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DNA integrity index as a potential molecular biomarker in colorectal cancer

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Abstract

Background: Efficient approaches for early detection of colorectal cancer offer opportunities to gain better treatment outcomes. Blood-based molecular biomarkers as DNA integrity index (DII) might represent a promising tumor marker in the future. The purpose of this study was to assess the clinical utility of the DII as a potential biomarker for colorectal cancer in 90 colorectal cancer patients, 30 patients with benign colorectal mass, and 30 age- and sex-matched healthy control subjects. PCR was used to assess the concentration of both ALU115 and ALU247. DII was calculated as the ratio of Q247/Q115.

Results: DII was significantly higher in colorectal cancer patients than both patients with benign colorectal mass and healthy controls. ROC curve was plotted using DII and the best cut-off was ≥ 0.60 with diagnostic sensitivity 93.0%, specificity 65.0%, PPV 80.0%, NPV 86.0%, and efficiency 82% with AUC (0.872) while the best cut-off for CEA was ≥ 1.4 ng/mL with diagnostic sensitivity 87.0%, specificity 60.0%, PPV 76%, NPV 75%, and efficiency 76% with AUC (0.79).

Conclusions: Our results suggest that DII is better than CEA as an early marker for colorectal cancer detection and may be used as a candidate biomarker for malignancy.

Keywords: Colorectal cancer, DII, ALU

Background

Colorectal (CRC) is ranked the third most frequent malignancy in men after lung and prostate tumors, and the second most commonly diagnosed malignancy in women after breast cancer and is considered the second leading cause of cancer mortalities [1]. The 5-year survival rate for CRC patients relies on the staging of the disease at diagnosis. Therefore, it is important to diagnose and manage CRC at the early stages to improve treatment outcomes and decrease cancer-related mortality [2].

Current screening approaches include stool-based tests and colonoscopy. Stool-based tests as fecal occult blood testing (FOBT) are cheap and non-invasive but with low sensitivity. Colonoscopy-guided biopsy is

the gold-standard screening and diagnostic approach with high specificity up to more than 95%, but it is invasive and necessitates bowel preparation and also may cause severe complications [3, 4]. Conventional tumor markers, as carcinoembryonic antigen (CEA) and carbohydrate antigen 19.9 (CA 19.9), have limited sensitivity and specificity limiting their efficacy in screening and diagnosis. Nevertheless, they are clinically used to detect prognosis, monitor disease progression, and response to treatment [5]. Since the conventional methods for CRC screening are either inefficient or invasive, there is a high need to offer more compliant and less-invasive screening methods with high sensitivity and specificity.

Cell-free DNA (CfDNA) has been suggested to be a promising tumor marker. However, its level is also elevated in various non-malignant disorders. Therefore, more specific approaches such as measuring the integrity of DNA have been proposed [6, 7]. This approach is

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based on the difference in length of cfDNA released from the cells according to the mechanism of cell death. Apoptosis, which usually occurs in normal tissues, results in short DNA fragments less than 180 bp, while necrosis, which occurs in tumor cells, produces longer fragments. The Arthrobacter luteus (ALU) repeats are 300 bp in length and are the most predominant repeated sequences in the human genome, with a copy number of 1.4×10^6 per genome. It accounts for more than 10% of the human genome [8]. Most studies used ALU quantitative polymerase chain reaction (PCR) for calculation of DNA integrity index (DII) that represents the ratio between ALU247 long fragments released from necrotic cells and ALU115 short fragments released from normal cells [4]. This study aimed to assess the clinical utility of DII as a potential biomarker for CRC and to evaluate its correlation with the traditional tumor marker (CEA).

Methods
The study included 90 adult CRC patients, 30 patients with benign colorectal mass, and 30 age- and sex-matched healthy controls. CRC patients who previously received chemotherapy/radiotherapy or undergone colorectal surgery as well as those who have other types of malignant tumors or a history of autoimmune diseases were excluded from this study. CRC and benign mass diagnosis were done using colonoscopy and biopsy followed by histopathological diagnosis. Magnetic resonance imaging (MRI) anorectal protocol and /or Pan-computerized tomography (CT) were done for CRC patients to detect metastasis. Written informed consent was obtained from each participant prior to participation in the study. Demographic and clinical characteristics of participants are shown in Table 1.

Sample collection
Three milliliters of whole blood were collected into a sterile plain vacutainer for PCR analysis. Double centrifugation was done within 4 h of collection. The first centrifugation was at $1600 \times g$ for 10 min, and the resultant supernatant was carefully harvested into another plain sterile tube and subjected to second centrifugation at $16000 \times g$ for 10 min. Serum was stored frozen at -80 °C until used for PCR. Another 3 mL of blood was collected into a plain vacutainer tube and centrifuged at $3000 \times g$ for 20 min and serum was used for the assay of CEA.

Assessment of CEA levels
CEA levels were measured using Cobas e411 immunoassay autoanalyzer (Roche Diagnostics, D-68305 Mannheim).

DNA extraction and ALU qPCR:
Deoxyribonucleic acid (DNA) extraction was performed from serum using QIAamp DNA blood mini kit (QIAGEN, Germany). Extracted DNA concentration was determined by measuring the absorbance at 260 nm using spectrophotometer. Meanwhile, the ratio of the absorbance at 260 and 280 nm (OD260/OD280) was used to assess the purity of extracted DNA. A ratio of about 1.8 was generally accepted.

Two PCR reactions were set for each sample. ALU 115 primers were used for the first reaction, and ALU 247 primers were used for the second reaction. The sequence of used primers are shown in Table 2.

Reaction volume was set as follows: 10 μL of the Maxima SYBR Green qPCR Master Mix, 0.6 μL of forward primer, 0.6 μL of reverse primer, 3.8 μL of RNASE free water, and 5 μL of DNA extract. PCR was performed on Applied Biosystem Step One Real-Time PCR System. Amplification was performed according to the following protocol: initial heat activation at 95 °C for 10 min, DNA denaturation at 95 °C for 30 s followed by annealing at 64 °C for 30 s and extension at 72 °C for 30 s for 40 cycles. PCR amplification was followed by melting curve analysis and gel electrophoresis. Calibration curves were constructed using Taqman Control Genomic Human DNA (Applied Biosystems, Thermofisher, USA) for the calculation of the concentration of both ALU115 and ALU247. Finally, DII was calculated as the ratio of Q247/Q115.

Statistical analysis
Data analysis was done using IBM SPSS statistics (V. 22.0, IBM Corp., USA, 2013). Data were presented as median and interquartile range for non-parametric data, mean, and standard deviation for parametric data, frequency, and percentage for qualitative data. Groups were compared using Kruskal-Wallis test then post hoc "Dunn's multiple comparison test" was used for pairwise comparison. Spearman's rank correlation coefficient (r_s) was used to test the correlation between numerical variables. The receiver operating characteristic (ROC) curve was plotted for diagnostic test evaluation. A p value < 0.05 was considered significant.

Results
Higher DII and ALU115, ALU 247, and CEA levels in CRC patients than controls
A significant difference was found between the three studied groups regarding ALU 115 ($p \leq 0.01$), ALU 247 ($p \leq 0.01$), DII ($p \leq 0.01$), and CEA ($p = 0.002$). Post hoc analysis revealed higher levels of ALU115 and ALU 247 in CRC patients than healthy controls ($p < 0.001$ for both), also higher levels were observed in pathological controls than healthy controls ($p = 0.001$ and $p = 0.009$,

Table 1 Demographic and clinical characteristics:

Parameter	CRC patients (n = 90)	Pathological controls (n = 30)	Healthy controls (n = 30)
Age (years) mean ± SD	52 ± 9.5	50 ± 4.7	51 ± 5.9
Sex			
Male (%)	48/90 (53%)	12/30 (40%)	21/30 (70%)
Female (%)	42/90 (47%)	18/30 (60%)	9/30 (30%)
Anemia			
Present (%)	66/90 (73.3%)	12/30 (40%)	0/30
Absent (%)	24/90 (36.7%)	18/30 (60%)	30/30 (100%)
Constipation			
Present (%)	57/90 (63.3%)	0/30	0/30
Absent (%)	33/90 (36.7%)	30/30 (100%)	30/30 (100%)
Bleeding per rectum			
Present (%)	51/90 (56.7%)	3/30 (30%)	0/30
Absent (%)	39/90 (43.3%)	27/30 (90%)	30/30 (100%)
Palpable mass			
Present (%)	3/90 (3.3%)	0/30	0/30
Absent (%)	87/90 (96.7%)	30 /30 (100%)	30/30 (100%)
Family history			
Present (%)	21/90 (20%)	6/30 (20%)	0/30
Absent (%)	69/90 (80%)	24/30 (80%)	30/30 (100%)
Tumor size (mean ± SD)	6.6 ± 1.9	NA	NA
Site of tumor		NA	NA
Left (%)	30/90 (33.3%)		
Right (%)	33/90 (36.7%)		
Rectum (%)	27/90 (30%)		
Grade of tumor		NA	NA
1 (%)	9/90 (10%)		
2 (%)	78/90 (86.7%)		
3 (%)	3/90 (3.3%)		
TNM stage		NA	NA
1 (%)	6/90 (6.7%)		
2 (%)	21/90 (23.3%)		
3 (%)	30/90 (33.3%)		
4 (%)	33/90 (36.7%)		
Lymph nodes metastasis		NA	NA
Present (%)	30/90 (33.3%)		
Absent (%)	27/90 (30%)		
Distant metastasis		NA	NA
Present (%)	33/90 (36.7%)		
Absent (%)	57/90 (63.3%)		

CRC colorectal cancer, *NA* non-applicable, *SD* standard deviation, *TNM* tumor size, node involvement, and metastasis status

respectively) while no difference was observed between CRC patients and pathological controls (p = 0.65 and p = 0.66, respectively) (Fig. 1 a, b). Additionally, higher DII was observed in CRC patients than in both pathological

controls (p = 0.037) and healthy controls ($p < 0.001$) while no difference was observed between pathological controls and healthy controls (p = 0.31) (Fig. 1c). Also, significantly higher levels of CEA were found in CRC patients compared to both pathological controls (p = 0.038) and healthy controls (p = 0.009) while no difference was observed between pathological controls and healthy controls (p = 0.71) (Fig. 1d).

DII and ALU 247 are elevated in early stages of CRC

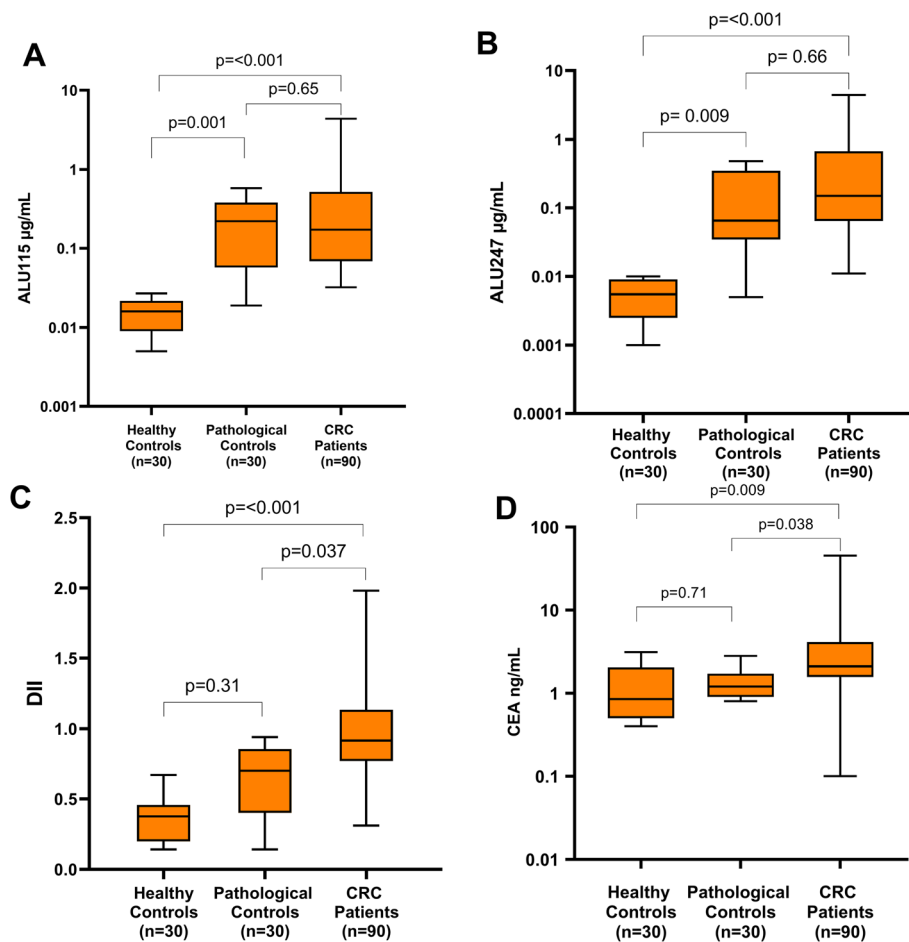To examine the usefulness of the different parameters in the early diagnosis of cancer colon, we compared

Table 2 Primers sequences

ALU 115 Forward primer (F_{115})	5′ CCTGAGGTCAGGAGTTCGAG′3
ALU 115 Reverse primer (R_{115})	5′ CCCGAGTAGCTGGGATTACA′3
ALU 247 Forward primer (F_{247})	5′ GTGGCTCACGCCTGTAATC′3
ALU 247 Reverse primer (R_{247})	5′ CAGGCTGGAGTGCAGTGG′3.

ALU the Arthrobacter luteus, *F* forward, *R* reverse

Fig. 1 Levels of ALU115 (**a**), ALU 247 (**b**), DII (**c**), and CEA (**d**) in CRC patients, pathological controls, and healthy controls

their levels in early stage CRC (stage I and II) to healthy controls. A significant higher DII and ALU 247 concentration in patients with early stages was observed compared to controls ($p = 0.01$ for both) while no difference was found regarding CEA and ALU115 concentrations ($p = 0.25$ and $p = 0.07$, respectively) (Fig. 2).

Comparison of different parameters between metastatic and non-metastatic CRC

To examine whether DII, ALU 115 levels, ALU 247 levels, and CEA levels are increased in metastatic cancer colon or not, we compared different parameters between CRC patients with metastasis and CRC patients with no metastasis. Only levels of DII and CEA were significantly higher in the metastatic CRC patients than the non-metastatic patients ($p = 0.004$ and $p = 0.04$, respectively) while no difference was observed in ALU115 and ALU 247 ($p = 0.44$ and $p = 0.49$) (Fig. 3).

DII correlated with CEA, tumor size, and tumor stage

Correlation study revealed significant positive correlation of DII with CEA ($r_s = 0.39$, $p = 0.03$) (Fig. 4a), tumor size ($r_s = 0.40$, $p = 0.02$) (Fig. 4b), and tumor stage ($r_s = 0.39$, $p = 0.03$) (Fig. 4c).

Diagnostic performance of DII in discriminating between CRC patients and both pathological and healthy controls

ROC curve was plotted to determine the diagnostic performance of DII in discriminating between CRC patients and healthy controls. The best cut-off was 0.55 with a diagnostic sensitivity of 93.3%, specificity of 90.0%, positive predictive value (PPV) of 96.5%, negative predictive value (NPV) of 81.8%, and efficiency of 92.5%. The area under the curve (AUC) was 0.95 with 95% CI of (0.89–1.0) (Fig. 5a).

Regarding the performance of DII in discriminating between CRC patients and pathological controls, the best cut-off was 0.66 with a diagnostic sensitivity of 86.6%, specificity of 50.0%, PPV of 85.0%, NPV of 63.0%,
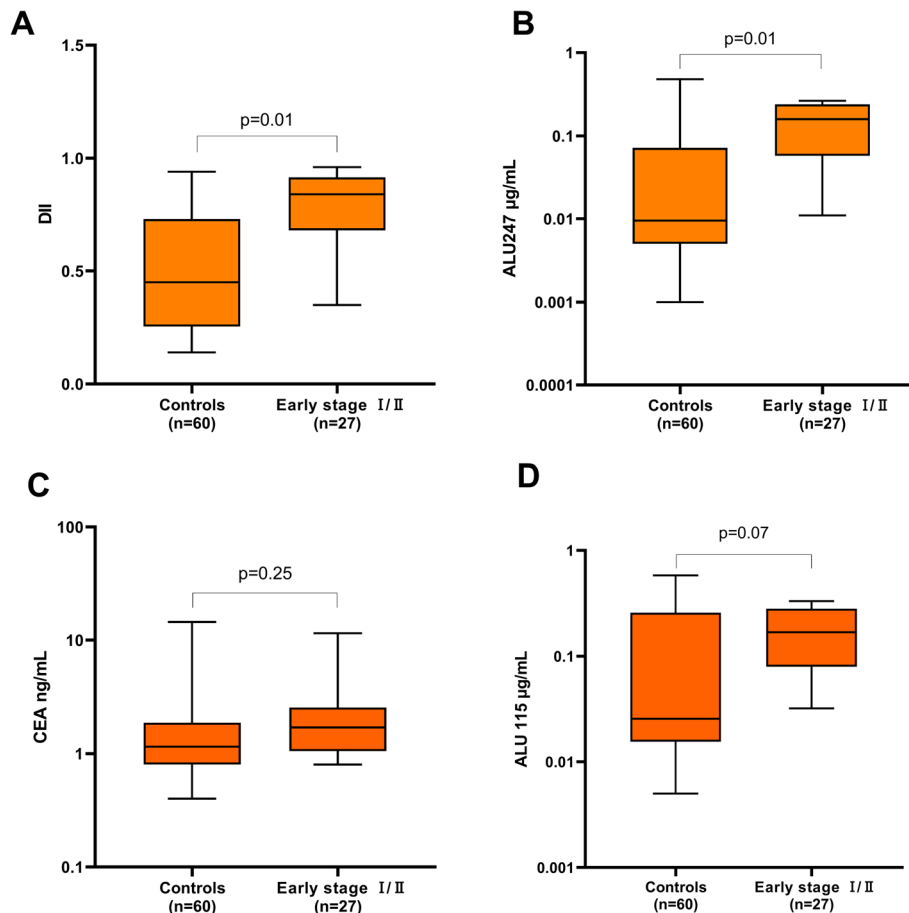
Fig. 2 Higher DII (**a**) and ALU 247 (**b**) levels in patients with early CRC stages while no difference was found regarding CEA (**c**) and ALU115 (**d**)

and efficiency of 80% with AUC of 0.792 and 95% CI (0.64–0.93) (Fig. 5b).

Another ROC curve was plotted for DII to discriminate CRC patients from all controls (both pathological and healthy), and the best cut-off was 0.60 with a diagnostic sensitivity of 93.0%, specificity of 65.0%, PPV of 80.0%, NPV of 86.0%, and efficiency of 82% with AUC of 0.872 and 95% CI (0.77–0.96) (Fig. 5c).

Discussion

Colorectal cancer remains a significant cause of morbidity and mortality worldwide with a high incidence rate. The discovery of non-invasive biomarkers for CRC detection with adequate sensitivity and specificity is a major challenge to reduce cancer-related morbidity and mortality. New non-invasive tests are under research to meet the balance between the increase of sensitivity and decreasing the need for unnecessary colonoscopies [9].

Circulating free DNA has been suggested to be a promising tumor marker. However, as cfDNA levels may also get elevated in various non-malignant disorders,

more specific approaches have been proposed. Among these approaches is the calculation of the DII. The DII describes the ratio of longer free DNA fragments to shorter free DNA fragments [6, 7]. In healthy individuals, cfDNA mainly originates from apoptotic cells which usually release DNA fragments of 185–200 bp. In contrast, cfDNA released from cancer cells is usually longer due to the pathologic cell death in tumors. Therefore, DNA integrity has the potential of being used for tumor detection and prognostic prediction [4]. DII calculation using fragments from GAPDH has been described by Van Beers et al. [10], also Salvianti et al. [11] has investigated the determination of DII targeting sequences in amyloid precursor protein.

The use of ALU repeats for determination of DII was first proposed by Umetani et al. [12]. ALU115 and ALU247 are 2 amplicons with a length of 115 and 247 bp. ALU115 and ALU247 are used to distinguish between DNA originating from apoptotic and necrotic cell death, respectively. Since the main source of short cfDNA (180–200 bp fragments) in healthy individuals
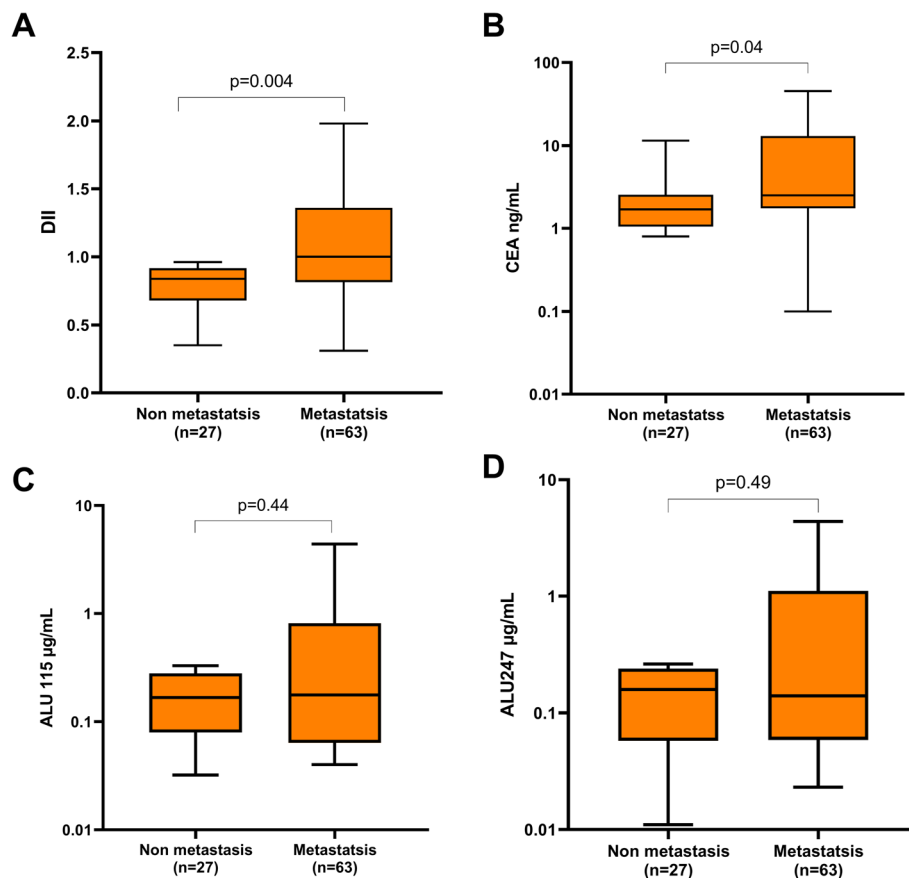
Fig. 3 Higher DII (**a**) and CEA (**b**) in metastatic CRC patients than in the non-metastatic patients while no difference was observed in ALU115 (**c**) and ALU 247 (**d**)

has been attributed to apoptotic cells, a majority of longer DNA fragments (ALU247) could represent a biomarker for malignant tumor detection. The annealing sites of ALU115 are within the ALU247 ones, the ALU115 primers could amplify both shorter (truncated by apoptosis, i.e., ALU115) and longer DNA fragments (ALU247), so results of ALU115 quantitation represent the total amount of cfDNA. However, ALU247 primers amplify only longer DNA fragments; therefore, results of ALU247 quantitation represent amounts of DNA released from necrotic cell death. DII is calculated as the ratio of longer to shorter ALU fragments (ALU247/ALU115) [12].
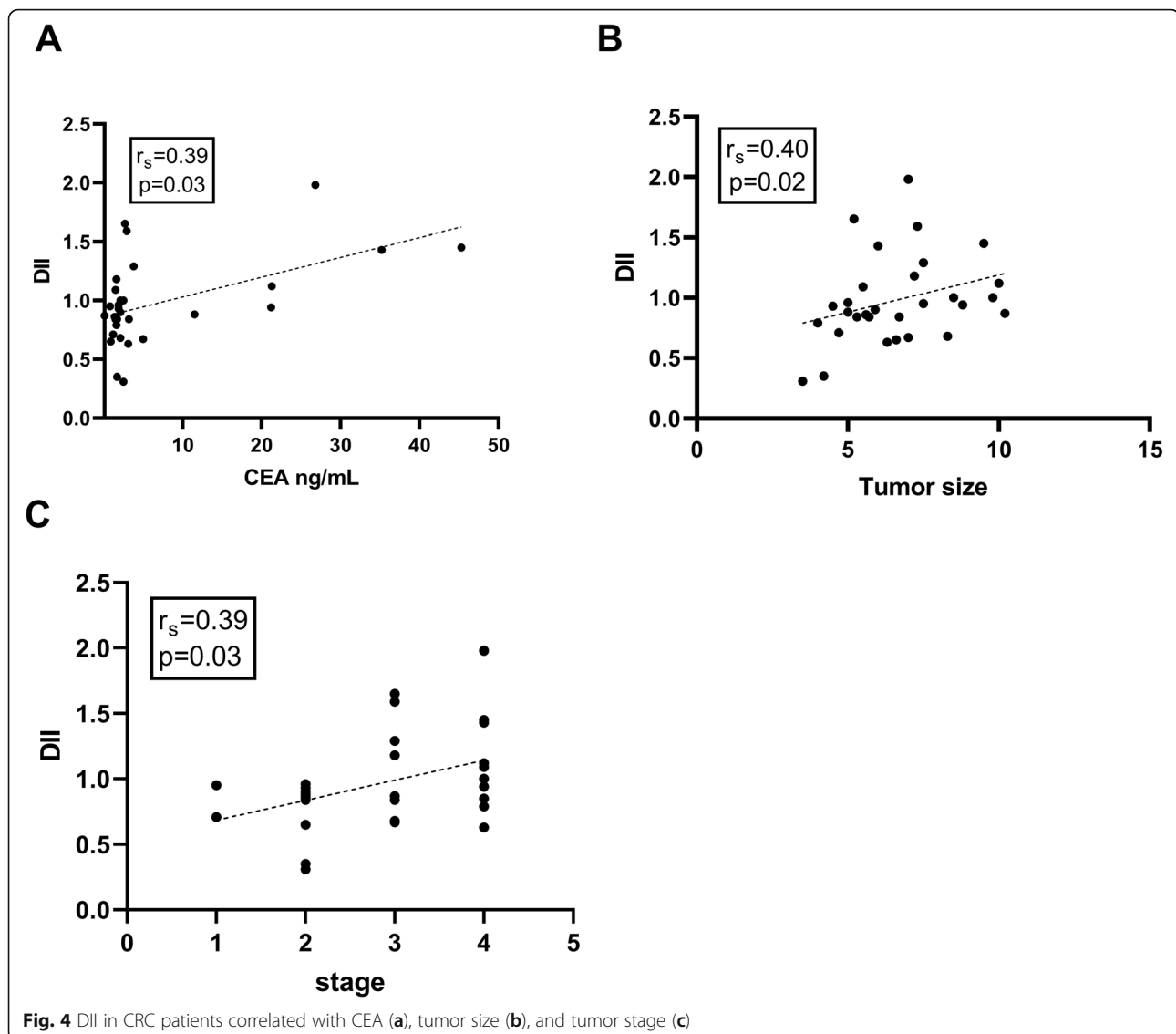
In this study, we chose ALU115 and ALU247 repeats to calculate the DII in CRC patients. We aimed to assess the clinical utility of DII as a potential biomarker for CRC and to evaluate its correlation with CEA, the conventional marker used for prognosis, and follow-up of CRC patients.

We found higher DII in CRC patients than both pathological controls and healthy controls. DII was higher in early stage CRC patients compared to healthy

controls and was also higher in the metastatic CRC patients compared to non-metastatic patients. Also, DII positively correlated with CEA, tumor size, and tumor stage.

Umetani et al. [12] investigated the DII in 32 CRC patients and 51 heathy controls in the USA and reported that DII was higher in CRC patients compared to healthy controls with an AUC of 0.78 for discriminating CRC patients from healthy individuals. Similarly, Leszinski et al. [6] assessed DII in 24 CRC patients, 11 patients with benign gastrointestinal diseases, and 24 healthy individuals in Germany, and reported higher DII in CRC patients compared to healthy controls with an AUC of 0.738 while no difference was observed between CRC patients and patients with benign gastrointestinal diseases. El-Gayar et al. [13] also assessed DII in 50 CRC patients and 20 healthy controls and reported that DII is higher in CRC patients compared to healthy individuals with AUC of 0.9.

As expected, the level of CEA was significantly higher in CRC patients than in both the healthy control group and the pathological control group. This came in

Fig. 4 DII in CRC patients correlated with CEA (**a**), tumor size (**b**), and tumor stage (**c**)

consistent with El-Gayar and his coworkers [13]. As for ALU 115 that represents absolute total DNA concentration, we found a significant increase in the level of absolute DNA concentration in CRC patients compared to healthy controls but non-significant difference was found between CRC patients and benign group. Similarly, El-Gayar et al. [13] and Hao et al. [14] found significantly higher levels of absolute DNA concentration in CRC group than healthy controls. However, in contrast to our findings, they found significantly higher levels of absolute DNA concentration in the CRC group compared to the benign group. This difference may be attributed to that previous studies were conducted on patients having poorly differentiated (grade III) tumors, with different histopathological states that might contribute to a significant increase in absolute cfDNA

levels in CRC patients compared to the benign group. However, in our study, most patients had moderately differentiated (grade II) mucinous adenocarcinoma.

Additionally, we observed a significant increase in absolute DNA concentration in a benign group compared to the healthy control group. This finding came in agreement with Mead et al. [15] and contrasted by Bedin et al. [7] and El-Gayar et al. [13]. Our findings are supported by the fact that any benign disease condition may be accompanied by some sort of inflammation that may cause elevation of total DNA in our pathological control group. However, the discrepancy with Bedin et al. [7] and El-Gayar et al. [13] in this regard may be attributed to difference in sample sizes, and Bedin and his colleagues [7] used plasma as source of cfDNA, the exclusion of presence of inflammatory conditions in
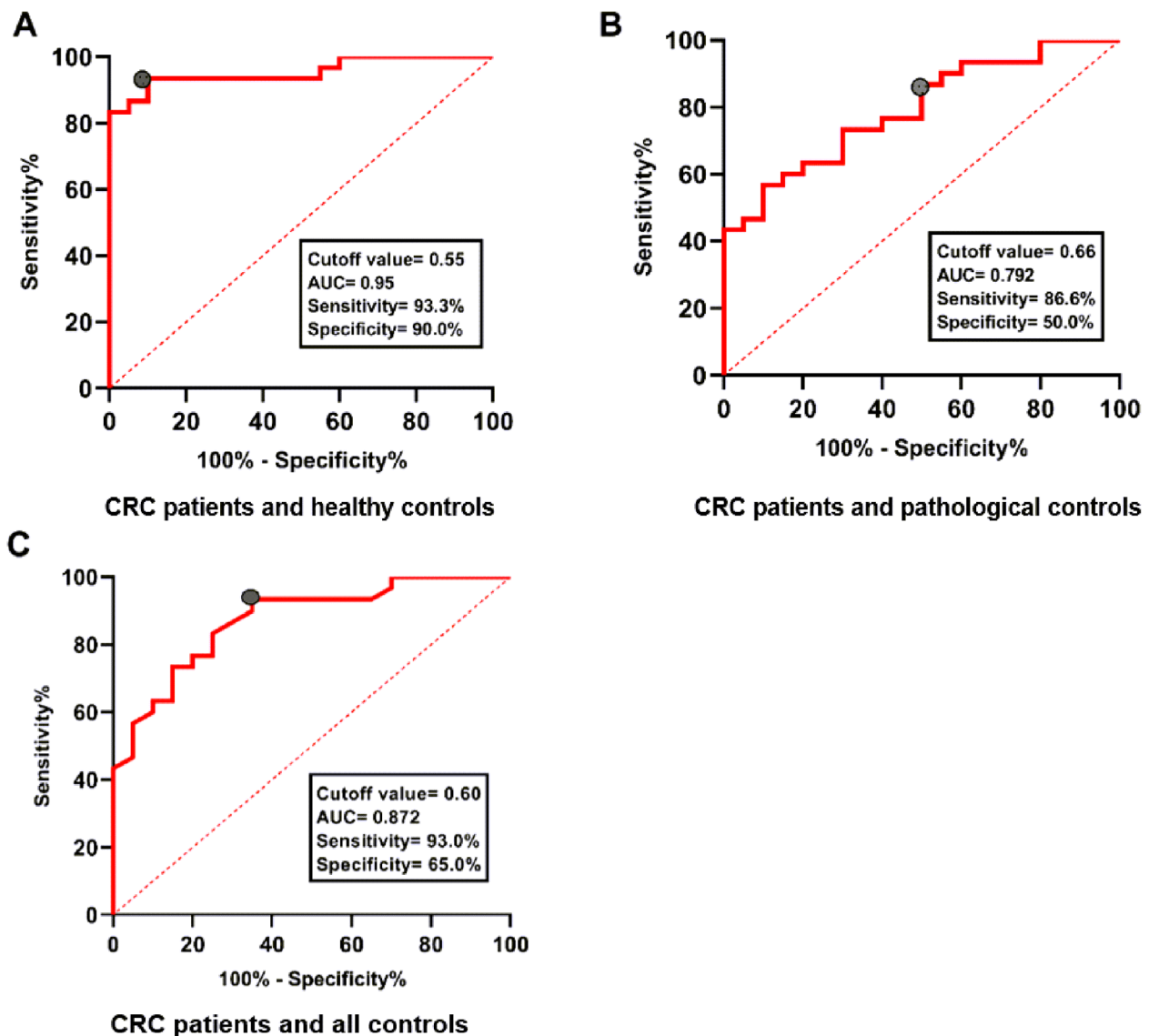
Fig. 5 ROC curve for DII for discriminating between CRC patients and healthy controls (**a**), CRC patients and pathological controls (**b**), and CRC patients and all controls (**c**)

both benign and healthy group was done by full history-taking only, and there were no investigations done to confirm absence of hidden inflammatory conditions in our study and other studies.

As for ALU 247 concentration, which represents necrotic long DNA fragments, there was a significantly higher concentration of ALU 247 in CRC patients compared to both healthy controls and pathological controls. No statistically significant difference was found between pathological controls and healthy controls. This came in agreement with Mead et al. [15] who found a significant difference between CRC patients compared to healthy controls.

To assess the clinical relevance of our studied markers as early markers for CRC, there was a statistically significant difference in the levels of DII and ALU247 between patients with early stages without evidence of metastasis (stages I and II) and controls. However, non-significant difference was found between the two groups as regards CEA and ALU115. This came in accordance with Umetani et al. [12] and Arakawa et al. [16] who found significantly higher levels of DII in patients with early stages of CRC than in healthy volunteers.

To predict the prognostic significance of CEA, ALU 115,247, and DII, we compared the previously mentioned markers with the status of metastasis; there were

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significantly higher levels of CEA and DII in patients with metastasis than those without metastasis. This suggests using both markers to differentiate between patients with early and late stages Also, a correlation study was done between the studied markers and both tumor size and stage. DII was found to have a significant positive correlation with tumor stage and tumor size while CEA was only found to have a significant positive correlation with tumor stage. Also, DII positively correlated with CEA. These findings came in agreement with El-Gayar et al. [13] and Arakawa et al. [16]. No significant difference was found between the metastatic and non-metastatic groups as regards ALU 115 and ALU 247. Similarly, El-Gayar et al. [13] found a non-significant difference in ALU 115 between the two groups.

Conclusions

Our study adds to previous research that suggests that DII is an early marker for CRC detection and may be used as a candidate biomarker for malignancy. Additionally, DII has prognostic significance as it correlated with tumor size and stage suggesting that it can be used to monitor disease progression and follow-up of the patients. Hence, this study highlights the utility of DII as a potential biomarker for CRC.

Abbreviations

ALU: Arthrobacter luteus; AUC: Area under the curve; CA 19.9: Carbohydrate antigen 19.9; CEA: Carcinoembryonic antigen; CfDNA: Cell-free DNA; CRC: Colorectal cancer; CT: Computerized tomography; DNA: Deoxyribonucleic acid; DII: DNA integrity index; MRI: Magnetic resonance imaging; NPV: Negative predictive value; PCR: Polymerase chain reaction; PPV: Positive predictive value; ROC: Receiver-operating characteristic; TNM: T = tumor size, N = node involvement, and M = metastasis status

Authors' contributions
KS designed the data collection tools, monitored data collection for the whole research, interpreted the data, and revised the paper. MA collected the samples, collected patients' clinical data, and staged the patients. RS, RA, and HH carried out the laboratory work and analyzed it. RS and RA carried out statistical analysis. RS, RA, HH, and MA drafted the paper. All authors read and approved the final manuscript.

Availability of data and materials
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate
The study protocol was approved by the ethical committee of the Faculty of Medicine, Ain Shams University. Ethical approval reference number FMASU MD214/2018. Written informed consent was taken from all subjects before participating in this study.

Consent for publication
Not applicable

Competing interests
The authors declare that they have no competing interests.

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