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Title:

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Date:

2022-08-01

Citation:

Cabalag, C. S., Yates, M., Corrales, M. B., Yeh, P., Wong, S. Q., Zhang, B. Z., Fujihara, K. M., Chong, L., Hii, M. W., Dawson, S. J., Phillips, W. A., Duong, C. P. & Clemons, N. J. (2022). Potential Clinical Utility of a Targeted Circulating Tumor DNA Assay in Esophageal Adenocarcinoma. *Annals of Surgery*, 276 (2), pp.E120-E126. <https://doi.org/10.1097/SLA.0000000000005177>.

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Potential Clinical Utility of a Targeted Circulating Tumor DNA Assay in Esophageal Adenocarcinoma

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Objective: To explore the clinical utility of circulating tumor DNA (ctDNA) in esophageal adenocarcinoma (EAC) by developing a cost-effective and rapid technique utilising targeted amplicon sequencing.

Summary of background data: Emerging evidence suggests that levels of ctDNA in the blood can be used to monitor treatment response and in the detection of disease recurrence in various cancer types. Current staging modalities for EAC such as computerised tomography of the chest/abdomen/pelvis (CT) and positron emission tomography (PET) do not reliably detect occult micro-metastatic disease, the presence of which signifies a poor prognosis. After curative-intent treatment, some patients are still at high risk of recurrent disease, and there is no widely accepted optimal surveillance tool for patients with EAC.

Methods: Sixty-two patients with EAC were investigated for the presence of ctDNA using a tumor-informed approach. We designed a custom targeted amplicon sequencing panel of target specific primers covering mutational foci in 9 of the most commonly mutated genes in EAC. Serial blood samples were taken before and after neoadjuvant treatment (NAT), and during surveillance.

Results: Somatic mutations were detected in pre-treatment biopsy samples of 55 out of 62 (89%) EAC patients. Mutations in *TP53* (80%) were

the most common. Out of these 55 patients, 20 (36%) had detectable ctDNA at baseline. The majority (90%) of patients with detectable ctDNA had either locally advanced tumors, nodal involvement or metastatic disease. In patients with locally advanced tumors, disease free survival (DFS) was more accurately stratified using pre-treatment ctDNA status [HR 4.34 (95% CI 0.93–20.21); $P = 0.05$] compared to nodal status on PET-CT. In an exploratory subgroup analysis, patients who are node negative but ctDNA positive have inferior DFS [HR 11.71 (95% CI 1.16–118.80) $P = 0.04$]. In blood samples taken before and following NAT, clearance of ctDNA after NAT was associated with a favourable response to treatment. Furthermore, patients who are ctDNA positive during post-treatment surveillance are at high risk of relapse.

Conclusions: Our study shows that ctDNA has potential to provide additional prognostication over conventional staging investigation such as CT and PET. It may also have clinical utility in the assessment of response to NAT and as a biomarker for the surveillance of recurrent disease.

Keywords: circulating tumor DNA, esophageal cancer, tumor staging

(*Ann Surg* 2022;276:e120–e126)

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Funding: This research was supported by a Grant-In-Aid (APP 1156945 to NJC, WAP, CPD and SJD) from the Cancer Council Victoria a research grant from the AVANT foundation, and a Translational Research Project grant (TRP15012 to WAP, NJC and CPD) from the Department of Health and Human Services acting through the Victorian Cancer Agency, Victoria, Australia. CSC is the recipient of, and supported by, The Alan & Kate Gibson Research Fellowship, 2018. NJC is supported by a Fellowship (MCRF16002) from the Department of Health and Human Services acting through the Victorian Cancer Agency, Victoria, Australia. PY is supported by grant funding administered by the Snowdome Foundation, Maddie Riewoldt's Vision and an NHMRC/MRFF investigator grant (#1195030). Data sharing statement: Forward and reverse primer sequences for amplicon design, individual participant clinico-pathological data, baseline variant calling, follow-up variant calling, and outcomes are provided in the supplementary tables, <http://links.lww.com/SLA/D402>. For raw sequencing data of individual patients, please submit a request to the corresponding author, Associate Professor Nicholas J Clemons.

The authors report no conflicts of interest.

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ISSN: 0003-4932/22/27602-e120

DOI: 10.1097/SLA.0000000000001577

Overall survival after treatment for esophageal adenocarcinoma (EAC) is amongst the lowest of any cancer type, with five-year survival rates below 20%.¹ Furthermore, approximately 60% of patients with apparent localised disease recur within 5 years of resection likely due to undetectable metastatic disease at the time of diagnosis.² This emphasises the importance of accurate tumor staging to optimise patient treatment and outcomes. Current staging for newly diagnosed EAC includes endoscopic ultrasound, computerised tomography (CT) of the chest/abdomen/pelvis and positron emission tomography (PET). Laparoscopy with peritoneal cytology is used for staging of gastro-esophageal junction tumors. A retrospective analysis of pre-operative staging of EAC by CT, endoscopic ultrasound and PET-CT estimated nodal staging accuracies of just 74–77% after validation with post-operative histological staging,³ raising the possibility of undetected lymph node involvement in up to 25% of patients. Similarly, recurrent EAC is often not diagnosed until the patients are symptomatic with a relatively large volume of disease.⁴ The ability to detect occult or low volume EAC not seen on current imaging modalities would allow for improved prognostication and could also act as a tool for monitoring response to second line therapies. Thus, a simple, accurate biomarker that can detect occult disease is likely to have substantial clinical impact.

One emerging strategy for sensitive, disease-specific monitoring in cancer is analysis of circulating tumor DNA (ctDNA). There are a number of studies suggesting the possible utility of ctDNA in monitoring response to treatment, disease progression and recurrence. ctDNA detection rates have been shown to

increase with more advanced disease stage and tumor burden across a number of cancer types.⁵ Furthermore, in patients treated with curative intent, a detectable post-treatment ctDNA is associated with inferior DFS.⁶

The aim of this pilot study was to ascertain the presence of ctDNA in the different stages of EAC patient treatment: at diagnosis, after neoadjuvant treatment or surgical resection, and during the surveillance period; and the relationship with cancer-specific outcomes. We used a unique custom-designed panel interrogating 9 commonly mutated driver genes in EAC using targeted amplicon sequencing (TA-seq).

METHODS

Patient Cohort and Sample Processing

Sixty-two EAC patients with clinical tumor stage of localised (cT1-cT2/N0; n = 7), locally advanced (cT3-cT4/N0 or N+,N0 n = 30, N+ n = 14), or metastatic disease (n = 11) were included into our study (Fig. 1). Neoadjuvant treatment (NAT) consisted of chemo-radiation with 5-FU/Cisplatin and 50.4 Greys or CROSS protocol.⁷ Tumor regression grade (TRG) was classified according to the modified 3-point Mandard scoring system.⁸ Tissue biopsies were collected during routine endoscopy and stored at -80°C until DNA extraction. Blood was collected in EDTA tubes and processed within two hours. Where possible, serial blood samples were collected from patients at baseline and at different stages of treatment, such as following NAT, surgery,

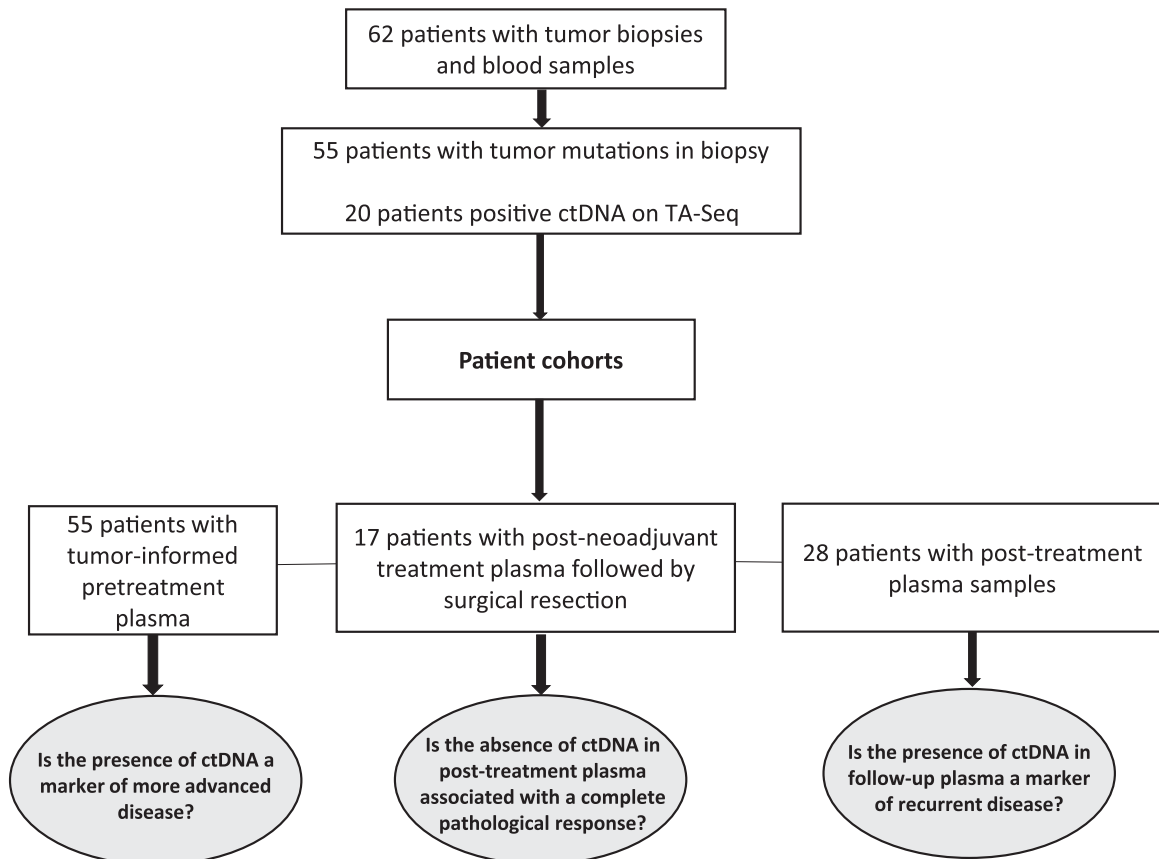


FIGURE 1. Study flow diagram showing patient cohorts used for analysis to address clinical questions. (TA-Seq: Targeted Amplicon sequencing). Clinical questions are circled in grey.

and during their remission period. Refer to supplementary methods, <http://links.lww.com/SLA/D401> for further details. Relevant ethics approval was granted by the Peter MacCallum Cancer Centre Human Research Ethics Committee (HREC numbers 10/108 and 18/211) and written informed consent was obtained from all patients.

Identification of Somatic Mutations by Targeted Amplicon Sequencing

The Catalogue of Somatic Mutations in Cancer database, as well as large EAC sequencing studies were used to identify recurrently-mutated genes and visualise mutation sites across them.⁹ Based on the frequency and site of mutations, primers were designed to amplify a panel of 104 amplicons covering frequently-mutated regions in 9 of the most frequently mutated genes in EAC (*APC*, *ARID1A*, *CDKN2A*, *KRAS*, *NRG1*, *PIK3CA*, *SMAD4*, *SMARCA4*, *TP53*). TA-seq was performed on matched tumor, white-blood cell and plasma samples in a multiplex fashion using the 48.48 Access Array system (Fluidigm, San Francisco, CA) with MiSeq (Illumina, San Diego, CA).

Statistical Analysis

Clinico-pathological patient data were summarised using descriptive statistics. Fisher's exact test was used to compare associations between categorical variables. DFS was calculated from the date of referral/diagnosis to the time of detection of clinical recurrence. Kaplan-Meier survival curves, hazard ratios and the corresponding 95% confidence intervals (CI) were calculated for locally advanced patients using GraphPad Prism 9.0. Log-rank test was performed to assess the statistically significant survival differences in the variables PET nodal status (node negative/positive) and ctDNA status (negative/ positive) respectively using the R package *survival* version 2.39-5. A subgroup analysis was also performed for the group of patients with PET node negative to assess their survival differences with respect to the ctDNA variable. Proportionality assumption was checked in all the analyses using the Grambsch-Therneau test¹⁰ and no violations were found. Statistical analyses were performed using GraphPad Prism 9 and R statistical software (Vienna, Austria), version 1.3.1056.¹¹ Atwo-tailed P-value of less than 0.05 was determined to be statistically significant difference for all the analyses.

RESULTS

ctDNA Improves Pre-treatment Tumour Staging

Pre-treatment tumor biopsies of 62 patients were analysed for somatic mutations using our customised gene panel. Among the 62 patients evaluated, 55 patients (89%) had an identifiable somatic mutation in their pre-treatment tumor biopsy in at least one gene of the 9-gene panel (Fig. 2A), with the most common mutation in *TP53* (80%). Of the 55 patients with an identified somatic mutation in the tumor biopsy, 20 (36%) were positive for ctDNA in their matched baseline blood sample as determined by TA-seq (Fig. 2A, green squares). For all baseline plasma samples, mean targeted sequencing coverage was 1730 fold (202–3289). Focussing only on patients with a detected tumor mutation (n = 55), the proportion of patients who were ctDNA positive increased in patients with metastatic disease (Fig. 2B).

We assessed the prognostic utility of pre-treatment ctDNA in locally advanced patients with EAC (n = 39) by comparing it to pre-treatment nodal status on PET-CT as a known prognostic indicator. Positive nodal status according to PET-CT did not significantly predict for inferior DFS in our cohort [HR 2.15 (95% CI 0.47–9.86); *P* = 0.29; Fig. 2C]. In the same population, patients who were positive for ctDNA at baseline were at higher

risk of recurrent disease [HR4.34 (95% CI 0.93 – 20.21); *P* = 0.05; Fig. 2D]. Importantly, we discovered in a sub-group analysis that nodal negative locally advanced patients who were positive for ctDNA suffer from earlier relapse [HR 11.71 (95% CI 1.16 – 118.80); *P* = 0.04; Fig. 2E]. Hence in patients with locally advanced disease, the use of ctDNA was a more accurate prognostic indicator over PET-CT alone and may also serve to identify a sub-group of node negative patients at high risk of recurrent disease.

Post-NAT ctDNA Status Before Surgery may Predict Pathologic Response

In a cohort of patients who underwent NAT, blood samples were taken pre-surgery (n = 17). The median time between the end of NAT and the post-treatment blood draw was 34days (range: 22–101 days). In pre-treatment ctDNA of patient P3, the variant allele frequency (VAF) of detected mutations decreased to undetectable levels post-NAT whilst the opposite occurred in P49 (Fig. 3A). We compared the relationship between post-NAT ctDNA status (positive or negative) and indicators of treatment response such as re-staging PET scan and TRG (Fig. 3B). In patients with detectable ctDNA post-NAT, five out of six patients had residual disease (TRG 2/3) on pathological examination. Interestingly, the restaging PET in one patient with residual disease showed a complete metabolic response. In patients with undetectable ctDNA post-NAT, 8 out of 11 patients had no residual disease (TRG 1). However, re-staging PET revealed only a partial metabolic response in four of these patients. Ultimately, there was no significant association between re-staging PET metabolic response and pathologic response (Fig. 3C, top panel). However, there was a significant association between patients with detectable ctDNA post-NAT and residual disease on pathological examination (*P* < 0.05, Fig. 3C, bottom panel).

ctDNA Status During Post-Treatment Surveillance is a Potential Biomarker for Recurrent Disease

Twenty patients with non-metastatic disease at diagnosis had post-treatment (after definitive chemoradiation or after surgical resection) follow-up plasma collections for ctDNA analysis. In patients with positive ctDNA after treatment whose disease recurred (n = 4), the median time from post treatment positive ctDNA detection to clinical/radiological detection of recurrent disease was 194 days (154–245 days). Importantly, this figure is likely to be an under-estimate as not all patients had regular blood collection intervals. Patients who become ctDNA positive during surveillance are at higher risk of relapse [HR7.85 (95% CI 1.23–50.10) *P* = 0.02], with the majority of patients succumbing to recurrent disease within 2 years from the end of treatment (Fig. 4A). Median follow-up was 457 days (30–1124). In three (P3, P5, P45) cases, ctDNA was detected before the onset of symptoms related to recurrent disease. For example, a rise of ctDNA VAF was indicative of recurrent metastatic disease inpatient P5 in the *absence* of any specific clinical symptomatology (Fig. 4B). ctDNA using ddPCR during the follow-up period had shown an increase in VAF of their truncal *TP53* mutation from 0% at 1 month after surgical resection to 0.8% and 23% at 4 and 8 months, respectively. Approximately 11 months after surgical resection, the patient was referred for a CT scan for nonspecific abdominal pain, which revealed hepatic metastasis confirmed on PET-CT.

DISCUSSION

In this pilot study, we have demonstrated potential clinically utility of ctDNA in the following ways: 1) baseline ctDNA status may sub-stratify patients with locally advanced disease

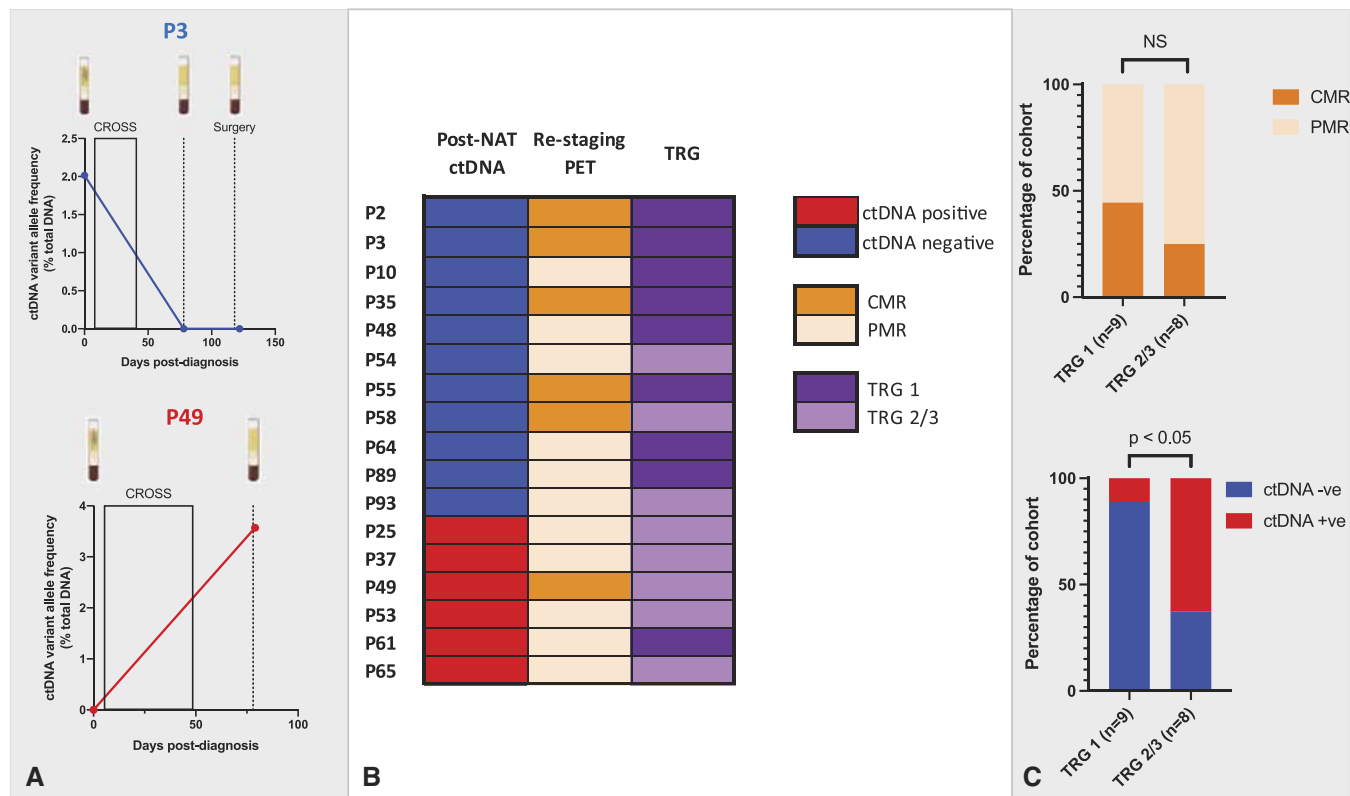


FIGURE 3. Post-neoadjuvant treatment (NAT) ctDNA status is a potential surrogate marker for pathologic response before surgical resection. (A) ctDNA VAF before and after neoadjuvant treatment in patients P3 and P49. Each vial represents time at which blood for ctDNA analysis was taken. (B) The relationship between post-NAT ctDNA status, re-staging PET scan and tumor regression grade (complete metabolic response;partial metabolic response;tumor regression grade-modified three-point Mandard score). (C) Comparison of PET (top panel) and ctDNA status (bottom panel) as markers of assessment after neoadjuvant treatment. Fisher exact test was used for comparison between groups. (NS: not significant).

and are consistent with two recently published studies of ctDNA in EAC.^{14,15} Another potential limitation is the fact that our panel of genes was curated from studies of EAC in patients originating from North America and Europe, thus possibly making it less applicable to patients of Asian ethnicity.

We have demonstrated that TA-seq can be used to detect ctDNA at diagnosis as well as in the post-treatment surveillance setting. Consistent with our results, Egyud et al.¹⁶ also found both the frequency of ctDNA detection, and also allele frequency, increased with more advanced disease. In another study by Ueda and colleagues conducted specifically in ESCC using a larger custom-built panel of 53 genes, 11/13 (83%) patients were positive for ctDNA, with TP53 being the most common mutation.¹⁷ Bettgowda et al.⁵ analysed 1000 genes and detected ctDNA in seven out of seven (100%) metastatic gastro-esophageal cancer patients and in 57% of local and locally advanced gastro-esophageal cancers. In Ueda's and Bettgowda's studies the rate of ctDNA detection is higher compared to ours and this is possibly due to the wider coverage of genes in their panel. Nevertheless, consistent with our findings, both studies demonstrate a higher detection rate in more advanced disease with mutations in TP53 being the most common.

Two recent studies of ctDNA in EAC^{14,15} used a more sensitive method of detection termed Cancer Personalized Profiling by deep Sequencing (CAPP-Seq) that utilises biotinylated oligonucleotides (known as 'selectors') to target the

exons of recurrently mutated genes based on the patient's unique tumor biopsy. Consistent with our results, they found that ctDNA positivity after curative intent-treatment was associated with inferior DFS. The increased sensitivity of the CAPP-seq technique, along with the much larger number of potential mutations screened, potentially explains why their ctDNA detection rate was significantly higher than ours. However, the cost of CAPP-Seq is considerably higher than TA-seq making it less cost-effective for routine screening and surveillance, especially in the case of EAC, where the dominant mutations are primarily in a relatively small number of genes. Importantly, 7 out of 10 of the most common gene mutations present at baseline and post-treatment from Ocock's study exist in our customised gene panel, thus reinforcing the clinical utility of a *targeted* ctDNA assay for use in EAC. Another limitation is the small number of genes in our current panel. However, we believe that our panel is an appropriate compromise between coverage of the most informative mutations and cost. Consequently, to detect ctDNA at baseline, we propose that a 'two-stage' approach using two different custom assays may be a potential strategy. Initially, the current assay could be used to identify the majority of patients with a mutation whilst the second assay consisting of a wider panel of genes could be used if the first assay is negative.

In summary, our study demonstrates the potential for ctDNA to add additional prognostic information in patients with node-negative locally advanced tumors and to identify

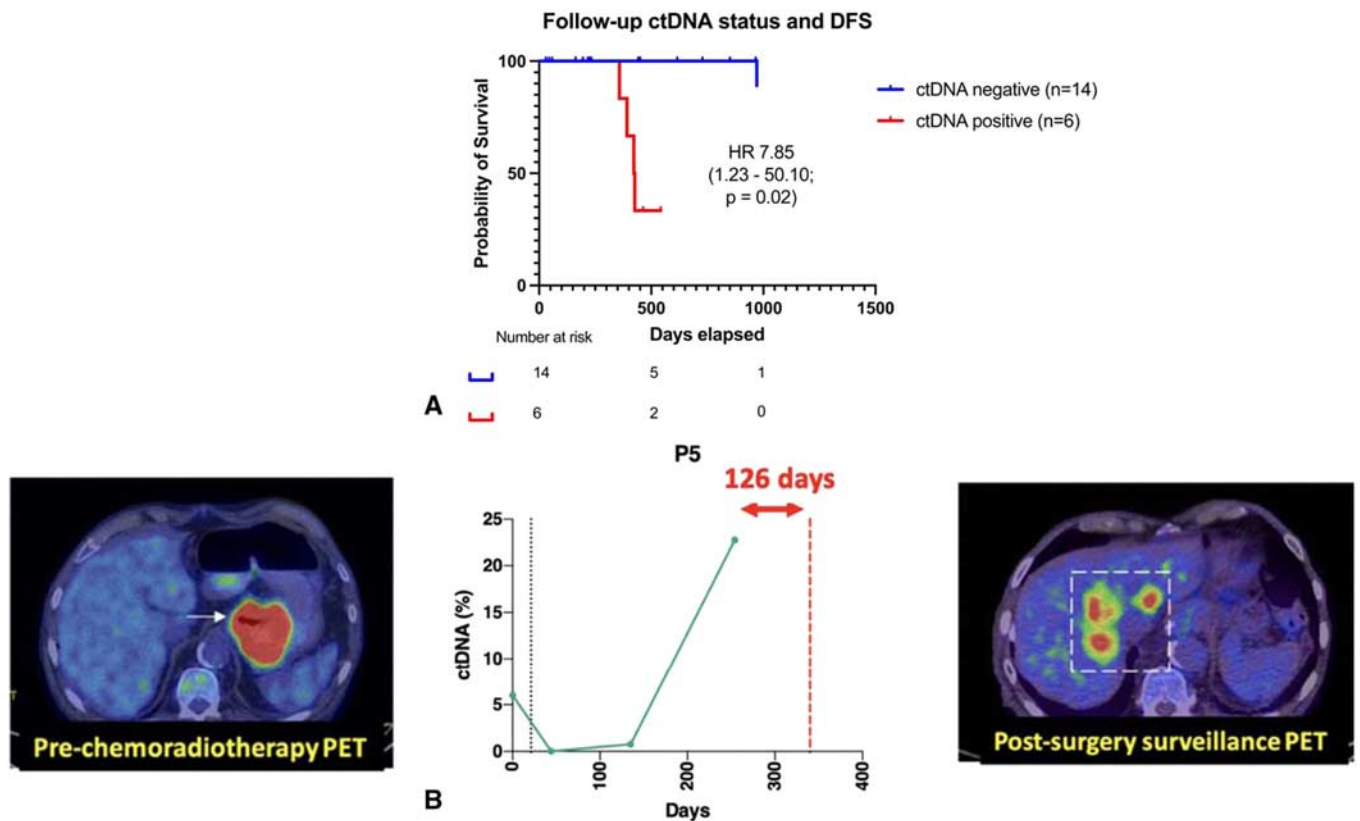


FIGURE 4. Use of ctDNA as a bio-marker for the surveillance of recurrent disease. (A) Kaplan Meier estimates of DFS in follow-up patients according to post-treatment ctDNA status. Time to disease recurrence was measured from the date of treatment completion (definitive chemoradiation or surgical resection). Hazards ratios (HR) were calculated using log-rank (Mantel-Cox) tests. (B) Detection of ctDNA can precede clinical detection of recurrent disease. Mean ctDNA VAF (ctDNA%) tracked over time (days) from diagnosis to clinical detection of recurrence in P5. The dotted black line indicates when surgical resection was performed. The red dashed line indicates when recurrence was detected by PET-CT. Blood collection was performed at 4 distinct time points and analysed for ctDNA using TA-Seq and ddPCR for the baseline blood sample and only ddPCR thereafter. The pretreatment PET image on the left demonstrates a locally advanced gastro-esophageal junction tumor (white arrow). The follow-up PET image on the right taken at 267 days after the start of treatment shows multiple hepatic metastases (white box).

patients at high risk of recurrent disease after curative-intent treatment. Our findings provide proof-of-principle and now require further studies to confirm the clinical utility of ctDNA in EAC. Future clinical trials should also further explore the role of ctDNA in surveillance after curative intent treatment and clarify the frequency of testing necessary for the optimal early detection of recurrent disease.

ACKNOWLEDGMENTS

Firstly, we acknowledge Professor David Watson for his advice with the initial study design and conception. We would like to acknowledge the staff of Professor Sarah Jane Dawson's laboratory in particular Rishu Agrawal, Maria Silva, Chen-Fang Weng, and Sarah Ftouni for their help with blood sample processing and sequencing. We thank the scientists at Molecular Genomics Core in particular Stuart Craig and Bernard Le for their assistance in next generation sequencing. Moreover, we also acknowledge Madawa Jayawardana for his help in the statistical analysis. Lastly, we would like to thank all the patients who have kindly donated their time, tissue and blood.

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