

Minireview

Circulating (cell-free) nucleic acids – A promising, non-invasive tool for early detection of several human diseases

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Abstract Circulating nucleic acids (CNA) are present in small amounts in the plasma of healthy individuals. However, increased levels of plasma CNA have been reported in a number of clinical disorders like cancer, stroke, trauma, myocardial infarction, autoimmune disorders, and pregnancy-associated complications. CNA has received special attention because of its potential application as a non-invasive, rapid and sensitive tool for molecular diagnosis and monitoring of acute pathologies and the prenatal diagnosis of fetal genetic diseases. This review throws light on the current status of blood CNA as a diagnostic marker and its potential as a powerful tool in the future.

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1. Introduction

The history of circulating nucleic acids (CNA) dates back to the 1940s, when Mandel and Mëtais reported in a French journal the presence of free nucleic acids in plasma [1]. They were able to detect free DNA and RNA in blood plasma of healthy individuals and patients. Unfortunately, their work went unnoticed probably because of a lack of clear understanding about CNA at that time. Barring two reports on auto-immune disorders, systemic lupus erythematosus (SLE) [2] and rheumatoid arthritis [3] there were no research articles published related to CNA for next thirty years. In 1977, Leon et al. reported high levels of CNA in patients of pancreatic cancer [4]. Interestingly, they even demonstrated that the plasma DNA levels in patients actually decreased after chemotherapy. The importance of CNA was recognized in 1994, when the presence of mutated oncogenes products (K-ras) was reported in the plasma of pancreatic cancer patients [5]. In the same year, the research group of Anker reported N-ras gene mutations in patients with myelodysplastic syndrome (MDS) [6]. In the next 5 years, several other reports followed related to

the detection of high fractional concentrations of tumor DNA in the plasma (or serum) of patients with various human cancers. Fetal-derived nucleic acids which enter maternal plasma during pregnancy have been shown to have potential uses in prenatal diagnostic strategies. The last decade has witnessed a great deal of interest in the subject of CNA with respect to the pathologies of cancerous and non-cancerous origin like diabetes, stroke, SLE, trauma, rheumatoid arthritis, inflammation, infection, etc.

The increased levels of circulating nucleic acids (DNA and RNA) in blood of patients indicated that CNA can be used as a non-invasive, rapid, sensitive and accurate method of diagnosis of several diseases. This review presents the existing knowledge on CNA and its future prospects as a diagnostic/prognostic marker. Research related to circulating nucleic acids recovered from other body fluids like urine, milk, cell-free bronchial lavage, etc. is rare and is not discussed here as it is beyond the scope of this review.

2. CNA in various human diseases

2.1. Cancer

It is known that the products of DNA/RNA in plasma actually arise from lysis of tumor cells. Activated oncogenes, mutations in tumor-suppressor genes like p53, chromosomal rearrangements and hyper-methylation of genes are some of the commonly found genetic variations in cancer. The first two genes whose mutations were studied in plasma of patients are (i) N-ras in myelodysplastic syndrome (MDS) and myelogenous leukemia [6] and (ii) K-ras in pancreatic [5] and colorectal cancer [7]. It has been suggested that p53 can be used as an early tumor marker to indicate recurrence or distant metastasis. In spite of their predominance, p53 alterations are difficult to detect because of widespread mutations along several exons and also due to low sensitivity in detection [8].

In plasma of non-Hodgkin's lymphoma patients, rearrangements in Ig heavy chain DNA sequences were detected. Similarly microsatellite instability, a feature frequently found in solid tumors, was first detected in plasma of small cell lung carcinoma patients [9] and in serum of head and neck carcinoma patients [10]. The sensitivity of detection of microsatellite alterations is also limited because of the fact that loss of heterozygosity (LOH) is more common than band shifts.

Alteration in global genome hypomethylation and hypermethylation of tumor suppressor genes at CpG islands in the promoter region, are frequently found in several types of tumors. The methylation pattern of mutated genes like p16, DAPK,

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Abbreviations: CNA, circulating nucleic acid; MI, myocardial infarction; SSc, systemic sclerosis; SCLC, small cell lung carcinoma; SLE, systemic lupus erythematosus; MSP, methylation specific PCR; NPC, nasopharyngeal carcinoma

GSTP1, O⁶-MGMT, etc. by methylation specific PCR (MSP) is proving to be a specific tool in lung cancer diagnosis [11,12]. Changes in hypermethylation pattern of DAP kinase gene in serum and plasma DNA of small cell lung cancer patients were noticed in 80% and 40% of these cases, respectively [13].

Several viruses have also been found to be associated with different cancers. CNA of Epstein Barr Virus (EBV) was first detected by Lo et al. in 1999 in nasopharyngeal carcinoma (NPC) patients [14]. They correlated the levels of circulating DNA with clinical stages of the disease. Similarly, human papilloma virus (HPV) was also detected in cervical cancer [15] and squamous head and neck cancer [16].

RNA has a high tendency to undergo degradation and is therefore not an attractive molecule as a diagnostic marker in plasma/serum. Although, tumor specific transcripts of tyrosinase mRNA were detected in melanoma patients using RT-PCR [17], however, two additional tumor markers of melanoma, gp-100 and MART-1 could not be amplified, indicating that sensitivity and specificity need to be evaluated carefully and sample size has to be increased, before concluding the validity of plasma RNA as a marker [18].

2.2. Prenatal diagnosis

Placenta is no longer thought to be an impermeable membrane; fetal DNA/RNA circulates freely in maternal plasma. Since, the rapidly growing fetus and placenta have ‘tumor-like’ characteristics, CNA of fetal origin can be expected to be found in maternal circulation. Based on this approach, for the first time Lo et al. demonstrated the presence of male fetal DNA in maternal plasma [19]. To prove that fetal cells in placenta are the source of CNA, they isolated plasma DNA from pregnant women carrying male fetus and after amplification of the Y-chromosome-specific gene sequences they correctly identified male fetuses in 80% of the cases [19]. Moreover, they also showed that fetal DNA rapidly cleared after delivery. Later, Lo suggested ‘hypomethylated msp1n’ as the first universal marker for fetal DNA [20]. Lo has done extensive work on circulating fetal DNA and its aberrations in pre-eclampsia (PE), chromosomal aneuploidies, RhD-genotyping, etc. Therefore, fetal DNA can be used for non-invasive diagnosis of paternally inherited diseases, pregnancy-associated complications and sex-linked disorders.

2.3. Other pathological disorders

The occurrence of CNA gained the interest of a wider scientific community investigating other pathological diseases like stroke, autoimmune disorders, myocardial infarction (MI), diabetes, trauma and even prion diseases. Lo’s group established a direct relationship between tissue injury in acute trauma and elevated plasma DNA levels [21]. Similarly, release of DNA into the peripheral blood can take place in acute stroke which involves CNS tissue damage. Till date there is no simple and accurate diagnostic blood test to determine the severity of stroke. Based on the report of Rainer in 2003 [22], it appears that a high correlation between plasma DNA concentration and the severity of stroke can prove to be useful in risk stratification.

Extending the argument of direct relation between extent of injury and increase in plasma DNA levels to cardiac tissue in MI patients, Chang et al. showed 10 times higher plasma

DNA concentrations (511 ng/ml) in MI patients than controls (36.3 ng/ml) [23]. Prolonged ischemia eventually leads to necrosis and at this time large amount of cell-free DNA is released into circulation. It appears that plasma DNA can be used along with the traditional markers of troponin and CK-MB (MB isoenzyme of creatine kinase) for diagnosis of MI [23].

Presence of high levels of circulating DNA in patients with SLE was first reported in 1966 [2]. Based on the different DNA banding patterns of lymphocytes and plasma, it was speculated that DNA found in circulation of SLE and systemic sclerosis (SSc) patients may be result of an actively produced ‘extrachromosomal DNA’ that may play an important role in the pathophysiology of autoimmune disorders [24].

Studies by Hoon have suggested that circulating DNA of different forms is very useful in staging, identifying disease progression and response to therapy in melanoma patients [25]. Tyrosinase mRNA was detected specifically detected in 67% of melanoma patients, but not in controls [26]. Detection of donor-derived DNA in plasma of transplant recipients offers a new method of monitoring transplant rejection. Bone allotransplants undergo rigorous processing and are considered as non-viable tissue. The donor genetic material may persist in circulation after bone allotransplantation [27].

3. Mechanisms of release of cell-free DNA into circulation

Although the evidences proving the presence of high levels of circulating DNA and RNA in plasma of patients is increasing day by day, the actual origin of CNA still remains enigmatic. In a healthy person, it is believed that CNA enters circulation via apoptosis of lymphocytes and other nucleated cells. Apoptosis as the primary source of CNA has been supported by the fact that normal plasma DNA on electrophoresis exhibits band sizes equivalent to a whole number multiple (1 – 5×) of nucleosomal DNA (185–200 bps) [28].

In cancer as well, apoptosis has been advanced as the possible origin of CNA on the basis of the fact that circulating DNA often represents ladder like electrophoretic pattern (e.g. as seen in pancreatic and lung cancer) which is similar to that shown by apoptotic cells [28,29]. It is important here to make a note of the fact that apoptosis is a mechanism supposedly lost by proliferating cancer cells and great efforts are needed to restore programmed cell death in malignant cells.

One of the hypotheses for the origin of CNA in cancer is based on “micrometastases” of tumor origin which are shed into circulation. Sorenson [30] and Chen [31] reported that the amount of DNA isolated from plasma of cancer patients was actually very high and did not correspond to the number of cancer cells present in the circulation. In other words, the number of cancer cells as per the amount of circulating DNA should have been 10000 cells per ml, while the authors detected much lower number of cells in plasma. Therefore, above hypothesis of micrometastases was also rejected.

High amounts of DNA found in plasma of patients with large or advanced/metastatic tumors [10,4] are thought to arise from tumor necrosis. However, it was noted that after radiation therapy, which is presumed to induce cell death/necrosis, circulating DNA levels surprisingly decreased in 90% of patients. Therefore, the hypothesis based on necrosis mediated

release of CNA in plasma became controversial. It was suggested by Leon et al. that the decrease in free DNA levels following radiotherapy may be due to arrest of cellular proliferation by radiation [4]. Cell-free nucleic acids also can contain small amounts of DNA of T-cell and mitochondrial origin.

Enzymes DNase I and II present in circulation degrade DNA and therefore minimal levels of plasma DNA are detected in healthy people. However, low activity of DNase I and II are seen in malignant diseases. This is supported by the fact that inhibitors of DNase have been detected in both tumors [32] and cells like thrombocytes [33], which explains why the elevated DNA levels in circulation are noticed. Spontaneous and active release of DNA by proliferating cancer cells is another possibility [34,35] that cannot be ignored as the activated lymphocytes are shown to release DNA in vitro [35].

As far as fetal DNA is concerned, there are three possible sources of fetal DNA entering maternal plasma: direct transfer of DNA, placenta and haematopoietic cells, of which placenta is believed to be the predominant source.

It is well known that RNA is very labile and easily degraded by ubiquitously present RNases. So, one would not expect cell-free RNA in plasma. Surprisingly, the presence of stable endogenous as well as exogenous circulating RNA in blood suggests that RNA perhaps contained in apoptotic bodies or bound to proteins/phospholipids and is therefore protected from degradation by nucleases [17]. Although, the origin of RNA entering circulation extends from tumor genes to house-keeping genes in healthy individuals, the knowledge on the source of CNA and more importantly the mechanism remains unanswered. An understanding of these issues will help in identifying the means of clinical application of CNAs. A schematic presentation of various possible pathways by

which cell-free DNA/RNA is released into circulation is given in Fig. 1.

4. Current methods of CNA analysis

Circulating DNA is commonly isolated using commercial kits like QIAmp 96 spin Blood DNA extraction kit supplied by Qiagen. Automated isolated systems like MagNa Pure LC [36] yield significantly higher copy numbers of DNA/RNA and seem to be better than manual methods. Earlier, plasma DNA below nanogram levels could not be detected using radio immunoassay (P^{32} DNA) [4], but now DNA upto picogram concentrations can be detected using PCR. Presently, real time quantitative PCR is used for amplifying and quantifying the circulating DNA/RNA of interest. Light-cycler based real-time PCR is rapid, eliminates carry-over contamination problems and does not require post-PCR processing [37]. Plasma DNA upto 10 ng/ml can be detected using simple fluorescent dyes, Pico Green reagent [38] and Hoechst 33258 [39]. Total plasma CNA (DNA + RNA) can be detected using SYBR Green II dye [40]. In general, plasma DNA concentrations of healthy individuals ranges from 10 to 50 ng/ml while that of patients is 100 ng/ml or above.

5. Future prospects

Methods of isolation and quantification of plasma/serum DNA/RNA are very crucial in analyzing data. We are in urgent need of standardization of techniques, careful evaluation and analysis of data according to common parameters like specificity and sensitivity. The issue of suitability of plasma

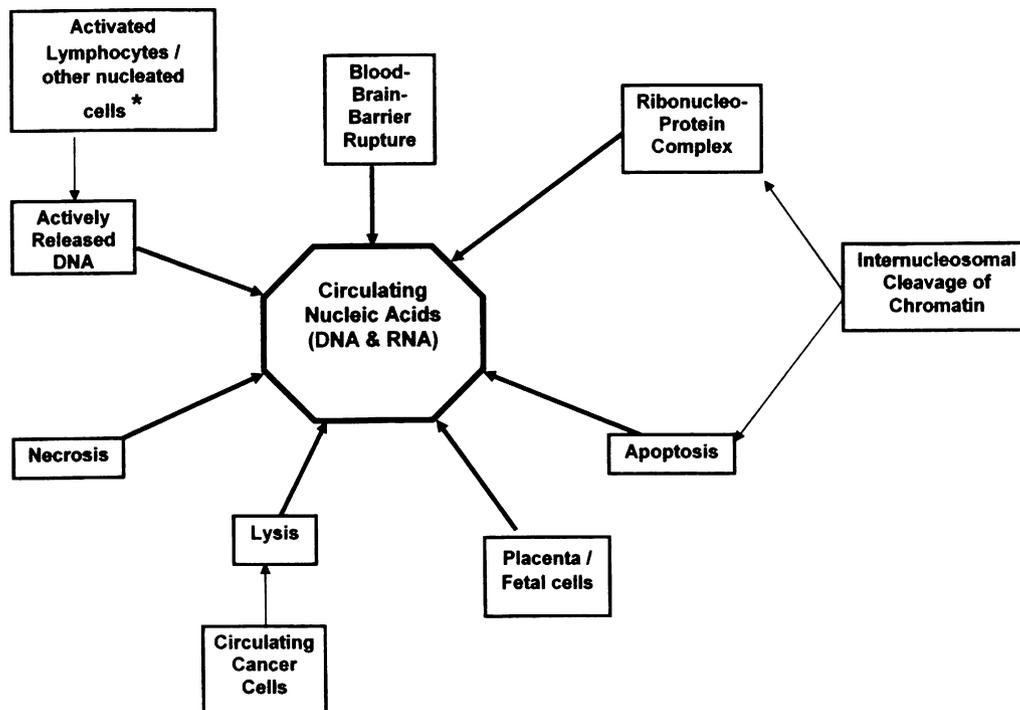


Fig. 1. Schematic presentation of various pathways by which nucleic acids are released into circulation. *Other nucleated cells include T-cells, haematopoietic cells, etc.

versus serum has to be resolved. Easy, cheap and faster techniques in future can make CNA identification and quantification a routine biochemical laboratory investigation. Plasma DNA/RNA testing can also eliminate the conventional methods of tissue biopsies, CT scan and expensive prenatal diagnostic tests like chorionic villus sampling (CVS) and amniocentesis. CNA detection is very challenging but has enormous utility if adequately managed.

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