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BIOMEDICAL SCIENCES

The contribution of the 20th century discoveries on the circulating DNA as biomarkers for cancer screening

GILDA ALVES, MARIANA CHANTRE & LUCAS DELMONICO

Abstract: Circulating DNA can be released in the biological fluids by a physiological process and by different pathological conditions. The first reports detecting circulating DNA in human plasma date from the late 40s. Even when specific pathological conditions were analyzed, the clinical importance of circulating DNA remained unclear. After PCR introduction, genetic and epigenetic alterations in circulating DNA gained more prominence for understanding the mechanisms of cancer development and progression. Nowadays, the circulating DNA assays are highlighted for their clinical relevance for cancer screening in liquid biopsy. In this review, we described the landscape of studies on circulating DNA isolated from human plasma or serum and the molecular tools used to obtain these findings throughout the 20th century and the current application in cancer.

Key words: Cancer, oncology, circulating DNA, liquid biopsy, plasma, serum.

INTRODUCTION

Liquid biopsy is an emerging field representing the circulating biomarkers that are found in the biological fluids. Circulating biomarkers can be represented by DNA, several types of RNA, lipids, or proteins. These molecules can be found circulating in aggregates or even as the cargo of the extracellular vesicles (EVs). Blood is normally the most examined fluid for liquid biopsy, but other fluids such as saliva, urine, cerebrospinal fluid (CSF), and nipple aspirate fluid (NAF) can be applied depending on the purpose. Circulating biomarkers can be purified or enriched from the whole biofluid or from a selected group of cells or just from a single cell present in these biofluids. The identification of the circulating biomarkers is a big deal in the scientific world regarding human health as well as our dear pets´ (Wiley et al. 2019, Marconato et al. 2019, Tagawa et al. 2019).

Currently, the research and application of liquid biopsy launches modern technologies, using powerful platforms and computers with databases called big data. However, to reach this point in the 21st century, the foundations of knowledge on circulating nucleic acid biomarkers were laid during the 20th century, which is the subject of this article. This review is especially based on historical publications that had constructed the road for the application of DNA biomarkers in cancer towards the 21st century.

THE DAWN OF CIRCULATING IN THE 20TH CENTURY

Before the discovery of the DNA structure by Watson and Crick in 1953, a landmark for molecular biology, the presence of circulating nucleic acids had already been observed in

human plasma from 10 healthy subjects and 15 subjects with pathological conditions, (Mandel & Métais 1948). The authors examined plasma since leukocytes in whole blood are rich in nucleic acids and could influence the assays. Plasma nucleic acid levels were expressed in phosphorus (P nucleic) content performed according to available techniques. Although plasma RNA levels were especially higher than plasma DNA levels, no significant changes were observed between healthy and pathological subjects examined. However, the authors pointed to a case of pregnancy in which plasma RNA levels were significantly higher compared to all other cases in the study. It is interesting to note that this preliminary study provides clues about the quantitative relevance of RNA in different biological processes.

After the DNA discovery, the following publications described the release and the uptake of DNA *in vivo* and *in vitro*. At least three decades were necessary to draw the full picture of circulating DNA. From the late 70s to the 90s, the circulating DNA was isolated from serum and from plasma. Controversy over how to isolate the circulating DNA had started. Collection, tubes, preservation, and all the parameters were under discussion. Some authors preferred serum as the fluid source; others preferred the plasma because of the concern that the clotting process might release small amounts of DNA, generating an artifact. Today, biotech companies sell long-term storage tubes for circulating DNA and RNA. In spite of not having long-term storage tubes, the scientific production of the circulating DNA in cancer emerged from simple in-house techniques, some of which are no longer in use and thus will probably remain unknown to the newer generations.

In 1966 Tan et al. verified the existence of DNA antibodies in the serum samples from patients with systemic lupus erythematosus (SLE) by agarose gel diffusion studies. This finding was confirmed by chemical assay for DNA detection. The clinical importance of this finding was unclear although the authors stated that the antibodies could be employed in a sensitive and specific method for detecting DNA in serum present in patients with SLE and other systemic diseases that are generally associated with cell deterioration (e.g. hepatitis, metastatic carcinoma, and miliary tuberculosis). The authors point out that the increase in cell death caused by skin rash in patients with SLE leads to the origin of circulating DNA. Further, Steinman (1984) using a multi-step DNA purification protocol (proteolysis, phenol saturation, adsorption and elution by hydroxyl apatite and urea, respectively) confirmed the presence of circulating DNA in the plasma from 10 patients with SLE. The authors also pointed out that the association with skin rash in SLE patients suggests that increased cell death may originate circulating DNA. The presence of circulating DNA in patients with SLE was later confirmed in the plasma of 10 patients. The DNA was purified by multiple steps, including proteolysis, phenol extraction, and hydroxyl apatite adsorption and elution (chromatography) in the presence of urea (Steinman 1984). The resulting double strand DNA (dsDNA) was radiolabeled by nick translation and then subjected to ultracentrifugation and treatment with S1-endonuclease (an endonuclease that degrades single-strand DNA (ssDNA) and RNA). The ultracentrifugation was a method frequently applied to separate DNA based on its density in a centrifuge according to the centrifugal force. These experiments verified the correspondence of the nucleotides between the plasma-derived DNA and human genomic DNA. Therefore, the plasma DNA was derived from human genome.

Circulating DNA (also called extracellular DNA) was observed in the media of

phytohemagglutinin (PHA) as well as other mitogen-stimulated culture of human lymphocytes up to 6 days (Rogers et al. 1972). The source of the secreted DNA was determined by stimulated cells that were pulsed with both [^{3}H]thymidine and [^{14}C]uridine in the culture day 3. The authors observed that between the cell culture days 4 and 6, the [^{14}C] counts in cellular DNA dropped, while the total [^{14}C] counts in DNA from the cells and the media had increased, representing the conversion of RNA degradation products into DNA. During this time a significant amount of [^{14}C]-labeled DNA was observed in the culture media while no [^{14}C]RNA was released. In these experiments, essentially no RNA release was detected although nowadays it is known that cells also release all sorts of RNA. Probably the detection of RNA secretion was limited by the methods. The examination of the released DNA suggested that it was complexed to protein or lipid (Rogers et al. 1972). In that paper, the authors discussed the possible roles in mitosis or mitosis failure trying to explain the DNA release that was observed; however, as concluded a few years later on, the cellular DNA release was normal.

More evidence of DNA release was produced through studies with frog heart auricles, with the first studies showing DNA release from eukaryote organ. The technique was summarized in a review of Stroun et al. (1977), which is to our knowledge the first review concerning eukaryotic circulating DNA. The organ was able to survive for several days in medium as evidenced by continued pulsation of the auricles. The sterile auricles were incubated for various periods of time, and the supernatant was removed and centrifuged at high speed for 12 hours to eliminate all cellular contaminants. DNA was isolated from both the supernatant and the auricles. The released DNA was dsDNA as observed by the UV absorption curve detected by the hyperchromic effect (increase of Ultraviolet absorbance at 260nm that occurs when the DNA duplex is denatured).

The spontaneous release of DNA by human blood lymphocytes in culture was reported by Anker et al. (1975). The released DNA was characterized by its typical hyperchromic effect, deoxyribose coloration, sensitivity to DNase digestion, and elution through hydroxyl apatite columns. All those methods together proved that DNA was excreted, in the shape of a double-stranded helix. The length of the released DNA was shorter than the cellular DNA. The extracellular DNA seemed to originate from living cells. In agreement, Olsen & Harris (1974) also said that it was unlikely that cell death could account for the appearance of the released DNA. They detected extracellular DNA derived from the rabbit spleen tissues that were cultured *in vitro*. The extracellular DNA was mainly dsDNA, showing a large spread in buoyant density gradient ultracentrifugation. The authors argued against cell lysis as the origin of extracellular DNA because the production of extracellular DNA was constant in several situations. Similar results were obtained with experiments with rat lymphocytes (Hoessli et al. 1977).

It is important to note that the PCR (polymerase chain reaction) thermocycling machine was invented by Kary Mullis (Mullis et al. 1986). Before PCR, tools of molecular biology were employed, as described in the aforementioned studies, but no DNA amplification was achieved rapidly and in large scale. The PCR technique, which is a common procedure nowadays, introduced a revolution in detecting low amounts of circulating DNA. In the pre-PCR era, the circulating DNA was reported in normal and in pathological conditions.

PRIMARY REPORTS ASSOCIATING DNA WITH CANCER

The first association of circulating DNA with cancer was not due to DNA secretion by the tumor cells, but rather the opposite. Polyoma virus DNA was transforming in a site other than the inoculation one, after injection into newborn and adult hamsters, suggesting that tumorigenic circulating DNA was transported in the blood or the lymphatic system and would later penetrate cells (Bendich et al. 1965). The authors speculated on the possibility of a mechanism of metastasis originated by the incorporation of the circulating transforming DNA by normal cells. In fact, some classes of viral DNA/RNA can promote cancer but the etiology of most cancers is more complex. Cancers caused by virus infection represent a fraction of the neoplastic processes. The next studies exposed the secretion of DNA by the cancer cells.

An amazing study (Davis & Davis 1973) with plasma samples from 418 individuals with variable pathological conditions (including 20 carcinomas and lymphomas) and healthy individuals was conducted in search of circulating DNA by counterimmunoelectrophoresis (a method used to evaluate the binding of an antibody to its antigen). Of the healthy individuals included in the study, only 1/83 (1.2%) of the healthy adults and 6/36 (16.7%) cord bloods (4/18 males and 2/18 females) were positive for plasma DNA. The rest included patients who underwent preoperative and postoperative surgical procedures (for major and minor surgery), in most of which circulating DNA was detected. Regarding the cancer cases, 2/20 cases with carcinoma (one bladder carcinoma and one lung carcinoma) were positive for DNA in the plasma and showed widespread dissemination. The authors considered unclear the reasons for DNA release, but they suggested that the phenomena would likely be due to tissue anoxia or tissue destruction. The prevalence of circulating DNA in the postoperative surgical patient was tolerated, arguing against the inherent pathogenicity of DNA.

CIRCULATING DNA IN CANCER PATIENTS

An important circulating DNA study focusing only on cancer patients was conducted using serum from 173 and 55 patients with different types of cancer and healthy subjects as controls, respectively (Leon et al. 1977). Using the radioimmunoassay technique, which consists of the competition of DNA in the test sample with I^{125}-iododeoxyuridine-labeled DNA for the anti-DNA antibody obtained from the serum (Leon et al. 1975), the authors found higher amounts of DNA in the serum from metastatic patients as compared to nonmetastatic patients (Leon et al. 1977). The mean of DNA concentration in normal controls was 13 ng/ml, whereas the mean observed in the patients was 80 ng/ml. The DNA concentrations were found in the range of zero to 50 ng/ml in 50% of the patients; while in the other 50%, the concentrations ranged between 50 to 5000 ng/ml. The authors also observed reduced DNA levels in the serum after radiation therapy and this finding was associated with improvement of some clinical condition, such as reduced tumor size and the reduction of pain. In contrast, increased DNA levels after radiation therapy was associated with failure in the response to the treatment and this profile was also observed in 13 of 17 patients who died within a year. Therefore, higher circulating DNA levels in the circulation of these patients were associated with relapse and worse prognosis.

Another DNA study using radioimmunoassay compared the levels of the circulating DNA found in the serum of patients with benign or

malignant gastrointestinal tract disease and determined the diagnostic values of those measures (Shapiro et al. 1983). The study enrolled 386 patients, in which those with benign disease (48%) showed mean DNA levels of 118 +/- 14 ng/ml, while those with malignant disease (52%) showed DNA values of 412 +/- 63 ng /ml, with statistically significant differences between these DNA concentration levels. The quantification of the circulating DNA showed the highest sensitivity for pancreas carcinoma; 90% of the patients showed DNA levels above 100 ng/ml. The parallel quantification of the circulating DNA levels and the carcinoembryonic antigen (CEA) serum test resulted in greater sensitivity and specificity, even when either biomarker alone exhibited low sensitivity (gastric carcinoma). The CEA is a classic serum biochemical marker used to aid in diagnosing and monitoring certain types of cancers, especially colorectal tumors (Kumar et al. 2005). The results (Shapiro et al. 1983) indicated that serum DNA concentration is significantly higher in cancer patients and moderately high in patients with benign disease. The diagnostic and prognostic values of circulating DNA were becoming evident.

In the study conducted by Stroun et al. (1987), circulating DNA was obtained from the plasma of 10 out of 37 patients with advanced malignant diseases, while DNA was not detected in the plasma of 50 normal controls. The detectable circulating DNA was found only in plasma of patients with advanced or progressing malignancies. The positivity was found in acute leukemia (2/3); plasmocytomas and lymphomas (1/9); lung cancer (1/7); various abdominal tumors (2/12); breast cancer (1/3); and metastases from unknown primary carcinomas (3/3). The purified circulating DNA was shown to be dsDNA and the concentration ranged from 0.15 to 12 µg/ml. As observed by agarose gel electrophoresis, the DNA length varied from 21 kb to less than 0.5 kb. The origin of the circulating human DNA genome was confirmed by hybridizing with a ^{32}P-labelled human DNA probe. The finding of substantial amounts of DNA in the plasma of 27% of the cancer patients investigated, plus its absence from the controls, suggested a positive correlation of circulating DNA with malignancy. Although the mechanism is still unknown, the cancerous cells secrete more DNA than normal cells.

DNA and RNA were found on the cell surface of tumor cells (Juckett & Rosenberg 1982). This observation was possible through experiments in which the authors used whole-cell electrophoresis on tumor cell suspensions with the intention of determining the charged molecules on the cell surfaces. For this purpose, the RNase and DNase enzymes were immobilized on agarose beads, were able to reduce the cells' mobility after incubation, corroborating that nucleic acids were anchored on the outside membrane surface. Surface nucleic acids were not identified by this electrophoresis approach on any normal cells that were studied. However, it was not possible to determine that tumor necrosis or cell apoptosis was responsible for at least part of the observed circulating DNA.

The origin of circulating DNA has been a matter of discussion (Stroun et al. 1987). Even today, the exact mechanism of the DNA release by the cells is not fully understood. But it is known that the constant releasing of DNA by lymphocytes is regulated by a homeostatic mechanism (Anker et al. 1976) and that the circulating DNA is not secreted naked. The circulating DNA was found complexed with proteins, lipids, and polysaccharides, and therefore the studies often apply purification methods to extract the DNA for quantification and for conducting the experiments. Actually, the purified circulating DNA may contain DNAs

other than the genomic, with the possibility that DNA derived from bacteria and viral particles might be coextracted.

SEQUENCE-SPECIFIC STUDIES IN CIRCULATING DNA AFTER PCR ERA

One problem in screening for a specific circulating cancer biomarker is that it might be diluted in a pool of DNAs from all sources; therefore, the application of enrichment techniques is currently recommended. In the 20th century, the enrichment techniques were not available, but since the 90s the PCR using sequence-specific primers to amplify mutant DNA was largely applied to identify oncogenes and tumor suppressor gene mutations. The discovery of most oncogenes and tumor suppressor genes is also a legacy of the 20th century. In some of these genes, the cancer-prone mutation is inherited but most cancers are sporadic, caused by carcinogenic agents in the environment. The studies that lead to their discovery are amazing and not systematic. Today, there are hundreds of known cancer driver genes. Among them can be highlighted the oncogenes of the *RAS* family: *NRAS*, *KRAS,* and *HRAS*.

Vasioukhin et al. (1994) investigated the presence of point mutations of the *NRAS* gene in the DNA of plasma, blood cells, and bone marrow from 10 patients diagnosed with myelodysplastic syndrome (MDS) or acute myelogenous leukaemia (AML). Sodium dodecyl sulfate (SDS) and proteinase K treatment, followed by phenol extraction and precipitation with ethanol, prepared the DNA isolation. The last step was ultracentrifugation for separation. The exon 1 of *NRAS* was amplified by PCR. The detection of point mutations was performed by the hybridization of PCR products with specific oligonucleotide probes. Mutations found in *NRAS* were detected in five cases and were present in the plasma DNA, contrasting with the infrequent presence in the DNA of either peripheral blood cells or bone marrow. Therefore, the authors described that these findings may indicate that the bone marrow biopsy or aspiration does not necessarily contain all the malignant clones causing the disease and that plasma could be a better source for detecting and monitoring myeloid disorders.

Mutations in *KRAS* were found in DNA extracted from the plasma of 14 patients with colorectal cancer (CRC) (Anker et al. 1997). Results were obtained by PCR and also by another PCR assay creating an enzyme restriction site in the absence of a *KRAS* mutation followed by direct sequencing and further cloning methods. Note that the authors used more than one method for confirmation of the results. The results revealed that 7 patients (50%) presented mutations at codon 12 in their primary tumor, and the same mutations were found in the plasma DNA of 6 patients (86%). No *KRAS* mutations were detected in the plasma specimens of 7 patients whose tumors were negative, as well as the plasma DNA of healthy controls. Therefore, the detection of mutations in *KRAS* in the plasma seemed to be more accurate in patients with CRC cancer. The results argued in favor of using plasma DNA for clinical applications.

A similar study was conducted by Kopreski et al. (1997). In this study, both plasma and serum were the source for screening the *KRAS* mutations. Plasma or serum were obtained from 31 patients with metastatic or unresected CRC and from 28 healthy controls. The extracted DNA was amplified using the two-stage PCR assay and digestion with restriction enzyme to enrich the mutated *KRAS*. Mutant *KRAS* DNA was identified in the plasma or serum of 12 (39%) patients. All mutations were confirmed by DNA sequencing. No *KRAS* mutation detection was

observed in any of the normal volunteers. In addition, 19 corresponding plasma/serum and tumor samples were screened for mutations in *KRAS*. Five cases (26%) were positive, although the mutations found did not coincide between the specimens. Since the beginning, plasma and/or serum were the preferred fluid in the liquid biopsy race; however, *KRAS* mutations were also screened in alternative fluids. Yakubovskaya et al. (1995) detected *KRAS* mutations in the sputum of patients with non-small cell lung cancer (NSCLC), and Tada et al. (1993) screened the pancreatic juice and the peripheral blood cells from patients with pancreatic cancer.

TP53 is the most famous tumor suppressor gene, encoding the protein p53. This protein is multifunctional and has a key role in preventing or promoting carcinogenesis when it is mutated. The protein p53 is so important that it was called "the molecule of the year" in 1993 (Koshland 1993). Mutated *TP53* was screened in plasma of patients with breast cancer (BC) and small cell lung cancer patients (SCLC) by the PCR-based method for detection of mutations, the PCR-SSCP (single-strand conformation polymorphism) (Silva et al. 1999). In this method, the amplified PCR product is denatured to ssDNA and then subjected to separation by electrophoresis in nondenaturing polyacrylamide gel. The ssDNA bands appear at different positions indicating the presence of mutations. In Silva's study, these bands were isolated from the gel and the DNA was sequenced for identifying the mutation. Tumor and plasma DNA of 25 patients (15 BC and 10 SCLC) was screened by PCR-SSCP and sequencing of exons 5, 6, 7, and 8 of *TP53*. Mutations in tumor DNA were observed in 6 cases, 3 (50%) of which were also identified in plasma of the same patients.

As expected, not only the oncogenes of the *RAS* family but also the tumor suppressor gene *TP53* was studied in the 90s. The list of the publications was increasing rapidly during that time. The studies were increasing the number of circulating genes to be evaluated in the same cohort (Esteller et al. 1999). Moreover, two other genetic alterations beyond the mutations were also studied. Promoter hypermethylation and microsatellites were at the spot as well, each one with different approaches.

The hypermethylation in the gene promoter CpG islands leads to gene expression suppression. The hypermethylation used to be studied applying methylation-sensitive restriction enzymes followed by PCR. These restriction enzymes are not able to cleave to the regions that contain the methylated-cytosine residues, leaving methylated DNA intact. However, unmethylated DNA is digested and the amplification products detected are methylated DNA. Nevertheless, the methylation-sensitive restriction method could create an artifact if the DNA was not fully digested by the restriction enzyme. To solve this problem, another method was created, the methylation-specific PCR (MSP-PCR). In this method, the unmethylated cytosines are chemically modified into uracil, and specific primers in the PCR assay for detecting methylated and unmethylated DNA are used (Herman et al. 1996).

The detection of aberrant promoter hypermethylation of tumor suppressor genes in serum and tumor DNA and paired normal tissues from 22 non-small cell lung cancer (NSCLC) patients was evaluated (Esteller et al. 1999). The methylation status of the tumor suppressor gene *CDKN2A/p16*^{INK4A}, the detoxification gene glutathione S-transferase P1 (*GSTP1*), and the DNA repair gene O6-methylguanine-DNA-methyltransferase (*MGMT*) were tested by MSP-PCR. Hypermethylation of at least one of these genes was detected in 15/22 (68%) NSCLC tumors but not in any paired

normal lung tissue. The hypermethylation in serum DNA was found in all tumor stages. Altogether, the results indicated that the detection of hypermethylation of cancer-related genes in serum might be useful for cancer diagnosis or the follow up of the treatment.

Microsatellites are short tandem repetitive sequences and the instability (MSI) in the size of tandem repeat is a characteristic of the CRC cancer associated with the hereditary non-polyposis colorectal carcinoma (HNPCC) syndrome (Peltomäki et al. 1993) caused by a genetic failure in the mismatch DNA repair system. In addition, MSI could be detected in the circulating DNA from other tumors having a role in the neoplastic progression. Microsatellites are also indicators of deletions in the genome when Loss of Heterozygosity (LOH) occurs, which is the loss of one microsatellite polymorphic allele. MSI was detected by PCR amplification of the microsatellite locus, and LOH was detected by the absence of amplification of the deleted allele.

In the study by Mao et al. (1994), paired normal and tumor specimens from 100 cancer patients were obtained for a huge microsatellite study. Thirty-five CRC, 23 NSCLC, 10 SCLC, and 32 bladder TCC (transitional cell carcinoma) were assessed, and 9 microsatellite biomarkers were tested. Each microsatellite marker was amplified in paired normal/tumor DNA by PCR. The electrophoresis of radioactive labeled products was performed on denaturing acrylamide gels, exposed to the film to reveal the bands. The authors found that 29% of head and neck cancers, 9% of NSCLC, 50% of SCLC, and 28% of bladder tumors exhibited microsatellite alterations with at least one marker. As a demonstration of the potential clinical application, the identical microsatellite alterations detected in the primary tumors were successfully identified in corresponding DNA extracted from the urine, sputum, and surgical margins from affected patients. Chen et al. (1996) described the microsatellite alterations detected in 3 markers in the plasma of 50% of SCLC patients. The microsatellite alterations (MSI and LOH) were present in 16/21 (76%) SCLC tumors and in 15/21 (71%) plasma samples. These alterations were also found in the sputum. In one case, the alteration was present only in the plasma DNA. Nawroz et al. (1996) tested 21 patients with primary head and neck squamous cell carcinoma (HNSCC) by PCR-based microsatellite screening based on the comparison of the patterns of DNA lymphocytes and the paired serum DNA. Patients were scored for alterations according to the presence of MSI or the LOH in serum at each of 12 microsatellite markers and then compared with primary tumor DNA. The results revealed that 6 out of 21 patients (29%) had one or more microsatellite alterations in serum, corresponding exactly to those in the primary tumors. All these 6 patients were diagnosed with advanced disease.

20th CENTURY CIRCULATING DNA OVERVIEW

In the last year of the 20th century, the circulation of DNA and RNA was the subject of a whole volume (volume 906, issue 1) of the *Annals of New York Academy of Sciences:* "Circulating Nuclei Acids in plasma or serum." The articles were great with far too much to be commented on here. The importance of this volume was that, definitely, it showed that the circulating DNA destiny was towards becoming biomarkers in cancer. A summary of the main achievements in circulating DNA is presented in Table I.

Table I. Landmarks in circulating DNA in the 20th century.

Year/Period	Observations	References
1948	Nucleic acids (P nuclei) is found in human plasma.	Mandel & Métais 1948.
1965	Circulating DNA can transform eukaryotic cells.	Bendich et al. 1965.
1966	Release of circulating DNA in serum of SLE patients.	Tan et al. 1966.
70s - 80s Characterization of circulating DNA	DNA release is normal and regulated. DNA is released in complexes. Cancerous cells release more circulating DNA than normal cells.	Rogers et al. 1972, Davis & Davis 1973, Olsen & Harris 1974, Anker et al. 1975, 1976, Leon et al. 1977, Stroun et al. 1977, 1987, Hoessli et al. 1977, Juckett & Rosenberg 1982, Shapiro et al. 1983, Steinman 1984.
90s Sequence specific studies	Screening of mutations in oncogenes and tumor, suppressor genes, microsatellites instability and DNA methylations in serum and plasma of cancer patients.	Koshland 1993, Peltomäki et al. 1993, Vasioukhin et al. 1994, Mao et al. 1994, Chen et al. 1996, Nawroz et al. 1996, Anker et al. 1997, Kopreski et al. 1997, Silva et al. 1999, Esteller et al. 1999.

SLE – systemic lupus erythematosus.

CIRCULATING DNA TECHNOLOGY IN THE 21st CENTURY

Currently, the circulating DNA is commonly referred to as cfDNA (circulating free DNA or cell-free DNA), and specific tubes for its best preservation have emerged, e.g., LbGard® Blood Tubes (Biomatrica), Cell-Free DNA Collection Tubes (Roche), Cell-Free DNA BCR® (Streck), and PAXgene Blood ccfDNA (Qiagen). All of these are able to preserve cfDNA for days (average 7-10) at room temperature (25ºC) without occurrence of cfDNA degradation.

Substantial advances occurred in the first decade of the 21st century with the release of the first data from The Cancer Genome Atlas (TCGA 2008) (NIH), and research on cfDNA grew significantly and was boosted with the introduction of more sensitive and specific sequencing platforms (Leighl et al. 2014). This is justified, especially by the search for point mutations in the circulating tumor DNA (ctDNA), part of the set of cfDNAs.

There are currently two validated tests for using cfDNA as research material: the Cobas EGFR Mutation Test v2 (Roche) and the Epi proColon® (Epigenomics AG). The Cobas EGFR Mutation Test v2 test is able to detect *EGFR* mutations (including deletions, insertions, and substitutions of exons 18 to 21) in cfDNA from NSCLC patients (Leighl et al. 2014, Sacher et al. 2016). According to the mutations detected by the test, the treatment can be replaced and targeted to the mutation present in the *EGFR* gene. The second test, Epi proColon® (Epigenomics AG), had its history started in 2003 (Lofton-Day & Lesche 2003), with the elaboration of the hypothesis that genes aberrantly methylated in the tumor

tissue could have the same epigenetic profile in the material circulating genetic. In 2008, the evaluation of more than 600 regions in genome-wide (promoter regions) revealed 45 genes capable of differentiating CRC from different colon pathological tissues, healthy tissues and peripheral blood lymphocytes. Based on three specific criteria for validation (the first, CRC discrimination of normal colon tissues with an AUC ≥0.70; the second, CRC discrimination of any other tissue with an AUC ≥0.65; and third, the lymphocytes had a methylation rate <10% for the evaluated gene), three genes (*TMEFF2*, *SEPT9* and *VTN*) showed to be sensitive for CRC detection, from their methylation positivity in the plasma DNA. Finally, the *SEPT9* gene proved to be the most specific and sensitive for all stages of the CRC evaluated, with an AUC> 0.80 (Lofton-Day et al. 2008). Further, for test's clinical validation, the first results demonstrated a sensitivity and specificity, 90% and 88%, respectively, for all stages of the 50 CRC evaluated (Warren et al. 2011). Currently, the test's false positive rate is 3%, meaning that out of 100 people tested, 3 with a positive result do not have cancer, but new trials have shown the low sensitivity to detect cases at an early stage (Song et al. 2017, Nian et al. 2017).

In addition to the tests cited above, there are two tests under development (under FDA approval), the FoundationOne® Liquid (Foundation Medicine) and Guardant360® (Guardant). The first test originated from a massive sequencing system with high sensitivity that allows the wide detection of genetic alterations, including variants, genomic rearrangements at low allele frequencies and copy numbers amplifications in the circulating DNA (Clark et al. 2018). From the analytical validation of 2,666 genomic alterations involved with the carcinogenic process, the test's primary results revealed a high agreement between the changes found in the tumor and those in the circulating DNA (75% for genomic changes and 83% for small mutations). For rearrangements at the frequency of 0.25%-0.50% and > 0.5%, the system was able to detect 95% and 99%, respectively. Surprisingly, of the 62 healthy volunteers analyzed, none of them were positive for the investigated changes, representing no false positive cases. From this analytical validation, the test consisted of sequencing 70 genes known to drive cancer growth, including homologous recombination deficiency genes, and reports the genomic biomarker for microsatellite instability. The second test has the same purpose; however, it emerged with the main objective of measuring genomic changes that would enable a specific target treatment (Collisson et al. 2015). The first data from the analytical and clinical validation, initially with the massive sequencing of 54 genes (512 exons), revealed a low false positive case rate (0.0001%), with an ability to recognize single-base variants at concentrations of 0.25%. Furthermore, the test application in 165 cancers (breast, lung, CRC, melanoma, genitourinary cancer, and other cancers types) paired with their respective circulating DNA, showed a genomic changes concordance with sensitivity and specificity of 85% and 99.6%, respectively (Lanman et al. 2015). Currently, the test relies on the sequencing of 73 driver genes to the tumor process, focusing especially on mutations that lead to resistance to therapies in NSCLC's cases (Thompson et al. 2016, Aggarwal et al. 2019).

Further, there is other major research aimed at creating new cancer diagnostic tests based on cfDNA. The largest and most robust study considered so far, called CancerSEEK that aims to detect cancer before any symptoms are noticed, showed its initial results in early 2018. The authors demonstrated that from the evaluation of genetic alterations in the ctDNA of

16 genes (*AKT1*, *APC*, *BRAF*, *CDKN2A*, *CTNNB1*, *EGFR*, *FBXW7*, *FGFR2*, *GNAS*, *HRAS*, *KRAS*, *NRAS*, *PIK3CA*, *PPP2R1A*, *PTEN*, *TP53*), and 8 circulating proteins (CA-125, CA19-9, CEA, HGF, Myeloperoxidase, OPN, Prolactin, TIMP-1) (including 1,005 patients with non-metastatic cancer of the ovary, liver, stomach, pancreas, esophagus, cervix, lung or breast) it is possible to identify solid tumors still in the early stages. CancerSEEK presented a median sensitivity of 43%, 73%, and 78%, for I, II, and III stage cancer, respectively (ranging from 98% in ovarian cancers to 33% in breast cancers), and specificity > 99%; only 7 of 812 healthy controls scored positive. The concordance between tumor tissue biopsy and ctDNA was 90% among the evaluated tumors (when concentrations were significant) (Cohen et al. 2018). The first phase of the CancerSEEK test, combined with PET-CT imaging, called DETECT-A, included 9,911 women with no previously diagnosed cancer. Of these cases, 134 (1.35%) had a positive component for the test and followed for comparison with the evaluation by PET-CT imaging. In the first year, CancerSEEK was able to detect 26 women with initial cancer, revealing a positive and negative predictive value of 19.4% and 99.3%, respectively. Seventeen of the 26 women carried early-stage cancers, and most of the them were subjected to surgery or undergoing chemotherapy treatment, suggesting that they benefited from the test (Lennon et al. 2020).

Another large study, funded by Grail Biotech, searched for methylation signatures in the cfDNA. The company's studies started with the total sequencing of the circulating genetic material derived from cancer patients, the so-called Circulating Cell-Free Genome Atlas (CCGA) (Aravanis et al. 2017). Since then, the company has focused on genomic changes in this material, and recently it was presented a gene panel with a high capacity for detecting tumors from the methylation profile present in cell-free DNA. The methylation panel studied included 103,456 distinct regions of the genome (17.2 Mb) and 1,116,720 cytosine-guanine dinucleotides (CpGs). The first results, in a cohort that included 6,689 participants [2,482 cancer (> 50 types of cancer), 4,207 non-cancer], revealed that the test had variable sensitivity rates for the different stages of the cancer evaluated (18%, 43%, 81% and 93% for stage I, II, III and IV, respectively). On the other hand, the specificity of the test for all tumors analyzed was 99.3% [95% confidence interval (CI): 98.3% to 99.8%], thus guaranteeing a false positive rate of 0.7%. Further, the test was able to determine the location of the known cancer in 93% of cases, suggesting that the need for whole body images could be reduced. (Liu et al. 2020).

Despite the evident importance in oncology practice, the biological role of circulating DNA is still unclear; The variety of cfDNA release sources associated to the different rates of uptake by cells makes it difficult to establish a specific biological function for cfDNA (Bronkhorst et al. 2019). The transfer of cfDNA fragments into the cells may be mediated by cell surface DNA-binding receptors in which the cfDNA can act into the nucleus or generate mononucleotides (Chelobanov et al. 2006). Moreover, cellular uptake of cfDNA can also be facilitated by the extracellular vesicles, such as exosomes which contains cfDNA bound to their exterior membranes (Fernando et al. 2017). Once into the cell, tumor-derived cfDNA carrying genetic alterations is able to promote oncogenic transformation (García-Olmo et al. 2010). Meanwhile, the biotechnology companies have taken advantage of circulating DNA to follow up cancer and other diseases. Table II shows the main technologies concerning the detection of circulating DNA in the 20th and the 21st centuries.

In conclusion, the circulating DNA discovery has a 20th century stamp, and what we have now

Table II. Technology evolution to detect and isolate circulating DNA.

Century	Methods
20th	Immunologic techniques with DNA antibodies DNA isolation procedure by proteolysis and phenol extraction Ultracentrifugation DNA quantification by ultraviolet absorbance at 260nm Radioimmunoassay technique Counterimmunoelectrophoresis Chromatography Enzyme digestion: DNase, RNase, S1-endonuclease dsDNA, ssDNA and RNA radioactivity labelling Cell culture/Organ culture PCR, PCR-SSCP, MSP-PCR In-house DNA sequencing
21st	Blood long-term preservation tubes Circulating DNA isolation kits Automatic DNA sequencing FDA approved tests: Cobas EGFR Mutation Test v2 (Roche) and the Epi proColon® (Epigenomics AG) Tests in development: FoundationOne® Liquid (Foundation Medicine), Guardant360® (Guardant), Grail Biotech and CancerSEEK (Thrive Earlier Detection Corp.)

dsDNA – double strand DNA, ssDNA – single strand DNA, PCR – polymerase chain reaction, PCR-SSCP - polymerase chain reaction - single-strand conformation polymorphism, MSP-PCR methylation-specific - polymerase chain reaction.

is the enormous biotech development, thanks to the pioneer researchers to be remembered.

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GILDA ALVES[1]
https://orcid.org/0000-0003-2246-719X

MARIANA CHANTRE[1]
https://orcid.org/0000-0002-7351-5588

LUCAS DELMONICO[2]
https://orcid.org/0000-0002-3753-9451

[1]Universidade do Estado do Rio de Janeiro/UERJ, Faculdade de Ciências Médicas, Departamento de Patologia e Laboratórios, Laboratório de Marcadores Circulantes, Avenida Prof. Manuel de Abreu, 444, 4° andar, Vila Isabel, 20550-170 Rio de Janeiro, RJ, Brazil

[2]Universidade Federal do Rio de Janeiro/UFRJ, Instituto de Biofísica Carlos Chagas Filho/IBCCF, Laboratório de Radiações em Biologia, Avenida Carlos Chagas Filho, 373, Bloco G, Sala G0-036, Cidade Universitária, 21941-902 Rio de Janeiro, RJ, Brazil

Correspondence to: **Gilda Alves**
E-mail: galvesbrown@gmail.com

Author contributions

GA - conceived the study and wrote the manuscript; MC - wrote the manuscript; LD - wrote the manuscript. All authors read and approved the final version.