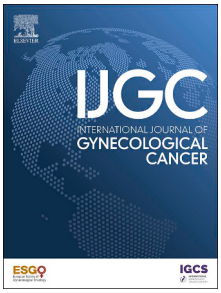
ORIGINAL RESEARCH

DNA-junction-based personalized liquid biopsy assays could inform management of aggressive endometrial cancer



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ABSTRACT

Objective: We investigated the clinical validity and feasibility of a DNA junction-based quantitative polymerase chain reaction assay to detect and quantitate circulating tumor DNA (ctDNA) in the blood of high-risk patients with endometrial cancer.

Methods: Whole genome sequencing tumor data was analyzed from 36 patients to select prominent somatic tumor-specific DNA junctions. Personalized quantitative polymerase chain reaction assays were developed for each junction and applied to available blood specimens to measure levels of ctDNA.

Results: Pre-surgical blood ctDNA was detected in 71% of the cases tested (56% early-stage vs 88% advanced-stage). In patients with available pre-amplified pre-surgical ctDNA ($n = 15$), pre-surgical ctDNA levels were elevated in patients with vital status or "alive with disease" or "died of disease" compared to patients with "no evidence of disease" ($p < .005$). Among 17 patients followed serially, ctDNA detection preceded clinical detection of recurrence in 5 of 8 cases and was concurrent in 1, with the caveat that imaging was rarely concurrent with blood draw timing.

Conclusions: Personalized junction-based measurement of ctDNA demonstrated promising clinical validity in pre-surgical prognosis and relapse prediction.

Keywords:
ctDNA; cfDNA; Personalized; Endometrial Cancer; Biomarker

WHAT IS ALREADY KNOWN ON THIS TOPIC

Circulating tumor DNA (ctDNA) testing appears to be a reasonable approach for endometrial cancer cases with aggressive features or late stage. Most previous studies have focused on single nucleotide variations to identify ctDNA.

WHAT THIS STUDY ADDS

Personalized panels of junction detection from somatic chromosome rearrangements show feasibility for pre-surgical prognostication and in post-surgical monitoring for recurrence.

HOW THIS STUDY MIGHT AFFECT RESEARCH, POLICY, OR PRACTICE

The findings in this study provide support for larger studies to precisely define ctDNA sensitivity and specificity from personalized junction ctDNA assays. These panels may be superior in (1) determining prognosis, (2) recurrence monitoring, and (3) recurrence detection lead-time as compared to current standard-of-practice.

INTRODUCTION

High-risk endometrial cancer is associated with poor prognosis. Indeed, the 5-year survival for endometrial cancer is 17% when distant disease is present at diagnosis[1] and around 50% in early stage when high-risk features are present (eg, non-endometrioid histology, diffuse lymphovascular space invasion, p53 abnormality, etc.).[1-4] The National Comprehensive Cancer Network (NCCN) guidelines recommend surveilling all patients with endometrial cancer with routine physical exams and Cancer Antigen 125 (CA125) monitoring; imaging is indicated for cases with elevated CA125 or symptoms suggestive of

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recurrence.[5] Although CA125 sensitivity for pre-surgical endometrial cancer is reasonable,[6] CA125 is elevated in only 10% of recurrent high-risk endometrial cancers.[7] Given the high rate of recurrence in high-risk endometrial cancer, there is a need to develop a sensitive biomarker for the detection of cancer recurrence and to investigate if earlier relapse detection is associated with improved patient outcomes.

Liquid biopsy via detection of circulating tumor DNA (ctDNA) within the background of total circulating cell-free DNA (cfDNA) is emerging as a tool in a variety of solid organ malignancies.[8-11] CtDNA testing has the potential to detect minimum residual disease (MRD) post-operatively and stratify patients by recurrence risk, thereby informing adjuvant therapy decisions.[12-16] Longitudinal ctDNA monitoring may detect recurrence earlier than standard-of-care radiographic imaging,[12] with reported lead-times of approximately 10 months in ovarian cancer,[17] 8.7 months in colorectal cancer,[18] and 4 months in gastric cancer.[19] Importantly, no studies have reported the converse to be true, that is, there are no data suggesting radiographic imaging can identify recurrent cancer earlier than ctDNA detection.

Most ctDNA detection approaches rely on hotspot mutations or broad genomic signatures, which can be less sensitive in heterogenous tumors like endometrial cancer. To address this gap, we developed a personalized approach of targeting somatic chromosomal junctions. This method of generating patient-specific, tumor-informed, junction-based assays, was previously applied in feasibility-pilot studies in ovarian cancer and endometrial cancer.[13,20] We gravitated to this approach because junction-based quantitative polymerase chain reaction (qPCR) assays can be very sensitive and leverage an abundant pool of junctions unique to each tumor. They can be multi-copy or even amplified without an association with clonal-hematopoiesis-related false positives.[21] In this study, we retrospectively and serially evaluated the feasibility of personalized ctDNA detection in patients with histologically high-risk stage I-IV endometrial cancer.

METHODS

Patient Characteristics

In this study, we investigated the feasibility and clinical validity of junction-based assays to measure ctDNA in the blood of patients with endometrial cancer within 2 clinical settings—pre-surgical and post-surgical serial monitoring. Patients were selected based on the presence of high-risk tumor characteristics and ctDNA sample availability among a cohort of 102 patients prospectively enrolled. See Supplementary Methods for greater detail on study inclusion criteria and cohort clinical description. All patients had provided informed written consent for 1 of 2 approved Mayo Clinic institutional review board (IRB) parent studies with enrollment dates specified: #15-005545 7/2016-5/2017 and #19-005326, 9/2019-6/2021. Inclusion criteria were: (1) Surgical staging with lymph node evaluation performed at one of the Mayo Clinic sites; (2) Age $\geq$18 years; (3) Preoperative biopsy showing grade 3 endometrial cancer, confirmed at final pathology; or grade 1 or 2 with preoperative imaging suggestive of gross extrauterine disease. Patients who declined enrollment or had invasive synchronous cancers were excluded. This report includes ctDNA data from 36 patients, combining 11 patients from an earlier IRB-approved cohort[13] and 25 additional cases with updated outcome data (Fig. S1).

Tumor Sequencing and ctDNA Assay Workflow

Blood and tissue were collected and processed as described previously.[13] Additional information on tissue and blood processing, next-generation sequencing (NGS), junction selection, and PCR details are available in the Supplementary Materials and Tables S1 to S4.[13,22]

Assay specificity and sensitivity analysis of ctDNA junction detection in pre-surgical blood was performed in 35 patients. For prognostic assessment of pre-surgical ctDNA, the lowest value qPCR cycle threshold (Ct) (value at which the signal crosses the Ct) obtained from any patient-specific junction result (ie, the most sensitive primer set for the individual patient ctDNA) was used. The Ct values of all pre-amplified cfDNA samples were derived from the means of 2 technical qPCR replicates. Feasibility of ctDNA junction detection for relapse detection was performed in 17 patients for whom at least 3 blood draws were available.

Statistical Analysis

Statistical significance of pre-surgical Ct values by vital status was assessed using a 2-tailed Mann-Whitney test in GraphPad Prism software V10.0.0. This analysis included 15 patients whose last recorded outcome was either "alive with disease" or "died of disease" compared to those with "no evidence of disease." Patients who died of causes other than endometrial cancer were omitted from the pre-surgical ctDNA analysis. Only pre-amplified samples were included in this analysis to ensure standardization of methodology for this grouping (Tables S2 and S5). Interquartile range (IQR first quartile-third quartile) was calculated using SAS version 9.4 (SAS Institute).

In accordance with journal policy, we will make our data available for independent verification by a team designated by the Editorial Board, either for further statistical analysis or to support reproducibility en external centers, upon request.

RESULTS

The clinical and pathological characteristics of the study patients are described in Tables S5 and S6. Half of the cases (18/36) were diagnosed with early-stage endometrial cancer I or II, while the other half were late-stage III or IV endometrial cancer. Among the 36 cases, histologic sub-types were distributed as follows: serous (n = 21), endometrioid (n = 5), carcinosarcoma (n = 4), clear cell (n = 4), and mixed endometrioid/serous (n = 2). Most patients had grade 3 disease (34/36), with 2 of 36 having grade 1 or 2 disease. Overall, 83% were considered at greater than intermediate risk according to European Society of Gynaecological Oncology/European Society for Therapeutic Radiology and Oncology/European Society of Pathology guidelines.[23] Using the definitions of the Cancer Genome Atlas Research[24,25] the patient group is comprised mostly of cancers with high copy number molecular classification (32/36) (Table S5). This sub-type is especially suited for ctDNA junction analysis because tumors contain many somatic chromosomal rearrangements. Patients were followed for a median of 27.9 months (IQR 20.0-34.0). During that time, 14 patients experienced recurrence (39%) with a

median time to recurrence of 18.6 months (IQR 13.7-23.1) after surgery. By the end of the study period, 18 were alive with no evidence of disease, 8 patients were "alive with disease", and 10 patients died during the study period: 6 attributed to endometrial cancer ("died of disease") and 4 to other causes or died of unknown cause (Table S5).

The ctDNA assay workflow for a representative case is illustrated in Figure 1A. Blood is obtained before the surgical removal of the primary tumor and at least once after surgery. In parallel, DNA from the tumor tissue is sequenced and high-confidence somatic DNA junctions identified bioinformatically.[13,21] Primers are designed for individualized patient junction panels comprised of 3 to 5 selected junctions in each case and tested on the total cfDNA via qPCR. CtDNA results were compared with patients' clinical histories to obtain clinical correlations.

Case EM129 is presented as an example of feasibility of the assay design process, as shown in Figure 1B to D. At surgery, EM129 had 100% myometrial invasion (MI), positive lymph nodes, lymphovascular space invasion (LVSI), and was categorized as a stage IVB tumor according to International Federation of

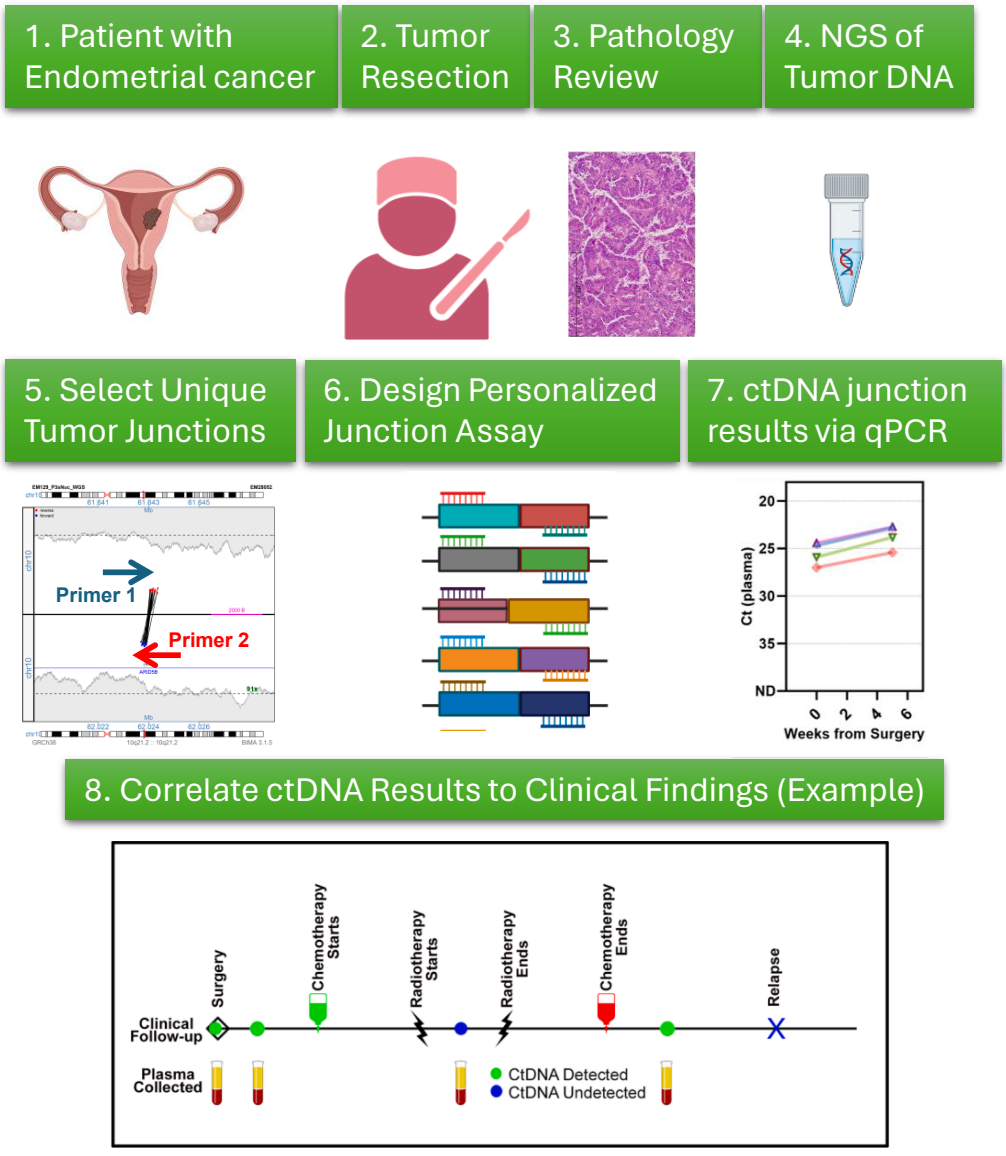

Figure 1 Study design.
A, Blood samples from endometrial cancer patients were collected before and after surgery. Tumor pathology was reviewed, DNA extracted, and sequenced (NGS) to identify somatic chromosomal rearrangements for personalized primer design and plasma ctDNA testing. CtDNA results were compared to clinical findings. **B-D**, Example case EM129: **B**, Genome U-Plot shows chromosomes with genomic position (X-axis), coverage (Y-axis), CNV (blue-gains, red-losses), and rearrangements and other alterations including findings from exome sequencing (lollipops); magenta lines indicate junctions, green line shows balanced junctions and transposon insertions. Selected junctions for ctDNA testing are highlighted. **C**, Junction plots display linked genomic regions (two reads linked with connecting line) and coverage (grey shading, with dashed line for overall average). Blue represents reads in the forward orientation; Red indicates reverse orientation. **D**, Longitudinal ctDNA detection using five tumor-specific junctions named for involved chromosomes; data points are means of two qPCR replicates.
Abbreviations: ctDNA, circulating tumor DNA; ND, not detected (Ct >40); NGS, next generation sequencing; qPCR, quantitative polymerase chain reaction.

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Gynecology and Obstetrics (FIGO) guidelines (Table S5). Sequencing of the primary EM129 tumor revealed a high copy number somatic state, with extensive copy gains throughout the genome, including copy gains on chromosome arms 8q and 19q (Fig. 1B), (Table S1). Of the 113 novel somatic DNA junctions identified (Table S3), 5 junctions were selected for ctDNA detection assays in the blood based on the criteria detailed in the Supplementary Methods (Fig. 1C) (Table S4). Junction 1a-3b predicts multiple copies of a junction between chromosomes 1 and 3 (1a-3b) at positions 27.5 Mb and 125 Mb, respectively (Fig. 1C). Primers and probes were designed for an amplicon of each selected junction and evaluated for sufficient sensitivity and specificity via qPCR on patient tumor DNA and controls (Table S4). In a multiplexed pre-amplification reaction, primers for all 5 EM129 junctions and NAGK control were combined with plasma processed for cfDNA for 10 cycles PCR (Table S2). The pre-amplified cfDNA was purified, concentrated, and used in the qPCR assay with 40 cycles. Five junctions were detected in the pre-surgical draw with an overall mean Ct value of 25.4 after normalization which persisted after surgery (Fig. 1D) (Table S4).

The ctDNA overall qualitative detection sensitivity of the junction-based method was calculated using 35 cases in which pre-surgical blood was available (Fig. S1). Our tumor-informed technique mandates previous tumor diagnosis, thus, "ctDNA detection sensitivity" mentioned should not be confused with early detection sensitivity. CtDNA was detected by personalized junction qPCR in 25 of 35 pre-surgical blood samples for an overall detection rate of 71% (Table S7). The detection of ctDNA was consistent with clinical indicators of aggressive disease, such as MI $\geq$50% and LVSI (Table S7). Patients with MI $\geq$50% were more likely to have detectable pre-surgical ctDNA (12/13, 92%) and the same was true for patients with presence of LVSI (10/12, 83%). In stage I/II endometrial cancer, 56% (10/18) of patients had detectable ctDNA pre-surgically, while 88% (15/17) of stage III/IV endometrial cancer patients had pre-surgical ctDNA detection (Table S7). Considering the 25 patients with detectable pre-surgical ctDNA, 9 patients recurred with median time to recurrence of 18.8 months (IQR 13.7-23.1). Of the 25 patients in whom both pre-surgical ctDNA and CA125 were evaluated, 17 had detectable ctDNA pre-surgically and 8 were positive for CA125 (normal: $<$34 IU), with 2 of those 8 having undetectable ctDNA.

The prognostic value of ctDNA levels in pre-surgical blood was assessed in 15 patients where ctDNA was detected after pre-amplification was performed and last vital status was "alive with disease" or "died if disease" compared to "no evidence of disease". Patient status was followed for a median of 26.5 months (IQR 19.1-29.4) (Table S5). To investigate if pre-surgical ctDNA levels of patients with "no evidence of disease" were lower than ctDNA from patients that experienced recurrence, we selected patient-representative Ct values and then compared them between the 2 groups. It is important to note that qPCR Ct value is negatively correlated with DNA levels, with higher Ct value indicating lower amount of DNA. The "no evidence of disease" group of 9 patients had a median Ct of 30.53 while the combined "alive with disease" and "died of disease" group of 6 patients had a median Ct of 24.72 (Fig. 2), suggesting more than 60-fold higher levels of ctDNA in plasma of patients "alive with disease" compared to patients with "no evidence of disease". The difference between

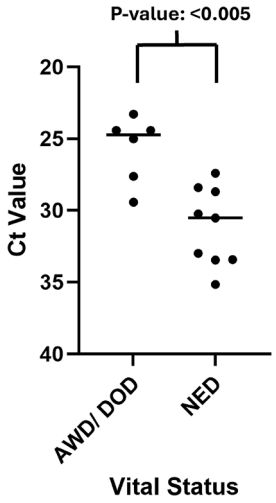

Figure 2 Plot of pre-surgical qPCR Ct values (y-axis).
AWD, alive with disease; Ct, Point at which signal crossed the cycle threshold in qPCR; DOD, dead of disease; NED, no evidence of disease; qPCR, quantitative polymerase chain reaction.
p-value calculated using Mann-Whitney unpaired 2-tailed *t* test. Ct$>$40 = Undetected ctDNA

the 2 groups was statistically significant via a *t* test ($p < .005$). Therefore, the approximated levels of ctDNA of the recurred patients are higher than those of the patients with "no evidence of disease" suggesting that ctDNA pre-surgical levels might have prognostic value.

Longitudinal screening for ctDNA using patient-specific junction qPCR assays was performed in 17 patients with $\geq$3 blood draws, totaling 85 draws. Median follow-up time for these patients was 25.6 months (IQR 19.1-28.1), during which 8 experienced clinically defined relapse of endometrial cancer with a median time to relapse at 16.1 months (IQR 13.2-21.1) after surgery (Fig. S1, Table S5). In 6 of the 8 relapsed cases, ctDNA was detectable in the post-surgical blood up to 85 weeks before recurrence, with ctDNA detected before imaging in 5 cases (Figs. 3A-E) and concurrent in 1 (Fig. 3F).

In patient EM129 (Fig. 3A), ctDNA was consistently detectable in all 5 post-surgical blood draws, while 4 pre-recurrence scans were negative, and 4 out of 5 CA125 tests were below the threshold until near recurrence at 115 weeks. In EM401 (Fig. 3B), ctDNA was detectable for 1 junction 20.7 weeks before a negative scan, and both tested junctions were highly detectable 10 weeks before the recurrence was detected by computed tomography scans. Similarly, case EM601 (Fig. 3C) had at least 1 junction from ctDNA that was detectable up to 39 weeks before a negative scan and up to 46 weeks before radiographic recurrence. Two cases (EM127 and EM122) showed detectable ctDNA in all junctions before convincing radiographic evidence of recurrence, scans taken shortly after those draws were deemed suspicious for recurrence, with CA125 still below the normal threshold of 35 IU (Fig. 3D and E). Notably, in case EM127 (Fig. 3D), post-surgical ctDNA is detected before the initiation of adjuvant therapy and decreased after the therapy, demonstrating therapeutic response. A similar pattern was observed in case EM109 (Fig. S2) where ctDNA persisted after surgery and dropped following adjuvant therapy.

In EM102, the recurrence was diagnosed concurrent with detection of ctDNA and increasing, but still $<$35 IU, CA125

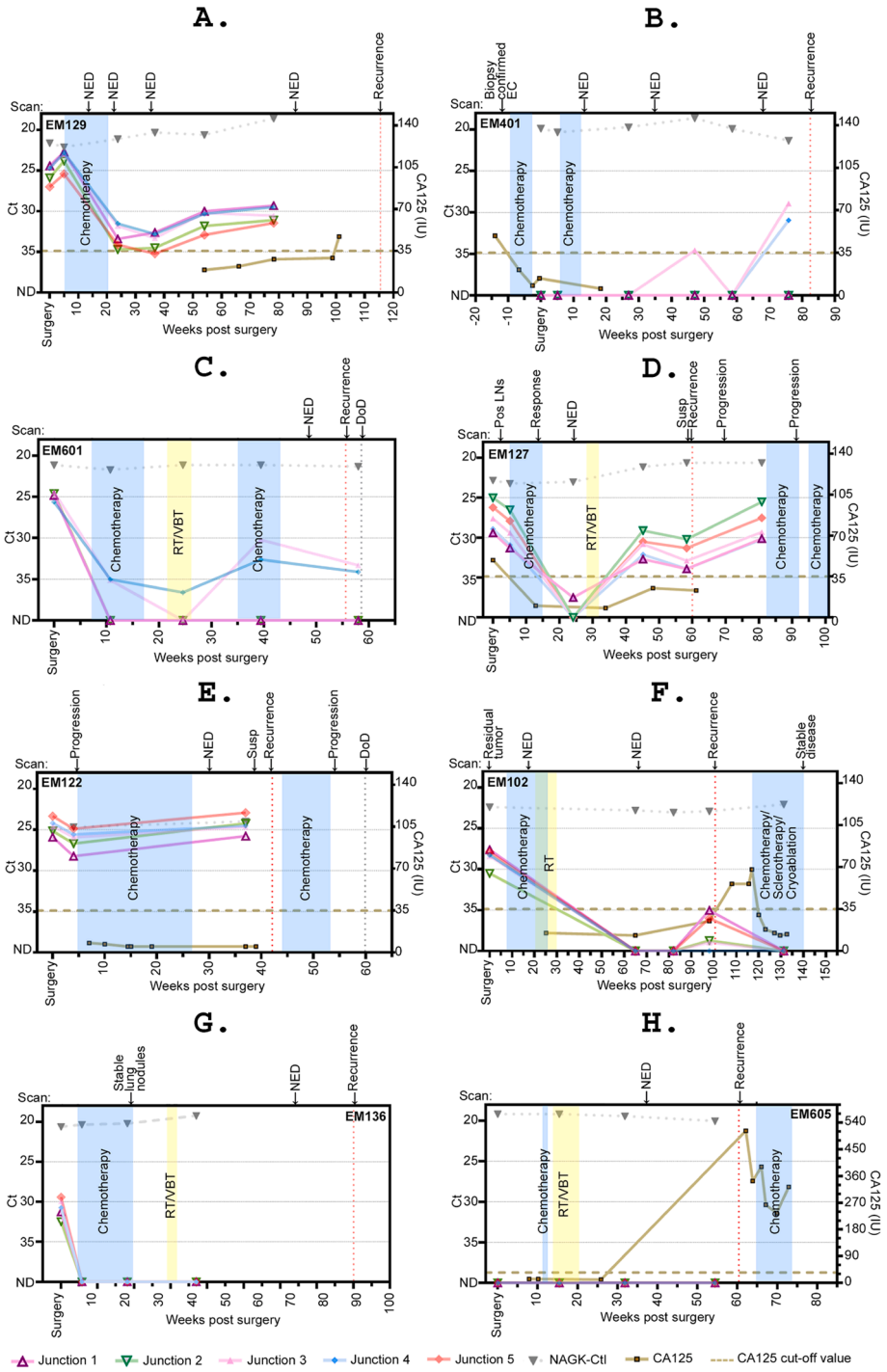

Figure 3 Patients in the secondary study with endometrial cancer recurrence.
Serial ctDNA detection and association with diagnosis of clinical recurrence. **A-F,** In 6 cases, ctDNA was detected in blood taken closest to when recurrence was diagnosed. **G-H,** In EM605 and EM136 ctDNA was not detected before recurrence. CA125 positivity threshold of 35 IU (right y-axis) indicated by dashed gold line in cases where CA125 was tested. CtDNA data points represent the mean of 2 technical qPCR replicates.
ctDNA, circulating tumor DNA; DoD, died of disease; N.D, not detected (qPCR Ct>40); NED, no evidence of disease; qPCR, quantitative polymerase chain reaction; RT, radiation therapy; Susp, suspicious for recurrence; VBT, vaginal brachytherapy.

(Fig. 3F), In EM136 was positive for ctDNA by each junction assay but tested negative for ctDNA by all applied junction assays in the available post-surgical blood (Fig. 3G). The last available blood draw preceded the date of recurrence by 10 months, and a more proximal blood draw to assess the reemergence of ctDNA was not available. In the last of the 8 cases with recurrence, EM605, the assay was not able to detect ctDNA either before or after surgery (Fig. 3H).

Ultimately, 5 of 8 patients with relapsed endometrial cancer had antecedent ctDNA and 1 with concurrent ctDNA, while only 3

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of 5 patients who were tested at or near recurrence had CA125 levels above the threshold (Fig. 3). The remaining 9 of the 17 cases with longitudinal follow-up did not relapse and all had undetectable ctDNA levels consistent with no evidence of endometrial cancer (Fig. 4).

DISCUSSION

Summary of Main Results

We assessed the ability of personalized junction panels to detect ctDNA and the correlation of the presence of ctDNA to outcome in both pre- and serial post-surgical scenarios. We found a high rate of ctDNA detection before surgery (71%). And there was a significant association between quantitative ctDNA detected before surgery and vital status. Assessment of serial draws suggested a lead-time for relapse detection in 5 cases.

Results in the Context of Published Literature

Our personalized DNA junction-based approach to assess pre-surgical ctDNA appears to have a higher rate of ctDNA detection than previously reported ctDNA assays. This may be attributable to the aggressive phenotype in our cohort compared to another study with cfDNA in predominately stage I endometrial cancer with endometrioid histology.[12] Another reason might be related to higher inputs of total cfDNA (Table S2) compared to other studies on ctDNA in endometrial cancer using 10 to 20 ng cfDNA,[12,26] 3 mL plasma,[27] or 200 μL plasma.[27,28] The inclusion of a pre-amplification step likely further enhanced sensitivity. Although studies have shown that ctDNA can be detected in the blood of patients with localized tumors,[12,29] not all tumors shed detectable ctDNA and such patients may not be candidates for following recurrence with ctDNA, especially in cases with less severe disease.[30] Alternatively, such cases might require more sensitive assays than what is currently available. Contrary to what was reported by Ashley and colleagues,[12] we did see a significant association of ctDNA levels before surgery and long-term patient outcome, as also reported by Casas-Arozamena and colleagues[31] While having detected pre-surgical ctDNA (vs undetected) was not associated with time to recurrence (Table S5) when focused on the subset of patients with pre-amplified detected ctDNA, there are significantly higher ctDNA values for patients who recurred.

In 6 of 8 relapsed endometrial cancer cases, ctDNA was detected prior or concurrent to the diagnosis of recurrence (Figs. 3 and 4). While a lack of aligned time points between blood draws and scans precludes a precise calculation of lead-time over conventional testing, the results show evidence supporting a lead-time of ctDNA detection before clinical detection of recurrence. Evidence for ctDNA lead-time was most striking in EM129, where the sustained ctDNA suggests continuing occult disease after adjuvant therapy that was undetected by conventional testing until recurrence diagnosis at 115 weeks post-surgery. These results are consistent with other data showing the ability of ctDNA to demonstrate lead-time to diagnosis of recurrence in endometrial cancer.[12,31] In other cancers, this lead-time has been found to be 4 to 10 months before radiographic detection following standard-of-care procedures.[17,18,32] Consistent with our findings in endometrial cancer, these cancers also have demonstrated prognostic value in post-surgical positive ctDNA.[33]

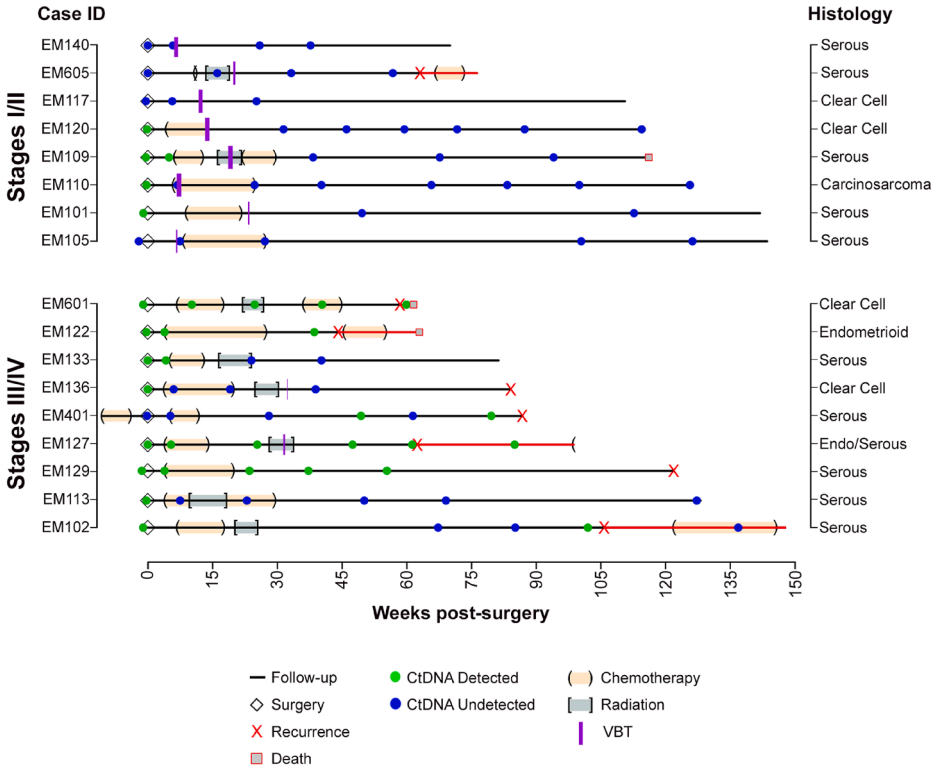

Figure 4 Swimmers plot.
Summary of ctDNA results for each patient with 3 or more blood draws followed longitudinally, associated with treatment and follow-up status. ctDNA, circulating tumor DNA; VBT, vaginal brachytherapy.

Strengths and Weaknesses

CtDNA assays have demonstrated utility as tumor-specific, highly-sensitive blood-based biomarkers and could supplement radiology, particularly in high-risk endometrial cancer. In some cases, ctDNA demonstrated a higher rate of detection than standard-of-care radiographic imaging or CA125 for detecting clinical recurrence in a longitudinal setting, albeit the sample size was small in this study. Cost is an additional benefit to this approach as compared to high-depth cfDNA sequencing. A personalized junction assay does have drawbacks, namely challenges in the adoption of the assay in a clinical lab environment and the need to sequence the tumors before panel construction. Detecting complex genomic rearrangements can be challenging, especially when they occur in repetitive or poorly mapped regions, or involve events like chromoplexy or chromothripsis. In our study, we identified multiple junctions per case, which allowed us to select high-coverage junctions more likely to be present across all tumor sub-clones and thereby improve ctDNA sensitivity. However, relying on single junctions may underestimate residual disease risk if not all sub-clones are represented. Our approach may be less effective for hypermutated tumors such as MSI or POLE, which typically lack translocations. For these cases, mutation panels may be more appropriate, though clonal hematopoiesis can complicate interpretation. Additionally, sequencing of future metastasis tumors may be advantageous to enable the panel to capture evolving clonal cell populations. Future studies benefiting from a larger cohort are needed to identify the contribution to prognosis etc. of ctDNA separately from other indicators of aggressive disease.

Implications for Practice and Future Research

With an increasing number of clinical trials to validate utility[34] and NCCN guideline adoption[35] of ctDNA detection or MRD, the methods used herein offer a personalized, highly-specific biomarker. In the future, the adoption of a personalized ctDNA-based assay could improve clinical practice by enabling physicians to monitor tumor progression or treatment response non-invasively and tailor treatment accordingly. Blood-based assays are logistically easier to accomplish and less expensive than imaging, and therefore ctDNA testing is well-positioned to allow closer monitoring of patients' disease course. Based on the evidence presented, clinical trials, which would increase case enrollment and blood draw frequency, are warranted to calculate any lead-time advantage of personalized ctDNA biomarkers over current standard-of-care disease detection measures.

CONCLUSIONS

Personalized ctDNA assays have demonstrated feasibility and clinical validity as tumor-specific, highly-sensitive blood-based biomarkers in high-risk endometrial cancer. In some cases, ctDNA demonstrated a higher rate of detection in the disease course than standard-of-care radiographic imaging or CA125 for predicting occult and recurrent disease. Based on the evidence presented, the value of pre-surgical ctDNA should be further investigated and high-volume clinical trials with standardized blood draw frequency are warranted to evaluate the rate of detection and potential lead-time advantage of ctDNA for clinical recurrence detection.

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Ethics Approval and Consent to Participate All methods were carried out in accordance with relevant guidelines and regulations, and all experimental protocols were approved by the Mayo Clinic Institutional Review Board studies: 15-005545 or 19-005326. Informed consent was obtained from all the participants and/or their legal guardians.

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Declaration of Competing Interest GV is the owner of WholeGenome LLC. ASM is supported by a Mark Foundation ASPIRE Award, NCI R21 CA251923, and Department of Defense W81XWH-22-1-0021 Concept Award. ASM also reports honoraria to his institution for participation in advisory boards from AbbVie, AstraZeneca, BeiGene, BMS, Genentech, and Inc., Janssen; Travel support and honoraria from Shanghai Roche Pharmaceuticals Ltd. and is non-remunerated member of the Mesothelioma Applied Research Foundation and Friends of Patan Hospital Board of Directors. No other authors report any conflicts of interest.

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Supplemental Material Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijgc.2025.102773.

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