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Circulating DNA in Cancer: An Overview

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Abstract

In the last decade, research has been focused on discovering markers which have high sensitivity and specificity to detect cancer. Molecular biologists have been trying to find markers which can be detected non-invasively. Circulating nucleic acids in plasma and serum of cancer patients and the mutations detected in them have gained immense importance in this field. Circulating nucleic acids (CNAs) include DNA, RNA, nucleosomal DNA, microRNA, viral DNA. This review focuses on circulating DNA, its origin and mechanism of release, its diagnostic and prognostic utility, association with any of the clinic pathological parameters and its role in monitoring treatment efficiency in cancer. However, due to lack of uniformity in laboratory techniques, variability in disease advancement, less number of samples in various studies, lack of evidences for the origin of circulating nucleic acids, the knowledge sequestered till now in this field has not been translated in to clinical practice. With more high throughput techniques, circulating nucleic acid levels and mutations in them may give rise to a new era of cancer diagnostics and therapy.

Circulating Nucleic Acids: Need as Cancer Markers

Cells in the body contain DNA and RNA, but these nucleic acids can also be found circulating freely in serum and plasma. Solid tumors undergoing uncontrolled proliferation of cells, angiogenesis, apoptosis are known to shed circulating nucleic acids into the blood. In the past one decade, the circulating nucleic acids have gained importance in tumor biology, because they offer a non-invasive approach for diagnosis, clinical assessment and follow-up of the treatment. Tissue biopsy, on the other hand, is an invasive and time taking procedure and can't be taken serially to monitor events of the disease.

However, there are various protein tumor markers which can be assessed using serum and plasma of cancer patients like, prostrate-specific antigen (PSA) in prostate cancer, the alpha-fetoprotein in hepatocellular carcinoma, the carcino embryogenic antigen (CEA) in colon cancer, the cancer antigen (CA)15.3 in breast cancer, and the CA19.9 in pancreatobiliary tumors [1]. But these are assessed through immunoassays and might lack sensitivity in non-secretory tumors and also may not discriminate tumor from inflammatory conditions. Moreover, there are other types of cancers which do not have any blood-based tumor markers. Furthermore, detecting tumor-specific mutations in CNAs not only provide diagnostic benefit but also help in deciding and monitoring the treatment. With such a backdrop, circulating nucleic acids from plasma and serum are emerging as a promising concept for use as a diagnostic or prognostic marker or for assessing the efficiency of any treatment of cancer.

Though the concept of circulating nucleic acids is gaining importance in the present decade, it was discovered by Mandel and Metais [2] in 1948, but their association with disease was not proposed at that time. However, in 1977, Leon et al. [3], found higher levels of CNA in plasma of patients affected by lung cancer than normal healthy individuals and those patients with higher levels of circulating DNA have worse prognosis. Stroun et al. [4] further suggested that primary tumor gave rise to CNAs. Later, by studies on pancreatic neoplasm [5,6] and acute myelogenous leukemia, presence of somatic mutations in plasma confirmed the fact that this CNA originated from tumor.

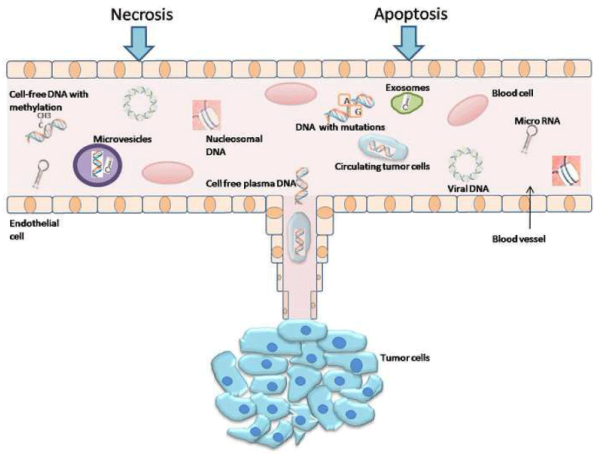

Figure 1: Circulating nucleic acids represent various types of nucleic acids from different sources. DNA from tumor is present as cell free form or in the intact tumor cells which get detached from the tumor. Apoptosis and necrosis are responsible for release of DNA from normal as well as tumor cells. Exosomes and microvesicles in peripheral blood, are formed from tumors and they carry nucleic acids. Viral DNA, micro RNA and nucleosomal DNA also contribute to the pool of nucleic acids in blood. The DNA present in the blood may have various genetic and epigenetic modifications.

Forms of Circulating Nucleic Acids

Circulating nucleic acids are present in varied forms. They can be present as cell free forms (cell–free circulating nucleic acids) which are

present as the naked DNA without any cell or associated protein, as circulating tumor cells (CTCs), which are directly being used for immunoassays, viral DNA [7], mitochondrial DNA [8], circulating cell free RNA [9] and miRNA [10]. Other bodies which carry fragments of DNA are exosomes and microvesicles [11,12]. The pictorial representation of the contributors of circulating nucleic acids is shown in Figure 1. Tumor DNA contributed by all of the above mentioned sources offer a unique opportunity for serially analyzing tumor genotype in a non-invasive manner. Thus, CTCs and circulating DNAs are often referred to as 'liquid biopsy'. This review however focuses on cell free circulating DNA or circulating tumor DNA, its characterstics and clinical significance.

Origin of Circulating DNA

Various hypothesis have been given which explain the origin of circulating DNA, but the topic is still found controversial. The circulating DNA are said to originate from processes like necrosis, apoptosis, direct active release from cells [13-15]. One of the hypotheses for the origin of cell-free circulating DNA in cancer is based on "micrometastasis" of tumor origin which are shed into circulation. Studies have reported higher amount of circulating DNA in cancer patients as compared to the corresponding number of cancer cells present in the circulation thus negating the hypothesis of micrometastasis [16,17].

Necrosis, is also thought to be responsible for high amounts of DNA fragments/circulating DNA in metastatic or advanced tumors [18,19]. But interestingly, it was reported that 90% of patients have decreased circulating DNA levels after radiation therapy, though radiation therapy is presumed to induce cell death/necrosis, probably due to cell proliferation arrest [3]. Thus, the hypothesis that necrosis of cells is responsible for release of DNA into plasma does not hold.

Apoptosis is also known to contribute to circulating DNA in healthy individuals as well as in cancer patients. During apoptosis, DNA is degraded in such a manner that nucleosomal DNA fragments are formed in multiples of 180-1000 kb length [19]. These fragments are further ingested by phagocytosis and digested by lysosomal enzymes and cleared from the bloodstream [20]. However, enzymatic action on these fragments is limited as this circulating DNA is assumed to be protected by some complexes and DNAase activity is also limited in plasma of cancer patients [16,17,21]. Thus, degraded DNA is not utilized by phagocytic cells as in normal cells. But on the other hand, in neoplastic diseases, the normal apoptotic mechanisms are lost, and fragments of variable sizes in blood stream are reported in literature, due to abnormal DNA degradation.

Some studies have also shown that cell free circulating DNA/nucleic acids are released actively from the cells specially lymphocytes [21-23].

Circulating DNA Concentration: Clinical Significance

High concentrations of circulating DNA have been reported in plasma and serum from cancer patients. In various cancers like colorectal [24-28], breast [29-34], liver [35-36], periampullary [28], pancreas [37], levels of circulating DNA was found to be significantly elevated in neoplastic condition than that in non-neoplastic conditions (healthy controls or/as diseased controls). Shaprio et al. [38], showed that patients with benign gastrointestinal diseases had a lower mean concentrations of plasma cell free DNA than cancer patients. In studies on hepatocellular carcinoma, circulating DNA levels can distinguish cancer patients from patients with hepatitis B and hepatitis C [35-36].

In contrast to these findings, there are few reports on lung cancer [39], ovarian cancer [40], periampullary cancer [28], which state that cell free circulating DNA cannot be used as a unique marker for cancer. Such discrepancies may arise due to variation in source of cell free DNA (serum or plasma), variation in storage and processing conditions. These variations are discussed later in the review.

Moreover, increased amount of plasma circulating DNA is observed as the tumor progresses [34,41] and high circulating DNA levels are found in patients with advanced disease [3,42,34] or metastasis [3,37], stage of the disease [43], tumor size [41]. Circulating DNA also showed high sensitivity to detect locally advanced disease [43]. In a recent study [44], more patients with advanced pancreatic, colorectal, ovarian, bladder, gastroesophageal, breast, melanoma, hepatocellular cancers were found to have detectable levels of circulating DNA while less patients with primary brain, renal, prostrate and thyroid tumors had detectable amount of circulating DNA. Cell free DNA levels were also found to correlate with shorter survival of patients [45,37].

Circulating DNA Integrity Index and its Clinical Significance

Different origins of circulating DNA contribute differently to the circulating DNA pool, high molecular weight long DNA fragments are known to be contributed by the necrosis of the tumor cells and low molecular weight small DNA fragments have been attributed to apoptosis phenomena [46]. In plasma and serum, Wang et al. [47] found that circulating DNA is present in different sizes and this difference can be used to calculate integrity index. Integrity index is the ratio between the long and short circulating DNA fragments. They suggested that integrity index can be a simple and inexpensive way to detect gynecologic and breast cancers. Briefly, integrity index is calculated by amplifying *Alu*- sequences [48] or by amplification of β-actin [49], GAPDH (glyceraldehyde-3-phosphate dehydrogenase) [50], Leptin [51], LINE1 (long interspersed nuclear elements) [52].

Integrity index of circulating DNA, has been found to be elevated in patients with different types of cancers like colorectal cancers [27,28], prostate cancer [53], hepatocellular cancer [35,36], acute leukemia [49], periampullary cancers [28], primary breast cancers [30], rectal cancers [54] and nasopharyngeal cancers [55]. Interestingly, in a review focused on circulating DNA integrity index, authors have highlighted the fact that DNA integrity index is more sensitive and specific than circulating DNA concentration in differentiating cancer patients from their respective non-neoplastic diseases and healthy controls [56]. Circulating DNA integrity index has been found to associate with tumor size, TNM stage, vascular invasion, lymph node involvement and distant metastasis in hepatocellular carcinoma [35,36]. Circulating DNA integrity index was found to be potential prognostic markers in patients with primary breast cancer [30].

Patients who responded to chemoradiotherapy, their pre and post treatment cell free DNA integrity indices were found to be statistically different whereas patients who did not respond, their pre and post treatment cell free DNA integrity indices did not differ [54]. Chan et al. [55] found that DNA integrity index not only got reduced in patients with nasopharyngeal carcinoma who responded successfully to radiotherapy but was also found to be associated with reduced probability of disease free survival at higher values. Another study by Gang et al. reported that integrity index of circulating DNA differ in pre and post-surgery samples of patients with renal cell carcinoma [50].

Genetic Alterations in Circulating DNA

Circulating DNA may come from tumor or non-tumor source. Moreover, this DNA has also been observed in normal individuals under physiological stress, trauma and exhaustive exercise or during inflammation [57]. Thus, the presence of tumor-specific genetic and epigenetic alterations in the circulating DNA provide evidence that the circulating DNA being analyzed have come from the tumor itself where these mutations must have occurred in a sequential manner to cause malignant transformation. Thus, methods for the detection of tumor-specific DNA variants have been developed.

K-ras is the most frequently mutated oncogene, reported in colon, pancreas, lung and thyroid tumors and can be used as a useful marker [58,59]. Kopreski et al. [59] detected *K-ras* mutations in plasma from 83% of patients whose tumors had such mutations. However, review of studies showed that the concordance with *K-ras* mutations as compared to the primary tumor was only 50%. Also, plasma DNA was found to have some other mutations also which were not present in the primary tumor, may be due to heterogenous tumor clones [61]. In a study, on metastatic colorectal cancer, Bettegowda et al. [44] showed that the circulatory tumor DNA had high sensitivity and specificity for detection of clinically relevant *KRAS* gene mutations. In a study on pancreatic cancer, the *K-ras* mutation rates in tissue and circulating DNA were 74.7% and 62.6%, respectively, and the concordance rate between them was 58 of 75 samples (77.3%) [62]. They also found that survival did not appear to differ by the presence of *K-ras* mutations in tissue DNA, but the survival of patients with *K-ras* mutations in circulating DNA was significantly shorter than that of patients without mutations. However in some studies, no correlation exists between *K-ras* mutational status in circulating DNA and clinicopathological parameters or survival [37].

In addition, most frequent mutations in colorectal cancers like *APC, K-ras, TP53*, *PIK3CA* and *BRAF*, were found to be more useful than using CEA and CA19-9 levels. [63]. In patients with carcinoma of breast, presence of amplified *HER2* in circulating cell free DNA during follow-up, have become a marker for prognosis and for response to treatment with monoclonal antibodies such as trastuzumab [64]. Many chemotherapeutic agents and targeted therapy are targeted on molecules which are an important part of certain pathways like *K-RAS, BRAF, EGFR* or *p53*. Analysis of circulating DNA key mutations in these in plasma DNA offers a non-invasive and quick way for predicting the response to treatment and monitoring the disease [65-66].

The LOH (Loss of Heterozygosity) status has been correlated to the disease stage and disease recurrence [67]. It is important to note that LOH at different loci are found in low molecular weight fraction. Thus, fractionation of circulating DNA is essential for achieving reliable results [68,69].

Hypermethylation of the promoters of various genes result in loss of transcription of the gene, leading to loss of gene expression. Examining these epigenetic changes in circulating DNA, may offer a possible method for early detection of cancers or analyzing the effect of the treatment. Some of the examples that link hypermethylated genes to cancer and detected in circulating DNA are-promoter hypermethylation of *ITIH5, DKK3, RASSF1A* tumor suppressor genes in breast cancer [70], *NPTX2* in pancreatic cancer [71], *UCHL1* (ubiquitin carboxyl-terminal hydrolase L1) in hepatocellular and esophageal carcinoma [72,73], *GSTP1* (Glutathione- S- transferase) gene in prostate cancer [74], SEPT9 (Septin) gene and *hMLH1* gene in colorectal cancer [75,76]. Some of the recent studies showing association of mutations in circulating DNA with disease progression, survival and follow-up of the patients undergoing treatment and assessing the acquired resistance are mentioned in Table 1.

Apart from differential diagnosis and association with clinicopathological parameters and survival, circulating DNA have been found to be useful in number of other applications also. The mutations detected in circulating DNA can be of help in deciding the type of therapy, predicting the response of treatment, for finding the reason for acquired resistance to therapy.

Study group	Year	Cancer type	Finding
Gonzalez-Cao M [86]	2015	Melanoma	BRAF V600E alleles in ct DNA can predict treatment outcome in melanoma patients
Takeshita T [87]	2015	Breast cancer	PIK3CA mutation found in ct DNA can predict relapse free survival and breast cancer specific survival in triple negative breast cancer patients
Bronte G [88]	2015	Colorectal cancer	Ct DNA can be used as tool to study primary and acquired resistance to anti-EGFR monoclonal antibodies
Garcia-Murillas I[89]	2015	breast cancer	Mutation tracking by sequencing analysis in circulating tumor DNA predicts relapse in early breast cancer and to monitor minimal residual disease
Powrozek T [90]	2015	Lung cancer	Methylation of DCLK1 promoter region in cell free DNA was found to be prognostic indicator.
Delmonico L [91]	2015	Breast cancer	Hypermethylation of p16 INK4A gene was found to discriminate breast cancer from impalpable breast lesions.
Sun W [92]	2015	Non-Small Cell Lung Carcinoma	Ct DNA has a high degree of specificity to detect EGFR mutations and also capable of monitoring disease progression during EGFR-TKI treatment.
Sorenson BS et al. [42]	2014	Non-Small Cell Lung Carcinoma	Observed acquired T790 mutation (in plasma DNA) after treatment with erlotinib
Salkeni MA et al [93]	2013	Glioblastoma	Mutated EGFR reported to have a poor prognosis and associated with chemoresistance and radioresistance
Schwarzenbach H et al. [94]	2012	Breast cancer	LOH at D12S1725 mapping to cyclin D2 gene locus, correlated significantly with shorter overall survival
Yoon KA et al. [76]	2009	Lung cancer	Found that circulating DNA levels can differentiate between lung cancer patients from healthy controls
Ligget T et al. [95]	2010	Pancreatic cancer	Differential methylation of cell free circulating DNA can differentiate between pancreatic cancer patients from chronic pancreatitis

Reinert T et al. [96]	2015	Colorectal cancer	Observed that circulating DNA analysis can be used to monitor disease burden following colorectal surgery
LoNigro C et al. [97]	2013	Melanoma	Methylated tissue factor pathway inhibitor2 (TFPI2) inserum is a biomarker of metastatic melanoma
Ponomaryova et al. [98]	2013	Lung cancer	Methylation in RARB2 and RASSF1 gene found in circulating DNA can be an important tool for diagnosis and post treatment follow-up
Mussolin et al. [99]	2013	Pediatric Lymphoma	Cell free plasma DNA was significantly was higher in lymphoma cases than in controls and these higher levels correlated with stage and survival in different sub types of lymphoma
Mouliere et al. [100]	2013	Colorectal cancer	High KRAS and BRAF mutation load was found in cancer patients than controls

Table 1: Summary of studies showing types of mutations detected in circulating DNA and their association with disease progression and treatment pattern.

Circulating DNA: Challenges Faced

Despite the numerous studies on this subject, there is no consensus about the correlation between circulating DNA concentration and tumor stage, location and size [41,42,77]. And despite various advancement in the techniques used to detect circulating DNA, it is not detectable in all cases. This may be due to different anatomical region of tumor, differences in the approach of tumor to the blood vessel, differing severity of the disease.

Both the levels of circulating nucleic acids and the presence of underlying mutation in this shed DNA have the potential to behave not only as diagnostic and prognostic marker but also as a tool to monitor the effect of any treatment given. Though, there are technical limitations regarding extraction of cell free DNA and subsequent processing, analysis of circulating nucleic acids/DNA may become easier with better high throughput techniques.

Such discrepancies may arise firstly due to difference in the use of serum and plasma as the starting material. Several anticoagulants (EDTA, heparin and lithium-heparin) which have been used may be responsible for variability in yield of circulating DNA. Secondly, variation in sample processing at the level of centrifugation steps, delay in sample processing and duration and condition of storage, all contribute to the disparity between various studies [61]. Also, some studies use quantitative methods and some use qualitative methods, which can again lead to contradicting results.

Whether plasma or serum DNA is the ideal candidate for studying circulating DNA has been a topic of debate. The concentration of DNA in serum has been reported to be 4-6 fold higher in serum as compared to that in plasma [78-81]. But the increased levels of DNA in serum may be so because of lysis of blood cells during storage and processing/separation of serum [56]. Studies reported that time delay and the storage temperature of blood before centrifugation had a significant impact on DNA concentration in serum and thus serum DNA can be of diagnostic value only if serum preparation is done at ambient temperature and minimizing the time period between blood collection and centrifugation. However, plasma was shown to be a better source of tumor derived circulating cell-free DNA than serum for the detection of mutations and it reflects probably the same concentration of circulating DNA as in circulation while serum DNA can get elevated not because of tumor DNA but because of clotting process [80,82-84].

With these contraindications in view, various projects are being run to set guidelines for sample collection, sampe processing and storage in order to analyze circulating DNA levels, its integrity and tumor associated mutations. One such project is SPIDIA (Standardization and improvement of generic pre-analytical tools and procedures for in-vitro diagnostics) funded by European Commission. This project focused on External Quality Assessmet (EQA) of pre-analytical steps of DNA/RNA analyses. As a part of this project, blood samples were sent to different laboratories where DNA/RNA is extracted and returned. The External Quality Assessment organizer assess the quality of DNA retreived from various laboratories. On the basis of these surveys, evidence-based guidelines will be set for the pre-analytical steps involved in DNA/RNA analysis [85].

With well standardized pre-analytical phase, and with more high throughput techniques to detect mutations, circulating cell free DNA concentration are finding relevance in personalized medicine. Circulating cell-free DNA and the molecular alterations associated with it may not only find importance as non-invasive diagnostic or prognostic marker but also as markers to monitor tumor burden during treatment.

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