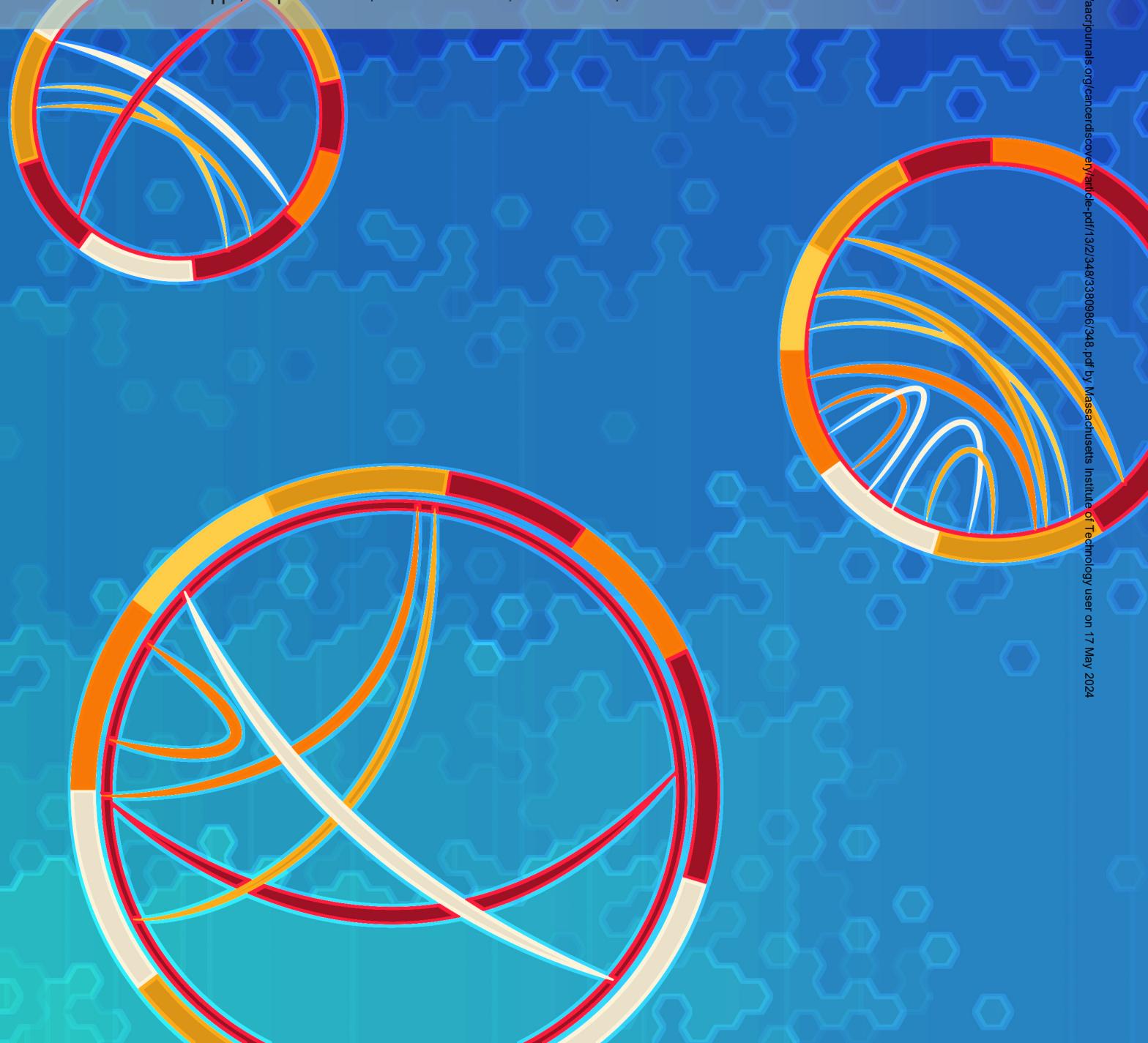


RESEARCH ARTICLE

MinimuMM-seq: Genome Sequencing of Circulating Tumor Cells for Minimally Invasive Molecular Characterization of Multiple Myeloma Pathology



Ankit K. Dutta^{1,2,3}, Jean-Baptiste Alberge^{1,2,3}, Elizabeth D. Lightbody^{1,2,3}, Cody J. Boehner^{1,2,3}, Andrew Dunford³, Romanos Sklavenitis-Pistofidis^{1,2,3}, Tarek H. Mouhieddine^{1,2,3}, Annie N. Cowan^{1,2}, Nang Kham Su^{1,2,3}, Erica M. Horowitz^{1,2}, Hadley Barr^{1,2}, Laura Hevenor^{1,2}, Jenna B. Beckwith^{1,2}, Jacqueline Perry^{1,2}, Amanda Cao^{1,2}, Ziao Lin³, Frank K. Kuhr⁴, Richard G. Del Mastro⁴, Omar Nadeem^{1,2}, Patricia T. Greipp⁵, Chip Stewart³, Daniel Auclair⁶, Gad Getz^{3,7}, and Irene M. Ghobrial^{1,2,3}



ABSTRACT

Multiple myeloma (MM) develops from well-defined precursor stages; however, invasive bone marrow (BM) biopsy limits screening and monitoring strategies for patients. We enumerated circulating tumor cells (CTC) from 261 patients (84 monoclonal gammopathy of undetermined significance, 155 smoldering multiple myeloma, and 22 MM), with neoplastic cells detected in 84%. We developed a novel approach, MinimuMM-seq, which enables the detection of translocations and copy-number abnormalities through whole-genome sequencing of highly pure CTCs. Application to CTCs in a cohort of 51 patients, 24 with paired BM, was able to detect 100% of clinically reported BM biopsy events and could replace molecular cytogenetics for diagnostic yield and risk classification. Longitudinal sampling of CTCs in 8 patients revealed major clones could be tracked in the blood, with clonal evolution and shifting dynamics of subclones over time. Our findings provide proof of concept that CTC detection and genomic profiling could be used clinically for monitoring and managing disease in MM.

SIGNIFICANCE: In this study, we established an approach enabling the enumeration and sequencing of CTCs to replace standard molecular cytogenetics. CTCs harbored the same pathognomonic MM abnormalities as BM plasma cells. Longitudinal sampling of serial CTCs was able to track clonal dynamics over time and detect the emergence of high-risk genetic subclones.

INTRODUCTION

Multiple myeloma (MM) is a largely incurable hematologic malignancy characterized by the aberrant proliferation of clonal plasma cells (PC) within the bone marrow (BM), which develops from the asymptomatic disease stages known as monoclonal gammopathy of undetermined significance (MGUS) and smoldering multiple myeloma (SMM). The initial diagnosis of MGUS or SMM remains an incidental process depending on the identification of increased monoclonal protein in the blood, yet BM biopsy is the gold standard for diagnosis and monitoring of progression to MM. BM biopsy is an intrusive and painful procedure for patients having an asymptomatic condition and does not allow for multiple samples to be acquired frequently over the course of disease development to monitor tumor dynamics. Moreover, next-generation sequencing (NGS) studies of MM have established a complex clonal architecture with the existence of genetic heterogeneity (1–7) and spatial heterogeneity (8, 9)

characterizing disease. Thus, localized BM biopsy alone is not able to represent the full pathology of disease. This presents a need for improvement in robust early detection methods that are tumor biology based, are able to capture the full temporal and spatial nature of disease and, ideally, are minimally invasive for patients.

Circulating tumor cells (CTC) are malignant cells that extravasate from the primary BM site to the peripheral blood (PB) and therefore are amenable to enrichment, enumeration, and molecular characterization of genetic biomarkers. They present an opportunity to assess the representation of BM tumor biology and are an emerging area of research as a marker of MM disease. As blood draws are readily possible and repeatable, there are many potential advantages for clinical use in settings of early detection, real-time monitoring, and assessment of treatment response and clonal evolution. Early investigations in the field have demonstrated the promise of liquid biopsy in all stages throughout MM, with enumeration studies highlighting the prognostic value of CTCs (10–15). Due to the rare nature of CTCs, low cell numbers can be recovered using enrichment strategies for downstream genomic profiling. In most cases, whole-genome amplification (WGA) is required to increase DNA template for input into library construction protocols. WGA has inherent issues such as allelic distortion/dropout and nonuniformity of coverage that can affect reliable mutation calling across the genome, thus limiting analyses of these samples to targeted approaches and considerations of multiple replicates and census-based mutation calling. Notwithstanding, targeted NGS studies using whole-exome or custom panels have demonstrated the ability of CTCs to successfully capture clonal driver mutations and copy-number variants of MM, similar to matched BM sample analyses (16–19), but fail to capture translocations. These findings suggest that not only enumeration but quantitative disease monitoring of patients may be possible at regular intervals. However, the reliability of genome-wide methods that can capture chromosomal

¹Center for Prevention of Progression of Blood Cancers, Dana-Farber Cancer Institute, Boston, Massachusetts. ²Department of Medical Oncology, Harvard Medical School, Boston, Massachusetts. ³Cancer Program, Broad Institute of MIT and Harvard, Cambridge, Massachusetts. ⁴Menarini Silicon Biosystems, Huntingdon Valley, Pennsylvania. ⁵Department of Laboratory Medicine and Pathology, Mayo Clinic Comprehensive Cancer Center, Rochester, Minnesota. ⁶Multiple Myeloma Research Foundation, Norwalk, Connecticut. ⁷Cancer Center and Department of Pathology, Massachusetts General Hospital, Harvard Medical School, Boston, Massachusetts.

Note: A.K. Dutta and J.-B. Alberge contributed equally to this work.

Corresponding Authors: Irene M. Ghobrial, Dana-Farber Cancer Institute, 450 Brookline Avenue, Boston, MA 02215-5450. Phone: 617-632-4198; Fax: 617-582-8608; E-mail: irene.ghobrial@dfci.harvard.edu; and Gad Getz, Broad Institute, 75 Ames Street, Cambridge, MA 02142. Phone: 617-714-7471; E-mail: gadgetz@broadinstitute.org

Cancer Discov 2023;13:348–63

doi: 10.1158/2159-8290.CD-22-0482

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translocations together with copy-number variants and mutations in a single unbiased assay has yet to be shown, and previous studies have been largely limited by low tumor purity and high DNA template input requirements.

Here, we describe a novel technique, minimally invasive multiple myeloma sequencing (MinimuMM-seq), which leverages advancements in tumor cell enrichment strategies, low input library construction, and tailored cancer genomics analyses to enable whole-genome sequencing (WGS) of CTCs for systematic genomic profiling of pathognomonic MM events. First, we establish the concordance of MinimuMM-seq results in paired BM and CTC samples from 24 patients. Subsequently, the clinical utility and performance are demonstrated in a cohort of PB-only CTC samples from 27 patients. We show that longitudinal serial sampling is able to unravel the clonal architecture and dynamics of CTC expansion and extinction over time for 8 patients. Finally, our method enables WGS-based risk classification of patients. This study provides proof of principle for a minimally invasive liquid biopsy-based screening method for the early detection and monitoring of MM.

RESULTS

Detection and Enumeration of CTCs Correlate with Precursor Disease Pathology

To evaluate the frequency of CTCs in precursor disease stages, PB was collected from 239 untreated patients across asymptomatic stages of MM, including 84 MGUS and 155 SMM, for the enrichment and capture of CTCs using the CellSearch platform with the Circulating Multiple Myeloma Cell Assay (Menarini Silicon Biosystems), which requires 4 mL of blood per test (Fig. 1A). The majority of precursor patients showed evidence of CTCs, with one or more CTCs detected in 82% of enrolled patients, with 75% of MGUS (63 patients) and 86% of SMM (134 patients) having successful enumeration (Fig. 1B). An increase in the number of CTCs was observed between MGUS and SMM disease stages, with a median count of 3 (range, 0–1,328) and 23 (range, 0–43,836), respectively ($P < 0.0001$; Fig. 1C). Peripheral blood from a cohort of newly diagnosed MM patients was also collected ($n = 22$), with a median enumeration of 324 (range, 1–32,071) at the overt disease stage (Supplementary Fig. S1A).

We next assessed whether CTC counts correlated with clinical parameters that are monitored for signs of progression or risk stratification in SMM to determine whether an association with current staging or disease risk is evident. A moderate positive correlation was observed between the number of CTCs enumerated from patients and their bone marrow PC (BMPC) percentage [$r = 0.47$; 95% confidence interval (CI): 0.29–0.61; $P < 0.0001$; Supplementary Fig. S1B], and to a lesser extent with the M-spike concentration ($r = 0.32$; 95% CI: 0.11–0.50; $P = 0.003$; Supplementary Fig. S1C). A proportion of patients with SMM in the cohort had clinical BM biopsy performed ($n = 94$) with results available for the assessment of risk classification using the International Myeloma Working Group 2/20/20 model (20). Enumeration of CTCs correlated with this model, in which a higher CTC count was associated with an increased risk group based on the three-risk factor model, with a median of 5, 24, and 170 CTCs detected in low-, intermediate-,

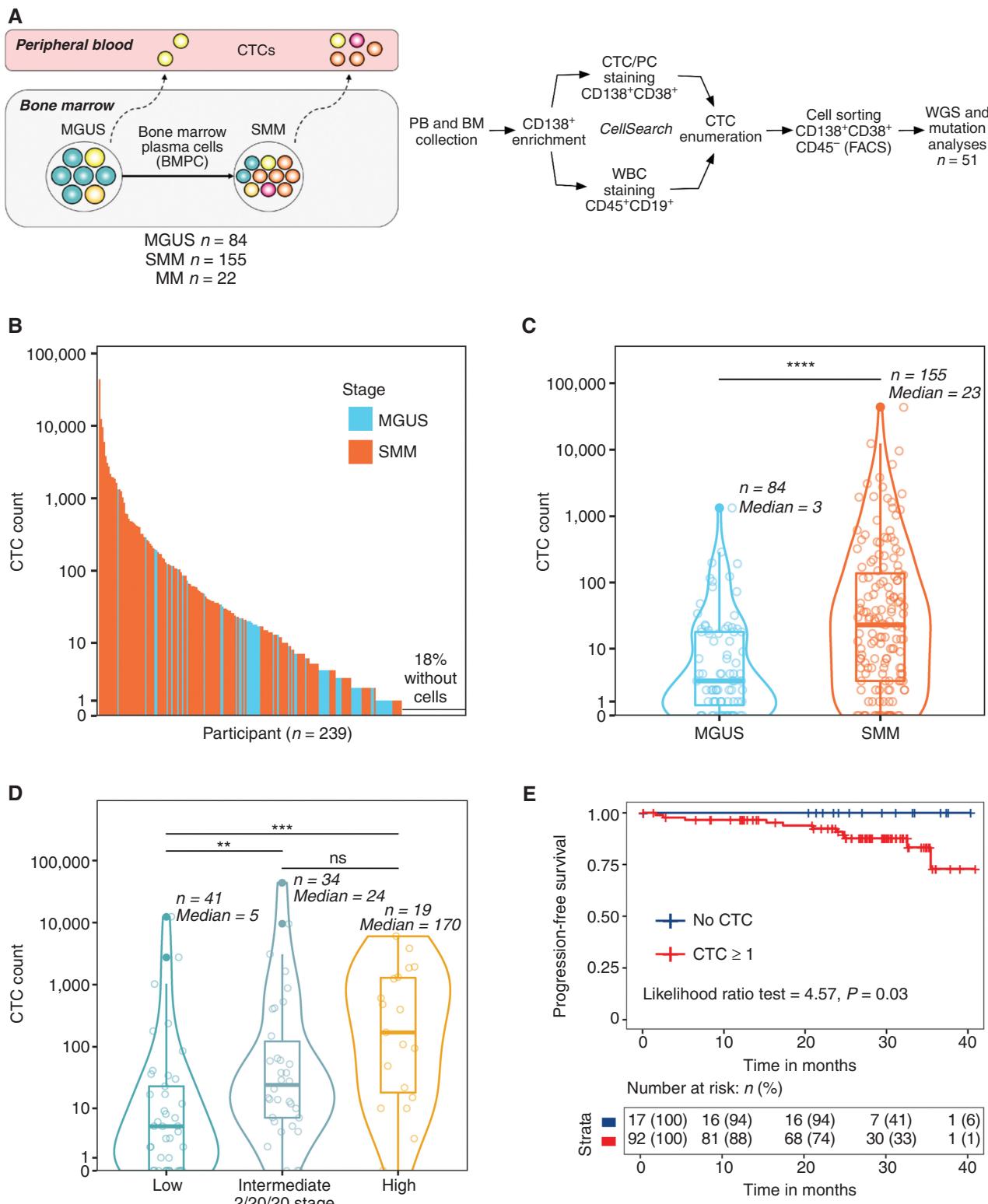
and high-risk SMM groups, respectively (Fig. 1D). We note that the median CTC count in low-risk SMM was similar to that found in MGUS, fittingly reflective of early disease categorization. CTC counts were found to be significantly increased at intermediate-risk ($P < 0.01$) and high-risk ($P < 0.01$) stages compared with the low-risk group. Survival analysis using the Kaplan–Meier method showed that SMM patients with detected CTCs have higher chances of progression (CTC count $\geq 1/4$ mL of blood, likelihood ratio test statistic 4.57, $P = 0.03$; Fig. 1E) over a median follow-up time of 27 months [interquartile range (IQR), 21–32 months]. The association remained true when comparing quartiles of CTC counts ($P = 0.04$; Supplementary Fig. S1D), and more specifically the lowest, Q1 (0–2 CTC/4 mL of blood), against Q2, Q3, and Q4 ($P = 0.005$; Supplementary Fig. S1E). Taken together, these data illustrate that as the tumor burden of disease increases, there is an increase in the release and trafficking of tumor cells from the BM to the PB, which may be predictive of disease staging and monitored by enumeration of CTCs.

MinimuMM-seq Reveals that CTCs Reflect the Major BM Clone and Could Replace Molecular Cytogenetics

Enumeration of CTCs provides a correlative measure of disease burden; however, molecular characterization can confirm tumor biology and MM-associated genetic alterations. We first characterized enriched CTCs that were isolated in our workflow to confirm they were malignant cells of good quality and morphology through imaging and ultralow pass (ULP) WGS and ichorCNA. CTCs were found to be intact and to harbor arm-level somatic copy-number abnormalities, in concordance with matched BM results (Supplementary Fig. S2A and S2B; Supplementary Materials and Methods). We next set to establish the minimum number of CTCs that could be recovered and sequenced, without loss of sensitivity for the detection of initiating events of MM, when the ground truth of BM fluorescence *in situ* hybridization (FISH) was known. Our quantitative evaluation revealed the lowest limit of detection when 50 CTCs were recovered (Supplementary Fig. S2C; Supplementary Materials and Methods).

With this foundation, we then investigated the clinical utility of genomic profiling of CTCs using standard WGS without WGA as an unbiased method to detect abnormalities present in CTCs. The clinical characteristics of all participants are highlighted in Supplementary Table S1, and sequencing metrics are summarized in Supplementary Table S2. Overall, a median of 394 CTCs (range, 41–75,000) and 11,700 BMPCs (range, 124–49,000 for matched sample patients) were isolated and sequenced (Fig. 2A) with paired germline samples as a reference normal comparison. Our enrichment approach yielded a median tumor purity of 99% (range, 48%–100%) and 98% (range, 45%–100%) for both BMPCs and CTCs, respectively, with quantification by ABSOLUTE (ref. 21; Fig. 2B).

To first illustrate that CTCs could deliver equivalent genomic yield compared with clinical BM testing, we collected matched PB and BM research samples at the same time point of clinic visit from 24 patients (2 MGUS, 15 SMM, and 7 MM). All patients with a BM biopsy had routine molecular cytogenetics performed at clinical pathology, and FISH analysis was recorded (Supplementary Materials and Methods).



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Figure 1. Detection and enumeration of CTCs correlate with MM disease pathology. **A**, Overview of the experimental workflow used to enumerate CTCs ($n = 261$) and characterize their genomic abnormalities ($n = 51$). FACS, fluorescence-activated cell sorting; WBC, white blood cell. **B**, Distribution of CTC counts across precursor disease stages of MM. Text indicates the proportion of participants with no CTCs detected. **C**, Box plot and density graphs of CTCs enumerated between disease precursor stages MGUS and SMM. **D**, Box plot and density graphs showing CTC counts associated with 2/20/20 SMM risk classification when available. **E**, Kaplan-Meier curves depicting probability of progression from SMM to overt myeloma or death based on CTC enumeration. ns, not significant; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

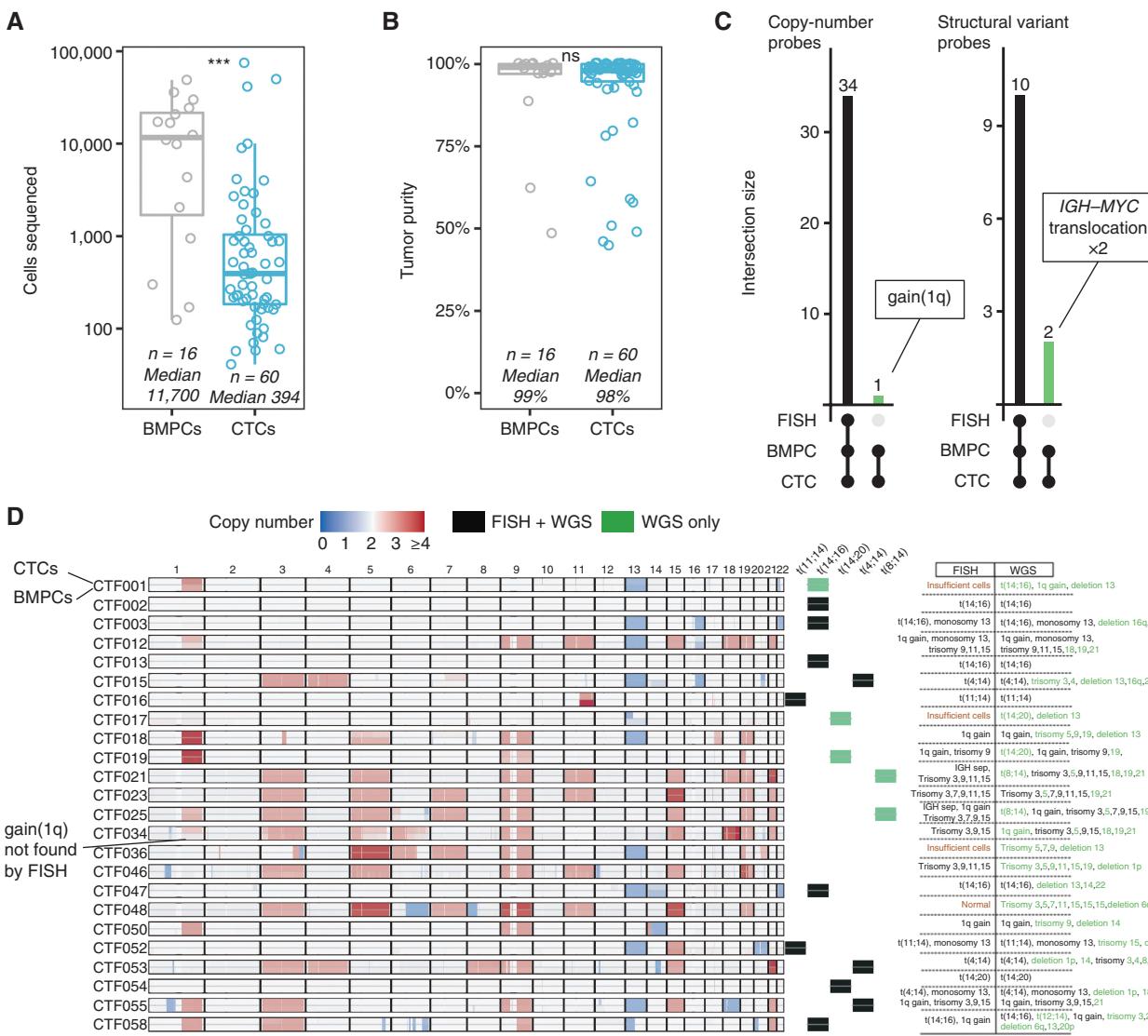


Figure 2. MinimuMM-seq reveals CTCs reflect the major BM clone and could replace molecular cytogenetics. **A**, Box plot of the number of cells sequenced for BMPCs (gray, left) versus CTCs (right, light blue) from this study. In 8 cases, selected CD138⁺ BMPC bulk genomic DNA was used. **B**, Tumor purity in BMPCs and CTCs assessed by the ABSOLUTE algorithm and based on copy-number abnormalities, and single-nucleotide variant multiplicity and fraction of alternate reads. **C**, Categorical classification of successful FISH probes by their presence or absence in the WGS data of BMPCs and CTCs. FISH failures and untested FISH probes are excluded from this graph. **D**, Genome-wide copy-number abnormalities heat map and translocation discovery (right) in participants with matched CTC and BM samples (n = 24), with comparison with clinical FISH report results. Each row is split into a top panel dedicated to CTC profiling and a bottom for BMPCs. Key differences between compartments are highlighted by an arrow or green text in the table. FISH failures/within normal limits are represented in red text in the table. ns, nonsignificant; ***, P < 0.001.

Clinical FISH reported that 11 patients (46%) harbored translocations, 9 patients (37%) had trisomies/copy-number changes, and 4 patients (17%) had inconclusive results, with BM biopsies showing PC infiltrations in the range of 5% to 80% (Supplementary Table S3). Head-to-head comparison of WGS with FISH probes that were tested showed sequencing of CTCs was able to detect 100% (N = 44) of abnormalities identified in clinical testing with copy-number and structural variant probes (Fig. 2C). Notably, new clinically reportable high-risk events of *IGH-MYC* [t(8;14)] translocations and 1q gain were distinguished in 3 patients by WGS, with concordance between CTC and BM compartments, but were

not found by FISH (n = 3, over 97 successful probes with negative results).

Overall comparison of large-scale events across all patients showed a high concordance of genetic profile between WGS and FISH. Matched CTCs and BMPCs were confirmed to originate from the same clonal expansion through B-cell receptor repertoire characterization (Supplementary Table S4). Genomic results demonstrate that CTCs are able to faithfully recapitulate all results obtained from ground truth BM, including translocations [t(11;14), t(14;16), t(14;20), t(4;14), and t(8;14)] and copy-number abnormalities (chromosomal trisomies, 1q gain, deletion 13q, and deletion 16q; Fig. 2D).

Moreover, WGS of CTCs was able to identify additional genetic events in 20 patients (83%; Fig. 2D, green text). In particular, 4 patients with SMM (CTF001, CTF017, CTF036, and CTF048) had FISH within normal limits or failure due to insufficient PC during clinical testing; however, analysis of CTCs was able to detect and recover cytogenetic abnormality results. In CTF001, we detected *MAF* translocation t(14;16), 1q gain, and copy-loss events on chromosomes 13 and 22. Interestingly, this patient had a BM biopsy with clinical FISH performed again 1 year after this sampling time point, being successful and reporting t(14;16), deletion 17p, monosomy 13, and 1q gain. In CTF017, a novel *IGH-MAFB* [t(14;20)] translocation and deletion 13q were detected. In CTF036, trisomies and deletion 13q were found, whereas in CTF048 trisomies and deletion 6q and 8p were identified. These results highlight that blood-based screening of CTCs can reliably detect alterations to replace BM, even when routine clinical BM fails, to assist with tumor classification.

MinimuMM-seq Enables Genomic Profiling of CTCs for Unbiased WGS-Based Molecular Analyses

As the characterization of CTCs proved feasible, we next explored the utility of PB sampling and WGS of CTCs as a diagnostic tool for minimally invasive detection of molecular events in patients in the absence of a BM reference. To extend our assessment and demonstrate the applicability of MinimuMM-seq as a clinical tool, we collected a validation cohort of prospective PB-only samples from 27 patients (3 MGUS, 22 SMM, and 2 MM).

We demonstrate comprehensive detection of chromosomal abnormalities, with the ability to detect key translocations and copy-number variants of MM across all patients using CTCs (Fig. 3A). Sixteen patients (59%) harbored translocations, including frequent and infrequent translocations such as t(11;14), t(4;14), t(14;16), t(14;20), t(6;14), and t(8;14). Eleven patients (41%) showed trisomies and copy-number changes including 1q gain, 1p deletion, odd chromosomal trisomies, and deletion 13q. Due to the asymptomatic nature of precursor patients, all were under careful clinical observation and most patients had a BM biopsy and FISH analyses performed at one time point during their ongoing monitoring, which was used as a known reference (Supplementary Table S5). Evaluation of WGS of CTCs in comparison with FISH probes that were tested on BM firmly showed that CTCs were able to detect 100% of abnormalities identified in clinical testing (Fig. 3B). In 6 patients (CTF022, 026, 027, 029, 040, and 044), no clinical FISH data were available (normal or insufficient cells); however, genomic characterization of CTCs revealed the presence of trisomies, t(14;16), rare *IGH-MAFA* translocation [t(8;14)], and deletion 1p and 13q, which are important cytogenetic abnormalities with a predictive value for the clinical understanding of a premalignant patient.

As such, we highlight two key examples of the application in blood sampling of SMM finding high-risk events. Participant CTF031's clinical testing showed t(14;16) and deletion of 8q, 13q, and 17p. Additionally, we found a sensitivity of WGS to layer in mutations with the detection of single-nucleotide variants (SNV) in *TP53*, inducing a likely pathogenic variant p.E285K (22). Notably, this result confers a biallelic double-hit event, which is a high-risk category for patients with MM that

conventional testing would not be able to uncover (Fig. 3C). From clinical reports, participant CTF025 harbored an *IGH* separation, a 1q gain, and trisomies. WGS of CTCs revealed the unknown translocation with chromosome 14 to be *IGH-MYC* [t(8;14)], with additional trisomies and *NRAS* hotspot mutation p.Q61H. Studies have shown *MYC-IGH* translocations as high-risk events compared with non-IgH *MYC* translocations, indicating an increased potential for progression from SMM to MM within 2 years (refs. 23, 24; Fig. 3D). Taken together, these results exemplify the resolution afforded by sequencing-based methods to characterize tumor biology for improved clinical decisions on early intervention strategies.

Longitudinal Liquid Biopsy and Serial WGS of CTCs Reveal Clonal Architecture and Evolutionary History

As PB continuously circulates, liquid biopsy sampling may be affected by the phenomena of spatial and temporal heterogeneity in CTC burden. In patients with matched samples, we investigated whether the major BM clone possessed the potential to extravasate and circulate or whether the preferential circulation of subclones occurred. We showed that large-scale copy-number and IgH translocations are shared between both BM and CTCs (Fig. 2D). We then calculated the sensitivity to detect point mutations in CTCs and BM given purity, ploidy, and sequencing depth of coverage (Supplementary Fig. S3A–S3C). Sensitivity was 99% (IQR, 92%–100%) for clonal events [cancer cell fraction (CCF) = 1], and 75% (IQR, 43%–94%) for mutations occurring $\geq 50\%$ CCF. Two patients with matched BMPCs fell below an average detection power of 80% for clonal events due to their low number of captured CTCs (Supplementary Fig. S3D). Total mutational load across all samples was in the range of 1,000 to 9,000 SNVs and short insertions or deletions (indels), in line with the expected burden from previous genome studies of MM PCs (refs. 2, 3, 25; Supplementary Fig. S3D). At the genome-wide scale, mutations were more likely to be shared between both compartments (Supplementary Fig. S3D), indicating the shared clonal history of BMPCs and CTCs. In participant CTF013, this is exemplified by a high mutation density at the upper diagonal (100% CCF), with t(14;16) and the first *KRAS* hotspot mutant (p.G12S) cluster (Fig. 4A, part i). A second high-density cluster was found with the second *KRAS* hotspot (p.G13D), having a vertical shift from the diagonal suggesting it is preferentially found in the PB compartment. Next, we used Phylogenetic NDT (26) to cluster mutations and copy-number abnormalities into branching subclones and found that *KRAS* p.G12S is indeed predicted to be a shared clonal event (green), whereas *KRAS* p.G13D belongs to a subclonal branch (blue, Fig. 4A, part ii; Supplementary Fig. S3E). This suggests that *KRAS* p.G13D confers an additional fitness advantage to tumor cells and indeed is more common than the p.G12S mutation in the MM disease setting (27).

As clonal complexity is not limited to spatial heterogeneity, but also temporal changes over time, we analyzed serial samples from 8 cases of SMM (CTF004, 013, 017, 019, 024, 027, 031, and 032), of which 7 patients remained under careful clinical observation without treatment (time range, 2–33 months). In all cases, we validated the stability of the major clone, with the clonal evolution and shifting dynamics of

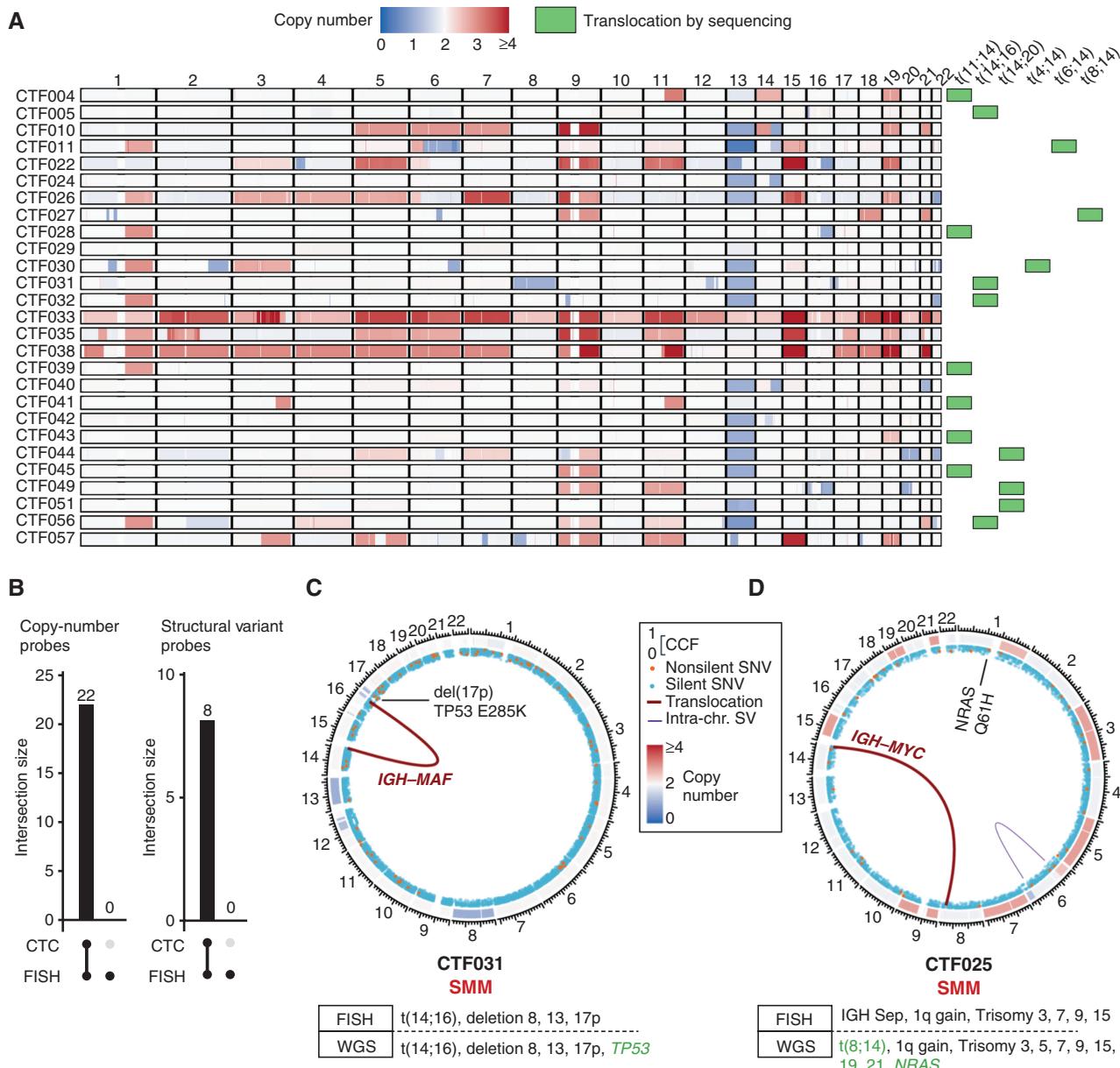


Figure 3. MinimuMM-seq enables the genomic profiling of CTCs for unbiased WGS-based molecular analyses. **A**, Copy-number heat map and translocation matrix from the discovery cohort of CTC-only sequencing ($n = 27$). **B**, For patients with longitudinal follow-up, concordance of previous FISH reports with this study's WGS results. FISH failures and untested FISH probes are excluded from this graph. **C** and **D**, Two examples of clinical relevance depicting mutations detected by MinimuMM-seq on CTCs. Both participants are clinically diagnosed as SMM, and our assay characterizes novel translocation (CTF025: IGH-MYC), gains and losses of chromosomes (CTF025: trisomies), as well as driver point mutations (CTF031: TP53 resulting in biallelic hit; CTF025: NRAS) using CTCs, which may refine patient tumor classification using blood-based sampling only. Intra-chr. SV, intrachromosomal structural variant.

CTC subclones over time (Supplementary Fig. S4A). Expansion of subclones harboring MM driver mutations such as KRAS (both G12 and G13 hotspots) and DIS3 was observed, indicating their potential to confer a selective advantage for clonal fitness and independence for circulation (Supplementary Fig. S4B). In an example case of CTF004, longitudinal sampling over 28 months demonstrated the reliability of our approach for diagnostic sampling based on liquid biopsy, with stability of mutation presence with clonal t(11;14) and gain of 11q validated at both time points (Fig. 4B, part i). Additionally, over time, an emergent subclone with increased

clonal fitness appeared harboring an additional deletion of 13q, growing from a CCF of 8% (blue; CI: 8%–9%) at T0 to CCF of 80% at T1 (CI: 75%–84%), whereas simultaneous extinction of the orange subclone was observed (Fig. 4B, part ii). These data demonstrate the use of CTCs to successfully track both acquisition of mutations and clonal dynamics of MM disease over time in a minimally invasive manner.

Of the 8 patients with serial blood collection, one SMM (CTF032) received early interventional treatment following first blood collection and CTC sequencing. After 4 months of therapy, the patient achieved a partial response with serum

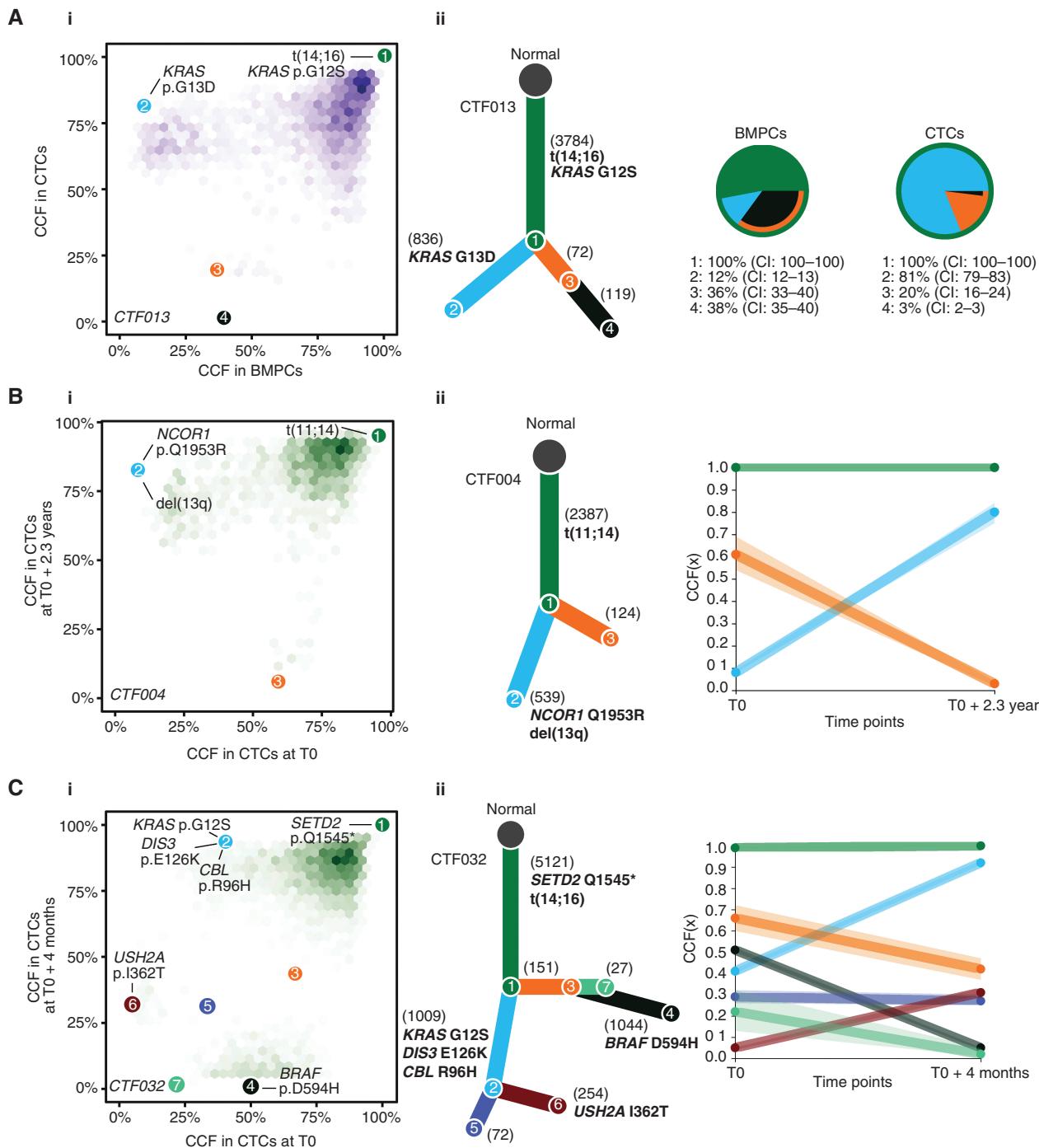


Figure 4. Serial WGS of CTCs reveals disease clonal architecture and evolutionary history. **A, (i)**, Comparison of mutation density occurring between BMPC (x-axis) and PB (CTCs, y-axis) compartments in SMM participant CTF013 estimated by ABSOLUTE. Recurrent mutations in MM are annotated. **(ii)**, Phylogenetic reconstruction of tumor architecture between BMPCs and CTCs of participant CTF013 after mutation clustering with PhylogeNDT shows the preferential circulation of subclones. **B and C, (i)**, Comparison of mutational density from longitudinal sampling of CTCs in SMM. In the example of participant CTF004 (**B**), serial blood was sampled at initial screening (x-axis) and at follow-up 2.3 years later (y-axis). For participant CTF032 (**C**), serial blood was sampled before (x-axis) and 4 months after start of treatment (y-axis). **B and C, (ii)**, Longitudinal reconstruction of CTC clonal evolution for participants CTF004 (**B**) and CTF032 (**C**).

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M-spike concentration decreased by 70% (1.16–0.37 g/dL) and CTC counts decreased by 86% (12,769–1,803); however, serial CTC profiling was able to give a readout of clonal tiding (i.e., switching of clones) with potential drug-related dynamics

evident in real-time, described here as an example. Within this period, an abundance of myeloma cells with *BRAF* p.D594H kinase-dead mutation (28) decreased from 51% (CI: 50%–52%) to 5% (CI: 4%–5%; Fig. 4C, parts i–ii). Simultaneously, a minor

clone (blue) present at baseline with an oncogenic KRAS p.G12S activating hotspot mutation showed a relative CCF increase from 41% (CI: 40%–42%) to 92% (CI: 91%–93%). The patient progressed to overt MM after the end of treatment, whereas PhylogicNDT modeling predicted that clones bearing KRAS p.G12S were selected in combination with other mutants that are part of the dark red branch also bearing USH2A p.I362T, a gene recurrently mutated in POEMS syndrome (29). Selection of the new fittest clone under treatment is revealed by our minimally invasive method before any potential resistance is clinically observed.

Genomic Profiling of CTCs Can Replace Clinical BM FISH for Risk Classification of Patients

In the genomics era, implementation of clinical sequencing to improve the stratification of patients based on genomic biomarkers has high potential, going beyond the use of correlative clinical markers alone in models such as the International Myeloma Working Group 2/20/20 for SMM (20) and International Staging System (ISS) for MM (30). Detection of chromosomal abnormalities has been implemented to extend both systems [which became the four-factor model and the Revised ISS (ref. 31), respectively]. The ability to use genomic profiling of CTCs for this stratification would provide a minimally invasive and repeatable solution to refine patient classification.

Sequencing of CTCs was able to detect all reported high-risk prognostic factors including t(4;14), t(14;16) 1q gain, del(13q), and del(17)p. Risk stratification carried out with WGS of CTCs, using available cytogenetic-based risk models (31, 32), showed matched patients would have been assigned to the correct risk group when BM FISH was successful. Additionally, the recovery of FISH failures (due to insufficient cells for testing) or additional diagnostic yield enabled attribution of clinical risk (Fig. 5A). Moreover, the detection of novel driver mutations (KRAS, NRAS, FAM46C), MYC rearrangements, and signatures (APOBEC activity) by NGS is also shown and could help in predicting which patients are at high risk of transformation. Similarly, in SMM patients in whom only CTCs were sampled, diagnostic yield was obtained from MinimuMM-seq, allowing for the assignment of risk classification (Fig. 5B). In 5 cases of MGUS (CTF026, CTF029, CTF044, CTF048, and CTF054), CTCs could also be used to obtain a genetic result. Usually, clinical BM is not performed at this very early stage due to low disease burden; as such, minimally invasive blood testing may be a rapid solution to overcome limitations of both sampling and patient discomfort for a closer observation of patients with MGUS. We note no cytogenetic risk association models for MGUS are present, but we observed trisomies, t(14;20), 1q gain, and deletion of 13q, potentially detailing high-risk patients. Taken together, these data show that genomic assessment of CTCs provides demonstrable results that match BM sampling and the potential of the power of their clinical application for cytogenetic assessment of patients.

CTC Sequencing Enables Cohort-Level Genomic Study of Mutational Signatures and Complex Structural Events

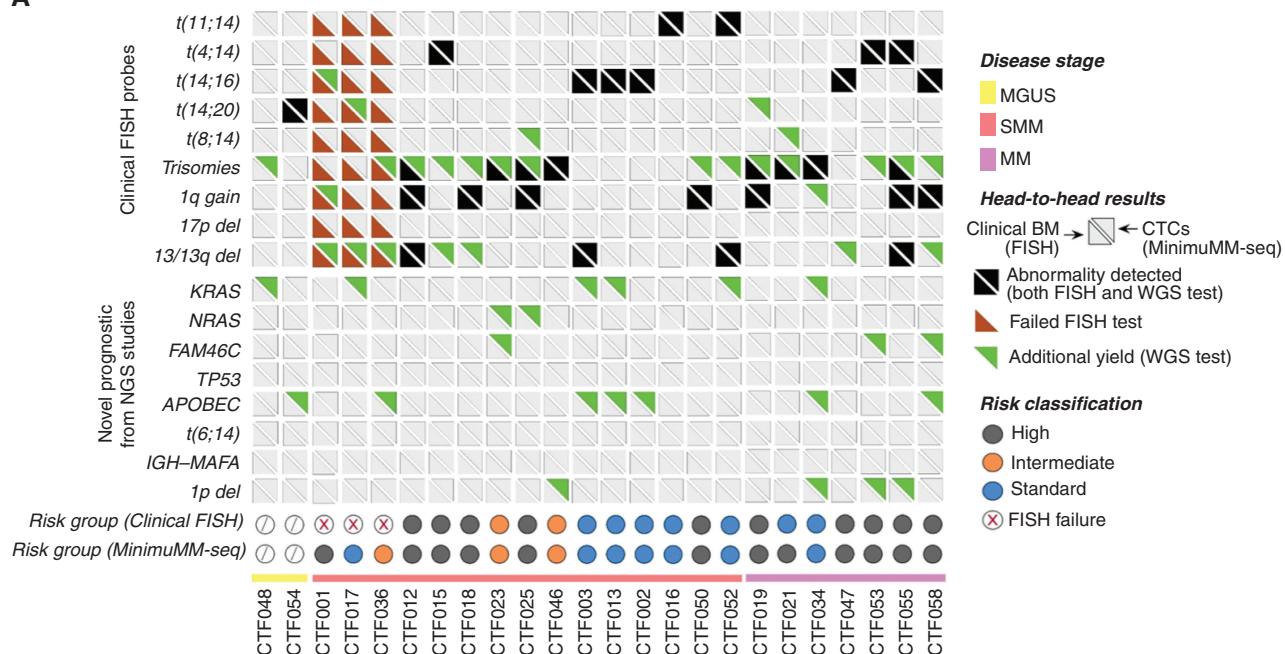
As WGS is an NGS technology that can sequence the entire genome, we reasoned that its application in CTCs may also

provide more comprehensive mutation data across all scales without prior knowledge. Mutation and structural variant counts are summarized in Supplementary Tables S6 and S7. Mutations in the RAS-MAPK pathway are the most prevalent and significantly mutated drivers in MM, with KRAS and NRAS mutated in 21% and 19.5% of patients with MM (1, 5, 6), respectively. We found clonal and subclonal MAPK mutations of KRAS and NRAS in 12 patients out of 51 (24%), including hotspot mutations of G12, G13, and Q61 (Supplementary Fig. S4C). We also found mutations in 6 driver genes of MM (BRAF, DIS3, FAM46C, KRAS, NRAS, and TP53) in 17 patients (33%; Supplementary Fig. S4D), with concordance between CTCs and matched BM. All mutations found in CTCs in driver genes were validated in the BM compartment by specifically looking for sequencing reads that support the mutant allele (Supplementary Fig. S4D and S4E).

In addition to the detection of somatic mutations, mutational processes and their distinctive signatures are important features in MM. Previous studies have revealed the contribution of AID, APOBEC, and aging signatures to the mutational spectrum of MM (2, 33, 34). Recent studies highlighted that they may have clinical importance in SMM (35, 36), with APOBEC shown to be enriched in those patients who progress or have a shorter time to progression. Mutation spectra and signature activity analysis reveals a comparable overlap of mutation types found between BM and CTCs (median cosine similarity 98%; IQR, 94%–99%; Supplementary Fig. S5A). Signatures extracted in our dataset comprised APOBEC, clock-like, and SBS9 as annotated by SignatureAnalyzer (Fig. 6A), with the comparable contribution of mutational processes to the CTC and BM compartments (Supplementary Fig. S5B). In line with the literature, we show all participants with APOBEC contribution of more than 25% to the mutation spectrum were of MAF subtype ($n = 7/51$, either IGH-MAF, MAFA, or MAFB). Finally, because WGS is not biased toward coding regions of the genome, we could decipher more complex structural events such as chromothripsis of chromosome 3 in CTF033 (Fig. 6B and C) and chromoplexy of chromosomes 7, 8, and 18 involving the MYC locus in matched BM and PB of CTF034 (Fig. 6D and E). Overall, we showed that additional novel information not found by routine clinical tests can be gained from CTCs that could be layered into patient assessment.

DISCUSSION

Liquid biopsy studies in MM have demonstrated the clinical potential of CTC assessment (37, 38) similar to that of CTCs in solid tumors. However, these early investigations focused on the predictive value of enumeration in disease staging and risk of progression or targeted genomics (16–19). As such, the full potential of genomic profiling of CTCs has yet to be realized, with no study implementing unbiased genome-wide methods that could capture any mutation type without prior knowledge. Here, we show proof of principle that both enumeration and molecular analyses of CTCs using WGS have clinical utility in MM. We demonstrate MinimuMM-seq for comprehensive genomic characterization of CTCs, which robustly matches data derived from BM samples, could be used as a surrogate

A

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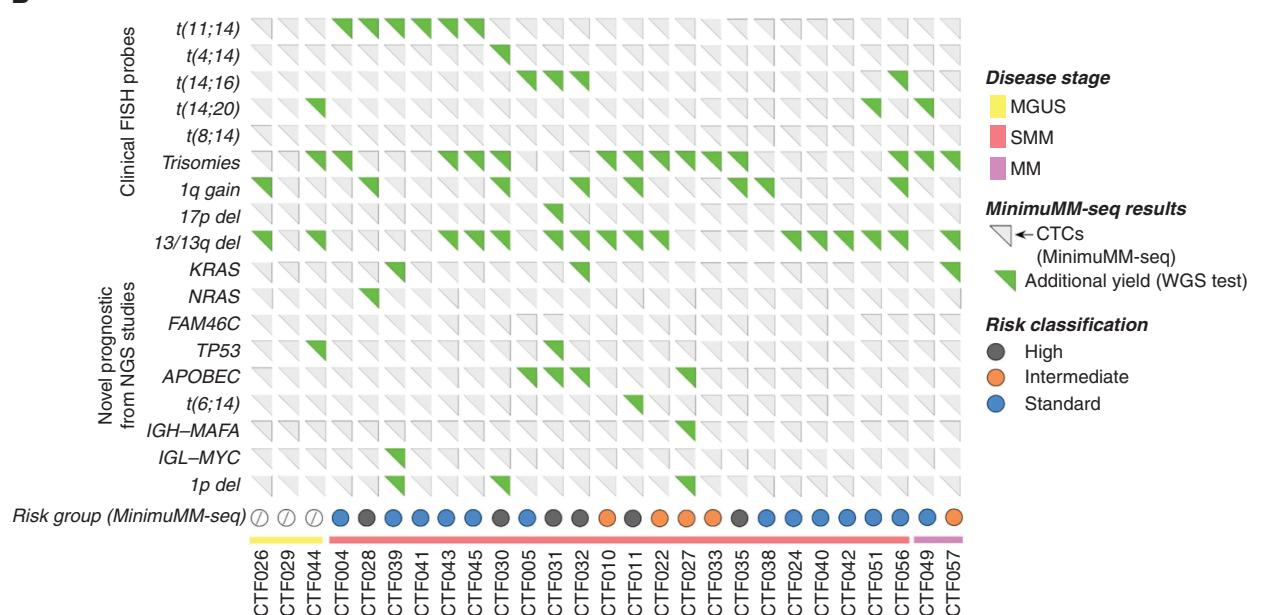
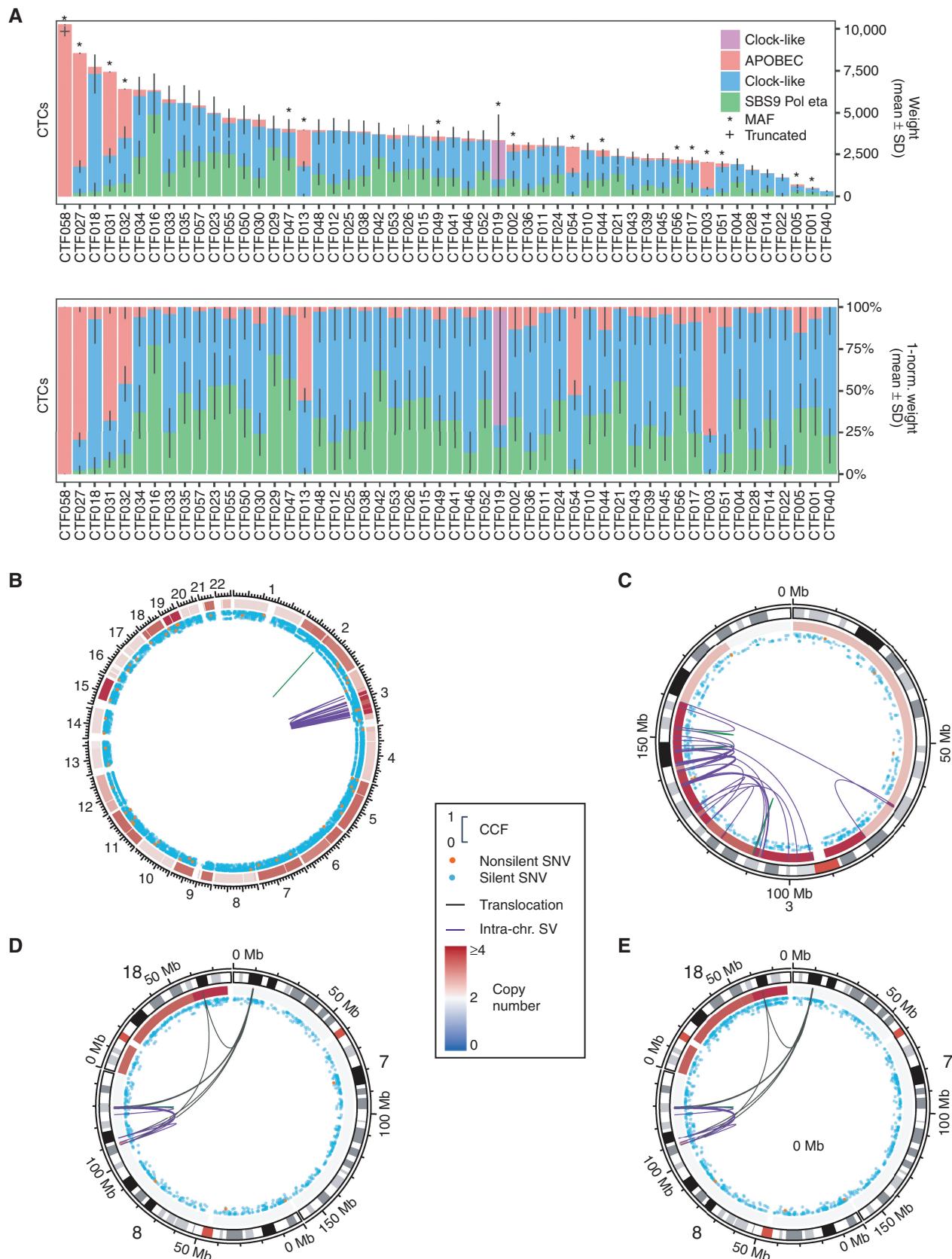
B

Figure 5. Genomic profiling of CTCs can be used to replace BM FISH for risk classification of patients. Matrix plots highlighting the diagnostic yield of MinimuMM-seq for 51 patients across the disease stages of MGUS, SMM, and MM. **A**, In 24 patients who had matched CTCs at the time of BM, head-to-head results detected by both FISH and WGS are highlighted in black, whereas additional yield detected by WGS is represented in green. The bottom rows show risk stratification enabled by results of CTC sequencing compared with standard clinical FISH. **B**, Abnormality detection and risk classification in the CTC-only cohort when only PB was sampled from 27 patients.

to accompany and possibly replace BM biopsies for the diagnosis and longitudinal monitoring of MM patients through pathognomonic variant detection (translocations and hyperdiploidy), supplanting the need for clinical variables or close observational monitoring of patients who are viewed as high risk.

The role of PB is well established in the clinical workup of precursor MM with the assessment of markers such as monoclonal protein, serum albumin, and light chain concentrations; however, it does not take into consideration tumor biology analytes. Enriching and selecting CTCs from a large background of mononuclear cells and subsequent extraction



of nucleic acids from this minute cell fraction pose significant technical challenges, precluding their extensive molecular characterization. Our study is the first to show intact CTCs can be purified and used to detect complex structural events and chromosomal abnormalities that are hallmarks of MM disease. This is a substantial advance demonstrating that initiating events of MM can be detected upon genomic profiling of CTCs *a priori* with similar sensitivity and specificity to clinical BM FISH. Importantly, our method can be the basis for clinical applications that use liquid biopsies to assay genomic biomarkers for diagnostic and prognostic purposes, in which screening and monitoring of MM clones can be achieved using blood sampling only.

The prevalence of MGUS in the general population is estimated to be 3% to 5% of individuals aged over 50 years, and SMM to be 0.53% of individuals aged over 40 years old (39–42). Screening may be especially important for the SMM stage, which is heterogeneous and captures multiple clinical trajectories of disease including “MGUS-like,” indolent, or “early MM-like” (43). Capturing high-risk patients before end-organ damage occurs is of especially high importance for decisions on clinical management strategies. Using MinimuMM-seq, we observed CTCs indeed represent the major BM clone upon blood sampling, with a high concordance of genomic profile between both compartments, illustrating that dominant clones in the BM possess the fitness to circulate and define the current tumors’ biology. Initiating mutational profiles included primary translocations of t(11;14), t(4;14), t(14;16), and t(14;20). A similar representation of the overall clonal complexity in PB compared with BM was observed in matched sample patients. Notably, in few cases we observed subclones with preferential circulation in the PB compartment, harboring driver mutations such as KRAS conferring improved fitness. This shows that blood sampling and screening of CTCs are sufficient to reliably identify mutations of the BM to screen and monitor patients for initiating clones and potentially emerging high-risk clones that may have more aggressive characteristics. Although genomic analyses of MM have not yielded targeted therapies to date [with the exception of venetoclax in t(11;14) patients], improved molecular profiling using CTCs and BM could indeed help to better stratify patients for clinical decisions using current models such as the four-risk factor 2/20/20 model (for SMM; ref. 20) or Revised ISS (31) or mSMART models (for MM; ref. 44). Targeting actionable mutations such as CDK, RAS/RAF, and t(11;14) is currently being investigated within the MyDRUG trial (NCT03732703).

Although BM remains the gold standard for diagnosis, recent studies have highlighted the importance of spatial heterogeneity in MM disease, a feature that is well known from functional imaging (9, 45, 46). As such, current methods of BM biopsy at a single iliac crest or sternal site may not accurately represent the full picture of tumor biology, potentially missing high-risk genomic events of prognostic interest such

as TP53 mutations (9). In this study, we showed that high-risk arm-level abnormalities of 1q gain and deletion of chromosome 13 could be found at a higher CCF in blood-derived CTCs than in BM biopsy samples of the same time point. Conversely, we did not detect any high-risk events occurring exclusively in BM. Furthermore, similar observations applied to driver mutations detected in KRAS and NRAS, which were all found to be of equal or higher clonality in the blood compared with BM. Thus, CTCs may assist in defining spatial genomic architecture and systemic disease through the detection of private mutations upon PB sampling not found in the standard BM biopsy site. This suggests that capturing CTCs could give us a major unexpected advantage over BM biopsies in that CTCs harboring such abnormalities lose dependence and diffuse into the blood from proliferative niches throughout the body before they settle and induce a clonal sweep and fixation at a primary or secondary regional site. Further studies of longitudinal abnormalities in matched BM and PB compartments will help elucidate this hypothesis.

MinimuMM-seq for CTCs also addresses the current challenge of repeated sampling of patients for the continuous monitoring of disease development and tumor evolution. CTCs at a single time point can provide an assessment of genomic profile and actionable mutations, whereas longitudinal blood collection at multiple time points can track mutation architecture and clonal dynamics through CTCs, similar to that of the major BM clone, in a minimally invasive manner. In particular, we showed a serial sampling of blood from untreated patients with SMM can be used to identify the clonal complexity, phylogeny, and evolution of CTCs, where emergence (of high-risk subclones with selective advantage) and extinction (of passenger subclones) are observed over time. Conceivably, this approach could also be applied in a treatment setting to provide evidence for response or resistance, restaging, and end-of-trial assessment in which BM is unable to be collected, and measure how the fitness landscape changes with treatment pressure. Indeed, in one patient with SMM who began receiving early interventional therapy, we show that longitudinal liquid biopsies monitoring CTCs provide information on clonal dynamics and selection of high-risk subclones that emerge in real time. This observation illustrates that dense sampling of blood for genomic profiling may assist in closely tracking patient response and tailoring changes given a patient’s specific evolving biology and clonal composition. As WGS becomes less expensive, we believe that this approach will be easily accessible and may become more affordable than traditional FISH for clinical application. Many tests using NGS, including germline WGS, have been introduced in recent years, and this category will likely continue to expand. Furthermore, at MRD testing time points, we observed CTCs are rarely present in the PB when patients were either MRD positive or MRD negative by NGS evaluation. This is similar to previous studies showing high false negativity of noninvasive MRD assessment

Figure 6. CTC sequencing enables the cohort-level genomic study of mutational signatures and complex structural events. **A**, Estimation of weight of mutational processes in the CTC sorted by known mutational signatures and represented in absolute (top) and 1-normalized values (bottom). Asterisks represent MAF-rearranged tumors. Plus (+) sign represents truncation for participant CTF058 (APOBEC weight $30,828 \pm 71$). SBS, single-base substitution. **B–E**, Characterization of complex structural variants from WGS data. Intra-chr. SV, intrachromosomal structural variant. **B** and **C**, CTF033 chromothripsis of the long arm of chromosome 3 at the genome scale (**B**) and zoomed on chromosome 3 only (**C**). **D** and **E**, Chromoplexy of chromosomes 7, 8, and 18 involving MYC on chromosome 8 in matched BM (**D**) and PB (**E**) from CTF034.

by monitoring CTCs as a measure of MRD burden after treatment compared with BM (15).

We also consider the limitations present in our study. Compared with higher burden SMM and overt MM, a minority of MGUS, lower risk SMM, and MRD status patients may be eligible for genomic characterization by MinimuMM-seq at the first sampling date; however, monitoring by enumeration and moving to genomic characterization when CTC numbers increase could coincide with the time at which such an assay is relevant in the disease course. In particular, patients on treatment and being assessed for MRD may benefit from a regular serial sampling of PB for CTC burden as an assessment of the duration of MRD status. Our current results suggest copy-number abnormalities face no lower limit of detection, whereas translocations could be detected in samples down to 50 CTCs and clonal mutations were reliably detected from around 300 CTCs. In this study, we relied on affinity-based selection of PCs ($CD138^+38^+$), and we note CTCs could be missed due to potential dynamic surface protein expression or clonogenic potential of $CD138^-$ cells (47–49). Whether the physical properties of PCs such as morphology could be a sensitive way to purify CTCs of premalignant disease remains to be tested. Although we focused on genetic alterations, future transcriptomics studies using single-cell RNA sequencing may also define phenotypic characteristics and traits that allow CTCs to extravasate, and whether high-risk or rare clones possess circulating specific functional features (17, 50–52).

MinimuMM-seq provides a foundation for minimally invasive detection, enumeration, and genomic interrogation of rare CTCs from the PB, illustrating the clinical potential of using liquid biopsies for monitoring and managing disease in MM. Addressing costs and strategies to bring WGS into the clinical standard of care will consequently provide an unbiased test to improve on and refine genomic biomarker assessment for patient diagnostics. Ultimately, CTCs possess great potential to enable precision oncology and prevention in MM and its precursor conditions.

METHODS

Patient Sample Collection

Blood samples from a cohort of 261 patients (84 MGUS, 155 SMM, and 22 MM) were prospectively collected from the Dana-Farber Cancer Institute Observational Precursor Crowd (PCROWD) study (NCT02269592) and the Plasma Cell Dyscrasias study. All patients provided written informed consent for the research use collection of PB and BM samples (Institutional Review Board #14-174 and #07-150). Research studies were carried out in accordance with the Declaration of Helsinki. PB was drawn into CellRescue Preservation Tubes (Menarini Silicon Biosystems) and kept at room temperature before processing on the CellSearch instrument. Samples were all processed within 96 hours from the time of collection. Patients with matched BM biopsies had routine clinical cytogenetics and FISH analysis performed at a molecular pathology laboratory (Supplementary Materials and Methods).

Enrichment and Enumeration of CTCs

From each patient, PB was collected in CellRescue Preservation Tubes (Menarini Silicon Biosystems) and processed on the CellSearch System (Menarini Silicon Biosystems) composed of the CELLTRACKS AUTOPREP System and the CELLTRACKS ANALYZER II using the

CELLSEARCH Circulating Multiple Myeloma Cell (CMMC) Assay (Menarini Silicon Biosystems) for the enrichment and enumeration of circulating MM cells. The sensitivity and linearity of circulating MM cells using the CellSearch System have previously been reported (11). Briefly, 4 mL of blood was removed from the CellRescue Preservation tube and processed on the CELLTRACKS AUTOPREP System using the CELLSEARCH CMMC Assay to enrich for myeloma cells following the manufacturer's recommendations. Myeloma cells were immunomagnetically captured using an anti-CD138 antibody conjugated with ferrofluid. All captured cells were stained with an anti-CD38 antibody conjugated with phycoerythrin (PE), an anti-CD19/CD45 antibody cocktail conjugated with allophycocyanin (APC), to differentiate leukocytes from the circulating myeloma cells. All cell nuclei were stained with DAPI and then fixed with paraformaldehyde. The cartridges containing the enriched myeloma cells were placed in the CELLTRACKS ANALYZER II, a semiautomated fluorescence microscope. The sample was then exported from CELLTRACKS ANALYZER II and imported into GateWorks software. The GateWorks software segmented the objects in the browser images, extracted object features, and “gated” those events that were most likely to be CMMCs for review by the user. The myeloma phenotype was $CD138^+/CD38^+/DAPI^+/CD19^-/CD45^-$. Any background cells such as leukocytes, which had the phenotype $CD138^-/CD38^-/DAPI^+/CD19^+/CD45^+$, were not counted as myeloma cells. Samples were then removed from the cartridges and stored in glycerol stocks at -20°C for subsequent downstream molecular characterization.

Sorting of CTCs

Upon enrichment of PB samples using The CellSearch System, ~100–10,000 leukocytes may remain in the background of the extracted sample; therefore, methods of cell sorting were used for sorting of pure CTCs to obtain high tumor fraction pools of CTCs. High-sensitivity fluorescence-activated cell sorting (FACS; BD Biosciences Aria II) was used to sort pure populations of tumor PCs and germline white blood cells based on immunophenotypic gating of $CD138^+38^+$ and $CD45^+CD138^-CD38^-$, respectively.

DNA Library Construction

Sorted samples underwent DNA purification (Thermo Fisher PicoPure DNA Isolation Kit) and library preparation using the NEBNext Ultra II FS DNA Library Prep Kit (New England Biolabs) with unique dual index adapters (NEBNext Multiplex Oligos) according to the manufacturer's instructions. Final library fragment sizes were assessed using the BioAnalyzer 2100 (Agilent Technologies), with yields quantified by a Qubit 3.0 fluorometer (Thermo Fisher Scientific) and qPCR (KAPA Library Quantification Kit).

DNA Sequencing and Genomic Data Analysis

Final sample libraries were normalized and pooled before WGS was performed on Illumina NovaSeq 6000 S4 flow cells, 300 cycles paired-end reads, at the Genomics Platform of the Broad Institute of MIT and Harvard. WGS analysis was performed on an in-house cloud-based HPC system for copy-number, mutation, and structural variant analyses, and detailed steps are described in the Supplementary Materials and Methods. Briefly, sequencing reads were aligned to the hg19 reference genome with the bwa mem v0.7.7 algorithm, duplicate reads were marked with MarkDuplicates from picard v1.457, indels were realigned with GATK 3.4 IndelRealigner, and base qualities were recalibrated with GATK 3.4 BaseRecalibrator software. Mutations were called with MuTect1 (53) and small indels with Strelka2 (ref. 54; for SNVs and indels) and were filtered (i) against a panel of normals (PoN), (ii) for potential technical artifacts (oxoG), and (iii) for multiple alignment with BLAT (Supplementary Materials and Methods). After copy-number normalization with AllelicCapSeg, ABSOLUTE (21) solutions were manually reviewed to estimate mutation CCF,

purity, and ploidy of tumor samples. Phylogeny reconstruction was performed with the PhylogenNDT (26) suite of tools with the following parameters: minimum CCF: 20%, minimum coverage: 10, number of iterations: 1,000. Mutational signatures were quantified with the SignatureAnalyzer (55, 56) method and $n = 100$ runs. Structural variants were detected and filtered as previously described (57).

Statistical Analyses

Quantitative bioclinical variables were described with median and IQR or absolute range or with mean and SD. The average difference between groups was assessed with the Kruskal-Wallis method for multiple group testing followed by Dunn *post hoc* tests and/or Wilcoxon test for two groups. When the mean is estimated from a random process, it is given with its 95% CI. Qualitative variables were described using the frequency of their respective modalities. Distinct distribution between groups was assessed with χ^2 Pearson test (or Fisher exact test if appropriate). Patients were stratified for progression-free survival by the presence of CTCs and by quartiles of CTC enumeration. Time to event was calculated from screening to clinical progression to MM, or death of any cause, whichever occurred first. The likelihood ratio test statistic is reported in the absence of an event in the nonprogressor group. P values were corrected for multiple testing with the Benjamini-Hochberg method. Adjusted P values under 0.05 were considered significant. All calculations were done using R 4.1.1 software.

Data Availability

Data and code used in the analysis are available at <https://github.com/jalberge/ms-cmmcs/> or in the Supplementary Materials and Methods. The sequencing data presented in the current publication have been deposited in and are available from the database of Genotypes and Phenotypes (dbGAP) under accession number phs003084.v1.

Authors' Disclosures

A.K. Dutta reports a patent for methods for characterization of CTCs pending and honoraria from Menarini Silicon Biosystems for a speaking engagement. J.-B. Alberge reports grants from the International Myeloma Society during the conduct of the study, as well as a patent for MinimuMM-seq pending. T.H. Mouhieddine reports other support from Legend Biotech outside the submitted work. F.K. Kuhr reports employment with Menarini Silicon Biosystems. R.G. Del Mastro reports employment with Menarini Silicon Biosystems. O. Nadeem reports personal fees from Janssen, Bristol Myers Squibb, Takeda, Adaptive Biotechnologies, and GPCR Therapeutics outside the submitted work. D. Auclair reports no conflict at the time of data generation and manuscript production and recent employment with AstraZeneca. G. Getz reports grants from the Adelson Medical Research Foundation and the SU2C Dream Team during the conduct of the study; grants from IBM and Pharmacyclics, and personal fees and other support from Scorpion Therapeutics outside the submitted work; a patent for MinimuMM-seq pending; and is an inventor on patent applications related to MSMuTect, MSMutSig, MSIDetect, Polysolver, and SignatureAnalyzer-GPU. I.M. Ghobrial reports grants from the Dr. Miriam and Sheldon G. Adelson Medical Research Foundation, the NIH, and Stand Up To Cancer, personal fees from Aptitude, GSK, Sanofi, Janssen, Takeda, Karyopharm, AbbVie, GNS, Cellecter, Medscape, Menarini Silicon Biosystems, Genentech, Adaptive, Bristol Myers Squibb, Curio Science, and Oncopeptides outside the submitted work, as well as a patent for MinimuMM-seq pending. No disclosures were reported by the other authors.

Disclaimer

Opinions, interpretations, conclusions, and recommendations are those of the authors and are not necessarily endorsed by Stand Up To Cancer, the Entertainment Industry Foundation, or the American Association for Cancer Research.

Authors' Contributions

A.K. Dutta: Conceptualization, funding acquisition, investigation, visualization, methodology, writing-original draft, writing-review and editing. **J.-B. Alberge:** Conceptualization, investigation, visualization, methodology, writing-original draft, writing-review and editing. **E.D. Lightbody:** Investigation, methodology, writing-review and editing. **C.J. Boehner:** Methodology, writing-review and editing. **A. Dunford:** Investigation, visualization, methodology, writing-review and editing. **R. Sklavenitis-Pistofidis:** Methodology, writing-review and editing. **T.H. Mouhieddine:** Methodology, writing-review and editing. **A.N. Cowan:** Methodology, writing-review and editing. **N.K. Su:** Methodology, writing-review and editing. **E.M. Horowitz:** Methodology, writing-review and editing. **H. Barr:** Methodology, writing-review and editing. **L. Hevenor:** Methodology, writing-review and editing. **J.B. Beckwith:** Methodology, writing-review and editing. **J. Perry:** Methodology, writing-review and editing. **A. Cao:** Visualization, methodology, writing-review and editing. **Z. Lin:** Methodology, writing-review and editing. **F.K. Kuhr:** Methodology, writing-review and editing. **R.G. Del Mastro:** Methodology, writing-review and editing. **O. Nadeem:** Funding acquisition, methodology, writing-review and editing. **P.T. Greipp:** Methodology, writing-review and editing. **C. Stewart:** Investigation, visualization, methodology, writing-review and editing. **D. Auclair:** Methodology, writing-review and editing. **G. Getz:** Conceptualization, supervision, investigation, visualization, methodology, writing-review and editing. **I.M. Ghobrial:** Conceptualization, supervision, funding acquisition, investigation, visualization, methodology, writing-review and editing.

Acknowledgments

We thank Anna V. Justis, PhD, for providing writing and editing support during the preparation of this article; Julian Hess, Liz Martin, Yo Akiyama, and Nicholas Haradhvala, PhD, for providing support and critical review of WGS data analysis; and Frances Tanne and the clinical laboratory team at Menarini Silicon Biosystems for processing the recovery of a batch of CellSearch samples. We acknowledge funding support from the Dr. Miriam and Sheldon G. Adelson Medical Research Foundation, the NIH (R01CA205954 and R35CA263817-01A1), an International Myeloma Society Career Development Award (J.-B. Alberge), and a Dana-Farber Cancer Institute Medical Oncology Grant Award (A.K. Dutta). This research was also supported by a Stand Up To Cancer Dream Team Research Grant (grant number: SU2C-AACR-DT-28-18). Stand Up To Cancer is a division of the Entertainment Industry Foundation. The indicated SU2C grant is administered by the American Association for Cancer Research, the scientific partner of Stand Up To Cancer.

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Received April 27, 2022; revised September 20, 2022; accepted November 9, 2022; published first December 7, 2022.

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