Int J Mol Epidemiol Genet 2018;9(1):1-12
www.ijmeg.org /ISSN:1948-1756/IJMEG0071847

Original Article

Mutation detection using plasma circulating tumor DNA (ctDNA) in a cohort of asymptomatic adults at increased risk for cancer

Anja Kammesheidt, Theresa R Tonozzi, Stephen W Lim, Glenn D Braunstein

Pathway Genomics, San Diego, CA 92121, USA

Received December 31, 2017; Accepted January 25, 2018; Epub February 5, 2018; Published February 15, 2018

Abstract: Purpose: The primary purpose of this study was to clinically evaluate circulating tumor DNA (ctDNA) with a nine gene, 96 mutation panel among subjects at increased risk for cancer with no previous cancer diagnosis. Subjects and Methods: DNA from 1059 asymptomatic subjects was analyzed for detection of low levels ctDNA using a blood plasma liquid biopsy assay. Subjects with detectable copies of ctDNA were asked to provide additional blood samples and relevant medical records throughout their one-year of participation. Subjects with a negative result were followed-up at one-year with a questionnaire. Results: Mutations were detected in 58 subjects and not detected in 1001 subjects. Among the subjects who tested positive for one or more mutations, four were diagnosed with cancer, two of which through study-triggered clinical follow-up. Two subjects who tested negative on the screen received an early cancer diagnosis over the course of the year. The sensitivity of the assay at a threshold of ≥2 copies in this population was 66.67% and specificity was 94.87%. While the negative predictive value was 99.8%, the positive predictive value was only 6.9% in this cohort. Analysis of buffy coat DNA from eight positive subjects, including one who was diagnosed with cancer, revealed matching mutations suggesting that the ctDNA could have been derived from clonal hematopoiesis. Conclusion: The observed false positive rate of ctDNA on a 96-mutation assay in an asymptomatic high-risk population is much greater than the true positive rate, limiting its usefulness as a cancer screening tool in its current form.

Keywords: Cell-free DNA, circulating tumor DNA, liquid biopsy, biomarkers, mutation, cancer, early detection of cancer

Introduction

Most cancers are caused by acquired mutations in the body's somatic cells. Investigating these mutations and their role in triggering the progression from benign to malignant lesions advances our understanding of tumor evolution which should lead to earlier detection and prevention [1]. Analysis of somatic mutations by measuring the circulating tumor DNA (ctDNA) component in peripheral blood ("liquid biopsy") can assist with noninvasive screening, treatment, and monitoring of cancer management [2-4]. Circulating tumor DNA (ctDNA) is a component of cell-free DNA (cfDNA) that can be detected and used as an indicator for the presence of a tumor [2-5].

Varying types of tumors have been shown to produce high levels of cfDNA in plasma [6-8], and several studies have identified mutations and quantified ctDNA in subjects with advanced stages of cancer or patients undergoing treatment [7-12]. As stage of disease increases, the prevalence of ctDNA also increases. Current advances in next-generation sequencing allows for detection of ctDNA at very low thresholds [3, 13-15] and for detection of ctDNA in the plasma of individuals with low tumor burden [16]. Demonstrating the utility of ctDNA in clinical practice as a biomarker for the presence of cancer or precancerous lesions in apparently "healthy", otherwise asymptomatic individuals has yet to be established with a large prospective study [2, 17].

In 2015, a pilot study (Chen et al., 2017, n=183 plasma samples from cancer patients and n= 102 healthy volunteers) was conducted to validate a panel of 96 mutations in a set of nine

genes (*BRAF, CTNNB1, EGFR, FOXL2, GNAS, KRAS, NRAS, PIK3CA* and *TP53*). The liquid biopsy assay had been validated at >78% analytical sensitivity for 2-5 ctDNA copies, >98% for 5-9 ctDNA copies, and 100% for >9 ctDNA copies [13]. In the pilot study, ctDNA was detected in 24.0% of cancer patients (14.7% stage I, 18.8% II, 33.3% III, 50.0% IV) and ctDNA was not detected in 96% of low risk subjects.

The present study describes the methods and results of a larger, prospective clinical study which evaluated the presence of one or more of 96 ctDNA mutations within a cohort of individuals at elevated risk for cancer over the general population, and who had as of enrollment, no previous personal cancer diagnosis.

Methods

Participants

Study subjects were recruited nationwide into the study between December 2015 and May 2017. The study documents were approved by Chesapeake IRB (Columbia, MD, USA) and the trial was registered at ClinicalTrials.gov (Identifier: NCT02612350). As part of the eligibility assessment, individuals had to be willing to review and sign the study consent form, provide contact information of their licensed physician for potential follow-up, and self-report demographic, medical, and risk factor information using a clinical health questionnaire. Those eligible were enrolled at two designated blood donation centers (San Diego Blood Bank, and LifeServe Blood Center Iowa), two conventions, or through an online process starting at the study website, and utilizing mobile phlebotomy services nationwide. Participants who were recruited online were provided a one-time small compensation with the result of the test.

Eligibility requirements for all potential participants included 18 years of age or older, absence of any previous cancer diagnosis (excluding basal cell carcinoma of the skin), and an increased risk of cancer. Increased risk of cancer was defined as having one or more of the following risk factors: reported chronic exposure to environmental and/or occupational toxins, carcinogens, radiation, tobacco smoke or other chemicals; strong family history of cancer; or previous identification of a pathogenic variant in a hereditary cancer gene (e.g. known carrier of a BRCA1/2, Lynch syndrome pathogenic genetic variant). For an individual less than 50 years of age without reported exposure or known carrier of a hereditary pathogenic cancer variant, two generations of family members on the same side with a diagnosis of cancer were required for enrollment (e.g. maternal grandmother and mother). Individuals 50 years of age and older were enrolled without any reported exposures or hereditary risks, as age was defined as a sufficient risk for developing cancer based on lifetime cancer incidence data (https://seer.cancer.gov/faststats/selections.php?series=age).

Blood collection

Participants were asked to provide a 20 milliliter (mL) blood sample collected in Cell-Free DNA BCT tubes (Streck, La Vista, NE, USA). Specimens were collected and shipped using manufacturer's storage and stability instructions. Blood collection tubes were typically received at the lab within 1-5 days of the blood draw. Validation of the Streck tubes and the molecular analysis system was completed by Pathway Genomics in accordance to Clinical Laboratory Improvement Amendments (CLIA) and College of American Pathologists (CAP) requirements.

Sequencing

Plasma separation, isolation, and processing were carried out at Pathway Genomics as previously described [13]. To separate plasma, both blood tubes were spun for 10 minutes at 2000× g at room temperature. Plasma layers were combined into a new tube, re-spun for 10 minutes at 2000× g at 4°C, then transferred again and frozen in 5 mL plus residual volume aliquots. For analysis, 5 mL of plasma was isolated using QIAamp Circulating Nucleic Acid Kit (Qiagen, Hilden, Germany). Quantity of cfDNA was measured using Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific, Waltham MA, USA). Library preparation was performed with 10-300 nanogram (ng) input DNA. The ctDNA was analyzed with the 96 mutation assay (CancerIntercept, Pathway Genomics, San Diego, CA, USA) which uses polymerase chain reaction amplification and mutation enrichment based on a multiplexed detection technology [18]. During the enrichment process wildtype DNA is

removed which lowers the necessary sequence depth. Next-generation sequencing was performed using Illumina MiSeq (Illumina, San Diego, CA, USA) [13]. The assay detects mutations in nine cancer driver genes (*BRAF, CTNNB1, EGFR, FOXL2, GNAS, KRAS, NRAS, PIK3CA,* and *TP53*) and has a >78% analytical sensitivity for 2-5 copies, >98% for 5-9 copies, and 100% for >9 copies across the cfDNA input range of 10-300 ng [13].

Thresholds

For the study, a detection threshold was established for reporting of a variant. Initial plasma samples with ctDNA copy numbers detected equal or greater than 5 mutant copies were classified as "positive". The mutant copies were also calculated as a percent of input cfDNA loaded into the assay (% ctDNA relative to input cfDNA), as input levels differ from person between 5 mL plasma specimens. Initial plasma samples with ctDNA copy numbers detected between 2-5 mutant copies were classified as "indeterminate" due to the known lower analytical sensitivity in this range. Participants with an initial sample in this indeterminate range were asked to provide a second blood sample to confirm the test result. The second result was utilized, with a repeat result of ≥2 copies classifying as screen-positive and those below as screen-negative. Subjects and their primary care providers were notified of the results in writing. In the event a screen was positive, both subject and their primary care provider were also contacted by an oncologist to review the test result, discuss medical history in depth, and provide guidance to the subject's provider about follow-up clinical screening.

Follow-up

Participants with detectable ctDNA were asked to provide additional blood samples to monitor ctDNA levels at 3-6 months and then a third sample at one-year of participation. When necessary for clinical confirmation, further medical records and medical test results were obtained with the patient's consent. Participants that were screen-negative were not retested within the one year follow-up. All subjects were contacted at one-year after their initial blood sample regarding changes in their cancer status using a questionnaire. Follow-up questionnaires were sent via an electronic questionnaire, or hardcopy. The initial study plan included follow-up of patients for up to 5 years, which may not be possible due to resource constraints.

Participation rate

Of the 1106 participants who submitted a blood draw, 1059 were included in the analysis. Twenty-six subjects initially enrolled into the study, who had submitted a blood sample, later turned out not to qualify for enrollment (e.g. previous cancer diagnosis revealed, or errors on family history reporting, or inability to verify medical history) and were therefore excluded from the result analysis. Lastly, ten subjects with an initial indeterminate result at <5 copies were removed, because no second plasma sample for confirmation could be obtained.

Statistical analysis

Demographic, reported exposure, clinical and family history variables were stratified by mutation status (positive or negative screen). Analyses were performed in R version 3.4.0 (Vienna, Austria). Two-sided independent t-tests were used to evaluate means. Pearson's Chi-square or Fisher's exact test (when cell counts were ≤1) were used to compare proportions. A $P \leq 0.05$ value was considered significant.

Sensitivity and specificity were determined at threshold levels of ≥2, 5 and 10 copies of detectable mutant DNA. For determination of false-positive and false-negative rates, we used a six-month post initial positive or negative ctDNA result as the cut-off period for a diagnosis of cancer.

Results

Demographics

Table 1 lists demographic and clinical characteristics categorized by negative or positive screen result. Wild type DNA only was present in 1001 (94.5%) of participants and ctDNA was detected in 58 (5.5%) participants. Among participants who had a negative test result, 37 participants had been initial "indeterminates", but screened negative on the repeat blood draw. Twenty-one of the 58 subjects were screen-positive with the repeat blood draw.

Table 1. Participant characteristics

Characteristic	Negative (Wild Type) n=1,001		Positive (Mutation Detected) n=58		*P*
	Participant Number	%	Participant Number	%	
Age (years)					
Mean	53.5	--	60.7	--	*P<0.001*
18-24	13	1.3	0	--	
25-34	112	11.2	1	1.7	
35-44	114	11.4	4	6.7	
45-54	214	21.4	11	19.0	
55-64	345	34.5	23	39.7	
65-74	173	17.3	14	24.1	
75+	30	2.3	5	8.6	
Two groups (years)					*P=0.02*
<50	330	33.0	10	17.2	
≥50	671	67.0	48	82.8	
Sex					*P=0.6*
Male	334	33.4	22	37.9	
Female	667	66.6	36	62.0	
Race/Ethnicity					*P=0.2*
African American	18	1.8	1	1.7	
Asian	19	1.9	5	8.6	
Caucasian	871	87.0	48	82.8	
Hispanic	60	6.0	3	5.2	
Mixed Race/Other	27	2.7	1	1.7	
Not Reported	6	0.6	0	--	
Smoking/Tobacco Use					
Self-Use					*P=1.0*
Yes	158	15.8	9	15.5	
No	843	84.2	49	84.5	
Secondhand Exposure					*P=0.7*
Yes	131	13.1	6	10.3	
No	870	86.9	52	89.7	
Prolonged exposure					*P=1.0*
Yes	287	28.7	17	29.3	
No	714	71.3	41	70.7	
Hormone Use					*P=1.0*
Male					
Yes	14	1.4	0	--	
No	320	32.0	22	37.9	
Female					
Yes	148	14.8	9	15.5	
No	519	51.8	27	46.6	
Health Condition					*P=0.4*
Yes	543	54.2	35	60.3	
No	458	45.8	23	39.7	
First Degree Family Member with Cancer					*P=0.6*
Yes	652	65.1	41	70.7	

No	345	34.5	17	29.3	
Unknown	4	0.4	0	--	
Second Degree Family Member with Cancer					*P=0.3*
Yes	596	59.5	29	50.0	
No	399	33.9	29	50.0	
Unknown	6	0.6	0	--	
Two Generations with Cancer					*P=0.4*
Yes	471	47.1	22	37.9	
No	523	52.2	36	62.1	
Unknown	7	0.7	0	--	
Germline Mutation Status					*P=0.02*
Yes Positive	136		2		
Yes Negative	23		3		
Not Tested	842		53		
Only Subjects with Known Germline Mutation Status	n=159		n=5		
Yes Positive	136	85.5	2	40.0	*P=0.03*
Yes Negative	23	14.5	3	60.0	

Table 2. Mutations detected at first blood draw

Mutation	Number of times detected
BRAF_e11a_G469A	1
BRAF_e15_K601E	1
BRAF_e15_V600E	1
GNAS_e8a_R201C	11
GNAS_e8a_R201H	21
KRAS_e2a_G12D	2
KRAS_e2a_G12R	1
KRAS_e2a_G13D	1
KRAS_e3a_Q61R	1
KRAS_e4a_A146V	1
NRAS_e2a_G12D	5
NRAS_e2a_G12S	1
TP53_e5a_R175H	2
TP53_e7a_G245D	2
TP53_e7a_G245S	2
TP53_e7a_R248Q	3
TP53_e7a_R248W	6
TP53_e7a_R249S	2
TP53_e8a_R273C	2
TP53_e8a_R273H	4
Total	70

Two thirds, 703 (66.4%) of the study subjects were female and 919 (86.8%) were Caucasian. The mean age of participants who had a negative result was significantly lower (53.5 years) than participants with a positive screen (60.7 years), (*P<0.001)*. There were no significant differences between those with a negative screen and detected mutations in terms of gender, ethnicity, tobacco use, exposure, health condition, or family history. Interestingly, the number of participants who had a previously known germline gene abnormality were proportionally greater among patients without ctDNA detected than in those in whom ctDNA was detected (159/1001 [15.9%] vs. 5/58 [8.6%], respectively); however, the counts in the screen-positive group are extremely small and the difference was only marginally significant (*P*=0.03). This may reflect the younger age of the patients who entered the study based upon known genetic mutations.

Mean cfDNA levels differed between the group of subjects with ctDNA detected compared to those without ctDNA (72.56 ± 31.06 ng/mL vs. 59.45 ± 24.48 ng/mL, respectively *P*=0.002 [**Table 3**]).

Mutation analysis

Most of the participants with a positive screen were over 50 years of age, (n=48, 82.8%). More than half of the subjects were female, (n=36, 62.0%) (**Table 1**), and that remains in proportion to the higher % of participating females in the study. In total, 70 mutations were detected in the 58 subjects with a positive screen on the

Table 3. Total cell free DNA (cfNA) detected in screen-negative and screen-positive subjects

	Negative (Wild Type) n=1,001	Positive (Mutation Detected) n=58
cfDNA		
Mean*	59.45 (range 12.60-197.40)	72.56 (range 13.8-187.8)
SD	24.48	31.06
SEM	0.77	4.08

Abbreviation: SD = Standard Deviation; SEM = Standard Error of the Mean. *The two groups mean input cfDNA amounts are statistically different (two-sided t-test with unequal variance, P=0.002).

first blood sample. These 70 mutations were represented by 20 of the 96 different mutations on the assay in 5 of the genes (*BRAF, GNAS, KRAS, NRAS, TP53*) (**Table 2**). Mutations in *GNAS* (45.7%) and *TP53* (32.9%) accounted for the majority of the observed mutations.

Serial sampling

Participants with an initial positive screening test were asked to provide at least two repeat blood draws throughout their one-year of participation after their initial blood draw date to determine changes in their screening results. Forty-three screen-positive participants (74.1%) submitted at least a second blood sample. Out of the five subjects who had a wild type result on their second test, one subject had ctDNA detected in the third sample, but the remaining subjects were not retested during the study. Eighteen submitted a third sample, 15 of which remained screen positive and three different subjects had a wild type result. The mean ctDNA levels increased slightly over time (**Table 4**).

Clinical observations of screen-positive subjects

Four of the screen-positive subjects were diagnosed with cancer in close proximity to the first or second blood draw, through study-triggered clinical follow-up in two patients and independently in two others (**Table 5**).

Subject 1 was a 51 year-old male, with a 0.023% *TP53* R248W mutation. He was subsequently diagnosed with adenocarcinoma of the prostate (Gleason 6) through study-triggered clinical follow-up. His second and third blood sample, five months prior to and three months after prostatectomy, showed relatively consistent mutation levels. He also received a melanoma diagnosis around the time of the prostate surgery. The melanoma was treated with local excision.

Subject 2 was a 75 year-old male, with a 1.742% *GNAS* R201H mutation. He was subsequently diagnosed with adenocarcinoma of the lung (stage I) through study-triggered clinical follow-up. His second blood sample, two months prior to a partial pneumonectomy had increased copies to 3.089%, but then dropped to 1.589% two months, and 1.885% eight months after the treatment. However, the mutation was also seen at equivalent 3.458% level in buffy coast DNA analyzed concurrent with the final blood draw.

Subject 3 was a 66 year-old female, with an initial indeterminate result of a 0.024% *KRAS* G12D mutation and 0.028% *TP53* R175H. After her second blood sample was obtained three months later, her mutations levels had increased to 0.058% and 0.025%, respectively. She was hospitalized with abdominal pain prior to second result and was diagnosed with stage IV pancreatic cancer. She died two months later.

Subject 4 was a 71 year-old male, with triple positive *TP53* mutations between 0.005% and 0.010%. He had known diabetes, hypertension and chronic kidney disease. He rapidly declined and died three months after his blood draw from probable myelodysplastic syndrome. A second blood draw was scheduled, but could not be completed before his death.

Among all 58 subjects with an initial positive result, two were lost to follow-up after their first sample and one declined further participation in the study. Lastly, the study was stopped before 11 and 35 of the initial screen-positive subjects could submit a second and third blood sample, respectively (**Table 4**).

In light of the consistent mutation levels in screen-positive patients, we initiated an analysis of buffy coat DNA at the time of blood redraw. For eight repeat samples, both plasma cfDNA and DNA from the buffy coat were analyzed to determine if plasma ctDNA variants

Table 4. Serial sampling results among screen-positive subjects

	Mutation Detected Participant Number n=58					
	Sample 1		Sample 2		Sample 3	
Liquid Biopsy First Sample Result	n=58	%	n=43	%	n=18	%
Number of Mutations Detected						
1	48	82.8	31	72.1	11	61.1
2	8	13.8	6	14.0	4	22.2
3	2	3.4	1	2.3	–	–
Mean abundance of mutant ctDNA relative to input cfDNA (%)	0.13 (70 mut.)	–	0.27 (46 mut)	–	0.52 (19 mut.)	–
Wild Type result (for repeat samples only)	–	–	5	11.6	3	16.7
Not collected	–	–	11	–	35	–
Loss to follow-up	–	–	4	–	5	–

Table 5. Circulating tumor DNA (ctDNA) levels and percent abundance mutant DNA relative to cell free DNA (cfDNA) in participants with a confirmed cancer diagnosis within one-year of DNA sampling

Subject	Age (years)/Gender	Number of Samples (Date of Collection)	Mutation	Copy Number	Abundance Mutant ctDNA relative to input cfDNA (%)	Pathology Result
1	51/Male	Sample 1 (Feb. 2016)	TP53_e7a_R248W	6.2	0.023	Adenocarcinoma of the prostate, Gleason 6 (Oct. 2016); melanoma (Nov. 2016).
		Sample 2 (July 2016)		7.5	0.028	
		Sample 3 (March 2017)		8.1	0.031	Sample obtained following prostatectomy (Dec. 2016); melanoma removed locally (Dec. 2016), no other treatment.
2	75/Male	Sample 1 (June 2016)	GNAS_e8a_R201H	218.6	1.742	Adenocarcinoma of the lung, stage 1 (Abdominal CT scan, Aug. 2016)
		Sample 2 (Aug. 2016)		629	3.089	
		Sample 3 (Dec. 2016)		248.4	1.589	Sample obtained following partial pneumonectomy (Oct. 2016)
		Sample 4 (June 2017)		253.2	1.882	
3	66/Female	Sample 1 (April 2016)	KRAS_e2a_G12D	2.5	0.024	
			TP53_e5a_R175H	2.9	0.028	
		Sample 2 (July 2016)	KRAS_e2a_G12D	15.6	0.058	Subject hospitalized Aug. 2016 and diagnosed with pancreatic cancer at workup.
			TP53_e5a_R175H	6.8	0.025	Subject died Oct. 2016 three months after second sample obtained.
4	71/Male	Sample 1 (June 2016)	TP53_e7a_G245D	2.2	0.005	Subject died Sep. 2016 from probable myelodysplastic syndrome.
			TP53_e7a_R248W	3.6	0.009	
			TP53_e8a_R273H	4.0	0.01	

could have originated from clonal hematopoiesis. In these cases, the DNA analyses confirmed mutations levels in both plasma and buffy coat. Buffy coat DNA was ascertained for one subject (Subject 2, described above) found to have a tumor detected. Only one (Subject 2) described above of these patients was found to have a tumor detected during the year of follow-up. Unfortunately, matching analysis could not be completed for all screen-positive participants.

One additional low level *GNAS* R201C screen-positive subject (Subject 5), a 49 year old female, was diagnosed with a brain meningioma during the one-year participation period, but this subject was not included as a true positive case since her meningioma was benign.

One other subject, (Subject 6), a 64 year-old female had an initial observed mutation level of 4.453% *NRAS* G12D and 0.019% *GNAS* R201H. She had a known fatty liver and prima-

ry biliary cholangitis diagnosis. Through the course of the year, serial sampling showed elevated *NRAS* levels at 6.815% (4.5-months), 7.737% (eight months) and 7.379% at the one-year time point with the other *GNAS* mutation remaining low. No cancer was diagnosed despite thorough clinical follow-up including abdominal and chest MRIs, colonoscopy, mammogram and a liver biopsy. The mutations were also confirmed in the buffy coat DNA (40.604% *NRAS* and 0.024% *GNAS*), analyzed concurrent with the last blood draw. The mutations were absent in genomic DNA from a cheek swab when analyzed with Sanger sequencing. Of note, N-RAS activation has been reported in biliary cholangitis diagnosis [19].

Clinical observations of screen-negative subjects

Two of the screen-negative subjects were diagnosed with cancer during the study. Subject 7, a 74 year-old male, had an indeterminate *TP53* G245D mutation, but screened negative on the second blood draw. He was classified as a screen-negative. He was diagnosed with a localized high-grade prostate cancer (Gleason 9) about a month after the first blood draw, and two months prior to the second blood draw. Subject 8, a 68 year-old male, had an indeterminate *TP53* R273H mutation, but screened negative on the second blood draw. He was classified as a screen-negative. He was diagnosed with a focal high-grade urothelial carcinoma about four months after the second blood draw.

One-year follow-up

As of December 22, 2017, 630 screen-negative subjects had participated in the study for one-year and were sent a follow-up questionnaire. Out of 630 subjects, 307 (48.7% response rate) had replied. One subject in addition to Subjects 7 and 8 previously identified declared a cancer diagnosis. Unfortunately, the information could not be verified. Carcinomas of the skin or other benign conditions were the only changes in health status among respondents over the year of participation. A screen-negative 64 year old female subject developed Non-Hodgkin B-cell follicular lymphoma during the one-year participation; however, driver mutations for lymphomas are not represented in the assay.

Sensitivity and specificity

Applying the available clinical data, the initially observed sensitivity and specificity at a threshold of greater than 2 copies were 66.67% and 94.87%, respectively. The positive predictive value (PPV) was 6.9% and the negative predictive value (NPV) was 99.8%. By increasing the threshold to 5 copies, the sensitivity and specificity were 50% and 96.97%, respectively. The PPV was 5.88% and the NPV was 99.8%. By increasing the threshold to 10 copies, the sensitivity dropped to 33.33%, specificity increased to 98.96%. The PPV was 8.33% and the NPV was 99.81%.

Discussion

Reliable, noninvasive methods for detecting cancer at early stages have the potential to drastically reduce cancer morbidity and mortality. A screening test would ideally have a high sensitivity for detecting cancer, be specific for cancer (i.e. a low false positive rate), have a high positive predictive value indicating that a positive result is most likely a true positive, be cost-effective, and positively affect clinical outcome. Our longitudinal prospective study was launched to further evaluate the performance of a multiple gene assay for detection of cancer using ctDNA as a biomarker. The screening test would have the advantages of easy blood-based sampling and a low price point, if successful in detecting early stage cancers.

In a study population of asymptomatic, high-risk individuals we found detectable ctDNA in 58/1059 (5.5%) of study subjects. Mutations were most commonly seen in *GNAS* and *TP53* genes, and most mutations detected during the one-year follow-up period remained relatively unchanged with each participant. Of the subjects with detectable ctDNA, four were diagnosed with cancer during the initial months of the follow-up. Two of the patients passed away due to cancer-related complications. The other two were successfully treated for early stage prostate or lung cancer. Among screen-negative patients, two were diagnosed with early stage prostate or bladder cancer within months after the blood draws. Both subjects had detectable ctDNA at their initial draw, but screened negative per the confirmatory sample. Overall, while the negative predictive value of the test was quite high, 99.8%, the positive

predictive value remained low (8.33%), even if we adjusted the reporting threshold to above 10 copies.

Others have identified somatic mutations in cancer genes in patients whose histology and/or diagnostic evaluation were normal. A prospective study (n=1,586) conducted by Gormally et al. found that *TP53* and *KRAS2* mutations in cfDNA were detectable in 3% and 1%, respectively, among subjects who did not develop cancer at follow-up. That study also found that among subjects with a cancer diagnosis and detectable mutations, the diagnosis occurred on average 20.8 (*TP53*) months and 14.3 (*KRAS2*) months after the plasma samples were obtained [20]. The findings reported here only cover the first one-year follow-up period, and ideally patients would be followed for up to 5 years.

In this cohort, the mean cfDNA levels were higher in subjects with detected ctDNA (P= 0.002) and the mean age of those with a positive screen test was significantly higher (P< 0.001). Fragments of ctDNA from sources other than malignancy could partly explain the false positives. Reasons for an upsurge of cell free DNA in the plasma may stem from programmed cell death or benign conditions such as exercise, myocardial infarctions, renal failure, and endometriosis [3, 21]. The false positives noted in our study may be from somatic DNA mutations due to the various conditions or processes known to be associated with elevated levels of cfDNA. Of interest, the highest mutant copy levels found in our study were present in a participant with fatty liver and primary biliary cholangitis (Subject 6). Analysis of the subject's genomic DNA from her buccal swab was wild-type for the mutations which were observed in both her cfDNA and buffy coat DNA. The high cell turnover from the inflammatory conditions and clonal hematopoiesis are likely the cause of the ctDNA presence in this case. Several subjects with false positives had gastro-intestinal symptomatology, primarily abdominal pain. Determining the clinical significance of these mutations in healthy individuals over time will complement the use of liquid biopsy for detection of cancer.

Furthermore, the majority of plasma cfDNA originates from hematological cells [22, 23]. In an analysis of whole-exome sequencing, Genovese et al. observed clonal hematopoiesis with somatic mutations in 10% of participants older than 65 years (n=12,380) [24]. One third of the subjects with detectable ctDNA in our study were over 65 years. These clonal expansions included cancer driver genes, and presence of these clones were found to be a strong risk factor for subsequent development of hematologic cancers though the conversion from clonal hematopoiesis to hematological cancer was low at 1.0% per year [24]. Preliminary results from a prospective cohort study indicated mutations detected in cfDNA arise in white blood cells, through clonal hematopoiesis, a process which increases with age [25]. Phallen et al. recently reported that the proportion of individuals with detectable gene alterations correlated with age and allele fractions of blood cell proliferation alterations in cfDNA were similar among the healthy population group and cancer patient group. Additionally, their sequencing method found alterations related to clonal hematopoiesis in 16% of healthy individuals and no alterations in genes related to solid tumors [26]. Lastly, Snyder et al. also found that cfDNA from healthy individuals primarily correlated with lymphoid and myeloid cell epigenetic features and cfDNA samples from five late-stage cancer patients were found to be of non-hematopoietic cell types (small cell lung cancer, squamous cell lung cancer, colorectal adenocarcinoma, hepatocellular carcinoma, and ductal carcinoma in situ breast cancer) [27]. In our small subset of positive subjects, buffy coat DNA showed identical mutations to the plasma ctDNA suggesting that the ctDNA was derived from clonal hematopoiesis rather than a tumor.

Limitations

Though our study sample size is not small, the study population may not truly represent those at significant risk for having developed a neoplasm at the time of sampling. First, risk factors were self-reported on the enrollment questionnaire and may be subject to reporting bias. Second, our sampling methods were not randomized; however, participants were enrolled at multiple centers and locations across the US, representing a diverse group of study subjects that can be generalized to patients undergoing routine cancer screening.

Study constraints did not allow matching of buffy coat DNA with plasma DNA in the remaining positive participants and therefore, we were

unable to determine which mutations detected were truly derived from a tumor for all screen-positive subjects. Any future cfDNA studies should analyze these two components of the blood together in order to differentiate the origins of the observed alterations, and ideally supplemented with tissue analyzed in the event of a biopsy. Since this study was conducted outside a centralized hospital network, but was dependent on the individual's personal physician-mediated follow-up, tissue biopsies were also not available for comparative analysis. A centrally controlled follow-up protocol for clinical screening with full body MRIs and relevant scans and standard procedures would also facilitate the assessment of ctDNA positive participants.

Removing specific genes and mutations now known to be common age-related mutations would enable us to lower the false positive rate in this assay. In addition, raising reporting thresholds would help screen out some of the low level ctDNA from benign conditions. Also, adding tests for copy number variation, insertions and deletions potentially could improve the clinical performance.

Conclusion

The need for early cancer detection is undiminished. Liquid biopsy assays provide a new way to investigate screening utility. Balancing assay cost and access with convincing positive and negative predictive values for broad cancer screening is a challenge. The Liquid Biopsies Working Group, consisting of members from the American Society of Clinical Oncology (ASCO) and CAP, are working to examine the evidence available on the pre-analytical, analytical, and clinical validity and utility of ctDNA-based assays.

While this 96 mutation assay in the setting of this study did not support high clinical utility for early detection, future improvements could easily be made to investigate further. Those include medical pre-screening of all participants; an expanded assay-content; matched plasma, white cell and tissue analysis to eliminate clonal hematopoiesis as the source of ctDNA and to correlate ctDNA with the actual mutations in the tumor tissue; sufficiently raised reporting thresholds coupled with enrollment of a larger patient population. While the required monetary investment in such sizable trials is not trivial, the possible benefit for early detection may ultimately outweigh those costs.

Acknowledgements

We wish to thank Matthew Collins, Juanita Thompson and Geni Constantino for expert assistance in processing the specimens, and Dr. Nilesh Dharajiya for reviewing the ctDNA results. This study was funded by Pathway Genomics, San Diego, CA 92121.

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Anja Kammesheidt, Pathway Genomics, 4755 Nexus Center Drive, San Diego, CA 92121, USA. Tel: 948-874-8044; E-mail: anja@pathway.com

References

[1] Vogelstein B, Papadopoulos N, Velculescu VE, Zhou S, Diaz LA Jr and Kinzler KW. Cancer genome landscapes. Science 2013; 339: 1546-1558.

[2] Heitzer E, Ulz P and Geigl JB. Circulating tumor DNA as a liquid biopsy for cancer. Clin Chem 2015; 61: 112-123.

[3] Diaz LA Jr and Bardelli A. Liquid biopsies: genotyping circulating tumor DNA. J Clin Oncol 2014; 32: 579-586.

[4] Esposito A, Bardelli A, Criscitiello C, Colombo N, Gelao L, Fumagalli L, Minchella I, Locatelli M, Goldhirsch A and Curigliano G. Monitoring tumor-derived cell-free DNA in patients with solid tumors: clinical perspectives and research opportunities. Cancer Treat Rev 2014; 40: 648-655.

[5] Lebofsky R, Decraene C, Bernard V, Kamal M, Blin A, Leroy Q, Rio Frio T, Pierron G, Callens C, Bieche I, Saliou A, Madic J, Rouleau E, Bidard FC, Lantz O, Stern MH, Le Tourneau C and Pierga JY. Circulating tumor DNA as a non-invasive substitute to metastasis biopsy for tumor genotyping and personalized medicine in a prospective trial across all tumor types. Mol Oncol 2015; 9: 783-790.

[6] Bortolin MT, Tedeschi R, Bidoli E, Furlan C, Basaglia G, Minatel E, Gobitti C, Franchin G, Trovo M and De Paoli P. Cell-free DNA as a prognostic marker in stage I non-small-cell lung cancer patients undergoing stereotactic body radiotherapy. Biomarkers 2015; 1-7.

[7] Bettegowda C, Sausen M, Leary RJ, Kinde I, Wang Y, Agrawal N, Bartlett BR, Wang H, Luber

B, Alani RM, Antonarakis ES, Azad NS, Bardelli A, Brem H, Cameron JL, Lee CC, Fecher LA, Gallia GL, Gibbs P, Le D, Giuntoli RL, Goggins M, Hogarty MD, Holdhoff M, Hong SM, Jiao Y, Juhl HH, Kim JJ, Siravegna G, Laheru DA, Lauricella C, Lim M, Lipson EJ, Marie SK, Netto GJ, Oliner KS, Olivi A, Olsson L, Riggins GJ, Sartore-Bianchi A, Schmidt K, Shih IM, Oba-Shinjo SM, Siena S, Theodorescu D, Tie J, Harkins TT, Veronese S, Wang TL, Weingart JD, Wolfgang CL, Wood LD, Xing D, Hruban RH, Wu J, Allen PJ, Schmidt CM, Choti MA, Velculescu VE, Kinzler KW, Vogelstein B, Papadopoulos N and Diaz LA Jr. Detection of circulating tumor DNA in early- and late-stage human malignancies. Sci Transl Med 2014; 6: 224ra224.

[8] Frattini M, Gallino G, Signoroni S, Balestra D, Lusa L, Battaglia L, Sozzi G, Bertario L, Leo E, Pilotti S and Pierotti MA. Quantitative and qualitative characterization of plasma DNA identifies primary and recurrent colorectal cancer. Cancer Lett 2008; 263: 170-181.

[9] Hamakawa T, Kukita Y, Kurokawa Y, Miyazaki Y, Takahashi T, Yamasaki M, Miyata H, Nakajima K, Taniguchi K, Takiguchi S, Mori M, Doki Y and Kato K. Monitoring gastric cancer progression with circulating tumour DNA. Br J Cancer 2015; 112: 352-356.

[10] Janku F, Angenendt P, Tsimberidou AM, Fu S, Naing A, Falchook GS, Hong DS, Holley VR, Cabrilo G, Wheler JJ, Piha-Paul SA, Zinner RG, Bedikian AY, Overman MJ, Kee BK, Kim KB, Kopetz ES, Luthra R, Diehl F, Meric-Bernstam F and Kurzrock R. Actionable mutations in plasma cell-free DNA in patients with advanced cancers referred for experimental targeted therapies. Oncotarget 2015; 6: 12809-12821.

[11] Wong SQ, Raleigh JM, Callahan J, Vergara IA, Ftouni S, Hatzimihalis A, Colebatch AJ, Li J, Semple T, Doig K, Mintoff C, Sinha D, Yeh P, Silva MJ, Alsop K, Thorne H, Bowtell DD, Gyorki DE, Arnau GM, Cullinane C, Kee D, Brady B, Kelleher F, Dawson MA, Papenfuss AT, Shackleton M, Hicks RJ, McArthur GA, Sandhu S and Dawson SJ. Circulating tumor DNA analysis and functional imaging provide complementary approaches for comprehensive disease monitoring in metastatic melanoma. JCO Precision Oncology 2017; 1: 1-14.

[12] Garcia-Murillas I, Schiavon G, Weigelt B, Ng C, Hrebien S, Cutts RJ, Cheang M, Osin P, Nerurkar A, Kozarewa I, Garrido JA, Dowsett M, Reis-Filho JS, Smith IE and Turner NC. Mutation tracking in circulating tumor DNA predicts relapse in early breast cancer. Sci Transl Med 2015; 7: 302ra133.

[13] Chen A, Braunstein G, Anselmo M, Jaboni J, Viloria F, Neidich J, Li X and Kammesheidt A. Mutation detection with a liquid biopsy 96 mutation assay in cancer patients and healthy donors. Cancer Translational Medicine 2017; 3: 39.

[14] Forshew T, Murtaza M, Parkinson C, Gale D, Tsui DW, Kaper F, Dawson SJ, Piskorz AM, Jimenez-Linan M, Bentley D, Hadfield J, May AP, Caldas C, Brenton JD and Rosenfeld N. Noninvasive identification and monitoring of cancer mutations by targeted deep sequencing of plasma DNA. Sci Transl Med 2012; 4: 136ra168.

[15] Newman AM, Bratman SV, To J, Wynne JF, Eclov NC, Modlin LA, Liu CL, Neal JW, Wakelee HA, Merritt RE, Shrager JB, Loo BW Jr, Alizadeh AA and Diehn M. An ultrasensitive method for quantitating circulating tumor DNA with broad patient coverage. Nat Med 2014; 20: 548-554.

[16] Chen KZ, Lou F, Yang F, Zhang JB, Ye H, Chen W, Guan T, Zhao MY, Su XX, Shi R, Jones L, Huang XF, Chen SY and Wang J. Circulating tumor DNA detection in early-stage non-small cell lung cancer patients by targeted sequencing. Sci Rep 2016; 6: 31985.

[17] Alix-Panabieres C, Schwarzenbach H and Pantel K. Circulating tumor cells and circulating tumor DNA. Annu Rev Med 2012; 63: 199-215.

[18] Thompson JD, Shibahara G, Rajan S, Pel J and Marziali A. Winnowing DNA for rare sequences: highly specific sequence and methylation based enrichment. PLoS One 2012; 7: e31597.

[19] Tabibian JH, O'Hara SP, Splinter PL, Trussoni CE and LaRusso NF. Cholangiocyte senescence by way of N-ras activation is a characteristic of primary sclerosing cholangitis. Hepatology 2014; 59: 2263-2275.

[20] Gormally E, Vineis P, Matullo G, Veglia F, Caboux E, Le Roux E, Peluso M, Garte S, Guarrera S, Munnia A, Airoldi L, Autrup H, Malaveille C, Dunning A, Overvad K, Tjønneland A, Lund E, Clavel-hapelon F, Boeing H, Trichopoulou A, Palli D, Krogh V, Tumino R, Panico S, Bueno-de-Mesquita HB, Peeters PH, Pera G, Martinez C, Dorronsoro M, Barricarte A, Navarro C, Quirós JR, Hallmans G, Day NE, Key TJ, Saracci R, Kaaks R, Riboli E and Hainaut P. TP53 and KRAS2 mutations in plasma DNA of healthy subjects and subsequent cancer occurrence: a prospective study. Cancer Res 2006; 6: 871-6.

[21] Anglesio MS, Papadopoulos N, Ayhan A, Nazeran TM, Noe M, Horlings HM, Lum A, Jones S, Senz J, Seckin T, Ho J, Wu RC, Lac V, Ogawa H, Tessier-Cloutier B, Alhassan R, Wang A, Wang Y, Cohen JD, Wong F, Hasanovic A, Orr N, Zhang M, Popoli M, McMahon W, Wood LD, Mattox A, Allaire C, Segars J, Williams C, Tomasetti C, Boyd N, Kinzler KW, Gilks CB, Diaz L, Wang TL,

Vogelstein B, Yong PJ, Huntsman DG and Shih IM. Cancer-associated mutations in endometriosis without cancer. N Engl J Med 2017; 376: 1835-1848.

[22] Lui YY, Chik KW, Chiu RW, Ho CY, Lam CW and Lo YM. Predominant hematopoietic origin of cell-free DNA in plasma and serum after sex-mismatched bone marrow transplantation. Clin Chem 2002; 48: 421-427.

[23] Tug S, Helmig S, Deichmann ER, Schmeier-Jurchott A, Wagner E, Zimmermann T, Radsak M, Giacca M and Simon P. Exercise-induced increases in cell free DNA in human plasma originate predominantly from cells of the haematopoietic lineage. Exerc Immunol Rev 2015; 21: 164-173.

[24] Genovese G, Kahler AK, Handsaker RE, Lindberg J, Rose SA, Bakhoum SF, Chambert K, Mick E, Neale BM, Fromer M, Purcell SM, Svantesson O, Landen M, Hoglund M, Lehmann S, Gabriel SB, Moran JL, Lander ES, Sullivan PF, Sklar P, Gronberg H, Hultman CM and McCarroll SA. Clonal hematopoiesis and blood-cancer risk inferred from blood DNA sequence. N Engl J Med 2014; 371: 2477-2487.

[25] Razavi P, Li BT, Hou C, Shen R, Venn O and Lim R. Cell-free DNA (cfDNA) mutations from clonal hematopoiesis: implications for interpretation of liquid biopsy tests. J Clin Oncol 2017; abstr 11526.

[26] Phallen J, Sausen M, Adleff V, Leal A, Hruban C, White J, Anagnostou V, Fiksel J, Cristiano S, Papp E, Speir S, Reinert T, Orntoft MW, Woodward BD, Murphy D, Parpart-Li S, Riley D, Nesselbush M, Sengamalay N, Georgiadis A, Li QK, Madsen MR, Mortensen FV, Huiskens J, Punt C, van Grieken N, Fijneman R, Meijer G, Husain H, Scharpf RB, Diaz LA Jr, Jones S, Angiuoli S, Ørntoft T, Nielsen HJ, Andersen CL and Velculescu VE. Direct detection of early-stage cancers using circulating tumor DNA. Sci Transl Med 2017; 9: 1-12.

[27] Snyder MW, Kircher M, Hill AJ, Daza RM and Shendure J. Cell-free DNA comprises an in vivo nucleosome footprint that informs its tissues-of-origin. Cell 2016; 164: 57-68.