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Detection of Circulating Tumor DNA in Plasma: A Potential Biomarker for Esophageal Adenocarcinoma

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Background. **Recent literature has demonstrated the potential of "liquid biopsy" and detection of circulating tumor (ct)DNA as a cancer biomarker. However, to date there is a lack of data specific to esophageal adenocarcinoma (EAC). This study was conducted to determine how detection and quantification of ctDNA changes with disease burden in patients with EAC and evaluate its potential as a biomarker in this population.**

Methods. **Blood samples were obtained from patients with stage I to IV EAC. Longitudinal blood samples were collected from a subset of patients. Imaging studies and pathology reports were reviewed to determine disease course. Tumor samples were sequenced to identify mutations. Mutations in plasma DNA were detected using custom, barcoded, patient-specific sequencing libraries. Mutations in plasma were quantified, and associations with disease stage and response to therapy were explored.**

Results. **Plasma samples from a final cohort of 38 patients were evaluated. Baseline plasma samples were ctDNA positive for 18 patients (47%) overall, with tumor allele frequencies ranging from 0.05% to 5.30%. Detection frequency of ctDNA and quantity of ctDNA increased with stage. Data from longitudinal samples indicate that ctDNA levels correlate with and precede evidence of response to therapy or recurrence.**

Conclusions. **ctDNA can be detected in plasma of EAC patients and correlates with disease burden. Detection of ctDNA in early-stage EAC is challenging and may limit diagnostic applications. However, our data demonstrate the potential of ctDNA as a dynamic biomarker to monitor treatment response and disease recurrence in patients with EAC.**

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The incidence of esophageal adenocarcinoma (EAC) in the United States and other Western countries has increased 300% to 600% during the past 40 years, with approximately 11,000 new cases annually in the United States alone.[1-3] Approximately 60% of new diagnoses are stage III and IV, where 2-year survival is less than 20%.[4,5] Even early-stage EAC can be aggressive, with 20% to 50% disease recurrence in patients with stage I and II cancer despite potentially curative perioperative therapy and resection.[4,6]

Most surgical candidates with EAC currently receive neoadjuvant chemotherapy with concurrent radiotherapy, because recent treatment protocols have improved response rates and 5-year survival among

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Abbreviations and Acronyms

ARID1A = AT-rich interactive domain-containing protein 1A
bp = base pairs
ctDNA = circulating tumor DNA
CTR = chemoradiotherapy
CT = computed tomography
cTNM = clinical tumor staging
Debarcer = De-Barcoding and Error Correction
EAC = esophageal adenocarcinoma
ERBB4 = receptor tyrosine-protein kinase erbB-4
FFPE = formalin-fixed paraffin-embedded
IGV = Integrated Genome Viewer
NGS = next-generation sequencing
PCR = polymerase chain reaction
PET-CT = positron emission tomography-computed tomography
POD = postoperative day
pTNM = pathologic tumor staging
SiMSen-Seq = Simple, Multiplexed, PCR-based barcoding of DNA for Sensitive mutation detection using Sequencing
TP53 = tumor protein 53
XRT = external beam radiotherapy
ypTNM = postneoadjuvant therapy tumor staging

select patients.[7,8] However, recurrence, detected by postoperative surveillance imaging or physical examination, is frequent.[6,9,10] Importantly, both modalities of diagnosing recurrence are inadequate at present. Because patient follow-up occurs at intervals of several months, there is potential for lead-time between recurrence and detection. In addition, imaging has reduced sensitivity in postoperative or irradiated tissues, is costly, and causes repeated radiation exposure. These barriers raise a clinical need for sensitive biomarkers that can be obtained at minimal risk and cost over short intervals.

A subset of patients will have disease progression despite therapy and are consequently not candidates for curative esophagectomy.[6] These patients may still benefit from palliative chemoradiation. This cohort commonly begins chemoradiation with the intent of undergoing resection, but metastatic disease subsequently develops in the interval between diagnosis and posttherapy restaging.[7] Retrospectively, these patients undergo weeks of ineffective chemoradiation, forgoing modified chemotherapeutic regimens or potentially curative esophagectomy plus adjuvant therapy.

Finally, patients who present with metastatic disease may benefit from biomarkers that improve clinicians' ability to prognosticate, allowing patients to make informed decisions regarding end-of-life care. Plasma-based biomarkers may permit real-time assessment of therapeutic response and guide oncologists to make appropriate decisions to continue, halt, modify, or restart therapeutic regimens.

The presence of circulating tumor (ct)DNA in plasma is well established.[11-13] In most patients, ctDNA represents a small fraction of the total circulating cell-free DNA[14] and is challenging to detect and quantify. Novel technologies, such as our sequencing approach,[15,16] enable detection of these rare tumor mutations in an abundance of normal circulating cell-free DNA. Several studies have indicated that the ctDNA burden correlates closely with overall disease burden.[11,14,17,18] Thus, ctDNA provides a potential "liquid biopsy" that can be used to monitor cancer progression and recurrence during treatment.

Several studies have looked at ctDNA in esophageal and gastrointestinal malignancies,[19-21] but these are small-scale or focus on esophageal squamous cell carcinoma. Because this novel concept has yet to be tested thoroughly in EAC, the objectives of this study were to detect and quantify stage-specific differences in EAC ctDNA and evaluate the utility of ctDNA for dynamic monitoring of therapeutic response and recurrence after resection.

Patients and Methods

Patient Recruitment

Patients were recruited from 2 sites: the University of Pittsburgh Medical Center and the University of Rochester Medical Center. All patients provided informed consent for use of their tissues and data in research projects, and all research was performed according to protocols approved by the Institutional Review Board at each participating institution. Patients were eligible for inclusion if they were undergoing medical or surgical treatment, or both, for any stage biopsy specimen-proven EAC. Samples were collected from each institution as follows:

- At the University of Pittsburgh, blood was drawn from patients who met inclusion criteria before biopsy or immediately before surgical resection. Tumor samples from the biopsy or resection specimen were obtained for DNA isolation and sequencing.
- At the University of Rochester, tumor tissue was obtained from the surgical pathology specimen or diagnostic biopsy specimen. Blood was obtained at an initial time as close as possible to the diagnosis, and longitudinal blood samples were obtained during scheduled visits with the surgical oncologist.

Data Extraction

Clinical data were extracted from patient medical records. Data included TNM category at diagnosis, type and fractions of chemoradiotherapy, imaging results, surgical reports, pathology results, and sentinel clinical events (recurrence, death). Data collection started at the time of diagnosis, and records were accessed at minimum every 6 months to update existing records.

Sample Preparation

BLOOD PROCESSING. Blood samples were drawn into tubes containing ethylenediaminetetraacetic acid and processed to separate plasma within 1 hour. Whole blood was centrifuged at 4°C for 10 minutes at 1600 relative centrifugal force. The plasma component was isolated and further centrifuged at 3600 relative centrifugal force for 10 minutes to remove all cells. Plasma was then aliquoted and frozen at −80°C until further use. Buffy coat, with a small amount of contaminating erythrocytes, was removed from the original spun sample and frozen at −80°C as a source of normal reference DNA.

EXTRACTION OF DNA FROM PLASMA AND BUFFY COAT. Frozen plasma samples were thawed at room temperature, and DNA was extracted using the QIAamp Circulating Nucleic Acid Kit (Qiagen, Hilden, Germany) using the vacuum protocol. Samples were eluted in 50 μL of buffer AVE (nuclease-free water with 0.04% sodium azide, Qiagen). Frozen buffy coat samples were thawed at room temperature, DNA was extracted using the QIAamp DNA Mini Kit (Qiagen), with 200 μL of buffy coat used for each extraction. Samples were eluted in 200 μL of buffer EB (10 mmol/L Tris-CL, pH 8.5; Qiagen). DNA concentrations were measured by fluorimetry (Qubit dsDNA HS Assay Kit; Thermo Fisher Scientific, Waltham, MA), and DNA was stored at −20°C until needed. If necessary, DNA was concentrated to approximately 5 to 20 ng/μL using a Vivacon 500 column with a 30,000 molecular weight cutoff (Sartorius, Göttingen, Germany).

EXTRACTION OF DNA FROM TUMOR SAMPLES. Tissue specimens were cut using a cryostat for frozen samples or microtome for formalin-fixed paraffin-embedded (FFPE) samples. One tissue section was stained with hematoxylin and eosin for pathologic verification of tumor, and 100 to 200 μmol/L of tissue was used for DNA extraction. Frozen tissue sections were cut directly into lysis buffer and extracted using the QIAamp DNA Mini Kit (Qiagen). Then, a 200 μL sample was processed and eluted in 200 μL of buffer EB. FFPE tissue sections were cut into 1.5 mL tubes and extracted using the QIAamp DNA FFPE Tissue Kit (Qiagen). Samples were eluted in 100 μL of buffer AE (10 mmol/L Tris-Cl, 0.5 mmol/L ethylenediaminetetraacetic acid, pH 9.0; Qiagen). The concentration of DNA was measured using a fluorometer (Qubit dsDNA HS Assay Kit, Thermo Fisher Scientific). Samples were diluted using buffer EB to obtain a final concentration of 5 to 20 ng/μL, then stored at −20°C until ready for use.

Identification of Mutations in Tumor Samples

Tumor DNA samples and matched normal DNA were sequenced using whole-exome or targeted next-generation sequencing (NGS) panels. Mutations in exome sequence data were identified as described previously.[22] Somatic mutations in targeted sequencing data were identified from .fastq files using the Mutect2[23] and Strelka[24] algorithms, and variant call files were generated. Anywhere from 1 to 15 mutations were selected per tumor, depending on the number of mutations identified, the observed variant allele frequency, and presence of the mutation in the Catalog of Somatic Mutations in Cancer database. Mutation sites in alignment files were visually checked in Integrated Genome Viewer (IGV; Broad Institute, Cambridge MA) to rule out obvious miscalls resulting from sequencing errors. If sufficient DNA was available, selected mutations were verified in the tumor using SiMSen-Seq (Simple, Multiplexed, PCR-based barcoding of DNA for Sensitive mutation detection using Sequencing), as described below.

Identification of Mutations in Plasma DNA

Mutations in plasma DNA were detected using digital polymerase chain reaction (PCR)[25] or a barcoded NGS method called SiMSen-Seq.[15,16] SiMSen-Seq is a PCR-based NGS library construction approach using molecular barcodes (random 12 base oligonucleotides) to tag each individual DNA target strand in the early PCR cycles of library construction. Barcoding greatly reduces background sequencing noise and enables variant allele detection at or below 0.1% allele frequency with 95% confidence.[16]

SiMSen-Seq Library Creation and Sequencing

SiMSen-Seq assays were designed to selected tumor mutations, tested, and validated as described previously.[15] All target regions were below 120 base pairs (bp) in length, but whenever possible, were kept below 80 bp (short assays). SiMSen-Seq libraries were generated from approximately 25 to 50 ng of tumor or plasma DNA. Custom libraries were created using specific assays designed to capture each tumor's selected mutations. Libraries were subsequently run on an MiSeq sequencer (Illumina, San Diego, CA) using single-end reads with 115 to 130 cycles, depending on amplicon length. Data (.fastq) files from the MiSeq runs were analyzed using a custom program called Debarcer (De-Barcoding and Error Correction) to calculate allele frequencies at each base of the amplicon.

A plasma DNA sample was considered positive if any previously called patient-specific mutation was present above background noise and with an alternative allele frequency of 0.05% or above. Patients with multiple mutations in the tumor required confirmation of 1 or more mutations in plasma to be considered positive.

Results

Samples from 63 patients were evaluated for use, and 8 were excluded due to insufficient normal DNA as a reference for sequencing. The remaining 55 tumor and matched normal samples were sequenced, and 8 were excluded because no mutations were identified. Of the remaining 47 patients, 29 had sufficient tumor DNA for verification of selected mutations using SiMSen-Seq, whereas 18 did not. Mutations did not verify in 7 patients, and these were excluded, leaving 40 patients with verified or presumed tumor mutations.

We selected 96 mutations from these 40 patients for development of sequencing assays, and at least 1 assay

Table 1. Frequency of Circulating Tumor DNA Positivity by Tumor Stage

Patients	Stage I	Stage II	Stage III	Stage IV	Total
All	1/7 (14)	4/8 (50)	8/17 (47)	5/6 (83)	18/38 (47)
Short amplicons	1/4 (25)	2/4 (50)	5/10 (50)	5/5 (100)	13/23 (57)
Long amplicons	0/3 (0)	2/4 (50)	3/7 (43)	0/1 (0)	5/15 (33)
Tumor mutations verified	1/4 (25)	2/4 (50)	5/9 (55)	4/4 (100)	12/21 (57)
Tumor mutations not verified	0/3 (0)	2/4 (50)	3/8 (37)	1/2 (50)	6/17 (35)
Short amplicons and verified mutations	1/4 (25)	2/4 (50)	5/9 (55)	4/4 (100)	12/21 (57)

Values are n (%).

was successfully developed for 39 of 40 patients (1-6 assays per patient). Baseline plasma samples were sequenced for all 39 patients, with 1 technical failure, leaving a final cohort of 38 patients with complete data (Supplemental Table 1). Of these 38 patients, 22 received no neoadjuvant therapy, and baseline plasma was collected immediately preoperatively. Pathologic (pTNM) staging was used to correlate stage with ctDNA amount.

Fourteen patients received neoadjuvant chemotherapy or chemoradiotherapy. For 9 of these, baseline blood samples were collected before or within 2 days of initiation of neoadjuvant therapy and clinical (cTNM) staging was used to correlate stage with ctDNA amount. For 5 patients receiving neoadjuvant therapy, blood was drawn at the time of procedure, and postneoadjuvant therapy (ypTNM) staging was used. Finally, 2 patients had documented metastases (stage IV) and underwent palliative therapy. Blood was collected from these patients before therapy initiation. This information is summarized in Supplemental Table 1.

Detection of ctDNA in Baseline Plasma Samples

The stage breakdown for the 38 patients was as follows: 7 stage I, 8 stage II, 17 stage III, and 6 stage IV. Baseline plasma samples were ctDNA positive for 18 patients (47%; Table 1), with tumor allele frequencies ranging from 0.05% to 5.30% (Figure 1; Supplemental Table 1). An example of a positive result is shown for a patient with stage IIB cancer (Supplemental Figure 1). Overall, the detection frequency of ctDNA increased with stage (Table 1), as did the mean quantity of ctDNA (Figure 1). Of note, short amplicons gave positive results in 13 of 23 patients (57%), whereas long amplicons were positive in only 5 of 15 cases (33%; Table 1). Similarly, for tumors with mutations verified by SiMSen-Seq, 12 of 21 plasma samples (57%) were positive vs 6 of 17 (35%) for samples in which DNA was not available for tumor verification (Table 1).

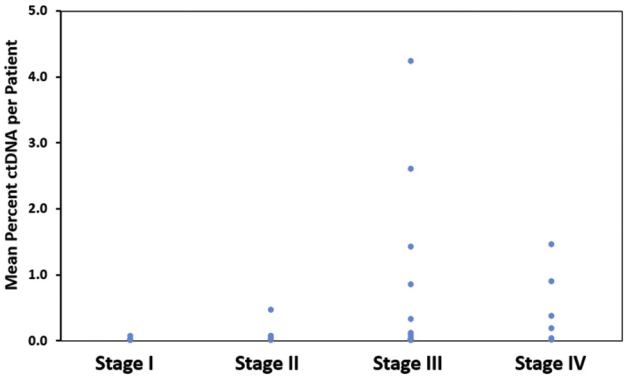

Figure 1. Mutant allele frequency in baseline plasma. Mean fraction (percent) of mutant tumor alleles detected in 38 patients with esophageal adenocarcinoma (EAC) separated by tumor stage at the time of blood draw. Higher stage at diagnosis is correlated with higher circulating tumor DNA (ctDNA) allele frequency (Spearman $\rho = 0.38$; $P = .019$).

Detection of Plasma DNA Longitudinally During Treatment Course

Longitudinal samples were available from 8 patients with verified mutations in their tumor. Two patients (M08 and M16) had metastatic disease at presentation and only baseline plasma was analyzed (both positive). Two additional patients (LA01 and LA08) demonstrated complete response to neoadjuvant therapy, followed by resection. In both cases, baseline plasma was negative for ctDNA, and these patients remain disease free after more than 500 days postoperatively. The 4 remaining patients were selected for sequencing of longitudinal plasma samples based on a potentially interesting clinical course and availability of plasma at key times. In total, 29 plasma samples were analyzed.

One patient (LA15; Figure 2) was diagnosed with cT3 N1 Mx EAC, and 2 tumor mutations, tumor protein P53 (*TP53*) and AT-rich interaction domain 1A (*ARID1A*), were identified. The patient underwent neoadjuvant chemoradiotherapy with cisplatin/paclitaxel and fractionated radiation (41.4 Gy in 23 fractions) over 5 weeks. ctDNA levels were initially positive (5.11% and 3.34%, respectively) 1 day into therapy, but were undetectable by the end of therapy and at a postneoadjuvant therapy positron emission tomography (PET)-computed tomography (CT), where partial response was noted by RECIST (Response Evaluation Criteria In Solid Tumors) criteria, maximum standardized uptake value decrease from 9 to 3.7, and no activity in 2 previously hot lymph nodes. The patient underwent an esophagectomy on postdiagnosis day 125. Pathologic stage was identical to clinical stage, and the resection pathologically R0. A follow-up visit on postoperative day (POD) 70 showed no concern for recurrence. Plasma DNA obtained on this date showed both previously identified mutations in circulation

(3.75%, 3.95%). Recurrence was subsequently documented on POD 80 when the patient presented to the emergency department with cerebral metastases causing intraparenchymal hemorrhage. The disease became widely metastatic, with ctDNA levels of both mutations increasing significantly despite palliative external-beam radiation, and the patient died on POD 235.

A second patient (LA15; Figure 3) was diagnosed with cT3 N1 M0 EAC and 2 tumor mutations, *TP53* and erbB-2 receptor tyrosine kinase 4 (*ERBB4*), were identified. The patient underwent neoadjuvant chemoradiotherapy with cisplatin/paclitaxel and fractionated radiation (41.4 Gy in 23 fractions) over 5 weeks. Plasma DNA showed the presence of both mutations at the beginning of neoadjuvant therapy (3.10%, 2.05%, respectively). Similar or increased ctDNA levels were also found toward the end of neoadjuvant therapy. A PET-CT after the neoadjuvant therapy showed progressive disease, with new focal peritoneal infiltration and ascites. Palliative chemotherapy was started on postdiagnosis day 148, with a pretreatment CT showing diffuse omental thickening and moderate ascites. Plasma DNA showed mutations present 30 days into palliative therapy, and a CT showed stable peritoneal disease and worse ascites. Both plasma markers decreased 1 month later, then rose slightly before a repeat CT showed a decrease in omental thickening plus resolution of ascites. Tumor markers continued to rise over the remaining month of palliative therapy, and after. A CT after treatment showed reaccumulation of ascites and worsened peritoneal disease on day 335. The patient died on day 369.

ctDNA levels in the remaining 2 patients were noninformative. One patient with stage IIIA cancer (LA06; Supplemental Figure 2) underwent neoadjuvant chemoradiotherapy but progressed. New metastatic lesions developed, and the patient was not offered an operation. This patient underwent palliative chemotherapy but continued to progress and died. The baseline plasma sample was positive for 3 mutations, but 6 additional samples taken during therapy were negative. A seventh sample taken after completion of palliative therapy was positive for ctDNA.

Finally, a patient with stage IIIB cancer (LA05; Supplemental Figure 3) underwent neoadjuvant therapy with a partial response in the primary tumor, followed by esophagectomy. Resection was R0, and pathologic stage with moderate (grade II of III) response was noted. The patient was clinically well 3 months postoperatively, but disease recurred on POD 160, diagnosed by PET-CT. All plasma samples were negative for ctDNA for 6 verified mutations.

Comment

The data from this large study evaluating ctDNA as a biomarker in EAC show that ctDNA is detectable in all stages of EAC and that the detection rate increases with stage. However, detection rates appear to be lower than for other tumor types.[11] Similarly, the fraction of ctDNA detected increases with tumor burden (stage), although there was substantial variability between individual

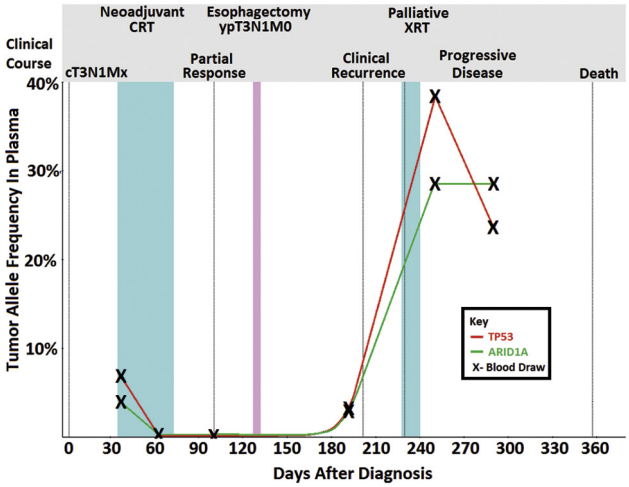

Figure 2. Levels of circulating tumor DNA (ctDNA), correlated with treatment course, in patient 1. (ARID1A, AT-rich interactive domain-containing protein 1A; CRT, chemoradiotherapy; TP53, tumor protein 53; XRT, external beam radiotherapy; ypTNM = postneoadjuvant therapy tumor staging.)

patients within each stage. This could reflect our using both clinical and pathologic staging of tumors, where clinical staging is known to have limited accuracy for EAC. Despite this limitation, our data agree with literature from other tumor types where detection rate and fraction of ctDNA both increase with stage.

During this project, our assay designs and methods evolved based on new literature and our own findings. Developing new digital PCR assays for each patient was time consuming and limited our analysis to only 1 or 2 mutations per patient owing to limited amounts of DNA. SiMSen-Seq overcame these problems but also evolved during the study. Most notably, PCR amplicon lengths for

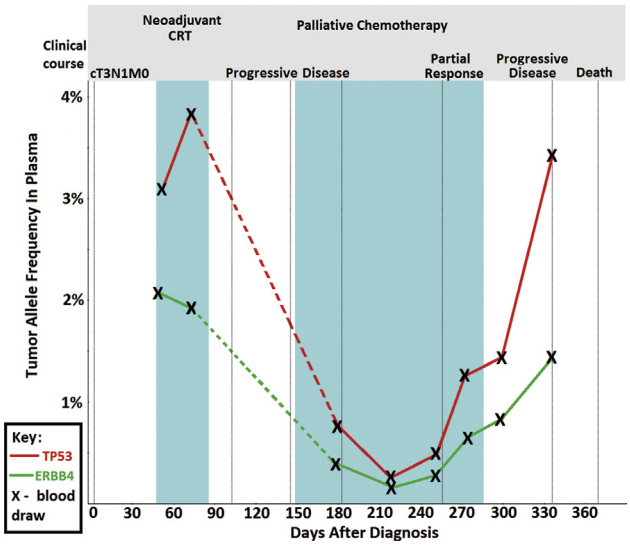

Figure 3. Levels of circulating tumor DNA (ctDNA), correlated with treatment course, in patient 2. (CRT, chemoradiotherapy; ERBB4, receptor tyrosine-protein kinase erbB-4; TP53, tumor protein 53.)

SiMSen-Seq assays were originally kept below 120 bp, because ctDNA averages approximately 160 bp in length.[26] However, it became clear that smaller amplicons (<80-85 bp) were detected more frequently than the larger amplicons, prompting our change in design criteria. In addition, although all tumor sequence data were reviewed in IGV, we discovered a relatively high failure rate when verifying the selected mutations.

Because DNA was not available for verification in all patients, our detection rates may be artificially low. When exploring only patients with verified tumor mutations and evaluated with short amplicons, the overall detection rate increased to 57%, including 100% detection in patients with stage IV cancer. Detection in patients with stage I and II cancer remained less than 50%; however, indicating that although an attractive concept, early detection of esophageal adenocarcinoma by liquid biopsy remains an elusive prospect. It will be interesting to see in future studies whether ctDNA positivity in early-stage disease correlates with prognosis. Even if sensitivity can be improved, successful implementation of a liquid biopsy screening test would require a dedicated assay panel designed to capture the mutational heterogeneity seen in EAC. This is further complicated by the fact that Barrett esophagus, the precursor to EAC, harbors similar mutational changes.[27]

Although screening may be a challenge, our longitudinal data demonstrate that ctDNA correlates with, and in some cases precedes by several weeks, disease response to therapy and progression and recurrence in otherwise asymptomatic EAC patients, as demonstrated by imaging, and thus may have clinical value. Successful monitoring of treatment response carries several important implications. First, it provides physicians with a rapid assessment of the success of the treatment choice and may allow for modification of therapeutic regimens if inadequate response is noted. Second, poor clinical response to neoadjuvant treatment may be identified early during therapy, thereby allowing changes in the treatment plan, including surgical management.

For patients undergoing potentially curative esophagectomy, local or distant recurrence is, unfortunately, common. Recurrence is currently identified by clinical symptoms, such as dysphagia and weight loss, or by surveillance imaging with CT and PET-CT. By contrast, our data indicate that ctDNA levels can be detected in some EAC patients before documentation of recurrence. Although lead time was only 10 days in this example, data from other tumor types have shown lead times as long as 15 months.[28]

Although limited data exist, patients with symptomatic EAC recurrence demonstrate poor survival and quality of life despite treatment.[9] Studying survival and quality of life is difficult if recurrence is diagnosed while a patient is asymptomatic. However, earlier diagnosis of recurrence may prompt earlier initiation of palliative therapy and confer a survival benefit. In addition, earlier detection of recurrence would allow patients and families to make better informed decisions regarding treatment plans and goals of care before manifestations of recurrence.

Using ctDNA for monitoring treatment response and recurrence in EAC has some limit ations. First, detection rates for patients with stage I cancer was 14%, limiting the utility of this test as a screening tool for primary disease. Early detection of local recurrence could be problematic, but most recurrences are nodal or systemic, where the tumor has access to lymphatic or systemic circulation.[29]

Second, differences in neoadjuvant treatment regimens and individual tumor biology may produce variable results regarding tumor response to therapy, timing, and amount of ctDNA released, which can confound the amount of ctDNA detected. This will become an important question if ctDNA detection becomes part of standard treatment protocols and is a future direction of study.

Finally, ctDNA is not always detectable, even in patients with progressive or recurrent disease. A negative ctDNA result is therefore much like a negative imaging result and needs to be interpreted with caution.

There are also limitations to the study itself. Initial plasma draws were obtained close to the time of diagnosis for all patients but occurred at the patient's discretion. This meant the plasma draw for some patients occurred before the initiation of therapy, whereas the draw for others occurred shortly after therapy commenced, and yet others were well into treatment. Draw times after therapy were highly variable as well. This confounder may impact our ability to detect and quantify ctDNA, and further studies need timely ascertainment of blood samples relative to clinically sentinel time points. In addition, the longitudinal blood draws occurred close to times of clinical importance in only 4 patients. This small data set limits our ability to draw conclusions regarding the use of ctDNA as a dynamic biomarker for monitoring EAC treatment response and recurrence. However, we do demonstrate that it has potential utility, and more standardized trials are needed to fully evaluate this.

In summary, we have explored the clinical value of ctDNA as a biomarker in EAC. Our data show that ctDNA is detectable at all stages of disease, that detection rates are higher with higher stage disease, and that ctDNA quantity increases with increasing stage. In addition, we show that quantification of ctDNA levels during the treatment course may be useful in some patients for determining response (or lack thereof) to therapy and for detection of tumor recurrence after definitive treatment.

Importantly, ctDNA levels appear to be dynamic and can precede imaging studies for monitoring response and recurrence. Thus, although ctDNA levels were not clinically informative in all patients in this study, our data support further investigation in larger cohorts. Future studies should evaluate the prognostic value of ctDNA in patients with early-stage disease, the value of response monitoring during neoadjuvant therapy, and lead time for recurrence detection compared with imaging studies.

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