Original Article

Quantitation of total Circulating Cell-free DNA as a Screening Modality for Cancer

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Abstract:

Aims: Early diagnosis and simple pan-cancer screening are two unmet diagnostic needs in cancer detection. We evaluated the clinical utility of total circulating cell-free DNA as a non-invasive dual-purpose pan-cancer screening and early-detection tool.

Material & Methods: The study recruited 159 newly diagnosed, therapy naïve patients spanning 16 cancer types as well as 12 asymptomatic (healthy) volunteers who provided signed informed consent. Ten mL of peripheral blood sample was collected from consenting participants, from which cfDNA was purified from the plasma. cfDNA concentrations were determined and correlated with disease status of each participant. Mann-Whitney U test was employed to determine statistical significance.

Results: Patients with Lymphoma were found to have significantly (p<0.05) elevated plasma cfDNA concentrations (15.61ng/µL) as compared to asymptomatic individuals (4.22ng/µL). No significant differences were determined between plasma cfDNA concentrations in diagnosed patients and asymptomatic individuals in any of the cancer types.

Conclusion: We hypothesize that analysis of larger sub-cohorts of individual cancer types in a larger multi-center study is required to unambiguously establish statistically significant differences (or lack of the same) in cfDNA concentrations between diagnosed cases and asymptomatic individuals.

Key Words: Circulating cell-free tumor DNA, cfDNA, ctDNA, Cancer, Liquid biopsy, as Cancer screening

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Introduction:

Higher rate of cancer mortality is attributed to late detection; in India, only 20-30% of all cancers cases are diagnosed at stage I or II (Rajpal et al., 2014).[1,2,3,4] Although there are several screening modalities that find use in early detection, they are usually restricted to a single type of cancer, e.g., mammograms for breast cancer, Pap smears for cervical cancer, PSA for prostate cancer, fecal occult blood tests for colon cancer, and LDCT (low-dose computed topography) for lung cancer. Additionally, although these screening tools are known to be effective, they are often associated with side effects such as radiation exposure (Dainiak et al., 2016; Zhou et al., 2016),[5,6] or significant false-positive rates such as in case of mammography for breast cancer, LDCT in lung cancer, and prostate-specific antigen (PSA) (Nelson et al., 2016a; Chou et al., 2011),[7,8] all of which have been reported with disastrous outcomes

(Nelsonet al., 2016b).[9] The need for a sensitive, accuratecost effective screening modality for early stage pan-cancer detection is an unmet and urgent clinical need and such a screen is key to improving outcomes. Several researchers have indicated the potential utility of analyzing circulating tumor biomarkers in peripheral blood, viz, Liquid Biopsy in diagnosis and early detection. (Crowley et al., 2013).[10] Among the many circulating tumor biomarkers qualitative and quantitative investigations of cell-free tumor DNA (cfDNA / ctDNA)finds use in diagnosis, monitoring and treatment of cancers (Diaz et al., 2014; Alix-Panabieres et al., 2016; Leung et al., 2016).[11,12,13] Quantitation of cfDNA appears to have utility as a surrogate marker of malignancy and has been studied in liver and lung cancers (Liao et al., 2015; Jiang et al., 2016).[14,15] cfDNA quantitation was reported to discern malignant breast cancer from benign nodules (Kohler et al., 2009; Hashad et al., 2010; Oliver et al., 2014).[16,17,18] Researchers have found that peripheral blood cfDNA concentrations was concordant with stage of cancer (Newman et al., 2014; Bettegowda et al., 2014).[19,20] Based on these reports, we hypothesized that quantitation of total cfDNA in peripheral blood was a simple, accurate, cost-effective means to detect presence of malignancy. To our knowledge there have been no such studies conducted in India so far. We investigated whether quantitation of total cfDNA correlated with disease status in confirmed cancers as well as asymptomatic individuals.

Material & Methods:

Ethical statement

This study was conducted following approval from the Institutional Ethical committees of Pravara Institute of Medical Sciences (PIMS), Loni, India. Participants provided signed informed consent for blood draw as well as for their deidentified information to be collected, stored and analyzed for research purposes.

Study population and design

This cross-sectional study included 171subjects divided into twogroups

Group I: included 12 healthy volunteers who were of matched age and sex, and their samples as well as data served as analytical controls.

Group II: included 159 newly diagnosed as well as previously diagnosed but therapy naïve patients with cancer of different types and all stages.

cfDNA purification and quantification

Peripheral blood samples (10 mL) were collected by venous puncture in EDTA vacutainers from all study participants. Within 1 hour of collection, the samples were centrifuged for 10 min at 3000 × g at 20°C. Collected samples were transported to Datar Genetics laboratories for analysis. The supernatant (plasma) were transferred to microcentrifuge tubes and stored at -80°C.cfDNA was isolated from plasma by means of a QIAamp Circulating Nucleic Acid Kit (Qiagen, Hilden, Germany) as per the manufacturer's instructions. Subsequently, the circulating cfDNA concentration was quantitated with the Invitrogen™ Qubit™ dsDNA HS (High Sensitivity) Assay Kit and stored at -80°C.

Statistical analysis

Patients were stratified according to age, gender and whether diagnosed cases or asymptomatic; diagnosed cases were further stratified according to cancer types. Mean values of cfDNA were calculated for each stratification sub-cohort and differences between cohorts determined.

Results:

Clinical characteristics

September 2016 to December 2017, 171 participants were recruited from the department OPD. All samples and medical records were anonymized. Patients with therapy naïve Stage I to

stage IV malignancy/cancer with primary or recurrent diseases was included in the study.

159 participants were diagnosed and therapy naïve, and 12 were asymptomatic. The age of the participants ranged between 20-81 years with a median age of 55 years. The cohort had a total of 80 males and 91 females, among whom 4 males were and 8 females were asymptomatic (Table 1).

cfDNA quantification

Plasma cfDNA levels in the cancer and control group is represented in Figure 1. The median cfDNA concentration for cancer group was 4.67 ng/mL (range1.53-90.24 ng/mL) and for asymptomatic group was 4.22ng/mL (range 3.2-14.4 ng/mL). The differences in median cfDNA concentrations were marginal and not statistically significant.

Among the cancer-type sub-cohorts, median cfDNA concentrations were: 3.87 ng/mL (head and neck), 3.54 ng/ mL (colorectal), 2.46 ng/mL (brain), 3.77 ng/mL (breast), 6.08 ng/mL (cervix), 15.61 ng/mL (lymphoma), 4.67 ng/mL (esophagus), 2.67 ng/mL (endometrium), 8.7 ng/mL (lung), 3.75 ng/mL (ovary), 15 ng/mL (leukemia), 11.52 ng/mL (urinary bladder), 19.52 ng/mL (liver), 4.09 ng/mL (prostate), 8.35 ng/mL (stomach), and 6.01 ng/mL (other). Amongst all the types of cancer, the plasma cfDNA level of the lymphoma group was significantly (p<0.05) higher than that of the control group (Figure 2).

Table 1: Patient data collected for the study

S.No.	Cancer type	No. of samples	Male		Female	
			No. of samples	Age range (years)	No. of samples	Age range (years)
1.	Asymptomatic	12	4	31-32	8	28-40
2.	Brain	1	1	42	-	-
3.	Breast	4	-	-	4	60-80
4.	Cervix	51	-	-	51	35-81
5.	Colorectum	6	4	28-74	2	50-75
6.	Endometrium	1	-	-	1	55
7.	Esophagus	8	5	48-67	3	65-70
8.	Head and Neck	64	53	40-80	11	40-76
9.	Leukemia	1	1	37	-	-
10.	Liver	1	1	70	-	-
11.	Lung	2	2	70-72	-	-
12.	Lymphoma	5	4	20-70	1	32
13.	Other	7	3	50-66	4	30-65
14.	Prostate	1	1	62	-	-
15.	RCC	1	1	55	-	-
16.	Ovary	3	-	-	3	30-65
17.	Stomach	2	-	-	2	45-78
18.	Urinary bladder	1	-	-	1	46

Figure 1: cfDNA levels in cancer and control group.

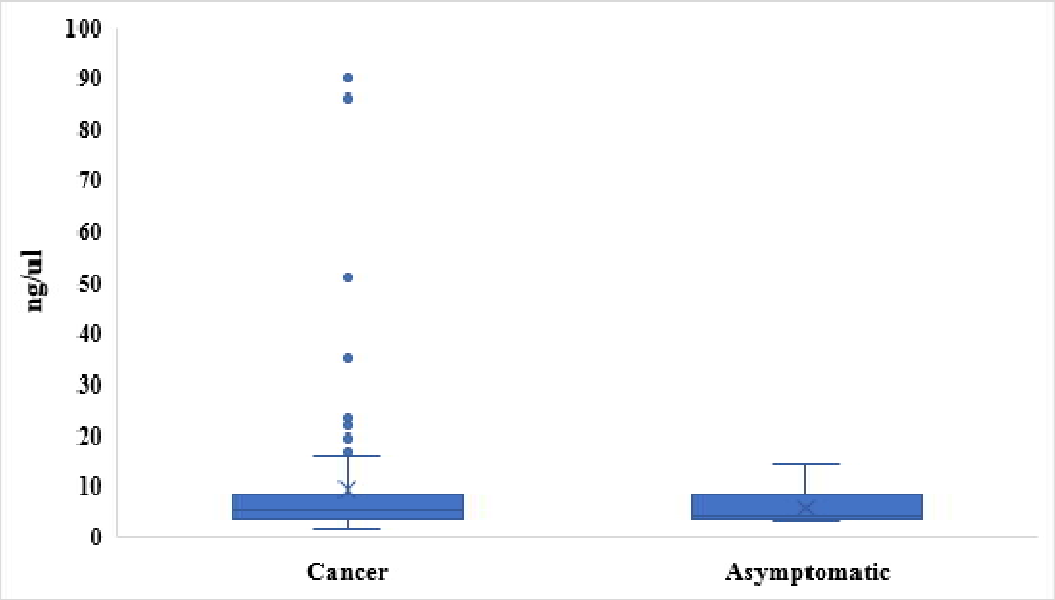
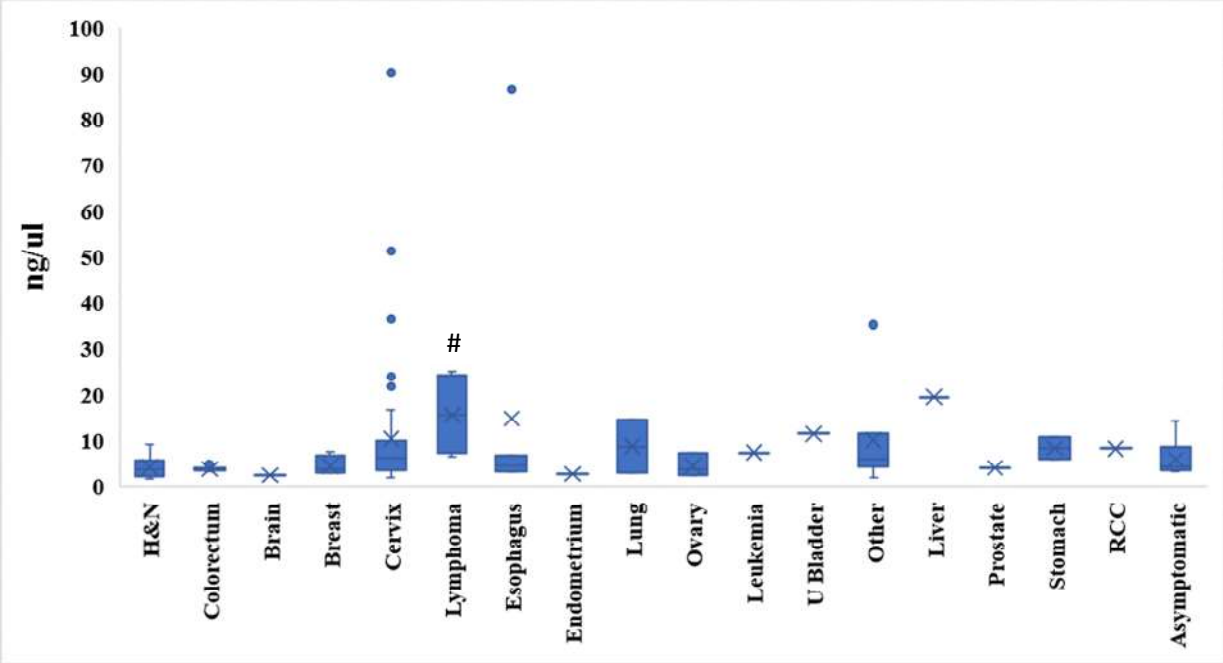


Figure 2: cfDNA levels in cancer subgroups and control group.



[#] indicates significance at <0.05.

Discussion:

There are presently ~2.5 million diagnosed cancer cases in India with an annual addition of ~700,000 new cases as well ~550,000cancer-related deaths annually, which is projected to increase to ~900,000 annual deaths by the end of this decade (Takiar et al., 2010).[1] The five most common cancers in India are those of breast (144,937; 14.3%), cervix uteri (122,844;12.1%), lip-oral (77,003; 7.6), lung (70,275; 6.9%) and colorectum (64,332; 6.3%), which comprise 47.2% of the 28 prevalent cancer types and contribute to ~300,000 deaths annually. (Sarnath and Khanna, 2014).[2] It has been hypothesized that ~60% of cancer deaths can be prevented with improvements in preventive facilities and screening programs for early detection and timely interventions in asymptomatic individuals. Likewise, curative therapies are most successful when cancer is diagnosed and treated at an early stage. Although technological advances in next-generation sequencing of circulating, tumor-derived nucleic acids hold promise for addressing the challenge of developing safe and effective cancer screening tests, cost feasibility for the same do not. A screening test should be safe, cheap, highly specific and sensitive, with a high predictive value that can easily and quickly be used in a large population to detect the disease with a proven benefit (Etzioni et al., 2003; Harris et al., 2006).[21,22] Analysis of circulating cell free tumor DNA has been the topic of significant research as an easily accessible biomarker for detection of cancers. While most studies have primarily focused on the qualitative aspects such as mutation profile and quantitation of mutation burden in cfDNA, limited number of studies have also evaluated the quantitation of total cfDNA as an indicator of malignancy. It has been suggested that the high turnover rates of cancer cells (proliferation + death due to apoptosis and necrosis) contributes to total cfDNA in peripheral blood, which otherwise originates from healthy dying cells in asymptomatic individuals; patients with cancer were thus hypothesized to have higher amount of total cfDNA (Vaart et al., 2010).[23] Prior studies have reported significant differences betweencfDNA from healthy individuals, benign conditions patients and diagnosed cancers (Board et al., 2008; Xie et al., 2004).[24,25] Frattini et al. (2008)[26] concluded that quantification of plasma DNA can confirm the presence of CRC and disease-free status or relapse after surgery. On the other hand, two separate studies reported that cfDNA quantitation was ineffective at discerning benign prostate hyperplasia from prostate cancer (Jung et al., 2004; Boddy et al., 2005).[27,28]

The aim of the present observational study was to evaluate the clinical utility of quantitative cfDNA analysis in detecting pan-cancers and discerning diagnosed cases from asymptomatic individuals. No significant difference was noted in cfDNA yield in cancer patients as compared with the control group. We quantitated cfDNA in population of mixed cancer types and observed that significant (p<0.05) differences were observed only in cases of Lymphoma.

We acknowledge the contribution of confounding factors that contribute to differences in these findings including, but not limited to, differences in collection tubes or protocols, variations in storage and transportation conditions, or DNA isolation and quantitation protocols. We also acknowledge the absence of statistically significant representation of all reported cancer types which may led to underrepresentation of cancer types where higher cfDNA concentrations may have otherwise been noted. Asymptomatic status of the 12 ‘healthy’ participants was determined on the basis of routine clinical investigations, which may have failed to detect latent or emergent

malignancies with no immediate clinical manifestation – in such cases, higher cfDNA concentrations may have contributed to an elevated baseline, especially considering the limited number of samples from asymptomatic individuals. Based on prior reports as well as our own observations of significant differences in cfDNA concentrations between asymptomatic and Lymphoma groups, further investigations are warranted with a larger population that better represents the various prevalent cancer types.

Conclusion:

In principle, quantitative analysis of cfDNA is a simple, non-invasive, rapid and cost-effective approach to screening multiple cancer types; the technology is easily scalable for higher sample throughput thus fulfilling important criteria for a pan-cancer screening test.

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