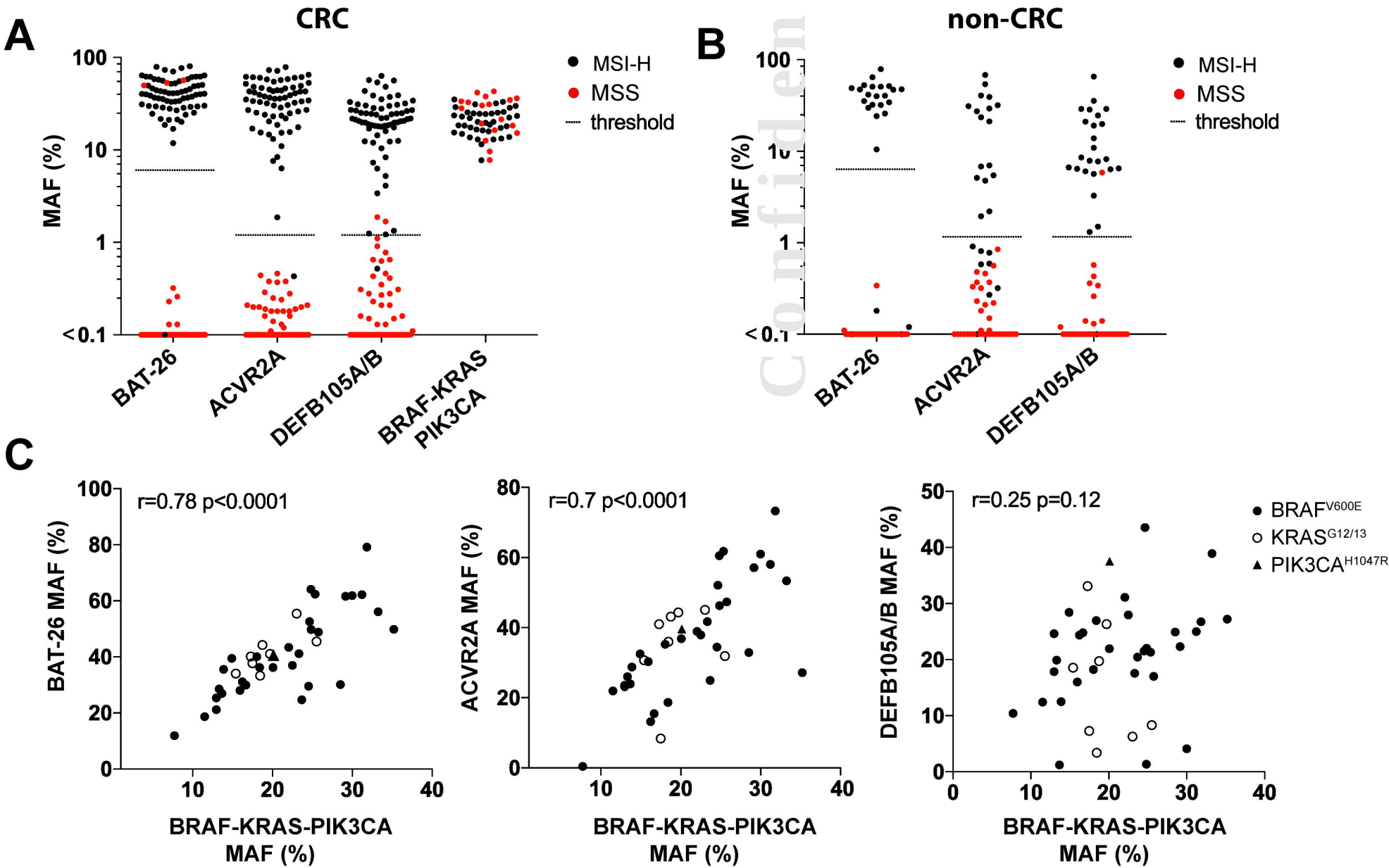
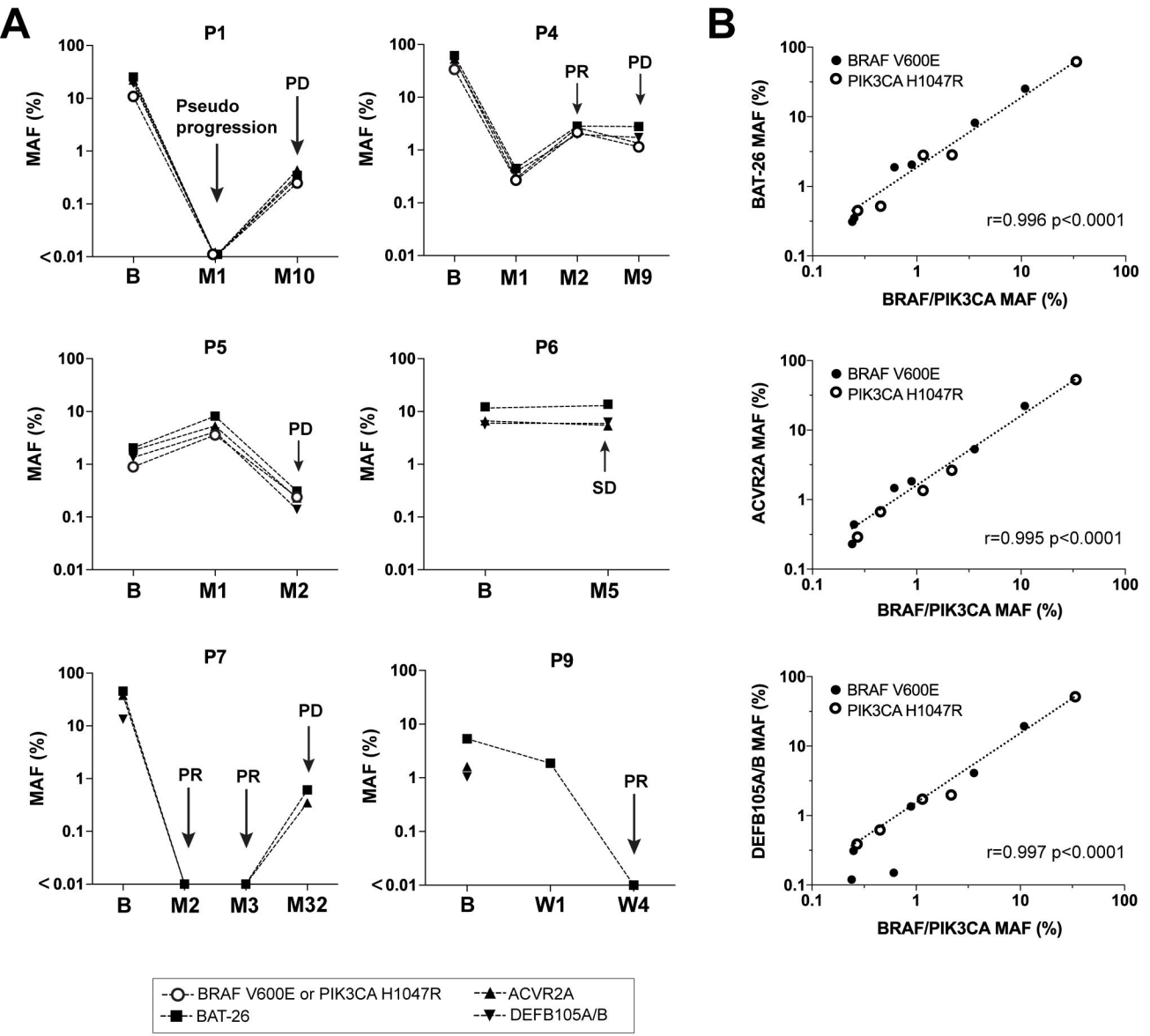


Fig. 3


A
P1
Pseudo progression
PD
MAF (%)
B
M1
M10
P4
PR
PD
M2
M9
P5
M2
P6
SD
M5
P7
PR
PR
PD
M3
M32
P9
W1
W4
B
BRAF V600E
PIK3CA H1047R
BAT-26 MAF (%)
BRAF/PIK3CA MAF (%)
r=0.996 p<0.0001
ACVR2A MAF (%)
r=0.995 p<0.0001
DEFB105A/B MAF (%)
r=0.997 p<0.0001
BRAF V600E or PIK3CA H1047R
BAT-26
ACVR2A
DEFB105A/B