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Multiplexed real-time polymerase chain reaction cell-free DNA assay as a potential method to monitor stage IV colorectal cancer

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ABSTRACT

Background: Liquid biopsy is a new area in cancer diagnostics that measures cell-free DNA in plasma from tumor that may serve as a monitoring tool in colorectal cancer patients.
Methods: Multiplexed real-time polymerase chain reaction based on multicopy retro-transposable elements (targeting 80 base pair and 265 base pair sequences and an internal-positive-control) was used to evaluate the ability of cell-free DNA concentration and DNA Integrity Index to discriminate cancer from healthy patients. A cohort of 40 healthy controls and 39 stage IV colorectal patient's plasma were interrogated. The potency of each biomarker was measured by using receiver operating characteristic curves and derived area under the curve measures.
Results: Significant differences in cell-free DNA concentration and DNA integrity index were observed between controls and stage IV patients with a limit of detection <0.1 pg/μL. Investigation of the ability of both biomarker candidates to differentiate cancer from healthy patients showed an area under the curve of 0.9891 and 0.9859 for 80 base pair and 265 amplicons respectively and 0.8603 for DNA integrity index-265/80.
Conclusions: After establishing differences in cell-free DNA levels between healthy and treated and untreated stage IV patients, the multiplexed real-time polymerase chain reaction measurements of retro-transposable elements in cancer patient plasma potentially possess the ability to monitor therapy responsiveness in near real time.

Introduction

Colorectal cancer is currently the second leading cause of cancer related mortality in the United States.[1,2] Each year nearly 1.4 million people are diagnosed with new cases of colon cancer worldwide, while >700,000 patients die from colorectal cancer during that same time period.[1–3] Fortunately, early detection, diagnosis, and treatment of colorectal cancer through endoscopic or surgical approaches frequently result in excellent long-term outcomes, with nearly a ninety percent 5-year survival.[2,3] Unfortunately, patients with locally advanced disease achieve less than optimal outcomes, with a lower than 70% 5-year survival and those with metastatic disease achieving a dismal 13% 5-year survival.[4] Even when adequately treated these colorectal cancer patients have nearly a 70% to 80% tumor recurrence or develop a secondary metastases with more than half occurring in the liver.[2,4]

The presence of circulating tumor cells and cell free DNA has long been recognized in cancer patients.[5,6] Liquid biopsy is a noninvasive method of studying alternations in the levels and nature of circulating cell free DNA.[7,8] These diagnostics tests can detect molecular alterations in tumor specific mutations including activating or resistance genes.[9–18] The majority of commercially available liquid biopsy techniques utilizes next generation sequencing to identify the presence of mutations present within the tumor, but without the need for a biopsy. However these methods are expensive, take significant time to complete, are

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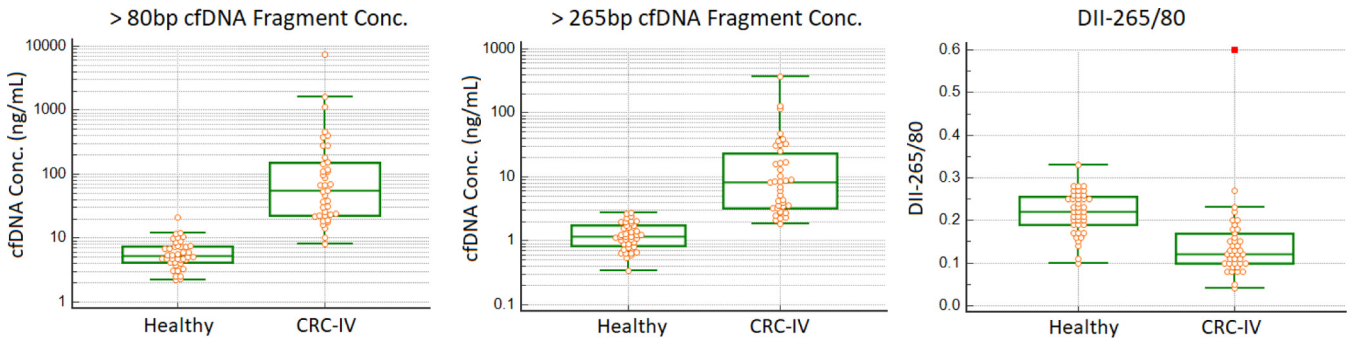

Fig 1. Box-and-whisker plot for cfDNA concentrations and DII-265/80. The central box represents the values from the 25 to 75 percentiles. The middle line represents the median.

Table I
Mean and standard deviation of plasma cfDNA concentrations of fragment sizes longer than 80 bp and 265 bp and DII-265/80

	Disease status	>80 bp (ng/mL)	>265 bp (ng/mL)	DII-265/80
Mean ± SD	Healthy controls ($n = 40$)	6.25 ± 3.49	1.31 ± 0.60	0.219 ± 0.050
	CRC-IV patients ($n = 39$)	351.49 ± 1,199.65	26.74 ± 63.04	0.145 ± 0.088

SD, standard deviation; *CRC-IV*, colorectal cancer stage IV.

associated with significant loss of DNA in preparation and the reproducibility of quantitative tumor burden measurements is unknown.[1,7,19,20] Cell free DNA (cfDNA) is the result of DNA apoptosis that can be found in the plasma of both normal and cancer patients.[7,9] The majority of cfDNA found in healthy patients originates from rapidly turning over cells from gastrointestinal tract and white blood cells. In cancer patients circulating tumor DNA is released from apoptosis or necrosis of tumor cells.[7,9] An attractive alternative approach to measuring cfDNA is the targeting of retro-transposable elements (RE) or Arthrobacter luteus (Alu) restriction endonuclease element because of their ubiquitous nature in the human DNA making them easily quantified by real-time polymerase chain reaction (qPCR) translating into an exquisitely analytical sensitive assay able to discern a picogram of DNA.

The present study was designed to evaluate the levels of cfDNA found in colorectal patients' plasma and compare them to cfDNA levels in healthy volunteers by a qPCR method. The assay utilizes a multiplexed qPCR, targeting 80 and 265 base pair (bp) sequences on 2 types of multi-copy retro-transposable elements (RE) comprising of >1,500 copies in the human genome, with an internal-positive-control.[21,22] A single-well multiplexed qPCR reaction allows simultaneous amplifications of the 3 targets in a 2-hour period with optimal qPCR efficiencies and high detection capability (level of detection <0.1 pg/μL, level of quantification = 0.61 pg/μL). Plasma cfDNA of 39 stage IV colorectal cancer patients were compared to plasma cfDNA of 40 healthy volunteers.

Methods and Materials

The study was performed after a full multi-institutional review board approval for a prospective collection of plasma from 40 healthy volunteers to compare with plasma from 39 stage IV colorectal patients' plasma specimens were collected serially from another study (NCT00984048) with the Jewish General Hospital in Montreal, Quebec, Canada. The 40 healthy plasma samples were collected from healthy volunteers undergoing screening colonoscopy, whose blood was collected at the time of screening colonoscopy. Prospective healthy volunteers were only enrolled, and their plasma evaluated, if their colonoscopy was completely clear with no evidence of polyp disease or colorectal cancer. The 39 colorectal cancer patients had histologic and radiographic diagnosis confirmation as stage IV colorectal cancer. All plasma specimens were stored at −80°C until cfDNA extraction.

We evaluated multiple qPCR target lengths to identify optimal efficacy (data not shown). A single-well multiplexed qPCR assay was then developed with the primers that can amplify 80 bp and 265 bp RE sequences in combination with an internal-positive-control. Purified cfDNA extracted from less than 0.5 mL of double-spun plasma using Quick-cfDNA Serum & Plasma Kit (Zymo-Research, Irvine, CA) was quantified with the developed multiplex qPCR assay. The patented primers for the 80 bp and 265 bp polymerase chain reaction target sequence sizes are utilized to amplify cfDNA fragment length longer than 80 bp and 265 bp, respectively. Therefore, the 80 bp target measures cfDNA concentration of >80 bp fragments in length, and the 265 bp target measures concentration of >265 bp fragments. The level of cfDNA fragmentation is assessed by the DNA integrity index (DII-265/80), which is a ratio of cfDNA concentration measured with 265 bp to cfDNA concentration measured with 80 bp. The qPCR measured quantities were normalized to plasma cfDNA concentration as follows: Plasma cfDNA concentration (ng/mL) = qPCR measured quantities (ng/μL) × extraction elution volume (μL) ÷ plasma extraction volume (mL). The resulting data was analyzed between the stage IV colorectal patients and the healthy controls. Mann-Whitney *U*-test was used to compare the differences in 80 bp concentration, 265 bp concentration and DII. Specificity and sensitivity of the association between either plasma cfDNA concentrations or DII and mCRC status were evaluated by analysis of the receiver operating characteristic area under the curve to demonstrate that cancer patients shed higher levels of cfDNA than normal, and could be used for measuring changes in tumor burden.

Results

We measured plasma cfDNA concentrations of >80 bp and >265 bp fragment lengths and DII-265 out of 80 of 40 healthy controls and 39 stage IV colorectal cancer (CRC) patients by the multiplex qPCR assay. Distributions of the obtained concentrations and DII are shown as box-and-whisker plots presented in Fig 1. Means (± standard deviation) of cfDNA concentrations and DII for mCRC

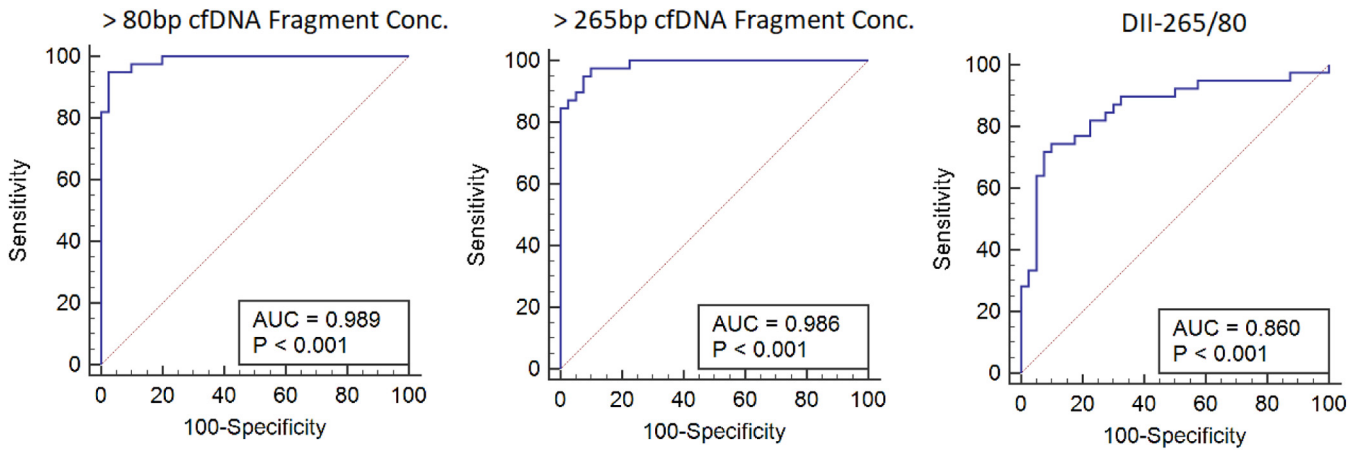

Fig 2. Receiver operating characteristic curve analysis of the cfDNA concentrations of fragment size longer than 80 bp and 265 bp and DII-265/80 in detecting CRC-IV.

Table II
Receiver operating characteristic curve analysis: sensitivities and specificities of 80 bp, 265 bp, and DII

	>80 bp	>265 bp	DII-265/80
AUC (95% CI)	0.9891 (0.974, 1)	0.9859 (0.969, 1)	0.8603 (0.773, 0.947)
P value	<0.001	<0.001	<0.001
Youden index: cutoff value	14.1 ng/mL	2.13 ng/mL	0.157
Sensitivity	94.87%	97.40%	74.36%
Specificity	97.50%	90.00%	90.00%

AUC, area under the curve; *CI*, confidence interval.

patients and the healthy controls are presented in Table I. The results showed an increase in cfDNA concentration of >80 bp and >265 bp fragments and a decrease in DII-265/80 in mCRC patients in comparison to healthy controls (Fig 1 and Table I). Mann-Whitney *U*-test revealed that plasma cfDNA was significantly elevated and more fragmented, as shown by lower DII-265 out of 80 values, in the CRC patients' plasma (80 bp: $P = 2.3 \times 10^{-20}$, 265 bp: $P = 8.4 \times 10^{-20}$, DII-265 out of 80: $P = 2.0 \times 10^{-9}$). The assay demonstrated a high correlation between stage IV colorectal cancer and elevated cfDNA levels. All final selected size markers and marker ratio displayed a clear discrimination with a high area under the curve: 80 bp = 0.9891 (r = 0.00755, $P < .0001$), 265 bp = 0.9859 (r = 0.00875, $P < .0001$), and DII-265/80 = 0.8603 (r = 0.0444, $P < .0001$; Fig 2). The sensitivity and specificity of the selected marker, 80 bp and 265 bp for the measurement of the cfDNA concentration exhibited above or equal to 90% when the cutoff values obtained from the Youden index of the receiver operating characteristic curve analysis were applied (Table II).

Discussion

The developed assay demonstrated high predictive capabilities in discriminating a group of 39 stage IV patients from 40 healthy control patients with cfDNA concentration and DNA integrity index (DII-265/80) values. Several potential applications have come to our attention during this initial analysis: (1) treatment monitoring for CRC stage IV patients, (2) a prognostic tool to help predict CRC patient outcomes, and (3) potentially an assessment tool for the measurement of minimal residual disease potentially serving as a tool to guide personalized and effective treatment.

The majority of cfDNA measured in normal healthy volunteers are the result of baseline apoptosis of gastrointestinal or white blood cells.[7,9] However, several groups have identified elevations in cfDNA from traumatic blood draws, exposure of plasma specimens to contaminants or plasma obtained from patients after moderate to excessive exercise stress.[7,9] This requires all patient blood specimens to be timed, drawn, processed, and handled meticulously under the prescribed conditions so as to not falsely alter patient cfDNA levels. Recent data on donor-derived cfDNA measurement in renal transplants has identified the majority (96%) of normal patients have <1% of cfDNA comprised of donor DNA and approximately 50% have <0.21% of donor DNA represented in cfDNA found in their blood stream.[23,24]

In cancer patients, the cfDNA contains DNA released from apoptosis or necrosis of tumor cells.[7,9] All cfDNA has a reasonably short half-life and will circulate in the blood stream for a matter of days before being broken down or excreted. cfDNA from apoptosis is typically fragmented into 180 bp, corresponding to the length of DNA wrapped around a nucleosome which is protected from degradation. Less frequently, cfDNA lengths corresponding to multimers of nucleosomes have been observed and thought to be originated from apoptosis. In cancer patients apoptotic fragment size has been observed to be more fragmented with an abundance of <100 bp fragments. Conversely, cfDNA in normal healthy patients are frequently found to be longer than 166 to 180 bp.[7,9,19] Several studies have confirmed the presence of elevated cfDNA levels at the time of diagnosis or post treatment, often corresponding to a poorer clinical prognosis making this a potentially informative marker in colorectal patients.[7,9,10,19,20]

The present study evaluates the use of highly abundant and repetitive RE for the detection of metastatic colorectal cancer in human plasma. The advantage of RE is they are ubiquitous in the human DNA and easily quantified by qPCR. This translates to a DNA sensitivity in the subpicogram quantities. The ubiquitous elements are genetic mutation free making this test agnostic to tumor histology. Exploitation of this 1,000-fold greater sensitivity, in combination with a 1- to 2-day turnaround may make this an ideal assay for evaluating therapeutic efficacy or defining drug futility. Our data in this preliminary study showed cell free DNA has a high predictive capacity in discriminating metastatic colorectal cancer

patients from healthy non-cancer controls. Additional studies will be required to compare the diagnostic capacity of cell free DNA to the sensitivity and specificity of standard diagnostic serum tests in colorectal cancer such as carcinoembryonic antigen, carbohydrate antigen (CA)19-9, CA72-4, and Ferratin. Similar work will also be required to assess the capacity of cell free DNA's correlation with tumor burden.

The currently developed diagnostic test based on two optimized principle RE is exquisitely sensitive for the measurement of cfDNA concentrations in stage IV colorectal patients and showed a promising potential to discriminate cancer patients from healthy normal individuals.

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Conflict of interest/Disclosure

Sudhir Sinha, Hiromi Brown, and Jonathan Tabak are partial owners of the technology and have a declared conflict of interest. Sinha has an ownership in Cadex Genomics Corp, Redwood City, CA.

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Discussion

Dr Emre Gorgun (Cleveland, OH): I would like to thank Dr Buell and associates for this clear presentation and the manuscript. I enjoyed the reading of the paper.

If traditional efforts and guidelines are not ideal for early detection of recurrences and optimal surveillance in colorectal cancers, a strong need for optimal methods such as genetic testing and platforms to be introduced into the clinical arena. There is a need for blood tests, for screening, and surveillance. Certainly a hot field. It would be great if it worked. Clearly, challenges to identify the specific tests that would be reliable and also valid.

I have the following questions.

I don't fully understand the technical details of your essay. I would appreciate it if you could provide more details. A simple question would be, if you compared predictive capacity of your assay to currently accepted clinical pathological predictors such as extent of metastatic burden, time to develop metastases, tumor differentiations, et cetera?

I am also wondering what the benefits of your test is compared to liquid biopsy with circulating tumor DNA or circulating tumor cells. This has been shown to be able to detect numerous mutations that can inform decisions about targeted therapy.

An additional question I have is the value of the liquid biopsy in earlier colorectal cancer stages, and even for precancerous conditions such as adenomas or polyps. This study shows that it can discriminate stage 4 from normal, although that is not that useful. It would be useful if you could distinguish stage 1 for adenoma, as mentioned earlier, from normal.

Additionally, it would be nice to show if you could use this to pick up tumor in someone who has cancer already. Could it be an adjunct or better than imaging for clinical staging? Could it be used for post-operative surveillance to detect recurrences?

What was the tumor burden in the stage 4 patients in your study for these 40 patients? If these were all large-volume mets, then they are little use for this test since if it would be picked up by

imaging anyway. If it could pick up microscopic disease earlier than an imaging test would or before even symptoms would occur.

Lastly, I would appreciate any input from the cost perspective, especially compared to current available markers such as CEA. Would you recommend this test to be widely used in your current practice for stage 4 colorectal cancers and others?

Dr Joseph Buell: Thank you very much for the kind thoughts. I will attempt to answer your questions in reverse order. When you look at average expense data for current next generation sequencing (NGS) technology, an average panel of genetic abnormalities where the cost of goods maybe somewhere between $500 and $1,000. The proposed diagnostic test in this study is based on RT-PCR that might cost between $50 and $150, so there's a considerable opportunity. More importantly the average turnaround time for NGS panels has been traditionally 4 weeks. However, some of the new platform technology has decreased this time down to 2 weeks. PCR technology is rapid with almost point of contact reporting.

I would like to invite you to review our data recently presented by the M.D. Anderson at ASCO. We have identified this technology is extremely powerful in identifying chemotherapeutic drug futility in stage IV colorectal patients. This study evaluated patient response to Stivarga being used as a third round agent for colorectal cancer. Our test clearly identified non-responsive patients at 4 weeks and even 2 weeks. So this technology offers the opportunity to evaluate chemotherapy futility after even a single dose of Stigarva avoiding any additional non-therapeutic dosing of chemotherapy.

In evaluating this circumstance, we performed a backward financial analysis that estimated the cost savings of avoiding additional non-therapeutic chemotherapy in non-responsive patients eliminating or alleviating additional non-effective chemotherapy in third round colorectal therapy. Just in this small and specific area of chemotherapeutic administration could save potentially $300 million per year in the U.S. healthcare system. So in this sense, this assay becomes an excellent tool to measure chemotherapy or even therapeutic futility.

As you indicate there are some limits to this technology. Obviously, we have identified stage IV colorectal cancer as an optimal area to utilize this diagnostic test and a robust area for further developing and commercializing this technology. Our technology has not yet been evaluated for the predictive capacity to identify individuals with early stage colorectal cancer or high-grade adenomatous disease. Clinical trials that verified the Cologuard technology took approximately 4,000 patients to be approved by the FDA at the cost of several millions of dollars.

So my answer to detection of early stage cancer or high-grade adenoma detection is still a potential, yes. I do believe that our current assay is highly sensitive and will be valuable in not only stage IV but also stage III patients allowing monitoring our patients for recurrence and/or response to therapy. The other critical area, which you have already mentioned about liquid biopsy is that individuals with elevations in cell-free DNA is not always a negative prognostic. It has been noted that there can be several variations in patterns of cell-free DNA among specific mutations measured by NGS technology. Some of these genetic elevations in cell-free DNA measured by NGS are protective or can be beneficial to an individual patient. These salutary genetic abnormalities have been correlated with improved survival.

So these are areas of active investigation that are underway where we will eventually correlate not only cell-free DNA level but also a diagnostic index, potentially giving physicians an ideal assay to assess how aggressive a tumor is going to be. But to achieve this insight we need to identify those areas that should be developed first, and I believe that it's going to be drug futility, followed then by a prognostic tool.

Lastly, this assay is based on two specifically designed primers exploiting the variations in apoptotic fragments in cancer patients compared to normal healthy patients. The primers were based on retransposable elements of Alu elements. This high replicable element found in all DNA and in specific cell-free DNA allows an exquisite sensitivity. Exploitation of the Alu elements rather than measuring specific genetic mutations also make this test tumor agnostic. This increases the level of detection from nanogram to picograms of DNA. This 1,000-fold increase in sensitivity becomes the principal advantage of this diagnostic assay. The current study was a preliminary analysis of our technology in stage IV colorectal cancer. These patients had radiologic measurable disease. So this study confirms our technology has significant sensitive level of detection and a highly accurate AUC for colorectal cancer far superior to standard tumor markers including CEA and CA19-9. In the next few years we will continue to development this technology in the area of monitoring therapeutic interventions, and especially chemotherapy.

Dr Sarkis Meterissian (Montreal, QC): Your presentation is extremely exciting for those of us who are working at identifying markers. I work on breast cancer, and we are trying to do the same with cell-free DNA. I am not as familiar, so excuse me for the question if it's too obvious. But in breast cancer, we are looking not only at cell-free DNA but we are also looking at genes in the cell-free DNA.

In colorectal cancer there are some papers on K-ras, in cell-free DNA. In patients who are resistant to K-ras treatment, they have K-ras mutants in their cell-free DNA. So, my question is— and I am curious to know in your research—where are you at in going one step further in looking at genes in the cell-free DNA as markers of resistance?

Dr Joseph Buell: I think if you look at the Gauradent assay which utilizes NGS technology evaluating individual gene expression, some of these panels include 20 to 30 specific genes. One of these assays under development is the OmniSeq which is probably another 2 years from commercialization for liver and pancreas cancer. In those areas, you are evaluating the variations in 20 specific genes, this panel of mutations will vary with therapeutic resistance or even recurrence with some genetic expressions being increased, some being decreased as well as the total quantity of cell-free DNA with expression of these individual genes.

If you think about this test, it is basically looking at cell-free DNA exploiting large and small apoptotic fragments, so it becomes a quick and very sensitive test to assess and follow the responsiveness. If you were to look at Guardant assay and you were to match it with cell-free DNA based on an Alu element, these would correlate very nicely. So this synergy is what we see as an excellent utility for this assay, because now we can have rapid, weekly or bi-monthly test to determine response, or detect a developing resistance. This might be the development of an aberrant clone that's going to change the nature of the tumor itself, or a secondary occurrence that has another genomic platform to it.

Dr Timothy M. Pawlik (Columbus, OH): It looks like your Kaplan-Meier curves reflect data from patients with advanced disease. The median survival seemed as if it was only 10 months or 11 months. Have you looked at data in patients with earlier stage resectable disease? The reason that I ask is that the role for additional adjuvant therapy among patients who have had an R0 and are NED is still somewhat controversial. Based on data from the EROTC trial, the effect of adjuvant may not be as dramatic as we think. Can a technique like you are proposing be utilized to help inform whether people should receive additional systemic chemotherapy once they have been rendered NED with surgical

therapy? Could we use data from your approach to tailor/individualize therapy for patients with colorectal liver metastasis?

Dr Joseph Buell: I think you hit the target directly. We have had the support of a phase I NCI grant and submitted a phase II grant attempting to address that very question. So if there is interest in combining our technology in another clinical trial or early investigation, we would like to proceed with such an event. We are open to evaluating our technology in any histology. Noting that the use of Alu elements make this a histologically agnostic assay, so it will work in any type of pathology.