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Original article

Tracking the history of circulating nucleic acids for cancer research in Brazil: A systematic review

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Abstract

Introduction: Circulating nucleic acids can be obtained by minimally invasive procedures based on liquid biopsy, which has emerged as a promising area of investigation for screening and monitoring cancer treatment. Currently, tests based on circulating nucleic acid analysis, specifically cell-free DNA (cfDNA), are commercially available for diagnostic and prognostic investigation of a number of neoplasms. Objective: To describe the research on circulating nucleic acid markers for cancer prospecting in Brazil, since this area has advanced rapidly in recent years. Methods: In this systematic review, we surveyed Brazilian publications in cancer research focused on cfDNA and cfRNA present in different fluids. Both MEDLINE-PUBMED and EMBASE databases were inspected using terms such as "circulating nucleic acids", "cancer", and "Brazil". Results: The search returned 326 articles, in which 28 Brazilian translational studies were eligible. Different methodologies were reported for different types of cancer, in which cfDNA from plasma was the most investigated biological material. Molecular investigations included quantification, somatic mutation, RNA expression, genotyping, microsatellites, blood protein interaction, and methylation. Discrepancies in the regional distribution of the studies were also observed. Conclusion: Studies on circulating nucleic acid markers have advanced significantly in the oncology field, but many others are needed to better address the clinical practice in Brazil.

Keywords: Brazil; Cancer; Circulating nucleic acids; cfDNA; cfRNA; Liquid biopsy.

Introduction

Cancer represents an important health problem worldwide. Tissue biopsies and surgical intervention are the standard strategies for the diagnosis and treatment of many cancer types. Although important, surgical interventions are very invasive procedures that may involve multiple risks. In this context, with the new technologies it is possible to track new potential cellular and genetic biomarkers that will improve this scenario, complementing the diagnosis, predicting the prognosis, and monitoring the treatment.[1] Further, since cancer is a systemic disease, these technologies can offer personalized and accurate medicine, contributing to an improved therapeutic strategy, even after surgical interventions.

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Liquid biopsy is a minimally invasive procedure that allows the screening of circulating biomarkers in the body fluids (e.g., blood, saliva, and urine), being successfully used for cancer analysis in recent years.[2] Circulating biomarkers can be molecular alterations found in circulating free or tumoral DNA and RNA (cfDNA and cfRNA), circulating tumor cells (CTCs), and extracellular vesicles (EVs); and which may be of clinical importance for cancer progression or to influence treatment.[3,4] The greater clinical interest in liquid biopsy is due to the possibility of finding great correspondence of the molecular alterations found in the circulating biomarkers reflected in the tumor tissue, thus reducing the number of invasive biopsies to monitor tumor process.

This systematic review found Brazilian publications in cancer research on circulating nucleic acids (CNAs) based on the bibliographic search from MEDLINE-PUBMED and EMBASE databases. The review focused on CNAs (cfDNA and cfRNA), since it is the most commonly studied area in liquid biopsy worldwide and approved commercial tests are available for diagnostic and prognostic investigation. As result of the search

strategy, 28 Brazilian studies on translational research met the eligibility criteria and were systematically described in this review.

Methods

Databases

The bibliographic search was performed in July 2020 in two databases: MEDLINE–PUBMED and EMBASE. The search strategy mixed generic terms, keywords, and index terms (MeSH [Medical Subject Headings] major topics, subheadings, and terms), subdivided into two synonymous term boxes (the first with terms about "circulating nucleic acids," and the second with terms about "cancer") and finally, a third restrictive search box for affiliation (Brazil OR Brasil). There were no restrictions on language and publication date. Regarding publications' variations (research articles, reviews, editorials, and abstracts), the search in the EMBASE database was restricted to research articles. Boolean operators (OR and AND) were used as connectives between searches. Lilacs and Cochrane databases and SciELO repository were consulted, but since no return from the search was obtained, they were excluded.

Inclusion and exclusion criteria

Based on the inclusion and exclusion criteria, two authors (M.C.J. and G.A.) independently selected the articles for full analysis. Review articles, editorials, abstracts, exclusive works on cell culture, trials, and articles of which the first and last authors were not Brazilian were excluded. The final two lists were compared and evaluated by a third author (L.D.).

Results

The initial search returned 326 articles, 28 of which were eligible according to the criteria selected for this study (Figure 1). To describe the articles, they were systematically reviewed and grouped by theme (Table 1). In 26/28 studies the plasma cfDNA was assessed, in 3/28 the serum cfDNA, in 2/28 the plasma RNA (1 microRNA and 1 with both microRNA and mRNA), in 1/28 cfDNA gastric wash, and in 2/28 urine cfDNA (Figure 2). Five studies included more than one fluid in their analyses. Brazilian regions were represented by São Paulo (SP), Rio de Janeiro (RJ), Alagoas (AL), and Paraná (PR). All these Brazilian studies will be presented by theme, as shown below.

cfDNA and blood protein interactions

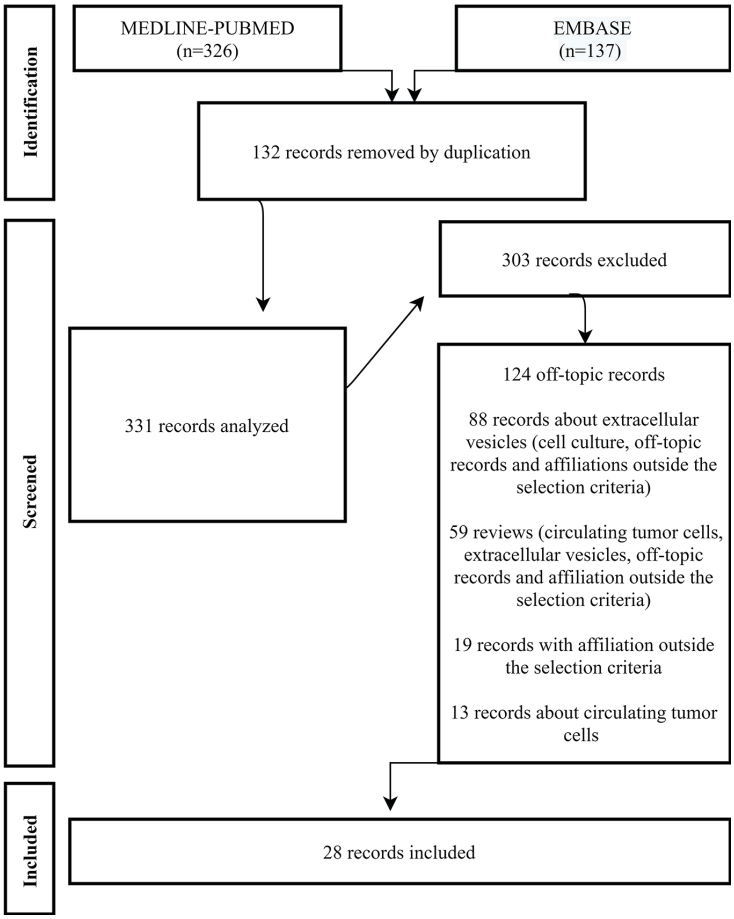
The first "made in Brazil" publication regarding cfDNA was a case report of a 65-year-old female diagnosed with small cell lung cancer in RJ.[5,6] Blood sample was collected before chemotherapy to obtain plasma cfDNA. The authors detected the formation of p53 protein-cfDNA complexes by electrophoretic-mobility shift and immunoblotting assays.

Quantification of cfDNA

Levels of CNAs in biofluids can be used to monitor minimal residual disease, relapses, and the response to therapy in oncological conditions.[7] The first Brazilian study evaluating plasma cfDNA quantity was conducted by Alves et al. in RJ using polymerase chain reaction (PCR) amplification of Long Interspersed Element-1 (LINE-1 or L1) and K562 DNA quantification standard to estimate the quantity of PCR products from glioma patients.[8] The authors suggested that the transposable elements L1 are usually released in body fluid, as they observed a greater amount of L1 PCR products in glioma patients (25 to 300μg/μL) compared to healthy controls (150μg/μL). Also in RJ, by real-time PCR-based cfDNA quantification, Machado et al. detected higher plasma cfDNA levels in pediatric patients with Burkitt lymphoma and diffuse large B-cell lymphoma at diagnosis compared to controls, and significantly decreased levels after therapy.[9]

In SP, three research groups have evaluated the cfDNA quantification in plasma from prostate cancer patients using different assays. Moreno et al. quantified plasma cfDNA using nanotechnology (Nanovue™-NV) compared with spectrophotometry (GeneQuant®).[10] The authors reported large variations values and no correlation between methods, concluding that nanotechnology was not reproducible to evaluate plasma DNA. Delgado et al. analyzed plasma cfDNA using GeneQuant® and real-time PCR at diagnosis, 3 and 6 months later.[11] The authors observed that at 3 and 6 months after diagnosis, 73.7% and 100% of the cfDNA fragments were, respectively, released from the apoptotic source (based on Alu repeat, 115-bp represented total cfDNA and 247-bp represented cfDNA from the non-apoptotic). Wroclawski et al. quantified plasma cfDNA using the PicoGreen assay and compared patients with controls (negative prostate biopsies).[12] Samples were drawn after biopsy and before treatment every 3 months during the 2-year period. The results revealed higher cfDNA concentration in patients compared to controls, and also, plasma cfDNA levels above 140 ng/mL

Figure 1. Flow diagram of the analyzed records



Authorship: The authors (2020).

Table 1. Summary of Brazilian studies on circulating nucleic acids that were included

Type of analysis	Cancer type	Biofluid	Methodology	Brazilian region (State)	References
cfDNA and blood proteins interactions	Small cell lung cancer (SCLC)	Plasma	Electrophoretic-mobility shift assay and immunoblotting	RJ	Kawamura et al., (1999, 2006)**5,6**
Quantification	Glioma	Plasma	PCR amplification	RJ	Alves et al., (2000)**8**
Quantification	Pediatric lymphoma	Plasma	Quantitative real-time PCR	RJ	Machado et al., (2010)**9**
Quantification	Prostate cancer	Plasma	Nanovue™-NV and GeneQuant	SP	Moreno et al., (2013)**10**
Quantification	Prostate cancer	Plasma	GeneQuant and quantitative real-time PCR	SP	Delgado et al., (2013)**11**
Quantification	Prostate cancer	Plasma	PicoGreen	SP	Wroclawski et al., (2013)**12**
Quantification	Colorectal cancer	Serum	Quantitative real-time PCR	AL	Filho et al., (2013)**13**
Quantification	Urothelial carcinoma of the bladder	Plasma	GeneQuant	SP	Almeida et al., (2016)**14**

Table 1 (cont.). Summary of Brazilian studies on circulating nucleic acids that were included

Type of analysis	Cancer type	Biofluid	Methodology	Brazilian region (State)	References
Quantification	Glioblastoma (GBM)	Serum	Qubit	RJ	Faria et al., (2018)**15**
Quantification	Gastric cancer	Plasma	GeneQuant	SP	Normando et al., (2018)**16**
Quantification	Bladder cancer	Plasma	Z-Scan	SP	Alves et al., (2019)**17**
Quantification	Metastatic ovarian carcinoma	Plasma	Quantitative real-time PCR	SP	Alves et al., (2020)**18**
Somatic mutations	Rectal adenocarcinoma	Plasma	ddPCR and NGS (SOLiD 4.0 platform)	SP	Carpinetti et al., (2015)**21**
Somatic mutations	Sarcoma	Plasma	ddPCR	SP	Ferreira et al., (2016)**22**
Somatic mutations	Non-small cell lung cancer (NSCLC)	Plasma	ddPCR	SP	Knebel et al., (2017)**23**
Somatic mutations	Colorectal cancer	Plasma	NGS (Ion Proton platform)	SP	Barros et al., (2018)**24**
Somatic mutations	Impalpable breast lesions	Plasma	Sanger sequencing	RJ	Delmonico et al., (2019)**25**
Somatic mutations	Colorectal cancer	Plasma	ddPCR and KRAS Screening Kit	SP	Knebel et al., (2019)**26**
Somatic mutations	Gastric adenocarcinoma	Plasma and gastric washes	NGS (Ion Proton platform)	SP	Pizzi et al., (2019)**27**
Somatic mutations	Wilms tumor	Plasma and urine	NGS (Ion Proton platform)	SP	Miguez et al., (2020)**28**
Genotyping and microsatellites assays	Head and neck squamous cell carcinomas (HNSCC)	Plasma	Automated sequencing	SP	Nunes et al., (2001)**30**
Genotyping and microsatellites assays	Breast cancer	Plasma and urine	PCR amplification	SP	Pinto et al., (2010)**31**
Genotyping and microsatellites assays	Non-small cell lung cancer (NSCLC) and Small cell lung cancer (SCLC)	Plasma and serum	PCR amplification	RJ	Cabral et al., (2010)**33**
Genotyping and microsatellites assays	Glioma	Plasma and serum	PCR amplification	RJ	Silva et al., (2013)**34**
Circulating RNA analysis	Breast cancer and breast lesions	Plasma	qRT-PCR and Artificial Neural Network (ANN)	SP	Pezuk et al., (2017)**35**
Circulating RNA analysis	Prostate cancer	Plasma	qRT-PCR	PR	Souza et al., (2017)**36**
DNA Methylation	Impalpable breast lesions	Plasma	Methylation-specific PCR	RJ	Delmonico et al., (2020)**38**

Abbreviations: AL, Alagoas; PR, Paraná; RJ, Rio de Janeiro; SP, São Paulo; PR, Paraná; ddPCR, Droplet Digital PCR; NGS, Next-Generation Sequencing.

showed a sensitivity of 66.2 % and a specificity of 87.9 % for prostate cancer diagnosis.

In AL, Filho et al. quantified the serum cfDNA by amplification of Alu repeats using quantitative real-time PCR from colorectal cancer (CRC) patients.[13] Increased levels of Alu115-qPCR and Alu247-qPCR were found in CRC non-operated patients compared to other groups, suggesting Alu-qPCR biomarkers to monitor CRC postoperative patients.

In SP, Almeida et al. quantified the plasma cfDNA levels from patients with urothelial carcinoma of the bladder using GeneQuant®.[14] Plasma was obtained before treatment and in different time points after the transurethral resection. The authors observed significant and higher mean plasma cfDNA concentrations in patients with microscopic hematuria compared to those without this condition, providing additional data for the bladder cancer prognosis.

Faria et al. compared serum cfDNA levels from patients with brain tumors eligible for intranasal delivery of perillyl alcohol (ITN-POH) with healthy controls, in RJ.[15] Samples were collected before treatment and periodically during ITN-POH, and cfDNA was quantified using Qubit®. Serum cfDNA levels from patients before ITN-POH administration were significantly higher than those found in controls. Patients who survived less than 6 months compared to those with longer survival showed higher serum cfDNA levels. Also, the ITN-POH therapy reduced cfDNA from patients, suggesting serum cfDNA to monitor brain tumor patients under ITN-POH treatment.

In SP, Normando et al. evaluated plasma cfDNA using GeneQuant® from patients diagnosed with gastric cancer.[16] Samples were collected before chemotherapy and every 3 months. The findings showed higher levels of plasma cfDNA in patients compared to controls. Also, patients who exhibited higher ctDNA levels at 3 months after chemotherapy presented a significantly lower disease-free survival (DFS), suggesting ctDNA levels to monitor disease recurrence after treatment.

Alves et al. used the Z-Scan technique to analyze cfDNA concentrations in plasma from patients diagnosed with bladder cancer, in SP, and differences in cfDNA concentrations during treatment were reported.[17]

Alves et al. analyzed the plasma ctDNA levels from patients with metastatic ovarian carcinoma, in SP.[18] Samples were drawn before treatment and afterwards each month for 6 months. Patients showing increased ctDNA from baseline after the first cycle of chemotherapy responded better to the treatment and had significant improvement in the DFS.

All of these cfDNA quantification aforementioned studies are consistent with those found in studies outside Brazil.[19,20]

Somatic mutations in cfDNA

Genetic mutations found in cfDNA can correspond to those found in the tumor tissue, thus reducing the need for invasive biopsies to monitor tumor dynamics. In SP, Carpinetti et al. analyzed the chromosomal rearrangements in the plasma cfDNA from patients with rectal adenocarcinoma.[21] Samples were obtained at diagnosis, during neoadjuvant chemoradiotherapy (nCRT), and during follow-up. cfDNA was detected using a QX200 ddPCR (Droplet Digital PCR) system and nested PCR assay. For chromosomal rearrangement assays, the DNA was sequenced in Next Generation Sequencing (NGS) platform. At least two distinct somatic rearrangements were found in the tumor samples from each patient that the authors later used to correlate with the plasma ctDNA. The authors observed that all patients showed undetectable or reduced ctDNA levels for the biomarkers after nCRT.

Also in SP, Ferreira et al. described a translocation t(11;22)(p13;q12) of the *EWS-WT1* gene fusion in plasma DNA using a QX200™ ddPCR assay from a 26-year-old male with a desmoplastic small round cell tumor (DSRCT).[22] Samples were collected periodically 3 years after surgery (4 time points) and no translocation was detected in the post-treatment, correspondent to the favorable clinical outcome.

In SP, Knebel et al. monitored *EGFR* gene mutations in plasma cfDNA from a 53-year-old woman diagnosed with non-SCLC.[23] Samples were isolated monthly after therapy (erlotinib and osimertinib) and *EGFR* mutations were evaluated by QX200™ ddPCR assays. After erlotinib progression, the authors found 3598 and 1564 copies/reaction for *EGFR*-exon19del and *EGFR*-T790M mutations, respectively. Both mutations were undetectable two weeks after osimertinib therapy. After 4 months, both mutations showed increased copies/reaction, suggesting acquired resistance (AR) to osimertinib, especially the *EGFR*-exon19del mutation.

Also in SP, Barros et al. investigated a panel of hotspot mutations in the plasma ctDNA from a 57-year-old man with metastatic CRC by NGS technology.[24] Samples were drawn before treatment and 5 times after surgery and chemotherapy. The authors detected a high frequency of *KRAS* (p.Gly12Val) and *TP53* (p.Arg175His) mutations in the first plasma sample. Both mutations frequencies were reduced 1 month after chemotherapy, but increased after the third month of treatment, showing ctDNA as a sensitive tool to monitor treatment response.

Delmonico et al. investigated the mutational profile of *PIK3CA* (exon 9 and 20), *TP53* (exon 5-8), and *CDKN2A* (exon 1, 2a, and 3) genes by Sanger sequencing in the plasma cfDNA from patients with impalpable breast lesions (BI-RADS 3 and 4), in RJ.[25] Samples were obtained before surgery and 5% and 24% of mutations were found in the cfDNA of women with benign lesions and malignant lesions, respectively. cfDNA mutations were found mostly in the *TP53* gene, especially in women exhibiting malignant lesions.

Knebel et al., in SP, evaluated *KRAS* mutations in the plasma ctDNA from a 61-year-old man with advanced CRC using QX200™ ddPCR and a *KRAS* screening commercial assay.[26] Samples were analyzed for almost 2 years and no *KRAS* mutations were found at the beginning of the cetuximab-based chemotherapy, but reaching to 33.8% about 2 months later.

Also in SP, Pizzi et al. evaluated plasma and gastric washes to assess the *TP53* mutations in the cfDNA using NGS platform from patients with gastric adenocarcinoma.[27] Samples were collected at diagnosis and after treatment. The authors found *TP53* mutations in 15.2% of gastric washes, with concordance between tumor biopsies in 82.6% of cases at diagnosis. Regarding plasma cfDNA, *TP53* mutations were found in 13% of cases, being 80.4% in concordance with biopsy samples. Post-treatment analyses revealed that only 6 cases had *TP53* mutations in tumor biopsy and 2 of them showed detectable mutation in gastric washes or plasma. Thus, the authors suggested both fluids to monitor tumor mutations and treatment response.

Miguez et al. evaluated the somatic mutations using NGS platform in the cfDNA from plasma and urine of 6 female patients with Wilms tumor in SP.[28] Before treatment, 5 patients showed at least 1 somatic mutation in tumor samples and also in body fluids, being reduced after chemotherapy.

Microsatellite assays with cfDNA

Alterations in the microsatellite's sequences are common in several kinds of cancer due to the genome instability.[29] In SP, Nunes et al. evaluated plasma cfDNA from patients with head and neck squamous cell carcinomas (HNSCC) to investigate microsatellite instability (MSI) and loss of heterozygosity (LOH).[30] Loss of heterozygosity and/or MSI were found in 64% of the tumor biopsies in at least 1 locus, in which 29% of these alterations were also detected in the plasma cfDNA, especially from those with advanced clinical stages. In SP, Pinto et al. investigated the induction of alkylating agent-based chemotherapy (ACHT) on MSI, before and after treatment, by analyzing plasma and urine cfDNA from untreated patients diagnosed with breast cancer.[31] The authors found at least 1 MSI or LOH event in 80% of both plasma and urine cfDNA samples at 6 months, especially for BAT40 and BAT26 markers, suggesting that ACHT could induce MSI.

Genotyping with cfDNA

The glutathione S-transferase (GST) gene family encodes metabolic enzymes involved in xenobiotic detoxification and therefore the GST polymorphisms may influence its activities.[32] In RJ, Cabral et al. evaluated the *GSTM1* and *GSTT1* genotypes by PCR amplification from plasma/serum cfDNA of lung cancer patients.[33] Lung cancer risk was significantly higher in tobacco smoker patients carrying *GSTM1* and *GSTT1* null genotypes. Also in RJ, Silva et al. analyzed the influence of *GSTM1* and *GSTT1* polymorphisms in plasma/serum cfDNA in response to ITN-POH administration from patients with malignant glioma.[34] Significant difference was found between patients and controls for *GSTT1* deletion. Patients with *GSTT1* null genotype had significantly lower survival rates, showing the influence of GST polymorphisms.

Circulating RNA analysis

RNA families are especially involved in gene expression process. In SP, Pezuk et al. compared plasma miRNAs from breast tumor patients (Breast Imaging-Reporting and Data System - BI-RADS 5 or 6) and controls (BI-RADS 1 or 2) using miRNome array, qRT-PCR, and the Artificial Neural Network (ANN).[35] Plasma was obtained before mammography and biopsy. As a result, the authors found miRNAs differentially represented between groups, with 46 over- and 9 underrepresented in the patients' samples. Regarding ANN, the authors found 92.46%, 87.50%, and 94.59% of accuracy, sensitivity, and specificity, respectively, suggesting this approach as a complementary method to classify BI-RADS 4 lesions. In PR, Souza et al. using *in silico* analysis compared circulating mRNAs and microRNAs from prostate cancer tissues and surrounding normal tissues (SNT) from the Cancer Genome Atlas (TCGA) database, and validated the results using the RT-qPCR assay.[36] The authors observed 2,267 genes and 49 miRNAs with differential expression between tumor and normal tissue samples. Additionally, the expression of 2 genes (OR51E2 and SIM2) and 2 cfmiRNA (miR-200c and miR-200b) were significantly associated with prostate cancer.

Methylation signature in cfDNA

Epigenetic inactivation by DNA methylation of tumor suppressor genes is associated with various neoplastic diseases due to increased genetic

instability.[37] Therefore, the methylation status in the promoter region of crucial genes should be monitored to provide additional information on cancer development and progress. Delmonico et al. investigated plasma cfDNA from women with and without impalpable breast lesions to detect the epigenetic alterations and observed that *ATM* and *p14*^{ARF} showed higher methylation rates in samples from women with malignant lesions and older than 50 years.[38] Thus, the authors described the potential detection of epigenetic changes in liquid biopsy to monitor the impalpable breast lesions.

Discussion

Liquid biopsy is a significant area of cancer studies with great prominence over the years, since it requires a minimally invasive procedure to track alterations in circulating biomarkers that potentially contribute to diagnosis, predict prognosis, and monitor response to treatment.[2,3] This systematic review described 28 Brazilian studies on circulating nucleic acids (CNAs) from cancer patients' samples with plasma and cfDNA the most investigated biological material, in which investigations included quantification (11 studies), followed by somatic mutation (8 studies), RNA expression (2 studies), genotyping, microsatellites, blood protein interaction (2 studies each one), and methylation (1 study).

Brazilian cfDNA quantification studies reported higher cfDNA levels in cancer patients compared to healthy volunteers. Decreases in cfDNA levels after treatment were also reported, being all these findings consistent with those found in studies outside Brazil.[19,20] Somatic mutations in the cfDNA samples reported in this review revealed molecular signatures that may be associated with tumor development, progression, and resistance to treatment. Additionally, Brazilian RNA-based studies reported differential expression between samples from cancer patients and healthy controls. RNAs are involved in multiple biological processes with a wide range of functions, in which dysregulations in RNA expression patterns are associated with pathological conditions. Circulating RNAs are usually found with differential expression profiles in cancer patients and may contribute to initiation and progression of oncological processes.[39]

Interesting to note, the studies were concentrated in the Southeastern region of Brazil, especially in São Paulo and Rio de Janeiro (17/28 and 9/28, respectively), followed by one study in Alagoas (Northeastern region of Brazil) and another one in Paraná (South region of the country). These regional discrepancies may reflect the differential distribution of financial resources for scientific research.

Conclusions

Liquid biopsy is a promising investigation area in cancer screening. In summary, this systematic review described 28 Brazilian studies on cfDNA and cfRNA from cancer patients' biofluids using different methodological assays, being cfDNA the most commonly analyzed CNAs. More studies on CNAs in cancer patients are needed to complement the clinical practice investigations in Brazil.

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