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Ultrasensitive detection of acute myeloid leukemia minimal residual disease using single molecule molecular inversion probes

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ABSTRACT

The identification of minimal residual disease is the primary diagnostic finding which predicts relapse in patients treated for acute myeloid leukemia. Ultrasensitive detection of minimal residual disease would enable better patient risk stratification and could open opportunities for early therapeutic intervention. Herein we apply single molecule molecular inversion probe capture, a technology combining multiplexed targeted sequencing with error correction schemes based on molecular barcoding, in order to detect mutations identifying minimal residual disease with ultrasensitive and quantitative precision. We designed a single molecule molecular inversion probe capture panel spanning >50 kb and targeting 32 factors relevant to acute myeloid leukemia pathogenesis. We demonstrate linearity and quantitative precision over 100-fold relative abundance of mutant cells (1 in 100 to 1 in 1,500), with estimated error rates approaching 1 in 1,200 base pairs sequenced and maximum theoretical limits of detection exceeding 1 in 60,000 mutant alleles. In 3 of 4 longitudinally collected specimens from patients with acute myeloid leukemia, we find that single molecule molecular inversion probe capture detects somatic mutations identifying minimal residual disease at substantially earlier time points and with greater sensitivity than clinical diagnostic approaches used as current standard of care (flow cytometry and conventional molecular diagnosis), and identifies persisting neoplastic cells during clinical remission. In 2 patients, single molecule molecular inversion probe capture detected heterogeneous, subclonal acute myeloid leukemia populations carrying distinct mutational signatures. Single molecule molecular inversion probe technology uniquely couples scalable target enrichment with sequence read error correction, providing an integrated, ultrasensitive approach for detecting minimal residual disease identifying mutations.

Intrtoduction

Acute myeloid leukemia (AML) is a highly aggressive, immature hematopoietic neoplasm with significant mortality and morbidity. Despite sustained improvements in our understanding of the mechanisms underlying this disease, persistent challenges regarding improving patient outcomes remain. Individuals with AML who can tolerate hematopoietic stem cell transplantation have enjoyed substantial improvements in disease-free and overall survival, but even so, clinical relapse is a significant challenge for many patients, particularly for those who receive only conventional chemotherapy treatments. Several independent studies have shown that the detection of minimal residual disease (MRD), defined as small numbers of neoplastic cells which persist after cancer therapy, is a key prognostic variable in predicting disease relapse in the post-treatment setting of AML.[1–5] In current practice, MRD may be assessed by a variety of disparate molecular or phenotypic assessment techniques including detection of abnormal immunophenotype by flow cytometry,[6,7] fluorescent *in situ* hybridization (FISH)/cytogenetics,[8] and real time polymerase chain reaction (RT-PCR) for individual AML-specific genetic

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lesions.[2,9,10] These methods have variable performance characteristics, and no single phenotypic or molecular signature of disease likely exists, ultimately making current patient stratification achievable with variable levels of success. Consequently, robust and standardized methods for identifying MRD are critically needed.[11]

For many hematologic disorders, next-generation sequencing has emerged as an appealing strategy for detecting and monitoring MRD.[12-14] In the case of AML, whole genome and exome sequencing[15-18] has revealed that the number of mutations occurring in AML is limited, with estimates of the mutational burden of any given malignancy being in the order of only 20 to 30 events.[15] This presents a special challenge in detecting meaningful genomic markers of AML MRD, particularly in sub-types that lack recurrent genomic fusions such as acute promyelocytic leukemia with *PML-RARA*. Successful strategies to detect AML MRD using high-throughput sequencing have consequently focused on 1 of 2 strategies. The first of these is focused deep sequence analysis of recurrent mutational hotspots, such as those occurring in *NPM1*,[19] *RUNX1*,[20] or *FLT3*.[21] Although potentially powerful molecular markers of malignancy, these variants are not necessarily present nor informative in all patients. Restricting analysis to single genes or sites also limits the ability to detect and monitor clonal heterogeneity, which is thought to be an important feature of AML pathogenesis.[22,23] The second strategy is to perform whole genome or whole exome sequencing of fulminant malignancy in order to catalog informative, patient-specific mutations which can be later targeted using personalized assays and followed over time.[24-26] Although more comprehensive, this latter strategy is both more costly and more time consuming,[27] and in practice also requires the subsequent validation of patient-specific assays for MRD detection.

To improve upon existing paradigms for conventional and next-generation sequencing-based AML MRD detection, herein we adapt single molecule molecular inversion probes (smMIPs)[28,29] in order to interrogate common genetic lesions in AML with ultrasensitive limits of detection. smMIP technology unites multiplexed targeted sequencing with an error correction strategy based on unique molecular identifiers (UMIDs),[30] degenerate oligonucleotide barcodes that mark sequence reads

Table 1. Patient specimens and clinical testing results.

Patient	Day from initial diagnosis	Days post-transplant	Molecular results	Flow cytometry results
	83	N/A**	*FLT3* and *NPM1* negative	NP*
	196	N/A	*FLT3* and *NPM1* negative	NP
1	329	N/A	*FLT3* Positive, 35%. *NPM1* Positive.	NP
	412	N/A	*FLT3* Positive, 15%. *NPM1* Positive.	NP
	531	N/A	*FLT3* Positive, 97%. *NPM1* Positive.	98% abnormal cells
	0	-97	*FLT3* Negative. *NPM1* Positive.	0.98% abnormal cells (regenerating blasts *vs.* MRD)
	69	28	NP	Negative for abnormal cells
2	125	84	NP	Negative for abnormal cells
	393	352	*FLT3* and *NPM1* negative	0.14% abnormal cells (suspicious but not diagnostic for MRD)
	397	356	NP	NP
	461	420	*FLT3* negative	Negative for abnormal cells
	579	538	NP	Negative for abnormal cells
	746	705	*FLT3* Positive, 10.46%	NP
	0	-115	*FLT3* and *NPM1* negative	NP
	94	-21	*FLT3* and *NPM1* negative	Negative for abnormal cells
	143	28	*FLT3* and *NPM1* negative	Negative for abnormal cells
	269	154	*FLT3* and *NPM1* negative	Negative for abnormal cells
3	346	231	*FLT3* and *NPM1* negative	Negative for abnormal cells
	437	322	*FLT3* and *NPM1* negative	Negative for abnormal cells
	542	427	*FLT3* and *NPM1* negative	Negative for abnormal cells
	584	469	*FLT3* and *NPM1* negative	Negative for abnormal cells
	599	484	*JAK2* mutation negative	NP
	269	-917	*NPM1* Negative.	NP
	1083	-103	*NPM1* Negative.	Negative for abnormal cells
4	1214	28	*NPM1* Negative.	Negative for abnormal cells
	1556	370	*NPM1* Negative.	Negative for abnormal cells

*Not performed. **Not Applicable. MRD: minimal residual disease.

derived from a common progenitor molecule and which allow for random sequencing errors to be eliminated through oversampling and consensus calling (Figure 1). When combined, these features enable simultaneous enrichment, detection, and quantitation of single nucleotide polymorphisms and small insertions and deletions in targeted genomic regions with a sensitivity greatly exceeding[27,28] the inherent error rate of next-generation sequencing (~2% per nucleotide).[31] These pilot studies define the performance characteristics of smMIP capture as a molecular diagnostic, and demonstrate the utility of the approach when applied in clinical practice.

Methods

Samples and Cell Lines

Residual, clinical samples were obtained and de-identified according to the University of Washington Institutional Review Board guidelines. This project was approved by the University of Washington Human Subjects Division and was conducted in accordance with the Declaration of Helsinki. A total of 25 samples, derived from prior, routine sampling from 4 patients were used in this pilot study (Table 1, *Online Supplementary Table S1*). All patients had a confirmed histologic or flow cytometry diagnosis of acute myeloid leukemia and were selected for further study if sufficient residual DNA (at least 500 ng) was available for analysis. Staging marrows from lymphoma patients under 40 years of age for whom myeloid flow cytometry was pre-screened as negative were used as normal bone marrow controls.

For linearity and sensitivity studies, suspensions of cell lines and normal human bone marrow were quantified using flow cytometry, combined in defined proportions, and incremental serial dilutions were prepared. Cell lines were obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen (cell lines KM-H2, OCI-AML3, and L1236, Braunschweig, Germany) or ATCC (cell line Raji, Manassas, VA, USA) and were cultured in accordance with supplier specifications. NA12878 genomic DNA was obtained from Coriell Biorepository (Camden, NJ, USA).

smMIP design, capture, and sequencing

A smMIP capture panel was designed against AML-relevant targets (Table 2, *Online Supplementary Table S2*), identifying polymorphisms carried in cell lines, and other clinically relevant genes (*ABL1, ALK, JAK2, NT5C2,* and *ROS1*) using the program Molecular Interaction Potential Generator (MIPgen).[32] As detailed in the *Online Supplementary Methods*, 500 ng genomic DNA was hybridized with the panel, exonuclease-treated, and PCR-amplified to generate sequencing libraries. Single libraries were prepared from all specimens in this study. Sequencing was performed using the 300 cycle Illumina NextSeq 500/550 High Output v2 kit (Illumina, San Diego, CA, USA).

Data analysis Pipeline

Sequencing data were analyzed as described in full in the *Online Supplementary Methods*. Briefly, reads were demultiplexed, regions corresponding to the smMIP backbone were removed, and read pairs self-assembled. After mapping to the human genome (hg37), reads corresponding to each smMIP were grouped based on whether their UMID was contained in 2, or more than 2 independent reads, with UMIDs represented in only 1 sequence read discarded. Single nucleotide polymorphisms and indels were called on these read groups using a "majority rule" approach. We applied an empiric site- and mutation-specific error model in order to assess the significance of the observed variation at each site in

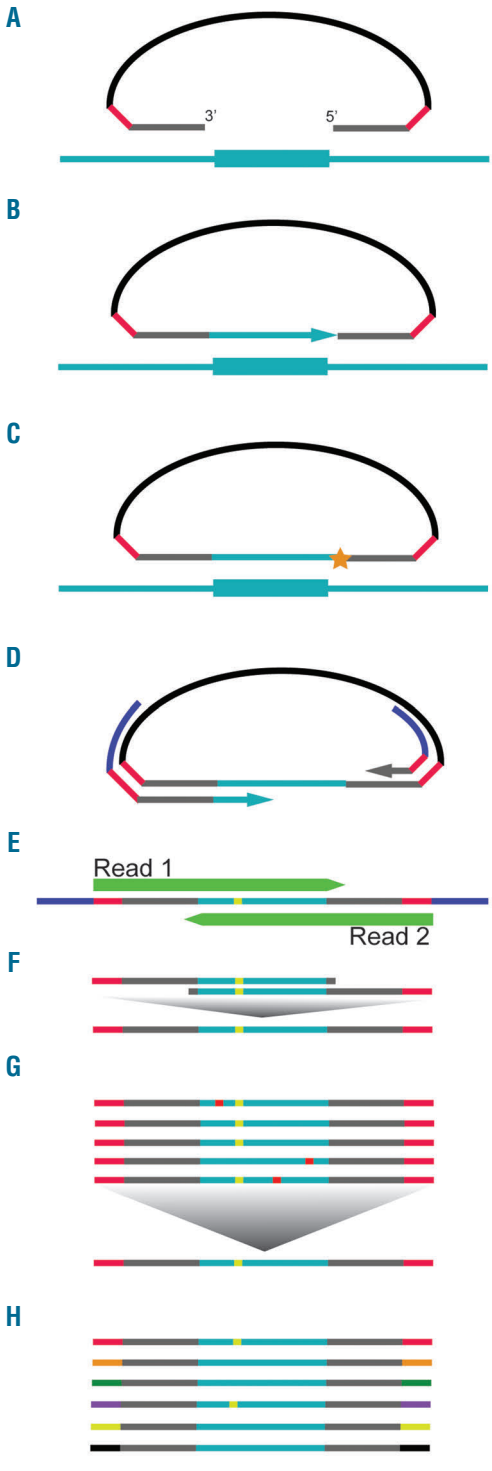

Figure 1. Overview of smMIP capture. (A) smMIPs are single-stranded (ss)DNA oligonucleotides with domains at both ends that are complimentary to genomic targets of interest (gray), each flanked by a 4bp fully degenerate UMID sequence (red), totaling an 8bp molecular tag, and linked by a backbone sequence common to all probes (black). After hybridization of probes to DNA, (B) DNA polymerase copies targeted genomic DNA by extension of the free probe arm and (C) the smMIP is joined into a covalently sealed circle by the action of DNA ligase. After exonuclease digestion of unbound probes and free genomic DNA, (D) PCR primed against the defined smMIP backbone is performed to amplify successful capture events and their identifying UMIDs. (E) Paired-end, high-throughput sequencing is performed. (F) Derived paired-end reads are merged into a single, contiguous read, providing inherent intra-read error correction. (G) A consensus sequence is generated out of all reads sharing a common UMID. Low prevalence, random errors are canceled out. (H) Extremely low variant allele frequencies can be accurately quantified by assaying the fraction of error-corrected reads bearing a mutation of interest out of the larger population of read groups derived from unique UMID-tagged smMIPs.

the smMIP panel, and sites with $P>0.005$ were excluded as potential artefacts.

In summary, the analysis pipeline expresses variant calls in terms of the number of unique smMIP capture events which are consistent with a given variant over the total number of smMIP capture events overlying that site, and assesses the statistical significance of individual variants.

Data availability

Sequence data generated for this study are available from the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) under study accession number SRP097634.

Results

smMIP panel design and performance

We designed a smMIP panel targeting the most recurrently mutated genes in AML[15,33] and other high-yield or actionable mutational targets, featuring a combination of full gene tiling and focused hotspot interrogation as appropriate to the selected targets (Table 2). After rebalancing the relative concentration of individual probes in the capture pool in order to promote evenness of performance, and subsequently removing smMIPs with persistently low performance (n=83), the final capture design included 511 probes which spanned a total of 50,176 base pairs (bp) of genomic DNA (*Online Supplementary Table S2*). With an allotment of $1.05X10^6 \pm 5.9X10^6$ (average ± standard deviation) reads per specimen, an average of 17,763 unique UMIDs were obtained per smMIP (range of 973 to 52,437), with an average of 11.2 sequence read pairs per UMID (Figure 2A, *Online Supplementary Figure S1*). Given our criteria for data filtering, these performance characteristics correspond to an average theoretical sensitivity of ~1 in 9,000 mutant alleles. Minor variations were seen among replicates, possibly due to differences in DNA quality.

Error reduction and error profile

In order to initially evaluate the performance of smMIP-mediated error correction compared to conventional sequencing we analyzed 2 specimens, reference cell line NA12878 and a bone marrow sample derived from a healthy donor, which were subjected to smMIP capture and sequencing. After masking sites of variation that were consistent with heterozygous or homozygous germline polymorphisms, we quantified the number of variant calls derived both from raw sequencing reads and after applying smMIP-mediated error correction (Table 3). Applying standard cutoffs for accepting variants with equal to or greater than 2% variant allele frequency[31] (*Online Supplementary Methods*), conventional deep sequencing registered an average of 1,047 ± 17 (average ± standard deviation) artefactual single nucleotide variant

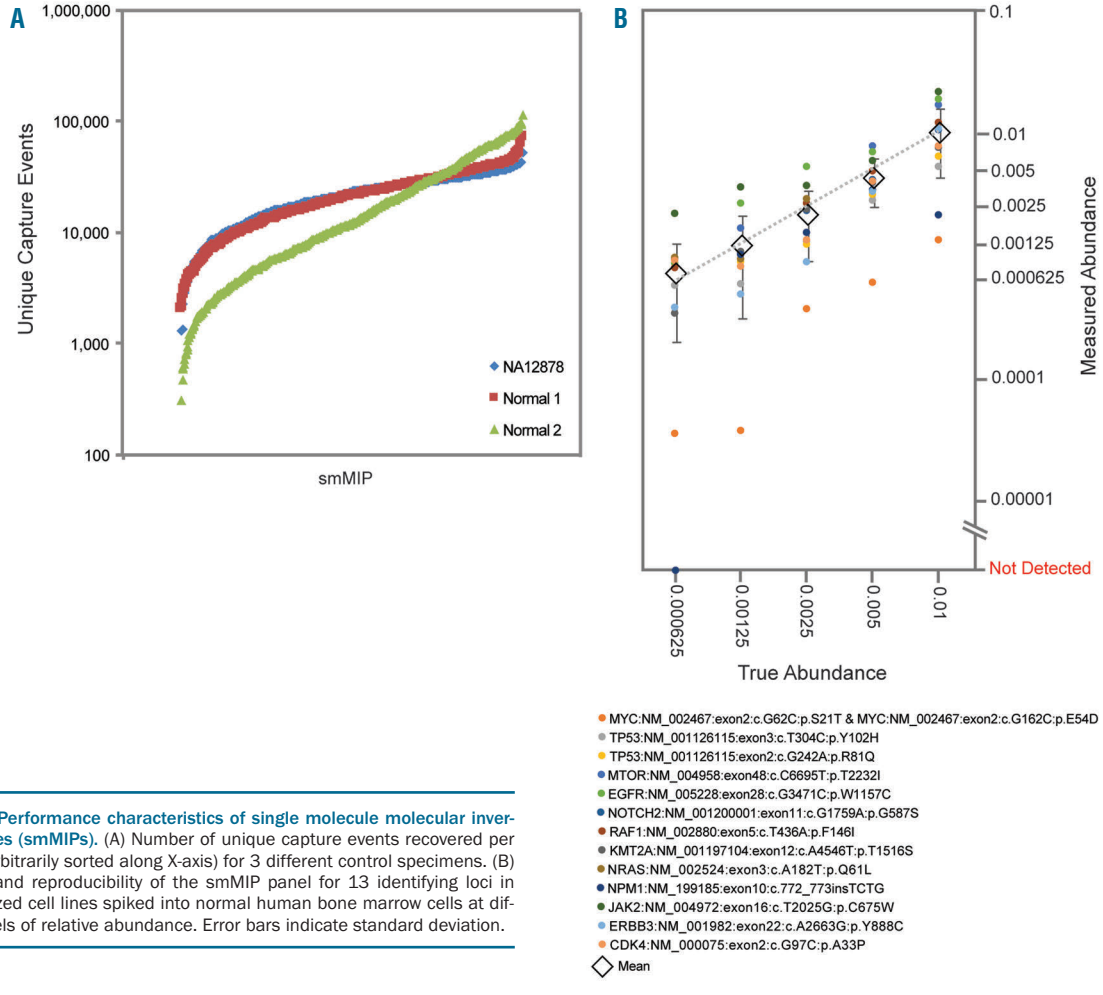

Figure 2. Performance characteristics of single molecule molecular inversion probes (smMIPs). (A) Number of unique capture events recovered per smMIP (arbitrarily sorted along X-axis) for 3 different control specimens. (B) Linearity and reproducibility of the smMIP panel for 13 identifying loci in immortalized cell lines spiked into normal human bone marrow cells at different levels of relative abundance. Error bars indicate standard deviation.

calls and 10 ± 2 indels, for a cumulative error frequency of 1 artefact reaching detection thresholds per 50 nucleotides sequenced. Using parameters enabled to detect low prevalence variation (*Online Supplementary Methods*), the conventional deep sequencing analysis approach increased errors considerably, resulting in 65,127 ± 1464 (average ± standard deviation) artefactual single nucleotide variants and 6,471 ± 334 (average ± standard deviation) artefactual indels per specimen, equating to an average of 1.42 different low prevalence variants detected per nucleotide sequenced. By contrast, using smMIP capture and associated analytic error modeling, we identified a total of 1,025±664 (average ± standard deviation) single nucleotide variants and 220±2 (average ± standard deviation) indels per specimen occurring at any level of detectable abundance. This equates to 1 error per 40 bp sequenced, comparable to error rates seen using deep sequencing with standard variant calling, and representing a 60-fold reduction in error rate over ultrasensitive mutation detection by conventional sequencing.

However, smMIP capture resulted in a non-uniform distribution of errors across the landscape of potential nucleotide substitutions (Table 3), for which there was a predominance of transversions involving cytosine and guanine. This spectrum of changes is consistent with a low frequency of oxidative DNA damage (G>T and C>A)[25,34] and spontaneous deamination of 5-methylcytosine (C>T and G>A),[28] which exist in template molecules prior to sequencing. Interestingly, an elevation of these error types was not observed for either of the conventional sequencing analysis approaches, presumably because DNA damage events are too low frequency to be detected, or are masked by random, higher frequency sequencing errors overlapping these sites. In light of these observations, we conclude that much of the low level variation detected by smMIP capture represents pre-analytic DNA damage, rather than sequencing errors per se. Removing these state changes from consideration, the error rate of smMIP capture is estimated to approach 1 error per 1,200 bp sequenced, an order of magnitude less than that seen with standard variant calling and more than 2 orders of magnitude less than seen for ultrasensitive calls made without error correction.

Linearity and quantitative precision

In order to assess the effectiveness with which smMIP capture was able to recover known mutations at various levels of relative abundance, we constructed a series of synthetic specimens where calibrated numbers of cells from normal human bone marrow were combined with lesser proportions of different immortalized human cell lines marked by identifying SNPs and indels.[35] We evaluated 2-fold serial dilutions, ranging from 1% to 0.0625% relative abundance, of 4 different cell lines representing a total of 14 identifying variants (although 2 of these variants, *MYC* c.G62C and c.G162C, were targeted by a single smMIP). We were able to recover all but 1 of the polymorphisms occurring at the lowest dilution examined (Figure 2B, *Online Supplementary Table S3*). Linearity and quantitation of variant allele frequency was achieved over 2 orders of magnitude, with consistency across samples and the individual mutations typed (coefficient of variation range of 0.41 to 0.74, depending on dilution). These data demonstrate that smMIP capture has both sensitivity and quantitative precision to at least 1 in 1,500 mutant alleles.

Detection of MRD in longitudinal patient samples using smMIP capture

To investigate the potential of smMIPs to identify MRD from patient samples, we examined a cohort of 4 patients for whom MRD had been evaluated clinically over multiple time points (Table 1). Two of the patients in our cohort relapsed during the period of sample collection, whereas the other 2 had negative MRD detection results at all time points. The AML at initial diagnosis from each of these patients was positive for *NPM1* mutation by conventional testing, providing a known molecular marker against which to benchmark performance. We subjected each specimen to smMIP capture and compared the ability of that approach to detect MRD with the results of conventional diagnostics that had been applied during the course of patient care (Table 1, Figure 3, *Online Supplementary Table S4*).

Table 2. smMIP panel design.

Gene	Capture Design	Nucleotides sequenced (bp)	Number smMIPs
ABL1	Hotspot	817	8
ALK	Hotspot	333	3
BRAF	Hotspot	222	2
CEBPA	Full Gene	203	2
DNMT3A	Hotspot	111	1
EZH2	Full Gene	3117	32
FAM5C	Full Gene	2583	28
FLT3	Hotspot	873	8
HNRNPK	Full Gene	2281	24
IDH1	Hotspot	111	1
IDH2	Full Gene	205	2
JAK2	Hotspot	120	1
KIT	Hotspot	1041	10
KRAS	Hotspot	333	3
NPM1	Hotspot	89	1
NRAS	Hotspot	222	2
NT5C2	Hotspot	444	4
PHF6	Full Gene	927	9
PIK3CA	Hotspot	333	3
PPM1D	Full Gene	1971	22
PTPN11	Full Gene	2418	24
RAD21	Full Gene	2410	25
RET	Hotspot	111	1
ROS1	Hotspot	333	3
RUNX1	Full Gene	1894	19
SMC1A	Full Gene	5060	53
SMC3	Full Gene	5147	52
STAG2	Full Gene	5163	53
TET2	Full Gene	6326	66
TP53	Full Gene	2069	22
U2AF1	Hotspot	222	2
WT1	Full Gene	1688	16
Cell line variants	Hotspot	999	9
Total	**N/A**	**50176**	**511**

N/A: not applicable; smMIPs: single molecule molecular inversion probes.

In 1 patient (patient 4), no low prevalence somatic mutations were identified in any specimen, consistent with the clinical diagnosis of the patient being free of detectable MRD. By contrast, and remarkably, for the remaining 3 patients multiple somatic mutations identifying the presence of MRD were observed at every time point interrogated, regardless of clinical diagnostic status. Between 4 and 7 variants were identified in these cases, representing mutations in coding, intronic, and UTR regions, to a minimum variant allele frequency of 0.02% (*Online Supplementary Table S4*). Despite their low prevalence, all reported variants occurred at levels significantly higher ($P<0.005$) than predicted under our empiric error models, and can therefore be ascribed to a biological, rather than artefactual, source.

In order to provide orthologous validation of MRD in specimens where abnormal cells were not identified by standard clinical diagnostics, we performed ultrasensitive detection of *NPM1* mutations using a previously described, targeted next-generation sequencing assay[19] (Figure 3, *Online Supplementary Table S4*). Although sensitivity of the NPM1 assay is validated only to a variant allele frequency of 0.03%, quantitation of *NPM1*-mutated cells by this independent assay closely mirrored the results obtained using smMIP capture, although at several time points smMIP capture identified the presence of MRD at levels occurring below the limits of detection of the targeted NPM1 assay. These results provide support for the clinical validity and quantitative measurement of low prevalence mutations detected by smMIP capture.

MRD heterogeneity in patient samples

AML is considered an oligoclonal disease, marked by the emergence of sublineages which evolve over time and which may exhibit different functional properties from one another,[36-38] and we therefore assessed our ability to identify genetically distinct subclones in our patient cohort. In 2 patient specimens we observed dynamic changes in the mutations seen over time, which is consistent with the recovery of discrete sublineages (Figure 3, *Online Supplementary Table S4*). In patient 2, we detected 2 low frequency variants in *TP53*, which initially emerged on day 579 following initial diagnosis and persisted through the next and final timepoint on day 746. However, these 2 mutations did not increase in abundance at the time that AML relapse was clinically diagnosed; by contrast, the 5 mutations which were originally identified in the patient's neoplasm markedly increased in prevalence. These finding suggest both that a discrete subclonal lineage of *TP53*-mutated hematogenic stem and progenitor cells[39] evolved in the patient over time, and that cells most closely related to the patient's original neoplasm, rather than this subclone, were responsible for the patient's relapse.

In patient 3, a different pattern was seen. One distinction is that the variant allele frequency of somatic muta-

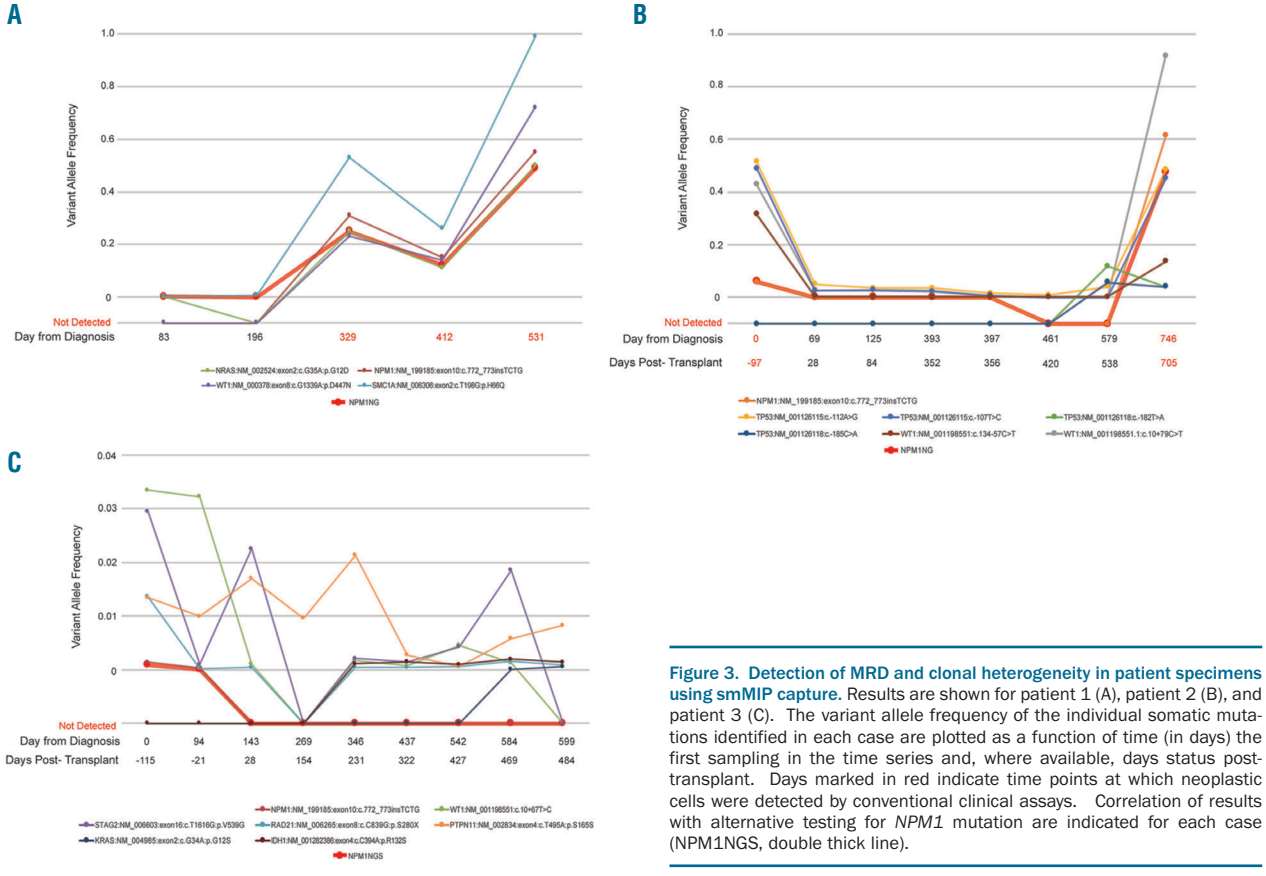

Figure 3. Detection of MRD and clonal heterogeneity in patient specimens using smMIP capture. Results are shown for patient 1 (A), patient 2 (B), and patient 3 (C). The variant allele frequency of the individual somatic mutations identified in each case are plotted as a function of time (in days) the first sampling in the time series and, where available, days status post-transplant. Days marked in red indicate time points at which neoplastic cells were detected by conventional clinical assays. Correlation of results with alternative testing for *NPM1* mutation are indicated for each case (NPM1NGS, double thick line).

tions identified in this background was considerably lower than for the other 2 patients, potentially reflecting that this patient did not undergo relapse during the period of study. Additionally, the relative abundance of individual mutations did not correlate in any obvious pattern, and instead fluctuated independently. These observations are consistent with the presence of multiple subclones carrying distinct identifying mutations, without any single lineage coming to dominate the environment.

Discussion

For next-generation sequencing applications intended to detect low prevalence variation in heterogeneous samples, inherent error rates of sequencing platforms rapidly become limiting by conventional workflows; even with the highest fidelity chemistries, single nucleotide variants occurring below ~2% relative abundance cannot be reliably distinguished from artefacts.[31] Various strategies for circumventing this problem have been demonstrated,[30,40-44] each with their own comparative advantages and disadvantages. smMIP technology uniquely couples scalable target enrichment with sequence read error correction, providing an integrated approach which is both facile and quantitative.[28] Moreover, due to the modular nature of smMIP assays, additional targets can be incorporated into an existing panel without necessitating assay redesign or resynthesis of existing probes, allowing for ready expansion.

In order to detect low abundance mutations which identify AML MRD, we have designed a smMIP panel targeted against coding genes and mutational hotspots relevant to AML pathogenesis, and have developed optimized protocols and analytic techniques to maximize sensitivity. Using cell line dilutions, we demonstrated that smMIP capture is able to interrogate relevant SNPs and indels occurring in AML with an average sensitivity of at least 1:1,500 mutant alleles. This sensitivity for low prevalence variation both exceeds that of conventional deep sequencing by 3 orders of magnitude,[27] and surpasses the limits of detection previously achieved using smMIP technology by 1 order of magnitude.[28,29] Nevertheless, the UMID counts obtained per smMIP enable theoretical limits of detection exceeding even these figures, in the order of 1 in 9,000 mutant alleles for a probe with average performance and up to 1 in 61,000 for the best performers. It is noteworthy that the levels of sensitivity achieved using smMIP capture approach those achievable by error corrected deep sequencing of single mutational targets in AML,[19,25] but the technology is distinguished from those methods in that ultrasensitive performance is obtained over a far greater breadth of coverage; in this application, greater than 50 kb of genomic sequence were interrogated. However, the sensitivity of smMIP capture scales linearly with reaction size; given sufficient quantities of DNA template and allocated sequencing coverage, the approach should be able to achieve detection of low prevalence mutations exceeding the limits practically demonstrated in the study herein.

A measurable amount of low prevalence genetic variation identified in our test materials corresponded to artefacts resulting from DNA deamination and oxidative damage.[25,28,34] In practice, these artefacts can be identified and distinguished from true mutations by capturing both strands of DNA independently and evaluating concur-

Table 3. Single nucleotide variant error rates before and after single molecule molecular inversion probe (smMIP)-mediated error correction.

	Conventional sequencing with 2% variant allele frequency calling		Conventional sequencing with ultrasensitive variant		smMIP-mediated ultrasensitive variant calling calling	
Error Type	Average errors per base	Standard deviation	Average errors per base	Standard deviation	Average errors per base	Standard deviation
A>N	$8.334x10^{-3}$	$2.401x10^{-4}$	1.182	$4.791x10^{-2}$	$1.303x10^{-3}$	$2.058x10^{-4}$
C>N	$3.537x10^{-2}$	$2.199x10^{-3}$	1.371	$1.150x10^{-2}$	$5.205x10^{-2}$	$2.712x10^{-2}$
G>N	$3.005x10^{-2}$	$2.853x10^{-3}$	1.419	$9.317x10^{-3}$	$3.540x10^{-2}$	$1.810x10^{-2}$
T>N	$1.452x10^{-2}$	$2.575x10^{-3}$	1.218	$5.325x10^{-2}$	$8.941x10^{-4}$	$1.073x10^{-4}$
A>C	$2.847x10^{-3}$	$3.430x10^{-5}$	0.281	$3.471x10^{-2}$	$3.087x10^{-4}$	$3.430x10^{-5}$
A>G	$2.881x10^{-3}$	$6.860x10^{-5}$	0.450	$7.683x10^{-3}$	$6.517x10^{-4}$	$1.715x10^{-4}$
A>T	$2.607x10^{-3}$	$1.372x10^{-4}$	0.451	$5.522x10^{-3}$	$3.430x10^{-4}$	$6.860x10^{-5}$
C>A	$2.708x10^{-2}$	$2.703x10^{-3}$	0.489	$5.452x10^{-3}$	$3.775x10^{-2}$	$2.190x10^{-2}$
C>G	$5.498x10^{-3}$	$4.582x10^{-4}$	0.447	$1.026x10^{-2}$	$3.665x10^{-4}$	$9.163x10^{-5}$
C>T	$2.795x10^{-3}$	$4.582x10^{-5}$	0.435	$6.689x10^{-3}$	$1.393x10^{-2}$	$5.315x10^{-3}$
G>A	$5.751x10^{-3}$	$4.012x10^{-4}$	0.461	$9.807x10^{-4}$	$1.092x10^{-2}$	$4.413x10^{-3}$
G>C	$9.451x10^{-3}$	$1.783x10^{-4}$	0.474	$2.675x10^{-3}$	$7.133x10^{-4}$	$4.458x10^{-4}$
G>T	$1.484x10^{-2}$	$2.274x10^{-3}$	0.484	$5.662x10^{-3}$	$2.376x10^{-2}$	$1.413x10^{-2}$
T>A	$8.869x10^{-3}$	$2.790x10^{-3}$	0.475	$8.583x10^{-4}$	$1.073x10^{-4}$	$3.576x10^{-5}$
T>C	$2.503x10^{-3}$	0	0.444	$7.832x10^{-3}$	$5.722x10^{-4}$	$1.431x10^{-4}$
T>G	$3.147x10^{-3}$	$2.146x10^{-4}$	0.299	$4.456x10^{-2}$	$2.146x10^{-4}$	$7.153x10^{-5}$
Total average errors per base	**$2.067x10^{-2}$**	**$3.255x10^{-4}$**	**1.285**	**$2.888x10^{-2}$**	**$1.966x10^{-2}$**	**$9.815x10^{-3}$**
Excluding C>A, C>T, G>A, G>T	**$9.677x10^{-3}$**	**$8.385x10^{-4}$**	**0.877**	**$3.01x10^{-2}$**	**$8.582x10^{-4}$**	**$1.480x10^{-4}$**

rence between strands at sites of variation.[29] However, this measure would require twice as many smMIPs and subsequent sequence coverage, with a negative practical impact on sensitivity. We have found that the incidence of these artefacts is non-limiting in our application, and have therefore elected for a minimally redundant capture design in order to maximize the detection of low prevalence alleles.

In comparison with existing, standard methods of clinical MRD detection, smMIP capture was able to identify mutations indicative of MRD with greater sensitivity and earlier in the course of patient care, up to 677 days before abnormal cells were detectable using standard of care diagnostics. In 3 of 4 patients with longitudinal specimens available, smMIP capture identified mutations consistent with MRD at all time points. Although the finding of low level neoplastic cells during remission is somewhat surprising, it is compatible with prior work by our group using ultrasensitive MRD detection methods targeting the *NPM1* locus in AML patients,[19] and also with the results of other groups using ultrasensitive sequencing approaches.[38] It has been argued that persistent, low level MRD indicates successful immune surveillance and suppression of abnormal cells rather than necessarily predicting the early stages of relapse.[11] This view is supported in our current study by the long periods of time over which background levels of MRD were identified without a marked increase in the size of the abnormal cell population. As such, a more informative biomarker for AML relapse may prove to be the growth kinetics of low prevalence abnormal cell populations,[19] a strategy which would be facilitated by the quantitative nature of smMIP capture.

smMIP capture has proven robust and has a workflow which is compatible with clinical implementation and timescales (2 days for library preparation and 1 to 2 days for sequencing, dependent on read requirements, and scalable computation time), and is expected to become more rapid with continued improvements to sequencing speed and throughput. However, there are limitations to the approach. One consideration applicable to all ultrasensitive approaches is the amount of sequencing power required to interrogate enough individual molecules to enable the detection of low prevalence mutations. In our studies, approximately 80 to 100 million sequence reads per specimen were needed to recover most unique capture events with a minimal redundancy of 2 more reads each. This currently incurs practical limits to the assay in terms of sequencing costs and also the computational time and resources required to analyze the data. Read requirements and computation time should scale linearly with the size of the capture design, so these limitations could be bypassed in exchange for a decreased breadth of the assay. It should also be noted that the number of reads required to detect a mutation by smMIP capture is inversely proportional to its variant allele frequency; although an average of 80 million reads were needed to identify mutations at a 0.0625% variant allele frequency, an average of only 6 million reads could reliably detect minor alleles at 1% relative abundance (*Online Supplementary Figure S2*). Detection of mutations occurring above the limits of detection by smMIP capture can consequently be achieved with more restricted read requirements. Separately, given the limited number of nucleotides which can be efficiently captured by an individual smMIP (~160 bp), it is also an inherent limitation of this approach that large-scale indels and more complex rearrangements, such as sizable internal tandem duplications, inversions, and translocations, cannot be recovered by the technology. Additionally, some regions of the genome are less amenable to smMIP capture due to relative guanine-cytosine (GC) content and other factors,[32] a bias which can be improved but not entirely corrected by empiric rebalancing, resulting in uneven performance for a minority of probes. Lastly, although our capture design is relatively large and is directed against high-yield targets, it is not comprehensive and it is likely that mutations identifying MRD will not be determined in all cases of AML. Based on published AML exome sequence data,[15] our capture panel is predicted to identify cancer-associated mutations in ~80% of AML specimens.

Despite these limitations, we have shown in this pilot study that smMIP capture is in principle well suited to the practical application of monitoring MRD in AML patients. Aside from providing ultrasensitive and quantitative detection of mutations, smMIPs can simultaneously interrogate a large number of high-yield mutational targets for MRD-identifying mutations. This combination of features enables screening for MRD without developing panels specific to individual patients,[24-26] and provides functional redundancy and added robustness, both in being able to identify multiple mutations which may serve as markers of disease, and in being able to monitor the emergence of genetically distinct AML subclones which may have different functional properties than the initial malignancy.[36,37] The ability to detect MRD-associated mutations with levels of sensitivity far exceeding those achievable by current approaches will require new criteria for assessing the significance of positive findings. Future work will incorporate larger numbers of patients and a greater variety of AML subtypes in order to correlate clinical outcomes with sequencing results, and to begin addressing this outstanding question.

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