

## RESEARCH ARTICLE

# Increased Free Circulating DNA Integrity Index as a Serum Biomarker in Patients with Colorectal Carcinoma

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## Abstract

**Background:** Cell-free DNA circulating in blood is a candidate biomarker for malignant tumors. Unlike uniformly truncated DNA released from apoptotic non diseased cells, DNA released from necrotic cancer cells varies in size. **Objectives:** To measure the DNA integrity index in serum and the absolute DNA concentration to assess their clinical utility as potential serum biomarkers for colorectal carcinoma (CRC) compared to CEA and CA19-9. **Materials and Methods:** Fifty patients with CRC, 10 with benign colonic polyps and 20 healthy sex and age matched volunteers, were investigated by real time PCR of ALU repeats (ALU q-PCR) using two sets of primers (115 and 247 bp) amplifying different lengths of DNA fragments. The DNA integrity index was calculated as the ratio of q-PCR results of ALU 247/ ALU 115bp. **Results:** Serum DNA integrity was statistically significantly higher in CRC patients compared to the benign and control groups ( $p < 0.001$ ). ROC curves for differentiating CRC patients from normal controls and benign groups had areas under curves of 0.90 and 0.85 respectively. **Conclusions:** The DNA integrity index is superior to the absolute DNA concentration as a potential serum biomarker for screening and diagnosis of CRC. It may also serve as an indicator for monitoring the progression of CRC patients. Combining CEA and CA19-9 with either of the genetic markers studied is better than either of them alone.

**Keywords:** Colorectal carcinoma - DNA integrity index - ALU q-PCR - CEA/CA19-9

*Asian Pac J Cancer Prev*, 17 (3), 939-944

## Introduction

Colorectal cancer (CRC) is a major health problem, in Europe more than one million individuals develop CRC every year (Ferlay et al., 2013). Colorectal cancer is a major cause of morbidity and mortality, globally ranking as the third most common tumor in men and the second in women and the fourth most common cancer-related cause of death (Labianca et al., 2013).

Small amounts of free DNA circulate in both healthy and diseased human serum. Tumor necrosis causes release of DNA of varying sizes, in contrast to apoptosis in normal cells that releases smaller and more uniform DNA fragments (Fong et al., 2009). DNA integrity index; represented as the ratio of longer to shorter DNA fragments; may be clinically useful as potential serum biomarker for cancer detection (Fong et al., 2009). Recently highly sensitive method was reported to measure the integrity of free circulating DNA in serum of patients with CRC by quantitative polymerase chain reaction (qPCR) for *Arthrobacter luteus* (ALU) repeats (Mead et al., 2011). The ALU is the most abundant short interspersed repeated sequence in the human genome, with a copy number of  $\sim 1.4 \times 10^6$  per genome (Wang et al., 2000).

The current study aimed at investigating the role of DNA integrity index as well as the concentration of circulating cell-free DNA in serum for screening, diagnosis and monitoring the progression of CRC.

## Materials and Methods

Eighty participants were enrolled in this study. They were all screened for inflammatory conditions and previous cancer by full history taking before consent. All subjects underwent endoscopic examination and accordingly they were selected. They comprised 20 age and sex matched volunteers without significant clinical findings, as the control group, 10 Patients diagnosed to have benign colonic polyps and 50 Patients with established colorectal cancer (CRC). All subjects were recruited from the National Cancer Institute and were diagnosed by histopathological examination of tumor biopsy taken during colonoscopy/sigmoidoscopy and were staged at time of diagnosis. Patients on chemotherapy or Radiotherapy were excluded. The study was approved by Cairo University Hospital research ethics committee and has been performed in accordance with the ethical standards of the Declaration of Helsinki. An informed consent was obtained from all participants. All laboratory

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tests were assayed in the Chemical Pathology Unit, National Cancer Institute, Cairo University.

### Specimen collection

Six ml of venous blood were collected from all participants in the study and divided as follows: 3 ml dispensed into a sterile plain vacutainer tube for DNA extraction and genetic studies and the remaining 3 ml were dispensed into another sterile plain vacutainer for measurement of tumor markers. Both were centrifuged (1000g for 15 minutes) within 4 hours of collection to separate serum and were stored frozen at -20 °C until the time of analysis.

### Laboratory investigations

**I- Tumor markers measurement:** Carcinoembryonic antigen (CEA) and CA19-9: Both tumor markers were measured by a solid-phase, two-site sequential chemiluminescent immunometric assay performed on Architect i 1000 SR autoanalyzer (Maestranzi et al., 1998). The analyzer and Kits were purchased from Abbott Architect diagnostics-USA.

### II- Molecular Studies:

**1- Genomic DNA extraction from the serum:** was done using QIAGEN DNA extraction minikit. (Catalog number: 51104/6). Lysis was done with the use of proteinase K enzyme followed by purification on QIAamp Mini spin columns in which the lysate buffering conditions were adjusted to allow optimal binding of the DNA to the QIAamp membrane. DNA was adsorbed onto the QIAamp silica membrane during a brief centrifugation step. Salt and PH conditions in the lysate ensure that proteins and other contaminants which might inhibit PCR were not retained in the QIAamp membrane. Removal of residual contaminants was done by washing DNA bound to the QIAamp membrane in 2 centrifugation steps using 2 different wash buffers AW1 and AW2 which significantly improves the purity of the eluted DNA. Finally the purified DNA was eluted from the QIAamp membrane in a concentrated form in AE buffer.

#### **2- Measurement of the quantity and quality of the DNA**

*i)* Quantification of DNA: The concentration of DNA was determined by measuring the absorbance at 260 nm (A260) using the Nanodrop spectrophotometer using the AE buffer as the blank. The concentration was displayed in ng/ul. *ii).* Purity of DNA: Samples purity was measured using the Nanodrop spectrophotometer.

The ratio of the readings at 260 nm and 280 nm (A260 / A280) provides an estimate of the purity of DNA. Pure DNA used in this study has an A260 / A280 ratio of 1.7 - 1.9.

#### **3- DNA Amplification and Detection Using Applied**

**Biosystems Step One Real-Time PCR System:** The power Syber Green PCR Master Mix kit Applied Biosystems (catalog number: 4344463); was used in the assay. The Power SYBR Green PCR Master Mix delivers highly sensitive nucleic acid quantitation of a target gene over a broad range of template concentrations. The master mix design also produces reliable DNA amplification results, with minimal variation in assay performance. In addition,

it includes AmpliTaq Gold DNA Polymerase, UP (Ultra Pure), a highly purified version of AmpliTaq Gold DNA Polymerase. The enzyme purification process minimizes non-specific, false positive DNA products due to potential bacterial DNA contamination during PCR.

Sequences of primers (Biosearch technologies) used in ALU genes were; for Alu 115 primer: Forward: 5'-CCTGAGGTCAGGAGTTTCGAG-3' and Reverse: 5'-CCCGAGTAGCTGGGATTACA-3' and for ALU 247:

Forward: 5'-GTGGCTCACGCCTGTAATC-3' and Reverse: 5'-CAGGCTGGAGTGCAGTGG-3'.

*Genomic DNA was obtained from Promega (catalog number: 115701).*

Real-time PCR amplification was performed by programming the computerized thermocycler as follows: pre-cycling heat activation of DNA polymerase at 95 °C for 15 min, followed 35 cycles of denaturation at 95 °C for 30 s, annealing at 64 °C for 30s, and extension at 72 °C for 30s in Applied Biosystem Real-Time PCR Detection System (Umetani et al., 2006). *Arthrobacter luteus* (ALU) repeats; being the most abundant repeated sequence in the human genome; had been used in our study to measure the DNA integrity in the serum by measuring the quantitative PCR for ALU247bp and ALU 115bp using a calibration curve created by performing qPCR on serially diluted genomic DNA. In this method we quantitated concentrations of Alu interspersed segment copy number in DNA of unknown sample by comparing the CT of the unknown sample against the standard curve with known copy numbers. DNA integrity index was calculated as ratio between Q247/Q 115 (Q247 and Q115 represent the ALU-qPCR results for sample x with ALU247 and ALU115 primers) (Cordaux and Batzer, 2009).

### Statistical methods

Data was analyzed using IBM SPSS advanced statistics version 20 (SPSS Inc., Chicago, IL). Numerical data were expressed as median, minimum and maximum as appropriate. Qualitative data were expressed as frequency and percentage. For non normally distributed quantitative data, comparison between two groups was done using Mann-Whitney test (non-parametric t-test). Comparison between 3 groups was done using Kruskal-Wallis test then post-Hoc "Scheffe test" was used for pairwise comparison. Spearman-rho method was used to test correlation between numerical variables. The Receiver Operating Characteristic (ROC) curve was plotted for diagnostic test evaluation. All tests were two-tailed. A p-value <0.05 was considered significant.

## Results

Fifty patients with histopathologically proven CRC, were 26 males and 24 females with a mean age of 49.4+14years. Patients with benign colonic lesions (50% Tubulovillous adenoma and 50% Colon polyp by histopathology) included 7 males and 3 females with a mean age of 33+6.5years, while the control group included 20 healthy individuals, they were 13 males and 7 females with mean age of 49.9 +11years. Considering

smoking; 14/50 CRC patients (28%) were smokers and 1/10(10%) patients with benign lesions were smokers, While 5/20(25%) of healthy controls were smokers.

The median levels of DNA integrity index, absolute DNA concentration, CA19-9 and CEA in the 3 studied groups are illustrated in Table 1, showing that the median levels of DNA integrity index and absolute DNA concentration were significantly higher in CRC patients as compared to the benign group and controls ( $p<0.001$  and  $0.002$  respectively). Meanwhile, the median levels of CA19-9 and CEA were significantly higher in CRC group than that in the benign and the control groups at  $p<0.001$ .

A statistically significant positive correlation existed between the DNA integrity index and the absolute DNA concentration ( $r=0.31$ ) ( $p=0.006$ ), along with the presence of statistically significant positive correlation between DNA integrity and the studied tumor markers; CA19-9 ( $p<0.001$ ) ( $r=0.4$ ) and CEA ( $P<0.001$ ) ( $r=0.45$ ).

Studying the markers in relation to risk factors of CRC, the median level of DNA integrity index was statistically significantly higher in non- smokers than that in smokers at  $p=0.006$ , however, all markers did not differ statistically in relation to positive family history or evidence of benign colonic lesion. Moreover, no statistically significant

**Table 1. Comparison between the Studied Markers in the 3 Studied Groups**

	CRC group (n=50) median (min.-max.)	Benign group (n=10) median (min.-max.)	Control group (n=20) median (min.-max.)	p-value
DNA integrity index	1.54 (0.7-3.1)a	0.3 (0.2-1.9)b	0.173 (0.1-1.35)b	$p<0.001$
Absolute...DNA concentration ng/ $\mu$ l	4.6 (1.1-48)a	2.0 (0.6-5.4)b	2.8 (0.8-23.5)b	$P=0.002$
CA19-9 U/ml	28.4 (0.8-5835)a	2.8 (0.7-25)b	9.9 (0.8-22)b	$p<0.001$
CEAng/ml	8.7 (0.9-2235)a	1.6 (0.8-2.1)b	1.7 (0.5-3.3)b	$p<0.001$

Groups bearing the same lintials were not statistically significantly different;  $P < 0.05$  is considered significant

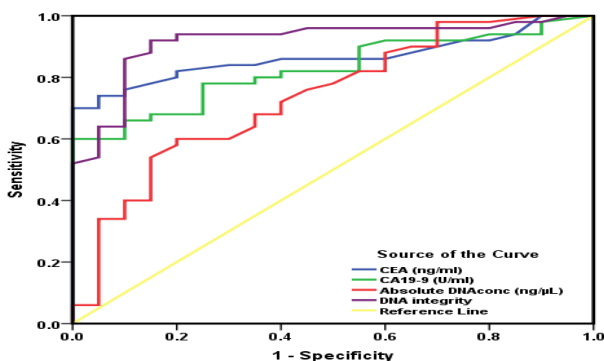
**Table 2. The edian levels of the Studied Markers in Relation to the Prognostic Factors**

		DNA integrity index	P value	Absolute DNA concentration ng/ $\mu$ l	P value	CA19-9U/ml	P value	CEA ng/ ml)	P value
		Median (min-max)		Median (min-max)		Median (min-max)		Median (min-max)	
Grade	Grade II (n=33)	1.08 (0.07-3.1)	$P=0.002$	3.6 (1.1-27.4)	$P=0.003$	24 (0.8-5835)	$P=0.28$	4.3 (0.9-2235)	$P=0.27$
	Grade III-IV (n=17)	2.03 (0.2-3.1)		11.6 (1.6-48)		38 (2.5-1300)		12.6 (1.2-246)	
Histo-pathological type	Adenocarcinoma (n=37)	1.35 (0.07-3.1)	$P=0.3$	4.1 (1.1-48)	$P=0.19$	27.4 (0.8-5835)	$P=0.9$	8.3 (0.9-2235)	$P=0.6$
	Mucinous adenocarcinoma (including Signet ring adenocarcinoma) (n=13)	1.7 (0.09-3.1)		10 (1.5-23.2)		31.2 (8.1-1300)		12.3 (0.9-130)	
	Proximal (n =25) (Cecum, ascending colon, transverse colon, descending colon)	1.7 (0.07-3.1)		4.8 (1.1-48)		31.2 (1.8-3099)		8.3 (0.9-246)	
Site of carcinoma	Distal (n=25) (Sigmoid colon. and rectum)	1.3 (0.4-3.1)	$P=0.6$	4.2 (1.5-18)	$P=0.4$	25 (0.8-5835)	$P=0.6$	9.3 (0.9-2235)	$P=0.9$
Lymph node status	Lymph node +ve (n=30)	1.5 (0.09-3.1)	$P=0.06$	4.6 (1.5-23.2)	$P=0.6$	33.2 (0.8-5835)	$P=0.18$	10.8 (0.9-2235)	$P=0.24$
	Lymph node -ve (n=20)	1.0 (0.07-2.1)		4.6 (1.1-48)		16.9 (1.8-1300)		4.2 (0.9-246)	
Status of distant metastasis	MO (No evidence of metastasis)(n=29)	0.8 (0.07-2.5)	$P=0.004$	4.2 (1.1-48)	$P=0.86$	24.9 (0.8-1300)	$P=0.2$	4.3 (0.9-246)	$P=0.44$
	M1 (evidence of metastasis) (n=21)	1.7(0.7-3.1)		5.3(1.5-23.2)		38(1-5835)		16.7 (0.9-2235)	

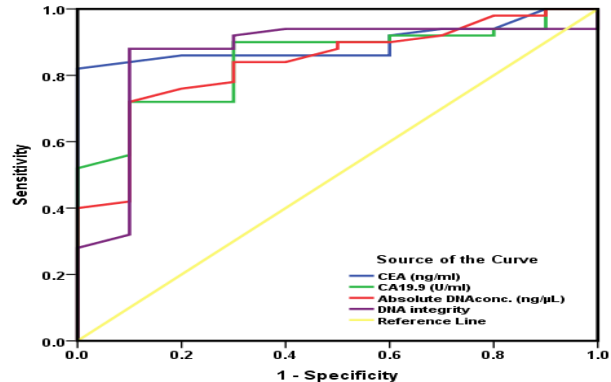
$P<0.05$  is considered significant

**Table 3. The Diagnostic Significance for Combined Markers to Diagnose the CRC among the Control and Benign Groups**

CRC among the control group						
Markers cutoff	AUC	Sensitivity	Specificity	Positive predictive value (PPV)	Negative predictive value (NPV)	Diagnostic accuracy
DNA integrity (0.41)	0.9	90%.	85%	93.90%	81%	92%
Absolute DNA concentration (3.3ng/μl)	0.73	68%	65%	82.90%	44.80%	57.2%.
CA19-9 (12.3 U/ml)	0.82	78%	75%	88.6%,	57.70%	78.1%.
CEA (2.1ng/ml)	0.86	82%	80%	91.1%,	64%	81.5%.
DNA integrity (0.41) and CA19-9 (12.3 U/ml)		96%	60%	85.7%,	85.70%	86%
DNA integrity (0.41) and CEA (2.1ng/ml)		98%	65%	87.5%,	92.90%	88.60%
Absolute DNA concentration (3.3ng/μl) and CA19-9 (12.3 U/ml)		96%	50%	90.60%	71.40%	88.30%
Absolute DNA concentration (3.3ng/μl) and CEA (2.1ng/ml)		100%	60%	92.60%	100%	93.30%
CRC among the benign group						
DNA integrity (0.55)	0.83	88%	90%	97.8%,	60%	88.30%
Absolute DNA concentration (2.35ng/μl)	0.83	84%	70%	93.30%	46.70%	81.70%
CA19-9 (7.35) U/ml)	0.84	88%	70%	93.6%,	53.80%	85%
CEA (2ng/ml)	0.89	84%	90%	97.70%	52.90%	85%
DNA integrity (0.55) and CA19-9 (7.35 U/ml)		96%	60%	92.3%,	75%	90%
DNA integrity (0.55) and CEA (2.0ng/ml)		98%	80%	96%	88.90%	92%
Absolute DNA concentration (2.35ng/μl) and CA19-9 (7.35 U/ml)		96%	50%	90.60%	71.40%	88.30%
Absolute DNA concentration(2.35ng/μl and CEA (2.0ng/ml)		100%	60%	92.60%	100%	93.30%



**Figure 1. ROC Curve for the Studied four Markers to Differentiate CRC Patients from Healthy Control subjects.** AUC of 0.9 for DNA integrity (shown as purple line),0.86 for CEA (shown as blue line),0.82 for CA19-9 (shown as green line) and 0.73 for absolute DNA concentration (shown as red line)



**Figure 2. Showing the ROC curve for the studied four markers to differentiate CRC from benign colonic patients.** AUC of 0.86 for DNA integrity (shown as purple line),0.89 for CEA (shown as blue line), 0.84for CA19-9 (shown as green line) and 0.83 for absolute DNA concentration (shown as red line)

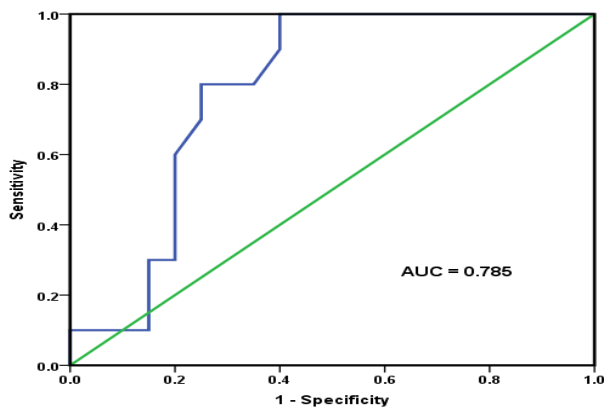
difference was found among the studied markers in different age or sex ( $p > 0.05$ ).

Studying the markers in relation to prognostic factors of CRC, showed that in more advanced grades (Grades III and IV), the median level of DNA integrity index and absolute DNA concentration demonstrated a statistically significantly higher median levels than that in grade II ( $P = 0.002$  and  $0.003$  respectively), however neither of the median levels of CA19-9 and CEA did. Patients with distant metastasis showed a statistically significantly higher median DNA integrity index than in non -metastatic patients ( $p = 0.004$ ). No statistically

significant difference was demonstrated in the median levels of the other studied parameters (Table2).

ROC curve analysis was plotted for each of the studied markers to evaluate its diagnostic efficacy in differentiation between the CRC and control groups, DNA integrity yielded the highest AUC (0.90) (Table3) (Figure 1). To differentiate between CRC and patients with benign colonic lesions, each of the studied markers showed high area under curve (AUC)  $> 0.80$  (ranging from 0.83-0.89) (Table3) (Figure 2). Only DNA integrity index was found to be able to differentiate between patients with benign





**Figure 3. Showing the ROC Curve for DNA Integrity to Differentiate Patients with Benign Colonic Lesion from Healthy Controls(AUC=0.78)**

colonic lesions from healthy controls with an AUC of 0.78, at a cutoff 0.28, it showed a sensitivity of 80% and a specificity of 75% (Figure 3). Upon combing of markers (considering that either of them is considered positive for cancer); to assess their diagnostic efficacy to differentiate between CRC and healthy controls and between CRC and benign colonic lesions patients they yielded sensitivities and diagnostic accuracies better than either of them alone (Table3).

## Discussion

Colorectal cancer is one of the few preventable cancers and the survival rate is closely related to the clinical and pathological stage of the disease at time of diagnosis (Otero et al., 2015), however, approximately 65 % of patients present with advanced disease (Luo et al., 2014). Efforts to develop better screening strategies; to improve patient survival rates; is mandatory (David et al., 2012). Unfortunately, the currently available serological markers for diagnosis and prognosis of CRC; such as CEA and CA19-9; have proven to be non-ideal (Wang et al., 2015).

A recent study in 2014 reported that free circulating DNA is considered to be a derivative of increased and abnormal apoptotic pathways in the cancerous lesions (Maio et al., 2014). The abnormal DNA degradation leads to increased DNA levels and DNA fragments of different sizes (Leszinski et al., 2013). Blood-based DNA integrity index; defined as the ratio of long to small fragments of cell-free circulating DNA; is known to be increased in various types of cancers including hepatocellular carcinoma (Chen et al., 2012), prostate cancer (Hanley et al., 2006), leukemia (Gao et al., 2010), melanoma (Pinzani et al., 2011), bladder Cancer (Casadio et al., 2013) and CRC (Leszinski et al., 2013). Moreover, a study in 2011, conducted on 76 patients who underwent chemoradiotherapy for CRC reported a significant decrease in DNA integrity index in patients with good response to chemotherapy versus non-responders (Agostini et al., 2011). The current study aimed at investigating the role of DNA integrity index as well as the concentration of circulating cell-free DNA in serum for screening, diagnosis and progression of CRC.

The median level of DNA integrity index was significantly higher in CRC group when compared to the control group ( $p < 0.001$ ). These results were supported by other studies (Umetani et al., 2006; da Silva et al., 2013; Leszinski et al., 2013, Hao et al., 2014). A statistically significant increase in median level of DNA integrity index between CRC group and the benign group ( $p < 0.001$ ) was found, which was also consistent with several studies (da Silva et al., 2013; Hao et al., 2014; Zaher et al., 2014), but was contrasted by one study (Leszinski et al., 2013), which reported a statistically non-significant difference between CRC and benign colonic patients ( $P = 0.14$ ). The current study failed to demonstrate a statistically significant difference in the median level of DNA integrity index between benign and control groups ( $P = 0.2$ ), this finding was opposed earlier by a study demonstrating a statistically significant difference between the benign group and healthy controls at  $p = 0.001$  (Mead et al., 2011), while comes in agreement with a more recent one (Zaher et al., 2014). As for absolute DNA concentration, its median value was statistically significantly higher in CRC as compared to the control ( $p = 0.004$ ) and the benign groups ( $p = 0.002$ ), close results were previously reported (Mead et al., 2011; Zaher et al., 2014). Nevertheless, this study reported a non-significant difference between the benign and control groups ( $p = 0.6$ ) which was contrasted by an earlier study (Mead et al., 2011), while comes in accordance with another (Zaher et al., 2014). The discrepancy between the results reported by different studies might be contributed to the difference in ethnic and racial groups studied, the differences in methodology and lack of standardization in these methodologies. Several studies use plasma to quantify the circulating cell-free DNA, while other studies use serum as a template. Moreover, some studies performed DNA extraction (Agostini et al., 2011) and measured the levels of circulating cell-free DNA by qPCR, while other studies use serum (Umetani et al., 2006) or plasma (Mead et al., 2011) as a direct template to quantify cell-free DNA.

In the current study, a significant positive correlation existed between DNA integrity index and absolute DNA concentration ( $P = 0.006$ ), which was previously suggested (Umetani et al., 2006).

On comparing the DNA integrity index and absolute DNA concentration, with tumor grade, advanced grades (Grades III and IV) showed a statistically significantly higher median values than patients with grade II at  $p = 0.002$  and  $0.003$  respectively. This was opposed by other studies that failed to demonstrate a significant difference in the DNA integrity index (Umetani et al., 2006) and absolute DNA concentration (Zaher et al., 2014) in different tumor grades.

Moreover, patients in the current study with distant metastasis at the time of presentation showed statistically significantly higher median level of DNA integrity index than that in non-metastatic patients at  $p = 0.004$ , which was opposed earlier (Zaher et al., 2014). No statistically significant difference exists in the median levels of neither DNA integrity index nor absolute DNA concentration in different states of lymph node, sites or types of the tumor ( $p > 0.05$ ) as was previously reported

(Zaher et al., 2014).

ROC curve for DNA integrity index, showed an AUC of 0.90 and at cutoff value of 0.41; it can differentiate CRC from healthy control with a sensitivity of 90% , a specificity of 85% and overall accuracy of 92% . Regarding absolute DNA concentration, ROC curve showed AUC = 0.73 and at cutoff value of 3.3 ng/ $\mu$ l had a sensitivity of 68%, specificity of 65% and diagnostic accuracy of 57.2%. These results were slightly different from a previous study in which ROC curve for DNA integrity index yielded an AUC of 0.78, at a cut off 0.22 showed a sensitivity of 56% and specificity of 90%, while for absolute DNA concentration, it showed AUC =0.75, at a cutoff value of 1.73ng/ $\mu$ l, showed a sensitivity of 40% and a specificity of 90% (Umetani et al., 2006).

Regarding CRC and the benign group; ROC curve for DNA integrity index showed an AUC of 0.86 , at cutoff 0.55, sensitivity was 88% , specificity was 90% and diagnostic accuracy was 88.3%. ROC curve for absolute DNA concentration yield an AUC of 0.83, at cutoff 2.35 ng/ $\mu$ l, it had a sensitivity of 84%, specificity of 70% and diagnostic accuracy of 81.7%, which suggested that DNA integrity index was superior to absolute DNA concentration considering the diagnosis of CRC among healthy control or benign subjects that comes in agreement with one study (Mead et al., 2011) and is contrasted by an earlier one concluding that DNA integrity index and absolute DNA concentration were equivalent to each other considering the diagnosis of CRC among healthy subjects (Umetani et al., 2006).

In this study, the currently used markers CA19-9 and CEA have a lower diagnostic value for CRC than both DNA integrity index and absolute DNA concentration, as was previously suggested (Mead et al., 2011).

We concluded that DNA integrity index could be clinically used as a serum biomarker in discriminating colorectal cancer patients from healthy subjects or patients with benign colonic polyp as well as serving as a potential indicator for the assessment of disease progression in CRC patients. Furthermore, Genetic markers (DNA integrity index or absolute DNA concentration) could be a clinically useful surrogate markers in CRC patients in combination with the conventional tumor markers (CEA and CA19-9), which was proved to be better than the use of either of them alone for the diagnosis of CRC. A simple blood draw test could even replace or minimize the use of the invasive colonoscopy, as the preferred mode for screening and diagnosis of CRC.

## Acknowledgements

To National Cancer Institute, Cairo University for providing the facilities during the research, including the laboratories, equipment, analyzers and consumables.

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