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Suspended in Blood and Circulating Within, Cell Free (cf) DNA Connects with a Vast Range of Adverse Human Health Conditions Including Cancer: A Review.

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ABSTRACT

Cell free DNA (cfDNA) is now emerging as potent biomarker in cancer diagnostics as well as an useful tool for non-invasive diagnostic methods related to a wide range of clinical conditions. Its extraction from blood is crucial to exploitation as a biomarker. Link of cfDNA has been established with a wide range of health conditions including autoimmune diseases, sepsis and trauma, heart diseases and dialytic processes. However, its utility in prenatal diagnosis has been one of the most widely investigated areas of research. Association of cfDNA with cancer and its methylation status as a potent prognostic marker is also assuming increasing significance in recent times. Its association with different forms of cancer with special reference to breast cancer is notable. A doubtful mammographic findings does indicate other testing that involve imaging techniques but the final diagnosis is established by a biopsy in particular to differentiate malignant from benign tumors. Although this is an established process that has considerably reduced mortality, several lacunae indicate the need for superior prognostic and diagnostic markers. In these contexts, cfDNA has been discussed in this review.

Keywords: cfDNA, cancer, blood, biomarker.

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INTRODUCTION

The actual credit for discovery of cell free DNA (cfDNA) goes to Mandel and Matais who managed to prove the presence of nucleic acid in the human circulatory system, mainly plasma (1). But their discovery went unidentified until 1977, when a paper suggested the potential application of cfDNA in tumour diagnosis and its management (2). Free DNA refers to those fragments of DNA found in various body fluids other than blood. Majorly cfDNA is found in plasma or serum, but some studies also report the presence of cfDNA in saliva (3, 4), synovial liquid (5), peritoneal fluid (6), cerebrospinal fluid (6) and urine (7, 8, 9, 10). On an average 30ng/ml of cfDNA is found in healthy individual but the concentration can vary from 0ng/ml to 100ng/ml (11). Apoptosis and necrosis are two major phenomena which contribute to the presence of cfDNA, although which one plays a major role over the other is still unclear. In a study of critically ill intensive care unit patients it was shown that the concentration of cfDNA was 16 fold greater in apoptosis as compared to necrosis (12). While in other study of liver damaged murine model, Jahr et al. (2001) showed that the increase in total cfDNA was only due to necrosis (13). Another process which adds up to the concentration of cfDNA is NETosis, a process where neutrophils discharge their extracellular traps. This discharged extracellular trap mainly contains DNA fragments (nuclear and mitochondrial) as well as histones and antimicrobial peptides (14, 15). When cfDNA extracted from children suffering from diabetes and Rheumatoid arthritis (RA) patients were resolved by gel electrophoresis, they were found to acquire ladder like pattern as seen in apoptotic cells (16).The size of the DNA fragments can also determine its origin; if the origin of cfDNA is from apoptosis the ladder fragments on the gel would be between 180-1000 bp in size. Whereas if the cfDNA originates from necrosis than it will form a smear on gel owing to incomplete and non-specific digestion, due to its higher size i.e. more than 10,000 bp (17). However, it is also believed that apoptosis and necrosis mediated contribution towards cfDNA is very less and negligible and it predominantly occurs as a result of spontaneous release of DNA from the living cells (18, 19). Majority of the cfDNA are found to be double stranded and contains various fragments of different sizes ranging from 0.18-21kb (20). Clinical studies have shown that majorly liver and to lesser extent kidney is responsible for the clearance of cfDNA (21). In an urine DNA examination, two different sizes of DNA fragments (one larger than 1 kb, while other fragment of size 150-250 bp) were allowed to pass through kidney membrane. When the urine was collected and centrifuged the supernatant consisted of the latter DNA fragments (9). Therefore the fact that mice and human kidney membrane is permeable to certain size of DNA is in complete agreement with the findings of Botezatu et al. 2000 (10). The role of plasma nucleases in the degradation of cfDNA has also been reported (22).

Extraction of cfDNA

The procedure of extraction or isolation of cfDNA is tedious as compared to normal DNA due to its very low concentration in plasma, serum and other body fluids. Studies have identified that the total mean concentration of cfDNA is found to be more in healthy male (1030 mg/ml) as compared to that of healthy females (440 ng/ml) (23, 24). The selection of type of protocol to extract cfDNA depends on the type of cfDNA present. If the cfDNA are bound to the cell surface than normal EDTA solution can be used to elute it. However more tightly bound cfDNA can be eluted with the treatment of trypsin.

The following protocols are mainly used to extract cfDNA from plasma/serum:

QIAamp method and modified QIAamp protocol

The QIAamp method is specifically designed for purification of bacterial, genomic, mitochondrial and viral nucleic acids. Nucleic acids bind specifically to the silica gel membrane while other contaminants pass through. The use of phenol chloroform for extra purification of nucleic acids is not required (25, 26).

Triton X-100 protocol

The blood samples once collected are hard centrifuged within 2 hours of collection. The supernatant is plasma/serum; to 500µl of the supernatant 5µl of triton X-100 is added. The solution is then heat denatured at 98^0C for 5mins and then immediately placed on ice for 5mins. After this, 500µl of phenol, chloroform and isoamyl alcohol (25:24:1) is added to the above solution and place in ice for 10mins. The solution is then centrifuged at 14,000g for 10mins. The aqueous phase is collected and mixed with $1/10^{th}$ volume of 3M

sodium acetate and 2.5 volume of 100% ethanol. The pellet formed after centrifugation is then air dried and suspended in 50µl water (27).

Modified phenol chloroform method

The blood samples collected are centrifuged twice; first at 1500g at 4^0C for 10mins and the second at 3000g at 4^0C again for 10mins. 1ml supernatant is then transferred to another tube and 100µl of a solution containing 250mmole/L of EDTA and 750mmole/L of NaCl is added. Further 100µl of 10% SDS and 20µl of 20mg/ml of proteinase K is also added and incubated for 2 hours at 56^0C. The proteins are precipitated by addition of 200µl saturated 6M NaCl solution. Equal volume 1:1 solution of phenol chloroform is added and incubated at room temperature for 5mins followed by centrifugation at 14,000g for 15mins. Supernatant is collected in another tube and extracted with ethanol. The solution is again centrifuged at 14,000g for 15mins and the pellet air dried and suspended in sterile distilled water (28).

The Nucleospin method

This is yet another very rapid method resulting in a high purity DNA yield. Fragmented DNA as small as 50–1000 bp can be purified with high efficiency by this method. Here, the columns has a special funnel design that allow elution volumes as small as 5µL (28).

Cell Free DNA and various diseases:

Autoimmune diseases

Systemic Lupus Erythematosis (SLE)

It is very well known by now that auto-antibodies can target DNA and the DNA-auto-antibody complex in the circulation plays a crucial role in SLE. The presence of DNA in the serum of patients suffering from SLE dates back to as early as 1966 (29). In a comparison study of 13 healthy individuals and 13 patients with SLE, blood samples of all the 26 patients were collected and analysed for anit-dsDNA, anti-ssDNA, nucleosome, anti-histone antibodies as well as cfDNA. It was demonstrated that cfDNA levels in SLE patients were significantly increased along with anti-ssDNA, anti-dsDNA and antihistone antibodies as compared to that of healthy controls (30). In a study, Raptis et al. 1980 performed a comparative study of healthy individuals, patients suffering from chronic inflammatory disease and SLE patients. This group analysed the presence of DNA in plasma in all three group of patients. The mean plasma DNA in healthy individual was found to be 266±57ng/ml, which was not significantly different from patients suffering from chronic inflammatory disease (209±14ng/ml) or patients suffering from SLE but on steroids (293±57ng/ml). However, the plasma DNA levels of two newly identified patients of SLE was considerably higher (4,024ng/ml and 2,437ng/ml) as compared to the above three groups, thus suggesting the presence of DNA in plasma in SLE patients (31).

Rheumatoid arthritis

In case of arthritis there are enough evidences which prove the presence of cfDNA. Leon et al. in his study of patients suffering from RA found increased levels of cfDNA in plasma as well as synovial fluid in comparison to that of healthy individual (32). The correlation of cfDNA obtained from synovial fluid and septic arthritis has also been established. In a study, cfDNA isolated from synovial fluid was compared with white blood cells, synovial white blood cells, C-reactive proteins, IL-6, IL-1 and TNF-α in patients suffering from joint effusion. The cfDNA levels (3286±386ng/ml) along with IL-6 and IL-1 were found to be elevated in patient suffering from septic arthritis as compared to that of non-infectious joint inflammation (1040±208ng/ml) (1).

Systemic sclerosis and Lupus

Galeazzi et al. had analysed cfDNA as well as DNA extracted from nucleated blood cells in systemic sclerosis and lupus patients. They confirmed the presence of elevated levels of cfDNA in these patients and also demonstrated a specific pattern of DNA sizing using gel electrophoresis. They claimed that SLE and SS patients have anomalous patterns of DNA in serum as well as in the buffy-coat (33). Mosca et al. claimed

otherwise and reported that there is no significant difference between the cfDNA levels of SS patients when compared to healthy individuals (34).

Sepsis and trauma

It is now evident that following severe trauma there is occurrence of systemic anti inflammatory response leading to organ failure. The organ failure in turn leads to increased cell death that cause an increased concentration of cfDNA in circulation. In a comparative study of 84 patients suffering from acute blunt traumatic injury and 27 healthy patients, it was found that plasma DNA was higher in trauma patients compared to healthy controls (35). Similar kind of results was found in a study which compared the plasma DNA levels of patients suffering from burns with healthy controls (36). Both the above mentioned studies used quantitative PCR method for identification of plasma DNA using for beta-globin as the target gene. Wijeratne et al. was successful in proving that cell free plasma DNA can be used a prognostic marker in assessing the clinical outcomes of intensive treatment unit patients (37). Cell free plasma DNA can also be used as a prognostic marker of mortality and sepsis in critically ill patients (38). It is found to be elevated in patients suffering from septic shock (39), organ failure (40) and patients needing mechanical ventilation (41).

Acute pancreatitis

Acute pancreatitis, also known as acute pancreatic necrosis is the sudden inflammation of pancreas. In a study, serum from 30 patients suffering from acute pancreatitis was collected on admission, after 1st, 4th and 7th day. Cell free DNA from serum was isolated using commercial kits and its correlation with pancreatitis was studied. The free serum DNA level was found to be higher in patients whose serum was drawn right after admission. As the disease progressed the serum DNA level was found to increase (serum DNA levels in patients with mild pancreatitis 0.059ng/ml while in acute pancreatitis 0.271ng/ml) and *vice versa* (42). The benefit of using serum and plasma DNA levels as a marker for acute pancreatitis has its own benefit over other biomarkers. The severity of the inflammation can be detected right from the first day of hospitalization; this was proved by a very recent study conducted by the same group (43). On the other hand there is another study which contradicts to the above findings and stating that there is no correlation between serum DNA and acute pancreatitis and that the reduction in serum DNA level is due to other necrotic and apoptotic pathways (44). The reason behind this contradiction may be different study design and smaller study population.

cfDNA in heart diseases

Cell free DNA can also be used as a prognostic marker in certain heart disorders such acute myocardial infarction (AMI). There are evidences which prove the role of cfDNA in detection of AMI. In a study, 13 patients admitted for AMI were assessed for creatine kinase, troponin and cell free DNA. it was concluded that free DNA levels where higher in patients suffering from AMI (2). Similar kind of findings were observed by another group which assessed the role of free DNA in patients suffering from AMI during hospitalization (45). In a more recent study, the cfDNA levels in patient with ST elevation myocardial infarction (STEMI) were examined and its correlation with other markers was established. This is the only study that used fluorometric assay for cfDNA detection while other studies used either gel electrophoresis or RT-PCR (46).

Role of cfDNA in prenatal diagnosis

Determining Fetal Sex

The discovery of fetal DNA in maternal DNA was a breakthrough in the field of prenatal diagnosis and was first noticed in the year 1969 (47) abrogating the use of invasive diagnostic techniques such as amniocentesis and chronic villus sampling (48). The fetal DNA originate from trophoblast cells lining and after trophoblast degradation, fragments of fetal DNA are released into maternal circulation (49, 50). The main advantage of fetal DNA is that it appears as early as in the 5th week of gestational period (51) and gets rapidly cleared after pregnancy (52). Whereas, the disadvantage of fetal DNA is that it is totally inseparable from maternal DNA and account for only 6-10% of the total DNA (depending on week of gestation) (53). The fetal DNA is not only present in maternal blood but is also seen in other maternal body fluids such as urine, peritoneal fluid, amniotic fluid and peritoneal fluid (53).

Genetic Disorders

Cell free DNA circulating in maternal plasma has also been used to determine many genetic disorders. The diagnosis of diseases such as anchondroplasia (54), myotonic dystrophy (55) and Huntington disease (56) are few examples which can be diagnosed by using cell free DNA. For the detection of β-thalassemia; Chiu et al. designed allele specific primers along with a fluorescent probe for the detection of single gene mutation within β-globin gene. When maternal plasma was taken as source of DNA and real time PCR was carried out, they were able to exclude fetal inheritance of this parentally transmitted mutation (57).

Obstetric Complications

As discussed earlier, detectable level of cell free DNA is found as early as in 5th week of gestational period. It is found to be at its peak during the last 8 weeks of pregnancy (58). The disruption of placenta causes increases in apoptosis of trophoblast cells (59). Increase in apoptosis in turn causes increase in cell free DNA levels in maternal blood as observed in pre-eclamptic (60) patients or in case of fetal growth restrictions (61). Measuring these levels of cfDNA may serve as a marker for placental abnormalities.

cfDNA as a bio-marker in dialytic process

cfDNA is also been found to be associated with various dialytic procedures. In the very first study for examining cfDNA in plasma, fresh blood was allowed to flow on the dialysis coil. It was found that the passage of blood through the coil causes rise in circulating DNA speculating leukocytes as the main source (62). In the year 1989, Fourine et al. confirmed the findings by using a detection method based on incorporation of radiolabeled nucleotides (63).

Ozkaya et al. (2009) studied the plasma cfDNA in children on peritoneal dialysis (PD) and found significantly elevated values in comparison to healthy children and a positive correlation with C- reactive protein levels in treated children (64). Samples of peritoneal effluent with regard to their content of cfDNA were examined in a study by Pajek et al. (2010) in order to analyze the impact of PD solution with different biocompatibility and cytotoxic properties on the peritoneal membrane cells. Two PD solutions were tested: a conventional lactate buffered, acidic solution and a novel, bicarbonate/lactate buffered, neutral solution low in glucose degradation products. A significant decrease in appearance of cfDNA in effluent was observed with the novel PD solution (65).

cfDNA and Cancer

It is well known that there are many tumour suppressor genes that regulate cell cycle and promote apoptosis. However, hypermethylation of such genes causes either loss of its integrity or reduction in gene expression. Measuring these methylated DNA in peripheral blood has a great potential to be used as prognostic biomarkers in cancer detection. In a comparison study between 72 healthy individuals and 102 patients with neuroblastoma it was found that DNA isolated from plasma as well as serum from 102 patients contained higher level of MYCN gene (66). Similar results were obtained in a study which targeted methylated DCR-2 gene in serum of 80 patients suffering from neuroblastoma as compared to 20 healthy individuals (67). In 2009, A. Misawa and colleagues screened serum DNA of 68 patients suffering from neuroblastoma and found higher level of the RASSF1A gene in serum DNA of 12 patients which was statistically associated with age, stage 4 and MYCN amplification (68).

Gliobalstoma multiforme (GBM) is one of the most aggressive forms of brain tumour and despite surgical resection, radiation and chemotherapy, it remains incurable. Iris Lavon et al (2010), screened 70 patients suffering from high grade astrocytomas or oligodendroglial tumours. DNA was extracted from whole blood, serum and tumour sections. The idea of the study was to evaluate whether cell free circulating DNA can be used as a non-invasive approach for detection of epigenetic alterations in brain tumours. Two sets of biomarkers were screened, namely MGMT and PTEN. Out of the 41 astrocytic tumour samples screened for MGMT methylation, 83% of the samples were in tumour-serum concordance. While for PTEN methylation the tumour-serum concordance was 76%. The findings for MGMT methylation in oligodendroglial tumours were similar to astrocytic tumours (69, 70). In another study, serum DNA was isolated from thirteen patients suffering from GBM; all the samples were targeted for EGFRVIII. Three DNA samples out of thirteen were

found to be carrying EGFRVIII deletion and the amount of EGFRVIII found in circulating DNA was found to be of significance with tumour age and also with the extent of tumour resection (71).

Majchrzak-Celinska et al (2013) attempted to assess the methylation status of few set of genes related to CNS cancers and tumours. They isolated serum DNA from 33 newly diagnosed and previously untreated patients suffering from different types of CNS cancers, namely brain tumour of glial origin, primary or recurrent gliobalstoma, astrocytoma or gliobalstoma. The methylation status of MGMT, RASSF1A, p15INK4B and p14ARF were checked methylation specific PCR. At least one promoter of the above mentioned four genes was found to be methylated in serum samples of the patients. MGMT was found to be methylated in almost 18% of the patients, while RASSF1A was found to be methylated in 47% of the total population. Likewise moderate percentage of methylation was found in the patient's serum for the remaining two promoters p15INK4B (12%) and p14ARF (41%) also. This study clearly indicated that circulating serum DNA can be screened for methylated markers and can be used as a biomarker in case of CNS cancers (72).

Page et al (2011), studied breast cancer related genes from cell free DNA. For this, they screened cell free DNA samples from 59 healthy female controls, and compared it with similar samples of 39 patients with benign breast disease, 6 patients of DCIS (Ductal carcinoma *in situ*). None of the samples were positive for HER2 gene in cell free DNA samples. But when the samples of follow-up patients were screened for the same gene, 8 out of 78 patients and 5 out of 30 patients from the metastatic group were positive for this gene (73). In other similar kind of study, a set of 11 genes, namely APC, ADAM23, CXCL12, ESR1, PGR B, CDH1, RASSF1A, SYK, TIMP3, BRMS1 and SOCS1 were screened simultaneously in tumour, plasma and peripheral blood cells in patients (n=34) suffering from primary breast cancer and compared with peripheral blood from 50 healthy individuals. Out of the 11 genes screened, none of the genes expression were in correlation when compared between cell free DNA and tumour DNA. However elevated level of certain genes (CXCL12, ESR1 and TIMP3) were found in cell free DNA when compared to healthy individuals (74). In a comparison study of CTC (circulating tumor cell) from peripheral blood and cell free DNA from serum of patients suffering from breast cancer, three breast cancer markers (APC, RASSF1A and ESR1) were found to be in correlation. Eighteen percentage of the breast cancer patients showed positive results for all three markers while 56% patients were positive for any of the 2 markers and 82% of them were positive for at least one of these molecular markers. The percentage showed an increasing trend in patients with stable disease and in patients with progressive disease respectively. When similar screening was done in healthy individuals, about 68% displayed negative results for all the three markers (75).

One of the studies screened around 56 genes in cell free DNA samples and compared it with tumour samples of patients suffering from breast cancer. Out of the 56 genes, RARβ2, ESR1, prB and PRPROX could be detected in cell free DNA samples of patients (76). Schwarzenbach et al (2012) showed a relation between loss of heterozygosity of tumour suppressor genes and cell free DNA. In breast cancer patients the cell free DNA showed losses of tumour suppressor genes TIG1, PTEN, Cyclin D2, RB1 and BRCA1, implying the importance of screening cfDNA for molecular markers (77). CST6 promoter mehtylation is very common in primary breast tumour and is also found to be methylated in circulating tumour cells. In a study the methylation pattern of CST6 promoter in cfDNA was analyzed. Twenty seven patients with stage I-III operable breast cancer and 46 patients with metastasis were screened for both methylated and unmethylated CST6 gene. In all, 29.6% of patients were found to be positive in stage I-III group while 13% were found positive for methylated CST6 promoter in metastatic group while none of the 37 healthy individuals showed any sign of CST6 methylation in their cfDNA samples (78).

The presence of vascular endothelial growth factor (VEGF) and its soluble counterpart's (sVEGFR1) presence in cfDNA have attracted a lot of attention as a biomarker. In a study, screening of VEGF and sVEGFR1 in cfDNA from breast cancer patients was carried out and a positive correlation was found between VEGF and cfDNA, whereas a negative correlation was found between VEGF and its soluble counterpart (79).

Domenyuk et al (2007) were the first group of researchers who suggested NFKB and DDR1 genes as a potential marker for some types of endometrial cancer (80). Role of cfDNA in endometrial cancer was studied by Dobrzycka et al (2010) wheer they found a complete correlation between KRAS mutation and cfDNA in patients suffering from type II endometrial cancer (81).

Cervical cancer is the principal cause of death due to cancer in women. Five-year survival rate ranges from 15-80% depending on the extent of the disease The cfDNA analysis of 93 cervical cancer patients showed an elevated methylation status of the following genes - CALCA, MYOD1, hTERT, PGR and TIMP3. Around 87% of the patients showed hypermethylation in any one of these genes and further, expression of the MYOD1 gene was found to be elevated in advanced stages of cancer. All these findings were in complete correlation with the corresponding tissue samples (82). Further addition of E-cadherin and H-cadherin (CDH1 and CDH13 respectively) to the set of molecular markers in cervical cancer was done by Widschwendter et al (2004). The methylation of both the genes was screened in cfDNA sample of 93 patients suffering from cervical cancer. Around 43% of the patients were positive for hypermethylation of both the genes. The survival rate increased with decrease in hypermethylation levels of both these genes in cfDNA (83).

Hypermehtylated RASSF1A gene is not only found in cfDNA samples of neuroblastoma and glioma patients but also in patients suffering from ovarian cancers. In a comparative study of 51 healthy individuals and 51 patients suffering from benign ovarian cancer, the RASSF1A gene was not methylated in healthy individuals whereas moderate (43.1%) level of methylation was found in cfDNA samples of the diseased group. Moreover, hypermethylation of RASSF1A gene was encountered at greater level in stage III and IV as compared to that in stage I and II (84). In one of the study, 126 patients suffering from ovarian cancer were screened to determine the significance between KRAS mutations, cfDNA and P53 antibodies. High grade cancer patients were encountered with both cfDNA and P53 antibodies; the overall survival rate of such patients was less as compared to those with negative tumors (85).

Hypermethylation of RASSF1A gene is very frequently encountered in hepatocellular carcinoma. One such study screened two different set of patients. In set-1, 63 patients suffering from HCC (Hepatocellular carcinoma) matched hepatitis B virus carriers as well as 50 healthy individuals were present whereas the second set comprised of 22 HCC patients detected through surveillance program. The cfDNA samples of about 93% patients suffering from HCC showed presence of hypermethylated RASSF1A gene and around 58% of HBV patients and 8% of healthy individuals showed similar type of results (86).

Differential methylation profiling of plasma DNA can detect ductal adenocarcinoma of the pancreas with significant accuracy. Out of the 56 frequently used markers, promoters of CCND2, SOCS1, THBS1, PLAU, and VHL genes were most methylated in cfDNA samples of 30 patients suffering from adenocarcinoma of pancreas (87).

Death-associated protein kinase (DAPK) and adenomatous polyposis coli gene (APC) have been recently shown to be associated with favourable outcome in patients with esophageal carcinoma, especially adenocarcinoma. To determine this hypothesis, cfDNA from 59 patients suffering from esophageal cancer were subjected to methylation specific PCR. A total of 61% of the patients had detectable levels of both methylated DAPK and APC genes; moreover combination of both markers increased specificity for differentiating between long and short survivors (88).

To determine the role of RUNX3 gene in gastric cancer, cfDNA of 65 patients suffering from the disease were collected at three different stages (preoperative, intraoperative and postoperative stage). A detectable level of methylated RUNX3 gene was found in almost 29% of the patient's cfDNA whereas the levels were found to decrease in postoperative patients. The methylation index of the gene was in complete correlation with histology, cancer stage and vascular invasion (89). In another study, cfDNA samples were collected from 53 patients suffering from gastric cancers and 21 healthy individuals. These samples were subjected to qPCR using β-actin primers to amplify short and long fragments. The DNA concentrations of both the fragments in were significantly high in gastric cancer patients as compared to healthy individuals (90). It may be concluded therefore that this plasma DNA concentration assay may serve as a new diagnostic marker for the screening and monitoring of patients with gastric cancer.

The presence of aberrantly methylated SEPT9 DNA in plasma highly correlates with the occurrence of colorectal cancer. cfDNA samples of 97 verified cases of colorectal cancers were screened for SEPT9 gene hypermehtylation using methylation specific PCR. This assay identified presence of SEPT9 gene hypermethylation in 72% of the patients (91). The most frequent promoter region to be hypermethylated in colorectal cancer was the CpG islands. In a study of 24 patients of colorectal cancer, 14 with metastasis and 20 healthy individuals, the serum DNA was isolated and screened for hypermehylated HLFT, HPP1/TPEF and

hMLH1 genes. The hypermehtylation of all the genes correlated with tumour size, metastatic disease and tumour stage (92, 93).

In one of the study the researchers screened BRAF and KRAS gene mutations in cfDNA of mice and human patients suffering from colorectal cancer. High levels of both the mutations were found in the resource population whereas the wild type of both the genes were detected in the non diseased population (94).

An activating point mutation of the BRAF oncogene results in a V600E amino acid missense mutation and is found in a majority of papillary thyroid carcinomas (PTC). In a study, BRAF mutation was checked in 28 cfDNA samples of patients suffering from benign and malignant thyroid disease. Five of 14 patients suffering from PTC tumors were positive for BRAF mutation and 3 of 14 patients were positive for BRAF mutation in both serum and tumor (95).

One study was aimed to quantify plasma circulating DNA level in patients with acute myeloid leukemia (AML) and to evaluate its clinical significance. 66 AML patients and 100 controls (60 healthy subjects for health examination, 20 cases of benign hematopathy, and 20 cases of solid tumors) were enrolled in this study. This study concluded that the quantification of plasma DNA may be useful for evaluating therapeutic effects and monitoring relapse in AML patients (96).

The epigenetic inactivation of genes play an important role in lung cancer. A study was designed to investigate the methylation status of seven genes: DAPK, p14, p16, APC1A, FHIT, RASSF1A and RARβ in cfDNA of 92 NSCLC patients. Majority of the patients showed at least presence of one epigenetic alteration in cfDNA samples. Out of the seven genes screened, most frequently hyper methylated ones were RASSF1A and p14 (ARF) (97).

APC is a gene frequently hypermethylated in gastric cancers or gastrointestinal tumours. The role of APC hypermethylation was also studied in cfDNA samples of 89 patients suffering from lung cancer. In all, 47% (42 out of the 89) patients screened carried detectable amount of methylated gene in cfDNA samples in contrast to its total absence in healthy individuals (98). Kimura et al (2004) screened 25 patients (12 on pre-treatment regimen while rest 13 on postoperative regimen) suffering from NSCLC for K-RAS mutations. The cfDNA was extracted and subjected to RFLP-PCR analyses. The results clearly indicated the presence of KRAS mutations in DNA samples. Moreover one of the patients on the postoperative regimen showed complete absence of KRAS mutation in cfDNA as compared to the preoperative results (99).

In a study, hypermethylation at APC, GSTP1, PTGS2, p14(ARF), p16(INK) and RASSF1A gene was analyzed using real-time polymerase chain reaction following methylation sensitive restriction endonuclease treatment in 73 patients with testicular cancer and 35 healthy individuals. The results were as expected; hypermethylation was more in patients than healthy individuals. APC - 57% and 6%, p16(INK)- 53% and 17%, p14(ARF)- 53% and 0%, RASSF1A- 47% and 0%, PTGS2- 45% and 0%, and GSTP1- 25% and 0% (100). Sensitive methylation-specific PCR was used to screen matched tumor and sediment DNA from preoperative urine specimens obtained from 50 patients with kidney tumors and representing all major histological types for hypermethylation status of a panel of six normally unmethylated tumor suppressor genes, namely VHL, p16/CDKN2a, p14ARF, APC, RASSF1A, and Timp-3. Hypermethylation of at least one gene was found in all 50 tumor DNAs (100% diagnostic coverage) and an identical pattern of gene hypermethylation was found in the matched urine DNA from 44 of 50 patients (88% sensitivity), including 27/30 cases of stage I disease. In contrast, hypermethylation of the genes in the panel was not observed in normal kidney tissue or in urine from normal healthy individuals and patients with benign kidney disease (100% specificity) (101). In another study, blood from healthy male donors (n = 40) and 83 patients with American Joint Cancer Committee (AJCC) stage I-IV prostate cancer (Pca) were analyzed. Mehtylation of RASSF1, RARB2, and GSTP1 genes were assessed using a methylation-specific PCR assay and checked for its sensitivity for detection of genetic or epigenetic changes in circulating DNA. The relation between circulating tumor-related DNA detection and prognostic factors was also investigated. This pilot study demonstrates that the combined circulating DNA multi-marker assay successfully identifies patients with PCa and may yield information independent of AJCC (American Joint Committee on Cancer) stage or PSA concentration (102). The profile of GSTP1 gene methylation in the extracellular DNA of PCa patients differed from the profiles characteristic of healthy donors and patients with BPH (benign-prostatic-hyperplasia) (103). The table 1 below summarises on the genes whose presence in cfDNA correlate with different types of cancer progression.

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Table 1: List of genes monitored while analyzing cfDNA in different types of cancer.

Type of Cancer	Extraction method used for cfDNA	Genes to be screened	Detection method used	Reference
Neuroblastoma	1	MYCN	PCR amplification	66
	1	DCR2	Real time PCR	67
		RASSF1A	PCR amplification	68
Gliomas		P16/1NK4α		69
		MGMT	Methylation specific PCR	69; 70
		P73		69
		RARβ		69
		EGFRvIII	Methylation specific PCR	71
	1	RASSF1A	Methylation specific PCR	72
Breast Cancer	1	HER2	Quatitative PCR	73
		LINE1		74
	1	ERβ	Real time qPCR	75
	1	RARβ2	MEthylation specific PCR	76
	1	Cylin D2	PCR based micro-satellite analysis	77
	1	CST6	Real time qPCR	78
	1	APC	Real time qPCR	75
		P16		74
		CDH1		74
	1	BRCA1	PCR based micro-satelliteanalysis	77
	1	VEGF	Real time qPCR	79
Endometrial Tumours	1	NFkβ	Methylation specific PCR	80
	1	DDR1	Methylation specific PCR	80
		P53-Ab	ÉLISA	81
Cervical Tumours	1	MYOD1	Methylation specific PCR	82
	1	CDH1/CDH13	Methylation specific PCR	83
Ovarian Tumours	1	RASSF1A	Methylation specific PCR	84
	1	KRAS	Real time qPCR	85
		P53-Ab	ELISA	85
Hepatocellular carcinoma	1	RASSF1A	Real time qPCR	86
	1	P53	Real time qPCR	86
Pancreatic cancer	2	CCND2	MSRE-PCR	87
	2	SOCS1	MSRE-PCR	87
	2	THBS1	MSRE-PCR	87
	2	PLAU	MSRE-PCR	87
	2	VHL	MSRE-PCR	87
Esophageal Tumours	1	DAPK	Methylation specific PCR	88
	1	APC	Methylation specific PCR	88
Stomach Tumours	1	RUNX3	RTQ-MSP PCR	89
	1	MGMT	Methylation specific PCR	90
	1	P15	Methylation specific PCR	90
	1	hMLH1	Methylation specific PCR	90
	1	CEA	Methylation specific PCR	90
	1	P16	Methylation specific PCR	90
	1	E-cadherin	Methylation specific PCR	90
	1	RARβ	Methylation specific PCR	90
	1	CDH4	Methylation specific PCR	90
Colorectal Cancer	1	SEPT9	Real time qPCR	91
	1	HPP1	Real time qPCR	92
	1	HLTF	Real time qPCR	92
	1	KRAS	Methylation specific PCR	93
	1	BRAF	Methylation specific PCR	22
Thyroid Tumors	1	BRAF	Real time qPCR	94
Leukemia	1	MGMT	Methylation specific PCR	95
	1	P53	Methylation specific PCR	96
NSCLC	1	RASSF1A	Methylation specific PCR	97
	1	APC	Real time qPCR	98
	1	P14	Real time qPCR	99
	1	KRAS	Real time qPCR	99
Testicular Tumors	1	APC	Methylation specific PCR	100
	1	GSTP1	Methylation specific PCR	100
	1	PTGS2	Methylation specific PCR	100
	1	P14	Methylation specific PCR	100
	1	P16	Methylation specific PCR	100
	1	RASSF1A	Methylation specific PCR	100
Kidney Tumors	3	VHL	Methylation specific PCR	101
	3	P16	Methylation specific PCR	101
	3	P14	Methylation specific PCR	101
	3	APC	Methylation specific PCR	101
	3	RASSF1A	Methylation specific PCR	101
	3	Timp-3	Methylation specific PCR	101
Prostate Cancer	1	RASSF1A	Real time qPCR	102
	1	RARB2	Real time qPCR	102
	1	GSTP1	Methylation specific PCR	103

Cell free DNA has all potential to be the basis of an extraordinarily simple blood test with superior sensitivity and specificity for screening patients will a wide range of diseases, specially cancer. Such tests are the need of the hour for prognostic and stages purposes in cancer such that they can effectively reflect treatment efficacy both in chemo prevention as well as therapeutic trials. However, the need of the hour of further study of this analyte and optimization of assays before they can be actually used in a therapeutic setting.

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