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Cell free DNA - A novel biomarker in the field of oncology: A comparative account in the cancer patients and healthy residents of Karachi city

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Abstract

The present study was carried out to ascertain the levels of cell free DNA (cf-DNA) in the serum samples of diagnosed cancer patients having various malignancies and healthy residents of Karachi City. Collection of blood samples was carried out with informed consent from diagnosed cancer patients and healthy human subjects at various hospitals of Karachi City. Serum was isolated and analyzed for cf-DNA with genomic DNA isolation accompanied with Isoamyl-Phenol-Chloroform purification. Positive percentage of cf-DNA was found to be 36.84% (14/38 samples) in the healthy subjects and 56.45% (35/62 samples) in the cancer patients. The detected mean level of cf-DNA in the control and cancer group was found to be 1758.8ng/mL and 5584.27ng/µl respectively. Significantly elevated levels of cf-DNA were detected in the cancer cases compared with the healthy subjects. Cancer cases of the oral cavity and pharynx and breast cancer generally were having higher mean cf-DNA concentrations followed by blood cancer cases. Higher serum cf-DNA levels in the cancer cases compared with the healthy subjects is associated with the level of damage caused to nuclear DNA in various malignancies. A simple cost-effective blood test for the application of novel cancer biomarker (cf-DNA) will assist clinicians to implement therapeutics in an efficient and effective way for the diagnosis, staging and prognosis of various cancers during cancer management.

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Introduction

Circulating DNA or cell free DNA (cf-DNA) is created during cell death and various malignancies in the body. This is named so because it contains DNA fragments that are present outside the nucleus, circulating freely in the blood stream. Cell death causes an increase in cf-DNA in cancer patients as well as in healthy individuals because of apoptosis of several hundred billions of cells on daily basis (Nagata *et al.,* 2010).

Patients with various types of tumors have been observed to have elevated cf-DNA levels compared with healthy subjects, (Jahr *et al.,* 2001, Schwarzenbach *et al.,* 2011). Healthy individuals generally have low levels of cf-DNA because of the efficient removal of dead cells from circulation by phagocytes (Elshimali *et al.,* 2013).

Concentrations of cf-DNA varies in different individuals at different stages of life and disease due to multiple factors. The most important factors which determine the levels of cf-DNA are age, tumor volume, tumor histology, lymph node metastasis and tumor progression (Park *et al.,* 2012).

Circulating DNA was first reported by Leon *et al.* (1977) in the serum samples of cancer patients. Later studies found tumor derived oncogene mutations in the serum and plasma of pancreatic cancer patients and acute myelogenous leukemia (Sorenson *et al.,* 1994; Vasioukhin *et al.,* 1994). Increase in the levels of cf-DNA in the serum indicates progression and metastasis of cancer which can be utilized as a diagnostic and prognostic tool in the clinical examination of various malignancies. Circulating nucleic acids have been found to be potent biomarkers in the diagnosis, staging and prognosis of various cancers in humans (Tong and Dennis 2005, Cabral *et al.,* 2010).

In healthy individuals, the concentrations of cf-DNA can be used to evaluate the level of damage caused to cellular DNA by various factors and to take preemptive measures to prevent the individual from various malignancies before its onset.

The present study aimed to evaluate the levels of cf-DNA in cancer patients and healthy humans so that it can best be utilized as a potent biomarker in the field of oncology for the diagnosis, staging and prognosis of various malignancies in the human body. Cell free DNA can be utilized in PCR analysis to ascertain the cause of various cancers at genetic level.

In light of the available literature on cf-DNA, it is evident that this potent biomarker has not properly utilized as a suitable diagnostic and prognostic tool in cancer management by medical practitioners.

The objective of the study was to ascertain the levels of cf-DNA in cancer patients and healthy humans and to evaluate any marked difference in the studied cohort. The detected concentrations of cf-DNA in the studied cancer types and healthy individuals can be used as diagnostic and prognostic biomarkers indicating the level of damage caused to cellular DNA by various factors.

This will help clinicians to efficiently utilize concentrations of cf-DNA as a cost-effective tool in the field of oncology. This study can be used as a baseline for the determination of exact or estimated threshold levels of cf-DNA beyond which the subject individual can be at high risk for the onset of various malignancies.

Materials and methods

Subjects and Sample Collection

Collection of blood samples were carried out with informed consent from diagnosed cancer patients (62 cases) of various hospitals and health care centers and from healthy individuals (30 samples) of Karachi City. Blood was collected in red top gel clot activator tubes. Serum was separated within 2 hours of collection by centrifugation at (1300 rpm: 5 min).

Isolation of cf-DNA

Total cf-DNA was isolated using genomic DNA kit YGE100 for the isolation of serum cf-DNA, accompanied with Phenol-Chloroform method.

The yield of cf-DNA in the serum samples with this method was significant as compared with other conventional methods in use.

The difference in various methods lies only in the buffer component of the kits which can be used variously according to the samples analyzed (blood or serum). Analysis of cf-DNA was carried out in light of the previously described methods (Ausubel *et al.,* 1995, Anker *et al.,* 1999, Rodrigo *et al.,* 2002). Methods used for the assessment of cf-DNA in plasma/serum have been diverse, and there is no universal standardized protocol established up till now (Wagner, 2012).

Cells (RBC) lysis buffer steps were excluded as the samples were pure serum. About 50µl of each serum sample was directly mixed with 100µL protein remove buffer and vortex for 10 seconds, followed by addition of RNA remove buffer (100µL) and then incubation on ice for 10 min. After incubation, the cell suspension was centrifuged at 14000 rpm for 10min.

The supernatant was shifted to another tube containing 800µL iso-propanol and mixed thoroughly by inverting the tube before placing it on ice for 10min. After wards, the pellet was collected by centrifugation of the tube at 14000rpm for 15min.

The pellet was rinsed with wash buffer (500µL; 70% ethanol) twice and air-dried for 10min for complete evaporation of ethanol. Finally, the cf-DNA pellet was dissolved in 50µL TAE buffer (2M Tris-Base pH 7.5,1M glacial acetic acid, and 10% of 0.5mM EDTA pH 8).

An equal amount of Isoamy l Phenol and Chloroform mixture was added to the isolated DNA and vortex at room temperature for 3-5 minutes and then centrifuged at 3000g, for 10min.

The supernatant cf-DNA containing layer was transferred to a clean 1.5mL tube while the lower white buffy layer was discarded. This step was repeated twice.

Quantification of cf-DNA with Nano Drop-2000/2000c Spectrophotometer

After the isolation and purification of cf-DNA, 2µL fraction of each sample was processed by Thermo NanoDrop-2000/2000c Spectrophotometer. A cf-DNA data file was generated, which showed the amount and purity of cf-DNA in each serum sample.

Results

Mean ages of the subjects in the cancer and control groups were 38.5 years and 33.3 years respectively. Sex ratio (Female /Male) was 10.5% (4/38) in control group and 43.5% (27/62) in cancer patients. Detection of cf-DNA was found to be 36.84% (14/38 samples) in control group and 56.45% (35/62 samples) in the cancer group. Elevated levels of cf-DNA were observed frequently in the cancer group compared with the normal group. The detected mean concentrations of cf-DNA in the control and cancer groups were 1758.8±3366ng/µl and 5584.27±5404ng/µl respectively.

Minimum and maximum detected levels of cf-DNA in the control and cancer groups were 17.4-8757.6ng/µl and 5381.3-18031.50ng/µl respectively.

Analysis of cf-DNA showed a high frequency and concentration in the cancer cases compared with the control group (Table 1, Fig. 1, 2). The major cancer sites in respect of the detected cf-DNA concentrations were found in the order of: oral cavity and pharynx > breast > blood cancer > bone tumor > female genital system > lymphatic system > digestive system > skin > respiratory system (Table 1, Fig.1). Order of the individual sub sites of cancers in respect of the decreasing mean levels of cf-DNA was: tongue > breast > ALL > CML > bone > vagina > NHL > stomach > AML > neck > rectum > esophagus > cheek > lung > colon (Table 1, Fig. 2).

There was no detection of cf-DNA in the cases of nasopharyx, oral mucosa, anal canal, larynx, cervix, ovary and HL. Cancer cases of the blood (CML, ALL and ALL) and Lymphatic system (NHL) generally were having higher mean levels of cf-DNA amongst

the analyzed sub sites of cancers which is indicative of the fact that the released cf-DNA ultimately come in the blood stream and adds to the net pool of cf-DNA. All of the analyzed major cancer sites and sub sites showed significantly higher levels of cf-DNA compared with the control group.

Table 1. Mean concentrations of cf-DNA in the major and sub sites of cancer cases.

Major Cancer Site				Cancer Sub Site			
S.No.	Major Cancer Site	Mean cf-DNA (ng/µl)	SD	S.No.	Cancer Sub Site	Mean cf-DNA (ng/µl)	SD
1	Oral Cavity and Pharynx	6886.8	6631.3	(I)	Nasopharynx	ND	
				(II)	Oral Mucosa	ND	
				(III)	Tongue	11478.1	4634.6
2	Digestive System	3921.7	4286.2	(I)	Oesophagus	3015.8	4265.0
				(II)	Stomach	7986.0	1514.8
				(III)	Colon	1931.9	3863.7
				(IV)	Rectum	4087.7	4087.7
				(V)	Anal Canal	ND	
3	Respiratory System	2211.0	3829.6	(I)	Larynx	ND	
				(II)	Lung	2948.0	4169.1
4	Skin	3409.8	4907.0	(I)	Cheek	2951.0	5111.3
				(II)	Neck	4327.2	4327.2
5	Breast	7742.8	5069.0	(I)	Breast	7742.8	5069.0
6	Female Genital System	2246.9	3891.7	(I)	Cervix	ND	
				(II)	Ovary	ND	
				(III)	Vagina	8987.6	0
7	Blood Cancer	7935.0	5386.6	(I)	ALL	9104.1	6393.7
				(II)	AML	7150.8	5325.2
				(III)	CML	9518.8	505.3
8	Lymphatic System	4419.0	4419.0	(I)	HL	ND	
				(II)	NHL	8838.1	0
9	Bone Marrow	9191.6	0	(I)	Ewing's Sarcoma	9191.6	0

Abbreviations: AML: Acute Myelocytic Leukemia; ALL: Acute Lymphoblastic Leukemia; CML: Chronic Myelocytic Leukemia; CLL: Chronic Lymphoblastic Leukemia; NHL: Non-Hodgkin's Lymphoma; HL: Hodgkin's Lymphoma; SD: Standard Deviation; ND: Not Detected.

Table 2. Cell free-DNA concentrations complete profile in normal healthy individuals.

Sample No	Sample Type	Conc. ηg/µl)	A260	A280	A260/ A280	A260/ 230	Factor
1.	Normal	ND	--	--	--	--	--
2.	Normal	627.0	12.540	7.042	1.78	1.84	50.00
3.	Normal	ND	--	--	--	--	--
4.	Normal	338.8	6.776	3.610	1.88	1.79	50.00
5.	Normal	ND	--	--	--	--	--
6.	Normal	ND	--	--	--	--	--
7.	Normal	7852.8	157.057	127.265	1.23	0.98	50.00
8.	Normal	34.5	0.689	0.371	1.86	0.58	50.00
9.	Normal	ND	--	--	--	--	--
10.	Normal	ND	--	--	--	--	--
11.	Normal	7472.3	149.447	119.111	1.25	1.02	50.00
12.	Normal	7767.65	155.353	131.807	1.18	0.91	50.00
13.	Normal	ND	--	--	--	--	--
14.	Normal	8342.0	166.840	113.715	1.47	1.26	50.00
15.	Normal	7880.3	157.606	111.089	1.41	1.13	50.00
16.	Normal	404.0	8.079	4.357	1.85	1.91	50.00
17.	Normal	ND	--	--	--	--	--
18.	Normal	ND	--	--	--	--	--
19.	Normal	18.7	0.374	0.211	1.77	0.34	50.00
20.	Normal	ND	--	--	--	--	--
21.	Normal	8687.5	173.75	168.271	1.03	0.99	50.00

Sample No	Sample Type	Conc. ηg/µl)	A260	A280	A260/ A280	A260/ 230	Factor
22.	Normal	8757.6	175.151	122.971	1.42	1.16	50.00
23.	Normal	ND	--	--	--	--	--
24.	Normal	ND	--	--	--	--	--
25.	Normal	ND	--	--	--	--	--
26.	Normal	17.4	0.348	0.189	1.84	0.33	50.00
27.	Normal	ND	--	--	--	--	--
28.	Normal	8633.4	172.668	123.679	1.40	1.09	50.00
29.	Normal	ND	--	--	--	--	--
30.	Normal	ND	--	--	--	--	--
31.	Normal	ND	--	--	--	--	--
32.	Normal	ND	--	--	--	--	--
33.	Normal	ND	--	--	--	--	--
34.	Normal	ND	--	--	--	--	--
35.	Normal	ND	--	--	--	--	--
36.	Normal	ND	--	--	--	--	--
37.	Normal	ND	--	--	--	--	--
38.	Normal	ND	--	--	--	--	--

Table 3. Cell free-DNA concentrations complete profile in cancer patients.

Sample No	Cancer Type	Conc. (ηg/µl)	A260	A280	A260/ A280	A260/ A230	Factor
1	Cheek	11804.1	236.083	156.222	1.51	1.34	50.00
2	Colon	ND	--	--	--	--	--
3	ALL	18017.0	360.340	341.542	1.06	1.00	50.00
4	ALL	ND	--	--	--	--	--
5	AML	ND	--	--	--	--	--
6	Anus	ND	--	--	--	--	--
7	Breast	8643.1	172.862	122.935	1.41	1.15	50.00
8	Breast	8302.2	166.045	118.823	1.40	1.12	50.00
9	Breast	ND	--	--	--	--	--
10	Stomach	5381.3	107.627	84.530	1.27	1.05	50.00
11	Stomach	8557.2	171.144	123.025	1.39	1.14	50.00
12	Breast	ND	--	--	--	--	--
13	Breast	8441.4	168.829	118.793	1.42	1.22	50.00
14	AML	9249.4	184.987	130.095	1.42	1.13	50.00
15	CML	10024.1	200.482	127.766	1.57	1.40	50.00
16	Cheek	ND	--	--	--	--	--
17	AML	9076.0	181.521	124.549	1.46	1.19	50.00
18	AML	17502.6	350.052	335.083	1.04	1.03	50.00
19	Neck	ND	--	--	--	--	--
20	CML	9013.5	180.270	130.398	1.38	1.08	50.00
21	AML	7822.2	156.445	106.911	1.46	1.25	50.00
22	Breast	8202.6	164.052	115.243	1.42	1.22	50.00
23	Lung	ND	--	--	--	--	--
24	Tongue	8110.1	162.203	114.820	1.41	1.20	50.00
25	Oral cavity	ND	--	--	--	--	--
26	Rectum	8175.4	163.507	112.498	1.45	1.22	50.00
27	AML	ND	--	--	--	--	--
28	Breast	9482.3	189.646	134.103	1.41	1.11	50.00
29	AML	8812.8	176.256	123.997	1.42	1.09	50.00
30	AML	10001.9	200.037	137.619	1.45	1.16	50.00
31	Colon	ND	--	--	--	--	--
32	Larynx	ND	--	--	--	--	--
33	Colon	ND	--	--	--	--	--
34	ALL	8432.8	168.656	120.852	1.40	1.17	50.00
35	Lung	8844.1	176.883	124.987	1.42	1.23	50.00

Sample No	Cancer Type	Conc. (ηg/µl)	A260	A280	A260/ A280	A260/ A230	Factor
36	AML	9040.8	180.816	132.348	1.37	1.05	50.00
37	Esophagus	ND	--	--	--	--	--
38	Stomach	9004.0	180.081	126.632	1.42	1.04	50.00
39	Breast	8585.1	171.701	125.739	1.37	1.09	50.00
40	Stomach	9001.4	180.028	170.346	1.06	1.01	50.00
41	Breast	18028.4	360.568	342.906	1.05	1.01	50.00
42	Rectum	ND					
43	Cervix	ND	--	--	--	--	--
44	Tongue	18031.5	360.630	340.819	1.06	1.00	50.00
45	Colorectum	9659.3	193.186	137.363	1.41	1.02	50.00
46	Lung	ND	--	--	--	--	--
47	NHL	8838.1	176.763	123.814	1.43	1.11	50.00
48	Cheek	ND	--	--	--	--	--
49	Ovary	ND	--	--	--	--	--
50	ALL	9966.9	199.338	135.286	1.47	1.20	50.00
51	Vagina	8987.6	179.753	132.774	1.35	1.05	50.00
52	AML	ND	--	--	--	--	--
53	Rectum	ND	--	--	--	--	--
54	Nasopharynx	ND	--	--	--	--	--
55	Esophagus	ND	--	--	--	--	--
56	Cheek	ND	--	--	--	--	--
57	Tongue	8292.6	165.851	115.685	1.43	1.19	50.00
58	Neck	8654.4	173.087	127.439	1.36	1.06	50.00
59	Cervix	ND	--	--	--	--	--
60	Ewing's Sarcoma	9191.6	183.831	137.532	1.34	0.99	50.00
61	HL	ND	--	--	--	--	--
62	Esophagus	9047.4	180.948	133.507	1.36	1.04	50.00

Legend: A 260 and A 280: Absorbance at wavelengths of 260nm and 280nm. A260/280: Ratio used for the assessment of purity of DNA. 260/230: Ratio of absorbance at 260 nm to 230nm, a secondary measure of the purity of DNA. Factor: Dilution factor of water added to the cf-DNA pellet, here used as 50µL.

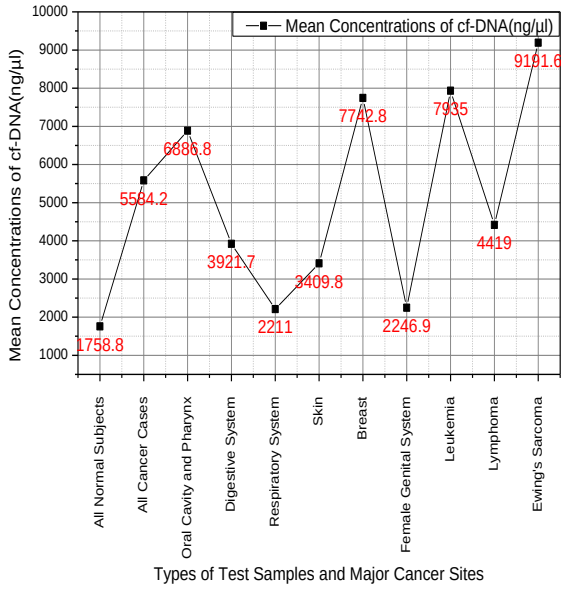

Fig. 1. Mean levels of cf-DNA in the Analyzed Normal Subjects and Major Cancer Sites.

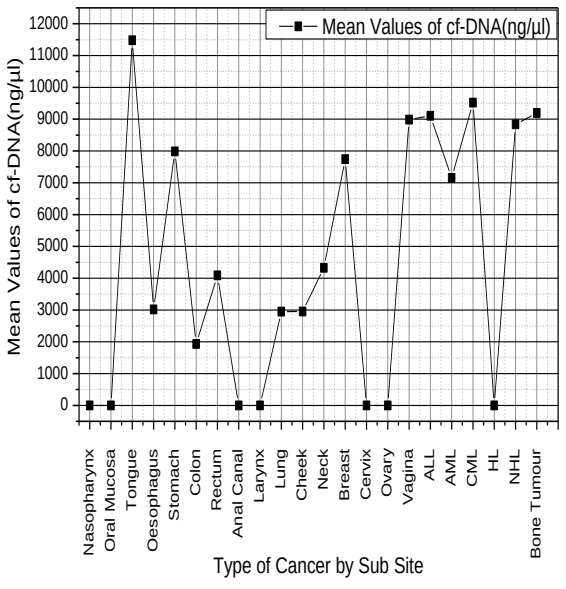

Fig. 2. Mean levels of cf-DNA in the Analyzed Sub Sites of Cancer Cases.

Discussion

Circulating or cell free DNA is released into the plasma and serum due to breakdown of nuclear DNA as a result of mutations and abnormalities in the body. Elevated concentrations of cf-DNA in the cancer cases indicate disease progression and act as a diagnostic tool in the clinical examination of various cancers. In the present study, a higher frequency cf-DNA concentration was observed in the cancer cases compared with the control group (Table 1, Fig. 1). This indicates an association of cf-DNA with the cancer cases and disease progression.

A previous study on serum cf-DNA in the control and cancer cases has reported cf-DNA levels of 13ng/mL and 180ng/mL respectively (Tabak *et al.,* 2004). The authors reported highest level of plasma cf-DNA in a pancreatic cancer patient with concentration of 1200ng/mL, while lowest concentration was found in the head and neck cancer (10ng/mL). Level of cf-DNA in the present study was found significantly higher compared with the reported study. The levels of circulating DNA in the cancer cases were higher than the control group, representing a similar pattern as compared with present study. Similarly, the mean concentration of cf-DNA in all of the studied major cancer sites and sub sites was found to be higher than the mean concentration found in the control group. This indicates a significant association between cf-DNA concentrations and risk of cancer.

The present study in the serum samples indicated elevated levels of cf-DNA in the studied cohort which is pertinent to a previous report (Lee *et al.,* 2001) in which a 20-fold higher cf-DNA level in fresh serum samples was observed compared to fresh plasma samples with a PCR-based method. In the cited study, the authors attributed the elevated cf-DNA levels in the fresh serum samples to the DNA released from white blood cells during blood clotting. The origin of cf-DNA has been associated with lymphocytes and other nucleated cells in healthy humans but its origin in various malignancies is not fully ascertained up till now (Ziegler *et al.,* 2002).

In our opinion, the probable origin of cf-DNA in cancer patients may have chemical basis and other mutagenic root factors which cause apoptosis and the release of higher cf-DNA concentrations in cancer patients. The increasing trend in various persistent environmental chemicals and the increased use of electromagnetic radiations in the modern world can damage the nuclear DNA which ultimately release out of the nucleus and circulate freely in the bloodstream in various malignancies (Lee *et al.,* 2001, Ziegler *et al.,* 2002).

Breitbach *et al.,* 2012 reported median circulating DNA concentration of 17 ng/mL in plasma samples of patients with solid tumors with a range of 0.5 to 1600ng/mL, which was 3-times higher as compared with the healthy humans. This supports the studied matrix (serum) in the present study that the cf-DNA levels whether analyzed in plasma or serum of cancer patients are found in elevated concentrations compared with the healthy humans. In this way, the cf-DNA seems to have a strong association with various malignancies and can be used as a diagnostic and prognostic biomarker for the clinical manifestation of tumor testing and staging.

Another study by Anile *et al.,* (2014) has reported cf-DNA level in preoperative lung cancer cases to be 23.07±7.4ng/mL and in the control group, the level was 7.5±3.4ng/mL (p = 0.0002). The levels increased postoperatively in one week (68.2±36.2ng/mL) while a decrease was observed after one month of surgery (9.6±3.1ng/mL). Our study conforms to this report in the sense that the levels of cf-DNA in the control group were lower than the pre and postoperative lung cancer cases. After one month of lung cancer surgery, the levels fell down and almost synchronous with the healthy individuals in the cited study which further strengthens the association of cf-DNA with metastasis and tumor progression. The potential of circulating nucleic acids in plasma and serum for the diagnosis, staging and prognosis of various malignancies has been reported by Nalini *et al.,* 2008; Ellinger and Bastian 2010; Gonzalez-Masia 2013; Catarino *et al.,* 2012).

The serum cf-DNA can be used in the PCR analysis for the ascertainment of mutations causing various cancers. The given discussion authenticates the role of cf-DNA as a potent biomarker in cancer analysis. Further research is recommended in this field to investigate the mutations taking place in the sequences of cf-DNA strands in various cancers so that it can be efficiently utilized for the clinical evaluation of various cancers in the future.

It is evident from the present study that cf-DNA has a significant association with various malignancies in the body and is found in elevated concentrations in the cancer cases compared with the control group. Cancer cases of the oral cavity and pharynx, breast and blood cancer cases generally have shown higher concentrations of cf-DNA than other types of cancers. The levels of cf-DNA indicate the level of damage of nuclear DNA which can be efficiently utilized in staging of cancer and as a diagnostic and prognostic tool in the clinical examination of various cancers. Application of novel cancer biomarker with better diagnostic specificity and sensitivity will assist clinicians to implement therapeutics in an efficient and effective way during cancer management.

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