

Circulating cell-free-DNA concentration is a good biomarker for diagnosis of colorectal cancer in Mexican patients

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ABSTRACT

Colorectal cancer (CRC) is the third most common cancer in the world. Overall survival is related to the clinical stage: more advanced stages show lower survival rates; therefore, they need to be monitored regularly with new, less invasive and more specific biomarkers. The concentration and integrity index of circulating cell-free DNA (ccfDNA) have been proposed as potential diagnostic and prognostic biomarkers for CRC, however, inconsistent results are still observed in different reports. Here we analyze these potential CRC biomarkers in a Mexican population. In this study, 124 patients with sporadic CRC and 37 healthy individuals were examined as a reference group. The ccfDNA was isolated from the plasma samples of all included subjects. The ccfDNA concentration was determined by fluorometry and the integrity index (ALU247/ALU115 ratio) by quantitative PCR amplification (qPCR) of ALU sequences. The results show that ccfDNA concentration was higher in CRC patients than in the reference group ($P=0.001$). The integrity index showed no significant differences between these groups ($P=0.258$), except for histological type ($P=0.012$). A higher ccfDNA concentration was also associated with patients younger than 50 years ($P=0.030$). The ccfDNA concentration showed significant discriminatory power (AUC: 0.854, C.I.: 0.78-0.92, $P=0.001$) between patients and the reference group and between tumor-node-metastasis (TNM) stages. In conclusion, ccfDNA concentration proves to be a good diagnostic biomarker for CRC patients, whereas the integrity index did not show diagnostic utility.

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Introduction

Colorectal cancer (CRC) is a worldwide health problem. In Mexico, 14,901 new cases of CRC were diagnosed in 2020, which means an incidence rate of 10.6/100,000 with a mortality rate of 5.4/100,000 inhabitants (1). Currently, CRC is the third most frequent cancer in the countries of the Americas (2), and the incidence and mortality rates worldwide were 19.5/100,000 and 9/100,000, respectively (3).

The overall survival of CRC tends to be reduced because most CRC cases are diagnosed at advanced stages, losing the opportunity to access timely treatment. About 50% of CRC patients die as a result of distant metastases

(4). The overall 5-year survival rate of CRC is 64.7%; however, survival is better in localized disease (90.6%) than in regional and metastatic disease (72.2% and 14%, respectively) (5). CRC is a silent disease characterized by the absence of early symptoms, so most diagnoses are made in the late stages of the disease (6). Available diagnostic methods such as colonoscopy and biopsy are the gold standard in the diagnosis of CRC; however, they are highly invasive processes, uncomfortable for patients and involve potential complications that limit the number of samples to be obtained from each patient; for all these reasons, their usefulness has been described as limited (7, 8). In these circumstances, there is an urgent need to find non-invasive and safe biomarkers that allow earlier diagnosis.

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In recent years, the study of circulating cell-free DNA (ccfDNA) has emerged as a liquid biopsy technique, which has several advantages over conventional biopsy, including its non-invasive nature, its ease of sample collection, which can be obtained repeatedly, and the detection of tumor heterogeneity, which is difficult to obtain in a biopsy of tumor tissue (9). ccfDNA has been found in different body fluids such as blood, urine, cerebrospinal fluid, saliva and pleural fluid (10). The ccfDNA is released into body fluids by two processes: active release and cell death (9). In active release processes, cells exchange genetic material, proteins and metabolites within microvesicles, exosomes and virtosomes, although these mechanisms are still poorly understood (11). In cell death processes, ccfDNA is released by apoptosis and necrosis. By apoptosis, DNA is degraded into regular fragments of 160-180 base pairs (bp) by caspase-activated DNase (CAD), whereas in necrosis DNA fragments vary from 200 to 21,000 bp (12, 13). In cancer patients, ccfDNA results from apoptosis and necrosis of tumor cells, whereas in healthy individuals, ccfDNA results mainly from apoptosis (12).

The ccfDNA of tumor cells contains valuable information about its concentration and integrity, which has been related to tumor burden; however, ccfDNA can also be used to determine specific mutations in oncogenes and tumor suppressor genes, insertions, deletions, translocations, copy number variation, microsatellite instability, and CpG island methylation of some regions (9).

Numerous studies have been conducted in order to evaluate the potential application of ccfDNA as a biomarker in cancer (14–21). Several reports find that ccfDNA concentration is increased in cancer patients compared to control groups; however, there are still several discrepancies on the concentration of ccfDNA and principally on the integrity index (cfDI) among different groups of patients (22, 23). Regarding the ccfDNA concentration, most of the inconsistencies are based on the variability of the quantified results (24), and some other reports still show variable specificity and sensitivity values (23–25). On the other hand, in studies of cfDI, it has been found that gender can affect cfDI values, although other authors insist that this is only a part of the inconsistencies (23). cfDI is usually obtained by amplification of short sequences within long sequences (direct ratio of ccfDNA concentration), from different genes such as *ALU*, *APP*, *ACTB*, *LINE1*, *HER2*, *BCAS*, *MYC* and *PI3K* (22), from which a cfDI ratio (between 0 and 1) should be obtained (22, 26–28). Small values of cfDI means higher DNA fragmentation, while, high values mean less fragmentation, however, in CRC both have been reported (29).

To better understand the value of ccfDNA concentration and cfDI, this study aims to evaluate its clinical utility as a diagnostic biomarker in Mexican patients with CRC.

Materials and Methods

Study population

The study was approved by the Ethical Committee 1305 (R-2017-1305-01) of West Biomedical Research Center, IMSS, and conducted according to national and international ethical standards. All the participants signed the informed consent for participation in this study. Between 2017 to 2020, 124 patients clinically diagnosed and histologically confirmed as sporadic colorectal adenocarcinoma were

studied according to the Clinical Practice Guidelines for colon and rectal cancer and the clinicopathological criteria of the Specialty Hospital of West National Medical Center of the Mexican Institute of Social Security (IMSS) in Guadalajara, Mexico. Tumor staging and pathologic grading were performed according to the tumor-node-metastasis (TNM) classification. The reference group included 37 unrelated healthy individuals and was not matched for age and sex with the patient group. Exclusion criteria for the patient and reference groups were: no family history of CRC or known hereditary cancer syndromes, infectious diseases, autoimmune diseases, inflammatory processes and pregnancy at the time of blood sampling.

Personal data, including sex, age, smoking and drinking habits, and clinical and pathologic characteristics of the patients were obtained from hospital records.

Blood specimen collection and processing

10 mL of peripheral blood was collected in PAXgene® Blood ccfDNA tubes (PreAnalytix GmbH, Feldbachstrasse). The plasma was separated by two centrifugation steps: 1900 x g for 15 min; the plasma was then transferred to a new tube and centrifuged at 1500 x g for 10 min to remove cell debris. The plasma was stored in 1 mL aliquots at -80 °C until further analysis.

Purification and measurement of ccfDNA concentration

1 mL of plasma was purified using the QIAamp circulating nucleic acid kit (QIAGEN Science, Inc, USA) according to the manufacturer's instructions. Total ccfDNA concentration was measured in a Qubit® 3.0 fluorometer (Invitrogen, Carlsbad, CA), using the Qubit® dsDNA HS assay kit (Life Technologies Corporation, Eugene, USA).

cfDI by quantitative PCR

To measure the concentration and determine the cfDI of ccfDNA fragments, amplification of ALU115 and ALU247 sequences was performed by qPCR. ALU115 amplifies short and long DNA fragments (derived from apoptosis and necrosis, respectively), whereas ALU247 amplifies only the long fragments derived from necrosis. For ALU115 amplification, the next primers were used: forward: 5'- TGAGGTCAGGAGTTCGAG -3' and reverse 5'- CCCGAGTAGCTGGGATTACA -3'. For ALU247 amplification: forward 5'- GTGGCTCACGCC-TGTAATC -3' and reverse 5'- CAGGCTGGAGTGCAG-TGG -3' (30). The qPCR was achieved on the instrument Light Cycler® 96 (Roche, Mannheim Germany) with the FastStart SYBR Green Master Kit (Roche, Mannheim Germany); the qPCR reaction was performed in 10 μ L of a total volume containing: 5 μ L 1 X FastStart SYBR Green Master Mix, 0.2 μ L of each primer (25 μ M), 3.6 μ L H₂O PCR grade and 1 μ L of the ccfDNA sample. Conditions of the qPCR reaction were: preincubation at 95 °C for 600 s, denaturation at 95 °C for 30 s, annealing at 65 °C for 30 s, and extension at 72 °C for 30 s for 35 cycles, and cooling at 40 °C for 10 s. All reactions were analyzed by duplicates and a negative reaction control was included in each experiment. The absolute amount of ccfDNA (ALU115 and ALU247) was determined from a standard genomic DNA curve with an initial concentration of 1000 ng to 1 ng. cfDI was determined by the ALU247/ALU115 ratio.

Statistical analysis

The Kolmogorov-Smirnov test was significant, so nonparametric statistical tests were used. The results of ccfDNA and cfDI concentrations are shown as medians with interquartile range (IQR, 25th to 75th percentile). Mann-Whitney U test was performed to compare two groups and Kruskal-Wallis to compare more than two groups. Evaluation of ccfDNA as a diagnostic biomarker was performed by receiver operating characteristic (ROC) curve analysis. All analyses were performed in SPSS 25.0 and graphs in GraphPad Prism 8.

Results

Characteristics of the subjects included in the study

Table 1 shows the demographic, clinical and pathological characteristics of the patients with CRC. The mean age in the CRC group was 57 years (± 12.38); 72.6% of patients with CRC were in advanced stages (TNM III+IV); 67.8% of the tumors were located in the rectum; 82.3% of the patients presented the no mucinous adenocarcinoma, and the adenocarcinoma with moderate tumor differentiation was more frequently observed.

ccfDNA concentration and cfDI between CRC and reference groups

Results of the ccfDNA concentration in the CRC patients and reference group are shown in Table 2 and Figure 1. In CRC patients, a significantly higher ccfDNA concentration was observed in comparison with the reference group ($P=0.001$) (Table 2), while no significant differences were observed among the TNM stages in patients with CRC ($P=0.274$) (Figure 1a). Amplification by qPCR using ALU115 or ALU247 primers did not show differences between the CRC patients and the reference group. Consequently, the cfDI showed no statistical difference between CRC patients and the reference group ($P=0.258$) (Figure 1b).

Effect of clinicopathological features on ccfDNA concentration and cfDI

Univariate analysis of the effect of age on ccfDNA concentration showed that patients under 50 years had a higher ccfDNA concentration than older patients ($P=0.030$) (Table 2). The cfDI does not evidence a significant difference among the TNM stages in the CRC patients ($P=0.483$) (Figure 1b). Univariate analysis of the effect of CRC histological type on the amount of ccfDNA amplified by ALU115 qPCR showed a significant difference in patients with mucinous adenocarcinoma compared with

Table 1. Demographic and clinical characteristics of CRC patients.

	CRC patients n=124 (100%)
Age in years	
Mean years old (\pm S.D)	57 (± 12.38)
<50	27 (21.8)
>50	97 (78.2)
Sex	
Female	53 (42.8)
Male	71 (57.2)
Diabetes Mellitus positive	
	21 (16.9)
Smoking status positive	
	63 (50.8)
Drinking status positive	
	70 (56.4)
Clinical stage TNM	
I+II	34 (27.4)
III	48 (38.7)
IV	42 (33.9)
Tumor location	
Right Colon	9 (7.2)
Left Colon	31 (25.0)
Rectum	84 (67.8)
Histological type	
No mucinous adenocarcinoma	102 (82.3)
Mucinous adenocarcinoma	22 (17.7)
Tumor differentiation	
Well-differentiated	15 (12.1)
Moderate	100 (80.6)
Poor	9 (7.3)

Where CRC: colorectal cancer; TNM: Tumor-node-metastasis.

CRC: Colorectal cancer; S.D.: Standard deviation, TNM: Tumor node metastasis.

the other histological type ($P=0.012$); such a difference was not observed when amplified by fluorometry. In these same patients (<50 years), we observed a significantly higher concentration of ccfDNA only in TNM stage IV ($P=0.041$) (data not shown).

ccfDNA concentration and cfDI by ROC analysis

ROC analysis lets us determine the discriminatory power of the ccfDNA concentration and the cfDI. The ROC analysis of the ccfDNA concentration achieved by fluorometry in 124 CRC patients and 37 healthy individuals showed a statistically significant difference, (AUC: 0.854, C.I.: 0.78-0.92, $P=0.001$) between these two groups (Figure 2). Regarding the cfDI, we did not observe the sta-

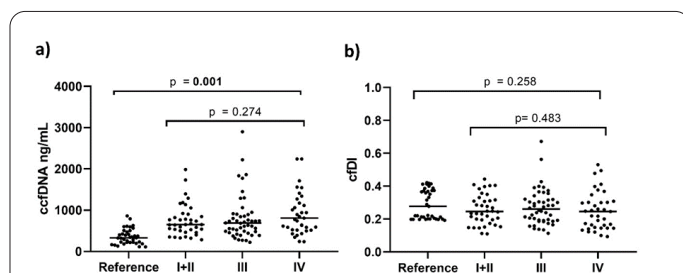


Figure 1. a) Comparison of ccfDNA concentration in CRC patients and the reference group ($P=0.001$), and between TNM stages ($P=0.274$). b) Comparison of cfDI in CRC patients with the reference group ($P=0.258$), and between TNM stages ($P=0.483$).

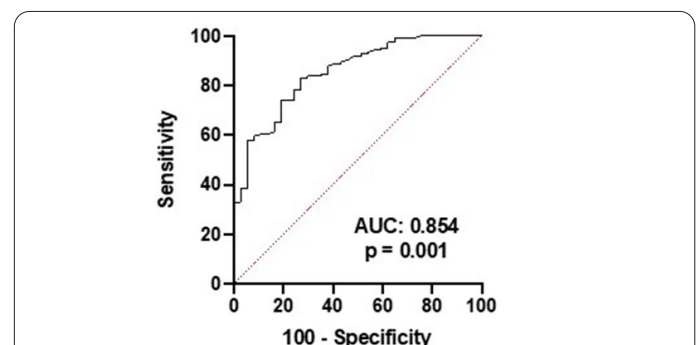


Figure 2. ROC curve of ccfDNA concentration in the CRC patients. The reference group was used as a comparison.

Table 2. Concentration and cfDI of ccfDNA in CRC patients and reference group, and its association with clinical and pathological characteristics.

	Concentration ccfDNA by fluorometry ng/mL Median (IQR)	<i>P</i> Value	ALU115 by qPCR ng/mL Median (IQR)	<i>P</i> Value	ALU247 by qPCR ng/mL Median (IQR)	<i>P</i> Value	cfDI of ccfDNA ALU247/ALU115 Median (IQR)	<i>P</i> Value
CRC vs. reference group								
CRC	700 (499-1042)	0.001	99 (57-206)	0.518	23 (13-50)	0.676	0.24 (0.17- 0.31)	0.258
Reference	330 (220-487)		87 (51-142)		26 (10-54)		0.27 (0.20-0.37)	
CRC characteristics								
Age in years								
<50	844 (557-1309)	0.030	85 (37-227)	0.732	18 (10-61)	0.547	0.23 (0.16-0.30)	0.678
>50	652 (465-920)		99 (61-206)		23 (14-50)		0.25 (0.18-0.33)	
Sex								
Men	733 (520-1089)	0.826	107 (57-211)	0.531	23 (13-56)	0.777	0.24 (0.16-0.30)	0.138
Female	669 (492-993)		86 (57-185)		23 (13-48)		0.26 (0.19-0.35)	
TNM stage								
I +II	652(502-922)	0.274	105(48-141)	0.432	25(13-36)	0.783	0.24(0.19-0.33)	0.483
III	688(460-899)		90(47-229)		21(13-58)		0.26(0.19-0.31)	
IV	808(521-1307)		121(65-229)		21(14-62)		0.24(0.14-0.30)	
Tumor location								
Colon	621 (452-894)	0.116	84 (39-151)	0.112	22 (10 -41)	0.151	0.25 (0.17-0.31)	0.940
Rectum	736 (518-1062)		104 (58-242)		25 (14-61)		0.24 (0.18-0.32)	
Histological type								
No mucinous adenocarcinoma	722 (476-1060)	0.679	89 (52-165)	0.012	22 (13-45)	0.119	0.25 (0.17-0.35)	0.113
Mucinous adenocarcinoma	638 (501-866)		171 (77-340)		36 (16-69)		0.21 (0.18-0.27)	

ccfDNA: circulating cell free DNA; qPCR: quantitative PCR; IQR: interquartile range; cfDI: circulating cell-free DNA integrity index; CRC: colorectal cancer, TNM: tumor node metastasis.

Table 3. Results of studies performed in CRC patients on concentration and cfDI of ccfDNA and their associations with clinical and biological features.

Study	Concentration of ccfDNA			Integrity index of ccfDNA				
	CRC vs:		Associations with:	Biomarker diagnostic	CRC vs:		Associations with:	Biomarker diagnostic
Umetani et al 2006 (30)	Control group	P=0.001	TNM stage	AUC=0.75*	Control group	P=0.001	TNM stage	AUC=0.78*
Schwarzenbach et al 2008 (37)	Reference group	P=0.001	Survival	ND	ND			ND
Mead et al 2011 (26)	Reference group	P=0.001		AUC=0.75*	Reference group	P<0.001		AUC=0.77*
Agostini et al 2011 (36)	Control group	P<0.001		AUC=0.92*	Control group	P<0.001		AUC=0.97*
da Silva et al 2013 (48)	Control group	P<0.05		ND	Control group	P=0.019		ND
Heitzer et al 2013 (38)	TNM-IV vs control	P=0.0001		ND	ND			ND
Yoruker et al 2015 (29)	ND			ND	Control group	P>0.05		ND
El- Gayar et al 2016 (28)	Bening vs control	P=0.002	Tumor grade	AUC=0.73*	Bening vs control	P<0.001	Tumor grade, metastasis	AUC=0.90*
Agah et al 2017 (39)	Control group	P<0.0001	Differentiation, lymphovascular invasion	AUC=0.87*	ND			ND
Bedin et al 2017 (43)	Control group	P=0.0001	TNM stage, survival	AUC=0.70*	Control group	P>0.05	TNM stage	AUC=0.76*
Kloten et al 2017 (13)	Reference group	P=0.001	Positive nodes, metastasis	ND	ND			ND
Zhong et al 2020 (49)	Control group	P<0.05	TNM stage, differentiation	AUC=0.77*	Control group	P<0.05	TNM stage, differentiation	AUC=0.80*
Saucedo-Sariñana et al 2022 (this study)	Reference	P=0.001	TNM stage, age	AUC=0.85*	Reference	P=0.258	Histological type	AUC=0.60

ND: non described; AUC: area under the curve; TNM: tumor node metastasis; CRC: colorectal cancer. Values in bold and marked with*: were statistically significant.

tistical difference between patients vs. the reference group (AUC=0.601, C.I.:0.50-0.70, P=0.063).

ROC analysis of the ccfDNA concentration between TNM stages showed a significant difference in TNM I+II (AUC: 0.829, C.I.: 0.73-0.92, P=0.001), TNM III (AUC: 0.834, C.I.: 0.75-0.91, P=0.001) and TNM IV stage (AUC: 0.896, C.I.: 0.83-0.96, P=0.001). (data not shown).

Discussion

Currently, liquid biopsies are highly reliable and easy to analyze diagnostic tools used for the evaluation of benign and malignant tumors. ccfDNA is released from dead cells by the normal process of apoptosis, but also include DNA released from tumor cells; therefore, the total concentration of ccfDNA is increased in several cancer types, including breast (31), lung (32), gastric (33), urologic (34), head and neck (35) and CRC (17, 26, 30, 36).

In this study, the ccfDNA concentration obtained by fluorometry was significantly higher in CRC patients than in the reference group (P=0.001), which is congruent with previous reports (13, 28, 30, 37–39) (Table 3).

On the other hand, the association analysis between ccfDNA concentration and clinical characteristics of the patients showed a significantly higher ccfDNA concentration in the group of patients younger than 50 years. This interesting finding seems to contrast with the well-accepted idea that, in older people, a higher amount of ccfDNA is released from different cell types in which catabolic processes such as cell lysis and apoptosis predominate.

Notably, and for several years now, different studies report that an increasing number of patients (1.4%/year) are diagnosed with early-onset CRC (under 50 years of age), while the incidence has decreased (3.1%/year) in people over 50 years of age (40). Yeo H. et al. in an extensive review including 369,796 patients with CRC found that early-onset CRC is more prevalent in black (14.6%) and hispanic (14.7%) patients, presenting with aggressive histology (high-grade morphology, signet ring cells, signs of perineural invasion and mucinous carcinomas), and frequently with distant disease (40, 41).

It is reasonable to assume that the increase in ccfDNA released by cancer cells in younger individuals, as observed in our study (by fluorometry), is the result of increased tumor activity, which would be congruent with the presence of early-onset CRC (diagnosed before the age of 50 years) and with more aggressive tumor characteristics. This assumption would imply that the amount of ccfDNA is not only related to the size of the tumor but also its aggressiveness.

In patients younger than 50 years, we also observed an association between ccfDNA concentration and more advanced TNM stages. Several previous reports have noted this association between ccfDNA concentration and TNM stages (30, 39, 42). Taken together, these findings are consistent with our presumption that a more severe pathologic process is at play in these patients.

As a diagnostic biomarker, ccfDNA concentration in CRC obtained by fluorometry was able to distinguish patients and individuals from the reference group with good discriminatory power (Figure 2). Although these results are very promising, we should remember that ccfDNA concentration may be increased in conditions other than cancer, such as stroke, inflammation, autoimmune di-

seases, exercise and trauma (27, 43). Therefore, the diagnostic ability of ccfDNA could probably be improved in combination with some other genetic biomarker (43).

Regarding cfDI, no significant differences were found between CRC patients and individuals in the reference group (P=0.258), except in the case of patients with mucinous adenocarcinoma (ALU115 amplification by qPCR) where a significant difference was observed (P=0.012). This histological type of CRC has a worse prognosis and has previously been associated with female sex, proximal colon location, advanced TNM stage, pericolic lymph node infiltration and larger tumors (44, 45). Mucinous adenocarcinoma has a substantial amount of extracellular mucin; this layer confers protection against the immune system and chemotherapy drugs (46, 47). Some other features of mucinous adenocarcinoma include microsatellite instability, mutations in *BRAF* and *KRAS*, a CpG island methylating phenotype, and more aggressive properties (47) with a worse prognosis in rectal cancer than in colon cancer (46).

Several previous reports found no significant difference in cfDI between patients with CRC (29, 43) (Table 3) or other cancer types (48, 49), versus control individuals; however, other authors state that there are clear differences (17, 26, 28, 30, 36, 48, 49). Such discrepancies may be due to the degree of fragmentation of ccfDNA and its probability of being amplified by qPCR using different ALU primers, which has a great impact on the concentration and cfDI.

Some authors have observed an underestimation in the amplification of ccfDNA fragments with the length between 100 and 500 bp. Mouliere et al (50) reported that up to 80% of ccfDNA is detected by amplifying sequences shorter than 100 bp, less than 20% of ccfDNA is detected from sequences of 150-250 bp, and only 1-5% from sequences >400 bp. Using conventional polymerases, the best amplification efficiency by qPCR is achieved with 150-300 bp fragments in genomic DNA, so ccfDNA fragmentation is a very important condition to consider (50). Sedlakova et al (51) found that, at fragments smaller than 150 bp, the quantification capability of qPCR decreases because the alignment sites for the primers can be broken. Therefore, it is imperative to design qPCR properly by selecting genes and amplicon lengths. Other factors to consider are variations in the methodology implemented for cfDI determination, and whether the sample is serum or plasma (22).

Here we analyzed the concentration of ccfDNA using two methods: fluorometry and qPCR (ALU115 amplification); the results show that the concentration of ccfDNA obtained by fluorometry is a more useful procedure to discriminate the diagnosis and severity of this disease (Table 2). Unlike quantitative PCR, the fluorometry technique allows the quantification of a greater amount of ccfDNA by means of a specific dye that is intercalated in the DNA, which makes it more specific and precise, in addition to being a fast and economical method (52, 53).

The determination of the cfDI by amplification of DNA fragments using the qPCR method was not able to distinguish patients from reference individuals; this result may be explained by the high fragmentation of the DNA sample, or by the methodology used. In this context, our results show that cfDI does not appear to be a good biomarker for CRC, in contrast to other studies highlighting

the potential use of cfDI (23, 26, 33).

Table 3 shows a compilation of reports in which the concentration and cfDI of ccfDNA in patients with CRC were studied. The results show significant differences in the concentration of ccfDNA but not regarding the cfDI between patients and reference or control groups. In several studies, an association with biological traits was established by the concentration or the cfDI of the ccfDNA.

Further studies are needed to confirm the diagnostic value of ccfDNA variables in CRC patients; moreover, it may be necessary to analyze ccfDNA concentration together with other biomarkers to increase its diagnostic sensitivity and specificity. A limitation of this study was the lack of follow-up of the patients studied, as well as obtaining other histopathological features.

Analysis of the results of this study showed an increase in ccfDNA concentration in the plasma of patients with CRC, demonstrating that it could be a good diagnostic biomarker. Significant differences in ccfDNA concentration were also found in patients younger than 50 years and according to histological type (mucinous adenocarcinoma). The ccfDNA concentration could be a valuable biomarker for predicting the therapeutic effects and progression of CRC.

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Conflict of interest

The authors declare no conflict of interest

Author's contribution

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Supervised, directed and managed the study; Mónica Alejandra Rosales-Reynoso, Patricio Barros-Núñez and Martha Patricia Gallegos-Arreola.

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